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State-of-the-Art Reviews: Diabetes

DIAGNOSIS AND TREATMENT OF PAINFUL DIABETIC PERIPHERAL NEUROPATHY

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ABSTRACT | Diabetic neuropathies are the most common chronic complications of diabetes, with an estimated lifetime prevalence exceeding 50% in people with diabetes. Among various forms of neuropathy, diabetic peripheral neuropathy (DPN) is the most common and has the strongest evidence base regarding therapeutic approaches. This American Diabetes Association clinical compendium summarizes the latest information about screening for, diagnosing, and treating painful DPN in routine clinical practice. It opens with an overview of the epidemiology of DPN, followed by a description of the pathophysiology of the disease and its often severely painful symptoms. The authors recommend a stepwise approach to effectively diagnose DPN and offer a novel perspective on the impact of social determinants of health on the development and management of DPN. They summarizes the latest guidance on effective therapies, including pharmacological oral and topical agents, nutraceutical products, and nonpharmacological therapies, including physical activity and dietary interventions, passive modalities, and energy or nerve stimulation techniques. Throughout the publication, the authors identify knowledge gaps that need to be addressed and advocate a personalized care approach to reduce the burden of painful DPN and optimize quality of life for individuals affected by it.

Diabetic neuropathy is one of the most prevalent chronic complications in adults with type 1 or type 2 diabetes while also affecting individuals with prediabetes and young people with diabetes, with an estimated lifetime prevalence exceeding 50% (1–4). Although the term "diabetic neuropathy" encompasses a broad spectrum of different neuropathic conditions, diabetic peripheral neuropathy (DPN) is the most common and most studied among them and has the strongest available evidence regarding therapeutic approaches (1).

A detailed epidemiological overview is beyond the scope of this monograph. However, understanding some of the key phenotypes and their associated differences in the risk of developing DPN is crucially important for busy clinicians who treat people with diabetes.

There are some epidemiological differences between DPN in type 1 versus type 2 diabetes, despite there being no major structural differences in nerve pathology. As demonstrated by the DCCT (Diabetes Control and Complications Trial) (5), the prevalence of DPN is low in individuals with newly

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diagnosed and early type 1 diabetes (<10 years' duration). The prevalence then increases with disease duration to up to 34% after ~25 years, as documented in the DCCT's observational follow-up EDIC (Epidemiology of Diabetes Interventions and Complications) study (2). Similar rates were reported earlier by the EURODIAB IDDM (European Insulin-Dependent Diabetes Mellitus Prospective Complications Study) (6) in randomly selected individuals with type 1 diabetes of similar duration from 16 European countries. Furthermore, data from contemporary cohorts reflective of current standards of care on both sides of the Atlantic have found similar results. For example, the T1D Exchange clinic network (7), consisting of >25,000 people with type 1 diabetes in >80 U.S.-based pediatric and adult endocrinology practices, and the large Scottish T1D Register (8), which includes all people with type 1 diabetes in Scotland, both of which phenotyped for DPN with the Michigan Neuropathy Screening Instrument (MNSI) questionnaire (9), found prevalence rates of 11–13% for symptoms of DPN, including pain, in their real-world cohorts. Interestingly, in addition to traditional risk factors such as glycemic control, age, and diabetes duration, cardiovascular risk factors (e.g., obesity, hyperlipidemia, hypertension, and smoking) and particularly socioeconomic risk factors have emerged as very strong predictors of DPN in type 1 diabetes (2,7,8).

In contrast, more than half of all individuals with type 2 diabetes develop signs and symptoms of DPN during their lifetime, as documented in several large observational or interventional cohorts (1,10–14). In fact, the prevalence of DPN is quite high, with rates of up to ~20–30% even in newly diagnosed and early type 2 diabetes, including in contemporary cohorts such as the >1,500 individuals with screen-detected type 2 diabetes in the Danish arm of the ADDITION (Anglo-Danish-Dutch Study of Intensive Treatment of Diabetes in Primary Care) trial (15) and >5,000 individuals with early type 2 diabetes (~4 years' duration) in the GRADE (Glycemia Reduction Approaches in Diabetes–A Comparative Effectiveness) trial (3). Both of these trials phenotyped participants for DPN using the MNSI. In type 2 diabetes, in addition to traditional DPN risk factors (e.g., glycemic control, age, and diabetes duration), racial/ethnic minority status also carries a higher DPN risk, including among American Indians.

Importantly, high prevalence rates of DPN similar to those observed in adults with early type 2 diabetes also have been observed in contemporary youth cohorts, particularly those with type 2 diabetes, as reported by the SEARCH (SEARCH for Diabetes in Youth) study (4), which included ~2,000 young people with type 1 or type 2 diabetes.

Among DPN symptoms, neuropathic pain, often severe, affects up to 30% of all individuals with DPN and is challenging to manage, resulting in increased risks of associated problems such as sleep disturbances, further reduced quality of life, polypharmacy, socioeconomic consequences (e.g., higher health care costs and reduced ability to work or perform daily activities), morbidity, and mortality (1,16–18). Given the epidemic explosion of diabetes in the United States (19) and worldwide (20), the high prevalence of this complication, and its clinical and socioeconomic consequences, effective therapeutic and preventive measures for DPN and DPN-related pain are of paramount importance.

This monograph offers clinicians up-to-date, evidence-based information regarding the mechanisms involved in inducing nerve fiber damage and neuropathic pain and the spectrum of risk factors and DPN phenotypes across the life span, as well as a novel discussion of the impact of social determinants of health (SDOH) on DPN development and management. It also provides busy clinicians with a customized, stepwise approach to effectively screen for and diagnose DPN in routine care. Additionally, it summarizes the latest guidance on effective pharmacological and nonpharmacological therapeutic strategies for painful DPN, including the respective roles of nutraceutical products, dietary modification, exercise, and new technologies. Finally, this publication outlines knowledge gaps that need to be targeted to identify modifiable risk factors, develop more sensitive assessments, and produce effective therapies to either prevent the progression of or reverse neuropathic disease. It advocates a personalized care approach to ultimately reduce sequelae and the related health care burden and optimize quality of life for people with diabetes and DPN.

PATHOPHYSIOLOGY OF DPN

Diabetes preferentially affects the peripheral nervous system (PNS), a likely reflection of the unique anatomy of the PNS (21). PNS axons are frequently ≥3 feet long and >20,000 times the length of their supporting cell bodies. PNS sensory neurons and their receptors lie outside the blood-brain barrier and are more vulnerable to injury secondary to diabetes than motor neurons, which lie within the barrier. Among the sensory neurons, there are small unmyelinated neurons known as C-fibers but also frequently called "small fibers." These fibers carry nociceptive information, particularly related to heat and pain, and constitute the majority of sensory axons in the PNS. The lack of myelin results in slow, continuous conduction of small fibers secondary to a uniform distribution of ion channels along the axon. In conjunction with small fibers

are small, thinly myelinated Aδ fibers, which relay information on touch, pressure, and cold, and fully myelinated fibers of different diameters, designated Aβ and Aα, which are responsible for vibratory and position sense. Collectively, these fiber types are known as large fibers. Myelin, provided by Schwann cells, ensheathes the axons of these fibers in a highly controlled manner, forming the nodes of Ranvier and paranodes, the sites of ion channels required for rapid nerve conduction and of tight junctions that protect large fibers from toxic substances (21).

Anatomical studies from sural nerve biopsies of patients with diabetic neuropathy align with their presenting symptoms (22). Early degeneration and loss of C fibers are evident in patients experiencing new-onset pain, burning, or prickling, which are known as dysesthesias, in their feet, followed by initial demyelination/remyelination of large fibers. As the disease progresses, large fiber axonal loss eventually occurs, and patients experience numbness and loss of proprioception in the feet that travels upward over time. This distal-to-proximal axonal loss and its accompanying symptoms are the hallmark of diabetic neuropathy (23).

Between 1970 to 2010, studies aiming to understand the pathophysiology of diabetic neuropathy focused on glucose dysregulation (24). In the polyol pathway, aldose reductase converts excess glucose to sorbitol, resulting in a series of downstream reactions that decrease sodium–potassium adenosine triphosphatase (ATP) activity, deplete nicotinamide adenine dinucleotide phosphate, and produce reactive oxygen species (ROS), impairing nerve function. Aldose reductase inhibitors were tested in 32 diabetic neuropathy clinical trials but unfortunately failed to improve nerve function.

Excess glucose also enters the hexosamine pathway, producing inflammatory by-products and activating protein kinase C (PKC) secondary to the accumulation of diacylglycerol. PKC activation, in turn, enhances insulin resistance, disrupts growth factor biology, and leads to vasoconstriction of nerve blood vessels. Similar to the aldose reductase trials, clinical trials of PKC inhibitors failed in human diabetic neuropathy.

Advanced glycation end products (AGEs), which bind receptors for AGEs (RAGEs), are another by-product of excess glucose. Activation of AGEs and RAGEs leads to downstream inflammation, ROS accumulation, and decreased blood flow to peripheral nerves. Although preclinical trials targeting activation of RAGEs were promising, available compounds were too toxic for human trials and remain in therapeutic development (25).

Many of the failed clinical trials occurred at the same time multiple, newer clinical trials were suggesting that glucose control alone was insufficient to prevent neuropathy in people with type 2 diabetes (26). There is now consensus that glycemic control alone cannot prevent the progression of DPN in patients with type 2 diabetes. The metabolic syndrome has emerged as a crucial risk factor for neuropathy based on data from multiple clinical studies in the United States (4,27–29), Denmark (15), Germany (30), the Netherlands (31), India (32), and China (33,34). The metabolic syndrome encompasses hyperglycemia, obesity, and dyslipidemia, and the risk of developing neuropathy increases with the number of these components present in an individual (27,28). These clinical trials led to new thinking about the pathophysiology of diabetic neuropathy focused on the idea that disruption in whole-nerve bioenergetics (i.e., how the nerve accesses energy along its entire length) is the crucial factor leading to disease (35).

Mitochondria are the energy-producing organelles in cells and use both glucose and lipids to produce ATP. In the PNS, mitochondria are primarily made in the cell body and are trafficked down long axons to provide energy for nerve function (36). In small fibers, mitochondria are found along the length of the axons. In large fibers, they are also present along the length of the axons but are particularly present at the nodes of Ranvier and the paranodes, where they assist in salutatory nerve conduction. In both fiber types, mitochondria produce ATP from glucose and lipids (21). ATP then supplies the needed energy for nerve impulses to travel the length of the axons and reach distal nerve terminals.

Under normal conditions, glucose and lipids undergo a series of distinct, highly regulated chemical reactions, ending with the transfer of the electron donors nicotinamide adenine dinucleotide and flavin adenine dinucleotide to the mitochondrial electron transport chain. These electron donors travel across the inner mitochondrial membrane and undergo oxidative phosphorylation, with generation of ATP for energy and small amounts of ROS as a by-product of the process. However, in the diabetic environment, excess glucose and lipids not only disrupt the normal pathways used for their own breakdown, but also produce excess electron donors that the mitochondria are unable to process. The result is bioenergetic failure (37) and the loss of normal mitochondrial membrane function (mitochondrial depolarization), decreased ATP production, impaired mitochondrial trafficking, and accumulation of ROS, leading to inflammation, endoplasmic reticulum stress, apoptosis of neurons, and axonal failure (38) (Figure 1).

With fewer functional mitochondria in the cell body and along the axons, energy-starved small and large nerve fibers lose their ability to function and undergo degeneration, with the axons farthest from the cell body (i.e., those in

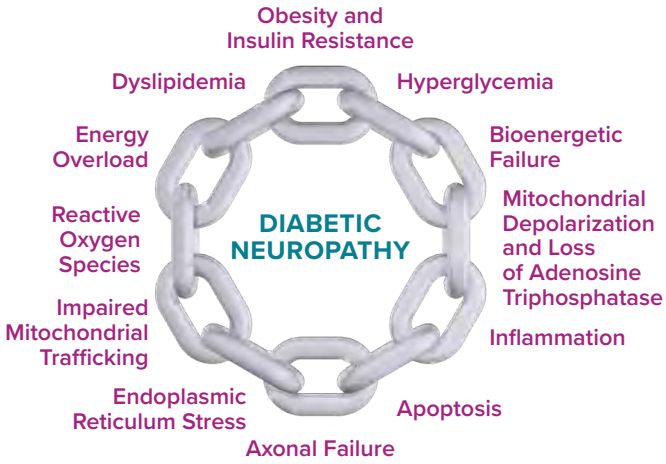

FIGURE 1 Chain of events underlying the pathophysiology of diabetic neuropathy. Components of the metabolic syndrome contribute to diabetic neuropathy by causing energy overload from excess glucose and lipids, leading to mitochondrial bioenergetic failure, with mitochondrial depolarization, loss of adenosine triphosphatase as an energy source, and accumulation of reactive oxygen species. This process leads to impaired mitochondrial trafficking from the cell body down the length of the axons, endoplasmic reticulum stress, apoptosis of neurons, and axonal failure.

the feet) being most vulnerable. This vulnerability occurs because fewer functional mitochondria successfully travel the long distance from the cell body along the entire length of the axons to their most distal terminals (Figure 2). Small fibers that convey pain and dysesthesias are particularly vulnerable to this energy loss. Schwann cells can provide energy-starved large, myelinated axons with some usable fuel, mitochondria, and protection from toxic substances (39), but small fibers lack this energy source and protection. This explains why small fibers are the earliest fibers to undergo injury secondary to diabetes and why pain and dysesthesias are frequently the first symptoms of DPN.

KEY POINTS

- Early injury and loss of small fibers, susceptible to energy flux, occur in people with diabetes, resulting in symptomatic pain and burning in their feet.
- As the disease progresses, larger nerve fibers also become injured by the lack of energy sources, and individuals experience numbness and loss of position sense in their feet.
- These signs and symptoms progress from the feet upward into the leg and reflect a distal-to-proximal fiber loss that is the hallmark of diabetic neuropathy.

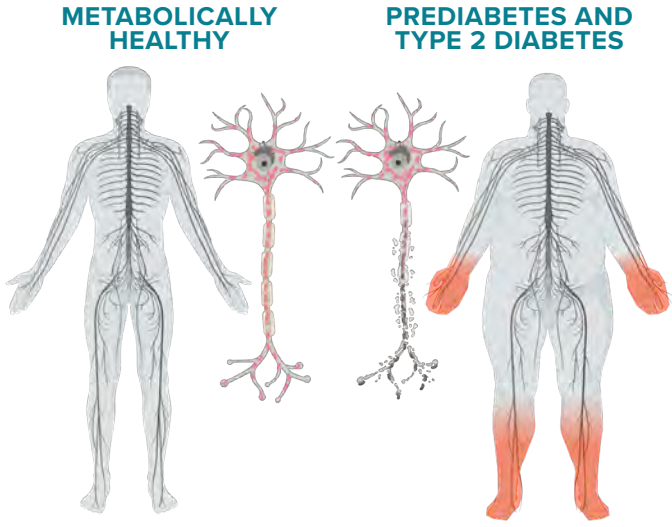

FIGURE 2 In metabolically healthy individuals (left), mitochondria produced in the neuron cell body traffic down the axons, providing energy for normal axonal function. In prediabetes and type 2 diabetes (right), the chain of injurious events leads to mitochondrial dysfunction, with adenosine triphosphatase loss and distal-to-proximal degeneration of energy-starved axons.

SCREENING AND DIAGNOSING DPN

As discussed in detail above, the hallmark clinical features of DPN are the result of progressive damage to and eventual loss of all populations of large and small myelinated and unmyelinated nerve fibers and related downstream effects. In diabetes, this process occurs in a specific symmetrical, distal-to-proximal pattern, starting at the tip of the toes and eventually progressing proximally. Thus, the entire constellation of symptoms and clinical signs associated with DPN follows the same pattern, creating the typical "stocking-and-glove" clinical presentation, which is an important diagnostic clue (Figure 3) (1).

A Customized, Stepwise Approach for Primary Care

Considering the high prevalence of DPN and the magnitude of its consequences, implementing effective screening strategies as part of routine clinical practice is key to ensuring its diagnosis at the earliest possible stage and the timely treatment of DPN pain, thereby preventing progression and the development of advanced complications, including limb amputations and death (1). All individuals should be assessed for DPN starting at diagnosis of type 2 diabetes and 5 years after the diagnosis of type 1 diabetes and at least annually thereafter.

Although the anatomy of the PNS, and hence the evaluation of the various nerve fiber populations, may be quite complex, there are several important tips that can

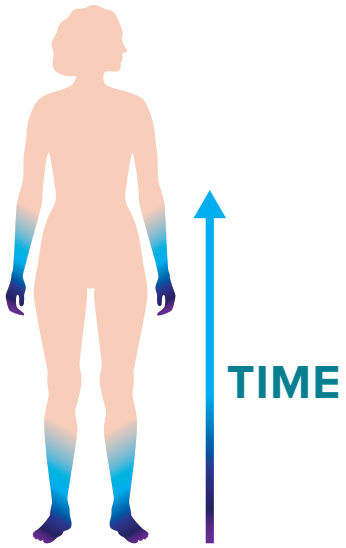

FIGURE 3 Early symptoms of diabetic peripheral neuropathy usually occur in the toes and fingertips, expanding proximally over time in a stocking-and-glove pattern. Sensations to stimuli such as vibration, pinprick, temperature, and monofilament testing all tend to diminish in this same pattern over time.

guide clinicians in implementing efficient and successful DPN screening and diagnosis procedures, as described in the American Diabetes Association's position statement on diabetic neuropathy (1). Importantly, when screening for DPN, one should keep in mind that each of the specific types of nerve fibers has a specific function and role; thus, targeted evaluations can be performed easily in the clinic. A stepwise approach to screening and diagnosis is shown in Figure 4 and described below.

1. Take a Targeted History

A targeted medical history may be obtained quickly during a routine clinic visit or may be ascertained from the electronic health record (EHR) by reviewing for the presence of several risk factors known to be strongly associated with DPN, including poor glycemic control, long duration of diabetes, older age (>70 years), tall stature, hypertension, obesity, and metabolic syndrome (1,28,40,41). In addition, a history of recent falls, particularly when no other risk factors for falls are apparent, may reflect gait and balance disorders that can be a direct consequence of the large-fiber dysfunction associated with DPN (42,43). Individuals with this type of nerve dysfunction are also at increased for other complications, including fractures and hospitalizations, and thus require more specific care.

2. Assess for DPN Symptoms

The symptoms associated with DPN are dependent on the type of fibers most affected initially (Table 1), although some individuals with DPN may be completely asymptomatic and thus may first present with advanced complications such as foot ulcers (1,44).

Small-Fiber DPN

Neuropathic pain is the key feature associated with the damage to small nerve fibers that usually occurs in the earliest stages of DPN (1,45). Neuropathic pain is largely a clinical diagnosis. Characteristically, it is described as burning, lancinating, tingling, and/or a shooting, electric shock–like sensation, occurring in varying combinations and typically worse at night. The pain may

TABLE 1 Symptoms and Clinical Signs of Diabetic Peripheral Neuropathy

	Symptoms	Function	Signs on examination (clinically diagnostic)*

Large, Myelinated Nerve Fibers	• Numbness
	• Tingling
	• Poor balance
		• Pressure
		• Balance
			• Ankle reflexes:
			  • Reduced
			  • Absent
			• Vibration perception:*
			  • Reduced
			  • Absent
			• 10-g monofilament sensation:*
			  • Reduced
			  • Absent
			• Proprioception:
			  • Impaired

Small Nerve Fibers	• Pain:
	  • Burning
	  • Electric shocks
	  • Stabbing
	• Hyperalgesia
	• Allodynia
		• Nociception
		• Protective sensation
			• Thermal (cold/hot) discrimination:*
			  • Reduced
			  • Absent
			• Pinprick sensation:*
			  • Reduced
			  • Absent

**Document impairment/loss in symmetrical, distal-to-proximal pattern.*

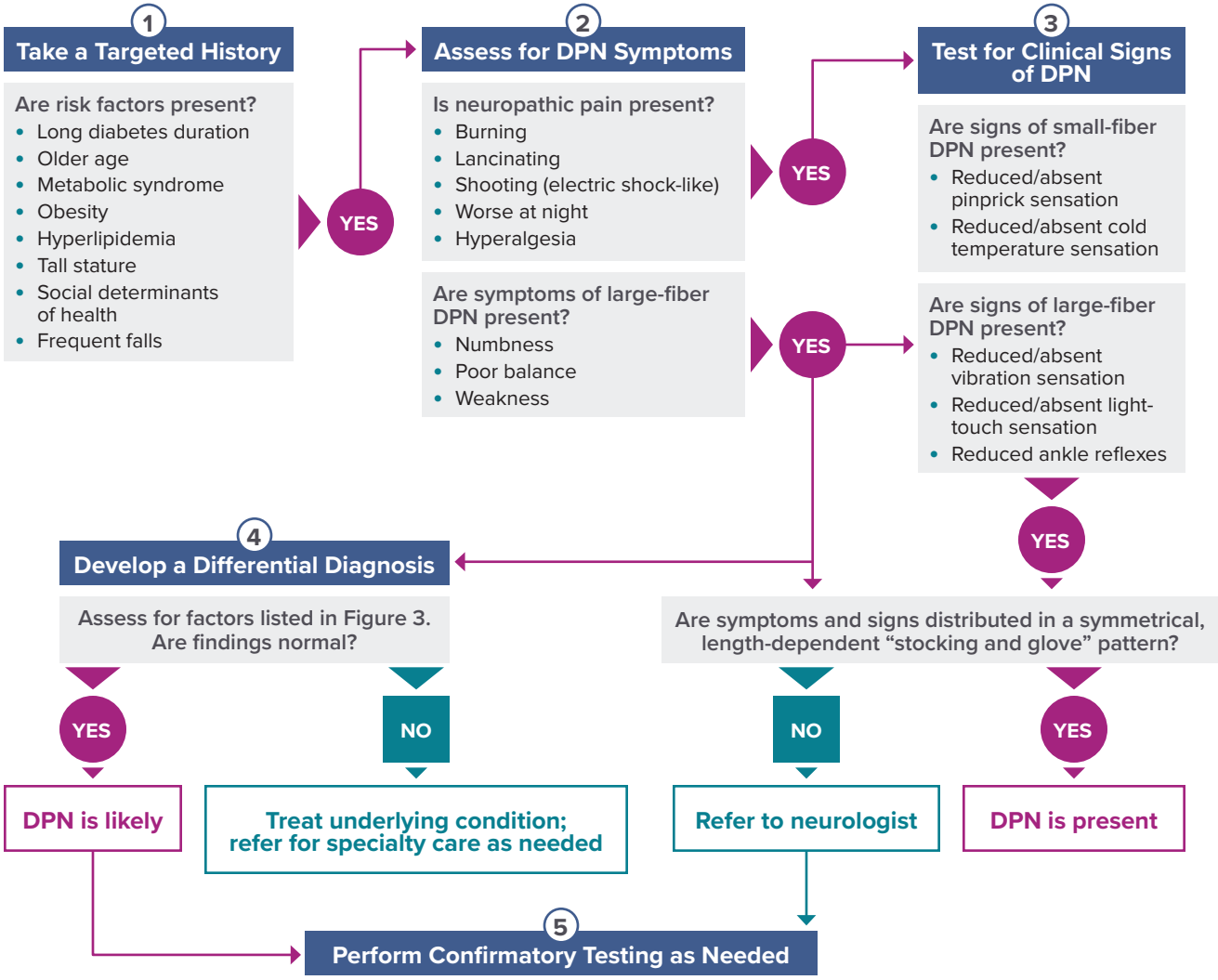

FIGURE 4 A stepwise approach to screening and diagnosing diabetic peripheral neuropathy.

be accompanied by dysesthesias such as an exaggerated response to painful stimuli (hyperalgesia) and/or pain evoked by contact with ordinarily unpainful stimuli such as socks, shoes, and bedclothes (allodynia) (1,46,47). Available evidence shows that up to 25–30% of people with diabetes will experience DPN pain, and because it heralds early disease, neuropathic pain may be present even in the absence of any neurological deficits (1,45,48). However, clinicians should also be aware that some individuals may not voluntarily report some symptoms, including pain, to their health care providers because of a variety of sociocultural factors (e.g., fear or being misunderstood or not taken seriously and misperception that treatments are unavailable or would not help) (45,49). Thus, DPN pain may be underreported.

Several other important tips may be helpful to clinicians when evaluating pain caused by DPN. Neuropathic pain may be the first symptom that prompts an individual to seek medical care, and it could be present in individuals with newly diagnosed diabetes or even prediabetes (1,46). Thus, the absence of a prior diagnosis of diabetes should not rule out the need for formal DPN screening, particularly in the presence of several of the risk factors mentioned above and with the typical clinical characteristics (Table 1) (1,46).

Women, members of some racial/ethnic minority groups, and individuals with type 2 diabetes appear to be at greater risk for developing DPN pain (45). Additionally, the direct and indirect economic burden associated with neuropathic pain is substantial. This pain may directly or indirectly interfere with daily activities or lead to loss of balance, disability, psychosocial impairment, sleep disturbances, and reduced health-related quality of life (1,46). Thus, there should be a strong suspicion of DPN in

individuals presenting with any of these complications.

Some individuals may present with sub-acute onset (over a period of days) and a clinical presentation dominated by foot and lower-limb pain, suggesting a prominent involvement of small fibers. The pain progresses over days to weeks to constant burning dysesthesias and allodynia involving the legs in a stocking distribution. Occasionally, the pain spreads to proximal sites, including the trunk, or it spreads more diffusely and is associated with several autonomic features (1). Despite the prominent pain, sensory loss may be mild or absent, there is no weakness, and reflexes are generally preserved. The sub-acute forms of DPN may be seen with acute weight loss or may be induced by diabetes treatment, developing within 2–4 weeks (occasionally up to 6 weeks) after the achievement of rapid and sustained glycemic control with insulin, oral antidiabetic agents, or dietary measures.

Large-Fiber DPN

Symptoms associated with the damage and loss of the large nerve fibers include numbness, tingling without pain, loss of protective sensation, and, in more advanced stages, poor balance (1,46), weakness, and unsteadiness that may lead to falls (1,23). Some individuals may also present with completely insensate, numb feet and may state in the clinic that their feet feel as if they are wrapped in wool or as if they are walking on thick socks (1,46).

Asymptomatic DPN

Clinicians should be also aware that up to half of all people with DPN may be either asymptomatic or, as previously mentioned, reluctant to report some symptoms (1,45). In such cases, neurological deficits may be discovered by chance during a routine clinical examination (1,46). Other individuals who are initially aware of neuropathic symptoms may become asymptomatic later in the course of the disease, as they experience severe sensory loss in all types of nerve fibers and develop insensate feet (1,46). A serious consequence of insensate feet is an increased risk for painless injury, leading to an increased risk for foot ulceration and amputation (1,50). For example, objects lodged in the shoe, including a wrinkled stocking; unrecognized, increased pressure during walking and weight bearing; or contact with very sharp or hot objects without the appropriate protection may produce blisters that erode through the skin and lead to more severe complications. It is the loss of the so-called "gift of pain" that causes people with plantar neuropathic ulcers to unknowingly walk on their lesions, inducing chronicity that is frequently complicated by infection (1).

In summary, periodic, flexible assessment for DPN risk factors and symptoms and their trajectories over time should be part of standard care for all people with diabetes.

3. Test for Clinical Signs of DPN

Similar to the DPN symptoms discussed above, the clinical signs of DPN are also characteristic of the type of nerve fiber deficits present and their progression (Table 1) (1,46).

Several well-established, effective clinical tests exist to assess small- and large-fiber function as part of routine clinical care and require only simple tools that can be carried easily in a lab coat pocket. Evaluation of DPN-associated small-fiber damage can be accomplished by testing a person's pinprick sensation using a sharp object such as a safety pin (discarded after one use) and temperature threshold sensation, which is mostly performed with a cold metal object such as a tuning fork (1,23). Evaluation of DPN-associated large-fiber damage involves assessing vibration perception using a 128-Hz tuning fork, proprioception, light-touch pressure with a 10-g monofilament on the dorsal aspect of the great toe, and bilateral ankle reflexes.

It is important to note that there are many monofilaments available, with varying diameters. Clinicians should use a standardized 10-g instrument that has been pretested to buckle at a 10-g force when applied to the site of interest and should apply the monofilament at the dorsal aspect of the great toe to ensure standardized assessment (1,46). Although the 10-g monofilament is arguably the most often used test to screen for DPN in routine care, its use alone is not recommended for effective screening or diagnosis, as the loss of light-touch sensation occurs in advanced stages of neuropathy, and relying solely on this test could miss opportunities to implement early preventive care measures in many people with DPN (1,46). Sensation testing with pin and vibration is more sensitive. Loss of ankle reflexes and weakness of small foot muscles and dorsiflexors occur earlier in the course of DPN (23).

All of these sensory modalities should be tested initially by application of the sensory stimulus to a body site where normal responses are expected, such as the forehead. Then the stimulus is applied to the great toe and then moved proximally up the limb to the level where the sensation is felt to be normal. In addition, for many of these evaluations (e.g., vibration perception using a 128-Hz tuning fork or the 10-g monofilament) using a blinded, forced-choice testing procedure will reduce the potential for bias and increase the sensitivity of the evaluations. This procedure involves applying a stimulus (either true vibration or just a touch with the tuning fork) at one of two times while a patient's eyes are closed and then asking

whether the patient felt the stimulus at time A or time B. Those with sensation loss may choose the incorrect time or state that they did not feel the stimulus either time. All of these assessments should follow the typical DPN pattern, starting distally (the dorsal aspect of the hallux) and moving proximally until a sensory threshold is identified, with the same evaluations being performed on both sides to confirm a symmetrical, distal-to-proximal distribution (Figure 3) (1,46).

A combination of at least two of these evaluations, with at least one targeting small fibers and one targeting large fibers, is recommended to screen for and diagnose DPN in routine clinical care (1,46). Several clinical scales combining symptoms and signs have been validated over time for the screening and diagnosis of DPN and can be used easily in routine care. These include the Toronto Clinical Neuropathy Score (51), the Utah Early Neuropathy Scale (52), the Neuropathy Disability Scale (53), or the previously mentioned MNSI (9). Similarly, there are several validated scales for neuropathic pain and its severity, including the McGill Pain Questionnaire and its more recent, shorter version known as the Douleur Neuropathique en 4 Questions (DN4) (54).

As DPN progresses, its symptoms and clinical deficits become more severe as a reflection of damage and loss of all nerve fibers, with sensory deficits also involving more proximal segments and including distal weakness with foot drop and variable degrees of autonomic dysfunction (1,55).

Although the norm in clinical care historically was to refer people with suspected DPN to a neurologist and to order electrophysiological testing to confirm the diagnosis, more recent evidence has shown that these measures are not necessary except in specific cases in which clinical features are atypical, onset is abrupt, and a different etiology is suspected, as described below (1,22,45). Indeed, specialized electrophysiological testing is usually not cost-effective, and its high associated costs and typically long waiting times would place unnecessary additional burden on both people with DPN and the health care system.

4. Develop a Differential Diagnosis

The presence of DPN is determined largely through clinical diagnosis based on the development of the symptoms and signs mentioned above (Table 1) in an individual with diabetes or prediabetes in whom other causes of neuropathy have been excluded (1,45). Therefore, a comprehensive differential (Figure 5) is needed initially in all individuals before a firm diagnosis of DPN is established, particularly given that there are many other forms of peripheral neuropathy that may either mimic or coexist with DPN and may be treatable.

FIGURE 5 Considerations for developing a differential diagnosis for diabetic peripheral neuropathy.

A careful medical history may unveil some of these conditions, including alcohol abuse or other toxic exposures, neoplasia with a history of chemotherapy, or amyloidosis (1,22,45). In addition, clinicians should order a comprehensive metabolic panel and screen for hypothyroidism by testing for thyroid-stimulating hormone. Consider as well the possible role of end-stage renal disease with uremia, particularly in individuals with type 1 diabetes, or perform serum immunoelectrophoresis with immunofixation to evaluate for a monoclonal gammopathy (1,22,45). Vitamin B12 deficiency is one of the most prevalent DPN mimics found in people with type 2 diabetes and can be completely reversed with treatment (1,22,45). Up to 30% of people with type 2 diabetes who are treated with metformin may develop B12 deficiency. Thus, laboratory screening for vitamin B12 levels (methylmalonic acid with or without homocysteine) is recommended at least annually in these individuals. Chronic inflammatory demyelinating polyradiculoneuropathy may also mimic or coexist with DPN, and a high level of suspicion should be raised by a sudden onset and rapid progression of symptoms and signs. Finally, more genetic forms of polyneuropathy are being discovered, and novel treatments have become available for disorders such as familial transthyretin amyloidosis. Thus, genetic testing could be also considered, although the role of routine genetic testing remains unclear.

5. Perform Confirmatory Tests as Needed

Confirmatory tests in ambiguous cases may include determining changes in nerve conduction studies to assess predominantly large-fiber dysfunction. These tests are performed with surface stimulating and recording techniques evaluating motor and sensory nerve fibers in the upper and lower limbs and usually demonstrate a decrease in sural nerve amplitude, followed by reductions in sensory and motor nerve conduction velocity (22). The gold standard for small-fiber neuropathy is assessment of intra-epidermal nerve fiber density measurements by skin punch biopsy. This test can be performed in the clinic if needed, but it is an invasive approach that is rarely necessary for routine diagnosis of DPN and is used primarily for research purposes (22). Other modalities may include quantitative sensory thermal thresholds for reduced cooling detection thresholds or elevated heat thresholds, laser Doppler flare imaging studies, or corneal confocal microscopy, although the latter, again, is largely reserved for research studies.

In summary, a thorough history and examination and routine screening laboratory testing are recommended to ensure that other etiologies, many of which may be treatable, are not contributing to the clinical presentation of DPN (1,22,45). Additionally, in atypical cases involving asymmetrical distribution of symptoms and clinical signs, a motor predominance, or an acute onset and rapid progression of signs such as severe weakness, a timely referral to a neurologist should be made, and cerebrospinal fluid examination by lumbar puncture for protein levels, genetic testing, and MRI imaging of nerve roots and peripheral nerves may be required.

KEY POINTS

- **DPN assessment should be performed annually starting at diagnosis for type 2 diabetes and 5 years after the diagnosis for type 1 diabetes. People with prediabetes and young people with symptoms or signs of DPN should also be screened.**
- **Assessment should include a detailed history and at least two sensation and reflex tests. Electrophysiological testing is rarely needed for people with typical signs and symptoms.**
- **A complex differential is recommended, and ambiguous or atypical cases should be referred to a neurologist and/or have additional testing.**

SOCIAL DETERMINANTS OF HEALTH AND THEIR IMPACT ON DPN

Diabetes continues to grow at an alarming pace, leading to excess morbidity and mortality in the United States and worldwide (19,56). Currently, 34.2 million Americans (10.5% of the U.S. population) have diabetes, with a disproportionate burden on racial and ethnic minorities and low-income populations (19,57). Despite an expanding arsenal of therapeutic options, only 26% of Americans are meeting combined targets for A1C, lipids, and blood pressure (58). Not unexpectedly then, DPN remains prevalent, affecting up to 50% of patients with diabetes (1). Recognizing that social factors are the root cause for health disparities, particularly for type 2 diabetes, the American Diabetes Association (ADA) in 2021 published a scientific review on SDOH and diabetes (59). This review highlighted the key social domains affecting diabetes incidence, prevalence, and outcomes (59). We seek here to complement this report with a focus on the impact of SDOH specifically on DPN.

Theoretical Frameworks: SDOH, Health Disparities, and Diabetes Risk

Health determinants historically have been classified into biological, clinical, and nonclinical factors (60). As health disparities worsen in the United States (61), research is unveiling the importance of nonclinical factors such as social, behavioral, and economic influences on both population and individual health. These factors, collectively known as SDOH, are defined by the World Health Organization (WHO) as "the conditions in which people are born, grow, live, work, and age; these circumstances are shaped by the distribution of money, power, and resources at global, national, and local levels" (62). Recognizing that up to 50–60% of health outcomes are attributed to SDOH (63), multiple frameworks have been developed to both categorize and describe the influence of SDOH on individual and population health outcomes (59). The most comprehensive framework was developed by the WHO in 2010 and highlights the causal effects of upstream structural determinants (e.g., socioeconomic context, political context, and socioeconomic position) on intermediary determinants (e.g., biological, behavioral, and psychosocial factors, as well as the health system), leading to health inequities (64). More recently, the Centers for Disease Control and Prevention's Healthy People 2020 (and now Healthy People 2030) framework grouped SDOH into five general domains, including the neighborhood and built environment, social and community context, economic stability, education access and quality, and health care access and quality (65), as shown in Figure 6. The common theme among all frameworks is that the inequitable distribution of SDOH is the fundamental basis for all health disparities (59).

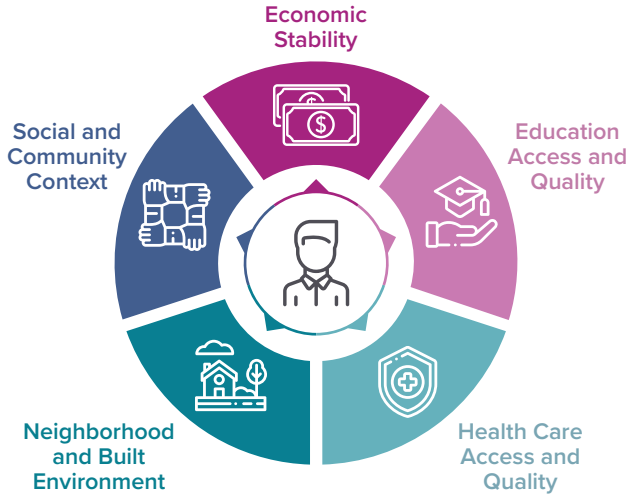

FIGURE 6 Healthy People 2030 framework for social determinants of health (65).

Diabetes, particularly type 2 diabetes, is especially influenced by SDOH. For example, people with a lower socioeconomic position, defined as a combination of education level, occupation, and income, have a 31–41% increased risk of developing diabetes compared to those with a higher socioeconomic position (66). Additionally, factors such as socioeconomic status (SES), food security, stable housing, health care access, and social support influence diabetes outcomes such as microvascular complications, cardiovascular disease, and mortality (59).

Impact of Key SDOH on DPN

Adverse distribution of SDOH has both direct and indirect impacts on proximal diabetes outcomes such as glycemic control (67), which in turn contribute to the pathogenesis of diabetes complications, as demonstrated in Figure 7. These indirect pathways, or mediators, include adverse care processes (e.g., preventive screenings for complications), poor access to health care (e.g., patient-centered care), or inadequate self-care behaviors (e.g., medication-taking) (67). Examples of negative diabetes outcomes resulting from the specific domains of SDOH are discussed below, with a particular focus on DPN.

Economic Stability

SES is a complex construct that commonly includes measures of income and occupation, both of which are closely intertwined with education (59). As described above, people with lower SES, as determined by poverty and low education status, are more likely to have type 2 diabetes. Furthermore, those with lower SES who have diabetes are more likely to have inadequate glycemic control (68). Unsurprisingly, then, individuals with type 1 diabetes living in more socially deprived areas in Scotland were found to be 2.17 times more likely to have DPN (8).

Education

Researchers conducting an analysis of the T1D Exchange clinic registry cohort in the United States found that lower individual educational attainment predicted DPN in type 1 diabetes, even when adjusted for glycemic exposure and vascular risk factors (7). Additionally, low educational status has been shown to be associated with the development of any microvascular complication in type 2 diabetes (69).

Health Care Access and Quality

In the United States, access to quality health care is highly dependent on having health insurance. This was particularly true in the era before passage of the Affordable Care Act; prior studies using data from 2011

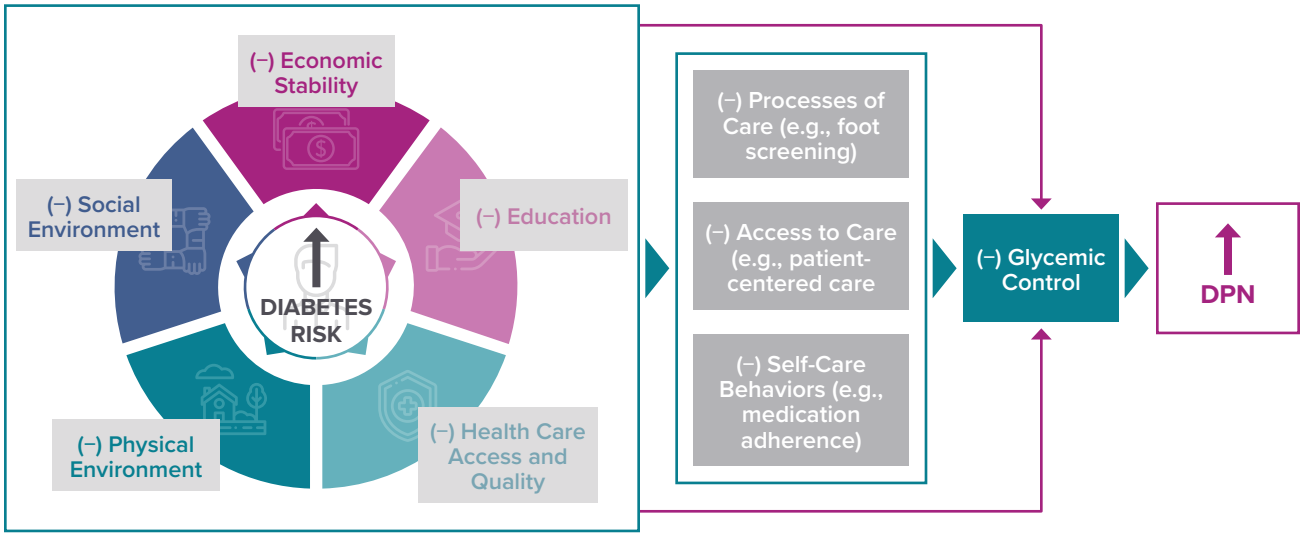

FIGURE 7 Framework for social determinants of health and their impact on diabetic peripheral neuropathy (adapted from refs. 65 and 67).

and 2012 demonstrated a lack of routine diabetes care measures such as assessment of glycemic control and preventive diabetes screenings, even when controlled for individual income (70). It was not surprising, then, that T1D Exchange researchers were able to demonstrate in their clinic registry cohort that DPN in type 1 diabetes was associated with having public or no insurance versus private insurance (7).

Physical and Built Environment

Stable housing, food security, and safe environments in which to engage in physical activity are crucial for individuals with diabetes to facilitate consistent medication use (e.g., the proper storage of insulin or noninsulin injectables) and self-care behaviors to achieve proximal glycemic outcomes (59). However, there is a lack of research evaluating the effects of the physical and built environment specifically on DPN.

Social Environment

Factors such as social context and social support have been shown to be important in improving glycemic control, self-care behaviors, and quality of life (59).There are also known cultural preferences in terms of support systems and interventions to improve support that include multifaceted approaches such as peer support, community health worker support, and support from the health care team, depending on the target population (59). However, there is a paucity of studies evaluating the impact of the social environment on DPN specifically.

Special Considerations

Racism as a Potential Root Cause for Health Disparities in Diabetes

Members of racial and ethnic minority groups historically have borne a disproportionate burden of diabetes and its associated complications (19,60). Biological factors such as alterations in glucose metabolism, insulin resistance, and obesity have been found to account for some of this increased risk (71). However, studies assessing race and ethnicity have found inconsistent findings, with many of the increased risks attributable to SDOH (72). With regard to DPN specifically, there have been no consistent findings of racial or ethnic differences in DPN prevalence rates (71,73).

With an increased understanding of the importance of SDOH in diabetes prevalence, incidence, and outcomes, the upstream social and political contexts that contribute to and perpetuate health disparities have been receiving increased attention. Specifically, the concept of racism, rather than race, as the root cause of the structural and social factors leading to socioeconomic deprivation, residential segregation, and discrimination has been proposed and warrants further evaluation (74).

Importance of Psychological Determinants of Health in Painful DPN

There is increasing evidence that psychological determinants such as emotional distress (75) and depression (76) significantly affect sleep and quality of life in painful DPN (77). It is recognized that there is a complex interplay

between SDOH, psychological determinants, and glycemic control (67). Further investigation to is needed to learn how these complex relationships extend to DPN, and particularly painful DPN.

The Way Forward

The writing group for the ADA's SDOH scientific review emphasized the need to evaluate the impacts of SDOH pathways on different populations with diabetes (59). Data specific to the effects of SDOH on DPN are limited but crucial to provide a comprehensive understanding of the contributors to this debilitating and common complication of diabetes. Because there are no clearly effective, disease-modifying agents for DPN (1), targeting proximal diabetes outcomes such as glycemic control, blood pressure control, and adequate treatment of lipid pathways remains the mainstay of therapy for prevention of DPN. Additional research on the impacts of SDOH on DPN risk and outcomes can provide clarification on potential high-yield intervention targets. Historically, our treatment strategies for DPN have focused on improving symptoms such as pain in individuals but are limited in terms of their effectiveness, with only 50% of people with DPN responding to such interventions (1). Ultimately, interventions that target more upstream social, political, and psychological causes of DPN may be more effective for a larger population.

In summary, DPN remains a common complication of both type 1 and type 2 diabetes, and early investigation in observational studies of large cohorts suggest that socioeconomic factors and health care accessibility are risk factors for DPN. A more comprehensive assessment of the impacts of social and psychological determinants of health on DPN is needed to better our understanding of potential therapeutic targets. It is likely that interventions that address more upstream causes of health disparities at structural and societal levels will be more effective for a larger population of patients at risk for DPN.

KEY POINTS

- **Discrepancies in SDOH are the basis for health disparities, particularly in diabetes, leading to an increased disease prevalence and incidence.**
- **Inequitable distribution of SDOH has both direct and indirect effects on diabetes outcomes, including glycemic control and cardiovascular outcomes.**
- **Social deprivation, lower educational status, and limited health care access are risk factors for DPN. Future exploration of other SDOH domains on DPN risk and progression should be pursued.**

TREATING PAINFUL DPN

As mentioned previously, ~30% of all individuals with DPN will experience painful symptoms that will require pharmacological and other treatments (78). Although painful DPN may occur in all age-groups, it is more common in older patients. Additionally, there are specific differences in pain phenotypes. For instance, in younger individuals with poorly controlled type 1 diabetes, pain may be present with no or very few other clinical signs (78,79), whereas older individuals are also likely to have large-fiber dysfunction, resulting in unsteadiness and gait disturbances, which can adversely affect activities of daily living (80). In a large, community-based study in the United Kingdom, painful DPN was more common in individuals with type 2 diabetes, and there was a weak association with older age (79). Given that the mean age of the 15,000 patients in this study was 61 years, this finding suggests that the management of painful DPN in the elderly requires special attention.

Pain Assessment

Successfully treating painful DPN requires the means to evaluate the effectiveness of each patient's therapeutic regimen. A large number of scales to assess pain severity have been proposed (81,82), including a visual analog scale (VAS) or a series of simple, orienting questions such as are found in the DN4 (54), and these approaches may be useful in clinical practice. More detailed assessment of neuropathic symptoms can be accomplished with tools such as the Neuropathy Total Symptom Score–6 or the modified Toronto Clinical Neuropathy Score) instruments (82–84).

Role of Glycemic Control

As described earlier, chronic hyperglycemia is a major contributory factor in the etiopathogenesis of the diabetic neuropathies; however, the importance of glycemic control in the management of painful symptoms is less clear. Early studies suggested that stable glycemic control with few excursions into hyperglycemia or hypoglycemia was associated with reduced pain scores as assessed on a VAS (78). Later, with the advent of continuous glucose monitoring, a small study confirmed that people with painful DPN have greater fluctuations in glucose and poorer overall glycemic control than matched subjects with painless neuropathy (85). Although there has not been, nor will there ever be, a randomized trial to test the hypothesis, an early step in the management of painful DPN should be to achieve optimal and stable glycemic control (86). Thus, the stability of glycemic control may

be more important than the actual level of control, as indicated by A1C, in the management of painful DPN.

Acute Painful Diabetic Neuropathy

Acute painful diabetic neuropathy is a rare but well-recognized and distinct variety of the sensory neuropathies. It is characterized by very severe neuropathic symptoms, as described earlier, that typically occur after a sudden change in glycemic control and has been described as occurring after normalization of blood glucose levels after simultaneous pancreas and kidney transplantation. It can also follow an episode of ketoacidosis, and in young people, particularly females, it may be associated with eating disorders (87). The prognosis of this acute form of painful neuropathy is good, typically with resolution of symptoms within 12 months. However, pharmacological treatment of the severe symptoms, as described below, is invariably required. This variety of neuropathy was originally called "insulin neuritis," but a more recent review by Gibbons and Freeman (88) suggested the term "treatment-induced neuropathy of diabetes," as insulin is not the only cause.

Chronic Painful Diabetic Neuropathy

This chronic painful neuropathy is common among people of all ages with either type 1 or type 2 diabetes. Many of these individuals will require treatment, and the commonly used therapeutic approaches are described below. It is important to remember that the natural history of this condition is usually characterized by the resolution of severe symptoms over a period of years (78), although data from reliable, long-term studies are lacking. Thus, regular review and adjustment of the therapeutic approach in each patient is essential.

Therapeutic Approaches

Because there are no pathogenetically oriented pharmacological treatments approved by the U.S. Food and Drug Administration (FDA), the treatment approaches described below target the symptoms but do not alter the natural history of neuropathy, which is one of progressive loss of nerve fibers in a distal-to-proximal manner. The following sections briefly describe the commonly used pharmacological agents, topical treatments, and physical therapies, as summarized in Figure 8. Limited space prevents us from including discussion of the many

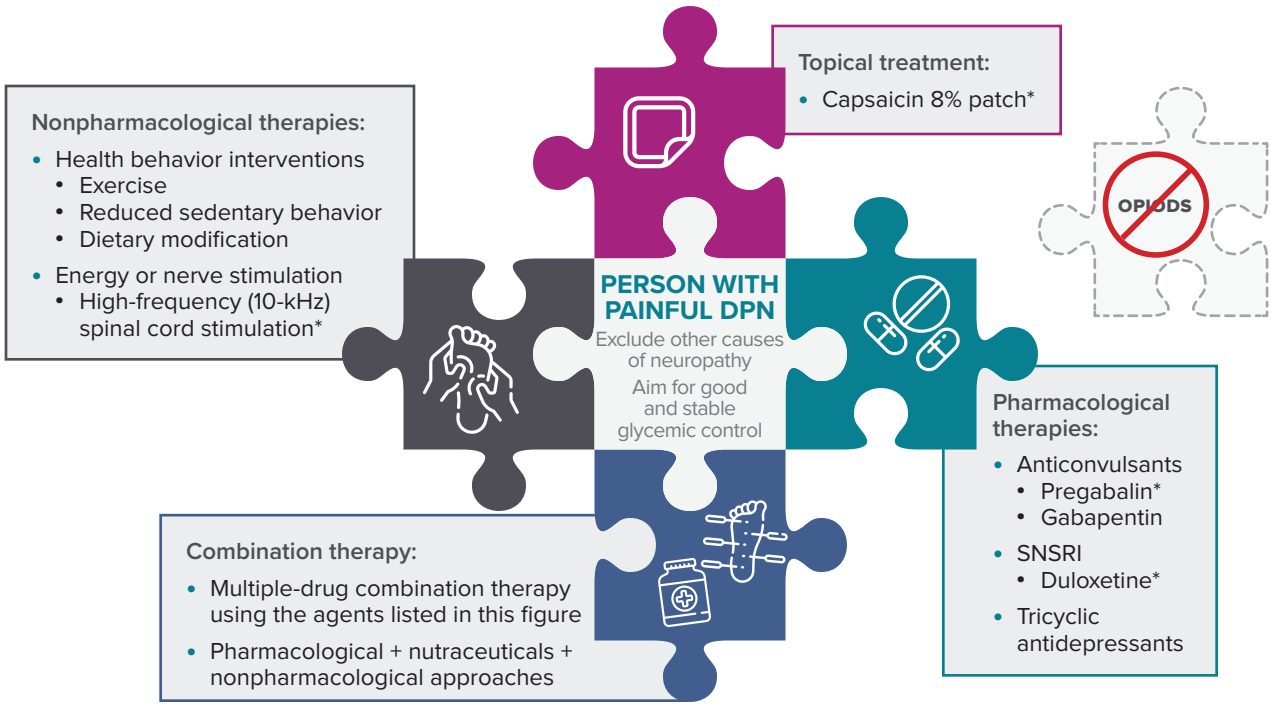

FIGURE 8 Recommended therapeutic approaches to painful diabetic peripheral neuropathy. Pharmacological therapy selection should be individualized based on factors such as comorbidities, cost, potential drug-drug interactions, and potential for adverse effects. Opioids are not recommended because of their high risk of addiction, abuse, and adverse effects. Topical capsaicin and a variety of nonpharmacological approaches are also available, and combination therapy may be needed. Not depicted are the neutraceuticals α-lipoic acid and benfotiamine, which are used in some countries but not approved in the United States. Individuals with severe pain that is refractory to other therapies should be referred to a specialist pain clinic. *U.S. Food and Drug Administration–approved for the treatment of painful diabetic peripheral neuropathy. SNSRI, selective norepinephrine and serotonin reuptake inhibitor.

other agents that have been proposed for the treatment of painful DPN. For a detailed discussion of this topic, readers are referred to recent systematic reviews and meta-analyses, position statements, and review articles (1,45,81,82,89).

FDA-Approved Pharmacological Treatments

There are three FDA-approved therapies for painful DPN: pregabalin, duloxetine, and tapentadol (1). However, the last is an opioid, and a systematic review after its approval questioned its effectiveness (90). This issue will be addressed in a separate section below.

Pregabalin

Pregabalin is a calcium channel α2-δ subunit ligand, the efficacy of which has been confirmed in a large number of studies (1,45,81,82,89,91). It has been extensively studied in painful DPN (1,82,91), and evidence suggests a dose-dependent response, with weaker effect at lower doses (92). It can be given twice, or, on occasion, three times daily, and most patients require 300–600 mg/day for symptomatic relief. However, because it is excreted virtually unchanged in the urine, great caution must be taken in individuals with renal impairment, especially those with an estimated glomerular filtration rate (eGFR) <45 mL/min/1.73 m^2. Adverse effects, which commonly include somnolence, dizziness, and peripheral edema, are more likely with higher dosages and also in the elderly.

As recommended by Freynhagen et al. (91), a low-and-slow dosing approach can limit these adverse effects. These authors also recommend starting with asymmetric dosing, with a larger dose being given in the evening. Up-titration should be based on pain relief. Thus, in a middle-aged patient with normal renal function, it is common to start with 75 mg twice daily and gradually increase the dose every 3–7 days to a maximum of 600 mg/day. Greater caution is required in elderly patients, who are more likely to have renal dysfunction and in whom side effects are more common; in these individuals, starting at 25 mg daily may be required.

Because pregabalin additionally has anxiolytic activity, it may be particularly helpful in individuals with marked anxiety, which is not uncommon among people experiencing neuropathic pain.

Duloxetine

Duloxetine is a selective norepinephrine and serotonin reuptake inhibitor and can be given once daily at a dose of either 60 or 120 mg. Its efficacy has been proven in a number of randomized controlled trials (RCTs), and it has been shown in head-to-head trials to have similar efficacy to other agents, including pregabalin and gabapentin (45). Its adverse effects are well recognized and include nausea, somnolence, and dizziness, among others (1,45,82).

Because duloxetine and pregabalin have different modes of action, the combination of these agents has been used with good effect in clinical practice. However, the largest comparative trial (93) did not show a significant difference between high-dose monotherapy with either agent and a standard-dose combination of both. However, there was a trend consistently favoring combination therapy over monotherapy.

Because duloxetine is also an antidepressant, it may be particularly helpful in people with painful DPN who also have depressive symptomatology.

Other Pharmacological Therapies

Amitriptyline

Amitriptyline and other tricyclic antidepressant agents have been used for many years in the management of neuropathic pain (1,45,81,82,89). However, adverse effects are common, especially in the elderly, and include anticholinergic effects such as dry mouth, urinary retention, and drowsiness. There are also warnings to use these agents with caution in the presence of ischemic heart disease or glaucoma.

Up to one in three patients cannot tolerate even the lowest dose of amitriptyline because of these predictable side effects. It can be given once daily, usually in the early evening, and the usual starting dose is 25 mg, increasing as necessary to a maximum of 150 mg. However, much lower doses must be used in older patients, and because of potential cardiovascular adverse effects, great caution should be taken in those with known ischemic heart disease.

Gabapentin

Gabapentin, similar to pregabalin, was first used as an anti-epileptic drug but has been shown to have efficacy in the management of painful DPN. However, it has a shorter half-life and therefore must be dosed three times daily. As with pregabalin, up-titration is recommended, but trials have shown that most patients require 1,800 mg daily in divided doses for pain relief, and, occasionally, patients need the maximum daily dose of 3,600 mg. Lower doses are recommended for individuals with a reduced eGFR (45).

The adverse effects of gabapentin are similar to those of pregabalin and include dizziness, somnolence, and, on occasion, gait disturbances. A recent systematic review comparing the efficacy and safety of gabapentin and duloxetine in painful DPN found no significant differences between the two drugs (94).

Opioids

Although several opioids have been shown to have some efficacy in the management of neuropathic pain in general, their associated high risks of addiction, abuse, sedation, and other complications such as somnolence, headache, and impaired gastrointestinal motility even in the short term are major barriers to their use. Additionally, it has been revealed recently that even the weak synthetic opioid agonists tramadol and the extended-release tapentadol pose the same risks with only modest evidence of efficacy in relieving severe neuropathic pain. Thus, given the evidence on risks versus potential benefits, none of these agents should be used in the treatment of painful DPN, and they should be avoided especially in the elderly.

Interestingly, a recently published randomized, double-blind, crossover clinical trial (95) paradoxically suggested that the opioid receptor antagonist naltrexone 2–4 mg daily might be effective in the treatment of painful DPN. Naltrexone showed comparable efficacy but greater safety and tolerability than amitriptyline in this trial. However, these preliminary results, although interesting, will need to be confirmed in larger trials.

Topical Therapies

A number of topical therapies have been proposed for the treatment of painful DPN over the years but are not widely used because the area of neuropathic pain can be quite extensive, involving not only both feet, but also the lower limbs, generally below knee level. The most studied of these products has been capsaicin, but its use has been restricted because of the need for frequent application and also the burning pain patients often experience when a capsaicin-containing patch is applied topically.

More recently, an 8% long-acting capsaicin patch has been developed and is now approved by the FDA for painful DPN based on data from two multicenter trials demonstrating effective pain reduction. Initial results are promising (96,97), and it is associated with fewer central adverse effects than the oral medications. However, its use may cause significant pain at the application site, it should be avoided in patients with active skin lesions, and its application requires suitable infrastructure to be available and must be performed in a physician's office.

Physical Therapies

Data from RCTs support the use of spinal cord stimulation (SCS) in the management of severe painful DPN. This and other energy or nerve stimulation treatments are discussed in detail in the section on nonpharmacological approaches to DPN and pain management.

Management of Painful DPN in the Elderly

As alluded to above, particular care is required in the management of painful DPN in elderly patients. Polypharmacy is common in the elderly, and this is especially true in those with diabetes, who may be on multiple therapies, including several oral antidiabetic agents, a statin, aspirin, and multiple treatments for other common comorbidities such as hypertension and cardiovascular disease. Indeed, an article published in 2016 by Jansen et al. (98) addressed the problem of inappropriate polypharmacy in older patients and proposed "deprescribing," a process of planned and supervised tapering of inappropriate medications.

As noted above, painful DPN is common in elderly patients with diabetes, and, in addition to painful symptoms, these patients may also have significant loss of large-fiber nerve function, which leads to unsteadiness and motor dysfunction, leading in turn to alterations in gait (78). These patients are therefore more prone to adverse effects of some of the drugs discussed above, particularly the tricyclic antidepressants, but also anticonvulsants and duloxetine at higher doses.

Moreover, older patients are also more likely to have other complications, including diabetic nephropathy, kidney disease, and cardiovascular complications. Therefore, marked caution must be taken when dosing drugs such as pregabalin and gabapentin, which should be started at lower doses than usual and with very careful up-titration. It is also well recognized that eGFR falls with age (99); thus, although a patient who is 80 years of age and has an eGFR of 50 mL/min/1.73 m2 may be considered as having "normal" renal function, extra care must still be taken when setting dosages for the drugs discussed above. Older patients with severe painful DPN not responsive to these drugs might benefit from referral to a pain clinic for possible SCS therapy (100).

Combination Treatment

Given the complexity of DPN-associated pain and the high risks for side effects of available pharmacological agents, particularly when higher effective doses are needed and in a patient population with multiple other comorbidities, consideration of combination therapy may be advisable. Combining two or three of the pharmacological agents discussed above that have a strong track record of benefit may enable better pain resolution at lower doses and with better tolerance, as shown in Figure 8 (1). Similarly, combining pharmacological with nonpharmacological treatment options may be more effective in a broader number of individuals (Figure 8).

Duration of Treatment

As noted previously, the natural history of painful DPN is not well studied, but it is clinically recognized that painful symptoms rarely persist for more than a few years. Thus, periodic review of pharmacological therapy is needed, and these agents must not be regarded as lifetime medications. It is recommended that, after 6 months of symptom relief, a slow and gradual reduction of drug treatment should be attempted. If symptoms reappear during this gradual reduction, a lower dose may be required for the next few months, when further review and attempted reduction should take place.

Future of Painful DPN Treatment

As summarized in recent reviews (45,81,89), a number of new agents are currently under investigation for the management of painful DPN. Moreover, phenotyping of patients with painful DPN may well lead to the development of more and better treatments for this condition. Many of the current pharmacological therapies are unsatisfactory, not only because of their adverse effects profile, but also because of their general poor efficacy and high numbers needed to treat to improve outcomes (81). Thus, more precise phenotyping of individuals with painful DPN might help to identify subgroups of patients who are more likely to respond to a given therapy.

KEY POINTS

- Effective pharmacological therapies for painful DPN include anti-epileptic agents (pregabalin and gapapentin) and anti-depressants (duloxetine and tricyclics), although often effective pain reduction requires higher doses that may be less tolerated or have a higher incidence of adverse effects.
- For those with severe painful symptoms not responding to a single agent, combination therapy with two to three agents may be effective at much lower doses, as well as combinations of pharmacological and nonpharmacological approaches. Such patients might also benefit from referral to a pain clinic if several attempts to control pain have failed.
- Given the high risk for complications and the modest evidence for benefit, the use of opioids is not recommended.
- Painful DPN is not uncommon in older people with diabetes, in whom caution must be taken with any prescribed medications. Starting at the lowest doses with slow up-titration is recommended in such cases.

ROLE OF NUTRACEUTICALS IN DPN AND PAIN MANAGEMENT

As discussed earlier, DPN remains the most relevant and prevalent clinical manifestation of diabetic neuropathy and predicts the development of neuropathic foot ulcers, all-cause mortality, and cardiovascular morbidity and mortality (1,101). Despite its major clinical impact, the condition still remains underdiagnosed and undertreated (102). Given that the efficacy of causal treatments of DPN and neuropathic pain is limited, there is an unmet need for adjunct treatments.

The term "nutraceutical" was coined in 1989 (103) as a portmanteau of "nutrition" and "pharmaceutical," but, to date, there are no internationally accepted definitions of the term or of related terms such as "functional food," "health food," or "herbal therapy" (104). It has been proposed that, unlike dietary supplements, nutraceuticals should not only supplement the diet, but also aid in the prevention and/or treatment of disease and/or disorder (105). However, nutraceuticals are not defined by U.S. law and usually would be categorized as dietary supplements and regulated by the FDA under the provisions of the Dietary Supplement Health and Education Act. According to that law, "Dietary supplements include a large, heterogeneous group of products intended to supplement the diet that are not better described as drugs, foods, or food additives. Supplements may contain, in whole or as a concentrate, metabolite, constituent, or extract, any combination of 1 or more vitamins, minerals, amino acids, herbs or other botanicals, and other substances used to increase total dietary intake, including enzymes, organ tissues, and oils. They must be intended for ingestion; sold in the form of capsules, tablets, soft gels, gel caps, powders, or liquids; and not be marketed as food items" (105).

Several nutraceuticals represent biofactors required by the body for its normal physiological functioning and may exert health-beneficial or disease-preventive biological activities. Essential biofactors are those that the organism cannot produce or cannot produce in sufficient quantity, thus requiring supplementation from external sources. Examples include vitamins (A, B1, B6, B9, B12, C, D, E, and K), minerals (selenium, magnesium, and zinc), fatty acids (α-lipoic acid [ALA], polyunsaturated fatty acids [PUFAs]), and amino acids (acetyl-L-carnitine) (106). Dietary supplementation with certain biofactors could be useful as a complement to established therapies for preventing and treating DPN because diabetes is associated with systemic deficits in several biofactors, but favorable effects have also been reported in the absence of such deficiencies (107).

Therapeutic Role of Nutraceuticals

In general, the management of DPN includes three cornerstones: *1*) causal treatment, including lifestyle modification, intensive diabetes therapy aimed at near-normoglycemia, and multifactorial cardiovascular risk intervention, *2*) pathogenetically oriented pharmacotherapy using specific nutraceuticals, and *3*) symptomatic treatment of neuropathic pain (102). The available evidence for the second pillar is outlined here. For this discussion, only RCTs of single compounds (monotherapy) were considered because use of combination nutraceuticals does not allow for separate assessment of their constituent parts, and uncontrolled studies are difficult to interpret given the lower quality of the evidence they yield.

The rationale for using nutraceuticals in DPN is primarily to favorably influence the underlying neuropathic process and its clinical consequences rather than only relieving symptomatic pain, which is usually the goal of analgesic therapy. The RCT results described below are also summarized in Table 2.

α-Lipoic Acid

Because oxidative stress plays a major role in the pathogenesis of diabetic neuropathy, the rationale for treatment using antioxidants such as ALA to diminish enhanced oxidative stress and thereby favorably influence DPN is obvious (108). Among the nutraceuticals reviewed herein, ALA has the best evidence in DPN. In summary, intravenous infusions of ALA (600 mg/day) ameliorated neuropathic symptoms and deficits (i.e., signs or impairments) after 3 weeks (108). Moreover, treatment for 5 weeks and 6 months using oral ALA 600 mg daily and twice daily, respectively, reduced the main symptoms of DPN, including pain, paresthesias, and numbness, to a clinically meaningful degree (108,109). Several meta-analyses confirmed the efficacy of ALA in symptomatic DPN (108). In the NATHAN (Neurological Assessment of Thioctic Acid in Diabetic Neuropathy) 1 trial, which included 460 patients with diabetes and mild to moderate, largely asymptomatic DPN, after 4 years of ALA treatment using 600 mg daily, neuropathic deficits (i.e., signs) were improved, suggesting a potential to favorably influence the underlying neuropathy, and the drug was well tolerated throughout the trial (108). Clinical and post-marketing surveillance studies have revealed a highly favorable safety profile (108).

ALA is approved and recommended by guidelines (101) as pharmacological therapy for the treatment of DPN in several countries, but not in the United States or Canada. The primary indication for ALA is symptomatic DPN, including not only neuropathic pain but also nonpainful symptoms such as paresthesias and numbness, particularly if these interfere with a patient's quality of life. On the other hand, based on the results of the NATHAN 1 trial (108), ALA (600 mg daily) can also be considered for long-term use ≥4 years in asymptomatic DPN to improve neuropathic signs (i.e., the underlying neuropathy) (108).

The usual dose is 600 mg daily, but higher doses (600 mg twice or three times daily) may occasionally be useful if symptom relief is only partial (≤30% reduction). The duration of RCTs using ALA in symptomatic DPN has been limited to ≤6 months, similar to those using analgesic drugs in painful DPN, which lasted ≤3 months. Nonetheless, these pharmacological therapies are being used for considerably longer periods in clinical practice if residual neuropathic symptoms or pain persist.

Theoretically, there is a rationale for using ALA and other nutraceuticals in combination with analgesic drugs to enhance efficacy and synergistically target the underlying neuropathy, but there are no RCTs to support this strategy.

Benfotiamine

Thiamine (vitamin B1) is a water-soluble vitamin that constitutes an essential cofactor of several enzymes involved in carbohydrate metabolism. Benfotiamine, a lipid-soluble allithiamine homolog, is a synthetic S-acyl derivative (prodrug) of thiamine and has been shown to inhibit the formation of AGEs (106). The BENDIP (Benfotiamine in Diabetic Polyneuropathy) study showed that neuropathic symptoms, with Neuropathy Symptom Score as the primary endpoint, were improved after 6 weeks of treatment using a benfotiamine dose of 300 mg twice daily but not 300 mg daily (102), while the smaller and shorter BEDIP (Benfotiamine in the Treatment of Diabetic Polyneuropathy: a Three-Week Randomized, Controlled Pilot Study) study found improvement in a score combining neuropathic symptoms and signs after 3 weeks of treatment with benfotiamine 400 mg daily (102). The incidence of adverse events did not differ between active and placebo treatment.

Benfotiamine is approved and recommended by guidelines (101) as pharmacotherapy for treatment of DPN in several countries, but not in the United States or Canada. Similar to ALA, the primary indication for benfotiamine is symptomatic DPN, including not only neuropathic pain but also nonpainful symptoms. However, the number of available RCTs is lower and their durations have been shorter than for ALA.

Based on the results of the BENDIP study (102), the appropriate dose of benfotiamine over the first 6

TABLE 2 Randomized Controlled Trials Using Nutraceuticals as Monotherapy for the Treatment of Diabetic Peripheral Neuropathy

Nutraceutical	Study	T1D, *n*/ T2D, *n*	Dose*	Duration	Effects	Adverse Events
α-Lipoic acid (ALA)	ALADIN (108)†	0/328	100/600/1,200/ placebo	3 weeks IV	Symptoms + Signs + QoL +	None
	ALADIN II (108)†	65‡	600/1,200/ placebo	2 years PO	NCS +	None
	ALADIN III (108)†	0/508	600 IV/1,800 PO/ placebo	3 weeks IV/ 6 months PO	Symptoms (+)/− Signs +/(+)	None
	ORPIL (108)†	0/24	1,800/placebo	3 weeks PO	Symptoms + QoL (+) Signs +	None
	SYDNEY (108)†	30/90	600/placebo	3 weeks IV	Symptoms + NSC + Signs +	None
	SYDNEY 2 (108)†	30/151	600/1,200/1,800/ placebo	5 weeks PO	Symptoms + NSC + Signs +	Dose-dependent GI symptoms
	NATHAN 1 (108)†	110/344	600/placebo	4 years PO	Signs + NCS −	SAEs slightly increased§
	El-Nahas et al. (109)	0/200	1,200/placebo	6 months PO	Symptoms + VPT +	Mild nausea
Benfotiamine	BENDIP (102)†	16/117	300/600/placebo	6 weeks	Symptoms + (PP) Signs −	None
	BEDIP (102)†	8/32	400/placebo	3 weeks	Symptoms/signs + Pain +	None
Vitamin B12	Didangelos et al. (112)	0/90	1/placebo	1 year	Symptoms + Signs − Pain + VPT + NCS +	None
Vitamin D	Karonova et al. (114)	0/67	40,000 IU/ 5,000 IU per week	24 weeks	Pain + Symptoms + Signs +	None
Vitamin E	VENUS (116)	300‡	400/placebo	1 year	Symptoms − Lancinating pain (+)	None
Acetyl-L-carnitine	Sima et al. (118)	1,257‡	3,000/placebo	1 year	Pain + VPT + NCS −	None
γ-Linolenic acid (GLA)	Keen et al. (119)	57/51	480/placebo	1 year	NCS + Signs +	None
	Won et al. (120)	0/100	320 GLA/ 600 ALA	12 weeks	Symptoms, pain noninferior‖	None
Magnesium	de Leeuw et al. (122)	110/0	300/ no supplement	5 years	DPN stage +¶ NCS +	GI symptoms

**Doses are mg/day except for Vitamin D, which is IU/week. †Summarized in review article. ‡Diabetes type not available. §Incidence: 38% (ALA) versus 28% (placebo), including cardiovascular and cerebrovascular disorders, infections, inflicted injuries, and fractures; deaths: 1.3% (ALA) versus 2.7% (placebo). ‖For GLA versus ALA. ¶Versus no supplement. + Indicates improvement compared to placebo. (+) Indicates trend toward improvement compared to placebo. − Indicates no difference compared to placebo. ALADIN, Alpha-Lipoic Acid in Diabetic Neuropathy; BEDIP, Benfotiamine in the Treatment of Diabetic Polyneuropathy: a Three-Week Randomized, Controlled Pilot Study; BENDIP, Benfotiamine in Diabetic Polyneuropathy; DPN, diabetic peripheral neuropathy; GI, gastrointestinal; IV, intravenous; NATHAN, Neurological Assessment of Thioctic Acid in Diabetic Neuropathy; NSC, nerve conduction studies; ORPIL, Oral Pilot; PO, oral administration; PP, per protocol analysis; QoL, quality of life; SAEs, severe adverse events; SYDNEY, Symptomatic Diabetic Neuropathy; T1D, type 1 diabetes; T2D, type 2 diabetes; VENUS, Vitamin E in Neuroprotection Study; VPT, vibration perception threshold.*

weeks is 300 mg twice daily. Whether this dose should be maintained during long-term treatment is currently being examined by the BOND study (110) assessing the effects of 1-year treatment with benfotiamine 300 mg twice daily on morphometric, neurophysiological, and clinical measures in individuals with type 2 diabetes and symptomatic DPN.

Vitamin B12

Vitamin B12 deficiency can have hematological or neurological consequences, including polyneuropathies (1). Because of the increased risk of a vitamin B12 deficiency associated with metformin treatment, the ADA recommends periodic measurement of vitamin B12 levels for metformin-treated patients (111). A recent 12-month RCT assessed the effects of oral vitamin B12 supplementation with 1,000 µg per day in metformin-treated people with type 2 diabetes and DPN who had low vitamin B12 levels (<400 pmol/L). Oral vitamin B12 treatment improved neurophysiological measures, pain score, sudomotor function, and quality of life, but not MNSI score (112). Vitamin B12 deficiency should be supplemented with oral vitamin B12 1,000 µg daily. The duration of supplementation depends on the cause and may be lifelong (e.g., in pernicious anemia or after bariatric surgery). Treatment of DPN with vitamin B12 in the absence of vitamin B12 deficiency is not indicated.

Vitamin D

Obesity, prediabetes, and type 2 diabetes constitute important risk factors for vitamin D deficiency. There is also accumulating evidence suggesting a link between low systemic vitamin D levels and DPN (113). In a randomized, open-label study in participants with type 2 diabetes and DPN, the majority of whom were vitamin D deficient, improvements in neuropathic symptoms and deficits were observed after 24 weeks of high-dose vitamin D treatment (40,000 IU/week) compared to a control group supplemented with vitamin D 5,000 IU/week (114). Thus, further studies are needed to define the exact role of vitamin D supplementation specifically in vitamin D–deficient people with DPN.

In general, vitamin D deficiency <50 nmol/L (20 ng/mL) is associated with fractures and bone loss. Severe vitamin D deficiency <30 nmol/L (12 ng/mL) dramatically increases the risk of excess mortality, infections, and many other diseases and should be avoided whenever possible (115). However, there is no international consensus on the optimal level for vitamin D supplementation, and recommendations range from 400 to 2,000 IU daily (115).

Vitamin E

Vitamin E is the most abundant liposoluble antioxidant, comprising eight fat-soluble compounds (four tocopherols and four tocotrienols) protecting cell membranes from oxidative stress. In a large clinical trial, vitamin E (200 mg of mixed tocotrienols twice daily) did not improve neuropathic symptoms over 1 year in people with DPN. However, in post hoc subgroup analyses, tocotrienols reduced lancinating pain among people with A1C levels >8% and normohomocysteinemia after 1 year (116). Based on these data, vitamin E cannot be recommended for DPN treatment.

Acetyl-L-carnitine

In humans, the metabolic pool of carnitine comprises nonesterified levo-carnitine (L-carnitine) and acyl carnitine esters, among which the amino acid acetyl-L-carnitine represents the greatest component. A Cochrane review analyzed four studies in participants with DPN (117). Although some favorable effects on pain and vibration perception threshold were reported (118), the evidence was rated as being of low certainty as to whether acetyl-L-carnitine causes a reduction in pain after 6 to 12 months of treatment in people with DPN (117).

Polyunsaturated Fatty Acids

An early RCT reported favorable effects of treatment with the PUFA γ-linolenic acid (GLA) for 1 year on multiple neurophysiological and clinical parameters in individuals with DPN (119). In a recent 12-week, multicenter, noninferiority RCT trial comparing the efficacy of GLA (320 mg/day) and ALA (600 mg/day) in participants with type 2 diabetes and painful DPN, both neuropathic symptoms and pain improved after 12 weeks, and GLA was noninferior to ALA in reducing pain intensity (120). Further studies are required before PUFAs can be recommended for DPN treatment.

Magnesium

Magnesium is the second most abundant intracellular divalent cation and is involved in several hundred metabolic reactions, in which it mainly serves as a cofactor and plays an important role in carbohydrate metabolism and cellular bioenergetics. Magnesium deficiency (serum levels <0.75 mmol/L) can lead to an enhanced neuromuscular excitability, including symptoms such as nervousness or cramps of both smooth and skeletal muscle (121).

Reduced magnesium intake and systemic magnesium levels are associated with both prediabetes and diabetes (121). However, there no evidence from placebo-controlled RCTs assessing the efficacy of

magnesium supplementation in DPN. In an open, low-quality RCT, individuals with type 1 diabetes, with or without DPN, were supplemented with magnesium (Group A), while another group did not receive magnesium (Group B). Magnesium in red blood cells increased to normal levels in Group A but remained low in Group B. After 5 years, DPN stages improved more often in Group A than in Group B (39 vs. 8%, respectively) and worsened more often in Group B than in Group A (12 vs. 61%, respectively) (122).

Magnesium supplementation has been recommended for people with diabetes and hypomagnesemia if other dietary approaches fail to balance magnesium status (121). Oral magnesium supplementation is safe in adults when used in dosages below the upper intake level of 350 mg daily, but because of its renal secretion, magnesium should be used with caution in individuals with kidney disease (123).

In summary, given that the efficacy of both causal therapies for DPN and symptomatic treatments for neuropathic pain is limited, there is an unmet need for adjunctive therapies. Experimental studies have indicated that diabetic neuropathy can be prevented or ameliorated by various nutraceuticals in animal models by interfering with the pathophysiology of the underlying condition. Some of these findings could be translated successfully into the clinical arena and confirmed in clinical trials of DPN.

The efficacy and safety of several nutraceuticals, including ALA, benfotiamine, vitamin B12, acetyl-L-carnitine, vitamin D, vitamin E, and the PUFA GLA have been studied in RCTs, some better designed than others. For clinical use, ALA and benfotiamine are licensed as drugs and approved for the treatment of DPN in several countries worldwide; however, they have not been approved for this use in the United States or Canada. ALA has the best evidence as therapy for symptom relief, highlighted by several meta-analyses. People with proven deficiencies in vitamins B and D and magnesium should receive supplementation to prevent worsening of DPN and other disorders.

The advantage of nutraceuticals is their excellent safety profile, but longer, well-designed, well-conducted confirmatory RCTs should be performed to establish the value of their use in DPN over the long term. Overall, nutraceuticals have the potential to favorably modify the natural history of DPN, and there is hope that, ultimately, they will contribute to expanding our therapeutic armamentarium against this common, debilitating, and potentially even life-threatening complication of diabetes.

KEY POINTS

- **Because the efficacy of both causal therapies for DPN and symptomatic treatments for neuropathic pain is limited, there is an unmet need for a holistic approach considering pathogenetically oriented adjunctive therapies.**
- **Based on evidence for efficacy in reduction of symptoms and excellent safety from RCTs, ALA and benfotiamine (currently approved in some countries, although not FDA-approved in the United States) may be added for the management of persistent neuropathic symptoms, including pain in DPN.**
- **Confirmatory RCTs should clarify the value of using nutraceuticals in DPN over the long term.**

NONPHARMACOLOGICAL APPROACHES TO DPN AND PAIN MANAGEMENT

The DCCT (124) showed in 1993 that tight glucose control reduces the risk of developing DPN in type 1 diabetes by >60%. The same has not proved true in type 2 diabetes, and, 30 years after the DCCT, no medication has been convincingly shown to retard the incidence of DPN or slow its progression (1). In this setting, nonpharmacological approaches that might alter the natural history of DPN or ameliorate neuropathic pain have received increasingly sophisticated evaluation. Treatments reviewed here fall broadly into three categories: health behavior interventions (HBIs), including exercise, dietary counseling, and their combination (Table 3); passive modalities, including massage and biofeedback; and nonpharmacological energy or nerve stimulation treatments.

Health Behavior Interventions

The DPP (Diabetes Prevention Program) and similar large, prospective RCTs demonstrated that a curriculum-based behavioral treatment combining dietary and exercise counseling delays the onset of type 2 diabetes (125,126). Physical activity has been shown to delay the onset and progression of DPN in individuals with type 2 diabetes or prediabetic metabolic syndrome, even in the absence of significant weight loss or improved glucose control (127). It is no surprise, then, that the most recent ADA position statement on the treatment of DPN (1) recommends lifestyle modifications that include supervised aerobic and/or resistance training, alone or in combination with dietary modifications based on those used in the DPP trial or based on a predominantly plant-based

TABLE 3 Lifestyle Interventions for Diabetic Peripheral Neuropathy

Intervention Type	Absolute Intensity	Intensity	Frequency/Duration	Modes
Aerobic exercise	Moderate (3.0–5.9 METs)/vigorous (≥6.0 METs) physical activity*	On a relative intensity scale of 0–10: • Moderate: 5 or 6 • Vigorous: 7 or 8	• 3–7 days/week • 150 min/week with no more than 2 consecutive days off	Brisk walking, running, cycling, swimming, or dancing
	High-intensity interval training (maximum effort over short time)	Maximum effort	Unknown	Running or cycling
	Light-intensity physical activity (1.6–2.9 METs)*	<5 on a relative intensity scale of 0–10	• Daily, multiple times throughout the day • Avoid being sedentary for >1 hour at a time except when sleeping	Slow walking, cooking, or light household chores
Resistance or strengthening exercise	Vigorous	6–8 repetitions of a weight that can be lifted ≤6–8 times	• 2–3 sessions on nonconsecutive days/week • 3–6 exercises of major muscle groups per session	Weight machines, whole body vibration, free weights, elastic bands, or body weight
	Moderate	15 repetitions of a weight that can be lifted ≤15 times		
Balance exercise			• 2–3 days/week	Tai Chi, single leg balance, or obstacle course
Anti-sedentary behavior				Wearable devices, coaching, or goal-setting
Diet modification				Calorie restriction, processed carbohydrate restriction, and emphasis on polyunsaturated fats and antioxidant foods

**MET, or metabolic equivalent, refers to the energy expenditure required to carry out a specific activity, with 1 MET equal to the rate of energy expenditure while sitting at rest.*

diet, for the treatment of DPN. The components of these programs are considered below.

Physical Activity and Exercise

Physical activity is defined as any movement that increases energy use. Exercise, a more structured form of physical activity, should be regarded as medical therapy and concurrently inhibits multiple established pathways in the pathogenesis of DPN.

Animal models of neuropathy in diabetes and prediabetic metabolic syndrome demonstrate that sustained exercise reduces hyperglycemia and consequent excess oxidative and nitrosative stress; improves mitochondrial bioenergetics in both the nerve cell body and the distal axon; enhances microvascular vasoreactivity and reduces nerve ischemia; increases axonal transport; opposes the inflammatory effects of obesity, lipotoxicity, and hyperlipidemia; and enhances nerve regeneration after metabolic injury. Broadly, outcomes in human DPN exercise trials can be placed in three categories: neuropathy progression (e.g., exam, nerve conduction studies, and cutaneous nerve fiber density); mobility, balance, and gait; and neuropathic pain and quality of life.

Neuropathy Progression Outcomes

High-quality evidence is lacking on the specific effects of exercise on objective measures of neuropathy progression in individuals with DPN. Exercise is perhaps the only intervention shown to improve the regenerative capacity of small-diameter cutaneous sensory axons in people with metabolic syndrome and those with diabetes. Using change in intraepidermal nerve fiber density from a 3-mm skin biopsy, either alone or obtained before and 1–3 months after experimental capsaicin axotomy, sustained mentored exercise has been shown to increase nerve fiber density and regenerative capacity in people with metabolic syndrome or early type 2 diabetes (128). Participants who show improvement in the greatest number of metabolic syndrome features demonstrate the greatest improvement in reinnervation rate.

Mobility, Balance, and Gait Outcomes

Exercise convincingly improves measures of mobility, balance, and gait and reduces fall risk in DPN (129). These improvements have been demonstrated with different modes of exercise training, including aerobic, resistance, balance, Tai Chi, and whole body vibration, as

well as combinations of these modalities (130). Generally, training improves function specific to the training discipline (e.g., therapy focused on balance improves measures of balance). However, aerobic training has also been shown to improve mobility, balance, and gait outcomes in DPN. In a controlled trial of multimodal aerobic training, moderate-intensity (50% heart rate reserve) or vigorous (75% heart rate reserve) exercise yielded equivalent benefits, suggesting that both training intensities promoted these improvements (131). Current exercise intensity and frequency recommendations for people with DPN mirror the U.S. Department of Health and Human Services' physical activity guidelines for Americans (132), although lower-intensity activity has also shown benefit and may be especially attractive for individuals unaccustomed to regular exercise.

Neuropathic Pain and Quality-of-Life Outcomes

Reduction of neuropathic pain is perhaps the most important outcome of interest to individuals with DPN. Randomized trials of scheduled aerobic exercise using modes such as cycling, treadmill, and progressive walking programs have reported significant improvement in pain scale scores, pain interference measures, and/or quality-of-life metrics over intervention periods ranging from 12 weeks to 4 years (133,134).

Less-intensive exercise modes may also show benefit in neuropathic pain. Passive whole body vibration is a new exercise mode in which participants stand on a vibrating platform and resist its effect to maintain an upright posture. It has been shown in small, sham-controlled, randomized trials to significantly improve neuropathic symptoms, pain, and health-related quality of life (135). Similarly, a Tai Chi regimen significantly improved DPN total symptom score, pain, and quality of life over the 12-week intervention, albeit with a 34% attrition rate (136).

Exercise Prescription and Safety

The potential benefits of exercise among people with DPN are increasingly clear. Clinically, a formal exercise prescription can stress the therapeutic value of exercise, encourage increased activity, and regulate progression to allow tissue adaptation without causing injury. Exercise prescriptions for individuals with DPN should include information on exercise type, mode, goal intensity, frequency, and duration (Table 3). Participation in weight-bearing exercise in individuals without severe foot deformity or peripheral vascular disease has been found to be safe. Individuals with DPN should seek medical clearance for cardiovascular risk before starting a formal exercise program.

Complications of exercise, including joint and muscle pain, hypoglycemia, orthostasis associated with autonomic dysfunction, and skin irritation, occur in more than one-third of mentored exercise participants with DPN and should be expected (137). Ongoing monitoring of neuropathic symptoms, glucose, heart rate, and blood pressure, as well as musculoskeletal and integumentary status, is recommended. Supervision by a physical therapist or exercise specialist is ideal to regulate progression and surveil for injury risks.

Reducing Sedentary Behaviors

Simply reducing sedentary behaviors (those that do not increase energy expenditure beyond the resting level) is another strategy that might improve DPN outcomes. The average awake sedentary time of adults >50 years of age is 8.3 hours (>500 minutes) daily. Restricted contraction of postural support muscles alters lipid metabolism and increases free fatty acid and adipokine release. Prolonged sitting also worsens insulin resistance.

Well-designed studies investigating the impact of reduced sedentary time on DPN outcomes are lacking, but epidemiological evidence linking inactivity to cardiovascular risk and poor health outcomes, independent of time spent in aerobic exercise, makes anti-sedentary behavioral modification an alluring future research direction (138). Wearable devices such as fitness trackers and smartphone applications, as well as research-grade accelerometers, make accurate measurement of sedentary behavior possible to provide feedback and facilitate goal-setting.

Dietary Counseling and Modifications

Dietary counseling as an isolated intervention for DPN has received only sparse investigation, but eating pattern modifications may prove effective as part of a more comprehensive lifestyle treatment regimen. Components of a healthy eating pattern ameliorate insulin resistance, improve glucose control, and promote anti-inflammatory effects in individuals with type 2 diabetes (139). Key components of a healthy eating pattern in the setting of DPN include calorie restriction, processed carbohydrate restriction, and emphasis on polyunsaturated fats and antioxidant foods.

Obesity, and especially abdominal adiposity, generate a potent pro-inflammatory state. Pro-inflammatory cytokines and free fatty acids released from enlarged adipocytes are neurotoxic to axons. Calorie restriction was more important than exercise for weight loss in the DPP and similar curriculum-driven HBI trials (125,126). Lipid metabolites and chronic cellular hyperglycemia activate pro-inflammatory cellular injury response pathways and contribute to oxidative stress, which inhibits mitochondrial function in distal axons.

Concentrated carbohydrates should be avoided, while emphasizing dietary sources of antioxidants such as green, leafy vegetables, berries, citrus fruits, and salmon. These dietary precepts are embodied in the low-fat and low-sugar eating pattern outlined in the DPP curriculum (125) and in predominantly plant-based diets such as the Mediterranean eating pattern (45% carbohydrate, 35–40% fat, and <10% of saturated fat) (139).

Passive Modalities

Thai massage (140) and other passive physical treatments and alternative medicine modalities have been reported in prospective studies to provide pain reduction or symptom improvement in DPN, but without the size and/or rigor of trial design or trial confirmation necessary to adequately evaluate their efficacy. These modalities include neuro-biofeedback (141), foot bath techniques (142), and static magnetic fields (143). None have been shown to alter the natural history of DPN progression.

Energy or Nerve Stimulation Treatments

In addition to HBIs and passive modalities, other nonpharmacological approaches to painful DPN that have received prospective evaluation include decompressive surgery and various forms of electrical modulation of nerve or other tissues, including transcutaneous electrical nerve stimulation (TENS), frequency-modulated electromagnetic stimulation, transcranial magnetic stimulation (TMS), and the previously mentioned SCS. These modalities have been extensively reviewed in practice guidelines, structured reviews (144), and detailed reports from the Agency for Healthcare Research and Quality (136). None of these treatments has been clearly shown to prevent or delay onset of DPN symptoms or to alter the natural history of DPN.

Electromagnetic Stimulation Modalities

In DPN, spontaneous ephaptic transmission from metabolically injured peripheral sensory afferent fibers generates sensation of neuropathic pain that often develops a chronic quality through central spinal sensitization. Various modes of intermittent electrical stimulation have been trialed to interrupt pain sensation. TENS and similar cutaneous nerve stimulation modes offer a competing sensory experience, whereas TMS and SCS modalities interrupt central processing of peripheral afferents. Although trial evidence for all of these modes is considered weak, centrally acting modes (i.e., TMS and SCS) appear more efficacious than peripherally acting modes. Several trials of electromagnetic stimulation have included change in exam or other measures of neuropathy severity as secondary or exploratory endpoints, but none has convincingly demonstrated neuropathy improvement with these therapies.

Spinal Cord Stimulation

SCS, in which stimulating electrodes are surgically implanted in the epidural space, is extensively used for pain reduction in failed back surgery syndrome and has been compared in clinical trials against best medical care (but not sham procedures) for treatment of painful DPN (145). These trials found large effect sizes favoring pain reduction with SCS over a 6-month follow-up period. Modest pain reduction appears durable. In longitudinal follow-up from a multicenter RCT of SCS, about one-third of recipients reported at least a 50% reduction in pain compared to baseline at 60 months (146).

Small, single-arm trials and RCTs of high-frequency (e.g., 10 kHz) SCS have reported 60–80% reduction in pain compared to best medical management, but also subjective improvements in sensory function. The strongest evidence for 10-kHz SCS comes from a recent multicenter RCT that randomized 216 patients with painful DPN who had not experienced improvement with at least one gabapentinoid and had a VAS score >50 mm to medical management alone or with 10-kHz SCS (100,147). Of the 104 participants assigned to SCS, 98 responded to temporary stimulation; 90 were implanted; 5 experienced implantation-related adverse events, with 2 requiring explants; and 74 reported a 50% reduction in baseline VAS pain at 6 months and 72 participants at 12 months, compared to 5 of 95 participants randomized to medical management alone. Control participants were allowed to cross over to 10-kHz SCS after 6 months of follow-up and showed similar significant improvement in pain measures as participants randomized to SCS at baseline. Quality-of-life measures improved significantly after 10-kHz SCS. Reported improvements in foot sensation on standardized exam for SCS participants suggest improved neurological function, but also raise concern about possible examiner and participant bias in a study that was not blinded or sham-controlled.

Risks for perioperative and long-term complications limit the appeal of SCS. Surgical and long-term complications of SCS specific to DPN have not been reported except in the context of clinical trials. However, a meta-analysis of 32 peer-reviewed longitudinal studies examining SCS for other indications found a complication rate of 21%, with chronic lead migration or infection requiring surgical revision or removal in 10% of recipients (148).

Transcranial Magnetic Stimulation

TMS using deep cortical stimulation has been reported in a 5-day, sham-controlled, crossover trial to provide significant short-term reduction in perceived neuropathic pain in people with DPN, with a return to baseline pain over 3 weeks (149).

Transcutaneous Electrical Nerve Stimulation

TENS in various forms, including via dermal pads, as an adjunct to amitriptyline, with electrode stockings, via percutaneous needles, and as frequency-modulated or pulsed stimulation (150) have been compared to sham treatment in short-duration randomized or crossover trials of up to 225 participants. A report by the Agency for Healthcare Research and Quality (136) and other structured reviews of these therapies concluded that TENS treatment was not clearly superior to sham for either pain or quality of life, with more rigorous and longer-duration trials yielding less apparent benefit (150).

Systematic Surgical Decompression

Systematic surgical decompression, in which individuals undergo surgery at multiple nerve sites in the leg that are considered common sites for compressive injury (e.g., the tibial nerve at tarsal tunnel and peroneal nerve at fibular head), has been reported prospectively in a small uncontrolled series (11 individuals with DPN) and a single-limb trial using the opposite leg as a control (42 individuals) (151). These studies reported improvement in neuropathic pain measures, with large effect sizes in the surgical subject or limb. However, poor design, treatment bias, lack of sham controls, lack of follow-up data on perioperative or long-term complications of surgery, and lack of confirmation from other trials substantially limit the ability to interpret these results.

Acupuncture

A 10-week RCT in 45 individuals with DPN and neuropathic pain compared acupuncture at traditional meridian-based sites to sham needling, using a VAS for neuropathic pain and the 36-Item Short-Form Health Survey as a measure of quality of life. It found only nonsignificant trends toward improvement in both measures over the treatment period (152).

Photon Stimulation

Photon stimulation describes a group of therapies in which pulsed infrared light or near-infrared laser energy is applied transcutaneously to neuropathy-affected skin with the goal of increasing blood flow and cellular and mitochondrial metabolism. Small, short-duration, sham-controlled, single-blind RCTs of the use of these technologies in DPN have shown a nonsignificant trend toward improved pain and quality-of-life measures (153).

In summary, clinical trial evidence for the efficacy of nonpharmacological therapies in DPN remains rudimentary. No energy or physical treatment modality has demonstrated the ability to sustainably alter the natural history of DPN. SCS and high-frequency SCS provide long-lasting reduction in neuropathic pain in people with DPN pain refractory to medical therapy and may be recommended in these situations. This potential benefit must be balanced against serious wound, infection, and equipment complications that affect 8–22% of people with SCS implants. Among modalities considered to be primary pain therapy, TMS consistently provides pain reduction, but there is no evidence that this improvement can be sustained. Other energy or physical treatment modalities have low-quality trial evidence and/or have not shown consistent neuropathic pain improvement and are not recommended.

HBIs that include a combination of regular aerobic, strengthening, and balance exercise; reduction of sedentary behavior; and dietary modification aimed at reducing calorie intake and increasing plant-based foods and polyunsaturated fats are recommended for every person with DPN. Intervention should be tailored to each patient's preferences and degree of physical conditioning to optimize adherence. Provision of exercise prescriptions and supervision by an exercise specialist to assess patients' baseline fitness and risk factors, regulate progression, and provide active encouragement can improve exercise behaviors and reduce the risk for exercise-associated injury, especially in people naive to exercise or with physical disabilities that increase their risk for falls.

KEY POINTS

- **Regular aerobic, strengthening, and balance exercise, alone or in combination; reduction of sedentary behavior; and dietary modification aimed at reducing calorie intake and increasing plant-based foods and polyunsaturated fats have all demonstrated positive outcomes for individuals with DPN.**
- **Exercise participation, guided by exercise prescription and supervision by an exercise specialist, is safe and can improve exercise behaviors and reduce the risk for exercise-associated injury.**
- **SCS and high-frequency SCS provide long-lasting reduction in neuropathic pain in people with DPN pain refractory to medical therapy but must be balanced against serious potential complications in this population.**

SUMMARY AND CONCLUSION

Our main objective in writing this monograph was to provide up-to-date information regarding painful DPN, including novel mechanisms and risk factors contributing to the contemporary prevalence trends for this serious and common complication of diabetes. Additionally, we sought to offer clear guidance to the greater diabetes care community on the best approaches for screening and diagnosis of DPN in individuals with type 1 diabetes, type 2 diabetes, or prediabetes, as well as on a broad spectrum of management strategies, with the ultimate goal of ensuring access to optimal, evidence-based DPN management for all people with this condition.

The symptoms associated with DPN are dependent on the type of fibers most affected initially, although some individuals with DPN may be completely asymptomatic and thus may first present with advanced complications such as foot ulcers. The small fibers that convey pain and dysesthesias are particularly vulnerable to the energy-starved environment of diabetes, which explains why small fibers are the earliest fibers to undergo injury secondary to diabetes. Thus, burning pain and dysesthesias are frequently the first symptoms of DPN. Both type 1 and type 2 diabetes increase the risk of developing painful DPN. Women, members of some racial/ethnic minority groups, and individuals with type 2 diabetes appear to be at greater risk for developing DPN pain.

Periodic, flexible assessment for DPN risk factors and symptoms and their trajectories over time should be part of standard care for all people with diabetes. Although earlier diagnosis of DPN could enable targeted treatment to prevent progression of the disease and other adverse outcomes, it remains underdiagnosed in many individuals with diabetes today. Thus, DPN assessment, including a detailed history and at least two sensation and reflex tests, should be performed annually starting at diagnosis of type 2 diabetes and 5 years after diagnosis of type 1 diabetes, including in young people. Electrophysiological testing is rarely needed for people with typical signs and symptoms of DPN. A complex differential is recommended, as nondiabetic neuropathies may coexist in individuals with diabetes and may be treatable. Additionally, more recent evidence from large cohorts has revealed that socioeconomic factors and health care accessibility are risk factors for DPN. Therefore, clinicians are encouraged to incorporate comprehensive assessment of the impacts of social and psychological determinants of health on DPN and to address these impacts as part of overall DPN management.

Because there are no FDA-approved, pathogenetically oriented pharmacological treatments to reverse DPN, current treatment approaches target prevention of DPN and relief of its symptoms. Prevention and management decisions should be individualized and might include intensively controlling blood glucose, as well as targeting other risk factors such as dyslipidemia, obesity, hypertension, and smoking, with both lifestyle and pharmacological interventions.

We have provided here a targeted DPN management strategy that includes the currently available oral (e.g., anticonvulsants and tricyclic antidepressants) and topical pharmacological agents with evidence of clinically meaningful pain reduction, as well as HBIs specifically for painful DPN (Figure 8). Dosing regimens should take into account individuals' age and comorbidities, and efforts should be made to use the lowest effective dose for a given agent alone or in combination to mitigate side effects, while avoiding opioid therapies entirely. Importantly, none of these agents should be regarded as lifetime medications, and periodic review of pharmacological therapy is recommended, with gradual reduction of drug treatment in response to symptom abatement over time. Particular care is required in the management of painful DPN in elderly patients, in whom polypharmacy is common and the risk of adverse effects is amplified.

Given their excellent safety profile, many nutraceuticals have been developed, some with proven benefit for relief of DPN pain symptoms, although the potential of these products to favorably modify the natural history of DPN remains to be proven in well-designed, well-conducted confirmatory RCTs. Several nonpharmacological approaches also might alter the natural history of DPN or ameliorate neuropathic pain. These include HBIs such as exercise, dietary modification, or their combination; passive modalities such as massage and biofeedback; and nonpharmacological energy or nerve stimulation treatments. These, too, deserve more study.

In conclusion, we encourage all clinicians who treat people with diabetes to strive for early recognition of DPN using the algorithms and tools we have described here and to bring to bear the requisite thoughtful clinical evaluation and implementation of a multilevel management strategy to ensure that all individuals with painful DPN benefit from optimal personalized care.

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Duality of Interest

R.P.-B. has served on advisory boards for Averitas Pharma, Boehringer Ingelheim, Novo Nordisk, and Nevro.

D.Z. has served as a consultant to Allergan, Bayer, Berlin-Chemie, Biogen, Cannaxan, Clexio, Grünenthal, Mitsubishi Tanabe, Mundipharma, Nevro, Novaremed, Novartis, Pathways Public Health, Pfizer, Procter & Gamble, Stada, Takeda, Viatris, and Wörwag; he has been a speaker for Astellas, AstraZeneca, Berlin-Chemie, Mundipharma, Pfizer, Sanofi, Takeda, Viatris, and Wörwag; and he has received research support from Mitsubishi Tanabe, Novartis, and Wörwag.

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Author Contributions

Lead author R.P.-B. reviewed all content, wrote the introduction and conclusion, and co-wrote with L.A. the section titled "Screening and Diagnosing DPN." A.J.M.B. wrote the section titled "Treating Painful DPN." E.L.F. wrote the section titled "Pathophysiology of DPN." R.L.M. and J.R.S. co-wrote the section titled "Nonpharmacological Approaches to DPN and Pain Management." K.M.-S. wrote the section titled "Social Determinants of Health and Their Impact on DPN." D.Z. wrote the section titled "Role of Neutraceuticals in DPN and Pain Management." All authors reviewed and edited the manuscript and approved the final version for publication. R.P.-B. is the guarantor of this work.

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FORUM REVIEW ARTICLE

Nox, Nox, Are You There? The Role of NADPH Oxidases in the Peripheral Nervous System

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Abstract

Significance: Reactive oxygen species (ROS) contribute to multiple aspects of peripheral nervous system (PNS) biology ranging from physiological processes (*e.g.*, axonal outgrowth and regeneration) to pathophysiology (*e.g.*, nerve degeneration). Although ROS are derived from multiple sources, NADPH oxidase (Nox) family members are dedicated to ROS generation. Noxs are expressed in the PNS, and their overexpression is associated with detrimental effects on nerve function and contributes, at least in part, to peripheral neuropathies.
Recent Advances: Of the seven members, studies mostly focused on Nox1, Nox2, and Nox4, which are expressed in the PNS in a cell-specific manner. We have also recently identified human Nox5 in sural nerve biopsies. When maintained at homeostatic levels, Noxs regulate several aspects of peripheral nerve health, most notably neurite outgrowth and axonal regeneration following nerve lesion. While Nox2 and Nox4 dysregulation is a major source of oxidative stress in PNS disorders, including neuropathic pain and diabetic peripheral neuropathy, recent evidence also implicates Nox1 and Nox5.
Critical Issues: Although there is compelling evidence for a direct role of Noxs on nerve function, little is known about their subcellular localization, intercellular regulation, and interaction. These, together with redox signaling, are considered crucial components of nerve redox status. In addition, the lack of isoform-specific inhibitors limits conclusions about the physiological role of Noxs in the PNS and their therapeutic potential in peripheral neuropathies.
Future Directions: Future research using isoform-specific genetic and pharmacological approaches are therefore needed to better understand the significance of Nox enzymes in PNS (patho) physiology. *Antioxid. Redox Signal.* 37, 613–630.

Keywords: NADPH oxidases (Nox), neuron, neuropathy, peripheral nervous system (PNS), reactive oxygen species, Schwann cells

Introduction

THE PERIPHERAL NERVOUS system (PNS) refers to the portion of the nervous system that lies outside the central nervous system (CNS) and serves as a connecting point between the CNS and peripheral tissues. It consists of a complex network of cranial and spinal nerves, composed of neurons, their axons, and supporting Schwann cells. Afferent sensory neurons and their associated axons transmit information from sensory receptors in the PNS back to the CNS, whereas motor efferent neurons and their axonal extensions transmit information from the CNS to the muscles and glands (38). This back-and-forth communication between the PNS and the CNS is pivotal for the physiological regulation of the internal system as well as the interactions with the external environment.

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The need to rapidly carry nerve impulses over long distances of up to 2 m or more in length poses a unique challenge to the PNS and makes it highly susceptible to metabolic, mechanical, toxic, and immune insults (141). When injured, the PNS exhibits abnormalities in nerve structure and function that disrupt the ability of the CNS to communicate *via* the PNS with effector organs and muscles.

Peripheral neuropathies are a heterogeneous group of disorders that occur secondary to peripheral nerve damage, leading to reduced quality of life (43). They affect more than 8% of the general population, and this number rises to 15% in patients 40 years or older (46). Peripheral neuropathies are often associated with axon degeneration, impaired regeneration, as well as disruptions in calcium (Ca^{2+}) signaling, electrophysiological function, mitochondrial function, and substrate utilization (38, 135, 141). Redox signaling modulates many of these processes, and accumulating clinical and preclinical evidence has shown increased nerve reactive oxygen species (ROS) following peripheral nerve dysfunction. However, untargeted antioxidant therapies to treat PNS disorders have only exhibited limited therapeutic potential in the clinical setting (83, 110).

In addition to the incorrect selection of antioxidant dosages and treatment durations, these failures are mainly attributed to the lack of antioxidant specificity against the ROS source(s) altered in a disease- and tissue-specific manner. Untargeted antioxidant treatment can result in off-target effects or lead to global suppression of ROS-producing enzymes, including those required for normal physiology (133). Therefore, it is critical to abandon the previous conventional approach that ''blindly'' targets all antioxidant activity. The new and needed way forward is to identify specific ROS sources that are altered during disease course, so that targeted antioxidant therapies can be developed to treat PNS diseases.

The major sources of ROS in peripheral nerves include mitochondria, xanthine oxidase, and NADPH oxidases (Noxs) (Fig. 1) (38, 124, 143). Among these sources, the Nox family of enzymes consists of seven members (Nox1–5 and dual oxidases [Duox] 1 and 2) specialized for ROS production (103). Although the role of Noxs in the PNS is not completely understood, evidence implicates Nox family members in cellular functions ranging from the immune response and neuronal development to pathophysiological involvement and neurodegeneration (35, 73, 96, 143). In this review, we first provide a general overview of ROS and antioxidant generation in the PNS. We then focus on Nox expression in the PNS, their physiological roles, how Nox-derived ROS contribute to PNS disorders, and the novel concepts of Nox signaling, which may be relevant to nerve redox homeostasis and PNS function.

ROS and Antioxidants: General Overview

ROS are a family of oxygen containing molecules resulting from cellular metabolism, which can avidly react with biomolecules, including nitric oxide, proteins, lipids, carbohydrates, and DNA (119). Because of their ability to modify biomolecules, ROS generation was initially considered

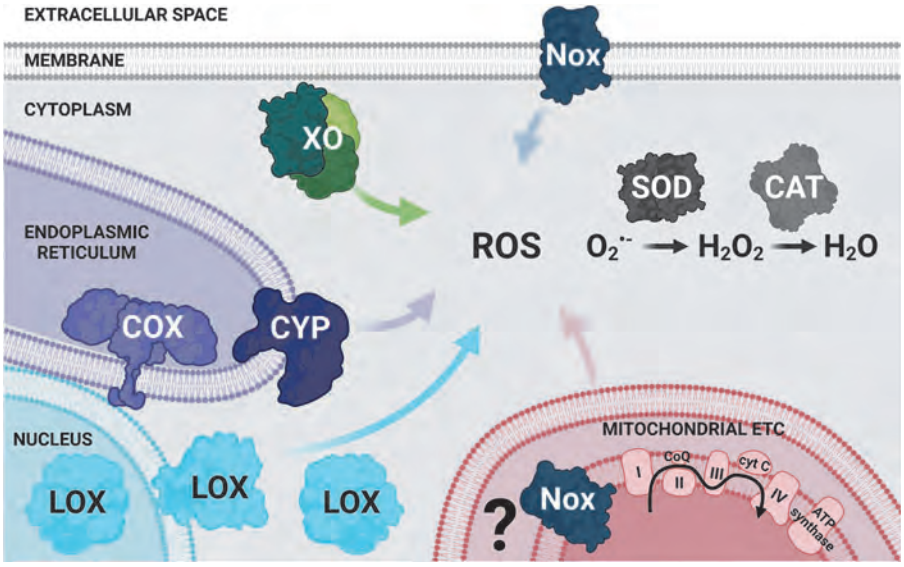

FIG. 1. ROS sources and metabolism in the PNS. The mitochondrial ETC is considered a major source of ROS in the PNS and can inadvertently lead to $O_2^{-\bullet}$ generation under physiological conditions (38). Other sources that generate ROS as a by-product of metabolism include XO located in the cytoplasm (116, 127), CYP and COX (128) that reside in the ER, as well as LOX (147) found in the nuclear and cytosolic regions and membranes (not shown in the figure for simplicity) (124). Noxs are a specialized source of ROS (120). They localize to the plasma membrane and intracellular compartments, including the mitochondria (shown in the figure with a question mark that highlights a possible localization to the inner mitochondrial space), the ER, and the nuclear envelope (not shown in the figure for simplicity). Under physiological conditions, $O_2^{-\bullet}$ generated by these ROS-producing enzymes is rapidly converted to the more stable and easily diffusible H_2O_2 by SOD. H_2O_2 is then detoxified by CAT to form H_2O (124). CAT, catalase; COX, cyclooxygenases; CYP, cytochrome P450 monooxygenases; ER, endoplasmic reticulum; ETC, electron transport chain; H_2O, water; H_2O_2, hydrogen peroxide; LOX, lipoxygenases; Nox, NADPH oxidase; $O_2^{-\bullet}$, superoxide anion; PNS, peripheral nervous system; ROS, reactive oxygen species; SOD, superoxide dismutase; XO, xanthine oxidase. Created with BioRender.com.

cytotoxic and believed to occur only under pathological conditions. However, it is now well established that homeostatic ROS levels are crucial for cellular physiology and regulate processes such as growth, apoptosis, signal transduction, cellular respiration, and host defense.

In the nervous system, ROS production is involved in blood pressure regulation, cognitive function, tissue repair, and immune response (103). However, when overproduced, these highly reactive molecules become deleterious and can promote cell damage in disease states. The increased understanding of both the beneficial and harmful effects of ROS is critical to the design of rationale, mechanism-based therapies for PNS disorders.

ROS are classified into two groups: radical and nonradical species. Radical ROS include superoxide anion ($O_2^{-\bullet}$), hydroxyl radical ($^{\bullet}OH$), and nitrogen-based species such as the nitric oxide radical ($NO^{\bullet}$), whereas nonradical ROS include hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and peroxynitrite ($ONOO^-$) (119). Many ROS types are involved in redox signaling, defined as the reversible redox modifications exerted by ROS to a specific biomolecule (14). As an example, $O_2^{-\bullet}$ exerts its effects at the site of generation and is rapidly converted to the more stable and easily diffusible H_2O_2; both $O_2^{-\bullet}$ and H_2O_2 are considered major ROS involved in redox signaling through iron/sulfur cluster modification and cysteine oxidation.

More potent oxidants such as $^{\bullet}OH$, exert irreversible redox reactions (14). In peripheral nerves, redox signaling is implicated in physiological processes, including axonal outgrowth and regeneration, as well as pathophysiology, including pain processing and nerve degeneration (35, 72, 143).

Intracellular ROS levels in the PNS are maintained in check *via* antioxidant defense mechanisms, which consist of enzymes and nonenzymatic scavengers. Enzymes are mainly under the control of the transcription factor NF-E2-related factor 2 (Nrf2) and include superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (71, 88, 137). On the contrary, nonenzymatic scavengers are primarily of dietary origins and include α-tocopherol (vitamin E), β-carotene, and ascorbate (vitamin C).

Neurons and Schwann cells can initially increase Nrf2-dependent antioxidant signaling in the face of cellular stressors at early disease stages before progression to irreversible damage (53, 137). Interestingly, we have shown that Schwann cells have a high basal antioxidant potential under physiological conditions, which is further increased during metabolic stress. This feature is thought to confer resistance to oxidative damage relative to the more vulnerable neurons (137).

An imbalance between ROS generation and the ability of the antioxidant defense mechanisms to clear excess ROS, or effectively repair the resulting damage, is deleterious and is associated with irreversible oxidative modifications to macromolecules as well as impaired redox signaling. These processes eventually lead to uncontrolled ROS generation commonly referred to as oxidative stress (119), an effect that has been implicated in nerve degeneration and PNS disorders.

Sources That Generate ROS As a By-Product of Metabolism in the PNS

Multiple cellular sources generate ROS in the PNS as a by-product of oxidative phosphorylation and metabolism. The mitochondrial electron transport chain is perhaps the most studied ROS source in the PNS, which can generate $O_2^{-\bullet}$ as a result of an electron leak leading to a 1-electron reduction from oxygen to $O_2^{-\bullet}$, instead of water (2).

Compared with other cell types, mitochondria make up half of the cytoplasmic volume of high energy consuming peripheral neurons, which require up to 4.7 billion adenosine triphosphate (ATP) molecules per second under normal physiologic conditions (153) to maintain their membrane potential across a large surface area (115). Because of this high mitochondrial content, the electron transport chain was long thought to be the major ROS source that sustains the oxidative potential in the PNS under physiological conditions besides being a substantial ROS source in PNS disorders (3, 16). Interestingly, Nox enzymes, mainly Nox4 and Nox5, might localize to mitochondria, turning the mitochondrial electron transport chain into both an oxidation target and an ROS source (10, 81, 93).

In addition to the mitochondrial electron transport chain, multiple enzymes in the PNS can produce ROS as by-products of their catalytic activities (Fig. 1). These include xanthine oxidase, located in the cytoplasm (116, 127), cytochrome P450 monooxygenases (unpublished data), and cyclooxygenases (128), residing in the endoplasmic reticulum (ER), as well as lipoxygenases (147), found in the nuclear and cytosolic regions and membranes (124). Studies have mostly evaluated the function of these ROS-generating enzymes in PNS pathophysiology and their role in normal PNS physiology is an avenue warranting further investigation.

NADPH Oxidases of the Nox Family As a Dedicated Source of ROS

In contrast to the other ROS-generating systems, NADPH oxidases of the Nox family (Nox) are transmembrane proteins with no known metabolic function, except catalyzing ROS generation across biological membranes (120). This occurs *via* electron transfer from NADPH as an electron donor. Two NADPH molecules transfer two electrons to flavin adenine dinucleotide, which passes the electrons to two iron-containing heme groups. The electrons are then transferred to oxygen as an electron acceptor, thereby yielding $O_2^{-\bullet}$ largely thought to be the major product of the electron transfer (Fig. 2). In most mammals, the Nox enzyme family consists of seven members: Nox1 through 5 and Duox 1 and 2. Interestingly, the Nox5 isoform is only expressed in higher mammals and is absent in rats and mice, which limits our understanding of its physiological and pathophysiological significance in humans (39).

The prototype NADPH oxidase, Nox2, originally discovered in neutrophils, is one of the best-characterized members of the Nox family and is critical for innate immunity (25). It consists of two transmembrane catalytic subunits ($gp91^{phox}$ [commonly referred to as Nox2], and the regulatory subunit $p22^{phox}$), three cytosolic subunits ($p47^{phox}$, $p67^{phox}$, and $p40^{phox}$), and a small Rho GTP-binding protein (Rac1 or Rac2). These subunits are disassociated in the inactive state, but assemble upon enzyme stimulation to produce $O_2^{-\bullet}$ (112).

While other Nox members share a certain extent of structural homology with Nox2, the mechanisms by which they are activated may vary; for example, similar to Nox2,

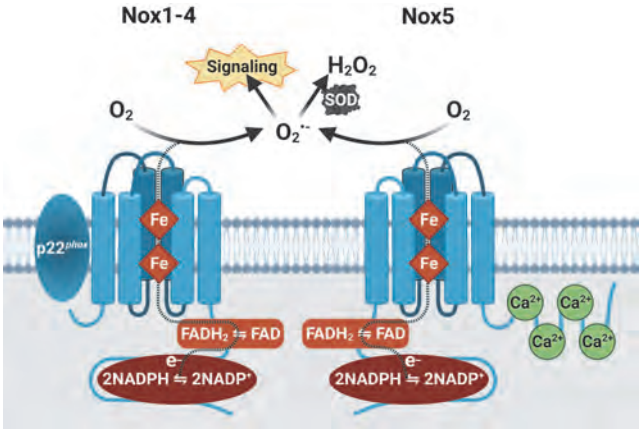

FIG. 2. NADPH oxidase family of enzymes as a dedicated source of ROS. Nox enzymes are a family of transmembrane proteins. They bind within their C-terminus, NADPH, which acts as the electron (e^-) donor. Two NADPH molecules transfer two e^- to FAD, also bound within the C-terminus, which passes the e^- to two Fe-containing heme groups. Finally, the e^- are transferred to O_2, the terminal acceptor, catalyzing the transformation to $O_2^{-\bullet}$, although Nox4 and Nox5 release H_2O_2. Nox1–4 share a certain extent of structural homology and all require interaction with the regulatory subunit $p22^{phox}$ to produce ROS. Nox5 does not require $p22^{phox}$, but is Ca^{2+}-dependent and thus contains N-terminal EF-hand domains with four Ca^{2+}-binding sites (103). Ca^{2+}, calcium; FAD, flavin adenine dinucleotide; Fe, iron; O_2, molecular oxygen. Created with BioRender.com.

Nox1–4 full activation also requires interaction with $p22^{phox}$ to produce ROS (112). Nox1 through 3 enzymes require cytosolic subunits ($p47^{phox}$ and $p67^{phox}$, or homologues) to form a fully functional enzyme complex (112). However, unlike the other isoforms, Nox4 is constitutively active and does not require any cytosolic subunits for full activation (92). Nox5 and Duox enzymes contain N-terminal EF-hand domains with four binding sites for Ca^{2+}, which is required for enzyme activation (103).

Under physiological conditions, Noxs are maintained at a relatively low level of constitutive activity to regulate redox signaling in the vicinity of target molecules. While Nox-dependent redox signaling mainly regulates cell differentiation, proliferation, and apoptosis, some Nox isoforms exert cell-specific functions. For example, as mentioned above, phagocyte Nox2 is heavily involved in the innate immune response, while Nox3 is highly expressed in the inner ear and plays a role in otoconia biogenesis (84).

With respect to the ROS type, Nox isoforms generally produce $O_2^{-\bullet}$ as their primary product. However, data have demonstrated that Nox4, Duox1, and Duox2 generate H_2O_2 (26, 105). Evidence also shows that H_2O_2 can be detected following Nox5 activation (20, 118). While the biochemical mechanism underlying H_2O_2 generation is unknown, a rapid conversion of $O_2^{-\bullet}$ into H_2O_2 before release from the enzyme has been suggested as a contributing factor. It has been further demonstrated that Nox4-dependent H_2O_2 generation relies on a histidine (His-222) residue, localized in the extracellular loop of the enzyme (105, 126), a mechanism that requires validation in the other isoforms.

In the following sections, we review Nox cellular distribution, as well as their physiological and pathophysiological involvement in the PNS. Because the roles of Nox3 and Duox1-2 in the PNS are unclear, we focus this review on Nox1, Nox2, Nox4, and Nox5.

Nox Expression in the PNS

Nox subcellular localization

Nox enzymes reside both at the plasma membrane and in intracellular compartments, including the ER, mitochondria, and the nuclear envelope (103). Under resting conditions, Nox2 is closely associated with $p22^{phox}$ and together primarily localize to intracellular and plasma membranes, while cytosolic subunits are typically located in the cytoplasm (102). Upon cell activation, the cytosolic subunits translocate to membrane-bound Nox2 to form a fully functional enzyme complex (102). Nox4 on the contrary is expressed intracellularly and can be found in the ER (146), the nuclear envelope (22), and the mitochondria (10). While both Nox1 and Nox5 have been identified in the plasma membrane (49, 130), reports have also detected Nox5 at several intracellular sites, including the perinuclear area, the mitochondria, and the ER (130).

Because of their high reactivity, this diverse subcellular localization of Noxs has several consequences, including how Nox isoforms influence redox signaling as well as how Nox isoforms function under normal and disease states. For example, Nox2 assembly and activation on the plasma membrane of neutrophils are essential for ROS generation and pathogen elimination at the injury site (36). A recent report identified mitochondrial Nox4 as an energetic sensor, whose activity is directly regulated by ATP in renal cells (117). These results suggest a close connection between ATP turnover and Nox4 redox signaling and could be of relevance to the PNS.

With respect to the PNS, axons are intimately associated with Schwann cells, and growing evidence highlights the importance of the Schwann cell/axon cross talk in peripheral nerve metabolic support (11). This in turn raises the possibility that ROS release from axons into the extracellular space may directly influence the surrounding Schwann cells and *vice versa*. Specifically, Nox4-derived oxidative stress in sensory dorsal root ganglion neurons is accompanied by Schwann cell injury and dysmyelination in a mouse model of neuropathic pain (72). While the underlying mechanism is unknown, one can speculate that Nox4-derived ROS in axons could damage Schwann cells in a paracrine manner, similar to what has been observed in the vasculature (12, 13, 98). This novel aspect of Nox signaling is discussed in detail below.

Nox cellular distribution in the PNS

Relative to the better characterized CNS (103), the cellular distribution of Nox enzymes in the PNS has not been comprehensively analyzed (Table 1). Data, however, demonstrate that Nox isoforms can be expressed simultaneously at multiple PNS sites (28, 73). In addition, it is generally thought that Nox expression in the PNS is maintained at low basal levels under physiological conditions, and upregulated in disease states (103).

Table 1. Nox Cellular Distribution in the Peripheral Nervous System

PNS cellular localization	*Nox isoforms*	*Accessory proteins*	*References*
Dorsal root ganglion neurons	Nox1, Nox2, and Nox4	$p22^{phox}$, $p47^{phox}$, and Rac1	(17, 59, 72, 111, 136)
Schwann cells	Nox1 and Nox4	Not identified	(28, 35)
Macrophages	Nox2 and Nox4	$p22^{phox}$ and $p47^{phox}$	(9, 28, 70)

Nox, NADPH oxidase; PNS, peripheral nervous system.

Dorsal root ganglion neurons express Nox1, Nox2, and Nox4 (59, 72, 136). Studies have also reported the expression of Nox accessory proteins $p22^{phox}$, $p47^{phox}$, and Rac1 in rodent primary cultures of dorsal root ganglion neurons (17, 111, 136).

As we mentioned above, Nox subcellular localization in dorsal root ganglion neurons is a crucial determinant of the downstream signaling effects of intracellular *versus* extracellular Nox-derived ROS. While this is still an area requiring further research, multiple studies have used dihydroethidium (DHE) for detecting $O_2^{-\bullet}$ in neuronal cultures or whole peripheral nerve samples (35, 57), which suggests a role for Nox-derived ROS in intracellular redox signaling. Increasing studies are, however, assessing ROS release into the extracellular space using cytochrome *c* assay and Amplex Red (23, 72), which may reflect a role in cell-to-cell communication and in the propagation of ROS signals into neighboring cells.

Schwann cells mainly express Nox1 and Nox4 (28, 35), with no available reports on Nox2 protein expression.

As a whole, Nox subcellular localization in Schwann cells remains largely unexplored. Recent data, however, have shown that Nox1 can generate both intra- and extracellular oxidants in Schwann cells in a neuropathic pain model. The authors suggest that intracellular ROS maintain mechanical allodynia, while extracellular H_2O_2 promotes Nox2-expressing macrophage recruitment to the perineurial space (28). Simultaneous expression of Schwann cell Nox1 and macrophage Nox2 in the PNS leads to a sustained feed-forward loop of oxidative injury. These results suggest a potential interaction between different Nox isoforms to regulate redox signaling, a novel concept that is further discussed below.

Macrophages mostly express Nox2 (28, 70), which is low in the absence of a stimulus (77). Once activated, reports demonstrate the Nox2-mediated oxidative burst at the injury site in rodent models of PNS disorders (28, 70). Besides Nox2, Nox4 is upregulated in spinal cord macrophages at late stages of neuropathic pain and, together with Nox2, modulates macrophage polarization (9). Whether Nox2 and Nox4 are simultaneously coexpressed in PNS macrophages and the significance of this coexpression on peripheral nerve health are areas requiring additional studies.

Nox Signaling in the PNS Under Physiological Conditions

Under physiological conditions, Nox enzymes regulate redox signaling by generating low ROS levels, in a spatially confined manner, which induces conformational changes in the target molecule, in turn impacting its interactions and downstream function (14). Signaling proteins containing active-site and structural cysteine residues are perhaps the most susceptible targets for redox modifications and include kinases, phosphatases, ion channels, and transcription factors (108). Oxidation targets that may be especially important for peripheral nerve function include redox switches Nrf2 and NFκB (69, 82), as well as sensory neuron ion transient receptor potential (TRP) channels (40).

The direct contributions of Nox enzymes to these processes in the PNS remain unclear and are not discussed in this review. Instead, we consider examples of cellular functions of Nox enzymes in neurite outgrowth and axon regeneration (Fig. 3).

Neurite outgrowth

Redox signaling is implicated in the regulation of neurite outgrowth, a highly coordinated process that allows exact pathfinding for developing and regenerating neurons in response to environmental cues (150). In this context, an early study showed that exposing cultured neurons to nerve growth factor (NGF) induces neurite outgrowth in an ROS-dependent manner (125). More recent data revealed that Rac1, the Nox cytosolic subunit, and increased Nox activity are both required for neurite outgrowth *in vitro* (78).

When evaluating the contribution of specific Nox isoforms to this process, Ibi *et al.* found that Nox1-dependent ROS suppress neurite outgrowth (58). However, studies in *Aplysia* bag cell neurons showed that Nox2 and its cytosolic subunit $p40^{phox}$ localize in growth cones, dynamic structures composed of an actin and microtubule cytoskeleton located at the tip of elongating neurites, which allows outgrowth and guidance to the proper target.

The authors suggest that Nox2/$p40^{phox}$ may modulate neurite outgrowth by oxidizing the actin cytoskeleton and changing its polymerization state (101). Findings in cultured hippocampal neurons further demonstrated that Nox2-derived H_2O_2 can also oxidize ryanodine receptors (RyR) localized in the ER, releasing Ca^{2+}. This Ca^{2+}-dependent process regulates the different aspects of neurite outgrowth, including axonal development and polarization (144). Together, these data suggest that physiological Nox levels are required for neurite outgrowth during development (Fig. 3A). Moreover, Nox isoforms (*e.g.*, Nox1 *vs.* Nox2) may have differential effects on neurite outgrowth, an idea that requires further research.

Tissue repair and axonal regeneration

As we mentioned above, neurons and their long axonal processes have high energy demands with considerable ATP consumption. Neuron maintenance and repair require efficient signaling over long distances, which can be achieved through bidirectional protein and organelle transport between cell body and axons (114).

Indeed, lesions to peripheral nerves trigger well-orchestrated cellular and molecular events, including an inflammatory response at the injury site to induce axonal

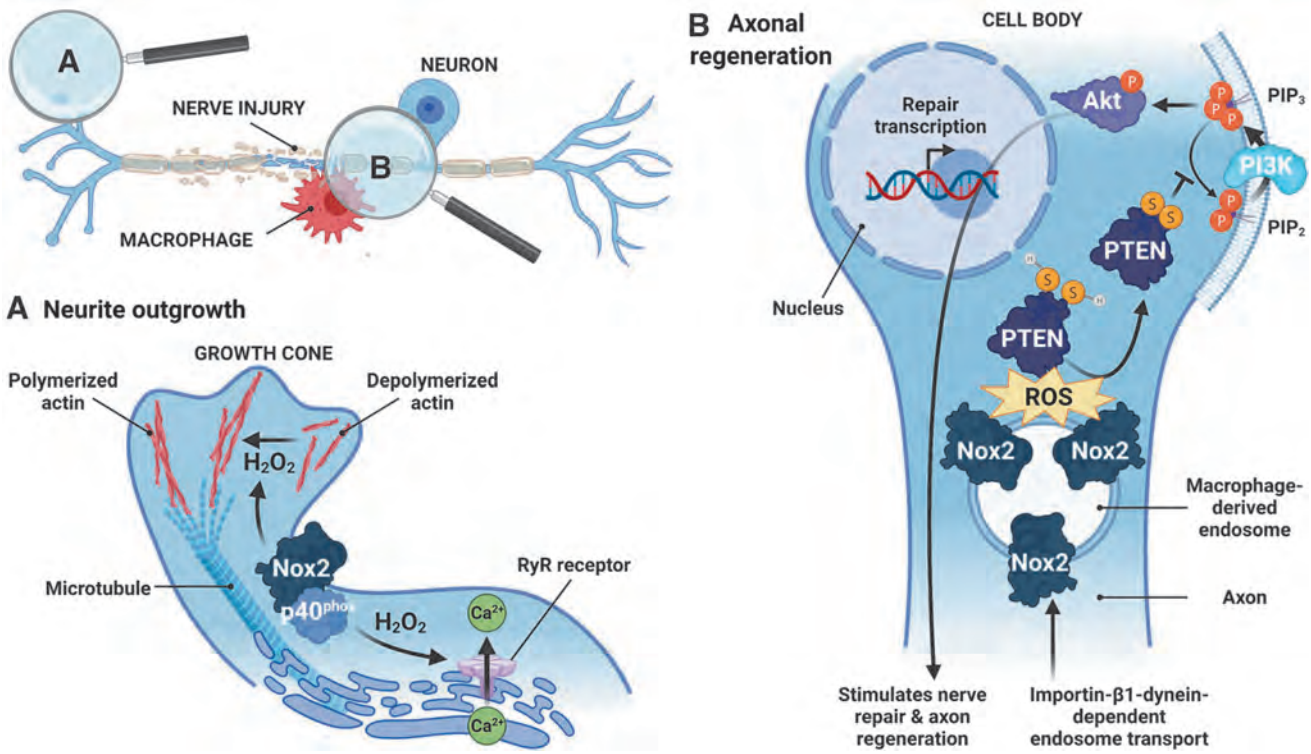

FIG. 3. NADPH oxidase signaling in the PNS under physiological conditions. Nox-derived ROS promote neurite outgrowth **(A)** and axon regeneration **(B)** under physiological conditions. **(A)** Nox2 and its cytosolic subunit $p40^{phox}$ localize in growth cones, dynamic structures composed of an actin and microtubule cytoskeleton located at the tip of elongating neurites, which allows outgrowth and guidance to the proper target. Nox2-derived H_2O_2 oxidizes the actin cytoskeleton, changing its polymerization state and favoring neurite outgrowth (101). Nox2-derived H_2O_2 can also oxidize RyR localized in the ER, releasing Ca^{2+}. This Ca^{2+}-dependent process regulates different aspects of neurite outgrowth, including axonal development and polarization (144). **(B)** Following peripheral nerve lesion, macrophages release exosomal Nox2, which is taken up by injured axons *via* endocytosis and retrogradely transported to the cell body in an importin-β1–dynein-dependent mechanism. Endosomal Nox2 oxidizes PTEN, triggering a disulfide bond (SS) formation, which inactivates PTEN. PTEN inactivation favors PIP_2 phosphorylation (P) to PIP_3 and PI3K-Akt pathway activation, promoting nerve repair and axon regeneration (50). PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol (3,4,5)-trisphosphate; PTEN, phosphatase and tensin homologue; RyR, ryanodine receptors. Created with BioRender.com.

regeneration (7). This inflammatory response favors a highly oxidizing environment, and accumulating evidence suggests that ROS are in turn essential for axonal regeneration and functional recovery after peripheral nerve injury (29, 50, 113). Earlier studies mainly examined axonal regeneration in *Drosophila* and zebrafish [reviewed in Terzi and Suter (129)], which consistently pointed to a key role of Duox-mediated ROS in axon reinnervation and wound healing (76, 104, 113).

More recently, studies in genetically modified mouse models revealed that macrophage-derived Nox2 promotes axon growth and regeneration in mouse dorsal root ganglion neurons (50). Hervera *et al.* found that exosomal Nox2 is taken up by injured axons *via* endocytosis and retrogradely transported to the cell body in endosomes in an importin-β1–dynein-dependent mechanism. Endosomal Nox2 oxidizes phosphatase and tensin homologue (PTEN), triggering a disulfide bond formation, which inactivates PTEN. PTEN inactivation favors phosphatidylinositol 4,5-bisphosphate (PIP_2) phosphorylation to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) and PI3K-Akt pathway activation, promoting nerve repair and axon regeneration (50).

The same team went on to show that *in vivo* activation of neuronal Nox2 promotes axonal regeneration and partial restoration of sensory nerve function after spinal cord injury (29).

Overall, these studies strongly support a role for Nox-dependent ROS as signaling molecules required for tissue regeneration after PNS injury (Fig. 3B). The idea of Nox shuttling from macrophages to damaged axons is a novel concept, which advocates a new way forward in our understanding of physiological redox signaling in the PNS (51). In addition, these results are particularly exciting in light of the growing interest in axo-glial metabolic communication (6) and require further investigation in *in vitro* and *in vivo* models of PNS disorders.

Nox Pathophysiological Involvement in PNS

Table 2 outlines the involvement of major Nox isoforms and their potential roles in PNS disorders detailed below.

Neuropathic pain

Neuropathic pain is a common and debilitating complication associated with a range of PNS disorders, including

Table 2. Nox Isoform Involvement in Peripheral Nervous System Disorders

PNS disorder	*Nox isoform(s) involved*	*Potential role(s)*	*References*
Neuropathic pain	(a) Nox1 (b) Nox2 (c) Nox4	(a) Macrophage infiltration; thermal and mechanical hyperalgesia (b) Central and peripheral immune regulation in pain sensitization after peripheral nerve injury (c) Peripheral pain processing, neuroinflammation, and dysmyelination	(28, 42, 59, 70, 72)
CIDP	Nox2	Unclear	(91)
CIPN	Nox4	Pain hypersensitivity; increased proinflammatory mediators	(96, 97)
DPN	(a) Nox2 (b) Nox4	(a) Pain processing and allodynia (b) Schwann cell injury; neurophysiological defects	(21, 35, 136)

CIDP, chronic inflammatory demyelinating polyneuropathy; CIPN, chemotherapy-induced peripheral neuropathy; DPN, diabetic peripheral neuropathy.

diabetic neuropathy, amputation, acute peripheral nerve injury, and chemotherapy-induced neuropathy (64). It presents as hypersensitivity with allodynia and hyperalgesia or continuous pain sensations (38). Current therapies only marginally provide relief due to the lack of understanding of pain processing (19).

Preclinical studies in animal models of neuropathic pain implicate oxidative stress as a prominent pathogenic factor in pain sensitization (73). Specifically, increasing evidence suggests that Nox enzymes contribute to pain processing (42, 70, 140), and the most studied isoforms are Nox1, Nox2, and Nox4 (Fig. 4). Nox2-dependent ROS are a key mediator of oxidative stress in pain sensitization after peripheral nerve injury (28, 70, 77, 86). Kallenborn-Gerhardt *et al.* also demonstrated that increased Nox2 signaling in infiltrating macrophages promotes dorsal root ganglion damage and neuropathic pain after peripheral nerve injury, an observation not present in Nox2-deficient mice (70). Besides its action on sensory neurons, Nox2 induction in infiltrating macrophages can also target Schwann cells, which in turn activate Nox1, as we discussed above (28).

In addition to its effect on peripheral immune cells, Nox2 can modulate neuropathic pain after peripheral nerve injury by its actions on microglia located in the dorsal horn of the spinal cord, a mechanism that requires Toll-like receptor 2 (TLR2)-dependent activation of the inflammatory response (77, 86). These findings suggest that Nox2-derived ROS in central and peripheral immune cells mediate, at least in part, neuropathic pain. This raises the possibility of inhibiting excess Nox2 activity as a meaningful therapeutic strategy. The caveat with this approach lies in the critical roles of Nox2-derived ROS in the innate immune response (133). In addition, we have discussed in a previous section the emerging role of Nox2 in axonal regeneration following PNS injury (50).

These results conflict with the findings that Nox2 mediates pain processing and suggest that the role of Nox2 may differ at different stages of peripheral nerve injury, which calls for caution in the use of Nox2 inhibitors as these may limit regeneration. Furthermore, genetic deletion of Nox2 functional subunits $p47^{phox-/-}$ and $gp91^{phox-/-}$ leads to arthritis, joint inflammation, and increased bone destruction (134). Thus, inhibiting Nox2 could be of limited therapeutic use for treating neuropathic pain due to its physiological roles in the immune response and axonal outgrowth (50).

Nox4 is also implicated in pain processing after peripheral nerve injury (42, 72, 140). Geis *et al.* found that Nox4 upregulation contributes to early neuropathic pain and increases proinflammatory cytokine release at the lesion site (42), worsening the pain sensation (121). While Nox4 genetic deletion prevented this acute neuropathic state, the authors found that pharmacological inhibition using the dual Nox1/4 inhibitor GKT136901 did not prevent pain in the later stages of neuropathy (42), suggesting that targeting Nox4 should occur early in the disease course.

Yet, another study showed that Nox4 upregulation in nociceptive primary afferent neurons maintains neuropathic

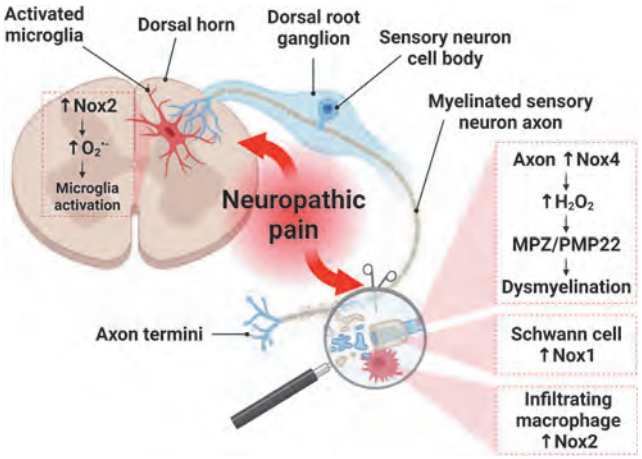

FIG. 4. Role of NADPH oxidases in pain processing. Nox2 induction in both central and peripheral immune cells contributes to neuropathic pain. In the dorsal horn of the spinal cord, Nox2 activation following peripheral nerve injury leads to $O_2^{-\bullet}$ generation, microglial activation, and pain hypersensitivity (77, 86). In addition, Nox2 upregulation in infiltrating macrophages promotes sensory neuron damage and neuropathic pain after peripheral nerve injury (70). Besides its action on sensory neurons, Nox2 induction in infiltrating macrophages can also target Schwann cells, which in turn activate Nox1 (28). This initiates a pro-oxidative feed-forward loop, leading to sustained macrophage infiltration to the damaged area, further exacerbating neuroinflammation and neuropathic pain (not shown in the figure). Nox4 is also involved in peripheral pain processing through H_2O_2 release in nociceptive primary afferent neurons, which is associated with neuropathic pain and dysmyelination, as evidenced by peripheral myelin protein MPZ and PMP22 degradation (72). Created with BioRender.com.

pain in both the subacute and late phases after peripheral nerve injury by mechanisms involving Schwann cell injury and dysmyelination, as evidenced by peripheral myelin protein MPZ and PMP22 degradation (72). This group went on to demonstrate, using mice with sensory neuron-specific Nox4 deletion, that the Ca^{2+}-binding protein S100A4 is an oxidation target of Nox4, which mediates hypersensitivity downstream of Nox4 (140).

These studies suggest that Nox4 signaling may vary during different stages of neuropathic pain. Future studies are needed to identify Nox4 signaling kinetics in neuropathic pain by direct comparisons across different clinically relevant animal models, on different genetic backgrounds, using pharmacological inhibitors and global and tissue-specific genetic manipulations (60) to identify potential therapeutic windows for pharmacological intervention.

In addition to the role of Nox1 in Schwann cells (28), Ibi *et al.* have demonstrated that Nox1-derived ROS mediate thermal and mechanical hyperalgesia in dorsal root ganglia using Nox1 knockout mice (59). Nox1-dependent pain processing is thought to involve the enhanced activity of the inflammatory sensor transient receptor potential vanilloid 1 (TRPV1) (59).

Chronic inflammatory demyelinating polyneuropathy

Chronic inflammatory demyelinating polyneuropathy (CIDP) is an autoimmune disease of the PNS (85), which typically presents as a slowly progressive and symmetric neuropathy, with impaired sensorimotor function (85). While immune therapies are generally effective, at least half of CIDP patients require prolonged treatment to prevent disease relapse (45). Identifying targetable pathways in CIDP is therefore needed to develop more effective therapies and to improve disease outcomes.

Numerous pathogenic mechanisms are implicated in human and mouse CIDP, including neuroinflammation, Schwann cell dysfunction, and oxidative stress (74). Because of its known role in neuroinflammation (122), it is perhaps not surprising that Nox2 activity is increased in granulocytes and monocytes of CIDP patients (91). Interestingly, treatment with intravenous immunoglobulin increases this activity compared with pretreatment values. The significance of this increased Nox2-mediated ROS following treatment remains unclear. However, another study found that Nox2 may play a role in combating neuroinflammation in multiple sclerosis (100). While a similar mechanism may occur in CIDP, future studies are required to validate this hypothesis in clinically relevant mouse models and determine the precise mechanisms downstream of Nox2.

Chemotherapy-induced peripheral neuropathy

Chemotherapy-induced peripheral neuropathy (CIPN), a disabling consequence of cancer therapies (109), is predominantly a sensory and painful neuropathy (89). Currently, there are no effective CIPN treatments beyond symptomatic relief, and a better understanding of disease pathogenesis is essential for developing much needed mechanism-based neuroprotective therapies.

One of the established mechanisms by which chemotherapeutic agents induce cancer cell apoptosis is *via* ROS generation (148). Yet, ROS production is not only restricted to the tumor environment, but can diffuse to the neighboring healthy cells and induce damage as well (79). Importantly, sensory dorsal root ganglion neurons and their axons, which are the first to be affected during CIPN, have low antioxidant potential and high mitochondrial content, and, unlike the CNS, lack a protective vascular barrier, rendering them more susceptible to oxidative damage (38, 137). While increased ROS levels and lipid peroxidation are present in the peripheral nerves of multiple experimental CIPN models (31, 33, 149), much less is known about the exact source of ROS in CIPN.

While some studies report ROS overproduction secondary to mitochondrial dysfunction (75, 94), there remains little direct evidence on the primary ROS source in the context of disease. With respect to Noxs, increased NADPH oxidase activity in the spinal cord of a rat model of CIPN results in neurotoxic peroxynitrite accumulation and CIPN development (32, 62). Miao *et al.* reported a role for Nox4 signaling in the dorsal root ganglion and the dorsal horn of the spinal cord during painful CIPN (96, 97). These findings suggest that Nox4 may be a primary source of ROS in CIPN. However, the lack of studies assessing changes in other Nox subunits, both in the CNS and PNS in the presence or absence of specific inhibitors, limits conclusions about the therapeutic potential of Noxs in CIPN.

Diabetic peripheral neuropathy

Diabetic neuropathy is a common and debilitating complication affecting more than 50% of all diabetic patients. While diabetes-induced nerve damage can present in multiple forms, the most common form is diabetic peripheral neuropathy (DPN), a length-dependent and symmetric peripheral nerve degeneration (37). There are no disease-modifying therapies for DPN beyond glycemic control, which often fails to slow or reverse progression, especially in prediabetes and type 2 diabetes (T2D) (15). It is therefore critical to identify specific pathogenic factors contributing to DPN development to develop targeted mechanism-based therapies.

Experimental and clinical data, including our own, have shown that oxidative stress is a major component of cellular and molecular injury in DPN (4, 35, 56, 136, 137, 154). Mechanisms of injury downstream of hyperglycemia converge and lead to oxidative stress. Moreover, high-fat diet (HFD)-fed mice, which develop robust peripheral neuropathy closely resembling human disease, have increased nerve oxidative stress (136). These findings indicate that other metabolic stressors, such as dyslipidemia, an independent DPN risk factor, may also induce ROS and contribute to DPN at early disease stages before progressing to overt T2D. However, untargeted antioxidant therapy, including our own clinical trial of allopurinol, α-lipoic acid, and nicotinamide (110), has only exhibited limited therapeutic potential (154).

Among the sources of ROS in the PNS, we have previously shown that Nox activity is increased in dorsal root ganglion neurons following multiple metabolic stressors, including hyperglycemia and hyperlipidemia (136, 138), implicating Nox enzymes in DPN pathogenesis. What is more important, however, is the specific Nox isoform altered in DPN-relevant cell types, which may underlie oxidative damage in DPN (summarized in Fig. 5).

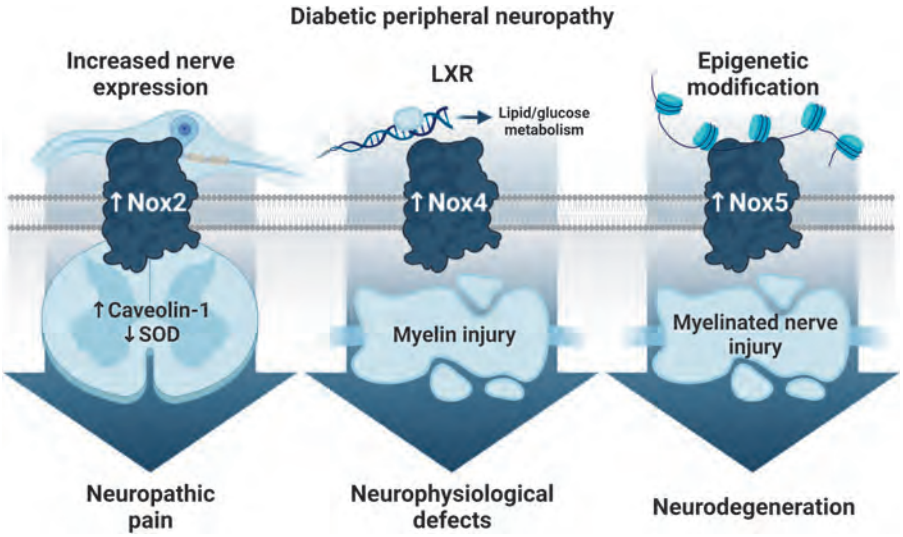

FIG. 5. Schematic summary of Nox-dependent mechanisms involved in diabetic peripheral neuropathy. Nox2, 4, and 5 are induced in peripheral nerves under diabetic conditions. Nox2 is activated in both dorsal root ganglion neurons and spinal cord. Increased spinal cord Nox2 is associated with reduced SOD activity and neuropathic pain (152). Nox2 is thought to mediate pain hypersensitivity by interacting with caveolin-1, a regulatory protein involved in lipid homeostasis (21). The LXR, a master regulator of lipid and glucose homeostasis, is inhibited in diabetes. This inhibition is accompanied by Nox4 induction in the sciatic nerves of type 1 diabetic mice and in cultured Schwann cells exposed to high glucose conditions. Nox4-derived ROS results in myelin injury and neurophysiological defects in hyperglycemia-induced peripheral neuropathy (35). The human Nox5 isoform is hypomethylated at the promoter region (47), which leads to increased Nox5 gene and protein expression in sural nerve biopsies of type 2 diabetic subjects with peripheral neuropathy (34). LXR, liver X receptor. Created with BioRender.com.

Studies show that Nox2 functional subunits $p47^{phox}$ and $gp91^{phox}$ are upregulated in the spinal cord of streptozotocin (STZ)-induced type 1 diabetic (T1D) rats, which develop neuropathic pain (152). These changes are associated with increased ROS generation and lipid peroxidation, and reduced SOD activity, ultimately resulting in tactile allodynia (152). Nox2-derived ROS also contributed to neuropathic pain in a T2D rat model rendered diabetic through an HFD and a single low STZ dose (21). Mechanistic analyses further showed that Nox2 induction in spinal cord microglia leads to chronic pain through a direct interaction with caveolin-1 (Cav-1), a regulatory protein involved in lipid homeostasis (21).

In line with these findings, we have previously shown that $p47^{phox}$ is increased in dorsal root ganglion neurons treated with oxidized low-density lipoproteins to mimic the dyslipidemic milieu in DPN (136). Taken together, these results provide supporting evidence for a role for Nox2 in the development of DPN and neuropathic pain, which may be conserved across diabetes type.

In addition to Nox2, we were the first to show that Nox4 is a major ROS source in peripheral nerves of STZ-induced T1D mice and that its pharmacological inhibition using GKT137831 prevents hyperglycemia-induced nerve dysfunction (35). Interestingly, we also showed that Nox4 mRNA levels were significantly increased in skin biopsies of T2D patients without clinical signs of DPN, an effect that was further enhanced in T2D patients with DPN (35). These clinical data are complemented by preclinical findings, which show increased Nox4-derived ROS in sciatic nerves from prediabetic HFD-fed mice even in the absence of hyperglycemia (unpublished data) and hyperglycemic T2D *db/db* mice (151).

Overall, these results support the hypothesis that Nox4-derived ROS are instrumental for human and murine DPN progression in prediabetes, type 1 diabetes, and T2D. Thus, in addition to hyperglycemia, additional studies exploring the link between Nox4 and different metabolic drivers of prediabetes, T2D, and DPN, such as dyslipidemia and insulin resistance, will be critical to validate Nox4 therapeutic efficacy in DPN.

Beyond Nox4, the human Nox5 isoform has emerged as a pathogenic factor in diabetic complications (30, 66). Particularly, data from humanized transgenic mice expressing Nox5 in different kidney cell populations have identified a role for Nox5 in promoting diabetic kidney disease, even in the absence of the Nox4 effect (66, 67). Consistent with these findings, our preliminary observations in sural nerve biopsies from T2D participants with DPN indicate that the human Nox5 promoter is hypomethylated (47), which promotes increased Nox5 gene and protein expression (34).

These data suggest that in addition to Nox2 and Nox4, Nox5-derived ROS may play a key role in human DPN development. We are currently examining the selective expression of Nox5 in Schwann cells and dorsal root ganglion neurons in transgenic mice to evaluate cell-specific Nox5 effects in the presence or absence of diabetes. These data will also allow us to evaluate the relative effects and the oxidation targets of Nox4 *versus* Nox5 in DPN.

Nox Inhibition As a Therapeutic Target for PNS Diseases

Given the emerging evidence implicating specific Nox isoforms as critical mediators of oxidative stress and nerve injury, Nox inhibition could be a promising therapeutic strategy to treat PNS diseases. Below we discuss these advantages.

Antioxidants

In the past two decades, untargeted antioxidant therapy aimed at neutralizing ROS overproduction was considered the only approach to reduce oxidative stress in the PNS. Although promising results were obtained using antioxidant therapy to treat PNS diseases in the experimental setting (18, 38), clinical trials, especially for DPN, were either of limited efficacy or were inconclusive (83, 110). This failure was mainly attributed to the lack of antioxidant specificity against the ROS source altered in a disease- and tissue-specific manner. In fact, it is thought that antioxidant supplementation could result in off-target effects or lead to global suppression of other ROS-generating enzymes or Nox isoforms, including those required for normal physiology (133).

Another limitation is related to the rapid oxidization by ROS, which leads to cellular damage even before initiation of antioxidant beneficial effects (103).

Nonspecific Nox inhibition

Diphenyleneiodonium (DPI) and apocynin are the two most commonly used nonspecific Nox inhibitors. Besides Nox inhibition, DPI inhibits other flavoproteins, such as xanthine oxidase and nitric oxide synthase (145). DPI as a therapy has been primarily studied in diabetic complications where it has cytoprotective effects in complication-prone tissues (61), including peripheral nerves (61, 68). However, its use *in vivo* is associated with insolubility and toxicity issues (133), rendering DPI a poor therapeutic option.

Apocynin may interfere with ROS detection by chemiluminescence and displays variable efficacy and potency (145). Unlike DPI, apocynin inhibition efficiency *in vitro* is low and very high concentrations are required for the antioxidant effect (52), which explains why it is more suitable and more commonly used *in vivo* (133, 145). Experimental evidence shows that apocynin alleviates neuropathic pain in the presence or absence of diabetes (48, 107). Apocynin treatment restored nerve conduction velocity and corrected blood flow and vascular conductance deficits in STZ-induced T1D rats (24). Although available safety data show low toxicity (123), to our knowledge, there are no studies addressing apocynin efficacy in patients with PNS disorders.

Specific Nox inhibition

A specific Nox inhibitor is essential to determine the therapeutic potential of targeting Noxs in PNS diseases. As opposed to global genetic deletion, administering a specific Nox inhibitor at a particular dose *in vivo* will be crucial for restoring Nox activity back to the homeostatic levels, rather than completely abolishing enzyme activity (134). Accordingly, recent high-throughput screening campaigns have effectively identified compound classes with enhanced selectivity against Nox enzymes, including the orally available, small-molecule Nox1/Nox4 allosteric inhibitors of the pyrazolopyridine chemical series: GKT136901 and its close analogue GKT137831. GKT compounds preferentially inhibit Nox1 and Nox4, and to a lesser extent Nox5 (1).

These dual Nox1/Nox4 inhibitors have gained considerable attention mainly because of their ability to prevent the development of diabetic complications, including DPN in the preclinical setting (30, 44). Specifically, we have shown that GKT137831 treatment improves nerve conduction velocity, sensorimotor deficits, and thermal sensitivity in neuropathic STZ-induced T1D mice, effects attributed to Nox4 inhibition (35). Experimental advances using GKT137831, particularly in the area of diabetic kidney disease, led to a randomized phase II trial in T2D participants with advanced diabetic kidney disease treated with a renin/angiotensin/aldosterone system inhibitor for 12 weeks (Genkyotex Innovation SAS; NCT02010242, GSN000200, completed).

The dual Nox1/Nox4 inhibitor had a favorable safety profile and improved several secondary outcome measures. Unfortunately, it did not effectively improve albuminuria, the primary outcome measure. Many reasons for drug failure were cited, such as inclusion of participants with very advanced kidney disease, short trial duration, low drug dose, as well as the heterogeneous nature of T2D-induced kidney disease relative to type 1 diabetes (133).

Accordingly, a new ongoing clinical trial addressing many of these concerns will shed light on the efficacy of GKT137831 in T1D patients with persistent albuminuria, using a longer treatment duration and a higher drug dose (27) (Genkyotex Innovation SAS; ACTRN12617001187336, UTN U1111-1187-2609). While DPN pathogenesis differs significantly between type 1 diabetes and T2D (15), experimental evidence suggests that Nox4 may be a viable therapeutic target for DPN conserved across diabetes type. It would therefore be interesting to evaluate the therapeutic efficacy of Nox4 inhibition using GKT137831 on DPN in type 1 diabetes, T2D, or preferably both.

In addition to DPN, the neuroprotective effects of the GKT compounds were tested in a rodent model of CIPN (97). GKT137831 improved mechanical and thermal sensitivity in CIPN rats, which was accompanied by reduced neuronal oxidative stress, proinflammatory cytokines, and increased Nrf2 signaling (97).

VAS2870 is a less specific pan-Nox inhibitor with a slight preference for Nox2 inhibition (5). While the therapeutic potential of VAS2870 is unknown for PNS dysfunction, reports show that VAS2870 treatment reduces neurodegeneration and improves neural function in a mouse model of acute ischemic stroke, an effect possibly mediated by Nox2 and/or Nox4 (80, 132). The therapeutic potential of VAS2870 is an area worthy of further investigation in PNS diseases, particularly in the context of neuropathic pain and CIDP, where Nox2 has emerged as a prominent pathogenic factor.

Novel Aspects of Nox Signaling: Potential Relevance to PNS Diseases?

Nox-derived ROS production in microparticles

Over the past decade, there has been a paradigm shift in PNS research, from focusing solely on neurons and their axonal extensions as an isolated system, to studying the interactions between axons and other nerve cell populations, namely Schwann cells and macrophages. Indeed, growing evidence points to the importance of Schwann cells and the Schwann cell/axon cross talk in axon viability and function (38, 135), such as through energy substrate transfer from Schwann cells to axons during periods of high energy demand and ROS scavenging by Schwann cells (6, 137).

Under conditions of metabolic dysfunction, however, studies show that Schwann cells can transfer lipotoxic species into the axon, promoting neurodegeneration (55, 135).

Interestingly, this shuttling activity can be mediated by Schwann cell-derived extracellular vesicles (EVs) under both normal and stress conditions (87, 142). EVs are a heterogenous group of membranous vesicles, released by all cells into body fluids or tissues (41). They mediate intercellular communication by transferring molecular cargo enriched with enzymes, nucleic acids, and metabolites to recipient cells (90). According to their biogenesis and size, EVs are commonly classified into three main types: exosomes (40–150 nm), originating from the endocytic pathway, microparticles (MPs; 100–1000 nm), derived from plasma membranes, and apoptotic bodies (50–5000 nm), released following apoptosis. EVs are implicated in neurodegeneration in disorders of the CNS [reviewed in Hill (54)].

In the PNS, most studies to date focus on the contribution of Schwann cell-derived exosomes to nerve regeneration after axonal injury and DPN through microRNA (miRNA), growth factor, and metabolite transfer (87, 142). Yet, the role of EVs in nerve redox signaling and the effect of oxidative stress on EV molecular cargo remain understudied.

Newly emerging ideas in the Nox field include the concept of intercellular ROS shuttling in MPs (98). The role of MPs has been generally studied in the context of vascular dysfunction, and increased MP levels positively correlate with adverse cardiovascular events and dyslipidemia (8, 106). Interestingly, increasing data implicate MPs in processes such as angiogenesis, vasorelaxation, inflammation, as well as oxidative stress (12, 95, 131), and further suggest that MPs may themselves be metabolically active and generate ROS (12).

Relevant to this review, multiple studies report that endothelium-derived MPs contain the Nox4 isoform as well as the regulatory subunit $p22^{phox}$ and produce Nox-dependent ROS (13, 63, 98). Secreted MPs can disrupt endothelial and vascular smooth muscle cell function in a paracrine/autocrine manner *via* a pro-oxidative feed-forward loop of injury, leading to apoptosis, inflammation, and impaired vascular tone (12, 13, 98).

Similar to the vasculature, it is tempting to speculate that disruption of the redox status between glial cells and axons, or the transfer of Nox-derived ROS from glia to axons *via* MPs, may contribute to nerve degeneration and PNS disorders (Fig. 6).

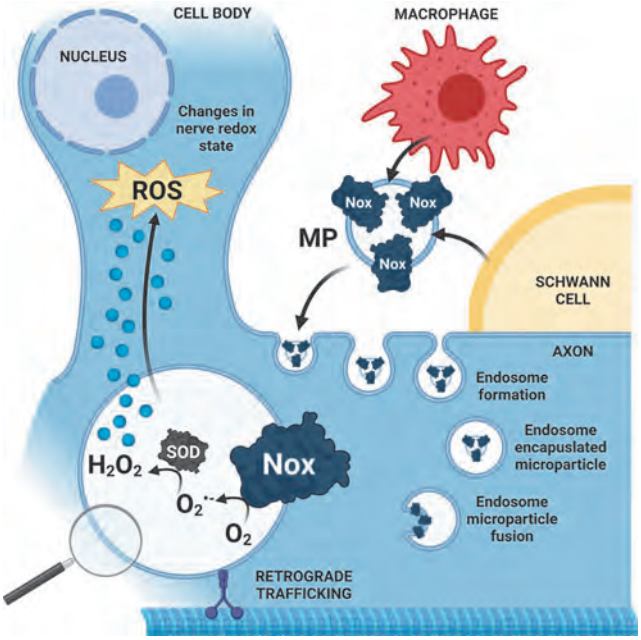

FIG. 6. Diagram summarizing our proposed mechanism of Nox-containing MPs in PNS diseases. We speculate that Nox-derived ROS are released from macrophages and/or Schwann cells *via* MPs, and engulfed by axons by endocytosis at the injury site. This ROS-producing MP transfer may disrupt the axo-glial redox state, contributing to nerve degeneration and PNS diseases. MP, microparticle. Created with BioRender.com.

Nox isoform interaction: is it biologically relevant?

As detailed above, PNS cell types simultaneously express several Nox isoforms, each exerting a distinct role (28). Emerging data suggest that these isoforms can interact and regulate ROS generation promoting sustained oxidative stress in disease states (98). Indeed, previous data reported the ability of Nox homologues such as Nox2 and Nox4 to dimerize (139). Furthermore, in cultured human endothelial cells with normal Nox2 and Nox4 expression, Nox5 knockdown is sufficient to abolish Nox-dependent ROS generation (99). In addition, this response is not accompanied by compensatory Nox2 or Nox4 increases, suggesting a potential regulatory effect of Nox5 on ROS generation.

More recently, Jha *et al.* reported similar results in transgenic models expressing human Nox5 in kidney mesangial cells, highlighting the ability of Nox5 to promote kidney disease progression, even in the absence of Nox4 upregulation (66). Perhaps more importantly, the same group showed that Nox5 may interact with Nox4 to regulate redox signaling in cultured renal cells exposed to metabolic stressors (65). While the precise mechanisms underlying these observations remain unclear, the findings do suggest, at least partly, an interdependent effect of Nox4 and Nox5 on the cellular oxidative response.

Thus, future studies are warranted to determine the relative Nox isoform expression and function in PNS cell types, their interactions, and their independent and interdependent effects on ROS levels. These findings will in turn be critical to understanding the cellular oxidative response and damage in PNS diseases.

Conclusion

In summary, oxidative stress research has evolved from the traditional view that ROS are exclusively harmful with focus on untargeted antioxidant therapies, to new advances centered on understanding the intricacies of redox signaling and the regulation of ROS sources in a cell- and disease-specific manner. The NADPH oxidase family, specialized for ROS generation, appears to be particularly important in the PNS for multiple cellular functions, ranging from neurite outgrowth and tissue repair to pathophysiological implications and neurodegeneration.

How specific Nox isoforms mediate peripheral nerve damage has fostered preclinical research examining the effect of Nox genetic and pharmacological manipulation on nerve function. While Nox inhibitors are currently being tested in the context of PNS diseases, these are not isoform

specific and may simultaneously inhibit several Nox isoforms. Thus, the development of isoform-specific Nox inhibitors with improved specificity is more promising for a better understanding of Nox biology in PNS health and disease.

The growing appreciation of the importance of axo-glial metabolic cross talk on nerve health is a recent area of interest among PNS researchers and may be of potential relevance to nerve redox status. Indeed, more research is needed in the area of Nox shuttling in MPs and how Nox isoforms interact to regulate nerve redox status and function in the axo-glial milieu. While much remains to be elucidated, increased experimental and clinical knowledge in the Nox field may facilitate the development of much needed mechanism-based therapies for the treatment of peripheral neuropathies.

Authors' Contributions

S.A.E. and E.L.F. contributed equally to the concept and design of the review. S.A.E. had final responsibility for the decision to submit for publication. S.A.E. completed the literature search, drafted, and, with E.L.F., finalized the review. M.G.S., A.A.E., and E.L.F. provided critical review of the article. M.G.S. created the figures, and, with S.A.E., conducted the literature search associated with the figures. All authors have reviewed and approved the article before submission. The review has been submitted solely to *Antioxidant & Redox Signaling* and is not published, in press, or submitted elsewhere.

Author Disclosure Statement

All authors declare no competing interests.

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Abbreviations Used

ATP = adenosine triphosphate
Ca^{2+} = calcium
CAT = catalase
CIDP = chronic inflammatory demyelinating polyneuropathy
CIPN = chemotherapy-induced peripheral neuropathy
CNS = central nervous system
COX = cyclooxygenases
CYP = cytochrome P450 monooxygenases
DPI = diphenyleneiodonium
DPN = diabetic peripheral neuropathy
Duox = dual oxidases
ER = endoplasmic reticulum
ETC = electron transport chain
EV = extracellular vesicle
FAD = flavin adenine dinucleotide
Fe = iron
H_2O = water
H_2O_2 = hydrogen peroxide
HFD = high-fat diet

Abbreviations Used (Cont.)

LOX = lipoxygenases
LXR = liver X receptor
MP = microparticle
Nox = NADPH oxidase
Nrf2 = NF-E2-related factor 2
O_2 = molecular oxygen
$O_2^{-\bullet}$ = superoxide anion
$^{\bullet}OH$ = hydroxyl radical
PIP_2 = phosphatidylinositol 4,5-bisphosphate
PIP_3 = phosphatidylinositol (3,4,5)-trisphosphate
PNS = peripheral nervous system
PTEN = phosphatase and tensin homologue
ROS = reactive oxygen species
RyR = ryanodine receptors
SOD = superoxide dismutase
STZ = streptozotocin
T1D = type 1 diabetic
T2D = type 2 diabetes
XO = xanthine oxidase

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FORUM REVIEW ARTICLE

A Role for Fatty Acids in Peripheral Neuropathy Associated with Type 2 Diabetes and Prediabetes

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Abstract

Significance: As the global prevalence of diabetes rises, diabetic complications are also increasing at an alarming rate. Peripheral neuropathy (PN) is the most prevalent complication of diabetes and prediabetes, and is characterized by progressive sensory loss resulting from nerve damage. While hyperglycemia is the major risk factor for PN in type 1 diabetes (T1D), the metabolic syndrome (MetS) underlies the onset and progression of PN in type 2 diabetes (T2D) and prediabetes.
Recent Advances: Recent reports show that dyslipidemia, a MetS component, is strongly associated with PN in T2D and prediabetes. Dyslipidemia is characterized by an abnormal plasma lipid profile with uncontrolled lipid levels, and both clinical and preclinical studies implicate a role for dietary fatty acids (FAs) in PN pathogenesis. Molecular studies further show that saturated and unsaturated FAs differentially regulate the nerve lipid profile and nerve function.
Critical Issues: We first review the properties of FAs and the neuroanatomy of the peripheral nervous system (PNS). Second, we discuss clinical and preclinical studies that implicate the involvement of FAs in PN. Third, we summarize the potential effects of FAs on nerve function and lipid metabolism within the peripheral nerves, sensory neurons, and Schwann cells.
Future Directions: Future directions will focus on identifying molecular pathways in T2D and prediabetes that are modulated by FAs in PN. Determining pathophysiological mechanisms that underlie the injurious effects of saturated FAs and beneficial properties of unsaturated FAs will provide mechanistic targets for developing new targeted therapies to treat PN associated with T2D and prediabetes. *Antioxid. Redox Signal.* 37, 560–577.

Keywords: fatty acids, neuropathy, diabetes, prediabetes, dyslipidemia

Introduction

DIABETES IS ONE of the fastest growing epidemics of the 21st century. Over 463 million adults worldwide currently have diabetes, and the number of diabetes cases is projected to reach 700 million by 2045 (66). This massive increase in diabetes cases over the next two decades will impact one tenth of the world's population, and result in a global rise in diabetes-related deaths and diabetic complications (132). Type 2 diabetes (T2D) is the predominant type of diabetes, accounting for 90%–95% of global diabetes cases, and is characterized by impaired insulin secretion and progressive insulin resistance (5, 66).

Before developing T2D, almost all individuals have prediabetes, a condition that precedes T2D and is a major risk factor for the development of T2D (41). In the United States

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alone, 1 in 3 adults have prediabetes placing them at risk of developing T2D. Although lifestyle interventions such as diet or exercise can slow the progression from prediabetes to T2D, 3%–11% of prediabetes cases progress to T2D each year (47, 100).

The global rise in both diabetes and prediabetes cases has led to a parallel increase in diabetic complications. Peripheral neuropathy (PN), the most prevalent complication of diabetes and prediabetes, occurs in ~50% of T2D as well as 30% of prediabetes patients (14, 49, 105). PN is characterized by progressive peripheral nerve damage that leads to tingling, numbness, and/or pain (35). The distal-to-proximal loss of normal sensation arises first in the feet and progresses proximally in a stocking-and-glove distribution.

The progressive loss of sensory function in PN causes severe morbidity and is a leading cause for nontraumatic amputations (88). In addition, the financial expenditure related to PN health care costs in the United States is >US$10.9 billion annually (49). Therefore, there is a pressing need to identify the factors that lead to PN in T2D and prediabetes to develop new and targeted therapies for PN; however, the molecular mechanisms that drive PN are incompletely understood.

Dyslipidemia is increasingly recognized as an important risk factor for PN in T2D and prediabetes (50, 75, 137). Broadly defined as an abnormal plasma lipid profile, dyslipidemia commonly develops in patients with T2D and prediabetes, is a component of the metabolic syndrome (MetS) (2), and is thought to play a major role in tissue-specific complications associated with metabolic diseases.

The plasma lipid profile is influenced by diet, in particular the dietary fatty acid (FA) level and composition (39). Consuming an unhealthy diet, such as the western diet, composed of foods rich in saturated FAs (SFAs), is associated with increased plasma lipid levels, elevated low-density lipoprotein cholesterol, and the development of dyslipidemia in both human subjects and preclinical animal models. The American Diabetes Association advises patients with T2D and prediabetes to reduce the dietary intake of saturated and trans-FAs, and supplement their diet with sources of monounsaturated FAs (MUFAs) and polyunsaturated FAs (PUFAs) (77).

Dietary FAs have emerged as both potential mediators and treatments for PN in T2D and prediabetes (25, 114, 144). The molecular mechanisms that underlie the injurious or beneficial effect of FAs on nerve function are an active area of study. Herein, we first review the clinical and preclinical studies that delineate the importance of dietary FAs in modulating peripheral nerve function in PN associated with T2D and prediabetes. Second, we discuss the metabolic effects of dietary FAs on the peripheral nervous system (PNS) in T2D and prediabetes. Finally, we detail the molecular effects of FAs on complex lipid synthesis, lipid metabolism, and mitochondrial function in the PNS.

Dietary FAs

FAs are essential molecules for the nervous system, and are critical for maintaining neuronal health and function (130). FAs play a number of important cellular functions ranging from substrates for mitochondrial FA β-oxidation and mediators of intracellular signaling to components of complex lipid species in plasma membranes and organelles (30). Circulating FAs are the major source of FAs for the nervous system, and can be obtained from the diet or synthesized through *de novo* lipogenesis FA synthesis. The physiological destination and functional role of FAs are defined by the structure of the FA hydrocarbon chain.

FAs are composed of an aliphatic hydrocarbon chain terminating in a carboxylic acid functional group. Although FAs tend to be hydrophobic in nature due to the hydrophobicity of the hydrocarbon chain, the carboxylic acid is a polar functional group that participates in FA β-oxidation (30). During β-oxidation, two carbons are removed from the carboxyl end of the FA to generate acetyl-CoA, which enters the tricarboxylic acid cycle (TCA) to produce cellular energy.

FAs are classified by hydrocarbon chain length and degree of saturation, which correspond to the number of carbons and double bonds in the hydrocarbon chain, respectively (Fig. 1). Short-chain FAs contain 2–4 carbons, medium-chain FAs are composed of 6–12 carbons, long-chain FAs consist of 14–18 carbons, and very long-chain (VLC) FAs contain 20–24 carbons. FAs also have varying degrees of saturation. SFAs lack double bonds and have fully saturated hydrocarbon chains, while unsaturated FAs have one or more double bonds (Fig. 1A–C). MUFAs contain one double bond typically between the 9th and 10th carbons in the hydrocarbon chain, which causes a kink in the FA structure (Fig. 1B). Among other types, PUFAs constitute omega-3 or omega-6, which indicates the location of the first double bond closest to the methyl group within the hydrocarbon chain (Fig. 1C).

The most common omega-3 FAs are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Bends in the hydrocarbon chain that result from a single double bond in MUFAs or multiple cis-double bonds in PUFAs play an important role in cellular membranes by preventing tight packing and improving membrane fluidity and curvature. The length and degree of saturation of FA acyl chains dictate the FA intracellular localization and function (1, 130). The hydrocarbon chain length of FAs dictates the intracellular localization and downstream metabolic and signaling pathways. The degree of saturation of a FA plays a key role in membrane fluidity and organelle dynamics.

There are two categories of FAs, including essential FAs, which must be obtained from the diet, and nonessential FAs, which may be synthesized by cells and tissues within the body. Essential FAs, including linoleic acid and linolenic acids, cannot be synthesized endogenously and must be obtained from dietary sources (28). These FAs play important roles in membrane fluidity, regulation of enzymatic reactions, and receptor signaling in the nervous system (130). Nonessential FAs can be either obtained from the diet or synthesized intracellularly through *de novo* lipogenesis pathways. Nonessential FAs include saturated, monounsaturated, and some polyunsaturated FAs.

Different types of foods contain distinct FA profiles (Fig. 1). Red meats, and processed and fried foods contain high levels of long-chain saturated FAs, whereas dairy products, such as milk and cheese, are rich in medium-chain saturated FAs (97). These foods are commonly found in the western diet, and have been associated with the development of both prediabetes and T2D.

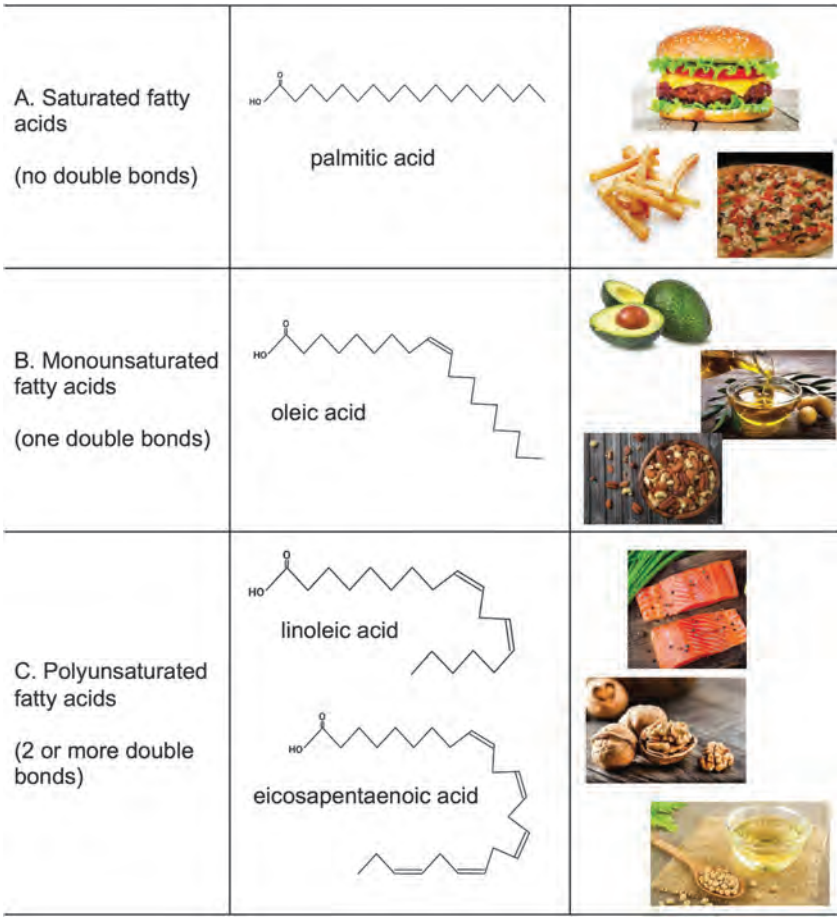


FIG. 1. The structural properties of FAs. (A) SFAs contain no double bonds and lack kinks in the hydrocarbon chain. Long-chain SFAs, such as palmitic acid, are found in foods, such as red meats, fried foods, and processed foods. **(B)** MUFAs, such as oleic acid, contain a single double bond that causes a kink in the hydrocarbon chain. Plant-based oils, nuts, and avocados are common dietary sources of MUFAs. **(C)** PUFAs contain two or more double bonds that create multiple kinks or bends in the hydrocarbon chain. For example, linoleic acid is an omega-6 PUFA that contains two double bonds, and eicosapentaenoic acid is an omega-3 PUFA that contains five double bonds. The major sources of PUFAs are fish, nuts, and plant-based oils. MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Nuts, seeds, avocados, and plant-based oils are rich in MUFAs, such as oleic acid and palmitoleic acid (69). These foods are commonly consumed at high levels in the Mediterranean diet, and improve metabolic function in patients with T2D and prediabetes (108, 139). Omega-3 FAs are commonly found in foods such as fish, flaxseed, and plant-based oils. In T2D and prediabetes, the western diet plays a major role in the development of dyslipidemia, and a high-fat diet (HFD) rich in SFAs underlies the onset and progression of PN in preclinical models of prediabetes (57, 94).

Aberrant dietary FA intake promotes dyslipidemia in patients with T2D and prediabetes. In healthy individuals, insulin release after a meal suppresses FA release from adipose depots in the body, and FA metabolism is under tight regulation. FAs are stored as energy in triglycerides in adipose tissue, and serve as fuel for the heart and muscles. In dyslipidemia, however, high plasma FA levels result from both the diet and FA mobilization from adipose tissue (32). This elevation in plasma FAs contributes to insulin resistance and also increases FA levels, promoting metabolic dysfunction in peripheral tissues, such as the PNS.

The PNS

The PNS consists of the somatic nervous system and the autonomic nervous system. Although both the somatic nervous system and autonomic nervous system are impacted by T2D and prediabetes, this review will focus on the somatic nervous system. The somatic nervous system, which consists of both motor and sensory nerves, is responsible for transmitting sensory information from the periphery to the central nervous system (CNS) (Fig. 2). Sensory neurons are afferent neurons that transmit sensory information from receptors in the sensory nerve terminals to the CNS, whereas motor neurons are efferent neurons that carry information from the CNS to muscles and organs within the body.

Axons from peripheral neurons are myelinated or unmyelinated. Myelination in the PNS is carried out by resident Schwann cells (SCs), which produce lipids and proteins to insulate the axons and facilitate efficient nerve conduction (109) (Fig. 2). SCs myelinate a single segment of an axon, called the internode, which is separated from other internodes by unmyelinated regions called Nodes of Ranvier. The myelin sheath is a lipid-rich multilamellar structure composed of closely packed lipid bilayers (104, 121). This lipid-rich myelin sheath is composed of 70%–85% lipids, and is attuned to changes in FAs and metabolic flux (40).

Myelinated and unmyelinated axons are bundled into nerve fibers surrounded by a layer of connective tissue called the endoneurium. Sensory nerve fibers, including Aα, Aβ, Aδ, and C fiber types, are each responsible for relaying specific types of sensory signals. The large, myelinated Aα fibers receive signals from the muscle spindle and Golgi tendon, and are responsible for proprioception required for spatial position and movement.

The Aβ fibers include large and mid-diameter myelinated fibers, which carry touch and pressure sensations as well as mechanoreceptor-mediated signals. Small afferent Aδ fibers

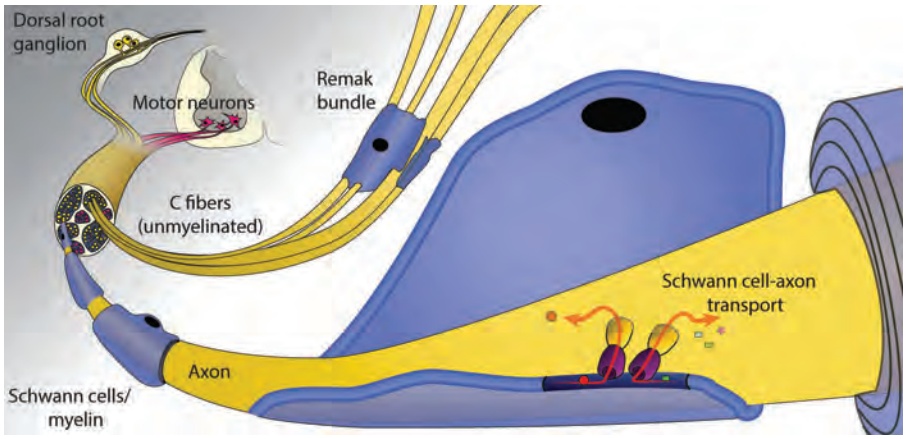


FIG. 2. The peripheral nervous system. The peripheral nerves are composed of nerve fibers containing axons and myelinating SCs. Motor neurons are protected by the spinal cord, whereas sensory DRG neurons are exposed to circulating metabolites, such as FAs. Some nerve fibers are myelinated by SCs to provide protection and support rapid nerve conduction velocity, while other fiber types, such as C fibers, are unmyelinated and more susceptible to metabolic injury. SCs may also contribute to metabolic injury by transporting toxic lipids and other metabolites to axons. Figure included with permission from Feldman *et al.* (38). DRG, dorsal root ganglion; SC, Schwann cell.

are thinly myelinated fibers, and transmit mechanoreceptor-triggered cold, touch, and pressure sensations. Finally, thin unmyelinated C fibers are <1 μm in length and are surrounded by nonmyelinating SCs that occur in groups, known as Remak bundles. These fibers serve as PNS nociceptors, and transmit heat, pain, and nociception sensory signals (37).

PN associated with diabetes and prediabetes tends to present as a loss of sensory function with only a small effect on motor function (38). The different anatomical locations of motor and sensory neuron cell bodies may contribute to the susceptibility of sensory neurons to diabetic metabolic flux (38). Motor neurons localized in the ventral horn inside the spinal cord are protected by the blood–brain barrier, whereas dorsal root ganglion (DRG) sensory neurons are located outside of the spinal cord, and lack either a blood–brain or blood–nerve barrier.

Consequently, unlike motor neurons, DRG sensory neurons are exposed to circulating metabolites, including FAs, glucose, and other metabolites, making them vulnerable to metabolic stressors and axonal injury in diabetes and prediabetes (38). Unmyelinated C nerve fibers are also particularly sensitive to injurious metabolites, such as FAs, likely secondary to the absence of a myelin sheath (Fig. 2) (38). Thus, the lack of protective barriers makes both DRG sensory neurons and C fibers more susceptible to metabolic injury.

The morphology of DRG sensory neurons also makes them more susceptible to injurious circulating metabolites. DRG neurons extend axons, which are among the longest axons in the body, extending up to 1 m in length from the cell body to the axon terminal. DRG neurons have a pseudounipolar morphology where the stem axon bifurcates into a peripheral axon branch and a central axon branch (91).

This unique morphology is key for transmitting sensory information from the peripheral axon branch to the bifurcation, and then to the CNS *via* the central axon branch (91). After a stimulus, action potentials are generated in the peripheral nerve branch and conducted toward the CNS. However, the extremely large axon to cell body ratio presents unique bioenergetics challenges for DRG neurons and axon health and function. To maintain energy homeostasis distally in the axon, DRG neurons employ mitochondrial axonal transport mechanisms to provide mitochondria throughout the entire length of the axon.

Clinical Data on the Impact of FAs on Neuropathy

Nerve injury from hyperglycemia was previously considered the principal molecular driver of PN, although the pathophysiology of type 1 diabetes (T1D) and T2D is distinct. Over the last decade, however, the multifactorial nature of T2D prompted clinical studies to investigate unique metabolic processes associated with PN in T1D and T2D. The concept that hyperglycemia is not the sole metabolic factor underlying PN in T2D was addressed in a Cochrane review evaluating the efficacy of glucose control for regulating PN in T1D compared with T2D subjects separately (13).

This systematic review compared 17 randomized, controlled studies consisting of subjects with T1D, T2D, or both. It found that glucose control significantly reduces PN development in T1D subjects, as identified by a decrease in abnormal nerve conduction studies and vibration thresholds. However, in subjects with T2D, glucose control had no significant impact on nerve conduction and vibration thresholds. These clinical studies suggest that the mechanisms underlying PN differ between T1D and T2D (12, 13).

In an effort to distinguish T2D risk factors that correlate with PN, the impact of the MetS on PN was evaluated (8). The MetS is a group of metabolic disorders that is highly prevalent in T2D subjects, ~73%–83% prevalence, and is a major risk factor for T2D (119, 142). To assess the effect of MetS components on PN, a cohort of 2382 subjects with PN assessments from the Health, Aging, and Body Composition (Health ABC) study were evaluated for prevalence of PN, after stratification for glycemic status and MetS components. Interestingly, although PN was more common in subjects with diabetes, MetS components, including prediabetes and obesity, were linked to secondary measures of PN (16).

In addition, PN was more prevalent in subjects with a higher number of MetS components independent of glycemic status (16). Another cross-sectional, population-based study consisting of 4002 subjects from China sought to identify individual MetS components associated with PN. In agreement with previous studies, diabetes and obesity were the major metabolic drivers of PN (11). The three components of MetS that correlated with PN in T2D were diabetes, prediabetes, and obesity, which all intersect with components of dyslipidemia (16, 17, 79).

The association of dyslipidemia with PN was evaluated in a longitudinal study, the Danish arm of Anglo-Danish-Dutch study of Intensive Treatment of Diabetes in Primary Care (ADDITION-Denmark). This study examined incident PN in T2D subjects at a 13-year follow-up appointment after enrollment in the ADDITION-Denmark study (6). The cohort of 1,445 subjects showed that obesity along with reduced levels of high-density lipoprotein cholesterol, a MetS component, is a substantial risk factor for PN in T2D. In parallel, a separate study on 5249 T2D subjects from the Danish Centre for Strategic Research in Type 2 Diabetes (DD2) Cohort found that PN is associated with MetS components, including elevated triglyceride levels, hypertension, and obesity (23).

To determine whether obesity is an independent risk factor for PN in nondiabetic individuals, another study evaluated the 138 obese subjects and 46 lean controls and the prevalence of PN (15). This study discovered that obesity in normoglycemic individuals associates with PN. Altogether, these studies indicate that MetS is a major risk factor for PN. Since dyslipidemia is commonly associated with MetS in T2D and prediabetes, we contend that dietary FA intake may contribute to PN in T2D.

Based on the emerging role of dyslipidemia in PN pathogenesis, another study determined whether lipid lowering statins would reduce the prevalence of PN in T2D subjects (124). Despite numerous studies showing an association of PN with dyslipidemia, the incidence of PN was similar in both T2D subjects with and without statin therapy (72). Therefore, the reduction of triglyceride and cholesterol levels by statins had little effect on PN. These results suggest that overall dyslipidemia is not responsible for PN in T2D subjects, but rather that specific lipid species underlie neuropathy progression.

To identify specific lipid metabolites that associate with PN in T2D, we evaluated global plasma metabolomics in a cohort of T2D subjects with and without PN compared with lean control subjects from ADDITION-Denmark (111). We found that individuals with T2D and PN had changes in plasma metabolites related to lipid and energy metabolism compared with T2D subjects without PN.

This prompted us to evaluate alterations in plasma lipid metabolites identified by the global metabolomics analysis. The abundance, chain length, and saturation of both plasma FAs and complex lipids were appreciably altered in T2D subjects compared with lean controls (111). T2D subjects shifted from beneficial VLC unsaturated FAs in the plasma to toxic long-chain saturated FAs. In addition, plasma complex lipids showed an increase in diacylglycerol and phosphatidylethanolamine species, and a decrease in phosphatidylcholine, sphingomyelin, ceramide, and acylcarnitine species (111).

A separate study comparing obese T2D subjects with and without PN showed that plasma serine and 1-deoxyhydroceramide levels are inversely correlated with quantitative C fiber assessment as a measure of PN, suggesting that 1-deoxyhydroceramides are potential mediators of PN in T2D and obesity (44). Collectively, these studies suggest that PN is associated with changes in plasma lipid species and metabolites of lipid metabolism in T2D and prediabetes.

Dietary supplementation studies with MUFAs or PUFAs in T2D and prediabetic subjects with PN show significant beneficial effects on metabolic parameters and nerve function. A European Prospective Investigation into Cancer and Nutrition (EPIC)-InterAct study showed that plant-based omega-3 and omega-6 PUFAs inversely correlate with T2D, suggesting that PUFAs improve metabolic function in T2D subjects (43). In this study, T2D was inversely correlated with plant-based omega-3 α-linoleic acid and omega-6 PUFA linoleic acid, while EPA and DHA had no association. These results highlight the need to evaluate the effect of individual FAs, not overall FA levels.

To evaluate the connection between dietary PUFA consumption and PN, a National Health and Nutrition Examination Survey study found that dietary PUFA intake is associated with lower incident neuropathy in diabetic subjects (129). In addition, individual FAs γ-linolenic acid (67, 70) and EPA (98) confer significant improvements in PN associated with T2D. A similar dietary supplementation paradigm in subjects with T1D, however, had no effect on nerve conduction or sensory nerve function, further supporting the idea that metabolic factors contributing to PN in T1D and T2D are unique (76).

In addition, a recent cross-sectional study on 147 T2D subjects showed that high dietary iron intake and a high iron:PUFA ratio correlate with PN, suggesting that the iron level determines the level of benefit conferred by dietary PUFAs (71). The effect of different types of FAs on nerve function is likely the result of altered lipid metabolism within the nerve. However, the molecular mechanisms that are differentially regulated by saturated and unsaturated FAs in the nerve are still an active area of research.

Preclinical Data on the Impact of FAs on Neuropathy

Similar to humans, obese and prediabetic mice develop PN associated with dyslipidemia and metabolic dysfunction (57, 94, 136, 137). Preclinical animal models are a valuable tool for identifying molecular mechanisms and lipid changes that associate with PN in prediabetes and T2D. Mice fed a HFD have impaired glucose tolerance, increased body weight, and a higher percentage body fat compared with their standard diet-fed counterparts (11, 57, 94).

The HFD mice also develop PN characterized by a reduction in sural and sciatic nerve conduction velocities, an increase in hind paw latency, and a decrease in C fibers, as measured by the intraepidermal nerve fiber density (IENFDs) in the hind paw (57, 94). Genetic models of T2D also consistently develop the same PN phenotypes, along with overt T2D characterized by hyperglycemia, dyslipidemia, and PN (96). Three common murine models of T2D are (i) *ob/ob* mice with leptin deficiency, (ii) *db/db* mice that lack the leptin receptor, and (iii) mice fed a HFD and treated with one low dose of streptozotocin (STZ).

Both prediabetic and T2D animal models are dyslipidemic, and align with the metabolic phenotypes in prediabetic or T2D humans. Using drugs to reduce free FAs in rodents improves nerve function. For example, pioglitazone treatment in *db/db* mice improved small fiber function as measured by sural nerve conduction velocity and IENFD (58, 62) and acipimox treatment of male Zucker *fa/fa* rats, a robust model of T2D when fed a HFD, restored nerve conduction velocities (81). Although these studies indicate that dyslipidemia and FAs contribute to PN development and progression in murine models, the molecular changes that underlie these beneficial effects on PN are not completely understood.

As a first step to identify dysregulated pathways that underlie PN in prediabetes and T2D, we performed lipidomics (94, 122) and transcriptomics (94) studies on peripheral nerves from HFD murine models of prediabetes and T2D. We integrated lipidomic profiles from one sciatic nerve and transcriptomics from the other sciatic nerve for each mouse. This analysis showed dysregulation of lipid pathways in both the HFD-fed prediabetic mice and the HFD-STZ T2D mice (94).

Specifically, we found increases in triglycerides containing SFAs as well as increases in diacylglycerol acyltransferase 2 (DGAT2), the enzyme that carries out the committed step in triglyceride synthesis. Lipid metabolism was also altered in the sciatic nerve from *db/db* mice (122). These studies indicate that high levels of SFAs in the HFD are incorporated into the nerve lipidome altering the sciatic nerve lipid profile, which may contribute to PN in prediabetes and T2D.

To determine whether the FA composition of the diet contributes to PN in the HFD murine models, we and others compared the effects of dietary SFAs and unsaturated FAs on metabolic and PN phenotypes in prediabetic and T2D mice (114). Mice fed a SFA-rich HFD from 6 to 16 weeks of age developed PN and metabolic dysfunction. However, mice switched from the SFA-rich HFD to a MUFA-rich HFD exhibited a significant improvement in nerve function, including increased sensory and motor nerve conduction velocities and restoration of C fibers as measured by increased IENFDs.

Similarly, the Yorek laboratory discovered the beneficial effects of Menhaden oil supplementation for improving nerve function in murine models of PN. Menhaden oil is composed of elevated levels of omega-3 PUFAs EPA and DHA. Supplementation of the HFD with Menhaden oil led to significant improvements in sensory and motor nerve conduction velocities, thermal nociception, and IENFDs in HFD- and low-dose STZ-treated Sprague Dawley rats and C57BL6/J mice (24, 29, 127).

Altogether, these results show that dietary FAs modulate nerve function in rodent models of PN associated with prediabetes and T2D. Dietary SFAs underlie PN in rodent models of prediabetes and T2D, while dietary MUFAs and PUFAs improve nerve function. The molecular mechanisms that underlie the differential regulation of nerve function by SFAs and MUFAs are not completely understood. One possibility is that SFAs impair lipid metabolism within the nerve resulting in an altered nerve lipid profile that drives PN development and progression. We will next detail the mechanisms by which SFAs and MUFAs/PUFAs might differentially regulate lipid metabolic pathways in the nerve.

FAs and Nerve Lipid Metabolism

Under homeostatic conditions, neuronal lipid metabolism is centered on a balance of lipid uptake, *de novo* lipogenesis, and FA β-oxidation to maintain neuronal health and function (104, 130). However, dyslipidemia leads to an accumulation of neurotoxic lipids, aberrant lipid lipogenesis, and impaired mitochondrial energy production. Dietary FAs are fundamental players in the development of dyslipidemia and drastically impact these lipid metabolism processes.

Adipose and liver regulate circulating FA levels through uptake, storage, and mobilization of triglycerides and FAs (Fig. 3). After meal consumption, dietary FAs are esterified into triglycerides and incorporated into chylomicrons in the intestine (53). The majority of these chylomicrons are taken up by tissues with high lipoprotein lipase activity, such as adipose tissue and muscle, whereas chylomicron fragments are taken up by the liver. These FAs are converted into triglycerides in very-low-density lipoproteins (VLDLs) or remain nonesterified FAs, which are then packaged into lipoprotein particles and transported through the bloodstream to peripheral tissues, such as the peripheral nerves (3).

In obesity, excessive intake of the foods rich in SFAs is associated with weight gain, impaired glucose tolerance, and increased body fat mass. This elevation in adiposity is often associated with adipose tissue dysfunction characterized by inflammation, adipocyte expansion, impaired insulin signaling, dysfunctional triglyceride storage, and increased FA mobilization (18). The liver also becomes dysfunctional in obesity with an overload of intrahepatic lipid and decreased production of VLDL triglycerides (82). Therefore, adipose tissue and liver dysfunction dictate changes in circulating FAs, and are closely associated with the development of dyslipidemia and obesity (95).

FA Uptake

Circulating FAs taken up by neurons and SCs are oxidized to generate cellular energy or converted into complex lipids to support the nerve. Circulating lipids, such as triglycerides, in lipoprotein particles are hydrolyzed into free nonesterified FAs by lipoprotein lipase localized in the peripheral nerves on the surface of cells within the endoneurium, the connective tissue that surrounds a myelinated nerve (33, 61). The free FAs are then transported into neurons and SCs, and the mode of transport is determined by the FA chain length (51). Short- or medium-chain FAs are passively diffused into cells by a membrane flip-flop mechanism. Conversely, long-chain FAs and VLC FAs must be transported into the neurons or SCs by FA transporters.

Two major FA transporters in peripheral nerves are FA translocase (CD36) and FA transport protein (FATP) (Fig. 4). Both CD36 and FATP have a high affinity for long-chain saturated and unsaturated FAs, and contribute to obesity and metabolic dysfunction (102). CD36 is localized on the cell membrane of neurons and SCs, and facilitates FA transport through a central FA transport tunnel (102). FATPs consist of a single transmembrane domain, an extracellular FA binding site that allows for FA transport, and an intracellular ATP binding site with acyl-CoA synthetase activity that converts incoming FAs into acyl-CoAs (85).

Once inside the cell, FA binding proteins (FABPs) facilitate intracellular FA distribution by acting as lipid

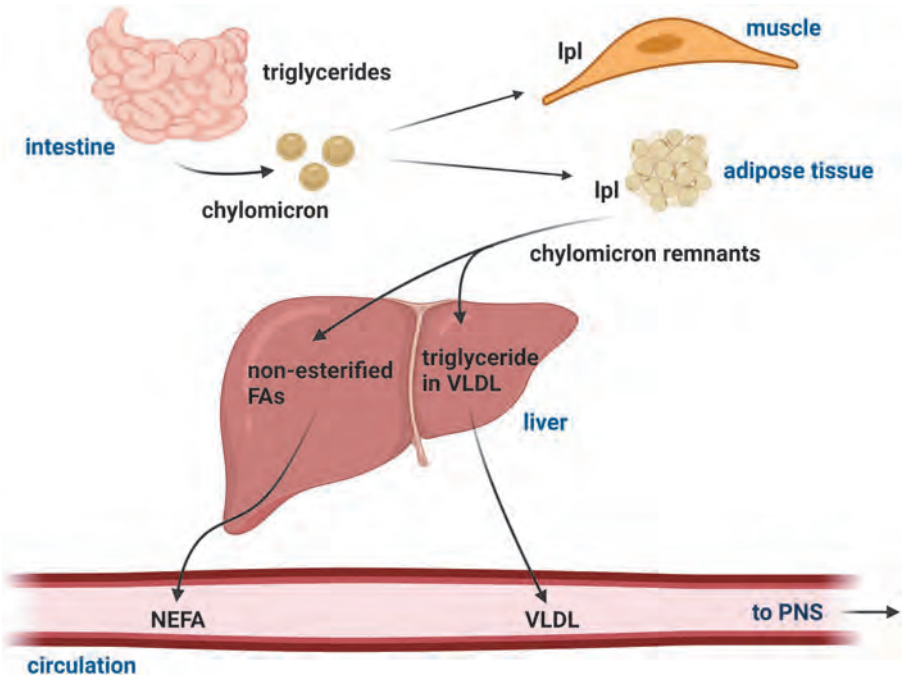


FIG. 3. FA and lipid metabolism. Dietary FAs are first processed through the intestine where they are converted into triglycerides and packaged into chylomicrons. Triglyceride-containing chylomicrons are then utilized by the adipose tissue or muscle, which are highly energy dependent. Here, triglycerides are broken down by LPL to release NEFAs to be metabolized for energy production. Fragments of chylomicrons, however, are taken up by the liver and metabolized into NEFAs, which can then be synthesized into triglycerides and packaged into VLDL, or released as NEFAs into the bloodstream. The peripheral nerves are exposed to VLDL and FAs in the bloodstream because the nerves are not protected by the spinal cord. FA, fatty acid; NEFA, nonesterified fatty acid; LPL, lipoprotein lipase; PNS, peripheral nervous system; VLDL, very-low-density lipoprotein. Created with Biorender.com.

chaperones to transport FAs to organelles (89, 90, 107). Only four of the six FABP isoforms are expressed in the nervous system, of which the peripheral myelin protein 2 (PMP2/M-FABP) isoform is found only in myelin in the peripheral nerves and the E-FABP isoform is mainly localized to neurons (133). The affinity of FABPs for FAs is proportional to FA hydrocarbon chain length and degree of saturation with the highest affinity for VLC FAs and fewer double bonds.

FA transporters in the dyslipidemic sciatic nerve are dramatically affected by circulating dietary FAs. Using a transcriptomics and lipidomics analysis, we recently found that the expression level of both lipoprotein lipase, *Lpl,* and *CD36* in the sciatic nerve is modulated by a HFD, indicating that the higher level of FAs in both prediabetes (94) and T2D (63, 101) alters FA uptake in PN. Although both neurons and SCs express CD36 and FATP, the known role of CD36 and FATP in dyslipidemic sensory neurons is limited. In SCs, CD36 and FATP mediate uptake of circulating FAs, which profoundly affects the myelin lipid composition by incorporating FAs into the myelin sheath (104, 143). In fact, ablation of CD36 perturbs nerve remyelination pathways after injury, suggesting that CD36 is essential for the proper maintenance of the myelin sheath (36, 104).

FABPs play an important role in PN associated with Charcot Marie Tooth disease (CMT) (59), but recent evidence indicates that FABPs also contribute to PN in T2D and prediabetes (56). The FABP PMP2 is expressed in myelinating SCs and facilitates structural stability in myelinated large axons by anchoring myelin lipid bilayers (116, 126, 131, 145). Mutant PMP2 causes severe demyelinating PN characterized by decreased nerve conduction velocities in CMT type 1A, emphasizing the importance of this FABP for nerve function (59). In addition to providing a scaffold for myelin lipid bilayers, PMP2 has a strong affinity for both cholesterol and FAs, and is likely to play a major role in FA cellular transport and metabolism in SCs (104, 110, 145).

Indeed, we identified a downregulation of *PMP2* gene expression in the sciatic nerve of *db/db* mice, indicating that elevated levels of circulating lipids in diabetic murine models impair nerve lipid homeostasis and potentially myelin structure (56). FABP5 is the major isoform expressed in DRG neurons and is also an important regulator of nerve function. Small nucleotide polymorphisms in FABP5 are associated with T2D, indicating that a loss of FABP function contributes to metabolic dysfunction (9). Indeed, omega-3 PUFAs increase FABP5 expression that provides neuroprotection after spinal cord injury. Increasing FABP5 levels in neurons also prevents palmitate-induced lipotoxicity (78).

FA transporters and FABPs are critical regulators of energy homeostasis in neurons, and contribute to the pathogenesis of diabetes, obesity, and their complications (74, 83). These studies suggest that dysregulation of FA transport by CD36, FATP, and FABPs may be a critical molecular target in PN associated with prediabetes and T2D.

FAs and Mitochondrial Function, Trafficking, and Dynamics in the PNS

After transport into the cell, FAs may be oxidized by mitochondrial β-oxidation to generate cellular energy in the form of ATP under homeostatic conditions. Synthesis of cellular ATP from FAs occurs in the mitochondria by sequential

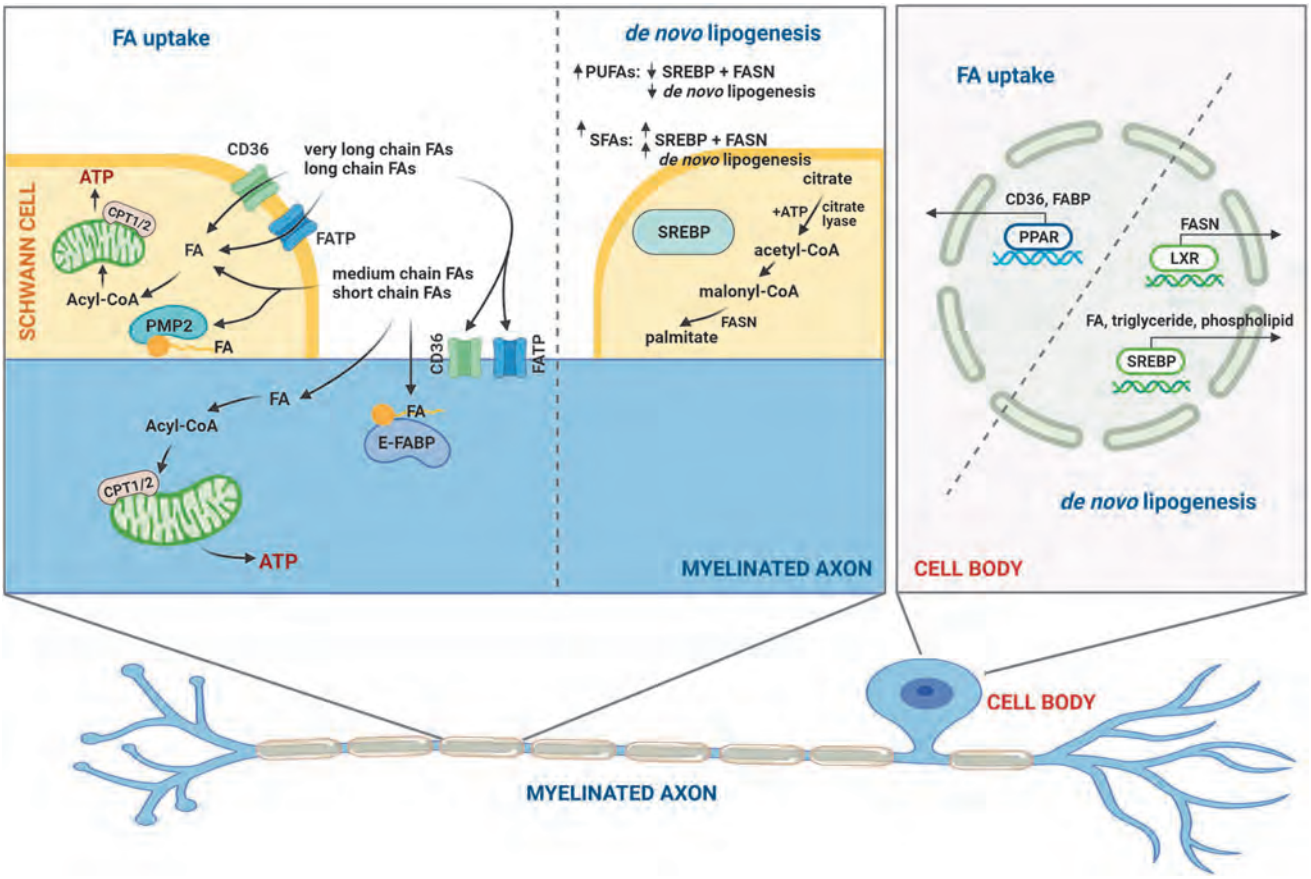


FIG. 4. FA uptake in neurons and SCs. FAs are transported into neurons and SCs through FA transporters, CD36 and FATP, whereas medium- and short-chain FAs enter cells by passive diffusion. FAs are then converted into acyl-CoAs and metabolized by mitochondria to produce ATP. Alternatively, FAs are bound by FABPs, and delivered to organelles or other cellular membranes. PMP2 is a FABP that is uniquely expressed in the myelin of the peripheral nervous system and plays an important structural role in the myelin sheath. E-FABP is localized to neurons and plays an important role in nerve function. SCs are also highly dependent on *de novo* lipogenesis regulated by transcription factor SREBP and FASN. *De novo* lipogenesis decreases in response to elevated levels of PUFAs and increases in response to SFAs. *De novo* lipogenesis is activated by LXR and SREBP transcriptional regulation, which increases the expression of FASN and complex lipid synthesis. Conversely, FA uptake pathways are upregulated by transcription factor PPARγ that increases the expression of CD36 and FABP. FABP, fatty acid binding protein; FASN, fatty acid synthase; FATP, fatty acid transport protein; LXR, liver X receptor; PPARγ, peroxisome proliferator-activated receptor gamma; SREBP, sterol regulatory element-binding protein. Created with Biorender.com.

reactions including FA β-oxidation, followed by the TCA, and finally oxidative phosphorylation. A detailed description of mitochondrial bioenergetics and dynamics in PN has been previously published (115). DRG neurons and SCs rely on mitochondrial ATP production to maintain nerve health and function (130). In addition to ATP production, DRG neurons require mitochondrial trafficking mechanisms to transport mitochondria throughout the axon and provide energy at distal regions of the axon. Mitochondrial fusion/fission events are also required to maintain energy balance (115).

We recently discovered that the effect of FAs on DRG neuron mitochondrial function is dependent on FA hydrocarbon chain length and degree of saturation (112–114). We observed a significant decrease in mitochondrial membrane potential in DRG neurons treated with diabetic concentrations of long-chain SFAs palmitate (C16:0) and stearate (C18:0), whereas shorter chain SFAs laurate (C12:0) and myristate (C14:0) did not affect mitochondrial depolarization (112, 113). The loss of mitochondrial membrane potential in long-chain SFA-treated DRG neurons was associated with loss of ATP in immortalized DRG neurons (113, 114).

In addition, long-chain SFA palmitate treatments profoundly affected DRG neuron mitochondrial bioenergetics marked by a significant and dose-dependent decrease in spare respiratory capacity, as well as an increase in mitochondrial uncoupling and proton leak (112). Interestingly, mitochondrial depolarization and ATP loss due to long-chain SFAs were completely abrogated with the addition of exogenous long-chain MUFA oleate (C18:1), potentially due to the formation of axonal lipid droplets (114).

These alterations in mitochondrial function were accompanied by impairments in axonal mitochondrial trafficking. Cell culture models of dyslipidemia show significant impairment in axonal mitochondrial trafficking in DRG axons (Fig. 5). DRG axons treated with long-chain SFAs palmitate (C16:0) and stearate (C18:0) had significant and dose-

dependent decreases in the percentage of motile mitochondria (Fig. 5A, D, E) (113). Shorter chain SFAs laurate (C12:0) and myristate (C14:0) had no effect on the level of mitochondrial trafficking in DRG axons (Fig. 5A–C) (113).

Long-chain MUFA oleate had no effect on mitochondrial trafficking, and supplementation of oleate into the palmitate treatments completely prevented the inhibition of mitochondrial trafficking caused by palmitate alone (Fig. 5A, F, G) (114). This suggests that long-chain SFAs with hydrocarbon chains >16 carbons in length have a detrimental effect on axonal mitochondrial trafficking and function, whereas shorter SFAs and MUFAs have no effect. Furthermore, the impairment in mitochondrial trafficking and function can be prevented by MUFA supplementation.

Mitochondrial fusion and fission dynamics are also profoundly impacted by complex lipids derived from FAs. Mitochondrial fission and fusion dynamics play an important role in regulating energy balance (115). Lipids including cardiolipin, phosphatidylethanolamine, phosphatidic acid, and diacylglycerols play a major role in mitochondrial fission/fusion, and the level of these lipids is modulated by FAs.

Cardiolipin is a mitochondria-specific phospholipid enriched with the essential FA linoleic acid that regulates mitochondrial fusion, fission, and function. The availability of linoleic acid modulates the level of cardiolipin in the mitochondria, which directly impacts mitochondrial fusion and fission dynamics and mitochondrial function (21, 45). Interestingly, despite elevated levels of linoleic acid in the HFD, there is a steep decrease in cardiolipin in the sciatic nerve of HFD-fed murine models of prediabetes (94), suggesting that increased linoleic acid levels in the HFD are not sufficient to maintain mitochondrial cardiolipin levels and prevent mitochondrial dysfunction caused by long-chain SFAs in the HFD.

Phosphatidylethanolamine, a phospholipid that is rich in the outer mitochondrial membrane and inner mitochondrial membrane, plays an important role in mitochondrial fusion. Phosphatidic acid stimulates mitochondrial fusion, whereas diacylglycerols promote fission. The interplay between these two lipid species tightly regulates fusion and fission dynamics (45). Although the effect of dietary FAs on mitochondrial fusion and fission remains incompletely characterized, mice fed a 60% HFD rich in SFAs exhibited significant alterations in the level of these complex lipids, indicating that changes in dietary FA composition may alter mitochondrial fusion and fission (94).

Much like DRG neurons, SCs can use mitochondrial β-oxidation of FAs to produce energy, but elevated levels of long-chain FAs result in SC mitochondrial dysfunction in PN associated with T2D and prediabetes. Elevated levels of long-chain FAs cause a loss of SC mitochondrial coupling efficiency and mitochondrial dysfunction (55). However, overexpression of long-chain acyl-CoA synthase 1 (Acsl1), which metabolically activates long-chain FAs for mitochondrial oxidation, is sufficient to rescue mitochondrial function. In the presence of elevated long-chain FAs, overexpression of Acsl1 in SCs alleviates oxidative stress, mitochondrial dysfunction, incomplete β-oxidation, and SC injury by improving metabolic activation of long-chain FAs (55).

Similarly, a murine model with SC-specific KO of the mitochondrial transcription factor A (TFAM) gene (*Tfam*), a transcription factor that is critical for mitochondrial biogenesis and function (73), developed severe PN (134). These TFAM SC knockout mice displayed mitochondrial dysfunction marked by a shift from lipid synthesis to FA β-oxidation (135). The loss of FA synthesis pathways significantly reduced crucial lipids in the myelin and instead triggered an elevation in acylcarnitine levels. These acylcarnitines were then excreted from the SCs triggering DRG neuron intracellular calcium flux and axonal degeneration. Therefore, functional SC mitochondria are critical for supporting peripheral neurons (134).

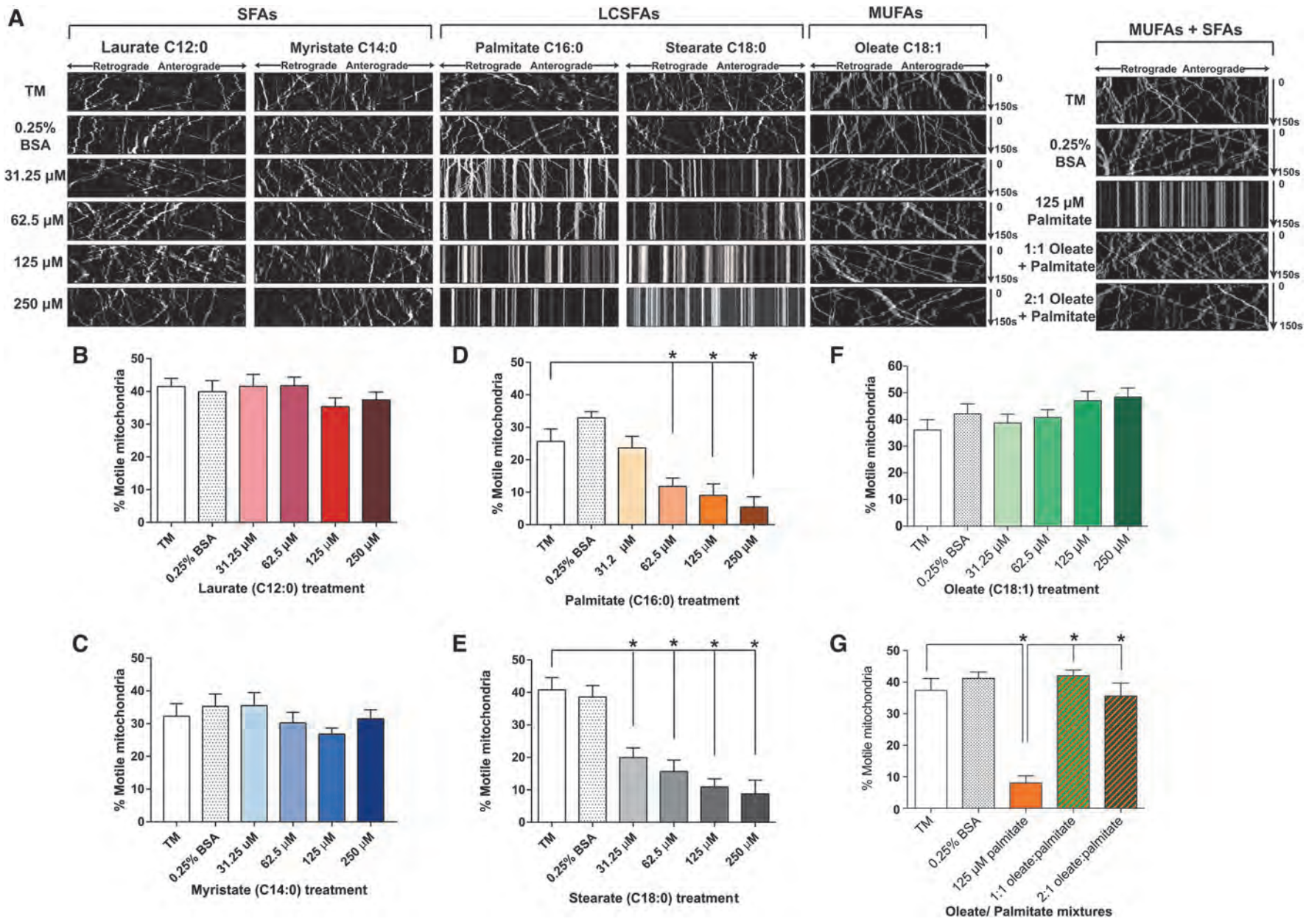
In conjunction with TFAM, two transcription factors, peroxisome proliferator-activated receptor coactivator 1 alpha (PGC-1α) and sirtuin 1 (SIRT1), play an important role in mitochondrial biogenesis. TFAM, PGC-1α, and SIRT1 respond to metabolic cues to preserve mitochondrial copy number and maintain efficient mitochondrial β-oxidation for ATP production. However, this pathway is compromised in PN associated with T2D (19, 34) and prediabetes (120). Conversely, caloric restriction enhances the mitochondrial biogenesis and mitochondrial oxidative capacity through the TFAM, PGC-1α, and SIRT1 pathway (34). These studies suggest that dysregulation of lipid uptake, β-oxidation, and mitochondrial biogenesis occurs in PN through the TFAM, PGC-1α, and SIRT1 pathway (19).

Oxidative Stress

Both hyperglycemia and dyslipidemia contribute to oxidative stress in diabetic PN (137, 138). Refer to Dr. Paul Fernyhough's Forum article for a detailed review about oxidative stress in T1D. Also, refer to Dr. Stephanie Eid's Forum article for an extensive review on oxidative stress in the PNS.

During β-oxidation, long-chain nonesterified FAs can also generate reactive oxygen species (ROS) in PN (125, 137).

FIG. 5. Axonal mitochondrial trafficking is differentially impacted by saturated and unsaturated FAs in sensory neurons. Mitochondrial trafficking is modulated by FA chain length and degree of saturation. Mitochondrial motility was recorded in live DRG neurons for 150 s. **(A)** Kymographs of mitochondrial motility were generated by stacking the fluorescence intensity from individual GFP-labeled mitochondria along the *y*-axis for each image taken during the 150 s video. Stationary mitochondria are depicted as straight vertical lines, while motile mitochondria are not straight lines. Shorter chain SFAs laurate **(B)** and myristate **(C)** had no significant effect on the number of motile mitochondria, while long-chain SFAs palmitate **(D)** and stearate **(E)** significantly impaired mitochondrial motility in a dose-dependent manner. **(F)** Increasing concentrations of MUFA oleate does not alter mitochondrial trafficking in DRG neurons and **(G)** prevents the impairment of mitochondrial trafficking caused by palmitate treatments alone. Values are expressed as mean ± SEM. $^*P < 0.01$, ordinary one-way ANOVA with Tukey's multiple-comparisons test. Figure included with permission from Rumora *et al.* (115).



Basal levels of nonesterified FAs in both neurons and SCs are utilized for ATP generation, but excessive levels of FAs trigger the production of superoxide and other ROS species due to leaky electrons from the electron transport chain (130). The generation of ROS within the nerve causes nerve injury by triggering apoptosis in DRG neurons (117, 118, 138). Dyslipidemic Zucker fatty (*fa/fa*) rats with increased circulating nonesterified FAs had lower sensory nerve conduction velocities, tactile allodynia, and thermal and mechanical hypoalgesia associated with oxidative–nitrosative stress within the nerve (81).

Reducing oxidative–nitrosative stress within the peripheral nerves significantly improved nerve function (81). Fortunately, unsaturated FAs may provide therapeutic benefits by reducing oxidative stress in murine models of diabetic PN (144). Omega-3 PUFAs in Menhaden oil significantly lower neuronal oxidative stress by scavenging free radicals (48), which stabilizes mitochondrial function (7). This also prevents the generation of proinflammatory modulators (48). These results suggest that modulating the redox state may be a feasible approach for future PN therapeutics.

De Novo Lipogenesis

Although cells and tissues mainly obtain FAs from dietary sources (26, 87), nonessential FAs can be synthesized intracellularly through *de novo* lipogenesis. The canonical purpose of *de novo* lipogenesis is to generate FAs for triglyceride synthesis and energy storage (4).

In the first step of lipogenesis, ATP-citrate lyase converts citrate to acetyl-CoA (Fig. 4). Acetyl-CoA is then carboxylated to malonyl-CoA by the rate-limiting enzyme acetyl-CoA carboxylase, which is activated by insulin during the fed state and impaired by glucagon during the fasting state (128, 140). Finally, FA synthase (FASN) converts malonyl-CoA to palmitate, which can be incorporated into triglycerides. These *de novo* lipogenesis enzymes are under the tight regulation of two transcription factors: sterol regulatory element-binding protein (SREBP1) and peroxisome proliferator-activated receptor gamma (PPARγ), both of which are highly regulated by FAs (Fig. 4).

Regulation of FASN expression by SREBP1 and PPARγ, and therefore *de novo* lipogenesis, is sensitive to changes in FA levels (Fig. 4). Unsaturated, but not SFAs decrease the nuclear level of SREBP1 in a length-dependent and saturation-dependent manner (52). PUFAs decrease the expression of SREBP1 and FASN, which results in a decrease in lipogenesis (68) (Fig. 6). PPARγ activity is also regulated by numerous FAs that activate PPARγ to stimulate lipid uptake and maintain lipid homeostasis (42, 106). In addition, PPARγ cooperates with another nuclear receptor, liver X receptor (LXR), to regulate FA metabolism (64), a cross-talk that is promoted by unsaturated FAs (141) (Fig. 4). In murine models of prediabetes, LXR agonists protect DRG neurons from the SFA-induced endoplasmic reticulum stress response and reduced allodynia (46).

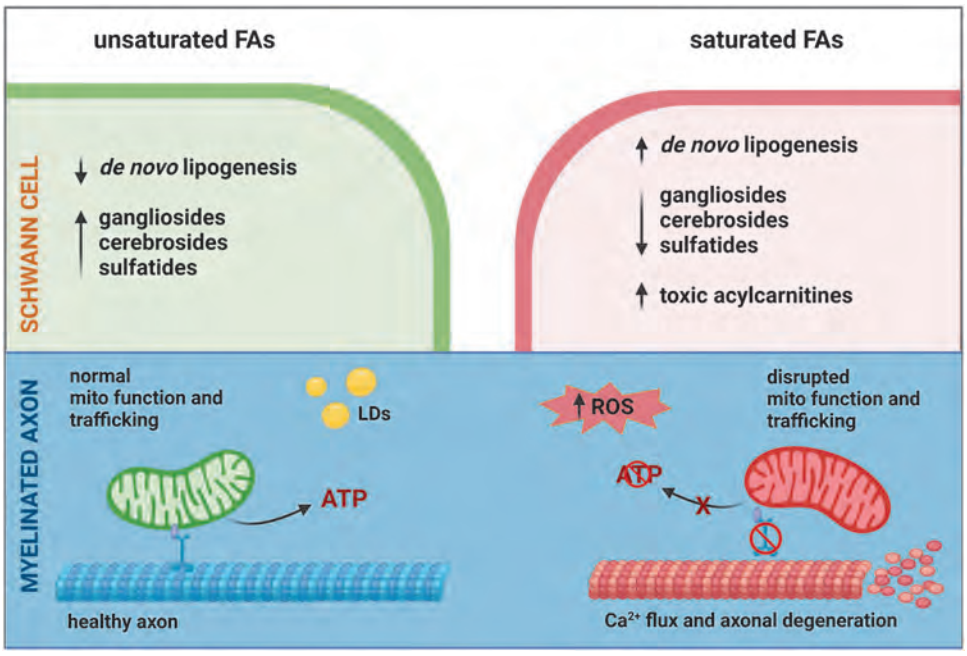


FIG. 6. The effect of saturated and unsaturated FAs on *de novo* lipogenesis, complex lipids, and mitochondria in neurons and SCs. Under basal conditions, *de novo* lipogenesis is not activated, and normal levels of complex sphingolipids, including glycosides, cerebrosides, and sulfatides, are maintained in SCs. Normal mitochondrial trafficking mechanisms, mitochondrial function, and ATP production also sustain DRG neuron health and function. Conversely, in dyslipidemia, elevated levels of SFAs trigger *de novo* lipogenesis and toxic acylcarnitine production in SCs, which can be shuttled to axons to contribute to axonal degeneration. In SCs, SFAs also lead to a decrease in complex sphingolipids essential for nerve function. In DRG neurons, SFAs impair mitochondrial trafficking mechanisms, mitochondrial function, and increase ROS. However, dietary supplementation with unsaturated FAs can potentially prevent mitochondrial bioenergetics and trafficking dysfunction by sequestering SFAs into lipid droplets. ROS, reactive oxygen species. Created with Biorender.com.

De novo lipogenesis is an essential process for myelinating SCs (87). SCs surround large-caliber axons, and produce myelin to insulate the axons and sustain saltatory conduction. Since myelination is an energetically expensive metabolic process, it requires careful coordination of RNA, protein, and lipid synthesis for membrane production (54, 86, 92, 103). FASN is critical for maintaining the lipid composition and myelination of the peripheral nerves (87).

In fact, SC-specific FASN depletion impairs PPARγ transcriptional regulation. Interestingly, increased FA uptake *via* rosiglitazone treatment compensated for insufficient FA synthesis in FASN-deficient mice. In line with these results, we have identified changes in PPARγ in prediabetic mice as well as an activation of PPARγ in the nerves of *db/db* mice receiving a rosiglitazone isomer, pioglitazone (58, 94). Therefore, altered *de novo* lipogenesis by insulin resistance and altered FA levels may contribute to PN in T2D and prediabetes.

Lipid Composition of DRG Neurons and Myelin

Careful coordination of FA uptake, β-oxidation, complex lipid synthesis, and *de novo* lipogenesis sustains the health and function of neurons and SCs within the peripheral nerves. Under homeostatic conditions, these lipid metabolic processes maintain the optimal proportion of lipids in the myelin and peripheral nerves to facilitate nerve conduction. Nerve tissue in the PNS contains free FAs, glycerolipids, sphingolipids, glycerophospholipids, cholesterol, and sterol lipids, which are uniquely distributed across each cell type (SC *vs.* neuron) and morphological localization (cell body *vs.* axon) (60, 93, 104).

These lipids are incorporated into membranes, lipid rafts, organelles, cytosolic lipid species, and myelin, and are critical for nerve function. To understand the pathogenesis of PN associated with T2D and prediabetes, it is critical to determine the effect of dietary FAs on peripheral nerve lipid composition. The FA properties of myelin can alter the speed and strength of sciatic motor nerve conduction (31), indicating that FAs associated with dyslipidemia may alter the nerve lipid composition and cause nerve injury. We contend that dyslipidemia alters the nerve lipidome, triggering injurious changes that affect myelination, sensory neuron function, and in turn, contribute to PN in T2D and prediabetes (94).

The myelin sheath is composed of 70%–85% lipids (104), and the major lipid classes in the myelin are 40% cholesterol, 40% phospholipids, and 20% glycolipids. The cholesterol and glycolipid levels are significantly higher in the myelin than in other tissues. Myelin phospholipid species include plasmalogen, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and sphingomyelin, while the glycolipids are mainly galactosylceramides.

Ethanolamine plasmalogen is the most abundant myelin phospholipid, but its role in the peripheral nerves is not fully understood. Several studies suggest that ethanolamine plasmalogen stabilizes the compact lipid structure in myelin membranes (80), prevents peroxidation of unsaturated myelin lipids (80), and mediates proper myelination within the peripheral nerves (27). This unique lipid profile is essential for maintaining myelin structure. Interestingly, many of these myelin lipids are altered in studies on the lipidomic alterations in whole nerves from HFD-fed mice (94).

Complex lipids within the myelin also contain a high level of VLC FAs compared with other tissues, which are important for maintaining the myelin integrity (22, 104, 123). Murine models deficient in ceramide synthase 2 (CerS2), the enzyme responsible for VLC sphingolipids synthesis, show that VLC sphingolipid deficiency is associated with severe multifocal detachment of myelin from the axon (65). Therefore, myelin integrity requires SC-mediated maintenance of VLC lipid levels within the myelin sheath. This study suggests that elevated levels of SFAs in dyslipidemia may impair CerS2 activity, thereby altering the level of VLC sphingolipids. Indeed, VLC glycosphingolipids including gangliosides (84), sulfatides (99), and galactosylceramides (99) were significantly depleted in murine models of T2D and prediabetes (Fig. 6).

Compared with myelin, the lipid content of DRG neurons is lower but plays an important role in sensory neuron function (10). The lipid composition of DRG neuron cell bodies and axons is distinct. Approximately 37% of the dry weight of the DRG neuron soma is lipid, consisting of 15.4% cholesterol, 4.8% galactolipid, and 57.1% phospholipids (10, 60). Fifteen percent of the dry weight of DRG axons is composed of lipids, which consists of 22.1% cholesterol, 7.7% galactolipid, and 56.4% phospholipid.

In both the DRG cell body and axons, the molar ratio of galactolipids was 2:1 with cerebrosides two times higher than that of sulfatides. Disruption of the DRG neuron lipidome in ApoE knockout mice is associated with severe peripheral sensory nerve defects and impaired electrophysiology (20), emphasizing the importance of the lipid composition for DRG neuron function.

Although the effect of dyslipidemia on complex lipid levels in DRG neurons is not completely understood, it is likely that dietary FAs also alter the complex lipid profile of DRG neurons in dyslipidemia. This contention is supported by evidence that SFAs and MUFAs profoundly and differentially effect sensory neurons both *in vivo* (120) and *in vitro* (112–114), and that IENFD is also modulated by the degree of FA saturation in HFD-fed mice (94). Specific sulfatide, cerebroside, and phospholipid species were also significantly reduced in DRG from diabetic mice. These studies suggest that elevated dietary FAs associated with dyslipidemia contribute to changes in complex lipid species within the nerve, and may contribute to PN in T2D and prediabetes.

Conclusions

Clinical studies have identified an association between PN and dyslipidemia in T2D and prediabetes, suggesting that FAs contribute to the development and progression of PN. Indeed, preclinical studies show that diets rich in injurious SFAs cause PN, while beneficial unsaturated FAs significantly improve nerve function in murine models of prediabetes and T2D. Peripheral nerve function is dependent on maintaining a specific lipid profile, which is regulated by dietary FA uptake, mitochondrial FA β-oxidation, oxidative stress, and *de novo* lipogenesis in DRG neurons and SCs (130). Molecular studies that determine the effect of SFAs and unsaturated FAs on these metabolic pathways and alterations in both neurons and SCs will greatly improve our understanding of the pathophysiological changes that underlie T2D and prediabetic PN.

Determining how these pathways alter the nerve lipidome in relation to PN in preclinical rodent models will provide insight into the lipid changes that underlie PN. In addition, axoglial interactions between DRG axons and SCs may regulate nerve function through the transfer of lipids or metabolites between the two cell types. Therefore, the biology of FAs is likely to play an important role in the molecular mechanisms that underlie PN in T2D and prediabetes.

Authors' Contribution

A.E.R. and E.L.F. planned, conceptualized, and wrote the article. A.E.R., B.K., and E.L.F. reviewed and edited the article. B.K. created the figures and contributed to writing the article. Each author reviewed, revised, and approved the submitted article.

Author Disclosure Statement

All authors declare no competing interests.

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Abbreviations Used

ACSL1 = long-chain acyl-CoA synthase 1
CD36 = FA translocase
CerS2 = ceramide synthase 2
CMT = Charcot Marie Tooth disease
CNS = central nervous system
DGAT2 = diacylglycerol acyltransferase 2
DHA = docosahexaenoic acid
DRG = dorsal root ganglion
EPA = eicosapentaenoic acid
FA = fatty acid
FABP = FA binding protein
FASN = FA synthase
FATP = FA transport protein
HFD = high-fat diet
IENFDs = intraepidermal nerve fiber density
LXR = liver X receptor
MetS = metabolic syndrome
MUFA = monounsaturated FA
PGC-1α = peroxisome proliferator-activated receptor coactivator 1 alpha
PMP2 = peripheral myelin protein 2
PN = peripheral neuropathy
PNS = peripheral nervous system
PPARγ = peroxisome proliferator-activated receptor gamma
PUFAs = polyunsaturated FAs
ROS = reactive oxygen species
SC = Schwann cell
SIRT1 = sirtuin 1
SREBP1 = sterol regulatory element-binding protein
STZ = streptozotocin
T1D = type 1 diabetes
T2D = type 2 diabetes
TCA = tricarboxylic acid cycle
TFAM = mitochondrial transcription factor A
VLC = very long chain
VLDL = very-low-density lipoproteins

Towards prevention of diabetic peripheral neuropathy: clinical presentation, pathogenesis, and new treatments

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Diabetic peripheral neuropathy (DPN) occurs in up to half of individuals with type 1 or type 2 diabetes. DPN results from the distal-to-proximal loss of peripheral nerve function, leading to physical disability and sometimes pain, with the consequent lowering of quality of life. Early diagnosis improves clinical outcomes, but many patients still develop neuropathy. Hyperglycaemia is a risk factor and glycaemic control prevents DPN development in type 1 diabetes. However, glycaemic control has modest or no benefit in individuals with type 2 diabetes, probably because they usually have comorbidities. Among them, the metabolic syndrome is a major risk factor for DPN. The pathophysiology of DPN is complex, but mechanisms converge on a unifying theme of bioenergetic failure in the peripheral nerves due to their unique anatomy. Current clinical management focuses on controlling diabetes, the metabolic syndrome, and pain, but remains suboptimal for most patients. Thus, research is ongoing to improve early diagnosis and prognosis, to identify molecular mechanisms that could lead to therapeutic targets, and to investigate lifestyle interventions to improve clinical outcomes.

Introduction

The prevalence of diabetes is increasing worldwide.[1] In 2019, 463 million people, or about 9·3% of the world's population, had either type 1 or type 2 diabetes and more than 4 million people died from diabetes-related complications.[1] Diabetes will affect an estimated 578 million people by 2030 and 700 million by 2045.[1] This growth is largely attributed to an increase in type 2 diabetes prevalence in ageing populations. Although diabetes prevalence will increase worldwide, the greatest increases are expected to occur in countries with economies transitioning from low-income to middle-income status, particularly in the Middle East and North Africa.[1] As the prevalence of diabetes increases, the burden of diabetes-related complications is also expected to grow.

Among diabetes complications, damage to the peripheral and autonomic nervous system is the most prevalent. Distal symmetric polyneuropathy, which presents as lower limb sensory loss followed by upper limb sensory loss, is the most common type of diabetic nerve damage. Distal symmetric polyneuropathy will be the focus of this Review and will be referred to as diabetic peripheral neuropathy (DPN). The effects of DPN on patient morbidity and quality of life are substantial; it predisposes to falls and superficial injuries, which can lead to infection and amputation.[2] Individuals with diabetes and DPN are at higher risk of all-cause and cardiovascular mortality than are individuals with diabetes but without DPN.[3]

Unfortunately, DPN management remains suboptimal. In people with type 1 diabetes, glucose control slows DPN progression.[4] However, in type 2 diabetes, glucose control only marginally affects DPN progression, especially when patients have metabolic syndrome. Therefore, managing DPN in people with type 2 diabetes currently revolves around weight loss and exercise to mitigate the metabolic syndrome.[5] Early intervention might slow DPN progression, making timely diagnosis crucial. In this Review, we will cover advances in epidemiology, clinical presentation, and diagnosis of DPN, highlighting emerging approaches for early detection. We will also outline new evidence on DPN pathophysiology. Additionally, we will address treatment and the research approaches into lifestyle interventions that could improve patient outcomes, as well as the obstacles that must be overcome to secure these outcomes.

Epidemiology and risk factors

Studies of DPN incidence and prevalence in people with diabetes most frequently use standardised measures of neuropathy, which combine symptoms and signs from physical examination findings. Cross-sectional and cohort studies done since 2017 have reported a DPN incidence of about 8·8 cases per 1000 person-years in individuals with type 1 diabetes[6] and 24–26·9 cases per 1000 person-years in individuals with type 2 diabetes[6–9] (table 1). A recent worldwide meta-analysis (29 studies, with 50 112 participants) found that individuals with type 2 diabetes had a higher DPN prevalence (31·5% [95% CI 24·4–38·6]) than those with type 1 diabetes (17·5% [13·1–36·5]).[18] Because diabetes duration is a strong DPN risk factor, DPN is present in fewer adolescents than adults.[9,11] However, the US SEARCH study reported substantial age-adjusted DPN prevalence in adolescents (type 2 diabetes, 17·7% *vs* type 1 diabetes, 8·5%).[11] Furthermore, DPN prevalence varies by country and can range from 1% to 80%.[16] This large variation probably arises from multiple factors, including disease severity, diabetes duration, DPN definition, and comorbid conditions predisposing to neuropathy, especially the metabolic syndrome.

Diabetes is the strongest risk factor for DPN, along with disease characteristics, such as diabetes duration and severity, measured by haemoglobin A_{1C} levels.[20,21] Additionally, several studies suggest an association between glycaemic variability and DPN presence,[22]

	Country	Study type and population	DPN measure	Prevalence
Abdel-Motal et al (2017)[10]	Algeria, Bahrain, Egypt, Libya, Jordan, Morocco, South Africa, and Sudan	Systematic review of 2243 people with type 1 diabetes	Multiple DPN measures	18·0%
Dabelea et al (2017)[9]	USA	Cross-sectional study of 1746 people with type 1 diabetes (mean age 17·9 years [SD 4·1]) and 272 young people with type 2 diabetes (mean age 22·1 years [SD 3·5])	MNSI-E	Type 1 diabetes, 8·5%; type 2 diabetes, 17·7%
Jaiswal et al (2017)[11]	USA	Cohort study of 1734 young people with type 1 diabetes (mean age 18·0 years [SD 4·0]) and 258 young people with type 2 diabetes (mean age 22·0 years [SD 3·5])	MNSI-E	Type 1 diabetes, 7·0%; type 2 diabetes, 22%
Cardinez et al (2018)[12]	Canada	Longitudinal study of 361 people with type 1 diabetes older than 50 years	MNSI-Q	42·7%
Ponirakis et al (2019)[13]	Qatar	Cross-sectional study of 1095 people with type 2 diabetes	Douleur Neuropathique en 4 questions	34·5%
Christensen et al (2020)[14]	Denmark	Longitudinal cohort study of 5249 people with type 2 diabetes	MNSI-Q	17·9%
Jeyam et al (2020)[15]	Scotland	Cohort study of 5558 people with type 1 diabetes	MNSI-Q	13%
Lu et al (2020)[16]	Argentina, Bangladesh, China, Germany, India, Italy, Kenya, Mexico, Pakistan, Poland, Russia, Serbia, Uganda, and Ukraine	Cross-sectional study of 2733 people with type 2 diabetes in clinics	Sensory symptoms for >3 months	Overall, 26·7%; Kenya, 0·6%; Ukraine, 79·6%
Mizokami-Stout et al (2020)[17]	USA	Cohort study of 5936 people with type 1 diabetes	MNSI-Q	11%
Sun et al (2020)[18]	Australia, Bangladesh, China, France, India, Iran, Malaysia, Nigeria, Sri Lanka, Sweden, Taiwan, Turkey, UK, and USA	Meta-analysis of 50 112 participants (comprising people with type 2 diabetes and healthy controls)	Multiple DPN measures	Type 1 diabetes, 17·5%; type 2 diabetes, 31·5%
Amutha et al (2021)[6]	India	Longitudinal cohort study of 3252 people with type 1 diabetes and 889 people with type 2 diabetes	Vibratory perception threshold	Incidence of type 1 diabetes, 8·8 cases per 1000 person-years; incidence of type 2 diabetes, 24·0 cases per 1000 person-years
An et al (2021)[7]	USA	Retrospective chart review of 135 119 people with type 2 diabetes in one health system	Medical diagnosis codes	Incidence: 26·9 cases per 1000 person-years
Aronson et al (2021)[8]	Canada	Cross-sectional study of 471 people with type 1 diabetes and 3903 people with type 2 diabetes	Modified Toronto Clinical Neuropathy Score	Type 1 diabetes, 16·7%; type 2 diabetes, 29·3%
TODAY Study Group (2021)[19]	USA	Cohort study of 674 young people with type 2 diabetes (mean age 14·0 years (SD 1·9)	MNSI-E, MNSI-QE	34·9%

DPN=diabetic peripheral neuropathy. MNSI-E=Michigan Neuropathy Screening Instrument Examination. MNSI-Q=Michigan Neuropathy Screening Instrument Questionnaire. MNSI-QE=Michigan Neuropathy Screening Instrument Questionnaire and Examination.

Table 1: **Prevalence and incidence of diabetic peripheral neuropathy, as reported by studies published since 2017**

although there is some discordance in the findings.[23] The UK Prospective Diabetes Study Group found that intensive glucose control lowered the relative risk of DPN onset in patients with type 2 diabetes, assessed by biothesiometer, but only after a 15-year follow-up;[24] however, substantial attrition was reported as a limitation in this study. The Rio de Janeiro Type 2 Diabetes Cohort Study found that glycaemic variability did not correlate with incident DPN, determined by clinical examination and neuropathic symptoms, over a median 9·3-year follow-up, although it did correlate with a composite outcome of both incident and worsening DPN symptoms.[25] Moreover, nerve damage might begin long before individuals with hyperglycaemia develop overt diabetes, and a growing body of evidence supports an association between prediabetes and early small-fibre symptoms.[26]

Control of hyperglycaemia affects DPN progression in patients with type 1 diabetes.[4] However, overall, glucose control might only moderately affect DPN onset and progression in patients with type 2 diabetes, suggesting the presence of additional risk factors. Clinical research has identified the metabolic syndrome as the crucial risk factor, which comprises, in addition to elevated fasting

	Country	Population	DPN measure	Findings
Hanewinckel et al (2017)[30]	Netherlands	Rotterdam Study of 908 participants	Symptom questionnaire, neurological examination, nerve conduction study	BMI independently associated with lower sural sensory nerve action potential (OR 1·53 [95% CI 1·13 to 2·09]) and peroneal compound motor action potential (OR 1·49 [1·11 to 1·99]) amplitudes
Jaiswal et al (2017)[11]	USA	SEARCH for Diabetes in Youth study of 1734 young people with type 1 diabetes and 258 young people with type 2 diabetes	MNSI-E; MNSI-Q	Higher LDL cholesterol, triglycerides, obesity, diastolic blood pressure, and lower HDL cholesterol were risk factors in young people with type 1 diabetes; lower HDL was a risk factor in young people with type 2 diabetes; poor glycaemic control correlated with DPN in type 1 diabetes (OR 1·53 [95% CI 1·24 to 1·88] but not in young people with type 2 diabetes (OR 1·05 [0·7 to 1·56])
Andersen et al (2018)[29]	Denmark	Danish arm of the Anglo-Danish-Dutch study of Intensive Treatment of Diabetes in Primary Care (ADDITION) of 1256 participants with type 2 diabetes without DPN at baseline	MNSI-Q	Baseline weight (HR 1·09 [95% CI 1·03–1·16]), waist circumference (HR 1·14 [1·05 to 1·24]), BMI (HR 1·14 [1·06 to 1·23]), log_2(methylglyoxal; HR 1·45 [1·12 to 1·89]), HDL cholesterol (HR 0·82 [0·69 to 0·99]), and LDL cholesterol (HR 0·92 [0·86 to 0·98]) were significantly associated with incident DPN
Callaghan et al (2018)[20]	China	Study of 4002 participants in Pinggu, China; 37·2% normoglycaemic, 18·9% with diabetes, and 44·0% prediabetic	MNSI-E; MNSI-Q	Diabetes (OR 2·60 [95% CI 1·77 to 3·80]), weight (OR 1·09 [1·02 to 1·18]), and the number of metabolic syndrome components (OR 1·17 [1·03 to 1·32]) were significantly associated with DPN
Kurisu et al (2019)[31]	Japan	625 participants (69% normoglycaemic, 12% with diabetes, and 19% prediabetic)	Interview, Achilles tendon reflexes, quantitative vibration threshold, nerve conduction study	Type 2 diabetes (OR 3·65 [95% CI 1·68 to 7·93]) and dyslipidaemia (OR 0·53 [0·30 to 0·96]) were significantly associated with DPN in multivariable models, but not prediabetes (OR 1·47 [0·69 to 3·12]) or waist circumference (OR 1·02 [0·95 to 1·10]); no parameters were associated with DPN when participants with type 2 diabetes were excluded
Schlesinger et al (2019)[32]	Germany	Cooperative Health Research in the Region of Augsburg (KORA) F4/FF4 cohort of 513 participants	MNSI-E	Overweight (OR 3·06 [95% CI 1·57 to 5·97]), obese (OR 3·47 [1·72 to 7·00]), and waist circumference (OR 1·22 [1·07 to 1·38]) were significantly associated with DPN in multivariable models; interaction analyses did not reveal any differences by diabetes status
Callaghan et al (2020)[33]	USA	University of Michigan bariatric surgery clinic study of 138 obese participants	Primary, Toronto consensus definition of probable DPN; secondary, IENFD in distal leg and four nerve conduction studies in sural, tibial, and ulnar nerves	BMI was comparable in obese participants with and without DPN (p=0·86); waist circumference (OR 1·39 [95% CI 1·10 to 1·75]), triglycerides (OR 1·31 [1·00 to 1·70]), systolic blood pressure (OR 2·89 [1·49 to 5·61]) were significantly associated with DPN
Callaghan et al (2020)[34]	USA	University of Michigan bariatric surgery clinic study of 138 obese participants	Primary, Toronto consensus definition of probable DPN; secondary, IENFD in distal leg and one nerve conduction study in sural nerve	Waist circumference (–1·48 [95% CI –2·38 to –0·57]), HDL cholesterol (–3·38 [–6·38 to –0·37]), and systolic blood pressure (2·30 [0·12 to 4·48]) were significantly associated with cognitive decline by National Institutes of Health Toolbox composite, after adjusting for age, Wide Range Achievement Test 4, and education level
Christensen et al (2020)[14]	Denmark	Danish Centre for Strategic Research in Type 2 Diabetes (DD2) cohort of 5249 participants with type 2 diabetes	MNSI-Q; Douleur Neuropathique en 4 Questions	In regression analyses, central obesity (waist circumference, waist-to-hip ratio, and waist-to-height ratio) was associated with DPN; triglycerides (≥1·7 mmol/L, aPR 1·36 [95% CI 1·17 to 1·59]), HDL cholesterol (<1·0 mmol/L for male, 1·2 mmol/L for female, aPR 1·35 [95% CI 1·12 to 1·62]), high-sensitivity C-reactive protein (≥3·0 mg/L, aPR 1·66 [95% CI 1·42 to 1·94]), and HbA_{1c} (≥78 mmol/mol [9·3%], aPR 1·42 [95% CI 1·06 to 1·88] were significantly associated with DPN
Reynolds et al (2020)[21]	India	Chennai study of 652 participants (20% normoglycaemic, 45% with diabetes, and 35·5% prediabetic)	Primary, MNSI combined index; secondary, MSNI-E, MNSI-Q, monofilament, biothesiometer	DPN prevalence increased with poorer glycaemic status (p<0·01), but not with number of metabolic syndrome components; in normoglycaemic participants, neuropathy prevalence increased as the number of metabolic syndrome components increased (p=0·04); diabetes (OR 3·41 [95% CI 1·28 to 9·11]), but not waist circumference (OR 1·00 [0·88 to 1·14]) significantly associated with DPN in multivariable models
van der Velde et al (2020)[35]	Netherlands	Maastricht Study of 2401 participants (59% normoglycaemic, 25% with diabetes, and 15·4% prediabetic)	Nerve conduction studies, vibration perception threshold, Douleur Neuropathique en 4 Questions	Fasting blood glucose was associated with worse peroneal (–0·17 [95% CI –0·21 to –0·13]) and tibial –0·18 [–0·23 to 0·14]) nerve conduction velocities. Larger waist circumference was associated with lower sural (0·08 [–0·13 to –0·02]) sensory nerve conduction velocity and higher vibration perception threshold (0·08 [0·04 to 0·13]); triglycerides, HDL cholesterol, LDL cholesterol, and systolic blood pressure were not associated with DPN.
Today Study Group (2021)[19]	USA	Treatment Options for type 2 Diabetes in Adolescents and Youth (TODAY) study of 674 young people with type 2 diabetes	MNSI-E; MNSI-Q	BMI (per 5 kg/m^2; HR 1·28 [95% CI 1·15 to 1·43]), HbA_{1c} (HR 1·26 [1·14 to 1·40]), male sex (HR 1·81 [1·25 to 2·62]), and age (HR 1·11 [1·01 to 1·21]) were significantly associated with DPN in multivariable models

We used the search terms "diabetic peripheral neuropathy" with "obesity, metabolic syndrome" and "risk factors, metabolic syndrome". We selected studies with more than 100 participants with type 1 diabetes, type 2 diabetes, or combined participants published since 2017. DPN=diabetic peripheral neuropathy. OR=odds ratio. MNSI-E=Michigan Neuropathy Screening Instrument Examination. MNSI-Q=Michigan Neuropathy Screening Instrument Questionnaire. LDL=low-density lipoprotein cholesterol. HDL=high-density lipoprotein cholesterol. HR=hazard ratio. IENFD=intraepidermal nerve fibre density. HbA_{1c}=haemoglobin A_{1c}. aPR=adjusted prevalence ratio.

Table 2: **Risk factors for diabetic peripheral neuropathy that are related to the metabolic syndrome, as identified from clinical studies published since 2017**

glucose, obesity, dyslipidaemia (high triglycerides, low high-density lipoprotein [HDL]), and hypertension. Obesity measured centrally by waist circumference or overall by BMI especially increases DPN risk, in both the context of diabetes and independent of glycaemia in adults[14,27–29] and in young people (table 2).[11,19]

Other risks factors are low HDL levels[11,14,28,29] and higher weight,[20,29] high triglycerides,[14,33] high blood pressure,[33] high concentrations of serum biomarkers of inflammation,[14] and high oxidative stress (ie, methylglyoxal).[29] Furthermore, the presence of more metabolic syndrome components increases the likelihood of DPN, particularly small-fibre symptoms, and shortens the time to symptom onset, suggesting a possible dose–response relationship in people with prediabetes[20,28] and diabetes.[21] Other independent risk factors include age and lack of physical activity.[14,27] Importantly, although the metabolic syndrome components are more frequent in type 2 diabetes than in type 1 diabetes, these components also increase DPN risk in individuals with type 1 diabetes.[11,36] Statin use has previously been reported to increase DPN risk; however, in a large nationwide registry done in Denmark, statin use was not linked to increased risk of DPN.[37]

A newly emerging area is the role of genetic risk factors in DPN. Although predominantly a metabolically acquired neuropathy, DPN is multifactorial and polygenic[38] risk factors are being identified. Single nucleotide polymorphisms (SNPs) that predispose to or are protective for DPN have been identified in metabolism and vasculature genes, but validation studies are needed. Genome-wide association studies (GWAS) have identified potential SNP risk modifiers of DPN.[39,40] For example, SNPs to mitogen-activated protein kinase 14 have been linked to DPN (rs3761980, rs80028505)[39] and foot ulcers (rs80028505)[40] in two independent cohorts of European descent. A multi-ancestry meta-analysis of data from patients with type 2 diabetes (n=228 499) versus healthy controls (n=1 178 783) resulted in a polygenic risk score, which was moderately, but significantly, associated with DPN.[38]

Epigenetic determinants of DPN are another emerging research avenue.[41,42] The epigenome is modifiable through environmental cues, including metabolism, and might constitute a mechanism of metabolically acquired DPN. In individuals with type 2 diabetes and DPN, there is decreased peripheral nerve genomic DNA methylation and loci-specific differential DNA methylation, supporting a role for epigenetic regulation in neuropathy development.[42] The role of DNA methylation in DPN development in people with type 1 diabetes is less clear.[41] Another putative but preliminary research area is the genetics and epigenetics of microRNAs in DPN.[43]

Overall, metabolic parameters—encompassing both diabetes and the metabolic syndrome—are strong DPN risk factors. Genetic and epigenetic factors remain in the research domain, although studies are starting to clarify their role in DPN development.

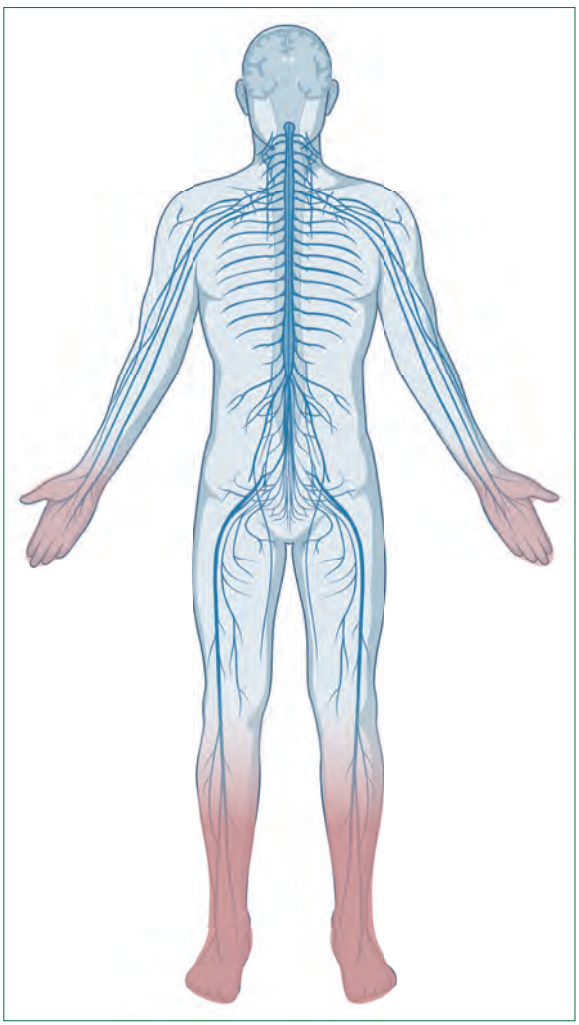

Figure 1: **Clinical presentation of diabetic peripheral neuropathy**
The most common manifestation of diabetic peripheral neuropathy is a distal symmetric polyneuropathy, which manifests in the lower limbs first, followed by the upper limbs in a so-called stocking-glove configuration. Signs and symptoms start in the toes (darker pink) and progress proximally towards the calves, at which point, nerve injury occurs in the fingers and moves up to encompass the hands (lighter pink).

Clinical presentation and diagnosis

Diabetes can cause several patterns of peripheral nerve injury, including DPN, autonomic neuropathy, radiculoplexus neuropathy, radiculopathy, and mononeuropathy. DPN, the focus of this Review, is the most common (figure 1).[44] DPN usually presents with sensory symptoms, which begin symmetrically in the toes and slowly advance up to the calves, before beginning in the fingers followed by the arms. Symptoms encompass numbness and tingling, and, in some patients, pain (burning, stinging, shooting, or deep aching). Neurological examination can reveal decreased sensation to multiple modalities, including vibration, pinprick, and proprioception, following the same distribution pattern as the symptoms. Some patients have symptoms (pain) and

Panel 1: Diagnosis of diabetic peripheral neuropathy

Diagnostic methods in routine clinical use

Clinical history

Diabetes is the most common cause of peripheral neuropathy. Long diabetes duration, uncontrolled diabetes (high levels of haemoglobin A_{1c}), obesity, dyslipidaemia, hypertension, and age are risk factors of DPN.

Clinical presentation

Positive symptoms (eg, tingling, burning, and lancinating pain) and negative sensations, such as numbness, usually occur in a symmetric distal-to-proximal formation, starting in the feet up to the calves, and then progressing to the fingers.

Physical examination

Temperature or pinprick sensation tests assess small fibre neuropathy. Vibration tests assess large fibre neuropathy. Monofilament tests assess ulcer risk. Small fibre neuropathy usually precedes large fibre neuropathy. Isolated small and large fibre neuropathy can also occur.

Methods for clinical diagnosis of atypical presentations or in research settings

Nerve conduction studies

Electrodiagnostic measures of nerve conduction velocities in sensory (sural, peroneal, tibial) and motor (peroneal, tibial) nerves using surface or needle electrodes.[44] Nerve conduction velocities and amplitudes drop with progressive large fibre neuropathy. Abnormality (velocity or amplitude) in the sural nerve with at least one other nerve conduction abnormality signifies large fibre neuropathy. Alternatively, a composite Z-score summation from multiple nerves is compared with normative values.

Intraepidermal nerve fibre density

Intraepidermal nerve fibre density is the gold standard for small fibre neuropathy.[44] Immunohistochemical tests can be done on skin punch biopsy, generally on the distal leg. Stained small fibres are counted and compared with normative values. Intraepidermal nerve fibre density drops with progressive small fibre neuropathy. Morphological (eg, fibre length, branching, axonal swellings), and molecular (eg, substance P, calcitonin gene-related peptide, and growth associated protein 43) changes can be assessed.

Diagnostic criteria

Hierarchical classification schemes rate the degree of certainty in the diagnosis, ranging from possible, to probable, to definite, using a combination of signs and symptoms (eg, the Toronto Consensus Panel).[44]

Diagnostic methods in research settings

Corneal confocal microscopy

Non-invasive imaging of corneal fibres, which are counted and compared with normative values.[44] Corneal confocal microscopy can also assess fibre length and branching. Studies suggest that decline in nerve fibre density can correlate with DPN progression. Sensitivity is 60·0–91·0% and specificity is 40·0–87·0%.[46]

Hand-held electrodiagnostic device

A handheld point-of-care device, which measures sural nerve conduction velocity and response amplitude in a few minutes.[47] Sensitivity is 84·3–90·5% and specificity ranges from 68·3 to 86·1%.

Sticker sweat detector

A sticker affixed to the plantar surface of the foot, which measures moisture (sweat) by turning from blue to pink.[47] Sensitivity ranges from 65·1 to 100·0% and specificity from 32·0 to 78·5%.

Instrument sweat detector

Measures conductance of chloride ions from sweat released from hands and soles of the feet after electrical stimulation of sweat glands. Measures sudomotor function in a few minutes.[47] Sensitivity is 87·5% and specificity 76·2%.

Diagnostic methods for painful DPN in research settings

Quantitative sensory testing

Standardised protocols can measure the response to well-defined sensory stimuli.[44] Parameters include thermal and mechanical detection; pain, vibration, and pressure pain thresholds; dynamic mechanical allodynia; and wind-up ratio, which assess function in all fibre types (Aβ large myelinated, Aδ thinly myelinated, and C unmyelinated).

Microneurography

A needle electrode measures spontaneous activity and stimulus-evoked of unmyelinated C fibres in peripheral nerves, usually peroneal.[48] Irregular so-called saw-tooth baselines in abnormal nerves have been observed. This is a labour-intensive technique, which requires cross-laboratory validation.

Hoffman-Reflex rate-dependent depression

Differentiates pain of spinal disinhibition origin from pain of peripheral origin by measuring the deep tendon reflex response neural pathways.[48]

Functional brain imaging (fMRI)

fMRI uses different protocols, including the BOLD response, arterial spin labelling, and connectivity analysis. This type of imaging relates these changes to both spontaneous and evoked pain states, illustrating the distributed cortical network involved in the discriminative and affective pain components, as well as the descending pain modulatory system.

signs (decreased pinprick sensation) attributable to injury of unmyelinated nerves, known as small fibres. Other patients experience symptoms (numbness) and signs (decreased vibration and proprioception) attributable to injury of large, myelinated nerves, known as large fibres. Pressure is perceived via thinly myelinated fibres, which fall under the large fibre category. Most patients exhibit both small and large fibre involvement. Motor signs and

***Panel 2*: Scales and questionnaires for clinical management of diabetic peripheral neuropathy**

Clinical scales

These scales measure a combination of signs and symptoms and are used to screen for and monitor diabetic peripheral neuropathy (DPN) progression.[49]

Diabetic Neuropathy Examination Score

Eight-item examination tool that consists of muscle strength; muscle jerk reflex at the triceps; pinprick in the index finger and great toe; and vibration, joint position, and touch in the great toe.

Michigan Neuropathy Screening Instrument Examination (MNSI-E)

A physical exam consisting of great toe vibration with a 128-Hz tuning fork, muscle jerk reflex at the ankle joint, monofilament testing, and foot exam (appearance, ulcerations).

Modified Toronto Clinical Neuropathy Score (mTCNS)

Includes six questions regarding impact of symptoms on daily living and examination of the loss of sensation to five modalities (touch, pinprick, temperature, vibration, and proprioception) in lower extremities. The mTCNS is a modification of the Toronto Clinical Neuropathy Score, which included ankle and knee jerk reflexes and did not include gradation of symptom impact or severity of sensory loss.

Neuropathy Impairment Score (NIS)

Examination-based measure that includes muscle strength in 24 muscle groups; jerk reflexes at the biceps, triceps, brachioradialis, quadriceps, and ankle; and sensation to touch, vibration, joint position in the index finger and great toe. The NIS-LL is a condensed measure focused on the lower extremities. The NIS-LL+7 was developed specifically to measure impairment in DPN and includes nerve conduction studies of sural, tibial, and peroneal nerves, vibratory threshold at the great toe using quantitative sensory testing, and heart rate response to deep breathing.

Total Neuropathy Score

Multimodality measure that includes history of sensory, motor, and autonomic symptoms; strength at major muscle groups; muscle jerk reflex at the ankle joint; vibratory threshold at the great toes bilaterally and right index finger using quantitative sensory testing; and nerve conduction studies of the bilateral sural nerves, right common peroneal, and bilateral posterior tibial nerves.

Utah Early Neuropathy Score

Examination-based measure that includes muscle strength at the great toes; extent of sensory loss to pinprick and vibration in the lower extremities; proprioception at the great toes; muscle jerk reflexes at the ankle joints; and presence of allodynia in the toes or foot.

Screening and assessment scales

Douleur Neuropathique en 4 Questions

Includes questions related to history of seven types of pain, loss of sensation to touch and pinprick, and hyperesthesia.[50]

Leeds Assessment of Neuropathic Symptoms and Signs Pain Scale

Includes five questions related to type of pain and associated signs and examination of decreased sensation to pinprick and hyperesthesia.[50]

Michigan Neuropathy Screening Instrument Questionnaire (MNSI-Q)

Includes 15 questions related to history and neuropathy symptoms.[50]

symptoms are much less common than are sensory symptoms, although motor involvement is often seen on electrodiagnostic testing.

The American Diabetes Association recommends evaluation of DPN in patients with type 2 diabetes at diagnosis and in patients with type 1 diabetes 5 years after diagnosis, and then annually thereafter for both patients with type 1 and type 2 diabetes.[5] Screening should comprise a detailed clinical history and examination, including assessment of temperature or pinprick sensation (small fibre), vibratory sensation (large fibre), and ability to perceive pressure using a 10 g monofilament (large fibre). Electrodiagnostic testing and referral to a neurologist are rarely needed, except for patients presenting with atypical features (eg, rapid onset, asymmetrical signs, non-length dependent neuropathy, or pronounced motor complications).

Clinical history and examination are the mainstays of clinical diagnosis. However, confirmatory testing is available for large fibre (electrodiagnostic) and small fibre (intraepidermal nerve fibre density) nerve injury. Electrodiagnostic tests have good test characteristics, with areas under the curve (AUCs) ranging from 0·76 to 0·90,[45] but defining the parameters that constitute an atypical test is challenging. Moreover, they do not assess small fibre involvement. Intraepidermal nerve fibre density has good test characteristics (AUCs 0·75 to 0·82)[45] but is invasive. Furthermore, many countries do not have the facilities to perform intraepidermal nerve fibre density assessments, and, where available, whether commercial laboratories perform comparably to academic centres remains uncertain. Overall, confirmatory testing is not usually needed for clinical management, but is used in clinical research and is helpful in the diagnosis of patients with atypical presentations (panel 1).

In addition to these diagnostic tests, clinical scales are also available to identify and monitor patients with neuropathy (panel 2). At least 18 such scales exist, mostly assessing similar domains (eg, sensory [large and small fibre], motor, autonomic and reflexes), but attribute variable weight to these domains.[49,51] Studies show that many of these scales have similar diagnostic characteristics regarding sensitivity and specificity. Similar to the aforementioned confirmatory tests, the diagnostic characteristics of clinician-rated scales are good.[52] A limitation of

the scales is the need for trained personnel for administering tests. However, the performance of these clinical scales is comparable to that of diagnostic testing, but without the associated costs, time, and discomfort.[45]

At least five neuropathic pain questionnaires are also available to identify patients with DPN experiencing neuropathic pain (panel 2).[53] Pain is often underreported and undertreated; therefore, identifying painful DPN is crucial for clinical management.[44,54] Importantly, not all pain in these patients is neuropathic in nature; therefore, distinguishing neuropathic pain (ie, pain arising from lesions or disease affecting the somatosensory nervous system) from other pain generators, such as joint pain, can inform clinical management.

DPN, and peripheral neuropathy more broadly, often remain undiagnosed. The reasons are manifold, but the lack of systematic and widespread screening contributes to this problem. Moreover, early diagnosis might improve prognosis, but current diagnostic methods are of suboptimal sensitivity. Therefore, new diagnostic tests for DPN are needed and should be rapid, sensitive, specific, and inexpensive. Emerging tests include corneal confocal microscopy and various point-of-care or rapid devices (panel 1).[47] Corneal confocal microscopy is less invasive than intraepidermal nerve fibre density and has acceptable diagnostic accuracy in most studies.[46] However, the test requires specialised personnel and equipment and, as a result, is primarily used in research settings. A hand-held electrodiagnostic device that measures nerve conduction velocity is quick, reliable, and inexpensive, and might have diagnostic accuracy nearly equal to nerve conduction studies.[47] A sticker developed for detecting sweat has not been studied as extensively as the hand-held electrodiagnostic device, but early results indicate its diagnostic test characteristics are modest at best.[47] Similarly, studies to date do not support an instrument designed to detect sweat as a reliable test to diagnose neuropathy.[47] In summary, although new diagnostic tests are being developed and tested, none is currently ready for clinical use.

DPN and cognitive impairment

Several clinical studies show that diabetes[55] and obesity[56] predispose individuals to cognitive impairment and dementia (eg, Alzheimer's disease) later in life. Even early in life, people with diabetes[55] or obesity[34] can have subtle, but detectable decline in cognition. These findings suggest that shared pathological processes might occur in the peripheral nervous system (PNS) and CNS. As in DPN, mitochondrial dysfunction and bioenergetic failure also occur in Alzheimer's disease.[57] Moreover, some risk factors are shared between DPN and cognitive impairment, and encompass both glucose and lipid metabolism.[55,56] In clinical studies of patients with type 1 diabetes or type 2 diabetes, DPN is a risk factor for cognitive impairment[58,59] and brain structural changes.[60] The possibility of shared or similar neurological damage in the periphery and centrally in diabetes is intriguing

DPN management

DPN prevention and treatment can be challenging. Although there is a salutary effect of glycaemic control for preventing DPN in patients with type 1 diabetes, multiple clinical trials have failed to show a similar effect of this intervention in patients with type 2 diabetes.[4] The guidelines from the American Diabetes Association for treating DPN recommend adding a healthy diet and exercise to glycaemic control as crucial therapeutic interventions for patients with type 2 diabetes with DPN. Thus, while glycaemic control is probably important in patients with type 2 diabetes, other interventions are also needed as standard of care.[5]

With respect to dietary modifications as a first-line treatment, weight loss improves the metabolic syndrome, making it an attractive intervention for DPN in patients with type 2 diabetes. Recent studies demonstrate the importance and limitations of this intervention. The Look Ahead study randomly assigned 5145 patients with diabetes to an intervention primarily focused on dietary weight loss for 9–11 years.[61] Dietary weight loss ameliorated Michigan Neuropathy Screening Instrument (MNSI) questionnaire scores, but did not change MNSI examination scores. Similarly, an observational study by our group demonstrated that a dietary weight loss intervention in severely obese participants (n=131, mean baseline BMI 40·8 kg/m²) had the same findings, with improved MNSI questionnaire scores but a stable MSNI examination.[62] Therefore, weight loss is a promising intervention for DPN,[63] but further improvements might require earlier or different intervention strategies. Exercise studies have been more limited, with smaller cohorts or without randomisation. Despite these limitations, studies have shown that patients with type 2 diabetes and DPN can show improvement in intraepidermal nerve fibre density with exercise.[64] Notably, exercise interventions lead to only minimal weight loss in patients with diabetes, indicating that, if exercise is effective in improving DPN, the mechanisms behind such improvements are possibly independent of weight loss.

There are no effective pharmacological interventions for DPN, although sodium-glucose cotransporter (SGLT)-2 inhibitors provide a novel direction. SGLT-2 inhibitors block glucose resorption in the kidneys, increasing glucose excretion, which lowers blood glucose concentrations. SGLT-2 inhibitors improve cardiovascular outcomes in patients with diabetes,[65] and recent animal studies indicate that SGLT-2 inhibitors could be promising for improving neuropathy outcomes, although they might be more effective in the setting of type 1 diabetes than in type 2 diabetes.[66] However, further studies on SGLT-2 inhibitors are warranted.

Given the lack of disease-modifying therapies, the clinical management of patients with DPN is focused on educating patients about the importance of good foot care, appropriate shoe wear, and an annual foot examination.[2] Another essential component of DPN management is the control of pain. Four drug classes are effective for painful DPN, including serotonin norepinephrine reuptake inhibitors, tricyclic antidepressants, gabapentinoids, and sodium channel blockers.[67] All these medications have similar effect sizes and differences across classes are minimal. Therefore, choosing a neuropathic pain medication should focus on factors beyond efficacy, including tolerability, contraindications, and cost (figure 2). Topical medications are also available, and capsaicin is the best studied.[67] Effect sizes for capsaicin are comparable with those of oral therapies. Overall, the effect sizes of all these medications are small, and only about one in seven patients with painful DPN experience pain relief,[44] emphasising the need for more effective pain interventions.

Behavioural interventions also exist for treating painful DPN. Exercise, cognitive behavioural therapy, and mindfulness have all been studied with early promising results.[69] Although definitive trials are needed, behavioural interventions might provide another avenue for treating pain, especially given the emerging evidence in other chronic pain conditions, such as fibromyalgia.[70] Combination therapy with a behavioural and a pharmacological intervention might be warranted to address painful DPN that is not responsive to one treatment modality alone.

Surgical interventions are also available for treating painful DPN. Unfortunately, the role of spinal cord stimulation remains unclear despite a recent randomised clinical trial of 216 participants.[71] Although 79% of participants responded in the intervention arm versus only 5% in the control arm, the results must be interpreted with great caution because this trial was an open label study without sham surgery in the control group. Future surgical intervention studies for painful DPN will need to include a sham control and masking to understand what role, if any, surgery has for treating painful DPN.

Although opioids, including tramadol and tapentadol, effectively reduce pain in DPN patients in the short term, there is no information regarding their long-term efficacy. Moreover, painful DPN is chronic, and long-term opioid use can be harmful and lead to dependence, drug overdoses, and death,[67,68] and should be avoided. Furthermore, multiple studies show that opioid treatment is common in patients with painful DPN, despite its lack of efficacy.[72,73] Many physicians prescribe tramadol or tapentadol as alternatives to other opioids, but recent evidence suggests that they have the same harmful long-term effects.[74] This evidence has led to guidelines recommending extreme caution when prescribing opioids for chronic non-cancer pain.[67,75]

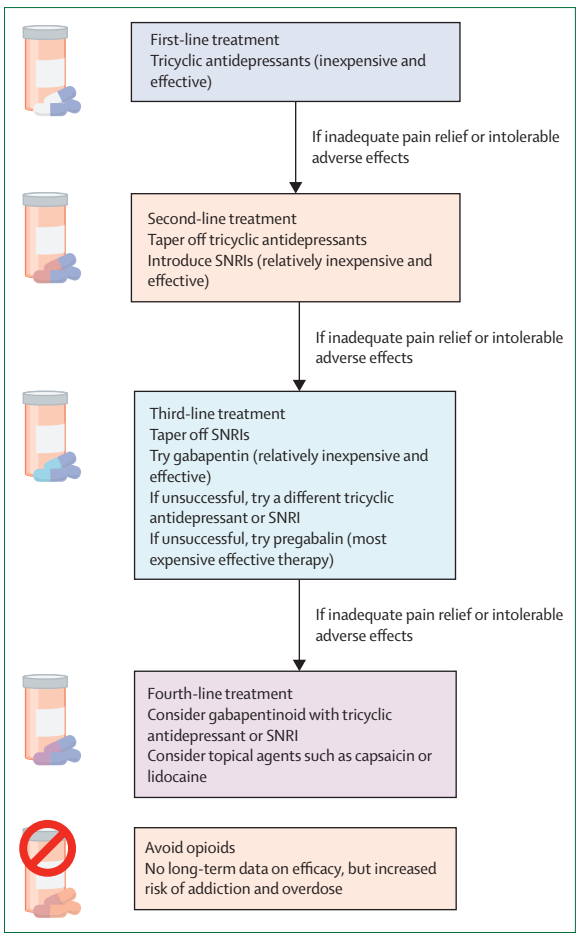

Figure 2: **Pain medication for patients with painful diabetic peripheral neuropathy**
Adapted from Callaghan and colleagues.[68] Tiered approach to pain management in patients with painful diabetic peripheral neuropathy. All medications (tricyclic antidepressants, SNRIs, gabapentinoids [eg, gabapentin, pregabalin], and sodium channel blockers [eg, lidocaine]) have similar effect sizes. Treatment selection should focus on alternative parameters such as tolerability and cost. SNRIs=serotonin norepinephrine reuptake inhibitors.

Improvements in the characterisation of pain presentations might help predict treatment response to pain medications. Characterisation methods include sensory profiles, such as pain or sensory quality and quantitative sensory testing, microneurography, and Hoffman-Reflex rate-dependent depression.[48] Quantitative sensory testing measures the perceptions evoked by distinct sensory stimuli; this test produces individual sensory profiles that can be used to stratify patients according to pathophysiological mechanisms.[76] Microneurography involves inserting an electrode into a peripheral nerve and measuring spontaneous and stimulus evoked activity of unmyelinated small fibres (autonomic and sensory). Hoffman-Reflex rate-dependent depression is a means of assessing spinal disinhibition from pain by measuring the same neural pathways that constitute the deep tendon reflex response. Studies using sensory profiling to predict treatment response are still

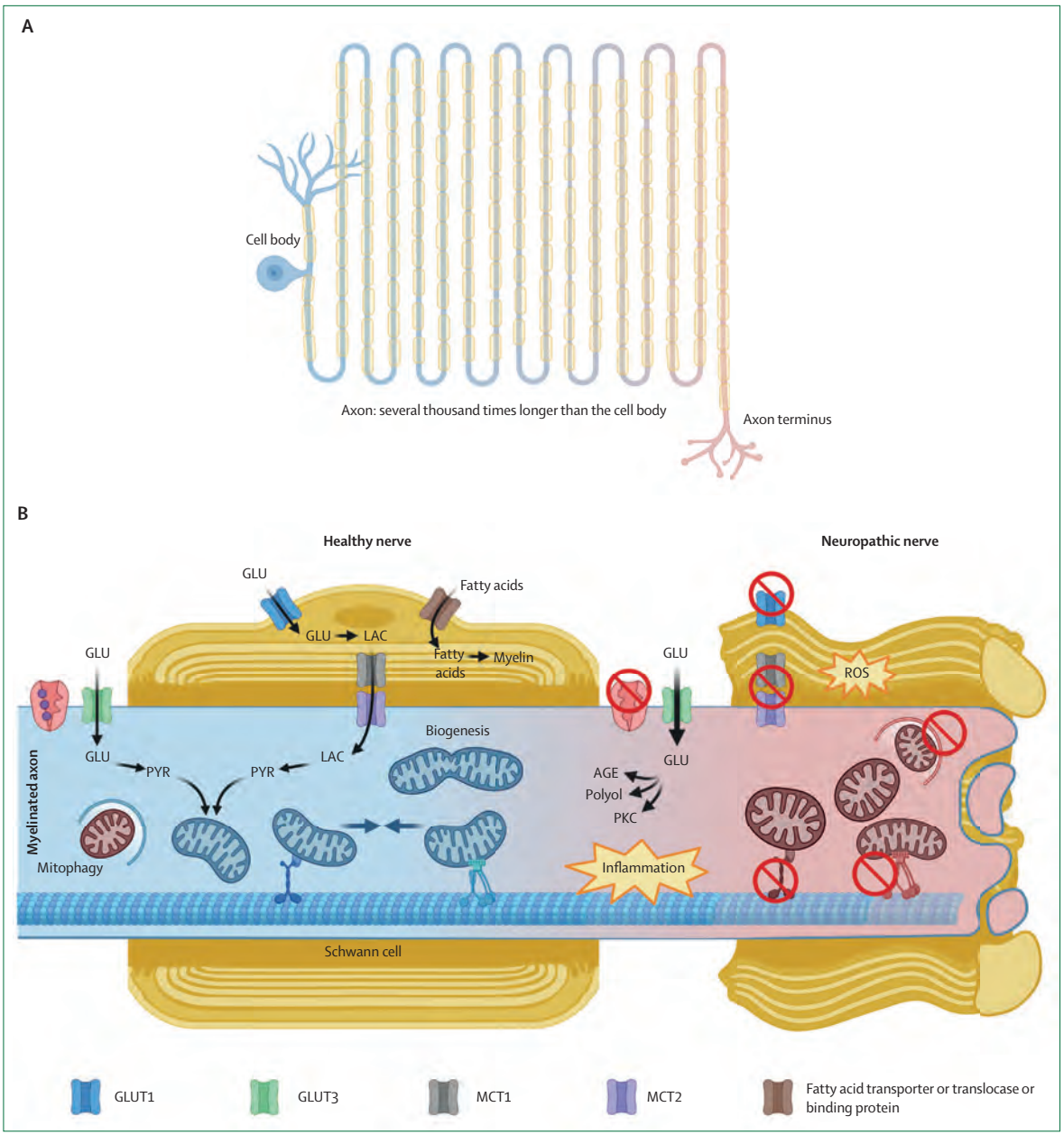

Figure 3: **Anatomy and pathophysiology of diabetic peripheral neuropathy**
(A) Peripheral neuron cell bodies are only microns in width, but axons are up to several feet in length. For nerve function, neurons traffic mitochondria from the cell body along the axons to areas of high energy demand. Failure to traffic mitochondria results in energy failure at axon termini followed by distal-to-proximal (pink to blue) nerve injury, which underlies the stocking-glove pattern of clinical signs and symptoms. (B) Homoeostatic conditions, healthy nerve (blue): axons take up glucose in an insulin-independent manner through glucose transporter 3 (GLUT3; green transporter), which is metabolised to pyruvate in the cytoplasm and further to ATP in mitochondria. Schwann cells take up glucose in an insulin-dependent manner through GLUT1 (blue transporter); some glucose is metabolised for energy use by Schwann cells, and some is metabolised to pyruvate and then lactate for transport to axons through the monocarboxylate transporter 1 (MCT1; grey, on Schwann cells) then MCT2 (purple, on axons). Schwann cells take up fatty acids using fatty acid transporters, translocases, or binding proteins, primarily for myelin production. Mitochondria are trafficked in anterograde or retrograde direction to areas of high energy demand. Mitochondria generate a mitochondrial membrane potential for ATP production, powering Na^+/K^+ ATPase channel activity (pink; purple spheres represent cations). Mitochondrial biogenesis can replenish dysfunctional mitochondria, which are eliminated by mitophagy. In pathological conditions, in a peripheral nerve (pink), hyperglycaemia increases glucose flux into axons through GLUT3; excess glucose that is not metabolised by glycolysis can activate other pathways, (eg, polyol, AGEs, and PKC pathways). Downstream, this activation triggers inflammation and disrupts Na^+/K^+ ATPase channel activity (no cations pumped, stop sign). Insulin resistance impairs insulin-dependent GLUT1-facilitated glucose uptake by Schwann cells, disrupting MCT-mediated fuel transport to axons and possibly the balance with lipid metabolism and myelin production. Hyperglycaemia and hyperlipidaemia also enhance oxidative stress and apoptosis in Schwann cells. Hyperlipidaemia disrupts mitochondrial biogenesis and trafficking in the axon, lowering the proportion and velocity of motile mitochondria. Palmitate also depolarises axonal mitochondria and reduces their ability to dissipate mitochondrial membrane potential to meet physiological energy demands. Mitophagy is also impaired. The figure was created by use of BioRender.com. AGE=advanced glycation end products. DPN=diabetic peripheral neuropathy. GLU=glucose. GLUT=glucose transporter. LAC=lactate. MCT=monocarboxylate transporter. PKC=protein kinase C. PYR=pyruvate.ROS=reactive oxygen species.

in their infancy,[44,77] and more work remains to integrate such measures into clinical trials. Hoffman-Reflex rate-dependent depression has been investigated in animals,[48] but future studies are needed to determine whether this test can predict treatment response in humans. If supported by future studies, pain characterisation might further our ability to address DPN pain by targeting patient-specific characteristics. Current guidelines recommend assessing patients for other factors that might affect pain perception, such as mood and sleep disorders, which helps select medications to manage these associated symptoms, possibly producing additional benefits for managing pain.[67]

Pathophysiology

Peripheral nerve anatomy poses challenges to well-orchestrated energy distribution and nerve function. Cell bodies of peripheral neurons are only microns in width, but their corresponding axons can be longer than a metre in length. Thus, for healthy nerve function, neurons need to traffic energy-producing mitochondria from the cell body along the axon, across large distances, to areas of high energy demand (figure 3A). When mitochondria do not traffic to the distal portion of a long axon or are dysfunctional upon arrival, subsequent energy failure and axonal injury occurs. This distal-to-proximal loss of energy supply to axons occurs in both type 1 and type 2 diabetes[78] and underlies the stocking-glove pattern of clinical signs and symptoms. Hyperglycaemia is present in both type 1 and type 2 diabetes; however, comorbid obesity and dyslipidaemia in type 2 diabetes additionally underscore the delicate balance between systemic and PNS metabolic requirements for nerve function. Thus, bioenergetic failure and mitochondrial dysfunction secondary to excessive circulating glucose and lipids might provide a unifying mechanism to explain both the pathology and clinical presentation of DPN. Axons are also dependent on glia for energy substrates and healthy function in the CNS;[79] however, the importance of this dependence in DPN pathophysiology has only been recognised recently.[80] Glucose enters both axons and supporting Schwann cells via specific glucose transporters. Through a process known as metabolic axoglial coupling,[80] Schwann cells can then supply energy-requiring long axons with fuel by transferring lactate through certain monocarboxylate transporters.[81] The metabolic syndrome results in poor glucose uptake in the PNS, which develops insulin resistance, similar to peripheral muscle and fat tissue.[82] This poor glucose uptake in turn disrupts axoglial coupling and depletes axonal energy stores, especially in the distal aspects of axons, leading to nerve injury and DPN over time. This novel idea underlying DPN pathogenesis bears out in preclinical studies. High-fat fed mice develop the metabolic syndrome, placing long axons in an energy crisis with resultant neuropathy, along with impaired axonal mitochondrial trafficking and biogenesis.[78]

High-fat feeding also depolarises energy-starved axonal mitochondria, further reducing their ability to provide ATP to meet physiological energy demands during nerve firing. As mitochondrial damage accumulates with disease progression, it triggers persistent damage due to energy failure,[83] oxidative[84] and endoplasmic reticulum stress,[85,86] and loss of normal ion flux.[83,87] Glucose accumulation in the nerve leads to an increase in glycation end products, and enhances the polyol, hexosamine, and protein kinase C pathways (figure 3B). These changes in nerve metabolism induce inflammation and disrupt osmotic balance, membrane resting potential, axon electrophysiology, and vascular function.[83]

Importantly, the effects of fatty acids on neuronal mitochondrial function are dependent on chain length and saturation; longer-chain, saturated fatty acids are more detrimental to neuronal mitochondria than their unsaturated counterparts.[88] This finding suggests that dietary interventions could slow DPN progression. In obese prediabetic rodents, supplementing the diet with unsaturated fatty acids ameliorates neuropathy, without reducing body fat mass or improving systemic insulin resistance.[88] This amelioration suggests local energy effects directly within the nerve. In type 2 diabetes[89] and obesity[90] studies in human beings, DPN correlates with distinct plasma signatures of specific lipid classes or species. Although causality in the DPN disease process has not been established, ceramides, sphingomyelins, and lipid signalling inhibitors are being investigated in animal models in the context of type 2 diabetes and obesity.[91]

Although research has focused on the investigation of direct metabolic dysfunction in DPN pathogenesis, other emerging directions focus on metabolism regulated through extracellular vesicles[92] and the gut microbiome.[93] We anticipate that these new areas will continue to converge on the basic tenet that neural anatomy results in unique energy demands which, if not met, can lead to injury and ultimately neuropathy.

DPN pain mechanisms

In patients with DPN, neuropathic pain (ie, pain arising from lesions or disease affecting the somatosensory nervous system), usually manifests as spontaneous burning pain, sometimes associated with hyperalgesia or allodynia, and often accompanied by sensory loss.[44] Determinants of painful DPN remain unknown; however, it likely results from the complex interactions of lesion characteristics (eg, severity), diabetes severity, the metabolic syndrome, genetic susceptibility, and environmental factors.[83]

Hyperexcitability of sensory neurons in the form of spontaneous activity and an enhanced response to sensory stimuli are thought to be key drivers of neuropathic pain in DPN. In the healthy nerve (figure 4 A, C), ion channels sense and transduce the initial stimulus to sensory receptors. Voltage-gated sodium channels then generate the action potential, which propagates along afferent axons

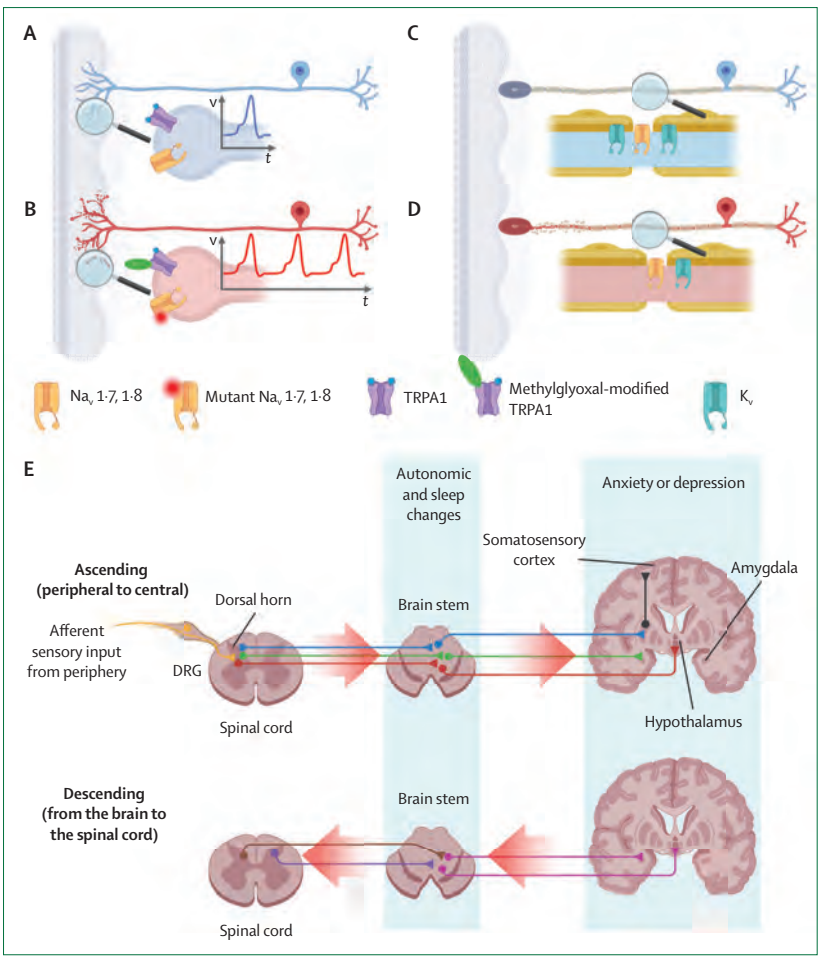

Figure 4: **pain mechanisms in diabetic peripheral neuropathy**
(A) Healthy unmyelinated C fibre (blue) has nerve endings embedded in skin. Inset: Voltage-gated sodium channels (eg, Nav1·7, 1·8) and ligand-gated channels (eg, TRPA1) transmit an afferent signal if the stimulus is higher than the channel threshold. (B) Neuropathic unmyelinated C fibre has distal-to-proximal length-dependent neurodegeneration, (red) starting in the nerve endings. Inset: Channel modification by reactive metabolites (eg, methylglyoxal, reactive species [green oval], or channel mutations [red shaded circle]) can lower the current threshold for generating an action potential in sensory neurons. Less intense stimulus now evokes an action potential and repetitive firing (red) on continued activation. (C) Healthy myelinated fibre has encapsulated nerve endings embedded in skin. Inset: Voltage-gated potassium (K_v; located at the juxtaparanode) and sodium (located at the nodes) channels propagate signals along nodes of Ranvier by saltatory conduction. (D) Neuropathic myelinated fibre has distal-to-proximal length-dependent neurodegeneration that starts in the encapsulated nerve ending. Inset: K_v channel expression level drops, leading to hyperexcitability. (E) CNS pain mechanisms. Top panel: ascending pathways of CNS dysfunction arising from peripheral to central pain signal amplification (red arrows). Sensory DRG (yellow) neurons carrying afferent signals connect with CNS neurons within the dorsal horn of the spinal cord, which relay within the brainstem to the brain. Different neuron colors represent various ascending pain pathways. Bottom panel: descending pathways of CNS dysfunction arising from the brain to pain signal amplification (red arrows) in the spinal cord. Different neuron colors represent various descending pain pathways. CNS pain pathways can give rise to autonomic dysfunction and anxiety, depression, and disturbed sleep. This figure was created by use of BioRender.com. DRG=dorsal root ganglion. K_v=voltage-gated potassium channel. Nav=voltage-gated sodium channel.

towards the CNS. Finally, within the spinal cord dorsal horn, ion channels trigger neurotransmitter release. Mutations, changes in expression, or post-translational modifications to ion channels alter their functional properties and cause hyperexcitability. Several ion channels are implicated in painful DPN (figure 4B, D), including voltage-gated sodium channels (Nav1·7, 1·8, 1·9),[44,83,94] voltage-gated potassium channels,[95] ligand-gated transduction channels such as TRPA1,[96] calcium channels (Cav3·2),[83] and hyperpolarisation-activated cyclic nucleotide-gated channels (HCN2).[97] Some of these channels have been validated in human studies of painful DPN (eg, voltage-gated sodium channels[94] and TRPA1),[96] whereas others only in animal models. Nevertheless, clinical studies targeting these channels (eg, ISRCTN68734605) have been initiated.[98]

Different mechanisms of ion channel-mediated hyperexcitability and pain can occur.[83] Increased Nav1·8 expression increases conduction in unmyelinated C fibre nociceptors, intensifying impulse transmission to the CNS.[83] Ion channel mutations resulting in gain of function (eg, through impaired inactivation) will lead to lowered action potential threshold and enhance spontaneous activity and firing frequency.[94] Post-translational modifications can also alter channel characteristics; for example, the modification of TRPA1 by methylglyoxal, an oxidised reactive metabolite that is elevated in diabetes, results in the activation of this channel (figure 4B).[83,96] TRPA1 senses noxious stimuli, such as exogenous reactive oxygen species and noxious cold. Therefore, oxidised (4-hydroxynonenal) or reactive oxygen species (H_2O_2) that are increased in diabetes can enhance this TRPA1 activation.

GWAS studies of painful DPN shed light on additional potential mechanisms; one study has identified that SNPs around the locus codifying for the mitochondrial phosphate carrier protein SLC25A3, which is involved in oxidative phosphorylation, increase the risk of neuropathic pain.[99] DOLORisk is a large multicentre observational study of patients with neuropathic pain, including patients with painful DPN, in which robust phenotyping data and gene sets are being collected for analysis to identify pathogenic SNPs.[100] Additionally, differences in circulating pro-inflammatory cytokines have been shown in clinical studies[101] and the involvement of the CXCR4 signalling axis has been described in experimental models.[102]

In addition to peripheral mechanisms, CNS dysfunction can also contribute to pain (figure 4E). Changes within the dorsal horn of the spinal cord further amplify nociceptive signals, contributing to painful DPN. This dysfunction includes altered dorsal horn neuron morphology and spinal disinhibition.[103] The descending pain modulatory system, mediated by projections from the brainstem, can either inhibit or facilitate transmission of nociceptive information within the dorsal horn. Conditioned pain modulation provides a means to test the integrity of the descending pain modulatory system and is predictive of treatment response.[2] Functional MRI studies suggest that facilitation via the ventrolateral periaqueductal grey is increased in patients with painful DPN versus those without painful DPN.[104] These changes are not restricted to neurons, as experimental models suggest a contribution of pro-inflammatory microglia.[105]

Search strategy and selection criteria

We search PubMed for articles published between Jan 1, 2017, and Dec 31, 2021, with the terms "diabetic peripheral neuropathy", "brain", "cognitive decline", "dementia", "differential diagnosis", "diagnosis", "epidemiology", "EWAS", "genetic risk", "GWAS", "hyperexcitability", "insulin resistance", "ion channel", "metabolic syndrome", "neuropathic", "pain", "painful", "pathophysiology", "prevalence", "scales", and "screening". Additionally, authors supplemented the search with references from the identified articles. There were no language restrictions. The final reference list was generated according to relevance to the topics covered in this Review.

Multimodal MRIs show structural and functional differences in the somatosensory cortex in patients with painful DPN versus those with non-painful DPN.[106] These changes could partly reflect deafferentation. Painful DPN is also associated with psychological changes and comorbidities, including anxiety, depression, and sleep problems, which can contribute to maladaptive pain states. Moreover, brain MRI findings might be useful to identify patients with painful DPN who are likely to respond to specific pain treatments.[107]

Conclusions and future directions

Unfortunately, many patients with diabetes struggle with DPN, a prevalent complication which, despite being long-recognised, still lacks disease-modifying therapies. DPN can develop despite well-controlled diabetes,[4] suggesting determinants of disease in addition to glucose parameters. A better understanding of modifiable risk factors such as the metabolic syndrome components,[11,14,20,27–29,33] could help in the identification of the patients most susceptible to DPN development. Awareness of modifiable risks also suggests potential interventions to slow DPN. Lifestyle interventions, such as diet and exercise, might slow DPN progression,[61,62,64] leading to stable disease, and the American Diabetes Association recommends diet and exercise as first-line prevention for DPN.[5] Because these interventions slow the progression of DPN, patients might benefit from early intervention. Thus, an earlier, more sensitive, and more specific diagnosis might help prognosis; however, clinical diagnosis has remained essentially unchanged over the past few decades and innovation is needed. The scarcity of effective therapies for painful DPN is a roadblock to pain management, and the long-term side-effects of opioids warrant a tiered approach, which should avoid opioid prescription. We anticipate that a deeper understanding of the pathogenesis of DPN and the mechanisms underlying distinct types of pain will lead to more effective treatment. This prevalent disorder needs a disease-modifying therapy, a goal that underlies all current and newly evolving DPN research.

Contributors

MAE, MGS, HA, DLB, VV, BCC, and ELF contributed to conceptualisation, writing, reviewing, and editing. All authors accept responsibility for the decision to submit for publication.

Declaration of interests

DLB reports grants from AstraZeneca, Lilly, and Diabetes UK, during the conduct of the study; has acted as a consultant on behalf of Oxford Innovation for Amgen, Bristows, LatigoBio, GSK, Ionis, Lilly, Olipass, Orion, Regeneron and Theranexus throughout 2020 and 2021, outside of the submitted work; and has a patent application "a method for the treatment or prevention of pain, or excessive neuronal activity, or epilepsy" (application number, 16/337,428) pending. BCC reports personal fees from Medical legal work, DynaMed, and Vaccine Injury Compensation Program; grants from Veterans Affairs; and grants and personal fees from the American Academy of Neurology, outside of the submitted work. All other authors declare no competing interests.

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PERSPECTIVE

Advances in diet-induced rodent models of metabolically acquired peripheral neuropathy

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ABSTRACT

Peripheral neuropathy (PN) is a severe complication that affects over 30% of prediabetic and 60% of type 2 diabetic (T2D) patients. The metabolic syndrome is increasingly recognized as a major driver of PN. However, basic and translational research is needed to understand the mechanisms that contribute to nerve damage. Rodent models of diet-induced obesity, prediabetes, T2D and PN closely resemble the human disease and have proven to be instrumental for the study of PN mechanisms. In this Perspective article, we focus on the development, neurological characterization and dietary fat considerations of diet-induced rodent models of PN. We highlight the importance of investigating sex differences and discuss some of the challenges in translation from bench to bedside, including recapitulating the progressive nature of human PN and modeling neuropathic pain. We emphasize that future research should overcome these challenges in the quest to better mimic human PN in animal models.

Introduction

The global prevalence of the metabolic syndrome (MetS; see Glossary, Box 1) has reached epidemic proportions (Saklayen, 2018). Thirty to sixty percent of patients with MetS are affected by peripheral neuropathy (PN; Box 1) (Feldman et al., 2019). For decades, extensive research focused on the role of hyperglycemia in PN development. However, we found that well-controlled glycemia only reduces PN incidence in type 1 diabetes (T1D; Box 1), but marginally improves onset and progression in prediabetes (Box 1) and type 2 diabetes (T2D; Box 1) (Callaghan and Feldman, 2013; Callaghan et al., 2012). Although T2D is the most common cause of PN, central obesity and dyslipidemia are PN risk factors, independent of glycemic status (Andersen et al., 2018; Callaghan et al., 2020). Thus, clinical studies support MetS more broadly as a major PN driver in prediabetes, T2 and obesity, highlighting the metabolically acquired and diet-induced nature of PN (Callaghan et al., 2018, 2016a,b; Christensen et al., 2020). Additionally, guidelines from the American Diabetes Association now recommend lifestyle interventions to improve MetS and prevent PN in prediabetic, T2D and obese patients (www.ada.org) (Pop-Busui et al., 2017).

Although MetS is increasingly recognized as an independent PN risk factor, the cellular and molecular mechanisms underlying disease onset and progression remain unclear. Animal models that closely mimic the human condition have been invaluable in gaining insight into PN pathogenesis. Our laboratory and others have developed rodent models fed a high-fat diet (HFD), which consistently induces MetS, including obesity, impaired glucose tolerance and dyslipidemia, as PN develops (Davidson et al., 2010; Guilford et al., 2011; O'Brien et al., 2020). In this Perspective article, we provide an overview of the guidelines for assessing PN in rodents. We then focus on diet-induced rodent models of obesity, prediabetes and T2D leading to PN, which reproduce human disease and have proven instrumental for studying PN mechanisms. We finally highlight the importance of sex differences and discuss outstanding challenges associated with the use of diet-induced PN models in research.

"Overall, implementing DiaComp recommendations standardizes neuropathy phenotyping, reducing lab-to-lab variation and facilitating the collection of rigorous, reproducible and translatable data, essential for enhancing our understanding of PN."

Guidelines for assessing PN in rodents

Rodent models are useful for studying PN etiology because they facilitate experiments that are not feasible in the clinical setting. The National Institutes of Health created the Diabetic Complications Consortium (DiaComp; www.diacomp.org) to identify new animal PN models and standardize neuropathy phenotyping to reduce lab-to-lab variation. DiaComp advises that a robust rodent PN model should exhibit essential pathological features of the human disease, including abnormal sensory symptoms (Fig. 1A,B; Box 1) such as allodynia, hyperalgesia and/or hypoalgesia; nerve conduction velocity (NCV; Box 1) deficits (Fig. 1C-E); and morphological evidence of intraepidermal nerve fiber density (IENFD; Box 1) loss (Juster-Switlyk and Smith, 2016) (Fig. 1F-H).

Based on DiaComp recommendations, neuropathy phenotyping first assesses thermal sensitivity as a measure of sensory dysfunction using tail-flick or hindpaw withdrawal tests. Alternatively, von Frey filaments (Box 1) can be used to quantitatively assess sensitivity to mechanical stimuli. Like humans, diabetic rodents first develop thermal hypersensitivity and mechanical allodynia followed by decreased sensitivity or hypoalgesia at later disease stages (Feldman et al., 2017). Next, neuropathy phenotyping records electrophysiological sciatic motor and sural sensory NCVs as measures of large nerve fiber impairment (Fig. 1). Lastly, quantifying IENFD in mice footpads serves as histological evidence

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Box 1. Glossary

Abnormal sensory symptoms: frequently experienced by diabetic patients with PN, including allodynia, a pain response to normally innocuous stimuli; hyperalgesia, increased sensitivity to painful stimuli; and/or hypoalgesia, decreased sensitivity to painful stimuli.
Dorsal root ganglia neurons: sensory neurons that relay information from the internal and external environments about nociception, touch, temperature or muscle length to the central nervous system.
Endoneurial microangiopathy: an abnormality of nerve microvessels including basement membrane thickening and endothelial cell hypertrophy, often accompanying PN development and progression (Fang et al., 2018).
Hydrogenated vegetable shortening: a type of fat used in rodent studies. Diets with vegetable shortening can be derived from partially hydrogenated soybean/palm oils or from partially hydrogenated soybean/cottonseed oils (Kubant et al., 2015).
Hyperphagia: excessive food intake, which in rodents is induced by a spontaneous mutation in the satiety factor leptin (*ob*/*ob* mice) or its receptor (*db*/*db* mice or Zucker diabetic fatty rats), leading to obesity and type 2 diabetes (T2D).
Intraepidermal nerve fiber density (IENFD): an assessment of small unmyelinated fibers. IENFD is a quantitative approach for the diagnosis of small-fiber neuropathy used in both the clinical and pre-clinical settings (Juster-Switlyk and Smith, 2016).
Metabolic syndrome (MetS): a cluster of metabolic risk factors that encompasses elevated fasting glucose (i.e. prediabetes leading to frank T2D), central obesity, dyslipidemia and hypertension (Saklayen, 2018).
Nerve conduction velocity (NCV): the speed at which an electrical impulse is transmitted through peripheral nerves. It is the gold standard for PN diagnosis in the clinical and preclinical settings and quantifies the extent of large myelinated nerve fiber dysfunction. NCV studies are reported in m/s and include sensory NCVs measured in the sural nerve following antidromic supramaximal stimulation at the ankle, in turn quantified by dividing the distance by the sensory nerve action potential take-off latency. Motor NCVs in the sciatic nerve are recorded at the foot dorsum following orthodromic supramaximal stimulation, first at the ankle then at the sciatic notch. Sciatic motor NCVs are quantified by subtracting ankle distance from notch distance and dividing by the difference in ankle and notch latencies (Hinder et al., 2017).
Nociception: the neurophysiological encoding of actual or potential tissue damage.
Peripheral neuropathy (PN): a debilitating degeneration of peripheral nerves in a distal-to-proximal manner, which can lead to chronic pain, non-healing ulcers and lower-limb amputations (Feldman et al., 2019).
Prediabetes: characterized by impaired glucose tolerance, often leading to frank T2D. Like T2D patients, prediabetic patients experience long-term complications, including nerve damage or peripheral neuropathy.
Type 1 diabetes (T1D): an autoimmune disease characterized by pancreatic β-cell destruction, which leads to insulin deficiency and hyperglycemia (DiMeglio et al., 2018). It accounts for up to 5-10% of all cases of diabetes.
Type 2 diabetes (T2D): a component of MetS characterized by hyperglycemia, impaired insulin signaling and dyslipidemia. It is the most common form of diabetes and, in addition to genetic factors, is primarily driven by lifestyle factors such as unhealthy diets and limited physical activity (Chatterjee et al., 2017).
von Frey filaments: used to quantify mechanical sensitivity ranging from hyperalgesia or allodynia to lack of sensation or hypoalgesia.

of small sensory nerve fiber loss (Hinder et al., 2017; Sullivan et al., 2007). These structural changes are paralleled by endoneurial microangiopathy (Box 1) (Fang et al., 2018) and inflammation (Pop-Busui et al., 2016) in peripheral nerves, which can be evaluated to further characterize the animal model. In addition to neuropathy phenotyping, it is important to metabolically profile obese, prediabetic and diabetic rodents, including body weights, glycemic status and insulin levels. Furthermore, our research has shown that dyslipidemia is an independent PN risk factor in obesity, prediabetes and T2D (Eid et al., 2019; O'Brien et al., 2020), and should therefore be examined through a basic lipid profile.

Overall, implementing DiaComp recommendations standardizes neuropathy phenotyping, reducing lab-to-lab variation and facilitating the collection of rigorous, reproducible and translatable data, essential for enhancing our understanding of PN.

Rodent models of obesity, prediabetes, T2D and PN

Because of their genetic similarities to humans, rodents are considered the model of choice in PN research and have considerably enhanced our understanding of human PN. In addition, they have been useful for evaluating responsiveness to novel or commonly prescribed therapeutic agents, identifying mechanisms of action in peripheral nerves (Eid et al., 2021a,b, 2020). Another advantage is the ability to genetically manipulate genes of interest in PN. Earlier animal studies mostly employed rat PN models (Jakobsen and Lundbaek, 1976; Yagihashi et al., 1979); however, focus has shifted recently to mice PN models (O'Brien et al., 2014) because they are more cost effective and have shorter breeding cycles. Obesity, prediabetes and T2D rodent models of PN include HFD-induced, spontaneous monogenic mutations and polygenic strains. Spontaneous mutations in leptin (*ob*/*ob* mice) or leptin receptor (*db*/*db* mice or Zucker diabetic fatty rats) induce T2D secondary to hyperphagia (Box 1). Although these models consistently develop PN and have been very useful (Eid et al., 2020; O'Brien et al., 2015), they are limited by not adequately modeling human PN progression. Most patients gradually develop hyperglycemia before overt T2D, whereas these T2D rodent models rapidly develop hyperglycemia, largely bypassing the prediabetic stage (Hinder et al., 2018; Oltman et al., 2005). Additionally, loss of leptin signaling may also confound translatability to individuals with prediabetes and T2D by differentially impacting glucose and lipid metabolism irrespective of obesity and T2D (Wang et al., 2014). By contrast, polygenic T2D mouse models gradually develop MetS components, closely mimicking the human disease (O'Brien et al., 2014). Surprisingly, there are no published reports of PN in these polygenic mice, although they hold great promise as potential models mirroring human development of MetS. Therefore, our laboratory is currently examining whether nerve dysfunction occurs in these mice.

Although genetic and spontaneous models are valuable research tools for studying PN, this Perspective article will focus on diet-induced PN models, which have an impressive array of experimental advantages.

Diet-induced rodent models of obesity, prediabetes, T2D and PN

HFD generates exemplary obesity and prediabetes models with PN. Our laboratory's clinical findings demonstrate that obesity and prediabetes are major PN drivers (Andersen et al., 2018; Callaghan et al., 2018, 2020, 2016b). Rodents fed increased dietary fat progressively display metabolic disturbances, including weight gain, insulin resistance, dyslipidemia and impaired glucose tolerance in the absence of hyperglycemia. These metabolic changes are often accompanied by compromised responses to stimuli, delayed sensory and/or motor NCVs, and IENFD loss, characteristic of human PN (Guilford et al., 2011; O'Brien et al., 2020).

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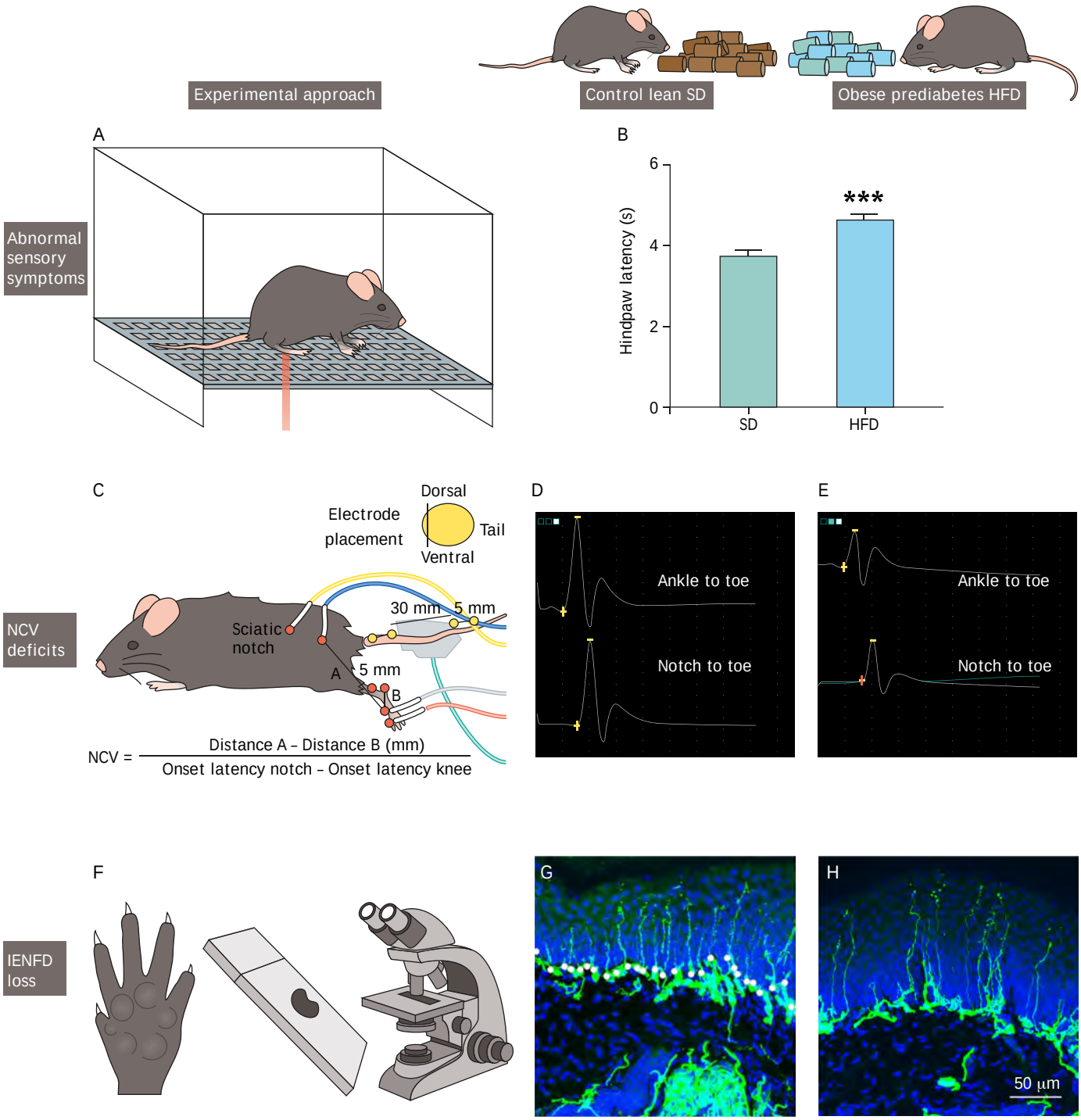

Fig. 1. Neuropathy phenotyping in diet-induced rodent models. (A) Abnormal sensory symptoms including allodynia, hyperalgesia and/or hypoalgesia are evaluated by testing rodents' sensitivity to a heat stimulus applied to the hindpaw. (B) Typical hindpaw withdrawal latency in 60% high-fat diet (HFD) versus standard diet (SD) mice at 36 weeks (***P<0.001) (reproduced with permission from O'Brien et al., 2018). (C) Electrode placement to record electrophysiological sciatic motor and sural sensory nerve conduction velocities (NCVs) as measures of large nerve fiber impairment [reproduced with permission from protocols.io (dx.doi.org/10.17504/protocols.io.7rbhm2n) under the terms of the Creative Commons Attribution License]. To calculate sciatic motor NCV (m/s), the difference between distance A and distance B (mm) is divided by the difference between the two onset latencies of the compound muscle action potentials (ms). (D,E) Typical sciatic motor traces recorded after stimulation at the ankle and at the notch in a control lean mouse placed on a SD (D) and in an obese prediabetes mouse placed on a HFD (E). (F) Quantifying intraepidermal nerve fiber density (IENFD) in mice footpads serves as histological evidence of small sensory nerve fiber loss. (G,H) Representative images of IENFD in SD (G) and HFD (H) mice at 36 weeks. Scale bar: 50 µm.

To date, most diet-induced murine studies have used the C57BL/6J strain (Coppey et al., 2018b; O'Brien et al., 2020). However, distinct background strains differentially impact the metabolic and neuropathic phenotypes in response to HFD (Hinder et al., 2017; Sullivan et al., 2007). To identify the optimal mouse strain in HFD-induced obesity, prediabetes and PN, our laboratory recently compared commonly used strains in diabetes research: BKS, BTBR and C57BL/6J. We found that C57BL/6J mice fed a HFD

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Eva Feldman (back) and Stéphanie Eid (front)

(54% kcal fat) develop the most robust obesity, prediabetes and PN phenotypes (Hinder et al., 2017), advocating it as the strain of choice in diet-induced PN models.

In addition to background strain, rodent age, as well as HFD duration, percentage and dietary fat source, impact the severity of nerve damage in diet-induced models. The percentage of fat content in HFD models can be fine-tuned to optimize the PN phenotype. Earlier studies utilized 42-54% HFD (Table 1); however, our most recent studies using 60% HFD between 11 and 31 weeks indicate that increasing the percentage of dietary fat induces a more pronounced metabolic phenotype, in agreement with The Jackson Laboratory reports (https://www.jax.org/jax-mice-and-services/strain-data-sheet-pages/phenotype-information-380050). Moreover, mice placed on a 60% HFD mice develop severe small- and large-fiber PN after 11 weeks of HFD (Table 1) (O'Brien et al., 2020, 2018). Overall, studies thoroughly comparing the effect of various fat percentages for short to long duration may differentially impact pain, NCVs and IENFDs and must therefore be rigorously accounted for and reported. Such studies will be essential to enhance recapitulation of human PN in animal models.

PN severity also depends on the source of dietary fat and saturation degree (Rumora et al., 2019; Yorek et al., 2017). Lard-derived HFD with 42-60% saturated fats induces obesity, prediabetes and key PN features (Anderson et al., 2014; O'Brien et al., 2020). Conversely, 54% HFD from hydrogenated vegetable shortening (Box 1) does not impair large or small nerve fiber function (Groover et al., 2013). Importantly, replacing lard-based HFD with diets rich in unsaturated fats, such as plant-based and fish oil fats, improves nerve function (Coppey et al., 2018a; Rumora et al., 2019). Our laboratory further shows that supplementing primary dorsal root ganglia neurons (Box 1) *in vitro* with unsaturated fats prevents saturated fat-induced mitochondrial dysfunction (Rumora et al., 2019). Together, these data suggest that fat saturation degree may differentially impact PN progression and that diets rich in unsaturated fats may be neuroprotective, potentially offering an effective lifestyle intervention.

Lastly, ketogenic diets consisting of 79-90% kcal plant-based fat, 8-9% protein and 0.3-3% carbohydrate improved nociception (Box 1) and increased IENFD without reversing pre-existing metabolic abnormalities (Cooper et al., 2018a). How a ketogenic diet improves nerve function remains unclear. However, it is thought that ketone bodies may promote axon growth (Cooper et al., 2018a) and reduce inflammation (Ruskin et al., 2009). These results suggest that ketogenic strategies may be promising in the treatment of metabolically acquired PN and should therefore be validated in the future.

"A better understanding of sex dimorphism in PN is critical for tailoring sex-specific therapeutic strategies."

Sex differences in rodent models of PN

Sex differences are evident in the prevalence, clinical manifestations and etiology of MetS in humans (Pradhan, 2014) and differential responsiveness to antidiabetic drugs (Franconi and Campesi, 2014). There is also increased prevalence of MetS postmenopause in female individuals, presumably due to estrogen deficiency (Lovre et al., 2016). However, clinical studies investigating sex as a potential differential PN risk factor are limited and inconclusive. One study reported that male T2D individuals were affected by PN earlier than females (Aaberg et al., 2008), which mirrors recent findings that female T2D individuals experience more frequent and intense neuropathic pain despite a milder PN phenotype (Abraham et al., 2018). However, larger prospective cohorts are required to validate these findings. Importantly, a better understanding of sex dimorphism in PN is critical for tailoring sex-specific therapeutic strategies. This view is in accordance with a recent National Institutes of Health policy, which now requires preclinical studies to account for sex differences, reflecting efforts to be more inclusive of unrepresented groups in clinical studies (Clayton and Collins, 2014).

In this vein, animal studies, which previously focused heavily on male rodents, are starting to investigate the role of sex dimorphism in obesity and prediabetes (Zore et al., 2018). Male rodents, such as C57BL/6J mice, are more susceptible to diet-induced obesity and prediabetes versus female littermates (Casimiro et al., 2021; Hwang et al., 2010; Yang et al., 2014). Additionally, inducing obesity

Table 1. High-fat diet (HFD) duration, percentage and dietary fat source impact the severity of small and large nerve fiber damage in diet-induced models

Chow		HFD paradigm (weeks)			Neuropathy phenotype			Reference
Fat %	Fat source	Initiation	Duration	Final age	Behavior	NCV	IENFD	
45%	Lard	3	34	37	Thermal hypoalgesia	↓ MNCV and SNCV	↓	Vincent et al., 2009
54%	Vegetable oil	7	8	15	Mechanical but not thermal allodynia	↓ MNCV but not SNCV	No change	Guilford et al., 2011
42%	Anhydrous milk fat	3-4	12	15-16	Mechanical and thermal allodynia	Not reported	Not reported	Gavini et al., 2018
60%	Lard	5	31	36	Thermal hypoalgesia	↓ MNCV and SNCV	↓	O'Brien et al., 2018

Neuropathy phenotyping reported was performed on male C57BL/6 mice at final age. IENFD, intraepidermal nerve fiber density; MNCV, motor nerve conduction velocity; NCV, nerve conduction velocity; SNCV, sensory nerve conduction velocity.

and glucose intolerance is more challenging in female Sprague-Dawley rats, requiring a higher dietary fat percentage and longer feeding duration relative to males (Coppey et al., 2018b). In line with an earlier report (Pettersson et al., 2012), our laboratory recently showed that HFD female mice are protected from insulin resistance early during HFD feeding versus male littermates (Elzinga et al., 2021). Interestingly, as in humans (Janssen et al., 2008; Lovre et al., 2016), estrogen exerts antidiabetic effects on MetS and T2D in rodents (Riant et al., 2009). Several mechanisms may contribute to these protective actions, including regulation of pro-inflammatory mediators and lipid metabolism (Bryzgalova et al., 2008; Stubbins et al., 2012), which are both crucial for nerve health (Mitro et al., 2017; Pop-Busui et al., 2016). There are clear differences between male and female obesity and prediabetes rodent models, which could have clinical diagnostic and management implications.

Although female mice are clearly protected from metabolic dysfunction, at least early in the disease course, sex-specific effects on PN are much less clear-cut. Our laboratory and others are addressing this issue by characterizing PN in two common animal models, C57BL/6J mice (Elzinga et al., 2021; Obrosova et al., 2007) and Sprague-Dawley rats (Coppey et al., 2018b). Our results in 60% HFD mice indicate that females develop PN to the same degree as males, despite early protection against insulin resistance (Elzinga et al., 2021), similar to findings in HFD-fed C57BL/6J mice and Sprague-Dawley rats that were treated with low-dose streptozotocin to generate a T2D model (Coppey et al., 2018b). Overall, these reports evaluated PN at a relatively late disease stage, and future studies investigating sex differences during the early stages of PN development are critical. Hormone levels may differentially affect the metabolic and neuropathic phenotypes; thus, it is also important to assess sex hormones in future studies.

Why female rodents still develop PN in obesity, prediabetes and T2D, despite a 'healthier' metabolic profile, at least early in the study, is unclear. Peripheral nerves in female mice still accumulate sorbitol and oxidative stress and exhibit poly (ADP-ribose) polymerase activation (Obrosova et al., 2007). Interestingly, these pathways mediate, at least in part, PN in male neuropathic mice (Feldman et al., 2017) and may be implicated in disease development in female mice. Our transcriptomic analysis of peripheral nerves from *ob/ob* mice identified dysregulation of inflammatory and immune response pathways in female mice (O'Brien et al., 2016), similar to our findings in peripheral nerve tissue from male mice (O'Brien et al., 2015). Although these results suggest similar mechanisms of nerve injury, regardless of sex, further research is required to define sex differences in PN, in rodents and humans, to develop effective disease-modifying therapies in both sexes, or tailor sex-specific therapies.

Challenges translating from bench-to-bedside

The limitations of diet-induced rodent models in accurately mimicking the metabolic aspect of human obesity and T2D have been previously laid out (Lai et al., 2014). In this section, we focus on challenges associated with modeling human PN.

Does diet-induced PN in rodents faithfully recapitulate gradual disease onset in humans?

The pathogenesis of prediabetes, T2D and PN in humans is driven by a complex interaction of environmental and genetic factors resulting in gradual disease progression over a long period of time (Wysham and Shubrook, 2020). Conversely, HFD rodent studies are often initiated at a young age roughly equivalent to that of a human teenager, as per The Jackson Laboratory reports (https://www.jax.org/news-and-insights/jax-blog/2017/november/when-are-mice-considered-old#:~:text=Mature%20adult%20mice%20range%20in,ranges%20from%2020%20%2D%2030%20years). Within only 4 weeks of diet, they display components of the MetS, including obesity and insulin resistance, although to variable extents (Wang and Liao, 2012). Similarly, HFD mice, within this 4-week timeframe, start to exhibit PN features, such as mechanical hypersensitivity (Groover et al., 2013), and an established PN phenotype is observed by 8 weeks of HFD (Cooper et al., 2018b; Guilford et al., 2011). Subsequently, the duration of most diet-induced rodent studies in PN research is often short, at a few weeks to a few months at most, which is roughly equivalent to human adult age (O'Brien et al., 2014) and therefore does not fully reflect the progressive nature of human PN. This is particularly true regarding neuropathic pain (discussed in detail below), which is commonly experienced by prediabetic and T2D patients (Abbott et al., 2011) and not accurately recapitulated in diet-induced models. Hence, these findings can potentially lead to inaccurate depiction of disease pathogenesis in humans, which should be validated in other robust PN models and clinical studies. This also applies to drug studies that are effective in diet-induced rodent models, but do not necessarily translate to improved outcomes for prediabetic and T2D patients.

Another translational roadblock is the more variable PN phenotype in humans versus a more uniform phenotype in rodents. As mentioned earlier, most rodent studies consist of same-sex animals on the same genetic background, which likely generate a similar level of nerve dysfunction. By contrast, distinct lifestyle factors and genetic predisposition in humans likely lead to variable PN presentation across prediabetic and T2D individuals (Feldman et al., 2019). Studies including different background strains within a single animal cohort are therefore essential to mimic patients with varying susceptibilities to metabolic disorders and PN.

"In light of the current obesity pandemic, increased preclinical knowledge from diet-induced rodents will accelerate the development of disease-modifying therapies for treating metabolically induced neuropathic pain, which remains largely understudied."

Neuropathic pain

Neuropathic pain is a common, and often the earliest, consequence of PN, affecting up to 50% of diabetic patients (Abbott et al., 2011). It manifests as hypersensitivity with hyperalgesia/allodynia or as spontaneous and continuous pain sensations (Feldman et al., 2017). Neuropathic pain is often accompanied by disturbed sleep, anxiety and depression, which reduce patients' quality of life (Sloan et al., 2018). Unfortunately, current therapies have limited efficacy due to incomplete understanding of the pathophysiological mechanisms of pain. Diabetic animal models, especially streptozotocin-induced T1D rodents, have been pivotal for studying pain processing and testing candidate therapeutics (Jaggi et al., 2011). However, much less is known on the interplay between components of MetS and neuropathic pain, mostly due to lack of an established animal model that consistently develops pain symptoms. Two recent studies reported contradictory findings regarding pain response in the commonly used C57BL/6J HFD mouse. One study examined the

effect of genetic differences among C57BL/6 mice from two different suppliers on pain behaviors following a 7-week plant-based HFD, observing that C57BL/6 mice from The Jackson Laboratory retained normal mechanical sensitivity, whereas C57BL/6 mice from Charles River Laboratories displayed pain behaviors (Cooper et al., 2018b). By contrast, another study observed that administering a Western diet containing 42% kcal from anhydrous milkfat for 12 weeks induced mechanical allodynia and thermal hyperalgesia in C57BL/6 mice from The Jackson Laboratory (Bonomo et al., 2020). In addition to different diet duration, a key difference between the two reports is dietary composition, particularly the high-sucrose (34%) and -cholesterol (0.2%) content of the Western diet, key drivers of MetS, which could account for the discrepancies in pain behaviors. Standardizing paradigms used to induce MetS, including HFD composition and duration, are required to establish a rodent model with reproducible and consistent pain phenotype, which should be a future avenue of work. Another critical consideration is including older animals to longitudinally assess pain behaviors that start early during HFD. As emphasized earlier, determining sex-specific pain responses is also key, especially because human studies show that T2D female individuals are more prone to pain than males (Abraham et al., 2018). In light of the current obesity pandemic, increased preclinical knowledge from diet-induced rodents will accelerate the development of disease-modifying therapies for treating metabolically induced neuropathic pain, which remains largely understudied.

Conclusions

PN is a frequent and complex complication of obesity, prediabetes and T2D, which requires better understanding to progress translational research and develop much needed mechanism-based therapies. Herein, we focused on diet-induced rodent models, which, although not perfect, share essential metabolic and neurological features with human obesity, prediabetes, T2D and PN. We, therefore, recommend them to researchers investigating nerve damage induced by MetS. In light of the growing obesity pandemic, diet-induced rodent studies have emphasized the importance of dietary interventions as a treatment for PN, in agreement with the American Diabetes Association recommendations. Moving forward, research should address the mechanisms underlying the beneficial effects of dietary interventions on PN, which will help inform the optimal dietary regimen and develop targeted therapies for PN patients unable or unwilling to engage in changes in dietary habits.

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Competing interests

The authors declare no competing or financial interests.

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Diabetes and dementia: Clinical perspective, innovation, knowledge gaps

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ABSTRACT

The world faces a pandemic-level prevalence of type 2 diabetes. In parallel with this massive burden of metabolic disease is the growing prevalence of dementia as the population ages. The two health issues are intertwined. The Lancet Commission on dementia prevention, intervention, and care was convened to tackle the growing global concern of dementia by identifying risk factors. It concluded, along with other studies, that diabetes as well as obesity and the metabolic syndrome more broadly, which are frequently comorbid, raise the risk of developing dementia. Type 2 diabetes is a modifiable risk factor; however, it is uncertain whether anti-diabetic drugs mitigate risk of developing dementia. Reasons are manifold but constitute a critical knowledge gap in the field. This review outlines studies of type 2 diabetes on risk of dementia, illustrating key concepts. Moreover, it identifies knowledge gaps, reviews strategies to help fill these gaps, and concludes with a series of recommendations to mitigate risk and advance understanding of type 2 diabetes and dementia.

1. Problem statement

The world faces a pandemic-level prevalence of type 2 diabetes. The 2021 global estimate is over 536 million affected people or about 10.5 % of the adult population,[1] up by a staggering 73 million in only two years from the 2019 estimate of 463 million (Fig. 1).[2] If trends persist, the world faces the prospect of 783.2 million affected people by 2045 or about 12.2 % of the population.[2] Estimated global total diabetes-related health expenditures were almost one trillion US dollars in 2021. In parallel with this massive burden of metabolic disease is the growing prevalence of dementia as the population ages. In 2019, 57.4 million people worldwide were living with dementia, which is projected to rise to 152.8 million cases by 2050.[3] Like diabetes, dementia poses a substantial socioeconomic burden, incurring around one trillion US dollars annually worldwide.[4] The situation is rendered graver still since dementia currently lacks effective disease-modifying treatments.[5]

The two health issues are intertwined. The Lancet Commission on dementia prevention, intervention, and care was convened in 2017[6] and again in 2020[7] to tackle the growing global concern of dementia by identifying risk factors. The Lancet Commission [6,7] and other studies increasingly suggest that diabetes[8–10] as well as obesity[11] and the metabolic syndrome[12] more broadly, which are frequently comorbid, raise the risk of developing dementia. There could therefore be grave consequences for the burden of dementia if the prevalence of diabetes[1] and obesity[13] increase further. Herein, we outline the studies of type 2 diabetes on risk of dementia; the review is not comprehensive, but rather serves to illustrate key concepts. Moreover, we identify knowledge gaps, review strategies that might help fill these gaps, and conclude with a series of recommendations to advance the field.

2. Type 2 diabetes and metabolic syndrome

Type 2 diabetes is characterized by insulin resistance, i.e., an impaired ability of the body to respond to insulin and metabolize glucose, leading to hyperglycemia, defined as a fasting glucose level greater than or equal to 100 mg/dL (Fig. 1). Additionally, patients with a glycated hemoglobin (HbA1c) higher than 6.5 % also meet the criteria of diabetes. In some instances, type 2 diabetes is preceded by prediabetes, an insulin resistant state with an elevated HbA1c of 5.7–6.4 %, which is above normal values but below the level defining type 2 diabetes. Type 2 diabetes is frequently co-morbid with obesity and the metabolic syndrome. Obesity can be characterized by generalized metrics, such as body mass index (BMI), or by parameters of central obesity, such as waist circumference. The metabolic syndrome encompasses a collection

Abbreviations: BMI, body mass index; MRI, magnetic resonance imaging; PET, positron emission tomography.

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of metabolic dysfunctions, which broadly include insulin resistance, obesity, dyslipidemia, and hypertension. The clinical criteria defining individuals with the metabolic syndrome require at least 3 out of 5 clinical findings that include elevated waist circumference, systolic or diastolic blood pressure, triglycerides, and fasting glucose and lower high-density lipoprotein cholesterol (Fig. 1).[14]

3. Dementia definitions

Dementia is an umbrella term, which encompasses several distinct clinical entities. It is characterized by a diminished ability to lead a normal life on a daily basis due to cognitive impairment from poor memory, executive function, and judgement, along with a decline in behavioral and social skills. Cognitive impairment progresses with time along a continuum, starting from mild cognitive impairment leading to frank dementia (Fig. 1). The most common dementia is Alzheimer's disease and the closely associated Alzheimer's disease-related dementias (ADRDs), which are defined by the presence of extracellular amyloid-β plaques and intracellular hyperphosphorylated tau tangles in the brain.[5] Vascular dementia occurs in 20 to 30 % of cases, with Lewy body and frontotemporal dementias accounting for 10–25 % and 10–15 % of cases, respectively. These dementias differ from Alzheimer's disease in neuropathology and the affected brain areas. For the purposes of this review, we will use the term "cognitive impairment" to refer to the clinical manifestation of dementia and will employ the general term "dementia" to refer to the link with diabetes.

4. Type 2 diabetes and the metabolic syndrome increase dementia risk

Patients with type 2 diabetes frequently develop neurological complications. Peripheral neuropathy and cardiac autonomic neuropathy are long known neurologic complications.[15] However, increasingly, evidence indicates that type 2 diabetes may also cause injury to the brain, possibly through similar pathological processes as occurring in peripheral nerves, which would manifest as cognitive impairment and, eventually, dementia. Indeed, clinical studies underscore a correlation between presence of peripheral neuropathy with the development of cognitive impairement.[16,17]

There are also important and shared pathological features between type 2 diabetes and dementia, which are both characterized by metabolic perturbations in the brain, e.g., insulin resistance,[18] altered glucose uptake and utilization.[19] These similarities in pathology are reflected in clinical studies that demonstrate an increased risk of dementia in individuals with type 2 diabetes and dementia.[9,10,20] Moreover, the Lancet Commission on dementia prevention, intervention, and care now recognizes diabetes as a well-established risk factor for dementia.[6,7] The onset of dementia in diabetes patients is gradual. It starts with subtle cognitive impairment, which, in progressive patients, develops into mild cognitive impairment followed by frank dementia, oftentimes as Alzheimer's disease.[21] This progressive cognitive impairment occurs in parallel with structural brain changes, as revealed by magnetic resonance imaging (MRI).[21] In addition to diabetes, obesity and hypertension, also components of the metabolic syndrome, are well-established risk factors for dementia based on the Lancet Commission[6,7] and independent studies.[22–24]

To estimate the contribution to dementia burden from diabetes, obesity, and hypertension, the Lancet Commission estimated the population attributable fraction (PAF), which represents the percentage of new cases that could be avoided if a specific risk factor was removed.[6,7] Their analysis leveraged risks from institutional guidelines, primary studies, and meta-analyses to calculate PAF for various risks on all-cause dementia. PAF for diabetes in later life was calculated as 1.1 %, 0.7 % for midlife obesity, and 1.9 % for hypertension. Additionally, physical

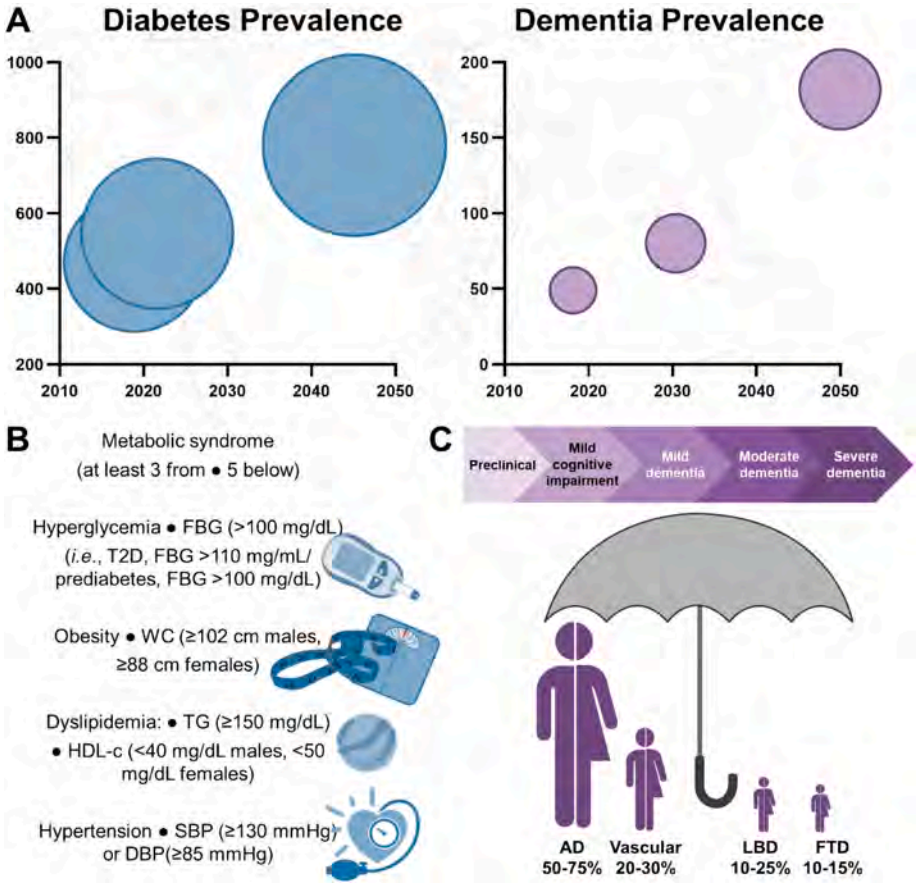

Fig. 1. Infographic of type 2 diabetes and dementia global prevalence and definitions.
(A) Diabetes prevalence in blue, y-axis represents millions of people, x-axis represents decades; circle area represents estimated global prevalence in 2019, 463 million people (9.3 % of global population) and 2021, 536.6 million people (10.5 % of global population) and projected global prevalence in 2045, 783.2 million people (12.2 % of global population); dementia prevalence in purple, y-axis represents millions of people, x-axis represents decades; circle area represents estimated global prevalence in 2018, 50 million people and projected global prevalence in 2030, 82 million people and 2050, 152 million people.[1,2,4] (B) Definitions of type 2 diabetes (T2D), prediabetes, and the metabolic syndrome.[14] DBP, diastolic blood pressure; FBG, fasting blood glucose; HDL-c, high-density lipoprotein cholesterol; SBP, systolic blood pressure; TG, triglycerides; WC, waist circumference. (C) Top: Continuum of cognitive impairment, beginning with an asymptomatic preclinical phase, progressing to mild cognitive impairment, and leading to frank dementia. Bottom: Umbrella of dementias, height represents proportion of clinical entity to total dementia prevalence. AD, Alzheimer's disease; FTD, frontotemporal dementia; LBD, Lewy body disease.[82] Figure contains elements from Biorender.com. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

inactivity, which is an aspect of poor metabolism and the modern sedentary lifestyle, has a PAF of 1.6 % for all-cause dementia.

In addition to the findings from the Lancet Commission on dementia prevention, intervention, and care, we present evidence for the link between type 2 diabetes and components of the metabolic syndrome to dementia from a series of illustrative studies. Cross-sectional evaluation of PREDIMED-PLUS, a study of 6823 older participants averaging 65 years of age, identified that an HbA1c cutoff of 7 % (i.e., 53 mmol/mol) impacted executive function in overweight or obese type 2 diabetes participants, after adjusting for education and additional clinical parameters.[20] Longitudinal assessment of The Atherosclerosis Risk in Communities, a study of 5099 older participants, over a median of 5 years found several risk factors for developing mild cognitive impairment, spanning presence of diabetes, diabetes duration, and poor diabetes control, following adjustments for education, among other parameters.[9] Longitudinal analysis of the Japan Public Health Center-Based Prospective Study over 23 years of follow-up reported that diabetes raised the risk of incident dementia based on 1244 participants, after adjusting for education and more variables.[10]

Other components of the metabolic syndrome, which are frequently comorbid with type 2 diabetes, similarly increase the chance of dementia. Cross-sectional analysis of our own Michigan cohort ($n = 184$) found that obesity, independent even of diabetes, compromised performance on the NIH Toolbox, following adjustments, in addition to educational attainment.[22] Meta-analysis of 21 longitudinal studies at least 2 years long reported obesity increased risk of developing dementia in participants below 65 years old but decreased the risk in participants older than 65 years.[23] Dyslipidemia emerged as a risk factor for cognitive decline at a 20-year follow-up in the ARIC study ($n = 13{,}997$) when adjusting for education and several additional parameters.[24]

Overall, these findings indicate that diabetes and components of the metabolic syndrome, i.e., obesity, hypertension, dyslipidemia, predispose individuals to dementia.

5. Major identified gaps limiting progress

Since diabetes and obesity are modifiable, they may constitute a potential avenue for lowering future incidence of dementia. Based on estimated PAFs, about 1.1 % of dementia cases may be preventable if later life diabetes could be avoided.[7] Preventing midlife obesity with a BMI above 30 kg/m^2 could similarly stop about 0.7 % of dementia cases.[7] Although the percentages of preventable dementia from diabetes, obesity, hypertension, and inactivity are low, they comprise a significant number of individuals, since annual global incidence rates are high. Thus, controlling these modifiable risk factors may pave a way forward for managing the anticipated burden of dementia. This is especially critical since there is evidence to suggest that age-specific incidence of dementia may currently be on the decline, especially in higher income countries,[7] possibly due to improved education, socioeconomic status, lifestyle, and healthcare.[6] However, we stand to lose the benefit from the current decreasing trend in dementia incidence if we cannot offset the rising prevalence of risk factors like type 2 diabetes[1,2] and obesity.[25,26]

The Lancet Commission summarized the available studies that examined modifiable risk from diabetes, obesity, hypertension, and inactivity.[7] Tentative evidence indicates that controlling hypertension with anti-hypertensive medications or exercise to battle inactivity may reduce dementia incidence; however, data regarding anti-diabetic medications and the long-term effects of weight loss are sparser and inconclusive.[7] The reasons anti-diabetic medications may not effectively lower dementia incidence may be manifold; possibly, once the diabetes disease process has started, it may already be too late to mitigate dementia risk due to irreversible damage to the brain. Longitudinal, prospective studies are needed to investigate early changes that occur to the brain during prediabetes and diabetes, both to document early injury and identify biomarkers for individuals most at-risk of dementia. Moreover, it is necessary to evaluate whether early interventions to prevent diabetes, rather than treating it once the disease has started, may more effectively prevent dementia.

Alternatively, diabetes is frequently comorbid with other components of the metabolic syndrome, such as obesity, which may still incur a risk of dementia to diabetic patients, even if they are taking anti-diabetic medications. Thus, a multi-pronged approach targeting multiple elements of the metabolic syndrome, and not just diabetes, may be needed. Another possibility is that some diabetes patients may be genetically predisposed to dementia and Alzheimer's disease. Indeed, although primarily a metabolically-acquired disease, polygenic risk, i.e., arising from multiple single nucleotide variants (SNPs) at several sites, exists for type 2 diabetes,[27] as well as, potentially, for peripheral diabetic neuropathy.[27] Dementia and Alzheimer's disease more specifically have well-established genetic risk.[5,28] Thus, it may be possible if type 2 diabetes and dementia share genetic risk, that some patients with type 2 diabetes may be genetically predisposed to dementia. If highly penetrant, this polygenic risk may not be non-modifiable if it is highly penetrant even by lifestyle changes. Alternatively, rather than being an inherent feature of diabetes biology, studies published to date on anti-diabetic drugs on dementia risk may not have been powered to detect an effect.[29] Larger, well-designed studies of anti-diabetic drugs may help address this possibility. Finally, dementia still lacks disease-modifying therapies; thus, targeted, mechanism-based therapies are needed, and understanding the shared pathophysiology between diabetes and dementia, for example using Omics, could help develop treatments.

Thus, although diabetes is now definitively recognized as a dementia risk factor, along with other components of the metabolic syndrome, the path forward remains challenging since the ability to modify dementia risk remains uncertain.

6. Innovative methods to address the knowledge gaps

In this section, we review research methods that may be applied to address the knowledge gaps in diabetes leading to dementia. The focus is on clinical studies in consented participants or on human tissue, although a rich literature on research models also exists. We cover brain imaging to monitor early changes that occur during the progression of disease from prediabetes to diabetes. Additionally, Omics technologies generate large datasets of genetic, epigenetic, transcriptomic, proteomic, and metabolic information to uncover the biology underlying the risk of dementia from diabetes.

6.1. Brain imaging

Reliable brain imaging biomarkers could prove highly transformative as biomarkers relevant to diabetes-related dementia. Non-invasive imaging of at-risk individuals could detect early changes to the brain prior to clinical manifestation of cognitive impairment. This could help as a diagnostic tool of at-risk individuals with prediabetes or diabetes or permit initiation of interventions, either possible lifestyle interventions or as-yet unavailable disease-modifying therapies, within this critical window for neuroprotection. Furthermore, as our understanding of mechanisms underlying diabetes-linked dementia progresses, imaging biomarkers could enable preventative strategies. Finally, imaging could track changes over time, providing a longitudinal measure of efficacy for any attempted intervention.

6.1.1. Brain magnetic resonance imaging

The current imaging technology, based largely on magnetic resonance imaging (MRI), is helpful for detecting brain changes. Difficulty, however, arises in determining whether these findings are a direct link between a comorbid condition like diabetes to dementia as opposed to noncontributory sequelae of a chronic condition. However, incorporating imaging techniques and biomarkers into modern clinical studies strengthens the causative link between diabetes and the progression to

dementia. For example, prediabetes[30,31] and diabetes[8] worsens signs of small vessel disease on MRI (white matter hyperintensities, lacunar and microinfarcts, microbleeds, or enlarged perivascular spaces) and alters brain volumes in regions subserving memory (e.g., hippocampus) (Table 1). In the Swedish National Study on Aging and Care-Kungsholmen, both prediabetes and diabetes correlated with elevated cognitive impairment over a mean follow-up of 9 years.[32] Prediabetes decreased total brain and white matter volume, whereas diabetic patients showed an increase in the volume of white matter hyperintensities over time. Similarly, in a subset of the Atherosclerosis Risk in Communities Neurocognitive Study, diabetic participants with HbA1c $\geq$7 % had smaller global and regional brain volumes as well as larger white matter hyperintensities versus diabetic participants with HbA1c <7 % or non-diabetic individuals.[33] However, here, and in other observational studies, direct causation between diabetes-related changes on MRI and cognitive performance could not be concluded, although the interaction persists after controlling for cardiovascular contributors.[33,34]

This link between diabetes, dementia, and concurrent pathophysiologic processes (e.g., neurovascular disease) is complex; however, some studies still demonstrate a strong direct link between diabetes and dementia. In participants from the Alzheimer's Disease Neuroimaging Initiative, there was an indirect association between type 2 diabetes with increased cortical atrophy (i.e., decreased baseline cortical thickness), which in turn correlated with greater cognitive decline.[35] Similarly, the Canadian Alliance for Healthy Hearts and Minds Study showed diabetes was linked to higher odds of cognitive impairment, even after controlling for dementia risk factors, such as education, ethnicity, smoking, hypertension, and vascular brain injury, suggesting diabetes impacts dementia via other factors or directly.[36]

6.1.2. Brain functional MRI

Several studies have utilized functional MRI (fMRI), to investigate the impact of diabetes on neural network activity in the context of dementia (Table 1).[37] fMRI detects changes in brain blood flow in participants in a resting state or while performing cognitive tasks in the MRI scanner. The increased regional blood flow serves as a surrogate of enhanced regional neural activity. Thus, assessing neural network function and connectivity by fMRI enhances structural findings from regular MRI imaging.

Participants with type 2 diabetes exhibit reduced connectivity of the default mode network and related structures on resting state fMRI, paralleling changes seen in cognitive and neurodegenerative disorders.[38,39] Furthermore, this change is more pronounced in participants with both type 2 diabetes and cognitive impairment.[37,40] Interestingly, a dose of intranasal insulin appears to restore connectivity between hippocampus and the default mode network.[41] In the majority of task-based fMRI studies, participants with type 2 diabetes tend to perform more poorly on recall tasks, with concomitant decreased activation in structures underpinning the tested cognitive functions.[37]

The inseparable link between diabetes and vascular pathology, and the reliance of most fMRI studies on blood-oxygen level-based contrast, advocates caution interpreting these nevertheless compelling studies. It is possible diabetes contributes to dementia via both microvascular insults causing downstream neurodegeneration, or directly through insulin resistance in neurons and impaired network function. Since fMRI studies rely on the link between neural activation and blood flow, disrupting this presumed relationship may undermine the conclusions of fMRI studies linking diabetes directly to neural function. Approaches to account for altered neurovascular coupling can be undertaken,[42] and further studies linking diabetes-related imaging changes and dementia will help tease apart this Gordian knot.

6.1.3. Brain positron emission tomography

Use of functional imaging may provide an additional dimension of data to link disease processes in diabetes with dementia and cognitive decline. Positron emission tomography (PET) is a modality that imparts a biochemical dimension to imaging data. PET can leverage ^{18}F-fluorodeoxyglucose ([^{18}F]-FDG) uptake (FDG-PET) as a measure of regional brain glucose metabolism. FDG-PET shows reduced uptake in brain regions of participants with prediabetes and diabetes similar to brain regions with glucose hypometabolism in participants with Alzheimer's disease (Table 1).[43–45] Moreover, markers of peripheral insulin resistance also correlate with impaired brain metabolism.[43] Interestingly, intranasal insulin enhanced cognition in some domains in a small pilot clinical study of Alzheimer's disease particiapants.[44] Studies are generally small and confounding parameters may limit interpretation, but their potential significance is intriguing.

PET imaging is also performed using radiolabeled compounds that bind to amyloid-β. This PET modality has been used to test the hypothesis that impaired insulin signaling directly exacerbates the formation of amyloid-β plaques and neurofibrillary tau tangles. An early study did not find that insulin resistance correlated to amyloid-β burden using both [^{11}C]-Pittsburgh Compound B (PiB)-PET and autopsy studies.[46] Indeed, in a subset of the Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability, individuals with positive PiB-PET had slightly better glucose homeostasis, but possibly not after adjusting for covariates.[47,48] However, in the Health2000 study, individuals with midlife insulin resistance associated with elevated PiB-PET positivity.[49] In the more recent MEMENTO cohort, diabetes was linked to elevated uptake of amyloid-β-binding radiotracers (^{18}F-florbetapir or ^{18}F-flutemetamol).[50] Diabetes appeared to contribute to neurodegeneration markers, which, in turn, correlated strongly to cognitive decline, even after controlling for Alzheimer's disease biomarkers and small vessel disease.[50]

The interplay between imaging modality and radiotracer properties, timing of imaging, and a host of other diabetes and dementia risk factors continues to make this field complex yet promising.

6.2. Omics technologies

Omics technologies generate large amounts of data in a system-wide manner by agnostically querying molecular pathways. They are extremely useful for shedding light on complex diseases or querying the intersection or shared biology between two conditions. We examined the literature and found applications of genomics, epigenomics, transcriptomics, proteomics, and metabolomics to elucidate the relationship between type 2 diabetes and dementia. Data from these studies can help address current knowledge gaps by evaluating shared genetic risk between type 2 diabetes and dementia, as well as shared biological pathways, which may provide insight for therapeutic approaches.

6.2.1. Genome-wide association studies (GWAS)

Although primarily considered a metabolically-acquired disease, type 2 diabetes does possess a component of polygenic inheritance.[27] Similarly, dementia, e.g., Alzheimer's disease, occurs from a combination of common and rare causative and risk genes of variable penetrance, either monogenic or belonging to a polygenic risk profile.[5] Heritability in Alzheimer's disease is 60 to 80 %;[5] however, since many variants are not causative, risk may be reduced by modifiable factors, such as metabolism or metabolic dysfunction, as occurs in type 2 diabetes.

Analyses of several GWAS studies have been performed to determine whether type 2 diabetes genes overlap with those for dementia, with both positive and negative findings (Table 2). Studies have found associations of genetic risk for type 2 diabetes and glycemic traits, e.g., HbA1c, fasting glucose, insulin resistance and β-cell dysfunction, with Alzheimer's disease,[51–54] dementia,[54] and cerebrovascular disease.[55] GWAS of lacunar stroke found association with type 2 diabetes.[56] Studies that examined functional and pathway enrichment identified shared and recurrent pathways in immune responses, cell signaling, neuronal plasticity and cellular processes.[52]

Conversely, some GWAS investigations yielded no correlations

Table 1
Overview of select imaging studies on type 2 diabetes and dementia.
Studies arranged chronologically, earliest to latest.

Reference	Link investigated	Study population	Methods	Main findings
MRI studies				
Schneider et al. 2017[33]	Prediabetes and diabetes on brain volume and subclinical CVD	Prediabetes (n = 514; m/f 197/317); diabetes (n = 602; m/f 149/453); controls (n = 597; m/f 241/356); The Atherosclerosis Risk in Communities Neurocognitive Study (ARIC-NCS); cross-sectional	MRI	Prediabetes and diabetes (HbA1c <7.0 %) do not differ significantly in brain volume or vascular pathology versus controls; diabetes (HbA1c ≥7.0 %) linked to smaller total brain and regional brain volumes (frontal, temporal, occipital, parietal lobes; deep gray matter; Alzheimer's disease signature region; hippocampus [all $p < 0.05$]), and higher burden of WMH ($p = 0.016$) versus controls; diabetes HbA1c ≥7.0 % versus <7.0 % linked to smaller total and regional brain volumes and higher WMH burden (all $p < 0.05$); participants with diabetes duration ≥10 years versus <10 years linked to smaller brain volumes and higher lacune burden (all $p < 0.05$); adjusted for age, sex, race/field center, education, smoking status, hypertension, cardiovascular disease, APOE4, and TIV (for volume outcomes)
Cui et al. 2019[31]	Structural subcortical gray matter changes in prediabetes and T2D	Prediabetes (n = 21; m/f 8/13); T2D (n = 21; m/f 7/14); age-, sex-, education-matched controls (n = 21; m/f 7/14); China; cross-sectional	MRI	Prediabetes and T2D linked to lower gray matter volume in the bilateral lateral hippocampi, left amygdala, and right putamen versus controls; postprandial blood sugar in T2D linked to lower gray matter volume in the left hippocampus; corrected for head size variation; age, sex, and education included as covariates
Dong et al. 2019[30]	HbA1c, cognitive function, and hippocampal subfields volumes in prediabetes and T2D	Prediabetes (n = 17 m/f 8/9); T2D (n = 21; m/f 10/11); controls (n = 22; m/f 12/10); China; cross-sectional	MRI, MoCA, Rey auditory verbal learning test, Stroop color and word tests, verbal fluency test, Trail Making Tests A & B	Total left hippocampal ($p = 0.046$) and left hippocampal tail ($p = 0.014$) volume differed across all groups; HbA1c correlated negatively with left hippocampal tail volume ($p = 0.009$); HbA1c correlated positively with executive dysfunction, assessed by trail making test B ($p = 0.0016$) and Stroop test C ($p = 0.001$); corrected for TIV; adjusted for age, sex, education, BMI, history of hypertension, cholesterol level, and APOE4
Marseglia et al. 2019[32]	Prediabetes and diabetes on cognitive decline and brain aging	Prediabetes (n = 947; m/f 326/621); diabetes (n = 242; m/f 127/115); controls (n = 1557; m/f 570/987); MRI subsample (n = 455); Swedish National Study on Aging and Care-Kungsholmen; 9-year longitudinal study	MRI, MMSE, dementia diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, 4th Ed.	Prediabetes and diabetes linked to faster cognitive decline over 9 years versus controls, adjusted for baseline age, sex, education, SES, BMI, smoking, alcohol consumption, physical activity, hypertension, heart disease, CVD, and APOE4; prediabetes linked to smaller TBTV, especially WMV, diabetes linked to higher WMH at baseline, diabetes linked to faster WMHV increase longitudinally, MRI adjusted for TIV and age, analyses adjusted for sex, education, SES, BMI, hypertension, and heart disease
Moran et al. 2019[35]	T2D on brain atrophy and cognitive decline	T2D (n = 124; m/f 85/39); controls (n = 693; m/f 390/303); Alzheimer's Disease Neuroimaging Initiative; 5-year longitudinal study	MRI, American National Reading Test, Rey Auditory Verbal Learning Test, Boston Naming Test, category fluency, Clock Drawing Test, Alzheimer's Disease Assessment Scale–cognitive subscale, Construction Praxis Test, Digit Span forwards and backwards tasks, Trail-Making Tests A & B, WAIS-R, Wechsler Memory Scale–Revised	T2D linked to lower baseline cortical thickness (p = 0.01); no direct T2D effect on decline in cortical thickness or cognition, but T2D linked to cognitive decline via baseline cortical thickness; T2D interacted with education, with lower T2D impact on baseline cortical thickness in participants with higher education; covariates were T2D, age, sex, education, APOE4, and cognition
Gerstein et al. 2021[36]	Diabetes on brain infarcts, small vessels, and cognition	Diabetes (n = 495; m/f 290/205); controls (n = 7238; m/f 3236/4002); Canadian Alliance for Healthy Hearts and Minds; cross-sectional	MRI, MoCA, Digit Symbol Substitution Test MRI	Diabetes linked to small vessel vascular brain injury (OR 1.52, 95 CI 1.15, 2.01) after adjusting for CVD risk factors and nonlacunar infarcts; diabetes linked to cognitive impairment (OR 1.27, 95%CI 1.03, 1.56) after adjusting for small vessel vascular brain injury

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Table 1 (*continued*)

Reference	Link investigated	Study population	Methods	Main findings
Grosu et al. 2021[34]	Prediabetes and diabetes on WMH	Prediabetes (n = 98; m/f 62/36); T2D (n = 51; m/f 37/14); controls (n = 239; m/f 123/116); Cooperative Health Research in the Region of Augsburg (KORA) FF4 study; cross-sectional		Prediabetes (p = 0.001) and T2D (p = 0.026) linked to higher WMH versus controls; OGTT 2-h serum glucose (p < 0.001), but not fasting glucose (p = 0.389) or HbA1c (p = 0.050), correlated positively with WMH; adjusted for age, sex, hypertension, LDL-c, BMI, smoking, and alcohol consumption
Functional MRI (fMRI)				
Zhang et al. 2015[41]	T2D on brain functional connectivity upon intranasal insulin	T2D (n = 14; m/f 8/6); controls (n = 14; m/f 4/10); USA; cross-sectional; NCT01206322	Resting-state BOLD fMRI, MMSE, Hopkins Verbal Learning Test-Revised, Trail-Making Tests A & B, Digit Span, Brief Visuospatial Memory Test-Revised, Verbal Fluency Task, Delis-Kaplan Executive Function System	Insulin increased connectivity between hippocampal regions and the medial frontal cortex (p = 0.03) and default mode network regions versus placebo in T2D participants; connectivity in T2D participants with versus without insulin was similar and lower than controls, respectively; connectivity correlated to cognition, which intranasal insulin enhanced in older T2D participants; adjusted for age, education, and race
Yang et al. 2016[40]	T2D with/without cognitive impairment on brain functional connectivity	T2D with cognitive impairment (n = 19; m/f 5/14); T2D without cognitive impairment (n = 19; m/f 7/12); controls (n = 19; m/f 8/11); China; cross-sectional	Resting-state fMRI, MoCA, MMSE, Activity of Daily Living	T2D impaired brain integrity, network, and connectivity to a greater extent in participants with versus without cognitive impairment, centered on changes in bilateral posterior cerebellum, right insula, default mode network, and control network; HbA1c and diabetes duration affected functional connectivity strength of specific brain regions; adjusted for age, sex, and education
Cheng et al. 2021[39]	T2D (without cognitive impairment and microvascular complications) on brain functional connectivity	T2D (n = 27; m/f 13/14); controls (n = 26; m/f 13/13); China; cross-sectional	Resting-state BOLD fMRI, MoCA, MMSE	T2D participants without cognitive impairment and microvascular complications had lower functional connectivity with posterior cingulate cortex (FC-PCC) in the anterior cingulate gyrus, right superior frontal gyrus, right medial frontal gyrus, and right angular gyrus, but higher FC-PCC in right superior temporal gyrus and calcarine fissure versus controls; FC in various regions correlated with HbA1c and diabetes duration; covariates were age, sex, education, and BMI
Guo et al. 2021[38]	T2D on brain functional connectivity	T2D (n = 60; m/f 39/21); controls (n = 33; m/f 18/15); China; cross-sectional	Resting-state BOLD fMRI, MoCA	T2D participants had lower functional connectivity strength in the bilateral fusiform gyri, right superior frontal gyrus, and right postcentral gyrus, but higher functional connectivity strength in the right supplementary motor area versus controls; T2D changed effective connectivity directionality between the left fusiform gyrus and bilateral lingual gyri and right medial frontal gyrus, as well as between the right superior frontal gyrus and bilateral frontal regions; triglyceride, insulin, and plasma glucose levels linked to abnormal effective connectivity of the left fusiform gyrus, while disease duration and cognitive function linked to abnormal effective connectivity of the right superior frontal gyrus in T2D; adjusted for age, sex, and education
Chen et al. 2014[83]	T2D and activation of working memory areas	Older participants with T2D without cognitive impairment (n = 30; m/f 13/17); controls (n = 37; m/f 18/19); China; cross-sectional	Task-based BOLD fMRI, visual n-back task	T2D participants had poorer response time/accuracy measures (p = 0.007) in the 1-back task; in the 1-back vs 0-back condition, T2D participants had reduced activation in the left inferior frontal gyrus; in the 2-back vs 0-back condition, T2D participants had reduced activation in left middle frontal gyrus and left superior frontal gyrus
Duarte et al. 2015[42]	Hemodynamic response function and neurovascular coupling in T2D	T2D without vascular lesions (n = 51; m/f 30/21); controls (n = 29; m/f 14/15); Portugal; cross-sectional	Task-based BOLD fMRI, block and event-related designs with deconvolution analysis	T2D participants had an altered hemodynamic response function, which

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Table 1 (*continued*)

Reference	Link investigated	Study population	Methods	Main findings
				may impact interpretation of BOLD signal in future fMRI studies
He et al. 2015[84]	T2D and activation of working memory areas in newly diagnosed participants	T2D, recent diagnosis in mid-life (n = 12; m/f 11/1); controls (*n* = 12; m/f 8/4); China; cross-sectional	Task-based BOLD fMRI, visual *n*-back task	No difference in task performance between groups; T2D participants had higher activation in right dorsolateral prefrontal cortex, left middle/inferior frontal gyrus, and left parietal cortex during 2-back task; suggestion of "compensatory" activation to complete task
Huang et al. 2016[85]	Cognitive task performance and areas of activation on fMRI in T2D	T2D (n = 18; m/f 6/12); controls (*n* = 18; m/f 6/12); China; cross-sectional	Task-based BOLD fMRI, n-back task, MoCA (Chinese revised), Wechsler Memory Scale (Chinese revised)	T2D participants had poorer performance on MoCA in visuospatial, attention, language, abstraction, and memory domains, as well as in total scores; T2D participants had poorer performance on Wechsler Memory Scale in a subset of mental control, visual recognition, visual reproduction, associative learning, touch test, understanding memory, numeric span, and memory quotient; T2D participants had poorer accuracy and reaction time in the 2-back task; control participants showed greater activation in bilateral dorsolateral prefrontal cortices and parietal cortex and supplementary motor area in the 1-back task, and additionally the premotor area and the precuneus in the 2-back task
Wood et al. 2016[86]	Cognitive task performance and areas of activation on fMRI in T2D	Twin pairs discordant for T2D; T2D (n = 22; m/f 12/10); controls (*n* = 22; m/f 8/14); Australia; cross-sectional	Task-based BOLD fMRI and visual memory encoding task, National Adult Reading Test -Revised, Wechsler Memory Scale 3 (mental control and digit span subtests), Wechsler Memory Scale 1 (paired associate learning subtest), Memory with Hopkins Verbal Learning Test-Revised, 5-minute recall for delayed Rey-Osterrieth Complex Figure, Cambridge Neuropsychological Automated Test Battery, visual paired associate learning	No within-pair differences in cognitive tasks or in memory encoding during fMRI; reduced activation for T2D in left angular gyrus, left supramarginal gyrus, and left middle temporal gyrus across all pairs; greater activation in T2D in bilateral superior parietal lobules, bilateral precunei, right inferior parietal lobule, and left occipital/fusiform/cuneus
PET				
Baker et al. 2011[43]	Brain glucose metabolism in cognitively normal T2D and prediabetes participants	T2D (n = 12); prediabetes (*n* = 11); controls (n = 6); USA; cross-sectional	Resting and activation [^{18}F]-FDG PET	Higher peripheral HOMA-IR linked to AD-like lower brain glucose metabolism in frontal, temporal-parietal, and cingulate regions, independent of age, 2-h OGTT, or APOE4; glucose metabolism pattern across the brain differed during the memory encoding task in T2D and prediabetes versus controls; unsure about adjustment parameters
Craft et al. 2012[44]	Insulin on cognitive performance and brain glucose metabolism in AD	AD high-dose insulin 40 IU (n = 38; m/f 20/18); AD low-dose insulin 20 IU (*n* = 36; m/f 22/14); AD placebo (n = 30; m/f 17/13); USA; cross-sectional; NCT00438568	[^{18}F]-FDG PET, ADAS-ADL, ADAS-cog	20 IU insulin improved delayed memory ($p < 0.05$); 20 and 40 IU insulin preserved caregiver-rated functional ability ($p < 0.01$); 20 and 40 IU insulin preserved cognition assessed by ADAS-cog score for younger participants and functional abilities by ADCS-ADL scale for adults with AD ($p < 0.05$); placebo group had lower [^{18}F]-FDG uptake in the parietotemporal, frontal, precuneus, and cuneus regions; adjusted for age and education
Thambisetty et al. 2013[46]	Insulin resistance on amyloid-β burden in AD	AD (*n* = 53; m/f 30/23); Baltimore Longitudinal Study of Aging; longitudinal	[^{11}C]-PiB PET	No significant correlations between OGTT to brain [^{11}C]-PiB amyloid-β
Ekblad et al. 2018[49]	Midlife insulin resistance on late-life brain amyloid	Older participants without dementia (*n* = 60; m/f 27/33); Health2000; longitudinal	[^{11}C]-PiB PET	Insulin resistance increases amyloid positivity (OR 11.1, 95%CI 1.9, 91.5; $p = 0.007$), independent of APOE4; higher midlife, but not late-life, continuous HOMA-IR increases brain amyloid at follow-up after multivariate adjustments for other cognitive and metabolic risk factors (β 0.11, 95%CI 0.002, 0.22; p = 0.04); adjusted for age, time from baseline to PiB scan, sex, education, APOE4,

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Table 1 (*continued*)

Reference	Link investigated	Study population	Methods	Main findings
				hypertension, BMI, HDL-c, and triglycerides
Kemppainen et al. 2018[47]	Brain amyloid load link to cognition and vascular risk factors	Participants at increased risk for dementia by CAIDE score ($n = 48$; m/f 26/22); Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability	MRI, [^{11}C]-PiB PET, neuropsychological test battery	PiB positivity linked to poorer executive functioning tests, APOE4, and slightly better glucose homeostasis; PiB positivity and negativity did not differ significantly in other cognitive domain scores or vascular risk factors; adjusted for age
Pekkala et al. 2020[48]	Peripheral insulin resistance and other T2D markers on brain amyloid	Participants at increased risk for dementia by CAIDE score ($n = 41$; m/f 20/21); Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability	[^{11}C]-PiB PET	Lower insulin, HOMA-IR, C-peptide, and plasminogen activator linked to amyloid positivity, but not after adjusting for multiple testing; no model found evidence for a link between amyloid status to fasting glucose or HbA1c
Frison et al. 2021[50]	T2D and associated markers on cognition	Total participants with mild or subjective cognitive complaints ($n =$ 2288; m/f 875/1413); T2D ($n = 254$; m/f 143/111); MEMENTO Cohort Study Group	MRI, [^{18}F]-FDG PET, [^{18}F]-florbetapir, neuropsychological test battery, MMSE, Free and Cued Selective Reminding Test, animal words, Rey-Osterrieth Complex Figure Test, Trail Making Test A & B	T2D link with lower cognition significantly mediated by higher neurodegeneration (standardized indirect effect −0.061, 95%CI −0.089, −0.032), but not by small vessel disease and AD markers; adjusted for age, sex, education, smoking, alcohol, hypertension, dyslipidemia, BMI, and APOE4
Képes et al. 2021[45]	Brain glucose metabolism in T2D versus non-T2D obesity	T2D (n = 51); non-T2D obese (n = 45); Hungary; cross-sectional	[^{18}F]-FDG PET, NeuroQ	T2D and non-T2D obese brain glucose metabolism did not differ by NeuroQ analysis; T2D had lower glucose metabolism in the precuneus and right superior frontal gyrus by voxel-based analysis versus non-T2D obese; correcting for pre-PET glucose level, hypometabolism only in the right superior frontal gyrus; in T2D, pre-PET correlated negatively with glucose metabolism in precuneus, left posterior orbital gyrus, right calcarine cortex, and right orbital part of inferior frontal gyrus; in non-T2D obesity, only the right rolandic (pericentral) operculum sensitive to pre-PET glucose level

[^{11}C]-PiB, Pittsburgh Compound B; [^{18}F]-FDG, fluorodeoxyglucose; AD, Alzheimer's disease; ADCS-ADL, Alzheimer's Disease Cooperative Study–activities of daily living; ADAS-cog, Alzheimer's Disease Assessment Scale–cognitive subscale; APOE4, apolipoprotein E4; BMI, body mass index; BOLD, blood-oxygen level dependent; CI, confidence interval; CVD, cerebrovascular disease; f, female; HbA1c, glycated hemoglobin; HDL-c, high-density lipoprotein cholesterol; HOMA-IR, Homeostatic Model Assessment for insulin resistance; HR, hazard ratio; LDL-c, low-density lipoprotein cholesterol; m, male; MMSE, Mini-Mental State Examination; MoCA, Montreal Cognitive Assessment; MRI, magnetic resonance imaging; OGTT, oral glucose tolerance test; OR, odds ratio; PET, positron emission tomography; SES, socioeconomic status; T2D, type 2 diabetes; TBTV, total brain tissue volume; TIV, total intracranial volume; WAIS-R, Wechsler Adult Intelligence Scale–Revised; WMV, white matter volume; WMH, white matter hyperintensity.

between genetic disposition for diabetes to cognitive impairment[57] or Alzheimer's disease.[58,59] One study found that type 2 diabetes, which was associated with some genetic risk, did predispose to cognitive impairment although it did not share genetic risk with dementia.[57] Negative findings employed a targeted panel of type 2 diabetes SNPs in participants with Alzheimer's disease versus genome-wide investigations, which may have missed less well-known SNP associations.[58] Alternatively, a negative study used GWAS from participants defined as having cognitive impairment, rather than dementia, which may constitute a less strongly defined genetic entity.[57]

However, overall, GWAS studies support the possibility that patients with type 2 diabetes may have some genetic risk for dementia. Diabetes could potentially prove a less modifiable risk for some individuals harboring genetic risk.

6.2.2. *Epigenome-wide association studies*

Epigenetic modifications, which are altered both by genetic and lifestyle factors, have also been investigated as contributors to pathological mechanisms driving cognitive impairment in type 2 diabetes and components of the metabolic syndrome (Table 3).[60,61] Indeed, aging alters methylation patterns[62,63] and changes in methylation are proposed as potential early biomarkers for Alzheimer's disease.[63,64] In a group of Mexican Americans with a high burden of the metabolic syndrome, there were changes in DNA methylation at multiple sites in the buffy coat from blood in participants with versus without cognitive impairment.[61] Pathway enrichment found that these differentially methylated sites were related to metabolic dysfunction and inflammation. Although the study did not investigate correlation between type 2 diabetes and dementia, it did suggest possible epigenetic differences in mild cognitive impairment related to peripheral metabolism. A longitudinal study in older participants with type 2 diabetes showed that over 18 months, participants who developed presymptomatic dementia had changes at 10 methylation sites in blood similar to those seen in participants with clinical Alzheimer's disease.[60] Overall, epigenetic studies demonstrate some changes, although validation studies are needed, including correlations specifically between type 2 diabetes with cognitive impairment and dementia.

6.2.3. *Transcriptomics*

There have been multiple studies investigating transcriptomic changes that are associated with cognitive impairment in type 2 diabetes and components of the metabolic syndrome (Table 3).[65–68] Neurons isolated from autopsy brains from participants with type 2 diabetes exhibit transcriptomic signatures indicative of impaired insulin signaling and metabolism along with neurodegeneration, including enrichment of Alzheimer's disease pathways.[65] When comparing transcriptomic data from type 2 diabetes tissue (brain and endothelial precursor cells) versus Alzheimer's disease tissue (brain), many

Table 2
Genome-wide association studies (GWAS) of type 2 diabetes and dementia.
Studies arranged chronologically, earliest to latest.

Reference	Link investigated	Datasets/study population	Methods	Main findings
Positive findings				
Hao et al. 2015[52]	T2D with AD	*T2D*: DIAGRAM; *AD*: IGAP	Overlapping SNPs with p = 0.01, expression quantitative trait loci, functional and pathway enrichment analysis	395 shared SNPs with the same risk allele for T2D and AD in pathways related to immune responses, cell signaling and neuronal plasticity, cellular processes; 532 shared SNPs with divergent risk alleles for T2D and AD
Karki et al. 2020[53]	T2D with AD	GWAS Catalog, GWAS Central, dbSNP, DisGeNET	Linkage disequilibrium analysis, variant prioritization, literature mining, coherently perturbed genes from gene expression meta-analysis	Identified *ABCG1*, *COMT*, *MMP9*, *SOD2* as potential genes with dual roles in T2D and AD
Pan et al. 2020[51]	Glycemic traits with AD	*T2D*: DIAGRAM and Meta-Analyses of Glucose and Insulin-related traits Consortium; *AD*: IGAP	Mendelian randomization	1 SD higher fasting glucose (OR 1.33, 95%CI 1.04, 1.68; p = 0.02) and lower HOMA-β-cell function (OR 1.92, 95%CI 1.15, 3.21; p = 0.01) causally linked to higher AD risk; no other significant links found
Georgakis et al. 2021[55]	T2D and glycemic traits with CVD, ICH, ischemic stroke and subtypes: large artery, cardioembolic, small vessel stroke, imaging markers of cerebral white matter integrity, brain atrophy	*T2D*: DIAGRAM; *HbA1c*: UK Biobank; *FG*: MAGIC; *ischemic stroke*: MEGASTROKE; *ICH*: International Stroke Genetics Consortium	Mendelian randomization	Genetic risk for T2D and higher HbA1c linked to higher risk of any ischemic stroke, large artery stroke, small vessel stroke; genetic risk for IR linked to higher risk of large artery and small vessel stroke; genetic risk for β-cell dysfunction linked to higher risk of small vessel stroke, ICH, lower gray matter, and total brain volume
Taylor et al. 2021[56]	Genetic basis of lacunar stroke	UK DNA Lacunar Stroke studies 1 and 2, collaborators within the International Stroke Genetics Consortium	Mendelian randomization, transcriptome-wide association study, colocalization	5 loci linked to lacunar stroke; 7 loci linked to cerebral white matter hyperintensity; 2 loci linked to monogenic lacunar stroke; expression of 6 genes linked to lacunar stroke; Mendelian randomization found lacunar stroke linked to T2D, elevated blood pressure, and smoking
Yu et al. 2022[54]	Stroke, diabetes, atherosclerosis, cholesterol level, and alcohol consumption with dementia or AD	UK Biobank, GWAS Catalog	Multi-trait colocalization analysis	T2D shared risk with dementia (*NAALAD2*) and AD (*CDC42BPB*)
Negative findings				
Chung et al. 2015[58]	T2D with AD and Parkinson's disease	AD (n = 400); PD (n = 500); controls (n = 500); case-control study; genotyped 32 variants from 11 genes and intergenic regions linked to T2D from GWAS (Genome.gov)	Mini-mental state examination (MMSE), Montreal Cognitive Assessment (MoCA), logistic regression models adjusted for age and sex	*KCNQ1* SNP linked to AD, but not after Bonferroni correction; *CDC123* SNP modest link to MMSE <26, CDKN2B SNPs modest link to MoCA <26 in PD but, not after Bonferroni correction; no other associations
Garfield et al. 2021[59]	Glycemia, cognitive function, brain structure, incident dementia	UK Biobank	Bidirectional Mendelian randomization, inverse-variance-weighted Mendelian randomization	No evidence of link between T2D and HbA1c genetic risk to measures of cognition (reaction time, visual memory, AD) and brain structure (white matter hyperintensity volume, hippocampal volume) in midlife
Ware et al. 2021[57]	T2D with cognitive impairment	Health and Retirement Study	Mendelian randomization	T2D and cognitive impairment did not share genetic risk; T2D had genetic risk and predisposed to cognitive impairment

AD, Alzheimer's disease; CI, confidence interval; CVD, cerebrovascular disease; DIAGRAM, DIAbetes Genetics Replication And Meta-analysis; FG, fasting glucose; HbA1c, glycated hemoglobin; ICH, intracerebral hemorrhage; IGAP, International Genomics of Alzheimer's Project; IR, insulin resistance; OR, odds ratio; SD, standard deviation; SNP, single-nucleotide variant; T2D, type 2 diabetes.

differentially expressed genes and related pathways overlapped, including those related to autophagy and the immune system.[66–68] However, it is important to note that these studies often combine datasets and/or have a limited number of samples.

6.2.4. *Proteomics & metabolomics*

Additional studies have focused on proteomic and metabolomic analyses to understand type 2 diabetes in the context of cognitive impairment (Table 3).[69] Many of these studies show changes in various apolipoproteins related to fat or cholesterol metabolism, as well as in proteins related to the immune system or inflammation, such as complement C4.[69] The recurrence of apolipoproteins as differential metabolites in Alzheimer's disease underscores APOE4 as a major risk gene.[28] Moreover, lipoproteins are defining metabolites in the metabolic syndrome.[14] In Mexican Americans, proteomics signatures, which adopt an inflammatory component in patients with diabetes, are predictive for mild cognitive impairement.[70] Additionally, when comparing proteomics signatures in type 2 diabetes participants with and without mild cognitive impairement,[71] proteins involved in fat metabolism as well as mitophagy or autophagy were differentially regulated. Conversely,

Table 3
Omics studies of type 2 diabetes and dementia.
Studies arranged chronologically, earliest to latest.

Reference	Link investigated	Datasets/study population	Methods used	Main findings
Epigenetics				
Lunnon et al. 2014[60]	T2D progression to pre-symptomatic dementia	T2D (n = 18); age-, sex-, education-matched controls (*n* = 18); Israel Diabetes and Cognitive Decline study; longitudinal	Differentially methylated sites in blood	10 CpG sites in the vicinity of loci previously implicated in neurodegeneration linked to conversion to pre-symptomatic dementia at 18-month follow-up; differentially methylated sites linked to methylation changes in clinical AD
Pathak et al. 2019[61]	Methylation with MCI	Mexican Americans with MCI (n = 45; m/f 14/31) and normal cognition (*n* = 45; m/f 13/32); Health & Aging Brains of Latino Elders; cross-sectional	Differentially methylated sites in blood cells (buffy coat) with adjusted p = 0.05, expression quantitative trait loci, functional and pathway enrichment analysis, Bayes gene enrichment	10 differentially methylated sites and 4 differentially methylated regions in Mexican Americans with MCI versus normal cognition; pathway enrichment found pathways related to neuronal cell death, metabolic dysfunction, and inflammation
Transcriptomics				
Caberlotto et al. 2019[66]	T2D and AD	T2D (*n* = 20), non-T2D (*n* = 12), T2D + AD (n = 6), non-T2DM + AD (n = 19); Hisayama study GSE36980	Gray matter from cortex (frontal and temporal) and hippocampi were used for microarray, DEGs identified by rank-based classification, network analysis, functional enrichment	Significant overlap between T2D and AD in pathways related to autophagy
Bury et al. 2021[65]	T2D and neuronal dysfunction	T2D (n = 6); age-, sex-matched controls (*n* = 6); Cognitive Function and Ageing Study neuropathology cohort, cross-sectional	Immuno-laser captured neuron, astrocytes, and endothelial cells used for microarray to identify DEGs, which were enriched using weighted gene co-expression network analysis, validated by NanoString	Changes to insulin signaling pathways were common across cell types in T2D; also, neurons were enriched in pathways related to aging and metabolism, including cell-cycle, cellular senescence, inflammation, and mitochondrial respiratory electron transport chain
Huang et al. 2021[68]	T2D and AD	NCBI GEO *T2D*: GSE43950 (endothelial precursor cells) *AD*: GSE28146 (hippocampus)	Microarray analysis, DEGs identified, functional enrichment analysis	64 key DEGs (enriched for immune-related pathways) and 3 coDEGs (*CACNA2D3*, *IER3*, *NUMB*) between T2D and AD
Shu et al. 2022[67]	T2D and AD	NCBI GEO, *T2D*: GSE161355 *AD*: GSE122063, GSE118553, GSE109887, GSE132903	Microarray analysis of temporal cortex from AD, T2D and matched controls, identified DEGs, pathway enrichment, functional enrichment	16 shared DEGs between T2D and AD, enriched in pathways related to apoptosis, autophagy, inflammation, and hemostasis
Proteomics and metabolomics				
Johnson et al. 2020[70]	T2D and depression on proteomics in MCI	Mexican Americans with T2D + depression (*n* = 85; m/f 19/66); T2D (*n* = 127; m/f 95/32); depression (*n* = 118; m/f 93/25); controls (n = 184; m/f 138/46); Health & Aging Brains of Latino Elders; cross-sectional	Proteomics on blood based on an electrochemiluminescence assay	T2D influences proteomic profile in MCI; profile has inflammatory component; T2D increases metabolic markers in the profile
Mindikoglu et al. 2020[72]	Intermittent fasting on metabolism and cognition	Healthy participants (n = 14; m/f 13/1) fasted for 14 h daily for 30 consecutive days	Proteomics on serum with/without depletion of abundant proteins, HPLC-MS/MS	Fasting serum proteomic signature upregulated in key regulatory proteins of glucose and lipid metabolism, insulin signaling, cognitive function, immune system, circadian clock, DNA repair, and cytoskeleton remodeling; serum proteome protective against diabetes, obesity, metabolic syndrome, AD, inflammation cancer
Yu et al. 2021[71]	T2D and MCI	T2D + MCI (n = 9; m/f 3/6); T2D (*n* = 10; m/f 4/6); NCT01830998	Proteomics on lysed platelets, labeling followed by LC-MS/MS	Differentially expressed proteins in T2D with and without MCI enriched for pathways related to fat metabolism and endocytosis; proteins linked to cognitive score involved in metabolic pathways and mitophagy/autophagy
Data integration				
Darst et al. 2019[73]	Genomics, longitudinal metabolomics, and AD risk factors	Participants (*n* = 1111; m/f 345/766); CSF metabolomics subset (*n* = 155; m/f 52/103); Wisconsin Registry for Alzheimer's Prevention; longitudinal	17 AD risk factors, plasma and CSF metabolomics, CSF AD biomarkers, genomics microarray, integrative network analysis, targeted mediation and interaction analyses	Multiple plasma and CSF metabolites clustered around AD risk factors; no gene directly clustered to an AD risk, indicating metabolites may mediate the AD risk factor-gene relationship; adjusted for several variables and multiple testing
Nugent et al. 2020[74]	T2D risk genes and glucose brain metabolism in aging and AD	[^{18}F]-FDG PET Alzheimer's Disease Neuroimaging Initiative (probable AD, *n* = 335; controls *n* = 386), Allen Human Brain Atlas gene expression	Generated gene expression map from gene expression atlas, correlated to [^{18}F]-FDG PET maps and T2D SNPs	15 risk genes correlated to [^{18}F]-FDG PET brain metabolism in controls versus probable AD participants after adjusting for multiple comparisons; 5 genes explained 72.5 % of glucose uptake

(*continued on next page*)

Table 3 (*continued*)

Reference	Link investigated	Datasets/study population	Methods used	Main findings
		database, Desikan–Killiany Atlas (anatomical)		variance across the control group regions, 4 genes accounted for 79.3 % across regions of the probable AD group; allele frequencies in 2 genes linked to differences in whole-brain glucose uptake

[^{18}F]-FDG, fluorodeoxyglucose; AD, Alzheimer's disease; CSF, cerebrospinal fluid; DEGs, differentially expressed genes; eQTL, expression quantitative trait loci; HPLC-MS/MS, high-performance liquid chromatography/tandem mass spectrometry; LC-MS/MS, liquid chromatography/tandem mass spectrometry; MCI, mild cognitive impairment; PET, positron emission tomography; SNP, single nucleotide polymorphism; T2D, type 2 diabetes.

intermittent fasting in healthy participants generated a proteomics signature indicative of protection against metabolic syndrome, inflammation, and Alzheimer's disease.[72]

6.3. Data integration approaches

Powerful bioinformatics tools facilitate the integration of multiple Omics datasets, which may yield additional insight into the connection between type 2 diabetes and dementia. In an integrative study of genomics and longitudinal metabolomics with Alzheimer's disease risk factors, network analysis revealed that multiple plasma and cerebrospinal fluid metabolites clustered around Alzheimer's disease risk factors.[73] Interestingly, no gene directly clustered to an Alzheimer's disease risk factor, suggesting that metabolites may mediate the Alzheimer's disease risk factor-gene relationship. Moreover, Omics datasets can be merged with spatial modalities, such as PET and MRI. A recent study of PET integrated with spatial gene expression and SNPs of Alzheimer's disease and type 2 diabetes yielded new mechanistic insights. The study found that 15 risk genes correlated to FDG-PET brain metabolism in controls versus probable Alzheimer's disease participants. Five genes explained 72.5 % of glucose uptake variance across the control group regions, whereas 4 genes accounted for 79.3 % across regions of the probable AD Alzheimer's disease. Changes were linked to expression of the TOMM40 gene (translocase of outer mitochondrial membrane 40 homolog), implicating mitochondrial dysfunction as a potential driver of disease.[74]

6.4. Other technologies

Additional technologies that may shed some light on potential mechanisms of type 2 diabetes in dementia include single-cell RNA-sequencing,[75] spatial transcriptomics,[76] and metagenomics (i.e., microbiome).[77] However, we did not find clinical studies or studies of human tissue using these methods in type 2 diabetes and dementia. Immunophenotyping may also be a relevant approach given the importance of "immunosenescence", a drop in immune function with aging, and "inflammaging", a low-grade chronic inflammation, in type 2 diabetes (obesity)[78,79] and dementia.[80,81]

7. Final recommendations

The Lancet Commission on dementia prevention, intervention, and care has posted its recommendations for all risk factors for dementia.[6,7] Here, we focus on recommendations specifically revolving around diabetes and components of the metabolic syndrome to mitigate personal risk. Moreover, we also post recommendations regarding future research directions to address the knowledge gaps involving diabetes and dementia.

7.1. Population-wide

Launch public health campaigns to raise awareness about the link between diabetes, obesity, hypertension, and sedentary lifestyle with dementia. Campaigns can encourage lifestyle changes linked to diet, exercise, and weight loss, which could mitigate dementia risk.

7.2. Individual-specific

Individuals can take precautions based on their personal lifestyle and health situation:

- Individuals with diabetes can adopt a healthy diet and exercise; presently, the Mediterranean diet is recommended, but there is no recommendation regarding the optimal type of exercise.[7] Evidence regarding anti-diabetic drugs on dementia risk remains sparse.
- Individuals with obesity can also adopt a healthy diet and exercise. Although short-term benefits of weight loss have been reported, evidence regarding the long-term benefits of weight loss on dementia risk remains sparse.[7]
- Individuals with hypertension can take prescribed anti-hypertensive medications to maintain systolic ($\geq$130 mmHg) or diastolic blood pressure ($\geq$85 mmHg) within healthy limits.[7]
- Prevention is better than treatment. Since anti-diabetic drugs may not lower risk of dementia, it may be too late to reverse risk from diabetes once the disease has progressed. Prevention strategies that avoid diabetes onset may be the best risk-modifying approach.
- In addition to changes regarding metabolism, education, mental activity and stimulation, and social engagement could enhance cognitive resilience and reserve.[6,7]

7.3. Research directions

The goal of research is to fill the current knowledge gaps to improve prospects for patients. For diabetes patients at risk of dementia, it will be necessary to:

- Conduct prospective, carefully-designed long-running studies to generate the needed data.
- Identify early changes in brain biomarkers in prediabetes and diabetes patients who are at elevated risk of dementia. Identify the early changes in the brain associated with imminent onset of dementia and correlate these changes to clinical and/or genetic variables.
- Develop a polygenic risk score in diabetes for greater risk of dementia to identify patients most predisposed to dementia onset and development.
- Conduct clinical studies that target the metabolic syndrome more broadly through diet, medical weight loss, bariatric surgery, and exercise to determine whether this multi-pronged approach more effectively prevents dementia onset than anti-diabetic drugs alone.
- Conduct clinical studies of early interventions against modifiable risk factors to assess whether dementia may be preventable if addressed early.
- Conduct research on shared molecular pathways to develop mechanism-based, potentially disease-modifying therapies.

CRediT authorship contribution statement

Masha G. Savelieff: Conceptualization, Writing – original draft,

Writing – review & editing, Visualization. **Kevin S. Chen:** Writing – original draft, Writing – review & editing, Funding acquisition. **Sarah E. Elzinga:** Writing – original draft, Writing – review & editing, Funding acquisition. **Eva L. Feldman:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare no conflicts of interest.

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Search criteria

For the "Innovative methods to address the knowledge gaps" section, we searched PubMed for English language articles involving human studies in July 2022 mostly published within the 5 past years using the terms "diabetes, dementia" or "diabetes, Alzheimer's disease" with "functional MRI", "PET", "GWAS", "EWAS", "epigenome-wide association studies", "transcriptomics", "proteomics", "metabolomics", "single-cell RNA-sequencing", "spatial transcriptomics", "metagenomics". Abstracts from the search results were assessed for their relevance to the review topic. Representative papers on "functional MRI", "PET", and "GWAS" were selected based on their overall quality and their relevance to the review topic; all relevant articles identified by the other search terms were included. Articles for the "Data integration approaches" subsection were obtained from reading abstracts from all other searches. For the "Type 2 diabetes and metabolic syndrome" section, which was not a comprehensive review of the literature, the authors used articles from their reference database.

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Patient and health care provider knowledge of diabetes and diabetic microvascular complications: a comprehensive literature review

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Abstract

Diabetic retinopathy, neuropathy, and nephropathy occur in more than 50% of people with diabetes, contributing substantially to morbidity and mortality. Patient understanding of these microvascular complications is essential to ensure early recognition and treatment of these sequalae as well as associated symptoms, yet little is known about patient knowledge of microvascular sequalae. In this comprehensive literature review, we provide an overview of existing knowledge regarding patient knowledge of diabetes, retinopathy, neuropathy, and nephropathy. We also discuss health care provider's knowledge of these sequalae given that patients and providers must work together to achieve optimal care. We evaluated 281 articles on patient and provider knowledge of diabetic retinopathy, neuropathy, and nephropathy as well as predictors of improved knowledge and screening practices. Results demonstrated that patient and provider knowledge of microvascular sequalae varied widely between studies, which may reflect sociocultural or methodologic differences. Knowledge assessment instruments varied between studies with limited validation data and few studies controlled for confounding. Generally, improved patient knowledge was associated with greater formal education, longer diabetes duration, and higher socioeconomic status. Fewer studies examined provider knowledge of sequalae, yet these studies identified multiple misconceptions regarding appropriate screening practices for microvascular complications and the need to screen patients who are asymptomatic. Further investigations are needed that use well validated measures, control for confounding, and include diverse populations. Such studies will allow identification of patients and providers who would benefit from interventions to improve knowledge of microvascular complications and, ultimately, improve patient outcomes.

Keywords Understanding · Nephropathy · Neuropathy · Retinopathy · Nurse · Pharmacist

Abbreviations

ADA	American Diabetes Association.
CI	confidence interval.
CKD	chronic kidney disease.
DKD	diabetic kidney disease.
GP	general practitioner.
HbA1c	hemoglobin A1C.
KAP	knowledge attitudes and practices.

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OR	odds ratio.
PRISMA	preferred reporting items for systematic reviews and meta-analysis.
T1D	type 1 diabetes.
T2D	type 2 diabetes.
UK	United Kingdom.
US	United States.

1 Introduction

The global prevalence of diabetes has reached epidemic proportions [1]. In 2019, 463 million people worldwide had either type 1 (T1D) or type 2 (T2D) diabetes [1], placing substantial socioeconomic burdens on health care systems globally. If trends persist, 700 million people will be affected by diabetes by 2045 [1]. The reasons for this growth are manifold. An increase in diabetes risk factors, such as obesity and a sedentary lifestyle, against the setting of aging conspire to increase diabetes onset and prevalence. Socioeconomic forces and demographic factors are also contributing to the rise in diabetes. Countries with growing economies, income levels, and populations, such as in the Middle East, North Africa, and Asia, are driving an increase in T2D prevalence [1]. By 2045, Pakistan will overtake the United States as the third largest population with diabetes.

Diabetes causes several microvascular complications, including retinopathy, nephropathy, and neuropathy, which increase morbidity and mortality. Diabetic retinopathy is the leading cause of moderate and severe vision impairment in working age adults [2]. Neuropathy similarly can impact as many as half of individuals with diabetes, which impairs gait and stability and increases the risk of foot ulcers, ultimately leading, if left untreated, to non-traumatic lower amputations [3]. Diabetic kidney disease (DKD), i.e., diabetic nephropathy, has an estimated prevalence of 25% of T1D and 30 to 40% of T2D patients [4], and can lead, in the end-stages, to death. Indeed, in 2019, 4.2 million people worldwide died from diabetes-related complications [1].

Unfortunately, the growing diabetes prevalence is driving an increase in diabetes-related complications. Long-term diabetic complications can be present at the time of or occur shortly after diabetes diagnosis. Since early treatment of diabetes is essential for preventing disability and death, it is important for patients to understand diabetes and its related complications. Knowledge can enhance self-management behaviors, ultimately improving outcomes among patients with diabetes [5]. However, little is known about patients' knowledge of diabetes sequalae, particularly microvascular complications. Health care providers can educate patients and recommend screening and treatment options for microvascular complications, helping patients with their medical care choices [6]. Yet, up to one-third of physicians do not recognize the signs of diabetic peripheral neuropathy, even in symptomatic patients [7]. Thus, characterizing health care provider knowledge of diabetic microvascular complications, particularly in low- and middle-income countries where diabetes prevalence is increasing most rapidly, can identify changes needed to health care systems to improve patient outcomes.

The aim of this literature review is to summarize knowledge of diabetes and diabetic microvascular complications among patients with diabetes and health care providers. This includes recent studies examining predictors of improved patient and provider knowledge as well as predictors of improved diabetic microvascular screening practices. Our goal, also, is to identify gaps in patient and provider knowledge to facilitate further studies of the relationship between patient and provider knowledge of diabetic microvascular complications.

2 Methods

We searched three electronic databases, PubMed, Cochrane, and CINAHL for articles published from the date of database inception until the end of search period, 20 July 2021. The key terms and synonyms used alone or in combination were: "patient", "caregiver", "individual", "provider", "doctor", "health care worker", "understanding", "knowledge", "diabetes", "neuropathy", "retinopathy", "nephropathy", and "kidney disease". Additional searches were conducted by scanning the references lists and citations of included articles to ensure all relevant studies were identified. Only peer-reviewed articles that investigated patient or health care provider knowledge of diabetic neuropathy, retinopathy, or nephropathy were included. Studies were excluded if the patient population did not have diabetes (i.e., community-wide sample), unless results from patients with diabetes could be separated using the data provided.

The search identified 13,128 potentially eligible studies once duplicates were removed. After screening based on title, abstract, and keywords, the eligibility of 358 full text articles was assessed, of which 102 were excluded. An additional 28 articles were identified by screening reference lists, resulting in a final literature sample of 284, as shown in PRISMA diagram (Fig. 1).

3 Patient knowledge of diabetes

Diabetes knowledge is generally assessed via multiple choice surveys, either self-administered or interviewer-administered, covering topics ranging from metabolic facts,

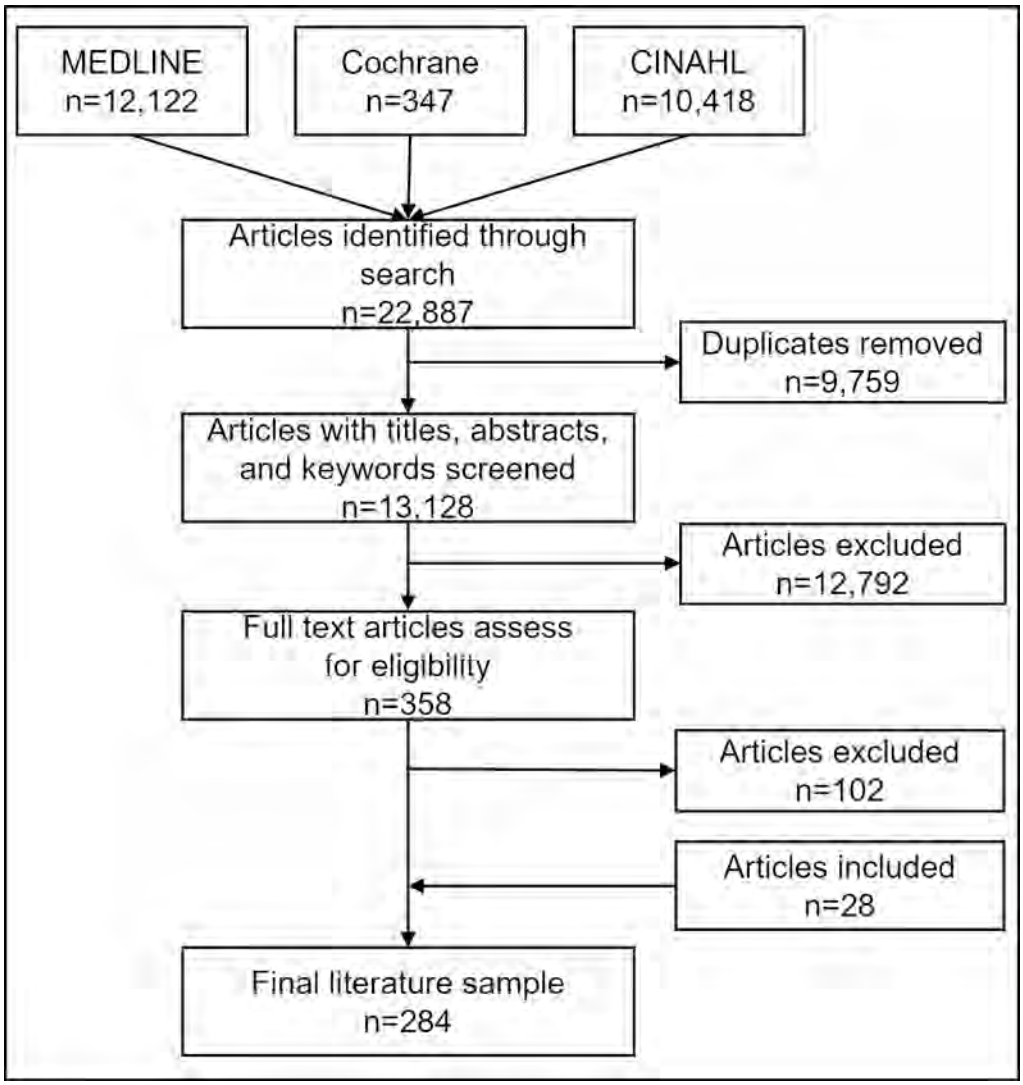

Fig. 1 Flow diagram of study inclusions. PRISMA criteria were adopted

hypoglycemia and hyperglycemia symptoms, medication usage, diet and exercise, among others [8]. Studies suggest that diabetes knowledge varies widely between study populations. In an early Scottish study, only 27/182 patients (15%) answered 11 questions correctly that investigators deemed essential diabetes knowledge [8]. Participants generally scored better on medication management questions than general metabolic and diabetes facts. The opposite was shown among patients with diabetes in Mexico who had a better knowledge of diabetes concepts than blood glucose self-monitoring and diabetes-related medication [9]. Diabetes knowledge was markedly better in a recent US study of 17 patients, with an average knowledge score of 60% [10]. Of note, however, the study was underpowered to assess predictors of diabetes knowledge.

A good body of evidence suggests that improved patient knowledge of diabetes is associated with better self-management of disease. This has been demonstrated using overall glucose control [11–13] and medication adherence [14] as surrogates of diabetes self-management. In a US study of 44 patients, those with better scores on a diabetes medication knowledge questionnaire had significantly lower HbA1c ($p < 0.0001$) [12]. This relationship between diabetes knowledge and diabetes control may also be upheld in select populations, such as patients with diabetes on dialysis [13]. Diabetes knowledge also aids in stricter adherence to medication, which is critical for disease management [14]. Overall, indications are that improved patient knowledge of diabetes can translate to better glycemic control and medication adherence, which likely reduce disease progression. Therefore, raising awareness and knowledge of diabetes could exert a meaningful and beneficial impact on disease self-management.

To identify patients with diabetes that would benefit from diabetes education, and hence improved disease control, it is essential to identify determinants of low diabetes knowledge. There are several parameters that potentially influence patients' knowledge of diabetes. Age is a well- and long-established predictor of diabetes knowledge. This relationship between greater diabetes knowledge and younger age has been noted in various populations, including in Mexico [9], Costa Rica [15], Kuwait [16], United Arab Emirates [17], and Singapore [18].

Additional clinical and/or demographic variables are linked to diabetes knowledge. Longer diabetes duration is implicated in better knowledge scores by some studies [17, 19], but not by others [16, 20], possibly due to confounding parameters. There is also discordance in studies investigating the impact of sex; diabetes knowledge was greater in females [21] or males [17, 22–25] depending on the study, and one found no difference [26]. However, overall, studies failed to adjust for education level and other confounding variables, which may have varied in these countries. Race/ethnicity is a possible contributor; studies to date suggest that patients who self-identify as White are likelier to have broader diabetes knowledge compared to some minorities [20, 27–30]. While this may indicate areas to improve through outreach in these populations, it is important to emphasize that most of these studies do not adjust for confounding, particularly by socioeconomic status, which limits its interpretation of this association. Further, some studies categorized participants by nationality, rather than by ethnicity [16, 17]. Overall, there is a need for better quality studies that adjust for confounding to identify predictive factors affecting diabetes knowledge to launch education campaigns likely to benefit those patients at highest risk.

As might be anticipated, formal education [9, 15–17, 19, 20, 23, 31], income [19], and health literacy [32, 33] influence diabetes knowledge. Importantly, delivering focused diabetes education can raise diabetes knowledge [11], paving a way forward for patients with lower knowledge on a trajectory for better disease control.

4 Knowledge of diabetic complications

Uncontrolled diabetes, i.e., elevated HbA1c and fasting blood glucose, is a significant risk factor for developing microvascular complications, including retinopathy, nephropathy [34], and neuropathy [3]. Several additional risks factors, such as obesity and dyslipidemia [35–37], which are frequent comorbidities in patients with diabetes, also raise the risk of microvascular complications, especially in patients with T2D. Therefore, lack of knowledge of diabetes leading to suboptimal self-care and poor disease control could potentially also increase the risk of diabetic complications. Indeed, just as patient knowledge of diabetes is generally suboptimal, recognition of diabetic complications, including microvascular complications, was less than 50% in some settings [23, 38].

In addition, and compounding the issue, providers do not universally address diabetic microvascular complications in patients [7, 39–42], even in patients clearly exhibiting symptoms e.g., neuropathy [7]. Since provider recommendations significantly influence patient care choices in diabetic microvascular complications [6, 43], it is important to understand the level of provider knowledge of microvascular complications. However, just as for patients, there is no comprehensive review of provider knowledge and predictors of provider knowledge of these diabetic complications. Herein, we provide our findings from a thorough literature review of both patient and provider knowledge, covering retinopathy, neuropathy, and nephropathy.

4.1 Retinopathy

Diabetic retinopathy is a leading global cause of preventable vision impairment and blindness. The damaging effects of diabetes on the eye can be prevented by early detection of retinopathy through screening and timely treatment of sight-threatening complications. Therefore, it is critical to identify approaches that improve patient knowledge leading to better health choices and care seeking behavior. We found literature regarding patient knowledge of retinopathy from many world regions, although the preponderance of data was from Saudi Arabia and India [40] with some studies from the US [44]. They highlight critical gaps in patient knowledge. Even in countries with provider guidelines, for instance by the American Diabetes Association (ADA) [45], to ensure retinopathy is evaluated in timely manner, patient adherence to screening is suboptimal [43], of which lack of knowledge of the potential harms of retinopathy may be a contributing cause. In a nationwide US study of 204,073 patients with diabetes, only 71.1% adhered to the retinal screening recommendations during a median 4.8-year follow-up [46]. Therefore, it is important to characterize the determinants leading to poor adherence, such as knowledge of retinopathy.

4.1.1 Instruments to assess retinopathy knowledge

Several formal Knowledge, Attitudes, and Practices (KAP) surveys have been developed to assess patient knowledge of retinopathy. A KAP survey is a quantitative or qualitative method to address a predefined question, what are patient and provider understanding of diabetic complications in this instance, through a standardized questionnaire. The

goal of KAP surveys is to reveal misconceptions or misunderstandings, which pose an obstacle to desirable activities or behaviors, i.e., screening, self-care, and appropriate management of diabetic complications in this review. KAP surveys can be structured, e.g., multiple choice, guided questions, or semi-structured, i.e., relatively more open-ended interviews. The KAP survey may be conducted by the investigator, either in-person or over the phone, or may be self-administered, online or by mail. To assess whether the KAP surveys will evaluate the intended topic, they are tested for internal consistency, reliability, and face validity and frequently pretested in a pilot group characteristic of the target population. Cronbach's alpha coefficient is a measure of test reliability or internal consistency for a set of scales or test items [60]. The coefficient ranges from 0 to 1; the coefficient is 0 when test items are independent from one another, but approaches 1 when test items have high covariances, i.e., they measure the same underlying concept, KAP in these instances. A Cronbach's alpha coefficient minimum of 0.65 and 0.8 is recommended.

KAP surveys for retinopathy span structured and semi-structured self- and investigator-administered questionnaires [47–55]. The internal consistency of some KAP surveys has been assessed and found to be acceptable with Cronbach's alpha coefficients ranging from 0.6 to 0.8 [56–59]. Some studies have evaluated face validity by consulting an expert panel [61, 62], whereas other have developed questionnaires based on reviews of the literature [62, 63]. Pretesting by leveraging a pilot group of volunteers outside of the study area or study cohort is a relatively widely adopted validation method [55, 58, 62]. Unfortunately, little information using the same retinopathy KAP survey in multiple settings or in multi-center studies is available therefore it is unclear how these measures perform across different patient populations [43].

Far less literature has been published regarding KAP surveys of retinopathy knowledge among providers. A search of the literature yields both self- [64–66] and investigator- [40, 67] administered structured questionnaires, all pretested in separate participant groups. One study evaluated KAP survey internal consistency with a moderate Cronbach's alpha coefficient (0.64) [65]. More commonly, informal surveys of provider knowledge of retinopathy using short one- or two-question queries are documented in the literature [68–70]. A couple of reports lack details regarding the instrument used [71, 72]. Thus, overall, there are few KAP survey instruments developed to assess provider knowledge of retinopathy.

4.1.2 Patient knowledge of retinopathy

KAP surveys of patient knowledge of diabetic complications suggest that retinopathy is the most recognized complication [31, 49, 63, 73]. Among Irish patients with diabetes, 92% of those with T1D and 83% with T2D knew retinopathy was a diabetes-related complication, compared to 71% of those with T1D and 53% of those with T2D for neuropathy [73]. Despite this, some populations, such as American Indians and Alaskan Natives, were unaware of the connection between retinopathy and diabetes [44] whereas other populations held misconceptions regarding causes, e.g., watching too much TV [6] or bad luck [52] **(**Table 1**)**. There is also a limited understanding by patients that retinopathy can be asymptomatic [48, 55, 74–76], including in select populations, such a 50% of females surveyed in New York City [77]. This belief may hold patients back from attending screening if they are unaware that they may be in the early asymptomatic stages of retinopathy. Indeed, in this survey of 150 low-income diabetic females from New York City, a fifth were unfamiliar with the type of provider required for an eye exam and 17% were unaware that annual eye exams were recommended [77]. Of those aware of the annual screening recommendation, only approximately a quarter had knowledge regarding the need for dilation of the pupils as a critical component of the eye exam. In a survey of patients with diabetes in rural India, less than a third were aware that eyes must be assessed on a regular basis [61].

In addition to knowledge gaps concerning causes and screening for retinopathy, there is a lack of knowledge regarding treatment options. Early surveys of urban populations suggest a significant proportion of patients with diabetes are unfamiliar with treatment options, with only around a fifth of respondents demonstrating correct knowledge [6]. This proportion was even lower, 5%, in a recent rural study in India [61]. It is unclear from our literature review whether knowledge has improved in urban populations in recent years. Furthermore, there is a low level of knowledge regarding the preventative, rather than curative, nature of treatments, such as laser and glucose control, which are erroneously thought to be curative [76, 78].

Previous studies also examined predictors of retinopathy knowledge, spanning demographic, clinical, and socioeconomic factors. Studies are discordant regarding age, with findings of greater knowledge in younger [69, 79] versus older [47, 61] patients with diabetes. In surveys that examined sex, being female was associated with greater knowledge of retinopathy [50]. In addition, patients with a longer duration of disease were more likely to understand diabetic retinopathy, and this was confirmed in multiple recent surveys [48, 56, 58, 69, 80]. Further, patients with a prior eye exam also had a greater knowledge of retinopathy [79].

Table 1 Summary of studies that investigated retinopathy knowledge among patients with diabetes and health care providers. Studies arranged alphabetically. Abbreviations: CI, confidence interval; GP general practitioner; KAP knowledge attitudes and practices; OR, odds ratio; T1D, type 1 diabetes; T2D, type 2 diabetes

Study	Country	Study Type & Population	Measure	Main Findings
Patients with Diabetes				
Addoor 2011 [83]	Malaysia	Cross-sectional 351 from 1 ophthalmology clinic	Study-created KAP survey	87% aware diabetes affects eye. Predictors of knowledge: duration of diabetes ($p < 0.01$), eye exam in last 6 months ($p < 0.04$)
Adriono 2011 [84]	Indonesia	Cross-sectional 196 from 3 primary care clinics	Study-created KAP survey	38% aware diabetes causes blindness. Prior exam linked to better knowledge ($p = 0.002$).
Ahmed 2017 [51]	Bangladesh	Cross-sectional 122 from 1 diabetes clinic	Study-created KAP survey	24% with poor knowledge about the effect of diabetes on the eye.
Al-Asbali 2020 [79]	Saudi Arabia	Cross-sectional 200 from 1 endocrine and 1 ophthalmology clinic	Study-created KAP survey	45% excellent knowledge. Predictors of knowledge: duration of diabetes ($p = 0.03$).
AlHargan 2019 [82]	Saudi Arabia	Cross-sectional 280 from 2 primary care clinics	Adapted KAP survey [148, 150]	88% knew diabetes affects the retina. Predictors of knowledge: formal education ($p < 0.01$), higher income ($p < 0.05$).
Almalki 2018 [56]	Saudi Arabia	Cross-sectional 253 T2D from 1 endocrinology clinic	KAP survey adapted from prior study [83]	64% knew diabetes affects the eye.
Alsaidan 2019 [70]	Saudi Arabia	Cross-sectional 174 T2D from 1 primary care clinic	Details not provided	82% aware diabetes affects eye. Predictors of knowledge: male gender ($p = 0.045$), well controlled T2D ($p = 0.021$).
Alwazae 2019 [57]	Saudi Arabia	Cross-sectional 404 from 4 clinics	Study-created KAP survey	73.5% with adequate knowledge.
Al-Yahya 2020 [151]	Saudi Arabia	Cross-sectional 313 from 52 primary care clinics	Validated KAP survey [53]	53% knew diabetes affects the eye. Predictors of knowledge: higher income ($p < 0.02$).
Alzahrani 2018 [61]	Saudi Arabia	Cross-sectional 377 from 38 primary care clinics	Study-created KAP survey	82% knew diabetes affects the eye.
Al Zarea 2016 [150]	Saudi Arabia	Cross-sectional 439 from 5 clinics	Study-created KAP survey	75% aware diabetes can cause eye disease.
Assem 2020 [58]	Ethiopia	Cross-sectional 230 from 1 diabetes clinic	Study-created KAP survey	52% with poor knowledge. Predictors of knowledge: urban residence ($p < 0.05$), income ($p < 0.05$), diabetes ($p < 0.05$), duration ($p < 0.01$).
Bakkar 2017 [148]	Jordan	Cross-sectional 237 T2D randomly selected from 3 cities	Study-created KAP survey	88% aware diabetes can affect the eyes. Predictors of eye knowledge: more than high school education ($p < 0.01$).
Balasubramanian 2016 [60]	India	Cross-sectional 105 from 1 clinic	Details not provided	76% aware diabetes affects the eye. Predictors of knowledge: education ($p < 0.05$)
Çetin 2013 [48]	Turkey	Cross-sectional 437 seen at 1 ophthalmology and 1 endocrinology clinic	Study-created questionnaire	88% knew diabetes affects eyes. 25% thought eye exams only necessary if having troubled vision or poorly controlled diabetes.
Das 2016 [49]	India	Cross-sectional 240 from 1 ophthalmology clinic	Study-created KAP survey	65% knew diabetic retinopathy affects the eyes. 42% disagreed that eyes could be affected, even if blood sugar was controlled. No significant predictors of knowledge.
Duan 2020 [68]	China	Cross-sectional 1972 in 1 community health system	Study-created KAP survey	62% knew diabetes affects eyes. Predictors of knowledge: younger age, male sex, higher education, longer diabetes duration (all $p < 0.01$).
Fallatah 2918 [54]	Saudi Arabia	Cross-sectional 380 from 1 ophthalmology clinic	Study-created KAP survey	92% aware diabetes affects eyes. Predictors of knowledge: formal education ($p < 0.05$), urban residence ($p < 0.05$).
Gillibrand 2000 [67]	UK	Cross-sectional 2,815 community patients not engaged in eye care	One knowledge question	18.3% did not know diabetes affects eyes.
Khandekar 2010 [47]	Oman	Cross-sectional 750 in 1 region	Study-created KAP survey	61% aware diabetes affects eyes.
Konstantinidis 2017 [52]	Switzerland	Cross-sectional 323 recruited from community pharmacies	Study-created questionnaire	96% aware diabetes can cause eye disease. 98% knew good glycemic control could prevent occurrence or deterioration of eyes.

Table 1 (continued)

Study	Country	Study Type & Population	Measure	Main Findings
Lian 2018 [74]	Hong Kong	Cross-sectional 2,593 at 2 clinics	Study-created questionnaire	11.5% knew retinopathy could be asymptomatic.
Livingston 1998 [78]	Australia	Cross-sectional 205 urban, 240 rural	Study-created knowledge score	37% aware eye problems can occur. Predictors of increased awareness: younger age: rural OR 2.89 [95% CI 1.36–6.06] urban OR 2.32 [95% CI 1.24–4.22]; eye exam in last 2 years: rural OR 1.89 [95% CI 1.04–3.42] urban OR 2.43 [95% CI 1.29–4.57].
Manu 2018 [75]	India	Cross-sectional 150 T2D from 1 hospital	Details not provided	58% aware diabetes affects the eye. No significant predictors of knowledge.
Mueke 2008 [89]	Myanmar	Cross-sectional 480 cared for by surveyed GPs	Study-created questionnaire	80.6% knew diabetes affects eyes. 90.4% agreed patients with diabetes should see an eye specialist.
Mumba 2007 [69]	Tanzania	Cross-sectional 316 at 1 diabetes clinic	One knowledge question	34% knew diabetes can damage eye.
Nathaniel 2015 [81]	Nigeria	Cross-sectional 225 at 1 endocrinology clinic	Study-created questionnaire	57% knew diabetes can affect eye.
Ovenseri-Ogbomo 2013 [152]	Ghana	Cross-sectional 360 at 1 diabetes clinic	Study-created questionnaire	49% knew diabetes can affect eye. No significant predictors of knowledge.
Pasagian-Macaulay 1997 [76]	US	Cross-sectional 150 women from 1 medical center	Study-created knowledge and belief score	17% did not know required frequency of eye exams. 40% knew controlling glucose was important. Formal education linked to greater knowledge ($p < 0.05$).
Rizwan 2004 [55]	Pakistan	Cross-sectional 132 from 1 ophthalmology clinic	Details not provided	57% knew diabetes affects the eye. 22% reported eye exams should occur once vision was affected.
Saikumar 2007 [153]	India	Cross-sectional 1,000 at 1 clinic	Study-created awareness score	84% aware diabetes can affect the eye. 46.9% knew related to glucose control. 50% thought routine eye exams not necessary.
Schmid 2003 [149]	Australia	Cross-sectional 68 T1D, 187 T2D in Diabetes Australia	Study-created questionnaire	96.2% knew diabetes causes eye problems.
Schoenfeld 2001 [86]	US	Cross-sectional 2,308 in 1 county	Study-created questionnaire	47% knew eye examinations were needed for people with diabetes.
Srinivasan 2017 [53]	India	Cross-sectional 288 from 1 ophthalmology clinic	Study created questionnaire	58% had poor knowledge.
Tajunisah 2011 [62]	Malaysia	Cross-sectional 137 from 1 ophthalmology clinic	Details not provided	86% aware diabetes can affect the eye. Predictors of knowledge: formal education ($p < 0.05$).
Vanugopal 2020 [59]	India	Cross-sectional 350 from 1 hospital	Study-created questionnaire	34% had adequate knowledge of diabetic retinopathy. Predictors of knowledge: formal education ($p < 0.001$).
Walker 1997 [6]	US	Cross-sectional 67 Black Americans with diabetes in New York	Study-created questionnaire	87% believed diabetic eye problems were symptomatic. 21% thought there were effective treatments.
Wang 2010 [85]	China	Cross-sectional 53 T1D 836 T2D from 1 endocrine and 1 general clinic	Study-created KAP survey	77% aware diabetes affects eyes. Prior exam linked to better knowledge ($p < 0.001$).
Whiting 1998 [77]	Australia	Cross-sectional 121 patients with retinopathy from 1 ophthalmology clinic	Study-created questionnaire	95% knew diabetes affects the eyes.
Zou 2017 [73]	China	Cross-sectional 519 with diabetes in 1 community	Study-created questionnaire	95% aware diabetes affects the eye, 12% aware it can be asymptomatic.
Health Care Providers				
Abdulsalam 2018[64]	Nigeria	Cross-sectional 105 physicians from 4 hospitals	Study-created KAP survey	36% perform eye exams, 90% do not use dilating eye drops
Abu-Amara 2019 [40]	Saudi Arabia	Cross-sectional 182 GPs, 115 internists	Study-created KAP survey	45% with poor knowledge.

Table 1 (continued)

Study	Country	Study Type & Population	Measure	Main Findings
Al Rasheed 2017 [63]	Saudi Arabia	Cross-sectional 142 family, 10 pediatric, 8 internists, 56 GPs	Study-created questionnaire	Knowledge linked to: family medicine subspecialty training ($p < 0.01$), years of practice ($p < 0.01$).
Al-Rashidi 2020 [71]	Saudi Arabia	Cross-sectional 76 GPs in 1 province	Previously used KAP survey [63]	37% performed dilated fundus exams.
Alhejji 2020 [97]	Saudi Arabia	Cross-sectional 141 GPs from 63 centers	Study-created questionnaire	56% with good knowledge.
Al-Wadaani 2012 [98]	Saudi Arabia	Cross-sectional 73 medical students	Study-created KAP survey	Moderate overall KAP score, linked to male sex ($p = 0.02$). 66% knew correct timing for eye exams.
Daly 2014 [92]	New Zealand	Cross sectional 287 nurses	Study-created survey	89% identified retinopathy as a diabetes complication. Predictors of knowledge: level of training ($p = 0.006$).
Delorme 1998 [65]	Canada	Cross-sectional 648 GPs, 96 trainees	Study-created questionnaire	Correct timing for screening in T1D: 74% vs. T2D: 82%. 33% knew macular edema could be asymptomatic.
Foster 1996 [95]	US	Cross-sectional 23 optometrists	Study-created survey	Low level of knowledge regarding need for dilated fundus exams.
Ghosh 2007 [154]	India	Cross-sectional 36 optometrists, 241 GPs	Study-created questionnaire	< 23% optometrists and < 33% GPs had acceptable knowledge regarding risk factors and management of diabetic retinopathy.
Goodman 1997 [39]	South Africa	Cross-sectional 12 doctors, 23 nurses	Study-created survey	100% knew diabetes affected the eye.
Khandekar 2008 [94]	Oman	Cross-sectional 42 ophthalmologists, 33 mid-levels, 12 GPs	Study-created questionnaire	Acceptable knowledge: 71% ophthalmologists, 54% mid-levels, 33% GPs.
Mueke 2008 [89]	Myanmar	Cross-sectional 100 GPs	Study-created questionnaire	Correct timing for screening in T1D: 2% vs. T2D: 93%.
Namperumalsamy 2004 [155]	India	Cross-sectional 199 paramedical personnel	Study-created questionnaire	88.5% knew diabetes could affect eyes. 20% knew uncontrolled diabetes is a risk factor. 75.9% unaware of treatments for retinopathy.
Raman 2006 [156]	India	Cross-sectional 159 GPs	Study-created questionnaire	54% aware patients with diabetes should have annual dilated eye exams.
Wright 2001 [157]	Australia	Cohort 310 optometrists	Study-created questionnaire	74.5% perform dilated exams on new patients with known diabetes.
Yan 2012 [96]	China	Focus groups 22 physicians, 25 village health workers	Study-created interview guide	Good overall knowledge, physicians did not dilate pupils to detect asymptomatic disease.

Examination of socioeconomic factors reveals some anticipated correlations with greater retinopathy knowledge, such as higher formal education [48, 56, 59, 61, 69, 77, 81–83], literacy [63], urban residency [58, 81], and income [58, 69, 83]. Additionally, speaking English, were English was not the person's first language, was also linked to greater knowledge of retinopathy [59, 79]. Only ten studies controlled for confounding. Among those that did, the following predictors of retinopathy knowledge remained significant: younger age [79], duration of diabetes [58, 84], a prior eye exam [79, 84–86], higher income [58], urban residency [58, 79], and greater formal education [61]. Six studies found no significant demographic or clinical predictors of increased retinopathy after controlling for confounding [47–49, 70, 74, 87].

Moreover, surveys have examined predictors of engaging in retinopathy screening behavior, which were multifactorial. From the literature we identified the main determinants for attending screening were younger age, female sex, and White race/ethnicity [88]. Diabetes characteristics also play a role in whether patients seek screening, such as more severe diabetes and comorbidities such as hypertension and hyperlipidemia, [85] and, overwhelmingly, longer disease duration [48, 74, 85, 89], although one study noted the opposite [90]. As might be expected, previous attendance for an eye exam [87], receiving a physician recommendation to attend for screening [43, 62, 75, 76, 85], and better knowledge of diabetes [53, 85, 91] and retinopathy [43, 51, 53, 57, 91, 92] also increase screening adherence. Lastly, as for determinants of retinopathy knowledge, greater formal education [48, 89, 90], urban residency [88], higher income

[92], and linkage into care/health insurance [92] was associated with an increased likelihood of screening attendance. Again, few studies adjusted for potential confounding.

Overall, our literature review identifies crucial gaps in patients' diabetic retinopathy knowledge. These span lack of awareness of the relationship of retinopathy to diabetes, occurrence of early asymptomatic disease, misconceptions regarding causes, and paucity of knowledge regarding therapies. Surveys have revealed several determinants of low retinopathy knowledge and screening, such as demographics (age, sex, ethnicity), diabetes duration, prior behavior, physician recommendations, and various socioeconomic factors. This could help identify patient populations, which would benefit from retinopathy education and outreach. Of note, several studies identified from urban, Western countries were conducted over two decades ago, and we could not ascertain whether patient KAP have since changed in the intervening years.

4.1.3 Provider knowledge of retinopathy

Provider knowledge of retinopathy is crucial for ensuring patients' optimal eye care because multiple studies support that physician recommendations are strong determinants of patients' adherence to screening guidelines [43, 62, 75, 76, 85]. Sixty to 100% of physicians [39, 66] and 50-75% of nurses and midlevel providers [67, 93] know diabetes can adversely affect the eyes. However, overall retinopathy knowledge can be poor among providers in some geographic areas. A survey of private sector non-ophthalmic providers (n = 355) in Saudi Arabia found a good level of diabetic retinopathy knowledge was only present in 54.3% of interviewees, along with a positive attitude among 31.3% and excellent practice among only 40.8% of interviewees [40]. We did not identify any studies that compared provider knowledge globally. We did, however, find evidence that ophthalmic specialists outperform non-specialists for detecting proliferative retinopathy from seven-view stereo fundus photographs and review of medical charts [94]; therefore, suboptimal retinopathy knowledge may be more of an issue among general doctors than eye experts. Since most patients receive their medical care first from their primary care physician, they must be knowledgeable of retinopathy to determine when a referral to an ophthalmologist is necessary.

Our search of the literature identified several points of provider knowledge limitations. These included a lack of awareness concerning what part of the eye diabetes affects [95], uncertainty regarding the tests used to diagnose retinopathy [64, 65, 72], as well as misconceptions regarding contraindications to diabetic fundoscopic exams, e.g., hypertension [96, 97]. As we had identified for patients, a small KAP survey also found lack of knowledge among providers regarding the existence of asymptomatic disease. This was noted by a small study of physicians (n = 22) and village health workers (n = 25) in rural China, which found most providers did not conduct a pupil dilation exam if the patient had no symptoms [97]. Similarly, a KAP survey of primary care physicians in Saudi Arabia (n = 216) found that only 46% were aware that patients initially exhibit no symptoms in the early stages of retinopathy [64]. An early investigation of KAP among Canadian general practitioners (n = 1,038) found that 27% overestimated the benefits of treatment, i.e., a false belief that laser photocoagulation improved rather than stabilize disease progression [66]. Lastly, we identified provider gaps in knowledge regarding gestational diabetes. Family-practice physicians (n = 224) were more likely to examine the eyes of patients with gestational diabetes for retinopathy compared to obstetrics/gynecology physicians (n = 184), as surveyed by mail [98, 99].

We found only scant information regarding predictors of greater provider knowledge of retinopathy. As might be anticipated, specialist training correlates with greater knowledge or ability to detect retinopathy, e.g., retinal specialists versus internists, diabetologists, and medical residents [94] or additional subspeciality training [64]. Longer duration of practice was also a determinant of greater knowledge [64]. Patient characteristics also contributed, with providers demonstrating better knowledge regarding the connection of retinopathy and T2D versus T1D and, as a result, providers more frequently referred patients with T2D versus T1D to ophthalmology [100]. In a KAP survey of medical students in Saudi Arabia, males scored higher on knowledge and practices whereas females scored better on attitudes [101].

Cumulatively, our search of the literature revealed some investigation of provider KAP, although recent studies were limited in scope and geographic location. Moreover, carefully adjusted studies for confounding factors are scarce.

4.2 Neuropathy

Peripheral neuropathy is an injury of the nerves, generally in a symmetric distal to proximal fashion, initiating in the feet and progressing to the calves [3]. In the later stages, the hands may also be affected. Neuropathy can impair gait and stability, increasing susceptibility to falls and secondary injury. Moreover, peripheral neuropathy can lead to non-healing foot ulcers, which may ultimately require lower limb amputation. Thus, it can significantly increase disability and lower quality of life, making it essential for patients to understand neuropathy. We searched the literature for studies that examined patient knowledge of neuropathy. Most studies were conducted in India and China, although studies were conducted across multiple other

countries [102–104]. Patient populations comprised both inpatients with diabetic ulcers as well as outpatients with diabetes lacking neuropathy symptoms. One 2000 US study of patients who were ADA members in an urban setting found that 27% of respondents reported they had not been advised or educated on diabetic neuropathy and foot complication by their health care provider [102]. Thus, gaps in patient knowledge of neuropathy may be substantial, even in patients belonging to an organization advocating and supporting diabetes research.

4.2.1 Instruments to assess neuropathy knowledge

We identified several instruments assessing neuropathy knowledge in the literature. The majority were KAP surveys focused on foot care and foot ulcer knowledge and practice, rather than neuropathy more broadly. KAP surveys were both in structured [105–107] and interview format questionnaires [108], either self- or investigator-administered [109]. In one study, the questionnaire was investigator-administered when the respondent was illiterate or physically unable to complete the survey but self-administered by the remainder of participants [110]. A few administered KAP surveys were adapted from prior surveys [111, 112], whereas a few were utilized in multiple studies [105, 113] or used prior instruments [106, 114]. Moreover, we found a KAP instrument that split the survey into basic and extended foot care practices [115]. The Patient Interpretation of Neuropathy (PIN) questionnaire evaluated both misperceptions about foot complications, patient knowledge of neuropathy and its link to complications, and foot self-care efficacy beliefs, among other concepts related to patient understanding of neuropathy [116].

A few studies evaluated parameters of KAP surveys for capturing patient knowledge. Several KAP surveys we identified were pretested [105, 106, 108, 112, 117], although one study was pretested in medical students instead of a population meeting the criteria of the study population [109]. Regarding, internal consistency of KAP surveys, they had Cronbach's alpha coefficients ranging from 0.72 to 0.86, which is rated as acceptable [115, 118, 119]. A couple of studies assessed face validity of the utilized KAP survey by a panel of medical experts [107, 108]. In addition to KAP surveys, we also found papers that leveraged scoring and/or scaling instruments to assess patient knowledge of diabetic foot care. These included diabetic knowledge [120] and foot care scores [103, 113, 120–124]. Finally, one study report provided no information regarding the employed instrument [125].

We found far fewer neuropathy KAP instruments for providers; however, they spanned structured and semi-structured questionnaires, which were self- [126–128] or investigator- [39, 93] administered. We noted some surveys were pretested [39], were assessed for face validity by experts, and evaluated for internal consistency by Cronbach's alpha coefficients (0.72 for junior doctors, 0.81 for nurses) [127]. Additionally, studies used previously validated instruments about KAP towards diabetes more broadly, e.g., Diabetes Self-Report Tool, Diabetes Basic Knowledge Tool [42, 128, 129].

4.2.2 Patient knowledge of neuropathy

Overall, evidence suggests patient knowledge of neuropathy ranges from 10 to 60% compared to 60–92% for retinopathy [73, 130], which may also be the case in providers, e.g., nurses [93]. Of the papers we assessed, we found a broad range of patient knowledge and practice behaviors in neuropathy, i.e., diabetic foot **(**Table 2**)**. Many reported less than adequate foot care behavior in diverse populations worldwide, urban and rural [104, 110, 122]. Moreover, some studies highlighted a disconnect in knowledge and practice. In a Saudi Arabian study of patients with T2D (n = 360), although 70% had knowledge of diabetic foot care, only 41.7% examined their feet, 41.4% washed them with warm water, 31.4% carefully dried them between the toes, and 33.1% used moisturizer [131]. We also noted some misconceptions regarding foot care; for example, qualitative interviews with people with diabetes in Jordan revealed the belief that there is no need to examine the feet if participants had no ulcers [132]. Appropriate education on diabetic neuropathy can have tangible effects on care adherence. A study of T2D patients with diabetic neuropathy (n = 104) found that foot care education enhanced attendance at yearly check-ups, as well as moisturizer use and appropriate shoe wear (all $p < 0.05$) [133]. Another study in Saudi Arabia similarly found that foot care practice was superior in T2D patients that received physician recommendations to examine their feet [131]. Therefore, it is essential for patients to understand neuropathy to adopt practices that improve foot care.

Across the studies, we identified multiple predictors of patient knowledge, which included demographic, clinical, and socioeconomic factors. The literature findings regarding sex were mixed. We found reports that neuropathy knowledge was greater in females [113] and, conversely, in males [117], and one study that did not find a relationship between neuropathy knowledge and sex [134]. Older age also associated with deeper knowledge of diabetic foot and neuropathy [108, 114]. Additionally, in a large Chinese study of patients with T2D (n = 5,961), disease characteristics had an influence on patient knowledge, including positive correlations with diabetes duration and regular diabetes care following multiple regression analysis [114]. Prior foot complications may impact neuropathy knowledge. In a Thai study,

Table 2 Summary of studies that investigated neuropathy knowledge among patients with diabetes and health care providers. Studies arranged alphabetically. Abbreviations: ADA American Diabetes Association; KAP, Knowledge, Attitudes, and Practices; T2D, type 2 diabetes

Study	Country	Study Type & Population	Measure	Main Findings
Patients with Diabetes				
Abu-Qamar 2014 [104]	Jordan	Qualitative interviews 7 patients with burn injuries	Study-created interview guide	Participants did not believe they needed regular food exams in the absence of ulcers.
Bohorquez Robles 2017 [114]	Mexico	Cross-sectional 200 T2D from 1 primary care clinic	Foot Care Knowledge and Practice Questionnaire [158]	52% had poor knowledge of foot self-care.
Chellan 2012 [107]	India	Cross-sectional 203 from 1 podiatry clinic	Previously validated KAP survey	Patients with foot ulcers were more likely to have poor knowledge ($p = 0.001$).
Corbett 2003 [119]	US	Randomized control trial 40 T2D with home care	Foot Care Knowledge Questionnaire [159]	Moderate baseline foot care knowledge. Educational intervention improved knowledge ($p < 0.01$).
De Sá Pilocarpo 2014 [113]	Brazil	Cross-sectional study 85 T2D from 2 primary care clinics	Previously used KAP questionnaire [159]	49.5% with limited foot care knowledge.
Desalu 2011 [102]	Nigeria	Cross-sectional 352 from 3 tertiary hospitals	Pre-tested questionnaire	46% with poor knowledge of diabetic foot care.
Foolchand 2013 [111]	Mauritius	Qualitative interviews 120 from 5 hospitals	Study-created interview guide	75% unaware of need for annual foot screening.
Hanley 2020 [131]	St. Kitts and Nevis	Cross sectional 210 from multiple health care settings	Adapted KAP questionnaire [107]	Average knowledge reported. No difference in knowledge based on amputation status.
Hasnain 2009 [101]	Pakistan	Cross-sectional 150 from 1 diabetic clinic	Study-created questionnaire	29.3% with good knowledge. Predictors of knowledge: formal education ($p < 0.01$).
Jain 2012 [106]	India	Cross-sectional 251 from multiple hospitals	Study-created questionnaire	62% had poor foot care knowledge.
Jinadasa 2011 [105]	Sri Lanka	Cross-sectional 110 with diabetic foot ulcers	Study-created questionnaire	52.7% with good footcare knowledge.
Khamseh 2007 [103]	Iran	Cross-sectional 148 T2D from 1 diabetes clinic	Study-created questionnaire	Predictors of knowledge: higher formal education ($p < 0.01$).
Lamchahab 2011 [121]	Morocco	Cross-sectional 91 hospitalized patients	Details not provided	85% did not pay attention to "warning signs" of foot injuries. Predictors of knowledge: formal education, socioeconomic status (both $p < 0.01$).
Li 2014 [110]	China	Cross-sectional 5,961 T2D from 144 hospitals	Summary of Diabetes Self-Care Activities	Overall medium level of foot care knowledge. Multivariate predictors of knowledge: female sex, older age, formal education, diabetes duration, regular diabetes care, prior education regarding diabetes complications (all $p < 0.001$).
Muhammad-Lutfi 2014 [130]	Malaysia	Cross-sectional 157 admitted with foot infections.	Previously used questionnaire [101]	58% with poor foot knowledge.
Naicker 2009 [120]	Malaysia	Cross-sectional 100 from 1 hospital	Preventative Measure Scale [160]	Poor overall foot knowledge.
Pollock 2004 [109]	UK	Cross-sectional 365 from a population-based diabetes register	Study-created questionnaire	Moderate overall knowledge. Predictors of knowledge: female gender ($p = 0.04$).
Pourkazemi 2020 [132]	Iran	Cross-sectional 375 T2D from 1 clinic	Study-created questionnaire	15% with good knowledge. Predictors of knowledge: female gender, duration of diabetes, urban residents, formal education, prior diabetic foot ulcer, prior amputation (all $p < 0.05$).
Rheeder 2008 [162]	South Africa	Cross-sectional 120 from 1 diabetes clinic	Modified questionnaire [109]	Participants with ulcer at-risk feet were less likely to inspect their feet daily ($p = 0.025$).
Sulistyo 2017 [115]	Thailand	Cross-sectional 81 from 1 clinic	Modified Diabetic Foot Care Knowledge Questionnaire [159]	58% with moderate, 39.5% poor knowledge.
Tuha 2021 [134]	Ethiopia	Cross-sectional 344 from 1 hospital	Details not provided	72.7% knew to inspect their feet for ulcers.
Health Care Providers				

Table 2 (continued)

Study	Country	Study Type & Population	Measure	Main Findings
Alotaibi 2017 [42]	Saudi Arabia	Cross-sectional 423 nurses at 1 hospital	Diabetes Basic Knowledge Test [161]	52.3% questions correct regarding diabetic foot care.
El Hajj 2018 [124]	Qatar	Cross-sectional 126 pharmacists	Michigan Diabetes Research and Training Center Diabetes Knowledge Test [162]	25% with moderately poor knowledge.

knowledge was lower among T2D patients with (n = 55) versus without ulcers (n = 110), which did not correlate with either foot care score or diabetes duration [120]. Conversely, a UK study of amputees at a foot clinic found a high level of foot care knowledge, which did not differ between patients with unilateral (n = 121) or bilateral (n = 22) amputations [121]. No differences were noted in KAP between patients with (n = 89) versus without (n = 121) amputation in a St. Kitts and Nevis [135]. We also identified that prior education on foot care [113, 114] and prior physician advice [131] was associated with greater neuropathy knowledge. As might be anticipated, multiple studies also found that higher levels of formal education enhanced knowledge of diabetic foot disease [105–108, 114, 125]. Lastly, higher socioeconomic status was linked to a greater knowledge [106, 125].

Although knowledge scores can correlate with practice behavior [136], as noted above, better neuropathy knowledge does not always lead to better foot care [131]. Thus, we also combed the literature for determinants of good foot self-care. Female sex was associated with greater foot self-care ($p < 0.035$) [137], an association that persists after controlling for confounders in the US, China, and Ethiopia [114, 137, 138]. Younger age is also a predictor of better care [103, 139], though one study noted no effect of age based on a 50-year-old cutoff [106]. Studies that identified predictors of greater knowledge through multiple regression analysis indicated a weak association of age with knowledge in a US study [103], but a strong association in other studies [114, 138]. A few studies examined the influence of race/ethnicity; analysis of T2D US participants from the Diabetes Attitudes, Wishes and Needs 2 study found that Black Americans spent more time on foot self-exam per week versus White or Chinese Americans, after controlling for income, age, education and diabetes type ($p < 0.05$) [140]. A US study of veterans with diabetes confirmed this association, with higher adherence to foot care among Black Americans as well as Hispanic patients when compared to White patients, in multiple regression analysis [103], as did a UK study across the general T2D population [30].

As with neuropathy knowledge, previous experiences with foot ulcers or amputations may also influence self-care practice [141]. In the study of US veterans, neuropathy symptoms, a foot ulcer in the previous year, or a prior amputation independently predicted more meticulous foot care [103]. Several studies highlighted that better diabetic foot education [103, 133, 137] and attention from a foot care professional [103, 137] also improved self-care practices. Finally, socioeconomic forces played a role in self-care behavior, including a higher formal education [106], urban residency [79, 136, 138], and income [141]. Factors may modify these relationships. For instance, in the US veteran study, years of schooling did not remain significant after multiple regression analysis [103], although it did in a Chinese study [114]. This emphasizes the importance of correcting for confounding factors in KAP surveys, as well the presence of additional potential contributors that may explain study differences.

4.2.3 Provider knowledge of neuropathy

Our comprehensive literature review found there were far fewer KAP studies of provider neuropathy knowledge versus of patients, and some studies were relatively small. Overall, the studies revealed significant knowledge gaps and misconceptions. A nationwide US study of health care professionals, which included general doctors (n = 250), specialists (n = 150), and nurses and/or physician assistants (n = 100), found 53% of survey participants held the belief that adequate glucose control could reverse peripheral neuropathy [126], despite the progressive nature of the disease and the presence of additional risk factors, such as central obesity [37]. Encouragingly, however, over half of providers expressed a desire for more information regarding several aspects of neuropathy, including its cause and how it induces pain or numbness. In a UK study, junior doctors and nurses scored poorly with regards to foot care, although they scored well on general diabetes knowledge [127], indicating a potential disconnect in understanding the link between diabetes and neuropathy. Moreover, neuropathy was the least recognized diabetes complication out of several micro- and macrovascular complications, including retinopathy and nephropathy, by nurses in Australia [93]. Conversely, a study of nurses in Saudi Arabia found neuropathy was recognized by 76% of participants, and most by nurses belonging to critical care units [42].

Our survey of the literature also uncovered a few trends in practice. A small 1997 study in Cape Town found that doctors and primary health care nurses (n = 22) did not usually assess for peripheral neuropathy (insensate foot, ulcers), unless the patient voiced a complaint [39]. A more recent KAP survey of pharmacists in Qatar found most counselled patients on foot exams and screening for neuropathic pain [128]. In a study that showed footage of a "patient" displaying signs of emerging peripheral neuropathy, only 42.2% of participating US primary care physicians (n = 192) indicated they would perform all essential components of a foot examination, whereas 21.9% stated they would perform none [7]. Additionally, providers were more likely to recommend all parts of a foot exam in male versus female, older versus younger, higher versus lower socioeconomic status patients, and in patients with signs of neuropathy compared to those without signs of neuropathy. We could not ascertain more current practices overall due to a lack of studies.

4.3 Nephropathy

Objectively, nephropathy, otherwise known as diabetic kidney disease (DKD), may be considered the most serious of diabetic microvascular complications. DKD is the progressive loss of kidney function secondary to diabetes, which manifests as microalbuminuria and renal inflammation [142]. In very advanced disease, the so-called end-stage renal disease, it can require renal replacement therapy, and, in the cases of failure, lead to death. Therefore, it is essential for patients with diabetes to be aware of DKD and take measures to prevent onset and/or slow progression. Unfortunately, our literature search did not yield many studies of nephropathy KAP, either among patients or providers. Therefore, this is a significant knowledge gap that requires addressing.

4.3.1 Instruments to assess nephropathy knowledge

Since we identified a few KAP studies of nephropathy in patients with diabetes in the literature, there were only a few instruments, some of which had been previously used to assess knowledge regarding kidney disease not necessarily related to diabetes [143, 144]. Only one mentioned face and content validity and internal consistency [143]. The scenario was similar for provider KAP instruments; some noted pretesting and assessment of internal consistency [145] and employed a previously published tool [146], but there was little data available overall. The lack of validated instruments hinders our ability to accurately assess KAP related to DKD and compare across populations.

4.3.2 Patient knowledge of nephropathy

Among the sparse studies we found, evidence suggests patient knowledge of nephropathy is less than that of retinopathy [73]. Moreover, there was a lack of studies regarding KAP in patients with diabetes and none adjusted for potential confounding **(**Table 3**)**. A study in Malaysia of patients diagnosed with diseases at risk of chronic kidney disease (diabetes, hypertension, heart diseases, obesity, n = 103), nephropathy knowledge was associated with being male, younger, formally educated, married, and of higher income (all $p < 0.05$), although none except marital status remained significant for practice behavior [147]. However, it was unclear from that study what the level of nephropathy knowledge and practice was specifically among patients with diabetes. A Fiji study of T2D patients with chronic kidney disease (n = 225) found KAP to be relatively good among participants, with high knowledge, attitude, and practice scores in 61.8%, 63.6%, and 88.4% survey respondents, respectively [143]. It is possible KAP scores were high due to the selected nature of participants, involving those with known T2D and nephropathy recruited from study site providing care for these specific conditions. In fact, patient KAP overall may be low. A study in India of only T2D patients (n = 323) found nephropathy knowledge was poor in 79% of survey respondents, and was associated with poor literacy, low socioeconomic status, and limited family income [144]. An Ethiopian study of patients with diabetes and hypertension (n = 208) found nephropathy knowledge to be low in 63.5% of participants [148]. Finally, one Australian study of patients with T1D (9%) and T2D (88%) and chronic kidney disease (n = 316) investigated the barriers to seeking appropriate care, which included inadequate knowledge of diabetes and nephropathy [149].

4.3.3 Provider knowledge of nephropathy

Nephropathy is relatively well recognized as a diabetes complication by Australian nurses (75% of survey participants), nearing their knowledge of retinopathy (89%) and far outpacing that of neuropathy (48%) and foot ulcers (43%) [93]. A KAP survey of Ethiopian health care professionals (n = 326) indicated 91% were aware of the association of diabetes and hypertension with chronic kidney disease, although there were some gaps, such as only 59% were aware that assessment of enhanced glomerular filtration rate was superior to serum creatinine alone for assessing nephropathy severity [150]. However, the KAP instrument used by this study only had a Cronbach's alpha coefficient of > 0.62; therefore, it may not have accurately captured KAP. The association between diabetes and nephropathy was also recognized by 88% of general practitioners in a Pakistani

Table 3 Summary of studies that investigated nephropathy knowledge among patients with diabetes and health care providers. Studies arranged alphabetically. Abbreviations: CKD, chronic kidney disease; GP, general practitioner; T2D, type 2 diabetes

Study	Country	Study Type & Population	Measure	Main Findings
Patients with Diabetes				
Alvis Zibran 2019 [139]	Fiji	Cross-sectional 225 with T2D and CKD from 1 hospital	Previously used KAP questionnaire [163]	61.8% with high knowledge.
Hussain 2019 [140]	India	Cross-sectional 323 T2D from 1 endocrinology clinic	Adapted CKD awareness questionnaire 164	21.4% had good knowledge. Predictors of knowledge: literacy, income, socioeconomic status (all $p < 0.05$).
Kumela Goro 2019 [144]	Ethiopia	Cross-sectional 208 with hypertension and diabetes from 1 hospital	Study-created questionnaire	63.5% with poor knowledge.
Lo 2017 [145]	Australia	Cross-sectional 308 patients with CKD and diabetes from 4 hospitals	Study-created questionnaire	43.5% cited inadequate knowledge of CKD and poor education about CKD as a barrier to care.
Health Care Providers				
Wolide 2020 [146]	Ethiopia	Cross-sectional 325 providers at 1 hospital and 3 private clinics	Study- created questionnaire	Predictors of knowledge: subspecialist provider ($p < 0.05$).
Wong 1999 [141]	US	Cross-sectional 216 GPs	Study- created questionnaire	91.4% with good risk factor knowledge.
Yaqub 2013 [142]	Pakistan	Cross-sectional 232 GPs in 1 city	Study-created questionnaire	80% knew risk factors for CKD, 41% were unsure when to refer to nephrology

study [146]. Overall, there is a lack of studies investigating DKD KAP in providers and only one adjusted for potential confounders using logistic regression [144].

With regards to DKD practices, in a small Cape Town study, 82% of doctors and primary health care nurses assessed nephropathy by urine protein, whereas only 27% assessed serum creatinine [39]. Moreover, providers were unaware that controlling hypertensive nephropathy is essential for reducing the risk of DKD. A 1999 US study of primary care physicians recruited from the American Medical Association database ($n = 211$) found nearly 98% of physicians assessed proteinuria and microalbuminuria at least as frequently or more frequently as the recommended guidelines at the time of the study, yet 39% chose an inappropriate test for monitoring [145].In addition, an Australian study found patients with DKD did not always receive the guideline recommended care, with nearly 40% of patients with a blood pressure > 140/90 mmHg despite strict blood pressure recommendations among patients with DKD [149]. However, further investigation into the reasons for this, including patient compliance with physician recommendations, was not provided.

5 Conclusion

Microvascular complications contribute to substantial morbidity and mortality among patients with diabetes, yet our comprehensive literature review found that studies examining patient and health care provider knowledge of these complications varies widely between microvascular complication and settings. Retinopathy had the largest number of studies and appears to be the most widely studied diabetic microvascular complication, yet nephropathy, which is a significant driver of diabetes-related mortality, is the topic of substantially fewer studies. Addressing this knowledge gap is essential to reduce mortality among patients with diabetes.

The current literature does offer insight into possible interventions for this patient population. Our literature review found that patients and providers often did not see the need to seek healthcare or screen for microvascular complications unless there are symptoms clearly consistent with diabetic sequalae [7, 64, 74]. This is a clear missed opportunity to reduce morbidity among patients with diabetes. As patients frequently cite health care providers as sources of information [85, 151], improving provider knowledge of diabetic microvascular complications and addressing barriers to patient education and risk factor modification may provide one avenue for improving patient outcomes.

Yet, it is challenging to know whether these interventions would be effective as our literature review noted significant discordance in findings across studies. This could

be due to the relatively small sample sizes of most studies, lack of adjusting for potential confounding parameters in most cases, or sociocultural differences between study sites. While there were studies that recruited from multiple cities or from organizational or national databases [113, 114, 152, 153], we did not identify any studies that recruited from more than one country. Therefore, it is difficult to draw firm conclusions whether differences in patient and provider knowledge are setting specific or related to methodology. Instruments used to assess patient and provider knowledge of microvascular complications varied substantially between studies and, while some authors included detailed regarding instrument validation, this was only moderately implemented. Consistent use of well-validated measures between study sites would address concerns regarding methodology as well as begin to highlight sociocultural differences between settings. Lastly, studies conducted in high-income settings have not been updated and there are few studies that have included the same study site over time. Therefore, we were unable to draw conclusions regarding temporal changes in patient and provider knowledge. To move forward and identify patient and provider populations that would benefit most from educational programs, updated studies using well-validated KAP surveys with results analyzed using more refined statistical tools are essential.

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The conundrum of diabetic neuropathies—Past, present, and future☆

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ABSTRACT

Diabetic neuropathy (DN) remains arguably the most prevalent chronic complication in people with both type 1 and type 2 diabetes, including in youth, despite changes in the current standards of clinical care. Additionally, emerging evidence demonstrates that neuropathy affects a large proportion of people with undiagnosed diabetes and/or prediabetes, as well as those with obesity. Here we summarize the latest epidemiology of DN, recent findings regarding the pathophysiology of the disease, as well as current outcome measures for screening and diagnosis, in research and clinical settings. The authors discuss novel perspectives on the impact of social determinants of health in DN development and management, and the latest evidence on effective therapies, including pharmacological and nonpharmacological therapies for neuropathic pain. Throughout the publication, we identify knowledge gaps and the need for future funding to address these gaps, as well as needs to advocate for a personalized care approach to reduce the burden of DN and optimize quality of life for all affected individuals.

1. Introduction

Diabetic neuropathy (DN) affects people in a myriad of ways including loss of sensation, loss of balance, severe pain, foot ulcers and amputations. Individuals with DN experience depression and anxiety, with poor quality of life and poor daily function. DN also affects the autonomic nervous system, with corresponding heart failure and even sudden cardiac death. More than $10 billion of annual healthcare costs are attributed to DN[1], underscoring the magnitude of this highly morbid disorder and the associated socioeconomic problems.

Among the various forms of DN, distal symmetric polyneuropathy (DPN) and diabetic autonomic neuropathies, particularly cardiovascular autonomic neuropathy, are by far the most studied, although emerging data highlight the impact of other forms of autonomic neuropathies such as gastrointestinal and urogenital autonomic neuropathies, on healthcare and patients' reported outcomes. The urogenital autonomic neuropathies are amply covered in a different manuscript in this same issue, to which the reader is referred to.

2. Epidemiology and risk factors

2.1. Distal symmetric polyneuropathy

DPN remains arguably the most prevalent chronic complication in people with both type 1 diabetes (T1D) and type 2 diabetes (T2D), including in youth. The estimated lifetime prevalence exceeds 50 % despite changes in the current standards of clinical care over time[2–5] with different rates of DPN progression depending on disease duration, population studied, and the DPN definition.[2,3,5,6] There are also epidemiological differences between DPN in T1D versus T2D, despite no major structural differences in nerve pathology, highlighting an area that deserves further targeted research.[5]

The Diabetes Control and Complications Trial (DCCT) and its observational follow-up, the Epidemiology of Diabetes Interventions and Complications (EDIC) study, the largest and best phenotyped TID cohort followed for ~40 years, demonstrated that despite a low DPN prevalence in early T1D, the prevalence increases steadily over time to

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~34 % after 25 years.[7] Similarly, a large cohort of randomly selected individuals with T1D from 16 European countries, the European Insulin-Dependent Diabetes Mellitus Prospective Complications Study (EURODIAB IDDM), reported similar mean DPN prevalence, but with significant DPN trends associated with age, diabetes duration, and hemoglobin A1c, as well as with hypertension and hyperlipidemia.[8]

Some may argue that neither the EURODIAB nor the DCCT cohorts are representative of contemporary populations, even though the vast majority of EDIC follow-up was performed after the DCCT lessons were implemented into clinical care.[9] More recent data reported from 2 large contemporary cohorts of ~6000 people with T1D each, the T1D Exchange in USA and the Scottish Register, demonstrated a prevalence of 11–13 % for symptomatic DPN based on the Michigan Neuropathy Screening Instrument (MNSI) questionnaire. These findings however must be considered in the context of solely using symptom as a diagnostic tool, compared to EURODIAB and DCCT, where clinical examinations were also performed.[10,11]

DPN prevalence is even higher in people with T2D. Data from several contemporary cohorts report DPN in ~20 % of individuals with newly diagnosed diabetes, despite progress in the standards of care, that increased to ~50 % after 10 or more years.[2–6] For instance in a large cohort of >1500 individuals with screen-detected T2D enrolled in the ADDITION-Denmark Study, DPN prevalence was ~13 % at baseline with cumulative incidence rates of 10 % over 13 years of follow-up.[12] Similarly, among a contemporary cohort of 5047 people with newly diagnosed T2D (mean duration of only four years) participating in the Glycemia Reduction Approaches in Diabetes - A Comparative Effectiveness (GRADE) trial, ~ 21 % of participants presented with DPN at baseline.[13] Conversely, a DPN prevalence rate as high as 50 % was reported in the Bypass Angioplasty Revascularization Intervention 2 Diabetes (BARI 2D) cohort that included ~2400 T2D participants with a mean diabetes duration of ~10 years.[14] Furthermore, unexpectedly high DN prevalence (both DPN and autonomic neuropathy) was reported in youth with T1D and especially T2D.[2,15,16]

The burden of DPN in both youth and adults is particularly alarming given the continuous rise in diabetes prevalence in USA and worldwide, including the disproportionately higher rates in minorities.[17,18] Up to 350 million people may develop DN and related comorbidities by 2045.[18] The true prevalence is likely higher when asymptomatic DPN is included as only 30 % of cases endorse typical DPN symptoms, including pain.[5] Therefore, timely identifying and addressing risk factors for DPN are imperative steps to reduce the burden of this devastating complication.

Traditional risk factors for DPN in T1D and T2D include glycemic control, age, diabetes duration, and height.[2,5,7,12] Additionally, cardiovascular risk factors (e.g., obesity, hyperlipidemia, hypertension, and smoking) are reported risk factors in several cohorts of both T1D and T2D.[2,5,10,19] These findings agree with several clinical studies in the United States, Europe, and Asia demonstrating the metabolic syndrome is a risk factor for DPN.[5,12,20,21] Obese individuals with normoglycemia have a higher prevalence of neuropathy versus non-obese individuals, suggesting that obesity alone may be sufficient to induce neuropathy, while glucose variability is also emerging as a potential risk factor for the development of DPN, particularly painful DPN.[22]

Social determinants of health (SDOH) are emerging as important diabetes complications risk factors, likely due to both the increased risk of diabetes mellitus as well as inadequate glycemic control.[23] However, in the USA, data regarding the association between DPN prevalence and sociodemographic characteristics, are limited. Data from the NHANES and the Atherosclerosis Risk in Communities (ARIC) cohorts show that non-Hispanic Blacks were more likely to have DPN on monofilament testing than non-Hispanic Whites, even after controlling for traditional risk factors, suggesting that race may also impact DPN development.[24] Yet data examining DPN prevalence and risk factors among racial/ethnic minorities, particularly Black Americans, are limited. Preliminary data from an ongoing study in Flint, Michigan with a predominantly Black, low-income patient population suggests that DPN is common but often underrecognized.[25] High prevalence of DPN has also been reported among native populations, including a cohort of Pima Indians in Arizona.[26] Although this increased burden among racial/ethnic minorities is likely due to an increased T2D and metabolic syndrome prevalence[23,27], these findings warrant further investigation. Additionally, U. S. racial/ethnic minority populations with DPN have worse diabetic foot ulcer outcomes and higher rates of amputation than non-Hispanic White Americans.[28–30] A recent modeling analysis using longitudinal data from the SEARCH for Diabetes in Youth suggested that unmeasured race and ethnicity-associated factors account for predicted DPN disparities in non-White versus White youth and young adults with diabetes, highlighting the need for further research in this area.[31] Teasing apart the association between DPN outcomes and race/ethnicity is challenging as it is likely confounded by socioeconomic status.[23] Yet, characterizing these relationships are essential to design interventions targeting patient outcomes.

More recently, the T1D Exchange cohort that collected data on several SDOH reported that both lower education and higher rates of public insurance options were associated with DPN in adults with T1D.[10] The role of SDOH was confirmed in a Scottish T1D cohort, which found that social deprivation led to a 2.17 higher odds of DPN.[11] In terms of psychological factors, both depression and anxiety have been found to be associated with DPN, particularly painful DPN.[32,33]

2.2. Epidemiology of pain in DPN

Up to 30 % of individuals with diabetes experience painful DPN and neuropathic pain may be the first symptom that prompts people to seek medical care.[2,4,34,35] A large community-based study in the U.K reported higher prevalence of painful symptoms (35 % vs 23 %) and painful DPN (22 % vs 13 %) in T2D compared to T1D respectively, using the neuropathy symptom and neuropathy disability scores.[36] In a recent cross-sectional, hospital-based, multicenter study including ~800 individuals with both T1D and T2D, the reported prevalence of painful DPN was 13 % using the grading system of the Neuropathic Pain Special Interest Group of the International Association for the Study of Pain.[37] Another recent large cross-sectional study in the Danish Centre for Strategic Research in Type 2 Diabetes cohort reported prevalence rates of DPN and painful DPN of 18 % and 10 %, respectively, using the MNSI questionnaire and the Douleur Neuropathique en 4 (DN-4) questionnaire.

Risk factors include female sex, age, duration of diabetes, and obesity.[38] Additionally, significant associations between painful DPN and psychosocial factors such as smoking, depression, and anxiety are emerging although the directionality is unclear.[39]

2.2.1. Knowledge gaps

- Understand what drives the high prevalence of DPN despite continuous refinements in the standards of diabetes care, and the progress in diabetes medications and diabetes technologies
- Recognize the relative contributions of various risk factors including SDOH, and the effects of modifying risk factors
- Understand differences between T1D and T2D DPN
- Identify gaps in the implementation of the current best practices
- Identify reasons for the high prevalence of DPN in youth

2.3. Diabetic autonomic neuropathy

The autonomic nervous system regulates many systems and organs through small C-fibers. Thus, diabetic autonomic neuropathy may manifest with a broad spectrum of signs and symptoms depending on the affected target system including cardiovascular autonomic neuropathy (CAN), gastrointestinal neuropathy, urogenital neuropathy, and others.[2]

2.3.1. Cardiovascular autonomic neuropathy

CAN is by far the most studied form of autonomic neuropathy.[2,3,40] The reported prevalence of CAN over time varies, and is contingent upon several factors including the definition of CAN, the population studied (e.g. observational cohorts vs interventional trials; T1D vs T2D vs prediabetes) and the study design (longitudinal vs cross-sectional).[2,40]

For instance, prevalence is very low in individuals with newly diagnosed T1D. This is documented by the primary prevention arm of the DCCT cohort, which similar to DPN, is arguably the best phenotyped cohort for CAN with cardiovascular autonomic reflex tests (CARTs), validated symptoms instruments, and electrocardiogram recordings obtained repeatedly during follow-up.[7,41] In DCCT/EDIC, the prevalence of CAN increased steadily over time to 44 % over 23 years of mean follow-up.[7] Similarly high prevalence was reported in other T1D cohorts including the EURODIAB IDDM, the Pittsburgh Epidemiology of Diabetes Complications Study, and the STENO T1D study.[2,40] While the argument may be made that these cohorts preceded the changes in standards of diabetes care, studies utilizing the T1D exchange network evaluated the contemporary prevalence of autonomic neuropathy based on the Survey of Autonomic Symptoms (SAS) in T1D adults with >5 years of diabetes duration. Autonomic symptoms were present in 17 % of participants who responded to the surveys and >70 % of those participants experienced moderate to severe symptoms.[42]

Prevalence as high as 60 % has been reported in earlier cohorts of individuals with long-standing T2D.[2,40] Additionally, recent data from the GRADE cohort[13] in the United States and the Anglo-Danish-Dutch Study of Intensive Treatment in People With Screen-Detected Diabetes in Primary Care (ADDITION) cohort[43] from Denmark demonstrated that up to 13 % of individuals with either short duration or newly diagnosed diabetes already have evidence of CAN. CAN was also reported in prediabetes in individuals with impaired glucose tolerance, insulin resistance, and/or the metabolic syndrome.[40,44] Moreover, an unexpectedly high prevalence of CAN was identified among youth with diabetes participating in the SEARCH for Diabetes in youth study.[16]

As with DPN, there are several traditional risk factors that are associated with CAN in diabetes including older age, longer diabetes duration, poor glucose control, diabetic kidney disease, hypertension, elevated triglycerides, and smoking. However, more recently, glucose variability, psychological factors (depression), and SDOH (lower income and education) are also emerging as less traditional risk factors for CAN in diabetes.[42]

2.3.2. Gastrointestinal autonomic neuropathy

Autonomic dysfunction in the gastrointestinal system may manifest as esophageal dysmotility, gastroparesis, constipation, diarrhea, and fecal incontinence.[2,3] Among gastrointestinal neuropathies, gastroparesis is by far most frequently encountered in clinical practice.

Earlier prevalence data on gastroparesis are sparse.[2] The reported cumulative incidence and prevalence of gastroparesis was 9.8 in women and 2.4 in men per 100,000 person-years and 37.8 for women and 9.6 for men per 100,000 person-years respectively in the only large community-based study in the United States.[45] The contemporary prevalence rates of confirmed gastroparesis due to T1D or T2D are low[2] but likely increasing based on the growing number of gastroparesis-related hospitalizations in the United States. However, given the increased use of many medications that may directly impact the gastrointestinal system motility, such as glucagon like peptide 1 receptor analogs (GLP1-RA), as well as opioids and more recently recreational marijuana, no well-designed studies have evaluated whether these cases are iatrogenic or due to diabetes.

2.3.3. Knowledge gaps

- Identify risk factors driving the heterogeneity in autonomic neuropathy risk, and of various forms of autonomic neuropathy between T1D and T2D
- Evaluate the impact of glucose variability on autonomic neuropathy, particularly CAN risk
- Understand the current epidemiology of diabetic gastroparesis and determine the percentage attributable to medication use

3. Pathophysiology/mechanisms for diabetic neuropathy

Diabetes can damage various components of the peripheral nerve, which comprises the cell body (dorsal root ganglion or anterior horn that originate in the spinal cord), its myelinated cell projections, as well as axonal extensions, innervating the periphery. The most common form of nerve injury is a progressive distal-to-proximal peripheral nerve loss that typically presents as sensory predominant.[34] Particularly, small unmyelinated nerve fibers, known as C-fibers or “small fibers”, which relay information related to heat discrimination and chemical pain are an early and frequent target of DPN.[5] Small, thinly myelinated Aδ fibers that carry signals related to touch, pressure, and cold are also commonly affected.[5] Only much later in the course of the disease is there evidence of large myelinated Aβ fiber (“large fiber”) dysfunction, responsible for vibratory and position perception.[5] Progressive sensory loss coupled with motor weakness at later disease stages leads to loss of sensation in the feet and predisposes to impaired balance and falls. [2]

The mechanisms promoting the onset and progression of DN are complex (Fig. 1). Fully understanding these mechanisms is essential for the successful development of disease modifying therapies, a goal that has been elusive so far.

In the last three decades, DN research, including our own, has focused on glucose and the streptozotocin (STZ)-induced T1D rat as the pre-clinical model of choice. These studies, amply discussed elsewhere,[46,47] provided insights into the molecular mechanisms that drive glucose pathogenesis in DPN which include: 1) the polyol pathway through its key enzyme, the aldose reductase, which results in a series of downstream reactions that decrease sodium–potassium adenosine triphosphatase (ATP) activity, deplete nicotinamide adenine dinucleotide phosphate, and produce reactive oxygen species (ROS); 2) the hexosamine pathway and protein kinase C (PKC) secondary activation generating inflammatory by-products, with subsequent insulin resistance, impairment in growth factor biology, and vasoconstriction of nerve blood vessels; 3) advanced glycation end products (AGEs) that bind the receptors for AGEs (RAGEs), leading to downstream inflammation, ROS accumulation, and decreased nerve blood flow; 4) cyclooxygenase (COX) mainly COX2, with downstream increased ROS leading to reduced nerve blood flow and neuronal dysfunction. Moreover, low-grade inflammation and its downstream impact on several of the pathways described above has emerged as a critical mechanism in the development of DN and painful DPN in several experimental and clinical studies.[48–54]

Promising results were achieved using aldose reductase inhibitors, PKC inhibitors, or RAGE inhibitors in animal studies, especially in the STZ-induced T1D rat. Unfortunately, singularly targeting each of these pathways either failed to reverse nerve damage in human DPN trials or was too toxic during therapeutic development.[2,5] Additionally, meta-analyses and systematic reviews of large T1D and T2D human trials that included DN outcomes reported that while tight glycemic control reduces DN incidence in T1D, it does not result in complete protection or reversal of disease, and it has a lesser effect in those with T2D.[2,5,6,55] Our recent clinical studies support these observations, implicating the metabolic syndrome, including dyslipidemia as major drivers of DPN in prediabetes and T2D, independent of glycemic status.[56,57] All these led to a paradigm shift in the DN field away from models based solely on glucose metabolism and the STZ-induced T1D rodent model towards understanding metabolic drivers in T1D versus T2D and global whole nerve metabolism.[21,55]

In a bedside-to-bench approach, recent studies employed non-genetic mouse models of prediabetes and obesity that when placed on a high saturated fat diet, develop features of prediabetes and the

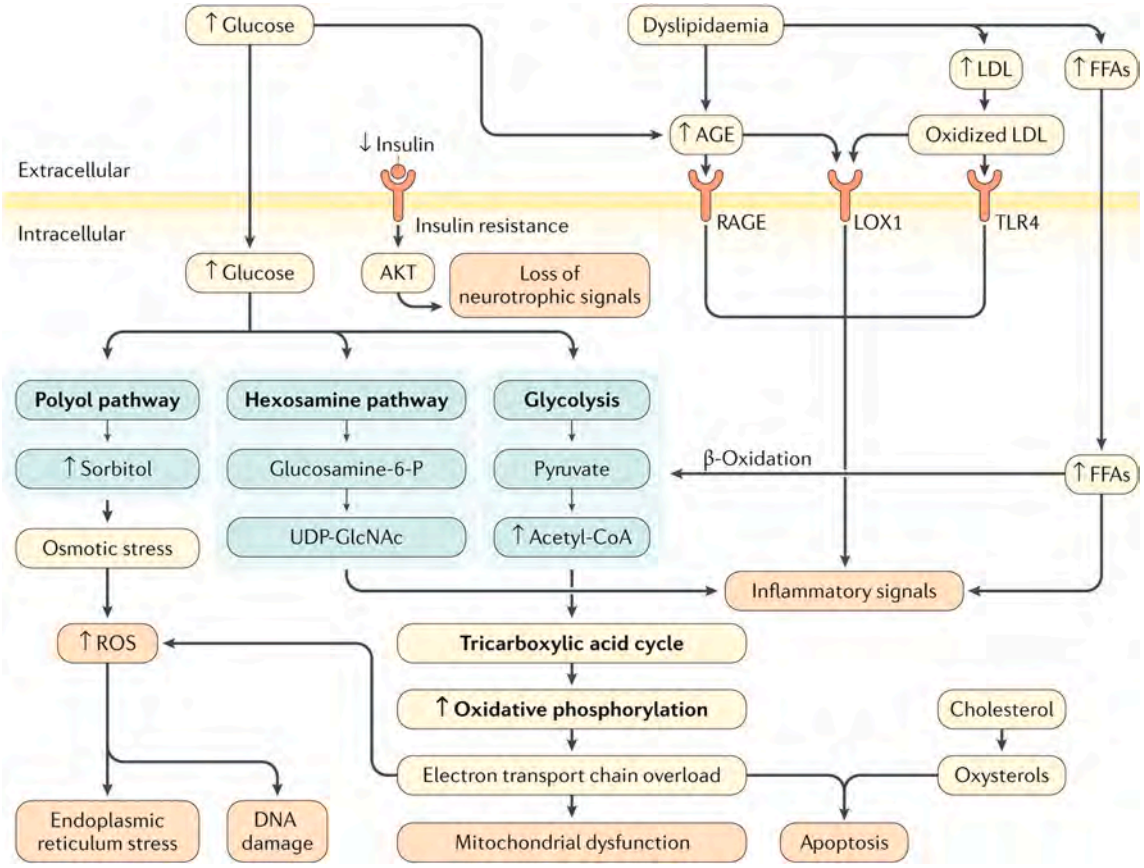

Fig. 1. Diabetic neuropathy pathogenesis. Hyperglycaemia and dyslipidaemia, together with altered insulin signalling, lead to several pathological alterations in neurons, glia and vascular cells that can lead to nerve dysfunction and ultimately, neuropathy, including DNA damage, endoplasmic reticulum stress, mitochondrial dysfunction, neurodegeneration and loss of neurotrophic signalling, and can trigger macrophage activation. The importance of these pathways in the development of neuropathy varies with cell type, disease profile and time, as distinct cell types are more or less susceptible to injury depending on the metabolic impairments. AGE, advanced glycation end-product; FFAs, free fatty acids; Glucosamine-6-P, glucosamine 6-phosphate; LDL, low-density lipoprotein; LOX1, oxidized LDL receptor 1; RAGE, AGE-specific receptor; ROS, reactive oxygen species; TLR4, Toll-like receptor 4; UDP-GlcNAc, uridine diphosphate *N*-acetylglucosamine.
Reproduced from: Feldman EL, Callaghan BC, Pop-Busui R, Zochodne DW, Wright DE, Bennett DL, Bril V, Russell JW, Viswanathan V. Diabetic neuropathy. Nat Rev. Dis Primers. 2019 Jun 13;5(1):42. doi: https://doi.org/10.1038/s41572-019-0097-9. PMID: 31197183; PMCID: PMC7096070.

metabolic syndrome with dyslipidemia, impaired glucose tolerance, insulin resistance, and neuropathy.[58–61] Using integrated lipidomic and transcriptomic profiling, we demonstrated that nerve triglyceride accumulation and triglyceride synthesis are key players in the pathogenesis of neuropathy, and a dietary reversal paradigm, aimed at correcting the lipid profile may be a promising non-pharmacological approach to improve nerve function in prediabetes and T2D.[59] Other studies using the same mouse model and in vitro DPN models unveiled the role played by mitochondria function. Under normal conditions, mitochondria use both glucose and lipids to produce ATP. However, in the diabetic environment, excess glucose and lipids disrupt the normal pathways used for their own breakdown, producing excess electron donors that overwhelm mitochondrial capacity, resulting in bioenergetic failure with mitochondrial depolarization, decreased ATP production, impaired mitochondrial trafficking, and accumulation of ROS, leading to inflammation, endoplasmic reticulum stress, apoptosis of neurons, and axonal failure.[5,62] With fewer functional mitochondria in the cell body and along the axons, energy-starved small and large nerve fibers lose their ability to function and undergo degeneration with the axons farthest from the cell body (i.e., those in the feet) and the smallest fibers that regulate pain and dysesthesia being most vulnerable.[5,46,63] Interestingly, switching saturated fatty acid-rich diets with diets rich in unsaturated fats from plant sources or fish oil improves nerve function[63,64] and prevents axonal mitochondrial dysfunction,[63,65] supporting a beneficial role for unsaturated fats as targeted therapy development for DPN.

Other emerging areas of interest are understanding the changes that occur in whole nerve metabolism, cell-specific changes in the nerve microenvironment during DPN, and how Schwann cell injury may impair energy substrate transfer[66] and/or extracellular vesicle secretion.[67] An improved understanding of axoglial cross-talk and nerve function will help inform development of future DPN therapies.

Overall, the DN field is moving from a nerve-centric focus on glucose alone to a new era of research centered on understanding the role of additional metabolic and inflammatory factors in DN pathophysiology, which could be instrumental to help develop mechanism-based therapies.

3.1. *Knowledge gaps*

- Develop experimental models that translate well to human disease
- Apply a precision approach to identify optimal therapeutic targets
- Understand how metabolic factors, other than hyperglycemia alone contribute to DN
- Interrogate the direct contribution of Schwann cells, and other cellular components of the nerve, including macrophages to DPN
- Understand differences in cellular and molecular mechanisms between DPN and autonomic neuropathies

4. Diagnosis and outcomes measures

4.1. Distal Symmetric Polyneuropathy

4.1.1. Diagnosis in clinical care

The hallmark symptoms and signs associated with DPN are the consequence of the progressive damage and loss of the various populations of nerve fibers, each with distinct roles and functions.[2,3] As highlighted in the recent American Diabetes Association Monograph on Diabetic Neuropathy, in diabetes, this process occurs in a specific symmetrical, distal-to-proximal pattern, starting at the tip of the toes and eventually progressing proximally.[5] The symptoms and clinical signs associated with DPN follow the same pattern, creating the typical "stocking-and-glove" clinical presentation, an important diagnostic feature.[5] A targeted history will unveil specific symptoms that include: a) neuropathic pain, a feature of small fiber damage; b) numbness and tingling without pain, features of small or large fibers damage; c) insensate, numb feet usually associated with more advanced mixed fiber damage.[2,3,5] In more advanced DPN, individuals present with reduced daily function with poor balance, falls or fractures, and an increased risk for painless injuries that lead to ulcers, infections, and amputations.[2,5] Neuropathic pain in DPN is typically experienced as burning, shooting, electric shock–like or lancinating, usually worse at night, and may be accompanied by dysesthesias such as an exaggerated response to painful stimuli (hyperalgesia) and/or pain evoked by contact with ordinarily unpainful stimuli such as socks, shoes, and bedclothes (allodynia).[2,5]

A large fraction of individuals with DPN may be asymptomatic and unaware or reluctant to report their condition.[2,5] Asking specific questions and performing a targeted examination are recommended. A focused examination effectively evaluates small and large fiber function. Small fiber testing includes assessment of pinprick sensation using a sharp object such as a safety pin and temperature threshold sensation with a cold metal object such as a tuning fork in the feet. Large fiber function is assessed using a 128-Hz tuning fork for vibratory sensation and a 10-g monofilament for light-touch pressure on the dorsal aspect of the great toe, and bilateral ankle reflexes (for predominantly large fibers).[2,5] Importantly, the 10-g monofilament alone should not be used to diagnose or exclude DPN as it detects only advanced neuropathy and individuals at increased risk of diabetic foot ulcerations.[2,3] Relying solely on the 10-g monofilament could miss the early stage of the disease which is most amenable to the therapeutic intervention to prevent progression of the disease.[2,3,5] The clinicians should combine at least two examinations of both small and large fiber nerve function to detect DPN in the clinical practice.[2] A comprehensive differential diagnosis is always needed, and readers are referred to published algorithms.[5]

4.1.2. Knowledge gaps/challenges in clinical care implementation

- Understand the barriers preventing the appropriate implementation of DPN screening and diagnosis in clinical care despite readily available recommended simple tests
- Understand why 10-g monofilament testing remains the most used screening test for DPN in primary care despite its low sensitivity and specificity for earlier stages of disease[2,3]
- Develop innovative educational methods and tools for implementing appropriate DPN diagnosis in the clinical practice.

4.1.3. Outcome measures in clinical research

As recommended by the Toronto Consensus on Diabetic Neuropathy, an outcome of confirmed DPN requires a combination of symptoms, signs and an abnormality of objective tests.[2,68] While clinical instruments that include any given combination of patient reported symptoms and clinical signs may perform well in large population studies assessing prevalence and incidence rates, different measures are needed for interventional trials. In fact, one of the most critical components for a successful path towards developing effective disease modifying therapies are having access to sensitive and specific outcome measures that correctly capture the natural history of the disease and detect repair in specific nerve fiber populations. This is particularly relevant given that there are currently no approved disease-modifying therapies for any forms of DN, and a very large number of clinical trials evaluating promising experimental targets for these conditions have failed.[2,5]

The MNSI, the modified Toronto Clinical Neuropathy Scale, the Neuropathy Disability Score, and the Utah Early Neuropathy Scale are validated clinical instruments that have been used in both observational and interventional studies. Among these, the MNSI has been most consistently used in large cohorts of people with T1D, T2D, the metabolic syndrome and in youth.[2] In addition, the Quality of Life in Neurological Disorders (NeuroQOL) and the Norfolk neuropathy instrument, are validated instruments that capture measures of quality of life specific to peripheral neuropathy in several domains including pain, lost/reduced feeling, diffuse sensory-motor symptoms, restrictions in activities of daily living, disruptions in social relationships and emotional distress.[2]

More objective measures include abnormalities in nerve conduction studies (NCS) and validated measures of small nerve fibers.[2] Mild DPN, may be characterized by decrease in sural nerve amplitude or mild reduction in sensory nerve conduction velocity,[34] while severe DPN includes NCS motor abnormalities or nonrecordable sensory NCS. NCS may be completely normal in those with primarily small fiber neuropathy and loss of small fibers typically precedes loss of large nerve fibers.[34] The gold standard for small fiber neuropathy is assessment of intra-epidermal nerve fiber density (IENFD) measurements by skin punch biopsy, while other measures include quantitative sensory testing for thermal thresholds for either elevated cooling or heat detection thresholds, and emerging corneal confocal microscopy.[34]

4.1.3.1. Painful DPN. Painful DPN is associated with depression, insomnia, and poor quality of life.[33,69] Visual analog scale (VAS), Likert scales, and the McGill Pain Questionnaire are used in many clinical studies and are considered as sensitive and validated tools for painful DPN.[70]

4.1.4. Knowledge gaps in clinical research

- Validate sensitive and specific outcome measures that may correctly capture the natural history of the disease and may detect timely repair in specific nerve fiber populations
- Reach consensus among the stake holders in the field to utilize uniform and adequate outcome measures across all interventional and/or observational studies
- Incorporate patient reported outcomes including psychological outcomes in DPN studies
- Develop reliable biomarkers to be implemented at the point of care

4.2. Diabetic autonomic neuropathy

4.2.1. Cardiovascular autonomic neuropathy

4.2.1.1. Screening and diagnosis in clinical care. Unlike DPN, individuals presenting with the earliest stages of CAN may be completely asymptomatic, making its early detection challenging. In more advanced stages, people may present with palpitations, or with dizziness, unsteadiness, fainting or syncopal episodes with sudden changes from supine to standing.[2] The earliest sign of CAN is reduced heart rate variability (HRV).[2,40] Later people may also present with resting fixed-rate tachycardia, changes in blood pressure (BP) regulation overnight (reverse/non dipping BP), orthostatic hypotension, and sudden premature death.[2,40] CARTs are considered the gold standard which evaluate changes in the heart rate and BP during clinical maneuvers such as deep

breathing, standing, and Valsalva.[40,68,71] Although CARTs could be ordered as part of clinical care, they are seldom used in practice due to inconsistent availability, costs, and perhaps lack of knowledge and understanding by most providers on how to best utilize the information. With the continuous development of several technologies including continuous glucose monitoring (CGM), the detection of CAN may enable their use to prevent hypoglycemia and glucose fluctuations in larger groups of people with diabetes,[40] thus reducing arrhythmia risk and other adverse clinical consequences (Fig. 2). Additionally, CAN is an important risk for heart failure, now the most prevalent cardiovascular complication in diabetes,[72] thus testing for CAN could allow implementation of more aggressive therapy in those most at risk of future complications.[72]

4.2.1.2. Knowledge gaps in clinical care.

- Implement successful providers' education on how to diagnose and effectively use CAN as a risk stratification tool to promote a personalized medicine approach towards the use of technologies and guideline-directed therapies

- Develop reliable CAN biomarkers to be used at the point of care

4.2.1.3. Outcomes measures in clinical research. The CARTs performed under deep breathing remain the gold standard outcome measure for CAN in research settings, given their refined standardized protocols that can be administered by technicians, as well as their easy scalability in larger cohorts.[2,40] CARTs are the most common CAN outcome measure in both interventional trials, including the DCCT, and observational cohorts such as EDIC. CARTs also allow for CAN staging, with one abnormal test indicative for early/subclinical CAN, while two or more abnormal tests indicative of definite CAN[2,40] although the evidence behind these recommendations is less clear. Recently, a plethora of recording devices including software operating with a "black-box" approach (output of data without reporting the algorithm that calculated the result) have emerged making it difficult to compare data sets and reproducibility across studies, particularly when few age-related normative data sets are available.

Indices of HRV, either in time (e.g. the standard deviation of normal RR intervals -SDNN, the root-mean square of the difference of successive RR intervals -rMSSD), or frequency domain (e.g. low, very low and high frequency power) indices, are emerging as sensitive and specific alternative CAN outcome measures,[2,73] including the HRV indices derived from standard 10-s 12-lead ECG recordings, that are much more feasible and easier to implement in larger and/or longitudinal cohorts.[2,13,73]

Cardiac sympathetic imaging using I-123 MIBG scintigraphy or ^{11}C-HED PET, 24-h BP profiles, muscle sympathetic nerve activity, or baroreflex sensitivity testing to assess cardiac vagal and sympathetic baroreflex function may have higher degrees of sensitivity or specificity based on the hypotheses being tested, and may be used in some research protocols, although these require sophisticated infrastructure, highly trained personnel, and are quite expensive and time-consuming.[2,40]

Similar to DPN, the value of patient reported outcomes has emerged for autonomic neuropathy, and several surveys were developed and validated over time. While the Autonomic Symptom Profile (ASP), the Composite Autonomic Symptom Scale (COMPASS), or the abbreviated COMPASS-31 are non-invasive and have high sensitivity and specificity, they are quite time-consuming or require complex scoring algorithms.[2,42] More recently, SAS was validated as a brief, specific, and sensitive measurement of symptomatic autonomic neuropathy in diabetes and used in several cohorts including the T1D Exchange.[42]

4.2.1.4. Knowledge gaps clinical research.

- Validate sensitive and specific outcome measures, with reliable intrasubject variability, that may correctly capture the natural history of the disease and may detect timely damage reversal

- Reach consensus among the stake holders in the field to utilize uniform and adequate outcome measures across all interventional and/or observational studies, as well as uniform technologies that make the testing algorithms readily available

- Incorporate patient reported outcomes including psychological outcomes

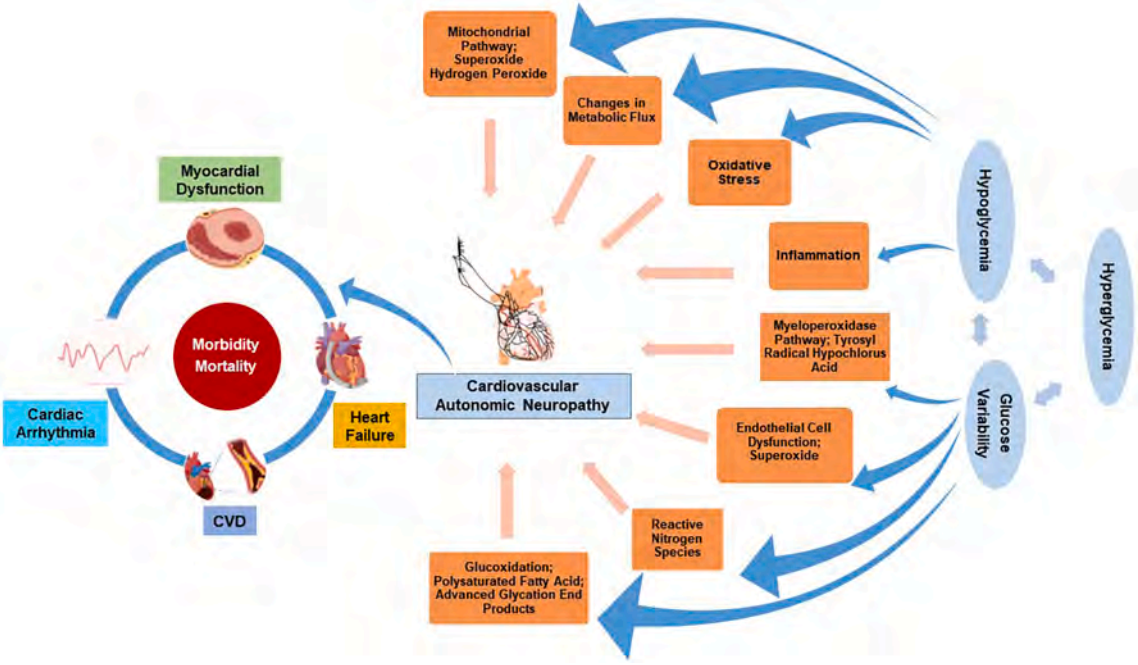

Fig. 2. Mechanisms and clinical consequences of cardiovascular autonomic neuropathy (CAN) in diabetes.

4.2.2. Gastroparesis

4.2.2.1. Diagnosis in clinical care. The clinical symptoms of gastroparesis may include early satiety, fullness, bloating, nausea, vomiting, dyspepsia, and abdominal pain. These symptoms are nonspecific and also poorly correlated with severity of gastroparesis and gastric emptying studies.[2] Among the clinical signs, individuals with gastroparesis may present with wide glucose fluctuations and frequent unexplained hypoglycemia after meals. Gastric emptying with scintigraphy of digestible solids at 15-min intervals for four hours after food intake is still considered the gold standard for diagnosis of gastroparesis. Optimization of glucose levels is utmost important before the test to avoid false positives[2] as both hypoglycemia and hyperglycemia have direct effects on gastric emptying.[74,75] Scintigraphic gastric emptying studies can be burdensome, time consuming, not readily available, and costly. Recently the use of 13C octanoic acid or an acetate breath test has been FDA approved, providing simpler alternatives.[2]

4.2.2.2. Outcomes measures for research. The scintigraphic gastric emptying study and 13C octanoic acid or acetate breath test have been widely used in the research setting with the latter being more feasible and available. In addition, collection of full thickness gastric tissue for detecting specific cellular changes associated with diabetic gastroparesis was developed by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Gastroparesis Clinical Research Consortium, although these studies are invasive, require specialized infrastructure and thus may be applicable only in selected situations. Validated questionnaires such as the Patient Assessment of Upper Gastrointestinal Disorders-Symptoms, the Gastroparesis Cardinal Symptom Index-Daily Diary, and the specific gastroparesis questions in the SAS, are widely used to assess the severity of gastroparesis symptoms in clinical research setting.[76]

5. DN management

5.1. Management of DPN

There is strong evidence that targeting near normal glucose prevents the onset and progression of DPN in T1D.[2,5] However, intensive glucose control does not reverse DPN even in T1D, and the effects of glucose control are less conclusive for DPN in T2D, as highlighted in several cohorts.[6] Likely reasons are the presence of several other risk factors and co-morbidities, as well as the complex mechanisms leading to DPN in T2D amply outlined above.

Besides glucose control, lifestyle and behavioral interventions have emerged as promising treatments for DPN prevention or even reversal.[5] For instance, Singleton and colleagues reported improvement in small nerve fiber function and reinnervation as assessed by IENFD with a lifestyle intervention comprised of diet and moderate intensity exercise similar to the intervention used in the diabetes prevention program.[77] A recent study from the Canadian Study of Longevity in T1D reported that individuals with diabetes who engaged in ≥150 min physical activity/week had a 12 % lower DPN incidence[78] providing additional insight on the potential role of lifestyle in the prevention of DPN. Moderate-intensity physical activity delays the onset and progression of DPN in individuals with T2D or prediabetic metabolic syndrome.[79] Multimodal aerobic training [moderate-intensity (50 % heart rate reserve) or vigorous (75 % heart rate reserve) exercise] in a controlled trial improves mobility, balance, and gait outcomes in DPN.[80] Additionally, dietary weight loss may help improve symptomatic DPN.[81]

Painful DPN management includes pharmacological, nutraceuticals, and non-pharmacological options.

Gabapentinoids, serotonin and norepinephrine reuptake inhibitors (SNRIs), tricyclic antidepressants (TCAs), and sodium channel blockers are all effective medication classes for the treatment of painful DPN. Few randomized controlled clinical trials report efficacy for opioids including tramadol or tapentadol. These trials were flawed by very high attrition rates and questionable study design.[2,5] Thus, given the evidence of the high risks of addiction, abuse and severe complications including death, compared to the low potential benefits, none of the opioids should be used in the treatment of painful DPN.[2,3] Yet despite these recommendations, the rates of opioid prescriptions for painful DPN remain unacceptably high, and interventions are needed to improve current clinical practice.

Among the nonpharmacological approaches, besides lifestyle interventions discussed above[6], a recent large randomized-controlled trial reported substantial pain relief and improvement in quality of life with high-frequency spinal cord stimulation in individuals with refractory painful DPN sustained over 12 months[5,82] that led to FDA approval. However, the lack of a sham arm means that this was an unblinded pain study with a high probability of a large placebo effect. Future more rigorous studies are needed to determine the role of spinal cord stimulation and other devices in the treatment of painful DPN.

5.2. CAN management

The conclusive data obtained by the DCCT and later during EDIC, as well as evidence from several smaller trials strongly support that tight control of blood glucose implemented early in the disease course is very effective for preventing CAN and slowing its progression in individuals with T1D.[2,7] The evidence for people with T2D continues to evolve.[2,3,6,40] Glucose control as part of a multifactorial intervention that targeted hyperglycemia, hypertension, dyslipidemia, and lifestyle, demonstrated a 63 % reduction in the rate of progression to CAN in a small T2D cohort participating in the STENO-2 trial.[83] Additionally, most recent analyses from the ACCORD trial, reported that after adjusting for multiple other risk factors, intensive glucose treatment reduced CAN risk by 16 % and the intensive BP intervention decreased CAN risk by 25 % as compared with the standard intervention group.[84] Considering that CAN was shown conclusively to predict cardiovascular mortality and cardiovascular events, reducing CAN incidence, would thus have a beneficial effect on the cardiovascular outcomes as well. Lifestyle intervention alone may also be beneficial as suggested by a recent pilot study from Germany that reported that high-intensity exercise training can improve indices of CAN over 12 weeks in overweight individuals with T2D.[85] These data are promising, however, we are still in need of larger studies on types and duration of exercise that demonstrate whether improvement in these measures can be sustainable over longer periods time.

Management of orthostatic hypotension involves both behavioral and pharmacological interventions.[2,3,40] Behavioral supportive measures include avoiding abrupt changes in body position, actions that elevate intra-abdominal and intra-thoracic pressures, or medications that would exacerbate hypotension, as well as raising the head of the bed during sleep, small and frequent meals to minimize postprandial hypotension or physical counter-pressure maneuvers such as leg crossing and squatting. Pharmacological therapy includes midodrine and droxidopa, both FDA approved for the management of orthostatic hypotension, or low dose fludrocortisone for use in individuals who fail non-pharmacological interventions but are limited by side effects.[2,3,40]

5.3. Gastroparesis management

Dietary changes and optimizing glucose control with reducing glucose variability may be effective in gastroparesis management.[2] Currently available diabetes technologies including sensor-augmented insulin pumps and/or semi closed-loop insulin pumps to improve glucose fluctuations and hypoglycemia, ultimately enhancing the gastric motility.[74,75] Withdrawal of medications that can slow down the gastric emptying, particularly opioids and marijuana, is also important.[2] To date, metoclopramide, a prokinetic agent, is the only medication

approved by the FDA for the treatment of gastroparesis. However given common extrapyramidal side effects, its use for more than five days is not recommended.[2]

5.4. Knowledge gaps

- Identify personalized prevention strategies using innovative technologies and artificial intelligence methods
- Understand why many if not most people are unable or unwilling to engage in lifestyle interventions
- Validate the cellular and molecular mechanisms by which lifestyle interventions improve nerve function could accelerate development of novel DPN treatments, beyond diet and exercise
- Develop effective disease modifying therapies for DPN, painful DPN, and CAN
- Identify sensitive biomarkers for DPN and pain phenotypes in DPN
- Implement effective providers' education on optimal pain management strategies
- Build quality improvement initiatives to avoid opioids in people with diabetes and DPN
- Build better infrastructure to enable implementation of lifestyle modifications for DPN or CAN
- Find adherence mechanisms to lifestyle strategies
- Understand the role of psychological factors
- Find effective and better tolerated treatments for postural hypotension or true gastroparesis
- Engage Pharma interest to develop new therapeutic agents for DN
- Develop cost-effective and scientifically sound large-scale pragmatic trials for DN in a real-world setting

Summary

We present here the most up-to-date facts on prevalence and incidence of various forms of DN, on potential mechanisms behind the development of DN as well as current management options. We also highlight knowledge gaps, current barriers in clinical practice for optimal DN management, including the need for development of disease modifying agents for management of all DN forms.

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State-of-the-Art Reviews: ALS and Brain Health

Amyotrophic Lateral Sclerosis 1

Emerging insights into the complex genetics and pathophysiology of amyotrophic lateral sclerosis

Stephen A Goutman, Orla Hardiman, Ammar Al-Chalabi, Adriano Chió, Masha G Savelieff, Matthew C Kiernan, Eva L Feldman

Amyotrophic lateral sclerosis is a fatal neurodegenerative disease. The discovery of genes associated with amyotrophic lateral sclerosis, commencing with *SOD1* in 1993, started fairly gradually. Recent advances in genetic technology have led to the rapid identification of multiple new genes associated with the disease, and to a new understanding of oligogenic and polygenic disease risk. The overlap of genes associated with amyotrophic lateral sclerosis with those of other neurodegenerative diseases is shedding light on the phenotypic spectrum of neurodegeneration, leading to a better understanding of genotype–phenotype correlations. A deepening knowledge of the genetic architecture is allowing the characterisation of the molecular steps caused by various mutations that converge on recurrent dysregulated pathways. Of crucial relevance, mutations associated with amyotrophic lateral sclerosis are amenable to novel gene-based therapeutic options, an approach in use for other neurological illnesses. Lastly, the exposome—the summation of lifetime environmental exposures—has emerged as an influential component for amyotrophic lateral sclerosis through the gene–time–environment hypothesis. Our improved understanding of all these aspects will lead to long-awaited therapies and the identification of modifiable risks factors.

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This is the first in a Series of two papers on amyotrophic lateral sclerosis

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Introduction

Amyotrophic lateral sclerosis is a fatal neurodegenerative disease affecting motor neurons in the brain, brainstem, and spinal cord.[1] The name derives from the muscle loss (amyotrophy) and axonal loss in the lateral spinal cord columns (lateral sclerosis) characteristic of the disease. Amyotrophic lateral sclerosis presents with progressive voluntary muscles weakness, which spreads to neighbouring body segments, typically leading to death from respiratory failure within 2–4 years from diagnosis. In addition to motor neuron loss, the major neuropathological findings are intracellular cytoplasmic inclusions of eosinophilic Bunina bodies and ubiquitinated TDP-43. There is also considerable phenotypic heterogeneity in disease presentation, involving cognitive and behavioural changes in up to 60% of patients and frontotemporal dementia in about 15% of patients.

Although there are several known genetic risks for amyotrophic lateral sclerosis, about 85% of cases do not have a single genetic cause;[2] thus, the pathophysiology of the disease remains incompletely understood, which is responsible, in part, for the absence of disease-modifying therapies. Currently, there are only two approved drugs of varying efficacy: riluzole and edaravone. Non-pharmacological multidisciplinary care can, in some cases, improve patient outcomes, including early non-invasive ventilation use and feeding tube insertion before substantial weight loss.[1]

The scarcity of treatments has spurred intense research into the complex genetics of amyotrophic lateral sclerosis and the pathomechanisms linked to known mutations. Improved knowledge of the genetic architecture could unlock the potential of genetic therapies. Additionally, an understanding of the effect of environmental exposures, diet, and lifestyle factors—cumulatively known as the exposome—on the risk of amyotrophic lateral sclerosis is needed to identify modifiable risk factors. This Series paper will highlight the latest advances from the past 5 years pertaining to the complex genetics, pathophysiology, therapeutic development, and exposome science of amyotrophic lateral sclerosis. It is accompanied by a second, more clinically focused, paper on clinical presentation, diagnosis, and prognosis.[1]

Genetic architecture

Amyotrophic lateral sclerosis is conventionally classified as familial or sporadic (panel 1). However, this simple subdivision ignores the complex genetic architecture of the disease (figure 1A–C), which is characterised by monogenic, oligogenic, and polygenic inheritance, gene penetrance, and heritability. Mendelian familial amyotrophic lateral sclerosis accounts for 10–15% of individuals with the disease, albeit with incomplete penetrance in most kindreds.[2,3] In the remaining 85%, large genome-wide association studies (GWAS) might be able to identify rare variants and so-called private mutations—ie, mutations found in a single family that might modulate disease risk and phenotypic presentation.[4]

The proportion of patients with disease that is familial is probably under-reported,[5] because of variation in the definition of familial amyotrophic lateral sclerosis.[6] Consensus criteria for familial amyotrophic lateral sclerosis were introduced nearly a decade ago, and are based on the likelihood that two or more family members carry the same disease-causing variant. Family size is key to this definition; in families with more than 17 members, there is about a 5% chance that two members will be affected, based on the overall lifetime risk of developing

Panel 1: **Glossary of terms**

Familial amyotrophic lateral sclerosis: classically, an inherited case of amyotrophic lateral sclerosis. Clinically defined on the basis of the likelihood that two or more family members carry the same disease-causing mutations.

Sporadic amyotrophic lateral sclerosis: classically, amyotrophic lateral sclerosis occurring in a patient without evidence that the disease was inherited. Nevertheless, shares several risk genes with familial amyotrophic lateral sclerosis.

Monogenic (mendelian) inheritance: the inheritance of a trait (or disease) defined by one gene. Inheritance might be autosomal or sex-linked; dominant (only one mutant allele must be inherited) or recessive (two mutant alleles must be inherited).

Oligogenic inheritance: the inheritance of a trait (or disease) defined by a few genes. This term is frequently used as an intermediate between monogenic and polygenic inheritance.

Polygenic inheritance: the inheritance of a trait (or disease) defined by the cumulative effect of many genes.

Gene penetrance: the proportion of individuals harbouring a mutant gene or gene variant that manifests a trait (or disease). High penetrance means that many individuals carrying the mutation will develop the trait (or disease); low penetrance means that few individuals will develop the trait (or disease).

Lifetime risk: the probability that a specific disease will occur in an individual or population within their lifetime.

Pathogenicity: a characteristic of a genetic variant that increases disease risk in an individual.

Heritability: measures the extent that variation in a trait (or disease) can be attributed to genetic versus environmental variation.

Gene–time–environment hypothesis of amyotrophic lateral sclerosis: posits that genetic predisposition interacts with environmental exposures over time leading to amyotrophic lateral sclerosis.

Multistep model of amyotrophic lateral sclerosis: posits that a series of steps—some genetic, some possibly environmental—leads to amyotrophic lateral sclerosis.

Genetic pleiotropy: the influence of one gene on two or more traits (or diseases).

Phenocopy: a trait (or disease) that has a similar phenotype to that associated with a specific genotype, but without harbouring that genotype.

Endophenotype: a neurobehavioural heritable trait that can be measured to assess genetic susceptibility for psychiatric illnesses.

Proband: an individual in a family with a heritable trait (or disease); generally, the proband is the first individual to seek medical attention for a genetic disease, although kindreds or ancestors might also manifest the disease.

amyotrophic lateral sclerosis (ie, one in 350).[5] Conversely, in a small family, if one parent carries a penetrant mendelian risk gene, the chance that other family members carry the allele is low, leading to an apparent sporadic case of disease.[7] Moreover, some genes for amyotrophic lateral sclerosis also cause frontotemporal dementia or other phenotypes; thus, there is an argument for including the identification of frontotemporal dementia in a kindred in the definition of familial amyotrophic lateral sclerosis, which would bring the percentage of amyotrophic lateral scletrosis cases due to familial disease closer to 20%.[5] Additionally, population studies on the family aggregation of neuropsychiatric conditions within kindreds of people with amyotrophic lateral sclerosis suggest that schizophrenia indicates familial amyotrophic lateral sclerosis, bringing the percentage closer to 30%.[5,8] Validation studies are needed to establish whether to include schizophrenia in kindreds in the familial definition of amyotrophic lateral sclerosis.

Genes associated with amyotrophic lateral sclerosis

Our current knowledge of validated genes for amyotrophic lateral sclerosis derives primarily from ancestral European (ie, Europe, the USA, Canada, and Australia) and Asian populations.[9] Although at least 40 genes have been associated with the disease, four genes account for about 48% of familial and about 5% of sporadic cases within populations of European origin.[10] These genes are *C9orf72*, *SOD1*, *TARDBP* (coding for TDP-43), and *FUS*, and they have lent important insights into the pathophysiology of amyotrophic lateral sclerosis.[11] New genes for amyotrophic lateral sclerosis have been identified in the past 5 years, including *TBK1*, *NEK1*, *CCNF*, *C21orf2* (also known as *CFAP410*), *ANXA11*, *TIA1*, *KIF5A*, *GLT8D1*, *LGALSL*, and *DNAJC7* (table),[2,12] which have highlighted important recurrent pathways and new avenues of research.

Importantly, the genes associated with amyotrophic lateral sclerosis vary in pathogenicity and their susceptibility risk; highly penetrant mutations generally lead to disease (eg, in *TARDBP*, *SOD1*, and *FUS*), whereas some variants associated with amyotrophic lateral sclerosis do not necessarily cause the disease but rather pose a risk of developing the disease (eg, *ANG*, *ATXN2*, and *DCTN1*; table). However, even causative mutations are not fully penetrant, and interactions with the environment modify the risk of developing the disease. Thus, genetic risk represents a continuum from high

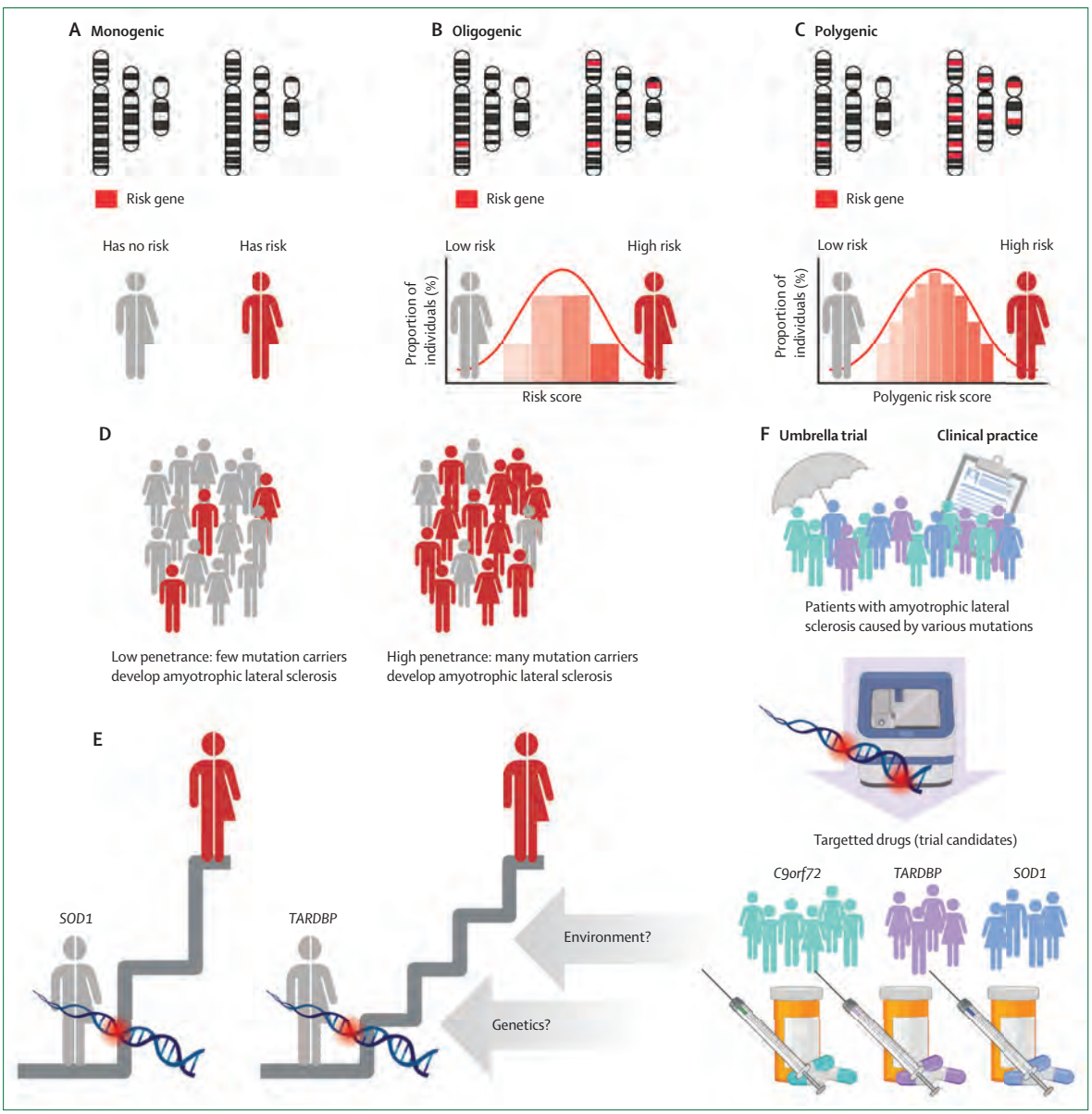

Figure 1: **The genetic architecture of amyotrophic lateral sclerosis**
The genetics of amyotrophic lateral sclerosis is characterised by (A) monogenic, (B) oligogenic, and (C) polygenic risk. Only three representative chromosomes are shown. (D) Genes for amyotrophic lateral sclerosis are not fully penetrant and the pathogenicity of some variants remains uncertain, complicating the full picture. (E) Overlaid over the genetic aspects are environmental factors, because heritability is incomplete. Thus, a multistep model for amyotrophic lateral sclerosis has emerged, which advocates that multiple steps are necessary for onset of the disease. The model posits that mutations with a larger effect require fewer steps for disease onset. Future work is needed to precisely define a step and establish when one has occurred (eg, genetic or environmental factors). (F) Several genetic therapies are under development (ie, in an umbrella trial stratified by molecular profile) and tailored precision treatments are future goals; thus, molecular profiling of patients with amyotrophic lateral sclerosis could become standard clinical practice. The figure was created in BioRender.

(rare mutations) to low (common variants). Even the largest genomics projects might not accurately identify rare intermediate-penetrance variants for amyotrophic lateral sclerosis due to the high lifetime risk and low frequency of pathogenic alleles.

Because precision treatments against specific disease-causing mutations are gaining importance as a therapeutic framework, distinguishing truly pathogenic versus benign variations is essential. Guidelines for interpreting the pathogenicity of variants exist (eg, the criteria by the American College of Medical Genetics and Genomics[13]), and resources such as ClinGen are available.[14] Establishing the pathogenicity of recently or newly identified genes for amyotrophic lateral sclerosis will pivot on segregation analysis, neuropathological signatures (eg, aggregates), or functional investigations in experimental models.[13] Large-scale analyses support a reoriented view of several genes and variants confined

For more on **ClinGen** see https://clinicalgenome.org/

For **data on genetic screening for patients with amyotrophic lateral sclerosis** see http://rshiny.tchpc.tcd.ie/users/dohertm7/journALS/App/

heavily to a single domain. A study of published data identified about 1% as pathogenic or probably pathogenic (111 mutations in 23 genes), 10% as benign or probably benign, and more than 89% as of uncertain significance. Of the pathogenic or probably pathogenic variants, 10% exhibited geographical heterogeneity underlining the population-specific and environmental interactions of variants for amyotrophic lateral sclerosis.

Oligogenic and polygenic models

Because mendelian inheritance only accounts for a proportion of cases, an oligogenic model of amyotrophic lateral sclerosis has emerged (ie, comprising a few risk genes).[15] Although oligogenic inheritance is reported in different populations, further studies are necessary. For example, a UK study of 100 participants with amyotrophic lateral sclerosis found that 13% harboured two pathogenic or probably pathogenic variants, which was associated with earlier disease onset (by 4 years) than in participants with only one pathogenic variant.[16] An Australian multicentre study of individuals with sporadic amyotrophic lateral sclerosis (n=616) found that 7% of participants had two or more variants, which was similarly associated with earlier disease onset than that in participants with no known variants.[15] By contrast, in an Irish population-based cohort study of both familial (n=50) and sporadic (n=394) cases, only 2% of patients harboured two or more known or potential variants for amyotrophic lateral sclerosis.[17]

Polygenic risk is assessed by linkage disequilibrium score testing and mendelian randomisation, which test associations between a particular disease or clinical phenotype with genetic variants. Analysis of GWAS data from 20 806 cases versus 59 804 controls found that amyotrophic lateral sclerosis shared polygenic risk with several traits: positive associations with smoking and moderate physical activity, and negative associations with cognitive performance and education.[18] Mendelian randomisation additionally identified a causal link between hyperlipidaemia and risk for amyotrophic lateral sclerosis. Indeed, a multi-ethnic GWAS identified variants in *ACSL5*, which encodes an enzyme involved in fatty acid β-oxidation and lipid biosynthesis, as a risk factor for amyotrophic lateral sclerosis.[19] Mendelian randomisation also suggested a causal association between genetically determined higher leukocyte count with lower risk of amyotrophic lateral sclerosis.[20]

Heritability

Strong evidence exists of an interplay between inherited and environmental factors, including for patients that carry a highly penetrant mutation.[21] Thus, heritability—ie, the extent that variation in disease risk is attributable to genetic variation—is an important concept in amyotrophic lateral sclerosis. Heritability estimates are population-specific, reflecting the underlying genetic substructure and gene–environment interactions. Assessment of heritability has relied on twin studies (38–78%),[22] large GWAS datasets (18%),[23] and population registers (53%).[3] In the Irish amyotrophic lateral sclerosis registry, the lifetime risk for a first-degree relative of a patient with amyotrophic lateral sclerosis, without known gene mutations associated with the disease, is 0·7% (and 1·4% if the genetic status is unknown).[3] This

	Year of discovery	Inheritance pattern	Familial ALS (%)*	Sporadic ALS (%)*	Function	Associated pathophysiology
ALS genes discovered since 2015						
ANXA11	2017	Autosomal dominant	~1%	~1·7%	Calcium-dependent phospholipid-binding protein; vesicle trafficking	Annexin A11 inclusions; impaired binding to calcyclin; putative LLPS
C21orf2 (also known as *CFAP410*)	2016	Not established	<1%	<1%	DNA damage repair (putative); actin structure	Cytoskeletal defects
CCNF	2016	Autosomal dominant	~1–3·3%	<1%	Component of an E3 ubiquitin ligase complex; cell-cycle regulation	Proteostasis defects
DNAJC7	2019	Not established	<1%	<1%	Heat shock protein co-chaperone	Not established
GLT8D1	2019	Autosomal dominant	<1%	<1%	Glycosyltransferase; unknown cellular function, widely expressed	Not established; localised to Golgi body, suggested role in impaired ganglioside synthesis and addition of O-linked β-N-acetylglucosamine
KIF5A	2018	Autosomal dominant	~0·5–3%	<1%	Kinesin microtubule motor protein	Cytoskeletal or trafficking defects
LGALSL	2015	Not established	<1%	<1%	Not established	Not established
NEK1	2015	Not established	~1–2%	<1%	Serine–threonine kinase; cell-cycle regulation; axonal development or guidance; axonal polarity; DNA damage repair	Putative DNA damage accumulation; protein aggregation
TBK1	2015	Autosomal dominant	~3%	<1%	Serine–threonine kinase; regulates innate immunity, autophagy, and cell-cycle	Autophagy; inflammation
TIA1	2017	Autosomal dominant	~2·2%	<1%	RNA-binding protein	Impaired RNA metabolism; LLPS

(Table continues on next page)

	Year of discovery	Inheritance pattern	Familial ALS (%)*	Sporadic ALS (%)*	Function	Associated pathophysiology
(Continued from previous page)						
ALS genes discovered before 2015						
ALS2	2001	Autosomal recessive	<1%	<1%	GEF	Vesicular trafficking defects
ANG	2006	Risk factor	<1%	<1%	Ribonuclease	Angiogenesis
ATXN2	2010	Autosomal dominant; risk factor	<1%	<1%	RNA-binding protein	Ribostasis defects; putative LLPS
C9orf72	2011	Autosomal dominant	40%	7%	Putative GEF, endosome trafficking, and autophagy regulation; DNA repair	Impaired RNA metabolism; impaired proteostasis or autophagy; intracellular trafficking; nucleocytoplasmic transport defects; LLPS; inflammation
CHCHD10	2014	Autosomal dominant	<1%	<1%	Mitochondrial protein localised to cristae junctions in the intermembrane space	Mitochondrial and bioenergetics dysfunction
CHMP2B	2006	Autosomal dominant	<1%	<1%	ESCRT-III complex component	Impaired proteostasis; vesicular trafficking defects
DCTN1	2003	Autosomal dominant; risk factor	<1%	<1%	Dynactin microtubule motor protein subunit	Axon trafficking defects
ELP3	2009	Not established	<1%	<1%	Histone acetyltransferase subunit of RNA polymerase II elongator complex	Ribostasis defects; cytoskeletal defects
FUS	2009	Autosomal dominant; autosomal recessive	4%	1%	RNA-binding protein; transcription regulation; splicing; RNA localisation and degradation; DNA repair	Ribostasis defects, nucleocytoplasmic transport defects, LLPS
HNRNPA1	2013	Autosomal dominant; risk factor	<1%	<1%	RNA-binding protein	Ribostasis defects, LLPS
HNRNPA2B1	2013	Autosomal dominant; risk factor	<1%	<1%	RNA-binding protein	Ribostasis defects, LLPS
MATR3	2014	Autosomal dominant	<1%	<1%	RNA-binding protein localised to nuclear matrix	Ribostasis defects
NEFH	1994	Autosomal dominant; risk factor	<1%	<1%	Neurofilament protein	Axon trafficking defects
OPTN	2010	Autosomal dominant; autosomal recessive	<1%	<1%	Coiled-coil containing protein regulating membrane trafficking, vesicle trafficking, and transcription activation	Autophagy; inflammation
PFN1	2012	Autosomal dominant	<1%	<1%	Actin-binding protein regulating actin polymerisation	Cytoskeletal or trafficking defects; impaired axon growth
SETX	1998	Autosomal dominant	<1%	<1%	Helicase	Ribostasis defects
SPG11	2010	Autosomal recessive	<1%	<1%	Putative transmembrane protein phosphorylated upon DNA damage	DNA damage
SOD1	1993	Autosomal dominant; autosomal recessive	12%	1–2%	Superoxide anion detoxifying enzyme	Proteostasis defects; oxidative stress; prion-like transmission; inflammation
SQSTM1	2011	Autosomal dominant	~1%	<1%	Ubiquitin-binding autophagy adaptor protein (regulates NF-κB)	Autophagy; inflammation
TARDBP	2008	Autosomal dominant; autosomal recessive	4%	1%	RNA-binding protein; transcription regulation; splicing, RNA localisation and degradation	Ribostasis, proteostasis, and nucleo-cytoplasmic transport defects; LLPS; prion-like transmission; inflammation
TUBA4A	2014	Autosomal dominant	<1%	<1%	Microtubule protein	Cytoskeletal or trafficking defects
UBQLN2	2011	X-linked, autosomal dominant	<1%	<1%	Ubiquitin-like protein (associates with proteasome and ubiquitin ligases)	Proteostasis defects; LLPS
VAPB	2004	Autosomal dominant	<1%	<1%	Plasma and intracellular vesicle membrane protein	Proteostasis defects
VCP	2010	Autosomal dominant	1%	1%	ATPase enzyme regulating protein degradation, intracellular membrane fusion, DNA repair and replication, NF-kB activation, and cell-cycle	Proteostasis defects; inflammation

Genes are listed alphabetically. Adapted from Chia et al.[2] ALS=amyotrophic lateral sclerosis. FTD=frontotemporal dementia. GEF=guanine nucleotide exchange factor. LLPS=liquid-to-liquid phase separation.
*Percentage of familial or sporadic ALS caused by mutations in the particular gene.

Table: **ALS mutations and associated pathophysiology**

lifetime risk equates to a heritability of 36·9% in the non-*C9orf72* population and 52·3% in the overall population. The missing heritability in these populations promotes a focus on epigenomics and environmental contributions. Several studies report changes to the epigenome that are linked to amyotrophic lateral sclerosis (eg, non-coding promoter and enhancer elements, and microRNAs).[24,25] Additionally, the epigenome, as an entity that is reprogrammable through environmental pressures, opens an avenue into exposome science. The gene–time–environment hypothesis of amyotrophic lateral sclerosis proposes a multistep model to account for the environmental effect on disease onset and progression.[21] In European and east Asian populations, the gene–environment interaction promotes disease in up to six steps, with fewer steps in patients harbouring known monogenic, penetrant mutations (eg, *C9orf72*, *SOD1*, *TARDBP*).[26,27] Future work is needed to precisely define a step and establish when one has occurred.[28]

Overall, on the basis of recent progress, we anticipate that genetic testing will become standard practice for profiling patients with amyotrophic lateral sclerosis and will identify known pathogenic mutations in up to 70% of familial and 15% of sporadic cases.[2] This practice will also lead to the discovery of novel mutations. Ultimately, case classification will shift to using mutation status rather than the concepts of familial and sporadic disease. However, genetic testing will require establishing the optimal approach, which will have to contend with the growing number of genes for amyotrophic lateral sclerosis, dealing with polygenic risk, and deciding whether to adopt whole-genome sequencing to address intronic variants that might contribute to the disease.

Genetic overlap with other neurodegenerative diseases

Amyotrophic lateral sclerosis is a clinically heterogeneous disease that extends beyond corticospinal structures.[29,30] Imaging shows thalamic and amygdala involvement as well as disrupted cortical functional networks in motor and extramotor domains (primarily involved in executive function and language),[31–33] whereas spatial domains are relatively preserved. Additionally, social, cognitive, and behavioural changes are common and mirror the behavioural variant of frontotemporal dementia.[34]

Clinical phenotypes of amyotrophic lateral sclerosis are modulated by some genetic variants;[2,35] *SOD1* variants primarily cause motor degeneration, whereas *FUS* mutations are associated with younger age of onset.[2] Additionally, some variants affect progression rate (eg, rapidly progressive SOD1^{A5V}, previously known as A4V). *C9orf72* repeat expansions are most strongly linked with cognitive and behavioural changes;[36] *FUS* and *TARDBP* mutations are also associated with dementia, as can some of the rarer mendelian mutations associated with amyotrophic lateral sclerosis. However, most affected patients with cognitive changes do not carry a known genetic variant. Moreover, several mutations that are risk factors for amyotrophic lateral sclerosis are genetically pleiotropic, and extramotor features of the disease overlap with those of other neurodegenerative diseases (panel 1).[8,37] *C9orf72* repeat expansions are the most common mutations occurring in Huntington's disease phenocopies—patients presenting with Huntington's disease without carrying the most characteristic Huntington's disease-associated mutation: *HTT* repeat expansions.[38] Conversely, in rare instances, patients with frontotemporal dementia or amyotrophic lateral sclerosis can harbour *HTT* repeat expansions concurrent with TDP-43 inclusions (the histopathological hallmark of amyotrophic lateral sclerosis), without defining Huntington's disease characteristics such as neostriatal atrophy.[39]

Although of uncertain clinical significance (because of the presence in individual patients in case reports), mutations in risk genes for amyotrophic lateral sclerosis (ie, *TIA1*, *TBK1*, *SQSTM1*, and *GRN*) are detected in patients with dementia with Lewy bodies, a clinically heterogeneous neurodegenerative disease.[40] A 32-CAG repeat expansion to *ATXN2* has been reported in a patient with both amyotrophic lateral sclerosis and spinocerebellar ataxia type 2;[41] intermediate 32-CAG repeats correlate with amyotrophic lateral sclerosis[42] but reside below the cutoff for spinocerebellar ataxia type 2,[43] suggesting a potential overlap between the two diseases. Additionally, pathogenic mutations to *KIF5A*, known to cause hereditary spastic paraplegia and Charcot-Marie-Tooth disease type 2, are also described in individuals with amyotrophic lateral sclerosis[4] and primary progressive multiple sclerosis,[44] although mutations occur in different *KIF5A* domains in those with hereditary spastic paraplegia compared with those with amyotrophic lateral sclerosis. Thus, the genotype–phenotype relationship among genetic mutations that cause neurodegenerative disease is highly complex. Research is needed to establish how the same genetic mutations diverge on distinct phenotypes and, on the other hand, how mutations to different genes converge on similar phenotypes—eg, mutations to distinct gene domains or overlap in the number of disease-causing repeats. Polygenic risk[18] and environmental influence[21] are possible factors, which are highly relevant to amyotrophic lateral sclerosis.

There is also emerging evidence of disease endophenotypes among family members of those with amyotrophic lateral sclerosis. Cohort studies describe family aggregation of neuropsychiatric disease, primarily psychosis and suicide, in kindreds of probands with amyotrophic lateral sclerosis.[45,46] Although *C9orf72* repeat expansions account for a proportion of aggregation, they are not overrepresented in individuals with typical schizophrenia.[47] Detailed family studies show a non-uniform distribution of neuropsychiatric conditions, which instead cluster in up to 30% of kindreds of patients

with amyotrophic lateral sclerosis,[8] suggesting genetic pleiotropy or oligogenic inheritance. There is also evidence of overlapping polygenic risk between amyotrophic lateral sclerosis and neuropsychiatric disease. Analysis of GWAS datasets from the Project MinE and the Psychiatric Genomics Consortium found 14% polygenic overlap between amyotrophic lateral sclerosis and schizophrenia.[48] Indeed, *GLT8D1*, a recently identified risk gene for amyotrophic lateral sclerosis, is also a schizophrenia risk gene.[49] These observations suggest that the pathogenic process underpinning some forms of amyotrophic lateral sclerosis disrupt specific brain network patterns.[50] This disruption might be mediated by developmental processes that render some brain networks more vulnerable, which manifests in various family members as neuropsychiatric phenotypes or later-onset neurodegeneration; however, further study is required to clarify any potential overlap of amyotrophic lateral sclerosis with neuropsychiatric disease.

Gene-based treatment strategies

The rising number of risk genes for amyotrophic lateral sclerosis, comprising gain-of-function and loss-of-function missense and nonsense mutations and repeat expansions, advocates for gene-based approaches for treatment. Rapid advances have been made in gene-based therapies, which comprise several techniques such as antisense oligonucleotides, RNA interference, gene replacement therapy, and genome editing (panel 2).[52] The optimal approach depends on the mutation and the distribution and amount of the encoded protein. Pathogenic gain-of-function mutations can be targeted by antisense oligonucleotides or RNA interference, but this strategy might be difficult in practice because many genes for amyotrophic lateral sclerosis are widely expressed and the wild-type protein performs essential functions. However, if the mutant protein is overexpressed, this approach could be feasible (eg, targeting mutant SOD1 protein aggregates). Loss-of-function mutations can be addressed by gene replacement therapy, which delivers a functional wild-type copy of the mutant gene. Finally, genome editing, although currently only in experimental stages, could potentially be leveraged to correct both gain-of-function and loss-of-function mutations and offer the ability to specifically target the mutant allele, overcoming the weakness of antisense oligonucleotides and RNA interference. Trial designs, such as umbrella trials, can leverage molecular phenotyping to select trial participants harbouring specific mutations targeted by a candidate gene therapy (figure 1F).

Antisense oligonucleotides

Antisense oligonucleotides are short, synthetic, single strands of oligonucleotides of around 20 chemically modified nucleotides with known in-vivo stability.[58] Because antisense oligonucleotides do not cross the blood–brain barrier, treating neurodegenerative disorders requires CSF delivery (eg, intrathecal or intracerebroventricular). Antisense oligonucleotides bind to target pre-mRNA or mRNA to reduce protein expression through two main mechanisms.[58] Duplex formation marks the target pre-mRNA or mRNA for degradation by endogenous ribonuclease H; alternatively, antisense oligonucleotides interfere with target pre-mRNA or mRNA translation or splicing, or both.[58] In individuals with amyotrophic lateral sclerosis, antisense oligonucleotides can potentially target *C9orf72*, *TARDBP*, *SOD1*, or *FUS* RNA foci. Several clinical trials of antisense oligonucleotides are underway in patients with amyotrophic lateral sclerosis (panel 2).[52,58] The *SOD1*-targeting tofersen (also known as BIIB067) was shown to be safe and to lower CSF SOD1 concentrations in a phase 1/2 trial, particularly in the high-dose group;[59] unfortunately, tofersen did not meet its primary endpoint in a phase 3 trial (NCT02623699). Another phase 3 trial of tofersen is also recruiting presymptomatic carriers of rapidly progressive *SOD1* mutations with blood-based biomarker evidence of disease through elevated neurofilament light chain concentrations (NCT04856982). This trial is following a framework of preventive therapy for highly penetrant *SOD1* mutation carriers. Phase 1 trials of antisense oligonucleotides designed to target *C9orf72* (BIIB078, NCT03626012; IWVE-004, NCT04931862) and *ATXN2* (BIIB105, NCT04494256) expansion repeats are also in the pipeline. Finally, a phase 1–3 trial targeting *FUS* is also ongoing (ION363, jacifusen, NCT04768972).

Pathophysiology

Despite tremendous progress, the pathophysiology of amyotrophic lateral sclerosis remains incompletely understood. However, as our knowledge of the genetic architecture deepens, we are discovering the molecular steps that various mutations take to converge on recurrent dysregulated nervous system pathways. The major shared pathological pathways in individuals with amyotrophic lateral sclerosis include impaired RNA metabolism, altered proteostasis or autophagy, cytoskeletal or trafficking defects, mitochondrial dysfunction, and compromised DNA repair (table; figure 2).[60,61] Among the most common genes for amyotrophic lateral sclerosis, mutant *C9orf72*, *TARDBP*, and *FUS* impair RNA metabolism; *C9orf72* repeat expansions, *TARDBP*, and *SOD1* also induce defects in protein homoeostasis. Mutant *SOD1* also triggers mitochondrial dysfunction and oxidative stress.[60]

Repeat expansions in *C9orf72* lead to mutant protein and haploinsufficiency from the wild-type allele; additionally, RNA transcripts of *C9orf72* expansions aggregate into toxic RNA foci, sequestering RNA-binding proteins and altering RNA metabolism.[60] Aberrant translation of *C9orf72* transcript expansions generates proteotoxic dipeptide repeats—eg, poly proline (P)–arginine (R) repeats (poly[PR]) and poly glycine (G)–arginine (R) repeats (poly[GR]).[60] TDP-43 cytoplasmic inclusions are an almost universal feature of amyotrophic lateral sclerosis, present in about 97% of cases.[62] Although

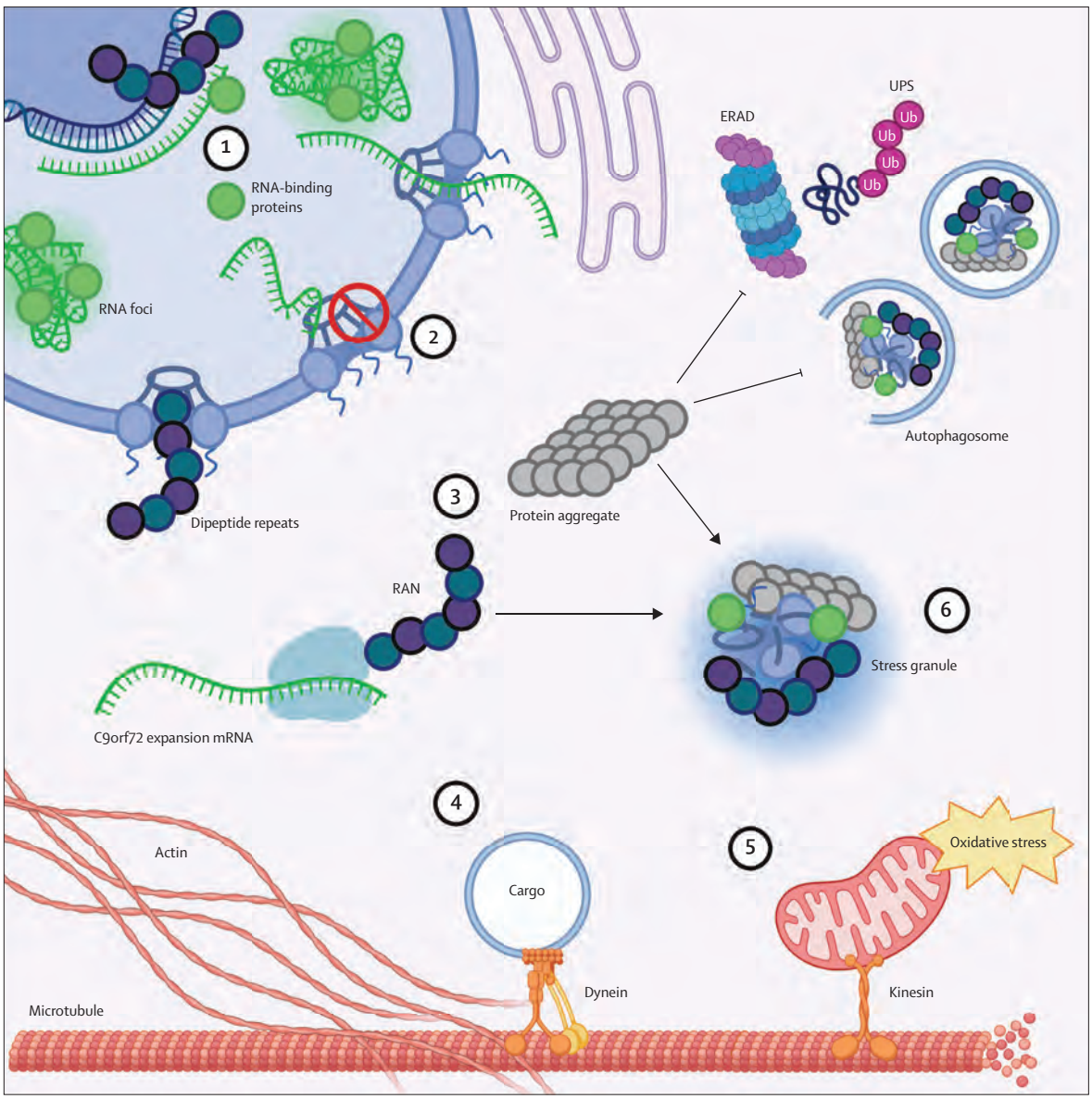

Figure 2: **The pathophysiology of amyotrophic lateral sclerosis**
Pathological pathways centre on impaired RNA metabolism, altered proteostasis or autophagy, cytoskeletal or trafficking defects, mitochondrial dysfunction, and compromised DNA repair. Numbering from top left downwards: (1) Mutant RNA-binding proteins (eg, FUS and TDP-43) disrupt RNA transcription and splicing. C9orf72 repeat expansion RNAs aggregate into RNA foci, sequestering RNA-binding proteins and impairing RNA metabolism. Additionally, haplo-insufficiency from the single remaining wild-type *C9orf72* allele leads to loss-of-function of native C9orf72 protein function, related to multiple mechanisms such as trafficking, autophagy, and DNA repair. (2) Mutant *C9orf72*, *FUS*, and *TARDBP* functionally impair nucleocytoplasmic transport and induce nuclear envelope morphology defects and cytoplasmic inclusions of nucleocytoplasmic transport components (eg, nucleoporins, importins, and RANs). (3) Repeat-associated non-AUG translation of *C9orf72* repeat expansions yields dipeptide repeats, which are toxic through several pathways, including protein aggregation, chromatin alterations, and DNA damage; impaired nucleocytoplasmic transport; and component sequestration. Additional cytoplasmic protein aggregation (eg, TDP-43 and SOD1) induces proteostasis and autophagy defects. Protein aggregates block the ERAD response and UPS, preventing aggregate clearance. Mutations to ubiquitination proteins (eg, CCNF and UBQLN2) additionally dysregulate the UPS. Protein aggregates and RNA-binding proteins also accumulate into stress granules, which become persistent in individuals with amyotrophic lateral sclerosis. Mutations to vesicle-forming proteins (eg, OPTN, VAPB, and VCP) disrupt vesicular transport and distribution, leading to dysfunctional autophagy and proteostasis. (4) Mutations to the tubulin transport machinery (eg, DCTN1, KIF5A, and TUBA4A) and actin (eg, PFN1) induce cytoskeletal or trafficking defects, which impair distribution of vital organelles throughout cells (eg, mitochondria and cargo-laden vesicles). (5) Protein aggregates (eg, TDP-43 and SOD1) and mutations to mitochondrial protein components (eg, CHCHD10) trigger mitochondrial and bioenergetics dysfunction and raise oxidative stress. (6) Liquid-to-liquid phase separation of aggregation-prone proteins (eg, FUS and TDP-43) drives formation of stress granules. This figure was created in BioRender. ERAD=endoplasmic reticulum-associated protein degradation. RAN=GTPase Ras-related nuclear protein. UPS=ubiquitin proteasome system.

***Panel 2*: Gene-based treatment strategies for amyotrophic lateral sclerosis**

RNA interference

Comprises two approaches: small interfering RNA (siRNA) and short hairpin RNA (shRNA).[51] siRNAs are generally duplexes of two strands of about 20 modified nucleotide base pairs long, that can be internalised into cells.[51] The strand of the siRNA complementary to the gene target binds to endoribonuclease Dicer protein and recruits argonaute proteins and target mRNA, generating an RNA-induced silencing complex (RISC). RISC cleaves the target gene mRNA, leading to gene knockdown.[51] shRNAs are hairpin structures of either natural or modified nucleotide bases, which can be delivered by viral vectors.[51] After internalisation into cells, shRNAs are first cleaved by endoribonuclease Dicer protein to remove the hairpin, and then follow the same pathway as siRNAs through RISC.[51]

RNA interference is approved by the US Food and Drug Administration (FDA) to treat hereditary transthyretin amyloidosis.[51] Strategies are being tested in experimental models of amyotrophic lateral sclerosis,[52] but have not yet entered clinical trials.

Gene replacement therapy

This approach uses viruses as vectors to provide patients harbouring loss-of-function mutations a functional copy of a gene.[52] Viruses can cross the brain–blood barrier and might consequently be administered intravenously, which is a considerable advantage. Currently, two vectors are employed, lentivirus, which delivers the replacement gene by mRNA, and adeno-associated virus (AAV), which delivers the replacement gene by cDNA.

Onasemnogene abeparvovec, an AAV9-mediated gene replacement therapy for *SMN1*, is approved by the US FDA. A phase 1, open-label, dose-escalation, clinical trial assessed a single intravenous injection of onasemnogene abeparvovec in children with the *SMN1* mutation (n=15; NCT02122952).[53] The treatment was safe and significantly improved motor function and survival (100% *vs* 8%) compared with historical cohorts. The extremely promising results warranted Fast Track, Breakthrough Therapy, and Priority Review designation by the FDA, culminating in approval for treating patients younger than 2 years and showing the feasibility of this approach for treating neuromuscular disease.

The most common mutations for amyotrophic lateral sclerosis (ie, *C9orf72*, *SOD1*, *TARDBP*, and *FUS*) result in toxic gain-of-function, and are therefore not amenable to gene replacement therapy. However, gene delivery of neurotrophic factors is being investigated in experimental models.[52] Moreover, less frequent but penetrant loss-of-function mutations might become viable candidates as research advances.

Genome-editing technologies

These technologies aim to correct a disease-causing genetic mutation in a patient; several technologies exist, but RNA-guided CRISPR-Cas9 is prominent due to its numerous advantages.[54] The CRISPR RNA guide targets the locus of interest by simple base pairing, which means that a guide can be designed to target any gene of interest.[54] Gene editing can modify chromosomal DNA, but that can have unintended consequences, such as unwanted deletions or chromosomal rearrangements.[55] CRISPR can do more targeted changes than other technologies can (eg, single-base editing),[54] which do not require a double-stranded DNA break. Additionally, CRISPR technology can modulate transcription and edit RNA, expanding its potential applications.[54]

There are no clinical applications of such technologies to date, but they are being tested in experimental models of amyotrophic lateral sclerosis against *SOD1* mutations and *C9orf72* repeat expansions.[52,56,57]

principally nuclear, TDP-43 is mislocalised to the cytoplasm in patients with amyotrophic lateral sclerosis, and is heavily post-translationally modified or truncated, or both.[63] Mislocalised TDP-43 impairs RNA splicing, for instance, of stathmin-2, a protein required for microtubule stability.[64] Diminished stathmin-2 concentrations lead to impaired axonal growth and motor neuron function.[64] Patients with amyotrophic lateral sclerosis and TDP-43 inclusions do not have FUS and SOD1 aggregates;[65] although both TDP-43 and FUS are RNA-binding proteins, which regulate transcription and RNA splicing, localisation, and degradation, there is little overlap between their binding targets.[66]

Of genes discovered in the past 5 years, research suggests involvement in RNA metabolism (*TIA1*), proteostasis or autophagy (*CCNF*, *NEK1*, *TBK1*), and cytoskeletal or trafficking defects (*ANXA11*, *C21orf2*, *KIF5A*).[12,60] The *DNAJC7*-mediated, *GLT8D1*-mediated, and *LGALSL*-mediated mechanisms of neurodegeneration are uncertain. DNAJC7 is a heat shock protein co-chaperone, which could possibly be linked to proteostasis or autophagy.[12] It is hypothesised that GLT8D1, a glycosyltransferase, might impair ganglioside biosynthesis and O-linked β-N-acetylglucosamine modification.[67] The cellular role of galectin-related protein (encoded by *LGALSL*) is completely unknown; however, galectins are galactoside-binding proteins. Therefore, the discovery of novel genes for amyotrophic lateral sclerosis might unlock as yet unknown research avenues and pathological processes.

Nucleocytoplasmic transport defects

Nucleocytoplasmic transport is a highly regulated process, which conveys RNA and protein cargo between the nucleus and cytoplasm.[68] This process is mediated by large, multi-subunit nuclear pore complexes consisting of nucleoporins, which act in concert with cytoplasmic importins (importing protein cargo from cytoplasm to nucleoplasm) and nuclear exportins (exporting protein

cargo from cytoplasm to nucleoplasm).[68] Transport directionality for protein cargo is governed by small GTP-binding nuclear Ran proteins by binding to importins and exportins. Studies report both morphological and functional defects in nucleocytoplasmic transport in animal and cell models of amyotrophic lateral sclerosis, also present in tissue from patients with sporadic or familial disease.[68] Specifically, nucleocytoplasmic transport and nuclear envelope morphology are impaired by *C9orf72* repeat expansions,[69,70] insoluble TDP-43 aggregates,[71] and mutant *FUS*.[72] In patients with amyotrophic lateral sclerosis with mutant *TARDBP* or sporadic disease, abnormal immunoreactivity against nucleoporins, importins, and GTP-binding nuclear Ran proteins is detected in motor cortex and spinal motor neurons, even independent of *C9orf72* repeat expansions.[71–73] Impaired nucleocytoplasmic transport might represent a universal pathology in neurodegenerative diseases, because it is also present in patients with Alzheimer's disease[74] and in those with Huntington's disease.[75]

C9orf72 dipeptide repeat proteins and neurotoxicity

Research is also uncovering the mechanism of toxicity of *C9orf72* repeat expansion-derived dipeptide repeats, which, in addition to impairing nucleocytoplasmic transport, alter chromatin structure.[76] Poly(PR) expression in mouse models produces neuronal loss and gliosis, resulting in motor and memory defects.[76] Poly(PR) binds to DNA and localises with heterochromatin, disrupting the condensed state, leading to aberrant histone methylation and altered gene expression.[76] Furthermore, poly(PR) produces nuclear lamina invaginations and impairs nucleocytoplasmic transport.[76] Poly(PR) also co-localises with heterochromatin in cortex from affected patients with the *C9orf72* repeat expansion.[76] These dipeptide repeats can trigger TDP-43 proteinopathy, forging a link between *C9orf72* repeat expansions and TDP-43 pathology.[77,78] Poly(GR) and Poly(GA) induce cytoplasmic TDP-43 inclusions;[77,78] additionally, poly(GR) sequesters nucleocytoplasmic transport proteins.[77] Encouragingly, an antisense oligonucleotide targeting *C9orf72* GGGGCC repeats reduces poly(GR) burden, TDP-43 pathology, and neurodegeneration.[77] Poly(GR) aggregates co-localise with TDP-43 inclusions in brain tissue from patients with amyotrophic lateral sclerosis, suggesting pathological involvement.[79] Importantly, studies are not fully concordant, possibly due to differing model systems; thus, these findings require further investigation.

Liquid-to-liquid phase separation

In addition to impaired nucleocytoplasmic transport, there is emerging interest in liquid-to-liquid phase separation (LLPS) in amyotrophic lateral sclerosis.[80] LLPS occurs when a homogeneous fluid separates into two liquid phases, forming a dynamic, organelle-like structure lacking a membrane.[80] This process is related to several pathophysiological processes in amyotrophic lateral sclerosis, including nucleocytoplasmic transport, RNA metabolism, DNA repair, protein aggregation, and axonal transport.[80] Stress granules are the most widely studied example of LLPS and form under cellular duress; normally, however, stress granules are dynamic and reversible once the cellular stress subsides. Yet, in amyotrophic lateral sclerosis, stress granule dynamics are impaired, leading to persistent granules of several RNA and protein aggregates, as well as TDP-43 and FUS, which possess so-called low-complexity domains that predispose to aggregation.[80] Arginine-rich *C9orf72* repeat expansion-derived dipeptide repeats undergo LLPS and induce stress granule assembly, impairing dynamics.[81] An in-vitro study on various cell types shows how LLPS occurs during increased cytoplasmic TDP-43 concentrations, even independent of stress granules, recruiting nucleoporins, importins, and GTP-binding nuclear Ran proteins.[82] Although *TARDBP*, *FUS*, and *C9orf72* are the major LLPS-related genes for amyotrophic lateral sclerosis, multiple, less common risk genes are also involved, such as *HNRNPA1*, *HNRNPA2B1*, *TIA1*, and *UBQLN2*.[80] Thus, LLPS is an exciting research direction, because it is shared by several risk genes and is also intertwined with well established pathophysiological mechanisms.

Cell-to-cell prion-like transmission

The low-complexity domains from TDP-43 and FUS contain prion-like motifs.[80] Self-propagating spread of amyloid β and tau is a well studied phenomenon in Alzheimer's disease. Cell-to-cell transmission of aggregation-prone proteins is a developing focus in amyotrophic lateral sclerosis research, including of wild-type and mutant SOD1,[83] dipeptide repeats,[84,85] and TDP-43.[86]

Inflammatory pathways

Dysregulated inflammatory pathways are a recurrent thread in patients with amyotrophic lateral sclerosis.[87] Central and peripheral inflammation are present in mutant *C9orf72*, *SOD1*, and *TARDBP* animal models and in patients with familial amyotrophic lateral sclerosis.[87] This pathophysiology is characterised by immune cell infiltration into the CNS, dysregulated peripheral immune cell counts, induction of an activated immune phenotype, and altered cytokine production (figure 3).[87] Cytotoxic CD8 T cells infiltrate the CNS of mutant *Sod1*^{G93A} mice and selectively destroy motor neurons; genetic ablation of this immune cell population slows motor neurodegeneration.[88] Furthermore, mutant *SOD1*^{G93A} CD8 T cells express increased concentrations of interferon γ, a cytokine linked to amyotrophic lateral sclerosis progression.[88] Patients with amyotrophic lateral sclerosis and loss of C9orf72 activity secondary to *C9orf72* repeat expansions lose the ability to regulate interferon production via the innate immune system (cGAS–STING pathway), leading to type 1 interferon-mediated systemic and CNS inflammation.[89]

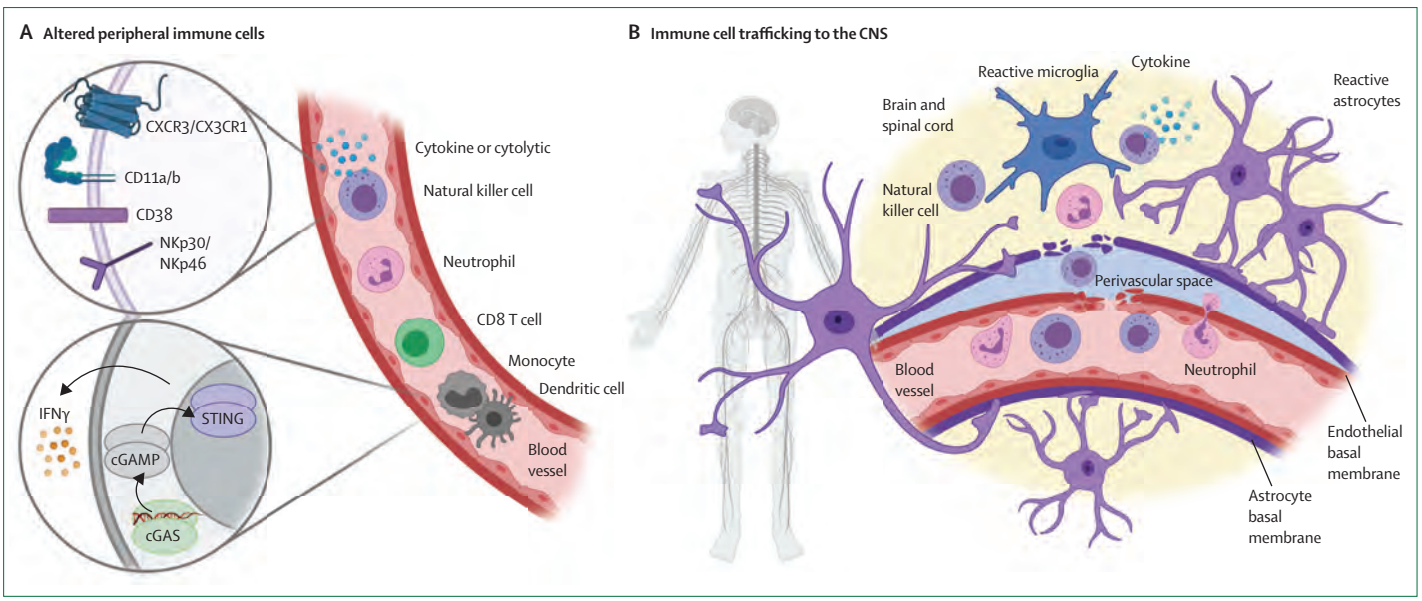

Figure 3: **Inflammatory pathways in amyotrophic lateral sclerosis**
(A) Various peripheral immune cell populations in blood have differential levels of expression in patients with amyotrophic lateral sclerosis, including innate (eg, neutrophils and natural killer cells) and adaptive (CD8 T cells) cells. In patients with amyotrophic lateral sclerosis, circulating natural killer cells over-express surface markers of cytotoxic function (eg, CD38, NKG2D, NKp30, and NKp46) and trafficking (eg, CD11a, CD11b, CXCR3, and CX3CR1). Circulating monocytes and dendritic cells expressing mutant TARDBP and C9orf72 repeat expansions increase IFNγ production. (B) Peripheral immune cells traffic to the CNS in patients with amyotrophic lateral sclerosis (eg, neutrophils and natural killer cells). This figure was created in BioRender. cGAMP= cyclic guanosine monophospate–adenosine monophosphate. cGAS=cGAMP synthase. IFNγ=interferon γ. STING=stimulator of interferon genes protein.

Similar increased interferon production is associated with TDP-43 pathology in cell and animal models of amyotrophic lateral sclerosis.[90] Blocking innate immunity signalling in mutant *Tardbp* mice normalises interferon concentrations, slows disease progression, and lengthens survival.[90] Simultaneous with the increase in cytotoxic immune cells, amyotrophic lateral sclerosis is characterised by decreased concentrations of immune-regulatory and anti-inflammatory Tregs[87] and CD4 T cells.[91] Additionally, less frequent mutations for amyotrophic lateral sclerosis induce inflammation, including those in *OPTN*, *SQSTM1*, *TBK1*, and *VCP*.[87] Thus, inflammation might modulate the progression of amyotrophic lateral sclerosis and survival. In patients with sporadic disease lacking any known genetic causes, the mechanism of immune dysregulation remains uncertain, although it is a characteristic feature.[91,92] Similar to amyotrophic lateral sclerosis with a determined genetic cause, patients with sporadic disease have altered peripheral immunity, induction of an activated immune phenotype, and changes in peripheral cytokine concentrations.[87]

Overall, the emerging research directions in the pathophysiology of amyotrophic lateral sclerosis are nucleocytoplasmic transport, LLPS, and cell-to-cell transmission. These pathways are interrelated and feed into other pathological aspects, such as abnormal ribostasis, proteostasis, and trafficking; mitochondrial dysfunction; DNA repair defects; and inflammation. Future work is needed to generate a holistic view of the pathophysiology of amyotrophic lateral sclerosis.

The exposome and amyotrophic lateral sclerosis

Although burgeoning genetic discoveries have deepened our understanding of the aetiology of amyotrophic lateral sclerosis, most cases are sporadic and do not have a known genetic cause. Moreover, incomplete heritability of known mutations suggests that environmental factors are involved.[21] This consideration has led to the gene–time–environment hypothesis, which suggests that genetic predisposition interacts with environmental exposures over time leading to the development of amyotrophic lateral sclerosis.[21] Thus, the role of an individual's cumulative lifelong exposure (the exposome) on the risk of amyotrophic lateral sclerosis represents a developing research direction to better understand the aetiology and identify modifiable risk factors to prevent disease. Furthermore, the multistep model also supports environmental effects in amyotrophic lateral sclerosis, because a series of steps are required for disease onset,[93] even in individuals with penetrant mutations.[26]

Several studies have investigated the exposome related to amyotrophic lateral sclerosis, which is broad and encompasses exogenous toxicant exposures (eg, environmental pollutants[94]), medical events (eg, brain trauma[94]), and lifestyle factors (eg, intense physical activity[95] and military service[94]). Some exogenous environmental exposures can increase the risk of disease or accelerate disease progression (appendix pp 3–6). A 2017 meta-analysis highlighted some commonly studied links between amyotrophic lateral sclerosis and the

See Online for appendix

environment (odds ratio>1), encompassing lead exposure, heavy metals, pesticides, agricultural chemicals, and solvents.[94] Studies in the past 5 years add to the growing literature of environmental risk factors for amyotrophic lateral sclerosis (appendix pp 3–6).

Importantly, not all exposome studies are concordant (appendix p 2), which might arise from different population sizes or characteristics (eg, location or genetics), exposure duration, adjustment parameters, and methodology (eg, historical estimates *vs* analyte measurements). Thus, despite a considerable body of evidence and identified links between amyotrophic lateral sclerosis and the environment, large prospective cohort studies are needed.[96] These studies will require detailed registries of patient medical information linked to personal data and occupational and residential history with banked biosamples. Studies should evaluate how the exposome modifies disease progression and outcomes,[97] as well as onset risk. Furthermore, environmental risks might not be geographically uniform, necessitating large prospective cohorts across diverse regions, possibly globally. Additionally, geographically distinct populations might also be genetically distinct, which could modify their exposure risk. Although gene–environment interaction studies have been done for single-gene candidates,[95] multiomics studies will be needed that bridge genetics[98] (ie, monogenic, oligogenic, and polygenic risk) with the exposome, to truly comprehend amyotrophic lateral sclerosis risk and progression.

Conclusions and future directions

Much progress has been made towards a more comprehensive picture of amyotrophic lateral sclerosis, aided by a new understanding of the complex genetics behind the disease and the discovery of novel disease mechanisms. The advent of genetic therapies has realised experimental and early clinical trials of genetic therapies. Our growing body of knowledge advocates for a shift in clinical practice, trial design, and emerging research questions. Regarding clinical practice, we anticipate genetic testing will become routine, with the profiling of patients by mutation or genetic or polygenic risk, rather than the previous dichotomisation of familial or sporadic. Genetic profiling should also be leveraged to transform how forthcoming clinical trials are conducted, especially for genetic therapies, by stratifying trial participants by mutation status. This stratification will also ultimately impact management, as we shift gears to a more tailored precision approach. For preventive therapies, improved predictive algorithms will identify individuals most at risk, as the understanding of penetrance and oligogenic or polygenic risk crystallises. This development will tie in with environmental factors; multiomics platforms could generate an integrated perspective on gene–exposome architecture rather than on individual genetic or exposome contributions.

Search strategy and selection criteria

We searched PubMed for English-language articles with the terms: "amyotrophic lateral sclerosis," "ALS," "motor neuron disease," "MND," "GWAS," "genetic," "risk," "oligogenic," "polygenic," "C9orf72," "SOD1," "TARDBP," "FUS," "TBK1," "NEK1," "CCNF," "C21orf2," "ANXA11," "TIA1," "KIF5A," "GLT8D1," "LGALSL," "DNAJC7," "genotype-phenotype," "Alzheimer's disease," "Huntington's disease", "Parkinson's disease," "pathophysiology," "mechanism," "nucleocytoplasmic transport," "liquid-to-liquid phase separation," "RNA splicing," "cell-to-cell transmission," "prion," "immune system," "gene therapy", "antisense oligonucleotide, "RNAi," "AAV9," "CRISPR," "exposure," "environment," "pollutant," "toxin," "metals," and "traffic." The search focused on articles published from Jan 1, 2016, to Oct 15, 2021, although well known and seminal older articles were also considered. We also included articles from the authors' personal reference lists. Articles were selected on the basis of relevance to this Review. Additionally, we searched the ClinicalTrials.gov registry using "amyotrophic lateral sclerosis" with "gene therapy", "antisense oligonucleotide, "RNAi," "small interfering RNA," "short hairpin RNA," "AAV9," and "CRISPR."

Machine learning and big data might play a part in these ambitious goals;[99] for instance, in prioritising genes for amyotrophic lateral sclerosis,[100] particularly in view of the disease's complexity. Emerging questions will continue to refine our picture of amyotrophic lateral sclerosis. Given the phenotypic spectrum of the disease and its overlap with other neurological diseases, and the genetic overlap among various conditions, should we switch to a molecular classification? Could we integrate such a classification with an exposome classification? These questions are not unique to amyotrophic lateral sclerosis, because most neurodegenerative diseases are sporadic. To meet the challenges of this complex disease, future studies will rely on large multicentre cohorts and integrated multiomics platforms, necessitating international collaborative projects. Findings from these collaborative projects will improve our understanding of disease pathogenesis and lead to much needed and long-awaited therapies.

Contributors

All authors contributed to conceptualisation, the writing of the original draft, and review and editing of later drafts.

Declaration of interests

SAG declares consulting fees from Biogen and ITF Pharma, a patent "Methods for treating amyotrophic lateral sclerosis", and participation on a Data Safety Monitoring Board for Watermark. OH declares consulting fees from Novartis, Cytokinetics, Denali Pharma, Stitching Foundation, and La Caixa; payment or honoraria from Biogen; participation on a Data Safety Monitoring Board for Accelsiors and steering committee for Cytokinetics; and is Editor-in-Chief for the journal *Amyotrophic Lateral Sclerosis and Frontotemporal Dementia*. AA-C declares consulting fees from Mitsubishi Tanabe Pharma, Biogen Idec, Cytokinetics, Wave Pharmaceuticals, Apellis, Amylyx, Novartis, and Eli Lilly. AC declares

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Amyotrophic Lateral Sclerosis 2

Recent advances in the diagnosis and prognosis of amyotrophic lateral sclerosis

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This is the second in a Series of two papers on amyotrophic lateral sclerosis

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The diagnosis of amyotrophic lateral sclerosis can be challenging due to its heterogeneity in clinical presentation and overlap with other neurological disorders. Diagnosis early in the disease course can improve outcomes as timely interventions can slow disease progression. An evolving awareness of disease genotypes and phenotypes and new diagnostic criteria, such as the recent Gold Coast criteria, could expedite diagnosis. Improved prognosis, such as that achieved with the survival model from the European Network for the Cure of ALS, could inform the patient and their family about disease course and improve end-of-life planning. Novel staging and scoring systems can help monitor disease progression and might potentially serve as clinical trial outcomes. Lastly, new tools, such as fluid biomarkers, imaging modalities, and neuromuscular electrophysiological measurements, might increase diagnostic and prognostic accuracy.

Introduction

Amyotrophic lateral sclerosis is a neurodegenerative disease characterised by progressive, painless muscle weakness due to motor neuron death in the brain and spinal cord.[1] Weakness begins in facial, tongue, and pharyngeal muscles in individuals with bulbar-onset disease, producing dysarthria and then dysphagia, or in distal upper-limb or lower-limb muscles in people with spinal-onset disease. Most patients with spinal-onset amyotrophic lateral sclerosis present with weakness in one body region that spreads over time to the same region on the contralateral side, as well as to regions rostral and caudal to the initial region of onset. Amyotrophic lateral sclerosis is now understood as a systems disease and there is substantial variation in clinical presentation, including of non-motor symptoms, behavioural changes, and cognitive decline (eg, frontotemporal dementia). Death generally occurs within 2–4 years of diagnosis from respiratory failure, although more slowly progressive forms of the illness occur in a small proportion of patients.

Diagnosis can be challenging, and the process has remained essentially unchanged in clinical practice in the past decade. No test or tool has replaced clinical history and examination for confirming diagnosis, even with the increased adoption of genetic testing. The typical median time between initial symptoms and a definitive diagnosis is 10–16 months,[2] due to the rarity of the disease, incomplete recognition of symptoms, and lack of early and appropriate specialist involvement.[3] Additionally, prognosis remains suboptimal because the determinants of disease progression are not fully known.

To facilitate earlier diagnosis and improve prognosis, research is ongoing into new diagnostic criteria and scoring systems, as well as emerging diagnostic and prognostic fluid biomarkers, imaging modalities, and electrophysiological measurements. This Series paper will highlight these emerging discoveries and focus on the most recent advances in diagnosis and prognosis within the past 5 years. This paper is accompanied by a research-focused Series paper,[4] which provides an update on the complex genetics, pathophysiology, therapeutic development, and exposome science of amyotrophic lateral sclerosis.

Epidemiology

Amyotrophic lateral sclerosis incidence and prevalence varies across the globe, and estimates are based on different data sources. The availability of registries in some countries enables more accurate calculations of incidence and prevalence, advocating for the need of population-based registries worldwide (panel 1). A recent meta-analysis of 110 incidence studies and 58 prevalence studies estimated an average global incidence of 1·59 (95% CI 1·39–1·81) and a prevalence of 4·42 (3·92–4·96) per 100 000 individuals.[11] Ancestral background and biological sex are linked to amyotrophic lateral sclerosis rates in an age-dependent manner.[12] Despite male predominance, heritability is greater in women, with the highest concordance in female–female parent–offspring pairs.[9] Male carriers of the *C9orf72* repeat expansion develop amyotrophic lateral sclerosis at an earlier age (by about 2 years) than female carriers do.[13] Thus, an intricate interplay between age, sex, and complex genetics drives the risk of amyotrophic lateral sclerosis.[12] These sex-dependent differences urge consideration of sex in preclinical and clinical research (to understand the basis of these effects), and in clinical trials for developing therapeutics.

Clinical presentation

Amyotrophic lateral sclerosis was historically considered a fairly uniform disease of progressive, painless weakness of voluntary muscles.[1] However, studies have redefined it as a complex disorder with considerable heterogeneity in clinical presentation, site of disease onset, and distribution

of upper and lower motor neuron signs (figure A, table 1). Recognition of these multiple heterogeneous presentations can facilitate early diagnosis and inform prognosis.[16] The Australian National Motor Neuron Disease (1677 patients with amyotrophic lateral sclerosis)[14] and Italian Piemonte and Valle d'Aosta registries (2839 patients with amyotrophic lateral sclerosis)[12,15] have documented this heterogeneity in presentations, which also correlate with median survival (figure B). Patients with bulbar-onset disease are at a greater risk of frontotemporal dementia than are patients with other presentations.[12] Additionally, less common presentations exist (eg, hemiplegic; table 1).[17] Furthermore, presentations can correlate with the timing of some treatments. In the Australian registry, feeding tube placement secondary to dysphagia occurs earlier in patients with bulbar-onset disease than in those with spinal-onset disease,[14] as was also reported in a European tertiary care cohort of people with amyotrophic lateral sclerosis.[18]

Thus, classification is based on clinical criteria, such as site of disease onset and distribution of upper and lower motor neuron signs.[16] Additional relevant clinical variables, such as age, sex, family history, progression rate, genetic profile, and presence of cognitive impairment and other non-motor symptoms, aid disease classification and can provide prognostic guidance.[19]

Non-motor symptoms

The concept of amyotrophic lateral sclerosis as a pure motor disease is now abandoned. In fact, it has been known for decades that executive dysfunction occurs in 50% and frontotemporal dementia in 15% of patients. Executive dysfunction is evaluated by a suite of neuropsychological tests (table 2),[20] and frontotemporal dementia in patients with amyotrophic lateral sclerosis is diagnosed by the revised Strong criteria.[25] The most characteristic cognitive changes in amyotrophic lateral sclerosis include impaired language function[22] and executive function deficits involving working memory, inhibition, set shifting, and fluency, whereas memory and spatial function are typically spared.[23] Patients also experience cognitive decline and neuropsychiatric symptoms, including apathy, disinhibition, irritability, loss of sympathy or empathy, perseveration, reduced concern for hygiene, and changes in eating habits. Similar clinical patterns are present in patients with frontotemporal dementia.[23] Additionally, many patients with amyotrophic lateral sclerosis have anxiety, depression, and sleep disorders.[26]

Executive dysfunction is a negative prognostic indicator and, if present, tends to worsen over time.[27] Cognitive impairment can later manifest even in patients who seem to be cognitively spared at diagnosis[27] and might be partly related to the worsening of motor function.[23] Thus, there is a growing need to incorporate an evaluation of cognitive function into the diagnosis and ongoing management of amyotrophic lateral sclerosis. These behavioural changes can also frustrate family members or caregivers and prevent the patient from accepting medical recommendations, emphasising the importance of addressing care preferences early in the disease.[28] These cognitive and behavioural symptoms can be accompanied by structural changes in extramotor domains of the brain.

The influence of genes on clinical phenotype

The discovery of mutant *SOD1* in a subset of patients with amyotrophic lateral sclerosis in 1993 suggested a potential genetic aetiology, which enhanced our understanding of risk factors and pathophysiology.[29] This possibility was strengthened in 2011 by the discovery of *C9orf72* repeat expansions in a larger proportion of patients, both with and without a family history of amyotrophic lateral sclerosis.[30] The genetic architecture of amyotrophic lateral sclerosis and nuances of familial versus sporadic disease are fully detailed in the accompanying research-focused Series paper.[4] More than 40 genes have been identified to date, which together

Panel 1: Global incidence

Standardised incidence

The standardised incidence of amyotrophic lateral sclerosis is similar among European populations (1·89 per 100 000 in Northern Europe, 1·71 per 100 000 in Western Europe, and 1·75 per 100 000 Southern Europe), and is higher than the standardised incidence in South American populations (1·59 per 100 000) and Asian populations (0·83 per 100 000 in East Asia, 0·94 per 100 000 in West Asia, and 0·73 per 100 000 in South Asia).[5] Standardised rates are highest in Oceanian populations (2·56 per 100 000) and north African populations (2·03 per 100 000).[5] There are no data on incidence for sub-Saharan Africa. Standardised incidence in North American populations is 1·79 per 100 000.[5]

Incidence by age

Incidence peaks between the ages of 60 and 75 years.[6] In the USA, the National ALS Registry, which is coordinated by the Centers for Disease Control and Prevention, reports a peak prevalence between the ages of 60 and 79 years.[7] Although global burden of amyotrophic lateral sclerosis is anticipated to increase due to the ageing of populations,[8] the Irish ALS Register did not observe a rise in incidence between 1995 and 2017.[9]

Incidence by sex

Sex plays a part in amyotrophic lateral sclerosis incidence and prevalence. In the Southeast England ALS Registry, the male-to-female ratio in incidence at younger ages (25–34 years) was 3·7, which narrows to 1·2 in the 65–74-year age group, but then grows slightly to 1·4 for those aged 75 years or older.[10] Sex differences in the prevalence of amyotrophic lateral sclerosis are present in the US National ALS Registry, which reports that 60% of people living with amyotrophic lateral sclerosis are male.[7] The Irish ALS Register reports a lifetime risk of 1:347 for males and 1:436 for females.[9]

account for about 15% of cases. Thus, genetic testing is a growing, albeit non-uniform, component of disease management. As the cost of genetic profiling drops, we anticipate earlier and broader adoption. First, detection of known pathogenic variants could complement and bolster diagnoses achieved by diagnostic criteria. Second, although most mutations converge on a typical phenotype, there are important prognostic implications for some mutant genes linked to unique features (table 3). For example, *ALS2*, *DCTN1*, *MATR3*, *OPTN*, and *SETX* mutations are associated with slower clinical trajectories than those in patients with other, more common, types of amyotrophic lateral sclerosis, information that is valuable to patients and their families. Furthermore, routine genetic profiling could move past the inadequate stratification of patients into sporadic or familial disease. Additionally, genetic profiling promotes precision medicine[33] and clinical trial stratification for targeted therapeutics (eg, gene therapies). Therefore, a genetic profile could potentially facilitate diagnosis, prognosis, and treatment for patients harbouring genetic mutations.

Diagnosis

Diagnostic criteria date back to the original El Escorial and later the revised El Escorial (Airlie House) and Awaji criteria. They rate the degree of diagnostic "certainty by clinical assessment alone" from possible to probable to definite amyotrophic lateral sclerosis, on the basis of the number of affected segments combined with clinical or electrophysiological findings, or both.[34–36] The El Escorial classification provides prognostic information because, for instance, definite amyotrophic lateral sclerosis progresses faster.[19] Although approaches that score the certainty of diagnosis solely by clinical assessment are reasonable (ie, possible amyotrophic lateral sclerosis), they can delay diagnosis and confuse patients, their families, and clinicians, who misinterpret these terms as meaning the diagnosis is improbable or incorrect.[37] In reality, nearly all patients diagnosed as having possible amyotrophic lateral sclerosis progress and ultimately die from the disease.

Emerging diagnostic criteria

To address these limitations, an international consensus group reconsidered criteria to improve the diagnostic process in the early stages of disease when clinical symptoms are minimal.[38] Recognising the broad heterogeneity in presentations, the Gold Coast criteria define amyotrophic lateral sclerosis by: (1) progressive motor impairment, documented by history or repeated clinical assessment, preceded by normal motor function; (2) upper and lower motor neuron dysfunction in at least one body region, or lower motor neuron dysfunction in at least two body regions; and (3) investigative findings that exclude alternative diseases.

Adoption of these simplified criteria abandons the previous diagnostic categories of possible, probable, and definite. The advent of these new criteria facilitates early and definitive diagnosis. An Australian study found that the diagnostic sensitivity of Gold Coast criteria (92%) was maintained irrespective of functional status, disease duration, or onset site, and was generally similar to that of the revised El Escorial (88·6%) and Awaji criteria (90·3%); however, the Gold Coast criteria were more sensitive and specific for identifying progressive muscular atrophy and for ruling out primary lateral sclerosis as a form of ALS, the latter of which meets the definition of possible amyotrophic lateral sclerosis in the revised El Escorial and Awaji criteria.[39] This finding was validated in a five-centre European study, which found consistent and improved sensitivity of the Gold Coast criteria, due to greater sensitivity for identifying progressive muscular atrophy.[40] Lastly, a Chinese study corroborated the greater sensitivity of the Gold Coast against the revised El Escorial and Awaji criteria,[41] suggesting that its diagnostic utility would be maintained in racially diverse populations. Importantly, the Gold Coast criteria were marginally less specific, which clinicians should bear in mind as they monitor their patients' disease course. However, overall, we anticipate that the new Gold Coast criteria will facilitate diagnosis and dispel uncertainty and confusion for patients and their families.

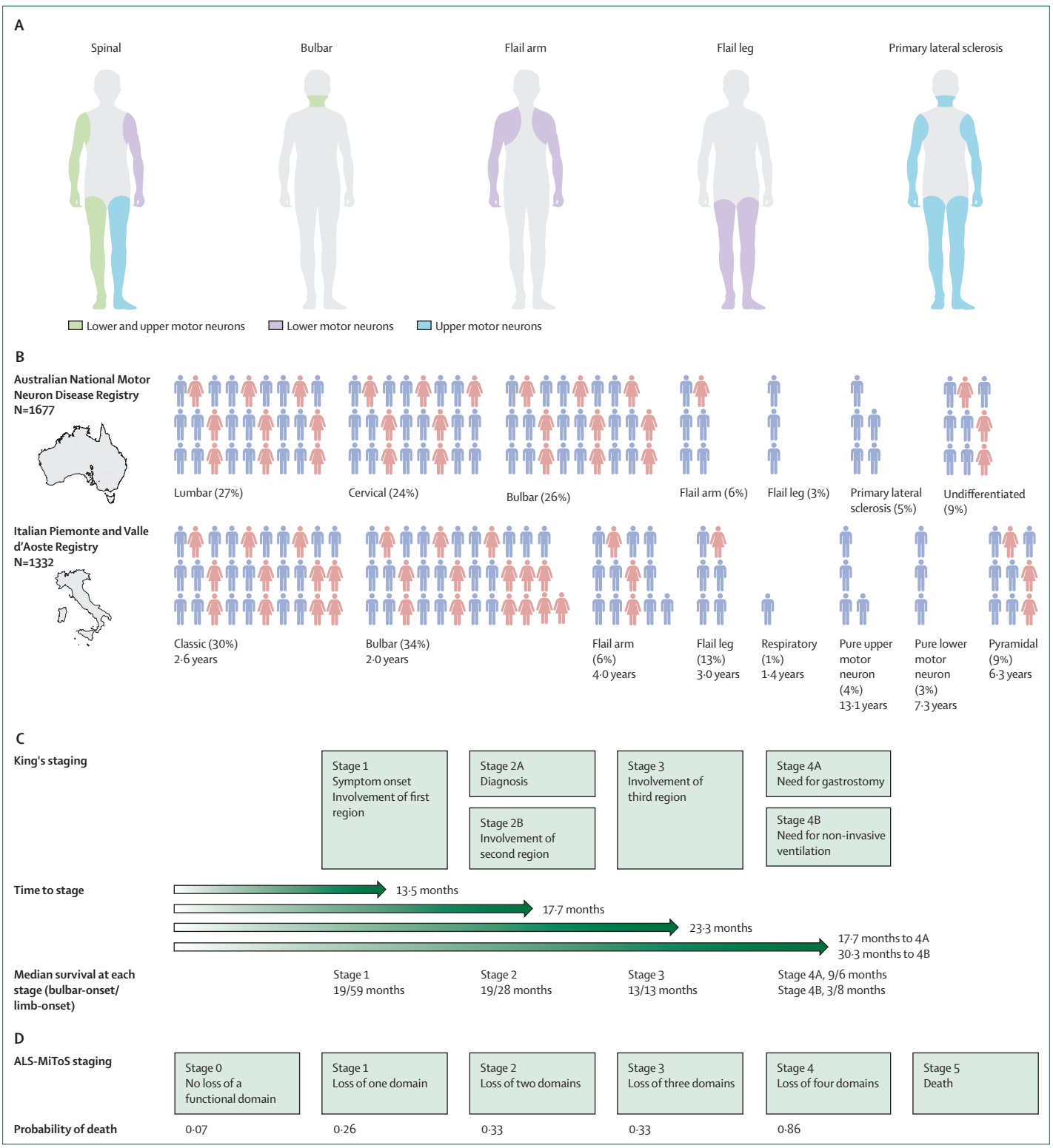
Clinical overlap with other neurodegenerative disorders

Amyotrophic lateral sclerosis is a multifaceted disease with remarkable heterogeneity of motor and non-motor features. This complexity contributes, in part, to the

Figure: **Heterogeneity in initial presentation and staging of amyotrophic lateral sclerosis**

(A) Involvement of motor neuron dysfunction at initial presentation in different presentations. Spinal-onset amyotrophic lateral sclerosis involves variable motor neuron dysfunction in a combination of limbs. Bulbar-onset amyotrophic lateral sclerosis involves motor neuron dysfunction in bulbar muscles (eg, facial, tongue, and pharyngeal). Flail-arm amyotrophic lateral sclerosis involves lower motor neuron dysfunction in the arms, although mild dysfunction of the upper motor neurons can occur in the legs too. Flail-leg amyotrophic lateral sclerosis often involves asymmetric lower motor neuron dysfunction in the legs. Primary lateral sclerosis mainly involves upper motor neuron dysfunction in the arms and legs or bulbar region, although restricted dysfunction of lower motor neurons can develop in the later disease stages or become more widespread if it transitions to amyotrophic lateral sclerosis, often within 4·5 years of symptom onset. (B) Distribution of amyotrophic lateral sclerosis presentations in the Australian National Motor Neuron Disease Registry (N=1677; each human figure represents one percentage point)[14] and distribution of amyotrophic lateral sclerosis presentations in the Italian Piemonte and Valle d'Aosta Registry (N=1332; each human figure represents one percentage point);[12,15] median survival in years is presented under each presentation. Note that the two registries use slightly different classification systems. (C) King's staging with four stages indicated (1, 2A/B, 3, 4A/B; blue); time to progress to stages and median survival at each stage (in months) for both bulbar-onset and limb-onset forms are also annotated. (D) ALS-MiToS staging with six stages indicated (0, 1, 2, 3, 4, 5; orange); staging is based on four functional domains from the ALSFRS-R: (i) movement (walking or self-care; ALSFRS-R question 6 or 8); (ii) swallowing (ALSFRS-R question 3); (iii) communicating (ALSFRS-R questions 1 and 4), and (iv) breathing (ALSFRS-R question 10 or 12). Intensifying colour indicates progression along stages for both King's and ALS-MiToS. ALS-MiToS=Amyotrophic Lateral Sclerosis Milano-Torino Staging. ALSFRS-R=amyotrophic lateral sclerosis functional rating score–revised.

difficulty of diagnosing the disease, which is rendered more challenging by its clinical overlap with other more common neurological and neuromuscular diseases (table 3). Additionally, *C9orf72* repeat expansions, the most common mutations associated with amyotrophic lateral sclerosis in populations of European descent, are



	Affected motor neurons	Progression	Additional features
Classic bulbar onset	Upper and lower motor neurons	Begins with dysarthria, then dysphagia, then spreads to the limbs	Might have unexplained weight loss; typically will benefit from earlier feeding tube placement *vs* those with limb-onset disease
Pseudobulbar palsy	Upper motor neurons	Prominent bulbar features that slowly spread to the limbs	Affects more females than males; longer survival than for other phenotypes; pseudobulbar effect
Progressive bulbar palsy	Lower motor neurons	Prominent bulbar features, which spread to limbs	Patients progress to ALS, although median survival can be longer than for those with classic bulbar-onset disease
Classic cervical onset	Upper and lower motor neurons	Typically, hand weakness that spreads to bulbar and lumbar regions	Trouble with hand dexterity or grip; split hand a prominent symptom
Classic lumbar onset	Upper and lower motor neurons	Typically, foot drop with weakness spreading to cervical and bulbar regions	Trouble with gait and a tendency to trip
Flail arm	Lower motor neurons in upper extremities; upper motor neurons in lower extremities	Symmetrical weakness in proximal upper limb (more so than in distal upper limb) that eventually spreads	Slower progression than for other presentations; affects more males than females
Flail leg	Lower motor neurons in lower extremities	Symmetrical lower-limb weakness	Lower motor neuron weakness usually, but upper motor neuron signs will often develop
Primary lateral sclerosis	Upper motor neurons	Might begin in any region and spread over time; if lower motor neuron signs develop within 4·5 years, diagnosis is amyotrophic lateral sclerosis instead	Normal life expectancy; exclude hereditary spastic paraparesis if the disease involves symmetrical lower-limb signs
Progressive muscular atrophy	Lower motor neurons	Might begin in any region and spread over time; if upper motor neuron signs develop within 4·5 years, diagnosis is amyotrophic lateral sclerosis instead	Male predominance; absence of upper motor neuron signs
Respiratory	Upper and lower motor neurons	Limb weakness follows respiratory involvement	Short survival
Hemiplegic	Unilateral upper motor neurons affected more than lower motor neurons	Often begins in leg and spreads to ipsilateral arm	Patients can have protracted disease course
Cachexia	Upper and lower motor neurons	Unexplained weight loss preceding presentation with classic limb-onset amyotrophic lateral sclerosis	Rapidly progressing disease

Classic amyotrophic lateral sclerosis refers to disease with combined upper and lower motor neuron dysfunction in the onset segment, which progressively spreads from region to region. Non-classical or atypical forms refer to phenotypes with predominance of upper or lower motor neuron dysfunction in a segment.

Table 1: **Clinical spectrum of amyotrophic lateral sclerosis**

among the strongest determinants of frontotemporal dementia. However, the clinical phenotypes present as a continuum from amyotrophic lateral sclerosis, to amyotrophic lateral sclerosis–frontotemporal dementia, to frontotemporal dementia, sometimes even within the same pedigree. Further complicating the situation, *C9orf72* repeat expansions are associated with movement disorders such as parkinsonism, essential tremor, and myoclonus,[42] in addition to cognitive impairment. The disease might present as atypical amyotrophic lateral sclerosis, which could contribute to a more difficult and lengthy diagnosis process. Therefore, awareness of additional manifestations of an amyotrophic lateral sclerosis mutation could facilitate early diagnosis. Additionally, *C9orf72* repeat expansions are the most frequent cause of Huntington's disease phenocopies (patients with the classic Huntington's disease phenotype but lacking characteristic *HTT* repeat expansions and inclusions).[43] Conversely, patients with amyotrophic lateral sclerosis might harbour *HTT* repeat expansions simultaneously with TDP-43 inclusions,[44] underscoring the complexity of genotype–phenotype associations. Understanding the spectrum of clinical presentations and overlap arising from mutations will expedite diagnosis. Finally, amyotrophic lateral sclerosis aggregates with neuropsychiatric illnesses, such as psychosis and suicidal ideation.[45] Amyotrophic lateral sclerosis and schizophrenia share a risk gene, *GLT8D1*,[46] as well as polygenic risk.[47] Therefore, in the family history of a patient with amyotrophic lateral sclerosis, it is not uncommon to find members with other neurodegenerative or psychiatric diseases.

Prognosis

Nearly every patient with amyotrophic lateral sclerosis asks a series of questions, including on the amount of time the patient has left to live. Access to reliable prognostic methods allows clinicians to give patients and

their families evidence-based answers. Despite important limitations,[48] clinicians and researchers currently rely on the revised functional rating score for amyotrophic lateral sclerosis (ALSFRS-R),[49] a scoring system that monitors the rate of disease progression. ALSFRS-R changes do not necessarily reflect improvement in disease; for instance, symptom management (eg, treating sialorrhoea) or medical decisions (eg, discontinuing non-invasive ventilation) affect the ALSFRS-R, even though there is no change in the patient's underlying disease. The multidimensionality of the ALSFRS-R limits its clinical usefulness, especially in clinical trials,[50] as well as its low responsiveness during plateau periods, which makes it hard to discern treatment effects in trials.[51] Clinicians also derive prognostic value from respiratory tests, such as forced vital capacity;[52] indeed, forced vital capacity is a predictive parameter in the European Network for the Cure of Amyotrophic Lateral Sclerosis (ENCALS) model.

Emerging prognostic methods

Scoring systems

The self-reported Rasch-Built Overall Amyotrophic Lateral Sclerosis Disability Scale (ROADS) was developed to overcome ALSFRS-R limitations by ensuring that symptom management or medical decisions do not ameliorate the disease score, which instead reflects true changes in disease progression.[53] Compared with the ALSFRS-R, the 28-question ROADS better captures functional changes because it accounts for function at the upper and lower ranges of disability. Additionally, the scale has high test–retest reliability and is designed for a 1-point change to represent the same change in function across the whole score spectrum. This new scale is not used in clinical practice as it requires validation; thus, whether ROADS will supplant or complement the ALSFRS-R requires further study.

Staging systems

A staging system identifies where an individual is in the disease course, thereby improving counselling and resource allocation. Staging systems are also useful in clinical trials to establish whether an intervention reduces advancement from less-severe to more-severe disease stages. The King's staging defines four progressive stages linked to survival (figure C) and can help in prognostication.[18] King's staging shows the different progression of patients as well. For instance, patients with bulbar onset require gastrostomy (stage 4A) before non-invasive ventilation (stage 4B), whereas non-invasive ventilation is usually needed before gastrostomy in patients with limb-onset disease. The Milano-Torino Staging for Amyotrophic Lateral Sclerosis (ALS-MiToS) places patients at one of six stages on the basis of select ALSFRS-R responses in four functional domains.[54] In ALS-MiToS, staging depends on the number of functional domains lost (figure D); stage 0 is no loss, a patient at stage 1 will have lost one functional domain, a patient at stage 2 will have lost two functional domains, and so on, with stage 5 representing death. Patients probably progress from stage to stage, as opposed to skipping stages, with increasing probability of death with each stage. The King's and ALS-MiToS systems are complementary; the King's staging system is superior for staging earlier in the disease course, whereas ALS-MiToS outperforms later in the disease course.[55] Although none of these instruments is used in clinical practice, both staging systems describe progression and survival, albeit with limitations,[56] and could be useful in clinical trials.[57]

ENCALS survival model

The ENCALS survival model is a recently developed approach for predicting survival in patients with amyotrophic lateral sclerosis, with non-survival defined as time to non-invasive ventilation for more than

	Signs and symptoms	Neuropsychological tests
Executive function		
Working memory	Unable to temporarily process, store, and use information with conscious awareness[20]	Digit span subtest (Wechsler Adult Intelligence Scale, fourth edition); Corsi block-tapping test or spatial span (Wechsler Memory Scale, third edition)
Inhibition	Inability to ignore stimuli, which can result in impulsive behaviour	Flanker task, continuous performance test, antisaccade task (NIH EXAMINER); Stroop test (Delis-Kaplan Executive Function System)
Set shifting	Inability to change attention and behaviour for different circumstances and demands,[20] causing rigid thinking and impairments in multitasking	Trail-making test (Delis-Kaplan Executive Function System); Wisconsin card sorting; set shifting test (NIH EXAMINER)
Fluency	Disorganised thoughts or inability to initiate tasks	Verbal and design fluency tests; category fluency
Language function		
Language impairment	Impairment in word naming, spelling, and grammatical processing	Psycholinguistic Assessments of Language Processing in Aphasia
Behaviour		
Apathy	Passivity and low levels of spontaneity and initiative, loss of interest and motivation for previously rewarding activities, and diminished social interest[21]	Beaumont Behavioural Inventory
Disinhibition	Impulsivity, low self-restraint, socially inappropriate behaviours, irritability, verbal or physical aggression, disinhibited emotional display, changes in sexual behaviour, and decline in personal hygiene[21]	Beaumont Behavioural Inventory
Loss of sympathy or empathy	Diminished response and understanding of the needs and feelings of others, reduced inter-relatedness and personal warmth, and emotional detachment[21]	Beaumont Behavioural Inventory
Perseveration and stereotyped or obsessive–compulsive behaviours	Simple repetitive movements, more complex ritualistic behaviours, and stereotypy of speech[21]	Beaumont Behavioural Inventory
Eating behaviours	Changed food preferences, and increased smoking, binge eating, hyperorality, and oral exploration of inedible items[21]	Beaumont Behavioural Inventory

Changes are shown along with associated symptoms and testing strategies.[20-24] NIH=National Institutes of Health.

Table 2: **Cognitive impairment and psychiatric comorbidities in patients with amyotrophic lateral sclerosis**

	Inheritance pattern	Proportion of familial cases	Proportion of sporadic cases	Associated clinical phenotype	Overlap with other diseases
ALS2	Autosomal recessive	<1%	<1%	Slowly progressive; infantile and juvenile forms mainly affect upper motor neurons; primary lateral sclerosis	Hereditary spastic paraparesis
ANG	Autosomal dominant; presence is a risk factor	<1%	<1%	Typical; bulbar-onset tendency; frontotemporal dementia	No overlap
ANXA11	Autosomal dominant	~1%	~1·7%	Not determined	Autoimmune diseases, sarcoidosis
ATXN2	Autosomal dominant; presence is a risk factor	<1%	<1%	Typical; early onset; phenotype modifer	Spinocerebellar ataxia
C9orf72	Autosomal dominant	40%	7%	Typical; frontotemporal dementia	Huntington's disease phenocopy, parkinsonism, essential tremor, myoclonus
C21orf2	Not determined	<1%	<1%	Typical; frontotemporal dementia	No overlap
CCNF	Autosomal dominant	~1·0–3·3%	<1%	Typical; frontotemporal dementia; primary lateral sclerosis	No overlap
CHCHD10	Autosomal dominant	<1%	<1%	Typical; frontotemporal dementia	Cerebellar ataxia, myopathy
CHMP2B	Autosomal dominant	<1%	<1%	Typical; progressive muscular atrophy	Frontotemporal dementia
DCTN1	Autosomal dominant; presence is a risk factor	<1%	<1%	Slowly progressive; juvenile	Perry syndrome (parkinsonism)
DNAJC7	Not determined	<1%	<1%	Not determined	No overlap
ELP3	Allelic	<1%	<1%	Typical	No overlap
FUS	Autosomal dominant or recessive, depending on variant; de novo	4%	1%	Typical or atypical; frontotemporal dementia; dementia; juvenile or adult onset	Essential tremor*
GLT8D1	Autosomal dominant	<1%	<1%	Typical; shorter or longer survival than typical ALS, depending on variant	Schizophrenia
GRN	Autosomal dominant; modifier	<1%	<1%	Earlier onset; shorter survival than typical ALS	Frontotemporal dementia, frontotemporal lobar degeneration, dementia with Lewy bodies*
HNRNPA1	Autosomal dominant; de novo; presence is a risk factor	<1%	<1%	Typical; cognitive impairment	Inclusion body myopathy
HNRNPA2B1	Autosomal dominant; presence is a risk factor	<1%	<1%	Typical; cognitive impairment	Inclusion body myopathy
KIF5A	Autosomal dominant	~0·5–3%	<1%	Early onset; longer survival than typical ALS	Charcot-Marie-Tooth disease type 2, primary progressive multiple sclerosis phenocopy,* hereditary spastic paraplegia

(Table 3 continues on next page)

23 h per day, tracheostomy, or death.[19] The model used data from 11 475 patients with amyotrophic lateral sclerosis from 14 centres at several European sites, and included 16 clinical predictors, of which only eight reached statistical significance (p<0·001), including age at onset, time to diagnosis, ALSFRS-R progression rate, forced vital capacity, bulbar onset, definite amyotrophic lateral sclerosis by revised El Escorial criteria, frontotemporal dementia, and *C9orf72* repeat expansion. These predictors define five survival groups: very short (predicted median survival 17·7 months); short (25·3 months); intermediate (32·2 months); long (43·7 months); and very long (91·0 months). The ENCALS survival model unlocks the potential for personalised prognosis, which is essential for a disease of such heterogeneity. The model accurately estimated the life expectancy of Stephen Hawking,[58] in stark contrast to the 2-year expectancy he was given at diagnosis.

Emerging diagnostic and prognostic biomarkers

Currently, the diagnosis of amyotrophic lateral sclerosis relies on an integrative approach, which leverages clinical history (eg, presenting illness and symptom evolution), physical examination (eg, testing strength and reflexes), and confirmatory tests (eg, electromyography).[59] Genetic testing is gaining traction but is not without caveats (table 3). Electromyography and nerve conduction studies are the mainstay of electrodiagnostic tests, although additional methods are available (panel 2). Although diagnosis remains suboptimal, there is an expanding toolbox of available methods and novel biomarkers.

	Inheritance pattern	Proportion of familial cases	Proportion of sporadic cases	Associated clinical phenotype	Overlap with other diseases
(Continued from previous page)					
LGALSL	Not determined	<1%	<1%	Early onset; typical	
MATR3	Autosomal dominant	<1%	<1%	Slowly progressive; typical or atypical; frontotemporal dementia; myopathy	Distal myopathy
NEFH	Autosomal dominant; presence is a risk factor	<1%	<1%	Typical	Charcot-Marie-Tooth disease type 2*
NEK1	Not determined	~1–2%	<1%	Not determined	No overlap
OPTN	Autosomal dominant or recessive, depending on variant	<1%	<1%	Slowly progressive; atypical	Open-angle glaucoma, Paget's disease
PFN1	Autosomal dominant	<1%	<1%	Typical	No overlap
SETX	Autosomal dominant	<1%	<1%	Slowly progressive; juvenile	Spinocerebellar ataxia, progressive motor neuropathy
SPG11	Autosomal recessive	<1%	<1%	Slowly progressive; juvenile, mainly affects upper motor neurons	Hereditary spastic paraparesis
SOD1	Autosomal dominant or recessive, depending on variant; de novo	12%	1–2%	Prominent lower motor neurons; cognitive impairment very rare	No overlap
SQSTM1	Autosomal dominant	~1%	<1%	Typical	Paget's disease, frontotemporal dementia, dementia with Lewy bodies*
TARDBP	Autosomal dominant or recessive, depending on variant; de novo	4%	1%	Typical; frontotemporal dementia	Supranuclear gaze palsy
TBK1	Autosomal dominant; de novo	~3%	<1%	Typical; frontotemporal dementia	Frontotemporal lobar degeneration, dementia with Lewy bodies*
TIA1	Autosomal dominant	~2·2%	<1%	Frontotemporal dementia	Dementia with Lewy bodies*
TUBA4A	Autosomal dominant	<1%	<1%	Typical; frontotemporal dementia	No overlap
UBQLN2	X-linked; autosomal dominant	<1%	<1%	Typical; juvenile or adult onset; frontotemporal dementia	Frontotemporal dementia*
VAPB	Autosomal dominant	<1%	<1%	Typical or atypical	Spinal muscular atrophy, essential tremor
VCP	Autosomal dominant; de novo	1%	1%	Typical; frontotemporal dementia	Inclusion body myositis with Paget's disease, parkinsonism, scapuloperoneal muscular dystrophy, dropped head syndrome

Adapted, with modifications, from Goutman et al (2018)[31] and Chia et al (2018).[32] Typical phenotype refers to the classic motor phenotype. ALS=amyotrophic lateral sclerosis. *Findings limited to few patients.

***Table* 3: Summary of genotype–phenotype correlations and their overlap with other diseases in people carrying genetic mutations associated with ALS**

Presently, most of these approaches are only used in the research setting and have not been validated for clinical use.

Neurofilaments

Neurofilaments are neuronal cytoskeletal proteins that control neuron shape. Two markers are being developed: phosphorylated neurofilament heavy chain (NfH) in CSF and neurofilament light chain (NfL) in plasma, serum, or CSF. Phosphorylated NfH concentrations and NfL concentrations are elevated in individuals with amyotrophic lateral sclerosis compared with healthy controls.[71] NfL concentrations also rise 1 year before phenoconversion in presymptomatic individuals harbouring an amyotrophic lateral sclerosis gene.[72] Higher NfL and phosphorylated NfH concentrations correlate with more aggressive disease and shorter survival, but are of low prognostic value.[71,73] Because baseline NfL concentrations are predictive of ALSFRS-R trajectory, incorporating them into mixed-effects models of ALSFRS-R slopes might lower the number of participants needed in clinical trials.[82] However, increased neurofilament concentrations are characteristic of neurodegenerative diseases generally,[83] although they might still be fairly diagnostic of amyotrophic lateral sclerosis;[73] thus, overall, neurofilaments remain of uncertain diagnostic and prognostic use alone, but could add value when combined with other methods.

Brain and spinal cord imaging

Functional and structural brain imaging is a rapidly growing field,[67] with considerable progress after the advent of multisite imaging protocols,[84] studies

Panel 2: Diagnostic and prognostic biomarkers for amyotrophic lateral sclerosis

Diagnostic methods in clinical use

Criteria: the most frequently used are the revised El Escorial[34] (ie, Airlie House)[36] and Awaji[35] criteria; these criteria rate the degree of diagnostic certainty (possible to probable to definite) on clinical assessment, on the basis of the number of affected segments or electrophysiological findings, or both.

Electrodiagnostic: needle electromyography recordings are used to confirm the presence and extent of lower motor neuron involvement.[59]

Ultrasound: lower motor neuron fasciculations are often an early sign[60] (method is not very specific, so differential diagnosis might be needed); ultrasound can also be used to localise specific muscle groups during needle electromyography.

MRI: can be used to exclude cerebral and spinal amyotrophic lateral sclerosis mimics.[59]

Genetic testing: around 40 genes associated with disease are currently known; genetic testing is burgeoning, but with caveats.

Diagnostic methods in the research setting

Criteria: Gold Coast criteria are simplified criteria to define amyotrophic lateral sclerosis, particularly in the early stages.[38]

Electrodiagnostic: the number of functioning lower motor neuron units can be quantified using various methods,[61] whereas upper motor neuron involvement can be assessed by cortical hyperexcitability through transcranial magnetic stimulation with some diagnostic utility (and also by spectral EEG mapping and magnetoencephalography, which are both novel techniques);[62–66] these techniques will be useful as adjuncts to existing methods, but require further research to evaluate their integration in clinical practice and to establish their sensitivity and specificity.

MRI and PET: advanced brain and spinal cord imaging offer some diagnostic insight;[67–69] these techniques will be useful as adjuncts to existing methods but require additional research to evaluate their integration in clinical practice and their sensitivity and specificity.

Fluid biomarkers: the focus is on neurofilaments, but other biomarkers have been reviewed[70] (neurofilaments have uncertain diagnostic utility);[71–73] such biomarkers could serve as adjuncts to other methods.

Prognostic methods in clinical use

Scoring: the revised functional rating score for amyotrophic lateral sclerosis (ALSFRS-R) is an established scoring system to monitor the rate of disease progression.[48,49]

Spirometry: respiratory tests, such as forced vital capacity, generate prognostic value.[52]

Prognostic methods in the research setting

Scales and scoring: the self-reported Rasch-Built Overall ALS Disability Scale captures functional changes at upper and lower disability ranges,[53] but requires validation.

Staging: the four-stage King's staging[18] and six-stage Milano-Torino Staging[54] systems are not used in clinical practice but might be useful in clinical trials;[57] patients progress across stages over the disease course and median survival drops from stage to stage.

Prediction models: the ENCALS model can predict individual patient survival by leveraging eight characteristics;[19] it is not in clinical use but could be useful for providing additional information to patients and their families.

Electrodiagnostic: hyperexcitability by transcranial magnetic stimulation has some prognostic utility;[62–66] it might be useful as an adjunct to existing methods but requires further research to evaluate its integration into clinical care.[74–76]

MRI and PET: advanced brain and spinal cord imaging offer some prognostic insight;[67,77–79] neuroimaging will be useful as an adjunct to existing methods but requires additional research to evaluate its integration in clinical practice.

Fluid biomarkers: the current focus is on neurofilaments, but various markers have been reviewed[70] (neurofilaments have some prognostic utility but it is generally low[71,80]); another new biomarker is neutrophil-to-lymphocyte ratio,[81] which positively correlates with shorter survival.

indicating feasibility for early diagnosis[68] and possibility of prognosis,[77,78] and for insight into pathogenesis—eg, quantifying brain atrophy and connectomics (ie, connections between brain regions). Spinal cord MRI is widely used to rule out diagnostic considerations other than amyotrophic lateral sclerosis,[59] but more advanced diagnostic[69] and prognostic[79] applications are emerging.[85]

MRI assesses tissue appearance, brain structure volumes, and diffusivity, among other factors (appendix). Routine MRI does not identify people with amyotrophic lateral sclerosis; findings, if present, might be higher corticospinal tract and corpus callosum intensity in patients with amyotrophic lateral sclerosis than in healthy controls.[86] A hypo-intensity of the cortical band along the precentral gyrus, called the motor band sign, might be characteristic of amyotrophic lateral sclerosis and can be detected by routine susceptibility-weighted images.[87] However, advanced MRI analyses generate deeper insights using post-image processing (eg, assessing brain volumes by mapping brain regions *vs* established clinical standards). Advanced MRI of patients with amyotrophic lateral sclerosis indicates, to variable degrees, atrophy in the precentral gyri, posterior cingulate cortex, thalamus, caudate, pallidum, putamen, hippocampus, and amygdala.[88] Additional MRI techniques include diffusion tensor imaging (DTI) and diffusion weighted imaging (DWI), which focus on white matter tracts. Studies consistently report changes to the

See Online for appendix

corticospinal tract, corticopontine tract, corticorubral tract, corticostriatal pathway, and corpus callosum.[88,89] Diffusion kurtosis, a DTI adjunct, is a newer, more sensitive neuroimaging technique of white matter abnormalities, which might more accurately identify patients with amyotrophic lateral sclerosis than DTI without kurtosis.[90] White matter changes are usually the earliest findings, followed by grey matter changes.[91] Spinal cord findings suggest a drop in corticospinal tract magnetisation transfer ratio and potential DTI changes, although progressive atrophy and cross-sectional area might be the most accurate biomarkers.[85]

The complexity of amyotrophic lateral sclerosis pathology advocates for multimodal MRI, which combines multiple MRI techniques. Multimodal MRI of both brain volume and white matter integrity has 85·7% sensitivity and 78·4% accuracy for discriminating scans from people with amyotrophic lateral sclerosis and healthy controls.[92] A multisite Italian study evaluated global and lobar connectivity in patients with amyotrophic lateral sclerosis using DTI, fractional anisotropy (a white matter tract integrity measure), and resting-state functional MRI.[93] The study found widespread connectomics dysfunction, with early degeneration of brain motor regions followed by a breakdown in functional connections, leading to cognitive decline.[93] Multimodal longitudinal MRI can monitor spatiotemporal spread via the brain connectome and potentially serve as a disease biomarker.[89] Finally, quantitative susceptibility mapping MRI measures iron accumulation in the motor cortex,[94] which can be coupled with white matter assessments (ie, DTI, DWI, or diffusion kurtosis) to identify early tract changes associated with metal toxicity in individuals with amyotrophic lateral sclerosis. Similarly, multimodal MRI of the spinal cord has leveraged fractional anisotropy, magnetisation transfer ratio, and cross-sectional area to build a survival prediction model.[79]

PET imaging is another modality that might facilitate diagnosis and prognosis (appendix pp 3–8). By use of [^{18}F]-fluorodeoxyglucose (FDG) PET, a two-site study reported hypometabolism in the frontal cortex and hypermetabolism in the temporal cortex, cerebellum, and brainstem in patients with amyotrophic lateral sclerosis.[95] [^{11}C]-peripheral-type benzodiazepine receptor (PBR28) PET brain uptake, a surrogate of microglial activation, is increased in the bilateral precentral and paracentral gyri of patients with amyotrophic lateral sclerosis compared with healthy controls, and colocalises with cortical thinning (as assessed by integrated MRI imaging)[96] but might not correlate with clinical progression.[96] Integrating the spinal cord with the brain in [^{18}F]-FDG PET allows differentiation of amyotrophic lateral sclerosis from mimics of the disease.[97]

Overall, tremendous progress has been made in advanced brain MRI and PET along with advanced spinal cord imaging, which could improve diagnosis[68,69] and prognosis.[77–79] Although we anticipate that imaging will be useful as an adjunct to existing methods, additional research is required to evaluate how to integrate imaging into clinical care. Furthermore, most imaging studies focused on individuals with amyotrophic lateral sclerosis versus healthy controls; however, future studies will need to include patients with mimic disorders to better evaluate sensitivity and specificity.[68,97]

Spectral EEG mapping and magnetoencephalography

Electrophysiological techniques are used to assess brain networks. High-density spectral EEG mapping measures the coherence of several frequency bands between brain regions, generating a functional measure of brain connectivity.[98,99] EEG changes occur to brain connectivity in both motor and non-motor systems, confirming that amyotrophic lateral sclerosis is not a pure motor disease, in agreement with MRI connectomics findings.[99] Magnetoencephalography shows that brain networks become increasingly connected during disease progression, indicating a dysfunctional, modified brain topology.[100] These findings are important because reorganisation of brain connections could potentially predict disease spread.[89] Connectomics studies are needed that combine multimodal MRI, high-density spectral EEG, and magnetoencephalography to further understand how brain structural changes and corresponding connectivity changes associate with the symptomatology and disease course. EEG and magnetoencephalography connectomics are novel techniques not presently in clinical use and their potential as diagnostic and prognostic tools is unknown.

Hyperexcitability

Excessive cortical excitability (ie, hyperexcitability) is increasingly recognised as a pathophysiological mechanism of the neurodegenerative cascade.[101] Clinically, hyperexcitability manifests as fasciculations combined with upper motor neuron features of increased tone and hyperreflexia.[102] Hyperexcitability is linked to excitotoxicity from excessive glutamate receptor activity at the synaptic cleft, leading to motor neuron death.[33,103] Cortical motor neuronal hyperexcitability can be captured by transcranial magnetic stimulation (TMS).[104] A TMS coil is placed over the motor cortex and responses are recorded from the contralateral hand in the abductor pollicis brevis muscle. TMS extracts measures of short-interval intracortical inhibition and facilitation that represent interneuron function.

There is a decrease in short-interval intracortical inhibition and increase in short-interval intracortical facilitation in presymptomatic individuals with amyotrophic lateral sclerosis.[105] TMS detects cortical hyperexcitability across a range of phenotypes and can differentiate amyotrophic lateral sclerosis from other disorders with high sensitivity (73·21%) and specificity (80·88%) at early disease stages.[62] TMS can also distinguish amyotrophic lateral sclerosis (with cortical hyperexcitability predominance) from primary lateral

sclerosis (with cortical inexcitability predominance).[63] TMS can also investigate pathological spread, using hyperexcitability as a surrogate by recording responses at the tibialis anterior in addition to the abductor pollicis brevis. Analysis of patients with amyotrophic lateral sclerosis shows that there is heterogeneity in cortical dysfunction by body region; cortical hyperexcitability predominates in the upper limbs and cortical inexcitability predominates in the lower limbs when compared with healthy controls.[64] Furthermore, cortical hyperexcitability correlates with the clinically affected body region; patients with amyotrophic lateral sclerosis exhibit focal asymmetry at the onset site early in the disease but widespread hyperexcitability alterations in late stages.[65] Cortical motor hyperexcitability might also detect cognitive dysfunction; cortical resting motor threshold distinguishes amyotrophic lateral sclerosis, amyotrophic lateral sclerosis–frontotemporal dementia, and frontotemporal dementia.[66]

The role of TMS in prognosis is less established than it is in diagnosis. A longitudinal study of participants with suspected amyotrophic lateral sclerosis found cortical hyperexcitability increases with longer disease duration, indicating a potential link to disease progression.[74] Cortical inexcitability might predict a poorer clinical trajectory, with inexcitability in all four limbs correlating with younger age, lower-limb onset, greater extent of functional disability, and more rapid disease progression.[75] Thus, cortical hyperexcitability might improve our ability to predict clinical outcomes. It could also serve as a biomarker for drug activity, such as in clinical trials of retigabine, an activator of voltage-gated potassium channels.[76]

Presently, TMS is not in clinical use, although it does appear to offer some diagnostic and prognostic utility and probably will be informative as an adjunct to pre-existing methods. However, future research will establish the full potential of TMS, and whether this novel electrophysiological assessment will become a fully accepted disease biomarker.

Machine learning

Amyotrophic lateral sclerosis is a highly heterogenous syndrome of genetic and unknown causes with diverse clinical presentations. Machine learning approaches can analyse large datasets (eg, clinical, demographic, electrophysiological, imaging, or morphology) in an agnostic, data-driven manner to develop diagnostic and prognostic models.[106] Tang and colleagues used clinical data encompassing 8000 patients, 3 million records, and 200 clinical features from the Patient Data Pooled Resource Open-Access ALS Clinical Trials database.[107] Their analysis yielded four consistent phenotypes, defined by slope change in ALSFRS-R, with more than 95% diagnostic accuracy on the basis of multivariate features. These investigators used deep learning modelling, a form of machine learning, for prognosis. Their modelling predicted patient survival in this cohort when incorporating TDP-43 aggregation and morphology, and MRI connectivity data with clinical characteristics.[89] Further research will establish whether machine learning can unlock a way forward for diagnosing and prognosticating at the individual level by integrating multidomain information.

Overview of prognostic and diagnostic tests

Overall, most novel diagnostic and prognostic tests for amyotrophic lateral sclerosis are limited to the research setting. Further studies are needed to establish whether these approaches will be useful in a real-world clinical setting. Such evaluation will entail studies enrolling participants with diseases mimicking amyotrophic lateral sclerosis and longitudinal studies against validated prognostic scales to evaluate their potential for improved diagnosis (sensitivity and specificity) and prognosis. Additionally, it will be necessary to identify how to apply findings made from large cohort studies to the diagnosis and prognosis of individual patients. Until more specific and sensitive tests are developed, the diagnosis of amyotrophic lateral sclerosis will remain an integrative and iterative process reliant on clinical history, physical examination, and confirmatory electrodiagnostic tests.

Conclusions and future directions

Although diagnosis and prognosis have remained essentially unchanged in the past decade (except for genetic testing), research is ongoing into new diagnostic and prognostic criteria, and biomarkers (eg, neurofilament, hyperexcitability, and imaging). Even within the realm of genetic testing, questions remain regarding variant pathogenicity, penetrance, and overlap with other neurological disorders. It is anticipated and hoped that

Search strategy and selection criteria

Between Aug 3 and Aug 12, 2021, we searched PubMed for English language articles published from Jan 1, 2016, to Oct 12, 2021, using the term "amyotrophic lateral sclerosis", and the terms "epidemiology"; "phenotype"; "diagnostic"; "cognition" and "cognitive"; "GWAS" plus each amyotrophic lateral sclerosis gene in turn; "neurofilaments", "Amyotrophic Lateral Sclerosis"[MeSH] AND "magnetic[title] OR mri[title]", "Amyotrophic Lateral Sclerosis"[MeSH] AND "connectome[title]", "Amyotrophic Lateral Sclerosis"[MeSH] AND "PET[title] OR positron[title]", "EEG", and "hyperexcitability"; and "prognosis". Additional searches were done during revisions between Nov 15 and Nov 19, 2021, using the terms "amyotrophic lateral sclerosis" and: "spinal cord", "multimodal MRI", and "PET"; "machine learning"; "biomarker", "fluid", "electrodiagnostic", and "electrophysiological". Additionally, authors used articles from their personal files and references from the identified articles. Articles were selected on the basis of relevance to this Series.

advances in these areas will expedite the diagnosis and prognosis of amyotrophic lateral sclerosis in the future. Faster diagnosis will allow clinicians to initiate care earlier, which might enhance effectiveness or ensure administration within a therapeutic window. Ultimately, insight into the long preclinical phase of amyotrophic lateral sclerosis will be necessary to truly facilitate early diagnosis.[108] Improved prognosis will give patients and their families a better understanding of the disease course, aiding medical decisions and planning. A major advance is the recognition of amyotrophic lateral sclerosis as a disease with both motor and non-motor features, which has implications for diagnosis, management, and prognosis. Importantly, cognitive symptoms are not presently considered in clinical criteria and scales, yet their integration might improve diagnosis and prognosis. We foresee that these and other future advances will lead to better care for patients with this disease.

Contributors

All authors contributed to the conceptualisation, writing of the original draft, and review and editing of the final manuscript.

Declaration of interests

SAG declares consulting fees from Biogen and ITF Pharma, a patent "Methods for treating amyotrophic lateral sclerosis", and participation on a Data Safety Monitoring Board for Watermark. OH declares consulting fees from Novartis, Cytokinetics, Denali Pharma, Stitching Foundation, and La Caixa; payment or honoraria from Biogen; participation on a Data Safety Monitoring Board for Accelsiors and steering committee for Cytokinetics; and is Editor-in-Chief for the journal Amyotrophic Lateral Sclerosis and Frontotemporal Dementia. AA-C declares consulting fees from Mitsubishi Tanabe Pharma, Biogen Idec, Cytokinetics, Wave Pharmaceuticals, Apellis, Amylyx, Novartis, and Eli Lilly. AC declares grants from Biogen to his institution, payments or honoraria from Biogen and Amylyx, and participation on a Data Safety Monitoring Board for Ely Lilly and ABScience and advisory board for Mitsubishi Tanabe, Roche, Denali Pharma, Cytokinetics, Biogen, and Amylyx. MCK has an honorary role as President of the Brain Foundation and as Editor-in-Chief of the Journal of Neurology, Neurosurgery and Psychiatry. ELF declares a patent "Methods for treating amyotrophic lateral sclerosis". MGS declares no competing interests.

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Amyotrophic lateral sclerosis

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Amyotrophic lateral sclerosis is a fatal CNS neurodegenerative disease. Despite intensive research, current management of amyotrophic lateral sclerosis remains suboptimal from diagnosis to prognosis. Recognition of the phenotypic heterogeneity of amyotrophic lateral sclerosis, global CNS dysfunction, genetic architecture, and development of novel diagnostic criteria is clarifying the spectrum of clinical presentation and facilitating diagnosis. Insights into the pathophysiology of amyotrophic lateral sclerosis, identification of disease biomarkers and modifiable risks, along with new predictive models, scales, and scoring systems, and a clinical trial pipeline of mechanism-based therapies, are changing the prognostic landscape. Although most recent advances have yet to translate into patient benefit, the idea of amyotrophic lateral sclerosis as a complex syndrome is already having tangible effects in the clinic. This Seminar will outline these insights and discuss the status of the management of amyotrophic lateral sclerosis for the general neurologist, along with future prospects that could improve care and outcomes for patients with amyotrophic lateral sclerosis.

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Introduction

Amyotrophic lateral sclerosis, a fatal CNS neurodegenerative disease, can be difficult to recognise, especially in the early stages. The disease is rare, and more common illnesses are frequently considered before amyotrophic lateral sclerosis, delaying diagnosis. However, the lifetime risk of the disease is approximately one in 350 people, although low life expectancy reduces the prevalence.[1] Recognition of phenotypic heterogeneity, and amyotrophic lateral sclerosis as a complex syndrome that frequently includes behavioural deficits, could help physicians better recognise it earlier in the disease course. Development of new diagnostic criteria and identification of genetic risk factors could also expedite the diagnostic process.[2] Regarding prognosis, a clearer understanding of the multisystem nature of amyotrophic lateral sclerosis, including cognitive dysfunction and behavioural changes, has important ramifications for caregiving support and end-of-life decision making. Moreover, newly developed predictive models, scales, and scoring systems can give patients with amyotrophic lateral sclerosis and their physicians a clearer idea of their disease course.[2] Advances in our understanding of disease pathophysiology are leading to mechanism-based and potentially disease-modifying therapies, currently in clinical trials. This Seminar will outline these topics and current clinical practice for amyotrophic lateral sclerosis, along with research advances, which could facilitate future improvements in diagnosis and prognosis for patients with amyotrophic lateral sclerosis.

Epidemiology

Incidence of amyotrophic lateral sclerosis rises with age and is highest between 60 years and 79 years,[3,4] although variation can occur by ancestral background.[5] Some studies show stable incidence over the past two or three decades,[1] whereas others report a possible increase.[6,7] Changes in perceived incidence could arise from improved diagnosis or changes in reporting standards over time, advocating the construction of well curated population registries. Whether the incidence of amyotrophic lateral sclerosis has changed in the past couple of decades is unclear, although it is anticipated to increase with an ageing population.[8] Prevalence of amyotrophic lateral sclerosis is also expected to increase due to an ageing population, in addition to improved management, which supports increased life expectancy.[8,9] However, it remains a relatively rare disease. Standardised global incidence of amyotrophic lateral sclerosis by meta-analysis is only 1·68 per 100 000 person-years of follow-up, but varies by region.[10] In populations of predominantly European descent, such as in Europe and North America, incidence is slightly higher than the global average, ranging from 1·71 per 100 000 to 1·89 per 100 000, and could even be higher within population-based studies.[11] Asian populations have lower incidences, varying from 0·73 per 100 000 in south Asia to 0·94 per 100 000 in west Asia, whereas Oceania

Search strategy and selection criteria

We searched PubMed for English language articles from Sept 15, 2021, to Oct 5, 2021, and then again in January, 2022, with the terms, in addition to "amyotrophic lateral sclerosis": "epidemiology"; "phenotype"; "diagnostic", "diagnosis", "cognition", and "cognitive"; "GWAS", "genetic", "risk", "oligogenic", "polygenic", and "heritability"; "mimic" and "GWAS" combined with every amyotrophic lateral sclerosis gene in turn; "pathophysiology", "mechanism", "nucleocytoplasmic transport", "cell-to-cell transmission", "immune system", "exposure", "environment", "pollutant", "toxin", "metals", and "traffic"; "prognosis", "scoring", "scaling", and "staging"; "multidisciplinary care", "riluzole", "edaravone", "non-invasive ventilation", and "gastrostomy"; and "gene therapy", "antisense oligonucleotide", "antibody", "immune", "clinical trial", "neurofilaments", "imaging", "PET", "connectome", "EEG", and "hyperexcitability". The search focused on articles published from Jan 1, 2017, to Jan 31, 2022, although older seminal articles were also considered. We also included articles from the authors' personal reference lists. Articles were selected on the basis of relevance to this Seminar. Additionally, we searched ClinicalTrials.gov for "amyotrophic lateral sclerosis".

***Panel 1*: Definitions of amyotrophic lateral sclerosis motor signs and phenotypes**

Lower motor neurons (LMN)
- Brainstem cranial motor nerve nuclei or anterior horn cells
- LMN dysfunction is characterised by muscle weakness, atrophy, and fasciculations

Upper motor neurons (UMN)
- Betz cells in layer V of the primary motor cortex
- UMN dysfunction is characterised by increased and pathological reflexes (including Hoffmann's sign, Babinski, and snout), pathological spread of reflexes, preserved reflexes in a weak limb, and spasticity

Bulbar amyotrophic lateral sclerosis
- Phenotype presents with weakness starting in the muscles controlling speaking and swallowing
- Both LMN and UMN signs are present

Pseudobulbar palsy
- A non-classical subset of bulbar onset, characterised by prominent bulbar features, predominantly from UMN signs, which slowly spread to limbs

Pseudobulbar affect
- Uncontrollable emotional outbursts, including laughing, crying, and excessive yawning

Classical amyotrophic lateral sclerosis
- Phenotype presents with muscle weakness starting in the limbs; both LMN and UMN signs are present

Cervical-onset amyotrophic lateral sclerosis
- A subset of classical amyotrophic lateral sclerosis with weakness commencing in the upper limbs, especially hand weakness

Lumbar-onset amyotrophic lateral sclerosis
- A subset of classical amyotrophic lateral sclerosis with weakness commencing in the lower limbs, especially foot drop

Flail arm
- Prominent LMN dysfunction initially causing proximal muscle weakness greater than distal muscle weakness in the arms
- Unlike progressive muscular atrophy, patients with flail arm do manifest progressive UMN dysfunction; this entity can also be referred to as brachial amyotrophic diplegia

Flail leg:
- LMN dysfunction causing muscle weakness in the legs; unlike progressive muscular atrophy, this phenotype does not generalise or generalises very slowly

Primary lateral sclerosis*:
- UMN dysfunction causing weakness in muscles controlling limbs, swallowing, and speaking
- Less commonly causes respiratory dysfunction

Pyramidal:
- Like primary lateral sclerosis but additionally eventually exhibiting LMN signs

Progressive muscular atrophy*:
- LMN dysfunction causing weakness in muscles controlling limbs, swallowing, speaking, and respiratory function

Respiratory onset
- LMN and UMN dysfunction causing weakness commencing in the respiratory muscles

Hemiplegic
- Predominantly UMN dysfunction causing muscle weakness in one side of the body

Cachexia
- Unexplained weight and muscle loss

*This Seminar considers primary lateral sclerosis and progressive muscular atrophy on the spectra of amyotrophic lateral sclerosis phenotypes, although they can also be considered as separate clinical entities.

universally has the highest incidence (2·25 per 100 000).[7,10] Incidence also varies by sex, with an overall standardised male-to-female ratio of 1·35, which is affected by age of onset.[12] Genetics also has a role; heritability is higher in mother–daughter pairs,[1] whereas the most common known amyotrophic lateral sclerosis risk gene, *C9orf72*, lowers onset age in men versus women.[13] Thus, amyotrophic lateral sclerosis arises from complex interrelationships between age, sex, and genetics,[14] which has implications for preclinical and clinical research, and clinical trials.

Clinical presentation

Phenotypic heterogeneity

Amyotrophic lateral sclerosis presents as a combination of upper motor neuron (UMN) and lower motor neuron (LMN) dysfunction, affecting the bulbar, cervical, thoracic, or lumbar segments.[2] This dysfunction leads to progressive weakness of voluntary skeletal muscles involved in limb movement, swallowing (dysphagia), speaking (dysarthria), and respiratory function, with different clinical presentations (panel 1). Sphincter and extraocular muscles are classically spared, although autonomic dysfunction in amyotrophic lateral sclerosis is increasingly recognised (eg, urinary urgency and incontinence).[15] Clinical weakness spreads contralaterally, rostrally, and caudally, most often in an anatomically contiguous manner. A 2018 survey of 470 patients with amyotrophic lateral sclerosis found that 85% had focal onset in one body segment, which progressed to the contralateral side and then to adjacent anatomical segments.[16] Spread of disease to non-contiguous segments was less common.

Amyotrophic lateral sclerosis presents as multiple phenotypes (figure 1A–B; appendix pp 4–6). Bulbar-onset and spinal-onset (cervical and lumbar) amyotrophic lateral sclerosis are the most common presentations, each constituting about a quarter of the cases. Less frequently, patients present flail arm and leg, primary lateral sclerosis, progressive muscular atrophy, respiratory onset, or hemiplegia.[12,13] This Seminar considers primary lateral sclerosis and progressive muscular atrophy on the spectra of amyotrophic lateral sclerosis phenotypes, although they could also be considered as separate clinical entities. Age, sex, and genetics also contribute to amyotrophic lateral sclerosis phenotypes. Women aged 60 years or older more commonly develop bulbar-onset amyotrophic lateral sclerosis, whereas men aged less than 60 years present with the classical phenotype. Pure UMN variants are more commonly seen in men and women aged less than 60 years. Flail arm, leg, and respiratory onset primarily develop in men, irrespective of age.[14] Specific genetic mutations favour certain phenotypes. One study of German and Chinese registries suggests that phenotypes could vary globally.[18] German patients with amyotrophic lateral sclerosis have an older onset age (66·6 years), a larger proportion of bulbar onset (35·9%), and a smaller male-to-female ratio (1·33) than do Chinese patients (53·2 years onset age; 22·8% bulbar; 1·51 male-to-female ratio).[18] Consensus phenotyping between registries would advance our knowledge of age, sex, genetics, racial, and ethnic contributions to phenotypes.

Cognitive and behavioural changes

Classically, amyotrophic lateral sclerosis was predominantly considered a disease of motor dysfunction (eg, dysarthria, dysphagia, and weakness of upper and lower limb muscles). However, cognitive and behavioural changes, which can occur early in the disease course,[19,20] are now recognised to occur in 35–50% of patients with amyotrophic lateral sclerosis.[21,22] Individuals with amyotrophic lateral sclerosis have loss of normal language and executive function (ie, poor working memory, inhibition, and fluency). Typically, more long-term memory and spatial domains remain intact.[21] Other behavioural changes include apathy, irritability, disregard for hygiene, and eating habit changes. Approximately 15% of patients with amyotrophic lateral sclerosis meet the diagnostic criteria for frontotemporal dementia.[20,23] Furthermore, depression, anxiety, and sleep disruptions occur in amyotrophic lateral sclerosis[24] along with pseudobulbar affect, which causes emotional lability.[16]

These cognitive and behavioural changes support the concept that amyotrophic lateral sclerosis is a global neurodegenerative disease along the same continuum as frontotemporal dementia (figure 1C). Transactive response DNA-binding protein 43 kDa (TDP43) proteinopathy, an almost universal finding in amyotrophic lateral sclerosis, is present in around 97% of patients and around 50% of patients with frontotemporal dementia. Mild deficits in executive function, language, and fluency have 100% specificity for TDP-43 pathology in non-motor brain regions corresponding to these domains.[25] Some patient characteristics, such as *C9orf72* status[26,27] and bulbar onset,[27] are strong determinants of cognitive impairment and could help the physician and patient to

See **Online** for appendix

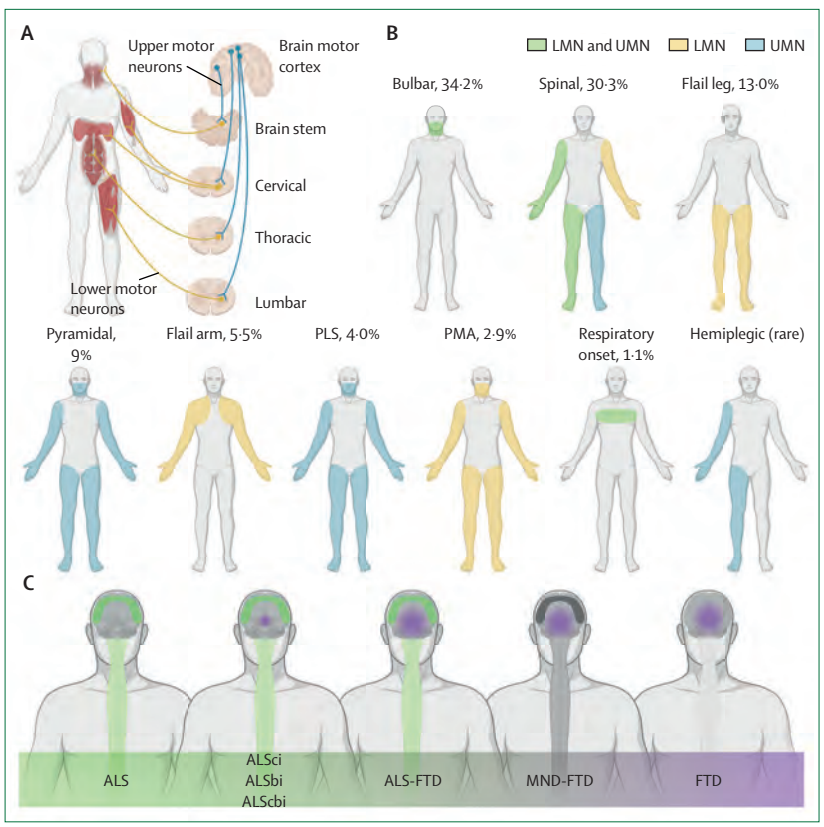


Figure 1: **Amyotrophic lateral sclerosis phenotypic variation and spectrum with frontotemporal dementia**
(A) Schematic showing UMNs (blue), which relay signals from the motor cortex to the LMNs (yellow; ie, cranial motor nerve nuclei in the brainstem and anterior horn cells in the spinal cord), which relay signals to the muscles. Motor neurons connecting within the brain stem innervate, among other muscles, cranial muscles. Initial UMN and LMN degeneration in the brain stem are linked to bulbar-onset amyotrophic lateral sclerosis. Motor neurons connecting within the cervical region of the spinal cord innervate, among other muscles, upper limb and respiratory muscles. Motor neurons connecting within the thoracic and lumbar regions of the spinal cord innervate, among other muscles, accessory respiratory, abdominal, and lower limb muscles. Initial UMN and LMN degeneration in the cervical and lumbar regions are linked to spinal-onset amyotrophic lateral sclerosis. (B) Patients with amyotrophic lateral sclerosis can present with signs of UMN (blue), LMN (yellow), and combined UMN and LMN (green) dysfunction. Most common amyotrophic lateral sclerosis phenotypic presentations are bulbar and classical spinal limb onset (cervical and lumbar). Less common amyotrophic lateral sclerosis phenotypic presentations are flail leg, pyramidal, flail arm, PLS, PMA, respiratory onset, and hemiplegic. Proportion of amyotrophic lateral sclerosis phenotypes shown in the figure as the percentage of a total representative amyotrophic lateral sclerosis population.[14,17] Pyramidal is predominantly UMN, but still exhibits some LMN signs, differentiating it from PLS (appendix pp 4–6). (C) Amyotrophic lateral sclerosis occurs on a continuum with frontotemporal dementia. Amyotrophic lateral sclerosis is on one end of the spectrum and presents with pure motor signs from UMN and LMN neurodegeneration. Frontotemporal dementia is on the other end of the spectrum and presents with behavioural and cognitive deficits from frontotemporal neurodegeneration. After pure amyotrophic lateral sclerosis are patients with amyotrophic lateral sclerosis not meeting frontotemporal dementia criteria, defined as ALSci, ALSbi, and ALScbi, followed by patients meeting frontotemporal dementia criteria (ALS–FTD). Patients on the remainder of the continuum have frontotemporal dementia but do not meet the criteria for amyotrophic lateral sclerosis. Some patients still have evidence of MND with frontotemporal dementia and patients with no MND signs have frontotemporal dementia. ALS=amyotrophic lateral sclerosis. ALSbi=amyotrophic lateral sclerosis behavioural impairment. ALSci=amyotrophic lateral sclerosis cognitive impairment. ALScbi=amyotrophic lateral sclerosis cognitive and behavioural impairment. FTD=frontotemporal dementia. LMN=lower motor neurons. MND=motor neuron disease. PLS=primary lateral sclerosis. PMA=progressive muscular atrophy. UMN=upper motor neurons.

***Panel 2*: Amyotrophic lateral sclerosis diagnosis**

Clinical history
- Symptoms (eg, weakness and time course)
- Family history of amyotrophic lateral sclerosis or other neurodegenerative diseases

Neurological examination
- Signs of upper motor neuron (UMN) and lower motor neuron (LMN) dysfunction in bulbar, cervical, thoracic, or lumbosacral segments (eg, hand weakness [split hand] and foot drop)
- Unexplained weight loss, cognition or executive functioning dysfunction, and pseudobulbar affect are additional signs

Electrodiagnostic testing
- Nerve conduction studies and needle electromyography to confirm LMN signs

Laboratory testing
- Serology should be normal except for elevated creatine phosphokinase concentrations, which can also lead to abnormal liver function tests

MRI
- Imaging the spinal cord by MRI is essential to rule out more common differential diagnoses (eg, disc herniation or cord compression)

Criteria
- Most neurologists use the revised El Escorial criteria[32]
- Classifies patients with amyotrophic lateral sclerosis as possible, probable, probable laboratory supported, and definite, on the basis of clinical presentation and electrodiagnostic findings

Revised El Escorial criteria

The presence of:
- LMN signs by clinical, electrodiagnostic testing, or neuropathological examination
- UMN signs by clinical examination
- Progressive symptom or sign spread within a region or to other regions, as determined by history or examination

With the absence of:
- Electrodiagnostic or pathological evidence of other diseases explaining LMN and UMN signs
- Neuroimaging evidence of other diseases explaining the observed clinical and electrodiagnostic signs

El Escorial diagnostic categories

Clinically definite
- Clinical evidence of UMN and LMN signs in the bulbar and two spinal regions, or
- UMN and LMN signs in three spinal regions

Clinically probable
- Clinical evidence of UMN and LMN signs in at least two regions with UMN signs rostral to LMN signs

Clinically probable—laboratory supported
- Clinical evidence of UMN and LMN signs in one region or UMN signs alone in one region, and
- LMN by electrodiagnostic criteria in at least two regions

Clinically possible
- Clinical evidence of UMN and LMN in one region, or
- UMN signs in two or more regions, or
- LMN signs are rostral to UMN signs

anticipate this complication. Furthermore, cognitive dysfunction and behavioural abnormalities might be prognostic of disease stage.[21] In a report of 146 patients with amyotrophic lateral sclerosis, cognition worsened in 30% after 6 months, even among patients that initially presented with normal cognition.[22] The patients who presented with cognitive decline had a more rapid clinical progression and shorter survival than those with normal cognition. Network analyses of brain MRIs show widespread disruption of motor and extramotor networks that correspond with amyotrophic lateral sclerosis phenotypes. Specifically, abnormal structural connectivity correlates with motor impairment, whereas disrupted functional connectivity aligns with changes in cognition and behaviour.[28]

Collectively, this new understanding of amyotrophic lateral sclerosis as a multisystem disorder underscores the importance of managing cognitive decline and neuropsychological problems (eg, depression, dysfunctional sleep, apathy, and irritability).[24] Importantly, when cognitive symptoms emerge, care teams should engage early with patients and their families to inquire about end-of-life care preferences to ensure the patient has an active role in these important conversations.

Diagnosis

Criteria

Patients with amyotrophic lateral sclerosis are unlikely to encounter a neurologist early in the diagnostic journey.[29,30] Therefore, there should be a low threshold for neurological referral when patients present with progressive dysarthria, dysphagia, limb weakness, or neuromuscular respiratory failure. The Amyotrophic Lateral Sclerosis Association's thinkALS tool[31] encourages early neurological referral to avoid unnecessary procedures, starts patients on disease-modifying treatments, and fast-tracks patient enrolment into clinical trials. Additional indications of a diagnosis of amyotrophic lateral sclerosis include unexplained weight loss, pseudobulbar affect, changes in cognition or executive functioning, and a family history of amyotrophic lateral sclerosis or other neurodegenerative

diseases. Clinical features that do not support a diagnosis include prominent sensory, sphincter, and autonomic nervous system dysfunction and anterior visual pathway abnormalities. A detailed neurological examination should identify signs of UMN and LMN dysfunction in bulbar, cervical, thoracic, or lumbosacral segments (panel 2).

Clinical history and neurological examination are accompanied by serological and electrodiagnostic testing. Patients with amyotrophic lateral sclerosis have normal serology, except for elevated creatine phosphokinase concentrations in some cases. Other abnormal serologies call into question an amyotrophic lateral sclerosis diagnosis. Nerve conduction studies exclude sensory nerve involvement and motor nerve conduction block. Needle electromyography can confirm LMN involvement, with the provision that testing of distal muscles, and muscles in the involved clinical segment, have the highest sensitivity.[33,34] Most neurologists still use the revised El Escorial criteria to subclassify amyotrophic lateral sclerosis, which categorises patients as possible, probable, probable laboratory supported, and definite amyotrophic lateral sclerosis, depending on clinical presentation and electromyography findings. The revised El Escorial criteria are most widely used (panel 2).[32]

Regarding advances in diagnostic criteria for amyotrophic lateral sclerosis, the Gold Coast criteria have been proposed to simplify and potentially replace the revised El Escorial and improve inter-rater reliability (appendix pp 7–9).[35] The Gold Coast criteria are primarily based on clinical presentation, although they do not consider cognitive changes, which the authors noted were covered by the 2017 Strong criteria.[36] Gold Coast classifies patients as having or not having amyotrophic lateral sclerosis, streamlining diagnostic certainty and eliminating confusion to patients and their relatives from El Escorial terminology. A comparison of the sensitivity and specificity of the various criteria reveal that Gold Coast criteria are the most sensitive, whereas El Escorial are the most specific (appendix pp 7–9). Additionally, the revised El Escorial criteria provide information that the Gold Coast criteria do not, such as the distribution of clinical segmental involvement, which is important for stratifying disease severity in patients with amyotrophic lateral sclerosis. Although the revised El Escorial criteria remain the mainstay of amyotrophic lateral sclerosis diagnosis, the field could be slowly moving towards simpler criteria, such as the Gold Coast.

Overall, early diagnosis of the disease is important. Educational efforts for physicians most likely to encounter patients with amyotrophic lateral sclerosis during initial symptom onset are essential to support prompt recognition of the disease and timely initiation of treatment. As simplified diagnostic criteria become more universally accepted, we anticipate that more practitioners will recognise and treat amyotrophic lateral sclerosis early in the disease course.

Cognitive assessment

Although not part of formal amyotrophic lateral sclerosis diagnostic criteria, it is essential to evaluate cognition and behaviour in patients with amyotrophic lateral sclerosis, despite potentially further fatiguing individuals undergoing long and complex clinical visits. Assessments of cognitive and behavioural impairment are essential as they relate to prognosis and progression rate, and thus inform clinical management.[21,22] Assessment of cognitive impairment in patients with amyotrophic lateral sclerosis should include multiple cognitive domains (eg, executive and language dysfunction, and social cognition).[37] Behavioural impairment (eg, apathy, disinhibition, loss of empathy, and compulsive behaviour) also affects the wellbeing of patients and family members and requires evaluation.

Some patients are diagnosed with frontotemporal dementia (amyotrophic lateral sclerosis–frontotemporal dementia, known as ALS–FTD), as defined by the criteria set by Neary and colleagues[38] or Rascovsky and colleagues.[39] For patients not meeting formal frontotemporal dementia criteria, the revised Strong criteria define patients with amyotrophic lateral sclerosis with cognitive dysfunction as amyotrophic lateral sclerosis cognitive impairment, with behavioural problems as amyotrophic lateral sclerosis behavioural impairment, or with both, as amyotrophic lateral sclerosis combined cognitive behavioural deficits (appendix p 10).[36] Several assessment batteries can classify these changes. The Edinburgh Cognitive and Behavioural Amyotrophic Lateral Sclerosis Screen (ECAS) is a validated, multidomain, assessment tool developed for patients with amyotrophic lateral sclerosis, which can be administered by neuropsychological and non-neuropsychological professionals.[37] ECAS, available in 23 languages, covers the largest number of amyotrophic lateral sclerosis-specific cognitive or behavioural assessment scales. Incorporating ECAS into management of amyotrophic lateral sclerosis has a positive effect on the quality of care by stimulating end-of-life care discussions, referrals to other services, and identifying caregiver support needs.[40]

The Amyotrophic Lateral Sclerosis Cognitive Behavioural Screen, available in three languages, can also identify cognitive and behavioural impairment and frontotemporal dementia in patients with amyotrophic lateral sclerosis.[37] The ALS–FTD questionnaire, completed by health-care professionals or caregivers to assess behavioural changes in patients with amyotrophic lateral sclerosis, is translated into nine languages and able to identify patients with behavioural variant frontotemporal dementia.[37] The Beaumont Behavioural Inventory is a screening tool developed in 2017 for evaluating behavioural impairment in patients with amyotrophic lateral sclerosis and might be more sensitive than the ALS–FTD questionnaire.[37]

Overall, cognitive symptoms should be recognised as a manifestation of amyotrophic lateral sclerosis, and

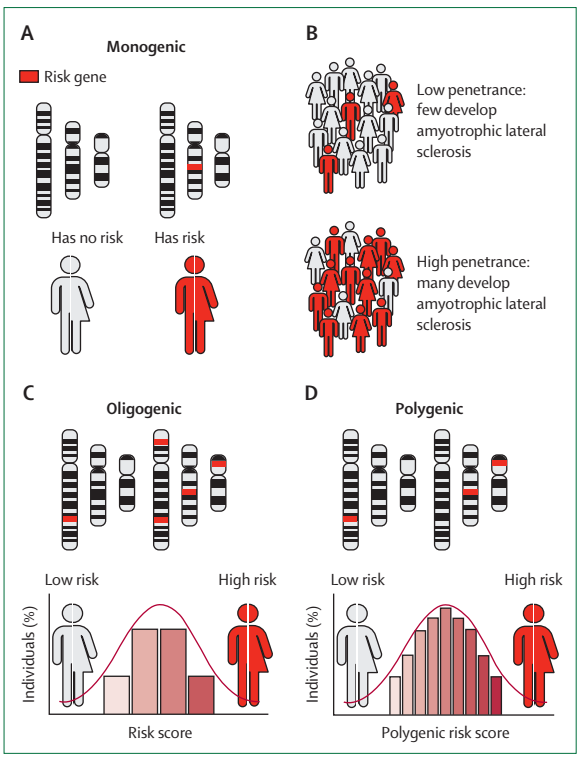


Figure 2: **Amyotrophic lateral sclerosis genetic architecture**
Adapted from Goutman et al.[2] Amyotrophic lateral sclerosis genetics is characterised by monogenic, oligogenic, and polygenic risk; figure featuring only three representative chromosomes (within each panel, chromosomes on the left for a person without the disease, on the right for a person with the disease). (A) Monogenic inheritance is characterised by inheritance of a single gene. (B) amyotrophic lateral sclerosis genes are not fully penetrant and pathogenicity of certain variants is uncertain. (C) Oligogenic inheritance is characterised by inheritance of several genes (four shown in the figure). (D) Polygenic inheritance is characterised by inheritance of many genes (nine shown in the figure).

properly identifying these symptoms improves disease management, counselling, and prognostication. Since cognitive symptoms can change with disease progression, regularly assessing them is crucial to best care for the patient. Future directions include standardising cognitive assessments for in-clinic screening, determining whether neuropsychologists should become part of the regular multidisciplinary team, and developing evidence-based treatments for cognitive impairment in amyotrophic lateral sclerosis.

Genetic architecture

Amyotrophic lateral sclerosis is currently classified as either familial or sporadic. Familial amyotrophic lateral sclerosis, which constitutes 10–15% of cases, is inherited from family members with amyotrophic lateral sclerosis and associated syndromes (eg, frontotemporal dementia).[41] About 70% of familial cases have mutations within known amyotrophic lateral sclerosis genes. Sporadic amyotrophic lateral sclerosis, which constitutes approximately 85% of the remaining cases, arises in patients without a family history of amyotrophic lateral sclerosis. About 15% of patients with sporadic amyotrophic lateral sclerosis harbour private pathogenic mutations (mutations limited to a single individual) to known amyotrophic lateral sclerosis genes, hence, they are without a family history of amyotrophic lateral sclerosis.[41] There is no known cause in the remaining 85% of sporadic cases of amyotrophic lateral sclerosis. Sporadic cases harbouring low penetrant mutations and belonging to small families, or having incomplete or poor knowledge of family history, could in fact be familial amyotrophic lateral sclerosis. Thus, familial amyotrophic lateral sclerosis might be under-reported and represent closer to 20% of cases.[42,43] As genetic testing becomes more widely implemented, and potential candidate therapies more targeted, it might become useful to drop the familial versus sporadic dichotomy of amyotrophic lateral sclerosis, in favour of genetically confirmed versus non-genetically confirmed amyotrophic lateral sclerosis (ie, presence versus absence of an amyotrophic lateral sclerosis mutation underpinning the molecular subclassification of the disease).

Genetic architecture of amyotrophic lateral sclerosis is highly complex and largely based on monogenic inheritance of rare variants (single disease-causing genes; figure 2A).[44] More than 40 amyotrophic lateral sclerosis-associated genes have been identified,[45,46] which vary in frequency, mode of inheritance (mostly dominant, rarely recessive), and penetrance (figure 2B; appendix pp 11–14). The most common and penetrant mutations are *C9orf72*, *TARDBP*, *SOD1*, and *FUS*,[45] although the frequency of genetic subtypes varies by population ancestry.[47] Some amyotrophic lateral sclerosis genes are not necessarily disease-inducing, but rather confer an increased risk of developing amyotrophic lateral sclerosis (*ANG*, *ATXN2*, and *DCTN1*).[45] Importantly, uncertainty remains on the relevance of some identified genes, which require further confirmation and replication efforts.[48] Consortia of amyotrophic lateral sclerosis genetics experts can curate and maintain an up-to-date list of amyotrophic lateral sclerosis genes as evidence emerges,[49] facilitating clinical translation for genetic testing. Since amyotrophic lateral sclerosis genetic architecture is complex, it is advisable that specialist amyotrophic lateral sclerosis centres perform genetic testing to avoid overdiagnosing or missing genetic amyotrophic lateral sclerosis. Of note, genetic testing in amyotrophic lateral sclerosis might not identify rare pathogenic variants (ie, allele frequency less than 1%).

In addition to primary monogenic inheritance in amyotrophic lateral sclerosis, interest in the effect on oligogenic and polygenic inheritance on disease risk has also gained traction. Several studies highlight that oligogenic inheritance, meaning a trait or disease controlled by inheritance of several genes, might have a role in amyotrophic lateral sclerosis risk and disease progression (figure 2C).[50,51] Genetic screening identified a subset of patients with sporadic amyotrophic lateral sclerosis harbouring two or more variants in amyotrophic

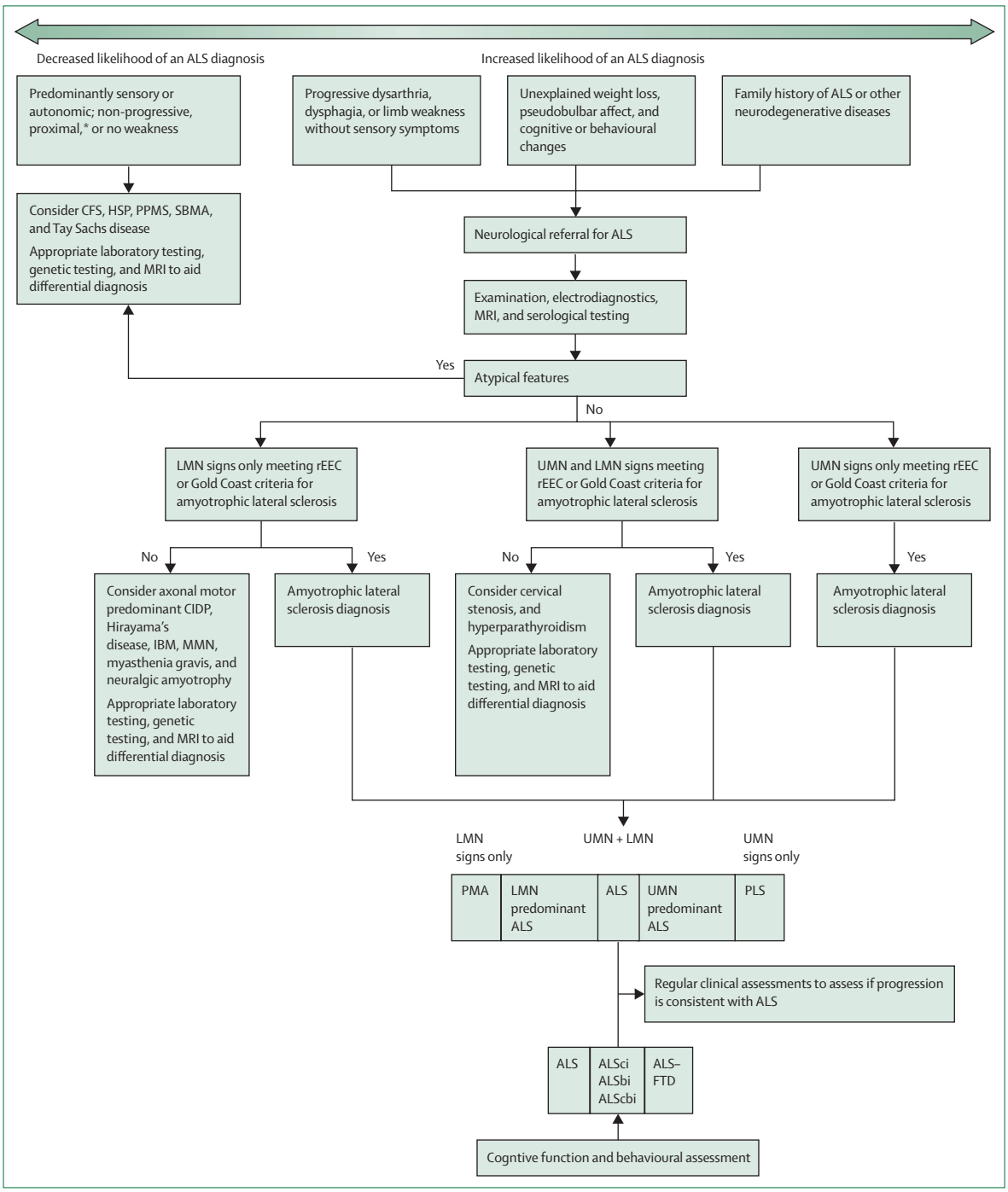


Figure 3: **Amyotrophic lateral sclerosis differential diagnosis**
Differential diagnosis, represented here by a flowchart for the classical process by use of symptoms and signs, is central to the diagnostic process in amyotrophic lateral sclerosis. At minimum, individuals suspected of the disease will undergo physical and neurological examinations, electrodiagnostic assessment, MRI of involved regions, and relevant serological testing. This figure is based on a summary of potential differential diagnoses for diseases more common or as common as amyotrophic lateral sclerosis (appendix p 10). Overlap of known amyotrophic lateral sclerosis genes with other diseases and syndromes also occurs (appendix pp 11–13). ALS=amyotrophic lateral sclerosis. CFS=cramp-fasciculation syndrome. CIDP=chronic inflammatory demyelinating polyneuropathy. HSP=hereditary spastic paraparesis. IBM=inclusion body myositis. LMN=lower motor neuron. MMN=multifocal motor neuropathy. MG=myasthenia gravis. PPMS=primary progressive multiple sclerosis. rEEC=revised El Escorial criteria. SBMA=spinobulbar muscular atrophy. UMN=upper motor neuron. *Several potential differential diagnoses present with proximal weakness and should be considered along with flail arm amyotrophic lateral sclerosis, which also presents with proximal greater than distal upper extremity weakness. Thus, check for increased proximal reflexes on examination and neurogenic motor unit action potentials on electromyography.

lateral sclerosis genes; these patients were more likely to have earlier onset disease versus patients with one or no variants.[50,51] Polygenic inheritance, arising from inheritance of multiple genetic variants, is also a component of amyotrophic lateral sclerosis genetic architecture (figure 2D).[52,53] Analysis of the genetic profiles identified shared polygenic risk of amyotrophic lateral sclerosis with traits and single nucleotide polymorphisms correlated with smoking status, physical activity, cognitive performance, and educational attainment,[52] as well as obesity-related traits, [52,53] particularly hyperlipidaemia. Our growing knowledge of the genetic architecture is due, in great part, to large collaborative projects, which are driving discovery in this relatively rare disease, such as the Amyotrophic Lateral Sclerosis Sequencing Consortium,[54] International Amyotrophic Lateral Sclerosis Genomics Consortium,[55] Genomic Translation for Amyotrophic Lateral Sclerosis Care Consortium,[54] Answer Amyotrophic Lateral Sclerosis Foundation,[54] and Project MinE.[54] We anticipate that these consortia will continue to deliver results and foster further investigation.

Importantly, amyotrophic lateral sclerosis is also characterised by incomplete heritability, meaning genetics does not fully account for all disease burden. Estimates vary, but most studies report heritability of 45–50% in amyotrophic lateral sclerosis parent–child dyads, driven largely by rare genetic variants.[1] However, heritability estimates can be as high as 66% in some dyad comparisons and as low as 37% in patients without a known genetic risk.[1] Several additional factors can account for missing heritability in amyotrophic lateral sclerosis,[56] such as alterations in the non-coding genome, structural variants,[57] epigenetic changes,[58] and environmental factors.[59] The contribution of the environment has led to the gene–time–environment hypothesis of amyotrophic lateral sclerosis,[60] which proposes that an interaction of genes and environment over time causes amyotrophic lateral sclerosis through a multistep process.[61] Evolving evidence shows that the environment effects amyotrophic lateral sclerosis risk and progression in a gene-dependent manner.

As therapeutics that target some genetic forms of amyotrophic lateral sclerosis become a possibility, genetic testing for all patients with amyotrophic lateral sclerosis will probably become standard practice. Future genetic treatments will increase the need for classifying and assessing genetic variants in amyotrophic lateral sclerosis. Additionally, partnership with genetic counsellors will expand to facilitate discussions of these complex results with patients and their families.[62]

Differential diagnosis and overlap syndromes

General physicians, and even specialist neurologists, might not initially recognise a diagnosis of amyotrophic lateral sclerosis in a patient with symptoms due to overlap of disease presentation with other conditions. Thus, classical differential diagnosis on the basis of clinical presentation is an important element of the diagnostic process in amyotrophic lateral sclerosis (figure 3; appendix pp 14–15).

Diseases more common than amyotrophic lateral sclerosis are often considered and thoroughly evaluated first, which ultimately delays an amyotrophic lateral sclerosis diagnosis. Conditions that most commonly mimic amyotrophic lateral sclerosis include multifocal motor neuropathy with conduction block, axonal motor-predominant chronic inflammatory demyelinating polyneuropathy, spinobulbar muscular atrophy, and inclusion body myositis.[63] Simultaneous cervical nerve root and spinal cord compression by disc herniations, tumours, or malformations might cause combined LMN symptoms in the arms and UMN symptoms in the legs, and be misdiagnosed as classical amyotrophic lateral sclerosis.[63] UMN-dominant amyotrophic lateral sclerosis or primary lateral sclerosis can be confused with hereditary spastic paraplegias or primary progressive multiple sclerosis. Additional, but rare, differential diagnoses include hyperparathyroidism and hexosaminidase A deficiency.[63] Since some of these disorders are treatable, these possibilities should be ruled out.

In conjunction with clinical presentation, genetic testing is increasingly used to explain disease cause and predict family risk. Risk amyotrophic lateral sclerosis genes can cause other syndromes or phenocopy alternative neurodegenerative diseases (appendix pp 11–13). *C9orf72* expansions, the most common amyotrophic lateral sclerosis gene, are linked to movement disorders[64,65] and phenocopy Huntington's disease in patients without huntingtin (*HTT*) expansions.[66] Conversely, patients with amyotrophic lateral sclerosis can have *HTT* repeat expansions simultaneously with TDP-43 inclusions.[67] Thus, patients could present with atypical amyotrophic lateral sclerosis, delaying diagnosis. Additional amyotrophic lateral sclerosis genes overlap with other syndromes and an improved understanding of the complexity of genotype–phenotype relationships will expedite the diagnosis of amyotrophic lateral sclerosis. Finally, the disease is associated with neuropsychiatric illnesses, such as psychosis and suicidal ideation,[68,69] thus, clinicians should obtain comprehensive detailed family history, not just of amyotrophic lateral sclerosis, but of neurodegenerative and neuropsychiatric illnesses.

Risk, progression, and pathophysiology

Identifying factors that increase amyotrophic lateral sclerosis risk and progression is central to patient diagnosis and care. Genetics are a major risk factor for amyotrophic lateral sclerosis (appendix pp 11–14). For instance, *C9orf72* expansions are penetrant and confer high risk, and are also associated with bulbar onset[14] and a decreased survival[70] in some studies. However, there are genetic mutations that confer risk but do not affect progression; therefore, risk and progression can be independent processes, and factors influencing either, or both, are an active area of research.[71]

A patient's cumulative environmental lifetime exposures, known as the exposome, is also increasingly recognised to confer amyotrophic lateral sclerosis risk and could accelerate disease progression.[72] Independent of whether risk is secondary to genetics or the exposome, a knowledge of amyotrophic lateral sclerosis pathophysiology will promote the development of novel treatment and prevention strategies, such as genetic therapies for asymptomatic carriers of highly penetrant pathogenic mutations.[73]

Molecular pathomechanisms

In amyotrophic lateral sclerosis, pathological processes arise from toxic gain-of-function or loss-of-function mutations to the approximately 40 known amyotrophic lateral sclerosis genes. Toxicity also occurs from aggregates of both wild-type and mutant proteins, which is a universal pathological feature in sporadic and familial amyotrophic lateral sclerosis.[74] Pathophysiological processes broadly fall into four major categories: impaired RNA metabolism, altered proteostasis or autophagy, cytoskeletal or trafficking defects, and mitochondrial dysfunction.[75] Several amyotrophic lateral sclerosis genes, including *C9orf72*, *TARDBP*, and *FUS*, impair RNA metabolism. Aggregation of the DNA and RNA binding proteins, TDP-43 and FUS, into inclusions impairs their normal function, causing broad changes to transcription and RNA processing. *TDP-43*, among several other amyotrophic lateral sclerosis genes, also dysregulates proteostasis and autophagy by preventing the clearance of damaged proteins. Multiple mutant amyotrophic lateral sclerosis genes, such as tubulin alpha 4a (*TUBA4A*) and profilin 1 (*PFN1*), induce cytoskeletal and tubulin defects, blocking axonal trafficking. Mitochondrial dysfunction, as triggered by *SOD1*, is a central characteristic for amyotrophic lateral sclerosis, which also increases oxidative stress.

Although much progress has been made, the full molecular underpinnings of the pathophysiology are incompletely understood. In addition to the major processes previously mentioned, TDP-43 and SOD1 aggregates also transfer from cell to cell in prion-like transmission,[76,77] which would propagate the pathology. *TARDBP*, *FUS*, and several other genes are linked to dysfunctional DNA repair in amyotrophic lateral sclerosis. For instance, loss of nuclear TDP-43 induces accumulation of double-stranded DNA breaks,[78] which would compromise genome stability. TDP-43 aggregates,[79] mutant *FUS*,[80] and *C9orf72* repeat expansions[81] also impair nucleocytoplasmic transport, the shuttling of cargo between the nucleus and cytoplasm.[79] Dipeptide repeat proteins derived from mistranslated *C9orf72* expansion transcripts are neurotoxic and might promote heterochromatin anomalies[82] and TDP-43 aggregation.[83]

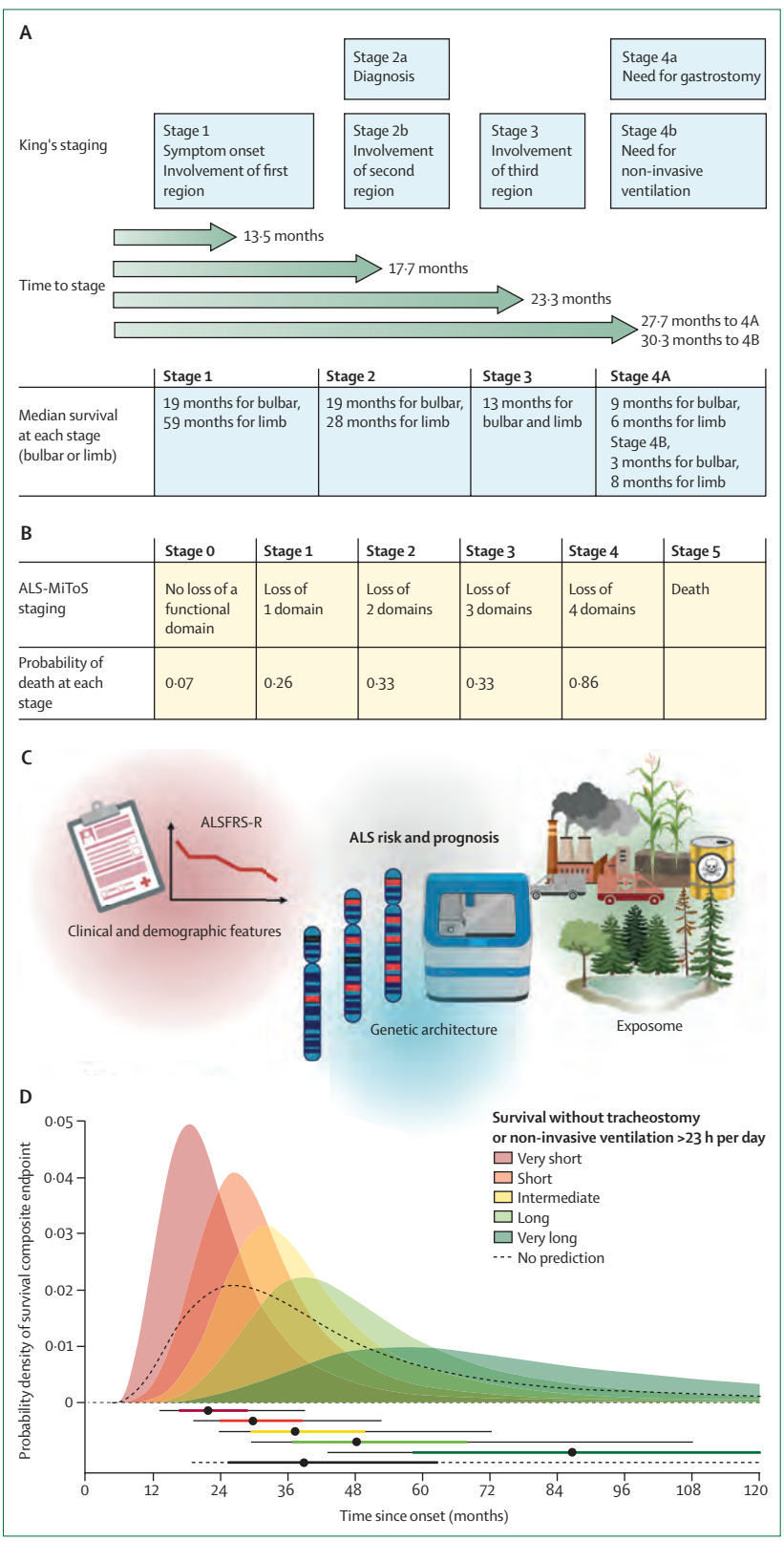
Central and peripheral inflammatory mechanisms are important contributors to amyotrophic lateral sclerosis,[84] both in the context of specific genetic mutations[85–87] and probably as a consequence of the general disease process in sporadic disease.[88,89] In amyotrophic lateral sclerosis, changes occur in specific immune cell populations,[88,89] their activation state,[88] and cytokine production.[86,87] Immune system involvement in amyotrophic lateral sclerosis is double-edged; a protective initial response is overcome by a destructive cytotoxic phase.[84] Hypermetabolism is also a broad characteristic,[90] both dependent and independent of mutations in amyotrophic lateral sclerosis, and metabolomics investigations[91] could provide information on the specific molecular changes that underscore disease progression. Pathways related to amyotrophic lateral sclerosis genes, inflammation, hypermetabolism, and other continued insights into the pathological mechanisms underlying the disease provide an essential knowledge base for therapeutic development and prevention strategies.

Environmental exposure

The gene–time–environment hypothesis of amyotrophic lateral sclerosis suggests that genetic susceptibility, age-related cellular damage, and a burden of environmental exposures combine to trigger amyotrophic lateral sclerosis.[60] Several lines of evidence support this model. First, genetic variants do not fully account for the disease.[92] Second, population-based modelling of amyotrophic lateral sclerosis indicates that disease occurs in a multistep process,[61] even in patients with highly penetrant monogenic mutations (eg, mutant *SOD1*).[93] Finally, a growing body of research supports the association of environmental exposures with disease risk, with a new focus on the amyotrophic lateral sclerosis exposome.[59]

The amyotrophic lateral sclerosis exposome is defined as the cumulative lifetime effect of environmental exposures, including lifestyle factors. Since the exposome involves exposures throughout a patient's lifespan, multiple study designs are needed to interrogate its role in amyotrophic lateral sclerosis. Many case-control studies have explored the relationship between occupational, residential, and avocational environmental risk factors on the risk of amyotrophic lateral sclerosis. Although studies leveraging population-based registries would provide a higher level of evidence, studies based on retrospective cohorts show reassuringly consistent results (appendix pp 16–17).

Of exposures with documented relevance to amyotrophic lateral sclerosis, plasma-persistent organic pollutants[94] and blood metals[95,96] correlate with disease risk and shortened survival.[72] Lifestyle factors associated with risk of amyotrophic lateral sclerosis include high cigarette pack-years, a low current BMI, and lifetime alcohol consumption.[97] Some relationships are dependent on *C9orf72* status,[97] showing an interaction between genes and environment. Physical activity as a risk is supported by several studies,[97,98] including analysis of the National Football League players.[99] Military service is also a recurring theme in risk assessments for amyotrophic lateral sclerosis.[100]



There are some important unanswered questions relating to the amyotrophic lateral sclerosis exposome. Are there periods of greater susceptibility to exposure throughout life, which increase the risk of amyotrophic lateral sclerosis? Will it be possible to adopt a preventative approach to the disease if modifiable risks are identified? Prospective studies using well curated population registries and biorepositories can help answer these questions and are a future goal of the field.[59]

Prognosis

Prognosis of amyotrophic lateral sclerosis is dependent on disease progression. Currently, clinicians monitor disease progression using the Amyotrophic Lateral Sclerosis Functional Rating Score-Revised (ALSFRS-R), a multidomain assessment that also serves as the gold standard for primary efficacy outcomes in clinical trials.[101] Respiratory function, which is a domain of the ALSFRS-R, provides prognostic information.[102] One shortcoming of the ALSFRS-R is that some subscores increase with symptom improvement despite continued underlying disease progression.[101,103] The Rasch-built Overall Amyotrophic Lateral Sclerosis Disability Scale was designed to specifically capture functional decline arising from the underlying disease course,[103] thereby overcoming the limitations of the ALSFRS-R. The Rasch-built Overall Amyotrophic Lateral Sclerosis Disability Scale awaits clinical validation before widespread adoption.

New staging examples have also been developed to inform prognosis. Patients assessed with the King's staging system[104] and Amyotrophic Lateral Sclerosis Milano-Torino Staging (ALS-MiToS)[105] system consistently

Figure 4: **Amyotrophic lateral sclerosis risk and prognosis**
(A) King's staging with four stages indicated (blue); time to progress to stages and median survival at each stage in months. (B) ALS-MiToS staging with six stages indicated (orange); staging based on four functional domains from the ALSFRS-R: (1) movement (walking and self-care; ALSFRS-R question 6 or 8); (2) swallowing (ALSFRS-R question 3); (3) communicating (ALSFRS-R questions 1 and 4); and (4) breathing (ALSFRS-R question 10 or 12). Intensifying colour indicates progression along stages for both King's and ALS-MiToS. (C) Schematic overview of factors that affect amyotrophic lateral sclerosis risk (onset) and prognosis, which include clinical and demographic features, genetic architecture (eg, rapidly progressive *SOD1*^{A5V} and slowly progressive *DCTN1* mutations), and exposome (eg, environmental exposures). (D) ENCALS prediction model of amyotrophic lateral sclerosis prognosis. Reproduced from Westeneng and colleagues,[70] with permission from Elsevier. The model defines five survival groups: very short (red; predicted median survival 17·7 months), short (orange; predicted median survival 25·3 months), intermediate (light orange; predicted median survival 32·2 months), long (light green; predicted median survival 43·7 months), and very long (green; predicted median survival 91·0 months). The dashed black line represents median survival without the use of the ENCALS prediction model, which is overly optimistic for patients with amyotrophic lateral sclerosis classified to the very short and short survival groups (ie, they end up with less time), and overly pessimistic for patients classified to long and very long groups (ie, they end up with more time). Horizontal bars have dots to represent median times to composite outcome, thick lines to represent probability IQR, and thin lines to represent 10–90% probability intervals to composite outcome. ALS=amyotrophic lateral sclerosis. ALS-MiToS=ALS Milano-Torino Staging. ALSFRS-R=ALS Functional Rating Score-Revised.

progress along stages that are associated with decreasing median survival (figure 4A, B). The King's staging system is more sensitive early in the disease course; the ALS-MiToS later in the disease course.[106,107] Neither staging system is yet in widespread clinical use.

Although median survival for amyotrophic lateral sclerosis is only 2–4 years, there is a broad distribution of individual patient survival, affecting both the clinician's ability to discuss and the patient's ability to understand disease prognosis. This variability is attributable to various factors that influence survival in amyotrophic lateral sclerosis (figure 4C), such as clinical and demographic features (eg, age at onset, site of onset, and presence of frontotemporal dementia), genetic architecture (eg, rapidly progressive *SOD1*^{A5V} and slowly progressive *DCTN1* mutations; appendix pp 14–15), and the exposome (eg, environmental exposures). The European Network for the Cure of Amyotrophic Lateral Sclerosis model was created to predict personalised survival (defined as survival without tracheostomy or non-invasive ventilation >23 h/day) based on eight parameters: onset age, time to diagnosis, ALSFRS-R progression rate, forced vital capacity, bulbar onset, definite amyotrophic lateral sclerosis by revised El Escorial criteria, frontotemporal dementia, and *C9orf72* repeat expansion (figure 4D).[70] Although not in routine clinical use, the European Network for the Cure of Amyotrophic Lateral Sclerosis prediction tool can potentially benefit patients by giving them a more accurate perspective of life expectancy.

Overall, accurate prognostication of the clinical course of the disease remains in its infancy since even predictions by the best models retain uncertainty. Thus, clinical care teams should advise patients and their families on the anticipated disease course and range of expected symptoms, with the caveat that these predictions can vary with each patient. Variation of disease phenotypes, even within the same family, attests to this unpredictability. Although clinical staging methods provide useful metrics for comparing participant stages in clinical research populations, their use in the clinic remains to be established.

Treatment

As amyotrophic lateral sclerosis remains incurable, treatment is focused on the use of disease-modifying therapies and maximising quality of life. The American Academy of Neurology, the European Federation of Neurological Societies, the UK National Institute for Health and Care Excellence,[108] and Amyotrophic Lateral Sclerosis Canada[109] have published evidence-based and expert consensus guidelines for managing amyotrophic lateral sclerosis, and supportive multidisciplinary care improves survival and quality-of-life for patients with amyotrophic lateral sclerosis (table 1).[110] The two medications with approval in some countries for slowing progression of amyotrophic lateral sclerosis are riluzole and edaravone. Riluzole, an antiglutamate agent, improves patient survival in clinical trials and postmarketing analyses, but whether this prolongation occurs at all stages of amyotrophic lateral sclerosis or just at advanced disease stages remains a topic of debate.[116,117] The antioxidant edaravone, given for 6 months, showed some efficacy in post-hoc analysis of the first phase 3 trial for participants, meeting the criteria of definite or probable amyotrophic lateral sclerosis (El Escorial and revised Airlie House diagnostic criteria), disease duration less than 24 months, forced vital capacity (lung function test) of more than 80%, and ALSFRS-R subscale scores all more than 2.[121] The trial was repeated prospectively with this defined patient population,[118,122] and again reported that edaravone slowed disease progression. However, this trial design could lack generalisability to the broader population of patients with amyotrophic lateral sclerosis and postmarketing analyses raise questions about edaravone's safety and benefits.[119,120] Thus, use of edaravone remains controversial and has not obtained worldwide approval. A combination of dextromethorphan and quinidine is approved in the USA for managing symptoms of pseudobulbar affect.[123] This drug is not marketed in all countries and alternative and more cost-effective treatments are available. Non-invasive ventilation also improves amyotrophic lateral sclerosis survival and quality of life.[124] For this reason, patients with amyotrophic lateral sclerosis should be regularly monitored for respiratory symptoms and undergo the appropriate respiratory assessments, such as overnight oximetry or measures for blood gas partial pressure of CO_2, blood bicarbonate concentrations, vital capacity, or maximum inspiratory pressure to confirm whether they qualify for non-invasive ventilation.[125]

Gastrostomy is also an effective therapy for supporting nutrition and is probably of greater benefit when established earlier in the disease course. Gastrostomy tubes can be inserted with percutaneous endoscopic gastrostomy, radiologically inserted gastrostomy, and per-oral image-guided gastrostomy placement with similar mortality.[126] Factors that are associated with a poor outcome after gastrostomy include use of non-invasive ventilation for more than 16 h/day, older age, BMI less than 20 kg/m², and recurrent accumulation of airway secretions.[127] High calorie nutrition has also been investigated for treating amyotrophic lateral sclerosis[90] and post-hoc analysis of a phase 3 trial suggests that it might be helpful for rapidly progressing patients,[128] although confirmatory trials are needed.

Several additional treatments are available (panel 3). Patients with amyotrophic lateral sclerosis might also contemplate alternative and off-label treatments, often found on the internet. Amyotrophic Lateral Sclerosis Untangled was conceived to provide a systematic review of unproven treatments. Care guidelines for amyotrophic lateral sclerosis encourage providers to have an open

For more on **Amyotrophic Lateral Sclerosis Untangled** see https://www.alsuntangled.com/

***Panel 3*: Treatments and interventions for management of amyotrophic lateral sclerosis**

Disease-modifying treatments

Disease progression

Only two drugs with regulatory approval are available, riluzole and edaravone. They are of marginal efficacy and only in select populations, and merely lengthen survival by a few months.[116–118] However, even within select populations, the efficacy of edaravone is contested.[119,120]

Symptomatic management

Comprehensive care

A multidisciplinary clinic plans the comprehensive, multidisciplinary care needed to manage symptoms in patients with amyotrophic lateral sclerosis. Care spans the management of respiration and oral symptoms (speech and swallowing); nutrition and gastrointestinal symptoms; pain and symptoms secondary to muscle loss; and cognition, mood, and behavioural changes.

Respiratory and oral symptoms

Bronchial secretions

Stop any provoking medications. Administer mucolytics if the patient exhibits sufficient cough flow, including N-acetylcysteine, anticholinergic bronchodilator, β-receptor antagonist and nebulised saline, furosemide, and guaifenesin. Mechanical or non-pharmacological approaches are also available, including manual assisted cough, mechanical insufflator–exsufflator, portable home suction device, and room humidifier. Additionally, patients are encouraged to remain hydrated or drink pineapple or papaya juice to break up secretions.

Dysarthria

Evaluate speech and language regularly and identify language impairments. Provide assistive communication tools, such as electronic writing, voice banking, and voice amplification devices.

Dyspnoea

Options include elevating the head of the bed, use of a hospital bed for elevation, non-invasive ventilation, and invasive tracheostomy ventilation.

Sialorrhea

Administer anticholinergics, such as amitriptyline, atropine ophthalmic drops, glycopyrrolate, and scopolamine patch. If sialorrhea is refractory to anticholinergics, botulinum toxin injections, external beam radiation therapy, and surgery can be considered. A portable suction device is a less aggressive approach. Dark grape juice and ginger tea are reported to decrease saliva production.

Nutrition and gastrointestinal symptoms

Constipation

Increase fluid and fibre intake or adjust enteral nutrition. Administer an osmotic or stimulant laxative. Increase physical activity.

Sources

EFNS Task Force on Diagnosis and Management of Amyotrophic Lateral Sclerosis,[62] the American Academy of Neurology,[111–113] the UK National Institute for Health and Care Excellence (NICE),[108] Amyotrophic Lateral Sclerosis Canada[109] and other Canadian guidelines,[114] and Bradley and Daroff's Neurology in Clinical Practice.[115] These therapies, in most cases, represent good clinical practice as few clinical trials involving patients with amyotrophic lateral sclerosis exist to provide a robust evidence base for these interventions.

dialogue about the use and risks of these treatments, especially as some can carry medical or financial risk.

Emerging directions in amyotrophic lateral sclerosis

Novel treatment approaches

Recognition of heterogeneity, genetics, and a deeper understanding of pathophysiology in amyotrophic lateral sclerosis brings new treatment approaches to the amyotrophic lateral sclerosis community. This recognition promotes new trial designs to address heterogeneity, genetic therapies, immune-targeting agents against inflammation, and stem cells to enrich the CNS environment.

New trial designs

New amyotrophic lateral sclerosis clinical trials can leverage a basket design of targeted agents against participant populations defined by phenotypes or genetics.[129,130] Novel platform trial designs simultaneously evaluate multiple therapies in distinct arms against a single placebo group, lowering the number of required participants and shortening trial duration.[129] Adaptive designs can further shorten trial duration by response-adaptive randomisation, which increases participant allocation to more promising treatment groups.[129] Several major trials with novel compounds and treatment approaches are currently underway (appendix pp 18–23).

Genetic therapies

There is a growing consensus that gene therapy is a promising avenue in amyotrophic lateral sclerosis. One strategy is silencing toxic gain-of-function genes by targeting mRNA and pre-mRNA with antisense oligonucleotides. The first clinical trial of the *SOD1* antisense oligonucleotide, BIIB067, showed safety, evidence of target engagement, and promising trends in exploratory secondary outcome measures.[131] However, the phase 3 clinical trial did not meet its primary efficacy outcome of slowing disease progression as measured by the ALSFRS-R, although cerebrospinal fluid (CSF) SOD1 protein and neurofilament concentrations were significantly decreased.[132] A new approach is earlier

intervention with BIIB067 during the presymptomatic phase of disease in mutant *SOD1* carriers (NCT04856982; appendix p 19). Clinical trials are also underway of antisense oligonucleotides that target other autosomal dominant gain-of-function mutations, including *C9orf72*, *FUS*, and *ATAXN2*.[133]

Antibodies

Monoclonal antibodies against mutant *C9orf72* and *TDP-43* are in preclinical development.[134] Several clinical trials have also been launched, but besides reporting safety, none were effective (eg, tocilizumab and ozanezumab).[134] A few antibody candidates are still in the clinical trial pipeline, including AP-101 against *SOD1* aggregates (NCT05039099), ANX005 against C1q protein (NCT04569435), and AT-1501 against CD40L protein (NCT04322149; appendix pp 18–23).

New anti-inflammatory therapies that target the immune system are also in the clinical pipeline (appendix pp 18–23). Phase 1/2 clinical trial results report that low-dose interleukin-2 is well tolerated and immunologically effective in increasing regulatory T-cell numbers, although its effect on progression of amyotrophic lateral sclerosis is still being evaluated in a phase 2b/3 trial (MIROCALS).[135] Autologous infusion of expanded Treg cells in a small patient cohort slowed disease progression.[136] Masitinib, a tyrosine kinase inhibitor, reduces microglial activation and showed promise in a phase 2/3 trial.[137] These reports underscore the feasibility of immune-targeting drugs as candidate therapies for amyotrophic lateral sclerosis.

Stem cells offer the unique opportunity to simultaneously target multiple dysregulated pathways while providing CNS neurotrophic support.[138] They can derive from diverse sources (eg, mesenchymal stem cells and neural progenitor cells [appendix pp 18–23]), each offering distinct advantages and disadvantages.[138] One meta-analysis concluded that adult stem cells are safe and well tolerated,[139] however, apart from a possible transient positive effect, trials have not shown long-lasting efficacy from stem cells.

Novel diagnostic biomarkers

There is an urgent need for amyotrophic lateral sclerosis biomarkers to expedite diagnosis, particularly in atypical phenotypes, and enable improved prognosis of disease course. Biomarkers can also refine clinical trial participant stratification, facilitate the estimation of progression rates, monitor target engagement, and detect early potential treatment effects.

Neurofilaments

CSF and plasma neurofilaments are well characterised and promising fluid biomarkers. Elevated CSF and plasma neurofilament light chain concentrations correlate with shorter survival, more aggressive disease phenotypes, and presence of *C9orf72* expansion.[140–142] Plasma neurofilaments are also elevated up to 5 years before disease onset in sporadic and familial cases of amyotrophic lateral sclerosis,[143,144] and indicate phenoconversion in clinically asymptomatic mutant *SOD1* carriers.[143] Some 2020 clinical trials support their use as pharmacodynamic markers of amyotrophic lateral sclerosis progression.[131,145]

Regarding brain imaging, although routine MRIs cannot diagnose amyotrophic lateral sclerosis, MRIs with quantitative analysis of fluid-attenuated inversion recovery can identify increased corticospinal tract and corpus callosum intensities in patients with amyotrophic lateral sclerosis.[146] More advanced structural and functional MRI techniques are not yet in routine clinical practice but might provide new diagnostic biomarkers. Examples include diffusion tensor imaging[147,148] and multimodal[147,149] approaches, such as quantitative susceptibility mapping to detect iron-related motor cortex changes, and connectome analyses of motor and non-motor networks. T1-weighted imaging and diffusion tensor imaging detect abnormalities (cortical and subcortical atrophy and white matter changes) already present in presymptomatic *C9orf72* repeat expansion carriers.[150] Although not a disease-specific biomarker, positron emission tomography by use of tracers to quantify brain metabolism ([^{18}F]-fluorodeoxyglucose) or glial activation ([^{11}C]-PBR28) provides new insights into disease mechanisms and could prove useful as pharmacodynamic indices in future clinical trials.[151,152]

Neurophysiological markers

Neurophysiological markers of disease-associated changes are currently available. Spectral electroencephalogram mapping reveals brain connectivity changes in amyotrophic lateral sclerosis, which correlate with MRI findings and could become useful, cost-effective markers of cortical network disruption.[153,154] Magnetoencephalography shows enhanced connectivity during progression of amyotrophic lateral sclerosis.[155]

Cortical motor neuronal hyperexcitability can sometimes be detected by routine transcranial magnetic stimulation (TMS); however, more often, refined techniques such as threshold-tracking TMS measuring short-interval intracortical inhibition and intracortical facilitation are necessary to detect subclinical UMN involvement.[156] Cortical hyperexcitability across phenotypes of amyotrophic lateral sclerosis distinguishes the disease from non-amyotrophic lateral sclerosis disorders, correlates with clinically affected body regions,[157] disease spread,[157] and cognitive dysfunction.[158] TMS might also have a role in prognosis, with increased cortical hyperexcitability associated with longer disease duration[159] and cortical inexcitability with poorer clinical trajectory.[160] Change in short-interval intracortical inhibition was the primary endpoint in a phase 2 amyotrophic lateral sclerosis trial of patients with amyotrophic lateral sclerosis given retigabine, a potassium channel activator, showing the potential of neurophysiological outcome measures as pharmacodynamic disease markers.[161]

LMN degeneration can be quantified by the non-invasive motor unit index, which correlates with the number of functioning motor units.[156] This index detects motor unit decline already in clinically unaffected muscle groups and can monitor motor unit loss over time. When used as an outcome measure in clinical trials, the index requires thorough qualification of the rater to ensure reliability.[162]

Conclusions

Amyotrophic lateral sclerosis remains difficult to diagnose and manage. This difficulty is due to heterogenous presentation and multiple disease phenotypes, and the overlap of symptoms and signs with other illnesses. Early in the diagnostic process, physicians should refer patients presenting with progressive dysarthria, dysphagia, limb weakness, or respiratory failure to a neurologist. This referral aligns with suggestions by advocate groups, as they lobby to help patients seek early treatment and enrol in clinical trials. Unfortunately, there are no effective disease-modifying drugs, and treatment revolves around multidisciplinary care to manage symptoms and aid end-of-life planning.

Research into improved diagnostic and prognostic tools could expedite diagnosis and give patients a better understanding of their disease course. Thus, we anticipate future directions in clinical management of the disease will move towards simpler diagnostic criteria, such as the Gold Coast criteria, and widespread genetic testing. Research will evaluate whether newly developed scoring, staging, and predictive tools will give patients meaningful and accurate insight into their anticipated clinical trajectory. Pathophysiology research and novel trial designs are developing rational, targeted candidates, which are passing through the clinical testing pipeline more efficiently. We anticipate that these research efforts will translate into improved outcomes for current and future patients with amyotrophic lateral sclerosis.

Contributors

All authors contributed to conceptualisation, writing of the original draft, and reviewing and editing it.

Declaration of interests

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REVIEW ARTICLE

ALSUntangled #67: rituximab

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Abstract

ALSUntangled reviews alternative and off-label treatments on behalf of people with ALS who ask about them. Here we review rituximab, a drug which specifically depletes B lymphocytes. We show a current lack of evidence for a role of these cells in ALS progression. The one patient we found who described using Rituximab for their ALS found no benefit. Given all this, and the known serious risks of rituximab, we advise against its use as an ALS treatment.

Keywords: *ALS, rituximab, neuroinflammation, off-label treatment*

Introduction

ALSUntangled reviews alternative and off-label treatments for ALS on behalf of people living with ALS (PALS). Here we review rituximab, for which we have had 445 requests (https://www.alsuntangled.com/future-reviews/).

Background

Rituximab is a first-generation chimeric monoclonal antibody generated by fuzing a rodent Fab domain with a human Fc domain (1). It selectively targets and rapidly depletes circulating CD20+ B lymphocytes, and via this action it is used to treat

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autoimmune diseases such as rheumatoid arthritis and hematological malignancies such as chronic lymphocytic leukemia (CLL) and non-Hodgkin's lymphoma (NHL) (1–3). Rituximab is also increasingly utilized as a second or third line treatment for autoimmune encephalitis, neuromyelitis optica, inflammatory neuropathy and myasthenia gravis (4–7).

Mechanism

Over the past few decades, evidence has emerged to support the downstream role of neuroinflammation in the progression of ALS. Microglial activation, perivascular infiltration of monocytes and T cells, and release of proinflammatory cytokines are pathological manifestations in ALS brain and spinal cord tissues (8,9). Microglia and macrophages are the primary sources of proinflammatory molecules (10). To date, no convincing evidence has emerged supporting a direct role of B cells in ALS.

B cells are part of the adaptive immune system and lead to the production of antibodies. CD20 is a marker expressed on most B cells, including immature, naïve and mature memory B cells (11). Mature CD19+/CD20+ memory B cells express IgG on cell surface and evolve into antibody secreting plasmablasts and plasma cells. CD19+ plasmablasts are located at the periphery and have various levels of CD20 expression (12). CD38+ plasma cells reside in the bone marrow and most also express CD19+ (13). Although antibodies against gangliosides and other proteins have been detected in PALS (14,15), the function of these antibodies in ALS pathogenesis is unclear; they may simply be an epiphenomenon of neurodegeneration, rather than a cause. Indeed, B cell surface markers (CD19, CD45, CD69) and serum immunoglobulin levels do not differ between $SOD1^{G93A}$ ALS mice and wild type mice (16). Moreover, depleting B cells from $SOD1^{G93A}$ mice does not affect limb strength, onset of limb paralysis or survival time (16,17). These studies suggest that B lymphocytes play a less pivotal role in ALS progression (16,17). However, in another study using this same animal model, transfer of IL-10+ B cells decreased myeloid-derived macrophages in the central nervous system and was associated with a trend toward improved neuromuscular function (though this did not prolong survival,18). Notably, the SOD1 ALS model may not be the idea disease model because only 20% of familial and 5% of sporadic PALS carry an SOD1 gene mutation. Further studies are required to clarify whether B cells play any significant role in ALS progression.

Based on the theory that rituximab might reduce neuroinflammation (albeit with no current evidence for a specific effect of B cells in this process), we assign a TOE "Mechanisms" grade of D.

Pre-clinical models

Recent pre-clinical studies show B cells are present in the meninges and likely derived locally from calvaria (19). Moreover, pre-B cells accumulate in the spinal cord meninges of mutant SOD1 mice (20). However, we did not find published studies examining rituximab treatment in preclinical ALS models. Unpublished work suggests that intrathecal administration of rituximab IgG Fc fragment to the presymptomatic $SOD1^{G93A}$ mice lowered B cell counts and extended survival by one month (the unpublished data were obtained from Dr. Rachel Lichtenstein, contributing author of this review). Thus, we assign a TOE "Pre-clinical" grade of D.

Cases

In the online community PatientsLikeMe, we found three people who reported taking rituximab for their ALS (https://www.patientslikeme.com/treatments/detail/rituximab). The only person who provided any details stated that they perceived no benefits from this treatment (https://www.patientslikeme.com/treatment_evaluations/browse?attribute=efficacy&brand=false&condition_id=9&id=7572&value=1). Therefore, we assign a TOE "Cases" grade of F.

Trials

We found no clinical trials using rituximab in PALS. We therefore assign a TOE "Trials" grade of U.

Risks, dosing, costs

There are no data examining risks in large numbers of PALS exposed to rituximab. Significant adverse events have been documented in patients with rheumatoid arthritis, systemic vasculitis and non-Hodgkin's lymphoma treated with Rituximab, especially in the COVID era. Infusion reactions are common, which include fever, headache, pruritus, flushing, and hypotension (21). These reactions are often mild to moderate; however severe anaphylactic reactions have been reported (22). Rituximab can reactivate previous hepatitis B and hepatitis C virus infection (23,24). Therefore, testing for hepatitis B and hepatitis C infection status are recommended before drug administration. Rituximab has also been reported to cause neutropenia (25,26) and hypogammaglobulinemia (27), which further increase the associated risk of infection. Finally, rituximab can lead to progressive multifocal leukoencephalopathy (PML), a rare but severe and fatal central nervous system infection caused by JC virus (28). In light of these findings, FDA has issued black box warnings for fatal infusion reactions, severe mucocutaneous reactions, PML, and reactivation of hepatitis B infection.

Table 1. Table of evidence grades for rituximab.

	Grade	Explanation
Mechanism	D	B cells are found in CNS meninges and could theoretically influence neuroinflammation. However, depleting B cells in the mutant SOD ALS mouse model did not change disease course.
Pre-Clinical	D	We did not fund published studies of Rituximab in pre-clinical models of ALS. Unpublished data suggest intrathecal administration of Rituximab Fc fragment to ALS mouse model reduces B cell number and modestly extends survival time.
Cases	F	The only person we could find who reported on their experience with Rituximab as an ALS treatment found no benefit
Trials	U	We found no clinical trials of Rituximab in PALS
Risks	F	There is no risk data on PALS being exposed to Rituximab. More than 5% of all exposed patients in other autoimmune conditions including autoimmune neurological disorders experienced serious adverse effects.

Rituximab has been reported to have similar side effects when it is used off-label in neurological conditions (29–31). Based on the frequency of serious adverse events in various populations, we assign a TOE Grade of F (Table 1).

When it is used in rheumatoid arthritis, rituximab is administered as two 1000 mg dose IV infusions separated by 2 weeks, followed by a repeat course every 24 weeks for responders (32). In ANCA-associated vasculitis, rituximab is given as 375 mg/m^2 every week for four weeks during the induction phase (33) followed by maintenance treatment of 500 mg every six months for two years or longer (34). Both dosing strategies have been utilized in autoimmune neurological diseases. Complete B cell depletion occurs within 14 days and can last for 6–12 months. Therefore, rituximab is typically re-dosed at a 6-month interval. Recommendations vary among different diseases regarding re-dosing intervals. A fixed 6-month repeat interval and repeat course based on circulating CD19+ B cell counts have both been utilized (34,35).

The cost of rituximab treatment, including the drug and the infusion, is approximately $1000 per 100 mg (information from UpToDate).

Conclusion

Neuroinflammation is associated with disease progression in PALS. Rituximab depletes a population of immune cells, so it could theoretically help with slowing progression. However, rituximab specifically acts on B cells and the importance of these specific cells in ALS progression is still unclear; further studies are needed to elucidate this. The one person we found who reported taking it for ALS perceived no benefit. We found no trials of rituximab in ALS. Considering the side effect profile and lack of evidence to support its efficacy, we do not currently recommend the use of rituximab as an ALS treatment.

Declaration of interest

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REPORT

ALSUntangled #68: ozone therapy

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Abstract

ALSUntangled reviews alternative and off-label treatments for people living with amyotrophic lateral sclerosis (PALS). Here we review ozone therapy. Ozone therapy has possible mechanisms for slowing ALS progression based on its anti-oxidant, anti-inflammatory, and mitochondrial effects. A non-peer-reviewed report suggests that ozone treatment may slow progression in a mTDP-43 mouse model of ALS. One verified "ALS reversal" occurred on a cocktail of alternative treatments including ozone. There are no ALS trials using ozone to treat PALS. There can be potentially serious side effects associated with ozone therapy, depending on the dose. Based on the above information, we support an investigation of ozone therapy in ALS cell or animal models but cannot yet recommend it as a treatment in PALS.

Keywords: *ALS, ozone therapy, oxidative stress, neurodegeneration, alternative therapy*

Introduction

ALSUntangled reviews alternative and off-label treatments on behalf of people living with amyotrophic lateral sclerosis (PALS). Here we review ozone therapy, for which we have had 556 requests (1).

Overview

Ozone is a gaseous molecule with a pungent smell. It is generated when diatomic oxygen (O_2) is exposed to an electrical field or ultraviolet light, which causes a portion of the diatomic oxygen molecules to split into individual oxygen atoms.

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These free oxygen atoms combine with diatomic oxygen molecules to form ozone (O_3). Ozone is an unstable molecule due to the weak bonds holding the third oxygen atom, rendering it a powerful oxidizing agent which is used to disinfect and sanitize water and hard surfaces (2).

There are currently no FDA-approved medical indications for ozone therapy. In fact, the FDA has advised against the medical use of ozone (3) and reportedly even shut down clinic advertisements touting ozone as a medical treatment (4). Nonetheless, different experimental and/or off-label methods have been and still are being employed to administer ozone to the human body, including local ozone injection as well as systemic administration *via* infusion of ozonized saline solution, rectal ozone insufflation, or using an ozone sauna, wherein a body part is bagged and exposed to ozone gas. Ozone autohemotherapy involves collecting venous blood from a patient, blending it with an oxygen/ozone mixture and then reinfusing it via the same vein (5). This review focuses on systemic ozone application (e.g. infusion, rectal insufflation, sauna, or autohemotherapy). Ozone therapy is different from hyperbaric oxygen therapy, which was reviewed separately in a previous ALSUntangled paper (6). Ozone is currently being offered as an ALS treatment on multiple websites (e.g. 7,8).

Mechanisms

Oxidative stress, neuroinflammation, and mitochondrial dysfunction are believed to play roles in ALS pathophysiology. There is some evidence that ozone therapy might modify these processes.

Antioxidant effects

Oxidative stress is an imbalance between production of damaging reactive oxygen species and their elimination by antioxidants. Oxidative stress can lead to protein misfolding and insoluble inclusions, which are associated with ALS (9,10). As a potent oxidizer, ozone can transiently worsen oxidative stress (11). However, this worsening in turn can activate the nuclear factor-related erythroid factor 2 (Nrf2) pathway, ultimately leading to the transcription of antioxidant response elements (AREs, 11,12). In small study of patients with multiple sclerosis, ozone therapy (20 ug/ml delivered rectally three times per week) was associated with increased markers of antioxidant activity and decreased markers of oxidative damage to lipids and proteins (12).

Anti-inflammatory effects

Neuroinflammation, characterized by microglial and astrocyte activation, as well as T lymphocyte infiltration, is associated with the progression of ALS (13). In rats, inhaled ozone can promote neuroinflammation (14). However, ozone delivered *via* injection to rats reportedly reduces pro-inflammatory cytokines by blocking the action of nuclear factor-kB (NF-kB) and promoting the Nrf2 pathway (15). In small numbers of patients with multiple sclerosis, ozone delivered rectally was associated with increased Nrf2 phosphorylation and decreased pro-inflammatory cytokine expression (12); ozone delivered via autohemotherapy was associated with increased expression of anti-inflammatory Treg cells (16).

Mitochondrial effects

Mitochondria produce the energy required for most of the cellular processes. Mitochondrial dysfunction is proposed to play a role in ALS progression (17). In animal models, ozone was shown to reduce mitochondrial damage in rat models of ischemia-reperfusion heart injury (18) and noise-induced hearing loss (19). It is not clear how similar the mitochondrial dysfunction seen in these animal models is to that seen in PALS.

Since ozone therapy is associated with reduced markers of oxidative stress and inflammation in small studies of humans with multiple sclerosis (12,16), ALSUntangled assigns a TOE "Mechanism" grade of A (Table 1).

Pre-clinical models

Ozone therapy has been studied in mSOD1 and mTDP43 mouse models of ALS. In mSOD1 mice, intraperitoneal injections of ozone for five consecutive days starting at symptom onset were associated with reduced markers of neuroinflammation and increased motor neuron counts in

Table 1. Table of evidence for ozone therapy.

	Grade	Explanation
Mechanism	A	Peer-reviewed publications show that ozone treatment is associated with decreased markers of oxidative stress and inflammation in patients with multiple sclerosis.
Pre-clinical	D	Non-peer- reviewed reports suggest that ozone therapy was associated with improved motor performance and prolonged survival in a mTDP43 mouse model of ALS.
Cases	C	One unpublished case report with validated diagnosis and improvements (however, ozone therapy was part of many treatments used)
Trials	U	Ozone therapy has not been studied in ALS trials
Risks	D	More than 0% but less than 5% of exposed patients experienced death or hospitalizations

some but not all areas of the brain (20). However, these injections had no effect on motor performance or survival (21). In 42-d-old mTDP43 mice, aerosolized ozone treatment for 4 h a day for 15 d was associated with improved motor performance and lengthened survival compared to animals treated with filtered air (22). Ozone therapy was also associated with several metabolic changes related to glucose regulation and insulin resistance in this mouse model (21,22). These mTDP43 studies were flawed by very small sample sizes and, as of this writing; they have not been published in peer-reviewed journals. Therefore, ALSUntangled assigns a TOE "Pre-Clinical Models" grade of D (Table 1).

Data in PALS

Cases

In the online community PatientsLikeMe, three members report receiving ozone therapy as a treatment for ALS. Two PALS completed treatment evaluations and both rated effectiveness as "slight" (23). We did not have records to verify the diagnoses nor the perceived benefits of ozone in these patients. Google search identified the website of Mr. Kim Cherry, who reports that his ALS reversed on a regimen of ozone treatments, in addition to hyperbaric oxygen therapy, various vitamins and supplements, detox, special diets, and attitude changes (24). His ALS diagnosis and his improvements have been independently verified by our group (25,26). Mr. Cherry's disease onset was mid-2010 and slowly progressed to his nadir in January 2012, at which time his ALSFRS-R score was 31. His ALSFRS-R score in August 2015 had improved to 47 (6). Associations like this do not prove causality.

Based upon these cases, we assign a TOE "Cases" grade of C (Table 1).

Trials

Ozone therapy has not been evaluated in an ALS clinical trial. As such, ALSUntangled assigns a TOE "Trials" grade of U (Table 1). Of potential interest, ozone therapy has been trialed in several other neurological conditions (reviewed in reference 11), including ischemic stroke (27), fibromyalgia (28), and multiple sclerosis (12,16,29). None of these trials produced results compelling enough to warrant FDA approval.

Dosing, risks, and costs

There is an online protocol for using ozone as an ALS treatment (30). However, it is not clear to us that this protocol has ever been studied so we do not know what benefits and/or side effects it might produce. Ozone therapy should never be administered by inhalation because of the risk of life-threatening pulmonary edema (3,31). The dose-effect relationship of ozone therapy delivered in other ways (autohemotherapy, ozonized saline solution, insufflation, etc.) is hormetic (32). This means that low doses can be anti-oxidant and anti-inflammatory, but higher doses can be toxic. According to the Madrid Declaration of ozone therapy (31), an online document written by scientists, dentists, pharmacists, and physicians with interests and experience in administering ozone, the potential therapeutic dosage for systemic treatment ranges between 5.0 and 6.0 mg per treatment, and concentrations of 10–50 μg/Nml are safe. Non-serious adverse events may occur at these doses, related to the administration technique. For example, side effects of autohemotherapy can include itching on lips and tongue, nausea, bad taste in the mouth, and dyspnea. Rectal insufflation can cause bloating and constipation. Higher doses of ozone may cause serious side effects, including stroke, myocardial infarction, and death (31). Given all this, if ozone therapy is at all useful in the treatment of ALS, the therapeutic dosing range is likely quite narrow. Because of the small risk of serious side effects including death, we assign a TOE "Risks" grade of D.

The cost of ozone therapy is variable. Clinics administering ozone intravenously charge anywhere from $100 to over $1000 per session (33). In our opinion, it is unlikely that insurance would cover this. In terms of devices currently on the market that can generate ozone by design or as a byproduct, FDA has regarded them as adulterated and/or misbranded if used or intended for use in any medical condition for which there is no proof of safety and effectiveness (3).

Conclusion

Ozone therapy has possible mechanisms for treating ALS. A preclinical study in very small numbers of mTDP43 mice (which has yet to be peer-reviewed) suggested benefits on motor function and survival (21,22); however, these benefits were not seen in mSOD1 mice (20). One verified "ALS reversal" occurred on a cocktail of alternative therapies including ozone (24); an association such as this does not prove causality. There have been no trials of ozone therapy in PALS. There may be potentially serious side effects associated with ozone therapy, depending on the dose (31). Based on all this, we support further investigation of ozone therapy in ALS cell or animal models, but we cannot yet recommend it as an ALS treatment.

Declaration of interest

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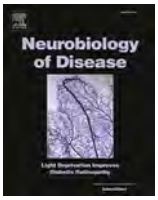

Glial-neuron crosstalk in health and disease: A focus on metabolism, obesity, and cognitive impairment

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ABSTRACT

Dementia is a complex set of disorders affecting normal cognitive function. Recently, several clinical studies have shown that diabetes, obesity, and components of the metabolic syndrome (MetS) are associated with cognitive impairment, including dementias such as Alzheimer's disease. Maintaining normal cognitive function is an intricate process involving coordination of neuron function with multiple brain glia. Well-orchestrated bioenergetics is a central requirement of neurons, which need large amounts of energy but lack significant energy storage capacity. Thus, one of the most important glial functions is to provide metabolic support and ensure an adequate energy supply for neurons. Obesity and metabolic disease dysregulate glial function, leading to a failure to respond to neuron energy demands, which results in neuronal damage. In this review, we outline evidence for links between diabetes, obesity, and MetS components to cognitive impairment. Next, we focus on the metabolic crosstalk between the three major glial cell types, oligodendrocytes, astrocytes, and microglia, with neurons under physiological conditions. Finally, we outline how diabetes, obesity, and MetS components can disrupt glial function, and how this disruption might impair glia-neuron metabolic crosstalk and ultimately promote cognitive impairment.

1. Introduction

The burden of dementia, defined as an impairment in mental capacity that interferes with daily function, is growing at a rapid pace as the population increases in size and age. Dementia is not one specific disorder but rather a constellation of signs and symptoms, which include poor judgement, faulty executive function, poor memory, and declining social skills. Alzheimer's disease (AD) is a complex neurodegenerative brain disease, which results in symptoms of dementia and constitutes the bulk of dementia cases. However, cognitive impairment occurs on a continuum, from mild cognitive impairment (MCI) leading up to the more serious loss of cognitive function present in frank AD. Although well-known AD risk genes exist, most notably apolipoprotein E ε4 (APOE ε4) (Serrano-Pozo et al., 2021), the vast majority of AD cases are sporadic and lack a known genetic determinant. Multiple clinical studies have identified associated AD risk factors, including components of the metabolic syndrome (MetS), such as diabetes (Biessels and Despa, 2018) and obesity (O'Brien et al., 2017). AD itself is also characterized by dysfunctional metabolism, including insulin resistance (Kim and Feldman, 2015; Kellar and Craft, 2020) and impaired glucose and lipid metabolism in the brain (Butterfield and Halliwell, 2019; Zhu et al., 2019). Thus, impairment of both systemic and brain metabolism are intimately linked to neurodegeneration and are important areas of ongoing inquiry.

This relationship between systemic and brain metabolism with neuronal health is intuitive. Neurons rely on well-coordinated bioenergetics to transmit signals, turnover neurotransmitters, and regulate synaptic and dendritic spine formation. Thus, breakdown in bioenergetics will impair each of these functions and would eventually lead to neurodegeneration and AD. Moreover, neurons are aided in these roles by glia, and AD is now increasingly believed to progress in a non-cell autonomous manner (Heneka et al., 2015), suggesting that AD entails a breakdown in axo-glial communication. However, the precise mechanisms remain to be elucidated.

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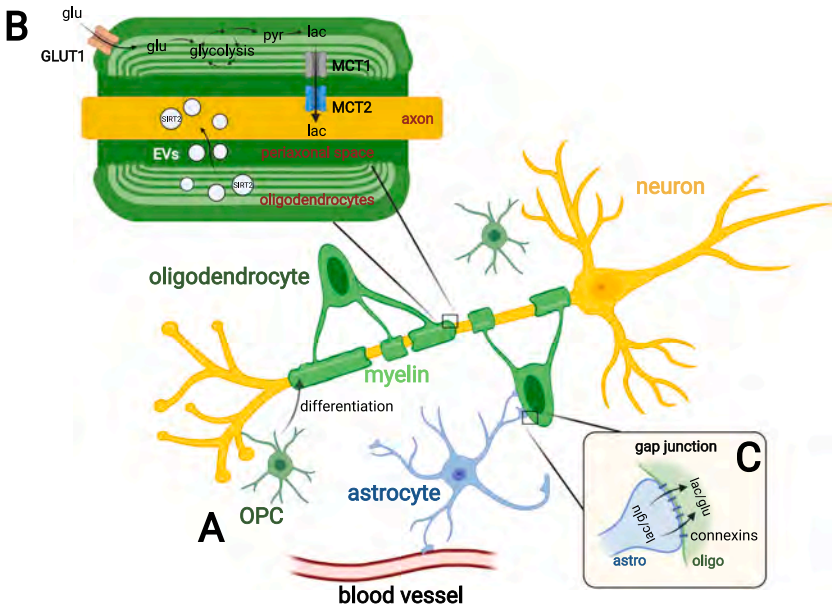

Fig. 1. Oligodendrocyte-neuron interactions under homeostatic conditions.
(A) Oligodendrocytes develop from the differentiation and maturation of oligodendrocyte precursor cells (OPCs) during embryogenesis and, to a lesser extent, in adulthood during myelin turn-over. The primary oligodendrocyte function is axon myelination in the CNS, which brings oligodendrocytes and axons into close contact. (B) Oligodendrocytes also metabolically support neurons. Glucose is taken up by oligodendrocytes through GLUT1 and undergoes glycolysis to pyruvate followed by conversion to lactate to provide metabolic aid to axons. Lactate is released from oligodendrocytes through MCT1 into the periaxonal space, where it enters the axon through MCT2. Oligodendrocytes also communicate with neurons by releasing EVs, which contain enzymes, such as SIRT2, a gluconeogenesis regulator, potentiating neuronal metabolic activity. (C) Oligodendrocytes use gap junctions to metabolically interact with both neurons and astrocytes by enhancing the exchange of lactate through connexins.
astro, astrocyte; CNS, central nervous system; EVs, extracellular vesicles; glu, glucose; GLUT1, glucose transporter 1; lac, lactate; MCT, monocarboxylate transporter; oligo, oligodendrocyte; OPC, oligodendrocyte precursor cell; pyr, pyruvate; SIRT2, NAD-dependent deacetylase sirtuin 2.

In this review, we present the studies supporting the evolving idea that disrupting axo-glial metabolic crosstalk can promote neurodegeneration and AD. First, we outline the clinical evidence demonstrating that systemic metabolic dysfunction increases the risk of AD. Next, we summarize the known mechanisms of axo-glial metabolic crosstalk under physiological conditions. Finally, we discuss how disruption of this crosstalk under pathological conditions promotes cognitive impairment.

2. MetS, diabetes, obesity, and cognitive impairment

Systemic metabolic dysfunction in humans is characterized by the presence of the metabolic syndrome (MetS). The MetS is defined as three out of five metabolic criteria: elevated waist circumference (≥102 cm males, ≥88 cm females; i.e., obesity), increased systolic (≥130 mmHg) or diastolic blood pressure (≥85 mmHg), increased triglycerides (≥150 mg/dL), elevated fasting blood glucose (>100 mg/dL; i.e., prediabetes, diabetes), and lower high-density lipoprotein cholesterol (HDL-c; <40 mg/dL males, <50 mg/dL females) (Grundy et al., 2005). Over the past decade, several clinical studies have shown that the components of MetS are dementia risks.

In diabetes patients with hyperglycemia, impairment commences very subtly as so-called diabetes-associated cognitive decrements but can progress to MCI or overt AD (Biessels and Despa, 2018). Cognitive impairment secondary to diabetes is accompanied by structural brain changes and pathological processes, such as central insulin resistance (Arnold et al., 2018), inflammation, and oxidative stress (Biessels and Despa, 2018). Cross-sectional analysis of the Spanish PREDIMED-PLUS study (n = 6823) found that older (mean 65 years), overweight or obese type 2 diabetes (T2D) participants with glycated hemoglobin (HbA1c) < 53 mmol/mol (7%), a hyperglycemia surrogate, had better executive function versus participants above this level, after various adjustments, including education level (Mallorquí-Bagué et al., 2018). The US ARIC study of older participants (n = 5099), spanning four states, identified risks for incident MCI at a 5-year median follow-up, which include diabetes (adjusted hazard ratio [HR] 1.14 [95% confidence interval (CI) 1.00, 1.31]), poor glycemic control based on HbA1c in diabetic individuals (HR 1.31 [95%CI 1.05, 1.63]), and longer diabetes duration (≥5 vs. <5 years; HR 1.59 [95%CI 1.23, 2.07]), adjusted for several covariates and education (Rawlings et al., 2019). Diabetes already in midlife can have far-reaching consequences in later-life; analysis of the Japan Public Health Center-Based Prospective Study (n = 12,219, aged 40–59 years) found that diabetes correlated positively with incident dementia risk (odds ratio [OR] 2.60 [95%CI 1.12, 6.03]. Thus, overall diabetes associates with dementia or MCI in cross-sectional and longitudinal studies and across diverse populations.

Several studies also report an association between dyslipidemia and central obesity with cognitive impairment and dementia. As with diabetes, obesity has pathological processes in common with AD, such as inflammation and mitochondrial dysfunction (O'Brien et al., 2017). In a cross-sectional US Michigan cohort (n = 184), obese, but normoglycemic, participants performed more poorly on the NIH Toolbox versus lean normoglycemic controls, after multiple adjustments, including education level (Callaghan et al., 2020). This study indicates that elevated waist circumference is a risk for cognitive impairment independent of hyperglycemia. A meta-analysis of 21 longitudinal studies with a minimum 2-year follow-up found that being overweight or obese correlated positively with incident dementia (risk ratio [RR] 1.41 [95%CI 1.20, 1.66] in participants less than 65 years old; interestingly, the trend reversed above 65 years of age (RR 0.83 [95%CI 0.74, 0.94]) (Pedditzi et al., 2016). Another analysis of ARIC (n = 13,997) found that elevated midlife total cholesterol, low density lipoprotein-c, and triglycerides correlated with more extensive cognitive decline at a 20-year follow-up, after adjusting for education along with multiple clinical and demographic variables (Power et al., 2018).

These findings indicate that early disruptions to systemic metabolism have long-lasting and progressive effects on cognition. However, the precise metabolic mechanisms leading to cognitive impairment remain incompletely understood. Since AD develops non-cell autonomously, we propose early events leading to cognitive impairments occur in concert with a breakdown in both neuronal and glial function, particularly their metabolic crosstalk. To set the framework, we will first review the homeostatic functions of glia-neuron interactions, followed by the mechanisms leading to their breakdown during pathological conditions of metabolic dysfunction.

3. Glia-neuron interactions in the healthy brain

Under homeostatic conditions, neurons are supported by central nervous system (CNS) glia, which mainly comprise oligodendrocytes, astrocytes, and microglia. Conventionally, oligodendrocytes myelinate CNS axons to expedite signal transmission, astrocytes primarily regulate

CNS blood flow and recycle neurotransmitters, and microglia, the resident immune cells, protect neurons from invading pathogens or brain damage via the inflammatory response. Recent evidence indicates, however, that glia, e.g., oligodendrocytes, also nurture axons by carefully orchestrated axo-glial metabolic crosstalk, in addition to their more traditional roles, e.g., myelin formation (Philips and Rothstein, 2017). The need to metabolically aid axons is intuitive; a resting cortical neuron in the human brain expends 4.7 billion ATP molecules per second, so the energy requirements are massive (Zhu et al., 2012). Although neurons have among the highest percent mitochondrial mass versus other cell types to meet these energy needs (Yu and Pekkurnaz, 2018), they lack significant energy storage capacity and rely on continuous glucose uptake. Thus, glia supplement axons with energy substrates during periods of especially high energy need (González-Gutiérrez et al., 2020), e.g., during high firing rates or during neurodevelopment, making glia-neuron metabolic crosstalk a central tenet of healthy brain functioning.

3.1. Oligodendrocyte-neuron interactions

Oligodendrocytes primarily function to myelinate CNS axons, facilitating saltatory signal transmission. Oligodendrocytes wrap around axons, bringing oligodendrocytes into very close proximity to axons (Fig. 1). Each oligodendrocyte can give rise to several myelin segments and support multiple axons (Philips and Rothstein, 2017). Oligodendrocytes develop from oligodendrocyte progenitor cells, a process called oligodendrogenesis, which starts in embryonic stages and slows with aging (Bergles and Richardson, 2015). However, oligodendrocyte progenitor cells are continuously present during life (Rivers et al., 2008) and de novo myelin deposition is a dynamic process, which, if impaired, can lead to cognitive impairment (Arai, 2020; Chen et al., 2021). Thus, oligodendrocyte-neuron interactions are critical to neuronal function and cognition.

Although the concept of metabolic support from glia to neurons was first advanced in astrocytes, the direct intimate contact of oligodendrocytes to axons renders them especially suitable for fulfilling the metabolic requirements of neurons (Philips and Rothstein, 2017). Studies in oligodendrocyte protein-specific knockout animals highlight oligodendrocyte support function beyond myelination. Mice lacking proteolipid protein (PLP) suffer axonal degeneration without changes to myelin compaction (Griffiths et al., 1998). In contrast, myelin basic protein (MBP) knockout does not induce axonal degeneration in mice but impairs myelin compaction (Griffiths et al., 1998). Further, identification of myelin-to-axon cytoplasmic channels, which are dependent on the oligodendrocyte protein 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP), suggests a potential route for transfer of contents to the axon (Snaidero et al., 2017). Overall, these findings demonstrate that axon degeneration can occur independent of myelin compaction, indicating a purpose for oligodendrocyte proteins, such as PLP, beyond myelin structure (Griffiths et al., 1998).

This view was reinforced by the discovery of the essential role of monocarboxylate transporters (MCTs) to axon health (Fig. 1). In a seminal paper, Lee et al. found MCT1 was highly enriched in oligodendrocytes, which they suggested was an important route oligodendrocytes leverage to metabolically support axons (Lee et al., 2012). In vivo, oligodendrocyte-specific MCT1 knockdown induces axon degeneration. Further, MCT1 inhibition ex vivo in organotypic spinal cord slices induces motor neuron death under glucose starvation, which is rescued by supplementing the culture media with lactate. Since lactate supplementation rescues the effect of MCT1 inhibition, the authors suggested that MCT1 transport of lactate from the oligodendrocyte to the axon is essential to neuronal health.

Inhibiting MCT1, 2, and 4 in the hippocampus in vivo impairs long-term memory formation, which can be rescued by lactate supplementation in the case of MCT1 and 4 inhibition, but not MCT2 inhibition (Suzuki et al., 2011). These data indicate a substantial reliance of neurons on MCT-mediated lactate uptake for memory retention (Fig. 1). Indeed, additional studies suggest neurons may even prefer lactate as an energy source over glucose, in both the intact (Wyss et al., 2011) and injured brain (Glenn et al., 2015). Additionally, inhibiting MCT1/MCT2 lowers axonal ATP levels in electrically stimulated axons (50 and 100 Hz) and alters the ATP to compound action potential ratio, lowering the capacity for electrical conduction (Trevisiol et al., 2017). Therefore, a growing body of evidence supports the concept that MCT-mediated lactate shuttling occurs from oligodendrocytes to axons and that lactate is an important energy source in the CNS, both in oligodendrocytes and neurons.

There are multiple examples of the role of lactate as an essential energy source in the brain (Hu and Wilson, 1997), both for oligodendrocytes as well as for neurons, directly and through oligodendrocyte-mediated lactate transfer. For example, lactate supplementation can

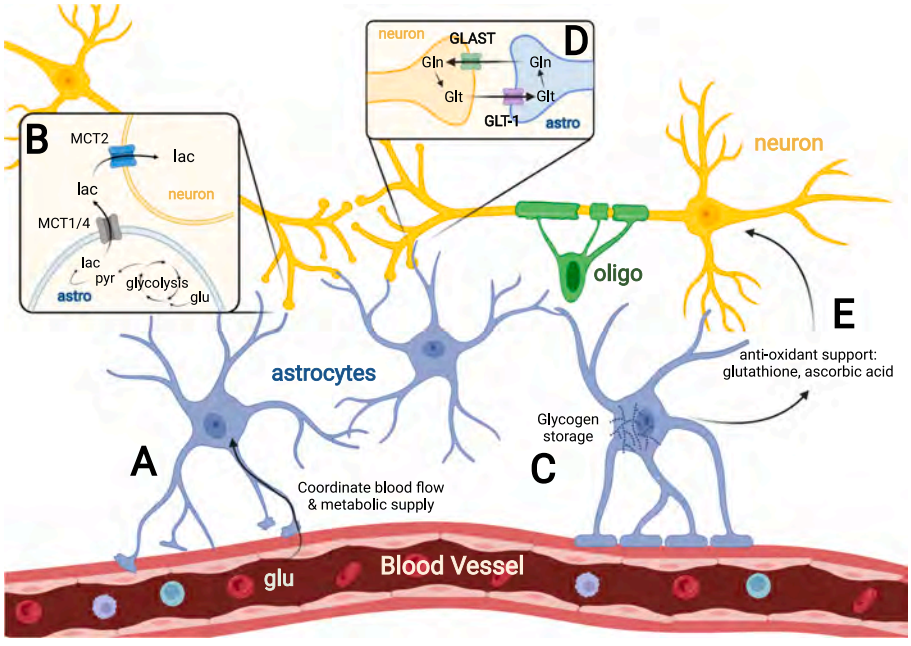

Fig. 2. Astrocyte-neuron interactions under homeostatic conditions.
(A) The primary function of astrocytes is to regulate CNS blood flow and recycle neurotransmitters. (B) Astrocytes take up glucose from blood vessels, which is metabolized to pyruvate by glycolysis, and then, in turn, to lactate. Astrocytes leverage the MCT1/MCT2 shuttle to transfer lactate to neurons, providing metabolic support to axons. (C) Moreover, astrocytes serve as an energy reserve through glycogen storage. (D) Astrocytes also play important roles through the recycling of glutamate/glutamine. First, astrocytes take up glutamate via GLT-1, which is converted to glutamine and transported to neurons via GLAST. Neurons can then convert glutamine back to glutamate to replenish their neurotransmitter pool. (E) Finally, astrocytes provide antioxidants to neurons, which prefer oxidative metabolism and generate relatively high reactive oxygen species levels.
astro, astrocyte; CNS, central nervous system; GLAST, glutamate aspartate transporter; gln, glutamine; glt, glutamate; glu, glucose; GLT-1, glutamate transporter 1; lac, lactate; MCT, monocarboxylate transporter; oligo, oligodendrocyte; pyr, pyruvate.

rescue low-glucose-induced impairment in oligodendrocyte differentiation in rat cerebellar or cortical slices, suggesting lactate is an important substrate during oligodendroglial myelination (Rinholm et al., 2011). In parallel, blocking oxidative phosphorylation in mature oligodendrocytes by COX10 knockout shifts metabolism to glycolysis and lactate utilization as a readily available energy source, which has no effect on myelination (Fünfschilling et al., 2012). Additionally, oligodendrocytes may import lactate independent from MCT1 through gap junctions composed of connexin hemichannels (Philips and Rothstein, 2017). Knocking out the expression of connexin hemichannels, which are mainly expressed by oligodendrocytes, induces neuronal vacuolation and variable degrees of dysmyelination (Odermatt et al., 2003). Even connexin hemichannel knockout in astrocytes triggers myelination defects and cognitive impairment in mice, suggesting the presence of gap junction crosstalk between astrocytes and oligodendrocytes (Lutz et al., 2009).

In addition to MCTs and the lactate axis, oligodendrocyte *N*-methyl-D-aspartate receptors (NMDARs) sense neuron-derived glutamate, a surrogate of signal transmission activity, which regulates the metabolic support offered by oligodendrocytes (Saab et al., 2016). Specifically, oligodendroglial NMDAR activation in optic nerves ex vivo enhances calcium influx and glucose uptake by oligodendrocytes via glucose transporter 1 (GLUT1), in turn augmenting lactate transfer to axons. Thus, an increase in neuronal transmission activates biochemical pathways in oligodendrocytes, which stimulates lactate transfer to energy-requiring axons through metabolic crosstalk.

Oligodendrocytes can additionally communicate with axons by secreting extracellular vesicles (EVs), which enter the periaxonal space and are internalized by neurons via endocytosis (Fig. 1) (Frühbeis et al., 2020). This EV-mediated oligodendrocyte-axon crosstalk enhances neuronal metabolism (Frühbeis et al., 2013), firing rates (Fröhlich et al., 2014Fröhlich, Kuo et al. 2014), and axonal transport (Frühbeis et al., 2020). Interestingly, PLP- and CNP-deficient oligodendrocytes secrete dysfunctional EVs, which are incapable of neuronal or axonal support (Frühbeis et al., 2020). Oligodendrocyte-derived EVs also transfer SIRT2 cargo, an NAD + -dependent deacetylase and regulator of gluconeogenesis, to axons, increasing axonal basal respiration and ATP production and levels in maturing neurons (Chamberlain et al., 2021). SIRT2 then boosts mitochondrial bioenergetics by deacetylating axonal mitochondria proteins, indicative of oligodendrocyte-axon metabolic crosstalk independent of myelin function.

Overall, evidence indicates that oligodendrocytes support neurons beyond myelin formation through metabolic coupling. This enforces the critical dependence of neurons on oligodendrocytes for the required energy substrates to maintain normal neuronal function and cognition.

3.2. Astrocyte-neuron interactions

Astrocytes primarily function to regulate CNS blood flow and recycle neurotransmitters. They help neurons maintain synaptic transmission and excitability via the primary astrocytic recycling pathway, the glutamate-glutamine cycle (Fig. 2) (Bélanger et al., 2011). Using this pathway, astrocytes take up glutamate via glutamate transporter 1, and convert it to glutamine which is transferred to neurons through the glutamate aspartate transporter. Neurons then convert glutamine back to glutamate, replenishing the neuronal neurotransmitter pool. Astrocytes interact directly with neuronal synapses, so-called tripartite synapses, to facilitate synaptic plasticity and transmission (Mederos et al., 2018). Hippocampal astrocytes also express functional acetylcholine receptors (Gahring et al., 2004; Shen and Yakel, 2012) and modulate neuronal cholinergic firing rates (Pabst et al., 2016). Thus, astrocytes may modulate neuronal activity through glutamate and cholinergic neurotransmitters (Maurer and Williams, 2017).

Astrocyte-neuron synapse interactions also couple neurons to the vasculature, facilitating astrocyte-coordinated changes to CNS blood flow and metabolite supply to the CNS through the vasculature (Bélanger et al., 2011). Astrocyte-mediated changes in blood flow are influenced, at least in part, by brain metabolism (Gordon et al., 2008), supporting metabolic links between neurons and astrocytes. Astrocytes residing near blood vessels increase glucose uptake from circulation in response to neuronal transmission and disseminate glucose and metabolites through connexins across astrocytic networks (Rouach et al., 2008).

In terms of metabolism, both astrocytes and neurons can use glucose and lactate as energy sources; however, they prefer different, though complementary, energy sources and pathways. Astrocytes primarily utilize glycolysis and produce lactate (Lovatt et al., 2007), whereas neurons prefer oxidative metabolism (Bélanger et al., 2011). Complementary energy generating pathways are critical to astrocyte-neuron metabolic interactions. Astrocytes consume a large portion of the brain glucose supply, especially during neural activation (Chuquet et al., 2010). However, neurons require a disproportionally large amount of energy to function versus other CNS cell types. Metabolic interaction partially explains this discrepancy; astrocytes sense glutamate, a surrogate of neuronal activity, which enhances their glucose consumption and subsequent lactate release to neurons through the astrocyte-neuron lactate shuttle (Pellerin and Magistretti, 1994). Thus, elevated astrocytic glucose metabolism serves to enhance lactate substrate transport into neurons, amplifying their energy reserves and aiding electrical activity. It is important to note that neuron and astrocyte populations are heterogeneous, varying in number and type by brain region and multiple additional factors; thus, their interactions likely also vary (Khakh, 2019).

While neurons prefer oxidative metabolism and generate relatively high amounts of reactive oxygen species, they are limited in intrinsic antioxidant production rendering them particularly susceptible to oxidative damage (Fig. 2). Astrocytes are less vulnerable to oxidative stress and also assist neurons partly by providing them with antioxidant precursors, e.g., glutathione (Bélanger et al., 2011), underscoring another instance of astrocyte-neuron interactions involving metabolism. The antioxidant glutathione is regenerated from its oxidized form using electrons from NADPH, which is itself generated from glucose processing via the pentose phosphate pathway (Dringen, 2000). Oxidative stress upregulates pentose phosphate pathway activity and NADPH levels in astrocytes (García-Nogales et al., 2003), augmenting their antioxidant capacity and ability to support neurons. Additionally, increased brain activity stimulates a shift in energy utilization from primarily glucose to lactate consumption in neurons concomitant with enhanced antioxidant ascorbic acid release from astrocytes (Castro et al., 2009), providing an additional route to astrocyte-mediated metabolic support.

Primarily located in astrocytes, glycogen is an important energy storage reserve in the brain that can protect neurons under hypoglycemic conditions (Brown, 2004). These astrocytic glycogen reserves are also a lactate source to neurons via the astrocyte-neuron lactate shuttle, further providing metabolic support to neurons and synaptic activity (Tekkök et al., 2005). Blocking astrocytic glycogenolysis impedes memory consolidation and long-term memory formation, implying bioenergetic support from glycogen is critical to cognitive function (Suzuki et al., 2011). Exogenous lactate reverses these effects, unless the neuronal lactate transporter MCT2 is blocked, indicating a critical role for astrocyte-neuronal metabolic lactate signaling long-term memory consolidation. Lastly, in addition to the significant metabolic interaction between astrocytes and neurons, astrocytes are also key mediators of metabolic support of oligodendrocytes to neurons, forming a neuron-oligodendrocyte-astrocyte axis (Amaral et al., 2013). The integrity of this critical trio is paramount to normal brain function.

3.3. Microglia-neuron interactions

Microglia are resident innate immune cells of the brain; however, their function extends well beyond immune surveillance and response to pathogens. During development, microglia support neurons by

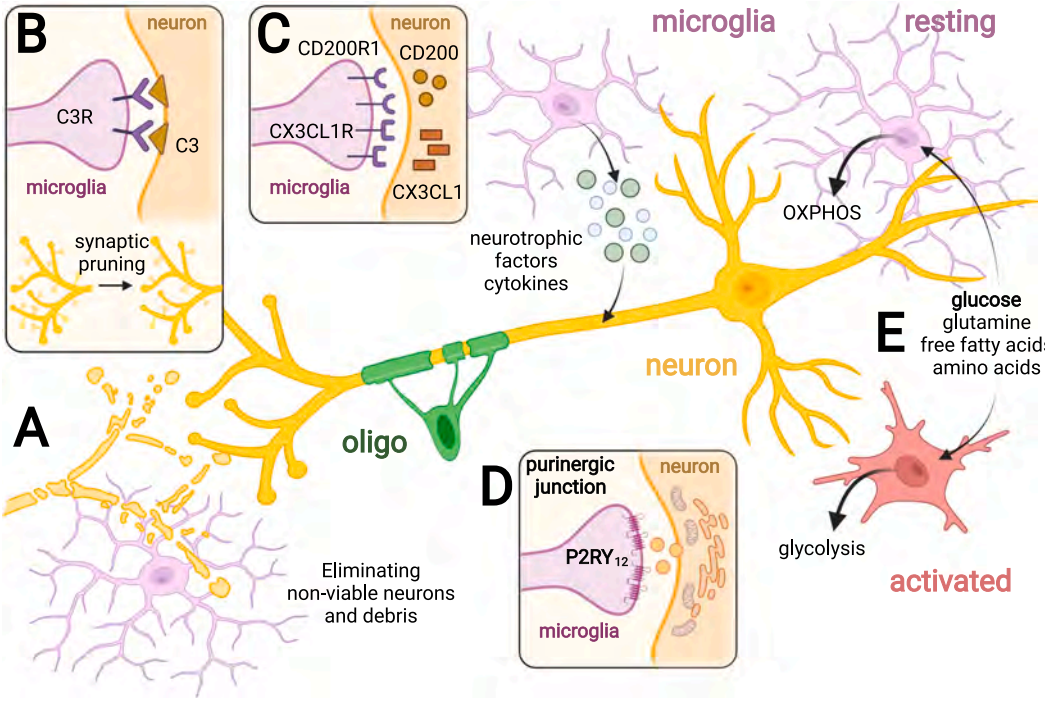

Fig. 3. Microglia-neuron interactions under homeostatic conditions.
The primary microglia function is as the resident innate immune cells of the brain; however, their function extends well beyond immune surveillance. (A) Microglia clear cellular debris from the CNS and eliminate non-viable neurons. (B) Microglia also prune neuronal synapses through C3-C3R interactions. (C) In addition, microglia-neuron interactions occur through other receptor-ligand interactions (CX3CL1-CX3CR1, CD200-CD200R, anti-inflammatory and house-keeping functions) and microglia-secreted neurotransmitters, neurotrophic factors, and cytokines, which bind to cognate receptors on neurons. (D) Regarding metabolic communication, one putative mechanism is through junction formation of neuronal somas to microglial purinergic receptors, e.g., $P2RY_{12}$, which are activated by ATP and may constitute a sensing mechanism of neuronal activity since these junctions are enriched with neuronal mitochondria and endoplasmic reticulum contacts. (E) Microglia prefer glucose as an energy substrate, which they take up through GLUTs, and metabolize through oxidative phosphorylation (OXPHOS) under homeostatic conditions (pink microglia). However, even though less efficient, activated microglia (red microglia) shift their metabolism to glycolysis while mounting a pro-inflammatory response. One putative reason is that microglia may utilize glycolysis-derived NADPH to generate reactive oxygen species for host defense.
C3, complement component 3; C3R, C3 receptor; CNS, central nervous system; CD200, Cluster of Differentiation 200 ligand; CD200R1, CD200 receptor 1; CX3CL1, fractalkine also called chemokine (C-X3-C motif) ligand 1; CX3CR1, fractalkine receptor also called CX3C chemokine receptor 1; glu, glucose; GLUT, glucose transporter; OXPHOS, oxidative phosphorylation; $P2RY_{12}$, purinergic receptor.

regulating neuronal survival and differentiation, eliminating non-viable neurons and pruning synapses (Fig. 3) (Schafer and Stevens, 2015; Mosser et al., 2017). Microglia-neuron crosstalk continues in the adult brain, serving a variety of purposes, including sensing neuronal activity and regulating synaptic plasticity (Marinelli et al., 2019). Microglia-neuron crosstalk is mediated by both direct contact (Cserép et al., 2021) and secreted signals (Marinelli et al., 2019). Microglia extend cellular processes that constantly surveil their environment, secret soluble signals that bind neuronal receptors, and express receptors to receive neuronal signals (Pósfai et al., 2019; Uweru and Eyo, 2019). Microglial processes communicate with neurons at multiple cellular anatomical locations, including somas, axons, and dendrites, allowing microglia to sense and respond to neuronal activity directly (Cserép et al., 2021). Here, we highlight the main mediators and key functions of microglial-neuronal crosstalk in development and the healthy adult brain.

Complement signaling though complement receptor 3 (C3R) on microglia and C3 from neurons contributes to developmental synaptic pruning (Fig. 3) (Schafer et al., 2012). In adulthood, aberrant complement activation is implicated in neurodegenerative disease (Dalakas et al., 2020). Other receptor-ligand pairs essential for homeostatic microglial-neuronal crosstalk involve fractalkine (CX3CL1; neurons) and the fractalkine receptor (CX3CR1; microglia), and CD200 ligand (neurons) and CD200 receptor (CD200R; microglia), which perform anti-inflammatory and house-keeping roles (Marinelli et al., 2019).

Microglia and neurons also communicate via secreted neurotransmitters, neurotrophic factors, cytokines, purines, and the purine derivative ATP (Marinelli et al., 2019). Microglia express serotonin, $GABA_B$, and glutamatergic receptors, which allows them to sense neuronal activity (Marinelli et al., 2019). They also express acetylcholine receptors, which, upon activation, exert anti-inflammatory and neuroprotective effects (Suzuki et al., 2006; Egea et al., 2015; Li et al., 2019b). Microglia also secrete neurotrophic factors (e.g., brain-derived neurotrophic factor) and cytokines (e.g., tumor necrosis factor alpha [TNF-α], interleukin-1β [IL-1β]), which bind to cognate receptors expressed on neurons (Pósfai et al., 2019). Purinergic receptor activation, e.g., P2XRs and P2YRs, evoke diverse microglial responses, such as migration upon ATP stimulation of $P2RY_{12}$ (Fig. 3) (Calovi et al., 2019; Illes et al., 2020). Microglial purinergic receptors form purinergic junctions with neuronal somas (Cserép et al., 2020). The exact function of these purinergic junctions remains under investigation, but microglia may leverage them to sense neuronal activity, since these junctions are comprised of neuronal mitochondria and endoplasmic reticulum contacts.

Diverse interactions between microglia and neurons contribute to proper nervous system development and help maintain homeostasis in the adult brain. In addition to interactions concerning neurotransmitters, neurotrophic factors, cytokines, and purinergic receptors, recent evidence also suggests potential for a metabolic aspect to microglia-neuron interactions. Most studies have defined the relationship between microglial activation state and metabolism, whereas less is known about microglia-neuron metabolic crosstalk. Microglia express multiple

GLUT receptors and utilize glucose as their main fuel source, preferentially using oxidative phosphorylation when in a homeostatic state (Bernier et al., 2020a, 2020b, Lauro and Limatola, 2020). However, although glucose is preferred, microglia can utilize glutamine, and possibly free fatty acids and amino acids, as fuel sources, making them metabolically versatile when glucose levels are low or under stressful conditions, for example when mounting an inflammatory response (Bernier et al., 2020a; Bernier et al., 2020b).

When microglia respond to a pro-inflammatory challenge, such as lipopolysaccharide stimulation, they transition from oxidative phosphorylation to glycolysis (Lauro and Limatola, 2020). This metabolic switch is also observed in activated peripheral macrophages (Liu et al., 2021). In a recent review on the topic, Bernier et al. proposed microglia require the glycolytic shift to mount a pro-inflammatory response (Bernier et al., 2020a, 2020b). Specifically, microglia might benefit from a glycolytic shift because NADPH can be used to generate reactive oxygen species for host defense, and metabolic intermediates can contribute to proliferation and cytokine production. Indeed, inhibiting glycolysis blocks lipopolysaccharide-induced primary microglial TNF-α, IL-1β, and IL-6 expression (Hu et al., 2020). Thus, the metabolic state of microglia regulates inflammatory signals, which can communicate with surrounding cells, including neurons.

How microglia might sense neuronal metabolism remains largely unknown. Microglia sense ATP- and activity-dependent neurotransmitter release from neurons, so, perhaps, they indirectly sense metabolic demands. One potential source of direct metabolic crosstalk exists at the microglial to neuronal somatic purinergic junction, where neuronal mitochondria aggregate, suggesting a potential mechanism of metabolic communication (Cserép et al., 2020).

Overall, microglial metabolic shifts are critical for pro-inflammatory activation, whereas microglia-neuron metabolic crosstalk remains poorly understood. However, given emerging evidence of potential metabolic communication, e.g., via purinergic receptors, research into microglial-neuron metabolic crosstalk constitutes an interesting research direction.

4. Glia-neuron interactions in the brain in the context of the MetS and dysfunctional metabolism

In the healthy brain, carefully orchestrated metabolic glia-neuron interactions occur to sustain normal brain functioning (Philips and Rothstein, 2017). Under conditions of the MetS, rising insulin resistance and excess energy substrates, both elevated glucose and lipid levels, perturb glial homeostasis and metabolism and induce neuroinflammation (Van Dyken and Lacoste, 2018; de la Monte and Grammas, 2019; O'Grady et al., 2019; Langley et al., 2020; Bouhrara et al., 2021). Additionally, metabolic dysfunction lowers brain expression of neurotransmitter receptors, e.g., acetylcholine receptors (Xu et al., 2020; Martinelli et al., 2021; Martins et al., 2021), which would lead to cognitive impairment. In parallel to MetS-induced glial disruption, we posit perturbations in glia-neuron interactions occur with a loss of energy substrate transfer to neurons in need of metabolic support. Ultimately, this would lead to failure of neuronal bioenergetics and signal transmission, and, eventually, neuronal loss and cognitive impairment. The following section summarizes the available studies on glia-neuron metabolic crosstalk occurring in the context of dysfunctional metabolism.

4.1. Oligodendrocyte-neuron interactions

As discussed in the section on homeostatic glia-neuron interactions, oligodendrocytes provide two pivotal supporting functions for neurons, myelination and metabolic support (Philips and Rothstein, 2017). With reference to myelination, diabetes, obesity, and MetS components negatively impact oligodendrocytes, leading to oligodendrocyte loss and loss of myelin integrity (Yoon et al., 2016; Kim et al., 2020). These pathological changes correlate with cognitive impairment in preclinical studies. In mouse models of obesity, high-fat diet (HFD) decreases myelin thickness, which correlates with poorer cognitive performance (Graham, Grabowska et al., 2019). Similarly, in type 2 diabetic mice, loss of white matter, a marker of brain demyelination, is associated with worsened cognitive function (Li et al., 2019a). In human clinical studies, patients with type 2 diabetes, like their murine counterparts, exhibit disrupted white matter networks that correlate with cognitive impairment (Zhang et al., 2016; Biessels and Despa, 2018). In parallel, obesity and insulin resistance are linked to lower myelin content in cognitively unimpaired adults (O'Grady et al., 2019; Bouhrara et al., 2021).

With reference to metabolic support, metabolomics analysis of central nervous system tissue from MetS animals fed a HFD demonstrates impaired metabolism occurs concurrently with oligodendrocyte loss, with a drop in tricarboxylic acid (TCA) cycle intermediates and changes in protein biosynthesis, glutathione metabolism, and the mitochondrial electron transport chain (i.e., oxidative phosphorylation) (Langley et al., 2020). These detrimental changes worsen over time. HFD feeding also promotes the loss of oligodendrocyte progenitor cells and reduces their differentiation in mouse models (Langley et al., 2020). In a *db/db* mouse model of obesity and type 2 diabetes, early changes in myelin and mitochondrial lipids occur in the brain prior to the onset of overt structural alterations (Palavicini et al., 2020). Although these studies in mouse models on oligodendrocytes are correlative and not causative, they suggest that systemic metabolic dysfunction adversely impact oligodendrocyte health, and possibly neuronal health in turn, and constitute interesting research avenues. Importantly, there is discordance across studies, which may arise from the rodent age, location of the sampled CNS matter, and differences in diet, which also require further inquiry.

The impact of hyperglycemia on oligodendrocytes is unclear. Chronic hyperglycemia does not affect viability, oxidative stress, or differentiation of oligodendrocyte progenitor cells in vitro (da Rosa et al., 2019). Yet a high-fat high-sucrose diet decreases the number of mature myelinating oligodendrocytes in mouse spinal cord in tandem with impaired TCA metabolism (Kim et al., 2020). Therefore, it is possible that mature oligodendrocytes, but not their progenitors, are susceptible to hyperglycemia. This idea is supported by data from type 1 diabetic rats with hyperglycemia. In these animals, the optic nerve is characterized by disorganized myelin and some demyelinated zones, indicating a potential oligodendrocyte loss and impaired function (Dorfman et al., 2015).

Regarding potential effects of metabolic dysfunction on oligodendrocyte-neuron metabolic crosstalk, neuronal expression of components of the lactate shuttle, MCT1 and MCT2, increase in the brain of obese mice after 12 weeks of HFD, as well as in the brain of type 2 diabetes and obesity mouse models (*ob/ob* and *db/db* mice) (Pierre et al., 2007). These findings likely represent an initial compensatory mechanism by the brain to overcome the bioenergetic crisis produced by systemic MetS conditions (Chomova, 2022). More recent MCT research has addressed changes in AD murine models over time, but data are lacking on HFD and MetS animals. MCT1, MCT2, and MCT4 expression and lactate levels decrease in APP/PS1 AD mice with cognitive impairment (Zhang et al., 2018), supporting an association between aberrant oligodendrocyte-neuron coupling and cognition. This association is further supported by studies showing brain MCT1 and MBP levels progressively decrease in older versus younger APP/PS1 AD mice, paralleling the trajectory of cognitive decline (Dong et al., 2018). Finally, AD also directly affects oligodendrocyte metabolism by altering the expression of glycolytic and ketolytic genes, impairing their ability to provide metabolic support and energy substrates to neurons (Saito et al., 2021). Analogous studies are required in MetS animal models to improve our understanding of systemic metabolic dysfunction on oligodendrocyte-neuron metabolic crosstalk.

It is also possible to draw parallels between oligodendrocyte-neuron interactions with Schwann cell-neuron coupling in the peripheral

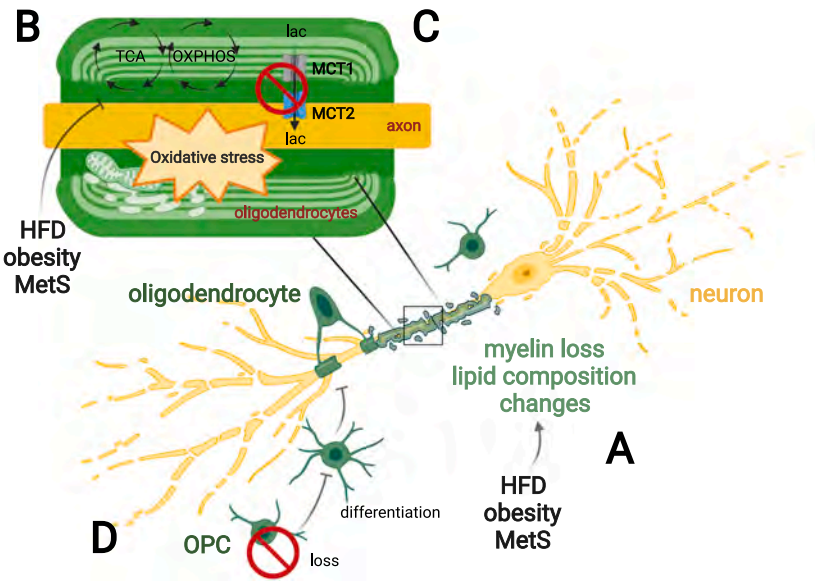

Fig. 4. Oligodendrocyte-neuron interactions under pathologic conditions of metabolic dysfunction.
(A) Diabetes, obesity, and MetS components negatively impact oligodendrocytes, which correlate with cognitive impairment. HFD promotes obesity and the MetS and induces oligodendrocyte loss and perturbed myelin structure and lipid composition. (B) Additionally, transcriptomics and metabolomics changes occur to multiple targets related to metabolism (drop in TCA cycle intermediates), mitochondrial biogenesis and function (changes in PGC-1α expression, drop in OXPHOS), and protein biosynthesis, along with upregulated endoplasmic reticulum stress and oxidative stress pathways (glutathione metabolism). (C) We hypothesize HFD also stimulates changes to MCT1, 2, and 4 expression and that oligodendrocytes experience a glycolytic shift to supply neurons with energy fuel under conditions of stress via metabolic coupling. (D) Lastly, HFD also promotes the loss of OPCs and reduces their differentiation, which would impair the ability to regenerate HFD-induced damage to myelin.
glu, glucose; HFD, high-fat diet; lac, lactate; MCT, monocarboxylate transporter; MetS, metabolic syndrome; OPC, oligodendrocyte precursor cell; OXPHOS, oxidative phosphorylation; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; TCA, tricarboxylic acid.

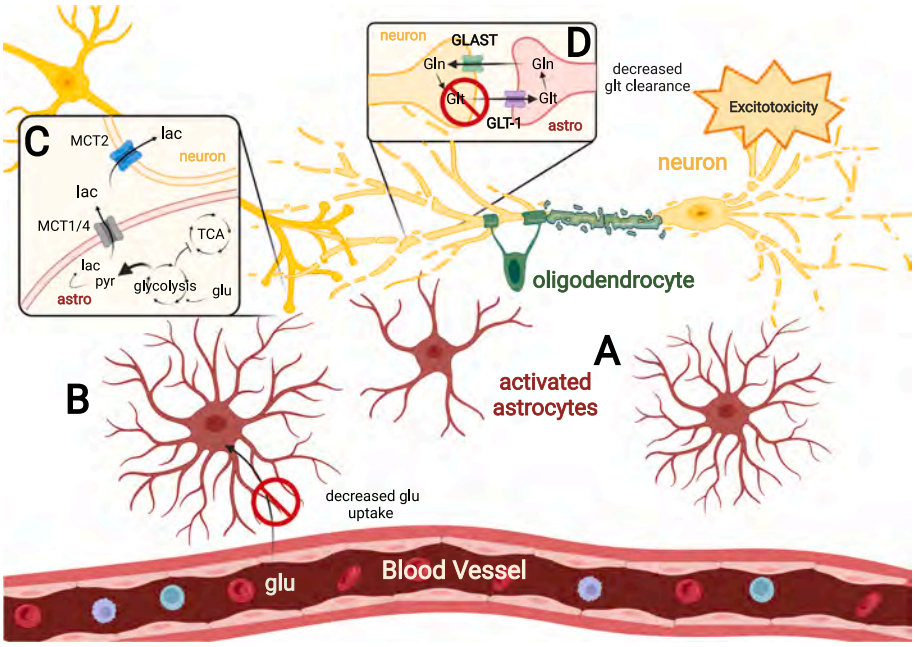

Fig. 5. Astrocyte-neuron interactions under pathologic conditions of metabolic dysfunction.
Diabetes, obesity, and MetS components negatively impact astrocytes and correlate with cognitive impairment. (A) Obesity promotes astrocyte reactivity (red astrocytes), morphologic changes, and inflammation. (B) Insulin resistance in the brain impairs the ability of astrocytes to take up glucose and maintain CNS glucose homeostasis. (C) Diabetes perturbs astrocytic metabolism. (D) Obesity and diabetes also impair the ability of astrocytes to clear glutamate, which subsequently suppresses neuronal transmission, and leads to excitotoxicity.
CNS, central nervous system; GLAST, glutamate aspartate transporter; gln, glutamine; glt, glutamate; glu, glucose; GLT-1, glutamate transporter 1; IR, insulin resistance; lac, lactate; MCT, monocarboxylate transporter; pyr, pyruvate; TCA, tricarboxylic acid.

nervous system. Schwann cell-restricted MCT1 knockout triggers sensory neuropathy and hypomethylation in aging mice along with perturbed myelin lipid composition (Jha et al., 2020b). Heterozygous MCT1 knockout worsens sensory and motor neuropathy and causes a thinning of myelin in STZ type 1 diabetic mice (Jha et al., 2020a). In models of peripheral nerve injury, Schwann cells shift their metabolism to glycolysis to supply neurons with energy fuel during repair, which specifically occurs through MCT1 and Schwann cell-axon metabolic coupling (Babetto et al., 2020). Indeed, heterozygous MCT1 knockout (Morrison et al., 2015) or pharmacological MCT1 inhibition (Babetto et al., 2020) impairs axon repair and/or accelerate degeneration. Cumulatively, these studies demonstrate how Schwann cells metabolically support neurons during pathological conditions, be it during diabetes- or mechanical injury-induced axon degeneration. Whether similar mechanisms operate in the CNS has not been investigated in this level of detail, to our knowledge.

In summary, diabetes, obesity, and MetS components disrupt oligodendrocyte metabolism and myelin integrity (Yoon et al., 2016; Kim et al., 2020), which we posit leads to a failure to provide adequate metabolic support to neurons and cognitive impairment (Fig. 4).

4.2. Astrocyte-neuron interactions

Astrocytes are central to normal brain physiology, glucose homeostasis, and energy regulation. Glucose is the primary energy source for the brain, and while neurons have high energy requirements, astrocytes are the principal cells responsible for glucose uptake and transfer of metabolic substrates, particularly lactate, to neurons. Although insulin is not required for glucose uptake by neurons, the brain is an insulin sensitive organ (Cai et al., 2018; Kim et al., 2019a) and develops insulin resistance in response to MetS, with changes in brain structure (Lu et al., 2021) (Fig. 5).

Astrocytes express insulin receptors and are insulin responsive, increasing glycogen storage following insulin treatment (Heni et al., 2011). In vivo studies using astrocyte-specific insulin receptor knockout mice show that astrocytes lacking insulin receptors are less capable of maintaining CNS glucose homeostasis, secondary to loss of hypothalamic astrocyte function, disrupting normal physiological responses to brain glucose levels (García-Cáceres et al., 2016). This is paralleled by a loss of normal glucose transport across the blood-brain barrier in these same animals, further emphasizing the crucial role astrocytes play in sensing systemic glucose (García-Cáceres et al., 2016). Insulin receptor knockout in astrocytes lowers ATP release and disrupts astrocyte-neuron energy exchange, which decreases dopamine release from dopaminergic neurons (Cai et al., 2018). This decreased dopamine release impacts cognition since knockout animals display depressive and anxiety. These preclinical observations support a growing literature on how systemic insulin resistance, a hallmark of dyslipidemia and the MetS, in parallel with aforementioned brain insulin resistance, develops over time and is associated with cognitive impairment and dementia (Kellar and Craft, 2020).

In the context of the MetS and astrocytes, two recent reports provide additional insight into this critical association. First, treating primary astrocytes with fatty acids to simulate dyslipidemia present in the MetS, lowers autophagic flux in astrocytes, a response likely dependent upon the brain region from which cells are isolated (Ortiz-Rodriguez and Arevalo, 2020). This response, along with blocking autophagy in astrocytes, is toxic, both to astrocytes and neurons. The second report highlights an intriguing response of the sympathetic nervous system to HFD animal models. HFD fed rats exhibit sympathetic neuron excitotoxicity, with increased astrocyte leptin receptor expression and decreased glutamate receptor and transporter expression (Liu and Zheng, 2019). Interestingly, changes in sympathetic nervous system function are common in obesity, components of the MetS, and dysfunctional metabolism (Liu and Zheng, 2019). The link between elevated sympathetic activity, cognitive impairment, and increased astrocyte leptin receptor expression is an interesting one deserving of further study (Knight et al., 2020). These two studies open new avenues of research as the field pursues a deeper understanding of the metabolic crosstalk between astrocytes and neurons during the MetS.

In comparison to the few studies outlined above, there is more established literature on the pathogenesis of AD and related dementias regarding changes in bioenergetics, mitochondrial function, and inflammation in astrocytes (Rodríguez-Arellano et al., 2016; Arranz and De Strooper, 2019). These changes in astrocyte physiology parallel those reported in astrocytes in response to obesity and systemic dysfunction, with well documented increases in inflammation and astrocyte reactivity, coupled with changes in morphology and function (Tomassoni et al., 2013; Koga et al., 2014; Tomassoni et al., 2020). What is less well studied is astrocyte biology in the context of diabetes-mediated cognitive impairment. While published research strongly supports our contention that diabetes will induce dysfunctional astrocyte-neuron metabolic coupling, there are only a few studies directly addressing this question. One such study is in the *db/db* mouse model of type 2 diabetes. Zhang et al. report increases in brain lactate and alanine levels and speculate these findings may signal a breakdown of the lactate-alanine shuttle between astrocytes and neurons, while concurrent changes in TCA metabolites suggest a metabolic switch in neurons from oxidative metabolism to anaerobic glycolysis (Zheng et al., 2017. The authors also report a decrease astrocyte clearance of glutamate, which subsequently suppresses GABA transmission to neurons and impairs synaptic plasticity. These findings associate with cognitive impairment in the *db/db* animals, as assessed by the Morris water maze (Zheng et al., 2017).

In summary, accumulating data support the hypothesis that metabolic imbalance between astrocytes and neurons promotes cognitive impairment in obesity and the MetS. Multiple preclinical and clinical studies also confirm that obesity, diabetes, and MetS components promote cognitive impairment, and that changes in astrocyte-neuron metabolic interactions are critical in other neurodegenerative diseases, including AD (Zulfiqar et al., 2019). Furthermore, several reports point to astrocyte-neuron metabolic coupling as critical for learning and memory under physiologic conditions. While more studies are needed to fully understand astrocyte-neuron metabolic interactions in cognitive impairment related to the MetS and metabolic dysfunction (Fig. 5), astrocytes are attractive therapeutic targets and the source of current and planned interventions for the treatment of cognitive impairment and associated dementias (Arranz and De Strooper, 2019).

4.3. Microglia-neuron interactions

Components of the MetS promote CNS neuroinflammation (Van Dyken and Lacoste, 2018), with microglial activation, elevated cytokine levels, oxidative stress, and blood-brain barrier disruption along with peripheral immune trafficking into the brain. Neuroinflammation contributes to pathology through activated microglia interactions with hypothalamic neurons leading to leptin and insulin resistance (Van Dyken and Lacoste, 2018; Robb et al., 2020) and with hippocampal neurons leading to cognitive decline (Cope et al., 2018). The role of microglia-neuron crosstalk in regulating metabolic disease is relatively well-established in the hypothalamus. However, the role of microglial metabolism and metabolic crosstalk in the hippocampus and on cognitive performance is not as well described.

Hypothalamic microglial pro-inflammatory activation contributes to neuronal stress and ultimately drives feeding behaviors and diet-induced obesity (Valdearcos et al., 2014; Valdearcos et al., 2017). Although the mechanism of the crosstalk remains elusive, Valdecaros et al. demonstrated that diphtheria toxin depletion of hypothalamic microglia prevents saturated fatty acid-induced neuronal stress (Valdearcos et al., 2014). Additionally, pharmacological microglial depletion in the context of saturated fatty acid treatment increases neuronal leptin responses and decreases intake of chow. In a later study, the authors show that inhibiting microglia-specific NF-κB inflammatory activation by genetic manipulation prevents HFD-induced hyperphagia and obesity (Valdearcos et al., 2017). Microglia, therefore, regulate systemic metabolic physiology by interacting with neurons in the medial basal hypothalamus (Robb et al., 2020). Along those lines, microglia also express leptin receptors, and knockout of myeloid leptin receptors reduces microglial morphological ramification in the hypothalamic paraventricular nucleus, disrupts hypothalamic neuronal circuitry, and induces hyperphagia and weight gain (Gao et al., 2018). This advocates a potential role for microglial leptin sensing in regulation of hypothalamic neurons and subsequent systemic metabolism. Dysregulated mitochondrial metabolism has further been implicated in HFD-induced hypothalamic microglial activation and subsequent obesity (Kim et al., 2019b). Finally, although microglia are the predominant activated immune cell contributing to hypothalamic neuroinflammation (Boura-Halfon et al., 2019), macrophages, the peripheral equivalent of microglia, also traffic into the CNS during the MetS (Van Dyken and Lacoste, 2018; Yang et al., 2019), including the hypothalamus based on $CD45^{high}$ expression (Lainez et al., 2018).

The influence of HFD consumption and obesity on microglia-neuron crosstalk in brain regions responsible for cognitive function and memory has not been as well studied as in the hypothalamus. The hippocampus, a limbic structure contributing to memory and learning tasks, displays microglial activation in mouse models of diet-induced obesity (Hao et al., 2016; Cope et al., 2018). Obesity is associated with hippocampal-dependent cognitive impairment in rodent models (Sobesky et al., 2014; Sims-Robinson et al., 2016; Cope et al., 2018). Moreover, microglia phagocytose synaptic spines, contributing to hippocampal-dependent cognitive impairment (Cope et al., 2018). While microglia play a role in obesity-induced cognitive impairment, the mechanisms of microglia-neuron crosstalk and the role of metabolism in this communication are unknown. Neuronal fractalkine and the microglial fractalkine receptor

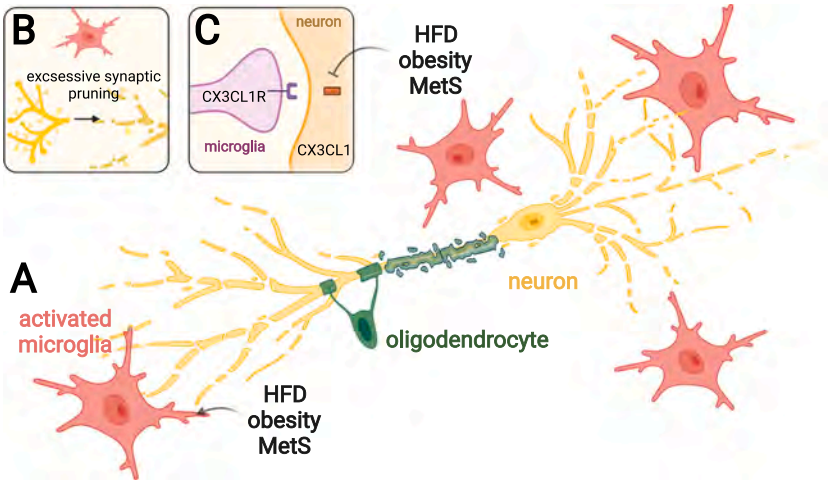

Fig. 6. Microglia-neuron interactions under pathologic conditions of metabolic dysfunction.
(A) Diabetes, obesity, and MetS components activate microglia, which leads to neuronal stress. (B) In the hippocampus, HFD induces aberrant and excessive microglial phagocytosis of synaptic spines, contributing to hippocampal-dependent cognitive impairment. (C) HFD decreases neuronal CX3CL1 and microglial CX3CR1, contributing to hippocampal dependent cognitive impairment.
CX3CL1, fractalkine also called chemokine (C-X3-C motif) ligand 1; CX3CR1, fractalkine receptor also called CX3C chemokine receptor 1; HFD, high-fat diet.

are decreased in a cognitively impaired diet-induced obese mouse models, and this dysregulated microglia-neuron interaction may contribute to cognitive impairment (Kawamura et al., 2021). However, fractalkine receptor deficiency using CX3CR1$^{+/-}$ mice in a diet-induced obesity model prevents microglial activation and hippocampal dependent deficits (Cope et al., 2018). Lastly, macrophages also infiltrate into the hippocampus and contribute to neuroinflammation (Buckman et al., 2014; Erion et al., 2014). Obesity-induced activation of NLR family pyrin domain containing 3 (NLRP3), a macrophage inflammasome component, in peripheral visceral adipose depots stimulates hippocampal microglia, contributing to cognitive impairment (Guo et al., 2020).

While the data collectively support a critical role for microglia in cognition (Fig. 6), future studies are needed to assess the role of microglial-neuron metabolic interactions on cognitive impairment.

5. Conclusions

Multiple clinical studies report diabetes, obesity, and MetS components are associated with cognitive impairment ranging from MCI to dementias, such as AD (Mallorquí-Bagué et al., 2018). These findings underscore the importance of metabolism in maintaining healthy cognitive function. Under normal physiological conditions, glia perform various supportive functions for neurons ranging from myelination and lactate supplementation by oligodendrocytes (Philips and Rothstein, 2017), replenishing of the neurotransmitter pool and energy storage and antioxidant supplementation by astrocytes (Bélanger et al., 2011), and synaptic pruning and immune functions by microglia (Mosser et al., 2017). Further, since neurons cannot store a significant amount of energy, they rely on glia for continuous metabolic support. States of diet-induced obesity or dysregulated metabolism lead to multiple pathologic changes in glia, including oligodendrocyte loss and impaired myelination (Kim et al., 2020), changes in astrocyte autophagy (Ortiz-Rodriguez and Arevalo, 2020) and neurotransmitter release (Zheng et al., 2017), and microglial activation (Valdearcos et al., 2017). Collectively, these pathological alterations impair glia-neuron metabolic interactions and lead to a failure in the energy supply chain to neurons, which may potentially result in neuronal damage leading to cognitive impairment.

Search terms

"astrocyte", "axo-glial", "brain", "cell", "central nervous system", "diabetes", "exosome", "glia", "metabolic syndrome", "microglia", "obesity", "oligodendrocyte", "peripheral immune cell", "Schwann cell"

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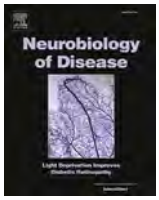

Review

Neurological sequela and disruption of neuron-glia homeostasis in SARS-CoV-2 infection

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ABSTRACT

The coronavirus disease 2019 (COVID-19) pandemic is responsible for 267 million infections and over 5 million deaths globally. COVID-19 is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a single-stranded RNA beta-coronavirus, which causes a systemic inflammatory response, multi-organ damage, and respiratory failure requiring intubation in serious cases. SARS-CoV-2 can also trigger neurological conditions and syndromes, which can be long-lasting and potentially irreversible. Since COVID-19 infections continue to mount, the burden of SARS-CoV-2-induced neurologic sequalae will rise in parallel. Therefore, understanding the spectrum of neurological clinical presentations in SARS-CoV-2 is needed to manage COVID-19 patients, facilitate diagnosis, and expedite earlier treatment to improve outcomes. Furthermore, a deeper knowledge of the neurological SARS-CoV-2 pathomechanisms could uncover potential therapeutic targets to prevent or mitigate neurologic damage secondary to COVID-19 infection. Evidence indicates a multifaceted pathology involving viral neurotropism and direct neuroinvasion along with cytokine storm and neuroinflammation leading to nerve injury. Importantly, pathological processes in neural tissue are non-cell autonomous and occur through a concerted breakdown in neuron-glia homeostasis, spanning neuron axonal damage, astrogliosis, microgliosis, and impaired neuron-glia communication. A clearer mechanistic and molecular picture of neurological pathology in SARS-CoV-2 may lead to effective therapies that prevent or mitigate neural damage in patients contracting and developing severe COVID-19 infection.

1. Introduction

Late in 2019, a new coronavirus emerged, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), triggering the coronavirus disease 2019 (COVID-19) pandemic. Globally, 267 million individuals have contracted SARS-CoV-2, which has killed over 5 million people, as of December 2021 (https://coronavirus.jhu.edu/map.html, n.d). SARS-CoV-2 is a single-stranded RNA beta-coronavirus, which causes a systemic inflammatory response, multi-organ damage, and respiratory failure requiring intubation in serious cases. The virus is especially dangerous to older populations and patients with co-morbidities, such as obesity and diabetes (Feldman et al., 2020). The pathophysiology remains incompletely understood; however, neurological involvement is increasingly evident. This has important health implications for SARS-CoV-2 survivors, due to the frequent long-lasting and potentially irreversible nature of neurologic sequalae. Moreover, as infections continue to rise, so will the burden of SARS-CoV-2-induced neurologic complications.

Therefore, understanding the spectrum of neurological disorders in response to SARS-CoV-2 is needed to manage COVID-19 patients (Ellul et al., 2020). This will facilitate recognition of nervous system injury secondary to SARS-CoV-2, which could expedite earlier treatment to improve outcomes. Furthermore, a clearer understanding of the neurological SARS-CoV-2 pathomechanisms could uncover potential therapeutic targets. To date, the evidence suggests SARS-CoV-2-triggered neurological damage occurs through several avenues. First, the virus exhibits neurotropism, enabling direct invasion of neural tissue (Song et al., 2021). Second, systemic cytokine storm and a hyperactive inflammatory immune response secondary to viral infection, can cause nerve injury (Thepmankorn et al., 2021). Additionally, local induction of neuroinflammation within the central nervous system (CNS) also contributes to neuronal damage. Importantly, pathological processes in

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neural tissue are non-cell autonomous and occur through a concerted breakdown in neuron-glia homeostasis. These processes span neuron axonal damage, astrogliosis, microgliosis, and impaired neuron-glia communication.

This review will cover the topic of SARS-CoV-2-triggered loss of neuron-glial homeostasis. We will describe the breadth of neurological presentation in COVID-19 patients and describe evidence from clinical and autopsy studies of virally induced neuronal loss, endothelial astrogliosis, and neuroinflammation. We will also outline in vitro and animal research demonstrating putative viral entry routes and neurotropism, followed by a focus on neuron-glial interactions. Lastly, we will round up the discussion with potential therapeutic avenues based on currently known SARS-CoV-2 pathophysiology.

2. Neurological manifestation in COVID-19 patients

The SARS-CoV-2 pandemic precipitated a now recognized global increase in the prevalence of well-characterized and rare neurologic sequelae of the CNS and peripheral nervous systems (PNS) in response to viral illness (Mao et al., 2020). One United Kingdom (UK) study systematically documented the various neurologic syndromes experienced by COVID-19 survivors. Neurologic complications included encephalopathy, encephalitis with documented CNS inflammatory changes, ischemic stroke, and Guillain-Barré Syndrome (GBS), among other miscellaneous syndromes (Paterson et al., 2020). The postulated SARS-CoV-2-mediated pathomechanisms for CNS and PNS injury comprise direct neurotropic invasion and parainfectious endothelial dysfunction, coagulopathy, hyperinflammation, and autoimmunity (Fig. 1A) (Mehta et al., 2020; Zubair et al., 2020).

Regarding endothelial dysfunction and coagulopathy, a prospective Belgian study of *post-mortem* brain magnetic resonance ($n = 62$) revealed subcortical micro- and macrobleeds, likely due to blood-brain barrier (BBB) breakdown (Coolen et al., 2020). This endothelial dysfunction, with or without direct viral infection, may underlie SARS-CoV-2 mediated CNS injury. SARS-CoV-2 related stroke syndromes tend to occur in the setting of markedly elevated D-dimer levels, frequently with concurrent large vessel and systemic venous thromboembolic events (Beyrouti et al., 2020). Notably, young patients suffer higher-than-expected stroke incidences, typically large vessel, which appears to implicate coagulopathy and endothelial dysfunction (Oxley et al., 2020). Antiphospholipid antibody syndrome from hypercoagulability has occurred in some cases, likely linked to pro-inflammatory cytokine states (Zhang et al., 2020).

In terms of immune response, *post-mortem* examination of six patients in Germany showed encephalitic and meningitic changes, with evidence of brainstem perivascular and inflammatory changes associated with neuronal loss (von Weyhern et al., 2020). Encephalitis, in particular, has figured prominently in SARS-CoV-2 mediated CNS injury. Acute demyelinating encephalomyelitis is a rare disorder lacking clear evidence of direct causality in SARS-CoV-2 injury. One autopsy study, however, illustrates some of the CNS inflammatory patterns seen in COVID-19-induced CNS injury, and sheds valuable insight into putative pathomechanisms. This *post-mortem* evaluation revealed hemorrhagic white matter lesions in the bilateral hemispheres, mostly subcortically, with macrophagic foci surrounding small vessels along with myelin breakdown and axonal damage (Reichard et al., 2020). Additionally,

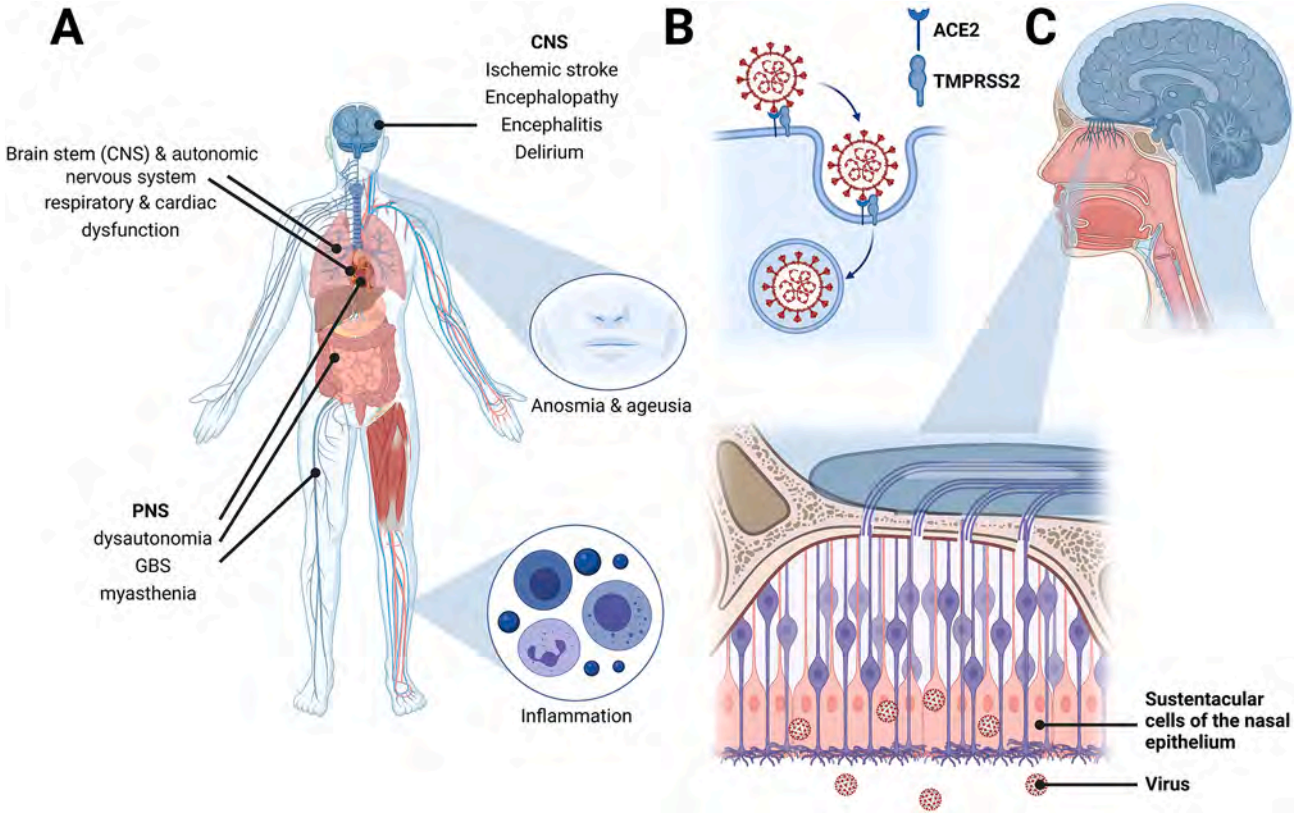

Fig. 1. SARS-CoV-2-induced neurological manifestations.
(A) SARS-CoV-2-induced neurologic complications can affect the CNS and PNS. Damage to the brain stem and autonomic nervous systems can lead to respiratory and cardiac dysfunction. Severe COVID-19 patients exhibit systemic inflammation, marked by elevated white blood cells and pro-inflammatory cytokines. Antibodies specific to PNS injury can be detected in blood, including IgG, anti-GM1, anti-GD1a, and anti-GD1b. (B) SARS-CoV-2 binds to ACE2 (shown in figure) or NRP1 receptors, and a serine protease, e.g., TMPRSS2, cleaves the complex, leading to viral internalization. (C) The virus infects sustentacular cells of the nasal epithelium. ACE2, angiotensin-converting enzyme 2; BBB, blood-brain barrier; CNS, central nervous system; GBS, Guillain-Barré Syndrome; NRP1, neuropilin-1; PNS, peripheral nervous system; TMPRSS2, transmembrane serine protease 2. Created, in part, with BioRender.com.

there was generalized widespread glial fibrillary acidic protein (GFAP) positive staining, indicative of astrogliosis, in white matter, but not in the hemorrhagic lesions. Furthermore, separate radiologic case reports of SARS-CoV-2-induced encephalitis document symmetric focal involvement of the bilateral thalami (Poyiadji et al., 2020) and the brainstem (Dixon et al., 2020). The neurologic UK study did not detect antibodies in COVID-19 patients with autoimmune encephalitis (Paterson et al., 2020). However, a number of studies have reported myelin oligodendrocyte glycoprotein (MOG)-associated demyelinating syndrome (Woodhall et al., 2020; Sinha et al., 2021), encephalitis (Peters et al., 2021), and optic neuritis (Sawalha et al., 2020) in the SARS-CoV-2 setting.

In the PNS, most neurological SARS-CoV-2-related clinical experience has focused on GBS, and numerous cases have been described (Padroni et al., 2020; Sedaghat and Karimi, 2020; Tiet and AlShaikh, 2020). As with most pathogen-mediated GBS, autoimmune cross-reactivity or molecular mimicry is thought to underlie SARS-CoV-2-related GBS. Antibodies specific to PNS disease are present in GBS cases post SARS-CoV-2, including IgG, anti-GM1, anti-GD1a, and anti-GD1b (Civardi et al., 2020; Dufour et al., 2021). One case of Miller Fisher syndrome with increased pro-inflammatory cytokine markers and positive anti-GD1b-IgG levels has been reported, supporting immune-mediated pathomechanisms (Gutiérrez-Ortiz et al., 2020).

Although it was initially thought that the pandemic would trigger a spike in GBS cases, data has shown otherwise. Instead, three studies found either stable incidence or a decline in GBS cases during the SARS-CoV-2 era (Keddie et al., 2021; Luijten et al., 2021; Umapathi et al., 2021). As with other novel viral pathogens, like Zika, there is an incontrovertible and emerging, albeit small, body of evidence that suggests a temporal link between COVID-19 infection and GBS (Aladawi et al., 2022). This seemingly contradictory finding lacks a definitive explanation, although putative reasons have been put forth. One suggestion for this discrepancy is the near-universal mask-wearing and stay-at-home orders of the pandemic (Foschi et al., 2021). It is postulated that this increased mask-wearing and limited social interaction reduced the incidence of non-SARS-CoV-2-related GBS, which would still constitute the bulk of total cases. Furthermore, it is possible that patients with milder GBS may not have sought medical attention to avoid a hospital setting. Limited hospital bed availability may have been another reason. Other explanations have also been explored (Foschi et al., 2021).

Of all post SARS-CoV-2 neurologic consequences, post-acute sequelae SARS-CoV-2 (PASC), also called long-COVID syndrome, remains the topic of greatest public interest, although the exact underlying pathomechanisms remain elusive. Patients report fatigue, cognitive slowing, and exertional intolerance, among many other symptoms. Some patients meet the criteria for orthostatic intolerance and postural tachycardia syndrome on formal autonomic testing (Shouman et al., 2021). It is suspected that SARS-CoV-2 binding to angiotensin-converting enzyme 2 (ACE2) receptors may disrupt the renin-angiotensin-aldosterone system, which regulates sympathetic outflow (Goldstein, 2021).

Therapeutic experience remains limited, although the neurologic UK study revealed that some encephalopathies improved without specific treatment (Paterson et al., 2020), while patients with inflammatory CNS syndromes improved with corticosteroids and/or immunoglobulin therapy.

3. SARS-CoV-2 neurotropism

Early in the pandemic, it was shown SARS-CoV-2 leverages the ACE2 receptor to facilitate entry into host cells, like its predecessors, SARS-CoV and Middle East respiratory syndrome coronavirus (Yan et al., 2020). Once the SARS-CoV-2 spike protein receptor-binding domain latches onto ACE2, proximal serine proteases, e.g., transmembrane serine protease 2 (TMPRSS2), cleave the spike-ACE2 complex, internalizing the virus (Fig. 1B) (Hoffmann et al., 2020). Tissue receptor expression dictates tropism; thus, widespread ACE2 expression across multiple tissues results in extensive SARS-CoV-2 tropism. ACE2 expression level in the CNS is moderate (Li et al., 2020a, 2020b); however, since the discovery that SARS-CoV-2 binds ACE2, additional receptors have been identified, including neuropilin-1 (NRP1), a glycoprotein involved in neurogenesis (Zhang et al., 2021a, 2021b). ACE2 and NRP1 are widely expressed in the brain; however, ACE2 is most highly expressed in endothelial vasculature and circumventricular organs (Hernández et al., 2021), whereas NRP1 expression is especially enhanced in the hippocampus, endothelial cells, mural cells, perivascular macrophages, and microglia (Davies et al., 2020).

Several mechanisms have been proposed for viral penetration into the brain. One suggested viral entry route is by disrupting the choroid plexus, thereby compromising the BBB and providing SARS-CoV-2 access to the CNS. The virus also triggers a pro-inflammatory response, which can render the BBB susceptible to damage, leading to immune cell infiltration into the brain along with viral penetration (Tremblay et al., 2020; Solomon, 2021). A possible avenue for dissemination throughout the CNS, once the virus has gained passage into the brain, is through the microvasculature. Autopsy examination of frontal cortex tissue from COVID-19 patients ($n = 17$) reveals an increase of so-called string vessels versus control tissue ($n = 23$) (Wenzel et al., 2021). String vessels are empty basement membrane tubes lacking endothelial cells, which are signs of capillary loss. The study found that M^{pro}, the primary SARS-CoV-2 protease, cleaves host endothelial NEMO (nuclear factor (NF)-κB essential modulator), leading to endothelial cell apoptosis, local hypoxia, and microglial and astrocytic reactivity (Wenzel et al., 2021). Therefore, microvascular pathology and endothelial dysfunction (Varga et al., 2020) in the brain may constitute a significant mode of viral dissemination and neuropathology in the brain.

Anosmia is a prominent symptom of COVID-19 infection, even in relatively mild cases. This promoted a putative mechanism of viral entry into the CNS intranasally through the olfactory epithelium, which expresses ACE2 (Fig. 1C) (Brann et al., 2020) and NRP1 (Cantuti-Castelvetri et al., 2020). In this proposed mechanism, the olfactory bulb would then serve as a conduit to the CNS, including ACE2-expressing circumventricular organs, such as the subfornical organ (de Melo et al., 2021), which are particularly vulnerable because they are not protected by the BBB. A recent study by Khan et al. of olfactory mucosa and whole olfactory bulb tissue from recently deceased COVID-19 patients ($n = 85$) detected SARS-CoV-2 primarily in the sustentacular cells of the mucosa (Khan et al., 2021). The virus was not detected in olfactory sensory neurons nor the olfactory bulb parenchyma (Khan et al., 2021), like another study, which only found sparse pathology of olfactory bulbs (Thakur et al., 2021). Thus, overall, an intranasal route into the CNS may be unlikely.

Far fewer studies have investigated SARS-CoV-2 penetration into the PNS, although case reports of peripheral neuropathies are reported within the same timeframe of COVID-19 infection (Padroni et al., 2020; Sedaghat and Karimi, 2020; Tiet and AlShaikh, 2020). PNS damage could occur secondary to SARS-CoV-2-induced inflammation, as occurs in the CNS. Alternatively, drawing parallels to other viruses, SARS-CoV-2 could be internalized into neurons through the endocytic pathway and hijack axonal trafficking to spread along the PNS (Fenrich et al., 2020).

Our understanding of SARS-CoV-2 neurotropism derives from both human, i.e., autopsy, and in vitro and mouse studies. Autopsy reveals invasion of the olfactory epithelium (Khan et al., 2021) and CNS (Mukerji and Solomon, 2021). Analysis of autopsy tissue from three COVID-19 patients demonstrated immunoreactivity against the viral spike protein in cortical neurons and endothelial cells, albeit to variable extents (Song et al., 2021). Cellular staining was perinuclear along with both intense puncta and diffuse cytoplasmic reactivity; subcortical microscopic ischemic infarcts were also present, which stained for viral proteins in the hyperacute stage. However, although neuroinflammation is a putative characteristic of severe COVID-19 infection, the areas that stained for viral protein were not infiltrated by lymphocytes or

leukocytes (Song et al., 2021), though another autopsy study noted some CD3+, CD4+, and CD8+ T cells in the parenchyma (Schurink et al., 2020).

Analysis of brain autopsy tissue ($n = 21$) for RNA detected the virus in eight samples, but only at low SARS-CoV-2 copies per cell (Puelles et al., 2020). Another study ($n = 18$ patients) similarly detected viral RNA in various brain regions in most samples (Solomon et al., 2020). Interestingly, viral protein was not detected in neurons, glia, endothelium, or immune cells. A larger analysis ($n = 40$) found both viral RNA and protein in 8 (20%) of brain autopsy samples and either RNA or protein in 21 (53%) specimens (Matschke et al., 2020). One study detected viral RNA, but no protein, in most brain autopsy samples ($n = 25$), but levels were far lower than in mucosal samples (Thakur et al., 2021). The authors concluded that direct viral neuroinvasion in the brain was unlikely due to the significantly lower viral RNA levels in the brain relative to the nasal epithelium, and that most CNS neuropathology results from systemic inflammation, possibly compounded by local hypoxia and ischemia. It is worth noting that peripheral immune cell infiltration into the CNS has been detected in the brain, but also to variable extents (Schurink et al., 2020; Song et al., 2021), and primarily in the brain stem (Matschke et al., 2020; Thakur et al., 2021).

Another point of consideration, which has limited our understanding of SARS-CoV-2 neurotropism in the brain from autopsy samples, is that sensitivity for detecting viral RNA may be higher than for viral proteins (Solomon et al., 2020; Thakur et al., 2021). However, RNA analysis from bulk brain tissue does not allow localization of the RNA to specific cell types, and the virus detected in the brain may be more prevalent in endothelial cells versus neurons or glia (Nuovo et al., 2021; Wenzel et al., 2021). However, this possibility requires further investigation and single-cell sequencing of brain autopsy tissue may be one path forward (Fullard et al., 2021).

Regarding the PNS, consecutive autopsies ($n = 35$) from femoral nerve tissue revealed neuritis in nine COVID-19 patients without evidence of direct SARS-CoV-2 invasion, likely indicating inflammatory- or immune-mediated PNS damage rather than tissue tropism (Suh et al., 2021), though further studies are necessary.

Beyond autopsy studies, brain organoids have also expanded our understanding of viral neurotropism (Ng et al., 2021). The first obstacles to viral penetration into the CNS are the BBB and blood-cerebrospinal fluid (CSF) barriers, which regulate entry of material into the brain. The blood-CSF barrier lines the choroid plexus, which produces CSF. Infection rate was significantly higher in choroid plexus (10–20%) versus cortical (<1.5%), hippocampal (<1.0%), hypothalamic (<1.5%), and midbrain (<1.5%) organoids generated from human induced pluripotent stem cells (hiPSCs) (Jacob et al., 2020). Similarly, analysis of cell cultures found choroid plexus epithelial cells were robustly infected with SARS-CoV-2, whereas neurons and astrocytes were sparsely infected. Another study of hiPSC-derived choroid plexus organoids observed higher ACE2 expression by mature choroid plexus cells but not by neurons or other cell types, which mirrored greater SARS-CoV-2 infectivity of choroid cells (13%), but not of neurons or glia (Pellegrini et al., 2020). Additionally, the live virus also structurally and functionally compromised the choroid plexus epithelial barrier, and preference for choroid plexus cells and glia by SARS-CoV-2 over neurons was also reported by McMahon and colleagues (McMahon et al., 2021). Although human studies suggest that viral entry may proceed primarily from systemic inflammation (Solomon, 2021; Thakur et al., 2021), brain organoid studies suggests= that direct viral infection and/or destruction of choroid plexus may be feasible. However, if this pathway does occur in vivo, it may be to an insignificant extent.

Some brain organoid studies have observed higher infection of neurons by SARS-CoV-2 (Song et al., 2021; Wang et al., 2021), especially mature, MAP2-positive neurons, leading to neuronal death and loss (Song et al., 2021). A single-cell RNA-seq study identified multiple clusters of neuronal, neuronal progenitor, and radial glial cell populations (Song et al., 2021). Overlap of SARS-CoV-2 transcripts occurred with all cell-types, but to variable extents among the multiple clusters. Viral infection also induced a transcriptomic shift to a hypermetabolic and hypoxic cellular state, indicating that SARS-CoV-2 may be hijacking cellular metabolism to replicate. Differences among brain organoid studies may derive from maturation state of the organoid, since differentiated neurons are more susceptible than neural progenitor cells (Song et al., 2021) and differentiation boosts infection of astrocytes (Wang et al., 2021).

Alternatively, variation in the hiPSC genetic background in genes linked to SARS-CoV-2 susceptibility could give rise to differential infectivity of neurons and various cell-types observed across organoid studies. For instance, the ApoE4 allele is a risk for severe COVID-19 infection (Kuo et al., 2020). Indeed, isogenic hiPSCs expressing ApoE3 versus ApoE4 demonstrates that ApoE4 renders differentiated neurons more prone to SARS-CoV-2 infection (2.1% ApoE4 versus 1.4% ApoE3) and viral-mediated neurite degeneration (Wang et al., 2021). ApoE4 in differentiated astrocytes similarly predisposes them to viral infection and a decrease in soma and process length. Another possibility for discordance in studies of cellular infectivity preference of SARS-CoV-2 is the viral strain. A German study concluded that a Düsseldorf isolate of SARS-CoV-2 preferentially targets neurons versus neuronal progenitor in human derived brain organoids (Ramani et al., 2020).

The striking differences between autopsy findings and in vitro brain organoids merits discussion. By and large, viral RNA or protein can be detected in brain tissue from COVID-19 patients, but only to low levels and their localization to specific cell types remains unclear, although endothelial cells may be a favored compartment versus neurons and glia (Nuovo et al., 2021; Wenzel et al., 2021). On the other hand, SARS-CoV-2 infection of neurons, glia, and choroid plexus cells is evident from brain organoid analysis. Several reasons may explain these disparate findings. If the virus does preferentially infect endothelial cells versus neurons and glia, microvasculature may be the preferred target in vivo. However, brain organoids are devoid of a comprehensive vasculature (Jacob et al., 2020; Song et al., 2021), and SARS-CoV-2 may invade neurons and glia in the absence of other targets, such as endothelial cells. Another possibility is differences in the viral load in vitro and in vivo. Brain organoids can be experimentally infected to a specified multiplicity of infection, at a burden that targets neurons and glia. However, the viral burden may be lower in vivo due to the BBB, despite damage from systemic inflammation, occurring at levels that do not infect neurons. Finally, viral attack is dynamic in vivo, and the temporal element is critical. COVID-19 patients may pass away before significant viral replication has occurred into the brain.

Cumulatively, however, human, in vitro, and animal data support invasion into the CNS. Possibly in humans, the dominant pathway is from systemic inflammation, though this does not fully exclude other less significant entry modes. Moreover, endothelial dysfunction in the brain is an important aspect of neuropathology secondary to SARS-CoV-2 infection. Additionally, the determinants of tropism among specific CNS cell-type still requires clarification. Understanding the determinants of infectivity could help identify patients at risk of developing COVID-19-mediated neurological disorders. If genetic background is a potential determinant of infection, it could explain variable susceptibly to SARS-CoV-2-induced neurological complications in COVID-19 patients. Finally, few studies have investigated neuroinvasion into the PNS, though SARS-CoV-2 may induce peripheral nerve damage.

4. SARS-CoV-2-induced disturbance in neuron-glial interactions

4.1. SARS-CoV-2 in the central nervous system

4.1.1. Astrocyte-neuron interactions

The brain is an incredibly complex organ; neurons are aided in their function by supportive glia, comprised primarily of oligodendrocytes, astrocytes, and microglia. Under homeostatic conditions, astrocytes

contribute structurally to BBB maintenance (Linnerbauer and Rothhammer, 2020) and are thus part of the brain's first line of defense from invading pathogens, including SARS-CoV-2 (Fig. 2). Astrocytes also form tripartite synapses with neurons, providing support through neurotransmitter regulation and metabolic coupling. Virally induced disruption of astrocyte function could thus impair both neuronal transmission and metabolism (Cotto et al., 2019; Sher et al., 2019).

Pathologic conditions, such as the presence of danger signals from viral invasion, called damage-associated molecular pattern molecules (DAMPs), induce astrogliosis, a neuroprotective phenotype characterized by morphological, transcriptomic, and biochemical reprogramming and glial fibrillary acidic protein (GFAP) upregulation (Fig. 3). However, astrogliosis can serve as a double-edged sword, promoting the disease process through a neurotoxic phenotype (Ding et al., 2021). In a longitudinal study of COVID-19 patients (n = 47) with varying disease severity, moderate and serious COVID-19 infection correlated with elevated plasma GFAP, and severe disease correlated with increased plasma neurofilament light chain (NfL), a marker of intra-axonal neuronal injury (Kanberg et al., 2021). In severe COVID-19 cases, plasma GFAP peaked earlier in the infection course, indicating initial astrogliosis, whereas NfL was persistently elevated, possibly from longer-lasting neuronal damage. Another study of hospitalized COVID-19 patients with (n = 34) and without (n = 94) neurological symptoms found elevated serum NfL levels, independent of neurologic presentation and uncorrelated to CSF NfL levels, possibly reflecting peripheral nerve damage in severe infection (Paterson et al., 2021). Serum NfL levels were not increased in COVID-19 community cases, suggesting mild disease may not produce nerve injury. Unlike some studies, however, GFAP was not prominent in CSF of patients with serious COVID-19, implying astrogliosis-induced neuronal damage may not always be part of SARS-CoV-2 pathophysiology. It is possible, however, that discrepancies between studies also arise from the time course of GFAP release, which is higher earlier in infection, leading to different results depending on when plasma/serum GFAP is sampled. Possibly, astrogliosis may be easier to detect in brain autopsy tissue. Indeed, investigation of brain samples from deceased COVID-19 patients (n = 43) documented astrogliosis through GFAP staining in 37 (86%) of cases (Matschke et al., 2020).

Another primarily astroglial protein, S100B, also has a putative role as a DAMP and correlates with various neural CNS injuries, from traumatic acute brain damage to neurodegenerative diseases (Michetti et al., 2019). In a small longitudinal study of COVID-19 patients with neurological manifestations involving the CNS and cytokine storm (n = 5), elevated serum S100B was coincident with cytokine release in three patients with acute leukoencephalitis. Though small, the study does suggest that cytokine storm correlates with leukoencephalitis, which injures astrocytes and induces BBB leakage (Perrin et al., 2021).

In addition to a shift in astroglial phenotypes, COVID-19 may lead to astrocytic and microglial proliferation, concomitant with a decrease in neuronal density, as noted in a small neurohistopathological study of brain autopsy material (n = 3 COVID-19, n = 3 control) (Boroujeni et al., 2021). The astrocytes and microglia also adopted a pro-inflammatory phenotype, a scenario suggesting inflammation-induced neuronal loss.

4.1.2. *Microglia-neuron interactions*

Microglia are the resident immune cells of the CNS (Prinz et al.,

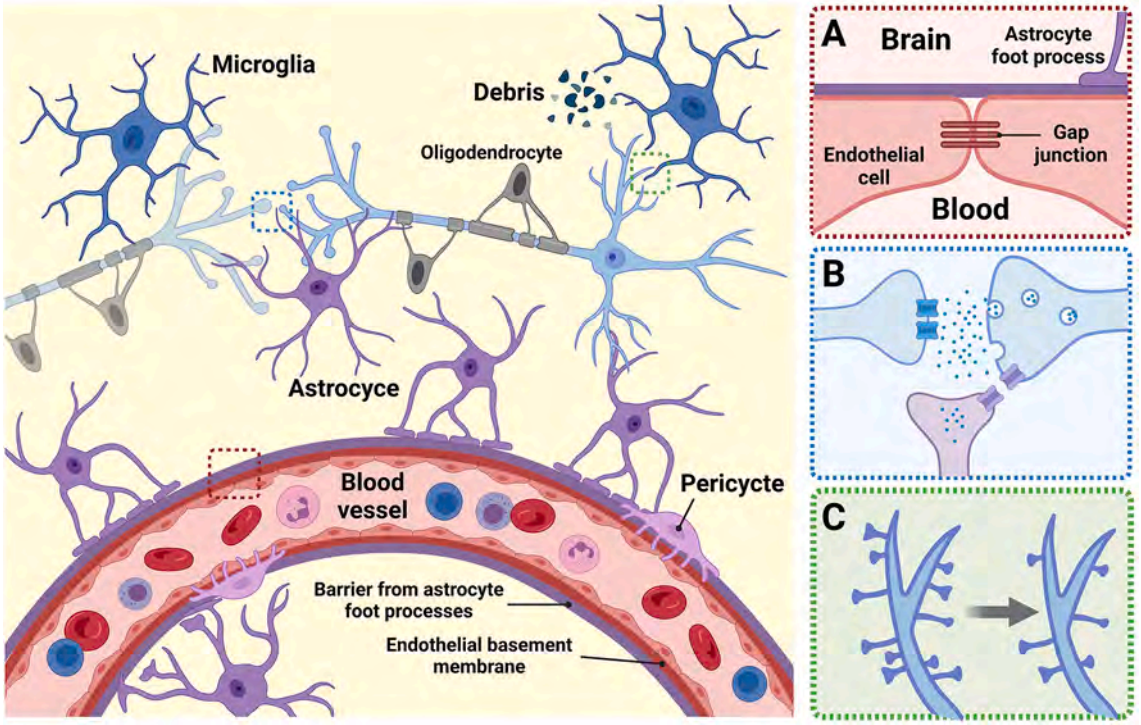

Fig. 2. Neuron-glia homeostasis in the healthy brain.
Overview figure of the central nervous system (CNS) under homeostatic conditions. Brain neurons are supported by glia, astrocytes, microglia, and oligodendrocytes. *Neurons (light blue)*: Functioning astrocytes and microglia support signal transmission in healthy neurons, which are wrapped with oligodendrocytes (grey). *Blood vessels*: Circulating species in blood vessels are separated from the brain by the blood-brain barrier (BBB; pink inset, A). The BBB is comprised of tight junctions between endothelial cells of the vasculature, along with structural reinforcement from the extracellular matrix from the basement membrane and astrocytic foot processes. The BBB regulates what species are permitted to traverse into the central nervous system (CNS). *Astrocytes (purple)*: Contribute structurally to the BBB through astrocyte foot processes, which form a layer against the endothelial basement membrane, defending the brain from invading pathogens. Inset, blue, B: Astrocytes (purple) form tripartite synapses with neurons (light blue) to provide neurotransmitter and metabolic support. *Microglia (dark blue)*: CNS resident immune cells, surveil for potentially harmful agents and clear the CNS of debris by phagocytosis. Inset, green, C: Microglia remodel neuronal circuits by pruning dendritic spines and synapses and promote myelin maintenance and remyelination. Created with BioRender.com.

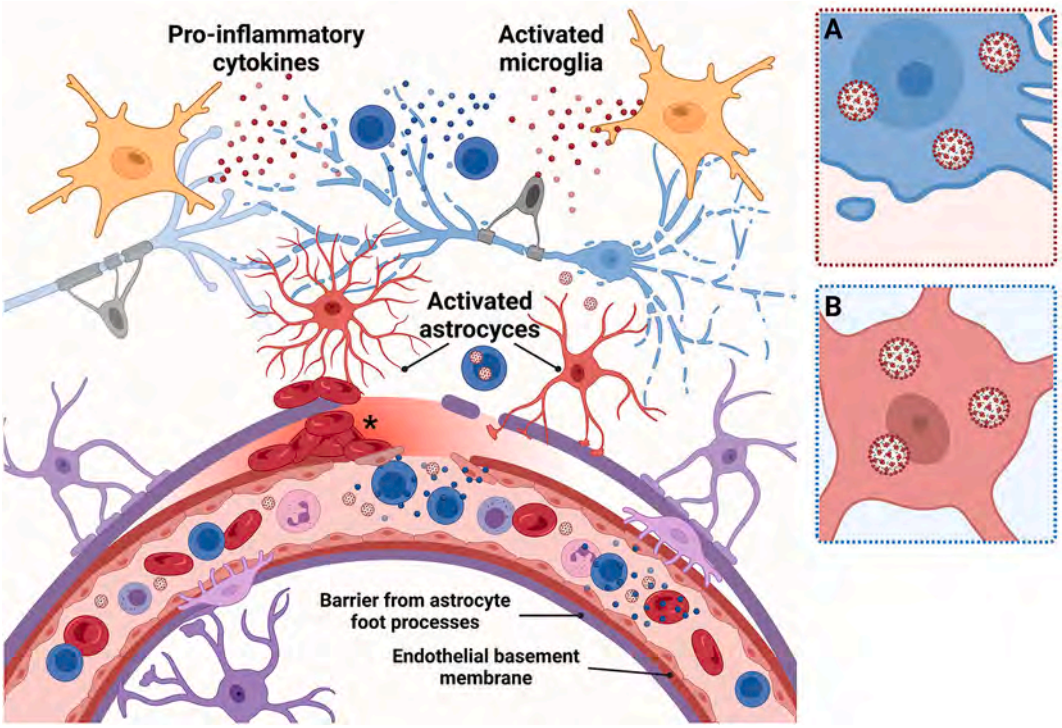

Fig. 3. Putative SARS-CoV-2-induced disruption of neuron-glia homeostasis.
Overview figure of the CNS under SARS-induced pathologic conditions. *Neurons (light blue)*: Excessive inflammation may cause neurons to degenerate. Inset, pink, A: SARS-CoV-2 may also potentially directly invade neurons, triggering apoptosis. *Blood vessels*: High levels of circulating pro-inflammatory cytokines (blue spheres) damage the BBB; peripheral cytotoxic T cells infiltrate the CNS, further augmenting pro-inflammatory cytokine levels. Circulating SARS-CoV-2 virions can also penetrate the CNS or hijack immune cells to enter. Microbleeds from SARS-CoV-2-induced endothelial dysfunction (asterisks) can also occur, causing CNS damage. *Astrocytes (purple and red, activated)*: SARS-CoV-2 triggers astrogliosis, which raises production of damage-associated molecular pattern molecules, e.g., GFAP. Astrogliosis may also contribute to the breakdown in BBB integrity, e.g., through astrocytic foot processes detachment. Inset, blue, B: SARS-CoV-2 may infect astrocytes, possibly depending on the genotype. *Microglia (orange)*: SARS-CoV-2 induces microgliosis, which elevates microglia-derived pro-inflammatory cytokines (red spheres). Both astrogliosis and microgliosis occur concomitant with neuronal loss. Created with BioRender.com.

2019). During homeostatic conditions in the adult brain, they surveil the CNS milieu for potentially harmful agents, e.g., infectious pathogens, tissue injury (Fig. 2). They also perform numerous "housekeeping" roles, such as clearing the CNS of debris by phagocytosis. Microglia also remodel neuronal circuits by pruning dendritic spines and synapses during learning and memory and participate in axonal myelin maintenance and remyelination. Therefore, microglia have a direct impact on neuronal health. Under pathologic conditions during infection, microglia launch an immune response in order to clear the invading pathogen from the CNS (Fig. 3) (Hatton and Duncan, 2019). This response is complex and involves variably activated microglia with anti- and pro-inflammatory phenotypes (Cherry et al., 2014). Unfortunately, in certain instances, an overly pro-inflammatory state can develop, which is toxic to neurons.

A comparable situation may occur during SARS-CoV-2, with a microglial-mediated local inflammatory response within the CNS, which is compounded by the presence of systemic hyperinflammation that can also spread throughout the brain (Solomon, 2021). *Post-mortem* evaluation of COVID-19 patients (n = 43) sheds light on this dual inflammatory process in the brain, which demonstrates both microglial activation and cytotoxic T lymphocyte infiltration, mostly in the brainstem and cerebellum (in up to 79% of cases) (Matschke et al., 2020). Analysis of cerebral autopsy samples (n = 3) also demonstrates activation of the NLRP3 inflammasome (Cama et al., 2021), a sensor of pathogen-associated molecular patterns (PAMPs) involved in the antiviral response (Zhao and Zhao, 2020). NLRP3 colocalizes with CD68+ macrophages in the brain and the periphery (lung) of the deceased, implicating a role for NLRP3 in SARS-CoV-2 pathology (Cama et al., 2021).

Single-nucleus RNA sequencing of brain samples for COVID-19 patients that died from severe infection confirm immunohistological microglia analyses. The brain transcriptome in COVID-19 (n = 8 COVID-19; n = 13 controls) reveals choroid plexus barrier disruption along with signaling cues into the CNS (Yang et al., 2021). Peripheral T cell infiltration is present along with resident microglia, which have adopted a phenotype reminiscent of neurodegenerative disease. Another transcriptomic analysis of three distinct brain areas (n = 5 COVID-19; n = 4 controls) found an influx of monocytes and macrophages in the choroid plexus along with a signature in cortical microglia linked to cellular activation, mobility, and phagocytosis (Fullard et al., 2021). In both instances, molecular traces of SARS-CoV-2 were not detected (Fullard et al., 2021; Yang et al., 2021), possibly suggestive of persistent inflammation and longer-lasting neural injury, even after the virus has cleared. Using a paradigm of spatial profiling by imaging mass cytometry at single-cell resolution, an evaluation was performed of autopsy brain stem and olfactory bulb (Schwabenland et al., 2021). Significant neuropathology was seen, involving axonal damage, astrocytosis, and BBB leakage, concomitant with viral antigens in ACE2+ vascular cells. Perivascular microglial nodules enriched with activated CD8+ T cells were also noted, which correlated with clinical measures of systemic inflammation.

Research of in vitro and in vivo models paints a similar picture. Exposure of rodent BV-2 microglia to SARS-CoV-2 spike protein increases tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), IL-1β, and inducible nitric oxide synthase (iNOS)/ nitric oxide (NO) production (Olajide et al., 2021). Microglial contact with spike protein also enhanced NLRP3 inflammasome and caspase-1 activity. Similar observations were made following exposure of human HMC3 microglia to

SARS-CoV-2 spike protein, leading to heightened TNF-α, IL-8, IL-1β, and reactive oxygen species generation and increased NOS and caspase-3/7 expression (Clough et al., 2021). This pro-inflammatory response was accompanied by microglial morphological changes along with mitochondrial fragmentation. In vivo, in SARS-CoV-2-infected K18-hACE2 transgenic mice (cytokeratin-18 gene promoter driven human ACE2 expression) and Syrian hamsters, microglia proliferated and increased TNF-α and IL-6 expression (Zhang et al., 2021b).

CNS microglia may also be activated by exosomes carrying viral material, rather than the virus itself. Exosomes are extracellular vesicles that bud off from cells and carry cargo to recipient cells. This intercellular communication mode can occur as part of normal physiologic function, or as a pathological process during disease states, including a viral infection. Circulating exosomes have been detected in patients in the context of SARS-CoV-2 infection, carrying cargo harboring SARS-CoV-2 RNA and a distinct proteomic signature strongly involved in host response to infection, immune processes, inflammation, and coagulation (Barberis et al., 2021). It is conceivable that exosomes can penetrate the CNS and activate microglia; indeed, an in vitro study of exosomes derived from HEK293T cells transfected with SARS-CoV-2 spike plasmid were found to transfect human microglial CHME3 cells (Mishra and Banerjea, 2021). Exosome cargo was enriched in microRNAs (miR) 148a and miR-590-3p. microRNAs are small non-coding RNAs that negatively regulate expression of target genes. The exosomes suppressed ubiquitin specific peptidase 33 (USP33) and interferon regulatory factor 9 (IRF9) expression in recipient microglia, the latter being involved in immune regulation.

5. Summary

Overall, human, in vitro, and animal evidence suggests a recurrent theme of astrogliosis and pro-inflammatory microglial activation in SARS-CoV-2 infection in the brain (Fig. 3). Some studies suggest these processes occur in tandem with neuron axonal damage and loss, possibly indicating neuronal injury arising secondary to a breakdown in neuron-glia homeostasis. Future studies are needed to better understand the molecular steps or mediators of neuron-glia or axo-glia communications during SARS-CoV-2 infection leading to brain damage, with the goal of developing mechanism-based therapies. Furthermore, understanding of potential neuron-oligodendrocyte interactions is lacking in the context of SARS-CoV-2. However, a study of another coronavirus, mouse hepatitis virus (MHV), suggests surviving oligodendrocytes post viral infection may prolong the inflammatory phase (Pan et al., 2020).

5.1. SARS-CoV-2 in the peripheral nervous system

In contrast to CNS studies, there is a paucity of reports in the PNS. However, PNS neurotropism is a facet of SARS-CoV-2 pathophysiology as evidenced by the emerging literature linking autoimmune peripheral neuropathies to COVID-19 infection (Padroni et al., 2020; Sedaghat and Karimi, 2020; Tiet and AlShaikh, 2020) and the presence of IgG, anti-GM1, anti-GD1a, and anti-GD1b antibodies (Civardi et al., 2020; Dufour et al., 2021). Elevated serum NfL hints at PNS damage during severe COVID-19 infection (Paterson et al., 2021). However, mechanistic studies in SARS-CoV-2 are lacking, although clinical findings suggest an immune-mediated component, at least in part.

Drawing from other neurotropic viruses, such as MHV, demyelinating strains are transported in a retrograde fashion from the brain via axons, inducing optic neuritis with macrophage infiltration and axonal demyelination and loss (Shindler et al., 2011). Human coronavirus OC43 can also spread via neuron axonal transport machinery (Dubé et al., 2018). Thus, similar modes of spread like MHV and OC43 have been proposed for SARS-CoV-2 (Li et al., 2020b); however, this is speculative, and experimental evidence for SARS-CoV-2 is presently missing. One study reported direct nociceptor infection on dorsal root ganglia by SARS-CoV-2, which suggests potential PNS neurotropism (McFarland et al., 2021). SARS-CoV-2 attack of sensory dorsal root ganglia nociceptors implicates potential pathways for pain during severe COVID-19, and possibly into the PASC post-infection phase.

Overall, more studies are needed and will likely evolve over time as we increase our understanding of PNS disorders secondary to SARS-CoV-2.

6. Potential therapeutic avenues of SARS-CoV-2-mediated neurologic injury

To our knowledge, there is no specific treatment protocol for preventing neurological complications in COVID-19 patients. Some neurological conditions that develop from SARS-CoV-2 infection improve without specific treatment or can otherwise be treated per standard of care, e.g., corticosteroids or immunoglobulins for autoimmune encephalitis (Paterson et al., 2020). Overall, therapeutic experience remains limited. Fortunately, current understanding of SARS-CoV-2 pathomechanisms suggests potential therapeutic avenues. First, lowering systemic and neural inflammation through broad-acting anti-inflammatory corticosteroid therapy, for example, could mitigate brain vascular and neural damage. Dexamethasone is part of the present repertoire of therapies being used to treat COVID-19 patients. In an open-label trial, dexamethasone reduced 28-day mortality in hospitalized COVID-19 patients on respiratory support, e.g., invasive mechanical ventilation, oxygen administration, but not in those off of respiratory support (Horby et al., 2021). Whether dexamethasone specifically prevents neurological injury from SARS-CoV-2 remains to be seen. Other approaches to lowering systemic inflammation include immunomodulating antibodies (Izda et al., 2021), e.g., tocilizumab, sarilumab.

The second potential therapeutic target, that of SARS-CoV-2 invasion and/or neuroinvasion, may be mitigated by blocking viral replication and penetration into cells (Izda et al., 2021). Replication-inhibitors include antivirals, such as remdesivir. Preventing cellular entry can be achieved by binding to the virus spike protein or by blocking the ACE2-protease machinery, which grants the virus entry into cells. Casirivimab and imdevimab are monoclonal antibodies that bind to SARS-CoV-2 spike protein and obstruct ACE-2 binding (Izda et al., 2021). The combined casirivimab and imdevimab cocktail is effective in preventive and therapeutic paradigms and lowers the incidence and severity of COVID-19 infection (O'Brien et al., 2021; Weinreich et al., 2021). However, the efficacy of this casirivimab/imdevimab cocktail at preventing neurological complications has not been studied. Other approaches of preventing SARS-CoV-2 entry into cells rely on pharmacological inhibition of ACE2-protease machinery (Zhang et al., 2021a). Such approaches have only had mixed success thus far, as with hydroxychloroquine.

Preclinical and clinical research is ongoing, and the need is great for more definitive COVID-19 therapeutics, specifically those aimed at neural protection.

7. Conclusion

It is now clear that SARS-CoV-2 induces a host of acute neurological complications of varying clinical severity. In some instances, these neurologic sequelae evolve into long-term and irreversible complications. As the virus becomes endemic (Emanuel et al., 2022), this could have ramifications for many recovering from COVID-19 infection, although emerging variants my not trigger as much neurological damage as the original and delta variants. Fortunately, the putative mechanism of COVID-19 induced acute neurologic injury is becoming more apparent, thus opening the window for therapeutic interventions. These pathologic mechanisms include systemically and locally induced inflammatory responses and/or endothelial dysfunction, potential direct viral neuroinvasion, astrogliosis, and pro-inflammatory microglial activation, all leading to neuronal injury. Thus, anti-inflammatories and monoclonal antibodies may help prevent or minimize neurologic damage in the context of acute infection, although formal studies are needed.

Moreover, a clearer mechanistic and molecular picture of neurological pathology in acute SARS-CoV-2 infection may lead to more effective therapies for mitigating neural damage in patients developing PASC (or long covid syndrome) post infection. For these patients, the landscape remains poorly defined, and therapies to date are primarily supportive. In summary, as COVID-19 infections continue to rise, the burden of SARS-CoV-2-induced acute neurologic disorders and PASC will also increase, necessitating a greater understanding of disease pathophysiology to enable the development of mechanism-based therapies targeting both the CNS and PNS.

Search terms

"COVID-19", "SARS-CoV-2", "astrocyte", "autopsy", "axon-glia", "brain", "cell", "central nervous system", "clinical", "exosome", "glia", "microglia", "neurotropism", "oligodendrocyte", "PASC", "peripheral", "peripheral nerve", "peripheral nervous system", "Schwann cell", "syndromes". Conducted initially during September and then again in December to update.

Declaration of Competing Interest

MGS, ELF, and AMS declare no conflicts of interest.

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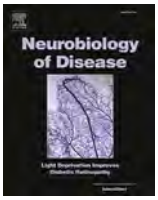

Stem cell therapy for central nervous system disorders: Metabolic interactions between transplanted cells and local microenvironments

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ABSTRACT

Stem cell therapy is a promising and rapidly advancing treatment strategy for a multitude of neurologic disorders. Yet, while early phase clinical trials are being pursued in many disorders, the mechanism of action often remains unclear. One important potential mechanism by which stem cells provide neuroprotection is through metabolic signaling with diseased neurons, glia, and other cell types in the nervous system microenvironment. Early studies exploring such interactions report normalization of glucose metabolism, induction of protective mitochondrial genes, and even interactions with supportive neurovasculature. Local metabolic conditions also impact stem cell biology, which can have a large impact on transplant viability and efficacy. Epigenetic changes that occur in the donor prior to collection of stem cells, and even during in vitro culture conditions, may have effects on stem cell biology that are carried into the host upon stem cell transplantation. Transplanted stem cells also face potentially toxic metabolic microenvironments at the targeted transplant site. Novel approaches for metabolically "preconditioning" stem cells prior to transplant harness metabolic machinery to optimize stem cell survival upon transplant. Ultimately, an improved understanding of the metabolic cross-talk between implanted stem cells and the local nervous system environment, in both disease and injury states, will increase the likelihood of success in translating stem cell therapy to early trials in neurological disease.

1. Introduction

Therapeutic options for neurologic disorders affecting the central nervous system (CNS) are at times hindered by the unknown or complicated mechanisms responsible for the underlying pathogenesis. While some CNS diseases have known genetic causes tied to a certain protein or pathway, many involve an intricate interplay between multiple cell types and metabolic processes within a complex microenvironment (Argueti-Ostrovsky et al., 2021; Guo et al., 2020; Le Gall et al., 2020; Mejzini et al., 2019). Similarly, traumatic brain and spinal cord injury, or vascular events such as stroke, induce a cascade of detrimental events that impact neurologic health (Delage et al., 2021; Mira et al., 2021; Uyeda and Muramatsu, 2020). As such, therapeutic approaches are required that offer multidimensional benefits to the diseased or injured nervous system.

Stem cell transplantation represents a promising opportunity to approach the treatment of CNS diseases and injury in a comprehensive, multifaceted manner. Many types of stem cells, including embryonic stem cells (ESCs), neural stem cells (NSCs), mesenchymal stem cells (MSCs), and induced pluripotent stem cells (iPSCs), are being evaluated in vitro, in vivo, and in translational clinical studies for their potential utility for a range of neurologic conditions (Chen and Feldman, 2017). These and other stem cell subtypes have entered the realm of early clinical trials for a variety of neurologic conditions, capitalizing on their proliferative capacity and adaptable biology. For amyotrophic lateral sclerosis (ALS), for example, intraspinal NSC transplantation has been

Abbreviations: ALS, amyotrophic lateral sclerosis; AMPK, AMP-activated protein kinase; CNS, central nervous system; COXIV, cytochrome c oxidase subunit 4; CREB, cAMP response element-binding protein; EPO, erythropoietin; ESC, embryonic stem cell; FAD, flavin adenine dinucleotide; FDG-PET, fluorodeoxyglucose-positron emission tomography; FOXO, forkhead box class O; GALE, urine diphosphate-galactose 4-epimerase; GLUT, glucose transporter; HIF, hypoxia-inducible factor; HLA, human leukocyte antigen; iPSC, induced pluripotent stem cell; PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator 1α; mTOR, mammalian target of rapamycin; NAD+, nicotinamide adenine dinucleotide; NRF-1, nuclear respiratory factor 1; NSC, neural stem cell; TOMM20, translocase of outer mitochondrial membrane 20; UDP, urine diphosphate; VEGF, vascular endothelial growth factor.

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evaluated in Phase 1 and 2 clinical trials (Feldman et al., 2014; Glass et al., 2016; Goutman et al., 2018). Additionally, several other stem cell types and delivery strategies are in various stages of development and clinical translation for ALS as well as a range of neurodegenerative conditions, including Alzheimer's disease, Huntington's disease, Parkinson's disease, and epilepsy (Bonaventura et al., 2021; De Gioia et al., 2020; Gonzalez et al., 2016; Goutman et al., 2019; Liu et al., 2021; Lunn et al., 2011; Reddy et al., 2020; Schweitzer et al., 2020; Zhao et al., 2021). Likewise, stem cell-based therapies are advancing for traumatic CNS injury (Bonilla and Zurita, 2021; Schepici et al., 2020; Silvestro et al., 2020; Younsi et al., 2021) and stroke (Azad et al., 2016; Hamblin and Lee, 2021; Kawabori et al., 2020).

For the majority of these clinical series, a precise mechanism of action for stem cells is not well established (Neal et al., 2018). Evidence is rapidly accumulating in support for metabolic drivers of pathology in nearly every neurologic disease, and thus metabolic pathways represent a promising window for stem cells to exert beneficial effects (Piers et al., 2020). Herein, we review the possible metabolic considerations associated with stem cell therapies, with particular emphasis on how stem cells impact the local environments and how metabolic implications of neurologic disease and injury states affect cell transplants.

2. Types of stem cells

Insight into the potential metabolic implications of the various stem cell classes used in transplantation research and translational applications first requires understanding of the origins and attributes of each stem cell type. ESCs are cells derived from the zygote or inner cell layer of the developing blastocyst, the former being totipotent (capable of differentiating to any cell type) and the latter being pluripotent (capable of differentiating to almost any cell type). In a similar vein, iPSCs are cultured cells (e.g., fibroblasts) that have been reprogrammed to express the Yamanaka factors (Oct3/4, Sox2, Klf4, c-Myc) and re-enter a state of pluripotency capable of differentiating into many cell types. NSCs, by contrast, are stem cells capable of proliferation but, upon differentiation, committed to neuroglial cell types. Similarly, MSCs are derived from a variety of tissues, such as bone marrow, and differentiate into mesodermal tissues, but have been coaxed into differentiating towards ectodermal and endodermal fates as well. Ultimately, the source and range of differentiation abilities should be considered when evaluating how stem cells may affect metabolic mechanisms in the host and vice versa.

3. Metabolic mechanisms by which stem cell transplants affect the host

The appeal of stem cells as a therapeutic tool is tied to the many complementary opportunities for neuroprotection made possible by their inherent properties. Stem cells proliferate, providing a self-renewing resource for therapeutic application. They can also differentiate into a range of cell types, and experimental paradigms are also now available to generate many neuronal subtypes from stem cells, such as motor neurons or GABAergic neurons (Ben-Shushan et al., 2015; Gupta et al., 2018; Ren et al., 2021; Shen et al., 2021). Another clear benefit of stem cell-based strategies is the ability to affect the host by a multitude of mechanisms, simultaneously and in a sustained manner (Chen et al., 2016; Pacheco-Herrero et al., 2021; Shinozaki et al., 2021; Wei et al., 2017a). Of course, the “holy grail” of regenerative approaches is replenishing a damaged cell population using stem cells. However, particularly in the nervous system, the ability to restore and rewire native neural circuits currently faces insurmountable challenges. Alternatively, stem cell differentiation into interneurons, glia, astrocytes, and other supporting cells offers a means to harness the full biological machinery of a complete cell to attenuate the progression of neurologic diseases. In this regard, stem cells can be employed to support neuromodulation, clear toxins, alter the extracellular matrix, facilitate vascular interactions, and regulate the immune system (Fig. 1). Importantly, stem cells are also capable of direct cell-cell communication (gap junctions, synapses, etc.), offer paracrine signaling and trophic support via secreted proteins and extracellular vesicles, and can be readily manipulated in vitro or in vivo to enhance expression of neuroprotective factors (Guy et al., 2019; Herman et al., 2021; McGinley et al., 2016; Willis et al., 2020).

One mechanism by which transplanted stem cells may benefit the host is by modulation or normalization of metabolic pathways. While the metabolic effects of stem cells have been explored in research fields such as cardiac, liver/pancreas, and hematopoietic stem cell transplants, they have not been well studied in the neurosciences. Limited reports, however, are beginning to provide insight into the implications of stem cells on glucose metabolism, mitochondrial function, and other neurovascular interactions.

3.1. Glucose metabolism

At a very basic level, the effect of stem cells on CNS glucose metabolism carries significant impact in the neurosciences. Neurons are known to rely chiefly on glycolysis and oxidative phosphorylation for their high energy demands, with minimal utilization of anaerobic forms of metabolism (Diaz-Garcia and Yellen, 2019). As a result, perturbations in glucose metabolism may have an outsized effect on neuronal function and survival. Transplanted stem cells, on the other hand, may normalize the glucose metabolism of neighboring cells and thus maximize neuronal survival in an otherwise hostile pathologic environment.

Stem cell transplantation studies in neurologic diseases have benefitted from ^{18}F-fluorodeoxyglucose positron emission tomography (FDG-PET) assessment of metabolic integrity. In a study of subventricular zone stem cells transplanted into Sprague-Dawley rats, striatal stem cell transplants were associated in increased FDG-PET signal, although inherent stem cell metabolism versus impact on surrounding host cells could not be parsed (Cicchetti et al., 2007). Interestingly, in a mouse model of temporal lobe epilepsy in which human ESCs were compared to GABAergic neuronal progenitors, restoration of glucose metabolism was only seen in ESC-implanted animals (Du et al., 2019). This appeared to be associated with ESC ability to differentiate down an astrocyte/glial lineage, which may represent a therapeutic strategy for epilepsy and other conditions in which normalization of the neuronal microenvironment is a central goal. Similarly, in a rat model of Huntington's disease, transplanted mouse iPSCs resulted in improved FDG-PET signal, along with elevated expression of neuronal, astrocyte, and microglial markers (Mu et al., 2014). These studies highlight the advantages of generating diverse cell types to act on the metabolism of diseased host cells.

Evidence for metabolic benefits in the CNS are seen in a handful of studies focused on stroke. In a middle cerebral artery occlusion (MCAO) rat model of ischemic stroke, transplantation of mouse iPSC and ESCs as well as rat NSCs into the ventricular space resulted in improved glucose uptake as measured by FDG-PET within the ischemic region (Wang et al., 2013; Zhang et al., 2015a). In a similar study, human NSCs promoted a restoration of glucose metabolism as measured by FDG-PET signal, and these stem cells had better ability to reduce stroke volume when the ischemic area was more modest in size (Daadi et al., 2013). Metabolic imaging to assess stem cell transplantation in early human trials for stroke confirm transplant feasibility as well as use of FDG-PET as a promising, non-invasive method for probing transplant viability and efficacy (Kondziolka et al., 2000).

Stem cell-associated changes in glucose metabolism are also seen in traumatic conditions. In a model of traumatic brain injury, intraparenchymal injections of rat hippocampal NSCs demonstrated restoration of FDG-PET signal at the injury site (Zhang et al., 2008). By contrast, in a hemisection model of spinal cord injury, glucose content at and around the injury site more closely paralleled that of untreated controls. Instead, ATP and lactate levels appeared to diminish within the

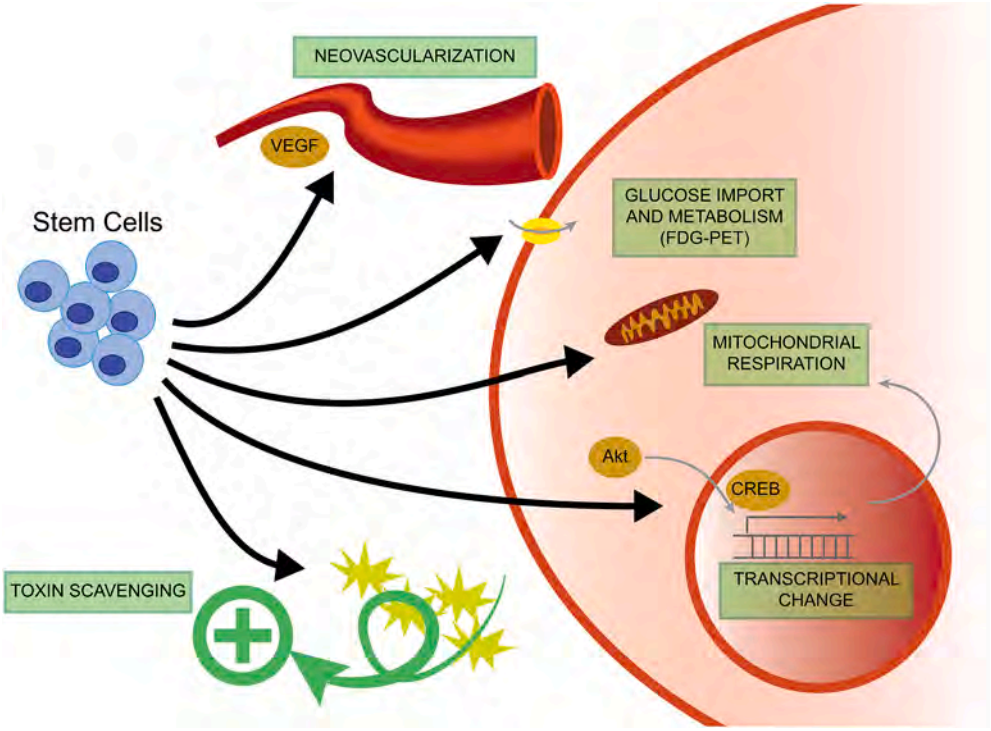

Fig. 1. Metabolic implications of stem cell therapies in the CNS. Although precise mechanisms have yet to be delineated, stem cells appear to have multimodal beneficial effects on neuronal metabolism. Stem cells may directly affect glucose metabolism and transport, which is often visualized using fluorodeoxyglucose-positron emission tomography (FDG-PET). Stem cells may also scavenge toxic metabolic byproducts. Mitochondrial proteins, which could mediate normalization of glucose metabolism and other pathways, represent a further downstream target of stem cell signaling. Secreted growth factors, such as VEGF, may also mediate metabolic repair via vascular remodeling.

injury site in animals receiving NSC transplants (Mautes et al., 2004). These changes were hypothesized to be a result of the metabolism of the transplanted cells themselves and their adaptation to the hostile, traumatized microenvironment. It is apparent that much remains unexplored when considering the effect of stem cells on the complex metabolic cascades following traumatic injury.

In many of the above studies, it is unclear how much of the normalized PET signal is performed by the transplanted cells directly or due to effects on native tissue. Transplanted stem cells may themselves contribute to changes in metabolic readouts to some degree. Nevertheless, FDG-PET is often used as a marker for more large-scale regional brain metabolism, and restoration of this signal is suggestive of rescued neuroglial populations. This is supported by histological correlation in the above studies that demonstrate restoration of host neuron and glial counts, and that changes in FDG-PET are more globally measured when compared to cell-specific PET imaging (Daadi et al., 2013; Zhang et al., 2008).

Additionally, given that neurons are reliant on glucose and that perturbations in glucose metabolism are known to exacerbate pathology seen in most neurologic diseases, the changes in FDG-PET shown in these studies could be due to intrinsic metabolic changes induced by stem cell grafts. In other words, rather than being merely a byproduct of rescued host population cell numbers, elevated FDG-PET signal may result from stem cells altering gene expression to increase glucose uptake in a more greatly elevated, hypercompensatory manner. Further mechanistic explorations are needed to elucidate how glucose normalization is mediated (e.g., signaling that alters neuronal or glial metabolism, alteration in microvascular blood flow, induction of glucose transporters, etc.) and better understand what downstream cellular components (e.g., mitochondrion) are involved in metabolic normalization.

3.2. Mitochondrial function

Stem cells may mediate normalization of glucose metabolism by altering mitochondrially expressed proteins in resident host cells. Using the MCAO stroke model in rats, a proteomic analysis identified 39 differentially expressed proteins upon treatment with mouse iPSCs. These included many mitochondrial proteins, such as TOMM20 (translocase of outer mitochondrial membrane 20) and GALE (urine diphosphate (UDP)-galactose 4-epimerase) (Chen et al., 2022). TOMM20 is a member of the mitochondrial translocase of the outer membrane, which functions to shuttle mitochondrial-targeted proteins to the mitochondrial matrix (Omura, 1998) and has been implicated in pathophysiology of Parkinson's disease (Franco-Iborra et al., 2018; Teixeira et al., 2016). GALE participates in the interconversion of UDP-galactose and UDP-glucose (Frey and Hegeman, 2013), important in the metabolism of galactose and generation of glucose substrates. Thus, transplanted iPSCs appear to directly affect mitochondrial physiology of host cells.

Similarly, in the APP/PS1 mouse model of Alzheimer's disease, murine NSCs increased mitochondrial DNA and normalization of PGC-1α (peroxisome proliferator-activated receptor-gamma coactivator 1α), NRF-1 (nuclear respiratory factor 1), COXIV (cytochrome *c* oxidase subunit 4), and other mitochondrial proteins (Zhang et al., 2015b). PGC-1α and NRF-1 are central transcriptional regulators of mitochondrial biogenesis and cellular energy metabolism (Li et al., 2017), and this is confirmed by electron microscopy in NSC-treated animals showing normalized mitochondrial morphology and numbers. Parallel findings were likewise found using a model of Huntington's disease treated with human adipose stem cells. Here, although mostly GABAergic neurons were formed, there appeared to be a restoration of Akt and CREB (cAMP response element-binding protein) signaling as well as PGC-1α (Lee et al., 2009). While further studies are required to identify factors mediating these stem cell-associated changes in mitochondrial function, these studies provide insight into the mechanisms by which stem cells promote neuroprotection by influencing metabolic pathways.

3.3. Neurovascular and other interactions

Stem cells may exert beneficial effects by modulating the metabolic response to pathologic injury in many other ways. For example, in a rat model of neonatal hypoxic-ischemic brain injury, transplanted MSCs appeared to attenuate the proliferation and activation of reactive astrocytes (He et al., 2019). This effect appeared to be mediated by stem

cell secretion of IL-6, which suppressed 5′ adenosine monophosphate-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) signaling in astrocytes. Importantly, these and the above discussed metabolic interactions could all occur simultaneously in a stem cell transplant treatment paradigm.

Transplanted stem cells may also exert beneficial effects indirectly, by first chiefly affecting neurovascular structures and blood flow, with metabolic changes a secondary benefit. In Alzheimer's disease models, many studies have shown that stem cells are associated with increased vascular endothelial growth factor (VEGF) expression (Li et al., 2018). In turn, higher VEGF levels are associated with greater glucose metabolism and neuroprotective effects (Wang et al., 2018), which may be mediated by neovascularization (Garcia et al., 2014). Hence, secreted factors derived from transplanted cells impact the supportive structures of neuronal metabolism, and stem cells can thus provide neuroprotection by both direct and indirect mechanisms simultaneously.

While the CNS is traditionally viewed as a privileged vascular environment, the benefits of stem cell transplants may have impact systemically as well. Metabolomic analysis in the MCAO model of ischemic stroke demonstrated that improvements after rat ESC transplants were associated with increased consumption of *N,N*-dimethylglycine, glucose, and formate, together with reduced excretion of lactate, alanine, glutamate, 3-hydroxybutyrate, glutathione, methionine, aspartate, fatty acyl chain, choline, glycerol, myoinositol, and glycerophosphocholine, as measured in peripheral serum (Gao et al., 2020). Whether these changes result from the stem cells directly and have a downstream impact on host neurons or whether these changes simply reflect rescue of native neuronal/glial populations is unclear, but these findings represent a metabolic signature which may have value as an accessible peripheral biomarker of disease and treatment response.

4. Host effects on stem cell transplant metabolism

While it is often the expressed goal for stem cell transplants to impact physiology of the host, the converse is emerging as an increasingly important area of study. With this in mind, the metabolic circumstances of donor tissue from which stem cells are derived become of great importance to the transcriptional and epigenetic signatures that are carried along with cell transplants. Once stem cells reach their destination, another consideration is how stem cells are impacted by the challenging microenvironment present in neurologic injury or disease (Frederiksen et al., 2020; Nguyen et al., 2019). While they can aid in detoxifying a potentially hostile disease microenvironment and confer several additional advantages, as mentioned above, stem cells must first be amenable to surviving within the immunologic and metabolic milieu that comprises that environment. Metabolic perturbations of the host, whether due to environmental factors (diet, exposures, etc.) or intrinsic neuropathologic disease processes, likewise impact the physiology of transplanted cells. Here we will summarize early work in understanding how destination metabolic microenvironments impact transplanted cells (Fig. 2), and approaches that build upon this knowledge to maximize the survival and therapeutic benefit of cell-based therapy.

4.1. Metabolic contributors to stem cell quiescence, pluripotency, proliferation, and differentiation

Metabolic disturbances can impact gene expression profiles of stem cells, and can thus influence the delicate balance between quiescence, self-renewal, and terminal differentiation. Interestingly, seemingly opposite dietary modifications, including both ketogenic/restricted calorie diets and high-fat diet, appear to increase stem cell self-renewal by convergent signaling onto common pathways engaged in fatty acid oxidation and peroxisome proliferator-activated receptors (Novak et al., 2021). However, high-fat diet or models of the “Western diet” appear to also impart a tendency towards inappropriate stem cell proliferation and carcinogenesis.

It is also clear that metabolic alterations have significant impact on stem cell epigenetic factors (Fawal and Davy, 2018; Ryall et al., 2015). Factors involved in oxidative phosphorylation act to modulate epigenetic changes (Tay et al., 2021). Sirtuin 1 activity, for example, is crucial for maintenance of pluripotency, participates in histone deacetylation, and is regulated by nicotinamide adenine dinucleotide (NAD+) concentrations that reflect stem cell metabolism (Correia et al., 2017; Fang et al., 2019). Histone and DNA methylation/demethylation by DNA methyltransferases and lysine-specific demethylase 1 also depends on one-carbon metabolism and the concentrations of flavin adenine

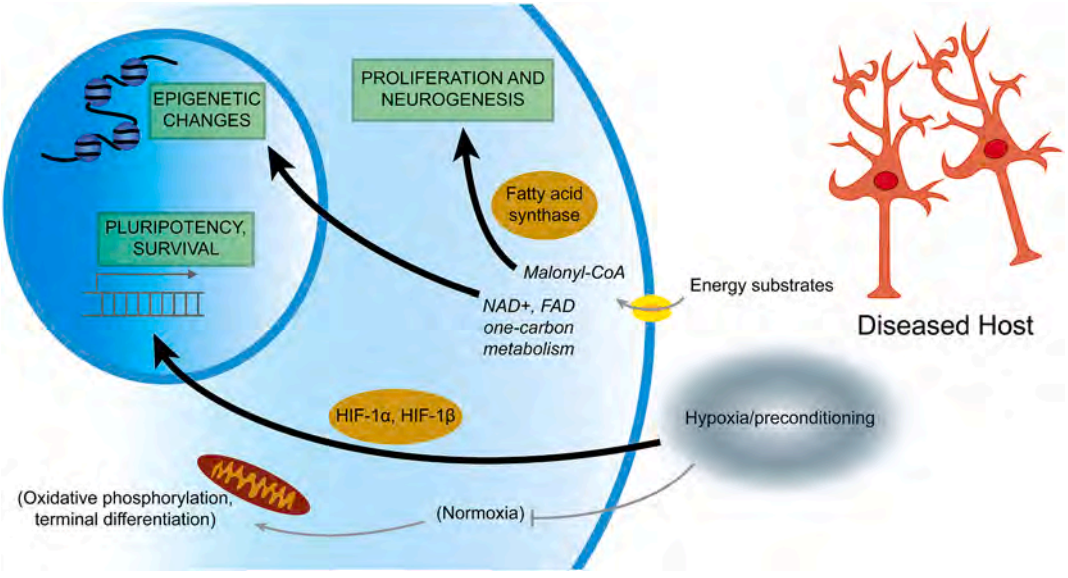

Fig. 2. Environmental and host metabolic influence on stem cells. Stem cells are affected by metabolic processes both from their source (carrying resultant epigenetic changes), metabolic changes resulting from in vitro culture, and also face pathologic metabolic signaling in the target tissue. Relative levels of energy substrates appear to influence the biology of proliferation and maintenance of a pluripotency, versus commitment towards terminal differentiation. Many epigenetic alterations resulting from metabolic conditions may also be carried with transplantation and may influence downstream efficacy. “Preconditioning” of stem cells prior to transplant, for example using relative hypoxia, may be a way to improve resilience of transplanted cells and maximize therapeutic benefits.

dinucleotide (FAD), again impacting expression of pluripotency versus differentiation genes (Castex et al., 2017; Ryall et al., 2015). Also, embryonic exposure to hyperglycemia appears to promote chromatin reorganization, histone H3 lysine 9 trimethylation, and global DNA methylation in NSCs (Shyamasundar et al., 2013). Thus, it is important to consider this metabolic "baggage" when establishing stem cell cultures or iPSC lines. Moreover, understanding the metabolic history of cell lines may lead to optimized transplantation paradigms and downstream studies.

Metabolic pathways additionally directly contribute to stem cell survival, maintenance of pluripotency, and the switch from quiescence to proliferation (Wanet et al., 2015). Signaling that involves forkhead box class O (FOXO), mTOR, AMPK, and sirtuin signaling pathways maintain a quiescent stem cell "pool" and minimize oxidative stress; however, the signaling of these pathways may be disrupted by changes in energy availability (Rafalski et al., 2012). Interestingly, fatty acid metabolism also appears to play a central role in stem cell biology. Malonyl-CoA reduces fatty acid oxidation which then promotes exit from quiescence into proliferation (Knobloch et al., 2017), and activity of fatty acid synthase also appears to promote adult neurogenesis and proliferation (Knobloch et al., 2013). The complexities of this area of study are only just beginning to be revealed, but knowledge of metabolic contributions to proliferation and differentiation may maximize stem cell survival and could be harnessed to improve treatment outcomes.

4.2. The stem cell niche and metabolic responses to culture and transplantation

The very act of in vitro culture and manipulation can impact stem cell metabolism and subsequent performance. Endogenous stem cells appear to exist in a specific niche with defined environmental factors and metabolic pathway utilization (Ottoboni et al., 2017; Rafalski et al., 2012). For example, certain stem cell populations appear to rely on glucose and preferentially utilize glycolysis over oxidative phosphorylation (Salazar-Noratto et al., 2020). This occurs in the face of relative hypoxia, whereas the switch to oxidative phosphorylation is linked to terminal differentiation in normoxic settings (De Filippis and Delia, 2011). The ability to expand and manipulate stem cell cultures in vitro prior to implantation is often cited as an advantage for stem cell-based approaches. However, keeping in mind the metabolic switch to oxidative phosphorylation is critical when considering that most in vitro culture of stem cells occurs at atmospheric oxygen levels. This exposure to elevated oxygen levels and switch to aerobic respiration may result in fundamental changes that might prove detrimental when cells are transplanted again into damaged, hypoxic host tissues and expected to proliferate (Sandvig et al., 2017).

Furthermore, the destination for cell transplants is often hostile, with altered blood flow, impaired nutrient and toxin shuttling, and inflammatory changes. A demonstration of these interactions was demonstrated using NSC transplants performed in a compression-based model of spinal cord injury in mice (Zhang et al., 2022). At baseline, transplanted NSCs tended to differentiate towards astrocytes in the presence of an M1 proinflammatory phenotype of surrounding microglia. By contrast, spinal cord injury in aldose reductase inhibition or in aldose reductase deficient mice favored an M2 microglial phenotype, associated with differentiation of NSCs towards a neuronal phenotype and improved motor function. Aldose reductase catalyzes the conversion of excess glucose to sorbitol in the polyol pathway, and has been implicated in activation of microglia (Chang et al., 2019). Thus, metabolic factors in stem cell transplant recipients clearly influence the inflammatory milieu, which in turn impacts the differentiation and survival of transplanted stem cells. These studies underscore the need for understanding stem cell interactions with host metabolic microenvironments in order to optimize the efficacy and translation of cell-based therapies.

4.3. Stem cell preconditioning

One domain in which metabolic contributors to stem cell performance, and indeed metabolic manipulation, has had greater study is in the realm of ischemic stroke (Bernstock et al., 2017; Yu et al., 2013). It is known that stem cells enter a hostile environment of hypoxia, excitotoxicity, and inflammation when transplanted acutely after stroke. As a result, there is a high degree of cell death for both endogenous and exogenous stem cells (Othman and Tan, 2020). Efforts to combat this are described as stem cell "preconditioning" using approaches such as genetic modifications (Wei et al., 2017a; Xue et al., 2019) or engineered biomaterials (Moshayedi et al., 2016). Alternatively, simple exposure to hypoxic culture conditions appears to result in transcriptional changes that improve metabolic profiles (Wei et al., 2017b). The mechanism underlying this observation is currently under investigation and may be multifactorial. Certainly, the activation of hypoxia inducible factors HIF-1α and HIF-1β is logical, with many potential downstream metabolically active targets, including VEGF, erythropoietin (EPO), sodium-calcium exchanger-1, protein kinase D1, lactate dehydrogenase A, and uncoupling protein 2 (Dehne and Brune, 2009; Greer et al., 2012; Semenza, 2011; Zhang et al., 2019). Interestingly, given the central role of glucose in stem cell and neuronal metabolism, the glucose transporters GLUT3 and glucose-6-phosphate transporter are also induced by HIF-1α after hypoxia (Thamotharan et al., 2013). Other mediators of stem cell preconditioning include EPO (Theus et al., 2008; Wei et al., 2012) or involve an increase in the formation of connexin hemichannels and ATP release (Jaderstad et al., 2010).

Further methods to induce stem cell preconditioning include exposure to compounds such as minocycline (Sakata et al., 2012b), doxycycline (Malik et al., 2013), interleukin-6 (Sakata et al., 2012a), adjudin (Zhang et al., 2017), resveratrol (Yao et al., 2021), or sodium butyrate/nicorandil (Hosseini et al., 2018), or even direct electrical stimulation (George et al., 2017). Again, growth factor secretion and/or angiogenesis appears to be engaged in these processes and are under further study. Notably, the AMPK activator metformin also appears to impart a beneficial effect on stem cell transplants. In an endothelin-1 rat model of stroke, co-treatment with metformin and human iPSC-NSCs resulted in improved proliferation, differentiation, and reduction of human leukocyte antigen (HLA)-A expression in stem cells (Ould-Brahim et al., 2018). Reduction in HLA-A or other antigen presenting molecules may help prevent graft rejection. While detailed metabolic studies of metformin and effects on transplanted stem cells were not performed, this study underscores the complex interplay between metabolism in the periphery, the CNS, injured tissue, and stem cells.

5. Conclusions

Stem cell therapies for neurologic conditions impart a range of metabolic effects for the CNS as well as for the stem cells themselves (Figs. 1 and 2). At baseline, the interaction between normal metabolism, impaired metabolism, and neurologic diseases is complex and poorly understood. Adding in stem cells, with their metabolic interactions with both local microenvironments as well as systemic processes, increases the combinatorial complexity of underlying metabolic and pathologic pathways. However, achieving therapeutic impact on host metabolism using the cellular capabilities of stem cells is a promising paradigm to address a wide range of neurologic conditions. Furthermore, emerging understanding regarding local environmental effects on stem cell metabolism may optimize the efficacy of stem cell treatments. It is apparent that much more detailed, high-quality research in this field is needed, and ongoing study is certain to yield great steps forward in enabling the translation of stem cell therapy for neurologic diseases and injury states. With this increased understanding of the interplay between stem cells and metabolic parameters in the brain, the success of stem cell therapy can ultimately be improved.

Search terms

A query in PubMed utilizing search terms “stem cell therapy” and “metabolism” and “nervous system” excluding “cancer” was performed screening for manuscripts describing use of stem cells as a therapeutic in CNS disorders with a potential metabolic mechanism of action. In relevant articles references were also screened for additional relevant papers.

CRediT authorship contribution statement

Stacey A. Sakowski: Conceptualization, Funding acquisition, Project administration, Visualization, Writing – original draft, Writing – review & editing. **Kevin S. Chen:** Conceptualization, Funding acquisition, Project administration, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

None.

Data availability

No data was used for the research described in the article.

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Chapter 13

Systems Biology to Address Unmet Medical Needs in Neurological Disorders

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Abstract

Neurological diseases are highly prevalent and constitute a significant cause of mortality and disability. Neurological disorders encompass a heterogeneous group of neurodegenerative conditions, broadly characterized by injury to the peripheral and/or central nervous system. Although the etiology of neurological diseases varies greatly, they share several characteristics, such as heterogeneity of clinical presentation, non–cell autonomous nature, and diversity of cellular, subcellular, and molecular pathways. Systems biology has emerged as a valuable platform for addressing the challenges of studying heterogeneous neurological diseases. Systems biology has manifold applications to address unmet medical needs for neurological illness, including integrating and correlating different large datasets covering the transcriptome, epigenome, proteome, and metabolome associated with a specific condition. This is particularly useful for disentangling the heterogeneity and complexity of neurological conditions. Hence, systems biology can help in uncovering pathophysiology to develop novel therapeutic targets and assessing the impact of known treatments on disease progression. Additionally, systems biology can identify early diagnostic biomarkers, to help diagnose neurological disease preceded by a long subclinical phase, as well as define the exposome, the collection of environmental toxicants that increase risk of certain neurological diseases. In addition to these current applications, there are numerous potential emergent uses, such as precision medicine.

Key words Alzheimer's disease, Amyotrophic lateral sclerosis, Diabetes, Inclusion body myositis, Neurodegenerative disease, Motor neuron disease, Obesity, Parkinson's disease, Peripheral neuropathy

1 Introduction

Neurological diseases constitute a significant burden of illness in the population. Worldwide, in 2016, neurological illnesses were the second most frequent cause of mortality, and first most significant contributor to disability [1]. Neurological disorders encompass a wide spectrum of neurodegenerative conditions, broadly characterized by injury to the peripheral and/or central nervous system (Fig. 1). Nerve damage can occur from aging, systemic diseases like diabetes, heritable genetic mutations, environmental

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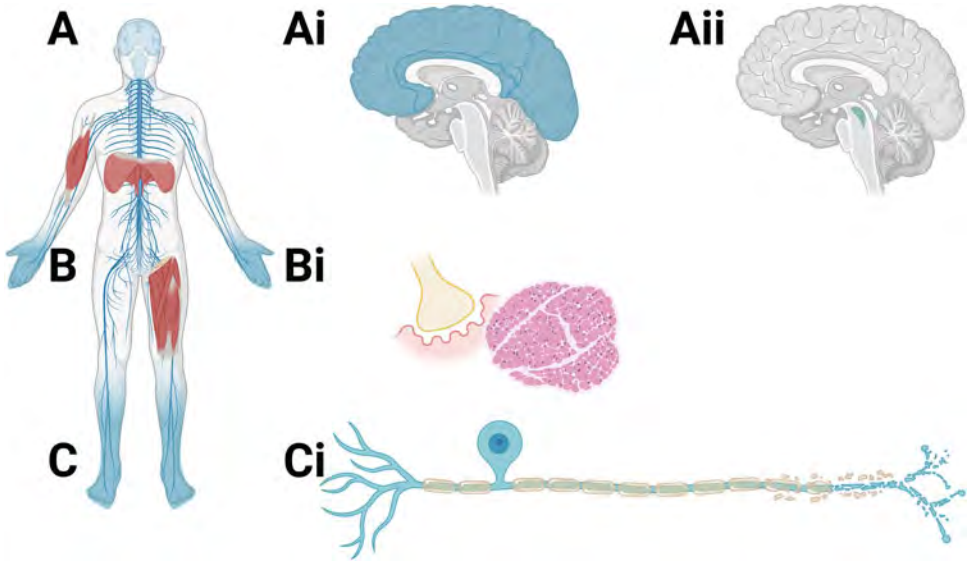

Fig. 1 Types of neurological diseases. Neurological diseases are characterized by damage to central and peripheral nervous tissue. Broadly, some categories of neurological disease include, (**a**) central neurodegenerative diseases, (**b**) motor neuron diseases, (**c**) peripheral neuropathies (PN). Central neurodegenerative diseases are characterized by neuronal loss in various areas of the brain, the cortex in Alzheimer's disease (blue shading; **ai**) and substantia nigra in Parkinson's disease (blue shading; **aii**). Motor neuron diseases lead to neurodegeneration of neuromuscular junctions (**bi**), which can lead to atrophy of limb and diaphragm muscles. Nerve damage (**ci**) in PN usually occurs in a symmetric, length-dependent manner (blue coloring; starting in the feet and progressing to the hands upon reaching the calves), including in the most common metabolically acquired diabetic PN. Sensory neuron degeneration also shown, distally (from axon termini) to proximally (toward cell body and dendrites). There can be overlap between these categories of neurological disease; for instance, a subset of patients with the motor neuron disease, amyotrophic lateral sclerosis, can also have central frontotemporal dementia in 15–20% of cases. (Created, in part, with BioRender.com)

exposures, or mechanical trauma. Although there are broad clinical and molecular differences, both within and between neurological diseases, complexity of pathogenesis is a unifying thread. It is also the principal reason that systems biology has gained traction in the recent decade as an important and central research tool for understanding neurological disease pathophysiology.

Disease complexity is evident in the heterogeneity of clinical presentation, breadth of etiology, non–cell autonomous nature, and cellular, subcellular, and molecular pathway diversity of each neurological illness. For example, amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease, characterized by motor neuron degeneration and consequent muscle wasting [2]. Regarding clinical presentation, ALS can manifest as bulbar or spinal onset, which influences the progression rate. Bulbar onset ALS presents as difficulty speaking, swallowing and breathing, and is fast progressing, whereas spinal onset initially presents as limb weakness, and is usually slow progressing [2, 3]. ALS phenotypes are dictated by

multiple patient clinical and genetics characteristics [4]. Additionally, although progression of symptomatic disease is relatively rapid, ALS has a long subclinical prodromal phase.

With regards to breadth of etiology, ALS is associated with close to 30 genetic mutations [5]; however, a known genetic mutation is present in only around 15% of ALS individuals. Thus, polygenic risk [6, 7], environmental exposure [8–10], and potential gene–environment interactions [11] have been proposed to account for cases lacking a known monogenic cause. In terms of a non–cell autonomous nature, although ALS is widely regarded as a motor neuron disease, there is a central neurodegenerative component, frontotemporal dementia, in around 15–20% of cases [2]. Additionally, immune system involvement is documented in ALS [12–17], which correlates with disease progression [14] and in a sex-dependent manner [15, 16].

Lastly, ALS is characterized by molecular heterogeneity in genetic [5–7], epigenetic [18–22], transcriptomic [23, 24], and metabolomic [25–27] signatures. Multiple biological processes are also involved, centered on excitotoxicity [28], mitochondrial dysfunction [29], and oxidative stress [30].

Although outlined here specifically for ALS, this level of heterogeneity and complexity of disease processes occurs in most neurological diseases, especially those of non-monogenic etiology. Clearly, appropriate analytical platforms are needed to uncover and dissect the numerous aspects present in these complex, multifactorial disorders. Systems biology can be leveraged to address this need, by agnostically querying molecular pathways in ach aspect of disease pathogenesis. This can shed light on mechanisms, correlate molecular signatures, that is, genomic, transcriptomic, epigenomic, proteomic, metabolomics, or multiomics, to clinical presentation to refine disease classification and diagnosis, and identify potential drug targets. This is especially essential for most neurological disorders, which remain recalcitrant to treatment. Technological advances also enable single-cell resolution, which is useful for non–cell autonomous diseases. Further, bioinformatics analysis can integrate multiomics datasets to gain additional insight.

This chapter will be subdivided by disease to illustrate how systems biology has advanced our understanding of neurological disorders in the recent decade. First, each subsection will provide an overview of the neurological disease, highlighting the complexity, which systems biology can help address. Second, each subsection will discuss the most salient studies shedding light specifically on the points stated above, namely, pathomechanisms, diagnosis/classification, and drug target identification. Rather than a comprehensive review, this chapter will highlight studies that deliver overarching messages, identify future areas of investigation, and serve as a guide to researchers leveraging or planning to leverage systems biology in their research endeavors. Additionally, the

chapter will emphasize metabolically acquired peripheral neuropathy and ALS, which are our areas of expertise, but will still illustrate systems biology examples in other neurological illnesses.

2 Peripheral Neuropathies

Peripheral neuropathies (PN) are a class of neurological diseases, which incur damage to the peripheral nerves [31]. The most common clinical presentation is a distal symmetric neuropathy, beginning in both feet, progressing distally to the calves, at which point it commences in the fingers and progresses distally to encompass both hands. Alternatively, PN can manifest focally as a mononeuropathy or a plexopathy, or to the autonomic nervous system as autonomic neuropathy. This chapter will refer to distal symmetric neuropathy as PN. The most frequent PN cause is metabolically acquired, secondary to either diabetes, prediabetes, or the metabolic syndrome [32]. Other causes include genetic mutations, chemotherapy, toxin exposure, infectious disease, vasculitis, mechanical injury, vitamin deficiencies, and immune-mediated disorders [33], although the cause is unknown in around 40% of PN cases, known as idiopathic PN [32]. This subsection will focus on metabolically acquired PN, as the most prevalent PN.

2.1 Metabolically Acquired Neuropathies

Diabetes, an elevated fasting blood glucose (hyperglycemia), and prediabetes, a state of impaired glucose tolerance, are extremely prevalent metabolic disorders. Diabetes itself can be subdivided into type 1 (T1D) diabetes, constituting around 5% of patients who lose pancreatic β-cells and no longer produce insulin. Patients with the more prevalent type 2 diabetes (T2D) develop insulin resistance and can no longer regulate glucose. In 2019, 463 million individuals had T2D globally, with 374 million individuals with prediabetes [34]. The metabolic syndrome, a constellation of obesity, dyslipidemia (abnormal blood lipid profile), and hypertension [35], also constitutes a massive and rising global epidemic [36, 37]. Up to 50% and 30% of T2D and prediabetes patients, respectively, develop PN [31]. PN also develops in obese individuals [38–53], even independent of hyperglycemia, and in proportion to the number of metabolic syndrome components [45, 47, 54, 55]. Frequently, however, T2D is comorbid with obesity and the metabolic syndrome [39, 56].

Metabolically acquired PN pathophysiology is complex, encompassing abnormal glucose- and lipid-centric pathways [31, 57], bioenergetics and mitochondrial defects [58, 59], oxidative stress [60], and inflammatory processes [61]. Moreover, PN progression may be non–cell autonomous, through a breakdown in neurometabolic coupling and crosstalk between axons and their supporting glia cells [62, 63]. Thus, PN development is highly

complex, advocating a systems biology approach to gain a deeper understanding of pathophysiology to develop mechanism-based treatments.

Indeed, we have conducted extensive informatics studies of PN in both mouse and human neuropathic nerve to identity recurrent pathways and possible routes to disease-modifying drugs. We have employed several mouse models of metabolically acquired PN [64]. Streptozotocin (STZ) destroys pancreatic β-cells, mirroring the T1D scenario. The *ob/ob* and *db/db* mice harbor spontaneous mutations to satiety regulating leptin and the leptin receptor, respectively, leading to over-eating, obesity, and a T2D phenotype. Alternatively, the high-fat diet (HFD) low-dose STZ mouse model of T2D was developed to more closely mirror diet-induced T2D with comorbid obesity in humans. Omitting STZ, and solely feeding mice HFD leads to an obese prediabetes model. Equipped with these models, we have leveraged systems biology approaches to address multiple questions.

2.1.1 What Is PN Pathophysiology?

Early studies employing gene expression microarray technology were conducted on human sural [65] and mouse sciatic nerve [66] (Fig. 2a). The human study was of sural biopsies from both T1D and T2D participants with PN, categorized as "progressors" (decrease in myelinated fiber density [MFD] as a measure of PN) versus "non-progressors" (no MFD change) over the course of a 52-week clinical trial [65]. Progressors differed in 532 differentially expressed genes (DEGs) from non-progressors; functional enrichment of DEGs identified pathways involving inflammatory responses and lipid metabolism, centered on apolipoprotein E (APOE), leptin, peroxisome proliferator-activated receptor gamma (PPARγ), JUN, and serpin family E member 1 (SERPINE1). A follow-up human study took a closer look at a subset of "regenerator" participants, which increased MFD during 1 year, indicative of nerve regeneration and improvement in PN [67]. Microarray analysis found regenerator sural nerves were upregulated in genes related to cell-cycle and myelin sheath functions, and downregulated in those related to immune/inflammatory pathways.

These findings were echoed in sciatic nerve from *db/db* T2D mice with PN, which revealed dysregulation of genes responsible for lipid and carbohydrate metabolism, PPAR signaling, apoptosis, and axon guidance [66]. Promoter sequence analysis demonstrated these changes were coregulated, indicative of structural changes of axonal degeneration involving lipid metabolism. PN is progressive and evolves over time. Gene expression microarray analysis of *ob/ob* T2D mice at earlier 5-week and later 13-week time points found 1503 and 642 DEGs, respectively, which were overrepresented in immune/inflammatory functions, especially at 5 weeks, suggesting an early and contributory role to PN onset [68]. Analysis of

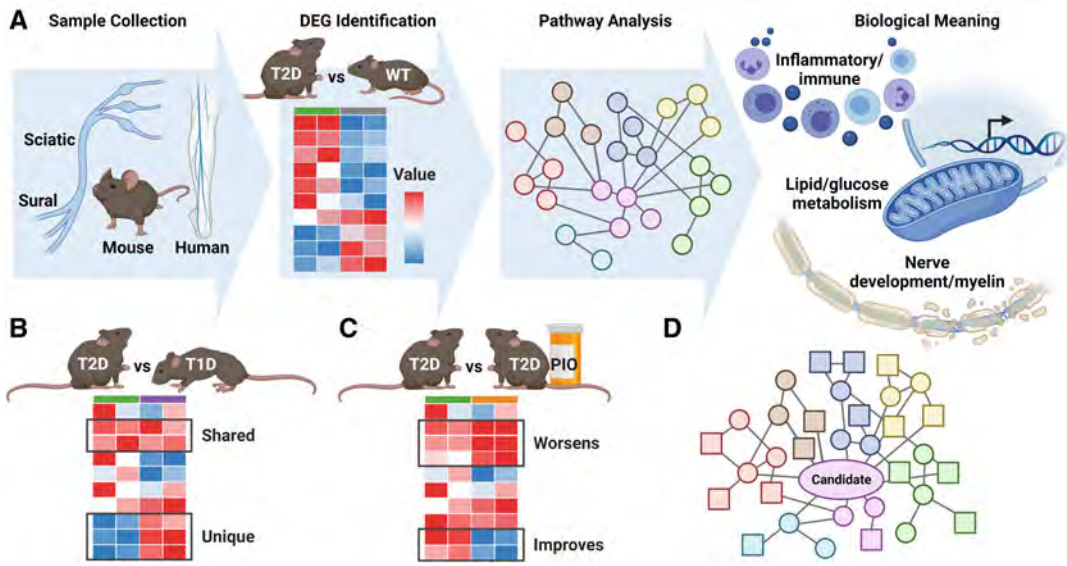

Fig. 2 Select systems biology applications to metabolically acquired peripheral neuropathy. Systems biology has manifold applications to address unmet medical needs for peripheral neuropathy (PN). (**a**) What is PN pathophysiology? Tissue samples (sural or sciatic nerve from mouse (animal model) or human with T2D, type 2 diabetes, versus WT, wild-type, in this example) are profiled by an omics platform (transcriptomics in this example). Next, differential species are identified (DEGs, differentially expressed genes, in this example). Pathway enrichment analysis of differential species lends biological insight. Thus, systems biology can uncover pathomechanisms, which can suggest therapeutic avenues. (**b**) What are shared and unique pathophysiology aspects in T1D versus T2D PN? Systems biology can differentiate pathomechanisms in T1D versus T2D PN, which can lead to tailored treatment regimens. (**c**) How does anti-type 2 diabetic drug treatment affect nerve health? Systems biology can shed insight on why current anti-type 2 diabetic drugs (PIO, pioglitazone, in this example) do not prevent PN onset and development (worsens PN by increasing expression of DEGs related to pathology, improves PN by decreasing or expression of DEGs related to pathology). (**d**) What new therapeutic targets for PN can systems biology identify? Multiomics systems biology can identity strong candidate targets for drug development (circles omics platform 1; squares omics platform 2). (Created, in part, with BioRender.com)

multiple transcriptomic datasets underscored inflammation as a recurrent theme in diabetic PN, particularly through toll-like receptor (TLR) signaling [69]. Knocking out TLR2/4 from a prediabetes HFD model slows the onset of PN, affirming immune system involvement early in pathogenesis.

2.1.2 What Are Shared and Unique Pathophysiology Aspects in T1D Versus T2D PN?

PN phenotype, that is, slowed nerve conduction velocities (NCV) in large fibers and intraepidermal nerve fiber (IENF) loss of small fibers, is similar in T1D and T2D. However, glucose control is more effective for slowing T1D versus T2D PN [70], suggesting possible pathophysiological differences (Fig. 2b). Transcriptomic analysis is an ideal tool for agnostically querying pathway differences and similarities between T1D and T2D PN. Comparison of

microarray results from STZ T1D versus *db/db* T2D sciatic nerve and kidney tissue from animals with PN and diabetic kidney disease, known as nephropathy, identified exceptionally high concordance among DEGs in diabetic nephropathy (94% of 2433 genes), but not in diabetic PN (54% of 1558 genes) [71]. These findings support the concept that distinct pathophysiology may underlie PN in T1D versus T2D, although transcriptional network analysis suggests the inflammatory Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway is shared, regardless of diabetes type. Expanding comparison of T1D versus T2D to include human sural (progressors and non-progressors) as well as mouse sciatic nerve (STZ T1D, *db/db* and *ob/ob* T2D), a total of eight microarray datasets were used to generate a merged transcriptional network with centrality analysis, which identified top and universally shared DEGs [72]. Pathway analysis discovered these shared DEGs to be connected to pathways involving liver X receptor (LXR)/retinoid X receptor (RXR) activation, adipogenesis, and glucocorticoid receptor signaling, as well as, as anticipated, multiple cytokine and chemokine pathways. However, although pathological pathways are shared, directionality of DEGs differed in human versus mouse samples. This may be related to the time course and PN stage, source tissue location (sural, which is more distal and affected earlier in disease course in humans, versus sciatic, which is more proximal and affected later in mice), or control comparisons (progressors versus non-progressors against neuropathic versus non-neuropathic) in humans versus mice.

Overall, however, these analyses identified highly recurrent pathways cross-species and in both T1D and T2D PN, as well as divergent pathways, which differed in T1D versus T2D.

2.1.3 How Does Anti-type 2 Diabetic Drug Treatment Affect Nerve Health?

There are several classes of anti-type 2 diabetic drugs; among them, thiazolidinediones are PPARγ agonists, which boost transcription of genes controlling glucose and lipid metabolism and improve insulin sensitivity [73]. In mice, pioglitazone and rosiglitazone, examples of thiazolidinediones, may improve some PN outcomes associated with T1D and T2D, though through an unknown mechanism. Systems biology is ideal for elucidating the mechanism of treatment-induced PN improvements (Fig. 2c). In an STZ T1D model, rosiglitazone (3 mg/kg) does not reverse hyperglycemia, but does lower nerve oxidative stress [74]. Regarding PN, it does not improve function of large myelinated nerve fibers assessed by measuring sural or sciatic NCVs, but does prevent loss of small unmyelinated fibers. This preservation of function is assessed by measuring thermal hypoalgesia (loss of sensation to heat) and anatomical quantitation of small unmyelinated intraepidermal nerve fiber density (IENFD). Gene expression microarrays identified 318 DEGs between T1D versus rosiglitazone-treated T1D

mouse sciatic nerve; analysis of DEGs collectively upregulated or collectively downregulated by rosiglitazone identified two transcription factor motifs linked to PN development. These transcription factor motifs were related to insulin-stimulated glucose metabolism, neurite outgrowth, growth factors, apoptosis, and survival, implicating these pathways in rosiglitazone's mechanism of action in PN.

We have also investigated the effect of pioglitazone on PN in *db/db* T2D mice. In this model, pioglitazone (15 mg/kg) normalizes fasting blood glucose, glycated hemoglobin (HbA1c), and triglycerides, and lowers plasma oxidative stress, but increases body weight [75]. Pioglitazone also prevents small fiber IENFD loss, but does not affect large fiber sural or sciatic NCVs. There were 4537 DEGs in sciatic nerve between *db/db* versus *db/db* pioglitazone using microarrays, and pathway analysis revolved around adipogenesis, adipokine signaling, and lipoprotein signaling. These pathways suggest nerve lipid accumulation, a possible reason for the blunted therapeutic response on large fiber PN. Bulk RNA sequencing (RNA-seq) of nerve and kidney followed by self-organizing maps found pioglitazone reversed mitochondrial dysfunction in both tissues, but only rescued cell death and inflammation in kidney [76]. In fact, pioglitazone may have even been detrimental to the inflammatory nerve response. Pathway crosstalk perturbation network modeling of this RNA-seq nerve dataset further identified glycolysis, gluconeogenesis, and carbohydrate metabolism as contributing to the return to health upon pioglitazone treatment [77].

Overall, our data implies inflammation may underlie large fiber dysfunction, since pioglitazone does not improve NCV nor reverse nerve inflammatory pathways. On the other hand, mitochondrial dysfunction may drive small fiber dysfunction in T2D PN, since pioglitazone improves IENFD and influences DEG expression related to mitochondria. The differential impact of pioglitazone on PN and diabetic nephropathy also upholds an important tenet in diabetes complications research, which states T2D induces distinct tissue-specific metabolic changes [78]. This suggests it may also require tissue-specific therapeutic solutions, rather than a "one size fits all" approach, which may have deleterious impact in one tissue, while improving another. These studies also highlight the power of systems biology to draw important and biologically relevant insight with potentially translational implications.

Although gene expression microarrays and bulk RNA-seq have been very instrumental to elucidating PN pathophysiology, the heterogeneity of cells in peripheral nerves presents limitations to these studies. To further unravel the cell-specific transcriptome and the cellular communications, single-cell RNA-seq (scRNA-seq) and spatial transcriptomics should be performed in future studies.

2.1.4 What New Therapeutic Targets for PN Can Systems Biology Identify?

Despite intensive research, PN remains untreatable. Effective anti-type 2 diabetic drugs improve glucose handling and systemic metabolic health; but none to date prevent PN onset and progression clinically in humans. Systems biology may aid in the discovery of potential PN therapeutics through agnostic query. Moreover, adopting a multiomics approach, by considering dysregulation on multiple levels, can strengthen identified candidates (Fig. 2d). Systems biology can also aid in the development of new drugs or repurposing of approved drugs by integrating gene expression datasets to develop connectivity maps, which correlates dysregulated transcriptome with drug databases [79]. Lipidomics of sciatic nerve of HFD-STZ T2D and HFD prediabetes mice found triacylglycerol and, to a lesser extent, diacylglycerol accumulation in neuropathic nerve, which was reversed upon a switch back to a regular diet [80]. Transcriptomics revealed "fat digestion and absorption" and "glycerophospholipid metabolism" as important pathways; integrated lipidomics-transcriptomics centered especially on three candidates, CD36 (lipid transport), LPL (lipoprotein hydrolysis), and DGAT2 (triacylglycerol synthesis). The study also confirmed DGAT2 was elevated in sural nerve biopsies from hyperlipidemic versus non-hyperlipidemic T2D participants. Although not in development for PN, inhibiting DGAT2 have been considered for treating non-alcoholic fatty liver disease [81], a common comorbid condition of obesity.

An epigenetic (DNA methylation) and transcriptomic analysis of human sural nerve biopsies from T2D PN participants by high versus low HbA1c found overlap between DEGs and differentially methylated genes, which integrated functional and network analysis found were related to immune response, extracellular matrix regulation, and PI3K-Akt signaling [82]. This study, for the first time, demonstrated that DNA methylation could constitute a mechanism regulating gene expression in PN, revealing a gene–environment interaction by integrating epigenomics with transcriptomics. Gene–environment interaction can be also investigated by integrating scRNA-seq with the assay for transposase-accessible chromatin-seq [83].

Both studies share the same weakness, namely, that causality cannot be inferred from these cross-sectional analyses. Thus, longitudinal bioinformatics analysis of nerve coupled with validation by knockout in mouse models will be required to select the best therapeutic candidates. Additionally, any developed drugs will require optimal pharmacological profiles to penetrate the nerve. Collectively, however, these studies demonstrate the power of systems biology to identify targets for therapeutic development.

2.1.5 What New PN Research Avenues Can Systems Biology Open?

In addition to the aforementioned established avenues systems biology has investigated in metabolically acquired PN, it is poised to shed light on emergent and future novel avenues. Although PN is primarily considered metabolically acquired in T2D, prediabetes, and obesity, a growing number of genome-wide association studies are identifying risk loci and generating polygenic risk scores to predict the chance of developing T2D PN [84, 85] (Fig. 3a). A systems biology approach of the microbiome through metagenome-wide association studies found some dysbiosis in T2D participants, which correlated with decreased universal butyrate-producing bacterial abundance and increased opportunistic pathogens [86]. Although far less investigated for PN (Fig. 3b) [87], butyrate is also key in obesity-driven PN [88]. The gut microbiome may modulate PN in STZ T1D rats [89] and enteric nerves in HFD prediabetic mice [90].

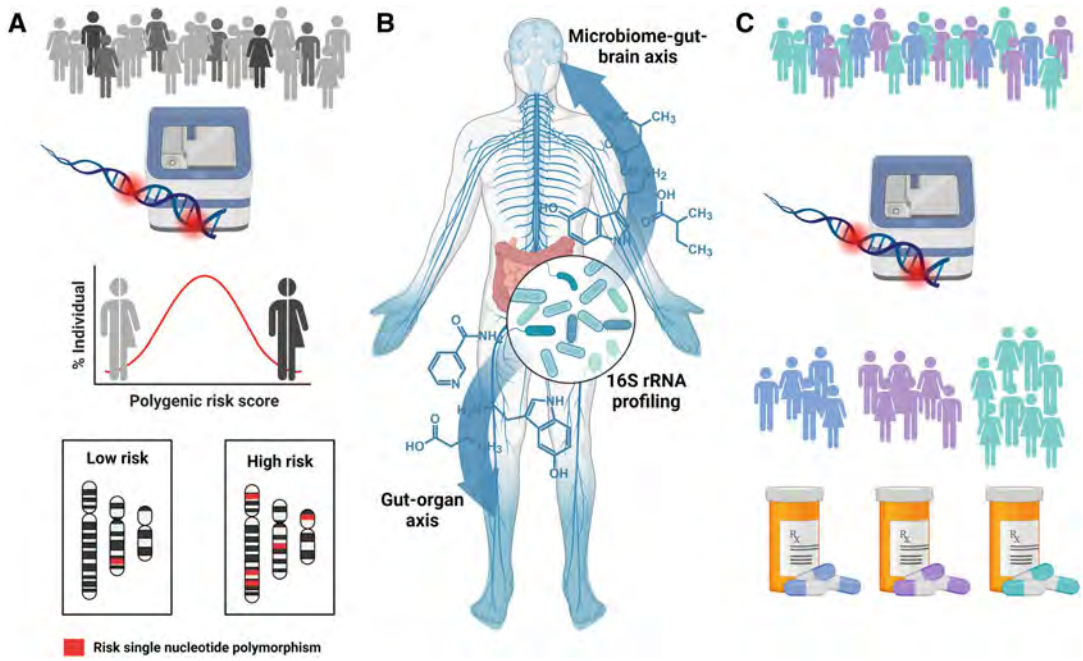

Fig. 3 Emergent and future systems biology research avenues in metabolically acquired peripheral neuropathy. Systems biology is poised to shed light on emergent and future novel research avenues in PN. (**a**) Genome-wide association studies to identify risk loci and generate polygenic risk scores (PRS) for PN. Genome sequencing a population and correlating to individuals with PN (dark grey figures) versus without (light grey) identifies risk loci and single nucleotide polymorphisms (SNPs). Low PRS (few risk SNPs) means a low chance to develop PN; high PRS (several risk SNPs) means a high chance to develop PN. (**b**) Metagenome-wide association studies to evaluate the contribution of the microbiome to PN. Microbiome can be sequenced by 16S profiling to identify microorganisms. Microorganisms and the gut secrete metabolites, which might affect the brain through the microbiome–gut–brain axis, or peripheral organs through the gut–organ axis. (**c**) Pharmacogenetics to identify patients that will respond to pain medications for painful PN. Genome sequencing a population can match identified SNPs with specific drugs. (Created, in part, with BioRender.com and ACD/ChemSketch)

An additional possible avenue for systems biology is in the search for effective treatments for painful PN (Fig. 3c), which occurs in a subset of T2D patients, who experience oftentimes debilitating pain over the course of neuropathy progression. Currently, only one in seven individuals get relief from the current standard treatments available for painful PN [32, 91]. One approach is to match PN sensory profiles with specific drug mechanism of actions, but this process is empirical and time consuming [32]. An unexplored avenue is pharmacogenetics, the intersection of genetic profiling with drug response [92]. In T2D, certain single nucleotide polymorphisms modulate antidiabetic drug efficacy [93, 94], and this may be the scenario in diabetic PN or painful PN. Indeed, in a small pilot study of amitriptyline, a tricyclic antidepressant, first-line painful PN treatment, participants with normal or ultrarapid metabolizer phenotypes had fewer side effects versus individuals with lower cytochrome p450 2D6 (CYP2D6) activity [95]. Thus, this is an unexplored and potentially valuable research avenue for selecting drugs in a precision systems biology–driven approach, which might bring relief to patients with painful PN.

2.2 Other Neuropathies

Although impaired metabolism is the most frequent PN cause, there are multiple other etiologies as outlined in the Subheading 1. In a sciatic transection model of nerve damage in rats, scRNA-seq disclosed that the main source of nerve factors following injury are Schwann cells and, unexpectedly, nerve mesenchymal cells, including from the endoneurium [96]. Integrated scRNA-seq-proteomic systems biology modeling predicted novel nerve mesenchymal cell-derived factors, which could potentially stimulate peripheral axon growth. In vitro validation of predicted factors in cultured sympathetic axons identified three factors, angiopoietin 1 (ANGPT1), C-C motif chemokine 11 (CCL11), and vascular endothelial growth factor C (VEGFC), which effectively stimulated outgrowth. This approach could make important discoveries for potential neuroregenerative therapies.

In a model of chronic autoimmune neuritis, an inflammation of the peripheral nerves, scRNA-seq was used to characterize immune cell populations [97]. Under homeostatic conditions in control mouse sciatic nerve, immune populations comprised nerve-resident homeostatic myeloid cells, which were transcriptionally distinct from central nervous system microglia. In contrast, scRNA-seq profiling of autoimmune neuritis sciatic nerve found that homeostatic myeloid cells were outnumbered by infiltrating lymphocytes, which restructured the local immune cell-to-cell interactome rather than single immune cell types. This discovery suggests a potential treatment targeting peripheral rather than resident lymphocytes or a therapeutic approach disrupting the dysregulated immune network, rather than specific immune populations.

Thus, systems biology can lend insight into the pathogenesis of various peripheral neuropathies, as attested by these two examples, unlocking possible therapeutic avenues.

3 Central Neurodegenerative Diseases

The most common neurodegenerative disorders of the central nervous system can be classified based on neuropathological protein aggregates, which cause nerve damage and neuronal loss. Broadly, they encompass amyloidoses, tauopathies, synucleinopathies, and TDP-43 proteinopathies, which occur in various brain regions and lead to neuronal loss and subsequent loss of nervous system function [98]. Most are incurable and treatment entails symptom management and palliative care, although there are new candidates in the preclinical and clinical pipeline [99, 100], such as gene therapy [101].

3.1 Alzheimer's Disease

Alzheimer's disease (AD) is the most common dementia, affecting 1 in 10 individuals 65 years and older [102]. In 2020, around 5.8 million people were living with AD in the USA, a number projected to increase to 13.8 million by 2050. It is familial and heritable in around 1–3% of cases, and sporadic in the other >95% [103]. AD is slowly progressive, with a long prodromal phase, sequentially followed by mild cognitive impairment, before frank mild, moderate, and severe dementia [102]. Monogenic and polygenic AD risks have been identified [104], as well as numerous modifiable [105, 106] and potential exposome [107, 108] risks. AD histopathology is defined by insoluble deposits of extracellular amyloid-β and intracellular hyperphosphorylated tau protein with oligomeric neurotoxic forms [109, 110]. Additionally, AD pathomechanisms include metabolic [111–113] and mitochondrial [114] derangements, protein aggregates [110], autophagy [115], neurotransmission breakdown [116], inflammation [117, 118], and oxidative stress [119]. There is also a non–cell autonomous component, and the brains' resident immune cells, microglia, may actively participate in AD pathogenesis [117, 118].

Systems biology has been widely used in AD research, for instance through precision medicine by leveraging genetic variants linked to neuroinflammation [120]. Herein, we will discuss four vignettes from transcriptomic, proteomic, metabolomic, and metagenomic perspectives, which uncovered exciting research avenues. Microglia and neuroinflammation have long been considered AD hallmarks; however, microglia appear to adopt a protective role, which ultimately fails, leading to neurodegeneration [118]. To shed deeper insight, Keren-Shaul et al. leveraged scRNA-seq to investigate microglia from transgenic AD mice with versus without knockout of an immune cell receptor triggering receptor expressed

on myeloid cells 2 (TREM2) [121]. TREM2 mutations significantly increase the risk of AD [122]. Indeed, scRNA-seq identified a microglia phenotype the authors referred to as "disease-associated microglia" (DAM), which are activated in a sequential two-step TREM2-independent and TREM2-dependent process [121]. DAMs localize near and phagocytose amyloid plaques, slowing disease progression. Thus, this analysis revealed heterogeneity in microglia phenotypes [123], in addition to evolution of their roles over time.

Bai et al. employed proteomics and phosphoproteomics of autopsy brain samples from AD patients along a spectrum of disease progression [124]. Mass spectrometry profiled 14,513 proteins and 34,173 phosphoproteins, of which 173 candidates in 17 pathways correlated with AD progression. These hits were validated in two independent cohorts, and comparison with cerebrospinal fluid suggested possible biomarker candidates. A similar metabolomic analysis in AD participants over time found correlations between specific lipid species with disease progression, amyloid burden in cerebrospinal fluid, and magnetic resonance imaging parameters [125]. Finally, gut microbiome dysbiosis has also been linked to neurodegenerative disease [126], although confounding parameters, such as diet, poses challenges. However, novel correlations between metagenome and ketogenic diet with mild cognitive impairment, a phase preceding frank dementia, suggests possible lifestyle and dietary interventions for slowing cognitive decline in AD [127, 128].

Thus, this overview exemplifies how systems biology can be capitalized to generate pathophysiological insights, generate biomarker panels, and unlock novel and paradigm-shifting therapeutic approaches in AD. The topic is discussed in greater detail in these recent reviews [120, 126, 129–131].

3.2 Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative disease characterized by dopaminergic neurons loss in the substantia nigra and a classical motor deficit phenotype [132]. It is the second most common neurodegenerative illness after AD, with 10–1500 prevalence per 100,000. However, clinical presentation is highly heterogeneous, and can also involve cognitive impairment, sleep, mood, and psychiatric disorders, autonomic dysfunction, pain, and fatigue. Monogenic [132] and polygenic [133–135] PD risks have been identified, as well as numerous modifiable and potential toxic environmental exposure risks [108, 132]. Histologically, Lewy bodies of α-synuclein deposits are present in various areas of the nervous system, which spread over the course of this slowly progressive disease [136]. As with AD, and even with peripheral neuropathies, such as metabolically acquired PN, mitochondrial dysfunction, inflammation, and oxidative stress [132, 137], as

well as protein aggregates and autophagy [115] are major pathological aspects. Additionally, PD pathogenesis progresses in a non–cell autonomous manner via astroglia [138].

As with AD, numerous system biology techniques have been applied to investigate PD and usher in precision medicine [139]. Of interest, lipid dysregulation has emerged as an important PD facet. Although lipid dysregulation is linked to neurodegenerative disease broadly, it has very direct links in PD through mutations to genes involved in lipid metabolism, such as glucosylceramidase beta (GBA), sphingomyelin phosphodiesterase 1 (SMPD1), galactosylceramidase (GALC), phospholipase A2 group VI (PLA2G6), and sterol regulatory element binding transcription factor 1 (SREBF1) [140]. Indeed, integrated proteomics/metabolomics and metabolomics analysis of plasma from PD participants indicate lipid dysregulation may also be key in sporadic cases [141, 142].

Metagenomic studies of PD have also been launched, due to the presence of α-synuclein fibril accumulation in the gastrointestinal tract [143], suggesting a possible causal relationship with microbiome dysbiosis. 16S ribosomal RNA profiling found associations of PD with increased *Akkermansia*, an intestinal mucin layer-degrading species, and decreased *Roseburia* and *Faecalibacterium*, short-chain fatty acid-producing species.

Though not comprehensive, these sample studies illustrate some applications of systems biology in PD, which are detailed extensively in these recent reviews [126, 144–147].

4 Motor Neuron Diseases

Motor neuron diseases are a broad class of disorders secondary to loss of motor neuron function in the brain and spinal cord. There are multiple well-known monogenic motor neuron diseases, such as spinal muscle atrophy; however, some, such as ALS, exhibit more complex genetic architectures, with risk factors from interactions with the exposome [148]. Like other neurodegenerative diseases of the central nervous system, motor neuron diseases are heterogeneous in clinical presentation. Additionally, there is overlap between motor neuron diseases and central neurodegenerative disease, such as ALS with frontotemporal dementia. Motor neuron diseases are also mostly lacking effective therapies, although many novel genetic approaches have recently emerged for monogenic cases [149].

The most common motor neuron disease is ALS. The clinical and molecular phenotype of ALS were outlined in the introduction to this chapter. It is a relatively rare disease, with an incidence of 1–2 per 100,000 per year, but is becoming increasingly prevalent as the population ages; ALS incidence is projected to rise by approx. 70% by 2040 [150]. It has a long prodromal period, but is relatively

rapidly progressive upon symptom onset and diagnosis, leading to death within 2–4 years. Thus, this highly heterogeneous fatal disease lacks early diagnostics and is without effective treatments. Numerous systems biology studies have been launched to address this unmet medical need. These studies are uncovering molecular pathways, to pinpoint actionable drug targets [151] and seek early plasma diagnostic biomarkers [152] and modifiable environmental risk factors [148], since certain environmental exposures increase ALS risk.

4.1 What Is ALS Pathophysiology?

Targeted molecular studies have identified important pathological aspects in ALS, which are highly shared with central neurodegenerative diseases. In ALS, this includes altered TDP-43 protein aggregates, autophagy, excitotoxicity, impaired metabolism, dysfunctional mitochondria, inflammation, and oxidative stress [27, 30, 153, 154]. Many studies have employed the mutant superoxide dismutase 1 (SOD1) mouse model ($SOD1^{G93A}$). However, mutant SOD1 is only present in around 12% of familial and 1–2% of sporadic ALS patients [5]. Thus, although the $SOD1^{G93A}$ mouse recapitulates many ALS features, it is limited, as are other genetic models, since only 15% of ALS cases have a known genetic etiology. Therefore, systems biology approaches can help agnostically query pathophysiology in sporadic ALS, in addition to genetic models (Fig. 4a).

Despite the breadth of identified genetic mutations (ca. 40 known mutations) in 15% of ALS cases and the fact that 85% cases are sporadic, TAR DNA-binding protein 43 (TDP-43) inclusion bodies are an almost universal finding in ALS histopathology [3]. TDP-43 regulates transcription, pre-mRNA splicing, and mRNA translation, as well as microRNA (miRNA) biosynthesis [155, 156]. Therefore, many omics studies have concentrated on dysregulation of the epigenome and transcriptome in ALS, including miRNAs [18], which have been a significant focus. miRNAs are short ~22 nucleotide-long noncoding RNAs, which degrade target mRNAs, blocking their expression and downstream effects in ALS, such as neuromuscular junction structure and function, neurogenesis, and inflammation [157].

Environmental exposure directly affects the cellular epigenome reflected in altered DNA methylation and histone acetylation. We investigated epigenetic regulation through DNA methylation in postmortem spinal cord tissue from sporadic ALS participants [158]. Global methylated (5mC) and hydroxymethylated (5hmC) cytosine were elevated in ALS spinal cord versus controls, indicating epigenome dysregulation. When we examined DEGs and differentially methylated genes by microarray, we found 251 shared hits, of which ~70% were hypermethylated, as aligned with global 5mC. Of 251, 112 were concordant, that is, hypomethylated/upregulated (51 genes) or hypermethylated/downregulated

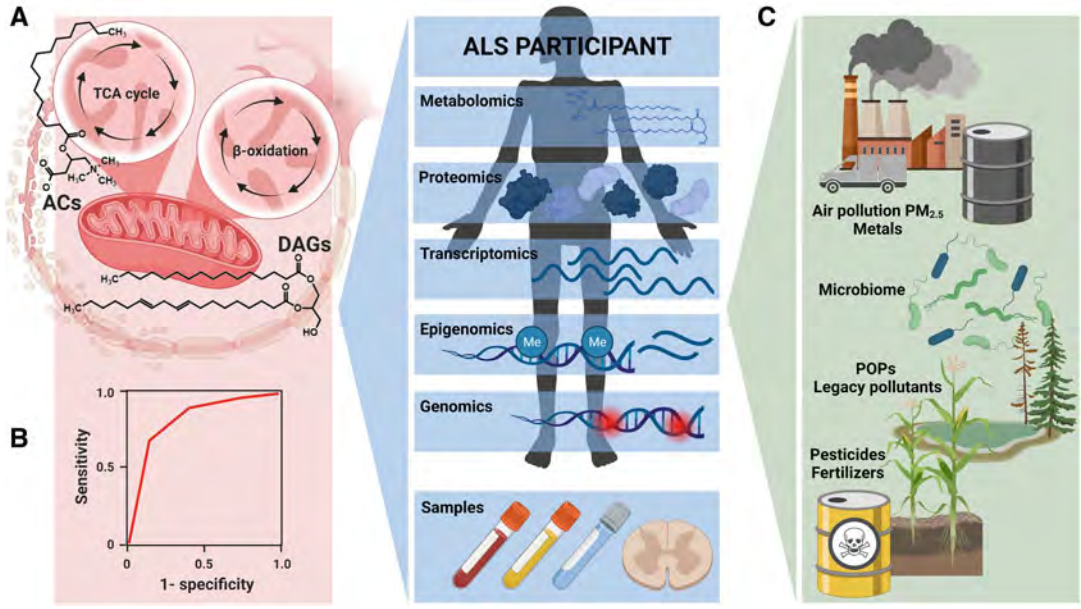

Fig. 4 Select systems biology applications to amyotrophic lateral sclerosis. Systems biology has manifold applications to address unmet medical needs for amyotrophic lateral sclerosis (ALS). Biosamples that can be analyzed include (left to right) blood, plasma, cerebrospinal fluid from consented participants, as well as postmortem spinal cord tissue. Biosamples from animal models can also be analyzed. Omics platforms include genomics (mutations, monogenic or polygenic, which correlate with ALS risk), epigenomics (DNA methylation, microRNA), transcriptomics (mRNA, long noncoding RNAs), proteomics (including phosphoproteins), and metabolomics and lipidomics. (**a**) What is ALS pathophysiology? Systems biology, by Omics analysis of ALS biosamples, can uncover pathomechanisms, which can suggest therapeutic avenues. In ALS, this includes altered mitochondrial and lipid metabolism (shown; AC, acylcarnitines; DAGs, diacylglycerols as examples), among other pathways (not shown). (**b**) Can we identify early ALS diagnostic biomarkers? ALS diagnoses are preceded by a long subclinical prodromal phase. Treatment may be more effective if initiated early; thus, systems biology, by omics analysis of ALS biosamples, can help by identifying early disease biomarkers using classifiers, shown for a receiver operating characteristic curve. (**c**) Can we define the ALS exposome? Systems biology can uncover environmental toxicants, which increase ALS risk, suggesting possible modifiable avenues. Examples include air pollution ($PM_{2.5}$, particulate matter 2.5 μm), metals, microbiome, POPs (persistent organic pollutants), pesticides, and fertilizer. This cumulative exposome over time interacts with genetic predisposition (polygenic risk), to alter ALS patient epigenome, transcriptome, proteome, and metabolome/lipidome, leading to disease onset and progression. (Created, in part, with BioRender.com)

(61 genes), and were enriched in biological pathways related to immune response, defense response, neuron adhesion, and plasma membrane part. Importantly, of the 112 candidates, 53 genes were cited at least once in PubMed, demonstrating the power of systems biology in one experiment to identify candidates from multiple publications. Our results additionally suggested myeloid or natural killer cell influx into ALS spinal cord, aligned with our findings in ALS participant blood samples [13, 15, 16].

We also analyzed miRNAs in sporadic ALS spinal cord by array profiling [20]. Globally, we saw reduced mature species levels, but no differences in immature transcripts, in ALS versus control, indicating impaired miRNA processing, which may be linked to TDP-43 lesions. Indeed, TDP-43 mislocalization to the cytoplasm alters miRNA profiles [19]. In sum, there were 90 differential miRNAs in ALS spinal cord versus control, 88 down- and 2 upregulated, which are annotated for pathways related to cell death, immune response, and brain development [20]. Enrichment analysis of biological functions of target mRNAs, both known and putative, corroborated immune and defense response, highlighting immune involvement in ALS [12].

In an exciting transcriptomic application, Maniatis et al. conducted spatiotemporal RNA profiling in spinal cord from $SOD1^{G93A}$ versus wild-type SOD1 mice at presymptomatic, onset, symptomatic, and end-stage time points to investigate the mechanism of "spread" in ALS neurodegeneration [23]. They found that microglial dysfunction preceded symptom onset and astroglial dysfunction in ALS, which occurred proximally to motor neurons. To complement their mouse work, they conducted a parallel analysis in human cervical and lumbar spinal cord tissue from sporadic ALS patients with bulbar ($n = 4$) and lower limb ($n = 3$) onset disease. As in mice, transcriptomic dysregulation was more pronounced near the site of symptom onset. Pathway analysis identified numerous biological processes, among them "ECM (extracellular matrix)–receptor interaction," "cell adhesion molecules," "axon guidance," and multiple immune "cytokine–cytokine receptor interaction," "chemokine signaling pathway," and "complement and coagulation cascades" in mouse and/or human, in alignment with our transcriptomics findings. Additionally, several metabolic pathways emerged, including "sphingolipid signaling pathway," "cholesterol metabolism," and "phosphatidylinositol signaling system." Indeed, impaired metabolism is an ALS hallmark and correlates with changes in basal metabolic rates in ALS cases [27].

Thus, we have also conducted a metabolomics analysis of ALS participant plasma versus controls [25]. Metabolites represent the cumulative effect of genetics, epigenetics, transcriptomics, and proteomics regulation, and also lend insight on potential environmental exposure through xenobiotics. Pathway analysis demonstrated that impaired lipid metabolism was a strong undercurrent in ALS, especially in complex "sphingomyelins," "ceramides," and "hexosylceramides" and β-oxidation intermediate "fatty acid metabolism (acyl carnitine, polyunsaturated)" species. Additionally, "creatine metabolism" was a top pathway, but is likely secondary to muscle wasting in ALS, as was xenobiotics "benzoate metabolism."

Moving forward, these studies will need validation in independent ALS cohorts and longitudinal profiling. However, they

underscore the ability of systems biology for putting into focus possible pathways, which may lead to therapeutic developments. Additionally, corroboration in model systems will be required to establish causality of any putative candidates.

4.2 Can We Identify Early ALS Diagnostic Biomarkers?

Although ALS lacks effective treatment, earlier intervention may help outcomes [159]. Unfortunately, ALS patients are generally diagnosed after symptom onset, sometimes even months following the initial symptoms. Therefore, earlier diagnosis could benefit patients if they can access treatment earlier. Along these lines, numerous studies have assessed potential ALS biomarkers in accessible biofluids, either cerebrospinal fluid or blood/plasma (Fig. 4b) [152]. Among candidates are neurofilament proteins, inflammatory molecules, and cystatin C, as well as molecules related to protein aggregates (TDP-43, SOD1) and genetic mutations (C9orf72 dipeptide repeats). Omics can also be employed to identify miRNA biomarkers; however, there is no consensus on a diagnostic miRNA panel, although manifold investigations have identified differential miRNAs in heredity and sporadic ALS versus healthy controls [22]. Metabolomics has similarly been proposed as a diagnostic toll; yet, again there is no consensus on a diagnostic metabolite panel, although altered lipid metabolism is a recurrent theme [25].

Most omics investigations of ALS biofluids have shared the same weakness, namely, that analyses were performed on samples from patients that had already developed symptoms and been diagnosed with ALS. One notable exception is a plasma metabolomics investigation by Bjornevik et al. of 5 large cohorts comprising over 318,000 participants with banked blood samples [160]. Participants that developed ALS after their blood sample had been banked were identified ($n = 275$), consented, and enrolled in the metabolomics analysis against matched controls ($n = 549$). The study found 31 differential metabolites in ALS versus controls, including many lipid species spanning diacylglycerols, triacylglycerols, phosphatidylcholines, cholesteryl ester, and sphingomyelin, as we had observed [25]. When participants were stratified by time of blood draw, there were 63 and 41 differential metabolites in samples collected less or more than 5 years, respectively, from the time of ALS diagnosis [160]. However, none of these metabolites remained significant after accounting for multiple comparisons, although penalized regression methods (lasso and elastic net) identified several metabolites, frequently lipids, which predicted ALS with moderate areas under the curve values ranging from 0.58 to 0.74. The authors suggested several study weaknesses, among them the large number of detected metabolites ($n = 404$) versus the relatively smaller sample size ($n = 275$), which limited statistical power. However, the study does illustrate a way forward for leveraging Omics to identify early ALS biomarkers, though the rarity of ALS poses significant challenges.

4.3 Can We Define the ALS Exposome?

There are well-documented ALS mutations [5]; however, the vast majority of ALS cases lack a known genetic etiology, despite numerous genome-wide association studies, whole genome studies, and exome sequencing studies. This has led to the emergence of the gene–time–environment hypothesis of ALS, which posits that cumulative environmental toxic exposures over time superimposes on genetic susceptibility to trigger disease onset and progression [11]. Thus, there has been significant interest in defining the ALS exposome, the collective of environmental exposures, which increases risk of disease (Fig. 4c) [148]. Indeed, our targeted environmental studies of potential environmental toxicants indicated a risk of ALS from pesticide, fertilizer, and persistent environmental pollutant exposures [8–10]. In our analysis of metals in teeth from ALS versus controls, we used laser ablation–inductively coupled plasma mass spectrometry to assess early exposure to metals [161]. Metal levels were elevated in cases versus controls, after adjusting for sex, smoking, occupational exposures, and ALS family history, findings corroborated for copper in teeth from $SOD1^{G93A}$ mice [162].

Exposome studies of ALS remain in the nascent stages. However, a high-profile metagenomic study in $SOD1^{G93A}$ versus wild-type SOD1 mice provided important insight on a possible causative role of gut microbiome on disease progression [163]. Longitudinal analysis revealed early gut dysbiosis occurred in ALS versus healthy mice, which centered around multiple species. Honing in on 11 candidates, Blacher et al. decolonized the gastrointestinal tract of $SOD1^{G93A}$ mice with antibiotics and inoculated mice individually with each of these focused species. Remarkably, inoculating $SOD1^{G93A}$ mice with *Akkermansia muciniphila* slowed disease progression and significantly increased survival. In addition to pinpointing the microbial species associated with disease severity, Omics (metabolomics) also elucidated a possible mechanism by identifying pathways centered on nicotinamide metabolism. Similar findings were corroborated in human ALS participants, whose microbiome could be differentiated from healthy controls by principal coordinate analysis, as were serum and cerebrospinal fluid analyses of nicotinamide levels. We have also conducted longitudinal microbiome investigations of $SOD1^{G93A}$ mice, similarly observing early gut dysbiosis, including of *Akkermansia muciniphila* [164]. Furthermore, gut dysbiosis correlated with immune cell infiltration into the brain and spinal cord.

Thus, systems biology can uncover how the exposome can exert an influence on ALS progression, with examples through the microbiome. However, broader studies involving additional candidate environmental pollutants, for example, air pollution, untargeted pollutant detection, are needed to fully define the ALS exposome.

5 Conclusions and Future Directions

In this chapter, we illuminated several recent studies, which employed systems biology in neurological disease for multifactorial goals, spanning pathophysiology, treatment response, therapeutic candidate identification, biomarker discovery, and exposome research. These studies demonstrate the ability of systems biology for advancing our understanding of neurologic diseases and suggest prospects for drug development, which is especially crucial since most diseases lack effective treatments.

Critically, more longitudinal studies are needed to identify the earliest pathological changes, which would allow therapeutic targeting of upstream events for disease-modifying treatments, rather than downstream events (palliative care) (Fig. 5a). This is challenging in human studies, especially during the prodromal phase [160], but can be readily accomplished in animal models [23, 163, 164]. Although the field of neurology is adopting these powerful Omics platforms, it lags behind the field of oncology, for example; however, it also ushers in the opportunity to learn from cancer research, clinical trials, and precision medicine for adoption in neurology. For example, Omics profiling has become well-entrenched in cancer research, where individuals with cancer are assigned specific treatments based on identified tumor mutations, and also participate in multiple novel clinical trials [165], a practice which could be adopted in neurological diseases, including the use of basket and umbrella trials (Fig. 5b). Indeed, investigators are implementing newer clinical trial designs, such as platform trials, for neurological diseases [166, 167], though this is lagging behind oncology trials.

Omics/multiomics profiling can be employed to develop molecular-based, rather than phenotype-based, diagnostic criteria and treatment selection for neurological illnesses (Fig. 5c). Although there are robust molecular tests for inherited PN, molecular tests for other neurological diseases, for example C9orf72 expansion in ALS, could also have diagnostic and treatment related implications. For instance, rather than bulbar versus spinal onset ALS, a molecular classification might additionally provide guidelines for future targeted treatment. This also includes generating molecular insight into idiopathic and sporadic neurological diseases. Finally, earlier studies during prodromal phases are needed to develop biomarkers or biomarker panels for early diagnostics of neurological illnesses (Fig. 5d). Omics/multiomics could bring the promise of precision medicine to bear in neurological diseases by matching patient profiles to approved drugs likely to be effective, for example, pain medications for painful PN (Fig. 5d). Thus, systems biology applications in neurological illnesses has a track record of success and a bright future for novel upcoming directions.

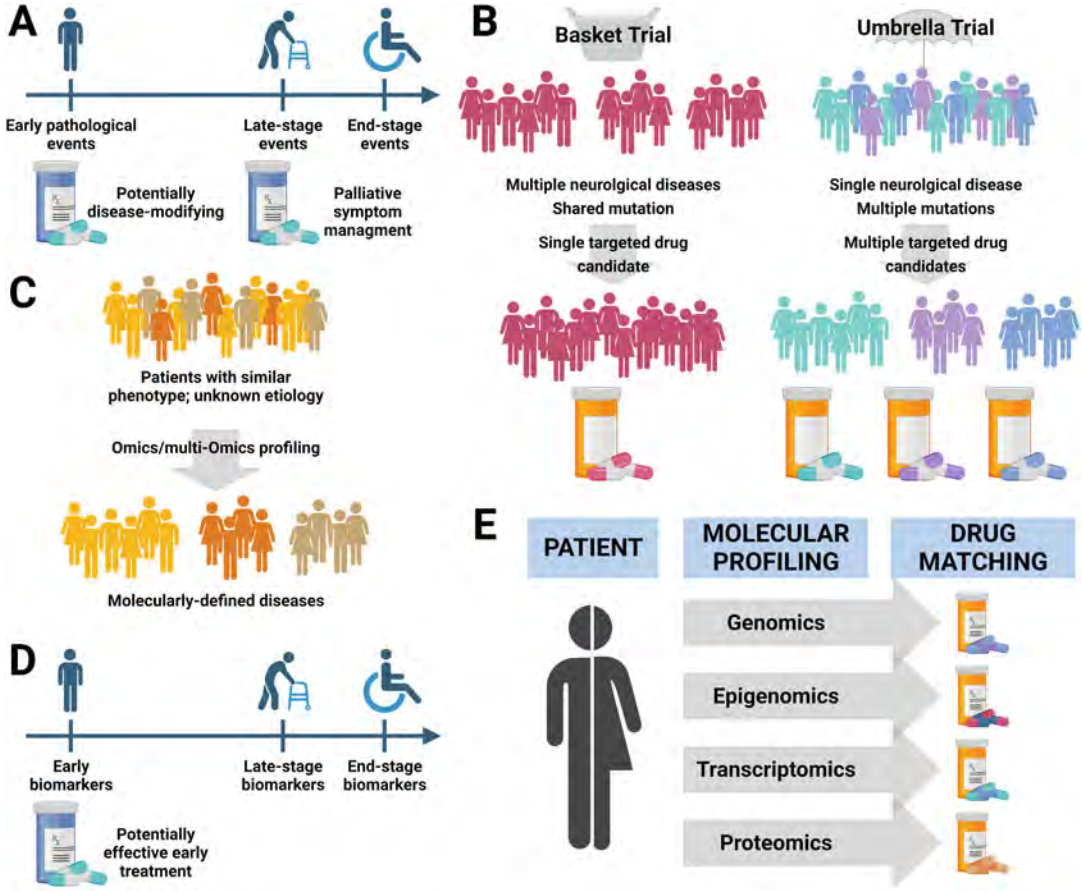

Fig. 5 Emergent and future systems biology research avenues in neurological diseases to address unmet medical needs. (**a**) Longitudinal studies to identify the earliest pathological changes, prior to symptom onset, to therapeutically target upstream (disease-modifying) rather than downstream (palliative care) events. (**b**) Omics/multiomics profiling to stratify participants for clinical trials of targeted, mechanism-based drug candidates. Basket trials comprise participants with multiple diseases harboring the same actionable mutation, and testing a single targeted drug candidate. Umbrella trials comprise participants with a single disease harboring multiple actionable mutations; participants are profiled and matched to a targeted drug candidate out of number of possible candidates. (**c**) Omics/multiomics profiling to develop molecular-based, rather than phenotype-based, diagnostic criteria and treatment selection for neurological illnesses. (**d**) Development of biomarkers or biomarker panels for early diagnostics of neurological illnesses. (**e**) Precision medicine to match patient omics/multiomics profile with approved drugs. (Created, in part, with BioRender.com)

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ORIGINAL ARTICLE

Epidemiology/Genetics

Dietary weight loss in people with severe obesity stabilizes neuropathy and improves symptomatology

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Abstract

Objective: The aim of this study was to determine the effect of dietary weight loss on neuropathy outcomes in people with severe obesity.

Methods: A prospective cohort study of participants attending a medical weight-management program was followed. Weight loss was achieved with meal replacement of 800 kcal/d for 12 weeks and then transitioning to 1,200 to 1,500 kcal/d. The co-primary outcomes were changes in intraepidermal nerve fiber density (IENFD) at the distal leg and proximal thigh. Secondary outcomes included nerve conduction studies, Michigan Neuropathy Screening Instrument questionnaire and exam, Quality of Life in Neurological Disorders, and quantitative sensory testing.

Results: Among 131 baseline participants, 72 (mean [SD] age: 50.1 [10.5] years, 51.4% female) completed 2 years of follow-up. Participants lost 12.4 (11.8) kg. All metabolic syndrome components improved with the exception of blood pressure. IENFD in the distal leg (0.4 [3.3], $p = 0.29$), and proximal thigh (0.3 [6.3], $p = 0.74$) did not significantly change. Improvements were observed on the Michigan Neuropathy Screening Instrument questionnaire, two Quality of Life in Neurological Disorders subdomains, and quantitative sensory testing cold threshold.

Conclusions: Dietary weight loss was associated with improvements in all metabolic parameters except blood pressure, and both IENFD outcomes remained stable after 2 years. Given that natural history studies reveal decreases in IENFD over time, dietary weight loss may halt this progression, but randomized controlled trials are needed.

INTRODUCTION

Neuropathy is a highly prevalent condition that results in pain, falls, and lower quality of life (1). Although diabetes has long been known to be the leading cause of neuropathy (2–4), obesity has recently emerged as an important risk factor (5–14). Furthermore, obesity is likely sufficient to cause neuropathy even in those with normal glucose control (7,9). In addition to hyperglycemia and obesity, other individual components of metabolic syndrome (hypertension, hypertriglyceridemia, and low high-density lipoprotein [HDL]

See Commentary, pg. 1990 (Dietary management of obesity-associated neuropathy: implications for clinical practice and trial design).

cholesterol) have also been shown to be associated with neuropathy (13). Unfortunately, despite multiple potentially modifiable risk factors, the only established disease-modifying therapy for neuropathy is glycemic control, which prevents neuropathy to a much larger degree in type 1 than in type 2 diabetes (14). We contend that newer interventions are needed to treat and prevent neuropathy.

Few studies, to our knowledge, have evaluated the effects of weight loss on neuropathy. Two uncontrolled studies have shown the potential for lifestyle interventions to improve neuropathy, but both primarily focused on exercise with only minimal weight loss (15,16). The most rigorous investigation to date, the Action for Health in Diabetes (Look AHEAD) study, randomized 5,145 participants with diabetes to 9 to 11 years of a lifestyle intervention designed to achieve and maintain weight loss compared with a diabetes support group (17). They found that the Michigan Neuropathy Screening Instrument (MNSI) questionnaire, but not the examination score, improved in those in the lifestyle intervention group, and that changes in weight, hemoglobin A_{1c} (HbA_{1c}), HDL cholesterol, and triglycerides were associated with changes in the MNSI questionnaire. No studies, to our knowledge, have investigated the effects of significant dietary weight loss on neuropathy outcomes in populations without diabetes or used the best quantitative measure of small fiber nerve injury, intraepidermal nerve fiber density (IENFD), as the primary outcome.

In a population with obesity with and without diabetes, we aimed to determine the effects of 2 years of a dietary weight-loss intervention on extensive neuropathy outcomes with the coprimary outcomes defined as IENFD at the distal leg and proximal thigh.

Study Importance

What is already known?

- ► Obesity is a consistent risk factor for neuropathy across many studies in different populations around the world.
- ► Metabolic syndrome and its individual components are also associated with neuropathy.
- ► Dietary weight loss has been demonstrated to improve questionnaire assessments of neuropathy in patients with diabetes but not in patients without diabetes, and no studies, to our knowledge, have used more comprehensive neuropathy phenotyping.

What does this study add?

- ► After 2 years, successful dietary weight loss in those with severe obesity leads to stable neuropathy as measured by our primary outcome (intraepidermal nerve fiber density).
- ► Successful dietary weight loss leads to improvements in secondary outcomes such as the Michigan Neuropathy Screening Instrument questionnaire, two Quality of Life in Neurological Disorders subdomains, and quantitative sensory testing cold threshold.
- ► Dietary weight loss also leads to stable cardiovascular autonomic neuropathy.

How might these results change the direction of research or the focus of clinical practice?

- ► Future randomized clinical trials are needed to confirm that dietary weight loss can stabilize neuropathy.
- ► If successful, dietary weight loss would become the second disease-modifying therapy for neuropathy along with glycemic control.
- ► Furthermore, studies are needed to compare the effectiveness of dietary weight loss, surgical weight loss, and exercise to allow clinicians to focus on the best intervention to prevent neuropathy.

METHODS

Population

From November 2010 to December 2014, we recruited participants with obesity attending the University of Michigan Weight Management Program and followed them for 2 years after starting a dietary weight-loss intervention. Inclusion criteria included being aged 18 years or older and having BMI ≥ 35 kg/m^2 or ≥ 32 kg/m^2 if they had one or more comorbidities (18). The intervention consisted of a very low-energy diet in the form of liquid meal replacement plus 2 cups of nonstarchy vegetables (~800 kcal/d) for approximately 12 weeks to promote a 15% weight reduction from the pre-dietary intervention weight. Participants were then slowly transitioned to a 1,000- to 1,200-kcal/d partial meal replacement diet until their desired weight loss was achieved. This consisted of three replacement products and 400 kcal of conventional food, consisting of half a plate of nonstarchy vegetables, 3 to 4 oz of lean protein, and half a cup of whole grain or fruit. Participants were counseled to perform 40 min/d of moderate activity including cardio and light strength training during the initial intensive dietary phase and then 60 min/d during the weight-loss maintenance phase.

This study was approved by the University of Michigan Institutional Review Board and registered on ClinicalTrials.gov (NCT02043457), and all participants signed informed consent documents.

Metabolic phenotyping

Participants underwent glucose tolerance testing (except those with a previous diagnosis of diabetes) and a fasting lipid panel at baseline and after 2 years. Participants also had blood pressure, height, weight, waist circumference, and BMI measurements taken monthly

throughout the study. Participants with diabetes also had a HbA_{1c} test. Diabetes and prediabetes were defined at baseline and after 2 years using HbA_{1c} and glucose tolerance testing, according to the American Diabetes Association (19).

Polyneuropathy definition (primary outcome)

Our coprimary outcome measures were the IENFD measured at the distal leg and proximal thigh. IENFD was evaluated using bright-field immunohistochemistry using an established protocol (20).

Secondary neuropathy outcomes

Our secondary outcome measures included 17 nerve conduction study (NCS) parameters from six different nerves (sural sensory, median sensory, ulnar sensory, peroneal motor, tibial motor, and median motor). NCS was performed using Viking on the Nicolet EDX electrodiagnostic system (CareFusion, San Diego, California). The MNSI questionnaire and examination (performed by a neuromuscular specialist) were completed as previously described (21). Quantitative sensory testing (QST) measurements of vibration and cold detection thresholds were performed using the Computer Aided Sensory Evaluator (CASE) IV (WR Medical Electronics Co., Maplewood, Minnesota). Quantitative sudomotor axon reflex testing (QSART) measurements were performed at the foot, distal leg, proximal leg, and arm using the Q-Sweat quantitative sweat measurement system (WR Medical Electronics). Monofilament testing was performed with a Semmes-Weinstein 5.07/10-g monofilament on the dorsum of the dominant great toe. Monofilament testing was normal if the participant felt eight or more out of ten responses, reduced for one to seven responses, and absent for zero responses. Clinical neuropathy was defined using the Toronto Consensus definition of probable polyneuropathy, which requires two or more of the following: neuropathy symptoms, abnormal sensory examination, and abnormal reflexes as determined by one of four neuromuscular specialists (22).

Patient-oriented neuropathy outcomes

The validated Quality of Life in Neurological Disorders (Neuro-QoL) instrument was used to measure neuropathy-specific quality of life, with higher numbers reflecting a worse quality of life (23). The validated short-form McGill Pain Questionnaire was employed to measure pain with a visual analog scale, a six-point rating scale of present pain intensity (PPI) score, and a four-point rating scale of 15 different neuropathic pain descriptors (McGill Pain score) (24).

Cardiovascular autonomic neuropathy outcomes

All cardiovascular autonomic neuropathy (CAN) tests were performed using the ANX 3.0 device (The Ansar Group, Inc., Philadelphia, Pennsylvania). Outcomes included three cardiovascular reflex tests (expiration to inspiration [E:I] ratio, 30:15 ratio, and the average of two Valsalva ratios), which are associated with mortality (25) and are considered the gold standard tests for autonomic neuropathy (26). Other measurements that were recorded included the resting median heart rate, frequency-domain measures (low-frequency area [LFA, measure of sympathetic activity], respiratory frequency area [RFA, measure of parasympathetic activity], and LFA/RFA [measure of sympathovagal balance]), time-domain measures (standard deviation [SD] of the normal-to-normal interval [sdNN]), and root mean square of successive differences of the normal-to-normal interval (rmsSD).

Statistical analysis

Descriptive statistics were used to characterize participants in terms of demographics, metabolic phenotyping, and neuropathy outcomes at baseline and after 2 years of follow-up. For continuous measurements, we determined the within-participant change during the study by subtracting baseline measurements from measurements taken after 2 years of follow-up.

We compared demographic information between participants who completed follow-up and those who did not using two-sample *t* tests for continuous covariates and Pearson χ^2 tests or Fisher exact tests for categorical covariates. Paired *t* tests were used to compare within-patient differences in continuous metabolic factors and all outcomes during follow-up. For ordinal outcomes, the Wilcoxon signed rank test was used to determine within-patient change during follow-up.

All analyses were completed using R version 3.4.2 (R Foundation, Vienna, Austria).

RESULTS

Population

During recruitment, the University of Michigan Weight Management Program enrolled 532 participants, including 394 who consented to be contacted about research studies and 131 who consented to our study. Of the 131 participants who completed baseline assessments, 72 completed assessments at 2 years. Reasons for attrition included the following: 16 participants decided to opt out of the study, 2 moved out of state, 1 died, 30 did not respond to multiple contacts, and 10 stopped participation for unclear reasons. Of the 59 who did not complete the neuropathy outcomes, 12 completed 2 years of follow-up with the weight-management program but did not want to complete the neuropathy outcomes. Of the remaining patients, median (interquartile range) follow-up in the weight-management clinic was 370 days (179-528 days).

Several outcome variables had missing information at baseline (V1) or at 2 years (V2): IENFD leg (V1:1,V2:6); IENFD thigh (V1:1,V2:8);

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TABLE 1 Demographics of primary cohort and those lost during follow-up

	All participants ($n = 131$)	Completed follow-up ($n = 72$)	Lost to follow-up ($n = 59$)	p value
Age, mean (SD)	49.1 (10.6)	50.2 (10.2)	47.8 (11.0)	0.19
Sex, *n* (%) female	72 (55.0%)	37 (51.4%)	35 (59.3%)	0.47
Race, *n* (%)				0.59
Asian	1 (0.8%)	0 (0.0%)	1 (1.7%)	
Black	10 (7.6%)	7 (9.7%)	3 (5.1%)	
White	118 (90.1%)	64 (88.9%)	54 (91.5%)	
Unknown	2 (1.5%)	1 (1.4%)	1 (1.7%)	
Ethnicity, *n* (%)				
Hispanic/Latino	2 (1.5%)	1 (1.4%)	1 (1.7%)	1
Smoking status, *n* (%)				0.39
Current smoker	3 (2.3%)	3 (4.2%)	0 (0.0%)	
Ex-smoker	42 (32.6%)	22 (31.0%)	20 (34.5%)	
Never smoker	84 (65.1%)	46 (64.8%)	38 (65.5%)	
Marital status, *n* (%)				0.52
Divorced	7 (5.6%)	4 (5.9%)	3 (5.3%)	
Married	94 (75.2%)	53 (77.9%)	41 (71.9%)	
Single	21 (16.8%)	11 (16.2%)	10 (17.5%)	
Separated	2 (1.6%)	0 (0.0%)	2 (3.5%)	
Widowed	1 (0.8%)	0 (0.0%)	1 (1.8%)	
Education, *n* (%)				0.09
Professional or graduate degree	51 (39.2%)	33 (45.8%)	18 (31.0%)	
College degree	54 (41.5%)	25 (34.7%)	29 (50.0%)	
Some college or vocational college	23 (17.7%)	14 (19.4%)	9 (15.5%)	
High school or less	2 (1.5%)	0 (0.0%)	2 (3.4%)	
Employment status, *n* (%)				0.03
Employed	101 (77.7%)	50 (69.4%)	51 (87.9%)	
Retired	19 (14.6%)	16 (22.2%)	3 (5.2%)	
Seeking work	2 (1.5%)	1 (1.4%)	1 (1.7%)	
Keeping house	4 (3.1%)	2 (2.8%)	2 (3.5%)	
Other	4 (3.1%)	3 (4.2%)	1 (1.7%)	
Insurance, *n* (%)				0.59
Blue Care Network (HMO)	77 (59.7%)	41 (56.9%)	36 (63.2%)	
Other	52 (40.3%)	31 (43.1%)	21 (36.8%)	

Abbreviation: HMO, health maintenance organization.

NCS parameters, including sural (V1:1), peroneal F wave (V2:1), median motor F wave (V2:2), and median motor conduction velocity (V2:1); QST cold threshold (V1:2, V2:2); QST vibration threshold (V1:1,V2:1); QSART parameters, including arm (V1:2,V2:3), proximal leg (V1:1,V2:7), distal leg (V1:1,V2:2), and proximal foot (V1:3,V2:4); MNSI questionnaire (V2:1); monofilament (V2:1); Neuro-QoL parameters, including social (V2:1), emotional (V2:1), and total (V2:2); CAN measures including E:I ratio (V2:2), 30:15 ratio (V1:1,V2:4), Valsalva ratio (V1:1,V2:3), RFA (V2:2), LFA (V2:2), sdNN (V2:2), rmsSD (V2:2), and resting median heart rate (V2:2); waist circumference (V2:7); triglycerides (V1:1,V2:7); HDL cholesterol (V1:1, V2:7); low-density lipoprotein (LDL) cholesterol (V1:2,V2:7); and fasting glucose (V1:12,V2:8). All patients had at least one measure of glycemic status at baseline, but three patients had no measure of glycemic status at 2 years.

Among those with complete follow-up, 19 (26.4%) had clinical neuropathy at baseline, and 14 (19.4%) had clinical neuropathy after 2 years. No difference in the dropout rate between those with and without neuropathy at baseline was observed ($p = 0.4$).

Demographics

At baseline, the mean (SD) age was 49.1 (10.6) years, and 55.0% of participants were female (Table 1). No significant demographic

TABLE 2 Change in metabolic factors after dietary weight-loss intervention

	Baseline	2-year follow-up	Change	*p* value (paired *t* test)
Weight (kg)	120.7 (23.0)	108.3 (22.3)	−12.4 (11.8)	<0.01
Height (cm)	171.7 (10.3)	171.8 (10.4)	0.1 (3.1)	0.86
BMI	40.8 (6.0)	36.5 (5.8)	−4.3 (3.8)	<0.01
Waist circumference (cm)	123.1 (15.0)	114.7 (15.7)	−9.0 (9.7)	<0.01
Systolic blood pressure (mmHg)	126.4 (11.0)	126.9 (13.9)	0.5 (12.5)	0.74
Diastolic blood pressure (mmHg)	64.4 (7.3)	68.2 (9.0)	3.8 (9.0)	<0.01
Triglycerides (mg/dL)	153.4 (78.3)	127.8 (65.1)	−27.1 (55.6)	<0.01
HDL (mg/dL)	45.3 (11.1)	51.0 (11.5)	5.2 (7.9)	<0.01
LDL (mg/dL)	97.5 (23.6)	98.1 (28.0)	1.1 (18.4)	0.64
Cholesterol (mg/dL)	172.2 (30.0)	174.6 (33.9)	0.7 (25.1)	0.83
Fasting glucose (mg/dL)	101.7 (23.9)	99.3 (23.6)	−7.5 (22.9)	0.02
2-hour glucose (mg/dL)	134.8 (57.3)	110.9 (34.4)	−21.8 (43.5)	<0.01
HbA_{1c} (%)	6.0 (0.9)	5.8 (0.7)	−0.3 (0.6)	0.01

Data given as mean (SD).

Abbreviations: HbA_{1c}, hemoglobin A_{1c}; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

differences were observed between those who completed follow-up compared with those who did not, with the exception of employment status (69.4% vs. 87.9%, $p = 0.03$).

Change in metabolic risk factors

With the exception of systolic blood pressure and LDL cholesterol, all metabolic parameters significantly changed after 2 years (Table 2). Among those with complete follow-up, 22.2% had diabetes, 37.5% had prediabetes, and 40.3% had normoglycemia at baseline. After 2 years, 14.7% had diabetes, 27.9% had prediabetes, and 57.4% had normoglycemia ($p < 0.01$). The median (interquartile range) weight loss comparing baseline with the end of the study was 5.5% (5.0%-14.7%) (Figure 1). At maximum weight loss, participants had lost 16.4% (13.0%-22.4%) of their weight. Comparing minimum weight to the end of the study, participants regained 8.5% (5.4%-13.9%) of their weight.

Change in coprimary outcomes

IENFD did not change significantly in the distal leg (0.4 [3.3] fibers/mm, $p = 0.29$) or proximal thigh (0.3 [6.3] fibers/mm, $p = 0.74$) after 2 years (Figure 2).

Change in secondary neuropathy outcomes

Of the 17 NCS parameters, significant changes were observed only in the ulnar sensory peak latency (0.1 [0.4] milliseconds, $p < 0.01$) and median sensory peak latency (0.2 [0.4] milliseconds, $p < 0.01$), which both worsened after 2 years (Table 3). The MNSI questionnaire (−0.6 [1.4], $p < 0.01$) improved, but there were no significant changes in the MNSI examination (0.04 [1.2], $p = 0.76$). The QST cold threshold (−2.0 [4.9] just noticeable difference, $p < 0.01$) improved, but there was no change in the QST vibration threshold (0.2 [4.2] just noticeable difference, $p = 0.77$). Monofilament and QSART measures were unchanged.

Change in patient-oriented neuropathy outcomes

The Neuro-QoL (−0.3 [1.4], $p = 0.06$), visual analog scale pain scores (−1.8 [29.3] mm, $p = 0.60$), McGill Pain scores (−0.8 [4.7], $p = 0.15$), and PPI (17.1% worsened, 12.9% improved, $p = 0.75$) were unchanged after 2 years (Table 3). However, the Neuro-QoL subdomains of pain (−0.4 [1.1], $p = 0.01$) and emotion (−0.7 [2.2], $p = 0.01$) were improved.

Change in CAN outcomes

No significant changes were seen in any of the CAN outcomes, including E:I ratio, 30:15 ratio, Valsalva ratio, RFA, LFA, RFA/LFA ratio, sdNN, rmsSD, or resting median heart rate (Table 3).

DISCUSSION

A successful dietary weight-loss intervention in those with severe obesity was associated with no change in our coprimary neuropathy outcomes (IENFD of the distal leg and proximal thigh). This sharply contrasts with the natural history of IENFD decline in those with small fiber neuropathy of any cause, including prediabetes

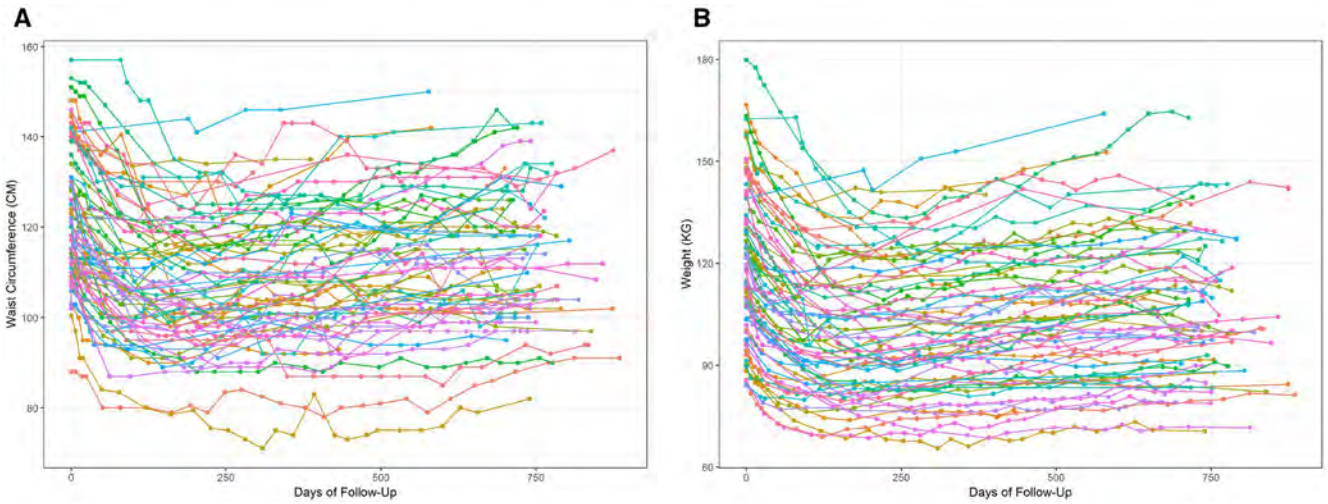

FIGURE 1 Change in obesity measures over 2 years. Longitudinal measures of waist circumference (A) and weight (B) during 2 years of follow-up after a dietary weight-loss intervention [Color figure can be viewed at wileyonlinelibrary.com]

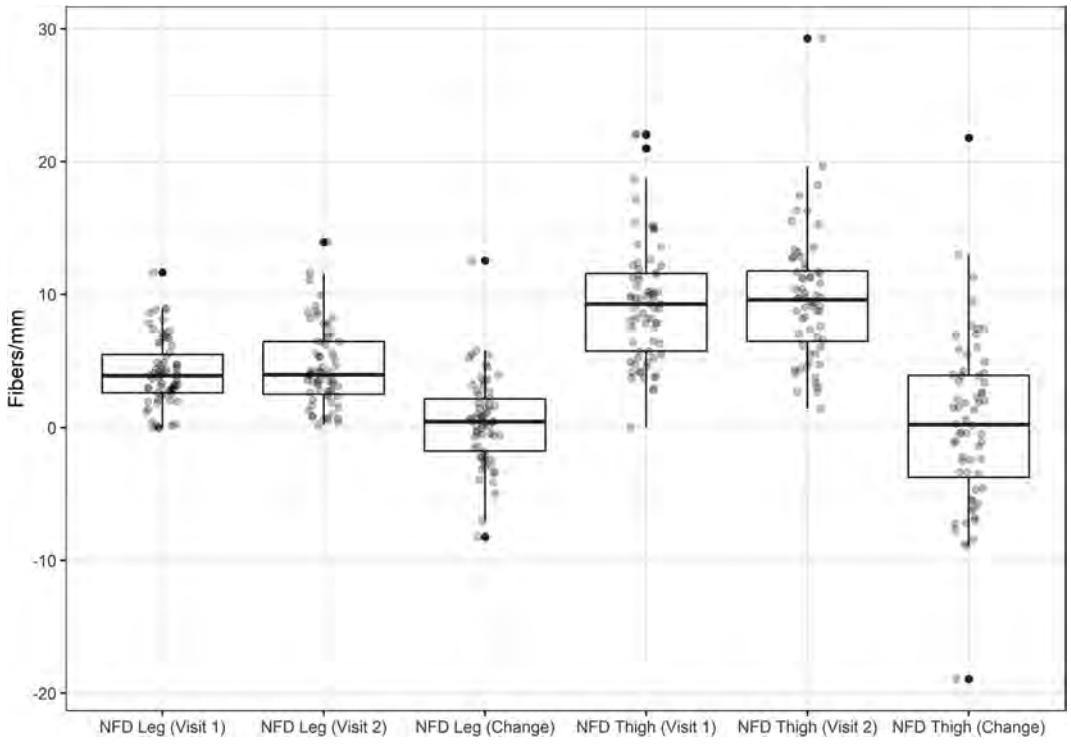

FIGURE 2 Change in IENFD (primary outcome) of the distal leg and proximal thigh after 2 years of a dietary weight-loss intervention. IENFD, intraepidermal nerve fiber density

and diabetes (27), but it is not as impressive as the improvements that have been reported with exercise intervention studies (15,16,28,29). Importantly, the natural history of IENFD decline in populations with obesity is unknown. Some secondary outcomes revealed improvements, specifically the MNSI questionnaire, QST cold threshold, and two subdomains of the Neuro-QoL, but NCS parameters and other secondary outcomes remained unchanged. Similar to neuropathy outcomes, CAN measures were also stable after 2 years.

This study is the second, to our knowledge, to evaluate the effects of a lifestyle intervention that was focused on dietary weight loss on neuropathy outcomes. The Look AHEAD study randomized more than 5,000 participants who had type 2 diabetes and overweight or obesity to a dietary weight-loss intervention for 9 to 11 years (17). Our study is complementary in that we were able to study a population with obesity that included those with normoglycemia and prediabetes in addition to those with diabetes. We also performed much more detailed neuropathy phenotyping.

TABLE 3 Changes in outcomes following weight loss

	Baseline	2-year follow-up	Change	*p* value (paired *t* test)
Neuropathy outcomes				
IENFD leg (fibers/mm)	4.1 (2.5)	4.6 (3.0)	0.4 (3.3)	0.29
IENFD thigh (fibers/mm)	9.4 (4.6)	9.7 (4.8)	0.3 (6.3)	0.74
NCS outcomes				
Ulnar peak latency (ms)	3.4 (0.4)	3.5 (0.4)	0.1 (0.4)	<0.01
Ulnar amplitude (μV)	28.0 (13.9)	25.0 (12.7)	−3.0 (12.5)	0.05
Peroneal distal motor latency (ms)	4.9 (0.9)	5.0 (0.7)	0.1 (0.8)	0.41
Peroneal amplitude (μV)	5.4 (2.6)	5.2 (2.9)	−0.2 (2.2)	0.38
Peroneal F (ms)	50.6 (5.8)	50.9 (5.7)	0.1 (5.9)	0.85
Peroneal CV (m/s)	44.7 (5.2)	44.0 (5.3)	−0.9 (5.7)	0.21
Sural peak latency (ms)	3.8 (0.4)	3.9 (0.4)	0.1 (0.4)	0.08
Sural amplitude (μV)	12.3 (6.9)	13.1 (8.8)	0.9 (7.9)	0.38
Tibial distal motor latency (ms)	4.8 (0.9)	5.1 (0.8)	0.2 (1.0)	0.08
Tibial amplitude (μV)	9.1 (5.3)	8.5 (4.5)	−0.5 (4.2)	0.30
Tibial F (ms)	53.1 (6.5)	52.3 (6.0)	−0.4 (6.6)	0.65
Median distal motor latency (ms)	3.9 (0.7)	3.9 (0.8)	0.001 (0.5)	0.98
Median motor amplitude (μV)	7.7 (3.3)	7.9 (3.3)	0.2 (3.6)	0.60
Median motor F (ms)	29.0 (2.7)	29.0 (3.6)	0.2 (2.8)	0.56
Median motor CV (m/s)	52.6 (5.2)	53.2 (6.3)	0.5 (6.2)	0.47
Median sensory peak latency (ms)	3.8 (0.6)	3.9 (0.6)	0.2 (0.4)	<0.01
Median sensory amplitude (μV)	28.8 (15.4)	28.0 (16.1)	−0.7 (5.8)	0.29
QST				
QST cold threshold	13.1 (4.8)	10.8 (4.4)	−2.0 (4.9)	<0.01
QST vibration threshold	17.8 (3.7)	17.9 (4.2)	0.2 (4.2)	0.77
QSART				
QSART arm	1.2 (1.1)	1.9 (6.7)	0.7 (6.9)	0.43
QSART proximal leg	0.5 (0.5)	1.4 (9.3)	1.0 (9.4)	0.41
QSART distal leg	0.5 (0.4)	1.3 (6.9)	0.9 (7.0)	0.31
QSART proximal foot	0.5 (0.5)	0.9 (4.7)	0.5 (4.8)	0.45
MNSI				
MNSI questionnaire	2.8 (2.5)	2.2 (2.2)	−0.6 (1.4)	<0.01
MNSI exam	1.0 (1.5)	1.1 (1.6)	0.04 (1.2)	0.76
Monofilament				
Normal	65 (90.3%)	66 (93.0%)	Worsened: 2 (2.8%)	1.00[#]
Reduced	5 (6.9%)	2 (2.8%)	Stable: 65 (91.6%)	
Absent	2 (2.8%)	3 (4.2%)	Improved: 4 (5.6%)	
Patient-oriented outcomes				
McGill Pain score				
McGill total	4.7 (6.0)	3.9 (5.7)	−0.8 (4.7)	0.15
McGill sensory	4.0 (5.0)	3.3 (4.6)	−0.7 (4.0)	0.12
McGill affective	0.6 (1.3)	0.5 (1.4)	−0.1 (1.3)	0.58
VAS total	19.6 (24.6)	17.8 (22.8)	−1.8 (29.3)	0.60
PPI				
No pain	54 (77.1%)	52 (72.2%)	Worsened: 12 (17.1%)	0.75[#]
Mild	9 (12.9%)	14 (19.4%)	Stable: 49 (70.0%)	

(Continues)

TABLE 3 (Continued)

	Baseline	2-year follow-up	Change	*p* value (paired *t* test)
Discomforting	7 (10.0%)	6 (8.3%)	Improved: 9 (12.9%)	
Neuro-QoL				
Neuro-QoL total	2.3 (1.8)	2.0 (1.1)	−0.3 (1.4)	0.06
Neuro-QoL pain	2.3 (1.8)	2.0 (1.5)	−0.4 (1.1)	0.01
Neuro-QoL reduced sensation	2.1 (2.3)	2.0 (2.4)	−0.1 (1.9)	0.65
Neuro-QoL sensory motor	1.9 (1.6)	2.0 (1.8)	0.04 (1.3)	0.80
Neuro-QoL social	2.1 (1.6)	2.0 (1.0)	−0.03 (1.9)	0.90
Neuro-QoL emotional	2.4 (2.6)	1.7 (1.0)	−0.7 (2.2)	0.01
Neuro-QoL activities of daily living	3.2 (3.0)	2.9 (2.1)	−0.3 (2.7)	0.34
CAN outcomes				
E:I ratio	1.13 (0.08)	1.19 (0.40)	0.07 (0.41)	0.17
30:15 ratio	1.40 (0.64)	1.38 (0.53)	−0.02 (0.46)	0.71
Valsalva ratio	1.48 (0.34)	1.54 (0.51)	0.07 (0.60)	0.35
RFA	10.5 (65.5)	26.5 (196.2)	15.8 (208.4)	0.53
LFA	7.5 (45.1)	57.9 (446.0)	50.2 (449.2)	0.35
LFA/RFA	3.1 (4.1)	5.4 (17.5)	2.2 (18.0)	0.31
sdNN	53.9 (31.1)	54.1 (40.2)	0.5 (48.7)	0.93
rmsSD	35.3 (32.7)	38.7 (49.4)	3.9 (59.5)	0.58
Median heart rate	66.8 (11.9)	67.6 (13.9)	0.5 (16.6)	0.80

Data given as mean (SD) unless otherwise indicated.

Abbreviations: CAN, cardiovascular autonomic neuropathy; CV, conduction velocity; E:I, expiration to inspiration; IENFD, intraepidermal nerve fiber density; LFA, low-frequency area, measure of sympathetic activity; LFA/RFA, low-frequency area/respiratory frequency area, measure of sympathovagal balance; MNSI, Michigan Neuropathy Screening Instrument; PPI, present pain intensity; NCS, nerve conduction study; Neuro-QoL, Quality of Life in Neurological Disorders, neuropathy-specific quality of life instrument; QSART, quantitative sudomotor axon reflex testing; QST, quantitative sensory testing; RFA, respiratory frequency area, measure of parasympathetic activity; rmdSD, root mean square of successive differences of the normal-to-normal interval; sdNN, SD of the normal-to-normal interval.

#*p* value represents results from Wilcoxon signed rank test.

Both Look AHEAD and our study found that dietary weight loss was associated with improvements on the MNSI questionnaire but not the MNSI examination. The consistency of these results provides more evidence for the benefits of dietary weight loss for peripheral nerves, but it also highlights the limitations of this intervention. Subjective measures such as the MNSI questionnaire improved, which we also observed for two Neuro-QoL subdomains. In contrast, objective measures of neuropathy, such as the MNSI examination, were largely stable. Our study included IENFD and NCS parameters that also demonstrated stability. Taken together, these studies indicate that dietary weight loss can halt the progression of neuropathy, if not lead to mild improvements, but that different interventions are likely needed if more dramatic improvement is the goal. Importantly, the natural history of small fiber neuropathy, regardless of cause, is to decline at a predictable rate (27); therefore, any intervention that leads to stability should be considered a success.

Other potential interventions to improve multiple metabolic risk factors and neuropathy outcomes include surgical weight loss, medication-induced weight loss, and exercise. To our knowledge, surgical weight loss was evaluated only in one small study of 12 participants before and after Roux-en-Y gastric bypass with a hint of efficacy (30). Medication-induced weight loss has not been studied, to our knowledge. In contrast, exercise has been the most studied of these interventions, including three uncontrolled studies and one randomized study (15,16,28,29). Two of these studies also had a dietary component, but weight loss was minimal: 0.1 and 1.1 decrease in BMI compared with 4.3 decrease in our study (15,16). Three of these exercise studies demonstrated improvements in IENFD outcomes, and the randomized trial revealed improvements in NCS and vibration thresholds. Taken together, the previous exercise studies indicate an improvement in neuropathy outcomes, whereas dietary weight loss demonstrates stability or mild improvement in subjective outcomes. However, more definitive studies comparing the effects of exercise and different weight-loss strategies (dietary, surgical, and medication induced) are needed before strong clinical recommendations can be made favoring one of these interventions. Given the modest effect size and adherence issues with dietary weight loss, future interventions may need to combine exercise and dietary weight loss or include dietary adjustments designed to improve adherence and/or to improve neuropathy through limiting certain metabolites that may lead to nerve injury.

Although IENFD and NCS parameters did not change after dietary weight loss, the MNSI questionnaire, two Neuro-QoL subdomains, and the QST cold threshold all demonstrated improvement after 2 years. This is an important finding because using more sensitive measures of neuropathy improvement has the potential to lead to more efficient clinical trials. We looked at several secondary outcome measures; therefore, these results should be considered hypothesis generating rather than definitive. On the other hand, the Look AHEAD study also demonstrated improvement in the MNSI questionnaire over the first couple of years as well as after 9 to 11 years of dietary weight loss (17). These results provide stronger justification for using the MNSI questionnaire as a sensitive measure of neuropathy improvement. Interestingly, the Neuro-QoL may also be a sensitive indicator of improvement, which could be important as this is also a patient-oriented outcome. Future studies are needed to determine whether the Neuro-QoL demonstrates sensitivity to neuropathy improvement. Despite these encouraging results, more sensitive biomarkers are needed to detect earlier changes in patients with neuropathy, which would enable more efficient clinical trials. Furthermore, the MNSI questionnaire and Neuro-QoL are more subjective neuropathy measures compared with IENFD and NCS, which may account for the differences observed with these outcomes.

Similar to neuropathy, CAN also demonstrated stability 2 years after dietary weight loss on all nine measures. Only one randomized trial, to our knowledge, investigated the effects of dietary weight loss in combination with exercise on CAN outcomes, and this was in a population of individuals with type 2 diabetes (31). The investigators found no improvement on the E:I ratio in the overall population, although diet and exercise did lead to improvement in women. Analogously to neuropathy, the natural history of CAN is to worsen over a 2-year period in those with diabetes (32). Therefore, the stability in CAN measures after dietary weight loss in both this study and our current study provides supporting evidence for a positive effect, but future randomized studies are needed especially because the natural history of CAN in populations with obesity is unknown. Comparable to neuropathy, most of the intervention studies have focused on the effects of exercise on CAN (33). These studies have generally showed improvement in CAN outcomes, but they are uniformly small, with varying outcomes and exercise regimens, which limits interpretability (28,34–38). Just like with neuropathy, studies that compare the effects of exercise with different weight-loss strategies are needed to guide clinical recommendations.

Interestingly, the improvement in neuropathy outcomes that we observed in humans with obesity after a dietary intervention has also been observed in obese mice. Mice on a high-fat diet developed neuropathy that was completely normalized after dietary reversal (39). Although we did not observe such robust results in humans, the dietary reversal was not nearly as complete as in the murine models, and the metabolic impairments were present for far longer in humans. Importantly, lipidomic analyses have shed light on potential biologic mechanisms of obesity-related neuropathy. Nerves from high-fat-fed mice with neuropathy contained an increase in triglycerides containing saturated fatty acids compared with nerves from control mice (40). Mice fed a high-fat diet consisting of saturated fatty acids developed neuropathy that was completely reversed by switching to a high-fat diet consisting of monounsaturated fatty acids (41). The monounsaturated fatty acid oleate also prevented defects in axonal mitochondrial transport and membrane potential that were present in sensory neurons treated with the saturated fatty acid palmitate. These results indicate that nerve-lipid signaling is an important factor in peripheral nerve injury (42).

Limitations of this study include the small sample size, which limits our power to detect small changes. However, we did observe significant changes in multiple secondary outcomes. We also had significant loss to follow-up, but only employment status was significantly different between the whole cohort and those who followed up after 2 years. We were unable to investigate longer-term effects of dietary weight loss on outcomes after 2 years, but our results are consistent with the Look AHEAD study, which followed participants for 9 to 11 years (17). Our pre-post intervention design does not allow for causal inferences. Generalizability to other populations, particularly those with different race/ethnicity and educational status, is unclear. Strengths of this study include the comprehensive metabolic and neuropathy phenotyping before and after a successful dietary weight-loss intervention.

After a dietary weight-loss intervention, participants with severe obesity had large improvements in multiple metabolic risk factors. Neuropathy, as measured by IENFD, and CAN were stable after 2 years, which is an improvement from the known natural history of decline in those with small fiber neuropathy from any cause (28). Randomized trials are needed to definitively address the effects of dietary weight loss on neuropathy and compare and contrast with other weight-loss measures and/or exercise.

CONFLICT OF INTEREST

BCC consults for DynaMed, performs medical legal consultations, including consultations for the Vaccine Injury Compensation Program, and receives research support from the American Academy of Neurology. AER consults for Nestle S.A., Rhythm Pharmaceuticals, and REWIND Inc. The other authors declared no conflict of interest.

CLINICAL TRIAL REGISTRATION

ClinicalTrials.gov identifier NCT02043457.

AUTHOR CONTRIBUTIONS

BCC was involved in the study design and interpretation of the statistical analysis and wrote the manuscript. ELR, MB, AER, CFB, and ELF were integrally involved in the study design, interpretation of the data, and critical revisions of the manuscript. ELR performed the statistical analyses. GA was involved in interpretation of the data and critical revisions of the manuscript. EVU and EC were involved in the study design and critical revisions of the manuscript.

DATA AVAILABILITY STATEMENT

Individual patient-level data will be available upon request.

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Inflammation, Hyperglycemia, and Adverse Outcomes in Individuals With Diabetes Mellitus Hospitalized for COVID-19

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OBJECTIVE

Diabetes mellitus (DM) is a major risk factor for severe coronavirus disease 2019 (COVID-19) for reasons that are unclear.

RESEARCH DESIGN AND METHODS

We leveraged the International Study of Inflammation in COVID-19 (ISIC), a multicenter observational study of 2,044 patients hospitalized with COVID-19, to characterize the impact of DM on in-hospital outcomes and assess the contribution of inflammation and hyperglycemia to the risk attributed to DM. We measured biomarkers of inflammation collected at hospital admission and collected glucose levels and insulin data throughout hospitalization. The primary outcome was the composite of in-hospital death, need for mechanical ventilation, and need for renal replacement therapy.

RESULTS

Among participants (mean age 60 years, 58.2% males), those with DM (*n* = 686, 33.5%) had a significantly higher cumulative incidence of the primary outcome (37.8% vs. 28.6%) and higher levels of inflammatory biomarkers than those without DM. Among biomarkers, DM was only associated with higher soluble urokinase plasminogen activator receptor (suPAR) levels in multivariable analysis. Adjusting for suPAR levels abrogated the association between DM and the primary outcome (adjusted odds ratio 1.23 [95% CI 0.78, 1.37]). In mediation analysis, we estimated the proportion of the effect of DM on the primary outcome mediated by suPAR at 84.2%. Hyperglycemia and higher insulin doses were independent predictors of the primary outcome, with effect sizes unaffected by adjusting for suPAR levels.

CONCLUSIONS

Our findings suggest that the association between DM and outcomes in COVID-19 is largely mediated by hyperinflammation as assessed by suPAR levels, while the impact of hyperglycemia is independent of inflammation.

As of October 2021, there have been >44 million confirmed cases and 700,000 deaths attributed to coronavirus disease 2019 (COVID-19) in the United States (1). The ongoing COVID-19 pandemic disproportionately affects individuals with diabetes

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mellitus (DM) (2). More than 40% of hospitalized individuals with COVID-19 have DM, which is a major risk factor for adverse outcomes in this patient population (2–7). The reasons underlying the susceptibility of individuals with DM to severe COVID-19 remain unclear.

DM is characterized by chronic low-grade inflammation (8), which promotes insulin resistance and hyperglycemia, processes important in the development of chronic complications (9). This chronic inflammatory state is thought to stimulate stronger immune and inflammatory responses in individuals with DM exposed to COVID-19 compared with those without DM, promoting cytokine release and hyperglycemic surges (2). Hyperglycemia further upregulates inflammatory and oxidative stress markers in a vicious cycle (10,11). The interplay between inflammatory cytokines and hyperglycemia may be a major factor in the development of multiorgan damage and mortality in individuals admitted for COVID-19 (12). Understanding the relationship among DM, inflammation, and hyperglycemia in individuals hospitalized for COVID-19 is instrumental in devising targeted strategies for improving outcomes in this high-risk patient population.

To that end, we leveraged the International Study of Inflammation in COVID-19 (ISIC), a large, multicenter, observational study of individuals admitted specifically for COVID-19 in whom inflammatory biomarkers were measured on admission. Our study objectives were to characterize the impact of DM on COVID-19–related outcomes in relation to inflammation, identify the determinants of risks in individuals with DM, and examine the interplay among inflammatory biomarkers, hyperglycemia, insulin use, and in-hospital outcomes.

RESEARCH DESIGN AND METHODS

ISIC

ISIC is a multicenter observational study with the primary objective of characterizing the role of inflammatory biomarkers in COVID-19–related adverse outcomes (13). Participating centers and site investigators are listed in the Supplementary Material. Institutional review board approvals and consent procedures were obtained separately at each site according to local institutional policies.

Study Design and Patients

Individuals were eligible if they met the following inclusion criteria: *1*) adult (≥18 years old) hospitalized specifically for COVID-19, *2*) confirmed severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection by RT-PCR testing of nasopharyngeal or oropharyngeal samples, and *3*) at least one blood sample collected during hospitalization. Individuals with a positive SARS-CoV-2 test who were hospitalized for non–COVID-19 reasons were excluded. All patients were monitored until hospital discharge or death. Extensive clinical data were collected through electronic health records using established data mining tools and reviewed for accuracy by at least two reviewers per site. All data were entered into the secure web-based repository REDCap.

Data collected were medical history, including DM type (type 1 or type 2); demographics; laboratory tests; medications; clinical characteristics; inpatient medical therapy; hospitalization course; and outcomes. DM was defined as a documented diagnosis in the medical record, treatment with hypoglycemic agents, or a hemoglobin A_{1c} (HbA_{1c}) ≥6.5% within 1 year before admission. Estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration equation.

The Michigan Medicine COVID-19 Cohort

The Michigan Medicine COVID-19 Cohort (M^2C^2) is the largest ISIC subcohort. The M^2C^2 comprises consecutive, systematically enrolled adults (≥18 years) with confirmed SARS-CoV-2 infection hospitalized specifically for COVID-19 at the University of Michigan from 1 February 2020 to 1 June 2021. In addition to the variables collected for ISIC, serial laboratory measurements, frequently monitored blood glucose levels, and daily insulin dose administered throughout hospitalization were collected for M^2C^2 as part of a standardized inpatient management protocol for hyperglycemia (14).

Biomarkers of Inflammation

Blood samples were collected and analyzed for several inflammatory biomarkers, including soluble urokinase plasminogen activator receptor (suPAR), interleukin-6 (IL-6), C-reactive protein (CRP), D-dimer, ferritin, lactate dehydrogenase, and procalcitonin levels within 48 h of admission. CRP, ferritin, D-dimer, lactate dehydrogenase, and procalcitonin levels were measured by the central laboratory at the respective institution of enrollment at the request of the clinical team. Residual samples were collected for suPAR and IL-6 measurement, which were measured in batches using a commercially available ELISA (suPARnostic ELISA [ViroGates, Birkerød, Denmark], Human IL-6 Quantikine QuicKit [R&D Systems, Minneapolis, MN]). Serum samples used for suPAR and IL-6 measurements were collected and kept frozen at −80°C until the time of measurement, which was no longer than 3 months. Samples underwent up to two thaw cycles. Both suPAR and IL-6 are highly stable in frozen samples stored for >5 years and are not affected by repeated freeze/thaw cycles. Technicians performing assays were blinded to clinical data.

Outcome Definitions

The primary outcome was the composite end point of in-hospital death, need for mechanical ventilation, and need for renal replacement therapy. Secondary

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**A complete list of the ISIC Study Group can be found in the supplementary material online.*

This article is part of a special article collection available at https://diabetesjournals.org/journals/collection/52/Diabetes-and-COVID-19.

outcomes included each individual component of the primary outcome.

Statistical Analysis

We included individuals hospitalized for COVID-19 during the period of 1 February 2020 to 1 June 2021, the date the database was locked (N = 2,044). We report clinical characteristics for the overall cohort stratified by DM history using categorical variables expressed as a number and percentage and continuous variables expressed as mean (SD) and median (25th–75th interquartile range) for normally and nonnormally distributed continuous data, respectively. The characteristics between individuals with and without DM were compared using χ^2 tests for categorical variables and unpaired t tests or Mann-Whitney U tests for normal and nonnormal continuous variables, respectively. The incidences of the individual outcomes (in-hospital death, need for mechanical ventilation, and need for renal replacement therapy) were compared between individuals with and without DM at admission using χ^2 tests.

DM and Biomarkers of Inflammation

To determine whether DM history was independently associated with higher thromboinflammation marker levels, separate linear regression models were created, with each biomarker as the dependent variable and DM, age, sex, BMI, race, hypertension, coronary artery disease, and heart failure as independent variables. Each model variable was standardized by subtracting the mean of each variable and dividing by its SD, resulting in a distribution with mean = 0 and SD = 1. Standardization was performed to compare the effect sizes for DM across each biomarker.

DM as a Risk Factor in Hospitalized Individuals With COVID-19

We examined the association between DM and the composite outcome using stepwise logistic regression models. Model 0 was unadjusted. Model 1 included age, sex, and race. Model 2 included the variables in model 1 as well as clinical characteristics, including BMI, history of hypertension, coronary artery disease, congestive heart failure, and admission eGFR. Model 3 included the variables in model 2 as well as suPAR, which was $\log_2$ transformed (interpreted as per 100% increase) given the nonnormal distribution. We repeated this analysis to explore the association between DM and each outcome individually. We then performed mediation analysis to assess whether the effect of DM on the composite outcome is mediated by suPAR, after adjusting for the clinical variables in model 3 (age, sex, race, BMI, and history of hypertension, coronary artery disease, congestive heart failure, and admission eGFR) (15).

In-Hospital Outcome Predictors in Individuals With DM and COVID-19

We used logistic regression to identify risk factors for the composite outcome among individuals with DM (n = 686). We first examined the association of each clinical characteristic and the composite outcome in univariable analysis. In addition to variables associated with the outcome in the univariable analysis, we included the following variables in the multivariable analysis on the basis of biologic plausibility and clinical knowledge: age, sex, race, BMI, smoking status, hypertension, coronary artery disease, heart failure, chronic kidney disease, admission eGFR, and glucose range at admission (<54–69, 70–180, 181–250, and >250 mg/dL). Biomarkers of inflammation ($\log_2$ transformed) were each examined in the multivariable risk model separately. We also explored risk factors for each individual outcome using the same multivariable risk model. Finally, we calculated the relative importance of clinical characteristics, biomarkers of inflammation, and glucose levels for predicting the composite outcome on the basis of the Gini index using a random forest approach (16).

Glucose, Insulin Use, and In-Hospital Outcomes

We used the M^2C^2 subset of ISIC (n = 1,608), in which serial data were collected, to determine whether glucose levels and insulin administered during hospitalization were associated with inflammatory biomarkers and outcomes. We assessed the following exposures: glucose level on admission, coefficient of variation for glucose during hospitalization, percentage of glucose measurements in target glucose range (70–180 mg/dL) during hospitalization per patient, percentage of glucose measurements >180 mg/dL during hospitalization per patient, and average daily amount of insulin administered during hospitalization per patient adjusted for body weight. The coefficient of variation is expressed as the SD divided by the mean of all glucose measurements during hospitalization. The average amount of insulin administered was calculated as the total insulin dose (units) divided by patient weight (kilograms) multiplied by the total number of in-hospital days. We used Spearman rank correlation to examine the correlation between each biomarker of inflammation with glucose coefficient of variation and the average insulin dose received during hospitalization. To assess the association between each exposure and the composite outcome, we used multivariable regression models. Each variable was modeled continuously and as a categorical variable using the following categories as references for each variable: 0 for glucose coefficient of variation, 100% for glucose values in the normal range, 0% for glucose values in the high range, and 0 units/kg/day for average insulin dose. For glucose variables, the coefficients are expressed as a 10-unit difference, whereas insulin dose is expressed as a difference in 0.1 unit/kg/day. Models were adjusted for age, sex, race, BMI, and history of hypertension, coronary artery disease, and congestive heart failure. Separate models were additionally adjusted for suPAR within 48 h of admission and corticosteroid use.

We performed a complete case analysis for multivariable models. There were no missing data for any demographic or clinical characteristic. A two-sided $P < 0.05$ was used to determine statistical significance. All analyses were performed using R 4.1.0 statistical software (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Study Cohort Characteristics

The overall cohort had a mean (SD) age of 60 (16) years, 42% were female, and 20.5% were Black. One-third of the cohort (n = 686, 33.6%) met the criteria for DM, of whom 98.5% had type 2 DM and 1.5% had type 1 DM. Compared with individuals without DM, those with DM were older (mean age 64 vs. 58 years) and were more likely to be Black

(27.6% vs. 16.9%), to be obese (mean BMI 33 vs. 31 kg/m^2), and to have a greater comorbidity burden, including hypertension (81.2% vs. 45.7%), coronary artery disease (24.6% vs. 8.9%), heart failure (17.2% vs. 7.7%), and chronic kidney disease (26.8% vs. 10.8%; $P < 0.001$ for all) (Table 1). On hospital admission, individuals with DM were less likely to present with fever (59.6% vs. 65.8%) but more likely to present with altered mental status (13.4% vs. 6.5%) compared with those without DM ($P < 0.001$).

DM and Biomarkers of Inflammation in Individuals With COVID-19

In unadjusted analyses, the levels of several inflammatory biomarkers, including suPAR, CRP, procalcitonin, and D-dimer, were higher on admission in individuals with DM than in those without DM (Table 1). In multivariable analyses, only suPAR levels were indepen-dently associated with DM (standardized β = 0.10 [95% CI 0.05, 0.15]) (Supplementary Table 1). On average, participants with DM had 20.7% higher suPAR levels than those without DM.

Associations Between DM and In-Hospital Outcomes

Overall, the primary composite outcome was observed in 647 (31.7%) individuals. There was a total of 288 (14.1%) in-hospital deaths, 550 (26.9%) individuals who required mechanical ventilation, and 182 (8.9%) individuals who required renal replacement therapy. In unadjusted analyses, individuals with DM had a significantly higher cumulative incidence of the primary composite outcome (37.8% vs. 28.8%; $P < 0.001$) as well as the individual components of in-hospital death (16.9% vs. 12.7%; $P = 0.01$), need for mechanical ventilation (31.6% vs. 24.5%; $P = 0.001$), and need for renal replacement therapy (12.4% vs. 7.1%; $P < 0.001$) compared with those without DM (Table 1). In multivariable analyses, adjusting for demographics (model 1) and clinical characteristics (model 2) heavily attenuated the association between DM and the primary outcome (adjusted odds ratio [aOR] 1.23 [95% CI 1.00, 1.52]), which became nonsignificant after including suPAR in the model (aOR 1.03 [95% CI 0.78, 1.37]) (Fig. 1). When these outcomes were examined individually, a similar pattern was seen (Fig. 1).

In mediation analysis, the average causal mediation effect (also known as indirect effect) of DM on the primary outcome through suPAR was significant ($P = 0.008$), while the average direct effect of DM on the primary outcome was not significant ($P = 0.73$). The proportion of the effect of DM on the primary outcome mediated by suPAR was 84.2%.

Predictors of the Composite Outcome in Individuals With DM and COVID-19

When examining predictors of the composite outcome in the subgroup of individuals with DM ($n = 686$), we found that higher BMI (aOR 1.18 [95% CI 1.06, 1.31]), lower eGFR (aOR 1.07 [95% CI 1.03, 1.10]), and admission glucose levels >180 mg/dL (aOR 1.85 [95% CI 1.20, 2.83]) were associated with the primary composite outcome (Supplementary Table 2). We found similar associations when examining outcomes individually, with a few notable exceptions (Supplementary Table 3). Older age was strongly associated with in-hospital death (aOR 1.44 [95% CI 1.17, 1.77]), and male sex was associated with the need for renal replacement therapy (aOR 2.33 [95% CI 1.31, 4.12]). Type 1 DM, prior insulin use, and medications for hyperglycemia were not associated with an increased odds in the primary outcome. Levels of all inflammatory biomarkers were associated with an increased odds of the primary outcome when examined separately in a multivariable model adjusted for demographic and clinical risk factors (Supplementary Table 2).

We identified suPAR level as the most important variable associated with the primary outcome in individuals with DM and COVID-19, followed by BMI, admission glucose, and age in descending order of importance (Fig. 2). Individuals with DM with a suPAR level <5.94 ng/mL (first quartile) had a 23.9% incidence of the primary outcome compared with 53.8% in individuals with suPAR ≥14.8 ng/mL (fourth quartile).

Glucose, Insulin, and Outcomes in Individuals With COVID-19

Among the M^{2}C^2 subset with longitudinal serial glucose and insulin data, we found only modest correlations between biomarkers of inflammation and both glucose coefficient of variation (r = 0.05–0.02) and average insulin dose (r = 0.09–0.02) during hospitalization (Supplementary Table 4). We also examined whether glucose ranges, glucose variation, and insulin requirements were associated with the primary outcome. The glucose coefficient of variation in individuals with DM was 17.0%, with an average of 53.9% of glucose measurements falling in the target range (70–180 mg/dL) and 44.8% of glucose values >180 mg/dL. The glucose coefficient of variation, a greater percentage of glucose values outside the target range, a greater percentage of high glucose values, and a higher required insulin dose were all associated with a greater odds of the primary outcome in individuals with DM (Fig. 3 and Supplementary Fig. 1). Per each 10% higher glucose coefficient of variation, the odds of the primary outcome was 1.30 (95% CI 1.11, 1.54]) (Supplementary Fig. 1). Per every 0.1 unit/kg/day of insulin administered, the odds of the primary outcome was 1.18 (95% CI 1.11, 1.25). Including suPAR or corticosteroid use in the models did not affect estimates significantly (Supplementary Table 5).

CONCLUSIONS

In this in-depth examination of the interplay among DM, inflammation, hyperglycemia, and outcomes in individuals hospitalized for COVID-19, we found that the impact of DM on outcomes is tightly linked to heightened inflammation. First, individuals with DM had a greater incidence of in-hospital outcomes and higher levels of inflammatory markers (notably suPAR) compared with those without DM. The association between DM and outcomes was abrogated, however, by including suPAR in the model, with mediation analysis suggesting that the effect of DM on outcomes is largely mediated by suPAR. Among individuals with DM, suPAR, BMI, admission glucose levels, and age were the most important risk factors (in that order). The correlation between inflammatory markers and hyperglycemia was modest at best, while hyperglycemia and higher insulin requirements during hospitalization were associated with worse outcomes. This association was

Table 1—Demographic and clinical characteristics by DM status

Variable	Overall cohort (*N* = 2,044)	Without DM (*n* = 1,358)	With DM* (*n* = 686)	*P*
Age (years), mean (SD)	60 (16)	58 (17)	64 (14)	<0.001
Male sex, *n* (%)	1,191 (58.2)	783 (57.7)	408 (59.5)	0.46
BMI (kg/m^2), mean (SD)	32 (9)	31 (9)	33 (9)	<0.001
Black race, *n* (%)	419 (20.5)	230 (16.9)	189 (27.6)	<0.001
History of tobacco use, *n* (%)	886 (43.3)	566 (41.7)	320 (46.6)	0.08
Comorbidities, *n* (%)				
Hypertension	1,177 (57.6)	620 (45.7)	557 (81.2)	<0.001
Coronary artery disease	290 (14.2)	121 (8.9)	169 (24.6)	<0.001
Congestive heart failure	223 (10.9)	105 (7.7)	118 (17.2)	<0.001
Chronic kidney disease	330 (16.1)	146 (10.8)	184 (26.8)	<0.001
End-stage renal disease on dialysis	56 (2.7)	17 (1.3)	39 (5.7)	<0.001
COPD	208 (10.2)	129 (9.5)	79 (11.5)	0.18
Asthma	288 (14.1)	201 (14.8)	87 (12.7)	0.22
Liver disease	61 (3.0)	34 (2.5)	27 (3.9)	0.10
Active malignancy	101 (4.9)	80 (5.9)	21 (3.1)	0.01
Admission eGFR (mL/min/1.73 m^2), mean (SD)	71 (32)	78 (30)	56 (31)	<0.001
Presenting symptoms, *n* (%)				
Fever	1,283 (62.8)	893 (65.8)	390 (56.9)	<0.001
Shortness of breath	1,466 (71.7)	976 (71.9)	490 (71.4)	0.88
Diarrhea	553 (27.1)	380 (28.0)	173 (25.2)	0.20
Altered mental status	180 (8.8)	88 (6.5)	92 (13.4)	<0.001
Laboratory data,† mean (SD)				
Hemoglobin (g/dL)	12.9 (2.4)	13.1 (2.5)	12.5 (2.3)	<0.001
White blood cell count (×10^3/μL)	7.4 (4.6)	7.2 (4.6)	7.8 (4.5)	0.010
Absolute neutrophil count (×10^3/μL)	5.6 (3.5)	5.5 (3.5)	5.9 (3.4)	0.006
Absolute lymphocyte count (×10^3/μL)	1.1 (2.4)	1.1 (2.5)	1.1 (2.3)	0.92
AST (IU/L)	63.8 (186)	66.4 (223.9)	58.7 (65.8)	0.40
ALT (IU/L)	51.6 (244.5)	55.3 (297.0)	44.2 (56)	0.34
Total bilirubin (mg/dL)	0.72 (1.09)	0.73 (1.24)	0.71 (0.72)	0.61
Glucose (mg/dL)	144 (84)	117 (37)	195 (118)	<0.001
HbA_{1c}†† (%)	7.0 (2.4)	5.9 (1.3)	8.0 (2.7)	<0.001
Glucose range at admission (mg/dL), *n* (%)				<0.001
<54	5 (0.2)	3 (0.2)	2 (0.3)	
54–69	12 (0.6)	8 (0.6)	4 (0.6)	
70–180	1,504 (73.6)	1,137 (83.7)	367 (53.5)	
181–250	171 (8.4)	34 (2.5)	137 (20.0)	
>250	136 (6.7)	13 (1.0)	123 (17.9)	
Inflammatory markers, median (IQR)				
SuPAR (ng/mL)	7.12 (5.24–10.54)	6.61 (4.99–9.54)	8.64 (5.97–12.11)	<0.001
CRP (mg/dL)	8.1 (4.2–15.4)	7.3 (3.8–14.5)	9.3 (5.2–17.2)	<0.001
Lactate dehydrogenase (IU/L)	373 (279–510)	373 (275–508)	375 (283–518)	0.76
IL-6 (pg/mL)	18.4 (12.5–96.5)	14.0 (12.5–94.8)	24.7 (12.5–99.3)	0.15
Procalcitonin (ng/mL)	0.17 (0.09–0.44)	0.15 (0.08–0.34)	0.23 (0.11–0.74)	<0.001
Ferritin (ng/mL)	680 (289–1,368)	670 (282.8–1,353.0)	694.5 (298–1,739)	0.52
D-dimer (FEU mg/L)	0.94 (0.54–1.92)	0.89 (0.52–1.77)	1.08 (0.59–2.2)	0.001
Outcomes, *n* (%)				
Composite outcome	647 (31.7)	388 (28.6)	259 (37.8)	<0.001
Need for mechanical ventilation	550 (26.9)	333 (24.5)	217 (31.6)	0.001
Need for renal replacement therapy	182 (8.9)	97 (7.1)	85 (12.4)	<0.001
In-hospital death	288 (14.1)	172 (12.7)	116 (16.9)	0.011

COPD, chronic obstructive pulmonary disease; FEU, fibrinogen-equivalent units; IQR, interquartile range. *Includes 30 (1.5%) individuals with type 1 DM and 275 (40.1%) individuals who required insulin. †First value within 48 h of presentation. ††HbA_{1c} measured within 1 year of hospital admission was available in 694 individuals (309 without DM and 385 with DM).

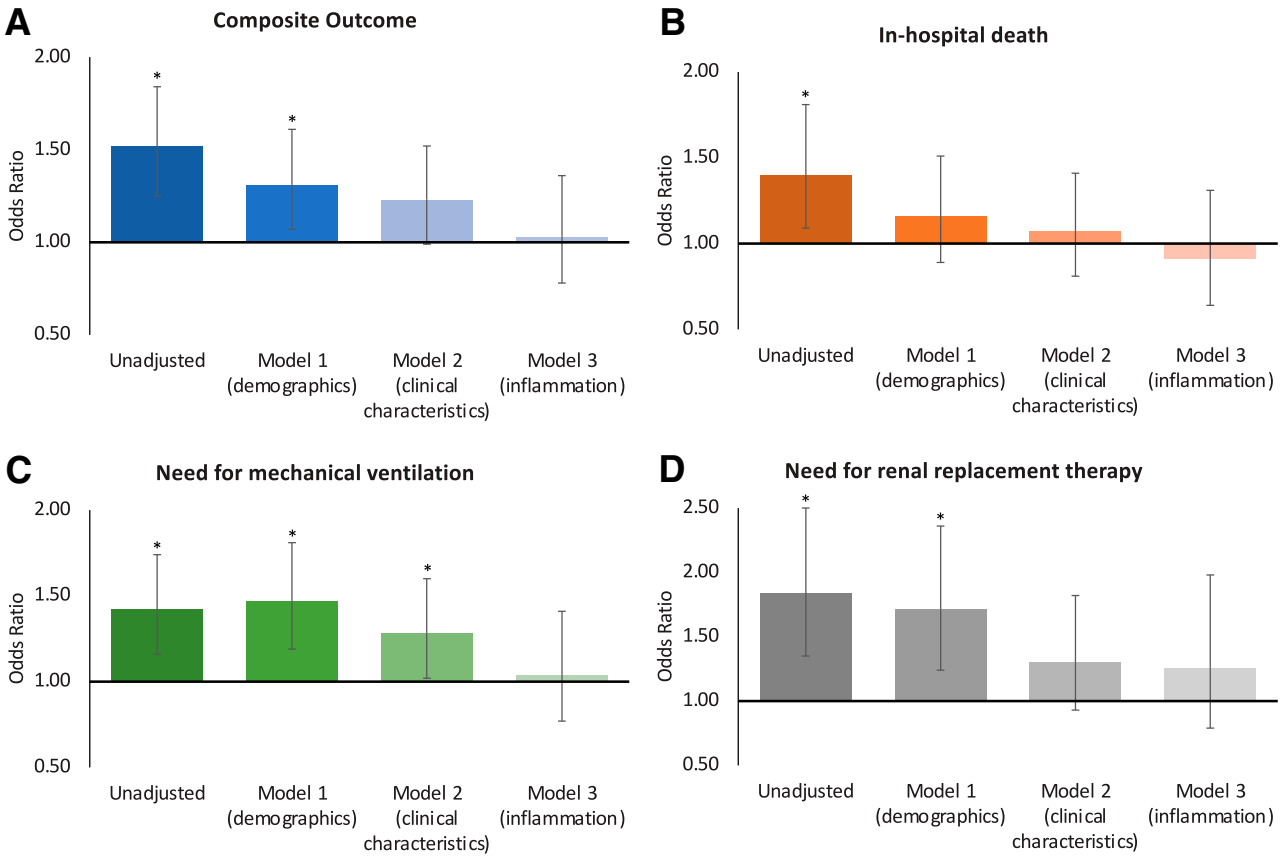

Figure 1—Risk of in-hospital outcomes in individuals with COVID-19 and with and without DM. The bar graphs depict the ORs comparing individuals with DM with individuals without DM (reference) and 95% CIs for the composite outcome (*A*) and the individual outcomes of in-hospital death (*B*), need for mechanical ventilation (*C*), and need for dialysis or continuous renal replacement therapy (*D*). Four different models were used: model 0 (unadjusted); model 1 (demographics) adjusted for age, sex, and race; model 2 (clinical characteristics) additionally adjusted for BMI and history of hypertension, coronary artery disease, and congestive heart failure (clinical characteristics); and model 3 (inflammation) further adjusted for suPAR level. *$P < 0.05$.

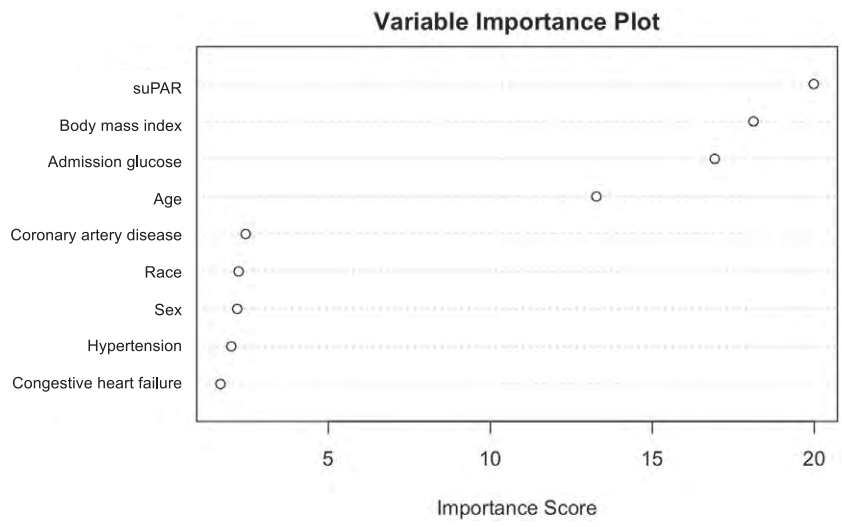

Figure 2—Variable importance plot to predict composite outcome in individuals with DM and COVID-19. The variable importance plot is based on the Gini index using a random forest approach. Shown are data from model 3 (adjusted for age, sex, race, BMI, admission suPAR, and history of preexisting coronary artery disease, hypertension, and heart failure) for predicting the composite outcome of in-hospital death, need for mechanical ventilation, and need for renal replacement therapy.

not attenuated after adjusting for suPAR, implying that hyperglycemia affects COVID-19–related outcomes through noninflammatory processes.

DM is a well-established risk factor for COVID-19 (2,17); however, the underlying mechanisms are unclear. In susceptible individuals, SARS-CoV-2 infection is thought to trigger a prolonged hyperinflammatory response, dubbed the cytokine storm (4,18–22). DM, as a chronic inflammatory condition, may predispose individuals to a heightened inflammatory response (23,24). Mitochondrial disruption, rather than changes to glucose metabolism, has been found to lead to altered T-cell cytokine production (notably by T-helper 17 cells) in type 2 DM (23). Consistently, we found that individuals with DM had higher levels of inflammatory biomarkers, including suPAR, CRP, procalcitonin, and D-dimer. After adjusting for comorbidities, we noted a singular

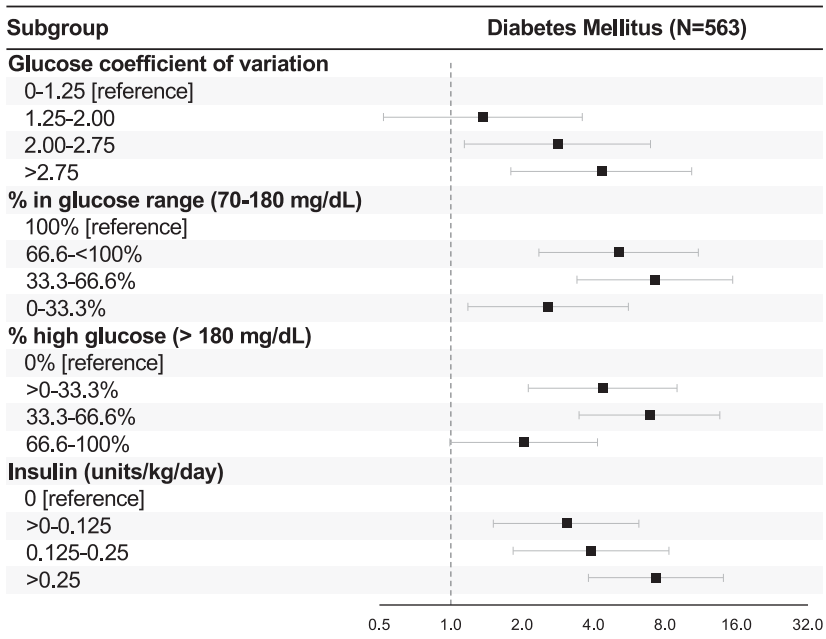

Figure 3—Associations among glucose, insulin, and combined outcome in individuals with DM in the M^2C^2 subset. The forest plot depicts the ORs and 95% CIs for the association among glucose, insulin, and the composite outcome of in-hospital death, need for mechanical ventilation, and need for renal replacement therapy stratified by DM among individuals with COVID-19 in the M^2C^2 subset (*n* = 1,608). All ORs are compared using the following reference categories for each variable: 0–1.25 for glucose coefficient of variance, 100% for glucose in range, 0% for high glucose, and 0 units/kg/day for insulin. The glucose coefficient of variation is calculated as the SD divided by the mean of all glucose measurements taken during hospitalization and then multiplied by 10. Percent in glucose range and high glucose are expressed as the percentage of all glucose measurements within each category during hospitalization. Insulin is calculated as the total amount of insulin (units) received during hospitalization divided by the patient's weight (kg) multiplied by the number of days in the hospital. Models were adjusted for age, sex, race, BMI, and history of hypertension, coronary artery disease, and congestive heart failure.

association between DM and suPAR, suggesting that suPAR represents the inflammatory biomarker most reflective of the hyperinflammatory state in DM and COVID-19. Our mediation analysis supports this finding in that we found that suPAR levels accounted for 84.2% of the effect of DM on the outcomes. Conversely, another study found that CRP accounted for only 32.7% (12).

SuPAR is an immune-derived signaling glycoprotein, which is notorious for its role in kidney disease (25–27), cardiovascular disease (28–30), and most recently, COVID-19 (13,31). Blood suPAR levels are notably high in individuals with type 1 or type 2 DM, even in the nonacute setting, and are strongly predictive of DM-related outcomes, such as nephropathy and atherosclerotic events (28,32,33). Several studies have identified a correlation between T-helper 17 cells and suPAR levels (34,35), which may explain the predilection for individuals with DM to have higher suPAR levels (23,36). SuPAR differs from other biomarkers of inflammation in that it is not an acute-phase reactant: Levels remain stable in highly proinflammatory situations, such as acute myocardial infarction or cardiac surgery (27). An increased suPAR level, however, is triggered by specific stimuli, such as smoking and RNA viruses (e.g., SARS-CoV-2), and is highly expressed in lung tissue (37). Accordingly, individuals with DM and COVID-19 have four- to eightfold higher suPAR levels (median 8.82 ng/mL) than healthy individuals (median 2.40 ng/mL). We found that suPAR was the most important predictor of outcomes in individuals with DM, which mediated at least 80% of the effect of DM on outcomes. Overall, these findings suggest that suPAR levels may reflect more specifically the burden of inflammation in COVID-19 compared with other biomarkers.

Hyperglycemia has traditionally been thought to be a major driver of inflammation through several mechanisms, including increased oxidative stress (8). In our study, hyperglycemia and higher insulin requirements are independently associated with in-hospital outcomes in individuals with DM and COVID-19, consistent with earlier studies (2,38). Surprisingly, we found only a weak correlation between suPAR or other inflammatory biomarkers with hyperglycemia, and the association between hyperglycemia and outcomes was not mitigated by adjusting for suPAR. The association between hyperglycemia and COVID-19–related outcomes likely occurs through mechanisms not reflected by inflammatory biomarkers. This is consistent with a study showing that nonmitochondrial glycolysis did not affect the inflammatory signature in type 2 DM (39). Whether aggressive glucose control would improve COVID-19–related outcomes remains to be shown in a clinical trial setting (14).

This study has several important strengths. It is the largest study to investigate the role of inflammatory biomarkers in individuals with DM hospitalized for COVID-19. In addition, in contrast with other studies, it includes a diverse cohort of individuals specifically hospitalized for COVID-19 rather than defined by SARS-CoV-2 positivity alone. Blood samples were collected on admission, without being confounded by anti-inflammatory therapies, and thus, reflect more accurately the inflammatory state. The clinical data were collected through careful and adjudicated review of individual medical records rather than through administrative data sets. The study benefited from standardized glucose and insulin data collected continuously throughout the hospitalization through the Michigan Medicine hyperglycemia management protocol.

This study also had some limitations. Given the small number of patients with type 1 DM in this cohort, the findings cannot be extended to these individuals. The diagnosis of DM was based on medical chart review and available HbA_{1c} levels at the time of admission; thus, it is possible that some individuals classified as not having DM could have had undiagnosed DM. Finally, mechanistic studies are warranted to validate the

inferences based on the epidemiologic observations noted in our study.

In summary, these data show that COVID-19–related in-hospital outcomes in individuals with DM are driven by a hyperinflammatory state reflected best by suPAR levels. SuPAR levels were the most important predictor of outcomes in individuals with DM, followed by obesity, hyperglycemia, and age. Hyperglycemia and higher insulin requirements correlated weakly with inflammatory biomarkers and were associated with outcomes independently of suPAR, suggesting that they likely impact outcomes through other mechanisms. Further study is needed to determine whether suPAR and hyperglycemia are therapeutic targets for the management of COVID-19 in individuals with DM.

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Data Sharing. Study protocol, statistical code, and data set summary data are available upon request after publication through a collaborative process. Data sets can be accessed upon approval of a submitted research proposal. Please contact penegonz@med.umich.edu for additional information.

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Neuropsychological Outcomes in Individuals With Type 1 and Type 2 Diabetes

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Objective: To determine the prevalence of neuropsychological outcomes in individuals with type 1 diabetes compared to individuals with type 2 diabetes or without diabetes, and to evaluate the association of diabetes status and microvascular/macrovascular complications with neuropsychological outcomes.

Patients and Methods: We used a nationally representative healthcare claims database of privately insured individuals (1/1/2001-12/31/2018) to identify individuals with type 1 diabetes. Propensity score matching was used as a quasi-randomization technique to match type 1 diabetes individuals to type 2 diabetes individuals and controls. Diabetes status, microvascular/macrovascular complications (retinopathy, neuropathy, nephropathy, stroke, myocardial infarction, peripheral vascular disease, amputations), and neuropsychological outcomes (mental health, cognitive, chronic pain, addiction, sleep disorders) were defined using ICD-9/10 codes. Logistic regression determined associations between diabetes status, microvascular/macrovascular complications, and neuropsychological outcomes.

Results: We identified 184,765 type 1 diabetes individuals matched to 524,602 type 2 diabetes individuals and 522,768 controls. With the exception of cognitive disorders, type 2 diabetes individuals had the highest prevalence of neuropsychological outcomes, followed by type 1 diabetes, and controls. After adjusting for the presence of microvascular/macrovascular complications, type 1 diabetes was not significantly associated with a higher risk of neuropsychological outcomes; however, type 2 diabetes remained associated with mental health, cognitive, and sleep disorders. The presence of microvascular/macrovascular complications was independently associated with each neuropsychological outcome regardless of diabetes status.

Conclusion: Microvascular/macrovascular complications are associated with a high risk of neuropsychological outcomes regardless of diabetes status. Therefore, preventing microvascular and macrovascular complications will likely help reduce the likelihood of neuropsychological outcomes either as the result of similar pathophysiologic processes or by preventing the direct and indirect consequences of these complications. For individuals with type 2 diabetes, risk factors beyond complications (such as obesity) likely contribute to neuropsychological outcomes.

Keywords: diabetes mellitus, regress analysis, big data and analytics, mental health, diabetes - quality of life

INTRODUCTION

Individuals with type 1 diabetes are at an increased risk for a number of microvascular and macrovascular complications, including retinopathy, neuropathy, nephropathy, stroke, myocardial infarction, peripheral vascular disease, and amputations (1). These complications result in substantial mortality, morbidity, and reduced quality of life (2). In contrast to these microvascular and macrovascular complications of diabetes, much less is known about the neuropsychological outcomes of type 1 diabetes including mental health, cognitive, chronic pain, addiction, and sleep disorders.

The current literature supports a higher burden of most neuropsychological outcomes in individuals with type 1 diabetes relative to the general population. The most well studied neuropsychological outcomes of type 1 diabetes are mental health disorders, specifically depression and anxiety. A systematic review found that the prevalence of depression and anxiety for individuals with type 1 diabetes is nearly three times that of the general population (3). Similarly, a systematic review found that individuals with type 1 diabetes had impaired cognitive function across broad categories including visual-spatial ability and memory (4). Multiple studies have also documented elevated rates of pain in adults and adolescents with type 1 diabetes (5, 6). In contrast to other neuropsychological outcomes, a systematic review found that there were similar rates of substance use between young adults with and without type 1 diabetes (7). Lastly, a meta-analysis found that children with type 1 diabetes get less sleep, adults with type 1 diabetes have lower quality sleep, and that type 1 diabetes is associated with higher rates of obstructive sleep apnea compared to the general population (8). However, these studies have four key limitations. Namely, their sample sizes were relatively small, they rarely investigated the role of microvascular and macrovascular complications, they were often focused on young individuals, and/or they lacked a control group (3–8). Furthermore, no previous study comprehensively evaluated the full spectrum of neuropsychological outcomes and few compared these complications for individuals with type 1 *vs.* type 2 diabetes. Our study fills these gaps in the literature using a large, nationally representative sample of privately insured individuals in the US.

Our objective was to describe and compare the prevalence of neuropsychological outcomes for individuals with type 1 diabetes, type 2 diabetes, and individuals without diabetes and to explore the independent effects of diabetes status and microvascular/macrovascular complications on these neuropsychological outcomes.

Abbreviations: ICD-9/ICD-10, International Classification of Diseases, 9^{th,} and 10th editions respectively.

METHODS

Population

We utilized the de-identified Optum Analytics database, which consists of detailed medical and pharmaceutical claims on tens of millions of insured individuals from 2001-2018. As the largest claims data repository in the United States, the demographic makeup of the Optum Analytics database closely matches those of the privately insured population. Using a validated ICD-9/ICD-10 code definition (9), we identified individuals with type 1 diabetes (250.x1, 250.x3, E10.xx) and type 2 diabetes (250.x0, 250.x2, E11.xx). For individuals with both type 1 and type 2 diabetes diagnosis codes, greater than 50% of one type of code determined individual diabetes type (9); this definition has sensitivity of 63% and positive predictive value of 94% for identifying individuals with type 1 diabetes, and a sensitivity of 100% and positive predictive value of 90% for identifying individuals with type 2 diabetes. The population was restricted to the first enrollment period for individuals with complete demographic and socioeconomic information.

Neuropsychological Outcomes

Neuropsychological outcomes were defined by aggregating across diagnoses specific to mental health, cognitive, chronic pain, addiction, and sleep disorders. These conditions were defined using ICD-9/ICD-10 codes (**Supplemental Table S1**) from the period of follow-up after diabetes diagnosis, or an analogous portion of follow-up in individuals without diabetes. Specifically, mental health disorders were determined as whether individuals had a diagnosis of anxiety, Attention Deficit Hyperactivity Disorder (ADHD), adjustment disorder, eating disorder, depression, Post-Traumatic Stress Disorder (PTSD), or other behavioral and emotional disorders (10–12). Cognitive disorders were determined as whether individuals had a diagnosis of dementia, mild cognitive impairment, Alzheimer's, or vascular dementia (13–15). Addiction disorders were determined as whether individuals had a diagnosis indicating dependence on alcohol, opioids, cocaine, sedatives, hallucinogens, nicotine, inhalant, other stimulants, and, other psychoactive and non-psychoactive chemicals (16). Chronic pain

disorders were determined as whether individuals had a diagnosis of chronic pain based on a previously validated definition that included postherpetic neuralgia, trigeminal neuralgia, HIV-associated pain, stroke-associated pain, chronic pain syndrome, lumbar radiculopathy, complex regional pain syndrome, spinal cord injury, surgically-induced pain, phantom limb, cervical radiculopathy, multiple sclerosis-associated pain, fibromyalgia, osteoarthritis, low back pain, migraine, rheumatoid arthritis, ankylosing spondylitis, psoriatic arthropathy, cancer pain, irritable bowel syndrome, painful bladder syndrome, and interstitial cystitis (17). In addition to the above conditions, chronic headache, chronic fatigue syndrome, temporomandibular joint disorder, and chronic pelvic pain (endometriosis or vulvodynia) were included as chronic pain conditions. Sleep disorders were determined as whether individuals had a diagnosis of insomnia, hypersomnia, sleep apnea, circadian rhythm disorders, or other sleep disorders (18).

Microvascular and Macrovascular Complications of Diabetes

We used ICD-9/ICD-10 codes to determine if individuals had microvascular or macrovascular complications of diabetes during the period of follow-up after diabetes diagnosis, or an analogous portion of total follow-up in individuals without diabetes (**Supplemental Table S1**). Microvascular complications included retinopathy, neuropathy and nephropathy (19, 20). Macrovascular complications included stroke, myocardial infarction, and peripheral vascular disease (14, 20, 21). Amputation was also included as a complication, but was not included as a microvascular or macrovascular complication since it results from both mechanisms.

Matching

Individuals with type 1 diabetes were matched to individuals with type 2 diabetes and non-diabetic controls stratified by age (0-20, 20-40, 40-60, 60+) using propensity scores within a caliper of 0.10 for individuals age 0-20 and within 0.01 for individuals age 20+ (22). Propensity scores were calculated based on individual age at study entry, sex, race/ethnicity, geographic region, education level, net worth, insurance plan type, high deductible health plan status, modified Charlson Comorbidity Index, starting year of enrollment, and length of follow-up. The modified version of the Charlson Comorbidity Index consisted of conditions that did not overlap with study outcomes. Specifically, the modified Charlson Comorbidity Index included congestive heart failure, chronic pulmonary disease, connective tissue disease, peptic ulcer disease, mild liver disease, paraplegia and hemiplegia, renal disease excluding diabetic nephropathy, cancer, liver disease, metastatic carcinoma, and HIV (23). Individuals with diabetes were also matched based on length of pre-diagnosis and post-diagnosis enrollment.

Based on the availability of well-matched controls without diabetes, each individual age 0-40 with type 1 diabetes was matched to 1 individual with type 2 diabetes and then independently matched with 1 non-diabetic control. For those age>40, each individual with type 1 diabetes was matched to 4 individuals with type 2 diabetes and then independently matched with 4 non-diabetic controls.

Statistical Analysis

Descriptive statistics were used to characterize the matched individuals, stratified by age. We used a Cochrane-Mantel-Haenszel test to compare the prevalence of each neuropsychological outcome and each microvascular/macrovascular complication stratified by diabetes type and age. Multivariable logistic regression was used to assess the association between diabetes status, microvascular and macrovascular complications, and each neuropsychological outcome (mental health, cognitive, chronic pain, addiction, and sleep disorders). Specifically, for each of the 5 outcomes, we fit a model as a function of diabetes status (type 1 diabetes *vs.* type 2 diabetes *vs.* non-diabetic controls) and presence of any microvascular or macrovascular complications, independent of diabetes status, stratified by age. Wald Tests were used to determine statistical significance of the effects of diabetes status and presence of microvascular or macrovascular complications on neuropsychological outcomes. Since very few individuals between the ages of 0-40 had cognitive disorders, we did not fit logistic regression models for those age strata.

To investigate the effects of distinct complications on each neuropsychological outcome, we fit additional models, first separating complications into microvascular, macrovascular, and amputations, and then another model including each specific complication as an individual covariate (retinopathy, neuropathy, nephropathy, stroke, myocardial infarction, peripheral vascular disease, and amputations).

For all hypothesis testing, statistical significance was determined using a P-value threshold of 0.05.

All analyses were performed using SAS version 9.4 (Cary, NC, USA).

This study was considered exempt by the Institutional Review Board of the University of Michigan.

RESULTS

Demographic and Socioeconomic Information of Matched Individuals

We identified 16,179 individuals aged 0-20 and 55,293 individuals aged 20-40 with type 1 diabetes that were each matched to 1 individual with type 2 diabetes and 1 individual without diabetes. Similarly, we identified 63,777 individuals aged 40-60 and 49,516 individuals age 60+ with type 1 diabetes that were each matched to 4 individuals with type 2 diabetes and 4 individuals without diabetes.

Descriptive statistics of the matched individuals' demographic, socioeconomic, and insurance plan information are presented in **Table 1**. Within age strata, individuals were closely matched in all characteristics. In individuals with type 1 diabetes, the mean follow-up length after diabetes diagnosis was 2.41 years (SD 2.94) for individuals ages 0-20, 1.56 years (SD 2.13) for individuals ages 20-40, 2.07 years (SD 2.67) for individuals ages 40-60, and 2.48 years (SD 3.04) for individuals ages 60+. In individuals with type 2 diabetes, the mean follow-up length after diabetes diagnosis was 2.42 years (SD 2.60) for individuals ages 0-20, 1.59 years (SD 1.93) for individuals ages 20-40, 2.07 years (SD 2.67) for individuals ages

TABLE 1 | Demographics of matched cohort, stratified by age and diabetes type.

Variable		age 0-20 (N=48,537)			age 20-40 (N=165,877)		
		Type 1 Diabetes (n=16,179)	Type 2 Diabetes (n=16,179)	No Diabetes (n=16,179)	Type 1 Diabetes (n=55,293)	Type 2 Diabetes (n=55,293)	No Diabetes (n=55,291)
Age	Mean years (SD)	14.1 (4.52)	14. (5.45)	14.1 (4.95)	31.0 (5.60)	30. (5.63)	30.9 (5.61)
Gender (%)	Female	52.0%	52.2%	51.9%	49.4%	49.8%	49.0%
	Male	48.0%	47.8%	48.1%	50.6%	50.2%	51.0%
Race (%)	Asian	3.2%	3.0%	3.4%	2.8%	3.0%	2.9%
	Black	10.7%	10.3%	10.7%	10.1%	10.0%	10.2%
	Hispanic	12.6%	12.6%	12.4%	10.2%	10.5%	10.8%
	White	73.5%	74.1%	73.5%	76.9%	76.5%	76.1%
State (%)	IL, IN, MI, OH, WI	16.2%	16.6%	16.2%	16.1%	15.9%	15.3%
	AL, KY, MS, TN	4.8%	4.7%	5.0%	5.6%	5.6%	5.6%
	NJ, NY, PA	9.4%	9.4%	9.4%	8.7%	9.2%	9.1%
	AZ, CO, ID, MT, NV, NM, UT, WY	7.2%	7.2%	7.2%	8.7%	8.6%	9.0%
	CT, ME, MA, NH, RI, VT	3.3%	3.4%	3.2%	2.8%	2.9%	3.1%
	AK, CA, HI, OR, WA	8.8%	8.6%	8.6%	8.2%	8.2%	8.6%
	DE, DC, FL, GA, MD, NC, SC, VA, WV	26.8%	26.7%	27.5%	25.2%	25.1%	25.0%
	IA, KS, MN, MO, NE, ND, SD	7.9%	7.8%	7.4%	10.5%	10.7%	10.3%
	AR, LA, OK, TX	15.5%	15.4%	15.3%	14.1%	13.8%	13.9%
Education Level (%)	Less than 12th Grade	0.6%	0.6%	0.7%	0.7%	0.8%	0.7%
	High School Diploma	29.9%	30.1%	30.2%	27.8%	27.6%	27.4%
	Less than bachelor's degree	52.5%	52.3%	51.9%	53.7%	53.2%	53.2%
	Bachelor's degree Plus	17.0%	17.0%	17.2%	17.8%	18.4%	18.7%
Net Worth (%)	<$25K	27.4%	27.2%	26.6%	33.2%	32.6%	32.8%
	$25K-$149K	24.1%	24.3%	24.1%	27.2%	27.0%	26.7%
	$150K-$249K	12.0%	11.8%	12.2%	11.7%	11.7%	11.8%
	$250K-$499K	16.9%	17.1%	17.0%	14.5%	14.7%	15.1%
	$500K+	19.5%	19.6%	20.1%	13.5%	14.1%	13.6%
Insurance Provider (%)	Exclusive Provider Organization	13.6%	13.7%	13.6%	12.2%	12.1%	12.6%
	Health Maintenance Organization	18.5%	18.1%	18.2%	20.3%	20.3%	19.1%
	Indemnity	0.0%	0.0%	0.0%	0.1%	0.1%	0.1%
	Other	0.1%	0.1%	0.1%	0.8%	0.9%	0.8%
	Point of Service	60.8%	61.1%	61.2%	58.1%	58.0%	58.8%
	Preferred Provider Organization	6.9%	7.0%	6.8%	8.5%	8.7%	8.6%
Customer Driven Health Plan Type (%)	Health Reimbursement Arrangement	6.0%	5.9%	6.1%	4.8%	4.9%	5.0%
	Health Savings Account	9.3%	9.4%	9.2%	7.5%	7.4%	8.0%
Micro/Macrovascular Complication	Any Complication (%)	7.8%	4.4%	0.6%	22.2%	8.9%	1.6%
	Retinopathy (%)	3.3%	0.5%	0.0%	12.9%	1.8%	0.0%
	Neuropathy (%)	1.4%	1.1%	0.2%	5.3%	2.7%	0.6%
	Nephropathy (%)	2.9%	1.6%	0.2%	7.8%	3.2%	0.4%
	Myocardial Infarction (%)	0.1%	0.1%	0.0%	0.4%	0.3%	0.1%
	Stroke (%)	0.1%	0.2%	0.1%	0.4%	0.4%	0.1%
	Peripheral Vascular Disease (%)	0.8%	0.8%	0.1%	2.5%	1.6%	0.4%
	Amputation (%)	0.4%	0.5%	0.1%	1.3%	0.9%	0.2%
Modified Charlson Comorbidity Score	Mean score (SD)	0.1 (0.34)	0.1 (0.35)	0.11 (0.35)	0.14 (0.42)	0.15 (0.4)	0.14 (0.41)
Years of Follow-up	Mean years (SD)	4.50 (3.77)	4.51 (3.54)	4.52 (3.78)	3.05 (2.93)	3.09 (2.80)	3.06 (3.04)

(Continued)

TABLE 1 | Continued

Variable		age 0-20 (N=48,537)			age 20-40 (N=165,877)		
		Type 1 Diabetes (n=16,179)	Type 2 Diabetes (n=16,179)	No Diabetes (n=16,179)	Type 1 Diabetes (n=55,293)	Type 2 Diabetes (n=55,293)	No Diabetes (n=55,291)
Years of Follow-up Pre-diabetes diagnosis)	Mean years (SD)	2.08 (2.31)	2.08 (2.20)	N/A	1.48 (1.)	1.50 (1.83)	N/A
Years of Follow-up Post-diabetes diagnosis	Mean years (SD)	2.41 (2.94)	2.42 (2.60)	N/A	1.56 (2.13)	1.59 (1.93)	N/A
Variable		**age 40-60 (N=573,874)**			**age 60+ (N=443,847)**		
		Type 1 Diabetes (n=63,777)	**Type 2 Diabetes (n=255,066)**	**No Diabetes (n=255,031)**	**Type 1 Diabetes (n=49,516)**	**Type 2 Diabetes (n=198,064)**	**No Diabetes (n=196,267)**
Age	Mean years (SD)	50. (5.65)	50.4 (5.64)	50.3 (5.64)	71.3 (7.42)	71.3 (7.34)	71.0 (7.15)
Gender (%)	Female	47.8%	47.7%	47.9%	52.6%	52.6%	52.3%
	Male	52.2%	52.3%	52.1%	47.4%	47.4%	47.7%
Race (%)	Asian	2.2%	2.3%	2.3%	2.8%	2.8%	3.0%
	Black	10.8%	11.0%	11.0%	14.6%	14.5%	14.4%
	Hispanic	8.2%	8.3%	8.4%	8.8%	8.8%	9.1%
	White	78.8%	78.5%	78.3%	73.8%	73.9%	73.5%
State (%)	IL, IN, MI, OH, WI	15.2%	15.2%	15.2%	13.3%	13.2%	13.0%
	AL, KY, MS, TN	5.9%	5.8%	5.8%	5.6%	5.6%	5.5%
	NJ, NY, PA	8.4%	8.4%	8.5%	11.6%	11.7%	11.1%
	AZ, CO, ID, MT, NV, NM, UT, WY	7.4%	7.5%	7.5%	7.2%	7.3%	7.9%
	CT, ME, MA, NH, RI, VT	3.5%	3.5%	3.6%	5.6%	5.7%	5.4%
	AK, CA, HI, OR, WA	8.4%	8.3%	8.4%	10.5%	10.5%	10.6%
	DE, DC, FL, GA, MD, NC, SC, VA, WV	28.2%	28.4%	27.8%	28.6%	28.5%	27.5%
	IA, KS, MN, MO, NE, ND, SD	10.1%	10.1%	9.9%	7.7%	7.7%	8.5%
	AR, LA, OK, TX	12.7%	12.9%	13.0%	9.6%	9.4%	10.1%
Education Level (%)	Less than 12th Grade	0.8%	0.8%	0.8%	1.3%	1.3%	1.3%
	High School Diploma	30.8%	30.9%	31.0%	36.0%	35.9%	36.0%
	Less than bachelor's degree	51.4%	51.0%	51.1%	49.7%	49.9%	49.6%
	Bachelor's degree Plus	17.1%	17.3%	17.2%	13.0%	12.9%	13.0%
Net Worth (%)	<$25K	20.0%	20.1%	20.2%	18.4%	18.2%	18.6%
	$25K-$149K	22.7%	22.6%	22.7%	21.2%	21.1%	21.4%
	$150K-$249K	13.5%	13.4%	13.8%	13.7%	13.8%	14.0%
	$250K-$499K	21.2%	21.1%	20.9%	21.1%	21.2%	21.2%
	$500K+	22.7%	22.7%	22.4%	25.6%	25.7%	24.9%
Insurance Provider (%)	Exclusive Provider Organization	10.2%	10.3%	10.5%	2.6%	2.6%	2.7%
	Health Maintenance Organization	23.4%	23.5%	22.8%	34.4%	34.2%	35.3%
	Indemnity	0.2%	0.2%	0.2%	3.8%	3.8%	3.9%
	Other	4.6%	4.6%	4.4%	33.7%	34.0%	32.0%
	Point of Service	52.4%	52.2%	52.9%	14.1%	14.1%	14.6%
	Preferred Provider Organization	9.2%	9.2%	9.2%	11.4%	11.4%	11.5%
Customer Driven Health Plan Type (%)	Health Reimbursement Arrangement	4.4%	4.5%	4.5%	1.2%	1.2%	1.2%
	Health Savings Account	7.2%	7.2%	7.4%	1.9%	1.8%	2.0%
Micro/Macrovascular Complication	Any Complication (%)	38.6%	21.9%	6.9%	53.6%	46.6%	25.4%
	Retinopathy (%)	21.2%	5.0%	0.0%	17.4%	7.8%	0.1%
	Neuropathy (%)	12.5%	7.9%	2.1%	15.5%	13.6%	4.7%
	Nephropathy (%)	13.6%	7.3%	1.9%	23.7%	22.7%	11.9%
	Myocardial Infarction (%)	2.0%	1.8%	0.9%	5.4%	5.2%	3.5%
	Stroke (%)	1.7%	1.7%	0.9%	6.1%	6.2%	4.6%
	Peripheral Vascular Disease (%)	9.1%	6.2%	2.1%	22.9%	20.3%	11.1%
	Amputation (%)	3.9%	2.4%	0.5%	6.2%	4.8%	1.8%

(Continued)

TABLE 1 | Continued

Variable		age 0-20 (N=48,537)			age 20-40 (N=165,877)		
		Type 1 Diabetes (n=16,179)	Type 2 Diabetes (n=16,179)	No Diabetes (n=16,179)	Type 1 Diabetes (n=55,293)	Type 2 Diabetes (n=55,293)	No Diabetes (n=55,291)
Modified Charlson Comorbidity Score	Mean score (SD)	0.32 (0.6)	0.3 (0.64)	0.32 (0.6)	0.64 (0.90)	0.65 (0.89)	0.65 (0.89)
Years of Follow-up	Mean years (SD)	3.8 (3.57)	3.87 (3.35)	3.85 (3.53)	4.21 (3.85)	4.20 (3.57)	4.10 (3.78)
Years of Follow-up Pre-diabetes diagnosis)	Mean years (SD)	1.79 (2.13)	1.78 (2.07)	N/A	1.7 (2.07)	1.73 (1.94)	N/A
Years of Follow-up Post-diabetes diagnosis	Mean years (SD)	2.07 (2.67)	2.08 (2.47)	N/A	2.48 (3.04)	2.47 (2.91)	N/A

N/A, Not Applicable.

40-60, and 2.47 years (SD 2.91) for individuals ages 60+. Matched individuals were roughly 50% female (within 3% in each group). Approximately 10% of matched individuals were black, except in the 60+ age strata, where approximately 15% of individuals were black. Individuals aged 0-40 were 10-12% Hispanic, while individuals age 40+ were 8-9% Hispanic.

Neuropsychological Outcomes

The unadjusted prevalence of each neuropsychological condition is presented in **Figures 1A–E**. Across all neuropsychological outcomes except cognitive disorders, individuals with type 2 diabetes had the highest prevalence, followed by individuals with type 1 diabetes and then individuals without diabetes (each Cochrane-Mantel-Haenszel Test P<.001). For cognitive disorders, individuals with type 1 diabetes had a higher prevalence than individuals with type 2 diabetes (each Cochrane-Mantel-Haenszel Test P<.001). In each age strata, chronic pain was the most prevalent condition, followed by mental health, sleep, addiction, and cognitive disorders. Cognitive disorders were rare in all age groups except in those greater than 60 years old.

Microvascular and Macrovascular Complications

The unadjusted prevalence of microvascular and macrovascular complications is presented in **Figure 2**. Individuals with type 1 diabetes had the highest prevalence of microvascular and macrovascular complications (ages 0-20: 7.8%, ages 20-40: 22.2%, ages 40-60: 38.6%, ages 60+: 53.6%), followed by individuals with type 2 diabetes (ages 0-20: 4.4%, ages 20-40: 8.9%, ages 40-60: 21.9%, ages 60+: 46.6%) and non-diabetic controls (ages 0-20: 0.6%, ages 20-40: 1.6%, ages 40-60: 6.9%, ages 60+: 25.4%) (Cochrane-Mantel-Haenszel Test: P<.001) (**Figure 2**). This trend was consistent for each individual complication and age strata (all P<.001).

Mental Health Disorders

The results of the mental health disorder models are presented in **Table 2**. Across age strata and after adjusting for the presence of microvascular/macrovascular complications, individuals with type 2 diabetes (ages 0-20: OR 1.31, 95% CI: 1.28-1.35; ages 20-40: OR 1.24, 95% CI 1.22-1.26; ages 40-60: OR 1.11, 95% CI: 1.10-1.12; ages 60+: OR 1.01, 95% CI: 1.01-1.02) and younger individuals with type 1 diabetes (ages 0-20: OR 1.14, 95% CI: 1.10-1.17; ages 20-40: OR 1.04, 95% CI 1.03-1.06) had significantly higher odds of mental health disorders compared to non-diabetic controls (**Table 2**). In contrast, older individuals with type 1 diabetes had significantly lower odds of mental health disorders compared to non-diabetic controls (ages 40-60: OR 0.98, 95% CI: 0.97, 0.99; ages 60+: OR 0.94, 95% CI: 0.93-0.95). In all age strata, individuals with type 2 diabetes also had significantly higher odds of mental health disorders compared to individuals with type 1 diabetes. After adjusting for diabetes status, the effects of microvascular and macrovascular complications were independently associated with an increased odds of mental health disorders (ages 0-20: OR 1.37, 95% CI: 1.28-1.46; ages 20-40: OR 1.31, 95% CI 1.28-1.34; ages 40-60: OR 1.43, 95% CI: 1.41-1.44; ages 60+: OR 1.61, 95% CI: 1.60-1.63).

Cognitive Disorders

The results of the cognitive disorder models are presented in **Table 2**. After adjusting for the presence of microvascular/macrovascular complications, both individuals with type 1 diabetes (ages 40-60: OR 0.89, 95% CI: 0.85-0.94; ages 60+: OR 0.87, 95% CI: 0.85-0.88) and individuals with type 2 diabetes (ages 40-60: OR 0.96, 95% CI: 0.93-0.99; ages 60+: OR 0.89, 95% CI: 0.88-0.90) had significantly lower odds of having a cognitive disorder compared to those without diabetes. However, there was no significant difference between individuals with type 1 diabetes and individuals with type 2 diabetes. In all individuals, after adjusting for diabetes status, the presence of microvascular/macrovascular complications were independently associated with an increased odds of cognitive disorders (ages 40-60: OR 2.45, 95% CI: 2.36-2.54; ages 60+: OR 2.16, 95% CI: 2.13-2.19).

Chronic Pain

The results of the chronic pain models are presented in **Table 2**. In all age strata and after adjusting for the presence of microvascular/macrovascular complications, individuals with type 2 diabetes (ages 0-20: OR 1.44, 95% CI: 1.41-1.48; ages 20-40: OR 1.34, 95% CI 1.33-1.36; ages 40-60: OR 1.12, 95% CI: 1.11-1.13; ages 60+: OR 1.05, 95% CI: 1.04-1.05) had significantly higher odds of chronic pain than both individuals with type 1 diabetes and those without

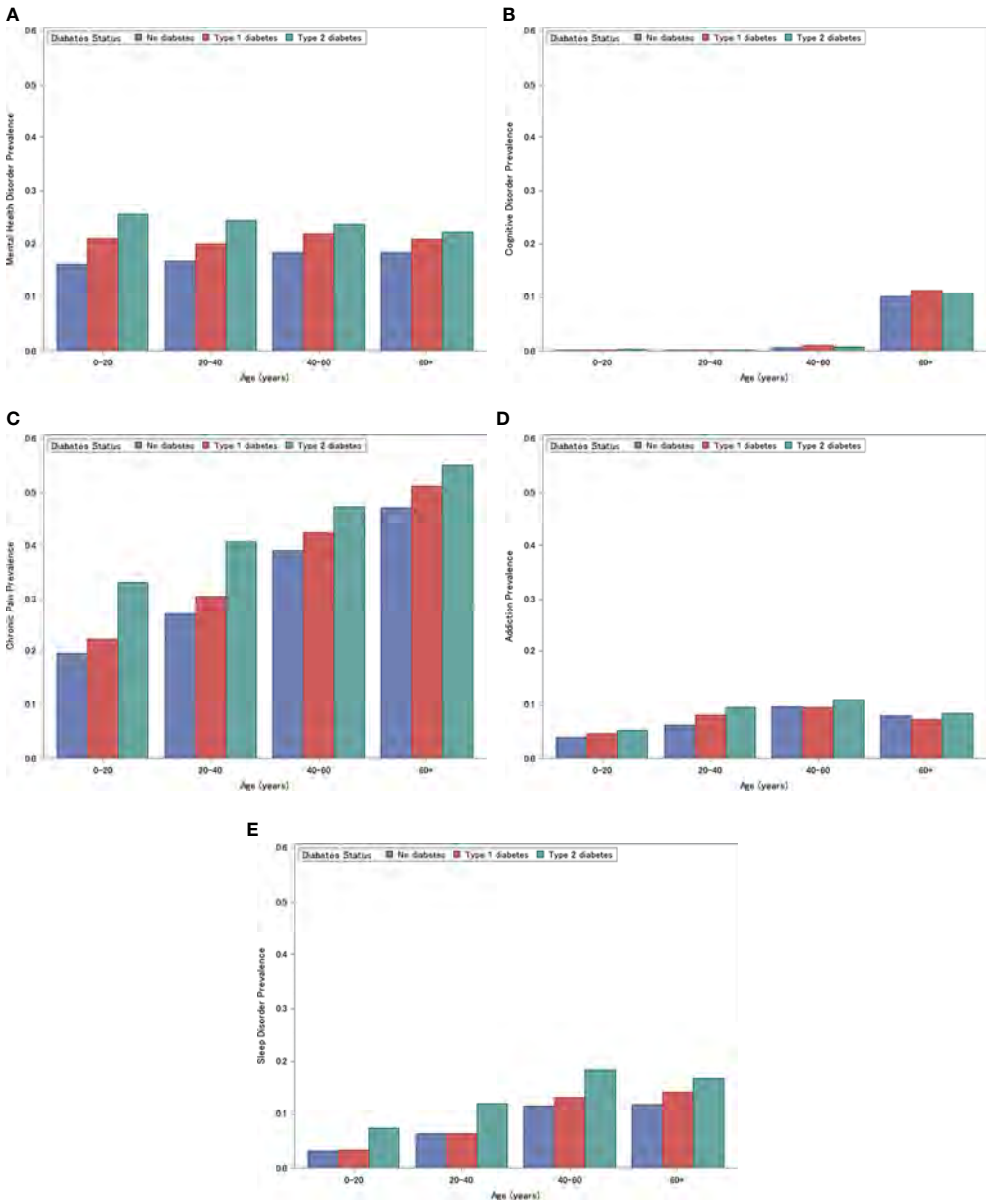

FIGURE 1 | Prevalence of Mental Health Disorders **(A)** Cognitive Disorders **(B)** Chronic Pain **(C)** Addiction **(D)** Sleep **(E)**. **(A)** Prevalence of Mental Health Disorders for individuals with type 1 diabetes, type 2 diabetes, and without diabetes, stratified by age (0-20, 20-40, 40-60, 60+ years). **(B)** Prevalence of Cognitive Disorders for individuals with type 1 diabetes, type 2 diabetes, and without diabetes, stratified by age (0-20, 20-40, 40-60, 60+). **(C)** Prevalence of Chronic Pain for individuals with type 1 diabetes, type 2 diabetes, and without diabetes, stratified by age (0-20, 20-40, 40-60, 60+). **(D)** Prevalence of Addiction for individuals with type 1 diabetes, type 2 diabetes, and without diabetes, stratified by age (0-20, 20-40, 40-60, 60+). **(E)** Prevalence of Sleep Disorders for individuals with type 1 diabetes, type 2 diabetes, and without diabetes, stratified by age (0-20, 20-40, 40-60, 60+).

diabetes. Compared to individuals without diabetes, individuals with type 1 diabetes aged 0-40 had significantly higher odds of chronic pain while individuals with type 1 diabetes aged 40+ had significantly lower odds of chronic pain than individuals with no diabetes (ages 0-20: OR 1.06, 95% CI: 1.03-1.09; ages 20-40: OR 1.02, 95% CI 1.01-1.04; ages 40-60: OR 0.94, 95% CI: 0.93-0.95; ages 60+: OR 0.92, 95% CI: 0.91-0.93). After adjusting for diabetes status, the presence of microvascular or macrovascular complications were independently associated with an increased odds of chronic pain amongst all age groups (ages 0-20: OR 1.46, 95% CI: 1.37-1.55; ages 20-40: OR 1.35, 95% CI 1.32-1.38; ages 40-60: OR 1.51, 95% CI: 1.50-1.53; ages 60+: OR 1.77, 95% CI: 1.75-1.78).

Addiction Disorder

The results of the addiction disorder models are presented in **Table 2**. After adjusting for the presence of microvascular/

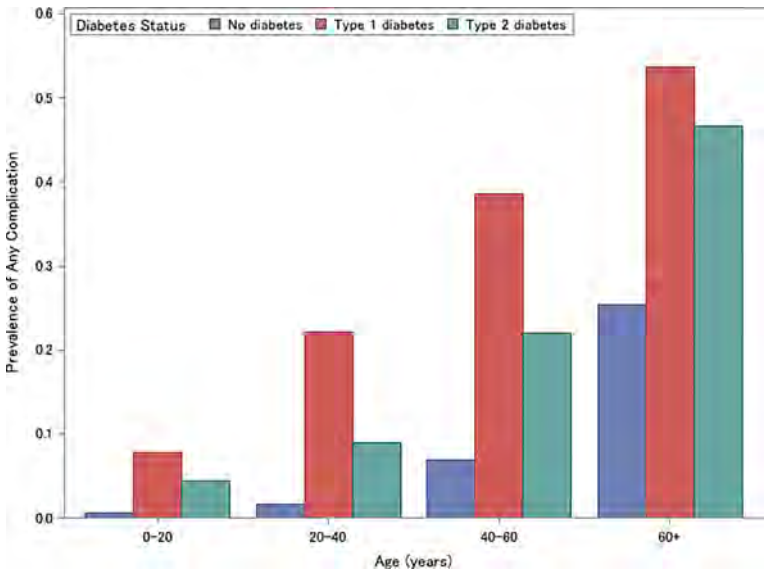

FIGURE 2 | Prevalence of Microvascular or Macrovascular Complications for individuals with type 1 diabetes, type 2 diabetes, and without diabetes, stratified by age (0-20, 20-40, 40-60, 60+).

TABLE 2 | The association between diabetes status and microvascular/macrovascular complications and neuropsychological outcomes stratified by age.

Model Outcome	Covariate	Ages 0-20 OR (95% CI)	Ages 20-40 OR (95% CI)	Ages 40-60 OR (95% CI)	Ages 60 + OR (95% CI)
Mental Health Disorder	Type 1 Diabetes (reference: no diabetes)	**1.14 (1.10, 1.17)** [a]	**1.04 (1.03, 1.06)** [a]	**0.98 (0.97, 0.99)** [a]	**0.94 (0.93, 0.95)** [a]
	Type 2 Diabetes (reference: no diabetes)	**1.31 (1.28, 1.35)** [a]	**1.24 (1.22, 1.26)** [a]	**1.11 (1.10, 1.12)** [a]	**1.01 (1.01, 1.02)** [a]
	Microvascular/Macrovascular complications (reference: none)	**1.37 (1.28, 1.46)**	**1.31 (1.28, 1.34)**	**1.43 (1.41, 1.44)**	**1.61 (1.60, 1.63)**
Cognitive Disorder	Type 1 Diabetes (reference: no diabetes)	N/A	N/A	**0.89 (0.85, 0.94)**	**0.87 (0.85, 0.88)**
	Type 2 Diabetes (reference: no diabetes)	N/A	N/A	**0.96 (0.93, 0.99)**	**0.89 (0.88, 0.90)**
	Microvascular/Macrovascular complications (reference: none)	N/A	N/A	**2.45 (2.36, 2.54)**	**2.16 (2.13, 2.19)**
Chronic Pain	Type 1 Diabetes (reference: no diabetes)	**1.06 (1.03, 1.09)** [a]	**1.02 (1.01, 1.04)** [a]	**0.94 (0.93, 0.95)** [a]	**0.92 (0.91, 0.93)** [a]
	Type 2 Diabetes (reference: no diabetes)	**1.44 (1.41, 1.48)** [a]	**1.34 (1.33, 1.36)** [a]	**1.12 (1.11, 1.13)** [a]	**1.05 (1.04, 1.05)** [a]
	Microvascular/Macrovascular complications (reference: none)	**1.46 (1.37, 1.55)**	**1.35 (1.32, 1.38)**	**1.51 (1.50, 1.53)**	**1.77 (1.75, 1.78)**
Addiction	Type 1 Diabetes (reference: no diabetes)	1.03 (0.97, 1.09)	**1.08 (1.06, 1.11)** [a]	**0.83 (0.82, 0.84)** [a]	**0.83 (0.81, 0.84)** [a]
	Type 2 Diabetes (reference: no diabetes)	**1.15 (1.09, 1.21)**	**1.24 (1.22, 1.27)** [a]	0.99 (0.98, 1.00) [a]	**0.94 (0.93, 0.95)** [a]
	Microvascular/Macrovascular complications (reference: none)	**1.51 (1.36, 1.68)**	**1.33 (1.29, 1.37)**	**1.58 (1.56, 1.60)**	**1.58 (1.56, 1.60)**
Sleep Disorder	Type 1 Diabetes (reference: no diabetes)	0.97 (0.91, 1.04) [a]	**0.92 (0.90, 0.95)** [a]	**0.93 (0.92, 0.94)** [a]	0.98 (0.97, 1.00) [a]
	Type 2 Diabetes (reference: no diabetes)	**1.54 (1.46, 1.63)** [a]	**1.39 (1.36, 1.42)** [a]	**1.25 (1.24, 1.26)** [a]	**1.14 (1.12, 1.15)** [a]
	Microvascular/Macrovascular complications (reference: none)	**1.54 (1.38, 1.72)**	**1.41 (1.36, 1.46)**	**1.49 (1.48, 1.50)**	**1.52 (1.50, 1.53)**

OR, Odds ratio; CI, confidence interval; N/A, Not Applicable.

Bold represents a statistically significant (P-Value<0.05) difference in odds between individuals with type 1 diabetes and without diabetes, between individuals with type 2 diabetes and without diabetes, and between individuals with and without microvascular/macrovascular complications based on Wald Tests.

[a]*Represents a statistically significant (P-Value<0.05) difference in odds between individuals with type 1 diabetes and individuals with type 2 diabetes based on Wald Tests.*

macrovascular complications, only individuals with type 2 diabetes aged 0-40 had significant differences in the odds of addiction compared to individuals with type 1 diabetes and those without diabetes (ages 0-20: OR: 1.15, 95% CI 1.09-1.21; ages 20-40: OR: 1.24, 95% CI 1.22-1.27). Individuals aged 40+ with type 1 diabetes had a significantly smaller odds of addiction compared to individuals with type 2 diabetes and without diabetes (ages 40-60: OR 0.83, 95% CI: 0.82-0.84; ages 60+: OR 0.83, 95% CI: 0.81-0.84). In contrast, for individuals 0-20 years old, there were no differences in odds of addiction between individuals with

type 1 diabetes and those without diabetes (OR 1.03, 95% CI 0.97-1.09), and individuals 20-40 years old experienced higher odds of addiction than individuals with no diabetes (OR 1.08, 95% CI 1.06-1.11). After adjusting for diabetes status, the presence of microvascular or macrovascular complications were independently associated with an increased odds of addiction (age 0-20: OR 1.51, 95% CI: 1.36-1.68; age 20-40: OR 1.33, 95% CI 1.29-1.37; age 40-60: OR 1.58, 95% CI: 1.56-1.60; age 60+: OR 1.58, 95% CI: 1.56-1.60).

Sleep Disorders

The results of the sleep disorder models are presented in **Table 2**. In all age groups and after adjusting for the presence of microvascular/macrovascular complications, individuals with type 2 diabetes had significantly higher odds of having sleep disorders compared to individuals without diabetes, (ages 0-20: OR 1.54, 95% CI: 1.46-1.63; ages 20-40: OR 1.39, 95% CI 1.36-1.42; ages 40-60: OR 1.25, 95% CI: 1.24-1.26; ages 60+: OR 1.14, 95% CI: 1.12-1.15). Individuals with type 1 diabetes ages 20-60 had significantly lower odds of sleep disorders than individuals without diabetes (ages 20-40: OR 0.92, 95% CI: 0.90-0.95; ages 40-60: OR 0.93, 95% CI: 0.92-0.94). After adjusting for diabetes status, the presence of microvascular or macrovascular complications was independently associated with an increased odds of sleep disorders across age strata (ages 0-20: OR 1.54, 95% CI: 1.38-1.72; ages 20-40: OR 1.41, 95% CI 1.36-1.46; ages 40-60: OR 1.49, 95% CI: 1.48-1.50; ages 60+: OR 1.52, 95% CI: 1.50-1.53).

Effect of Specific Microvascular and Macrovascular Complications

When separating the effects of complications into microvascular, macrovascular, and amputations, nearly all effects remained positive and statistically significant, as detailed in **Supplementary Table S2**. Macrovascular complications had the largest effect size for 14 out of 18 comparisons. The models with individual complication effects, detailed in **Supplementary Table S3**, revealed that amongst microvascular complications, neuropathy had the largest effect size for 17 out of 18 comparisons. Amongst macrovascular complications, stroke had the largest effect size for 12 out of 18 comparisons.

DISCUSSION

To our knowledge, this is the largest US study to examine the prevalence of neuropsychological outcomes among a nationally representative population of privately insured individuals with type 1 and type 2 diabetes and controls without diabetes. Furthermore, we are unaware of studies that have evaluated the independent effects of diabetes status and microvascular/macrovascular complications on these neuropsychological outcomes. We found that the prevalence of neuropsychological outcomes (mental health, chronic pain, addiction, and sleep disorders) was higher in individuals with type 2 diabetes compared to type 1 diabetes, and in individuals with type 1 diabetes compared to those without diabetes. For cognitive disorders, microvascular complications, and macrovascular complications, the prevalence was highest in those with type 1 diabetes, followed by those with type 2 diabetes and then those without diabetes. Microvascular and macrovascular complications were consistently associated with higher odds for all five neuropsychological outcomes, independent of diabetes status. Interestingly, after adjusting for the presence of microvascular and macrovascular complications, individuals with type 1 diabetes had similar odds of developing neuropsychological outcomes compared to those without diabetes (no odds ratios >1.15). In contrast, individuals with type 2 diabetes are more likely to experience mental health, chronic pain, and sleep disorders even after adjusting for microvascular and macrovascular complications.

Despite a higher prevalence of neuropsychological outcomes, we found that individuals with type 1 diabetes had similar or reduced odds of developing all neuropsychological outcomes compared to individuals without diabetes, after adjusting for the presence of microvascular and macrovascular complications. Thus, microvascular and macrovascular complications likely play a fundamental role in the development of neuropsychological outcomes in individuals with type 1 diabetes. One explanation of our results is that the same pathophysiologic processes that drive microvascular and macrovascular complications also drive neuropsychological outcomes. For instance, individuals with a longer duration of diabetes or worse glycemic control are more likely to develop complications, and these same factors may also increase the risk of neuropsychological outcomes. Unfortunately, our database does not contain information on diabetes duration or severity to address this important question. Another possibility is that the complications themselves lead to worse neuropsychological outcomes, either directly through downstream consequences that result from these complications or indirectly through reduced quality of life and disease burden. A combination of these two explanations is likely and should be the focus of future studies. In addition, future studies should focus on the role of neuropathy and stroke as these were the individual microvascular/macrovascular complications that resulted in the highest odds of neuropsychological outcomes. Furthermore, since microvascular and macrovascular complications are more common in individuals with type 1 diabetes and are a major driver of the higher prevalence of neuropsychological outcomes in these individuals, our results highlight the importance of preventing these complications.

In contrast, after adjusting for the presence of microvascular and macrovascular complications, individuals with type 2 diabetes were still at higher risk for developing three neuropsychological outcomes: mental health disorders, chronic pain, and sleep disorders, compared to both individuals with type 1 diabetes, and individuals without diabetes. These results indicate that factors beyond microvascular and macrovascular complications likely contribute to the development of these wide-ranging neuropsychological conditions in individuals with type 2 diabetes. Since individuals with type 2 diabetes have a higher prevalence of metabolic risk factors than individuals with type 1 diabetes and the general population, these other metabolic factors may contribute to the higher prevalence of neuropsychological outcomes.

Supporting this hypothesis, metabolic risk factors other than hyperglycemia have been shown to be associated with multiple neuropsychological outcomes. Specifically, meta-analyses demonstrated associations between obesity, metabolic control and mental health disorders such as anxiety and depression (24, 25). Similarly, a meta-analysis revealed associations between overweight and obesity with chronic pain (26). Moreover, obesity also increases the likelihood of lower quality sleep and sleep apnea (27). Given the robust literature linking obesity and other metabolic risk factors with neuropsychological outcomes and the high prevalence of these comorbidities with type 2 diabetes, the higher prevalence of neuropsychological outcomes in the type 2 compared to the type 1 diabetes population is at least partially explained.

Another possibility is that individuals that have or are susceptible to neuropsychological outcomes may be more likely to develop type 2 diabetes. Though the majority of the literature focuses on risks in individuals that already have type 2 diabetes, a systematic review (28) found that depressed adults have a 37% increased risk of developing type 2 diabetes. While demographic factors are also different between type 1 and type 2 diabetes populations, our comparisons are adjusted for many key factors including age, sex, race, ethnicity, and socioeconomic status. Given that microvascular and macrovascular complications are not the sole driving force behind neuropsychological outcomes in individuals with type 2 diabetes, studies are needed to determine the other key risk factors including demographic factors.

Individuals that experienced any microvascular complications, macrovascular complications or amputations had higher odds of having each neuropsychological outcome, suggesting that these complications are the primary driver for a wide range of neuropsychological outcomes, regardless of diabetes status. Although macrovascular complications were less prevalent than microvascular complications, macrovascular complications were associated with a higher odds of neuropsychological outcomes compared to microvascular complications in 14 out of the 18 models we evaluated. The macrovascular and microvascular complications having the largest associations with neuropsychological outcomes were stroke (12 out of 18 comparisons) and neuropathy (17 out of 18 comparisons) respectively. These results are congruent with previous studies that have found that dementia, mental health disorders, chronic pain, and sleep disorders were common in individuals following a stroke (29–33). In addition, neuropathy has been previously linked to chronic pain, various mental health disorders, sleep disorders, lower cognitive performance, and inhalant addiction (34–39). Given that individuals with these complications have a higher risk for these neuropsychological outcomes, preventing or improving complications such as neuropathy or stroke in individuals with diabetes may simultaneously improve their neuropsychological prospects, and therefore, should be the focus of future studies.

Limitations of the current study include possible disease misclassification using ICD-9/ICD-10 codes. However, many of our definitions have been validated with high positive predictive values. Separately, claims data lack the necessary detailed clinical information to assess the severity of microvascular/macrovascular complications, neuropsychological conditions, and diabetes. In addition, our analyses may have differentially captured severe neuropsychological outcomes, as only such cases would prompt a visit to a provider and result in a diagnostic code. Furthermore, the generalizability to other populations such as those that are not privately insured is unclear. On the other hand, the large-scale claims data allowed us to identify a wide range of neuropsychological outcomes across many age ranges, including older populations with type 1 diabetes.

In summary, individuals with type 1 diabetes have a higher prevalence of neuropsychological outcomes compared to those without diabetes. However, after adjusting for the presence of microvascular or macrovascular complications, type 1 diabetes was not associated with an increased odds of neuropsychological outcomes compared to individuals without diabetes. Furthermore, microvascular and macrovascular complications are independently associated with neuropsychological outcomes. Specifically, we identified stroke and neuropathy as major risk factors for most neuropsychological outcomes. Therefore, prevention of microvascular and macrovascular complications will likely reduce neuropsychological outcomes either as the result of similar pathophysiologic processes or by preventing the direct and indirect consequences of these complications. In contrast, individuals with type 2 diabetes were at increased odds of multiple neuropsychological outcomes compared to those with type 1 diabetes, even after adjusting for presence of microvascular/macrovascular complications. This indicates that in individuals with type 2 diabetes, other factors (such as obesity) may lead to neuropsychological complications. Alternatively, it is possible that neuropsychological complications may result in type 2 diabetes onset.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: BC is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Requests to access these datasets should be directed to bcallagh@med.umich.edu.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of the University of Michigan (HUM00176199). Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

NP was involved in the data management, study design, statistical analysis, interpretation of data, and wrote the manuscript. ER was involved in the study design,

interpretation of the statistical analysis, and critical revisions of the manuscript. MB was involved in the study design, interpretation of the data, and critical revisions of the manuscript. KM-S was involved in data interpretation and manuscript revision. DA was involved in data interpretation and manuscript revision. JL was involved in data interpretation and the critical revisions of the manuscript. RP-B was involved in data interpretation and the critical revisions of the manuscript. EF was involved in the interpretation of the statistical analysis and critical revisions of the manuscript. BC was involved in the study design, interpretation of the statistical analysis and critical revisions of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.834978/full#supplementary-material

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Plasma Metabolomics and Lipidomics Differentiate Obese Individuals by Peripheral Neuropathy Status

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Abbreviations: BCAA, branched-chain amino acid; BMI, body mass index; DAG, diacylglycerol; FBG, fasting blood glucose; HbA_{1c}, glycated hemoglobin A_{1c}; HDL-c, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; IWMC, Investigational Weight Management Clinic; LDL-c, low-density lipoprotein cholesterol; MetS, metabolic syndrome; NCEP, National Cholesterol Education Program; O2PLS, 2-way orthogonal partial least squares; OGTT, oral glucose tolerance test; OR, odds ratio; PCA, principal component analysis; PKC, protein kinase C; PLS-DA, partial least squares–discriminant analysis; PN, peripheral neuropathy; T2D, type 2 diabetes; TAG, triacylglycerol; UPLC-MS/MS, ultrahigh performance liquid chromatography–tandem mass spectroscopy; VIP, variable importance in projection; WC, waist circumference.

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Abstract

Context: Peripheral neuropathy (PN) is a frequent prediabetes and type 2 diabetes (T2D) complication. Multiple clinical studies reveal that obesity and dyslipidemia can also drive PN progression, independent of glycemia, suggesting a complex interplay of specific metabolite and/or lipid species may underlie PN.

Objective: This work aimed to identify the plasma metabolomics and lipidomics signature that underlies PN in an observational study of a sample of individuals with average class 3 obesity.

Methods: We performed plasma global metabolomics and targeted lipidomics on obese participants with (n = 44) and without PN (n = 44), matched for glycemic status, vs lean nonneuropathic controls (n = 43). We analyzed data by Wilcoxon, logistic regression, partial least squares–discriminant analysis, and group-lasso to identify differential

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metabolites and lipids by obesity and PN status. We also conducted subanalysis by prediabetes and T2D status.

Results: Lean vs obese comparisons, regardless of PN status, identified the most significant differences in gamma-glutamyl and branched-chain amino acid metabolism from metabolomics analysis and triacylglycerols from lipidomics. Stratification by PN status within obese individuals identified differences in polyamine, purine biosynthesis, and benzoate metabolism. Lipidomics found diacylglycerols as the most significant subpathway distinguishing obese individuals by PN status, with additional contributions from phosphatidylcholines, sphingomyelins, ceramides, and dihydroceramides. Stratifying the obese group by glycemic status did not affect discrimination by PN status.

Conclusion: Obesity may be as strong a PN driver as prediabetes or T2D in a sample of individuals with average class 3 obesity, at least by plasma metabolomics and lipidomics profile. Metabolic and complex lipid pathways can differentiate obese individuals with and without PN, independent of glycemic status.

Key Words: complex lipid, diacylglycerol, metabolomics, lipidomics, obesity, polyneuropathy

Peripheral neuropathy (PN) is a common prediabetes and the most common type 2 diabetes (T2D) complication (1). Multiple clinical studies have identified factors beyond glycemia that underlie PN onset and progression (2), specifically, components of metabolic syndrome (MetS) (3-5). Furthermore, we have shown in several population studies that MetS components are PN risk factors, independent of glycemic status (6-8). MetS encompasses a collection of conditions, which include obesity, dyslipidemia, insulin resistance, and hypertension, which frequently occur together. The criteria defining individuals with MetS are 3 out of 5 from the following: elevated waist circumference (WC; ≥ 102 cm men, ≥ 88 cm women), systolic (≥ 130 mm Hg) or diastolic blood pressure (≥ 85 mm Hg), triacylglycerols (TAGs, ie, triglycerides; ≥ 150 mg/dL), and fasting glucose (> 100 mg/dL) and lower high-density lipoprotein cholesterol (HDL-c; < 40 mg/dL men, < 50 mg/dL women) (9).

In our most recent clinical study, we identified WC, defined by National Cholesterol Education Program (NCEP) criteria, as the primary anthropometric PN driver, even in normoglycemic obese individuals (10, 11). This finding suggests that central obesity itself is a sufficient condition for PN development. This observation was replicated in our preclinical study of diet-induced obesity in mice, which also drives PN development independent of T2D status (12). In this integrated lipidomic-transcriptomic study, we found that high-fat diet dysregulated the nerve lipidome during PN progression. The sciatic nerve from obese prediabetic mice with PN exhibited a distinct lipid profile compared to lean mice without PN, which centered on differential TAG species and expression of diacylglycerol acyltransferase 2, the enzyme catalyzing the final and committed step in TAG synthesis. While the study suggested that lipidome dysregulation is a critical PN feature in obese murine models, whether these results translate to humans is unknown.

To overcome this gap (13), we undertook an observational study to characterize the metabolomic and lipidomic plasma profiles in a sample of individuals with average class 3 obesity with (n = 44; obese_PN) and without PN (n = 44; obese_No_PN), matched for glycemic status, vs lean controls without PN (n = 43). In this clinical cohort, metabolomic profiles between obese vs lean individuals correlated most strongly with gamma-glutamyl and branched-chain amino acid (BCAA) metabolism and within obese participants by PN status by alterations in polyamine, purine biosynthesis, and benzoate metabolism (a xenobiotics subpathway). Lipidomic profiles identified TAGs as strongly correlating with obesity compared to lean individuals, regardless of PN status. However, significant differences were present in diacylglycerols (DAGs) and in several complex lipid subpathways in obese individuals without PN compared to their obese counterparts with PN, supporting a role for dysregulation of specific lipid species in PN development. Stratifying the obese group by glycemic status did not affect discrimination by PN status. These results suggest obesity may be as strong a PN driver as prediabetes or T2D in individuals with class 3 obesity, at least by plasma metabolomics and lipidomics profile.

Materials and Methods

Study Participants and Diagnoses

Participants were recruited as part of two separate clinical trials from the University of Michigan Bariatric Surgery Clinic (1) and Investigational Weight Management Clinic (IWMC) (2), respectively. In parallel, lean controls were recruited for each trial. Controls did not have any MetS

components, assessed by clinical testing, by the NCEP/Adult Treatment Panel III criteria. NCEP/Adult Treatment Panel III definitions for MetS included WC (> 102 cm men, > 88 cm women), systolic (> 130 mm Hg) or diastolic blood pressure (> 85 mm Hg), TAGs (> 150 mg/dL), HDL-c (< 40 mg/dL men, < 50 mg/dL women), and fasting glucose (> 100 mg/dL) (3). Baseline plasma samples were obtained from all participants. The present baseline observational study combines the demographic data from both studies and includes obese participants without PN (n = 44) and with PN (n = 44) and lean controls without MetS (n = 43) (Table 1).

The aim of this observational study was to identify differential plasma metabolites and lipids by PN status in obesity, independent of glycemic status. Lean controls were normoglycemic, as determined by clinical testing, and did not meet any criteria for prediabetes or T2D. Lean controls had fasting blood glucose (FBG) of less than 100 mg/dL, 2-hour glucose of less than 140 mg/dL following a 75-g oral glucose tolerance test (OGTT) or a glycated hemoglobin (HbA1c) of less than 5.7% (39 mmol/mol). The obese participants with and without PN were matched for glycemic parameters. Prediabetes was defined based on an FBG (100-≤ 125 mg/dL) or a 2-hour glucose of 140 to 199 mg/dL following an OGTT or HbA_{1c} of 5.7% (39 mmol/mol) to less than or equal to 6.4% (46 mmol/mol). T2D was defined as an FBG greater than 126 mg/dL or a 2-hour glucose greater than or equal to 200 mg/dL after an OGTT, according to the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (14). T2D was also determined based on a known diabetes diagnosis and/or medications or an HbA_{1c} greater than or equal to 6.5% (48 mmol/mol). All participants without a known diagnosis of T2D had an OGTT, and HbA_{1c} status was collected from available medical records if performed within 6 months of the study visit. Participants underwent PN diagnosis according to the Toronto consensus definition of probable PN, which requires 2 or more of the following: PN symptoms, abnormal sensory examination, and abnormal reflexes, as determined by 1 of 4 neuromuscular specialists (15). All participants gave their written informed consent for these studies, which were approved by the University of Michigan Institutional Review Board (HUM00092638 for the bariatric surgery clinic, HUM00039723 for the IWMC study).

Study Design

For each participant, we collected demographics (age, sex), anthropometric measures (body weight, height,

Table 1. Participant demographics at time of plasma collection for global metabolomics and lipidomics analysis

Clinical parameter	Lean (n = 43)	Obese_PN (n = 44)	Obese_No_PN (n = 44)	*P* Obese_PN vs Lean	*P* Obese_No_PN vs Lean	*P* Obese_PN vs Obese_No_PN
Age, mean (SD), y	43.93 ± 12.28	53.16 ± 8.68	52.75 ± 8.42	< .001	< .001	.98
Sex						
Female	35 (81.40%)	24 (54.55%)	24 (54.55%)	.011	.011	≥ .99
Male	8 (18.60%)	20 (45.45%)	20 (45.45%)	.011	.011	≥ .99
BMI, mean (SD)	22.89 ± 2.06	45.03 ± 6.84	43.03 ± 6.22	< .001	< .001	.21
Body weight, mean (SD), kg	64.43 ± 10.01	137.32 ± 29.94	123.88 ± 21.91	< .001	< .001	.015
WC, mean (SD), cm	80.64 ± 7.12	136.47 ± 16.92	124.49 ± 16.11	< .001	< .001	< .001
Blood pressure, mean (SD), mm Hg						
Systolic	108.40 ± 10.50	135.00 ± 15.39	130.50 ± 11.12	.001	< .001	.22
Diastolic	66.30 ± 9.73	70.02 ± 11.17	71.36 ± 10.67	.23	.069	.82
Cholesterol, mean (SD), mmol/l	181.81 ± 41.06	157.25 ± 41.60	162.27 ± 36.91	.013	.062	.83
TAGs, mean (SD), mmol/L	72.42 ± 22.34	161.09 ± 113.90	151.25 ± 94.48	< .001	< .001	.86
HDL-c, mean (SD), mmol/l	67.19 ± 16.23	43.34± 12.36	41.57 ± 9.99	< .001	< .001	.80
LDL-c, mean (SD), mmol/L	102.77 ± 28.83	85.16 ± 34.91	99.26 ± 44.57	.069	.90	.18
FBG, mean (SD), mg/dL	85.07 ± 6.46	128.61 ± 37.80	131.71 ± 66.70	<0.001	< .001	.95
Type 2 diabetes (yes/no)	0/43	29/15	25/19	< .001	< .001	.051
Prediabetes (yes/no)	0/43	7/37	13/31	.012	< .001	.20
Statin use (yes/no)	0/43	22/22	21/23	< .001	< .001	≥ .99
β-blocker use (yes/no)	0/43	18/26	17/27	< .001	< .001	≥ .99

Age, BMI, body weight, WC, blood pressure (systolic, diastolic), cholesterol, TAGs, HDL-c, and LDL-c were analyzed by one-way analysis of variance with post hoc analysis with Tukey test; sex (female, male), diabetes status, prediabetes status, statin use, and β-blocker use were analyzed by Fisher test.

Abbreviations: BMI, body mass index; FBG, fasting blood glucose; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; obese_PN, obese with peripheral neuropathy; obese_No_PN, obese without peripheral neuropathy; TAGs, triacylglycerols; WC, waist circumference.

body mass index [BMI], WC), vitals (systolic and diastolic blood pressure), and a fasting lipid profile (TAGs, total cholesterol, HDL-c, and low-density lipoprotein cholesterol [LDL-c]). For the primary study outcome, participants provided a plasma sample for metabolomics and lipidomics analysis before weight-loss intervention. Participants were asked to fast for 12 hours overnight and abstain from alcohol, smoking, medication use, and caffeine, and avoid vigorous exercise out of their normal routine. Participants with T2D were asked to monitor their blood sugar and adjust medication if needed. Blood was drawn from fasted participants using good clinical practice into EDTA tubes, which were temporarily kept at 4 °C for a maximum 2 hours' duration. Tubes were then centrifuged (2000*g*, 10 min, 4 °C) and the plasma supernatants were saved in cryovials, which were directly transferred for storage at –80 °C.

Plasma Untargeted Metabolomics and Targeted Lipidomics Analysis

For metabolomics and lipidomics, plasma samples were shipped on dry ice to Metabolon, where they were stored at –80 °C. Untargeted metabolomics is a system-wide technique that agnostically and systematically detects and identifies metabolites from a biosample. Owing to the technical methodology, metabolomics generally captures polar, water-soluble metabolites, including more polar lipids. Thus, metabolomics analyses also contain certain lipid classes. Lipidomics is a technique that detects and identifies nonpolar as well as polar species, with an emphasis on lipid species. Targeted lipidomics specifically detects a predetermined lipid species panel.

Global untargeted metabolomics analysis was conducted by ultrahigh performance liquid chromatography–tandem mass spectroscopy (UPLC-MS/MS), using published Metabolon protocols (16, 17). Briefly, recovery and internal standards were added to plasma samples for evaluating extraction efficiency and instrument performance, respectively. Metabolites were extracted with methanol and analyzed by reverse-phase UPLC-MS/MS (positive and negative ion modes) and hydrophilic interaction chromatography UPLC-MS/MS. Metabolites were identified by automated ion peak comparison from each sample to a reference library of authenticated chemical standards with specific mass-to-charge ratios and retention times, followed by data curation. Each metabolite within a sample was quantified by its area under the curve and normalized to account for day-to-day variation by equating the metabolite median across all samples that day to 1 and normalizing the metabolite within each sample proportionately against the median.

The targeted Complex Lipid Panel was performed by differential mobility spectroscopy by Sciex SelexION at Metabolon. Differential mobility spectroscopy separates species beyond differences in mass-to-charge ratios and retention time, such as, additionally, by size and shape, facilitating lipid identification, even of highly similar species. Briefly, lipids were extracted from plasma in the presence of internal standards by butanol-methanol extraction (18), dried under nitrogen, and reconstituted in a dichloromethane:methanol solution containing ammonium acetate. Samples were analyzed via both positive and negative mode electrospray MS. Each lipid species concentration was quantified by a ratio of its sample signal intensity to an assigned internal standard, multiplied by the internal standard concentration in that sample. Each lipid class concentration was calculated by summing all lipids belonging to that class. Each fatty acid composition was calculated through the proportion of each class composed of individual fatty acids.

Metabolite Data Sets and Imputation Method

In sum, we detected 842 named metabolites from the metabolomics analysis and 983 named lipid species from the lipidomics analysis, both from Metabolon's curated databases (19). We excluded from further analysis any metabolites not present in at least 80% of samples (ie, overall missingness > 20%), yielding 604 metabolites and 858 lipids (19). Missing values for metabolites retained in our analysis were imputed to the minimum observed value for each metabolite, per Metabolon protocols (16, 17).

Statistical Analysis

Descriptive analysis

Descriptive summaries of demographic and clinical characteristics were calculated for the following 4 groups: obese vs lean, obese with PN vs lean, obese without PN vs lean, and obese with vs without PN. Fisher exact tests and one-way analysis of variance with post hoc Tukey tests were used to determine the pairwise differences between groups (obese vs lean, obese with PN vs lean, obese without PN vs lean, and obese with vs without PN).

Identification of differential metabolites and lipids

Multiple approaches were employed to identify metabolites and lipids that statistically significantly differed between the groups (obese vs lean, obese with PN vs lean, obese without PN vs lean, and obese with vs without PN). Wilcoxon rank sum tests, referred to as *unadjusted*, were used to identify significant unadjusted differences in abundance for each metabolite and lipid between groups. Multivariable

logistic regression models, referred to as *adjusted*, were created to determine the association between groups and each natural log-transformed and standardized metabolite and lipid, after adjusting for age and sex. Separate logistic regression models were created for each metabolite/lipid and for each comparison (obese vs lean, obese with PN vs lean, obese without PN vs lean, and obese with vs without PN). For both unadjusted (Wilcoxon rank sum tests) and adjusted (logistic regression) approaches, statistically significantly different metabolites and lipids were defined as those with a corresponding Benjamini-Hochberg–corrected *P* value of less than .05. The resulting adjusted and unadjusted *P* values were visualized using volcano plots and Manhattan plots, respectively.

Partial least squares-discriminant analysis (PLS-DA) was also performed on metabolites and lipids separately by using the R package mixOmics (20, 21). This dimensionality reduction tool identifies metabolite/lipid patterns that statistically significantly contributed to group separation as determined using a variable importance in projection (VIP) score greater than 1 as the cutoff.

We also performed group-lasso on log-transformed and standardized metabolites and lipids separately using the R package gglasso (22). Specifically, we used a 5-fold cross-validation to optimize the tuning parameter corresponding to a sparse model that was within 1 SE of the minimum cross-validation error. We then refit the group-lasso model to adjust for age and sex, and therefore generate the final model. Group-lasso results are represented by heatmaps with significant metabolites/lipids having βvalues greater than 0 or Manhattan plots with odds ratios (OR) greater than 1.

Prediabetes and type 2 diabetes status analysis

Despite matching, we reanalyzed the data stratified by glycemic status to determine whether there was any effect from plasma metabolomic and lipidomic profiles in prediabetes or T2D compared to the overall obesity profiles on PN separation. Specifically, we employed Wilcoxon rank sum tests and evaluated Benjamini-Hochberg–adjusted *P* values to identify differential metabolites/lipids between obese participants with and without T2D and with and without prediabetes. In addition, we used principal component analysis (PCA) to visualize groups by glycemic status in the entire cohort.

Metabolism and complex lipid pathway analysis

Pathway enrichment analysis was conducted by our in-house R package richR (https://github.com/hurlab/richR/). Superpathway and subpathway annotations were from Metabolon and were employed as background pathways. PLS-DA– and group-lasso–selected statistically significant metabolites were assessed for overrepresentation within each subpathway. A hypergeometric test was conducted for each candidate subpathway, which was considered statistically significant for *P* less than .05.

Two-way orthogonal partial least squares (O2PLS)

Two-way orthogonal partial least squares (O2PLS) was employed to integrate metabolomics and lipidomics to identify highly interassociated metabolites and lipids of biological significance (23), using an R package OmicsPLS (24). The network was built from 604 metabolites with established links to 858 lipids. Metabolomics and lipidomics data were scaled and transformed, according to published methods (25). The loading values for the joint covariance were extracted to identify highly correlated metabolites and lipids. The final metabolite-lipid correlation network was generated using the top 100 correlations between the 50 metabolites and 50 lipids with the highest loading values.

Spearman correlation analysis

Spearman rank correlation was calculated to determine correlation in metabolites and lipids in the obese participants with vs without PN. Heat maps were used to display the significant correlations (adjusted $P < .05$) for positive (strongest with the value of 1) and negative (strongest with the value of –1) correlations.

Statistical software

All statistical and prediction analyses were completed using the R statistical computing software version 4.0.2.

Results

Clinical Characteristics of the Obese Groups Compared to the Lean Group

In this observational study (see Table 1), age and sex differed in both obese groups vs lean, but did not differ between obese participants with vs without PN. All lean individuals were normoglycemic. Since obese participants were matched based on their glycemic status, the obese with vs without PN groups did not differ in the prevalence of prediabetes and T2D. Both obese groups had higher anthropometric measures (body weight, BMI, WC), systolic blood pressure (all $P < .001$), and TAGs ($P < .001$) and lower HDL-c ($P < .001$) vs lean controls. The mean BMIs of the obese group with PN and obese group without PN were 45.03 ± 6.84 and 43.03 ± 6.22, respectively, making these obese groups, on average, groups with class 3 obesity. There was a significantly lower total cholesterol level in obese participants with PN vs lean controls ($P = .013$) and a trending lower cholesterol level in obese participants without PN vs lean controls ($P = .069$). This, presumably, is

because most obese individuals were on statins for hyperlipidemia (cholesterol level by statin use, "yes" vs "no," $P = 9.4 \times 10^{-6}$ by Wilcoxon in the obese vs lean comparison, $P = .00051$ by Wilcoxon in the obese with vs without PN comparison). Despite statin management, obese individuals had significantly higher plasma TAGs vs controls, which may be partly due to prevalent β-blocker use (26). While statins affect the lipidomic profile in obese participants, it does not affect PN development (27). WC was higher in obese participants with vs without PN, as anticipated (10, 11), as was body weight. Otherwise, importantly, obese groups with and without PN did not differ significantly in any other metabolic metric, including BMI, total cholesterol, TAGs, HDL-c, LDL-c, prediabetes status, and T2D status, underscoring our hypothesis that specific metabolite and/or lipid species may underlie PN rather than global dyslipidemia and/or glycemia alone.

Plasma Metabolomics Differs in Obese Groups vs Lean Group

We first examined metabolite differences between the obese group as a whole (obese groups both with and without PN combined) vs the lean controls. Descriptive analyses are listed for Wilcoxon (19) and logistic regression (19). PLS-DA clearly separated obese from lean participants with 205 metabolites satisfying VIP greater than 1 (Fig. 1A) (19), whereas group-lasso identified 47 significant metabolites (Fig. 1B) (19).

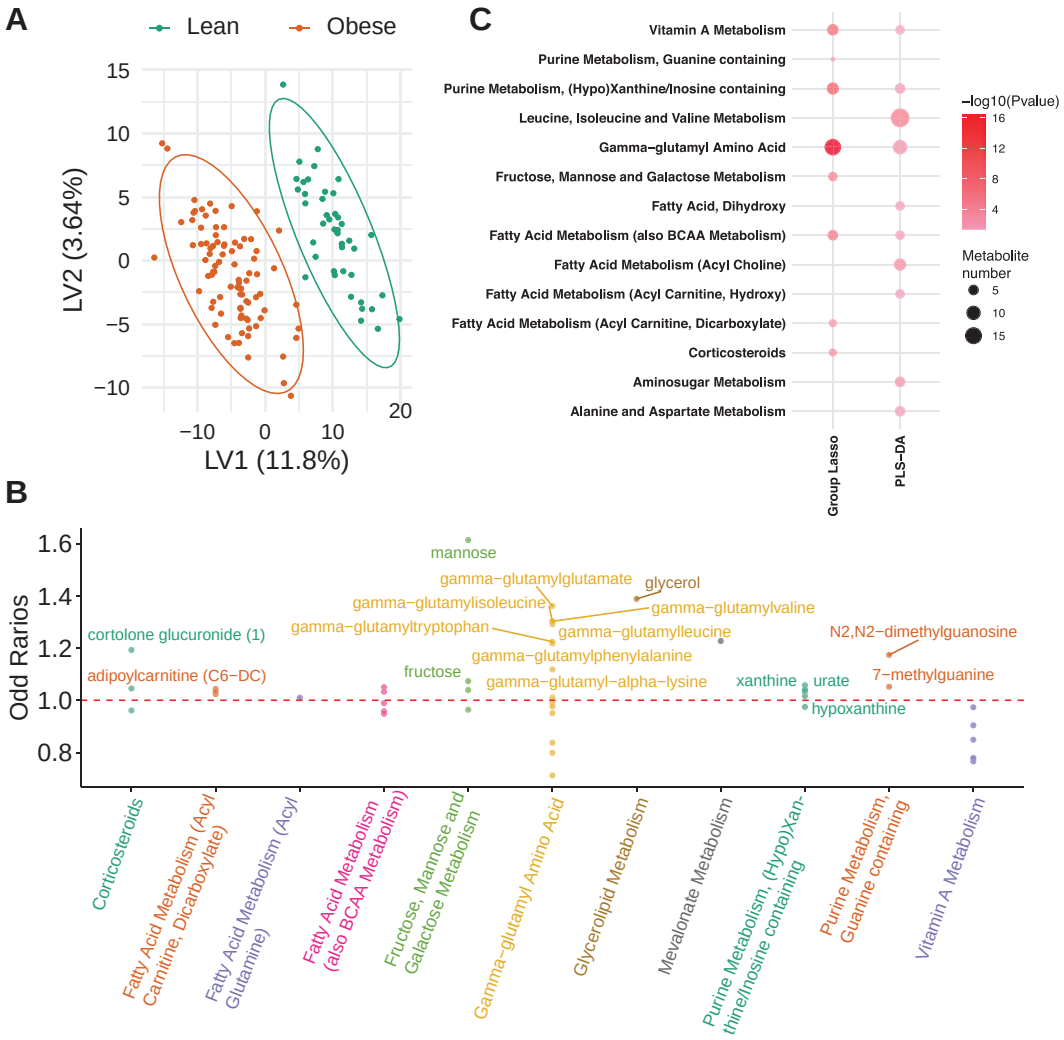

Figure 1. Metabolomics analysis in obese vs lean participants. A, Partial least squares–discriminant analysis (PLS-DA) fully separated obese (red, n = 88) from lean (green, n = 43) participants, and selected 205 significant metabolites that contributed to the separation with variable importance in projection (VIP) greater than 1. B, Group-lasso selected 11 subpathways containing 47 significant metabolites with an odds ratio (OR) greater than 1 (metabolite higher in obese) or OR less than 1 (metabolite lower in obese), adjusted for age and sex. C, Pathway analysis of PLS-DA– and group-lasso–selected metabolites. The circles represent selected enriched subpathways. Circle color indicates significance level from most (red) to least (lightest pink) significant. Circle size represents the number of selected metabolites belonging to the enriched subpathways. The subpathways also encompass several lipid pathways because the global metabolomics analysis detects some lipids.

PLS-DA and group-lasso overlapped in several subpathways, including the most significant "gamma-glutamyl amino acid," but also uniquely selected others, including several fatty acid metabolism subpathways (Fig. 1C).

Plasma Lipidomics Differs in Obese Groups vs Lean Group

We observed more differences in lipid species than in general metabolites between the obese group as a whole (both groups) vs lean group; descriptive analyses are listed for Wilcoxon (19) and logistic regression (19). Interestingly, although PLS-DA fully resolved obese from lean participants by metabolites (Fig. 1A), the separation was less pronounced by lipids, although many more fulfilled VIP greater than 1 (Fig. 2A (19);). Moreover, group-lasso selected 668 statistically significant lipids (Fig. 2B) (19). Among lipid subpathways, PLS-DA and group-lasso were fully discordant (Fig. 2C); PLS-DA selected "TAG ester" (highest significance) and "DAG ester," whereas group-lasso selected complex lipids, such as "dihydroceramide" and "ceramide."

We next analyzed lipid species by chain length and saturation by generating heat maps of $\log_2$-transformed levels of lipids selected by PLS-DA (Fig. 3). TAGs and DAGs of all chain lengths and saturation were elevated in obese vs lean groups (see Fig. 3A). When we examined complex lipids, we found that the obese group was characterized by higher levels of free fatty acids, dihydroceramides, and ceramides of all chain lengths and saturation, and lower levels of cholesterol esters, hexosylceramides, lactosylceramides, lysophosphatidylcholines, lysophosphatidylethanolamines, phosphatidylcholines, phosphatidylethanolamines, and phosphatidylinositols (see Fig. 3B).

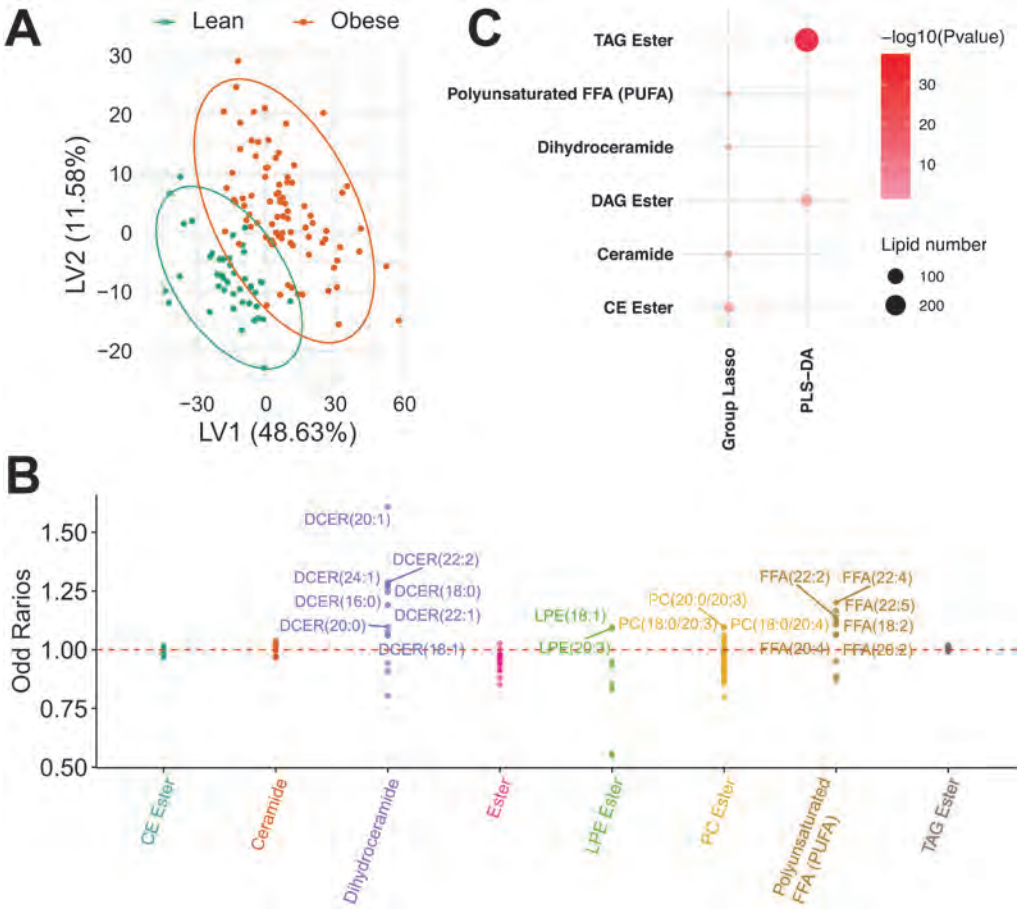

Figure 2. Lipidomics analysis in obese vs lean participants. A, Partial least squares–discriminant analysis (PLS-DA) partly separated obese (red, n = 88) from lean (green, n = 43) participants, and selected 388 significant lipids that contributed to the separation with variable importance in projection (VIP) greater than 1. (B) Group-lasso selected 8 subpathways containing 668 significant lipids with an odds ratio (OR) greater than 1 (lipid higher in obese) or OR less than 1 (lipid lower in obese), adjusted for age and sex. PLS-DA and group-lasso both selected more lipids than metabolites (Fig. 1A and 1B) in obese vs lean comparisons. C, Pathway analysis of PLS-DA– and group-lasso–selected lipids. The circles represent selected enriched subpathways. Circle color indicates significance level from most (red) to least (lightest pink) significant. Circle size represents the number of selected lipids belonging to the enriched subpathways.

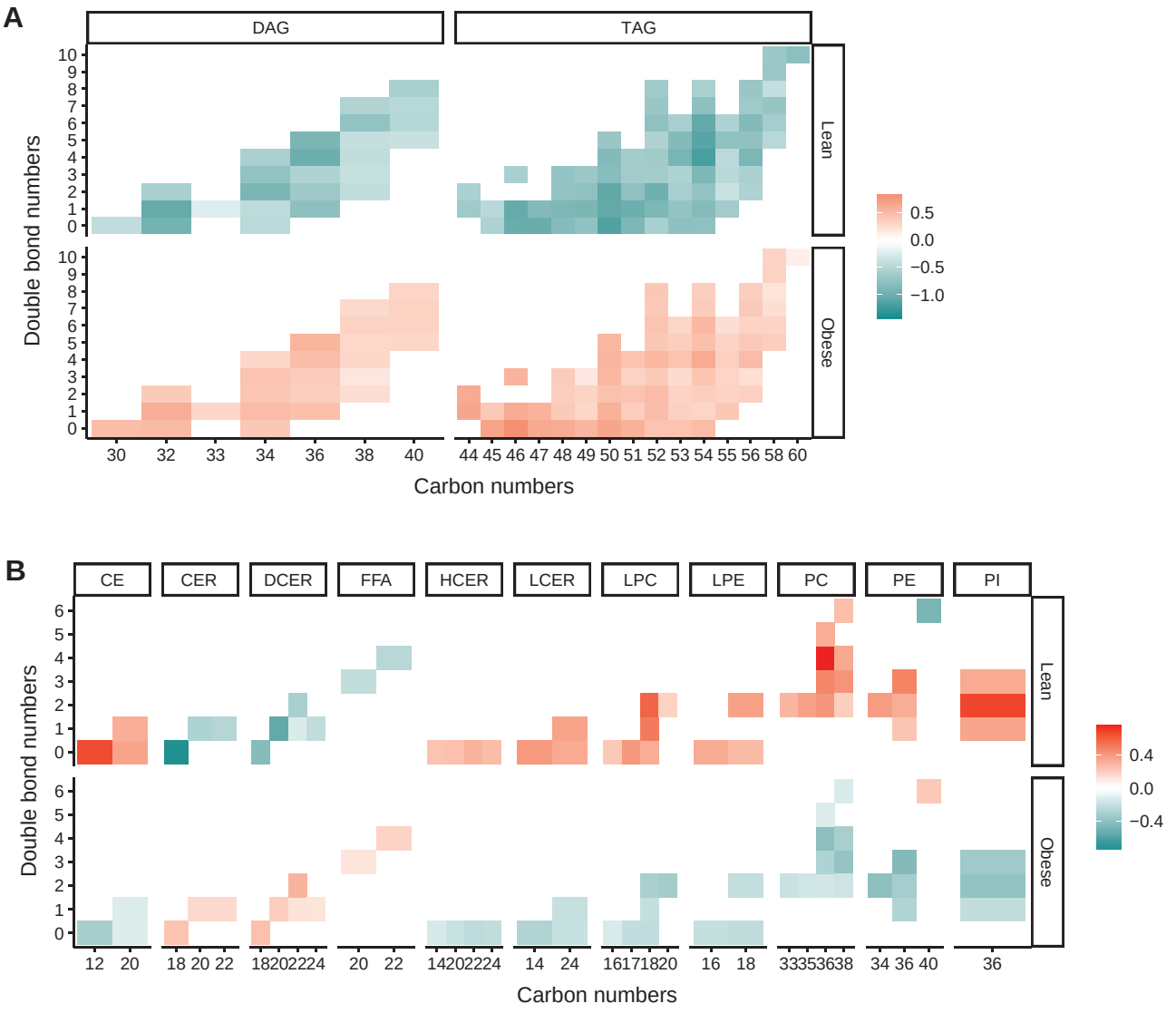

Figure 3. Lipid abundance heat maps by chain lengths and saturation in obese vs lean participants. Heat maps of log_2-transformed abundances of lipids selected by partial least squares–discriminant analysis (PLS-DA) for A, triacylglycerols (TAGs) and diacylglycerols (DAGs); and B, cholesterol esters (CE), ceramides (CER), dihydroceramides (DCER), free fatty acids (FFA), hexosylceramides (HCER), lactosylceramides (LCER), lysophosphatidylcholines (LPC), lysophosphatidylethanolamines (LPE), phosphatidylcholines (PC), phosphatidylethanolamines (PE), and phosphatidylinositols (PI).

Plasma Metabolomics Differ in Obese Groups With and Without Peripheral Neuropathy vs Lean Controls

We next stratified the obese group by PN status and examined how obese participants with and without PN differed in plasma metabolomic profile compared to lean controls. Descriptive analyses for obese with PN vs lean and obese without PN vs lean were conducted by Wilcoxon and logistic regression (19). As expected in both instances, there was a good separation of obese with PN vs lean and obese without PN vs lean by PLS-DA (19); however, the 2 obese groups did not differ from lean participants in all the same metabolites (19). Group-lasso selected 45 and 46 statistically significant metabolites in obese with PN vs lean and obese without PN vs lean comparisons, respectively (19). In subpathways, obese with PN and obese without PN both differed from lean controls most significantly in "gamma-glutamyl amino acid," as expected (19), and were discordant in only a few subpathways.

Plasma Lipidomics Differ in Obese Groups With and Without Peripheral Neuropathy vs Lean Controls

Descriptive analyses by Wilcoxon and logistic regression were conducted to identify lipid differences for obese with PN vs lean and obese without PN vs lean (19). When we

compared obese groups by PN status to lean controls, we again did not see a complete group separation by lipids by PLS-DA but when compared to the number of metabolites, more lipids had VIP greater than 1 (19). Lipid species differing between obese with PN vs lean were highly shared with obese without PN vs lean. Group-lasso selected 670 and 165 statistically significant lipids in obese with PN vs lean and obese without PN vs lean comparisons, respectively (19). In obese with PN vs lean, "TAG ester" by PLS-DA was most statistically significant and encompassed the most lipid species; in obese without PN vs lean, "DAG ester" (group-lasso) was most statistically significant while "TAG ester" (PLS-DA) comprised the most lipids (19).

Plasma Metabolomic and Lipidomic Profiles Separate Obese Groups With and Without Peripheral Neuropathy

We next examined the metabolomic and lipidomic profiles of obese individuals with vs without PN (descriptive statistics in [19]). PLS-DA separated the 2 groups based on metabolites and lipids (Figs. 4A-5A) (19), but not to the same extent as obese vs lean individuals (Figs. 1A-2A), suggesting fewer differences in plasma metabolome and lipidome by PN status as opposed to obesity status. PLS-DA (VIP > 1) (19) and group-lasso (OR > 1 or OR < 1) selected 218 and 58 metabolites, respectively, and 256 and 249 lipids, respectively (Figs. 4B-5B) (19). By subpathway, "benzoate metabolism" contained the largest metabolite numbers and greatest statistical significance by PLS-DA and group-lasso overlap (Fig. 4C), but other subpathways were also mutually selected. The lipid subpathways, most significantly different by PN status, were primarily selected by group-lasso and comprised "DAG ester" and "PC (phosphatidylcholine) ester" (Fig. 5C). "Sphingomyelin" and "ceramide" subpathways differentiated PN status by both PLS-DA and group-lasso. Overall, complex lipid subpathways were most discriminating for obese participants with vs without PN.

We also examined lipid species by chain length and saturation using heat maps of abundances of lipids selected by PLS-DA (Fig. 6). Saturated TAGs (no double bonds) and highly polyunsaturated and longer-chain TAGs tended to be higher in obese participants with PN vs without PN (see Fig. 6A). Among the complex lipids, obese participants with PN had lower ceramide and sphingomyelin levels across the whole spectrum of chain lengths and saturation vs obese participants without PN (see Fig. 6B). Trends in dihydroceramides differed across the 2 obese groups; dihydroceramides of lower carbon and double bond numbers were elevated in obese participants without PN, whereas dihydroceramides with more double bonds were elevated in obese participants with PN.

Effect of Prediabetes and Type 2 Diabetes Status on Peripheral Neuropathy Status

There were no differences in prediabetes or T2D prevalence in the obese group with vs the obese group without PN because we matched participants for glycemic status (see Table 1). However, there were also no differences in basic lipid profiles (cholesterol, TAGs, HDL-c, LDL-c) between either obese group by PN status, although there were overall distinctions in total complex lipid profiles. Therefore, despite matching for prediabetes and T2D in the 2 obese groups, we reanalyzed the data stratified by glycemic status to evaluate if there was an influence from plasma metabolomic and lipidomic profile in the prediabetes and T2D setting compared to the overall obesity profiles on PN separation. First, we examined differential metabolite/lipid abundance by Wilcoxon within the obese groups, using a cutoff of adjusted *P* less than .05. By T2D status, 12 metabolites differed in obese participants with vs without T2D, of which 10 were shared with the 331 metabolites that differed in the obese vs lean comparison, and 2 were unique (annotated as "shared" and "unique" in [19]). Glucose was among the shared metabolites and was elevated in obese vs lean participants and in obese with vs without T2D, as anticipated. There were no differential metabolites by prediabetes status, and no differential lipids by either prediabetes or T2D status. Therefore, within the obese group, few metabolites differed in abundance by T2D status, but many differed from lean participants. When we examined by PN status, within either the obese groups with vs without PN, there were no differential metabolites or lipids by either prediabetes or T2D status. Overall, prediabetes and T2D status only marginally affected metabolite and lipid abundance by Wilcoxon.

Next, we examined group clustering by unsupervised PCA of the entire (obese and lean) cohort, stratifying obese participants, both with and without PN, by prediabetes and T2D status. We found that PCA of metabolites or lipids clustered obese participants with or without T2D together but still separated the obese participants (either with/without T2D) from lean participants (19). Moreover, there was no separation of participants with vs without T2D by PN status. Thus, in this highly obese sample of individuals (average class 3 obesity, average BMI 44.04) composed of normoglycemic, prediabetic, and diabetic participants, metabolic and lipidomic profiles by obesity status can separate participants from lean controls, independent of the glycemic state. As expected, we also determined that PCA of metabolites or lipids did not separate obese participants with or without prediabetes (19). As with the T2D analysis, there was no separation of participants with vs without prediabetes by PN status. Overall, at least by

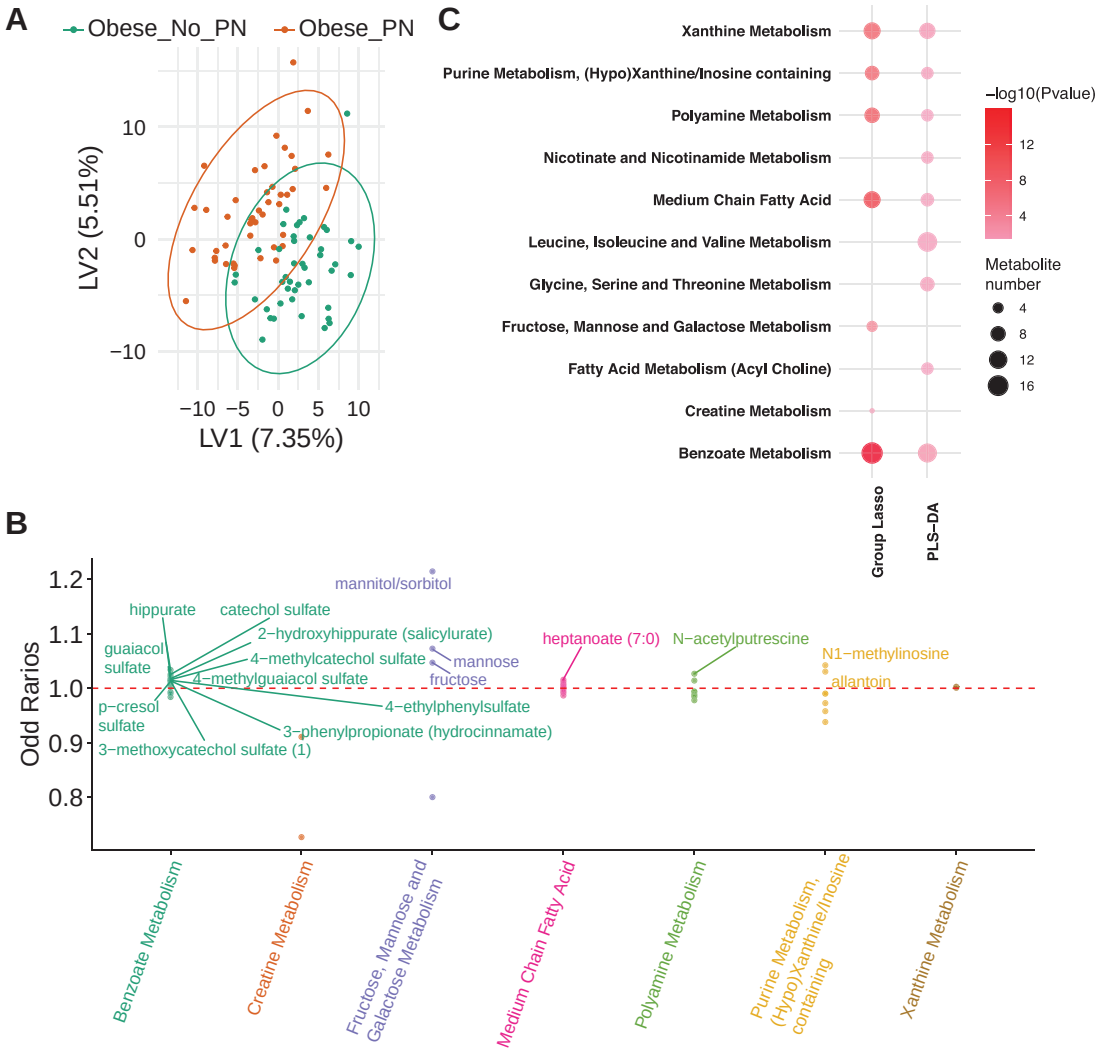

Figure 4. Metabolomics analysis in obese_PN vs obese_No_PN participants. A, Partial least squares–discriminant analysis (PLS-DA) partly separated obese_PN (red, n = 44) from obese_No_PN (green, n = 44) participants, and selected 218 significant metabolites that contributed to the separation with variable importance in projection (VIP) greater than 1. B, Group-lasso selected 7 subpathways containing 58 significant metabolites with an OR greater than 1 (metabolite higher in obese) or OR less than 1 (metabolite lower in obese), adjusted for age and sex. C, Pathway analysis of PLS-DA– and group-lasso–selected metabolites. The circles represent selected enriched subpathways. Circle color indicates significance level from most (red) to least (lightest pink) significant. Circle size represents the number of selected metabolites belonging to the enriched subpathways. The subpathways also encompass several lipid pathways because the global metabolomics analysis detects some lipids.

plasma metabolomic and lipidomic profiles, obesity and T2D differentiate PN status to similar extents in a sample of individuals with average class 3 obesity.

Plasma Metabolomic and Lipidomic Correlation Network

O2PLS integrated metabolites with lipids to construct a metabolite-lipid correlation across the obese vs lean comparison (Fig. 7) (19). The metabolite-lipid correlation network was constructed from correlations between the 50 metabolites and 50 lipids with the highest loading values. These 50 metabolites and 50 lipids generated 2500 statistically significant correlations (adjusted $P < .05$), of which the top 100 correlations are represented in the correlation network, which contains 13 metabolites and 50 lipids (Fig. 7). Candidates from the most statistically significant metabolite subpathways "gamma-glutamyl amino acid" (ie, gamma-glutamyl valine, gamma-glutamyl leucine) and "leucine, isoleucine and valine metabolism" (ie, leucine, 1-carboxyethylvaline) correlated to lipids from the most statistically significant subpathways lipid pathway "TAG ester." Spearman correlation was performed between metabolites and lipids in the obese group with vs the obese group without PN comparisons (Fig. 8). A cluster of positive correlations occurred between ceramides, dihydroceramides, and sphingomyelins with 2-hydroxynervonate, β-hydroxyisovalerate, 2-hydroxymyristate, α-ketobutyrate, and

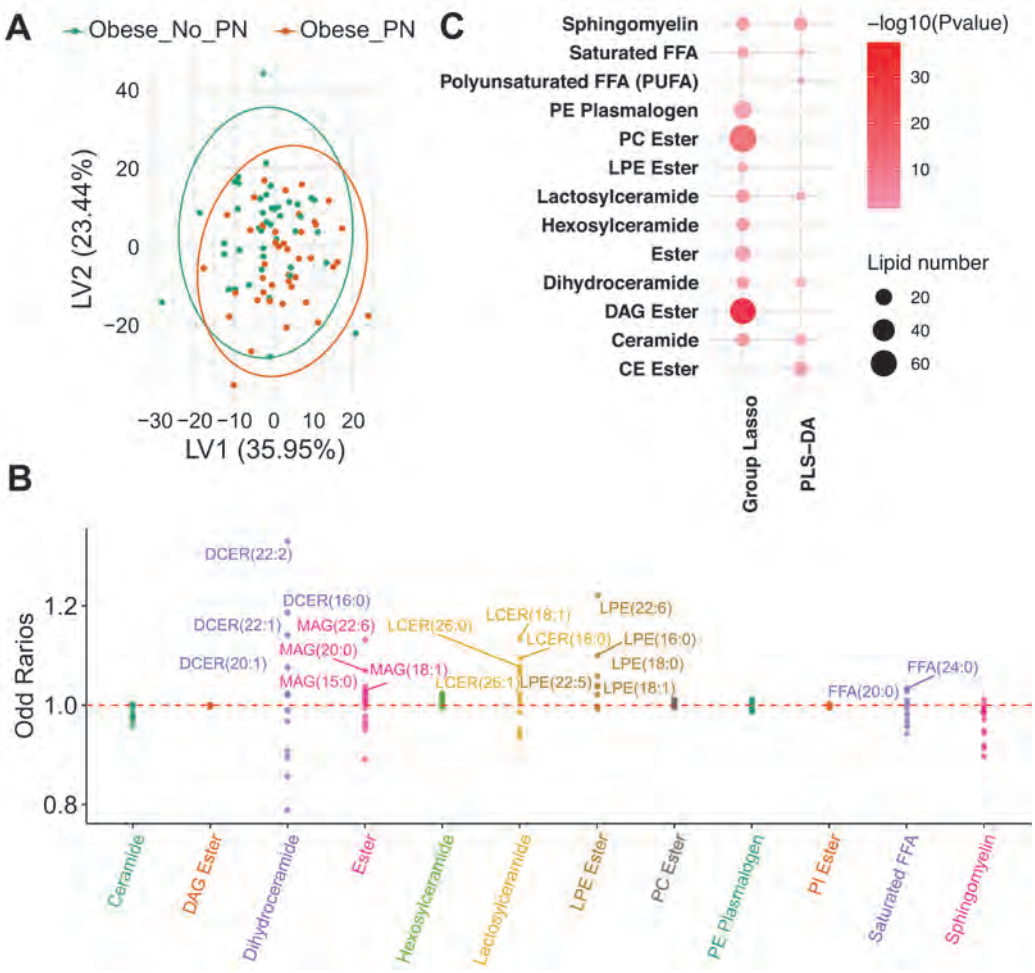

Figure 5. Lipidomics analysis in obese_PN vs obese_No_PN participants. A, Partial least squares–discriminant analysis (PLS-DA) did not separate obese_PN (red, n = 44) from obese_No_PN (green, n = 44) participants, but selected 256 significant lipids that differed between groups with variable importance in projection (VIP) greater than 1. B, Group-lasso selected 12 subpathways containing 249 significant lipids with an odds ratio (OR) greater than 1 (lipid higher in obese) or OR less than 1 (lipid lower in obese), adjusted for age and sex. C, Pathway analysis of PLS-DA– and group-lasso–selected lipids. The circles represent selected enriched subpathways. Circle color indicates significance level from most (red) to least (lightest pink) significant. Circle size represents the number of selected lipids belonging to the enriched subpathways.

2-hydroxybutyrate/2-hydroxyisobutyrate, which negatively correlated with other complex lipids.

Discussion

In the present observational study, we performed plasma global metabolomics and lipidomics to identify species and pathways correlating with PN status in a sample of individuals with average class 3 obesity. Since our focus was obesity as a PN risk factor, we matched our obese groups with and without PN for glycemic status before our data analysis. However, we also conducted a post hoc subanalysis by prediabetes and T2D since dysglycemia remains a major risk factor for PN. The obese participants as a whole exhibited the anticipated characteristics vs the lean participants, for example, elevated body weight, BMI, WC, systolic blood pressure, and TAGs, as well as lower HDL-c. WC was higher in obese participants with vs without PN, as anticipated (10, 11), as was body weight. Otherwise, there were no additional demographic differences, allowing us to identify specific metabolic and lipidomic signatures underlying PN status in obese individuals.

As a first step, we examined the metabolites and lipid species that differed between the obese vs lean groups. We focused on subpathways because metabolites/lipids can exist within more than one network, which are further interconnected. The most prominent subpathway differentiating obese and lean individuals, selected by both PLS-DA and group-lasso, was "gamma-glutamyl amino acid," which belongs to the gamma-glutamyl cycle responsible for glutathione antioxidant synthesis and degradation (28). Gamma-glutamyl transferase, a key gamma-glutamyl cycle enzyme, correlates positively with TAGs, BMI, and blood pressure and is linked to oxidative stress in obesity

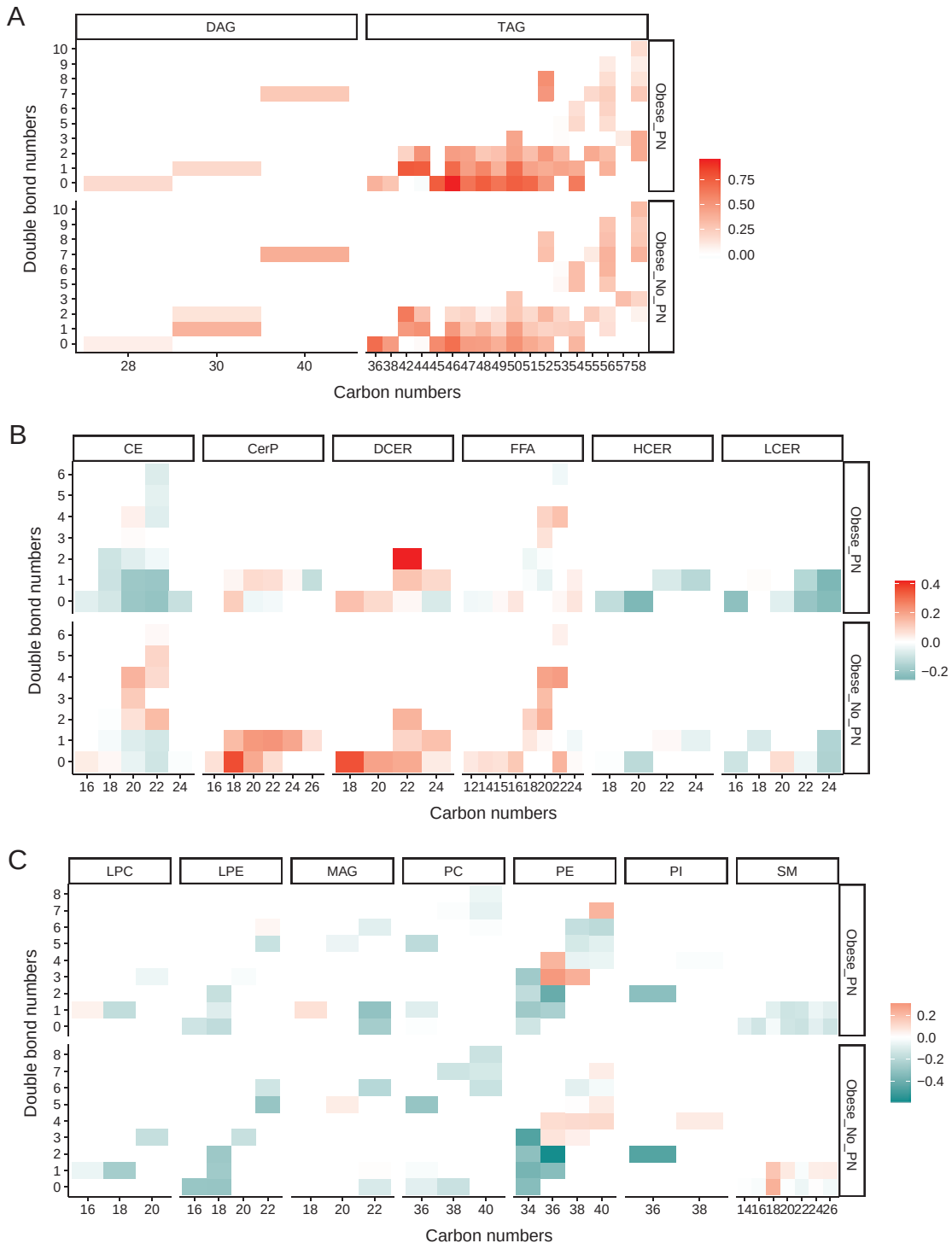

Figure 6. Lipid abundance heat maps by chain lengths and saturation in obese_PN vs obese_No_PN participants. Heat maps of $\log_2$-transformed abundances of lipids selected by partial least squares–discriminant analysis (PLS-DA) for A, triacylglycerols (TAGs) and diacylglycerols (DAGs); B, cholesterol esters (CE), ceramides (CER), dihydroceramides (DCER), free fatty acids (FFA), hexosylceramides (HCER), lactosylceramides (LCER); and C, lysophosphatidylcholines (LPC), lysophosphatidylethanolamines (LPE), monoacylglycerols (MAG), phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylinositols (PI), and sphingomyelins (SM).

and MetS (29) and also potentially to T2D (30). Other emergent pathways when comparing the metabolome of obese vs lean participants were related to "leucine, isoleucine and valine metabolism" (or BCAA metabolism), as well as BCAA related to fatty acid metabolism. BCAA metabolism is centered around protein synthesis (through mechanistic target of rapamycin [mTOR]), glucose regulation, neurotransmission modulation, adiposity, and satiety,

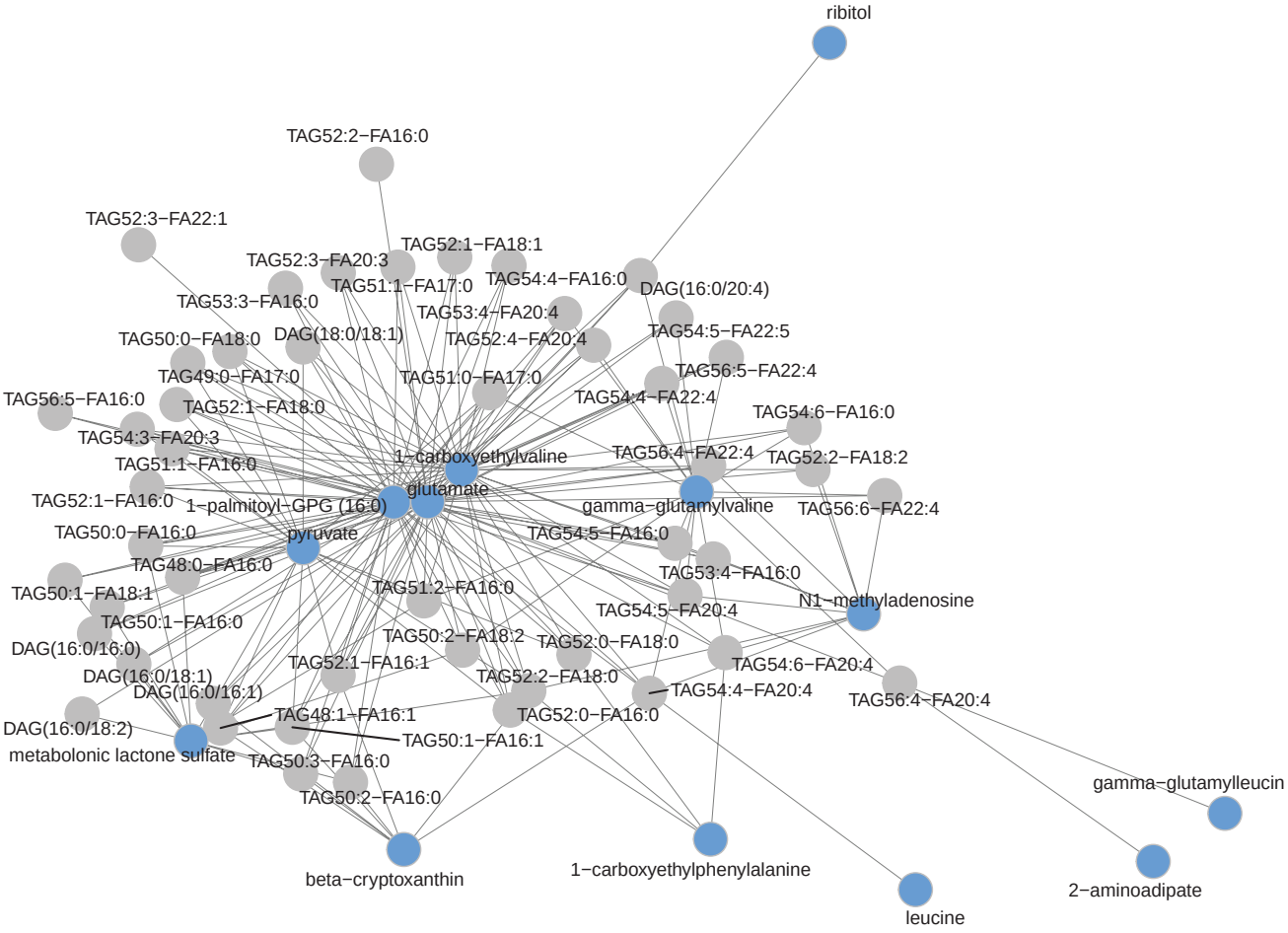

Figure 7. Integrated metabolomics and lipidomics in obese vs lean groups. An integrative method, 2-way orthogonal partial least squares (O2PLS), was used to construct a metabolite-lipid correlation network using correlations between the 50 lipids and 50 metabolites with the highest loading values. These 50 metabolites and 50 lipids generated 2500 statistically significant correlations (adjusted $P < .05$), of which the top 100 correlations are represented in the correlation network, which contains 13 metabolites (blue circles) and 50 lipids (gray circles). Candidates from the most significant metabolite subpathways "gamma-glutamyl amino acid" (ie, gamma-glutamyl valine, gamma-glutamyl leucine) and "leucine, isoleucine and valine metabolism" (ie, leucine, 1-carboxyethylvaline) correlated to lipids from the most significant subpathways lipid pathway "TAG ester".

and is linked to obesity, where it correlates with insulin resistance and T2D-onset risk (31). Plasma metabolomics of participants of the Susceptibility to Particle Health Effects, miRNAs and Exosomes study (n = 1391) identified the highest association of BMI with aromatic amino acid and BCAA metabolism, after adjusting for age, sex, and smoking status (32). An integrative metabolomics and genome-wide association study in a population of Korean women (n = 77) concluded elevated BMI disrupted BCAA and aromatic amino acid catabolism, as well as β-oxidation, lipid metabolism, urea cycle, and purine/pyrimidine metabolism (33). Finally, our global metabolomics identified a number of fatty acid metabolism pathways that differed by obese vs lean status, as anticipated.

When we specifically examined lipids between the obese groups and the lean group, the largest and most significant pathway selected was "TAG ester," followed by "DAG ester." This agrees with the basic blood lipid profiles from our obese groups, which were higher in TAGs specifically containing long-chain fatty acids vs the lean group. Interestingly, group-lasso did not select the TAG subpathway, possibly because it contains the most candidates, at 518, and there may not have been sufficient significant TAGs for group-lasso to select the entire subpathway. On the other hand, group-lasso, which places greater emphasis on subpathway structure than PLS-DA, identified subpathways that were more centered on complex lipids, such as ceramides and dihydroceramides, as well as cholesteryl esters.

Our findings are aligned with several published studies, which have also investigated the correlation of plasma lipidomics signatures with obesity metrics, such as BMI, WC, and insulin resistance. Lipidomics analysis of plasma from participants enrolled in the Australian Diabetes, Obesity and Lifestyle Study (n = 10 339) and validated in the Busselton Health Study (n = 4207) found 577 lipids

Figure 8. Correlation analysis of metabolomics to lipidomics in obese_PN vs obese_No_PN groups. Spearman correlation was performed between metabolites (along the vertical y axis) and lipids (along the horizontal x axis) in the obese_PN vs obese_No_PN comparisons. Heat map shows significant correlations (adjusted $P < .05$) for positive (red, strongest with the value of 1) and negative (blue, strongest with the value of −1) correlations. A cluster of positive correlations occurs between CERs, DCERs, and SMs with 2-hydroxynervonate, β-hydroxyisovalerate, and 2-hydroxymyristate, which are negatively correlated with other lipid species. Additionally, CERs, DCERs, and SMs positively correlate with α-ketobutyrate and 2-hydroxybutyrate/2-hydroxyisobutyrate, which are negatively correlated with phospholipids (PCs, PEs), CEs, and LPEs. CE, cholesterol ester; CER, ceramide; DCER, dihydroceramide; FFA, free fatty acid; LCER, lactosylceramide; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamines; SM, sphingomyelin; TAG, triacylglycerol.

correlated with BMI, after adjusting for age and sex (34). Differential lipids dropped to 508, after additionally adjusting for total cholesterol, HDL-C, and triglycerides. TAGs and sphingolipids, especially shorter-chain ceramides and sphingomyelins, positively correlated with BMI, whereas gangliosides and hexosylceramides correlated negatively. In particular, Cer (18:1/18:0) and Cer (18:1/20:0) increased proportionately with BMI (both $P < 10^{-10}$) (34); similarly, we had a statistically significant increase in Cer (18:1/18:0) and Cer(18:1/20:0) in obese participants with P values of 3.53×10^{-10} and 1.27×10^{-4}, respectively, by Wilcoxon, along with several other statistically significant shorter-chained ceramides. These findings were mirrored in a cohort of 2302 ethnically Chinese Singaporeans, where ceramides associated positively with BMI, central obesity, measured by WC, and homeostatic model assessment of insulin resistance (HOMA-IR), whereas hexosylceramides associated negatively, after adjusting for age, sex, HDL-c, LDL-c, and triglycerides (35). Associations were also chain length and saturation dependent; as in other studies, shorter-chain ceramides, including Cer (18:1/18:0) and Cer (18:1/20:0), were elevated with BMI. In contrast, a smaller study (n = 28, BMI > 25; n = 23 lean) concluded a plasma lipidomic signature of higher BMI was characterized by elevated TAGs and lower plasmalogens, and lacking any association with sphingolipids (36). A plasma TAG signature also correlates with insulin resistance and T2D (37).

Our main interest focused on global metabolomic and complex lipidomic differences in obese participants by PN status. With regard to the metabolome, we identified 4 unique metabolomics signatures that are associated with PN in obese participants, "purine metabolism," "polyamine metabolism," "benzoate metabolism," and "xanthine metabolism." Purine metabolism is centered on the breakdown of adenosine monophosphate and guanosine monophosphate, which are involved in cellular energy metabolism and adenosine 5′-triphosphate synthesis. In alignment with these data, we previously identified altered nerve bioenergetics and decreased adenosine 5′-triphosphate levels (38-40) associated with PN in mouse models of prediabetes and T2D and in cultured primary sensory neurons treated with saturated fatty acids (41). Polyamines, such as spermidine, are part of "polyamine metabolism" and have proautophagy, immunomodulatory, and neuroprotective properties, and are present in high levels in the brain, where they sustain neuronal health (42). Polyamines have not been well studied in PN, although supplementation of polyamine biosynthesis precursors, L-arginine (43) and agmatine (44), are beneficial in treating neuropathic pain in diabetes rodent models. Two xenobiotics superpathways were identified in "benzoate metabolism," possibly from benzoate additives in food, and "xanthine metabolism," which contains several natural products and metabolized byproducts from coffee and tea, as well as pharmaceutical medications. Why metabolites from these pathways are associated with PN in only our obese group is unknown. One intriguing possibility lies in the idea that these food additives and byproducts alter the microbiome, which in turn is associated with PN (45), an area of active research by several groups (46, 47).

When examining distinct lipidomic signatures, there were more signaling and complex lipid classes associated with the obese group with PN. These results suggest that signaling dysregulation and complex, and possibly lipotoxic, lipids may in part underlie PN onset and progression. Among the largest and most significant signaling lipids were the DAGs, bioactive lipids that participate in several neurovascular mechanisms, such as blood flow and conduction velocity, through protein kinase C (PKC) activation (48). While earlier clinical trials of PKC inhibitors failed in the treatment of PN in diabetes cohorts (49), more recent efforts have focused on selective inhibition of different PKC isoforms for treating PN (50). Among the complex lipids, "ceramide," "dihydroceramide," "lactosylceramide," and "sphingomyelin" are associated with obese participants with PN vs those obese participants without PN. This is of significant interest, and aligned with our prior studies, which found sphingolipid levels were altered in obese individuals with PN (51, 52).

Ceramides and dihydroceramides are a family of bioactive lipids that modulate apoptosis, senescence, and stress responses (53). As such, they are lipotoxic and accumulate in tissues or plasma during obesity and insulin resistance (54). Ceramide biosynthesis occurs through multiple pathways (53); however, under conditions of excessive dietary TAG intake, de novo synthesis may dominate (55), leading to an increase in dihydroceramides. Ceramides and dihydroceramides are linked to PN in patients with diabetes (56), and could be recurrent features in neuropathies, generally, for example, in hereditary neuropathies, such as in hereditary sensory and autonomic neuropathy type 1 (52). When comparing T2D participants with and without PN, we previously observed trends in ceramide acyl chain lengths (51), which influence their biological properties (57). The contributions of the various ceramide and dihydroceramide species length and saturation that contributed to PN status in this sample of individuals with average class 3 obesity are a future area of interest, along with a greater understanding of these lipotoxic lipids in prediabetes, T2D, and PN.

Sphingomyelins are important constituents of myelin-ensheathed nerves; however, they are less studied in obesity and diabetes, and associated PN (58). One study found higher sphingomyelins in cerebrospinal fluid from

participants with acquired demyelinating peripheral neuropathies compared to axonal neuropathies (59). In neuropathic sural nerve, a loss of long-chain sphingomyelins occurs compared to healthy nerve and brain tissue (60). As with ceramides and dihydroceramides, the discovery of significant sphingomyelin species associated with PN opens a new avenue of research aimed at understanding PN pathogenesis.

When we integrated metabolites with lipids to build an O2PLS correlation network for all obese participants (both groups) vs the lean group, the most significant metabolite subpathways gamma-glutamyl amino acid (ie, gamma-glutamyl valine, gamma-glutamyl leucine) and BCAA (ie, leucine, 1-carboxyethylvaline) correlated to lipids from the most significant lipid subpathways, TAGs, suggesting that global dysregulation of both the metabolome and lipidome occurred in concert in all obese participants. When we performed Spearman correlation analyses between metabolites and lipids in the obese groups based on PN status, we found a cluster of positive correlations between ceramides, dihydroceramides, and sphingomyelins to 2-hydroxy fatty acids, namely 2-hydroxynervonate, β-hydroxyisovalerate, and 2-hydroxymyristate. 2-Hydroxy fatty acids condense with ceramides and sphingolipids to generate 2-hydroxy fatty acid–ceramides and 2-hydroxy fatty acid–sphingolipids, which are important for normal neural function (61). Additionally, ceramides, dihydroceramides, and sphingomyelins positively correlated with α-ketobutyrate and 2-hydroxybutyrate. α-Ketobutyrate is the oxidized form of α-hydroxybutyrate, which is linked to insulin resistance and impaired glucose regulation, particularly in nondiabetes populations, and could constitute a link to dyslipidemia (62).

Our study has limitations. First, although it involved a real-life cohort, which is a strength, most participants were on statins to manage their hyperlipidemia, which affected their basic plasma lipid profiles, for example, cholesterol. Thus, data analysis could place a greater emphasis on accounting for medication use, that is, statins, β-blockers, antidiabetic medications. However, since statin use does not affect PN status in T2D (27) and was evenly balanced between both obese groups with and without PN in the present study, we anticipate that the identified metabolites and lipids present in the obese PN participants represent an association with PN. In addition, though our primary focus was on the effects of obesity on PN, insulin resistance and hyperglycemia are frequently comorbid conditions with obesity in patients. However, our study was limited by a lack of sensitive measures of insulin resistance, like HOMA-IR. Sex and age differences were not evaluated in this study, which could affect the plasma lipidome (63-66). Sex and age differences could be relevant to this study, particularly regarding the sex and age imbalance in lean vs obese participants, though our logistic regression and group-lasso models accounted for these factors as covariates. Importantly, the study also faced a tissue issue, since plasma may not be representative of nerve tissue-specific metabolome and lipidome differences, which is not an accessible tissue in human participants. Additionally, the study was not longitudinal, rendering it difficult to make causal inferences. Furthermore, this study was in individuals with severe obesity, and whether these results generalize to other populations is unknown.

Finally, our sample size included 88 obese individuals and 43 lean controls, which likely limited our power to detect small differences in metabolites and lipids between groups with vs without PN. This was even more pronounced in our subanalysis by prediabetes (n = 15) and T2D (n = 43) status. Analysis of larger cohorts may find that metabolic and lipidomic profiles in T2D may outperform obesity profiles for PN separation. Although we statistically accounted for the number of comparisons, their sheer number, due to a large number of detected metabolites and lipids relative to the number of study participants could led to some false associations. Future studies are needed to confirm our results.

In conclusion, when compared to lean individuals, this observational study identified differences in the metabolome and lipidome of a sample of individuals with average class 3 obesity, especially gamma-glutamyl amino acid and BCAA metabolism and TAG lipid metabolism. When obese individuals were stratified by PN status, possible novel research avenues emerged in the metabolome with polyamine biosynthesis, as well as better established defects in bioenergetics through purine biosynthesis. With respect to the lipidome, bioactive and complex lipids distinguished the obese group with PN from the obese group without PN. It will be critical, moving forward, to identify trends in chain length and saturation level of specific bioactive lipid classes and particular species that contribute to PN. We anticipate lipid signaling and complex lipids may be exciting avenues of investigation for PN associated with metabolic dysfunction, that is, obesity, prediabetes, and T2D. Inhibitors of complex lipid signaling, such as ceramides (67, 68), are an active area of research as antiobesity, anti-T2D, and anti-insulin resistance therapies, highlighting the potential significance of this research direction in PN.

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A High-Fat Diet Disrupts Nerve Lipids and Mitochondrial Function in Murine Models of Neuropathy

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As the prevalence of prediabetes and type 2 diabetes (T2D) continues to increase worldwide, accompanying complications are also on the rise. The most prevalent complication, peripheral neuropathy (PN), is a complex process which remains incompletely understood. Dyslipidemia is an emerging risk factor for PN in both prediabetes and T2D, suggesting that excess lipids damage peripheral nerves; however, the precise lipid changes that contribute to PN are unknown. To identify specific lipid changes associated with PN, we conducted an untargeted lipidomics analysis comparing the effect of high-fat diet (HFD) feeding on lipids in the plasma, liver, and peripheral nerve from three strains of mice (BL6, BTBR, and BKS). HFD feeding triggered distinct strain- and tissue-specific lipid changes, which correlated with PN in BL6 mice versus less robust murine models of metabolic dysfunction and PN (BTBR and BKS mice). The BL6 mice showed significant changes in neutral lipids, phospholipids, lysophospholipids, and plasmalogens within the nerve. Sphingomyelin (SM) and lysophosphatidylethanolamine (LPE) were two lipid species that were unique to HFD BL6 sciatic nerve compared to other strains (BTBR and BKS). Plasma and liver lipids were significantly altered in all murine strains fed a HFD independent of PN status, suggesting that nerve-specific lipid changes contribute to PN pathogenesis. Many of the identified lipids affect mitochondrial function and mitochondrial bioenergetics, which were significantly impaired in *ex vivo* sural nerve and dorsal root ganglion sensory neurons. Collectively, our data show that consuming a HFD dysregulates the nerve lipidome and mitochondrial function, which may contribute to PN in prediabetes.

Keywords: dyslipidemia, prediabetes, mitochondria, obesity, neuropathy, lipidomics, high-fat diet, metabolic syndrome

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Abbreviations: CL, cardiolipin; DG, diacylglycerol; DGAT2, diacylglycerol acyltransferase 2; DRG, dorsal root ganglion; FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenyl-hydrazone; HFD, high-fat diet; IENFD, intraepidermal nerve fiber density; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; NCV, nerve conduction velocity; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; plasmenyl-PE, plasmenyl-phosphatidylethanolamine; plasmenyl-PC, plasmenylphosphatidylcholine; PLS-DA, Partial least squares-discriminant analysis; PN, peripheral neuropathy; PS, phosphatidylserine; SM, sphingomyelin; TG, triglyceride; T2D, type 2 diabetes; VIP, variable importance in projection.

INTRODUCTION

Peripheral neuropathy (PN) is a common and highly morbid complication of prediabetes and type 2 diabetes (T2D) (Feldman et al., 2019). PN presents as a distal to proximal loss of sensation in the extremities with pain as a frequent feature (Feldman et al., 2019). While the pathogenesis of PN is incompletely understood, impaired peripheral nervous system bioenergetics under conditions of excess energy substrate is a central characteristic of PN (Feldman et al., 2017). In parallel, recent clinical studies highlight components of the metabolic syndrome as PN risk factors (Callaghan et al., 2016a; Callaghan et al., 2016b), suggesting lipids, including triglycerides (TGs), contribute to peripheral nervous system energy overload (Wiggin et al., 2009; Andersen et al., 2018).

Murine models of diet-induced obesity develop features of prediabetes and PN like that seen in humans; however, the genetic background of each mouse strain affects the degree of metabolic dysfunction and type of nerve fibers affected (Montgomery et al., 2013). Large nerve fibers confer proprioceptive information related to position and movement whereas small afferent Aδ fibers and unmyelinated C-fibers are responsible for temperature, pain, and nociceptive sensations. Prediabetes and T2D PN result from a combination of large and small fiber dysfunction. We recently reported the effects of high-fat diet (HFD) feeding on three mouse strains (BL6, BTBR, and BKS). Mice on the BL6 background gained weight throughout the 36-weeks study and developed features of the metabolic syndrome as well as large and small fiber PN similar to what is observed in humans with prediabetes (Hinder et al., 2017). In contrast, HFD-fed BTBR mice developed large fiber PN only and gained weight at the same rate as standard diet (SD)-fed BTBR for the first 24 weeks of the study. The final strain of mice fed a HFD, the BKS mice, also developed large fiber PN only but required genetic manipulation of the leptin receptor to gain weight from study onset.

The goal of the current study was to assess the association between disruptions in lipid composition and PN metabolic risk factors and disease severity. Because lipid levels profoundly impact mitochondrial bioenergetics (Rumora et al., 2018), we postulated that distinct nerve lipid levels would associate with PN under varying conditions of metabolic dysfunction. We conducted untargeted lipidomics of nerve, liver and plasma from HFD-fed BL6, BTBR and BKS mice and observed distinct changes in nerve, liver and plasma lipids in all three strains. Unique changes in mitochondrial lipid levels were observed within the nerves of HFD-fed BL6 mice with PN, the only strain that developed both large and small nerve fiber dysfunction. Further evaluation of mitochondrial bioenergetics in *ex vivo* sural nerves and sensory dorsal root ganglion (DRG) neurons from BL6 animals showed impaired mitochondrial bioenergetics, suggesting a role for nerve-specific lipid signatures in the pathogenesis of PN.

MATERIALS AND METHODS

Mouse Model Description

Mouse strains included i) BKS-*wt* (C57BLKS/J #000662, Jackson laboratory, Bar Harbor, ME), ii) B6-*wt* (C57BL/6J #000664, Jackson Laboratory), and iii) BTBR-*wt* (BTBR T+ Itpr3tf/J #002282, Jackson Laboratory). Mice from each strain were randomly assigned to two groups at 4 weeks of age and fed either a standard diet (SD) (#D12450-B, 10% kcal fat, Research Diets, New Brunswick, NJ) or a 54% HFD (#05090701, 54% kcal fat from lard, Research Diets) for 32 weeks, leading to six groups of male mice with 12 mice/group (HFD BKS, SD BKS, HFD B6, SD B6, HFD BTBR, SD BTBR). The fatty acid composition of each diet is provided in **Supplementary Table S1**. At the study end at 36 weeks of age, sciatic nerve, footpads, plasma, and liver samples were collected. Terminal metabolic measurements included body weight, fasting blood glucose, glucose tolerance, and glycated hemoglobin, as well as terminal neuropathy measurements including assessments of sural and sciatic nerve conduction velocities (NCV), measures of large fiber function, and intraepidermal nerve fiber density (IENFD), a measure of small nerve fiber function, were evaluated at 36 weeks of age. Plasma insulin, cholesterol, and triglycerides were also measured by Mouse Metabolic Phenotyping Centers (MMPC; Vanderbilt University, Nashville, TN; University of Cincinnati, Cincinnati, OH). All metabolic and neuropathy measurements were reported previously (Hinder et al., 2017). Herein, we conducted a follow-up untargeted lipidomics analysis on sciatic nerve, plasma, and liver from each group of mice. Mice were housed in a pathogen-free environment and animal husbandry was conducted by the University of Michigan Unit for Laboratory Animal Medicine. Animal protocols followed Diabetic Complications Consortium Guidelines (https://www.diacomp.org/shared/protocols.aspx) and were approved by the University of Michigan University Committee on Use and Care of Animals.

Untargeted Lipidomics Profiling

Four sciatic nerves from each group of mice were selected blindly and submitted to the Michigan Regional Comprehensive Metabolomics Resource Core (MRC2; www.mrc2.umich.edu) for untargeted lipidomics, which was conducted as described previously (Sas et al., 2018). Briefly, lipids were extracted from each sample (plasma, homogenized sciatic nerve, or homogenized liver) according to a modified Bligh-Dyer protocol. Purified lipids from samples and quality controls were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). LipidBlast (http://fiehnlab.ucdavis.edu/projects/LipidBlast) was used to identify lipids and MultiQuant (SCIEX, Concord, Canada) was used for lipid quantification. A total of 967 lipid species were detected within the sciatic nerve (Positive - 579; Negative—388), 1,339 lipid species were detected in the liver (Positive- 799; Negative—540), and 956 lipid species were detected in the plasma (Positive - 603; Negative—353).

Untargeted Lipidomics Data Preprocessing and Analysis

Missing values in the raw data were imputed with the K-nearest neighbor method and normalized to internal standards using the R package pamr with the function pamr. knnimpute (https://www.rdocumentation.org/packages/pamr/versions/1.55/topics/pamr.knnimpute) (Troyanskaya et al., 2001). Euclidian was used

as the distance metric (Troyanskaya et al., 2001). At least one internal standard for each lipid class was included in the analysis (**Supplementary Tables S2–S4**). Lipid species with a coefficient of variation >30% were removed and then lipid species from positive and negative ion modes were merged into a single dataset. Lipids measured in both positive and negative modes were assigned an average value from both modes. Lipid species with an odd number of carbons were removed because odd-chain lipids are rarely synthesized in mammalian systems and are typically obtained from the diet or by gut microbiota (Venn-Watson et al., 2020; Ampong et al., 2022). We also did not identify any significant changes in branched lipids in this study. To summarize lipid levels per class, the total values of lipid species in each class were summed and then $\log_2$-transformed. Heatmaps were generated to visualize the profiling pattern of each lipid class across different tissue and genetic background groups. Pearson correlation coefficients were calculated for each shared lipid species between different tissues (O'Brien et al., 2020). Lipid heatmaps were not displayed in the figures if the HFD compared to the SD had no significant impact on tissue lipid levels in a particular strain of mice.

Identifying Important Lipid Species

A *t*-test was performed for each lipid species to determine significant differences between the HFD and SD groups. Lipid species with a p-value < 0.05 were deemed significant differential lipids. Partial least squares-discriminant analysis (PLS-DA) was also performed with mixOmics package (Rohart et al., 2017), to identify lipid species that carry the greatest class-separating information, represented by the first latent variable (Brereton and Lloyd, 2014). Tenfold cross-validation was used to select the tuning parameter (the number of components) for PLS-DA with the minimal overall error rate. Once the optimal number of components was decided, the PLS-DA was refit to the full dataset to obtain the final model. Score plots were generated to illustrate the difference between HFD versus SD for each genetic background (BKS, BL6, BTBR). The variable importance in projection (VIP) score for each lipid species was calculated as a weighted sum of the squared correlations between the PLS-DA components and the original lipid species (Galindo-Prieto et al., 2014). Lipid species with a VIP score >1 were selected as the important species, which contribute highly to group separation (Cho et al., 2008). All the above analyses were performed using R v3.5 (https://www.R-project.org/).

Mitochondrial Bioenergetics Analysis

Ex vivo mitochondrial bioenergetics analysis was conducted on whole sural nerve tissue and primary DRG neurons dissected from 20-week HFD- versus SD-fed BL6 mice using an XF24 Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA, United States). Bioenergetic analysis was conducted 3–6 h post mortem for both primary DRG neuron cultures and whole sural nerve. Whole sural nerves were dissected from four mice/group, placed in optimized energetics media, and arranged on an islet capture screen (Pooya et al., 2014). For DRG neuron cultures, DRG were extracted, dissociated into a single-cell suspension, and plated on a laminin-coated Seahorse plate, as described previously (Rumora et al., 2018; Rumora et al., 2019a; Rumora et al., 2019b). Whole sural nerves were then challenged by sequential addition of mitochondrial drugs in the following order: i) 12.6 μM oligomycin, ii) 20 μM carbonyl cyanide-4-(trifluoromethoxy)phenyl-hydrazone (FCCP), and iii) 2 μM antimycin A. DRG neurons were challenged with the consecutive injection of i) 1.25 mM oligomycin, ii) 100 or 600 nM FCCP, and iii) 1 mM antimycin A. All bioenergetics measurements were recorded by the Seahorse XF analyzer and bioenergetics parameters were analyzed using mitochondrial drug response curves, as described previously (Rumora et al., 2018). Results were normalized to tissue weight and mitochondrial copy number (see below). Data analysis was conducted on GraphPad Prism using one-way ANOVA with a Tukey *post*-test for multiple comparisons, two-way ANOVA with Bonferroni *post*-test for multiple comparisons, or unpaired *t*-test (Festing and Altman, 2002).

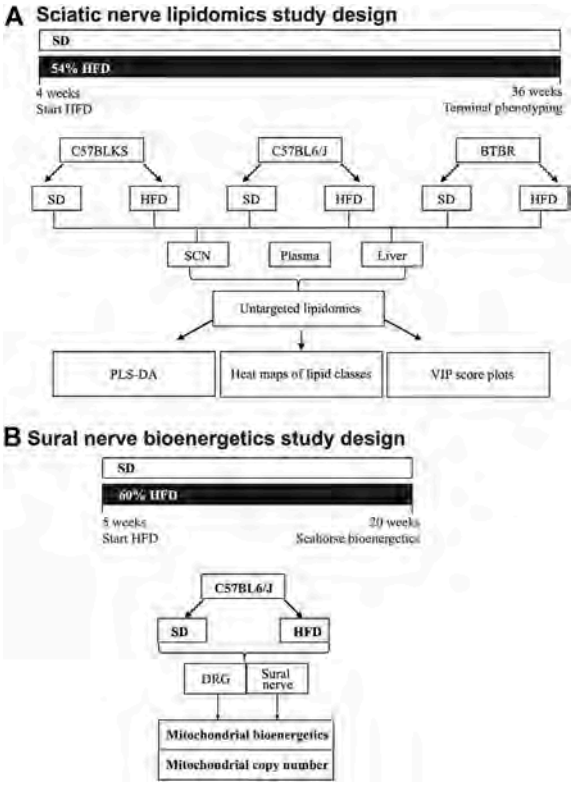

FIGURE 1 | Study paradigm and workflow for lipidomics **(A)** and mitochondrial bioenergetics analysis **(B)**. **(A)** Three strains of mice (C57BLKS/J, C57BL6/J, and BTBR) were divided into two groups per strain and fed either a standard diet (SD) or 54% high-fat diet (HFD). Mice were fed SD or HFD for 32 weeks and phenotyped for metabolic and neuropathy parameters at 36 weeks. At 36 weeks, sciatic nerve, liver, and plasma were collected and processed for LC-MS/MS lipidomics analysis. Lipidomics data were subjected a bioinformatics pipeline including PLS-DA, heat maps, and VIP score plots. **(B)** DRG neuron and sural nerve mitochondrial bioenergetics were evaluated in BL6 mice fed a SD or 60% HFD from 5 to 20 weeks. At 20 weeks of age, mitochondrial bioenergetics and mitochondrial copy number were assessed in DRG neurons and whole sural nerves from HFD BL6 mice compared to SD BL6 mice.

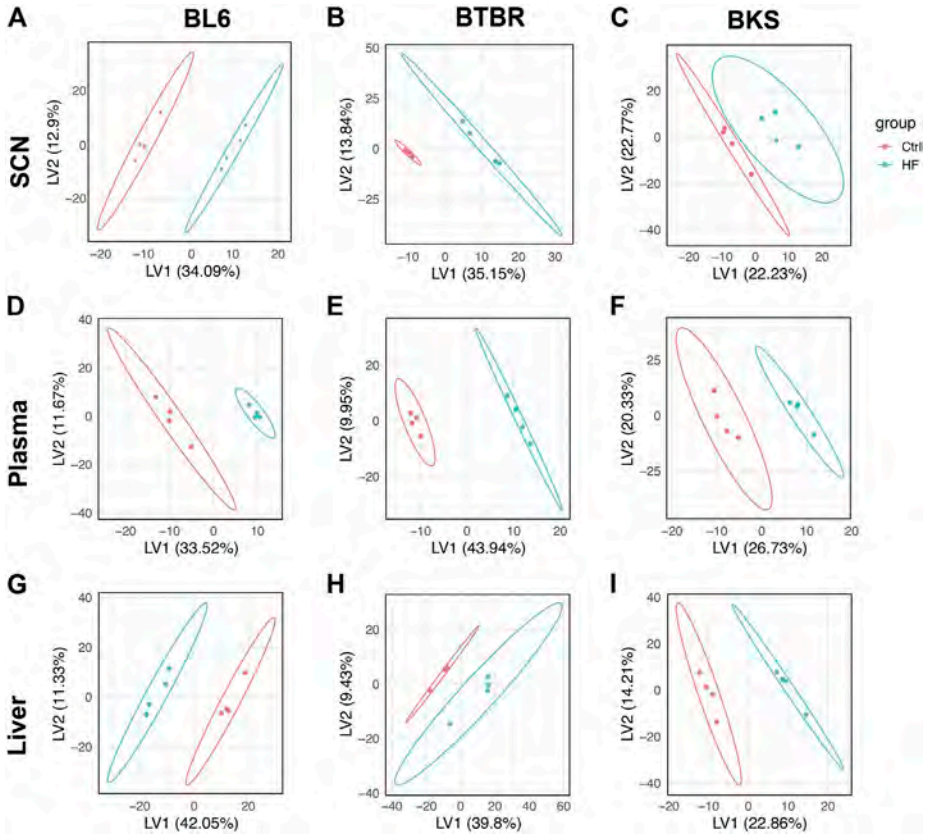

FIGURE 2 | Score plots of lipid changes across species. Partial least squares-discriminant analysis (PLS-DA) showed strain-dependent separation of lipid species between the SD (red) and HFD mice (blue); dots represent individual mice. BL6 mice with weight gain, dyslipidemia, and both large fiber and small fiber PN **(A)** and BTBR mice with weight gain and large-fiber PN **(B)** showed distinct separation of sciatic nerve (SCN) lipids between SD and HFD groups compared to BKS mice with large fiber PN without weight gain **(C)**. Whereas, clear separation between plasma **(D–F)** and liver lipids **(G–I)** was visible across murine strains.

Mitochondrial Copy Number Analysis

The sural nerve mitochondrial copy number was evaluated in HFD- versus SD-fed BL6 mice, as previously described (Rumora et al., 2018). Briefly, DNA was isolated using the AllPrep DNA/RNA Mini Kit (Qiagen, Germantown, MD, United States) from the sural nerves, which were used for mitochondrial bioenergetics analysis. Quantitation of mitochondrial cytochrome b (*cytob*) and nuclear tyrosine 3-monooxygenase/tryptophan five-monooxygenase activation protein (*Ywhaz*) was evaluated using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) on a StepOnePlus Real-Time PCR system (Thermo Fisher Scientific), as described previously (Rumora et al., 2018). The standard curve method was used for *cytob* and *Ywhaz* gene quantitation.

RESULTS

Tissue Lipidomics Profiling of HFD BL6, BTBR, and BKS Mice

Untargeted lipidomics was performed on the sciatic nerve, plasma, and liver from BL6, BTBR, and BKS mice fed either SD or 54% HFD for 36 weeks (**Figure 1A**). PLS-DA score plots showed a clear separation between lipid species in the sciatic nerve of HFD BL6 mice with large and small fiber PN and HFD BTBR mice with large fiber PN, versus SD BL6 and SD BTBR mice without PN (**Figures 2A,B**). Sciatic nerve lipid profiles from HFD-fed BKS mice that had no weight gain compared to SD-fed animals show less separation between HFD and SD score plots (**Figure 2C**). Elevated plasma insulin levels and large fiber PN, based on slowed sciatic and sural nerve conduction velocities, were present in all strains. However, only BL6 mice fed a HFD had highly significant weight gain throughout the entire study (8-, 16-, 24-, 36- weeks) compared to SD-fed BL6 animals. At the study end, these animals also had statistically elevated cholesterol levels and low IENFDs, a marker of small fiber PN (**Supplementary Table S5**) (Hinder et al., 2017). HFD-fed BTBR mice gained weight at a similar rate as SD-fed animals for the first 24 weeks of the study, and only at the 36-weeks time point were significantly heavier than their SD-fed counterparts. These HFD animals had higher levels of fasting glucose than the SD-fed animals with no changes in lipid levels. In contrast both SD- and HFD-fed BKS mice gained weight at equivalent rates and had no evidence of elevated cholesterol or fasting glucose (**Supplementary Table S5**) (Hinder et al., 2017). The greater separation between the score plots of sciatic nerve lipids from

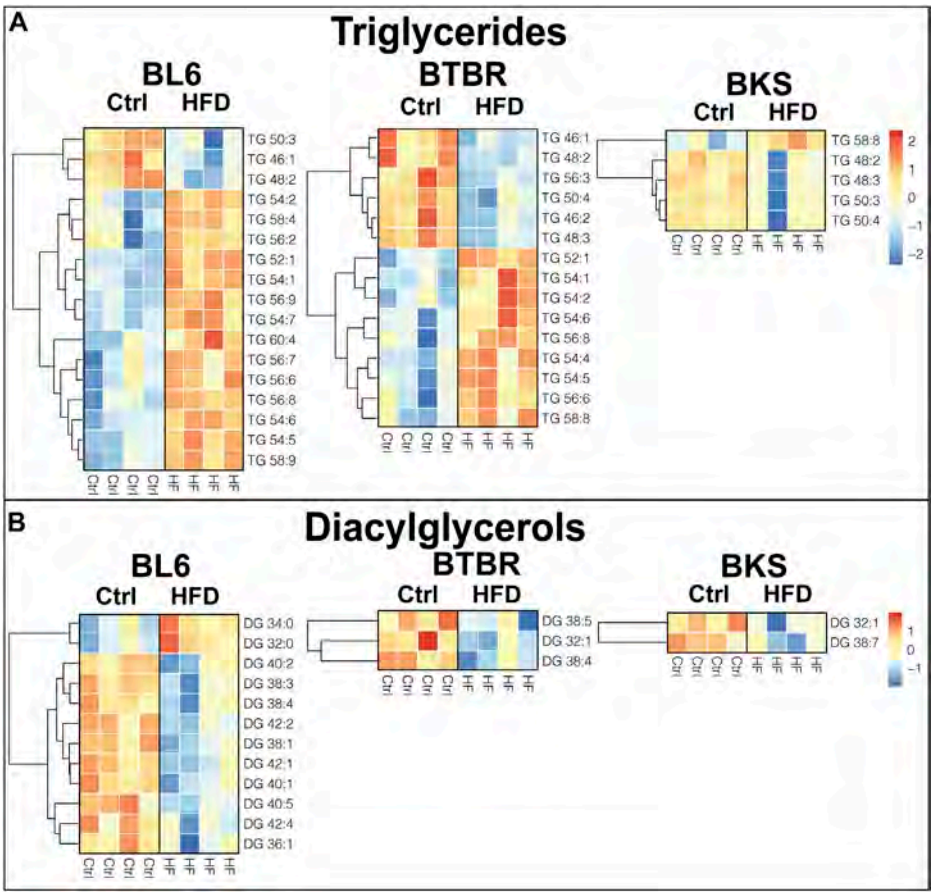

FIGURE 3 | Heat maps of neutral lipids in the sciatic nerve of BL6, BTBR, and BKS mice fed the SD or HFD. **(A)** Sciatic nerve triglyceride (TG) chain length and degree of saturation were significantly altered in HFD sciatic nerve from BL6 and BTBR mice. **(B)** Sciatic nerve diacylglycerols (DGs) were significantly altered by the HFD in all three strains. *t*-test, *p*-value < 0.05.

HFD- vs SD-fed BL6 and BTBR mice shows that changes in nerve lipid composition are associated with distinct PN phenotypes and metabolic changes including weight gain, fasting glucose, and plasma insulin (**Supplementary Table S5**) (Hinder et al., 2017). Unlike the strain-dependent sciatic nerve lipid profiles, the liver and serum lipid profiles had a distinct separation between HFD versus SD groups, regardless of strain (**Figures 2D–I**). These results suggest that tissue-specific sciatic nerve lipid profiles are associated with distinct types of PN as defined by large and small nerve fiber involvement and metabolic dysfunction.

Triglycerides and Diacylglycerols

To identify tissue-specific changes in lipid profiles that associate with PN, we generated heat maps of significantly altered ($p < 0.05$) lipid species in mice fed a HFD compared to SD. Out of a total of 57 detected TG species in the sciatic nerve, 17 were altered in BL6 mice, 15 in BTBR mice, and five in BKS with HFD feeding (**Figure 3A**). The chain length and saturation degree of sciatic nerve TGs were also altered by a HFD. Both BL6 and BTBR strains fed a HFD experienced weight gain by 36 weeks, developed at least two measures of metabolic dysfunction, and exhibited a higher abundance of sciatic nerve long-chain TGs, which contrasted with a higher level of shorter-chain TGs in BL6 and BTBR mice fed a SD. The highly abundant sciatic nerve long-chain TGs in HFD-fed BL6 and BTBR also showed a higher degree of acyl chain unsaturation. These HFD-induced changes in nerve TGs correlated with large fiber PN in both BL6 and BTBR mice. Conversely, sciatic nerve from BKS mice that did not gain weight and developed only one measure of metabolic dysfunction, showed changes in TG level but no distinct changes in TG chain length or saturation.

Diacylglycerols (DGs) were significantly altered in the sciatic nerve of all three strains of mice fed a HFD. A total of 35 DGs were analyzed in the sciatic nerve, of which 12 in BL6, three in BTBR, and two in BKS sciatic nerve were significantly affected by consuming a HFD (**Figure 3B**). Only sciatic nerve from BL6 mice with both large and small fiber PN showed changes in DG chain length, while BTBR and BKS mice had an overall decrease in DGs with HFD feeding compared to their respective SD controls. Interestingly, changes in chain length were opposite in DGs versus TGs, with BL6 sciatic nerve displaying greater shorter-chain and lower longer-chain DG levels in animals fed a HFD compared to animals on a SD. Collectively, these results suggest that elevated long-chain TGs and shorter-chain DGs in sciatic

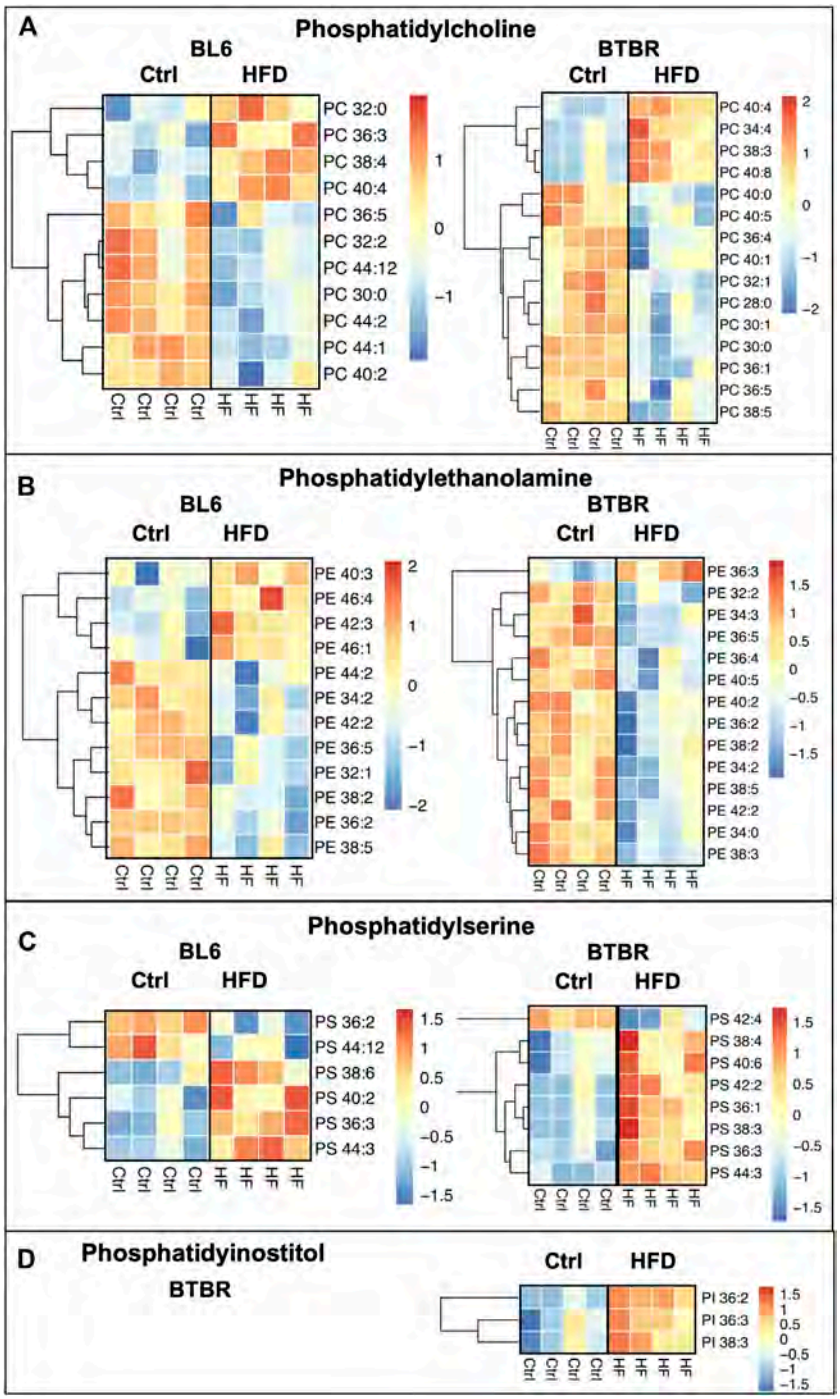

FIGURE 4 | Heat maps of BL6, BTBR, and BKS sciatic nerve phospholipids. All phospholipid levels including **(A)** phosphatidylcholine (PC) **(B)** phosphatidylethanolamine (PE) **(C)** phosphatidylserine (PS) and **(D)** phosphatidylinsotitol (PI) were altered by the HFD in BL6 and BTBR mice. *t*-test, *p*-value < 0.05.

nerves correlate with weight gain, metabolic dysfunction, and large and small fiber PN in HFD BL6 mice after 36 weeks.

Phospholipids

HFD feeding drastically dysregulated phospholipids in the sciatic nerve of BL6 models with large and small fiber PN and BTBR models with large fiber PN, which both experienced weight gain and metabolic dysfunction (**Figure 4**). However, phospholipids were unaffected in the sciatic nerve of HFD-fed BKS mice, the strain that did not gain weight and developed less metabolic dysfunction with a HFD. Collectively, these findings suggest a role for phospholipids in large fiber PN pathogenesis associated with

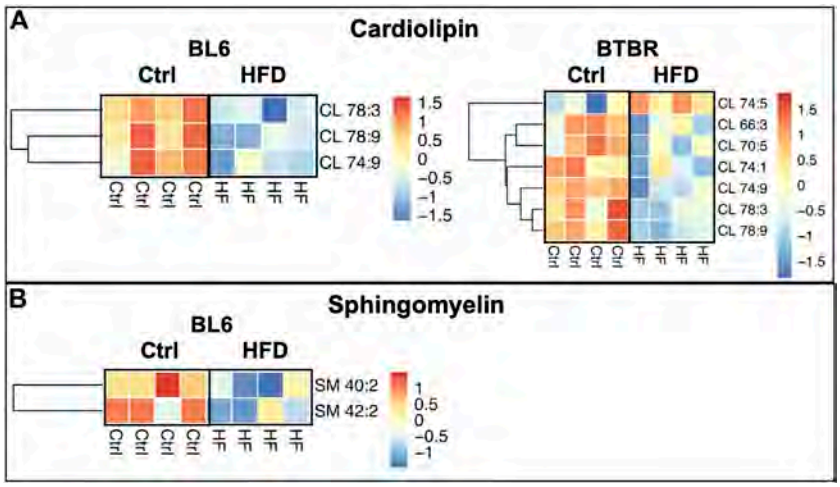

FIGURE 5 | Heat maps of BL6 and BTBR sciatic nerve cardiolipin (CL) and sphingomyelin (SM). HFD BL6 and BTBR mice show global decreases across all **(A)** CL and **(B)** SM lipid species. *t*-test, *p*-value < 0.05.

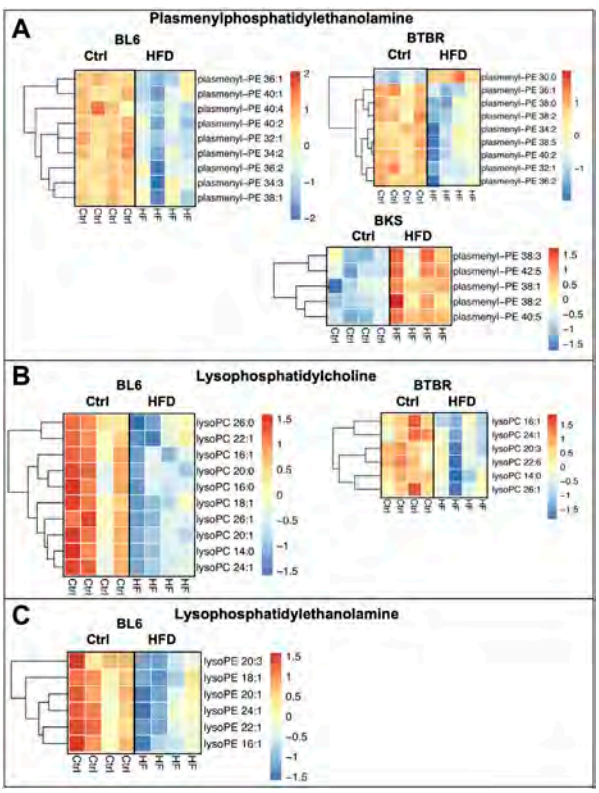

FIGURE 6 | Heat maps of BL6, BTBR, and BKS sciatic nerve plasmalogens and lysophospholipids. **(A)** HFD BL6 and BTBR mice show a global decrease in plasmenyl-phosphatidylethanolamine (plasmenyl-PE) whereas BKS mice have an increase in plasmenyl-PE. **(B)** Lysophosphatidylcholine (LPC) species were also significantly decreased in HFD BL6 and BTBR mice. **(C)** LPE species were decreased exclusively in the sciatic nerve of HFD BL6 mice only. *t*-test, *p*-value < 0.05.

metabolic dysfunction. Within the sciatic nerve, BL6 mice had a higher level of short-chain phosphatidylcholines (PCs) and long-chain phosphatidylethanolamines (PEs) with HFD feeding versus SD mice (**Figures 4A,B**). Although the levels of certain PC and PE species were also affected in HFD-fed BTBR sciatic nerve, there were no distinct changes in the chain length. In both BKS and BTBR mice fed a HFD, levels of specific phosphatidylserine (PS) species were altered, but without discernable changes in chain length (**Figure 4C**). Decreases in phosphatidylinositol (PI) species were exclusive to HFD-fed BTBR sciatic nerve (**Figure 4D**).

Levels of mitochondrial phospholipid cardiolipin (CL) were significantly reduced within the sciatic nerves of BL6 and BTBR mice after HFD feeding whereas sphingomyelin (SM) levels were only reduced in the sciatic nerves from HFD-fed BL6 mice (**Figures 5A,B**). Conversely, a HFD did not affect SM and CL levels in the sciatic nerves of BKS mice. Since CL and SM were only reduced in sciatic nerve from animals that gained weight and were metabolically dysfunctional, these phospholipids may play an important role in PN pathogenesis associated with metabolic dysfunction.

Lysophospholipids and Plasmalogens

The HFD feeding significantly altered lysophospholipid and plasmalogen lipids in the sciatic nerve for all three strains of mice when compared to SD (**Figures 6A–C**). Lysophosphatidylcholine (LPC) and plasmenyl-phosphatidylethanolamine (plasmenyl-PE) lipid species were significantly decreased in the sciatic nerves of BL6 and BTBR mice fed a HFD compared to SD. The HFD-fed BKS mice with no metabolic dysfunction displayed a significant decrease in LPC and an increase in plasmenyl-PE. A reduction in lysophosphatidylethanolamine (LPE) in the sciatic nerve occurred only in BL6 mice fed a HFD, and not the two other

strains, suggesting an association with LPE and both large and small fiber PN in the murine model that most closely replicates the human condition.

Liver and Plasma Lipid Profiles

The sciatic nerve lipidome is modulated by changes in plasma lipid levels, whereas the liver is a major regulator of circulating plasma lipid levels (O'Brien et al., 2017; O'Brien et al., 2020). Therefore, we compared the liver and plasma lipid levels across all murine strains with HFD versus SD feeding and found major changes in lipid profiles regardless of murine strain. Although no significant differences in plasma TG level were detected in all murine strains (**Supplementary Table S5**), the chain length of plasma TGs changed with HFD feeding. The BL6 and BTBR mice, but not the BKS animals, had significant elevations in long-chain TGs in plasma after HFD feeding compared to SD groups (**Supplementary Figure S1**). The levels of several plasma DG and cholesterol ester species were uniquely altered in HFD BTBR mice after HFD feeding (**Supplemental Figure 1B-C**). Plasma phospholipid levels were changed across all murine strains fed the HFD but showed no significant difference in chain length or degree of saturation (**Supplementary Figure S2**). Interestingly, plasma SM levels were elevated in all HFD-fed murine strains including HFD BL6 mice, which contrasted with decreased SM levels in the HFD BL6 sciatic nerve (**Supplementary Figure S3**). The levels of plasma plasmenyl-PE, plasmenylphosphatidylcholine (plasmenyl-PC), LPE, and LPC were also significantly upregulated or downregulated by the HFD feeding depending on the murine strain (**Supplementary Figure S4**).

The liver had distinct changes in neutral lipids including significant increases in TG and DG chain length, as well as an overall decrease in DGs, in BL6 fed a HFD compared to a SD diet (**Supplementary Figure S5**). Conversely, the levels of liver TGs were significantly decreased in BTBR and BKS mice fed a HFD (**Supplementary Figure S5**). Phospholipids were also significantly altered in the liver of all three mouse models. The level of PCs and PEs were significantly decreased in the liver of all three murine strains with HFD feeding (**Supplementary Figure S6**). Other phospholipid groups including PI, PS, phosphatidylglycerol, and phosphatidic acid were also decreased in a strain-dependent manner in the liver of these mice (**Supplementary Figure S6**). As in the sciatic nerve, there was a significant decrease in CL, plasmenyl-PC, LPC, and LPE in the liver of BL6 mice fed a HFD (**Supplementary Figures S7A, S8A–D**). The levels of specific species of SM, plasmalogens, and lysophospholipids were altered in certain strains of HFD mice [SM (BL6, BKS), plasmalogens (BL6, BTBR, BKS), and lysophospholipids (BL6, BTBR)], but there were no distinct changes in chain length or degree of saturation.

Top Lipids Contributing to Peripheral Neuropathy in BL6 HFD-Fed Animals

We have previously reported that HFD feeding of BL6 mice leads to metabolic dysfunction and large and small fiber PN that most closely resembles that seen in humans (Hinder et al., 2017; Rumora et al., 2019b; O'Brien et al., 2020). To determine lipids most significantly linked to pathogenesis of both large and small fiber PN, we identified the lipid species in sciatic nerves, plasma, and liver that contributed the most to diet-induced group separation among BL6 animals by VIP plots and correlation coefficient analysis. A total of 166 sciatic nerve lipids, 141 plasma lipids, and 240 liver lipids had VIP values greater than 1. The top 20 lipids with the highest VIP values were 9 TGs, four PCs, four plasmenyl-PEs, one PS, one PE, and one CL species, which were significantly altered in sciatic nerves (**Figures 7A–C**). Lipid VIP scores for each tissue are provided in **Supplementary Tables S6–S8**. Plasma lipids were also significantly impacted by HFD feeding including five LPCs, five SMs, three PCs, two plasmenyl-PCs, two LPEs, one PE, one CL species, and one cholesterol ester. Important liver lipids affected by HFD feeding included six PEs, 4 TGs, three PCs, two CLs, one LPE, one PS, one phosphatidic acid, one DG, and one phosphatidylglycerol species. We next assessed lipid correlations across tissues and found, among the 35 differentially altered lipids, plasma and liver had greater overlap in shared lipids versus sciatic nerve (**Figure 7D**). Finally, we directly compared the liver, plasma, and sciatic nerve lipid levels in BL6 mice. Interestingly, lipid levels in the sciatic nerve were distinct from lipid levels in the plasma or liver in BL6 mice fed the SD and the HFD (**Supplementary Figures S9A,B**).

To identify lipid changes that contribute to PN in the different mouse strains, we compared sciatic nerve lipids with VIP >1 across the three strains of mice. We identified 33 shared lipid changes between all murine strains, 57 shared lipid changes between sciatic nerve from BL6 and BTBR mice, and 20 shared lipid changes in sciatic nerve from BL6 and BKS mice (**Supplementary Figures S10** and **Supplementary Table S9**). All HFD murine strains developed large fiber neuropathy and had changes in the level of neutral lipids (triglycerides and diacylglycerols) indicating that changes in neutral lipid species may contribute to large nerve fiber damage. HFD BL6 and BTBR mice that developed large fiber neuropathy associated with metabolic dysfunction shared many lipid changes in lysophospholipids and plasmalogens that were less distinct in HFD BKS mice, indicating that these lipid species may contribute to large fiber neuropathy in metabolic dysfunction.

HFD Impairs Mitochondrial Bioenergetics Within DRG Neurons and the Sural Sensory Nerve

Since essential mitochondrial phospholipids, including PE, PC, PI, PS, and CL, were significantly altered in sciatic nerves of BL6 mice fed a HFD, we next evaluated the impact of HFD on *ex vivo* mitochondrial function. Mitochondrial bioenergetic analyses were performed on the DRG sensory neurons and the sural sensory nerve. DRG neurons showed significant increases in basal respiration and ATP production with no discernable change in coupling efficiency at rest (**Figures 8A–C**). DRG neurons from HFD-fed BL6 mice challenged with both 100 and 600 nM FCCP had significantly higher maximum spare respiratory capacity relative to the BL6 DRG neurons from SD, but loss of spare respiratory capacity at 600 nM FCCP (**Figures 8D,E**). Basal ATP production and coupling efficiency were significantly reduced in BL6 sural nerves from

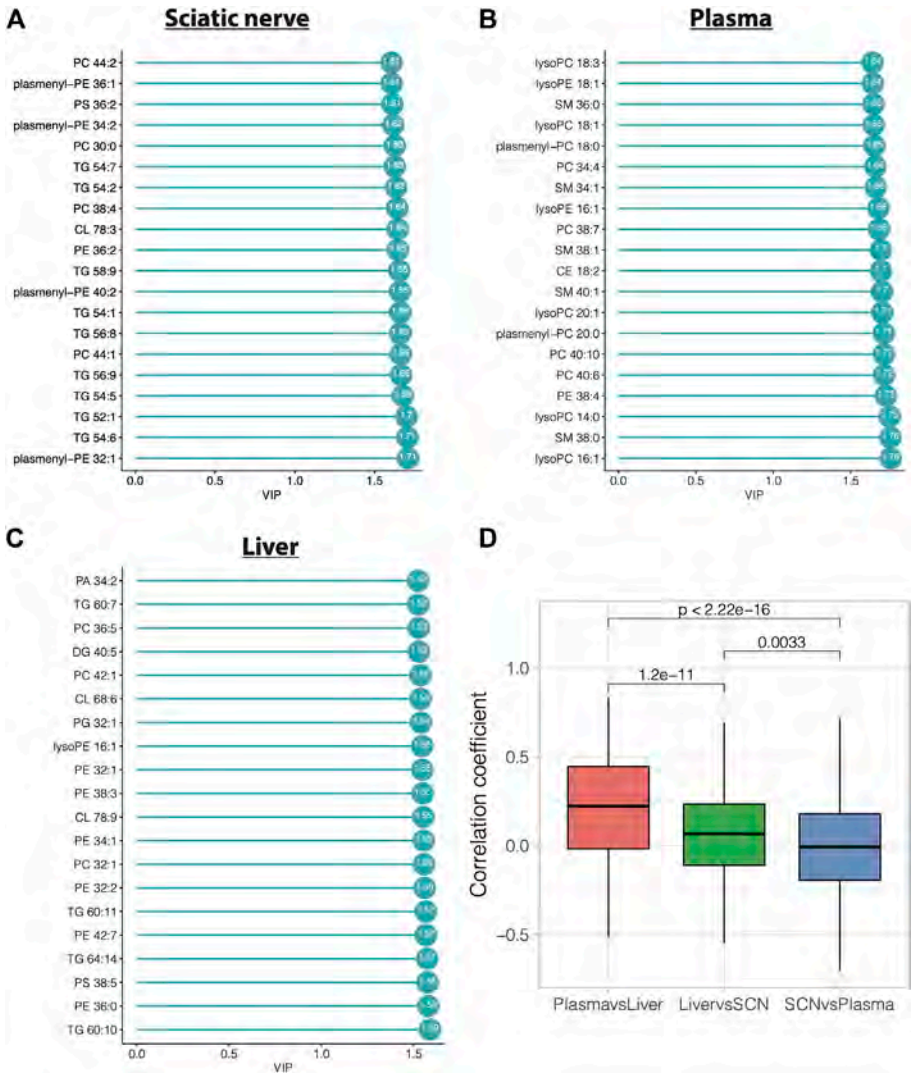

FIGURE 7 | Variable importance in projection (VIP) score plots of the top 20 PLS-DA lipids in **(A)** sciatic nerve, **(B)** plasma, and **(C)** liver, that separate HFD BL6 mice from SD mice. **(D)** Pearson correlation coefficients for each shared lipid species between plasma vs liver, liver vs sciatic nerve, and sciatic nerve vs plasma.

HFD-fed animals, while the basal respiration was not impacted, compared to sural nerves from animals fed a SD (**Figures 8F–H**). Sural nerve mitochondria also showed a significant decrease in maximum respiratory capacity and spare respiratory capacity in HFD-fed versus SD animals (**Figures 8I,J**). Mitochondrial copy number was also significantly lower with HFD feeding in sural nerves but not DRG neurons (**Figure 8K**). These results indicate that a HFD induces DRG and sural nerve mitochondrial dysfunction, correlating with altered mitochondrial lipid levels, which may contribute to the loss of sensory nerve function in PN.

DISCUSSION

Multiple clinical studies identify components of the metabolic syndrome, including dyslipidemia and elevated TGs, as important PN risk factors (Wiggin et al., 2009; Andersen et al., 2018). Preclinical research shows these same risk factors adversely impact axonal mitochondrial trafficking and bioenergetics (Rumora et al., 2018; Rumora et al., 2019a; Sajic et al., 2021) resulting in bioenergetic failure in distal peripheral nerve axons and PN (Feldman et al., 2017). Despite the relevance of lipids, both as PN risk factors and in PN pathogenesis, the precise circulating and nerve lipid species most important to PN remain unknown. Importantly, the correlation of plasma and liver lipidome to nerve lipidome is also incompletely understood, despite the fact that a circulating lipidomic signature correlated to that identified in the nerve could serve as a disease biomarker. Thus, we undertook a systematic study of sciatic nerve, plasma, and liver lipidomics of three HFD-fed mouse models of varying metabolic and neuropathic phenotypes. The first model, HFD-fed BL6 mice, gain weight and develop insulin resistance, dyslipidemia and both large and small fiber PN, metabolic and PN features similar to those reported in humans with prediabetes

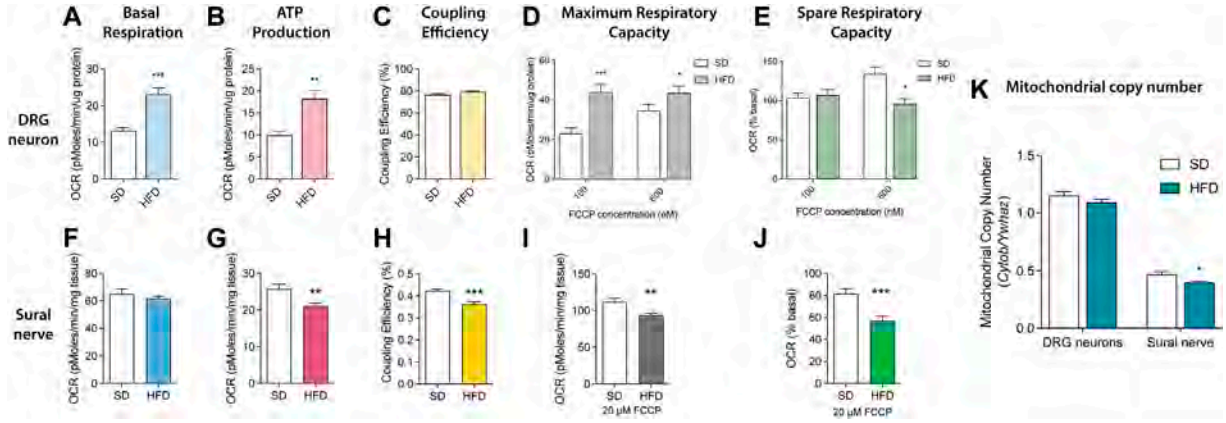

FIGURE 8 | Mitochondrial bioenergetics in SD vs HFD DRG neurons and sural nerves. Resting bioenergetics parameters including **(A)** basal respiration and **(B)** ATP production were significantly increased in DRG neurons from HFD mice compared to SD mice, whereas **(C)** coupling efficiency was unaffected. **(D)** HFD DRG neurons challenged with 100 and 600 nM FCCP had increased maximum respiratory capacity, but significantly diminished spare respiratory capacity with 600 nM FCCP **(E)** relative to SD DRG neurons. The HFD had no effect on sural nerve **(F)** basal respiration but significantly reduced both **(G)** ATP production and **(H)** coupling efficiency compared to the SD sural nerve. Challenging the HFD sural nerve with 20 μM FCCP significantly impaired both **(I)** maximum respiratory capacity and **(J)** spare respiratory capacity compared to SD sural nerves. **(K)** Mitochondrial copy number was reduced in HFD sural nerve. Data mean ± SEM, n = 15 mice/group sural nerves; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

(Hinder et al., 2017). The second model, BTBR mice, are resistant to weight gain until 36 weeks but develop insulin resistance, hyperglycemia and large fiber PN. Lastly, the third model, BKS mice fed a HFD diet develop only insulin resistance and large fiber PN without gaining weight. Although large fiber PN was detected in all strains of mice by 36 weeks of age, only BL6 mice consistently developed the diet-induced metabolic dysfunction and sensory PN that closely mimics the human condition. We, therefore, reasoned that comparing the lipid signatures in these different strains with varying metabolic and neuropathic phenotypes would identify tissue-specific lipids important in PN pathogenesis.

We found that score plots of sciatic nerve lipids separated BL6 and BTBR mice fed a HFD from their SD counterparts, aligning with the presence of weight gain and large fiber PN at 36 weeks in these HFD-fed strains. In contrast, BKS animals did not experience this same diet-mediated separation in sciatic nerve lipids and in parallel did not gain weight with a HFD. These data show that nerve-specific lipid changes correlate with weight gain from HFD feeding and support the idea that diet-induced lipid changes impact tissue function, especially in tissues with diverse lipid composition, such as the peripheral nervous system (Surma et al., 2021). We also discovered distinct changes in the sciatic nerve lipidome of HFD-fed BL6 and BTBR mice with large fiber PN, including neutral lipids (TGs, DGs), phospholipids, lysophospholipids, and plasmalogens. However, changes in nerve SMs and LPE levels were unique to HFD-fed BL6 mice who robustly model large and small fiber PN and are the only strain to develop plasma dyslipidemia. These nerve lipid classes may selectively contribute to small fiber nerve damage commonly associated with obesity, the metabolic syndrome, and prediabetes (Palavicini et al., 2020). Liver and plasma lipid profiles, that presumably dictate the sciatic nerve lipidome, also changed significantly in response to a HFD in all mouse strains. Since none of these plasma or liver changes were specific to animals with varying degrees of PN and metabolic dysfunction, it suggests that nerve-specific lipid changes specifically contribute to PN. Many of the nerve lipids identified in BL6 mice fed a HFD are critical for mitochondrial function; indeed, sural nerves from BL6 mice fed a 60% HFD were characterized by a loss of respiratory capacity in both the basal resting and energetically challenged states. Collectively, these results indicate that changes in the peripheral nerve lipidome associate with specific PN phenotypes (large and/or small fiber dysfunction) and likely contribute to mitochondrial dysfunction in PN.

An accumulation of long-chain TGs and short-chain DGs in the sciatic nerve was associated with PN in HFD BL6 mice with small and large fiber PN and in BTBR mice with large fiber PN, indicating that long-chain fatty acids from the diet are incorporated into TGs, mobilized into the plasma, and integrated into the sciatic nerve lipidome (Tracey et al., 2018). These results are consistent with previous studies showing elevated TGs in the sciatic nerve of neuropathic BL6 mice fed 60% HFD (O'Brien et al., 2020). In fact, gene expression of diacylglycerol acyltransferase 2 (DGAT2), the rate-limiting enzyme for TG biosynthesis was significantly increased in the sciatic nerve of these neuropathic BL6 mice fed 60% HFD. DGAT2 was also elevated in sural sensory nerve biopsies from T2D humans with PN, suggesting that nerve TG synthesis is elevated in PN (O'Brien et al., 2020). In the current study, TGs also displayed longer hydrocarbon chains and a greater degree of unsaturation in the HFD BL6 sciatic nerve, consistent with findings in plasma of type 2 diabetic human subjects with dyslipidemia and progressively worsening diabetic complications (Afshinnia et al., 2018; Afshinnia et al., 2019). The increase in TG hydrocarbon chain unsaturation in the sciatic nerve might be a compensatory mechanism to replace saturated TG hydrocarbon chains with polyunsaturated hydrocarbon

chains in an attempt to prevent nerve lipid peroxidation (Bailey et al., 2015; Ackerman et al., 2018). Alternatively, the observed mobilization of long-chain saturated fatty acids from TGs can uncouple the mitochondrial membrane and impair mitochondrial oxidative phosphorylation, which could have contributed to the observed nerve injury (Murray et al., 2011).

In contrast to TGs, saturated DGs, including DGs 30:0–34:0, were significantly elevated in the sciatic nerve of HFD BL6 mice. The predominant DG species in standard rodent sciatic nerve are unsaturated DGs 38:4 (18:0/20:4) and 34:1 (16:0/18:1) (Eichberg and Zhu, 1992). Therefore, our findings indicate that HFD consumption triggers the incorporation of saturated fatty acids, such as palmitic acid and stearic acid, into sciatic nerve DGs in HFD BL6 mice with small and large fiber PN and dyslipidemia. This accumulation of saturated DGs in the sciatic nerve may underlie nerve damage by mediating lipotoxicity, mitochondrial dysfunction, endoplasmic reticulum stress, or apoptosis (Akoumi et al., 2017).

Our findings suggest that redirecting lipid biosynthetic pathways away from TG/DG synthesis could provide a viable therapeutic approach to treating PN. In support of this idea, inhibiting DGAT and lipin1, a DG synthesizing enzyme, promotes axon regeneration in peripheral neurons by reducing TG/DG synthesis and stimulating phospholipid synthesis (Yang et al., 2020). Furthermore, modulating DG levels in the sciatic nerve of STZ-treated rats confers neuroprotection and improves PN measures (Wang et al., 2021). Future preclinical studies focused on nerve-specific TG/DG biology in the setting of dyslipidemia and metabolic dysfunction could facilitate the development of targeted interventions for the treatment of PN.

Phospholipids, including PE, PC, PS, and CL, were significantly altered in the sciatic nerve of both HFD-fed BL6 and BTBR mice but not BKS mice, indicating a major shift in the nerve phospholipid content in response to HFD feeding that correlates with weight gain. These results parallel previous studies in murine models and humans with metabolic syndrome, prediabetes, and T2D (O'Brien et al., 2020; Rumora et al., 2021), suggesting that changes in nerve phospholipids contribute to large fiber PN pathogenesis in these disease states. Phospholipids make up approximately 57% of lipids in the cell bodies and axons of peripheral neurons and 40% of lipids in the myelin sheath (Calderon et al., 1995; Poitelon et al., 2020; Hornemann, 2021). Alterations in phospholipid levels can trigger aberrant changes in cellular signaling (Nishizuka, 1992), cell membrane structure (Kuge et al., 2014), and membrane dynamics in neurons (Tracey et al., 2018). Importantly, phospholipids are a major constituent of the mitochondrial membrane and play an integral role in regulating mitochondrial function.

The most abundant phospholipids in the inner mitochondrial membrane (PE, PC, CL) (Basu Ball et al., 2018) were those most changed in HFD BL6 sciatic nerve in this study, supporting the idea that these phospholipid changes could alter mitochondrial bioenergetics (Schenkel and Bakovic, 2014). Changes in the levels of inner mitochondrial membrane PE, PC, and CL result in the improper assembly of the mitochondrial electron transport supercomplexes, impairing oxidative phosphorylation (Tasseva et al., 2013). Changes in CL are of particular interest since CL is exclusively found in mitochondria and modulates the assembly of respiratory chain supercomplexes III and IV (Zhang et al., 2005), mitochondrial membrane potential (Ghosh et al., 2020), mitochondrial bioenergetics (Paradies et al., 2014), reactive oxygen species production (Falabella et al., 2021), and apoptotic signaling and mitochondrial dynamics (Falabella et al., 2021). The shift in PS and PE lipids, as well as the loss of CL, within the sciatic nerves of HFD BL6 mice may destabilize mitochondrial respiratory chain complexes, thereby reducing the efficiency of oxidative phosphorylation and injuring the peripheral nerves.

The most distinct lipid change was a global decrease in lysophospholipids (LPC, LPE) and plasmenyl-PE in the sciatic nerve of HFD BL6 and BTBR mice compared to BKS mice. A decrease in lysophospholipids was recently reported in both sciatic nerve (O'Brien et al., 2020) and plasma (Guo et al., 2021) from mice and humans, respectively, with PN and metabolic disease. Elevated levels of LPC are also implicated in neuropathic pain associated with chemotherapy-induced neuropathy (Rimola et al., 2020) and other painful neuropathies (Inoue et al., 2008). Lysophospholipids are generated from the hydrolysis of phospholipids (Tan et al., 2020), leading to elevated nitric oxide levels, which may damage peripheral nerves (Wang et al., 2013). Interestingly, LPCs and LPEs, were only decreased in HFD-fed BL6 and HFD-fed BTBR sciatic nerves emphasizing the possibility that sciatic nerve lysophospholipid levels may be strain-dependent (Hinder et al., 2017) and may contribute to the observed differences in PN among the three strains. The HFD BL6 sciatic nerve had the highest number of altered LPC species and was the only strain with HFD-induced alterations in LPE species indicating that altered lysophospholipids levels may contribute to small and large fiber PN associated with metabolic dysfunction. LPC levels are reportedly increased during painful PN (Wang et al., 2013), indicating that a loss of sensory function may be associated with the distinct decrease in LPC species in HFD-fed BL6 mice. Plasmenyl-PE is a plasmalogen, a family of lipids that contain arachidonic acid, a known mediator of nervous system lipid-signaling pathways (Murphy, 2017), membrane trafficking, and inflammatory pathways (Tracey et al., 2018). Critical for the formation of membrane rafts in the nervous system (Poitelon et al., 2020), loss of plasmalogen plasmenyl-PE in peripheral nerves may alter the lipid composition of myelin and ultimately lead to nerve damage.

Only two lipid species, SM and LPE, were dysregulated exclusively in the sciatic nerve from HFD BL6 mice with weight gain, dyslipidemia, and small and large fiber PN that mimics the human condition, compared to sciatic nerve from BTBR or BKS mice. Both SM and LPE were significantly decreased, indicating that the loss of SM and LPE within the sciatic nerve may contribute to small fiber damage within the nerve. This is supported by reports showing decreased plasma SM levels in patients with T2D (Rumora et al., 2021) and obesity (Guo et al., 2021). SM is an important nerve lipid of the myelin sheath, which protects and supports sensory nerve fibers

(Poitelon et al., 2020; Hornemann, 2021). Although the role of LPE in the peripheral nervous system is less studied, changes in LPE levels are reported in other neurological disorders, including Alzheimer's disease (Liu et al., 2021; Llano et al., 2021), emphasizing the importance of this lipid for proper nervous system function.

In the current study, HFD significantly impacted the liver and plasma lipid profile in all three murine strains, irrespective of PN. Since the peripheral nerves rely on both *de novo* lipogenesis and lipid uptake from circulation (Tracey et al., 2018; Poitelon et al., 2020), the saturation and chain length of circulating dietary fatty acids and complex lipids can influence the nerve lipidome. We have shown switching mice from a saturated fatty acid-rich HFD to a monounsaturated fatty acid-rich HFD rich significantly improves nerve function (Rumora et al., 2019b), likely because monounsaturated fatty acids restore mitochondrial function following saturated fatty acid-induced mitochondrial dysfunction (Rumora et al., 2018; Rumora et al., 2019a; Rumora et al., 2019b). Previous studies also describe elevated plasma and liver TGs in HFD-fed BL6 mice with PN, consistent with reports showing higher TGs in plasma of dyslipidemic rodents (Lupachyk et al., 2012), and plasma of diabetic (Wiggin et al., 2009; Callaghan et al., 2011; Smith and Singleton, 2013) and obese subjects with PN (Guo et al., 2021). However, we observed no strain-dependent differences in plasma or liver lipid classes that were unique to the HFD-fed BL6 mice or BTBR HFD-fed animals. These data suggest that nerve-specific lipid changes are a more important driver of PN pathogenesis than plasma or liver lipid signatures. In support of this idea, a recent study showed that statins alter circulating lipid levels in a T2D patient cohort from the ADDITION-Denmark study but have no effect on PN (Kristensen et al., 2020).

Lipids profoundly influence mitochondrial bioenergetics (Hinder et al., 2012; Aon et al., 2014); therefore, we determined whether changes in the peripheral nerve lipidome correlate with mitochondrial function distally in the *ex vivo* sural sensory nerve and proximally in sensory DRG neurons from HFD-fed BL6 mice. Although untargeted lipidomics was conducted on sciatic nerves to provide sufficient tissue for the lipidomic analysis, prediabetic and T2D PN is primarily a sensory neuropathy (Feldman et al., 2017), so mitochondrial bioenergetic analyses were performed on the sural sensory nerve and DRG sensory neurons. Since HFD-fed BL6 mice robustly mimic PN in humans with metabolic dysfunction, we postulated that *ex vivo* sural nerve and DRG neurons from HFD-fed BL6 mice would model changes in mitochondrial function that underlie diet-induced small and large fiber PN pathogenesis. Basal ATP production, coupling efficiency, and mitochondrial copy number were reduced in the sural nerves from HFD-fed animals, suggesting that mitochondrial energy production is compromised due to uncoupling of ATP production from mitochondrial respiration, as well as fewer mitochondria (Chowdhury et al., 2013). Challenging these sural nerves with mitochondrial uncoupler, FCCP, revealed a decrease in both maximum respiratory capacity and spare respiratory capacity, indicating the inability to increase ATP production to match increased energy demand.

In contrast, DRG neurons cultured from HFD-fed BL6 mice had significant increases in basal respiration and ATP production under resting conditions with no changes in coupling efficiency (Rumora et al., 2018), suggesting that mild uncoupling doesn't occur despite the increase in basal respiration. The lack of uncoupling could in part prevent the formation of reactive oxygen species. The loss of spare respiratory capacity suggests the DRG neuron mitochondria are already functioning at maximum capacity and cannot increase energy output to meet increased energy demands. Elevated ATP production in DRG neurons may therefore be a compensatory mechanism to increase mitochondrial content and mitochondrial-derived ATP distally in the sural nerve, to maintain at least partial nerve function (Feldman et al., 2017).

Our study had several limitations. First, we used two different HFD paradigms including a 54% HFD for lipidomics studies versus a 60% HFD for mitochondrial bioenergetics. Since we previously showed lipid changes in the sciatic nerve of mice fed a 60% HFD by 16 weeks of age were similar to mice fed the 54% HFD at 36 weeks of age (O'Brien et al., 2020), we postulated that changes in mitochondrial bioenergetics would be similar across the two HFD paradigms. Future studies will test the effect of the two different HFD paradigms on mitochondrial bioenergetics in whole sural nerve and DRG neurons. Second, we were unable to determine the acyl chain composition of lipids in the untargeted lipidomics analysis. Targeted lipidomics platforms will be used in future studies to identify structural changes in sciatic nerve, liver, and plasma lipid species. It will be interesting to determine whether HFD impacts the identity of the acyl chains of key sciatic nerve lipid species we identified in this study. Further studies are also needed to determine the significance of odd chain lipids in HFD fed mice. Additionally, we will conduct transcriptomics on nerve, liver, and plasma from each mouse strain to assess changes in genes related to *de novo* lipogenesis and other metabolic pathways in each tissue. A third limitation of this study is the use of the sciatic nerve for untargeted lipidomics versus DRG neurons and sural nerve for mitochondrial bioenergetics analysis. Future directions will use targeted lipdomics or MALDI-MSI to correlate changes in mitochondrial bioenergetics function with lipid changes in DRG neurons and sural nerve. A fourth limitation was our limited number of biological samples (4 samples/tissue type) for the untargeted lipidomics analysis. Future studies will evaluate lipidomics changes with a greater number of tissue samples per group and will be analyzed using q-value statistical analysis to show variance across samples.

In conclusion, HFD feeding of different mouse models with varying degrees of PN and metabolic dysfunction produced significant remodeling of the sciatic nerve lipidome and aberrant mitochondrial bioenergetics. Of the three mouse strains (BL6, BTBR, BKS), HFD-fed BL6 mice develop large fiber and small fiber PN and metabolic dysfunction that most closely resembles the human condition. These animals showed significant changes in neutral lipids, phospholipids, lysophospholipids, and plasmalogen levels in the sciatic nerve. Both SM and LPE were significantly altered in sciatic nerves only in the HFD-fed BL6 animals, indicating the importance of these

lipids for maintaining small fiber nerve function. Although plasma and liver lipids were significantly impacted by the HFD across all murine strains, the plasma and liver lipid changes were not biomarkers of PN. The loss of mitochondrial bioenergetics capacity in the sensory sural nerves from HFD-fed BL6 mice differed from HFD-fed BL6 DRG neurons, which showed increased ATP production, potentially as a compensatory mechanism to restore ATP production distally in the injured nerve. Future studies will focus on determining lipid changes that damage specific subsets of nerve fibers, including small and large nerve fibers, as a potential pathogenic mechanism underlying specific PN phenotypes. Additionally, identifying the specific lipid species that drive mitochondrial dysfunction and nerve damage may provide novel therapeutic targets for PN associated with prediabetes and T2D.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author. Raw lipidomics data files are publicly available at the DOI: 10.5281/zenodo.6814022.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Michigan University Committee on Use and Care of Animals.

AUTHOR CONTRIBUTIONS

AR, KG, LH, and EF designed the study; AR, KG, LH, PO'B, and JMH conducted the research and collected data; KG, LH, JMH, and JH performed the statistical analyses; AR and EF wrote the original draft; AR, KG, LH, PO'B, JMH, JH, and EF reviewed and edited the manuscript; EF supervised the manuscript and provided project administration. All authors contributed to the manuscript preparation, edited the manuscript, and approved the submitted manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.921942/full#supplementary-material

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RESEARCH ARTICLE

Serum lipidomic determinants of human diabetic neuropathy in type 2 diabetes

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Abstract

Objective: The serum lipidomic profile associated with neuropathy in type 2 diabetes is not well understood. Obesity and dyslipidemia are known neuropathy risk factors, suggesting lipid profiles early during type 2 diabetes may identify individuals who develop neuropathy later in the disease course. This retrospective cohort study examined lipidomic profiles 10 years prior to type 2 diabetic neuropathy assessment. **Methods**: Participants comprised members of the Gila River Indian community with type 2 diabetes ($n = 69$) with available stored serum samples and neuropathy assessment 10 years later using the combined Michigan Neuropathy Screening Instrument (MNSI) examination and questionnaire scores. A combined MNSI index was calculated from examination and questionnaire scores. Serum lipids (435 species from 18 classes) were quantified by mass spectrometry. **Results**: The cohort included 17 males and 52 females with a mean age of 45 years (SD = 9 years). Participants were stratified as with (high MNSI index score > 2.5407) versus without neuropathy (low MNSI index score ≤ 2.5407). Significantly decreased medium-chain acylcarnitines and increased total free fatty acids, independent of chain length and saturation, in serum at baseline associated with incident peripheral neuropathy at follow-up, that is, participants had high MNSI index scores, independent of covariates. Participants with neuropathy also had decreased phosphatidylcholines and increased lysophosphatidylcholines at baseline, independent of chain length and saturation. The abundance of other lipid classes did not differ significantly by neuropathy status. **Interpretation**: Abundance differences in circulating acylcarnitines, free fatty acids, phosphatidylcholines, and lysophosphatidylcholines 10 years prior to neuropathy assessment are associated with neuropathy status in type 2 diabetes.

Introduction

Peripheral neuropathy is a common complication of type 2 diabetes, ranging in prevalence from 10 to over 50% in various cohorts.[1–6] Peripheral neuropathy symptoms manifest as a loss of sensation and pain in a length-dependent manner.[1] Peripheral neuropathy in type 2 diabetes remains recalcitrant to effective treatment; glucose control only marginally prevents neuropathy onset and development.[7] This has spurred interest in identifying metabolic factors early during type 2 diabetes, including modifiable factors, which identify

patients most at risk to implement lifestyle interventions in those patients.

Over the past decade, numerous studies have provided evidence that obesity and dyslipidemia are important neuropathy risk factors,[8] independent, even, of glycemic status. In the Danish ADDITION type 2 diabetes cohort, peripheral neuropathy is associated with decreased high-density lipoprotein cholesterol.[3] This relationship was also present in older populations, in the Health, Aging and Body Composition study,[9] and in youths, in the SEARCH for Diabetes in Youth study.[5] Elevated triglycerides associated with peripheral neuropathy in the Danish Centre for Strategic Research in Type 2 Diabetes cohort[10] and risk for nontraumatic lower-extremity amputations in the DISTANCE study.[11] In these investigations, measurements were limited to a basic lipid profile. Recent mass spectrometry advances allow the identification and quantification of a larger array of lipids, termed the lipidome, from biosamples. These lipidome studies can identify relevant disease biomarkers at a more granular level and shed light on pathogenesis to develop rationalized, targeted therapies.[12–17]

Previously, we reported serum abundance differences in the lipidome in type 2 diabetes patients, which correlated with diabetic kidney disease (DKD) one decade later.[13] In cross-sectional studies, we also reported stepwise trends in plasma abundance in free fatty acids, acylcarnitines, diacylglycerols, sphingomyelins, and various additional complex lipids by carbon and double bond number in type 2 diabetes patients with compared to those patients without neuropathy.[18] We similarly found lipidomic signatures differentiated obese patients with versus without peripheral neuropathy, independent of glycemic status,[19] a finding replicated in preclinical animal models.[15,20,21] Collectively, these observations suggest that free fatty acids, acylcarnitines, and complex lipids may be differentially linked with a known diagnosis of neuropathy. However, they do not address whether the serum lipidome can predict future incident neuropathy.

This present retrospective cohort study profiled free fatty acid, acylcarnitine, and complex lipid abundance in 18 lipid classes by carbon number and saturation level in individuals with type 2 diabetes 10 years prior to their neuropathy assessment. The objective was to evaluate correlations between serum lipidomics profile to future incident peripheral neuropathy development. The peripheral neuropathy stage was stratified by Michigan Neuropathy Screening Instrument (MNSI) index scores (low, score $\leq$ 2.5407; high, score > 2.5407), an index that combines the MNSI physical examination score and MNSI symptom questionnaire. Study participants are members of the Gila River Indian community and comprise one of the longest-running studies of type 2 diabetes.[22] Since we found that the lipidome early during type 2 diabetes correlates with the onset and progression of DKD severity a decade later,[13] we anticipated a relationship would also emerge with neuropathy and specific lipid profiles. Indeed, we identified abundance differences in circulating acylcarnitines, free fatty acids, phosphatidylcholines, and lysophosphatidylcholines, which were linked to neuropathy severity 10 years later in this type 2 diabetes cohort. This is, to our knowledge, the first study to demonstrate that serum lipidomic signatures can associate with the presence and severity of future peripheral neuropathy.

Participants and Methods

Participant population and diabetic peripheral neuropathy diagnosis

Study population details and participant recruitment are published elsewhere.[23] Briefly, the population comprised American Indians from the Gila River Indian Community who were participating in a longitudinal study of diabetes and its complications (n = 169). They were recruited between 1996 and 2001 for a randomized, double-blind, placebo-controlled clinical trial to assess the efficacy of an angiotensin receptor blocker on the development and progression of DKD in type 2 diabetes (ClinicalTrials.gov, NCT00340678).[23] Of the 169 clinical trial participants,[23] 89 were subsequently enrolled in a long-term observational study, which included a neuropathy evaluation (Fig. 1). Of these, 69 participants met the eligibility criteria for this study, which included the availability of a stored serum sample 10 years prior to the neuropathy evaluation by MNSI examination and MSNI questionnaire.[24,25] All participants meeting the eligibility criteria were included in this study.

The MNSI examination consists of a foot exam, which assesses ulceration, vibration, and ankle reflexes, and scores 0 to 8. The MNSI questionnaire consists of 15 self-administered questions related to symptoms and clinical history and scores of 0–15. The combined MNSI index score (hereafter referred to as MSNI index score) is calculated by a weighted sum of the individual four MSNI examination domains (the eight scores for left and right foot are combined to give four) and 15 MSNI questionnaire components. The weight of components is regression coefficients from a multivariable logistic regression model, which most accurately predicts definite neuropathy.[25] An MNSI index cutoff of >2.5407 indicates the presence of neuropathy.

Participants did not have a baseline MNSI examination or questionnaire. To evaluate baseline status, the risk of baseline of diabetic neuropathy was estimated using a "diabetic neuropathy prediction risk score".[26] This method

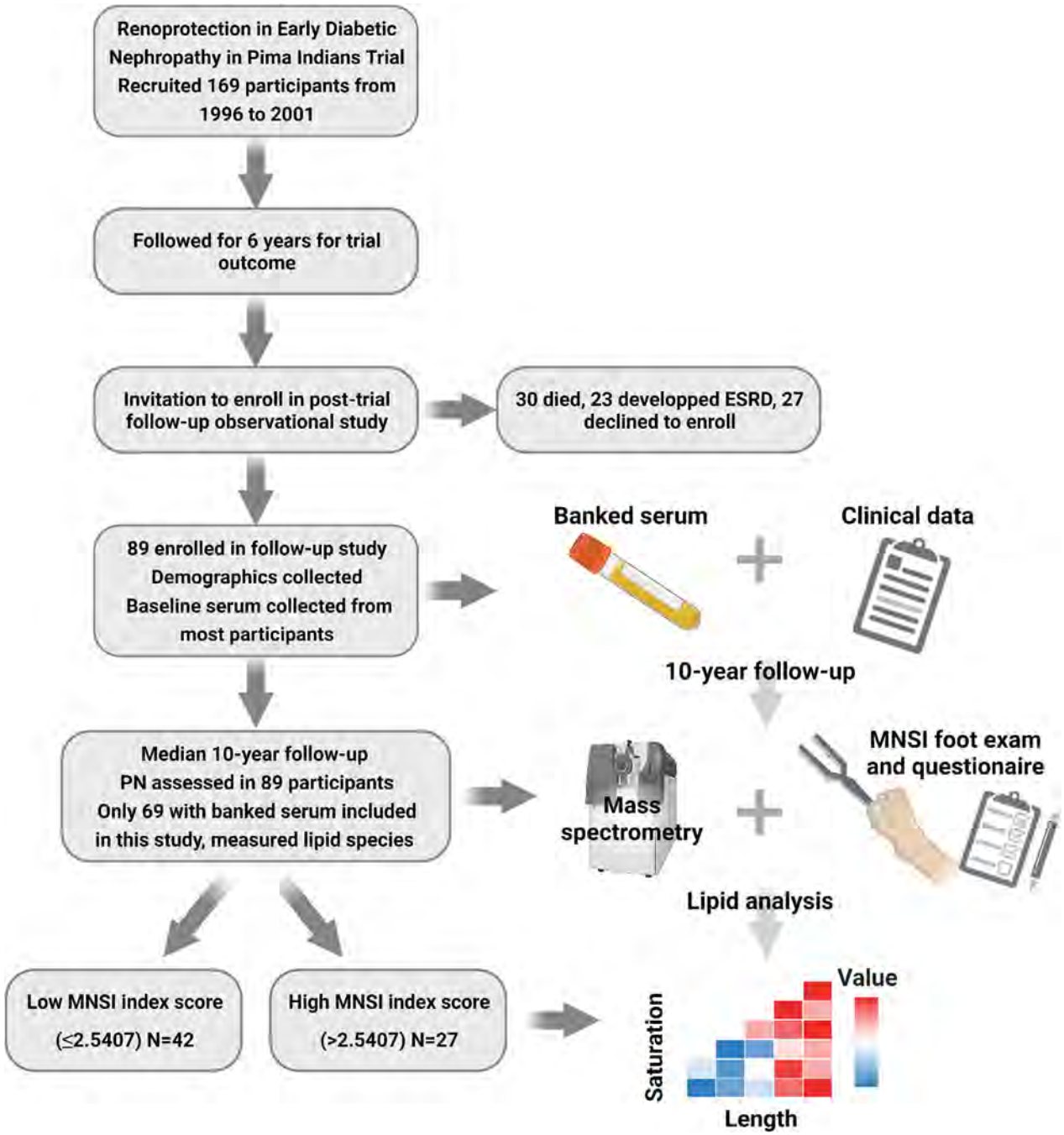

Figure 1. Flow diagram of the study strategy. Participants (n = 169) were originally recruited for the Renoprotection in Early Diabetic Nephropathy in Pima Indians trial from 1996 to 2001. Participants were followed for 6 years to assess the trial outcome. Of the original 169 clinical trial participants, 89 were subsequently enrolled in a long-term observational study, which banked baseline serum and collected baseline clinical data (age, sex, height, weight, BMI, blood pressure, heart rate, diabetes duration, FPG, HbA_{1c}, total cholesterol, triglycerides, GFR, urine ACR, and medication use). At a mean 10-year follow-up, all 89 participants were reexamined, and peripheral neuropathy was assessed by MNSI examination (foot ulceration, vibration, and ankle reflexes) and questionnaire. Of these, 69 participants met the eligibility criteria for this study, which included the availability of a stored serum sample 10 years prior to the neuropathy evaluation. Banked serum from 10 years prior was analyzed by mass spectrometry; 435 lipids from the 18 classes were quantitated and their abundance by chain length and saturation were analyzed. ACR, albumin creatinine ratio; ESRD, end-stage renal disease; FPG, fasting plasma glucose; GFR, glomerular filtration rate; HbA_{1c}, glycated hemoglobin; MNSI, Michigan Neuropathy Screening Instrument; PN, peripheral neuropathy. Figure created in BioRender.com.

leverages an artificial intelligence neural network-based approach to predict neuropathy status from clinical risk factors, including age, height, weight, diabetes duration, glycated hemoglobin, urine albumin-to-creatinine ratio, and total cholesterol. The model achieves over 70% accuracy for predicting neuropathy status, as assessed by continuous-scale vibration perception threshold using a neurothesiometer.[26] Based on the diabetic neuropathy prediction risk score, the probability of baseline neuropathy was only 5.5% (SD = 1.0%) in patients with low MNSI index and 5.7% (SD = 0.7%) in patients with high MNSI index, which did not differ significantly ($p = 0.367$). Therefore, participants likely did not have neuropathy at baseline.

The clinical data at the time of serum sample collection were used to describe baseline participant characteristics. The study was approved by the Institutional Review Board #0000006 at the National Institute of Diabetes, Digestive, and Kidney Diseases, Bethesda, Maryland. All participants gave signed informed consent prior to their participation in the study.

Sample preparation and mass spectrometry

Samples were prepared and mass spectrometry quantified lipids from 18 classes per our published protocols.[12,14,27] Details of sample preparation and lipid analyses are provided in Supplemental Methods.

Measured lipids

Four hundred and thirty-five lipids were quantitated from the 18 classes (Table S1). Classes that consisted of two or fewer lipid species (monoacylglycerols, plasmenylphosphatidylcholines, phosphatidic acids, phosphatidylglycerols, phosphatidylserines, ceramide phosphates) were eliminated. After combining the different mass spectrometry adducts of the same feature, 236 unique lipids were included in the final analysis, including 16 free fatty acids (6.8%), 76 glycerolipids (32.2%; diacylglycerols and triacylglycerols), 12 cholesteryl-esters (5.1%), 83 phospholipids (35.1%; phosphatidylcholines, phosphatidylethanolamines, lysophosphatidylcholines, lysophosphatidylethanolamines, plasmenylphosphatidylethanolamines, phosphatidylinositols), 20 sphingomyelins (8.5%), and 29 acylcarnitines (12.3%).

Quality control

A pool of study samples was injected at the beginning and after every 20 mass spectrometry runs in the lipidomic study, and after every 15 mass spectrometry runs in the acylcarnitine study, to assess the stability of measures over time and identify any batch effects.

Statistical analysis

Mean (±SD) or frequency (percentage) was used to describe normally distributed continuous and categorical variables, respectively. The median and interquartile ranges were used to describe nonnormally distributed continuous and categorical variables, respectively. Participant baseline characteristics for normally distributed continuous variables were compared using the *t* test for two groups, whereas Kolmogorov–Smirnov test compared skewed continuous variables and chi-square to compare categorical variables. Lipidomics data were prepared for analysis by batching and sum normalizing the raw peak intensities by lipid species within each lipid subclass, which were logit transformed and *z*-score standardized.[12] Models were adjusted by age, sex, body mass index (BMI), and systolic and diastolic blood pressure, glycated hemoglobin (HbA_{1c}), statin use, and use of other lipid-lowering agents due to their established association with neuropathy, and with lipid class carbon number and number of double bond due to test the effect of chain length and saturation status with the outcome, that is, the presence of neuropathy (high MNSI index >2.5407) or the absence of neuropathy (low MNSI index ≤2.5407). We used backward elimination of nonsignificant covariates. We also performed a Pearson's correlation analysis treating the MNSI index as a continuous variable correlated to each lipid within each lipid class and to each component of the metabolic syndrome.

Results

Cohort characteristics

Sixty-nine participants with type 2 diabetes, including 17 males and 52 females, received a neuropathy assessment. Mean age was 45 ± 9 years (±SD) and mean BMI was 36.0 ± 7.5 kg/m^2 in females and 34.8 ± 7.5 kg/m^2 in males ($p = 0.575$). When we assessed neuropathy at the 10-year follow-up, there were 27 participants with neuropathy (i.e., with a high MNSI index score of >2.5407) and 42 participants without neuropathy (i.e., with a low MNSI index score of ≤2.5407; Table 1). Systolic ($p = 0.010$) and diastolic ($p = 0.006$) blood pressure were significantly greater in participants with versus without neuropathy.[13,28] Additionally, the median urine albumin creatinine ratio (ACR) was 15 mg/g in participants without neuropathy, which is within the normal range. This contrasts with a median ACR of 54 mg/g in participants with neuropathy, which indicates microalbuminuria and DKD onset and is significantly elevated versus the group without neuropathy ($p = 0.005$). The correlation of DKD with peripheral neuropathy is anticipated, based on our

Table 1. Participant characteristics by neuropathy status.

Variables	Without Neuropathy MNSI index ≤ 2.5407	With Neuropathy MNSI index > 2.5407	*p* value
N	42	27	
Age (years)	46 ± 8	45 ± 9	0.666
Male sex (%)	8 (19.0)	9 (33.3)	0.179
Height (m)	1.6 ± 0.1	1.7 ± 0.1	0.168
Weight (kg)	92 ± 20	100 ± 25	0.160
Body mass index (kg/m^2)	35.0 ± 6.3	36.8 ± 8.9	0.316
Systolic blood pressure (mmHg)	117 ± 14	126 ± 14	**0.010**
Diastolic blood pressure (mmHg)	74 ± 8	79 ± 7	**0.006**
Pulse (/min)	72 ± 9	74 ± 9	0.381
Diabetes duration (years)	15.5 ± 5.4	15.6 ± 6.4	0.925
Fasting plasma glucose (mg/dL)	192 ± 78	223 ± 96	0.147
HbA_{1c} (mmol/mol)	72.7 ± 18.2	81.4 ± 14.9	0.102
HbA_{1c} (%)	8.8 ± 2.2	9.6 ± 1.8	0.102
Total cholesterol (mg/dL)	162 ± 41	165 ± 37	0.788
Triglyceride (mg/dL)	184 ± 160	195 ± 240	0.839
GFR (mL/min)	146 ± 45	162 ± 62	0.264
Urine albumin creatinine ratio (mg/g)[#]	15 [5–53]	54 [18–155]	**0.005**
Intervention arm (%)	25 (59.5)	12 (44.4)	0.220
Medication			
Antihypertensive (%)	17 (40.5)	10 (37.0)	0.775
Metformin (%)	25 (59.5)	18 (66.7)	0.550
Insulin (%)	17 (40.5)	11 (40.7)	0.983
Oral hypoglycemic (%)	31 (73.8)	24 (88.9)	0.128
Statins (%)	9 (21.4)	6 (22.2)	0.938
Other lipid-lowering (%)	10 (23.8)	7 (25.9)	0.842

Significant *p* values are in bold. Data represented as mean ± SD (for continuous variables) or frequency (percentage; for categorical variables), except nonnormally distributed data, for example, urine albumin creatine ratio[#], represented as median [interquartile range]. ANOVA assessed normally distributed continuous variables; Kolmogorov–Smirnov test assessed skewed continuous variables; chi-square test assessed categorical variables. ANOVA, analysis of variance; GFR, glomerular filtration rate; HbA_{1c}, glycated hemoglobin; MNSI, Michigan Neuropathy Screening Instrument.

earlier study of Pima participants.[6] There were no other significant differences in baseline characteristics by neuropathy status. However, there were several trends of increased fasting plasma glucose (FPG), HbA_{1c}, BMI, total cholesterol, and triglycerides in participants with versus without neuropathy, as anticipated based on uncontrolled diabetes, obesity, and dyslipidemia being known peripheral neuropathy risk factors.[3,9,29–31] Analysis of MNSI index to components of the metabolic syndrome yielded the same results, with the only significant correlation a positive association between MNSI index and diastolic blood pressure (Table S2).

Baseline acylcarnitine and free fatty acid abundance associated with future neuropathy status

We next conducted lipidomics on serum, which had been banked one decade earlier from these participants to identify early lipidomic changes correlating with neuropathy development. Participants with significantly reduced overall baseline abundance of medium-chain (C6–C14) acylcarnitines correlated with the development of peripheral neuropathy at a 10-year follow-up ($p < 0.001$; Fig. 2A), that is, had high MNSI index scores. Species-specific differences by chain length and saturation level were nonsignificant, likely attributable to a lack of statistical significance from limited power of low sample size (Fig. 2B). However, the trend for all individual medium-chain species with 0, 1, or 2 double bonds followed the aggregate trend and were lower in participants with versus without neuropathy. In short-chain acylcarnitines, there were nonsignificant trends for reduced L-carnitine and C4 and elevated C2 and C3 in participants with versus without neuropathy. In long-chain acylcarnitines, there were also trends for decreased unsaturated species and increased saturated species by participants with versus without neuropathy. Elevated aggregate free fatty acid values correlated with participants that developed neuropathy ($p = 0.042$; Fig. 3A). When we examined individual free fatty acids by carbon and double bond numbers, there were no significant differential levels in single species (Fig. 3B). However, long-chain (C20–C24) saturated and unsaturated free fatty acids had nonsignificant trends for being generally elevated in participants with neuropathy compared to participants without neuropathy. Differences in free fatty acids with 16 and 18 carbons were far less pronounced. Finally, correlation analysis of the MNSI index to lipids similarly found significant correlations between acylcarnitines ($p = 0.015$) and free fatty acids ($p = 0.006$) (Fig. S2A and B).

Overall, at baseline, lower aggregate medium-chain acylcarnitines and higher aggregate free fatty acids levels in serum were associated with the presence and severity of peripheral neuropathy in participants with type 2 diabetes at the 10-year follow-up.

Baseline phosphatidylcholine and lysophosphatidylcholine abundance associated with future neuropathy status

Of the complex lipids, we found that baseline abundance differences in phosphatidylcholines and lysophosphatidylcholines are associated with future peripheral neuropathy status. Overall, decreased phosphatidylcholine aggregate abundance correlated with neuropathy in participants at

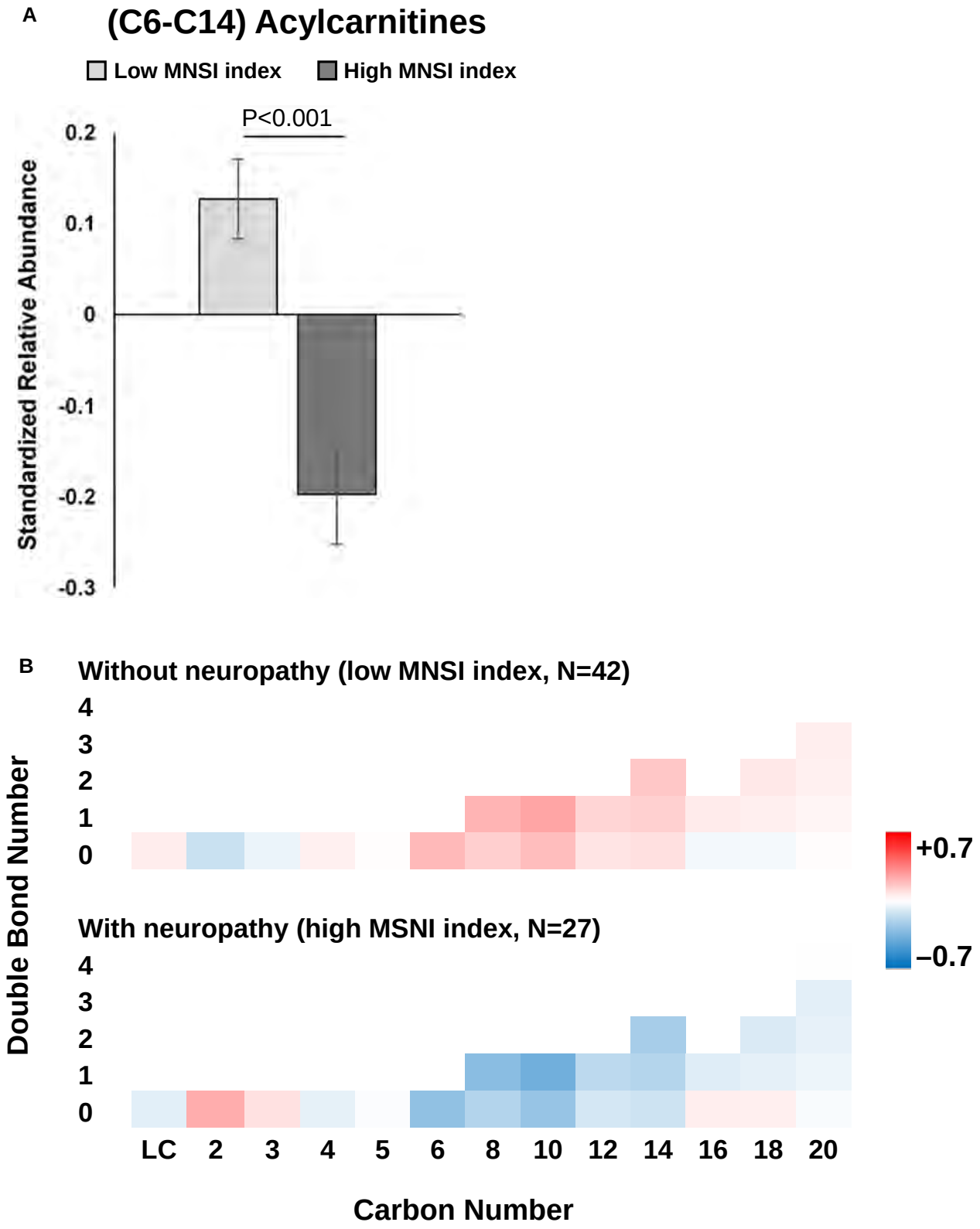

Figure 2. Acylcarnitine abundance by neuropathy status. (A) Overall, mean C6–C14 acylcarnitine abundance was significantly decreased in participants with (dark gray, high MNSI index) versus without neuropathy (light gray, low MNSI index; $p < 0.001$). (B) Heatmap of acylcarnitine abundance by chain length (carbon number) and saturation (double bond number) for participants with ($n = 27$, high MNSI index) versus without neuropathy ($n = 42$, low MNSI index). The scale represents acylcarnitines species that are increased (red) or decreased (blue) in groups. A and B are based on generalized linear mixed models with carbon number, double bond number, and MNSI index groups as main effect variables and the MNSI index group by carbon number interaction, adjusted for other covariates. B, bars represent *z*-score standardized mean values ± SEM. LC, L-carnitine; MNSI, Michigan Neuropathy Screening Instrument.

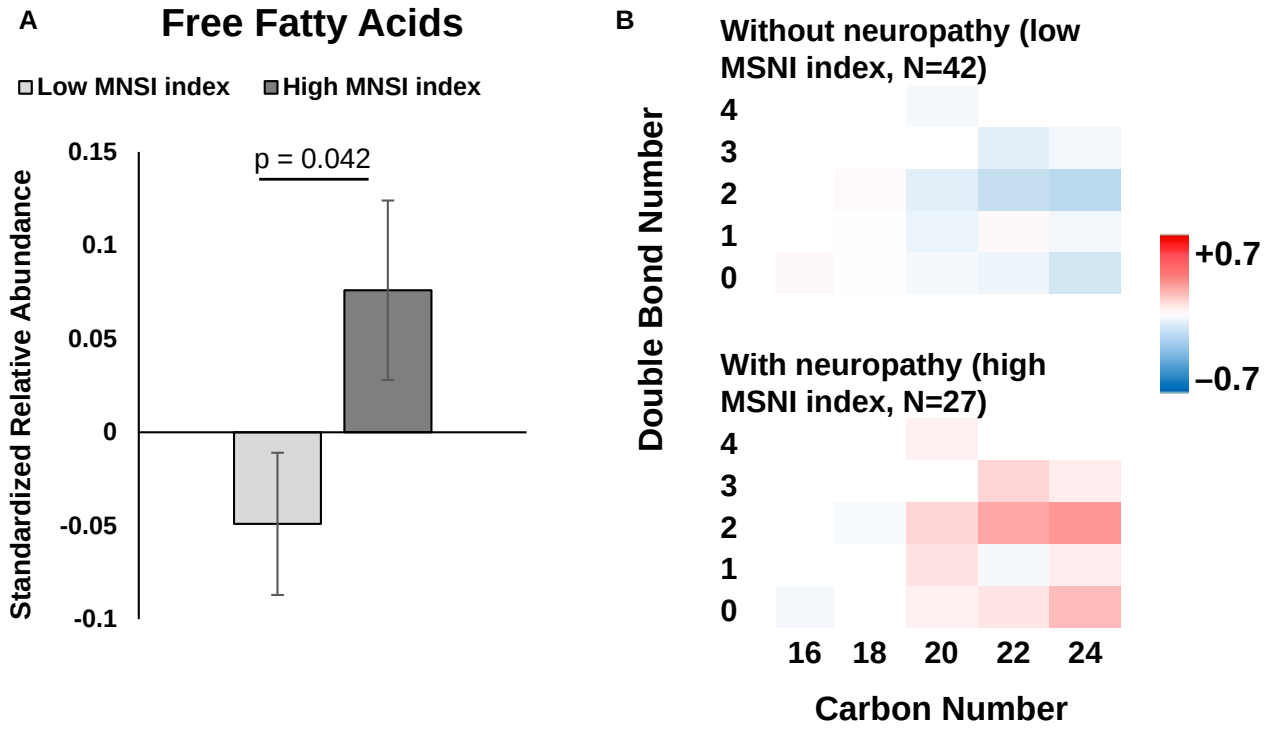

Figure 3. Free fatty acid abundance by neuropathy status. (A) Overall, mean free fatty acid abundance was significantly increased in participants with (dark gray, high MNSI index) versus without neuropathy (light gray, low MNSI index; $p = 0.042$). (B) Heatmap of free fatty acid abundance by chain length (carbon number) and saturation (double bond number) for participants with ($n = 27$, high MNSI index) versus without neuropathy ($n = 42$, low MNSI index). The scale represents acylcarnitines species that are increased (red) or decreased (blue) in groups. (A and B) are based on generalized linear mixed models with a double bond number, carbon number, and MNSI index groups as main effect variables. (B) bars represent *z*-score standardized mean values ± SEM. MNSI, Michigan Neuropathy Screening Instrument.

the 10-year follow-up ($p = 0.016$; Fig. 4A). Although nonsignificant, most mono- and polyunsaturated phosphatidylcholines were diminished in banked serum from participants that eventually developed neuropathy (i.e., high MNSI index; Fig. 4B). In contrast, the class level of lysophosphatidylcholine abundance was significantly higher in participants with versus without neuropathy ($p = 0.017$; Fig. 5A). This observation is aligned with lower phosphatidylcholines in the neuropathy group, since lysophosphatidylcholines are generated from phosphatidylcholines by phospholipase A2-mediated removal of a fatty acid chain. There were no significant chain length- and saturation-dependent differences by neuropathy status (Fig. 5B). Broadly, individual lysophosphatidylcholine species followed the aggregate trend, with few exceptions, most notably the saturated C18 species. As anticipated, correlation analysis of the MNSI index to lipids identified a significant correlation to lysophosphatidylcholines ($p = 0.006$) (Fig. S2C).

Cumulatively, of complex lipids, differential baseline serum phosphatidylcholine and lysophosphatidylcholine levels correlated with the presence and severity of peripheral neuropathy one decade later in Pima participants with type 2 diabetes. Abundances of other signaling and complex lipids did not significantly affect future neuropathy status (Figs. S1 and S2D–L). Among these lipid classes, none of the covariates were used to adjust models associated with lipid level variation.

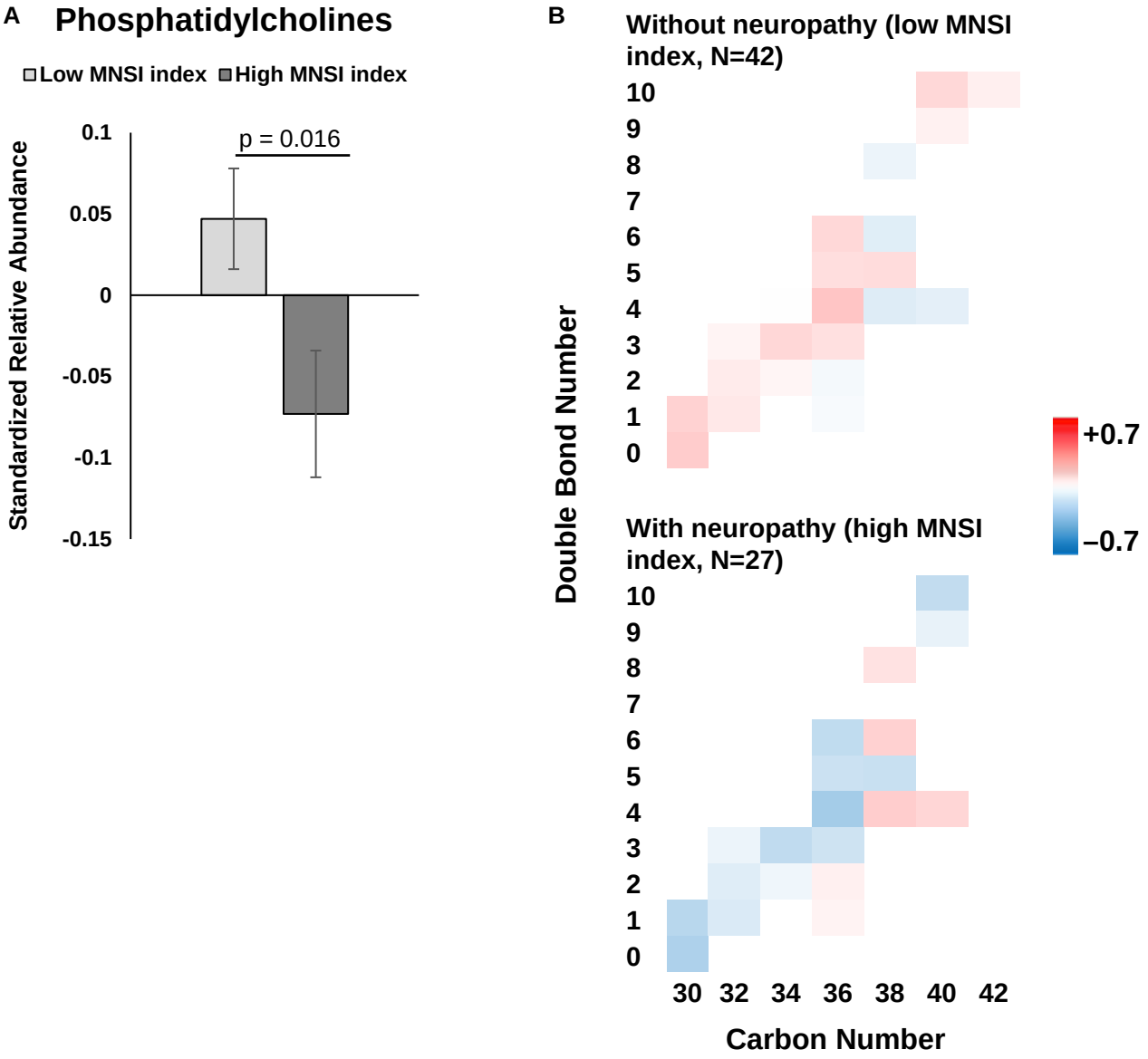

Figure 4. Phosphatidylcholine abundance by neuropathy status. (A) Overall, mean free fatty acid abundance was significantly decreased in participants with (dark gray, high MNSI index) versus without neuropathy (light gray; low MNSI index; $p = 0.016$). (B) Heatmap of phosphatidylcholine abundance by chain length (carbon number) and saturation (double bond number) for participants with ($n = 27$, high MNSI index) versus without neuropathy ($n = 42$, low MNSI index). The scale represents acylcarnitines species that are increased (red) or decreased (blue) in groups. A and B are based on generalized linear mixed models with a double bond number and MNSI index groups as main effect variables, adjusted for other covariates. B, bars represent *z*-score standardized mean values ± SEM. MNSI, Michigan Neuropathy Screening Instrument.

Discussion

Herein, for the first time, to our knowledge, we demonstrate that a serum lipidomics signature associates with future incident peripheral neuropathy. Specifically, in Pima participants with type 2 diabetes, decreased baseline serum medium-chain acylcarnitines and increased free fatty acids associated with peripheral neuropathy, were assessed by the MNSI index, one decade later. Participants that developed neuropathy also had lower phosphatidylcholines and higher lysophosphatidylcholines versus participants that did not develop neuropathy. The abundance of other lipid classes did not significantly vary with neuropathy. These findings indicate lipid changes related to impaired mitochondrial β-oxidation in participants that develop peripheral neuropathy.

Diabetes is the greatest risk for peripheral neuropathy,[4,31] although dyslipidemia,[3,5,8–11] obesity,[2,3,30] and additional

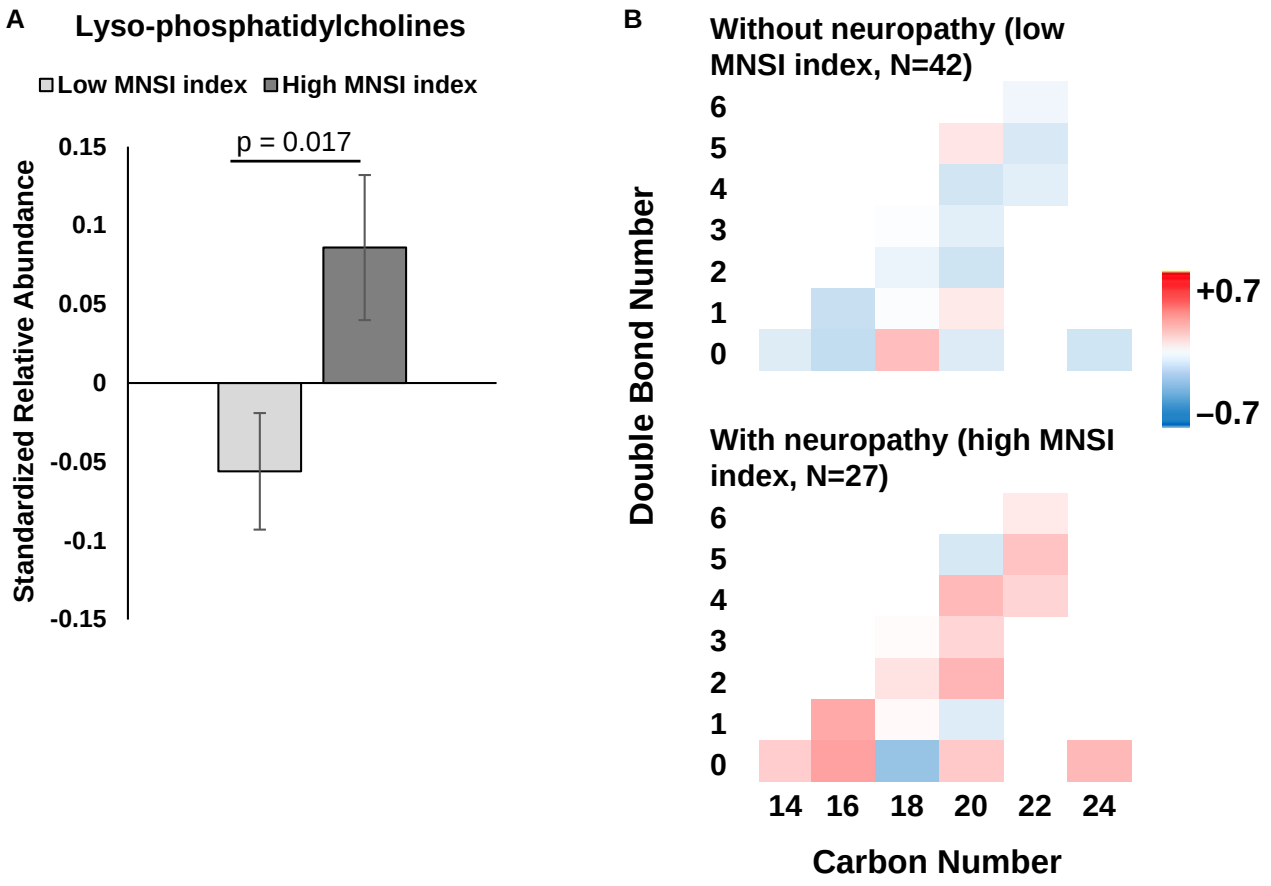

Figure 5. Lysophosphatidylcholines abundance by neuropathy status. (A) Overall, mean free fatty acid abundance was significantly increased in participants with (dark gray, high MNSI index) versus without neuropathy (light gray, low MNSI index; $p = 0.017$). (B) Heatmap of lysophosphatidylcholine abundance by chain length (carbon number) and saturation (double bond number) for participants with ($n = 27$; high MNSI index) versus without neuropathy ($n = 42$, low MNSI index). The scale represents acylcarnitines species that are increased (red) or decreased (blue) in groups. A and B are based on generalized linear mixed models with a double bond number and MNSI index groups as main effect variables, adjusted for other covariates. B, bars represent *z*-score standardized mean values ± SEM. MNSI, Michigan Neuropathy Screening Instrument.

components of the metabolic syndrome, such as hypertension,[30,32] are additional important metabolic risk factors. Herein, systolic and diastolic blood pressure were elevated in participants with versus without neuropathy.[13,28] Moreover, microalbuminuria and DKD at baseline correlated with neuropathy at the follow-up in Pima participants with type 2 diabetes.[6] However, there were no significant differences in baseline glycemic (FPG, HbA_{1c}) and basic lipid (triglycerides, total cholesterol) profiles or anthropometric measures (BMI). This may be due to the relatively smaller sample size in the group with ($n = 27$) versus without ($n = 42$) neuropathy or the presence of additional risk factors beyond glycemic and basic lipid profiles, such as intermediate and complex lipids, which may associate with future incident neuropathy. We previously noted no differences in basic lipid, glycemic, or anthropometric metrics in type 2 diabetes participants with ($n = 49$) versus without neuropathy ($n = 48$) in our larger ADDITION study.[18] Since there were no differences in basic lipid profiles, we next investigated the lipidomics profiles from stored serum baseline samples.

We found a specific lipidomics profile of differential aggregate levels of decreased medium-chain acylcarnitines, increased free fatty acids, decreased phosphatidylcholines, and increased lysophosphatidylcholines associated with future incident neuropathy in Pima participants with type 2 diabetes. This agrees with metabolomics and lipidomics profiles of incident peripheral neuropathy in the Danish ADDITION cohort,[18] which collected plasma at the time of neuropathy assessment. Participants with neuropathy had a trending increase in triacylglycerols in both the ADDITION and Pima studies. We saw abundance variation by carbon and double bonds in diacylglycerols and other sphingo- and phospholipids in both the Pima and ADDITION studies, although trends were more uniform in ADDITION, especially for diacylglycerols,

sphingomyelins, ceramides, and phosphatidylethanolamines.[18] Differences between the two studies may have arisen from the distinct temporal assessments of lipid signatures relative to peripheral neuropathy development in the two studies, from our smaller sample size in the current study, or from natural variation in the populations.

Few other studies have compared plasma or serum lipidomics of participants with type 2 diabetes to peripheral neuropathy status. We recently assessed cross-sectional lipidomics in obese participants, independent of glycemic status, and found peripheral neuropathy was characterized by differential diacylglycerols, phosphatidylcholines, sphingomyelins, ceramides, and dihydroceramides.[19] Ziegler et al. analyzed cross-sectional plasma signatures from participants from the German Diabetes Study with recent-onset type 2 diabetes (n = 95) to cardiac autonomic neuropathy.[33] Several phosphatidylcholines and sphingomyelins correlated inversely with cardiac autonomic neuropathy in participants with type 2, but not type 1, diabetes, concluding this may arise from dyslipidemia as a major driver of nerve damage secondary to type 2 diabetes.[33]

We have also reported lipidomic analyses of other diabetic complications in the Pimas, including nephropathy[13] and retinopathy.[34] The neuropathy lipidomic signatures from the current study were distinct to both the DKD and the retinopathy signatures, although signatures in all three diabetic complications centered around impaired β-oxidation. This aligns with our mouse data of tissue-specific metabolic and lipidomic differences in nerve, kidney, and retina in type 2 diabetes.[16,35] Overall, these studies underscore the importance of baseline plasma lipidomics profiles on the development, even a decade later, of type 2 diabetes complications,[13,34] including peripheral neuropathy.

Lipids are a diverse class of molecules with numerous biological functions, especially in the nervous system. Herein, the lipidomic signatures associated with neuropathy in Pima participants with type 2 diabetes centered on lipids related to mitochondrial function. Indeed, dysfunctional mitochondrial dynamics underpin diabetic neuropathy,[36] including dyslipidemia, which impairs mitochondrial trafficking[20,21,37] and bioenergetics, leading to energy failure and resulting nerve injury.[37] In the current study, serum linked to neuropathy was characterized by elevated free fatty acids and diminished medium-chain acylcarnitines. Free fatty acids are an important energy source through mitochondrial β-oxidation. Fatty acids are shuttled into mitochondria by conjugating with L-carnitine, forming acylcarnitine intermediates, which are converted back to the fatty acid acyl within mitochondria, where they are metabolized by β-oxidation.[38] Free fatty acids accumulation and decreased medium-chain acylcarnitines suggest blockade in fatty acid to acylcarnitine conversion and disrupted mitochondrial β-oxidation, a scenario likely arising from fatty acid substrate excess,[38] as occurs in dyslipidemia.

Complex phospholipids, such as the ratio of phosphatidylcholines to phosphatidylethanolamines, dictate membrane curvature, regulating mitochondrial biogenesis and bioenergetics.[39,40] Herein, reduction phosphatidylcholines signals potential changes to mitochondrial structure and, in turn, mitochondrial function. Moreover, lysophosphatidylcholines, which were elevated in aggregate in participants that developed neuropathy, correlate with insulin resistance[41] and are linked to retinal neurodegeneration in preclinical studies.[42] Lysophosphatidylcholines are also precursors to lysophosphatidic acid, which is related to neuropathic pain,[43] a frequent symptom in type 2 diabetes patients and peripheral neuropathy. Although lipidomics identified differential abundance in these lipid species in participants with type 2 diabetes and neuropathy, preclinical studies are needed to infer causality from these specific lipids and/or elucidate pathomechanisms.

This study has several strengths. The Pima Indian type 2 diabetes cohort is long-established and very homogenous and has been deeply phenotyped for several diabetic complications.[13,28,34] The great extent of homogeneity minimizes potential confounders, creating a unique opportunity to explore the biology of neuropathy in a type 2 diabetes cohort. Physical examinations and data collection followed well-specified research protocols, resulting in high-quality data. Additionally, the mass spectrometry quality control protocol ensured high-quality lipidomic data on a large array of lipid classes, with low coefficients of variations and minimal to no batch-to-batch variability.

This study also has limitations. Serum lipidomics can identify biomarkers of diabetic peripheral neuropathy, but the relationship between serum versus nerve tissue lipids and damage is uncertain. Our study did not assess nerve conduction velocities as a neuropathy outcome; however, the MNSI index has good diagnostic characteristics for neuropathy (area under the curve of 0.86 for the receiver operating characteristic curve).[44] Moreover, the study did not assess baseline MNSI index; however, diabetic neuropathy prediction risk scores indicate participants likely did not have neuropathy at baseline.[26] As an observational study, we cannot infer causality between lipid species abundances to later development of neuropathy; however, our observations align with our preclinical model studies, which inferred causality.[15,20,21] The cohort is relatively small and findings will need replication in larger cohorts, although findings in this cohort have broadly been confirmed in other populations,[18] especially for DKD.[13,45] Our study also noted multiple interesting non-significant trends, which larger studies might be powered to rigorously assess. Our study was too small for analyses

by sex, though sex-dependent differences in the plasma lipidome have been reported.[46–48] Finally, as with other Omic studies, our lipidomics platform generated a relatively large number of lipids; hence, applying traditional statistical methods to individualized lipids is limited by our sample size and hence the potential of false discovery. To overcome these limitations, we continuously applied data reduction strategies. This included applying intralipid class mixed models, which reduced multiplicity and potential for false discovery, and enhanced statistical power. Yet, the approach retained deep pathophysiological insight by accounting for lipid profiles in relation to neuropathy phenotype.

We conclude that aggregate abundance differences in circulating medium-chain acylcarnitines, free fatty acids, phosphatidylcholines, and lysophosphatidylcholines, early in the course of the disease are linked to the later development of human type 2 diabetic neuropathy. Our findings have important clinical implications. They suggest a potential diagnostic route through biomarker discovery and risk stratification to identify type 2 diabetes patients at the highest risk of peripheral neuropathy, facilitating better management in this patient subset. Additionally, further research validating and delineating the relationship of serum lipid species to neuropathy in preclinical models could enhance our understanding of pathogenesis and open avenues for targeted therapeutic development. Importantly, since lipidomics highlighted impaired mitochondrial β-oxidation, it suggests that conventional lipid-lowering medication, such as statins and fenofibrates, which act through cholesterol and apoprotein synthetic pathways, may be ineffective for treating neuropathy. Indeed, statin use does not appear to impact neuropathy onset.[49] As an alternative approach, this study underscores a possible need for therapeutics that optimize fatty acid metabolism and enhance mitochondrial β-oxidation.

Acknowledgments

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Conflicts of Interest

BCC declares consulting fees from Dynamed. All other authors have nothing to disclose.

Authors' Contribution

FA designed the lipidomic study, prepared serum samples for mass spectrometry, analyzed and interpreted the data, and wrote the first draft. TMR performed serum sample preparation for lipidomic analysis and mass spectrometry runs. TS retrieved mass spectrometry data. JB performed serum sample preparation for lipidomic analysis and mass spectrometry runs. MGS interpreted data and wrote the first draft. HCL and RGN contributed to clinical study design, data collection, and manuscript drafting. GM performed statistical analyses and contributed to manuscript drafting. SP contributed to the lipidomic study design, mass spectrometry, data interpretation, and manuscript drafting. ELF trained clinical research coordinators on MNSI administration and oversaw data collection, contributed to the lipidomic study design, data interpretation, manuscript drafting, and secured study funding. All authors critically evaluated the paper and have approved the final version. ELF is the guarantor.

Data Availability Statement

Anonymized data will be shared by request from any qualified investigator.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 Identified lipids by adduct, mass-to-charge ratio (*m*/*z*), and retention time (RT) in positive and negative modes. The mass accuracy was ±0.001 Da in positive mode and ±0.005 Da in negative mode, with an overall mass error of <2 ppm. CE, cholesterol ester; CerP, ceramide-phosphate; CL, cardiolipin; DAG, diacylglycerol; FFA, free fatty acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MAG, monoacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; pPC, plasmenyl-phosphatidylcholine; pPE, plasmenyl-phosphatidylethanolamine; SM, sphingomyelin; TAG, triacylglycerol.

Table S2. Correlation of MNSI index with baseline variables. *p = 0.017; BMI, body mass index; DBP, diastolic blood pressure; FPG, fasting plasma glucose; MNSI, Michigan Neuropathy Screening Instrument; SBP, systolic blood pressure.

Figure S1. Various lipid class abundance by neuropathy status. Heatmap of lipid abundances reveals no statistically significant differences in participants with (n = 27; high MNSI index) versus without neuropathy (n = 42; low MNSI index) for (A) sphingomyelins (SM), (B) phosphatidylinositols (PI), (C) lysophosphatidylethanolamines (LPE), (D) plasmenyl-phosphatidylethanolamines (pPE), (E) cholesteryl-esters (CE), (F) phosphatidylethanolamines (PE), (G) diacylglycerols (DAG), (H) triacylglycerols (TAG).

Figure S2. Correlation between neuropathy severity and lipids. Heatmap of Pearson correlation coefficients of neuropathy severity (MNSI index) with each lipid by lipid class by carbon number (x-axis) and double bond number (y-axis) for (A) acylcarnitines (AC), (B) free fatty acids (FFA), (C) lysophosphatidylcholines (LPC), (D) phosphatidylcholines (PC), (E) triacylglycerols (TAG), (F) phosphatidylinositols (PI), (G) cholesteryl-esters (CE), (H) diacylglycerols (DAG), (I) lysophosphatidylethanolamines (LPE), (J) phosphatidylethanolamines (PE), (K) plasmenyl-phosphatidylethanolamines (pPE), and (L) sphingomyelins (SM).

The Impact of the COVID-19 Pandemic on Ethnic Minority Groups With Diabetes

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Major ethnic disparities in diabetes care, especially for intermediate outcomes and diabetes complications, were evident prior to the coronavirus disease 2019 (COVID-19) pandemic. Diabetes is a risk factor for severe COVID-19, and the combination of these ethnic disparities in diabetes care and outcomes may have contributed to the inequity in COVID-19 outcomes for people with diabetes. Overall, ethnic minority populations have suffered disproportionate rates of COVID-19 hospitalization and mortality. Results from the limited number of studies of COVID-19 in ethnic minority populations with diabetes are mixed, but there is some suggestion that rates of hospitalization and mortality are higher than those of White populations. Reasons for the higher incidence and severity of COVID-19–related outcomes in minority ethnic groups are complex and have been shown to be due to differences in comorbid conditions (e.g., diabetes), exposure risk (e.g., overcrowded living conditions or essential worker jobs), and access to treatment (e.g., health insurance status and access to tertiary care medical centers), which all relate to long-standing structural inequities that vary by ethnicity. While guidelines and approaches for diabetes self-management and outpatient and inpatient care during the pandemic have been published, few have recommended addressing wider structural issues. As we now plan for the recovery and improved surveillance and risk factor management, it is imperative that primary and specialist care services urgently address the disproportionate impact the pandemic has had on ethnic minority groups. This should include a focus on the larger structural barriers in society that put ethnic minorities with diabetes at potentially greater risk for poor COVID-19 outcomes.

A significant body of evidence indicates that many of the most vulnerable in our society have faced the greatest burden of both direct and indirect consequences of the coronavirus disease 2019 (COVID-19) pandemic, including the elderly, people living in deprived areas, people with learning difficulties, the homeless, and migrants. In particular, ethnic minority populations in many European countries and North America are at greater risk of infection and severe outcomes due to COVID-19 (1–3), although recent data on later waves and variants suggest some of these disparities have reduced for some minority groups (4).

There is now strong evidence that having a long-term condition, such as diabetes, increases risk of severe COVID-19 outcomes. Due to the greater prevalence of comorbidities in some ethnic minority populations (5), this may be one factor explaining outcome disparities between ethnic groups (Fig. 1). However, despite the large volume of literature and many reviews focusing on the association between ethnicity and COVID-19, there is, to our knowledge, far less attention paid to

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Figure 1—Potential reasons for COVID-19 disparities among ethnic minority populations.

examining differences in COVID-19 outcomes by ethnic groups in patients with diabetes.

Therefore, this Perspective summarizes the literature on prepandemic ethnic disparities in diabetes care as well as the wider context of disrupted diabetes care during the pandemic. It also provides an overview of the limited literature on ethnic disparities in COVID-19 in people with diabetes. Finally, this article concludes with a summary of potential explanatory mechanisms for ethnic disparities in COVID-19, interventions implemented to improve diabetes outcomes during the pandemic, structural determinants that require addressing, and recommendations for future research.

PREPANDEMIC DISPARITIES IN DIABETES CARE

Prior to the COVID-19 pandemic, numerous studies had reported major ethnic and socioeconomic disparities in diabetes care, particularly for intermediate outcomes and diabetes complications. For patients with type 1 diabetes, inequalities in access to technology and insulin pumps also exist. The combination of these ethnic disparities in diabetes care and outcomes has been a significant contributor to the predictable and undesirable inequity in COVID-19 outcomes for people with diabetes.

Internationally, ethnic and socioeconomic disparities in intermediate diabetes outcomes have been repeatedly demonstrated. For example, a 2013 U.S. study found that the Hispanic population was less likely to receive five diabetes quality-of-care recommendations, including twice-yearly HbA_{1c} tests and yearly foot exam, dilated eye exam, cholesterol test, and flu vaccination (6). Black patients were also less likely to receive HbA_{1c} tests, eye exams, and flu vaccination, and Asian patients were less likely to receive HbA_{1c} tests and annual foot and eye examinations. These differences were eliminated after adjusting for insurance status and education.

Similarly, in a nationally representative study of 164 English primary care practices, Black patients and people from the lowest socioeconomic status (SES) groups had higher HbA_{1c} than White patients and people from the highest SES groups (7). In a systematic review of 24 studies, Black patients were ∼30% less likely to have HbA_{1c} and blood pressure control but were ∼20–50% more likely to receive eye or foot examinations than White patients (8). A high school degree or higher was associated with a greater likelihood of HbA_{1c} control and eye examination. Other social determinants of health (i.e., income, access to care, and food insecurity) were also related, albeit more weakly, to these intermediate outcomes.

The results of disparities in diabetes quality of care and intermediate outcomes have translated into well-characterized ethnic and socioeconomic disparities in eye, kidney, and cardiovascular disease. Black, Latino, and Asian patients all have higher rates of diabetic eye disease than non-Hispanic White patients (8). Additionally, Black, Latino, Asian, Filipino, and mixed-ethnicity patients all have been found to have higher rates of end-stage renal disease than non-Hispanic White patients within the Kaiser Permanente Northern California integrated health care delivery system (9). Furthermore, in a 2020 study of people with type 2 diabetes, South Asian patients had a higher cardiovascular disease risk than people of White ethnicity (10).

Studies in China, Sweden, and the U.S. have identified an association between low SES and increased diabetes complication rates. For example, in a Chinese cohort of over 25,000 patients with type 2 diabetes, the least educated population groups had the highest risk for cardiovascular disease, cerebrovascular disease, and retinopathy, and the lowest household income population had the highest rate of retinopathy and neuropathy (11). In a study of over 24,000 Swedish patients with type 1 diabetes, similarly, the risk of death was three times

higher among patients in the two lower income quintiles compared with the highest income quintile, and the risk for cardiovascular disease and stroke was twofold (12). Higher education was also associated with a lower risk of stroke. In the U.S., risk of death was ~1.5 times greater among those with the lowest education level and without measures of financial wealth, such as home ownership (13). Importantly, accounting for U.S. socioeconomic differences by ethnicity narrows the excess mortality attributed to diabetes for White and Hispanic populations but not for non-Hispanic Black populations (14).

IMPACT OF THE PANDEMIC ON DIABETES CARE FOR ETHNIC MINORITIES

Data from England suggest there was between a 74% and 88% reduction in care processes during the pandemic compared with prepandemic levels (15). Other work has also suggested reduced consultation rates and hospital admissions, 30% reduction in diabetes diagnoses, and 70% reduction in HbA_{1c} testing during the pandemic (16). Older patients from deprived areas experienced the largest reductions in health checks, but high-quality data on the differences by ethnic groups are lacking.

The few studies that have examined the impact of the pandemic on diabetes care have suggested that there have been no ethnic disparities in diabetes care. For example, in the U.K., a study of 618,161 people with type 2 diabetes from 1,744 general practices identified similar lower rates of performing health checks and prescribing diabetes medications by ethnicity (15). Interestingly, a study of a large academic U.S. health system reported a higher rate of eye exams among Black, Asian, and Hispanic patients with diabetes compared with White patients and no difference in HbA_{1c} and nephropathy testing (17). Of note, a major shift to telemedicine occurred during the pandemic, and one study of 1,292 patients did not identify ethnic disparities in telemedicine use for subspecialty diabetes care but did report lower rates of telemedicine use among people with a non-English primary language and older adults (18).

ETHNIC DISPARITIES IN COVID-19 OUTCOMES IN PEOPLE WITH DIABETES

Diabetes is a risk factor for severe COVID-19, including intensive care unit (ICU) admission and death (19). However, despite multiple publications reporting the association of ethnicity with COVID-19 outcomes in ethnic minority populations, very few studies have examined differences in COVID-19 outcomes by both ethnicity and diabetes (Table 1). The largest is a National Population Database study, which included 264,390 people with type 1 diabetes and 2,874,720 people with type 2 diabetes in England (20). Overall, in individuals with type 1 diabetes, South Asian, Black, mixed, and other ethnic groups had significantly higher mortality risk from COVID-19 versus White populations (20). For people with type 2 diabetes, risk of in-hospital mortality was greater for Asian, Black, and mixed ethnic groups but not for the other ethnic groups (20). The other ethnic groups included Vietnamese, Japanese, Filipino, Malaysian, and other ethnicities.

Another U.K. study of over 19,000 COVID-19 high-dependency-unit and ICU admissions found that of 3,524 patients with diabetes, 58.5% were White, 21.9% were Asian, 11.6% were Black, and 8% were mixed or other ethnicity. There was no evidence of a difference in COVID-19 mortality associated with type 2 diabetes in subgroups defined by ethnicity (21). Other U.K. data include a small, single-center study of 39 patients with end-stage renal disease secondary to diabetic kidney disease, which reported a higher prevalence of patients of African-Caribbean ethnicity hospitalized with COVID-19 (60%) versus White or other ethnic groups (22).

In a multisite, cross-sectional, observational study of 113 people in the U.S. with type 1 diabetes (n = 58 hospitalized), people of ethnic minority background (Black, Hispanic, and other) with confirmed COVID-19 were, on average, 3.63 times more likely to be hospitalized than non-Hispanic White patients (23). The same research group also subsequently reported a cohort of 180 patients with type 1 diabetes and laboratory-confirmed COVID-19, and they found that Black patients were significantly more likely to present with diabetic ketoacidosis (DKA) than non-Hispanic White patients (24). Additionally, in a multisite prospective study of 137 service locations and 313 patients with type 1 and type 2 diabetes, compared with the White group, the Black group was significantly associated with hospitalization and worsening illness severity within 114 days of a positive COVID-19 test (25). However, a retrospective study of 6,104 people with type 2 diabetes from an ethnically diverse southern U.S. sample found 239 (39%) tested positive for COVID-19, but there were no significant differences in mortality between Black and White patients (26).

In a retrospective study from the University of Michigan, among those with diabetes, in the whole cohort Black patients were significantly more likely to be tested for COVID-19 and have positive test results than White patients; however, hospitalization, ICU admission, and mortality were not different for White and Black COVID-19 patients with type 2 diabetes (5). Another multisite study of 4,413 COVID-19 patients with type 2 diabetes admitted to New York hospitals reported that most patients were non-White (67.3%), with 27.1% Black, 17.2% Hispanic, and 24.9% multiethnic or other ethnicity (27). There was no significant association between ethnicity and mortality (27). Similarly, a nationwide retrospective cohort study in the U.K. (n = 19,256) reported no difference in mortality associated with type 2 diabetes in subgroups defined by ethnicity (21).

Overall, results from the few studies of COVID-19 in ethnic minority populations with diabetes are mixed, but there is some suggestion that the rates of hospitalization and mortality are higher than those of White populations. Although a number of larger population-level studies have been performed, these were all conducted in the U.K. or U.S., and there is a need for evidence from other countries, particularly lower- and middle-income countries. In addition, further work is needed to examine disparities for more granular ethnic groups (e.g., breaking down South Asian into Indian, Pakistani, etc.), although efforts may be hampered by poor ethnicity coding in routine health care data in many countries.

Table 1—Summary of studies on COVID-19–related outcomes by ethnic group in patients with diabetes

Reference	Country	Design	Population	Results
Corcillo et al. (22)	U.K.	Not stated	Type 1 and 2 diabetes	High prevalence of patients of Afro-Caribbean ethnicity hospitalized with COVID-19; 73% and 54% prevalence in renal transplant and hemodialysis groups, respectively. The mortality rate of the cohort was 36%.
Holman et al. (20)	U.K.	Population cohort	Type 1 and 2 diabetes	In individuals with type 1 diabetes, South Asian (HR 1.57, 95% CI 1.16–2.12), Black (HR 1.77, 95% CI 1.25–2.49), mixed (HR 1.77, 95% CI 1.25–2.49), and other ethnic groups (HR 1.89, 95% CI 1.03–2.37) had significantly higher mortality risk from COVID-19 than White populations. For people with type 2 diabetes, risk of in-hospital mortality was greater for Asian (HR 1.08, 95% CI 1.01–1.15), Black (HR 1.63, 95% CI 1.51–1.77), and mixed ethnic groups (HR 1.30 95% CI 1.10–1.55) but not for the other ethnic groups (HR 1.01, 95% CI 0.86–1.18).
Dennis et al. (21)	U.K.	Retrospective cohort	Type 2 diabetes	No difference in mortality associated with type 2 diabetes in subgroups defined by ethnicity.
Crouse et al. (26)	U.S.	Retrospective data registry	Type 2 diabetes	In COVID-19–positive patients (n = 604), diabetes was associated with increased mortality (OR 3.62, 95% CI 2.11–6.2), adjusting for age, sex, ethnicity, obesity, and hypertension. Ethnicity was not an independent predictor of mortality.
Ebekozien et al. (24)	U.S.	Observational	Type 1 diabetes	Non-Hispanic Black (11.7 [IQR 4.7]) and Hispanic (9.7 [IQR 3.1]) patients had higher median HbA_{1c} than White patients (8.3 [IQR 2.4]). More non-Hispanic Black (55%) and Hispanic (33%) patients presented with DKA than White patients (13%). Adjusting for confounders, non-Hispanic Black patients continued to have greater odds of presenting with DKA than non-Hispanic White patients (OR 3.7, 95% CI 1.4–10.61).
Gold et al. (63)	U.S.	Prospective	Type 1 and 2 diabetes	Among 305 patients, 121 (39.7%) had diabetes, of whom 103 (41.7%) were of Black ethnicity and 56 (32.0%) were of other ethnicities. Clinical outcomes (discharged alive, still hospitalized, needed invasive mechanical ventilation, and death) were similar between Black and non-Black individuals.
Gregory et al. (25)	U.K.	Prospective cohort	Type 1 and 2 diabetes	Compared with patients without diabetes, patients with type 1 diabetes had adjusted ORs for hospitalization risk of 3.90 (95% CI 1.75–8.69) and worsening illness severity of 3.35 (95% CI 1.53–7.33). For patients with type 2 diabetes, adjusted ORs were 3.36 (95% CI 2.49–4.55) for hospitalization, 3.42 (95% CI 2.55–4.58) for worsening illness severity, and 3.21 (95% CI 1.54–6.70) for death. Adjusted risk of worsening illness severity for Black versus White ethnic groups was 1.88 (95% CI 1.47–2.41).
Gu et al. (5)	U.S.	Retrospective cohort	Type 2 diabetes	In the COVID-19–positive cohort (n = 1,139), Black patients were more likely to be hospitalized (OR 1.72, 95% CI 1.15–2.58). No difference was found in ICU admission by ethnicity after adjusting for covariates. Type 2 diabetes was associated with hospitalization in White (OR 2.59, 95% CI 1.49–4.48) but not Black (OR 1.17, 95% CI 0.66–2.06) patients.

Continued on p. 5

Table 1—Continued

Reference	Country	Design	Population	Results
Kabarriti et al. (47)	U.S.	Retrospective	Type 1 and 2 diabetes	Of 5,902 patients who tested positive for COVID-19, 509 (8.6%) were non-Hispanic White, 1,935 (32.8%) were non-Hispanic Black, 1,905 (32.3%) were Hispanic, 171 (2.9%) were Asian, and 1,382 (23.4%) were other/unknown/decline to answer. Overall, 74 (14.5%) non-Hispanic White, 162 (8.4%) non-Hispanic Black, 175 (9.2%) Hispanic, 36 (21.1%) Asian, and 388 (28.1%) unknown/other ethnic group patients had diabetes. Overall, in adjusted analysis, Hispanic or non-Hispanic Black patients had significantly improved survival versus non-Hispanic White patients.
Myers et al. (27)	U.S.		Type 2 diabetes	Of 4,413 patients examined by multivariate analysis, male sex, older age, and admission hyperglycemia associated with increased mortality and intubation but not ethnicity, insurance type, or HbA_{1c} level.
O'Malley et al. (23)	U.S.	Multisite observational	Type 1 diabetes	Of 58 hospitalized COVID-19 patients, 26 were admitted to ICU and 5 male patients died. Hospitalization was more likely for increasing age, minority ethnicity (OR 3.63, 95% CI 1.42–9.70), and cardiovascular disease.
Price-Haywood et al. (64)	U.S.	Retrospective	Diabetes (does not state which type)	Of 3,481 COVID-19 patients, 2,451 (70.4%) were non-Hispanic Black and 1,030 (29.6%) were non-Hispanic White. Overall, 454 (18.5%) Black and 112 (10.9%) White patients had diabetes. In adjusted analysis, Black ethnicity was not associated with mortality.

IQR, interquartile range.

POTENTIAL PREDISPOSING FACTORS FOR ETHNIC DISPARITIES IN COVID-19

Ethnic disparities in rate, severity, and mortality from COVID-19 infection were evident early in the pandemic. Understanding the impact of ethnicity on COVID-19–related outcomes was initially hampered by a lack of data reporting patient ethnicity. However, with a greater appreciation, as the pandemic evolved, of associations of ethnicity with poorer COVID-19 outcomes, studies are now shedding light on the potential factors predisposing ethnic minorities to poorer clinical COVID-19 outcomes. The exact reasons for the higher incidence and severity of COVID-19–related outcomes in minority ethnic groups are complex and involve social, economic, cultural, and lifestyle factors and pathophysiological differences (28). These parameters then modulate, within ethnic populations, exposure to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), vulnerability to the disease, social environment, control measures, and biological differences (Fig. 1).

Increased Exposure to COVID-19

Greater exposure to the virus raises the likelihood individuals will contract COVID-19. During the pandemic, workers with essential occupations had increased frequency of contact with other individuals, for example, workers in health care settings, those who work indoors in crowded spaces, or workers making frequent contact with multiple people, such as those in transport and retail. Indeed, ethnic minority groups were overrepresented in a nationwide investigation of occupations classified as essential during the pandemic (29). This included medical staff, such as registered nurses, medical assistants, and bus drivers. A study of frontline health care workers (n = 99,795) versus the general community (n = 2,035,395) found higher risk of a positive COVID-19 test result for frontline health care workers (hazard ratio [HR] 3.40) after adjusting for multiple parameters, including their elevated likelihood of receiving a COVID-19 test (30). Secondary post hoc analyses found that frontline minority workers are at increased risk of a positive COVID-19 test compared with White coworkers. In New York City (NYC), frontline workers were overrepresented in Black (29.4%) and Hispanic (35.5%) populations (31). Therefore, one possible reason predisposing ethnic minorities to poorer outcome is greater exposure to the virus through overrepresentation of these groups in essential frontline jobs.

Increased Vulnerability to COVID-19

Another potential contributing factor to adverse COVID-19 outcomes, which was considered relatively early in the pandemic, is the high prevalence of comorbid conditions in ethnic minority populations (32), which is associated with increased hospitalization rate, ICU admission, and mortality. A study of UK Biobank data with 5,623 COVID-19 cases found that BMI was associated with a positive test in all patients, but that higher BMI (>30 kg/m^2) was a stronger determinant of a positive test in Black and minority ethnic patients than in White patients (33). Evaluation of NYC data found a relationship in the two boroughs with the highest COVID-19 mortality rate and the highest obesity rate, which was

most prevalent among Black and Hispanic residents (34). Notably, however, the study did not fully adjust for all potential risk factors. Another NYC study found that poorer neighborhoods had greater hypertension risk among Black residents (relative risk [RR] 3.4) and diabetes risk among Hispanic residents (RR 5.5) compared with residents of poorer White neighborhoods (31). Furthermore, poorer predominantly Black and Hispanic neighborhoods had more ICU admissions than poorer predominantly White neighborhoods. In addition, a nationwide U.S. county-level investigation found, after multiple adjustments, that counties with higher percentages of Black residents had a greater prevalence of comorbid conditions and COVID-19 diagnoses (RR 1.24) and deaths (RR 1.18) (35).

Disadvantaged Social Environment

Beyond potential health determinants, such as comorbidities, on COVID-19 outcomes for Black and Hispanic patients, several socioeconomic factors have been investigated. Urban setting, household crowding, income, lack of transportation, health insurance availability, and health care access could conceivably impact COVID-19 outcomes (29–31,35,36), although there is some discordance across studies (37). These determinants may also be differentially present by ethnicity, although few COVID-19 studies have been stratified by ethnicity.

Analysis of NYC electronic health records (n = 23,300) found a correlation between social disadvantage (compared by quintiles) and higher likelihood of Black or Hispanic ethnicity, of chronic comorbid conditions (e.g., diabetes, obesity, and hypertension), and higher hospitalization risk from COVID-19 (38). County-level analysis also found, after multiple adjustments, correlations between counties with greater burden of COVID-19 cases and the proportion of Black and Hispanic residents and counties with greater burden of COVID-19 deaths and the proportion of Black and Native American residents (36). A greater proportion of multiunit households and households lacking a vehicle correlated positively with cases and deaths, respectively. Another study also found a relationship between public transportation use (i.e., household lacking vehicles) (39) and mean household size (40) with COVID-19 cases, which are likely contributors in disadvantaged communities. In a nationwide U.S. county-level study, lack of health insurance was a greater risk factor for COVID-19 diagnosis in counties with a percentage of Black residents above the national average (35).

In addition to housing, transportation, income, and insurance status, opioid and other substance use disorders are contributors to adverse COVID-19 outcomes in the U.S. Nationwide, Black individuals with opioid use disorders (odds ratio [OR] 4.16) and substance use disorders (OR 2.17) were at higher COVID-19 risk than White individuals (41), although the analysis did not adjust for comorbidities.

Poor Control Measures

Control measures are used to limit viral spread in the community through awareness campaigns. However, ethnic minorities may experience language barriers, lower access to public health messaging, poor uptake or access to screening, and structural discrimination, leading to suboptimal control measures. County-level analysis by a U.S. study found that individuals with limited English proficiency were more likely to suffer COVID-19 deaths after multiple adjustments (36). In a nationwide study, ethnic minority status and language subindex correlated positively with COVID-19 incidence and mortality rates after adjusting for population density, urbanicity, and COVID-19 testing rate (42). Regarding uptake of public health messaging, a cross-sectional U.S. survey of 1,435 adults found that non-Hispanic White (30.7%) and Asian (25.0%) participants were likelier to correctly answer all 14 questions concerning COVID-19 versus Hispanic (19.7%) and non-Hispanic Black (15.8%) participants (43). Thus, it is possible that preventative control measures through public health campaigns are failing in ethnic minority communications.

An additional possible predisposing factor is medical mistrust, which can result from prior experience of unethical medical practices. Though understandable, this mistrust can represent a barrier to seeking medical care in the present. In a California cohort of Black individuals with HIV (n = 101), most (97%) held at least one general COVID-19 mistrust belief and over 50% held COVID-19 vaccine or treatment hesitancy beliefs (44). In a large U.K. study of 32,361 adults in the University College London COVID-19 Social Study, distrust of vaccines was higher among people of ethnic minority backgrounds than among White people (45). Therefore, overall, in addition to greater exposure and susceptibility, people from ethnic minorities may also be predisposed to more serious COVID-19 outcomes due to poorer control measures.

Biological Differences

Finally, it is important to note that although systematic reviews and meta-analyses associate ethnicity with poorer COVID-19 outcomes, some studies report no disparities or even protective effects (46–49). Nevertheless, in studies that do identify risk in Black, Hispanic, or other minority ethnic groups, this risk persists even after adjusting for comorbidities and socioeconomic factors (2,50–53). This suggests there are biological differences underlying differential responses of ethnicity to COVID-19. One possibility, given its pivotal role in promoting viral entry into host cells, is differential ACE2 expression by ethnicity (54) as well as of other molecules related to SARS-CoV-2 pathophysiology, e.g., immune function. Another possible aspect is single nucleotide polymorphisms in genes involved in SARS-CoV-2 infection, such as TMPRSS2, in addition to ACE2 (55,56). Caution in interpretation is required, however, because these potential mechanisms require further work. It is also possible that plausible biological mechanisms will be politicized to minimize criticisms of structural racism, which underpins social inequalities driving long-standing health inequalities in ethnic minority populations.

Overall, emerging evidence implicates multiple potential determinants of ethnic disparity in COVID-19 outcomes. Moving forward, a better understanding will be facilitated by stratifying study data sets by ethnicity (the most granular ethnic group data should be used when available). Additionally, most studies to date have been correlative. Although it will be challenging, future investigations should establish causality in order to implement evidence-based policies to alleviate disparities.

NEXT STEPS AND FUTURE RESEARCH PRIORITIES

As we now plan for recovery, improved surveillance, and risk factor management, it will be imperative that primary and specialist care services urgently address the disproportionate impact the pandemic has had on ethnic minority groups. Recommended interventions for diabetes patients during the pandemic, which has disproportionately impacted ethnic minority populations, focused on individual-level strategies and barriers to diabetes care (57–60) but excluded the larger structural barriers, which placed ethnic minorities with diabetes at particular risk for poor COVID-19 outcomes from the outset. These barriers include structural inequities in adequate housing, food, education, employment opportunities, and neighborhood resources, among others, which are important determinants of health for both diabetes and COVID-19 independently but particularly for individuals in high-risk populations within both groups (e.g., ethnic minorities).

Prioritizing affordable housing, a living wage, economic investments in ethnic minority communities, and policy changes to mitigate ethnic residential segregation are some long-term strategies to address the underlying housing and related economic challenges faced by ethnic minorities, particularly low-income minorities. Implementing these changes would strengthen the resilience of this population in weathering pandemics and natural disasters as well as in managing chronic diseases, such as diabetes, which require stable housing, refrigeration (for many types of insulin storage), and safe spaces for physical activity as part of optimal self-management.

Structural interventions, like equal access to high-quality education and employment opportunities, implementing a living wage, and job retraining for those impacted by the changing economy or who are reentering the workforce, can affect the short-term and long-term health trajectories of ethnic minority individuals with diabetes. Regular employment usually provides access to health insurance (for U.S. residents), and the use of supporting federal policies to provide paid sick leave and quarantine leave for COVID-19 is another way, along with pandemic mitigation, to ensure individual assistance reaches patients.

The pandemic also impacted food insecurity, which saw a large rise during this period. Before the pandemic, over 35 million Americans faced food insecurity, which rose to over 50 million, disproportionately affecting families and children (61). Food insecurity also disproportionately impacts ethnic minorities, low-income individuals, and other socially marginalized populations. Previous studies showed that for patients with diabetes, food insecurity is associated with worse diabetes self-management, up to twice the odds of poor diabetes control, and higher hospitalization rates (62). Supporting economic policies that reduce poverty and food policies that reduce food insecurity, such as increased eligibility for the Supplemental Nutrition Assistance Program in the U.S., will help improve diabetes outcomes.

As we move into the recovery phase, postpandemic research should focus on developing and evaluating interventions to address these disparities in the short and long term for all ethnic minority populations with long-term conditions such as diabetes. This should involve both individual-level and wider systems-level approaches. In addition, a number of other priorities require further attention. Given limited published reports, there is a need to utilize large population cohorts to further examine disparities in COVID-19 outcomes in ethnic minority populations with diabetes. It will also be important to further examine the impact of the pandemic on diabetes care for ethnic minorities, as limited work has been done in this area. Other outstanding issues include whether there are therapeutic differences in patients from ethnic minority populations with diabetes who were admitted with COVID-19 or who are positive for COVID-19 compared with White populations and if there are differential effects of therapies in ethnic minority groups with diabetes. Are new models of care, such as telemedicine, widening health disparities for ethnic minority populations with diabetes?

In summary, the structural inequities highlighted by the coronavirus pandemic, which have driven many of the disparities in COVID-19 morbidity and mortality, have exacerbated existing health disparities in diabetes among ethnic minority populations. While there have been many helpful guidelines and approaches for diabetes self-management as well as outpatient and inpatient care during the pandemic, few have recommended addressing structural drivers. Only by taking a long-term, holistic view of health and health care will we, and particularly our most vulnerable populations, be better able to weather future pandemics.

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Cutting-Edge Research: ALS and Brain Health

BRAIN
ORIGINAL ARTICLE

Metabolomics identifies shared lipid pathways in independent amyotrophic lateral sclerosis cohorts

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease lacking effective treatments. This is due, in part, to a complex and incompletely understood pathophysiology. To shed light, we conducted untargeted metabolomics on plasma from two independent cross-sectional ALS cohorts versus control participants to identify recurrent dysregulated metabolic pathways. Untargeted metabolomics was performed on plasma from two ALS cohorts (cohort 1, $n = 125$; cohort 2, $n = 225$) and healthy controls (cohort 1, $n = 71$; cohort 2, $n = 104$). Individual differential metabolites in ALS cases versus controls were assessed by Wilcoxon, adjusted logistic regression and partial least squares-discriminant analysis, while group lasso explored sub-pathway level differences. Adjustment parameters included age, sex and body mass index. Metabolomics pathway enrichment analysis was performed on metabolites selected using the above methods. Additionally, we conducted a sex sensitivity analysis due to sex imbalance in the cohort 2 control arm. Finally, a data-driven approach, differential network enrichment analysis (DNEA), was performed on a combined dataset to further identify important ALS metabolic pathways. Cohort 2 ALS participants were slightly older than the controls (64.0 versus 62.0 years, $P = 0.009$). Cohort 2 controls were overrepresented in females (68%, $P < 0.001$). The most concordant cohort 1 and 2 pathways centred heavily on lipid sub-pathways, including complex and signalling lipid species and metabolic intermediates. There were differences in sub-pathways that were enriched in ALS females versus males, including in lipid sub-pathways. Finally, DNEA of the merged metabolite dataset of both ALS and control cohorts identified nine significant subnetworks; three centred on lipids and two encompassed a range of sub-pathways. In our analysis, we saw consistent and important shared metabolic sub-pathways in both ALS cohorts, particularly in lipids, further supporting their importance as ALS pathomechanisms and therapeutics targets.

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Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal and progressive motor neuron disease,[1] which lacks effective treatments or cures. Therefore, understanding the disease mechanisms[2] is important to identifying novel therapeutic targets. ALS pathogenesis is complex and influenced by genetic,[3] epigenetic[4] and environmental[5,6] factors. An organism's metabolome derives from the cumulative effect of genetic, epigenetic, transcriptomic and proteomic forces, superimposed with environmental effects. Thus, metabolomics is a useful tool for investigating complex diseases that arise from multiple influences,[7] including ALS.[8] Moreover, the metabolome can reflect dysregulation from pathological processes, providing clues to potential treatment avenues. Specifically, in ALS, oxidative stress was identified as a disease characteristic through the observation of oxidized metabolites, e.g. nitric oxide and its toxic metabolite, peroxynitrite,[9] or oxidized lipids.[10] This line of research supported antioxidants as an ALS therapeutic, which led to clinical trials of edaravone,[11] which culminated in US Food and Drug Administration (FDA) approval, constituting one of only two drugs available for treating ALS.

To date, a handful of studies have employed untargeted metabolomics to identify differential metabolites and metabolic pathways in ALS versus control participants.[12–15] These studies uncovered multiple dysregulated pathways, including lipid,[14,16,17] amino acid,[14,18–21] and polyamine[18] metabolism. Although extremely insightful, these studies were limited to approximately 400 metabolites or less. Seeking further understanding, we recently utilized a commercial untargeted metabolomics platform of up to 3300 detectable compounds to yield insight into ALS mechanisms.[22] Our analysis included 899 metabolites, which spanned both novel and previously identified sub-pathways in ALS. Additionally, replication metabolomics studies are lacking in ALS, posing a significant roadblock, which prevents metabolomics applications in ALS from making truly meaningful advances.

In the current study, we sought to overcome this roadblock by performing a metabolomics analysis of an independent replication cohort using the same commercial platform as our initial cohort.[22] Replication cohorts are essential to understanding the reproducibility of metabolomics for identifying potential disease biomarkers for diagnostic applications[8] and pathomechanisms for drug development, which is especially pertinent for a complex, heterogeneous disease like ALS. We report the first ALS metabolomics replication study, to our knowledge, to identify potentially important, recurrent metabolic pathways and build prediction models from two independent cohorts to assess the feasibility of metabolomics for discovery in ALS. We identified shared and important pathways in the original and replication cross-sectional ALS cohorts using knowledge-based enrichment analysis, which centred on fatty acid and sphingomyelin metabolism as well as creatine and xanthine metabolism. We also combined the two datasets and implemented differential network enrichment analysis (DNEA),[23] a data-driven approach, which does not rely on known pathway annotation, allowing the method to uncover new potential metabolite correlations. Lastly, we generated prediction models leveraging metabolic data from the original cohort, which we employed to predict cases in the replication cohort. Collectively, these data confirm an association between distinct metabolite and lipid signatures in ALS and uncover new areas of research into ALS pathogenesis, biomarker identification and therapy development.

Materials and methods

Participants and biosamples

Our enrollment strategy is published.[6,22] Briefly, we recruited ALS patients older than 18 years and able to communicate in English seen at the University of Michigan Pranger ALS Clinic. Control participants were also recruited through the University of Michigan Institute for Clinical and Health Research. Participants provided their age, sex, height, and weight and underwent a clinical examination, including assessment of the ALS functional rating score–revised (ALSFRS-R) and other ALS characteristics. Participants also provided plasma samples, which were non-fasted, because it was deemed unethical to request ALS patients to fast. Studies show lack of dietary effects on the plasma lipidomics profile[24] and low intra-individual variation in non-fasted plasma[25]; thus, deep grained plasma metabolomics/lipidomics signatures of disease exist, independent of diet. We collected plasma samples from unfasted ALS participants via peripheral venipuncture, centrifuged at 2000*g* for 10 min at 4°C, aliquoted into cryovials and stored at −80°C, following good clinical practice. Samples were collected in exactly the same manner for cohort 1 and cohort 2, following a standard operating procedure conforming to the Centers for Disease Control and Prevention guidelines. Additionally, all samples were stored at −80°C, to minimize changes to sample metabolite composition.[26] Verbal and written informed consent were obtained from all participants and the study was approved by the University of Michigan institutional review board (HUM00028826).

Metabolomic profiling

Untargeted metabolomics profiling of plasma samples was performed by ultra-high performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS) by Metabolon (Durham, NC).[27,28] Multiple recovery and internal standards were added to plasma samples for evaluating extraction efficiency and instrument performance, respectively, before the sample extraction process using methanol. Following sample extraction, metabolites were analysed by reverse-phase UPLC-MS/MS, in both positive and negative ion mode, and hydrophilic interaction chromatography UPLC-MS/MS. In addition to the spiked internal standards within each sample, a pooled 'technical replicate' generated from all study samples was periodically injected into the UPLC-MS/MS to assess instrument performance and calculate overall process and platform variability. Metabolites were identified by retention time/index, mass-to-charge ratio, and chromatographic data against authenticated standards and validated by Metabolon through data curation. Day-to-day variability was accounted for by rescaling the daily median for each metabolite to one and scaling that metabolite within each sample proportionately against the median. Missing values were replaced by the minimal value detected for that metabolite in the entire cohort, per Metabolon protocols.[27,29]

Metabolites detected in >80% of samples (missingness <20%) were included in downstream analyses. This differed from our recent publication of the original cohort,[22] which was more focused on the discovery of ALS mechanisms and hypothesis development, and included metabolites detected in >60% of samples (missingness <40%). In this present analysis, we were more interested in rigorous test reliability and thus employed more stringent missingness criteria, reanalysing the original cohort 1 and analysing the replication cohort 2 with the missingness cutoff <20%. Missingness was generally low (Supplementary Tables 1 and 2), and most metabolites were detected in 99.5–100% of samples

(Supplementary Fig. 1). Thus, despite the more stringent criteria of metabolites with missingness <20% in this analysis, the overlap with metabolites with missingness <40% from the previous analyses was high and similar metabolites were selected using the two missingness cutoffs (Supplementary Fig. 2).

Differential metabolite identification

Analytical methods

The statistical analysis plan in this report follows our recent publication,[22] but draws upon cases and controls from both the original cohort (cohort 1 ALS cases, cohort 1 controls)[22] and a new second replication cohort (cohort 2 ALS cases, cohort 2 controls). Thus, ALS case and control demographics were summarized and compared using chi-square and Wilcoxon rank-sum tests, as appropriate, across these four groups. Some missing demographic data from the initial publication have since been obtained, accounting for slight differences in the previous and current reported medians for cohort 1. Metabolite missingness was summarized across the original and replication datasets. As in our prior publication, we performed a series of case and control metabolite analyses and then compared the overlap in selected metabolites (Supplementary Fig. 3). An 'unadjusted' model used the non-parametric Wilcoxon rank-sum test to compare non-normally distributed metabolites with Benjamini–Hochberg *P*-value correction for multiple comparisons. An 'adjusted' model used logistic regression, adjusted for age, sex and body mass index (BMI) and regressed each natural log-transformed and standardized metabolite against case/control status. Participants were dropped from the analysis if they were missing a BMI value. *P*-values were adjusted for multiple comparisons using Benjamini–Hochberg correction.

Partial least squares-discriminant analysis (PLS-DA) was performed using the R package mixOmics[30] separately on the original and replication case and control cohorts (Supplementary Fig. 3). Differences in metabolites between cases versus controls were visualized with score plots of the variable importance in projection (VIP).[31,32] Metabolites with significant contributions to group separation had VIP > 1. The 10-fold cross-validation to select the tuning parameter for the PLS-DA analysis is shown in Supplementary Fig. 4A and B. To assess similarities in sub-pathways selected in the original and replication cohorts, we again used group lasso, adjusted for age, sex and BMI, using the gglasso R package with natural log-transformed and standardized metabolite data (Supplementary Fig. 3). Five-fold cross-validation was used to select the tuning parameter corresponding to a sparse model within 1 standard error (SE) of the minimum cross-validation error. Once the tuning parameter, corresponding to the group lasso penalty was finalized, group lasso was refit to the full dataset to obtain the final model. The five-fold cross-validation to select the tuning parameter for the group lasso analysis is shown in Supplementary Fig. 4C and D.

Overlapping metabolites and sub-pathways selected by each model (Wilcoxon, logistic regression, PLS-DA, group lasso) from each original cohort 1 and replication cohort 2 were represented in Venn diagrams.

Metabolite correlation with ALSFRS-R

Heatmaps were generated from the relative abundance of the 20 top differential metabolites in ALS versus controls, shared by both cohort 1 and cohort 2, as a function of the ALSFRS-R score at the time of plasma collection. Relative abundances were scaled by row. ALS participants were sorted by $\log_2$(ALSFRS-R), from high to low score.

Construction of case prediction models

To examine the feasibility of predicting metabolite-based ALS cases, machine learning classification models were constructed using PLS-DA, group lasso and random forest (RF). Prediction accuracy was calculated by the area under the curve (AUC) for each model, which were visualized through receiver operating characteristic (ROC) curves generated by the R package pROC.

Metabolism pathway analysis

We used the R package richR (https://github.com/hurlab/richR/) for pathway enrichment analysis (Supplementary Fig. 3). Sub-pathways were annotated by Metabolon. Over-represented sub-pathways were determined from the metabolites selected by unadjusted Wilcoxon, adjusted logistic regression, PLS-DA and group lasso models. A hypergeometric test was performed for each candidate sub-pathway. Sub-pathways with a *P*-value <0.05 were deemed significantly enriched.

Sex sensitivity analysis

To assess the impact of the sex imbalance, we performed a sex sensitivity analysis. Briefly, the original datasets were separated based on sex, each of which was analysed for differential metabolites and enriched pathways between case and control using the above analyses. Then, the results were compared between male and female at the metabolite- and pathway-levels.

Statistical software

All statistical and prediction analyses were performed using R statistical computing software.

Differential network enrichment analysis

To further understand the metabolic alterations underlying ALS, we merged the two datasets (cohort 1 and 2) and analysed them using the data-driven DNEA approach.

Metabolite selection and data treatment

The cohort 1 and 2 datasets, containing the same metabolite compound identifiers, were merged, numbering 954 total metabolites. Drug-related metabolites and metabolites with missingness <20% in either or both datasets were excluded, leaving 640 total metabolites. Missing values for these metabolites were imputed using each metabolite's minimum value. All measurements were subsequently log-transformed.

Covariate adjustment, autoscaling and dataset merging

We assessed batch effects within and between runs from both cohorts using principal components analysis (PCA). For each dataset separately, metabolites were linearly adjusted for age, sex and BMI. Out of 525 participants, only 15 had missing BMI values. Several imputation methods to approximate BMI values were tested, including simple (mean/median imputation) and machine-learning (linear regression, random forest, support vector regression) approaches. A linear model using the abundance of a select number of metabolites that correlated with BMI generated the best results. Thus, BMI values were imputed using linear models

based on the top 13 and 23 metabolites from cohort 1 and cohort 2, respectively, which correlated most strongly with BMI. After adjustment, each cohort 1 and cohort 2 dataset was separately autoscaled (mean centred and scaled by the standard deviation) to obtain an N(0,1) distribution for each metabolite. The adjusted and normalized datasets from the two original and replication cohorts were finally merged into a single dataset. PCA analysis demonstrates elimination of batch effects (Supplementary Fig. 5).

Differential network enrichment analysis method

We employed DNEA to identify metabolite subnetworks that differentiate ALS from control samples. DNEA methodology has been described previously.[23,33] Briefly, DNEA computes a partial correlation network using metabolomics data from two conditions (i.e. ALS and control) jointly. Due to an imbalance in the number of samples in ALS versus control groups, we employed a subsampling procedure coupled with partial correlation network estimation to obtain robust network edges, as described in Iyer *et al.*[23] The network was then clustered into densely-connected metabolite subnetworks. Next, we performed an enrichment analysis using the NetGSA method,[34] which takes into account differential metabolite abundances as well as the differences in network structure between cases and controls. DNEA results consist of computed subnetworks and their respective *P*-values and false discovery rate (FDR)-adjusted *q*-values, which correspond to significant subnetwork differences between cases and controls. We applied DNEA to the full dataset as opposed to each dataset separately to have a sufficient sample size, *n*, compared with the metabolite count, p, for the partial correlation network computation.

Data availability

Anonymized data will be shared by request from any qualified investigator.

Results

Original and replication ALS cohorts reflect typical ALS populations

The original cohort included 125 ALS and 71 control participants, and the replication cohort included 225 ALS and 104 control participants (Table 1). Age at plasma collection differed between groups overall ($P = 0.013$); replication ALS cases were slightly older than replication controls ($P = 0.009$). Replication ALS cases also had a lower proportion of females versus replication controls. There were no statistically significant differences in BMI or race between the four groups. There were no differences between the original and replication ALS groups in ALS family history, age at diagnosis, El Escorial criteria, onset segment, ALSFRS-R at plasma collection, time between symptom onset and diagnosis, time between diagnosis and blood draw and percentage of participants with feeding tubes. The original and replication ALS cohorts both reflect a typical ALS population, with median diagnostic ages of 62.0 and 63.0 years, intervals between symptom onset and diagnosis of 1.01 and 1.07 years and onset segment of bulbar 30.4 and 26.7%, cervical 30.4 and 38.8% and lumbar 39.2 and 34.9% for the original and replication cohorts, respectively. Plasma was collected within [median (25th–75th percentile)] 0.57 (0.36–0.75) years of diagnosis in the original cohort 1 and within 0.60 (0.36–1.12) years of diagnosis in the replication cohort 2.

Metabolite profiling in original and replication cohorts

In the original cohort 1, we identified and evaluated 1051 known metabolites by descriptive statistics by case/control status (Supplementary Table 1), of which 258 had missingness >20% and were removed from further analyses. In the replication cohort 2, we identified and evaluated 1019 known metabolites by descriptive statistics by case/control status (Supplementary Table 2), of which 315 had missingness >20% and were removed from further analyses. Metabolites identified in the original but not the replication cohort are listed in Supplementary Table 3. Conversely, metabolites unique to the replication cohort are listed in Supplementary Table 4. Metabolites were matched by ChemID, since Metabolon changed some metabolite names between the two cohorts. In our prior publication,[22] very few drug metabolites satisfied missingness criteria. Those that did, such as riluzole, only correlated weakly with other measured metabolites and therefore exerted a minimal impact on metabolite associations. Furthermore, another study demonstrated there were no differences in metabolites identified after washing out typical ALS drugs.[35] Thus, we further excluded any drug metabolites, which will not unduly bias case/control associations. A total of 793 and 703 metabolites for the cohorts 1 and 2, respectively, were used for differential metabolite and pathway analysis.

Differential metabolites in ALS cases versus controls

Wilcoxon identified 268 and 335 significant differential metabolites in the original and replication cohorts, respectively (adjusted *P*-value <0.05), visualized by volcano plot (Supplementary Fig. 6). Of these, 101 were unique to the original cohort and 168 to the replication cohort and 167 were shared (Supplementary Fig. 7A). Next, logistic regression, adjusted for age, sex and BMI, identified 289 and 317 metabolites in the original and replication cohorts, respectively, presented in Manhattan plots at the sub-pathway level (Supplementary Fig. 8). Of these, 99 were unique to the original cohort, 127 to the replication cohort, and 190 were shared (Supplementary Fig. 7B). There were differences among metabolites selected by Wilcoxon versus age-, sex- and BMI-adjusted logistic regression (Supplementary Fig. 9), suggesting that clinically important variables in ALS, such as age, sex and BMI, may significantly affect metabolite relationships between cases versus controls.

PLS-DA identified 275 and 230 metabolites in the original and replication cohorts (Supplementary Fig. 7C), respectively, with VIP >1 that separated cases from controls (Fig. 1A and B). Of these, 136 were unique to the original cohort and 91 to the replication cohort, whereas 139 were shared by both cohorts (Supplementary Fig. 7C). The top 50 metabolites, with the highest VIP and largest contribution to case/control separation, are presented in a VIP score plot (Fig. 1C and D). Among these top 50 metabolites, 19 were common between the original and replication cohorts. As previously reported,[22] we also performed group lasso because it considers sub-pathway structure to evaluate significant ALS and control metabolite differences. Age-, sex- and BMI-adjusted group lasso identified 251 and 299 differential metabolites [odds ratio (OR) $\neq 1$] in the original and replication cohorts, respectively (Supplementary Fig. 7D), which represented 35 and 42 sub-pathways, respectively. Of these metabolites, 127 were unique to the original cohort and 175 to the replication cohort, whereas 124 were shared (Supplementary Fig. 7D). Heatmaps visualize relative abundance

Table 1 Participant demographics

Covariate	Original Cohort 1		Replication Cohort 2		P-value
	ALS cases ($n = 125$)	Controls ($n = 71$)	ALS cases ($n = 225$)	Controls ($n = 104$)	
Age at plasma collection, years[a]	63.0 (53.3–69.6)	60.5 (53.4–64.8)	64.8 (56.7–71.3)	62.0 (55.7–67.5)	0.013
Sex[a]					<0.001
Female	51 (40.8)	29 (39.4)	101 (45)	71 (68.0)	
Male	74 (59.2)	42 (60.6)	124 (55)	33 (32.0)	
BMI at study entry, kg/m^2[b]	25.8 (23.0–29.7)	27.3 (24.6–31.1)	26.0 (22.6–30.2)	27.3 (24.3–30.4)	0.051
Race[a]					
Asian	0 (0)	0 (0)	3 (1.3)	3 (2.9)	
Black or African American	2 (1.6)	2 (2.8)	5 (2.2)	6 (5.8)	
Not reported				1 (0.9)	
White or Caucasian	123 (98.4)	69 (97.1)	217 (96.5)	94 (90.4)	
Family history of ALS					0.68
No	112 (89.6)		195 (86.7)		
Yes	10 (8.0)		25 (11.2)		
Unknown	2 (1.6)		4 (1.8)		
Missing	1 (0.8)		1 (0.4)		
ALSFRS-R at plasma collection	33 (27–37)		31 (25–36)		0.11
Age at diagnosis, years[a]	62.2 (52.7–68.7)		63.8 (54.6–70.7)		0.24
El Escorial criteria[a]					0.17
Suspected	3 (2.4)		13 (5.8)		
Possible	19 (15.2)		30 (13.0)		
Probable, LS	37 (29.6)		59 (26.3)		
Probable	42 (33.6)		60 (26.8)		
Definite	24 (19.2)		63 (28.1)		
Onset segment[a]					0.30
Bulbar	38 (30.4)		60 (26.7)		
Cervical	38 (30.4)		87 (38.8)		
Lumbar	49 (39.2)		78 (34.9)		
Time between diagnosis and blood draw, years[a]	0.57 (0.36–0.75)		0.61 (0.36–1.13)		0.33
Time between symptom onset and diagnosis, years[a]	1.01 (0.68–1.51)		1.07 (0.70–1.84)		0.53
PEG tube present, %	8 (6.4)		27 (12.0)		0.56

Table of descriptive statistics for the overall participant study population. Continuous variables represented as the median (25th–75th percentile) and for categorical variables as *n* (%). *P*-values correspond to Wilcoxon rank-sum test for continuous variables and chi-squared test for categorical variables. LS = lab supported; NA = not available; PEG = percutaneous endoscopic gastrostomy.

[a]Median, 25th percentile and 75th percentile are computed using all cases and controls (no missing subjects).

[b]Median, 25th percentile and 75th percentile are computed using 119 original cohort cases, 67 original cohort controls (six cases and four controls are missing), 218 replication cohort cases, 84 replication cohort controls (seven cases and three controls are missing).

differences between ALS versus controls for all group lasso metabolites in the original and replication cohorts (Supplementary Fig. 10). Among the top 50 metabolites, 14 were common between the original and replication cohorts.

Finally, Venn diagrams illustrate the unique number of metabolites for each analysis and for each cohort (Supplementary Fig. 9).

Differential metabolites correlate with ALSFRS-R

To assess whether metabolites correlate with clinical status, we plotted heatmaps of the relative abundance of the 20 top differential metabolites in ALS versus controls, shared by both cohort 1 and cohort 2, as a function of the ALSFRS-R score at the time of plasma collection (Fig. 2). When we sorted ALS participants by ALSFRS-R from high to low score, this generated an overall trend of decreasing metabolites with worsening ALS status. Interestingly, and aligned with the significant sub-pathways, several sphingolipids were among the metabolites that correlated with ALSFRS-R, such as lignoceroyl sphingomyelin (d18:1/24:0), sphingomyelin (d18:1/20:0, d16:1/22:0) and sphingomyelin (d18:1/14:0, d16:1/16:0), as well as acylcarnitines [e.g. lignoceroylcarnitine (C24)]. However, although sphingomyelin abundance is elevated in ALS cases versus participants, these specific sphingolipid species were lower in patients with more advanced disease.

Case prediction models

We leveraged our two independent cohorts to construct case prediction models. The original cohort 1 dataset was used to build PLS-DA, group lasso and RF prediction models to identify ALS cases in the replication cohort 2. Prediction accuracy was calculated by AUCs and visualized by ROC curves (Fig. 3). Metabolites that contributed to models are outlined in Supplementary Tables 5–7. Group lasso and PLS-DA had very similar AUCs (0.945 and 0.944, respectively), which were slightly higher versus RF (AUC of 0.903). As anticipated, creatine and creatinine were among the most strongly contributing metabolites to all three models, likely secondary to muscle loss and not to the causative disease process. Thus, they may not be specific to ALS.

Metabolites related to antioxidant defense and polyamine metabolism were high on the list in the group lasso model, amino acid metabolism in the PLS-DA model and xenobiotics and amino acids in the RF model. Additional strongly influential metabolites (top 10) include sphinganine and sphingadienine (members of 'Sphingolipid Synthesis') in the group lasso prediction model,

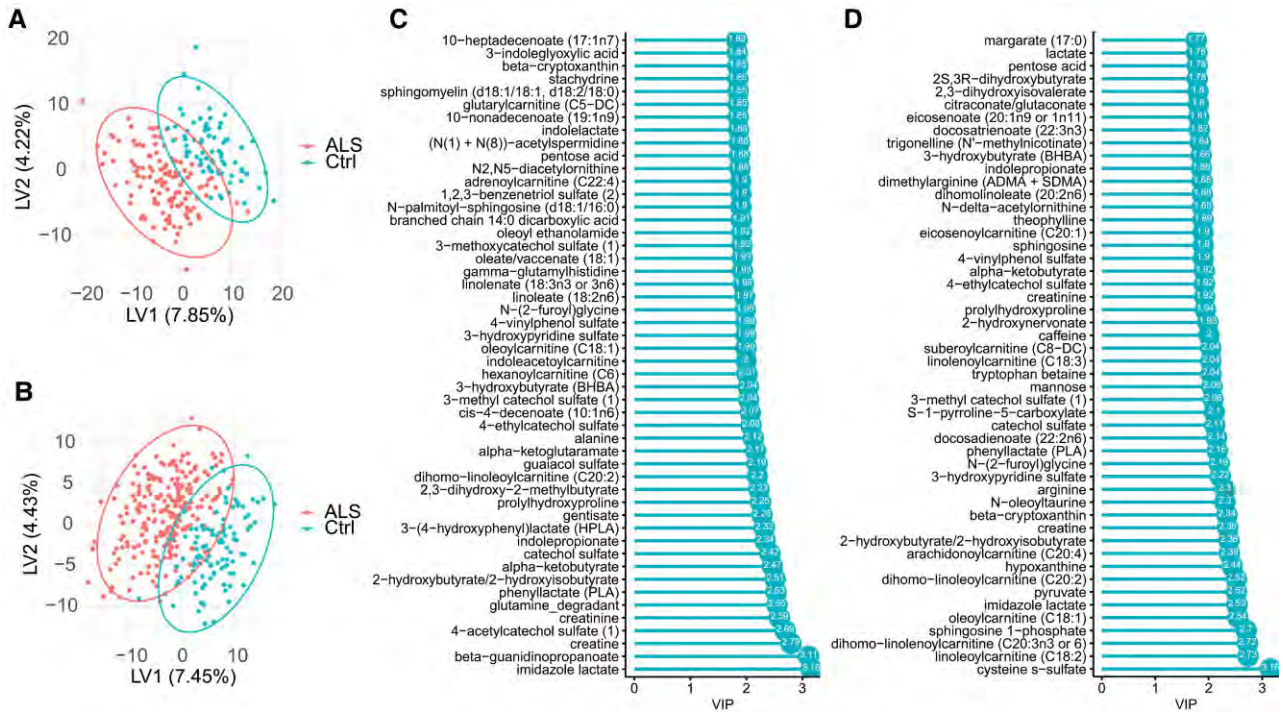

Figure 1 PLS-DA analysis of ALS cases versus controls for original and replication cohorts. (**A** and **B**) PLS-DA score plot of ALS cases (red) versus controls (blue) for (**A**) original cohort 1, and (**B**) replication cohort 2, individually; each dot represents an individual participant. (**C** and **D**) The VIP score plot of the top 50 PLS-DA metabolites, which most significantly separate ALS cases from controls for (**C**) original cohort 1 and (**D**) replication cohort 2, individually. A total of 275 (original) and 230 (replication) metabolites had VIP >1. Among the top 50 PLS-DA metabolites, 19 were shared between the original and replication cohorts. LV = latent variables.

which were less discriminating in the PLS-DA and RF models. Nevertheless, several sphingomyelins, e.g. sphingomyelin (d18:1/18:1, d18:2/18:0) and sphingomyelin (d18:2/16:0, d18:1/16:1), were in the top 100 metabolites of the PLS-DA and RF models. Thus, overall, all models performed well and could predict ALS cases with good accuracy. This supports metabolomics as a feasible approach for ALS biomarker discovery, although future studies will need to include disease mimics to assess specificity.

Differential sub-pathways in ALS cases versus controls

As before,[22] we next performed pathway enrichment analysis to identify over-represented sub-pathways based on differential metabolites identified by the various analysis methods. In the original cohort, Wilcoxon, adjusted logistic regression, PLS-DA and group lasso enriched 12, 13, 12 and 23 sub-pathways, respectively (Fig. 4). For the replication cohort, Wilcoxon, adjusted logistic regression, PLS-DA and group lasso enriched 16, 13, 14 and 25 sub-pathways, respectively (Fig. 4). There were few fully concordant sub-pathways that were selected across all four analytical methods in both the original and replication cohorts. However, the concordant sub-pathways selected by multiple methods in both cohorts centred heavily on lipid sub-pathways, including 'long chain saturated fatty acid', 'long chain polyunsaturated fatty acid (n3 and n6)', 'long chain monounsaturated fatty acid', 'fatty acid metabolism (acyl carnitine, polyunsaturated)' (Supplementary Fig. 11) and 'sphingomyelins'. With few exceptions, acylcarnitines, which are partially metabolized intermediates, of all chain lengths and saturation level were elevated in ALS cases versus controls. 'Xanthine' and 'creatine' were also repeatedly selected.

There were sub-pathways that were highly selected by multiple analytical methods but were mostly consigned to one cohort or the other. For instance, lipid 'ceramides', 'hexosylceramides', antioxidant 'gamma-glutamyl amino acid' and xenobiotics 'benzoate' were widely selected in the original cohort, whereas lipid 'sphingosines', 'fatty acid metabolism (acyl carnitine, hydroxyl)', 'fatty acid metabolism (acyl carnitine, dicarboxylate)' and energy 'TCA (tricarboxylic acid) cycle' were selected in the replication cohort. There was overlap in some of these sub-pathways between the two cohorts. The most significant sub-pathway was 'sphingomyelins' by group lasso of the original cohort, which was also selected in the replication cohort.

Sensitivity analysis for sex imbalances

The original cohort 1 was well-balanced for sex; however, females were overrepresented in the control group of the replication cohort 2, which led to differences in the sex composition of the original and replication cohort. Therefore, we conducted a sensitivity analysis to evaluate whether the imbalance contributed to uncertainty in the outcomes. Moreover, we[36,37] and others[38] have noted that sex is an important clinical factor in ALS; thus, analysis by sex may yield additional insight. We repeated the Wilcoxon, logistic regression, PLS-DA and group lasso analyses in male cohort 1 ($n = 74$ ALS, $n = 42$ control) and female cohort 1 ($n = 51$ ALS, $n = 29$) separately, which were compared to the combined original cohort 1, and additionally in male cohort 2 ($n = 123$ ALS, $n = 33$ control) and female cohort 2 ($n = 101$ ALS, $n = 70$ control) separately, which were compared to the combined replication cohort 2 (Fig. 5 and Supplementary Fig. 12).

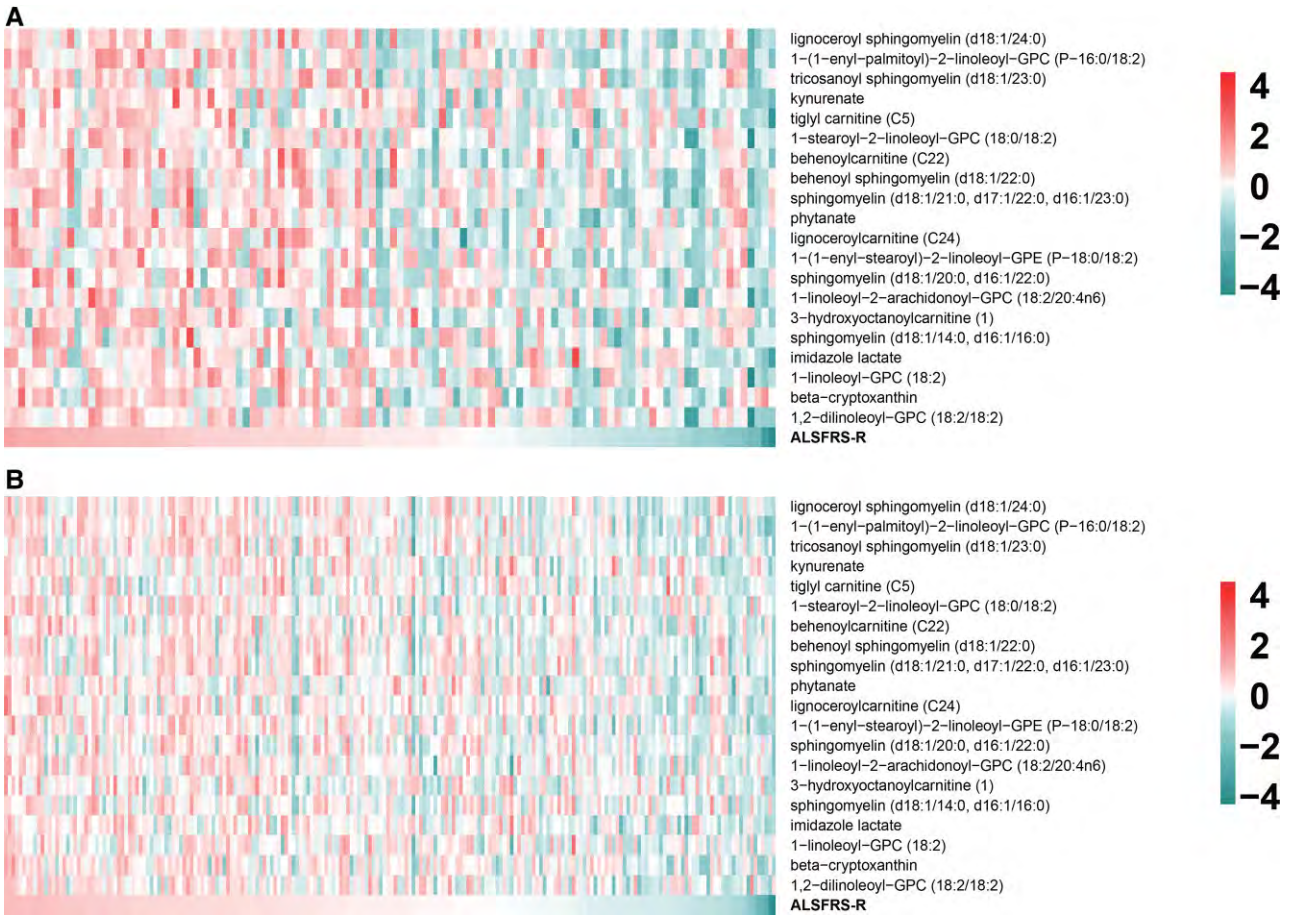

Figure 2 Correlation of metabolite abundance with ALSFRS-R. Cross-sectional heatmap visualization of the 20 top differential metabolites in ALS versus controls, shared by both cohort 1 and cohort 2, as a function of ALSFRS-R score at the time of plasma withdrawal. The relative abundance of the metabolites significantly correlated with ALSFRS-R in both (**A**) cohort 1 and (**B**) cohort 2. Relative abundances and $\log_2$(ALSFRS-R) were scaled by row. ALS participants were sorted by $\log_2$(ALSFRS-R), from high score (*left*, pink) to low score (*right*, green), i.e. progressive disease, which results in an overall trend of decreased metabolites.

Some interesting observations arose from the sex sensitivity analysis. Although 'fatty acid metabolism (acyl carnitine, polyunsaturated)' was almost invariably selected by all analysis methods, in both sexes, certain lipid focused sub-pathways were selected more frequently and/or significantly in ALS females, either in the original or replication cohorts, such as 'long chain saturated fatty acid', 'long chain polyunsaturated fatty acid (n3 and n6)', 'long chain monounsaturated fatty acid', 'sphingosines' and 'diacylglycerol'. One exception stood out in 'sphingomyelins', which was more often and/or significantly selected in ALS males from either the original or replication cohorts. These sex-dependent lipid differences, especially in sphingomyelins, were present irrespective of health or disease, when we analysed the control and ALS groups separately by male versus female, within both cohort 1 and cohort 2 (Supplementary Fig. 13). We found overall sphingomyelin abundance was lower in healthy males versus females,[39,40] which persisted in ALS, albeit to a lesser extent, presumably due to dysregulated sphingomyelin metabolism in ALS (Supplementary Fig. 14 and Supplementary Tables 8 and 9).

Another interesting finding was 'benzoate metabolism', which was selected in our original but not our replication cohort (Fig. 4). Examination of the sex analysis suggests this might arise due to sex imbalance in the replication cohort. When the original cohort was stratified by sex, benzoate metabolism was most frequently and/or significantly selected in ALS males (Fig. 5). Therefore, benzoate metabolism may not have been selected in the replication cohort because it was underrepresented in control males.

Differential network enrichment analysis

We employed DNEA to identify metabolite subnetworks that differentiate ALS from control samples. DNEA is a data-driven, functional enrichment approach, which facilitates discovery of novel, functionally active metabolite modules, without depending on prior knowledge of biochemical interactions. Additionally, it identifies biochemical interactions that are present in either healthy or ALS samples, or present in both. DNEA is especially useful for metabolites with incomplete pathway knowledge, e.g. lipids and exogenous metabolites. DNEA also eliminates batch effects between the two ALS cohorts ($n = 349$) and control cohorts ($n = 174$) by adjusting and autoscaling data. Once the datasets were merged, DNEA analysis identified a total of 15 different metabolite subnetworks, nine of which were significantly enriched at the 0.01 FDR cutoff (Supplementary Tables 10 and 11, Fig. 6 and Supplementary Figs 15–23).

The most significant subnetwork S1 comprised candidates from a variety of sub-pathways, encompassing 'benzoate metabolism' and 'food component/plant', both related to xenobiotics as well as

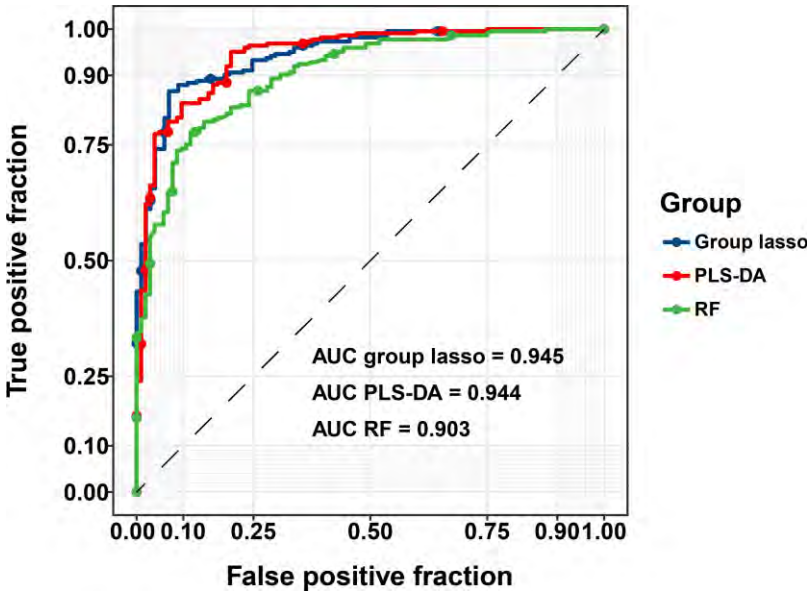

Figure 3 Case prediction models. ROC curves for ALS case prediction models generated by group lasso, PLS-DA and RF from cohort 1 applied to cohort 2. Prediction accuracy was calculated for each model by the AUC.

fatty acid and amino acid metabolites (Supplementary Fig. 15). The second most significant subnetwork S2 was primarily contributed by xenobiotics sub-pathways, 'benzoate metabolism' and 'xanthine metabolism' (Supplementary Fig. 16). Regarding lipid-centric transformations, long-chain and various fatty acid and complex lipid metabolic sub-pathways contributed the most metabolites to subnetworks S3, S8 and S9 (Fig. 6 and Supplementary Figs 17, 22 and 23). These subnetworks contained more ALS edges, indicating biochemical interactions that were present in the pathological condition. Subnetwork S8 contained a large number of complex lipids, mostly sphingomyelins, but also ceramides and dihydrosphingomyelins. Subnetwork S9 encompassed multiple species in signalling, e.g. diacylglycerols and complex lipid sub-pathways, e.g. phosphatidylcholines, phosphatidylethanolamines, phosphatidylinositols and lysophospholipids.

In contrast to these more focused aforementioned subnetworks, subnetworks S5 (Supplementary Fig. 19) and S7 (Supplementary Fig. 21) converged on metabolites from diverse super-pathways. Subnetwork S7 was particularly interesting, embodying several metabolic ALS characteristics, e.g. energy (TCA cycle), amino acid, antioxidants and creatine. Purine/pyrimidine[41] and xenobiotics metabolism were also featured. Subnetwork S5 similarly had a diverse profile.

Discussion

In the current study, we compared the metabolome of a second new ALS and control cohort to our previously published original cohort 1 ($n = 125$ ALS, $n = 71$ control), where we reported multiple ALS-associated metabolites and pathways.[22] Our goal was to identity recurrent dysregulated metabolites and pathways in ALS. The original investigation was a hypothesis-generating study; thus, it employed a less stringent missingness criteria of 60% cutoff.[22] Our replication effort reanalysing cohort 1 and analysing new cohort 2 ($n = 225$ ALS, $n = 104$ control) employed a more rigorous missingness criteria of 80% cutoff to identify the metabolites and pathways most strongly associated and replicated in ALS. We leveraged the two independent cohorts to examine recurrent metabolites and pathways, correlate metabolites to clinical status and build prediction models. As a final step, we merged the original and replications cohorts in a data-driven DNEA analysis to derive a more integrated view of plasma metabolome structure in ALS. This approach does not rely on prior biochemical knowledge, but rather clusters metabolites into subnetworks dictated solely by experimental measurements, in this case, by partial correlations between metabolites across samples.

When we examined the overlap in original and replication cohorts by metabolites, many were shared, but several also were not shared. For the PLS-DA and group lasso analyses, which rank metabolites by VIP and OR, the overlap between cohorts was 19 and 14 metabolites, respectively, when restricting our analysis to the top 50 metabolites. Additionally, when we examined the top 20 differential metabolites in ALS versus controls, which were shared by cohort 1 and cohort 2, we found that metabolite abundance decreased with decreasing ALSFRS-R score and advancing disease. Interestingly, this was the case for several sphingomyelin species, although sphingomyelin abundance generally correlated positively with ALS status. It is possible that a temporal element in sphingomyelin levels may exist,[42] with an initial rise followed by a dip, which might account for the pattern seen with ALSFRS-R score. Therefore, though only cross-sectional, this finding indicates that metabolomic profiles may correlate with clinical status in ALS, and future longitudinal studies could inform the importance of critical metabolites in the disease course.

Furthermore, even though metabolite overlap was not fully concordant between cohort 1 and cohort 2, the cohort 1 metabolite dataset could be used to construct several prediction models, which identify ALS cases in the replication cohort. Our AUCs are higher than previously reported in ALS studies,[13,14] possibly due to our larger metabolite dataset and sample numbers[13,14] or because we analysed metabolomics profiles from symptomatic ALS participants.[13] Our prediction model results support the viability of utilizing metabolomics for ALS biomarker discovery, shown, for the first time,

Figure 4 Pathway enrichment of Wilcoxon-, adjusted logistic regression-, PLS-DA- and group lasso-selected metabolites for original and replication cohorts. Significantly enriched sub-pathways from metabolites selected by Wilcoxon, adjusted logistic regression, PLS-DA and group lasso models illustrated in dot plots for original cohort 1 and replication cohort 2. Rich factor refers to the proportion of selected metabolites relative to total sub-pathway metabolites. Metabolite number (node size) refers to the number of sub-pathway metabolites. Node colour indicates the significance level according to $-\log_{10}$(P-value).

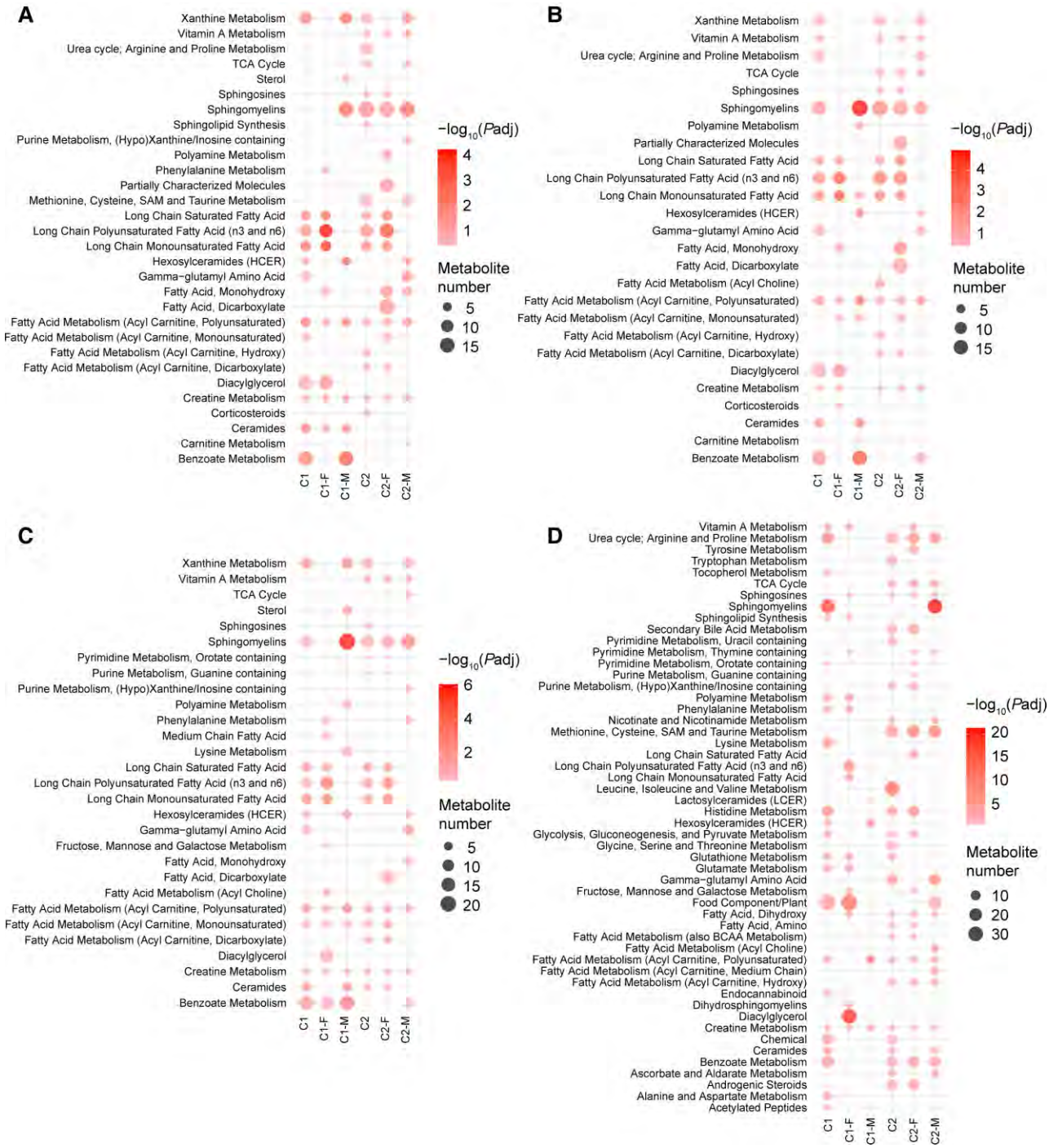

Figure 5 Pathway enrichment by sex of Wilcoxon-, adjusted logistic regression-, PLS-DA- and group lasso-selected metabolites for original and replication cohorts. Significantly enriched sub-pathways by sex from metabolites selected by (**A**) Wilcoxon, (**B**) adjusted logistic regression, (**C**) PLS-DA and (**D**) group lasso models illustrated in dot plots for original cohort 1 and replication cohort 2. Rich factor refers to the proportion of selected metabolites relative to total sub-pathway metabolites. Metabolite number (node size) refers to the number of sub-pathway metabolites. Node colour indicates the significance level according to $-\log_{10}$(P-value). C1, cohort 1; C1-F, cohort 1 females; C1-M, cohort 1 males; C2, cohort 2; C2-F, cohort 2 females; C2-M, cohort 2 males.

using two independent cohorts. Future research will need to include disease mimics to evaluate specificity for ALS versus possible differential diagnoses to determine the diagnostic utility in a real-world setting.

Although identifying individual differential metabolites sheds insight on pathogenesis and may identify new biomarkers, metabolites exist along a series of biochemical transformations, which contribute to a biological process. Therefore, pathway analysis of all significant differential metabolites emphasizes metabolic networks rather than discrete metabolites and provides more meaningful insight on entire pathway dysregulation in disease pathogenesis. As with individual metabolites, there were shared

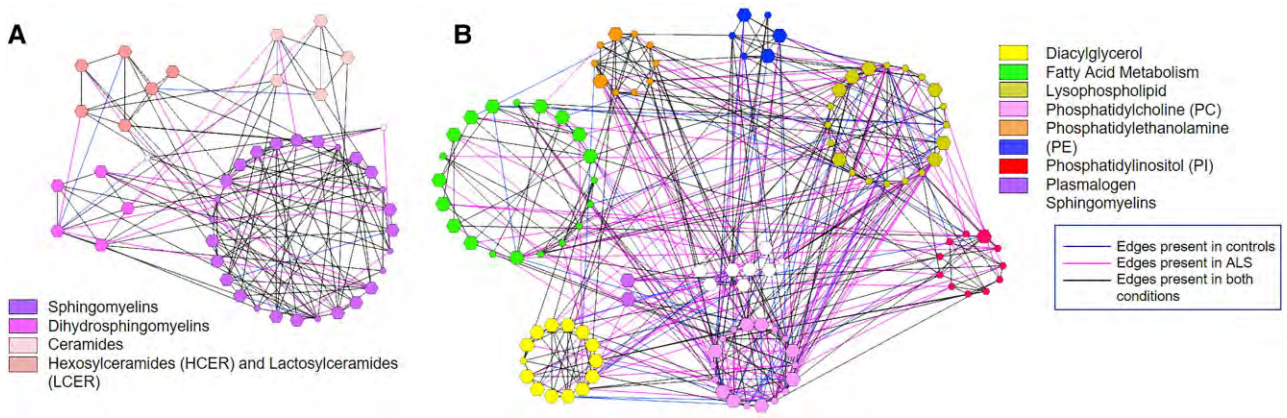

Figure 6 DNEA analysis subnetworks S8 and S9 overview. Subnetworks (**A**) S8 (Padj = 1.11×10^{-3}) and (**B**) S9 (Padj = 2.74×10^{-3}) from the data driven DNEA analysis. Nodes represent metabolites, which are colour-coded by sub-pathway; larger nodes indicate metabolites higher in ALS; smaller nodes indicate metabolites lower in ALS. Only metabolites from sub-pathways contributing three or more metabolites to the subnetworks are colour-coded; metabolites from sub-pathways contributing less than three metabolites are white. Edges present in controls, blue; edges present in ALS, pink; edges present in both, black. Fully annotated subnetworks S8 and S9 are available in Supplementary Figs 22 and 23 and member metabolites and their direction change and significance are in Supplementary Table 10.

sub-pathways identified within the original and replication cohorts, but also sub-pathways that were unique to either cohort.

Sub-pathways consistently selected in both the original and replication cohort centred heavily on lipid metabolism. The most significant sub-pathway 'sphingomyelin', selected by group lasso in the original cohort as well as the three other methods, continued to be selected in the replication cohort, but not by group lasso. Other recurrent lipid pathways in the original and replication cohorts included various long-chain fatty acids, acyl intermediates and ceramides. Similarly, in the DNEA analysis, subnetworks S3, S8 and S9 were highly populated by lipid species.

With regards to subnetwork S8, this subnetwork contained mostly elevated sphingomyelins, dihydrosphingomyelins, ceramides and hexosylceramides (significant) in ALS, which is aligned with other studies of ALS participant plasma,[13,14,35] spinal cord[43,44] and CSF,[16] and ALS mouse studies.[17,43–45] Impaired sphingolipid metabolism is a central, and at this point relatively well-validated, aspect of ALS pathogenesis, although the details and underlying aetiology, especially in sporadic disease, remain incompletely understood. The increase in ceramides and glucosylceramides in ALS has been linked to enhanced glucocerebrosidase activity in mutant superoxide dismutase 1 ($SOD1^{G93A}$) mice,[44] although conversely glucosylceramide synthase expression is upregulated in $SOD1^{G86R}$ mouse muscle.[42] These differences may arise from distinct model systems, but also from natural evolution during the disease course.[42] Nevertheless, this dysfunction in sphingolipid synthesis could be integral and potentially causative to disease progression, as seen in a model of monogenic childhood-onset ALS with mutant serine palmitoyltransferase subunit 1 (*SPTLC1*).[46] In an induced pluripotent stem cell ALS model, allele specific mutant *SPTLC1* knock-in increases sphinganine and ceramide levels versus wild-type allele.[46] Indeed, we observed elevated sphinganine and ceramides in our sporadic ALS cohort, indicating impaired sphingolipid metabolism could be central to ALS pathogenesis. Ceramides are pro-apoptotic and potentially excito-[43] or neurotoxic,[47] contributing to neurodegeneration (Fig. 7). Additionally, high fatty acid levels (see below) increase ceramides and possibly dihydroceramide intermediates[48] and sphingomyelins,[49,50] as observed in both the original and replication cohorts and the combined ALS dataset.

When examining subnetwork S9, this subnetwork contained various mostly downregulated complex lipids in ALS, though only some phosphatidylcholines and lysophospholipids were significantly decreased. Our findings agree with some reports,[45] but conflict with others,[13,16] which may arise from the specific phosphatidylcholine species[16] or the stage in ALS development.[13] Phosphatidylcholines and phosphatidylethanolamines are primarily synthesized from diacylglycerols through the Kennedy pathway[51] and phosphatidylinositols from diacylglycerol intermediates.[52] Phosphatidylcholines, phosphatidylethanolamines and phosphatidylinositols are important membrane constituents; loss of phosphatidylethanolamines is especially central to mitochondrial dysfunction through impaired mitochondrial membrane curvature, fission/fusion and bioenergetics.[53] In addition to their role in membrane structure, phosphatidylcholines and phosphatidylethanolamines are precursors to diverse signalling molecules, e.g. diacylglycerols,[51] which control various biological processes, such as proliferation, survival and migration.[54] Our study emphasizes the importance of continued research in the area of complex and bioactive lipids and lipid signalling in ALS.

Subnetwork S3 comprised free long-chain fatty acids of all saturation levels (saturated, monounsaturated, polyunsaturated; highly significant), which were universally elevated in ALS participants. Subnetwork S9 encompassed increased fatty acid intermediate acylcarnitines (significant), linked to β-oxidation, along with raised diacylglycerols (significant) in ALS plasma. Though noted in some studies,[13,17] these observations regarding free fatty acids, acylcarnitines, and diacylglycerols in symptomatic sporadic ALS, are relatively novel and align with our growing understanding of ALS 'hypermetabolism'.[55]

ALS 'hypermetabolism' is characterized by elevated resting energy expenditure,[55] possibly related to glucose uptake,[56] and low BMI and fat-free mass; thus, β-oxidation may be very relevant to ALS pathogenesis (Fig. 7). Increased levels of non-metabolized free fatty acids and partially metabolized intermediate acylcarnitines could indicate dysfunctional or at capacity β-oxidation,[57] which ties in with impaired fatty acid uptake and utilization as well as mitochondrial dysfunction. A study found $SOD1^{G93A}$ mice cleared triacylglycerol from plasma post feeding to a greater extent versus control mice,[58] which could elevate plasma free fatty acids,

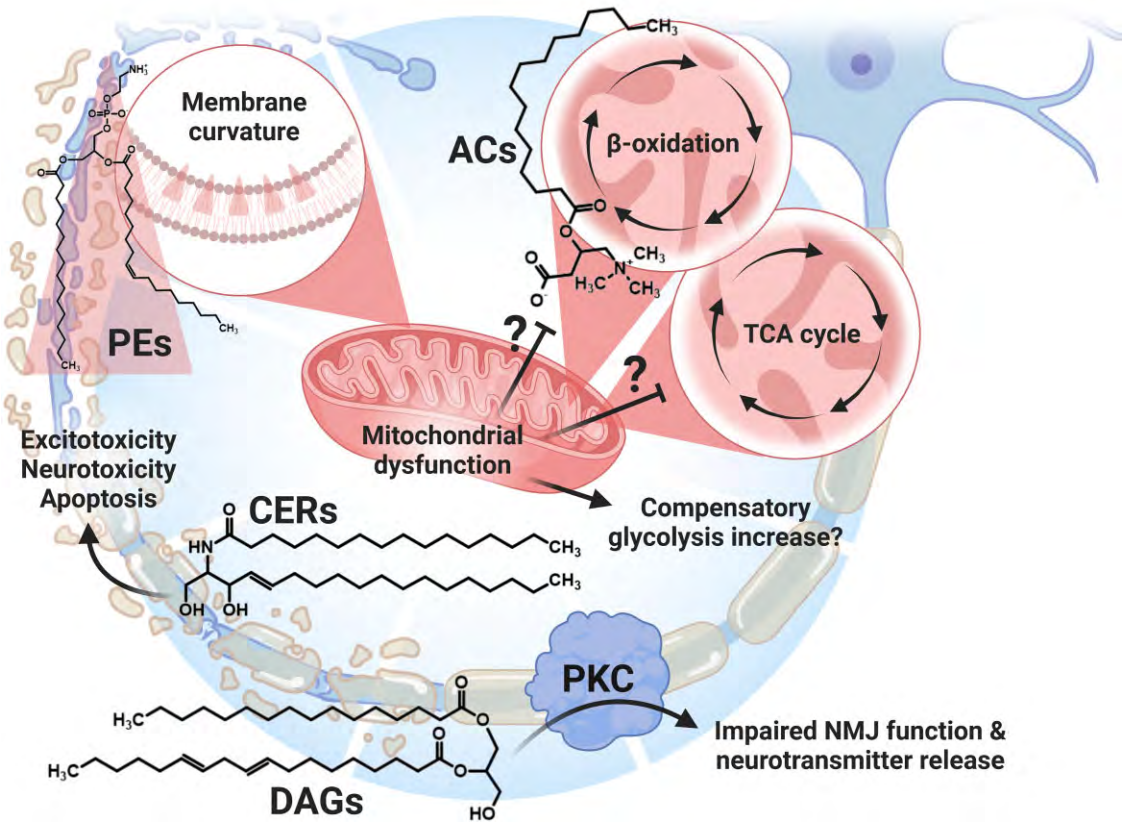

Figure 7 Potential mechanisms of lipid and energy dysregulation in ALS. Plasma metabolomics in two independent ALS cohorts revealed recurrent dysregulation in pathways related to lipid metabolism and energy. Lipids encompassed classes associated with multiple biological processes. Acylcarnitines (ACs) were mostly and significantly upregulated in ALS and could be indicative of impaired β-oxidation, which also feeds into the TCA cycle. TCA metabolites also differentiated ALS from control plasma; however, there was no pattern of up versus downregulation across specific TCA species. Impaired β-oxidation and TCA metabolism could lead to a compensatory increase in glycolysis. Dysfunctional sphingolipid metabolism leads to elevated ceramides (CERs), which are excito- and neurotoxic and trigger apoptosis. Various phospholipids, important membrane constituents, were mostly downregulated in ALS plasma, though only phosphatidylcholines attained significance. Phosphatidylethanolamines (PEs) regulate membrane curvature, and hence mitochondrial function, and were non-significant subnetwork S9 members. Diacylglycerols (DAGs), which can be synthesized from phosphatidylcholines and phosphatidylethanolamines, were universally upregulated and mostly significant in ALS plasma. Diacylglycerols activate protein kinase C (PKC), which has diverse biological properties, including important roles in the peripheral nervous system related to synaptic transmission and neuromuscular junction function. Future investigation will be required to evaluate these pathways in ALS relevant tissues, i.e. motor nerves, spinal cord, frontal and temporal brain lobes, and longitudinally, to assess whether these transformations are causative or downstream of pathogenesis. NMJ = neuromuscular junction. Created, in part, with ACD/ChemSketch and BioRender.com.

as we observed, if tissues do not compensate by enhanced uptake. In both our original and replication cohorts, acylcarnitines of all lengths and saturation levels were universally elevated in ALS, with few exceptions, indicating an overall lack of catabolic β-oxidation. Enhanced muscle lipolysis is reported in SOD1^{G86R} mice,[59] along with increases in mRNA[59] and protein[60] levels of β-oxidation enzymes, such as carnitine palmitoyltransferases (CPTs) and fatty-acid-binding proteins. In a mutant TAR DNA-binding protein 43 (TDP-43) fly model, acylcarnitine accumulation was observed, along with differential carnitine palmitoyltransferase expression.[61] It is possible that hypermetabolism may revolve around glycolysis as a compensatory mechanism[62] to impaired β-oxidation.

Subnetworks S5 and S7 contained metabolites from several subpathways related to ALS pathogenesis, including energy metabolism, which, as noted above for β-oxidation, appears to be a central ALS theme. Among them were metabolites related to TCA and glycolysis energy metabolism,[13,14,16,17,35,63] as well as amino acid metabolism, e.g. histidine, lysine, 'leucine, isoleucine and valine' (otherwise known as branched-chain amino acid)[18,20,21] and creatine.[14,19,64] Purine/pyrimidine[41] and xenobiotics metabolism were also featured. Members of the glycolysis, gluconeogenesis and pyruvate metabolism sub-pathways were mostly elevated in ALS, including glucose, lactate and pyruvate metabolites, whereas the level of TCA substrates and intermediates did not follow very significant or evident trends within this S7 subnetwork. The literature also reports elevated glycolysis,[35,65] although it is less clear regarding TCA metabolism.[66] From our own results, it is not possible to deduce a pathway direction for these interrelated metabolites in glycolytic and TCA metabolism. It may be difficult to infer shifts in metabolic processes from steady state metabolite evaluations by metabolomics, and fluxomics are needed to elucidate TCA metabolite flux in ALS.[67] Additionally, some amino acid sub-pathways in subnetwork S7 may also eventually feed into energy metabolism, e.g. glycine, serine and threonine, tyrosine and phenylalanine metabolism.[68]

Creatine was significantly increased, and creatinine significantly decreased in ALS, as in the literature,[14,19] but is likely linked to muscle loss as part of the disease process. Oxidative stress is an extremely prominent ALS characteristic,[69] which led to one of only

two FDA-approved ALS therapies, edaravone.[11] Gamma-glutamyl amino acid, glutathione metabolism and vitamin A were selected in both the original and replication cohorts and were mostly downregulated in ALS. Deficits in antioxidants have previously been noted in ALS, such as for glutathione,[70,71] tocopherol[14] and ascorbate.[19,63] However, since clinical trials of antioxidant therapies have been unsuccessful[72] and edaravone only minimally slows ALS progression, antioxidant dysregulation is likely not causative and may occur downstream of disease initiation, like creatine/creatinine.

Sex is an important clinical variable in ALS.[73] We recently reported that elevated neutrophil counts correlated with ALS progression, but that the trend was especially pronounced in female participants.[36] We also found that sex influenced the association of the natural killer cell cytotoxicity marker, NKp30, with ALS progression by ALSFRS-R score.[37] These findings implicate sex differences in immune system aspects of ALS. We took advantage of the sex imbalance in the control group of cohort 2 in the replication to conduct a sensitivity analysis and examine potential sex differential effects on plasma metabolomics in ALS. We found that although lipid metabolism pathways continued to be selected in both males and females in both cohorts, certain sub-pathways were more significant in females, such as long-chain fatty acid metabolism, diacylglycerols and sphingosines, whereas others were more important in males, e.g. sphingomyelin. Several xenobiotics pathways, such as benzoate and xanthine metabolism, were also more often and/or significantly selected in male ALS participants. Metabolic differences among ALS males and females could explain variation in published studies and may be important considerations for any therapeutics targeting metabolism in ALS. These differences could also yield new avenues of research to better understand why the incidence of ALS is highest in males.[74]

Our study has strengths and limitations. Among the strengths are the extensive number of detectable and identifiable metabolites in the metabolomics platform and large size of both cohorts. Additional strengths include the validation design of the original study in this replication effort, especially with the more stringent missingness criteria, and analyses using several statistical methods, including the data driven DNEA approach. Despite numerous strengths, our study has weaknesses. Most salient to a neurodegenerative disease is the tissue issue, since plasma may not necessarily reflect the peripheral and central nervous system milieu. Furthermore, since plasma collection was cross-sectional, the study does not establish causality among any of the observed metabolic sub-pathways. The study also does not correlate the metabolome with clinical progression, although this is a future area of investigation, which is required to delineate correlative from causative shifts in metabolic profiles in ALS. However, the long prodromal phase in ALS poses significant challenges. From a technical perspective, both our cohorts consisted mainly of White participants and our replication study was not well balanced for sex, although this limitation prompted us to conduct analyses for sex differences. Additionally, plasma samples were not collected from fasted participants because it was deemed an unethical request. Finally, batch effects were present since samples were run a year apart, although data were autoscaled for the DNEA analysis, removing any batch influence.

Overall, our metabolomics replication study highlighted recurrently dysregulated lipid metabolism in multiple sub-pathways, indicative of altered β-oxidation, mitochondrial bioenergetics and complex lipid signalling. Targeted lipidomics looking at specific lipid classes and lipid species along the ALS continuum could provide new insight into disease pathogenesis. Future directions could also address metabolic flux to move past steady state evaluations. Preclinical work along a time continuum could shed additional insight on early metabolic changes and in motor nerves and spinal cord tissue. The concurrent dysregulation in transcriptome and epigenome in ALS may also advocate a multi-omics approach.

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Competing interests

S.A.G. has acted as a medical advisor for Biogen and ITF Pharma and served on a DSMB. The other authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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Tofacitinib Suppresses Natural Killer Cells *In Vitro* and *In Vivo*: Implications for Amyotrophic Lateral Sclerosis

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Amyotrophic lateral sclerosis (ALS) is a fatal and incurable neurodegenerative disease with few therapeutic options. However, the immune system, including natural killer (NK) cells, is linked to ALS progression and may constitute a viable therapeutic ALS target. Tofacitinib is an FDA-approved immunomodulating small molecule which suppresses immune cell function by blocking proinflammatory cytokine signaling. This includes the cytokine IL-15 which is the primary cytokine associated with NK cell function and proliferation. However, the impact of tofacitinib on NK activation and cytotoxicity has not been thoroughly investigated, particularly in ALS. We therefore tested the ability of tofacitinib to suppress cytotoxicity and cytokine production in an NK cell line and in primary NK cells derived from control and ALS participants. We also investigated whether tofacitinib protected ALS neurons from NK cell cytotoxicity. Finally, we conducted a comprehensive pharmacokinetic study of tofacitinib in mice and tested the feasibility of administration formulated in chow. Success was assessed through the impact of tofacitinib on peripheral NK cell levels in mice. We found tofacitinib suppressed IL-15-induced activation as measured by STAT1 phosphorylation, cytotoxicity, pro-inflammatory gene expression, and pro-inflammatory cytokine secretion in both an NK cell line and primary NK cells. Furthermore, tofacitinib protected ALS neurons from NK cell-mediated cytotoxicity. In mice, we found tofacitinib bioavailability was 37% in both male and female mice; using these data we formulated mouse containing low and high doses of tofacitinib and found that the drug suppressed peripheral NK cell levels in a dose-dependent manner. These results demonstrate that tofacitinib can suppress NK cell function and may be a viable therapeutic strategy for ALS.

Keywords: ALS, NK cells, immune system, tofacitinib, JAK/STAT

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease resulting in death of the motor neurons (1). The average patient lifespan is 2 to 4 years from disease diagnosis, and few therapeutic options exist. However, an increasing body of literature suggests that the immune system is involved in the pathogenesis of ALS (2, 3), with specific immune cell populations likely contributing to disease progression in ALS mouse models (4–9). Similarly, in human ALS patients, changes in peripheral immune cell numbers and activation state correlate with disease progression (10–13). Unfortunately, suppressing the immune system can have unintended and sometimes fatal consequences. General immune suppression increases susceptibility to pathogens and cancer (14); it may also accelerate ALS progression (15, 16) since several immune populations perform protective functions, which slow disease progression (10, 11). The loss of protective immune cell populations likely explains previous failures of immunosuppressive drugs for ALS. Conversely, certain immune cell populations accelerate ALS (6–10, 12, 13); thus, targeting these specific immune populations may be a more nuanced and potentially effective approach for slowing disease progression than global immune suppression.

Generally, immune cells do not attack the body's own cells under homeostatic conditions. However, natural killer (NK) cells destroy the body's own cells when they become cancerous, infected, or damaged (17, 18). NK cells may also contribute to ALS progression (7, 8, 10, 13); we and others have found elevated NK cell levels in the peripheral blood of ALS patients (10, 11) and NK cells accumulate in the spinal cord of ALS mice (7, 8, 19). Moreover, during ALS, motor neurons stop expressing major histocompatibility complex proteins, which mark them as self, protecting them from NK cell-mediated cytotoxicity (20, 21). This suggests a particular vulnerability of motor neurons to NK cells in ALS. Finally, NK cells drive a pro-inflammatory microglia phenotype and simultaneously suppress protective regulatory T cells during ALS (7). Thus, drugs targeting NK cells may prove a viable therapeutic option for ALS, both by blocking NK cell cytotoxicity as well as preventing a pro-inflammatory cascade in the central nervous system.

Tofacitinib is a small molecule pharmaceutical approved for treating multiple immune disorders, including rheumatoid arthritis (22), ulcerative colitis (23), and psoriasis (24). The drug suppresses pro-inflammatory immune activation by blocking the JAK/STAT pathway of the adaptive immune system (25, 26) while preserving innate immune activity and regulatory function (27, 28). However, cytokines associated with NK cell survival and function, including IL-15, signal through the JAK/STAT pathway as well (29–32) and would also be blocked by tofacitinib (33). Indeed, several studies suggest tofacitinib suppresses NK cell numbers in the peripheral blood of mice (34, 35) and humans (36). However, little research has been performed to examine the impact of tofacitinib on NK cell activation. Thus, tofacitinib could potentially block NK cell cytokine production and cytotoxicity in addition to lowering overall levels, providing added benefits as an ALS treatment. In addition, tofacitinib would target NK cells and pro-inflammatory pathways while preserving protective immune function (28) thus overcoming previous failures of previous immune-based therapies for ALS (37).

The present study therefore evaluated the ability of tofacitinib to suppress NK cell cytokine expression and cytotoxicity *in vitro*, both in an NK cell line and in primary NK cells derived from ALS participants. We also investigated whether tofacitinib suppressed NK cell cytotoxicity to inducible neurons (iNeurons) differentiated from ALS patient-derived inducible pluripotent stem cells (iPSCs). Finally, we examined tofacitinib pharmacokinetics in mice as well as the impact *in vivo* on the immune system, since these data have not been previously established and are crucial to future preclinical studies of ALS. Our study found that tofacitinib suppresses NK cell cytotoxicity and cytokine production *in vitro* and suppresses NK cell levels *in vivo* in mice after oral administration in food. These data demonstrate that tofacitinib may be a viable ALS treatment and establish a foundation for future preclinical studies.

METHODS

Study Participants

Healthy control participants without a history of neurodegenerative disease, chronic inflammatory disease, collagen vascular disease, or immunomodulatory medication use were recruited through the University of Michigan Institute for Clinical & Health Research. In parallel, ALS participants meeting a diagnosis of ALS by El Escorial Criteria were recruited during clinical visits at the University of Michigan Pranger ALS Clinic as previously described (13). All study participants provided oral and written informed consent and the study received ethics board approval by the University of Michigan Medical School Institutional Review Board (HUM00028826).

Cell Lines and Primary Human NK Cells

Cell Lines

The NK-92 NK cell line (ATCC Cat# CRL-2408, RRID : CVCL_3755) and K-562 leukemia cell line (ATCC Cat# CCL-243, RRID : CVCL_0004) were acquired from ATCC (Manassas, VA). NK-92 cells were grown in NK media [Alpha's Modification of Medium Essential Eagle media (STEMCELL Technologies cat #36453) supplemented with 12.5% horse serum (Gibco cat #16050122), 12.50% fetal bovine serum (FBS, Sigma Aldrich cat #F4135), 1% penicillin/streptomycin (Gibco cat #15140122), 0.2 mM myo-inositol (Sigma-Aldrich cat #17508), 0.02 mM folic acid (Sigma-Aldrich cat #F8758), and 0.1 mM β-mercaptoethanol (Sigma cat #M7522) and 645.2 nM IL-2 (PeproTech cat #200-02)]. K-562 cancer cells were grown in K-562 media [Iscove's Modified Dulbecco's Medium (STEMCELL Technologies cat #36150) with 10% FBS and 1% penicillin/streptomycin (Gibco cat #15140122)]. Human-derived iPSC lines #1021 (control) and #265 (sporadic ALS, sALS) were obtained from the University of Michigan ALS Biorepository (38). Control and ALS iPSCs were used to generate iNeurons by suppressing the polypyrimidine-tract-binding (PTB) protein, as previously described (39). Briefly, iPSCs

were cultured on poly-D-lysine (50 μg/L, Sigma cat #p1149)/laminin (1:100, Sigma cat #L2020) coated plates in iPSC media [E8 media (Gibco cat #A1517001) supplemented with iROCK Y27632 (Fisher cat #BDB562822)] in 6-well plates at a density of $1x10^5$ cells/well. The following day (Day 1), the media was changed to iNeuron media #1 [E8 media supplemented with 1X N2 supplement (Gibco, cat #17502-048), 1X NEAA supplement (Gibco cat #11140-050), 10 ng/mL BDNF (Peprotech cat #450-02), 10 ng/mL NT3 (Peprotech cat #450-03), 0.2 μg/mL mouse laminin (Sigma cat #L2020), 2 mg/mL doxycycline (Sigma cat #D3447)]. On Day 2, the cells were changed to iNeuron media #2 [½ E8, ½ DMEM/F12 (Gibco cat #11320-033), 1X N2 Supplement, 1X NEAA supplement, 10 ng/mL BDNF, 10 ng/mL NT3, 0.2 μg/mL laminin, 2 mg/mL doxycycline]. On Day 3, cells were changed to iNeuron media #3 [Neurobasal-A (Gibco cat #12349-015), 1X B27 supplement (Gibco cat #17504-044), 1X Glutamax supplement (Gibco cat #35050-061), 10 ng/mL BDNF, 10 ng/mL NT3, 0.2 μg/mL mouse laminin, 2 mg/mL doxycycline]. Additional media #3 was added on Day 6 and Day 8. iNeurons were differentiated for 10 days prior to treatment.

Primary NK Cells

10 mL of whole blood was collected from control and ALS participants, as previously described (10, 12, 13). NK cells were enriched using RosetteSep Human NK isolation cocktail (STEMCELL Technologies cat #15025) and cultured in NK media supplemented with 645.2 nM IL-2 or IL-2 + 2.33 nM IL-15 (PeproTech cat #200-15) for co-culture assays. All cells were grown at 37°C in 5% CO_2.

NK-92 IL-15 Stimulation and Tofacitinib Treatment Paradigms

NK-92 cells were cultured using two IL-15/tofacitinib paradigms (**Figure 1A**). In the first paradigm (P1), NK-92 cells were cultured for two hours with 2.33 nM IL-15 in serum-free NK media prior to overnight treatment with 50 nM tofacitinib (Selleckchem cat #CP-690550). In the second treatment paradigm (P2), NK-92 cells were cultured overnight with 50 nM tofacitinib in serum-free NK media prior to two-hour culture with 2.33 nM IL-15. 50 nM concentration of tofacitinib was used based on previous *in vitro* immune studies (40, 41). For each treatment paradigm, three groups of NK-92 cells were generated: cells receiving no IL-15 and no tofacitinib (Unstimulated), cells receiving only IL-15 (Stimulated) or cells receiving IL-15 stimulation and tofacitinib treatment (Treated). NK-92 cells were then collected, washed, and analyzed for STAT1 phosphorylation (P-STAT), cytotoxicity towards K-562 cells, granzyme B and perforin expression, or cytokine gene expression (see below).

Primary NK Cell Stimulation and Tofacitinib Treatment

$1x10^5$ human primary NK cells were cultured in a 48-well plate (Corning cat #3524) in NK media supplemented with 645.2 nM IL-2 + 2.33 nM IL-15 ± 50 nM tofacitinib for two hours at 37°C in 10% CO_2. Unlike NK-92 cells, primary NK cells were provided additional IL-2 cytokine stimulation to enhance survival in culture (data not shown) (42). The cells were washed and pelleted for subsequent western blot, quantitative real-time PCR (qRT-PCR), and cytotoxicity analysis.

Western Blot Analysis

NK-92 or primary NK cells were lysed in RIPA buffer (Pierce/Thermo Fisher Scientific cat #89901) supplemented with cOmplete mini, EDTA-free protease inhibitors (Roche cat #11836170001), sonicated, and centrifuged. Protein samples were resolved by SDS-PAGE in 10% acrylamide gels, transferred to Immobilon-FL PDVF membranes (Millipore cat #IPFL00010), and immunoblotted with the indicated primary antibodies: rabbit anti-STAT1 (D1K94) (Cell Signaling Technologies, CST, cat #14994S), rabbit anti-P-STAT1 (Y701) (CST cat #9167S), and rat anti-α-tubulin (Abcam cat #ab6160; RRID : AB_305328). Goat anti-rabbit (cat #7074S; RRID : AB_2099233) and anti-biotin horseradish peroxidase (HRP)-linked (cat #7075P5) secondary antibodies were used at 1:2000 (CST) and anti-rat IgG HRP-conjugated secondary antibody was used at 1:5000 (R&D cat #HAF005; RRID : AB_1512258). Densitometric analysis was performed Quantity One v.4.6.5 (Bio-Rad).

Cytotoxicity Assays

NK-92 Cells and K-562 Cells

Pre-treated NK-92 cells (Paradigm 1 or 2) were plated at a density of $1x10^6$ NK-92 cells and co-cultured with $1x10^5$ K-562 cells for two hours at a 10:1 ratio in a final volume of 500 μL followed by flow cytometric analysis of K-562 and NK-92 cell viability dye levels (positive levels indicating cell death, see below).

NK-92 Cells and iNeurons

$1x10^6$ NK-92 cells were starved for 2 hours in serum-free NK media, then treated with 50 nM tofacitinib or vehicle (dimethyl sulfoxide, DMSO) for 30 minutes prior to stimulation with 2.33 nM IL-15 for 4 hours (similar to the intervention treatment paradigm, P2). Treated NK-92 cells were re-suspended in 0.5 mL iNeuron media #3 and co-cultured for 2 hours with 10-day old iNeurons plated at $2x10^5$ cells/well (NK-92:iNeuron = 5:1). At the end of the incubation, the media containing the NK-92 was removed and the iNeurons were washed with 1X PBS and released from the plates with Accutase (Innovative Cell Technologies cat #AT-104) for viability analysis *via* flow cytometry (see below).

Primary NK Cells and K-562 Cells

$1x10^5$ human primary NK cells were co-cultured with $1x10^5$ K-562 cells at a 1:1 ratio were seeded to a 48-well plate (Corning) with NK media for 2 hours at 37°C in 5% CO_2 under one of three conditions: with 645.2 nM IL-2, with IL-2 + 2.33 nM IL-15, or with IL2 + IL-15 + 50 nM tofacitinib. Following co-culture, conditioned media was then collected and stored for subsequent analysis (see below). K-562 and primary NK cells were then washed with flow buffer [1000 mL 1X PBS + 20 mL FBS + 0.01 g

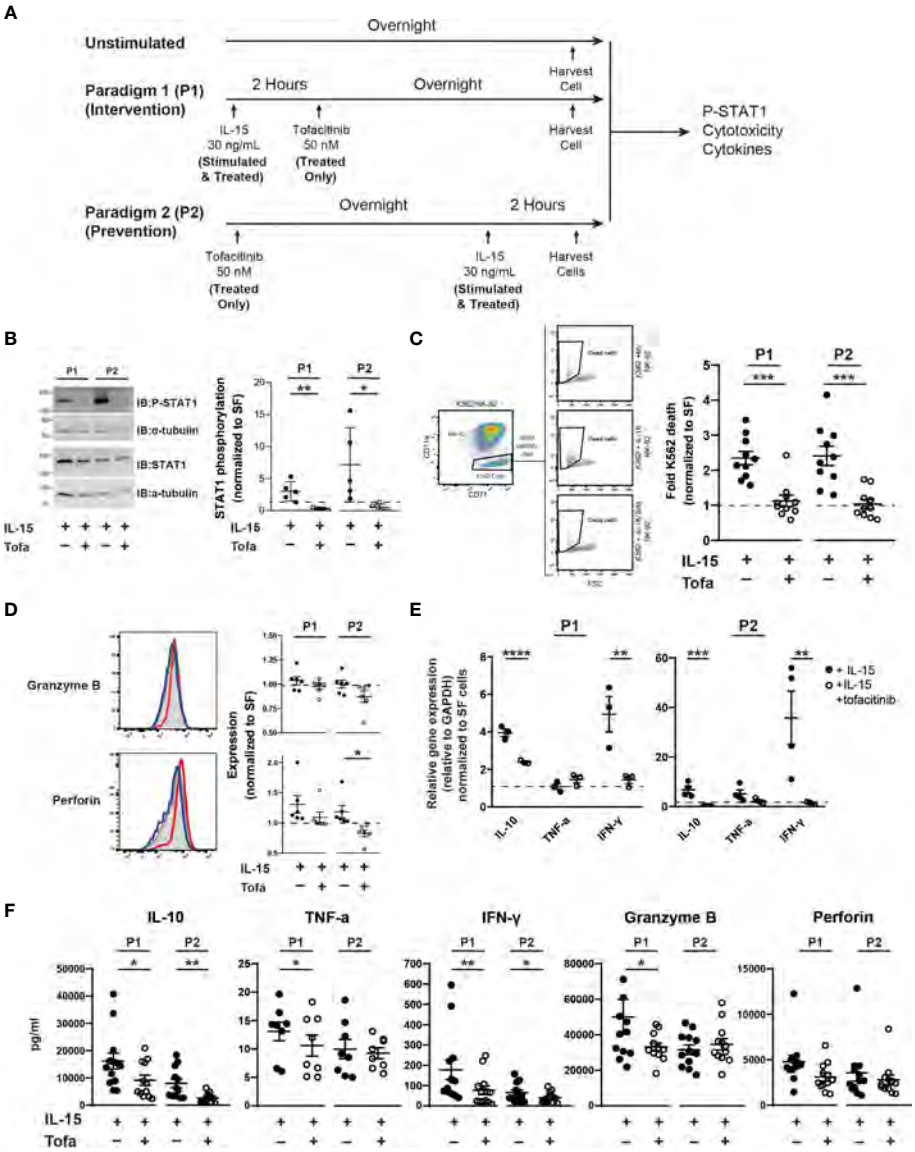

FIGURE 1 | Tofacitinib inhibits NK-92 cells function *in vitro*. **(A)** NK-92 cells were cultured with serum-free media under two culture paradigms: Paradigm 1 (P1) intervention treatment, whereby NK-92 cells were treated with IL-15 for two hours and then cultured overnight in the presence of tofacitinib (Treated), and Paradigm 2 (P2) prevention treatment where NK-92 cells were treated overnight with tofacitinib prior to two-hour IL-15 stimulation. **(B)** Stimulated and Treated NK-92 cells were assessed for STAT1 phosphorylation (P-STAT) following both culture paradigms. Representative immunoblots for Stimulated and Treated P-STAT1 and α-tubulin (internal reference) are shown. Quantitative data represent densitometric analysis, where total STAT1 and P-STAT1 signals were first normalized to α-tubulin then to P-STAT1 levels in Unstimulated cells; n = 5 independent experiments. **(C)** Unstimulated, Stimulated, and Treated NK-92 cells were co-cultured with K-562 cancer cells (10:1 ratio) following initial P1 or P2 culture paradigms. Flow cytometry was used to identify K-562 cells in the co-culture, and cell death was assessed by viability dye staining. K-562 cell death was quantitated for Stimulated and Treated NK-92 cells and normalized to Unstimulated NK-92 cells; n = 10 independent experiments. **(D)** Expression of the intracellular NK cell proteins perforin and granzyme B was determined by flow cytometry on Unstimulated, Stimulated, and Treated NK-92 cells using intracellular flow cytometry following P1 and P2 culture paradigms. Representative histograms are shown; gray peaks = Unstimulated NK-92 cells, red peaks = Stimulated NK-92 cells, blue peaks = Treated NK-92 cells show MFI. MFI from Stimulated and Treated cells were normalized to protein levels in Unstimulated NK-92 cells for quantitation; n = 6 independent experiments. **(E)** Gene expression of IL-10, TNF-α, and IFNγ cytokines was assessed in Unstimulated, Stimulated, and Treated NK-92 cells following P1 and P2 paradigms using qRT-PCR. Data for Stimulated and Treated cells were normalized to GAPDH expression then to the Unstimulated cells; n = 3-4 independent experiments. **(F)** Extracellular expression of IL-10, TNF-α, IFN-γ, granzyme B, and perforin was assessed for Stimulated and Treated NK-92 cells using a CD8/NK cell multiplex analysis following P1 and P2 treatment paradigms (n = 8-13 independent experiments). For all experiments, quantitative data is shown as the mean ± SEM; **(A–E)** comparisons were made by Student's t-test; **(F)** comparisons were made using a paired t-test or a Wilcoxon test based on normality of the data. Horizontal dashed lines represent normalized Unstimulated NK-92 cell levels. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

NaN_3] and plated for analysis of both cell types using flow cytometry and viability dye (see below).

Quantitative Real-Time PCR (qRT-PCR)

RNA was isolated from NK-92 or primary NK cells using the RNeasy isolation kit (Qiagen cat #74104) and with RNA/DNA/RNase-free DNase treatment (Qiagen cat#79254). cDNA was generated using 0.5 μg of NK-92 RNA or 40 ng primary NK cell RNA and 5X iScript RT (Bio-Rad cat#1708840) in a 20 μL reaction following the manufacturer's protocol. The reactions were run in a PTC-200 Peltier Thermal Cycler (MJ Research). qRT-PCR was performed in triplicate using 10-μL reactions consisting of sequence specific TaqMan™ primers for human IL-10 (Hs00961622_m1), TNF-α (Hs00174128), IFN-γ (Hs00989291_m1), GAPDH (Hs02758991_g1), and yWHAZ (Hs03044281_g1); 2X gene expression Master Mix (Applied Biosystems/Thermo Fisher Scientific, cat #4369016) and 2 μL cDNA. C_T values were used to calculate ΔC_T and $\Delta\Delta C_T$ using GAPDH or yWHAZ as the internal references. Data were expressed as the mean of the relative quantity of gene expression ($2^{-\Delta\Delta CT}$).

Multiplex Analysis of Cytokines and Secreted Proteins

The release of cytokines and pro-apoptotic factors by NK-92 and primary NK cells was assessed using the LEGENDplex Human CD8/NK Cell Panel (Biolegend, cat# 740267) according to the manufacturer's instructions. In brief, fluorescent beads were incubated with conditioned media from NK-92 or primary NK cells co-cultured with K-562 cancer cells, and flow cytometry (see below) was used to quantify IL-10, TNF-α, IFN-γ, granzyme B, and perforin in the conditioned media.

Mice

Mice were purchased from Jackson Laboratory (Bar Harbor, ME). Male and female C57BL/6 mice (Stock #000664; RRID : IMSR_JAX:000664) were used for initial tofacitinib pharmacokinetic assays. For tofacitinib efficacy studies, male and female non-carrier, wild-type (WT) control littermates of $SOD1^{G93A}$ ALS mice were used (B6.Cg-Tg(SOD1*G93A)1Gur/J; Jackson Stock #004435). All mice were housed under specific pathogen-free conditions. Animals were fed 5L0D chow *ad libitum* when not treated. All mouse studies were performed in accordance with University of Michigan Institutional Animal Care & Use Committee approved protocols (approval #PRO00010247). Mouse studies were conducted in accordance with the United States Public Health Service's policy on Humane Care and Use of Laboratory Animals.

Tofacitinib Administration to Mice and Plasma Collection for Pharmacokinetic Assays

Tofacitinib was suspended at 2 mg/mL in PBS containing 5% DMSO and 10% PEG-400, which was administered by intravenous (IV) injection (10 mg/kg, 10 mice) or *per os* (PO) *via* gavage (20 mg/kg, 10 mice). At the given time points (0.083, 0.167, 0.25, 0.5, 1, 2, 4, 7, 16, and 24 hours), blood samples were collected using heparinized calibrated pipettes. Samples were centrifuged at 2000*g* for 10 minutes. Subsequently, plasma was collected from the upper layer and frozen at -80°C for later analysis.

Liquid Chromatography

Sample Preparation

To precipitate plasma proteins, 150 μL of acetonitrile containing internal standard and 30 μL of ice-cold acetonitrile were added to 30 μL of plasma. The mixture was vortexed for 10 minutes and centrifuged at 15,000 x g for 10 minutes. The supernatant was transferred to a 96-well plate (Fisher Scientific) for liquid chromatograph-tandem mass spectrometry (LC–MS/MS).

Sample Specificity

The chromatograms of blank plasma versus blank plasma spiked with internal standard (CE302) showed that the blank plasma did not interfere with tofacitinib and internal standard determination.

Calibration Curve

Analytical curves were constructed with 12 nonzero standards by plotting the tofacitinib peak area ratio to the internal standard versus the concentration in plasma. The concentration range was evaluated from 1 to 10000 ng/mL for drug level quantification in plasma. A blank sample (matrix sample processed without internal standard) was used to exclude contamination or interference. The curve was built with linear regression with weighing ($1/X^2$). The linearity of the relationship between peak area ratio and concentration was demonstrated by the correlation coefficients (r = 0.9990).

Quality Control (QC) Samples

The accuracy and precision were evaluated at four concentration levels (2 ng/mL, 400 ng/mL, 4500 ng/mL, and 9000 ng/mL) with three individual replicates at each concentration. The QC stock solution was prepared from separate weighing. QC samples were prepared at four levels (2 ng/mL, 400 ng/mL, 4500 ng/mL, and 9000 ng/mL). QC samples were run before, in the middle, and after running the samples. At least 50% of QCs at each level were within 15% of their nominal concentration. The intra-batch precision was calculated and expressed as relative standard deviation. Data indicate that the assay method was reliable and reproducible.

Analysis

Tofacitinib concentrations in mouse plasma were determined by a liquid chromatography tandem mass spectrometry (LC–MS/MS) method developed and validated for this study. The LC-MS/MS method was preformed using an AB-4500 Qtrap (Sciex, Concord, ON, Canada) mass spectrometer with electrospray ionization source interfaced with a Shimadzu high-performance LC system. Separation was performed on an XBridge C18 column (50 × 2.1 mm ID, 3.5 μm; Waters, Milford, MA, USA) at a flow rate of 0.4 mL/minute. The mobile phase consisted of A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid). The gradient was 0.0-0.5 minutes, 2% B;

0.5-2.0 minutes, 2-95% B; 2.0-3.6 minutes, 95% B; and 3.6-4.1 minutes, 95-2% B. The mass spectrometer was operated in positive mode with multiple reaction monitoring for analysis. The multiple reaction monitoring transitions were m/z 313.1 > 173.1 for tofacitinib and 455.2 > 425.2 for the internal standard. The gas temperature was 500°C with an ionspray voltage of 5500 V, gas 1 and gas 2 of 30 psi, and curtain gas of 30 psi. Analyst Software (version 1.6) from Applied Biosystems (MDS SCIEX; Carlsbad, CA, USA) was used to control the LC-MS/MS system, as well as for data acquisition and processing. All pharmacokinetic parameters were estimated using non-compartmental analyses with Phoenix WinNonlin software (Certara, Princeton, NJ).

Tofacitinib Mouse Chow

Low-dose (5 mg/kg) and high-dose (30 mg/kg) chow was manufactured by Research Diets (New Brunswick, NJ). To determine the initial tofacitinib content in the chow, chow from two cages of mice was weighed daily for a week to determine the daily chow consumption per cage. The average chow consumption for each mouse was then calculated, and low- and high-dose chow was formulated based on average consumption. For the peripheral immune analysis, male and female mice were placed on low-dose, high-dose, or normal chow (control animals) for two weeks prior to harvest.

Blood Leukocyte Collection

Peripheral immune cells were harvested as previously described (9). At the time of harvest, mice were euthanized with sodium pentobarbital, whole blood was collected from the vena cava and measured using a 1 mL syringe (BD Biosciences, Franklin Lakes, NJ), and transferred to a BD Vacutainer® blood collection tube (BD Biosciences) coated with 3.6 mg of EDTA. Red blood cells were lysed with 9.5 mL red blood cell lysis buffer [150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA (Thermo Fisher Scientific) with 13.8 mM HEPES, pH 7.2-7.5 (Thermo Fisher Scientific)] for 20 minutes on a rocker at room temperature. Leukocytes were pelleted (1000 rpm, 10 minutes, 4°C, with brake), supernatant siphoned off, washed twice with flow cytometry buffer [1X PBS, 2% FBS (Thermo Fisher Scientific), 0.1% NaN_3] and resuspended in 1 mL flow cytometry buffer. Cells were counted by hemocytometer (Hausser Scientific, Horsham, PA), and kept on ice until staining for flow cytometry.

Flow Cytometry

Intracellular Perforin and Granzyme B Staining of NK-92 Cells

All samples were washed and resuspended at a density of $\leq 10^6$ cells/25 µL, plated in U-bottom 96-well plates (Fischer cat #07-200-760), and spun down at 1200 rpm for 10 minutes. Samples were incubated with 10 µg/mL human TruStain FcX™ blocking solution (Biolegend cat #422302; RRID : AB_2818986) at 4°C for 30 minutes prior to immune staining. Following blocking stage, NK-92 cells were stained with CD56 and HLA and washed with flow buffer (PBS + 2% FBS). Cells were then permeabilized using Cytofix/cytoperm (BD cat #554714; RRID : AB_2869008) and stained with antibody for perforin (Biolegend cat #353303; RRID : AB_10915476) and granzyme B (Biolegend cat #515403; RRID : AB_2114575). Control stains were performed with non-specific IgG antibody (Biolegend cat #400111; RRID : AB_2847829 and #400137). Cells were then transferred to polystyrene tubes (12x75 mm) (BD Biosciences) and analyzed on a BD LSRFortessa™ flow cytometer with FACSDiva™ software (BD Biosciences) and FlowJo (FlowJo, Ashland, OR).

NK-92 and Primary NK Cytotoxicity Assays

Following collection and wash, co-cultures of NK and K-562 cells were incubated with Fixable Viability Dye eFluor™ 506 (1:500) (eBioscience cat #65-0866-14) during the blocking stage. Samples were then incubated with surface stains CD56 (Biolegend cat #318318; RRID : AB_604107), CD11a (Biolegend cat#301207; RRID : AB_10660819), and CD71 (Biolegend cat #334110; RRID : AB_2563117) at 4°C for 30 minutes. K-562 cells were identified as CD56-, CD11a-, CD71+. Samples were fixed using Stabilizing Fixative (BD cat #338036; 1:3 dilution).

iNeurons

All samples were washed, resuspended at a density of $\leq 10^6$ cells/25 µL, plated in U-bottom 96-well plates, and blocked at 4°C for 30 minutes prior to staining. Cells were then stained for annexin V (Invitrogen cat #12-8102-69) and with the viability dye 7-amino-actinomycin D (7AAD; Invitrogen cat #00-6993-50), according to the manufacturer's instructions, and fixed for analysis, as above, consistent with previously reported protocols in neurons (43, 44).

Mouse Peripheral Immune Cells

All samples were washed and resuspended at a density of $\leq 10^6$ cells/25 µL, plated in U-bottom 96-well plates (Fischer), and spun down at 1200 rpm for 10 minutes. Samples were incubated with 10 µg/mL mouse TruStain FcX™ blocking solution (Biolegend cat #101320; RRID : AB_1574975) at 4°C for 30 minutes prior to staining. Samples were incubated with antibodies against myeloid and lymphoid surface markers as previously described (9). In brief, cells were stained with antibodies against CD45, CD11b, Ly6C, and Ly6G to analyze myeloid populations and CD45, CD3, CD4, CD8, NK1.1, and CD49b to analyze lymphocyte and NK cell populations.

Statistics

All statistics were performed using GraphPad Prism version 8.0.0 (San Diego, CA). All datasets were assessed for normality using normality using Shapiro-Wilk (45). For comparing between two groups, either a two-tailed Student's t-test (normally distributed data) or Mann-Whitney (non-normally distributed data) were used. For cytokine expression by NK-92 cells a paired t-test or a Wilcoxon test (paired data with non-normal distribution) was used when data was not normally distributed. A Wilcoxon test was used for comparing NK-92 cytotoxicity to iNeurons and comparing primary NK cell cytotoxicity to K-562 cancer cells. For comparing peripheral immune cell levels, two-way ANOVA with multiple comparisons was used. *P*-values < 0.05 were considered statistically significant.

RESULTS

Tofacitinib Inhibits NK-92 Cells Function *In Vitro*

Previous studies demonstrate that tofacitinib lowers NK cell levels *in vivo* (34–36, 46); however, little is known about the effect of tofacitinib on NK cell activation. Blocking NK cell activation in addition to lowering levels could potentially increase tofacitinib efficacy for treating NK cell-mediated diseases, such as ALS. Thus, we explored whether tofacitinib reduces NK cell cytotoxicity, cytokine production, and trafficking *in vitro*. We initially employed a commercially available NK cell line, NK-92 cells, and two treatment paradigms to explore the impact of tofacitinib on NK cell function (**Figure 1A**). In the first treatment paradigm (P1, intervention treatment) NK-92 cells were activated with IL-15 for two hours (30–32) and cultured overnight in the presence of tofacitinib. In the second paradigm (P2, prevention treatment), NK-92 cells were first pre-treated with tofacitinib overnight and then activated for two hours with IL-15. In parallel, Unstimulated cells (which received no IL-15 stimulation, serum stimulation, or tofacitinib treatment) were also cultured overnight and analyzed simultaneously with P1 or P2 NK-92 cells. For both paradigms, NK-92 cells were further divided into two groups: NK-92 cells cultured with IL-15 without tofacitinib (Stimulated) and NK-92 cells cultured with both IL-15 and tofacitinib (Treated). This resulted in a total of five NK-92 groups (Unstimulated, P1 Stimulated, P1 Treated, P2 Stimulated, and P2 treated) that were analyzed in parallel.

First, we confirmed that tofacitinib treatment blocks IL-15 signaling in NK-92 cells in both intervention and prevention paradigms. Tofacitinib disrupts JAK/STAT signaling by blocking STAT protein phosphorylation (47). Therefore, we measured phosphorylated STAT1 (P-STAT1) levels in Unstimulated, Stimulated, and Treated NK cells and normalized values levels in Untreated NK-92 cells to account for run-to-run variation. Immunoblot analysis indicated that tofacitinib treatment significantly reduced P-STAT1 in both culture paradigms (10-fold in P1 and 13 fold in P2, **Figure 1B**) demonstrating that tofacitinib blocks IL-15 signaling in NK-92 cells.

We next tested whether tofacitinib treatment suppresses NK cell function by assessing cytotoxicity, the intrinsic ability of NK-92 cells to eliminate other cells. NK-92 cells in both intervention and prevention treatment paradigms (Unstimulated, Stimulated, or Treated) were co-cultured with K-562 leukemia cells. K652 cell death, as measured by cellular viability dye *via* flow cytometry, was used to quantify NK-92-killing activity (48) (**Figure 1C**); cytotoxicity of Stimulated and Treated NK-92 cells was normalized to Unstimulated NK-92 cells. We found that IL-15-treated NK-92 cells doubled the rate of K-562 cancer cells killing under both paradigms. However, under both intervention and prevention treatment paradigms, blocking IL-15 with tofacitinib significantly reduced the ability of NK-92 cells to induce K-562 cell death, showing that tofacitinib reduces the ability of NK cells to eliminate target cells. To ensure that tofacitinib is suppressing NK-92 cytotoxicity rather than reducing cellular viability, we also examined NK-92 survival using viability dye. We found no significant differences in NK-92 viability following tofacitinib treatment (data not shown).

To support these findings, we next examined the impact of tofacitinib on NK-92 expression of intracellular granzyme B and perforin, both of which play key roles in NK cell-mediated cytotoxicity (49). Intracellular granzyme B and perforin levels from Unstimulated, Stimulated, or Treated NK-92 cells (**Figure 1D**) were quantified by the median fluorescent intensity (MFI) from intracellular flow cytometry; values from Stimulated and Treated NK-92 cells were then normalized to the MFI from Unstimulated NK-92 cells. We found that tofacitinib significantly lowered intracellular perforin levels in the prevention paradigm (P2), with a trending reduction in the intervention paradigm (P1). Tofacitinib also induced a trending reduction towards reduced granzyme B levels. These data suggest tofacitinib may reduce NK cell cytotoxicity, partly by suppressing the expression of proteins that induce target cell death.

In addition to direct cytotoxicity, we examined the ability of tofacitinib to suppress NK cell cytokine production, which enhances neuroinflammation in ALS (7). NK-92 cells were cultured under both paradigms, and cytokine IL-10, TNF-α, and IFN-γ mRNA expression levels were measured by qRT-PCR and normalized to Unstimulated cells. Overall, *TNF-α*, *IL-10*, and *IFN-γ* gene expression increased in both paradigms following IL-15 stimulation, but this increase was reversed by tofacitinib treatment (**Figure 1E**). To confirm these findings, we next examined the secretion of pro-inflammatory and pro-apoptotic factors using a bead-based multiplex analysis paired with flow cytometry. Conditioned media from Stimulated and Treated culture conditions was analyzed for levels of IL-10, TNF-α, IFN-γ, granzyme B, and perforin. Similar patterns of NK cell suppression were seen following both tofacitinib treatment paradigms. As observed with qRT-PCR, both IL-10 and IFN-γ levels were suppressed during the P1 and P2 treatment paradigms (**Figure 1F**). Similarly, granzyme B and perforin expression were suppressed following the P2 treatment paradigm, demonstrating that tofacitinib suppresses the release of pro-inflammatory and pro-apoptotic factors by NK-92 cells. Together, these data demonstrate that tofacitinib suppresses the ability of NK cells to generate cytokines in addition to blocking their cytotoxicity in response to pro-inflammatory stimulation.

Next, we explored whether tofacitinib protects neurons from NK cell-mediated cytotoxicity in an *in vitro* ALS model. To do so, we co-cultured NK-92 cells with iPSC-derived iNeurons. Two iNeuron cell lines were used: one derived from a control participant and one derived from an ALS participant (50). Following differentiation and ten days of growth, iNeurons were co-cultured for four hours with IL-15-stimulated NK-92 cells with and without tofacitinib. Annexin V and 7AAD were used as markers to quantitate iNeuron cell death by flow cytometry (43, 44), and mCherry was used to identify iNeurons within the co-culture (**Figure 2A**). However, dead NK-92 cells autofluoresce and appear positive for mCherry, and NK-92 cells cultured in iNeuron media #3 display increased NK cell death. Thus, dead NK-92 cells also appeared

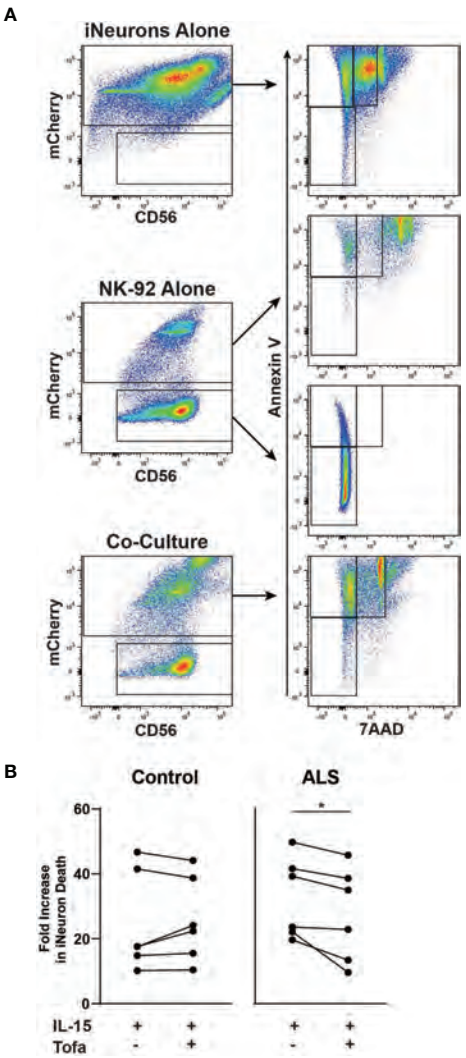

FIGURE 2 | Tofacitinib decreases the cytotoxicity of NK-92 to motor neurons in an *in vitro* ALS model. iNeurons were differentiated from control- or ALS-participant derived iPSCs and were co-cultured for four hours with pretreated NK-92 cells (IL-15 ± tofacitinib); cell death was quantitated by flow cytometry. **(A)** Gating strategy for quantitating cell death of iNeurons cultured alone, of NK-92 cells cultured alone, and iNeuron and NK-92 cell co-culture. Dead iNeurons were characterized by positive fluorescence levels of annexin V (apoptosis) and moderate levels of 7AAD viability dye (cell death). **(B)** The rate of iNeuron death was quantitated in control- and ALS-derived iNeurons following co-culture with NK-92 cells; data were normalized to cell death rates from co-culture with Unstimulated NK-92 cells; n = 6 independent experiments. Comparisons were by paired t-test to assessed paired, non-normally distributed data. *$P < 0.05$.

within the Annexin V+ and 7AAD+ gates for iNeurons, potentially skewing the data. To account for this this, we compared Annexin V and 7AAD flow plots for mCherry+ iNeurons versus mCherry+ NK-92 cells cultured alone in iNeuron media and found that NK 7AAD fluorescence levels for NK cells were higher than that of iNeurons. Thus, dead iNeurons were identified based on moderate 7AAD staining; cell death rates for iNeurons co-cultured with Stimulated and Treated NK-92 cells were then examined. We found that control-derived iNeurons showed similar rates of cell death whether cultured with or without tofacitinib treatment (**Figure 2B**). In contrast, there was a significant reduction in the rate of cell death for ALS-derived iNeurons that were cultured with Treated NK cells versus Stimulated NK cells. Together these results demonstrate that tofacitinib can protect ALS neurons from NK cell-mediated cytotoxicity.

Tofacitinib Inhibits Primary NK Cells Function *Ex Vivo*

We next extended these findings to determine whether tofacitinib suppresses cytotoxicity in primary NK cells from control and ALS participants (see **Table 1** for demographics). Primary NK cells were isolated from the peripheral blood of control and ALS participants. We first confirmed that tofacitinib suppresses JAK/STAT signaling in primary NK cells from control and ALS participants, similar to NK-92 cells. As measured by STAT1 phosphorylation *via* immunoblotting, tofacitinib significantly reduced P-STAT1 levels in primary ALS and control NK cells versus those stimulated with IL-2 and IL-15 (**Figure 3A**). Next, cytokine gene expression was examined in control and ALS primary NK cells following cytokine stimulation and treatment (**Figure 3B**). In ALS NK cells, tofacitinib treatment significantly decreased *TNF-α* and *IFN-γ* expression from primary ALS NK cells. A similar trend was observed in the cytokine gene expression of control primary NK cells. As with the NK-92 cell line, we also examined whether tofacitinib inhibits the release of pro-inflammatory and pro-apoptotic factors using a multiplex analysis. Conditioned media from primary NK cells cultured with IL-15 with and without tofacitinib was analyzed for the secretion of TNF-α and IFN-γ (**Figure 3C**). There was a trend towards reduced TNF-α secretion from primary NK cells isolated from control participants and reduced TNF-α and IFN-γ secreted from primary NK cells isolated from ALS participants.

Finally, we examined whether tofacitinib suppresses the cytotoxicity of primary NK cells isolated from control and ALS participants. After isolation, primary NK cells were co-cultured with K-562 target cancer cells, and the rate of K-562 cell death was used to quantitate primary NK cell cytotoxicity. Since K-562 viability can fluctuate, the rate of K-562 cell death in co-culture was normalized to the rate of K-562 cells cultured alone (**Figure 3D**). The cytotoxicity of both control and ALS primary Stimulated NK cells (IL-2 + IL-15) did not significantly differ from NK cells stimulated with IL-2 alone (data not shown). In contrast, primary NK cells treated with tofacitinib displayed significantly lower cytotoxicity to K-562 cells than Stimulated NK cells (**Figure 3E**). As with NK-92 cells, exposure to tofacitinib did not alter the viability of primary NK cells (data not shown). Together, these results demonstrate that primary NK cells are already stimulated in the peripheral blood of control and ALS participants, but tofacitinib can nevertheless suppress primary NK cell cytotoxicity by inhibiting JAK/STAT signaling.

TABLE 1 | Subject demographics for primary NK cell analyses.

	Western Blot		Cytokine Gene Expression		Cytokine Protein Secretion		Cytotoxicity	
	Control (n = 3)	ALS (n = 4)	Control (n = 3)	ALS (n = 8)	Control (n = 5)	ALS (n = 4)	Control (n = 12)	ALS (n =32)
Age(Mean ± SD) years	70.5 ± 5.3	60.7 ± 13.4	66.2 ± 10.9	65.0 ± 8.2	69.32 ± 3.9	65.61 ± 6.7	61.7 ± 13.3	64.2 ± 9.0
Sex (%) male	66.6	25.0	33.3	37.5	60.0	50.0	50.0	56.3
ALSFRS-R at Blood Draw	N/A	23.0 ± 7.7	N/A	28.4 ± 10.3	N/A	35.8 ± 6.1	N/A	26.1 ± 8.3
Site of Onset	N/A	Bulbar (50.0%) Cervical (25.0%) Lumbar (25.0%)	N/A	Bulbar (25.0%) Cervical (25.0%) Lumbar (50.0%)	N/A	Bulbar (50.0%) Cervical (25.0%) Lumbar (25.0%)	N/A	Bulbar (18.8%) Cervical (34.4%) Lumbar (46.8%)
Race	White (100%) Black (0%)Asian (0%)Not reported (0%)	White (100%) Black (0%)Asian (0%)Not reported (0%)	White (100%) Black (0%)Asian (0%)Not reported (0%)	White (75.0%) Black (25.0%) Asian (0%)Not reported (0%)	White (100%) Black (0%)Asian (0%)Not reported (0%)	White (100%) Black (0%)Asian (0%)Not reported (0%)	White (100%) Black (0%)Asian (0%)Not reported (0%)	White (90.6%) Black (9.4%) Asian (0%)Not reported (0%)
Ethnicity	Not Hispanic (100%)Hispanic (0%)Not reported (0%)	Not Hispanic (100%)Hispanic (0%)Not reported (0%)	Not Hispanic (100%)Hispanic (0%)Not reported (0%)	Not Hispanic (100%)Hispanic (0%)Not reported (0%)	Not Hispanic (100%)Hispanic (0%)Not reported (0%)	Not Hispanic (100%)Hispanic (0%)Not reported (0%)	Not Hispanic (100%)Hispanic (0%)Not reported (0%)	Not Hispanic (100%)Hispanic (0%)Not reported (0%)
Time from Onset to Collection (Mean ± SD) years	N/A	5.0 ± 4.2	N/A	7.7 ± 6.8	N/A	2.7 ± 1.6	N/A	4.1 ± 3.9
Time from Diagnosis to Collection (Mean ± SD) years	N/A	3.6 ± 3.4	N/A	5.2 ± 6.7	N/A	1.6 ± 1.5	N/A	2.7 ± 3.6

N/A, not applicable.

Tofacitinib Pharmacokinetics in Mice

Next, we wanted to optimize tofacitinib pharmacokinetics to administer to ALS mice in future studies, since NK cells are implicated in ALS pathogenesis (7, 13). However, symptoms in ALS mice do not emerge until after 90 days of age, and their lifespan is about 160 days of age in low-copy mouse strains (9). Therefore, preclinical studies of tofacitinib in SOD1^{G93A} mice will require long-term treatment. Unfortunately, previous studies testing tofacitinib in mice were either short-term studies with daily gavage (35) or longer-term studies using osmotic minipumps (51). For long-term diseases, administering tofacitinib daily by gavage is not logistically feasible and can induce significant animal losses (52). Conversely, minipumps cannot administer high treatment doses and require repeated surgeries. Thus, we wished to evaluate the efficacy of tofacitinib formulated into chow as a method of long-term administration for future preclinical studies.

Although tofacitinib pharmacokinetics had been performed in rats and human participants, little data are available in mice. Therefore, our first step was to assess tofacitinib pharmacokinetics after a single orally (PO, *per os*, gavage) or intravenously (IV) administered tofacitinib dose to male and female C57BL/6 mice. Blood was collected at multiple time points (5, 10, 15, 30, 60, 120, 240, 420, 960, 1440 minutes) post IV and PO administration and the kinetics of blood tofacitinib assessed. Tofacitinib bioavailability was roughly 37% in both male and female mice, *i.e.*, roughly 37% of the initial dose administered orally reaches the peripheral blood (**Table 2**). Interestingly, we observed that other pharmacokinetic parameters differed between male and female mice. For instance, the maximal plasma level was higher in female versus male mice after both IV and PO dosing, while drug clearance was higher in male mice. These data indicate that tofacitinib can be administered orally by chow with similar drug uptake in males and females, though there may be sex-specific differences in drug metabolism resulting in altered plasma levels.

Efficacy of Low- and High-Dose Tofacitinib in Mouse Chow

Finally, we tested tofacitinib efficacy formulated in chow on NK cell levels in mice. Based on the pharmacokinetic data, chow was formulated to deliver a daily dose of 5 mg/kg (low-dose) or 30 mg/kg (high-dose) to male and female WT control mice (*i.e.*, WT littermates on an SOD1^{G93A} background) for two weeks. At the end of the treatment period peripheral immune cell levels were assessed by flow cytometry for the percentage and total number of NK cells, neutrophils, Ly6C- monocytes, Ly6c+ monocytes, CD4 T cells, and CD8 T cells. Both low- or high-dose tofacitinib treatment significantly lowered NK cell percentage in a dose-dependent manner (**Figure 4A**). Moreover, both doses significantly reduced total NK cell counts in peripheral blood versus normal chow, and there was a trend towards fewer circulating NK cells in of high- versus low-dose mice (**Figure 4B**). In contrast, tofacitinib treatment did not significantly reduce the percentage or total number of neutrophils, Ly6C+ monocytes, CD4 T cells, or CD8 T cells. Interestingly, high-dose mice had significantly lower percentage and total number of circulating Ly6C- monocytes, which is consistent with our previous study utilizing NK cell depletion (13). Together, these results demonstrate that tofacitinib can be

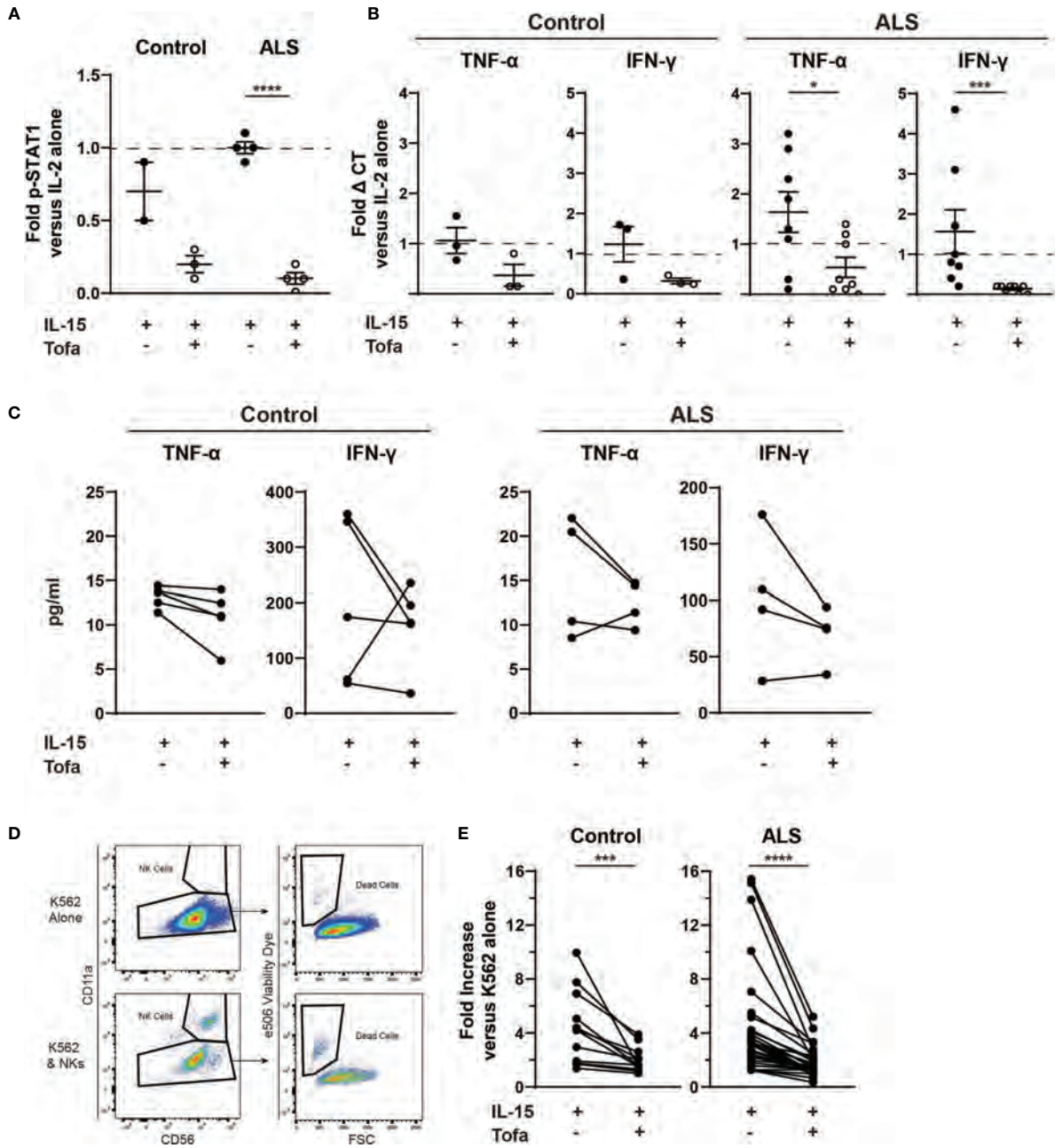

FIGURE 3 | Tofacitinib inhibits primary NK cells *in vitro*. Primary NK cells were enriched from the whole blood of control and ALS participants. **(A)** P-STAT1 was assessed using Western blot. Primary NK cells were incubated with IL-2 alone, IL-2 + IL-15, or IL-2 + IL-15 + tofacitinib for 2 hours. Protein extracts were resolved by SDS-PAGE and immunoblotted for total STAT1, phosphorylated STAT1 (P-STAT1), and α-tubulin (internal reference). Graph represents densitometric analysis where total STAT1 and P-STAT1 signals were normalized to α-tubulin then normalized to NK cells receiving IL-2 alone; n = 2-4 participants. **(B)** Cytokine gene expression was assessed using qRT-PCR for *TNF-α* and *IFNγ*. Data were normalized to yWHAZ expression and normalized to the IL-2 NK cells; n = 3-8 participants. **(C)** Extracellular expression of TNF-α and IFN-γ was assessed for primary NK cells cultured with IL-15 ± tofacitinib (n = 5 control and n = 4 ALS). **(D)** Primary NK cells were assessed for cytotoxicity. Primary NK cells were cultured for two hours with K-562 cancer cells (1:1 ratio) + IL-15 ± tofacitinib; K-562 cell death was assessed using flow cytometry to quantitate e506 viability dye fluorescence. **(E)** Data were quantitated by normalizing to K-562 cells cultured without NK cells; n = 12 control and n = 32 ALS. For **(A, B)**, data are presented as mean ± SEM with dashed line showing cells cultures with IL-2 alone; comparisons were made by Student's t-test. For **(C)**, comparisons were made using a paired t-test. For **(E)**, comparisons were made by Wilcoxon test to assessed paired, non-normally distributed data. *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$.

TABLE 2 | Tofacitinib pharmacokinetic parameters in plasma following IV and PO administration.

Route Unit	Sex	Dose mg/kg	C0/Cmax ng/mL	Tmax h	$AUC_{(0-24)}$ h*ng/mL	$AUC_{(0-inf)}$ h*ng/mL	t½ h	CL/CL_F mL/h/kg	Vss/Vz_F mL/kg	%F %
IV	M	10	3554.4	N/A	676.76	678.09	1.85	14747.23	39278.78	N/A
PO	M	20	960	0.25	505.74	508.48	0.91	39332.99	51817.31	37.4
IV	F	10	6516.9	N/A	980.54	982.90	0.72	10173.94	10549.83	NA
PO	F	20	1114.3	0.167	713.26	719.57	0.90	27794.47	36093.12	36.4

IV, intravenous, PO, per os; C0, concentration at time 0; Cmax, maximum observed concentration; Tmax, time to reach Cmax; $AUC_{(0-24)}$, area under the concentration-time curve from time zero to 24 hours; $AUC_{(0-inf)}$, area under the concentration-time curve from time zero to infinite; CL, systemic clearance; CL_F, apparent clearance; Vss, volume of distribution at steady state; Vz_F, volume of distribution associated with the terminal elimination phrase; terminal elimination half-life (t½) was calculated based on data points (≥3) in the terminal phase with correlation of coefficient >0.90; %F, bioavailability; N/A, not applicable.

administered in chow mouse models and suppresses NK cell levels in a dose-dependent manner.

DISCUSSION

Previous studies have shown that tofacitinib treatment suppresses NK cell levels (34–36, 46), but there is limited information on the impact of tofacitinib on NK cell function (41). Similarly, preclinical mouse disease studies of tofacitinib have been short-term or used administration routes unsuitable to long-term studies. Addressing these shortcomings is of utmost importance to test tofacitinib for regulating NK cell function and counts in future preclinical ALS studies, since NK cells are implicated in ALS progression (7, 13). Therefore, in the current study, we evaluated tofacitinib on NK cell function *in vitro* and conducted a pharmacokinetic study *in vivo*. We used the pharmacokinetic data to formulate tofacitinib in chow, a suitable format for long-term oral administration. We evaluated the impact of a 2-week tofacitinib chow regimen on circulating NK cell levels in WT mice. We found tofacitinib suppressed IL-15-mediated JAK/STAT pathway stimulation, cytotoxicity to cancer cells and iNeurons, granzyme B and perforin expression, and cytokine expression in the NK-92 cell line. Importantly, in the context of ALS, tofacitinib also significantly lowered cytotoxicity of IL-2/IL-15-stimulated primary NK cells isolated from ALS participants and healthy controls, as well as cytokine levels. Finally, tofacitinib bioavailability was similar in male and female mice, although there were sex differences in some parameters; formulation in chow at both low- (5 mg/kg) and high-dose (30 mg/kg) tofacitinib after 2 weeks lowered peripheral NK cell levels in WT control mice.

These findings suggest that tofacitinib may be a viable therapeutic strategy to regulate the NK cell population in ALS. NK cells accumulate in the spinal cord of ALS mice (8, 13, 19, 53). In individuals with ALS, NK cells are increased in the peripheral blood (10, 11) and co-localize with motor neurons

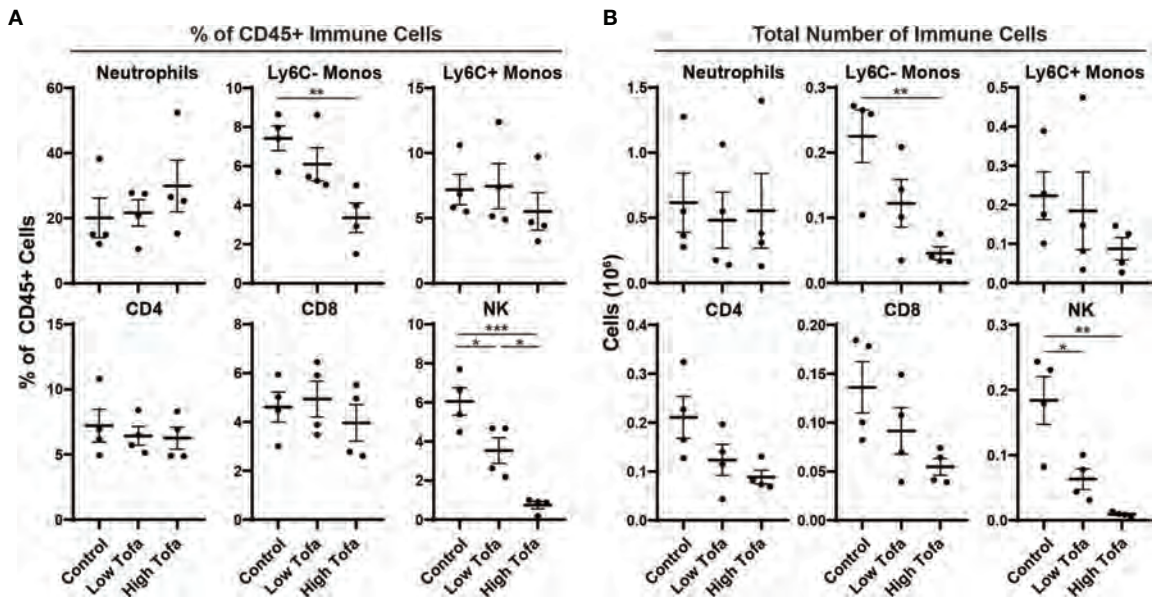

FIGURE 4 | Impact of orally administered tofacitinib on peripheral immune populations in mice. WT control mice (half male and half female) were treated for two weeks with low- (5 mg/kg) and high- (30 mg/kg) dose tofacitinib administered in standard chow. Immune cells were then analyzed in peripheral blood using flow cytometry; **(A)** percentage of all CD45+ immune cells as well as **(B)** total numbers of cells was examined for six immune populations. Data show mean ± SEM. Comparisons by ANOVA; n = 4 mice per group. **P*<0.05, ***P*<0.01, ****P*<0.001; monos, monocytes.

in *postmortem* spinal cord tissue, driving microglial activation *via* IFN-γ expression (7). Indeed, as ALS progresses, motor neurons lose surface markers, which protect against NK cells-mediated cytotoxicity (20), rendering them more susceptible to attack. In addition to increased NK cell levels, they are also more highly activated in individuals with ALS, correlating with disease progression (13). Depleting NK cells from $SOD1^{G93A}$ ALS mice extends survival (7, 13). Thus, reducing NK cell levels, blocking NK cell cytotoxicity, and suppressing IFN-γ release from NK cells may slow motor neuron loss and suppress central nervous system inflammation, increasing survival in ALS.

Previous studies have established that tofacitinib blocks immune cell activation and activity by interfering with the JAK/STAT pathway (25), promoting pro-inflammatory cytokine signaling between cells (27, 54, 55). Blocking cytokines, such as IFN-γ, with tofacitinib prevents inflammatory T cell activation, which effectively treats autoimmune disorders, such as rheumatoid arthritis, ulcerative colitis, and psoriasis (22–24). In the case of NK cells, blocking JAK/STAT signaling suppresses the IL-15 pathway (33), which is a crucial mediator of both NK cell survival and activation (29, 56, 57). This likely explains the reduced peripheral NK cell levels observed after tofacitinib treatment in both mouse and humans (34–36, 46). However, in addition to maintaining NK cell homeostasis, IL-15 is also a potent stimulator of NK cell function (30–32). Thus, tofacitinib should also block IL-15-mediated NK cell activation. Our current study definitively shows this: tofacitinib treatment suppresses pro-inflammatory cytokine production and cytotoxicity in both NK-92 cells and primary NK cells. This is particularly salient to ALS, since direct NK cell cytotoxicity as well as IFN-γ production likely contribute to disease progression (7). The importance of these findings is further corroborated by our results showing that tofacitinib significantly reduces NK cell cytotoxicity towards iNeurons generated from ALS patient-derived iPSCs.

The current study also suggests that tofacitinib suppresses NK cell levels in an ALS mouse model. However, although preclinical mouse models are crucial for evaluating drug efficacy *in vivo*, no comprehensive studies have examined tofacitinib pharmacokinetics nor the long-term impact of the drug on peripheral immunity in mice. There are challenges associated with long-term drug administration. One possible solution is the use of osmotic minipumps as it has been previously shown (51). However, minipumps typically administer either a low drug dose over a long period of time or a high dose over a short period of time. A higher dose of tofacitinib, such as 30 mg/kg, would require frequent pump replacement and multiple surgeries. Not only is this logistically difficult, but frequent surgeries would be potentially life-threatening for mice in advanced stages of disease. In contrast, daily oral tofacitinib administration tofacitinib to mice *via* gavage is not logistically feasible over long time periods either as the rate of death associated with technique is 15% over a six week period (35, 52). Moreover, these previous studies did not examine tofacitinib pharmacokinetics – particularly bioavailability – meaning the final concentration in the peripheral blood following oral administration was not known. In the present study we found that tofacitinib bioavailability in mice (around 37%) differed from humans, where bioavailability is 74% (58). Perhaps unsurprisingly, bioavailability in mice is closer to that in rats (29%) (59). Interestingly, we found that plasma tofacitinib levels differed between male and female mice, even after IV administration, suggesting the sexes may clear the drug at different rates. These sex-specific tofacitinib pharmacokinetics differences are potentially important for future ALS treatment, since we have previously described sex-based immune differences in ALS (12, 13).

Consistent with other methods of tofacitinib administration, treating mice orally with tofacitinib in chow successfully suppressed circulating NK cell levels in a dose-dependent manner in WT mice on a $SOD1^{G93A}$ genetic background. Together with our *in vitro* findings, these results suggest that tofacitinib modulates NK cell levels and activity and should be tested in preclinical mouse models of ALS. However, an in-depth series of studies will be required, as the mechanisms of NK cell involvement in ALS is incompletely understood. One mechanism by which NK cells contribute to ALS is the destruction of damaged motor neurons within the CNS, as motor neurons are uniquely vulnerable to NK cells during disease progression (20). Alternatively, NK cells may be involved in other disease mechanisms. NK cells may play an important role in driving early microglial activation (7) which has been implicated in ALS pathology (60–62), and they may also contribute to peripheral nerve damage in ALS, as increased expression of major histocompatibility complex I was associated with slower disease progression in mouse models of ALS (63). The role of NK cells in the loss of neuromuscular junction (NMJ) integrity during ALS has also not been examined.

Preclinical studies must also account for the impact of tofacitinib on other immune cell populations that modulate ALS progression, both in the periphery and the CNS. Immune cells are both protective and destructive in ALS (2, 10), so preserving protective immune function is of the utmost importance when designing and utilizing immune-based therapies. While the current study demonstrated that tofacitinib suppresses NK cell numbers in the peripheral blood, changes were also observed in other cell populations. Ly6C- monocytes, which patrol the body and are involved in fibrosis and wound repair (64), were significantly reduced in mice treated with the higher tofacitinib dose; analogous monocytes in human patients may have a protective effect (65). Similarly, there was a trend towards reduced CD4 and CD8 T cell levels following tofacitinib treatment, particularly in mice treated with the high dose. While these observations did not reach statistical significance, it is important to account for these changes, as these immune cell types play a central role in ALS, in particularly CD4 T cells (4, 10). Since the cellular lifespan of NK cells (66–68) is much shorter than that of T cells (69) it may be possible to preserve T cell levels by utilizing on/off drug treatment cycles. This possibility should also be explored in future clinical trials.

In addition, preclinical tofacitinib studies should account for the impact of sex in ALS mouse models. Though the impact of sex on tofacitinib-NK cell interaction was not explored in the current study, we have previously demonstrated that sex alters the impact of several immune cell populations in ALS, including

NK cells (12, 13). Moreover, depletion of NK cells also impacts male and female mice differently, both in terms of survival and neuroinflammation (13); therefore, tofacitinib studies should likewise account for sex differences given the reduction in peripheral NK cell levels following tofacitinib treatment. Altogether, *in vivo* tofacitinib studies in ALS mice should examine a myriad of mechanisms and factors including peripheral and CNS immune cell populations, peripheral and CNS gene expression, motor neuron survival, and NMJ integrity. Studies will need to examine drug dosing, drug timing, and will need to account for the impact of sex.

The current study does have several limitations. First, primary NK cells are more difficult to culture than NK-92 cells and were therefore not co-cultured with iNeurons. iNeurons are also not motor neurons, thus, it is unclear to what degree ALS iNeurons recapitulate true motor neurons *in vivo*. Moreover, in mice, we only examined the effect of tofacitinib on NK cell numbers rather than on NK cell function. Many of the *in vitro* assays require large cell numbers, and tofacitinib treatment reduced overall NK cell levels, making these analyses *in vivo* difficult. Finally, while we have previously shown that both age and sex alter the activity of immune cells during ALS, including NK cells (12, 13), the present study did not explore the impact of these factors on tofacitinib suppression of NK cells. Nonetheless, our results conclusively show that tofacitinib suppresses NK cell function *in vitro*, suppresses NK cell levels *in vivo*, and can be administered orally in chow for use in preclinical ALS mouse models. These findings also indicate tofacitinib may be used to treat long-term diseases mediated by NK cell function, such as ALS.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Michigan Medical School Institutional Review Board. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by University of Michigan Institutional Animal Care & Use Committee.

AUTHOR CONTRIBUTIONS

CF-R, AM, BM, and EF designed the overall study. CF-R, AM, BM, JF, IW-D, CEP, ST, and CP performed *in vitro* studies using NK-92 cells. CF-R, BM, IW-D, ST, and CP performed iNeuron co-culture assays. CF-R, BM, JF, IW-D, CEP, ST, and CP performed *ex vivo* studies using primary NK cells. BM, SG, and EF wrote the IRB protocol. SG and EF recruited human participants for the study. JF and CEP worked with the pharmacokinetic core for the mouse pharmacokinetic studies. BM, JF, and CEP performed the mouse immune studies. CF-R, AM, and BM analyzed the data. CF-R, AM, BM, JF, IW-D, CEP, ST, CP, SG, and EF wrote the manuscript with substantial input from all authors. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: BM, SG, and EF are listed as inventors on a patent, Issue number US10660895, held by University of Michigan titled "Methods for Treating Amyotrophic Lateral Sclerosis" that targets immune pathways for use in ALS therapeutics.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL ARTICLE

Associations of self-reported occupational exposures and settings to ALS: a case–control study

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Abstract

Background Environmental exposures contribute to the pathogenesis of amyotrophic lateral sclerosis (ALS), a fatal and progressive neurological disease. Identification of these exposures is important for targeted screening and risk factor modification.

Objective To identify occupational exposures that are associated with a higher risk of ALS using both survey and standard occupational classification (SOC) coding procedures, and to highlight how exposure surveys can complement SOC coding.

Methods ALS participants and neurologically healthy controls recruited in Michigan completed a detailed exposure assessment on their four most recent and longest held occupations. Exposure scores were generated from the exposure survey, and occupations were assigned to SOC codes by experienced exposure scientists.

Results This study included 381 ALS and 272 control participants. ALS participants reported higher duration-adjusted occupational exposure to particulate matter (OR = 1.45, 95% CI 1.19–1.78, $p < 0.001$), volatile organic compounds (OR = 1.22, 95% CI 1.02–1.45, $p = 0.029$), metals (OR = 1.48, 95% CI 1.21–1.82, $p < 0.001$), and combustion and diesel exhaust pollutants (OR = 1.20, 95% CI 1.01–1.43, $p = 0.041$) prior to ALS diagnosis, when adjusted for sex, age, and military service compared to controls. In multivariable models, only occupational exposure to metals remained significant risk (OR = 1.56, 95% CI 1.11–2.20, $p = 0.011$), although in an adaptive elastic net model, particulate matter (OR = 1.203), pesticides (OR = 1.015), and metals (1.334) were all selected as risk factors. Work in SOC code "Production Occupations" was associated with a higher ALS risk. SOC codes "Building and Grounds Cleaning and Maintenance Occupations", "Construction and Extraction Occupations", "Installation, Maintenance, and Repair Occupations", and "Production Occupations" were all associated with a higher exposure to metals as determined using survey data.

Discussion Occupational exposure to particulate matter, volatile organic compounds, metals, pesticides, and combustion and diesel exhaust and employment in "Production Occupations" was associated with an increased ALS risk in this cohort.

Keywords Amyotrophic lateral sclerosis · Occupation · Risk factors · Exposome · Metals

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that results in degeneration of the motor neuron cells located in the brain, brainstem, and spinal cord causing painless progressive weakness involving cranial and limb muscles along with respiratory failure (Goutman 2017). This relentless progression leads to death within 2–4 years from symptom onset for most individuals that develop this disease. In addition to the motor involvement, up to half of patients with ALS will manifest cognitive changes. The major pathologic hallmark of ALS is aggregation of transactive response (TAR)

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DNA-binding protein 43 (TDP-43) in multiple brain areas. Approximately 85% of ALS is considered sporadic with no one single mutation underlying the disorder. Among the remaining 15% of familial cases, the most common genetic form is secondary to a hexanucleotide expansion in chromosome 9 open reading frame 72 (*C9orf72*). While the full picture of what causes ALS is incomplete, a combination of genetic and environmental factors is strongly implicated as underlying disease risk and progression (Goutman 2017).

The fact that ALS exhibits incomplete heritability and follows a multistep model of disease adds credence to the hypothesis that environmental exposures contribute to disease (Al-Chalabi and Hardiman 2013; Al-Chalabi et al. 2014). A recent meta-analysis identified exposures to lead, heavy metals, pesticides, agricultural chemicals, solvents, and electric shock as strong ALS risk factors (Wang et al. 2017). Other risk factors are also linked to ALS including smoking, military service, and physical activity (Al-Chalabi and Hardiman 2013). Our group has shown that self-reported residential pesticide exposure and concentrations of persistent organic pollutants in blood are associated with a higher odds of having ALS (Su et al. 2016; Yu et al. 2014). Further, we have shown that higher concentrations of these persistent organic pollutants in blood are associated with a faster disease progression. (Goutman et al. 2019)

Identifying the specific exposures that contribute to ALS risk is a critical step toward better understanding disease pathogenesis and developing mechanism-based therapies. This knowledge will point to specific exposures that should be avoided to decrease ALS risk and prevent disease (Goutman and Feldman 2020). The occupational setting is an important exposure environment, and occupational exposures to metals including lead, pesticides, silica, asbestos, organic dust, contact with animals or fresh animal products, endotoxins, polycyclic aromatic hydrocarbons, and diesel motor exhaust have all been associated with an increased ALS risk (Visser et al. 2019; Malek et al. 2014; Dickerson et al. 2019). Specific occupational sectors associated with an increased ALS risk include mechanics, manufacturing, mechanical, military, painting, precision metal, and/or construction industries (Andrew et al. 2020, 2017; Fang et al. 2009). Additional investigations are needed to elucidate which occupations have a high ALS risk and the job- and task-specific exposures that increase this risk.

The overall goal of this work is to identify occupational exposures that contribute to the risk of developing ALS. We also show how survey data can be used to develop occupational exposure scores for use in ALS disease risk models and demonstrate how these scores complement traditional job codes by providing greater specificity and personal-level details that may affect exposure and risks.

Methods

Participants

All patients with an El Escorial diagnosis of ALS seen at the University of Michigan (UM) Pranger ALS Clinic were asked to participate as ALS participants. Controls were identified for this study using an online recruitment database hosted by the Michigan Institute for Clinical & Health Research, which allows University of Michigan research teams to contact individuals that express interest in research participation. Interested controls were selected if they met inclusion criteria and fit the demographic ranges of ALS participants. Controls were excluded if they had a neurodegenerative condition or had a first- or second-degree blood relative with ALS. All participants were older than 18 years and provided verbal and written consent in English, and controls received $50 compensation for study participation and donated blood and urine samples at enrollment. The study received Institutional Review Board approval (HUM28826). Details of this study are previously published. (Su et al. 2016; Yu et al. 2014; Goutman et al. 2019).

Survey administration and follow-up

Following consent, participants were provided a written questionnaire and completion instructions. In the event a questionnaire was not returned, follow-up phone calls were placed to the participants to encourage completion; for ALS participants, survey completion was also encouraged at follow-up clinic visits. In circumstances where a response was incomplete or illegible, follow-up phone calls were placed to the participants. In some deceased ALS participants or those with severe dysarthria, next of kin were able to provide clarification to responses.

Survey description and exposure scores derivation

The survey was constructed from instruments available from the Agency for Toxic Substances and Disease Registry (ATSDR) (ATSDR 2000) and input from experts trained in exposure science. The questionnaire queried exposures at four jobs: the most recent; the job before the most recent; and the next two longest held jobs. For ALS participants, jobs that began after onset of symptoms were excluded, as were jobs for ALS participants without an onset date. Each respondent was also asked to provide a complete job history including job title, description, and years worked.

The questionnaire data provided insight into the 9 *exposure types*: particulate matter (PM), volatile organic compounds (VOCs), pesticides, metals, biologicals, combustion/

diesel exhaust, electromagnetic radiation, radiation, and corrosives (Table S1). Within each exposure type, we developed multiple *exposure factors* to assess the potential for exposure from specific sources or activities. For example, PM exposure may be contributed by occupational tasks, such as welding, handling dust-generating materials (e.g., talc, powders, fibers), and being exposed to diesel exhaust. Further, chronic exposure will be increased with jobs held for long periods.

The exposure factors were quantified using the survey data, typically using responses to combination of questions (Table S1). For example, occupational PM exposure utilized two factors (likelihood of general PM exposure, and exposures to specific PM sources), and a total of 18 survey questions. This analysis was repeated for each qualifying job reported by each participant. An *occupational exposure score* was obtained for each exposure type and job by summing the exposure factor scores weighted by our assessment of the contribution of the factor to the overall exposure; these were subsequently normalized (0 indicated no exposure potential; 1 indicated the highest possible exposure potential) to facilitate comparisons among exposure types. Finally, the *duration-adjusted occupational exposure score* used the occupational exposure score multiplied by the duration of the job, based on the individual's job history (job start and end dates). Thus, the *occupational exposure score* represents whether the participant was ever exposed on a particular job, whereas the duration-adjusted occupational *exposure score* accounts for the duration of exposure on up to four jobs.

Assignment and review of job codes

Job titles and descriptions for all occupations ($N = 2169$, prior to removing excluded jobs, such as those occurring after symptom onset or consent) were processed using two automated job coding platforms: Standardized Occupation Coding for Computer-assisted Epidemiological Research (SOCcer (Russ et al. 2016), available via National Cancer Institute, https://soccer.nci.nih.gov/soccer/); and The National Institute for Occupational Safety and Health (NIOSH) Industry and Occupation Computerized Coding System (NIOCCS; available from https://www.cdc.gov/niosh/topics/coding/code.html). For SOCcer, model version 2.0 was selected and the input file included job titles and job tasks. The system returned 10 SOC codes per job and the fit score for each. For NIOCCS, the 2010 coding scheme was selected, and the input file included Industry Title, Occupation Title, and Job Duties. Both packages were accessed in April 2020. Because both platforms provided the SOC (Standard Occupational Classification), we elected to use SOC as the coding mechanism. The SOCcer and NIOCCS output files were then merged, resulting in multiple SOCs for each of the participants' jobs. Because SOCs assigned by automated platforms can involve considerable uncertainty, particularly for exposure assessment purposes, we subjected results to a series of validation steps, as discussed below. These steps were performed blinded to ALS or control participant status.

The assigned job codes/titles were first prioritized for manual review using a priority score of 0, 1, 2 or 3, representing "indeterminate," "low," "medium," or "high" priority for review, respectively. Indeterminate was assigned (22 jobs) if the self-described job title and description were judged insufficient to utilize the SOCcer or NIOCCS procedure (although these still provided a result in many circumstances), e.g., titles of "CEO," "owner," "division controller," "management," or "engineering equipment officer" without additional job descriptors were judged insufficient to allow classification. Low priority was assigned if SOC codes were consistent with high fit, or if there was little potential for significant occupational exposure based on the job title, job description and SOC codes. Second, participants with job titles that suggested low priority but with descriptors that provided supporting information received an additional point (no score exceeded 3), e.g., a job title of secretary (ordinarily receiving a score of 1) for an individual who worked in a military facility was assigned a score of 2. This review was facilitated by sorting by the initially assigned SOCcer codes. Third, we identified jobs for manual review by considering those jobs with priority scores of 2 or 3 if the SOCcer and NIOCCS algorithm outcomes did not agree ($n = 382$), and all jobs with priority scores of 2 or 3 if the SOC fit score was low (< 0.3; $n = 298$). For these jobs, we examined the SOC assignment, and the respondent-provided job titles and descriptors. In some circumstances, we overrode the SOC assignment, drawing first from the top 100 SOC codes assigned, but utilizing additional SOC codes if none of the top 100 were appropriate.

To maintain independence from the survey-derived exposure scores detailed in the previous section, the validation steps did not use survey information. An exposure scientist (C.G.) provided both the initial prioritization and the manual review, which was then checked by a second exposure scientist (S.A.B.), and in a few circumstances, the assignment was revised. After this review, we estimated the misclassification rate using a randomly selected subset (excluding participant jobs that were manually revised and indeterminate jobs). In this subset of jobs ($n = 117$), 8.6% ($n = 10$) were judged to be incorrect. However, all of these jobs occurred in the low priority set. While our analysis is limited in sample size and other regards, it suggests that while the automated coding procedures have a non-negligible misclassification rate, most mistakes occur in occupations with relatively little potential for occupational exposure.

Data management

All survey data were entered into Redcap by research staff. A number of quality assurance techniques were employed: (1) Redcap logic and data validation tools were utilized; (2) in a random sample of surveys, data were double entered and reviewed; and (3) audits of data were performed to ensure logical responses. Participant were contacted if appropriate for any follow-up questions.

Statistical analysis

Demographic characteristics of study participants were collected and responses for occupational exposure scores were tabulated by ALS and control status and by demographic characteristics (age, sex, education) for all participants. Continuous data were summarized by mean, standard deviation, median, and interquartile range, and categorical data by counts and percentages. Differences between groups were determined via *t*-test for continuous variables and chi-square tests for categorical variables. For eight subjects with missing military service, single imputation with the mode was used.

Occupational exposure score analysis

Descriptive statistics and missingness for each occupational exposure score were summarized by ALS-control status. Differences between ALS participants and controls were evaluated using permutation tests. Occupational exposure scores were regressed one-at-a-time against ALS/control status adjusted for age (quartiles), sex, and military service to identify associations between exposure and ALS risk. Nonlinearity in the one-at-time associations between exposure scores and ALS/control status was assessed using generalized additive models adjusted for age (quartiles), sex, and military service. We also considered both an unpenalized and adaptive elastic net penalized multivariable logistic regression model including all nine occupational exposure scores adjusted for age (quartiles), sex, and military service to account for moderately high correlations among several exposures. All analyses were performed for both the occupational exposure score and duration-adjusted occupational exposure scores. We present the duration-adjusted occupational exposure scores in the main text given that they account for the duration of exposure and provide the non-duration-adjusted scores in the supplement.

SOC code analysis

The number of unique ALS participants and controls, and the corresponding job-years worked, was tabulated within each two-digit SOC code. One sample test of proportions was performed for an enrichment of ALS participants relative to the overall distribution of ALS and control participants in the study population. For each two-digit SOC code, unadjusted and adjusted logistic regression models were used to associate job-years worked with ALS/control status. We then fitted an adjusted logistic regression model with adaptive lasso penalization including all two-digit SOC codes simultaneously to select the two-digit SOC codes associated with ALS risk. From there, job-years corresponding to the selected two-digit SOC codes associated with a higher odds of being an ALS participant were then subdivided into job-years worked 10 years prior to symptom onset (ALS participants) or survey consent (controls), 10–20 years prior to symptom onset/survey consent, and more than 20 years prior to symptom onset/survey consent, and subsequently inputted into adjusted logistic regression models to potentially identify important windows of exposure. Models were adjusted for age (quartiles), sex, and military service. The selected two-digit SOC codes were subdivided into six-digit SOC codes to see if particular types of jobs within the two-digit SOC code were driving the association.

Joint analysis with exposure scores and SOC codes

Aggregated six-digit SOC codes were clustered based on the average exposure for the nine occupational exposure scores using the Euclidean distance as the distance metric to identify patterns of exposure by more granular occupational categorizations. Descriptive statistics for selected occupational exposure scores were tabulated for each two-digit SOC code. Associations between years worked in each two-digit SOC code and the selected occupational exposure scores were estimated using linear regression models and additive models, adjusted for age (quartiles), sex, and military service. The two-digit SOC codes associated with ALS/control status were subdivided into six-digit aggregated SOC codes, and then fit using linear regression models associating job-years worked within the six-digit SOC code and selected occupational exposure scores adjusted for age (quartiles), sex, and military service.

Analyses were performed for both occupational exposure score and duration-adjusted occupational exposure scores. Exposure scores were calculated using Excel and subsequent statistical analyses were performed in R.

Results

Participants

Between June 30, 2010 and February 12, 2020, completed surveys were received by 653 individuals: 381 from ALS and 272 from control participants (Table 1). This

Table 1 Participant Demographics

Covariate	Overall ($N=653$)	ALS ($N=381$)	Controls ($N=272$)	P value
Age at survey consent (years)	62.5 (55.1–69.2)	63.0 (55.5–70.0)	61.2 (54.5–68.3)	0.025
Sex				0.059
Female	316 (48.4)	172 (45.1)	144 (52.9)	
Male	337 (51.6)	209 (54.9)	128 (47.1)	
Military Service				0.066
Neither	556 (85.1)	320 (84.0)	236 (86.8)	
Enlisted	89 (13.6)	61 (16.0)	28 (10.3)	
Missing	8 (1.2)	0 (0.0)	8 (2.9)	
Education				< 0.001
High School or less	129 (19.8)	106 (27.8)	23 (8.5)	
Some Postsecondary	200 (30.6)	123 (32.3)	77 (28.3)	
Bachelor's Degree	168 (25.7)	87 (22.8)	81 (29.8)	
Graduate Degree	149 (22.8)	61 (16.0)	88 (32.4)	
Missing	7 (1.1)	4 (1.1)	3 (1.1)	
El Escorial Criteria				
Suspected		12 (3.2)		
Possible		42 (11.0)		
Probable, Lab Supported		105 (27.6)		
Probable		127 (33.3)		
Definite		95 (24.9)		
Onset Segment				
Bulbar		110 (28.9)		
Cervical		130 (34.1)		
Lumbar		139 (36.5)		
General		2 (0.5)		
Family History of ALS				
No		333 (87.4)		
Yes		33 (8.7)		
Unknown		12 (3.1)		
Missing		3 (0.8)		
Time Between Symptom Onset and Diagnosis (years)*		1.04 (0.66–1.77)		

Table of descriptive statistics for the study population. For continuous variables, Median (25th–75th percentile), and for categorical variables, N (%). P values for continuous and categorical variables correspond to analysis of variance tests and chi-squared tests, respectively

*Median and Interquartile Range calculated for 380 ALS participants, with one ALS case having a missing diagnosis date

represents a 55% survey participation rate for the ALS group. Participation numbers by age category are shown in Table S2. ALS participants were slightly older than controls, 63.0 versus 61.2 years ($p=0.025$) and had a smaller percentage of females, 45.1% versus 52.9% ($p=0.059$). Educational attainment was statistically different as well with ALS participants having a larger percentage of high school or less education, 27.8% versus 8.5% ($p<0.001$). ALS participants reflected a typical population with 18.4% meeting a definite El Escorial Diagnosis, 28.9% with bulbar onset, and 1.04 years median time from symptom onset to diagnosis.

Occupational exposure scores

Participants provided self-reported occupational exposure histories for up to 4 jobs, the most recent (but before symptom onset for ALS), the one before the most recent, and the other 2 longest held jobs; a total of 1,867 unique jobs were reported after excluding jobs occurring after symptom onset (or after consent for controls). For jobs meeting the above criteria, ALS cases provided an average of 2.63 jobs and controls had 3.18 jobs. ALS participants had an average work duration of 31.0 years and control participants 28.6 years.

The median occupational exposure score and duration-adjusted occupational exposure score for all exposure types were 0, meaning that the questionnaire responses did not indicate PM, VOC or other exposures for most jobs and participants i.e., only a subset had occupational exposure based on survey responses (Tables 2, S3, Fig. 1). Tables S3 and 2 and Fig. 1 highlight the upper percentile of exposure scores between ALS and controls participants and shows significant differences in mean occupational exposure and duration-adjusted occupational exposure scores for ALS versus control participants for PM, VOCs, pesticides, metals, and combustion/diesel exhaust. For the occupational exposure scores and duration-adjusted occupational exposure scores, Spearman's correlations were highest for PM and metals ($R=0.73$), PM and combustion ($R=0.55$), PM and VOCs ($R=0.53$), and VOCs and metals ($R=0.60$) (data not shown).

Differences in mean occupational exposure and duration-adjusted occupational exposure scores were seen when stratifying the population by sex (Tables S4 and S4a) and by education (Tables S5 and S5a). Overall, men and those with a high school or lower education are more likely to report occupational exposures.

ALS status and occupational exposure

Differences in duration-adjusted exposure scores by ALS/control status were adjusted for age, sex, and military service using univariate logistic regression models to show how a one standard deviation increase in the occupational exposure score changes the odds of having ALS (Table 3). For the duration-adjusted occupational exposure scores, after adjusting for age, sex, and military service, PM (OR = 1.45, 95% CI 1.19–1.78, $p<0.001$), VOCs (OR = 1.22, 1.02–1.45, $p=0.029$), metals (OR = 1.48, 95% CI 1.21–1.82, $p<0.001$), and combustion/diesel exhaust (OR = 1.20, 95% CI 1.01–1.43, $p=0.041$) were all associated with increased ALS risk. In the multivariable logistic regression model, only occupational exposure to metals was significantly associated with ALS risk (OR = 1.56, 95% CI 1.11–2.20, $p=0.011$), while unexpectedly, occupational exposure for corrosives was associated with a decreased risk (OR = 0.77, 95% CI 0.62–0.96, $p=0.021$) (Table 3). Due to the correlated self-reported exposure scores, we utilized an adaptive elastic net model to account for mixtures, which selected PM (OR = 1.203), pesticides (OR = 1.015), metals (OR = 1.334), and corrosives (OR = 0.864). Overall, PM, VOCs, metals, and combustion/diesel exhaust were consistently identified as risk factors for ALS. As a sensitivity check, the model was rerun with age as a continuous variable and there were no significant differences compared to the model with age represented as quartiles. Similar models for the occupational exposure scores are presented in Table S6.

Metals subcomponent scores

As metal exposures were the strongest risk factor across all models, we next examined the factors that determined these scores for both ALS and control participants. Figure 2 shows the overlap of individual metal subcomponent scores. The largest self-reported metal exposure, either alone or in a mixture, was to welding ($n=170$), followed by lead ($n=125$). Interestingly, 66 participants reported an isolated exposure to welding without other concurrent metals. We also evaluated the years worked for each job by each individual metal subcomponent score. For our cohort, this represented the period from 1950 to the present. Jobs with metal exposures spanned the full

Table 2 Duration-adjusted occupational Exposure Scores

Exposure	Duration-adjusted occupational exposure score												*P*-value
	ALS ($N=381$)						Control ($N=272$)						
	N	Mean	SD	Q75	Q90	Q95	*N*	Mean	SD	Q75	Q90	Q95	
Particulate Matter (PM)	381	0.15	0.25	0.19	0.50	0.77	272	0.08	0.17	0.07	0.30	0.47	0.00
Volatile Organic Compounds (VOCs)	381	0.15	0.24	0.25	0.44	0.76	272	0.11	0.20	0.14	0.37	0.53	0.01
Pesticides	373	0.11	0.28	0.00	0.61	0.96	269	0.06	0.21	0.00	0.10	0.57	0.04
Metals	381	0.13	0.23	0.21	0.45	0.66	272	0.06	0.15	0.00	0.25	0.39	0.00
Biological Exposures	376	0.02	0.11	0.00	0.00	0.00	270	0.01	0.10	0.00	0.00	0.00	0.83
Combustion and Diesel Exhaust	379	0.14	0.33	0.00	1.00	1.00	270	0.08	0.26	0.00	0.11	1.00	0.02
Electromagnetic Exposure	381	0.10	0.27	0.00	0.48	0.93	272	0.07	0.22	0.00	0.00	0.80	0.12
Radiation	381	0.07	0.24	0.00	0.11	0.83	272	0.06	0.21	0.00	0.05	0.55	0.42
Corrosives	378	0.07	0.21	0.00	0.33	0.60	271	0.07	0.19	0.00	0.31	0.58	0.86

For all scores, minimum value is 0 and maximum is 1. Median for all scores is 0

N number, *SD* standard deviation, *Q* quartile

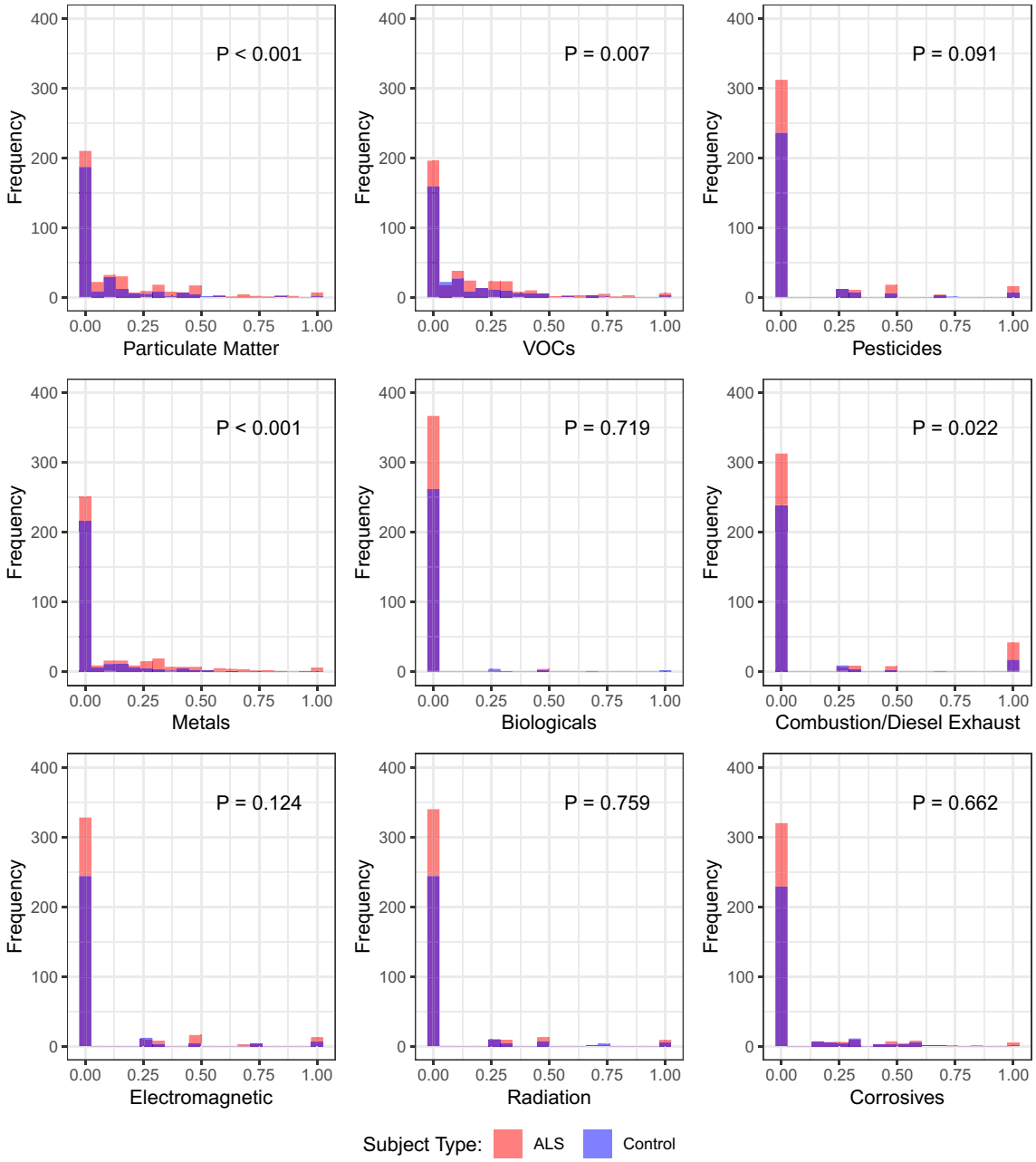

Fig. 1 Occupational Exposure Score Histograms by ALS and Control. Overlapping histograms showing the distribution of occupational exposure scores for ALS (red) and control (blue) participants. Differences in occupational exposure scores between ALS and control participants were evaluated using permutation tests. *VOCs* volatile organic compounds; *P* p value

time period (1950–present), with the exception of arsenic where exposure was mostly reported from about 1970 to 2010 (Fig. S1). Lastly, we looked at each individual metal exposure on ALS risk and found that exposure to both iron (OR = 2.25, $p = 0.006$) and welding fumes (OR = 1.97, $p = 0.003$) were significant (Table S7).

SOC codes

SOC codes are commonly used to assign individuals to occupations and exposure categories (Buckner-Petty et al. 2019). Of the universe of 465 SOC job codes, 374 were assigned to the 1867 occupations in our cohort; these were

Table 3 ALS and control logistic regression models

Exposure score	Duration-adjusted occupational exposure scores						
	Univariate model			Multivariable model			AEN
	OR	95% CI	*P*-value	OR	95% CI	*P*-value	OR
Particulate matter (PM)	1.45	1.19–1.78	< 0.001	1.23	0.88–1.71	0.224	1.203
Volatile organic compounds (VOCs)	1.22	1.02–1.45	0.029	1.03	0.80–1.34	0.819	1.000
Pesticides	1.18	0.99–1.40	0.061	1.06	0.87–1.29	0.581	1.015
Metals	1.48	1.21–1.82	< 0.001	1.56	1.11–2.20	0.011	1.334
Biologicals	1.01	0.86–1.19	0.868	0.95	0.80–1.14	0.605	1.000
Combustion and diesel exhaust	1.20	1.01–1.43	0.041	1.02	0.82–1.27	0.851	1.000
Electromagnetic radiation	1.09	0.92–1.29	0.342	0.90	0.72–1.12	0.324	1.000
Radiation	1.07	0.91–1.26	0.391	0.97	0.81–1.17	0.757	1.000
Corrosives	1.01	0.86–1.19	0.910	0.77	0.62–0.96	0.021	0.864

Single exposure score logistic regression and multivariable logistic regression models where the outcome is ALS/control status, the variables of interest are the occupational duration-adjusted exposure scores, and the covariates are age, sex, and military service. The duration-adjusted occupational exposure scores are weighted by occupation duration

AEN adaptive elastic net, *OR* odds ratio, *CI* confidence interval

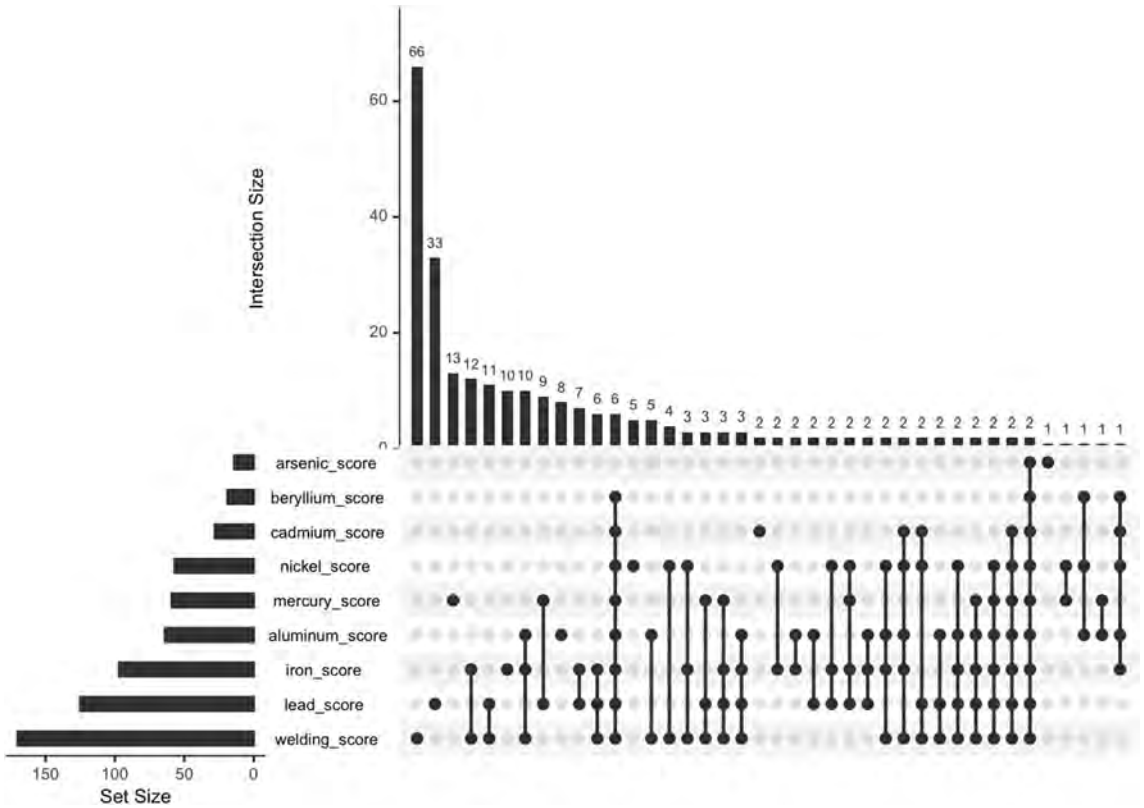

Fig. 2 Self-reported occupational exposure responses to metal subcomponents. Upset plot showing the intersection of the subcomponent questions that comprise the metal score, for each job, for ALS and control participants combined

further reduced to 75 aggregated codes (73 codes were represented in ALS cases and 72 codes in controls (Table S8). These were further aggregated to the first 2 digits (representing industry) due to small counts in some codes. The average job-years reported for the four self-reported jobs meeting the inclusion criteria for ALS participants was 31.1 and for controls was 28.6; the difference in years is consistent with the slight difference in age in ALS versus

Table 4 Job-Years and counts by ALS and control status

SOC Code	Occupational category	*N* (ALS)	Job-Years (ALS)	*N* (controls)	Job-Years (controls)	% Job-Years ALS	% ALS	*P*-value
51-0000	Production Occupations	78	1359.3	34	323.8	80.8	69.6	0.016
49-0000	Installation, Maintenance, and Repair Occupations	31	524.7	17	157.2	76.9	64.6	0.465
53-0000	Transportation and Material Moving Occupations	31	438.7	15	138.9	75.9	67.4	0.234
47-0000	Construction and Extraction Occupations	33	606.8	15	197.1	75.5	68.8	0.187
45-0000	Farming, Fishing, and Forestry Occupations	5	116.5	7	41.3	73.8	41.7	0.256
17-0000	Architecture and Engineering Occupations	40	709.4	24	339.0	67.7	62.5	0.529
41-0000	Sales and Related Occupations	64	849.9	55	458.2	65.0	53.8	0.353
35-0000	Food Preparation and Serving-Related Occupations	35	332.3	33	180.4	64.8	51.5	0.269
43-0000	Office and Administrative Support Occupations	108	1997.3	83	1088.6	64.7	56.5	0.608
37-0000	Building and Grounds Cleaning and Maintenance Occupations	27	294.4	20	172.0	63.1	57.4	0.884
25-0000	Education, Training, and Library Occupations	39	844.9	34	505.6	62.6	53.4	0.408
31-0000	Healthcare Support Occupations	18	232.6	15	144.5	61.7	54.5	0.725
39-0000	Personal Care and Service Occupations	16	252.1	14	174.1	59.1	53.3	0.584
21-0000	Community and Social Services Occupations	10	164.0	8	124.2	56.9	55.6	0.815
13-0000	Business and Financial Operations Occupations	32	535.6	32	439.7	54.9	50.0	0.205
23-0000	Legal Occupations	6	146.8	8	137.9	51.6	42.9	0.283
11-0000	Management Occupations	65	1158.0	78	1176.8	49.6	45.5	0.002
33-0000	Protective Service Occupations	8	120.0	11	151.5	44.2	42.1	0.167
27-0000	Arts, Design, Entertainment, Sports, and Media Occupations	15	231.8	21	297.7	43.8	41.7	0.061
29-0000	Healthcare Practitioners and Technical Occupations	27	566.7	39	781.2	42.0	40.9	0.006
55-0000	Military Occupations	8	30.1	8	55.6	35.1	50.0	0.614
15-0000	Computer and Mathematical Occupations	15	183.0	19	367.3	33.3	44.1	0.117
19-0000	Life, Physical, and Social Science Occupations	12	143.2	29	328.9	30.3	29.3	0.000

control participants. The total number of ALS and controls and the respective job-years is shown in Table 4. Occupations with the highest percentage of job-years in the ALS group were "Production Occupations" (51-0000, 80.8%), "Installation, Maintenance, and Repair Occupations" (49-0000, 76.9%), and "Transportation and Material Moving Occupations" (53-0000, 75.9%). Compared to the study population (58% ALS and 42% control participants), there were significant differences in job-years worked between ALS and controls for four SOC codes (% job-year ALS, p value): 51-0000 "Production Occupations" (80.8%, $p = 0.016$); 11-0000 "Management Occupations" (49.6%, $p = 0.002$); 29-0000 "Healthcare Practitioners and Technical Occupations" (42.0%, $p = 0.006$); and 19-0000 "Life, Physical, and Social Science Occupations" (30.3%, $p < 0.001$).

We next considered job-years worked in each two-digit SOC category on ALS risk (Table 5). In an unadjusted analysis, "Production Occupations" were associated with an increased ALS risk for every 5 years worked (OR = 1.25, 95% CI 1.09–1.42, $p = 0.001$), while three of the two-digit SOC categories were associated with a decreased ALS risk for every 5 years worked: "Computer and Mathematical Occupations" (OR = 0.81, 95% CI 0.67–0.98, $p = 0.029$), "Life, Physical, and Social Science Occupations" (OR = 0.72, 95% CI 0.56–0.92, $p = 0.010$), and "Healthcare Practitioners and Technical Occupations" (OR = 0.89, 0.80–0.99, $p = 0.032$). These effects remained statistically significant after adjusting for sex, age, and military service: ALS risk remained elevated for "Production Occupations" (OR = 1.22, 95% CI 1.07–1.40, $p = 0.003$), while risk was decreased for "Management

Table 5 Job-years worked with two-digit SOC codes associated with ALS Risk: single SOC code models

Two-Digit SOC Code	Description	Unadjusted				Adjusted			
		OR	95% CI	*P*-value	*P*-value (BH)	OR	95% CI	*P*-value	*P*-value (BH)
11-0000	Management Occupations	0.92	0.85–1.01	0.075	0.246	0.90	0.82–0.98	0.015	0.084
13-0000	Business and Financial Operations Occupations	0.97	0.86–1.10	0.664	0.770	0.95	0.83–1.08	0.408	0.587
15-0000	Computer and Mathematical Occupations	0.81	0.67–0.98	0.029	0.187	0.78	0.65–0.94	0.010	0.084
17-0000	Architecture and Engineering Occupations	1.09	0.95–1.25	0.209	0.474	1.07	0.93–1.23	0.373	0.572
19-0000	Life, Physical, and Social Science Occupations	0.72	0.56–0.92	0.010	0.111	0.73	0.57–0.94	0.014	0.084
21-0000	Community and Social Services Occupations	0.99	0.77–1.26	0.915	0.945	0.99	0.77–1.27	0.947	0.947
23-0000	Legal Occupations	0.95	0.77–1.18	0.670	0.770	0.94	0.76–1.17	0.590	0.646
25-0000	Education, Training, and Library Occupations	1.03	0.93–1.15	0.552	0.746	1.04	0.93–1.16	0.478	0.611
27-0000	Arts, Design, Entertainment, Sports, and Media Occupations	0.91	0.77–1.07	0.248	0.474	0.92	0.79–1.09	0.341	0.561
29-0000	Healthcare Practitioners and Technical Occupations	0.89	0.80–0.99	0.032	0.187	0.91	0.82–1.01	0.090	0.319
31-0000	Healthcare Support Occupations	1.04	0.81–1.33	0.758	0.830	1.11	0.86–1.42	0.434	0.587
33-0000	Protective Service Occupations	0.88	0.68–1.14	0.331	0.544	0.86	0.67–1.12	0.260	0.543
35-0000	Food Preparation and Serving-Related Occupations	1.10	0.86–1.41	0.437	0.670	1.14	0.88–1.47	0.318	0.561
37-0000	Building and Grounds Cleaning and Maintenance Occupations	1.06	0.85–1.32	0.623	0.770	1.07	0.85–1.34	0.552	0.635
39-0000	Personal Care and Service Occupations	1.01	0.83–1.23	0.945	0.945	1.03	0.85–1.26	0.747	0.781
41-0000	Sales and Related Occupations	1.08	0.94–1.23	0.267	0.474	1.07	0.94–1.23	0.296	0.561
43-0000	Office and Administrative Support Occupations	1.07	0.98–1.15	0.124	0.316	1.08	0.99–1.17	0.085	0.319
45-0000	Farming, Fishing, and Forestry Occupations	1.14	0.79–1.64	0.478	0.687	1.13	0.78–1.63	0.531	0.635
47-0000	Construction and Extraction Occupations	1.16	0.99–1.36	0.072	0.246	1.14	0.96–1.34	0.128	0.319
49-0000	Installation, Maintenance, and Repair Occupations	1.21	0.99–1.47	0.059	0.246	1.16	0.95–1.41	0.139	0.319
51-0000	Production Occupations	1.25	1.09–1.42	0.001	0.024	1.22	1.07–1.40	0.003	0.073
53-0000	Transportation and Material Moving Occupations	1.19	0.97–1.46	0.096	0.276	1.18	0.96–1.44	0.120	0.319
55-0000	Military Occupations	0.61	0.26–1.46	0.268	0.474	0.42	0.13–1.29	0.129	0.319

Single exposure score logistic regression models where the outcome is ALS/control status, the variables of interest are the number of job-years worked within two-digit SOC codes, and the covariates are age, sex, and military service. Interpretation of odds ratios (OR) correspond to 5 additional years worked within the respective SOC code

CI confidence interval, *BH* Bejamini–Hochberg

Occupations" (OR = 0.90, 0.82–0.98, $p = 0.015$), "Computer and Mathematical Occupations" (OR = 0.78, 95% CI 0.65–0.94, $p = 0.010$), and "Life, Physical, and Social Science Occupations" (OR-0.73, 95% CI 0.57–0.94, $p = 0.014$). After correction for multiple comparisons, "Production Occupations" remained significant in unadjusted models (p = 0.024) and was marginally significant in adjusted models ($p = 0.073$). As a sensitivity check, the model was rerun with age as a continuous variable and there were no significant differences compared to the model with age represented as quartiles.

Exposure scores and SOC codes

We explored the relationships of the occupational exposure scores to SOC codes via a dendrogram using the occupational exposure score and duration-adjusted occupational exposure scores (Fig. 3A, B). The occupational exposure score dendrogram showed a small cluster (bottom of plot) including "Trades, plumbing," "Trades, electrical and sheet metal," "Mechanic, vehicle," and "Operator, welding and metals." The duration-adjusted occupational exposure score dendrogram showed a small cluster (top of plot) containing

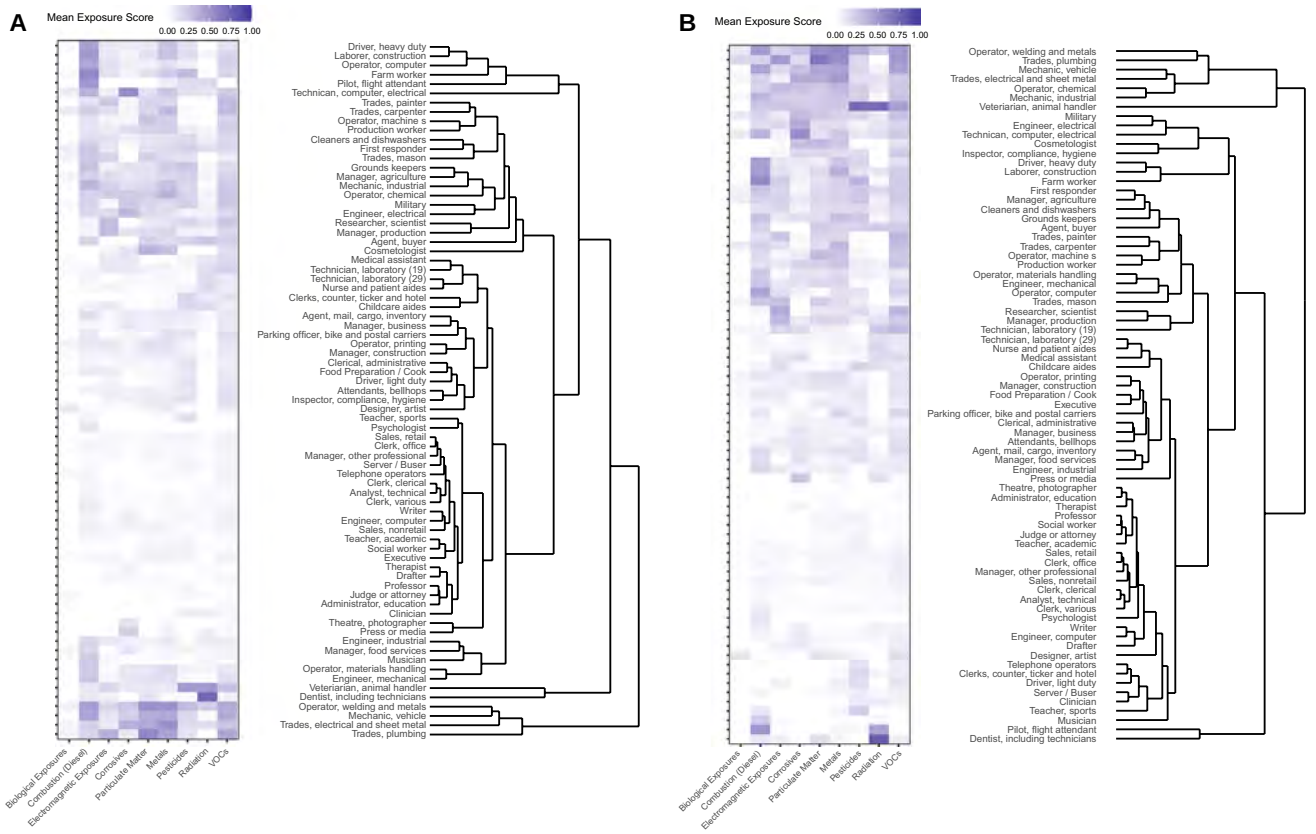

Fig. 3 Occupational exposure scores by aggregated SOC clusters. Dendrograms of the standard occupational classification (SOC) codes by **A** occupational exposure score and **B** duration-adjusted occupational exposure scores

"Veterinarian, animal handler," "Mechanic, industrial," "Operator, chemical," "Trades, electrical and sheet metal," "Mechanic, vehicle," "Trades, plumbing," and "Operator, welding and metals." These clusters were overall characterized by higher exposure scores. The lack of other clear smaller clusters highlights that SOC codes alone do not capture the full range of exposures that occur in the occupational setting, and that self-reported details (e.g., in the questionnaire) can be highly informative.

Because occupational exposure to metals was significantly associated with ALS risk in all models (Table 6), we next compared the metals occupational exposure scores by SOC code. The five occupations with the highest mean metals occupational exposure scores were: 49-0000 "Installation, Maintenance, and Repair Occupations;" 51-0000 "Production Occupations;" 55-0000 "Military Occupations;" 47-0000 "Construction and Extraction Occupations;" and 17-0000 "Architecture and Engineering Occupations." Several occupations with overall lower scores had a large range of exposure scores, e.g., 11-0000 "Management Occupations" had scores ranging from 0 to 1.00. This reflects the large range of tasks and activities that can fall into the two-digit SOC classifications, e.g., some managers work exclusively in offices with no exposure to metals, while managers in services, trades or production settings may experience relatively high exposure.

Linear models adjusted for age, sex, and military service were next developed to understand the association between duration of each SOC code and metals duration-adjusted occupational exposure. For every 5 years of work, the standard deviation changes in metals duration-adjusted occupational exposure scores were as follows: 13-0000 "Business and Financial Operations Occupations" (beta = −0.08, 95%CI −0.14- −0.02 $p = 0.006$); 15-0000 "Computer and Mathematical Occupations" ($\beta = -0.08, -0.16$ to -0.01, $p = 0.037$); 37-0000 "Building and Grounds Cleaning and Maintenance Occupations" ($\beta = 0.12$, 95% CI 0.02–0.22, $p = 0.020$); 41-0000 "Sales and Related Occupations" ($\beta = -0.09$, 95%CI −0.15- −0.04, $p = 0.002$); 47-0000 "Construction and Extraction Occupations" ($\beta = 0.14$, 95% CI 0.08–0.20, $p < 0.001$); 49-0000 "Installation, Maintenance, and Repair Occupations" ($\beta = 0.15$, 0.07–0.22, $p < 0.001$); and 51-0000 "Production Occupations" ($\beta = 0.18$, 0.14–0.23, $p < 0.001$) (Table 7). A similar analysis for the metal occupational exposure score is presented in Table S9. Unsurprisingly, these data indicate that occupational

Table 6 Metals occupational exposure scores associated with SOC codes

Two-Digit SOC	Description	*N*	Mean	SD	Min	Q25	Q50	Q75	Max
49-0000	Installation, Maintenance, and Repair Occupations	55	0.31	0.36	0	0	0.33	0.48	1.00
51-0000	Production Occupations	148	0.25	0.32	0	0	0.00	0.46	1.00
55-0000	Military Occupations	17	0.25	0.33	0	0	0.00	0.33	1.00
47-0000	Construction and Extraction Occupations	63	0.19	0.30	0	0	0.00	0.33	1.00
17-0000	Architecture and Engineering Occupations	100	0.15	0.28	0	0	0.00	0.18	1.00
33-0000	Protective Service Occupations	23	0.15	0.18	0	0	0.00	0.28	0.61
45-0000	Farming, Fishing, and Forestry Occupations	12	0.12	0.20	0	0	0.00	0.13	0.61
19-0000	Life, Physical, and Social Science Occupations	52	0.08	0.19	0	0	0.00	0.00	0.67
11-0000	Management Occupations	191	0.08	0.22	0	0	0.00	0.00	1.00
37-0000	Building and Grounds Cleaning and Maintenance Occupations	50	0.08	0.23	0	0	0.00	0.00	1.00
53-0000	Transportation and Material Moving Occupations	54	0.06	0.16	0	0	0.00	0.00	0.72
25-0000	Education, Training, and Library Occupations	107	0.05	0.17	0	0	0.00	0.00	0.78
31-0000	Healthcare Support Occupations	46	0.05	0.15	0	0	0.00	0.00	0.56
13-0000	Business and Financial Operations Occupations	86	0.04	0.15	0	0	0.00	0.00	1.00
21-0000	Community and Social Services Occupations	33	0.04	0.10	0	0	0.00	0.00	0.33
27-0000	Arts, Design, Entertainment, Sports, and Media Occupations	47	0.03	0.11	0	0	0.00	0.00	0.44
43-0000	Office and Administrative Support Occupations	303	0.02	0.11	0	0	0.00	0.00	1.00
29-0000	Healthcare Practitioners and Technical Occupations	116	0.02	0.09	0	0	0.00	0.00	0.61
35-0000	Food Preparation and Serving-Related Occupations	85	0.02	0.13	0	0	0.00	0.00	0.89
41-0000	Sales and Related Occupations	153	0.02	0.08	0	0	0.00	0.00	0.61
15-0000	Computer and Mathematical Occupations	50	0.01	0.06	0	0	0.00	0.00	0.33
23-0000	Legal Occupations	20	0.00	0.00	0	0	0.00	0.00	0.00
39-0000	Personal Care and Service Occupations	29	0.00	0.00	0	0	0.00	0.00	0.00

N number, *SD* standard deviation, *Min* minimum, *Q* quartile, *Max* maximum

exposure to metals is more likely to be reported in "Construction and Extraction," "Installation, Maintenance, and Repair," and "Production Occupations."

Discussion

Understanding non-genetic ALS risk factors is critically important to identify factors that increase disease risk, the underlying mechanisms, and potential preventative strategies. Our analysis of occupational exposures, based on a comprehensive survey and job classification coding, found that self-reported exposure to metals and a history of working in "Production Occupations" (SOC 51-0000)—which includes production workers, welders and metal, machine, printing, and chemical operators—increased ALS risk. ALS risk also increased with self-reported occupational exposures to particulate matter, volatile organic compounds, pesticides, metals, and combustion/diesel exhaust in univariate and adaptive elastic net models.

Our findings are consistent with other published reports, which demonstrate that survey-based tools (Morahan and Pamphlett 2006; Bonvicini et al. 2010) and structured interviews (Malek et al. 2014; McGuire et al. 1997) are informative for examining ALS environmental risk factors. Studies on occupational risk factors differ in terms of design, job and exposure ascertainment, and other factors, and results are not always consistent. Table 8 presents the key findings from several occupational exposure studies and their alignment with the current report. A large population-based study in the Netherlands, Ireland, Apulia, Lombardy, and Piedmont and Valle d'Aosta in Italy including 1157 ALS participants showed that occupational exposures to silica, asbestos, organic dust, contact with animals or fresh animal products, endotoxins, polycyclic aromatic hydrocarbons and diesel motor exhaust were all associated with an increased ALS risk (Visser et al. 2019). Our univariate analyses provided consistent findings for exposure to particulate matter (which includes exposures to silica) as well as combustion products and diesel exhaust. A study in Pennsylvania, United States showed that occupational exposures to metals and pesticides increased ALS risk (Malek et al. 2014). A study in Australia showed that men who worked with metals, chemicals/solvents, and herbicides/pesticides and women who reported a higher exposure to chemicals/solvents had a higher risk of ALS (Pamphlett 2012). Another Australian study showed that male technicians and trade workers, machinery operators and drivers, and laborers had an

Table 7 Metal duration-adjusted occupational exposure score association with SOC codes

Two-Digit SOC Code	Description	Metal duration-adjusted occupational exposure score			
		B	95% LCL	95% UCL	*P*-value
11-0000	Management Occupations	– 0.03	– 0.07	0.01	0.173
13-0000	Business and Financial Operations Occupations	– 0.08	– 0.14	– 0.02	0.006
15-0000	Computer and Mathematical Occupations	– 0.08	– 0.16	– 0.01	0.036
17-0000	Architecture and Engineering Occupations	0.01	– 0.05	0.07	0.687
19-0000	Life, Physical, and Social Science Occupations	0.01	– 0.08	0.11	0.780
21-0000	Community and Social Services Occupations	– 0.04	– 0.16	0.07	0.456
23-0000	Legal Occupations	– 0.07	– 0.17	0.04	0.207
25-0000	Education, Training, and Library Occupations	– 0.01	– 0.06	0.04	0.758
27-0000	Arts, Design, Entertainment, Sports, and Media Occupations	– 0.02	– 0.09	0.06	0.652
29-0000	Healthcare Practitioners and Technical Occupations	– 0.01	– 0.05	0.04	0.832
31-0000	Healthcare Support Occupations	0.02	– 0.10	0.13	0.761
33-0000	Protective Service Occupations	0.08	– 0.04	0.20	0.173
35-0000	Food Preparation and Serving-Related Occupations	– 0.04	– 0.15	0.07	0.443
37-0000	Building and Grounds Cleaning and Maintenance Occupations	0.12	0.02	0.22	0.020
39-0000	Personal Care and Service Occupations	– 0.04	– 0.13	0.05	0.391
41-0000	Sales and Related Occupations	– 0.09	– 0.15	– 0.04	0.002
43-0000	Office and Administrative Support Occupations	– 0.03	– 0.07	0.01	0.135
45-0000	Farming, Fishing, and Forestry Occupations	0.01	– 0.13	0.14	0.937
47-0000	Construction and Extraction Occupations	0.14	0.08	0.20	0.000
49-0000	Installation, Maintenance, and Repair Occupations	0.15	0.07	0.22	0.000
51-0000	Production Occupations	0.18	0.14	0.23	0.000
53-0000	Transportation and Material Moving Occupations	– 0.05	– 0.13	0.03	0.213
55-0000	Military Occupations	– 0.02	– 0.32	0.28	0.893

Single two-digit SOC logistic regression models and generalized additive models where the outcome is the metal duration-adjusted occupational exposure score, the variables of interest are the number of job-years worked within each two-digit SOC code, and the covariates are age, sex, and military service. Interpretation of coefficient is in in terms of 5 year increments corresponding to standard deviation changes in occupational metal score

LCL lower confidence limit, *UCL* upper confidence limit

increased risk of ALS and that truck driving as an occupation was associated with a higher ALS risk (Pamphlett and Rikard-Bell 2013).

The multivariable models showed that self-reported occupational metals exposure was most strongly linked to ALS risk. This could be related to the type of correlated exposures experienced in certain occupations where participants with higher metals occupational exposure scores also reported higher exposures to particulate matter, volatile organic compounds, and corrosives. This is unsurprising as workers are often exposed to mixtures, particularly in certain trades, production/manufacturing, and service industries (Mixed Exposures Research Agenda 2004). In some respects, this is similar to our findings for mixtures of persistent organic pollutants in our cohort (Goutman et al. 2019)—participants are exposed to polychlorinated biphenyls, brominated flame retardants, and organochlorine pesticides, for example, yet pesticides alone carry the highest risk. In this present study, metals may be the most critical component of a larger occupational exposure mixture. This should lead us to focus on the types of mixtures and resulting injuries to the central nervous system in future research focused on understanding ALS pathogenesis. This mixture effect led to the adaptive elastic net analysis that showed overall consistency with the univariate models, again with occupational metals exposure carrying the strongest association.

Among the individual metals/tasks, iron and welding fume exposure were the most significant; these exposures were also among the most common. Welding has been linked to ALS in prior studies (Gunnarsson et al. 1992; Strickland et al. 1996; Armon et al. 1991). Dickerson et al. did not find iron to be a risk factor in Denmark (Dickerson et al. 2020). Although we did not find a significant association between occupational lead exposure and ALS, this has been shown in other survey-based studies, e.g., Dickerson et al. used a job-exposure matrix in a Danish population

Table 8 Summary of ALS occupational case/control studies (by publication year)

References	Year	# Cases	# Controls	Population	Occupational exposure	Risk	Consistent with present study
Gresham et al. (1986)	1986	66	66	San Diego, California, USA	Heavy metals	↔	○
Armon et al. (1991)	1991	47	47	Rochester, Minnesota, USA	Work in welding in males	↑	●
Gunnarsson et al. (1992a)	1992	92	372	Sweden	Work in welding	↑	●
Chancellor et al. (1993)	1993	103	103	Scotland	Lead	↑	○
					Solvents	↑	●
					Pesticides	↔	◐
					Mineral	↔	–
Strickland et al. (1996)	1996	25	25	Minneapolis, Minnesota, USA	Exposure to welding	↑	●
McGuire et al. (1997)	1997	174	348	Western Washington, USA	Metals	↔	○
					Solvents, self-reported in women	↑	●
					Agricultural chemicals in men	↑	◐
Kamel et al. (2002)	2002	109	256	New England, USA	Lead	↑	○
Morahan and Pamphlett (2006)	2006	179	179	Australia	Metals	↔	○
					Solvents	↑	●
					Pesticides	↑	◐
					Work in construction	↑	○
					Work in mechanics and repairers	↑	○
Fang et al. (2009a)	2009	109	253	Northern New England, USA	Work in precision production	↑	●
					Work in farming, forestry, fishing	↔	●
					Work in management	↔	●
Bonvicini et al. (2010)	2010	41	82	Northern Italy	Pesticides	↑	◐
Pamphlett (2012)	2012	787	778	Australia	Metals in men	↑	●
					Solvents	↑	●
Kamel et al. (2012)	2012	41	84,698	USA	Pesticides	↑	◐
Pamphlett and Rikard-Bell (2013aa)	2013	611	775	Australia	Work in professional	↓	–
					Work in technicians and trade	↑	●
					Work in machinery operators and drivers	↑	●
					Work in laborer	↑	–
Malek et al. (2014)	2014	66	66		Metals	↑	●
					Pesticides	↑	◐

and found that occupational exposure led to increased ALS risk (Dickerson et al. 2019), and both Kamel et al. in a New England population (Kamel et al. 2002) and Chancellor et al. in a Scottish population (Chancellor et al. 1993) found that self-reported occupational exposure to lead increased ALS risk. Of note, other studies, like ours, report no association with occupational lead exposure (Gunnarsson et al. 1992; Gresham et al. 1986).

The questionnaire data included 66 jobs for welding where no other metal exposure was reported (Fig. 2). Since welding requires the use of metals (OSHA 2021), this may mean that participants were not aware of their exposure.

Table 8 (continued)

				Pittsburgh and Philadelphia, Pennsylvania, USA	Organic solvents	↔	○
					Aromatic solvents	↔	○
					Electrical/electronic equipment or machinery or electromagnetic fields	↔	●
					Work in precision tool manufacturing	↑	●
Peters et al. (2017a)	2017	5020	25,100	Sweden	Work in glass, pottery, and tile	↑	–
					Work in textile	↓	–
					Metals	↔	○
					Solvents	↑	●
					Lead	↑	○
Andrew et al. (2017)	2017	295	224	Vermont, USA	Mercury	↔	●
					Cooling/cutting lubricants	↔	–
					Pesticides	↑	◐
Dickerson et al. (2018)	2018	1639	151,975	Denmark	Diesel exhaust	↑ in males	●
Visser et al. (2019)	2019	1157	2922	Netherlands, Ireland, Apulia, Lombardy, and Piedmont and Valle d'Aosta in Italy (Euro-MOTOR cohort)	Silica	↑	–
					Asbestos	↔	–
					Organic dust	↑	–
					Contact with animals or fresh animal products	↔	●
					Endotoxins	↑	–
					Polycyclic aromatic hydrocarbons	↑	–
					Diesel motor exhaust	↑ at low levels	●
Dickerson et al. (2019)	2019	986	93,522	Denmark	Lead	↑ in males	○
Andrew et al. (2020)	2020	188	376	New Hampshire, Vermont, and Ohio, USA	Work in mechanics	↑	○
					Work in painting	↑	○
					Work in manufacturing	↔	○
					Work in construction	↑	○
					Work in agricultural	↑	○
Filippini et al. (2020)	2020	95	135	Northern and Southern Italy	Work in manufacturing	↔	○
					Work in welding	↔	○
					Metals	↑	●
					Pesticides	↔	◐
					Solvents	↔	○
					Electromagnetic fields	↔	–
Dickerson et al. (2020)	2020	1639	168,194	Denmark	Chromium	↔	–
					Iron	↔	–
					Nickel	↔	–

↑ increased risk, ↓ decreased risk, ↔ no difference in risk, *NE* not evaluated, ● risk consistent with current study, ◐ risk partially consistent with current study, ○ risk not consistent with current study, – not evaluated

[a]Due to the number of exposures analyzed in the referenced study, only outcomes highlighted in the present study are listed

Plotting metal exposure by years the on the job, we saw that essentially all metals (except for arsenic) continue to be reported to the present, suggesting that metal exposure continues to be an actionable risk factor. Our finding that welding and iron exposure are ALS risk factors may indicate that workers are exposed to other metals without their knowledge, that welding and iron exposure represent surrogates for some other exposure, or that mixtures of metals play a greater role on ALS risk compared to an individual metal, similar to what we have seen in our analysis of teeth (Figueroa-Romero et al. 2020).

Among the job codes, "Production Occupations" was most strongly associated with ALS risk. This is a diverse category that includes production workers, welders, and metals, machine, printing, and chemical operators. ALS risk is highly variable in this group, potentially reflecting the variability of occupational exposures to metals in this code. For example, in this job code, most participants had a zero metal exposure score, while a small group of participants accounted for the bulk of exposure. High metals occupational exposure scores also occurred for workers in other job codes, e.g., "Building and Grounds Cleaning and Maintenance Occupations," "Construction and Extraction Occupations," and "Installation, Maintenance, and Repair Occupations," demonstrating the value of complementing the job codes with personal-level exposure data. Our findings are consistent with other studies. Andrew et al. found that working in mechanics, painting, or construction increased ALS risk (Andrew et al. 2020), and, in a separate study, that working in construction, manufacturing, mechanical, military, or painting occupations increased ALS risk (Andrew et al. 2017). In parallel, Fang et al. also found that construction and precision metal workers were at an increased ALS risk (Fang et al. 2009).

We examined the job titles and tasks linked to the metals occupational exposure scores, and graded each exposure as probable, possible or unlikely based on this information. For example, lead exposure was reported by individuals who had worked in construction (e.g., builders, painters, pipefitters, electricians, plumbers, remodelers, handymen), boat restorers, maintenance workers, some automotive shop and factory workers, some workers in steel and metal industries (metallurgist, welders), and X-ray technicians. In these industries, lead exposure can occur from lead in paint, plumbing, solder, and other materials. However, self-reported lead exposure for a subset (17%) of workers did not appear concordant with reported job titles and tasks (e.g., some truckers and property managers), while a smaller subset (9%) did not report lead exposure although it may have occurred (some skilled trades). Workers reporting mercury exposure included dentists and dental staff, and some production, waste and engineering workers; this is reasonable given mercury in dental amalgam and some (older) electrical switches and other equipment. Again, a subset (20%) of self-reported mercury exposures seemed unlikely, for example, education and most metal workers. For cadmium exposure, a wide range of workers reported exposure, e.g., researchers, engineers, automotive workers, painters, and some production and metal workers. While cadmium has been used in metal plating, paints and coatings, the likelihood of this exposure is difficult to assess based on the survey data. For arsenic, self-reported exposure was reported by only six workers. Again, exposure is difficult to confirm; arsenic is present in some agricultural chemicals and pressure treated wood, but no farmers, construction, building or grounds workers reported this exposure. While any survey will have issues of accuracy, omissions, recall bias, etc., our results suggest a reasonable degree of consistency for the more common and recognized metal exposures (e.g., lead), but also the challenge for other metals. Arsenic exposure, for example, might be better handled by questions to construction and agricultural workers such as "did you "handle treated wood?," although this would increase the complexity and length of the survey.

Our findings add to a growing literature of potential occupational ALS risk factors. Importantly, it should be noted that all studies are not uniform. For example, a nested case–control study in Sweden did not show that occupational exposure to metal was an ALS risk factor (Peters et al. 2017). Further, a study of 1 million participants from a cancer prevention cohort study did not show an increased ALS risk among farmers, electricians, and welders (Weisskopf et al. 2005). Like the large ALS occupational risk assessment in the prospective Netherlands Cohort Study and the Western Washington study, we find that ALS participants have a lower educational attainment compared to controls (McGuire et al. 1997; Koeman et al. 2017). However, while we find occupational exposures to metals increase ALS risk, the Netherlands study did not. An important difference is that we used individual reporting as opposed to only assigning risk based on a job-exposure matrix. This is a strength for our study as reported exposures are not uniform across each job code.

Also of note, independent of case status, men and those with high school or lower secondary education report higher occupational exposures. These groups may require public health attention to lessen exposure risks. McGuire et al. (McGuire et al. 1997) found in their ALS case and control cohort that men had higher exposures to agricultural chemicals. Our results were partially consistent with pesticide exposure showing a small risk in the adaptive elastic net model, although those working in farming, fishing, and forestry did not have a significant association, which could be due to incomplete case capture, or changes in in occupational exposures since the publication of that study

in 1997. These findings should be interpreted cautiously as we have previously shown higher levels of organochlorine pesticides in ALS participants. We also did not find that occupational exposure to electromagnetic radiation increases ALS risk, which could be due to the low number of individuals reporting this exposure in our cohort, most of whom were health care workers. A recent meta-analysis suggests that electromagnetic radiation exposure slightly increases ALS risk (Jalilian et al. 2021), although reports included in the meta-analysis were mixed, indicating that the risk is not uniform across all studies.

In prior studies, including our own, pesticide exposure was identified as an ALS risk factor (Al-Chalabi and Hardiman 2013; Su et al. 2016; Kamel et al. 2012). Specifically, an exposure history to agricultural chemicals increased ALS risk for individuals in western Washington State (McGuire et al. 1997). Bonvicini and colleagues used a questionnaire to show that pesticide exposure increased ALS risk in a northern Italian population (Bonvicini et al. 2010). Morahan and colleagues used a questionnaire to show that ALS risk in Australia was associated with solvent/chemical exposure, herbicide/pesticide exposure, and industrial herbicide/pesticide exposure (Morahan and Pamphlett 2006). Although pesticide exposure is a small risk factor in the adaptive elastic net logistic regression model using the duration-adjusted occupational exposure scores, this finding is not present in other models. This is possibly because this exposure may largely be occurring outside the workplace, and because we captured relatively few participants with occupational pesticide exposure, such as individuals in the farming industry. Thus, further work exploring exposure across multiple settings (occupational and residential) is needed. Additionally, prospective cohorts of individuals that have higher exposures to pesticides would be beneficial.

We used a combined approach of self-reported occupational exposures and occupational histories, augmented by expert assessment, to identify ALS occupational risk factors. Occupational histories, self-reported exposure assessments, and expert assessment are the main strategies used in retrospective case–control studies examining occupational exposures (Ge et al. 2018; Teschke et al. 2002). All techniques are especially challenging when retrospectively identifying disease risk factors with a long latency period (Ge et al. 2018). Occupational histories—a listing of job titles and responsibilities—have several limitations impacting reliability, especially when the job title does not reflect the work performed (Teschke et al. 2002). Nonetheless, occupational histories can help identify certain at-risk occupations, which in turn can highlight mixtures of chemicals typically used in that occupation without zeroing in on a specific chemical or exposure (Teschke et al. 2002). Generic job-exposure matrices (JEMs) share limitations of occupational histories by not capturing a full range of exposures or homing in on a specific risk (Teschke et al. 2002). Self-reported exposures, subject to recall bias, can outperform JEMs as they provide individualized data on job activities. With self-reported exposures there is no gold standard for comparison, e.g., participants may not know the names of chemicals to which they were exposed (Teschke et al. 2002). The performance of these techniques can improve by both focusing on a specific set of exposures and complementing expert assessment with self-reported exposures, the study design with the highest accuracy (Ge et al. 2018; Teschke et al. 2002). Thus, this was our approach.

Outside of ALS, this study has other important findings. Automated systems that assign SOC codes to occupations are a useful tool, especially when a large number of occupations require classification. However, despite using two separate systems from NIH and CDC, additional input from exposure scientists was needed, consistent with other findings related to these auto-coding systems (Buckner-Petty et al. 2019). We also found that the SOC coding is insufficient to account for exposures, especially in certain occupations where the same job code can encompass very diverse occupational settings.

This study has several strengths. First, we captured a large number of participants in Michigan, a diverse state with an historical agricultural and industrial legacy. Second, the questionnaire obtained detailed self-reported information on exposures. We identified at-risk occupations via SOC coding and showed that complementing SOC codes with self-reported exposures is meaningful, thus addressing the variability of exposures across a job code. Hand curation by exposure scientists provided further refinement of the automated SOC coding. Overall, our approach combining expert assessment with self-reported exposures and targeted automated SOC codes was consistent with best practices identified in the literature (Ge et al. 2018).

This study also has limitations. Selection bias is possible as not all persons with ALS seen in our clinic enrolled in this study. The participation rate of 55% is consistent with other large ALS cohorts, including the National ALS Registry (completion rate of 43.6–49.2%) (Bryan et al. 2016). Further, the control population was based on altruism. While our control population was more highly educated compared to controls, this could represent a true difference, especially as polygenic factors associated with higher educational attainment are associated with a lower ALS risk (Bandres-Ciga et al. 2019). There is no gold standard for self-reported exposure assessment, and recall bias may influence results. As military service is a recognized ALS risk factor (Al-Chalabi and Hardiman 2013), we elected to adjust models for military service history, but did not include self-reported military exposures in the exposure scores. Also, while only a subset of individuals experienced work-related exposures to particulate matter (PM), volatile organic compounds (VOCs),

metals and other contaminants, this is expected and reflects the contemporary distribution of job types. These are still important findings as the identification of environmental ALS risk factors, even in small groups, helps identify modifiable factors that could be used to better understand how to prevent disease in certain population groups. We do not differentiate between full- and part-time employment and only considered job-years in the analysis. This should not result in exposure misclassification as exposure scores were based on participants' responses to questions relevant to exposures, e.g., do they work with specific chemicals? First, most of these questions were "yes/no", and quantification of exposure (like a dose or concentration) from such survey questions is not possible. Second, the survey questions were repeated for up to four different jobs, from which we calculated an overall (duration-adjusted) occupational exposure score. The survey responses suggest that most participants had had several jobs that tended to be similar, e.g., staying in the service or educational sector, thus likely diminishing the potential effect of a part-time versus full-time position. Third, in most cases, the job title, descriptors, and survey questions suggested that most individuals described full-time jobs, although we do not have direct evidence. Finally, we believe that differences in exposure contrast across jobs in the different sectors (e.g., as a mechanic, food preparation, or office worker) likely exceed the difference that might result due to whether an individual works 20, 30 or 40 h. It is important to note too that analyses that did not consider duration of each occupation showed similar results. Finally, we focused on occupational exposures, whereas non-occupational exposures do occur and may also contribute to ALS risk.

Conclusion

Self-reported occupational exposures to particulate matter, volatile organic compounds, metals, and combustion and diesel exhaust are identified as ALS risk factors. The greatest risks were self-reported occupational metals exposure among exposure types, and production occupations among job codes. Overall, these data provide important insights into the occupational exposures and settings that increase ALS risk. Further investigations are encouraged to understand the mechanisms that lead to this increase in risk. Additionally, these data may be informative for ALS prevention strategies designed to limit exposures, especially for people most at risk of developing ALS.

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Data availability Sharing of non-identifiable data will be considered at the reasonable request of a qualified investigator.

Declarations

Conflict of interest SAG sat on an advisory board for Biogen and IFT Pharma and serves on a DSMB. The other authors declare they have no competing interests.

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Association Studies Article

miRNA analysis reveals novel dysregulated pathways in amyotrophic lateral sclerosis

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease. Its complex pathogenesis and phenotypic heterogeneity hinder therapeutic development and early diagnosis. Altered RNA metabolism is a recurrent pathophysiologic theme, including distinct microRNA (miRNA) profiles in ALS tissues. We profiled miRNAs in accessible biosamples, including skin fibroblasts and whole blood and compared them in age- and sex-matched healthy controls versus ALS participants with and without repeat expansions to chromosome 9 open reading frame 72 (*C9orf72*; C9-ALS and nonC9-ALS), the most frequent ALS mutation. We identified unique and shared profiles of differential miRNA (DmiRNA) levels in each C9-ALS and nonC9-ALS tissues versus controls. Fibroblast DmiRNAs were validated by quantitative real-time PCR and their target mRNAs by 5-bromouridine and 5-bromouridine-chase sequencing. We also performed pathway analysis to infer biological meaning, revealing anticipated, tissue-specific pathways and pathways previously linked to ALS, as well as novel pathways that could inform future research directions. Overall, we report a comprehensive study of a miRNA profile dataset from C9-ALS and nonC9-ALS participants across two accessible biosamples, providing evidence of dysregulated miRNAs in ALS and possible targets of interest. Distinct miRNA patterns in accessible tissues may also be leveraged to distinguish ALS participants from healthy controls for earlier diagnosis. Future directions may look at potential correlations of miRNA profiles with clinical parameters.

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease affecting motor neurons in the spinal cord, brainstem and brain (1). Motor neuron loss results in skeletal muscle atrophy and weakness, ultimately leading to respiratory failure and death within 2–4 years of diagnosis (1). ALS is familial in ∼15% of patients and sporadic in the remaining 85%. Mutation to chromosome 9 open reading frame 72 (*C9orf72*) is the most common among the approximate 40 genes associated with ALS (2). Only two drugs, riluzole (3) and edaravone (4), are approved by the US Food and Drug Administration for treating ALS, but they only slow disease progression modestly. Clinical trial evidence suggests that treatment may be more effective if initiated earlier, including with edaravone, methylcobalamin and combined taurursodiol-sodium phenylbutyrate (4–6). However, this idea remains unproven, further emphasizing the critical need for novel ALS therapeutic targets as well as biomarkers to facilitate earlier diagnosis.

Unfortunately, the complex molecular mechanisms underlying ALS hinder these goals (2). Epigenetics and RNA processing are strong undercurrents in ALS pathogenesis since inclusion bodies of the protein TAR DNA-binding protein 43 (TDP-43) are an almost universal pathologic finding in ALS (2). TDP-43 binds DNA and RNA in cells, regulating transcriptional repression, pre-mRNA splicing, mRNA translation and microRNAs (miRNAs) biogenesis and processing (7,8). miRNAs are approximately 22 nucleotide-long non-coding RNAs that negatively regulate gene expression by destabilizing mRNA, which modulates numerous physiological processes (9–12). miRNAs are highly expressed in the nervous system (13,14) and may play a role in ALS pathogenesis through altered RNA and protein metabolism, neuromuscular junction structure and function, neurogenesis and inflammation (15). Additionally, miRNAs are dysregulated in ALS mouse models (16) and human tissues (17,18) and may reflect disease state and progression (19).

miRNA analysis in ALS can thus be leveraged for a dual purpose, providing both biological insight into disease mechanisms and serving as a diagnostic biomarker. The goal of the current investigation was to understand differences in miRNA levels across two tissues, skin fibroblasts and whole blood (WB), from *C9orf72* positive (C9-ALS) and negative (nonC9-ALS) participants against age- and sex-matched controls (Fig. 1). Pathway analysis yielded biological insights that were conserved or unique across

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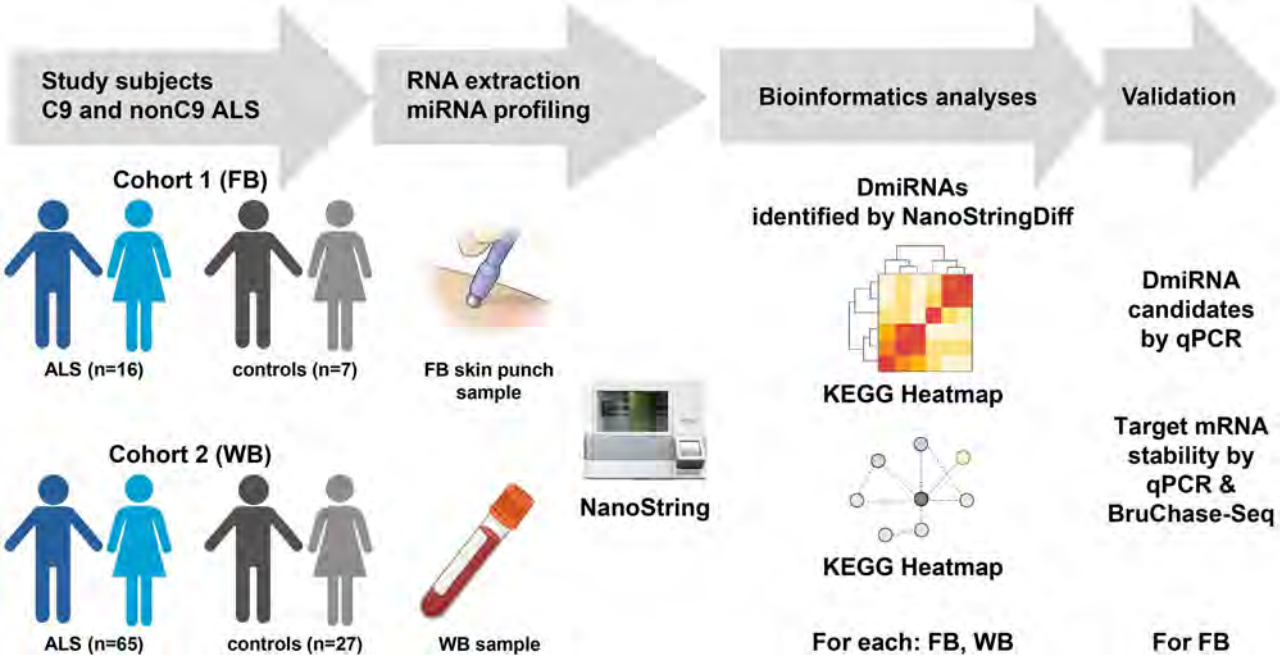

Figure 1. Study design. miRNA levels in fibroblasts (FB) and WB were evaluated from two cohorts of C9-ALS and nonC9-ALS participants versus control samples using NanoString. Differential miRNAs (DmiRNAs) were identified by NanoStringDiff and compared across tissue type and in C9-ALS and nonC9-ALS versus controls. DmiRNAs were validated by qPCR and their predicted target mRNAs by BruChase-Seq. Biological meaning from DmiRNAs was inferred by pathway analysis, and random forest was applied to leverage DmiRNAs as biomarkers. KEGG, Kyoto Encyclopedia of Genes and Genomes. Generated in part using BioRender.com.

tissue types and genetic backgrounds associated with ALS and provided information for future investigations.

Results

Cohort

Fibroblasts were obtained and cultured from C9-ALS ($n = 8$), nonC9-ALS ($n = 8$) and controls without any neurological disorders ($n = 7$). WB samples were collected from a larger cohort, including15 C9-ALS, 50 nonC9-ALS and 27 control participants. Twelve out of 23 fibroblast samples were from the same subjects included in the WB samples. Demographics and clinical characteristics of the cohort, stratified by biosample, are outlined in Table 1.

miRNA expression varies by tissue type

The two tissues were selected based on accessibility since we sought to leverage miRNA profiles as a potential diagnostic tool. Profiling indicated that fibroblasts and WB (all fractions including both plasma and blood cellular components) miRNAs had distinct non-overlapping miRNA expression profiles (Supplementary Material, Fig. S1A). Within each tissue type, group-specific samples (i.e. C9-ALS, nonC9-ALS, controls) only clustered with fibroblasts (Supplementary Material, Fig. S1B, left panel). There was a high degree of heterogeneity among the WB C9-ALS, nonC9-ALS and control groups (Supplementary Material, Fig. S1B, right panel).

C9-ALS and nonC9-ALS have differential miRNA levels in fibroblasts and WB

We identified differential miRNA (DmiRNA) levels in C9-ALS and nonC9-ALS versus controls using NanoStringDiff (20) (Supplementary Material, Tables S1 and S2). Raw count data were normalized with the positive and negative control probes as well as the reference probes, embedded in the NanoString nCount system, which showed consistent patterns across samples (Supplementary Material, Fig. S2). NanoStringDiff models count data using a generalized linear model of the negative binomial family and the likelihood ratio test, which has superior performance for identifying differentially expressed genes (20). We found a total of 62 fibroblast DmiRNAs (31 upregulated and 31 downregulated) in C9-ALS versus control samples (Fig. 2A). In nonC9-ALS fibroblasts, there were 55 DmiRNAs, including 38 increased and 17 decreased miRNAs versus control samples (Fig. 2B). WB also had slightly more DmiRNAs in C9-ALS than in nonC9-ALS versus control samples. C9-ALS WB had 62 DmiRNAs, of which 42 were increased and 20 were decreased in C9-ALS (Fig. 2C). Analysis of nonC9-ALS WB samples produced a total of 44 DmiRNAs, the majority of which ($n = 40$) were increased in nonC9-ALS, with only 4 decreased relative to controls (Fig. 2D).

C9-ALS and nonC9-ALS have shared and unique DmiRNAs in fibroblasts and WB

C9-ALS and nonC9-ALS shared four DmiRNAs (miR-30b-5p, miR-30c-5p, miR-484, miR-92a-3p) across both fibroblasts (decreased miRNA levels) and WB (increased miRNA levels) (Fig. 2E, Supplementary Material, Tables S1 and S2). In fibroblasts, 30 DmiRNAs overlapped between C9-ALS and nonC9-ALS out of 87 total DmiRNAs (34%), which differed in ALS versus control samples. All shared fibroblast DmiRNAs had the same direction of change in C9-ALS and nonC9-ALS versus controls. In WB, 24 DmiRNAs overlapped between C9-ALS and nonC9-ALS out of 82 DmiRNAs (29%), which differed in ALS versus control samples. All shared WB DmiRNAs differed in ALS versus control in the same direction.

C9-ALS and nonC9-ALS share DmiRNA-regulated biological pathways in fibroblasts and WB

Functional enrichment analysis identified 102 overrepresented biological pathways from statistically significant DmiRNAs in

Table 1. Demographics and clinical characteristics of ALS participants and controls

		Fibroblasts				WB			
		NonC9-ALS	*C9-ALS*	*Control*	*P-value*	*NonC9-ALS*	*C9-ALS*	*Control*	*P-value*
N		8	8	7		50	15	27	
Age	Years (mean ± SD)	59.4 ± 6.8	58.2 ± 5.9	58.4 ± 7.2	0.71	62.9 ± 12.0	57.6 ± 5.7	61.6 ± 10.4	0.21
Sex	Male	5	4	4	0.88	25	6	15	0.63
	Female	3	4	3		25	9	12	
Race	White	8	8	7		49	14	23	
	African American	.	.	.		.	.	4	
	Other/Not reported	.	.	.		1	1	.	
Onset segment	Bulbar	1	1	.	1.00	21	3	.	0.27
	Cervical	2	2	.		13	5	.	
	Lumbar	5	5	.		16	7	.	
Initial El Escorial criteria	Definite	11	2	.	0.42	.	.	.	0.59
	Probable	15	8	.		6	5	.	
	Probable, lab supported	20	4	.		2	3	.	
	Possible/suspected	4	1	.		.	.	.	
	Missing	.	.	.		.	.	.	
Symptom duration	Days (mean ± SD)	1268 ± 830	589 ± 53	.	0.04*	1062 ± 930	579 ± 316	.	0.08
ALS-FRS	Points (median + IQR)	36.0 (34.8–40.0)	36.0 (34.5–40.5)	.	0.65	36.0 (33.0–41.0)	35.0 (33.0–44.5)	.	0.50

The significant differences among the groups (NonC9-ALS, C9-ALS and Control) were tested using one-way ANOVA for continuous variables and Chi-Square test for categorical variables with the significance cutoff of 0.05. * indicates P-value < 0.05. SD, Standard Deviation. ALS-FRS: Amyotrophic Lateral Sclerosis Functional Rating Scale. IQR, Inter-Quartile Range.

fibroblasts and WB (Fig. 3). Of these, 44 enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were shared across all four groups (Fig. 3; Supplementary Material, Table S3). There were 54 KEGG pathways shared by C9-ALS and nonC9-ALS fibroblasts, including 'proteoglycans in cancer' and 'ErbB signaling pathway' as the top two most significant ones (Table 2). In WB, 56 KEGG pathways were shared by C9-ALS and nonC9-ALS, including 'proteoglycans in cancer', 'morphine addiction' and 'GABAergic synapse' (Table 3).

To identify the main themes of these enriched pathways, we built an association network using our in-house tool richR from the significant pathways in fibroblasts and WB. The generated network clusters enriched pathways with similar gene content. Highly interconnected subnetworks are highlighted by distinct colors (Fig. 4). In both C9-ALS (Fig. 4A) and nonC9-ALS (Fig. 4B) networks, subnetworks were centered around pathways related to neuronal functions (e.g. 'glutamatergic synapse', 'dopaminergic synapse', 'long-term potentiation'), cancer or cell proliferation (e.g. 'glioma', 'ErbB signaling pathway') and metabolism and inflammation (e.g. 'mTOR signaling pathway').

Fibroblast DmiRNAs inversely correspond to target mRNA stability

miR-186-5p and miR-16-5p were underrepresented in C9-ALS fibroblasts, while miR-543 was overrepresented in nonC9-ALS fibroblasts by NanoString analysis (Fig. 2A and B). To validate these findings, we used quantitative real-time PCR (qPCR) to quantify miR-186-5p and miR-16-5p in C9-ALS and miR-543 in nonC9-ALS fibroblast RNA (Supplementary Material, Fig. S3). As anticipated, miR-186-5p levels were lower in C9-ALS versus control fibroblasts ($P = 0.0236$). Similarly, miR-16-5p transcripts were lower in C9-ALS versus control fibroblasts, although this only approached statistical significance ($P = 0.0599$). In nonC9-ALS fibroblasts, miR-543 was higher than in controls ($P = 0.0423$), whereas it did not statistically differ in C9-ALS versus control fibroblasts ($P = 0.2773$) as expected.

miRNAs negatively regulate their mRNA targets. We previously analyzed the stability of fibroblast RNA from C9-ALS and nonC9-ALS samples by Bru-seq and BruChase-seq, techniques that quantitatively measure mRNAs stability (21,22). We performed a correlation analysis between DmiRNA levels with their predicted mRNA target stability in fibroblasts corresponding to a subset of the published cohort (Fig. 5). Inverse Spearman correlations were observed for 22 DmiRNAs, including miR-1246 and miR-515-5p, and 35 mRNAs, including growth arrest and DNA-damage-inducible, beta (GADD45B) and inositol hexakisphosphate kinase 2 (IP6K2) (Fig. 5). We validated the stability of GADD45B mRNA, a predicted miR-515-5p target with the largest fold-change, and of IP6K2 mRNA, a predicted miR-1246 target with the third largest fold-change, in C9-ALS fibroblasts by qPCR (Fig. 6). miR-1246 was highly upregulated in C9-ALS fibroblasts, and, as anticipated, the stability of its mRNA IP6K2 target was significantly diminished versus controls ($P = 0.0181$). Similarly, miR-515-5p transcripts were increased in C9-ALS fibroblasts, which is reflected in a lower GADD45B mRNA stability relative to controls, although this only approached statistical significance ($P = 0.0555$).

Random forest analysis of DmiRNAs

We next performed a random forest analysis on DmiRNAs from nonC9-ALS WB samples ($n = 80$) to determine whether miRNAs can classify nonC9-ALS participants from controls. There were too few C9-ALS samples ($n = 16$) for a random forest analysis. We ran the analysis in three formats, DmiRNAs identified by NanoStringDiff ($n = 46$), an additional DmiRNA set identified by a different analysis tool ($n = 60$), nSolver from NanoString, and the DmiRNAs ($n = 11$) overlapping between the two tools. Overlapping DmiRNAs produced a receiver operating characteristic (ROC) curve with the greatest area under the curve (AUC) of 0.831 [95% Confidence Interval (CI) 0.734–0.929; Fig. 7A], possibly because employing overlapping DmiRNAs minimized noise. The next best ROC had AUC 0.778 (95%CI 0.664–0.892), using nSolver (Fig. 7B). Last was the ROC generated using DmiRNAs identified by NanoStrongDiff (AUC 0.761, 95%CI

Table 2. Top functions of differential miRNAs from fibroblasts

Type	KEGG ID	Pathway description	#miRNAs	#genes	Adjusted *P*-value
C9-ALS	hsa05205	Proteoglycans in cancer	45	147	2.81E−10
	hsa04012	ErbB signaling pathway	44	71	1.18E−06
	hsa04152	AMPK signaling pathway	45	99	1.30E−06
	hsa04512	ECM-receptor interaction	38	57	5.98E−06
	hsa04390	Hippo signaling pathway	46	109	6.24E−06
	hsa00512	Mucin type O-Glycan biosynthesis	25	21	7.77E−06
	hsa05200	Pathways in cancer	48	273	7.77E−06
	hsa00061	Fatty acid biosynthesis	20	9	1.12E−05
	hsa04550	Signaling pathways regulating pluripotency of stem cells	46	103	1.23E−05
	hsa04261	Adrenergic signaling in cardiomyocytes	45	105	2.94E−05
	hsa04915	Estrogen signaling pathway	43	71	5.11E−05
	hsa04360	Axon guidance	45	95	5.11E−05
	hsa04510	Focal adhesion	44	149	5.87E−05
	hsa04015	Rap1 signaling pathway	46	151	5.87E−05
	hsa04150	mTOR signaling pathway	44	51	6.26E−05
	hsa05214	Glioma	44	49	6.26E−05
	hsa04350	TGF-beta signaling pathway	41	59	7.58E−05
	hsa04520	Adherens junction	44	59	7.58E−05
	hsa05215	Prostate cancer	44	69	1.22E−04
	hsa04151	PI3K-Akt signaling pathway	47	231	1.34E−04
nonC9-ALS	hsa00512	Mucin type O-Glycan biosynthesis	23	20	3.43E−07
	hsa05205	Proteoglycans in cancer	38	135	3.43E−07
	hsa04012	ErbB signaling pathway	37	68	9.17E−07
	hsa04360	Axon guidance	36	91	4.44E−06
	hsa05032	Morphine addiction	36	65	5.11E−06
	hsa04520	Adherens junction	39	58	6.67E−06
	hsa05031	Amphetamine addiction	33	49	2.33E−05
	hsa04152	AMPK signaling pathway	38	90	2.33E−05
	hsa04014	Ras signaling pathway	41	153	2.79E−05
	hsa04915	Estrogen signaling pathway	35	65	3.85E−05
	hsa04727	GABAergic synapse	36	60	3.85E−05
	hsa04724	Glutamatergic synapse	37	82	3.85E−05
	hsa05231	Choline metabolism in cancer	38	76	7.47E−05
	hsa04261	Adrenergic signaling in cardiomyocytes	37	99	9.34E−05
	hsa00310	Lysine degradation	33	34	1.29E−04
	hsa05211	Renal cell carcinoma	35	52	1.43E−04
	hsa05212	Pancreatic cancer	35	49	2.08E−04
	hsa04015	Rap1 signaling pathway	38	140	2.84E−04
	hsa05200	Pathways in cancer	40	261	3.13E−04
	hsa04350	TGF-beta signaling pathway	31	56	3.71E−04

0.645–0.877; Fig. 7C). In all instances, miR-26a-5p emerged as the top candidate, which differentiated nonC9-ALS from control samples, followed by miR-30c-5p (Fig. 7D). This result suggests that the small subset of common DmiRNAs identified by the two tools have the most classifying power between ALS and control. Pathway analysis of genes regulated by miR-26a-5p identified enriched pathways, including 'protein processing in endoplasmic reticulum' (hsa04141, $P = 6.09e{-}06$), 'hippo signaling pathway' (hsa04390, $P = 4.04e{-}05$) and 'biosynthesis of unsaturated fatty acids' (hsa01040, $P = 0.00046$).

Discussion

Familial ALS comprises ∼15% of all cases and the most common genetic mutation is a hexanucleotide repeat expansion in *C9orf72* (2). The underlying disease etiology remains unknown in the remaining 85% of sporadic ALS patients. Cytoplasmic TDP-43 aggregates in motor neurons are an almost universal feature in ALS (2), although mutations to TAR DNA binding protein 1 (*TARDBP*) itself (gene encoding TDP-43) are uncommon. TDP-43 regulates miRNA processing by affecting the stability of or binding to Drosha or Dicer (7,8). Thus, altered RNA metabolism, including of miRNAs, may be generally disrupted in ALS. In the current study, we found that fibroblasts and WB from C9-ALS and nonC9-ALS patients exhibited a distinct DmiRNA profile versus controls. There were more DmiRNAs in C9-ALS ($n = 114$, total fibroblasts and WB) than in nonC9-ALS ($n = 91$) samples, possibly because of the comparative heterogeneity of nonC9-ALS samples. We also identified DmiRNAs by tissue type in fibroblasts (31 increased, 31 decreased) and WB (42 increased, 22 decreased) from C9-ALS and nonC9-ALS patients. Of these, four DmiRNAs were common to both tissues and groups (C9-ALS, nonC9-ALS) and may represent an ALS-specific panel of miR-30b-5p, miR-30c-5p, miR-484 and miR-92a-3p. Functional

Table 3. Top functions of differential miRNAs from WB

Type	KEGG ID	Pathway description	#miRNAs	#genes	Adjusted *P*-value
C9-ALS	hsa05205	Proteoglycans in cancer	57	157	1.74E−08
	hsa04012	ErbB signaling pathway	54	72	3.40E−06
	hsa05032	Morphine addiction	54	72	3.40E−06
	hsa04390	Hippo signaling pathway	53	113	7.86E−06
	hsa04727	GABAergic synapse	51	67	9.30E−06
	hsa05200	Pathways in cancer	61	286	2.39E−05
	hsa04360	Axon guidance	56	97	6.49E−05
	hsa00512	Mucin type O-Glycan biosynthesis	31	22	6.57E−05
	hsa00533	Glycosaminoglycan biosynthesis—keratan sulfate	17	12	8.38E−05
	hsa05100	Bacterial invasion of epithelial cells	51	63	8.95E−05
	hsa05211	Renal cell carcinoma	48	54	9.14E−05
	hsa04520	Adherens junction	51	60	9.14E−05
	hsa04120	Ubiquitin mediated proteolysis	52	106	9.14E−05
	hsa04015	Rap1 signaling pathway	57	157	9.85E−05
	hsa04350	TGF-beta signaling pathway	48	62	1.28E−04
	hsa04068	FoxO signaling pathway	53	104	1.40E−04
	hsa05033	Nicotine addiction	46	31	2.13E−04
	hsa00310	Lysine degradation	48	38	2.28E−04
	hsa04919	Thyroid hormone signaling pathway	55	90	2.28E−04
	hsa04724	Glutamatergic synapse	56	86	2.75E−04
nonC9-ALS	hsa05032	Morphine addiction	30	68	1.57E−11
	hsa05205	Proteoglycans in cancer	34	140	4.15E−11
	hsa04727	GABAergic synapse	28	63	3.95E−08
	hsa04360	Axon guidance	33	92	4.79E−08
	hsa04390	Hippo signaling pathway	33	102	4.79E−08
	hsa04012	ErbB signaling pathway	34	68	4.79E−08
	hsa04015	Rap1 signaling pathway	36	147	1.87E−07
	hsa05200	Pathways in cancer	37	255	6.01E−07
	hsa04014	Ras signaling pathway	37	150	1.33E−06
	hsa00512	Mucin type O-Glycan biosynthesis	20	20	1.39E−05
	hsa04724	Glutamatergic synapse	33	77	3.45E−05
	hsa04510	Focal adhesion	35	139	3.52E−05
	hsa04919	Thyroid hormone signaling pathway	35	81	4.76E−05
	hsa04512	ECM-receptor interaction	27	51	5.02E−05
	hsa05211	Renal cell carcinoma	27	50	7.22E−05
	hsa05214	Glioma	28	46	9.21E−05
	hsa05030	Cocaine addiction	26	34	1.47E−04
	hsa04350	TGF-beta signaling pathway	29	53	1.47E−04
	hsa04068	FoxO signaling pathway	32	91	1.47E−04
	hsa05100	Bacterial invasion of epithelial cells	30	55	1.49E−04

enrichment identified 102 biological pathways overall, of which 44 were shared across C9-ALS and nonC9-ALS in both tissues versus controls. Network analysis centered on pathways related to neuronal function, metabolism and cellular proliferation and structure. Finally, random forest of DmiRNAs produced an ROC curve with an AUC of 0.831 for differentiating ALS patients from control participants.

Previously, we profiled miRNA (18,23) and mRNA (23) in *post mortem* spinal cord tissue from sporadic ALS patients. We found that only miR-142-5p and miR-155-5p were upregulated in sporadic ALS spinal cord, but 88 miRNAs were downregulated, with miR-577 and miR-935 as the most suppressed in ALS (18). None of the spinal cord miRNAs overlapped with both fibroblast and WB miRNAs in this study. It is unclear whether differences in miRNAs between accessible biospecimens versus spinal cord tissue in ALS stems from disease pathology or from tissue-specific biomarkers independent of the disease process. Functional pathway analysis of spinal cord miRNAs highlighted cellular regulation and proliferation and immune response (18,23), which partially overlaps with fibroblast and WB miRNA pathways.

The role of miRNAs in ALS was first highlighted in mutant Superoxide Dismutase 1 ($SOD1^{G93A}$) mice, which had upregulated skeletal muscle-specific miR-206 later in disease (24). Progression was slowed following miR-206 knockout. Several studies have corroborated overrepresentation of miR-206 in ALS muscle and plasma (17), but it is non-specific to ALS since it is also altered in muscular dystrophies (25) and other neurodegenerative diseases (26,27). This underscores the importance of including biosamples from similar yet distinct diseases to rule out miRNAs with significant overlap, which only a few studies have considered (28–30). Aside from miR-206, several other miRNAs are up- or downregulated in ALS, but with very little overlap across studies, which may be attributed to several factors. First, most studies recruited sporadic ALS patients at distinct stages of the disease, resulting in heterogeneous populations. Second, study sample sizes were small and likely unrepresentative of larger populations

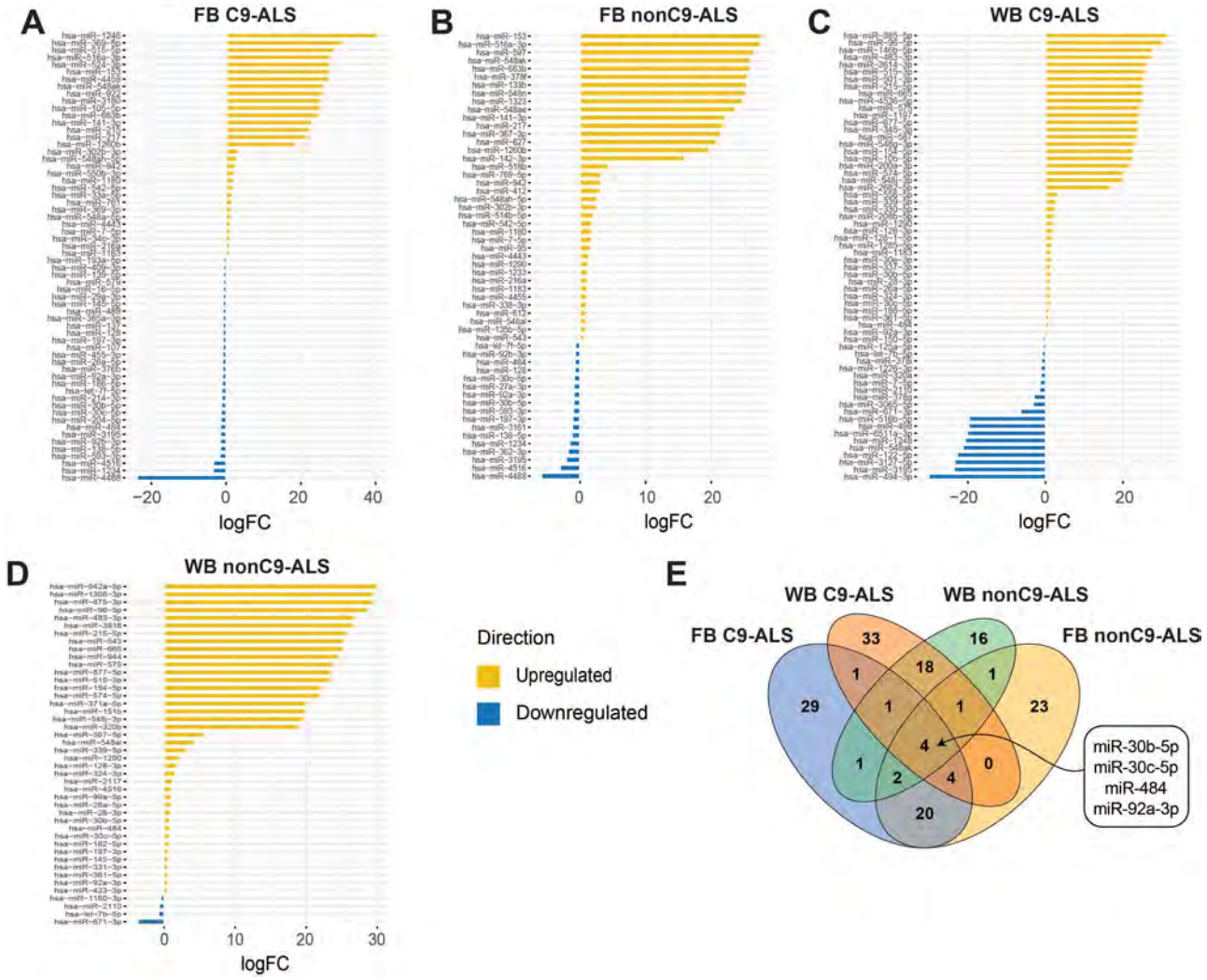

Figure 2. Fibroblast and WB DmiRNAs from C9-ALS and nonC9-ALS versus control participants. Fold-change (*x*-axis) of differential miRNAs (DmiRNAs; $P < 0.05$; *y*-axis) identified by NanoStringDiff. DmiRNAs that increased in ALS versus controls in yellow; DmiRNAs that decreased in ALS versus controls in blue. Plots for fibroblasts (FB) in (**A**) C9-ALS and (**B**) nonC9-ALS versus controls; plots for WB in (**C**) C9-ALS and (**D**) nonC9-ALS versus controls; yellow, upregulated in ALS versus controls; blue downregulated in ALS versus controls. (**E**) Venn diagram of the number of shared and unique DmiRNAs between fibroblasts and WB for C9-ALS and nonC9-ALS groups.

(17). Finally, studies profile different tissues. To overcome these limitations, we included both C9-ALS and nonC9-ALS from two tissues, fibroblasts and WB, from a relatively large total number of ALS samples ($n = 81$).

We report more DmiRNAs in C9-ALS than in nonC9-ALS versus controls with tissue-specific differences, although there was some overlap. This is aligned with other studies (31,32), which identified a 30-panel DmiRNA signature in familial ALS versus controls, but only 2 DmiRNAs in sporadic ALS versus controls, which the authors concluded could have arisen from greater heterogeneity of sporadic ALS. Another study of ALS muscle versus plasma found some but incomplete overlap in miRNA profiles (33), as we observed, indicating variation by tissue. Moving forward, it will be important to define the best tissue for analysis and stratify a sufficient sample size by genetic mutation to validate a strong ALS miRNA panel.

Currently, there is no sensitive molecular diagnostic test for ALS. With the goal of bridging this knowledge gap, we examined ALS-associated miRNA profiles in accessible WB, an immune cell-containing biofluid. Immune system dysfunction is a recurrent ALS theme (34). ALS participants exhibit distinct circulating immune cell populations versus healthy controls (35–38), advocating WB as a diagnostic medium for an ALS test. miRNAs are also attractive because they circulate widely in WB (39) and may represent a snapshot of disease status, including ALS (17). We performed random forest of all DmiRNAs from nonC9-ALS WB, which generated an ROC curve with an AUC of 0.832. The top candidate was miR-26a-5p, followed by miR-30c-5p, which most robustly differentiated ALS patients from control participants. Pathway analysis of miR-26a-5p-regulated genes yielded 'protein processing in endoplasmic reticulum', 'hippo signaling pathway' (see below) and 'biosynthesis of unsaturated fatty acids'.

No study has identified an miRNA panel unique to nonC9-ALS. Our top two candidates, miR-26a-5p and miR-30c-5p, were identified in a study of 56 sporadic ALS WB samples (40) but were downregulated rather than upregulated. Other studies have noted miR-26a-5p downregulated in muscle (41) or upregulated in serum (42) in ALS. Therefore, there is a lack of consensus in the literature arising from the heterogeneity of sporadic ALS and small sample sizes. We did not evaluate miRNA changes longitudinally over the disease course, which may be an additional factor affecting consensus among results. Indeed, a pilot study

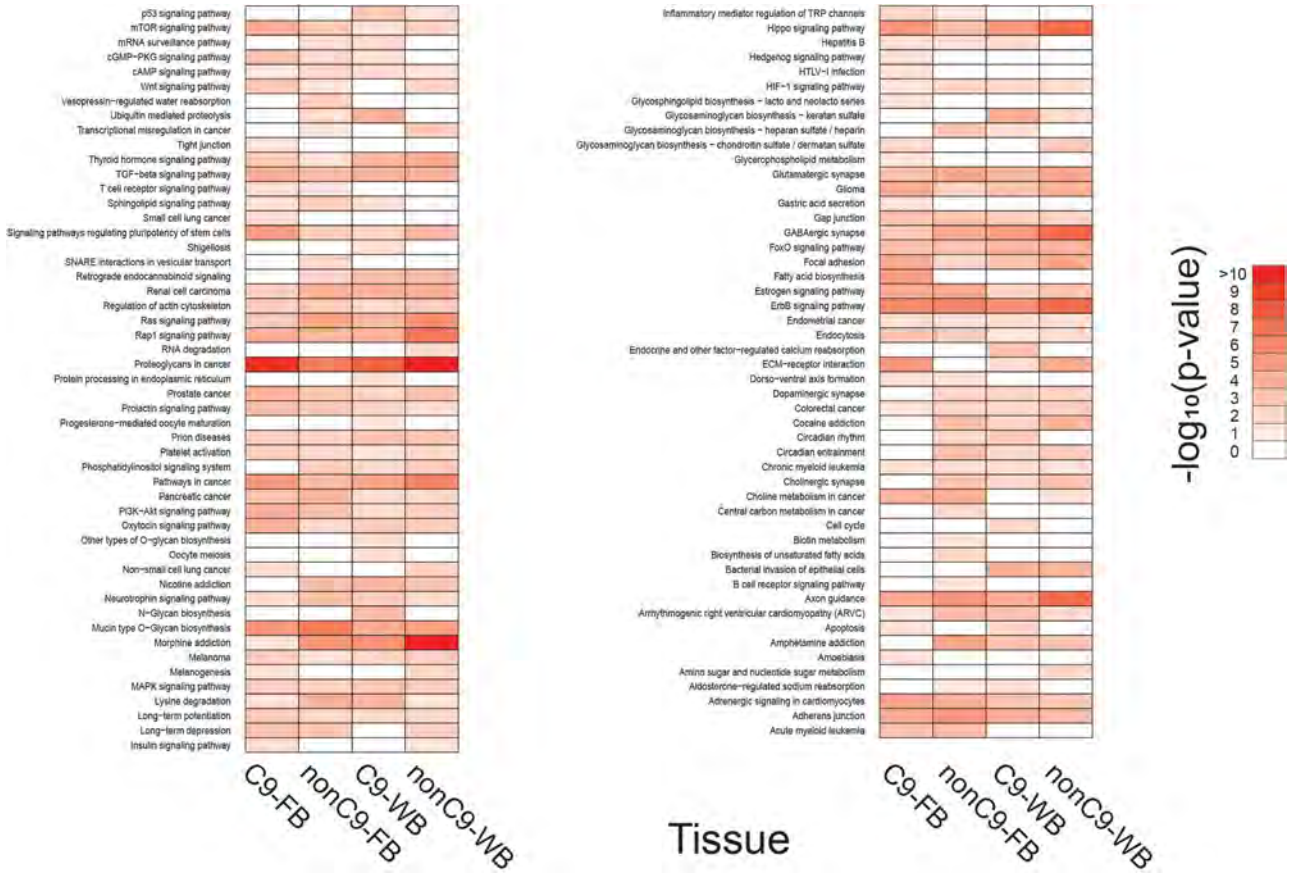

Figure 3. KEGG pathway analysis of DmiRNAs. Heat-map of significantly enriched KEGG pathways identified for each of the DmiRNA datasets represented by a $\log_{10}$-based color and number index. FB, fibroblasts.

suggests that miRNAs correlate with and change over time in relationship to clinical parameters (30). Furthermore, miRNAs have multiple targets, so there may be overlap in downstream biological pathways even if the overlap between DmiRNAs by studies is lacking.

Thus, after analyzing DmiRNAs, we next examined their target mRNAs to infer downstream biological pathways. miRNAs negatively regulate their targets by recruiting Argonaute proteins and assembling into an RNA-induced silencing complex, cleaving the target mRNA (43). We leveraged our Bru-seq and BruChase-seq dataset to correlate DmiRNAs to target mRNA stability (21,22). Bru-seq and BruChase-seq label nascent mRNA followed by a pulse with an orthogonal label after a time delay to assess stability (21). Less stable mRNAs decrease in level to a greater extent than more stable mRNAs over time. As anticipated, we found that C9-ALS fibroblast miRNA levels from this study correlated inversely with mRNA target stability from C9-ALS fibroblasts from our previously published study (21). Presumably, high-level miRNAs degraded their target mRNAs relatively rapidly, rendering them of lower stability, whereas low-level miRNAs degraded their tagets relatively more slowly, rendering them of higher stability. DmiRNA-to-mRNA correlations did not attain 100%, likely due to the presence of alternative mRNA-destabilizing or degrading pathways, such as decapping and base modifcations (44,45). We validated the DmiRNA-to-mRNA correlations by qPCR for two target mRNAs, GADD45B and IP6K2.

However, miRNAs have multiple targets, so we performed functional enrichment of all mRNA targets of significant DmiRNAs. We then clustered biologically enriched pathways by network analysis to identify the main themes. The subnetworks, along with the highly inter-connected nodes, provided interesting insight into ALS pathology. The largest inter-connected subnetwork in C9-ALS (shaded purple) contained several cancer KEGG pathways mostly shared by fibroblasts and WB, including 'pathways in cancer', 'ErbB signaling pathway' and 'MAPK signaling pathway'. There were also cancer KEGGs unique to fibroblasts, such as 'small cell lung cancer'. JAK–STAT signaling is central to 'pathways in cancer', which is overrepresented in C9-ALS samples. We previously found that JAK–STAT signaling is also prominent upon analysis of mRNA from sporadic ALS spinal cords (23). In an *in vitro* ALS model, we found that tofacitinib-mediated blocking of JAK–STAT signaling in natural killer cells inhibits their ability to attack motor neurons (46). Collectively, these results suggest that this pathway may serve as a therapeutic target in ALS, although further studies are warranted.

ErbBs are also of particular interest; they influence cell survival and proliferation and activate MAPK and PI3K-Akt signaling (47). ErbB is activated by its ligand neuregulin 1, which has lower expression in spinal cords from ALS patients and $SOD1^{G93A}$ mice, a model of familial ALS (48). Promoting neuregulin 1 expression in $SOD1^{G93A}$ animals slows disease progression in females. In humans, a Japanese family with familial ALS harbors mutant *ERBB4*, with a diminished capacity for neuregulin 1-mediated activation (49). MAPK signaling was also featured in this subnetwork (50); inhibiting p38 MAPK rescues retrograde axonal transport in $SOD1^{G93A}$ ALS mice (51), whereas blocking AMPKα prevents hydrogen peroxide-induced apoptosis of $SOD1^{G93A}$ embryonic neural stem cells (52). These examples illustrate how our pathway

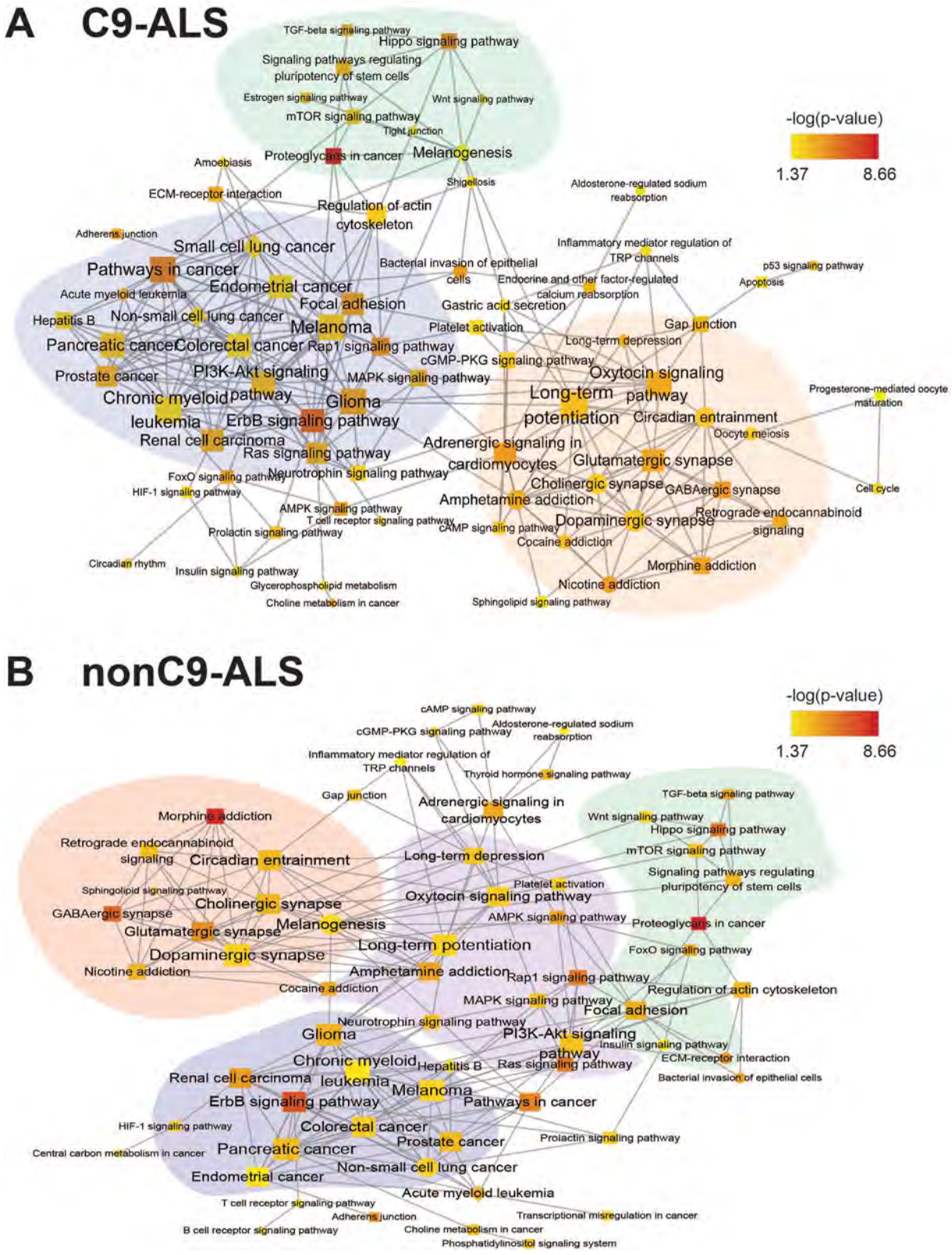

Figure 4. KEGG pathway association networks. Significantly enriched KEGG pathways were combined and visualized in a network for (**A**) C9-ALS and (**B**) nonC9-ALS samples. KEGG pathways are represented by nodes; shared gene content between pathways are represented by edges. Within the network, node shape indicates the tissue source of the enriched pathways: diamond, fibroblasts only; circle, WB only; square, both fibroblasts and WB. Node color is based on $-\log_{10}$ (*P*-value). Node size corresponds to the number of connections each node has. All networks were organized by the inverted self-organizing map layout with minimal manual node rearrangement for visibility. Highly inter-connected subnetworks were identified by Cytoscape MCODE and are highlighted by various colors. Single nodes, which are not connected to other nodes, were excluded from this network visualization.

Gene	DmiRNA	hsa-miR-105-5p	hsa-miR-107	hsa-miR-1246	hsa-miR-1260b	hsa-miR-138-5p	hsa-miR-204-5p	hsa-miR-217	hsa-miR-26a-5p	hsa-miR-29a-3p	hsa-miR-33a-5p	hsa-miR-34c-3p	hsa-miR-369-3p	hsa-miR-4458	hsa-miR-455-3p	hsa-miR-515-5p	hsa-miR-548a-5p	hsa-miR-548ah-5p	hsa-miR-548ak	hsa-miR-663b	hsa-miR-7-5p	hsa-miR-761	hsa-miR-922
	Stability \ miRNA-FC	25.35	-0.89	40.41	18.45	-1.57	-1.41	21.90	-0.94	-0.73	1.75	1.07	1.43	27.69	-0.92	29.00	1.36	2.97	26.21	24.71	1.17	1.59	25.79
ATP2B1	1.65								-0.66														
BPNT1	-1.58					-0.64										-0.7							
CASP7	-1.52									-0.62													
CCDC59	-2.26																						-0.68
CEBPD	-3.60	-0.69																-0.62					
CFDP1	-1.91																	-0.61	-0.65				
COQ5	-1.87																	-0.63					
DDIT4	-4.16																-0.62						
EDN1	-1.69															-0.62							
EED	-1.51					-0.61						-0.65									-0.66		
EIF4EBP1	-2.54					-0.74																	
FAM103A1	-1.57													-0.63									-0.63
FAM214A	-1.72			-0.61																			
FAM46A	-1.80	-0.67																				-0.69	
GADD45B	-3.45															-0.64							
GREM2	1.91		-0.61																				
GRPEL2	-1.79																		-0.62				
HIST1H2AH	-3.05																-0.65						
HIST1H2BC	-1.70												-0.62										
IP6K2	-1.84			-0.65																			
KCNE4	-2.88																	-0.61					
KRCC1	-2.27												-0.64										
LBH	-1.54				-0.64																		
MCL1	-1.96							-0.68															
MEIS2	-1.78										-0.7			-0.77			-0.74		-0.85				-0.69
MRPS18A	-1.94																			-0.67			
PLK2	-1.85							-0.64															
PRDM4	-1.55																					-0.64	
PRKRIP1	-2.08				-0.69																		
PSPH	-1.63																-0.68						
SLC4A1AP	-1.56						-0.61																
STX8	-1.90					-0.61																	
SUMF1	-1.72											-0.65	-0.62										
TMPPE	1.66														-0.63								
ZNF654	-1.54																						-0.66

Figure 5. Identifying potential DmiRNA as regulators of mRNA stability in fibroblasts. miRNA fold-change (FC) for differential miRNAs (DmiRNAs; top row) in C9-ALS fibroblast. Predicted target mRNA (first column) stability values (mature/nascent; second column) are from our previously published study (21). Values in other table cells are the significant Pearson correlation coefficients between each pair of DmiRNA and its predicted mRNA targets; color represents the degree of differential expression in fold-changes (red up-regulation in ALS, blue down-regulation in ALS). Stability-FC and miRNA-FC were scaled independently.

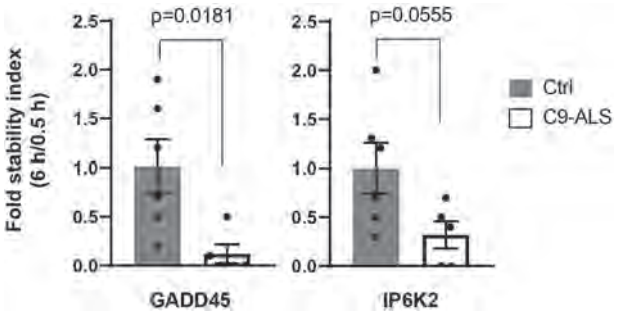

Figure 6. Validation of target mRNA in fibroblasts. Target mRNA in fibroblasts were validated by qPCR. Results were normalized to yWHAZ and presented as fold-change calculated by the $2^{-\Delta C_T}$ method for GADD45 [C9-ALS, $n = 5$; Control (Ctrl), $n = 6$], IP6K2 (C9-ALS, $n = 5$; Ctrl, $n = 6$). Transcript stability was determined as the ratio between transcript abundance at 6 h (pulse/chase) to 0.5 h (pulse) and compared with Ctrl. Experiment performed in duplicate; analysis by Student's *t*-test; data represented as mean ± standard error of the mean.

analysis can identify possible therapeutic ALS targets, e.g. ErbB, MAPK, which the literature shows can be pursued as potential therapies in pre-clinical studies with ALS mouse models.

There were two other highly inter-connected C9-ALS subnetworks with signaling pathways known to be dysfunctional in ALS. One subnetwork (shaded orange) was heavily centered on pathways in both fibroblasts and WB related to neuronal function and signaling, such as 'glutamatergic synapse', 'GABAergic synapse' and 'long-term potentiation'. 'Sphingolipid signaling pathway' was also featured, which we have identified as a recurrent dysregulated pathway in sporadic ALS by metabolomics analysis of plasma (53,54). This pathway is also linked to familial ALS through mutations to *SPTLC1*, involved in sphingolipid synthesis (55,56). Additionally, the orange subnetwork contained some pathways that were WB predominant, e.g. 'dopaminergic synapse', 'cholinergic synapse'. The second subnetwork (shaded green) contained pathways previously identified in ALS and linked to development, e.g. 'Wnt signaling pathway' (57) and 'hippo signaling pathway' (58), and is aligned with recent evidence of widespread neural network disruption in ALS (59).

Finally, when we examined the network constructed from nonC9-ALS KEGG pathways, similar clusters emerged as for C9-ALS. These spanned a cancer-predominant subnetwork (shaded blue) and a development subnetwork (shaded green). The orange C9-ALS subnetwork splits into two subnetworks in sporadic nonC9-ALS, one involving pathways related to neuronal function (shaded pink), which is highly interconnected with a second subnetwork encompassing various signaling pathways (shaded purple). Of pathways related to ALS, 'PI3K-Akt signaling pathway' (60) appeared in the nonC9-ALS network and clustered with MAPK. 'FOXO signaling pathway' (61) was linked with the neurodevelopment subnetwork in nonC9-ALS, rather than the cancer subnetwork in C9-ALS. Another notable difference in the nonC9-ALS network is greater integration of 'regulation of actin cytoskeleton' into the green development subnetwork, which bridges the development and cancer subnetworks in familial C9-ALS. Several ALS mutations have been identified in genes that regulate actin cytoskeleton (62), such as profilin 1 (*PFN1*), a regulator of actin polymerization (63), and *ALS2*, encoding the protein alsin, which has a RhoGEF domain for regulating Rho and actin dynamics (64). Although preliminary, these studies corroborate the overrepresented terms in our pathway analysis of miRNAs in ALS and may suggest therapeutic targets.

This study benefited from several strengths. First, the ability to access to two tissues. Second, ALS patients were stratified by the

most prevalent *C9orf72* alteration and examined DmiRNAs in the context of both familial and sporadic ALS. Third, DmiRNAs were identified by a multiplexed, untargeted approach, NanoString and rigorously analyzed by NanoStringDiff. This study also suffered limitations. Even with the large number of samples, the study still was not powered to detect sex differences, an important consideration in ALS, which is more prevalent in men (1). Moreover, familial ALS samples were limited to *C9orf72* carriers, which comprise only half of familial cases. The qPCR validation of select DmiRNAs was done using a single reference rather than multiple references.

One major goal of this study was to seek ALS biomarkers in accessible tissue. Our top candidates, miR-26a-5p and miR-30c-5p, did not find overlap with all other ALS miRNA reports in the literature. The challenges faced for identifying a unifying miRNA biomarker panel in ALS are substantial. ALS is highly heterogeneous, with variation in clinical presentation (e.g. onset segment, speed of disease progression, disease stage), underlying genetic cause (with over 40 identified ALS mutations) and a possible environmental contribution (1,2). It is possible that this considerable extent of heterogeneity may defy determination of an ALS-specific miRNA panel. Nevertheless, our study underscores the importance of miRNAs for understanding ALS pathophysiology by providing miRNA pathway analysis of one of the largest ALS sample sizes to date, which uncovered both well-known, corroborating, pathways and less-known pathways of potential future interest.

Materials and Methods

Study participants and samples

All patients 18 years and older and able to communicate in English attending the University of Michigan Pranger Multidisciplinary ALS clinic were invited to submit samples to the University of Michigan ALS Patient Repository (UMAPR), an Institutional Review Board (IRB)-approved repository. Control participants were recruited separately. All participants provided written informed consent. For this study, participant-provided biospecimens were retrieved from UMAPR, which met the following criteria: diagnosis of ALS based on the Gold Coast criteria (65) and further classified by the initial and/or revised EI Escorial criteria (66). ALS participants were distributed between the sporadic ALS cohort (nonC9-ALS), by selecting patients without a family history of ALS and negative for *C9orf72* expansion, and the familial ALS cohort (C9-ALS), by selecting patients with *C9orf72* expansion determined by published methods (67). Age- and sex-matched healthy controls were then selected. Skin punch biopsy from the forearm was collected using standard protocols and participant-derived fibroblasts were isolated as previously reported (21). Fibroblasts were cultured in fibroblast media: Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/l D-glucose, with glutamine/without pyruvate (Gibco, Thermo Fisher Scientific, Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific), 1X Glutamax-1 (Gibco, Thermo Fisher Scientific) and 1X MEM NEAA (Gibco, Thermo Fisher Scientific). Approximately 5 mL of WB was collected by standard venipuncture into PAXgene Blood RNA Tubes (cat # 762125, Qiagen, Germantown, MD, USA; for RNA isolation) and another 5 mL into EDTA tubes (plasma isolation by centrifugation); all tubes were stored at −80°C. The overall experimental outline is presented in Fig. 1.

RNA extraction and quality determination

Total RNA for miRNA profiling was isolated from fibroblasts using the miRNeasy Mini Kit (cat # 217004, Qiagen) and from WB with the PAXgene Blood miRNA Kit (cat # 63134, Qiagen), according to the manufacturer's instructions. RNA was concentrated and purified using the Zymo RNA Clean & Concentrator-5 (cat # R1016, Zymo Research, Irvine ,CA, USA). RNA concentration was determined with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific, Wilmington, DE, USA) and quality was assessed by the 260/280 and 260/230 nm ratios. RNA integrity was measured using the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA).

miRNA expression profiling

Profiling was performed by the NanoString nCounter Human v2 (on fibroblasts) and v3 (on WB) miRNA Expression Panels (NanoString Technologies, Seattle, WA, USA), as previously described (68). Briefly, this assay detects 800 endogenous miRNAs, 5 reference transcripts, plus 6 positive and 6 negative controls. About 150 ng of total RNA per sample was used as input for the nCounter Human miRNA sample preparation and hybridized for 16 h at 65°C. Subsequently, the strip tubes were placed into the nCounter Prep Station for automated sample purification and subsequent reporter capture. Each sample was scanned for 555 fields of view on the nCounter Digital Analyzer and data were extracted using the nCounter RCC Collector (both NanoString). A quality control step identified and eliminated unrelated samples (outliers) using nSolver Analysis Software v3.1 (NanoString), according to manufacturer's instruction. Principal component analysis visualized and examined the overall variation across samples. Next, data were analyzed with NanoStringDiff, an R package specifically designed for NanoString nCounter data (20). Data were normalized using positive controls, negative controls and reference genes (ACTB, B2M, GAPDH, RPL19, RPLP0) embedded in the nCounter system, and differential log fold-changes and multiple testing adjusted statistical significance q-values were obtained.

Pathway analysis

DIANA-miRPath v3.0 (69) was used to predict DmiRNA gene targets and characterize their biological functions and pathways using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway terms. KEGG pathways with false discovery rate (FDR) < 0.05 were considered significantly enriched among the DmiRNAs from each tissue and genotype (C9-ALS or nonC9-ALS). To identify the overall theme of these enriched pathways, an association network for each tissue was generated from the gene-content overlap among the KEGG pathways using *richR*, our in-house analysis R package (https://github.com/hurlab/richR). The inter-relationship among the significant pathways within each tissue was visualized in Cytoscape (70) and highly-inter-connected pathway clusters were detected by MCODE (71), a Cytoscape application for network cluster analysis.

Fibroblast labeling and sequencing and mRNA stability

Fibroblast mRNA stability from controls ($n = 3$), nonC9-ALS ($n = 4$) and C9-ALS ($n = 4$) participants was determined by 5-bromouridine sequencing (Bru-seq) and 5-bromouridine-chase sequencing (BruChase-seq), as previously described (21). Briefly, fibroblasts were grown to confluency in duplicate in 150 mm petri dishes (Falcon/Corning, Corning, NY, USA) in fibroblast media: DMEM with 4.5 g/l D-glucose, with glutamine/without pyruvate (Gibco, Thermo Fisher) supplemented with 10% heat inactivated FBS (Gibco, Thermo Fisher), 1X Glutamax-1 (Gibco, Thermo Fisher) and 1X MEM NEAA (Gibco, Thermo Fisher), and 5-Bromouridine (2 mM; cat # 850187, Sigma-Aldrich, Burlington, MA, USA) was added to both petri dishes and incubated for 0.5 h

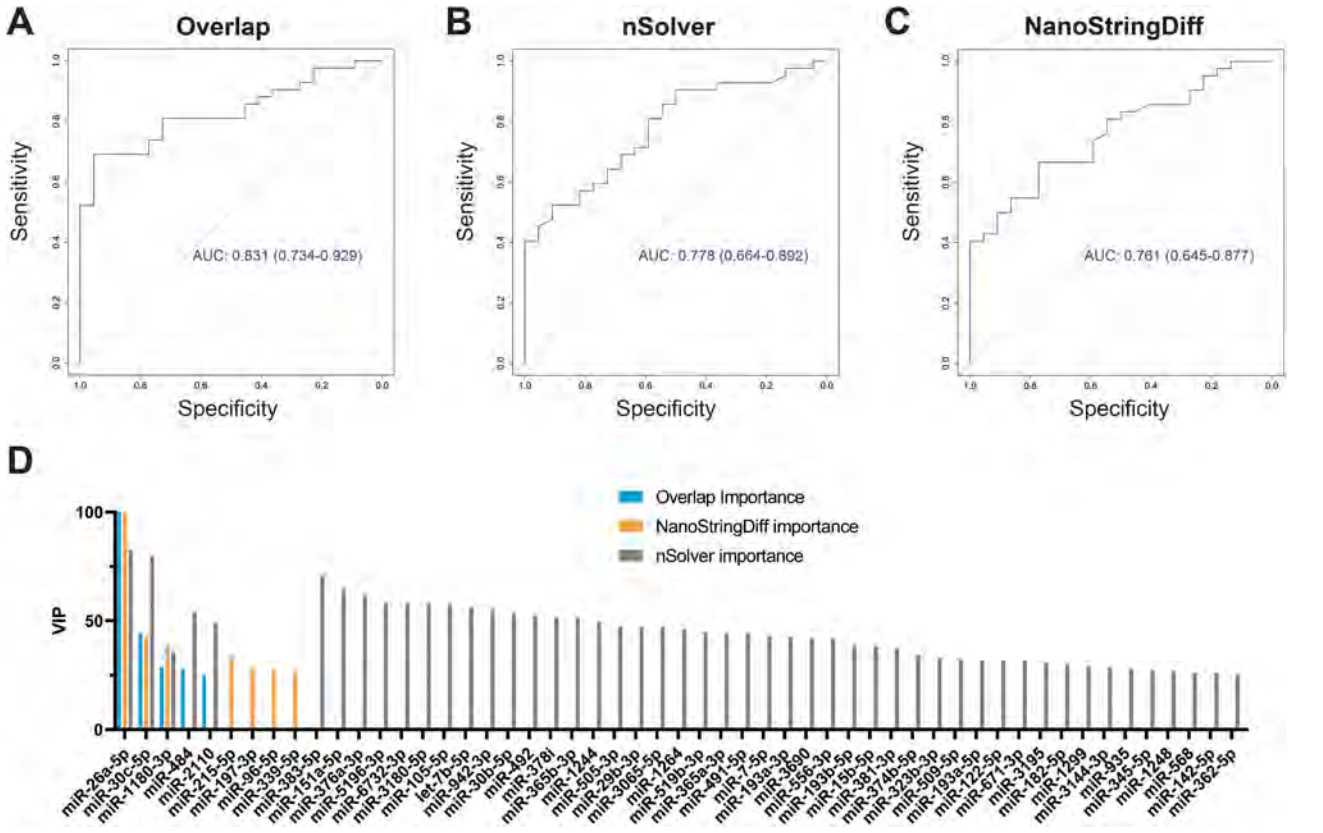

Figure 7. Random forest analysis using overlapping DmiRNAs. ROC curves for random forest analysis of (**A**) overlap DmiRNAs (shared by NanoStringDiff and nSolver) with an AUC of 0.831 (95% CI 0.734–0.929), (**B**) nSolver AUC 0.778 (95%CI 0.664–0.892) and (**C**) NanoStringDiff AUC 0.761 (95%CI 0.645–0.877). (**D**) Variable importance in projection plot ranking importance of DmiRNAs to all three classifiers, overlap (blue), NanoStringDiff (orange) and nSolver (grey). miR-26a-5p was the top candidate, followed by miR-30c-5p, across all methods.

at 37°C in 5% CO_2 to label cells, which were either harvested (pulsed, first petri dish) or chased for 6 h with uridine (20 mM, second petri dish; cat # U3750, Sigma-Aldrich) at 37°C in 5% CO_2. Cells were harvested in 3 mL of QIAzol (cat # 79306, Qiagen) and total RNA was isolated by phenol/chloroform extraction and resuspended in 100 μL of diethyl pyrocarbonate water. Bru-labeled RNA was immunoprecipitated with mouse anti-BrdU antibody (clone 3D4; cat # 555627, BD Pharmingen, Franklin Lakes, NJ, USA) conjugated to goat anti-mouse Dynabeads (cat # 11033, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Strand-specific DNA libraries were prepared with the Illumina TruSeq Kit (Illumina, San Diego, CA, USA) and sequenced on an Illumina platform, as previously described (21). RNA stability was calculated as the ratio of transcript abundance at 6 h versus 0.5 h. We used a cutoff of ≥1.5-fold-change in stability of mRNA transcripts.

Quantitative real-time PCR

For miRNA, 30 ng of total RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (cat # 4368814) with Megaplex Primer Pools, Human Pools A v2.1 (cat # 4401009). cDNA was preamplified using TaqMan PreAmp Master Mix (cat # 4391128) and Megaplex PreAmp Primers, Human Pool A v2.1 (cat # 4399233). qPCR was performed with TaqMan Universal PCR Master Mix (cat # 4318157) and TaqMan microRNA Assays (cat # 4427975, all Thermo Fisher Scientific, Waltham, MA, USA). Each sample was run in triplicate. Transcripts levels were calculated by the $\Delta\Delta C_T$ method and normalized to RNU48. The control group in each comparison was used as a calibrator ($\Delta\Delta C_T = 0$, $2^{\wedge}(-\Delta\Delta C_T) = 1$). Amplification efficiency for each miRNA was assessed by technical duplicates method on a Bio-Rad Real-Time PCR System (Bio-Rad, Ann Arbor, MI, USA).

For mRNA stability, cDNA was generated using iScript Reverse Transcription Supermix (cat # 1708841, Bio-Rad) on the entire anti-Bru-immunoprecipitated pulse and pulse/chased RNA (Fibroblast labeling and sequencing and mRNA stability section). qPCR was performed on 2 μL of template with TaqMan Gene Expression Master Mix (cat # 4369016, Thermo Fisher Scientific) and TaqMan probes (Thermo Fisher Scientific) for GADD45B, IP6K2a and YWHAZ (normalization reference). The PCR program was 120 s at 50°C, 10 s at 95°C, 40x cycles (15 s at 95°C, 60 s at 60°C) on an Applied Biosystems StepOne Real-Time PCR system (Thermo Fisher Scientific). Transcript stability was determined as the ratio between transcript abundance at 6 h (pulse-chase/pulse) to 0.5 h (pulse).

Random forest

The Classification And REgression Training (CARET) package from R was used to build a random forest classification model (72). Ten-fold cross-validation was performed to construct and evaluate the model. The ROC curves were plotted for all folds' prediction using the pROC package from R (73). Pathway analysis for miR-26a-5p was derived using mirPath (69). For DmiRNAs identified by nSolver, nCounter data by sample were normalized to the top 100 most highly expressed miRNAs across all samples and differential levels were examined by nSolver differential expression testing menu between groups. miRNA ratios between groups and statistical significance *P*-values were obtained, which were adjusted

for multiple testing by the p.adjust function in R to calculate FDRs.

Statistical analysis

Raw data were analyzed using NanoStringDiff software to identify statistically significant DmiRNAs ($P < 0.05$) using normalized counts between sample groups. Data were expressed as mean ± standard error of the mean. Prism 5.01 (GraphPad, San Diego, CA, USA) was also used to analyze statistical significance ($P < 0.05$, two-tailed Student's *t*-test).

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. The authors declare that they have no competing interests related to the direct applications of this research.

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RESEARCH ARTICLE

Occupational history associates with ALS survival and onset segment

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Abstract

Objective: To identify associations between occupational settings and self-reported occupational exposures on amyotrophic lateral sclerosis (ALS) survival and phenotypes. *Methods*: All patients seen in the University of Michigan Pranger ALS Clinic were invited to complete an exposure assessment querying past occupations and exposures. Standard occupational classification (SOC) codes for each job and the severity of various exposure types were derived. Cox proportional hazards models associated all-cause mortality with occupational settings and the self-reported exposures after adjusting for sex, diagnosis age, revised El Escorial criteria, onset segment, revised ALS Functional Rating Scale (ALSFRS-R), and time from symptom onset to diagnosis. Multinomial logistic regression models with three categories, adjusted for age, assessed the association between occupational settings and exposures to onset segment. *Results*: Among the 378 ALS participants (median age, 64.7 years; 54.4% male), poorer survival was associated with work in SOC code "Production Occupations" and marginally with work in "Military Occupation"; poor survival associated with self-reported occupational pesticide exposure in adjusted models. Among onset segments: cervical onset was associated with ALS participants having ever worked in "Buildings and Grounds Cleaning and Maintenance Occupations," "Construction and Extraction Occupations," and "Production Occupations"; bulbar onset with self-reported occupational exposure to radiation; and cervical onset with exposure to particulate matter, volatile organic compounds, metals, combustion and diesel exhaust, electromagnetic radiation, and radiation. *Conclusion*: Occupational settings and self-reported exposures influence ALS survival and onset segment. Further studies are needed to explore and understand these relationships, most advantageously using prospective cohorts and detailed ALS registries.

Keywords: *Amyotrophic lateral sclerosis, occupation, survival, phenotype*

Introduction

Amyotrophic lateral sclerosis (ALS) is a complex and fatal neurodegenerative disease caused by genetic and non-genetic factors. Of the non-genetic factors, exposures in occupational, residential, and avocational settings linked to ALS risk include pesticides and metals exposure (1). Identifying and confirming risk factors are critical to pinpoint modifiable ALS risks. Recently we reported that occupational exposure to particulate matter, volatile organic compounds, combustion and diesel exhaust, and especially metals, along with a history of working in production occupations, correlated with increased ALS risk (2). We have found that persistent organic pollutant exposure associates with ALS risk (3) and survival (4). Therefore, identifying links between occupational factors and ALS survival and onset segment could have important implications on understanding how exposures influence the progression and presentation of disease. This study uses a prospective ALS cohort to explore the association of ALS survival and phenotype with occupational histories and self-reported exposures.

Methods

Participants

Full cohort details were previously published (3–6). Briefly, all patients attending the University

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of Michigan Pranger ALS Clinic were invited to join this study provided they were older than 18 years. Participants were required to carry a diagnosis of ALS and to provide written informed consent in English. Participants were enrolled between June 2010 and March 2020. The study received Institutional Review Board (IRB) approval.

Data collection and processing

Participants completed a paper survey requesting information on four previous occupations: the current or most recent job, the job prior to the current/most recent, and the other two longest held jobs. For each job, participants provided the job title and description, and answered detailed prompts about occupational exposures using instruments modified from the Agency for Toxic Substances and Disease Registry (2). All responses were assessed for completeness and accuracy, and follow-up phone calls for clarifications were made as needed. Responses were entered into an electronic database and checked at random to ensure correct data entry. Jobs and job years following ALS symptom onset were removed. Standardized Occupation Classification (SOC) Coding was completed and exposure scores were derived for each job (2). Briefly, SOCs were initially generated using both National Institutes of Health (NIH) (SOCcer model version 2.0 (7), available via National Cancer Institute, https://soccer.nci.nih.gov/soccer/) and Centers for Disease Control (CDC) (NIOCCS; 2010 coding scheme available from https://www.cdc.gov/niosh/topics/coding/code.html) systems. SOCs that were discordant, had low confidence scores, could not be assigned using the two coding systems, occurred in a military setting, or had high exposure risk were reviewed by two exposure scientists (C.G., S.A.B.) for accuracy. Coding was blinded to clinical outcome data. Next, survey questions were assigned to nine exposure categories (particulate matter, volatile organic compounds, pesticides, metals, biologicals, combustion and diesel exhaust, electromagnetic radiation, and corrosives) and combined to generate composite exposure scores, as detailed previously (2).

Participant demographics (age, sex, race), date of symptom onset, date of diagnosis, and onset segment were abstracted from the survey and medical records. Participants were followed prospectively and last contact (death or censoring) was recorded. Surveys were returned by March 2020 and prospective survival data were collected through July 2021.

Statistical analysis

Demographic, clinical, and occupational characteristics for the study population were calculated. For each of the nine occupational exposure scores (2), Kaplan–Meier curves were generated and log-rank tests ascertained differences in survival for individuals with or without the respective exposures. To understand associations between occupational history and ALS survival, Cox models regressed job-years worked in each individual SOC code against survival post-diagnosis adjusted for sex, diagnosis age (quartiles), log-transformed time between symptom onset and diagnosis, revised El Escorial criteria (definite or not), onset segment (bulbar or not), and revised ALS functional rating scale (ALSFRS-R) at first visit (quartiles). The Benjamini–Hochberg procedure with a false discovery rate threshold of 0.2 determined significance after correcting for multiple testing (8). An adaptive lasso penalized Cox regression model with the same adjustment variables above fit job-years worked in all SOC codes simultaneously against survival. Because the distribution of job-years worked was right-skewed, Cox models were re-run using binary indicators of ever having worked in each SOC code as a sensitivity analysis. To estimate the mixture effect corresponding to all two-digit SOC codes simultaneously conditional on adjustment covariates, we use a Cox proportional hazards model with the framework of quantile g-computation (9). Non-parametric bootstrap is used to obtain standard error estimates, and are subsequently used to construct confidence intervals and p values. Quantile g-computation is implemented for Cox Proportional Hazards models in the qgcomp package in R. For occupational exposure scores, Cox models were fit for each exposure score with survival as the clinical endpoint adjusted for the same covariates as the SOC survival models. After the single exposure models, an unpenalized multivariable Cox model and an adaptive lasso penalized Cox model simultaneously modeled all exposures with survival, again adjusted for covariates. Because the occupational exposure scores were right-skewed, we also considered an unpenalized multivariable Cox regression model where exposure scores were treated as a binary variable (zero exposure vs. non-zero exposure), as a sensitivity check.

Also, as a small subset of participants survived much longer than average, survival analyses were repeated after restricting to those less than five years post-diagnosis to ensure that long survivors were not overly influential.

Next, occupational association with onset segment (bulbar, cervical, lumbar) was explored. Job-years and number of unique jobs worked grouped by onset segment were tabulated. Age-adjusted univariate multinomial logistic regression models associated job-years worked in each SOC code, and binary indicators of ever working in a job code, against onset segment. Age-adjusted

multinomial logistic regression associated continuous and binarized occupational exposure scores with onset segment. The Benjamini–Hochberg procedure with a false discovery rate threshold of 0.2 determined significance after correcting for multiple testing.

Analyses were performed in R version 4.1.1. Key packages used were glmnet version 4.1–2, nnet version 7.3–16, survival version 3.2–13, and survminer version 0.4–9.

Results

Participants

Of the 378 ALS participants with demographic, ALS phenotyping, and occupational exposure data, 96.3% completed all occupational exposure data from the questionnaire (Table 1).

Occupational settings and exposures

Of the 23 reported two-digit SOC codes, "Education, Training, and Library Occupations," "Sales and Related Occupations," "Management Occupations," "Production Occupations," and "Office and Administrative Support Occupations" accounted for the most job-years. "Construction and Extraction Occupations," "Healthcare Practitioners and Technical Occupations," "Education, Training, and Library Occupations," "Farming, Fishing, and Forestry Occupations," and "Legal Occupations" had the highest average job-years per participant (Supplemental Table 1).

Table 1. Participant demographics and results of univariable unadjusted Cox proportional hazards models.

Covariate	ALS Cases ($N = 378$)	HR	95% CI	p value
Age at diagnosis (years)[a]	64.7 (57.5–71.2)	1.03	(1.01, 1.04)	<0.001
Status				NA
Censored	92 (24.3)			
Observed Death	286 (75.7)			
Race				NA
American Indian and Alaska Native	1 (0.3)			
Black or African American	3 (0.8)			
White or Caucasian	374 (98.9)			
Sex				
Female	172 (45.5)	0.65	(0.51, 0.82)	<0.001
Male	206 (54.5)	Ref		
Military Service				
Enlisted	60 (15.9)	0.96	(0.70, 1.32)	0.807
Neither	318 (84.1)	Ref		
Education				
High-school or Less	106 (28.0)	Ref		
Some College, Associate's Degree	122 (32.3)	0.68	(0.51, 0.91)	0.010
Bachelor's Degree	85 (22.5)	0.55	(0.39, 0.76)	<0.001
Graduate Degree	61 (16.1)	0.52	(0.36, 0.75)	0.001
Missing	4 (1.1)			
Smoking Status				
Non-smoker	172 (45.8)	Ref		
Current Smoker	29 (7.7)	0.57	(0.35, 0.92)	0.022
Former Smoker	173 (45.8)	1.01	(0.79, 1.28)	0.965
Missing	4 (1.1)			
Revised El Escorial Criteria				
Possible	41 (10.8)	0.43		<0.001
Probable, LS	104 (27.5)	0.52		<0.001
Probable	127 (33.6)	0.67		0.008
Definite	94 (24.9)	Ref		
Suspected	12 (3.2)	0.28		<0.001
Onset Segment				
Bulbar	110 (29.1)	Ref		
Cervical	130 (34.4)	0.44	(0.33, 0.59)	<0.001
Lumbar	138 (36.5)	0.54	(0.40, 0.72)	<0.001
Time between symptom onset and diagnosis (years)[b]	1.04 (0.67-1.76)	0.75	(0.65, 0.87)	<0.001
Initial ALSFRS-R[c]	37 (33-41)	0.97	(0.95, 0.98)	<0.001

Table of descriptive statistics for the overall ALS participant study population. For continuous variables, median (25^{th}–75th percentile), and for categorical variables, N (%). Hazard ratios, 95% confidence intervals, and p values correspond to univariable unadjusted Cox proportional hazards models.

ALS: amyotrophic lateral sclerosis; ALSFRS-R: Revised ALS Functional Rating Scale; CI: confidence interval; HR: hazard ratio; LS: lab supported; Ref: reference category.

[a]Interpretation of hazard ratio is for one year change in age at diagnosis.

[b]Interpretation of hazard ratio is for one log-year change in time between symptom onset and diagnosis.

[c]About 369 out of 378 have observed ALSFRS-R. Interpretation of ratio is for one point change in ALSFRS-R.

Exposure to particulate matter, volatile organic compounds, and metals were most frequent (Supplemental Table 2).

Survival associations

We first evaluated associations between occupation and ALS survival. In adjusted models, an additional five years worked corresponded to a hazard ratio (HR) of 1.06 (95% CI 1.00–1.12, p value = 0.040, q-value = 0.416) in "Production Occupations" (Table 2). No SOC codes were significant after multiple comparisons correction. Furthermore, no occupations were selected by adaptive lasso regression. The estimated hazard ratio corresponding to a per quartile increase in the joint exposure to all two-digit SOC codes conditional on adjustment covariates is 0.99 (95% CI: 0.76, 1.29; $p = 0.948$), suggesting that there is no evidence of an occupational mixture effect on ALS survival. Although not statistically significant, perhaps owing to only eight participants, "Military Occupation" correlated with a marginally significant shorter survival in unadjusted models (HR = 2.04, 95% CI 0.87–4.81, p value = 0.103, q-value = 0.572; adjusted HR = 2.22, 95% CI 0.97–5.07,

Table 2. Job-years worked with two-digit SOC codes associated with survival: single SOC code models.

		Unadjusted Model				Adjusted Model			
Two-digit SOC Code	Description	HR	95% CI	p value	Q-Value (BH)	HR	95% CI	p value	Q-Value (BH)
11-0000	Management Occupations	1.00	(0.93, 1.08)	0.977	0.981	0.99	(0.92, 1.08)	0.894	0.987
13-0000	Business and Financial Operations Occupations	1.05	(0.96, 1.13)	0.277	0.647	1.02	(0.94, 1.11)	0.690	0.987
15-0000	Computer and Mathematical Occupations	0.92	(0.76, 1.13)	0.446	0.855	0.87	(0.69, 1.09)	0.214	0.614
17-0000	Architecture and Engineering Occupations	0.92	(0.84, 1.01)	0.087	0.572	0.96	(0.87, 1.06)	0.442	0.923
19-0000	Life, Physical, and Social Science Occupations	0.82	(0.61, 1.09)	0.169	0.602	0.77	(0.57, 1.04)	0.086	0.416
21-0000	Community and Social Services Occupations	1.06	(0.9, 1.25)	0.501	0.871	1.09	(0.92, 1.3)	0.332	0.764
23-0000	Legal Occupations	0.85	(0.67, 1.08)	0.183	0.602	0.83	(0.65, 1.06)	0.141	0.539
25-0000	Education, Training, and Library Occupations	1.02	(0.94, 1.1)	0.651	0.871	1.02	(0.94, 1.1)	0.608	0.987
27-0000	Arts, Design, Entertainment, Sports, and Media Occupations	0.92	(0.78, 1.08)	0.309	0.647	0.89	(0.76, 1.05)	0.180	0.590
29-0000	Healthcare Practitioners and Technical Occupations	0.98	(0.9, 1.07)	0.706	0.871	1.00	(0.92, 1.09)	0.987	0.987
31-0000	Healthcare Support Occupations	1.02	(0.83, 1.26)	0.844	0.925	0.89	(0.71, 1.12)	0.321	0.764
33-0000	Protective Service Occupations	1.05	(0.88, 1.25)	0.574	0.871	1.04	(0.87, 1.25)	0.638	0.987
35-0000	Food Preparation and Serving Related Occupations	**1.21**	**(1.06, 1.39)**	**0.006**	0.146	1.15	(0.98, 1.34)	0.081	0.416
37-0000	Building and Grounds Cleaning and Maintenance Occupations	0.97	(0.83, 1.13)	0.730	0.871	1.00	(0.86, 1.17)	0.967	0.987
39-0000	Personal Care and Service Occupations	0.96	(0.83, 1.12)	0.611	0.871	0.89	(0.77, 1.02)	0.091	0.416
41-0000	Sales and Related Occupations	0.98	(0.9, 1.06)	0.627	0.871	1.00	(0.92, 1.08)	0.959	0.987
43-0000	Office and Administrative Support Occupations	1.04	(0.99, 1.09)	0.124	0.572	1.00	(0.95, 1.06)	0.934	0.987
45-0000	Farming, Fishing, and Forestry Occupations	1.10	(0.95, 1.28)	0.211	0.608	1.01	(0.87, 1.17)	0.896	0.987
47-0000	Construction and Extraction Occupations	0.95	(0.86, 1.04)	0.294	0.647	0.98	(0.89, 1.08)	0.651	0.987
49-0000	Installation, Maintenance, and Repair Occupations	1.02	(0.92, 1.13)	0.757	0.871	1.03	(0.93, 1.15)	0.567	0.987
51-0000	Production Occupations	**1.06**	**(1, 1.12)**	**0.042**	0.484	**1.06**	**(1.00, 1.12)**	**0.040**	0.416
53-0000	Transportation and Material Moving Occupations	1.00	(0.9, 1.12)	0.981	0.981	1.01	(0.9, 1.13)	0.918	0.987
55-0000	Military Occupations	2.04	(0.87, 4.81)	0.103	0.572	**2.22**	**(0.97, 5.07)**	**0.058**	0.416

Single standard occupational classification (SOC) Cox models where outcome is survival post-diagnosis in years for the number of job-years worked within two-digit SOC codes. Covariates in adjusted models are sex, age at diagnosis (quartiles), log-transformed time between symptom onset and diagnosis, El Escorial criteria (definite or not), onset segment (bulbar or not), and ALSFRS-R at first visit (quartiles). Interpretation of hazard ratios (HR) correspond to five additional years worked within the SOC code. BH is the Benjamini–Hochberg p value. Statistically significant or marginally significant values in bold.

Table 3. Occupational exposure scores associated with survival.

	Univariate Model with Exposure Scores as Continuous Variable				Multivariable Model with Exposure Scores as Continuous Variable			Adaptive lasso
Exposure Score	*N*	HR	95% CI	*p* value	HR	95% CI	*p* value	HR
Particulate Matter	378	1.02	(0.9, 1.16)	0.771	0.97	(0.74, 1.27)	0.835	1.000
Volatile Organic Compounds	378	1.14	(1.01, 1.29)	0.038	1.07	(0.89, 1.29)	0.451	1.000
Pesticides	370	1.29	(1.15, 1.46)	0.000	1.25	(1.09, 1.44)	0.002	1.246
Metals	378	1.11	(0.97, 1.26)	0.134	1.08	(0.85, 1.38)	0.541	1.000
Biologicals	373	1.01	(0.88, 1.14)	0.934	0.98	(0.86, 1.11)	0.750	1.000
Combustion/Diesel Exhaust	376	0.95	(0.84, 1.08)	0.426	0.97	(0.84, 1.12)	0.674	1.000
Electromagnetic radiation	378	1.06	(0.94, 1.2)	0.340	1.08	(0.93, 1.25)	0.315	1.000
Radiation	378	0.98	(0.87, 1.1)	0.725	0.93	(0.81, 1.06)	0.271	1.000
Corrosives	375	0.93	(0.81, 1.07)	0.298	0.88	(0.74, 1.04)	0.140	1.000

Results of three types of Cox models for survival post-diagnosis in years: univariate models with occupational exposure scores as a continuous variable; multivariable models with occupational exposure scores as a continuous variable, and adaptive lasso penalized models with occupational exposure scores as a continuous variable. Covariates in all models include sex, age at diagnosis (quartiles), log-transformed time between symptom onset and diagnosis, El Escorial criteria (definite or not), onset segment (bulbar or not), and initial ALSFRS-R (quartiles). CI: confidence interval; HR: hazard ratio.

value = 0.058, q-value = 0.416). Excluding long surviving participants did not significantly alter the results (Supplemental Table 3). In models further adjusted for education and smoking status, the HR for "Production Occupations" was no longer significant (HR = 1.04, 95% CI 0.98–1.11, *p* value = 0.207); however, occupation and education were correlated (Supplemental Table 4).

We next investigated associations between self-reported occupational exposure and survival (Table 3). Kaplan–Meier plots for each occupational exposure category differed significantly only for pesticides, which had a median survival of 1.49 years (IQR 1.14–2.43) versus 2.32 years (IQR 1.39–3.92; *p* value = 0.046) for those without this exposure (Figure 1, Supplemental Figure 1). In multivariable models and adaptive lasso, mortality rate increased for exposures to pesticides (HR = 1.25, 95% CI 1.09–1.44, *p* value = 0.002 and HR = 1.246, respectively). Results were similar after adjusting for education and smoking status (HR = 1.26, 95% CI 1.09–1.46, *p* value = 0.002) (Supplemental Table 5). The ever-exposed (binary model, occupational exposure score > 0) sensitivity analysis showed similar results (Supplemental Table 6). Across all models, occupational exposure to pesticides consistently correlated with poorer ALS survival. Multivariable models excluding long surviving participants also yielded similar results (Supplemental Table 7).

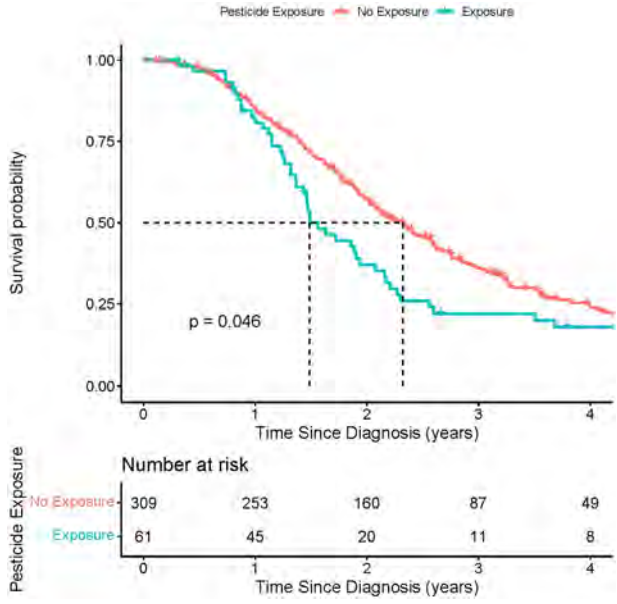

Figure 1. Kaplan–Meier survival plot by occupational pesticide exposure. Kaplan–Meier survival plot comparing ALS participants who report occupational exposure to pesticides (blue line; median survival = 1.49 years) versus those who do not report occupational pesticide exposure (red line; median survival = 2.32 years; *p* value = 0.046).

Onset segment associations

The association of onset segment with occupational settings and exposures was investigated using multinomial logistic regression models. Initial models examined duration of each occupation by SOC code (Table 4, Supplemental Figure 2). For every five years of work in "Construction and Extraction Occupations," the odds of cervical compared with lumbar onset increased 31% (OR = 1.31, 95% CI = 1.02–1.68, *p* value = 0.031, q-value = 0.846) after adjusting for age at diagnosis. This same association was seen when considering whether a participant "ever-worked" in an occupation (Supplemental Table 8).

Similar analyses correlated several occupational exposure scores with onset segment (Table 5, Figure 2). Using the continuous self-reported occupational exposure scores adjusted for age at diagnosis, for every five years of work: the odds of bulbar onset disease relative to lumbar onset was 1.38 (95% CI 1.01–1.88, *p* value = 0.040, q-value = 0.103) for radiation exposure; the odds of cervical onset relative to lumbar onset was higher for exposures to particulate matter (OR = 1.49, 95%

Table 4. SOC codes associated with onset segment.

		Unadjusted model with SOC as continuous variable								Adjusted model with SOC as continuous variable							
		Bulbar vs lumbar				Cervical vs lumbar				Bulbar vs lumbar				Cervical vs lumbar			
Two-digit SOC Code	Description	OR	95% CI	p value	Q-Value (BH)	OR	95% CI	*p* value	Q-Value (BH)	OR	95% CI	*p* value	Q-Value (BH)	OR	95% CI	*p* value	Q-Value (BH)
11-0000	Management Occupations	1.02	(0.87, 1.2)	0.786	0.974	1.04	(0.89, 1.21)	0.656	0.974	1.00	(0.85, 1.18)	0.954	0.979	1.05	(0.9, 1.23)	0.533	0.846
13-0000	Business and Financial Operations Occupations	0.98	(0.82, 1.19)	0.867	0.974	0.93	(0.76, 1.13)	0.460	0.961	0.96	(0.8, 1.16)	0.705	0.979	0.93	(0.75, 1.14)	0.460	0.846
15-0000	Computer and Mathematical Occupations	1.03	(0.7, 1.51)	0.875	0.974	1.03	(0.71, 1.48)	0.888	0.974	0.99	(0.68, 1.46)	0.972	0.979	1.04	(0.72, 1.51)	0.836	0.979
17-0000	Architecture and Engineering Occupations	1.01	(0.84, 1.2)	0.951	0.974	0.94	(0.78, 1.13)	0.506	0.966	1.01	(0.84, 1.21)	0.922	0.979	0.93	(0.77, 1.12)	0.436	0.846
19-0000	Life, Physical, and Social Science Occupations	0.47	(0.15, 1.47)	0.196	0.855	0.90	(0.59, 1.36)	0.609	0.966	0.45	(0.14, 1.51)	0.198	0.846	0.87	(0.57, 1.31)	0.500	0.846
21-0000	Community and Social Services Occupations	0.74	(0.39, 1.41)	0.364	0.855	1.05	(0.76, 1.45)	0.776	0.974	0.70	(0.36, 1.34)	0.279	0.846	1.04	(0.75, 1.44)	0.836	0.979
23-0000	Legal Occupations	1.00	(0.74, 1.34)	0.974	0.974	0.77	(0.45, 1.34)	0.358	0.855	0.99	(0.73, 1.33)	0.941	0.979	0.77	(0.44, 1.36)	0.371	0.846
25-0000	Education, Training, and Library Occupations	0.97	(0.83, 1.13)	0.669	0.974	0.93	(0.79, 1.09)	0.372	0.855	0.94	(0.81, 1.1)	0.469	0.846	0.94	(0.8, 1.1)	0.424	0.846
27-0000	Arts, Design, Entertainment, Sports, and Media Occupations	1.00	(0.77, 1.32)	0.972	0.974	0.83	(0.56, 1.22)	0.335	0.855	1.02	(0.77, 1.35)	0.884	0.979	0.83	(0.56, 1.23)	0.352	0.846
29-0000	Healthcare Practitioners and Technical Occupations	1.10	(0.89, 1.36)	0.361	0.855	1.10	(0.9, 1.35)	0.355	0.855	1.11	(0.9, 1.37)	0.317	0.846	1.10	(0.9, 1.35)	0.345	0.846
31-0000	Healthcare Support Occupations	1.16	(0.85, 1.57)	0.343	0.855	0.65	(0.33, 1.27)	0.207	0.855	1.15	(0.85, 1.57)	0.362	0.846	0.66	(0.33, 1.3)	0.229	0.846
33-0000	Protective Service Occupations	0.68	(0.27, 1.69)	0.404	0.885	1.06	(0.72, 1.55)	0.777	0.974	0.68	(0.28, 1.64)	0.387	0.846	1.09	(0.74, 1.6)	0.662	0.979
35-0000	Food Preparation and Serving Related Occupations	1.01	(0.76, 1.34)	0.957	0.974	0.83	(0.58, 1.19)	0.306	0.855	1.01	(0.76, 1.34)	0.961	0.979	0.82	(0.56, 1.2)	0.298	0.846
37-0000	Building and Grounds Cleaning and Maintenance Occupations	0.96	(0.66, 1.38)	0.806	0.974	1.09	(0.81, 1.47)	0.578	0.966	1.02	(0.7, 1.47)	0.930	0.979	1.07	(0.79, 1.45)	0.671	0.979
39-0000	Personal Care and Service Occupations	0.77	(0.52, 1.15)	0.209	0.855	0.85	(0.63, 1.15)	0.303	0.855	0.78	(0.52, 1.15)	0.209	0.846	0.86	(0.63, 1.17)	0.325	0.846
41-0000	Sales and Related Occupations	1.05	(0.88, 1.26)	0.565	0.966	1.02	(0.85, 1.22)	0.807	0.974	1.06	(0.88, 1.27)	0.522	0.846	1.02	(0.85, 1.22)	0.821	0.979
43-0000	Office and Administrative Support Occupations	1.03	(0.92, 1.15)	0.601	0.966	0.99	(0.89, 1.11)	0.865	0.974	1.01	(0.9, 1.13)	0.882	0.979	1.00	(0.89, 1.12)	0.979	0.979
45-0000	Farming, Fishing, and Forestry Occupations	0.99	(0.7, 1.41)	0.965	0.974	0.92	(0.62, 1.37)	0.685	0.974	0.96	(0.67, 1.36)	0.810	0.979	0.92	(0.62, 1.37)	0.682	0.979
47-0000	Construction and Extraction Occupations	1.21	(0.93, 1.57)	0.158	0.855	**1.32**	**(1.03, 1.68)**	**0.026**	0.855	1.22	(0.94, 1.6)	0.136	0.846	**1.31**	**(1.02, 1.68)**	**0.031**	0.846
49-0000	Installation, Maintenance, and Repair Occupations	0.86	(0.63, 1.18)	0.351	0.855	1.13	(0.94, 1.37)	0.197	0.855	0.85	(0.63, 1.15)	0.281	0.846	1.15	(0.94, 1.39)	0.173	0.846
51-0000	Production Occupations	1.11	(0.97, 1.27)	0.113	0.855	1.09	(0.96, 1.25)	0.187	0.855	1.11	(0.97, 1.26)	0.144	0.846	1.10	(0.96, 1.26)	0.165	0.846
53-0000	Transportation and Material Moving Occupations	0.94	(0.75, 1.18)	0.604	0.966	0.89	(0.7, 1.13)	0.328	0.855	0.92	(0.73, 1.16)	0.500	0.846	0.89	(0.69, 1.13)	0.340	0.846
55-0000	Military Occupations	4.64	(0.36, 59.7)	0.239	0.855	2.51	(0.17, 36.84)	0.502	0.966	3.93	(0.29, 53.92)	0.306	0.846	2.79	(0.18, 43.39)	0.463	0.846

Results of multinomial logic regression models associating standard occupational classification (SOC) codes with onset segment. Shows unadjusted models with SOC codes treated as total years of exposure and adjusted models with SOC codes treated as total years of exposure Interpretation of odds ratios correspond to five additional years worked within the respective SOC code. Adjusted multinomial logistic regression models are adjusted with age at diagnosis (quartiles). BH: Benjamini–Hochberg; CI: confidence interval; OR: odds ratio. Statistically significant results are bolded.

Table 5. Occupational exposure scores associated with onset segment.

	Exposure score as a continuous variable							
	Bulbar vs lumbar				Cervical vs lumbar			
Exposure Score	OR	95% CI	*p* value	Q-Value (BH)	OR	95% CI	*p* value	Q-Value (BH)
Particulate Matter	1.11	(0.83, 1.49)	0.476	0.659	**1.49**	**(1.15, 1.92)**	**0.002**	0.043
VOCs	1.04	(0.79, 1.38)	0.770	0.866	**1.32**	**(1.03, 1.69)**	**0.027**	0.103
Pesticides	0.97	(0.72, 1.3)	0.828	0.877	**1.25**	**(0.98, 1.59)**	**0.077**	0.173
Metals	1.06	(0.8, 1.4)	0.687	0.825	**1.31**	**(1.02, 1.68)**	**0.033**	0.103
Biological Exposures	1.01	(0.68, 1.51)	0.959	0.959	1.31	(0.96, 1.8)	0.089	0.177
Combustion (Diesel)	1.15	(0.87, 1.52)	0.335	0.502	**1.30**	**(1.01, 1.67)**	**0.040**	0.103
Electromagnetic	1.24	(0.93, 1.67)	0.145	0.260	**1.39**	**(1.06, 1.83)**	**0.016**	0.103
Radiation	**1.38**	**(1.01, 1.88)**	**0.040**	0.103	**1.43**	**(1.06, 1.92)**	**0.018**	0.103
Corrosives	1.09	(0.82, 1.45)	0.538	0.692	1.19	(0.92, 1.53)	0.178	0.292

Interpretation of odds ratios correspond to a standard deviation increase in the respective occupational exposure score. Adjusted multinomial logistic regression models are adjusted for age at diagnosis (quartiles). BH: Benjamini–Hochberg; CI: confidence interval; OR: odds ratio. Statistically significant or near significant results are bolded.

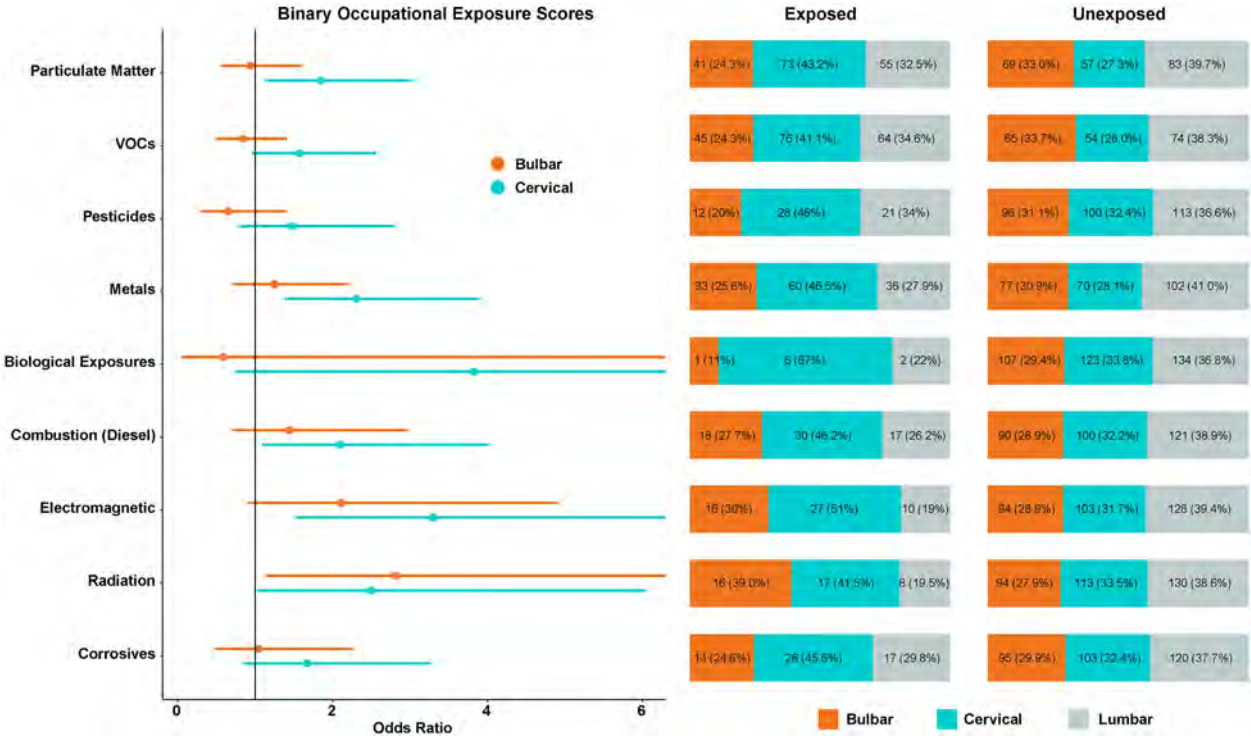

Figure 2. Onset segment multinomial logistic regression model and distribution. On the left, forest plot of the adjusted multinomial logistic regression models from Table 5 showing the odds ratio for bulbar and cervical onset with lumbar onset as the reference. On the right, the distribution of each onset segment by exposed and unexposed.

CI 1.15–1.92, *p* value = 0.002, q-value = 0.043), volatile organic compounds (OR = 1.32, 95% CI 1.03–1.69, *p* value = 0.027, q-value = 0.103), metals (OR = 1.31, 95% CI 1.02–1.68, *p* value = 0.033, q-value = 0.103), combustion and diesel exhaust (OR = 1.30, 95% CI 1.01–1.67, *p* value = 0.040, q-value = 0.103), electromagnetic radiation (OR = 1.39, 95% CI 1.06–1.83, *p* value = 0.016, q-value = 0.103), and radiation (OR = 1.43, 95% CI 1.06–1.92, *p* value = 0.018, q-value = 0.103). Pesticides (OR = 1.25, 95% CI 1.02–1.68, *p* value = 0.077, q-value = 0.173) and biological exposures (OR = 1.31, 95% CI 0.96–1.80, *p* value = 0.089, q-value = 0.177) also favored cervical onset after FDR correction. Overall, findings were similar for logistic regression models that considered exposure scores as binary, ever-exposed variables (Supplemental Table 9).

Discussion

Previously, we have shown that plasma-persistent organic pollutant levels correlate with ALS survival (4) and self-reported occupational exposures associate with ALS risk (2). Here, we investigated whether occupational settings, via SOC codes, and self-reported exposures are linked to survival and onset segment and found several associations in this Michigan ALS cohort. First, regarding survival, we identified that work in "Production

Occupations" correlated with a decrease in ALS survival. Lack of significance following multiple comparison testing could be related to small sample size rather than from a lack of an effect. In addition, work in "Military Occupation" associated with a large reduction in survival, although the effect was only marginally significant. Military service is a known ALS risk (10) and raises the possibility of additional or cumulative exposures that may impact disease progression. Future research with larger sample sizes is encouraged to determine if these marginal associations, including the joint effect of military and other exposure types, are confirmed. Larger samples might also reveal interaction effects, such as between education and occupation, which would better identify culpable exposures and potentially lead to interventions to reduce ALS risk.

Few studies have explored the role of occupation on ALS survival. In the Cancer Prevention Study cohort, occupations associated with increased ALS mortality included programmers and laboratory technicians in males and machine assemblers in females (11). A mortality study showed an excess number of ALS deaths among persons working in the computer and mathematical, architecture and engineering, legal, and education, training, and library fields (12). However, few studies have explored the survival duration by occupational exposures.

The "Production Occupations" SOC code encompasses a wide range of occupational settings and tasks, e.g. assemblers, fabricators, and operators in food, metal, plastic, printing, textile, wood, and other industries. These workers may experience exposures to metals, fumes, and industrial chemicals and may perform repetitive and strenuous tasks. Regarding "Military Occupations," veterans are exposed to a range of hazards, including herbicides, pesticides, heavy metals, combustion products, jet fuels, and chemicals released from burn pits (10), many already linked to ALS risk (13,14). Thus, workers in such specific SOCs have increased likelihood of exposure to certain hazards, e.g. metals, fumes, and other toxics. Although two-digit SOC job codes are very broad and only a subset of workers in a particular code is likely to receive a specific exposure, we still found that several SOCs were correlated with ALS survival.

We next examined self-reported exposure and survival. Our analysis showed that self-reported pesticide exposure is associated with poorer survival, a finding consistent across several different analytical models. This aligned with our previous reports, which identified persistent organic pollutants including pesticides in blood as both ALS risk factors (3) and determinants of disease progression and survival (4). Moreover, our methods showed consistency with studies utilizing self-reported questionnaires to identify occupational exposures (15, 16). Pesticides in current use, e.g. organophosphates, can cause widespread occupational exposure to farm workers, building and grounds maintenance staff, and production and distribution workers in the agrichemical industry, which can be assessed by self-reported questionnaires. Recently, a study of 94 ALS participants recruited from the United States National ALS Registry reported no survival association with occupational exposure to agricultural chemicals based on exposure classification by a single industrial hygienist (17). Our study classified exposures based on assessments from two trained assessors using self-reported data, methods established in the literature (15, 16). However, since we did not classify exposures with an industrial hygienist, we could not confirm or account for the intensity of particular exposures, e.g. frequency, duration and level of exposure events, and recall bias are always a concern with retrospective studies.

Regarding our second study goal, the linkage of SOC or self-reported exposure with onset segment, we identified several important associations in this cohort. Specifically, cervical onset disease correlated with "Construction and Extraction Occupations" when job duration was considered, and with "ever-working" in "Buildings and Grounds Cleaning and Maintenance Occupations," "Construction and Extraction Occupations," and "Production Occupations." Workers in these occupations typically perform strenuous and repetitive physical movements, especially in the upper body. Whether this leads to injury in the cervical motor neurons or reflects the physical activity risk with ALS is unknown. Strenuous physical activity associates with ALS risk, most recently shown via genetic risk (18) and a survey of vigorous leisure-time physical activity in the National ALS Registry (19). Professions characterized by strenuous physical activity linked to ALS include manual laborers (20–24), professional athletes (25–28), and military veterans (29, 30). With respect to correlations with onset segment, in contrast to our findings, a recent Maltese study found that workers in construction occupations (31) and agricultural workers in Brittany, France (24) tended to have bulbar onset. In professional Italian soccer players, bulbar onset was predominant, although the numbers were small (25). In Israeli triathletes, bulbar onset was more frequent (32). This paucity of studies warrants further research, especially given the possible cumulative impact of physical activity with other exposure types.

When we examined the relationship of self-reported exposures instead of SOC codes to segment onset, radiation exposure was linked to bulbar and cervical onset disease as opposed to lumbar onset disease. Radiation can cause injury to the

brainstem and spinal cord; the cervical spine may be more susceptible to damage compared with the thoracic spine due to lower dispersion of radiation across the tissue (33), which might explain the pattern we observed. This exposure only occurred in a subset of workers in the "Healthcare Practitioners and Technical Occupations" and "Healthcare Support Occupations." In contrast, most other occupational scores correlated with cervical onset disease, which may reflect a cumulative effect in individuals exposed to pollutants in occupational settings with strenuous upper body physical activity. An Australian study did find that workers exposed to metals tended to have limb-onset disease (16).

Causes of phenotypic heterogeneity in ALS remain largely unknown. Some genetic mutations are associated with predisposition to certain onset segments, but these are largely in rare genes (13, 14). Therefore, the finding that some occupations lead to a higher odds of cervical onset disease is of interest in terms of our appreciation for why disease starts in certain areas of the nervous system.

This study has strengths. The use of expert assessment of occupation with SOC codes and self-reported occupational exposures improves the accuracy of assigning exposures (34, 35). Moreover, the dual approach of leveraging self-reported exposures and exposure scores in addition to SOC codes overcomes the weaknesses of using SOC codes alone. Occupational exposure scores yield insight beyond the occupational SOC codes, which tend to be broad and encompass diverse workplace activities and exposure types. Thus, sole reliance on SOC codes can lead to exposure misclassification, including both false positives and false negatives. For example, workers in managerial roles in job codes usually associated with specific exposures, e.g. volatile organic compounds in the petrochemical industry, may not actually be exposed if not working at the production site. Conversely, some workers in "Management Occupations" or "Architectural and Engineering Operations" working in production facilities or at construction sites may potentially be exposed to hazards. Thus, we accounted for such situations by considering both SOC codes and self-reported exposures. Moreover, this allowed us to investigate environmental effects in ALS, which is a rare disease, making large prospective cohorts difficult. Furthermore, our analysis included many ALS participants recruited from a single center, which captured a large fraction of ALS cases in Michigan. Next, the consistency of results across multiple analyses improved the confidence of our results.

This study also has limitations. Although all patients seen in our Pranger ALS Clinic are invited to participate, ALS is not a reportable disease in the State of Michigan and therefore we were unable to recruit from all incident cases in the State. Furthermore, we could not account for bias that exists if people in certain occupations preferentially seek care at another center or choose not to receive care at all. Furthermore, we are unable to account for bias if certain occupations or occupational exposures are differentially associated with a rapid disease progression, if individuals do not survive to diagnosis, or if individuals are so overwhelmed at the time of diagnosis that they choose not to enroll in observational research, i.e. recruitment bias. In addition, we could neither isolate nor confirm the exposures associated with a specific job from the SOC code. For example, physical activity is linked to ALS through polygenic risk (18); however, the association we identified of survival with "Production Occupations" may be linked to the level of regular physical activity required to perform the job as opposed to the job setting. This might be addressed by adjusting our analyses for activity level, but we did not collect data on occupational physical activity levels. Models that adjusted for education and smoking did not show the same association with "Production Occupations." Workers in this occupational category typically report lower educational attainment, which could suggest confounding in prior studies that associated educational attainment with increased ALS risk. In this case, the true driver may be workplace exposures to chemicals, particles, and other physical stressors. Although we show associations with pesticides, we did not capture specific pesticide formulations, which have changed considerably over time. In our study, many workers had long job tenures and often transitioned to similar jobs, which could involve the same exposure types. Thus, older workers may have had occupational exposure to both "legacy" pesticides, like organochlorines, as well as currently used pesticides. Also, it is extremely challenging to quantify occupational exposure over the life course, although a combination of job history, self-reported exposures, and biological monitoring can help address this important gap. Further unknown is whether exposures are only relevant in certain critical exposure windows. Leveraging prospective cohorts of workers in specific occupations provides a possible alternative, but very large sample sizes would be needed given the low incidence of ALS. In addition, creative approaches are also needed to collect the necessary data given the fragmented reporting of this disease.

Conclusion

Few prior studies have linked occupational job or exposures to ALS survival. Working in "Production Occupations" and self-reported occupational exposure to pesticides worsened ALS survival, as did "Military Occupations" although this

occupation was only marginally significant. In addition, several occupations associated with typically strenuous physical activity were associated with cervical onset disease. Occupational settings and self-reported occupational exposures have important associations with ALS phenotypes. These intriguing findings regarding the impact of occupations and occupational exposures on ALS survival and phenotypes help build the case for prospective cohorts and registries to better assess disease risk.

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Author contributions

S.A. Goutman, MD, MS: Drafting/revising the manuscript for content, study concept and design, analysis and interpretation of data.

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Declaration of interest

SAG served on a DSMB. The other authors declare they have no competing interests.

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Data availability statement

Sharing of non-identifiable data will be considered at the reasonable request of a qualified investigator.

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CLINICAL RESEARCH ARTICLE

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Body mass index associates with amyotrophic lateral sclerosis survival and metabolomic profiles

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Abstract

Introduction/Aims: Body mass index (BMI) is linked to amyotrophic lateral sclerosis (ALS) risk and prognosis, but additional research is needed. The aim of this study was to identify whether and when historical changes in BMI occurred in ALS participants, how these longer term trajectories associated with survival, and whether metabolomic profiles provided insight into potential mechanisms.

Methods: ALS and control participants self-reported body height and weight 10 (reference) and 5 years earlier, and at study entry (diagnosis for ALS participants). Generalized estimating equations evaluated differences in BMI trajectories between cases and controls. ALS survival was evaluated by BMI trajectory group using accelerated failure time models. BMI trajectories and survival associations were explored using published metabolomic profiling and correlation networks.

Results: Ten-year BMI trends differed between ALS and controls, with BMI loss in the 5 years before diagnosis despite BMI gains 10 to 5 years beforehand in both groups. An overall 10-year drop in BMI associated with a 27.1% decrease in ALS survival ($P = .010$). Metabolomic networks in ALS participants showed dysregulation in sphingomyelin, bile acid, and plasmalogen subpathways.

Discussion: ALS participants lost weight in the 5-year period before enrollment. BMI trajectories had three distinct groups and the group with significant weight loss in the past 10 years had the worst survival. Participants with a high BMI and increase in weight in the 10 years before symptom onset also had shorter survival. Certain metabolomics profiles were associated with the BMI trajectories. Replicating these findings in prospective cohorts is warranted.

KEYWORDS
amyotrophic lateral sclerosis, body mass index, metabolism, prognosis, survival

Abbreviations: AFT, accelerated failure time; ALS, amyotrophic lateral sclerosis; BMI, body mass index; GEE, generalized estimating equation; LDL-C, low-density lipoprotein cholesterol; OR, odds ratio; rEEC, revised El Escorial criteria; SOD1, superoxide dismutase 1.

1 | INTRODUCTION

Amyotrophic lateral sclerosis (ALS) diagnosis is preceded by a presymptomatic phase, characterized by initiation of the disease process but lacking pronounced clinical symptoms.[1–3] ALS patients frequently experience a rapid decrease in body mass index (BMI) and the rate of loss early in the disease course is a strong prognostic factor.[4] Therefore, BMI loss may reflect an early and presymptomatic manifestation of disease. Indeed, individuals with ALS develop BMI loss many years before symptom onset.[5] Additionally, lower BMI earlier in life may both increase ALS risk[5–9] and decrease ALS survival.[5,10]

BMI decreases in ALS patients are linked to lower energy intake from dysphagia and higher energy expenditure,[11,12] including hypermetabolism, altered glucose and lipid metabolism, and mitochondrial dysfunction.[13] Perturbations in metabolism in ALS are supported by correlations in basic lipid profiles with risk and outcomes. Increased low-density lipoprotein cholesterol (LDL-C) and apolipoprotein B levels years before ALS diagnosis are associated with a higher future risk of ALS onset[14]; higher levels of both at diagnosis also associated with a lower risk of death.[15] However, basic lipid profiles do not capture the full spectrum of metabolic changes that occur in disease. Rather, the metabolome and lipidome, the cumulative profile of all metabolites and lipids, may more comprehensively reflect the metabolic state. Indeed, metabolomics profiles correlate with BMI[16–18] and disease phenotypes, such as cardiometabolic risk.[16,17] In the future, metabolomics signatures may be useful in combination with BMI as predictors of disease outcomes.[16]

However, the correlation of BMI with metabolomics profile and disease outcomes has not been investigated in ALS. Thus, our goal in this study was to leverage our case/control study to examine trends in BMI trajectory in ALS vs control participants correlated with survival and metabolomics profile.

2 | METHODS

2.1 | Participants and samples

Recruitment and data collection procedures are published.[19–22] Briefly, all patients seen at the Pranger ALS Clinic at University of Michigan with an ALS diagnosis, age at least 18 years, and ability to consent in English were asked to participate. Neurologically healthy controls, recruited through population outreach, completed the same procedures. All participants provided oral and written informed consent and the study was approved by the institutional review board of the University of Michigan. Demographic characteristics and available previous heights and weights from the medical records of the participants were obtained, as were ALS disease characteristics according to the Revised El Escorial criteria (rEEC).[23] Participants were asked to self-report height in feet and inches and weight in pounds 10 years ago, 5 years ago, and at the present time. For ALS participants, present weight was typically equivalent to weight at diagnosis since enrollment occurred shortly after diagnosis. BMI was calculated from height and weight as follows: weight (kg) / [height (m)]^2.[24] ALS participants with an interval of more than 5 years from symptom onset to diagnosis were not included in the analysis as the goal was to investigate presymptomatic differences in BMI. A subset of participants provided plasma for metabolomics analysis, as described elsewhere.[25,26]

2.2 | Descriptive analysis

Descriptive statistics were calculated for demographic characteristics including age, sex, ALS disease-onset segment frequencies, and disease duration (time from symptom onset to diagnosis). Study population differences were compared between BMI groups by analysis-of-variance and chi-square tests. Lin's concordance correlation coefficient quantified agreement between available self-reported and measured BMIs.

2.3 | BMI progression analysis and group assignment

Generalized estimating equations (GEEs) with unstructured correlation structure assessed differences in BMI changes for ALS and control participants, while accounting for within-participant correlation between self-reported BMI measurements.[27] The GEE outcome was self-reported BMI and the covariates were interaction terms between ALS/control status and the three time-points adjusted for age and sex at study entry. Differences in average BMI rate of change between ALS and controls were assessed using the Wald test and performed with the R geepack package (R Foundation for Statistical Computing, Vienna, Austria).[28]

After subtracting self-reported BMI 10 years before consent from all time-points, *k*-means clustering for longitudinal data (kml R package[29]) grouped ALS cases based on their self-reported changes in BMI, for use in ALS survival models. This subtraction step ensured that the *k*-means procedure clustered exclusively on BMI changes over time, rather than differences in baseline BMI. After considering two to six clusters, the selected number of clusters maximized the Calinski and Harabasz criterion,[30] a measure of between-cluster variation relative to within-cluster variation for longitudinal data.[31] The distance metric used for clustering was Euclidean distance with Gower adjustment.[31]

2.4 | Survival analysis

Kaplan-Meier plots of survival from diagnosis by cluster were produced. Cox proportional hazards models determined associations between cluster groups and ALS survival, defined as the time from diagnosis to death. Associations were adjusted for sex, age, baseline BMI (ie, 10 years earlier), onset segment, diagnosis rEEC, and time from symptom onset to diagnosis. Proportional hazards assumptions were checked using global and individual Schoenfeld tests with graphical assessment of the rescaled Schoenfeld residuals over time. Due to

proportional hazards violations in some models, accelerated failure time (AFT) models were constructed.

2.5 | Sensitivity analyses

Sensitivity analyses for incomplete BMI data (inverse probability weighted models) and nonlinear effects of BMI were performed (Supplemental Methods). $P < .05$ was considered statistically significant for the analyses.

2.6 | Metabolomics data analysis

Plasma samples from ALS participants were analyzed by Metabolon (Morrisville, North Carolina), with data published previously in case-control analyses.[25,26] Plasma samples were nonfasting as this was considered unethical for many ALS participants. Metabolomics analysis included data set normalization, computing correlations between BMI and metabolites, and selecting metabolites associated with BMI trajectory via least absolute shrinkage and selection operator (lasso) regression (Supplemental Methods). To identify highly interconnected metabolic modules, further analysis included construction of a partial correlation network using a previously published sparse partial correlation algorithm,[32–34] followed by consensus clustering.[35] Finally, group-penalized lasso regression (group lasso) models were created to identify metabolic modules associated with BMI clusters (Supplemental Methods). Group lasso[36] is a generalization of lasso regression, which has the advantage of incorporating before information on the grouping structure of the covariates, that is, the metabolic modules in this instance. Analyses were performed with R version 4.0.2.

3 | RESULTS

3.1 | Participants

For those with BMIs observed at all three time-points, ALS participants represented a typical patient population, according to onset age, distribution of segment onset, among other variables. Controls (n = 266) were slightly younger than cases (n = 381) (Table 1). Two ALS participants with an uncertain onset segment and one control with a BMI greater than 100 kg/m^2 labeled as an outlier were removed from subsequent analysis.

3.2 | BMI trends in cases vs controls

Lin's concordance correlation coefficient showed consistency between self-reported and measured BMI values (Supplemental Results). ALS and control participants reported BMI increases in the

TABLE 1 Participants' demographics

Covariate	Overall (n = 647)	ALS cases (n = 381)	Controls (n = 266)	*P* value
Mean age at survey consent, years	63.3 (56.5-69.9)	64.9 (57.6-71.4)	61.3 (55.2-68.2)	<0.001
Sex				0.143
Female	317 (49.0)	177 (46.5)	140 (52.6)	
Male	330 (51.0)	204 (53.5)	126 (47.4)	
Last contact event				NA
Death		251 (64.9)	NA	
Censored		130 (34.1)	NA	
Original and/or revised El Escorial criteria				NA
Possible/suspected		53 (13.9)	NA	
Probable, LS		104 (27.3)	NA	
Probable		123 (32.3)	NA	
Definite		101 (26.5)	NA	
Onset segment				NA
Bulbar		113 (29.7)	NA	
Cervical		126 (33.1)	NA	
Lumbar		142 (37.3)	NA	
Time between symptom onset and diagnosis (years)		1.01 (0.64-1.66)	NA	NA

Note: For continuous variables, data expressed as median (25th to 75th percentile); for categorical variables, data expressed as number (%). *P* values for continuous and categorical variables correspond to analysis of variance and chi-square tests, respectively.
Abbreviations: ALS, amyotrophic lateral sclerosis; LS, laboratory supported; NA, not applicable.

TABLE 2 Accelerated failure time model

	Percent change in survival	LCL	UCL	*P* value
Age at entry (years)	−1.0	−1.9	−0.2	0.016
Symptom onset to diagnosis (log years)	17.3	3.3	33.2	0.014
Baseline BMI	−1.0	−2.7	0.8	0.278
Decrease BMI trajectory	−27.1	−42.6	−7.3	0.010
Increase BMI trajectory	−7.1	−25.2	15.5	0.509
Male	0.1	−16.1	19.4	0.994
Cervical onset	41.0	13.0	76.0	0.002
Lumbar onset	21.3	−1.4	49.3	0.068
rEEC possible/suspected	88.3	41.9	149.7	0.000
rEEC probable	23.4	−0.7	53.3	0.058
rEEC probable, laboratory supported	61.6	28.5	103.1	0.000

Abbreviations: BMI, body mass index; LCL, lower confidence limit; rEEC, revised El Escorial criteria; UCL, upper confidence limit.

10- to 5-year period before study entry (Figure S1). Unlike controls, however, ALS cases had an overall BMI decrease in the 5-year prior-to-study entry time window. The age- and sex-adjusted GEE model showed average ALS BMI change from −5 years to 0 year was 1.75 kg/m^2 (95% confidence interval [CI], 1.35 to 2.16 kg/m^2; $P < 1 \times 10^{-17}$), but was only 0.02 kg/m^2 for controls (95% CI, −0.35 to 0.40 kg/m^2; $P = .9$). Thus, ALS participants report BMI loss occurring 5 years before diagnosis/study entry, whereas control participants had no significant BMI change during the same time-frame. The kml algorithm applied to the ALS participant BMI trajectories identified three clusters, defined as decrease, mild decrease, and increase BMI groups (Supplemental Results, Figure S2, and Table S1).

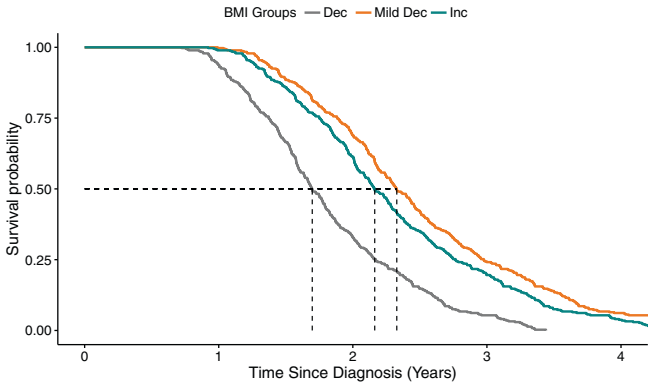

FIGURE 1 Accelerated failure time model plots. Covariate adjusted survival curves corresponding to the unweighted accelerated failure time model with body mass index (BMI) cluster groups. The estimated median survival time is 1.7 years for the decrease BMI group, 2.33 years for the mild decrease BMI group, and 2.16 years for the increase BMI group. Dec, decrease; Mild dec, mild decrease; Inc, increase.

3.3 | Survival analysis

Unadjusted Kaplan-Meier survival analysis showed decreased absolute median survival times for the decrease BMI cluster (Figure S3). Some Cox models violated proportional hazards by Schoenfeld residuals, so AFT models were constructed. After adjusting for age, sex, baseline BMI (ie, 10 years earlier), onset segment, rEEC, and time from symptom onset to diagnosis, participants in the decrease BMI cluster had a 27.1% shorter survival (95% CI, −42.6% to −7.3%; $P = .010$) vs the mild decrease group (Table 2 and Figure 1). Results were similar in missing BMI data sensitivity analyses and when using base BMI as a categorical variable (see Supplemental Results, Figures S4 and S5, and Tables S2, S3, S4, and S5). Interestingly, in sensitivity analyses for interaction effects between baseline BMI and change in BMI over time, ALS participants with an obese baseline BMI and increase BMI trajectory had shorter survival, similar to participants in the decrease BMI trajectory group (Table S5).

3.4 | Metabolites associated with BMI trajectory

Metabolomic differences by BMI cluster (decrease, mild decrease, increase) were investigated for the 207 participants with available previously published untargeted metabolomics.[25,26] The final curated data set included 607 metabolites from plasma collected near the time of diagnosis. Associations of individual metabolites with BMI trajectory groups are presented in the Supplemental Results and Tables S6 and S7.

The partial correlation network was constructed using recently published data from 349 ALS participants,[26] of whom 207 were also in this analysis. Including additional samples generated a more informative network because partial correlation methods are sensitive to sample size. The resulting partial correlation network contained 600 metabolites connected by 887 edges (false discovery rate–adjusted $P < .1$), of which 31 had a negative partial correlation coefficient. Seven metabolites did not have any significant correlations and were not included in the network. Consensus clustering identified 26 metabolic modules spanning 555 highly connected metabolites. The remaining 45 metabolites did not

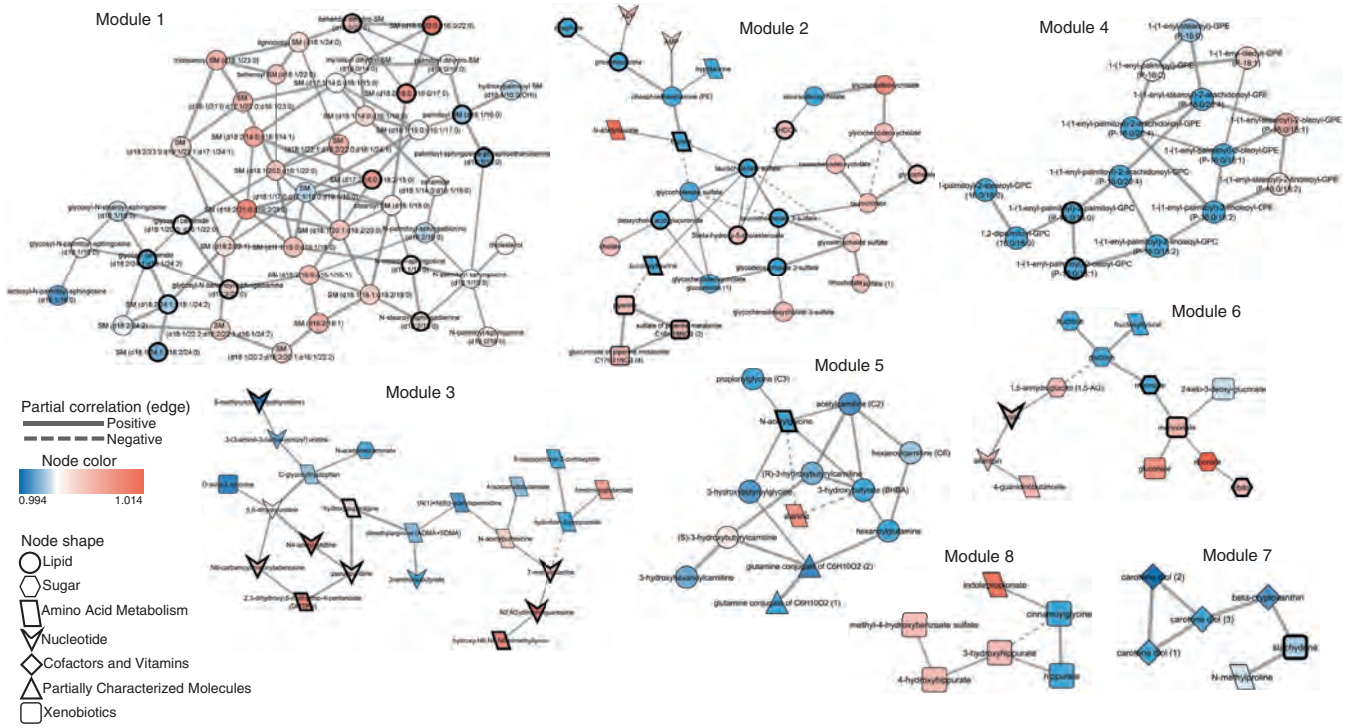

FIGURE 2 Metabolic modules associated with body mass index (BMI) trajectory. Eight metabolic modules containing 152 total metabolites associated with BMI trajectory in group lasso regression models. Node color indicates odds ratio (OR) from group lasso: OR > 1 indicates association with the increase BMI cluster (red node), OR < 1 indicates association with the decrease BMI cluster (blue node). Nodes with a bold border correlate significantly with current BMI (false discovery rate < 0.05). Node shape indicates the subpathway to which the metabolite belongs. Solid edge between metabolites indicates positive partial correlation coefficient. Dashed edge indicates negative partial correlation coefficient.

TABLE 3 Metabolomics modules from group lasso associate with BMI trajectory groups

Metabolic module	Number of nodes (metabolites)	Number of edges	Average degree[a]	Metabolic pathways
1	47	88	3.76	Ceramides, sphingomyelins
2	30	41	1.367	Bile acid metabolism, amino acid and purine metabolism
3	22	23	2.09	Amino acid, nucleotide metabolism
4	15	21	2.8	Plasmalogens, lyso-plasmalogens, phosphatidylcholines
5	13	18	2.77	Fatty acid metabolism (acyl carnitines, acyl amino acids)
6	13	12	1.85	Carbohydrate, amino acid, nucleotide metabolism
7	6	6	2	Vitamin A metabolism, amino acid metabolism
8	6	6	2	Benzoate metabolism, amino acid metabolism

[a]Average degree represents the average number of connections each node (metabolite) makes within the module and indicates the network/module density.

cluster due to poor connectivity. Metabolic module size ranged from 5 to 66 metabolites (Figure 2).

Group lasso selected eight modules containing 152 metabolites, which associated with the decrease and increase BMI clusters (Figure 2, Table 3, and Table S8), with odds ratios (ORs) ranging from 0.92 to 1.1 (Table S9). The largest module 1 (47 metabolites) included ceramides and sphingomyelins, of which 36 had OR > 1, indicating associations with the increase BMI cluster. The second largest module 2 (30 metabolites) included primary and secondary bile acid metabolites, taurine and its derivatives, AMP, ADP, and sterols. Primary bile

acids associated with the increase BMI cluster (OR > 1), whereas most secondary bile acids and taurine metabolites associated with the decrease BMI cluster (OR < 1). Module 3 (22 metabolites) primarily contained amino acid and nucleotide metabolites, half of which associated with the decrease BMI cluster. Module 4 (15 metabolites) was composed of plasmalogens, lyso-plasmalogens, and phosphatidylcholines, 11 of which associated with the decrease BMI cluster. Module 5 (13 metabolites) had mostly acyl carnitines, acyl amino acids, and some other amino acid metabolites, which mostly associated with the decrease BMI cluster. The remaining smaller module 6 (13 metabolites; sugar and nucleotide metabolites, xenobiotics, amino-sugar), module 7 and module 8 (6 metabolites each; xenobiotics, cofactors, vitamins, modified amino acids) contained various metabolites.

Overall, these results suggest that unique metabolomic profiles correlate with BMI trends in participants with ALS, especially metabolism centered on ceramides, sphingomyelins, and primary and secondary bile acids.

4 | DISCUSSION

This study adds to the growing body of evidence that presymptomatic BMI loss is linked to ALS risk and survival. We have shown that ALS participants are characterized by significant BMI loss at 5 years, but not 10 years, before study entry as compared with control participants. A decrease in BMI trajectory was associated with shorter survival in ALS, which also correlated with a distinct metabolomic profile. Our study also suggests that BMI loss may occur during the presymptomatic phase of ALS leading up to diagnosis. Several other studies have similarly shown BMI decrease preceding ALS diagnosis, out to 10 years before onset,[5] and even within the decades preceding ALS.[6,9] Although we found BMI trajectories differed over the 10-year window, absolute BMI did not vary between ALS and control participants 10 years before study entry when participants would have had a mean age of 54.9 (ALS) and 51.3 (controls) years. In contrast, other studies reported that lower mid- to late-life BMI increases ALS risk,[8,9,37] although one study reported ALS survival depends on BMI change, not on BMI before or at diagnosis.[4] Another recent study has suggested that BMI in ALS patients diverges from controls 10 years before disease onset.[38]

Next, we found that that ALS participants with a 10-year decrease BMI trend had shorter survival. Our results are consistent with several studies demonstrating that a drop in BMI before ALS diagnosis correlates with poorer survival.[4,5,9,39] In particular, an analysis of the Piemonte and Valle d'Aosta Register for ALS showed that BMI loss at diagnosis was more prognostic of survival than BMI either before or at diagnosis.[4] However, because there is literature showing that BMI is an ALS risk factor,[8,9,37] we conducted sensitivity analyses to assess the interaction of baseline BMI with BMI trajectory. We found that normal baseline BMI lengthened survival in the decrease BMI trajectory group, whereas obese baseline BMI shortened survival in the increase BMI trajectory group. Baseline BMI only marginally influenced survival in the mild decrease BMI trajectory group. Interestingly, the European Prospective Investigation into Cancer and Nutrition study also showed that obese females had shorter survival, which did not reach significance,[22] but the Piemonte and Valle d'Aosta Register showed no impact of BMI on survival.[4]

The reasons for survival differences by BMI or BMI change in ALS are not known. However, the prevailing theories are related to impaired energy homeostasis,[11] with lowered energy intake fighting against higher energy expenditure. Dysphagia is a frequent cause of reduced energy intake; however, in ALS, BMI loss also occurs independent of dysphagia,[4,39] indicating the presence of significantly elevated energy expenditure. Indeed, one study showed that hypermetabolism was more frequent in ALS than in control participants and correlated inversely with survival.[12] Resting energy expenditure may additionally interact with BMI and fat mass to influence survival in ALS.[40,41]

In our study, we employed data-driven network analysis to identify highly interconnected metabolic modules and we assessed their correlation with BMI trajectory groups. The largest of these, module 1, contained ceramides (13 species) and sphingomyelins (33 species). The latter were primarily associated with the increase BMI group. In earlier work, we and others found that sphingomyelins also differ in analyses of ALS vs control participant plasma.[25,26,42–45] Further, one recent study reported that higher sphingomyelin levels may correlate with faster disease progression.[45] Sphingomyelins are a large class of lipids that have structural roles in cell membranes and lipid rafts, and, through hydrolysis to ceramides, with signaling activity, for example, pro-apoptotic, excitotoxic, neurotoxic.[46,47] Impaired sphingomyelin metabolism may be an integral factor in ALS as supported by investigations of genetic models.[48] Of the 47 metabolites in module 1, only 13 correlated significantly with BMI at diagnosis, suggesting associations of the remaining 34 metabolites with BMI trajectory may be related to the ALS disease process.

The second largest module, module 2, mostly contained primary and secondary bile acids, which generally associated with the increase BMI trajectory, in addition to metabolites of methionine, cysteine, *S*-adenosyl methionine, and taurine metabolism and oxidative phosphorylation. Nearly half of the metabolites in this module also correlated significantly with diagnosis BMI (13 species). Bile acids play important roles in nutrient absorption, regulation of cholesterol metabolism, and systemic energy expenditure,[49] so the correlation with BMI trajectory herein is unsurprising. Interestingly, although not present in the module, two bile acids, ursodeoxycholic and its taurine derivative tauroursodeoxycholic acid (taurursodiol), showed some efficacy in ALS clinical trials.[50–53]

Module 3 contained modified amino acids and nucleotide derivatives spanning 22 species evenly split between the decrease and increase BMI groups, of which 9 correlated significantly with diagnosis BMI. Module 4 contained several bioactive lipids, plasmalogens (10 species), lyso-plasmalogens (3 species), and phosphatidylcholines (2 species), which associated mostly with the decrease BMI group, that is, poorer survival. Only two species were significantly linked with diagnosis BMI. We[26] and others[42,45,54,55] have shown that phosphatidylcholines differentiate ALS from control participants, in particular phosphatidylcholine 36:4.[45,54]

Modules 5 and 6 comprised candidates related to energy metabolism. Module 5 contained four short-chain acyl-carnitines intermediates, which all save one correlated with the decrease BMI group. We previously reported acyl-carnitines, along with free fatty acids, contributed to the discrimination between ALS vs control participants,[25,26] which we attributed to either dysfunctional or at-capacity β-oxidation.[56] Modules 6, 7, and 8 contained few metabolites equally divided in their correlation with either the decrease or increase BMI trajectory group, suggesting ALS status may be a stronger determinant of these metabolites than BMI trajectory.

Overall, across some modules, such as module 5, there were more metabolites from various biochemical pathways relating to energy utilization (eg, fatty acid β-oxidation) that are more discerning of ALS vs control participants than of BMI trajectories. These findings suggest that ALS status is a major determinant of energy metabolism. One possibility is that metabolites correlate with fat mass loss in ALS patients,[57] an idea supported by studies where ALS polygenic risk associates with body fat percentage in addition to BMI.[58,59] Interestingly, neither creatine nor creatinine were among the metabolites correlating with BMI change or diagnosis BMI, indicating weight changes may be more pronounced for fat mass than muscle mass. However, lacking body composition measures, we could not evaluate this possibility in this study.

This study has limitations. Participants self-reported weight, potentially incurring recall bias; however, Lin's concordance correlation coefficient was high for participants with available weight, indicating good recall. Our study did not query weight at frequent intervals, so we cannot determine whether BMI loss in ALS participants was linear in the 5 years before study entry or more pronounced closer to diagnosis. It is also possible we failed to detect an onset in BMI changes between the 10- to 5-year window before diagnosis due to the lack of granular BMI information. Next, we only asked participants to report current height, and use this for BMI calculations at all time-points. However, such changes in height over the life course are not anticipated to cause bias in statistical models.[60] We also did not collect a dietary or physical activity survey for this analysis. Additionally, our metabolomics analysis was untargeted, and thus did not measure all metabolites in every relevant biochemical pathway. Although BMI analysis was longitudinal, metabolomics analysis was cross-sectional. Plasma samples for untargeted metabolomics were nonfasted for ethical reasons, as noted in our earlier publications.[25,26]

In conclusion, we found that ALS participants have distinct BMI trajectories vs controls, with the most significant BMI drop occurring within 5 years before diagnosis. ALS participants with normal baseline BMI and decrease BMI trajectory, or baseline obese BMI and increase BMI trajectory, have shorter survival. BMI trajectories correlate with metabolic changes, especially with sphingomyelins and bile acids.

AUTHOR CONTRIBUTIONS

Stephen Aaron Goutman: Conceptualization; data curation; funding acquisition; investigation; project administration; writing – original draft; writing – review and editing. **Jonathan Boss:** Formal analysis; writing – review and editing. **Gayatri Iyer:** Formal analysis; writing – review and editing. **Hani Habra:** Formal analysis; writing – review and editing. **Masha Savelieff:** Writing – review and editing. **Alla Karnovsky:** Formal analysis; writing – review and editing. **Bhramar Mukherjee:** Conceptualization; formal analysis; writing – review and editing. **Eva L Feldman:** Funding acquisition; investigation; project administration; resources; supervision; writing – review and editing.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

DATA AVAILABILITY STATEMENT

Deidentified data may be made available to qualified investigators upon reasonable request.

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We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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cGAS/STING and innate brain inflammation following acute high-fat feeding

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Obesity, prediabetes, and diabetes are growing in prevalence worldwide. These metabolic disorders are associated with neurodegenerative diseases, particularly Alzheimer's disease and Alzheimer's disease related dementias. Innate inflammatory signaling plays a critical role in this association, potentially *via* the early activation of the cGAS/STING pathway. To determine acute systemic metabolic and inflammatory responses and corresponding changes in the brain, we used a high fat diet fed obese mouse model of prediabetes and cognitive impairment. We observed acute systemic changes in metabolic and inflammatory responses, with impaired glucose tolerance, insulin resistance, and alterations in peripheral immune cell populations. Central inflammatory changes included microglial activation in a pro-inflammatory environment with cGAS/STING activation. Blocking gap junctions in neuron-microglial co-cultures significantly decreased cGAS/STING activation. Collectively these studies suggest a role for early activation of the innate immune system both peripherally and centrally with potential inflammatory crosstalk between neurons and glia.

KEYWORDS

cGAS/STING, acute, innate inflammation, microglia, high fat diet

Introduction

Global incidences of obesity, prediabetes, and diabetes are increasing worldwide (1, 2). Obesity rates have burgeoned in recent years, growing to pandemic proportions (3). Global diabetes rates topped 463 million in 2019, with an estimated additional 374 million people having impaired glucose tolerance and prediabetes (1). This alarming rise in the rates of obesity and metabolic disease predispose individuals to complications of

the central nervous system (CNS), including mild cognitive impairment, Alzheimer's disease or Alzheimer's disease related dementias (AD/ADRD) (4–6).

Chronic inflammation and immune system dysregulation are common in individuals with obesity and in individuals who fall along the continuum of metabolic dysfunction from prediabetes to frank type 2 diabetes (7). Previous studies investigating the effects of metabolic dysfunction on the CNS report dysregulation of immune and inflammatory mechanisms, typically increased glial activation and elevated production of CNS pro-inflammatory proteins and mediators (8–10). Specifically, a high-fat diet (HFD) in mice induces an inflammatory phenotype in microglia, the resident immune cells of the CNS (11, 12). Additionally, HFD or other CNS pro-inflammatory events increase trafficking of peripheral immune cells into the brain (13–15), further promoting neuroinflammation.

Although evidence supports a role for CNS inflammation in obesity, prediabetes, and diabetes, previous studies primarily focus on later disease time points, and few have investigated how HFD-induced obesity and prediabetes impact short-term inflammatory changes. Innate inflammatory pathways with an acute response to damage or danger signals may potentially respond to metabolic stress to mediate early CNS responses to HFD. In a dysmetabolic environment, elevated fatty acids can activate innate inflammatory mechanisms and upregulate pro-inflammatory cytokine production (16, 17). This in turn up-regulates downstream feed-forward mechanisms, such as signaling through the interferon-α receptor (18), which further contributes to a pro-inflammatory environment.

One innate inflammatory pathway implicated in the cellular response to metabolic dysfunction is the cGAS/STING (cyclic GMP-AMP/stimulator of interferon genes) pathway (19–21). cGAS/STING is a cytosolic double-stranded DNA (dsDNA) sensing pathway, which responds to viral or bacterial dsDNA as well as self dsDNA, *e.g.*, from damaged nuclei or mitochondria *via* cGAS and working through its adaptor molecule STING and transcription factors interferon regulatory factor 3 (IRF3) and nuclear factor kappa beta (NFkB) to upregulate pro-inflammatory gene expression. In the periphery or peripheral cells, HFD or treatment with the saturated fatty acid palmitate upregulates cGAS/STING signaling (22). cGAS/STING also contributes to pro-inflammatory feed forward mechanisms *via* inflammatory crosstalk between neighboring cells *via* gap junctions (23). Further, cGAS/STING is implicated in the pathology of CNS neurodegenerative diseases, such as AD/ADRD (24–26), Parkinson's disease (27), and amyotrophic lateral sclerosis (28), and may thus constitute a "bridge" between metabolic dysfunction and cognitive impairment.

In the current study, we examined CNS activation of the cGAS/STING pathway in mice fed a high fat diet (HFD) for 3 d. We focused our studies on the primary immune cells of the brain, microglia, capable of inflammatory crosstalk with neurons *via* gap junctions (23). We leveraged our HFD mouse model, which develops obesity and prediabetes along with cognitive impairment upon acute and chronic feeding (29). We observed systemic changes in metabolic and inflammatory responses, with impaired glucose tolerance, insulin resistance, and alterations in peripheral immune cell populations after just 3 d of HFD. We also identified central inflammatory changes, with microglial and cGAS/STING pathway activation. Additionally, in our neuron-microglial co-culture system, reducing cell to cell inflammatory crosstalk by blocking gap junctions significantly reduced cGAS/STING activation. These findings support an early role for cGAS/STING in response to HFD *via* neuron-glial inflammatory crosstalk and suggest a pivotal role for acute activation of innate immune mechanisms in the CNS in response to global metabolic dysfunction.

Materials and methods

Experimental animals and study design

Four-wk-old C57BL/6J male mice obtained from The Jackson Laboratory (catalog # 000664; Bar Harbor, ME). Animals were housed with no more than five littermates per cage in a pathogen free room at 20 ± 2 °C with a 12 h light/dark cycle at the University of Michigan Unit for Laboratory Animal Medicine and monitored daily by veterinary staff. Animals were provided food and water *ad libitum* and a minimum of one enrichment item (nestlet and/or enviropak). Following a 1 or 2 wk acclimation period, animals were assigned randomly to their respective diets (Research Diets, New Brunswick, NJ) as follows: standard diet (SD; 10% calories from fat; catalog # D12450J) or high-fat diet (HFD; 60% calories from fat; catalog # D12492). A subset of animals were used for cognitive phenotyping (see puzzle box), which was performed on day 2 of diet and for a duration of 3d. Animals were sacrificed (detailed below) on the final day of puzzle box (4 d on diet). For all other animals, after 3 d on diet mice underwent glucose tolerance testing (see metabolic phenotyping) and were sacrificed (detailed below) the next day (4 d on diet). Four hours prior to euthanasia, animals were fasted and a subset of animals within both the SD and HFD groups were given intraperitoneal injection of either saline (5 mL/kg body weight [BW]) or lipopolysaccharide (LPS; catalog # tlrl-3pelps, Invivogen, San Diego, CA) at a dose of 500 μg LPS/kg BW in total volume of 5 mL/kg BW saline. At terminal, animals were euthanized *via* intraperitoneal injection of 150 mg/kg pentobarbital (Fatal-Plus, Vortech Pharmaceuticals, Dearborn, MI). Blood was removed from the vena cava and animals were perfused with phosphate buffered saline prior to removal of tissues. Cortex tissue was used to determine *ex vivo* CNS insulin sensitivity using western blotting, plasma and hemi-brains for immunophenotyping using flow cytometry, plasma for inflammatory cytokines using ELISA, hemi-brains for microglial morphology using

immunohistochemistry, and hippocampal tissue for cGAS/STING pathway protein expression using Western blotting (all methods detailed below). The University of Michigan's Institutional Animal Care and Use Committee approved all animal protocols (PRO0010039).

Metabolic phenotyping and immunophenotyping

Glucose tolerance testing (GTT) was performed after 3 d of diet as previously (30, 31). Briefly, 10% D-glucose at 1g glucose/1kg body weight was injected intraperitoneally after a 4 h fast and glucose measurements taken at baseline and 15-, 30-, 60-, and 120-min post injection. Blood glucose levels were determined from a tail blood sample using a glucometer (AlphaTRAK, Abbot Laboratories, Chicago, IL) and appropriate glucose strips (Zoetis, Parsippany, NJ).

After 4 d HFD feeding, immunophenotyping was performed on peripheral blood samples and on CNS tissue using flow cytometry (32) to determine circulating immune cell populations, as previously published (32, 33). Fluorescently labeled leukocytes were classified by staining with antibodies (Biolegend, San Diego, CA) for well-characterized surface markers (Table 1). Briefly, doublets were excluded using forward scatter width (FSC-W) and forward scatter height (FSC-H) where events farther than 10% from the diagonal were excluded. In both tissue types, lymphocytes were characterized as CD45+, SSC-low cells expressing CD3 and either CD4+ or CD8+, while NK cells were characterized as CD45+, SSC-low, CD3–, NK1.1+, and CD49b+. B cells in the periphery were characterized as CD45+, SSC-low, CD3-, and CD19+ and were not detectible in the CNS. Myeloid populations in the blood were characterized as CD45+ and CD11b+: neutrophils were Ly6G+ while monocytes were Ly6G– and either Ly6C– or Ly6C+. In the CNS, myeloid cells were CD45+, CD3–, CD19. Ly6G+ cells were identified as neutrophils, Ly6G-, CD11b+, CD45-high, and Ly6C+ were identified as Ly6C+ monocytes, and Ly6G-, CD11b+, CD45-mid cells were identified as microglia. In both tissue types, monocytes, microglia, and neutrophils were further assessed for F4/80 or CD11c surface expression by their median fluorescent intensity as a proxy for activation state. A FACSAria II (BD Biosciences, San Jose, CA) was used to run samples and FlowJo software (FlowJo, Ashland, OR) to analyze results.

Microglial morphology

As previously (34), we performed analysis of microglial morphology for three regions of the hippocampus, the hilus, molecular layer, and CA1 regions. In brief, hemi-brains were dissected and fixed for 48 h in 4% paraformaldehyde. Following a sucrose gradient (10%, 20%, and 30% for 24 h each), hemi-brains were embedded in OCT and frozen at -80°C. Brains were sectioned (50 μm) and stained (rabbit anti-Iba1, 1:1000; catalog # 019-19741, Wako, Richmond, VA) in 6-well plates in floating tissue sections. Secondary antibody (anti-rabbit Alexa-fluor Plus 594, 1:2000; catalog # A32740, Invitrogen) and Hoechst nuclear stain were applied, and sections were mounted using ProLong Gold (Invitrogen). A Leica Stellaris 8 Falcon Confocal Microscope and a 40X oil immersion objective was used to take Z-stack images (30 μm). Images were processed with Imaris Software (Oxford Instruments) and open microscopy environment TIF files used to analyze microglial territorial volume, cell volume, percent occupied volume, average branch length, maximum branch length, minimum branch length, number of end points, and number of end points using a modified 3DMorph script in MATLAB (MathWorks, Natick, MA), as previously published (34).

TABLE 1 Flow cytometry antibodies for blood and CNS immune cell characterization.

	BV421	FITC	PE	PerCP-5.5	APC	PE-Cy7	APC-Cy7
Lymphoid (Blood and CNS)	CD8	CD3	Nk1.1	CD19	CD45	CD49b	CD4
CD4 T-cells	+	+	–	–	+	–	+
CD8 T-cells	+	+	–	–	+	–	–
NK cells	–	–	+	–	+	+	–
B cells (CNS; not detectible)	–	–	–	+	+	–	–
Myeloid (Blood)	Cd11c	Ly6c	F4/80	CD3/CD19	CD45	Ly6G	CD11b
Neutrophils	MFI	–	MFI	–	+	+	+
Ly6C- Monocytes	MFI	–	MFI	–	+	–	+
Ly6C+ Monocytes	MFI	+	MFI	–	+	–	+
Myeloid (CNS)							
Neutrophils	MFI	–	MFI	–	+	+	+
Ly6C+ Monocytes	MFI	+	MFI	–	+	–	+
Microglia	MFI	–	MFI	–	+/-	–	+

+/- (with/without).

Ex vivo insulin stimulation

On day 4 of diet after sacrifice and perfusion, right cortex was dissected and placed in a 12-well plate containing media (Neruobasal, 5% pen-strep, MN additives (Sigma, St Louis, MO); 10 mg/mL bovine serum albumin, 10 mg/mL apo-transferrin, 0.1 mg/mL biotin, 15 mg/mL D-galactose, 0.63 mg/mL progesterone, 16 mg/mL putrescine, 50 µg/mL selenium, 50 µg/mL β-estradiol, 50 µg/mL hydrocortisone, 16 mg/mL catalase, 2.5 mg/mL SOD). Tissue was finely minced with scissors and split into two microcentrifuge tubes (one for unstimulated control and one for insulin) containing 300 µL media. Tubes were placed into an incubator (37°C, 5% CO_2) for 30 min. Following the 30 min incubation, insulin (20 nM) or an equivalent volume of media was added to the appropriate tubes. Tubes were returned to the incubator for 45 min and inverted several times every 10-15 min. Following the 45 min incubation, tubes were spun down (1 min, 4°C, 17,000 g), media removed, and tissue snap frozen in liquid N_2. Tissue was maintained at -80°C for later Western blot (WB) analysis.

ELISA and WB

On day 4 of diet, blood was collected, and plasma isolated for inflammatory cytokine analysis *via* ELISA. ELISA was performed for TNF-α and MCP-1 by the University of Michigan Rogel Cancer Center Immunology Core. Cortex and hippocampal tissue as well as neuronal and microglia cells were homogenized in RIPA buffer (Pierce, Rockford, IL) with protease inhibitors (Roche Diagnostics, Indianapolis, IN), sonicated, and centrifuged (30 min, 4°C, 13,300 rpm) in preparation for WB, which was performed as previously published (35, 36). All samples were normalized for equal protein concentration prior to loading. Nitrocellulose membranes were blocked (Tris buffered saline [TBS], 0.01% Tween-20, 5% bovine serum albumin [BSA]) for 2 h, primary antibodies (varying concentrations in TBS, 0.01% Tween-20, 5% BSA) were incubated overnight at 4°C, and secondary antibodies (varying concentrations in TBS, 0.01% Tween-20, 5% milk) were incubated for 1.5 h at room temperature. SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) or Clarity Max (Biorad, Hercules, CA) was used to visualize signal and images were captured by a ChemiDoc (Biorad) or with x-ray film. Images were analyzed using ImageJ (37) or Image Lab software (Biorad). Insulin signaling primary antibodies were: pAkt (catalog # 4060), Akt (catalog # 4691), pIRS-1 (pSer307, catalog # 2381; pSer636/639, catalog # 2388), IRS-1 (catalog # 3407), all from Cell Signaling Technologies (Danvers, MA) and diluted at 1:1000. cGAS/STING pathway primary antibodies (Cell Signaling Technologies) were: cGAS (catalog # 31659S; 1:1000), STING (catalog # 50494S; 1:1000), pIRF3 (S396; catalog # 4947S; 1:500), total IRF3 (catalog # 4302S; 1:500), and NFκβ (catalog # 8242P; 1:500). Tubulin (catalog # ab6160; 1:20000; AbCam, Cambridge, MA) or histone (catalog # NB 100-56347; Novus Biologicals, Littleton, CO) were used as loading controls. IgG conjugated with horse radish peroxidase secondary antibodies used were anti-rabbit (catalog # 7074), anti-mouse, (catalog # 7076), and anti-rat (catalog # 7077S) all Cell Signaling Technologies.

Cell culture

Partially immortalized human hippocampal neurons (38) and an immortalized human microglia cell line (catalog # T0252; Applied Biological Materials, Richmond, BC, Canada) were used for *in vitro* studies. Cells were maintained in growth media in 6-well plates until 80-85% confluent. Neuron growth media was: N2b medium (customized media from Cytivia, Marlborough, MA) with 0.2 µM beta-estradiol (catalog # E4389; Sigma) and 10 µg/mL fibroblast growth factor basic (catalog # GF003AF; Millipore, Burlington, MA) and 1% heat-inactivated fetal bovine serum (FBS; catalog # MT35016CV; Corning, Corning, NY). Microglia growth media was: PriGroIII (catalog # TM003; Applied Biological Materials, Richmond, BC, Canada) and 10% non-heat inactivated FBS or DMEM (catalog # BW12741F; Lonza, Quakertown, PA) and 10% heat inactivated FBS for cytosolic dsDNA qPCR experiments. At 60-80% confluence, neurons were changed to differentiation media (NSDM, custom media, Cytiva, Global Life Sciences Solutions, Marlborough, MA for 8 d (39)). On differentiation day 9 for neurons and at 80-85% confluence for microglia, media was changed to treatment media (differentiation media without insulin for neurons and growth media without FBS for microglia) 5 h prior to experimental treatments. Following this, cells were treated with either palmitate alone (62.5 µM in microglia or 250 µM in neurons) or palmitate plus insulin (50 nM, both cell types) for 24 h (35, 40). At 24 h, cultures were washed, and cells were fixed for cytosolic dsDNA determination *via* immunocytochemistry or qPCR (below) or isolated for cGAS/STING pathway protein determination by WB (above).

Cytosolic dsDNA *via* qPCR

Cytosolic DNA isolation was performed as previously published (41). In brief, cells were lysed with RIPA buffer (Invitrogen, Waltham, MA), centrifuged (10 min, 4°C, 700*g*), and supernatant used to quantify and normalize protein concentrations. The pelleted nuclei/whole cell fraction was saved for downstream analysis. Normalized protein concentrations of the supernatant were spun further (30 min, 4°C, 10,000*g*) and the pellet (cytosolic fraction) saved. The pelleted nuclei/whole cell fractions and the pelleted cytosolic fractions were used to isolate DNA using a commercially

available kit (catalog # 80004, All prep DNA, RNA, and Protein mini kit; Qiagen, Germantown, MD). Nuclear (18S; 5'-TAG AGG GAC AAG TGG CGT TC-3' [forward] and 5'-CGC TGA GCC AGT CAG TGT-3' [reverse]) and mitochondrial DNA (cytochrome oxidase I; 5'- GCC CCC GAT ATG GCG TTT-3' [forward] and 5'- GTT CAA CCT GTT CCT GCT CC -3' [reverse]) were run on the nuclei/whole cell fractions and pelleted cytosolic fractions using qPCR SYBR green primers (above). Levels of cytosolic DNA were quantified using the ddC_T method (42), with the nuclei fraction used to normalize the cytosolic fraction and the mean ΔC_T of the BSA controls as the calibrator for all samples.

Puzzle box

To assess possible changes in cognition, we performed a modified version of the puzzle box task (43). In this task, mice are intrinsically motivated to move from the light area of the puzzle box into the dark area. On day 2 of diet, puzzle box testing was carried out over a period of 3 d, with a series of three single tasks repeated for a total of three replicates over the first 2 d. The single tasks were then combined into a 'complex' task, which was performed once on day 2 and 24 h later on day 3. Latency to 'escape' or to enter the dark area of the box was recorded for each of the tasks. Animals were allowed 5 min to perform each task. If the mouse was unable to escape the light area of the box after 5 min, it was removed from the box and its time recorded as 5 min.

Statistical analysis

We previously established that a sample size of n=8 per group (30, 44) provides adequate power to detect significant metabolic differences between groups. Statistical analyses were performed using Prism 9 (GraphPad Software, La Jolla, CA) using either t-test or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons. Alternatively, analysis of microglial morphology and CNS immunophenotyping data was performed using SAS 9.4 (SAS Institute, Cary, NC) using the Proc Mixed function. Anderson-Darling, D'Agostino-Pearson omnibus, Shapiro-Wilk, and Kolmogorov-Smirnov tests were used to determine normality, and non-normal data was log transformed to achieve normality. Statistical tests and software used for each analysis (glucose tolerance test, immunophenotyping, etc.) and the corresponding results section/figure are detailed in Supplemental Table 1. Statistical significance was defined as $p<0.05$ and trends as $p<0.10$. Unless otherwise indicated, results are presented as mean ± standard error of the mean (SEM).

Results

Acute HFD impairs metabolic but not cognitive responses

We previously showed that chronic HFD induces obesity and prediabetes (29), however little is known about the acute metabolic, inflammatory, and cognitive effects of HFD. Therefore, we examined the impact of acute HFD on both metabolic and cognitive function. To do so, BL6 mice were placed on either a HFD or a sucrose matched 10% fat standard diet (SD) for 4 d. GTT was performed on 3 d and mice were harvested for blood and tissue analysis on 4 d (Figure 1A). Within just 3 d, we observed HFD impaired glucose tolerance, with higher blood glucose levels at all time points of the glucose tolerance test, as well as a higher area under the curve versus SD mice (Figures 1B, C). We and others also previously observed CNS insulin resistance in mice following chronic HFD feeding (29, 45). However, changes in response to acute HFD were unknown. To investigate this, we measured the responsiveness of *ex vivo* brain tissue to insulin by assessing phosphorylation of critical insulin signaling proteins (46, 47). After 3 d of HFD feeding, we observed changes in cortex insulin sensitivity, with decreased phosphorylated protein kinase B (pAkt)/total Akt (Figure 1D; Supplemental Figure S1A) and decreased insulin receptor substrate 1 (IRS1) phosphorylation [pIRS-1(S307)]/total IRS-1 in response to insulin stimulation (Figure 1E; Supplemental Figure S1B).

In addition to metabolic shifts, we and others have shown that chronic HFD also induces cognitive impairment (29, 48, 49), although cognitive changes in response to acute HFD were less clear. Here we performed puzzle box testing, a behavioral task which primarily tests executive function, to assess possible changes in cognition after 3 d on diet. However, we did not detect any differences in behavior between HFD and SD mice (Supplemental Figure S2). Overall, 3 d of HFD induces systemic and central metabolic changes related to glucose tolerance and insulin sensitivity, without a detectable impact on cognition within this timeframe.

Acute HFD alters peripheral and central immune cell populations

We and others have previously reported that chronic HFD also induces changes in circulating inflammatory profiles (33, 48). Using ELISA to examine inflammatory cytokine concentrations and flow cytometry to examine circulating and CNS immune cell populations, we observed changes to plasma inflammatory profiles after 4 d of HFD similar to those seen in long-term HFD feeding (Supplemental Figure S3). Specifically,

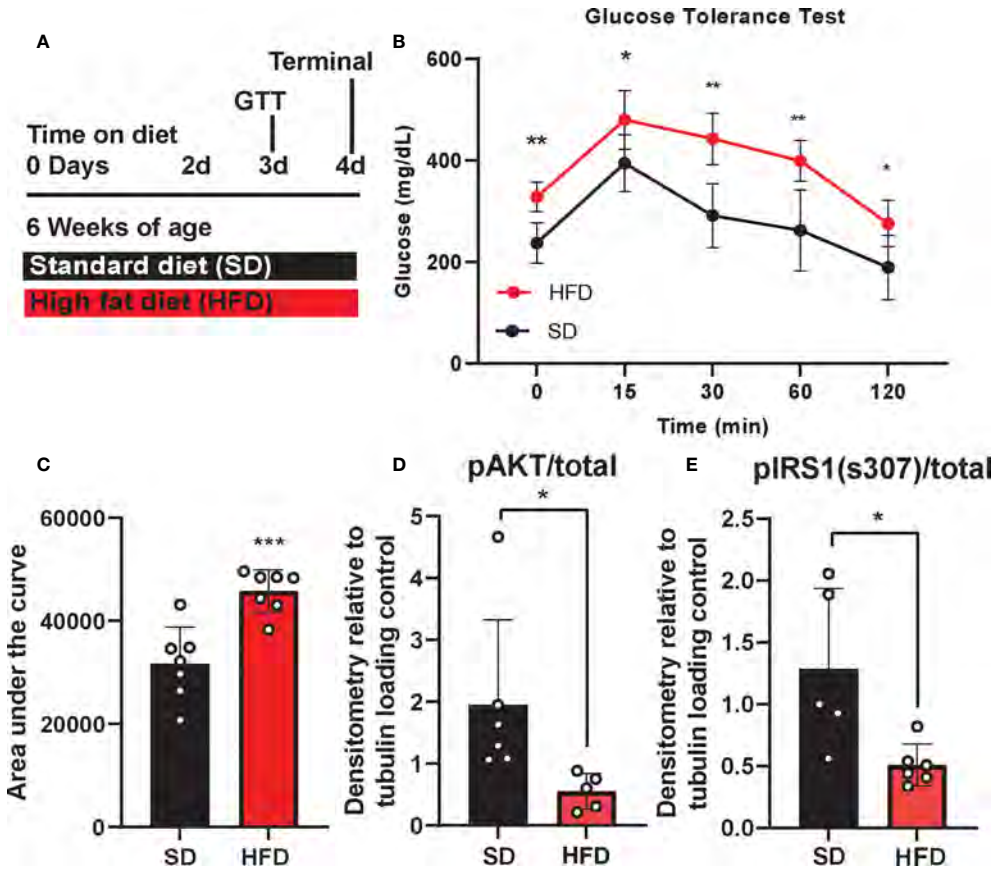

FIGURE 1
Experimental timeline and metabolic phenotyping. Experimental timeline **(A)**, glucose tolerance test, (GTT; **B**), area under the curve **(C)**, and cortex response to insulin stimulation **(D, E)** in male BL/6 mice fed standard diet (SD) or high fat diet (HFD). Protein expression normalized to tubulin and relative to unstimulated control; *p<0.05, **p<0.01, ***p<0.001.

HFD mice had a trending increase in the number of CD4 T-cells (Supplemental Figure S3C), and a significant increase in B-cells (number and % of leukocytes; Supplemental Figures S3F, G) versus SD animals. HFD mice also had a trend for lower Ly6C+ monocytes compared to SD mice (Supplemental Figure S3J). There was no difference due to diet in any of the other measured immune cell populations including CD8 T cells, natural killer cells, Ly6C+ monocytes, Ly6C- monocytes, or neutrophils (Supplemental Figures S3A, B, D, E, G-I, K-S). We also measured plasma inflammatory cytokine levels in HFD and SD mice after injection with either saline or lipopolysaccharide (LPS). LPS robustly increased circulating TNF-α and MCP-1 concentrations (Supplemental Figure S4); there was no effect of diet.

To understand CNS specific changes in immune cell populations, we repeated our experiment in a separate cohort of HFD versus SD mice, both in control treated (saline injection) and in response to immune challenge (LPS injection). When lymphoid populations in the CNS were examined, HFD increased leukocytes (Supplemental Figures S5A, B) and decreased CD8 T-cells (% of leukocytes; Supplemental Figure S5D) versus SD animals and LPS injection had no effect (Supplemental Figure S5). CD4 T-cell levels were low/not detectable and HFD did not impact the numbers or percentages of CNS natural killer cells (Supplemental Figures S5C, E-H). In CNS myeloid cell populations (Figure 2 and Supplemental Figure 6), total immune cell levels and surface marker expression were impacted by 4 d HFD. Neutrophil, microglia, and Ly6C+ monocyte levels were examined as well as expression of CD11c and F4/80, markers of activation and differentiation. HFD mice had more neutrophils (numbers and %; Figures 2A, B) and a greater number of microglia, which also had a trending increase in size as measured by a larger forward side scatter (Figures 2C, I) suggesting activation. There were no differences due to LPS treatment or due to diet for neutrophil or microglial F4/80 or CD11c expression, or for the percentage of microglia (Figures 2C, D, F-H). LPS administration did

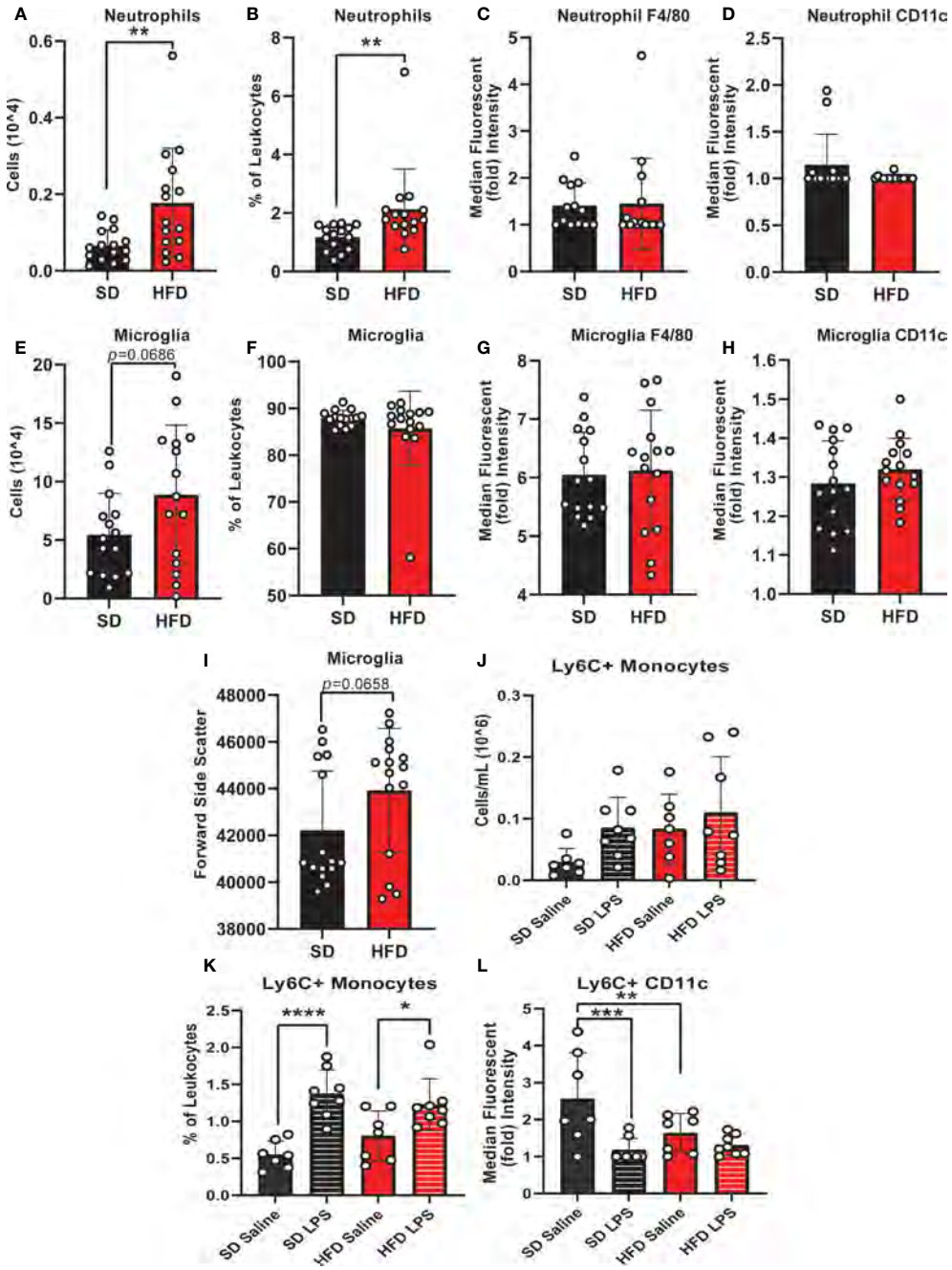

FIGURE 2
CNS immunophenotyping of myeloid cells by flow cytometry. Data represented as neutrophils (number of cells, % of cells, F4/80 expression, and CD11c expression; **A-D**), microglia (number of cells, % of cells, F4/80 expression, CD11c expression, and forward side scatter; **E-I**), and Ly6C + monocytes (number of cells, % of cells, and CD11c expression; **J-L**) in male BL/6 mice fed standard diet (SD) or high fat diet (HFD) who were administered saline or LPS (lipopolysaccharide). In the absence of differences between saline and LPS, data for each dietary group were combined and are presented as SD *vs.* HFD alone; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

impact LyC6+ monocyte numbers and surface expression of CD11c, which was further altered by diet (Figures 2K, L).

Monocyte CD11c expression can indicate a change in monocyte activation, and activation can promote monocyte differentiation into a microglial-like phenotype (32, 50, 51). We observed that LPS increased CNS monocytes in both HFD and SD animals; however, monocyte CD11c expression was lower in response to saline injection in HFD versus SD mice.

In contrast, LPS decreased monocyte CD11c expression in SD but not HFD mice. Increased numbers of microglia and decreased expression of CD11c on monocytes in the absence of increased monocyte numbers likely indicates that HFD promotes monocyte conversion into a more microglial-like phenotype, which LPS fails to further promote. Cumulatively, our findings indicate that acute HFD of only 3 d produces changes in peripheral and central immune cell populations. In this setting, LPS stimulation differentially impacts CNS immune cell dynamics, *i.e.*, monocyte to microglial shifts, in HFD versus SD.

Acute HFD activates hippocampal microglia

Since we observed changes in microglia numbers and size upon 4 d of HFD in our CNS immunophenotyping data, we were interested in further interrogating acute inflammatory microglial changes. We therefore assessed microglial morphology (34) as a proxy of activation in an area of the brain critically important for learning and memory, the hippocampus. Mice were administered HFD or SD with or without LPS stimulation for 4 d, and microglia morphology was examined using confocal microscopy. Three days of HFD shifted the morphology of hippocampal microglia to a state indicative of activation (Figures 3A–D) where microglia of HFD mice given saline (Figure 3C) appeared to have a larger soma size and more ameboid-like shape with fewer and shorter processes compared to microglia of SD mice given saline (Figure 3A). Additionally, microglia in SD (Figure 3B) and HFD (Figure 3D) mice given LPS appeared to take on an activated morphology similar to HFD mice given saline. Indeed, when quantifying these morphological changes, we observed that HFD lowered the ratio of three-dimensional space occupied by the microglia to its perimeter (ramification index; Figure 3E) versus SD mice. Interestingly, administering LPS to SD mice caused the microglia to have a decreased ramification, indicating activation. However, administering LPS to HFD mice did not change their ramification index; this inability of HFD microglia to respond to LPS stimulation may suggest they are activated under basal conditions to such a degree that further stimulation cannot provoke an appropriate immunological response to cellular insult or injury. Like the ramification index, HFD microglia had shorter average branch length (Figure 3F), shorter maximum branch length (Supplemental Figure S7D), and shorter minimum branch length versus SD microglia (Supplemental Figure S7F). LPS stimulation did not affect territorial volume (Supplemental Figure S5A), average branch length (Figure 3F), and maximum branch length (Supplemental Figure S7D). While HFD mice had a greater overall cell volume compared to SD mice, there no effect of LPS (Supplemental Figure S7B). Between groups differences in the number of microglial branch points and end points were varied and dependent upon hippocampal region (Supplemental Figures S7C, E). Thus, acute 3 d HFD activates hippocampal microglia and renders them less able to mount a response to additional stimulation, *e.g.*, to LPS.

Acute HFD activates cGAS/STING signaling

The deleterious role of cGAS/STING inflammatory signaling in obesity and metabolic dysfunction is well established in the periphery, particularly in adipose tissue) (19, 21). However, little is known about its role in this context in the CNS. Therefore, next we wanted to establish the effects of HFD feeding on hippocampal cGAS/STING pathway protein expression (Figure 4; Supplemental Figure S8). To do so, we took hippocampal tissue from SD and HFD animals fed diet for 4 d, homogenized it, and performed Western Blotting. We observed that HFD of only 4 d already acutely upregulated expression in the hippocampus of the dsDNA sensing cGAS and its adaptor molecule STING (Figures 4A, B; Supplemental Figures S8A, B). However, HFD did not promote phosphorylation or change expression of the cGAS/STING pathway transcription factors IRF3 (Figures 4C–E; Supplemental Figures S8C–E) and NFkβ (Figures 4F; Supplemental Figures S8C–E). When activated, IRF3 and NFkβ act as canonical transcription factors and move from the cytosol to the nucleus to induce gene transcription. Therefore, a lack of changes these transcription factors in bulk tissue is perhaps not surprising. Differences in cytosolic *vs.* nuclear localization are likely present, as have been observed by others in culture and in microglia (21, 26). Together, these data further suggest an early upregulated and pro-inflammatory phenotype involving the cGAS/STING pathway after only 3 d on HFD diet.

We previously showed that *in vitro* treatment of neurons with insulin or palmitate for 24 h produces insulin resistance, providing a cell culture model of prediabetes, with the expected changes in cellular signaling pathways (40, 52). We adopted this same approach to establish the contribution of various CNS cell types, namely neurons and microglia, to cGAS/STING pathway activation. Using a partly immortalized human hippocampal cell line and an immortalized human microglial cell line, we first established the presence of cytosolic DNA in response to palmitate and insulin treatment. Our data show a trending increase in cytosolic nuclear DNA (18s) in both neurons and microglia in response to palmitate or combined insulin and palmitate treatment (Figures 5A, C). However, there were no differences in either cell type in response to stimulation for cytochrome oxidase I DNA, a marker of mitochondrial DNA (Figures 5B, D). Of note, only trending differences in cytosolic nuclear DNA and a lack of differences in mitochondrial DNA were likely due to low sample sizes and a high degree of

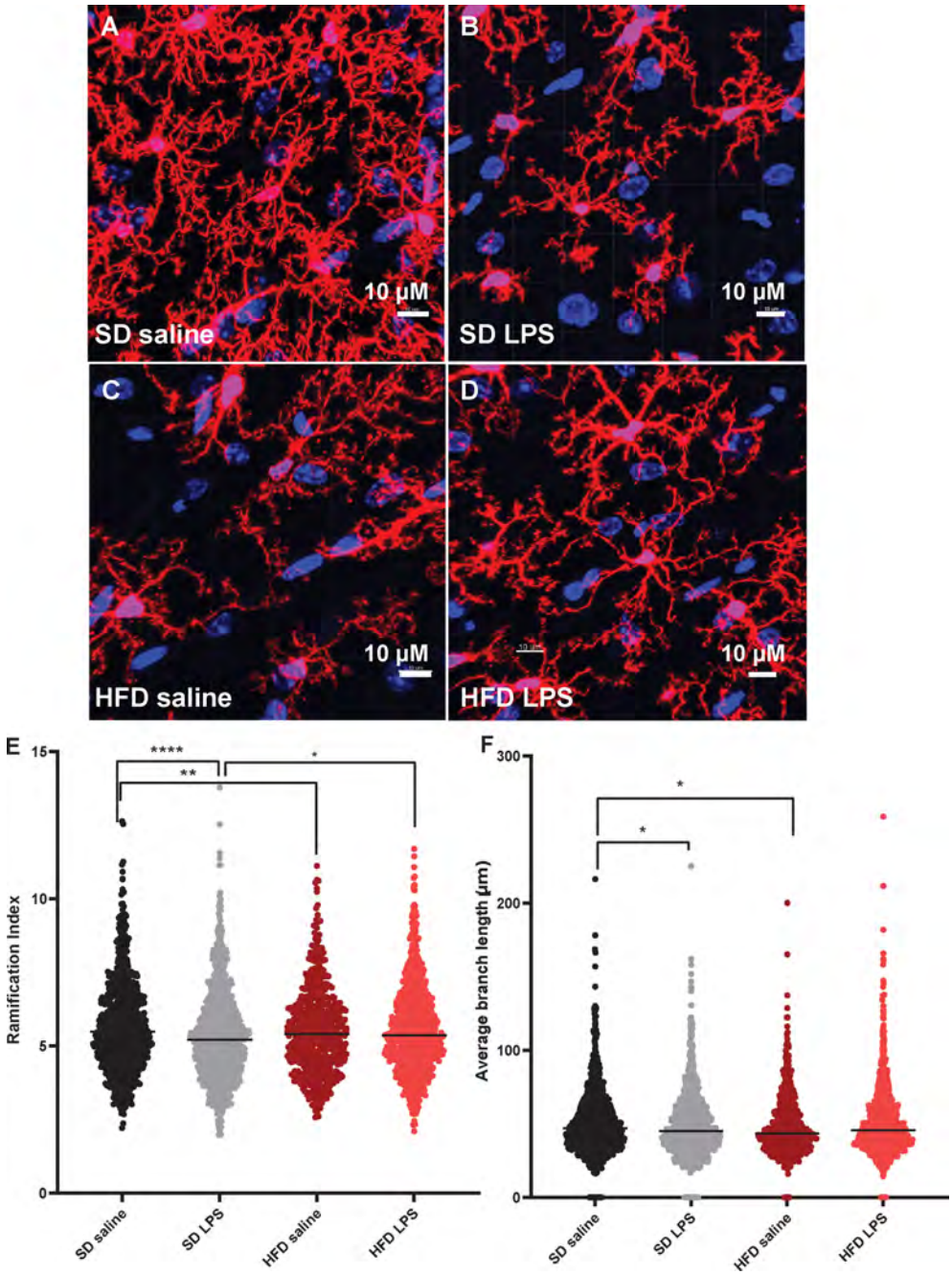

FIGURE 3
Microglial morphology. Representative images of IBA-1 microglia (red stain) in male BL/6 mice fed standard diet (SD) or high fat diet (HFD) who were administered saline or LPS (lipopolysaccharide; **A-D**). Quantification of microglia ramification index **(E)** and average branch length **(F)**. In the absence of differences between saline and LPS, data for each dietary group were combined and are presented as SD *vs.* HFD alone; *p<0.05, **p<0.01, ****p<0.0001.

variability between replicates. Future studies could address how obesogenic conditions might cause genomic damage and the role of mitochondrial *vs.* genomic or nuclear damage on cGAS/STING signaling in the CNS. We next assessed cGAS/STING pathway protein expression in both cell types. There was a robust response in microglia, with a significant increase in STING, pIRF3, and NFκβ, in the presence of either palmitate alone or combined insulin and palmitate for 24 h (Figure 6; Supplemental Figure S9). We also found a trending increase in cGAS protein expression in response to acute treatment for 24 h

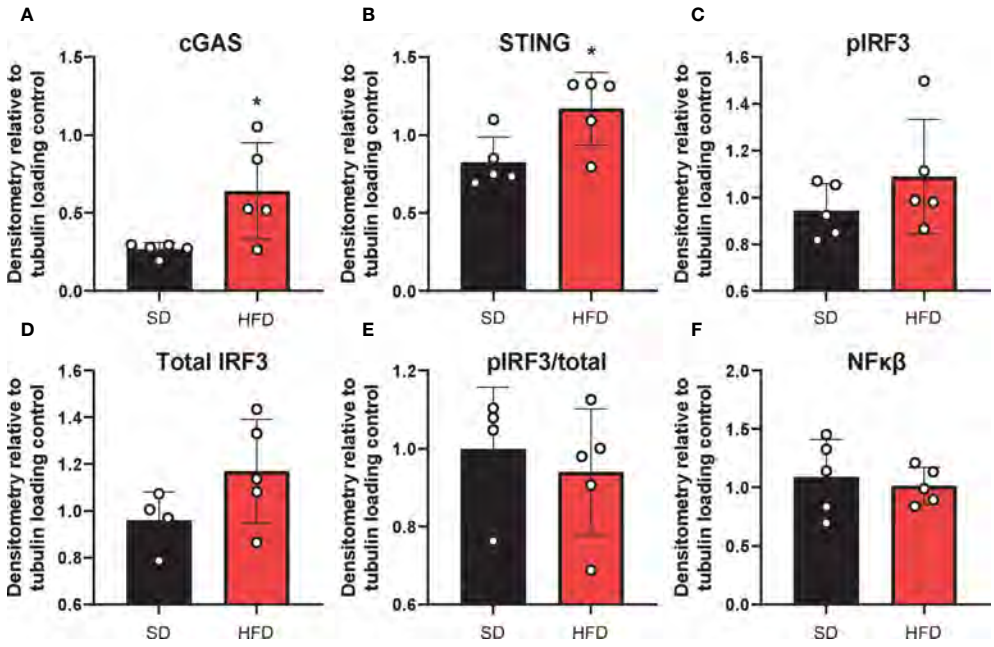

FIGURE 4
Hippocampal cGAS/STING protein expression. Expression in male BL/6 mice fed standard diet (SD) or high fat diet (HFD). Data represented as cGAS **(A)**, STING **(B)**, pIRF3 **(C)**, total IRF3 **(D)**, pIRF3/total **(E)**, and NFκβ **(F)** relative protein expression. Protein expression quantified as average band intensity relative to tubulin loading control; *$p<0.05$.

with either palmitate alone or combined insulin and palmitate in hippocampal neurons (Figure 6A; Supplemental Figure S9A).

Finally, we assessed cGAS/STING pathway activation in co-culture to evaluate the contribution of inflammatory crosstalk on potential pathological mechanisms *via* gap junctions in the CNS. Inflammatory crosstalk (53) is vital for normal intercellular communication (54). However, aberrant inflammatory crosstalk in the CNS (either *via* glia-glia or glia-neuron signaling) may promote pathological inflammatory mechanisms. Indeed, it plays a role in neurodegenerative diseases, such as AD/ADRD (55–57), and gap junctions facilitate transfer between cells of the cGAS/STING second messenger, cyclic GMP-AMP (cGAMP) (23, 56). To determine whether gap junctions mediate inflammatory crosstalk, we co-cultured neurons and microglia in the presence or absence of a gap junction inhibitor (CBX; carbenoxolone). Co-cultures were pre-treated with either the saturated fatty acid palmitate or the combination of insulin to mimic obesogenic prediabetic conditions. Our data (Figure 7; Supplemental Figure S10) show that treating co-cultures with transfection reagent alone did not change cGAS (Figure 7A; Supplemental Figure S10A), STING (Figure 7B; supplemental Figure S10B), or NFκβ protein expression (Figure 7D; Supplemental Figure S10D) in the presence of the gap junction inhibitor, carbenoxolone. However, we observed a significant increase in co-cultures pre-treated with insulin and palmitate for 24 h then stimulated with the dsDNA analog, poly dA:dT (poly deoxyadenylic-deoxythymidylic acid sodium salt), which was completely reversed in the presence of carbenoxolone (Figure 7C; Supplemental Figure S10C). In aggregate, these data suggest that cGAS/STING inflammatory crosstalk between CNS cells, *e.g.*, neurons and microglia, in response to metabolic injury is mediated, at least in part, by gap junctions.

Discussion

Metabolic dysfunction, in the form of chronic obesity, prediabetes, or diabetes, induces peripheral and central inflammation which correlate with cognitive impairment (58, 59). However, early inflammatory events secondary to obesity- or prediabetes that might contribute to cognitive impairment remain uncertain. The innate immune cGAS/STING pathway is dysregulated in cognitive impairment and neurogenerative disease (24, 26) and by responding to excess saturated fatty acids may connect metabolic dysfunction to inflammation in the CNS (19–21). In the current study, we examined the effect of acute HFD on peripheral and CNS inflammation, cognition, and CNS cGAS/STING activation. Our data show that acute HFD for only 3 d causes peripheral and central metabolic and immunologic changes indicative of insulin resistance and an acute pro-inflammatory response,

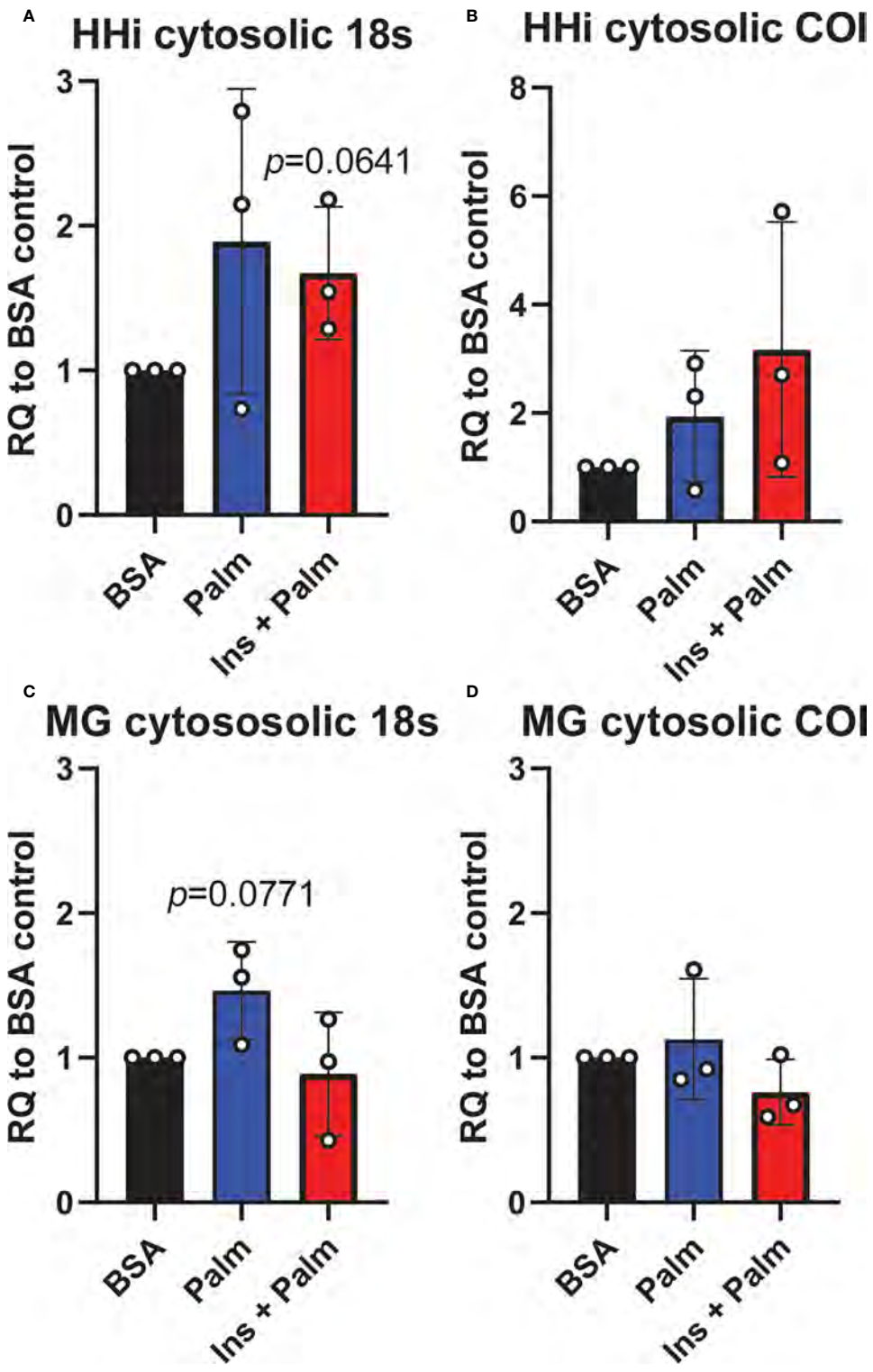

FIGURE 5
Cytosolic DNA concentrations. Relative quantity (RQ) of cytosolic DNA (nuclear and mitochondrial) in partially immortalized human hippocampal neurons (HHi; n=3, **A, B**) and in a human microglial cell line (MG; n=3, **C, D**). Cells treated with palmitate (Palm; HHi=250 µM, microglia=62.5 µM, 24h) or a combination of insulin and palmitate (Ins + Palm; above palmitate concentrations + 50 nM insulin, 24h). Values relative to BSA controls.

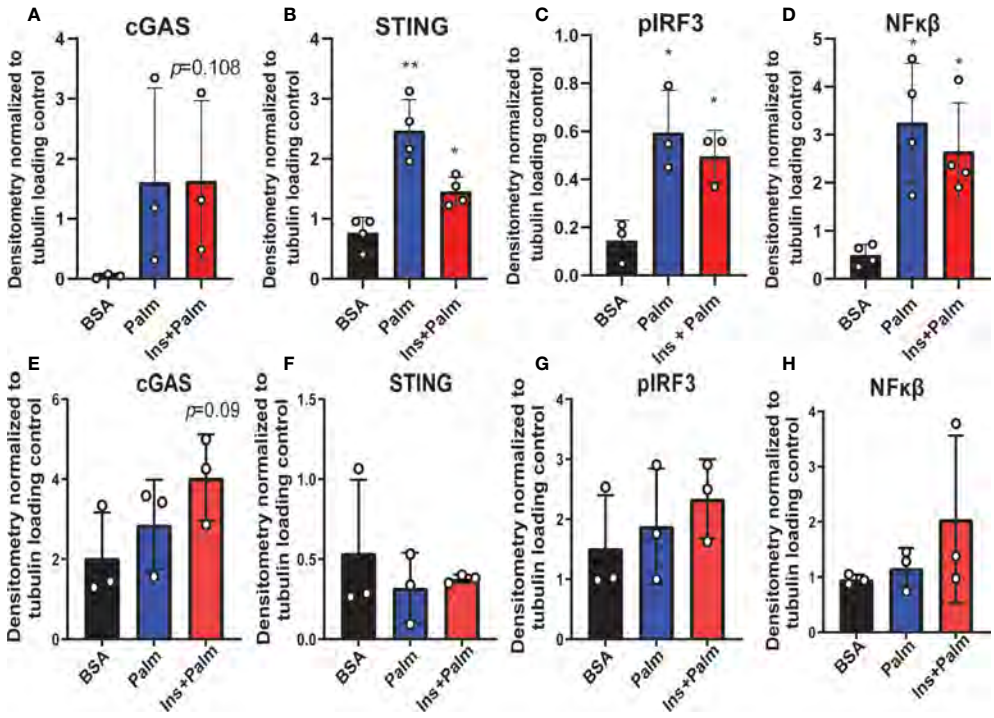

FIGURE 6
Neuronal and microglial cGAS/STING protein expression. Expression in a human microglial cell line (n=3 biological replicates, **A-D**) and a human hippocampal neuronal cell line (n=3 biological replicates, **E-H**) treated with either palmitate alone (Palm; HHi=250 µM, MG=62.5 µM, 24h) or a combination of insulin and palmitate (Ins+Palm; above concentrations of Palm+50nM insulin, 24h). Relative protein expression quantified as average band intensity relative to tubulin loading control; *p<0.05, **p<0.01.

though changes in cognition were not detected. Additionally, acute HFD activates CNS microglia, as measured by changes in cell size and morphology, and promotes cGAS/STING signaling. This immune response was mirrored *in vitro* under conditions of metabolic injury, particularly in microglia, as well as in in neuron-microglia co-culture and was blocked by a gap junction inhibitor. Overall, our findings indicate that inflammation and cGAS/STING activation are early responses to HFD, potentially through direct gap junction-mediated neuron-microglia crosstalk in the CNS.

We found that short-duration HFD induced acute peripheral and CNS metabolic changes in mice, specifically impaired glucose tolerance and insulin resistance. These findings are aligned with another study of 3 d of HFD feeding, which similarly saw impaired glucose homeostasis (60). These changes are also consistent with literature regarding chronic HFD, *i.e.*, of a few to several weeks, that report increases in body weight and impaired glucose tolerance (29, 30). We further show that both peripheral and CNS immune cell populations are dysregulated after only 3 d on HFD. Specifically, HFD increased circulating and CNS lymphocytes and neutrophils. We also observed that acute HFD decreased circulating Ly6C+ monocytes and Ly6C+ monocytes in the CNS had lower CD11c expression. Concurrent with increased CNS microglia, these data suggest that HFD promoted of monocyte recruitment to the CNS and monocyte conversion to a more microglia-like phenotype. Moreover, LPS failed to mount a further immune response in HFD, indicating peripheral and CNS immune cells are activated to such a degree by HFD that LPS is unable to provoke an appropriate response. Our findings are broadly aligned with the acute impact of HFD on the CNS, where others have reported increased levels of inflammatory cytokines after 3 d on diet (12). Furthermore, it is frequently reported that chronic HFD feeding induces an inflammatory phenotype (33, 48, 61).

HFD-induced pro-inflammatory responses through upregulated cGAS/STING signaling in peripheral tissues has been proposed as a potential pathological mechanism in obesity and prediabetes/diabetes (19, 21, 41). As an intracellular pattern recognition receptor, cGAS/STING is widely expressed by innate cells of the CNS, including microglia (62, 63), which canonically senses cytosolic dsDNA of viral or bacterial origin (64). However, the cGAS/STING pathway can also be activated by cytosolic self dsDNA released under conditions of metabolic stress, such as by saturated fatty acid overload (20, 64). Indeed, HFD fed mice have elevated adipose (41) and liver (65) STING levels. In endothelial cultures, the long-chain saturated fatty acid

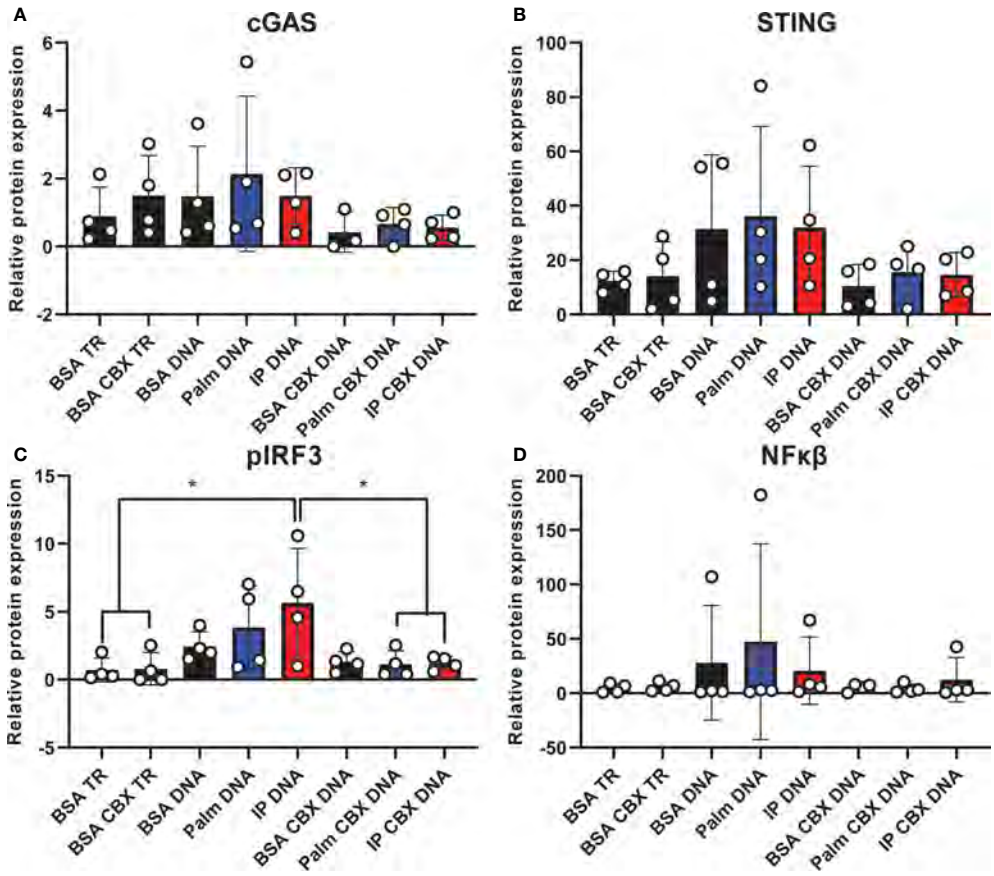

FIGURE 7
Co-culture cGAS/STING protein expression with and without gap junction inhibitor. Expression in a human hippocampal and human microglial cell line co-culture (n=4). Cells pretreated with bovine serum albumin (BSA; 31.25 μM, 24h) as a control, palmitate (Palm; 31.25 μM, 24h), or a combination of insulin and palmitate (IP; 31.25 μM palmitate and 50 nM insulin, 24h) +/- the gap junction inhibitor carbenoxolone (CBX; 150 μM), then stimulated with the dsDNA analog Poly dA:dT (DNA; 1μg/mL). Protein expression of cGAS **(A)**, STING **(B)**, pIRF3 **(C)**, and NFκβ **(D)** quantified quantified as average band intensity relative to histone loading control; *$p<0.05$. TR, transfection reagent.

palmitate activates cGAS/STING and induces inflammation (21, 41). Further, STING deficiency partially reverses HFD-induced weight gain, decreases plasma free fatty acids and adipose macrophage infiltration, and improves impaired insulin sensitivity and glucose tolerance (41).

While there is ample evidence to suggest a role for cGAS/STING in obesity and prediabetes/diabetes in the periphery, the role of cGAS/STING in the brain is less clear. We observed cGAS/STING was upregulated in the hippocampus of HFD animals versus SD controls. We previously established that our HFD feeding paradigm induces obesity, prediabetes and cognitive impairment with chronic HFD in mice (29). While here we did not observe cognitive impairment after only 3 d of HFD, our findings suggest HFD promotes an acute and early CNS pro-inflammatory programming that precedes or initiates the cascade of processes leading up to neurodegeneration and cognitive impairment with chronic HFD. Conversely, others have reported changes in cognition after acute HFD feeding (66–68). Differences may have arisen from variations in model system (mouse versus rat), animal age (5 wk versus 12 wk) or testing modality (puzzle box versus contextual fear conditioning versus radial arm maze) (66–68). Moreover, it is possible that cognitive differences in HFD versus SD animals in only measurable upon additional stimulation, *e.g.*, by LPS (66). Therefore, the temporal evolution of cognitive impairment upon acute HFD requires further study.

In alignment with our findings of early cGAS/STING activation, cGAS/STING is implicated in frank dementia, such as AD/ADRD (24, 26). In the brains of AD models, cGAS/STING is increased and improving DNA damage/repair by NAD+ supplementation normalizes cGAS/STING levels, reduces inflammation, and improves behavioral outcomes (24). Furthermore, cGAS/STING may be involved in AD *via* interaction with one of the key pathological AD proteins, tau. Specifically, tau activates cGAS/STING *via* binding to polyglutamine binding protein 1, which is essential for tau-

mediated cGAS/STING activation, specifically in microglia (26). In a Parkinson's disease mouse model, knocking out cGAS/STING signaling rescues the inflammatory phenotype, prevents loss of dopaminergic neurons, and improves motor deficits (27). In amyotrophic lateral sclerosis, the critical disease protein TDP-43 promotes the release of mitochondrial dsDNA into the cytosol, which subsequently activates the cGAS/STING pathway and promotes neurodegeneration (28).

We observed that acute HFD was sufficient to activate hippocampal microglia, which were unable to respond to additional stimulus in the form of LPS injection. Further, using an established *in vitro* model of metabolic injury, we observed a stronger response of the cGAS/STING pathway in microglia compared to neurons. This was anticipated, as cGAS/STING pathway proteins are highly expressed in microglia (62). Moreover, as we observed, HFD induces an inflammatory phenotype in hippocampal microglia (11, 12), and inflammatory microglia play critical roles in AD/ADRD pathology and related neuroinflammation (69–71). cGAS/STING activation primarily results in type 1 interferons (IFN) pro-inflammatory cytokine production, which acts to further stimulate cytokine release, *e.g.*, of IL-1β, IL-6, TNF-α (72). Excessive cGAS/STING activation contributes to pathological mechanisms, often mediated in the CNS by microglia (72, 73). This cGAS/STING activation and subsequent IFN release structurally and functionally injures neurons (72). Our findings indicate that microglia may be constituently activated under HFD conditions in the hippocampus, are less able to respond to inflammatory stimulus, and may contribute to CNS neuroinflammation, neurodegeneration, and eventual cognitive decline.

The immune system has multiple functions, including to induce inflammation, recruit immune cells, initiate protective cellular programs (including metabolic processes), preserve homeostasis, and maintain tissue functions (74). To perform these functions, it partly relies on inflammatory crosstalk, such as gap junctions (53), for intercellular communication (54). This crosstalk may become dysregulated upon chronic inflammatory activation, such as occurs in obesity and prediabetes, and thus is a potential mechanism promoting disease progression. In our co-culture model of human hippocampal neurons and microglia, we showed activation of the cGAS/STING pathway is strongly reduced in the presence of a gap junction inhibitor. These data show that gap junction mediated cGAS/STING crosstalk is a mechanism by which cGAS/STING inflammatory signaling can be promoted in the CNS in the presence of metabolic insults. In fact, gap junctions are relevant to neurodegenerative diseases, such as AD. Gap junctions are elevated near Aβ plaques (75, 76), and their blockade slows disease progression (55). Further, immune responses and cytokines can regulate gap junctions during insult, infection, or injury (77, 78). cGAS/STING has been shown to utilize gap junctions as an inflammatory crosstalk mechanisms in HEK cells and murine fibroblasts (23). Specifically, in response to cytosolic dsDNA, cGAS triggers production of its second messenger, cGAMP (20), which can travel to neighboring cells *via* gap junctions and stimulate downstream cytokine production by activating STING and pIRF3 (23). This represents a source of direct cell-to-cell crosstalk, contributing to inflammatory activation in neighboring cells, possibly furthering pathological processes. While our data support a role for gap junctions in promoting inflammatory crosstalk, it is unclear which cell types are the primary source of this inflammation. Future studies using single cell sequencing and cGAS cell specific knock out models are currently underway to better understand how different cell types contribute to this inflammation and the downstream effects they might have on cognition.

However, our study had some limitations. First it was carried out in male animals only. We (79) and others (80) have shown that male and female animals have sexually dimorphic responses to high fat diet feeding, particularly early in the paradigm. Additionally, there are known differences between males and females in terms of immune function and inflammation (81, 82), including in microglia (83). These differential effects also potentially impact cognition, as some have shown a differential effect of sex on cognitive outcomes (84). As mentioned above, no differences were observed between groups for puzzle box performance. However, motivation to escape in the puzzle box task is primarily driven by the animal's fear and anxiety in brightly lit spaces (85). Additional non-cognitive tasks that more directly measure anxiety under a similar motivation, such as the open field task (86), would allow for discrimination between a lack of cognitive deficits *vs.* overall anxiety in the animals and should be considered for future studies.

Overall, our data indicate that acute HFD feeding promotes early dysregulated glucose and insulin metabolism in the periphery and CNS. HFD feeding also causes an acute pro-inflammatory response, including microglial and innate inflammatory cGAS/STING pathway activation in the brain. Our *in vitro* data in neurons and microglia further point to a critical role for microglia in promoting this pro-inflammatory phenotype and indicate that gap junction may, at least in part, mediate cGAS/STING signaling, participating in inflammatory spread in the CNS.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by The University of Michigan's Institutional Animal Care and Use Committee approved all animal protocols (PRO0010039).

Author contributions

SE, RH, and EF designed the studies. IW-D, ST, and BM performed the immunophenotyping. BK, JH, FM, IW-D, ST, RH, and SE contributed to the tissue processing. BK and SE performed the *ex vivo* insulin stimulation and western blotting. IW-D sectioned and stained the images for microglial morphology. SE and RH imaged and analyzed the microglial morphology data. CP and SE performed the cell culture and subsequent PCR and western blotting. SE performed statistical analyses. SE wrote the manuscript. RH, EF, BK, and BM edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1012594/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Representative insulin signaling western blot images. Representative unaltered images of western blots quantified in main figure 1 (w/link color) for cortex insulin signaling protein expression of AKT (pAKT and total AKT; **A**) and IRS-1 (pIRS-1(pS307) and IRS-1; **B**) in male BL/6 mice fed standard diet (SD) or high fat diet (HFD) and +/- acute insulin treatment.

SUPPLEMENTARY FIGURE 2
Cognition as measured by puzzle box testing. Data represented as single tasks **(A-D)**, and the combination of the single tasks into a complex task **(E)** in male BL6 mice fed high-fat diet (HFD) versus standard diet (SD).

SUPPLEMENTARY FIGURE 3
Peripheral immunophenotyping by flow cytometry. Data represented as leukocytes (number of cells **A**), CD4 T-cells (% of cells or number of cells; **B, C**), CD8 T-cells (% of cells or number of cells; **D, E**), B-cells (% of cells or number of cells; **F, G**), natural killer cells (% of cells or number of cells; **H, I**), Ly6C+ monocytes (% of cells or number of cells; **J, K**), Ly6C- monocytes (% of cells or number of cells; **L, M**), neutrophils (% of cells or number of cells; **N, O**), Ly6C+ monocytes CD11c expression (median fluorescent (fold) intensity; **P**), Ly6C+ monocytes F4/80 expression (median fluorescent (fold) intensity; **Q**), Ly6C- monocytes CD11c expression (median fluorescent (fold) intensity; **R**), and Ly6C- monocytes F4/80 expression (median fluorescent (fold) intensity; **S**) in male BL6 mice fed high-fat diet (HFD) versus standard diet (SD); *p<0.05.

SUPPLEMENTARY FIGURE 4
Plasma inflammatory cytokines as measured by ELISA. Data represented as plasma TNF-α (pg/mL; **A**) and plasma MCP-1 (pg/mL; **B**) concentrations in male BL6 mice fed high-fat diet (HFD) versus standard diet (SD), administered either saline or lipopolysaccharide (LPS); **p<0.01, ****p<0.0001.

SUPPLEMENTARY FIGURE 5
CNS immunophenotyping of lymphoid cells by flow cytometry. Data represented as leukocytes (number of cells or % of cells; **A, B**), CD8 T-cells (number of cells or % of cells; **C, D**), CD4 T-cells (number of cells or % of cells; **E, F**), and natural killer cells (number of cells or % of cells; **G, H**) in male BL6 mice fed high-fat diet (HFD) versus standard diet (SD). There were no differences between animals administered saline *vs.* lipopolysaccharide (LPS), therefore SD and LPS animals were combined within their appropriate dietary groups; *p<0.05.

SUPPLEMENTARY FIGURE 6
Representative flow cytometry panels for CNS myeloid cells. Data represented as CD11c or F4/80 surface expression on neutrophils **(A)**, microglia **(B)**, and monocytes **(C)** in male BL6 mice fed high-fat diet (HFD; black) versus standard diet (SD; red) given saline. For monocytes, solid lines represent mice given saline and dashed lines represent mice given lipopolysaccharide. Grey peaks represent IgG control antibody.

SUPPLEMENTARY FIGURE 7
Microglial morphology. Quantification of microglial territorial volume (μm^3; **A**), cell volume (μm^3; **B**), number of end points **(C)**, maximum

branch length (μm; **D**), number of end points **(E)**, and minimum branch length (μm; **F**) in male BL6 mice fed high-fat diet (HFD) versus standard diet (SD) administered saline or LPS (lipopolysaccharide; **A-D**). Quantification of microglia percentage occupied volume **(E)** and average branch length **(F)**. For A, B, D, and F quantification was performed on individual cells per image (n=3 images for the CA1 and molecular layers of the hippocampus and n=2 images for the hilus). For C and E quantification was performed by combing all images per hippocampal region. In the absence of differences between saline and LPS, data for each dietary group were combined and are presented as SD *vs.* HFD alone; *$p<0.05$, **$p<0.01$, *** $p<0.001$, ****$p<0.0001$.

SUPPLEMENTARY FIGURE 8

Representative hippocampal cGAS/STING western blot images. Representative unaltered images of western blots quantified in main figure 4 (w/link color) for hippocampal cGAS/STING pathway protein expression of cGAS **(A)**, STING **(B)**, pIRF3 **(C)**, IRF3 **(D)**, NFκβ **(E)**, and tubulin **(F)** in male BL/6 mice fed standard diet (SD) or high fat diet (HFD).

SUPPLEMENTARY FIGURE 9

Representative neuron and microglia cGAS/STING western blot images. Representative unaltered images of western blots quantified in main figure 6 (w/link color) for cGAS/STING pathway protein expression of cGAS **(A)**, STING **(B)**, pIRF3 **(C)**, NFκβ **(D)**, and tubulin **(F)** in palmitate (Palm) and insulin (Ins) stimulated neuronal and microglial cell lines. Conditions in bold are those used for analysis.

SUPPLEMENTARY FIGURE 10

Representative co-culture cGAS/STING western blot images. Representative unaltered images of western blots quantified in main figure 7 (w/link color) for cGAS/STING pathway protein expression of cGAS **(A)**, STING **(B)**, pIRF3 **(C)**, NFκβ **(D)**, and histone **(F)** in in palmitate (Palm) and insulin and palmitate (IP) stimulated neuronal and microglial cell line co-culture +/- the gap junction inhibitor carbenoxolone (CBX; 150 μM). Co-cultures were further stimulated with the dsDNA analog Poly dA:dT (DNA; 1μg/mL). Conditions in bold are those used for analysis. TR; transfection reagent.

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CLINICAL AND TRANSLATIONAL MEDICINE

WILEY

RESEARCH ARTICLE

Monoclonal antibody-mediated immunosuppression enables long-term survival of transplanted human neural stem cells in mouse brain

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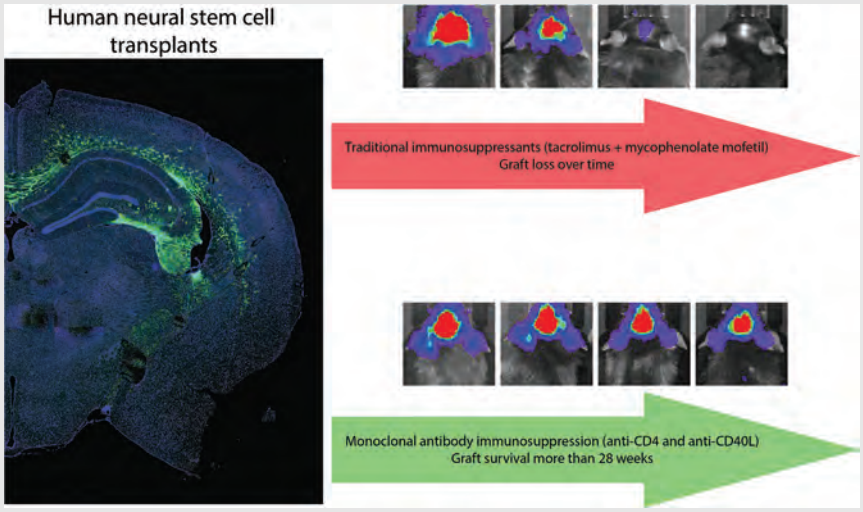
Graphical Abstract



- Traditional immunosuppression with tacrolimus and mycophenolate mofetil did not sustain human neural stem cell xenograft survival in mouse brain for more than 2 weeks.
- Immunosuppression with monoclonal antibodies targeting CD4 and CD40L enabled long-term persistence of human stem cell transplants.
- Transplants survive past 28 weeks in C57BL/6 mice and the 5XFAD animal model of Alzheimer's disease.

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Abstract

Background: As the field of stem cell therapy advances, it is important to develop reliable methods to overcome host immune responses in animal models. This ensures survival of transplanted human stem cell grafts and enables predictive efficacy testing. Immunosuppressive drugs derived from clinical protocols are frequently used but are often inconsistent and associated with toxic side effects. Here, using a molecular imaging approach, we show that immunosuppression targeting costimulatory molecules CD4 and CD40L enables robust survival of human xenografts in mouse brain, as compared to conventional tacrolimus and mycophenolate mofetil.

Methods: Human neural stem cells were modified to express green fluorescent protein and firefly luciferase. Cells were implanted in the fimbria fornix of the hippocampus and viability assessed by non-invasive bioluminescent imaging. Cell survival was assessed using traditional pharmacologic immunosuppression as compared to monoclonal antibodies directed against CD4 and CD40L. This paradigm was also implemented in a transgenic Alzheimer's disease mouse model.

Results: Graft rejection occurs within 7 days in non-immunosuppressed mice and within 14 days in mice on a traditional regimen. The addition of dual monoclonal antibody immunosuppression extends graft survival past 7 weeks ($p < .001$) on initial studies. We confirm dual monoclonal antibody treatment is superior to either antibody alone ($p < .001$). Finally, we demonstrate robust xenograft survival at multiple cell doses up to 6 months in both C57BL/6J mice and a transgenic Alzheimer's disease model ($p < .001$). The dual monoclonal antibody protocol demonstrated no significant adverse effects, as determined by complete blood counts and toxicity screen.

Lisa M. McGinley and Kevin S. Chen contributed equally to this work.

Conclusions: This study demonstrates an effective immunosuppression protocol for preclinical testing of stem cell therapies. A transition towards antibody-based strategies may be advantageous by enabling stem cell survival in preclinical studies that could inform future clinical trials.

KEYWORDS
Alzheimer's disease, antibodies, cell tracking, immunosuppression therapy, monoclonal, stem cell transplantation

1 | BACKGROUND

Transplantation of human stem cells in the central nervous system (CNS) represents a rapidly developing, multifaceted approach for the treatment of neurological disorders.[1,2] Preclinical studies show that stem cell transplantation can improve disease-related pathology and neurologic function, and advances have led to clinical testing in patients with stroke, Parkinson's disease, amyotrophic lateral sclerosis and Alzheimer's disease.[3–6] Established human neural stem cell (hNSC) lines are increasingly utilized in clinical trials[7–11]in lieu of autologous sources of stem cells (e.g., mesenchymal stem cells). There are many benefits to using a hNSC line, including commitment to neuronal/glial fates, the ability to characterize and/or modify cells ex vivo, and amenability to large-scale production, thus reducing cost and increasing accessibility for patients. However, US Food and Drug Administration approval for biologic therapies requires preclinical efficacy testing in small animals, necessitating xenogeneic transplant paradigms. Given this requirement, host rejection of transplanted cells and the reliance on immunosuppressive agents to prevent an immune response, even in human allogenic transplant paradigms, remain significant challenges.[12–15]

Traditionally considered an 'immunoprivileged' site, it is now known that CNS antigens are transported to peripheral lymph nodes, and activated T cells can cross the blood brain barrier.[16,17] In the context of xenogeneic cell transplantation in the CNS, recipient $CD4^+$ T cells appear to play a major role, capable of recognizing donor antigens, triggering the adaptive immune response and consequent graft rejection.[18–20] Costimulatory signalling, for example via CD40-CD40L interactions, appears to be a necessary component of xenograft rejection.[21,22] This represents a substantial hurdle to ensuring robust survival of human cells in xenograft experimental models.[21,23] Immunosuppressive drug regimens derived from clinical protocols for solid organ transplant have been deployed to prevent xenograft rejection and include steroids and inhibitors of calcineurin, inosine monophosphate dehydrogenase, interleukins and tumour necrosis factor α.[24,25] In terms of xenogeneic grafts, the long-term effectiveness of these immunosuppressive drugs in preventing cytotoxic immune-mediated rejection of human stem cell transplants in the CNS is not well established. Furthermore, standard non-specific immunosuppression paradigms often do not sufficiently overcome host immune responses, with transient survival of transplanted human cells and subsequent graft rejection within 4–6 weeks in rodents.[26–32] More effective and specific immunosuppressive regimens are needed for robust xenograft survival in rodent models to extend experimental timelines, enable evaluation of maximum therapeutic effects and mitigate effects of stem cell loss.

Emerging approaches include the use of monoclonal antibodies (mAbs) targeted to specific immune cell populations and costimulatory pathways involved in the immune response.[33–37] In preclinical studies, various combinations of mAbs have been used to promote immune tolerance in transplantation experiments. Although this approach seems promising, the application of long-term mAb-based immunosuppressive regimens to CNS stem cell transplantation therapy is unknown. This prompted us to investigate a mAb-based immunosuppressive regimen consisting of anti-CD4 and anti-CD40L mAb costimulatory blockade in the context of preclinical intracranial hNSC transplantation. Using real-time in vivo bioluminescent imaging (BLI), we demonstrate that this mAb-based immunosuppressive regimen enables robust and durable survival and extensive migration of a clinically relevant hNSC line following intracranial transplantation in both normal and Alzheimer's disease mouse models.

2 | METHODS

2.1 | In vitro generation of hNSC-luc$^{\pm}$/green fluorescent protein$^{\pm}$ cells

hNSC lines (HK532-CAG-IGF1, previously assessed for intracranial transplantation)[38,39] were supplied by Seneca

BioPharma, Inc. (Germantown, USA) and cultured as previously described.[40,41] Stable reporter gene expression was achieved in hNSCs with a lentivirus vector (LV-Luc2-P2A-EmGFP, # LV050-L, Imanis Life Sciences, Rochester, USA) encoding firefly luciferase (luc) and emerald green fluorescent protein (GFP). Briefly, lentivirus was added to hNSCs at approximately 70% confluence in growth media and incubated for approximately 16 h in normal culture conditions (5% O_2, 5% CO_2, 37°C). After incubation, cells were washed three times in growth media to remove virus. Transduction efficiency was monitored under fluorescence microscopy for GFP expression. Luciferase expression was measured at 48 h post-transduction using a luciferase activity assay per manufacturer protocols (Promega, Madison, USA). For transplantation, a bank of hNSC-luc$^+$/GFP$^+$ transduced at multiplicity of infection (MOI) 25 was expanded and stored in liquid nitrogen.

2.2 | Stem cell transplantation

We assessed viability of hNSC transplants in C57BL/6J mice (Jackson Laboratory, Bar Harbor, USA) as well as the 5XFAD transgenic mouse (Jackson Laboratory), a commonly used model of Alzheimer's disease.[42–44]These mice harbour two humanized transgenes: (1) the amyloid-β precursor protein gene with the Swedish, Florida and London mutations and (2) the presenilin-1 (*PSEN1*) gene harbouring the M146L and L286V mutations. It is important to note here that we[45] and others[46] have demonstrated that the neurological and behavioural phenotypes on the C57BL/6J genetic background are less severe and occur at later timepoints when compared to that observed on the original C57BL6/SJLF1 hybrid background.[42] Mice were randomly assigned to treatment groups for each experiment. Sample sizes are specified in figures and/or figure legends, and outliers are included. Personnel performing BLI, immunohistochemistry, microscopy, complete blood counts (CBCs), flow cytometry, ELISA and toxicity screen analysis were blinded to the treatment groups. All animal procedures were approved by the University of Michigan Institutional Animal Care and Use Committee (PRO00010247) and performed according to University of Michigan guidelines and state and federal regulations, including the NIH Guide for the Care and Use of Laboratory Animals.

Intracranial transplantation was performed on 8-10-week-old male C57BL/6J or 5XFAD mice (Jackson Laboratory) using our established stereotactic approach.[39–41,47] Briefly, mice were anesthetized with 2% isoflurane and placed in a standard Kopf stereotactic frame (David Kopf Instruments, Tujunga, USA). hNSC-luc$^+$/GFP$^+$ vials were thawed and cultured in N2b Growth Media (Seneca Biopharma) supplemented with basic fibroblast growth factor. At the same passage number, cells were harvested with 0.25% trypsin followed by addition of soybean trypsin inhibitor (Invitrogen, Waltham, USA, 0.5 mg/ml). hNSCs were pelleted by centrifugation, then resuspended in hibernation media (Seneca BioPharma) and trypan blue exclusion ensured transplantation of >90% viable cells. hNSCs were delivered by bilateral injection to the fimbria fornix of the hippocampus at three sites per hemisphere (2 μl hNSC-luc$^+$/GFP$^+$, total six injections) delivered to the following coordinates (bregma/lateral/ventral): −0.82/0.75/2.5, −1.46/2.3/2.9, −1.94/2.8/2.9 mm. Cell concentrations varied depending on the experiment and intended cell dose (see figure legends). Final cell doses ranged between 1.8×10^5 and 9.6×10^5 total cells per animal. Each injection was administered over 120 s followed by a 120 s delay prior to needle withdrawal. Cell viability was reassessed by trypan blue exclusion post-transplantation to ensure adequate cell survival throughout the procedure.

2.3 | Immunosuppression treatments

Body weights were collected weekly for all mice for accurate dosing. For the initial comparison of tacrolimus with mycophenolate mofetil (Tac/MMF) versus mAbs in C57BL/6J, mice were either non-immunosuppressed or received one of several immunosuppression regimens. For Tac/MMF treatment, beginning 1 week preoperatively, mice received intraperitoneal MMF (30 mg/kg daily, Genentech USA Inc., South San Francisco, USA) until post-operative day (POD) 7 and intraperitoneal Tac (5 mg/kg daily, Astellas Pharmas US Inc., Northbrook, USA) beginning 1 week preoperatively and continuing daily for the study duration. For animals in this experiment comparing Tac/MMF to mAbs, induction of immunosuppression by mAb therapy (20 mg/kg each, intraperitoneal) was begun on the day of surgery and given for three daily consecutive doses. Subsequent maintenance mAb treatment was given every 7 days thereafter for the study duration. Depleting mAb treatment targeted CD4 (Clone GK1.5, Rat IgG2b,κ, 20 mg/kg, Bio X Cell, Lebanon, USA) and CD40L (Clone MR-1, Armenian Hamster IgG, 20 mg/kg, Bio X Cell). The Tac/MMF/mAb groups received the above regimens in combination.

In subsequent experiments, mAb immunosuppression began on the day prior to surgery and induction dosing continued for four daily consecutive doses followed by maintance therapy every 7 days for the study duration. This mAb regimen was administered to C57BL/6J and 5XFAD mice for hNSC dosing and long-term tracking experiments. For comparison of both mAbs versus single mAb treatment, mice received the same mAb treatment as above, compared with anti-CD4 mAb alone (20 mg/kg),

or anti-CD40L mAb alone (20 mg/kg) on the same dosing schedule.

2.4 | BLI

For in vitro imaging, hNSC-luc$^+$/GFP$^+$ were plated in a black-walled, clear bottom 96-well plate at various cell concentrations 1–2 days before imaging. Control groups included unlabelled hNSC (prepared identically to hNSC-luc$^+$/GFP$^+$) and dead hNSC-luc$^+$/GFP$^+$ (cells subjected to heat shock at 70°C for 3 min, verified by trypan blue staining). On the day of imaging when cells were approximately 70%–80% confluent, D-Luciferin was added at 150 μg/ml 2 min prior to imaging. Imaging was performed using the bioluminescence protocol with open emission, an exposure time of 30 s, medium binning and 1.5 cm subject height.

In vivo BLI was performed in the Center for Molecular Imaging at the University of Michigan using the IVIS Spectrum in vivo Imaging System (PerkinElmer, Waltham, USA). The IVIS Spectrum was initialized at the start of each imaging session to cool the charge-coupled device camera to −90°C. Mice received a single 100 μl D-Luciferin intraperitoneal injection (resuspended at 40 mg/ml in 1× PBS) 10 min prior to imaging. Anesthesia was induced with inhaled isoflurane (2% in 100% oxygen) 6 min prior to imaging and maintained at 1.5% isoflurane for the duration of the procedure. At 2 min prior to imaging, mice were placed in prone position on the heated imaging platform inside the IVIS Spectrum chamber with integrated gas anesthesia provided through a nose cone. The following IVIS acquisition settings were used throughout the study: Exposure time 180 s; F/Stop 1; Medium Pixel Binning; Field of View C; Subject height 1.50 cm. Both Luminescent and Photograph Imaging modes were selected to render a quantitative bioluminescent signal expressed in photons/second overlaid on a photographic image of the animal under white light. BLI analysis was performed using the Living Image Software (IVIS Imaging Systems). IVIS Spectrum-generated images were analysed using automatically generated contour regions of interest with a 10% threshold to eliminate background noise. Total flux (photons/second) data were collected and aggregated throughout the study.

2.5 | Tissue and blood collection, histology, flow cytometry, ELISA and CBCs

Mice were euthanized by intraperitoneal pentobarbital overdose (FatalPlus, Vortech Pharmaceuticals, Dearborn, USA), and whole blood was collected from the inferior vena cava using a 23-gauge needle. For CBCs, 100 μl of blood was placed into an EDTA-coated 100-μl microvette tube, gently rolled to mix and maintained at room temperature. Automated CBC analysis (element HT5, Heska, Loveland, USA) was performed at the In Vivo Animal Core at the University of Michigan within 4 h of blood draw. Following blood collection, mice were perfused with saline followed by 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose and cryosectioned (coronal, 40 μm sections). Transplanted hNSCs were visualized in brain sections by using the 488 nm filter for GFP for hNSC-luc$^+$/GFP$^+$ or by immunostaining with a primary antibody for human nuclei (HuNu; MAB1281, Millipore, Burlington, USA) for unlabelled hNSCs. Additional characterization of hNSC grafts used primary antibodies towards Nestin (ABD69, Millipore), glial fibrillary acidic protein (GFAP, Z0334, Dako, Glostrup, Denmark), Mouse IgG (4410, Cell Signaling, Danvers, USA), Mouse IgM (13-5790-82, Invitrogen), Mouse IgA (NB7506, Novus, Weldon Spring, USA), CD4(STJ114879-50, St. John's Laboratory, London, UK), CD40L (STJ114971-50, St. John's) and Iba1(091-19741, Wako, Richmond, USA) as previously described.[39] All fluorescent sections were counterstained with Hoechst (Pierce, Walthmam, USA).

To assess systemic toxicity associated with dual mAb treatment and/or hNSC transplantation, samples of brain, heart, liver, kidney, and pancreas of hNSC-treated animals were submitted to the University of Michigan Unit for Laboratory Animal Medicine In Vivo Animal Core. Hematoxylin/eosin-stained sections of formalin-fixed paraffin-embedded tissue from each organ were reviewed by a board-certified veterinary pathologist for evience of organ toxicity or damage, blinded to experimental groups.

A CD40L enzyme linked immunosorbent assay (ELISA) kit (Abcam, Waltham, USA) was utilized to quantify CD40L levels in serum of mAb treated animals. Briefly, following the manufacturer's protocol, samples were diluted 1:2 and placed into a 96-well plate pre-coated with anti-CD40L antibodies. Sandwich ELISA was performed by then incubating with biotin-conjugated anti-CD40L antibody, followed by Streptavidin-horseradish peroxidase and tetramethylbenzidine substrate. The reaction was then stopped by Stop solution, and absorbance in each well was read on a spectrophotometer at 450-nm wavelength (Synergy HTX, Agilent, Santa Clara, USA). Levels were compared to absorbances from a serially diluted standard of mouse CD40L provided in the kit.

To assess efficacy and off-target effects of mAb treatment, serum samples were taken 8 days after the above four dose induction regimen of anti-CD4 alone, anti-CD40L alone or both mAbs, then processed for flow-cytometry (without hNSC transplants). Briefly, red cells were lysed

in blood samples, and peripheral immune cells were isolated by centrifugation. Cells were resuspended in flow cytometry buffer and Fc receptors blocked (TruStain FcX blocking solution, BioLegend, San Diego, USA). Cells were then stained with APC-CD45, FITC-CD3, BV421-CD8 and APC-Cy7 CD4 antibody (BioLegend) at 1:100 dilution, then fixed with BD Stabilizing Fixative (BD Biosciences, Franklin Lakes, NJ). Flow cytometry was performed on a BD LSRFortessa flow cytometer with FACSDiva software (BD Biosciences) and analysed with FlowJo software (FloJo LLC, Ashland, USA). Lymphocytes were initially gated by low side scatter properties then gated for $CD4^+$ T cells, and the number of $SSC^{low}CD4^+$ cells were counted as a percentage of total lymphocytes as has been reported previously.[48–52]

2.6 | Morris water maze

The Morris water maze is a well-established method of assessing spatial memory in animal models. Briefly, at 11 months of age, animals are placed in a pool of opaque water with visual cues placed around the perimeter of the pool. Groups included wild-type mice (WT) with no treatment as well as 5XFAD mice with no treatment, 5XFAD mice that received biweekly injections of saline or CD4/CD40L mAb. In groups receiving saline or mAb injections, intraperitoneal injections began 4 weeks prior to initiation of behavioural testing. A submerged hidden platform is placed in one quadrant, which provides an escape from water. The location of the hidden platform can be deduced by spatial relationship to the surrounding visual cues, and latency for animals to swim towards and find the hidden platform reduces over repeated trials as animals learn the spatial relationship between the platform and visual cues (four trials per day for 12 days). A positive control with visible platform is performed on the 14th day. Subsequently, to test long-term reference memory, probe trials (with the platform removed) were conducted prior to start of training on day 4 and 24 h after the end of training (day 13). Time spent probing each quadrant of the pool is measured: mice that are successful in spatial learning and memory will spend a disproportionate amount of time searching for the platform in the quadrant where it was previously placed, whereas impaired spatial memory results in only 25% of time spent in the correct quadrant as a matter of chance.

2.7 | Statistical analysis

All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software Inc., La Jolla, USA), or R. Statistical significance was determined using an alpha-level of 0.05. Brown-Forsythe F-tests were used to compare variances and determine distribution. Data were analysed by parametric *t*-test, one-way analysis of variance (ANOVA) with Tukey's post-test for comparisons of multiple groups, or Pearson's correlation. Analysis of repeated BLI measurements was performed using a linear mixed effects model with random mouse-specific intercepts to determine the association between changes in total flux during follow-up as a function of treatment group. Specifically, the mixed models included a treatment effect, a linear follow-up time effect and a time by treatment group interaction effect. The mixed effects models were fit using the lmerTest package in R software version 3.5.2, and model parameter estimates were determined using the maximum likelihood method.[53] *T*-tests calculated using Satterthwaite's degrees of freedom method were evaluated to assess differences in total flux between treatment groups during follow-up. Since total flux was heavily skewed, outcomes were log transformed as log(Flux+1). We performed an available-case analysis and included all information in the mixed effects models, even those without complete data. Comparison between mouse strains was performed using Wilcoxon-Mann-Whitney Tests. Exact *n* values, *p* values and test specifics for each experiment are included in the figure legends.

3 | RESULTS

3.1 | Using BLI to assess transplanted cell survival

Using a molecular imaging approach, we modified hNSCs with a lentiviral vector to induce stable expression of luc and GFP reporters. GFP reporter expression was visualized in transduced hNSCs confirming transduction (Figure 1A), and expression increased with escalating MOI. For MOI selection, luciferase expression was measured in modified hNSCs, where highest activity was observed at MOI 25 (Figure 1B). There was also negligible effect on cell viability at this MOI, whereas higher MOI reduced cell viability (Figure 1C). Based on these data, a large bank of hNSC-luc^+/GFP^+ transduced at MOI 25 was generated for all further experiments.

We next used optical imaging to evaluate the sensitivity and specificity of bioluminescence in hNSC-luc^+/GFP^+ in vitro. Cells were serially diluted within the range of 5×10^2 to 3×10,[6] and luciferin was added prior to imaging for bioluminescence (Figure 1D). Bioluminescent signal was increased at greater cell densities. Media only and hNSC-luc^+/GFP^+ cells without luciferin were included as negative controls and produced no signal. To assess if

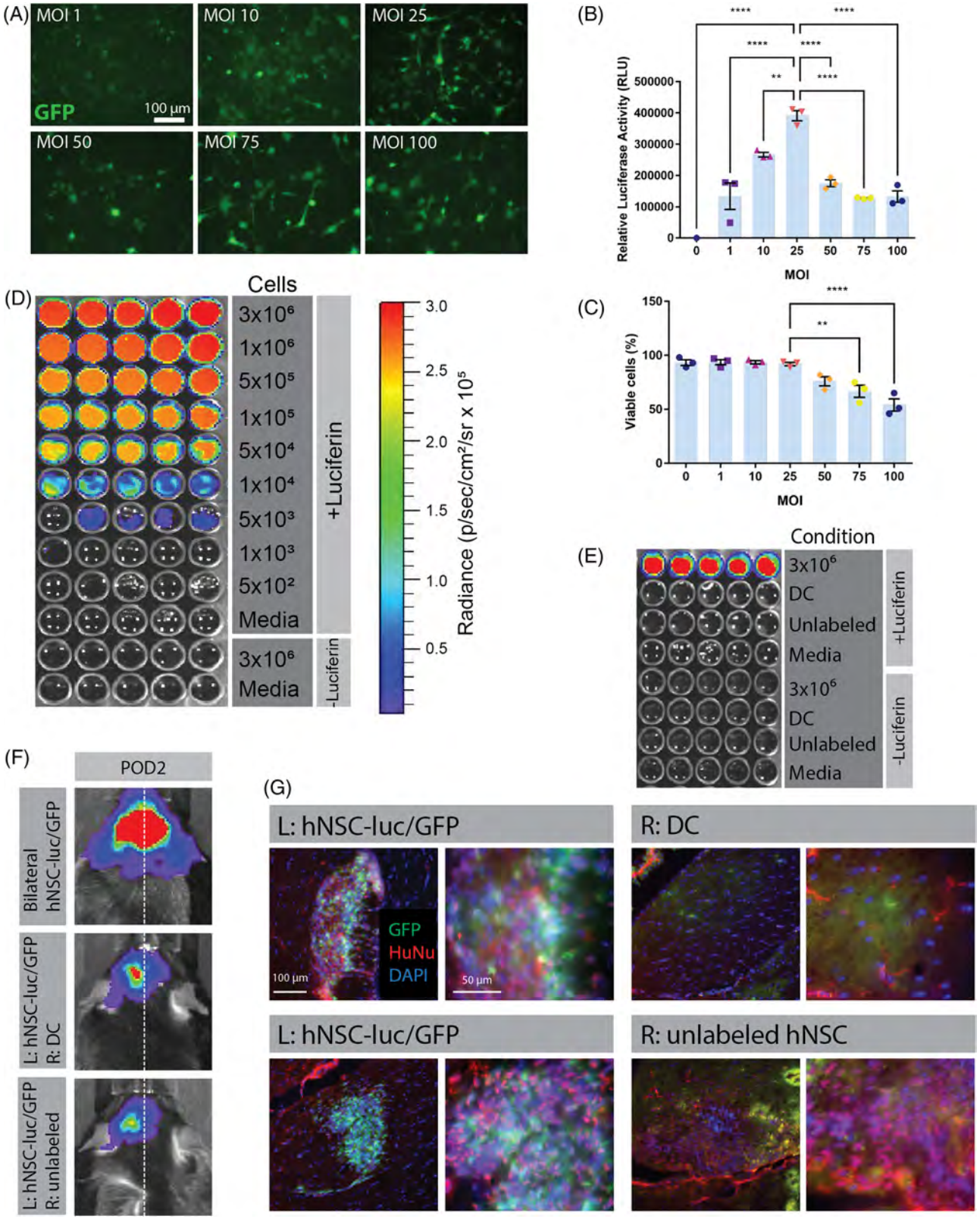

FIGURE 1 Development and validation of bioluminescent imaging (BLI) to assess transplanted human neural stem cell (hNSC) graft viability in vivo. (A) Fluorescence microscopy of green fluorescent protein (GFP) expression in hNSCs modified to express a dual reporter luc$^+$/GFP$^+$ vector at increasing multiplicity of infection (MOI) 48 h post-transduction. Luciferase assay (B) and trypan blue exclusion viability assay (C) of hNSC-luc$^+$/GFP$^+$ performed at 72 h post-transduction. (D) In vitro BLI of hNSC-luc$^+$/GFP$^+$ cells at concentrations ranging from 3×10^6 to 5×10^2 per well, with no luciferin and media only controls. (E) In vitro BLI of unlabelled hNSC and dead hNSC-luc$^+$/GFP$^+$ (DC), with hNSC-luc$^+$/GFP$^+$ cells as a positive control. (F) In vivo BLI detection of transplanted hNSC-luc$^+$/GFP$^+$ in 8-week-old C57BL/6J mice on

signal is specific to viable, labelled cells, unlabelled hNSC and dead hNSC-luc$^+$/GFP$^+$ cells were also imaged and did not produce bioluminescent signal (Figure 1E).

To evaluate bioluminescence in vivo, we injected hNSC-luc$^+$/GFP$^+$ bilaterally into the fimbria fornix of 8-week-old C57BL/6J mice and imaged on POD 2. Control groups included mice with unilateral injection of live hNSC-luc$^+$/GFP$^+$ and contralateral injection of either unlabelled hNSC or dead cells. In bilaterally transplanted mice, hNSC-luc$^+$/GFP$^+$ cells were detectable as substantial bilateral BLI signal (Figure 1F). In unlabelled and dead cell groups, bioluminescent signal was present unilaterally at the site of live hNSC-luc$^+$/GFP$^+$ cell transplants, while the contralateral side exhibited no signal. Histological analysis confirmed that hNSC-luc$^+$/GFP$^+$ grafts in postmortem mouse brain co-localized with BLI signal (Figure 1G). Here, GFP$^+$ and HuNu$^+$ cells were visualized at the site of hNSC-luc$^+$/GFP$^+$ transplants and BLI signal. Unlabelled hNSCs showed HuNu labelling alone, and dead cell grafts showed no HuNu or GFP signal.

Together, these initial proof-of-concept experiments confirmed that BLI can detect hNSC-luc$^+$/GFP$^+$ cells in vitro and in vivo and indicate that bioluminescent signal is specific to viable, labelled cells, thereby validating BLI as a tool to assess in vivo survival of transplanted cells.

3.2 | Anti-CD4 and anti-CD40L mAbs enable robust hNSC survival compared to traditional immunosuppression

We next used in vivo BLI tracking of hNSC survival in real time to compare immunosuppression protocols in C57BL/6J mice. For this purpose, mice were randomly assigned to the following groups: no immunosuppression, Tac/MMF, Tac/MMF/mAb or mAbs alone. hNSC-luc$^+$/GFP$^+$ cells were transplanted into the fimbria fornix of the hippocampus, and mice were imaged on POD 2 and weekly until end point, approximately 7 weeks post-transplantation. Induction Tac/MMF treatment began with daily administration starting 7 days before transplant and continuing 7 days after transplant, with Tac continuing daily until study endpoint. Induction of mAb immunosuppression began with daily administration of anti-CD4 and anti-CD40L starting on day of transplanted for three doses, followed by administration every 7 days until study endpoint.

Robust BLI signal indicating graft survival was observed in all groups 2 days post-operatively (Figure 2A). Signal loss rapidly declined after POD 7 in non-immunosuppressed mice and at POD 14 in mice on a regimen of Tac/MMF. However, the addition of anti-CD4 and anti-CD40L mAbs preserved bioluminescent signal, and hence graft survival, to the terminal time point of 7 weeks.

At POD 10, graft loss appeared associated with IgG and CD4 positive staining. Animals treated with traditional Tac/MMF were compared to animals receiving combined Tac/MMF and mAb, and both demonstrated accumulation of Iba1 positive microglia at graft sites (Figure S1A), suggesting microglial reaction to hNSC grafts was not impacted by mAb. We did not detect significant CD40L at cell grafts in either group (Figure S1A). By contrast, staining for Mouse IgG as well as CD4 was only seen in Tac/MMF treated animals and was not detectable in mAb treated mice (Figure S1B). There was no detectable staining of IgM or IgA (data not shown). Higher magnification images demonstrated IgG staining on the surface of many GFP$^+$ stem cells in animals treated with Tac/MMF, suggesting host-generated antibodies were reactive to xenograft surface epitopes prior to clearance of grafts (Figure S1C). While rare inclusion bodies showed IgG staining in mAb treated animals, this was not associated with GFP-expressing stem cells. This appears consistent with prior work describing a role for a humoural response as well as CD4$^+$ T cell response to xenografts in mice.[16] The difference in Mouse IgG detection could be a result of anti-CD40L mAb activity in the periphery and interference with B cell Ig class switching.[40,54]

Total BLI flux was quantified for all time points (Figure 2B) and was significantly increased over follow-up time in the mAb groups as compared to non-immunosuppressed and Tac/MMF groups ($p < .001$). No significant difference in flux was observed between Tac/MMF/mAb and mAb alone groups. Of note, in the Tac/MMF group, signal was present in only two mice from POD 14 onward, representing a survival rate of 20% ($n = 10$ total). Conversely, robust bioluminescent signal was present throughout the experiment in all mice on mAb regimens, both in the mAb alone ($n = 7$ euthanized at POD 14, $n = 5$ euthanized POD 49) and the combined Tac/MMF/mAb groups ($n = 7$ euthanized

post-operative day (POD) 2 after bilateral injection of 3.6×10^5 hNSC-luc$^+$/GFP$^+$, or unilateral injection of 1.8×10^5 hNSC-luc$^+$/GFP$^+$ (L: left side) with contralateral injection of 1.8×10^5 DC or unlabelled hNSC transplants ($n = 2$ per group). (G) Representative POD 2 immunohistochemical (IHC) images showing the fimbria fornix target area in C57BL/6J mice, with hNSC-luc$^+$/GFP$^+$ grafts expressing GFP (green) and human-specific nuclear antibody HuNu (red), with contralateral staining of DC or unlabelled hNSC. Data presented as mean ± standard error of the mean (S.E.M.) for luciferase activity and cell viability analysed by ANOVA with Tukey's post-test for comparisons of multiple groups. $^{**}p < .01$; $^{****}p < .0001$. DC, dead cells; POD, post-operative day

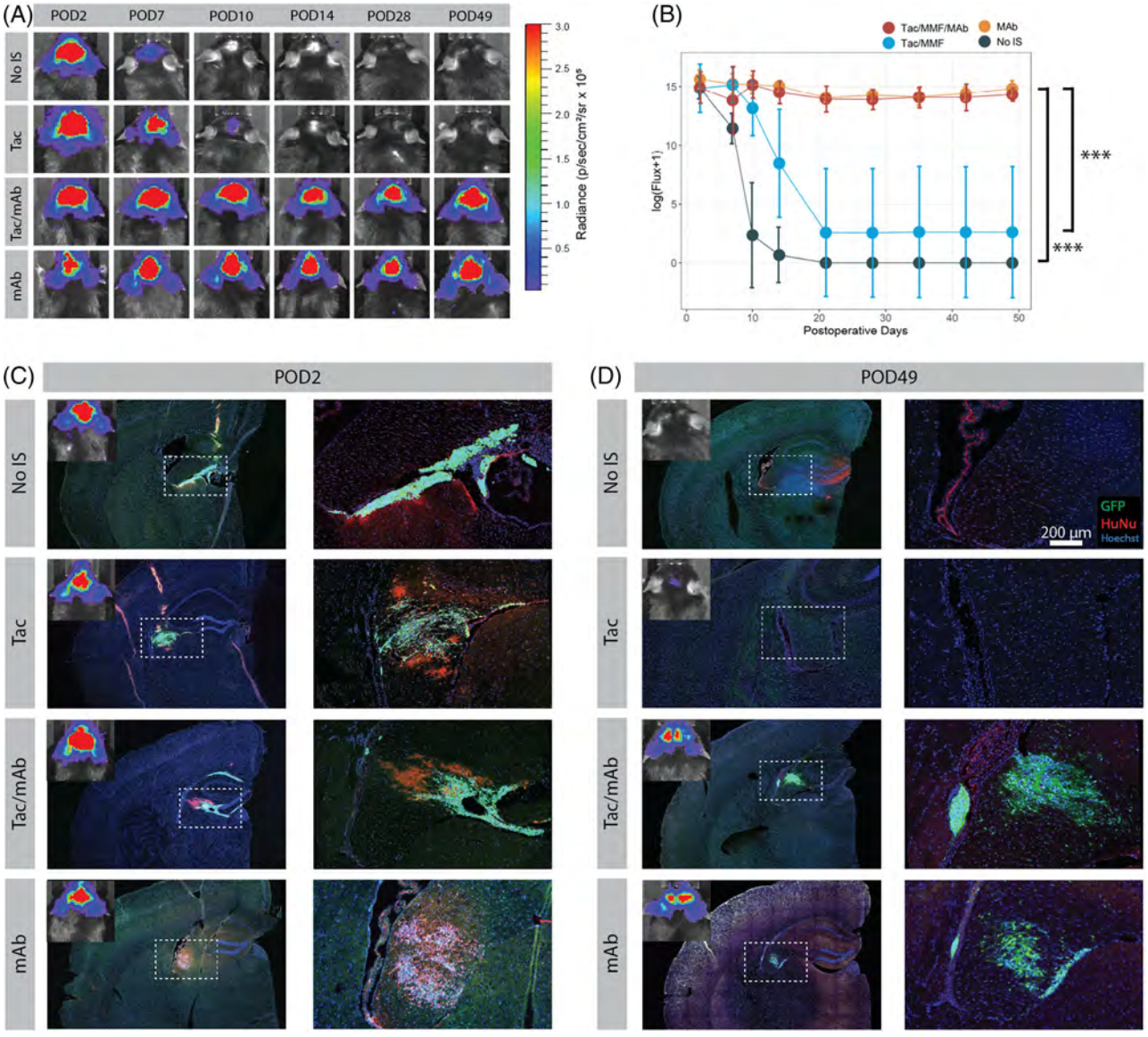

FIGURE 2 Assessment of immunosuppression protocols using bioluminescent imaging (BLI) to track transplanted hNSC graft survival. (A) Serial BLI detection of transplanted hNSC-luc$^+$/green fluorescent protein (GFP)$^+$ in C57BL/6J mice (3.6×10^5 total cells) receiving no immunosuppression (No IS), tacrolimus with mycophenolate mofetil (Tac/MMF), Tac/MMF in combination with mAbs against CD40L and CD4 (Tac/MMF/mAbs) or only mAb against CD40L and CD4 (mAbs). (B) BLI signal quantification for all mice at all-time points demonstrates significant maintenance of BLI signal in mAb treated groups versus Tac/MMF alone or non-immunosuppressed groups. (C and D) immunohistochemical (IHC) images showing GFP$^+$ grafts (green) colocalized with HuNu (red) in the fimbria fornix target area at POD 2 and POD 49 (endpoint). Starting sample size: $n = 10$ in No IS group, $n = 12$ in all other groups. Subsets were euthanized for IHC at POD2 (2 from No IS group and 3 from all other groups) and POD10 (2 from No IS group and 3 from all other groups, IHC data not shown). Remaining animals were used for each BLI data point until study end (POD 49). Data presented as mean ± standard deviation (SD) for repeated BLI measures, analysed by linear mixed effects model, ***$p < .001$. HuNu, human nuclei; IS, immunosuppression; POD, post-operative day

at POD 14, $n = 5$ euthanized at POD 49), representing a survival rate of 100%. This indicates that a regimen of only CD4/CD40L mAbs is sufficient to support graft survival.

To confirm the BLI results, transplanted hNSC-luc$^+$/GFP$^+$ grafts were also evaluated histologically directly following POD 2 and POD 49 imaging. At the earlier POD 2 time point, bioluminescent signal corresponded with GFP$^+$ cells and HuNu$^+$ immunostaining in all groups, where sizeable grafts were visible in the fimbria fornix target region (Figure 2C). These cells stained strongly for the neuronal marker Nestin, with the occasional expression of astrocyte marker GFAP (Figure S1D), consistent with our previous characterization of this cell line.[40] By POD 49, the non-immunosuppressed and Tac/MMF groups that had lost bioluminescent signal also exhibited no human-specific immunostaining or GFP$^+$

TABLE 1 Complete blood count (CBC) analysis. (A) Whole blood CBC analysis in C57BL/6J mice 8 weeks post-transplantation of 360k human neural stem cell (hNSC)-luc$^+$/green fluorescent protein (GFP)$^+$. CBC panel measures and data (mean ± standard deviation) for each experimental group. (B) CBC measures were compared between groups, and statistically significant comparisons are highlighted

A. CBC results					
Measure	**Normal range**	**No IS ($n = 7$)**	**Tac ($n = 6$)**	**Tac mAb ($n = 7$)**	**mAb ($n = 7$)**
White blood cell count - WBC ($10^3/\mu l$)	1.8–10.7	3.123 ± .5019	1.007 ± .5417	1.194 ± .7348	3.28 ± 1.469
Neutrophil count - NE ($10^3/\mu l$)	.1–2.4	.8957 ± .5754	.2067 ± .1122	.2643 ± .1295	.6986 ± .3076
Lymphocyte count - LY ($10^3/\mu l$)	.9–9.3	2.119 ± .4143	.755 ± .4117	.8714 ± .5934	2.471 ± 1.165
Monocyte count - MO ($10^3/\mu l$)	.0–.4	.09143 ± .02545	.03 ± .0228	.04429 ± .02637	.09429 ± .07829
Eosinophil count - EO ($10^3/\mu l$)	.0–.2	.01 ± .005774	.008333 ± .007528	.008571 ± .006901	.01429 ± .01272
Basophil count - BA ($10^3/\mu l$)	.0–.2	.004286 ± .005345	.003333 ± .008165	.004286 ± .005345	.002857 ± .00488
Red blood cell count - RBC ($10^6/\mu l$)	6.36–9.42	8.579 ± .31	8.478 ± .8597	9.18 ± .7665	9.134 ± .4404
Hemoglobin - HB (g/dl)	11.0–15.1	11.69 ± .3891	10.97 ± 1.234	11.91 ± .4634	11.73 ± .6317
Hematocrit - HCT (%)	35.1–45.4	37.01 ± 1.471	35.63 ± 3.976	38.17 ± 1.608	39.47 ± 2.391
Mean cell volume - MCV (fL)	45.4–60.3	43.14 ± .5442	42 ± .6663	41.71 ± 1.875	43.2 ± .8794
Mean cell hemoglobin - MCH (pg)	14.1–19.3	13.64 ± .1902	12.9 ± .2098	13.01 ± .7151	12.83 ± .4751
MCH concentrate - MCHC (g/dl)	30.2–34.2	31.59 ± .3805	30.78 ± .4167	31.21 ± 1.014	29.73 ± .9759
Red cell distribution width - RDW (%)	12.4–27.0	17.74 ± .4894	17.18 ± .7305	18.24 ± 1.247	18.04 ± .4036
Platelet count -PLT ($10^3/\mu l$)	592—2972	439.9 ± 197.1	377.3 ± 250.2	472.3 ± 249.2	662.3 ± 105.1
Mean platelet volume - MPV (fL)	5.0–20.0	4.543 ± .4198	4.717 ± .6401	4.514 ± .7988	4.271 ± .1799

B. CBC statistics							
Group comparison	**WBC**	**NE**	**LY**	**MO**	**EO**	**BA**	**RBC**
No IS versus Tac	****	***	***	*	ns	ns	ns
No IS versus Tac MAb	***	***	**	*	ns	ns	ns
No IS versus MAb	ns	ns	ns	ns	ns	ns	ns
Tac versus Tac MAb	ns	ns	ns	ns	Ns	ns	*
Tac versus MAb	****	**	****	**	Ns	ns	ns
Tac MAb versus MAb	****	*	****	*	Ns	ns	ns

*Significant ($p < .0332$) by one-way ANOVA.
**Significant ($p < .0021$) by one-way ANOVA.
***Significant ($p < .0001$) by one-way ANOVA.

cells (Figure 2D). Weakly GFP$^+$ and HuNu$^+$ debris was observed in the target areas, indicative of graft rejection. Conversely, the retention of bioluminescent signal in mAb groups corresponded with intact HuNu$^+$/GFP$^+$ cells in the fimbria fornix target region, demonstrating robust viability of transplanted hNSC-luc$^+$/GFP$^+$ cells.

We also analysed CBC profiles on whole blood from mice that underwent hNSC transplantation and BLI (Table 1). Decreased white blood cell counts, including neutrophils, lymphocytes and monocyte counts (with preserved lymphocyte:neutrophil ratios) were observed in Tac/MMF and Tac/MMF/mAb groups as compared to non-immunosuppressed controls, indicating a more global immunosuppression relative to non-treated, non-immunosuppressed controls. However, mice receiving only mAbs with hNSC transplants maintained normal CBC profiles comparable to non-immunosuppressed mice. Eosinophil, basophil, red blood cell and platelet counts were normal for all hNSC transplant groups regardless of immunosuppressant. These data indicate that mAb immunosuppression mediates a targeted immunosuppressant activity sufficient to preserve human hNSC survival without detrimental impact to peripheral CBC profiles.

Together, these data show that a regimen of anti-CD4 and anti-CD40L mAbs extends the survival of intracranial human stem cell transplants through at least 7 weeks in C57BL/6J mice and does not negatively impact CBCs compared to the conventionally used immunosuppressive agents Tac/MMF, offering an enhanced immunosuppression method for xenografts in mice.

3.3 | Both anti-CD4 and anti-CD40L mAbs are required for transplanted hNSC graft survival

Although anti-CD4 or anti-CD40L mAbs have been used in combination with other agents to promote the short-term acceptance of transplanted organ and cell grafts in mice,[33,55,56] they have not well studied individually in the context of CNS xenograft transplantation. Therefore, we next questioned if suppression of both $CD4^+$ and $CD40L^+$ cells is required, or if administration of a single mAb is sufficient for long-term transplanted hNSC graft survival. To determine this, hNSC-luc^+/GFP^+ cells were transplanted intracranially to C57BL/6J mice on a regimen of anti-CD4 mAb alone, anti-CD40L mAb alone, or a combination of both anti-CD4 and anti-CD40L mAbs. Induction mAb treatment began 1 day prior to transplant for 4 daily doses, and continued every 7 days until study endpoint. Serial BLI imaging was again used to track hNSC survival over time and was performed beginning on POD 2 and weekly or biweekly until end point, approximately 28 weeks post-transplantation (Figure 3A). Mice receiving both mAb retained BLI signal strength through 28 weeks, indicative of hNSC graft survival. However, similar graft survival was not maintained in the anti-CD4 alone and anti-CD40L alone groups. This was also reflected in the signal quantification (Figure 3B), where total flux was similar for all groups at POD 2 then separated longitudinally, with the dual-mAb group retaining significantly increased signal throughout the 28-week time span as compared to anti-CD4 alone ($p < .001$) or anti-CD40L alone ($p < .001$).

To confirm and characterize the effect of mAb immunosuppression on the host immune response, we also assessed $CD4^+$ cells and soluble CD40L levels in peripheral blood. Here, a separate cohort of animals received anti-CD4 alone, anti-CD40L alone or dual mAb therapy on an induction schedule of four daily doses of mAb. Flow cytometry for $CD4^+$ cells was performed on peripheral whole blood collected 1 week after the induction regimen of mAb. Mice in the anti-CD4 alone and dual mAbs groups displayed depletion of $CD4^+$ cells, relative to mice that were treated with anti-CD40L alone and untreated controls (Figure S2A). Numbers of $CD40L^+$ T cells were poorly detected by flow cytometry even in untreated animals (Figure S2B) likely due to the transient nature of surface CD40L expression with T cell activation.[57,58] Therefore, serum was taken from animals after completion of BLI studies above and analysed for soluble CD40L. ELISA analysis of soluble CD40L levels in serum collected at experimental endpoint (28 weeks) for hNSC-transplanted animals, only mice treated with anti-CD40L, either alone or in combination with anti-CD4 mAb, displayed depletion of soluble CD40L, relative to controls (Figure S2C).

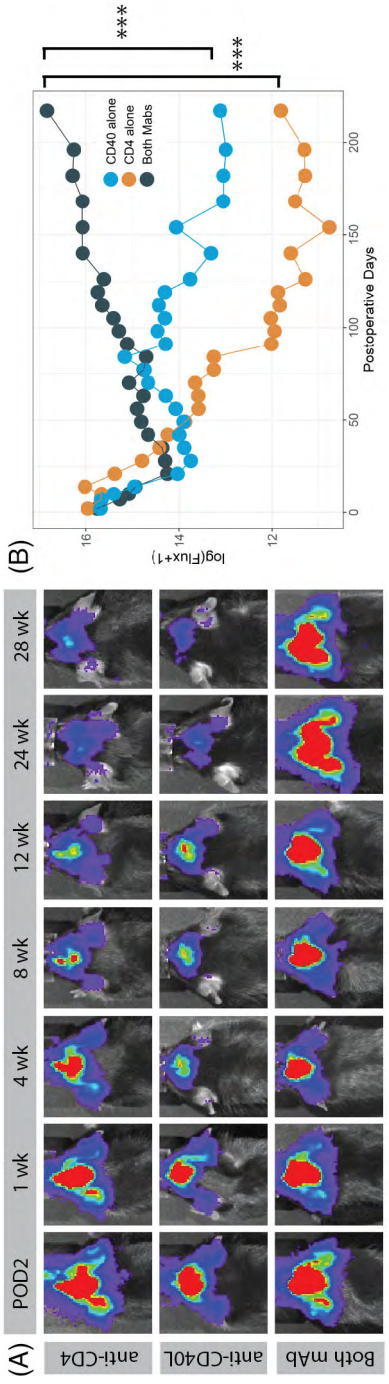

FIGURE 3 Both anti-CD4 and anti-CD40L mAbs are required for human neural stem cell (hNSC) graft survival. (A) Serial bioluminescent imaging (BLI) detection of transplanted hNSC-luc^+/green fluorescent protein $(GFP)^+$ in C57BL/6J mice (3.6×10^5 total cells) receiving anti-CD4 mAb, anti-CD40L mAb or both mAbs. (B) BLI signal quantification for all mice at all-time points. Dual mAb treatment resulted in significantly greater persistence of BLI signal. Sample size: $n = 5$ animals per group. Data presented as mean for BLI measures, error bars omitted for clarity, analysed by linear mixed effects model, $^{***}p < .001$. POD, post-operative day, wk = week

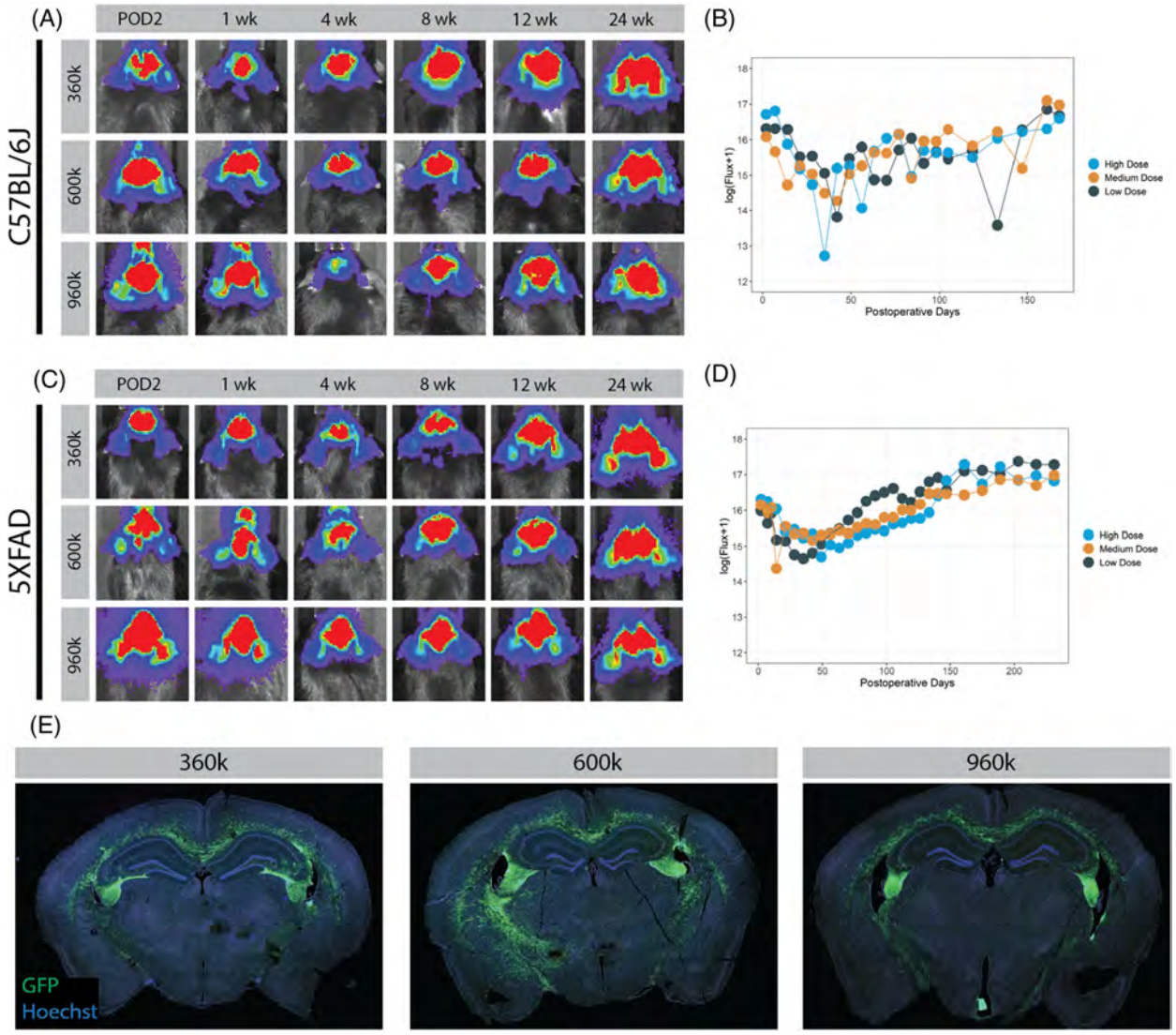

FIGURE 4 Long-term bioluminescent imaging (BLI) tracking of transplanted human neural stem cell (hNSC) in C57BL/6J and 5XFAD. Serial BLI detection and signal quantification of 3.6×10^5, 6.0×10^5 or 9.6×10^5 hNSC-luc$^+$/green fluorescent protein (GFP)$^+$ cells transplanted in C57BL/6J mice (A and B) and 5XFAD Alzheimer's disease mice (C and D) on a dual mAb immunosuppression protocol of anti-CD4 and anti-CD40L. No biologically relevant statistical differences in BLI flux are seen between cell dose groups. (E) Representative immunohistochemical (IHC) images demonstrate GFP$^+$ hNSC-luc$^+$/GFP$^+$ grafts in the fimbria fornix target area at endpoint in C57BL/6J mice. Sample size: $n = 5$ animals per treatment dose ($n = 15$ animals for C57BL/6J and another 15 for 5XFAD). Data presented as mean for BLI measures, error bars omitted for clarity, analysed by linear mixed effects model. POD, post-operative day

3.4 | CD4 and CD40L mAb regimen is effective at multiple cell doses and in a transgenic mouse model

After demonstrating that dual mAb-based immunosuppression is more effective than Tac/MMF-based regimens or each mAb alone, we next investigated this method of immunosuppression using multiple cell doses in a transgenic mouse model. Induction of immunosuppression by daily dual mAb therapy was again performed starting 1 day prior to transplant for four doses, followed by maintenance dosing every 7 days until study endpoint. Long-term graft survival was initially assessed in 8-week-old C57BL/6J mice. Multiple doses of hNSC-luc$^+$/GFP$^+$ cells were transplanted bilaterally into the fimbria fornix at increasing doses of 3.6×10^5 (low dose), 6.0×10^5 (medium dose) and 9.6×10^5 (high dose) total cells, and longitudinal BLI was performed to track graft survival. Robust BLI signal indicative of hNSC graft survival was present up to the study endpoint 24 weeks post-transplantation (Figure 4A). While a statistical difference in the BLI signal was seen in comparing the medium and the low dose cell groups ($p = 0.035$), this difference was thought to be attributable to outlier BLI flux timepoints in the low- and high-dose

groups and deemed not biologically meaningful (Figure 4B). Functionally, there was no difference in BLI signal between administered cell doses.

Using an identical experimental design, we assessed mAb immunosuppression in 8-week-old 5XFAD mice, a commonly used model of Alzheimer's disease. Escalating doses of $3.6 \times 10,^5$ 6.0×10^5 and 9.6×10^5 total cells were transplanted bilaterally into the fimbria fornix, and weekly BLI was again performed to track graft survival over time until the study termination at approximately 8 months post-transplantation (data concordant with C57BL/6J mice up to 24 weeks presented here). Here, similar long-term hNSC graft survival was achieved in 5XFAD mice, where robust BLI signal was present in all three cell dose groups until study endpoint (Figure 4C). Again, while statistical differences were noted between groups in effect of treatment dose over time (low- vs. medium-dose, $p = .013$; low- vs. high-dose, $p < .001$), stratification and correlation of BLI signal to treatment group could not be consistently identified (Figure 4D).

To address the question of differential hNSC survival rates between strains, we performed a comparison of within-individual percent change of log (Flux+1) between first measurement to post-operative day 161 (latest shared time point) using Wilcoxon-Mann-Whitney tests. There were no significant differences between C57BL/6J and 5XFAD animals in low dose ($p = .73$), medium dose ($p = .56$) or high dose ($p = .53$) groups, or even all groups combined ($p = .61$).

BLI data were confirmed histologically, where GFP^+ hNSC grafts were visible in the fimbria fornix target of C57BL/6J (Figrue 4E). Whole brain imaging revealed significant migration of hNSCs from the site of transplantation at the fimbria fornix throughout the white matter tracts, including the corpus callosum. This migratory action may account for the apparent BLI signal increase over the long-term period of 6 months. Histopathologic toxicity screening (blinded to treatment group) was performed at 6 months after transplant of C57BL/6J animals receiving low, medium and high dose hNSC grafts and dual mAb immunosuppression. This revealed 'no findings suggestive of treatment-related toxic effect in the experimental group, based on hematologic, gross pathology and histological evaluation of representative organs' (Table S1, full report available upon request).

We were further able to confirm in 5XFAD mice that the known behavioural deficit in hippocampal-based memory tasks was not affected by injection regimen or mAb treatment. Delayed learning curve during training period for Morris Water Maze was noted in all 5XFAD animals as compared to WT controls, regardless of whether animals received mAb, saline injections alone or no treatment (Figure S3A). Further, there was no difference in 5XFAD groups during probe trials as measured by time spent exploring the target quadrant (Figure S3B). Together, these data demonstrate that dual mAb immunosuppression supports robust hNSC survival at multiple cell doses for up to 6 months in C57BL/6J mice and up to 8 months in the 5XFAD transgenic model of Alzheimer's disease.

4 | DISCUSSION

A significant obstacle facing the widespread adoption of stem cell therapy for neurological diseases is transplant rejection by the host immune system and eventual graft failure.[16,59] As progress is made in developing cell-based treatments, reliable methods to overcome host immune rejection are of paramount importance. In the present research, we show that an immunosuppressive regimen of depleting anti-CD4 and anti-CD40L mAbs results in robust long-term persistence of hNSC grafts, superior to the traditional agents Tac/MMF. Cell survival requires both antibodies for maximal efficacy, persists across escalating stem cell doses and is applicable to disease models. The mAb regimen is also more effective and less toxic than conventional immunosuppressive agents. Overall, this study supports the use of dual mAbs as an alternative immunosuppression method for study of human stem cell therapeutics in preclinical animal disease models.

For cell-based therapy to succeed in clinical translation, transplanted cells must survive. In allogenic transplant paradigms, a variety of strategies have been pursued to achieve this. Prior work has utilized mesenchymal stem cells, given the relative ease of sourcing from bone marrow and early thought that mesenchymal stem cells could avoid immune rejection.[60] However, follow-up studies demonstrated that allogenic mesenchymal stem cell transplants still elicited an immune response and graft rejection in the 'immune privileged' CNS.[61–63] Furthermore, while mesenchymal stem cells can be transdifferentiated to neuron-like cells in vitro, their ability to recapitulate neurons in vivo after transplantation has been less studied.[64,65] Alternatively, the advent of induced pluripotent stem cell technology brings the promise of autologous cell transplants, but currently these approaches are labour- and cost-intensive and likely to be prohibitive for large clinical trials and population scale therapy. Therefore, as a more practical paradigm, hNSC lines represent a promising 'off the shelf' therapeutic option in neurologic disorders given the capacity for large-scale expansion and commitment to neuroglial differentiation. These advantages have led to many early phase trials utilizing hNSC lines.[7–11]

In preclinical testing for future therapies, hNSC studies encounter the added barrier of requiring xenograft animal models to demonstrate efficacy even prior to invoking

an allograft paradigm in human trials. A number of studies use immunodeficient or humanized animal strains,[66] yet this broad approach removes a critical contribution of the immune system to underlying disease pathophysiology of the animal model.[67] Many groups utilize traditional pharmaceutical-based immunosuppression based on solid organ transplant protocols. As an example, preclinical studies supporting Phase 1 and 2 clinical trials of a human spinal cord stem cell line in amyotrophic lateral sclerosis utilized Tac/MMF.[68–71] Yet these drugs proved to be insufficient in long-term xenograft models. This is consistent with our own published work in various mouse models where immunosuppression with a combination of Tac/MMF resulted in low survival rates and graft clearance within 8 weeks.[41,47] Furthermore, long-term use of these drugs may directly impact the fidelity of the underlying disease model,[72,73] and prolonged use of these drugs is associated with toxic side effects, including elevated risk of infections, diabetes, hypertension, cardiovascular disease, nephrotoxicity and neurotoxicity.[74] Therefore, a robust immunosuppressive regimen that ensures hNSC survival without impacting the model phenotype is critically needed.

In the current study, dual mAb therapy resulted in cell survival in 100% of transplanted animals for at least 6–8 months in both C57BL/6J mice and an aggressive model of AD, with no disruption in peripheral blood profiles or other organ toxicity. There was also no detrimental impact to the behavioural phenotype under study. We expect this targeted approach is a major advance enabling the study of a wide range of stem cell therapies in the CNS. $CD4^+$ T cells play a central role in cytotoxic rejection of transplanted cells, particularly more mature or differentiated cells that up-regulate major histocompatibility complex class I and II in the setting of local inflammation.[21,24,75,76] Therefore, depleting $CD4^+$ T cells targets a fundamental immune rejection mechanism. CD40L also appears to be a critical component of the immune recognition and rejection of stem cell xenografts, as demonstrated by our data showing mAbs targeting both CD4 and CD40L are required for optimal cell survival. We did not detect significant changes in membrane-bound CD40L with peripheral or infiltrating T cells, and hypothesize that CD40L plays a role in antigen presentation in the periphery for activation of stem cell-responsive immune cells.[77–79] However, constitutive expression of CD40L in licensed T cells within rejecting grafts appears unnecessary for immune clearance, at least in the CNS.[80] Our observed CD4 and mouse IgG staining at the xenograft site are likely downstream effects of CD40L activation, which is curtailed with dual mAb therapy. At the same time, in contrast to broad pharmacologic immunosuppression, this targeted approach appears to minimize collateral adverse effects. This is evidenced by reduced alterations in CBC profiles of mAb-treated animals versus those receiving Tac/MMF, as well as lack of histopathologic toxicity. A prior study by Ager et al.[81] reported survival of CNS xenografts for up to 6 weeks in two AD mouse models utilizing mAbs targeting LFA-1, CD40 and CTLA-4, representing an improvement over traditional immunosuppressant drugs. A number of alternate mAb-based approaches have been pursued targeting the T cell costimulatory mechanism, including targeting CD4 alone,[20,31,82] CD2/T-cell receptor $\alpha\beta$,[83] CD25,[84] IL-2R,[85] CTLA-4/MR1[86] and B7/LFA-1,[80,87] although these studies did not interrogate cell survival for more than 4–18 weeks. Here, our data demonstrate that anti-CD4 mAb alone still results in a significant degree of graft loss over the long-term, while dual mAbs produces robust graft survival for more than 28 weeks.

Although we found statistical differences in BLI signal over time with varying cell doses in C57BL/6 and 5XFAD mice, variability in BLI quantification curves precludes conclusions on biological relevance. Some variability in BLI signal is likely related to migration and spread of luminescent cells along white matter tracts in the brain. Furthermore, it is possible that a maximal cell dose was reached, and additional cell survival at higher cell number/concentration could not be supported in the transplant site.[88] We are performing additional dosing studies using an unmodified hNSC cell line to further investigate and identify the maximal tolerated dose in AD mice. As this approach is extended to other disease models, the preservation of therapeutic benefit as well as underlying animal model phenotype will need to be validated for each cell line/model in the presence of dual mAb-based immunosuppression.

The approach presented here is readily translatable to clinical trials of stem cell therapy. Therapies targeting CD4 (clenoliximab, keliximab, zanolimumab) and CD40(teneliximab) are pending or already under investigation in human trials.[36,89,90] Still, several obstacles remain in clinical application of stem cell therapies for neurological diseases. Although we saw no behavioural difference with mAb treatment in our AD mouse model, given the complex interaction of the nervous system and the immune system, global impacts of chronic mAb treatment especially in humans will need further study. Furthermore, while we modified transplanted cells to express luc/GFP for in vivo BLI, alternate methods will be required for non-invasive cell tracking in humans. Current techniques, such as superparamagnetic iron oxide labelling or use of radiotracers, can become diluted over time and are also limited by the inability to distinguish between live cells versus phagocytosed dead cell debris.[91,92] Thus, future studies must also address a viable cell tracking approach in humans.

The present study has a number of limitations and is focused on optimizing survival of hNSC grafts in murine models, including 5XFAD model of Alzheimer's disease. Our data demonstrate cell survival in an AD mouse model; still, many questions regarding therapeutic benefit of hNSCs and potential mechanisms of action remain to be answered in ongoing larger-scale preclinical studies. We show that dual mAb therapy and the anxiety from repeated handling/injections do not adversely affect the fidelity of the cognitive deficits seen in this model.[42] However, these studies were not designed or powered to assess efficacy of hNSCs as a therapy for Alzheimer's disease. Our studies with mAb immunosuppression are limited to a single human NSC line that is currently under therapeutic development for AD as part of the NIA Alzheimer's Drug Development Program (5U01AG057562). Larger-scale efficacy studies are ongoing, enabled by discoveries presented here. Feasibility must also be established for other cell types of interest, such as those derived from iPS or ES cells. We also altered our cell line to express luciferin and GFP. We therefore cannot conclude that graft survival was not purely mediated by immune tolerance to expression of luciferin or GFP specifically, as opposed to other human epitopes on the transplanted cells.

While we demonstrated superiority of dual mAb treatment to traditional immunosuppression, it remains unclear if maintenance mAb treatments are required indefinitely. Ongoing weekly antibody infusions would be challenging for patients, although infusion in human subjects could be extended based on antibody pharmacokinetics[93] or using techniques to extend antibody half-life[94] Animal data suggest cessation of immunosuppression results in graft loss even at later time points,[32] although scant human data show persistence of some cells after a limited course of immunosuppression.[95]

As mAb mature in the clinical realm, their use and potential drawbacks will require long-term study. Although current solid organ/bone marrow transplant patients carry elevated risk of infection and medication side effets with current immunosuppressants, ongoing depletion of CD4 and CD40L in humans is also likely to carry some risk. While we did not see significant adverse events in our well-controlled animal population, infection risks and adverse effects of sustained CD4/CD40L depletion will need to be accounted for and compared to traditional regimens in any future application in humans.[96,97] Given the mechanism of targeting costimulatory activation in the presence of transplanted stem cell antigen presentation, it is theoretically possible host tolerance could be induced or that T cells could be rendered anergic to transplanted antigens.[80,87] In future studies, if a limited course of mAb therapy could be defined that preserves stem cell viability, side effect profiles and translational potential could be improved even further.

The results presented here are important for several reasons. Establishing an efficacious targeted immunosuppression treatment that ensures hNSC graft survival enables preclinical investigation and translation of cell-based therapeutics in the CNS. We have demonstrated durable xenograft cell survival over an extended period of at least 28 weeks, longer than other reported studies.[31,32] This informs future preclinical studies that may require longer timelines and serves as a guideline for cellular therapies that translate to early clinical trials. As the advantages of stem cell-based treatment paradigms become increasingly utilized, the robust immunosuppression regimen outlined here will optimize long-term rates of success.

5 | CONCLUSIONS

mAbs (anti-CD4 and anti-CD40L) enable long-term (>6 months) survival of hNSCs grafted into brains of laboratory mice and a mouse model of Alzheimer's disease. This facilitates robust preclinical study of cell-based therapy for CNS disorders and fast-tracks translation of stem cell therapy to early phase clinical trials.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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