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Advances in understanding the pathogenetic mechanisms of neurodevelopmental disorders and neurodegenerative disease - the environment as a putative risk factor

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Published in
Frontiers in Neurology
Frontiers in Neuroscience

ISSN 1664-8714
ISBN 978-2-8325-3269-0
DOI 10.3389/978-2-8325-3269-0

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Advances in understanding the pathogenetic mechanisms of neurodevelopmental disorders and neurodegenerative disease - the environment as a putative risk factor

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Citation

Lavezzi, A. M., Colizzi, M., Lein, P. J., eds. (2023). *Advances in understanding the pathogenetic mechanisms of neurodevelopmental disorders and neurodegenerative disease - the environment as a putative risk factor*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-3269-0

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frontiers | Frontiers in Neurology

TYPE Editorial
PUBLISHED 03 August 2023
DOI 10.3389/fneur.2023.1259772

OPEN ACCESS

EDITED AND REVIEWED BY
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RECEIVED 16 July 2023
ACCEPTED 25 July 2023
PUBLISHED 03 August 2023

CITATION
Lavezzi AM, Colizzi M and Lein PJ (2023) Editorial: Advances in understanding the pathogenetic mechanisms of neurodevelopmental disorders and neurodegenerative disease — The environment as a putative risk factor.
Front. Neurol. 14:1259772.
doi: 10.3389/fneur.2023.1259772

Editorial: Advances in understanding the pathogenetic mechanisms of neurodevelopmental disorders and neurodegenerative disease — The environment as a putative risk factor

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KEYWORDS

neurodevelopmental disorders, neurodegenerative disease, environmental factors, Parkinson's disease, Alzheimer's disease, sleep disorders

Editorial on the Research Topic
Advances in understanding the pathogenetic mechanisms of neurodevelopmental disorders and neurodegenerative disease — The environment as a putative risk factor

Introduction

Neurodevelopmental disorders are generally influenced by not only genetic, but also intrauterine and extrauterine factors that affect the fetal-maternal environment and/or brain development that continues after birth (1). Specific genetic polymorphisms may increase susceptibility to environmental factors that alter the trajectory of brain development via diverse molecular mechanisms (2). In particular, pre- and post-natal exposure to neurotoxic metals, pesticides, persistent organic pollutants, and other chemicals is increasingly recognized as involved in the pathogenesis of neurodevelopmental disorders, such as autism, deficiency attention/hyperactivity disorders, and even fetal and infant death, including SIUDS (Sudden Unexplained Intrauterine Death Syndrome) and SIDS (Sudden Infant Death Syndrome) (3–9).

Similar evidence has been found also for neurodegenerative disorders such as Parkinson's, Alzheimer's disease, and chronic multiple sclerosis (10–12). In fact, especially in the context of specific genetic vulnerability, exposure to such environmental factors across the lifespan increases the likelihood of neurodegenerative processes. Combining research outputs from studies of both neurodevelopment and neurodegeneration may help advance

our understanding of the complex phenomena that modulate brain structure and function throughout life, with implications for health and disease.

Thus, it is essential to study the ethio-pathogenetic and anatomo-pathological aspects of neurodevelopmental disorders and neurodegenerative disease with particular attention to the study of specific biomarkers useful for diagnostic and prognostic purposes.

The goal of the Research Topic "*Advances in understanding the pathogenetic mechanisms of neurodevelopmental disorders and neurodegenerative disease — The environment as a putative risk factor*" was to collect contributions from expert authors in order to advance the state of knowledge regarding the pathogenetic mechanisms by which various factors, including drugs, diet, genetic, and environmental factors, interact to increase individual risk for neurological disorders and diseases across the lifespan.

This Research Topic was proposed in particular to focus on the following subtopics:

- Genetic background that may increase susceptibility to environmental factors that alter nervous system development
- Molecular and neuropathological features of neurological disease across the lifespan
- Approaches for identifying specific genetic substrates and environmental factors that alter brain development to cause disease
- Mechanisms by which genetic and environmental factors interact to increase risk of neurological disease
- Neuropathology of unexplained perinatal deaths, considering also the interaction between environmental risk factors and brain developmental defects
- Proposal of evidence-based prevention and management strategies to decrease the incidence of these pathologies.

Published articles

In order to summarize the contributions to this Research Topic, we have grouped the articles in two main sections: one dedicated to new perspectives useful both for preventive and diagnostic purposes of various neurological disorders and a second focused on new biomarkers which can help in predicting Alzheimer's and Parkinson's diseases.

Section 1- Original perspectives on pathogenesis of sleep disorders, cognitive alterations and neurodegenerative processes

The articles are distributed in the following subsections:

(a) New indicators of neurodegenerative processes

- Through a meta-analysis approach, Zhang L. et al. highlighted a positive association between exposure to first-generation antiepileptic drugs (such as valproate, carbamazepine, and clonazepam) and increased risk of dementia.
- Li W. et al., in an original study found that dietary inflammation and blood inflammation indexes are negatively associated with cognitive function in an elderly American population.
- The experimental research of Li G.-S. et al. explored the ultrastructural pathological changes of the neurovascular unit (NVU), a structural and functional complex that plays an important role in the coupled interaction between neural activity and microcirculation, and consequently in the pathophysiological mechanism of many cerebral disorders. The study disclosed the presence of NVU destruction in the development stage of the cervical spondylotic myelopathy (CSM), the most common cervical spinal cord disorder among the elderly population.

(b) Involvement of environmental factors in neurological disorders

- Calderón-Garcidueñas et al. envisaged how exposures to high concentrations of particulate matter (PM2.5), ultrafine particulate and industrial nanoparticles, can damage the nervous system dating back to in prenatal life, playing a significant role in the development of neurodegenerative processes and sleep disorders.
- The adverse impact of exposure to neurotoxic metal mixtures on brain connectivity was illustrated by Invernizzi et al. through neuroimaging studies on a wide set of young people. They demonstrate that various metal mixtures may alter the brain development by modifying the global and local connectivity. These changes may potentially lead to alterations in cognition and neurobehavior in adolescents and young adults.
- Damage from toxic metals was also demonstrated by Vegard et al.. The aim of this study was to investigate whether early-life exposure to toxic metals and essential elements can adversely affect nervous system development. These authors demonstrated an association between second trimester maternal blood levels of copper and manganese and increased risk of Cerebral Palsy, which is the most common motor disability in childhood, the causes of which are currently only partly known.
- The study of Humphreys and Valdés Hernández, based on a systematic review and meta-analysis, supports the hypothesis that prenatal polycyclic aromatic hydrocarbon exposure negatively affects cognitive function and increases the risk of neurodegeneration in humans.
- Spencer et al. proposed an intensive analysis of published geographic clusters of conjugal cases, single-affected twins, and young-onset cases of Amyotrophic Lateral Sclerosis (ALS), to identify which environmental factors may trigger motor neuron disease. The study highlights that exposure to naturally occurring or synthetic hydrazine-related chemicals, acting alone or in the presence of a genetic susceptibility, is associated with the development of clinical ALS.

Section 2- New diagnostic parameters for the early identification of individuals at risk of Parkinson's and Alzheimer's diseases

- New biomarkers for predicting the occurrence of Parkinson's disease (PD) have been highlighted by Zhang P. et al. They used bioinformatics analysis of the immune system to show that the analysis of four immune infiltration-related genes (precisely SYT1, NEFM, GAP43, and GRIA1) identified individuals at risk of PD before the onset of motor symptoms.
- The relation between hypertension and increased risk of AD has been underlined by Sáiz-Vazquez et al. in original research based on the analysis of information predominantly obtained by meta-analyses of primary studies worldwide.
- In an explorative study, Tsai et al. investigated the associations between sleep parameters measured using polysomnography and plasma levels of selected biomarkers of neurodegenerative diseases in patients with suspected obstructive sleep apnea (OSA) to assess the relationships between sleep disorders and the risk of Alzheimer's disease development. The results reveal that individuals at high-risk have significantly higher mean values for various indices of sleep-disordered breathing and arousal responses than those at low-risk.

Conclusions

We believe that the above contributions, although heterogeneous in their approach, collectively broaden the current knowledge on the pathogenetic mechanisms of neurodevelopmental disorders and neurodegenerative diseases, which often involves complex interactions between genetic and non-genetic factors, including exposure to environmental contaminants. In conclusion, we hope these articles will prove useful for improving current diagnostic criteria and preventive strategies and also for providing impetus for further research in the field of this Research Topic.

Author contributions

AL: Conceptualization, Data curation, Validation, Writing—original draft, Writing—review and editing. MC: Conceptualization, Data curation, Validation, Writing—original draft. PL: Conceptualization, Supervision, Validation, Writing—original draft, Writing—review and editing.

Acknowledgments

The Research Topic Editors express sincere gratitude to the authors who accepted the invitation to contribute to this collection and the reviewers for their significant comments, so elevating the quality of the submitted articles. Finally, they thank Frontiers and, in particular, the Editorial Office for the essential support.

Conflict of interest

MC has been a consultant/advisor to GW Pharma Limited, GW Pharma Italy SRL, and F. Hoffmann-La Roche Limited outside of this work.

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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

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frontiers | Frontiers in Neuroscience

TYPE Original Research
PUBLISHED 16 November 2022
DOI 10.3389/fnins.2022.1031180

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

RECEIVED 29 August 2022
ACCEPTED 25 October 2022
PUBLISHED 16 November 2022

CITATION
Li G-S, Wang X-X, Tan R-B, Wang K-H, Hu X-s and Hu Y (2022) Ultrastructural destruction of neurovascular unit in experimental cervical spondylotic myelopathy. *Front. Neurosci.* 16:1031180. doi: 10.3389/fnins.2022.1031180

Ultrastructural destruction of neurovascular unit in experimental cervical spondylotic myelopathy

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Background and purpose: The pathogenesis of cervical spondylotic myelopathy (CSM) remains unclear. This study aimed to explore the ultrastructural pathology of neurovascular unit (NVU) during natural development of CSM.

Methods: A total of 24 rats were randomly allocated to the control group and the CSM group. Basso–Beattie–Bresnahan (BBB) scoring and somatosensory evoked potentials (SEP) were used as functional assessments. Hematoxylin–eosin (HE), toluidine blue (TB), and Luxol fast blue (LFB) stains were used for general structure observation. Transmission electron microscopy (TEM) was applied for investigating ultrastructural characteristics.

Results: The evident compression caused significant neurological dysfunction, which was confirmed by the decrease in BBB score and SEP amplitude, as well as the prolongation of SEP latency ($P < 0.05$). The histopathological findings verified a significant decrease in the amount of Nissl body and myelin area and an increase in vacuolation compared with the control group ($P < 0.05$). The TEM results revealed ultrastructural destruction of NVU in several forms, including: neuronal degeneration and apoptosis; disruption of axonal cytoskeleton (neurofilaments) and myelin sheath and dystrophy of axonal terminal with dysfunction mitochondria; degenerative oligodendrocyte, astrocyte, and microglial cell inclusions with degenerating axon and dystrophic dendrite; swollen microvascular endothelium and loss of tight junction integrity; corroded basement membrane and collapsed microvascular wall; and proliferated pericyte and perivascular astrocytic endfeet. In the CSM group, reduction was observed in the amount of mitochondria with normal appearance and the number of cristae per mitochondria ($P < 0.05$), while no substantial drop of synaptic vesicle number was seen ($P > 0.05$). Significant narrowing of microvascular lumen size was also observed, accompanied by growth in the vascular wall area, endothelial area, basement membrane thickness, astrocytic endfeet area, and pericyte coverage area (rate) ($P < 0.05$).

Conclusion: Altogether, the findings of this study demonstrated ultrastructural destruction of NVU in an experimental CSM model with dorsal–lateral compression, revealing one of the crucial pathophysiological mechanisms of CSM.

KEYWORDS

ultrastructural pathology, neurovascular unit, chronic, compressive, spinal cord injury, cervical spondylotic myelopathy, ultrastructural evidence

Introduction

Cervical spondylotic myelopathy (CSM) is the most common cervical spinal cord disorder among the elderly population (Amenta et al., 2014). The chronic compression to the cervical spinal cord ultimately causes structural and functional neurovascular destruction in the forms of ischemia, blood–spinal cord barrier (BSCB) disruption, apoptosis of neuron and oligodendrocyte, and axonal demyelination (Baron and Young, 2007; Kalsi-Ryan et al., 2013). However, the vast variation of clinical symptoms and functional presentation among the population of CSM could not be well explained. A good understanding of pathophysiology in CSM is therefore required.

The neurovascular unit (NVU) is a structural and functional complex that plays a vital role in the coupled interaction between neural activity and microcirculation (Lok et al., 2007; Iadecola, 2017; McConnell et al., 2017; Huang et al., 2021). Numerous studies have demonstrated that NVU destruction is involved in the pathological and pathophysiological mechanism of CNS disorders such as brain trauma (Armstead and Raghupathi, 2011; Lok et al., 2015), cerebral ischemia injury (Cai et al., 2017; Zhao et al., 2020), and degenerative cerebral disorders (Cai et al., 2017; Yu et al., 2020). The intercellular connection and interaction among microvascular endothelial cells, perivascular astrocytes, and pericytes regulate the structural and functional integrity of BSCB (Hawkins and Davis, 2005; Sá-Pereira et al., 2012; McConnell et al., 2017). The intercellular coupling between neuron and neuroglial cells has an essential effect on balancing ionic homeostasis, regulating axoplasmic neurotransmission and synaptic re-uptake, and insulating axons to promote nerve conduction velocity (Lok et al., 2007; Guo and Lo, 2009). Thus, the multicellular and multicomponent NVU constitutes a complexed neural–vascular network that is responsible for not only oxygen and nutrition delivery but also intercellular signaling coupling and maintenance of the microenvironmental homeostasis for neural activity (Sá-Pereira et al., 2012; McConnell et al., 2017; Yang et al., 2020; Zhao et al., 2020). In fact, NVU destruction develops prior to motor neuron degeneration (Miyazaki et al., 2011), and NVU remodeling or repair could improve functional recovery (Lake et al., 2017; Chio et al., 2019; Ye et al., 2021). It suggests that NVU could be an early and effective target of treatment. A recent study reported that NVU destruction following the chronic compressive spinal cord injury may lead to impairment of endothelial cell, defect of tight junction, degeneration of neuron and axon, and swelling of astrocyte endfeet and mitochondria (Xu et al., 2017). However, the ultrastructural evidence of NVU is scarce and far from being able to reveal the underlying pathophysiology of CSM (Xu et al., 2017; Guo et al., 2021).

This study explored the ultrastructural pathology of NVU during the natural development of CSM. Pathological staining [hematoxylin–eosin (HE), toluidine blue (TB), and Luxol fast blue (LFB)] and transmission electron microscopy (TEM) examination were used to comprehensively investigate the (ultra)pathological characteristics of NVU in an experimental rat CSM model. Ultrastructural characteristics and notable changes in different aspects of NVU following chronic dorsal–lateral cervical cord compression at the C5 level will be thoroughly examined.

Materials and methods

Experimental materials and animal model establishment

A total of 24 female adult Sprague–Dawley (SD) rats (180–250 g) were divided into the CSM group (n = 12, cervical spinal cord compression for 2 months) and control group (n = 12). All the animal-handling procedures were in accordance with the Guide for the Care and Use of Laboratory Animals, approved by the local Committee on the Use of Live Animals.

In the CSM group, a water-absorbing and progressively expandable synthetic polyurethane polymer sheet (Fulin Ltd., Shenzhen, China) of 3 × 1 × 1 mm was used as an implant material to create compression on the spinal cord (Long et al., 2013). In brief, the rats received general anesthesia with a mixture solution of 10% ketamine and 2% xylazine (Sigma Chemical Co., St. Louis, MO, USA) intraperitoneally. The location of cervical spine levels was identified by X-ray fluoroscopy (**Figure 1A**). The thin polymer sheet was carefully

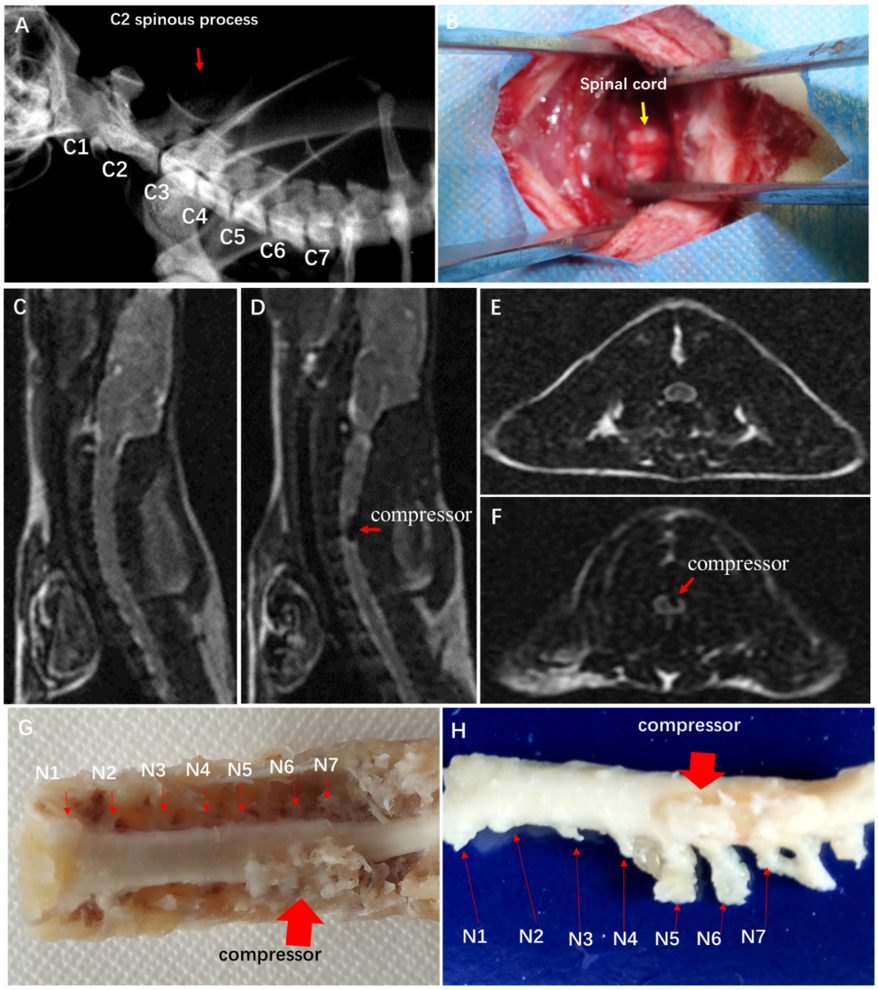

FIGURE 1
Establishment of experimental CSM rat model. **(A)** Spine location in X-ray fluoroscopy. **(B)** Surgical exposure at C4 and C5 spinal levels. Sagittal MRI T2-weighted images of control group **(C)** and CSM group **(D)**. Axial MRI T2-weighted images of control group **(E)** and CSM group **(F)**. **(G,H)** View of specimen after compression.

implanted into the left side of the spinal canal at the C5 level (Figure 1B). The rat after implantation fully recovered from the surgery on a heating bed and was then sent back to the cage freely for food and water. All of the animals in this study survived at the end of observation.

To confirm spinal cord compression, MRI T1- and T2-weighted images were obtained with a 3.0-T MR imager (Discovery MR 750, GE Medical Systems, Milwaukee) 2 weeks after the implantation. Hemorrhage and edema in the epidural space were observed.

Neurological function assessment

Locomotor function was evaluated by using 21-point Basso–Beattie–Bresnahan (BBB) scoring system in the open field (Basso et al., 1996). The evaluation time point was set at 1 day, 3 days, 7 days, 14 days, 21 days, 1 month, and 2 months postoperatively. Two equally trained spinal surgeons, who were not involved in previous implantation surgeries, were invited to independently evaluate the locomotor function of all rats. The average score was calculated to depict the dynamic change of motor function.

Sensory functional integrity was evaluated by somatosensory evoked potentials (SEP). To elicit cortical SEP, a constant current stimulation with a frequency of 5.1 Hz and a pulse duration of 0.2 ms was applied to the tibial nerve. The cortical SEP was recorded from the skull at location Cz–Fz in the 10–20 system. SEP signals were amplified 100,000 times band-passed between 20 and 2,000 Hz by an electrophysiological measurement system (YRKJ-G2008, Zhuhai, China). The latency and amplitude of the SEP waveform were analyzed after averaging 200 trials.

Tissues preparation and histopathological examination

All samples from both groups underwent satisfactory euthanasia with sodium pentobarbital solution 2 months postoperatively. Half of the samples from each group were designated to histopathological examination, while the other half reserved for TEM examination.

For histopathological examination, the whole cervical spinal cord was carefully harvested and fixed with 4% phosphate buffer liquid in formaldehyde solution. Then, a 5-mm-long cord block at the C5 level was longitudinally cut and embedded in paraffin. Five-micrometer slides of transverse cord were continuously sectioned for histopathological and immunohistochemical (IHC) staining.

A total count of 12 sectioned specimens were stained with HE (Sigma Chemical Co., St. Louis, MO, USA), TB, and LFB (Sigma Chemical Co., St. Louis, MO, USA). All images of the cords were acquired with a microscope (FV-1000, Olympus, Japan). The number of large motor neurons was counted in the ventral horn of the gray matter at ×10 view for all specimens using ImageJ 1.47v (National Institutes of Health, USA). Nissl body area was examined at ×40 view. After LFB staining, the blue color intensity (×20 view ImageJ) in the posterior funiculus indicated the content of myelin.

Transmission electron microscopy examination

For TEM examination, the specimen sections were stained with uranyl acetate and alkaline lead citrate for observation with TEM (JEM-1400). Images acquired from the imaging system (GATAN 832) were used to investigate ultrastructural features of neurovascular cytohistology.

Electron micrographs (4,008 × 2,672 pixels, 6.384 × 4.252 μm, 1.6 nm/pixel) of capillaries from the CSM group and control group were compared. Micrographs of microvessels in cross section were taken at 15,000× to measure the area of lumen, endothelial cell, microvascular wall (lumen area was excluded), astrocytic endfeet, and the circumference of microvessel and pericyte with "freehand selections" tool. The pericyte coverage rate (percentage) of microvessel abluminal surface and the total length of the inner pericyte processes around each microvascular abluminal surface were divided by the perimeter of microvessel in 60,000× magnification micrograph. The integrity of tight junction was evaluated by the number of mitochondrial cristae and synaptic vesicle under 100,000× magnification. The tight junctions were recognized as intercellular space when the fluid space is wider than 50 nm.

Statistical analysis

Sample sizes were determined prior to the experiment. All data are presented as the mean values ± standard error of the mean (SEM). All statistical analyses were performed using SPSS 25.0 software (IBM Corp., Armonk, NY, USA). Comparisons between groups were made using *t*-test of two independent samples. $P < 0.05$ was deemed statistically significant.

Results

Image verification of spinal cord compression

Hypointense changes on T1WI and T2WI were observed in the CSM group. The compressor posed dorsal–lateral compression at C5–C6 level and caused evident cord deformation, without spinal cord edema, hemorrhage, and intramedullary cavity (**Figures 1C–F**). The expanded compressor was encompassed by pseudomembrane inside the spinal canal (**Figures 1G,H**). A sunken C5 spinal cord was seen on the dorsal–lateral dissected specimen (**Figure 1H**).

Neurological dysfunction after chronic cervical cord compression

The CSM group showed significantly declined BBB scores in contrast to the control group with normal BBB scores at different evaluation time points ($P < 0.05$) (**Figure 2A**). Ipsilateral upper limb weakness with paw contractures, fore–hind limb discordance, and trunk imbalance were observed in the CSM group. The spontaneous locomotor function recovery was observed 1–2 months postoperatively, before the locomotor function appeared to be in a steady state (16.8 ± 2.0) (**Figure 2A**).

Somatosensory evoked potential examination confirmed sensory dysfunction in the CSM group (**Figure 2B**). In the CSM group, there were delayed latency (9.46 ± 1.40 ms at 2 months postoperatively vs. 8.21 ± 1.32 ms preoperatively, $P < 0.05$) and decreased amplitude (2.88 ± 1.21 uv at 2 months postoperatively vs. 4.40 ± 1.93 uv preoperatively, $P < 0.05$) (**Figure 2C**).

Motoneuronal and axonal degeneration after chronic compression

In the control group, numerous large motor neurons with a high amount of Nissl body and clear nucleus were identified in the ventral horn (**Figures 3A1,A2**). In contrast,

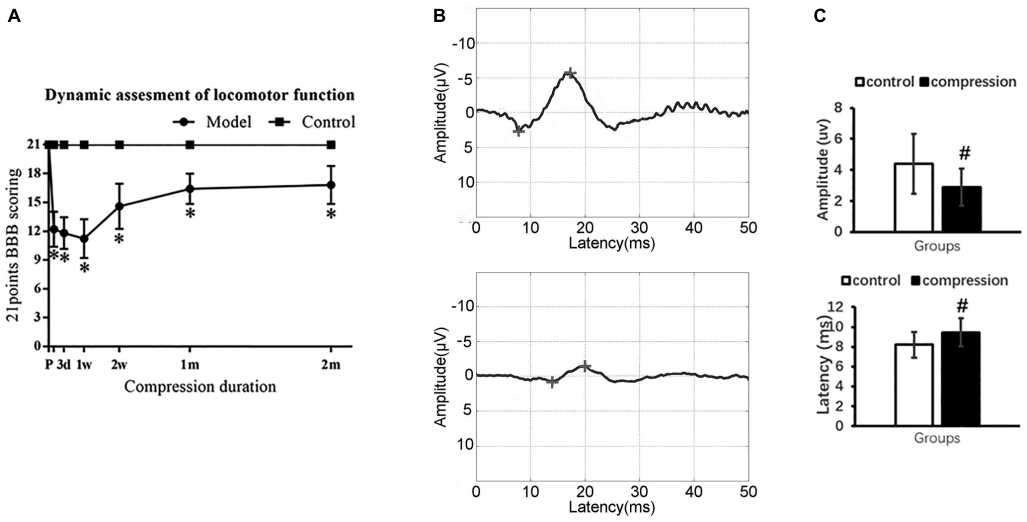

FIGURE 2
Neurological dysfunction after compression. **(A)** Locomotor evaluation with BBB scores. **(B)** Representative SEP waveforms. **(C)** Values of SEP amplitude and latency in two groups. "*" compare with the control group at the same time point for BBB scores ($P < 0.05$); "#" compared with the control group for SEP ($P < 0.05$); CSM group ($n = 12$), control group ($n = 12$).

neuronal death and loss of neuron number were remarkable in the CSM group (Figures 3B1,B2). Disappearance or loss of Nissl body was rather obvious in the large motor neuron (Figures 3B1,B2). Statistical analysis demonstrated a significant decline in Nissl body size (2,753 ± 234 μm^2 in the CSM group vs. 7,952 ± 543 μm^2 in the control group, $P < 0.05$) (Figure 3A3). Meanwhile, vacuolization was seen in the ventral horn of the CSM group (Figure 3B1). A significant increment in the vacuole area was found (66.44 ± 3.20 in the CSM group vs. 46.84 ± 2.90 in the control group, $P < 0.05$) (Figure 3B3).

Compared with clear neural fiber derived from the dorsal horn in the control group (Figures 4A1,A2), disrupted neural fiber with increased number of vacuoles was identified in the CSM group (Figures 4B1,B2). A significant increase in the vacuole area around the axon in the posterior funiculus was seen in the CSM group (63.80 ± 3.46) compared with that in the control group (46.64 ± 2.87, $P < 0.05$) (Figure 4A3). LFB staining also showed obvious vacuole formation and a significant decrease in myelin area (27,721 ± 1,587 μm^2 in the CSM group vs. 42,960 ± 2,153 μm^2 in the control group, $P < 0.05$) (Figure 4B3).

Ultrastructural destruction to neurovascular unit after chronic compression

In the control group, the Nissl body (Nb), chromatin, and primary lysosome (L) were evenly distributed in karyoplasm (Figures 5A1,A2). The neuropils with several bundles of neurofilaments were clearly distinguished in the gray matter. The mitochondria appeared with clear cristate. In the CSM group, the neurons presented degenerative ultrastructural changes, which were characterized with dense and dark chromatin, plenty of phagolysosomes and autophagic vacuoles, dystrophic neurites, and swelling and disappearance of cristate of mitochondria (Figures 5B1,B2). In particular, increased dense cytoplasm, karyoplasm chromatin condensation and densification, organelles aggregation, and ultrastructural destruction such as nuclear membrane breakdown and nuclei–cytoplasm separation were the most prominent morphological ultrastructural characteristics of apoptotic neuron (Figures 5B1,B2). In addition, a close contact of neuron, oligodendrocyte (Oli), astrocyte endfeet (Ae), and microvessels was seen in the CSM group, which was considered one of the NVU paradigms (Figures 5B1,B2).

In the control group, the axons of normal myelinated neural fibers have an even and pale axoplasm with clearly identifiable neurofilaments and microtubules. Some of the mitochondria have distinct cristate (Figure 6A1). The myelinated axons were encompassed with visible lamellae of the myelin sheath. In the CSM group, the swollen axons were found with atrophic axoplasm and cavitation, surrounded by absolutely disorganized and disrupted outer and inner loops of myelin sheaths. Myelin sheath splitting was frequently observed in a large proportion in the neural fibers. Neurofilaments and microtubules were difficult to recognize in the axoplasm in the majority of the neural fibers. Mitochondria were swollen, and mitochondrial cristate disappeared or vacuolized in the CSM group (Figure 6B1).

In the control group, numerous axonal terminals synapsed with dendrite spine and formed mostly asymmetric synapse along with a plenty of spherical synaptic vesicles (Figure 6A2). The axonal terminal axoplasm mainly contained

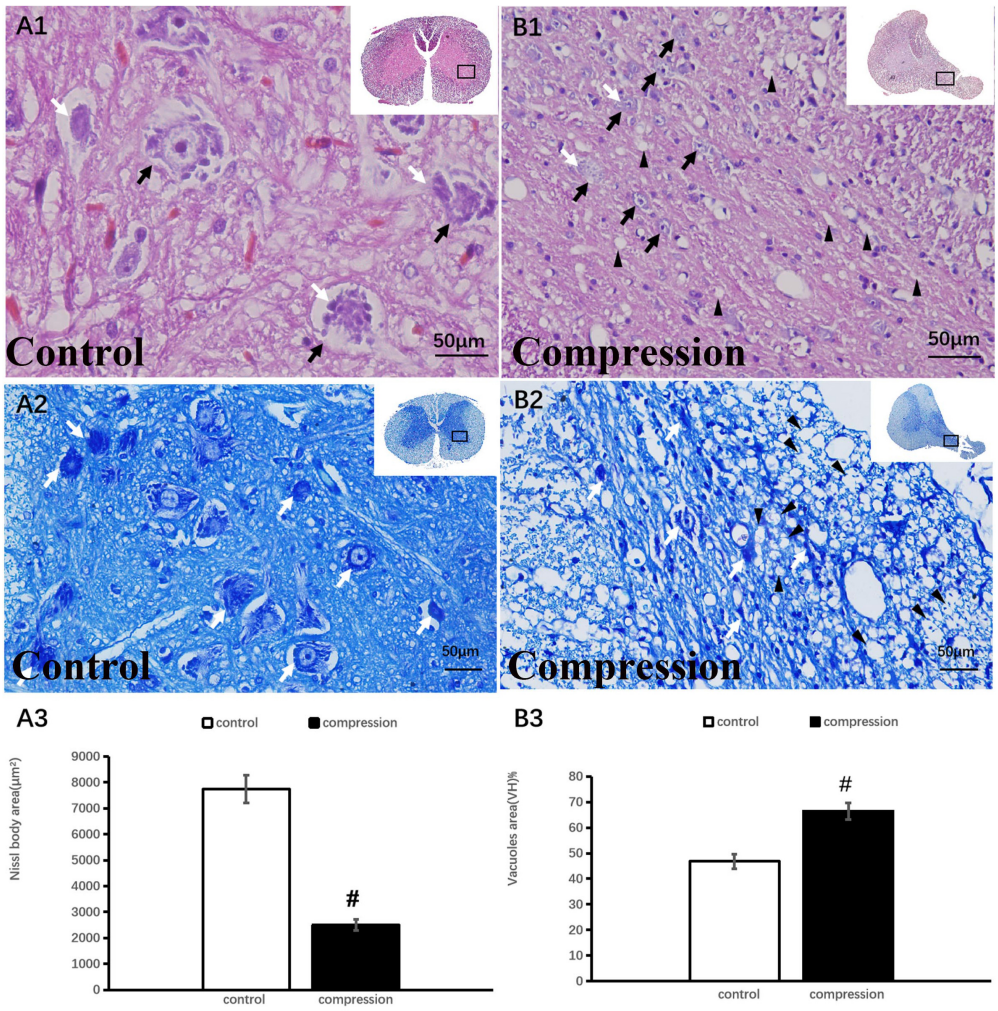

FIGURE 3
Neuronal degeneration verified by HE and TB staining. **(A1,A2)** Numerous large motor neurons with rich Nissl body were identified in the ventral horn. **(B1,B2)** Loss of neuron and Nissl body with vacuolization in the ventral horn. Black arrow indicates large motor neuron, black arrowhead indicates vacuoles, and white arrow indicates Nissl body. A significant decline in Nissl body **(A3)** and increment in vacuoles **(B3)** in the compression group ($P < 0.05$). "#" significant difference between CSM group and control group ($P < 0.05$, $n = 6$ per group).

neurofilaments and abundant mitochondria with clear cristate (Figure 6A3). In the CSM group, the axonal terminal appeared to be degenerately changed. Observation included disruption of axonal terminal membrane, disorganized neurofilament, and swelling and vacuolar degeneration of the mitochondrial cristae (Figures 6B2,B3). The proportion of normal mitochondria (59.38 ± 7.76 in the CSM group vs. 91.21 ± 8.94 in the control group) and the number of mitochondrial cristate per mitochondria (4.30 ± 1.93 in the CSM group vs. 7.60 ± 1.72 in the control group) decreased significantly ($P < 0.05$) (Figures 6D,E), whereas no significant difference in the number of synaptic vesicles and total mitochondria (96.97 ± 16.03 in the CSM group vs. 130.84 ± 19.88 in the control group, $P > 0.05$) was found (Figures 6C,F).

In the white matter of the control group, the fibrous astrocytes showed oval nuclei contour with relatively even and lower electron density of karyoplasm and cytoplasm, along with a thin and condense rim of heterochromatin beneath the karyolemma (Figure 7A1). Organelles such as short cisternae of granular endoplasmic reticulum (GER), free glycogen granules, and mitochondria were sparsely distributed in the cytoplasmic matrix, whereas a few of neurofilaments bundles were the most prominent component in the perikaryal cytoplasm. In particular, the astrocyte endfeet (Ae) was seen closely surrounding the microvascular wall (Figures 7B1,B2). In contrast, increased electron density of karyoplasm and cytoplasm was seen in the CSM group (Figure 7B1). The cytoplasm of astrocytes appeared to be filled with degenerating axon (Figure 7B1). The chromatin of oligodendrocytes is

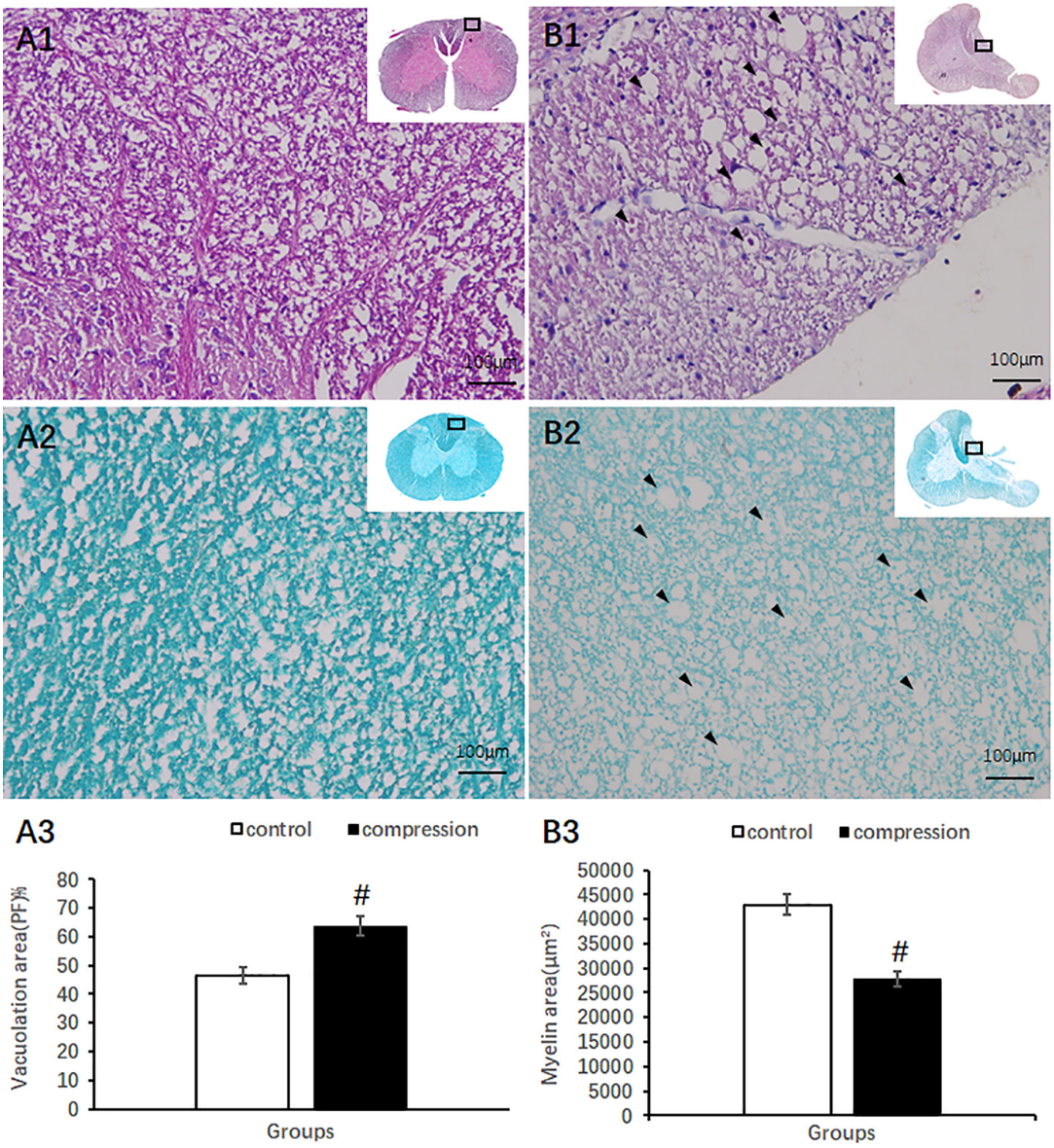

FIGURE 4
Axonal degeneration in the posterior funiculus after compression revealed by HE and LFB staining. Organized neural fiber derived from the dorsal horn (**A1**, HE staining) and myelin sheath stained in dark blue in the control group (**A2**, LFB staining). **(B1,B2)** Vacuoles formation and a decrease in myelin staining in the posterior funiculus of the CSM group. Vacuolation area increased **(A3)** and myelin area decreased **(B3)** significantly in the CSM group ($P < 0.05$, marked with "#" $n = 6$ per group).

clumped and circulating along the karyolemma or scattering throughout the karyoplasm (Figure 7A2). The cytoplasm contains numerous short GER, abundant free ribosomes, well-developed Golgi apparatus (G), and relatively smaller mitochondria. Tight connections between the cytomembrane of oligodendrocytes and some myelinating axons were found in the control group (Figure 7A2). After compression, dissolved karyolemma, degenerating axon, and myelin sheaths inclusions were clearly observed (Figure 7B2).

The microglial cells in the resting state from the CSM group appeared to have elongated nuclei outline, higher density of clumped chromatin, and slightly denser cytoplasm compared with oligodendrocytes in the control group (Figure 7A3). Also, the perikaryal cytoplasm from the CSM group was distended by phagocytosed material, some of which appeared to degenerate myelin and dystrophic dendrite (Figure 7B3). Lipofuscins and autophagy cavitation were seen in the perikaryon (Figure 7B3).

Destruction of the vascular elements and collapse of vascular contour were evident in the CSM group (Figures 8A1,B1).

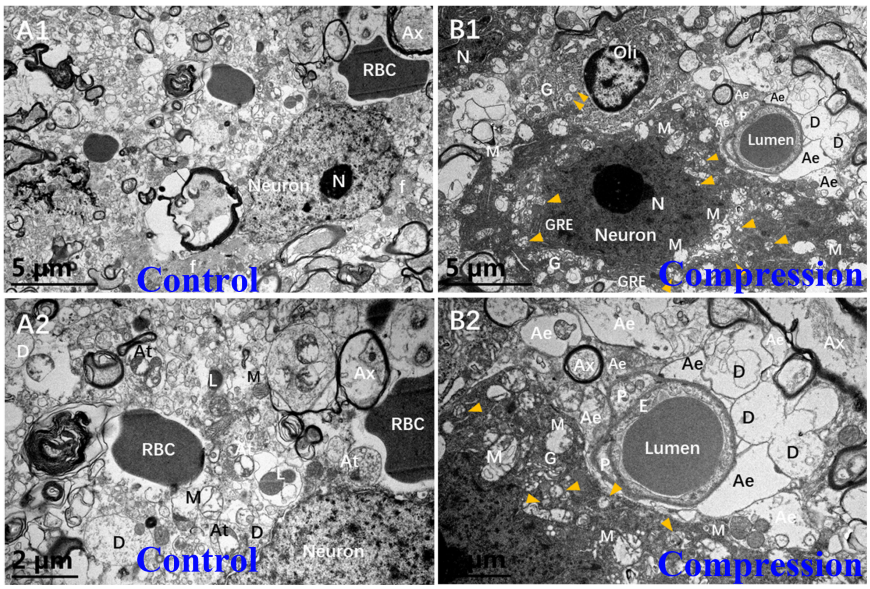

FIGURE 5
Comparison of ultrastructural evidence of neuron, NVU degeneration, and apoptosis between samples from the control group **(A1,A2)** and CSM group **(B1,B2)**.

Compared with the control group, significant increases were found in the vascular wall area (79.34 ± 14.14 vs. 46.57 ± 10.43, $P < 0.05$) (Figure 8C), endothelium area (73.45 ± 6.58 vs. 58.83 ± 4.35, $P < 0.05$) (Figure 8E), and BM thickness (215.30 ± 52.32 vs. 106.70 ± 29.54, $P < 0.05$) (Figure 8G), while a significant decrease was found in lumen size (17.72 ± 4.14 vs. 53.43 ± 10.43, $P < 0.05$) (Figure 8D). In the control group, the endothelium has distinct and integrated nuclei, karyoplasm, and organelles (Figures 8A1,A2), which was obviously different from the fuzzy and loose appearance of endothelium in the CSM group (Figures 8B1–B3). Loss of electron density of cytoplasmic matrix, mitochondria swelling, disappearing mitochondria, and vacuolation were the main observations of endothelium in the CSM group (Figures 8B2,B3). Such swelling appearance was further proved by the increased area of endothelium in the vascular wall under a chronic compression condition (Figure 8E). After compression, disruption of the interacting plasm membranes of tight junctions and caveolae-like enlargement of the intercellular space were clearly observed (Figures 8B3,F). In the control group, the BM's electron density was even lower (Figures 8A2,A3). The contour's corrugated deformation was consistent with the distorted and collapsed vascular profile. Compared with the control group, the thicker BM in the CSM group was scattered with an increased density of electron granules that appeared to be corroding change (Figures 8B3,G).

Unlike the flattened and elongated nuclei of endothelium, pericytes have more roundish or oval nuclei contour (Figures 9A,B). Similar to the oligodendrocyte, the chromatin is quite clumped adjacent to karyolemma and throughout karyoplasm (Figure 9A). In contrast, loss of chromatin was observed in some of the pericytes in the CSM group (Figure 9B). Long and narrow cytoplasmic processes of pericytes crawled and circumvoluted affixed the long axis of microvessels, constituting another physical barrier that strengthened the vascular wall (Figure 9B). The outmost barrier, referred to as astrocytic endfeet, was seen closely affixed to the pericyte or BM. Pericyte vascular coverage rate (%) and pericytes area (μm^2) were defined as the percentage of ensheathed pericyte length and the area attached to the vascular wall's long axis, respectively. A significant increment can be seen in the pericyte vascular coverage rate (%) (67.02 ± 4.83 in the CSM group vs. 36.35 ± 3.56 in the control group, $P < 0.05$) and pericytes area (71.48 ± 8.34 in the CSM group vs. 54.41 ± 6.62 in the control group, $P < 0.05$) (Figures 9C,D).

Astrocytic endfeet appeared to be irregularly morphed in cross section with lower electron density (Figures 8A2,B2). In the CSM group, mitochondria expanded and cristae disappeared in most of the perivascular astrocytic endfeet (Figure 8B2), accompanied by a significant increase in perivascular astrocytic endfeet area (30.62 ± 5.03 in the CSM group vs. 21.04 ± 5.40 in the control group, $P < 0.05$) (Figure 8H).

Discussion

This study comprehensively disclosed the ultrastructural characteristics of NVU and its components' critical changes after chronic dorsal–lateral compressive spinal cord injury.

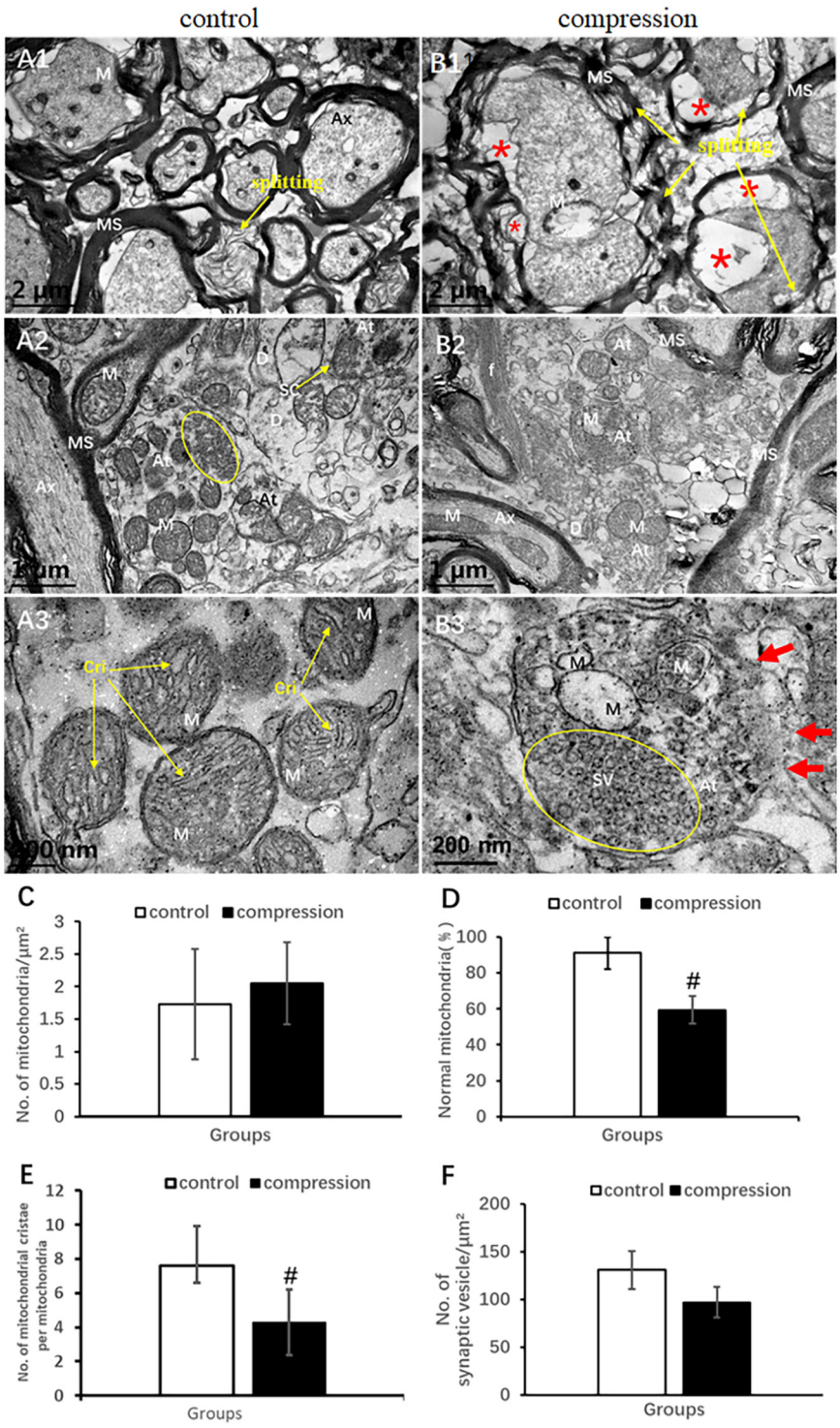

FIGURE 6
Comparison of ultrastructural destruction of axon and axonal terminal between samples from the control group **(A1–A3)** and CSM group **(B1–B3)**. Increased total number of mitochondria **(C)** but significantly decreased number of mitochondria with normal appearance **(D)** and mitochondrial cristae **(E)** in the CSM group. **(F)** No significant decrease in the number of synaptic vesicles after compression. Ax, axon; MS, myelin sheath; f, filament; At, axonal terminal; SC, synaptic cleft; SV, synaptic vesicle; D, dendrites; M, mitochondria; Cri, mitochondrial cristate; yellow oval include synaptic vesicle "*" axonal cavitation; "#" significant difference between compression and control group ($P < 0.05$, $n = 6$ rat/group).

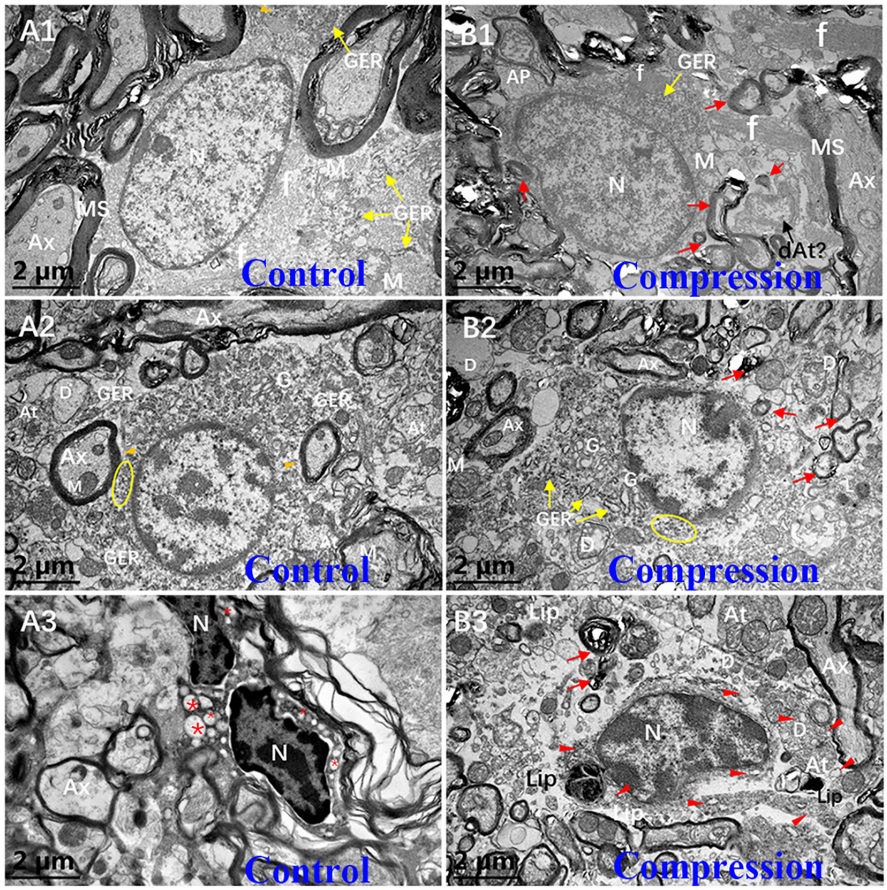

FIGURE 7
Ultrastructural evidence of neuroglial cells degeneration. Astrocyte **(A1,B1)**, oligodendrocytes **(A2,B2)**, and microglial cells **(A3,B3)** in the control and CSM groups, respectively. N, nucleus; Ax, axon; MS, myelin sheath; f, filament; At, axonal terminal; D, dendrite; GER, granular endoplasmic reticulum; Lip, lipofuscin; L, lysosome; "*" empty phagocytic inclusion; yellow oval includes abundant of free ribosomes; orange arrowhead indicates a few of free glycogen granules in astrocyte; and red arrow indicates degenerating myelin.

Ultrastructural observation revealed a series of pathological NVU changes in addition to decreased BBB score, prolonged SEP latency, and reduced SEP amplitude. The NVU destruction appeared with the following ultrastructural characteristics: neuronal degeneration and apoptosis; disruption of axonal cytoskeleton and myelin sheath with dystrophy of axonal terminal; degenerative oligodendrocyte, astrocyte, and microglial cell inclusion with degenerating axon and dystrophic dendrite; swollen microvascular endothelium and loss of tight junction integrity; corroded basement membrane and collapsed microvascular wall; proliferated pericyte and perivascular astrocytic endfeet; and swollen mitochondria in neuron, axon (axonal terminal), and astrocyte. The results of this study have explicitly and systematically demonstrated the ultrastructural destruction of each NVU component in the experimental rat CSM model, which may provide a profound understanding of pathophysiology of CSM. It would help build a research platform for investigating the neurovascular mechanisms behind NVU and finding potential treatment targets to promote an effective therapeutic strategy for CSM.

The surgery-induced compression to the cervical cord at the C5 spinal level was verified at 2 weeks postoperatively using MRI. Neurological dysfunction indicated by changes in BBB score and SEP measurement further validated the establishment of the CSM model as previously reported (Long et al., 2013). It was noted that rats could develop spontaneous functional recovery in 2 months after compressive spinal cord injury, which is different from human. However, behavior, electrophysiology, and pathological findings are useful in simulation of CSM.

In this study, axonal degeneration and demyelination in the CSM group were demonstrated by HE and LFB staining in the posterior funiculus. The ultra-pathological results further verified the loss of Nissl body and large motor neuron in general observation of histopathological findings. The apoptotic changes in large motor neuron in ventral horns during early and mediate phase included increased density of cytoplasm, condensation and densification of karyoplasm chromatin,

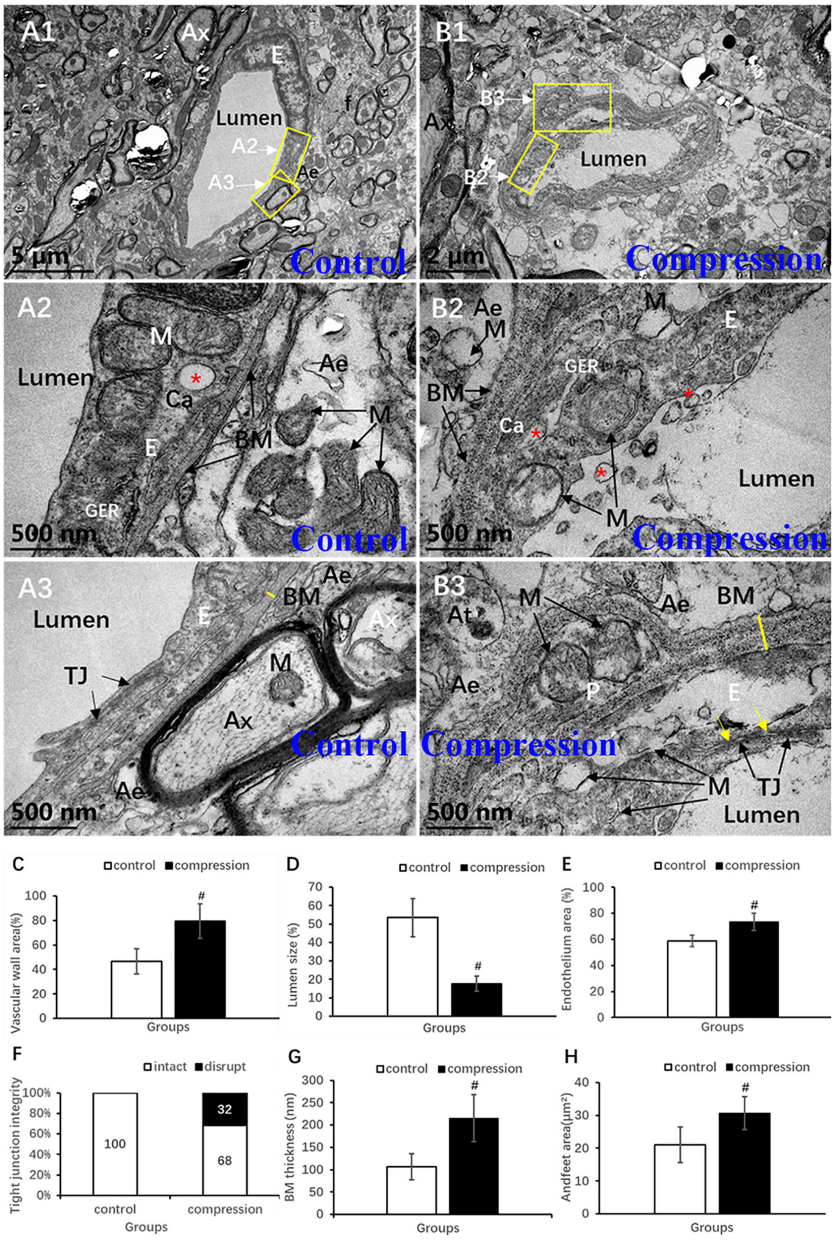

FIGURE 8
Ultrastructural destruction of microvascular and vascular elements. **(A1–A3)** Normal ultrastructure of microvascular and vascular elements in the control group. **(B1–B3)** Ultrastructural destruction of microvascular and vascular elements in the CSM group. Comparison of characteristics between two groups: vascular wall area **(C)**, endothelium area **(E)**, Tj disruption **(F)**, BM thickness **(G)**, endfeet area **(H)**, and lumen size **(D)**. Ax, axon; E, endothelial cells; p, pericyte; BM, basal membrane; Tj, tight junction; M, mitochondria; Ae, astrocyte endfeet; f, filament; "*" Ca, caveolae; yellow arrow indicates disruption of interacting plasm membranes of Tj; "#" significant difference between CSM and control group ($P < 0.05$, $n = 6$ per group).

swelling mitochondria and disappearance of its cristate, breakdown of nuclear membrane and separation of nuclei-cytoplasm, as well as plenty of phagolysosomes and autophagic vacuoles (**Figures 5B1,B2**). These changes proved that neurons were undergoing apoptosis due to chronic and persistent compression, which is one of the typical neuropathological impairments responsible for neurological dysfunction in CSM (Baptiste and Fehlings, 2006; Yu et al., 2009; Kalsi-Ryan et al., 2013; Karadimas et al., 2013, 2015; Akter et al., 2020). Although ischemia injury and inflammatory impairment have been proposed as the main pathophysiological factors for neuronal apoptosis (Kalsi-Ryan et al., 2013; Karadimas et al., 2015), the underlying mechanisms are still not entirely clear. It is worth noting that the decrease in the number of mitochondria and

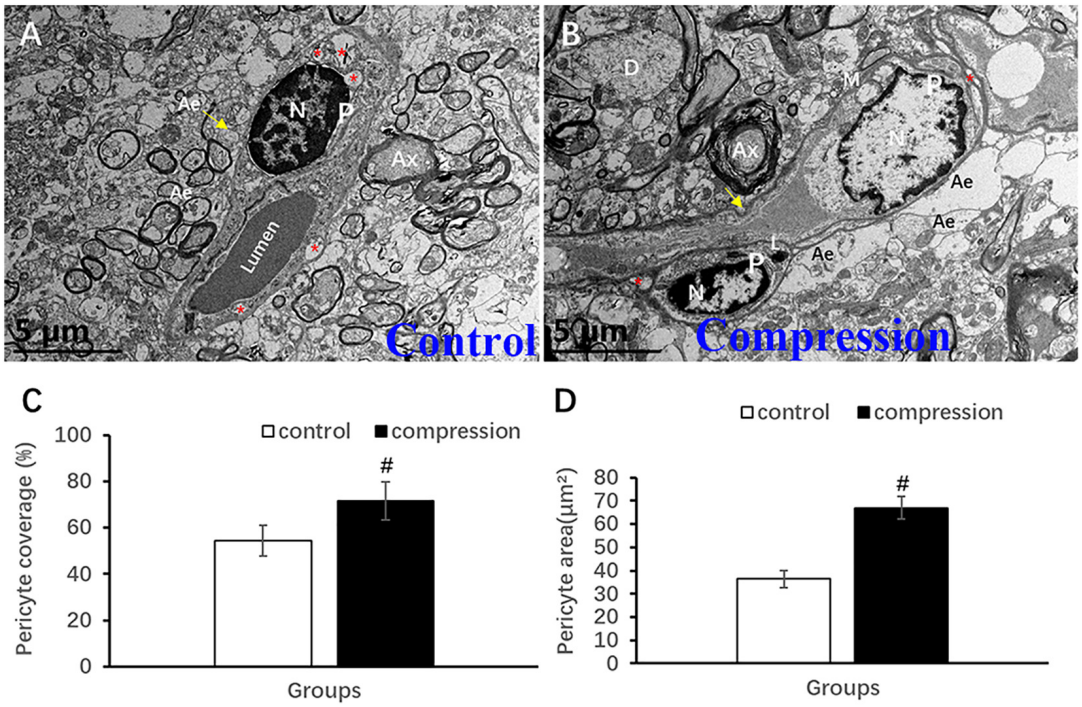

FIGURE 9
Ultrastructural evidence of pericytes degeneration. **(A)** Control group. **(B)** CSM group. Comparison of pericyte vascular coverage rate **(C)** and pericytes area **(D)**. N, nucleus; P, pericyte; Ax, axon; Ae, astrocyte endfeet; D, dendrite; "*" indicates caveolae. $^{\#}P < 0.05$ compared with the control group, $n = 6$ per group.

mitochondrial cristate was the most important characteristic in the degenerative or apoptotic large motor neuron. Thus, more investigations could be focused on the mitochondria-induced pathway in neuronal apoptosis to further elucidate the pathophysiological mechanism of CSM.

Disruption of axolemma, disorganized neurofilament (**Figures 6B2,B3**), and decreased number of normal mitochondria and mitochondrial cristate in the axonal terminal provided additional particulars for axonal degeneration. The ultrastructural findings were consistent with the axonal degenerative changes revealed by histopathological staining, including increased vacuolation area and decreased myelin area. It may provide an explanation for the underlying pathomechanism of sensory dysfunction, i.e., abnormal SEP latency and amplitude. The axonal terminal (synapse) carrying the neurotransmitter by synaptic vesicle enables interneuron information transmission (Harris and Littleton, 2015). Meanwhile, such context may also have a great impact on mitochondrial viability/renewal and thereby energy supply for synaptic plasticity, axoplasmic transportation, and neurotransmission (Todorova and Blokland, 2017). In brief, these findings may provide ultrastructural evidence for further interpreting the early consensus that long-lasting static and repetitive compression accumulatively produces stretch-associating injury to the axon cytoskeleton (Shi and Pryor, 2002; Baptiste and Fehlings, 2006), disturb axoplasmic transportation, and thereafter cause axonal degeneration, axonal terminal degeneration, and synaptic dysfunction in CSM (Zhou et al., 2015).

In the present study, increased perivascular astrocytic endfeet area was thought to be an astrocytic reaction to chronic compressive injury. Astrocytes can help maintain ion balance around neurons, constitute the BSCB by perivascular astrocytic endfeet, regulate microcirculation (Marina et al., 2020), and mediate transsynaptic signaling in physiological conditions (McConnell et al., 2017). It allows intercellular exchange of other small neutral molecules through aquaporins (Hladky and Barrand, 2016). But the reactive astrocytic is regarded as a detrimental factor that prevents axonal sprouting and plasticity (Okada et al., 2018). It is interesting to note that the astrocytes have numerous inclusions in their cytoplasm, which appeared to be degenerating axon with myelin sheath and axonal terminal (**Figure 7B1**). Thus, it could be speculated that astrocyte might expedite axonal degeneration or participate in clearing the degenerative axon in chronic compressive circumstances.

In contrast to astrocyte, the oligodendrocytes can be identified by a high density of clumped chromatin, rich and denser cytoplasm, and rich organelles (**Figure 7A2**). In the gray matter, the oligodendrocyte was frequently seen surrounding the neuron (**Figure 5B1**), which was called

satellite oligodendrocytes, and it also aided in maintaining the normal function of neuron. But in the white matter, the oligodendrocytes defined as interfascicular oligodendrocytes were arranged in rows alongside the axon, providing glucose for neuronal axons under poor nutrition conditions (Hamanaka et al., 2018). The apoptotic changes, which were rather distinct in some of the oligodendrocytes, included strikingly dissolved/disrupted karyolemma, degenerating axon, and myelin sheaths inclusions (**Figure 7B2**). They indicate that chronic compression may induce the apoptosis of oligodendrocyte. Our findings further support the previous conclusions that apoptosis of oligodendrocyte may lead to axonal demyelination, neuronal impairment, and neurological dysfunction in CSM (Kim et al., 2003; Baptiste and Fehlings, 2006; Yu et al., 2009, 2011; Karadimas et al., 2010, 2013; Kalsi-Ryan et al., 2013).

In the CSM group, we also found that perikaryal cytoplasm was distended by phagocytosed material, some of which appeared to be degenerating myelin, dystrophic dendrite, lipofuscins, and autophagy cavitation (**Figure 7B3**). The findings indicate that microglial cells were involved in promoting phagocytosis and clearing degenerated neural tissues. Hence, microglial cells often serve as "daring vanguard" or "maintainer" who could detect any subtle damage in microenvironment and clear the degenerated tissues and injury debris (Liu L. R. et al., 2020). Moreover, neuroinflammatory impairments regulated by microglial cell are one of the key pathophysiological causes that lead to neuronal and neuroglial cell death (Glass et al., 2010; Savage et al., 2018). In immunological and inflammatory environment, microglial cells may develop into M1-like phenotype (classical activated macrophages) or M2-like phenotype (alternative activated macrophages) (Orihuela et al., 2016). M1-like phenotype is associated with secretion of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) and is regarded as a detrimental phenotype. Meanwhile, M2-like phenotype is able to promote anti-inflammatory cytokine expression such as interleukin-10 (IL-10), transforming growth factor beta (TGF-β), and glucocorticoids and is regarded as a beneficial phenotype (Liu L. R. et al., 2020). In an experimental rat CSM model, M1-like phenotype microglial cell existed chronically and may be responsible for neuronal and axonal degeneration, while M2-like phenotype appeared only temporally (Hirai et al., 2013). Thus, inducing the development of M2-like phenotype while inhibiting M1-like phenotype by changing the cellular microenvironment to prevent inflammatory impairment seems to be a promising therapeutic strategy for CSM (Plastira et al., 2019).

Blood–spinal cord barrier is one of the key components of NVU that is comprised of, from inside to outside, tightly connected endothelial cells, the encapsulated basement membrane, the adhesive pericyte, and the enwrapping astrocytic endfeet (Hawkins and Davis, 2005; Choi and Kim, 2008). The interactions and reciprocation among those components construct the functional integrity of BSCB (Choi and Kim, 2008). BSCB disruption is one of the key pathophysiological processes in the CSM (Karadimas et al., 2013; Blume et al., 2020), and the magnitude of BSCB disruption was strongly correlated with the severity of myelopathy (Blume et al., 2020). In this study, microvascular ultrastructural destruction induced by chronic compression was characterized by swollen endothelial cell, expanded and corrugated basement membrane, and collapsed microvascular contour and luminal stenosis (**Figures 8B2,B3**). The findings were consistent with the previous ultrastructural features depicted (Xu et al., 2017). However, this study presented the swollen mitochondria with disrupted mitochondrial cristae in the endothelial cells as a potential pathomechanism responsible for endothelial dysfunction. We also observed disrupted integrity of tight junctions in the forms of disruption of interacting plasm membranes and caveolae-like enlargement of intercellular space (**Figure 8B3**). Such disrupted integrity leads to the increase in BSCB permeability. Tight junctions are the main inter-endothelium connections that strengthen the physical barrier that controls paracellular substance diffusion through the vascular wall into the parenchyma of spinal cord. The tightly connected endothelial cells and tight junction gateways enable physiological transcellular flux that is responsible for nutrition delivery from peripheral blood and metabolism substance transfer from the neural tissues (Bartanusz et al., 2011; Daneman and Prat, 2015).

Our findings verified that chronic compression led to the corrosion and expansion of basement membrane (**Figure 8B3**). In addition, the abnormally thickened basement membrane was arranged in a loose pattern and had lower electron density and indistinct lamellar structure (**Figure 8B3**). This indicates the swollen expansion rather than the proliferation of basement membrane in chronic compressive circumstances. The basement membrane, mainly composed of laminin and collagen IV (Hallmann et al., 2005), encapsulates the microvascular endothelial cell. Besides, deformation contour of basement membrane was also thought to contribute to the collapse of microvascular wall. Accordingly, it can be inferred that the collapse of microvascular wall and stenosis of vascular lumen may reduce spinal cord perfusion. It is known that extracellular matrix enzymes such as matrix metalloproteases (MMP) are essential in regulating the metabolic balance of basement membrane and extracellular matrix. However, an intemperate expression of MMP-9 may speed up degradation process of basement membrane and lead to NVU destruction (del Zoppo, 2010).

Pericytes are embedded in the basement membrane, and they wrapped around the abluminal surface of microvessel,

maintaining structural and functional integrity of microvessel (Brown et al., 2019; Liu Q. et al., 2020). In the present study, the cytoplasmic processes of pericytes were observed to crawl and circumvolute affixed the long axis of microvessel (**Figure 9B**), constituting another physical barrier for the vascular wall. In the CSM group, loss of chromatin may indicate degeneration in some of the pericytes (**Figure 9B**) and could be associated with increased microvascular permeability (Armulik et al., 2010). A recent study also demonstrated that pericyte degeneration reduced microvascular blood flow and oxygen supply, leading to NVU dysfunction and neurodegeneration (Kisler et al., 2017). We also found that pericyte vascular coverage rate and pericytes area increased significantly in the CSM group (**Figures 9C,D**). Similar findings from the previous study showed that the coverage rates of pericytes along the long axis of vascular wall were 54 and 71% in the control and CSM groups, respectively, exceeding the 22–32% coverage rate of cerebral capillary surface (Fisher, 2009). Meanwhile, it remains unanswered whether such a difference was a compensatory proliferating response to chronic compression. In addition, caveolae-like vesicle/vacuoles were also commonly seen in the cytoplasm of pericytes as in the endothelium in both groups (**Figures 9A,B**), suggesting that pericyte may participate in transcellular exchange between the blood and the spinal cord parenchyma (Xu et al., 2017). Besides, pericyte could promote development of endothelial cells (Hellström et al., 2001), maturation of microvasculature (Fisher, 2009), and repairment of destructed NVU components potentially on behalf of the stem cell (Fisher, 2009). In brief, pericyte plays an important role in the regulation of microvessel and microcirculation homeostasis (Sá-Pereira et al., 2012).

Compared with the progressive neurological deterioration of CSM in human, rats usually show spontaneous functional recovery in 2 months, disallowing a long-term study over 2 months. Clinical trials are needed for a translation study (Nishida et al., 2012). The present study applied dorsal–lateral compression to the spinal cord as animal CSM model. It is a question of whether the NVU changes would vary after ventral compression was applied. Further development of animal models with ventral compression is needed to observe variation of NVU changes in different compression types.

Conclusion

In summary, we have established explicit and systematic ultrastructural evidence of NVU destruction in the present experimental CSM model with dorsal–lateral compression. The ultrastructural changes have the following characteristics: (1) neuronal degeneration and apoptosis; (2) disruption of axonal cytoskeleton (neurofilaments and microtubules) and myelin sheath with dysfunction mitochondria; (3) disruption of axolemma and dystrophy of axonal terminal with dysfunction mitochondria; (4) degenerative oligodendrocyte, astrocyte, and microglial cell inclusion with degenerating axon and dystrophic dendrite; (5) swollen endothelium with dysfunction mitochondria and loss of tight junction integrity; (6) expanded and corrosive change of basement membrane with collapsed contour of microvascular wall; (7) increment in pericyte vascular coverage rate (area); and (8) increased perivascular astrocytic endfeet area with significant dysfunction mitochondria. Above all, neuronal and axonal degeneration and ultrastructural destruction of cellular constituents and organelles, such as dysfunction of mitochondria, were most evident in the present study. Microvascular collapse and compensatory changes in the forms of expanded basement membrane and proliferated pericytes and astrocytic endfeet were remarkable. These characteristics may inspire further pathophysiological investigation on the potential target NVU component of treatment to promote an effective therapeutic strategy.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

This animal study was reviewed and approved by the Committee on the Ethics of Animal Experiments of the Affiliated Hospital of Guangdong Medical University.

Author contributions

G-SL and YH were involved in the study design and drafted the manuscript. G-SL, X-XW, K-HW, and X-SH were involved in the experimental implementation. G-SL and R-BT were involved in the data acquisition. G-SL analyzed the data. YH reviewed the manuscript. All authors read and approved the final version of the manuscript.

Funding

This work was supported by the National Key Research and Development Program of China (2021YFF0501604), National Natural Science Foundation of China (82072507), and Natural Science Foundation of Guangdong Province, China (2018A0303130105 and 2016A030313679). The funders had no roles in the design of the study, conduction of the experiment,

data collection and analysis, decision to publish, or preparation of the manuscript.

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

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frontiers | Frontiers in Neurology

TYPE Original Research
PUBLISHED 30 November 2022
DOI 10.3389/fneur.2022.1038735

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Dementia and Neurodegenerative Diseases, a section of the journal Frontiers in Neurology

RECEIVED 07 September 2022
ACCEPTED 04 November 2022
PUBLISHED 30 November 2022

CITATION
Tsai C-Y, Wu S-M, Kuan Y-C, Lin Y-T, Hsu C-R, Hsu W-H, Liu Y-S, Majumdar A, Stettler M, Yang C-M, Lee K-Y, Wu D, Lee H-C, Wu C-J, Kang J-H and Liu W-T (2022) Associations between risk of Alzheimer's disease and obstructive sleep apnea, intermittent hypoxia, and arousal responses: A pilot study. *Front. Neurol.* 13:1038735. doi: 10.3389/fneur.2022.1038735

Associations between risk of Alzheimer's disease and obstructive sleep apnea, intermittent hypoxia, and arousal responses: A pilot study

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Objectives: Obstructive sleep apnea (OSA) may increase the risk of Alzheimer's disease (AD). However, potential associations among sleep-disordered breathing, hypoxia, and OSA-induced arousal responses should be investigated. This study determined differences in sleep parameters and investigated the relationship between such parameters and the risk of AD.

Methods: Patients with suspected OSA were recruited and underwent in-lab polysomnography (PSG). Subsequently, blood samples were collected from participants. Patients' plasma levels of total tau (T-Tau) and amyloid beta-peptide 42 ($A\beta_{42}$) were measured using an ultrasensitive immunomagnetic reduction assay. Next, the participants were categorized into low- and high-risk groups on the basis of the computed product ($A\beta_{42} \times$ T-Tau, the cutoff for AD risk). PSG parameters were analyzed and compared.

Results: We included 36 patients in this study, of whom 18 and 18 were assigned to the low- and high-risk groups, respectively. The average apnea–hypopnea index (AHI), apnea, hypopnea index [during rapid eye

movement (REM) and non-REM (NREM) sleep], and oxygen desaturation index (≥3%, ODI-3%) values of the high-risk group were significantly higher than those of the low-risk group. Similarly, the mean arousal index and respiratory arousal index (R-ArI) of the high-risk group were significantly higher than those of the low-risk group. Sleep-disordered breathing indices, oxygen desaturation, and arousal responses were significantly associated with an increased risk of AD. Positive associations were observed among the AHI, ODI-3%, R-ArI, and computed product.

Conclusions: Recurrent sleep-disordered breathing, intermittent hypoxia, and arousal responses, including those occurring during the NREM stage, were associated with AD risk. However, a longitudinal study should be conducted to investigate the causal relationships among these factors.

KEYWORDS

obstructive sleep apnea, Alzheimer's disease, sleep-disordered breathing, total tau, amyloid beta-peptide 42, arousal response

Introduction

The prevalence of obstructive sleep apnea (OSA) in the general population ranges from 9 to 38% (1). OSA is characterized by repetitive upper airway collapse, which leads to intermittent hypoxia, recurrent arousal responses, and sleep fragmentation (2). OSA is associated with a 1.26-fold risk (95% CI: 1.05–1.50) of cognitive impairment and dementia and has been linked to memory dysfunction (3, 4). One review reported that 11–71% of patients with cognitive impairment have OSA (5). In another study, more than 90% of the enrolled patients with dementia had diagnosed OSA, and 39.1% of the cases of diagnosed OSA were severe (6).

Studies have provided possible explanations of the pathological mechanisms underlying the relationship between OSA and dementia. For example, one study indicated that the respiratory events associated with OSA—namely episodes of apnea and hypopnea—affect cerebral circulation as well as cerebrovascular responses and result in hypercapnia and concomitant hypoxia (7). Intermittent hypoxia is associated with elevated reactive oxygen species formation, which can cause oxidative stress; oxidative stress can lead to inflammatory cytokine activation and, in turn, cerebral neuron impairment (8). In addition, hypercapnia and hypoxia can induce arousal responses, which are associated with fragmented sleep and an increased risk of cognitive impairment (9, 10). Therefore, exploring the effects of the various responses induced by OSA stimuli on individuals' risk of developing neurodegenerative diseases is crucial.

OSA severity is classified using the apnea–hypopnea index (AHI), which is the total number of respiratory events during sleep time. Although it can be used to assess sleep-disordered breathing, the AHI may not be entirely suitable for evaluating all phenotypic subtypes of OSA (11). For example, the AHI does not sufficiently reflect the pattern or level of sleep fragmentation or the effect of arousal responses, although these factors are correlated. Frequent arousal, which leads to sleep fragmentation, can result in neurodegeneration and is associated with dementia (12). Another study recruited male participants and investigated the relationships between brain cortical thickness and sleep parameters measured using polysomnography (PSG) among the participants with severe OSA (13). Those results indicated that the arousal index (ArI) and respiratory arousal index (R-ArI) values of the patients were significantly and negatively correlated with the cortical thickness of the prefrontal and parietal cortex areas, which may elucidate some of the underlying mechanisms of cognitive dysfunction. Additionally, researchers have established numerous animal models to investigate the association between arousal indices and biomarkers of neurodegenerative diseases. In a mouse model of Alzheimer's disease (AD), for example, increased amyloid beta peptide (Aβ) deposition was determined to be related chronic sleep fragmentation induced by intermittent nocturnal arousals (14). In other studies, sleep arousal significantly increased both Aβ and tau protein levels in the interstitial fluid of mice and reduced the clearance efficiency of these proteins in animal models (15, 16). However, the associations among sleep-disordered breathing, arousal responses, and the risk of AD development remain unclear and warrant further investigation.

This explorative study, conducted in Taiwan, investigated the associations between sleep parameters measured using PSG and plasma levels of biomarkers of neurodegenerative diseases. We also compared the sleep parameters of the patients in the low- or high-risk groups, who were grouped on the basis of biomarker levels. These aims to determine the relationships between sleep disorders and the risk of AD

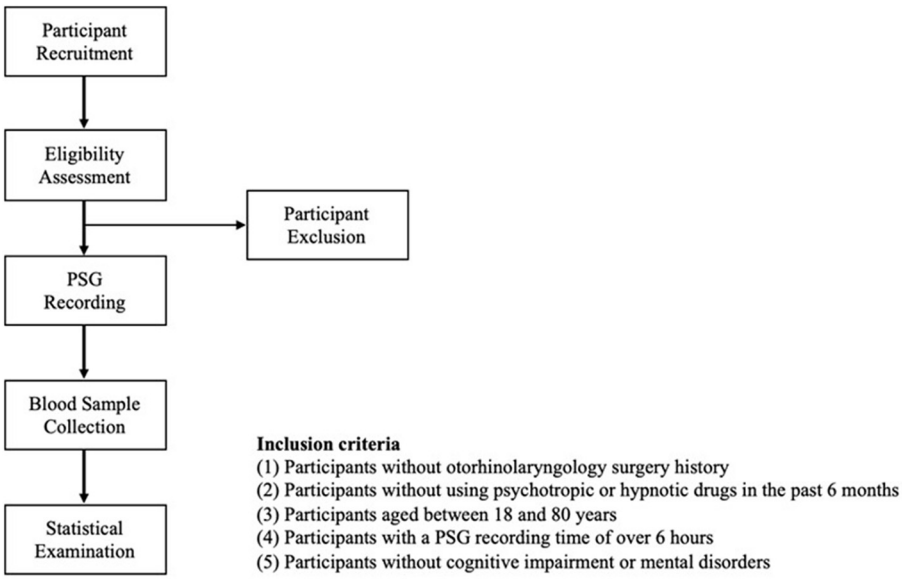

FIGURE 1
Participant recruitment flowchart. PSG, polysomnography.

development. The findings of this study may help elucidate the effects of the accumulation of neurochemical biomarkers on arousal responses.

Methods

Ethics

The study protocol was approved by the Joint Institutional Review Board of Taipei Medical University (TMU-JIRB: N201912097), and the study was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from each participant before data collection. Participant enrollment, PSG outcome access, and blood sample collection were performed in accordance with approved guidelines.

Study population and procedure

Patients with suspected OSA were recruited and referred to the Sleep Center of Taipei Medical University Shuang Ho Hospital (New Taipei City, Taiwan). The participants were recruited from June to October 2018 and from July 2020 to March 2022. The recruitment criteria were as follows: (1) no history of otorhinolaryngological surgery for OSA, (2) no use of psychotropic or hypnotic drugs in the prior 6 months, (3) an age between 18 and 80 years, (4) a PSG recording of over 6 h, (5) no diagnosis of neurological disorders (e.g., AD, dementia, epilepsy, and Parkinson's disease) and other comorbidities (e.g., cardiovascular disease, renal failure, and metabolic syndrome), and (6) absence of cognitive symptoms. Figure 1 presents a flowchart of the study procedure. We arranged for the eligible participants to undergo overnight PSG at the Sleep Center. We collected blood samples from each patient in the morning at a fixed time (6:30 a.m.) after they underwent PSG to determine their plasma levels of neurochemical biomarkers. All the collected data were statistically analyzed.

PSG

The PSG examination was performed using a ResMed Embla N7000 (ResMed, San Diego, CA, USA) and an Embla MPR (ResMed Global Supplier Alliance, Sydney, Australia) at the Sleep Center. The collected images were scored using RemLogic software (version 3.41; Embla Systems, Thornton, CO, USA) by certified PSG technologists who undergo monthly interscoring training. All the scoring rules were established in accordance with the 2017 guidelines of the American Association of Sleep Medicine (17). To ensure the consistency of the scoring, all the scoring outcomes were examined by another technologist, and any inconsistencies were resolved through discussion. Regarding the PSG parameters of interest, we collected the participants' sleep architecture–related, respiratory event–related, hypoxemia-related, and arousal-related indices. OSA severity was categorized as normal (AHI $<$ 5 events/h), mild (AHI $=$ 5 to 15 events/h), moderate (AHI $=$ 15 to 30 events/h), or severe (AHI $\geq$ 30 events/h) (18). All arousal

events were categorized as spontaneous arousal, respiratory arousal (apnea or hypopnea related), limb-movement arousal (single, periodic, or respiratory-related movement), or snore arousal on the basis of cause. The ArI value was the sum of all arousal values. In particular, abrupt alterations in electroencephalography caused by alpha (8 to 12 Hz), theta (4 to 8 Hz), or high-frequency (>16 Hz, but not with a spindle pattern) bands were scored. Alterations were recorded only if they continued for more than 3 s (≥10 s of stable sleep preceding the alterations). Next, we calculated the respiratory event–related and arousal-related indices during the rapid eye movement (REM) stage, the non-REM (NREM) stage, and total sleep time. Briefly, certified PSG technologists first examined apnea, hypopnea, oxygen desaturation (≥3%), arousal events, and sleep stages. Next, the scoring system automatically used different sleep periods to calculate the indices. The apnea index (AI) is defined as apnea events divided by total sleep time. AI_{NREM} is defined as the apnea event that occurred during the NREM period divided by NREM time. AI_{REM} is defined as the apnea event that occurred during the REM period divided by REM time. All arousal subtypes were classified on the basis of factors induced before the arousal event. For instance, respiratory arousal was defined as respiratory events, including apnea and hypopnea, that cause arousal, whereas spontaneous arousal was defined as the occurrence of arousal without the induction of any particular factor.

Blood sample collection and processing

The blood samples of the participants were collected using a procedure described in another study (19). Approximately 16 mL of venous blood was collected from each participant and stored in a lavender-top tube coated with tripotassium ethylenediamine tetra-acetate. Within 1 h of collection, the samples were centrifuged at 2,500 *g* for 15 min at room temperature. The extracted plasma was then aliquoted into cryovials, frozen at −80°C, and delivered to MagQu (New Taipei City, Taiwan) to test the levels of neurochemical biomarkers.

Measurement of neurochemical biomarkers in blood samples

The participants' plasma levels of total tau (T-Tau) and $A\beta_{42}$ were determined using an ultrasensitive immunomagnetic reduction (IMR) assay. The procedure and technical details of the assay were reported in another study (20). The biomarkers $A\beta_{42}$ and T-Tau were assayed using different reagents (MagQu, catalog number: MF-AB2-0060 and MF-TAU-0060). Mixtures of various volumes of reagents and samples were analyzed using a SQUID-based alternating-current magnetosusceptometer (model XacPro-S, MagQu, New Taipei City, Taiwan). The plasma levels of T-Tau protein and $A\beta_{42}$ were quantified on the basis of the IMR signals generated by interaction between IMR reagents and target proteins; these signals were then converted into concentrations on the basis of the characteristic curves of each protein. The cutoff value for the computed product of $A\beta_{42}$ and T-Tau for identifying AD (382.68 pg^2/mL^2, 92% accuracy in identifying AD) was established in another study (21). In this study, we used this established cutoff value to separate the recruited participants into two groups on the basis of whether they were at low or high risk of developing AD.

Statistical analysis

All the statistical analyses were performed using SPSS version 20 (SPSS, Chicago, IL, USA). The Shapiro–Wilk test was first conducted to examine the normality of the derived parameters. Student's *t*-test and the Mann–Whitney *U* test were used to identify between-group differences in the mean values of the continuous variables with normal distributions (Shapiro–Wilk test, $p > 0.05$) and non-normal distributions (Shapiro–Wilk test, $p < 0.05$), respectively. Categorical variables were analyzed using the chi-squared test. Next, to explore the associations between the variables of the low-risk group (computed products ≤ 382.68 pg^2/mL^2) and those of the high-risk group (computed products > 382.68 pg^2/mL^2), we used simple and multivariable logistic regression models with adjustment for age, sex, and body mass index (BMI). The results are reported as crude or adjusted odds ratios (ORs) with 95% CIs. The level of significance was set to $p < 0.05$.

Results

Demographic characteristics of enrolled participants

Table 1 presents the demographic data of the participants. The low- and high-risk groups consisted of 18 participants each. No significant between-group differences in body-related parameters were identified. Regarding neurochemical biomarkers levels, compared with the low-risk group, the high-risk group had significantly higher levels of T-Tau and $A\beta_{42}$ as well as a higher average ratio ($A\beta_{42}$/T-Tau) and product ($A\beta_{42}$ × T-Tau) of the two. In addition, the OSA severity distributions of the groups did not differ significantly ($p = 0.11$); most of the participants in both groups had severe OSA (low-risk group: 61.11%; high-risk group: 88.89%).

Sleep parameters

Table 2 presents the participants' sleep architecture parameters, oximetry parameters, and sleep-disordered

10.3389/fneur.2022.1038735

TABLE 1 Comparison of demographic characteristics of the low- and high-risk groups.

Categorical variable	Low-risk group ($n = 18$)	High-risk group ($n = 18$)	p
Age (y)[a]	51.72 ± 11.4	52.78 ± 11.24	0.90
Sex (male/female)[b]	10/8	13/5	0.30
BMI (kg/m^2)[a]	29.5 ± 5.22	28.62 ± 3.82	0.62
Neck circumference (cm)[a]	41.67 ± 10.44	38.56 ± 3.05	0.60
Waist circumference (cm)[a]	95.36 ± 20.19	96.72 ± 9.76	0.99
Biomarker levels[a]			
T-Tau (pg/mL)	20.31 ± 2.62	27.39 ± 4.57	<0.01
$A\beta_{42}$ (pg/mL)	15.83 ± 0.72	16.98 ± 0.85	<0.01
$A\beta_{42}$/T-Tau	0.79 ± 0.1	0.63 ± 0.08	<0.01
$A\beta_{42}$ × T-Tau (pg/mL)^2	322.02 ± 46.75	467.79 ± 102.24	<0.01
OSA severity[b]			0.11
Normal, *n* (%)	1 (5.56%)	–	
Mild, *n* (%)	4 (22.22%)	–	
Moderate, *n* (%)	2 (11.11%)	2 (11.11%)	
Severe, *n* (%)	11 (61.11%)	16 (88.89%)	

BMI, body mass index; OSA, obstructive sleep apnea.
Data are expressed as means ± standard deviations.
[a]Differences between groups were assessed using Mann–Whitney U test.
[b]Differences between groups were assessed using the chi-squared test.

TABLE 2 Comparison of sleep parameters of the low- and high-risk groups.

Categorical variable	Low-risk group ($n = 18$)	High-risk group ($n = 18$)	p
Sleep architecture parameters			
Sleep efficiency (%)	79.16 ± 15.53	77.55 ± 13.37	0.39
Wake (% of SPT)	17.46 ± 14.49	17.08 ± 11.16	0.75
NREM (% of SPT)	71.97 ± 11.57	70.32 ± 10.41	0.67
REM (% of SPT)	10.55 ± 6.81	12.6 ± 5.52	0.33
WASO (min)	60.04 ± 48.7	57.65 ± 32.42	0.82
TST (min)	288.97 ± 57.25	285.21 ± 49.4	0.54
Oximetry parameters			
Mean SpO_2 (%)	95.19 ± 1.63	93.98 ± 2.1	0.06
Minimum SpO_2 (%)	83.17 ± 7.65	76.39 ± 8.61	<0.05
ODI-3% (events/h)	25.16 ± 15.11	47.16 ± 16.56	<0.01
SDB indices (events/h)			
AHI	28.55 ± 15.56	52.59 ± 17.28	<0.01
AI	6.33 ± 8.03	19.53 ± 16.4	<0.01
AI_{NREM}	5.38 ± 8.76	19.39 ± 17.5	<0.01
AI_{REM}	12.26 ± 15.45	21.34 ± 18.01	0.13
HI	22.22 ± 13.23	33.07 ± 14.39	<0.05
HI_{NREM}	21.2 ± 13.3	33.38 ± 14.66	<0.05
HI_{REM}	27.24 ± 21.18	33.05 ± 20.47	0.41

SPT, Sleep period time; NREM, non-rapid eye movement; REM, rapid eye movement; WASO, wake time after sleep onset; TST, total sleep time; SpO_2, peripheral arterial oxygen saturation (as measured using pulse oximetry); ODI-3%, oxygen desaturation index ≥3%; SDB, sleep-disordered breathing; AHI, apnea–hypopnea index; AI, apnea index; AI_{NREM}, apnea index in non-rapid eye movement stage; AI_{REM}, apnea index in rapid eye movement stage; HI, hypopnea index; HI_{NREM}, hypopnea index in non-rapid eye movement stage; HI_{REM}, hypopnea index in rapid eye movement stage.
Data are expressed as means ± standard deviations.
Differences between groups were assessed using the Mann–Whitney U test.

breathing indices. Regarding peripheral arterial oxygen saturation (SpO_2) indices, compared with the low-risk group, the high-risk group had significantly higher oxygen desaturation index values (≥3%, ODI-3%; $p < 0.01$) and lower minimum SpO_2 ($p < 0.05$). Regarding sleep-disordered breathing indices, the low-risk group had significantly lower AHI, AI, and hypopnea index (HI) values than did the high-risk group (AHI: 28.55 ± 15.56 vs. 52.59 ± 17.28 events/h, $p < 0.01$; AI: 6.33 ± 8.03 vs. 19.53 ± 16.4 events/h, $p < 0.01$; HI: 22.22 ± 13.23 vs. 33.07 ± 14.39 events/h, $p < 0.05$). Regarding the indices calculated during NREM sleep, the high-risk group had significantly higher mean AI_{NREM} and HI_{NREM} values. No significant between-group difference in sleep-disordered breathing indices calculated during REM sleep was identified.

Arousal parameters

Table 3 presents the participants' arousal-related parameters during the REM and NREM stages and total sleep time. The ArI and R-ArI of the low-risk group were significantly lower than those of the high-risk group (ArI: 17.44 ± 8.94 vs. 28.46 ± 15.95 events/h, $p < 0.05$; R-ArI: 8.87 ± 6.44 vs. 20.93 ± 14.93 events/h, $p < 0.01$). Regarding NREM sleep parameters, the ArI_{NREM} and R-ArI_{NREM} of the high-risk group were significantly higher than those of the low-risk group (ArI_{NREM}: 16.59 ± 8.35 vs. 28.91 ± 16.97 events/h, $p < 0.05$; R-ArI_{NREM}: 8.05 ± 6.14 vs. 21.2 ± 15.95 events/h, $p < 0.01$). In addition, the high-risk group had a significantly higher mean R-ArI_{REM} than did the low-risk group ($p < 0.05$). However, no significant between-group differences in the types of spontaneous arousal were observed.

Elevated sleep-disordered breathing indices are associated with a higher risk of AD development

Table 4 presents the associations among the sleep-disordered breathing indices of the low- and high-risk groups determined using the logistic regression models. An increase of 1 event of ODI-3% per hour was associated with a 1.10-fold higher OR (95% CI: 1.03–1.17, $p < 0.01$) and a 1.13-fold higher OR (95% CI: 1.05–1.21, $p < 0.01$) for developing AD in the crude model and the model adjusted for age, sex, and BMI. Similarly, we

TABLE 3 Comparison of arousal indices of the low- and high-risk groups.

Variable (events/h)	Low-risk group ($n = 18$)	High-risk group ($n = 18$)	p
ArI	17.44 ± 8.94	28.46 ± 15.95	<0.05
ArI_{NREM}	16.59 ± 8.35	28.91 ± 16.97	<0.05
ArI_{REM}	23.54 ± 17.3	25.36 ± 13.55	0.73
Sp-ArI	6.61 ± 4.45	5.51 ± 5.01	0.27
Sp-ArI_{NREM}	6.64 ± 4.48	5.62 ± 5.12	0.25
Sp-ArI_{REM}	9.09 ± 9.17	4.31 ± 5.78	0.05
R-ArI	8.87 ± 6.44	20.93 ± 14.93	<0.01
R-ArI_{NREM}	8.05 ± 6.14	21.2 ± 15.95	<0.01
R-ArI_{REM}	12.44 ± 15.49	19.12 ± 13.93	<0.05
Sn-ArI	0.26 ± 1.11	0.45 ± 1.32	0.62
Sn-ArI_{NREM}	0.26 ± 1.08	0.47 ± 1.39	0.57
Sn-ArI_{EM}	0.31 ± 1.3	0.34 ± 1.0	0.62
L-ArI	1.64 ± 2.05	1.63 ± 2.14	0.79
L-ArI_{NREM}	1.69 ± 3.7	1.55 ± 2.93	0.71
L-ArI_{REM}	1.72 ± 2.03	1.58 ± 2.09	0.63

ArI, arousal index; ArI_{NREM}, arousal index in non-rapid eye movement stage; ArI_{REM}, arousal index in rapid eye movement stage; Sp-ArI, spontaneous arousal index; Sp-ArI_{NREM}, spontaneous arousal index in non-rapid eye movement stage; Sp-ArI_{REM}, spontaneous arousal index in rapid eye movement stage; R-ArI, respiratory arousal index; R-ArI_{NREM}, respiratory arousal index in non-rapid eye movement stage; R-ArI_{REM}, respiratory arousal index in rapid eye movement stage; Sn-ArI, snore arousal index; Sn-ArI_{NREM}, snore arousal index in non-rapid eye movement stage; Sn-ArI_{REM}, snore arousal index in rapid eye movement stage; L-ArI, limb movement arousal index; L-ArI_{NREM}, limb movement arousal index in non-rapid eye movement stage; L-ArI_{REM}, limb movement arousal index in rapid eye movement stage.
Data are expressed as means ± standard deviations.
Differences between groups were assessed using the Mann–Whitney U test.

TABLE 4 Odd ratios (ORs) associated with the sleep-disordered breathing indices of the low- and high-risk groups.

Variable (events/h)	Crude OR (95% CI)[a]	Adjusted OR (95% CI)[b]
Oximetry parameter		
ODI-3% (events/h)	1.10 (1.03–1.17)**	1.13 (1.05–1.21)**
SDB index (events/h)		
AHI	1.10 (1.03–1.18)**	1.13 (1.05–1.21)**
AI	1.06 (1.01–1.10)*	1.10 (1.01–1.2)*
AI_{NREM}	1.09 (1.01–1.18)*	1.09 (1.01–1.18)*
AI_{REM}	1.03 (0.99–1.08)	1.04 (0.99–1.09)
HI	1.06 (1.0–1.12)*	1.12 (1.02–1.22)*
HI_{NREM}	1.07 (1.01–1.13)*	1.12 (1.02–1.22)*
HI_{REM}	1.01 (0.98–1.05)	1.03 (0.98–1.08)

ODI-3%, oxygen desaturation index ≥3%; SDB, Sleep-disordered breathing; AHI, apnea–hypopnea index; AI, apnea index; AI_{NREM}, apnea index in non-rapid eye movement stage; AI_{REM}, apnea index in rapid eye movement stage; HI, hypopnea index; HI_{NREM}, hypopnea index in non-rapid eye movement stage; HI_{REM}, hypopnea index in rapid eye movement stage.
[a]Simple logistic regression models.
[b]Multivariable logistic regression models adjusted for age, sex, and body mass index.
*p < 0.05; **p < 0.01.

determined the statistically significant ORs for developing AD for each event-per-hour increase in the following parameters: AHI (crude OR 1.10, 95% CI 1.03–1.18, $p < 0.01$; adjusted OR 1.13, 95% CI 1.05–1.18, $p < 0.01$), AI (crude OR 1.10, 95% CI 1.02–1.20, $p < 0.05$; adjusted OR 1.10, 95% CI 1.01–1.2, $p < 0.05$), and HI (crude OR 1.06, 95% CI 1.0–1.12, $p < 0.05$; adjusted OR 1.12, 95% CI 1.02–1.22, $p < 0.05$). Similar results were obtained when the analysis was restricted to parameters measured during NREM sleep (AI_{NREM}: crude OR 1.09, 95% CI 1.01–1.18, $p < 0.05$; adjusted OR 1.09, 95% CI 1.01–1.2, $p < 0.05$; HI_{NREM}: Crude OR 1.07, 95% CI 1.01–1.13, $p < 0.05$; adjusted OR 1.12, 95% CI 1.02–1.22, $p < 0.05$).

Elevated arousal indices are associated with a higher risk of AD development

Table 5 presents the associations among the arousal indices of the low- and high-risk groups determined using the logistic regression models. Every event-per-hour increase in ArI and R-ArI was associated with a 1.08-fold higher OR (95% CI: 1.01–1.15, $p < 0.05$) and 1.16-fold higher OR (95% CI: 1.03–1.31, $p < 0.05$) for developing AD, respectively. After adjustment for age, sex, and BMI, each event-per-hour increase in ArI and R-ArI was significantly associated with a 1.08-fold higher OR (95% CI: 1.0–1.16, $p < 0.05$) and 1.16-fold higher OR (95% CI: 1.02–1.31, $p < 0.05$) of developing AD, respectively. When the analysis was restricted to arousal indices measured during NREM sleep, we identified similar trends in ArI_{NREM} and R-ArI_{NREM} (ArI_{NREM}: crude OR 1.08, 95% CI 1.01–1.16, $p < 0.05$; adjusted OR 1.08, 95% CI 1.01–1.16, $p < 0.05$; R-ArI_{NREM}: crude OR 1.15, 95% CI 1.03–1.3, $p < 0.05$; adjusted OR 1.15, 95% CI 1.02–1.29, $p < 0.05$).

Associations between sleep parameters and computed product ($A\beta_{42}$ × T-Tau)

Table 6 presents a summary of the associations between the computed product and sleep parameters, including oximetry, sleep-disordered breathing, and arousal, determined using multivariable linear regression after adjustment for age, sex, and BMI. Each additional ODI-3% and AHI event occurring per hour of sleep was significantly associated with an elevated level of the computed product [ODI-3%: 0.41, 95% confident interval (CI): 0.08 to 0.74, $P < 0.05$; AHI: 0.40, 95% CI: 0.06–0.74, $P < 0.05$]. For the arousal index, every event-per-hour increase in R-ArI and R-ArI_{NREM} was significantly associated with an increased level of the computed product

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TABLE 5 Odd ratios associated with arousal indices of the low- and high-risk groups.

Variable (events/h)	Crude OR (95% CI)[a]	Adjusted OR (95% CI)[b]
ArI	1.08 (1.01–1.15)*	1.08 (1.0–1.16)*
ArI_{NREM}	1.08 (1.01–1.16)*	1.08 (1.01– 1.16)*
ArI_{REM}	1.01 (0.97–1.05)	1.0 (0.96–1.05)
Sp-ArI	0.95 (0.82–1.1)	0.98 (0.84–1.14)
$Sp\text{-}ArI_{NREM}$	0.95 (0.83–1.1)	0.99 (0.85– 1.15)
$Sp\text{-}ArI_{REM}$	0.92 (0.83–1.01)	0.92 (0.84–1.02)
R-ArI	1.16 (1.03–1.31)*	1.16 (1.02–1.31)*
$R\text{-}ArI_{NREM}$	1.15 (1.03–1.3)*	1.15 (1.02– 1.29)*
$R\text{-}ArI_{REM}$	1.03 (0.98–1.08)	1.03 (0.98–1.09)

CI, confidence interval; ArI, arousal index; ArI_{NREM}, arousal index in non-rapid eye movement stage; ArI_{REM}, arousal index in rapid eye movement stage; Sp-ArI, spontaneous arousal index; $Sp\text{-}ArI_{NREM}$, spontaneous arousal index in non-rapid eye movement stage; $Sp\text{-}ArI_{REM}$, spontaneous arousal index in rapid eye movement stage; R-ArI, respiratory arousal index; $R\text{-}ArI_{NREM}$, respiratory arousal index in non-rapid eye movement stage; $R\text{-}ArI_{REM}$, respiratory arousal index in rapid eye movement stage.
[a]Simple logistic regression models.
[b]Multivariable logistic regression models adjusted for age, sex, and body mass index.
*$p < 0.05$.

TABLE 6 Associations between sleep parameters and computed product ($A\beta_{42}$ × T-Tau).

Variable (events/h)	Beta coefficient (95% confidence interval)
Oximetry parameters	
ODI-3% (events/h)	0.41 (0.08 to 0.74)*
SDB indices (events/h)	
AHI	0.40 (0.06 to 0.74)*
AI	0.21 (−0.12 to 0.54)
AI_{NREM}	0.20 (−0.13 to 0.54)
AI_{REM}	0.19 (−0.16 to 0.54)
HI	0.35 (−0.03 to 0.72)
HI_{NREM}	0.36 (−0.0 to 0.72)
HI_{REM}	0.17 (−0.25 to 0.59)
Arousal indices (events/h)	
ArI	0.26 (−0.07 to 0.59)
ArI_{NREM}	0.26 (−0.06 to 0.59)
ArI_{REM}	0.08 (−0.3 to 0.45)
Sp-ArI	−0.11 (−0.46 to 0.24)
$Sp\text{-}ArI_{NREM}$	−0.12 (−0.47 to 0.23)
$Sp\text{-}ArI_{REM}$	−0.12 (−0.46 to 0.21)
R-ArI	0.34 (0.02 to 0.66)*
$R\text{-}ArI_{NREM}$	0.35 (0.04 to 0.67)*
$R\text{-}ArI_{REM}$	0.13 (−0.24 to 0.51)

ODI-3%, oxygen desaturation index ≥3%; SDB, sleep-disordered breathing; AHI, apnea–hypopnea index; AI, apnea index; AI_{NREM}, apnea index in the non-rapid eye movement stage; AI_{REM}, apnea index in rapid eye movement stage; HI, hypopnea index; HI_{NREM}, hypopnea index in non-rapid eye movement stage; HI_{REM}, hypopnea index in rapid eye movement stage. ArI, arousal index; ArI_{NREM}, arousal index in non-rapid eye movement stage; ArI_{REM}, arousal index in rapid eye movement stage; Sp-ArI, spontaneous arousal index; $Sp\text{-}ArI_{NREM}$, spontaneous arousal index in non-rapid eye movement stage; $Sp\text{-}ArI_{REM}$, spontaneous arousal index in rapid eye movement stage; R-ArI, respiratory arousal index; $R\text{-}ArI_{NREM}$, respiratory arousal index in non-rapid eye movement stage; $R\text{-}ArI_{REM}$, respiratory arousal index in rapid eye movement stage.
Multivariable linear regression models were adjusted for age, sex, and body mass index.
*$p < 0.05$.

(R-ArI: 0.34, 95% CI: 0.02–0.66, $P < 0.05$; AHI: 0.35, 95% CI: 0.04–0.67, $P < 0.05$).

Determination of odds ratio by using different cutoff points

To enhance the robustness of the observed outcomes, we employed another cutoff for the product (Aβ42 × T-Tau; 403.72 pg^2/mL^2), which was determined using the IMR technique (22). According to this cutoff, 14 and 22 patients were included in the high- and low-risk groups, respectively. Supplementary Tables S2, S3 present associations among the sleep-disordered breathing indices and arousal indices, respectively, in the low- and high-risk groups determined using logistic regression models. Significant associations among ODI-3%, AHI, HI, and HI_{NREM} were observed in both crude and adjusted models (adjusted for age, sex, and BMI). In terms of the arousal effect, R-ArI was significantly associated with the risk of AD in both crude and adjusted models. When the analysis was restricted to only the NREM period, significant associations were observed between ArI_{NREM} and $R\text{-}ArI_{NREM}$ in both crude and adjusted models.

Discussion

Although OSA has been determined to be associated with the formation and accumulation of neurochemical biomarkers, the relationship between the clinical symptoms of OSA, such as intermittent hypoxia and arousal responses, and levels of neurochemical biomarker has not been explored. Therefore, we compared the sleep parameters (measured using PSG) of individuals at low and high risks of developing AD and explored the associations between the participants' sleep parameters and neurochemical biomarker levels. The results reveal that the high-risk group had significantly higher mean values for various indices of sleep-disordered breathing and arousal responses than the low-risk group. We determined that increased risk of developing AD was associated with various arousal response and sleep-disordered breathing indices.

The mean AI, HI, AI_{NREM}, HI_{NREM}, ODI-3%, and AHI of the low- and high-risk groups differed significantly despite the lack of significant differences between the OSA severity distributions of the groups. Moreover, significant and positive associations were observed among the AHI, ODI-3%,

and computed product. These findings are consistent with those of studies investigating the association between oxygen desaturation and elevated levels of neurochemical biomarkers (23, 24) that serve as indicators of hypoxia, a major risk factor for neurochemical biomarker accumulation. Hypoxia causes neuronal apoptosis and tau hyperphosphorylation (25). One study reported a significant association between high AHI values and neurochemical biomarker levels in American patients with severe OSA without dementia (26). Another prospective study analyzed the sleep disorder characteristics of 298 older women (aged $\geq$ 65 years) without dementia and reported that an increased oxygen desaturation index ($\geq$15 events/h) was associated with the risk of mild cognitive impairment or dementia after adjustment for age, BMI, and ethnicity (27). Hypercapnia, another key risk factor of OSA, can cause deterioration of the functional and anatomic status of cerebral vessels, which may lead to AD (28). Taken together, the available evidence suggests that sleep-disordered breathing is related to an individual's risk of developing AD.

Regarding arousal responses, the patients in the high-risk group had significantly higher ArI, R-ArI, ArI_{NREM}, and R-ArI_{NREM} values than did those in the low-risk group. Moreover, R-ArI and R-ArI_{NREM} values were positively associated with the increased computed product. These results may be attributed to the pathogenic mechanisms of sleep arousal. Specifically, arousal responses refer to abrupt alterations between sleep and fractional wakefulness (29). Recurrent sleep arousal can interrupt the sleep cycle, alter the sleep architecture, and affect the metabolism of neurodegenerative biomarkers (30). They may also be attributed to the tendency of arousal to disrupt the clearance of neurotoxic proteins, resulting in the increased formation of amyloid plaques and the hyperphosphorylation of tau protein strands in the brain, as demonstrated in another study (31). One review similarly concluded that sleep fragmentation and nighttime awakening were associated with AD progression (32). In the present study, the groups' mean arousal indices measured during NREM sleep differed significantly. This may be explained by the underlying mechanisms of slow-wave sleep, which only occurs in the NREM stage and is associated with the modulation of neurochemical biomarkers; that is, arousals during NREM sleep are likely to interfere with the clearance of neurotoxic proteins. Studies have indicated that lessened or unstable NREM sleep increases the level of neurochemical biomarkers, which is consistent with the findings of the present study (33, 34). Collectively, the results of the present study indicate that the high-risk group had higher mean values for the selected arousal indices because arousal responses may disrupt the clearance of neurotoxic proteins, resulting in sleep fragmentation and, in turn, an increased risk of developing AD.

We further explored the relationships between sleep parameters and the risk of AD development by using logistic regression models with and without adjustment for demographic characteristics. Our findings indicate that frequent respiratory events, intermittent hypoxic episodes, and respiratory arousal responses were significantly associated with an increased risk of developing AD. These findings may be attributable to various risk factors, including hypoxia, oxidative stress, and sleep cycle fragmentation. One review elucidated the pathological roles of hypoxia in AD, which include facilitating the accumulation of neurotoxic proteins, enhancing the hyperphosphorylation of tau protein, diminishing the function of the blood–brain barrier, and accelerating neurodegeneration (35). Research has demonstrated the relationship among AD pathology, oxidative stress, and oxidative damage to the brain (36). Another study reported that sleep discontinuity interfered with the clearance of neurotoxic proteins from the central nervous system by the glymphatic system (37). Similarly, another study suggested that sleep disturbance may cause systemic inflammation, thereby increasing Aβ accumulation, which is thought to be a driver of AD pathogenesis (38). Taken together, the findings of this study reveal that high indices of sleep-disordered breathing and hypoxia and a high frequency of respiratory arousal are associated with an increased risk of developing AD.

The present study has some strengths. First, this study analyzed data derived from patients without cognitive symptoms and observed positive associations among sleep-disordered breathing events, neuron biomarker levels, and AD risk. These outcomes are in accordance with the findings of several studies indicating an association of OSA with AD risk (23). Moreover, in contrast to previous studies analyzing sleep parameters in patients with AD (6, 39), this pilot study focused on patients without any cognitive impairment symptom, and the findings of this study may help in understanding the relationship between OSA and AD risk. Another major finding of this pilot study is the positive associations between the respiratory arousal frequency and neuron biomarker levels. Previous studies have investigated the associations between arousal responses and cognitive impairment or different neuron biomarker plasma levels in children (40, 41). The results of this study demonstrated that respiratory arousal events were associated with elevated neuron biomarker levels and thus increased AD risk in adult patients without cognitive impairment. The findings of this pilot study suggest that sleep-disordered breathing and related arousal responses may increase neuron biomarker levels and thus AD risk.

This study has several limitations that should be addressed. First, although individuals with diagnosed AD were excluded from this study, we did not perform neuropsychological evaluations to assess the brain function of the enrolled participants. Moreover, we did not enroll patients who had cognitive symptoms or were diagnosed as having neurological disorders. The mean age of the enrolled patients was 52.25 years; thus, they may have a relatively low risk of cognitive

impairment (42). Nevertheless, future studies should investigate the associations among PSG parameters, cognitive questionnaire responses, and biomarker levels to enhance the robustness of our results. Next, during the recruitment process, the presence of genes associated with neurodegenerative diseases was not determined to be predictive of an individual's risk of developing neurodegenerative diseases. However, the potential effects of genetic factors (e.g., *ApoE4*) on the participants' baseline levels of neurochemical biomarkers and the risk of AD development must be taken into account (43). We did not measure the levels of biomarkers in the participants' cerebrospinal fluid to compare against their levels in the plasma samples, and only one cross-sectional measurement was performed. We were therefore unable to track shifts in biomarker levels over time or evaluate the potential causal relationship between sleep parameters and individuals' risk of AD development. Next, this study did not measure other biomarkers in plasma, such as p-tau 181, $A\beta_{40}$, or neurofilament light chain protein. These related biomarkers may help identify the risk of AD and thus the associations between OSA and AD. Although we observed positive associations between sleep-disordered breathing indices and the computed product ($A\beta_{42} \times$ T-Tau), we did not measure the levels of inflammatory biomarkers (i.e., glial fibrillary acidic protein). Examination of the level of this biomarker can help in identifying the relationships among hypoxia, inflammation, and neuron impairment. These limitations should be addressed in long-term follow-up studies involving cohorts of patients with OSA.

Another limitation of this study is that it included a small sample of participants enrolled from a single sleep center. Such a small sample size may affect the generalization of our results to different populations. In addition, we recruited individuals only from a single region in Taiwan as the study population. The relationships between arousal indices and neurochemical biomarkers should be further explored in multicenter studies. Previous studies have indicated that the ratio of slow oscillations in the N3 stage was linked to cognitive impairment or AD risk (44). However, this study calculated only the sleep index during the NREM period to eliminate the first-night effect of PSG instead of splitting them into N1, N2, and N3 stages. The first-night effect, resulting from the sleep laboratory environment or PSG devices, may reduce slow-wave sleep and thus cause the incorrect estimation of sleep parameters in some particular sleep stages (e.g., sleep indices in the N3 stage may be overestimated due to the short N3 period) (45). However, respiratory and arousal events occurring in the N3 stage may be crucial risk factors interrupting neuron biomarker clearance. Therefore, researchers should include a large sample and individuals of different ethnicities as well as perform multiple-night PSG to increase the number of participants and investigate sleep parameters in each sleep stage but with the elimination of the first-night effect.

Conclusion

In this study, drawing on the PSG data and plasma levels of selected biomarkers of a sample population from northern Taiwan, we observed that the group that was at high risk of developing AD (patients with computed products $>$ 382.68 pg^2/mL^2) had higher mean values for several sleep-disordered breathing and arousal indices (during both the REM and NREM stages) than did the group that was at low risk of developing AD. In addition, higher values for sleep-disordered breathing indices, namely AI, HI, AI_{NREM}, HI_{NREM}, AHI, and ODI-3%, were associated with an increased risk of AD. Arousal responses, especially respiratory arousal responses, were also associated with an increased risk of AD. Moreover, sleep-disordered breathing indices (AHI and ODI-3%) and respiratory arousal indices (R-ArI and R-ArI_{NREM}) were positively associated with the computed product. These results indicate that respiratory events, intermittent hypoxia, and arousal responses, including those that occur during the NREM stage, are associated with an increased risk of developing AD. However, the causal relationships among these factors must be further explored through a longitudinal study.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the corresponding author without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Joint Institutional Review Board of Taipei Medical University TMU-JIRB: N201912097. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualized and designed the study: C-YT, S-MW, and W-TL. Data curation and investigation: Y-CK, C-RH, W-HH, Y-SL, and C-MY. Analyzed the data and drafted the paper: C-YT, S-MW, Y-CK, Y-TL, and MS. Critically revised the manuscript and provided essential intellectual contributions: W-TL, AM, C-MY, DW, H-CL, and C-JW. Project administration: K-YL, J-HK, and W-TL. All authors have approved the final version of the manuscript for publication.

Funding

This study was supported by the Ministry of Science and Technology of Taiwan (Grant Number: MOST 111-2634-F-002 -021-) and Taipei Medical University Shuang Ho Hospital (Grant Number: 108TMU-SHH-08). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

We appreciate the contributions of all the participants in this study, and we thank Wallace Academic Editing for editing this manuscript.

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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2022.1038735/full#supplementary-material

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frontiers | Frontiers in Neurology

TYPE Systematic Review
PUBLISHED 10 January 2023
DOI 10.3389/fneur.2022.1052333

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Dementia and Neurodegenerative Diseases,
a section of the journal
Frontiers in Neurology

RECEIVED 23 September 2022
ACCEPTED 06 December 2022
PUBLISHED 10 January 2023

CITATION
Humphreys J and Valdés Hernández MdC (2023) Impact of polycyclic aromatic hydrocarbon exposure on cognitive function and neurodegeneration in humans: A systematic review and meta-analysis.
Front. Neurol. 13:1052333.
doi: 10.3389/fneur.2022.1052333

Impact of polycyclic aromatic hydrocarbon exposure on cognitive function and neurodegeneration in humans: A systematic review and meta-analysis

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Introduction: This article documents an emerging body of evidence concerning the neurological effect of polycyclic aromatic hydrocarbon (PAH) exposure with regard to cognitive function and increased risk of neurodegeneration.

Methods: Two electronic databases, PubMed and Web of Science, were systematically searched.

Results: The 37/428 studies selected included outcomes measuring cognitive function, neurobehavioral symptoms of impaired cognition, and pathologies associated with neurodegeneration from pre-natal (21/37 studies), childhood (14/37 studies), and adult (8/37 studies) PAH exposure. Sufficient evidence was found surrounding pre-natal exposure negatively impacting child intelligence, mental development, average overall development, verbal IQ, and memory; externalizing, internalizing, anxious, and depressed behaviors; and behavioral development and child attentiveness. Evidence concerning exposure during childhood and as an adult was scarce and highly heterogeneous; however, the presence of neurodegenerative biomarkers and increased concentrations of cryptic "self" antigens in serum and cerebrospinal fluid samples suggest a higher risk of neurodegenerative disease. Associations with lowered cognitive ability and impaired attentiveness were found in children and memory disturbances, specifically auditory memory, verbal learning, and general memory in adults.

Discussion: Although evidence is not yet conclusive and further research is needed, the studies included supported the hypothesis that PAH exposure negatively impacts cognitive function and increases the risk of neurodegeneration in humans, and recommends considering the introduction of a variable "rural vs. urban" as covariate for adjusting analyses, where the neurological functions affected (as result of our review) are outcome variables.

KEYWORDS

polycyclic aromatic hydrocarbon (PAH), cognition, neurological, neurodegeneration, neurobehavioral, meta-analysis, systematic literature search

Introduction

Exposure to air pollution in the environment is now recognized globally by governments, leading research scientists, and civil society as one of the greatest public health hazards of the 21st century (1). Legislation such as "The UK National Air Quality Strategy" (2), and the European Commission's "Fourth Daughter Directive" (3) have introduced standards to monitor and limit levels of air pollutants posing the greatest risk to human health. Polycyclic aromatic hydrocarbons (PAHs) are a group of pollutants included in such legislation. PAHs are atmospheric organic compounds composed of two or more benzene rings arranged in a variety of different configurations. PAH compounds also include functional derivatives of the PAHs only containing carbon and hydrogen atoms (e.g., nitro-PAHs) and the heterocyclic analogs (e.g., aza-arenes) (4). Over 100 different PAHs were already identified by the beginning of the 21st century (4), and now the list exceeds 300, with an exact number still to be determined, as those studied are mainly selected based on the instrumentation available to each research group and reference standards (5). They are discharged from anthropogenic sources (Supplementary Figure 1), involving the incomplete combustion and pyrolysis of hydrocarbons, predominantly found in coal, oil, wood, and petrol. PAHs exist in the atmosphere in a gaseous state or are adsorbed to particulate matter. Over 80% of particulate-bound PAHs are associated with particulate matter of an aerodynamic diameter $\leq 2.5\,\mu m$ ($PM_{2.5}$) (6). However, a large number have been also identified in tobacco smoke (5). The study of PAHs and their impact on health has been compounded by their ubiquitousness and the numerous and widespread sources in which they can be found, some of which are also affected by other air pollutants. As PAHs are rather present as part of complex mixtures in air, water, soil, and food, their identification and characterization, for studying their effect on human health, is challenging (5).

Research surrounding PAH exposure and acute short-term health effects in humans has, thus, far focused on vulnerable individuals with pre-existing health conditions: thrombotic effects in individuals with pre-existing coronary heart disease and impaired lung function in asthma sufferers (7). Chronic long-term exposure has implicated PAH's reactive metabolites as having the ability to bind to proteins and DNA and exert carcinogenic effects (8). Such biochemical disruption and cellular damage have been most extensively researched in occupational studies, whereby high exposure has been associated with increased incidence of lung, bladder, skin, and gastrointestinal cancer (8–11). Additionally, decreased immune function, developing cataracts, and having kidney or liver damage, including jaundice, have also been associated with high exposure (5, 12). Whilst extensive research exists surrounding PAH's genotoxic and carcinogenic properties, an emerging body of evidence concerns PAH's neurotoxic effect through the induction of oxidative stress, inflammation (13), and vascular injury within the brain (14). Recently, research has emerged associating PAH exposure with impaired cognitive function and increased risk of neurodegeneration.

To the best of our knowledge, from the large body of literature on the influence of air pollution on human health, the implications of PAH exposure specifically, on cognitive function and neurodegeneration in humans, have not been systematically reviewed. Prior reviews have addressed the implications of PAH exposure on general health (15, 16) and its carcinogenic outcomes (17, 18). The reviews which have made cognitive function and neurodegeneration the outcome of interest include exposures to a vast mixture of air pollutants (19–22). Therefore, we aim to disentangle the unique neurotoxic effect of PAHs in specific age groups and cognitive-related functions to provide evidence for cognitive research and more vigilant monitoring and tighter restrictions on the main sources of emission, tailored to each age group, given the differential factors affecting the various stages of brain development. The Department for Environment, Food and Rural Affairs currently considers annual monitoring of concentrations of one PAH, benzo(a)pyrene (B(a)P), to be a sufficient representation of all atmospheric PAHs, and classifies the potential effect on human health of PAHs collectively, as six compounds, categorized as probably or possibly carcinogenic. No mention is made of the adverse neurological impact (2). A possible explanation is the consideration of concentration levels that constitute a risk for cancer, below which the effect

Abbreviations: AD, Alzheimer's disease; ADHD, Attention deficit hyperactivity disorder; APOE4, Apolipoprotein E4; $A\beta_{1-42}$, Amyloid beta protein fragment 1–42; α-synuclein, Alpha-synuclein; B[a]P, Benzo[a]pyrene; BDNF, Brain-derived neurotrophic factor; CEREB IgG, Cerebellar antigen;CO, Carbon monoxide; CSF, Cerebrospinal fluid; ETS, Environmental tobacco smoke; IFN γ, Interferon gamma; IgA, Immunoglobulin A; IgG, Immunoglobulin G; IgM, Immunoglobulin M; IL β, Interleukin beta; IL 2, Interleukin 2; IL 6, Interleukin 6; IL 10, Interleukin 10; IQ, Intelligence quotient; MBP, Myelin basic protein; MBP IgA, Myelin basic protein immunoglobulin A; MBP IgG, Myelin basic protein immunoglobulin G; MCP-1, Monocyte chemoattractant protein-1; MOG IgG, Myelin oligodendrocyte glycoprotein immunoglobulin G; MOG IgM, Myelin oligodendrocyte glycoprotein immunoglobulin A; NHANES, National Health and Nutrition Examination Survey; Non-p-tau, Non-phosphorylated tau; NO_x, Nitrogen oxide species; NO_2, Nitrogen dioxide; OZ IgA, Occludin/zonulin immunoglobulin A; OZ IgG, Occludin/zonulin immunoglobulin G; PAH, Polycyclic aromatic hydrocarbons; PAH-DNA adducts, Polycyclic aromatic hydrocarbon- DNA adducts; PD, Parkinson's disease; PM, Particulate matter; $PM_{2.}5$, Particulate matter with an aerodynamic diameter > 2.5 micrometers; PM_{10}, Particulate matter with an aerodynamic diameter > 10 micrometers; p-tau, Phosphorylated tau; S-100 IgG, Astrocytic protein immunoglobulin G; S-100 IgM, Astrocytic protein immunoglobulin M; TDP-43, Transactive response DNA binding protein 43; TRAP, Traffic-related air pollution.

of these pollutants can pass inadvertently. The UK national air quality objective for B(a)P is 0.25 ng m^{-3}. However, emissions of B(a)P have been increasing since 2008 and have exceeded this limit in multiple locations at multiple time points (23). Atmospheric PAH concentrations are subject to seasonal variation and climate (24), as seen in pollution level charts that are used by studies to stratify exposure. While such stratification may add granularity to the data, it is often unrealistic given urban movement and the effect of different local government policies e.g., transportation. A more robust stratification would be to contrast urban and rural areas, where the pollution levels known to widely differ. Therefore, a further aim is to explore the difference in PAH concentration in rural vs. metropolitan areas and the influence this could have on cognitive function and neurodegeneration to inform further studies.

Methods

Eligibility criteria

This review was conducted in line with the PRISMA guidelines (25). Studies included were observational cohort studies of both male and female humans. Time of exposure was inclusive of the gestational period and stretched throughout life until death. Exposure quantification was limited to studies that measured the level of exposure to ambient PAHs or $PM_{2.5}$ through environmental air sampling or spatiotemporal modeling. Measures of exposure also included concentration of PAH metabolites in urine and dosimetry of PAH-DNA adducts from DNA extracted from white blood cells. Outcomes included involved a formal assessment of cognitive function, neurobehavioral symptoms of impaired cognition, and pathologies associated with neurodegeneration. Reports were limited to published scientific articles written in the English language. No publication dates were imposed. Studies were excluded if they did not fulfill the inclusion criteria, were not in humans, or where PAH exposure was measured as a component of the diet, environmental tobacco smoke (ETS), or traffic related air pollution (TRAP). Exposure through diet and ETS is not an appropriate representation of a major source of atmospheric PAH that can be geographically differential (i.e., in terms of urban vs. rural areas) or influential in both short- and long-term exposure. Moreover, prior research has elucidated contaminating pollutants within TRAP composition detrimentally affecting cognitive function, and the effect of diet-related benzo[a]pyrene, dibenz[a,h]anthracene, and benzo[h]fluoanthracene in human health and cognition (e.g., learning and memory functions). The inclusion of such studies would confound results and prevent us from elucidating the specific impact of ambient PAH on cognition.

Information sources

Studies were identified by searching electronic databases PubMed (1984–2021) and Web of Science (1979–2021). Given the environmental changes seen as the consequence of lockdown policies and movement restrictions mainly in the period April 2020 to December 2021, publications that reflected or analyzed the environmental effect of this "abnormal" period were excluded. "Polycyclic aromatic hydrocarbons" in addition to the following search terms: "brain," "neurological," "cognitive," "cognition," "neurodegenerative," "neurodegeneration," "neurodevelopment," and "neurodevelopmental" were used to identify articles in both databases. Limitations applied to the search included only the fields "Title" and "Abstract" being searched. In Web of Science, the document type "Articles" was applied. In PubMed, an additional limitation of species, "Humans," was applied. Eligibility assessment was performed independently in an unbiased standardized manner by one reviewer. Ambiguity concerning the inclusion or exclusion of a study was resolved by a second reviewer being consulted and a consensus taken. Initial screening was performed by reviewing the title and abstract, after which, the full manuscript was reviewed.

Data collection process

A data extraction sheet was developed and pilot tested on five randomly selected included studies, before being refined accordingly. One review author extracted data from the included studies, a second was consulted where ambiguity arose surrounding the appropriate data to extract. One author was contacted through email to provide numerical data that had only been presented graphically. Information extracted from studies comprised sample size, sample characteristics, ratio between sexes, mean age, age range, comorbidities, air pollution component, time of exposure, air pollution data acquisition method, and outcome measure.

Risk of bias in individual studies

The risk of bias was assessed in line with the QUADAS[1] guidelines (University of Bristol, 2003). To ascertain the risk of bias within each study included, one reviewer working independently extracted the following information: participant inclusion/exclusion criteria explained, participant withdrawals from the study explained, use of/comparison with a control/low

1 QUADAS-2. URL http://www.bristol.ac.uk/population-health-sciences/projects/quadas/quadas-2/.

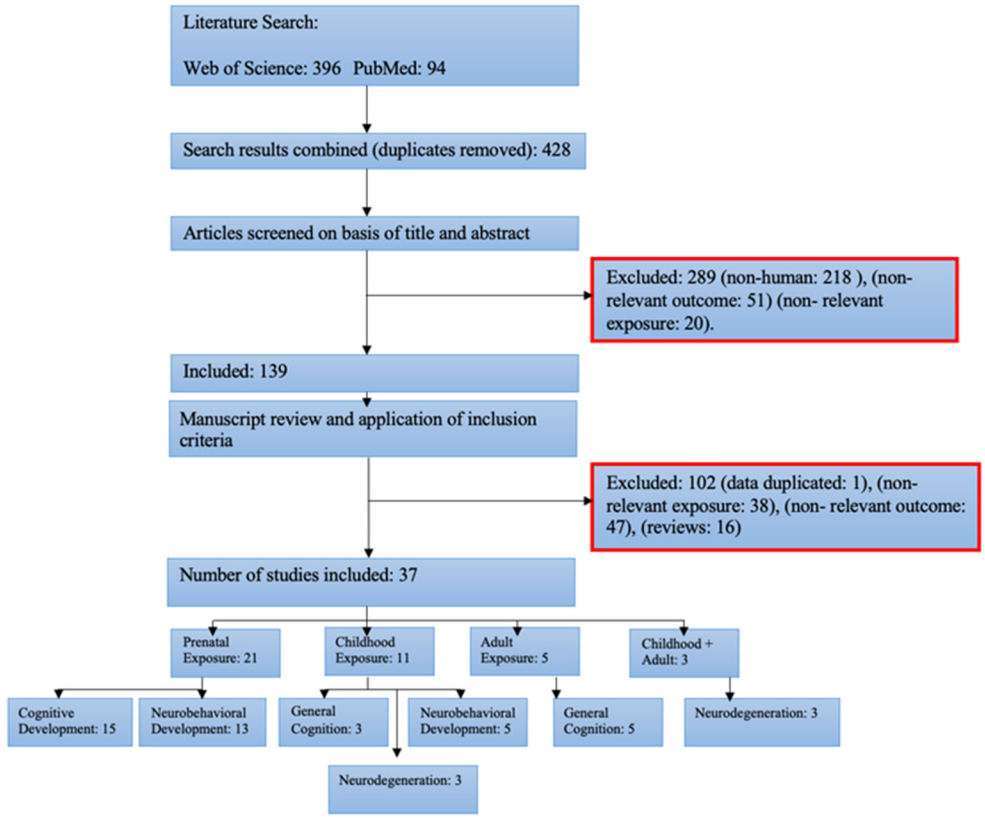

FIGURE 1
Flow chart of the search, study inclusion, and subgroup division.

exposure population, confounding variables identified, appropriate method/analysis to adjust for confounding variables, outcome assessors aware of exposure status of study participant, intermediate or unexpected results explained/reported, and whether or not the methods of the study were reproducible.

Methods of analysis

Studies included were divided into four subgroups depending on the time at which the exposure was measured: pre-natal, childhood, adult, and finally childhood + adult. The category childhood + adult included studies of young individuals from a wide age range, some exposed during childhood, and others where exposure extended through to adulthood, not analyzed separately. Subsequently, studies were further divided into categories depending on the outcome measured: cognitive abilities, neurobehavioral development, or neurodegeneration. This subgroup division was conducted to adjust for heterogeneity between studies. The meta-analysis was performed by extracting odds ratios and 95% confidence intervals (CI) for the effect sizes of reported outcomes or calculating them from the parameters and data given in the original publications using the Practical Meta-Analysis Effect Size Calculator by David B. Wilson, from (https://www.campbellcollaboration.org/escalc/html/EffectSizeCalculator-OR-main.php). Results were double-checked using the following online resources: (https://www.gigacalculator.com/calculators/odds-ratio-calculator.php) and effect size converter (https://www.escal.site/). Forest plots were used to visualize differences in effect sizes between studies within the same subgroup.

Results

Study selection

The search of Web of Science and PubMed provided a total of 490 citations. After adjusting for duplicates, 428 remained. Subsequent screening of the title and abstract resulted in a further 289 being discarded. Of the remaining 139, a further 102 were excluded upon further examination of the full manuscript

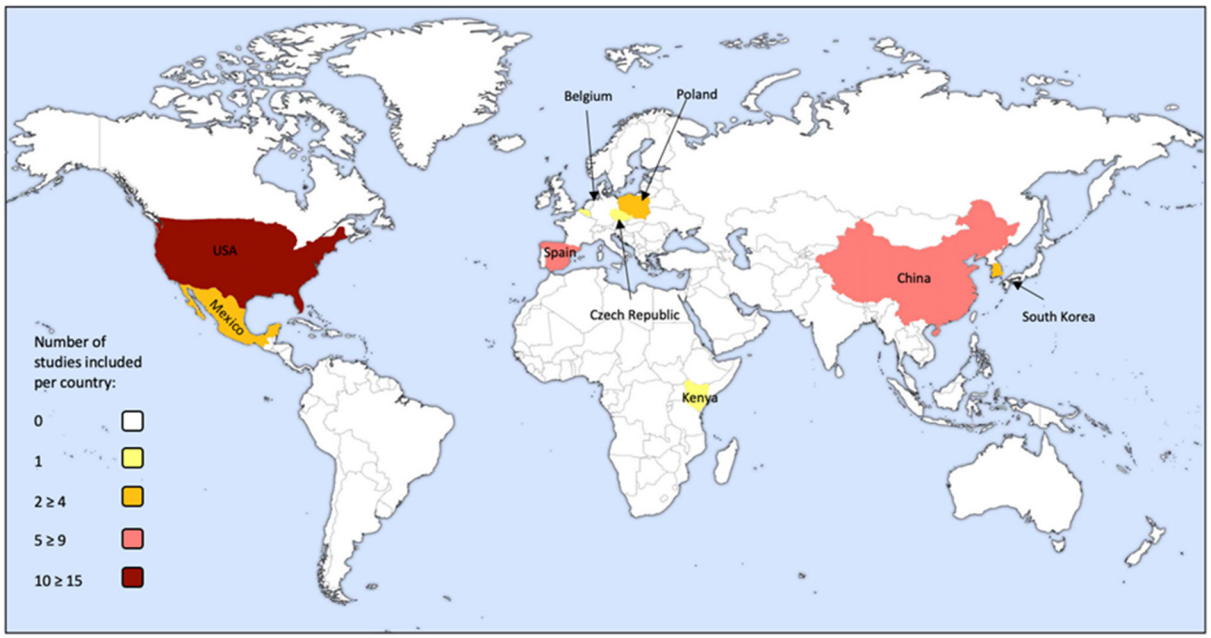

FIGURE 2
Global distribution of the population cohorts in each of the 37 studies included in this review (figure made using: Biorender.com).

and application of inclusion criteria. One study (26) reported data from a previous study (27). Sixteen were reviews and did not include any primary data, 47 reported outcomes not relevant to cognitive function or neurodegeneration, and in 38 the recorded exposure to PAH was not in keeping with the specified criteria, resulting in a total of 37 studies included in this review. Subgroup division resulted in 21 pre-natal exposure studies, 15 concerning cognitive development outcomes, and 13 on neurobehavioral development, seven included measures of both. From 11 childhood exposure studies, three were on general cognition, five on neurobehavioral development, and three on neurodegeneration. There were also five adult exposure studies all with outcomes of general cognition, and three childhood + adult studies all with measures of neurodegeneration. One study included measured outcomes for both pre-natal and childhood exposure (28) and two studies involved two different study cohorts: one exposed only during childhood and the other included a mix of childhood and adult-exposed subjects (29, 30). Figure 1 depicts the flow chart for study inclusion and subgroup division. The full dataset can be found at the following link: https://doi.org/10.7488/ds/3031.

Study characteristics

The 37 studies involved populations from nine countries (Figure 2). Study population characteristics including sample size and mean age (±SD) are displayed in Figure 3.

Eleven studies included cohorts from the USA. Six of them selected participants from the Columbia Center for Children's Environmental Health cohort; however, each study selected different subgroups of the population and measured different outcomes (31–36). Two studies selected a subgroup of participants from the National Health and Nutrition Examination Survey 2001–2002 (NHANES) (37), one of which included additional participants from the NHANES 2003–2004 cohort (38). The remaining three studies involved cohorts from the Childhood Autism Risks from Genetics and the Environment Study (28), the Adolescent Brain Cognitive Development Study (39), and the Asthma Coalition on Community, Environment and Social Stress project (40). Eight studies reported results from populations in China. One study involved a Taiyuan population (41) in addition to two selecting different subgroups from the Taiyuan Mother and Child Cohort Study (42, 43). Three involved populations were from Tongliang (27, 44, 45), and the remaining two were from Shanxi province (46) and Qingdao City (47).

Five studies involved populations from Spain. Two involved a subgroup from the Infancia y Medio Ambiente Project (48, 49) and three studies from the Brain Development and Air Pollution Ultrafine Particles in School Children project (50–52).

Four studies reported on populations in Poland, three including participants from the Krakow Study (53–55) and one from the Polish Mother and Child Cohort Study (56). Four studies reported on populations in Mexico. All involved Mexico City residents (57), where two refer to

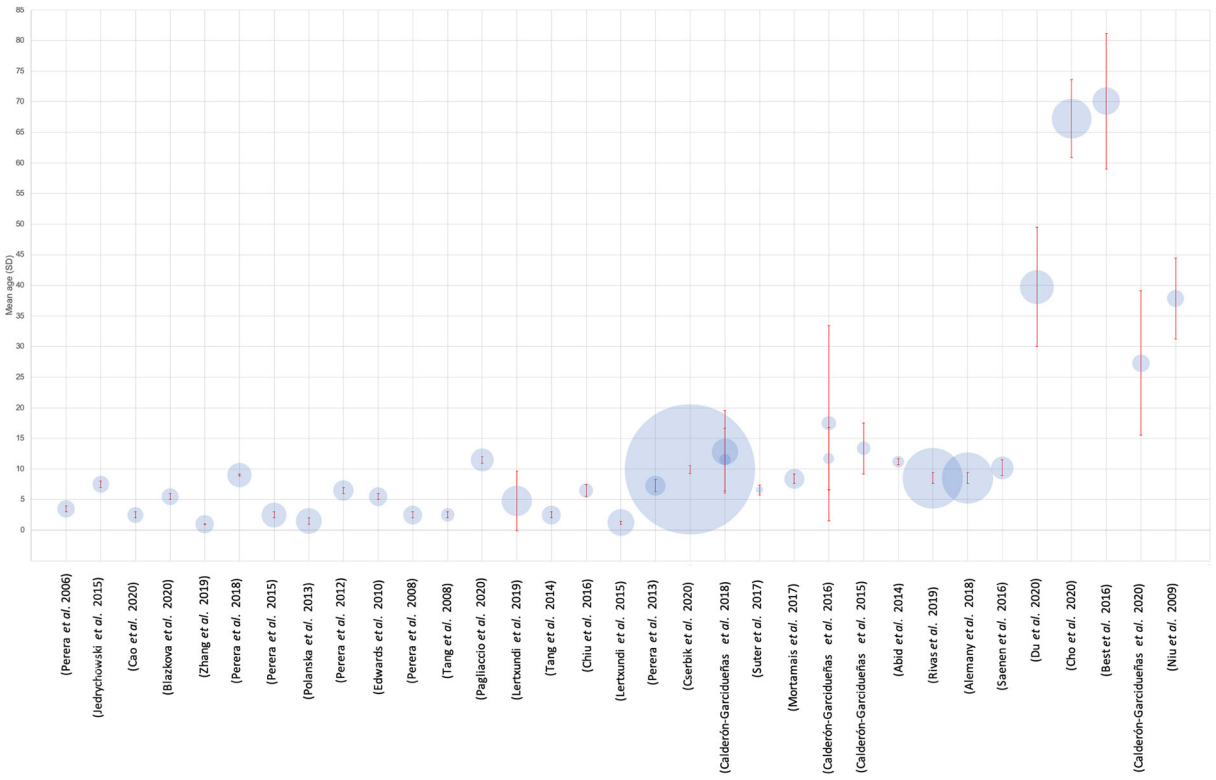

FIGURE 3
Characteristics of the study population involved in each study. Circle size is representative of the sample size. Red bars indicate mean age and SD. Four studies were omitted from this analysis due to insufficient data (28, 34, 43, 60).

six Mexican cities (29, 58), and another included details of three small Mexican cities (30). Two studies involved a Korean population (59, 60). Further individual studies included populations from the Czech Republic (61), Kenya (62), and Belgium (63).

Exposure assessment

Of the 37 studies included, seven measured exposure through environmental PAH sampling, five by environmental $PM_{2.5}$ sampling, seven by $PM_{2.5}$ spatiotemporal modeling, 10 by concentrations of PAH metabolites in urine, and eight using dosimetry to measure PAH-DNA adducts (Figure 4).

Outcome assessments

Outcomes included 21 different tests measuring cognitive function, nine different tests measuring neurobehavioral symptoms of impaired cognition, and three different measures of pathologies associated with neurodegeneration (Figure 5).

Pre-natal exposure

Association between pre-natal PAH exposure and cognitive abilities in childhood

Children with a high pre-natal PAH exposure were found to have a delay in overall child intelligence [OR = 1.75, 95% CI, 1.11–2.71) (54), mental development [OR = 0.65 (32)], and average overall development (27, 44) (OR = 0.84, 95% CI, 0.52–1.36; OR = 1.85, 95% CI, 1.13–3.01, respectively). Specifically, the greatest negative effects reported were in verbal IQ (OR = 3.45, 95% CI, 0.95–12.49) (53) and language (OR = 5.99, 95% CI, 1.88–19.02) (47). However, the latter could not be confirmed in five out of six studies (27, 42, 44, 45, 56). Two studies analyzed the effect of PAH on general cognitive abilities with contradictory results: one (31) reported a negative effect (OR = 2.89, 95% CI, 1.33–6.25) while another (56) reported no effect. PAH effect on impaired motor development was inconclusive, as confirmed by four studies (27, 42, 44, 45) (OR = 0.95, 95% CI, 0.58–1.53; OR = 1.91, 95% CI, 1.22–2.97; OR = 1.63, 95% CI, 1.00–2.65; OR = 1.82, 95% CI, 3.21–1.03, respectively), whereas three others could not confirm it. No association was found between PAH exposure and developmental motor ability (56),

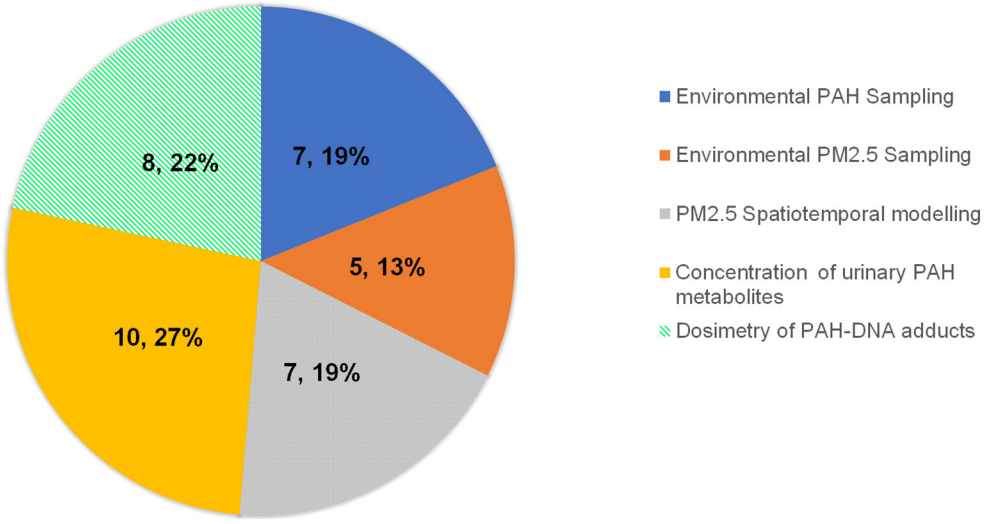

FIGURE 4
Pie chart representing the proportion of included studies measuring exposure to PAH as a measure of environmental PAH sampling, environmental $PM_{2.5}$ sampling, $PM_{2.5}$ spatiotemporal modeling, concentration of urinary PAH metabolites, and dosimetry of PAH-DNA adducts.

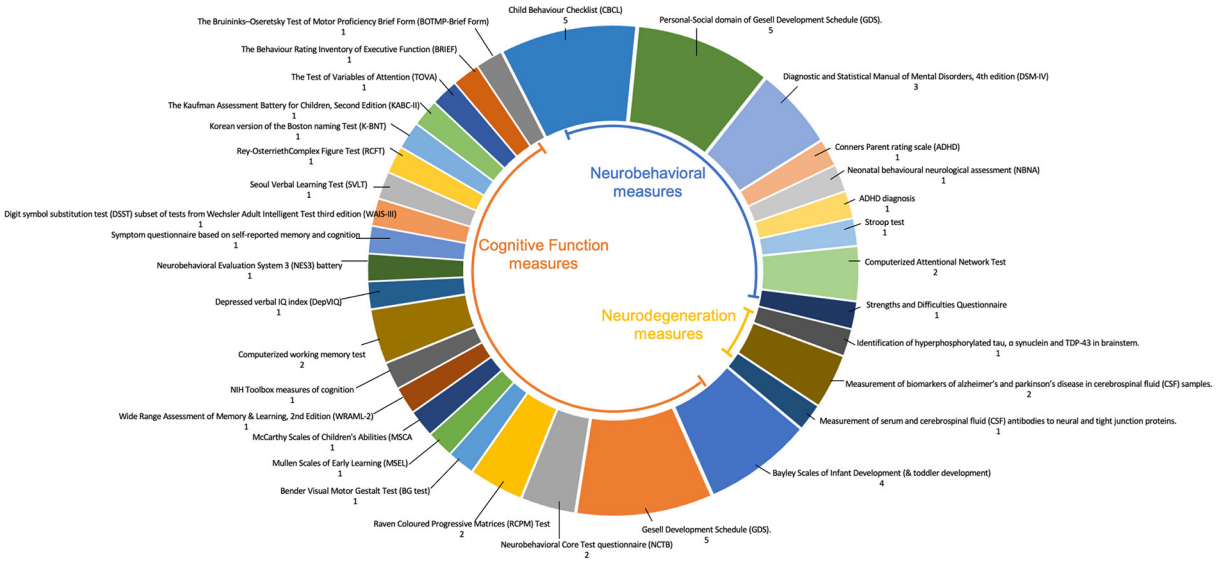

FIGURE 5
Pie chart representing the number of studies using different tests to measure outcomes. Measures include cognitive function, neurobehavioral symptoms of impaired cognition, and pathologies associated with neurodegeneration.

fine and gross motor abilities (47), and psychomotor abilities (31). Only one study reported the effect of PAH and reduced adaptive development (27) (OR = 1.77, 95% CI, 1.09–2.88) while four out of five studies reported no association with adaptive domains (42, 44, 45, 47). Size effects reported by the studies mentioned are graphically represented in Figure 6 and listed in Table 1.

Association between pre-natal PAH exposure and neurobehavioral development

Children with a high pre-natal PAH exposure were found to exhibit externalizing and internalizing behavioral problems (OR = 2.49, 95% CI, 1.57–3.95; OR = 2.39, 95% CI, 1.51–3.79, respectively) (55), and infants exhibited a decrease in behavioral development (OR = 2, 95% CI, 1.27–3.15) (43).

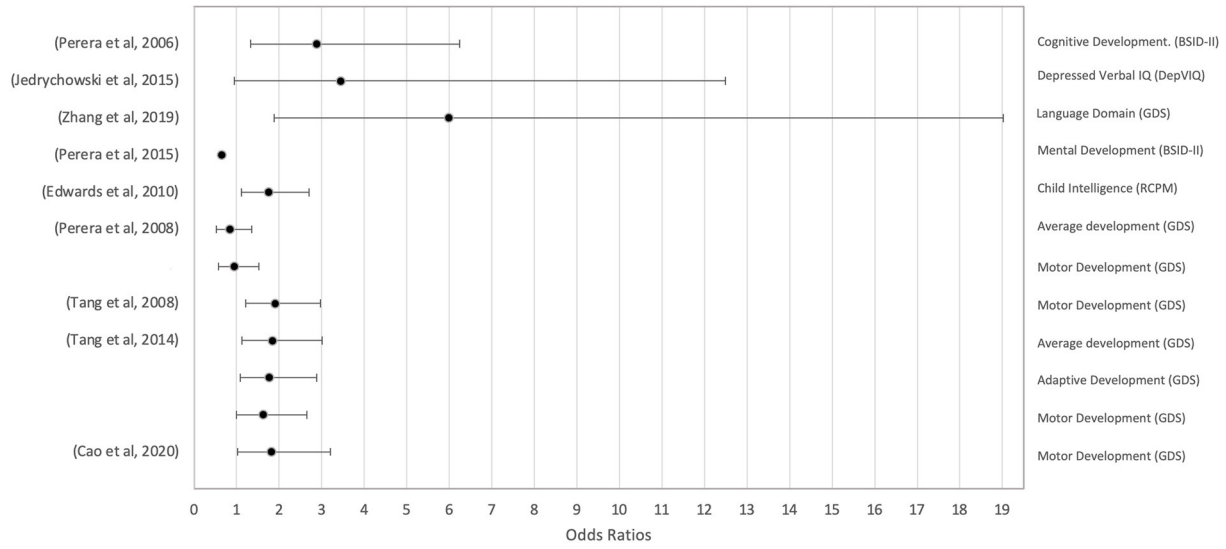

FIGURE 6
Forest plot of the calculated odds ratios (OR) and 95% confidence intervals (95% CI) for the association between pre-natal exposure to PAH and measures of cognitive development. Bayley Scales of Infant Development—revised (BSID-II), Depressed Verbal IQ Index (DepVIQ), Gesell Development Schedule (GDS), Raven Colored Progressive Matrices Test (RCPM), Wechsler Intelligence Scale for Children IV (WISC-IV). One study had insufficient data to calculate 95% CI (32).

Associations with anxious/depressed behavior were found in three out of four studies (33, 34, 55) (OR = 8.89, 95% CI, 1.7–46.51; OR = 8.14, 95% CI, 1.21–54.94; OR = 1.7, 95% CI, 1.08–2.68, respectively), with no association found by one study (36). Three out of five studies reported a negative effect on children's attentiveness (33, 35, 36) (OR = 1.34, 95% CI, 0.85–1.83; OR = 2.02, 95%, 1.35–3.03; OR = 3.79, 95% CI, 1.14–12.66) whilst two (34, 55) reported no effect. The report from one study (55) about the effect of both withdrawn/depressed and aggressive behavior (OR = 2, 95% CI, 1.27–3.16; OR = 2.29, 95% CI, 1.45–3.62, respectively) was contradicted by another study (36) that reported no effect for either. The latter (36) did, however, report the effect of impaired thought problems (OR = 1.95, 95% CI, 1.3–2.91) which was contradicted by the former (55). Only one out of seven studies reported an association between PAH and social problems (55) (OR = 1.57, 95% CI, 1.00–2.48), and the remaining six reported no effect (27, 36, 42, 44, 45, 47). Two studies (36, 55) found no effect on rule breaking behavior or somatic complaints. One study (35) reported no associations with attention deficit hyperactivity disorder (ADHD) index scores or hyperactive compulsive behavior, nor did another from the same research group (31) regarding total behavioral problems. Studies reporting neurobehavioral effects are reported in Table 2, and effect sizes are depicted in Figure 7.

Association between pre-natal $PM_{2.5}$ exposure and cognitive abilities and neurobehavioral development in childhood

A study (40) examined high $PM_{2.5}$ exposure during early, mid, and late pregnancy with measures of full-scale IQ score, inattentiveness, and adverse memory performance. Boys highly exposed during late pregnancy exhibit lower IQ and inattentiveness when exposure was from mid to late pregnancy. Girls highly exposed during early to mid pregnancy exhibited adverse memory performance. No effect was reported for the remaining domains analyzed by this study (40).

The finding of impaired motor development (48) was not supported by a subsequent study conducted by the same group (49), which reported, however, impaired memory in boys (49). From studies analyzing the impact of pre-natal $PM_{2.5}$ exposure on cognition and neurobehavioral development (Table 3), no effect was found on visual-motor functioning (61), general cognitive ability (28, 49), mental status (48), non-verbal intelligence (61), adaptive function or autism spectrum disorder (28), nor on verbal, perceptive manipulative, and numeric development (49).

TABLE 1 Studies with measured pre-natal PAH exposure on cognitive abilities in childhood.

References	Sample size	Sample characteristics	Male: female	Mean age (SD)	Age range	Comorbidities	Air pollution data acquisition method
Perera et al. (31)	183	Children 3 years of age, mothers 18–35 years, non-smoking, free of diabetes, hypertension, or known HIV, African American and Dominican women residing for a minimum of a year in Washington Heights, Harlem, or the South Bronx in New York City	84:99	3.5 (0.5)	3 years to 3 years 12 months	N/A	Environmental samples analyzed for 8 PAHs
Jedrychowski et al. (53)	170	Children 7 years of age, mothers ≥18 years of age, non-smoking, singleton pregnancies, no history of illicit drug use, pregnancy related diabetes, or hypertension, no current occupational exposure to PAH or any other known developmental toxicants, and have been resident in Krakow, Poland for a minimum of a year	80:90	7.5 (0.5)	7 years to 7 years 12 months	N/A	Cord blood PAH–DNA adduct
Zhang et al. (47)	211	Infants 12 months of age, free from delivery injuries, neonatal problems, acquired disabilities, developmental dysplasia or other developmental defects, mothers resident in Qingdao city, China for at least 3 years, free from diabetes, known HIV and known neuropsychiatric disease.	192:156	1.0 (0.083)	1 year to 1 year 1 month	N/A	Cord blood benzo(a)pyrene-DNA adducts (ng/mL)
Perera et al. (32)	380	Children 2 years of age, mothers 18–35 years, non-smoking, free of diabetes, hypertension, or known HIV, African American and Dominican women residing for a minimum of a year in Washington Heights, Harlem, or the South Bronx in New York City.	N/A	2.5 (0.5)	2 years to 2 years 12 months	N/A	PAH/aromatic DNA adducts in umbilical cord blood samples
Polanska et al. (56)	406	Children 1–2 years of age, mothers had single pregnancy up to 12 weeks of gestation, no assisted conception, no pregnancy complications, no chronic disease, resident in Poland	192:214	1.5 (0.5)	1 year to 2 years 12 months	N/A	1-hydroxypyrene metabolites in mothers' urine
Edwards et al. (54)	214	Children 5 years of age, mothers ≥18 years of age, non-smoking, singleton pregnancies, no history of illicit drug use, pregnancy related diabetes, or hypertension, no current occupational exposure to PAH or any other known developmental toxicants, and have been resident in Krakow, Poland for a minimum of a year	103:111	5.5 (0.5)	5 years to 5 years 12 months	N/A	Environmental samples analyzed for 8 PAHs
Perera et al. (44)	217	Children 2 years of age, born between either March to June 2002 or March to May 2002, mothers ≥20 years, non-smoking, resident within 2 km of Tongliang power plant	113:104	2.5 (0.5)	2 years to 2 years 12 months	N/A	Cord blood benzo(a)pyrene-DNA adducts (ng/mL)
Tang et al. (45)	110	Children 2 years of age, born between March and June 2002, mothers ≥20 years, non-smoking, resident within 2 km of Tongliang power plant	54:56	2.5 (0.5)	2 years to 2 years 12 months	N/A	Cord blood benzo(a)pyrene-DNA adducts (ng/mL)
Tang et al. (26)	215	Children 2 years of age, born between either March to June 2002 or March to May 2002, mothers ≥20 years, non-smoking, resident within 2 km of Tongliang power plant	106:109	2.5 (0.5)	2 years to 2 years 12 months	N/A	Cord blood benzo(a)pyrene-DNA adducts (ng/mL)

(Continued)

TABLE 1 (Continued)

References	Sample size	Sample characteristics	Male: female	Mean age (SD)	Age range	Comorbidities	Air pollution data acquisition method
Cao et al. (42)	158	Children 2 years of age, mothers ≥18 years of age, non-smoking, resident in Taiyuan, Shanxi province, China for a minimum of 1 year	82:76	2.5 (0.5)	2 years to 2 years 12 months	N/A	The sum of the maternal concentrations of eleven urinary PAHs metabolites Σ-OHPAHs

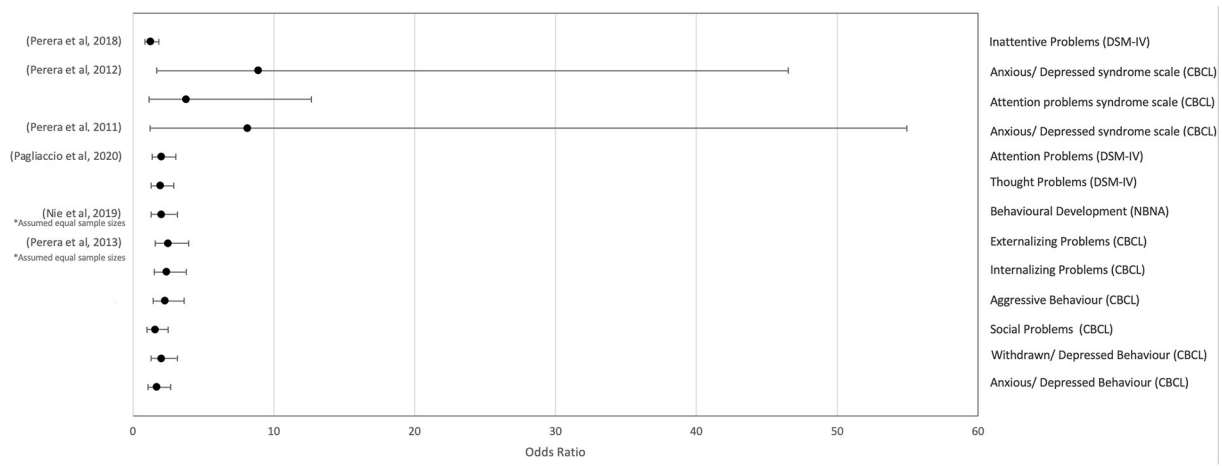

FIGURE 7
Forest plot of the calculated odds ratios (OR) and 95% confidence intervals (95% CI) for the association between pre-natal exposure to PAH and measures of neurobehavioral development. Gesell Development Schedule, Diagnostic (GDS) and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV), Child Behavior Checklist (CBCL), Neonatal Behavioral Neurological Assessment (NBNA). *Sample sizes were assumed equal.

Childhood

Association between childhood PAH exposure and cognitive abilities and neurobehavioral development

Children exposed to high levels of PAH post-natally exhibited lower general cognitive ability and delayed impaired memory (62). Increased inattentiveness was reported by two studies (50, 62), but this finding was contradicted by one study (51). The negative effect of post-natal PAH exposure was not observed in all memory domains. A study (50) found an association between impaired working numeric memory but not on working verbal memory. Short-term memory was not found affected either (62).

The association with ADHD diagnosis reported by one study (38) was not supported by two other studies (50, 51). Neither study found an effect on learning performance (38, 62) or an association with visual spatial skills, non-verbal test performance, executive function, motor performance (62), or behavioral problems (50). Studies reporting childhood PAH exposure can be found in Table 4.

Association between childhood $PM_{2.5}$ exposure and cognitive abilities and neurobehavioral development

From the four studies reporting post-natal $PM_{2.5}$ exposure on general cognition and neurobehavior (Table 5), one reported that children exposed to high levels of $PM_{2.5}$ post-natally displayed impaired selective and sustained attention (63), but this finding was contradicted by two studies (39, 52) reporting no effect on inattentiveness or attention and executive function, respectively. A study's report of impaired visual information processing speed (63) was again contradicted by another (39) reporting no association with processing speed. No association was found with working memory (39, 52), episodic memory, language (39), cognitive ability, adaptive function, or autism spectrum disorder (28).

Association between childhood $PM_{2.5}$ exposure and neurodegeneration

Only three studies by the same research group analyzed post-natal $PM_{2.5}$ exposure to neurodegeneration (Table 6). Two

TABLE 2 Studies with measured pre-natal PAH exposure on neurobehavioral development.

References	Sample size	Sample characteristics	Male: female	Mean age (SD)	Age range	Comorbidities	Air pollution data acquisition method
Perera et al. (35)	351	Children 9 years of age, mothers 18–35 years, non-smoking, free of diabetes, hypertension, or known HIV, African American and Dominican women residing for a minimum of a year in Washington Heights, Harlem, or the South Bronx in New York City.	163:188	9.01 (0.19)	9 years to 9 years 12 months	N/A	Cord blood benzo(a)pyrene-DNA adducts (ng/mL)
Perera et al. (33)	253	Children 6–7 years of age, mothers 18–35 years, non-smoking, free of diabetes, hypertension, or known HIV, African American and Dominican women residing for a minimum of a year in Washington Heights, Harlem, or the South Bronx in New York City.	131:122	6.5 (0.5)	6 years to 7 years 12 months	N/A	Environmental samples analyzed for 8 PAHs
Perera et al. (34)	215	Children 3 years 9 months to 5 years 11 months of age, mothers 18–35 years, non-smoking, free of diabetes, hypertension, or known HIV, African American and Dominican women residing for a minimum of a year in Washington Heights, Harlem, or the South Bronx in New York City.	87:128	4.8 (not reported)	3 years 9 months to 5 years 11 months	N/A	Cord blood benzo(a)pyrene-DNA adducts (ng/mL)
Pagliaccio et al. (36)	319	Children 11 years old, mothers 18–35 years, non-smoking, free of diabetes, hypertension, or known HIV, African American and Dominican women residing for a minimum of a year in Washington Heights, Harlem, or the South Bronx in New York City.	177:142	11.5 (0.5)	11 years to 11 years 12 months	Early life stress	Environmental samples analyzed for 8 PAHs
Nie et al. (43)	247	Infants 3 days of age, mothers ≥18 years, non-smoking, no chronic disease or family history of neurological disease, single gestational viable fetus, who delivered in the Sixth Hospital of Shanxi Medical University and the Eighth People's Hospital of Taiyua, resident in Taiyuan for at least a year	132:115	3 days (not reported)	3 days	N/A	Urinary metabolite concentrations of 2-hydroxyfluorene
Perera et al. (55)	248	Children from Krakow, Poland, mothers ≥18 years, non-smoking	122:126	7.28 (0.98)	6 years to 9 years 12 months	Maternal psychological distress	Personal air monitoring analyzing concentrations of 8 PAHs
Tang et al. (45)	110	Children 2 years of age, born between March to June 2002, mothers ≥20 years, non-smoking, resident within 2 km of Tongliang power plant	54:56	2.5 (0.5)	2 years to 2 years 12 months	N/A	Cord blood benzo(a)pyrene-DNA adducts (ng/mL)
Zhang et al. (47)	211	Infants 12 months of age, free from delivery injuries, neonatal problems, acquired disabilities, developmental dysplasia or other developmental defects, mothers resident in Qingdao city, China for at least 3 years, free from diabetes, known HIV, and known neuropsychiatric disease.	192:156	1.0 (0.083)	1 year to 1 year 1 month	N/A	Cord blood benzo(a)pyrene-DNA adducts (ng/mL)
Perera et al. (44)	217	Children 2 years of age, born between either March to June 2002 or March to May 2002, mothers ≥20 years, non-smoking, resident within 2 km of Tongliang power plant	113:104	2.5 (0.5)	2 years to 2 years 12 months	N/A	Cord blood benzo(a)pyrene-DNA adducts (ng/mL)

(Continued)

TABLE 2 (Continued)

References	Sample size	Sample characteristics	Male: female	Mean age (SD)	Age range	Comorbidities	Air pollution data acquisition method
Tang et al. (27)	215	Children 2 years of age, born between either: March to June 2002 or March to May 2002, mothers ≥20 years, non-smoking, resident within 2 km of Tongliang power plant	106:109	2.5 (0.5)	2 years to 2 years 12 months	N/A	Cord blood benzo(a)pyrene-DNA adducts (ng/mL)
Cao et al. (42)	158	Children 2 years of age, mothers ≥18 years of age, non-smoking, resident in Taiyuan, Shanxi province, China for a minimum of 1 year	82:76	2.5 (0.5)	2 years to 2 years 12 months	N/A	The sum of the maternal concentrations of 11 urinary PAHs metabolites Σ-OHPAHs
Perera et al. (31)	183	Children 3 years of age, mothers 18–35 years, non-smoking, free of diabetes, hypertension, or known HIV, African American and Dominican women residing for a minimum of a year in Washington Heights, Harlem, or the South Bronx in New York City	84:99	3.5 (0.5)	3 years to 3 years 12 months	N/A	Environmental samples analyzed for 8 PAHs

of them found that children highly exposed to $PM_{2.5}$ postnatally exhibited lower amyloid beta protein fragment 1–42 ($A\beta_{1-42}$) and brain-derived neurotrophic factor (BDNF) (29, 30), with one finding, in addition, higher interferon (IFN) γ concentrations in cerebrospinal fluid (CSF) (30). No effect was found with regard to concentrations of biomarkers: non-phosphorylated tau (non-p-tau), vitamin D, tau phosphorylated at threonine 181 (30), cellular prion protein, total tau, interleukin (IL) β, leptin (29, 30), total alpha- synuclein (α-synuclein), oligodendrocyte α-synuclein, hyperphosphorylated tau, tumor necrosis factor alpha, IL 2, IL 6, IL 10, or monocyte chemoattractant protein-1 (MCP-1) (29). Of 33 antibodies to neural and tight junction proteins, actin immunoglobulin G (IgG), occludin/zonulin (OZ) immunoglobulin A (IgA), OZ IgG, myelin oligodendrocyte glycoprotein (MOG) IgG, MOG immunoglobulin M (IgM), myelin basic protein (MBP) IgA, MBP IgG, astrocytic protein (S-100) IgG, S-100 IgM and cerebellar antigen (CEREB) IgG in serum, and MBP antibodies in CSF were higher in children exposed to high levels of PAH compared to controls (58).

There was one account of impaired cognitive disturbances (60), which was contradicted by two reports of no association with cognitive dysfunction (37, 59) and by additional individual accounts of no effect in approximate number system functioning (41) (i.e., digit span, digit symbol, number of dots tests), confrontational word retrieval, verbal fluency, delayed reaction time between congruent and incongruent stimuli, visual attention, and task switching (59). Mood state, attention/response speed, manual dexterity, or perceptual motor speed were not found associated with PAH exposure in adulthood (41, 46). However, it must be noted that two investigations were population studies (37, 59), while the other three (41, 46, 60) were occupational health studies on brain effects of PAHs in coke ovens (41, 46) or oil spill (60) workers, who are exposed to very high levels of PAHs, especially high molecular weight compounds including benzo(a)pyrene and other compounds with five to six or more hydrocarbon rings. A summary of the population characteristics of the five studies exploring adult PAH exposure and general cognition can be found in Table 7.

Adult

Association between adult PAH exposure and cognitive abilities

Adults highly exposed to PAH exhibited impaired auditory memory (41, 46), individual accounts of memory disturbances (60), and impaired verbal learning and memory (59). However, there was no effect on working memory and executive function, visuospatial memory/attention and planning (59), or visual perception memory (41, 46).

Childhood and adult

Association between childhood and adult $PM_{2.5}$ exposure and neurodegeneration

Details from the three studies reporting on cohorts inclusive of participants exposed to $PM_{2.5}$ only during childhood and some participants through to adulthood can be found in Table 8. In a cohort of mixed exposure to $PM_{2.5}$, the presence of neurodegenerative biomarkers phosphorylated tau (p-tau), α-synuclein, and transactive response DNA-binding protein 43

TABLE 3 Studies with measured pre-natal $PM_{2.5}$ exposure on cognitive abilities and neurobehavioral development in childhood.

References	Sample size	Sample characteristics	Male: female	Mean age (SD)	Age range	Comorbidities	Air pollution data acquisition method
Blazkova et al. (61)	169	Children 5 years of age, born in the summer 2013 to winter 2014, non-smoking mothers, resident in Karvina and Ceske Budejovice, Czech Republic	78:90	5.5 (0.5)	5 years to 5 years 12 months	Viral diseases otitis bronchitis GIS HCD	Analysis of 11 OH-PAHs in urine
Kerin et al. (28)	325	Children 2–5 years, resident in catchment area of 20 counties in northern California, the central valley and parts of Los Angeles metropolitan area, US, complete history of environmental air exposure, lived with at least 1 biological parent who speaks English or Spanish.	281:44	(not reported)	2 years to 5 years 12 months	N/A	Residential addresses inputted into Tele Atlas database and software
Lertxundi et al. (49)	560	Children 4 years male, mothers ≥16 years, resident in Valencia, Sabadell, and Gipuzkoa in Spain	560:00	4.8 (4.9)	4 years to 4 years 12 months	N/A	Land use regression models
Lertxundi et al. (48)	438	Children aged ∼15 months age, mothers ≥16 years, singleton pregnancies	198:240	1.25 (0.25)	1 year 1 month to 1 year 6 months	N/A	Environmental samples from digital DHA-80 high-volume aerosol samplers
Chiu et al. (40)	119	Mothers ≥18 years, at 28.4 ± 7.9 weeks gestation between August 2002 and January 2000 in Boston.	00:119	6.5 (0.98)	6 years to 7 years 3 months	N/A	Use of a hybrid satellite based spatio-temporal prediction model and residential address during pregnancy

(TDP-43) was confirmed in brainstems (57). The faster increase in concentrations with regard to the age of non-p-tau in CSF was also associated with increased exposure (30). However, no association was found with regard to the concentration of total and oligomer α-synuclein in CSF (29).

Risk of bias within studies

All studies included were of high quality with reproducible accounts of the method employed to assess relevant outcomes, and the inclusion/exclusion criteria used to select the study population were explained in sufficient detail (refer to detailed QUADAS tool responses in the publicly available data at https://datashare.ed.ac.uk/handle/10283/3892). Where applicable, all studies provided explanations for participant withdrawal, which were unrelated to both the exposure and the outcome being measured and reported intermediate or unexpected results. Approximately 54% of studies involved the use of a comparison with a low exposure or control population either by dichotomizing exposure data or using a demographically matched control population. The remaining 46% of studies assessed PAH exposure as a continuous variable. All studies correctly identified confounding variables, and the method and analysis were adjusted accordingly. There was, however, a considerable risk of information bias amongst studies, with only 16% of studies reporting the outcome assessor to be blinded and unaware of the exposure status of the study participant. Seventy-three percent of the studies provided no indication as to whether they were or not blinded, and in 11% the outcome assessors were confirmed not blinded.

Discussion

This review found sufficient evidence that pre-natal PAH exposure negatively impacts cognitive function with specific regard to child intelligence, mental development, verbal IQ, memory impairment, average overall development, child attentiveness, behavioral development, and externalizing, internalizing, anxious, and depressed behavioral problems.

Evidence concerning exposure during childhood and as an adult with cognitive function was insufficient to conduct a meta-analysis, due to a reduced number of studies, low consistency, and high heterogeneity in results. However, associations can be observed such as exposure during childhood with lowered cognitive ability, impaired child attentiveness, and exposure as an adult manifesting in memory disturbances

TABLE 4 Studies with measured childhood PAH exposure on cognitive abilities and neurobehavioral development.

References	Sample size	Sample characteristics	Male: female	Mean age (SD)	Age range	Comorbidities	Air pollution data acquisition method
Suter et al. (62)	31	Children aged 5–12 resident in Nairobi, Kenya, Infected with HIV and previously enrolled in the Optimizing HIV-1 Therapy Study	N/A	6.6 (0.8)	5 years to 12 years 12 months	HIV	Concentration of urinary PAH metabolite 1-hydroxypyrene (1-OHP)
Mortamais et al. (51)	242	Children 7–10 years, resident and enrolled in one of 40 schools in Barcelona, Spain, no dental braces	123:119	8.4 (0.8)	7 years to 10 years 12 months	N/A	Environmental air sampling
Abid et al. (38)	83	Children 6–15 years of age, part of a civilian population resident in the US	58:25	11.2 (0.5)	6 years to 15 years 12 months	N/A	Urinary metabolite concentrations of 2-napthol
Alemany et al. (50)	1589	Children aged 7–11, attending one of 38 schools in Barcelona, Spain, and one school in the adjacent municipality, Sant Cugat del Vallés	831: 758	8.52 (0.87)	7 years to 11 years 12 months	APOE e4 allele	Environmental samples analyzed for 7 PAHs

with specific regard to auditory memory and verbal learning and memory.

Studies concerning PAH exposure during childhood and as an adult were scarce, but an increased risk of neurodegeneration was found through the presence of neurodegenerative biomarkers and increased concentrations of cryptic "self" antigens in serum and CSF, indicative of the neuroinflammatory pathology which precedes Alzheimer's disease (AD) and Parkinson's disease (PD).

It is known that some pathways of aryl-hydrocarbon neurotoxicity are common for PAHs, TCDD, dioxin-like agents, polyphenols, and similar xenobiotics. A review of the neuropathological mechanisms of PAHs highlights that these, together with their metabolites, may cross the blood–brain barrier causing neurological abnormalities that may include neuronal damage, impaired neurotransmitter regulation, parasympathetic dysregulation, and neurodegeneration (65). Preclinical studies hint at a common neuropathological mechanism of PAH action being the binding of these compounds to the aryl-hydrocarbon receptor (AhR), a cytosolic transcription factor that initiates a complex pathway leading to alteration of gene regulation. AhR is also present in neural cells and can be involved in the mechanisms leading to PAH-induced neurological disorders (65).

This review differentially addressed the neurological impact of PAHs in three different domains, namely, cognitive abilities, neurobehavioral development, and neurodegeneration, and can be used as evidence for policy surrounding the monitoring of PAHs specifically. In addition, it raises awareness of the potentially confounding effect that different ambient PAH concentrations, in metropolitan and rural settings, can have on research assessing outcomes concerned with cognitive function and neurodegeneration in studies. It was not possible, however, to conclude on the differential impact of PAHs acquired mainly from outdoor sources from those acquired from indoor sources.

A previous review on the impact of $PM_{2.5}$ in disease incidence did not stratify patients by age nor considered differences between urban and rural areas, rather stratifying studies by the pollution level in which the country was considered (i.e., "lightly polluted" vs. "heavily polluted") (64). Other reviews have highlighted general adverse health conditions such as chronic asthma, increased incidence of premature death and hospital admissions (15), and kidney and liver damage (16). Some focused specifically on the carcinogenic properties and resulting incidence of the lung (17), urinary tract (18), and skin and gastrointestinal tract cancers (16). Those that focused on the neurological impact of air pollution concerned a diverse mixture of compounds. One specifically focused on non-communicable diseases and the roles of nitrogen dioxide (NO_2), nitrogen oxide species (NO_x), carbon monoxide (CO), and $PM_{2.5}$ (19). Another (20) raises awareness concerning ambient pollution's adverse effect on cognitive decline and impairment, concurring with findings from (22), where the emphasis was on ozone, $PM_{2.5}$, and PM_{10}. A study (21) reported NO_2, NO_x, black carbon, and PMs as potential risk factors for AD, PD, and multiple sclerosis. Despite the outcomes assessed being orientated toward neurological health, the exposures measured either include multiple pollutants or compounds NO_2, NO_x, CO, PM, or black carbon, around which extensive research already exists and has culminated in tight air quality restrictions and monitoring, which is closely adhered to by governing bodies. This review raises awareness of the neurological impact PAH

TABLE 5 Studies with measured childhood $PM_{2.5}$ exposure on cognitive abilities and neurobehavioral development.

References	Sample size	Sample characteristics	Male: female	Mean age (SD)	Age range	Comorbidities	Air pollution data acquisition method
Cserbik et al. (39)	10, 343	Children aged 9–10 years, resident in one of 21 study sites in the US	5,410: 4,933	9.93 (0.64)	9 years to 10 years and 12 months	N/A	Ensemble-based model approach combining aerosol optical depth models, land-use regression, and chemical transport models
Kerin et al. (28)	325	Children 2–5 years, resident in catchment area of 20 counties in northern California, the central valley and parts of Los Angeles metropolitan area, US, complete history of environmental air exposure, lived with at least one biological parent who speaks English or Spanish	281:44	(Not reported)	2 years to 5 years 12 months	N/A	Residential addresses inputted into Tele Atlas database and software
Rivas et al. (52)	2,221	Children 7–10 years old, attending one of 39 schools in Barcelona, Catalonia, Spain, without special needs	1,133: 1,088	8.5 (0.9)	7 years to 10 years 12 months	N/A	Land use regression models
Saenen et al. (63)	310	Children in grades 3–6 in three primary schools, Flanders, Belgium.	158: 152	10.2 (1.3)	N/A	N/A	Chronic exposure: spatial temporal interpolation method to model the daily residential exposure. Recent exposure (at schools): portable devices

has, independent of other pollutants, the importance of which is paramount with the current health impacts of PAHs in the UK air quality strategy detailed as "possibly" or "probably" carcinogenic, detracting from the seriousness of their impact on neurological human health (2). This review proceeds to categorize outcomes into subgroups depending on the time of exposure to provide further insight into the demographics of the individuals most vulnerable to the pollution levels reported and to differentiate between the areas of cognitive function and neurodegeneration most impacted, elucidating the potential mechanisms of neurotoxicity. The observation that the most profound effect of PAH exposure culminates from the pre-natal period is in keeping with prior research, showing the fetal brain to be more vulnerable to environmental toxic insult than the adult. The increased permeability of the not yet fully formed blood–brain barrier combined with the rapid brain growth during the second trimester means the period of most intense construction and brain architecture is also the time the brain is most vulnerable to the passage of toxins and neurotoxicity (66). Overall, this review has systematically located, summarized, and meta-analyzed evidence about the potent neurotoxicity of direct or indirect exposure to PAHs across the human lifespan, highlighting the need for more well-designed epidemiological studies.

Limitations

Studies included in the analysis were limited to those written in the English language. Publication bias and selective reporting within studies cannot be discarded, nor can indexing issues, in which the search terms may have failed to identify relevant studies.

The study populations included only originated from nine countries, of which the UK was not one. Findings are therefore limited to the environments and seasonal variations in climate found in these countries, and no specific recommendations for the UK, where the present review was conducted, can be made. The studies included also involved the use of different subgroup samples from the same large cohort, due to the necessity and availability of a limited number of longitudinal study databases. Sampling bias cannot, therefore, be disregarded.

Other polluting aryl-hydrocarbons present in the air, in particulate matter ($PM_{2.5}$) and diets, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and its congeners, dibenzofurans and dioxin-like polychlorinated biphenyls, have been reported to induce similar neurotoxicity and neurological disorders to PAHs. The concomitant exposure to these compounds, which are ubiquitously present as persistent organic pollutants, could have confounded the measured

TABLE 6 Studies with measured childhood $PM_{2.5}$ exposure on neurodegeneration.

References	Sample size	Sample characteristics	Male: female	Mean age (SD)	Age range	Comorbidities	Air pollution data acquisition method
Calderón-Garcidueñas et al. (30)	1) 426 2) 81	Children admitted to Mexico City hospital, resident in Mexico City Metropolitan area (MCMA) and other small cities in Mexico	1) 256:161 2) 44:33	1) 13.36 (8.82) 2) 11.54 (5.1)	(Not reported)	Lymphoblastic leukemia	Environmental air sampling, for regulating levels above the USEPA standards
Calderón-Garcidueñas et al. (22)	1) 73 2) 126	Children admitted to Mexico City hospital, resident in Mexico City Metropolitan area (MCMA) and other small cities in Mexico	1) 42:31 2) 59:70	1) 11.7 (5.14) 2) 17.49 (15.98)	(Not reported)	Lymphoblastic leukemia	Environmental air sampling
Calderón-Garcidueñas et al. (58)	111	Children within 5 miles of Mexico City Metropolitan Area (MCMA) or small control cities in Mexico (Zacatlán and Huachinango, Puebla; Zitaácuaro, Michoacaán; Puerto Escondido, Oaxaca; Chalma, Veracruz; Tlaxcala, Tlaxcala), No ETS exposure, lived within 5 miles of an air monitoring station	54:57	13.37 (4.2)	(Not reported)	N/A	Environmental air sampling

effects of PAHs reported in the studies reviewed. If the primary sources did not disentangle their effects, it is possible that some of the meta-analyzed results embody added effects of these aryl-hydrocarbons on ambient PAHs.

To adjust for heterogeneity, studies were stratified depending on the time point of exposure and outcome assessed; however, this did not account for heterogeneity between evaluators and instruments used, mainly due to the limited number of sources analyzed. In addition to this, the use of five different measures to quantify levels of PAH exposure, as well as the inclusion of quantification of $PM_{2.5}$ as a measure, resulted in heterogeneity in exposure measurement instruments and the inclusion of potential contaminating compounds within $PM_{2.5}$, which would confound results. Finally, there were insufficient data to calculate 95% CI for one study (32), and the request for numeric data from another study received no response (60); hence, the report of an effect on memory and cognitive disturbances was inferred from a figure with no confirmation from the raw data.

Future research

The role of biological sex in the neurotoxic effects of PAH exposure requires further investigation. Sex stratification of data concerning memory impairment in pre-natally exposed populations was contradictory. Further accounts of memory impairment following both childhood and adult exposure should be dichotomized to examine sensitivity between sexes. Pre-natal PAH exposure's effect on motor development was an area of controversy. Additional research is required in this domain to eliminate ambiguity. Individual reports of a lack of association with motor performance and perceptual motor speed, respectively, were inadequate to clarify such controversy or draw any conclusions.

In addition to this, a more thorough examination of the timescale of PAH exposure is needed, utilizing a smaller scale to determine critical windows.

Stratification by pregnancy term elucidated differential full-scale IQ, inattentiveness, and memory performance results. No effect on the concentration of non-p-tau in CSF was reported following childhood exposure, however when looked at in a mixed cohort of childhood and adult exposure, as association in relation to age progression was reported, indicative of a critical window of exposure.

Furthermore, gene-environment interactions need further analysis, for example PAHs effect on the brain of genetically susceptible populations, such as carriers of the APOE4 allele.

Repeated future analysis on longitudinal cohorts is required to examine the impact of sustained high PAH exposure or subsequent markedly reduced exposure, the effects such fluctuations can have on cognitive function and neurodegeneration, and whether some adverse effects from pre-natal or early life exposure are recoverable.

Future research should identify and analyze the individual contributions and specific synergistic combinations of PAHs on neurological health. This would differentiate and determine the most neurotoxic PAHs and provide evidence for updates in policy, requiring the monitoring

TABLE 7 Studies with measured adult PAH exposure on cognitive abilities.

References	Sample size	Sample characteristics	Male: female	Mean age (SD)	Age range	Comorbidities	Air pollution data acquisition method
Du et al. (46)	697	Employed at a coking plant in Shanxi province, China for minimum of 1 year	470:227	39.73 (9.74)	24–64 years 12 months	N/A	The sum of the concentrations of 11 urinary PAHs metabolites Σ-OHPAHs
Cho et al. (59)	949	≥50 year-old individuals, no known neurological diseases, resident in Seoul, Incheon, Wonju, and Pyeongchang, Republic of Korea.	421:528	67.24 (6.39)	≥50 years	Hypertension diabetes dyslipidemia angina myocardial infarction	Concentrations of urinary PAHs metabolites including: 1-hydroxypyrene
Ha et al. (60)	565	Volunteers in the Hebei Spirit oil spill, 2007, near the shore of Taean, Korea.	275:288	N/A	N/A	Asthma	1-hydroxypyrene and 2-naphthol metabolites in urine
Niu et al. (41)	176	Male 23–48-year-old coke oven workers Taiyuan, China, employed for a minimum of 1 year, middle school educated.	176:00	37.86 (6.61)	23 years to 48 years 12 months	N/A	Concentration of urinary PAH metabolite 1-hydroxypyrene (1-OHP)
Best et al. (37)	454	≥60-year-old individuals without known neurological diseases, resident in 15 randomly selected states in the US	221:233	70.1 (0.02)	≥ 60 years	Hypertension thyroid disease stroke kidney disease liver disease	The sum of the concentrations of eight urinary PAHs metabolites (Σ-OHPAHs)

TABLE 8 Studies with measured cohorts inclusive of childhood and adult $PM_{2.5}$ exposure on neurodegeneration.

References	Sample size	Sample characteristics	Male: female	Mean age (SD)	Age range	Comorbidities	Air pollution data acquisition method
Calderón-Garcidueñas et al. (57)	186	Metropolitan Mexico City residents, acute cause of death not involving the brain, autopsies were performed 3.7 ± 1.7 h after death, autopsy material examined between 2004 and 2008	162:186	27.29 (11.8)	11 months to 41 years	N/A	Ministry of environment of Mexico city monitoring stations
Calderón-Garcidueñas et al. (30)	1) 426 2) 81	Children admitted to Mexico City hospital, resident in Mexico City Metropolitan area (MCMA) and other small cities in Mexico	1) 256:161 2) 44:33	1) 13.36 (8.82) 2) 11.54 (5.1)	N/A	Lymphoblastic leukemia	Environmental air sampling, for regulating levels above the USEPA standards
Calderón-Garcidueñas et al. (22)	1) 73 2) 126	Children admitted to Mexico City hospital, resident in Mexico City Metropolitan area (MCMA) and other small cities in Mexico	1) 42:31 2) 59:70	1) 11.7 (5.14) 2) 17.49 (15.98)	N/A	Lymphoblastic leukemia	Environmental air sampling

of additional PAHs, rather than only B(a)P. Additional research into the threshold at which PAH is capable of exerting neurotoxic effects would inform policy, with scientific backing to implement a safe limit with regard to neurological health and update the limit of 0.25 $ng.m^{-3}$ B(a)P, which was set only with regard to carcinogenic properties. Furthermore, more studies are needed concerning populations in the UK, to account for the local environmental, climate, and seasonal variations capable of altering PAH's neurotoxic properties.

Data availability statement

The datasets presented in this study can be found in online repositories. The name of the repository and

accession number can be found below: Edinburgh DataShare, https://datashare.ed.ac.uk/, https://doi.org/10.7488/ds/3031.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Funding

JH was funded by the College of Medicine and Veterinary Medicine at The University of Edinburgh and MV was funded by The Row Fogo Charitable Trust (Grant No. BROD.FID3668413). The study was conducted at the Dementia Research Institute at the University of Edinburgh, which receives its funding from UK DRI Ltd. Company No. 11045257 | Charity No. 1179589, funded by the UK MRC, Alzheimer's Society and Alzheimer's Research UK. This study has been also supported by the EPSRC-funded project Lifecourse of Place: how environments throughout life can support healthy aging (Ref ES/T003669/1). None of the funding bodies mentioned were involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

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/fneur.2022.1052333/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Anthropogenic sources (industrial, mobile, domestic and agricultural sources) of PAH release into the environment (figure made using: Biorender.com).

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frontiers | Frontiers in Neurology

TYPE Systematic Review
PUBLISHED 11 January 2023
DOI 10.3389/fneur.2022.1065335

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Dementia and Neurodegenerative Diseases, a section of the journal Frontiers in Neurology

RECEIVED 09 October 2022
ACCEPTED 21 December 2022
PUBLISHED 11 January 2023

CITATION
Sáiz-Vazquez O, Puente-Martínez A, Pacheco-Bonrostro J and Ubillos-Landa S (2023) Blood pressure and Alzheimer's disease: A review of meta-analysis.
Front. Neurol. 13:1065335.
doi: 10.3389/fneur.2022.1065335

Blood pressure and Alzheimer's disease: A review of meta-analysis

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Background: Alzheimer's disease (AD) is a neurological disorder of unknown cause, resulting in the death of brain cells. Identifying some of the modifiable risk factors for AD could be crucial for primary prevention and could lead to a reduction in the incidence of AD.

Objective: This study aimed to perform a meta-meta-analysis of studies in order to assess the effect of blood pressure (BP) on the diagnosis of AD.

Method: The search was restricted to meta-analyses assessing high systolic BP (SBP) and diastolic BP (DBP) and AD. We applied the PRISMA guidelines.

Results: A total of 214 studies were identified from major databases. Finally, five meta-analyses (52 studies) were analyzed in this review. Results confirm that high SBP is associated with AD. The exploration of parameters (sex, age, study design, region, and BP measurements) shows that only region significantly moderates the relationship between BP and AD. Asian people are those whose SBP levels >140 mmHg are associated with AD. BP is associated with AD in both people aged ≤65 years and those aged ≥65 years and in cross-sectional and longitudinal studies. In the case of DBP, only women are at a higher risk of AD, particularly when its levels are >90.

Conclusion: SBP is associated with both cerebrovascular disease and AD. Therefore, future studies should use other uncontrolled factors, such as cardiovascular diseases, diabetes, and stroke, to explain the relationship between SBP and AD.

KEYWORDS

Alzheimer's disease, blood pressure, systo-diastolic hypertension, risk factor, meta-analysis

1. Introduction

There are 55 million people affected by dementia worldwide (1). Alzheimer's disease (AD) is the most common cause of dementia, accounting for up to 75% of all dementia cases (2). The prevalence of AD increases every year in individuals between the ages of 65 and 85 years (3), and by the year 2050, the worldwide prevalence of AD will grow four-folds,

to 106.8 million (range 47.2–221.2) (4). While between the ages of 65 and 74 years, about 10% of people have AD, and in those over 85 years old, the risk increases by 50% (3). According to estimates by the World Health Organization (WHO), the projected global prevalence of AD by 2050 will increase by 110% from 2010 (5).

Alzheimer's disease is a neurological disorder of unknown cause, resulting in the death of brain cells (3). AD is the most common cause of cognitive impairment (6). AD is characterized by hallmark pathological changes such as extracellular Aβ plaques and intracellular neurofibrillary pathology, which selectively affect specific subclasses of neurons and brain circuits. While dementia is a general term, Alzheimer's disease is a specific brain disease. It is marked by symptoms of dementia that gradually get worse over time (7). Dementia is a rather broad syndrome of global cognitive decline. However, AD first affects the part of the brain associated with specific cognitive functions, such as language (aphasia), motor skills (apraxia), and perception (agnosia) (8, 9). Moreover, in AD, early symptoms often include changes in memory, thinking, and reasoning skills (10).

Some of the first symptoms that occur with AD (neuropsychiatric) are a direct cause of early institutionalization (11). In AD, there is an identity loss (12) and worsening in the physical and social areas (11), along with the progressive deterioration of basic cognitive (episodic memory, linguistic, and spatial orienting) and executive functions (inhibitory abilities and the visuospatial functions) (13). Emotional and mental health problems (e.g., delusions and hallucinations, abnormal behaviors, or physical violence and hitting) are common, cause distress to caregivers, and may be amenable to treatment (14, 15). All these symptoms affect the quality of life and activities of daily living in individuals diagnosed with this disease (15).

The most important non-modifiable risk factor for developing AD is age. Many cardiovascular risk factors increase with age, such as high blood pressure (BP), which, in turn, could affect the mechanisms that lead to impairment in the brain (16).

According to Ballard et al. (17), the development of dementia is associated with not only genetic factors but also acquired factors (i.e., hypertension) that could predict a higher risk of AD. In this study, we particularly focused on analyzing high BP as a risk factor for the development of AD (18, 19). The overall prevalence of high BP in adults is 25%, with more than 50% of those individuals over 60 years (20). Vascular risk factors like BP could change the anatomy of the human body by modifying vascular walls or causing ischemia and cerebral hypoxia, which may consequently lead to the development of AD (21). Furthermore, BP could generate dysfunction in the blood–brain barrier, which has been associated with the genesis of AD (22). Studies on the relationship between BP and AD have yielded inconsistent results, showing an association between AD and high BP, or no significant association between these variables (23–25). For example, Mielke found that systolic hypertension was associated with an increased risk of AD. However, the authors did not find an association between diastolic hypertension and AD (22).

Findings also established that the association between AD and hypertension was determined by age of onset (early-onset AD $\leq$ 65 years and late-onset AD $\geq$ 65 years). In fact, AD has been classified as presenile or early onset ($\leq$65 years) and as senile or late onset ($\geq$65 years) that tend to be sporadic and slow moving (26). However, it is still not clear in the current literature whether age moderates the relationship between BP and AD. Indeed, some researchers have indicated that elevated BP occurring in either middle age or late life may be involved in the development of AD (23, 27, 28). Also, one study concluded that high systolic BP (SBP) and diastolic BP (DBP) were related to worse cognitive function for persons aged 65–74 years. However, in older age ($\geq$75), higher SBP and DBP were related to adequate cognitive function (29).

Other studies have studied the relationship between hypertension and gender. Gillis and Sullivan (30) concluded that women are more likely to be prehypertensive than men. Furthermore, Anstey et al. (31) concluded that hypertension in middle-aged women was associated with greater cognitive impairment and AD. However, recent studies have shown that the prevalence of hypertension is higher in men before the sixth decade of life, although it increases in women after menopause (32).

Related to regions due to the high incidence of hypertension in developed countries, studies are aimed at prevention strategies (33, 34). In addition, the earlier onset and more aggressive development of AD in the young population have been identified as risk factors for hypertension in these countries (35).

The literature refers to various degrees of hypertension. This study was based on the cutoff points established by the International Society of Hypertension (ISH) (36). On the one hand, the ISH establishes the following measures for SBP: elevated (130–139 mmHg), grade 1 (140–159 mmHg), and grade 2 (160–179 mmHg). On the other hand, there are also three cutoff measurements for DBP: elevated (85–89 mmHg), grade 1 (90–99 mmHg), and grade 2 (100–109 mmHg) (36, 37). Mielke et al. (38) concluded that SBP measurements greater than 160 mmHg were associated with greater cognitive impairment in the elderly, which may lead to AD. Similarly, according to Launer et al. (23), elevated midlife SBP $>$ 160 mmHg and DBP $\geq$ 90 mmHg were particularly associated with an increased risk of AD.

Furthermore, longitudinal (39, 40) and cross-sectional (41, 42) studies have been used to identify risk factors and elucidate some characteristics of AD. To this end, we aggregated data from longitudinal and cross-sectional studies and used meta-analytic equation modeling to test for causal relationships. One major advantage of meta-analytic equations is that it allows an integration of the given data from all studies into one model

and specify models that have not been tested in the primary studies (43).

Based on the results and evidence of other articles and meta-analyses, we aimed to perform a meta-meta-analysis of longitudinal and cross-sectional studies to test the association between BP (high SBP and high DBP) and the risk of AD. We also aimed to pool findings separately from cross-sectional and longitudinal studies and assess the effect of BP on the risk of subsequent diagnosis of AD.

2. Materials and methods

2.1. Data collection

The search was restricted to meta-analyses assessing high SBP and DBP and AD. We applied the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (44). The literature searches were carried out in five electronic databases, including ISI Web of Science, Scopus, PubMed, Elsevier Science Direct, and Google Scholar. No publication date was set. The list of keywords was generated through a system of successive approximations: "blood pressure" and "Alzheimer's disease" and "meta-analysis." A Google Scholar search was also performed but was limited to the title. The literature search was carried out in English and Spanish.

2.2. Inclusion criteria

The procedures applied to carry out this meta-meta-analysis were as follows: (1) search and selection of meta-analyses assessing high SBP and DBP and AD and (2) selection of primary studies contained in the meta-analyses and the deletion of duplicates.

Meta-analyses and primary studies that met each of the following criteria were selected: (1) meta-analysis and primary studies that measured the relationship between hypertension (high SBP and DBP) and the risk of AD; (2) meta-analysis and primary studies reported data that allowed the estimation of a pooled effect size; (3) meta-analysis and primary studies that diagnosed AD through clinical examination, using defined diagnostic criteria, DSMV (9) and NINCDS-ADRDA (45); (4) meta-analysis and primary studies that reported the sample size; and (5) meta-analysis and primary studies written in English or Spanish.

To avoid bias in eligible studies, all abstracts were independently reviewed by two investigators (O.S. and A.P.). After excluding all irrelevant abstracts, the remaining articles were analyzed, and data precision was examined in detail. In meta-analysis where relevant data were lacking ($k = 1$), the authors were contacted to request additional data to be subsequently added to the meta-analysis. Then, duplicate reports were excluded to pool the primary studies. After all meta-analyses and primary studies were selected, a third researcher independently extracted the highlighted data (S.U.). Information on all data collected from the primary studies included in the meta-analysis is presented in the Supplementary Table 1.

2.3. Quality assessment

The qualities of the meta-analyses were independently coded by two co-authors using the 11-item Assessment of Multiple Systematic Reviews (AMSTAR) tool (46), which has been shown to have a good inter-rater agreement, reliability, and content validity (46, 47). Total scores for the meta-analyses were calculated as the sum of the 11 items on a binary scale. Quality classifications were set as low quality (0–4), moderate quality (5–8), and high quality (9–11).

2.4. Statistical analysis

Initially, we reported the associations between hypertension and AD for each primary study included in the previous meta-analysis (see Supplementary material).

Then, for this review of meta-analyses, first, we calculated the cumulative incidence ratio [or log risk ratio (LnRR)] of AD for both SBP and DBP for each primary study. Second, we identified separate effect sizes for SBP and DBP measurements and their relationships with the risk of AD. Third, study outcomes were grouped according to the definition of BP (SBP or DBP) and the measurement of hypertension established by the ISH: (1) SBP: elevated (130–139 mmHg), grade 1 (140–159 mmHg), and grade 2 (160–179 mmHg), and (2) DBP: elevated (85–89 mmHg), grade 1 (90–99 mmHg), and grade 2 (100–109 mmHg) (36, 37). Heterogeneity between study samples was assessed using Cochran's Q statistic (48). The I^2 statistic was calculated to express the fraction of variation between studies that was due to heterogeneity. The I^2 statistic explains the percentage of variance in the observed effects due to variance in the true effects. An I^2 value $<25\%$ was considered low heterogeneity, between 25 and 50% was considered moderate heterogeneity, and $>50\%$ was considered high heterogeneity (48). Statistical significance was set at $p \leq 0.05$. Data were analyzed using Comprehensive Meta-Analysis version 3.1 (Biostat Inc, NJ, USA) (49). Additionally, to test for the possibility of publication bias, we computed the Egger regression test. Results revealed no evidence for a publication bias (50).

For each primary study included in the meta-analysis, we calculated the following (see Table 1): (a) k or number of studies, (b) effect size, (c) 95% confidence interval (95%CI)

TABLE 1 Characteristics of the population of the AD and BP studies.

References	Variable[a]	Design[b]	*K*[c]	Regions (*N*)[d]	Sample[e]	% F[f]	Age[g]	SBP/DBP[h] measure/ mmHg	Results	Effect size[i]			AMSTAR[j] scores
										Effect size (RR)	95 % CI LLIC∼ULIC	*p*	
Lennon et al. (22)	SBP	L (13–22)	6	EU (2), NA (2), AS (2)	AD *n* = 2,208	47.3	M = 56.87	>140 mmHg	> SBP > AD	1.18	1.02–1.35	0.021	10
					HC *n* = 852,683			>160 mmHg	> SBP > AD	1.25	1.06–1.47	0.006	
								>90 mmHg	> DBP > AD[k]				
Xu et al. (51)	SBP	L (1–21)	39	EU (15), NA (20), AS (8), AF (1),	AD *n* = 21,359	50.5	M = 71.8	>140 mmHg	> SBP > AD	0.87	0.70–1.0	0.000	10
					HC *n* = 1,421,593								
	DBP		5		AD *n* = 743			>90 mmHg	> DBP = AD	1.14	0.89–1.39	0.028	
					HC *n* = 11,653								
Meng et al. (52)	SBP	L (10)	1	EU (1)	AD *n* = 79	100	*M* = 45	>140 mmHg	>SBP > AD	1.77	0.93–3.37	0.082	10
					HC *n* = 707								
Guan et al. (53)	SBP	L (2–27)	4	EU (2), NA (1), AS (1)	AD *n* = 176	56.3	40–92	>160 mmHg	>SBP and DBP =AD	1.01	0.87–1.18	0.850	9
	DBP				HC *n* = 7,283			>85 mmHg					
Wang et al. (54)	SBP	T	2	EU (1), NA (1)	AD *n* = 385	39	<65	>140 mmHg	>SBP = AD	1.50	0.56–4.04	0.036	10
					HC *n* = 3,626			>160 mmHg					
							≥65	>160 mmHg	>SBP = AD	1.00	0.79–1.25	0.180	
							65–75	>160 mmHg	>SBP = AD	1.01	0.66–1.53	0.215	
							75–85	>160 mmHg	>SBP > AD	1.07	0.63–1.82	0.052	
	DBP		2	EU (1), NA (1)	AD *n* = 385		<65	>90 mmHg	–	1.70	0.80–3.60	–	

(Continued)

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TABLE 1 (Continued)

References	Variable[a]	Design[b]	K[c]	Regions (N)[d]	Sample[e]	% F[f]	Age[g]	SBP/DBP[h] measure/ mmHg	Results	Effect size[i]			AMSTAR[j] scores
										Effect size (RR)	95 % CI LLIC~ULIC	*p*	
					HC $n = 3626$		≥65	>90 mmHg	>DBP = AD	0.75	0.43–1.32	0.066	
							65–75	>85 mmHg	>DBP = AD	0.71	0.30–1.67	0.616	
							75–85	>90 mmHg	>DBP = AD	0.52	0.32–0.85	0.267	

[a]Variable: SBP, systolic blood pressure; DBP, diastolic blood pressure.
[b]Design: T, cross-sectional; L, longitudinal.
[c]K: Number of studies.
[d]Regions: N, number of independent studies; EU, European Union; NA, North America; AS, Asia; AF, Africa.
[e]Sample: AD, participants with Alzheimer's disease; HC, health control participants.
[f]%F: percentage of women.
[g]M, mean of age.
[h]Study outcomes were grouped according to the measurement of hypertension: (1) SBP > 140 mmHg and >160 mmHg, (2) DBP > 85 mmHg and 90 mmHg [reference guides: (36, 37)].
[i]CI: 95% confidence interval; RR: risk ratio.
[j]AMSTAR, Assessing the Methodological Quality of Systematic Reviews. https://amstar.ca/Amstar_Checklist.php.
[k]Given that two studies used odds ratios and the others hazard ratios, the authors could not compute summary estimates.

of the effect, and (d) *p* (two-tailed significance) (55). We used a random-effect model for the calculation of pooled effect estimates. Then, to assess the heterogeneity of our results, subgroup analyses were performed to examine the differential effects of type of BP: (1) SBP, (2) DBP, and (3) BP (total) on the risk of AD. We did not assume a common among-study variance component across subgroups. High-resolution forest plots were also developed separately with random effects.

Additionally, moderating variables were selected based on substantive considerations and the availability of data across studies included in the meta-analysis. We anticipated interstudy heterogeneity as there was some variation between studies according to the study design (longitudinal *k effect size* = 29 vs. cross-sectional *k effect size* = 46) and the measures of SBP (>140 mmHg *k effect size* = 52 and >160 mmHg *k effect size* = 8) and DBP (>85 mmHg *k effect size* = 2 and >90 mmHg *k effect size* = 9). Finally, we also considered whether age at exposure assessment (early age of onset ≤65 *k effect size* = 39 vs. late age of onset or ≥65 *k effect size* = 36) could account for heterogeneity in associations. When possible, we used separate summary measures for early- and late-life measures of BP. Otherwise, BP in early life or late life was defined according to the mean of age. Moreover, we also analyzed the sex (male or female) in the different BP measurements. In the same line, we also analyzed the continent where the sample was recruited (Europe, Asia, and North America) in the different BP measurements.

3. Results

A total of 214 studies were identified from major databases: 61 in ISI Web of Science, 55 in Scopus, 17 in PubMed, 79 in Elsevier Science Direct, and 2 in Google Scholar. In total, 189 articles were excluded from this review for various reasons: (a) $k = 89$ were duplicates and (b) $k = 100$, in which no information was provided on the relationship between BP and AD.

A total of 25 meta-analyses were eligible for inclusion in this review of meta-analyses. Of these meta-analyses, 20 were excluded: (a) $k = 14$ studies were duplicated data; (b) $k = 2$ were systematic reviews about other issues; (c) $k = 2$ aimed to study the effect of antihypertensives on AD; and (d) $k = 2$ aimed to study genetic factors (Figure 1).

Table 1 summarizes key features of the included primary diagnosis, design, number of primary studies, regions of origin of the study, sample size, gender, mean age, results, effect sizes of the relationships between BP and AD, and AMSTAR scores. Although the meta-meta-analyses were based on the criteria established by ISH, the studies only showed values for the following cutoff points: SBP (>140 mmHg and >160 mmHg) and DBP (>85 mmHg and >90 mmHg). Eggers' test was not

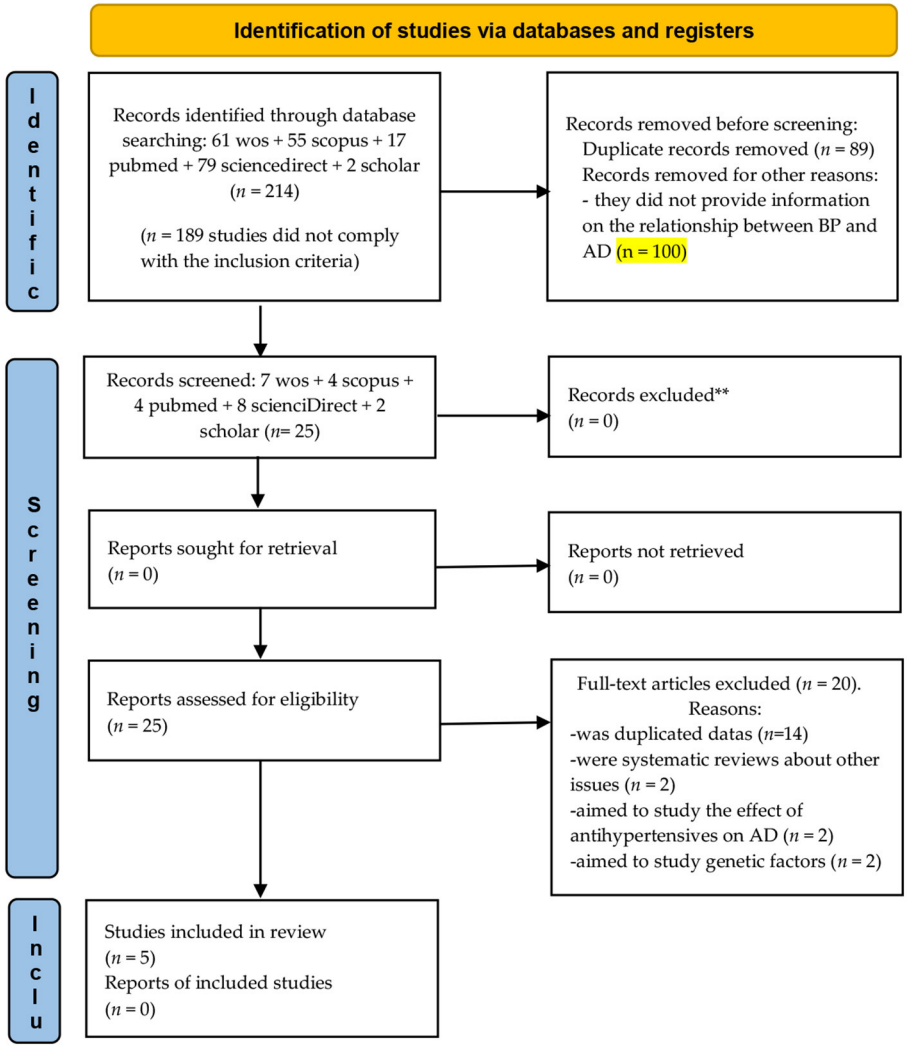

FIGURE 1
Flowchart depicting the selection of articles for our meta-analysis. From Page et al. (56).

significant: the intercept (B0) is 0.47, Se = 0.28, 95%CI (−0.09, 1.04), with $t = 1.65$, df = 73, indicating no publication bias.

3.1. BP and AD: Heterogeneity analysis

A total of 75 effect sizes were extracted from a total of five meta-analyses that included $k = 52$ primary studies. Also, 60 effect sizes provided information about high SBP and risk of AD (80%); $k = 11$ about high DBP (14.7%); and $k = 4$ about the combined effect (5.3%) (Supplementary Table 1).

For the pooling LnRR analysis, we analyzed primary studies. The total effect size was LnRR = 0.07, Se = 0.02 (0.031, 0.125), Z = 3.27, $p = 0.001$, and heterogeneity was high (Qb = 415.56, df = 74, $p = 0.0000$; $I^2 = 82.19$). These findings suggest that heterogeneity of effect may be present in some analyses.

3.2. Systolic blood pressure and AD

Four meta-analyses examined the relationship between high SBP and AD. The meta-analyses carried out by Lennon et al. (22) ($k = 11$ effect sizes; $N = 7{,}666$; $n = 1{,}520$ participants with AD and high SBP; $nHC = 6{,}146$ HC participants), Xu et al. (51) ($k = 40$ effect sizes; $N = 1{,}443{,}213$; $n = 17{,}113$ participants with AD and high SBP; $n = 1{,}426{,}100$ HC participants), Meng et al. (52) ($k = 1$ effect size; $N = 786$; $n = 79$ participants with AD and high SBP; $n = 707$ HC participants), and Wang et al. (54) ($k = 8$ effect sizes; $N = 5{,}885$; $n = 385$ participants with AD and high SBP; $n = 5{,}500$ HC participants) compared HC and AD subjects with high SBP. Only two of them (22, 52) found significant associations between high SBP and the risk of AD (Figures 2–4).

The total random effect of the high SBP value was $k = 60$ effect sizes; $N = 1{,}457{,}550$ participants; $nAD = 19{,}097$

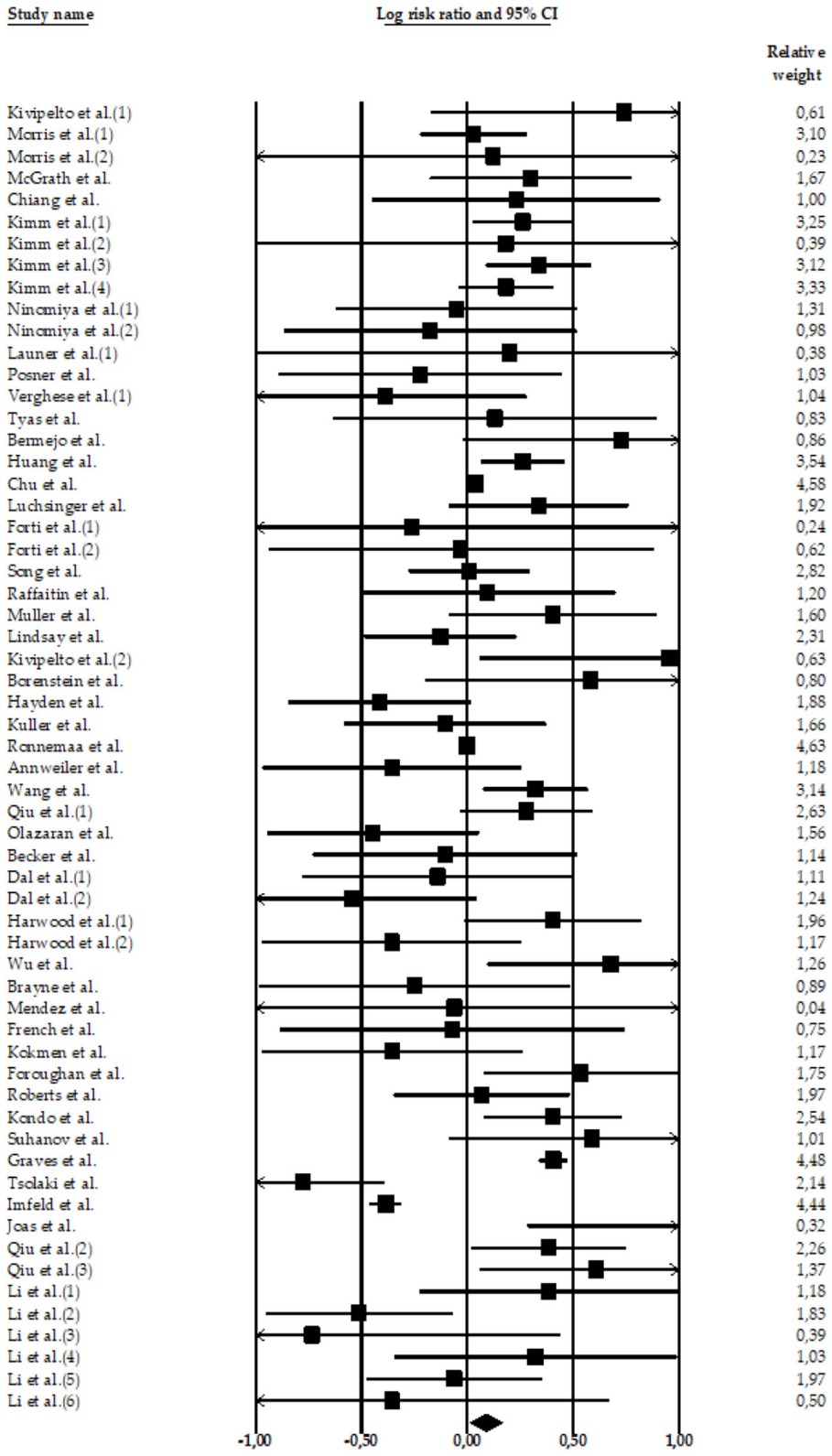

FIGURE 2
Forest plot of the meta-analysis of incidence rates of AD in participants with high SBP. Individual and pooled estimates of the association between measures of hypertension and AD. The size of the box representing the point estimate for each study in the forest plot is proportional to the contributing weight of that study estimate to the summary estimate.

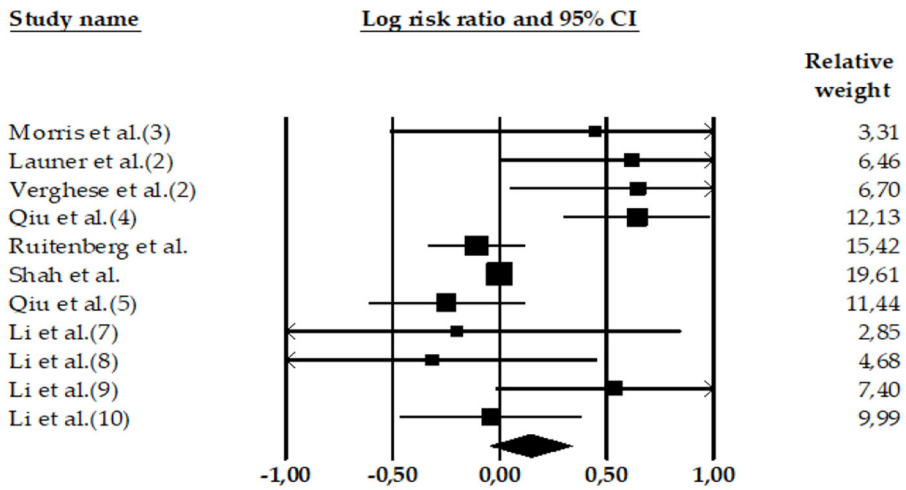

FIGURE 3
Forest plot of the meta-analysis of incidence rates of AD in participants with high DBP. Individual and pooled effect estimates of the association between DBP hypertension and AD. The size of the box representing the point estimate for each study in the forest plot is proportional to the contributing weight of that study estimate to the summary estimate.

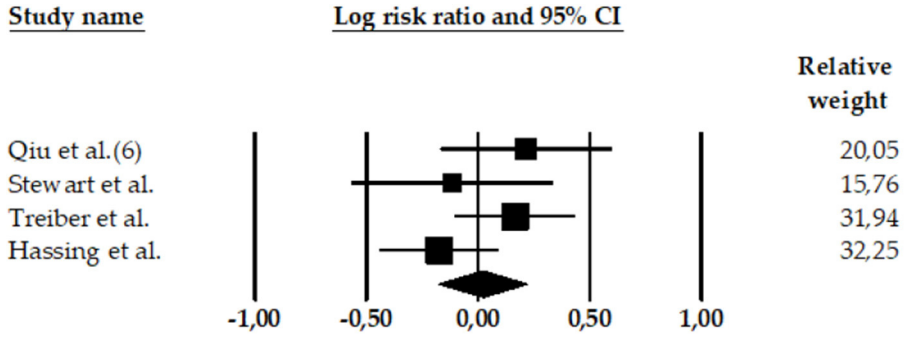

FIGURE 4
Forest plot of the meta-analysis of rates of AD in participants with high BP (high SBP and high DBP). The size of the box representing the point estimate for each study in the forest plot is proportional to the contributing weight of that study estimate to the summary estimate.

participants; *nHC* = 1,438,453 (LnRR = 0.09, 95%CI = 0.013–0.166, $Z = 2.28$, $p = 0.022$) (see Table 2). The heterogeneity was high: Q-value= 380.08, df = 59, and $I^2 = 84$.

3.3. Diastolic blood pressure and AD

Three meta-analyses showed the relationship between DBP and AD: Lennon et al. (22) ($k = 1$ effect size; $N = 378$; $n = 78$ with AD and high DBP; $n = 300$ HC participants), Xu et al. (51) ($k = 5$ effect sizes; $N = 12,225$; $n = 497$ with AD and high DBP; $n = 11,728$ HC participants), and Wang et al. (54) ($k = 5$ effect sizes; $N = 7,745$; $n = 306$ with AD and high DBP; $n = 7,439$ HC participants). None of the three meta-analyses show significant associations between high DBP and AD.

Consistently, our results ($k = 11$ effect sizes; $N = 20,348$; *nAD* = 881; HC = 19,467) did not find an association between high DBP and the risk of AD (LnRR = 0.15, 95% CI = −0.045 to 0.338, $Z = 1.50$, $p = 0.133$) (see Table 3). The heterogeneity was high: Q-value = 29.99, df = 10, and $I^2 = 66.65$.

3.4. High SBP and high DBP studies: Combined effect sizes

A meta-analysis reported a combined effect size for high SBP and high DBP (97). This study ($k = 4$ effect sizes; $N = 7,494$; $n = 211$ with AD and high SBP/DBP; $n = 7,283$ HC participants) found a non-significant association between high SBP and high DBP and AD (LnRR = 0.02, 95% CI = −0.179 to 0.222, Z =

TABLE 2 Individual and pooled estimates of the association between high SBP and AD.

References	Sample	Statistics for each study						
		LnRR	*Se*	*Ve*	*LLIC*	*ULIC*	*Z*	*p*
Lennon et al. (22)								
Kivipelto et al. (1) (18)	AD $n = 48$	0.74	0.47	0.22	−0.174	1.658	1.59	0.113
	HC $n = 1,400$							
Morris et al. (1) (25)	AD $n = 324$	0.03	0.13	0.02	−0.221	0.280	0.23	0.817
	HC $n = 378$							
Morris et al. (2) (25)[a]	AD $n = 54$	0.12	0.79	0.63	−1.430	1.674	0.15	0.877
	HC $n = 378$							
McGrath et al. (57)	AD $n = 81$	0.30	0.24	0.06	−0.174	0.775	1.24	0.215
	HC $n = 1,440$							
Chiang et al. (58)	AD $n = 64$	0.23	0.35	0.12	−0.448	0.910	0.67	0.505
	HC $n = 292$							
Kimm et al. (1) (59)	AD $n = 282$	0.26	0.12	0.01	0.030	0.495	2.21	0.027
	HC $n = 821$							
Kimm et al. (2) (59)	AD $n = 164$	0.18	0.60	0.36	−1.000	1.364	0.30	0.762
	HC $n = 821$							
Kimm et al. (3) (59)[a]	AD $n = 274$	0.34	0.13	0.02	0.088	0.584	2.66	0.008
	HC $n = 821$							
Kimm et al. (4) (59)[a]	AD $n = 206$	0.18	0.11	0.01	−0.041	0.405	1.60	0.109
	HC $n = 821$							
Ninomiya et al. (1) (60)	AD $n = 6$	−0.05	0.29	0.08	−0.619	0.516	−0.18	0.859
	HC $n = 149$							
Ninomiya et al. (2) (60)[a]	AD $n = 17$	−0.17	0.35	0.12	−0.865	0.516	−0.50	0.621
	HC $n = 177$							
Total (22)		**0.20**	**0.06**	**0.00**	**0.090**	**0.307**	**3.58**	**0.000**
Xu et al. (51)								
Launer et al. (1) (27)	AD $n = 81$	0.20	0.61	0.37	−0.996	1.394	0.33	0.744
	HC $n = 2.137$							
Posner et al. (24)	AD $n = 257$	−0.22	0.34	0.12	−0.892	0.446	−0.65	0.513
	HC $n = 1.259$							
Verghese et al. (1) (61)	AD $n = 65$	−0.39	0.34	0.11	−1.049	0.278	−1.14	0.255
	HC $n = 406$							
Tyas et al. (39)	AD $n = 35$	0.13	0.39	0.15	−0.634	0.897	0.34	0.737
	HC $n = 685$							

(Continued)

TABLE 2 (Continued)

References	Sample	Statistics for each study						
		LnRR	*Se*	*Ve*	*LLIC*	*ULIC*	*Z*	*p*
Bermejo et al. (62)	AD $n = 113$	0.73	0.38	0.15	−0.020	1.475	1.91	0.056
	HC $n = 3.824$							
Huang et al. (63)	AD $n = 612$	0.26	0.10	0.01	0.064	0.460	2.60	0.009
	HC $n =$ 142.744							
Chu et al. (64)	AD $n = 10$	0.04	0.02	0.00	0.009	0.069	2.54	0.011
	HC $n = 153$							
Luchsinger et al. (65)	AD $n = 246$	0.34	0.22	0.05	−0.087	0.760	1.56	0.120
	HC $n = 1.138$							
Forti et al. (1) (66)	AD $n = 18$	−0.26	0.77	0.60	−1.777	1.254	−0.34	0.735
	HC $n = 466$							
Forti et al. (2) (66)	AD $n = 30$	−0.03	0.46	0.21	−0.939	0.878	−0.07	0.948
	HC $n = 238$							
Song et al. (67)	AD $n = 416$	0.01	0.15	0.02	−0.276	0.296	0.07	0.946
	HC $n = 2.790$							
Raffaitin et al. (68)	AD $n = 134$	0.10	0.31	0.10	−0.509	0.700	0.31	0.757
	HC $n = 7.087$							
Muller et al. (69)	AD $n = 147$	0.41	0.25	0.06	−0.085	0.896	1.62	0.105
	HC $n = 1833$							
Lindsay et al. (70)	AD $n = 194$	−0.13	0.18	0.03	−0.486	0.231	−0.70	0.485
	HC $n = 4.088$							
Kivipelto et al. (1) (71)	AD $n = 48$	0.96	0.46	0.21	0.060	1.851	2.09	0.037
	HC $n = 1.449$							
Borenstein et al. (72)	AD $n = 90$	0.58	0.40	0.16	−0.196	1.361	1.47	0.143
	HC $n = 1.859$							
Hayden et al. (73)	AD $n = 104$	−0.42	0.22	0.05	−0.847	0.016	−1.89	0.059
	HC $n = 3.264$							
Kuller et al. (74)	AD $n = 330$	−0.11	0.24	0.06	−0.582	0.372	−0.43	0.665
	HC $n = 2.807$							
Ronnemaa et al. (75)	AD $n = 127$	0.00	0.09	0.01	−0.182	0.182	0.00	1.000
	HC $n = 2.268$							
Annweiler et al. (76)	AD $n = 70$	−0.36	0.31	0.10	−0.968	0.254	−1.14	0.253
	HC $n = 498$							
Wang et al. (77)	AD $n = 8.488$	0.32	0.13	0.02	0.076	0.568	2.57	0.010
	HC $n =$ 1.230.400							

(Continued)

TABLE 2 (Continued)

References		Statistics for each study						
	Sample	*LnRR*	*Se*	*Ve*	*LLIC*	*ULIC*	*Z*	*p*
Qiu et al. (1) (78)	AD $n = 333$	0.28	0.16	0.03	−0.034	0.590	1.74	0.081
	HC $n = 1.301$							
Olazaran et al. (79)	AD $n = 68$	−0.45	0.26	0.07	−0.946	0.054	−1.75	0.080
	HC $n = 1.376$							
Becker et al. (80)	AD $n = 48$	−0.11	0.32	0.10	−0.729	0.518	−0.33	0.740
	HC $n = 288$							
Dal et al. (1) (81)	AD $n = 40$	−0.14	0.32	0.11	−0.775	0.496	−0.43	0.668
	HC $n = 576$							
Dal et al. (2) (81)	AD $n = 67$	−0.54	0.30	0.09	−1.134	0.045	−1.81	0.070
	HC $n = 781$							
Harwood et al. (1) (82)	AD $n = 202$	0.41	0.21	0.05	−0.011	0.822	1.91	0.056
	HC $n = 392$							
Harwood et al. (2) (82)	AD $n = 188$	−0.36	0.31	0.10	−0.969	0.256	−1.14	0.254
	HC $n = 84$							
Wu et al. (83)	AD $n = 201$	0.68	0.30	0.09	0.095	1.261	2.28	0.023
	HC $n = 391$							
Brayne et al. (84)	AD $n = 18$	−0.25	0.37	0.14	−0.983	0.486	−0.66	0.507
	HC $n = 340$							
Mendez et al. (85)	AD $n = 50$	−0.06	2.02	4.07	−4.015	3.891	−0.03	0.976
	HC $n = 407$							
French et al. (86)	AD $n = 76$	−0.07	0.42	0.17	−0.887	0.742	−0.17	0.861
	HC $n = 102$							
Kokmen et al. (87)	AD $n = 203$	−0.36	0.31	0.10	−0.972	0.258	−1.14	0.256
	HC $n = 415$							
Foroughan et al. (88)	AD $n = 42$	0.54	0.23	0.05	0.078	0.995	2.30	0.022
	HC $n = 115$							
Roberts et al. (89)	AD $n = 151$	0.07	0.21	0.04	−0.348	0.483	0.32	0.750
	HC $n = 264$							
Kondo et al. (90)	AD $n = 60$	0.41	0.16	0.03	0.082	0.729	2.46	0.014
	HC $n = 120$							
Suhanov et al. (91)	AD $n = 127$	0.59	0.34	0.12	−0.086	1.262	1.71	0.087
	HC $n = 260$							
Graves et al. (92)	AD $n = 18$	0.43	0.03	0.01	0.339	0.472	11.90	0.000
	HC $n = 340$							
Tsolaki et al. (93)	AD $n = 65$	−0.77	0.19	3.86	−1.161	−0.391	−3.94	7.829
	HC $n = 69$							

(Continued)

TABLE 2 (Continued)

References		Statistics for each study						
	Sample	*LnRR*	*Se*	*Ve*	*LLIC*	*ULIC*	*Z*	*p*
Imfeld et al. (94)	AD $n = 3.541$	−0.38	3.75	1.41	−0.459	−0.312	−10.26	0.000
	HC $n = 7.086$							
Total (52)		**0.05**	**0.05**	**0.00**	**−0.038**	**0.146**	**1.16**	**0.246**
Meng et al. (52)								
Joas et al. (95)	AD $n = 79$	1.59	0.67	0.45	0.285	2.902	2.39	0.017
	HC $n = 707$							
Wang et al. (54)								
Qiu et al. (2) (96)	AD $n = 150$	0.61	0.28	0.08	0.060	1.159	2.18	0.030
	HC $n = 1.270$							
Qiu et al. (3) (96)[a]	AD $n = 124$	0.39	0.19	0.03	0.019	0.751	2.06	0.039
	HC $n = 441$							
Li et al. (1) (97)	AD $n = 14$	0.39	0.31	0.10	−0.225	0.995	1.24	0.216
	HC $n = 530$							
Li et al. (2) (97)	AD $n = 19$	−0.51	0.23	0.05	−0.953	−0.069	−2.26	0.024
	HC $n = 733$							
Li et al. (3) (97)	AD $n = 37$	−0.73	0.60	0.36	−1.908	0.440	−1.23	0.220
	HC $n = 530$							
Li et al. (4) (97)[a]	AD $n = 31$	0.32	0.34	0.12	−0.346	0.990	0.95	0.345
	HC $n = 733$							
Li et al. (5) (97)[a]	AD $n = 4$	−0.06	0.21	0.04	−0.476	0.352	−0.29	0.770
	HC $n = 733$							
Li et al. (6) (97)[a]	AD $n = 6$	−0.36	0.52	0.27	−1.384	0.670	−0.68	0.496
	HC $n = 530$							
Total (55)		**0.08**	**0.16**	**0.03**	**−0.241**	**0.399**	**0.48**	**0.629**
Total random		**0.09**	**0.04**	**0.00**	**0.013**	**0.166**	**2.28**	**0.022**

[a]Measures SBP > 160.

0.21, $p = 0.835$) (see Table 4). The heterogeneity was medium: Q-value = 4.52, df = 3, and $I^2 = 33.69$.

3.5. Subgroup analyses

Results of the subgroup analysis on the primary outcomes are presented in Table 5. Study outcomes were grouped by definition of hypertension and measures of BP (e.g., SBP, DBP, or total BP). Notably, 60 effect sizes examined SBP at both grades (22): 52 effect sizes examined only grade 1 (>140 mmHg) (51, 54) and 8 effect sizes examined only grade 2 (>160 mmHg) (53). Eleven effect sizes examined DBP at both grades: 2 effect sizes examined DBP using a cutoff point of >85 mmHg (51, 54) and 9 effect sizes >90 mmHg. Four effect sizes combined both types of hypertension (53). Moderator analyses were performed comparing effect sizes according to sex (men and women), age (≤65 and ≥66), study design (cross-sectional or C and longitudinal or L), and regions (Europe, Asia, and North America).

The results of pooling studies that reported RRs for a total score of BP showed that sex, age, and design did not moderate the relationship between hypertension and AD risk (Qb: $p \leq 0.50$). These results indicate that the risk of AD in participants with hypertension did not change significantly according to sex, age, and study design groups. However, it can be observed that there are significant relationships between different categories of the variables such as sex, age, study design, and AD (*Z*: $p \leq 0.50$). Findings revealed a significant relationship only between being women and a greater risk of AD ($p = 0.008$). Age was

TABLE 3 Individual and pooled estimates of the association between high DBP and AD.

References	*Sample*	Statistics for each study						
		LnRR	*Se*	*Ve*	*LLIC*	*ULIC*	*Z*	*p*
Lennon et al. (22)								
Morris et al. (3) (25)	AD $n = 78$	0.44	0.49	0.24	−0.513	1.402	0.91	0.363
	HC $n = 300$							
Xu et al. (51)								
Launer et al. (2) (27)	AD $n = 87$	0.62	0.31	0.10	0.005	1.236	1.98	0.048
	HC $n = 2.137$							
Verghese et al. (2) (61)	AD $n = 65$	0.65	0.31	0.09	0.048	1.246	2.12	0.034
	HC $n = 406$							
Qiu et al. (4) (78)	AD $n = 87$	0.64	0.17	0.03	0.303	0.981	3.71	0.000
	HC $n = 1.301$							
Ruitenberg et al. (98)	AD $n = 107$	−0.11	0.11	0.01	−0.331	0.120	−0.92	0.359
	HC $n = 6.985$							
Shah et al. (99)	AD $n = 151$	0.00	0.01	0.00	−0.010	0.010	0.00	1.000
	HC $n = 899$							
Total (52)		**0.27**	**0.15**	**0.02**	**−0.019**	**0.554**	**1.83**	**0.068**
Wang et al. (54)								
Qiu et al. (5) (96)	AD $n = 245$	−0.25	0.19	0.03	−0.613	0.116	−1.34	0.182
	HC $n = 2,249$							
Li et al. (7) (97)	AD $n = 22$	−0.20	0.53	0.28	−1.245	0.848	−0.37	0.710
	HC $n = 2.605$							
Li et al. (8) (97)	AD $n = 28$	−0.31	0.39	0.15	−1.086	0.457	−0.80	0.424
	HC $n = 1.321$							
Li et al. (9) (97)[a]	AD $n = 4$	0.54	0.28	0.08	−0.018	1.091	1.90	0.058
	HC $n = 905$							
Li et al. (10) (97)[a]	AD $n = 7$	−0.04	0.22	0.05	−0.464	0.383	−0.19	0.850
	HC $n = 359$							
Total (54)		−0.04	0.15	0.02	−0.339	0.263	−0.25	0.805
Total random		**0.15**	**0.10**	**0.01**	**−0.045**	**0.338**	**1.50**	**0.133**

[a]Measures DBP > 90.

also associated with increased risk of AD in early ($p = 0.008$) and late ($p = 0.047$) age of onset, and this association was also significant in cross-sectional ($p = 0.021$) and longitudinal ($p = 0.013$) studies. Regions moderated the association between BP and AD. The risk of AD was greater in studies that used samples from Asia and North America than those performed in Europe.

Results did not find significant differences in the risk of AD according to the measures of SBP (>140 and >160 mmHg) and DBP (>85 and >90 mmHg). Similarly, sex, age, design, and region did not moderate the relationship between SBP and DBP and the risk of AD, except sex in the case of DBP. Results found that women showed a stronger risk of developing AD than

TABLE 4 Individual and pooled estimates of the association between high BP and AD.

References	*Sample*	Statistics for each study						
		LnRR	*Se*	*Ve*	*LLIC*	*ULIC*	*Z*	*p*
Guan et al. (53)								
Qiu et al. (6) (19)	AD $n = 75$	0.22	0.20	0.04	−0.168	0.599	1.10	0.272
	HC $n = 719$							
Stewart et al. (100)	AD $n = 35$	−0.12	0.23	0.05	−0.566	0.333	−0.51	0.611
	HC $n = 1.778$							
Treiber et al. (101)	AD $n = 65$	0.17	0.14	0.02	−0.103	0.434	1.21	0.227
	HC $n = 3.634$							
Hassing et al. (102)	AD $n = 36$	−0.17	0.14	0.02	−0.441	0.092	−1.28	0.199
	HC $n = 1.152$							
Total random		**0.02**	**0.10**	**0.01**	**−0.179**	**0.222**	**0.21**	**0.835**

men. It is also observed that only in longitudinal studies and Asia regions, significant associations were found between SBP and AD.

According to measures of SBP (>140 and >160 mmHg), results indicated that SBP had no significant differences in effect sizes on the risk of AD at different sexes, ages, and designs. However, for SBP > 140 mmHg, there was evidence of heterogeneity between regions in RRs of AD. Asian countries showed stronger effect sizes between SBP and risk of AD than European and North American countries. Also, results found that elevated SBP (>160 mmHg) was significantly associated with AD risk in the young elderly (≤65), longitudinal studies, and in Europa and Asia.

For DBP (>85 and >90 mmHg), there was evidence of heterogeneity between the sexes. Women with elevated DBP (>90 mmHg) showed a greater risk of AD than men. Furthermore, there were no significant differences in AD risk according to age, design, and region.

Finally, age and region did not moderate the relationship between the combined effects of BP and the risk of AD.

4. Discussion

This study analyzes the association between high BP and the risk of AD. This is the first study to evaluate this relationship by identifying previous meta-analyses and analyzing primary studies worldwide. The present study summarized the information on meta-analyses of hypertension (DBP and SBP) and AD and expanded the findings from individual studies. In this study, 52 primary studies and 75 effect sizes were extracted. Furthermore, we included some moderator variables between high DBP and high SBP and AD, such as sex, age, study design, regions, and measures of SBP and DBP.

Overall, results suggest that hypertension is associated with an increased risk of AD ($RR = 1.08$, 95%CI: 1.032, 1.13, $Z = 3.273$, $p = 0.001$). It indicates that the risk of AD increases by 8% for patients with SBP.

In this study, 46 primary studies and 60 effect sizes extracted from four meta-analyses (22, 51–53) confirm the relationship between high SBP and AD ($RR = 1.09$, 95%CI: 1.013, 1.181, $Z = 2.285$, $p = 0.022$). These results indicate that participants with high SBP increase the rate risk of AD by 9% and support findings of previous studies, suggesting that there were consistent demonstrations of a relationship between SBP and the risk of developing AD. In this vein, research demonstrated that high SBP could increase the risk of AD since it could cause neurobiological alterations (deposits of beta-amyloid protein), which lead to lesions in the brain, such as cerebral atrophy, senile plaques, and neurofibrillary tangles, which could be explanatory factors of the development of AD (103, 104). Other studies also suggest that high SBP could cause brain vascular injury, leading to increased flow of blood, cerebral patency, and cerebral amyloid angiopathy which were also associated with a higher risk of AD (105–107). However, our analysis cannot underlie the pathophysiology of AD and could only define SBP as a risk factor.

The relationship between high DBP and AD was studied through $k = 8$ primary studies and eleven effect sizes (three meta-analyses) (22, 51, 54). Findings did not find a significant association between high DBP and the risk of AD. Nevertheless, according to previous studies, these results could be explained by confounding due to associations between BP and advanced disease or other unknown modifiable risk factors (108–110).

TABLE 5 Effects of sex, age, design, and regions in different types of SBP (>140 and >160 mmHg) and DBP (>85 and >90 mmHg).

Group by		Statistics for each study									
		Effect sizes	*LnRR*	*Se*	*Ve*	*LLIC*	*ULIC*	*Z*	*p*	I^2	*Qb*
BP (all types)											
	Sex										
	Men	54	0.06	0.04	0.00	−0.023	0.140	1.407	0.159	72.01	1.867, $p = 0.172$
	Women	21	0.16	0.06	0.00	0.041	0.274	2.657	0.008	88.38	
	Age										
	≤65	36	0.09	0.03	0.00	0.024	0.160	2.645	0.008	58.70	0.280, $p = 0.596$
	≥65	39	0.07	0.03	0.00	0.001	0.132	1.984	0.047	88.11	
	Design										
	C	46	0.06	0.03	0.00	0.010	0.120	2.303	0.021	87.61	0.744, $p = 0.389$
	L	29	0.11	0.04	0.00	0.023	0.197	2.484	0.013	36.48	
	Regions										
	Europe	23	−0.05	0.03	0.00	−0.113	0.025	−1.244	0.214	87.66	20.65, $p = 0.0001$
	Asia	15	0.19	0.04	0.00	0.115	0.284	4.627	0.000	58.27	
	North America	37	0.11	0.04	0.00	0.038	0.190	2.939	0.003	62.02	
SBP											
>140		52	0.08	0.04	0.01	−0.007	0.158	1.786	0.074	86.01	0.948, $p = 0.330$
>160		8	0.19	0.11	0.01	−0.027	0.407	1.720	0.085	3.14	
	Sex										
	Men	42	0.08	0.05	0.01	−0.015	0.174	1.649	0.099	67.99	0.107, $p = 0.744$

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TABLE 5 (Continued)

Group by		Statistics for each study									
		Effect sizes	*LnRR*	*Se*	*Ve*	*LLIC*	*ULIC*	*Z*	*p*	I^2	*Qb*
	Women	18	0.11	0.06	0.01	−0.012	0.221	1.158	0.079	88.94	
>140	Men	35	0.06	0.05	0.01	−0.045	0.162	1.11	0.267	71.87	0.237, $p = 0.626$
	Women	17	0.09	0.06	0.00	−0.025	0.222	1.565	0.118	89.81	
>160	Men	7	0.21	0.11	0.01	−0.009	0.426	1.880	0.060	15.65	0.018, $p = 0.895$
	Women	1	0.18	0.11	0.01	−0.041	0.405	1.601	0.109	0.000	
	Age										
	≤*65*	29	0.101	0.07	0.01	−0.034	0.250	1.495	0.135	54.50	0.133, $p = 0.715$
	≥*65*	31	0.07	0.07	0.01	−0.063	0.207	1.040	0.298	90.29	
>140	≤65	25	0.08	0.08	0.01	−0.084	0.234	0.927	0.354	49.01	0.000, $p = 0.987$
	≥65	27	0.08	0.07	0.01	−0.067	0.221	1.048	0.295	91.54	
>160	≤65	4	0.26	0.10	0.01	0.070	0.455	2.667	0.008	23.26	1.854, $p = 0.173$
	≥65	4	0.01	0.17	0.03	−0.318	0.334	0.047	0.962	0.00	
	Design										
	C	41	0.06	0.05	0.01	−0.031	0.152	1.294	0.196	88.23	1.336, $p = 0.248$
	L	19	0.16	0.07	0.01	0.018	0.302	2.206	0.027	35.78	
>140	C	41	0.06	0.05	0.00	−0.032	0.152	1.290	0.198	88.23	0.517, $p = 0.472$
	L	11	0.14	0.10	0.01	−0.052	0.327	1.425	0.154	50.73	
>160	C	–	–	–	–	–	–	–	–		–
	L	8	0.21	0.07	0.01	0.065	0.356	2.834	0.005	3.14	

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TABLE 5 (Continued)

Group by		Statistics for each study									
		Effect sizes	*LnRR*	*Se*	*Ve*	*LLIC*	*ULIC*	*Z*	*p*	I^2	*Qb*
	Regions										
	Europe	18	0.03	0.09	0.01	−0.148	0.198	0.284	0.777	89.30	5.785, $p =$ 0.055
	Asia	14	0.27	0.09	0.01	0.095	0.436	3.044	0.002	60.41	
	North America	28	0.01	0.07	0.01	−0.130	0.152	0.156	0.876	64.11	
>140	Europe	17	0.00	0.09	0.01	−0.187	0.176	0.057	0.955	89.62	5.985, $p = 0.050$
	Asia	11	0.29	0.10	0.01	0.091	0.493	2.854	0.004	63.14	
	North America	24	0.01	0.08	0.01	−0.143	0.160	0.109	0.913	67.66	
>160	Europe	1	0.61	0.28	0.08	0.060	1.159	2.176	0.030	0.00	3.562, $p = 0.169$
	Asia	3	0.23	0.08	0.01	0.067	0.389	2.771	0.006	9.15	
	North America	4	0.01	0.17	0.03	−0.318	0.334	0.047	0.962	0.00	
DBP											
>85		2	0.21	0.24	0.06	−0.266	0.680	0.859	0.390	61.98	0.067, $p = 0.795$
>90		9	0.14	0.11	0.01	−0.081	0.358	1.236	0.217	69.65	
	Sex										
	Men	8	−0.01	0.06	0.01	−0.13	0.118	−0.109	0.913	39.20	13.37, $p = 0.0001$
	Women	3	0.62	0.15	0.03	0.307	0.927	3.897	0.0001	0.00	
>85	Men	2	0.22	0.29	0.08	−0.344	0.782	0.763	0.446	61.98	–
	Women	–	–	–	–	–	–	–	–	–	
>90	Men	6	−0.02	0.05	0.01	−0.126	0.079	−0.452	0.641	35.53	16.052, $p = 0.0001$
	Women	3	0.62	0.15	0.02	0.321	0.915	4.081	0.0001	0.00	

(Continued)

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TABLE 5 (Continued)

Group by		Statistics for each study									
		Effect sizes	*LnRR*	*Se*	*Ve*	*LLIC*	*ULIC*	*Z*	*p*	I^2	*Qb*
	Age										
	≤65	4	0.21	0.18	0.03	−0.133	0.552	1.198	0.231	85.01	0.131, $p = 0.717$
	≥65	7	0.12	0.16	0.03	−0.196	0.442	0.756	0.449	39.41	
>85	≤65	–	–	–	–	–	–	–	–	–	–
	≥65	2	0.22	0.29	0.08	−0.344	0.782	0.763	0.446	61.98	
>90	≤65	4	0.21	0.18	0.03	−0.147	0.574	1.160	0.246	85.01	0.245, $p = 0.621$
	≥65	5	0.08	0.21	0.04	−0.334	0.485	0.363	0.716	36.35	
	Design										
	C	5	0.26	0.14	0.02	−0.015	0.537	1.854	0.064	82.58	1.345, $p = 0.246$
	L	6	0.01	0.17	0.023	−0.317	0.334	0.052	0.958	28.15	
>85	C	–	–	–	–	–	–	–	–		–
	L	2	0.22	0.29	0.08	−0.344	0.782	0.763	0.446	61.98	
>90	C	5	0.26	0.14	0.02	−0.013	0.530	1.864	0.062	82.58	2.450, $p = 0.118$
	L	4	−0.15	0.21	0.05	−0.575	0.282	−0.671	0.502	0.00	
	Regions										
	Europe	3	0.12	0.19	0.04	−0.253	0.498	0.638	0.523	87.13	0.074, $p = 0.786$
	Asia	–	–	–	–	–	–	–	–	–	
	North America	8	0.19	0.15	0.02	−0.109	0.487	1.241	0.215	49.06	
>85	Europe	–	–	–	–	–	–	–	–	–	–
	Asia	–	–	–	–	–	–	–	–	–	
	North America	2	0.22	0.29	0.08	−0.344	0.782	0.763	0.446	61.98	
>90	Europa	3	0.12	0.21	0.04	−0.278	0.525	0.604	0.546	87.13	0.041, $p = 0.840$

(Continued)

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TABLE 5 (Continued)

Group by		Statistics for each study									
		Effect sizes	*LnRR*	*Se*	*Ve*	*LLIC*	*ULIC*	*Z*	*p*	I^2	*Qb*
	Asia	–	–	–	–	–	–	–	–	–	
	North America	6	0.18	0.19	0.04	−0.193	0.554	0.946	0.344	53.09	
BP (combined effects)											
	Sex										
	Men	4	0.02	0.10	0.01	−0.179	0.222	0.209	0.835	33.68	–
	Women	–	–	–	–	–	–	–	–	–	
	Age										
	≤65	3	−0.05	0.12	0.02	−0.289	0.192	−0.387	0.669	27.19	0.978, $p = 0.323$
	≥65	1	0.17	0.18	0.03	−0.182	0.513	0.934	0.350	0.00	
	Design										
	C										–
	L	2	0.02	0.10	0.01	−0.179	0.222	0.209	0.835	33.69	
	Regions										
	Europe	2	−0.01	0.19	0.04	−0.383	0.383	−0.026	0.979	62.61	0.522, $p = 0.770$
	Asia	1	−0.12	0.32	0.10	−0.736	0.503	−0.368	0.713	0.00	
	North America	1	0.16	0.26	0.07	−0.339	0.670	0.643	0.520	0.00	

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For instance, secondary diseases, such as obesity, cardiovascular diseases, silent infarcts, and vascular risk factors (111) or type 2 diabetes (103, 108, 109), could be closely related to the development of AD. Hence, in these cases, it is not clear if hypertension is directly related to the risk of AD or whether AD is indirectly motivated by a secondary disease (110). Finally, there was a small number of studies analyzing DBP and AD in comparison with SBP, and in consequence, it is possible that we did not have sufficient statistical power to obtain a significant pooled estimate of the association between DBP and AD.

Related to the combined BP hypertension, only a meta-analysis (53) with four independent studies and effect sizes compared the incidence of AD between subjects with and without hypertension. These studies found that high BP is not associated with an increased risk of AD. This result is contradictory to the general view on the association between risk for AD and hypertension. For example, Guan et al. (53) highlighted that AD and hypertension are independent diseases with some common etiopathogenesis, which is a risk factor in AD.

To explore the influence of other research parameters in the relationship between high SBP and high DBP with AD, we analyzed different moderators: sex, age, study design, and region. This study does not find differences in the risk of AD according to the type of measure of SBP (>140 and >160 mmHg) and DBP (>85 and >90 mmHg). Total scores reveal significant differences between men ($RR = 0.99$, 95%CI: 0.887, 1.125, $Z = -0.109$, $p = 0.913$) and women ($RR = 1.85$, 95% CI: 1.359, 2.527, $Z = 3.897$, $p = 0.001$) (rate risk of AD increases by 85%) in the relationship of high DBP and AD, but not between SBP and AD. Specifically, the data suggest that women with high DBP (>90 mmHg) had an increased risk of AD compared with men ($RR = 1.86$, 95%CI: 1.379, 2.498, $Z = 16.05$, $p = 0.001$), which increase the rate risk of AD by 86%. These results have been shown in previous studies that worked with different samples (women and men), where AD was also associated with high DBP mainly in women (107, 108). For instance, Benetos et al. (112) found that DBP in women is associated with a higher cardiac output, pulse pressure, and heart rate (HR) factors that are related to a higher risk of AD (63.8%).

Total scores of BP show that age is associated with increased risk of AD in the early and late age of onset ($RR = 1.10$, 95%CI: 1.024, 1.174, $Z = 2.645$, $p = 0.008$; $RR = 1.07$, 95%CI: 1.001, 1.141, $Z = 0.047$, $p = 0.047$), with the rate risk of AD increases by 10% and 7%. However, the age of onset (early onset ≤65 years and late onset ≥65 years) does not moderate the relationship between high SBP/DBP and AD, showing similar effect sizes for both categories. Related to the measure of BP, this study found that elevated SBP > 160 mmHg was associated with the risk of AD in the young elderly (≤65 years), but not in those ≥65 years of age. In this vein, several studies have found that hypertension has different impacts on cognitive function at different ages (19, 22, 110). Current literature indicates that hypertension is a risk factor for cognitive decline in midlife and young old age but may be protective against cognitive decline in late life (22). For example, some authors concluded that high BP at the early age of onset impacted cognitive functions and increased the risk of developing AD in older age (19, 113). Iadecola et al. (114) also found that hypertension in early onset is associated with a higher risk of AD. Therefore, changes in BP may be due to hemodynamic regulation being altered by neurodegenerative processes in the years preceding disease onset (22).

The only variable that moderates the relationship between BP and AD is the region. We observe a higher risk of AD in Asia with SBP >140 mmHg ($RR = 1.34$, 95%CI: 1.096, 1.637, $Z = 2.854$, $p = 0.004$) compared with European ($RR = 0.99$, 95%CI: 0.829, 1.193, $Z = -0.057$, $p = 0.955$) and North America ($RR = 1.01$, 95%CI: 0.866, 1.174, $Z = 0.109$, $p = 0.913$). Therefore, the rate risk of AD in Asia increases by 34%. These results are related to the findings of some studies. During the past four decades, the highest BP measurements worldwide have shifted from high-income countries to low-income countries, such as South Asia and Africa (115), which could explain our results (116, 117). On the one hand, several authors suggest that recent lifestyle changes in Asia countries, such as diet, changing demographics, urbanization, environmental interactions, and other factors, may help explain this relationship (117). On the other hand, one study with data from 90 countries showed that the percentage of people with hypertension receiving treatment increased in both high-income and low- and middle-income countries, but the gap between them widened (118). Moreover, our results also show that the risk of AD related to SBP > 160 mmHg in Europe ($RR = 0.61$, 95%CI: 0.060, 1.159, $Z = 2.176$, $p = 0.030$) and Asia ($RR = 0.23$, 95%CI: 0.067, 0.389, $Z = 2.771$, $p = 0.006$) is significant. However, North America ($RR = 0.01$, 95%CI: −0.318, 0.334, $Z = 0.047$, $p = 0.962$) did not find a significant relationship. Despite these results, the strength of the association between SBP (>160 mmHg) and AD risk is similar in the three regions.

Finally, results do not find differences in the effect size of the association between high SBP and DBP and the risk of AD according to the type of design (cross-sectional and longitudinal). Our results found an association between BP and the risk of AD in both types of studies. However, findings confirm that the relationship between higher SBP and AD is only significant in longitudinal studies and with SBP > 160 mmHg ($RR = 1.23$, 95%CI: 1.067, 1.428, $Z = 2.834$, $p = 0.005$), so the rate risk of AD increases by 23%, while high DBP (>85 and >90 mmHg) is not related to increased AD risk. In this vein, previous work found differences according to the type of design that may result in part from the use of different definitions of hypertension and non-uniform measures of high or low BP. In this study, we use standardized criteria to define BP (SBP > 140/160 mmHg and DBP > 85/90 mmHg) and AD (clinical criteria) which could explain that there are no differences according to the study design. After controlling

for this confounding factor, the effect size of longitudinal studies is higher in all the BP and SBP measures, although the differences do not reach significance. Longitudinal studies provide an opportunity to assess the temporal relationship between BP and AD and the length of follow-up remains relevant since hypertension could render individuals more vulnerable to comorbid conditions, such as cerebrovascular disease, that confer greater risk for AD during long periods of follow-up.

However, there are some limitations to our study. The key limitation is that only a small number of studies examined the association between DBP, both types of BP combined, and AD compromising the generalizability of the results. Furthermore, it is likely that due to the procedure used in this meta-meta-analysis, some primary studies were not included. Another challenge was that studies reported outcomes using different metrics (OR, HR, and RR). Likewise, not all the cutoff points established by ISH could be analyzed since the stages of SBP $\geq$ 130–139 and DBP $\geq$ 100 could not be defined due to the lack of primary studies. Other confounders may also influence the study's findings. For example, results were not adjusted for other risk variables including cardiovascular disease, stroke, alcohol consumption, smoking, kidney disease, and many others. Also, two studies did not report the mean age of the sample, and they were not included in the moderator analysis. Moreover, the relationship between hypertension and AD could not be thought of as binary but rather as a dynamic one, changing with life stage and disease state. Hence, a single measurement of BP may not accurately reflect the participant's average BP measurements. Additionally, data on the age at the onset of hypertension and years of living with the condition may be important in clarifying temporal relationships between hypertension and AD. Also, we did not examine the potentially modifying impact of antihypertensive therapy on the relationship between hypertension and AD. In addition, another limitation is the absence of studies from South America and Australia. Finally, we did not include educational level as a moderator variable since the external validity of some of the results has been questioned. The primary studies contained in this meta-analysis used very different forms of measurement. For instance, some studies analyzed education using individual (i.e., no formal education, mandatory education, secondary studies, university studies) (79, 88) or community-based samples (i.e., family education level, region, or country) (80, 88), quantitative (linear relation between the number of years of education and the risk of dementia) (81, 83) or qualitative measures (a threshold effect at a given level of education) (86), and composite measures (i.e., socioeconomic status, SES defines education plus income) (67, 119) that show different results. Therefore, we should interpret our results cautiously.

Several strengths of our review of a meta-analysis should be emphasized. First, most prior studies were drawn from general community samples or non-AD-specific studies (vascular dementia, cortical dementia, or dementia in general), whereas the current study relied on AD. Second, we add to the current literature by analyzing 52 primary studies extracted from the previous meta-analysis increasing the statistical power of our results. Third, we analyzed the impact of different moderators (sex, age, study design, region, and measures of SBP/DBP) to explore the influence of other research parameters in the relationship between high SBP and DBP and AD. Finally, we want to focus on effect sizes since the statistical significance should never be interpreted as evidence that an effect had clinical importance. It is important to note that the effect sizes were "relatively small" and the variation is great within the same meta-analysis. Therefore, the clinical significance and practical importance of these results should be considered in relation to the patient's status, goals, and clinician experience.

As a practical implication, this study suggests that high SBP could be a risk factor for AD. There is limited evidence that single cardiovascular risk factors affect AD risk, but the strength of the association is influenced greatly by changing the parameters of the risk factors and in particular by identifying interactions between the factors. Future research should confirm this and determine whether stabilizing BP might be a target to slow or decline the development of AD.

5. Conclusion

This study analyzes the association between SBP/DBP/combined BP and the risk of developing AD. A total of five meta-analyses and 52 primary studies were analyzed in this review of meta-analysis. Our study found that SBP is associated with an increased risk of AD by 11%, although no association was found for DBP. Measures of SBP >140, SBP >160, DBP >85, and DBP >90 do not moderate the relationship between SBP and DBP and AD. Moderator analysis (sex, age, study design, region, and measures of SBP/DBP) shows a significant association between high DBP (>90) and AD in women. The age of onset (early-onset AD $\leq$65 years and late-onset AD or senile AD $\geq$65 years) did not moderate the relationship between SBP and DBP and AD. Finally, regarding the type of study, there were no differences in the association between BP and AD between longitudinal and cross-sectional studies. However, Asian countries showed stronger effect sizes between SBP > 140 and risk of AD than European and North American countries. Future work should use other uncontrolled factors (e.g., cardiovascular diseases, diabetes, and stroke) to explain the relationship between high BP and AD.

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.

Author contributions

OS-V and AP-M conceived and designed the analysis, collected the data, contributed data or analysis tools, performed analysis, and wrote the paper. JP-B wrote the paper. SU-L contributed data or analysis tools, performed analysis, and wrote the paper. All authors take full responsibility for the data, the analyses and interpretation, and the conduct of the research, full access to all of the data and the right to publish any and all data. All authors contributed to the article and approved the submitted version.

Funding

This study was partially funded by grant Y133GI awarded by the University of Burgos (Spain) to the Social Inclusion and Quality of Life (SIQoL) research group. This work was partially supported by FEDER funds and the Spanish State Research Agency (Projects PID2019-104263RB-C44 and PDC2021-121021-C22) and the Regional Government of "Castilla y León" and FEDER funds (Project BU056P20).

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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2022.1065335/full#supplementary-material

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frontiers | Frontiers in Neuroscience

TYPE Original Research
PUBLISHED 26 January 2023
DOI 10.3389/fnins.2023.1083928

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

RECEIVED 29 October 2022
ACCEPTED 09 January 2023
PUBLISHED 26 January 2023

CITATION
Zhang P, Zhao L, Li H, Shen J, Li H and Xing Y (2023) Novel diagnostic biomarkers related to immune infiltration in Parkinson's disease by bioinformatics analysis.
Front. Neurosci. 17:1083928.
doi: 10.3389/fnins.2023.1083928

Novel diagnostic biomarkers related to immune infiltration in Parkinson's disease by bioinformatics analysis

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Background: Parkinson's disease (PD) is Pengfei Zhang Liwen Zhao Pengfei Zhang Liwen Zhao a common neurological disorder involving a complex relationship with immune infiltration. Therefore, we aimed to explore PD immune infiltration patterns and identify novel immune-related diagnostic biomarkers.

Materials and methods: Three substantia nigra expression microarray datasets were integrated with elimination of batch effects. Differentially expressed genes (DEGs) were screened using the "limma" package, and functional enrichment was analyzed. Weighted gene co-expression network analysis (WGCNA) was performed to explore the key module most significantly associated with PD; the intersection of DEGs and the key module in WGCNA were considered common genes (CGs). The CG protein–protein interaction (PPI) network was constructed to identify candidate hub genes by cytoscape. Candidate hub genes were verified by another two datasets. Receiver operating characteristic curve analysis was used to evaluate the hub gene diagnostic ability, with further gene set enrichment analysis (GSEA). The immune infiltration level was evaluated by ssGSEA and CIBERSORT methods. Spearman correlation analysis was used to evaluate the hub genes association with immune cells. Finally, a nomogram model and microRNA-TF-mRNA network were constructed based on immune-related biomarkers.

Results: A total of 263 CGs were identified by the intersection of 319 DEGs and 1539 genes in the key turquoise module. Eleven candidate hub genes were screened by the R package "UpSet." We verified the candidate hub genes based on two validation sets and identified six (SYT1, NEFM, NEFL, SNAP25, GAP43, and GRIA1) that distinguish the PD group from healthy controls. Both CIBERSORT and ssGSEA revealed a significantly increased proportion of neutrophils in the PD group. Correlation between immune cells and hub genes showed SYT1, NEFM, GAP43, and GRIA1 to be significantly related to immune cells. Moreover, the microRNA-TFs-mRNA network revealed that the microRNA-92a family targets all four immune-related genes in PD pathogenesis. Finally, a nomogram exhibited a reliable capability of predicting PD based on the four immune-related genes (AUC = 0.905).

Conclusion: By affecting immune infiltration, SYT1, NEFM, GAP43, and GRIA1, which are regulated by the microRNA-92a family, were identified as diagnostic biomarkers of PD. The correlation of these four genes with neutrophils and the microRNA-92a family in PD needs further investigation.

KEYWORDS

Parkinson's disease, bioinformatics analysis, immune infiltration, weighted gene co-expression network analysis (WGCNA), hub genes, gene set enrichment analysis

Introduction

Neurological disorders, as the main cause of disability worldwide, impose a major financial burden (Feigin et al., 2019). Compared to other neurological disorders, Parkinson's disease (PD) has shown the fastest growth in prevalence and incidence in recent years (Bloem et al., 2021). The main characteristic features of PD include progressive loss of neurons in specific areas of the substantia nigra and the presence of Lewy bodies in the brain (Wakabayashi et al., 2013), which lead to dysfunction in patients. There have been significant advances in the treatment of PD, such as dopamine substitution and deep-brain stimulation, which retard symptom progression and improve quality of life for decades after disease onset (LeWitt and Fahn, 2016; Limousin and Foltynie, 2019). However, PD eventually leads to severe disability, which remains a healthcare challenge. Therefore, modifying PD progression and delaying disability are still key problems that need to be solved. When early clinical signs are inadequate for diagnosis of PD, in particular a lack of typical motor symptoms, diagnosis is often delayed and misdiagnosis may occur (Feigin et al., 2019; Bloem et al., 2021). By the time a diagnosis of PD is made, a substantial proportion of neurons in the brain have been lost (Gaenslen et al., 2011; Postuma and Berg, 2016). Thus, new diagnostic methods, including biomarkers that can identify individuals at risk and early before clinical manifestation of the onset of motor symptoms are needed.

In recent years, growing evidence has indicated that the immune system is involved in the pathophysiology of PD and increases the progression of PD (Öberg et al., 2021; Zhang et al., 2021). For instance, immune infiltrating cells CD8+ and CD4+ T cells, which were significantly different in PD samples compared with control animal models (Brochard et al., 2008; Harms et al., 2017), were related to dopaminergic neuron cell loss in the PD group (Brochard et al., 2008; Williams et al., 2021). Notably, a high proportion of substantia nigra CD8 T-cell infiltration has been considered an early alteration in PD, even occurring before death of dopamine neuronal cells and α-synuclein aggregation, which is also associated with progression of PD (Galiano-Landeira et al., 2020). However, the pathological mechanism underlying immune infiltration in PD lacks comprehensive evidence. Thus, understanding the mechanism of immune regulation in PD and identifying reliable biomarkers related to immune regulation can guide clinical diagnosis and immune strategies for treatment of the disease.

MicroRNA (miRNA), a small single-stranded non-coding RNA molecule, binds to mRNA and induces mRNA degradation and translational repression for posttranscriptional regulation of gene expression. Recent studies have elucidated that dysregulated expression of miRNAs plays a substantial role in regulating PD (Briggs et al., 2015; Nair and Ge, 2016). For instance, miR-153 can significantly reduce expression of synuclein-alpha (SNCA) (Je and Kim, 2017), which has been confirmed to be relevant to the pathogenesis of PD (Lesage et al., 2020; Kung et al., 2022). Moreover, miRNAs are not only related to dopaminergic neuron survival (Kabaria et al., 2015) and neuroinflammation (Yao et al., 2018), but can also serve as diagnostic biomarkers (Shu et al., 2020) and therapeutic tools for PD (Gan et al., 2019; Nies et al., 2021). Therefore, miRNAs can provide useful insight into the pathophysiology of PD to identify new therapeutic targets and strategies to slow or reverse neurodegeneration.

In recent years, with the development of microarray technology, bioinformatics analysis has been widely applied to identify potential novel biomarkers and reveal key pathways to explore the pathogenesis and drug targets of different diseases (Zhou et al., 2021). In this study, we conducted systematic bioinformatics analysis to identify novel immune infiltration-related diagnosis genes and understand the potential immune mechanism during the development of PD.

We integrated three datasets from the Gene Expression Omnibus (GEO) database, including 38 substantia nigra samples from the PD group and 29 normal samples. Differential expression gene (DEG) analysis of the integrated dataset comparing PD samples with normal controls, Gene Ontology (GO) functional analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, weighted gene co-expression network analysis (WGCNA), and protein–protein interaction (PPI) analysis were successively performed. Next, six hub genes were identified after validation using another two cohorts. The diagnostic effectiveness of the hub genes, gene set enrichment analysis (GSEA) of the hub genes, and the correlation between the hub genes and immune infiltration type were investigated. Then, four immune infiltration-related marker genes were confirmed. Finally, a nomogram model and miRNA-TF-mRNA network were constructed based on four immune infiltration-related marker genes, constituting potential biomarkers for the early diagnosis and treatment of PD.

Materials and methods

Data collection and preprocessing

We used the keyword "Parkinson's disease" to search the GEO[1] (Clough and Barrett, 2016). Five datasets were downloaded,

1 https://www.ncbi.nlm.nih.gov/geo/

and those datasets met of the following inclusion criteria: (1) related to *Homo sapiens*; (2) datasets containing Parkinson's patients and control subjects; and (3) tissue derived from the substantia nigra.

We downloaded the original data files (*.CEL) of the four datasets (GSE8397, GSE20163, GSE20164, and GSE20292), which were all based on the GPL96 (HG-U133A) Affymetrix Human Genome U133A Array (Affymetrix, Santa Clara, CA, United States). The GSE26927 dataset, including 12 PD substantia nigra tissue samples and 8 normal substantia nigra samples, was based on the GPL6255 platform Illumina humanRef-8 v2.0 expression beadchip (Illumina Inc., Bethesda, MD, United States). The GSE8397 dataset (GPL96, HG-U133 A chips) consisted of 47 individual brain tissue samples, including substantia nigra tissues of 24 patients with PD and 15 control samples. GSE20163 contained 17 substantia nigra tissue samples, including 9 patients with PD and 8 normal substantia nigra tissue samples. GSE20164 consisted of 11 substantia nigra tissue samples, including 5 PD samples and 6 normal samples. GSE20292 consisted of 29 substantia nigra tissue samples, including 11 PD samples and 18 normal samples. GSE26927 was used to validate the hub genes. The raw data files (*.CEL) of the four datasets (GSE8397, GSE20163, GSE20164, and GSE20292) were processed by the "affy" package (version 1.74.0) (Gautier et al., 2004). The robust multichip average (RMA) method was used for the above four datasets to obtain the gene expression matrix after background correction, normalization and calculation of expression values in the "affy" package.

We used the "limma" package (version 3.52.2) of *R* software[2] (ver. 4.2.0) (Ritchie et al., 2015) to match the identity document (IDs) of the datasets with that of the gene (gene symbol) based on each platform annotation file; empty probes that did not match the gene symbol were removed. If multiple probes corresponded to the same gene symbol, the maximum expression value was taken as its expression value. The gene expression profiles of GSE8397, GSE20163, and GSE20164 were integrated by the "limma" package in *R*. The combined dataset is processed using the surrogate variable analysis (SVA) package (version 3.44.0) (Leek et al., 2019) to remove batch effects and other unwanted variations in high-throughput experiments. **Supplementary Table 1** shows the detailed information of the five datasets, and a flow chart of the study design is shown in **Figure 1**.

Analysis of differentially expressed genes (DEGs)

The "limma" package was used to normalize the integrated dataset with the 'normalize Between Arrays' function in *R* software (version 4.2.0) and then screen DEGs between 38 patients with PD and 29 normal substantia nigra tissues from control patients with the threshold of adjusted *p*-value < 0.05 and $|\log2$ Fold change (FC)$| > 0.5$. Heatmap and volcano plots of DEGs were created by the "pheatmap" package (version 1.0.12) and the "ggplot2" package (version 3.3.6) in *R* software (version 4.2.0), respectively.

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses

We examined GO functions from three classifications, including biological processes (BP), cellular component (CC), and molecular function (MF), to further explore the potential molecular mechanisms of DEGs. KEGG was used to integrate pathway information for those DEGs. The R project was used to perform GO and KEGG analyses based on the "clusterProfiler" (Wu et al., 2021), "org.Hs.e.g.db" (Carlson et al., 2019), "ggplot2" (Villanueva and Chen, 2019), "enrichplot" (Yu, 2019), and "DOSE" (Yu et al., 2015) packages, and adjusted $p < 0.05$ was considered to be statistically significant.

Construction of weighted gene co-expression network analysis (WGCNA) and identification of hub modules

Weighted gene co-expression network analysis, a systematic biology approach, was utilized to construct a co-expressed gene network and to explore genes closely associated with the clinical phenotype. First, according to variation across samples in the integrated dataset, the top 5000 genes were imported into WGCNA using the "WGCNA" package (version 1.71) (Langfelder and Horvath, 2008). Second, all samples were clustered, and discrete samples were removed to ensure the reliance of the network construction results. Third, the soft threshold parameter was calculated, and the optimal parameter β was selected to form the scale-free network based on the scale independence and mean connectivity. According to the suitable power of $\beta = 7$ ($R^2 = 0.85$), the topological overlap matrix (TOM) and corresponding dissimilarity (1-TOM) were calculated. Fourth, through the dynamics cut tree algorithms, hierarchical clustering genes were identified, and then similar genes were classified into the same modules based on the TOM-based dissimilarity measure.

Each module of the gene dendrogram contained at least 50 genes, and similar modules were merged, with a height cutoff of 0.25. Finally, the module membership (MM) and gene significance (GS) were measured. The relevance between module eigengenes (MEs) and clinical traits was assessed by the Pearson correlation test and was shown with a heatmap to identify the most significant modules associated with MEs. Significant module genes were selected for further analysis.

Construction of a protein–protein interaction (PPI) network and identification of candidate hub genes

The "VennDiagram" package (Chenn, 2018) (version 1.7.3) was used to obtain intersecting common genes (CGs) between DEGs and the genes in the most significant module of WGCNA. The PPI network of CGs was analyzed with the Search Tool for the Retrieval of Interacting Genes (STRING[3]; version 11.0) online

2 https://www.r-project.org/

3 www.string-db.org

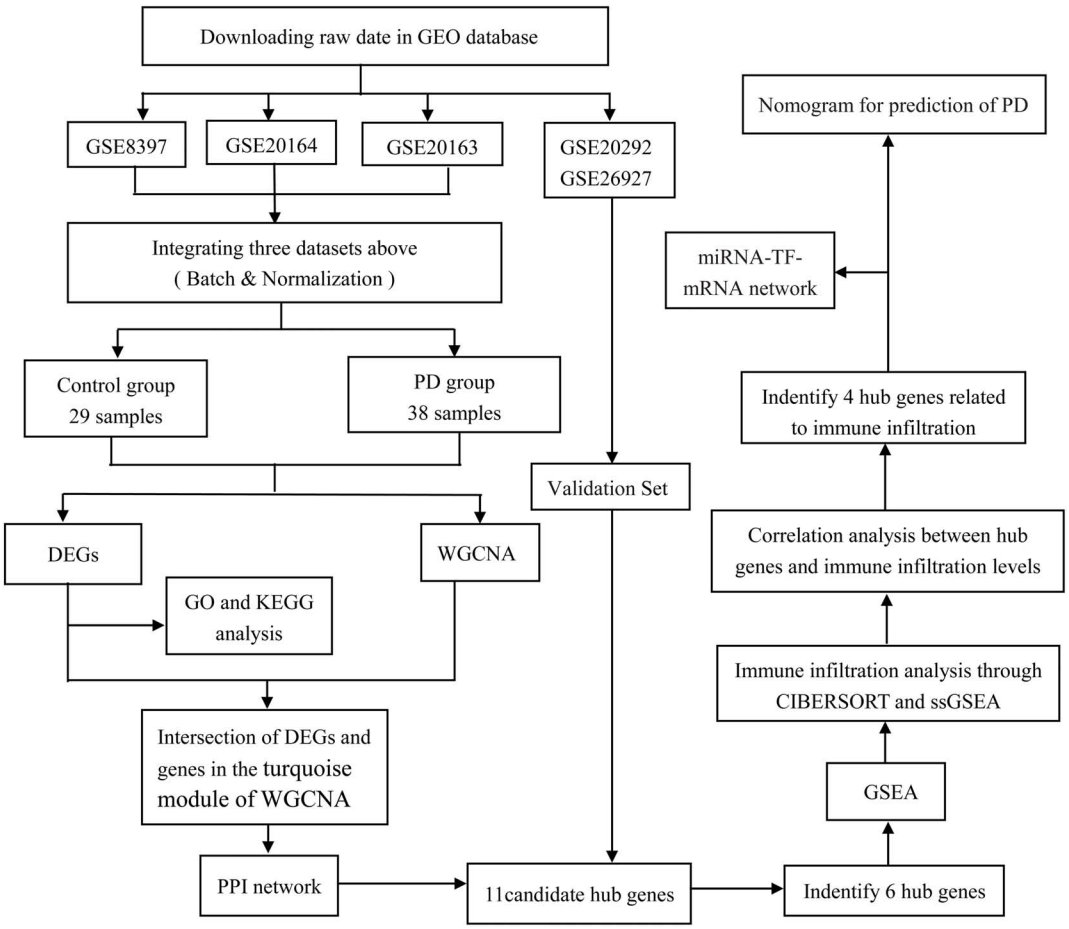

FIGURE 1
Flowchart of the study. GEO, gene expression omnibus; DEGs, differentially expressed genes; WGCNA, weighted gene co-expression network analysis; ssGSEA, single-sample gene set enrichment analysis; GSEA, gene set enrichment analysis; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein–protein interaction; PD, Parkinson's disease; TF, transcriptional factor.

database (Szklarczyk et al., 2019). In addition, we selected PPI interaction pairs with a significance cutoff of interaction score over 0.4 while hiding disconnected nodes in the network. Finally, the results of the PPI network were visualized in Cytoscape (Shannon et al., 2003) (version 3.9.1). We selected the CytoHubba (version 0.1) plugin in Cytoscape by nine algorithms, namely, maximal clique centrality (MCC), maximum neighborhood component (MNC), node connection degree (Degree), edge percolated component (EPC), BottleNeck, closeness, radiality, stress, and betweenness, to detect the top 30 genes by each approach from the PPI network. Then, the number of nodes for each gene (PPICount) was calculated, and the top 30 genes with the largest nodes were obtained in *R* software. For this study, nine approaches of CytoHubba and PPIcount were used to screen candidate hub genes by the "UpSetR" package (Gehlenborg, 2019) (version 1.4.0) in *R*.

Identification of hub genes and diagnostic implications of hub genes for PD

Expression of candidate hub genes was extracted from GSE20292 and GSE26927. The difference between PD and normal samples of each candidate hub gene was calculated and visualized by the "ggpurb" package (Kassambara, 2020). Candidate hub genes that were statistically significant in both training and validation sets were considered hub genes. A p-value of <0.05 was considered significant.

To evaluate the ability of the hub genes in both the training and validation sets to identify PD, the "pROC" package (Robin et al., 2011) was used to conduct receiver operating characteristic (ROC) curve analysis. The area under the curve (AUC) value was used to examine the diagnostic effectiveness in discriminating PD from control samples in both training and validation sets.

Single-gene gene set enrichment analysis (GSEA)

Through the median expression value of six hub genes (SYT1, GAP43, SNAP25, GRIA1, NEFL, and NEFM), we divided the 38 PD substantia nigra tissues into low- and high-expression groups based on each hub gene. Then, to further explore the function of hub genes, single-gene GSEA was implemented by the ordered gene expression matrix based on the Pearson correlation between each hub gene and other genes in *R* software using the "clusterProfiler" (Wu et al., 2021) and "enrichplot" (Yu, 2019) packages. A p-value of <0.05 was considered significant.

Evaluation of immune cell infiltration by ssGSEA and CIBERSORT

Single-sample gene set enrichment analysis (ssGSEA) was implemented to estimate infiltration levels of 16 immune cells and 13 immune functions on the basis of expression profiling between the PD and control samples using the "GSVA" package (version 1.40.1) (Hänzelmann et al., 2013). The "pheatmap" package (version 1.0.12) (Kolde, 2019) was used to visualize the heatmap of 29 immune cells and immune functions. The correlation heatmap between risk scores and the scores of 16 immune cells and 13 immune functions was generated using the "corrplot" package (Version 0.90) (Wei and Simko, 2021). Boxplots were separately generated using the "ggpubr" package (version 0.4.0) (Kassambara, 2020) and the "reshape2" package (Wickham, 2007). We used Spearman correlation analysis to reveal the correlations between the candidate hub genes and 29 immune cells and immune functions.

In addition, the CIBERSORT algorithm (Newman et al., 2015) was applied to quantify and calculate the proportion of 22 types of infiltrating immune cells among the merged expression profile; CIBERSORT filters the samples at $p < 0.05$. A bar plot was generated to show the percentage of 22 types of immune cells in each sample. The heatmap, violin plot, and correlation heatmap were generated using the "pheatmap" package (Kolde, 2019), "vioplot" package (version 0.3.7) (Adler and Kelly, 2020), and "corrplot" package (Wei and Simko, 2021) by the R program, respectively.

Correlations between the candidate hub genes and 20 types of immune infiltrations were calculated and visualized by using Spearman correlation analysis. Based on ssGSEA and CIBERSORT, four hub genes that were most relevant to immune infiltration were selected.

Construction of a nomogram model for PD

We established a nomogram model based on four immune-related biomarker genes for predicting the occurrence of PD using the "rms" package (Harrell, 2021). The accuracy of the nomogram was assessed through calibration curve analyses. In addition, the AUC value was utilized to quantify the predictive performance of the nomogram model based on ROC curve analyses using the "ROCR" package (Sing et al., 2005).

Analysis of transcription factors (TFs) and miRNAs of immune-related hub genes

We searched the target miRNAs of immune-related biomarker genes in the miRWalk (Dweep et al., 2011), RNAInter (Kang et al., 2022), and TargetScan (Agarwal et al., 2015) databases and retained common miRNAs. Moreover, we determined the transcription factors (TFs) for the hub genes in the Enrichr database[4] and screened the results with a p-value < 0.05. Finally, a miRNA-TF-mRNA regulatory network was constructed using Cytoscape.

4 http://amp.pharm.mssm.edu/Enrichr/

Statistical analysis

We used version 4.2.0 of *R* software (limma, ggpurb, pheatmap, violplot, corrplot package, and so on) for all statistical analyses. Student's t test was applied to compare the mean difference between groups. Correlation between variables was determined using Pearson's or Spearman's correlation test. Two-tailed p-values < 0.05 were considered significant.

Results

Identification of DEGs

Differentially expressed genes of the integrated dataset were analyzed using the "limma" package. A total of 319 genes were differentially expressed between 38 PD samples and 29 normal substantia nigra tissue samples, with 45 genes being upregulated and 274 downregulated. The volcano plot and heatmap of DEGs are shown in **Figures 2A, B**, respectively.

GO and KEGG analyses of DEGs

Differentially expressed genes were assessed through GO and KEGG analyses to explore the biological functions associated with PD. KEGG enrichment analysis results showed that DEGs are mainly related to synaptic vesicle cycle, phagosome, collecting duct acid secretion, gap junction, GABAergic synapse, and Parkinson's disease, among others (**Figures 3A–C** and **Supplementary Table 2**). GO enrichment analysis indicated DEGs to be associated with neurotransmitter transport, vesicle-mediated transport in synapse, synaptic vesicle cycle, regulation of neurotransmitter levels, and regulation of trans-synaptic signaling, among others, in biological processes (BP) analysis. Cellular component (CC) analysis showed the DEGs to be mainly enriched in presynapse, neuronal cell body, transport vesicle, distal axon, and exocytic vesicle, among others. The top five significant terms enriched in molecular function (MF) analysis were structural constituent of cytoskeleton, ATPase-coupled ion transmembrane transporter activity, ATPase activity (coupled to transmembrane movement of ions, rotational mechanism), proton-transporting ATPase activity (rotational mechanism), and GTPase activity (**Figure 3D** and **Supplementary Table 2**).

Construction of a weighted co-expression network and identification of hub modules

The variance of all genes in integrated dataset was calculated, and the top 5000 variant genes were selected for analysis using the "WGCNA" package. We performed clustered hierarchically analysis of all samples to remove outliers by setting the threshold value to 50, and no outlier samples were removed (**Figure 4A**). The power of $\beta = 7$ (scale-free $R^2 = 0.885$) was selected as the soft threshold to ensure a scalefree network (**Figure 4B**). As shown in **Figure 4C**, 12 modules were finally identified after merging similar modules in the cluster tree by setting the threshold to 0.25. The correlation between module eigengene (ME) values and clinical features is presented

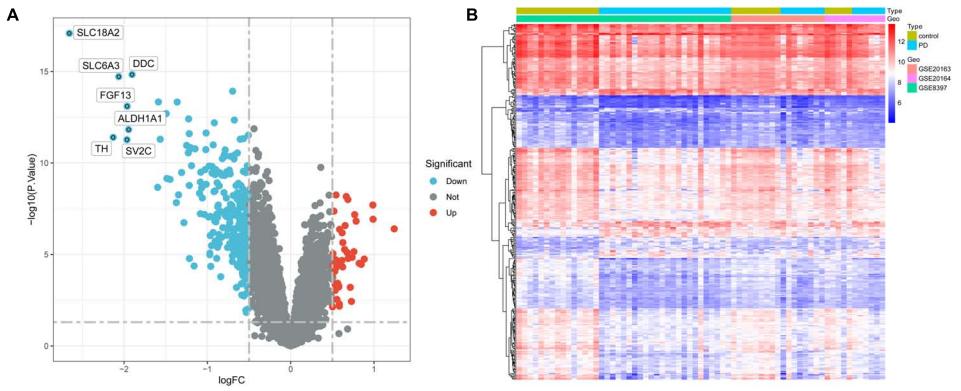

FIGURE 2
Identification of DEGs in the integrated dataset. **(A)** Volcano plot of all DEGs. The tomato nodes represent upregulated DEGs with p-value < 0.05 and logFC > 0.5; the cyan nodes represent downregulated DEGs with p-value < 0.05 and logFC < −0.5. **(B)** Heatmap of DEGs in PD samples vs. normal samples. Each row of the heatmap represents one gene, and each column represents one sample. The red and blue colors represent gene expression levels corresponding to upregulated and downregulated expression. DEGs, differentially expressed genes; FC, fold change; PD, Parkinson's disease.

in Figure 4D. Seven modules exhibited significant correlation with PD ($p < 0.05$), and the turquoise module represented the highest negative correlation with PD compared ($r = 0.62$; $p = 3E\text{-}8$). The correlation between different modules was illustrated through the cluster diagram and heatmap (Figure 4E). Moreover, 400 genes were randomly selected in *R* software to draw a heatmap of the weighted gene co-expression correlations to further illustrate the correlation between different modules (Figure 4F). GS and MM of all modules were calculated to draw scatterplots. As expected, a significant correlation existed in the turquoise module MM and GS ($|\text{cor}| = 0.72$, $p < 1E\text{-}200$, Figure 4G), including 1593 genes, which were most significantly associated with PD and selected for further analysis.

PPI network and candidate hub genes

Venn analysis was performed based on the DEGs screened from the integrated dataset and the genes in turquoise module, and 263 common genes (CGs) genes were found (Figure 5A). The PPI network of 263 CGs was constructed to investigate the relationships of those genes at the protein level and obtain candidate hub genes using Cytoscape according to the STRING database (Figure 5B). The top 30 genes with the largest number of adjacent nodes were screened as PPICount, including SNAP25, SYN1, SYT1, SNCA, GAP43, GRIA1, STXBP1, RAB3A, TH, and CALB1 (Figure 5C and Supplementary Table 3). Then, we applied nine algorithms to calculate the score of each node gene using the CytoHubba plug-ins of Cytoscape and selected the top 30 genes of each algorithm (Supplementary Table 3). Finally, we screened 11 intersected genes (SNAP25, SNCA, SYT1, ENO2, GRIA, STXBP1, SYN1, TH, NEFM, GAP43, and NEFL) through 10 approaches by "UpSet" in the *R* package (Figures 5D, E). All these intersected genes that were defined as candidate hub genes were found to be downregulated.

mRNA expression of hub genes in patients

The mRNA expression results for the candidate hub genes in the GSE20292 indicated SNAP25, SNCA, SYT1, GRIA, NEFM, GAP43, and NEFL to be expressed at significantly lower levels in the PD group than the control group (Figure 6A, $p < 0.05$). No significant differences were found in mRNA expression of ENO2, STXBP1, SYN1, and TH. In addition, we verified expression of marker genes in the GSE26927 dataset, and SNAP25, SYT1, GRIA, NEFM, GAP43, NEFL, and TH expression was significantly lower in PD patients than normal samples (Figure 6B, $p < 0.05$). Finally, SNAP25, SYT1, GRIA, NEFM, GAP43, and NEFL, which could effectively differentiate PD patients from controls ($p < 0.05$), were selected and considered as the hub genes and potential biomarkers for PD.

Diagnostic effectiveness of hub genes for PD

Receiver operating characteristic curves analyses were used to examine the accuracy of the six potential biomarker genes to diagnose PD, with AUC values of 0.896 (GAP43), 0.837 (GRIA1), 0.775 (NEFL), 0.762 (NEFM), 0.740 (SNAP25), and 0.890 (SYT1), respectively, in the training set (Figure 7A). As shown in Figure 7B, the AUC values of GAP43, GRIA1, NEFL, NEFM, SNAP25, and SYT1 were 0.722, 0.808, 0.813, 0.773, 0.788, and 0.788, respectively, in the validation set (GSE20292). Figure 7C indicates that the AUC for all genes was greater than 0.7 in the GSE26927 dataset. The above evidence suggests that GAP43, GRIA1, NEFL, NEFM, SNAP25, and SYT1 can be used as diagnostic biomarkers for differentiating PD patients from normal controls.

Single-gene GSEA

Parkinson's disease substantia nigra tissues were divided into two subgroups based on the median expression of the six hub genes. Then, we utilized single-gene GSEA to explore potential signaling pathways of the potential biomarker genes. The top five pathways enriched for potential biomarker genes are illustrated in Figures 7D–I. After comprehensive analysis, we found low NEFM expression to be associated with immune responses (B-cell receptor signaling pathway, Th1 and Th2 cell differentiation,

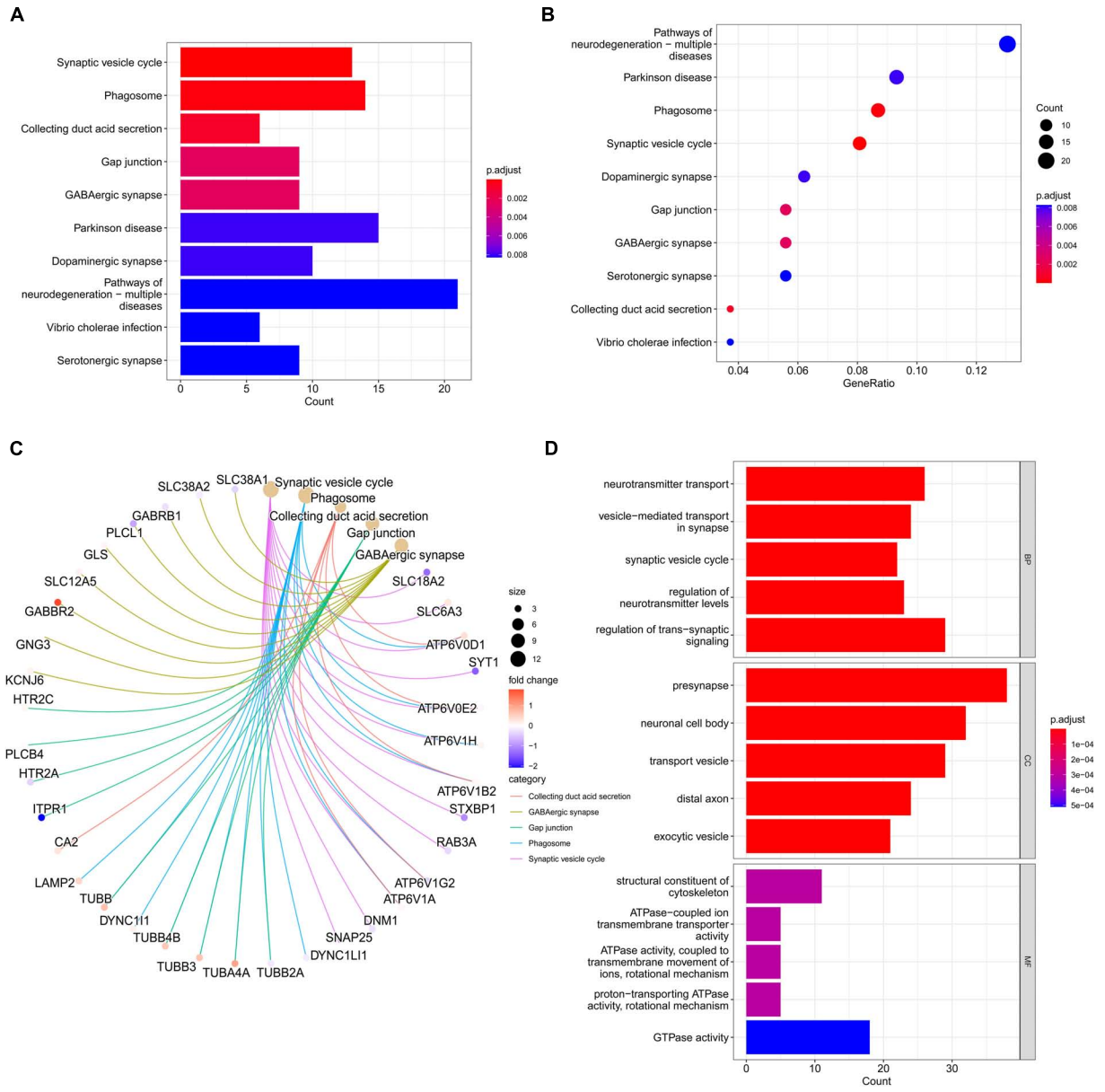

FIGURE 3
Gene ontology and KEGG pathway enrichment analyses of DEGs. **(A)** Barplot of KEGG analysis based on the obtained 263 genes. **(B)** The bubble diagrams show the top ten significantly enriched terms in KEGG analysis. The *X*-axis is the GeneRatio (gene count/gene size) of the term, and the *Y*-axis denotes the name of the term. The darker the color is, the smaller the adjusted *p*-value is. **(C)** Subnetwork showing the top five KEGG pathways and related genes. **(D)** The top 5 terms for BP, CC, and MF with $p < 0.05$ are shown. DEGs, differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, gene ontology; BP, biological processes; CC, cell component; MF, molecular function.

etc.) and various immunologic disease pathways (systemic lupus erythematosus, intestinal immune network for IgA production, etc.), and the neuroinflammation response (NF-kappa B signaling pathway, IL-17 signaling pathway, etc.). The remaining five hub genes are also involved in several immune response pathways or various immunologic disease pathways, including viral protein interaction with cytokine and cytokine receptor (GAP43, GRIA1, NEFL, SNAP25, and SYT1), primary immunodeficiency (GAP43, GRIA1, NEFL, SNAP25, and SYT1), autoimmune thyroid disease (GRIA1, NEFL, SNAP25, and SYT1), and maturity-onset diabetes of the young (GAP43, NEFL, and SNAP25), among others. The details of the KEGG pathway results for the six hub genes are shown in Supplementary Table 5. The above results suggest that these potential marker genes may influence PD development through immune-related pathways.

ssGSEA of immune infiltration

We evaluated the samples in the integrated dataset by ssGSEA to quantify the immune infiltration and enrichment scores of 29 immune cells and immune-related functions. A heatmap was drawn to investigate correlations between substantia nigra tissue samples with or without PD and immune cells (Supplementary Figure 1A). In the PD group, immune cells such as B cells, neutrophils, plasmacytoid dendritic cells (pDCs), T follicular helper

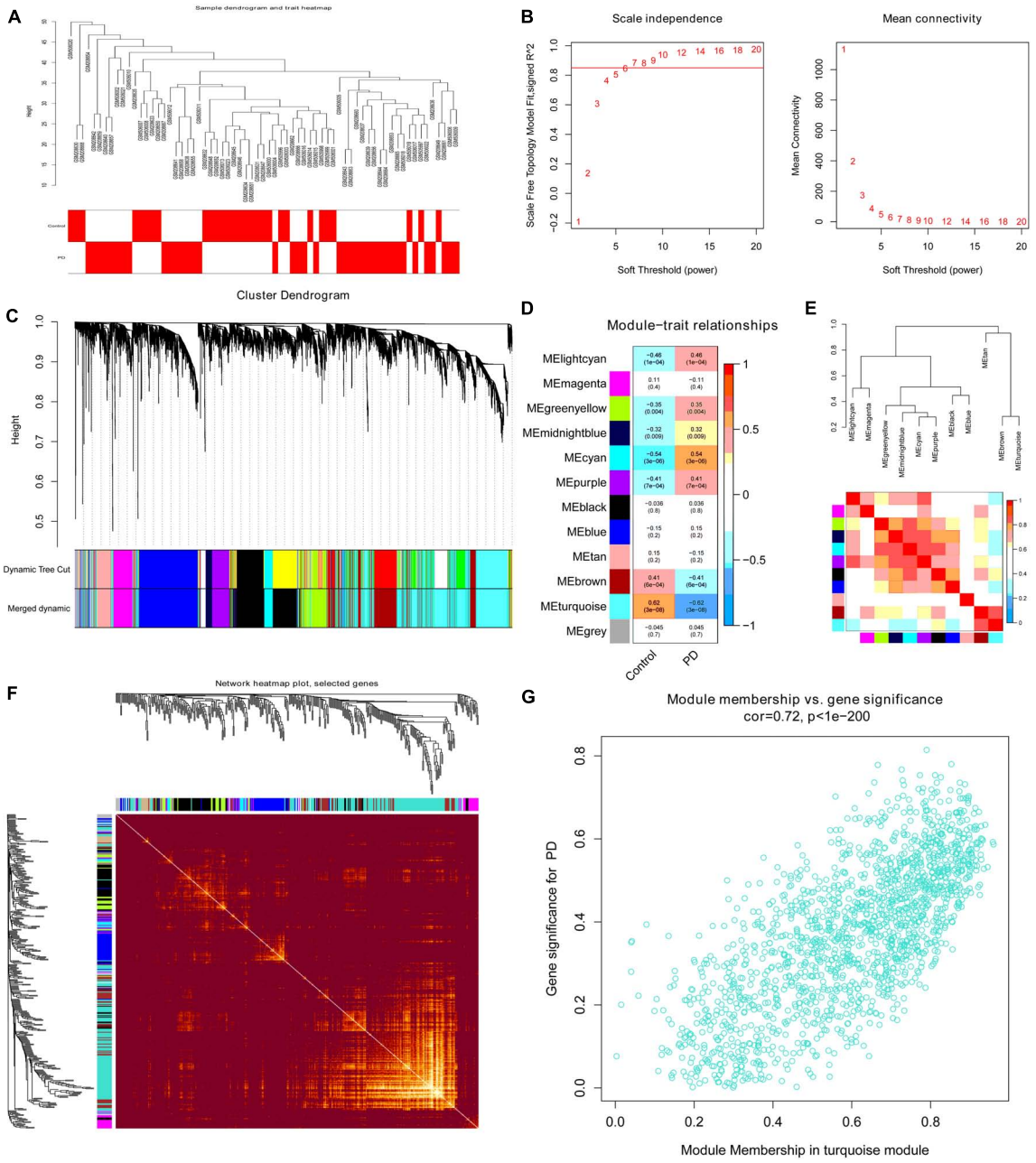

FIGURE 4
The WGCNA process for the integrated dataset. **(A)** Clustering dendrogram of 38 PD substantia nigra tissue and 29 normal substantia nigra tissue gene expression patterns. **(B)** Analysis of the scale-free fit index *(left)* and the mean connectivity *(right)* for various soft-thresholding powers; the power value β was set as 7 for further analysis. **(C)** Clustering dendrograms of 5000 genes based on a dissimilarity measure (1-TOM). Seventeen co-expression modules were constructed with various colors under the gene tree, and similar modules were merged into twelve modules with a height cutoff of 0.25. Each color represents one module. **(D)** Heatmap of associations between modules and clinical traits. Correlation coefficients and p-values are shown in each cell, which were obtained by the intersection of rows and columns. The turquoise module correlated significantly with PD. **(E)** Visualization of the eigengene dendrogram and eigengene adjacency heatmap. Red indicates more similarity, and blue indicates less similarity. **(F)** Visualization of 400 random genes from the WGCNA network using a heatmap plot to depict the TOM among all modules included in the analysis. A redder background indicates a higher module correlation. **(G)** Scatter plot of module membership vs. gene significance for PD in the turquoise module. WGCNA, weighted gene co-expression network analysis; TOM, topological overlap matrix; PD, Parkinson's disease.

cells (Tfhs), tumor-infiltrating lymphocytes (TILs), and regulatory T cells (Tregs) had higher ssGSEA scores ($p < 0{:}05$, Figure 8A). Moreover, the box plot illustrated that immune pathways such as APC_co_stimulation and CCR were associated with elevated ssGSEA scores in the PD group, whereas Type_I_IFN_Reponse immune function was lower in the PD group ($p < 0{:}05$, Figure 8B). The corHeatmap of immune-related functions result showed that check-points were positively related with T_cell_co-stimulation and T_cell_co-inhabition ($r = 0.84$ and 078, respectively, Supplementary Figure 1B). Similarly, parainflammation had a significant positive correlation with CCR ($r = 0.78$, Supplementary Figure 1B). Immune cells such as neutrophils, pDCs, T_helper_cells were positively related with TILs ($r = 0.77$, 0.73, and 0.73, respectively, Supplementary Figure 1C), whereas aDCs were negatively related to

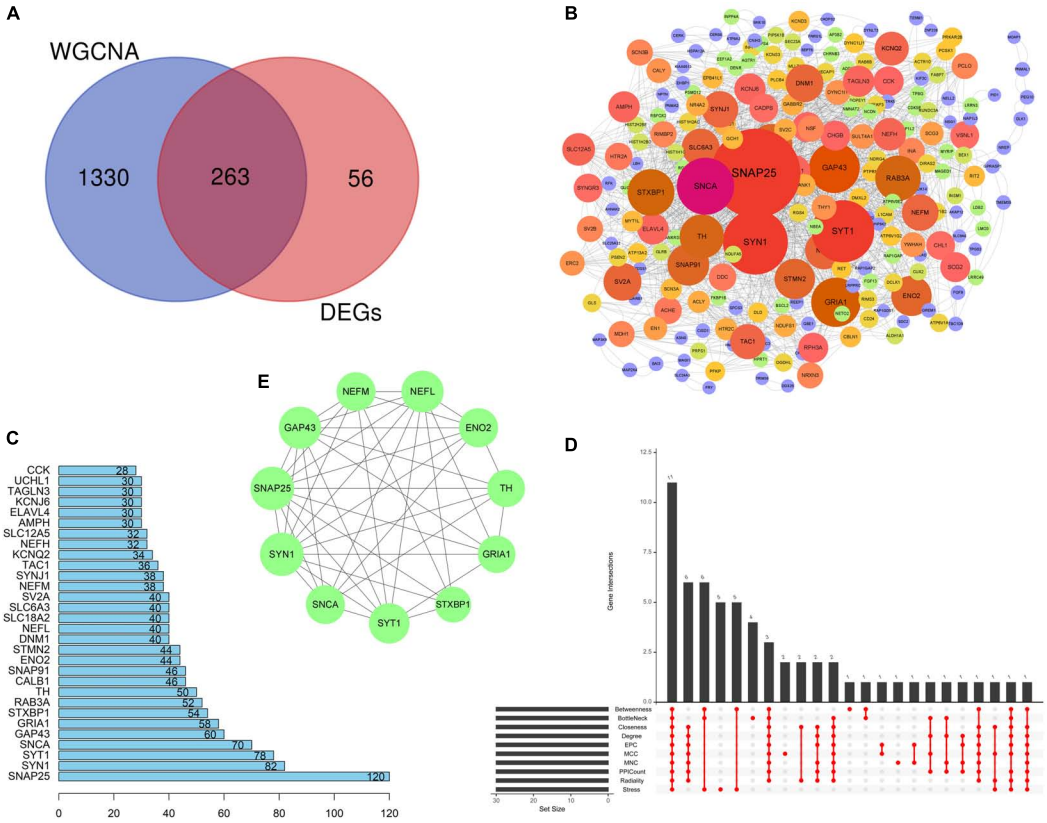

FIGURE 5
Protein–protein interaction network and identification of candidate hub genes. **(A)** Venn plot showing the intersection between the DEGs and genes in the turquoise module, and 263 CGs were obtained. **(B)** PPI network of CMs by Cytoscape. The size and gradient color of circles are adjusted by the degree value, which reflects the connectivity between nodes. The size of circles has a positive correlation with the degree value. **(C)** PPI Count, revealing the number of adjacent nodes of the top 30 genes (ranked from low to high) based on the PPI network. **(D)** UpSet plot showing the intersection of ten algorithms, namely, MCC (top 30), MNC (top 30), Degree (top 30), EPC (top 30), BottleNeck (top 30), Closeness (top 30), Radiality (top 30), Stress (top 30), and Betweenness (top 30), and PPI Count (top 30). **(E)** Eleven candidate hub genes. All these candidate hub genes were found to be downregulated. PPI, protein–protein interaction; CGs, common genes; MCC, maximal clique centrality; MNC, maximum neighborhood component; EPC, edge percolated component.

Tregs ($r = -0.42$, Supplementary Figure 1C). Correlation analysis showed that most of immune cells had a negative correlation with all of the hub genes (Figure 8C and Supplementary Table 4).

CIBERSORT analysis of immune infiltration

We performed CIBERSORT analysis to assess infiltrating levels of 22 immune cells in PD samples and normal samples by *R* software. The heatmap showed the relationship between immune cells and all of the samples filtered (Supplementary Figure 2A). The correlation heatmap of 22 types of immune cells demonstrated that T cells regulatory were positively related with T cells CD8 ($r = 0.56$, Supplementary Figure 2B) but that activated dendritic cells and M1 macrophages had a negative correlation ($r = -0.70$); activated mast cells were negatively related to resting mast cells ($r = -0.69$). The violin plot of the immune cells showed that PD patients had a higher level of neutrophils, activated NK cells and monocytes ($p < 0:05$, Figure 9A). Supplementary Figure 2C illustrates the proportion of each type of immune cell in each sample. Pearson correlation analysis revealed a negative correlation for neutrophils with four downregulated genes, including NEFM, GRIA1, SYT1, and GAP43 (Figure 9B and Supplementary Table 4). Combined with above two methods, four genes (NEFM, GRIA1, NEFL, and SYT1) were strongly negatively related to immune infiltration, especially neutrophils, and regarded as marker hub genes related to immune infiltration.

Construction of a nomogram model to predict occurrence of PD

The marker hub genes, namely, NEFM, GRIA1, NEFL, and SYT1, were then used to construct a nomogram model to predict PD occurrence (Figure 10A). We also utilized calibration plots to confirm the performance of this nomogram model, with a sufficient degree of fit for predicting the incidence of PD (Figure 10B). The AUC was 0.905, suggesting that the predictive model had high predictive accuracy (Figure 10C).

MiRNA–TF–mRNA regulatory network analysis based on marker genes

After miRNA and TF pairs were predicted based on the four marker hub genes, we constructed a miRNA-TF–mRNA network, including 70 miRNAs common in three databases (miRWalk,

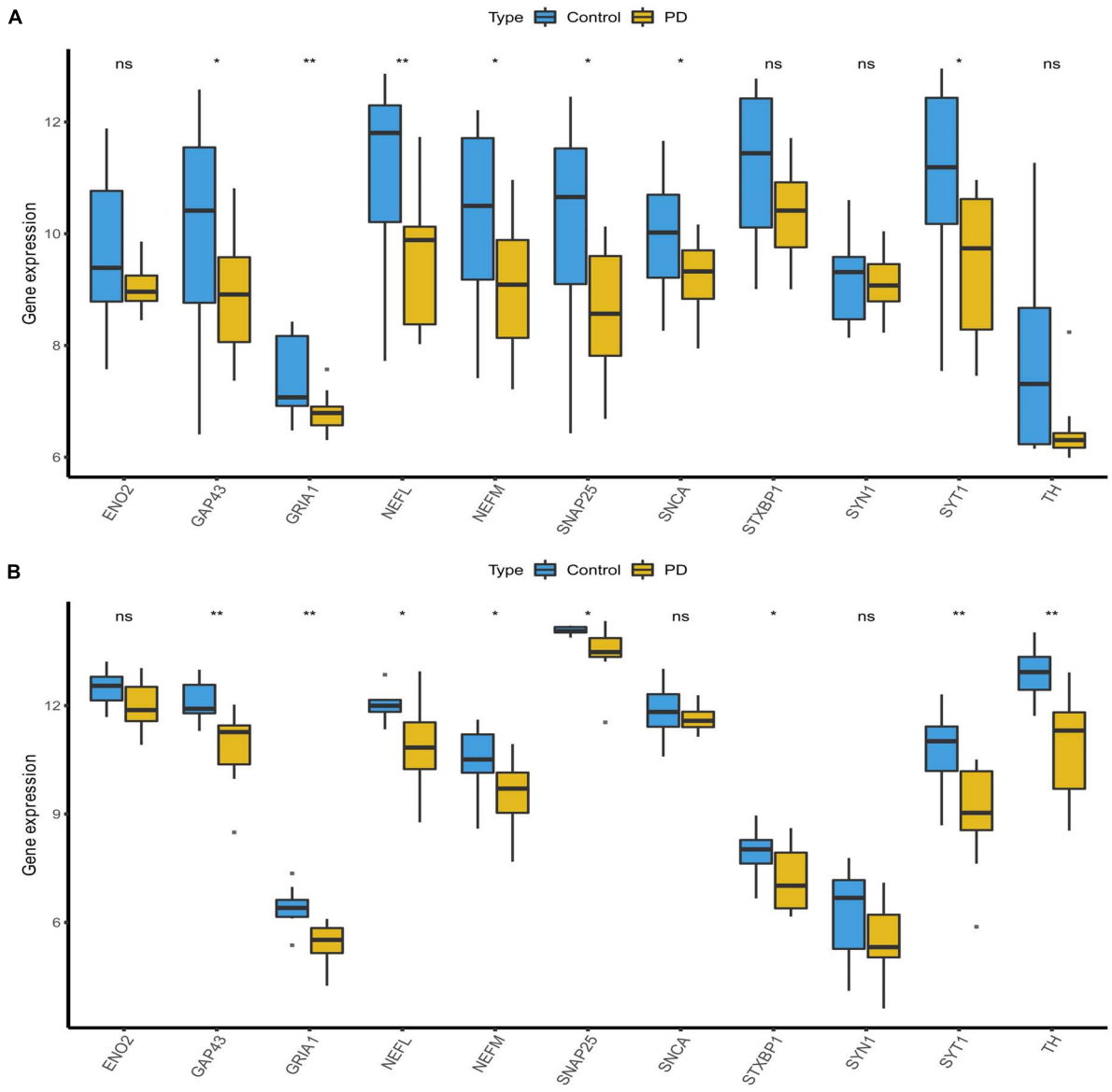

FIGURE 6
Validation of candidate hub genes. **(A)** Validation of candidate hub genes in the GSE20292 dataset. **(B)** Validation of candidate hub genes in the GSE20681 dataset (*$p < 0.05$, **$p < 0.01$). PD, Parkinson's disease.

RNAInter and TargetScan database), 10 TFs, and 4 downregulated marker genes. The regulatory network included 69 nodes and 81 edges, as established through Cytoscape 3.9.1 (Figure 11). Within the network, expression of all marker genes is regulated by hsa-miR-92a-3p, hsa-miR-92b-3p, and hsa-miR-25-3p.

Discussion

Parkinson's disease is the most common movement disorder, though its mechanisms have not been fully clarified. This present study used bioinformatics analysis to identify immune infiltration-related marker genes, which were used to construct a nomogram model for the early prediction of PD and miRNA-TF-mRNA network analysis to explore potential therapeutic targets for PD. We obtained a total of 319 DEGs based on the integrated dataset. WGCNA was performed and confirmed the turquoise module as the key module correlating with PD. Intersecting DEGs and genes in the turquoise module were obtained; a total of 263 CMs were screened, which were then used for PPI network construction. We then used 10 approaches based on Cytoscape software and obtained 11 candidate hub genes. Moreover, those 11 genes were validated in the GSE20292 and GSE26927 datasets, with 6 genes, namely, SNAP25, SYT1, GRIA, NEFM, GAP43, and NEFL, screened. ROC analysis demonstrated the effective diagnostic of those six hub genes in PD, suggesting potential for distinguishing PD patients from normal controls.

Six hub genes were selected for further single-gene GSEA by dividing the PD samples into two subgroups based on median expression of the six hub genes. We found that low expression of hub genes was mainly related to the immune response and immune diseases. Then, we analyzed differences in immune cell infiltration level between the substantia nigra of PD samples and healthy brain tissue. Our result indicates that the proportion of neutrophils, monocytes, and activated NK cells were significant higher in PD

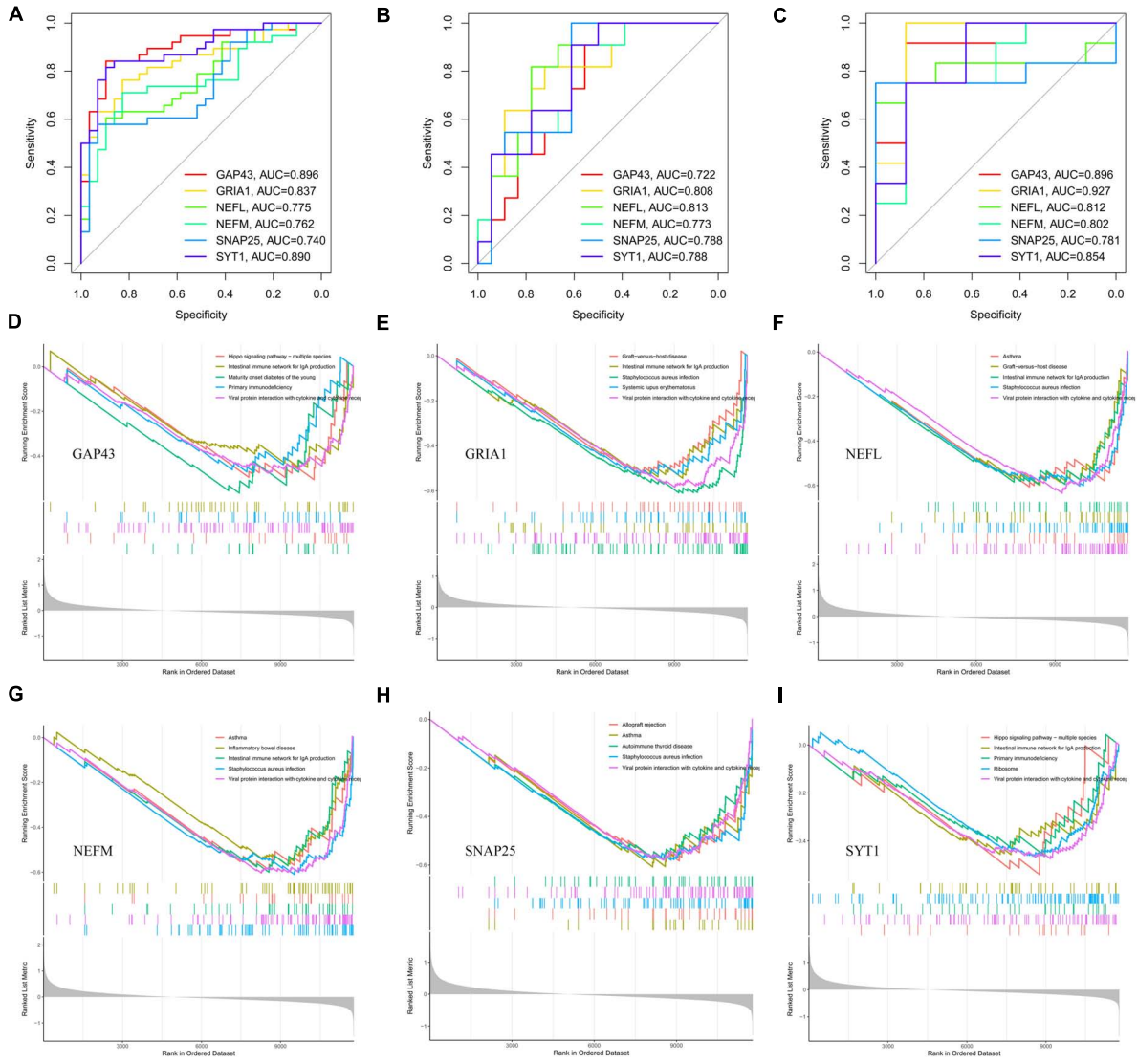

FIGURE 7
Diagnostic efficacy of potential biomarkers (GAP43, GRIA1, NEFL, NEFM, SNAP25, and SYT1) for prediction of PD and GSEA based on expression levels of those potential biomarkers. ROC analysis of six hub genes (GAP43, GRIA1, NEFL, NEFM, SNAP25, and SYT1) for diagnosing PD in the integrated dataset **(A)** and the validation sets GSE20292 **(B)** and GSE26927 **(C)**. Single-gene GSEA-KEGG pathway analysis of GAP43 **(D)**, GRIA1 **(E)**, NEFL **(F)**, NEFM **(G)**, SNAP25 **(H)**, and SYT1 **(I)**. PD, Parkinson's disease; GSEA, gene set enrichment analysis; ROC, receiver operator characteristic.

samples than in controls by CIBERSORT analysis. The ssGAEA algorithm also showed that B cells, neutrophils, pDCs, Tfhs, TILs, Treg, APC_co_stimulation, and CCRs had higher ssGSEA scores in the PD group but that immune function of Type_I_IFN_Reponse was lower in the PD group. The above results revealed that the immune cells (DC, NK cells, T cells, CCRs, neutrophils, and monocytes) play a vital role in the pathogenesis of PD.

Overexpressed α-synuclein induces infiltration of pro-inflammatory monocytes through C-C chemokine receptor type 2 (CCR2) in the CNS, whereas deletion of CCR2 prevents this and subsequent dopaminergic neuronal death in the progression of PD (Harms et al., 2018). Dysregulation of peripheral human monocytes in PD has also been observed (Grozdanov et al., 2014), subsequently inducing a higher infiltration level of monocytes in the cerebrospinal fluid than in the control group (Schröder et al., 2018). The correlation of circulating monocytes and immune cells may be that chemokines lead to increased blood–brain barrier permeability, invasion of peripheral monocytes into the CNS, and infiltration of immune cells (Harms et al., 2018). However, the potential role monocytes in the pathogenesis of PD in humans has not yet been investigated, and further research is warranted.

Increasing evidence suggests that significantly increased T cells (CD8 and CD4) are present in the substantia nigra of PD patients compared with control subjects (Theodore et al., 2008; Harms et al., 2017), and T-cell responses were connected with dopaminergic neuron cell loss (Brochard et al., 2008; Lira et al., 2011; Williams et al., 2021). A growing body of evidence shows that Treg cells, Foxp3-expressing CD4+ CD25+ T lymphocytes, play an important role in immune regulation (Noack and Miossec, 2014). Treg cells protect neurons by inhibiting microglial oxidative stress and inflammation in the central nervous system (CNS) (Reynolds et al., 2007). Huang et al. (2017) revealed that dopaminergic neuronal protection of Treg cells is achieved *via* interaction between CD47 and signal regulatory protein α

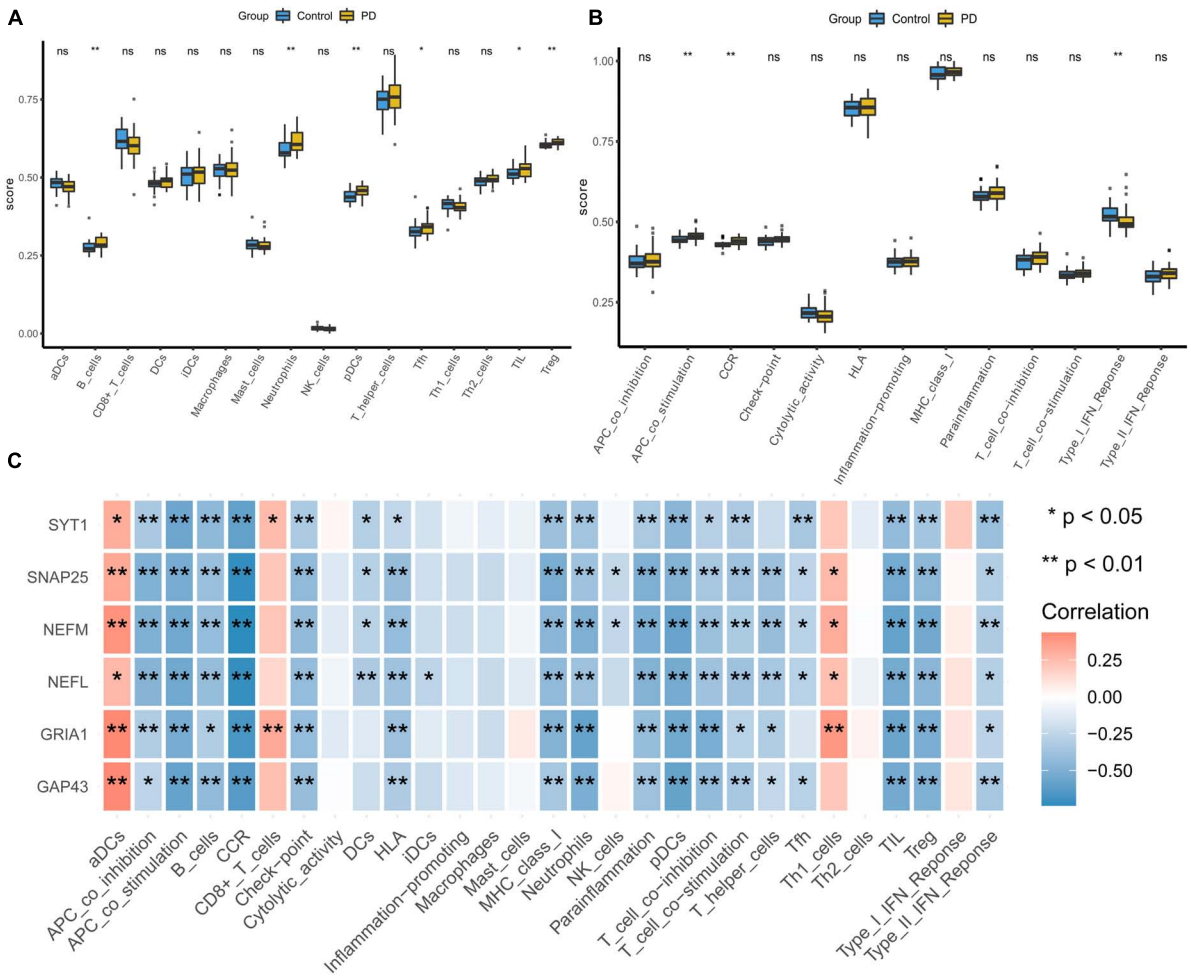

FIGURE 8
Visualization and evaluation of immune infiltration levels based on ssGSEA. **(A)** Comparison of 16 immune cells between PD samples and control samples. **(B)** Box diagram of different immune function expression levels in the PD and control groups. **(C)** Pearson correlation analysis of 29 types of immune cells and immune-related functions with hub genes (*$p < 0.05$ and **$p < 0.01$). PD, Parkinson's disease.

(SIRPA) in PD processes. Moreover, dysfunction of Treg cells decreases the ability to suppress the function of effector T cells in PD, which may accelerate its progression (Saunders et al., 2012).

T follicular helper cells, which are essential in B-cell activation, are a specialized subtype of CD4+ T cells that are expressed at significantly higher levels in PD patients than in controls (Zhao et al., 2020). In addition, Tfh cells promote Th-17-induced neuroinflammation by inducing inflammatory B-cell responses in the CNS and increasing disease severity (Quinn et al., 2018). Such progression of disease severity can be reduced by inhibiting Tfh cells in the CNS.

Combining two methods, ssGSEA and CIBERSORT, we found that neutrophils were differentially expressed between PD samples and normal samples. The PD-relevant immune response is related not only to changes in brain immune cells and neuroinflammation but also to changes in the peripheral blood system. A recent study reported higher neutrophil counts in PD compared to controls and a decreased lymphocyte count (Jensen and Jacobs, 2021). The neutrophil-to-lymphocyte ratio, which has proven prognostic value in infection, inflammatory diseases and several types of cancers, is significantly higher in PD patients than the peripheral immune profile (Akıl et al., 2015; Muñoz-Delgado et al., 2021). Such an elevated neutrophil population leads to mitochondrial changes, increased markers of oxidative stress, and overexpression of nitric oxide, suggesting that neutrophils participate in the pathological progression of PD (Vitte et al., 2004).

Plasmacytoid dendritic cells can regulate the immune response by producing large amounts of cytokines, particularly type I interferons, which induce B cells to differentiate into plasma cells and produce immunoglobulin (Jego et al., 2003; Poeck et al., 2004; Menon et al., 2016), activate NK-cell cytolytic activity (Colonna et al., 2004), and affect T-cell functions (Agnello et al., 2003). The former is supported by studies in other disease, whereas a direct influence of pDCs in PD remains to be confirmed.

Similarly, Alzheimer's disease (AD), the most common neurodegenerative disease, is also closely related to immune infiltration (Hu and Wang, 2021; Qian et al., 2022; Zhang et al., 2022). A previous study revealed that some specific immune cells in brain tissue, including Treg cells, activated NK cells, and neutrophils, were significantly more or less abundant in patients with AD than in healthy controls (Hu and Wang, 2021), which is consistent with the results of this study. The infiltration levels of some immune cells, such as pDCs, macrophages, and basophils, were altered in the brain

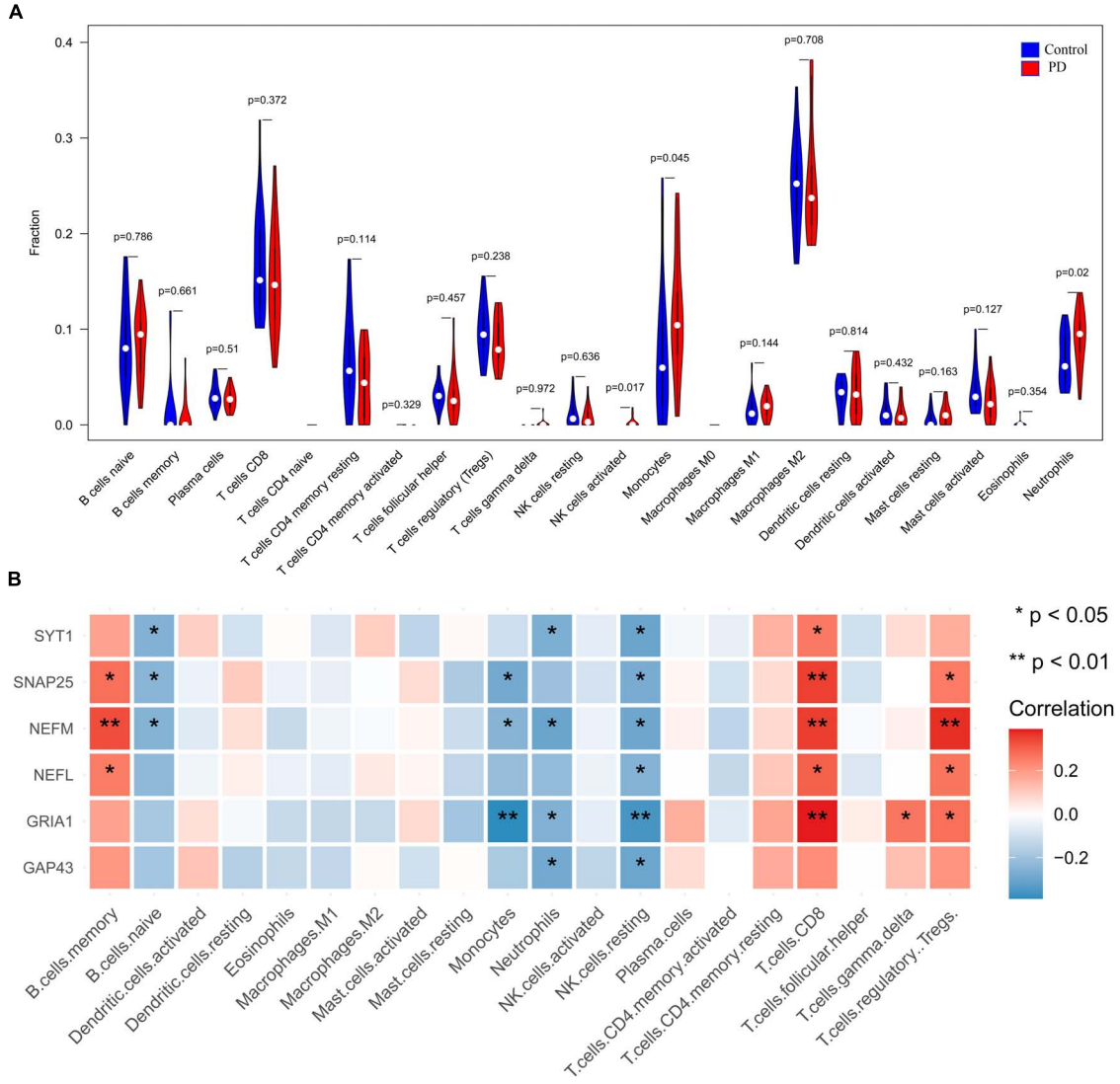

FIGURE 9
Visualization and evaluation of immune infiltration levels based on the CIBERSORT algorithm. **(A)** Comparison of 22 immune cells between PD samples and control samples. **(B)** Pearson correlation analysis of immune cell infiltration with hub genes (*$p < 0.05$ and **$p < 0.01$). PD, Parkinson's disease.

tissue samples of PD and AD patients compared to healthy controls (Qian et al., 2022; Zhang et al., 2022). The potential reasons for this were that the brain tissue samples were derived from different regions and that the pathological mechanisms of these two diseases are not completely the same. The roles of immune infiltration in neurodegenerative diseases still require further investigation.

Moreover, we identified the relationship between the six genes (NEFM, GRIA1, NEFL, SYT1, NEFL, and SNAP25) and immune cell type by CIBERSORT and ssGSEA, and NEFM, GRIA1, NEFL, and SYT1 were found to be closely related to immune cells. KEGG analysis results showed DEGs to be enriched in the synaptic vesicle cycle. Disorders of the synaptic vesicle cycle participate in the pathogenesis of PD and play a critical role in degeneration of dopaminergic neurons. Synaptotagmin-1 (SYT1), a potential target in treating nervous system disorders, regulates neuron exocytosis and the synaptic vesicle cycle (Mingazov and Ugrumov, 2016; Liu and Kaeser, 2019). It has been demonstrated that by sponging miR-34-5p, overexpression of SYT1 has a neuroprotective effect in a mouse model of PD (Shen et al., 2021).

Growth-associated protein-43 (GAP-43), also known as neuromodulin, a marker of synaptic formation and neuronal elongation, plays an essential role in the early stage of nervous system development. PD patients have significantly lower expression levels of GAP-43 in dopaminergic neurons than age-matched controls, which results in reduced regenerative capacity in dopaminergic neurons, as well as involvement of GAP43 downregulation in glial PD pathophysiology (Saal et al., 2017; Chung et al., 2020). Another study revealed that an enriched environment promotes GAP-43 upregulation to induce plastic brain changes and prevent dopaminergic cell loss on the progression of neuronal impairment related to PD (Yuan et al., 2018).

Emerging evidence supports that GluA1 (also known as GRIA1), a subunit of a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, mediates synaptic plasticity, thereby playing a critical role in brain function and dysfunction (Qu et al., 2021). The GluA1-homomeric form, a calcium-permeable AMPA receptor subtype, induces trafficking and insertion of AMPARs (AMPA receptors) in synapses (Zhang and Abdullah, 2013;

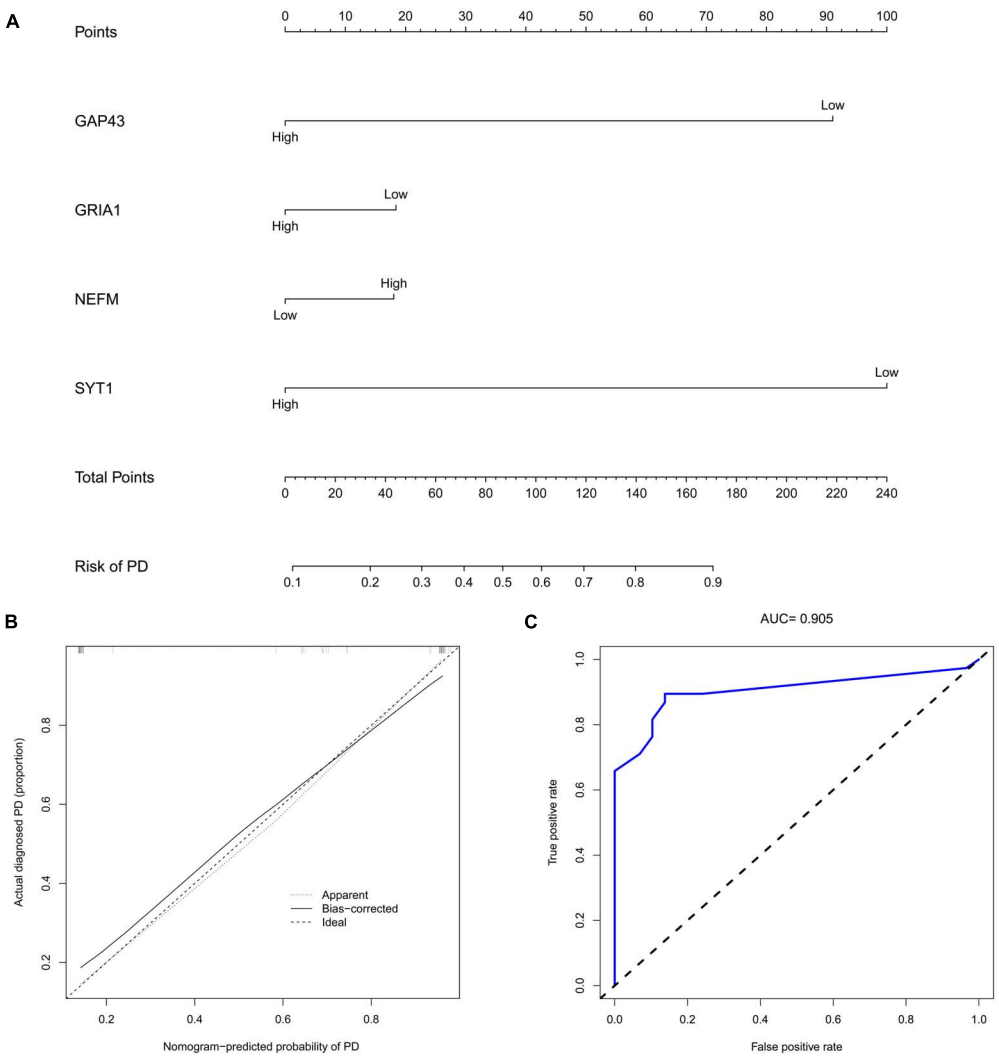

FIGURE 10
Construction of the nomogram. **(A)** Construction of a nomogram for immune-related hub genes (GAP43, GRIA1, NEFM, and SYT1) for predicting the occurrence of PD. **(B)** Calibration curve estimates the prediction accuracy of the nomogram for PD patients. **(C)** The area under the curve *(AUC)* was 0.905. PD, Parkinson's disease; AUC, area under the curve.

Tamano et al., 2018). Defects in regulated AMPAR trafficking can lead to movement disorders, which may be involved in the pathogenesis of PD (Tamano et al., 2018). One study demonstrated that the mechanism for the treatment of Alzheimer's disease (Qu et al., 2021) is mainly related to upregulation and phosphorylation of GluA1.

Neurofilament proteins, composed of a triplet of neurofilament medium chain (NFM), heavy chain (NFH), and light chain (NFL) proteins according to their molecular weight, are commonly used as reliable biomarkers for neurodegenerative pathology (Zucchi et al., 2020). It has been demonstrated that NEM, also known as NEFM, is linked to regulatory functions in dopaminergic neurotransmission (Kim et al., 2002) and is associated with the immune response (Barboni et al., 2014; Li et al., 2021). Increased levels of NEFM have been detected in various neurological diseases, such as brain damage (Martínez-Morillo et al., 2015), schizophrenia spectrum disorders (Runge et al., 2022), and amyotrophic lateral sclerosis (Häggmark et al., 2014). However, the association of NEFM with PD has not been reported previously and requires further investigation.

We also investigated the associations between four biomarker genes and AD. NEFM (Mirza and Rajeh, 2017; Hu et al., 2020) and SYT1 (Mirza and Rajeh, 2017) were notably downregulated in AD compared with control brain tissues in previous studies, demonstrating that these two genes are linked to the pathogenesis of AD. In a postmortem study, the hippocampal expression levels of GRIA1, GAP-43, and NEFM were significantly decreased in AD patients compared with controls (Chowdhury et al., 2020). In addition, the CSF levels of both GAP-43 and SYT1 significantly increased in patients with dementia due to AD, implying that these genes can potentially be used as biomarkers of synaptic dysfunction to predict the progression of AD (Öhrfelt et al., 2016; Qiang et al., 2022). The above evidence identifies four biomarker genes associated with AD; however, their roles in this disease still require further research.

Various biomarkers for early PD diagnosis have been proposed (Surguchov, 2022), but they remain investigational and need further confirmation. Recent studies have demonstrated that exosomes mediate the transfer of α-synuclein protein to brain cells, providing a potential mechanism for the propagation of pathological α-synuclein aggregation in brain cells and the acceleration of pathology in PD

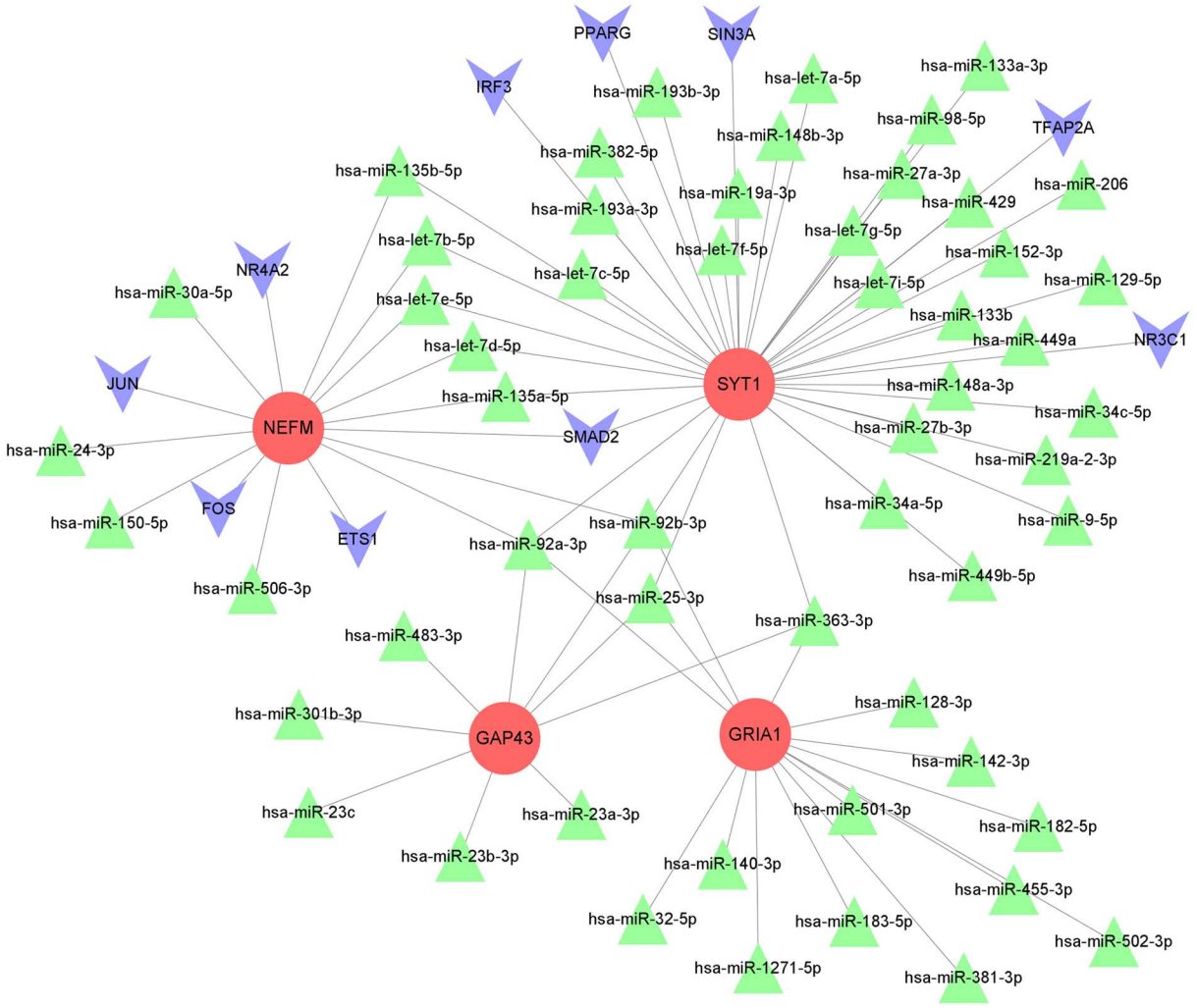

FIGURE 11
The miRNA-TF-mRNA interaction network. The red circles represent marker hub genes, the blue triangles represent miRNAs, and the green inverted cones indicate TFs. mRNAs, messenger RNAs; miRNAs, microRNAs; TFs, transcription factors.

(Pinnell et al., 2021; Valencia et al., 2022). α-Synuclein aggregation in CSF as detected by protein misfolding cyclic amplification (PMCA) and real-time quaking-induced conversion (RT-QuIC) had high diagnostic accuracy (AUC 0.93 and 0.89, respectively) in distinguishing PD patients from controls (Kang et al., 2019). Pathological α-synuclein in plasma neuron-derived exosomes can serve as a biomarker to differentiate PD patients from healthy controls (Kluge et al., 2021). Moreover, CNS-derived exosomes in plasma were significantly higher in PD patients than in controls, whereas the performance of plasma exosomal α-synuclein was only moderate (AUC 0.654). We should conduct further research to shed more light on this extraordinary phenomenon. Whether the four biomarker genes drive this discrepancy by interacting with or regulating α-synuclein aggregation in fluid or neuron-derived exosomes and lead to this discrepancy also remains to be explored.

Next, the four immune-related biomarkers were selected as key genes for further miRNA-TF-mRNA network analysis. Taking the miRNA-TF-mRNA network into account, we explored the regulatory mechanisms of NEFM, GRIA1, NEFL, and SYT1, which might be regulated by hsa-miR-92a-3p and hsa-miR-92b-3p. Our findings indicate that hsa-miR-92a is the hub miRNA in both regulatory and co-expression networks and has a strong functional role in PD. Based on integrated network analysis, hsa-miR-25-3p and hsa-miR-363-3p were also identified as bridges connecting to GRIA1, NEFL, and SYT1. Campos-Melo et al. demonstrated that miR-92a-3p is expressed in motor neurons of the spinal cord and can directly downregulate NEFM. MiR-92a is also able to repress translation of GluA1 receptors to block homeostatic scaling in rats (Letellier et al., 2014). Moreover, researchers have found that an inhibitor of miR-363 increases the expression level of GAP43 in glioma cells (Conti et al., 2016). Interestingly, miR-25, miR-92a-3p (miR-92a-1 and miR-92a-2), and miR-363 all belong to the miR-92 family, a group of highly conserved miRNAs (Olive et al., 2010). MiR-92a may be viewed as a potential therapeutic target for PD. Previous studies have revealed aberrant expression of miR-92a in various cancers, and it exerts its function in tumors mainly by promoting cell proliferation, invasion and metastasis and inhibiting apoptosis (Dou et al., 2020; Feng et al., 2021). Overexpression of miR-92a suppresses immune cell function in many kinds of malignant tumors (Dou et al., 2020; Feng et al., 2021). However, the regulatory mechanisms of miR92a have rarely been studied in PD. Thus, we speculate that the miR92a family may regulate the identified biomarkers to participate in immune

infiltration in PD, and further studies to explore the pathophysiology of the miR92a family in PD are required in the future.

Finally, we developed a nomogram to predict the occurrence in PD patients based on the four immune-related biomarkers. The results showed that the nomogram model had excellent individual predictive effects. Therefore, this nomogram may provide new insight and contribute to accurate diagnosis of PD, particularly for early stage PD.

To the best of our knowledge, this is the first diagnostic nomogram to predict PD based on GEO datasets. However, there are still some limitations in this study. First, although we performed rigorous bioinformatics analysis and external validation to verify expression of the hub genes and their predictive power in PD diagnosis, the results need to be thoroughly investigated in *in vitro* experiments. Second, there are few datasets of miRNA expression in the substantia nigra of PD, and the relationship of miRNAs and immune infiltration should be investigated. Further studies are warranted to focus on miRNAs with mechanisms in PD. Finally, although a nomogram to predict PD is presented, this analysis was based on genome-wide expression of the substantia nigra from postmortem brains, and detection of substantia nigra mRNA expression in practical applications is difficult to implement. Future research should focus on comparing gene expression and immune infiltration patterns between different neurodegenerative diseases, enabling the identification of early stage disease biomarkers that can improve the understanding of the pathophysiology of neurodegenerative diseases and facilitate the application of timely symptomatic interventions. Nevertheless, this study provides new insight into exploring the mechanism of PD and PD diagnosis.

Conclusion

In conclusion, this study not only suggests that immune cell infiltrates are associated with PD but also presents four effective diagnostic immune-related biomarkers for PD patients. We also predict that the miRNA-92a family might target these immune-related biomarkers in regulating PD. Our research provides further insight into potential therapeutic targets for PD.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Author contributions

PZ, YX, and LZ contributed to the design and initiation of the study. PZ, YX, LZ, HoL, and JS collected and analyzed the data. PZ and YX drafted the manuscript. LZ, HoL, JS, and HuL critically revised the content. All authors reviewed and revised the final manuscript.

Funding

This work was supported by the Medical Health Science and Technology Project of Tianjin of China (Grant no. TJWJ2021YJ007) and Science and Technology Project of Beichen District of Tianjin (Grant no. SHGY-2021025).

Acknowledgments

We acknowledge all developers of the *R* programming package and the GEO database for providing their platforms as well as their contributors for uploading meaningful datasets.

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/fnins.2023.1083928/full#supplementary-material

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frontiers | Frontiers in Neuroscience

TYPE Original Research
PUBLISHED 06 February 2023
DOI 10.3389/fnins.2023.1098441

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

RECEIVED 14 November 2022
ACCEPTED 17 January 2023
PUBLISHED 06 February 2023

CITATION
Invernizzi A, Rechtman E, Oluyemi K, Renzetti S, Curtin P, Colicino E, Ambrosi C, Mascaro L, Patrono A, Corbo D, Cagna G, Gasparotti R, Reichenberg A, Tang CY, Smith DR, Placidi D, Lucchini RG, Wright RO and Horton MK (2023) Topological network properties of resting-state functional connectivity patterns are associated with metal mixture exposure in adolescents. *Front. Neurosci.* 17:1098441. doi: 10.3389/fnins.2023.1098441

Topological network properties of resting-state functional connectivity patterns are associated with metal mixture exposure in adolescents

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Introduction: Adolescent exposure to neurotoxic metals adversely impacts cognitive, motor, and behavioral development. Few studies have addressed the underlying brain mechanisms of these metal–associated developmental outcomes. Furthermore, metal exposure occurs as a mixture, yet previous studies most often consider impacts of each metal individually. In this cross–sectional study, we investigated the relationship between exposure to neurotoxic metals and topological brain metrics in adolescents.

Methods: In 193 participants (53% females, ages: 15–25 years) enrolled in the Public Health Impact of Metals Exposure (PHIME) study, we measured concentrations of four metals (manganese, lead, copper, and chromium) in multiple biological media (blood, urine, hair, and saliva) and acquired resting–state functional magnetic resonance imaging scans. Using graph theory metrics, we computed global and local efficiency (global:GE; local:LE) in 111 brain areas (Harvard Oxford Atlas). We used weighted quantile sum (WQS) regression models to examine association between metal mixtures and each graph metric (GE or LE), adjusted for sex and age.

Results: We observed significant negative associations between the metal mixture and GE and LE [βGE = −0.076, 95% CI (−0.122, −0.031); βLE= −0.051, 95% CI (−0.095, −0.006)]. Lead and chromium measured in blood contributed most to this association for GE, while chromium measured in hair contributed the most for LE.

Discussion: Our results suggest that exposure to this metal mixture during adolescence reduces the efficiency of integrating information in brain networks at both local and global levels, informing potential neural mechanisms underlying the

developmental toxicity of metals. Results further suggest these associations are due to combined joint effects to different metals, rather than to a single metal.

KEYWORDS

resting state–fMRI, graph theory, global and local efficiency, topological network properties, exposure, neurotoxic metals

Introduction

Exposure to neurotoxic metals and their impact on the brain is a growing worldwide health concern (Carmona et al., 2021). Metals such as lead and manganese have been shown to readily pass the blood-brain barrier and accumulate within various brain areas, where they exert neurotoxic effects (Balali-Mood et al., 2021; Carmona et al., 2021) and are associated with altered neurotransmission, disrupted synaptic structure (Sadiq et al., 2012; Karri et al., 2016; Carmona et al., 2021; Goel and Aschner, 2021) and accelerated neurodegeneration (Caito and Aschner, 2015; Karri et al., 2016; Lim et al., 2019; Twohig and Nielsen, 2019). These among others key features of the above mentioned metals contributed to define the brain as the target organ for exposure (Chandra et al., 1983; Caito and Aschner, 2015; Gilani et al., 2015; Chen et al., 2022). Growing research has identified adolescence as a critical window (Schalbetter et al., 2022) that is vulnerable to environmental exposure including metals (Rechtman et al., 2020). Few studies investigated the neural mechanisms of metal neurotoxicity throughout this extended window of vulnerability. Findings from these studies have linked metal exposure with alterations in regional brain volume (Claus et al., 2012, 2014; Horton et al., 2014, 2018; Levin-Schwartz et al., 2021; Heng et al., 2022; Migneron-Foisy et al., 2022), and brain metabolite concentrations (Trope et al., 2001; Meng et al., 2005; Thomason et al., 2019, 2021; Cecil, 2022) during this period. This heightened vulnerability may be due to rapid growth and differentiation of the brain throughout childhood. Neurotoxic exposures during this critical period can also disrupt behavioral, cognitive, and motor development (Claus et al., 2012, 2014; Horton et al., 2018; Rechtman et al., 2020; Levin-Schwartz et al., 2021; Heng et al., 2022). Despite the breadth of research on the developmental effects of childhood metal exposure, the underlying brain mechanisms behind these observed metal-associated outcomes are still not clear.

Over the past decade, increasing use of functional magnetic resonance imaging (fMRI) provides insight into the mechanisms linking metal exposure and alterations in brain functions (Horton et al., 2014). In particular, resting-state functional MRI - task-independent assessment of spontaneous fluctuations in blood oxygen level dependent (BOLD) signal from the brain at rest–has emerged as a novel tool in pediatric populations to investigate the intrinsic functional connectivity of the brain. Different from task-based fMRI which requires participants to engage or respond to stimuli (Canario et al., 2021), in rs-fMRI participants are instructed to simply lay still in the scanner with their eyes closed, while allowing their mind to roam freely (i.e., not focusing their thought on anything in particular). This facilitates research in younger populations, who may have difficulty completing complex tasks in the scanner (Canario et al., 2021). Results from rs-fMRI studies have shown a topological organization of the brain in a highly efficient manner with a high level of local clustering, together with long-distance connections (van den Heuvel et al., 2009). Graph theory analysis of rs-fMRI data characterizes the topological organization of the brain at rest (Wang et al., 2010) using metrics such as global and local efficiency, which quantify how efficient the brain is at integrating information at global and local levels, respectively (Wang et al., 2010). Global efficiency (GE) provides an indication of how efficiently the information is integrated and exchanged between the different regions of the brain (Stanley et al., 2015; Rakesh et al., 2020). In contrast, local efficiency (LE) measures the ability of the brain to perform functionally specialized and segregated processing within a network, requiring densely interconnected regions within modules (Stanley et al., 2015; Rakesh et al., 2020). Previous results have demonstrated the utility to characterize the topological network organization of the brain by using graph metrics based on rs-fMRI and link them with human behavior (Xu et al., 2015; Liu et al., 2022), cognition (van den Heuvel et al., 2009; Uehara et al., 2013), and diseases (Liu et al., 2008; Supekar et al., 2008). Recent studies have used rs-fMRI to demonstrate intrinsic functional connectivity patterns in *a priori* selected brain regions associated with early life exposure to individual metals (i.e., lead, manganese) (de Water et al., 2018, 2019; Thomason et al., 2019). Our data-driven graph theory approach builds on this foundational research by informing potential neural mechanisms underlying the developmental toxicity of metal mixture exposure during adolescence.

To investigate the impact of metal exposure on the brain, it is critical to consider not only single metal exposures but the mixture of co-occurring neurotoxic metals (Bauer et al., 2020). Historically, studies measure individual chemical concentrations in individual biological media (i.e., blood, urine, etc.). These exposure biomarkers are used as surrogates of total exposure from the environment. However, metals distribute unevenly among biological media that represent different aspects of each chemical's toxicokinetics. Therefore, each medium provides complementary information on different biological processes. Recent studies have started to combine information from multiple biomarkers using statistical methods, like multi-media biomarker approaches, that resulted in an improved measure of the total body burden and thus improved exposure characterization (Levin-Schwartz et al., 2020, 2021; Bauer et al., 2021). Exposure, defined as metal mixtures, has been observed to more negatively impact neurodevelopment than exposure to a single metal component (Claus et al., 2012, 2014; Freire et al., 2018; Horton et al., 2018). Therefore, examining the effects of metal mixtures on brain function is crucial to better understand the real-world impact of metal exposure on cognition and behavior. In this study, we will use an integrated measure of metal mixtures across multiple media, called multi-media biomarker (MMB) (Levin-Schwartz et al., 2020), to analyze the impact on the brain of each metal across multiple media.

In this study, we investigate how metal exposure impacts brain network properties in adolescents. We use graph theory metrics to quantify how the brain integrates globally (GE) and locally segregates (LE) information and assess associations between these metrics with metal mixture exposure. To define our metal mixtures, we measured concentrations of four metals [lead (Pb), manganese (Mn), chromium (Cr), and copper (Cu)] in four biological media (blood, urine, hair, and saliva) from 193 adolescent participants living nearby ferro-manganese industry/alloy plant activity in northern Italy enrolled in the Public Health Impact of Metals Exposure (PHIME) study. Then, using weighted quantile sum (WQS) regression, a statistical method commonly used to assess the impact of chemical mixtures on various health outcomes (Tanner et al., 2019), we examined associations between the metal mixture and each graph metric (GE and LE), adjusting for sex and age. This paper contributes to further understanding the impacts of environmental exposures to a mixture of neurotoxic metals in developmental windows like adolescence.

Materials and methods

Participants

The Public Health Impact of Metal Exposure (PHIME) cohort investigates associations between metal exposure from anthropogenic emissions and developmental health outcomes in adolescents and young adults living nearby the ferro-manganese industry in northern Italy. Details of the study have been described elsewhere (Lucchini et al., 2012a; Lucas et al., 2015). Inclusion criteria were: birth in the areas of interest; family residence in Brescia for at least two generations; residence in the study areas since birth. The exclusion criteria were: having a neurological, hepatic, metabolic, endocrine, or psychiatric disorder; using medications (in particular with neuro-psychological side effects); having clinically diagnosed motor deficits or cognitive impairment and having visual deficits that are not adequately corrected. Detailed description of this recruitment process and study design can be found in previous publications (Lucchini et al., 2012a,b). A convenience based sample of 202 participants (53% female, ages 15–25 years) were selected to participate in a multi-modal magnetic resonance imaging (MRI) study, PHIME-MRI. They completed multimodal MRI scans, neuropsychological tests, including measures of IQ [Kaufman Brief Intelligence Test, Second Edition (KBIT-2)] (Kaufman and Kaufman, 2014; Reynolds et al., 2014), memory and motor functions. All participants satisfied eligibility criteria for MRI scanning [i.e., metal implants or shrapnel, claustrophobia, no prior history of traumatic brain injury, body mass index (BMI) $\leq$40]. Mn, Pb, Cr, and Cu were measured in saliva, hair, blood and urine, for each PHIME-MRI participant. Complete exposure data (i.e., all metals in all media for a total of 16 components), MRI and covariates data were available for 193 participants included in this analysis. A total of 193 participants were included in this analysis, 9 were missing at least one biological marker (**Supplementary Figure 1**).

Written informed consent was obtained from parents, while participants provided written assent. Study procedures were approved by the Institutional Review Board of the University of California, Santa Cruz and the ethical committees of the University of Brescia, and the Icahn School of Medicine at Mount Sinai.

Biomarker measures of exposure

Biological samples including venous whole blood, spot urine, saliva and hair were collected from each subject upon enrollment, as described in detail in previous studies (Smith et al., 2007; Eastman et al., 2013; Lucas et al., 2015; Butler et al., 2019). Complete overview of biomarkers can be found in **Supplementary Figure 1** and **Table 1**. Biological samples were processed and analyzed for metal concentrations using magnetic sector inductively coupled plasma mass spectroscopy (Thermo Element XR ICP-MS), as described elsewhere (Smith et al., 2007; Eastman et al., 2013; Lucas et al., 2015; Butler et al., 2019).

MRI and fMRI data acquisition

Magnetic resonance imaging (MRI) and functional MRI (fMRI) data acquisition was performed on a high-resolution 3-Tesla SIEMENS Skyra scanner using a 64-channel phased array head and neck coil, at the Neuroimaging Division of ASST Spetali Civili Hospital of Brescia. For each participant, a high-resolution 3D T1-weighted structural scan was acquired using a MPRAGE sequence (TR = 2.4 ms, TE = 2.06 ms, TI = 230 ms, acquisition matrix = 256 $\times$ 256 and 224 sagittal slices with final voxel size = 0.9 mm^3). Fifty contiguous oblique-axial sections were used to cover the whole brain where the first four images were discarded to allow the magnetization to reach equilibrium. For each subject, a single 10-min continuous functional sequence using a T2*weighted echo-planar imaging (EPI) sequence (TR = 1.0 ms, TE = 27 ms, 70 axial slices, 2.1 mm thickness, matrix size 108 $\times$ 108, covering the brain from vertex to cerebellum) was acquired. During resting-state scans, lights of the MRI room were off and participants were instructed to stay awake, relax and daydream (not think about anything) with their eyes open. They were presented with an image of a night skyline figure projected on a MRI compatible monitor. Padding was used for comfort and reduction of head motion. Earplugs were used to reduce noise. Data were read by a board-certified radiologist to determine quality and possible incidental findings–no findings were reported.

fMRI data analyses

Image pre-processing, global and local efficiency calculations, and statistical analyses were performed using SPM12 (Wellcome Department of Imaging Neuroscience, London, UK), Brain Connectivity toolbox (Rubinov et al., 2009; Rubinov and Sporns, 2010) and customized scripts, implemented in MatLab 2016b (The Mathworks Inc., Natick, MA, USA) and R (v3.4).

Image preprocessing

For each subject, the structural magnetic resonance image was co-registered and normalized against the Montreal Neurological Institute (MNI) template and segmented to obtain white matter (WM), gray matter (GM) and cerebrospinal fluid (CSF) probability maps in the MNI space. FMRI data were spatially realigned, co-registered to the MNI-152 EPI template and subsequently normalized utilizing the segmentation option for EPI images in SPM12. All normalized data were denoised using ICA-AROMA (Pruim et al.,

TABLE 1 Metal concentrations (Mn, Pb, Cr, and Cu) measured in blood, urine, hair, and saliva collected from 193 adolescents participants PHIME-MRI included in the current study.

Medium*	Metal	% > LOD	LOD mean ± SE	GM	GSD
Saliva	Lead	90.6	0.05 ± 0.003	0.19	3.07
	Chromium	91.7	0.13 ± 0.003	0.50	3.69
	Manganese	96.4	0.08 ± 0.001	3.13	2.97
	Copper	97.4	0.35 ± 0.025	8.63	2.35
Blood	Lead	100	0.16 ± 0.003	8.84	1.56
	Chromium	62.7	0.19 ± 0.008	0.34	4.54
	Manganese	100	0.49 ± 0.018	8.45	1.49
	Copper	100	1.09 ± 0.035	586.94	1.30
Hair	Lead	100	0.003 ± 0.0001	0.09	2.97
	Chromium	100	0.004 ± 0.0001	0.04	2.57
	Manganese	100	0.005 ± 0.0003	0.06	2.58
	Copper	100	0.04 ± 0.002	9.96	1.62
Urine	Lead	98.4	0.06 ± 0.003	0.36	2.17
	Chromium	96.9	0.09 ± 0.004	0.28	3.07
	Manganese	80.3	0.11 ± 0.003	0.24	3.69
	Copper	100	0.30 ± 0.009	6.01	1.85

GM, geometric mean; GSD, geometric standard deviation; LOD, limit of detection; SE, standard error of the mean. *Metrics used to measure metal concentrations within each medium were: blood and saliva (ng/mL), hair (ug/g), urine (ug/mL).

2015). Additionally, spatial smoothing was applied (8 millimeters) to the fMRI data. As further quality check of fMRI data, large head motion in any direction or rotation (>3 mm or 3°) was used as exclusion criteria in our study–no participants were excluded in this study. No global signal regression was applied.

Based on the Harvard-Oxford (Desikan et al., 2006) atlas, 111 regions of interest (ROI; 48 left and 48 right cortical areas; 7 left and 7 right subcortical regions and 1 brainstem) were defined. In this atlas, the brain areas were defined using T1-weighted images of 21 healthy male and 16 healthy female subjects (ages 18–50). The T1-weighted images were segmented and affine-registered to MNI152 space using FLIRT (FSL), and the transforms then applied to the individual brain areas' label. Finally, these were combined across subjects to form population probability maps for each ROI (Desikan et al., 2006). For each ROI, a time-series was extracted by averaging across voxels per time point. To facilitate statistical inference, data were "pre-whitened" by removing the estimated autocorrelation structure in a two-step generalized linear model (GLM) procedure (Monti, 2011; Bright and Murphy, 2015). In the first step, the raw data were filtered against the 6 motion parameters (three translations and three rotations). Using the resulting residuals, the autocorrelation structures present in the data were estimated using an Auto-Regressive model of order 1 [AR (1)] and then removed from the raw data. Next, the realignment parameters, white matter (WM) and cerebrospinal fluid (CSF) signals were removed as confounders on the whitened data.

Graph theory metrics/Network properties

Global and Local Efficiency (GE and LE) were computed using the Brain Connectivity toolbox (Rubinov et al., 2009; Rubinov and Sporns, 2010) on the defined ROI time course data per subject. GE and LE build on the concept of efficient integration of communication in a network at local (LE) and whole (GE) level. Based on the average inverse shortest path length in the brain or network, GE is defined as the inverse of the average characteristic path length between all nodes in the networks (Latora and Marchiori, 2001; Bullmore and Sporns, 2012). For each individual node defined as ROI, the shortest number of steps required to go from one node to another was computed. Then, the average number of shortest steps to all defined nodes was computed separately for each node. To correct for the total number of connections between nodes, the inverse of the average number of shortest steps for each node was summed across all network nodes and normalized. LE is computed on the neighborhood of each single ROI/node (Rubinov et al., 2009; Rubinov and Sporns, 2010) and defined as the inverse of the shortest average path length of all neighbors of nodes among themselves (Latora and Marchiori, 2001). First we identified a set of nodes which are directly connected with a given node, then we removed that node from the identified subgraph and calculated the averaged shortest path between all remaining nodes. GE and LE are scaled measures ranging from 0 to 1, with a value of 1 indicating maximum GE/LE in the brain.

Statistical analyses

Descriptive statistics

Visual inspection and descriptive statistics (geometric mean, geometric standard deviation and Pearson's correlation) were used to characterize the metal concentrations in different media. All descriptive statistical analyses were performed using R version 4.2.1.

Multi-media biomarker (MMB) approach

To examine associations between our multi-media metal mixture (four metals, four media) and graph theory outcomes (GE and LE),

we used a WQS-based multi-media biomarker (MMB) approach (Lee et al., 2019; Levin-Schwartz et al., 2021). **Figure 1** shows the complete flowchart of the analysis performed. Briefly, WQS is a data driven, mixture-based ensemble modeling strategy that tests for associations between the combined effect of multiple, correlated variables and an outcome of interest. The WQS MMB approach builds on WQS, by incorporating exposure information across different biological media, providing an integrated estimate of total bodily exposure to a given chemical as well as identifying the chemical-matrix specific combination that contributes most to the overall association with the graph theory based outcomes (GE and LE) (Levin-Schwartz et al., 2021). The MMB WQS is hierarchical with two levels. The first level estimates a weighted index across all biological media for a single metal and the outcome (i.e., Pb MMB = blood Pb, urine Pb, saliva Pb, hair Pb). Our model estimated across 50 bootstrap samples, and 100 repeated holdouts (Tanner et al., 2019) for each individual MMB. By using the repeated holdouts WQS (Tanner et al., 2019), the data are randomly partitioned 100 times to produce a distribution of validated results where the mean is taken as the final estimate. The directionality of the association of the WQS index was constrained to be negative. Note that the WQS assumptions of linearity and directional homogeneity were validated through visual inspection of residuals (Levin-Schwartz et al., 2021). The second level estimates a weighted index across the different metals (i.e., Pb MMB, Mn MMB, Cr MMB, Cu MMB; (Levin-Schwartz et al., 2019, 2021)). First level MMBs are included in the WQS regression model predicting the association between the metal biomarker "mixture" and GE or LE. A significance test for the WQS index provided an estimate of the association with the metal mixture, while the weights associated with each metal MMB provided an indicator of each individual metal contribution to the overall effect. All weights are constrained to sum to one, enabling sorting by relative importance. Metals that impact the outcome have larger weights; factors with little or no impact on the outcome have near-zero weights. These models were adjusted for age and sex, and prior to model estimation, all exposures were grouped into deciles.

Results

Demographic and exposure characteristics

This study included 193 participants (53% female) living in Northern Italy, with an average age of 19.2 years (range = 15–25). Metal concentrations in the different media are reported in **Table 1** while Pearson's correlations between them is reported in **Supplementary Figure 3**.

First level MMBs and brain topological properties

We first examined the association between each individual metal in all media with GE and LE (**Figure 2**). For all metals, urinary metal concentrations contributed most to the association between the first level MMB (i.e., individual metal in each matrix) and GE. Urinary Pb contributed 46% of the association between Pb exposure and GE. Urinary Mn, Cr and Cu contributed 51, 34, and 68%, respectively to the association with GE. For LE, the most heavily weighted metal-matrix combination differed by metal; blood Pb concentration

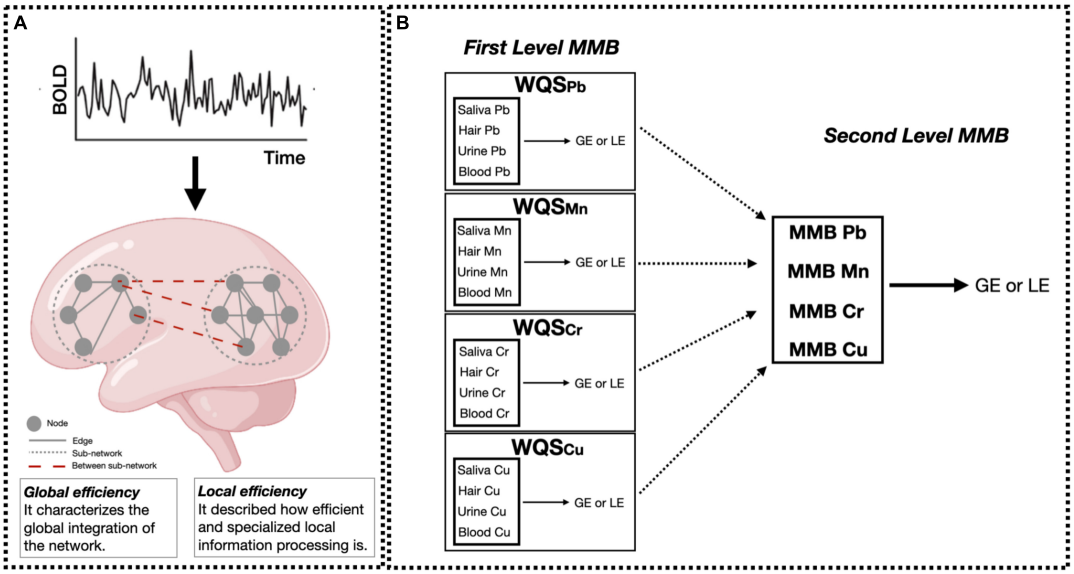

FIGURE 1
Data analysis flowchart. **(A)** Resting-state fMRI data were preprocessed and the averaged time-series were extracted using the Harvard-Oxford atlas. Then, global and local efficiency (GE and LE, respectively) metrics were computed for each participant using graph theory. Small solid gray circles represent nodes of the graphs (brain regions), while gray connecting lines are the edges of the graph (functional connections). Larger dotted circles represent segregated sub-graphs/networks (functional network characterized by highly connected brain areas), while dashed red lines are the within-network connections at the whole brain level. Panel **(B)** shows the two hierarchical levels of analysis performed using the MMB WQS approach to measure the effect of Pb, Mn, Cr, Cu on brain metrics (GE and LE). At the first level, WQS was performed to measure and derive the MMB metric for each metal individually (Pb, Mn, Cr, Cu) on brain metrics (GE and LE). Then, the joint effect of Pb, Mn, Cr, Cu on brain metrics was assessed by applying WQS to Pb, Mn, Cr, and Cu MMBs. All models were adjusted for sex and age. Figure adapted from Levin-Schwartz et al. (2019) and Rakesh et al. (2020).

contributed most to the association with LE (34%). Hair Mn and hair Cr contributed 31 and 43% to the association with LE. Urine Cu contributed the most to the Cu-LE association. Beta coefficients and 95% confidence intervals obtained for each individual MMB WQS model are reported in **Figure 2** and **Supplementary Figure 2**.

Second level MMB and brain topological properties

Results from second level repeated holdout WQS analyses revealed associations between the overall metal mixture and GE and LE (**Figure 3A**), and the contribution of each metal MMB to these associations (**Figure 3**). We observed significant negative associations between the combined metal mixture MMB and both GE [$\beta_{GE} = -0.076$, 95% CI (−0.122, −0.031); **Figure 3A**], and LE [$\beta_{LE} = -0.048$, 95% CI (−0.095, −0.006); **Figure 3A**]. We observed that Cr and Pb contributed most to the association between the combined metal mixture and GE (29%; **Figure 3B**), whereas Cr contributed most to the association with LE (38%; **Figure 3C**).

Discussion

This is the first study to use rs-fMRI to investigate global and local connectivity in adolescents exposed to a neurotoxic metal mixture. Using graph-theory based network metrics and a multimedia biomarker (MMB) approach, we observed a significant negative association between exposure to a mixture of five neurotoxic metals (lead, manganese, copper, chromium, and zinc) and global efficiency (GE), with lead and chromium contributing most to this association. Significant negative associations between the metal mixture and both GE and LE were found [$\beta_{GE} = -0.076$, 95% CI (−0.122, −0.031); $\beta_{LE} = -0.048$, 95% CI (−0.095, −0.006)]. We also observed that urinary lead and chromium contributed most to the association with GE (29 and 24%, respectively); while hair chromium contributed most to the association with LE (38%). Overall, our results substantiate previous findings of associations between metal exposure and altered brain connectivity, and further suggest that environmental exposure to a mixture of neurotoxic metals during adolescence reduces the brain ability to efficiently integrate and segregate information, highlighting the need to further study the impacts of environmental exposures in developmental windows like adolescence (Golub, 2000; Spear, 2007; Rechtman et al., 2020). Furthermore, our results suggest these associations are due to the combined joint effects of multiple metals, rather than to a single metal, emphasizing the importance of analyzing metal mixtures to better understand the real-world impact of metal exposure on brain health.

Our findings show that urinary lead and chromium were the top contributing metals in the association between the metal mixture and GE, and hair chromium contributed most to the association with LE, suggesting that urine and hair may be critical biomarkers for estimating the effects of metal mixtures on brain connectivity and further, these metals may exert a greater influence on global and/or local functional connectivity across/within topological brain networks. Lead exposure is known for causing the disruption of neuronal activity, in particular to alter the release and storage of neurotransmitters from the presynaptic nerve endings, that may change the developmental processes of synapse formation in children and young adults and results in altered brain functions (Bressler and Goldstein, 1991). Previous human neuroimaging studies have observed associations between lead exposure and altered structural connectivity and functional activation patterns in both children and adults (Thomason et al., 2019, 2021; Cecil, 2022). In particular, Thomason et al. (2019) found prenatal lead (Pb) exposure was associated with altered age-related intrinsic functional connectivity patterns in developing fetuses. Furthermore, previous studies in animal models have found lead exposure to disrupt multiple neurotransmitter systems (Goel and Aschner, 2021) (e.g., glutamatergic, dopaminergic, cholinergic), as well as neurotransmitter and synaptic function in various areas of the brain, including the hippocampus (Sadiq et al., 2012; Carmona et al., 2021), and prefrontal cortex (Mansouri et al., 2013). Therefore, our finding of lead being a top contributor to the negative association between the metal mixture and global efficiency, could in part be explained by its impact on structural connectivity (e.g., white matter integrity) and synaptic function and neurotransmission within/across the brain.

The underlying mechanism for neurotoxicity of chromium is still not fully understood (Xu et al., 2021). Increased oxidative stress, chromosome disruptions and DNA-adduct formation are some of the many cellular damages found to be caused by high level exposure to Cr in the brain (Wise et al., 2022). While there are no neuroimaging studies investigating the impact of chromium exposure to date, previous studies in humans have observed evidence linking chromium exposure to neurological and psychiatric conditions, including olfactory dysfunction, autism spectrum disorder, and acute schizophrenia (Watanabe and Fukuchi, 1981; Kitamura et al., 2003; Saghazadeh et al., 2020; Wise et al., 2022). These findings suggest an impact of chromium exposure on underlying neurobiological function. Furthermore, previous studies across various animal models have observed brain-wide neurodegeneration following chromium exposure, again suggesting an impact of chromium exposure on neurobiological function *via* its neurodegenerative effects (Soudani et al., 2012; Hao et al., 2017; Wise et al., 2022). Therefore, our finding of chromium being a top contributor to the negative association between the metal mixture and both local and global efficiency is consistent with these prior studies suggesting its widespread neurodegenerative effects, which could potentially contribute to changes in functional connectivity across brain networks.

Several studies have also detailed the potential synergistic neurotoxic effects of certain metals upon co-exposure, based on their unique chemical properties and similar neurobiological mechanisms of action (de Andrade et al., 2021). Metals within our mixture that have been shown to produce such synergistic neurotoxic effects include lead and manganese (Tao et al., 1999; Chen et al., 2016; Lu et al., 2018), whose co-exposure has been observed to increase disruptions to neurodevelopment in both animal (Chandra et al., 1981, 1983; Shukla and Chandra, 1987; Levin-Schwartz et al., 2021) and human studies (Kim et al., 2009; Claus et al., 2012; Lin et al., 2013; Levin-Schwartz et al., 2021). de Water et al. (2018) found that early postnatal manganese (Mn) concentrations were associated with altered intrinsic functional connectivity within cognitive control and motor brain areas of adolescents. Additionally, in another study, de Water et al. (2019) found prenatal Mn concentrations were associated with reduced intrinsic functional connectivity of brain areas involved in emotion processing and regulation in children. Furthermore, co-exposures of certain metals have been reported

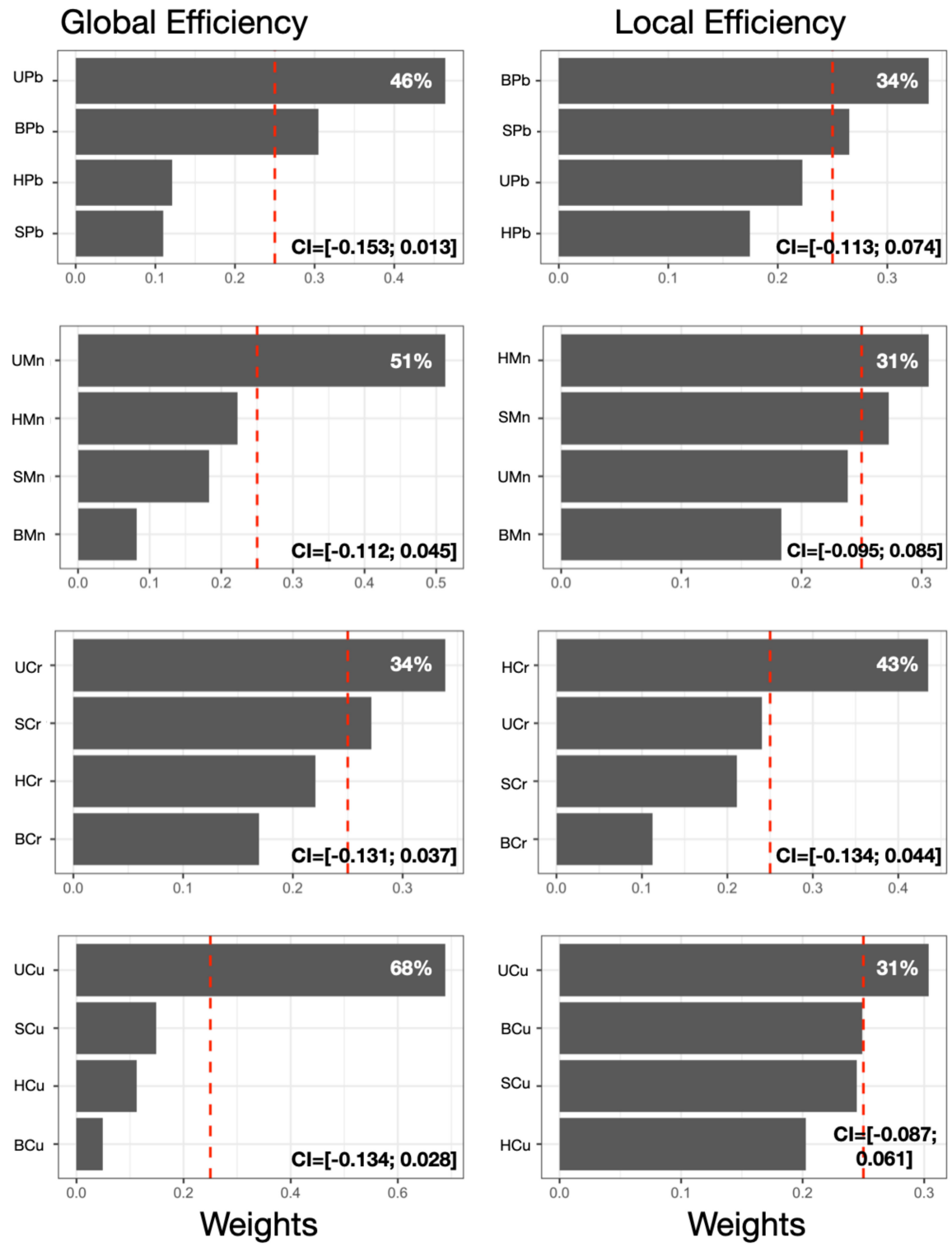

FIGURE 2
First level MMB models. Results obtained from the MMB WQS association between each metal's respective exposure biomarker (e.g., blood Pb) and GE or LE was estimated among 193 adolescents included in the current study. Bar plots show estimated weights for each component of the mixture in the WQS regressions. Red dotted lines represent the significant thresholds for each WQS model. 95% confidence intervals obtained for each individual MMB WQS model are reported. All models were adjusted for sex and age. Components abbreviations: the first letter represents the medium (S, saliva; B, blood; U, urine; H, hair) and the second and third letters represent the metals (Mn, manganese; Pb, lead; Cr, chromium; Cu, copper).

to potentially increase accumulation, retention and distribution of individual metal components in animal models (Chen et al., 2016). In particular, manganese has been shown to increase accumulation of various metals in the brain, notably lead (Chandra et al., 1983; Chen et al., 2016), and copper (Mercadante et al., 2016). Therefore, while lead and chromium were found to contribute most to the association between the metal mixture and GE, and chromium contributed most to the association with LE, the higher influence of these metals may

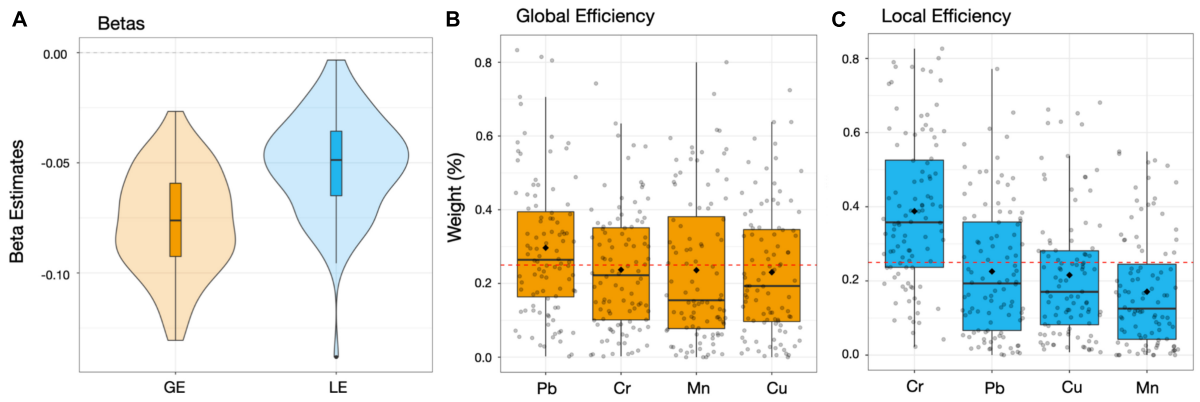

FIGURE 3
Second level MMBs approach. Beta coefficients **(A)** and weights **(B,C)** obtained from the WQS association between each MMB metal and GE or LE was estimated among 193 adolescents included in the current study. Panel a reports the beta coefficients for GE (orange) and LE (blue), respectively. In panels **(B,C)**, data points indicate weights for each of the 100 holdouts; box plots show the 25, 50, and 75th percentiles while the whiskers show the 10 and 90th percentiles of weights for the 100 holdouts. Diamonds show the mean weights for the 100 holdouts. Dotted lines indicate the thresholds. Mn, manganese; Pb, lead; Cr, chromium; Cu, copper.

be due to synergistic interactions with other metals in the mixture (e.g., manganese). This possibility highlights the importance of analyzing metal mixtures rather than single metals in environmental epidemiological studies, as the influence of a single metal exposure may be affected by other metals an individual is exposed to. Further, our findings show that urine contributed most to the association between both lead, chromium and GE, and hair contributed most to the association between chromium and LE, suggesting that urine and hair may be critical for estimating the effects of metal mixtures on brain connectivity. Previous studies that analyzed one metal at the time, have indicated blood as the most reliable biomarker to assess lead exposure (Levin-Schwartz et al., 2021), and blood has also been used previously as an exposure biomarker for other metals such as chromium (Alexopoulos et al., 2008; Wise et al., 2022) and manganese (Levin-Schwartz et al., 2021). By using novel techniques like MMB WQS, we can increase the accuracy in measuring mixture effects compared to individual biomarkers and provide a data-driven biomarker selection (Levin-Schwartz et al., 2019, 2020). Finally, as previous neuroimaging studies have mainly examined associations between brain function and a single metal exposure, future studies should aim to utilize metal mixtures to better account for these potential synergistic effects due to metal co-exposure, which would ultimately help better understand the real-world impact of metal exposure on the brain.

Limitations

In this study, while we found robust associations between metals and GE and LE metrics, our small sample size resulted in relatively small effect sizes (**Figure 3**). While it would be beneficial to repeat our analysis in a larger dataset, to our knowledge no such dataset with multi-media biomarkers and fMRI data exists. Further, we assumed that all metals have a linear association with both global and local efficiency metrics. Our MMB WQS approach does not assume linear associations between outcomes but only considers additive effects. Future studies should investigate non-linear associations between outcomes and possible multiplicative effects. Finally, MMB WQS might suffer from overfitting issues, since two WQS models are performed on the same set of data. To compensate for this, we split our data into training and testing datasets in both MMB WQS analysis levels.

Conclusion

Using a multimedia biomarker (MMB) approach, we were able to estimate the associations between a complex metal mixture and brain metrics. This method allows us to leverage the complementary information provided by each medium on different biological processes and therefore, to improve the exposure characterization. Our findings that urine contributed most to the associations between both lead and chromium and GE, and hair contributed most to the associations between chromium and LE, suggests that urine and hair may be critical overlooked biomarkers for estimating the effects of metal mixtures on brain connectivity. Given our results, we suggest that future neuroimaging studies on metal mixture exposure aim to collect multiple media, including urine and hair specimens, to explore the effects of metal mixtures on the brain. Altogether, our research supports the notion of adolescence being a timepoint of vulnerability to environmental exposures. More specifically, our results suggest that the adolescent brain connectivity is vulnerable to metal mixture exposures during this period. Given that adolescence is a period of rapid brain development, our results suggest that metal exposure may have the potential to alter neurodevelopment *via* changes to global and local connectivity. These connectivity changes may potentially lead to alterations in cognition and neurobehavior in adolescence. Therefore, future environmental neuroimaging studies should focus on adolescents to further characterize how metal mixture exposure during this period can lead to potential alterations in brain development (e.g., brain volume, functional connectivity), and ultimately neurobehavior and cognition.

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 the Institutional Review Board of the University of California, Santa Cruz and the Ethical Committees of the University of Brescia, and the Icahn School of Medicine at Mount Sinai. Written informed consent to participate in this study was provided by the participants or their legal guardian/next of kin.

Author contributions

All authors listed in this work have made a substantial contribution to the concept or design of the article, the acquisition, analysis, or interpretation of data for the article, drafted the article or revised it critically for important intellectual content, approved the version to be published, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Funding

The authors would like to acknowledge support from the National Institutes of Environmental Health Sciences (NIEHS) grant numbers R01 ES019222, R01 ES013744, and P30ES023515, and the European Union through its Sixth Framework Programme for RTD (contract number FOOD-CT-2006-016253).

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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2023.1098441/full#supplementary-material

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frontiers | Frontiers in Neuroscience

TYPE Review
PUBLISHED 13 February 2023
DOI 10.3389/fnins.2023.1005096

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

RECEIVED 27 July 2022
ACCEPTED 09 January 2023
PUBLISHED 13 February 2023

CITATION
Spencer PS, Palmer VS, Kisby GE, Lagrange E, Horowitz BZ, Valdes Angues R, Reis J, Vernoux J-P, Raoul C and Camu W (2023) Early-onset, conjugal, twin-discordant, and clusters of sporadic ALS: Pathway to discovery of etiology *via* lifetime exposome research.
Front. Neurosci. 17:1005096.
doi: 10.3389/fnins.2023.1005096

Early-onset, conjugal, twin-discordant, and clusters of sporadic ALS: Pathway to discovery of etiology *via* lifetime exposome research

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The identity and role of environmental factors in the etiology of sporadic amyotrophic lateral sclerosis (sALS) is poorly understood outside of three former high-incidence foci of Western Pacific ALS and a hotspot of sALS in the French Alps. In both instances, there is a strong association with exposure to DNA-damaging (genotoxic) chemicals years or decades prior to clinical onset of motor neuron disease. In light of this recent understanding, we discuss published geographic clusters of ALS, conjugal cases, single-affected twins, and young-onset cases in relation to their demographic, geographic and environmental associations but also whether, in theory, there was the possibility of exposure to genotoxic chemicals of natural or synthetic origin. Special opportunities to test for such exposures in sALS exist in southeast France, northwest Italy, Finland, the U.S. East North Central States, and in the U.S. Air Force and Space Force. Given the degree and timing of exposure to an environmental trigger of ALS may be related to the age at which the disease is expressed, research should focus on the lifetime exposome (from conception to clinical onset) of young sALS cases. Multidisciplinary research of this type may lead to the identification of ALS causation, mechanism, and primary prevention, as well as to early detection of impending ALS and pre-clinical treatment to slow development of this fatal neurological disease.

KEYWORDS

genotoxin, gyromitrin, agaritine, monomethylhydrazine (MMH), methylazoxymethanol (MAM), lifetime exposome, motor neuron disease

Introduction

Amyotrophic lateral sclerosis (ALS) has an average clinical onset age of 58–60 years, an annual incidence rate of 1–2.6 cases/100,000 persons and a prevalence of ∼6 cases/100,000 (Talbott et al., 2016), with higher rates among males (GBD 2016 Motor Neuron Disease Collaborators, 2018). Inherited forms of the disease account for 5–10% of cases in the U.S., whereas the balance has no clearly defined causation (Mehta et al., 2018). While almost 50 ALS gene mutants are recognized (Smukowski et al., 2022), with many more suspected, the vast majority of ALS cases occurs sporadically. Young-onset ALS refers to patients with initial symptom onset before age 45, the percentage of which has declined markedly since 1850, with a greater proportion on the continents of Africa, Asia, and South America perhaps related to nutritional and occupational factors (Gouveia and de Carvalho, 2007; Turner et al., 2012). The etiology of sporadic ALS (sALS) is often attributed with little evidence to undefined environmental agents acting on a genetic susceptibility to the disease (Al-Chalabi et al., 2010; Vasta et al., 2021; Goutman et al., 2022). Viruses, fungi, cyanobacteria, heavy metals, pesticides, persistent toxicants, solvents, electromagnetic radiation, electric shock, cigarette smoke, DNA damage, impaired DNA repair, epigenetic changes, immune dysfunction, endocrine abnormalities, excessive exercise, professional soccer, and trauma, have all been associated with sALS (Tandan and Bradley, 1985; Chiò et al., 2005; Armon, 2009; Pritchard and Silk, 2014; Alonso et al., 2015; Coppedè and Migliore, 2015; Harwood et al., 2016; Schwartz and Klug, 2016; Riancho et al., 2018; French et al., 2019a; Filippini et al., 2020; Andrew et al., 2022; Re et al., 2022), but convincing evidence of a causative role for any of these factors has yet to be demonstrated. Among heavy metals, lead has been proposed as a risk factor for ALS (Kamel et al., 2002; Fang et al., 2010; Meng et al., 2020; Andrew et al., 2022) but the association is considered weak (Wang et al., 2014; Newell et al., 2021). One group has proposed sALS arises from an opportunistic fungal infection (French et al., 2019a,b), and another has published evidence of mixed fungal and bacterial infection in the CNS of a small number of sALS cases (Alonso et al., 2017, 2019).

While mutations are suspected and often found in familial ALS, the operation of culpable trans-generational environmental exposures cannot be excluded (Johansen et al., 2021). For example, in the case of the former ultra-high incidence of familial and sporadic ALS among the Chamorro people of Guam, where the disorder was first thought to result from a dominantly inherited genetic trait (Plato et al., 1969), disease rates fell steadily in the second half of the twentieth century such that the high incidence had essentially disappeared (Garruto et al., 1985; Chen, 1995). A comparable reduction of high-incidence ALS has occurred in the Kii Peninsula of Honshu Island, Japan, and in Papua, Indonesia, in the western half of the island of New Guinea (Kihira et al., 2005; Spencer et al., 2005; Okumiya et al., 2014). Given the disparate genetic origins of Chamorro, Japanese, and Papuan New Guinean people, coupled with the absence of any consistent mutant genotype associated with these disappearing hyperendemic foci of ALS, the etiology of Western Pacific ALS appears to be dominated by environmental factors, particularly the gradual loss of traditional food and medicinal practices that accompanied societal modernization (Spencer et al., 2020). The only known exogenous factor of consequence arising from traditional practices is to the neurotoxic seed of cycad plants (*Cycas* spp.), which were formerly used for food (Guam) or medicine (Guam, Kii-Japan and Papua-Indonesia), as recorded on film[1]. These practices resulted in systemic exposure principally to methylazoxymethanol (MAM, the aglycone of cycasin and neocycasins) and also to beta-*N*-methylamino-L-alanine (L-BMAA), compounds with genotoxic, epigenotoxic and other toxic actions on the developing and adult mammalian nervous system (Spencer et al., 2020). Guam ALS was significantly correlated with the concentration of cycasin—but not with L-BMAA—in cycad flour used for food by Chamorros (Román, 1996). A diet of Chamorro-prepared cycad flour induced unilateral arm weakness and neuropathological changes thought to be reminiscent of ALS (Dastur, 1964), mice fed washed cycad pellets developed an ALS-like syndrome, with loss of motor neurons and later loss of dopaminergic innervation of the striatum (Wilson et al., 2002), and cycasin is known to be responsible for induction of hindlimb stiffness and weakness, muscle wasting, and spinal lesions in cattle and goats grazing on cycad leaves or seed (Shimizu et al., 1986; Spencer et al., 2022b). Formaldehyde is a metabolite of both L-BMAA and MAM (Spencer et al., 2020), variably identified as a risk factor for ALS (Weisskopf et al., 2009; Pinkerton et al., 2013; Seals et al., 2017) and the potential role of formaldehyde in relation to neuronal DNA damage and ALS has been discussed (Spencer, 2018).

Beyond the Western Pacific, L-BMAA and formaldehyde have been reported to be highly associated with sALS (French et al., 2019b). L-BMAA is produced by prokaryotic (cyanobacteria) and eukaryotic (diatoms and dinoflagellates) microorganisms across the globe (Delzor et al., 2014; Nunes-Costa et al., 2020). However, L-BMAA was excluded as a risk factor in a hotspot of sALS in the French Alps, where an association was made with consumption of poisonous wild *Gyromitra* mushrooms (Lagrange et al., 2021b). Species of these fungi contain hydrazones that are metabolized to monomethylhydrazine (MMH), a genotoxic that produces a pattern of DNA damage comparable to that of MAM. Hydrazine-related chemicals occur worldwide as natural products of certain bacteria, fungi, plants, and marine organisms; in synthetic form, they are used to produce certain pharmaceuticals and agrochemicals; in the manufacture of paints, inks, and organic dyes; in the preparation of polyurethane coatings and adhesives; as corrosion inhibitors in water treatment; to remove solids in steam generators; as oxygen scavengers; as reducing agents for metal recovery, as propellants for jet aircraft, rockets and spacecraft, and, formerly (1950s–70s) for artillery (World Health Organization, 1987; Knapton and Stobie, 1993; ATSDR, 1997; European Chemical Agency on request of the European Commission, 2011; Nguyen et al., 2021; Spencer and Kisby, 2021). Given the recently discovered association between food-derived MMH and sALS in France (Lagrange and Vernoux, 2020; Lagrange et al., 2021b), further research on the possible etiologic relationship between hydrazine-related chemicals and sALS is merited.

This paper proposes a strategy to maximize the possibility of discovering exogenous factors potentially underpinning the etiology of sALS. Once these factors have been identified, prevention can follow, either in primary form (avoidance of exposure) or, in theory, secondarily *via* post-exposure therapeutic blockade of neuropathogenesis. Two principles underlie the proposed research approach: (a) focus on conjugal, single-affected twin, young-onset, migrant, and geographically clustered sALS cases and (b) lifetime

1 https://vimeo.com/1621281

search for atypical exposures to chemical agents of all types (exposome), especially those relevant to the nervous system (neural exposome) and, in particular, to substances chemically related to those implicated in Western Pacific ALS. The present paper addresses the first point and asks whether, among published ALS cases, there are plausible potential links between sALS and a history of exposure to the many sources of hydrazine-related chemicals.

Materials and methods

Literature citations were retrieved from online databases (principally PubMed Central®) using Boolean search procedures. Search terms included: ALS, Lou-Gehrig disease; motor neuron(e) disease; epidemiology, clusters, cases, and studies of neurodegenerative disease; intoxication/poisoning linked to hydrazine; hydrazine-related chemicals; cycads; mushrooms/fungi; gyromitrin; pesticides; agrochemicals; organic solvents; jet airplane fumes; ALS-related regional geographic information, populations, customs, practices, military service, and war. Focus was limited to conjugal, single-affected twin, young-onset, migrant, and clustered sALS cases, and their reported and potential association with chemicals of natural and synthetic origin.

Results

Environmental etiology: Migrant ALS cases

Western Pacific ALS declined after WWII in all three affected populations (Spencer et al., 2020). Best studied in Guam, mean ALS incidence among males (65/100,000) and females (35/100,000) peaked in 1950–54 and then began to decline steadily through the end of the twentieth century when high-incidence motor neuron disease had disappeared (Plato et al., 2003). While the motor neuron was clinically typical of ALS elsewhere, the disorder was sometimes associated with parkinsonism and dementia in more elderly subjects, mean incidence rates of which peaked in males in 1960–64 (50/100,00) and females in 1970–74 (25/100,000), before declining in concert with ALS (Plato et al., 2003). Signs of parkinsonism have been reported in 30% a of population-based cohort of ALS patients (Calvo et al., 2019), and dementia is commonly associated with ALS, particularly in patients with a hexanucleotide repeat expansion (*C9ORF72)* in the non-coding region of chromosome 9 open reading frame 72 (Abramzon et al., 2020).

An important feature of the Western Pacific ALS Parkinsonism-Dementia Complex was the acquisition of neurodegenerative disease by persons who had migrated to, and resided for decades within, communities with a high incidence of the disease (Garruto et al., 1981; Kokubo et al., 2022). Also noteworthy was the development of ALS in persons years or decades after migration as children or teenagers from a hotspot of the Western Pacific disease (Reed and Brody, 1975; Garruto et al., 1980; Yoshida et al., 1998; Tsunoda et al., 2017). Taken together with the steady progressive decline (from 1950s to 2000) and virtual disappearance of ALS on Guam (Garruto et al., 1985; Chen, 1995) and in the two other high-incidence disease foci (Kihira et al., 2005; Spencer et al., 2005; Kuzuhara, 2007; Doi et al., 2010; Okumiya et al., 2014), these epidemiological observations provided powerful evidence in support of an environmental etiology of motor neuron disease; furthermore, since the ALS focus among former hunter-gatherers in western New Guinea was present before the introduction of any human-made chemicals, a natural causal agent seemed likely (Gajdusek and Salazar, 1982) and was subsequently linked to the medicinal use of the toxic pulp of cycad seed (Spencer et al., 1987b, 1991). The progressive decline of the traditional use of cycad seed for food and oral medicine was also, respectively, associated with the disappearance of high-incidence ALS on Guam and in the Kii Peninsula of Honshu, Japan (Spencer et al., 1987a, 2020).

Young-onset sALS

Western Pacific ALS cases

While Western Pacific ALS was clinically indistinguishable from ALS elsewhere, it is considered part of an ALS/Parkinsonism-Dementia Complex (ALS/PDC). ALS affected young subjects (20 years and older), while atypical parkinsonism with dementia (P-D), or dementia alone, affected older persons, with all such phenotypes in at least one instance occurring in the same Guam family, while some other cases exhibited mixed forms of the neurodegenerative disease complex (Spencer et al., 2020). While unproven, it seems plausible that persons with larger exposures to the culpable environmental agent(s) developed motorsystem disease at an early age (expressed clinically as ALS) while lesser-exposed subjects who survived fatal motor neuron loss developed mixed forms of ALS/PDC expressed clinically later in life. Additionally, on Guam in particular, many with and without ALS/PDC had a stationary pigmentary retinopathy (Campbell et al., 1993), which was replicated in laboratory species treated with cycasin or MAM at an age equivalent to the second trimester of human pregnancy (Spencer, 2020; Kisby and Spencer, 2021). While the exposure age for acquisition of neurodegenerative disease in later life is unknown, subjects who moved from the high-incidence focus in Kii-Japan developed motor neuron disease 1–7 decades later (Yoshida et al., 1998; Tsunoda et al., 2017), while exposure to Guam during adolescence/young adulthood, but not childhood, correlated strongly with ALS/PDC (Borenstein et al., 2007).

U.S. Veterans

In 1999, ALS cases were described among young American servicemen in an age group in which the disease is usually rare. A 2003 paper reported a nationwide epidemiologic case ascertainment study of ALS occurrence during the 10-year period since August 1990 among U.S. military subjects who served in the Gulf War (August 2, 1990, through July 31, 1991) during which the U.S. and its allies fought Iraq. A significant elevated risk of ALS was found among all U.S. personnel deployed to the Gulf region and was especially high among deployed Air Force personnel (Horner et al., 2003). During 8 post-War years, 17 of 20 Gulf War Veterans (GWV) were diagnosed with ALS before age 45 years (Haley, 2003). Although a 2005 study found an excess risk for ALS generally associated with U.S. military service (Weisskopf et al., 2005), the excess risk of ALS among 1991 GWVs was limited to the decade following the War (Horner et al., 2008). Whereas ALS prevalence among GWV was 5.8 per 100,000 over 10 years after the Gulf War, there was a significantly higher prevalence (19.7 per 100,000 persons over 14 years) among a somewhat older group of U.S. Veterans deployed in support of post-9/11 conflicts (Sagiraju et al., 2020). ALS prevalence (33/2/100,000)

was significantly higher in Air Force personnel relative to that of other service branches, and among tactical operation officers in comparison with general and administrative officers. Tactical operations officers consisted primarily of pilots, aircraft crew, missile, and combat operations staff. These data suggest that environmental concerns should be explored among those who routinely work with jet aircraft and develop ALS at a young age (Hayes et al., 2021; Andrew et al., 2022).

Interview of 82 mostly young (<45 years old) Gulf-deployed and non-Gulf-deployed U.S. Veterans with ALS diagnosed between 1998 and 1999 revealed familial cases in 10% and one deployed and 2 non-deployed subjects with prior service on Guam, plus a Guam-born Chamorro (Palmer and Spencer, 2002). Principal findings are shown in **Box 1**. Ten percent of deployed GWVs in this study were fighter pilots or involved in aircraft maintenance.

One Gulf-deployed Veteran not included in this preliminary study was a 32-year-old American F16 fighter pilot who flew 44 combat missions in Gulf War Operation Desert Storm, was diagnosed with ALS at age 37, and died 9 years later (Miller, 2022; Netter and Taylor, 2005). During Desert Storm operations, the U.S. Air Force relied heavily on the F16 Fighting Falcon, a multirole jet fighter uniquely equipped with an Emergency Power Unit (EPU) powered by the monopropellant H-70, which contains 70% hydrazine (N_2H_4) and 30% water by weight (Anon, 2022s). With the exception of 12 F16 fighter jets used by the Royal Bahrani Air Force, none of the Allied Countries (Canada, France, Italy, Kuwait, Qatar, Saudi Arabia, U.K., United Arab Emirates) used this jet fighter during Operation Desert Storm (Anon, 2022m). Iraq had experimented with hydrazine rocket fuels, including unsymmetrical dimethylhydrazine, but the U.S. Department of Defense concluded these fuels were not used by Iraq during the Gulf War. Thus, while it appears unlikely that non-U.S. Coalition service members were exposed to hydrazine rocket fuels during the 1991 Gulf War (Brown, 2006), prior investigations of toxic exposures among U.S. GWVs have failed to recognize the potential for exposures to hydrazine associated with powering up or servicing the F16 EPU (Suggs et al., 1979; Anon, 2022h; Cenciotti, 2022). Such potential exposures from F16 EPUs continue to this day; 4,600 F16s had been built by 2012, while improved versions will continue to be constructed for export customers (Bahrain, Slovakia, Bulgaria, Taiwan, Morocco, and Jordan) through 2026 (Anon, 2022k). Small numbers of F-16 variants are used for non-flying ground instruction of maintenance personnel (Anon, 2022k). Hydrazine is also used to fuel an auxiliary power unit on the Eurofighter Typhoon, but use of hydrazine in the European Union beyond 2025 is predicted to be banned (Anosseir, 2021).

ALS clusters and hotspots

Studies on the incidence of ALS and the identification of geographical clusters have taken place in many Western countries, including the USA (Taylor and Davis, 1989; Sienko et al., 1990; Turabelidze et al., 2008; Caller et al., 2009; Reddy, 2020), Italy (Giagheddu et al., 1983, 1993; Grainieri et al., 1989; Uccelli et al., 2007), Sweden (Gunnarsson et al., 1996), Finland (Sabel et al., 2003), Denmark (Johansen et al., 2021), United Kingdom (Mitchell et al., 1998; Scott et al., 2009), Greece (Kalfakis et al., 1991), and France (Boumédiène et al., 2011; Lagrange et al., 2021b). While an aggregation of patients in a given geographic area theoretically may occur by chance or be the consequence of a statistical bias in the process of patient selection (Malaspina et al., 2002), their intensive study has the potential of discovering important environmental associations, as demonstrated by the experience with Western Pacific ALS. Noteworthy is that a cluster across generations might arise from a genetic factor, an established local practice, or a geographically restricted exposure (Malaspina et al., 2002).

France and Italy

Environmental factors were examined closely in a hotspot of sALS in Savoie in southeast France (Lagrange et al., 2021b). After excluding a role for heavy metals, pesticides, garden chemicals, and L-BMAA in drinking water, a case-control study of 14 sALS patients among part-time and full-time residents of a ski-resort hamlet revealed the prior food use of wild mushrooms (*Gyromitra* spp., including the Snow Morel *G. gigas*) (Miller et al., 2020). Subsequent investigation revealed that ALS patients also consumed the poisonous False Morel, *G. esculenta.* Half of the ALS cases reported an acute illness following ingestion of *gyromitres* 5–20 years prior to onset of muscle weakness. Control subjects had also collected and eaten wild mushroom species, but not *G. esculenta*. Banned for sale in France, *G. esculen*ta (False Morel, Brain mushroom, Turban fungus) contains gyromitrin (*N*-methyl-*N*-formylhydrazine) and eight additional homologous hydrazones that generate genotoxic MMH (Nagel et al., 1977; Liener, 1986; Trestrail, 1994, 2000) which, like cycad-derived MAM (Matsumoto and Higa, 1966; Nagata and Matsumoto, 1969; Nair, 1990) and nitrosamines (Shank and Magee, 1967) produces carbon (methyl) free radicals that methylate DNA and RNA.

While the generality of the following observation is unknown, samples of *G. esculenta* collected in southern France at middle altitudes (900–1,200 m, Mt. Aigoual, Gard/Lozère; Espinouse Mountains, Hérault) reportedly have higher MMH concentrations (200–350 mg MMH) than concentrations (50–60 mg MMH) found in specimens at higher altitudes (2,200 m) in the Pyrénées Orientales, France (Lac des Bouillouses). Prolonged desiccation (76 days for > 6 months) resulted in the concentration of MMH stabilizing around 300–450 mg/kg of dry specimen (equivalent to about 15–30 mg MMH/kg of reconstituted fresh specimen) (Andary et al., 1985). Collection of wild mushrooms for food is locally popular on the other side of the Alps, in northwest Italy (Piedmont, Aosta Valley) (Anon, 2022t); ALS incidence is high (Marin et al., 2017), and hotspots of sALS have been reported in Cuneo and Vercelli (Migliaretti et al., 2013), Briga Novarese, Trino and Tronzano, and Vercellese (Uccelli et al., 2007; **Figure 1**), and confirmed in Acqui Terme, province of Alessandria (*vide infra*) (Vasta et al., 2018), regions (province of Biella) that harbor MMH-generating fungi, notably *G. esculenta* (Pers.) Fr. (Anon, 2022n), that can trigger acute poisoning (Andary et al., 1985; GBIF, 2022). Today, food use of *Gyromitra* spp. in Piedmont is reportedly almost non-existent (Nicola Sitta, personal communication, February 2022).

On the island of Sardinia (**Figure 1**), the distribution of ALS was non-homogenous (more cases in rural areas) and more common among farmers and shepherds with low levels of education (Giagheddu et al., 1983, 1993; Grainieri et al., 1989). While no association has been made between ALS and food use of mushrooms outside of southeast France, wild morels are among the fungi collected in Sardinia (Anon, 2020), and many authors have warned that some MMH-generating False Morels can be mistaken for the highly sought-after True Morel (*Morchella esculenta*) (Gone 71°N, 2022; **Figure 2**). In recent decades, there has been a

BOX 1 ALS among U.S. Gulf War Veterans.

Principal findings of a preliminary 2002 investigation of exposures of U.S. Gulf War Veterans (GWV, *n* = 33 males) who were deployed to S.W. Asia during the period August 1, 1990–July 31, 1991) and U.S. Gulf-era Veterans (GEV, *n* = 49 males) not deployed to S.W. Asia, who were diagnosed with definite ALS (D-ALS) or probable ALS (P-ALS) between 1991 and 2001 (Palmer and Spencer, 2002). (Supported by Department of Veterans Affairs Cooperative Studies Program #500). Report available on request from Corresponding Author.

- Approximately 10% of ALS cases were familial, three others had seen service on Guam and one was Guam-born.
- Similar numbers of D-ALS and P-ALS among GWV and GEV serving in the Army, Navy or Air Force.
- Similar dates of diagnosis for D-ALS and P-ALS, with peak incidence in 1988–99 for both groups.
- One third of D-ALS and P-ALS involved with aircraft, land-based vehicles or construction.
- Chemical exposures/activities included pesticides, organic solvents, jet fuel, welding, soldering, others.
- Voluntary skin applications included a shampoo containing neurotoxic zinc pyridinethione.
- Physical exposures included radiation (radar, microwave), electromagnetic fields, electric shocks.
- Biological exposures included vaccines, sand flies, mosquitoes; several cases of prior Lyme disease.
- Physical exposures included survival training in tropics, long-distance running and physical exhaustion.
- Oral exposures included contaminated/malodorous water and large quantities of diet and other sodas.
- One familial case collected and ate mushrooms like his uncle: both developed ALS at 37–38; years of age.
- Conjugal pair, electric mechanic/industrial cleaner and wife exposed to his chemical-saturated clothing.

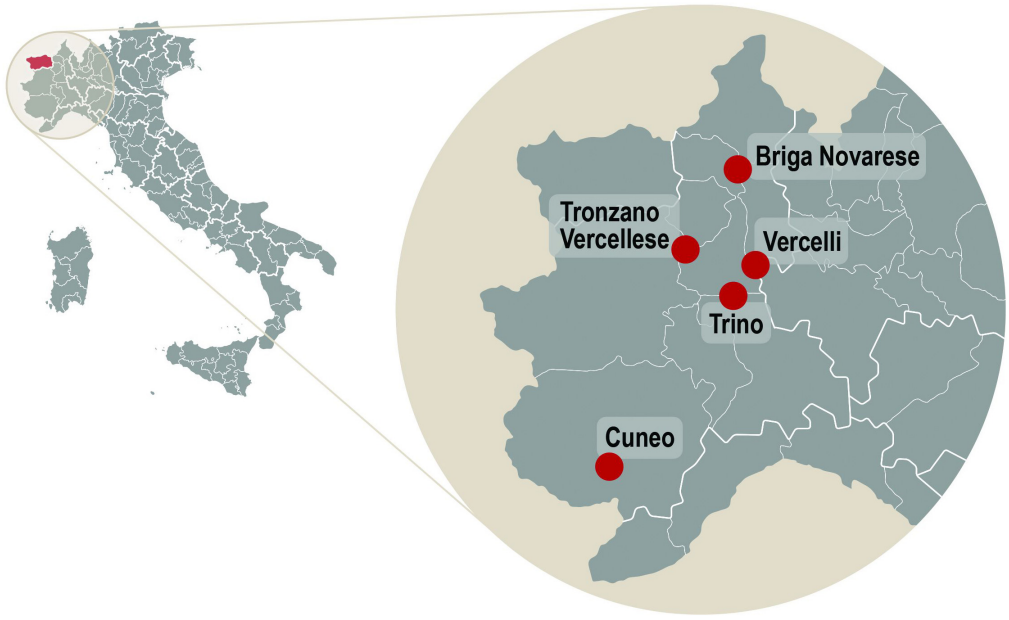

FIGURE 1
Left image: Map of Italy (including the islands of Sardinia **center left** and Sicily, **bottom**), showing the Aosta Valley in red **(upper left)** and nearby ALS hotspots **(right)** in Piedmont. The town of Acqui Terme is located ~50 miles due east of Cuneo.

sharp rise in foraging for wild mushrooms across Sardinia along with an increase in the number of acute (but unspecified) intoxications, few of which have proved fatal (Comandini et al., 2018). Noteworthy is that images #11 and #21 in an historical treatise on the hypogeous fungi of Sicily and Sardinia (Mattirolo, 1900) depict False Morels (*Gyromitra* spp.).

Denmark, Sweden, and Finland

The incidence and prevalence of ALS is high in the Danish Faroe Islands (Johansen et al., 2022), particularly the southernmost island of Suðuroy, where the prevalence is three times higher than the nationwide prevalence (Johansen et al., 2021). While familial clustering (14%) was in excess of that expected for ALS on Suðuroy, a result suggestive of genetic contribution, environmental factors were not excluded or sought. In Sweden, compared to the rest of the population, agricultural work was significantly more common among cases in an ALS cluster in the county of Skaraborg (Gunnarsson et al., 1996), where *G. esculenta* was formerly eaten (Denchev et al., 2013; Svanberg and Lindh, 2019). Additionally, as discussed elsewhere (Spencer, 2019), the birth location of a cluster of ALS subjects in Finland (Sabel et al., 2003) corresponded to a region of False Morel consumption promoted by wartime-associated food shortages. Between 2000 and 2015, almost 20% of mushroom-identified calls to the Finnish Poison Information Center involved acute food poisoning from *G. esculenta* (Tähkäpää et al., 2020).

United Kingdom

Clusters of ALS have been reported in areas of southeast England and East Lancashire (Mitchell et al., 1990, 1998; Scott et al., 2009). *G. esculenta,* which has a localized U.K. distribution in coniferous regions, has been reported in these regions (Anon, 2022j,o). The East Lancashire focus of ALS in Addington/Owaldtwistle is the location of the urban Foxhill Nature Reserve where mushroom hunters are taught to identify poisonous species (Cruces, 2010). The U.K. lists 4 *Gyromitra* spp. and 36 *Agaricus* spp. (BMS, 2022), the latter containing various concentrations of

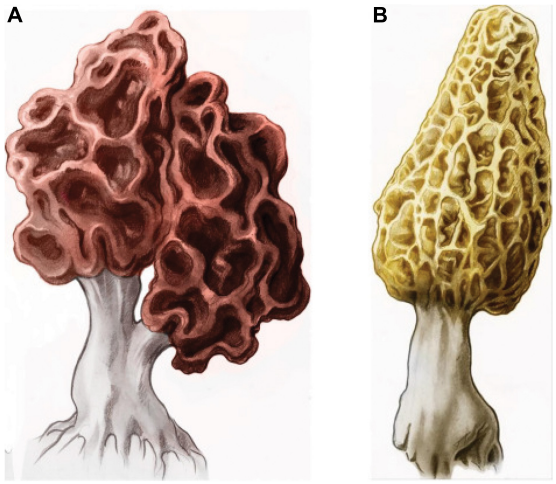

FIGURE 2
(A) False Morel (*Gyromitra esculenta*). **(B)** True Morel (*Morchella esculenta*). Reproduced from Arłukowicz-Grabowska et al. (2019) under Creative Commons license. Mistaken identification of morels can lead to group poisoning events, as occurred in February 2019 among diners at a Michelin-starred restaurant in Valencia, Spain. https://www.thelocal.es/20190221/michelin-starred-valencia-restaurant-closed-amid-fears-diner-died-from-poisonous-mushroom/. In 2020, the U.S. issued an alert given the history of *G. esculenta* contaminating imported commercial shipments of dried and canned *M. esculenta* (FDA, 2020). A 2-year survey showed 21% of the True Morel and 15% of the wild mixed mushrooms were contaminated with toxic look-alike species (Gecan and Cichowicz, 1993). An epidemiologic study of ALS in southern Greece showed an overrepresentation of farmers among patients and an aggregation of cases in the region of Cephalonia (Kalfakis et al., 1991), where morels occur (Denchev et al., 2013). See also: how to identify a real morel from a false morel. Available at: https://www.youtube.com/watch?v=OXePLfjCUeI.

the phenylhydrazine derivative agaritine [*N*-(γ-L(+)-glutamyl)-4-hydroxymethylphenylhydrazine] (Schulzová et al., 2009).

Spain

A 2018 study identified three ALS clusters in agriculture-intensive areas of north/northeast Catalonia (Povedano et al., 2018), two of which are proximate to national parks with edible True Morels (*M. dunalii*) and several poisonous hydrazinic fungi (*Agaricus* spp.) (Sierra and Valverde-Valera, 2019), one of which [*A. bitorquis* (Qu l.) Sacc.] had (in the Czech Republic) a very high content of agaritine (Schulzová et al., 2009). The Catalonian authors proposed their results were consistent with exposure to agricultural pesticides, as well as to air pollution, but dietary factors were not addressed. Among wild mushrooms in Catalonia are the *bolet de greix* (fat mushrooms), namely *G. esculenta and G. gigas*, that grow beneath pine trees during Spring, and *G. infula,* which is found in the autumn (Anon, 2011). *G. esculenta,* while recognized to be poisonous, reportedly has been consumed since ancient times in the Valleys of the Pyrenees (Anon, 2022e). In recent times, *G. esculenta* has been sold in a market called *Mercat del bolet de Cal Rosal* located in Olvan (Bergueda) (R.V.A., personal observation), which joins the Pyrenees and Central Depression and is within the north-south cluster of ALS in the Barcelona region. Further north in the Pyrenees, *G. esculenta* and *G. infula* are recorded in the Ordesa y Monte Perdido National Park (Pancorbo et al., 2017).

Southwest Central France

Three ALS clusters in Nouvelle Aquitaine (formerly Limousin) were geostatistically linked to the presence of paper paste and water-treatment plants; the authors suggested that heavy use of chemicals and water in these plants would create habitats favorable to cyanobacteria and, hence, the generation of L-BMAA (Boumédiène et al., 2011). Formaldehyde is heavily associated with paper or paperboard production, while hydrazine is mainly used for eliminating oxygen in water for steam generation in the paper industry (Korhonen et al., 2004). Hydrazine is widely employed in thermal engineering as an anticorrosive agent; when preserving and passivating equipment, a large volume of wastewater containing a high concentration of hydrazine (about 100 mg/dm3) is formed after chemical cleanings (Gogolashvili et al., 2001). In the Limousin study, the Standardized Incidence Ratio for ALS exceeded unity (but without statistical significance) for water-treatment stations that used an active sludge system, which cannot handle hydrazine (Farmwald and MacNaughton, 1981). Also noteworthy is the strong mushroom culture in Limousin that includes distinguishing edible (notably *Boletus edulus,* or *cèpe*) from toxic varieties (Harley, 2021), including *G. esculenta* (Taylor, 2008). A 2003–2011 population-based study of >5 million inhabitants in 10 *départements* (the name assigned to the largest unit of government in France) in 5 regions of France found the "possible over-incidence" of ALS (8 cases vs. 2 expected) in one *département* (Haute Vienne, town of Rochechouart) of Nouvelle Aquitaine (Boumédiene et al., 2022). Among seven areas subject to complete the robust analysis, only one "definite cluster of ALS" was identified, namely that associated with *gyromitres in* the French Alps (Lagrange et al., 2021b).

United States Mainland

MMH-related mushroom poisoning is recorded in many countries, including the USA, notably the State of Michigan (Trestrail, 1994; Hatten et al., 2012; Brandenburg and Ward, 2018; Horowitz et al., 2022; **Figure 3**) in the Midwest, which has among the highest prevalence of ALS in the nation[2]. Self-identified clusters of ALS are common in Michigan, such as three friends with proximate childhood homes who later developed ALS around the same time (Feldman, 2000). A case-control study of ALS in Michigan (Yu et al., 2014) that sought information on occupational and residential exposures, residence location, exercise and sports, body weight, tobacco use, military experience, and family history, found an association with fertilizers and pesticides and no association with smoking, occupational exposures to metals, dust/fibers/fumes/gas and radiation, and physical activity. Questions related to diet were not included in this study.

A 1989 study found evidence for clustering of ALS in northeast Wisconsin adjacent to northwestern Lake Michigan (Taylor and Davis, 1989; **Figure 3**), where wild morel season begins in May (Anon, 2022d); while *G. esculenta* can be found (Anon, 2022l), *G. brunnea* is most common (Volk, 2002; Anon, 2022l). Additionally, a 1990 case-control study found a small cluster of ALS cases among long-term residents of Two Rivers, Manitowoc County, Wisconsin. Physical trauma, the frequent consumption of freshly caught Lake Michigan fish, and a family history of cancer were reported more often by case patients than control subjects (Sienko et al., 1990). While diet was among many factors examined, it is not clear whether

2 https://www.pbs.org/video/als-xmw7rw/

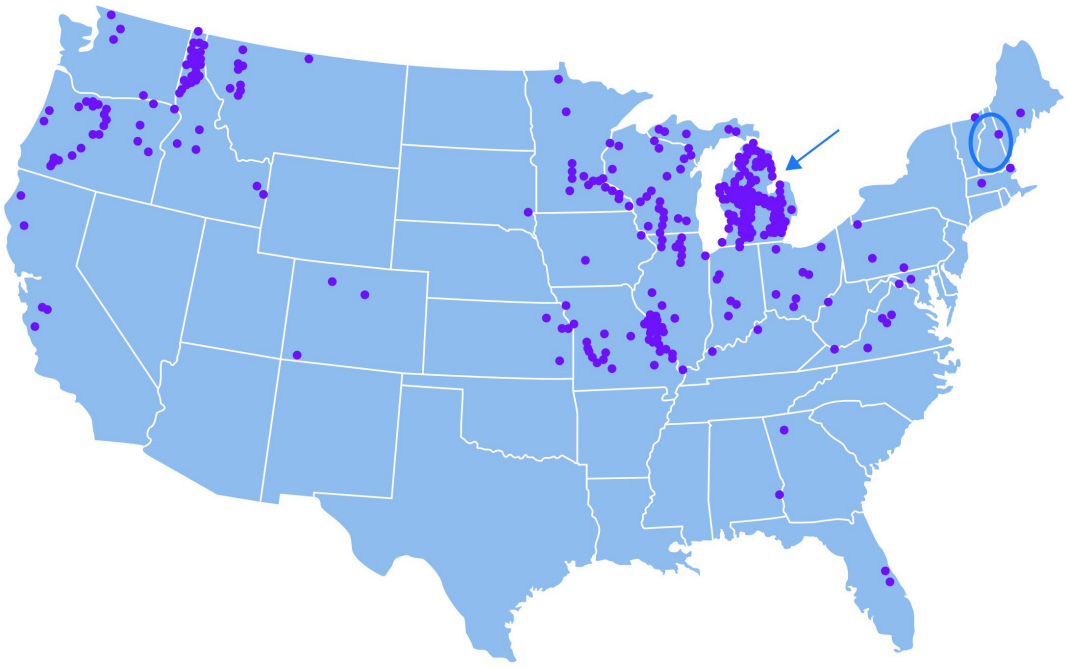

FIGURE 3
Map of USA in which each dot represents one call to a U.S. poison center regarding ingestion of a mushroom species containing monomethylhydrazine (MMH) over the 10-year period 2001–2011. Redrawn from Hatten et al. (2012). A 2006 paper from the North American Mycological Association tabulated reports of acute illness from *Gyromitra* spp. (including *G. brunnea, esculenta, gigas, montana*) from Arkansas, Idaho, Iowa, Massachusetts, Michigan, Montana, Quebec, Oregon, and Washington (Beug et al., 2006), and an Asian couple west of the North American Rockies (Leathem and Dorran, 2007). Several MMH reports originated from the northwestern United States (notably Oregon, Idaho), northcentral and central states, including Wisconsin (adjacent to Michigan) Missouri (southernmost cluster of dots) and a few from New England states, including New Hampshire (*oval*). **(Upper-right)** The highest concentration of MMH-related calls came from Michigan (*arrow*) in the Great Lakes region of the upper Midwestern United States, the home of several species of poisonous False Morels (Mushroom Observer, 2015). Areas of Michigan suspected to have a high ALS incidence include the towns of Cadillac and Greenville, and Newaygo County (Anon, 2022b). Mesick, a town 19 miles northwest of Cadillac, holds morel-hunting contests at the May Annual Mesick Mushroom Festival (Anon, 2022p). The National Morel Mushroom Festival takes place in Boyne City in northern Michigan (National Morel Mushroom Festival, 2022). These locations are all in northern Michigan's Lower Peninsula where wild morels have been mapped (Morels, 2022). Note that fungi take up not only metals from soil but also persistent environmental pollutants, including organochlorine pesticides, polychlorinated biphenyls, and brominated flame retardants (Moeder et al., 2005; Rodríguez-Rodríguez et al., 2012; Bokade et al., 2021), certain members of which were elevated in the blood of Michigan patients with ALS (Su et al., 2016).

their research instrument queried the popular practice in Wisconsin of collecting and eating wild morels (Craddock, 2017; Anon, 2022f).

A 2009 study of nine patients in Enfield, New Hampshire (**Figure 3**), revealed an incidence of sporadic sALS that was 10–25 times the expected incidence of 2/100,000/year (Caller et al., 2009, 2013). The patients lived close to Lake Mascoma, one of several New Hampshire waterbodies with higher-than-average mercury concentrations (Neils and Nelson, 2018) and a history of algal blooms. The authors suggested the ALS hotspot might arise from chronic exposure to cyanobacterial neurotoxins (such as L-BMAA) in association with aerosols, fish consumption or ingestion of lake water. Uninvestigated is whether these cases engaged in the Spring harvesting of True Morels (*M. esculenta*) (Anon, 2016; **Figure 2**) but collected and consumed *G. esculenta* either deliberately or in error (De Román et al., 2006), a subject addressed by the Northern New England Poison Center (Colin, 2019). Other ALS clusters have been reported in eastern and northwestern Vermont, Maine and western New Hampshire (Caller et al., 2013), where seasonal collection of wild morels is also popular (Garrett, 2012; Anon, 2022r).

A 2008 epidemiologic investigation of ALS in Jefferson County, Missouri (**Figure 3**), identified a small cluster of patients around a lead smelter area (Taylor and Davis, 1989). Mushrooms flourish in smelter areas, where they take up heavy metals such as lead (Spencer and Palmer, 2021). In the case of *G. caroliniana* (Big Red) (Kuo, 2021), which is distributed statewide in Missouri (Anon, 2022c), fungal metal uptake might require continuous production of hydrazine-related compounds to store the potentially toxic elements in the form hydrazine-metal chelates (Govindarajan et al., 1995). Selenium is of interest in this regard because this metal has been linked to ALS in South Dakota USA (where *Gyromitra* spp. also occur Miller et al., 2020) and in Reggio Emilia Italy (Kilness and Hichberg, 1977; Vinceti et al., 2010a,b), where education on False Morels has been posted (Cocchi and Siniscalco, 2022). *G. caroliniana* is widely available in North Carolina, where bulbar presentation of ALS occurred in three geographically proximate long-term residents (Hochberg et al., 1974).

Conjugal ALS

Conjugal cases are important because of the possibility of identifying a history of common environmental exposures with potential relevance to etiology (Godeiro-Junior et al., 2009), as demonstrated by the association of cycad toxins with Western Pacific ALS, especially on Guam (Spencer et al., 2020). Outside of Guam, where conjugal cases of ALS were reported in 1975 (Reed et al., 1975), diagnoses of ALS in couples is rare (Dewitt et al., 2012). Through

2021, there were reports of at least 20 conjugal ALS pairs in the literature. There was an important geographical cluster of 10 pairs in southeast France (Camu et al., 1994; Corcia et al., 2003; Lagrange et al., 2021b), 5 pairs in Italy (Paolino et al., 1984; Poloni et al., 1997; Rachele et al., 1998; Chiò et al., 2001; Bersano et al., 2014; Vasta et al., 2018), 2 pairs each from Brazil (Godeiro-Junior et al., 2009) and the UK (Orrell et al., 1996; Fernandes et al., 2017), one pair each from Libya (Maloo et al., 1989) and Spain (Martínez Matos et al., 1986), and 4 pairs from the United States (Chad et al., 1982; Cornblath et al., 1993; Palmer and Spencer, 2002). No conjugal case was shown to be consanguineous and, prior to ALS diagnosis, all pairs had lived together for at least 10 years and sometimes much longer. These reports raise the possibility of shared exposure to unknown environmental risk factors; unfortunately, they have been often dismissed as coincidental, random associations.

France

Two reports of geographic clustering of conjugal ALS cases in southeast France are of singular importance (Camu et al., 1994; Corcia et al., 2003; **Figure 4A**). In total, 18 patients representing 9 couples (cases) presented with ALS between January 1975 and December 1999. Eight patients had disease onset between 1975 and 1992, while 10 were diagnosed during or after 1994. The mean age of onset was 65 years (range, 41–85 years), and the mean interval between onset of spousal ALS was 8 years (range, 1–19 years). Disease onset was spinal in 60% and bulbar in one third. There was no known consanguinity between affected spouses, and there was no major predominance of a given occupation or any specific environmental exposure that could be identified. The mean conjugal lifetime before the first ALS case was 10 to > 40 years (mean: 25 years), which is consistent with the long-latent period for post-exposure development of Western Pacific ALS (Spencer et al., 2020). Three of the conjugal cases resided in Drôme *département* and two of these lived in Valence, a town in Auvergne-Rhone-Alpes not far removed from the *département* of Savoie to the northeast, the location of a cluster of ALS patients in Montchavin (Lagrange et al., 2021b), including a conjugal case (**Figure 4A**), all of which reported a history of food use of *gyromitres*. The University of Illinois Natural History Survey Fungarium lists genetically confirmed examples of *G. gigas* in the S.E. French alpine region (**Figure 4B**); an equivalent map for *G. esculenta* is not yet available.

Italy

One of the 5 reported conjugal Italian cases resided in a hotspot of ALS in Acqui Terme, a town situated in the Monferrato area of the province of Alessandria, Piedmont, Italy (Bersano et al., 2014; Vasta et al., 2018; **Figure 1**, right). In 2005, spinal-onset ALS was diagnosed in a 63-year-old male and, 3 years later, bulbar-onset ALS in his 68-year-old non-consanguineous wife. Genetic screening of both patients revealed no ALS-associated mutant genes. Environmental histories identified no common exposures to radiation, food-borne pathogens, cosmetics, drugs, or pesticides in agricultural environments; nor were there exposure risks from smoking, intense physical activity, or trauma. While the etiology was not identified, it is noteworthy that a May 16, 2020 article in the local Acqui Terme newspaper *L'Ancora* described an undefined syndrome associated with food use of *Gyromitra* spp. (*Falsa Spugnola*), which contains high concentrations of "*giromitrina* (a hydrazine mixture)." An article on page 9 described two "*Serate Micologiche*" (Evenings Mycological) devoted to fungal toxicology organized from the Punto Cultura Association, with the patronage of the municipality of Acqui Terme and the Province of Alexandria.

Conjugal ALS was also reported to affect a married couple, members of which originated from different regions of Italy and lived in a small Piedmont town of 19,571 inhabitants (Chiò et al., 2001), a population approximating that of Acqui Terme (**Figure 1**, right). The husband was diagnosed with ALS in 1994 (age 61 years), the wife in 1999 (age 53 years). He had used "solvents: nitro-compounds and dimethylketone." Prior to their diagnoses, both had engaged in various jobs including, between 1989 and 1993, the collaborative maintenance and operation of a gasoil-powered central heating system that served the apartments of the house in which they resided. Their chemical exposure during this period was unstated, but it is of potential interest that hydrazine solutions are used to control oxygen corrosion in boiler systems (Scrivenand and Winter, 1978).

Three other Italian reports describe non-consanguineous conjugal cases of ALS in the second half of life. Two conjugal pairs involved residents of Sardinia (**Figure 1**, left). One pair (from

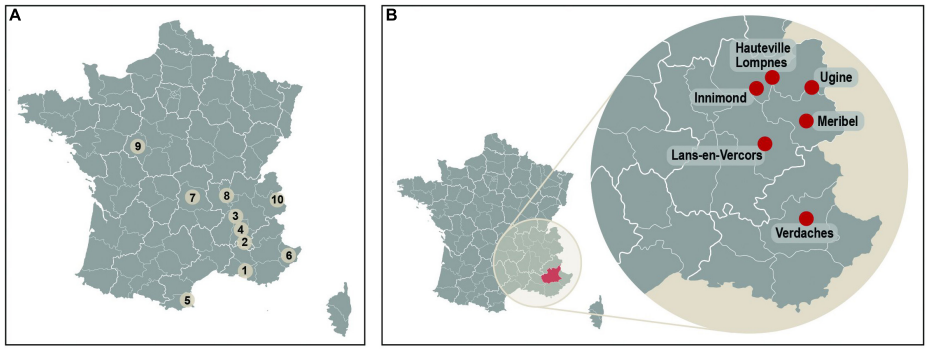

FIGURE 4
(A) Geographic distribution of the conjugal amyotrophic lateral sclerosis cases. The French territory is divided on the map into its different *départements*. Each conjugal case is represented by its number in the text. #1, Bouches-du-Rhône; #2-4, Drôme; #5, Pyrénées-Orientales; #6, Alpes-Maritimes, #7, Puy-de-Dôme; #8, Rhône; #9 Indre-et-Loire, #10 Montchavin-Les Coches. Modified from Corcia et al. (2003) to include data from Lagrange et al. (2021b). **(B)** Location of genetically confirmed samples of *G. gigas* Krombe. Cooke collected from woodland conifers in S.E. France. Left: Alpes-de-Haut-Provence, Colmars, Ratery. Right: Ain, Hauteville-Lompnes, Col de la Berche; Ain, Innimond, Plaine du Bief; Isere, Lans-en-Vercors, Combe de Servagnet; Savoie, Ugine, La Mollette; Savoie, Les Allues, Altiport de Méribel; Alpes-de-Haut, Verdaches, Haut-Bès. (Sourced from the Illinois Natural History Survey Fungarium, University of Illinois, January 2022).

Barbagia, province of Nuoro) was involved in sheep breeding (Paolino et al., 1984), another lived next to a distillery (Rachele et al., 1998). The third pair lived adjacent to a metallurgical plant in a heavily farmed area of southern Italy (location not stated). Affected couples had lived together for 3–4 decades (Poloni et al., 1997; Rachele et al., 1998), with common exposure to water and garden vegetables reportedly resulting in no significant exposure to heavy metals, pesticides or "known toxic substances" (Poloni et al., 1997). Couples were considered to have conjugal ALS by chance (Paolino et al., 1984; Rachele et al., 1998; Chiò et al., 2001).

Other countries

Three papers describe three conjugal cases of ALS in mainland USA (Chad et al., 1982; Cornblath et al., 1993; Palmer and Spencer, 2002), but none investigated common environmental exposures and one attributed the conjugal association to chance (Cornblath et al., 1993). A conjugal case occurred in a >20-year married couple; the husband worked for the U.S. military as an electrical mechanic with large air conditioners and refrigeration and had chemical-saturated clothing washed by his wife (Palmer and Spencer, 2002). Common exposures were also not explored for (a) an elderly Libyan couple who had lived together for 40 years (30 years in Benghazi) and developed ALS within 15 months of each other (Maloo et al., 1989) or (b) for another conjugal ALS couple from Spain (Martínez Matos et al., 1986). Another study lacking an exposure history described two couples with conjugal ALS from Brazil; one couple had lived together for 40 years, and the second for 20 years in the countryside outside Sao Paulo (Godeiro-Junior et al., 2009). Near simultaneous onset of motor weakness occurred in the 7th decade of life of a couple living in Scotland; since no common environmental history was found, conjugal ALS was assumed to have occurred by chance (Fernandes et al., 2017). Studies of ALS in the Lothian lowlands of Scotland between 1961 and 1981 did address potential environmental exposures to the extent of employment history (Holloway and Mitchell, 1986). None of the foregoing studies explored food-related exposures.

Twins discordant for ALS

Twins with only one ALS-affected subject can serve as matched-pair case-controls for study of disease etiology (Schlesselman, 1982). A 1997 British (England and Wales) study of 70 pairs of monozygotic and dizygotic twins assessed the environmental exposure history of pairs of twins discordant for ALS (Graham et al., 1997). Seventy-seven probands were identified, of which 26 were monozygotic and 51 dizygotic, with deaths between 1979 and 1989. Four monozygotic probands were concordant, but two probands came from a family known to have familial ALS. The content of the questionnaire and interviews of surviving relatives was not stated, but the results suggest the authors focused on occupation. Although analysis of the exposure history of discordant monozygotic twins would be of primary interest, small subject numbers required the authors to include both discordant monozygotic and dizygotic twins in their analysis. The strongest and most highly significant association (OR 7.0, 95% CI 1-33-89-90, p = 0.006) was with a history of regular car/vehicle maintenance (Graham et al., 1997). Noteworthy is that hydrazine was used to fuel racing cars, funny cars and dragsters in the 1960s (with events in the U.S. and U.K.) (Cook, 2018; Anon, 2022a), and several individuals with a history of motor racing developed ALS (Spencer, 2019), as did a Gulf War Veteran with familial ALS (Palmer and Spencer, 2002). The British twin study also found a significant association between motor neuron disease and a history of occupational paint usage (OR = 3.75; 95% CI 1-0-17 1, P = 0.022) (Graham et al., 1997). Hydrazide compounds are widely used in paint and adhesive thermoset applications, including latent hardeners for epoxy resins and as crosslinking agents in acrylic emulsions (Hara, 1990; ATSDR, 1997).

Close-proximity ALS groups

There are several brief reports of unrelated people living or working in close proximity who developed ALS at approximately the same time (**Table 1**).

A 2007 report described three friends who grew up in the same village in southeast England, began to play soccer at age 15/16, continued together at a moderately high level for many years (sometimes in the soccer league/team), and 10–20 years after they had stopped playing soccer, developed symptoms of ALS (no family history of ALS) within a few years of each other (Wicks et al., 2007). This account was preceded and followed by reports of an elevated risk of ALS among Italian First and Second Division soccer players. Whereas no cases of ALS were found during the 1970–1979 period, the standardized morbidity ratio was significantly increased for the periods 1980–1989 and 1990–2001. Moreover, a dose-response relationship between the duration of professional football activity and the risk of ALS was found (Chiò et al., 2005), with a younger age at onset of symptoms in soccer players born in more recent years (Vanacore et al., 2018). The mean age at diagnosis was 45.0 years, > 20 years earlier than that for the general population (Pupillo et al., 2020). No cases of ALS were found among professional basketball players or cyclists, suggesting that the physical activity of soccer players *per se* was not causally related (Chiò et al., 2009), although a possible role for repetitive head trauma during play could not be excluded (Beghi, 2013). The Italian and English reports also raised the possibility of repetitive player contact with pesticidal chemicals used on soccer pitches (Vanacore et al., 2006).

Discussion

We propose intensive study of young-onset, conjugal, twin-discordant and clusters of sALS as a method to discover and identify exogenous agents with the potential to trigger motor neuron disease. Among such agents, acting alone or in the presence of a genetic susceptibility, there is evidence that sufficient exposure to naturally occurring or synthetic hydrazine-related chemicals is associated with the development of clinical ALS years or decades later. While this association requires confirmation, it is known that hydrazines and MAM form carbon-centered free radicals (potent alkylating agents) that can methylate DNA in the O^6-, N7-, and C8-positions of guanine (Kobayashi and Matsumoto, 1965; Matsumoto and Higa, 1966; Shank and Magee, 1967; Albano et al., 1989; Gamberini and Leite, 1993; Kisby et al., 1999; Spencer et al., 2015). The accumulation of DNA lesions is responsible for the teratogenic, mutagenic, hepatotoxic, carcinogenic, and neurotoxic properties of MAM (Laqueur, 1977; Sieber et al., 1980; Kisby and Spencer, 2011). Neurons are proposed to be susceptible to O^6-methylguanine because the specific DNA-repair enzyme O^6-methylguanine methyltransferase (MGMT) is

TABLE 1 Close-proximity ALS case groups.

	ALS grouping	Location	Common exposures	Environmental factors	References
A	Ranchers (n = 3)	South Dakota, USA	Proximate residences	High selenium soil content	Kilness and Hichberg, 1977
B	Males (n = 3)	North Carolina USA	Common residence	No association proposed	Hochberg et al., 1974
C	Mail clerks (n = 3)	Florida, USA	A11 died within 10-years period	No association proposed	Sanders, 1980
D	Three (2M, 1F)	Montreal, Canada	Apartment complex	No association proposed	Melmed and Krieger, 1982
E	Teachers (n = 3)	Ohio, USA	Same classroom	No association proposed	Hyser et al., 1987
F	NASA staff (n = 7)	California, USA	Common workplace	No association proposed	Noack, 2016; NASA Ames Astrogram, 2018
G	Population	Missouri, USA	Lead smelter proximity	Small cluster	Turabelidze et al., 2008
H	Three friends (3F)	Michigan, USA	Childhood proximity	No association proposed	Feldman, 2000

Rows: (A) Region has a high soil content of selenium, one of many elements taken up and concentrated by mushrooms (Spencer and Palmer, 2021). (B) Unstated occupational, travel, dietary and animal-exposure histories similar to that of local people. Hunting for True Morels and avoiding False Morels is an annual pastime between March and May in North Carolina (Johnson, 2020). (C) *G. montana* was sold to unnamed restaurants in Florida (Bergo, 2021) and poisonings from unnamed wild mushrooms are recorded in the State (Kintziger et al., 2011). (D) Ashkenazi Jews. Wild mushrooms, including morels, are considered kosher and, as such, have served as an important component of food for Ashkenazi and other Jewish people (Gelman, 2019). (E) No unusual shared dietary habits or medications, different residential locations, and no other social, occupational or environmental interactions. Hydrazine-related chemicals may be used in schools (EPA, 2006; NIOSH, 2006). (F) The National Aeronautics and Space Administration (NASA) Ames Research Center led development of the liquid hydrazine-propelled *Pioneer 10* spacecraft launched in 1972 (NASA, 2022), and hydrazine sulfate and ammonium hydrazinium sulfate were subjects of five papers listed by Ames published between 1993 and 1998 (Spencer, 2019). (G) A small but significant cluster (p = 0.04) was detected around the lead smelter area (Wang et al., 2014; Meng et al., 2020); lead is taken up by mushrooms (Spencer and Palmer, 2021). (H) Three friends whose childhood homes were located proximate to the County High Point of Kalamazoo, Michigan. Kalamazoo is a popular location for collection of wild morel mushrooms (see: https://www.mlive.com/news/kalamazoo/2016/04/planning_a_morel_mushroom_hunt.html).

downregulated in mid to late S-phase of the cell cycle (Mostofa et al., 2018) such that post-mitotic cells have low MGMT levels (Kisby and Spencer, 2021). Noteworthy is that changes in gene and protein expression of MGMT have been found in Alzheimer disease (Oláh et al., 2015; Chung et al., 2022; Kisby et al., 2022).

ALS and botanical exposures to hydrazinic chemicals

Fungal hydrazine-related compounds include agaritine in *Agaricus* spp. (Schulzová et al., 2009) and gyromitrin in *Gyromitra* spp. (Monographs, 1987; **Figure 5**) and certain other fungal genera (*vide supra*) (Trestrail, 2000). The association between food use of *gyromitres* and sALS in Savoie, France (Lagrange and Vernoux, 2020; Lagrange et al., 2021b) receives support both from the discovery of multiple conjugal cases clustered in adjacent French *départements* and in pockets of ALS to the east in Piedmont, Italy, where *gyromitres* are consumed. Since *G. gigas* reportedly contains 1,500-fold lower concentrations of gyromitrin than that of *G. esculenta* (Viernstein et al., 1980), the latter constitutes a substantially greater health threat and, thus, this highly poisonous species may have contributed to ALS cases in both Savoie and Piedmont, Italy. Urgently needed are precise analytical methods (e.g., HPLC-MS/MS) to quantify gyromitrin and minor hydrazones in *Gyromitra* spp. under specified environmental conditions. Focused research is merited in these regions to test this hypothesis in retrospective and, potentially, prospective studies. There is also justification to explore whether other pockets of sALS are linked to deliberate or mistaken food use of False Morels (Cruces, 2010; Beug, 2014; Pulse, 2022), especially in northern Michigan, USA, given the State's relatively frequent occurrence of acute MMH poisoning (**Figure 3**) and high prevalence of ALS (Feldman, 2000). In Europe and Scandinavia, most acute MMH intoxications occur from consumption of False Morels collected in the conifer forests of Germany, Poland, Sweden, and Finland (Horowitz et al., 2022). The birth location of a cluster of ALS subjects in Finland (Sabel et al., 2003) corresponds to a region of False Morel consumption (Spencer, 2019). *Gyromitra esculenta,* among other species, if found on conifers (mostly pines) across Europe, Scandinavia, and North America, parts of Central (Mexico, Costa Rica) and South America (Argentina, Chile) and the Caribbean (Dominican Republic, U.S. Virgin Islands), parts of Asia (Georgia, Japan, Kazakhstan, Pakistan, Russia), including India (Jammu and Kashmir) and China (Heilongjiang), Northern Africa (Algeria, Morocco, Canary Islands), Australia, and New Zealand (Minter, 2018).

There are numerous factors that determine the toxic effects of fungal hydrazones in False Morels (Nordic Council of Ministers, 1995). The concentration of the principal hydrazone gyromitrin (**Figure 5**) is said to vary across *Gyromitra* species, age, geographic location, elevation, temperature (Anon, 2022q) and, conceivably, the soil content of metal elements and atmospheric humidity. All parts of the fungus are potentially toxic, including the stipe and cap that are used as food. The concentration of gyromitrin changes according to the method and duration of mushroom preservation (refrigeration, canning, desiccation) (Pyysalo, 1976). Fresh European specimens of *G. esculenta* may contain 1,200–1,600 mg/kg gyromitrin, while concentrations in dried tissue have ranged between 14.7 to >6,400 mg/g (Nagel et al., 1977). False Morel food preparation (cutting, washing, boiling, frying) reduces gyromitrin concentration and, since MMH boils at 87.5°C, fumes with potential for acute human intoxication are released into the air (Anon, 2022j). The amount, frequency and duration of consumption determines the dosage of hydrazones received by the consumer and the total amount of MMH (via *N*-methyl-*N*-formylhydrazine) generated by acid hydrolysis in the stomach (Nagel et al., 1977; Garnier et al., 1978). Symptoms of acute MMH intoxication appear 6–14 h after ingestion or 2–8 h after inhalation, with the lethal dose in micrograms/kg estimated to be 10–30 for children and 20–50 for adults (Trestrail, 2000). Whatever the route of exposure, gastrointestinal illness may be accompanied by hemolysis and, in severe intoxication, hepatorenal toxicity with jaundice, liver failure, delirium, seizures, coma, and death (Trestrail, 2000).

Individual susceptibility to acute MMH toxicity seems to vary widely (Miller et al., 2020), but no formal studies have been carried out to establish this widely held impression. The toxin affects the liver, central nervous system, and sometimes the kidneys. As with

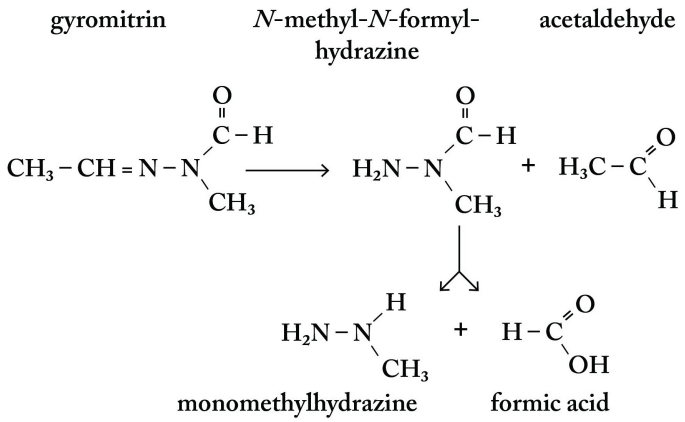

FIGURE 5
Metabolic pathway to monomethylhydrazine (MMH) of the major hydrazone gyromitrin in *G. esculenta*. MMH has acute neurotoxic properties but, like cycad-derived methylazoxymethanol, also induces DNA lesions (O^6- and N^7-methylguanine) that are poorly repaired in neurons and are proposed to result in multiple downstream cellular effects linked to neurodegenerative diseases, such as ALS.

MAM-liberating cycad seed, acute food poisoning from ingestion of *G. esculenta* takes the form of vomiting and diarrhea several hours after consumption, often followed by dizziness, lethargy, vertigo, tremor, ataxia, nystagmus, and headaches and fever. Severe cases may exhibit delirium, muscle fasciculation and seizures, mydriasis progressing to coma, circulatory collapse, and respiratory arrest 5–7 days of ingestion (Michelot and Toth, 1991). Acute poisoning by *Gyromitra* spp., which is similar in character to the toxic effects of hydrazine propellants (Azar et al., 1970), is scarce in Western Europe and more frequent in Eastern Europe (Patocka et al., 2012; National Research Council [NRC], 2022).

In other alpine countries (Switzerland, Monaco, Italy, Liechtenstein, Austria, Germany, and Slovenia) and beyond (Eastern Europe, Russia), *G. esculenta* has various local names (Michelot and Toth, 1991; Anon, 2022i; GBIF, 2022) and mushroom culinary traditions vary (Peintner et al., 2013). Other fungi reported to contain gyromitrin include (*Cudonia circinans* syn. *Leotia circinans*), *L. lubrica* (Jelly Baby), *Helvella crispa* (Elfin Saddle), *H. lacunose* (Slate Gray Saddle), *H. elastica* (Elastic Saddle), and *H. macropus* (Felt Saddle) (Andary et al., 1985; Flume, 2022).

Wild hydrazinic mushrooms consumed in Sicily include *H. crispa* (*spugnola crespa, funci di chiddi rizzi, munachessi*) and two species of *Agaricus* (*funcia campagnola, funcia picurina*) are eaten raw or cooked (Lentini and Venza, 2007). This may be relevant to the spatio-temporal and spatial high-incidence clusters of sALS on the southeastern flank of Mt. Etna in Sicily that have been linked to a possible etiologic role for metals in volcanic ash (Nicoletti et al., 2016; Boumediene et al., 2019). Importantly, fungi are able to accumulate metals from contaminated soils and even have a role in soil bioremediation (Spencer and Palmer, 2021).

There appears to be a narrow margin for ingestion of *Gyromitra* spp. between individual tolerance and development of acute illness; symptoms may follow an "all or none" pattern both in monkeys and humans (Miller et al., 2020; National Research Council [NRC], 2022). Nothing is known about individual susceptibility, but age, physical health and gene status are possible variables, such that individuals with fast acetylator hepatic metabolism may be able to mitigate the potential acute and delayed effects of consuming False Morels (Lagrange and Vernoux, 2020). Genetic variation in expression of microsomal cytochrome P450 isoenzymes also has relevance since (in rat liver) hydrazine is metabolized and detoxified by CYP2E1, CYP2B1, CYP1A1/2, ultimately yielding molecular nitrogen (IARC, 1999). Subject medication may be another variable (Lagrange and Vernoux, 2020) since hydrazine-derived drugs also generate DNA lesions (Mathison et al., 1994) and alter microbiome composition (Wei et al., 2010). Vitamin B6 status is also significant since gyromitrin binds to and inhibits pyridoxal phosphokinase, the enzyme responsible for transforming dietary pyridoxine into active pyridoxal 5-phosphate, without which glutamic acid decarboxylase cannot convert glutamate to the neurotransmitter γ-aminobutyric acid (GABA). The resulting depletion of GABA promotes CNS excitation and seizures (Horowitz et al., 2022). While severe MMH poisoning from consumption of *Gyromitra* spp. is well documented, some people can consume appropriately prepared False Morels for years/decades without experiencing acute illness (List and Luft, 1967; Trestrail, 1994). Indeed, in Finland, local *G. esculenta* are commercially available for sale to the public according to strict regulations set by the Ministry of Trade and Industry of Finland and accompanied by precise food preparation instructions from the Finnish Food Authority (FFA) (Anon, 2022j; Pulse, 2022). Given that gyromitrin residues may remain when these instructions are followed, the FFA advised in 2019 against consumption by pregnant and breast-feeding women, and children (FFA, 2022). This appears to have been driven by concerns relating to the long-recognized carcinogenic and teratogenic potential of fungal hydrazines in laboratory animals (Toth, 1991, 1993; Toth and Gannett, 1994). Outside of Scandinavia, the sale of False Morels is generally banned (Germany, Switzerland) but still are consumed by some in Bulgaria and Spain,[3] and they are available on-line for purchase from Piliakalni, Lithuania (Morel Mushrooms, 2022).

The foregoing makes it apparent that as-yet-undefined conditions must be fulfilled before the toxic properties of MMH are expressed clinically as an acute illness and, presumably, in the context of ALS, if indeed there is not only an association (Lagrange et al., 2021b) but an actual cause-effect relationship with ingestion of *Gyromitra* spp. Testing that relationship requires a detailed understanding of the

3 https://en-academic.com/dic.nsf/enwiki/182112

lifetime exposome prior to onset of motor symptoms, not the rather superficial knowledge of exposures gained through epidemiological surveys of ALS, which have tended to emphasize occupational exposures (Holloway and Mitchell, 1986; Chancellor et al., 1993; Graham et al., 1997; GBD 2016 Motor Neuron Disease Collaborators, 2018) and have rarely examined dietary history. An example of studying individual subjects with uncommon forms of ALS is a 56-year-old man with a history of poisoning from eating either crude or undercooked false morels: he developed muscle cramps, nausea and vertigo, and a rapidly evolving/sub-acute upper and lower motor neuron syndrome with significant weakness in all four limbs and bulbar region; however, 6 months later, his condition plateaued, and he began a progressive recovery over subsequent years to reach normal neurological status with no electromyographic evidence of denervation (Lagrange et al., 2021a). Note that certain other *Gyromitra* spp. (*G. ambigua*, *G. infula*) are thought to contain gyromitrin (Brooks and Graeme, 2016), while other fungi contain related toxic azo/azoxy compounds (Andary et al., 1985; Flume, 2022), including the Stump Puffball [*Apioperdon* (formerly *Lycoperdon*) *pyriforme*] and Yellow Puffball (*Calvatia rubroflava*), which are considered to be edible (Blair and Sperry, 2013).

ALS and non-botanical exposures to hydrazinic chemicals

There is potential for significant non-botanical exposures to hydrazine-related chemicals in industrial, agricultural and military settings. Their principal applications include chemical blowing agents (40%), agricultural pesticides (25%), and water treatment (20%) (Wiser, 2022). In the military and in aerospace, hydrazine has been used in various rocket fuels, in fuel cells to power an experimental Army truck (Anon, 2022g) and to fuel the EPUs of the NASA Space Shuttle, the Lockheed U-2 Spy Plane, and the General Dynamics F-16 fighter jet (Mayfield, 2022). Given America's heavy use of F-16s in the 1991 Gulf War and the potential for hydrazine exposure of those who flew and serviced these aircraft, there is reason to explore a possible association with the high incidence of ALS among relatively young subjects who served in the U.S. Air Force at that time (Horner et al., 2008). The proposal that inhalation of aerosolized cyanotoxins (notably L-BMAA) in desert sands was a significant factor for the development of Gulf War-related ALS (Cox et al., 2009) cannot explain why the disease only developed in isolated members of the U.S. military. For reasons unknown, even during later periods (2002–2005), ALS continued to impact the U.S. Air Force more than other armed services (Sagiraju et al., 2020).

Some ALS researchers have identified agrochemicals, notably the broad class of pesticides, as plausible causal factors for sALS among farmers and other rural residents (Granieri et al., 1988; Poloni et al., 1997; Govoni et al., 2005; Gams et al., 2018; Povedano et al., 2018; de Jongh et al., 2021). Hydrazine compounds are used as active ingredients in combination with other agricultural chemicals, including insecticides, miticides, nematicides, fungicides, antiviral agents, attractants, herbicides, and plant growth regulators (Toki et al., 1994). Specifically, it is noteworthy that a synthetic hydrazine-related compound (maleic hydrazide: 1,2-dihydro-3,6-pyridazinedione), a plant-growth regulator synthesized in 1947 (Schoene and Hoffman, 1949) and introduced in the U.K. in 1984 (EFSA, 2016), served as a first-generation plant regulator for turf management in sports fields (Anon, 2015). Use of a hydrazine-related compound to control turf on sports fields has obvious potential relevance to the higher risk for ALS reported among relatively young professional and amateur soccer players (Vanacore et al., 2006; Chiò et al., 2009; Beghi, 2013). Factors relevant to this consideration include: the stability of maleic hydrazide in (simulated) sunlight, its slow degradation *in situ*, the lack of product odor, and the potential for exposure through inhalation and dermal contact (PubChem, 2022), and (b) the paucity of genetic fast acetylators in European (5–10%) as compared to Japanese (45%) populations (Koizumi et al., 1998). Hydrazine-related chemicals have also found use as herbicides (Metribuzin, Paclobutrazol) and fungicides (Triademifon) (Toki et al., 1994), and nitrosamines (*vide supra*) are released from recycled rubber crumb used in artificial turf (United States Environmental Protection, 2022; van Bruggen et al., 2022).

Occupational exposure to hydrazine and nitrosamines has occurred in the leather industry (de la Burde et al., 1963; Lahiri et al., 1988), which historically has had elevated rates of ALS (Hawkes and Fox, 1981; Buckley et al., 1983), a subject discussed elsewhere (Spencer, 2019), as well as an excess of stillbirths and an increased number of congenital malformations (Hawkes et al., 1989). Textile workers, who work with azo (hydrazone) dyes (Benkhaya et al., 2020) have also been identified as possible subjects at risk for ALS (Abarbanel et al., 1985, 1989). In the 1970s, azo dyes that form aromatic amines were used in hair rinses and tints (Ames et al., 1975; Yung and Richardson, 2004), which has potential relevance to the report of an elevated risk of ALS among hairdressers (Chió et al., 1991; D'Ovidio et al., 2017).

ALS exposome research

Experience with migrants to and from Guam demonstrates that years or decades separate the timing of exposure to an environmental trigger (cycad genotoxins) and clinical onset of motor neuron disease. Additional experimental evidence indicates that exposure to the culpable agent (primarily cycasin) can occur prior to or after birth (Spencer, 2020), during infancy or in adolescence, although the latter may be a period of greatest vulnerability (Spencer et al., 2020). Detailed assessment of an ALS patient's exposure to extrinsic factors from conception to onset (lifetime exposome) is a daunting task (Maguire, 2017). In general, one searches both for unusual high exposures to commonly encountered exogenous agents as well as exposures to agents to which few are in contact, whether the agent itself or the history of exposure deviates substantially from the norm. Such exposures, even to a single chemical class (such as hydrazine-related compounds), may involve a wealth of natural and synthetic substances deployed in multiple loci that cannot be addressed by conventional epidemiologic methods. Indeed, the "neural exposome," a construct recently introduced by the NIH-National Institute of Neurological Disease and Stroke, comprises not only exogenous factors but also behavioral and endogenous components (Tamiz et al., 2022). When available, the science of exposomics may employ internal and external exposure assessment methods (Vasta et al., 2021). While research on the selected group of ALS cases highlighted here may be questioned, it is important to recognize that major discoveries of the causes of both acute and chronic neurologic diseases have been made by intensive study of very small numbers of patients. For example, end-stage L-dopa-responsive parkinsonism in a handful of post-teenage males was

traced to drug use of a meperidine analog contaminated with methylphenyltetrahydropyridine (MPTP) (Langston, 2017).

Since onset of sALS in younger vs. older persons might plausibly result from a higher dosage of culpable agent(s) at a critical point in life, patients who are diagnosed with sALS in their second to fourth decade represent invaluable subjects for research investigation. Such cases are also more likely to have living parents and older siblings able to describe the ALS subject's environmental exposures that occurred from conception onward. Our experience has taught that interviews with the patient and their family members are best conducted in a semi-structured manner (Palmer and Spencer, 2002) without rigid adherence to a research instrument with pre-formed questions that often reflect researcher interests or biases, such as the commonly perceived overarching etiologic relevance to sALS of workplace chemical exposure. The power of a non-biased approach to the acquisition of local knowledge was demonstrated by several Guam Chamorros who suggested six decades ago that food use of cycad was responsible for *leetiko* (ALS) in their community, an observation that led to the identification in cycad seed of glucosides of methylazoxymethanol (MAM) and the non-protein amino acid alpha-amino-beta-methylaminopropionic acid (Whiting, 1988), later named L-BMAA (Nunn, 2017). Over the course of six NIH-sponsored cycad conferences (1962–1972), discovery that MAM (like MMH) is a genotoxin with carcinogenic properties, prematurely diverted interest away from neurology and toward cancer biology. Since MAM exposures induce DNA damage in both cycling and non-cycling cells (i.e., neurons), this may result in tumorigenesis of the former and degeneration of the latter. Concurrent study of cancer and sALS may thus be merited in the search for genotoxic mechanisms as discussed elsewhere (Bharucha et al., 1983; Eizirik and Kisby, 1995; Spencer et al., 2012; Fang et al., 2013; Bertuzzo et al., 2015; Gibson et al., 2016; Taguchi and Wang, 2017).

Evidence from studies of familial ALS and sALS suggests that an interplay among DNA damage, altered DNA repair, and changes in epigenetic pattern, contribute to neurodegeneration (Goutman et al., 2022). Genome-wide DNA methylation age is the most consistently altered epigenetic signature in ALS. In twins with ALS, there was a much greater between-co-twin difference in DNA methylation age in a late-onset sALS twin set compared to an early-onset sALS twin set (Tarr et al., 2019). Noteworthy is that hydrazine induced hypomethylation of c-jun and p53, and hypermethylation of c-Ha-ras and DNA methyltransferase, in rodent liver (Kuppusamy et al., 2015). The experimental DNA-damaging activity of hydrazine-related compounds, including hydrazine hydrate and 1,2-methylhydrazine (which is metabolized to MAM), is well established in the cancer literature (Pollard and Zedeck, 1978; Parodi et al., 1981), and experimental systemic treatment of young adult mice with MAM induces brain transcriptional profiles associated with both cancer and neurodegenerative disease (Kisby et al., 2011).

Future research direction

We propose there are special opportunities for discovery of environmental factors associated with/causal of ALS from intensive analysis of the lifetime exposome of patients with young-onset sporadic disease, as well as very rare ALS-discordant twin and conjugal ALS cases (Spencer et al., 2019, 2022a). Since the lifetime probability of conjugal ALS (in Britain) has been estimated as 1/510,000 couples (~0.75 couples/year) (Cornblath et al., 1993; Fernandes et al., 2017), less rare, young onset sALS cases (45 years of age and younger) represent priority research subjects. While sALS occasionally occurs in the second decade of life, such cases should also be screened for mutant genes since juvenile-onset genetic forms of ALS occur in children and subjects < 25 years of age (Turner et al., 2012). Genetic screening, exposome analysis and the timing of exposure may also reveal evidence of postulated gene-environment interactions in ALS (Bradley et al., 2018; Goutman et al., 2022). Environmental exposures should cover both synthetic and natural agents, including mycotoxins, given their potential relevance to the etiology of ALS (Alonso et al., 2015; Reid, 2017, 2020; French et al., 2019a,b).

Retrospective analysis of sALS clusters and geographically proximate cases is another valuable research strategy. This approach requires collaboration among ALS neurologists, epidemiologists, mycologists, analytical chemists, toxicologists, and other specialists, often with input from members of ALS-affected communities. Given the recent recognition of an association between sALS and prior False Morel poisoning in the French Alps (Lagrange et al., 2021b), an association that should be tested in other populations, prospective research strategies should include long-term follow-up of patients with a history of acute MMH poisoning identified by poison control centers. While the focus here is on the environmental etiology of ALS and its potential relationship to hydrazine-related chemicals, the hypothesis may be pertinent to other neurological disorders, since cycad-associated Western Pacific ALS phenotypes included atypical parkinsonism and progressive Alzheimer-like dementia (Borenstein et al., 2007; Spencer et al., 2020).

Author contributions

PS drafted the manuscript. VP provided the Gulf War research data. GK contributed to genotoxic data. EL, J-PV, CR, JR, and WC provided the data on ALS in the French Alps. RV contributed to data on mushroom occurrence in Spain. BH provided the data on U.S. cases of monomethylhydrazine poisoning. All authors edited and/or read the manuscript.

Acknowledgments

We thank Dr. Andrew Miller, Mycologist and Director of the Herbarium/Fungarium, University of Illinois for information on the distribution of *Gyromitra gigas* in southeast France. We thank Dr. Nicola Sitta for information on the current food use of *Gyromitra* spp. in Piedmont, Italy. We thank Kim Coverdale of the U.K. Wildlife Trust and David Earl of Lancashire County Council for their efforts to identify fungal genera in the Foxhill Nature Reserve. We thank Mr. Alden Dirks, University of Michigan for sharing his unpublished data on the gyromitrin content of U.S. samples of *G. esculenta* (detected) and *G. gigas* (not detected). We also thank the Oregon Mycological Society for their interest and information.

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

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frontiers | Frontiers in Neuroscience

TYPE Original Research
PUBLISHED 21 February 2023
DOI 10.3389/fnins.2023.1117056

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience

RECEIVED 06 December 2022
ACCEPTED 07 February 2023
PUBLISHED 21 February 2023

CITATION
Li W, Li S, Shang Y, Zhuang W, Yan G, Chen Z and Lyu J (2023) Associations between dietary and blood inflammatory indices and their effects on cognitive function in elderly Americans.
Front. Neurosci. 17:1117056.
doi: 10.3389/fnins.2023.1117056

Associations between dietary and blood inflammatory indices and their effects on cognitive function in elderly Americans

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Objective: To determine the correlations between dietary and blood inflammation indices in elderly Americans and their effects on cognitive function.

Methods: This research extracted data from the 2011–2014 National Health and Nutrition Examination Survey for 2,479 patients who were ≥60 years old. Cognitive function was assessed as a composite cognitive function score (Z-score) calculated from the results of the Consortium to Establish a Registry for Alzheimer's Disease Word Learning and Delayed Recall tests, the Animal Fluency test, and the Digit Symbol Substitution Test. We used a dietary inflammatory index (DII) calculated from 28 food components to represent the dietary inflammation profile. Blood inflammation indicators included the white blood cell count (WBC), neutrophil count (NE), lymphocyte count (Lym), neutrophil–lymphocyte ratio (NLR), platelet–lymphocyte ratio (PLR), neutrophil–albumin ratio (NAR), systemic immune-inflammation index [SII, calculated as (peripheral platelet count) × NE/Lym], and systemic inflammatory response index [SIRI, calculated as (monocyte count) × NE/Lym]. WBC, NE, Lym, NLR, PLR, NAR, SII, SIRI, and DII were initially treated as continuous variables. For logistic regression, WBC, NE, Lym, NLR, PLR, NAR, SII, and SIRI were divided into quartile groups, and DII was divided into tertile groups.

Results: After adjusting for covariates, WBC, NE, NLR, NAR, SII, SIRI, and DII scores were markedly higher in the cognitively impaired group than in the normal group ($p < 0.05$). DII was negatively correlated with the Z-score when combined with WBC, NE, and NAR ($p < 0.05$). After adjusting for all covariates, DII was positively

correlated with SII in people with cognitive impairment ($p < 0.05$). Higher DII with NLR, NAR, SII, and SIRI all increased the risk of cognitive impairment ($p < 0.05$).

Conclusion: DII was positively correlated with blood inflammation indicators, and higher DII and blood inflammation indicators increased the risk of developing cognitive impairment.

KEYWORDS

cognitive function, DII, blood inflammation indicators, NHANES, regression analysis

Introduction

With the advent of an aging society, the cognitive decline of the elderly has become a social problem that is important to all humankind and needs to be solved (Hebert et al., 2013; Wang et al., 2021). The number of people living with dementia is increasing worldwide (Afzal et al., 2014). The increasing incidence rates of cognitive impairment and dementia will lead to an increased incidence of various geriatric diseases, which will greatly increase medical investment and impose a heavy socioeconomic burden (Mangialasche et al., 2009; Ruangritchankul et al., 2020). There is now evidence that cognitive impairment is related to human inflammation (Ferrucci and Fabbri, 2018; Irwin and Vitiello, 2019; Barter et al., 2021; Minhas et al., 2021). An adjustable, controlled intake of anti- or proinflammatory foods may be able to modulate the inflammatory state of the body, thereby positively impacting human cognitive function (Aquilani et al., 2020, 2022; van't Klooster et al., 2020). The foods that people consume in daily life have complex constituents, which make it necessary to explore the impacts of certain combinations of dietary conditions on inflammation and cognitive impairment in humans (Watson et al., 2022). The dietary inflammatory index (DII) is calculated by combining various food components and is a recognized indicator of overall dietary inflammation (Shivappa et al., 2017, 2019; Ryu et al., 2019). There is evidence that DII is negatively correlated with cognitive function (Hayden et al., 2017; Frith et al., 2018; Shin et al., 2018), and studies have also found that DII has no significant effect on cognitive function (Zabetian-Targhi et al., 2021). There is a need to systematically explore the relationship between DII and cognitive function.

The blood inflammation indicators analyzed in the study were collected during physical examinations and recorded in the National Health and Nutrition Examination Survey (NHANES) database. Platelets, platelet–lymphocyte ratio (PLR), and neutrophil–lymphocyte ratio (NLR) have been found to be positively associated with the risks of cerebrovascular and cardiovascular disease (Trakarnwijitr et al., 2017; He et al., 2019). The systemic inflammatory response index [SIRI, calculated as neutrophil count (NE) × (monocyte count)/(lymphocyte count) (Lym)] and the systemic immune-inflammation index [SII, calculated as (peripheral platelet count) × NE/Lym] may be associated with age-related diseases such as those of the cerebrovascular and cardiovascular systems (Jin et al., 2021; Xu et al., 2021). However, there is little comprehensive evidence of the relationships between DII and the white blood cell count (WBC), NE, Lym, NLR, PLR, neutrophil–albumin ratio (NAR), SII, and SIRI, or of their synergistic effects on cognitive function.

We therefore used NHANES data to investigate the relationships of DII and a blood inflammation index with cognition in older Americans, and to explore possible ways to reduce the occurrence of cognitive impairment.

Materials and methods

Data source

The data used in our study were derived from the NHANES public database in the United States. All participants provided written informed consent (Wu et al., 2021). There is a dedicated system management system that is responsible for data collection and updates in the NHANES, and the survey data and project information are updated regularly on the website and can be accessed by the public for free (Wu et al., 2021).

Participants

Data on the DII, blood inflammation index, and cognitive performance test scores were obtained from the NHANES for the period from 2011 to 2014 (Yang et al., 2020). All participants or their guardians signed an informed-consent form. We only included people aged 60 years or older, and after further exclusion screening, 2,479 cases were finally included (**Figure 1**).

Calculation of DII

This study analyzed 28 of the 45 food components from the original DII: carbohydrates, protein, total fat, alcohol, fiber, cholesterol, saturated fat, MUFA, PUFA, n-3 fatty acids, n-6 fatty acids, niacin, vitamin A, thiamin (vitamin B1), riboflavin (vitamin B2), vitamin B6, vitamin B12, vitamin C, vitamin D, vitamin E, Fe, Mg, zinc, selenium, folic acid, beta-carotene, caffeine, and energy. There is evidence that DII is still useful for predicting overall inflammation when only information on fewer food components is available (Shivappa et al., 2014a). DII calculations were based on a 24-h dietary recall interview or food record of the participant or their guardian (Shivappa et al., 2014b; Wirth et al., 2017). There are standard reference values for each food parameter in the world

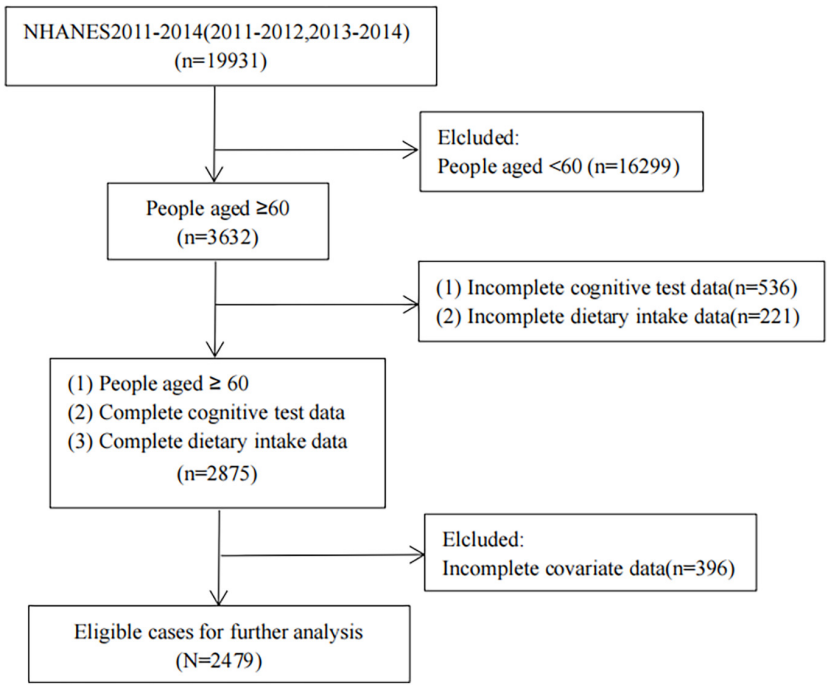

FIGURE 1
Case inclusion process.

database. The 24-h dietary recall data were multiplied by standard food parameters from the world database to obtain individual dietary inflammation composite cognitive function scores (Z-scores) relative to the standard global average. We transformed this value into a percentile to reduce bias. Each percentile was doubled, and then 1 was subtracted from it. The percentage values for each food parameter were then multiplied by their respective "overall food parameter-specific inflammatory effect scores" to obtain individual food-specific DII scores. Finally, the DII scores for all individual food components were summed to obtain the "overall DII score" for each person (Shivappa et al., 2014a).

Cognitive function

The cognitive function assessment consisted of the following four tests: Consortium to Establish a Registry for Alzheimer's Disease Word Learning (CERAD-WL) test, Animal Fluency (AF) test, Digit Symbol Substitution Test (DSST), and CERAD Delayed Recall (CERAD-DR) test. The CERAD-WL test requires participants to recall as many words as possible after reading ten unrelated words aloud in different orders for a total of 30 points. The CERAD-DR test was administered after the AF test and DSST. Participants were asked to recall words on the CERAD-WL test, which was used to assess transient and delayed learning ability (Rosen, 1983).

The Animal Fluency (AF) tests. Participants were asked to name as many animals as possible within 1 min. The absolute verbal fluency and executive function of the participants were examined (Clark et al., 2009; Sutin et al., 2022).

In the DSST, we asked participants to copy the corresponding symbols into the boxes next to the numbers within 2 min for a total of 133 points (Brody et al., 2019). This test examines the executive function and working memory capacity of the participants.

Composite cognitive function score

To exclude uneven differences in individual cognitive scores, we used a Z-score consisting of the CERAD-WL test, CERAD-DR test, AF test, and DSST as the total globally standardized cognitive function score. The Z-score was calculated as $Z = (x\text{-}m)/\sigma$, where x is the raw score, m is the overall mean, and σ is the overall SD. A Z-score of<-1 is taken to indicate that the person has cognitive impairment (Wirth et al., 2017; Frith et al., 2018; Zhang et al., 2022).

Blood inflammation indicators

Data on WBC, NE, Lym, NLR, PLR, NAR, SIRI, SII were extracted from the NHANES database or calculated using extracted peripheral blood counts (Wu et al., 2021).

Covariates

The possible effects of the following confounders were assessed: age (continuous), sex (male and female), race (Mexican American, other Hispanic, non-Hispanic white persons, non-Hispanic black persons, and non-Hispanic American), marital status (married/living with a partner, widowed/divorced/separated,

TABLE 1 Characteristics of participants [mean (SE)/N (%)].

	Normal cognitive performance (*N* = 2,053)	Low cognitive performance (*N* = 426)	*P*
Age (year)	68.551 (0.205)	73.568 (0.592)	<0.0001**
Gender			1
Male	985 (46.49)	240 (46.50)	
Female	1,068 (53.51)	186 (53.50)	
Race			<0.0001**
Mexican American	161 (2.90)	56 (9.05)	
Non-Hispanic white persons	1,116 (82.46)	131 (54.34)	
Non-Hispanic black persons	420 (6.70)	133 (19.52)	
Other Hispanic	163 (2.57)	85 (13.58)	
Other races	193 (5.37)	21 (3.50)	
Material status (%)			<0.001**
Married/living with partner	1,250 (66.92)	218 (52.36)	
divorced and separated and widowed	657 (27.26)	179 (41.35)	
never married	146 (5.82)	29 (6.28)	
Educational level (%)			<0.0001**
less than 9th grade	106 (3.01)	157 (27.67)	
9–11th grade	239 (8.89)	92 (19.51)	
high school graduate/ged or equivalent	496 (21.78)	90 (25.08)	
some college or a degree	655 (33.33)	56 (17.48)	
college graduate or above	557 (32.98)	31 (10.26)	
Body mass index (%)	29.098 (0.251)	28.382 (0.603)	0.325
Ever told you had high blood pressure (%)			<0.0001**
Yes	1,246 (55.82)	298 (72.16)	
No	807 (44.18)	128 (27.84)	
Doctor told you have diabetes (%)			<0.0001**
Yes	416 (17.23)	147 (32.24)	
Borderline	101 (4.46)	16 (2.96)	
No	1,536 (78.31)	263 (64.81)	
CERAD-WL	20.492 (0.236)	13.757 (0.324)	<0.0001**
CERAD-DR	6.619 (0.110)	3.462 (0.115)	<0.0001**
AF	18.941 (0.173)	11.491 (0.203)	<0.0001**
DSST	55.865 (0.458)	24.456 (0.658)	<0.0001**
The composite cognitive score Z-score	0.549 (0.032)	−1.455 (0.029)	<0.0001**

CERAD-WL, the Consortium to Establish a Registry for Alzheimer's Disease Word Learning; CERAD-DR, the Consortium to Establish a Registry for Alzheimer's Disease Delayed Recall; AF, the Animal Fluency. DSST, Digit Symbol Substitution Test. *$P < 0.05$ and **$P < 0.01$.

and unmarried), education level (less than 9th grade, 9–11th grade, high school graduate/GED or equivalent, some college or a degree, and college graduate or above), BMI (continuous), hypertension (yes and no), and diabetes (yes, borderline, and no).

Statistical analysis

We calculated new sample weights for the data analysis (Liu et al., 2013). If continuous variables did not conform to the normal distribution, they were represented by median (interquartile range) values; otherwise mean (SE) values were used. Regarding intergroup comparisons of baseline data, weighted-sample independent *t*-tests were used for continuous variables, while chi-square tests were used for categorical variables. WBC, NE, Lym, NLR, PLR, NAR, SII, SIRI, and DII were initially considered as continuous variables. In the logistic regression, WBC, NE, Lym, NLR, PLR, NAR, SII, and SIRI were divided into quartile groups (Q1, Q2, Q3, and Q4), and DII was divided into tertile groups (T1, T2, and T3) (Brody et al., 2019). The logistic regression model

TABLE 2 Comparison of dietary inflammatory index and blood inflammatory indicators between the cognitive impairment group and the normal group.

	Total	The composite cognitive score Z-score		
		Normal cognitive performance	Low cognitive performance	*P*-value
DII	1.353 (0.073)	1.279 (0.079)	2.040 (0.100)	<0.0001**
WBC	6.955 (0.080)	6.906 (0.083)	7.407 (0.156)	0.004**
NE	4.209 (0.051)	4.167 (0.054)	4.606 (0.123)	0.003**
Lym	1.916 (0.033)	1.912 (0.032)	1.953 (0.072)	0.513
NLR	2.502 (0.041)	2.464 (0.039)	2.850 (0.138)	0.008**
PLR	131.252 (2.095)	131.166 (2.146)	132.054 (3.497)	0.79
NAR	0.101 (0.001)	0.095 (0.001)	0.104 (0.002)	<0.001**
SII	561.714 (11.226)	554.512 (11.386)	628.510 (26.768)	0.01*
SIRI	1.440 (0.030)	1.417 (0.031)	1.658 (0.082)	0.009**

DII, dietary inflammatory index; WBC, white blood cell; NE, neutrophil count; Lym, lymphocyte count; NLR, neutrophil–lymphocyte ratio; PLR, platelet-lymphocyte ratio; NAR, neutrophil-albumin ratio; SII, systemic immune inflammation index; SIRI, system inflammation response index. *$P < 0.05$ and **$P < 0.01$.

was adjusted for sex, age, race, marital status, education level, BMI, hypertension, and diabetes. Significant results were indicated by $p < 0.05$. All analyses were performed using R software.

Results

General characteristics

The study finally included 2,479 individuals aged ≥60 years. The Z-scores indicated that 426 participants had low cognitive function and 2,053 had normal cognitive function. According to Z-scores, the low-cognitive-function group was older and had higher rates of non-Hispanic black persons, divorced/separated/widowed, lower education levels, hypertension, and diabetes than the normal group (Table 1).

Comparisons of DII, WBC, NE, Lym, NLR, PLR, NAR, SII, and SIRI between low-cognitive-function group and controls

No differences in LE and PLR were found between the cognitively impaired and normal groups. Patients in the cognitively impaired group had higher DII ($p < 0.0001$), WBC ($p = 0.004$), NE ($p = 0.003$), NLR ($p = 0.008$), NAR ($p < 0.001$), SII ($p = 0.01$), and SIRI ($p = 0.009$) than the normal group (Table 2).

Correlations of DII, WBC, NE, Lym, NLR, PLR, NAR, SII, and SIRI with the Z-score

Multiple linear regression analysis was performed to analyze the Correlations of DII, WBC, NE, Lym, NLR, PLR, NAR, SII, and SIRI with the Z-score. DII combined with Lym, NLR, PLR, SII, and SIRI, were no correlation with Z-scores after adjusting for all of the abovementioned covariates ($p > 0.05$). However, DII (β = −0.091, $p < 0.0001$) combined with WBC (β = −0.028, $p = 0.012$), NE (β = −0.036, $p = 0.003$), and NAR (β = −1.776, $p < 0.001$) were negatively correlated with Z-scores (Table 3).

Performance of DII on WBC, NE, Lym, NLR, PLR, NAR, SII, and SIRI in cognitive impairment

The relationship between DII and blood inflammation indices (WBC, NE, Lym, NLR, PLR, NAR, SII, and SIRI) in the cognitively impaired group was further investigated using multiple linear regression. After adjusting for covariates, DII was found to be positively correlated with SII (β = 27.476, $p = 0.047$). No significant associations were found between DII and the other inflammation indicators ($p > 0.05$) (Table 4).

The role of inflammation indicators in patients with cognitive impairment

A logistic regression approach was used to explore the association between inflammation scores and cognitive impairment risk. Q1 was the reference for all comparisons. First, after adjusting for all the confounding factors that we accounted for, the relationship between inflammatory indicators (DII, WBC, NE, Lym, NLR, PLR, NAR, SII, and SIRI) and the risk of cognitive impairment risk was examined independently (Table 5). The results indicated that T2 and T3 of DII (T2: OR = 1.879, 95% CI = 1.242–2.842; T3: OR = 2.661, 95% CI = 1.745–4.058), Q4 of WBC (OR = 1.778, 95% CI = 1.022–3.096), Q4 of NLR (OR = 1.671, 95% CI = 1.162–2.403), Q4 of NAR (OR = 1.656, 95% CI = 1.094–2.509), Q4 of SII (OR = 1.717, 95% CI = 1.092–2.700), and Q4 of SIRI (OR = 1.563, 95% CI = 1.082–2.258) were risk factors for cognitive impairment. DII combined with WBC, NE, Lym, NLR, PLR, NAR, SII, and SIRI were tested in models 1–8, respectively. Although the combinations of DII and WBC, NE, Lym, and PLR were not found to be significantly associated with the risk of cognitive impairment, higher DII with NLR, NAR, SII, and SIRI significantly increased the risk of cognitive impairment ($p < 0.05$) (Table 6).

TABLE 3 Relationship between DII and cognitive function after binding of each blood inflammatory index separately.

	The composite cognitive score Z-score	
	95% CI	*P*
Model 1		
DII effects	−0.091 (−0.118, −0.065)	<0.0001**
WBC effects	−0.028 (−0.050, −0.007)	0.012*
Model 2		
DII effects	−0.092 (−0.118, −0.065)	<0.0001**
NE effects	−0.036 (−0.060, −0.013)	0.003**
Model 3		
DII effects	−0.094 (−0.120, −0.068)	<0.0001**
Lym effects	−0.025 (−0.077, 0.028)	0.339
Model 4		
DII effects	−0.094 (−0.120, −0.068)	<0.0001**
NLR effects	−0.012 (−0.036, 0.011)	0.284
Model 5		
DII effects	−0.094 (−0.120, −0.069)	<0.0001**
PLR effects	0 (0.000, 0.001)	0.268
Model 6		
DII effects	−0.091 (−0.118, −0.063)	<0.0001**
NAR effects	−1.776 (−2.732, −0.821)	<0.001**
Model 7		
DII effects	−0.094 (−0.120, −0.067)	<0.0001**
SII effects	0 (0.000, 0.000)	0.279
Model 8		
DII effects	−0.094 (−0.120, −0.067)	<0.0001**
SIRI effects	−0.019 (−0.053, 0.016)	0.279

Data are all adjusted by age, sex, race, marital status, education level, body mass index, hypertension, diabetes. Models 1–8 show the effects of DII and WBC, NE, Lym, NLR, PLR, NAR, SII, and SIRI on cognitive function, respectively. DII, dietary inflammatory index; WBC, white blood cell; NE, neutrophil count; Lym, lymphocyte count; NLR, neutrophil–lymphocyte ratio; PLR, platelet-lymphocyte ratio; NAR, neutrophil-albumin ratio; SII, systemic immune inflammation index; SIRI, system inflammation response index. *$P < 0.05$ and **$P < 0.01$.

TABLE 4 Relationship between DII and each blood inflammatory index in a cognitively impaired population.

	The composite cognitive score Z-score		
	β	95% CI	*P*
WBC	0.13	(−0.062, 0.321)	0.174
NE	0.092	(−0.031, 0.214)	0.135
Lym	0.027	(−0.034,0.088)	0.370
NLR	0.068	(−0.036, 0.172)	0.188
PLR	2.716	(−1.489, 6.921)	0.195
NAR	0.003	(0.000, 0.006)	0.075
SII	27.476	(0.360, 54.593)	0.047*
SIRI	0.052	(−0.066, 0.171)	0.370

Data are all adjusted by age, sex, race, marital status, education level, body mass index, hypertension, diabetes. DII, dietary inflammatory index; WBC, white blood cell; NE, neutrophil count; Lym, lymphocyte count; NLR, neutrophil–lymphocyte ratio; PLR, platelet-lymphocyte ratio; NAR, neutrophil-albumin ratio; SII, systemic immune inflammation index; SIRI, system inflammation response index. *$P < 0.05$.

Discussion

We systematically explored the relationships between dietary inflammation, blood inflammation indicators, and cognitive impairment. Our study found that DII combined with WBC, NE, NLR, NAR, SII, and SIRI were considerably higher in the low-cognitive-ability group than in the normal group. DII, WBC, NE, NLR, NAR, SII, and SIRI were negatively correlated with Z-scores. DII combined with WBC, NE, and NAR were all negatively correlated with Z-scores. DII was positively correlated with blood inflammation indicators. Older adults with higher levels of DII and blood inflammation indicators (NLR, NAR, DII, and SIRI) were at a higher risk of cognitive impairment.

In the elderly, the body becomes less functional and more susceptible to inflammation (Tangestani Fard and Stough, 2019). Human inflammation indicators (NLR, PLR, NAR, SII, and SIRI) have been indicated to be potentially related to various health hazards in the elderly, including cardiovascular and cerebrovascular diseases (Trakarnwijitr et al., 2017; He et al., 2019; Dong et al., 2020; Jin et al., 2021; Li et al., 2021; Xu et al., 2021). Platelets, PLR, NLR, and NAR are associated with the risks of stroke and cardiovascular disease (Trakarnwijitr et al., 2017; He et al., 2019). Our results found that DII combined with WBC, NE, NLR, NAR, SII, and SIRI were considerably higher in the cognitive-impairment group than in the normal group. DII, WBC, NE, Lym and NAR were negatively correlated with Z-scores, which was similar to the results of previous studies (Xu et al., 2021).

DII represents the combined inflammatory profile of the human diet, and the relationship between DII and many risk factors has been demonstrated for age-related diseases (Frith et al., 2018). Our study found that DII was significantly associated with SII. Although no significant correlation was found with other blood inflammation indicators, SII is a more-reliable and representative marker of inflammation, so we believe that the evidence here is sufficient and convincing. However, our conclusion differed from those of previous studies, including that of Wang et al. (2022), who found that DII was significantly correlated with SIRI but not with SII. The possible reasons for this are that the previous study involved Chinese subjects, and Chinese and American diets are very different, there are various racial differences, and the Montreal Cognitive Assessment scale differs from the Z-score calculated by our CERAD-WL, CERAD-DR, AF, and DSST. However, both studies suggested that DII contributes to chronic inflammation development in humans.

The correlation between DII and cognitive impairment has been previously explored in different regions. Hayden et al. (2017) found that DII scores were positively associated with the risk of developing cognitive impairment, Shin et al. (2018) found that higher DII indicated higher cognitive impairment risk, and Frith et al. (2018) also found that higher DII scores were negatively associated with cognitive impairment risk. Our study found that DII, WBC, NE, NLR, NAR, SII, and SIRI were all negatively associated with Z-scores. DII combined with WBC, NE, and NAR were negatively correlated with Z-scores. This suggests that DII

TABLE 5 The effect of dietary inflammatory index and blood inflammation indicators on cognitive function was analyzed by logistic regression.

	The composite cognitive score Z-score	
	OR (95% CI)	*P*
DII effects		
T2 (0.74∼2.60) vs. T1 (≤ 0.74)	1.879 (1.242,2.842)	0.005**
T3 (> 2.60) vs. T1 (≤ 0.74)	2.661 (1.745,4.058)	<0.001**
WBC effects		
Q2 (5.60∼6.70) vs. Q1 (≤ 5.60)	1.136 (0.678,1.903)	0.608
Q3 (6.70∼8.10) vs. Q1 (≤ 5.60)	1.299 (0.769,2.196)	0.307
Q4 (> 8.10) vs. Q1 (≤ 5.60)	1.778 (1.022,3.096)	0.043*
NE effects		
Q2 (3∼3.90) vs. Q1 (≤ 3)	0.830 (0.481,1.432)	0.481
Q3 (3.90∼5) vs. Q1 (≤ 3)	1.145 (0.726,1.805)	0.539
Q4 (> 5) vs. Q1 (≤ 3)	1.558 (0.912,2.661)	0.099
Lym effects		
Q2 (1.50∼1.90) vs. Q1 (≤ 1.50)	0.826 (0.535,1.277)	0.368
Q3 (1.90∼2.30) vs. Q1 (≤ 1.50)	0.639 (0.409,0.997)	0.048*
Q4 (> 2.30) vs. Q1 (≤ 1.50)	1.133 (0.779,1.648)	0.493
NLR effects		
Q2 (1.53∼2.07) vs. Q1 (≤ 1.53)	0.969 (0.647,1.451)	0.871
Q3 (2.07∼2.88) vs. Q1 (≤ 1.53)	1.212 (0.788,1.863)	0.359
Q4 (> 2.88) vs. Q1 (≤ 1.53)	1.671 (1.162,2.403)	0.008**
PLR effects		
Q2 (92.71∼117.20) vs. Q1 (≤ 92.71)	1.018 (0.744,1.393)	0.903
Q3 (117.20∼150) vs. Q1 (≤ 92.71)	0.799 (0.540,1.181)	0.242
Q4 (> 150) vs. Q1 (≤ 92.71)	1.033 (0.695,1.535)	0.864
NAR effects		
Q2 (0.07∼0.09) vs. Q1 (≤ 0.07)	0.853 (0.530,1.372)	0.489
Q3 (0.09∼0.12) vs. Q1 (≤ 0.07)	1.111 (0.754,1.638)	0.575
Q4 (> 0.12) vs. Q1 (≤ 0.07)	1.656 (1.094,2.509)	0.020*
SII effects		
Q2 (320∼452.57) vs. Q1 (≤ 320)	1.405 (0.920,2.146)	0.108
Q3 (452.57∼653.05) vs. Q1 (≤ 320)	0.865 (0.543,1.376)	0.518
Q4 (> 653.05) vs. Q1 (≤ 320)	1.717 (1.092,2.700)	0.022*
SIRI effects		
Q2 (0.76∼1.13) vs. Q1 (≤ 0.76)	1.334 (0.805,2.209)	0.245
Q3 (1.13∼1.69) vs. Q1 (≤ 0.76)	0.997 (0.665,1.496)	0.988
Q4 (> 1.69) vs. Q1 (≤ 0.76)	1.563 (1.082,2.258)	0.020*

Data are all adjusted by age, sex, race, marital status, education level, body mass index, hypertension, diabetes. DII, dietary inflammatory index; WBC, white blood cell; NE, neutrophil count; Lym, lymphocyte count; NLR, neutrophil–lymphocyte ratio; PLR, platelet-lymphocyte ratio; NAR, neutrophil-albumin ratio; SII, systemic immune inflammation index; SIRI, system inflammation response index. *$P < 0.05$, **$P < 0.01$.

and blood inflammation indices can synergistically serve to affect cognitive function.

Logistic regression was used to further investigate the synergistic effect of blood inflammation index and DII on cognitive impairment risk. The results indicated that older adults with higher DII and levels of blood inflammation indicators (NLR, NAR, DII, and SIRI) were at a higher risk of cognitive impairment, which was similar to the results of previous studies (Jin et al., 2021). The possible mechanism is that inflammation indicators can cross the blood–brain barrier to inflame nerves, leading to neurodegeneration (d'Avila et al., 2018; Godos et al., 2020; Leng and Edison, 2021). A comprehensive assessment of diet and blood inflammation can help us take early steps to develop a rational dietary intervention plan and protect cognitive function.

TABLE 6 Synergistic effects of dietary inflammatory index and blood inflammation indicators on cognitive function.

	The composite cognitive score Z-score	
	OR (95% CI)	*P*
Model 1		
DII effects		
T2 (0.74∼2.60) vs. T1 (≤ 0.74)	1.812 (1.199,2.739)	0.008**
T3 (> 2.60) vs. T1 (≤ 0.74)	2.583 (1.686,3.957)	<0.001**
WBC effects		
Q2 (5.60∼6.70) vs. Q1 (≤ 5.60)	1.142 (0.675,1.933)	0.597
Q3 (6.70∼8.10) vs. Q1 (≤ 5.60)	1.301 (0.769,2.201)	0.302
Q4 (> 8.10) vs. Q1 (≤ 5.60)	1.699 (0.970,2.975)	0.062
Model 2	−0.165 (−0.282, −0.048)	0.008*
DII effects		
T2 (0.74∼2.60) vs. T1 (≤ 0.74)	1.825 (1.207,2.761)	0.007**
T3 (> 2.60) vs. T1 (≤ 0.74)	2.638 (1.728,4.029)	<0.001**
NE effects		
Q2 (3∼3.90) vs. Q1 (≤ 3)	0.789 (0.453,1.375)	0.378
Q3 (3.90∼5) vs. Q1 (≤ 3)	1.111 (0.703,1.754)	0.632
Q4 (> 5) vs. Q1 (≤ 3)	1.478 (0.852,2.564)	0.152
Model 3		
DII effects		
T2 (0.74∼2.60) vs. T1 (≤ 0.74)	1.920 (1.255,2.937)	0.005**
T3 (> 2.60) vs. T1 (≤ 0.74)	2.678 (1.728,4.152)	<0.001**
Lym effects		
Q2 (1.50∼1.90) vs. Q1 (≤ 1.50)	0.792 (0.499,1.256)	0.298
Q3 (1.90∼2.30) vs. Q1 (≤ 1.50)	0.627 (0.390,1.008)	0.053
Q4 (> 2.30) vs. Q1 (≤ 1.50)	1.094 (0.741,1.615)	0.629
Model 4		
DII effects		
T2 (0.74∼2.60) vs. T1 (≤ 0.74)	1.835 (1.203,2.801)	0.008**
T3 (> 2.60) vs. T1 (≤ 0.74)	2.654 (1.716,4.105)	<0.001**
NLR effects		
Q2 (1.53∼2.07) vs. Q1 (≤ 1.53)	0.987 (0.659,1.477)	0.945
Q3 (2.07∼2.88) vs. Q1 (≤ 1.53)	1.198 (0.771,1.861)	0.396
Q4 (> 2.88) vs. Q1 (≤ 1.53)	1.687 (1.176,2.418)	0.007**
Model 5		
DII effects		
T2 (0.74∼2.60) vs. T1 (≤ 0.74)	1.877 (1.231,2.860)	0.006**
T3 (> 2.60) vs. T1 (≤ 0.74)	2.640 (1.717,4.058)	<0.001**
PLR effects		
Q2 (92.71∼117.20) vs. Q1 (≤ 92.71)	1.008 (0.745,1.365)	0.954
Q3 (117.20∼150) vs. Q1 (≤ 92.71)	0.824 (0.557,1.218)	0.307
Q4 (> 150) vs. Q1 (≤ 92.71)	1.046 (0.702,1.558)	0.814
Model 6		
DII effects		
T2 (0.74∼2.60) vs. T1 (≤ 0.74)	1.815 (1.198,2.749)	0.008**
T3 (> 2.60) vs. T1 (≤ 0.74)	2.610 (1.712,3.980)	<0.001**

(Continued)

TABLE 6 (Continued)

	The composite cognitive score Z-score	
	OR (95% CI)	*P*
NAR effects		
Q2 (0.07∼0.09) vs. Q1 (≤ 0.07)	0.830 (0.509,1.354)	0.430
Q3 (0.09∼0.12) vs. Q1 (≤ 0.07)	1.056 (0.712,1.564)	0.773
Q4 (> 0.12) vs. Q1 (≤ 0.07)	1.577 (1.045,2.379)	0.032*
Model 7		
DII effects		
T2 (0.74∼2.60) vs. T1 (≤ 0.74)	1.639 (0.995,2.702)	0.052
T3 (> 2.60) vs. T1 (≤ 0.74)	2.140 (1.325,3.458)	0.005**
SII effects		
Q2 (320∼452.57) vs. Q1 (≤ 320)	1.513 (0.929,2.464)	0.089
Q3 (452.57∼653.05) vs. Q1 (≤ 320)	0.944 (0.589,1.513)	0.794
Q4 (> 653.05) vs. Q1 (≤ 320)	1.996 (1.251,3.183)	0.007**
Model 8		
DII effects		
T2 (0.74∼2.60) vs. T1 (≤ 0.74)	1.883 (1.243,2.851)	0.005**
T3 (> 2.60) vs. T1 (≤ 0.74)	2.627 (1.727,3.997)	<0.001**
SIRI effects		
Q2 (0.76∼1.13) vs. Q1 (≤ 0.76)	1.371 (0.879,2.138)	0.151
Q3 (1.13∼1.69) vs. Q1 (≤ 0.76)	0.853 (0.529,1.378)	0.491
Q4 (> 1.69) vs. Q1 (≤ 0.76)	1.679 (1.052,2.680)	0.032*

Data are all adjusted by age, sex, race, marital status, education level, body mass index, hypertension, diabetes. DII, dietary inflammatory index; WBC, white blood cell; NE, neutrophil count; Lym, lymphocyte count; NLR, neutrophil–lymphocyte ratio; PLR, platelet-lymphocyte ratio; NAR, neutrophil-albumin ratio; SII, systemic immune inflammation index; SIRI, system inflammation response index. *$P < 0.05$ and **$P < 0.01$.

This study had some limitations. First, because the study had a cross-sectional design, it was not possible to infer causal relationships between dietary and blood inflammatory indicators, and cognition. Second, dietary inflammatory indicators calculated from dietary intake data obtained from 24-h dietary recall might not accurately reflect individual dietary intakes and are subjected to recall bias. Third, we did not identify the cause of any impairment, such as Alzheimer's disease, Lewy-body dementia, or vascular dementia.

Our study also shows strengths and important originality. First of all, the study has a rich sample size and is analyzed only in older adults over 60 years of age, which has a strong social significance. In addition, the study used relatively accurate dietary data. Finally, the cognitive impairment composite -z score was created by summing the z scores [(individual test score - mean score)/SD] of these three individual tests (DSST, AFT, CERAD), with good sensitivity and avoiding ceiling and floor effects. For cognitive purposes our findings emphasize the importance of an anti-inflammatory diet with clinical implications.

In conclusion, we found that dietary and blood inflammation indicators were negatively associated with cognitive function in an elderly American population, and that dietary inflammation indicators were also negatively associated with cognitive function when combined with blood inflammation indicators. DII was positively correlated with blood inflammation. Older adults with higher DII and blood inflammation indicator levels were at a higher risk of cognitive impairment. An ideal dietary intake among older adults was associated with improved cognitive function, and future studies should therefore further investigate the interrelationships and the mechanisms underlying their effects on cognition.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: https://www.cdc.gov/nchs/nhanes/index.htm.

Ethics statement

The data used in our study were derived from the NHANES public database in the United States. All participants provided written informed consent. The patients/participants provided their written informed consent to participate in this study.

Author contributions

WL, SL, and WZ: conceptualization. WL: methodology and data curation. YS: software. WL, SL, and GY: validation. WL, WZ, and ZC: writing – original draft preparation. WL and WZ: writing – review and editing. JL: visualization. All authors have read and agreed to the published version of the manuscript.

Funding

This study was supported by Guangdong Provincial Key Laboratory of Traditional Chinese Medicine Informatization (2021B1212040007), National Key R&D Program of China (2020YFC2005700), and Key Science & Technology Brainstorm Project of Guangzhou (202103000027).

Acknowledgments

We thank Zhang Jing (Shanghai Tongren Hospital) for his work on the NHANES database. His outstanding work, nhanesR package and webpage, makes it easier for us to explore NHANES database.

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.

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frontiers | Frontiers in Neurology

TYPE Review
PUBLISHED 27 February 2023
DOI 10.3389/fneur.2023.1117695

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Dementia and Neurodegenerative Diseases, a section of the journal Frontiers in Neurology

RECEIVED 06 December 2022
ACCEPTED 01 February 2023
PUBLISHED 27 February 2023

CITATION
Calderón-Garcidueñas L, Torres-Jardón R, Greenough GP, Kulesza R, González-Maciel A, Reynoso-Robles R, García-Alonso G, Chávez-Franco DA, García-Rojas E, Brito-Aguilar R, Silva-Pereyra HG, Ayala A, Stommel EW and Mukherjee PS (2023) Sleep matters: Neurodegeneration spectrum heterogeneity, combustion and friction ultrafine particles, industrial nanoparticle pollution, and sleep disorders—Denial is not an option. *Front. Neurol.* 14:1117695. doi: 10.3389/fneur.2023.1117695

Sleep matters: Neurodegeneration spectrum heterogeneity, combustion and friction ultrafine particles, industrial nanoparticle pollution, and sleep disorders—Denial is not an option

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Sustained exposures to ubiquitous outdoor/indoor fine particulate matter ($PM_{2.5}$), including combustion and friction ultrafine PM (UFPM) and industrial nanoparticles (NPs) starting *in utero*, are linked to early pediatric and young adulthood aberrant neural protein accumulation, including hyperphosphorylated tau (p-tau), beta-amyloid ($A\beta_{1-42}$), α-synuclein (α syn) and TAR DNA-binding protein 43 (TDP-43), hallmarks of Alzheimer's (AD), Parkinson's disease (PD), frontotemporal lobar degeneration (FTLD), and amyotrophic lateral sclerosis (ALS). UFPM from anthropogenic and natural sources and NPs enter the brain through the nasal/olfactory pathway, lung, gastrointestinal (GI) tract, skin, and placental barriers. On a global scale, the most important sources of outdoor UFPM are motor traffic emissions. This study focuses on the neuropathology heterogeneity and overlap of AD, PD, FTLD, and ALS in older adults, their similarities with the neuropathology of young, highly exposed urbanites, and their strong link with sleep disorders. Critical information includes how this UFPM and NPs cross all biological barriers, interact with brain soluble proteins and key organelles, and result in the oxidative, endoplasmic reticulum, and mitochondrial stress, neuroinflammation, DNA damage, protein aggregation and misfolding, and faulty complex protein quality control. The brain toxicity of UFPM and NPs makes them powerful candidates for early development and progression of fatal common neurodegenerative diseases, all having sleep disturbances. A detailed residential history, proximity to high-traffic roads, occupational histories, exposures to high-emission sources (i.e., factories, burning pits, forest fires, and airports), indoor

PM sources (tobacco, wood burning in winter, cooking fumes, and microplastics in house dust), and consumption of industrial NPs, along with neurocognitive and neuropsychiatric histories, are critical. Environmental pollution is a ubiquitous, early, and cumulative risk factor for neurodegeneration and sleep disorders. Prevention of deadly neurological diseases associated with air pollution should be a public health priority.

KEYWORDS

air pollution, Alzheimer's, nanoparticles, nanoneuropathology, PM2.5, sleep disorders RBD, OSA, depression

1. Introduction

Chronic exposures to outdoor concentrations of $PM_{2.5}$ above WHO air quality guidelines (annual 5 $\mu g/m^3$) caused 6.4 million premature deaths and 93 billion days lived with illness in residents worldwide in 2019 (1). Exposures to traffic-generated pollutants, residency close to high-traffic roads, incomplete combustion emissions, firepit emissions, and *in vitro* experimental PM exposures of neural tissues, among other sources, have all been associated with extensive neural damage and increases in neurodegenerative diseases, including AD, PD, and ALS for the last two decades (2–17). Millions of US residents are exposed to wild forest fires and live near high-volume traffic roads and traffic-related air pollution (TRAP) (18, 19). Disadvantage populations, including minorities and low-income individuals, are exposed to high TRAP pollution (19, 20).

This study focuses on how incomplete combustion species and friction-derived and industrial-sourced nanoparticles reach neural tissues and damage target organelles in the nervous system; how these UFPMs and NPs travel in the brain and affect brain hubs with extensive communications and key roles in the integration of critical information, including sleep (21–23). For this study, we would be using either UFPMs and/or NPs, since our focus is on particle size, i.e., $\leq$100 nm.

The identification of the initial neuropathological stages of Alzheimer's disease (hyperphosphorylated tau and amyloid beta) (24) in 202/203 Metropolitan Mexico City forensic autopsies, with an average age of 25.4 $\pm$ 9.2 years, including 44 children with an average age of 12.89 $\pm$ 4.9 years, and the progression of the disease by the second and third decades of life, along with the concomitant development of PD and TDP-43 pathology in young urbanites, are at the core of our research efforts and our deep interest in comparing sleep disorders in patients with AD, PD, FLTD, and ALS, the involvement of aberrant neural proteins, and the presence of UFPM and NPs in sleep hubs in young highly exposed to air pollution cohorts (9–11, 24–34).

Populations that are exposed chronically to high concentrations of outdoor and indoor $PM_{2.5}$ are at higher risk of developing early diagnostic and neurodegenerative hallmarks, and the fact that they overlap from the earliest ages strongly suggests that there is a common denominator affecting the protein neural structures. UFPM and NPs could be the causative agents in association with genetic, epigenetic, and other environmental variables, and damaged sleep hubs, and resulting sleep disorders could be early findings (35–43).

Millions of people worldwide are exposed to outdoor and indoor environmental fine particulate matter ($PM_{2.5}$) and nanosize PM $\leq$ 100 nm [ultrafine particulate matter (UFPM) and industrial nanoparticles (NPs)]. Metal combustion and friction-derived UFPM and NPs are identified in brain organelles starting *in utero* and are directly responsible for intense oxidative stress, protein misfolding, protein aggregation, and fibrillation. AD, PD, and ALS are associated with exposure to air pollutants. Sleep disorders are strong predictors of fatal neurodegenerative disorders.

2. Particulate matter pollution, what is it? How do we measure it? Why nanosize PM is key?

Particulate matter (PM) consists of a mixture of microscopic solids and aerosols (liquid droplets) of different sizes and compositions found in the air. Different sizes of PM are based on their aerodynamic diameters: PM_{10} (mass of PM with an aerodynamic diameter $<$10 μm); fine or $PM_{2.5}$ (particles $<$2.5 μm), and ultrafine particles (UFPM, with an aerodynamic diameter $<$0.1 μm). PM differs in chemical composition, size, shape, morphology, and air lifetime, depending mainly on their origin, which in turn can be primary or secondary. Particles emitted directly into the atmosphere are primary PM, while those formed within the atmosphere from a number of processes such as nucleation, condensation, and/or chemical reactions of gas-phase species are secondary PM, mainly gaseous air pollutants (44). PM_{10} and $PM_{2.5}$ are our current indicators for PM pollution worldwide, particularly in highly polluted urban areas (i.e., Metropolitan Mexico City, Figure 1). Routine measurements of UFPM are neither common nor enforced, despite it being well-recognized that they can reach alveoli, circumvent primary airway defenses, and carry numerous toxic organic and inorganic compounds (44, 45).

Notably, while PM_{10} and $PM_{2.5}$ ambient concentrations and their regulatory compliance with air quality standards are determined by mass-based methods, UFPMs have negligible mass, making them very difficult to measure. UFPMs are quantified by number concentration, which in many cases

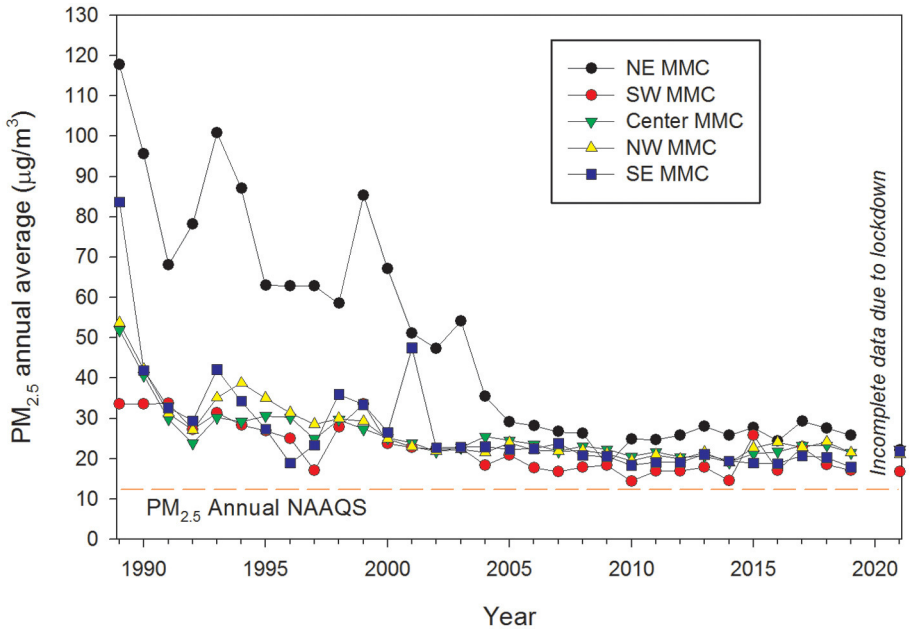

FIGURE 1
Time series trend of annual mean 24-h $PM_{2.5}$ concentrations, averaged over 3 years, for five representative monitoring stations in MMC from 1990 to April 2020 and their comparison with the respective annual USEPA NAAQS. Data were processed and evaluated from measurements reported by the manual PM network of the Secretaría del Medio Ambiente del Gobierno de la Ciudad de México (SEDEMA) under a 6-day sampling schedule. Annual means from the years before 2004 were estimated from available information on PM_{10} since 1990 and the mean slope of the correlation PM_{10} vs. $PM_{2.5}$ between 2004 and 2007. Source of data: http://www.aire.cdmx.gob.mx/default.php#.

do not correlate with the mass concentrations reported as PM_{10} or $PM_{2.5}$ (46–48). We currently do not have worldwide ultrafine particle matter regulations (21, 22). Although some countries have guidelines for UFPMs in terms of particulate number concentrations, their focus has been on short-term exposures in occupational environments and for specific materials, thus they do not apply to outdoor or indoor environments. Available measurement systems for UFPMs include condensation particle counters, electro-mobility spectrometers, diffusion battery counters, and photoelectric nucleus counters (44–49).

As road traffic and uncontrolled small combustion sources generate a significant number of nanoparticles, heavily polluted urban areas are suffering from strong UFPM problems (48). Metropolitan Mexico City (MMC) has experienced a dramatic increase in the number of vehicles in the last 20 years. Before 2000, CO and $PM_{2.5}$ levels in MMC were among the highest levels registered in North America. However, due to actions to reduce traffic pollution, UFPM particle number concentrations (PNC) from the mid-2000s on, have been reduced to around 30,000 cm^{-3} (50). Using a non-linear correlation model between PNC, CO, and $PM_{2.5}$ concentrations obtained from short-term monitoring studies, we have estimated that in the 1990s, PNC in MMC was around 300,000 cm^{-3} (50–54). Figure 2 shows the estimated annual average UFPMs number trend coupled with the CO annual median for MMC from 1989 to 2021 (50). We assumed that $PM_{2.5}$ and CO could be reasonable proxies of vehicular emissions and incomplete combustion processes in the urban area. Typical particle number concentrations measured in 44 urban areas worldwide are in the order of $\sim 5 \times 10^3$ to $\sim 8 \times 10^4$ cm^{-3} with extremes above 1×10^5 cm^{-3} in China and India (48).

3. Nanoparticles, metals, metalloids, and plastics. How harmful? How early? Where do they go in the brain? How relevant are systemic inflammation and neuroinflammation in neurodegeneration and their association with air pollution?

Nanoparticles, regardless of composition or shape, go everywhere in the body, cross all biological barriers, and go through paracellular pathways, including tight junctions, adherens junctions, and cytoskeletons (55–62). Their small size facilitates their absorption capabilities and their passage through membranes (5, 63–71); red blood cells (RBCs) and white blood cells (WBCs) are very efficient transporters of UFPM and NPs because they can reach any place, including the brain (12, 71). Their portals of entry (55) are key to understanding the importance of inhalation and ingestion of NPs and their direct brain entrance through the olfactory region and access to the trigeminal nerve. The inhalation entry starts in the nasal mucosa and continues to the alveolar space and the enormous lung capillary bed with the transport of UFPM and NPs through RBCs and WBCs and their free systemic circulation transportation. The massive amount of NPs

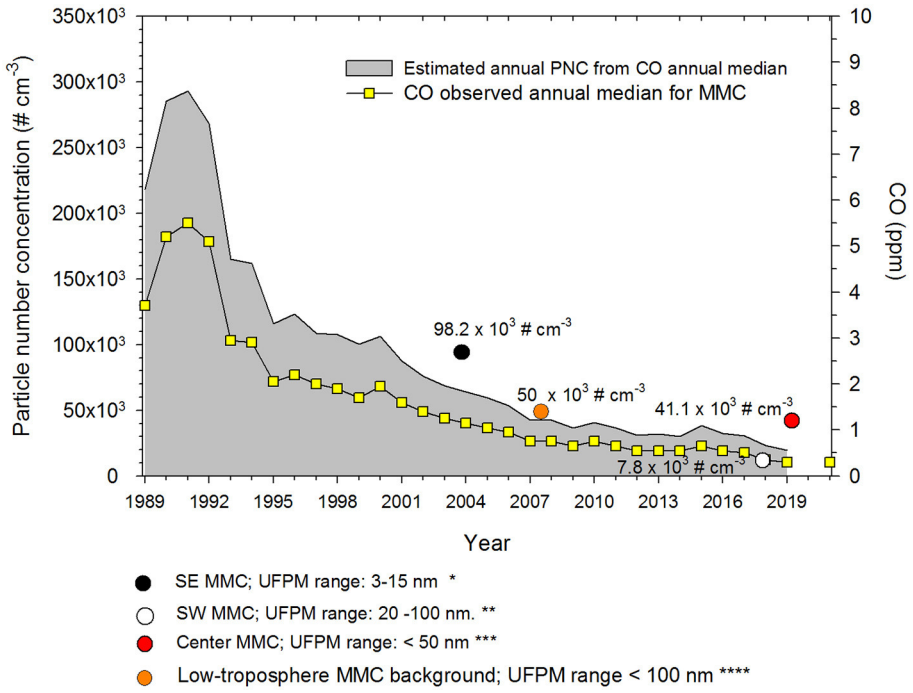

FIGURE 2
Trends of estimated PNCs and the associated annual medians of 1-h average CO for five representative monitoring stations of the MMC from 1989 to 2021. The colored circles in the figure correspond to the medians of PNCs measured by the authors referenced in (51*), (52**), (53***), and (54****). CO data source: http://www.aire.cdmx.gob.mx/default.php.

we ingest every day have direct access to the intestinal epithelium and submucosa, causing significant damage to the paracellular structures and allowing direct entry of NPs to the enteric nervous system (ENS) (72).

The neurovascular unit (NVU) (73), defined as *a complex functional and anatomical structure integrated by endothelial cells, capillaries, arterioles, a basal lamina covered by pericytes, smooth muscle cells, and neural cells including neurons, interneurons, astrocytes, and an extracellular matrix,* is a direct UFPM/NP target, a critical observation explaining the extensive capillary and small arteriole vascular damage starting *in utero* and in childhood upon PM air pollution exposures (9, 10, 12, 55). As described by Schaeffer and Iadecola (73), NVU damage, regardless of the source, has serious effects on neurovascular regulation and coordination of vascular responses to central and peripheral signals, which are critical to maintaining brain homeostasis. NVU damage predicts neurodegeneration (21, 55, 72–74).

The detrimental impact of NPs on the brain includes high production of reactive oxygen species, neural inflammation, depletion of anti-oxidative enzymes, DNA damage, apoptosis, structural cell damage, including organelles, nuclei, tight junctions, adherens junctions, endothelial damage, and dysfunction (5, 55, 59, 62, 64, 70, 75–78).

Particularly relevant to this study is the fact that UFPM/NPs are very effective in their capacity to aggregate, conglomerate, and produce protein folding, destabilization, and fibrillation (5, 61, 63, 65, 67, 69, 70, 79–82). John et al. (81) referred to large nanostructures of ≥20 nm affecting the kinetic peptide aggregation, thus size and shape matter. They also discussed how NPs serve as a surface for the adsorption of peptide monomers and facilitate nucleation to oligomers and fibril formation (81). Mohammad-Beigi et al. (82) discussed how α-synuclein undergoes interactions with NPs and how these interactions can be prevented by the characteristics of the protein corona acquired during the exposure of NPs to serum proteins. When α-synuclein and polyethylenimine-coated carboxyl-modified polystyrene NPs (PsNPs-PEI) interact, the NP surface promotes the primary nucleation step of amyloid fibril formation, thus key to pathological fibrillation, serum proteins modulate the complex interplay between NPs and amyloid proteins (82).

NP/UFPM interactions with brain cells are complex, and variables such as the nature of the protein corona, bioavailability, biodistribution, size, shape, charge, composition, cell and organelle targets, and certainly portal of entry are all impacting the extent and type of brain damage.

An interesting and concerning factor in UFPM composed of iron (magnetite and maghemite) is precisely their magnetic properties (5, 83–85). In the study by Shu et al. (85), the superparamagnetic NPs could respond to an external magnetic field, and magnetic NPs could be seen setting down in the magnetic pole regions (see Figure 4d of that study). This magnetic cell settling does, in fact, occur in MMC residents, as we have documented the phenomenon in electron micrographs [(55), Figure 3B]. The issue is more than a sporadic finding; MMC brains contain significant concentrations of magnetic NPs measured as saturation remanent magnetization (SIRM), being highest in the cerebellum (10). The

cerebellum in young MMC residents shows extensive vascular pathology and cerebellar endothelial erythrophagocytosis (50) and significant atrophy by volumetric brain MRI in young MMC residents (39).

Systemic inflammation and endothelial dysfunction are very relevant to air pollution exposures, as shown by our laboratory in Mexico City children (86), along with nasal inflammation, DNA nasal epithelial damage (87–90), and CSF inflammation (43). Systemic inflammation and endothelial dysfunction have been described in 295 pregnant women (91) with strong associations between increases in soluble vascular adhesion molecule-1 (sVCAM-1) levels for each 10 $\mu g/m^3$ increase in PM_{10} concentration, strongly suggesting that inflammation and endothelial dysfunction have a key role in modulating the detrimental effects of air pollution exposure during pregnancy, as shown recently in our laboratory with the extensive presence of nanoparticles of industrial origin in placentas of all ages and brain fetal tissues (12).

Oxidative stress and inflammation are common denominators of particulate matter (PM) air pollution exposures (92), including PM containing polycyclic aromatic hydrocarbons (PAHs) at low exposure settings. Occupational exposures are equally important for both systemic and neural inflammation and neurodegeneration (93, 94). Orysiak et al. (93) described a significant increase in proinflammatory cytokines in firefighters, along with respiratory inflammation, a piece of information that is very significant given the massive exposure of the US population to forest fires (18) and traffic air pollution (19). Thus, the report of Huang et al. (94) on neuroinflammation in the 2001 World Trade Center (WTC) responders is not a surprise, nor is the increment in suicides among the same responder population (95–97). The expected responses of the highly PM-exposed WTC responders were precisely what researchers are publishing 22 years later and what we commented within hours of the tragic event: acceleration of neuroinflammation, neurodegeneration, and suicides, as we see in Mexico City residents, more pronounced in APOE4 carriers, and associated with dose and routes of exposure key for both WTC responders and MMC residents (9–12).

Monitoring systemic inflammation in children should be a health priority since ambient air pollution impacts inflammatory responses from childhood (86, 98). Certainly, UFPM/NPs play a key role in both systemic and neural inflammation (12, 14, 16, 17, 21, 45, 50, 55, 62, 64), and diesel and Fe-NPs cause significant damage to neural cells under experimental conditions (62, 64). The issue also applies to industrial NPs consumed worldwide in massive amounts, i.e., titanium oxide NPs (99). Rolo and coworkers (99) have an excellent review of the TiO_2-NPs in foods causing *oxidative stress, cytotoxicity/apoptosis/cell death, inflammation, cellular and systemic uptake, genotoxicity, and carcinogenicity,* and although the authors made a plea to support limiting the use of TiO_2-NPs in food, we are aware as toxicologists that the food industry will be reluctant to follow-up on the recommendations. Thus, although the literature supports the multiple pathways UFPM/NPs are capable of causing systemic and neural damage through oxidative stress, neuroinflammation, mitochondrial function, neurodegeneration, *via* excessive activation of cellular prion protein signaling, hippocampal-impaired neurogenesis and synaptic plasticity, abnormal peptidomic responses, apoptosis, and necrosis (100–105), we still do not have NPs and UFPM regulations in the United States, and we need to establish clear correlations between PM exposures, neurodegeneration, and inflammation (106–109).

4. Development of Alzheimer's and Parkinson's diseases and TDP-43 pathology in children and young adult MMC residents. The diagnostic neural abnormal proteins are present and overlap from childhood and are key for the diagnosis of early sleep disturbances

In 2002, we described the association between the neuropathological hallmarks of Alzheimer's disease and air pollution exposures in our laboratory, stating: *Neurodegenerative disorders such as Alzheimer's may begin early in life with air pollutants playing a crucial role* (37). *Two* decades later, we have robust evidence to support this statement in populations exposed to high levels of $PM_{2.5}$ and UFPM/NPs. Our studies demonstrate the development of AD, PD, and TDP-43 pathology starting in childhood, and it is corroborated clinically with the progressive cognitive deterioration, abnormal gait and equilibrium, brainstem-evoked auditory potentials, olfactory deficits, sleep abnormalities, and brain MRI cortical, subcortical, and cerebellar atrophy in seemingly healthy individuals (36–42). Low CSF concentrations of amyloid β_{1-42} and BDNF differentiate children exposed to MMC air pollution from low pollution controls (43).

We have identified p-tau, the presence of Aβ and α-synuclein, and abnormal TDP-43 expression in 202 MMC forensic autopsies from residents who died in accidents, homicides, and suicides aged 25.3 ± 9.2 years (9, 10). Extensive, early, and progressive neurovascular unit damage and key organelle ultrastructural pathology were associated with metal- and metalloid-rich UFPM/NPs, making solid UFPM/NPs an agent for brain pathology in MMC subjects (9–12).

Figure 3 illustrates the two key AD neuropathology markers, namely, hyperphosphorylated tau and beta-amyloid, in MMC residents per decade, including 44 children (9). We thoroughly studied the extra neural tissues and confirmed there were no gross and/or light microscopy abnormalities.

As seen in Figure 3, every child had h-tau pre-tangle stages in the 1st decade of life, and by the 2nd decade, we documented neurofibrillary (NFT) tangles I–V (24). Subjects in the 4th decade were clearly in NFT I–V stages, and pre-tangle stages could no longer be identified. In contrast, Aβ progressed slowly and remained in the early phases. Interestingly, in our autopsy studies (9, 10), apolipoprotein E allele 4 (APOE4) carriers of the strongest Alzheimer's disease genetic risk factor (110–113) had higher AD Braak stages and the highest risk for suicide associated with lower cumulative exposures to $PM_{2.5}$ vs. APOE3 carriers. A finding in keeping with the literature regarding the higher risk of carrying two copies of ε4 allele increasing the

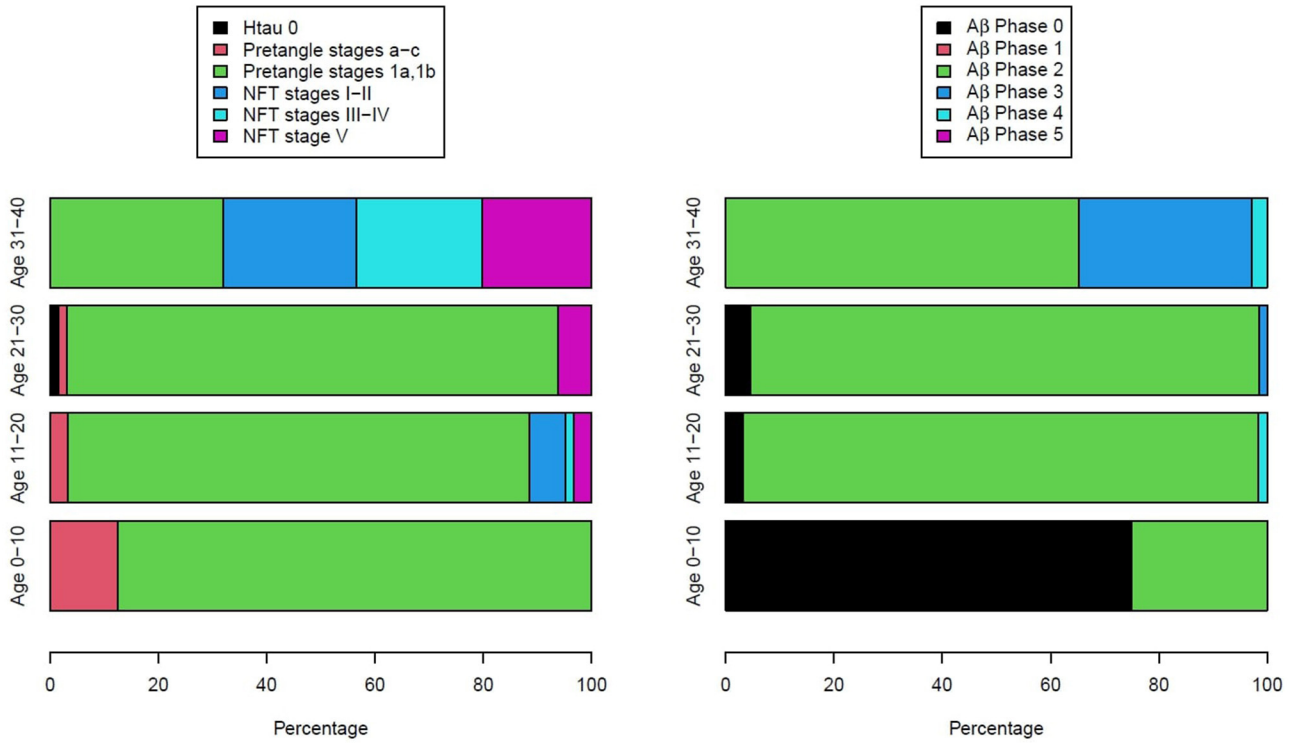

FIGURE 3
Two hundred and three forensic autopsies were staged for Alzheimer's disease using p-tau and $A\beta_{1-42}$ (9). Subjects were 25.4 ± 9.2 years old and causes of death were related to car accidents, homicides, and suicides; 202/203 had AD pathology, including the youngest subject, an 11-month-old baby.

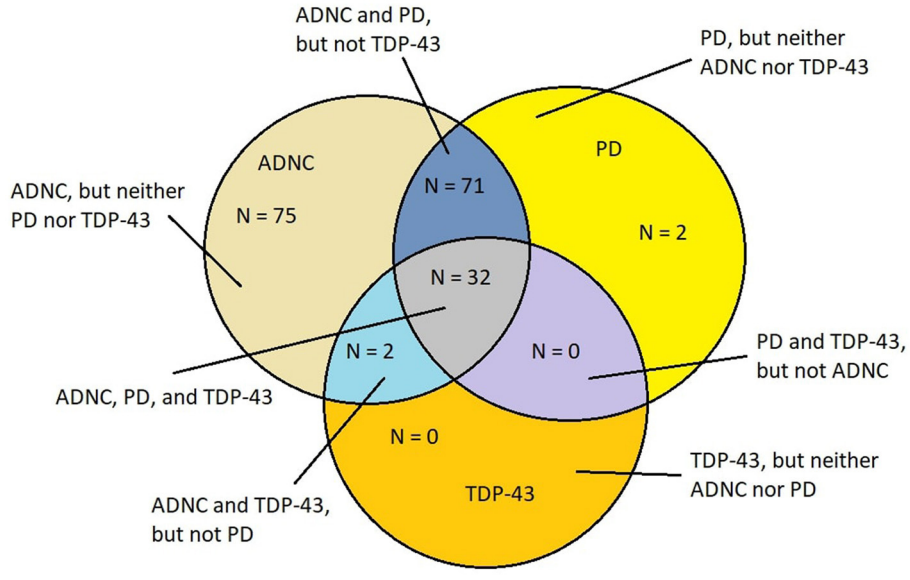

FIGURE 4
Venn diagram showing the overlap of neurodegenerative fatal diseases in a cohort of 186 MMC residents with an average age of 27.3 ± 11.8 years (10). Alzheimer's disease neuropathology changes (ADNC) (h-tau and Aβ) were present in each case.

AD risk up to 15-fold versus an APOE3 carrier in European ancestry subjects (114). APOE is a key protein in the equation of AD risk, neuroinflammation, oxidative stress, and metals (35, 112–114). The study by Tcw et al. (115), relating local APOE haplotype and the ε4-specific amino acid changes to important deficits in lipid metabolism dysregulation, glial activation, and inflammation, is of considerable interest in the setting of air pollution (9).

The overlap of AD, PD, and TDP-43 pathology is remarkable in young MMC residents as it is extraordinarily similar to the mixed protein pathologies described in elderly demented patients diagnosed with AD, FTLD, LBD, PD, ALS, and cerebral amyloid angiopathy (CAA), white matter rarefaction (WMR) pathology, and in the younger than 60-year patients who are AD demented (25–34, 116–119). Metha and Schneider (118) illustrated the overlapping neuropathology in a Venn diagram, which was further discussed by Jellinger (119, 120). It is clear that in elderly populations, AD is a heterogeneous disease, and co-pathologies (119), including LBD and TDP-43 pathology, and cerebrovascular lesions, are critical for the clinical picture, imaging, and laboratory results (25, 119–128). Figure 4 illustrates the aberrant neural protein overlap in MMC young without extra neural pathology (10).

All major neuropathological hallmarks of AD, PD, FTLD, and ALS are identified in young urbanites, from brainstem p-tau and diffuse amyloid plaques in an 11-month-old baby to extensive cortical p-tau in carriers of APOE4 alleles in 15-year olds. Common findings in MMC residents include p-tau in substantia nigrae and lack of nuclear TDP-43 in cortical motor neurons, lower motor neurons for cranial nerves III, V, and XII, and cervical motor neurons in teens and young adults (9–11). Hyperphosphorylated tau is definitely the major aberrant protein in highly exposed air pollution young urbanites (36). Figure 5 shows the overlap of aberrant neural protein in MMC young residents (10), compared to elderly subjects in the key work of Karanth et al. (25).

Cerebrovascular pathology involving small and large cerebral vessels, with lesions ranging from gross and microscopic infarcts, atherosclerosis, and arteriolosclerosis, is commonly attributable to aging and independently associated with a higher risk of AD in elderly subjects (116, 118–120, 128). Strikingly, we have described extensive brain capillary and arteriole endothelial pathology and abnormal NVU in MMC dogs, children, and fetal brains in weeks 12–15 (12, 129). In dogs and children, capillaries displayed abnormal tight junctions—a critical component of the NVU (129), decorated with UFPM/NPs, and white matter extensive perivascular damage with leaking capillaries and arterioles displaying extravascular lipids and erythrocytes. The endothelial basement membranes are thickened and display beta-sheet structures, and the perivascular glial sheet is focally absent. NPs (10–48 nm) are localized in endothelial cells (EC), pericytes, and across the basement membranes. Endothelial damage associated with NPs is detected very early and worsens with age in children and teens with high PM exposures (Figure 6) (129).

We strongly suggest that neural abnormal protein overlap could be explained by the presence of UFPM/NPs in critical hubs with portals of entry, emission sources, cumulative exposures, size, shape, surface charge, chemical composition, biomolecular corona proteins, target organelles, cellular toxicity, axonal anterograde and retrograde transport, trans-synaptic movements, and a number of genetic (i.e., APOE4 carrier status) and environmental factors (*in utero* exposures), comorbidities, etc., accounting for the neural damage and the heterogeneity of neurodegenerative diseases (12–17, 25–34, 113, 116–129).

The nanosize PM is composed of metals including Fe, Ti, Hg, Cu, Al, and Bi; post-transition metals, i.e., Al and Pb; alkaline earth metals, i.e., Ba; and non-metallic chemical elements such as Si are identified in every organelle in neurons, glial cells, microglia, and endothelial cells in Mexico City residents (10). Mitochondria and endoplasmic reticulum (ER), as well as the mitochondria-ER membrane contacts (MERC), are key NPs targets, and abnormal MERCs are common in highly exposed subjects (55, 56, 129). UFPM/NPs are also localized in the nuclear matrix—in close contact with heterochromatin—and nuclear pores. The outstanding accumulation of NPs in endolysosomes and specific structures like neuromelanin has great relevance in targeted neurodegenerative processes, including PD (Figure 7) (10, 55, 56, 130).

The spectrum of metals and metalloids is critical for brain targets. We are identifying Fe-based, highly magnetic UFPM along with metals commonly associated with electronic waste, such as elongated TiO2 NPs (131). Shredding of e-waste is an extensive source of NPs in the United States (131), and very high concentrations of lead, for example, 2.9 μg-lead m^3, are common 1.8 m away from the shredder operator, with extensive metal surface contamination reaching up to 250,000 particles cm^3 with fine $PM_{2.5}$ up to 171 μg m^3, and both failing to return to background levels after 40 min of inactivity, as described by Ceballos et al. (131). As stated by Frazzoli et al. (132), *the aggressively extractive advanced technology industry thrives on the intensive use of non-renewable resources and hyper-consumeristic culture* and unfortunately, the health impact on the brain is detrimental. Figures 8, 9 show the metal and metalloid profiles in individual UFPM/NPs in neural and vascular cells analyzed by energy-dispersive X-ray spectrometry (EDX).

UFPM/NPs in targeted organelles with critical functions, including the assembly of proteins, lipid synthesis, regulation, transportation, clearing of damaged organelles *via* lysosomal degradation, inter-organellar communication, Ca^{2+} storage, transport and signaling, apoptosis, autophagy, stress responses, and formation and activation of inflammasomes, are at the core of the nanoneuropathology, as shown by the myriad of interesting studies focusing on alterations of mitochondria, MERCS, ERs, mitochondria-lysosome connections, neuromelanin, and nuclear pores (133–142).

Neurodegenerative diseases are heterogeneous, multisystem disorders with multiple abnormal proteins frequently associated with cognitive impairment and sleep disorders. The heterogeneity includes clinical-brain imagen variants that complicate diagnoses and putting forward we still have limitations for clinical and neuropathology diagnostic criteria (25–34, 116–129). AD, PD, and TDP-43 pathology start in childhood in populations with high exposures to $PM_{2.5}$ (for this review, concentrations above the USEPA annual standards of 12 μg/m^3) and UFPM and NPs.

5. Neurodegeneration spectrum heterogeneity, quadruple neural abnormal proteins, and sleep disorders

At the core of this study are the neuropathology spectrum heterogeneity and the overlap in neuropsychiatric outcomes, including sleep disorders (25–34, 117–128, 143–166). There is consensus that for specific sleep disorders, i.e., rapid eye

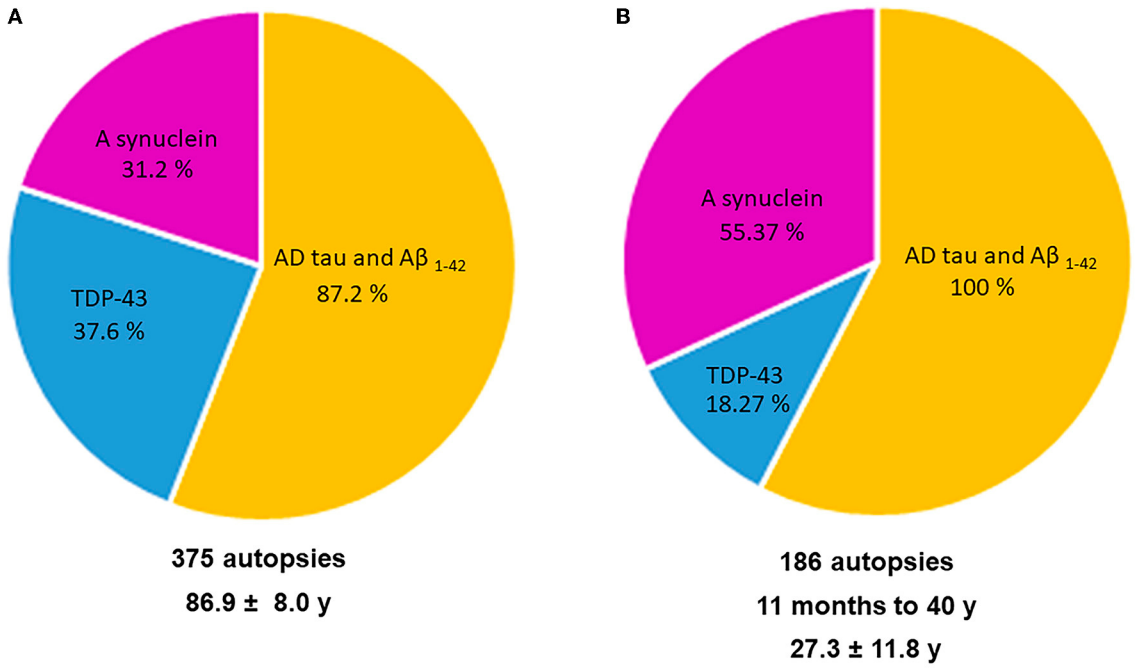

FIGURE 5
(A, B) A comparison in aberrant neural proteins between the young MMC 186 autopsy cohort (10) and Karanth et al. (25) 375 autopsies with an average age of 86.9 ± 8.04 years including subjects with normal cognition, mild cognitive impairment (MCI), impaired (but not MCI), and dementia.

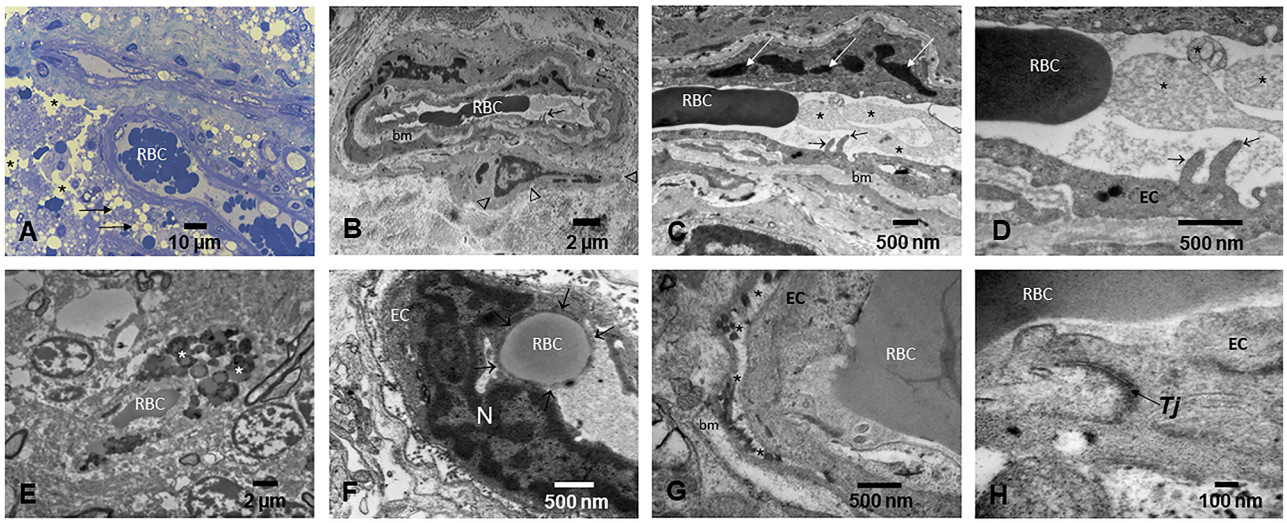

FIGURE 6
Light and electron microscopy of the brain in different anatomical locations in MMC young residents. **(A)** Thirteen-year-old girl's olfactory bulb showing small blood vessels with red blood cells (RBC) in the lumen and prominent endothelial cells. A significant number of perivascular vacuolated foamy cells (short arrows) and extensive areas of vacuolated neuropil (*) are observed. **(B)** A frontal blood vessel showing two luminal RBCs and prominent endothelial extensions into the lumen (short arrow). The basement membrane (bm) is thickened, and a pericyte (arrow heads) is identified. **(C)** The activated endothelial cell sends filopodia into the lumen (short arrows), while the lumen is occupied by ghost cell fragments (*), seen in higher magnification in **(D)**. **(E)** Small frontal blood vessel containing one single luminal RBC and extravascular numerous lysosomal structures containing lipids and NPs (*). **(F)** Cerebellar blood vessel showing a typical RBC endothelial phagocytosis. The RBC is sequestered by the EC and surrounded by EC cytoplasm (short arrows). **(G)** Small blood cortical vessel with luminal RBCs closely in contact with the EC. The EC basement membrane is detached from the cell (*), and an accumulation of lysosome-like structures is seen. The basement membrane (bm) shows focal thickening. **(H)** A close-up of a tight junction Tj—a key structure in brain endothelial cells—showing poorly defined integrity (arrow).

movement sleep behavior disorder (RBD), the association with synucleinopathies, i.e., PD, LBD, or multiple system atrophy (MSA), is supported (143–146, 152–160, 162–166). RBD is regarded clinically as preceding the appearance of motor symptoms and cognitive decline by several decades and the overlap of neurodegenerative diseases is certainly present as magnifically

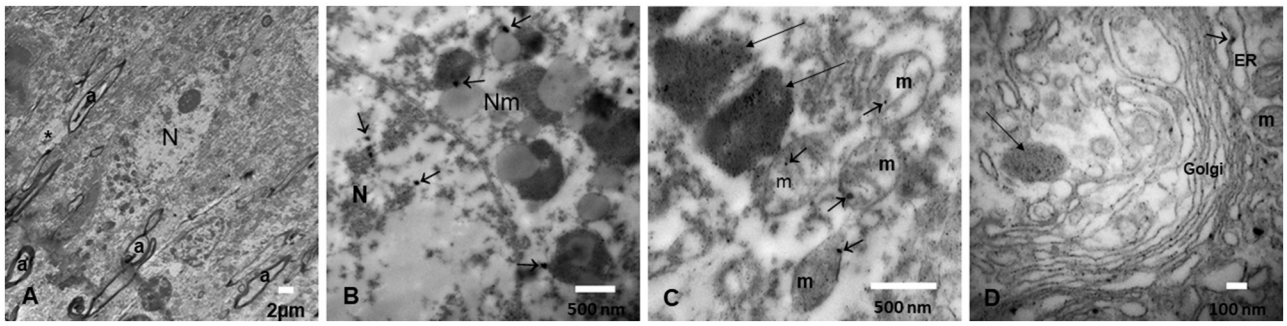

FIGURE 7
(A) Locus coeruleus (LC) neuron surrounded by loose neuropil (*) and myelinated axons (a). **(B)** An LC neuron with neuromelanin (Nm) structures containing nanoparticles (arrows) and similar NPs identified inside the nucleus (N). **(C)** Numerous NPs are identified both in large endolysosomal structures (long arrows) and in mitochondria (m) (short arrows). **(D)** The Golgi apparatus is a target of NPs, as well as the endoplasmic reticulum dilated structures (ER) (short arrows) and the lysosomal structures (long arrows).

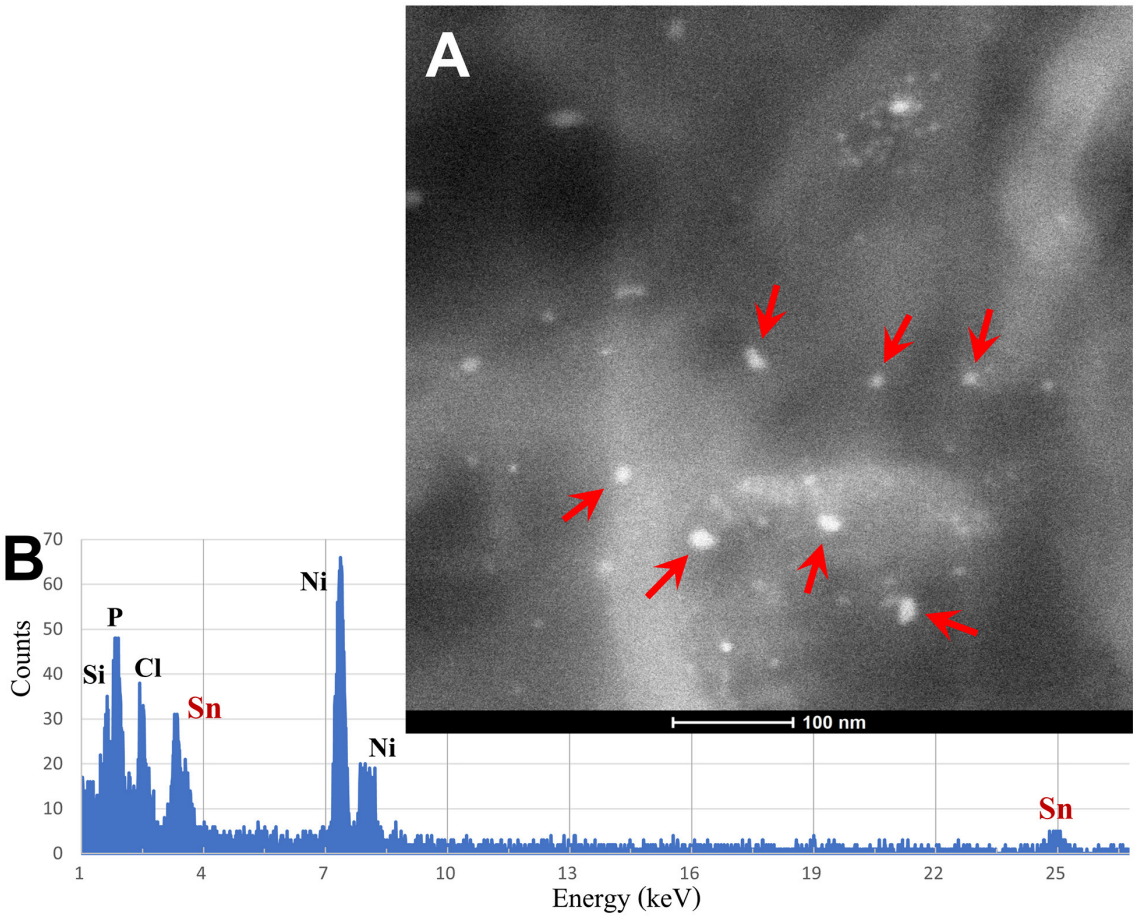

FIGURE 8
(A) Using the transmission electron microscopy (TEM) Z-contrast technique, metallic nanoparticles are documented in brain tissues. Only the nanoparticles marked with red arrows are Sn-NPs, while the rest are Fe-NPs. **(B)** The presence of Sn-NPs is verified through the acquired energy-dispersive X-ray spectrometry (EDX) that shows the tin metal (Sn) peak.

shown by Boeve and collaborators (26). LBD, LBD and AD, MSA, AD, and progressive supranuclear palsy (PSP) were diagnosed at autopsy in patients with a clinical diagnosis of PD, cognitive impairment, and autonomic dysfunction (29). Further support for evidence of dopaminergic and cholinergic system alterations in neuroimaging is present in the literature (143, 144).

There is a complex etiopathogenesis involved in the association of sleep disorders and diseases such as PD. As discussed by

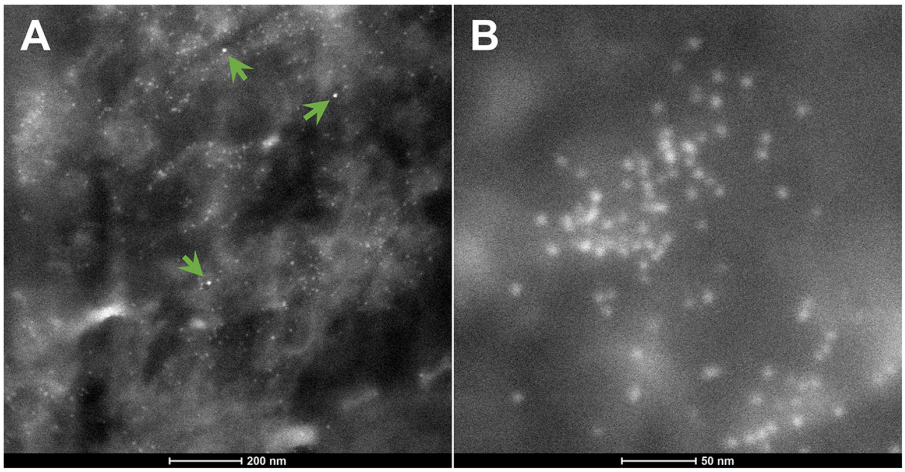

FIGURE 9
(A) The green arrows indicate three Hg-NPs that stand out from surrounding iron NPs. **(B)** An area of Fe-NPs. These NPs are magnetic.

Mizrahi-Kliger et al. (147), patients with PD exhibited at least four distinct pathways to explain their sleep problems: (i). a path directly associated with their PD synucleinopathy with regional involvement, (ii). medical therapy, (iii). degeneration of non-dopaminergic cells altering the circadian rhythm, and (iv). damage to brainstem dopaminergic neurons and its effect on the basal ganglia (147). Thus, common sleep complaints are linked to complex etiopathogenesis in the context of synucleinopathies, along with poor sleep quality associated with depression, PTSD, mood disorders, and excessive daytime sleepiness (148, 149, 156).

How much should we be concerned about sleep complaints and neurodegeneration? The answer is that we should be concerned depending on the patient, age, gender, clinical history, and how many risk factors, *including environmental factors*, are impacting neurodegenerative processes. The issue is relatively easy when we deal with a diagnosed elderly patient with AD or FTLD (151, 156, 157, 161), but not if we have a young adult resident in a polluted city or with occupational exposures (36).

It is in the younger adult population that learning about the etiopathogenesis of sleep disorders in AD, FTLD, PD, and movement disorders is helpful (24–34, 161, 167–169). Standlee and Malkani (169) underlined the mechanisms by which movement disorders are associated with sleep and circadian rhythm disruption, sleep fragmentation, insomnia, and excessive daytime sleepiness. It is worth emphasizing, as these authors did (169), the extensive involvement of brainstem nuclei regulating sleep and wakefulness in neurodegenerative processes.

At this time, it is unclear if sleep disturbances precede the common clinical neurodegenerative symptoms (i.e., cognition deficits) or if the sleep problems are some of the initial, early manifestations of neurodegenerative processes. Sleep complaints and neurodegeneration may be bidirectional. The sleep literature has addressed the abovementioned concerns in many different ways. For example, Zamore and Veasey (170) addressed chronic sleep disruption and neural damage, focusing on key variables, including duration and type of sleep disruption, age at which sleep loss occurs, neuronal populations responding to the injury, and the presence of genes involved in neurodegenerative processes. Sleep disruption impacts cognitive targets, such as episodic memory and sustained vigilance, pre- and post-synaptic impairment, the release of inflammatory cytokines and chemokines from microglia, and in transgenic 3×Tg-AD mice models, daily sleep-wake rhythm chronic fragmentation, increases in brain amyloid-beta (Aβ) levels, and neuroinflammation (170, 171). Grigg-Damberger and collaborators (172) discussed acceleration of mild cognitive impairment (MCI) and dementia in patients with sleep-wake disorders and the removal of Aβ in non-rapid eye movement stage 3 sleep and fragmented or insufficient sleep leading to accumulation of abnormal neural proteins in preclinical stages. Burke et al. (173) explored the association between sleep disturbance and brain volumes in 1,533 subjects (cognitively normal/cognitively impaired or demented) using a single question from the Neuropsychiatric Inventory Questionnaire (NPI-Q): "Does the patient awaken you during the night, rise too early in the morning, or take excessive naps during the day?" The sleep disturbance was rated in a binary fashion (yes/no). Subjects with a yes answer to the NPI-Q question had a lower total brain, hippocampal volume and frontal and temporal lobe gray matter volume. The authors concluded as follows*: These findings suggest that disrupted sleep is associated with atrophy across multiple brain regions and ventricular hydrocephalus ex vacuo*. We will add that since the brain MRI findings take years to evolve, it is possible that the atrophic brain changes preceded the sleep disturbances. The direct relationship between subcortical wake-promoting damaged neurons and sleep phenotypes has been described by Oh et al. (174), in patients with AD and progressive supranuclear palsy (PSP). In fact, in 19 subjects, aged 70 ± 7.7 years at their demise, neuronal counts in three wake-promoting nuclei, namely, noradrenergic locus coeruleus [LC], orexinergic lateral hypothalamic area [LHA], and histaminergic tuberomammillary nucleus [TMN], were correlated with decreased homeostatic sleep drive. The authors suggested subcortical wake neurons correlate with sleep phenotypes in a

number of neurodegenerative diseases, an observation that has practical and immediate applications (174).

On the other side of the relationship between sleep disorders and neurodegeneration, we find the literature on the brain impact of obstructive sleep apnea (OSA) and intermittent hypoxia as risk factors for preclinical AD and the incidence and progression of cognitive deficits (175–180). There is strong data on OSA producing intermittent hypoxia and sleep disruption and the observation that patients with OSA have higher serum levels of amyloid-beta and total tau and neuronal-derived Aβ and tau exosomes, going hand in hand with changes in sleep architecture (175, 176). There is strong evidence for the role of OSA as a risk factor for cognitive deficits (177) and the risk of developing or having OSA is significantly higher in patients with MCI or who are demented (178). It is also clear that several pathways are involved in neuropathological processes, including dysregulation of the orexinergic system and cerebral β-amyloid metabolism (179), major changes in CSF production, circulation, and glymphatic system abnormalities, which are crucial for the removal of metabolic waste (180). The American Thoracic Society workshop on the link between obstructive sleep apnea and neurocognitive impairment (181) concluded there is a strong biological plausibility but insufficient data to prove bidirectional causality of the associations between OSA and aging brain pathology. Thus, future research needs to address sleep disorders, oxidative stress, and accelerated brain aging (182, 183).

The WHO Mental Health Action Plan 2013–2030 emphasizes depression, affecting 4% of the population, ∼280 million people, as an important cause of disability worldwide (184). Depression should be considered in the setting of sleep–wake disorders, anxiety, stress, burnout, and suicide (185, 186). Insomnia and mental health conditions coexist among US college students: depressed students (adjusted odds ratio, 9.54; 95% CI, 4.50–20.26) had significantly higher odds of insomnia, which were also significantly higher among employed students (odds ratio, 2.10; 95% CI, 1.05–4.18) (187). Sleep and mental disorders are related, and in the case of major depressive disorder (MDD), insomnia seems to be a comorbid disorder (188). The relationship between depression and sleep is highly concerning for sleep physicians given the association between depression and neuropathology (189). Villela Nunes and coworkers (189) examined the autopsies in 741 Brazilian non-demented individuals with an average age of 72.2 ± 11.7 years and major depressive disorder (MDD) (7.3%), late-life MDD (LLD) (10.8%), and depressive symptoms (DS) close to death (22.7%). Remarkably, all three correlated with small vessel disease: LLD and DS with brain infarcts and LBD, and DS with beta-amyloid plaques and amyloid angiopathy (189). Therefore, in fact, depression could be considered a premorbid neurodegeneration in elderly people (189), and it is associated with insomnia in young individuals (187).

The issues of sleep outcomes, sleep deprivation, sleep spindles, and neurodegeneration are critical to the bidirectionality of the relationship (190–194). The relationship between fast-frequency sleep spindles, aging, AD, and glial activation is very interesting and opens up the possibility of establishing an early marker associated with microglia dysfunction, synaptic loss, p-tau, and memory impairment. Sleep spindle deficits are a good example of the opportunity of using sleep variables as early AD biomarkers in aging and as trackers of AD progression (190–192). Furthermore, slow oscillations, sleep spindles, and their coupling during non-REM sleep are useful in experimental AD mouse models and could apply to patients with AD as key biomarkers and as guides to identify translationally relevant biomarkers and early intervention strategies to prevent or delay AD progression (193, 194).

Most of the associations between neurodegenerative processes have been done with particulate matter, especially UFPM and NPs, due to their capacity to travel to the brain and be localized in every organelle and cellular compartment (4, 5, 9–11, 16, 21, 22, 44, 137); however, the atmospheric chemistry is very complex and has to be seen as a continuum connecting emissions through chemistry and transport, as discussed by Finlayson-Pitts (195). Toxicologists and atmospheric chemistry researchers are working to understand sources, chemical characteristics, relationships between different pollutants, and transformations, as they are major challenges in air quality control and climate research (196, 197).

There has been a significant reduction in the solid fraction of PM in the United States and Europe; however, the generation of UFPM by nucleation of organic vapor during the dilution of the exhaust remains a serious issue (198), and the carbon UFPM from brakes, tires, and road wear will remain a problem even if we accomplish a fully electric vehicle fleet (198, 199). Furthermore, exposure to microplastics and nanoplastics is ubiquitous, and these nanoplastics can reach the brain and induce oxidative stress (57, 58).

Sleep is impacted in every neurodegenerative disease and has a robust link with depression. For a number of patients with sleep disorders, there is a close association between sleep complaints and the development and progression of well-characterized proteinopathies. It is important to determine if a sleep disorder is a consequence of the neurodegenerative process or if it plays a key role in the development of the neurodegenerative process itself. The bidirectionally/interplay between sleep and neurodegeneration makes sleep a critical physiological process subject to study in young populations with high risk for neurodegenerative pathologies.

6. Summary

1. Sleep disorders are common in neurodegenerative diseases, and the presence of targeted sleep problems associated with a high risk of development of common proteinopathies, along with significant associations between sleep deprivation, obstructive sleep apnea, intermittent hypoxia, cognitive deficits, pre-clinical AD, and other neurodegenerative pathologies, make sleep and neurodegeneration a focus for exploration in a number of patients sent to sleep laboratories (200).

2. Sustained and significant exposures to high concentrations of $PM_{2.5}$ and UFPM/NPs are likely to play a significant role in the developing of neurodegenerative processes, dating back to *in utero* exposures. The presence of quadruple abnormal neural proteins starting in MMC infants and progressing as the subjects remain in the polluted environment should be of deep concern for health workers and has serious implications, including sleep disorders, for millions of people residing in such places.

3. We have shown the overlap of AD, PD, and TDP-43 pathology in highly exposed Mexico City children and young adults and the similarity of the overlap five decades later, when the patients are in terminal stages. We support that nanosized PM plays a key role in brain protein alterations and the complex subsequent cellular pathology.

4. Sleep disorders affect individuals of all ages with serious consequences across professions, including sleep deprivation in physicians (201). Our children are sleeping less, and there is a strong association between adverse childhood experiences and age-specific insufficient sleep duration in US youth, with serious repercussions in adulthood (202–208). There are also significant differences in sleep duration for US children depending on ethnicity and socioeconomic status (SES): among 9–13-year olds; black children sleep fewer hours compared to white, and poor children compared to higher-income children (204). Across the US, children sleep much less than what pediatricians recommend according to age, and minorities and disadvantaged children accumulate risk factors detrimental to their health (205). Moreover, lack of sleep increases the risk for addiction in adolescents based on chronic sleep loss and circadian misalignment (208). A potential association between inadequate sleep duration and changes in telomere length raises significant concerns related to cellular function (209).

5. The relationship between air pollution, sleep, neurodegeneration, depression, and suicide (210, 211) should encourage health workers to know about combustion and friction UFPM sources and engineered NPs (food products, cosmetics, toothpaste, sun protectors, surface disinfectants, paints, and e-waste). The presence of zinc, silver, copper, gold, selenium, and calcium NPs as potential food additives for animals (212), nanoplastics in drinking water (213), the massive presence of nanometric particle fraction of TiO_2 in the food industry, and $Fe_3\ O_4$ magnetic nanoparticles from food production, processing, storage, and detection, make constant exposures to NPs a serious health issue (76, 214).

6. The problem of human exposure to ultrafine particle pollution is solvable. We are knowledgeable of the cellular effects under experimental conditions and their intracellular and key organelle presence in the brains of urbanites (5, 10, 45, 48, 57–59, 62–71, 81, 82, 84). We also know the main emission sources and the technological options to control them (27, 215–217). The cost-benefit ratio is in favor of raising awareness (the role of our study) and taking action. We need a broader concern and awareness and the will to protect public health from deadly UFPM and industrial nanoparticles. We are also facing a lack of support for research from sleep medical societies. Denial is not an option.

Author contributions

LC-G, RT-J, and PM: conception and design of the study. LC-G, RT-J, PM, GG, RK, AG-M, RR-R, GG-A, DC-F, EG-R, RB-A, and HS-P: acquisition and analysis of data. LC-G, AA, RK, PM, ES, and GG: drafting of the text, writing, critical analysis, and preparing the figures. PM: statistical analysis. RT-J: air pollutant data. All authors contributed to the article and approved the submitted version.

Conflict of interest

AA works for the Sacramento Metropolitan Air Quality Management District.

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.

Publisher's note

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frontiers | Frontiers in Neurology

TYPE Systematic Review
PUBLISHED 22 March 2023
DOI 10.3389/fneur.2023.1133816

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Dementia and Neurodegenerative Diseases, a section of the journal Frontiers in Neurology

RECEIVED 29 December 2022
ACCEPTED 28 February 2023
PUBLISHED 22 March 2023

CITATION
Zhang L, Jiang H-y and Liu W-j (2023) Anti-seizure medication exposure and the risk of dementia: A meta-analysis of observational studies. *Front. Neurol.* 14:1133816. doi: 10.3389/fneur.2023.1133816

Anti-seizure medication exposure and the risk of dementia: A meta-analysis of observational studies

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Objective: There is growing evidence of a relationship between anti-seizure medication (ASM) use and the risk of dementia. This study examined this association using a meta-analysis approach.

Methods: PubMed, EMBASE, and Cochrane Library were systematically searched for peer-reviewed observational studies published up to February 2023. Study quality was evaluated using the Newcastle-Ottawa Scale, and an overall odds ratio (OR) was pooled using fixed or random-effects models.

Results: The analysis included 9 publications with 10 studies. The results showed that overall ASM exposure was associated with an increased risk of dementia [OR: 1.09, 95% confidence interval (CI): 1.03–1.15; $P = 0.003$] in general population. However, this association disappeared (OR: 1.02, 95% CI: 0.97–1.07; $P = 0.361$) when the study data adjusted for drug indications were pooled. Subgroup analysis based on individual drugs found only a positive association among those exposed to valproate, carbamazepine, and clonazepam. Furthermore, an increased risk was found in patients with bipolar disorder exposed to ASMs (OR: 1.43, 95% CI: 1.07–1.92; $P = 0.015$).

Conclusions: The statistically significant association between ASM and dementia in general population may be driven by unmeasured confounding or several individual first-generation ASMs. However, a higher risk of dementia was observed among bipolar disorder patients treated with ASMs. Given the few included studies and evidence of high heterogeneity, further larger, prospective studies that control for important confounders are needed to verify our findings.

KEYWORDS

anti-seizure, second generation, cognitive, systematic, meta-analysis

1. Introduction

Dementia is a progressive neurodegenerative disease characterized by progressive cognitive and functional decline constituting one of the leading causes of disability worldwide (1). It mainly affects older people, especially those over 65 years old (2). With the growing aging population, the number of people with dementia is predicted to triple to an estimated 152 million worldwide by 2050 (3). Considering the lack of treatment options, recognition of the risk factors of dementia may help to prevent the disease and could also inform appropriate interventions. Modifiable risk factors, including hypertension, infection,

mental disorders, diabetes, and smoking, account for around 35% of dementia cases (4). Therefore, decreases in the incidence of dementia are partially attributable to avoiding some of these risk factors (5).

Anti-seizure medication (ASM) are widely used to treat epilepsy and bipolar disorder (6). While effective, they have been linked to negative clinical outcomes, such as increased risks of cognitive decline (7), cardiovascular disease (8), and fracture (9). Increasing numbers of epidemiological studies (10–18) have investigated the risk of dementia in ASM users; however, the results have been controversial. Some found an increased risk of dementia with ASM exposure, whereas others revealed no association. In the earliest cohort study, Carter et al. (10) reported that ASM use was associated with an increased risk of dementia; in three other large studies (14, 15, 18), however, dementia was not associated with ASM use. The findings of three studies (11, 12, 17) focusing on patients with bipolar disorder also conflicted. Because the various factors associated with ASM exposure (i.e., type of ASM and participants) may alter the risk of dementia differentially, these factors should be evaluated. Due to the increasing use of ASMs, determining the long-term effects of these drugs on dementia is important. The purpose of this systematic literature review and meta-analysis is to assess whether ASMs exposure increases the incidence of dementia.

2. Methods

Preferred Reporting Items for Systematic Reviews and Meta-analysis framework guidelines (PRISMA) were followed for this meta-analysis.

2.1. Data sources and search strategy

A comprehensive literature search of the PubMed, EMBASE, and Cochrane Library databases was conducted on February 2, 2023, according to the PRISMA statement, with no year restrictions. The search incorporated index terms (Mesh) and free text words for the search concepts: (antiepileptic AND antiseizure AND anticonvulsant AND valproic acid AND paraldehyde AND phenobarbitone AND levetiracetam AND lorazepam AND carbamazepine AND phenytoin AND midazolam AND lidocaine AND fosphenytoin AND bumetanide) AND (dementia OR Alzheimer OR frontotemporal dementia OR cognitive dysfunction OR cognitive impair OR cognitive decline OR vascular dementia OR multiinfarct dementia OR neurodegenerative diseases OR neurocognitive disorders) AND (risk OR ratio OR prospective studies OR epidemiologic studies OR case-control studies OR cohort studies). An additional search was conducted in the bibliographies of relevant articles and relevant reviews.

2.2. Selection criteria

The studies were assessed by two independent reviewers who determined whether the studies met the inclusion criteria. Observational studies were included if they were: (1) a peer-reviewed study with a case–control or cohort design published in English, (2) included ASM exposure preceding a diagnosis of dementia, (3) included participants 18 years or older, (4) explored the association between ASM exposure and the risk of dementia, and (5) provided sufficient data to allow the calculation of risk estimates if adjusted data were not provided. Case reports, case series, animal studies, editorials, reviews, and meta-analyses were excluded. Studies that considered dementia as comorbidity and not as an outcome were also excluded.

2.3. Data extraction

Two authors extracted information from all selected studies using piloted data extraction sheets. Any discrepancies in the extracted data were resolved by a third author. The following information was collected from each study: author, publication year, study location, sample demographics, information on ASM exposure, diagnostic criteria for dementia, number of subjects in each group, statistical adjustments, and study quality.

2.4. Risk of bias and quality assessment

The quality of the included observational studies was assessed using the Newcastle-Ottawa Scale (NOS) (19), which is recommended by the Cochrane Handbook for Systematic Reviews of Interventions. The assessment focuses on three major areas: the study population selection, the comparability between the two groups, and the ascertainment of exposure (for case-control studies) or the outcome of interest (for cohort studies).

2.5. Statistical analysis

We used the STATA ver.16.0 (StataCorp., College Station, TX, USA) to perform meta-analysis. A random-effects model was used to pool the odds ratios (ORs) and 95% confidence intervals (CI) of individual studies; such models are optimal in terms of allowing the results to be generalized because they can deal with potential heterogeneity (20). ORs were considered as approximations of relative risks (RRs) or hazard ratios (HRs) because the dementia outcome under study is rare in all populations and subgroups under review. Splitting one study into several estimates leads to substantially more weight being assigned to this study in the meta-analysis, especially in a random-effects model. Therefore, we used a fixed-effects model to produce a pooled OR if more than three estimates from one study were provided, and then included this pooled OR in the meta-analysis. The I^2 statistic was used to assess between-study heterogeneity; The I^2 values were classified into four groups: of 0–29%, 30–49%, 50–74%, and 75–100%, representing very low, low, medium, and high inconsistency, respectively (21). Funnel plots and Egger's test were used to test the presence of potential publication bias within this review (22, 23). All

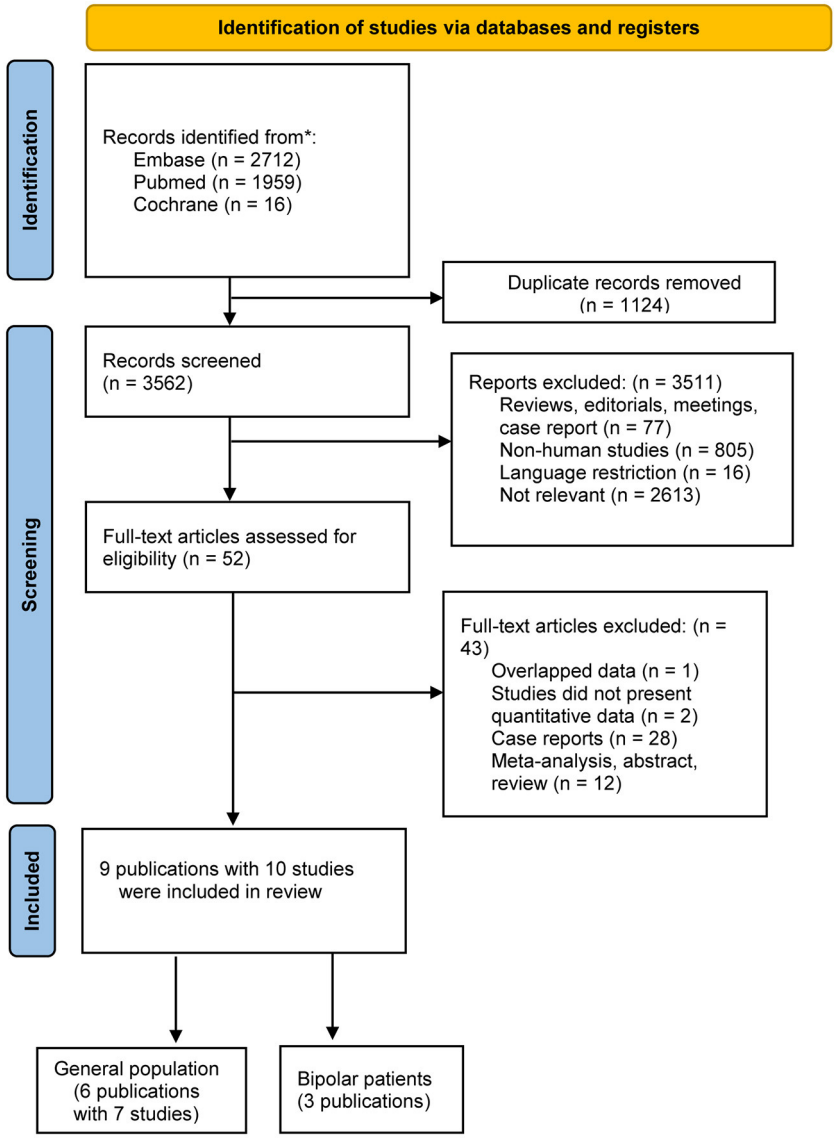

FIGURE 1
Flow chart of the search process and study selection.

the statistical tests were bilateral, and P-values < 0.05 indicated considered significant.

3. Results

3.1. Search results

After using the keywords, 4,687 records were identified in the initial search. Of these, 1,124 were duplicates, and 3,511 records were not relevant to the Research Topic after title and abstract screening, leaving 52 potentially eligible studies for which the full text was reviewed. Based on the inclusion and exclusion criteria, 9 publications with 10 studies were eligible for inclusion; all nine (10–18) were observational studies. Figure 1 is a flow diagram of the literature search and selection process.

3.2. Study characteristics

Table 1 summarizes the nine studies considered in this analysis. The studies included 1,629,213 participants from three different continents: five studies from Europe (13–16, 18), two from North America (10, 11), and two studies from Asia (12, 17). The publication year ranged from 2007 to 2022, and the sample sizes of the included studies ranged from 5,158 to 353,576. Exposure to ASMs was assessed using interviews or a drug prescription database. Three studies (11, 12, 17) assessed the use of ASMs and the development of dementia in individuals with bipolar disorder, and the remaining study evaluated this association in a general population. Regarding study quality, the mean NOS score for the nine studies was 8.3, indicating the high-quality of the included studies (Table 1). The score breakdown is given in Supplementary Tables S1, S2.

TABLE 1 Characteristics of the included studies.

References	Location, setting	Study design	Age	Ascertainment of antiepileptic exposure	Outcome measurement	Number of participants	Confounding adjusted	Quality
Carter et al. (10)	Canada, population-based	Cohort, general population	>65	Clinical examination or questionnaire	Modified mini-mental state examination or clinical examination	Exposed 67 Non-exposed 5,309	Age, sex, baseline 3MS score, head trauma, and stroke	6
Gerhard et al. (11)	USA, population-based	Cohort, patients with bipolar disorder	≥50	Pharmacy claims	ICD-9-CM	Exposed 20,778, Non-exposed 18,119	Gender, ethnicity, age, Medicaid eligibility, long-term care residency, depression, anxiety, alcohol-related disorders, drug-related disorders, arrhythmia, heart failure, myocardial infarction, other acute ischemic heart disease, other chronic ischemic heart disease, hypertension, cerebrovascular disease, diabetes mellitus, Parkinson's disease, antidepressant use, antipsychotic use, use of anti-anxiety medications	9
Tsai et al. (12)	Taiwan, population-based	Cohort, patients with bipolar disorder	≥20	Pharmacy claims	ICD-9-CM	Valproate exposed 1,792, Non-exposed 3,366	Age; sex; obesity; length of hospital admissions because of bipolar disorder; and the use of lithium, carbamazepine, antipsychotics, or benzodiazepine derivatives	9
Taipale et al. (13)	Finland, population-based	Case-control, general population	NA	Pharmacy claims	Hospital discharge register	Case 20,325, Control 81,300	Polypharmacy, stroke, depression, cardiovascular diseases, diabetes, and epilepsy	8
	German, population-based	Case-control, general population	≥60	Pharmacy claims	ICD-9	Case 70,718, Control 282,858	Polypharmacy, stroke, depression, cardiovascular diseases, diabetes, and epilepsy	8
Coupland et al. (14)	England, population-based	Case-control, general population	≥55	Pharmacy claims	Clinical codes or prescriptions	Case 58,769, Control 225,574	Body mass index, calculated as weight in kilograms divided by height in meters squared, smoking status, alcohol consumption, Townsend deprivation score, ethnic group, coronary heart disease, atrial fibrillation, heart failure, hypertension, hyperlipidemia, diabetes, stroke, transient ischemic attack, subarachnoid hemorrhage, renal disease, asthma, chronic obstructive pulmonary disease, anxiety, depression, bipolar disorder, schizophrenia, severe head injury, cognitive decline/memory loss, antihypertensive drugs, aspirin, hypnotics, anxiolytic drugs, non-steroidal anti-inflammatory drugs, statins, and with matching by age, sex, general practice, and calendar time	8

(Continued)

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TABLE 1 (Continued)

References	Location, setting	Study design	Age	Ascertainment of antiepileptic exposure	Outcome measurement	Number of participants	Confounding adjusted	Quality
Jacob et al. (15)	German, population-based	Case-control, general population	≥60	Pharmacy claims	ICD-10	Case 50,575, Control 50,575	Epilepsy, hypertension, diabetes, hyperlipidemia, coronary heart disease, stroke including transient ischemic attack, intracranial injury, depression, bipolar disorder, mental and behavioral disorders due to use of alcohol, migraine, osteoporosis, prescription of benzodiazepines, prescription of antidepressants, and prescription of antipsychotics	8
Schnier et al. (16)	Wales, population-based	Cohort, general population	≥60	Pharmacy claims	ICD-10	563,151	Sex	6
Moon et al. (17)	Korean, population-based	Cohort, patients with bipolar disorder	≥50	Pharmacy claims	ICD-10	VPA exposed 1,785, Non-exposed 2,378	Diabetes, alcohol-related disorder, and use of anti-epileptics	7
Mur et al. (18)	UK, population-based	Cohort, general population	≥55	Pharmacy claims	Clinical codes or prescriptions	Case 2,124, Control 169,652	No	6

3.3. Meta-analysis

3.3.1. Association between ASM use and dementia among general population

The results of all analyses are listed in Table 2. Six studies measured the relationship between overall ASM exposure and the risk of dementia among the general population. A meta-analysis of these studies with 9 estimates indicated that overall ASM exposure was significantly associated with an increased risk of dementia (OR: 1.09, 95% CI: 1.03–1.15; $P = 0.003$) (Figure 2). High heterogeneity was observed among these studies ($I^2 = 85.6\%$). As shown in Supplementary Figure S1, we did not find any evidence of publication bias (Begg's test, $P = 0.3$).

A subgroup analysis by study design found a significant association in cohort studies (OR: 1.12, 95% CI: 1.02–1.23; $P = 0.02$; $I^2 = 73.3\%$), but a non-significant trend toward an increased risk of dementia in case–control studies (OR: 1.07, 95% CI: 1–1.16; $P = 0.059$; $I^2 = 89.3\%$).

Considering the number of adjustment variables revealed a significantly increased dementia risk in those studies adjusting for fewer than five variables (OR: 1.12, 95% CI: 1.02–1.23; $P = 0.02$; $I^2 = 73.3\%$), but no significant association in those adjusting for more than five (OR: 1.07, 95% CI: 1–1.16; $P = 0.059$; $I^2 = 89.3\%$).

When we grouped studies by ASM type, significant associations were observed for those using valproate (OR: 1.47, 95% CI: 1.29–1.67; $P < 0.001$; $I^2 = 63.2\%$), carbamazepine (OR: 1.11, 95% CI: 1.03–1.19; $P = 0.004$; $I^2 = 56.6\%$), or clonazepam (OR: 1.21, 95% CI: 1.11–1.32; $P < 0.001$; $I^2 = 29.2\%$), but no significant association was observed for those using levetiracetam (OR: 1.25, 95% CI: 0.85–1.85; $P = 0.253$; $I^2 = 80.9\%$), topiramate (OR: 1.11, 95% CI: 0.84–1.47; $P = 0.452$; $I^2 = 0\%$), lamotrigine (OR: 1.05, 95% CI: 0.91–1.21; $P = 0.527$; $I^2 = 0\%$), gabapentin (OR: 0.76, 95% CI: 0.49–1.18; $P = 0.225$; $I^2 = 92.3\%$), pregabalin (OR: 0.84, 95% CI: 0.64–1.11; $P = 0.227$; $I^2 = 73.1\%$), primidone (OR: 1.25, 95% CI: 0.95–1.64; $P = 0.11$; $I^2 = 80.1\%$), or phenytoin (OR: 1.05, 95% CI: 0.92–1.19; $P = 0.465$; $I^2 = 32.3\%$).

3.3.2. Association between ASM use and dementia among patients with bipolar disorder

Three studies compared the risk of dementia in bipolar disorder patients who were and were not exposed to ASMs; the combined OR of dementia was 1.43 (95% CI: 1.07–1.92; $P = 0.015$; $I^2 = 85.9\%$) (Figure 3). When our analysis limited to studies only evaluated valproate; the combined OR of dementia was 1.62 (95% CI: 1.38–1.89; $P < 0.001$; $I^2 = 0\%$).

4. Discussion

This meta-analysis of current observational evidence suggests that the statistically significant association between ASM use and dementia in general population can be partially explained by unmeasured confounding. However, subgroup analyses based on individual ASMs found that only valproate, carbamazepine, and clonazepam were associated with an increased risk of dementia. Furthermore, we found that bipolar disorder

TABLE 2 Meta-analysis for studies included in the analysis.

Subgroup analysis	Number of studies	Number of estimates	Pooled RR (95% CI), I^2 statistics (%)	Model used
General population	6	9	1.09 (1.03–1.15); $I^2 = 85.6\%$	Random effects
Study design				
Cohort	3	3	1.12 (1.02–1.23); $I^2 = 73.3\%$	Random effects
Case-control	3	6	1.07 (1–1.16); $I^2 = 89.3\%$	Random effects
No. of adjustment variables				
<5	3	3	1.12 (1.02–1.23); $I^2 = 73.3\%$	Random effects
≥5	3	6	1.07 (1–1.16); $I^2 = 89.3\%$	Random effects
Type of AEDs				
Valproate	3	6	1.47 (1.29–1.67); $I^2 = 63.2\%$	Random effects
Carbamazepine	3	6	1.11 (1.03–1.19); $I^2 = 56.6\%$	Random effects
Clonazepam	3	6	1.21 (1.11–1.32); $I^2 = 29.2\%$	Random effects
Levetiracetam	2	2	1.25 (0.85–1.85); $I^2 = 80.9\%$	Random effects
Topiramate	2	2	1.11 (0.84–1.47); $I^2 = 0\%$	Random effects
Lamotrigine	2	2	1.05 (0.91–1.21); $I^2 = 0\%$	Random effects
Gabapentin	2	2	0.76 (0.49–1.18); $I^2 = 92.3\%$	Random effects
Pregabalin	2	2	0.84 (0.64–1.11); $I^2 = 73.1\%$	
Primidone	3	6	1.25 (0.95–1.64); $I^2 = 80.1\%$	Random effects
Phenytoin	2	5	1.05 (0.92–1.19); $I^2 = 49\%$	Random effects
Bipolar disorder	3	4	1.43 (1.07–1.92); $I^2 = 85.9\%$	Random effects
Type of AEDs				
Valproate	2	3	1.62 (1.38–1.89); $I^2 = 0\%$	Random effects

patients who were prescribed ASM showed an increased risk of dementia.

The impact of ASM use on cognitive function is controversial. Theoretically, ASMs can adversely affect cognitive functions by suppressing neuronal excitability or enhancing inhibitory neurotransmission (7, 24); however, several studies (25–27) have shown that exposure to several ASMs was associated with improved cognitive function because they also induce the neurogenesis of neural progenitor/stem cells both *in vitro* and *in vivo* (28). Consistent with the findings of these preclinical studies, the results of clinical studies that assessed the effects of ASMs on cognitive function or dementia varied. Furthermore, previous reviews (7, 24) have summarized this relationship, but failed to provide an overall estimate of the effects of ASMs on cognitive function or dementia. The authors noted that first-generation drugs had negative effects on cognitive function, but they were not found to increase the risk of dementia.

Although these modifying effects of ASMs on dementia are biologically plausible, the results of the included studies were discordant, as reflected in the high heterogeneity in the overall meta-analysis. This heterogeneity could not be accounted for in the subgroup analyses based on study design, location, or quality; number of adjustments; drug indications; and individual drugs. The existence of clinical heterogeneity should lead to a degree of statistical heterogeneity in the results.

Most of the studies in our overall analysis drew conclusions based on general-population data and did not consider the drug indications. However, epilepsy was shown to be associated with an increased risk of dementia (29). It is reasonable to speculate that this association may be overestimated if the studies did not adjust for this potential confounder. To minimize the effect of indication, we conducted a subgroup analysis based on the number of adjustment variables and found no significant association after we combined the estimates from the included studies adjusted for the drug indication. In addition to epilepsy, ASMs are commonly prescribed to treat bipolar disorder, depression, and other mental disorders (6). Previous meta-analysis have demonstrated that bipolar disorder is associated with an increased risk of dementia (30). Three included studies (11, 12, 17) focused on patients with bipolar disorder and used non-exposed patients as negative controls to minimize the effects of indication. In our meta-analysis, we observed an ∼ 1.43-fold increase in the risk of dementia in patients with bipolar disorder who were exposed to ASMs.

The high heterogeneity of the overall analysis may also arise from the types of ASM. In our subgroup analysis of individual ASMs, only valproate, carbamazepine, and clonazepam, which are first-generation ASMs, were found to increase the risk of dementia. Previous studies demonstrated that the main cognitive effects of ASM use were impaired attention, vigilance, and psychomotor

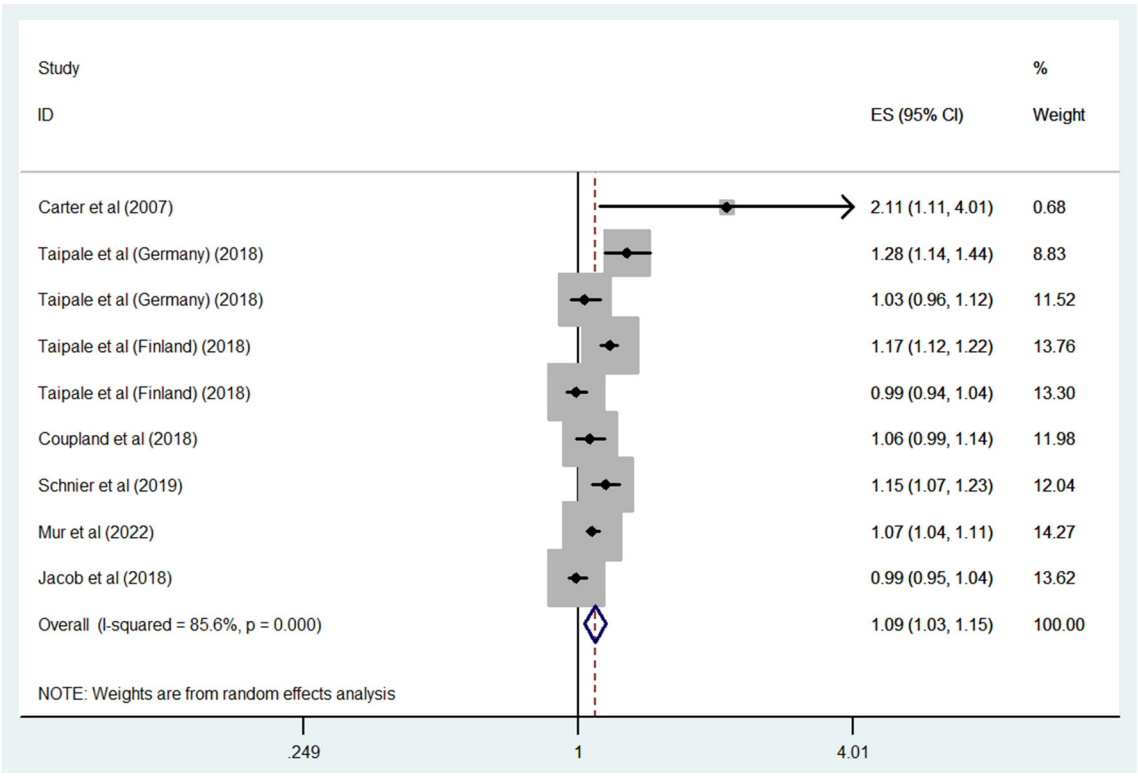

FIGURE 2
Forest plot of the overall risk of dementia in relation to ASMs use among the general population.

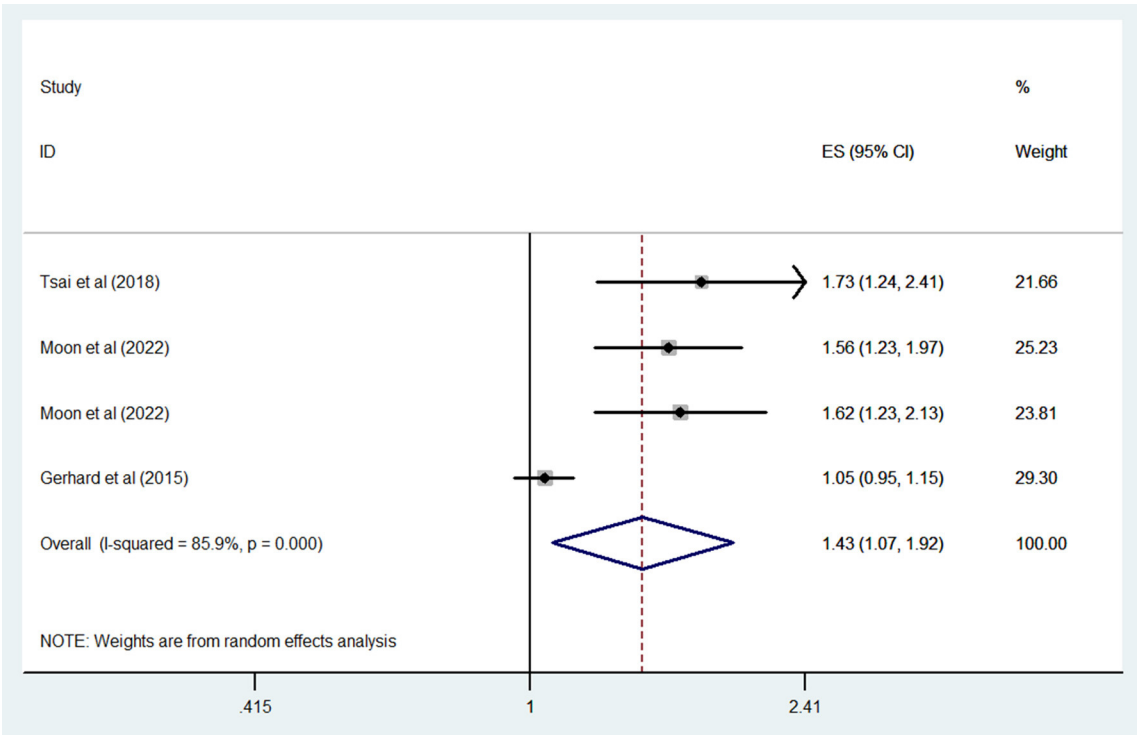

FIGURE 3
Forest plot of the overall risk of dementia in relation to ASMs use among patients with bipolar disorder.

speed. One double-blind, placebo-controlled study (31) reported convincing evidence of improved motor skills after discontinuing valproate in patients with epilepsy. Two double-blind, placebo-controlled studies (32, 33) involving epilepsy patients on ASM monotherapy (mainly carbamazepine or valproate) observed that drug discontinuation significantly improved performance in tests that required complex cognitive processing under time pressure. However, most studies (25, 34, 35) tend to report little or no cognitive impairment associated with pregabalin or gabapentin in people with partial epilepsy. Consistent with the cognitive findings in epilepsy patients, our individual ASM analysis found that newer ASMs act more favorably on dementia risk compared with first-generation drugs. Recently, preclinical studies demonstrated a protective effect of levetiracetam on cognitive function. In the transgenic mice models of Alzheimer's disease, a low dose of levetiracetam could alleviate cognitive decline, through suppression of proinflammatory cytokines expression and inhibition of abnormal tau hyperphosphorylation (36, 37). In clinical study, levetiracetam improved performance on spatial memory and executive function tasks in patients with Alzheimer's disease (38). However, the beneficial role of levetiracetam on dementia was not detected in our analysis. Hence, our results of individual ASM on risk of dementia may be limited by sample size and need further investigation to clarify this issue.

To our knowledge, this meta-analysis is the first to explore the association between ASM use and dementia risk. The strengths of this work are the comprehensive search and the rigorous systematic review and meta-analysis of all relevant reports to date. We also performed several additional analyses to test the robustness of the results. Nonetheless, there are several limitations to this meta-analysis. First, residual confounders are always a concern in epidemiological observational studies. Second, all of the included studies considered Western populations and not subjects from Asia or Africa, which may have affected the generalizability of our results. Third, information on the dose of ASM used in the included studies could not be extracted; therefore, any exposure parameter possibly associated with dementia could not be defined.

In summary, this systematic review and meta-analysis only observed a greater risk of dementia with the use of valproate, carbamazepine, or clonazepam in general population. We also found that ASMs are associated with an increased risk of dementia in bipolar disorder. However, large, well-designed, prospective cohort studies that consider a greater number of confounding factors are warranted to verify our findings.

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.

Author contributions

LZ and H-yJ searched the library, wrote the manuscript text, extracted data, and reviewed all articles. W-jL designed the manuscript. All authors reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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/fneur.2023.1133816/full#supplementary-material

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frontiers | Frontiers in Neurology

TYPE Original Research
PUBLISHED 17 August 2023
DOI 10.3389/fneur.2023.1124943

OPEN ACCESS

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RECEIVED 16 December 2022
ACCEPTED 11 July 2023
PUBLISHED 17 August 2023

CITATION
Weyde KVF, Winterton A, Surén P, Andersen GL, Vik T, Biele G, Knutsen HK, Thomsen C, Meltzer HM, Skogheim TS, Engel SM, Aase H and Villanger GD (2023) Association between gestational levels of toxic metals and essential elements and cerebral palsy in children.
Front. Neurol. 14:1124943.
doi: 10.3389/fneur.2023.1124943

Association between gestational levels of toxic metals and essential elements and cerebral palsy in children

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Introduction: Cerebral palsy (CP) is the most common motor disability in childhood, but its causes are only partly known. Early-life exposure to toxic metals and inadequate or excess amounts of essential elements can adversely affect brain and nervous system development. However, little is still known about these as perinatal risk factors for CP. This study aims to investigate the associations between second trimester maternal blood levels of toxic metals, essential elements, and mixtures thereof, with CP diagnoses in children.

Methods: In a large, population-based prospective birth cohort (The Norwegian Mother, Father, and Child Cohort Study), children with CP diagnoses were identified through The Norwegian Patient Registry and Cerebral Palsy Registry of Norway. One hundred forty-four children with CP and 1,082 controls were included. The relationship between maternal blood concentrations of five toxic metals and six essential elements and CP diagnoses were investigated using mixture approaches: elastic net with stability selection to identify important metals/elements in the mixture in relation to CP; then logistic regressions of the selected metals/elements to estimate odds ratio (OR) of CP and two-way interactions among metals/elements and with child sex and maternal education. Finally, the joint effects of the mixtures on CP diagnoses were estimated using quantile-based g-computation analyses.

Results: The essential elements manganese and copper, as well as the toxic metal Hg, were the most important in relation to CP. Elevated maternal levels of copper (OR = 1.40) and manganese (OR = 1.20) were associated with increased risk of CP, while Hg levels were, counterintuitively, inversely related to CP. Metal/element interactions that were associated with CP were observed, and that sex and maternal education influenced the relationships between metals/elements and CP. In the joint mixture approach no significant association between the mixture of metals/elements and CP (OR = 1.00, 95% CI = [0.67, 1.50]) was identified.

Conclusion: Using mixture approaches, elevated levels of copper and manganese measured in maternal blood during the second trimester could be related to increased risk of CP in children. The inverse associations between maternal Hg and CP could reflect Hg as a marker of maternal fish intake and thus nutrients beneficial for foetal brain development.

KEYWORDS

toxic metal, essential element, cerebral palsy (CP), pregnant women, brain develeopment, The Norwegian Mother, Father, and Child Cohort Study (MoBa), Medical Birth Registry of Norway (MBRN)

1. Introduction

Cerebral palsy (CP) is defined as a "group of permanent disorders of the development of movement and posture" caused by a non-progressive lesion in the developing brain occurring before 2 years of age (1). Cerebral palsy is the most common motor disability in childhood, with a prevalence of about 0.2 percent (2), but affecting up to one in 10 extremely preterm born children (3). About 30% have severe gross- and fine motor impairments, being unable to walk without assistive devices or being dependent on a wheelchair and/or unable to use their hands independently. In addition, associated neurodevelopmental disorders and difficulties are common, including epilepsy, intellectual disability, eating difficulties, speech and communication difficulties as well as pain and musculoskeletal complications (4). The pathophysiology underlying CP is varied and complex, and the resulting brain injuries include brain malformations, white and grey matter injuries, though congenital malformations are identified in a minority of cases (5). CP is further classified into subtypes based on the dominating clinical symptom, i.e., spasticity, dyskinesia, or ataxia. Based upon the timing of the insult, CP is classified as postneonatal occurring from day 28 after birth to the age of 2 years, or congenital CP. Whereas the event leading to postneonatal CP is usually clearly identified, the aetiology underlying congenital CP is more obscure (6).

Genetic causes of CP are rare, but genetic factors may interact with risk factors, as the prevalence of CP is elevated among close relatives (7). Rather than being a direct cause of CP, genetic factors may interact with other risk factors, such as preterm birth, foetal growth restriction, placental dysfunction, and hypoxic ischemic insults during delivery, leading to neonatal encephalopathy and seizures (8). However, in the majority of congenital CP cases the aetiology still remains unexplained (5).

In utero exposure to chemicals may alone, or interacting with other factors, interfere with normal brain development leading to an early brain insult and CP. Earlier studies have found increased risk of CP diagnosis in children prenatally exposed to pharmaceuticals such as paracetamol or aspirin, as well as environmental toxicants such as pesticides and perfluoroalkyl substances (PFASs), which are known or suspected to adversely affect brain development (9–11).

Several toxic metals, such as mercury (Hg) and lead (Pb), can contribute to neurodevelopmental disorders, and neurological and motor impairments in children (12). Children and foetuses are especially vulnerable to such exposures, due to the rapid development of the brain and nervous system, lack of detoxifying enzymes and underdeveloped blood-brain barrier (13). Thus, toxic metals in maternal blood can pass the placenta and reach the foetal brain, and adversely affect foetal development of the brain and nervous system and later functioning (14–16).

In contrast to toxic (non-essential) metals, essential elements, such as copper (Cu), cobalt (Co), selenium (Se), zinc (Zn), magnesium (Mg), and manganese (Mn), are important in human physiological and biochemical processes (17). A healthy, nutrient-rich diet during pregnancy is imperative to ensure a healthy development of the foetus (18). Pregnant women are at increased risk of micro- and macronutrient deficiency due to increased demands from the foetus (19). Essential elements generally have a narrow optimal dose range, and both excessive and insufficient intake may adversely affect health (17, 20, 21).

Although there are uncertainties as to whether gestational exposure to toxic metals and essential elements are associated with risk of neurological disorders like CP in the child, there is some evidence that elevated exposure to the toxic metals Pb, Hg, arsenic (As) and the essential element Mn can impair motor function in children and adolescents (22–27). For example, symptoms of chronic Hg intoxication in childhood includes muscular hypotonia, tremor, ataxia, and coordination problems (28), and prenatal exposure to Hg has been related to poorer motor function and gross motor skills (29). A study from Japan reported a high incidence of CP following pollution of wastewater with methylmercury (MeHg) leading to high concentrations in local fish and seafood ingested by the local population, including pregnant women (30). Similar to MeHg, Pb is an established developmental neurotoxicant (31). Bansal et al. (32) found increased blood Pb concentrations in children with CP, compared to controls. However, few or no previous studies, to our knowledge, have investigated associations between gestational levels of toxic metals and essential elements, and CP diagnosis in the child.

Studies of CP diagnoses requires very large sample sizes, due to the heterogeneity and the low prevalence of CP. The present study aims to address this question by using data from a large population-based cohort, the Norwegian Mother, Father, and Child Cohort Study [MoBa; Magnus et al. (33)]. Most studies on health effects from chemical exposure have been limited to only a few exposures (34), however human populations are not exposed to only one metal at the time, but rather to a mixture of multiple metals. Metals, as well as essential elements, can act jointly (additively), or they can interact antagonistically or synergistically, yielding potentially different effects on development and health compared to when the metals/elements are considered alone (29, 35). Investigating the associations of combinations of metals/elements with health outcomes is therefore critical when it comes to research on environmental factors and children's health (36, 37).

The aim of the present study is to investigate the associations between gestational levels of toxic metals and essential elements, individually and as mixtures, and risk of CP diagnoses in children using a prospective, population-based birth cohort.

2. Methods

2.1. Study sample

2.1.1. The Norwegian Mother, Father, and Child Cohort Study (MoBa)

Participants in the current study were selected from NeuroTox, a sub-study to MoBa aimed at investigating the association of prenatal exposure to environmental toxicants and risk for neurodevelopmental and neurological disorders in children. MoBa is a population-based pregnancy cohort study conducted by the Norwegian Institute of Public Health. Participants were recruited from all over Norway during 1999–2008. The women consented to participation in 41% of the pregnancies. The cohort now includes 114,500 children, 95,200 mothers and 75,200 fathers (33). Blood samples were obtained from both parents during pregnancy and from mothers and children (umbilical cord) at birth (38). The current study is based on version 12 of the quality-assured data files. The establishment of MoBa and initial data collection was based on a license from the Norwegian Data Protection Agency and approval from The Regional Committees for Medical and Health Research Ethics. The MoBa cohort is now based on regulations related to the Norwegian Health Registry Act. The Norwegian Patient Registry (NPR) has approved the linkage between NPR and MoBa, identifying cases with a diagnosis of CP. Linkage between MoBa and the Cerebral Palsy Registry of Norway (CPRN) was also used to identify cases. The CPRN is a consent-based national medical quality register established in 2006 containing clinical data on individual children born from 1996 onwards (39). The Medical Birth Registry of Norway (MBRN) is a national health registry containing information about all births in Norway.

2.1.2. Cases and controls

In total, 247 MoBa children with CP diagnoses (one or more registrations of ICD-10 codes G80.0-G80.9; 40) in the CPRN (39) or the NPR (41) were identified. For children with recorded CP diagnoses in the NPR, but who were not previously captured by the CPRN (39), the diagnoses were validated according to the standard procedures of the CPRN. The inclusion criteria in the present study were (Figure 1): Singleton, alive at 2 years of age, born 2002 or later, available record from the MBRN, available maternal MoBa questionnaire 1 (gestations week 15), no registration of Downs syndrome and available maternal whole blood samples (gestations week ~18). The final sample consisted of 144 CP diagnostic cases and their mothers. Of the CP cases, 59 were categorised as hemiplegic, 43 as diplegic, 16 quadriplegic, 2 choreo-athetotic, 13 dystonic, 7 ataxic and 4 unknown or unclassifiable. The CP cases were analysed together, as there is often overlap in presentation and clinical significance between them (6).

The control population in the NeuroTox project was designed to be used for other outcomes as well, including autism spectrum disorders (ASD), attention deficit/hyperactivity disorder (ADHD), and epilepsy. This control group was randomly sampled from the eligible MoBa sample, frequency matched with birth year and child sex to all diagnostic cases in NeuroTox applying the same inclusion criteria as for the cases. Since CP, and especially ADHD and ASD, are more prevalent among boys (42), more male than female controls were sampled. The final control group consisted of 1,082 children and their mothers. The high case-control ratio leads to increased statistical power when the prevalence of cases is small (43). In addition, this study was a part of a larger studies on neurodevelopment and neurological outcomes in children, where control group was designed to fit all cases groups such as ADHD, ASD and epilepsy, where the prevalence in children is higher than that of CP (44).

The current study was approved by The Regional Committees for Medical and Health Research Ethics (ref. no. 2012/985-1). Parents enrolled in MoBa gave written consent for the use of this data.

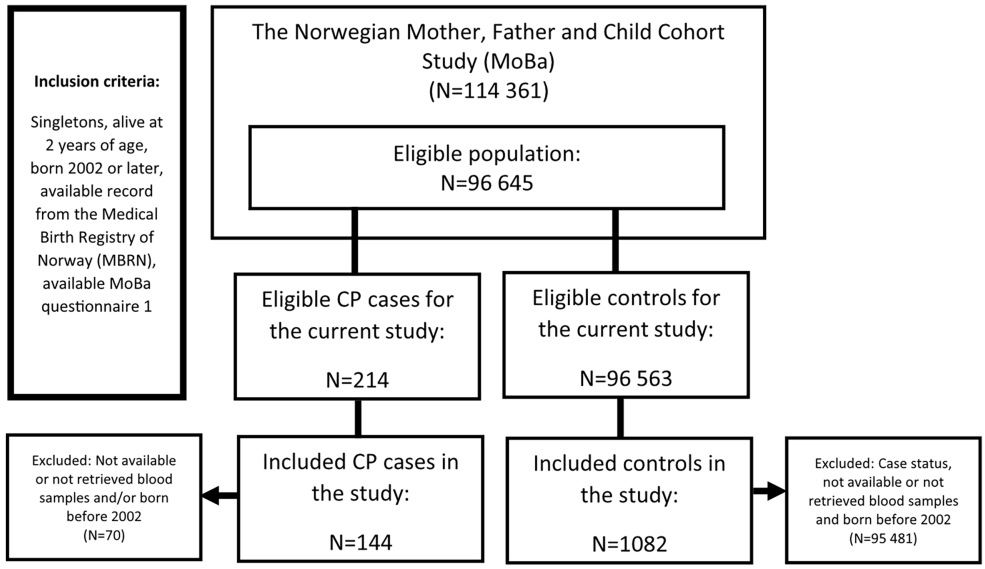

FIGURE 1
Flow chart showing the selection of cases and controls in a nested case-control study of cerebral palsy in the Norwegian Mother, Father, and Child Cohort Study (MoBa), 2002–2006.

2.2. Measurement of toxic metals and essential elements in maternal blood

The present study used maternal blood samples from around gestation week 18. Details about the sampling procedure and handling and storage in the MoBa biobank are described in detail elsewhere (38). Eleven toxic/non-essential metals and essential elements were determined in maternal whole blood, using inductively coupled plasma-sector field mass spectrometry (ICP-SFMS). These included the toxic metals As, cadmium (Cd), caesium (Cs), Pb and Hg, and the essential elements Co, Cu, Mg, Mn, Se, and Zn. Hg and As were measures of total Hg and total As, containing both inorganic and organic forms. In the Norwegian population, these measures will to a large degree reflect organic forms from intake of fish and seafood (45). Most samples (n = 1,121) were analysed at ALS laboratory group's lab in Sweden, and some (n = 105) were analysed at the University of Lund (Sweden). Internal quality control samples and procedure blanks were analysed along with each batch of samples to ensure high quality of the determinations throughout the project. Additionally, reference samples were included (Seronorm Trace Elements whole blood L-1, SERO AS, Billingstad, Norway) that were used as project-specific quality control (QC) samples. Case, control, and QC samples were randomized to batch and blinded to the analysist. Details on analytical procedures, limits of detection (LOD), limits of quantification (LOQ) and quality control are presented in Supplementary materials 1 and 3. For most metals/elements, concentrations above LOQ are reported, but for As, Cd, Pb, and Hg, concentrations above LOD are reported. Metal/element concentrations are given in µg/L, except for Mg, which is given in mg/L.

Due to issues related to project design and logistics, the blood samples were pulled from the biobank and analysed for metals and elements in three separate analytical rounds (Supplementary material 1). In addition, some samples were analysed at the University of Lund in another MoBa sub-study (~round 4). To account for analytical variation across analytical rounds, the metal/element concentrations were normalised for each participant using the QC samples (Seronorm reference material) analysed in each of the analytical rounds. The approach used was similar to the scaled variation of the Ratio-G batch adjustment described in Luo et al. (46). Let M be the measured metal/element concentration i for each participant j. M*ij is then the analytical round adjusted metal/element concentration, and is calculated as (Eq. 1):

$$M^{*}ij = Mij \times \left(\text{meanQCl} / \text{meanQClk}\right), \quad (1)$$

where meanQCl represents the geometric mean of metal/element i in reference samples across all analytical rounds, and meanQClk represents the geometric mean of metal/element i in reference samples from analytical round k (i.e., in the analytical round in which sample of participant j was measured).

2.3. Covariates

The covariates in the present study were obtained from three prenatal MoBa questionnaires completed in gestation weeks 15, 22, and 30 (33) and from the MBRN. The following covariates were considered: from MoBa: maternal education (up to and including 4 years of university/college vs. 5 years of university/college or more), maternal smoking during pregnancy (daily/sometimes vs. no), maternal seafood consumption during pregnancy obtained from the food frequency questionnaire (gestation week 22), and maternal pre-pregnancy body mass index; from MBRN: child sex, child birth year (2002–2005 vs. 2006–2009), parity (0 vs. 1^{+}), maternal age at delivery (in years), gestational age, and birth weight. A minimal adjustment set was identified using directed acyclic graphs made at dagitty.net [DAGs; Textor et al. (47); see Supplementary material 11 and Table 1], and included maternal education, maternal seafood intake during pregnancy, maternal age at delivery, maternal smoking during pregnancy, and parity. Sex and birth year were additionally included as covariates, since these variables are important in relation to CP and metal/element exposure, respectively.

TABLE 1 Descriptive information for controls, cases, and the total study sample in a nested case-control study of cerebral palsy in the Norwegian Mother, Father, and Child Cohort Study (MoBa), 2002–2006.

	Controls	CP cases	Total
	N = 1,082	*N* = 144	*N* = 1,226
Maternal age [mean (SD)][a]	30.0 (4.5)	30.4 (4.7)	30.1 (4.5)
Seafood intake, pregnancy [mean (SD)][a]	36.5 (21.8)	33.7 (21.2)	36.2 (21.8)
Missing	128	15	143
Maternal folate intake, pregnancy [mean (SD)][b]	511.2 (272.3)	547.5 (277.8)	515.5 (273.1)
Missing	242	31	273
Gestational age, days [mean (SD)][b]	279.6 (11.7)	265.0 (31.3)	277.9 (16.0)
Missing	4	1	5
Parity[a]			
0	459 (42.4%)	80 (55.6%)	539 (44.0%)
1^{+}	623 (57.6%)	64 (44.4%)	687 (56.0%)
Maternal education[a]			
<5 year university	357 (33.8%)	49 (34.8%)	406 (33.9%)
≥5 year university	698 (66.2%)	92 (65.2%)	790 (66.1%)
Missing	27	3	30
Maternal smoking, pregnancy[a]			
No	937 (86.6%)	132 (91.7%)	1,069 (87.2%)
Yes	145 (13.4%)	12 (8.3%)	157 (12.8%)
Maternal pre-pregnancy BMI[b]	23.4 (5.9)	24.5 (7.1)	23.5 (6.1)
Birth year[a]			
<2006	867 (80.1%)	65 (45.1%)	932 (76.0%)
≥2006	215 (19.9%)	79 (54.9%)	294 (24.0%)
Sex[a]			
Boys	744 (68.8%)	83 (57.6%)	827 (67.5%)
Girls	338 (31.2%)	61 (42.4%)	399 (32.5%)

[a]Adjustment variables in the analyses.
[b]Not part of minimal adjustment set.

2.4. Statistical analyses

Preliminary analyses (see Supplementary material 12) indicated that some metal/element outliers could influence the estimates of the individual metal/element-CP relationships. To deal with outliers, a winsorization approach was used (48) where metal/element concentrations below the first and above the 99th percentiles were replaced with the value of the first and 99th percentile, respectively. Then all metal/element concentrations were natural log transformed to reduce skewness.

Multiple imputation ($M = 20$) was used to replace missing values with Amelia II in R (49). As, Cd and Co had some missing values due to concentrations below LOD or LOQ. Therefore, lower (≈0) and upper (LOQ for Cd and Co; LOD for As) bounds were specified for these variables in the imputation. Missing Mg and Cs (not analysed at the University of Lund) were imputed based on their log-normal distributions. We also imputed missing covariates (see Tables 1, 2). Imputations were based on the following variables: CP diagnoses, log-transformed metal/element concentrations, maternal age, maternal smoking during pregnancy, parity, maternal education, child sex, maternal pre-pregnancy BMI, maternal seafood intake during pregnancy, birth year, gestational age in days, and total maternal folate intake during pregnancy. Kernel density plots were used to confirm that the imputed values seemed reasonable.

Correlations in one of the imputed datasets among the measured toxic metals and essential elements in maternal blood were investigated using Spearman correlation.

All regression models are based on multiple imputed data, unless otherwise mentioned, and adjusted for child sex, birth year, parity, maternal education, maternal smoking during pregnancy, maternal age at delivery, and maternal seafood consumption during pregnancy.

2.4.1. Identifying important metals/elements in the association with CP

Simultaneously including multiple correlated exposure variables can produce unstable estimates and inflated standard errors when running traditional regression models (50). To overcome this limitation, a method for regularization and variable selection was used: elastic net regression (51) (see Supplementary material 2), to identify metal/element exposures important for CP. The covariates were not penalized. Elastic net regression is a suitable method to identify the most important elements in relation to the outcome within a mixture, which then can be used to characterise in the independent exposure-response relationships of the selected mixture member(s) (50).

To ensure the robustness of the elastic net results, stability selection was performed. In stability selection, variables that are only weakly related to the outcome are more likely to be filtered out, due to more noise being introduced into the data (52). In short, random sampling from the original data with replacement was done 200 times, yielding 200 new datasets. In each of these datasets, 20 multiple imputed (MI) datasets were made. Elastic net was run in every MI dataset, and it was calculated how often, on average, the exposures were selected. Thus, each randomly drawn dataset yielded selection probability estimate for each exposure. The mean of the selection probabilities across the 200 randomly drawn datasets was then calculated. A permutation procedure was used to calculate p-values from the elastic net regression with stability selection.

The selection probabilities and p-values were in combination used as an indication of the strength of the association between exposure and outcome. Exposures with p-value ≤0.05 (and a high selection probability; >0.6) were selected for further analyses and entered into multivariable, adjusted logistic regression models (co-adjusted for other selected exposures) in order to obtain odds ratios (ORs) for CP. The regression model was run in each imputed dataset, and resulting ORs (no CIs or p-values were considered) were combined using Rubin's rules (53). For reason of comparison with the variable selection results, we ran multivariable adjusted linear regression models with all individual metal/elements. Estimates are given as OR per interquartile increase in metal/element.

Whether the associations between selected individual exposures and risk for CP deviated from a monotonic dose-relationship was further investigated using multivariable adjusted natural splines with knots at 10th, 50th, and 90th percentiles. A model with the exposure as linear term was then tested against a model with the exposure modelled as splines using likelihood ratio (lr) tests.

2.4.2. Identifying important two-way interactions

In order to detect possible multiplicative two-way interactions between individual metals/elements, we performed the elastic net stability selection described above. Previous studies have found associations with CP for sex and parental education (42, 54, 55). Thus, we also investigated interaction (effect measure modification) by child sex and maternal education (as proxy for SES). All independent variables were standardized. Pairwise interaction terms between all the metals/elements and between metals/elements and sex and maternal education were generated. The selected interaction terms (with p-value ≤0.1 and high selection probability) were included in a logistic regression model and visualized using line graphs.

2.4.3. Assessment of total metal/element mixture effect using quantile g-computation

The effect of individual exposures may be small and difficult to identify, while the joint effect of multiple chemicals in the mixture can cause stronger effects than that of single exposures (35). Therefore, analyses to investigate the joint effects of three mixtures were performed using three models: (1) a mix containing all 11 metals/elements (MixAll), (2) a mix containing five toxic metals (As, Hg, Cd, Cs and Pb; MixTox), and (3) a mixture containing six essential elements (Mn, Cu, Co, Se, Mg and Zn; MixEssential). For this purpose, quantile g-computation (qgcomp) analyses with the R package qgcomp (56) were used. In this approach the exposure variables are used to construct a weighted exposure index of the mixture, reducing dimensionality and possible multicollinearity problems. The index is included in a regression model along with covariates of interest, yielding an overall effect estimate for the mixture. A one-unit increase in the mixture corresponds to all metals/elements in the mixture increasing by one unit. Weights are constructed to represent the relative strength of each exposure in relation to the outcome. The model was run in each imputed dataset, with exposures categorized as quartiles. The estimates with 95% CIs were combined using Rubin's rules (53). Additionally, the interactions identified in the elastic net analysis were tested, along with potential non-linear effects of the mixture using the qgcomp.boot function.

TABLE 2 Batch adjusted metal concentrations (μg/L or mg/L) for controls, cases, and the total study sample in a nested case-control study of cerebral palsy in The Norwegian Mother, Father, and Child Cohort Study (MoBa), 2002–2006.

	Controls						CP cases						Total					
	N = 1,082						*N* = 144						*N* = 1,226					
	Mean (SD)	Min	10%	50%	90%	Max	Mean (SD)	Min	10%	50%	90%	Max	Mean (SD)	Min	10%	50%	90%	Max
Hg (ug/L), adjusted	1.4 (0.9)	0.1	0.6	1.2	2.5	8.4	1.2 (0.7)	0.1	0.3	1.0	2.2	3.5	1.4 (0.9)	0.1	0.5	1.1	2.4	8.4
As (ug/L), adjusted	2.4 (3.1)	0.1	0.6	1.6	4.8	51.9	2.1 (2.3)	0.2	0.8	1.4	3.7	14.9	2.4 (3.0)	0.1	0.6	1.6	4.8	51.9
Missing[a]	12						0						12					
Cd (ug/L), adjusted	0.3 (0.3)	0.0	0.1	0.2	0.5	3.0	0.2 (0.2)	0.0	0.1	0.2	0.3	2.1	0.2 (0.3)	0.0	0.1	0.2	0.5	3.0
Missing[a]	22						0						22					
Pb (ug/L), adjusted	10.0 (5.5)	2.0	5.6	9.0	15.1	85.8	9.8 (7.6)	1.0	4.9	8.7	14.4	86.0	10.0 (5.8)	1.0	5.5	8.9	15.0	86.0
Mn (ug/L), adjusted	11.2 (8.8)	3.4	6.6	9.8	15.2	162.7	13.4 (12.9)	1.9	6.7	10.2	19.4	105.1	11.5 (9.4)	1.9	6.6	9.8	15.5	162.7
Se (ug/L), adjusted	92.3 (20.4)	45.8	71.6	89.7	116	303.5	91.9 (18.7)	56.7	69.3	90.0	121.7	144.1	92.3 (20.2)	45.8	71.1	89.7	117.0	303.5
Co (ug/L), adjusted	0.3 (0.9)	0.0	0.1	0.2	0.4	29.3	0.2 (0.2)	0.0	0.1	0.2	0.3	1.4	0.3 (0.9)	0.0	0.1	0.2	0.4	29.3
Missing[a]	32						1						33					
Cs (ug/L), adjusted	2.4 (0.9)	0.9	1.5	2.3	3.4	8.4	2.3 (0.8)	0.8	1.4	2.2	3.3	5.3	2.4 (0.9)	0.8	1.5	2.2	3.3	8.4
Missing[a]	103						2						105					
Cu (ug/L), adjusted	1,583 (243)	778	1,300	1,551	1891	3,178	1,623 (282)	939	1,277	1,609	1980	3,069	1,588 (248)	778	1,297	1,554	1903	3,178
Zn (ug/L), adjusted	5,491 (1088)	1,641	4,090	5,460	6,837	10,294	5,203 (909)	1,432	4,056	5,240	6,264	7,433	5,457 (1072)	1,432	4,086	5,436	6,751	10,294
Mg (mg/L), adjusted	30.3 (3.5)	18.3	25.9	30.3	34.8	45.0												

[a]Missing was due to values below level of detection.

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2.4.4. Sensitivity analyses

Sensitivity analyses were performed in regression models of selected metals only. Analyses were stratified by children of mothers with folate intake during pregnancy below (N = 613) or above (N = 613) the median. Analyses were restricted to children born to term (born in gestational week 37 or later; N = 1,138), children not born small for gestational age [SGA; Maršál et al. (57); N = 1,199], and children of mothers who did not smoke during pregnancy (N = 1,069). The main analysis was also done without winsorizing (N = 1,226) and on complete cases (subjects without missing values; N = 1,002). In addition, due to multiple comparison, the results from the elastic net stability selection were assessed controlling the false discovery rate (fdr) at $\alpha = 0.05$ ($\alpha = 0.1$ for the interactions), according to a method proposed by Ahmed et al. (58) (see Supplementary material 2 for details).

All analyses were done in R version 4.0 (59), using the packages *glmnet* (60), *qgcomp* (56), *forestplot* (61), *ggplot2* (62), *sjPlot* (63), *ggExtra* (64), *ggeffects* (63), *stargazer* (65), *mitools* (66), *Amelia II* (49), *reshape* (67), *and splines* (68). Example scripts for the analysis are available at https://osf.io/5867z/. Data from MoBa and MBRN used in this study are managed by Norwegian Institute of Public Health and can be made available to researchers, provided approval from the Regional Committees for Medical and Health Research Ethics (REC), compliance with the EU General Data Protection Regulation (GDPR) and approval from the data owners. The consent given by the participants does not open for storage of data on an individual level in repositories or journals. Researchers who want access to data sets for replication should apply through helsedata.no. Access to data sets requires approval from The Regional Committee for Medical and Health Research Ethics in Norway and an agreement with MoBa.

3. Results

The characteristics of the study population is presented in Table 1. Compared to mother of controls, mothers of children with CP had a lower seafood consumption during pregnancy and had slightly higher pre-pregnancy BMI. A larger proportion of children with CP were first-borns, were girls and had lower birth weight and gestational age than compared to the controls (Table 1), which is in line with previous literature (69, 70). Table 2 and Supplementary material 4 present the distribution of adjusted and non-adjusted (original) gestational concentrations of metals/elements, respectively.

Spearman correlations between the log-transformed concentrations of the metals and elements showed low to moderate correlations (Figure 2), with the highest correlations being those between As and Hg (r = 0.59) and Mg and Zn (r = 0.53).

Using elastic net models in conjunction with stability selection and the permutation approach, maternal levels of Cu (p = 0.018, P_{sel} = 0.88), Hg (p = 0.019, P_{sel} = 0.87) and Mn (p = 0.055, P_{sel} = 0.79); had the strongest associations with odds of CP in children. These associations did not remain when comparing with p-values adjusted for multiple comparisons ($p > p_{FDR}$; Figure 3 and Supplementary material 5). When Mn, Hg and Cu were included in the same multivariable adjusted, logistic regression models, this resulting in the following effect estimates (ORs; per interquartile range increase in exposure): Higher maternal levels of Cu (OR = 1.40) and Mn (OR = 1.2) were associated with an increased risk of CP in the child, whereas Hg was associated with a lowered risk (OR = 0.68) (Figure 4 and Supplementary material 6). A similar pattern was observed in multivariable adjusted logistic regression models of single exposures: Hg [OR = 0.69, 95% CI = (0.51, 0.92)], Mn [OR = 1.30, 95% CI = (1.00, 1.50)] and Cu [OR = 1.50, 95% CI = (1.10, 2.00)] (Figure 4 and Supplementary material 6).

Modelling the relationship between the selected metals/element exposures and CP as natural splines with knots at 10th, 50th, and 90th percentiles, indicated no departure from linearity in the relationship between prenatal levels of Cu, Mn and Hg and odds of CP in the child (Supplementary material 6).

Restricting the analyses to children of non-smokers, children born at term, non-SGA children, or complete cases only, the effect estimates remained relatively unaffected (Supplementary material 14 and Supplementary material 7). When stratified by median maternal folate intake during pregnancy, the results in the lower intake group attenuated somewhat, whereas the higher intake group tended to be further away from one.

Several two-way interactions were identified: Cu*Pb (p = 0.017, P_{sel} = 0.89;), Cd*Cu (p = 0.065, P_{sel} = 0.82), Maternal education*Cu (p = 0.059, P_{sel} = 0.79), Hg*Mg (p = 0.025, P_{sel} = 0.79), Maternal education*Hg (p = 0.061, P_{sel} = 0.74;), Cd*Pb (p = 0.088, P_{sel} = 0.73), Child sex*Cu (p = 0.090; p_{FDR} = 0.010), Cu*Mn (p = 0.096, P_{sel} = 0.72), and Co*Hg (p = 0.084, P_{sel} = 0.70) (Figure 5 and Supplementary material 8). None of the relationships remained when controlling for multiple comparisons ($p > p_{FDR}$; Figure 5 and Supplementary material 6). The identified interaction terms (i.e., $p \leq 0.10$) are visualized using stratified, linear regression plots in Figure 6. The plots show, for example, that the positive relationship for Cu with CP was larger for lower Pb levels and higher levels of Cd, and for children of less educated mothers, and for boys. The relationship between Mn and CP was stronger for higher prenatal levels of Cu. The inverse relationship for Hg was largest in children of less educated mothers, and for lower levels of Mg.

Using qgcomp, there was no significant association between the metal/element mixture [MixAll—OR = 1.00, 95% CI = (0.67, 1.50), Supplementary material 6]. When restricting the analysis to the toxic metals mixture (MixTox) there was an inverse association with CP [OR = 0.77, 95% CI = (0.56, 1.00); Figure 4 and Supplementary material 6], with Hg being the largest contributor to this association (Supplementary material 13). The association did not remain after excluding Hg [OR = 0.83, 95% CI = (0.61, 1.12)], or when comparing with false discovery rate for multiple comparisons (Supplementary material 6). No significant effect was found for the essential elements mixture [MixEssential—OR = 1.30, 95% CI = (0.93, 2.50), Supplementary material 6]. The MixAll model including all the interactions identified using elastic net regression did not find significant effects [OR = −0.05, 95% CI = (−0.3, 0.27)], nor did the model accounting for nonlinear effects [quadratic fit OR = 0.004, 95% CI = (−0.008, 0.008)].

4. Discussion

The present study is among the first to investigate the associations between multiple toxic metals and essential elements and their mixtures measured in maternal blood during pregnancy and risk of CP diagnosis in the child within a population-based birth cohort. As

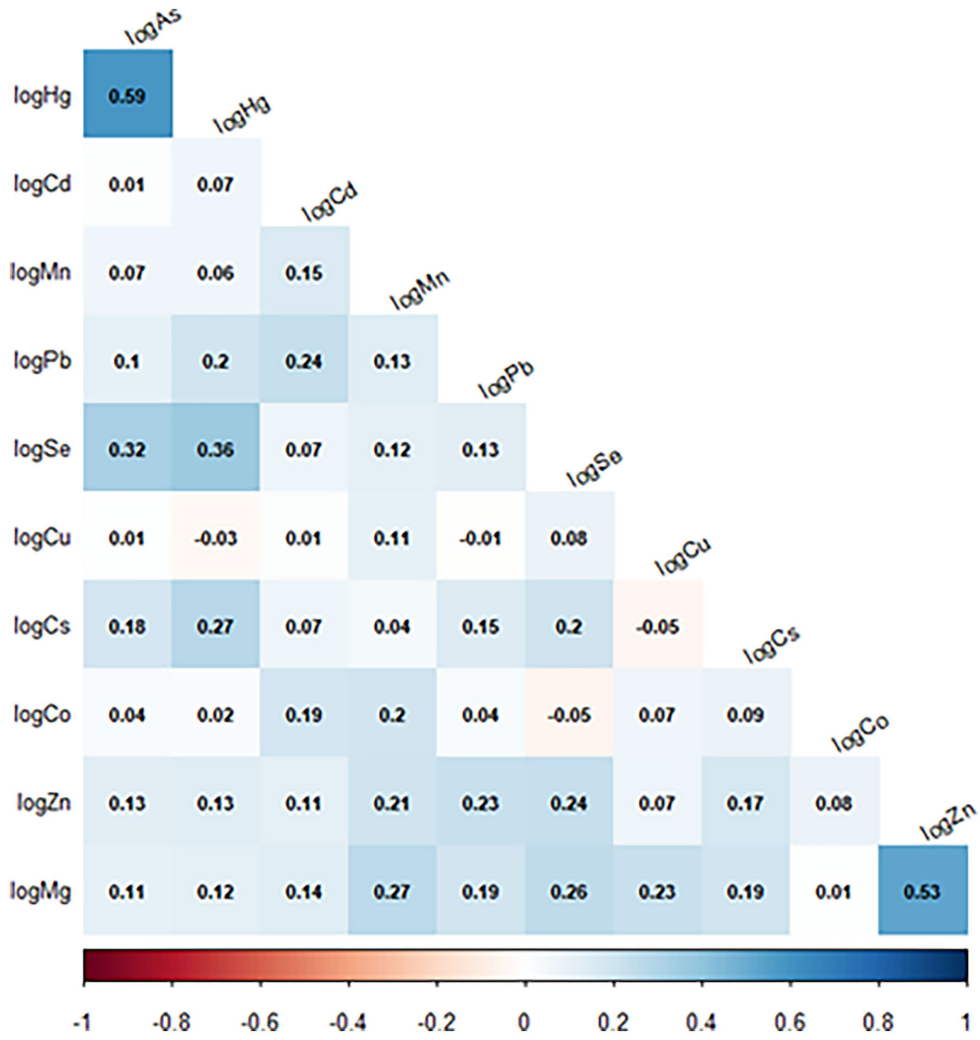

FIGURE 2
Spearman correlation between the metals/elements (adjusted concentrations) in a nested case-control study of cerebral palsy in the Norwegian Mother, Father, and Child Cohort Study (MoBa), 2002–2006. *N* = 1,226. Arsenic (As); cadmium (Cd); cesium (Cs); cobalt (Co); copper (Cu); lead (Pb); magnesium (Mg); manganese (Mn); mercury (Hg); selenium (Se); zinc (Zn).

a first mixture approach, a penalized regression analysis was used to identify potentially important elements in the mixture in relation to risk of CP. Mn, Cu, and Hg appeared the most important. Follow-up logistic regression models of these individual elements indicated an increased risk of CP in the child associated with increased maternal levels Cu and Mn. In addition, maternal level of Hg was inversely related to CP. Using a similar approach, several two-way interactions were identified among metals/elements that appeared important for risk of CP, as well as effect measure modification by child sex and SES. The second mixture approach, investigating the joint effect of the mixture(s), revealed no significant effect for the metal/element mixture, but the mixture containing toxic metals revealed an inverse association with CP, in which Hg appeared to have the most influential, though it must be noted that since it will be missing co-confounding or co-exposure effects, it might not be very reliable. The discrepancy between the effects of the overall (MixAll) and toxic (MixTox) mixtures could point to antagonism between essential and toxic metals. None of the associations for selected metals/elements, two-way interactions or the joint mixture effect remained after adjusting for multiple comparisons. Therefore, the results must be interpreted with caution. Still, highlighted exposures herein should be considered important candidates for further studies of metal/element exposure and risk of CP in children, especially since very little knowledge exists to date on associations between toxicant and micronutrient levels during perinatal development and risk of CP.

The main source of Cu exposure in humans is food, and in some cases, drinking water (71). Cu is an essential trace mineral for many important enzymes and proteins in living organisms (72). It is important for foetal and child development, but excess levels can be toxic (17). There is evidence to support an adverse effect of Cu in human neurological disorders, such as Alzheimer, Huntington, and Menkes diseases (73). Possible mechanisms for Cu toxicity include its contribution in the formation of reactive oxygen species that modify the structure and/or function of essential biomolecules (72). Little is known about prenatal Cu and CP, and the present study is, to our knowledge, among the first to investigate and report this association. Only very few studies have investigated potential adverse effects of Cu on brain development, especially when it comes to psychomotor

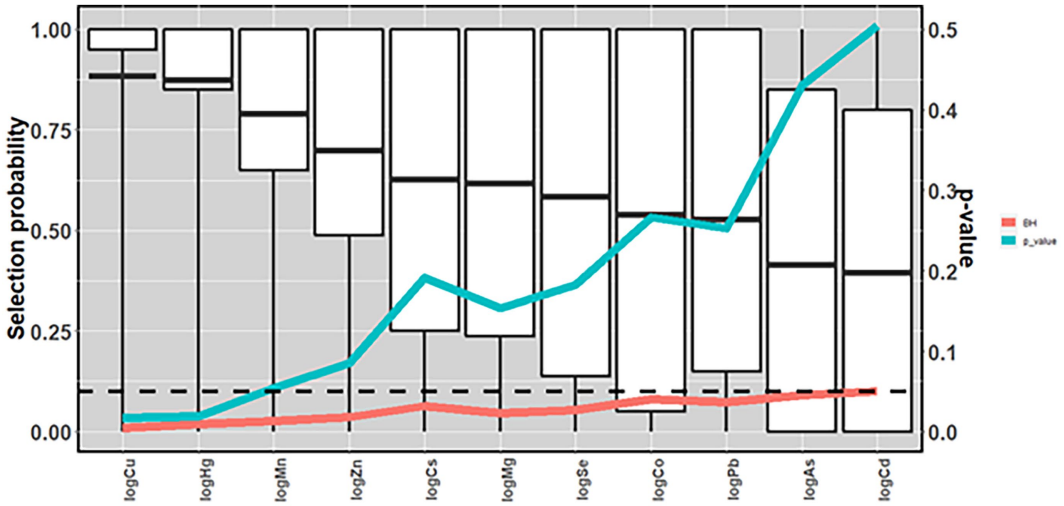

FIGURE 3
Mean selection probability (boxplot) in a nested case-control study of cerebral palsy in the Norwegian Mother, Father, and Child Cohort Study (MoBa), 2002–2006. *N* = 1,226. Based on elastic net regression in 2000 datasets (20 multiple imputed datasets in each of 100 randomly drawn datasets with replacement), and calculated *p*-values [based on 240,000 elastic net runs (once in each of 10 multiple imputed datasets in each of 20 randomly sampled datasets with replacements in each of 1,200 permuted datasets)], and Benjamini and Hochberg false discovery rate thresholds. All analyses adjusted for maternal age, parity, maternal smoking during pregnancy, maternal education, sex, and birth year. Arsenic (As); Benjamini and Hochberg false discovery rate thresholds (BH); cadmium (Cd); cesium (Cs); cobalt (Co); copper (Cu); lead (Pb); magnesium (Mg); manganese (Mn); mercury (Hg); selenium (Se); zinc (Zn).

development (74, 75). Elevated airborne Cu exposure at school at ages 8 years and 12 years was associated with poorer motor performance and altered basal ganglia structure and function of the brain (76). Partly consistent with our findings, Amorós et al. (77) found inverse associations between Cu measured in maternal blood during the first trimester, and scores on neuropsychological development at one and 5 years. Also, in line with our findings where the risk of CP was higher among boys than compared to girls in relation to maternal Cu levels, Amorós et al. (77) found the associations to be strongest for boys at age 1 year. A stronger susceptibility for males to a range of toxicants is also found in several other studies (78). Further, the association between Cu and CP in the present study was strongest for lower concentrations of Pb, higher concentrations of Cd, and higher concentrations of Mn. Previous studies have shown that the toxicity of a toxicant can depend on the presence of other toxicants or elements (37, 79). Further, the effect of Cu was mainly found in children of less educated mothers, but not for children of highly educated mothers. Higher SES is associated with healthier lifestyle and living conditions (80), which might attenuate the adverse effects of increased Cu in the body. For example, studies have reported effect measure modification by SES in the relationship between Pb and adverse neurodevelopment, and it has been hypothesized that this might be due to differences in genetic susceptibilities, environmental enrichment, or stress (81).

Since preterm birth is an important predictor of CP (82), it was of special interest to see whether the effect estimates changed when children born before term were excluded from the analyses. For Cu, the estimate was slightly attenuated, indicating that the association for Cu might be stronger in preterm born children.

The maternal blood concentration of Cu in the present study (mean = 1,588 μg/L) was comparable to those in other studies, such as studies of pregnant women from Northern Norway (mean = 1,670 μg/L), Poland (mean = 1,694 μg/L), and Republic of Korea (mean = 1,650 μg/L) (83–85). Thus, the present study indicates that Cu blood levels during pregnancy might be associated with CP, even at concentration ranges commonly seen in populations.

For the trace element Mn measured in pregnancy, a positive association (increased risk) was identified with CP in the child. The main source of Mn exposure in humans is diet, and some industrial occupations (i.e., mining, welding and steel production) also represent a risk for increased Mn exposure by inhalation (86). As for Cu, excessive exposure can be neurotoxic and Mn is an established developmental neurotoxicant (31), although there is no clearly established mechanistic underpinning for its neurotoxicity. Mn could act through substituting calcium (Ca), and thus interfere with dopaminergic synaptic transmission, disruption of ATP synthesis in the mitochondria, and oxidization of dopamine, leading to increased intracellular oxidative stress (87, 88).

In adults, inhalation of Mn can lead to a condition called manganism, which is characterized by tremors, difficulties walking, and facial spasms. There is limited knowledge about early-life Mn exposure and neurological outcomes, but there is some evidence of adverse impact on cognition and behaviour (35, 89). Prenatal Mn concentrations in blood is associated with reduced birth weight and head and chest circumference (90). A South Korean study measured blood levels of Mn in pregnant women at term, and found associations with mental and psychomotor development at child age 6 months (91). A study on Italian adolescents found associations between increased soil Mn concentrations and impaired motor coordination and hand dexterity, and positive associations between blood and hair Mn concentrations and tremor intensity (22). Another study of children in Bangladesh found no associations between Mn in drinking water and Mn in blood and urine, and between Mn and motor function (23). However other neurodevelopmental outcomes (i.e., impaired cognitive function and academic achievement, internalizing

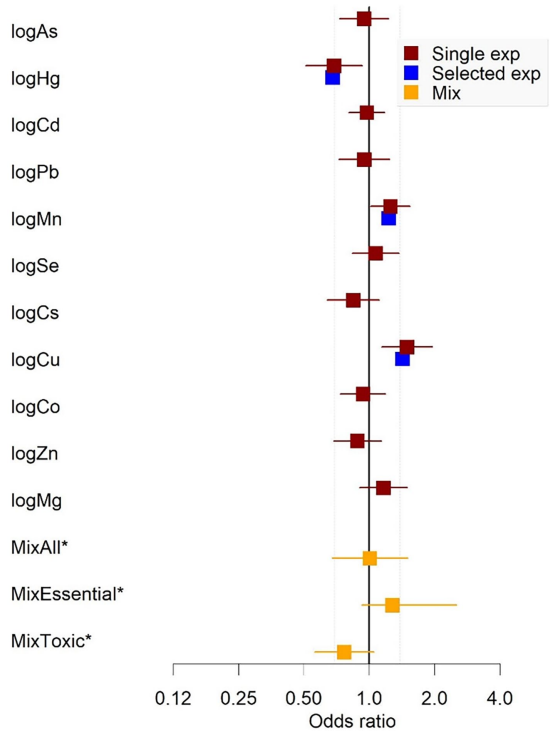

FIGURE 4
Estimates from logistic regression models of single exposures (with 95% CI), selected exposure (co-adjusted) and joint mixture exposure in a nested case-control study of cerebral palsy in the Norwegian Mother, Father, and Child Cohort Study (MoBa), 2002–2006. *N* = 1,226. Odds ratios for single metals/elements are per interquartile range increase in exposure. Yellow squares are estimates from the quantile *g*-computation analyses and represent ORs per one quartile increase in mix. *MixAll = all 11 metals/elements; MixEssential = Co, Cu, Mn, Se, Mg, Zn; MixToxic = As, Hg, Cd, Pb, Cs. All models were fit in 20 multiply imputed datasets, adjusting for maternal age, parity, maternal smoking during pregnancy, maternal education, sex, and birth year. The estimates were combined using Rubin's rules. Arsenic (As); cadmium (Cd); cesium (Cs); cobalt (Co); copper (Cu); lead (Pb); magnesium (Mg); manganese (Mn); mercury (Hg); selenium (Se); zinc (Zn).

and externalizing classroom behaviour) have been reported with elevated Mn exposure (92–94). The present study adds to the findings that increased Mn exposure is associated with adverse neurological effects in children. Contrary to some studies [i.e., (90)], the association in the present study was linear (positive), and not U- or inverted U-shaped. The maternal Mn concentrations in the present study (arithmetic mean = 11.5 μg/L, median = 9.8 μg/L, range: 1.9–163 μg/L) was similar or lower than reported in other studies general female United States population [median = 9.7 μg/L; Oulhote et al. (95)]; pregnant women during first trimester in the United States [median = 9.0 μg/L; Ashley-Martin et al. (96)]; and in mid-to-late-term pregnant Japanese women [median = 16.1 μg/L; Nakayama et al. (97)], and was within what is considered the normal range of 4 to 15 μg/L (87) [but higher in pregnant women (98)]. Thus, the present study indicates that elevated maternal levels of Mn in the second trimester can be associated with risk for CP, even at variation within normal concentration ranges.

The effect size of Cu and Mn found in the present study (OR for an increase in interquartile range = 1.40 and 1.20) is comparable to effect sizes of other reported risk factors for CP, such as low and high maternal age, low SES, and maternal hypertension during pregnancy (8).

Increased prenatal Hg exposure was associated with lowered odds of CP in the child. Much of the previous research indicates harmful effects of Hg on various human health outcomes, including adverse neurodevelopmental outcomes impaired motor function in children (25, 99). For example, cohort studies in the Faroe and Seychelles Islands found that increased prenatal MeHg exposure was related to lower scores on tests of motor function, coordination, fine motor skills, and motor speed in children (100–102). One of the most well-known examples is the MeHg poisoning in Minamata Bay in the 1950s (103). Hundreds of people died, after ingestion of MeHg-contaminated fish and shellfish from the Minamata Bay. Many people, especially children that were exposed to MeHg *in utero,* also displayed various adverse neurological effects, some similar to symptoms of CP. The Hg concentrations in Minamata were, however, much higher (i.e., hair concentrations of total Hg measured in 1960 was 15 times higher in Minamata than in the Kumamoto, a city located on the same island) than in the present study (104).

The inverse association between prenatal Hg exposure and odds of CP in the present study was unexcepted. This relationship also appeared to be driven by children of less educated mothers. Several studies report higher seafood intake in the higher SES strata, resulting in an elevated Hg prenatal exposure compared to the lower SES strata (105–107) since seafood is an important source of Hg (20). In a study of pregnant women from MoBa, Hg concentrations in blood were positively associated with total fish and seafood intake (45). Among the well-educated in the present study, the mothers of controls and CP cases ate similar amounts of seafood and Hg levels were approximately similar (Supplementary material 10). Among the less-educated, however, mothers of children with CP reported eating less seafood than mothers of controls, and they also appeared to have lower Hg blood concentrations than the mothers of controls (Supplementary material 10). In this study the analyses were adjusted for estimated maternal seafood intake. Even though it is possible that our results were biased by residual or unmeasured confounding, it could be speculated that if the Hg concentrations in blood is an even better marker of seafood intake than maternal, self-reported FFQ-based estimates of fish and seafood intake. If so, the seemingly lowered CP risk associated with increasing maternal Hg concentrations could in fact reflect increased intake of seafood and its beneficial nutrients for brain development (e.g., polyunsaturated fatty acids and iodine) (108).

The toxic metal mixture (MixTox) was inversely associated with risk of CP. This was mainly due to the inverse association between Hg and CP, reflected in the large negative weight for Hg (Supplementary material 13). There were no association between the essential element mixture (MixEssential) and CP or the total mixture and CP. Supplementary material 13 shows that the metal/element weights tend to go in opposite directions. In addition, some of the two-way interactions show that the effect of one metal or element is attenuated for certain concentration ranges of another metal or element (i.e., Cu and Pb). Nevertheless, the total mixture (MixAll) might have a stronger impact in other populations exposed to higher levels of toxic metals and/or with inadequate intake of essential

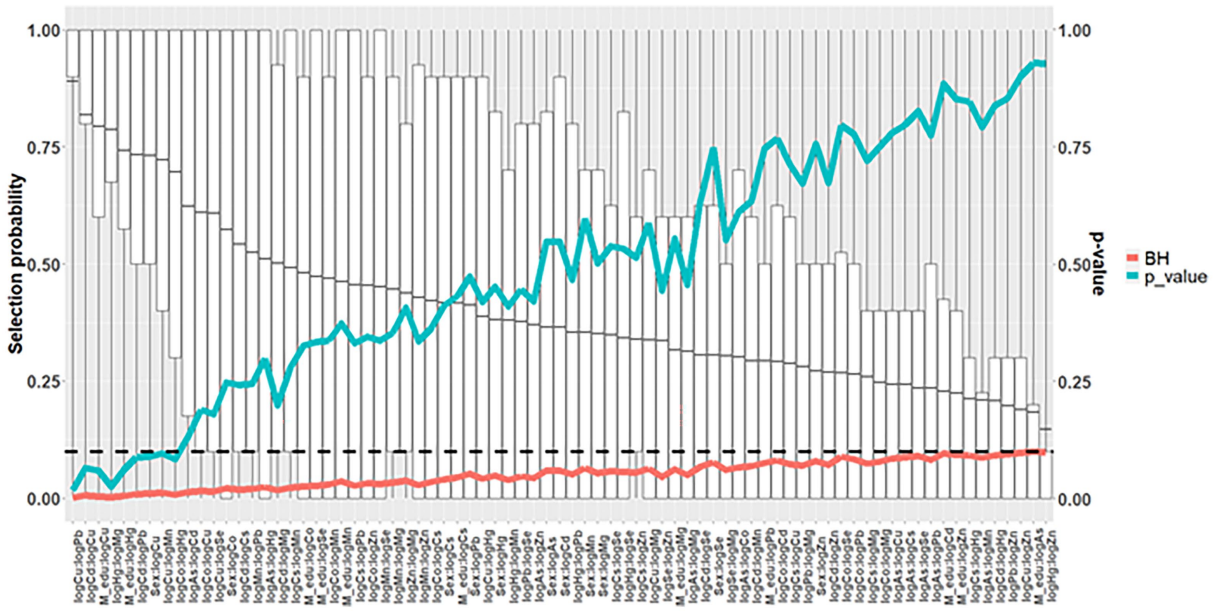

FIGURE 5
Mean selection probability (boxplot) for two-way interaction terms in a nested case-control study of cerebral palsy in the Norwegian Mother, Father, and Child Cohort Study (MoBa), 2002–2006. *N* = 1,226. Based on elastic net regression in 4000 datasets (20 multiple imputed datasets in each of 200 randomly drawn datasets with replacement; alpha = 0.9), and calculated *p*-values [based on 1,000,000 elastic net runs (once in each of 5 multiple imputed datasets in each of 20 randomly sampled datasets with replacements in each of 10,000 permuted datasets)], and Benjamini and Hochberg false discovery rate thresholds. All analyses adjusted for maternal age, parity, maternal smoking during pregnancy, maternal education, sex, and birth year. Arsenic (As); Benjamini and Hochberg false discovery rate thresholds (BH); cadmium (Cd); cesium (Cs); cobalt (Co); copper (Cu); lead (Pb); magnesium (Mg); manganese (Mn); mercury (Hg); selenium (Se); zinc (Zn).

elements or other micronutrients (e.g., folate), as they could have antagonistic effects.

This study has several strengths. First, it is among the first and largest studies of the association between prenatal exposure to toxic metals and essential elements, and CP diagnoses in children. Since only a relatively small percentage of children are diagnosed with CP (109), large studies are needed to identify a sufficient number of cases. The MoBa cohort was well suited for this purpose. Second, the scientific literature has called for investigation of chemical mixtures, as was done in the present study, which represent a more relevant exposure-scenario than assessing one or very few toxicants (37). By additionally assessing multiple two-way interactions between metals/elements, a more nuanced picture of the associations studied could be given. Third, our study was nested within a well-characterized prospective birth cohort with extensive questionnaire data that enabled us to obtain a wide range of relevant information on covariates.

Our study has some limitations. One limitation concerns self-selection bias into MoBa, resulting in larger proportion of older mothers with high education and healthier lifestyle than the general population (110). A second limitation is the lack of information on iron status. Iron is a main determinant for absorption of Mn and Cd in the body (79). Unfortunately, good measures of maternal iron levels were lacking in the present study so there is uncertainty regarding how well the levels of these metals reflect environmental exposure. Third, despite being one of the largest studies of its kind to date, the statistical power enabling identification of small to medium effect sized associations with intrauterine metal/element levels is probably restricted by the relatively low number of CP cases. Thus, future studies should strive to increase the case sample size.

5. Conclusion

When investigating the associations between gestational levels of 11 toxic metals and essential elements, within normal population ranges, Cu, Hg, and Mn were found to be associated with CP in children. Higher maternal levels of the essential elements Cu and Mn were associated with increased risk of CP in the child. While the total mixture effect was not found to be significant, counterintuitively, an inverse association between maternal Hg levels and risk of CP was also observed, and this association was mainly found in the lower SES strata. However, the inverse association reported herein should not be interpreted as a protective effect of Hg, but rather that Hg could be acting as a marker of seafood intake and nutrients that are beneficial for brain development. Disentangling adverse neurodevelopment of Hg or other contaminants originating from seafood intake and SES remains a great challenge within environmental epidemiology. The etiology of CP is a complex and multifactorial disease. Considerable research effort remains to elucidate the role of toxicants and micronutrients and their interactions during perinatal development in the etiology of CP in children, including increased attention to Cu and Mn. Consortium studies would be preferred, in order to produce larger case groups.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: data from the Norwegian Mother, Father and Child Cohort Study (MoBa) and the Medical Birth Registry of Norway

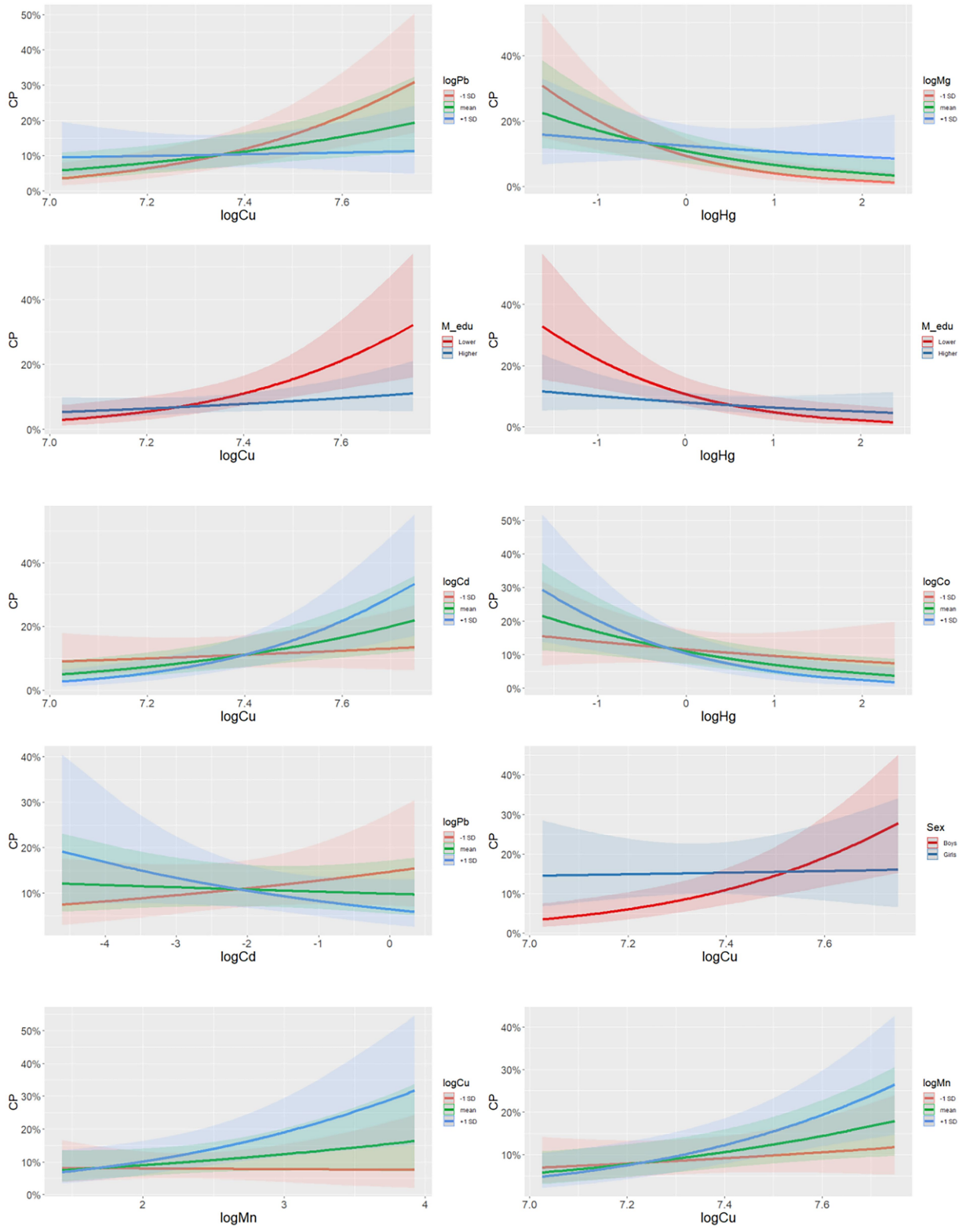

FIGURE 6
Graphs of selected two-way interaction terms in a nested case-control study of cerebral palsy in the Norwegian Mother, Father, and Child Cohort Study (MoBa), 2002–2006. *N* = 1,226. Based on a single imputed dataset. All analyses adjusted for maternal age, parity, maternal smoking during pregnancy, maternal education, sex, and birth year. *N* = 1,226. Arsenic (As); cadmium (Cd); cerebral palsy (CP); cesium (Cs); cobalt (Co); copper (Cu); lead (Pb); magnesium (Mg); manganese (Mn); mercury (Hg); selenium (Se); zinc (Zn).

(MBRN) used in this study are managed by Norwegian Institute of Public Health and can be made available to researchers, provided approval from the Regional Committees for Medical and Health Research Ethics (REC), compliance with the EU General Data Protection Regulation (GDPR) and approval from the data owners. The consent given by the participants does not allow for storage of data on an individual level in repositories or journals. Researchers who want access to data sets for replication should apply through helsedata.no. Access to data sets requires approval from the Regional Committees for Medical and Health Research Ethics in Norway and an agreement with MoBa. Requests to access these datasets should be directed to helsedata.no, service@helsedata.no.

Ethics statement

The studies involving human participants were reviewed and approved by The Regional Committees for Medical and Health Research Ethics. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

KW: writing—original draft, formal analysis, and methodology. AW: writing—second draft, review, formal analysis, and editing. PS, GA, TV, HK, CT, HM, and TS: writing—review and editing. GB and SE: writing—review and editing and methodology. HA: writing—review and editing and PI NeuroTox. GV: conceptualization, supervision, writing—review and editing, and methodology. All authors contributed to the article and approved the submitted version.

Funding

This study was funded by the Research Council of Norway's "NeuroTox" project (grant no. 267984/E50). The research is also conducted as part of the project Center for Global Health Inequalities Research (CHAIN) at the Norwegian University for Science and Technology (NTNU) financed by the Research Council of Norway (project no. 288638). The Norwegian Mother, Father, and Child Cohort Study (MoBa) is supported by the Norwegian Ministry of Health and Care Services and the Ministry of Education and Research, NIH/NINDS (grant nos. 1 UO1 NS 047537-01 and 2 UO1 NS 047537-06A1).

Acknowledgments

The authors are grateful to all the participating families in Norway who take part in this ongoing cohort study.

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/fneur.2023.1124943/full#supplementary-material

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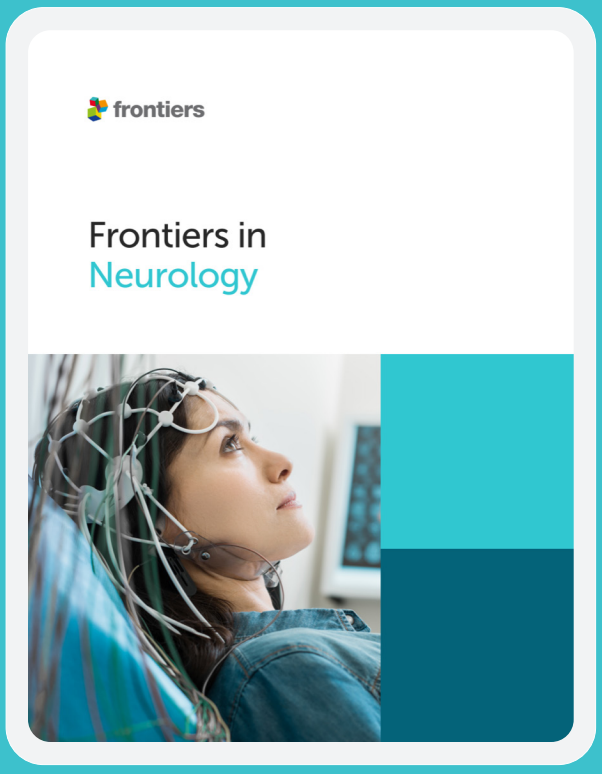

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