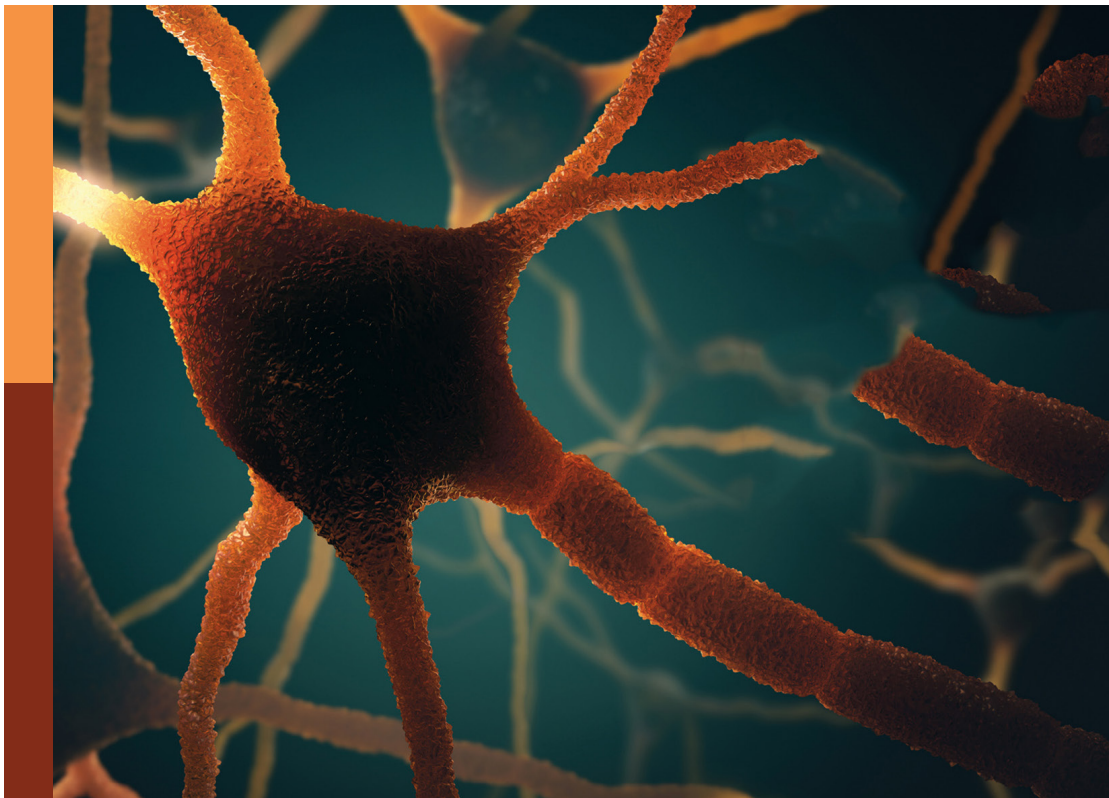
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Bioactive compounds, lifestyle factors and neurodegenerative diseases

Edited by
Binosha Fernando, Stephanie R. Rainey-Smith and Tejal Shah

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Bioactive compounds, lifestyle factors and neurodegenerative diseases

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frontiers in Aging Neuroscience

REVIEW
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Multiple Roles in Neuroprotection for the Exercise Derived Myokine Irisin

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Exercise has multiple beneficial effects on health including decreasing the risk of neurodegenerative diseases. Such effects are thought to be mediated (at least in part) by myokines, a collection of cytokines and other small proteins released from skeletal muscles. As an endocrine organ, skeletal muscle synthesizes and secretes a wide range of myokines which contribute to different functions in different organs, including the brain. One such myokine is the recently discovered protein Irisin, which is secreted into circulation from skeletal muscle during exercise from its membrane bound precursor Fibronectin type III domain-containing protein 5 (FNDC5). Irisin contributes to metabolic processes such as glucose homeostasis and browning of white adipose tissue. Irisin also crosses the blood brain barrier and initiates a neuroprotective genetic program in the hippocampus that culminates with increased expression of brain derived neurotrophic factor (BDNF). Furthermore, exercise and FNDC5/Irisin have been shown to have several neuroprotective effects against injuries in ischemia and neurodegenerative disease models, including Alzheimer's disease. In addition, Irisin has anxiolytic and antidepressant effects. In this review we present and summarize recent findings on the multiple effects of Irisin on neural function, including signaling pathways and mechanisms involved. We also discuss how exercise can positively influence brain function and mental health via the "skeletal muscle-brain axis." While there are still many unanswered questions, we put forward the idea that Irisin is a potentially essential mediator of the skeletal muscle-brain crosstalk.

Keywords: neuroprotection, myokine, exercise, skeletal muscle, brain

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INTRODUCTION

Over millions of years humans have evolved and developed the ability to run on two legs. Massive skeletal muscle development was paralleled with crucial changes in the brain (Noakes and Spedding, 2012), to facilitate the complex cognitive processing needed for running (Mendoza and Merchant, 2014). Thus, running did not only help skeletal muscle development but also promoted maturation of the brain (Schulkin, 2016). Evolutionary findings indicate a powerful crosstalk between muscle and brain. Several studies have provided evidence that exercise has beneficial effects on cognition and mental health in humans and also rodents models (Pietropaolo et al., 2008; Fuss et al., 2010; Mattson, 2012; Deslandes, 2014), including reducing the risk of developing neurodegenerative disorders associated with aging (Ahlskog et al., 2011). Exercise also improves several basic physiological functions such as appetite and sleep (Kline, 2014; Thackray et al., 2016).

Skeletal muscle tissue adapts to external stimuli, it has a high energy demand and actively controls metabolic homeostasis (Carbone et al., 2012). Skeletal muscle cells can also function as secretory organs in response to different stimuli such as exercise and cold exposure (Cannon and Kluger, 1983; Sepa-Kishi et al., 2017). Myokines are a group of cytokines and other small proteins that are synthesized and secreted by skeletal muscle cells upon muscular contraction. Myokines participate in building communications channels between skeletal muscle and other tissues (see reviews Trayhurn et al., 2011; Pedersen and Hojman, 2012). While the expression and secretion of myokines are mostly induced by muscle contraction, baseline expression of myokines in skeletal muscle conducts differentiation, proliferation, and regeneration of muscle cells (Clow and Jasmin, 2010; Broholm et al., 2011; Petersson et al., 2013).

Fibronectin type III domain-containing protein 5 (FNDC5) is a transmembrane protein that was discovered in 2002 (Ferrer-Martinez et al., 2002; Teufel et al., 2002). Initially, the main functions of FNDC5 were determined to be myocyte differentiation and development (Ferrer-Martinez et al., 2002). Then in 2012, it was discovered that FNDC5 is cleaved by enzymatic action, and a segment of the protein becomes a secreted peptide named Irisin (Bostrom et al., 2012). Interestingly, Irisin secretion is potently induced by exercise (Bostrom et al., 2012; Wrann et al., 2013). Furthermore, recent findings have shown that Irisin influences expression of different neuronal genes that mediate neuronal plasticity (Wrann et al., 2013; Forouzanfar et al., 2015), and that can potentially counteract neurodegeneration (Lourenco et al., 2019).

In this review we summarize recent finding on different signaling pathways and neural processes that are affected by the myokine FNDC5/Irisin in the brain. We specifically focus on Irisin as an important mediator of the communication between skeletal muscle and the brain. We also discuss the clinical significance of exercise and its impact on neurological disorders and mental health.

IMPACT OF EXERCISE ON SKELETAL MUSCLE

The Skeletal muscle has been shown to change in structure/function in response to exercise (Hoppeler, 1986; Matsakas et al., 2012). As a heterogenous tissue, skeletal muscle is composed of different fibers. Based on metabolic activity, muscle fiber types include oxidative (slow twitch, type I), oxidative-glycolytic (fast twitch, type IIa) and glycolytic (fast twitch, type IIX/IIb) in rodents and humans (Bottinelli et al., 1994; Schiaffino and Reggiani, 1994). Type I fibers have abundant mitochondria, exhibit high aerobic metabolism and oxidative enzymes activity. Type IIX/IIb fibers depend on glycolysis and anaerobic metabolism (Pette, 1985). As an intermediate, type IIa fibers use both aerobic and anaerobic metabolism for generating energy (Bourdeau Julien et al., 2018). The type of activity and exercise induce transition of fibers from fast-to-slow or slow-to-fast (Pette and Staron, 2000).

Additionally, skeletal muscles can act as secretory organs (Pedersen and Hojman, 2012; Egan and Zierath, 2013). As such, the skeletal muscle synthesizes and secretes cytokines and other peptides collectively named "myokines" (Pedersen and Hojman, 2012). Myokines employ autocrine, paracrine, and/or endocrine strategies to mediate different functions at cellular level on skeletal muscles and other organs (Pedersen and Hojman, 2012; Huh, 2018). For example, myokines control differentiation, growth, and regeneration of muscle fibers through autocrine mechanism (McPherron et al., 1997; Burks and Cohn, 2011; Petersson et al., 2013). Further, evidence shows that endocrine mechanisms of action mediate the functional association between skeletal muscle and other organs, including the brain. From adaptation to physiological changes to protecting cellular function depend on myokines acting in an endocrine fashion (Mohr et al., 1997; Marasco et al., 2018). Different factors such as exercise, pathological conditions and hormonal level stimulate myokine synthesis and secretion (Steensberg et al., 2000; Ciaraldi et al., 2016; Roberts et al., 2017). Exercise (aerobic and resistance) stimulates synthesis and secretion of different myokines into the circulatory system (**Table 1**).

The Central nervous system (CNS) is strongly influenced by myokines (Qin et al., 2013; Briken et al., 2016). For example, skeletal muscle derived BDNF, IL-6, FGF-21 and Irisin can cross the blood-brain barrier (BBB) and directly affect the neural function and activity, influencing synaptic plasticity and protecting neurons against degeneration (Banks et al., 1994; Pan et al., 1998; Hsuchou et al., 2007; Ruan et al., 2019). Furthermore, Irisin is a recently discovered exercise-induced myokine that controls a wide range of cellular signaling in different organs. Irisin is cleaved from the transmembrane protein FNDC5 during aerobic and/or resistance exercise in rodents and humans (Kraemer et al., 2014; Kim et al., 2015; Zhao et al., 2017; Shirvani and Rahmati-Ahmadabad, 2019). In mice, 72% of Irisin is derived from skeletal muscle and 28% from adipose tissue (Bostrom et al., 2012). In the next section we will focus on muscle derived FNDC5/Irisin and how exercise regulates its expression. We will also review signaling pathways related to FNDC5/Irisin in skeletal muscle and CNS.

THE MYOKINE IRISIN

Structure

In 2002, two research groups introduced FNDC5 as a protein involved in myoblast differentiation, initially named Peroxisomal protein (PeP) (Ferrer-Martinez et al., 2002; Teufel et al., 2002). Early analyses indicated high expression of FNDC5 in skeletal muscle, heart, and brain (Ferrer-Martinez et al., 2002). FNDC5 is a type I membrane protein that has 209 and 212 amino acids in rodents and humans, respectively, with the main difference located in the extracellular N-terminal segment (**Figure 1A**). FNDC5 is composed of an N-terminal signal sequence, a fibronectin III (FNIII) domain, an unknown domain, a hydrophobic transmembrane domain, and a cytoplasmic C-terminal (Bostrom et al., 2012; Schumacher et al., 2013) (**Figure 1B**).

TABLE 1 | Myokines and type of exercise that induce their secretion in rodents and humans.

Myokine	Type of exercise	Species	References
Irisin	Aerobic/Resistance	Rodents/Human	Wrann et al., 2013; Huh et al., 2015; Tsuchiya et al., 2015; Tine Kartinah et al., 2018
Fibroblast growth factor-21 (FGF21)	Aerobic/Resistance	Rodents/Human	Kim et al., 2013; Kim and Song, 2017
Vascular endothelial growth factor (VEGF)	Aerobic/Resistance	Rodents/Human	Lloyd et al., 2003; Gavin et al., 2007; Delavar et al., 2014
Growth differentiation factor 15 (GDF-15)	Aerobic	Human	Kleinert et al., 2018
Brain derived neurotrophic factor (BDNF)	Aerobic/Resistance	Rodents/Human	Jimenez-Maldonado et al., 2014; Church et al., 2016; Kim et al., 2020; Nilsson et al., 2020
Decorin	Aerobic/Resistance	Rodents/Human	Kanzleiter et al., 2014; Marqueti et al., 2018
Leukemia inhibitory factor (LIF)	Aerobic/Resistance	Rodents/Human	Broholm and Pedersen, 2010
Interleukin-6 (IL-6)	Aerobic/Resistance	Rodents/Human	Trenerry et al., 2011; Chowdhury et al., 2020
Interleukin-15 (IL-15)	Aerobic/Resistance	Rodents/Human	Quinn and Anderson, 2011
Meteorin-like (Metrnl)	Aerobic	Rodents/Human	Rao et al., 2014; Bae, 2018
Myonectin	Aerobic	Rodents/Human	Otaka et al., 2018; Pourranjbar et al., 2018

The N-terminal signal peptide is an endoplasmic reticulum (ER) transport signal needed for FNDC5 maturation and cleavage (Nie and Liu, 2017).

Different stimuli such as exercise and cold exposure induce FNDC5 cleavage at the ectodomain portion (Bostrom et al., 2012; Lee et al., 2014). The cleaved part produces a soluble segment named Irisin that consists of 112 amino acids. Protein sequence analysis of FNDC5 shows the cleavage site at position 28–29 in rodents (**Figure 1C**). However, in humans, the cleavage site is predicted in positions 31–32 (**Figure 1C**). Further proteolytic cleavage is performed between positions 140–141 (Nie and Liu, 2017). Therefore, the secreted part Irisin includes the N-terminal, FNIII domain and C-terminal tail (residues 29–140). Irisin molecular weight is ∼12 kD however analysis by X-ray crystallography showed dimerization of Irisin through FNIII domain (Schumacher et al., 2013). Other studies have shown a range of molecular weight from ∼12 to ∼35 kD (Bostrom et al., 2012; Nie and Liu, 2017). This difference may represent dimerization and post-translational modifications such as *N*-glycosylation, which can alter the number and/or structure of glycans attached to the protein (Nie and Liu, 2017; Korta et al., 2019). For example, after complete deglycosylation, Irisin was detected at ∼12 kD in human plasma (Jedrychowski et al., 2015) or 15 kDa in mice (Zhang et al., 2014). Other studies however, showed that deglycosylation reduced Irisin molecular weight below 12 kD (Schumacher et al., 2013; Nie and Liu, 2017). The signal sequence has a crucial role in post-translation modification of FNDC5. Removing the signal peptide, N-terminal, or cleavage site has significant effects on FNDC5 glycosylation, process that influences the stability of the protein and secretion Irisin (Nie and Liu, 2017). *N*-glycosylation of Irisin may have an important role in browning of white adipose tissue (Zhang et al., 2014). Therefore, while many important details are still unclear, glycosylation of Irisin not only changes its molecular mass but also can influence its stability and activity.

Several differences between rodent and human FNDC5 were found using genomic and transcriptomic analyses. For example, human *fndc5* (gene) has an ATA as a start codon instead of ATG as in rodents (Raschke et al., 2013). Further, the human *fndc5* transcript (with ATA as a start codon) has been reported to translated into protein with low efficiency (Raschke et al., 2013). In genes with non-AUG start codon, hairpin loop formation in downstream of the start codon increases the efficiency of translation (Kozak, 1990). Recently, it was shown that there are several transcripts for human *fndc5* in skeletal muscle, due to non-canonical start codon (Albrecht et al., 2020). Further, the first ATG codon is downstream of the ATA codon and could be used as start codon to translate into the short version of FNDC5 (Raschke et al., 2013).

Using transcriptome profiling through RNA-sequencing (RNA-Seq), FNDC5 expression was analyzed in different tissues from both male and female juvenile mice (C57BL/6 strain), and humans (Functional Annotation of the Mammalian Genome/Genotype-Tissue Expression Project) (GTEx Consortium, 2015)[1]. Transcriptome analysis showed that in humans and mice the gene *fndc5*/*Fndc5* has its highest expression in skeletal muscle, heart, and several regions in the brain, including hippocampus, cortex, medulla oblongata and in particularly high level, the human cerebellum (**Figure 2**). This pattern raises interesting questions about the possible role of FNDC5/Irisin in modulating the essential cerebellar function of motor control and planning, though future research is needed to expand these findings. Furthermore, this differential expression of FNDC5/Irisin might be regulated through various signaling pathways in different tissues and possibly in an

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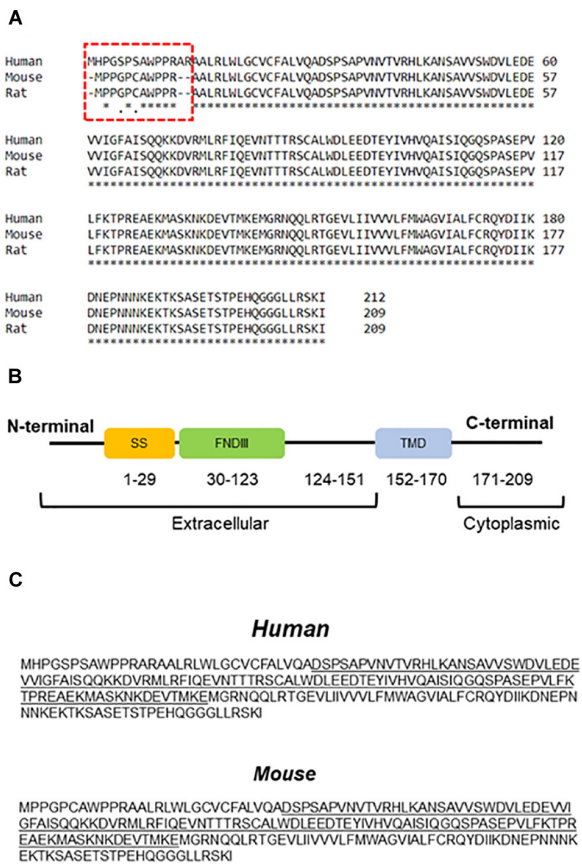


FIGURE 1 | Amino acid sequence and protein structure of FNDC5. **(A)** Comparison between FNDC5 amino acid sequences from different species. The highlighted region depicts N-terminus region of the protein. **(B)** Schematic structure of FNDC5. SS, signal sequence; FNDIII, fibronectin domain III; TMD, transmembrane domain. **(C)** FNDC5 and Irisin (underlined) amino acid sequence in human and mouse.

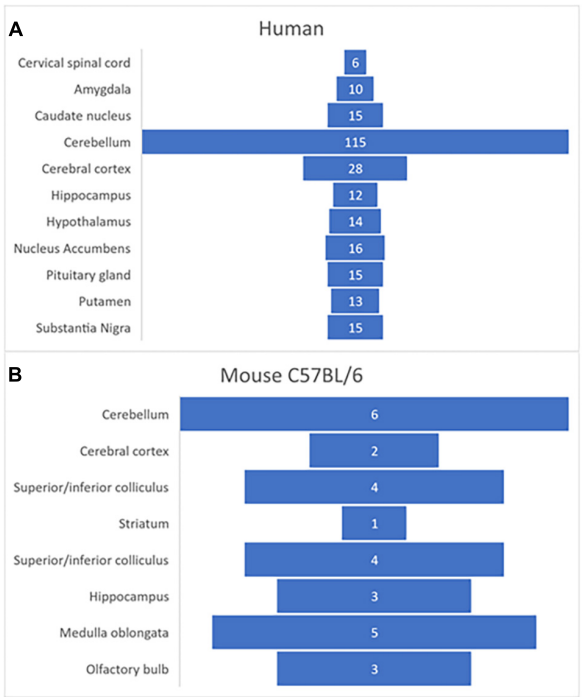


FIGURE 2 | Expression of *fndc5* gene in human **(A)** and C57BL/6 mouse **(B)** brain. Data was obtained from publicly available databases from GTEx Consortium (2015; Perez-Lopez et al., 2018). The numbers represent TPM (transcripts per million) units.

activity dependent manner. The following section discussed recent advances on the mechanisms involved in regulating FNDC5/Irisin expression.

Regulation of the Expression of FNDC5/Irisin

Expression in Skeletal Muscle

Both the expression of FNDC5 protein and secretion of Irisin are regulated by different external and internal stimuli, although basal expression of *Fndc5* (gene) is different in different types of muscle fibers. For example, FNDC5 has a higher expression in slow twitch fibers in comparison to fast type fibers, and after 3 weeks of aerobic exercise (i.e., running wheel), FNDC5/Irisin expression increased in both fiber types (Roca-Rivada et al., 2013). Further studies have shown that FNDC5 expression is indeed modulated by the type and duration of exercise (Bostrom et al., 2012; Ellefsen et al., 2014; Tiano et al., 2015).

While the skeletal muscle showed abundant expression of FNDC5 in both rodents and humans (Bostrom et al., 2012; Huh et al., 2012), other studies have shown that different tissues from the CNS to placenta express FNDC5 (Varela-Rodriguez et al., 2016). However, the secretion of Irisin is restricted to few tissues. Namely, the majority of Irisin is secreted into the peripheral blood from skeletal muscle during exercise (Bostrom et al., 2012), while another important source are the subcutaneous and visceral adipose tissues (Bostrom et al., 2012; Roca-Rivada et al., 2013). Therefore, Irisin is not only secreted as a myokine from skeletal muscles, but also it has the potential to be released into the peripheral blood as an adipokine.

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is the main regulator of FNDC5 in skeletal muscles in rodents and humans (Bostrom et al., 2012; Huh et al., 2014b). As co-activator, PGC-1α interacts with a wide range of transcription factors and it is expressed in high energy demand tissues such as skeletal muscle, heart, and brain (Lin et al., 2002; Buroker et al., 2008; Katsouri et al., 2016). PGC-1α is involved in different responses to distinct stimuli, controls mitochondrial biogenesis and glucose/fatty acid metabolism (Lehman et al., 2000; Finck and Kelly, 2006). In response to exercise, PGC-1α expression is increased in skeletal muscle which expedites mitochondrial biogenesis and switching of fast to slow twitch fibers (Lin et al., 2002; Koves et al., 2005).

Exercise induces the expression of PGC-1α in skeletal muscles, in rodents and humans (Little et al., 2010; Wrann et al., 2013). During exercise, muscle contraction needs Ca^{2+} influx to function properly. Rising Ca^{2+} influx in skeletal muscle

augments PGC-1α expression and activity (Baar et al., 2002). Therefore, the expression level of PGC-1α could be a marker for exercise and skeletal muscle contraction (Egan et al., 2010; Egan and Zierath, 2013; Brandt et al., 2017). Furthermore, increasing PGC-1α in skeletal muscle in response to exercise might be a strategy for balancing energy influx (Olesen et al., 2010).

PGC-1α binds and/or activates transcription factors that differentially induce FNDC5 expression (for review see Handschin and Spiegelman, 2006). For example, endurance exercise (voluntary running wheel), induces FNDC5 expression in skeletal muscles (quadriceps) through PGC-1α/estrogen-related receptor alpha (ERRα) pathway (Wrann et al., 2013). Further, it has been shown that cAMP response element-binding protein (CREB) act as a transcription factor that controls FNDC5 expression in C2C12 myotubes by binding to PGC-1α (Yang et al., 2018). Importantly, aerobic exercise can activate CREB in skeletal muscle (Popov et al., 2019). Activation of cAMP signaling during exercise in skeletal muscle activates CREB in response to metabolic adaptation (Berdeaux and Hutchins, 2019).

Treatment with retinoic acid (RA) increases FNDC5 expression in differentiated C2C12 myocytes (Amengual et al., 2018). RA is a natural ligand for retinoid X receptor (RXR). RXR is a ligand−activated transcription factor and binds to retinoic acid−responsive elements (RARE) in the regulatory sequences of genes dependent and independent of PGC-1α (le Maire et al., 2012). In C2C12 myocytes, induction of FNDC5 by RA is independent from PGC-1α (Amengual et al., 2018). Mouse *Fndc5* gene has an RXR binding site in the promoter region (Seifi et al., 2014). Further investigations are needed to uncover the regulatory role of RXR in inducing FNDC5 expression in skeletal muscles.

Different proteins and conditions can suppress the expression of PGC-1α/FNDC5 in skeletal muscle. For example, PGC-1α expression in skeletal muscle is reduced in humans and diabetic models in rodents (Jove et al., 2004; Mensink et al., 2007). Reduced expression of PGC-1α in diabetic models correlates with low Irisin level in serum, while FNDC5 expression in skeletal muscle does not change (Kurdiova et al., 2014). Similarly, the protein Mothers against decapentaplegic homolog 3 (SMAD3) can suppress PGC-1α and FNDC5 expression in C2C12 mouse myoblasts, while in *Smad3*$^{-/-}$ mice, aerobic exercise increased serum Irisin in comparison to wild-type mice (Tiano et al., 2015). Myostatin is another factor which mediates the expression of PGC-1α/FNDC5 in skeletal muscle (Ge et al., 2017). Neutralizing myostatin in skeletal muscle increases PGC-1α and FNDC5 expressions at mRNA level (Shan et al., 2013). Myostatin is a myokine that inhibits myoblast differentiation, thus showing opposite actions to FNDC5 (Rios et al., 2001). Fasting for 48 h reduced FNDC5 expression in skeletal muscle and reduced circulating Irisin in serum, while intraperitoneal (i.p.) injection of insulin for 14 days showed similar effects on FNDC5 and Irisin levels in skeletal muscle and plasma (Varela-Rodriguez et al., 2016).

Membrane receptors transduce the information from external environment to the nucleus. Some receptors are internalized into the cytoplasm through endocytosis mechanism after binding to their ligands. Lourenco et al. (2019) showed that FNDC5/Irisin bound to unknown receptors on the surface of hippocampal neurons and astrocytes in culture. This evidence suggests a putative mechanism of endocytosis initiated by FNDC5/Irisin binding to its membrane receptor in the CNS, however, this is still unclear and such receptor has not been characterized. Further, Irisin uptake into A549 cells (human lung carcinoma cell line) is blocked by nystatin, an inhibitor of endocytosis (Chen et al., 2017). At the cellular level, Irisin influences endocytosis and exocytosis mechanisms differently. For example, in isolated mouse pancreatic islet cells, Irisin increased insulin secretion (i.e., exocytosis) in response to glucose (Zhang et al., 2018). Moreover, recombinant Irisin (50 nM) induced the secretion of lactate from primary human adipocytes in culture and stimulated glucose uptake in skeletal muscle (Huh et al., 2014a). Similarly, subcutaneous injection of Irisin increased glucose uptake in the brain which suggests it increased the endocytosis of glucose transporters (Wang and Pan, 2016).

In summary, diverse physiological conditions and environmental stimuli can modulate PGC-1α/FNDC5 pathway in skeletal muscle cells.

Expression of FNDC5/Irisin in the Brain

FNDC5 is highly expressed in several regions of the brain of rodents and humans (**Figure 2**). Interestingly, in rodent brains Irisin was detected in especially high level in Purkinje cells of the cerebellum and vestibular nuclei of the medulla oblongata, together with other areas such as hippocampus and cortex (Dun et al., 2013). In primates Irisin is highly expressed in hypothalamic arcuate and ventromedial nuclei (Wahab et al., 2019). However, the roles of endogenous FNDC5/Irisin in the CNS remain to be fully characterized.

Similar to skeletal muscle, FNDC5 expression in the brain is modulated by different physiological conditions and environmental stimuli. For example, hippocampal FNDC5 is increased with aerobic and resistance exercise in rodents (Wrann et al., 2013; Nokia et al., 2016), while it is not known if exercise modulates FNDC5 expression in other brain regions. Interestingly, environmental enrichment (EE) increases FNDC5 expression in the prefrontal cortex (Yu K. W. et al., 2020). Studies have shown EE protected neurons against injuries, induced neurogenesis, and increased brain activity (Zhang et al., 2017; Wang C. J. et al., 2019). Pathological conditions also change FNDC5 expression in different regions of the brain. In Alzheimer's disease (AD) for example, FNDC5 expression is decreased not only in the hippocampus but also in prefrontal cortex (Lourenco et al., 2019). In individuals suffering from post-stroke depression, Irisin levels are low in the peripheral blood (Tu et al., 2018). In addition, Irisin administration into the lateral ventricle or hippocampus suppresses depression, acute stress-induced anxiety, and memory impairment (Siteneski et al., 2018; Jodeiri Farshbaf et al., 2020). Chronic treatment with insulin increases FNDC5 expression in the hypothalamus which is responsible for feeding behavior and energy homeostasis. More details of FNDC5/Irisin involvement in disease are in section "Role of FNDC5/Irisin in Neurological and Neuropsychiatric Disorders" of this review.

Details of how the expression of FNDC5/Irisin in the brain is regulated are emerging. It has been shown that PGC-1α controls FNDC5 expression in the hippocampus and primary neurons in culture (Wrann et al., 2013). In the hippocampus, this depends on the cAMP/PKA signaling pathway (Lourenco et al., 2019). Further, in the P19 cell line as well as differentiated C2C12 myocytes, RA treatment increased FNDC5 expression (Ostadsharif et al., 2011). Similarly, Lactate released from skeletal muscle during exercise induces FNDC5 expression in the hippocampus (El Hayek et al., 2019). Further analysis has shown a putative ERRα binding element (ERRE) located upstream to the *fndc5* promoter (Wrann et al., 2013). In primary cortical neurons, PGC-1α activated ERRα and increased FNDC5 expression, which then negatively fed back onto PGC-1α/ERRα (Wrann et al., 2013). Additional analysis of upstream regulatory sequences of the *fndc5* promoter could uncover putative binding elements that could be differentially regulated by activators, repressors, and transcription factors. This could elucidate different stimuli that could influence *fndc5* expression in different tissues and conditions.

Signaling Pathways Involved and Function

Skeletal Muscle

FNDC5 was initially introduced as a regulator of myoblast differentiation (Ferrer-Martinez et al., 2002). *Fndc5* knockdown in muscle stem cells decreased the expression of myogenic genes and myotube formation without any effects on muscle growth (Lee et al., 2019). Recently, it was shown that inhibiting *Fndc5* expression induces autophagy and causes skeletal muscle atrophy (Pan et al., 2019). Further, loss-of-function mutation in the *Fndc5* gene in skeletal muscles decreases maximal oxygen consumption during aerobic exercise (Xiong et al., 2019). In addition, the mutant *Fndc5* mice showed higher glucose level after aerobic exercise in skeletal muscles while the blood glucose level did not change during fasting (Xiong et al., 2019). *Fndc5* overexpression in hind-limb muscle increases glycogen content in muscle and Irisin level in peripheral blood (Farrash et al., 2020). Modulation of FNDC5 level in skeletal muscle correlates with secreted Irisin level in mice and C2C12 cells (Tiano et al., 2015; Chen et al., 2019; Rodriguez Lanzi et al., 2020). Human clinical studies have shown that single nucleotide polymorphism in *Fndc5* is related to insulin level and sensitivity (Staiger et al., 2013).

Circulating Irisin acts via autocrine mechanisms on skeletal muscle fibers. For instance, Irisin induced muscle hypertrophy through activation of protein kinase B (also known as Akt), mammalian target of rapamycin (mTOR) and extracellular signal-regulated kinases (ERK) (Reza et al., 2017). Irisin-dependent hypertrophy was controlled by promoting protein synthesis and preventing protein turnover in skeletal muscle fibers. Interestingly, resistance exercise induced muscle hypertrophy as well through activation of mTOR signaling in mice and humans (Ogasawara et al., 2016; Song et al., 2017). This effect of resistance exercise on muscle mass through mTOR signaling pathway might be mediated by Irisin but this needs further investigation. In a recent human study, Irisin seemed to be a marker for improved muscle strength (Planella-Farrugia et al., 2019).

Irisin has been shown to control metabolism and energy expenditure in muscle cells. For instance, treating C2C12 cells with recombinant Irisin (5 nM) increased metabolism in a time and dose dependent manner. In the short-term (1–4 h), Irisin increased glycolytic metabolism whereas in long-term (24 h), incubation with recombinant Irisin resulted in increased mitochondrial biogenesis and oxidative metabolism (Vaughan et al., 2014). Further, recombinant Irisin increased glucose and lipid uptake in primary human skeletal muscle cells. Specifically, Irisin increased glucose transporter-4 (GLUT4) and hexokinase (HK) expression which are responsible for glucose uptake and use (Vaughan et al., 2014). Glycogen phosphorylase (PYGM), rate-limiting enzyme of glycogenolysis, was decreased by Irisin (50 nM) treatment in human skeletal muscle (Huh et al., 2014b).

One of the main downstream targets for FNDC5 in skeletal muscle cells is BDNF (Wrann et al., 2013; Wrann, 2015). The main roles for BDNF are regulating muscle regeneration and adapting myotubes to different metabolic conditions (Omura et al., 2005). BDNF also increases mitochondrial content and oxidative phosphorylation in C2C12 myotubes (Yang et al., 2019). Based on this evidence, Irisin may be important for metabolic flexibility and adaptation in skeletal muscles.

Both aerobic and resistance exercise types induce metabolic adaptation in skeletal muscles. AMP-activated protein kinase (AMPK) is the main cellular sensor for energy depletion and has a crucial role in metabolic adaptation (Winder and Hardie, 1996). Using high amounts of ATP during contraction increases AMP/ATP ratio in muscle cells which triggers AMPK activation and induces glucose uptake through GLUT-4 (Wright et al., 2004). In differentiated L6 muscle cells, acute treatment with Irisin (20–200 ng) increases glucose uptake through activation of AMPK after 15 min and up to 180 min (Wright et al., 2004). Injecting recombinant Irisin (100 μg/kg i.p. for 28 days) increases FNDC5 expression in skeletal muscle (Colaianni et al., 2015). Interestingly, acute exercise does not change AMPK level and activity in skeletal muscle, in fact acute exercise modulates glucose uptake and metabolism independent from AMPK pathway in skeletal muscle fibers (Mu et al., 2001; McConell et al., 2005). Acute exercise increases plasma levels of Irisin (∼100 ng/ml) in men without any change in women (Loffler et al., 2015). Therefore, Irisin may have an important role in inducing AMPK signaling pathway in skeletal muscle. Further, chronic activation of AMPK triggers mitochondrial biogenesis in skeletal muscle cells (Winder et al., 2000). Indeed, AMPK activates PGC-1α through direct or indirect pathways (Canto et al., 2009). A recent study showed that PGC-1α protein level was not changed after acute exercise while mRNA level greatly increased in skeletal muscle (Safdar et al., 2018). Interestingly, PGC-1α expression of mRNA and protein increased 1–3 h of recovery after acute exercise in skeletal muscles (Peng et al., 2017; Safdar et al., 2018). Chronic exercise training for 28 days gradually increased PGC-1α expression in skeletal muscle (Park et al., 2020). This evidence suggests that AMPK is upstream of FNDC5 expression in skeletal muscle cells (Lally et al., 2015).

Therefore, AMPK may induce glucose uptake and glycolytic metabolism in skeletal muscle to restore ATP generation, and Irisin could be a mediator of metabolic adaptation in skeletal muscle during exercise. Interestingly, treatment for 24 h with Irisin in C2C12 cells increased oxygen consumption and ATP level (Vaughan et al., 2014). Therefore, it is possible to speculate that exercise and released Irisin impact glycolytic and oxidative metabolism during metabolic adaptation to maintain ATP levels. However, the signaling cascade including AMPK/PGC-1α/FNDC5 needs to be better understood in the context of skeletal muscle physiology.

Brain

Several studies have shown that the levels of FNDC5/Irisin can alter specific gene expression in neurons. For instance, the gene *Fndc5* can influence the differentiation of mouse embryonic stem cells to neural cells (Hashemi et al., 2013; Forouzanfar et al., 2015). Further, *Fndc5* overexpression induces the expression of *Bdnf*, Neuronal PAS Domain Protein 4 (*Npas4), cFos, and Arc* in primary cortical neurons (Wrann et al., 2013). Intriguingly, intracerebroventricular (i.c.v.) administration of Irisin (1 ng) in male mice increases BDNF mRNA level in the hippocampus but it decreases it in the prefrontal cortex (Siteneski et al., 2018).

An important question that remains unanswered is whether Irisin can control the expression of FNDC5 in the brain. A recent *in vivo* study showed that i.c.v. administration of Irisin leads to a short time decrease in FNDC5 expression in the prefrontal cortex and hippocampus after 1 h. However, the expression of FNDC5 was enhanced in the hippocampus without change in the prefrontal cortex 6 h after Irisin administration (Siteneski et al., 2018). The mechanisms of action for this change are not fully understood.

Additionally, Irisin can influence the function and activity of glial cells in different conditions. In pathological conditions, glial cells initiate the neuroinflammation responses to injuries through expression of cytokines such as IL-6 and tumor necrosis factor-α (TNF-α). Intravenous (I.V) injection of recombinant Irisin decreased the number of active microglia and TNF-α expression in the middle cerebral artery occlusion (MCAO) model (Li et al., 2017). Further, recombinant Irisin decreased TNF-α induced apoptosis in SH-SY5Y cells (Huang et al., 2020). Similarly, Irisin counteracted several changes induced in a model of streptozotocin-induced diabetes in mice. For instance, glial fibrillary acidic protein (GFAP), a marker for active astrocyte, was decreased in the hippocampus of Irisin-treated diabetic mice, change that was paralleled with a reduction in IL-6 level (Wang K. et al., 2019). Importantly, Irisin immunostaining has been observed in both neurons and glial cells (Aydin et al., 2014), including expression on the surface of astrocytes (Lourenco et al., 2019). Physiological concentration of Irisin (50–100 nM) increases proliferation of mouse H19-7 hippocampal cell lines through activating STAT3 signaling pathway (Moon et al., 2013). Further, in the focal cerebral ischemic stroke model, Irisin reduces the number of active microglia which protect neurons against inflammation (Li et al., 2017). This is important considering that neuroinflammation is one of the most important factors that accelerates brain injury in case of stroke (see review Jayaraj et al., 2019). In cultured astrocytes, Irisin increases ATP level and GLUT-4 expression (Wang and Pan, 2016).

Irisin was able to protect PC12 neuronal cells against cell death induced by oxygen/glucose deprivation (Li et al., 2017). Similarly, in an oxygen/glucose deprivation *in vitro* model, Irisin treatment protected neurons against apoptosis through an inflammatory signaling pathway (Peng et al., 2017). Furthermore, FNDC5 can bind to the N-terminus region of the amyloid precursor protein (APP) (Noda et al., 2018). APP is cleaved by β/γ-secretases and produces amyloid beta (Aβ) deposits which are one of the hallmark pathological signs of AD (Chow et al., 2010). Co-transfection of FNDC5 and APP into human embryonic kidney 293 (HEK293) cells decreased Aβ production (Noda et al., 2018). Therefore, this evidence suggests that FNDC5/Irisin may contribute to reduce APP cleavage and consequent Aβ secretion. More details of the involvement of FNDC5/Irisin in mechanism of neurodegenerative disorders are discussed in Section "Role of FNDC5/Irisin in Neurological and Neuropsychiatric Disorders" of this review.

Extensive research has shown that exercise increases memory and cognition (reviewed in Pedersen and Saltin, 2015; Vecchio et al., 2018). A recent report has shown that Irisin injected directly into the dentate gyrus (DG) in the hippocampus, increases long term potentiation (LTP) in male rats (Mohammadi et al., 2019). Moreover, blocking FNDC5/Irisin decreased the maximal LTP induced at Schaffer-CA1 synapses in mouse hippocampal slices (Lourenco et al., 2019). Inducing LTP correlates with synaptic plasticity and memory formation in the hippocampus, therefore, exercise may have beneficial effects on memory by altering mechanisms of synaptic plasticity, at least in the hippocampus. While these data are promising, the role of FNDC5/Irisin in mediating the beneficial effects of exercise on memory needs to be further investigated.

Different types of exercise induce the expression of FNDC5/Irisin in the CNS. Bostrom et al. (2012) showed that exercise (30 days of voluntary running or swimming for 14 days) can induce FNDC5 expression in skeletal muscles, and Irisin secretion into the peripheral blood in mice and human subjects. Further, FNDC5 expression also increased in the hippocampus of running mice, which results in increased BDNF expression (Wrann et al., 2013). These results confirmed previous studies showing that exercise increases BDNF level in the hippocampus (Neeper et al., 1995; Vaynman et al., 2004; Sleiman et al., 2016). This is relevant because hippocampal BDNF is associated with memory formation and cognition, and exercise and BDNF have crucial roles in inducing hippocampal neurogenesis, process that is also involved in memory function (reviewed in Spalding et al., 2013; Choi et al., 2018; Gonzalez et al., 2019). BDNF expression in the hippocampus is influenced by different factors that could mediate the connection between exercise and neurogenesis (Horowitz et al., 2020). For example, the PGC-1α/FNDC5/BDNF axis is activated in the hippocampus by voluntary running (Wrann et al., 2013). Moderate aerobic exercise increases PGC-1α/FNDC5/BDNF axis through activating AMPK signaling

pathway in the hippocampus (Azimi et al., 2018). Interestingly, it has been shown that muscle contraction through electrical stimulation under anesthesia induces FNDC5/BDNF expression in the hippocampus without any change in skeletal muscle (Azimi et al., 2018).

As Irisin, lactate is released from skeletal muscle during exercise and can cross the BBB potentially acting as a neuroprotector (Berthet et al., 2009; Newman et al., 2011). In addition, lactate could be used as energy source by neurons (Quistorff et al., 2008). Interestingly, i.p. injection of lactate induces FNDC5/BDNF expression in the hippocampus (El Hayek et al., 2019). Similarly, Irisin i.p. injection (0.5 μg/g of body weight), used to imitate exercise, increases BDNF expression in the brain (Natalicchio et al., 2020). Therefore, this evidence indicates that Irisin (acting on unknown receptors in the brain) triggers BDNF expression and release, which could lead to memory and learning improvement. Furthermore, beside improving learning and memory, BDNF is also important for the brain to adapt metabolic challenges. Treatment with BDNF (10–40 ng/ml) increases mitochondrial biogenesis to maintain ATP level in hippocampal neurons in culture, by increasing the expression of PGC-1α (Cheng et al., 2012). This was also shown after chronic aerobic exercise in mice (8 weeks with a running wheel), which increased mitochondrial biogenesis through increasing PGC-1α in different regions of the brain (Steiner et al., 2011). In addition, BDNF induces glucose transportation in cortical neurons (Burkhalter et al., 2003), and exercise-induced hippocampal neurogenesis depends on BDNF controlled neuronal bioenergetic (reviewed in Mattson, 2012). One of the possibly involved signaling pathways, which has crucial role in memory and cognition, is the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway (Shu et al., 2013). Curiously, treadmill exercise activates PI3K/Akt signaling pathway in the hippocampus (Wang and Baek, 2018). A recent study showed that human recombinant Irisin activates Akt in the brain (Li et al., 2017). PI3K/Akt signaling pathway is also activated by BDNF in neurons (Chen et al., 2013). Activation of PI3K/Akt controls glucose homeostasis, mitochondrial biogenesis, and integrity in neurons (Pearson-Leary et al., 2018). In other tissues such as injured lung alveoli, myocardial cell, and chondrocytes Irisin controls mitochondrial integrity and function (Chen et al., 2017; Wang et al., 2018, 2020). Nevertheless, the direct impact of the FNDC5/Irisin on mitochondrial integrity and function in neurons and/or glial cells remains to be fully elucidated.

A recent interesting discovery about Irisin function is related to autophagy (reviewed extensively in Pesce et al., 2020). Autophagy is an intracellular process that occurs to eliminate misfolded proteins and injured organelles, and in general to maintain the cellular homeostasis (reviewed in Glick et al., 2010). Autophagy is also involved in the pathology of neurodegenerative disorders such as AD, amyotrophic lateral sclerosis (ALS), and familial Parkinson's disease (PD) (Nixon, 2013). Recent studies have shown that exercise stimulates autophagy in a variety of tissues, including skeletal muscles (Salminen and Vihko, 1984; Grumati et al., 2011), and several myokines have been recognized as autophagy regulators (Pesce et al., 2020). Irisin has been associated to autophagy induction in several cell types including myocytes, cardiomyocytes (Li et al., 2018), and hepatocytes (Bi et al., 2019, 2020). Further, Irisin increased autophagy in hepatic ischemia-reperfusion (IR) model and mitigated liver injury (Bi et al., 2020). Meanwhile, the knock down of FNDC5 results in autophagy and fatty acid oxidation impairment in mice, and enhancement of lipogenesis via the AMPK/mTOR pathway (Liu et al., 2016). However, future studies are needed to elucidate the potential role of FNDC5/Irisin in controlling autophagy in the brain. The implications for neurodegenerative disorders are extensive.

SEX DIMORPHISM IN EXERCISE AND FNDC5/IRISIN

Sexual dimorphism has been shown in a variety of physiological responses, pathological conditions and even in the effect of therapeutic agents. In neuroscience research, the role of sex hormones on neuronal activity and functions has not been studied in extensive detail. In addition to brain, skeletal muscles and myokines have also shown sex-dependent effects/functions in rodents and humans. In this section we review potential roles for estrogen, as one of the most prevalent circulating sex hormones, in modulating FNDC5/Irisin expression and function in skeletal muscle and CNS.

Sex Hormones and Skeletal Muscles

Steroid sex hormones include estrogens, androgens, and progestogens. Aside from their well-known fundamental roles in reproduction, they contribute to a variety of other physiological responses and exert their actions via autocrine, endocrine, and paracrine pathways.

Progesterone is initially made from cholesterol during the steroid sex-hormone biosynthesis pathway. Progesterone is then converted to testosterone and androstenedione which is ultimately converted into estrogens and estrone (reviewed in Schiffer et al., 2019). 17β-estradiol (E2) is the most abundant estrogen in females and its level in peripheral blood change during the human menstrual cycle and rodent estrous cycles (Nilsson et al., 2015). Most of the effects of E2 are mediated by estrogen receptor (ER) α and β, both members of the nuclear receptor family and located intracellularly (reviewed in Hewitt and Korach, 2018). A second family of ERs has been found at the cell membrane (mER). These mERs signal via modulation of intracellular signaling pathways (Soltysik and Czekaj, 2013). Additionally, based on their sequence similarity to ERα, the orphan nuclear receptors called estrogen-related receptors α and β (ERRα and ERRβ) were first identified. Then a third type was found (ERRγ) and together these three receptors form the ERR subfamily of the steroid nuclear receptor superfamily (Giguere et al., 1988; Zhang and Teng, 2007). Activation of ERα induces the expression of ERRα (Hu et al., 2008). The ERRs contain DNA-binding domains that target the receptor to a specific DNA sequence (TCAAGGTCA) called the estrogen-related response element (ERRE) (Vanacker et al., 1999). Interestingly, ERR

deficiency in skeletal muscle impairs regeneration after injury, while treatment with E2 stimulate its regeneration and growth (Velders et al., 2012; LaBarge et al., 2014).

Studies have shown ERRα controls metabolic homeostasis through regulating mitochondrial biogenesis, oxidative phosphorylation, and Krebs cycle (Huss et al., 2004; Dufour et al., 2011). ERRα is activated by binding to PGC-1α as a co-activator (Schreiber et al., 2004). *Fndc5* itself has an ERRE at 6 kb upstream of its promoter (Wrann et al., 2013). Therefore, it is possible to infer that ERRα activation or induction could influence FNDC5/Irisin levels. Indeed, studies have shown that the serum Irisin concentration is positively correlated with E2 levels in humans (Huh et al., 2012). Similarly, Irisin level in serum is lower in amenorrheic women in comparison to eumenorrheic young women (Singhal et al., 2014). In amenorrheic women E2 level is low because of less endogenous estrogen production). Furthermore, E2 is decreased during aging which correlates with Irisin decline and muscle mass loss (Brown, 2008; Huh et al., 2012). E2 may thus activate ERα/ERRα axis which could increase FNDC5 expression in skeletal muscle and Irisin level in serum. However, ovariectomy (OVX), which is the surgical removal of one or both ovaries, results in increased FNDC5 expression in skeletal muscle and Irisin level in serum (18 weeks after surgery) without affecting PGC-1α expression (Zugel et al., 2016). While these findings are unexpected based on previous human studies showing E2 and Irisin correlation, this discrepancy may be related to OVX-induced obesity (Asarian and Geary, 2006). In fact, circulating level of Irisin is higher in obese women (Zugel et al., 2016). Further, Irisin could also be secreted from adipose tissue in addition to skeletal muscle (Roca-Rivada et al., 2013).

E2 levels can also affect the main regulators of FNDC5 expression in skeletal muscle, namely AMPK and PGC-1α. For example, treatment with E2 increases AMPK phosphorylation in skeletal muscle and C2C12 cells (D'Eon et al., 2008; Rogers et al., 2009). Also, PGC-1α expression in skeletal muscle is decreased 9 weeks after OVX but recovered with E2 treatment (Capllonch-Amer et al., 2014). In human studies AMPK and PGC-1α expression in skeletal muscle changes over time after menopause. Studying samples from post menopause subjects showed that E2 treatment induces AMPK/PGC-1α axis in skeletal muscle only in early post menopause cases (less than 6 years post-menopause) (Park et al., 2017). Also, E2 treatment increases PGC-1α expression in skeletal muscle in men (Maher et al., 2010).

Skeletal muscle BDNF production has been shown to respond to metabolic adaptation in a sex-dependent manner (Yang et al., 2019). Further, plasma levels of BDNF and E2 have been shown to be positively correlated (Pluchino et al., 2009). Interestingly, a few exercise studies in rodents and humans have shown sex and gender-dependent differences. For example, aerobic exercise for 6 months does not result in increased Irisin level in young women with lower E2 level or amenorrheic subjects, in comparison to young women with normal menstrual period (Singhal et al., 2014). Furthermore, OVX mice subjected to exercise showed decreased endurance without changes in skeletal muscle mass, while mimicking estrous cycle with E2 injections recovered their deficiency for endurance exercise (Nagai et al., 2016). Based on this evidence, we could speculate that Irisin secretion/function may crosstalk with estradiol and/or ERs actions in female individuals. Our own data has shown interesting results when probing the anti-stress properties of irisin in male/female mice. We recently showed that Irisin successfully prevented the acute stress induced memory impairment observed in male mice only, with no protective effects in female mice (Jodeiri Farshbaf et al., 2020).

Sex Hormones and FNDC5/Irisin in the Brain

Strong evidence of sex dimorphism in the CNS has been shown at different levels, ranging from molecular, circuit-base and behavior. To date, morphological differences, cognitive functions and different signaling pathways involved, they have all been shown to be influenced by sex. As discussed earlier, there have been a few previous reports showing a possible sex-specific relation between FNDC5/Irisin and related signaling pathways, and the effect of exercise. For instance, Wrann et al. (2013) showed that ERRα can modulate PGC-1α and FNDC5 expression in the hippocampus of male mice. Thus, treating primary cortical neurons with an ERRα inhibitor (XCT-790), decreased *fndc5* expression (Wrann et al., 2013). Remarkably, other studies have shown that PGC-1α can have sex-dependent effects. For example, nigral dopaminergic neurons of PGC-1α null mice display robust ultrastructural alterations in intracellular organelles such as ER and mitochondria, changes that result in increased vulnerability to α-synuclein specifically in male mice (Ciron et al., 2015). Also, PGC-1α deficiency leads to earlier onset and death in male animal models of ALS and male human subjects (Eschbach et al., 2013). Further, activation of the AMPK pathway protects memory function only in females in a mouse model of AD, while increasing dysfunction in males (DiTacchio et al., 2015).

As mentioned, PGC-1α binds to ERRα which is influenced by E2. OVX females show a reduced expression of PGC-1α in the brain (Zawada et al., 2015). In addition, mitochondrial function and dynamic are also affected in OVX females, effect that is reversed with E2 treatment (Yao et al., 2012). Treatment with E2 also increases PGC-1α expression and ameliorates mitochondrial dysfunction in Leber's hereditary optic neuropathy (Giordano et al., 2011). Our studies have also shown that Irisin differentially rescues short-term memory in male versus female mice (Jodeiri Farshbaf et al., 2020). Different studies have shown that BDNF expression decreases in the brain after OVX, and E2 treatment leads to its recovery (Singh et al., 1995; Fortress et al., 2014). Other reports have corroborated these findings by showing that the protein level of BDNF is not increased in OVX female rats subjected to voluntary running wheel exercise for ∼2 weeks (Rashidy-Pour et al., 2019). This is relevant considering that after menopause, the risk of neurological and psychological disorders is increased in women (Brinton, 2008). Diverse studies have shown that E2 can have neuroprotective properties (Bonnefont et al., 1998; Platania et al., 2005). Further, astrocytes express α and β estrogen receptors (Kuo et al., 2010) and play a critical role in E2-induced neuroprotection (Spence et al., 2011). Several studies have shown that E2 induced the expression of various genes critical for neuroprotection such as nerve growth factor

(NGF), BDNF and glial cell line derived neurotrophic factor (GDNF) (Platania et al., 2005; Xu et al., 2013). In addition to genomic changes, non-genomic pathways such as ERK and Akt mediate neuroprotection in response to E2 treatment (Nicole et al., 2001; Dhandapani et al., 2005). Therefore, E2 can mediate neuroprotection not only through neuronal pathways but also by mediating genomic and non-genomic mechanisms of action in astrocytes. An important question remains unanswered, does Irisin require glial cells to exert its putative neuroprotective functions?

Abruption of peripheral estrogens level and E2-dependent signaling pathways in the brain may play central role in increasing susceptibility of post-menopause women to neurological diseases. Because FNDC5/Irisin is one of the factors influenced by E2 levels, this suggests that FNDC5/Irisin might have an important role in protecting neurons against aging related CNS issues.

ROLE OF FNDC5/IRISIN IN NEUROLOGICAL AND NEUROPSYCHIATRIC DISORDERS

Different studies have shown that exercise can have neuroprotective effects in conditions of neurodegenerative diseases (reviewed in Ahlskog et al., 2011). Further, in mental health disorders exercise can also be used as a therapeutic strategy for prevention and treatment (Zschucke et al., 2013). While exercise can change and modify the expression of several genes in different tissues that can eventually affect the CNS, we will focus primarily on the skeletal muscle-brain axis and the potential for FNDC5/Irisin as a mediator of beneficial effects (**Figure 3** and following sections).

FNDC5/Irisin in Neurological Diseases

Exercise induced neuroprotection has been demonstrated in rodents and humans. However, the underlying mechanisms and pathways involved are far from fully understood. In a landmark study, Lourenco et al., showed that the expression of FNDC5 in the hippocampus is decreased in both AD patients and AD mouse models (Lourenco et al., 2019). AD is the most prevalent neurodegenerative disorder in elderly people (Prince et al., 2013), therefore there are tremendous efforts dedicated to device therapeutic alternatives to delay or counteract AD's devastating consequences. It has been also shown that AD patients have less circulating Irisin in their cerebrospinal fluid (CSF) (Lourenco et al., 2019, 2020). FNDC5 also decreases Aβ production by binding to the N-terminus of APP (Noda et al., 2018). While Aβ oligomers decrease the expression of components of the PGC-1α/FNDC5/BDNF axis in neuro-2a (n2a) cells, mice, and humans (Xia et al., 2017; Lourenco et al., 2019).

Several studies have shown that exercise can delay or improve the memory decline in AD patients (Winchester et al., 2013; Jia et al., 2019). Lourenco et al. (2019) showed that using neutralizing circulating Irisin antibodies suppressed exercise-induced memory improvement in AD mouse model. Furthermore, in PD exercise not only improved memory deficit but also has impacted psychological indicators such as depression, anxiety, and psychosis (Tanaka et al., 2009; Petzinger et al., 2013). While no studies have directly indicated a role for FNDC5/Irisin in alleviating PD pathology, it has been shown that PGC-1α and BDNF (upstream and downstream from FNDC5/Irisin, respectively) can alleviate PD symptoms (St-Pierre et al., 2006; Palasz et al., 2020). For example, aerobic exercise increases BNDF protein level in the substantia nigra of a PD model mouse, which leads to protection against further degeneration (Lau et al., 2011). BDNF level in peripheral blood and brain is decreased in PD rodent models and humans (Wang et al., 2016; Lin et al., 2017). In addition, PGC-1α expression is reduced in human PD brain samples (Zheng et al., 2010). Further, in animal models of PD overexpressing PGC-1α levels can result in suppression, while knocking out PGC-1α can accelerate neurodegeneration (St-Pierre et al., 2006; Jiang et al., 2016). Based on this evidence, FNDC5 could have an important role in alleviating PD pathology.

Exercise is showing promise as an effective strategy to protect neurons against degeneration not only in AD and PD, but also in Huntington's disease (HD). Expansion of CAG repeats in Huntingtin (*HTT*) gene leads to neurodegeneration in striatum in HD (Vonsattel and DiFiglia, 1998). As in AD and PD, BDNF level is decreased in HD mouse model and human samples (Zuccato et al., 2001, 2005). Voluntary running wheel increases BDNF level in striatum and frontal cortex of HD mice, which results in improvement of behavioral deficits and cognitive decline (Pang et al., 2006). In the R6/2 HD model, PGC-1α expression is suppressed which leads to increased neurodegeneration in the striatum (Cui et al., 2006). Overexpression of PGC-1α in striatum and cortex ameliorate HD pathology in mice (Tsunemi et al., 2012). Interestingly, in HD model PGC-1α expression is decreased in skeletal muscle (Chaturvedi et al., 2009). FNDC5 expression and Irisin secretion from skeletal muscle are processes downstream from PGC-1α expression, it is currently unknown how this is regulated in skeletal muscle of PD subjects. Taken together, these results suggest that FNDC5/Irisin could have a potential role in rescuing HD pathology and symptoms.

FNDC5/Irisin has also shown neuroprotective properties in conditions of ischemia. In fact, Irisin treatment protected hippocampal neurons against injury and cell death in cerebral ischemia models through different signaling pathways and mechanisms such as Akt, ERK1/2, Notch, TLR4/MyD88 and by protecting BBB from disruption (Li et al., 2017; Guo et al., 2019; Jin et al., 2019; Yu Q. et al., 2020). FNDC5 expression in skeletal muscle and circulating Irisin levels were also decreased by cerebral ischemia (Yu Q. et al., 2020). In addition, different types of exercise have been shown to protect brain against ischemic insult. For example, acute treadmill running prior to stroke protected the brain by enhancing angiogenesis in rats (Pianta et al., 2019). Similarly, treadmill running for 14 days protected prefrontal cortex neurons against cerebral ischemic induced apoptosis (Pianta et al., 2019). Importantly, neutralizing Irisin in peripheral blood using an antibody, eliminated the positive effect

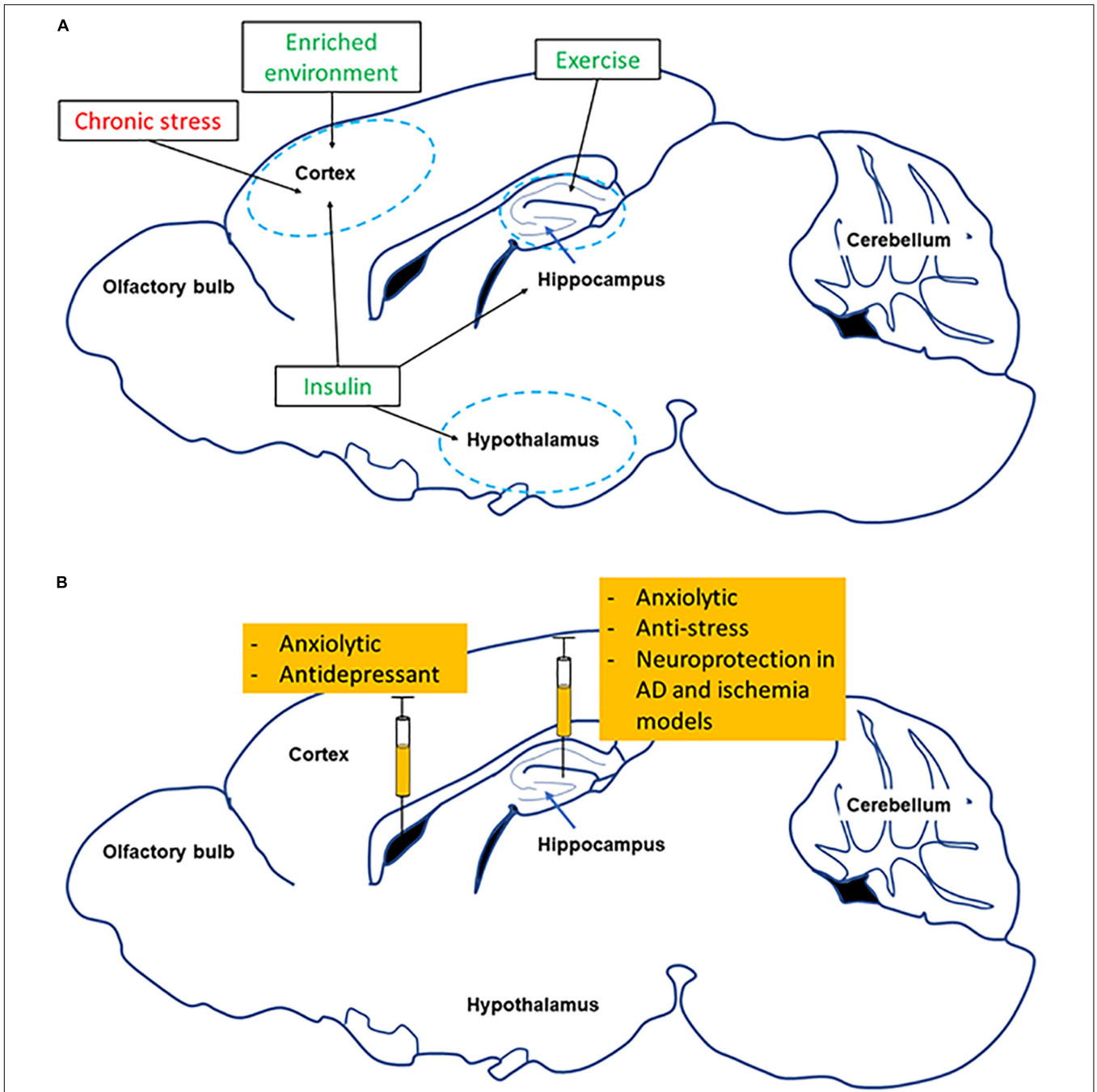


FIGURE 3 | Summary of effects of changing FNDC5/Irisin levels in different brain regions. **(A)** Shows how several environmental factors lead to alterations in FNDC5/Irisin levels in different brain regions (red symbolizes reduction, green means increase). **(B)** Shows the effect of directly manipulating levels of FNDC5/Irisin on neurobehavior and neuroprotection.

that exercise had on neuroprotection against cerebral ischemia (Li et al., 2017).

FNDC5/Irisin in Neuropsychiatric Disorders

Neuropsychiatric disorders such as depression, anxiety, and schizophrenia, are high prevalent (Kessler et al., 2005). Thus, finding successful treatments against them is one of the main current challenges in neuroscience research. Exercise has been introduced as a potential treatment for preventing and alleviating neuropsychiatric disorders (Swenson et al., 2020). As revealed by several recent studies, FNDC5/Irisin could act as mediator for the skeletal muscle-brain axis that could be used as a therapeutic avenue in neuropsychiatric disorders.

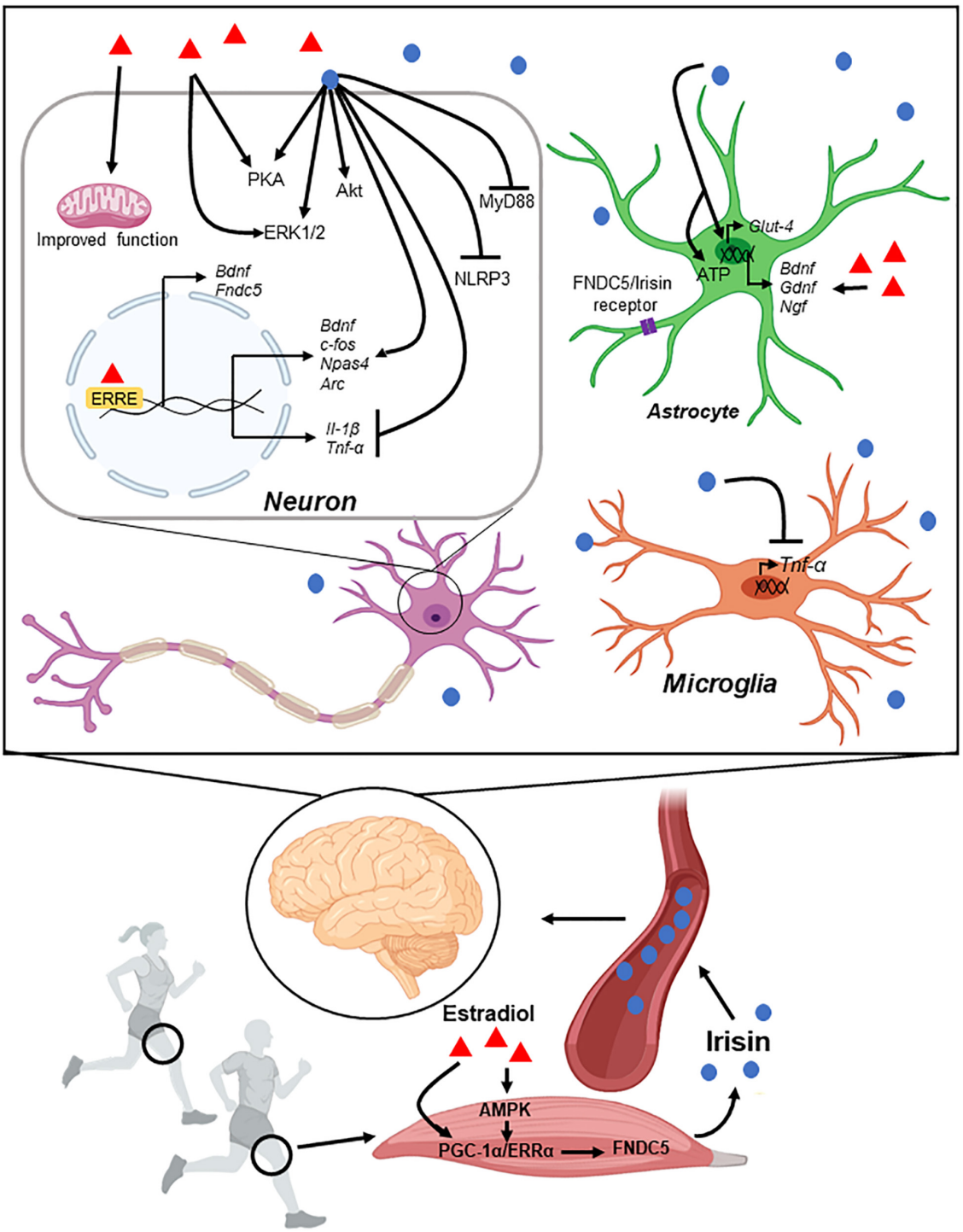


FIGURE 4 | Schematic representation of muscle-brain connection through Irisin. Enhancement in FNDC5 expression by exercise leads to Irisin secretion into the peripheral blood. Irisin crosses the blood brain barrier and reaches different regions of the brain. Irisin controls gene expression and different signaling pathways in neurons and glial cells (see main text for details).

I.c.v. injection of Irisin decreases immobility time in tail suspension and forced swimming tests in male mice, change that could be interpreted as anti-depressant in animal models (Siteneski et al., 2018). Similarly, Irisin level in prefrontal cortex is decreased by chronic stress in male Sprague–Dawley rats. Specifically, Irisin levels were correlated with chronic

stress-induced depression behavior and recombinant Irisin injection for 14 days suppresses depressive-like behavior through controlling glucose metabolism in prefrontal cortex (Wang and Pan, 2016). Further, a human study showed that post-stroke depression is associated with decreased serum Irisin levels (Tu et al., 2018). The expression of PGC-1α is decreased in the hippocampus of mice with depressive-like behavior (Fu et al., 2020). BDNF level in the hippocampus and peripheral blood is correlated negatively with the severity of depression in rodents and humans (Lee et al., 2007; Serra et al., 2018). As discussed in previous sections, exercise induced FNDC5/Irisin increases BDNF expression in different regions of the brain, especially hippocampus.

Irisin injection into the brain decreases anxiety-like behavior in male mice (Siteneski et al., 2018). Our own data has shown that acute stress-induced anxiety like behavior is suppressed by injection of Irisin into the hippocampus in male mice only, without affecting female mice (Jodeiri Farshbaf et al., 2020). Further studies have shown that Irisin level is reduced in serum in subjects diagnosed with anxiety disorders (Szilasi et al., 2017). This is relevant considering different studies showing that exercise can reduce anxiety (reviewed in Anderson and Shivakumar, 2013). Furthermore, PGC-1α and BDNF levels have also been altered in rodents with anxiety-like behavior (Govindarajan et al., 2006; Szalardy et al., 2018), and both PGC-1α and BDNF can have anxiolytic properties (Agrawal et al., 2014; Xie et al., 2019). Exercise also reduces panic disorder, which is a type of anxiety disorder, through increasing BDNF concentration in serum (Strohle et al., 2010).

DISCUSSION

Altogether, there is solid evidence of the exercise-induced functional connection between skeletal muscles and the brain, and FNDC5/Irisin is a likely mediator. The multiple endocrine actions of Irisin in the CNS highlight the importance of this circulating myokine in neuroprotection against different injuries and insults, including neurodegenerative disorders.

Different myokines are released from skeletal muscles in conditions of physical activity and several can reach the CNS, likely mediating the multiple beneficial effects associated with exercise (see **Table 1**). Irisin is a recently discovered myokine that is secreted form skeletal muscle during exercise and can cross the blood brain barrier. Irisin and its precursor, FNDC5, have important roles in development and regeneration of muscle, and other metabolic processes (**Figure 4**). However, only recently FNDC5/Irisin has been investigated as possible mediator of exercise-induced benefits for brain function. Specifically, Irisin release induces BDNF expression in the hippocampus leading to improvement in learning and memory, and protection against injuries such as ischemia, acute stress, and neurodegenerative disorders such as AD. Intriguingly, several sex-depended differences have been described in the context of exercise effects on health and cognitive function, mirroring the specific effects of FNDC5/Irisin. These findings warrant the need for expanding our research to include sex as a biological variable in the context of exercise promoted neuroprotection.

One of the areas of potential therapeutic relevance is the discovery of the receptor for Irisin in the brain, either in neurons and/or glial cells, and the specific mechanisms underlying its neuroprotective effects. To date, the only FNDC5/Irisin receptor that has been characterized occurs in bone and adipose tissue cells (Kim et al., 2018). It is possible to speculate about a similar type of receptor present in the CNS and with that knowledge many possible pharmacological interventions can be devised. This could be greatly relevant for aging related conditions such as neurodegenerative diseases, including AD. In this context, the possible identification of Irisin as an important mediator of the "muscle-brain axis" could have several implications for therapeutics development. The results summarized in this review indicate that FNDC5/Irisin could potentially be used as mimetic of exercise, in cases for example that physical activity is not recommended or not possible. Moreover, understanding what roles FNDC5/Irisin play as exercise intermediary could further cement exercise as non-pharmacological alternative intervention for individuals at risk, and even general aging population. Furthermore, the current knowledge in the field strongly indicates that Irisin (and possibly other myokines) could be considered and explored as a biomarker of unhealthy aging and neurodegeneration. This could have large consequences for public health initiatives considering that AD and related dementias affect to a large group of the aging population, and while enormous efforts have been dedicated to find a cure, there is currently no available treatment.

AUTHOR CONTRIBUTIONS

MJF and KA devised the original plan for the review. MJF wrote the initial draft, sketched initial figures, and revised the final draft. KA edited first draft and figures and finalized the manuscript and figures. Both authors contributed to the article and approved the submitted version.

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High-Fat Diet Alleviates Neuroinflammation and Metabolic Disorders of APP/PS1 Mice and the Intervention With Chinese Medicine

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Alzheimer's disease (AD) is a progressive neurodegenerative disease caused by the complex interaction of multiple mechanisms. Recent studies examining the effect of high-fat diet (HFD) on the AD phenotype have demonstrated a significant influence on both inflammation and cognition. However, different studies on the effect of high-fat diet on AD pathology have reported conflicting conclusions. To explore the involvement of HFD in AD, we investigated phenotypic and metabolic changes in an AD mouse model in response to HFD. The results indicated there was no significant effect on Aβ levels or contextual memory due to HFD treatment. Of note, HFD did moderate neuroinflammation, despite spurring inflammation and increasing cholesterol levels in the periphery. In addition, diet affected gut microbiota symbiosis, altering the production of bacterial metabolites. HFD created a favorable microenvironment for bile acid alteration and arachidonic acid metabolism in APP/PS1 mice, which may be related to the observed improvement in LXR/PPAR expression. Our previous research demonstrated that Huanglian Jiedu decoction (HLJDD) significantly ameliorated impaired learning and memory. Furthermore, HLJDD may globally suppress inflammation and lipid accumulation to relieve cognitive impairment after HFD intervention. It was difficult to define the effect of HFD on AD progression because the results were influenced by confounding factors and biases. Although there was still obvious damage in AD mice treated with HFD, there was no deterioration and there was even a slight remission of neuroinflammation. Moreover, HLJDD represents a potential AD drug based on its anti-inflammatory and lipid-lowering effects.

Keywords: Alzheimer's disease, high fat diet, neuroinflammation, metabolism, Huanglian Jiedu decoction, gut microbiota

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INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the pathological hallmarks of progressive cognitive decline, extracellular accumulation of amyloid-β (Aβ) plaques and intracellular accumulation of neurofibrillary tangles. Diet and nutrition display potential for non-pharmacological AD prevention (Hill et al., 2019); however, different studies examining

the effect of a high-fat diet (HFD) on AD pathology in AD models have reported conflicting conclusions. Ordinarily, systemic inflammation and obesity caused by HFD are likely to interfere with immunological processes of the brain and further promote disease progression (Heneka et al., 2015; Nam et al., 2017). And study showed that the adversely affect can be reversed by low-fat diet, at least partially, even after 7 months of HFD feeding when neuropathological burden was reasonably advanced (Walker et al., 2017). However, other studies found that HFD have no effect on Aβ or tau (Studzinski et al., 2009; Herculano et al., 2013; Knight et al., 2014) or cognitive performance (Elhaik Goldman et al., 2018). In all the related research, potential genetic variability in different mouse strains and the age-related factors may explain the high variability of the results. In APP knock-in mouse model, carrying a humanized Aβ sequence with the Swedish mutation (KM670/671NL), this paper shown that high fat diet treatment, despite inducing obesity and a T2DM peripheral phenotype, failed to trigger AD pathology (Salas et al., 2018). HFD treated Tg2576 mouse had no effect on the level of amyloid beta$_{1-42}$ in the cortex and shown more anxious, but had better learning which related to the improvements of BBB function (Elhaik Goldman et al., 2018). HFD had only a limited effect upon learning and memory in C57BL/6 mice despite broad peripheral changes (Mielke et al., 2006; Kesby et al., 2015). These data therefore suggest that different modes of memory are differentially sensitive to the effects of a high-fat diet. Research on the longitudinally the effect of a high-fat diet in both control non-Tg and 3xTgAD mice found that effects on memory were transient as at the age of 11–12 and 15–16 months no difference in time spent in the target quadrant was observed between 3xTgAD or non-Tg mice on either a control or high-fat diet (Knight et al., 2014).

A number of factors have been proposed to cause HFD-induced damage to the brain, especially in response to aging, including oxidative stress, insulin resistance, inflammation, and changes in vascularization/BBB integrity (Freeman et al., 2014). Therefore, it is necessary to explore the specific effect of HFD on metabolism in AD. HFD-related obesity has been reported by many studies to be involved in the regulation of gut microbiota, cholesterol circulation, and liver damage (DeAngelis et al., 2005; Kim et al., 2015). Because total serum cholesterol levels represent the most general change among the effects of high-fat diet (Guay et al., 2012), cholesterol that is excreted with bile acids (BAs) is a relevant host factor that modulates the gut microbiota (Martinez et al., 2013). Therefore, we hypothesized that altered gut microbiota was the more important metabolic change in response to HFD intervention. Emerging evidence from recent studies indicates that neurodegenerative diseases might be directly associated with the impairment of gut microbial flora (Li B. et al., 2019). Disturbances in the microbiota influence the formation of the amyloid beta peptide and exacerbate neurodegeneration through the upregulation of neuroinflammatory processes (De-Paula et al., 2018). The "gut-brain metabolic axis" facilitates bidirectional chemical communication between the central and enteric nervous systems (Nicholson et al., 2012; Wang and Wang, 2016). This metabolic axis is thought to be involved in the regulation of multiple host metabolic pathways in which the levels of short-chain fatty acids, hormones, neurotransmitters, amines, lipid metabolites, and others are regulated by gut microbiome activity (Nicolas and Chang, 2019).

The goal of the present study was to characterize diet-related effect on AD models and the therapeutical effects of HLJDD. In the present study, we performed a detailed examination of the effects of HFD on AD pathological characteristics, including the cognitive capacity, pathological slices and the neuroinflammation. Neurotransmitters, PUFAs and their derivatives in the central nervous system (CNS), BAs in the periphery were systematically detected by the UPLC-MS/MS to identify the general metabolic dysfunction, which have been as factors that may lead to impaired cognitive function. In addition, gut microbiota was analyzed to reveal the vital function of the gut-brain metabolic axis in HFD treated AD mice.

Abbreviations: AD, Alzheimer's disease; HFD, high-fat diet; HLJDD, Huanglian Jiedu decoction; Bas, bile acids; MWM, Morris water maze; SCFAs, short-chain fatty acids; Ber, berberine; Don, donepezil; 24S-HC, 24-Hydroxycholesterol; AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; OTUs, operational taxonomic units; ACE, abundance-based coverage estimator.

MATERIALS AND METHODS

Animals

Four-month-old SPF grade male C57BL/6J-TgN (APP/PS1) transgenic mice and C57BL/6J wild type mice (Beijing HFK Bioscience CO., LTD, Production license number SCXK 2014-004) were used in this study. All experiments and animal care in this study were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and the Provision and General Recommendation of Chinese Experimental Animals Administration Legislation. The study was approved by the Institutional Animal Care and Use Committee of the Beijing animal science CO., LTD and the animal ethics approval number was IACUC-2018100605. Animals were housed in a single cage with chow and water *ad libitum* and a 12 h light-dark cycle and kept under a consistent temperature of 21°C. Wild type mice were assigned to the normal group and fed a normal chow diet. APP/PS1 mice were randomly allocated into one of 6 groups: one was fed a normal chow diet (the AD group), and the others were fed a HFD containing 63.6% basic feed, 15% lard, 20% sucrose, 1.2% cholesterol, and 0.2% cholate (Beijing Keao Xieli Feed CO., LTD). One of the HFD experimental groups was the AD + HFD group, and the other four were HLJDD-L, HLJDD-H, berberine (Ber), and donepezil (Don) groups. The Huanglian Jiedu decoction was prepared in our laboratory as previously described (Yang et al., 2013). Our research was a preventive protocol, and the gavage doses were 172 mg/kg/d (HLJDD-L) and 344 mg/kg/d (HLJDD-H) for 6 mouths. Oral gavage of 50 mg/kg/d berberine (H51022193) and 2 mg/kg/d donepezil (1705051) once daily was used as a positive control. Animal weights were recorded every 3 weeks.

Morris Water Maze Test

The MWM test was performed to assess spatial memory. In acquisition trial, gently place the mouse into the water, facing the edge of the pool. If the mouse finds the platform before the 90 s cut-off, allow the mouse to stay on the platform for 10 s then return it to its home cage. Otherwise, place the mouse on the platform and allow it to stay there for 20 s. The mouse was trained in different direction. Repeat for all mice in the trail in the next 3 days. In probe trial, remove the platform from the pool and the test time was 60 s. Escape latencies, time spent or distance traveled in the target quadrant and platform-crossing times were recorded and analyzed using the analysis management system (Beijing Zhongshi Kechuang CO., LTD).

Tissue Collection

After the MWM test, all animals had a 2-day rest, and then mice were deeply anesthetized via an intraperitoneal injection of sodium pentobarbital for sampling of heart blood. Brains were removed and immediately frozen in dry ice prior to storage at −80°C for subsequent metabolic analyses. For immunohistochemical analyses, removed brains were postfixed in 4% paraformaldehyde (PFA) solution and then immersed in a 4% PFA solution. Feces were collected before the day animals were euthanized, and the intestinal contents were also collected when animals were dissected and frozen at −80°C.

Immunohistochemistry

Coordinates of the targets in mouse brains were determined using The Rat Brain in Stereotaxic Coordinates of Paxinos and Watson (fifth edition, page 53–63). Three-millimeter-thick brain tissues were dehydrated using gradient alcohol solutions, embedded in paraffin and cut into 4-μm sections, and dehydrated. Thin sections were dewaxed and rehydrated in distilled water for hematoxylin and eosin, Congo red and immunohistochemistry staining.

We performed antigen retrieval with citrate buffer (pH 6.0) treatment, and sections were blocked with endogenous peroxidase and washed in phosphate buffered saline (PBS) (pH 7.4) 3 times. Then, sections were blocked in 5% normal goat serum and 1% bovine serum albumin (BSA) with 0.3% Triton-X 100 in PBS for 30 min at room temperature. Sequential incubation with the primary antibodies, rabbit anti-beta amyloid 1–42 (1:1000, 1% BSA, 0.3% Triton-X100/PBS, ab201060), rabbit anti-GFAP (1:1000, 1% BSA, 0.3% Triton-X100/PBS, ab7260) and rabbit anti-MHC class II antibody (1:200, 1% BSA, 0.3% Triton-X100/PBS, ab180779), was performed overnight at 4°C. After washing in PBS five times, sections were sequentially incubated with HRP anti-rabbit IgG (peroxidase) polymer (PV-9001, Beijing Zhongshan Golden Bridge Biotech, China) for 20 min. Color development was performed by adding freshly prepared diaminobenzidine (DAB) for 30 s. Sections were counterstained in hematoxylin for 5 min, dehydrated, cleared and sealed with neutral gum. A slice scanner (Aperio, American) was used for recording and analysis.

Biochemical Parameters

Brain tissue was homogenized in liquid nitrogen and subpacked. Then, NP-40 protein lysis buffer was added. After centrifugation, the supernatant was removed. Inflammatory factors, including TNF-α, IL-1β, and IL-6, were detected using ELISA kits (VAL609, VAL601, and VAL604).

Meso scale discovery (MSD) multispot assay system proinflammatory panel 1 (mouse) kits (K15048D) were used to measure ten inflammatory cytokines (IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-12p70, and TNF-α) in serum. Total serum levels of cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), free fatty acids (FFAs), albumin, and C-reactive protein (CRP) were determined using an automatic biochemical analyzer (AU480, Beckman Coulter, Inc., Brea, CA, USA).

RT-PCR

Total RNA was extracted from brain tissue using TRIzol reagent (ELK Biotechnology, China) according to the manufacturer's instructions. RNA concentrations were equalized and converted to cDNA using the EntiLink™ 1st Strand cDNA Synthesis Kit (ELK Biotechnology, China). Gene expression was measured using a StepOne™ Real-Time PCR system. The sequences of primers used in these experiments are listed in the **Supplementary Material**.

Western Blot Analysis

Brain tissues were lysed in precooled RIPA buffer with the protease inhibitor PMSF (Amresco), and protein concentrations were determined using a BCA protein assay kit. Protein samples were separated on 12% sodium dodecyl sulfate polyacrylamide gels electrophoresis (SDS-PAGE) and transferred onto NC membranes. Then, membranes were blocked in 5% non-fat milk for 30 min at room temperature and incubated with primary antibodies (rabbit polyclonal NR1H3 antibody, 1:1000; rabbit polyclonal PPARγ antibody, 1:4000; mouse monoclonal APOE antibody, 1:5000; rabbit polyclonal 5 lipoxygenase antibody, 1:200; rabbit polyclonal COX2 antibody, 1:1000) overnight at 4°C. Membranes were then washed and incubated with HRP-conjugated goat anti-rabbit and HRP-conjugated goat anti-mouse (1:10,000) secondary antibodies for 40 min at room temperature followed by development using ECL detection. The obtained bands were then scanned and analyzed using ImageJ software, and band density (IOD) was assessed using Total Lab Quant V11.5 (Newcastle upon Tyne, UK).

Metabolic Phenotyping

Neurotransmitters

Brain tissue (50 mg) was weighed, and 1 mL of 80% acetonitrile solution (containing 0.2% formic acid) was added and centrifuged at 3,000 rpm, 0°C for 60 s, and ultrasonication was performed for 60 s. Samples were centrifuged again at 12,000 rpm, 0°C for 15 min. Then, 10 μL of 1% ascorbic acid, 10 μL of internal standard solution and 380 μL of methanol (with 0.2% formic acid) were added to 100 μL of supernatant, which was vortexed for 180 s and centrifuged at 12,000 rpm at 0°C

for 15 min. After centrifugation, the supernatant was used for UPLC–MS/MS analysis. Each standard was precisely weighed, and 50% methanol solution was added to prepare the 2 mg/mL mixed standard stock solution. According to the abundance of each substance in the sample, each stock solution was precisely added at a corresponding volume into the mixed standard. The standard was diluted with 50% methanol, and the standard synephrine was accurately weighed and prepared in a 10 μg/mL internal standard solution. All solutions were stored at −80°C.

Gradient chromatographic separation was performed on a Waters PFP C18 column (2.1 × 100 mm, 1.7 μm). Mobile phase A consisted of 0.05% formic acid in acetonitrile. Mobile phase B consisted of 0.05% formic acid in water. Gradient elution was as follows: 0–4 min, 2% A; 4–6 min, 2–95% A; and 6–10 min, 95–95% A. The column temperature was 40°C, the flow rate was 0.3 mL/min, and the injection volume was 5 μL. Mass spectrometry analysis was performed using an electrospray ion source (ESI), and detection was performed in the multireaction ion monitoring (MRM) positive mode. The temperature was 550°C, the curtain gas (CUR) pressure was 35 V, the atomization gas pressure (GS1) was 55 psi, and the auxiliary gas flow rate (GS2) was 55 psi. The MRM ion pair transitions and collision energy levels of each component are listed in the **Supplementary Material**.

Polyunsaturated Fatty Acids and Metabolites

Sample preparation referenced a previous report (Yue et al., 2007). Briefly, 1 ng of 20-HETE-d6, 1 ng PGE_2-d5 and 100 ng AA-d8 internal standard solutions were added. After vortex mixing, nitrogen blowing was performed by heating in 37°C water. Finally, the sample was redissolved in 50 μL of methanol and analyzed by LC-MS/MS. Standards of each polyunsaturated fatty acid and their metabolites were accurately weighed and diluted with methanol solution to prepare the mixed lipid peroxide standard stock solution of 200 ng/mL and PUFA standard stock solution of 200 μg/mL. Standards of 20-HETE-d6, PGE_2-d5, and AA-d8 were weighed and prepared in 500, 500 ng/mL and 5 μg/mL internal standard stock solutions. All solutions were stored at −80°C.

Gradient chromatographic separation was performed on a Waters BEH C_{18} column (2.1 × 100 mm, 1.7 μm). Mobile phase A consisted of 0.05% formic acid in acetonitrile. Mobile phase B consisted of 0.05% formic acid in water. Gradient elution was as follows: 0–8 min, 60% A; 8–8.5 min, 60–75% A; and 8.5–11.5 min, 75–100% A. The column temperature was 40°C, the flow rate was 0.25 mL/min, and the injection volume was 5 μL. Mass spectrometry analysis was performed using ESI. Detection was performed in MRM negative mode. The temperature was 550°C, CUR pressure was 35 V, GS1 was 55 psi, and GS2 was 55 psi. MRM ion pair transitions and collision energy levels of each PUFA and its metabolites are listed in the **Supplementary Material**.

Bile Acids

Serum samples were melted at 4°C, and simple protein precipitation using methanol was used as follows: 130 μL of methanol was added to 50 μL serum samples, spiked with 20 μL of IS, and the sample was vortexed for 20 min and centrifuged at 12,000 rpm and 4°C for 5 min. The supernatant was collected, and 5 μL was injected into the LC–MS/MS system for analysis. Bile acid standard stock solutions were prepared by dissolving the respective 17 BAs in the appropriate amount of methanol to obtain mixed stock solutions of 1 mg/mL. Then, the solution was further diluted with methanol:water (3:1) to obtain a series of standard stock solutions. CA-d4 and LCA-d4 mixed solutions were prepared in methanol and further diluted with methanol to obtain a 2 μg/mL internal standard solution.

Chromatographic separation was performed on an ACQUITY HSS T3 column (2.1 × 100 mm, 1.8 μm). The mobile phase consisted of H_2O:acetonitrile (10:1, containing 1 mmol/L ammonium acetate) (A) and acetonitrile:isopropanol (1:1) (B) at a total flow rate of 0.26 mL/min. The gradient profile for the LC pumps under the chromatography conditions were as follows: 0–1 min, 100% A; 1–2 min, 100–60% A; 2–6 min, 60–50% A; 6–8 min, 50–30% A; 8–9 min, 30–0% A; 9–12 min, 0%; 12–12.1 min, 0–100% A; and 12.1–13 min, 100% A. The injection volume of all samples was 5 μl. The column temperature was set at 50°C. For MS detection, the ESI source was operated in the negative ion mode to produce MS/MS spectra. The parameters were as follows: capillary 2 kV; cone 21 V; source temperature 150°C; desolvation temperature 600°C; cone gas flow 150 L/Hr; and desolvation gas flow 1,000 L/Hr. The MRM ion pair transitions and collision energy levels of each component are listed in the **Supplementary Material**.

Short-Chain Fatty Acids

Fecal short-chain fatty acids (SCFAs) were also analyzed. Briefly, 1,000 μL of ethanol (containing 0.5% hydrochloric acid, *v/v*) and 10 μL of internal standard (acetic acid-d4) were added to 25 mg of each sample. The mixture was vortexed and ultrasonicated for 40 min and centrifuged at 14,000 rpm for 10 min. Supernatants were analyzed by Agilent 7890-5977 gas chromatography–mass spectrometry (Agilent Technologies, Santa Clara, CA, USA).

The chromatographic column was a DB-FFAP (30 m × 0.25 mm i.d., 0.25 μm film, Agilent Technologies). One microliter of sample was automatically injected in the splitless mode, which was maintained at 250°C. Helium was used as a carrier gas at a flow rate of 1 mL/min in constant flow mode. The oven program was set at an initial temperature of 50°C for 2 min and was then increased to 120°C at a rate of 15°C/min, to 170°C at a rate of 5°C/min, and was finally increased to 240°C at a rate of 15°C/min and held at 240°C for 3 min. The electron impact ionization was 70 eV, and data were acquired in full scan mode and SIM mode. Identification of each SCFA was confirmed by comparing the mass spectra and retention times. Acquired tandem mass spectra were matched against the NIST Standard reference database 1A. The quantifiers for acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid and acetate d3 (ISTD) were m/z 43, 74, 43, 60, 60, 60, 60, and 46.

Microbial Diversity Analysis

Intestinal content was collected when animals were dissected and was stored at −80°C. DNA extraction and PCR amplification were subsequently performed. Microbial DNA was extracted from intestinal content samples using the E.Z.N.A.® soil

DNA Kit (Omega Biotek, Norcross, GA, U.S.) according to the manufacturer's protocols. The final DNA concentration and purification were determined by a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA), and DNA quality was checked by 1% agarose gel electrophoresis. The V3–V4 hypervariable regions of the bacterial 16S rRNA gene were amplified with the primers 338F (5′- ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′- GGACTACHVGGGTWTCTAAT-3′) by a thermocycler PCR system (GeneAmp 9700, ABI, USA). PCRs were performed in triplicate in a 20 μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA. The resulting PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor™-ST (Promega, USA) according to the manufacturer's protocol.

Illumina MiSeq sequencing was performed using purified amplicons, which were pooled in equimolar amounts and subjected to paired-end sequencing (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Processing of sequencing data involved using raw fastq files, which were demultiplexed, quality-filtered by Trimmomatic and merged by FLASH with the following criteria: (i) reads were truncated at any site receiving an average quality score <20 over a 50-bp sliding window; (ii) primers were exactly matched, allowing 2 nucleotide mismatches, and reads containing ambiguous bases were removed; and (iii) sequences whose overlap was longer than 10 bp were merged according to their overlapping sequence. Operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff using UPARSE (version 7.1 http://drive5.com/uparse/), and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed by the RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Silva (SSU123) 16S rRNA database using a confidence threshold of 70%.

Statistical Analysis

For all experiments, at least three replicates were analyzed. All quantitative data are expressed as the mean ± standard error of the mean (SEM) and were analyzed using unpaired Student's *t*-test for two groups and one-way ANOVA for five groups, as indicated, followed by the LSD and Games-Howell post-test. Statistical calculations were performed using IBM SPSS Statistics software. Differences with statistical significance are denoted by $p \leq 0.05$. Histograms were drawn using GraphPad Prism (San Diego, CA).

Data Availability

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

RESULTS

Pathology and Biochemical Parameters in APP/PS1 Mice

HFD had no Effect on Cognitive Impairment or Cerebral Pathology in APP/PS1 Mice

In the MWM test, the sequential changes in the average escape latency during spatial acquisition training are shown in **Figure 1A**. AD mice displayed markedly longer average escape latency compared to normal control mice on days 2 and 4, but AD + HFD mice were not significantly different. In the spatial probe test, the platform crossing number in AD and AD + HFD mice was lower compared to that in the control group. The percent distance and time spent in the target quadrant were significantly lower than those in the Nor group (**Figure 1B**). However, MWM test results in the AD + HFD group were similar to those in the AD group, indicating that there was impairment of spatial memory capacity in AD mice with no aggravation in response to treatment with a high-fat diet.

In HE staining of brain tissue, morphological lesions were not apparent in all groups. Next, we evaluated Aβ deposition in the brain by Congo red staining. The AD and AD + HFD groups exhibited obvious Aβ deposition. This result was in agreement with the immunohistochemistry in which the levels of Aβ were significantly increased in APP/PS1 mice compared to the Nor group (**Figure 1C**). Abnormal accumulation of Aβ induces excessive activation of microglia in the brain, which is associated with neuronal injury in AD. We next assessed whether a high-fat diet promotes inflammation in the brain. By immunohistochemistry staining, GFAP and major histocompatibility complex (MHC) class II expression were restricted to the hippocampal area in wild type mice but were overexpressed in the hippocampus and cortex of AD and AD + HFD mice. Both microglia and astrocytes present antigens via MHC class II molecular complexes with self-peptides to cause infiltration of $CD4^+$ T cells in drainage lymphoid tissues that stimulate the adaptive immune response in the CNS (Das and Chinnathambi, 2019). Microglial cells are first primed by intracellular accumulation of Aβ, and prior to plaque deposition, classical markers of microglial activation, such as MHC class II, inducible nitric oxide synthase, and CD40, are already upregulated in the hippocampus of transgenic mice. The inducible, neuron-specific cyclooxygenase 2 (COX-2) enzyme is upregulated and specifically expressed by neurons in close relationship with Aβ-bearing cells (Ferretti et al., 2012).

A High-Fat Diet Relieves Inflammation and Oxidative Stress in Brain Tissue

To further assess the effect of high-fat diet on brain inflammation, we measured the mRNA expression of different proinflammatory cytokines, namely, *IL-1β, TNF-α, IL-6, IL-12A, IL-12B, IFN-γ,* and *MCP-1*, that were upregulated in the AD and AD + HFD groups. Surprisingly, we found that the mRNA levels of proinflammatory cytokines were significantly reduced in response to high-fat diet exposure (**Figure 1D**). Inflammatory cytokine levels were also detected by ELISA, and TNF-α and

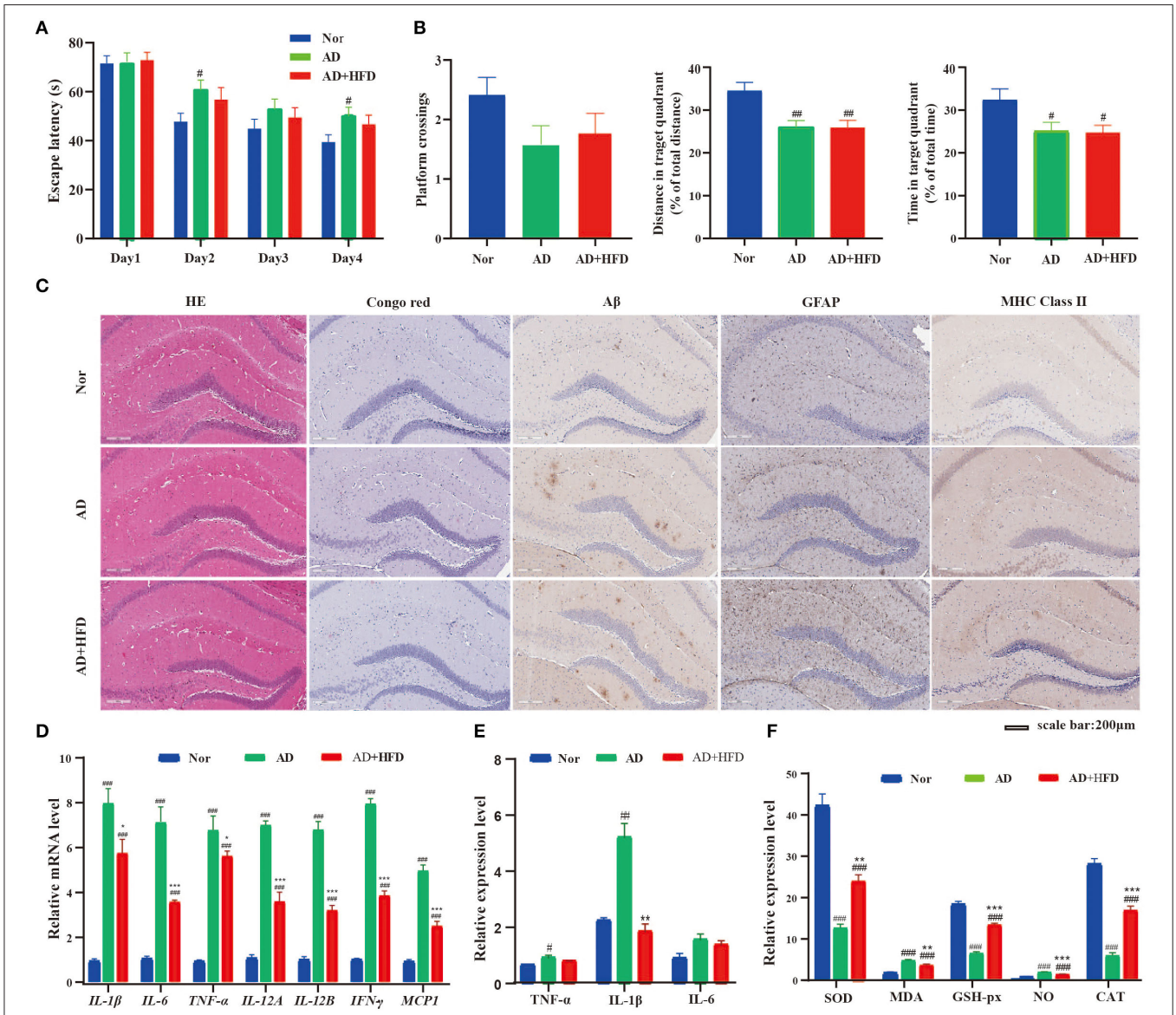


FIGURE 1 | The effect of HFD on APP/PS1 mice cognitive impairment and cerebral pathology. **(A)** Escape latency during spatial acquisition training, **(B)** the platform crossing number, distance traveled percentage in the target quadrant and time spent percentage in the target quadrant in the spatial probe test. HFD had no effect on Aβ level, but HFD could relieve the inflammation in APP/PS1 mice. **(C)** The HE and Congo red stain in mice brain tissue, Aβ deposition was determined by Congo red stain and immunohistochemical technique. And the level of GFAP and MHC class II in brain tissue detected by immunohistochemical method. The expression of inflammatory cytokine detected by RT-PCR **(D)** and Elisa **(E)** method, respectively. And the indicators of oxidative stress **(F)** in brain tissue. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ (compared to Nor group); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (compared to AD group).

IL-1β were significantly increased in the AD group, while IL-1β levels were significantly reduced in the AD + HFD group (**Figure 1E**). Taken together, these results confirm that the effects of relieving inflammation might be associated with high-fat diet exposure.

Indicators of severe oxidative stress, that is, SOD, CAT, and GSH-px, exhibited significantly decreased expression in the AD and AD + HFD groups, and the levels of NO and MDA were strikingly elevated (**Figure 1F**). MDA is a marker for lipid peroxidation in the brain, and higher levels in APP/PS1 mice indicated disordered lipid metabolism. Compared to AD mice, the above indexes were significantly reversed in the AD + HFD group, suggesting that a high-fat diet may moderate oxidative stress to some extent.

High-Fat Diet Increases Body Weight, Serum Lipid Levels, and Inflammation

We next investigated whether HFD treatment was sufficient to trigger obesity and hyperlipidemia in APP/PS1 mice. After 6 months of high-fat diet, there was obvious weight gain in AD

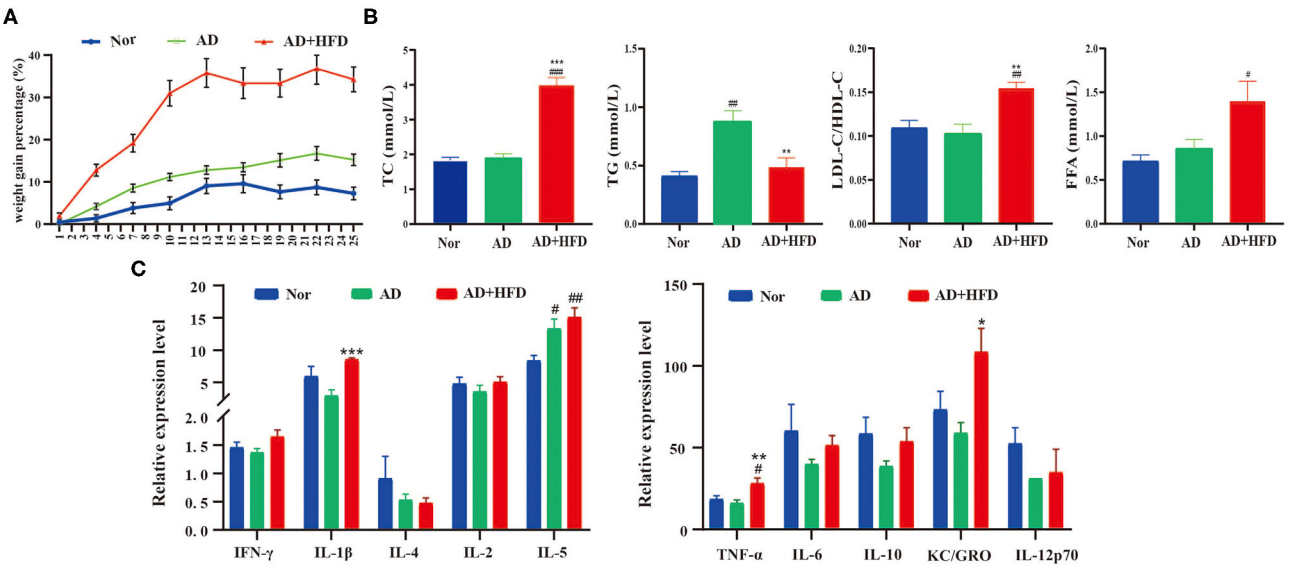


FIGURE 2 | The effect of high fat diet on APP/PS1 mice body weights, serum lipid and inflammation level. **(A)** The weight gain percentage with either normal diet or HFD for 6 months. **(B)** The level of total cholesterol, triglyceride, ratio of LDL-C/HDL-C, and FFA in mice serum. **(C)** Serum inflammatory cytokines were detected by MSD cytokine assays. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ (compared to Nor group); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (compared to AD group).

+ HFD mice at a rapid pace compared to mice on chow diet (**Figure 2A**). Serum TC, TG, the ratio of LDL-C/HDL-C and FFAs were also largely increased, indicating that high-fat diet induced hyperlipidemia in AD + HFD mice. The concentration of serum TG increased markedly, suggesting abnormal lipid metabolism in the AD group (**Figure 2B**). Because obesity was accompanied by peripheral inflammation, we next sought to study the impact of HFD on peripheral inflammatory tone. MSD cytokine assays provide a rapid and convenient method for measuring the level of protein targets. The results revealed that IL-5 was significantly increased in APP/PS1 mice, while TNF-α, IL-1β, and KC/GRO were markedly increased in the AD + HFD group compared to the AD group (**Figure 2C**). This finding indicates that there was inflammation in the serum of HFD mice.

Alzheimer's-Associated Metabolic Alteration

High-Fat Diet Modulates Neurotransmitter and Amino Acid Metabolism in Brain Tissue

Amino acids have roles in neuronal signaling, energy production, and nitrogenous waste production and elimination. Aromatic amino acids in the brain function as precursors for the monoamine neurotransmitters serotonin and catecholamines (Fernstrom and Fernstrom, 2007). Brains and serum from patients with AD exhibit many alterations in amino acid levels and metabolism (Pan et al., 2016; Griffin and Bradshaw, 2017; Cui et al., 2020). Compared to the normal group, dopamine, acetyl choline, and serotonin were all decreased in APP/PS1 mice (**Figure 3A**), and acetyl choline was significantly different as well. GABA, glutamine, phenylalanine, lysine, asparaginate, proline, and alanine were significantly elevated in AD and AD + HFD mice. Additionally, serotonin and hypoxanthine were significantly decreased in AD mice, while glycine, tryptophan and methionine were significantly increased. Serine, levodopa, lysine, glutamine, and hypoxanthine were significantly increased in the HFD group compared to AD mice; however, methionine was significantly decreased.

All four principal nuclei of the monoaminergic systems undergo significant degeneration in AD (Trillo et al., 2013). In addition, bioanalysis of serotonin, dopamine and norepinephrine in the plasma after 8 weeks of HFD revealed that serotonin was significantly decreased in obese mice (Kim et al., 2013). In this experiment, we observed reductions in serotonin, 5-hydroxyindoleacetic acid and dopamine in APP/PS1 mice. The concentration of the sulfur-containing amino acid methionine was significantly decreased, and cysteine was significantly increased in AD + HFD mice compared to AD mice. Studies previously identified increased methionine levels in the CSF of MCI subjects (Kaddurah-Daouk et al., 2013), and dietary methionine supplementation resulted in increased Aβ and phosphorylated tau levels in the brain and cognitive impairment in wild type mice. In addition, cysteine supplementation caused a slight decrease in mTOR activity and inhibited the reduction in ROS in animals restricted by methionine (Gomez et al., 2015).

High-Fat Diet Increases Brain Cholesterol Levels

Cholesterols play a key role in the maintenance of brain health, and sufficient availability of cholesterol for synapse formation is a critical step in the structural and functional development of the nervous system (Hussain et al., 2019). Since peripheral and central cholesterol pools are not readily interchangeable, the brain has to produce cholesterol by *de novo* synthesis. In our research, cholesterol was significantly

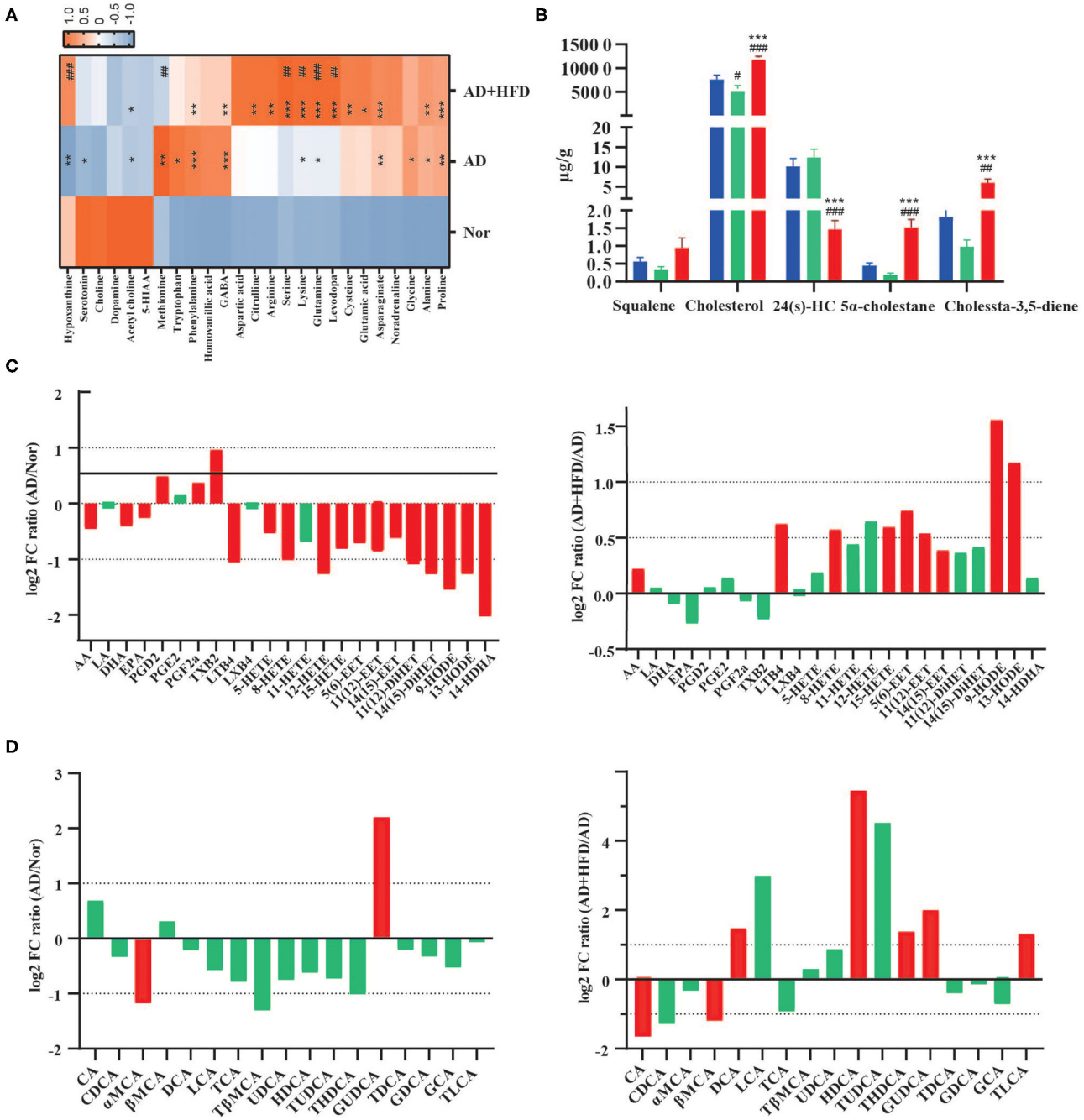


FIGURE 3 | High fat diet altered the metabolite in AD mice. **(A)** The neurotransmitters and amino acids metabolism in brain tissue. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (compared to Nor group); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ (compared to AD group). **(B)** The level of cholesterol and its related compounds in brain tissue. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ (compared to Nor group); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (compared to AD group). **(C)** High fat diet-dependent changes in modulated polyunsaturated fatty acids and peroxilipidos metabolism in brain tissue samples (Log_2 FC). **(D)** The concentration of 17 bile acids are dynamically changed in the serum (Log_2 FC). The column in red had significant difference and the column in green had no significant difference. Data are presented as mean ± standard error of mean. $^{\#}$compared to Nor group; *compared to AD group.

decreased in AD mice, but cholesterol and its precursor, squalene, were significantly increased in AD + HFD mice. Two reduction products of cholesterol, 5α-cholestane and cholesta-3,5-diene, were also present at higher levels in the AD + HFD group (**Figure 3B**). 24-Hydroxycholesterol (24S-HC) is the main mediator of cholesterol removal in the brain and represents classical feedback regulation of cholesterol homeostasis. 24S-HC serves as an activator of the nuclear transcription factors

liver X receptors alpha and beta, which increase expression of cholesterol transport genes, including ABCA1 in both neurons and glia and ApoE in astrocytes (Martin et al., 2014). It serves as an intercellular signal that controls the delivery of astrocytic-derived cholesterol to neurons (Sun et al., 2016). Increased 24S-HC levels in both plasma and cerebrospinal fluid have been observed at early stages of AD, and as the disease progresses, 24S-HC levels decrease, possibly reflecting extensive neuronal loss and resultant loss of the CYP46A1 enzyme. Combined with the higher levels of squalene, lower levels of 24S-HC suggested that synthesis of cholesterol may be improved or that elimination may be suppressed by a high-fat diet.

High-Fat Diet Modulates Polyunsaturated Fatty Acid and Peroxilipid Metabolism

PUFA levels and the signaling pathways they regulate are altered in various neurological disorders, including AD and major depression diseases. PUFAs participate in signal transduction, either directly or after enzymatic conversion to a variety of bioactive derivatives (mediators) (Bazinet and Laye, 2014). In our research, arachidonic acid (AA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) were significantly decreased in APP/PS1 mice. The mediators PGD2, PGE2, PGF2α, and TXB2, which are produced by COX and are highly proinflammatory, were elevated relative to the normal group. However, LTB4, HETEs, EETs, DiHETs, and 14-HDHA were significantly decreased. Of note, HFD regulated both the pro- and anti-inflammatory lipid mediators AA, LTB4, 5-HETE, 15-HETE, 5(6)-EET, 11(12)-EET, 14(15)-EET, 9s-HODE, and 13s-HODE, and these findings were significantly reversed in response to high-fat diet treatment (**Figure 3C**).

High-Fat Diet Affects Peripheral Bile Acid Levels in APP/PS1 Mice

BAs are the end products of cholesterol metabolism produced by human and gut microbiome cometabolism. A total of 17 bile acids were detected in mouse serum; among them, GDCA, GCA and TLCA were semiquantitatively detected due to their low levels in mouse serum. Compared to the normal group, GUDCA was significantly increased and α-MCA was significantly deceased in the AD group. CA and βMCA were decreased by HFD treatment; however, the levels of DCA, HDCA, THDCA, GUDCA, and TLCA were significantly increased. Compared to the normal group, α-MCA was significantly decreased in APP/PS1 mice. However, compared to chow-fed controls, DCA, HDCA, GUDCA, and TLCA were significantly increased by HFD treatment (**Figure 3D**).

Bile acids may be helpful to further diagnose AD in plasma along with a broader pattern of biomarkers (Marksteiner et al., 2018). It was previously reported that decreased serum levels of a liver-derived primary BA and increased levels of bacterially produced secondary BAs and their conjugated forms were associated with reduced cognition (MahmoudianDehkordi et al., 2019). The ratios of secondary to primary BAs (DCA:CA, LCA:CDCA) were associated with worse cognition, and these ratios were increased in AD + HFD mice in our study. However, HFD also improved functional BAs, such as UDCA, HDCA and their taurine-conjugated BAs, which are considered neuroprotective agents (Yanguas-Casás et al., 2014; Li C. X. et al., 2019).

High-Fat Diet Alters the Gut Microbiome in APP/PS1 Mice

Increased permeability of the gut and blood-brain barrier induced by microbiota dysbiosis may mediate or affect AD pathogenesis and other neurodegenerative disorders, especially those associated with aging (Jiang et al., 2017). In this study, the composition of the gut microbiome was characterized using traditional ecological measures, including OTUs, alpha diversity, and beta diversity. Taxon analysis revealed that the final OTU dataset for the Nor, AD and AD + HFD groups consisted of 895 OTUs classified into 172 genera, 75 families, 39 orders, 22 classes, and 12 phyla. For community richness estimates, we used the abundance-based coverage estimator (ACE) and Chao; these measurements use non-parametric modeling to calculate a conservative estimate of total OTU richness for each participant. The microbiome in mice fed a high-fat diet exhibited reduced richness, with ACE significantly decreased in the AD + HFD group compared to the control group. For community diversity metrics, we used the Simpson and Shannon indexes, and there was no significant decrease between the AD and AD + HFD groups (**Supplementary Figure 1**). With respect to beta diversity, Bray-Curtis-based principal coordinate analysis revealed distinct clustering of microbe communities among all three groups (**Figure 4A**). There was a difference between normal and AD mice, and remarkable changes in the microbiota community structure were induced by HFD intervention.

At the phylum level, AD and AD + HFD exhibited a decreased abundance of *Bacteroidetes* and increased abundance of *Firmicutes* compared to the normal group (**Figure 4B**). The Wilcoxon rank-sum test found that *Verrucomicrobia* was significantly decreased in both the AD and AD + HFD groups. In addition, *Tenericutes* and *Cyanobacteria* were also decreased in the AD + HFD group compared to the AD group. It was reported that Bacteroidetes and Verrucomicrobia are decreased in patients with AD, whereas Actinobacteria are slightly more abundant (Zhuang et al., 2018). Previous findings suggested that the production of neurotoxins by the phylum *Cyanobacteria* may play a role in the onset and development of cognitive dysfunctions (Mancuso and Santangelo, 2018). The present results indicated an alteration in the types of bacteria in both AD mice and HFD intervention mice. At the family level (**Figure 4C**), *Prevotellaceae* was significantly decreased and *Rikenellaceae* was significantly increased in AD mice. *Lactobacillaceae* was significantly increased and *Ruminococcaceae, Rikenellaceae,* and *Bacteroidaceae* were significantly decreased in the AD + HFD group compared to the AD group. Similarly, at the genus level (**Figure 4D**), *Prevotellaceae_UCG-001* was less abundant and *unclassified_f_Lachnospiraceae* and *Alistipes* were more abundant in AD mice. Compared to AD mice, *Lactobacillus* was increased, and *Bacteroides* and *Alistipes* were significantly decreased in AD + HFD mice. One study showed that Bacteroides and Alistipes were more abundant in patients with AD (Vogt et al., 2017).

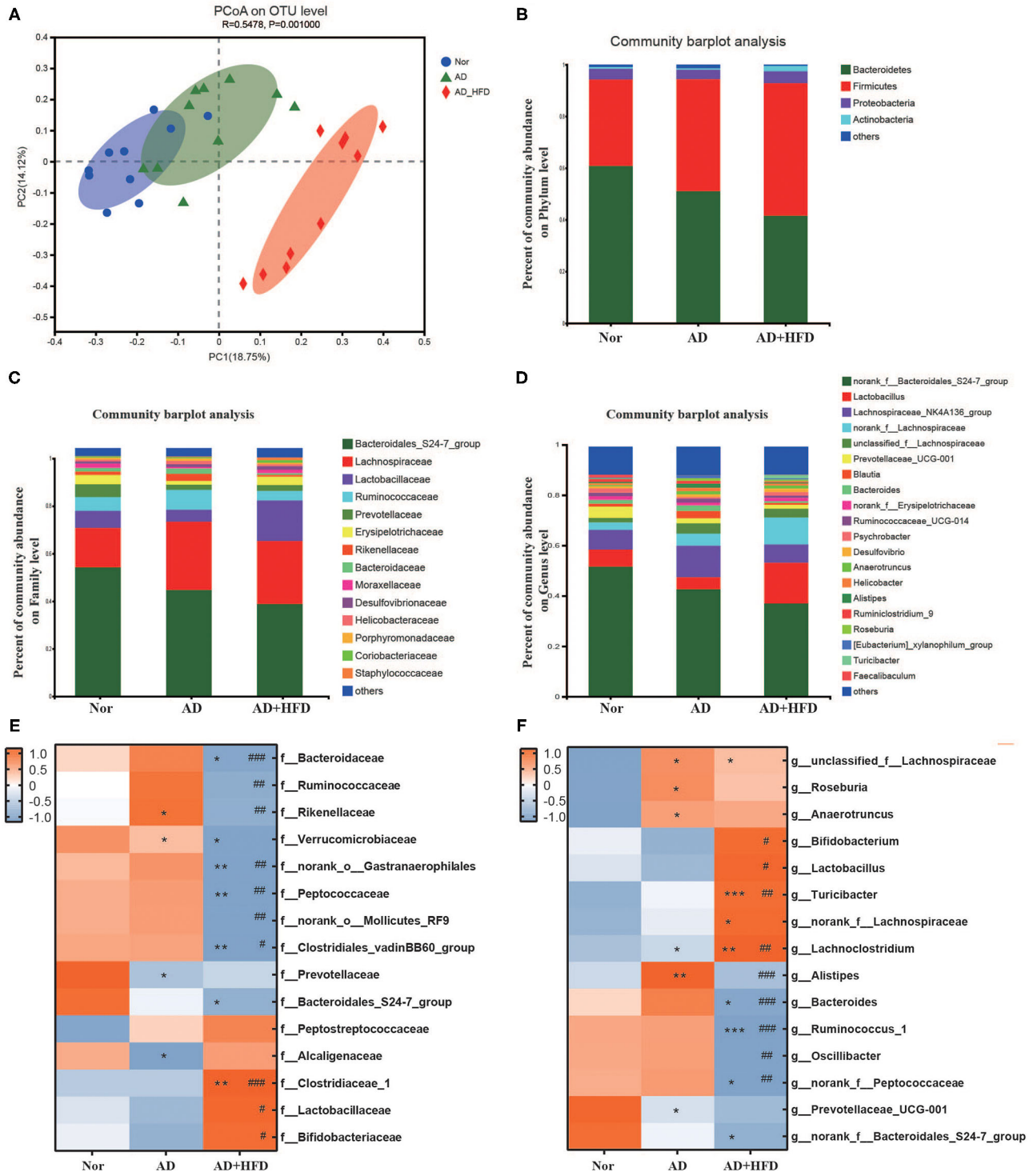


FIGURE 4 | High fat diet altered the fecal microbiota in APP/PS1 mice. **(A)** PcoA based on the distance matrix of Bray-Curtis dissimilarity of the gut microbiota communities in the Nor, AD, and AD + HFD mice on the operational taxonomic unit (OTU) level. **(B–D)** The relative abundance of gut microbes of Nor, AD, and AD + HFD groups on Phylum, Family and Genus level, respectively. Heatmap of significant gut microbiota changes represented at the Family **(E)**, Genus **(F)** level. Colors on the heatmap indicate the relative abundance of gut microbiota. Gut microbiota with significant changes were chosen using a Wilcoxon rank-sum two-tailed test with $P < 0.05$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (compared to Nor group); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ (compared to AD group).

Notably, in our present research, HFD suppressed changes in Alistipes and *Bacteroides*.

Furthermore, the top 15 taxonomic genera that significantly differed in abundance among the Nor/AD/AD + HFD groups at the family and genus levels were analyzed ($P < 0.05$, Student's *t*-test). At the family level (**Figure 4E**), *Prevotellaceae* and *Bacteroidales_S24-7_group* were decreased in APP/PS1 mice, and *Peptostreptococcaceae* was enriched. Consistently, at the genus level (**Figure 4F**), *Prevotellaceae_UCG-001* and *norank_f_Bacteroidales_S24-7_* were decreased, whereas *unclassified_f_Lachnospiraceae, Roseburia,* and *Anaerotruncus* were increased in AD and AD + HFD mice. A high-fat diet alters the composition of the gut microbiota with a dramatic reduction in various bacterial groups, such as *Bacteroides, Ruminococcus_1, Oscillibacter,* and *norank_f_Peptococcaceae.* Increases in *Bifidobacterium, Lactobacillus, Turicibacter, norank_f_Lachnospiraceae,* and *Lachnoclostridium* were observed in AD + HFD mice compared to AD mice.

The gut microbiota of Tg mice was highly dynamic, in great contrast to WT mice (Wang et al., 2019). In concert with the BA profile, the gut microbiota was partly restored by HFD. It was reported that *Oscillibacter* and *Alistipes* were negatively correlated with cognitive abilities, and *Lactobacillus* and *Bifidobacterium* were positively correlated with spatial learning and memory. In contrast, *Lactobacillus* and *Bifidobacterium* were negatively correlated, and *Oscillibacter*, *Alistipes*, and *Anaerotruncus* were positively correlated with proinflammatory factors (Wu et al., 2019; Xu et al., 2020). Therefore, these data demonstrated that HFD modulates neuroinflammation by altering microbiota abnormalities in APP/PS1 mice. Conversely, Akkermansia muciniphila treatment reversed high-fat diet-induced metabolic disorders, including fat mass gain, metabolic endotoxemia, adipose tissue inflammation, and insulin resistance (Everard et al., 2013), and our study revealed that *g_Akkermansia* was decreased in HFD mice. Furthermore, the main products of the fermentation of dietary fibers by anaerobic intestinal microbiota, SCFAs, were significantly decreased in response to high-fat diet (Wan et al., 2019). SCFAs can stimulate the NLRP6 inflammasome and ameliorate impairment of the intestinal epithelial barrier, resulting in protection against high-fructose diet-induced hippocampal neuroinflammation and neuronal loss (Li J. M. et al., 2019). Our research analyzed SCFAs in fecal samples and found that SCFAs were significantly decreased in APP/PS1 mice and that the total levels of SCFAs were decreased in response to high-fat diet treatment. AA and IBA were also markedly decreased by HFD intervention (**Figure 5A**).

Network Visualization Analysis of Metabolite-Protein Interaction During HFD Intervention in AD

To explore the effect of altered metabolites during AD progression, we analyzed the correlation between metabolites and functional protein markers. Briefly, the differential metabolites in the AD and AD + HFD groups were searched in the HMDB database to identify their related enzymes. Then, the enzyme was connected with functional proteins using the STRING database. As a result, we found that AA and most of its mediators were related to COX-2 (also known as PTGS2), GSH-px (also known as GPX3) SOD, TNF and APOE. Cholesterol was related to PPARγ, APOE, LXRβ (also known as NR1H2), TNF, and IFNγ. There was also a potential correlation between CA and PPARγ, APOE, LOXβ, and acetic acid, as well as PPARγ and APOE (**Figure 5B**).

For further verification, brain tissues from 10-month-old APP/PS1 mice were collected and subjected to RT-PCR and western blot analyses. AA generated PGs and TXs through the COX-2 enzyme. LTs, LXs and HETEs are converted by lipoxygenase (LOX) enzymes. Western blot results showed that HFD reversed the elevation of COX-2 and 5-LOX levels in the brains of AD mice compared to the Nor group (**Figure 5C**). PGs and TXs, as well as COX-2, were increased, suggesting increased inflammation in APP/PS1 mice, and HFD relieved neuroinflammation in the brain. Next, we investigated whether the expression of APOE, LXR and PPARγ was changed in APP/PS1 mice. The results showed that *APOE* levels were increased in the AD and AD + HFD groups; however, *LXRβ* and *PPARγ* were significantly decreased. Compared to AD mice, *APOE* was significantly decreased in response to HFD treatment, and the expression of both *LXRβ* and *PPARγ* was increased (**Figure 5D**). Western blotting revealed that the variation in the protein levels was the same as that of the gene expression levels (**Figure 5E**). LXRs are transcription factors that control the expression of gene products involved in cholesterol homeostasis. Several direct LXR target genes are intimately linked to cholesterol transport, such as *ABCA1* and *APOE*. PPARγ induced cholesterol removal from macrophages through a transcriptional cascade mediated by the nuclear receptor LXRα (Chawla et al., 2001). In addition, reports have indicated that the regulation of the PPARγ-LXR-ApoE cascade may represent a significant molecular connection between adipocyte TG and cholesterol homeostasis (Yue and Mazzone, 2009).

HLJDD Attenuates Pathology in HFD-Exposed AD Mice

Our previous studies of the underlying therapeutic mechanism of HLJDD for the treatment of AD were associated with alleviating oxidative stress, inflammation, neurotransmitters, and energy metabolism (Gu et al., 2018, 2020; Wu et al., 2020). HLJDD also moderately improved cognitive competence in AD + HFD mice at a high dose (**Figure 6A**). In practical terms, the average escape latency was significantly decreased in the HLDD-treated group compared to the AD + HFD group on days 3 and 4 in the MWM. The percent distance, time spent in the target quadrant and number of platform crossings in the HLJDD group were increased, suggesting that cognitive ability was improved by HLJDD intervention. Aβ levels were markedly decreased in the hippocampus, illustrating that HLJDD may suppress Aβ deposition (**Figure 6B**). The expression of GFAP and MHC class II was reduced, and inflammation was reduced in brain tissue. Furthermore, *TNFα* and *INFγ* were significantly decreased in HLJDD-H mice. Moreover, indicators of oxidative stress were also moderately inhibited in high-dose HLJDD mice (**Figure 6C**).

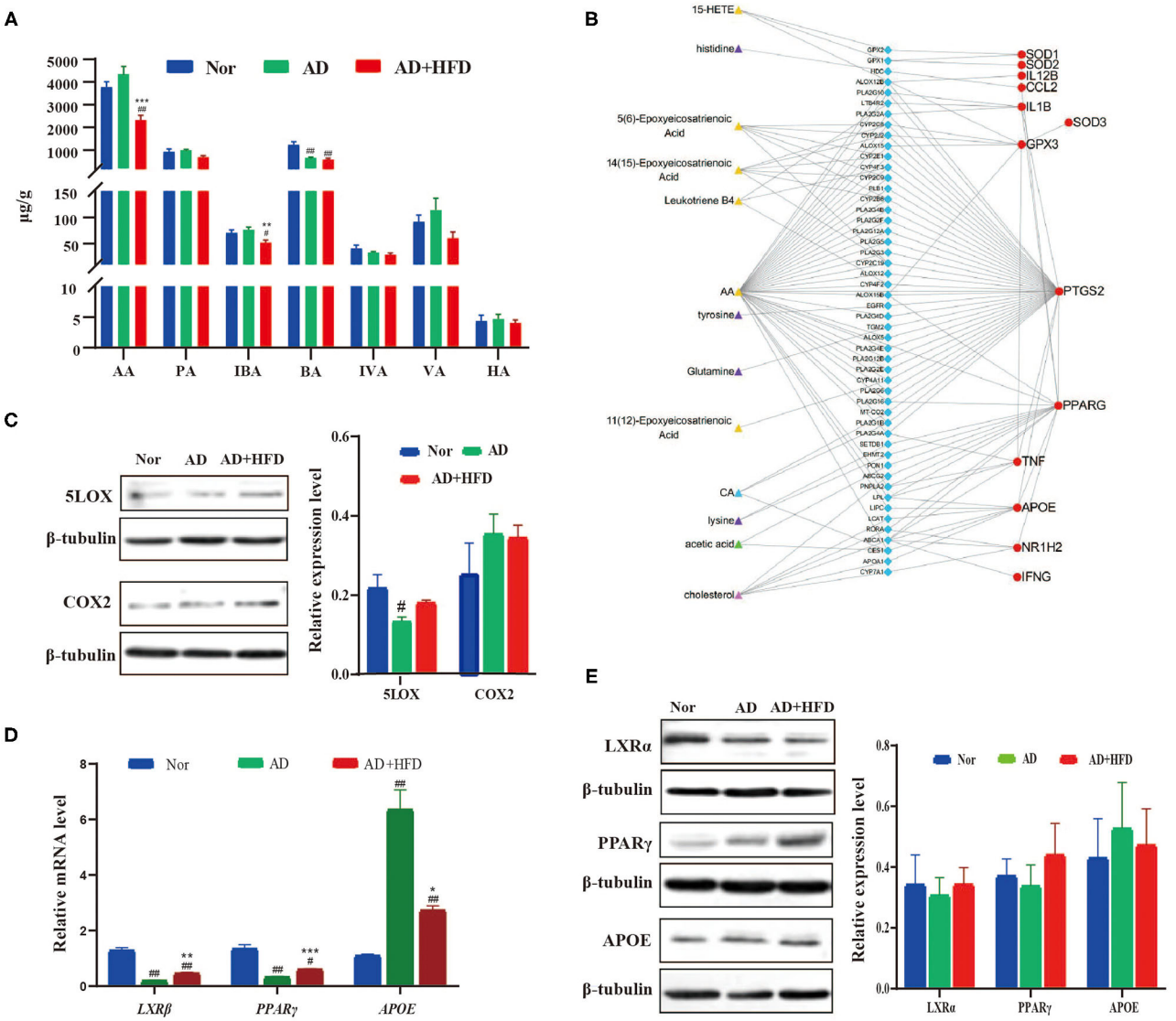


FIGURE 5 | Network visualization analysis of metabolite-protein interaction in high fat treated mice. **(A)** The SCFA level changed by high fat diet. **(B)** Network visualization analysis of metabolite–enzyme (blue) –protein (red) interaction. The metabolites in which were significant changed in AD + HFD mice compared with AD mice. And the related protein detected by RT-PCR **(C)** and Western blot **(D,E)**.

Additionally, weight gain was attenuated by drug intervention, and the serum lipid levels and cytokine IL-1β and KC/GRO levels in serum were lower compared to AD + HFD mice (**Supplementary Figure 2**).

HLJDD reversed the levels of lysine and glutamine, which were increased in the AD + HFD group, and HLJDD-L decreased the levels of levodopa (**Supplementary Table 1**). Cholesterol in brain tissue was also decreased by HLJDD (**Supplementary Figure 3A**). The high dose of HLJDD significantly decreased the levels of PGE2, PGF2a, and TXB2, indicating a neuroprotective effect (**Supplementary Table 2**). The expression of COX-2 and 5-LOX in the different groups had the same tendency as the downstream metabolites of PUFAs. However, the expression of LXR, PPAR and APOE was not significantly different (**Supplementary Figure 3**). In the periphery, HLJDD regulated BA and SCFA levels, as well as the gut microbiome. Specifically, CDCA, αMCA, TβMCA, TDCA, GDCA, GCA, TLCA, AA, and BA levels were reversed by HLJDD treatment (**Supplementary Table 3**, **Supplementary Figure 2D**). *Parasutterella, Alloprevotella, Alistipes, Rikenella*, and *Caproiciproducens* levels were also reversed in response to HLJDD (data not shown).

DISCUSSION

A high-fat/glucose diet was previously reported to induce biochemical changes related to increased AD biomarker burden and cognitive impairment (Lin et al., 2016; Kothari et al.,

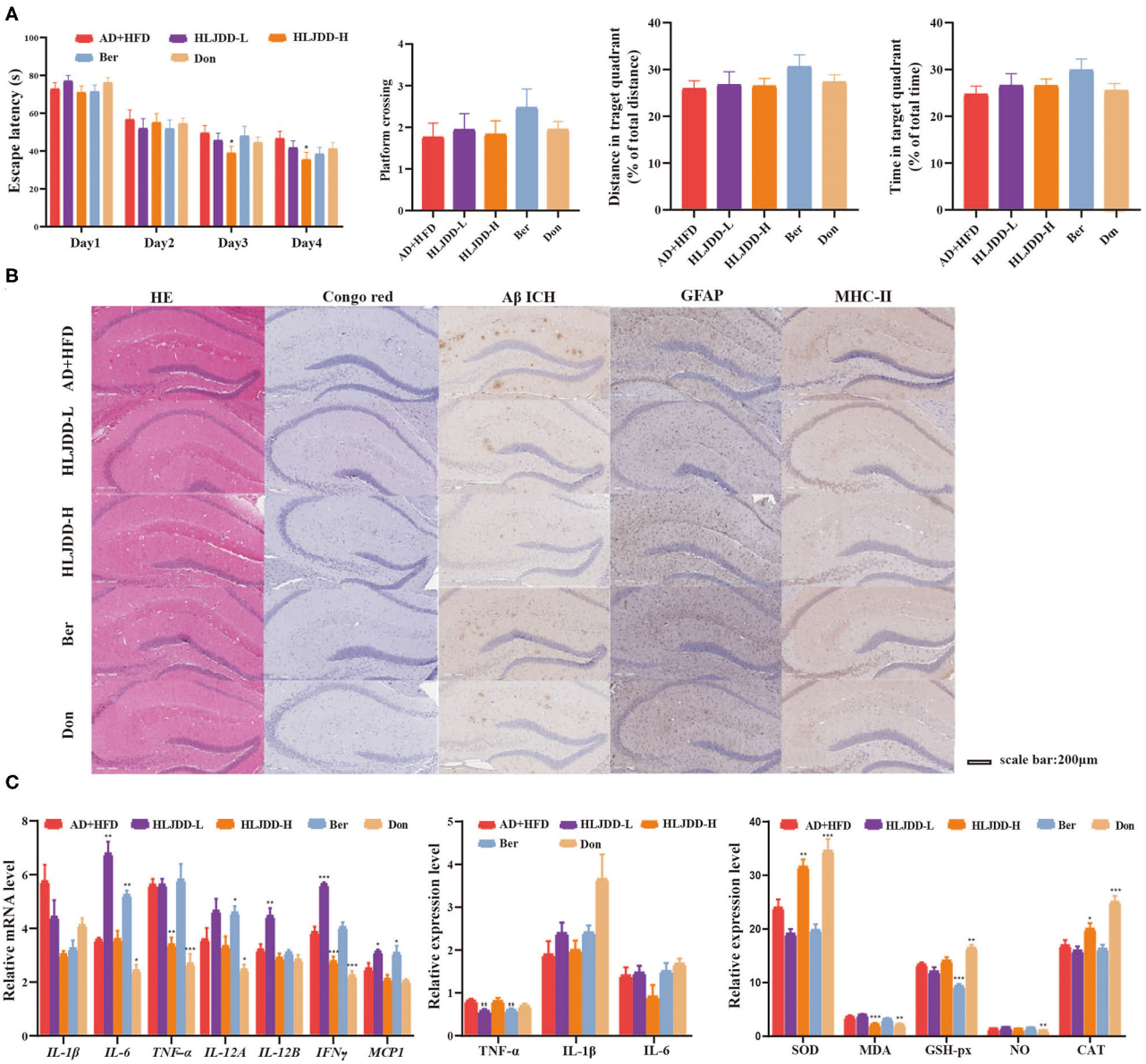


FIGURE 6 | The effect of HLJDD on high fat diet treated APP/PS1 mice. Escape latency during spatial acquisition training **(A)**, the platform crossing number, distance traveled percentage in the target quadrant and time spent percentage in the target quadrant in the spatial probe test. **(B)** The HE and Congo red stain in mice brain tissue, Aβ deposition, the level of GFAP, and MHC class II in brain tissue detected by immunohistochemical method. **(C)** The expression of inflammatory cytokine detected by RT-PCR and Elisa method, respectively. And the indicators of oxidative stress in brain tissue. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (compared to AD+HFD group).

2017; Hill et al., 2019). However, other data have suggested no major effects of high-fat diet treatment on hippocampal neuroinflammation (Cremonini et al., 2019) or even a protective effect induced by HFD through mechanisms that involve improved BBB properties and brain morphology, as well as higher insulin receptor RNA expression and higher HDL cholesterol without a reduction in $A\beta_{1-42}$ parenchymal load (Elhaik Goldman et al., 2018). To date, it is not possible to establish a causal relationship between diet and the development of AD with certainty because there are still many confounding factors and biases (David et al., 2014). More broadly, diet-related diseases will benefit from elucidating the links among nutritional, biliary and microbial dynamics (Grimm et al., 2013).

In the present study, cognitive competence and Aβ plaques were not aggravated in APP/PS1 mice by treatment with a high-fat diet. Endogenous substances, such as neurotransmitters and amino acid levels, were changed, and cholesterol and AA were increased in the CNS. Cholesterol affects Aβ generation,

aggregation and subsequent neurotoxicity, as it is the primary component of lipid rafts in which the AD-associated proteins APP, β-secretase, and γ-secretase have been found (Geifman et al., 2017). While it is quite clear that pharmacological reduction of cholesterol levels in cells with normal cholesterol content inhibits amyloidogenesis in a variety of cell types and systems, it remains unclear whether conclusions from these studies can be extrapolated to the disease or normal aging contexts, especially taking into account the human studies showing that cholesterol levels are reduced in normal and pathological aging brains (Martin et al., 2014). The assumption about statins reducing the risk of AD (Zissimopoulos et al., 2017) is currently difficult to maintain because the reduction in AD risk varies across statin molecule status, sex, and race/ethnicity (Downs, 2018). Cholesterol levels in the serum/plasma and brain of patients with AD do not support cholesterol as a causative factor in AD (Salas et al., 2018). Furthermore, our study showed that microglia and astrocytes were activated and that the mRNA expression levels of inflammatory cytokines were inhibited in AD + HFD mouse brain tissue. Although originally identified for their role in cholesterol homeostasis, LXRs suppress inflammation through multiple direct and indirect mechanisms, some involving transactivation and others involving transrepression (Schulman, 2017; Fessler, 2018). Activation of PPARs reduces inflammation and confers neuroprotection, in part through their ability to minimize cell death and reduce mitochondrial dysfunction. PPAR activation may also enhance axonal growth and remyelination (Mandrekar-Colucci et al., 2013), as well as reduce Aβ levels in cell culture and AD animal models (Sastre et al., 2006). The pleiotropic effects of PPARγ activation are a promising efficacious candidate for neuropathologies (Villapol, 2018). Therefore, we cannot conclude that the high level of cholesterol in the AD + HFD group exacerbated AD symptoms.

Gut microbiota may affect brain function and behavior through the microbiota-gut-brain axis in a bidirectional interplay with both top-down and bottom-up regulation (Wang and Wang, 2016; De-Paula et al., 2018). Microbial involvement in the development of Aβ pathology (Harach et al., 2017) may contribute to neuroinflammation in AD (Giau et al., 2018). Alteration of the gut microbiota composition leads to the peripheral accumulation of phenylalanine and isoleucine, which stimulates the differentiation and proliferation of proinflammatory T helper 1 cells, which are associated with M1 microglia activation and contribute to AD-associated neuroinflammation (Wang et al., 2019). Host-microbe metabolic axes are thought to be involved in the regulation of multiple host metabolic pathways in which the levels of hormones, neurotransmitters, amines, SCFAs, lipid metabolites, and others are regulated by gut microbiome activity (Nicholson et al., 2012; Erny et al., 2017). As far as the HFD was concerned, alteration of the gut microbiota and subsequent changes in both the serum and brain BA profiles are mechanistically involved in the development of AD (Jia et al., 2020). An increased DCA:CA ratio in both the serum and brain was significantly associated with a worse cognition, and downstream effects might affect metabolic homeostasis and/or signaling functions in the human brain (MahmoudianDehkordi et al., 2019). In our study, the ratios of secondary to primary BAs, which reflected differences in gut microbiome enzymatic activity leading to altered production of secondary BAs, were increased in the AD and AD + HFD groups. Our study suggested that HFD might change CNS inflammation and metabolism partly by restoring the gut microbiota.

Bile acids and SCFAs have been reported to act as important regulators involved in the mechanisms by which gut microbiota affect lipid metabolism (Granado-Serrano et al., 2019). In our research, *Alistipes* was positively correlated with SCFAs and negatively related to DCA. *Oscillibacter* was positively related to IVA and negatively related to FFAs. Interestingly, *Clostridiales* was closely related to BAs, and most of the bacteria from *Ruminococcaceae* were negatively related to secondary bile acids, as the results of the correlation analysis show in **Supplementary Figure 1B**. However, the *Lachnospiraceae* family, such as *g_Lachnoclostridium, g_Marvinbryantia,* and *g_Lachnospiraceae_UCG-001*, is positively related to BAs. It was clear that microbial disturbances correlate with altered bile acid metabolism during the progression of various diseases and conditions (Joyce and Gahan, 2017). Other studies have shown that the gut microbiota also regulates the metabolism of bile acids that are ligands for the nuclear receptor farnesoid X receptor and the membrane receptor GPCR5, which are involved in the regulation of glucose, lipids, and energy balance (Pathak et al., 2018). In addition, our research showed that SCFAs were present at lower levels in AD + HFD mice. The depletion of butyrate-producing microbes by antibiotic treatment reduced epithelial signaling through the intracellular butyrate sensor PPARγ (Byndloss et al., 2017), which induced expression of genes involved in enterohepatic circulation and bile acid synthesis (Wang et al., 2018). As signaling molecules, SCFAs could adjust lipogenesis, gluconeogenesis and cholesterol synthesis notably through the G-protein coupled receptors GPR43/FFAR2 and GPR41/FFAR3 (Schoeler and Caesar, 2019). Dietary SCFA supplementation prevented and reversed high-fat diet-induced metabolic abnormalities in mice by decreasing PPARγ expression and activity (den Besten et al., 2015). As the main products of the fermentation of dietary fibers by anaerobic intestinal microbiota, SCFAs was a key note in the communication of gut and brain (Dalile et al., 2019).

CONCLUSIONS

Our study showed that there were obvious cognitive impairment and Aβ plaques in APP/PS1 mice, regardless of their diet. However, neuroinflammation was slightly relieved by HFD treatment, which might have been mediated by increased levels of LXR/PPAR and altered gut microbes. It was further shown that a high dose of HLJDD exerted more effective anti-inflammatory and lipid-lowering effects, with potential effects on the pathophysiological cascade of Alzheimer's disease.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI SRA BioProject repository, accession number is PRJNA724708.

ETHICS STATEMENT

The animal study was reviewed and approved by Beijing animal science Biotechnology Co., Ltd.

AUTHOR CONTRIBUTIONS

BB, BL, and HZ conceived the study concept and design and are guarantors of the article. HZ, XF, and JZ contributed to the data collection and elaboration, writing, and approval of the manuscript. XG, XW, HW, NS, and JY contributed to the methodology, discussion and writing and approval of manuscript. YY and FG contributed to the immunohistochemistry and network visualization analysis. YZ contributed to the valuable revision of the manuscript. The corresponding author attests that all listed authors meet authorship criteria and that no others meeting the criteria have been omitted.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.658376/full#supplementary-material

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Nocturnal Sleep Problems Mediate the Impact on Quality of Life of Early Morning Off in Parkinson's Disease

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Background: Early morning off (EMO) refers to off-states in the morning in people diagnosed with Parkinson's disease (PwPD). This study determined the clinical manifestations of EMO and the association with nocturnal sleep problems and quality of life (QOL) in Chinese PwPD.

Methods: In this multicenter, observational, cross-sectional study, data concerning the clinical manifestations of EMO were collected from PwPD in Shanghai by questionnaire. The stepwise logistic regression was performed to analyze the potential risk factors, as well as whether EMO was an independent risk factor for functional dependency in daily life. The mediation analyses were conducted to evaluate whether nocturnal sleep problems might mediate the association between EMO and the QOL.

Results: Among the 454 subjects evaluated, EMO occurred in 39.43% of PwPD across all disease stages. The prevalence of EMO increased as the Hoehn and Yahr stage increased and was observed in 35.60% of patients in stages 1–2.5 and 48.85% of patients in stages 3–5. EMO was associated with non-motor symptoms (NMSs). The predominant NMSs associated with EMO were nocturnal sleep problems (98.90%), mood/cognition impairment (93.90%), decreased attention/memory (91.60%), gastrointestinal symptoms (91.60%), and urinary urgency (90.50%). The QOL of PwPD with EMO was significantly reduced ($P < 0.001$). Moreover, nocturnal sleep problems might partially mediate this relationship (indirect effect: $\beta = 13.458$, 95% boot CI: 6.436, 22.042).

Conclusion: PwPD have EMO throughout all stages of the disease. Patients with EMO have severe motor symptoms and NMSs. EMO decreases the QOL in PwPD and this relationship is partially mediated by nocturnal sleep problems. In light of these findings, it is suggested that recognition and appropriate treatment of EMO and nocturnal sleep problems could improve the management of PwPD.

Keywords: sleep disturbance, early morning off, quality of life, Parkinson's disease, China

INTRODUCTION

Parkinson's disease (PD) is a common movement disorder. Clinical manifestations of PD are heterogeneous and cover a broad spectrum of manifestations ranging from motor symptoms to non-motor symptoms (NMSs). These include bradykinesia, rigidity, tremor, cognitive impairments, and mood disorders (Ascherio and Schwarzschild, 2016; Tysnes and Storstein, 2017). Several NMSs of PD predate the movement symptoms by decades. Early morning off (EMO) is a motor complication in people diagnosed with Parkinson's disease (PwPD) (Chapuis et al., 2005). Since there was no established definition of EMO, the presence of EMO was identified in patients who present with poor motor or NMSs on morning waking, while related symptoms could be alleviated after taking the first dosage of the dopaminergic drug. EMO is related to the concentration of anti-Parkinson's medications in the serum, which is at a low level during the night and can be relieved until the first dose of dopaminergic therapy; sometimes it takes longer than usual to restore the ON-state from the first dose of dopaminergic therapy (Stocchi et al., 2019). Rotigotine was an efficacious treatment to reduce early morning motor functioning, reduced sleep disturbances, and improved quality of life (QOL), as it has relevant clinical implications and recommendations (Ray Chaudhuri et al., 2016).

Patients with EMO complain of slowness or stiffness in the early morning and experience an "off" state during the remaining daytime hours. It has been confirmed that EMO has a substantial impact on QOL by A Rizos group (Rizos et al., 2014). Occasionally, patients report that EMO-induced dystonia with pain causes nocturnal sleep problems and imposes additional burdens on caregivers (Rizos et al., 2016; Han et al., 2020). Nocturnal sleep problems are common NMSs in PwPD that negatively impact the QOL of patients (Stefani and Högl, 2020). EMO has been reported to be associated with nocturnal sleep problems and reflects the nocturnal decline in dopamine levels with insufficient night-time storage (Stocchi et al., 2019). However, objective evidence of how nocturnal sleep problems correlated or regulates the impact of EMO on QOL is limited. Exploring the effects of nocturnal sleep problems on this relationship may reveal important implications for early interventions designed to enrich and prolong the QOL of PwPD.

In this multicenter, observational, cross-sectional study, we determined the clinical characteristics of EMO in PwPD and verified the association with nocturnal sleep problems and QOL, to provide a comprehensive understanding of the EMO phenomenon in Chinese PwPD.

METHODS

Study Design and Participants

We conducted a multicenter, observational, outpatient-based, cross-sectional study that was approved by the Research Ethics Committee of Xin Hua Hospital affiliated to Shanghai Jiao Tong University School of Medicine and the Ethics Committees of other sites. After obtaining informed consent, PwPD were consecutively recruited and diagnosed according to the Movement Disorder Society PD criteria (Postuma et al., 2015). Patients with secondary parkinsonism, stroke, brain tumor, or an alternative cause for parkinsonism symptoms were excluded. We did not set an exclusion criterion based on sleep.

Clinical Assessment of the Motor Symptoms and NMSs of PwPD

Patients were recruited from 12 sites. This study screened 462 patients and enrolled 454 patients. Eight patients were excluded as they did not meet the inclusion criteria. A structured interview for clinical and demographic variables was performed. All PwPD were evaluated during the medication "on" period. The symptoms of PwPD were measured with the following scales: the Unified Parkinson's Disease Rating Scale (UPDRS), modified Hoehn–Yahr (HY) stage, Mini-Mental State Examination (MMSE), and the Non-Motor Symptom Scale (NMSS) (Chaudhuri et al., 2007). The nocturnal sleep problems of patients were assessed with the Parkinson's Disease Sleep Scale (PDSS). The QOL of patients was assessed with the Parkinson's Disease Questionnaire-39 (PDQ-39).

Since there is no established definition of EMO in PwPD, the presence of EMO was identified using a two-step process during a structured clinical interview in the first instance, as described by Rizos et al. (Rizos et al., 2014).

The interview included specific questions based on the definition of the wearing-off state upon morning awakening, while related symptoms could be alleviated after taking the first dosage of the dopaminergic drug and supplemented by the following: (1) how the patient is upon awakening (morning "on" vs. morning "off" stages), (2) response to UPDRS item 35 [presence of early morning dystonia (historical information)], (3) response to PDSS item 14 (do you feel tired and sleepy after awakening in the morning?), and (4) whether these symptoms improve after a morning dose of dopaminergic drug (Rizos et al., 2014).

Statistical Analysis

The statistical analysis was performed using SPSS 26.0 (IBM. Corp., Armonk, New York, USA). Categorical variables were compared using the Pearson's chi-square test, the Mann–Whitney *U* test, or Student's *t*-test. A binary logistic regression model was performed to evaluate the factors that could independently associate with EMO. A linear regression of PDQ-39 total scores was performed with different variables. The level of significance was set at $P < 0.05$. Odds ratios (ORs) are presented with 95% CIs.

Mediation analyses were conducted with the SPSS mediation modeling software, PROCESS, to determine whether the severity of nocturnal sleep problems mediated the association between EMO and QOL. The analysis considered the total effect of EMO (with or without EMO) on QOL (PDQ-39) and the indirect effect mediated by PDSS total scores. A bootstrap estimation approach with 5,000 samples was used to measure the indirect effect with 95% CIs. The indirect effect was considered significant when the 95% CIs did not contain zero.

RESULTS

A total of 454 PwPD were enrolled in this study. The mean age of the patients was 68.97 ± 8.620 years and 56.17% were males (**Table 1**). The mean disease duration of PwPD was 5.954 ± 4.762 years. The mean modified HY stage was 2.269 ± 0.874 (stage 1–2.5, $n = 323$; stage 3–5, $n = 131$). The mean UPDRS total score and UPDRS part III scores were 51.02 ± 29.340 and 28.10 ± 16.506, respectively. The mean NMS total score was 58.36 ± 48.676, and every component NMS score (from I to IX) was presented in **Table 1**.

The prevalence of patients with EMO was 39.43% ($n = 179$). The EMO group had a tendency to be older at the time of this study (70 ± 9.787 years vs. 68.23 ± 7.693 years, $P = 0.008$). Disease duration and family history did not exhibit significant differences between the two groups (**Table 1**). EMO was present in 28.14% of patients in stage 1–2, 46.03% of patients in stage 2.5–3, and 79.41% of patients in stage 4–5 (**Figure 1A**). PwPD with EMO were more likely to have a lower QOL, especially in advanced HY stages (**Figure 1B**). The reason for the distinction between stage 2.5 and below and stage 3 is based on the Chinese guidelines for the treatment of PD (fourth edition) [Chinese guidelines for the Treatment of Parkinson's disease (fourth edition)., 2020]. The EMO group also had a higher dose of levodopa-equivalent dose (LED) (631.078 ± 383.730 vs. 519.636 ± 330.735, $P = 0.003$), higher dose of L-dopa medication (500.150 ± 250.496 vs. 426.872 ± 207.557, $P = 0.003$), higher dose of monoamine oxidase-B (MAO-B) inhibitor (10.00 ± 4.364 vs. 7.554 ± 3.378, $P = 0.018$), higher UPDRS-III scores (36.68 ± 18.909 vs. 22.52 ± 11.78, $P < 0.001$), higher NMS scores (88.09 ± 58.448 vs. 39.00 ± 27.283, $P < 0.001$), higher PDSS scores (23.84 ± 9.903 vs. 11.40 ± 7.132, $P < 0.001$), lower MMSE scores (21.80 ± 6.271 vs. 26.85 ± 3.422, $P < 0.001$), and higher PDQ-39 scores (63.98 ± 31.520 vs. 25.68 ± 20.207, $P < 0.001$). Also, there were statistical differences in each item in PDSS and NMS between two groups (**Table 1**). The percentage of NMSs between the two groups was significantly different. The prevalence of NMSs in the EMO group was higher in contrast to the non-EMO group, such as sleep disturbance/fatigue (98.90 vs. 91.30%, $P < 0.001$), depressed mood/cognitive impairment (93.90 vs. 74.50%, $P < 0.001$), decreased attention/memory (91.60 vs. 74.90%, $P < 0.001$), gastrointestinal symptoms (91.60 vs. 80.00%, $P < 0.001$), urinary urgency (90.50 vs. 72.70%, $P < 0.001$), cardiovascular symptoms (76.00 vs. 37.5%, $P < 0.001$), sexual dysfunction (59.20 vs. 8.00%, $P < 0.001$), perceptual problems/hallucinations (58.70 vs. 25.10%, $P < 0.001$), and miscellaneous symptoms (86.00 vs. 69.50%, $P < 0.001$) (**Figure 1C**).

The logistic regression analysis of EMO using a likelihood ratio forward selection showed that several factors contributed to EMO, including PDSS scores (OR = 1.196, CI = 1.156–1.238, $P < 0.001$), PDQ-39 scores (OR = 1.045, CI = 1.031–1.060, $P < 0.001$), UPDRS III scores (OR = 1.048, CI = 1.030–1.066, $P < 0.001$), NMS total scores (OR = 1.016, CI = 1.008–1.024, $P < 0.001$), MMSE scores (OR = 0.887, CI = 0.837–0.940, $P < 0.001$), and total LED (OR = 0.999, CI = 0.998–1.000, $P = 0.001$; **Table 2**). We further performed the regression analysis on the sub-items of NMSS and PDSS with EMO to determine which items in NMSS and PDSS are the core driving factors of EMO. The results showed that PDSS-5 (OR = 3.282, CI = 2.267–4.752, $P < 0.001$), PDSS-13 (OR = 1.627, CI = 1.269–2.087, $P < 0.001$), PDSS-14 (OR = 2.571, CI = 1.968–3.359, $P < 0.001$), PDSS-15 (OR = 2.108, CI = 1.443–3.081, $P < 0.001$), NMS-II (OR = 0.923, CI = 0.884–0.963, $P < 0.001$), and NMS-VIII (OR = 1.142, CI = 1.083–1.204, $P < 0.001$) were core driving factors of EMO (**Table 3**).

When the PDSS scores were entered as mediators of the EMO and PDQ-39, the total effect was significant ($\beta = 38.294$, $P < 0.001$), the indirect effect was significant ($\beta = 13.458$, $P < 0.001$), and the direct effect remained significant ($\beta = 24.836$, $P < 0.001$). Therefore, we inferred that nocturnal sleep problems partially mediated the effect of EMO on QOL (**Figure 1D**).

DISCUSSION

Previous studies have focused on the effects of daytime wearing-off on PwPD (Stacy et al., 2005; Stacy, 2010). In this study, we found that EMO was observed in all stages of PD, even in 28.14% of patients in stages 1–2. In light of these findings, we suggested that EMO should be the focus of attention during the management of all stages of PD. The factors that are associated with EMO are complicated. According to the statistical results in our cohort, we showed that motor symptoms are associated with EMO. There were deteriorating motor symptoms and a more advanced HY stage in the EMO group than the non-EMO group, which is consistent with the results suggested by Onozawa et al. (Rizos et al., 2016). Therefore, patients with EMO should undertake medical intervention to counteract the disease symptoms. We also found that the total LED was a factor associated with EMO. It might be caused by people with worse

TABLE 1 | Clinical characteristics of all PD patients with and without EMO groups.

	Mean ± SD			
Variable	**With EMO (*n* = 179)**	**Without EMO (*n* = 275)**	**Total (*n* = 454)**	***P*-value**
Male (%)	54.2	57.45	56.17	0.493
Age at study, y	70 ± 9.787	68.23 ± 7.693	68.97 ± 8.620	0.008
Age of PD onset, y	63.81 ± 10.945	62.04 ± 9.711	62.73 ± 10.239	0.063
Disease duration, y	6.164 ± 5.449	5.818 ± 4.266	5.954 ± 4.762	0.979
Modified Hoehn-Yahr stage	2.534 ± 0.978	2.096 ± 0.751	2.269 ± 0.874	<0.001
UPDRS Total	70.44 ± 32.154	38.37 ± 18.57	51.02 ± 29.340	<0.001
UPDRS III	36.68 ± 18.909	22.52 ± 11.78	28.10 ± 16.506	<0.001
PDSS total score	23.84 ± 9.903	11.40 ± 7.132	16.3 ± 10.313	<0.001
PDSS-1	2.15 ± 1.134	1.55 ± 1.205	1.79 ± 1.212	<0.001
PDSS-2	1.90 ± 1.232	0.92 ± 1.257	1.30 ± 1.336	<0.001
PDSS-3	2.26 ± 1.250	1.84 ± 1.413	2.0 ± 1.365	0.002
PDSS-4	1.59 ± 1.003	0.48 ± 0.905	0.92 ± 1.090	<0.001
PDSS-5	1.45 ± 0.989	0.29 ± 0.652	0.74 ± 0.981	<0.001
PDSS-6	1.72 ± 1.190	1.06 ± 1.182	1.32 ± 1.228	<0.001
PDSS-7	0.98 ± 1.104	0.28 ± 0.693	0.56 ± 0.901	<0.001
PDSS-8	2.46 ± 1.143	2.11 ± 1.412	2.25 ± 1.323	0.005
PDSS-9	1.82 ± 1.229	0.61 ± 1.080	1.09 ± 1.283	<0.001
PDSS-10	1.28 ± 1.045	0.31 ± 0.757	0.70 ± 1.001	<0.001
PDSS-11	1.39 ± 0.996	0.56 ± 0.900	0.89 ± 1.021	<0.001
PDSS-12	1.22 ± 1.052	0.22 ± 0.607	0.61 ± 0.949	<0.001
PDSS-13	1.52 ± 1.177	0.49 ± 0.998	0.90 ± 1.183	<0.001
PDSS-14	2.18 ± 1.066	0.67 ± 1.093	1.26 ± 1.310	<0.001
PDSS-15	1.16 ± 1.032	0.22 ± 0.636	0.59 ± 0.935	<0.001
NMS Total	88.09 ± 58.448	39.00 ± 27.283	58.36 ± 48.676	<0.001
NMS-I	3.44 ± 3.892	1.43 ± 2.623	2.22 ± 3.329	<0.001
NMS-II	14.21 ± 10.132	8.74 ± 7.467	10.89 ± 9.012	<0.001
NMS-III	20.58 ± 18.101	8.03 ± 10.588	12.97 ± 15.293	<0.001
NMS-IV	3.29 ± 4.916	0.90 ± 2.240	1.84 ± 3.727	<0.001
NMS-V	9.32 ± 8.227	3.92 ± 4.864	6.05 ± 6.919	<0.001
NMS-VI	10.12 ± 8.175	4.71 ± 5.209	6.84 ± 7.048	<0.001
NMS-VII	10.20 ± 8.801	5.86 ± 7.377	7.58 ± 8.238	<0.001
NMS-VIII	7.35 ± 9.102	0.70 ± 3.390	3.32 ± 7.078	<0.001
NMS-IX	9.30 ± 8.631	4.84 ± 5.457	6.60 ± 7.213	<0.001
MMSE score	21.80 ± 6.271	26.85 ± 3.422	24.86 ± 5.351	<0.001
PDQ-39 score	63.98 ± 31.520	25.68 ± 20.207	40.78 ± 31.438	<0.001
Total LED, mg	631.078 ± 383.730	519.636 ± 330.735	563.575 ± 356.363	0.003
L-dopa medication, mg	500.150 ± 250.496	426.872 ± 207.557	457.931 ± 229.331	0.003
DA medication, mg	21.159 ± 38.517	32.159 ± 44.483	27.822 ± 42.530	0.252
MAO-B inhibitor, mg	10.00 ± 4.364	7.554 ± 3.378	8.210 ± 3.800	0.018

PD, Parkinson's disease; EMO, early morning off; PDSS, Parkinson's disease sleep scale; PDQ, Parkinson's disease questionnaire; UPDRS, unified Parkinson's disease rating scale; NMS, non-motor symptom; MMSE, minimum mental state examination; DA, dopamine agonists; MAO-B, monoamine oxidase-B; LED, levodopa equivalent dose.

EMO have worse motor symptoms and so are on higher LED dosage. Besides, the patients with EMO take a higher dosage of L-dopa medication than the non-EMO group, since they need to take a high dose of L-dopa medication to deviate their symptoms and control their condition. This study also showed that the dose of MAO-B inhibitor was higher in the EMO group than the non-EMO group; however, this study did not further explore which type of MAO-B inhibitor was more effective. The other types of MAO-B inhibitor, such as Rasagiline, were rarely used in our research than Selegiline, which was widely taken in PwPD in China. But some studies proved that Rasagiline was efficacious in reducing "off" time and in improving EMO but also some NMS, thus enhancing the therapeutic approach to PD (Stocchi et al., 2015). The most used dopamine agonists (DA) recorded in this study was Pramipexole, whereas new types of DA, such as Ropinirole and Rotigotine, were barely used in clinical practice

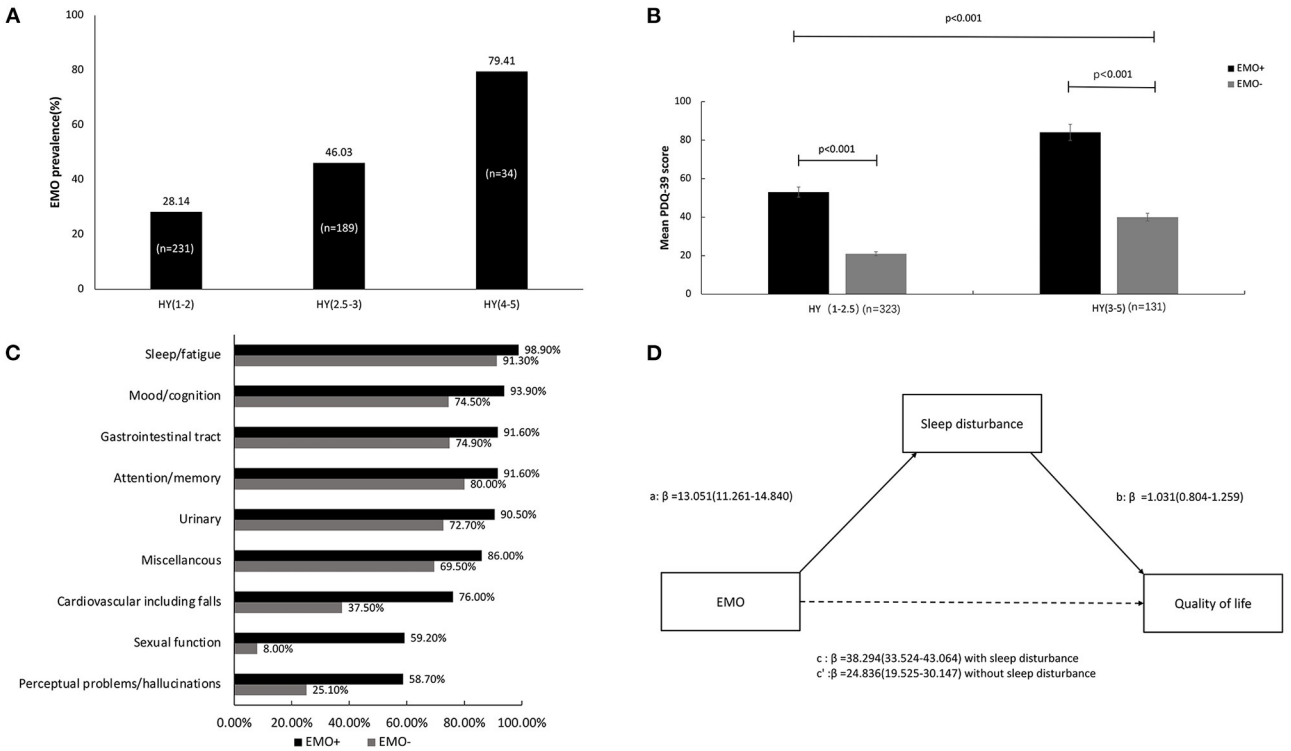


FIGURE 1 | The clinical manifestations of EMO and the association with nocturnal sleep problems and quality of life (QOL) in Chinese PWPD. **(A)** The prevalence of EMO in different stages of HY. **(B)** The comparison of mean PDQ-39 scores in different stages of HY between the EMO and non-EMO groups. **(C)** The proportion of non-motor symptoms between the EMO and non-EMO groups. **(D)** The mediation effect of nocturnal sleep problems on the association between EMO and QOL.

TABLE 2 | Multivariate binary logistic regression analyses of the factors associated with EMO.

Variables	OR (95% CI)	*P*-value
PDSS score	1.196 (1.156–1.238)	<0.001
PDQ-39 score	1.045 (1.031–1.060)	<0.001
MMSE	0.887 (0.837–0.940)	<0.001
UPDRS III	1.048 (1.030–1.066)	<0.001
NMS Total	1.016 (1.008–1.024)	<0.001
Total LED	0.999 (0.998–1.000)	0.001

PDSS, Parkinson's disease sleep scale; PDQ, Parkinson's disease questionnaire; MMSE, minimum mental state examination; UPDRS, unified Parkinson's disease rating scale; NMS, non-motor symptom; LED, levodopa equivalent dose.

TABLE 3 | Multivariate binary logistic regression analyses of sub-items in NMS and PDSS associated with EMO.

Variables	OR (95% CI)	*P*-value	Standardized Coefficients
PDSS-5	3.282 (2.267–4.752)	<0.001	0.643
PDSS-13	1.627 (1.269–2.087)	<0.001	0.318
PDSS-14	2.571 (1.968–3.359)	<0.001	0.682
PDSS-15	2.108 (1.443–3.081)	<0.001	0.385
NMS-II	0.923 (0.884–0.963)	<0.001	0.403
NMS-VIII	1.142 (1.083–1.204)	<0.001	0.519

EMO, early morning off; PDSS, Parkinson's disease sleep scale; NMS, non-motor symptom.

in China. In this study, there was no difference in the dose of DA medication between the two groups. Therefore, the impact of varieties of anti-PD medication on EMO needs a long-term clinical follow-up to clarify.

Another factor associated with EMO was NMSs. The EMO group had higher NMS scores than the non-EMO group (**Table 1**). Common NMSs include sleep disturbance/fatigue (98.90%), depression (93.90%), decreased attention/memory (91.60%), gastrointestinal symptoms (91.60%), and urinary urgency (90.50%). Rizos et al. (Rizos et al., 2014) reported that the common NMSs consist of urination (61.3%), anxiety (49.7%), drooling (46.6%), pain (46.6%), low mood (45.5%), and paresthesia (42.6%). Hence, the NMSs of the EMO group were wide-ranging and should be evaluated. Moreover, nocturnal sleep problems, such as an NMS, were more significant in the EMO group. Although the EMO group had higher NMS scores of each item than the non-EMO group, our results cannot directly conclude that which NMS can distinguish patients with EMO and non-EMO, which requires prospective studies to prove. Moreover, further analysis showed that sexual dysfunction (NMS-VIII) and cognitive impairment (NMS-II) in the NMS are the main factors that affect EMO. Therefore, our results

indicated that EMO could be a clinical marker of, not only sleep problems but also possibly a "dysautonomia/cognitive" aggressive phenotype and help raise awareness for clinical practice implications.

In this study, we verified that EMO significantly decreases the QOL of PwPD and that this is partially mediated by nocturnal sleep problems. Our results showed that the EMO group had higher PDQ-39 scores, thus they have an inferior QOL compared with the non-EMO group. Nocturnal sleep problems can also have a negative impact on QOL (Gómez-Esteban et al., 2011; Schrempf et al., 2014), and this study revealed that nocturnal sleep problems were the factors associated with EMO. We, therefore, analyzed the effect of nocturnal sleep problems and EMO on QOL. Nocturnal sleep problems on QOL were revealed to have a mediating effect according to this study. The QOL of patients with EMO could be improved not only by the management of EMO but also by the treatment of nocturnal sleep problems. At the same time, we should monitor the sleep quality of PD patients with EMO who do not suffer from nocturnal sleep problems and be alert to their occurrence. Therefore, both motor and NMSs of patients with EMO should be evaluated.

There were several limitations in this study. First, this study was retrospective, so the causality between EMO, nocturnal sleep problems, and QOL could not be confirmed. It is crucial to conduct prospective studies for verification. Second, the patients were evaluated at their "on" state, which largely required them to recall symptoms, which might lead to recall bias. Finally, due to the lack of a standard for accurate quantitative evaluation of EMO, there is a margin of error in the accuracy assessment.

This cross-sectional non-interventional study provides prevalence information regarding EMO phenomena in Chinese PwPD. This study provided a good characterization of motor function, NMSs, and sleep disorder characterization of EMO vs. non-EMO. It indicated that EMO is a common symptom in all stages of PD and is associated with higher disease burden and poor QOL in Chinese PwPD, and that sleep disorders may play a contributing role to EMO. This study established that nocturnal sleep problems mediate the detrimental effects on QOL in PD caused by EMO. These findings are important to create more awareness of EMO, screen for EMO, and especially sleep disorders in clinical practice. Further research should confirm causality and the impact on sleep interventions in EMO.

DATA AVAILABILITY STATEMENT

The original contributions generated for this study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Research Ethics Committee of Xin Hua Hospital affiliated to Shanghai Jiao Tong University School of Medicine. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YuZ and ZZ: analysis and interpretation of data and drafting and critical revision of the manuscript. SG, YiZ, LH, YaZ, HW, JZ, FW, CZ, DS, and WW: enrollment of patients and critical revision of this manuscript. DH and ZL: critical revision of this manuscript and supervision of this study. For the detailed information for members of the Shanghai Parkinson's Disease Study Group, see **Supplementary Material 1**. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Cerebral Cortex Apoptosis in Early Aged Hypertension: Effects of Epigallocatechin-3-Gallate

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This study aimed to investigate cerebral cortex apoptosis on the early aged hypertension and the effects of green tea flavonoid epigallocatechin-3-gallate (EGCG). Twenty-four rats were divided into three groups: a control Wistar-Kyoto group (WKY, n = 8), a spontaneously early aged hypertensive group (SHR, n = 8), and an early aged hypertension with EGCG treatment group (SHR-EGCG, n = 8; daily oral EGCG 200 mg/kg—94%, 12 weeks). At 48 weeks old, blood pressures (BPs) were evaluated and cerebral cortexes were isolated for TUNEL assay and Western blotting. Systolic, diastolic, and mean blood pressure levels in the SHR-EGCG were reduced compared to the SHR. The percentage of neural cell deaths, the levels of cytosolic Endonuclease G, cytosolic AIF (Caspase-independent apoptotic pathway), Fas, Fas Ligand, FADD, Caspase-8 (Fas-mediated apoptotic pathway), t-Bid, Bax/Bcl-2, Bak/Bcl-xL, cytosolic Cytochrome C, Apaf-1, Caspase-9 (Mitochondrial-mediated apoptotic pathway), and Caspase-3 (Fas-mediated and Mitochondria-mediated apoptotic pathways) were increased in the SHR relative to WKY and reduced in SHR-EGCG relative to SHR. In contrast, the levels of Bcl-2, Bcl-xL, p-Bad, 14-3-3, Bcl-2/Bax, Bcl-xL/Bak, and p-Bad/Bad (Bcl-2 family-related pro-survival pathway), as well as Sirt1, p-PI3K/PI3K and p-AKT/AKT (Sirt1/PI3K/AKT-related pro-survival pathway), were reduced in SHR relative WKY and enhanced in SHR-EGCG relative to SHR. In conclusion, green tea flavonoid epigallocatechin-3-gallate (EGCG) might prevent neural apoptotic pathways and activate neural survival pathways, providing therapeutic effects on early aged hypertension-induced neural apoptosis.

Keywords: EGCG, hypertension, neural apoptosis, neural survival, caspase-independent apoptosis

INTRODUCTION

Hypertension is considered as the common cause of mortality (ESC/ESH, 2018). In hypertension, the brain suffers oxidative stress and inflammatory processes, which lead to neurodegeneration characterized by the progressive loss of neurons (Gąsecki et al., 2013; Hou et al., 2018). Neural apoptosis was reported in either hypertension or aged hypertension (Mignini et al., 2004; Poulet et al., 2006; Li et al., 2012, 2016; Gąsecki et al., 2013). Neural apoptosis in hippocampi is not found in the young (16-week-old) hypertensive brain, is further enhanced in the mature (32 week-old) hypertensive brain, and remarkably augmented in aged (64-week-old) hypertension (Li et al., 2016). Neural apoptosis is promoted by several major apoptotic pathways, including Endonuclease G (EndoG) and Apoptosis-inducing factor (AIF)-related Caspase-independent apoptotic pathway, Fas-mediated Caspase-dependent apoptotic pathway, and mitochondrial-mediated Caspase-dependent apoptotic pathway (Yakovlev and Faden, 2004). EndoG is not only the mitochondrial specific endonuclease but also the Caspase-independent factor, released into the cytosol in brain damage (Yakovlev and Faden, 2004). AIF is a mitochondrial oxidoreductase, also discharged to cytosol in response to death stimuli (Yu et al., 2010). During neural apoptosis, the cytosolic EndoG and AIF translocate into the nucleus, activating the neural Caspase-independent apoptotic manner through large-scale DNA loss and chromatin condensation (Bastianetto et al., 2011). To the best of our knowledge, the alterations of EndoG and AIF-related Caspase-independent apoptotic pathway in the early aged (48-week-old) hypertensive brain have not been investigated yet.

Fas-mediated Caspase-dependent apoptotic pathway in the brain is commenced by the binding of Fas ligand (FasL) and Fas receptor, which in turn recruits Fas-associated protein with death domain (FADD). Then, FADD activates Caspase-8, followed by Caspase-3 cleavage to execute the neural apoptosis (Choi and Benveniste, 2004). Caspase-8 also cleaves Bid to form truncated Bid (t-Bid) which translocates to mitochondria, playing as the intracellular apoptotic signal from the Fas-mediated Caspase-dependent apoptotic pathway to the mitochondrial Caspase-dependent apoptotic pathway (Akhtar et al., 2004). In the early aged hypertensive cerebral cortex, the alterations of the Fas-mediated apoptotic pathway have still not been explored.

The mitochondrial-mediated Caspase-dependent apoptotic pathway in the cerebral cortex is promoted by Bcl-2 pro-apoptotic factors in which t-Bid induces the oligomerization of Bax, Bak, and Bad. Those factors (t-Bid, Bax, Bak, and Bad) augment the release of Cytochrome *C* from mitochondria to the cytosol (Akhtar et al., 2004). Cytosolic Cytochrome *C* binds Apaf-1 to activate Caspase-9 and then Caspase-3, promoting the neural apoptotic process (Czabotar et al., 2014). On the contrary, in the Bcl-2 family-related pro-survival pathway, Bcl-2 family pro-survival factors (Bcl-2, Bcl-xL, and p-Bad) prevent the activities of Bax, Bak, and Bad, thereby reducing the levels of cytosolic Cytochrome *C* and preventing neural apoptosis (Akhtar et al., 2004; Czabotar et al., 2014). Bad is phosphorylated to form p-Bad, then binds with protein 14-3-3, activating pro-survival activity (Akhtar et al., 2004). Previous studies showed that Bcl-2 and Bcl-xL were decreased whereas Bax, cytochrome *C*, and caspase-3 were not increased in young about 4 weeks old but increased in mature hypertensive brain indicating that hypertension activated the mitochondrial-mediated Caspase-dependent apoptotic pathway and suppressed the Bcl-2 pro-survival pathway in the brain (Li et al., 2012, 2016).

Sirtuin 1 (Sirt1), a key factor of mitochondrial biogenesis, has been proven to provide neuroprotective effects by regulating several mechanisms, including the activation of neural survival pathways (Pfister et al., 2008). The PI3K/AKT related pro-survival pathway is the main pathway which protects the brain against neurodegeneration (Ersahin et al., 2015). Once triggered by many trophic factors, the phosphorylated PI3K (p-PI3K) activates the phosphorylation of AKT (p-AKT), promoting the downstream components to control the neural survival in the brain (Brunet et al., 2001; Ersahin et al., 2015). Besides, Sirt1 has been reported to activate p-AKT to enhance neural survival and reduce neural apoptosis in previous studies (Li and Wang, 2017; Teertam and Prakash Babu, 2021). Furthermore, p-AKT enhances the phosphorylation of Bad to form p-Bad, activating Bcl-2 family-related pro-survival members such as Bcl-2 and Bcl-xL to prevent neural apoptosis (Brunet et al., 2001). A study showed that hypertension reduced the phosphorylation of AKT, which, thereby inhibited the PI3K/AKT related pro-survival pathway in the brain of Stroke-Prone Spontaneously Hypertensive Rats (Yoshitomi et al., 2011).

Epigallocatechin-3-gallate (EGCG)- $C_{22}H_{18}O_{11}$, the primary flavonoids extraction from green tea are characterized by anti-oxidant and anti-inflammation properties (Singh et al., 2011; Li et al., 2019). EGCG was reported to reduce blood pressure (BP) and improve memory functions in hypertensive individuals (Wang et al., 2012; Yi et al., 2016). Besides, studies have indicated that EGCG was able to attenuate neural apoptosis after stroke or traumatic brain injury (Itoh et al., 2013; Nan et al., 2018), but no previous research investigated the effects of EGCG on the hypertension-promoted cerebral cortex neural apoptosis.

The effects of EGCG on neural apoptotic and pro-survival pathways in the early aged hypertensive brain have remained unclear. We hypothesized that cerebral cortex apoptosis on the early aged hypertension is enhanced as well as that EGCG might prevent neural EndoG and AIF-related Caspase-independent apoptotic pathway, Fas-mediated Caspase-dependent apoptotic pathway, and mitochondrial-mediated Caspase-dependent apoptotic pathway, as well as enhance Bcl-2 family-related and Sirt1/PI3K/AKT related pro-survival pathways, in the cerebral cortex under early aged hypertension.

MATERIALS AND METHODS

Animals

The male normotensive Wistar-Kyoto rats (WKY, n = 8) and male spontaneously hypertensive rats (n = 16) at 28-weeks old were purchased. All of the rats were housed at 22–24°C, 12 h light/dark cycle. They were freely accessed the standard chow (Lab Diet 5001; PMI Nutrition International, Brentwood, MO,

United States), water *ad libitum*. The procedures were approved by the Institutional Animal Care and Use Committee of China Medical University, Taichung, Taiwan.

Animal Experimental Protocol

At 36 weeks old, spontaneously hypertensive rats were randomly allocated into a spontaneously early aged hypertensive group (SHR; $n = 8$) and an early aged hypertension with EGCG treatment group (SHR-EGCG; $n = 8$). Rats in the SHR-EGCG group were treated daily by oral TEAVIGO (EGCG 94%; 200 mg/kg/day, once/day, between 7 and 17 am, 12 weeks), whereas the WKY and SHR groups did not receive any treatment. TEAVIGO, a highly purified green tea extract with 94% EGCG, was purchased from Healthy Origins, Pittsburgh, PA, United States.

Blood Pressure Measurement

The BP-98A non-invasive BP system (a tail-cuff method, Softron, Tokyo, Japan) was used to evaluate systolic BP (SBP), diastolic BP (DBP), mean BP (MBP), and pulse pressure (PP) of rats from 32 weeks old to 48 weeks old, 4 weeks once and after EGCG treatment for at least 1 h. In each instance, BP measurements (SBP and DBP) were repeatedly recorded three times, then the average of them was calculated and regarded as SBP and DBP in each rat. MBP was calculated based on formula [MBP = DBP + 1/3 (SBP–DBP)]. Pulse pressure is defined as SBP–DBP. Values of SBP, DBP, MBP and PP in each group were expressed as means ± SEM from $n = 8$ of different rats in WKY, SHR or SHR-EGCG group.

Brain Tissue Preparation

Rats were weighed before they were anesthetized by inhaled isoflurane 2% in a 70% oxygen mixture. Brains were removed, washed in ice-cold saline, and weighed. The cerebral cortex was dissected and divided into two sections for TUNEL assay-DAPI staining and Western blotting.

TUNEL Assay and DAPI Staining

A neutral formalin solution was used to fix the cerebral cortex tissues. The samples were deparaffinized and rehydrated, incubated with proteinase K, then washed three times with PBS, soaked in permeabilization solution and blocking buffer, followed by 02 washes with PBS. Then, the slides were immersed in terminal deoxynucleotidyl transferase (TdT) and fluorescein isothiocyanate-dUTP conjugated using the *in situ* Cell Death Detection Kit, Fluorescein (Roche Applied Science), followed by washing with PBS. The slides were mounted by using DAPI fluoromount G (Southern Biotech, Birmingham, AL, United States). TUNEL-positive nuclei fluoresced bright green at 450–500 nm. The DAPI-positive nuclei were fluoresced by blue light at 340–380 nm. All counts were independently conducted by two people in a blinded manner.

Western Blotting Analysis

The cerebral cortex tissues were homogenized in tissue protein extraction reagent (T-PER, Thermo Scientific) with protease inhibitors cocktail (Roche Applied Science, Germany). The protein extracts were obtained and then centrifuged at 12,000 *g* for 40 min to collect the supernatant. The Bio-Rad Protein assay (Hercules, CA, United States) was used to measure the protein concentrations of the cerebral cortex tissue. Protein samples (100 μg/well) were cataphoresized on a 12% SDS-PAGE at 80 V in the first 20 min, 100 V in the next 2 h, followed by transfer to polyvinylidene difluoride membranes (Millipore, Bedford, MA, United States) at 100 V in 90 min. Membranes were blocked in 5% skimmed milk at 25°C for 1 h, then were incubated overnight at 4°C with primary antibodies which were diluted 1,000 times, including EndoG, AIF, FasL, Fas, FADD, t-Bid, Bax, Bak, Bad, Bcl-2, Bcl-xL, p-Bad, 14-3-3, Cytochrome C, Apaf-1, Caspase-8, Caspase-9, Caspase-3, PI3K, p-PI3K, AKT, p-AKT, α-tubulin (Santa Cruz Biotech), Sirt1 (Cell Signaling Tech, Danvers, MA, United States), and COX IV (Taiclone Biotech Corp., Taipei, Taiwan). We washed the immunoblots four times with TBST. The immunoblots then were immersed 1 h in the HRP-conjugated secondary antibody solution (Santa Cruz), which was diluted 5,000 times, followed by 04 washes with TBST. The protein intensities were visualized using an enhanced chemiluminescence HRP substrate kit (Millipore Corporation) and the Fujifilm LAS-3000 system (Tokyo, Japan). The density of the bands was quantified by densitometry using Gel-Pro Analyzer densitometry software (Media Cybernetics, Silver Spring, MD, United States). The protein levels based on density of the bands was compared among groups expressed as fold changes relative to the control group and mean values ± SEM ($n = 6$ of different rats in each group).

Statistical Analysis

The data of bodyweight, brain weight, TUNEL-positive cell percentages, and protein levels among WKY, SHR, and SHR-EGCG groups were compared by one-way ANOVA with the *post hoc* test. To compare BP, we repeated the measurement of ANOVA which was used with two factors (group and week-old) and the Bonferroni-adjusted multiple comparison test. We used SPSS 22.0 software for analysis. $P < 0.05$ was considered as a significant difference.

RESULTS

Experimental Animal Characteristics

Body weight and brain weight were unchanged among the WKY, SHR, and SHR-EGCG groups (**Table 1**). The heart rate in the SHR group was higher than those in the WKY group, whereas the heart rate in the SHR-EGCG group was lower than those in the SHR group (**Table 1**). During the entire study period, the levels of SBP, DBP, MBP, and PP in the WKY group were significantly lower than those in SHR and SHR-EGCG groups (**Figure 1**). However, after 8 weeks of EGCG treatment, the levels of SBP and MBP in the SHR-EGCG group were significantly reduced when compared to the SHR group (**Figure 1**). After 12 weeks of EGCG treatment, the levels of SBP, DBP, and MBP in the SHR-EGCG group were significantly reduced when compared to the SHR group (**Table 1** and **Figure 1**). The PP levels were unchanged

TABLE 1 | Characteristics of the experimental animal groups.

	WKY	SHR	SHR-EGCG
Number of animals	8	8	8
Body weight, g	403 ± 4	404 ± 6	412 ± 6
Brain weight, g	2.25 ± 0.02	2.29 ± 0.04	2.33 ± 0.04
Heart rate, beats/min	277 ± 10	387 ± 16*	341 ± 7*#
Systolic blood pressure, mmHg	112 ± 4	194 ± 2*	174 ± 2*#
Diastolic blood pressure, mmHg	89 ± 5	156 ± 2*	138 ± 5*#
Mean blood pressure, mmHg	95 ± 4	169 ± 2*	150 ± 3*#
Pulse pressure, mmHg	24 ± 2	39 ± 2*	36 ± 5*

Values are expressed as means ± SEM.
WKY, Wistar Kyoto rats; SHR, spontaneously early aged hypertensive rats; SHR-EGCG, spontaneously early aged hypertensive rats with Epigallocatechin-3-gallate (EGCG) treatment.
**p < 0.05 in comparison between the SHR group or SHR-EGCG group and WKY group.*
#p < 0.05 in comparison between the SHR-EGCG group and SHR group.

between the SHR and SHR-EGCG groups after 12 weeks of EGCG treatment (**Table 1** and **Figure 1**).

Neural Cell Deaths Comparison Among Three Groups

To determine the effects of EGCG on neural cell deaths in early aged hypertension, TUNEL assay and DAPI staining were carried out on cerebral cortex tissues from WKY, SHR, and SHR-EGCG groups. The number of TUNEL-positive neural cells in the SHR group were significantly increased when compared to the WKY group. On the contrary, the number of TUNEL-positive neural cells in the SHR-EGCG group were significantly reduced in comparison with the SHR group (**Figure 2**).

EndoG and AIF-Related Caspase-Independent Apoptotic Pathway

To evaluate the EGCG effects on neural EndoG and AIF-related Caspase-independent apoptotic pathway in early aged hypertension, Western blotting was carried out to evaluate the protein distributions of EndoG and AIF between mitochondria and cytosol in the cerebral cortex tissue among the WKY, SHR, and SHR-EGCG groups. The protein ratios of cytosolic EndoG to mitochondrial EndoG and cytosolic AIF to mitochondrial AIF in the SHR group were significantly greater than those in the WKY group. In contrast, those ratios in the SHR-EGCG group were significantly lower than those in the SHR group (**Figure 3**).

Upstream Components of Fas-Mediated Caspase-Dependent Apoptotic Pathway

In order to determine the EGCG effects on the upstream components of neural Fas receptor Caspase-dependent apoptotic pathway in early aged hypertension, we evaluated the protein expressions of FasL, Fas, and FADD in the cerebral cortex tissues from the WKY, SHR, and SHR-EGCG groups. The protein expressions of FasL, Fas, and FADD in the SHR group were significantly increased in comparison with the WKY group. However, those levels in the SHR-EGCG group were significantly reduced in comparison with the SHR group (**Figure 4**).

Upstream Components of Mitochondrial-Mediated Caspase-Dependent Apoptotic Pathway and Bcl2 Family-Related Pro-survival Pathway

To determine the effects of EGCG on the upstream components of mitochondrial-mediated Caspase-dependent apoptotic pathway and Bcl-2 family-related pro-survival pathway in the early aged hypertensive brain, Bcl-2 family-related pro-apoptotic factors (t-Bid, Bax, Bak, and Bad) as well as Bcl-2 family-related pro-survival factors (Bcl-2, Bcl-xL, p-Bad and 14-3-3) expressions in cerebral cortex tissue from WKY, SHR, SHR-EGCG groups were evaluated by Western blotting. The levels of t-Bid, Bax/Bcl-2, Bak/Bcl-xL, but not Bad in the SHR group were significantly higher than those in the WKY group, whereas those levels in SHR-EGCG group were significantly lower than those in the SHR group. Bad levels were unchanged among the WKY, SHR, and SHR-EGCG groups. In contrast, the protein levels of Bcl-2, Bcl-xL, p-Bad, and 14-3-3, as well as pro-survival indices Bcl-2/Bax, Bcl-xL/Bak, and p-Bad/Bad, were significantly reduced in the SHR group when compared to the WKY group, whereas those indices were significantly increased in the SHR-EGCG group when compared to the SHR group (**Figure 5**).

Downstream Components of Fas-Mediated and Mitochondrial-Mediated Caspase-Dependent Apoptotic Pathway

To determine whether EGCG attenuates downstream components of neural Fas-mediated and mitochondrial-mediated Caspase-dependent apoptotic pathways in early aged hypertension, Western blotting was carried out to compare the protein expressions of Caspase-8 (Fas-mediated), mitochondrial Cytochrome *C*, cytosolic Cytochrome C, Apaf-1, Caspase-9 (mitochondrial-mediated), and Caspase-3 (Fas-mediated and mitochondrial-mediated) in cerebral cortex tissues among WKY, SHR, and SHR-EGCG groups. The levels of Caspase-8 (Fas-mediated), Apaf-1, Caspase-9, cytosolic Cytochrome C to mitochondrial Cytochrome C ratio (mitochondrial-mediated), Caspase-3 (Fas-mediated and mitochondrial-mediated), in the SHR group were significantly increased when compared to the WKY group. However, those levels in the SHR-EGCG group were significantly reduced in comparison with the SHR group (**Figure 6**).

Sirt1/PI3K/AKT-Related Pro-survival Pathway

To evaluate the effects of EGCG on neural pro-survival pathway under early aged hypertension, the levels of Sirt1, PI3K, p-PI3K, AKT, and p-AKT in the cerebral cortex tissue from WKY, SHR, and SHR-EGCG groups were determined by Western blotting. The levels of Sirt1, p-PI3K to PI3K ratio and the p-AKT to AKT

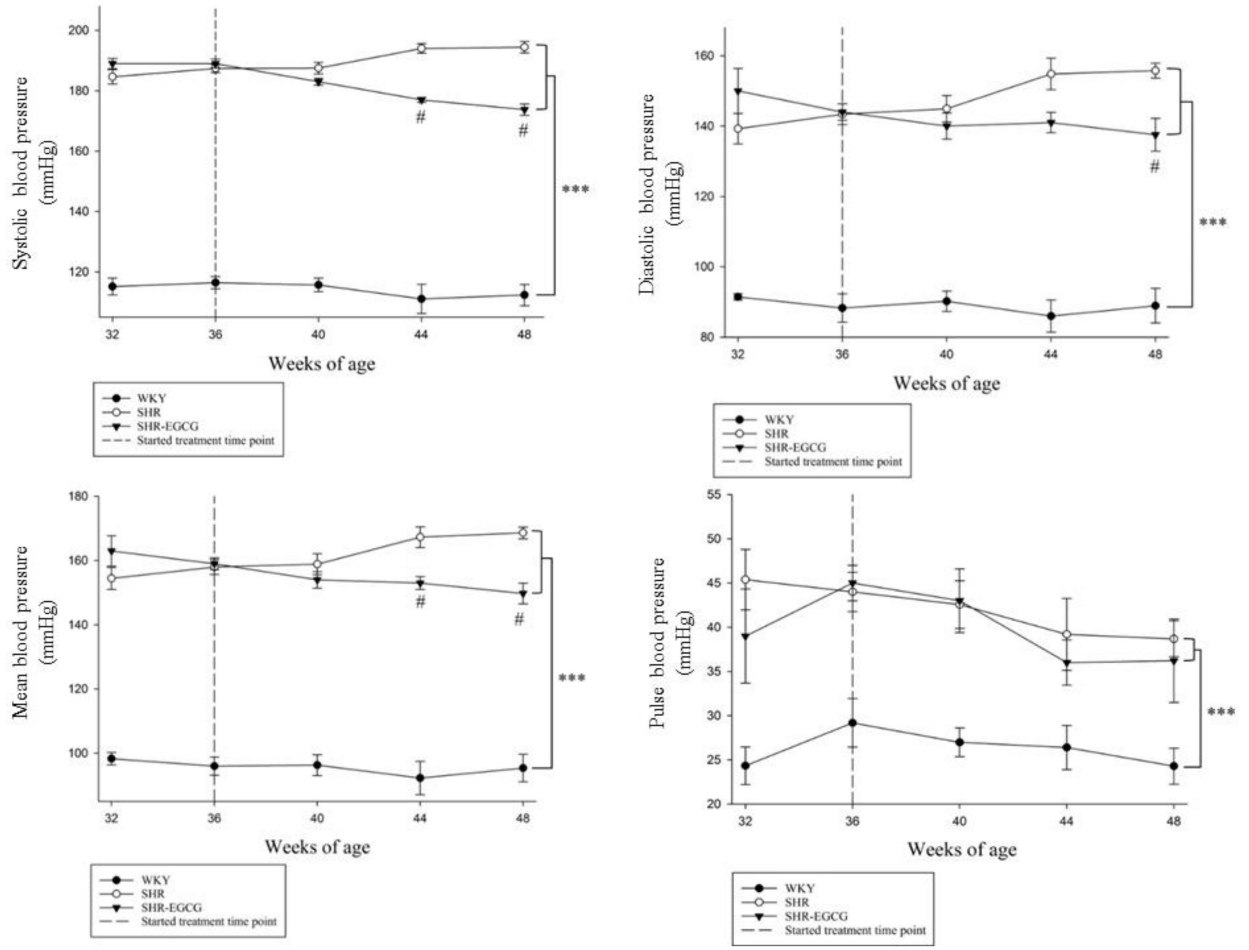


FIGURE 1 | The line chart of systolic blood pressure (SBP), diastolic blood pressure (DBP), mean blood pressure (MBP), and pulse pressure (PP) levels with measurement time points in a control Wistar Kyoto (WKY) group, a spontaneously early aged hypertensive (SHR) group and an early aged hypertension with Epigallocatechin-3-gallate (EGCG) treatment (SHR-EGCG) group. Data were expressed as means ± SEM. ***$p < 0.001$ in the comparison between the SHR group or SHR-EGCG group with the WKY rat group. #$p < 0.05$ in the comparison between the SHR-EGCG group and the SHR group.

ratio in the SHR group were significantly reduced in comparison with the WKY group. However, those levels in the SHR-EGCG group were significantly increased when compared to the SHR group (**Figure 7**).

DISCUSSION

In the current study, the new findings were found as follows: (1) Early aged hypertension activated neural EndoG and AIF-related Caspase-independent, Fas-mediated Caspase-dependent, and mitochondrial-mediated Caspase-dependent apoptotic pathways as well as inhibited Bcl-2 family-related and Sirt1/PI3K/AKT related pro-survival pathways in the cerebral cortex. (2) EGCG treatment for 12 weeks decreased systolic blood pressure, diastolic blood pressure, and mean blood pressure in early aged hypertensive rats. (3) EGCG treatment reduced early aged hypertension-induced TUNEL positive cells in the cerebral cortex; (4) EGCG treatment reduced the early aged hypertension-activated EndoG and AIF-related Caspase-independent apoptotic pathway, in which the evidence was based on decreases in ratios of cytosolic EndoG to mitochondrial EndoG as well as cytosolic AIF to mitochondrial AIF. (5) EGCG treatment reduced early aged hypertension-activated Fas-mediated Caspase-dependent apoptotic pathway, in which the evidence was based on decreases in expression levels of FasL, Fas, FADD, Caspase-8, and Caspase-3 in the cerebral cortex. (6) EGCG treatment attenuated early aged hypertension-activated neural mitochondrial-mediated Caspase-dependent apoptotic pathway, in which the evidence was based on decreases in expression levels of t-Bid, Bax/Bcl-2, Bak/Bcl-xL, Apaf-1, cytosolic Cytochrome *C*, Caspase-9, and Caspase-3 in the cerebral cortex; (7) EGCG treatment enhanced Bcl-2 family-related pro-survival protein levels (Bcl-2, Bcl-xL, p-Bad, 14-3-3) and Sirt1/PI3K/AKT related pro-survival protein levels (Sirt1, p-PI3K/PI3K, p-AKT/AKT) in the early aged hypertensive cerebral cortex. Taken our findings and the previously apoptotic theories together, we drew the hypothesized diagram (**Figure 8**),

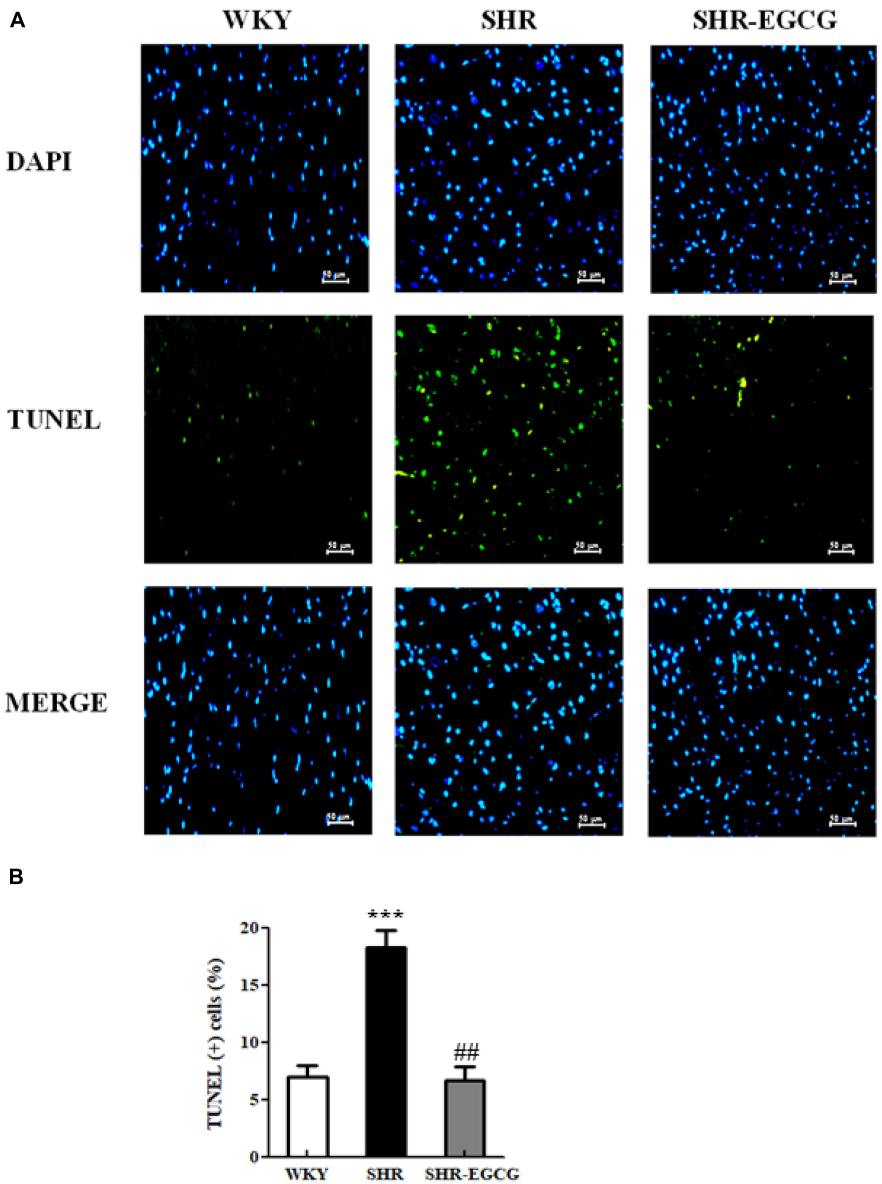


FIGURE 2 | The TUNEL-positive neural cells in a control Wistar Kyoto (WKY) group, a spontaneously early aged hypertensive (SHR) group, and an early aged hypertension with Epigallocatechin-3-gallate (EGCG) treatment (SHR-EGCG) group. **(A)** Representative photomicrographs of DAPI staining (top, blue spots) and TUNEL assay (bottom, green spots) in cerebral cortex sections from the WKY, SHR, and SHR-EGCG groups (400× magnification). **(B)** The bar chart represents the percentage of TUNEL-positive neural cells relative to total DAPI-stained cells and expresses as mean values ± SEM ($n = 6$ in each group). ***$p < 0.001$ in the comparison between the SHR group and WKY rat group; ##$p < 0.01$ in the comparison between SHR-EGCG group and SHR group.

which suggests that the cerebral cortex EndoG and AIF-related Caspase-independent, Fas-mediated Caspase-dependent and mitochondrial-mediated Caspase-dependent apoptotic pathways were augmented by early aged hypertension and were attenuated by EGCG treatment. In contrast, the cerebral cortex Bcl-2 family-related and Sirt1/PI3K/AKT related pro-survival pathways were suppressed by early aged hypertension and were enhanced after EGCG treatment. Our study found that EGCG appeared to attenuate neural apoptosis and enhance neural survival in the early aged hypertensive cerebral cortex.

Chronic hypertension or aged hypertension is a high-risk factor of neurodegeneration (Kruyer et al., 2015). Neural apoptosis of the brain was augmented by oxidative stress in hypertension or aged hypertension (Poulet et al., 2006; Li et al., 2012). A previous study showed that the Bax/Bcl-2 ratio was increased in the mature hypertensive cerebral cortex (Li et al., 2012). Another study observed the elevated levels of Bax and Cytochrome *C* proteins along with the reduced levels of Bcl-2 protein in the mature or aged hypertensive brain when compared to the age-match normal brain (Li et al., 2016).

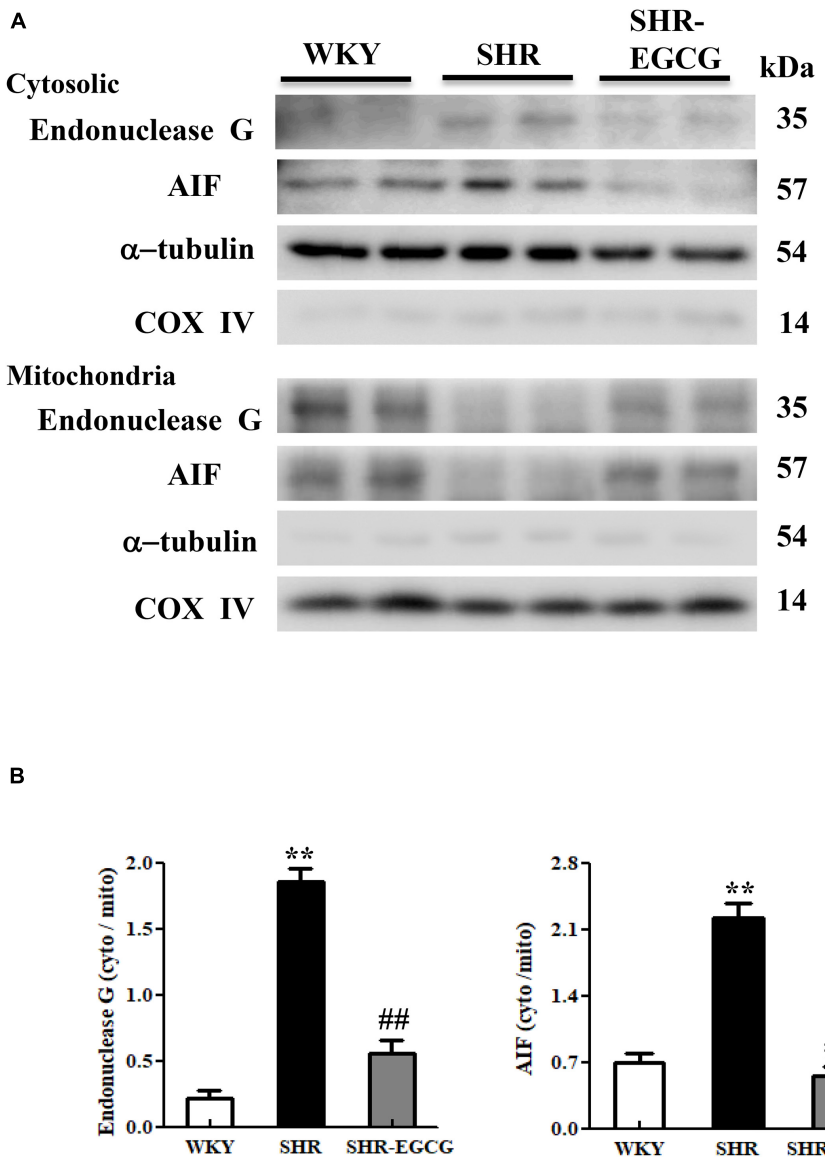


FIGURE 3 | The Caspase-independent Endonuclease G (EndoG) and Apoptosis-inducing factor (AIF) apoptotic pathway in a control Wistar Kyoto (WKY) group, a spontaneously early aged hypertensive (SHR) group and an early aged hypertension with Epigallocatechin-3-gallate (EGCG) treatment (SHR-EGCG) group. **(A)** Representative Western blotting images of cytosolic AIF, mitochondrial AIF, cytosolic EndoG and mitochondrial EndoG in the cerebral cortex tissues from WKY, SHR, and SHR-EGCG groups with α-tubulin as an internal control. **(B)** Bars represent the protein quantification of cytosolic EndoG to mitochondrial EndoG as well as cytosolic AIF to mitochondrial AIF normalized by α-tubulin and COX IV, expressed as fold changes relative to the WKY group and mean values ± SEM ($n = 6$ in each group). $^{**}p < 0.01$ and $^{***}p < 0.001$ in the comparison between the SHR group and WKY rat group; $\#\#p < 0.01$, in the comparison between the SHR-EGCG group and SHR group.

Besides, hypertension has been shown to reduce the protein levels of PI3K and p-AKT in the cerebral cortex of stroke-prone spontaneously hypertensive rats (Yoshitomi et al., 2011). Consistent with those data, we found that early aged hypertension not only activated the mitochondrial-mediated Caspase-dependent apoptotic pathway but also inhibited Bcl-2 family-related and PI3K/AKT related pro-survival pathways in the cerebral cortex. Furthermore, our study additionally clarified that early aged hypertension also augmented the EndoG and AIF-related Caspase-independent and Fas-mediated Caspase-dependent apoptotic pathways. This new finding added more evidence of hypertension-induced neural apoptosis *via* EndoG/AIF-related and Fas-mediated pathways.

Our study found that the systolic blood pressure, diastolic blood pressure, and mean blood pressure in hypertensive rats were reduced by 12 weeks of EGCG treatment. In agreement with our findings, a previous study showed that

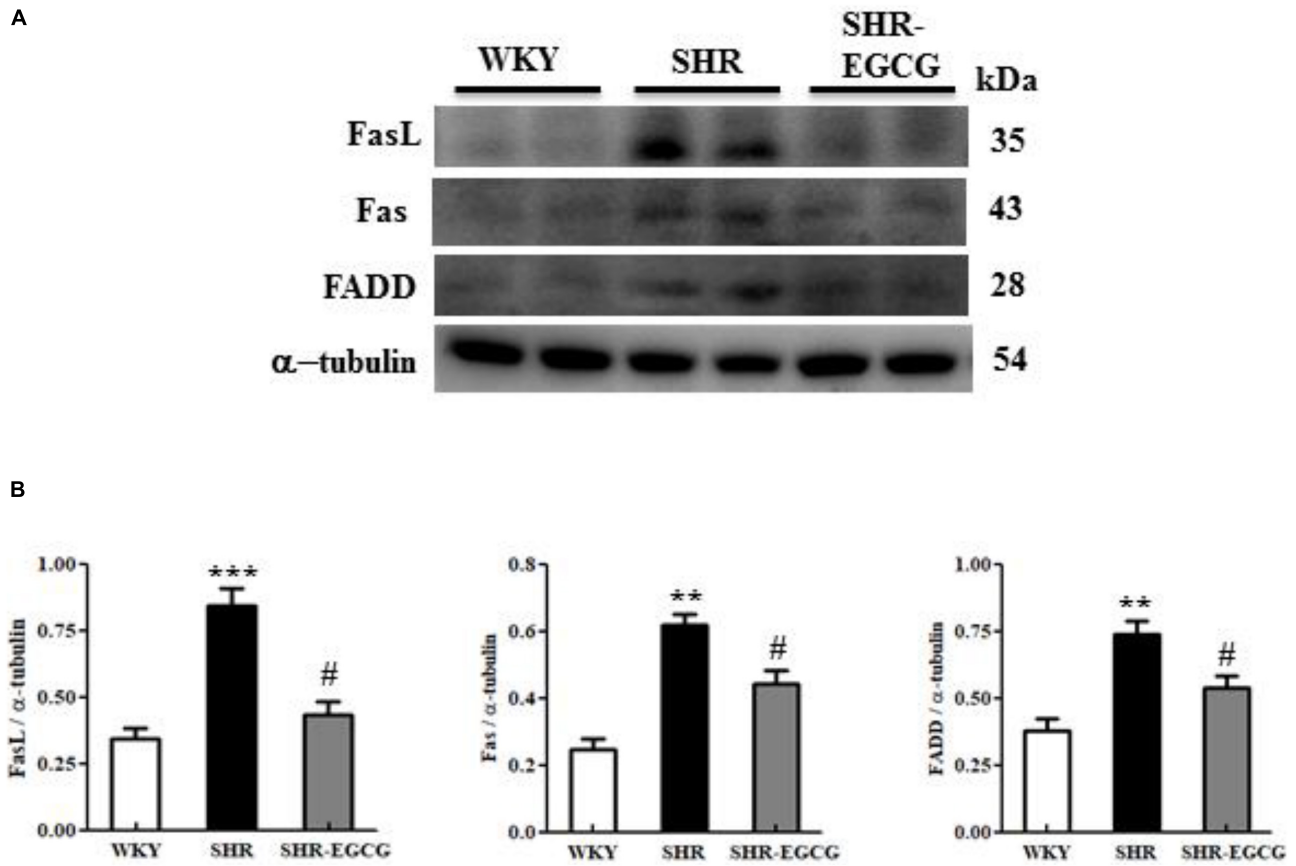


FIGURE 4 | The upstream components of the Fas-mediated Caspase-dependent apoptotic pathway in a control Wistar Kyoto (WKY) group, a spontaneously early aged hypertensive (SHR) group and an early aged hypertension with Epigallocatechin-3-gallate (EGCG) treatment (SHR-EGCG) group. **(A)** Representative Western blotting images of Fas ligand (FasL), Fas receptor (Fas), and Fas-associated death domain (FADD) in the cerebral cortex tissues from the WKY, SHR, and SHR-EGCG groups with α-tubulin as an internal control. **(B)** Bars represent the protein quantification of FasL, Fas, and FADD normalized by α-tubulin, expressed as fold changes relative to the WKY group and mean values ± SEM (n = 6 in each group). $^{**}p < 0.01$ and $^{***}p < 0.001$ in the comparison between the SHR group and the WKY rat group. $\#p < 0.05$ and $\#\#p < 0.01$, in the comparison between SHR-EGCG group and SHR group.

EGCG treatment decreased blood pressure *via* balancing neurotransmitters and cytokines in the hypothalamic paraventricular nucleus (Yi et al., 2016). Another study observed that EGCG treatment attenuated the endothelial dysfunction in vessels, leading to reduced blood pressure in spontaneously hypertensive rats with myocardial ischemia/reperfusion injury (Potenza et al., 2007). Besides, a study suggested that the renin-angiotensin system, a major hormone system that regulates blood pressure, was inhibited by EGCG treatment *in vitro* (Li et al., 2013). Taken together, our findings supported that EGCG treatment could reduce blood pressure.

The current study demonstrated that EGCG treatment reduced the percentage of neural cell deaths in the early aged hypertensive cerebral cortex, suggesting that EGCG treatment could attenuate neural deterioration in the cerebral cortex against early aged hypertension. Consistent with our study, a previous study reported that 20 mg/kg EGCG intraperitoneal administration suppressed the TUNEL-positive neural cells in the brain of ischemic stroke rats (Nan et al., 2018). Additionally, another study observed that 2 mg/kg/day EGCG subcutaneous injection attenuated the TUNEL-positive hippocampal cells in aging mice (He et al., 2009). Therefore, we confirmed that EGCG treatment could provide the protective effects on neural cells in the cerebral cortex under early aged hypertension.

As reported in this study, EGCG treatment prevented overactive EndoG and AIF-related Caspase-independent apoptotic pathway in early aged hypertension, as evidenced by reductions in the ratios of cytosolic EndoG to mitochondrial EndoG as well as cytosolic AIF to mitochondrial AIF proteins in the early aged hypertensive cerebral cortex after EGCG treatment, suggesting that EGCG treatment could alleviate the translocation of EndoG and AIF from neural mitochondria to neural cytosol. Our study is the first to report the EGCG treatment attenuates early aged hypertension-induced EndoG and AIF caspase-independent pathway in the cerebral cortex. A previous study investigating different organs with different disease models has reported that

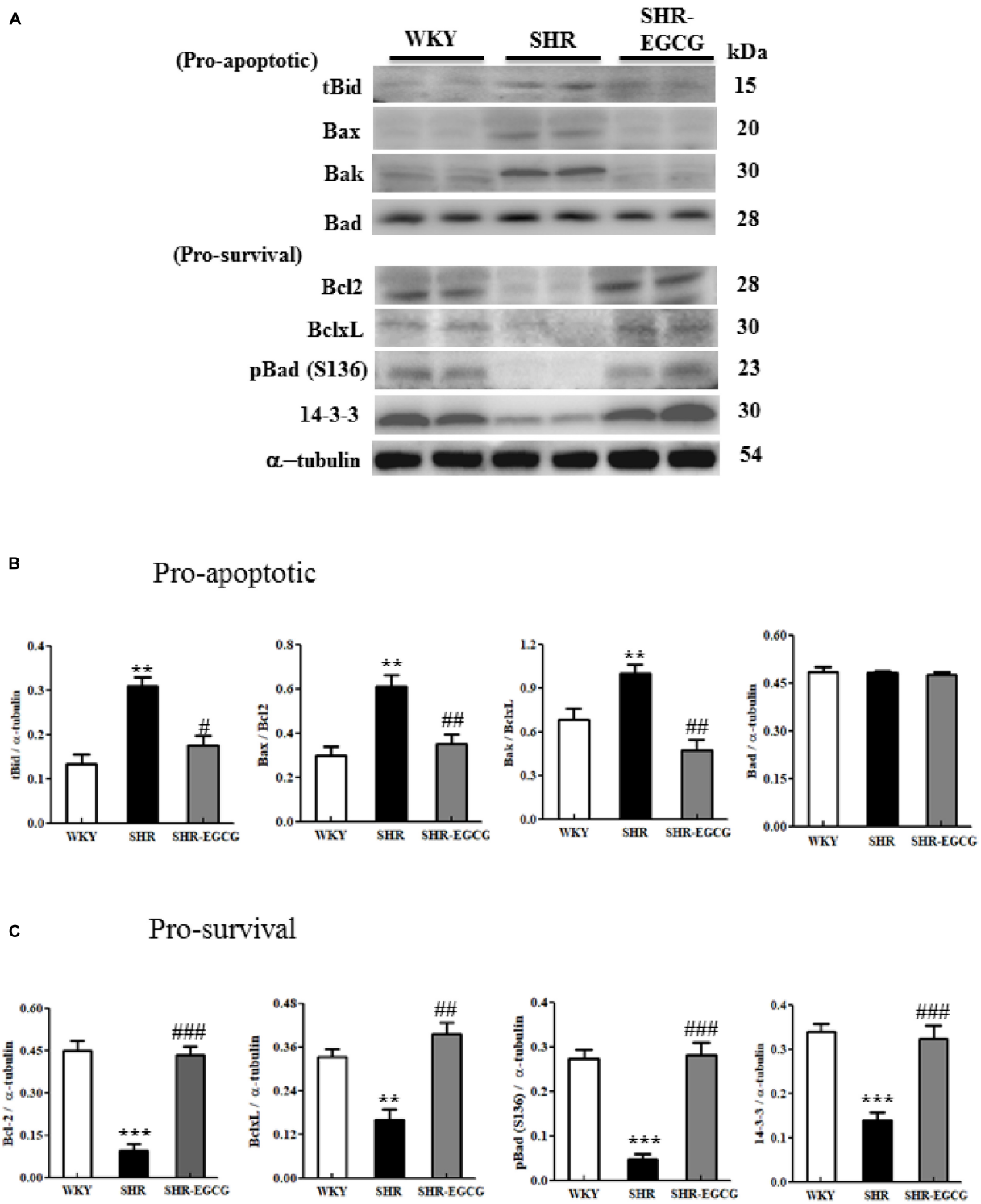


FIGURE 5 | The upstream components of the mitochondrial-mediated Caspase-dependent apoptotic pathway in a control Wistar Kyoto (WKY) group, a spontaneously early aged hypertensive (SHR) group and an early aged hypertension with Epigallocatechin-3-gallate (EGCG) treatment (SHR-EGCG) group. **(A)** Representative Western blotting images of the truncated BH3 Interacting Domain Death Agonist (t-Bid), Bcl-2 associated protein X (Bax), Bcl-2 homologous antagonist/killer (Bak), Bcl-2 associated agonist of cell death (Bad), B-cell lymphoma 2 (Bcl-2), B-cell lymphoma-extra large (Bcl-xL), p-Bad, and 14-3-3 in the cerebral cortex tissues from the WKY, SHR, and SHR-EGCG groups with α-tubulin as an internal control. **(B,C)** Bars represent the protein quantification of t-Bid, Bax/Bcl-2, Bak/Bcl-xL, and Bad, as well as Bcl-2, Bcl-xL, pBad and 14-3-3 normalized by α-tubulin, expressed as fold changes relative to the WKY group and mean values ± SEM ($n = 6$ in each group). $^{**}p < 0.01$ and $^{***}p < 0.001$, in comparison between the SHR and WKY rat group; $\#p < 0.05$, $\#\#p < 0.01$, and $\#\#\#p < 0.001$ in comparison between the SHR-EGCG group and SHR group.

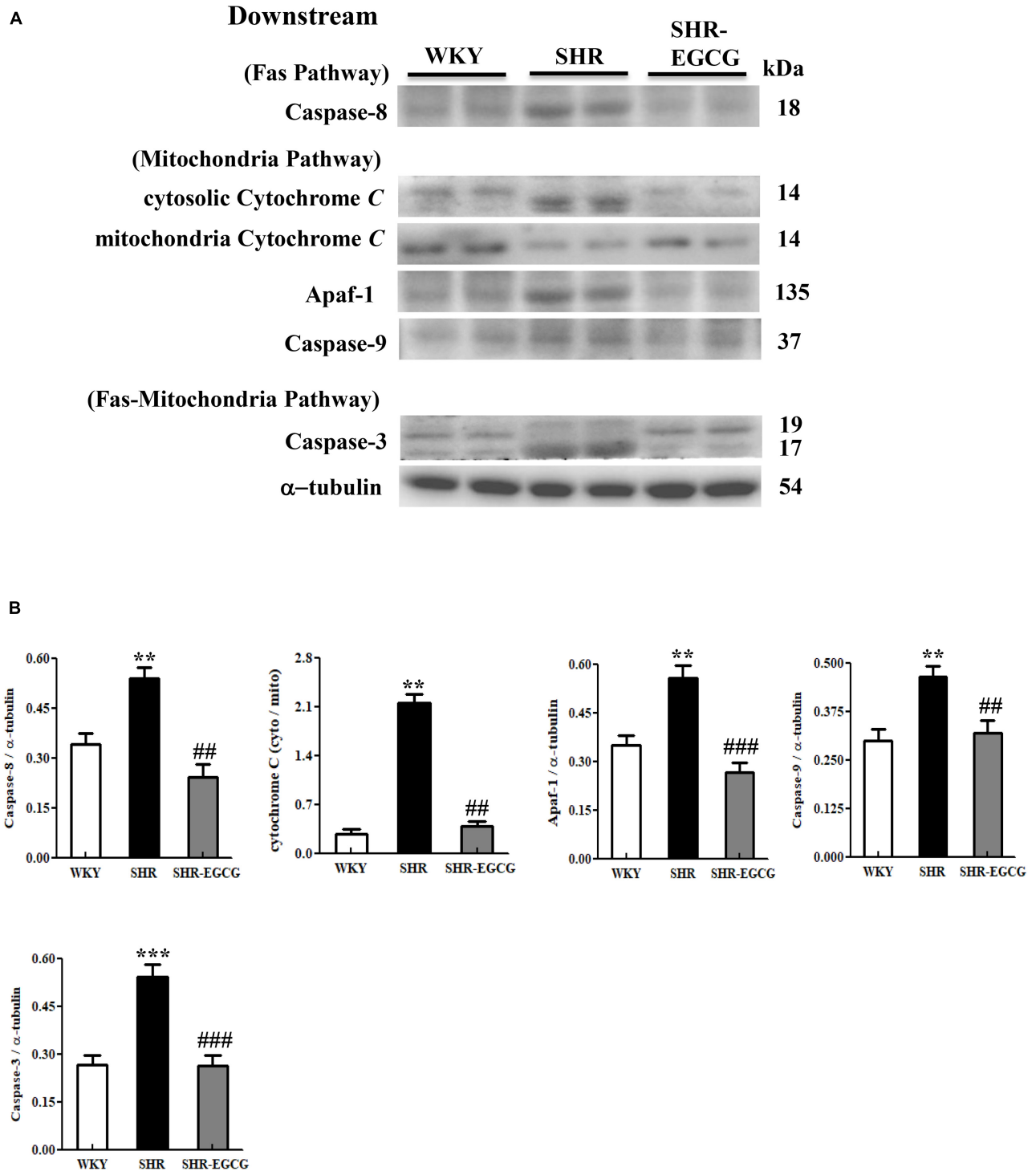


FIGURE 6 | The downstream components of the Caspase dependent-apoptotic pathway in a control Wistar Kyoto (WKY) group, a spontaneously early aged hypertensive (SHR) group and an early aged hypertension with Epigallocatechin-3-gallate (EGCG) treatment (SHR-EGCG) group. **(A)** Representative Western blotting images of Caspase-8 (Fas downstream), cytosolic Cytochrome C, mitochondrial Cytochrome C, Apaf-1, Caspase-9 (Mitochondrial downstream), Caspase-3 (Fas and Mitochondrial downstream) in the cerebral cortex tissues from the WKY, SHR, and SHR-EGCG groups with α-tubulin as an internal control. **(B)** Bars represent the protein quantification of Caspase-8, cytosolic Cytochrome C to mitochondrial Cytochrome C ratio, Apaf-1, Caspase-9, Caspase-3 normalized by α-tubulin, expressed as fold changes relative to the WKY group and mean values ± SEM ($n = 6$ in each group). $^{**}p < 0.01$ and $^{***}p < 0.001$ in comparison between the SHR group and WKY rat group; $\#\#p < 0.01$, $\#\#\#p < 0.001$ in comparison between the SHR-EGCG group and SHR group.

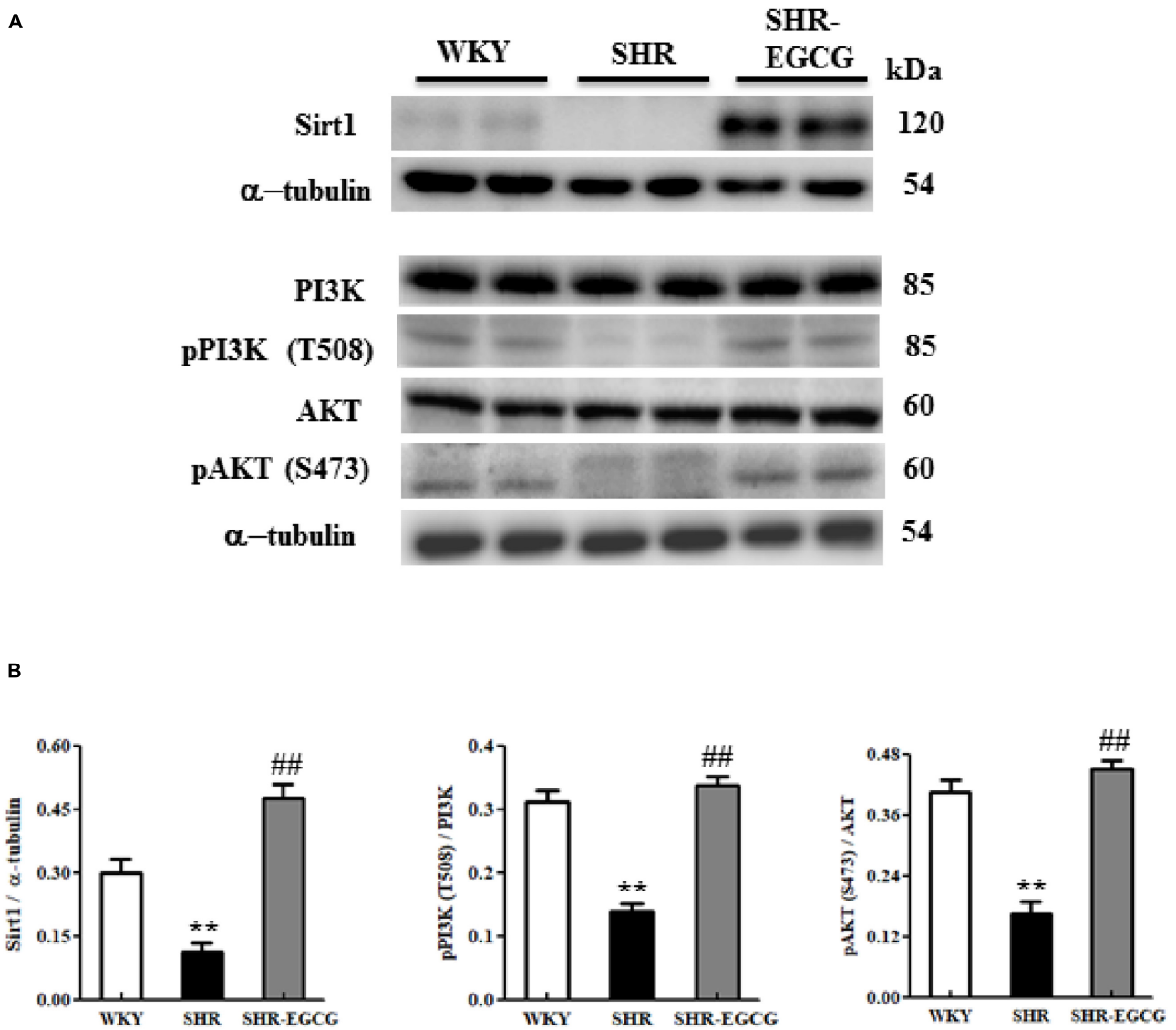


FIGURE 7 | The Sirt1/PI3K/AKT-related survival pathway in a control Wistar Kyoto (WKY) group, a spontaneously early aged hypertensive (SHR) group, and an early aged hypertension with EGCG treatment (SHR-EGCG) group. **(A)** Representative Western blotting images of Sirt1, PI3K, p-PI3K, AKT, and p-AKT in the cerebral cortex tissues from the WKY, SHR, and SHR-EGCG groups with α-tubulin as an internal control. **(B)** Bars represent the protein quantification of Sirt1, p-PI3K/PI3K, and pAKT/AKT normalized by α-tubulin, expressed as fold changes relative to the WKY group and mean values ± SEM (n = 6 in each group). $^{**}p < 0.01$, in comparison between the SHR group and WKY rat group; $\#\#p < 0.01$, in the comparison between the SHR-EGCG group and SHR group.

EGCG treatment reduced the level of AIF protein in the rats model with nicotine-induced cardiac apoptosis (Nacera et al., 2017). Of note, due to its permeability through the brain-blood barrier (Bastianetto et al., 2011), EGCG treatment could directly adjust neuronal mitochondrial membrane permeability and calcium homeostasis, which thereby may prevent the release of EndoG and AIF from mitochondria and inactivate Caspase-independent apoptotic pathway (Campos-Esparza et al., 2009).

Our results indicated the new finding that EGCG treatment was able to prevent the early aged hypertension-activated Fas-mediated Caspase-dependent neural apoptotic pathway, as evidenced by the down-regulated levels of FasL, Fas, FADD, Caspase-8, and Caspase-3 in the hypertensive cerebral cortex. Supportively, a previous study observed that 1 μM EGCG treatment reduced the mRNA level of Fas ligand in the neuronal cell line (SH-SY5Y cells) *in vitro* (Weinreb et al., 2003). In addition, EGCG has been reported to decrease Caspase-8 protein levels in radiation-induced hippocampal apoptosis *in vivo* (El-Missiry et al., 2018) and nitrite oxide-induced neuronal cell death (PC12 cells) *in vitro* (Jung et al., 2007). Therefore, EGCG treatment could diminish the early aged hypertension-activated Fas-mediated Caspase-dependent neural apoptotic pathway in the cerebral cortex.

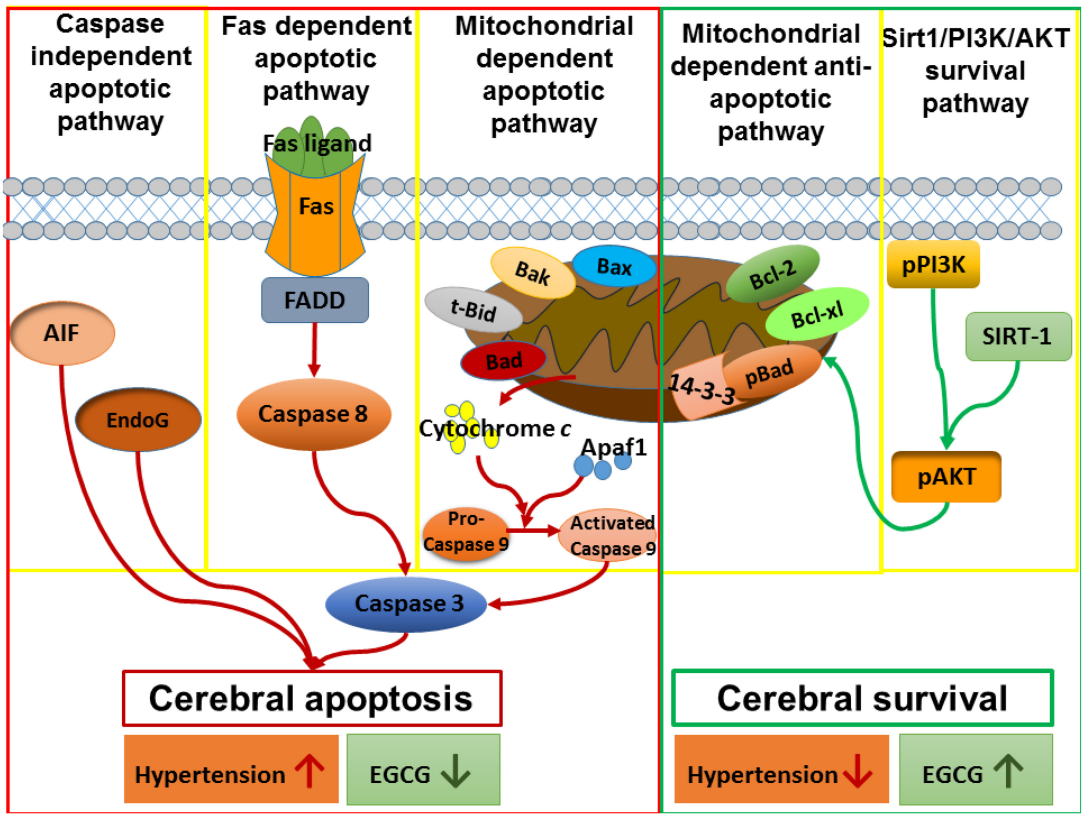


FIGURE 8 | The hypothesized diagram. We proposed that the cerebral cortex EndoG and AIF-related Caspase-independent, Fas-mediated Caspase-dependent and mitochondrial-mediated Caspase-dependent apoptotic pathways were promoted in early aged hypertension, whereas those pathways were suppressed after EGCG treatment. This hypothesis was evidenced by which early aged hypertension increased the levels of EndoG, and AIF-related Caspase-independent apoptotic proteins (EndoG, AIF), Fas-mediated Caspase-dependent apoptotic proteins (FasL, Fas, FADD, Caspase-8, Caspase-3), and mitochondrial-mediated Caspase-dependent apoptotic proteins (Bax, Bak, t-Bid, Cytochrome C, Apaf-1, Caspase-9, Caspase-3), whereas EGCG treatment reduced those levels in the early aged hypertensive brain. On the contrary, the Bcl-2 family-related and Sirt1/PI3K/AKT related pro-survival pathways were inhibited in the early aged hypertensive brain, while those pathways were enhanced by EGCG treatment. This hypothesis was evidenced in which early aged hypertension suppressed Bcl-2 family-related pro-survival proteins (Bcl-2, Bcl-xL, pBad, 14-3-3) and Sirt1/PI3K/AKT related pro-survival proteins (Sirt1, p-PI3K/PI3K, p-AKT/AKT), whereas EGCG treatment enhanced those levels in the early aged hypertensive brain.

In the present study, we found that EGCG inactivated both upstream components (t-Bid, Bax/Bcl-2, and Bak/Bcl-xL) and downstream components (cytosolic Cytochrome *C*, Apaf-1, Caspase-9, Caspase-3) of the mitochondrial-mediated Caspase-dependent apoptotic pathway in the early aged hypertensive cerebral cortex. Supportively, previous studies showed that EGCG attenuated the overexpression of Bax, Caspase-9, Cytochrome *C* protein levels in the radiation-induced hippocampal apoptosis *in vivo* (El-Missiry et al., 2018) and nitrite oxide-induced neuronal cell death (PC12 cells) *in vitro* (Jung et al., 2007). Another study observed that EGCG decreased the Bax protein level in the cerebral ischemia brain (Nan et al., 2018). Hence, we suggested that EGCG could significantly reduce the early aged hypertension-activated mitochondrial-mediated Caspase-dependent apoptotic pathway in the brain.

We identified that EGCG treatment activated the Bcl-2 family-related pro-survival pathway against early aged hypertension in the cerebral cortex, as evidenced by the up-regulated levels of Bcl-2, Bcl-xL, pBad, and 14-3-3. Although there was no previous evidence that mentioned the effects of EGCG on Bcl-2 family-related pro-survival pathway in the early aged hypertensive brain, previous studies showed that EGCG enhanced Bcl-2 protein levels in the cerebral ischemia brain (Nan et al., 2018) and in radiation-induced hippocampal apoptosis (El-Missiry et al., 2018). Likewise, EGCG has been reported to increase the mRNA and protein levels of Bcl-2 and Bcl-xL in quinolinic acid-induced neuronal cell death (N18D3 cells) *in vitro* (Jang et al., 2010). Those data suggested that EGCG treatment could enhance the Bcl-2 pro-survival pathway in the brain against early aged hypertension.

Moreover, in this study, EGCG activated the Sirt1/PI3K/AKT pro-survival pathway by increasing Sirt1 levels as well as promoting the phosphorylation of PI3K and AKT in the hypertensive cerebral cortex. Supportively, a study has shown that EGCG reduced neural apoptosis in the cerebral ischemic brain by enhancing the neural PI3K/AKT pathway (Nan et al., 2018). Another study reported that the activation of the PI3K/AKT pathway by EGCG protected the brain against amyloid-beta

accumulation, which thereby slowed down Alzheimer's disease progression (Yamamoto et al., 2017). In addition, EGCG has been shown to directly improve neural survival in the aging hippocampus, both *in vivo* and *in vitro,* by enhancing the PI3K/AKT pathway (Ortiz-López et al., 2016). Therefore, we hypothesized that EGCG not only reduced neural cell loss but also enhanced neural survival in the early aged hypertensive cerebral cortex through regulating the Sirt1/PI3K/AKT pathway. Of note, Sirt1 not only activates p-AKT, but also play a key role in mitochondrial quality-control. The activation of Sirt1 might attenuate the damage on neural mitochondrial biogenesis, and thus, mitigate neural apoptosis in the cerebral cortex (Li and Wang, 2017; Teertam and Prakash Babu, 2021). This question need to be addressed in further studies.

Study's Limitation

There were several limitations in our study which need to be mentioned. Firstly, 6% of unknown micronutrients in the chosen-EGCG product (TEAVAGO) might induce the apoptotic effects on the hypertensive cerebral cortex, leading to reduce the anti-apoptotic effects of EGCG treatment observed in this study. Secondly, the TUNEL assay cannot distinguish apoptosis from necrosis, and thus, we were unable to rule out hypertension-related neural necrotic cell deaths. Finally, the effects of EGCG on neural apoptosis in the early aged hypertensive cerebral cortex noted here cannot be distinguished or attributed to specific factors because EGCG treatment affects multiple factors such as anti-hypertension, enhanced-neuronal stem cells, anti-inflammation, anti-oxidative stress, and baroreflex regulation (Itoh et al., 2013; Legeay et al., 2015; Li et al., 2019). Our study proved the therapeutic effects of EGCG on early aged hypertension-induced neural apoptosis in the cerebral cortex, but cannot provide the cause-effect of why EGCG treatment could reduce neural apoptosis and enhance neural survival in the early aged hypertensive cerebral cortex. Thus, the mechanisms of EGCG against neural apoptosis in the early aged hypertensive brain need to be clarified in further studies.

Conclusion and Perspective

Since early aged hypertension augments neural apoptosis in the cerebral cortex, chronic hypertensive patients should be aware of the progressive development of neurodegenerative diseases. It is difficult to extract the cerebral cortex tissues from the human brain, and thus, the cerebral cortex in the spontaneously early aged hypertensive rats with EGCG treatment here should provide a critical explanation on how EGCG treatment prevents apoptosis-related cerebral cortex disorders in hypertensive patients. If EGCG prevents the neural apoptosis in the hypertensive cerebral cortex, EGCG may slow down the progression of early aged hypertension-induced neurodegeneration. Our findings demonstrated that EGCG reduced hypertensive-augmented neural apoptosis in the cerebral cortex through preventing overactive neural EndoG and AIF-related Caspase-independent apoptotic pathways, Fas-mediated Caspase-dependent apoptotic pathway and mitochondrial-mediated Caspase-dependent apoptotic pathways as well as enhancing the cerebral cortex Bcl2-related and PI3K/AKT-related pro-survival pathways. Thus, we might further hypothesize that EGCG represents the natural therapeutic agent for attenuating early aged hypertension-induced cerebral cortex apoptosis, which might prevent the early aged hypertension-augmented neurodegeneration. Regarding clinical applications, although EGCG treatment reportedly reduced blood pressure and improved neurobehavioral performance in patients (Legeay et al., 2015; Cascella et al., 2017), the dosage of EGCG used was varied. Additionally, it was unclear whether EGCG treatment could reduce neurodegenerative progression in early aged hypertensive patients. Therefore, clinical studies are required to determine the long-term effects of EGCG treatment with dosage comparison in neurodegenerative patients.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://1drv.ms/x/s!Ag29XwtG6G8vg8sqI-Zhf9FiX1DtgQ.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of China Medical University.

AUTHOR CONTRIBUTIONS

S-DL, Z-YC, A-LY, and X-BW: conceptualization. M-HH, S-YT, A-LY, and S-DL: experimental resources. M-HH, S-YT, Y-YL, and A-LY: performing the experiments. M-HH, S-YT, and S-DL: data analysis. NTN, S-HY, and Y-JC: writing—original draft preparation. S-DL, NTN, and Z-YC: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Moderate-Intensity Physical Activity, Music and Art Activities Preserved Cognitive Health in Older Adults: An Argument for Social Prescribing Solution

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Introduction: Rates of dementia are projected to increase over the coming years as global populations age. Without a treatment to slow the progression of dementia, many health policies are focusing on preventing dementia by slowing the rate of cognitive decline with age. However, it is unclear which lifestyle changes in old age meaningfully reduce the rate of cognitive decline associated with aging.

Objectives: Use existing, multi-year longitudinal health data to determine if engagement in a variety of different lifestyle activities can slow the rate of cognitive decline as older adults age.

Method: Data from the English Longitudinal Study of Aging was analyzed using a quasi-experimental, efficient matched-pair design inspired by the clinical trial methodology. Changes in short-term memory scores were assessed over a multi-year interval for groups who undertook one of 11 different lifestyle activities, compared to control groups matched across confounding socioeconomic and lifestyle factors.

Results: Two factors, moderate-intensity physical activity and learning activities, resulted in significant positive impact on cognitive function.

Conclusion: Our analysis brings cognitive benefit arguments in favor of two lifestyle activities, moderate-intensity physical activity and learning activities, while rejecting other factors advanced by the literature such as vigorous-intensity physical activity. Those findings justify and encourage the development of new lifestyle health programs by health authorities and bring forward the new health system solution, social prescribing.

Keywords: cognitive function, dementia, Alzheimer's disease, modifiable lifestyle factors, longitudinal observational studies

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1. INTRODUCTION

The number of older adults over the age of 65 is increasing globally, which is resulting in a range of challenges for healthcare systems and providers (Lunenfeld, 2008; Christensen et al., 2009). Rates of dementia—a disease that primarily affects older adults—are projected to increase from about 40 million people currently to 125 million by 2050. No cure exists for dementia and patients require increasing levels of labor-intense care as their symptoms progress, making dementia by far the most expensive end-of-life disease to treat (Kelley et al., 2015). Fortunately, even relatively modest delays in the onset and progression of dementia can result in significant global reductions in this disease; one estimate is that the incidence of AD would be reduced by approximately 8.6%, or 9.2 million cases if the onset of the disease was delayed by just 1 year (Brookmeyer et al., 2007).

Current pharmacological treatments are ineffective at delaying the onset of various dementias (Birks, 2006; McShane et al., 2019). Most medical recommendations, therefore, focus on preventative health measures (i.e., Social Prescribing; Fancourt et al., 2020), including physical activity, proper diet, and social and cognitively stimulating activities, to maintain cognitive health (Lang et al., 2008; De Oliveira et al., 2014; Norton et al., 2014; Deckers et al., 2015). Although these recommendations are good for maintaining overall physical health, the scientific evidence for maintaining cognitive health is unclear (Christie et al., 2017). Supporting evidence comes largely from observational studies, which often cannot determine whether engagement in a particular activity (e.g., physical exercise, music) has a causal benefit on cognitive health or only a correlational association. Data from randomized control trials have established causal effect between several activities and aging brain health such as physical activity (Cheng, 2016; Ishimaru et al., 2020), meditation and music (Alain et al., 2019). However, these studies are difficult to run and can have their methodological limitations (e.g., poor cross-sectional representation) (Harada et al., 2017).

Here, we investigated a large longitudinal, cross-sectional health dataset, the English Longitudinal Study of Aging, proposing new analytical methods that minimize the effect of confounding variables. Our analysis allowed us to determine if causal inference deems engagement in certain lifestyle activities to have benefit on cognitive health as people age. Based on previous finding (Christie et al., 2017), we hypothesized that engaging in physical activity and learning activities (e.g., art and music classes) would lead to improved cognitive health outcomes.

2. METHOD

2.1. Participants and Sample Data

We used data from the English Longitudinal Study of Ageing (ELSA), a longitudinal study of community-dwelling people aged 50 and older in England (Steptoe et al., 2013). Participants recruited using multistage-stratified probability sampling. The cohort was first assessed in 2002/2003, and was re-examined in eight examinations (waves), every 2 years. The examination dates are 2004/2005 (wave 2), 2006/2007 (wave 3), 2008/2009 (wave 4), 2010/2011 (wave 5), 2012/2013 (wave 6), 2014/2015 (wave 7), 2016/2017 (wave 8), and 2018/2019 (wave 9). For the purpose of this study, we drew 4,091 participants from waves 1–7 that did not have a missing value in their key variables. **Figure 1** shows the age distribution of samples at baseline, wave 1. The statistics for other demographics, social engagement, physical activity, and cognitive function measures are reported in **Table 1**. Further information on the instrumentation, sampling, recruitment, and data collection procedures can be found at the ELSA's project website https://www.elsa-project.ac.uk/study-documentation.

The outcome variable to assess changes in cognitive function over time was memory index (MI), a computed variable within ELSA comprising three memory subtests on immediate, delayed, and prospective memory. MI ranges from 0 to 27, with 0 zero representing the lowest performance.

Candidate variables were selected a priori to explore causal relation with the outcome variable. Candidate variables included mild/moderate/vigorous physical activity, group membership, tobacco use, contact frequency, friendship quality, and number of close friends. Mild/moderate/vigorous physical activity variables were measured using a 4-point scale (range "more than once a week" to "hardly ever"). Examples of mild activities included laundry and home repairs; moderate intensity activity included gardening, cleaning the car, walking at moderate pace, dancing, and stretching exercises; vigorous intensity included running/jogging, swimming, cycling, aerobics/gym workout, tennis, and digging with a spade. Group membership variables asked the participants to indicate if they are a member of any sports club, social club, church, and if they participate in any learning activities. In particular, learning activities refer to participation in any education, art, or music groups, or evening classes. The values for these measures are 0 and 1 corresponding to No and Yes. Tobacco use was assessed by asking the respondent how many cigarettes they smoke per weekday. To measure contact frequency, respondents were asked how often they meet up with their friends, using a 6-point scale (range "Three or more times a week" to "Less than once a year or never"). Friendship quality captures the perceived social support by the respondents; they were asked how much they think their friends understand their feelings, using a 4-point scale (range "a lot" to "not at all"). As another measure of social support, respondents were asked how many of their friends they would say they have a close relationship with.

Confounding variables are mediator variables that affect both a given candidate variable (which is suspected to be causal) and the outcome variable. Failing to control for confounding variables may result in a misleading correlation, i.e., a possibly strong correlation that is not due to some causal relationships, damaging the validity of the analysis (Spirtes et al., 2000). The confounding variables controlled for in this study include general health and demographics. General health measures the overall health of the participants; The respondents were asked how their health is in general, using a 5-point scale (range "very good" to "very bad"). The demographics used in this study are age, gender, and

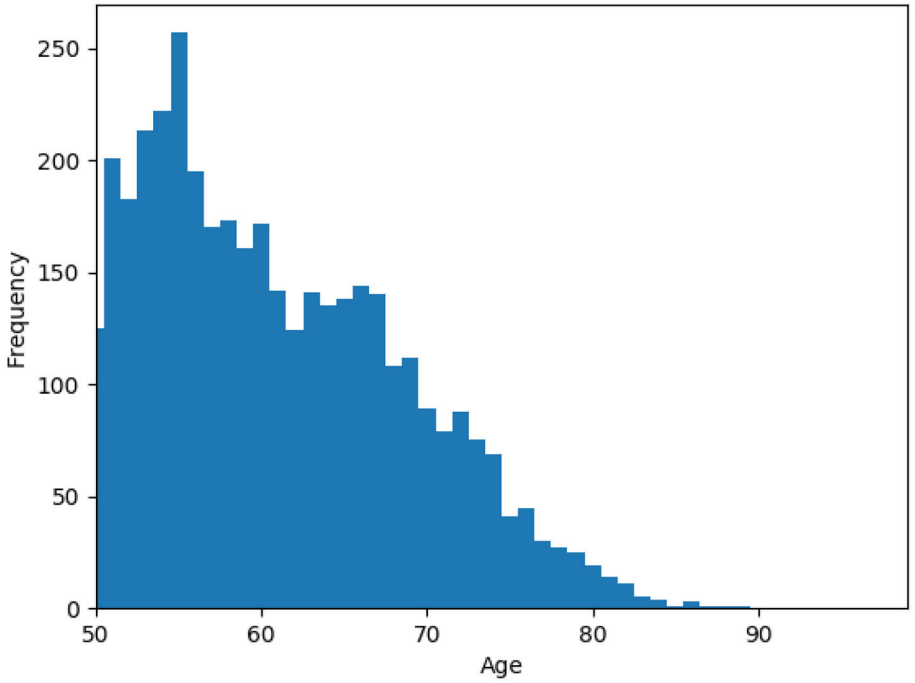


FIGURE 1 | Histogram of the age variable for samples at wave 1.

socioeconomic status. Socioeconomic status is a derived variable computed based on other collected economic variables which indicate the decile of the net total wealth.

2.2. Data Analyses

We employed a Quasi-experimental design (QED) based on matched pair design to study the effect of candidate variables on MI. In this design, samples are paired randomly such that one sample has been exposed to a potential cause X_i, known as the treatment, and the other sample has not been exposed to it, while both samples are constrained to be similar with respect to the other potential causes and confounders (Kuhn, 1955; Miettinen, 1968; Mansouri et al., 2018). These constraints guarantee that the effect of the other observed variables on the outcome is controlled, and the random pairing minimizes the effect of latent (unobserved) variables on the outcome.

Figure 2 provides an overview of the analysis method. (i)The method iterates over all candidate variables, for each wave. (ii) Experimental Design: The treatment and control groups were defined based on the value of the candidate variable for the current and the two previous waves. (iii) Cost Matrix Calculation: A cost matrix was calculated, specifying the similarity between each pair of samples from the treatment and control groups. (iv) Matching: A set of matchings were established such that the total dissimilarity cost between matched pairs was minimized while constraining the base memory index to be equal for each pair. If for a treatment sample, two or more control samples have the same minimum distance, one was chosen randomly. (v) Sets of pairs obtained from each wave were merged, and a statistical test was performed to determine the significance of the mean difference of the outcome variable between the treatment and the control cases of the matched pairs. (vi) Finally, after computing the p-values for each variable and each wave, all computed p-values were adjusted for multiple hypothesis tests (Benjamini and Hochberg, 1995). Details of the method are provided in the following subsections.

2.3. Preprocessing

In order to prepare the data for the matched pairs design, a series of pre-processing operations was performed. Variables were normalized to their standard score, with the mean and standard deviation estimated across all samples and all waves. A binary version of the candidate variables X was generated in order to efficiently distinguish between the treatment and control cases. For continuous variables, namely the number of close friends and the number of cigarettes smoked per weekday, based on domain knowledge and variable nature, two thresholds were selected; all values above the higher threshold were converted to one and all values below the lower threshold were converted to zero. For the discrete variables, only one threshold was used for binarization.

2.4. Identifying Treatment and Control Groups

To capture the change in cognitive health over time, we consider the difference in MI from one wave to the next one as the outcome. Hence, the treatment group for a candidate variable in a particular wave is defined as samples where the candidate variable is zero at the initial wave and one for the two consecutive waves. Similarly, the control group consists of samples where the

TABLE 1 | Descriptive statistics for demographics, candidate treatment variables, and the cognitive function measure used in the analysis.

	Range		W1	W2	W3	W4	W5	W6	W7
Sex	58% female								
Age	50–99	Mean	60.31						
		Std	8.35						
Socioeconomic status	1–10	Mean	6.32	6.24	6.2	6.03	5.94	6.04	5.96
		Std	2.73	2.77	2.77	2.77	2.78	2.71	2.71
Physical health	1–5	Mean	2.53	2.57	2.02	2.68	2.72	2.82	2.87
		Std	1.08	1.05	0.82	1.04	1.05	1.06	1.06
Mild physical activity	1–4	Mean	1.39	1.28	1.32	1.35	1.38	1.36	1.43
		Std	0.86	0.72	0.79	0.83	0.86	0.86	0.93
Moderate physical activity	1–4	Mean	1.62	1.59	1.6	1.68	1.76	1.8	1.88
		Std	1	0.98	1.01	1.07	1.12	1.16	1.22
Vigorous physical activity	1-4	Mean	2.93	2.95	3.01	3.03	3.1	3.13	3.21
		Std	1.26	1.25	1.25	1.24	1.21	1.21	1.18
Friendship Quality	1–4	Mean	1.87	1.88	1.83	1.86	1.88	1.87	1.8
		Std	0.78	0.76	0.75	0.76	0.76	0.75	0.73
Number of close friends	0–98	Mean	3.22	3.88	4.43	3.65	3.67	3.61	3.74
		Std	3.66	4.45	6.6	2.97	3.16	2.96	3.44
Contact frequency	1–6	Mean	2.46	2.5	2.48	2.49	2.49	2.51	3.73
		Std	1.02	1.04	1.03	1.07	1.08	1.09	0.56
Sport club membership	0–1	Mean	0.26	0.25	0.25	0.25	0.24	0.25	0.26
		Std	0.44	0.44	0.43	0.44	0.43	0.43	0.44
Church membership	0–1	Mean	0.23	0.24	0.23	0.24	0.23	0.24	0.24
		Std	0.42	0.43	0.42	0.43	0.42	0.42	0.43
Social club membership	0–1	Mean	0.2	0.2	0.19	0.2	0.2	0.19	0.19
		Std	0.4	0.4	0.39	0.4	0.4	0.39	0.39
Learning activities	0–1	Mean	0.18	0.16	0.15	0.14	0.15	0.15	0.15
		Std	0.39	0.37	0.36	0.35	0.36	0.36	0.36
Smoking	0–60	Mean	1.78	1.49	1.3	1.17	1.05	0.92	0.78
		Std	5.67	5.2	4.78	4.66	4.31	4.04	3.72
Memory index	3–24	Mean	14.55	14.88	14.93	14.76	14.62	14.59	13.95
		Std	3.17	3.21	3.34	3.38	3.54	3.65	4.04

candidate variable is zero for both the previous wave and the current wave. The reason for defining one treatment and control group per wave is to control the impact of time-dependent latent variables such as age and social scale events.

2.5. Pairing

In this step, samples from treatment and control groups are paired together. To control the effect of confounding variables, the features of each pair are constrained to have a minimum distance. A hard constraint was imposed on the base memory index to make them equal for the treatment and control pairs. A sample is represented by a vector consisting of the other potential causes and the confounding variables. The distance between two samples is defined as the Euclidean distance of their corresponding vectors. In cases where multiple pairings with the same distance are possible for a sample, one is randomly selected to add the randomness component to the matched pairs design. Once the distance between all pairs is calculated, Hungarian algorithm (Kuhn, 1955) is applied to get an optimal pairing.

To define the pairing process formally, we introduce the following notation; $[Y^i]^t (1 \leq t \leq T, 1 \leq i \leq N)$ is the target value of sample i in wave t, where T is the number of time points, and N is the number of samples; $[X_j^i]^t (1 \leq t \leq T, 1 \leq i \leq N, 1 \leq j \leq M)$, is the *jth* candidate variable of sample i in wave t, where M is the number of candidate variables; and $[Z_j^i]^t (1 \leq t \leq T, 1 \leq i \leq N, 1 \leq j \leq K)$, is the j^{th} confounding variable of sample i in wave t, where K is the number of confounding variables.

As the input, the Hungarian algorithm takes the cost matrix specifying the cost of each pairing. Output of the algorithm is a matching where the total cost is minimized. As usually the size of treatment and control groups are different, the number of matched pairs is the minimum size of treatment and control group. The more similar a pair, the lower the cost. The Hungarian algorithm solves the pairing of treatment and control cases as an Assignment problem:

$$u^* = \text{argmin}_u \sum_{n_t} \sum_{n_c} D\left(i, i', t\right) u_{ii'} \quad \text{such that } u_{ii'} \in \{0, 1\} \quad and \quad \sum_{i=1}^{n_t} u_{ii'} = 1 \quad and \quad \sum_{i'=1}^{n_c} u_{ii'} = 1 \tag{1}$$

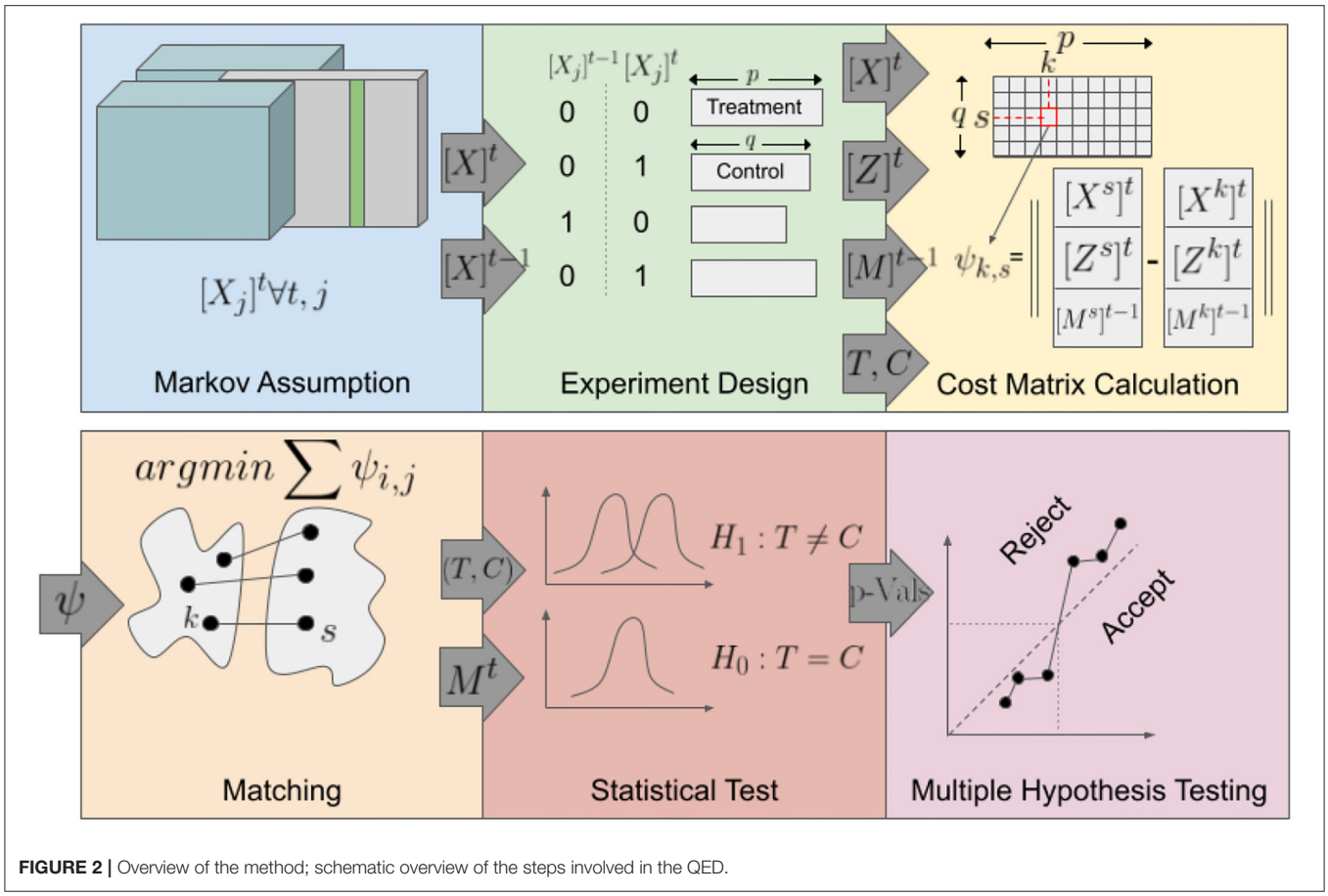


FIGURE 2 | Overview of the method; schematic overview of the steps involved in the QED.

where u^* is the optimal assignment, n_c is the number of control samples and n_t is the number of treatment samples. In this formulation, $u_{ii'} = 1$ indicates that sample i of treatment set and sample i' of control set are paired, and $u_{ii'} = 0$ indicates otherwise. $D(i, i', t)$ is the matching distance between treatment sample i and control sample i' at time t, for variable v is composed of three components:

$$D(i, i', t, v) = D_{\text{confounders}}(i, i', t) + D_{\text{treatments}}(i, i', t, v) + \beta * D_{\text{target}}(i, i', t) \quad (2)$$

where β is the weight given to the target variable during the matching. The reason for allocating a component for each of the confounders, treatment, and target variable is that a high number of variables in one category does not overshadow the variables in other categories. A high weight is used for D_{target} to make sure that the two matched samples have similar conditions at the baseline in terms of the target variable. The subcomponents are defined as following:

$$\begin{aligned} D_{\text{target}}\ (i, i', t) &= \left[Y^i\right]^{t-2} - \left[Y^{i'}\right]^{t-2} \\ D_{\text{Confounder}}\ (i, i', t) &= \sum_{j=0}^{K-1} \left(\left[Z_j^i\right]^t - \left[Z_j^{i'}\right]^t \right) \\ D_{\text{treatment}}\ (i, i', t, v) &= \sum_{\substack{j=0 \\ j \neq v}}^{M-1} \left(\left[X_j^i\right]^t - \left[X_j^{i'}\right]^t \right) \end{aligned} \quad (3)$$

2.6. Statistical Test

Considering that the values for the outcome are continuous and skewed, the potential causes are binary, and the samples are paired, the Wilcoxon Signed-Rank test is used to evaluate the impact of a potential cause. The Wilcoxon Signed-Rank test for our QED evaluates the null hypothesis that there is no significant difference in the change of MI because of the value of the candidate variable. This provides us with one p-value for each candidate variable and each wave.

2.7. Adjusting the *P*-Values

Evaluating the causality of multiple candidate variables for MI produces a large number of hypotheses. This increases the chance of some of these hypotheses being incorrectly accepted by chance, which is known as the multiple comparisons problem. A standard approach to counteract the multiple comparisons problem is to adjust p-values and the threshold to accept them based on the false discovery rate. We use the Benjamin-Hochberg adjustment method for this purpose.

The outline of our approach is presented is Algorithm 1. The source code is made publicly available to facilitate the usage and reproducibility of the experimental results[1].

[1]https://github.com/causal-discovery-elsa/causal_discovery_elsa.

TABLE 2 | *P*-values and sample sizes associated with hypothesis testing across all waves and merged population.

Variable		Wave 3	Wave 4	Wave 5	Wave 6	Wave 7	Merged
Mild physical activity	*p*-value	0.4962	0.8931	0.299	0.8187	0.9885	0.57589
	N	42	38	68	65	71	284
Moderate physical activity	*p*-value	0.2108	0.0099	0.0004	0.1319	0.1584	0.00001
	N	113	117	79	171	199	679
Vigorous physical activity	*p*-value	0.7364	0.7865	0.6463	0.1102	0.8064	0.93253
	N	223	195	175	149	201	943
Number of close friends	*p*-value	0.4692	0.8062	0.1122	0.9159	0.6736	0.83818
	N	197	18	11	17	14	257
Friendship quality	*p*-value	0.0930	0.9169	0.6184	0.0542	0.9365	0.78565
	N	195	187	137	153	150	822
Social contact frequency	*p*-value	0.9325	0.9325	0.9325	0.9325	0.9325	0.93253
	N	209	233	211	221	13	887
Church membership	*p*-value	0.6618	0.7400	0.9119	0.9141	0.2600	0.77176
	N	59	32	38	36	50	215
Smoking	*p*-value	0.0686	0.2410	0.6534	0.2558	0.8125	0.89711
	N	21	20	11	9	13	74
Gym membership	*p*-value	0.7910	0.1725	0.3899	0.3402	0.2567	0.88873
	N	128	124	105	109	124	590
Social club membership	*p*-value	0.3264	0.7986	0.9106	0.1456	0.8096	0.84209
	N	114	87	94	103	94	492
Learning activities	*p*-value	0.1588	0.8668	0.0017	0.1918	0.0191	0.00030
	N		78	72	77	63	369

We can see that the p*-values for learning activities and moderate-intensity physical activity are statically significant, implying an association between the two variables and the cognitive function measure, MI. N, sample size.*

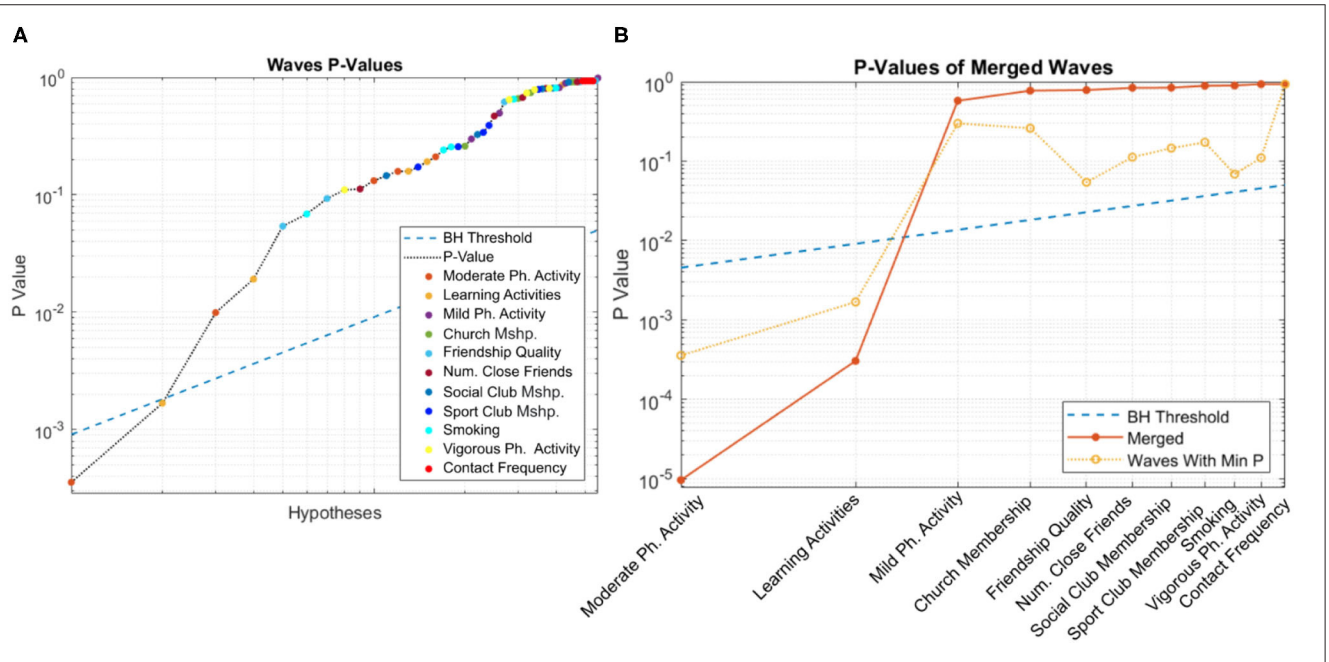


FIGURE 3 | Distribution of *p*-values for **(A)** individual and **(B)** merged populations. In both cases, learning activities and moderate-intensity physical activity pass multiple hypothesis testing.

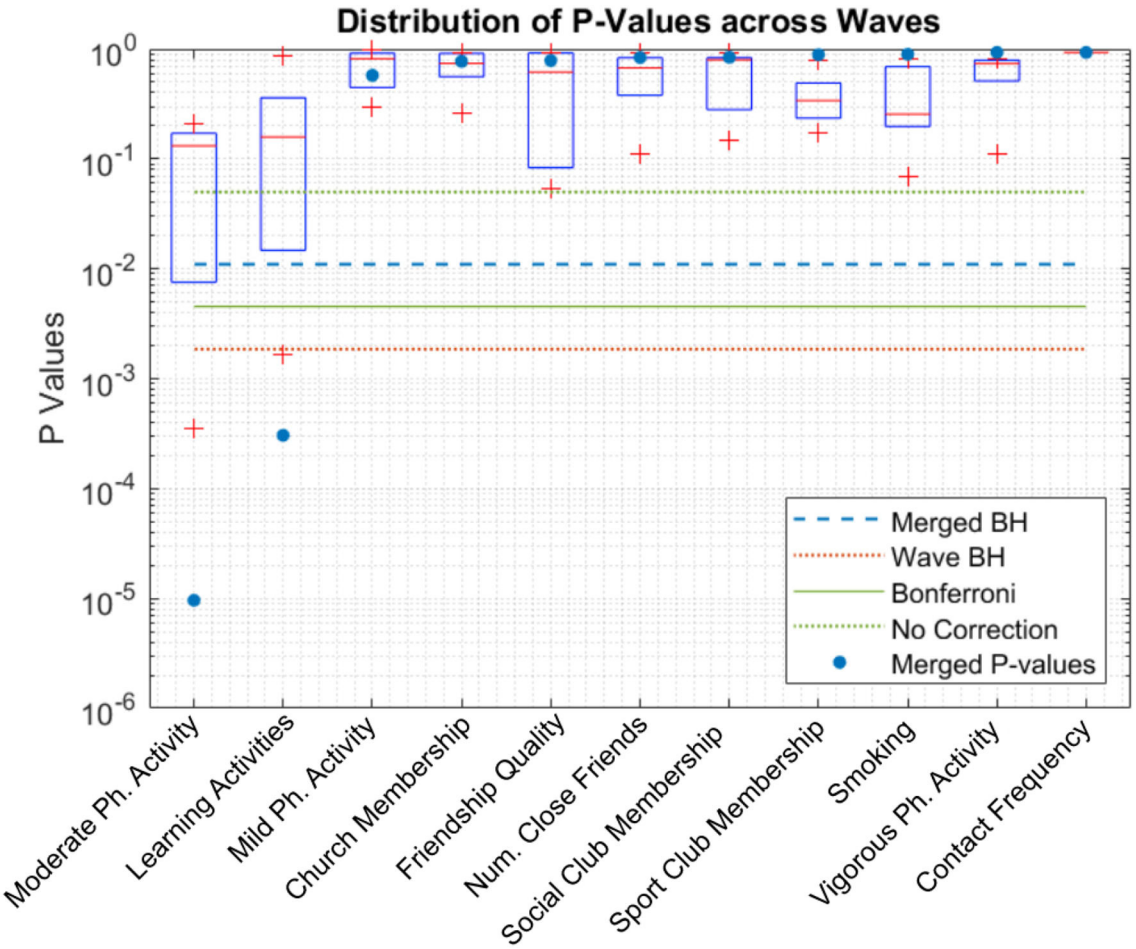


FIGURE 4 | Boxplot of *p*-values of each candidate. Learning activities and moderate-intensity physical activity pass the statistical test after *p*-value adjustment even for the more conservative adjustment method, Bonferroni. "Merged BH" and "Wave BH" indicate the significance thresholds for Benjamini-Hochberg method performed on *p*-values of merged waves and individual waves, respectively.

3. RESULTS

The set of *p*-values for each combination of one variable and one wave and also for each variable in the merged population is provided in **Table 2**. The *p*-value for individual waves was computed based on the treatment and control group identified at each wave, whereas the last column shows the *p*-value for the merged population across all five waves. The sample sizes used to compute the *p*-values are presented below each variable.

Figure 3A shows the curve corresponding to the *p*-values of candidate variables across all waves, with the blue line depicting the Benjamini-Hochberg adjusted threshold. Among the candidate variables, learning activities and moderate-intensity physical activity pass multiple hypothesis testing. The distribution of *p*-values is very healthy, with a long linear trail of evenly distributed *p*-values, as would typically be observed from random variables, and ending with few exponentially smaller *p*-values corresponding to statistically significant factors and their covariates (Oyeniran and Chen, 2016).

The corresponding curve for the merged population is shown in **Figure 3B**. As before, the blue line depicts the BH-adjusted 5% significance threshold. Consistent with the previous figure, both learning activities and moderate-intensity physical activity pass multiple hypothesis testing.

Figure 4 shows the boxplots of *p*-values for each candidate variable. Horizontal lines depict various thresholds for rejecting the null hypothesis without correction for familywise error, Bonferroni correction (Oyeniran and Chen, 2016) and Benjamini-Hochberg correction. Learning activities and moderate-intensity physical activity remain significant even at the more conservative Bonferroni level.

Finally, to evaluate the magnitude of the improvement expected from each treatment, we measured the mean difference in MI between treatment and control group pairs (**Figure 5**). Blue and red bars correspond to the merged population analysis and the per-wave analysis, respectively; in the latter case, bar height is the average of observed improvement overall five waves. For the merged population analysis, the two highest bars again

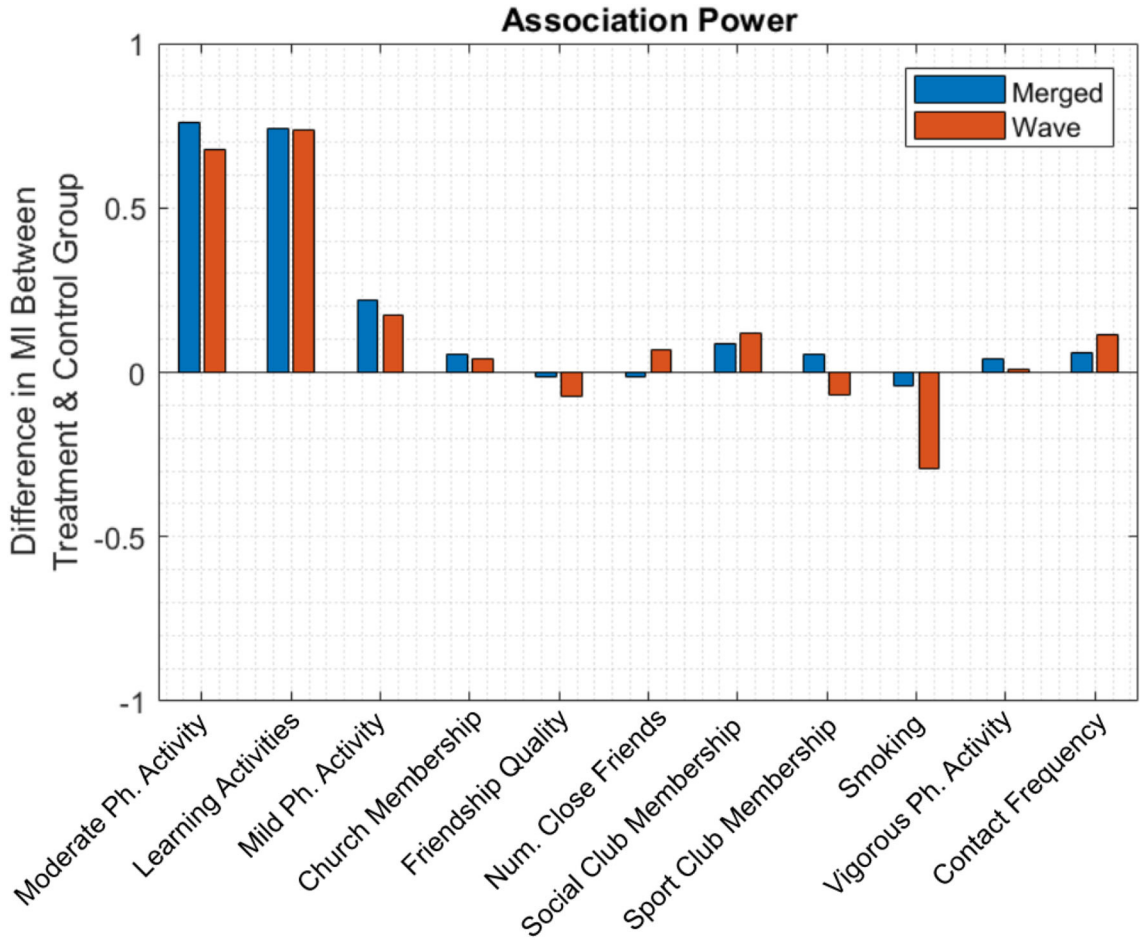


FIGURE 5 | The mean difference in MI between treatment and control group. Engagement in moderate-intensity physical activity and learning activities leads to approximately 0.75 points increase in MI.

correspond to the two previously identified significant candidate variables. Although significant, the benefit from each of these activities was relatively modest, leading to an improvement in MI of approximately 0.75 points on a 27-point scale.

In the per-wave analysis, smoking had a relatively large negative value, suggesting that MI decreased as the number of cigarettes smoked increased. However, due to a relative lack of participant data, this comparison lacks statistical power and was not identified as a statistically significant factor.

4. DISCUSSION

For many years, researchers have sought to ascertain whether certain lifestyle changes (e.g., physical activity) in later life can preserve cognitive health during aging. Here, our findings settle part of this debate by analyzing a large, longitudinal study of aging containing detailed information on cognitive function and real-world setting engagement in various life activities. To date, many of the existing studies of lifestyle effect on cognitive health have focused on correlational changes in cognition over time (Sona et al., 2012). Inferring causation from such studies is challenging because confounding effects cannot be ruled out. Causal discovery strives to identify cause-effect relations between a set of treatment and outcome variables, where any change in a causal treatment would result in a change in the outcome (Nguyen, 2018). The gold standard method to test causality is with randomized control trials, but RCT studies are notoriously difficult to conduct in the field of longitudinal cognitive health. QED is a competent replacement for randomized controlled trials in the evaluation of causal hypotheses using observational data when the sample size is not a limiting factor. QEDs are designed to deal with the lack of random assignment in observational data by sampling from the population such that the impact of selection bias and confounding is minimized. In this study, we adopted a QED based on matched pair design to investigate the effect of undertaking and maintaining changes in various lifestyle factors and how these factors influenced long-term cognitive health.

The results of these analyses revealed only two lifestyle activities that led to improved long-term cognitive outcomes: moderate-intensity physical activity and engagement in learning activities, such as education, art, music groups, or evening classes. Specifically, older adults who engaged in moderate-intensity

Algorithm 1: Causal Discovery Algorithm.

Input: candidate causal variables X, confounding variables Z, outcome variable Y
Output: A subset of X that have causal association with the target variable

```
X = preProcess(X);
mergedPvals=[];
for each indVar in X do
    mergedTargetValues = [];
    for each waveNum in [3,4,5,6,7] do
        treatmentGroups = getTreatmentGroups(X,indVariable, waveNumber);
        C= ComputeCostMatrix(X, Z, treatmentGroups, indVariable, waveNumber);
        matchedPairs = performMatching(C);
        targetValues = getTargetValues(Y,treatmentGroups, matchedPairs, waveNumber);
        mergedTargetValues.append(targetValues);
        pval = computePValue(targetValues["control"], targetValues["treatment"]);
        pVals[indVariable].append(pval);
    pval = computePValue(mergedTargetValues["control"], mergedTargetValues["treatment"]);
    mergedPvals.append(pval);
finalPvals= adjust the p-values using Benjamin Hochberg method;
return indVars that have significant p-values;
```

physical activities, such as gardening and walking, for a period of at least 4 years with a frequency of at least three times per week, gained one-third standard deviations from their baseline of memory score. Similarly, older adults who participated in learning activities for at least 4 years experienced a similar level of improvement in their cognitive health.

Broadly, these findings dovetail with past studies that sought to determine how lifestyle activities affect cognitive aging. For example, a recent meta-analysis by Woods et al. (2012) determined that cognitive stimulation improves cognition in individuals with mild cognitive impairment and moderate dementia over and above any benefit from medication. Likewise, studies of same-sex twins found that participation in leisure activities during early and middle adulthood—and participation in intellectual-cultural activities in particular—was associated with a reduced risk of Alzheimer's disease (Crowe et al., 2003).

In the present study, other lifestyle activities previously proposed as beneficial for healthy cognitive aging, such as active social participation and vigorous-intensity physical activity, did not pass hypothesis testing. We do not rule out the possibility that these and other activities may also have a positive effect on cognitive aging, which was not observed in the present study given certain limitations outlined below. Likewise, several additional issues remain to be investigated, including whether cognitive benefits are affected by the vigor or frequency of learning activities; whether exercise and learning activities are tapping into overlapping or separable neurcognitive circuitry; the added cognitive health benefit, if any, of continued engagement across longer time scales; of engagement in combinations of multiple life activities; of the duration of the cognitive protective factor following discontinuation of a given life activity; and understanding how these cognitive benefits change across older adults' lifespans.

A critical piece of converging evidence lies in determining the neuroanatomical effects of lifestyle activities and how these affect brain structure and function over time. Moderate exercise and learning activities likely both recruit wide-scale neuronal involvement for a range of activities that can include movement-related learning and delicate motor regulation. We consider it plausible that exercise and learning activities induce neuroplastic changes in the brain that counter age-related cognitive decline. Similar neuroplastic explanations have been advanced to account for cognitive improvements in other pathological populations (Särkämö et al., 2014, 2016) and healthy populations (Hötting and Röder, 2013; Moussard et al., 2016; Alain et al., 2019) and may be instantiated through a combination of increased synaptic and dendritic receptors, and changes in neuronal growth factors (Kraft, 2012; Hötting and Röder, 2013; Moreno and Bidelman, 2014); for reviews of the neurobiology of plasticity in aging see Freret et al. (2015) and Gelfo et al. (2018). Future studies will help clarify whether moderate exercise and learning activities are tapping into separable underlying mechanisms or whether these activities (and others) are accessing a unified underlying mechanism that can promote healthy cognitive aging.

As with any longitudinal study, our analysis suffers from a number of limitations. First, the data in this study was self-reported and retrospective, and could therefore be different from participants' actual behaviors. This can be exacerbated when the measurement techniques change over time, leading to a wrong inference of having found a causal relation while a developmental change actually occurring. Second, the study design included only individuals who participated in all seven waves of the ELSA (to mitigate attrition bias) and who engaged in a new lifestyle activity for at least two subsequent waves (to demonstrate a treatment effect). This may limit the extent to which the present findings apply to the broader public and may have increased the incidence of Type-II error. To increase generalizability, further replication studies with a wider variety of data sources will be required.

Nevertheless, the present results represent an important, causal discovery analysis on a large, longitudinal population. They confirm the significance of the relationship between moderate-intensity physical activities, learning activities, and long-term cognitive functioning. They also largely rule out important alternatives (i.e., spurious correlations) such as engaging in greater levels of physical activity and community classes simply because they have greater cognitive health to do so. We encourage additional research in this space and the development of community health guidelines to promote healthy cognitive aging.

5. CONCLUSION

Typical onset of dementia is at approximately age 80, and with the oldest of the baby boom generation approaching this age, there is a narrowing window with which to proactively maintain cognitive health. The results from the present study brings an answer to the baby boom generation's key question, engagement in moderate-intensity physical activity and learning activities may provide modest but significant protection against cognitive decline in aging. Therefore, we recommend the mobilization of this knowledge into community initiatives aimed at older adults and developing health policy encouraging programs such as social prescribing.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found at: https://www.elsa-project.ac.uk/.

AUTHOR CONTRIBUTIONS

AA had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. ME and SM: study concept and design. AA, GC, and MM: drafting of the manuscript. AA and MM: statistical analysis. ME, SM, and GC: obtained funding. MA: administrative and technical support. ME and SM: study supervision. All authors acquisition, analysis, or interpretation of data, critical revision of the manuscript for important intellectual content. All authors provided input into the design, edited and revised the manuscript, and read and approved the final manuscript.

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ORIGINAL RESEARCH
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The Effect and Mechanism of Cholesterol and Vitamin B_{12} on Multi-Domain Cognitive Function: A Prospective Study on Chinese Middle-Aged and Older Adults

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Background: Nutrients are associated with cognitive function, but limited research studies have systematically evaluated on multi-domain cognitive function. The aim of this study was to investigate the effect and mechanism of specific nutrient on multi-domain cognitive function, and provide nutrition guidance for improving cognitive function.

Methods: Participants were selected based on a multicenter prospective study on middle-aged and older adults in China. Global cognitive function was evaluated by the Mini-Mental State Examination (MMSE). Nutrients intake was assessed according to food frequency questionnaire and China Food Composition Database, and principal component analysis was performed to extract nutrient patterns. Associations between specific nutrients and cognitive function were assessed using log-binomial regression. Restricted cubic spline was used to illustrate the dose-response relationship of nutrients with multi-domain cognitive function. Mediation analysis was used to determine the mechanism of nutrients in cognitive function.

Results: Four nutrient patterns were identified (vitamin-mineral, protein-carbohydrate, fatty acid-vitamin E, and cholesterol-vitamin B_{12}), and only a nutrient pattern rich in cholesterol and vitamin B_{12} was found associated with cognitive function (RR = 0.891, 95%CI = 0.794–0.999). In multi-domain cognitive function, dietary cholesterol and vitamin B_{12} were related to better performance of visual memory function ($P = 0.034$, $P = 0.02$). In dose-response relationship, it suggested a U-shaped association between vitamin B_{12} and MMSE ($P = 0.02$) within a certain range.

Conclusions: Dietary intake rich in cholesterol and vitamin B_{12} was associated with better cognitive function, and vitamin B_{12} had a U-shaped dose-response relation with MMSE. Thus, ensuring moderate cholesterol and vitamin B_{12}intake may be an advisable strategy to improve cognitive function in middle-aged and older adults.

Clinical Trial Registration: EMCOA, ChiCTR-OOC-17011882, Registered 5th, July 2017-Retrospectively registered, http://www.medresman.org/uc/project/projectedit.aspx?proj=2610

Keywords: mild cognitive impairment, cholesterol, vitamin B12, dose-response relation, mediation analysis

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INTRODUCTION

Alzheimer's disease is one of the most common neurodegenerative diseases, characterized by progressive impairment in cognitive function, learning ability, memory function, and executive reasoning (Cui et al., 2020). Mild cognitive impairment (MCI) is a transitional stage between normal aging and dementia, 60–100% can develop into Alzheimer's disease (AD) within 5–10 years (Xue et al., 2018). According to the latest research, the overall prevalence of MCI and AD was estimated to be 15.5 and 3.9%, respectively, which imposed a considerable burden on individuals and society in China (Jia et al., 2020). Thus, early intervention on cognitive function can slow down the progress of AD efficiently (Fiorini et al., 2020).

In the past two or three decades, dietary habits in China have been experiencing a great change, particularly consumption of meat and sodium (Zhou et al., 2019). For example, residents in Beijing experienced a transition from traditional grain-based to animal-sourced diet (Xiong et al., 2019). Meantime, dietary habit transition leads to change in nutrient intake. Various studies have verified a strong link between nutrients and cognitive function, and most studies only focused on a single nutrient, while a single nutrient was not sufficient to affect cognitive function (Shi et al., 2019). Nutrient pattern may be more appropriate to examine the cumulative beneficial effect and common mechanism of nutrients in cognitive function, and it could explore the bioactive components of foods and potential interactions (Scarmeas et al., 2018). In Sweden, recent studies have found that nutrient pattern may play important roles in brain heath, but it still lacked relevant research in Chinese populations (Prinelli et al., 2019). Besides, the effects of nutrients on cognitive function may vary with intake doses, which revealed potential non-linear relationships between nutrients and cognitive function (Chianese et al., 2017). A dose-response meta-analysis showed a significant non-linear relationship between polyunsaturated fatty acids and MCI (Zhang et al., 2016). A prospective cohort study also found a non-linear relationship between dietary Mg intake and cognitive function in adult women aged 65–79 in the United States (Lo et al., 2019). Therefore, it is necessary to focus on the non-linear relationship between nutrients and cognitive function to provide a precise dietary recommendation.

Nowadays, increasing studies elucidated the mechanisms of nutrients affecting cognitive function, such as oxidative stress, inflammatory processes, and Aβ or tau protein accumulation (Poulose et al., 2017). Recently, cholesterol and its oxygenated derivatives were found to play an important role in cognitive impairment, such as 27-hydroxycholesterol (27-OHC) and 24S-hydroxycholesterol (24S-OHC) (Wang et al., 2016a). An animal research study proved that high fat/cholesterol diet resulted in increasing level of tau phosphorylation and memory loss (Bhat and Thirumangalakudi, 2013). Because of their role in homocysteine metabolism, the mechanisms of vitamin B_{12} affecting cognitive function were briefly summarized as cerebrovascular effects, activation of tau kinases, or inhibition of methylation reactions (Smith and Refsum, 2016). It showed that both cholesterol and vitamin B_{12} could affect cognitive function through tau phosphorylation; however, the findings are inconsistent and fragmented (Vauzour et al., 2017). Therefore, it is necessary to perform mediation analysis to verify the role of cholesterol oxygenated derivatives or tau phosphorylation between nutrients and cognitive function, and to provide an important basis for exploring the common mechanism between nutrients and cognitive function.

This study was performed mainly to demonstrate the dose-response relationship between specific nutrient in nutrient patterns and multi-domain cognitive function based on a multicenter prospective study, and explore the potential mechanisms, which could provide scientific dietary recommendations for middle-aged and older Chinese populations.

METHODS

Participants

The Effects and Mechanism Investigation of Cholesterol and Oxysterol on Alzheimer's disease (EMCOA) study was a multicenter prospective study on community-dwelling volunteers initiated by Capital Medical University in 2014–2015 (Yu et al., 2018). In this study, participants aged 50 and 70 years were recruited and followed for an average of 2 years. Participants who had neuropsychiatric problems (e.g., depression, schizophrenia, drug addiction or other), severe diseases (e.g., visual impairment, hearing loss, or other severe organ dysfunction), who used dietary supplements or drugs affecting cognitive function, who were untraceable or failure to accomplish were excluded. Finally, 2,546 middle-aged and older eligible adults were selected for the demonstration of the relationship between specific nutrients in nutrient pattern, food composition, and multi-domain cognitive function. A subgroup that included 104 participants was randomly selected to explore the mechanism of nutrients in cognitive function. The details are shown in **Figure 1**. Face-to-face interviews were conducted by a survey team composed of clinical neuropsychologists and research investigators who have been trained in the details of the measurements and questionnaires. The assessments of diet and cognitive function was conducted initially, and then after 2 years. The EpiData software was used for parallel double data entry.

Abbreviations: 27-OHC, 27-hydroxycholesterol; 24S-OHC, 24S-hydroxycholesterol; ACME, average causal mediation effect; AD, Alzheimer's disease; Aβ, β-amyloid; AVLT-LR, auditory verbal learning test-long recall; AVLT-IR, auditory verbal learning test-immediate recall; AVLT-SR, auditory verbal learning test-short recall; BMI, body mass index; CFCD, China Food Composition Database; CVD, cerebrovascular diseases; DSTB, Digit span backwards; DSTF, Digit span forwards; EMCOA, effects and mechanism of cholesterol and oxysterol on Alzheimer's disease; FFQ, Food Frequency Questionnaire; LMT-IR, logical memory test-immediate recall; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; MUFA, monounsaturated fatty acid; NC, normal cognition; PCA, principal component analysis; PMT, picture memories test; p-tau, phosphorylated tau; PUFA, polyunsaturated fatty acid; RCA, restricted cubic spline; SCWT-IT, Stroop Color-Word Test Interference Trial; SFA, saturated fatty acid; TMT, Trail Making Test; t-tau, total tau; WMS-RC, Wechsler Memory Scale-Revised of China.

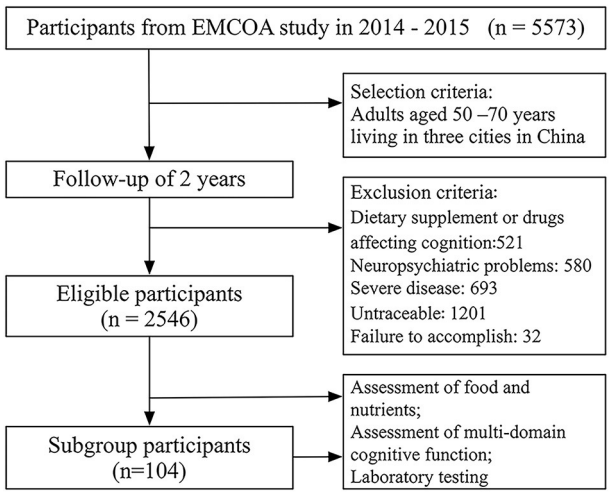


FIGURE 1 | Flow diagram.

Assessment of Food Composition and Nutrients

Dietary intake was assessed with a semiquantitative Food Frequency Questionnaire (FFQ) (Clare et al., 2017), which included 85 different foods types of 33 items. The participants were requested to state the frequency (per year, month, week, or day) and amount (in grams, bowls, etc.) of food intake for each food item on baseline, and follow up to make sure they were on the same food regimen during the study. For each food reported, food models and an album with over 50 photos of the most common dietary products were used as helpful tools to determine the amounts of food portions. Nutrient intake was calculated by multiplying the nutrient content of each food of specific portion size by the frequency of consumption and then summed over all food items from the China Food Composition Database (CFCD) (Rong et al., 2017). In this study, the following nutrient intake was calculated: protein, carbohydrate, dietary fiber, cholesterol, saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA), vitamin A, vitamin B_1, vitamin B_2, niacin, vitamin B_6, folic acid, vitamin B_{12}, vitamin C, vitamin E, magnesium, iron, zinc, selenium, copper, and manganese. The food composition included meat, milk and its products, eggs, grain, fruits, vegetables, etc.

Assessment of Cognitive Function

The global cognitive function of the participants was assessed by Mini-Mental State Examination (MMSE). MMSE is commonly used as a simple tool to screen for cognitive impairment based on education level. The diagnostic criteria used for MCI screening were as follows: (1) illiterate individuals $\leq$ 19 points, (2) individuals with 6 or less years of education $\leq$ 22 points, (3) individuals with 7 or more years of education $\leq$ 26 points. Then, if the participants were suspected of having MCI based on their MMSE performance, they would be comprehensively examined by neurologists to establish a clinical diagnosis (Wong et al., 2016; Yin et al., 2018; Jiang et al., 2020).

Comprehensive neuropsychological measures were used to assess the multi-domain cognitive function (Yu et al., 2018). Verbal memory function was assessed by Auditory Verbal Learning Test (AVLT) using summarized scores of immediate recall (AVLT-IR), short recall (AVLT-SR), and long recall (AVLT-LR) (Hong et al., 2015); attention/processing speed/executive function was assessed by Logical Memory Test—immediate recall (LMT-IR) (Wang et al., 2016b), Digit Span Forward (DSTF), and Digit Span Backward (DSTB) (Darby et al., 2017), and Trail Making Tests (TMT) A and B (Wei et al., 2018) from Wechsler Memory Scale Revised for China (WMS-RC); flexibility of cognitive function was assessed by the Stroop Color-Word Test-Interference Trial (SCWT-IT) (Bondi et al., 2002); and visual memory function was assessed by the Picture Memories Test (PMT) from WMS-RC (Krakowski and Czobor, 2011). The measurements were performed in a quiet and private room by trained technicians.

Covariates

The questionnaires on sociodemographic included age, sex, education (in years), body mass index (BMI); lifestyle risk factors included smoking, drinking, and reading habit; and medical history included hypertension (measured blood pressure > 140/90 mmHg or antihypertensive medication), hyperlipidemia (total cholesterol $\geq$ 6.2 mmol/L or low-density lipoprotein $\geq$ 4.1 mmol/L or high-density lipoprotein < 1 mmol/L or triglycerides $\geq$ 2.3 mmol/L or lipid-lowering medication), diabetes (fasting glucose $\geq$ 7 mmol/L or antidiabetic medication), and cerebrovascular diseases (CVD). Smokers were defined as those who have smoked at least one cigarette per day in the last 6 months. Alcohol drinkers were defined as those who have consumed any type of alcoholic beverage at least once a week in the last 6 months. Weight and height were measured according to World Health Organization recommendations, and BMI was calculated as weight in kilograms divided by height in meters squared (kg/m^2) (Physical Status, 1995).

Laboratory Testing

After at least 12 h of fasting, blood samples were collected in 6 ml tubes containing coagulant and inverted 8–10 times. Serum was obtained by centrifugation at 3,000 rpm at 4°C for 10 min, and stored at −80°C until use. The serum concentrations of 27-OHC and 24S-OHC were measured by high performance liquid chromatography–mass spectrometry (HPLC-MS). A standard curve was generated and used to calculate the concentrations of 27-OHC and 24S-OHC with internal standard, according to the method previously described (Bandaru and Haughey, 2014). Serum total tau (t-tau) and phosphorylated tau (p-tau) were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit, and the concentration of the samples was determined by comparing the standard curve (Tang et al., 2019).

Statistical Analysis

Statistical analyses were performed using STATA version 15.0 (Stata Corp, College Station, TX, United States), R statistical software version 3.6.1 (R Foundation, Vienna, Austria), and IBM SPSS Statistics 23.0 software (SPSS, Chicago, IL, United States). A two-sided $P \leq 0.05$ was considered statistically significant.

Continuous variables were expressed as median (interquartile range) and compared by Mann–Whitney *U*-test. Categorical variables were presented as number (percentages) and compared by chi-square test or Fisher's exact test. Principal component analysis (PCA) was performed to identify nutrient patterns. Varimax rotation was used in component analysis, and eigenvalues > 1 were considered as principal component (Makura-Kankwende et al., 2020). Log-binomial regression was used to estimate the relationship between nutrient patterns and MCI (Palta et al., 2018). Multiple linear regression was used to illustrate the linear relationship between specific nutrients in nutrient patterns and multi-domain cognitive function. Restricted cubic spline (RCA) was used to flexibly model the dose-response relationship and explore the potential non-linear association between specific nutrients in nutrient patterns and multi-domain cognitive function (Desquilbet and Mariotti, 2010).

RESULTS

Characteristics of Participants

General characteristics, nutrients intake, and multi-domain cognitive function were compared between participants with mild cognitive impairment (MCI) and those with normal cognition (NC). The details are shown in **Supplementary Table 1**. Of the 2,546 participants, 54.6% were women. It showed that the difference of age between the MCI and NC groups was significant, but the median was same ($P = 0.016$). Compared with NC, participants in the MCI group had a worse performance in multi-domain cognitive function ($P < 0.001$). No significant differences were observed in the other characteristics.

Association Between Nutrient Patterns and MCI

Four independent nutrient patterns were identified by principal component analysis (PCA) (**Table 1**). The first nutrient pattern was identified as "Vitamin-Mineral," explained 36.4% of the variance and was illustrated by intakes of vitamin A, vitamin B_2, vitamin B_6, folic acid, vitamin C, magnesium, iron, zinc, copper, and manganese. The second nutrient pattern was identified as "Protein-Carbohydrate," explained 21.6% of the variance, and was illustrated by intakes of protein, carbohydrate, dietary fiber, and vitamin B_1. The third nutrient pattern was identified as "Fatty Acid-Vitamin E," explained 17.2% of the variance, and was illustrated by intakes of SFA, MUFA, PUFA, and vitamin E. The fourth nutrient pattern was identified as "Cholesterol-Vitamin B_{12}," explained 12.2% of the variance, and was illustrated by intakes of cholesterol and vitamin B_{12}.

Log-binomial regression was used to determine the effects of the different nutrient patterns on mild cognitive impairment (MCI). The results showed that only the Cholesterol-Vitamin B_{12} nutrient pattern (RR = 0.891, 95%CI = 0.794–.999, $P = 0.048$) was negatively associated with MCI (**Table 2**).

TABLE 1 | Principal component analysis of nutrient patterns.

Variables	Vitamin-mineral	Protein-carbohydrate	Fatty acid-vitamin E	Cholesterol-vitamin B_{12}
Protein	0.260	**0.702**	0.401	0.449
Carbohydrate	0.145	**0.945**	0.079	0.083
Dietary fiber	0.261	**0.841**	0.112	−0.027
Cholesterol	0.036	0.159	0.132	**0.842**
SFA	0.040	0.191	**0.735**	0.574
MUFA	0.051	0.068	**0.965**	0.016
PUFA	0.021	0.204	**0.816**	0.429
Vitamin A	**0.900**	0.146	−0.024	0.225
Vitamin B_1	0.392	**0.810**	0.169	0.253
Vitamin B_2	**0.864**	0.260	0.085	0.275
Niacin	0.600	0.442	0.480	0.206
Vitamin B_6	**0.922**	0.210	0.072	0.063
Folic acid	**0.917**	0.317	0.151	0.068
Vitamin B_{12}	0.121	0.013	0.066	**0.861**
Vitamin C	**0.944**	0.184	−0.017	−0.022
Vitamin E	0.259	0.185	**0.872**	−0.058
Magnesium	**0.671**	0.524	0.451	0.093
Iron	**0.903**	0.385	0.084	0.055
Zinc	**0.706**	0.556	0.283	0.284
Selenium	0.579	0.507	0.251	0.521
Copper	**0.763**	0.519	0.322	0.096
Manganese	**0.693**	**0.611**	0.304	0.033
Iodine	0.552	−0.055	0.070	−0.062

Bold entries indicate measures with high loadings on each factor.
SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

Association Between Cholesterol or Vitamin B_{12} and Multi-Domain Cognitive Function

Multiple linear regression models were used to further examine the association between baseline cholesterol or vitamin B_{12} and follow-up multi-domain cognitive function. The models were adjusted for age, sex, education, BMI, smoking, drinking, hypertension, hyperlipidemia, diabetes, CVD, and other nutrient patterns (**Table 3**).They showed that cholesterol was positively associated with PMT ($\beta = 0.137$; $P = 0.034$), and that vitamin B_{12} was also related to better performance in PMT ($\beta = 0.08$; $P = 0.02$).

Restricted cubic spline analysis was performed to simulate the non-linear dose-response relationship between cholesterol or vitamin B_{12} and multi-domain cognitive function. The models were adjusted for age, sex, education, BMI, smoking, drinking, hypertension, hyperlipidemia, diabetes, CVD, and other nutrient patterns (**Table 4**). The results showed that vitamin B_{12} had a non-linear relationship with MMSE ($P = 0.020$). Regarding the dose-response curve, it emerged a *U*-shaped relationship

TABLE 2 | Log-binomial regression of nutrient patterns and MCI.

Nutrient patterns	RR (95% CI)	*P*-value
Unadjusted		
Vitamin-mineral	1.024 (0.933, 1.122)	0.622
Protein-carbohydrate	0.970 (0.877, 1.073)	0.557
Fatty acid-vitamin E	0.994 (0.901, 1.097)	0.907
Cholesterol-vitamin B_{12}	0.892 (0.796, 1.000)	0.051
Model 1		
Vitamin-mineral	1.016 (0.926, 1.115)	0.734
Protein-carbohydrate	0.967 (0.873, 1.072)	0.527
Fatty acid-vitamin E	0.993 (0.900, 1.095)	0.885
Cholesterol-vitamin B_{12}	0.894 (0.797, 1.003)	0.055
Model 2		
Vitamin-mineral	1.021 (0.931, 1.119)	0.665
Protein-carbohydrate	0.963 (0.868, 1.068)	0.477
Fatty acid-vitamin E	0.994 (0.902, 1.096)	0.906
Cholesterol-vitamin B_{12}	0.891 (0.794, 0.999)	0.048*

Model 1 was adjusted for age, sex, education, BMI, smoking, and drinking; Model 2 was adjusted for Model 1 and hypertension, hyperlipidemia, diabetes, and CVD.
MCI, mild cognitive impairment; BMI, body mass index; CVD, cerebrovascular diseases; RR, risk ratio; CI, confidence interval.
$^*P < 0.05$, $^{**}P < 0.001$.

TABLE 3 | Linear relationship between cholesterol or vitamin B_{12} and multi-domain cognitive function.

Variables	Cholesterol		Vitamin B_{12}	
	β (95% CI)	*P*-value	β (95% CI)	*P*-value
MMSE	0.024 (−0.053, 0.100)	0.547	0.023 (−0.018, 0.064)	0.269
AVLT-IR	0.024 (−0.153, 0.200)	0.792	0.027 (−0.067, 0.122)	0.567
AVLT-SR	0.072 (−0.020, 0.164)	0.125	0.037 (−0.012, 0.086)	0.139
AVLT-LR	0.005 (−0.098, 0.108)	0.930	0.003 (−0.058, 0.052)	0.915
SDMT	0.154 (−0.253, 0.560)	0.459	−0.012 (−0.229, 0.205)	0.913
LMT	−0.029 (−0.234, 0.177)	0.784	0.034 (−0.076, 0.143)	0.544
TMTA	0.018 (−0.898, 0.934)	0.970	0.045 (−0.444, 0.535)	0.856
TMTB	−0.640 (−3.255, 1.975)	0.631	−0.326 (−1.723, 1.071)	0.647
DSTF	−0.010 (−0.061, 0.041)	0.697	0.013 (−0.014, 0.040)	0.353
DSTB	−0.027 (−0.074, 0.020)	0.260	−0.019 (−0.044, 0.007)	0.149
PMT	0.137 (0.010, 0.263)	0.034*	0.080 (0.013, 0.148)	0.020*
SCWT-IT	0.447 (−0.284, 1.179)	0.230	0.042 (−0.348, 0.432)	0.832

Adjusted for age, sex, education, BMI, smoking, drinking, hypertension, hyperlipidemia, diabetes, CVD, and other nutrient patterns using multiple linear regression models.
MMSE, Mini-Mental State Examination; AVLT-IR, Auditory Verbal Learning Test—immediate recall; AVLT-SR, Auditory Verbal Learning Test—short recall; AVLT-LR Auditory Verbal Learning Test—long recall; SDMT, Symbol Digit Modalities Test; LMT, Logical Memory Test; TMTA (B), Trail Making Test A (B); DSTF, digit span test forwards; DSTB, digit span test backwards; PMT, Picture Memories Test; SCWT-IT, Stroop Color-Word Test Interference Trial; BMI, body mass index; CVD, cerebrovascular diseases; CI, confidence interval.
$^*P < 0.05$. $^{**}P < 0.001$.

between vitamin B_{12} and MMSE when the intake was <3 μg/day (**Figure 2**). No dose-response relationship between cholesterol and multi-domain cognitive function was found.

TABLE 4 | Dose-response relationship between cholesterol or vitamin B_{12} and multi-domain cognitive function.

Variables	Cholesterol		Vitamin B_{12}	
	P-overall	*P*-non-linear	*P*-overall	*P*-non-linear
MMSE	0.221	0.111	0.036*	0.020*
AVLT-IR	0.172	0.092	0.144	0.107
AVLT-SR	0.204	0.313	0.587	0.966
AVLT-LR	0.482	0.295	0.894	0.767
SDMT	0.896	0.801	0.661	0.483
LMT	0.988	0.936	0.529	0.417
TMTA	0.633	0.435	0.713	0.563
TMTB	0.559	0.356	0.658	0.516
DSTF	0.957	0.990	0.096	0.067
DSTB	0.650	0.771	0.718	0.654
PMT	0.308	0.868	0.161	0.712
SCWT-IT	0.924	0.866	0.550	0.349

Adjusted for age, sex, education, BMI, smoking, drinking, hypertension, hyperlipidemia, diabetes, CVD, and other nutrient patterns using restricted cubic spline (RCS) models.
MMSE, Mini-Mental State Examination; AVLT-IR, Auditory Verbal Learning Test—immediate recall; AVLT-SR, Auditory Verbal Learning Test—short recall; AVLT-LR Auditory Verbal Learning Test—long recall; SDMT, Symbol Digit Modalities Test; LMT, Logical Memory Test; TMTA (B), Trail Making Test A (B); DSTF, digit span test forward; DSTB, digit span test backward; PMT, Picture Memories Test; SCWT-IT, Stroop Color-Word Test Interference Trial; BMI, body mass index; CVD, cerebrovascular diseases; CI, confidence interval.
$^*P < 0.05$, $^{**}P < 0.001$.

Mediation Effect Between Cholesterol or Vitamin B_{12} and Cognitive Function

A total of 104 participants were selected in the subgroup to elucidate how cholesterol and vitamin B_{12} affect cognitive function. The characteristics between overall samples used in previous analysis (n = 2546) and subgroup (n = 104) were demonstrated in **Supplementary Table 2**. It was showed that there was a significant difference in sex, BMI or drink habit between subgroup and overall, so these variants were adjusted in the analysis subsequently.

In mediation analysis, the average causal mediation effect (ACME) between cholesterol or vitamin B_{12} and MMSE was not significant when 27-OHC, 24S-OHC, t-tau, or p-tau was used as a mediator ($P > 0.05$). None of the mediators had significance in the relationship between nutrients and cognition. The details are shown in **Supplementary Table 3**.

DISCUSSIONS

In this prospective study based on middle-aged and older adults, we examined the effect of nutrient patterns on cognitive function. Four nutrient patterns were identified, and only the Cholesterol-Vitamin B_{12} pattern was associated with MCI (RR = 0.891, 95%CI = 0.794, 0.999). In multi-domain cognitive function, cholesterol and vitamin B_{12} were positively correlated with PMT ($P = 0.034$, $P = 0.02$). Furthermore, vitamin B_{12} was non-linearly associated with MMSE and the dose-response curve emerged U-shaped ($P = 0.02$). Generally, this study provided a perspective

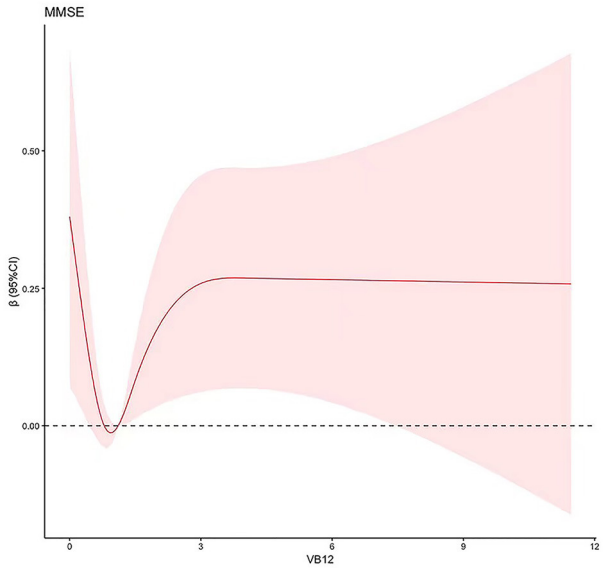


FIGURE 2 | Dose-response relationship between vitamin B_{12} and MMSE. Adjusted for age, sex, education, BMI, smoking, drinking, hypertension, hyperlipidemia, diabetes, CVD, and other nutrient patterns using RCS models. The shaded area represents the estimated relative risk and the 95% CI. MMSE, Mini-Mental State Examination; BMI, body mass index; CVD, cerebrovascular diseases; CI, confidence interval.

that nutrition, as a modifiable factor, could be an applicable and critical strategy to improve cognitive function.

In this study, a diet rich in cholesterol was associated with better cognitive function. Similarly, a study based on Framingham Heart Study showed that lower total cholesterol was correlated with poorer performance on cognitive function, and another cohort study showed that cholesterol was associated with deceased risk of dementia (Elias et al., 2005; Mielke et al., 2005). Moreover, cholesterol intake restriction had been removed from the 2015–2020 Dietary Guidelines in America, which indicates that the effect of cholesterol on cognitive function is complicated, and it was still difficult to make a conclusion whether it was harmful or beneficial (Williams et al., 2015). In multi-domain cognitive function, dietary cholesterol was found to be associated with PMT in this study. PMT was a subtest of WMS-RC, which was used to assess visual memory function. Little research has examined the relationship between dietary cholesterol and visual memory function, and a study has found that a mild increase in low-density lipoprotein cholesterol or high-density lipoprotein cholesterol level is related to a better visual memory (Kinno et al., 2019; Guo et al., 2020). Therefore, maintaining moderate cholesterol consumption may be advisable for cognitive function.

In this study, vitamin B_{12} was non-linearly associated with MMSE when the intake dose was within a certain range, which suggested that it was important to ensure adequate consumption of vitamin B_{12}. A cross-sectional study in Ireland also proposed that dietary vitamin B_{12} was related with MMSE in older adults, indicating that vitamin B_{12} may be a protective factor for cognitive function (O'Connor et al., 2020). Meantime, cross-sectional data from a nationally representative study in the United States showed that low vitamin B_{12} was related to cognitive impairment independently among older adults (Molloy, 2020). Therefore, ensuring adequate vitamin B_{12} intake might be an effective intervention to maintain cognitive function. In addition, in this study, vitamin B_{12} was related to a better performance in PMT. Although no direct evidence between vitamin B_{12} and visual memory had been found, a community-based cross-sectional study demonstrated that elevated homocysteine was associated with poorer performance in visual memory test (Ai-Vyrn et al., 2008). More studies are needed to be conducted on the possible mechanisms between vitamin B_{12} and visual memory function.

In this study, the cholesterol-vitamin B_{12} nutrient pattern was associated with MCI, which led us to consider the common mechanism of cholesterol and vitamin B_{12} in cognitive function. However, no statistical evidence for the hypothesis that cholesterol and vitamin B_{12} could work together to affect cognitive function through cholesterol oxygenated derivatives or tau phosphorylation. First, mediation analysis can only identify mediators that had a linear mediation effect, but the biological process was complex, and the relationship might be non-linear. Next, cholesterol and vitamin B_{12} may affect cognitive function through other mechanisms. For instance, it has been revealed that both cholesterol and vitamin B_{12} could influence cognitive function *via* Aβ metabolism (Alam et al., 2017; Wang et al., 2019). What is more, an inflammatory effect played an important role in the process of dietary cholesterol or vitamin B_{12} affecting cognitive function (Chen et al., 2018; Ma et al., 2019). Additionally, increasing research studies examined the role of epigenetics in biological mechanism of AD. As an essential cofactor of homocysteine metabolism, vitamin B_{12} is integral to DNA methylation and consequently involved in the pathology of AD (Athanasopoulos et al., 2016). Recently, a cohort study in Scotland also found that cholesterol was associated with apolipoprotein E (APOE) methylation (Mur et al., 2020). Therefore, future studies may focus on exploring the common mechanism of cholesterol and vitamin B_{12} affecting cognitive function, especially in the role of epigenetics.

In this study, the nutrient pattern characterized as cholesterol and vitamin B_{12} was related to lower risk of MCI, and the possible biological explanation for this beneficial effect may come from the high loadings of cholesterol and vitamin B_{12}. Mostly, dietary cholesterol and vitamin B_{12} are derived from meat, eggs, and milk. In this study, we explore the relationship of meat intake with cognitive function further, and it was found that meat intake was correlated with lower risk of MCI (**Supplementary Table 4**) and better performance of global cognitive function (**Supplementary Table 5**). Similarly, a longitudinal study on community dwellers in the state of New York also demonstrated that higher meat consumption was associated with better cognitive performance (Crichton et al., 2015). Meantime, a prospective study on oldest old Chinese population suggested that participants who consumed more meat were less likely to develop cognitive impairment (An et al., 2019). However, a cohort study from Sweden showed that adherence to a diet low in meat might contribute to healthy cognitive aging, the

inconsistent results indicated that native conditions are needed to be considered in dietary instructions (Titova et al., 2013). The inconsistent evidence provides us a hypothesis that the effect of meat on cognitive function was complicated, and that it may be different regarding local conditions. In general, meat should be consumed appropriately, for it is a risk factor in other diseases such as cardiovascular disease.

This study has several strengths. First, the data in this study were based on a prospective study with a relatively large sample size. Second, cognitive function was assessed through multiple scales, such as verbal memory cognitive, attention/processing speed/executive function, flexibility cognitive function, and visual memory function, which would reflect cognitive function more comprehensively. Third, not only the linear effects of nutrients but also the non-linear effects on multi-domain cognitive function were evaluated. The limitations of this study are as follows: First, the number of APOE-ε4 alleles is a major non-modifiable risk factor for AD, but we did not measure the APOE genotype in this study. Recently, a national wide cross-sectional study in China observed that lifestyle such as dietary pattern was associated with cognitive function regardless of APOE genotype (Jin et al., 2021). In order to confirm the relationship between dietary intake and cognitive function, the research team will further explore it. Second, cognitive function was assessed by scales instead of clinical examination, and education level can significantly affect the performance in completing the scales. Thus, we adjusted education level in each statistical model to compensate for this limitation. Besides, the mediation effect of oxysterols or tau protein between cholesterol or vitamin B_{12} and MMSE was only examined by statistical analysis, but the biological process was complex, and the relationship might be non-linear. Therefore, animal experiments and *in vitro* studies are still needed to elucidate the molecular pathway and physiological regulation underlying the observed associations in the next step.

CONCLUSIONS

In summary, the study provided evidence that a nutrient pattern rich in cholesterol and vitamin B_{12} was associated with lower risk of MCI and better performance in visual memory function. Vitamin B_{12} was non-linearly associated with MMSE, and the curve emerged *U*-shaped when the intake was within a certain range, which suggested that it was essential to establish a dose-response relationship for optimal dietary recommendations. Thus, ensuring appropriate cholesterol and vitamin B_{12} intake in the diet may be an effective strategy to prevent MCI in middle-aged and older adults. As a modifiable factor in cognitive function intervention, scientific dietary guidelines are critical.

DATA AVAILABILITY STATEMENT

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Capital Medical University (2013SY35). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

RX conceived and designed the study. LW and KL performed statistical analysis and wrote the manuscript. XZ, YW, WL, TW, LH, and MJ helped in the collection of data and conducted the experiments. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.707958/full#supplementary-material

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REVIEW
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Intake of Products Containing Anthocyanins, Flavanols, and Flavanones, and Cognitive Function: A Narrative Review

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The purpose of this review is to examine human research studies published within the past 6 years which evaluate the role of anthocyanin, flavanol, and flavanone consumption in cognitive function, and to discuss potential mechanisms of action underlying any observed benefits. Evidence to date suggests the consumption of flavonoid-rich foods, such as berries and cocoa, may have the potential to limit, or even reverse, age-related declines in cognition. Over the last 6 years, the flavonoid subgroups of anthocyanins, flavanols, and flavanones have been shown to be beneficial in terms of conferring neuroprotection. The mechanisms by which flavonoids positively modulate cognitive function are yet to be fully elucidated. Postulated mechanisms include both direct actions such as receptor activation, neurotrophin release and intracellular signaling pathway modulation, and indirect actions such as enhancement of cerebral blood flow. Further intervention studies conducted in diverse populations with sufficient sample sizes and long durations are required to examine the effect of consumption of flavonoid groups on clinically relevant cognitive outcomes. As populations continue to focus on adopting healthy aging strategies, dietary interventions with flavonoids remains a promising avenue for future research. However, many questions are still to be answered, including identifying appropriate dosage, timeframes for intake, as well as the best form of flavonoids, before definitive conclusions can be drawn about the extent to which their consumption can protect the aging brain.

Keywords: flavonoids, cognition, Alzheimer's disease, anthocyanins, flavanols, flavanones, dementia

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INTRODUCTION

Flavonoids are a class of polyphenols found in plant-based foods and are categorized into six major subclasses: anthocyanins, flavanols, flavonols, flavanones, flavones, and isoflavones (Spencer, 2008). Fruits and vegetables, wine, tea, and cocoa contain high concentrations of flavonoids (Somerset and Johannot, 2008). Structurally, flavonoids consist of two aromatic carbon rings (benzopyran A and C rings), and a benzene (B) ring, and may be divided into various subgroups based on the degree of

the oxidation of the C ring, the hydroxylation pattern of the ring structure, and the substitution of the three-position. Once ingested, flavonoids undergo extensive metabolism in the small and large intestine, in the liver, and within cells, resulting in forms in the body distinct from those consumed in foods (Manach et al., 2005).

The reduction in risk of cardiovascular disease and some cancers afforded by intake of flavonoids is well known (Kampa et al., 2007; Chong et al., 2010), and more recently, there has been attention directed toward their potential to improve cognitive performance in older adults, and thereby potentially protect against neurodegenerative diseases such as Alzheimer's disease (AD) (Bergland et al., 2019; Chai et al., 2019). In light of the projections indicating rapid increases in the prevalence of AD, and in the absence of successful treatments, alternative measures to slow the development and progression of the disease are imperative: flavonoid intake is being investigated as one such alternative measure.

Studies using animal models [reviewed in Nehlig (2013), de Andrade Teles et al. (2018), Hajialyani et al. (2019)] have demonstrated that flavonoids may slow or even reverse the effects of aging and dementia of which AD is the most common form. For example, in a rat model of aging which demonstrates inflammation, oxidative stress, memory and synaptic dysfunction, intraperitoneal injection of 100 mg/kg anthocyanins daily for 7 weeks, significantly improved spatial learning and memory ability, as well as short term memory (Rehman et al., 2017). Moreover, spatial learning and memory impairments were prevented in 4-month-old male senescence-accelerated mouse prone-8 (SAMP8) animals, orally administered 0.05 or 0.1% green tea catechins in drinking water for 6 months (Li et al., 2009). SAMP8 is typically characterized by early onset of learning and memory deficits along with spontaneous overproduction of soluble Aβ in the brain, a hallmark of AD.

While existing animal studies provide important insights into the neuroprotective properties of flavonoids and their underlying mechanisms, these studies cannot provide information regarding potential effects on more complex human cognitive functions such as abilities underpinned by language, and those supported by multifaceted organization of information, i.e., executive function. Consequently, clinical studies are required to determine if the beneficial effects observed in animal models are replicable in human populations.

The purpose of this narrative review is to examine human research studies published within the past 6 years (2014–2020 inclusive), to evaluate associations between intake of products containing the flavonoid subclasses of anthocyanins, flavanols, and flavanones and cognitive function (summarized in **Supplementary Table 1** and **Table 1**). Potential mechanisms underlying any observed benefits will also be discussed. Studies preceding this timeframe have been comprehensively reviewed elsewhere; e.g., Spencer, 2010; Lamport et al., 2012; Crichton et al., 2013. We focused on the three subclasses of anthocyanins, flavanols, and flavanones as the effects of the additional three flavonoid subclasses (flavones, flavonols, and isoflavones) have not been reported in human studies published within the 6-year timeframe of this narrative review.

METHODS

A computer-based search of PubMed was conducted for relevant articles published between 2014 and 2020 inclusive, with cognitive function as the primary or secondary outcome. Search terms were: (flavonoids or anthocyanins or flavanols or flavanones) and (cognition or cognitive function). The search was limited to articles in humans, published in English. Studies that specifically focused on the cognitive effects of flavonoids either in extract or whole food form were included.

Titles and abstracts were screened by the first author to ensure they examined the relationship between one of the flavonoid subclasses of interest (anthocyanins, flavanols, or flavanones) and cognition in adults. Articles were included if they reported prospective, retrospective or intervention studies and were either cross-sectional or longitudinal in nature. There were no strict criteria relating to the inclusion of controls or the quality of these controls with respect to matching for other macro or micronutrients. Studies were excluded if they; (1) were not peer-reviewed, (2) were a conference proceeding abstract, (3) were a review or theoretical article, (4) were published in a language other than English, (5) were conducted in animal models, or (6) were conducted in children (age <18 years). The reference lists of included articles were screened and additional articles which met inclusion criteria were added via hand search. Included studies are presented in **Supplementary Table 1** and **Table 1**.

ANTHOCYANINS

Anthocyanins are a class of flavonoids concentrated in purple and red fruits. In summary, we have reviewed four acute studies (<24 h) and eight chronic studies (duration between 12 and 24 weeks) investigating cherry, blackcurrant, bilberry, and blueberry anthocyanins in participants with varying cognitive status, ranging from cognitively normal (both young and older adults), self-perceived cognitive decline, and objectively determined mild cognitive impairment (MCI), to diagnosed dementia.

A 12-week randomized clinical trial conducted in 49 adults (24 intervention group, 25 control group) over the age of 70 years with mild to moderate AD, found consumption of 200 ml/day of cherry juice resulted in improvements in verbal fluency, short term memory, and long term memory compared to the control group, which consumed a juice with negligible anthocyanin content. The intervention group also had a reduction in systolic blood pressure, with a trend for reduction in diastolic blood pressure. However, markers of inflammation (CRP, C-reactive protein; IL-6, Interleukin 6) were not altered (Kent et al., 2017); suggesting either the mechanism of action of anthocyanins is not through the inflammatory pathway, or perhaps more likely, that the advanced disease stage which is accompanied by significant pathological hallmarks and inflammation, masked any potential anti-inflammatory effects conferred by the cherry juice. Of note, pre-clinical studies have indicated that anthocyanins from berry fruits may require only a few weeks to accumulate in brain regions associated with cognition (Willis et al., 2009).

TABLE 1 | Observational human studies published over the past 6 years investigating flavonoid consumption and cognitive outcomes.

Title, reference	Cohort information	Study design	Flavonoids investigated	Outcome measure(s)	Main finding(s)
Long-term dietary flavonoid intake and change in cognitive function in the Framingham Offspring Cohort (Shishtar et al., 2020)	$N = 1779$ Age: ≥45 Cognitively normal Framingham, MA, United States	Prospective cohort study Median 11.8-year follow-up	Total flavonoids, flavanones, flavan-3-ols, flavonoid polymers, flavonols, flavones, and anthocyanins	Verbal memory, visual memory, verbal learning, attention and concentration, abstract reasoning, language, visuoperceptual organization, and global function	Nominally significant trends observed between slower decline in global function, verbal and visual memory with higher flavonol and flavan-3-ol intakes; slower decline in visual memory with higher total flavonoid and flavonoid polymer intake; and slower decline in verbal learning with higher flavanol intake
The efficacy of cocoa polyphenols in the treatment of mild cognitive impairment: a retrospective study (Calabro et al., 2019)	$N = 55$ Age: 56–75 Amnestic MCI Messina, Italy	Retrospective cohort study over 1 year	Mexenion® composed of cocoa polyphenols (240 mg/sachet)	MMSE	Participants were divided into two groups depending on change in MMSE score at 1-year follow-up: Group 1 = stable or improved, Group 2 = decreased by at least three points. Group 1 had a significantly higher rate of Mexenion® intake than Group 2
Chocolate intake is associated with better cognitive function: The Main Syracuse Longitudinal Study (Crichton et al., 2016)	$N = 968$ Age: 23–98 Syracuse, NY, United States	Cross-sectional study	Chocolate intake divided into categories: less than once a week, once per week, more than once per week	Maine-Syracuse Longitudinal Study neuropsychological test battery: Composite domains global, visual-spatial memory and organization, scanning and tracking, verbal episodic memory, and working memory. Wechsler Adult Intelligence Scale Similarities Test MMSE	Chocolate consumption was significantly and positively associated with the global composite, visual-spatial memory and organization, scanning and tracking, verbal episodic memory, working memory, Wechsler Adult Intelligence Scale Similarities Test, and MMSE

MCI, Mild Cognitive Impairment; MMSE, Mini-Mental State Examination.

Twelve-week consumption of two cups of Montmorency tart cherry juice per day resulted in better performance on the paired associates learning task which assesses visual memory (mean difference of −8.5; 95% −12.5 to −4.5; $p = 0.02$), and significantly better movement time scores on the Reaction Time test which measures speed of response (mean difference of −10.4; 95% −13.4 to −7.5; $p = 0.03$), compared to the control group. This study was conducted in 37 adults aged between 65 and 80 with normal cognitive function. There were no significant improvements in working memory and strategy use, and visual attention. It is, however, important to note, that the intervention group had significantly decreased physical activity level whilst the control group had significantly increased cholesterol intake over the intervention period (Chai et al., 2019): factors which may have impacted the outcome measures.

In contrast, Caldwell et al. (2016) found no change in acute memory following consumption of a 300 ml serving of cherry juice, either administered as a single serving at time "0," or three 100 ml servings at "0," 1, and 2 h, with cognitive testing occurring at time "0" and 6 h post-intervention, in six young healthy participants, five older adults, and five older adults with dementia. The cognitive tests assessed verbal learning and memory, higher executive function, and speed of processing. The serving of cherry juice contained approximately 55 mg of anthocyanins; a lower dose compared to other food-based studies. The cognitive tests administered may not have adequately assessed the cognitive domains affected by acute anthocyanin consumption. Moreover, testing at 2 rather than 6 h post-intervention may better reflect the physiological absorption of anthocyanins in the small intestine.

In an intervention trial undertaken in 36 healthy young participants (aged 18–35 years), individuals consumed one of three different treatment drinks once, with at least 7 days of washout between consumption of each drink. The drinks contained either 0 mg of polyphenols (control), 525 ± 5 mg of polyphenols per 60 kg of bodyweight from an anthocyanin-enriched blackcurrant extract, or from 142 ml of a cold-pressed blackcurrant fruit juice. Participants underwent 10 min of cognitive testing before consumption of each treatment drink, followed by additional cognitive testing 60 min post-consumption, comprising seven repetitions of the 10-min battery. Improvements in accuracy on the Rapid Visual Information Processing task were found after supplementation with the extract drink ($p = 0.011$), and improvements in reaction time on the digit vigilance task after supplementation with the juice treatment, at repetitions 1 ($p = 0.028$), 4 ($p = 0.011$), and 7 ($p = 0.038$). The juice drink also induced a number of neuroendocrinological and physiological effects including increase in blood glucose concentration compared with control. These results suggest that the degree of processing and the cultivar of blackcurrant fruit used substantially alters the neuroendocrinological and cognitive benefits conferred (Watson et al., 2015).

A pilot study measuring cognition as a secondary endpoint following 16-week consumption of two Medox® capsules per day (total daily intake of 320 mg anthocyanin from bilberry and blackcurrants) found improved verbal memory

scores in the subdomains of learning ($p = 0.016$), recall ($p < 0.001$), and recognition ($p = 0.047$). Improved cognitive speed ($p < 0.001$) and inhibition ($p = 0.044$) were also observed, whilst no improvements in executive functioning were seen. The intervention group was comprised of eight participants with MCI, and 19 with stable non-obstructive coronary artery disease, as the study was designed to investigate supplementation in those with increased risk of dementia. However, the control group did not undergo cognitive testing; consequently, these results should be interpreted with caution due to the lack of a comparison group (Bergland et al., 2019).

Several randomized, double-blinded, placebo-controlled studies to evaluate the effects of blueberry consumption on cognition have been conducted. In 112 adults aged 65–80, Whyte et al. (2018) investigated the effect of 6-month consumption of; (1) whole wild blueberry powder containing 1.35 mg total anthocyanin per dose (WBP500), (2) whole wild blueberry powder containing 2.7 mg total anthocyanin per dose (WBP1000), or (3) purified extract containing 7 mg total anthocyanin per dose (WBE111), compared to placebo. Cognitive testing occurred at baseline, 3 and 6 months using a battery targeting episodic memory, working memory, and executive function. At the 3-month testing point, delayed word recognition and short-term spatial episodic memory were significantly better in the WBE111 treatment group compared to control. There were no significant differences at 6 months, or with consumption of the WBP500 and WBP1000 at any timepoint. It should be noted, however, that the quantity of anthocyanins administered is relatively small compared to other studies. Miller et al. (2018) recruited 13 men and 24 women between the ages of 60 and 75 to consume either 24 g of freeze-dried blueberries (containing 19.2 mg/g anthocyanins), or placebo for 90 days, with cognitive testing conducted at baseline, 45, and 90 days. Participants in the blueberry group showed a greater reduction in switch stimuli errors (measure of executive function) across visits compared to the control group [$F(2,70) = 3.587$; $p = 0.033$; $\eta p^2 = 0.09$]. Participants in the blueberry group also made fewer repetition errors on the California Verbal Learning Test free recall (measure of verbal memory) at 90 days compared to baseline, whilst the control group demonstrated increased errors compared to baseline [$F(1,35) = 5.024$; $p = 0.031$; $\eta p^2 = 0.126$]. No effect of the intervention was seen on tests of psychomotor speed, short-term memory, spatial cognition, or attention.

A single-blind, randomized, placebo-controlled, between-subjects study investigated executive function changes over a 6-h period following consumption of a 400 mL smoothie containing equal blueberry, strawberry, raspberry, and blackberry ($n = 20$), or placebo ($n = 20$) in 20–30 years old. Following berry intervention, participants maintained accuracy in executive function, up to and including the 6-h testing point, measured using the Modified Attention Network Task (MANT) and Task Switching Task (TST). Participants demonstrated quicker response times in the MANT conducted at the 2- and 4-h post-smoothie consumption cognitive assessments, and in the TST at 6-h post-smoothie consumption. The results of this study suggest that berry-related benefits were more evident during periods of fatigue, with placebo participants showing decreased performance across the 6 h as they became cognitively fatigued. Whilst the placebo was matched to the intervention for sugar content and sweetness, it was not matched for berry flavor, thus participants may have guessed which treatment they received, potentially leading to performance being influenced by placebo effect (Whyte et al., 2019).

A cross-over randomized controlled trial was conducted in 18 older adults (aged 60–75) using a flavonoid-rich blueberry beverage drink (579 mg of antho- and pro-cyanidins), or sugar-matched control. Cognitive function was assessed at baseline, 2- and 5-h post-beverage consumption. Whilst there was no significant effect of consumption of the flavonoid-rich beverage on global cognition compared to the control drink, there was a decline in performance in the control group from the 2-h to the 5-h assessment [$F(1,36) = 4.60$; $p = 0.04$], which was not observed in the intervention group. There were also no significant differences between groups in performance on the 14 tasks that comprised the global cognitive function score; although some associations were approaching significance, suggesting that the study may be underpowered (Dodd et al., 2018).

Sixty-five men and women aged 62–80 years completed a 24-week randomized double-blind, placebo-controlled trial consuming either daily fish oil, daily blueberry (providing 269 mg anthocyanin per day), both fish oil and blueberry, or control. Participants all reported mild, age-related cognitive decline, but lacked a diagnosis of MCI or dementia. No effect on motor speed, working memory, learning and retention, or lexical access was observed for the fish oil group, compared to placebo, at the end of the 24-week intervention, or at 48-week follow-up. There was improved discrimination in recognition memory for the blueberry treated group at 24-weeks, indicating improved resistance to interference of extraneous material in memory; however, this benefit was not maintained at week 48. Unexpectedly, there was no effect on any cognitive domain of the combined fish oil and blueberry treatment (McNamara et al., 2018).

In a randomized, double-blind placebo-controlled trial of 16 older adults (aged 68–92 years) with MCI, functional magnetic resonance imaging (MRI), conducted during a working memory task, was assessed pre- and post- 16-weeks of blueberry supplementation (providing 269 mg anthocyanins daily). Whilst there was no clear indication of working memory enhancement associated with supplementation, enhanced neural response during the working memory task was observed in the blueberry treatment group compared to placebo. Accuracy for the treatment group in the *1*-back task was trending toward significance ($p = 0.08$; participants had to recognize when the currently displayed letter was the same as the letter displayed one item previously), with the authors stating that the large observed effect size ($d = 1.02$) indicated that increasing the sample size from 8 to 17 per group would have been sufficient to achieve significance at $p = 0.05$ with power = 0.80 (Boespflug et al., 2018).

Change in performance on cognitive tests was not significantly different between placebo and blueberry supplementation

treatment groups in a 12-week double-blind randomized controlled trial of 26 cognitively normal participants aged over 65 years. Participants underwent computerized tests of psychomotor function, visual processing, executive function, verbal and spatial memory, and working memory. The percentage change in performance on the *2*-back test did, however, show weak evidence for improvement in the blueberry treatment group compared to placebo (Bowtell et al., 2017).

Of the 12 studies reviewed in this section, six reported improvements in at least one cognitive domain following anthocyanin consumption (four chronic studies and two acute), whilst six studies showed no improvement or change compared with placebo (four chronic studies and two acute). Cognitive domains impacted by anthocyanin intake included verbal fluency, short term memory, long term memory, visual memory, speed of response, accuracy, verbal memory, and executive function. Several cognitive domains including verbal memory and executive function were observed as being both impacted by anthocyanin intake in some studies but not affected in others; the possible reasons for these disparate results including cohort differences and dose are discussed in the "Discussion."

FLAVANOLS

Chocolate and cocoa products are a rich source of flavonoids with flavanols, and in particular epicatechin, being the most common type present in cocoa. High levels of flavanols are also found in tea, red wine, and fruits such as grapes and apples. In summary, we have reviewed two observational studies, six acute studies (<24 h) and seven chronic studies (duration between 1 and 6 months) investigating flavanols from fruit and vegetables, grape juice, cocoa, and apples in mainly cognitively normal participants (both young and older adults), with one MCI cohort.

A recently published intervention study of 211 healthy older adults (50–75 years) found a significant intake level-dependent treatment effect, compared to placebo, on hippocampal-dependent list-learning performance, following 12-weeks of flavanol intake (260, 510, or 770 mg/day cocoa flavanols). However, object recognition and prefrontal cortex-dependent list sorting performance did not improve. Eight weeks post-cessation of flavanol consumption, there were no observable effects remaining on list-learning performance in the intervention group compared to placebo. Notably, the object recognition task was newly developed, and data analysis suggested the task was so difficult that the majority of participants performed no better than chance; thus, failure to observe associations with flavanol intake may have been due to the psychometric properties of this outcome measure (Sloan et al., 2021).

Gratton et al. (2020) showed that flavanol intake can improve efficiency in blood oxygenation during hypercapnia (carbon dioxide-dependent increase in cerebral blood flow), and that this is likely to contribute to improvements in cognitive function, only when cognitive demand is high; significant associations were only observed in the Double Stroop Task and not in the standard Stroop Task. This study was conducted in 18 healthy males (18–45 years) using an acute, randomized, placebo-controlled, double-blind, crossover design, with high or low cocoa flavanol drink, and cognitive function assessed pre- and 2-h post-consumption (using a Modified Stroop Task). One advantage of this study is the intervention and placebo were matched for macronutrient and micronutrient content, including caffeine. With the cohort comprising only young males, future work should be extended to include females as well as older at-risk populations.

A study retrospectively analyzing data from 55 participants with amnestic MCI (29 males and 26 females, aged 56–75) found dietary supplementation with cocoa flavonoids was associated with slowed cognitive decline. Specifically, participants were divided into two groups depending on change in Mini-Mental State Examination (MMSE) score at 1-year follow-up (Group 1 = stable or improved, Group 2 = decreased by at least three points). Intake of the commonly used nutraceutical Mexenion® was examined. Mexenion® is composed of cocoa polyphenols (240 mg/sachet), *Bacopa monnieri* (110 mg/sachet), group B vitamins, vitamin E, and folic acid. Group 1 had a significantly higher rate of Mexenion® intake compared to Group 2 which demonstrated decline in cognitive function at 1-year follow-up [$\chi 2 = 13.79$, $p < 0.001$; 29]. However, given the formulation of Mexenion®, it is not possible to determine directly if the cocoa polyphenols present were responsible for the observed benefit.

Neshatdoust et al. (2016) conducted a randomized, controlled, double-masked, crossover dietary intervention involving 40 cognitively normal participants, aged 68.3 ± 3.0, who consumed either a high flavanol cocoa drink (494 mg flavanols) or a low flavanol cocoa drink (23 mg flavanols) for 28 days. After the first intervention period, there was a 4 week wash out prior to switching treatment. A cognitive battery measuring global executive function, and blood sample collection was completed pre-intervention, post-first intervention, and at the end of the study. Intake of the high flavanol cocoa drink resulted in significantly better performance on the global executive function measures ($p < 0.01$), and an increase in serum brain-derived neurotrophic factor (BDNF) levels ($p < 0.01$), relative to the low flavanol cocoa drink intervention. BDNF is a member of the neurotrophin family of growth factors that help to stimulate and control neurogenesis. The authors additionally conducted a randomized, controlled, dose-dependent, parallel designed trial in 154 participants (aged 26–70 years) with a high flavonoid fruit and vegetables (F&V) diet, or a low flavonoid F&V diet, compared to a habitual control diet. Participants consumed two portions of F&V for the first 6 weeks, followed by four portions for the next 6 weeks, and finally six portions for the remaining 6 weeks, this equated to 3, 6, and 7 mg/d in the low flavonoid intake group, and 49, 121, and 198 mg/d in the high flavonoid intake group. All F&V were provided to the participants and effort was taken to match the intake of other bioactive components of F&V such as carotenoids, vitamin C, and folate. Cognitive testing and blood sample collection occurred at weeks 0, 6, 12, and 18. Results showed significantly better performance on the global executive function measures at 12 and 18 weeks

for the high flavonoid F&V diet group relative to both the low flavonoid F&V diet and the habitual control diet ($p < 0.01$). Furthermore, serum BDNF was significantly higher at 18 weeks in the high flavonoid F&V diet group relative to the habitual control diet ($p < 0.001$). The authors concluded that increased brain and peripheral BDNF expression could be mediating the improvements observed in cognitive function (Neshatdoust et al., 2016).

Flavanol-rich chocolate has been shown to counteract the effects of sleep deprivation on working memory in women but not men. Sixteen women and 16 men participated in a randomized, double-blind, crossover study, undergoing four sets of cognitive testing, two after a night of undisturbed sleep and two after a night of total sleep deprivation, with a week between each of the four testing sessions. Participants consumed either a flavanol-poor chocolate bar or a flavanol-rich chocolate bar before each of the testing sessions. Accuracy in the *2*-back task was significantly higher in the sleep deprivation condition when the flavanol-rich chocolate was consumed, compared to the flavanol-poor chocolate, in women only ($p = 0.04$). Consumption of the flavanol-rich chocolate also produced positive effects on blood pressure, flow-mediated dilation, and pulse-wave velocity, which were all impaired following sleep deprivation (Grassi et al., 2016).

The dentate gyrus (DG), a brain region in the hippocampal formation, demonstrates functional decline with aging. In a controlled randomized trial of 37 cognitively normal adults aged 50–69 years, Brickman et al. (2014) showed that consumption of a high cocoa-containing diet for 3 months enhanced performance on a novel object recognition task which localizes function to the DG (high flavanol reaction time = 1,997 ms; low flavanol reaction time = 2,627 ms; t31 = 2.17; $p = 0.038$; Cohen's $d = 0.816$). There was no effect of the diet on delayed retention. The high flavanol group consumed two 450 mg supplements per day, and the low flavanol group consumed a total of 45 mg divided into two doses. The high flavanol group also demonstrated a significant increase in cerebral blood volume in the DG and the downstream subiculum in the body of the hippocampus, with the changes in object recognition task performance correlating with these cerebral blood volume increases. The authors concluded that DG dysfunction is a driver of age-related cognitive decline, and flavanol consumption may be a method to ameliorate this process.

The effect of consumption of a high flavanol cocoa drink compared to a low flavanol cocoa drink on cognitive function was investigated in 90 cognitively normal elderly individuals in a double-blind, controlled, parallel-arm study. Participants were randomly assigned to groups consuming either a drink containing 993 mg (high flavanol), 520 mg (intermediate flavanol), or 48 mg (low flavanol) cocoa flavanols, daily for 8 weeks. No change in MMSE score in response to the three treatments was observed. However, better performance on the Trail Making Test A and B was observed after consumption of the high and intermediate flavanol drinks, compared to the low flavanol drink ($p < 0.001$). Verbal Fluency Test scores were also significantly better after consumption of the high flavanol drink, compared to the intermediate and low flavanol drinks ($p < 0.001$). Additionally, significant improvements in insulin resistance, blood pressure, and lipid peroxidation were seen in the high and intermediate flavanol groups, in comparison to the low flavanol group; highlighting potential mechanistic pathways through which cocoa flavanols exert positive effects on cognition (Mastroiacovo et al., 2015).

By contrast, Massee et al. (2015) found daily consumption of cocoa supplementation (250 mg catechin polyphenols and 5.56 mg caffeine), for 4 weeks, had no effect on cognitive performance in 40 young cognitively normal adults. However, acute consumption, with cognitive testing conducted 3 h after first supplementation, was associated with significant improvement on Serial Sevens performance, in the first of three cycles of cognitive testing. Notably, the intervention duration was half that of Mastroiacovo et al. (2015), whilst the quantity of flavanols consumed was considerably less than the intermediate flavanol drink in that study. Additionally, participants in the current study were considerably younger with mean age 24.13 ± 4.47, and it is possible that by starting cognitive testing 3 h post-flavanol consumption the peak in flavanol concentration may have been missed.

A study investigating the effect of cocoa flavanol intake combined with exercise on executive function found that, whilst flavanol intake increased cerebral oxygenation during the executive function task, there was no impact on cognitive performance. Furthermore, exercise alone improved cognitive function, however, there was no additive effect of the flavanol consumption when combining the two conditions. The executive function task comprised the Stroop test, and potentially this short cognitive task was not sufficiently challenging for the young, healthy cohort to show improvements. The cohort comprised 12 males with a mean age of 30 ± 3 years, and the study was randomized, double-blind, and crossover in design (flavanol/placebo intake occurred 100 min preceding a 30 min time trial), with flavanol consumption of 904 mg (treatment), or 15 mg (control drink) (Decroix et al., 2016). Furthermore, a study investigating cognitive function an hour after consumption of either a low flavanol containing white chocolate, a medium flavanol containing milk chocolate, or a dark chocolate containing high flavanol levels, found no improvements on a computerized test battery (Cogstate Ltd.). The authors did, however, observe enhanced vascular endothelial function following the high flavanol chocolate condition which was reflected by improvements in brachial artery flow-mediated dilation. The cohort was limited to 12 post-menopausal women, but included strict control of the type, composition, and energy content of the chocolate used to ensure consistency between conditions (Marsh et al., 2017).

A cross-sectional study undertaken on 968 community-dwelling participants between the ages of 23 and 98 years, drawn from the Maine-Syracuse Longitudinal Study, showed that eating chocolate more than once a week was associated with better performance on a global cognitive composite ($p < 0.001$), as well as better visual-spatial memory and organization ($p < 0.001$), working memory ($p = 0.048$), scanning tracking ($p < 0.001$), and abstract reasoning ($p < 0.001$), compared to eating chocolate less than once a week. There were no associations observed between

chocolate intake and verbal memory. An important point to note, however, is that chocolate intake was not differentiated according to type, i.e., dark, milk, or white, all of which contain varying quantities of flavanols (Crichton et al., 2016).

Apple skin contains high concentrations of the flavanol (−)-epicatechin and the flavonol quercetin. In a randomized, controlled, crossover design study of 30 cognitively normal participants, with mean age 47.3 ± 13.6, individuals received the following treatments; (1) control: low flavonoid, low nitrate, (2) apple: high flavonoid, low nitrate, (3) spinach: low flavonoid, high nitrate, and (4) apple + spinach: high flavonoid, high nitrate (the authors were also assessing effects from nitrates). There was a minimum washout period of 1 week between treatments. Relative to control, all treatments augmented nitric oxide status acutely, but had no effect on cognitive function 150 min post-intervention. The cognitive battery assessed "Quality Working Memory," "Power of Attention," and "Continuity of Attention," and cognitive effects may have been evident with tasks measuring additional domains. The "younger" age range of participants and absence of cognitive impairment may also account for the lack of observed effect. Furthermore, this study assessed the acute effects of flavonoid intake, raising the possibility that longer-term consumption is required to induce cognitive benefit (Bondonno et al., 2014).

A 6-month bicentric, randomized, double-blind, placebo-controlled trial conducted in 215 cognitively normal participants (aged 60–70) receiving 600 mg/day of a flavanol-rich extract from grape and blueberry, demonstrated improvements in verbal episodic and recognition memory (VRM)-free recall in the treatment group, with no effect observed on Cambridge Neuropsychological Test Automated Battery (CANTAB) Paired Associate Learning (PAL), a measure of visual-spatial learning and episodic memory. However, there was a subgroup with "advanced cognitive decline" who showed better VRM-delayed recognition, post-flavanol treatment, only when the cohort was stratified into quartiles using baseline PAL score. The authors summarized that the study demonstrated a positive effect of flavanol intake on episodic memory in otherwise cognitively normal older adults with a lower level of memory performance (Bensalem et al., 2019).

Moreover, a randomized, placebo-controlled, double-blind, counterbalanced-crossover study of 20 healthy young adults (mean age 21.05 ± SD 0.89) found 230 mL of purple grape juice significantly improved reaction time, compared to placebo, on a composite attention measure ($p = 0.047$), 20 min after consumption. However, there was no effect on attention accuracy, or on measures of memory (Haskell-Ramsay et al., 2017).

Of the 15 studies reviewed in this section, 11 found improvements in at least one cognitive domain following flavanol consumption (two observational studies, six chronic, and three acute intervention studies). Of these 11 studies, flavanol intake was associated with improvements in global cognition as well as the cognitive domains of visual-spatial memory and organization, working memory, abstract reasoning, accuracy, reaction time, executive function, episodic memory, verbal fluency, and recognition memory. There were no common methodological approaches amongst the studies reporting positive effects compared to those reporting no effect, with a wide range of age groups included, varying flavanol doses utilized and two studies incorporating the additional variables of sleep deprivation and exercise. Factors likely impacting the varying results reported are discussed in greater detail in the "Discussion."

FLAVANONES

Orange juice is one of the most commonly consumed juices throughout the world and is a rich source of flavanones, particularly hesperidin and narirutin. As one of the most readily absorbed flavonoid subclasses, flavanones have been shown to cross the blood-brain barrier. In summary, we have reviewed two acute studies (<24 h) and one chronic study (duration 8 weeks) investigating flavanones from orange juice in cognitively normal participants (both young and older participants), and one prospective cohort study examining long-term intake of total and the six classes of dietary flavonoids over a follow-up period of up to 15 years, in dementia-free individuals aged 45 years and over.

High flavanone (HF; 305 mg) 100% orange juice and a low flavanone (LF; 37 mg) orange-flavored cordial were consumed daily for 8 weeks by 37 cognitively normal older adults (mean age 67 years) in a crossover, double-blind, randomized study, with a 4 weeks wash out period between treatments. After 8 weeks of consuming the HF drink, global cognitive performance was significantly better than after the LF drink intervention ($t = 2.86$; $p < 0.05$). In other cognitive areas, the HF drink reduced the decline in performance associated with LF beverage consumption. Further, performance was better when the HF drink was consumed in the first arm of the trial than when the LF drink was consumed first, indicating effects of the HF drink may have continued into the second arm of the intervention (Kean et al., 2015).

The effect of drinking a HF orange juice (272 mg) compared with placebo has been investigated acutely, with cognitive testing conducted at 2- and 6-h post-consumption, in a randomized, double-blind, counterbalance study, with a 2-week wash out period. This study was conducted in 22 healthy males between 30 and 65 years of age. The HF drink contained 220.46 mg hesperidin, 34.54 mg narirutin, and other flavonoids (17.14 mg). Performance on the Simple Finger Tapping test, a measure of psychomotor speed (involves pressing the "2" key as many times with the index finger over a 10 s period) was better, following the HF drink, relative to placebo [$F(1,20) = 8.32$; $p < 0.01$]. Additionally, the change in performance from baseline to 6 h post-consumption on Continuous Performance Task accuracy, a measure of psychomotor speed, was significantly different across the treatments, with fewer errors observed when the HF drink had been consumed. This test involves random presentation of letters at a rate of one every 250 ms for a duration of 6 min, and participants are required to press the space bar when a letter other than "X" is presented (Alharbi et al., 2016).

Lamport et al. (2016) investigated acute effects of HF juice consumption (orange and grapefruit juice) compared to a concentrated cordial drink containing zero flavonoids. The HF

drink contained considerably less total flavanones than the previously mentioned studies, with 70.5 mg present (42.15 mg hesperidin, 17.25 mg naringin, 6.75 mg narirutin, and 4.3 mg caffeic acid); however, the drink also contained additional flavanones in greater proportions, for example naringin. Twenty-four young adults between 18 and 30 years of age underwent cognitive testing at baseline and 2 h post-drink consumption, and a further 16 participants underwent functional MRI assessment at baseline, 2- and 5-h post-drink consumption to measure cerebral blood flow (CBF). The authors observed significantly improved performance on the Digit Symbol Substitution Test (DSST) 2 h after consumption of the HF drink, with no improvements following consumption of the control drink [$F(1,23) = 10.76, p < 0.01$]; however, other cognitive tests showed no improvements. Regional perfusion in the inferior frontal gyrus and middle frontal gyrus of the right hemisphere was significantly higher 2 h post-consumption of the HF drink compared with the control drink. There were no differences in regional perfusion 5 h after consumption of either drink. The inferior frontal gyrus has been implicated in tasks that require inhibition, planning, decision making, and other aspects of executive function, examined by the DSST. Stronger cognitive effects could have occurred more than 2 h post-drink consumption, as was seen in the previously mentioned studies, yet this was not assessed in the current study. However, increased CBF was observed at 2 h and not 5 h post-consumption in the current study, suggesting that the time course by which flavonoids in orange and grapefruit juice exert their effect may differ from orange juice alone. It is not possible, however, to determine a direct link between increased CBF and better cognitive function in the current study due to the separate cohorts utilized to investigate these outcomes.

All three studies reviewed thus far in this section reported improvements in at least one cognitive domain following flavanone consumption (one chronic study and two acute). Global cognition as well as the cognitive domains of psychomotor speed, and executive function were positively impacted by flavanone consumption. All three studies also reported no associations of flavanone intake with episodic memory and verbal memory. These three studies were conducted in cohorts of distinct age groups of cognitively normal adults, ranging from 30 to over 65 years. It appears therefore, that flavanone intake yields positive effects on cognition regardless of age. Further discussion of these results in conjunction with the results of the anthocyanin and flavanol studies is included in the "Discussion."

Finally, a recent study assessed the association of long-term intake of total and six classes of dietary flavonoids (flavanones, flavan-3-ols, flavonoid polymers, flavonols, flavones, and anthocyanins) and decline in cognitive function, over a median follow-up period of up to 11.8 years, in 1779 participants who were dementia free, aged ≥45 years at commencement, and who attended at least two study assessments (approximately 4 years apart). The findings did not support a clear association, with nominally significant trends observed between (1) higher flavonol and flavan-3-ol intakes, and slower decline in global function, verbal and visual memory; (2) higher total flavonoid and flavonoid polymer intakes and slower decline in visual memory; and (3) slower decline in verbal learning with higher flavanol intake (Shishtar et al., 2020). The age range of participants, and sensitivity of cognitive measures used, may have impacted the study findings.

MECHANISMS OF ACTION

Having a clear understanding of how these flavonoid subclasses are modulating cognitive function is required for furthering the use of these compounds as interventions which can be recommended by health professionals. For example, knowledge of a compound's mechanism of action enables better dosing through monitoring of the compound's effects on the target pathway in the patient. There is a relative paucity of data in humans, with the majority of mechanistic studies to date, having been undertaken in animal models. Nevertheless, these animal studies provide clues regarding potential mechanistic pathways in humans. Collectively, the published studies implicate a number of candidate mechanisms underlying the beneficial effects of consumption of these flavonoid subclasses on cognitive function, including, modulation of intracellular signaling pathways, altered CBF, and conferring protection against neurotoxins and neuroinflammation.

Modulation of Signaling Cascades

The flavonoid subclasses of anthocyanins, flavanols, and flavanones modulate several neurological processes via their interaction with signaling pathways involved in neuronal survival and function, upregulation of proteins important for synaptic plasticity and neuronal repair, and inhibition of neuropathological processes which occur in brain regions typically implicated in AD pathogenesis.

Various flavonoids interact with important cell survival signaling pathways including the phosphatidylinositol-3 kinase/Akt (PI3K/Akt), extracellular signal-regulated protein kinase (ERK), and protein kinase C pathways [PKC; (Levites et al., 2002; Spencer, 2007; Vauzour et al., 2007; Williams et al., 2008)]. Flavonoids have also been shown to modulate the cell death pathways mediated by p38 and c-Jun N-terminal kinase (Hwang and Yen, 2009) to confer protection against neurodegeneration. These interactions are summarized in **Figure 1**.

The activation of cell survival pathways by flavonoids involves upregulation of anti-apoptotic and pro-survival genes, and inhibition of pro-apoptotic proteins (Levites et al., 2002; Spencer, 2007). Targets include inhibition of apoptosis signal-regulating kinase 1 (ASK1; Vauzour et al., 2007), and non-transcriptional inhibition of BclxL/Bcl2-associated death promoter (Bad), BCL2-associated X protein (Bax), and murine double minute 2 (Mdm2; Levites et al., 2002). Flavonoids are also proposed to confer cell survival through activation of cAMP response element-binding protein (CREBP) phosphorylation (Williams et al., 2008) and increases in levels of BDNF (Williams et al., 2008). As mentioned previously, BDNF is a neurotrophin required for the development and maintenance of the nervous system (Schindowski et al., 2008). BDNF levels are known to decline during aging, and their levels have been shown to correlate

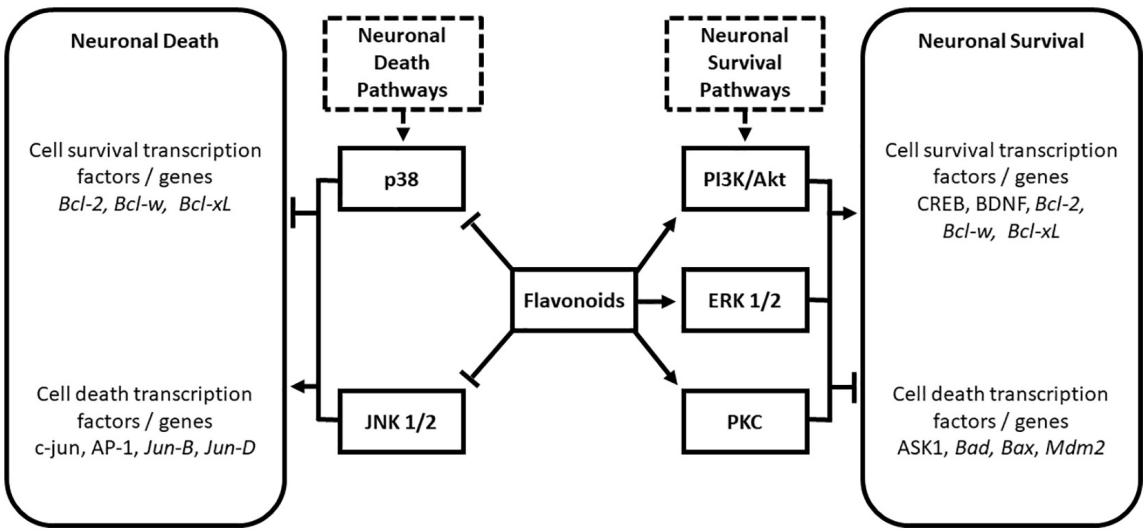


FIGURE 1 | Modulation of neuronal death and survival pathways by the flavonoid subclasses of anthocyanins, flavanols, and flavanones. Modified from Solanki et al. (2015). AP-1, activated protein 1; ASK1, apoptosis signal-regulating kinase 1; Bad, BclxL/Bcl-2-associated death promoter; Bax, Bcl-2-associated X protein; Bcl-2, B-cell CLL/lymphoma 2; Bcl-w, BCL2-like 1; BDNF, Brain-derived Neurotropic Factor; CREB, cAMP response element-binding protein; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; Mdm2, murine double minute 2; PI3K/Akt, phosphatidylinositol-3 kinase/Protein kinase B; PKC, protein kinase C.

with learning and memory (Garzon et al., 2002; Hwang et al., 2006; Laske et al., 2006). JNK and p38 are strongly linked to transcription-dependent apoptotic signaling (Mielke and Herdegen, 2000) via the activation of c-Jun and other activated protein 1 (AP-1) proteins including Jun-B and Jun-D (Behrens et al., 1999): flavonoids have been shown to impact these pathways at multiple levels to confer antiapoptotic activity (Ramiro-Puig et al., 2009).

The inflammatory cascade is believed to play a critical role in the development of chronic low grade inflammatory diseases such as AD. Reductions in blood levels of inflammatory markers such as CRP, IL-6, and tumor necrosis factor alpha (TNF-α) have been shown following flavonoid consumption, suggesting anti-inflammatory activity (Carey et al., 2014; Macready et al., 2014). Furthermore, flavonoids have the capacity to downregulate the activity of pro-inflammatory transcription factors such as NF-κB, nuclear factor erythroid 2-related factor 2 (Nrf2) and STAT (signal transducers and activators of transcription), through their influences on a number of glial and neuronal signaling pathways (Hamalainen et al., 2007; Risitano et al., 2014).

Mitochondria control cellular energy status, reactive oxygen species (ROS) production, and apoptosis, all of which are important for determining lifespan, and mitochondria are therefore, proposed to act as central organelles in the regulation of aging and neurodegeneration. Mitochondria constitute the major source of superoxide and other ROS within most tissues. AMP-activated protein kinase (AMPK), a key cellular regulator of energy metabolism, has been implicated in the regulation of mitochondria function. Damaged mitochondria are the major sources of ROS in cells and are implicated in many neurodegenerative diseases including AD. Activated AMPK can decrease intracellular ROS by inhibiting NADPH (nicotinamide adenine dinucleotide phosphate) oxidase activity, or by increasing the expression of antioxidant enzymes such as superoxide dismutase-2 and uncoupling protein-2 (Manczak et al., 2006). Flavonoids may ameliorate mitochondrial dysfunction and increase oxidative defense mechanisms by activating AMPK (Cordero-Herrera et al., 2013).

Indirect Benefits to Cerebrovasculature

Consumption of flavonoids could improve cerebrovascular outcomes including CBF. Whilst the mechanisms require elucidation, they may be related to increases in the pool of bioavailable nitric oxide (NO; Bondonno et al., 2012), which is a key signaling molecule responsible for mediating vascular function changes (Heiss et al., 2007). Flavonoids may enhance NO levels by preventing NO breakdown, which could occur by a direct reaction with superoxide and other ROS and/or inhibition of the enzymes that produce these molecules [xanthine oxidase, lipoxygenase, and NADPH oxidase; (Nijveldt et al., 2001; Mladenka et al., 2010)]. One mechanism by which NO is synthesized is by the action of endothelial nitric oxide synthase (eNOS) on arginine, and flavonoids may enhance NO production by increasing eNOS activity or enhancing expression. Decreases in blood pressure, improvements in endothelial function (Bondonno et al., 2012), and improvements in CBF have all been observed in association with an increase in NO status (Francis et al., 2006). This is consistent with literature showing natural products that enhance brain metabolism or elevate CBF are effective at augmenting cognitive function during prolonged, effortful, cognitive processing (Owen and Sunram-Lea, 2011), with reduced blood flow to the brain associated with cognitive impairment (Farkas et al., 2002).

Moreover, there is evidence to show that increasing glucoregulatory control via improved insulin sensitivity can improve cognitive function, with intake of flavonoids being shown to increase insulin sensitivity, as well as insulin and glucose levels (Russo et al., 2019). Attenuation of decline in blood glucose concentrations has been observed following blackcurrant (Watson et al., 2015) and cranberry (Wilson et al., 2008; Torronen et al., 2010) consumption, which suggests glucose regulation may be an additional mechanism through which flavonoids confer cognitive benefit, particularly in the immediate postprandial period where the majority of improvements in cognitive performances were observed.

DISCUSSION

Evidence accumulated to date suggests the consumption of products containing anthocyanins, flavanols, and flavanones, such as berries and cocoa, throughout life, may have the potential to limit or even reverse age-related declines in cognition and memory, and potentially delay the onset and progression of neurodegenerative diseases such as AD. Over the last 6 years, the flavonoid subgroups of anthocyanins, flavanols, and flavanones have been shown to be beneficial in terms of conferring neuroprotection and cognitive benefit. Of note, however, methodological disparities hinder comparison of results between studies. For example, a vast range of flavonoid-containing products utilized in interventions of varying durations, and differences in comprehensiveness of neuropsychological assessments, as well as heterogeneity of cohorts, are all likely to impact findings.

Of the literature reviewed here, acute studies ranged in duration from 60 min to 6 h, whilst the longer-term studies ranged from 28 days to 6 months, with 3 months being the most common timeframe employed. All except two intervention studies included control groups consuming a placebo, with these two studies comparing cognitive function at baseline to end point (16 weeks or 6 h). Whilst studies were included in the current review regardless of placebo being matched with the intervention condition for macro- and micro- nutrient content, this should be a consideration when designing future interventions, to ensure observed effects are due to the flavonoid content and not additional components (e.g., caffeine which has independent effects on alertness, mood, arousal, and concentration). The quantity of flavonoid treatment in the reviewed studies also varied greatly, with some participants consuming 900 mg daily or greater, and others under 100 mg. Interestingly, a study including groups of participants consuming 993 mg or 520 mg daily, for 8 weeks, found both quantities were associated with improvements in measures of attention, visual search and scanning, sequencing and shifting, and psychomotor speed, but only the 993 mg group also demonstrated increased performance in verbal fluency (Mastroiacovo et al., 2015).

A range of cognitive domains have been shown to be beneficially affected in the reviewed studies; summarized in **Figure 2**. Acute supplementation with the three flavonoid

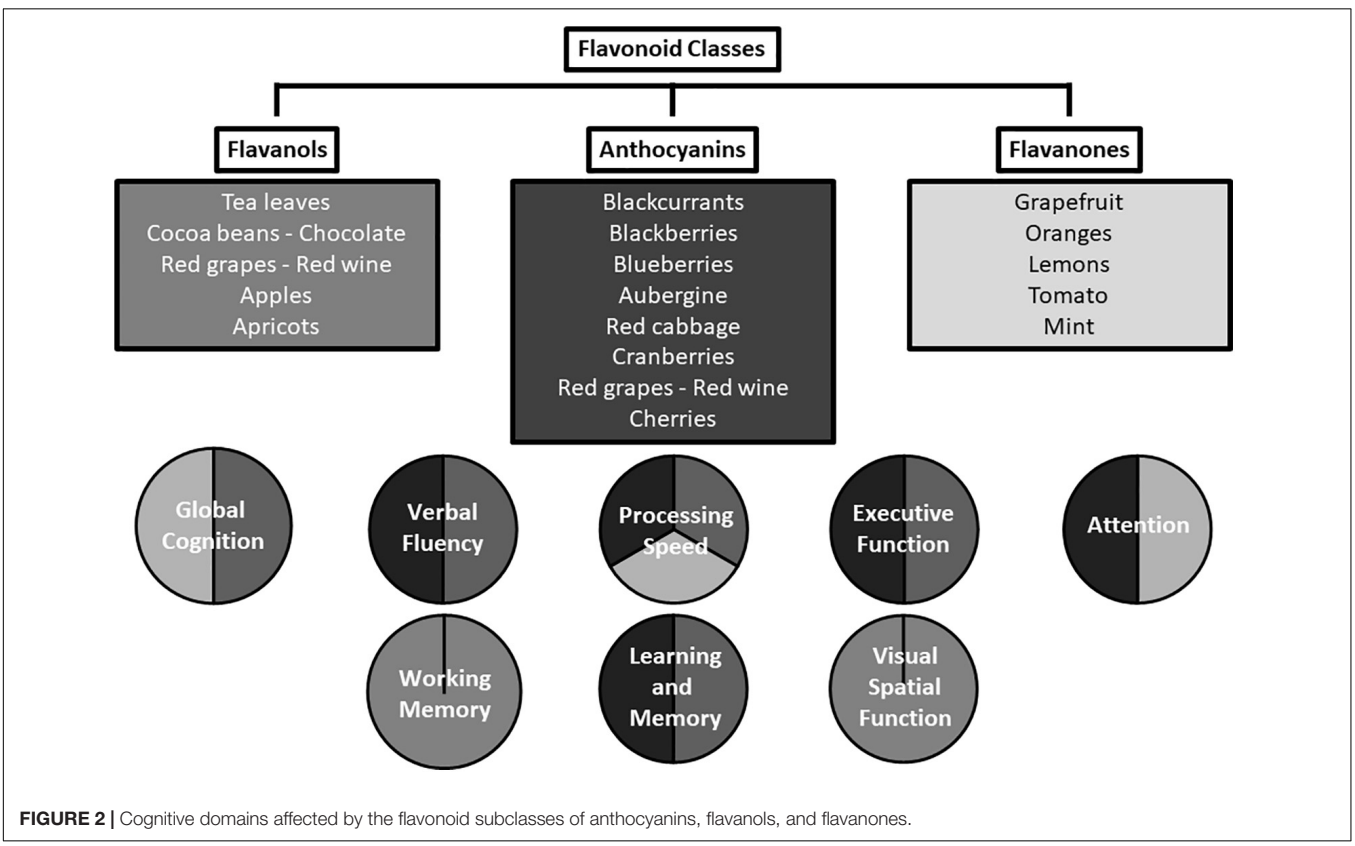


FIGURE 2 | Cognitive domains affected by the flavonoid subclasses of anthocyanins, flavanols, and flavanones.

subclasses investigated was associated with positive effects in tests measuring psychomotor speed, executive function, and attention. Longer-term supplementation was associated with positive effects in tests of episodic memory, global cognition, verbal fluency, psychomotor speed, and verbal memory, in multiple studies. Although a particular task may have a primary focus such as verbal fluency or episodic memory, a range of processes may support the primary focus, e.g., memory, processing speed, and motor function. Potentially, flavonoids could be exerting greater effects on relatively effortful tasks and less effect on simpler tasks. This notion is consistent with the results of Bondonno et al. (2014), who utilized a computerized cognitive assessment battery which was not heavily loaded with cognitive processing tasks, and yielded no significant associations. Furthermore, Gratton et al. (2020) showed improvements in cognitive function only when cognitive demand is high; significant associations were only observed 2 h following high flavanol consumption in the Double Stroop condition and not in the standard word and color Stroop conditions. A wide range of tasks which fully assess cognitive ability (multiple domains and tasks of varying complexity) needs to be employed by studies attempting to fully characterize the effects of flavonoids on cognition.

The mechanisms by which the flavonoid subclasses of anthocyanins, flavanols, and flavanones modulate cognitive function are yet to be fully established. Available evidence plausibly suggests these flavonoid subclasses can promote beneficial effects via both direct (i.e., through receptor activation, neurotrophin, and signaling pathway modulation) and indirect actions (i.e., enhancement of CBF). Although, it should be noted that the majority of mechanistic studies to date have been conducted in animal models, and more human studies are required to draw definitive conclusions.

Wide inter-individual variability in flavonoid absorption and metabolism has been reported, with a number of factors including age and gender affecting these metabolic processes. Flavonoids initially undergo extensive metabolization by phase I and II metabolism which occur predominantly in the gastrointestinal tract and liver. Hepatic perfusion and morphology reduce as part of the aging process, which has been suggested to reduce phase I and II metabolism of flavonoids. Flavonoid absorption occurs in both the small and large intestines, with a high percentage reaching the colon where there is exposure to colonic microbiota (Williamson and Clifford, 2010). The microbiome plays a key role in catabolizing unabsorbed flavonoids into smaller molecules which may become bioavailable. It is conceivable that heterogeneity in flavonoid absorption and metabolism may diminish beneficial associations between intakes and cognitive outcomes reported in studies. Ideally, urinary flavonoid excretion results would be taken into account in analyses, and to inform personalized health goals.

FUTURE RESEARCH

Overall, additional prospective studies conducted in diverse populations, and adequately powered intervention studies with long durations, are required to thoroughly examine the effect of consumption of the flavonoid subclasses of anthocyanins, flavanols, and flavanones on clinically relevant cognitive outcomes. Moreover, the characterization of appropriate dosage, timeframes for intake, and form of flavonoids, remains to be fully determined. Whilst animal models have shown promising results with interventions of timeframes such as 2 months, this represents a considerably larger percentage of total lifespan for animals than it does for humans, and a longer time-frame could be needed to show enhanced outcomes in human trials. Both acute and chronic effects of these flavonoid subclasses also need to be investigated using neuroimaging techniques in conjunction with cognitive and physiological measures to further elucidate the underlying biological mechanisms. Furthermore, it should be acknowledged that studies in cognitively normal adults are unlikely to demonstrate large improvements in cognitive function, and therefore it is imperative that suitable, sensitive cognitive tests are utilized. There is currently no clear evidence regarding the specific domains of cognition and memory that these flavonoid subclasses impact, with future studies requiring a range of cognitive domains to be investigated in order to determine those most likely to benefit. If emerging evidence continues to suggest significant cognitive benefit, another important consideration is the optimum age for the initiation of supplementation of flavonoid intake. Indeed, the neuropathological hallmarks of AD begin to accumulate 15–20 years before symptoms manifest, implying that the optimal age for supplementing flavonoid consumption could be middle-age or younger.

CONCLUSION

As populations continue to focus on developing strategies to promote healthy aging, dietary interventions with flavonoids represents a promising avenue for future research. However, many questions still need to be answered before a definite conclusion can be made regarding the extent to which consumption of anthocyanins, flavanols, and flavanones can protect the aging brain, and intake can be included in public health dietary recommendations.

AUTHOR CONTRIBUTIONS

SG and SR-S contributed to the conception and design of the work. SG prepared the first draft of the manuscript. SR-S, MW, CB, and RM revised the manuscript. All authors approved the final version of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.640381/full#supplementary-material

Supplementary Table 1 | Intervention human studies published over the past 6 years investigating flavonoid consumption and cognitive outcomes.

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REVIEW
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Potential of Sorghum Polyphenols to Prevent and Treat Alzheimer's Disease: A Review Article

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Alzheimer's disease (AD) is characterized by the excessive deposition of extracellular amyloid-beta peptide (Aβ) and the build-up of intracellular neurofibrillary tangles containing hyperphosphorylated tau proteins. This leads to neuronal damage, cell death and consequently results in memory and learning impairments leading to dementia. Although the exact cause of AD is not yet clear, numerous studies indicate that oxidative stress, inflammation, and mitochondrial dysfunction significantly contribute to its onset and progression. There is no effective therapeutic approach to stop the progression of AD and its associated symptoms. Thus, early intervention, preferably, pre-clinically when the brain is not significantly affected, is a better option for effective treatment. Natural polyphenols (PP) target multiple AD-related pathways such as protecting the brain from Aβ and tau neurotoxicity, ameliorating oxidative damage and mitochondrial dysfunction. Among natural products, the cereal crop sorghum has some unique features. It is one of the major global grain crops but in the developed world, it is primarily used as feed for farm animals. A broad range of PP, including phenolic acids, flavonoids, and condensed tannins are present in sorghum grain including some classes such as proanthocyanidins that are rarely found in others plants. Pigmented varieties of sorghum have the highest polyphenolic content and antioxidant activity which potentially makes their consumption beneficial for human health through different pathways such as oxidative stress reduction and thus the prevention and treatment of neurodegenerative diseases. This review summarizes the potential of sorghum PP to beneficially affect the neuropathology of AD.

Keywords: Alzheimer's disease, sorghum, polyphenols, antioxidant, amyloid-beta, tau, mitochondrial dysfunction, flavonoids

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INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by different neuropathological features including excessive accumulation of Aβ peptides outside of neurons and the hyperphosphorylated form of tau protein inside neurons (Duyckaerts et al., 2009). Currently, more than 50 million people worldwide and more than 440,000 Australians are living with AD

(Dementia Australia, 2018a; WHO, 2021). The number of affected people worldwide is expected to reach 152 million by 2050. According to the Australian Bureau of Statistics in 2017, AD was the first leading cause of mortality in Australian women and second leading cause of mortality in all Australians (Australian Bureau of Statistics, 2017). The estimated cost of dementia in Australia was more than $15 billion in 2018 and it will be more than $18.7 billion by 2025, and more than $36.8 billion by 2050 (The National Centre for Social and Economic Modelling NATSEM, 2016).

Despite advances in medicine and drug therapies, a disease-modifying treatment of AD is still not available. Recently, the new drug (Aduhelm) has been approved by the FDA as a modulator of amyloid plaques in the brain but its efficacy on memory and cognition is yet to be determined (Alexander et al., 2021; Canady, 2021). Current drug interventions only temporarily delay the progression of some of the cognitive symptoms of AD. Acetylcholine is a major neurotransmitter in the brain which has limited reserves in the AD brain where the enzymes cholinesterase breaks down the beneficial acetylcholine. Thus, cholinesterase inhibitors including acetylcholinesterase and butyrylcholinesterase inhibitors (AChEI and BChEI) help to attenuate the AD symptoms. The U.S. Food and Drug Administration (FDA) has approved five symptomatic drugs for the treatment of AD, including three cholinesterase inhibitors (rivastigmine, galantamine, donepezil) and memantine and memantine mixed with donepezil (Alzheimer's Association, 2019). These treatments reduce the rate of progression of cognitive decline by increasing the neurotransmitters in the brain, but they are unable to prevent irreversible damage to neurons (Sivaraman et al., 2019). Therefore, there is a serious need to find a reliable intervention that can prevent or slow AD progression.

Natural sources of antioxidants have been identified as a promising preventive or therapeutic avenue for neuroprotection. Polyphenols (PP) are the most abundant antioxidants in the diet (Kulshreshtha and Piplani, 2016). Diets rich in PP are considered neuroprotective due to their capacity to affect several cellular pathways, that contribute to the pathogenesis of AD, will be discussed below (Malar and Devi, 2014; Lakey-Beitia et al., 2015; Omar et al., 2017).

Sorghum grain is a natural source of antioxidants with strong anti-inflammatory activities (Burdette et al., 2010; Xiong et al., 2019). The profile of PP of sorghum is unique and epidemiological evidence have demonstrated these PP may have specific health benefits such as superior chemoprotective properties and strong anti-inflammatory activity which are not provided by PP in other grains, such as rice, oats, and wheat (Awika, 2011). In addition, several PP of sorghum are thought to beneficially interfere with pathological changes in AD, such as Aβ and tau accumulation in *in vitro* and *in vivo* level (Rossi et al., 2008; Jabir et al., 2018) However, research is yet to identify the effect of sorghum PP on AD pathology.

This review will focus on the current evidence and potential mechanisms for protective effects of sorghum PP on the pathology of AD.

ALZHEIMER'S DISEASE AND ASSOCIATED PATHOLOGICAL HALLMARKS

Dementia is a group of disorders characterized by progressive cognitive impairment which affects daily living activities (Roman, 2002). Dementia is considered as one of the most serious health and social concerns of the century. It has major impacts on individuals, carers, families, and societies.

Alzheimer's disease is the most prevalent form of dementia (Puglielli et al., 2003) with the clinical symptoms of progressive memory decline and other cognitive functions, eventually leading to an inability to do daily tasks and a reliance on care (Long and Holtzman, 2019). It is pathologically characterized by the accumulation of extracellular Aβ oligomers, hyper-phosphorylation of intracellular neurofibrillary tangles (NFTs; tau protein) and neuroinflammation in the brain (Sadhukhan et al., 2018). Other primary changes of AD include increased oxidative stress, mitochondrial dysfunction, and neuroinflammation (Mecocci et al., 2018). A diagram of the generally accepted hypothesis for AD is shown in **Figure 1**.

Aβ Accumulation

According to the amyloid hypothesis (Hardy and Higgins, 1992), the primary cause of AD is the accumulation and deposition of oligomeric or fibrillar Aβ peptides. The Aβ peptide consists of 38–42 amino acids that are derived from amyloid precursor protein (APP); a transmembrane protein that has two competing pathways. In the non-amyloidogenic pathway, it is cleaved by α-secretase, to produce the secretory fragment sAPPα (see **Figure 2**). The candidate enzymes for α-secretase which are from the a-disintegrin and metalloprotease (ADAM) family include ADAM17, ADAM9, and ADAM10 (Buxbaum et al., 1998; Lammich et al., 1999; Fahrenholz et al., 2000; Asai et al., 2003). Among these enzymes, ADAM10 is suggested as the most physiologically relevant α-secretase in neurons (Anders et al., 2001; Kuhn et al., 2010). In the amyloidogenic pathway, APP is initially cleaved by β-secretase (BACE1), then γ-secretase, resulting in the generation of Aβ peptides (Gandy et al., 1994). The non-amyloidogenic pathway is beneficial since sAPPα has neuroprotective activity (Corrigan et al., 2011). In contrast, in the amyloidogenic pathway, an over-production of Aβ and its accumulation results in cytotoxicity (Chasseigneaux and Allinquant, 2012; Paroni et al., 2019). The length of the Aβ peptide influences this toxicity, where $A\beta_{42}$ (42 amino acids) is more cytotoxic than $A\beta_{40}$ and $A\beta_{43}$ (Fu et al., 2017). This is because $A\beta_{42}$ has a higher hydrophobicity and thus higher propensity to aggregate by hydrophobic bonding into toxic oligomers compared with $A\beta_{40}$ peptide (Vion et al., 2018). Several inherited and environmental factors such as APP, presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*), gene mutations, deficit Aβ clearance, oxidative stress and mitochondrial dysfunction might be contributing factors to the over-production and accumulation of Aβ (Mao and Reddy, 2011;

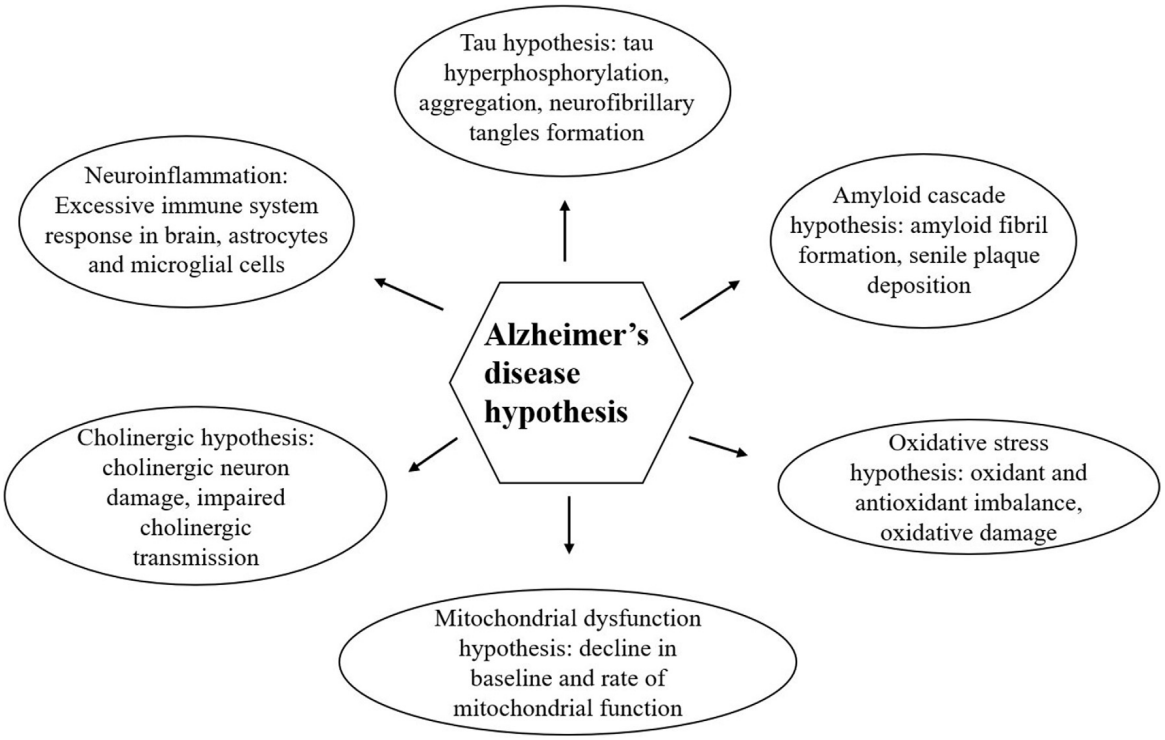


FIGURE 1 | Alzheimer's disease (AD) hypotheses (Swerdlow, 2018; Altinoglu and Adali, 2020). The six most common AD hypotheses include the amyloid cascade hypothesis, tau hypothesis, neuroinflammation, cholinergic hypothesis, mitochondrial dysfunction hypothesis, and oxidative stress hypothesis which are explained separately through the article. All the these mechanisms can interact with each other.

Hernández-Zimbrón and Rivas-Arancibia, 2015; Zuo et al., 2015; Paroni et al., 2019). Although amyloid deposition is always seen in AD patients, its pathogenic role is still unclear (Modrego and Lobo, 2019). While many questions still remain unanswered regarding the pathogenesis of AD, the amyloid hypothesis is still the most accepted theory to describe the associated neuropathological events.

Tau Proteins

Tau proteins are phosphoproteins present in all cells of the central nervous system (CNS; Lionnet et al., 2018). The main function of tau is the modulation of microtubule stability which forms the main pathway for intracellular protein trafficking (Mandelkow, 1998; Buée et al., 2000). But in AD, abnormal hyperphosphorylation of tau leads to its dysfunction, resulting in impairment of the transport system, the cytoskeleton, intracellular signaling, and mitochondrial integrity (Mandelkow, 1998; Iqbal et al., 2005). Hyperphosphorylated tau proteins dissociate from the microtubule (**Figure 3**) and bind with each other, forming paired helical filaments (PHFs). These accumulate, resulting in the characteristic NFTs seen in AD pathology (Gamblin et al., 2003).

There are other alternative hypotheses where Aβ plaques and NFTs may be formed independently and may be the products of dementia and not the cause (Hardy and Selkoe, 2002; Selkoe and Hardy, 2016). To date, there is no satisfactory hypothesis that can fully explain the exact mechanism of Aβ and tau accumulation, aggregation, and subsequent toxicity.

Neuroinflammation

The neuroinflammation is a response of the innate immune system within the brain as shown by an increased level of activated microglia and astrocytes, activated complement proteins and cytokines (Heneka et al., 2015; Zhang and Jiang, 2015). In AD, Aβ plaques and NFTs exacerbate any chronic inflammatory state, resulting in the increased action of cytokines (interleukin 1, tumor necrosis factor), prostaglandins, growth factors, thromboxanes, and ROS. These, in turn, enhance the APP processing, increasing $A\beta_{42}$ levels in brain (Meraz-Ríos et al., 2013). Aβ also activates proinflammatory cytokines and some pro-inflammatory enzymes, such as cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and nuclear factor kappa B (NF-κB). This Aβ-linked inflammatory response has been claimed to lead the neuronal damage in AD (Meraz-Ríos et al., 2013; Heneka et al., 2015; Zhang and Jiang, 2015). Accumulating evidence suggests that neuroinflammation is a major contributor of AD onset and progression (Heneka et al., 2010). Long-term consumption of nonsteroidal anti-inflammatory drugs (NSAIDs) can delay the onset or progression of AD, which also supports the role of neuroinflammation in AD (Ali et al., 2019).

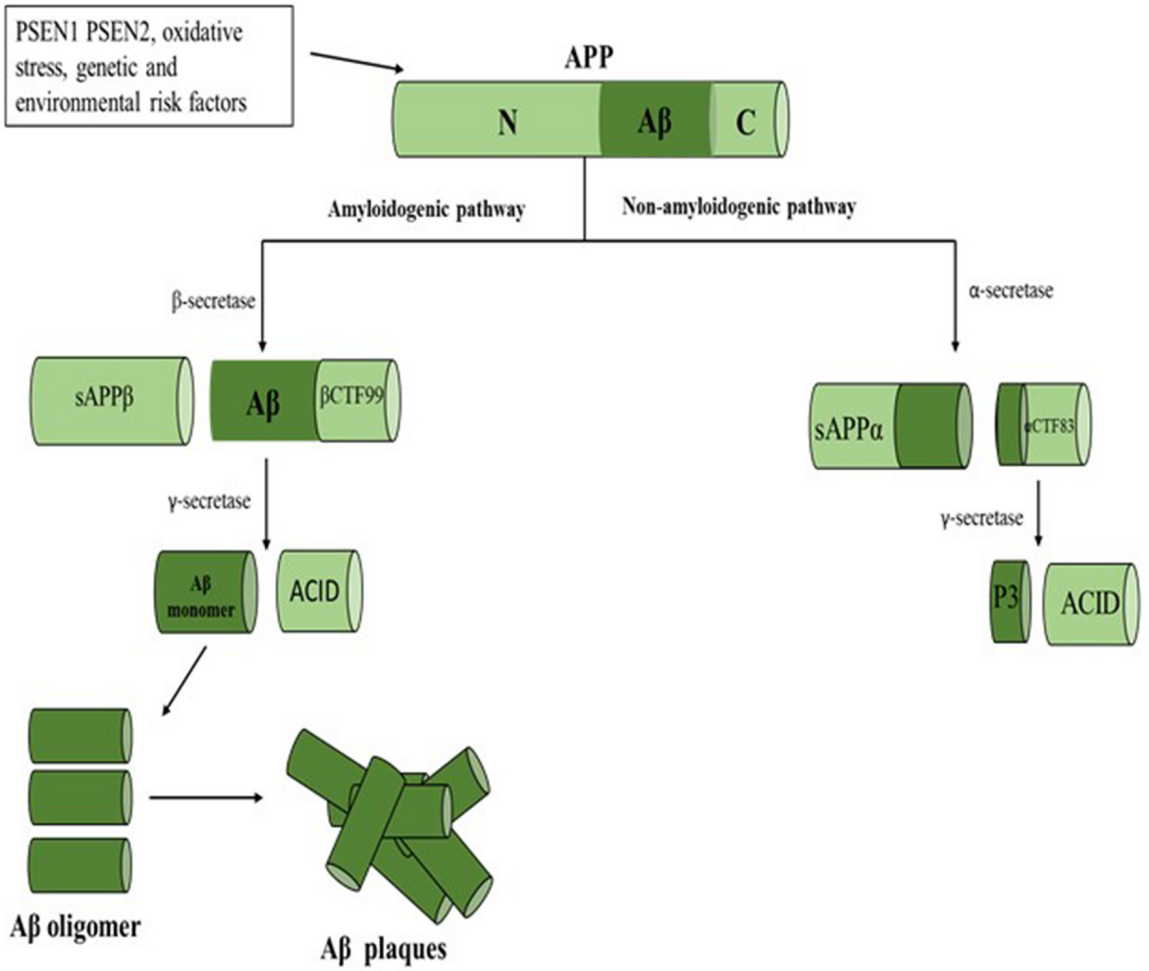


FIGURE 2 | Schematic of Aβ hypothesis. APP is sequentially cleaved *via* amyloidogenic or non-amyloidogenic pathways. In the non-amyloidogenic pathway, it is cleaved by α-secretase, resulting in the production of the sAPPα. In the amyloidogenic pathway, APP is initially cleaved by β-secretase, then γ-secretase, resulting in the production of Aβ peptides. Abbreviations: APP, amyloid precursor protein; *PSEN1*, presenilin 1; *PSEN2*, presenilin 2; Aβ, amyloid beta.

Oxidative Stress as Another Major Contributor to AD Pathology

A growing body of literature indicates that oxidative stress is another pathophysiological feature of the AD brain (Good et al., 1996; Agostinho et al., 2010). Oxidative stress is the imbalance between the formation and detoxification of reactive oxygen species (ROS; Adwas et al., 2019). These ROS are normally produced as by-products of oxygen metabolism which utilizes both free radicals and non-free radical oxygen intermediate species, such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radical ($^\bullet OH$), and singlet oxygen ($^1O_2$). These ROS are known to damage many biomolecules including DNA, RNA, protein, and lipids (Pham-Huy et al., 2008). Studies suggest that oxidative stress could: (a) be a consequence of Aβ deposition; (b) induce the production of Aβ; (c) be a combination of both: (a) and (b) (Sonnen et al., 2008; Tamagno et al., 2012). ROS are produced *in vivo* during oxidation and are involved in the progression of various health problems including cellular aging, mutagenesis, cardiovascular problems, diabetes, and neurodegeneration (Halliwell and Gutteridge, 1999; Moskovitz et al., 2002).

A high intake of foods rich in antioxidants may be beneficial to attenuate the ROS-associated problemsbased on the result of human dietary intervention studies (Lobo et al., 2010; Liu et al., 2018).

Mitochondrial Dysfunction as One of the Suggested Contributors of AD Pathology

Mitochondria are responsible for energy homeostasis in cells. Their dysfunction may contribute to the progression of several diseases including cancer, cardiovascular diseases, diabetes and neurodegenerative diseases (de Moura et al., 2010; Wen et al., 2016). A large body of research indicates that dysfunctional mitochondria play an important role in the pathogenesis of AD (Bhatti et al., 2017).

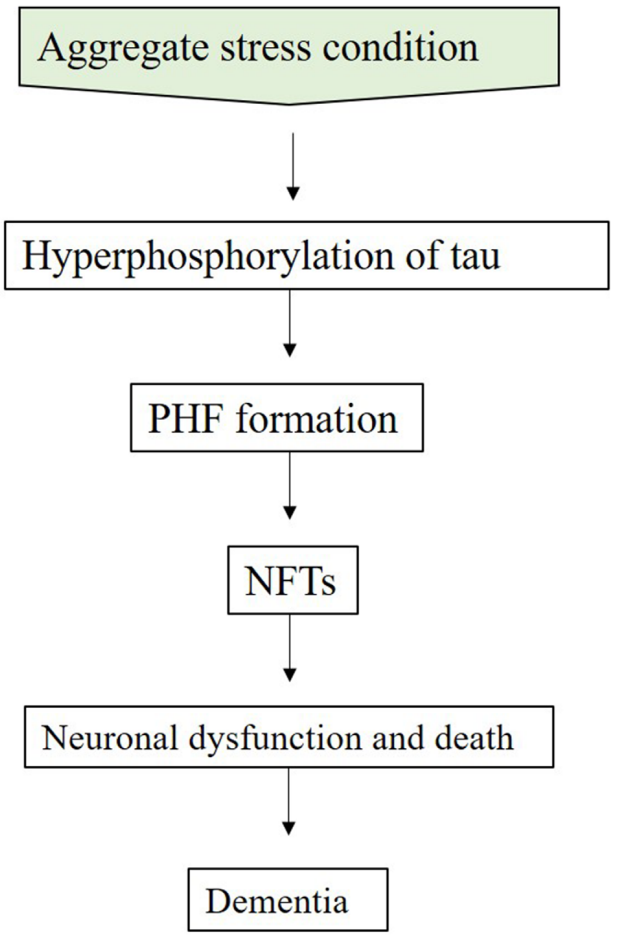


FIGURE 3 | Schematic of tau hypothesis. Aggregate stress condition is a condition in which Aβ aggregation alters the kinase/phosphatase activity that can lead to hyperphosphorylation of tau resulting in PHF formation which consequently leads to neuronal dysfunction and dementia (Verwilst et al., 2018). Abbreviations: PHF, paired helical filament; NFT, neurofibrillary tangles.

The initial level of mitochondria functions as well as its rate of decline influences AD onset and progression (Swerdlow et al., 2014). When mitochondrial function falls below a critical threshold, abnormal tau phosphorylation processes, amyloid plaque generation, synaptic degeneration, and oxidative stress can result (Lezi and Swerdlow, 2012). Several essential mitochondrial functions such as biogenesis, fission/fusion, and bioenergetics are also associated with AD. This makes mitochondrial dysfunction an important factor to consider in AD pathogenesis and its prevention (Lezi and Swerdlow, 2012; Nicolson, 2014; Flannery and Trushina, 2019).

RISK FACTORS FOR ALZHEIMER'S DISEASE

Risk factors are the specific conditions of an individual's lifestyle along with genetic, gender, and environmental factors that determine the likelihood of developing AD (Alzheimer's Association, 2018). The risk factors of AD can be divided into two main groups. Modifiable risks, are those that can be reduced through specific actions such as lifestyle changes. These risks contribute to 40% of AD cases (Livingston et al., 2020). Non-modifiable risks are those that cannot be changed including parental dementia, genetic risk factors and etc. (Alzheimer's Association, 2018).

Modifiable Risk Factors

There is compelling evidence that smoking, high blood pressure, diabetes, high cholesterol, and obesity significantly increase the risk of AD (Prince et al., 2014). Hormones (testosterone and estrogen) can have a neuroprotective effect through regulating Aβ, thus, age-related decline in these hormones can affect cognitive ability and therefore increase the risk of developing AD (Verdile et al., 2014). Traumatic brain injury (TBI) is also reported to increase AD risk (Alzheimer's Association, 2018).

Lifestyle factors including a healthy diet; adequate physical exercise, good sleep hygiene and cognitive training have been shown to reduce the risk of developing AD (Bauer and Morley, 2018). Conversely psychological factors (e.g., depression, anxiety, and stress) and vascular risk factors contribute to an increased risk of AD. A diet with high PP and high antioxidant activity can thus be considered as an approach to assist the prevention of chronic diseases, especially AD.

Non-modifiable Factors

Age is the main risk factor for AD. As people age, the risk of AD increases exponentially, as shown in several population based studies (Corrada et al., 2010). Apolipoprotein E *(APOE)* ε4 allele is the major genetic risk factor, which increases the probability of developing AD (Thakur et al., 2019). The effects of *APOE* ε4 on cognitive ability are variable from person to person (Prince et al., 2014; O'Donoghue et al., 2018). The *APOE* gene is present in chromosome 19 (Dementia Australia, 2018b). In humans, there are three common alleles: ε2, 3 and 4. Each individual carries two apolipoprotein genes which can be the same type (ε2, 2; 3, 3 or 4, 4), or a combination of two types (ε2, 3; 2, 4; 3, 4; Dementia Australia, 2018b). Individuals with at least one ε4 have a 2 to 3-fold risk of AD while those with two ε4 alleles (4, 4) rarely escape the disease. Compared to the other APOE alleles, the higher risk of developing AD in ε4 alleles is associated with an earlier age of AD onset (Alzheimer's Association, 2018). This higher risk is three fold for one copy of ε4 allele and 12 fold for two copies (Alzheimer's Association, 2018). In contrast, *APOE ε2* carriage has a neuroprotective effect relative to *APOE ε3* and *APOE ε4*. Carrying the double-barrelled *APOE ε4* combination is fortunately uncommon, affecting only about 2% of the population, whereas about 25% of people carry a single copy of *APOE ε4* (Alzheimer's Association, 2018).

NATURAL PRODUCTS TO EFFECTIVELY COMBAT ALZHEIMER'S DISEASE

Nutraceuticals ("nutrition" + "pharmaceutical") with diverse compositions of plant secondary metabolites may hold great potential forpreventing and treating chronic diseases such as

AD. These secondary metabolites, below a toxic dosage, usually do not have the side effects seen in synthetic drugs and are more widely available through the agri-food system. Some plant secondary metabolites such as PP perform beneficial physiological acts through specific mechanisms such as targeting enzymes and receptors. Epidemiological and preclinical studies have shown the protective effect of nutraceuticals such as fatty acids and polyphenolics (PP) found in fruits, vegetables, herbs, and nuts against neurodegeneration, to improve memory and cognitive function (Cole et al., 2005; Miller et al., 2017). In light of the literature, dietary PP, one of the richest sources of antioxidant activity in the human diet have become a topic of great current interest as potential neuromodulator agents to attenuate pathological hallmarks of AD. The rationale for this is their potential protective activities such as blood-brain-barrier (BBB) penetration capacity, oxidative stress attenuation, and Aβ aggregation inhibition (Mendes et al., 2018). Taken together, these potential therapeutic effects of PP indicate great potential of this class of phytochemicals to be investigated as a protective agent for AD (Panza et al., 2018).

Polyphenols and Their Anti-Alzheimer's Disease Potential

Polyphenols are naturally occurring compounds and secondary metabolites of plants mostly produced in response to major stress (Pandey and Rizvi, 2009; Isah, 2019). They protect plants against biotic (living beings present in an ecosystem e.g., fungi, bacteria, and protists), and abiotic (non-living components e.g., water, soil, air, sunlight, temperature, and minerals) stressors (Rauf et al., 2019) acting as antioxidants, antimicrobials, and photo-absorption molecules. Thus, they defend plants from pathogens, ultraviolet radiation damage and predators such as insect pests (Beckman, 2000). Moreover, they are involved in the structural strength of plants during growth (Pandey and Rizvi, 2009). Polyphenols have received special attention from researchers due to their antioxidant activities which enable them to scavenge free radicals formed during the pathological processes of diseases such as cancer, cardiovascular diseases, and neurodegenerative disorders (Lakey-Beitia et al., 2015). They also have anti-inflammatory activity that is important in reducing oxidative stress thus conferring potential protective effects against the neurodegenerative process (Masci et al., 2015).

Polyphenols have demonstrated that they provide their neuroprotection through antioxidant, cholinergic, Aβ, and tau aggregation pathways *in vitro* and *in vivo* (Omar et al., 2017). The PP attenuate Aβ toxicity and oxidative stress in neurons by decreasing the Aβ aggregation and increasing the scavenging of free radicals, as shown in animal and cell culture studies (Dore et al., 1999; Agostinho et al., 2010; Mathiyazahan et al., 2015; Bai et al., 2017; Hwang et al., 2017). Polyphenols donate electrons to the free radicals to neutralize them, which is important to decrease the levels of ROS within cells (Lobo et al., 2010). Additionally, there is some evidence from cellular and animal model studies that PP may inhibit the $A\beta_{42}$ toxicity (Bastianetto et al., 2008; Hugel and Jackson, 2015). Decrease in the hyperphosphorylation of tau protein, the formation of NFTs, and inflammation in *in vitro* and *in vivo* studies upon addition of PP has also been demonstrated (Mendes et al., 2018).

The basic structure of PP includes two aromatic rings linked through a pyran ring (Ross and Kasum, 2002). There structures are very complex, with the two main categories of PP are flavonoids and non-flavonoid compounds (El Gharras, 2009; see **Figure 4**). Flavonoids contain 15 carbon atoms. They are soluble in water and characterized by two benzene rings connected through a three-carbon chain. Flavonoids are sub-divided into anthoxanthins (flavones, flavonols, flavanols, isoflavonoids, flavanones), and anthocyanins (Lakey-Beitia et al., 2015). Non-flavonoid PP are phenolic acids, stilbenes, curcuminoids, lignans, and tannins (Lakey-Beitia et al., 2015).

It has been hypothesized that the, the anti-amyloidogenic activity of PP is due to their physicochemical features, including the aromatic rings, molecular planarity, hydrogen bond formation, internal double bonds, and molecular weights below 500 g/mol (Lakey-Beitia et al., 2015). All these features are important for the inhibition of the amyloidogenic APP processing to reduce amyloid load, by activating α-secretase and inhibiting β- and γ-secretase (Lakey-Beitia et al., 2015).

Several *in vitro* and *in vivo* studies reported that PP-rich extracts from plants, like fruits and herbs, possess neuroprotective activities (Dai et al., 2006; Rossi et al., 2008; Loef and Walach, 2012; Hassaan et al., 2014; Dal-Pan et al., 2017; Omar et al., 2017; Polito et al., 2018). For instance, *in vitro*, *in vivo* and clinical studies showed the neuroprotective action of berry fruits through their polyphenolic contents (Vepsalainen et al., 2013; Wong et al., 2013; Subash et al., 2014). Other *in vitro* and *in vivo* studies indicate that pomegranate which is also rich in PP has the potential to attenuate AD progression by its anti-inflammatory and anti-Aβ accumulation activity (Hartman et al., 2006; Rojanathammanee et al., 2013). Moreover, extracts of other PP-rich fruits such as apple, banana, orange, grape, citrus fruit, and walnut have been also shown to inhibit Aβ neurotoxicity and oxidative stress as demonstrated by several *in vitro* studies (Chauhan et al., 2004; Heo et al., 2008; Toda et al., 2011; Lian et al., 2016; Braidy et al., 2017a). In one animal model study, PP-rich fruits such as Palm fruit could attenuate oxidative stress (Subash et al., 2015). Of particular interest to this current review is that several *in vivo* studies have reported cognition and memory enhancement activities of grapes, citrus fruit, walnut and buckwheat extracts (Wang et al., 2010; Choi et al., 2013; Lian et al., 2016; Braidy et al., 2017a; Pandareesh et al., 2018). *in vitro* investigation of the effect of a PP-rich extract of perennial buckwheat reported attenuation of Aβ toxicity in plasma (Liang et al., 2017). Another *in vitro* study using PP-rich extract of the herb *Patrinina villosa* Juss has shown a significant inhibitory effect on Aβ plaque aggregation (Bai et al., 2017). A cell culture study on twenty different South African medicinal PP-rich plants against AD reported the effectiveness of *Xysmalobium undulatum*, *Cussonia paniculata*, and *Schotia brachypetala* in decreasing the production of Aβ in comparison to other investigated extracts (Thakur et al., 2019). Moreover, based on the dietary intervention animal study of Ingale and Kasture (Ingale and Kasture, 2017), PP rich extract of purple passionflower could enhance

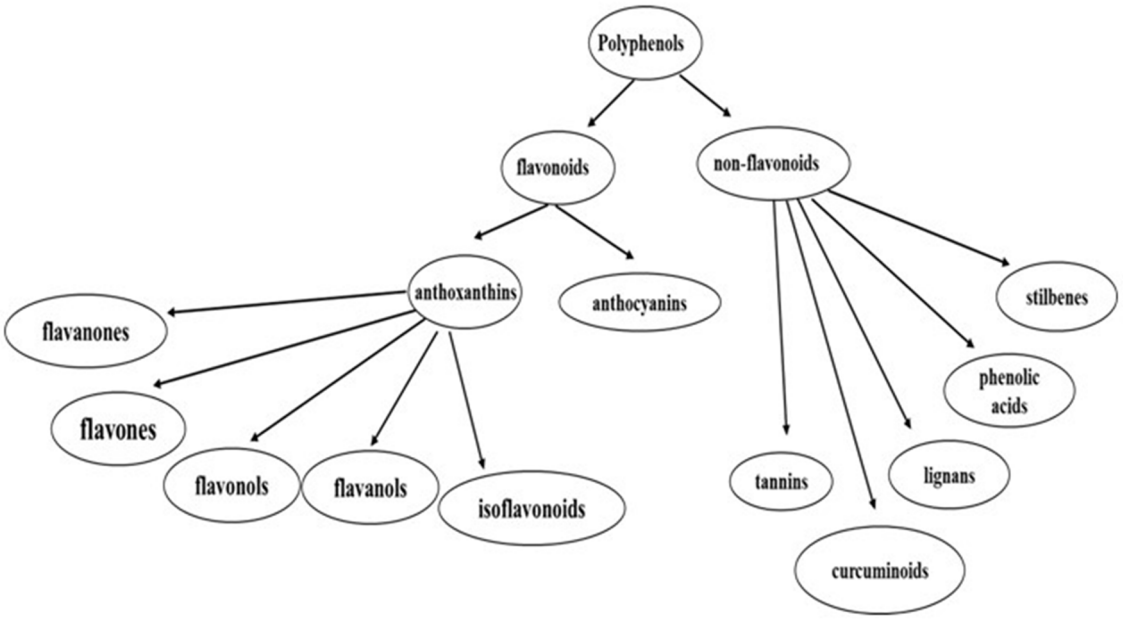


FIGURE 4 | Polyphenols classifications. PP are divided into two main groups of flavonoids and non-flavonoids (El Gharras, 2009; Lakey-Beitia et al., 2015).

cognitive function. Animal model studies, *Capparis spinose*, *Caesalpinia crista*, *Iris germanica*, and *Paeonia suffruticosa* could attenuate inflammation and Aβ aggregation through their polyphenolic contents, and make positive changes in cognition and memory (Costa et al., 2016; Gu et al., 2016; Borhani et al., 2017).

In vitro studies have reported that dietary drinks such as the crude juice of broccoli sprouts (Masci et al., 2015), tea (Polito et al., 2018), coffee (Ishida et al., 2018), and red wine (Dhir, 2018) are protective against Aβ-induced cytotoxicity and apoptotic cell death. They have been shown to attenuate mitochondrial dysfunction and hyperphosphorylation of tau proteins through their polyphenolic content (Lakey-Beitia et al., 2015; Sawikr et al., 2017; Polito et al., 2018).

Whole grain consumption as part of a healthy diet has been reported to be protective against several chronic diseases (Miller et al., 2000; Slavin, 2003; Aune et al., 2016). The health benefits of whole grains are in part due with their PP and the associated antioxidant activity (Slavin et al., 1997; Miller et al., 2000; Slavin, 2003; Tian et al., 2019).

Among whole grains, sorghum has some unique features that make it very attractive for neuroprotection studies. It is an inexpensive and abundant grain with a wide range of varieties, some of which are very high in PP content (including PP that are very rarely found in other plant food) and antioxidant activity. Several *in vitro* and *in vivo* studies have reported beneficial effects of sorghum PP on chronic diseases such as diabetes and cardiovascular disease, both of which are as risk factors of AD (Kim and Park, 2012; Suganyadevi et al., 2013; Stefoska-Needham et al., 2015; de Morais Cardoso et al., 2017; Moraes et al., 2018).

SORGHUM

General Characteristics of Sorghum

Sorghum (**Figure 5**) is the fifth most-produced cereal crop in the world (Awika and Rooney, 2004). It is adaptable to grow in drought and hot climates. Thus, it is usually grown in warm semi-arid and arid areas across the globe (de Morais Cardoso et al., 2017). Sorghum grain has been mostly used as livestock feed and in the biofuel industry (de Morais Cardoso et al., 2017). Sorghum is gluten-free and low-fat while being high in protein and fiber. It has a high antioxidant and anti-inflammatory potential due to its bioactive compounds such as polyphenolics (Awika and Rooney, 2004; de Morais Cardoso et al., 2017).

Classification of Sorghum

Sorghum grain has been classified into three different groups based on extractable tannin content. Sorghum type I (low tannins extracted by 1% acidified methanol), type II (tannins extractable in 1% acidified methanol and not methanol alone), and type III (tannins extractable in both acidified methanol and methanol alone; Awika and Rooney, 2004).

Another common way of sorghum classification is based on its grain color and its total polyphenols. Sorghum has varieties with pigmented and non-pigmented precarps. White sorghum has no tannins or anthocyanins and has a very low level of total PP. Red sorghum (red pericarp) has a considerable amount of extractable PP without any tannins. Black sorghum (black pericarp) has a large amount of anthocyanins and finally, the brown sorghum (pigmented testa, different degrees of pericarp pigmentation) contains significant levels of tannins (Awika and Rooney, 2004). The concentration of flavonoid in sorghum is related to the pericarp color, pericarp thickness, and presence



FIGURE 5 | Different genotypes of sorghum grain: black pericarp, red pericarp, white pericarp, brown pericarp, and orange pericarp varieties. Selection of the most potent varieties of sorghum is crucial for health and medical-related purposes. Picture adopted from Barmac (2021).

of testa (Taleon et al., 2012). However both environmental and genetic factors influence the phenolic level and profiles of sorghum grain (Awika and Rooney, 2004).

Sorghum PP and Health Benefits

A limited numbers of studies such as Awika et al. (Awika and Rooney, 2004; Yang et al., 2009; Awika, 2017; Girard and Awika, 2018) and Cardoso (de Morais Cardoso et al., 2017) have investigated the potential benefit of sorghum on health and disease prevention. According to their findings, sorghum should be considered as a health-beneficial grain, not just a low-value cereal grain. Sorghum has shown a positive impact on glycemic control, colonic microbiota, cholesterol attenuation, cardiovascular disease, anti-mutagenicity, and anti-inflammatory activity (Stefoska-Needham et al., 2015; de Morais Cardoso et al., 2017) which are all risk factors of AD. Below, we summarize information on the effect of sorghum on some chronic disease and their relation to AD.

Sorghum Protects Thyroid Gland Function and Combats Obesity

Sorghum is rich in manganese which is essential for thyroid hormone homeostasis. It facilitates the proper regulation of the thyroid gland promoting weight loss through regulating fat metabolism (Kangama, 2017). Moreover, sorghum contains a slow-digestible starch relative to other cereal crops which is also helpful to control obesity through slow glucose release and therefore modulation of food intake (Girard and Awika, 2018; Hasek et al., 2018). These beneficial characteristics of sorghum in controlling thyroid function (van Osch et al., 2004; Tan and Vasan, 2009; Chaker et al., 2016) and obesity (Alford et al., 2018) are considered as important risk factors of AD and thus can be very useful in its prevention.

Prevention of Cancer

The sorghum bran layer is rich in antioxidants which may reduce the risk of cancer by eliminating the possibility of free radical damage to DNA (Kangama, 2017). Sorghum extracts have been shown to have an antiproliferative effect on cancer cells (de Morais Cardoso et al., 2017). Suganyadevi et al. (2011) found that the red sorghum anthocyanin has anti-proliferative activity on a breast cancer cell line (Devi et al., 2011). Similar studies have shown the ability of sorghum extractto inhibit of cell proliferation and increase cell cycle regulator leukemia (Woo et al., 2012), breast (Park et al., 2012a), colon (Suganyadevi et al., 2011), and liver (Suganyadevi et al., 2011) cells. This characteristic is potentially beneficial to fight against diseases in which excessive free radicals play a major role including. AD (Kamath et al., 2004). The antioxidant and neuroprotective activity of the red dye extract from sorghum stem on cyclophosphamide-induced oxidative stress in rat brain is attributed its high level of phenolic and antioxidant activities (Oboh et al., 2010).

Managing Diabetes

Some varieties of sorghum grain possess a high amount of tannins which interact with starch and inhibits its digestion thus beneficially regulating blood glucose and insulin levels (Kangama, 2017). It has been suggested that sorghum has anti-diabetic and hypoglycemic effects through the regulation of insulin sensitivity *via* peroxisome proliferator-activated receptor gamma (PPAR-γ; Park et al., 2012b). Another study indicated that the hypoglycemic effect of sorghum extract is associated with hepatic gluconeogenesis not the glucose uptake of skeletal muscle (Kim and Park, 2012). Sorghum also can reduce both glucose and insulin responses (Poquette et al., 2014) and promote glucose and insulin homeostasis (Moraes et al., 2018). As diabetes could increase the risk of developing AD and declining cognitive function, anti-diabetic agents such as sorghum PP could potentially attenuate the AD pathological pathways (Arvanitakis et al., 2004; Hölscher, 2011).

Anaemia Prevention

The high level of iron and copper in sorghum helps the generation of red blood cells and improves the blood circulation and growth of cells and decreases the probability of getting anemia (Kangama, 2017). According to a population-based study, anemia is also considered as a risk factor of AD in the elderly and therefore preventing anemia would contribute to decreasing the risk of developing AD (Beard et al., 1997).

Assisting With Digestion and Cardiovascular Diseases Prevention

Sorghum assists with the proper function of the digestive system through its dietary fiber content (Kangama, 2017). This helps to control bloating, constipation, diarrhea, and excess gas. Moreover, having a high level of fiber in the diet decreases cholesterol uptake binding bile acids in the small intestine and preventing them from entering the blood-stream which is helpful for the prevention of cardiovascular which includes atherosclerosis, and stroke (Knopp et al., 1999; Kangama, 2017). Cardiovascular disease is considered an important risk factor for AD and thus its prevention will help reduce its risk (Meyer et al., 2000; Tosto et al., 2016; Tini et al., 2020).

The Anti-Alzheimer's Disease Potential of Sorghum Polyphenolics

Some varieties of sorghum possess up to 6% (w/w dry basis) of phenolic compounds which is the highest level in any cereal grain (Su et al., 2017). Almost all classes of the phenolic compounds are present in sorghum (Awika and Rooney, 2004) including phenolic acids, flavonoids, tannins, and stilbenes (**Tables 1**, **2**; Vanamala et al., 2017). The bran fraction of sorghum has the highest concentration of PP thus processing to remove the bran (decortication), will notably decrease the potential health benefits of the grain and therefore un-decorticated sorghum (whole grain) is recommended for consumption (Girard and Awika, 2018; Ashley et al., 2019).

Based on the literature, almost all the polyphenolic compounds of the various sorghum genotypes have antioxidant activity which may be effective for the attenuation of AD pathological hallmarks (Awika and Rooney, 2004). Among all the PP of sorghum (**Tables 1**, **2**), caffeic acid, trans-resveratrol, quercetin, catechin, cinnamic acid, cyanidin, apigenin, and kaempferol have gained the most attention for AD prevention and treatment (Rossi et al., 2008; Jabir et al., 2018). Snow et al. (2019) showed that PP exert their anti-AD properties primarily through prevention of aggregation of Aβ fibrils and tau protein NFTs. The presence of hydroxyl groups adjacent to aromatic rings may enhance the inhibition of Aβ/tau aggregation (Snow et al., 2019) by reducing the secondary folding of β-sheet structures which are characteristic of Aβ plaques and NFTs. For example, this property is found in proanthocyanidins, which are highly effective in reducing plaques and tangles in the brain as well as in improving short-term memory. Concluding from this article, sorghum PP such as epicatechin, luteolin, quercetin, etc. with adjacent hydroxyl groups can provide Aβ/tau disaggregation (**Figures 6**, **7**).

Collectively the functions of sorghum PP include a combination of antioxidant, anti-amyloid, anti-tau, anti-inflammatory, AchEI and BChEI activities (Rossi et al., 2008; Omar et al., 2017; Jabir et al., 2018).

TABLE 1 | Reported flavonoids in sorghum (Vanamala et al., 2017).

Class	Compound
Proanthocyanidins	(3-Deoxyanthocyanidins)
	Apigeninidin
	Luteolinidin
	7-methoxyapigenindin
	5-methoxyluteolinidin
	malvidin
Flavones	apigenin
	luteolin
	tricin
Flavanones	naringenin
	eriodictyol
	eriodictyol 5-glucoside
Flavonols	kaempferol 3-rutinoside-7-glucuronide
	quercetin 3,4'-dimethyl ether
Dihydroflavonols	taxifolin
	taxifolin 7-glucoside
Flavan-3-ols	catechin
	epicatechin
	procyanidins

TABLE 2 | Reported non-flavonoid in sorghum (Vanamala et al., 2017).

Class	Compound
Phenolic acids	Protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, p-Coumaric acid, o-Coumaric acid, Ferulic acid, Gallic acid, gentisic acid, Caffeic acid, Cinnamic acid, Hydroxybenzoic acid, Salicylic acid, Syringic acid, Sinapic acid
Stilbenes	trans-resveratrol, trans-piceid

In summary, sorghum has a diverse polyphenolic profile depending on its genotype. According to *in vitro* and *in vivo* studies, several PP of sorghum have the potential to act as an anti- AD agent through different pathways such as free-radical scavenging, inhibition of Aβ and tau aggregation. The neuroprotection potential of important single PP of sorghum is illustrated in the subsequent sections.

Flavonoids

Proanthocyanidins

Pigmented sorghums are rich in anthocyanins some of which are rarely found elsewhere in plants kingdom (Su et al., 2017). High levels of different anthocyanins were detected in a red seed variety PI297139 (1,461.4 ± 98.7 mg/kg), followed by two brown varieties PI221723 and PI35038 (1,376.4 ± 33.2, 937.3 ± 29.4 mg/kg, respectively) and a yellow variety PI229838 (574.8 ± 105.4 mg/kg; Su et al., 2017).

Anthocyanins are divided into the sugar-free proanthocyanidins aglycons and the anthocyanin glycosides (Khoo et al., 2017). The most common anthocyanins found in the plant include cyanidin, delphinidin, pelargonidin, peonidin, malvidin, and petunidin (Khoo et al., 2017). Anthocyanins have several health benefits, where their intake is associated with a lower risk of several chronic diseases due to vasoprotective and anti-inflammatory activities (Lietti and Picci, 1976), antioxidant (Ali et al., 2018), anticancer, chemoprotective (Karaivanova et al., 1990), and hypoglycemic properties (Tsuda et al., 2003). In addition, anthocyanins also are beneficial in the progression/managing/controlling of cardiovascular diseases and HIV-1 (Nakaishi et al., 2000; Stintzing et al., 2002; Cooke et al., 2005; Jang et al., 2005; Julie Beattie and Duthie, 2005; Talavéra et al., 2006).

Anthocyanins are a type of PP with the ability of crossing the blood brain barrier (BBB; Belkacemi and Ramassamy, 2016). It is also reported that anthocyanins from anthocyanin-enriched bilberry and blackcurrant extracts can regulates the APP processing and spatial memory of a mouse model of AD (Vepsäläinen et al., 2013). Additionally, they could alleviate cognitive dysfunction and neuroinflammation in APP/PSEN1 transgenic mice model of AD (Li et al., 2020). According to the cell culture study of Belkacemi and Ramassamy, a mixture of anthocyanins and proanthocyanidins could beneficially affect various mechanisms involved in AD development such as through inhibition of Aβ toxicity and tau phosphorylation, prevention of oxidative stress, and amelioration of mitochondrial dysfunction (Belkacemi and Ramassamy, 2016).

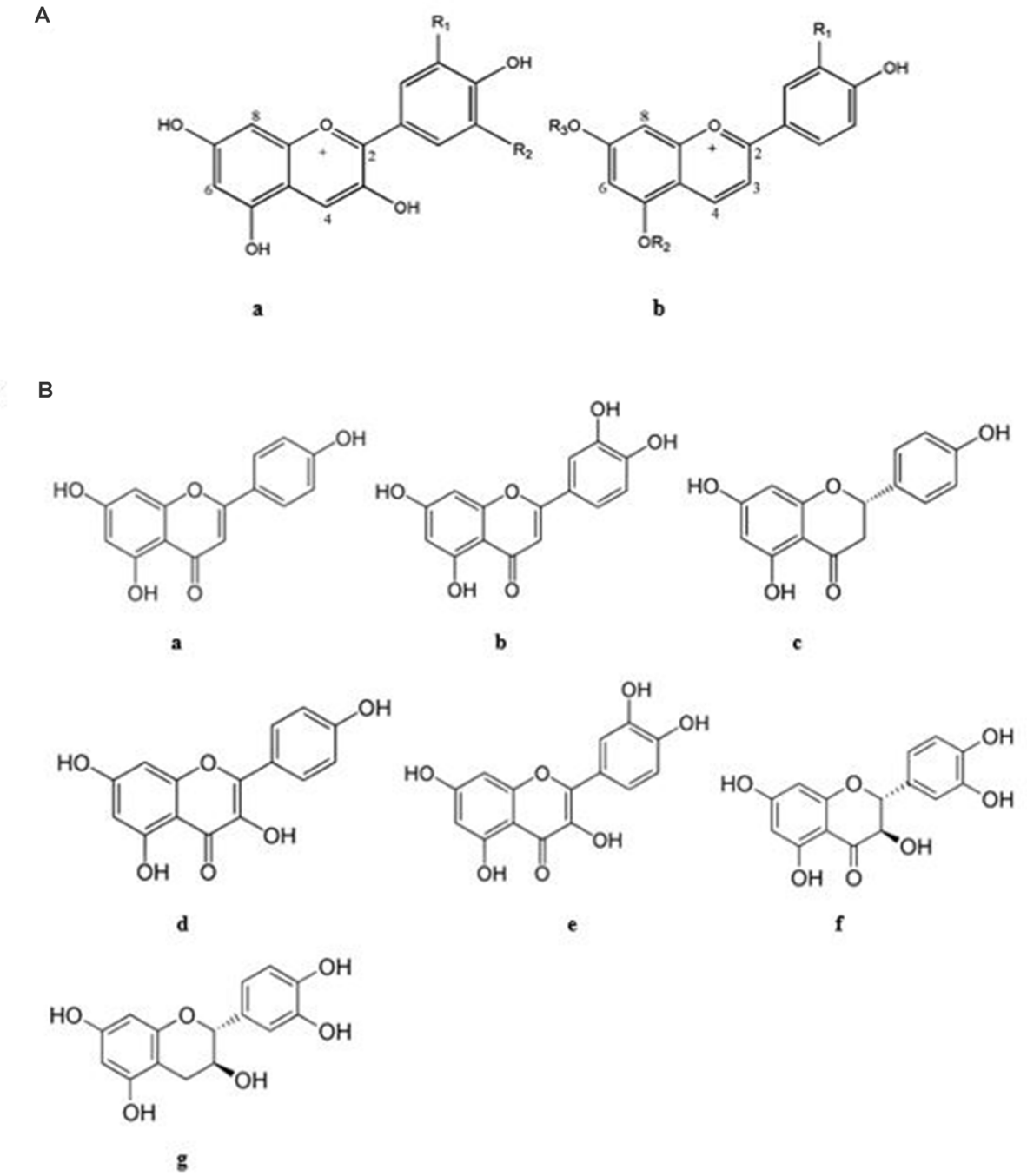


FIGURE 6 | Chemical structure of important flavonoids of sorghum with anti-AD activities. **(A)** Anthocyanidins (**a**: $R_1 = R_2 = OCH_3$: malvidin, R1 = OH, R2 = H: cyanidin, R1=OCH_3, R_2 = H: peonidin, **b**: R_1 = H, R_2 = H, R_3= H: apigeninidin, R_1 = H, R_2 = Glc, R_3= H: apigeninidin-5-glucoside, R_1 = H, R_2 = H, R_3=CH_3: 7-methoxyapigenindin, R_1 = OH, R_2 = H, R_3= H: luteolinidin, R_1 = OH, R_2 = Glc, R_3= H: luteolinidin-5-glucoside, R_1 = OH, R_2=CH_3, R_3= H: 5-methoxyluteolinidin), **(B)** others (**a**: apigenin **b**: luteolin **c**: naringenin **d**: kaempferol **e**: quercetin **f**: taxifolin **g**: catechin; Awika et al., 2005; Lakey-Beitia et al., 2015; Vanamala et al., 2017; Jabir et al., 2018).

The most common group of anthocyanins found in sorghum are the 3-deoxyanthocyanidins (3-DXA) and their derivatives (Hipskind et al., 1990; **Figure 6A**). Sorghum is the only common dietary source of the 3-DXA. The percentage of the 3-DXA in sorghum is dependent on the genotype (Awika and Rooney, 2004; Su et al., 2017). The recent animal study of Arbex et al. (2018) suggested that 3-DXA have a significant anti-inflammatory effect, thus protecting against one of the main hallmarks of AD (Arbex et al., 2018). The 3-DXAs are also reported to have antioxidant and anti-carcinogenic

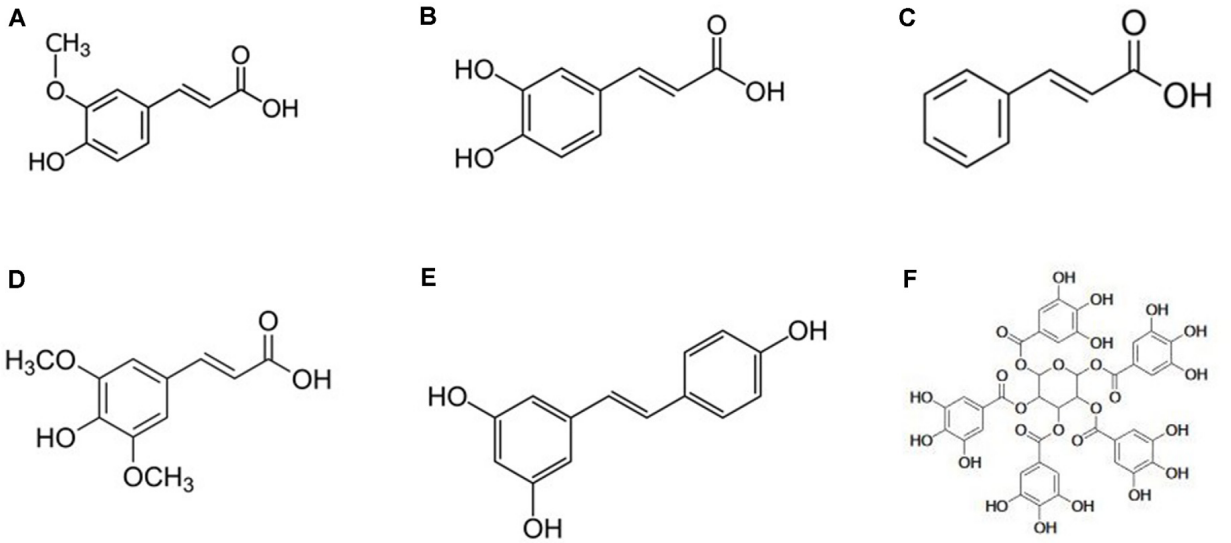


FIGURE 7 | Chemical structure of important non-flavonoids of sorghum with anti-AD activities (Lakey-Beitia et al., 2015; Girard and Awika, 2018; Jabir et al., 2018; **A**: ferulic acid, **B**: caffeic acid, **C**: cinnamic acid, **D**: sinapic acid, **E**: resveratrol, and **F**: tannins).

activities (Fratianni et al., 2007; Suganyadevi et al., 2011; Makanjuola et al., 2018). However, the data on the bioactivity of 3-DXA are very limited (Dore et al., 1999; Mathiyazahan et al., 2015). Also, there is lack of clinical trial data on the neuroprotective activity of anthocyanins and specifically 3-DXA.

Two main 3-DXAs of the sorghum are luteolinidin and apigeninidin which are orange and yellow colored, respectively. These two 3-DXAs which lack oxygen at the C-3 position are very rare in nature and also differ from the more common proanthocyanidins. The lack of oxygen at C-3 is associated with their high stability in light, heat, and change in pH (Suganyadevi et al., 2013; **Figure 6A**). Also, the molar absorptivity of the 3-DXA is higher compare to other proanthocyanidins except for cyanidin-3,5-diglucoside and therefore 3-DXA will be absorbed higher than other anthocyanins (Awika et al., 2004).

Flavones

Flavones are an important subgroup of flavonoids that are abundant in plants, especially herbs and cereal grains such as maize and sorghum (42–386 μg/g; Jiang et al., 2016). Common flavones include apigenin, luteolin, tangeritin, and chrysin (Singh et al., 2014; Kawser Hossain et al., 2016). Flavones have beneficial functions not only for the plant but also for human health. They possess strong antioxidant activity, which makes them potentially beneficial for the prevention and treatment of several chronic diseases including cancer, coronary heart disease, and neurodegenerative disease (Martens and Mithöfer, 2005; Singh et al., 2014).

Apigenin

Apigenin is present in several plants including sorghum with the reported concentration of 0.36–2.09 μg per gram of dry grain (Bradwell et al., 2018). This flavone from sorghum is known to be a strong antioxidant (Makanjuola et al., 2018). It has been shown to protect neurites and cell viability by enhancing the cytokine and nitric oxide release in inflammatory cells which may prevent or slow the progression of AD (Balez et al., 2016). Several studies reported the anti-inflammatory effects of apigenin in human and animal experiments (Liang et al., 1999; Rezai-Zadeh et al., 2008; Zhang et al., 2014). In one study, they induced inflammation by adding lipopolysaccharide to human and mouse macrophages then checked the effect of apigenin by PrimePCR array and through examining different mechanisms. They identified major target genes regulated by apigenin in lipopolysaccharide-mediated immune response (Zhang et al., 2014).

According to the apigenin-treated animal study of Zhao et al. (2013), in a double transgenic mouse model of AD, apigenin ameliorated the APP processing and Aβ toxicity through the regulation of BACE1 level and the reduction of Aβ deposition. They also showed apigenin beneficially reduced oxidative stress and reduced memory impairment, reduced of oxidative stress through the Morris water maze performance test (Zhao et al., 2013). It is also reported that oral administration of apigenin ameliorated the learning and memory deficits of Aβ-induced mice by attenuating oxidative damage, enhancing cholinergic neuronal transmission, and maintaining the BBB integrity in the cerebral cortex (Liu et al., 2011).

Luteolin

Luteolin is a flavone class of flavonoids found in several plants including sorghum (Lin et al., 2008). The concentration of luteolin in sorghum grain is (0.84–5.57 μg/g, dry basis) depending on the variety and environmental factors (Bradwell et al., 2018). Luteolin showed strong antioxidant and neuroinflammation activities in *in vitro* and *in vivo* studies (Paterniti et al., 2014; Kwon, 2017). Based on the

animal model study of Wang et al. (2016) which was performed through Morris water maze and probe tests, luteolin (10 and 20 mg/kg) significantly attenuates spatial learning deficiencies and memory impairment. Furthermore, the animal behavioral tests study of Yu et al. (2015) found that luteolin (200 mg/kg) attenuates Aβ-induced learning and memory impairment through increasing the regulation of the cholinergic function and attenuation of oxidative stress. According to another *in vivo* study, luteolin could improve basal synaptic transmission and enhance the long-term potentiation (which is considered as a cellular correlate of learning and memory) through high frequency stimulation in the dental gyrus of the rat hippocampus (Xu et al., 2010). Moreover, daily oral administration of luteolin (50, 100, and 200 mg/kg) demonstrated a significant reduction of Aβ aggregation, oxidative stress, and inflammatory reaction in the hypoperfused rat brain (Fu et al., 2014).

Flavanones

Flavanones are a subgroup of flavonoids commonly available in grains and especially sorghum and some fruits such a citrus fruit (Tomás-Barberán and Clifford, 2000; Duodu and Awika, 2019). Common flavanones include hesperidin, naringenin, isosakuratenin, and eriodictyol (Das et al., 2019).

Some varieties of sorghum such as yellow sorghum possess high levels of flavanones mainly eriodictyol and naringenin; up to 1,800 μg/g depending on genotypes and environmental factors. The levels of reported flavanones in sorghum grain are much higher than in citrus fruit (400–600 μg/g) which has previously been considered as a main source of these compounds (Duodu and Awika, 2019).

According to cell culture studies, flavanones have several potential health benefits including neuroprotection potential through scavenging ROS (Lu et al., 2010), inhibiting the H_2O_2-induced neurotoxicity, increasing catalase activity, attenuating the intracellular free Ca^{2+}, and decreasing the mitochondrial membrane potential (Hwang and Yen, 2008).

Naringenin

Naringenin is a compound that belongs to the flavanone group of flavonoids. It is available in several plant food including citrus fruits and sorghum (Manchope et al., 2017). Several *in vivo* and *in vitro* studies reported anti-inflammatory and antioxidant activity of naringenin (Heo et al., 2004; Manchope et al., 2017). The study of Khajevand-Khazaei et al. (2018) suggested positive effects of naringenin for the alleviation of lipopolysaccharide-induced cognitive deficits in rats; through enhancing the spatial recognition memory in Y maze, discrimination ratio in the object discrimination task, and retention in the passive avoidance test. Lipopolysaccharide and naringenin were administrated daily in a dose of 167 μg/kg and 25, 50, or 100 mg/kg, respectively (Khajevand-Khazaei et al., 2018). Naringenin is able to pass through the BBB and thus can act on the CNS. It has been shown to increase Aβ degrading enzymes through increasing M2 microglia polarization and inhibiting $A\beta_{42}$ -induced M1 microglia activation in primary cultured cortical microglia (Yang et al., 2019). Two other *in vivo* studies also showed the ability of naringenin to improve learning and memory function through alleviation of oxidative stress and reducing apoptosis as evidenced by the Morris water maze test conducted in a rat model of AD (Ma et al., 2013; Ghofrani et al., 2015).

Flavonols

Flavonols are another subgroup of flavonoids which commonly available in onions, leeks, broccoli, blueberries and also abundant in cereal such as quinoa, barley, and sorghum (Awika, 2011; Pérez-Chabela and Hernández-Alcántara, 2018).

Common dietary flavonols include myricetin, quercetin, and kaempferol (Aherne and O'Brien, 2002). Flavonols are reported to have several health benefits including inhibiting low-density lipoprotein oxidation and thus reduced risk of atherosclerosis and general cardio protection effects (Giovinazzo and Grieco, 2019), cancer (Ali et al., 2008; Szliszka et al., 2011), and neurogenerative disease such as brain vascular atrophy, mild cognitive impairment (MCI), and AD (Patel et al., 2008).

Kaempferol is found in a variety of plants including sorghum (Przybylska-Balcerek et al., 2019). Epidemiological evidence suggests a positive relationship between the high intake of kaempferol and a reduced risk of developing several chronic diseases such as cancer, cardiovascular disease, and neurodegeneration (Calderon-Montano et al., 2011). Moreover, many studies have demonstrated that kaempferol has a wide range of pharmacological properties, including antioxidant (Tatsimo et al., 2012), anti-inflammatory (Devi et al., 2015), antimicrobial (Tatsimo et al., 2012), anticancer (Yoshida et al., 2008; Chen and Chen, 2013), cardioprotective (Xu et al., 2006; Choi et al., 2015), antidiabetic (Zhang and Liu, 2011; Alkhalidy et al., 2018), and neuroprotective activities (Kim et al., 2010; Calderon-Montano et al., 2011). An *in vivo* study by Cheng et al. (2018) reported significant anti-neuroinflammatory effects of kaempferol through high-mobility group protein 1 release and decreasing the toll-like receptor-4/myeloid differentiation factor 88 which was induced by lipopolysaccharide in the brains of mice. The effective doses of kaempferol were 50, or 100 mg/kg for 7 days (Cheng et al., 2018). Kaempferol has also been shown to have an anti-apoptotic activity in Aβ-induced neuroblastoma cell lines which can be beneficial for the prevention and treatment of AD (Kim et al., 2019). Another study in the transgenic Drosophila model of AD indicated that administration of 10, 20, 30, and 40 μM of kaempferol for 30 days could delay memory loss, reduce oxidative stress and AChE activity, and therefore is a potential therapeutic agent for AD (Beg et al., 2018).

Quercetin, a flavonoid found in various foods including sorghum, possesses strong antioxidant activity (Zhang et al., 2011). It demonstrates anti-inflammatory activities, the mechanism of which is through inhibition of the NF-κB pathway (Comalada et al., 2005), anticancer activities through a variety of mechanisms (Xing et al., 2001), attenuation of high cholesterol (Lu et al., 2010), protection form viral infections (Davis et al., 2008; Gonzalez et al., 2009), reduced risk of diabetes (Vessal et al., 2003), and cardiovascular diseases (Kleemann et al., 2011). Several of these effects can in turn potentially reduce the risk of AD (Zaplatic et al., 2019). Pre-treatment of hippocampal cell cultures with quercetin considerably

attenuates Aβ-induced cytotoxicity, protein oxidation, lipid peroxidation, and apoptosis (Ansari et al., 2009). Thus, quercetin might be protective against Aβ toxicity by regulating oxidative stress (Ansari et al., 2009). Moreover, quercetin deceases ROS which is a major contributor to AD (Zaplatic et al., 2019). It also decreases extracellular Aβ, AChE level, tau toxicity, and microgliosis (Sabogal-Guaqueta et al., 2015). Additionally, quercetin improved learning and memory function in aged 3xTg-AD mice determined through the elevated plus-maze test (Orhan et al., 2007; Sabogal-Guaqueta et al., 2015).

Dihydroflavonols

Dihydroflavonols also referred to as flavanonols, are a subgroup of flavonoids available in some plants including sorghum (Gujer et al., 1986). Common dihydroflavonols include taxifolin, dihydrokaempferol, and dihydromyricetin (Espargaro et al., 2017; Sunil and Xu, 2019). They have several reported potential health benefits including antiallergic and anti-inflammatory activities (Ayoub et al., 2018). Moreover, they show strong free radical scavenging activity and protect neuronal cells from oxidative damage *in vitro* (Gong et al., 2009). Dihydroflavonols also demonstrated the capacity to inhibit Aβ aggregation, a main pathological hallmark of AD (Espargaro et al., 2017).

Taxifolin is a flavonoid with strong anti-inflammatory and antioxidant activities (Topal et al., 2016; Wang et al., 2018). It is available from different natural sources such as onion, milk thistle, and sorghum (Sunil and Xu, 2019). It significantly attenuated Aβ-induced cognitive impairment and neuronal cell death which was measured through novel object recognition tasks and the spatial memory in a mice model of AD (Wang et al., 2018). The *in vivo* study of Saito et al. (2017) showed the capacity of taxifolin in the improvement of cognitive and cerebrovascular functions which was evaluated by the water maze test and monitoring the cerebral blood flow changes in the cerebral amyloid angiopathy model of mice.

Flavan-3-ols

Flavan-3-ols are a subgroup of flavonoids available in several natural sources including teas, apples, beer, wine, and cereals like sorghum (Yao et al., 2004; Rao et al., 2018). Flavan-3-ols have a variety of health beneficial effects including reducing metabolic syndrome risk (Yang et al., 2012), antioxidant activity (Castillo et al., 2000), anti-cancer characteristic (Lei et al., 2016) and neuroprotective potential (Bastianetto et al., 2006). Common flavan-3-ols are catechin, epigallocatechin, epicatechin, epicatechin 3-gallate, epigallocatechin 3-gallate (EGCG), and theaflavin (Yao et al., 2004).

Catechins are common in tea, cocoa, berries, and sorghum. They have potent antioxidant and anti-inflammatory activities as reported by several *in vitro* and *in vivo* studies (Higdon and Frei, 2003; Abd El-Aziz et al., 2012; Zanwar et al., 2014; Stohs and Bagchi, 2015). Apart from radical scavenging activity, catechins modulate mitochondrial functions, activate survival genes, and also fight against Aβ-induced cognitive deficit and neurotoxicity through their antioxidant activity (Heo and Lee, 2005; Ban et al., 2006; Haque et al., 2008). Therefore, catechins are receiving great attention as potential neuroprotective agents (Mandel and Youdim, 2004).

Non-flavonoids

Phenolic Acids

Phenolic acids are the simplest naturally available PP (Tsao, 2010). The natural sources of phenolic acids include fruits, vegetables, and cereals, especially sorghum (Klensporf-Pawlik and Aladedunye, 2017; Ratnavathi, 2019). The phenolic acids of sorghum are mostly benzoic or cinnamic acid derivatives (Calviello et al., 2007). Phenolic acids are reported to have strong antioxidant (Sroka and Cisowski, 2003) and anti-inflammatory activities (Kang et al., 2015) as well as other health benefits including neuroprotective activities (Saibabu et al., 2015).

Cinnamic acid is an aromatic carboxylic acid (see **Figure 7**) with many beneficial effects. Several studies have shown the anti-microbial, antioxidant (Sova, 2012; Guzman, 2014), anti-cancer (De et al., 2011; Su et al., 2015), anti-atherogenic (Lapeyre et al., 2005), anti-tuberculosis (De et al., 2012), and anti-fungal (Tawata et al., 2014) effects of cinnamic acid. Cinnamic acid treatment in a mouse model of AD significantly reduced the Aβ plaque formation and improved the cognitive function through PPARα activation to stimulate lysosomal biogenesis. Additionally, cinnamic acid treatment improved the memory and behavioral performance in the mouse model of AD (Chandra et al., 2019). Interestingly, cinnamic acid derivatives have been also reported to act as cholinesterase inhibitors thus may have therapeutic effects on AD through this mechanism (Lan et al., 2017; Chen et al., 2018).

Ferulic acid (FA) is the most abundant phenolic acid found in sorghum and is also suggested to have strong anti-inflammatory activity (Sosulski et al., 1982; Lempereur et al., 1997; Sgarbossa et al., 2015; Ratnavathi, 2019). Due to its chemical structure (**Figure 7**), FA possesses a strong free radical scavenging ability (Srinivasan et al., 2007). The antioxidant effect of FA has been shown to be effective against several chronic diseases such as cancer (Rocha et al., 2012), cardiovascular (Ardiansyah et al., 2008), diabetes (Jung et al., 2007), and cellular oxidative stress (Calabrese et al., 2008). The efficacy of FA has been investigated against several neurodegenerative pathologies, particularly in AD. According to the finding, it could inhibit fibril formation (Ono et al., 2005) and protect neurons against Aβ-induced oxidative stress and neurotoxicity *in vitro* (Sultana et al., 2005). Moreover, the *in vivo* study of Yan et al. (2001) demonstrated that long-term administration of FA induces resistance to Aβ toxicity in the brain likely through its antioxidant and anti-inflammatory. These results indicate that FA at a dosage of 5.3 mg/kg/day could be beneficial for the prevention and treatment of AD (Yan et al., 2013).

Caffeic acid (CA) is a hydroxycinnamic acid derivative which is commonly found in fruits, herbs, and grains, especially sorghum. It has strong antioxidant and anti-inflammatory activities (da Cunha et al., 2004; Gülçin, 2006; Priebe et al., 2014). According to the *in vivo* study of Kim et al. (2015), CA was administrated to a Aβ-injected mouse model of AD at an oral dose of 50 mg/kg/day for 2 weeks. The cognitive impairment was assessed by different behavioral tests. The result demonstrated

the ability of CA to enhance memory and cognitive impairment through inhibition of lipid peroxidation and NO production (Kim et al., 2015).

Sinapic acid (SA) is another phenolic acid present in sorghum that is suggested to have anti-inflammatory and neuroprotective activity (Yun et al., 2008; Zare et al., 2015). The neuroprotective examination of SA (10 mg/kg/day for 7 days) in an Aβ-induced mouse model of AD showed a strong attenuation of glial cell activation and memory impairment in a passive avoidance task. Moreover, SA attenuated neuronal cell death and cognitive dysfunction through its antioxidant and anti-inflammatory activities (Lee et al., 2012).

Stilbenes

Stilbenes are important group of non-flavonoid PP produced by plants in response to major stress, especially, fungal infection, and UV radiation (Varoni et al., 2016). Stilbenes contain two benzene rings connected by ethanol or ethylene molecule (Yu et al., 2005). Stilbenes are present in some plants such as grapes, berries, and sorghum (Yu et al., 2005; Reinisalo et al., 2015). According to the literature, they are protective against cancer, cardiovascular disease and age-related disease through their antioxidant and anti-inflammatory activities (Reinisalo et al., 2015; Sirerol et al., 2016). More than 400 stilbenes are available in nature and the most well studied ones are resveratrol, pterostilbene, piceatannol, and pinosylvin (Sirerol et al., 2016).

Resveratrol, well known as a PP from grapes, is also found in sorghum grain. It is one of the most studied stilbenes for neuroprotection and AD prevention (Dal-Pan et al., 2017). Resveratrol is classified as a non-flavonoid PP. There are two isomers of this compound in plants, *trans*-resveratrol and *cis*-resveratrol, along with their glucosides, *trans*-piceid, and *cis*-piceid (Varoni et al., 2016). Clinical trials have shown the beneficial effects of resveratrol on neurological disorders, cardiovascular disease and diabetes biomarkers (Berman et al., 2017). Currently, resveratrol is considered as a nutraceutical due to its many therapeutic effects including the regulation of caloric restriction, anti-inflammatory, and antioxidant activities (Salehi et al., 2018; Banez et al., 2020). Numerous cell culture and animal studies of resveratrol have demonstrated its anti-inflammatory, antioxidant, anti-Aβ aggregation and anti-abnormal tau phosphorylation properties (Savaskan et al., 2003; Lagouge et al., 2006; Rege et al., 2015; Wang et al., 2016; He et al., 2017). Antioxidant and anti-inflammatory activity of resveratrol could increase the clearance of Aβ, and modulate oxidative stress, neuronal energy homeostasis, and apoptosis (Bastianetto et al., 2015). Resveratrol also assists synaptic plasticity and neuroprotective kinases activities (Bastianetto et al., 2015). It is also reported to provide its neuroprotective activity through the activation of SIRT1, an enzyme that deacetylates proteins related to cellular regulation (Lagouge et al., 2006).

Tannins

Tannins are a group of non-flavonoid PP with many biological activities specifically binding to precipitate proteins and other organic molecules (Hagerman and Butler, 1989). They protect plant from predation and also help plant growth (Ferrell and Richard, 2006). They are distributed in many plants including fruits, beverages and grains such as grape, coffee, tea, wine, cacao and sorghum (Lamy et al., 2016). Brown colored sorghum varieties are known to have a high antioxidant capacity due to their higher tannin content, which is not present in all genotypes of sorghum regardless of whether they are colored or not (Awika et al., 2004). Tannins have strong antioxidant and anti-inflammatory activities (Braidy et al., 2017b). They are reported to reduce hyperphosphorylation of tau proteins in *in vitro* study (Yao et al., 2013). Moreover, oral administration of tannins in a transgenic mouse model of cerebral amyloidosis demonstrated an improvement in object recognition and spatial reference memory (Mori et al., 2012) and also they showed to inhibit the β-secretase activity *in vitro* and therefore they have significant preventative potential against AD (Mori et al., 2012). Tannins also demonstrated a significant inhibitory effect against AChE and BChE (Türkan et al., 2019). Additionally, the study of Park et al. (2019) reported strong cognitive and memory enhancing activities of tannins in a rat model *via* avoidance and the water maze task.

CONCLUSION

Currently available medication for AD is extremely limited in efficacy, therefore more studies should be conducted to discover new preventative and therapeutic agents. Recently, researchers have focused more on identifying treatments that can attenuate AD pathological hallmarks, rather than focusing on the treatments which only target the disease symptoms. Available symptomatic treatments such as AchEIs just attenuate symptoms temporarily by increasing the neurotransmitters in the brain without altering the disease progression path. For this purpose, studies on the effects of natural products such as polyphenolic antioxidants on AD pathological hallmarks are appearing in the scientific literature with increase regularity. One of the most highly concentrated food sources of antioxidant activity is sorghum grain which in colored gain varieties is due to high levels PP including 3-deoxyanthocyanidins, not found in any other common food.

To the best of our knowledge, there is no study on the effects of sorghum PP on AD pathology, therefore, the present review has illustrated the potential of sorghum PP as therapeutic agents against AD pathological hallmarks. This review has highlighted the unique chemistry and potential health beneficial properties of sorghum PP that can be leveraged to promote this under-utilized grain as a healthy food source.

As discussed throughout this review, numerous single PP have been studied and have demonstrated potential anti-AD effects in cellular and animal studies through a wide range of different mechanisms. However, a mixture of PP as found in an extract of sorghum grain could provide an additive or even synergistic multi-target therapeutic efficacy (Wang et al., 2014; Caruana et al., 2016; Andrade et al., 2019; Ayaz et al., 2019; Habtemariam, 2019).

Based on a variety of cell culture and animal model studies, sorghum PP have demonstrated several beneficial properties

against some of the cellular pathways that contribute to AD pathogenesis. Among all the sorghum PP, caffeic acid, trans-resveratrol, quercetin, catechin, cinnamic acid, cyanidin, apigenin, and kaempferol have gained the most attention for their potential for AD prevention and treatment. However, the above-mentioned PP are not unique to sorghum. We hypothesise that the unique sorghum PP such as 3-DXA, and the complex mixtures of PP in sorghum grain extracts may collectively exert powerful synergistic effects on the inhibition of neurotoxic aggregation of Aβ and tau which initiate AD pathology.

Further studies to identify the specific mechanisms by which sorghum PP provide any neuroprotective activities are now necessary. One target mechanism is the antioxidant pathway in which the PP-rich extract of sorghum might reduce AD-associated oxidative stress. Both *in vitro* and *in vivo* animal model studies should be performed to gain as much evidence as possible before making recommendations for follow-on clinical trials. Moreover, anti-amyloidogenic, anti-tau/phospho tau, and anti-inflammatory mechanisms related to AD require further investigation. The new knowledge from these future studies may produce the high level of evidence require to confirm that the PP-rich extract from sorghum grain is a high efficacy preventative and therapeutic agent against AD.

AUTHOR CONTRIBUTIONS

NR, WMADB, EH, HS, SJ, SG, and RM substantially contributed to the conception and design of the article and interpreting the relevant literature. NR (PhD candidate), wrote the first draft of the manuscript. NR, WMADB, EH, HS, SJ, and RM revised it critically for important intellectual content. All authors contributed to the article and approved the submitted version.

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Higher Coffee Consumption Is Associated With Slower Cognitive Decline and Less Cerebral Aβ-Amyloid Accumulation Over 126 Months: Data From the Australian Imaging, Biomarkers, and Lifestyle Study

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Background: Worldwide, coffee is one of the most popular beverages consumed. Several studies have suggested a protective role of coffee, including reduced risk of Alzheimer's disease (AD). However, there is limited longitudinal data from cohorts of older adults reporting associations of coffee intake with cognitive decline, in distinct domains, and investigating the neuropathological mechanisms underpinning any such associations.

Methods: The aim of the current study was to investigate the relationship between self-reported habitual coffee intake, and cognitive decline assessed using a comprehensive neuropsychological battery in 227 cognitively normal older adults from the Australian Imaging, Biomarkers, and Lifestyle (AIBL) study, over 126 months. In a subset of individuals, we also investigated the relationship between habitual coffee intake and cerebral Aβ-amyloid accumulation ($n = 60$) and brain volumes ($n = 51$) over 126 months.

Results: Higher baseline coffee consumption was associated with slower cognitive decline in executive function, attention, and the AIBL Preclinical AD Cognitive Composite (PACC; shown reliably to measure the first signs of cognitive decline in at-risk cognitively normal populations), and lower likelihood of transitioning to mild cognitive impairment or AD status, over 126 months. Higher baseline coffee consumption was also associated

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with slower Aβ-amyloid accumulation over 126 months, and lower risk of progressing to "moderate," "high," or "very high" Aβ-amyloid burden status over the same time-period. There were no associations between coffee intake and atrophy in total gray matter, white matter, or hippocampal volume.

Discussion: Our results further support the hypothesis that coffee intake may be a protective factor against AD, with increased coffee consumption potentially reducing cognitive decline by slowing cerebral Aβ-amyloid accumulation, and thus attenuating the associated neurotoxicity from Aβ-amyloid-mediated oxidative stress and inflammatory processes. Further investigation is required to evaluate whether coffee intake could be incorporated as a modifiable lifestyle factor aimed at delaying AD onset.

Keywords: coffee, Alzheimer's disease, AIBL, Australian Imaging Biomarkers and Lifestyle flagship study of ageing, caffeine, cognitive decline, Aβ-amyloid, dementia

INTRODUCTION

Worldwide, a high proportion of adults drink coffee daily, making it one of the most popular beverages globally. Coffee contains a range of bioactive compounds, including caffeine, chlorogenic acid, polyphenols and small amounts of vitamins and minerals (Spiller, 1984). Epidemiological studies suggest coffee has beneficial effects on various conditions including stroke (Larsson and Orsini, 2011), heart failure (Mostofsky et al., 2012), cancers (Wang et al., 2016), diabetes (Akash et al., 2014), and Parkinson's disease (Ross et al., 2000). Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive impairment of learning, memory and other cognitive deficits, with extracellular deposition of Aβ-amyloid (Aβ) protein within the brain leading to neuroinflammation, synaptic loss and neuronal death (Villemagne et al., 2013). Several studies suggest a protective role of coffee, with reduced risk of mild cognitive impairment (MCI) and AD reported (van Gelder et al., 2007; Eskelinen et al., 2009; Arab et al., 2011; Liu et al., 2016; Wierzejska, 2017; Wu et al., 2017). However, there are limited longitudinal data from cohorts of cognitively normal older adults describing associations of coffee consumption with distinct domains of cognition, and concurrently investigating potential neuropathological mechanisms underpinning any such associations.

Results from a meta-analysis conducted in 2016, which included nine published prospective cohort studies, found drinking one to two cups of coffee daily was associated with lower incidence of cognitive disorders (i.e., cognitive decline, cognitive impairment, AD, and all cause dementia) compared with less than one cup; studies ranged in follow-up from 1.3 to 28 years (Liu et al., 2016). Moreover, a recent cross-sectional analysis found self-reported lifetime intake of two or more cups of coffee per day was associated with lower rates of "Aβ positivity" (presence of significant brain Aβ), compared to less than two cups per day, in 411 non-demented older adults. However, current coffee intake was not related to "Aβ positivity" in this cohort, and neither current, nor lifetime intake, was related to cerebral mean cortical thickness or white matter hyperintensity volume (Kim et al., 2019). A second study investigating the effect of self-reported coffee consumption on brain volume found both high coffee consumption ($\geq$ 4 cups per day) *and* low coffee consumption (no coffee or $<$ 1 cup per day) to be associated with larger hippocampal volume cross-sectionally (p for test for quadratic trend = 0.001). There were no associations with volumes of the neocortex and striatum (accumbens, putamen, globus pallidus) or total intracranial volume. The cohort was homogeneous in nature, with all participants young Caucasian females (aged 23.2 $\pm$ 2.7 years), right handed with university education, and no history of smoking or drug/alcohol abuse (Perlaki et al., 2011). Conversely, higher self-reported coffee consumption (>3 cups/day compared to 0–1 cup/day) has also been associated cross-sectionally with smaller hippocampal volume, in 2914 individuals aged 59.28 $\pm$ 7.2 years (p = 0.033) (Araujo et al., 2016). However, this association was only observed when body mass index, previous coronary heart disease, alcohol consumption, and smoking were included in the statistical model, along with age, sex, and educational attainment (Araujo et al., 2016). Well-designed longitudinal cohort studies are necessary to further understand the influence of coffee consumption on AD phenotype and rates of decline in different cognitive domains.

The aim of the current study was to investigate the relationship of self-reported habitual coffee intake to rates of decline in varying cognitive domains, assessed using a comprehensive neuropsychological battery, over 126 months in 227 older adults classified as cognitively normal at baseline. Furthermore, we aimed to investigate whether habitual coffee intake was associated with rates of cerebral Aβ-amyloid deposition, or brain volume atrophy over the same time-period, in subsets of 60 and 51 participants, respectively. These investigations were conducted using data from the well-characterized Australian Imaging, Biomarkers and Lifestyle study of ageing (AIBL) (Fowler et al., 2021).

MATERIALS AND METHODS

Participants

This report describes data from 227 cognitively normal participants who were enrolled at the Perth site of the AIBL study

(Fowler et al., 2021) and who completed the Commonwealth Scientific and Industrial Research Organisation (CSIRO) food frequency questionnaire (FFQ). The AIBL study is a longitudinal study of cognitively normal individuals, as well as those with MCI and AD who are being assessed for prospective research into aging and AD. MCI and AD participants were excluded from the current analysis as the CSIRO FFQ requires estimations of food intake over the previous year, and there is potential for misclassification due to limited accuracy in recall.

All AIBL volunteers were aged 60 years and above at baseline, and excluded if they had a history of non-AD dementia, schizophrenia, bipolar disorder, significant current depression, Parkinson's disease, cancer (other than basal cell skin carcinoma) within the last 2 years, symptomatic stroke, insulin-dependent diabetes, uncontrolled diabetes mellitus or current regular alcohol use exceeding two standard drinks per day for women or four per day for men. Further details regarding recruitment, assessment, inclusion and exclusion criteria are described in Fowler et al. (2021). The AIBL study is approved by the institutional ethics committees of Hollywood Private Hospital, Edith Cowan University, St Vincent's Health and Austin Health (Fowler et al., 2021). Written informed consent was obtained from each participant prior to undertaking any procedures related to the study.

Food Frequency Questionnaire

The CSIRO FFQ quantifies intake of over 200 foods and beverages (Lassale et al., 2009). The online version of the CSIRO FFQ was administered at the participant's residence, over the phone or at our research institution. Online delivery of the questionnaire ensures completion of all questions as participants cannot proceed without finishing each section. Participants were able to complete the questionnaire in multiple sessions to prevent non-completion due to fatigue. An individual's habitual coffee intake was determined as grams per day from responses to the question "How often do you drink coffee" with response options of number of cups consumed and frequency of consumption, e.g., "2 times per week," "1 time per day," etc. No delineation between caffeinated and de-caffeinated coffee, or methods of preparation (latte, cappuccino, no milk, soy milk, etc.) was possible from the FFQ responses. The FFQ was completed a mean of 3.38 years $\pm$ 0.9 standard deviations after the baseline assessment.

Cognitive Assessments

A comprehensive neuropsychological battery of well-validated measures was administered according to standard protocols (described previously; Fowler et al., 2021) at baseline and up to seven additional assessments, 18 months apart, for up to 126 months. The battery assessed six cognitive domains—episodic recall memory, recognition memory, executive function, language, attention and processing speed, and the AIBL Preclinical Alzheimer Cognitive Composite (AIBL PACC; Donohue et al., 2014). Composite scores were calculated for each of these cognitive domains by first converting raw scores for individual measures to overall sample-based Z scores, then averaging Z scores for the relevant measures to compute a single composite score for each individual for that domain. Neuropsychological tests were assigned to one of the six cognitive domains via consensus among neuropsychologists, psychologists and neurologists involved in the AIBL study. **Supplementary Table 1** lists the neuropsychological tests used to construct the cognitive domain composite scores. A "change" in clinical classification variable was also computed by assigning each participant a "0" if they remained cognitively normal from baseline throughout their study participation, and a score of "1" if they converted to MCI or AD status.

Positron Emission Tomography

A subset of 60 participants underwent Positron Emission Tomography (PET) brain scans to quantify cerebral Aβ-amyloid burden at baseline, and on up to seven additional occasions, 18 months apart, for up to 126 months. Imaging with PET was conducted using 11C-PiB, 18F-NAV4694, or 18F-flutemetamol at the Western Australia PET and Cyclotron Service, Sir Charles Gairdner Hospital, and Oceanic Medical Imaging, Perth. Methodology for each tracer has been previously described (Bourgeat et al., 2018). Briefly, PET images were analyzed using CapAIBL (Bourgeat et al., 2015) and Aβ-amyloid burden was expressed using the Centiloid (CL) scale (Bourgeat et al., 2018). The CL scale provides a single continuous scale across the different Aβ-amyloid imaging tracers, where a value of "0" represents the typical Aβ-amyloid burden in young controls, and "100" the typical Aβ-amyloid burden seen in mild AD patients (Klunk et al., 2015). In addition to the continuous CL scale, Aβ-amyloid burden was also expressed as a categorical variable, defined as follows: "negative" = < 15 CL, "uncertain" = 15 CL to 25 CL, "moderate" = 26 CL to 50 CL, "high" = 51 CL to 100 CL, and "very high" = > 100 CL.

Magnetic Resonance Imaging

A subset of 51 participants underwent Magnetic Resonance Imaging (MRI) brain scans at baseline, and on up to seven additional occasions, 18 months apart, to quantify gray matter, white matter, and hippocampal volumes. Participants underwent a 3D T1-weighted magnetization-prepared rapid acquisition gradient-echo sequence using the following acquisition parameters: in-plane resolution 1 X 1 mm, slice thickness 1.2 mm, repetition time (TR)/echo time (TE)/inversion time (TI) = 2,300/2.98/900, flip angle 9°, and field of view (FOV) 240 X 256. Magnetization-prepared rapid acquisition gradient-echo images for all participants were segmented into white matter, gray matter, and cerebrospinal fluid using an implementation of the expectation maximization algorithm (Van Leemput et al., 1999). Hippocampal extraction was performed using a multi atlas approach based on the Harmonized Hippocampus Protocol (Boccardi et al., 2015). All measures were corrected for total intracranial volume.

Statistical Analysis

All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL) version 25. A p-value of < 0.05 determined a significant result.

Means, standard deviations and percentages are provided for the whole cohort and the brain imaging subsets (**Table 1**).

TABLE 1 | Descriptive statistics for the cognitively normal cohort and subsets with brain imaging.

	Whole cognitively normal sample $n = 227$	Subset with Aβ-PET imaging $n = 60$	Subset with MR imaging $n = 51$
Age, y	69.71 ± 6.0	70.44 ± 5.6	69.92 ± 5.2
Sex, male; n (%)	92 (40.5)	28 (46.7)	22 (49.0)
Presence of *APOE* ε4 allele; n (%)	61 (26.9)	23 (39.0)	22 (43.1)
Years of education, ≤ 12 y; n (%)	113 (49.8)	30 (50.0)	24 (47.1)
Daily coffee intake, g	280.90 ± 323.0	236.01 ± 232.6	237.02 ± 237.2
Time from baseline assessment to FFQ completion, y	3.38 ± 0.9	2.49 ± 1.3	2.67 ± 1.2
Daily energy intake, KJ	2243.30 ± 677.0	2429.83 ± 817.0	2327.08 ± 795.2

Unless otherwise described, data are presented as mean ± standard deviation of the mean.
APOE, Apolipoprotein E; FFQ, food frequency questionnaire; g, grams; KJ, kilojoules; MR, magnetic resonance; PET, positron emission tomography; y, years.

Normality of the data was tested using the Kolmogorov–Smirnov test statistic. Cognitive domains and MRI brain region volumes were natural log transformed as necessary to better approximate normality.

A series of repeated measures linear mixed model (LMM) analyses (using maximum likelihood estimation and an unstructured covariance matrix) were conducted to examine the relationship between coffee intake and time with respect to change in cognitive function. Coffee intake, age, Apolipoprotein (*APOE*) ε4 allele carrier status (presence or absence of ε4 allele; the most common genetic risk factor for AD; genotype determined as previously described (Fowler et al., 2021)), sex, education level (≤ 12 years and > 12 years), energy intake, and time from baseline to FFQ completion were entered as main effects, participant as a random factor, and coffee intake*time as an interaction term. Models were then re-run with the cognitive function dependent variable exchanged for individual MRI brain region volumes or continuous PET Centiloid; however, education level was excluded from these neuroimaging models. Scanner location was included as a confounder in the MRI volume LMMs due to multiple scanners being utilized for MRI.

Multinomial logistic regression models calculated odds ratios for the investigation of the likelihood of exhibiting change in clinical classification from cognitively normal to MCI or AD, with increasing coffee intake. Multinomial logistic regression models additionally calculated odds ratios for the investigation of the likelihood of exhibiting "moderate," "high," or "very high" brain Aβ burden compared to "negative" burden over time, with increasing coffee intake. These models included the same covariates as the LMM analyses excluding education level.

All participants irrespective of their number of cognitive assessments or brain scans were used in the LMMs to reduce parameter variation, however, only those with two or more assessments were used in the calculation of the time-related changes dependent on coffee consumption. The number of brain imaging scans and cognitive assessments completed at each timepoint is reported in **Supplementary Table 2**.

RESULTS

The current cohort comprised 227 participants (40.5% male) with an average age of 69.7 years at baseline. Almost 27% were carriers of the *APOE* ε4 allele (genotypes ε2/4, ε3/4, or ε4/4) and nearly 50% had 12 or less years of education. Of the 60 participants included in the PET imaging analysis, 46.7% were male with an average age of 70.44 years, and almost 40% were carriers of the *APOE* ε4 allele. Of the 51 participants included in the MRI analysis, all were also included in the PET imaging analysis (supplemented by an additional $n = 9$ who did not undergo MRI). Forty-nine percent were male with an average age of 69.92 years, and over 43% were *APOE* ε4 carriers (**Table 1**).

Habitual coffee intake was positively associated with the cognitive domains of executive function ($p < 0.01$), attention ($p < 0.05$), and the AIBL PACC ($p < 0.01$), such that higher coffee consumption was associated with slower cognitive decline in these domains over 126 months (**Table 2**). Within the executive function, attention and AIBL PACC models, the following covariates contributed to variance in the outcome: for executive function, *APOE* ε4 allele carriage ($F = 4.421$, $p = 0.037$); for

TABLE 2 | Results of linear mixed models examining the association between coffee intake, cognitive decline, brain Aβ-amyloid accumulation, and brain volume atrophy in the cognitively normal cohort over 126 months.

Change in cognitive domain or brain imaging measure	Slope estimate	F Statistic	*p*-value
Episodic recall	1.042[c]	0.395	0.532
Recognition	1.004[c]	0.079	0.780
Executive function	1.009[c]	7.974	**0.006****
Language	1.003[c]	0.432	0.513
Attention	0.974[c]	4.284	**0.044***
AIBL PACC	1.039[c]	9.954	**0.003****
Aβ-amyloid Burden[a]	4.328	5.978	**0.017***
Cortical gray matter volume[ab]	−2.033	2.940	0.138
White matter volume[ab]	−2.476	0.002	0.964
Left hippocampal volume[ab]	0.996[c]	0.136	0.713
Right hippocampal volume[ab]	0.997[c]	0.243	0.624

Model includes coffee intake, age, APOE ε4 allele carrier status, energy intake, education level, time from baseline to FFQ completion, and sex as main effects.
*Bold indicates statistical significance (*p < 0.05, **p < 0.01).*
AIBL, Australian Imaging, Biomarkers, and Lifestyle study; APOE, Apolipoprotein E; FFQ, food frequency questionnaire; PACC, Preclinical Alzheimer Cognitive Composite.
[a]*Does not include education level in model.*
[b]*Scanner location was included as a confounder in model.*
[c]*Cognitive domains and hippocampal brain region volumes were natural log transformed, and estimates were back transformed.*

attention, age, and education ($F = 4.779$, $p = 0.030$; $F = 6.335$, $p = 0.013$, respectively); for the AIBL PACC, sex, age, and education ($F = 8.987$, $p = 0.003$; $F = 30.131$, $p < 0.001$; $F = 20.655$, $p < 0.001$, respectively). Moreover, odds ratios showed that higher coffee consumption was associated with lower risk of converting from cognitively normal to MCI or AD status over 126 months ($\beta = -0.004$; $p = 0.006$).

Daily coffee intake was also associated with cerebral Aβ-amyloid burden, such that higher coffee consumption was associated with slower Aβ-amyloid accumulation over 126 months ($p < 0.05$; **Table 2**). Within the model, the following covariates contributed to variance in the outcome: energy intake, and time from baseline to FFQ completion ($F = 4.860$, $p = 0.030$; $F = 4.865$, $p = 0.034$, respectively). Furthermore, odds ratios showed that higher coffee consumption was associated with lower risk of transitioning to "moderate," "high," or "very high" cerebral Aβ-amyloid burden status over 126 months compared to not transitioning ($\beta = -0.005$, $p = 0.035$). Graphical representation of the change in cerebral Aβ-amyloid burden over 126 months, across coffee intake tertiles, demonstrates higher overall rates of Aβ-amyloid accumulation in those participants with "low" coffee intake (**Figure 1**). Of note, there were no significant differences in baseline Aβ-amyloid across the tertiles of coffee intake.

There were no associations observed between coffee intake and atrophy in gray matter, white matter, or hippocampal volume over 126 months.

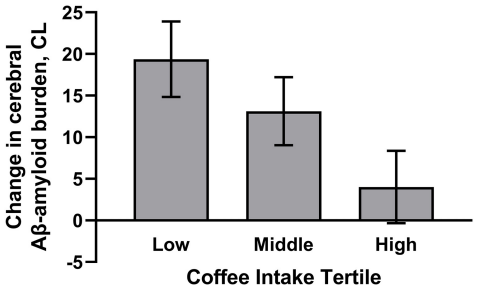


FIGURE 1 | Plot demonstrates higher habitual coffee intake was associated with slower cerebral Aβ-amyloid accumulation. Graphical representation of mean change in cerebral Aβ-amyloid accumulation, ± SD, over 126 months, across tertiles of coffee intake (Low tertile = 0–26 g/day; Middle tertile = 36–250 g/day; High tertile = 360–750 g/day). Model includes age, apolipoprotein E (*APOE*) ε4 allele carrier status, sex, energy intake, and time from baseline to dietary questionnaire completion. Cerebral Aβ-amyloid burden was measured using positron emission tomography and expressed using the Centiloid (CL) scale.

DISCUSSION

The aim of this study was to investigate the relationship between habitual coffee intake and decline in multiple cognitive domains, over 126 months, in 227 cognitively normal individuals, accompanied by examination, in a subset of these individuals, of whether coffee intake was associated with rates of cerebral Aβ-amyloid accumulation, or brain volume atrophy.

Our results showed higher coffee consumption was associated with slower cognitive decline, specifically in the executive function and attention domains. Furthermore, higher consumption of coffee was also associated with slower decline in the AIBL PACC, which has previously been shown to reliably measure the first signs of cognitive decline in at-risk cognitively normal populations (Donohue et al., 2014). Consistent with these findings, higher coffee consumption was additionally associated with lower risk of transitioning from cognitively normal to MCI or AD status over the same time-period of 126 months.

In the neuroimaging subset, higher coffee intake was associated with slower cerebral Aβ-amyloid accumulation over 126 months, as well as lower risk of progressing to "moderate," "high," or "very high" Aβ-amyloid burden status over the same time-period. By contrast, coffee consumption was not associated with rates of brain volume atrophy.

The observed effect size estimates suggest that if the average cup of coffee made at home is 240 g, increasing intake from one to two cups per day could potentially provide up to 8% decrease in executive function decline over an 18-month period, and up to 5% decrease in cerebral Aβ-amyloid accumulation over the same time period. However, we readily acknowledge, that further longitudinal observational and intervention studies are required to substantiate this suggestion.

Whilst coffee consumption has previously been linked to decreased risk of MCI and AD (Solfrizzi et al., 2015; Wierzejska, 2017; Wu et al., 2017), until now, no prior studies have characterized the relationship of coffee intake to *rates* of decline in multiple cognitive domains. Nevertheless, consistent with the longitudinal findings of the current study, in a *cross-sectional* analysis of community-dwelling Dutch older adults ($n = 2914$; mean age 59.28 ± 7.2 years), consumption of > 3 cups of coffee per day was associated with better performance on an executive function measure (Araujo et al., 2016). However, the authors reported that this level of coffee consumption was also associated with worse performance on information processing speed and delayed memory measures, compared with 0–1 cups per day. The authors proposed an explanation of their findings whereby the neuro-stimulant effects of coffee on memory function are short-acting, and participants had not consumed coffee for at least 60 min preceding cognitive assessment (Araujo et al., 2016). In contrast to the current report, a study of Finnish twins found coffee consumption was not an independent predictor of cognitive performance in old age, among 2606 middle-aged twins assessed in 1975 and 1981, with a median follow-up of 28 years (Laitala et al., 2009). Cognitive status, however, was assessed using a brief telephone interview comprising a combination of a screen to identify potential dementia cases (TELE) and the Telephone Interview for Cognitive Status (TICS). Furthermore, coffee intake was assessed by the question "How many cups of coffee do you drink daily?" with respondents advised to answer "zero" if they did not drink coffee daily. In the present study, coffee intake over the previous year was used to generate a daily intake value on a continuous scale, such that drinking coffee less frequently than once a day would still yield an intake level; thereby generating a more realistic coffee consumption variable for analyses. Similar to the Finnish study, a study of

3494 Japanese males born between 1900 and 1919 and residing in Hawaii in 1964, reported that coffee intake was not associated with risk of cognitive impairment 25 years later. Like the Finnish study, cognition was not assessed using a comprehensive battery, instead a single screening test, the Cognitive Abilities Screening Instrument (CASI) was utilized, with impairment defined as scoring < 74 out of 100. Moreover, coffee intake was assessed via 24-h dietary recall, and the very specific cohort inclusion criteria limits the generalizability of results (Gelber et al., 2011).

Beyond the current study, only one other published report has examined the relationship between coffee intake and brain Aβ-amyloid (Kim et al., 2019). Consistent with the results of the current longitudinal study, where higher coffee intake was associated with slower cerebral Aβ-amyloid accumulation, this other study, comprising cross-sectional analysis of 411 non-demented older adults from South Korea, found lifetime intake of two or more cups of coffee per day was associated with lower rates of "Aβ positivity" (presence of significant brain Aβ-amyloid), compared to less than two cups per day. However, current coffee intake was not related to "Aβ positivity" in this South Korean cohort (Kim et al., 2019). MRI outcomes were also examined in this cross-sectional study, and consistent with our findings, neither current, nor lifetime coffee intake, was related to cerebral mean cortical thickness or white matter hyperintensity volume (Kim et al., 2019).

Precisely which constituents of coffee contribute to the positive outcomes described in the current study, and by others, remains to be determined. Yet, studies conducted in animal models of AD provide preliminary evidence to suggest the observed benefits are not due to caffeine alone. Both caffeine and crude caffeine (CC), a by-product of the coffee de-caffeination process, partially prevented memory impairment in AD mice, with CC producing a greater effect than pure caffeine. However, only CC consumption reduced the $A\beta_{1-42}$ levels and the number of Aβ-amyloid plaques in the hippocampus of these animals. Nevertheless, *in vitro*, both caffeine and CC were shown to protect primary neurons from Aβ-induced cell death, and to suppress Aβ-induced caspase-3 activity (Chu et al., 2012). Multiple components of coffee, including cafestol and kahweol, have also been show to induce Nrf2 (Nuclear factor-erythroid factor 2-related factor 2) activation in *C. elegans* (Higgins et al., 2008). Nrf2-dependent upregulation of proteasomal activity has been directly implicated in protection against Aβ-mediated toxicity in cell culture models (Park et al., 2009); however, further work is needed to determine whether this is a related mechanism in humans. Moreover, dietary supplementation with Eicosanoyl-5-hydroxytryptamide (EHT), a minor component of coffee, for 6–12 months in rats, substantially ameliorated cognitive impairment, tau hyperphosphorylation, and elevated levels of cytoplasmic Aβ, potentially through its ability to increase phosphoprotein phosphatase 2A (PP2A) activity. These findings suggest EHT may also make a substantial contribution to the neuroprotective benefits associated with coffee consumption (Basurto-Islas et al., 2014).

The stimulant effect of coffee is attributed to the pharmacological activity of caffeine, acting as an antagonist of adenosine receptors in the brain (de Paulis et al., 2002). Adenosine regulates several physiological functions including sleep, cognitive performance, and memory, and its main role is to regulate neuronal excitatory synaptic transmission by inhibitory A_1 receptors, and synaptic plasticity via facilitatory A_2 receptors (Merighi et al., 2021). The effects of A_1 and A_2 receptor antagonists have been tested in animal models, where inactivation of both A_1 and A_2 receptors was shown to counteract age-related cognitive decline in rats (Prediger et al., 2005) and enhance release of several neurotransmitters including acetylcholine (Boison, 2006). Long-term caffeine administration to amyloid-beta precursor protein (APP) overexpressing mice has been shown to induce significant improvement in multiple cognitive tasks, as well as reduce levels of soluble Aβ-amyloid fragments compared to control mice (Arendash et al., 2006). The average daily intake of caffeine per mouse (1.5 mg) was the human equivalent of 500 mg caffeine, the amount typically found in five cups of coffee per day. Furthermore, caffeine reduced the production of $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides in neuronal cell cultures from these same APP overexpressing mice (Arendash et al., 2006). Involvement of A_1 receptors has also been observed in APP processing and tau phosphorylation *in vitro*, and the presence of A_1 receptors in degenerating neurons with neurofibrillary tangles and Aβ-amyloid plaques in the hippocampus and frontal cortex suggest that these receptors may play a role in the pathogenesis of AD (Angulo et al., 2003). Whilst the current study cannot provide evidence that caffeine is causing the positive effects observed, the blocking of adenosine receptors by caffeine, leading to a decrease in Aβ-amyloid in the brain, and subsequent reduction of tau hyperphosphorylation is a potential mechanism that warrants further investigation.

There are some limitations that should be considered when interpreting our findings. Although we adjusted for known potential confounding factors there is a possibility of residual confounding due to other factors which were not measured. There is also the possibility of measurement error or recall bias with respect to the dietary data, which is especially true for questionnaire-based dietary exposures. However, coffee intake is less prone to misreporting due to its long-term habitual nature. Moreover, participants with cognitive impairment at baseline were excluded from the current analysis to reduce the likelihood of measurement error or recall bias. No data on mid-life coffee consumption was obtained from participants, consequently, potential deleterious or beneficial effects of coffee intake at midlife cannot be assessed in the current study. Finally, in our study it was not possible to differentiate between intakes of caffeinated or de-caffeinated coffee, nor to determine the potential consequences of varying methods of coffee preparation (e.g., brewing method, with or without milk or sugar etc.) on the associations observed. Nevertheless, strengths of our study include the long duration of follow-up, and comprehensive characterization of the cohort, including concurrent assessment of both multiple domains of cognition and neuroimaging biomarkers.

In summary, our results support the notion that habitual coffee intake may be a protective factor against AD. Specifically, increased coffee consumption could contribute to slower

cognitive decline potentially by slowing the rate of cerebral Aβ-amyloid accumulation, and in doing so, ameliorate the associated neurotoxicity from Aβ-amyloid-mediated oxidative stress and inflammatory processes. Additional longitudinal observational and intervention studies are required to validate our findings and confirm this hypothesis. Such confirmation would corroborate the idea that coffee intake could be incorporated as a modifiable lifestyle factor aimed at delaying AD onset.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the AIBL data are available only to authorized users. Requests to access the datasets should be directed to the following online form: https://ida.loni.usc.edu/collaboration/access/appApply.jsp?project=AIBL.

ETHICS STATEMENT

The study involving human participants was reviewed and approved by the Ethics Commitees of Hollywood Private Hospital, Edith Cowan University, St Vincent's Health and Austin Health. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SG, SR-S, KT, CM, PM, CR, DA, and RM designed research. SG conducted research, primary responsibility for final content, and analysis for the manuscript. VV, JF, VD, and PB oversaw collection of and analyzed imaging data. SG and SR-S wrote the manuscript. VV, JF, VD, PB, KT, CF, CM, PM, CR, DA, and RM edited the final manuscript. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.744872/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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A Synergistic Combination of DHA, Luteolin, and Urolithin A Against Alzheimer's Disease

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Alzheimer's disease (AD) is a devastating neurodegenerative disorder and the most common form of dementia worldwide. The classical AD brain is characterized by extracellular deposition of amyloid-β (Aβ) protein aggregates as senile plaques and intracellular neurofibrillary tangles (NFTs), composed of hyper-phosphorylated forms of the microtubule-associated protein Tau. There has been limited success in clinical trials for some proposed therapies for AD, so attention has been drawn toward using alternative approaches, including prevention strategies. As a result, nutraceuticals have become attractive compounds for their potential neuroprotective capabilities. The objective of the present study was to derive a synergistic nutraceutical combination *in vitro* that may act as a potential preventative therapy for AD. The compounds of interest were docosahexaenoic acid (DHA), luteolin (LUT), and urolithin A (UA). The cell viability and cytotoxicity assays MTS and LDH were used to evaluate the compounds individually and in two-compound combinations, for their ability to inhibit $A\beta_{1-42}$-induced toxicity in human neuroblastoma BE(2)-M17 cells. The LDH-derived% protection values were used in the program CompuSyn v.1.0 to calculate the combination index (CI) of the two-compound combinations. The software-predicted potentially synergistic ($CI < 1$) two-compound combinations were validated using CellTiter Glo assay. Finally, a three-compound combination was predicted ($D_5L_5U_5$) and shown to be the most effective at inhibiting $A\beta_{1-42}$-induced toxicity. The synergistic combination, $D_5L_5U_5$ warrants further research for its mechanism of action; however, it can serve as a basis to develop an advanced functional food for the prevention or co-treatment of AD.

Keywords: Alzheimer's disease, docosahexaenoic acid, *in vitro*, Luteolin, synergistic nutraceutical combinations, urolithin A

INTRODUCTION

Plants, the immobile life on earth, have the inherent ability to synthesize defensive secondary metabolites, commonly known as phytochemicals, to withstand attacks by different organisms such as pathogens, insects, and herbivores. These phytochemicals have proven bioactivity through modulating molecular targets in living beings (Efferth and Koch, 2011). These bioactive phytochemicals are used in traditional medicine in China and Sri Lanka, and Ayurveda in India. These medicine systems use herbal mixtures consisting of many different herbs to treat diseases (Thomas and Egon, 2011).

The unraveling of complex disease mechanisms in modern medicine by technological advancement has immensely contributed to a greater understanding of drug interactions and usage of drug combinations in therapeutic regimes. In combination drug therapies, the simultaneous action of drugs in low doses increases therapeutic efficacy and decreases toxicity effects and drug resistance (Sun et al., 2016). Combination drug therapies are widely researched in treating diseases such as cancer (De Kok et al., 2008), human immunodeficiency virus (HIV) infection (Moreno et al., 2019), and many other ailments. Furthermore, combining natural compounds is popularizing in dealing with medical conditions where there is a shortage of discovery and approval of new drugs, and the existing monotherapies have shown limited therapeutic efficacy (Patti et al., 2017; Santana-Gálvez et al., 2019).

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and is the second major cause of death in Australia. The classical AD brain is characterized by extracellular deposition of amyloid-β (Aβ) protein aggregates as senile plaques and intracellular neurofibrillary tangles (NFTs), composed of hyper-phosphorylated forms of the microtubule-associated protein Tau. Amyloid beta peptides are formed by the normal metabolic processing of amyloid precursor protein (APP). The predominant (90%) Aβ peptides are $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$, respectively, with the latter being the most toxic (Selkoe, 2001; Murphy and Levine, 2010; Tiwari and Kepp, 2016).

It has been reported that by February 2020, there were 121 drugs studied in 136 AD therapeutic trials (Cummings et al., 2020). However, considering the past decade, many clinical trials have failed outright while the efficacy and effect size have been problematic in the ones that have indicated a positive outcome (Banik et al., 2015). There has been no new drug approved within the past 16 years until the controversial approval of the drug Aducanumab recently in 2021 (Rabinovici, 2021).

Due to the toxicity associated with the use of currently available drugs and their limited therapeutic effectiveness, the purposed drugs for AD are being repositioned as combinations (Cummings et al., 2019; Kabir et al., 2020). Considering the multifactorial nature of AD, combinations of therapeutic agents may be effective than monotherapies. One study reported that a drug combination of two approved drugs, acamprosate and baclofen synergistically protected rat cortical neurons and human brain-derived microvascular endothelial cells against Aβ oligomer-induced toxicity (Chumakov et al., 2015). Furthermore, this combination has alleviated cognitive deficits in an acute $A\beta_{25-35}$ peptide injection mouse model and a mutant APP transgenic mouse model (Chumakov et al., 2015). Many studies and clinical trials have been conducted for AD drug combinations, to name a few, the N-methyl-D-aspartate (NMDA) receptor antagonist Memantine with various Acetyl Cholinesterase inhibitors such as Memantine and Rivastigmine (Dantoine et al., 2006; Riepe et al., 2007), Memantine, and Donepezil (Tariot et al., 2004; Cummings et al., 2006) and Memantine and Galantamine (Simoni et al., 2012).

Prevention of AD has become an important consideration, particularly since disease-modifying treatment trials have proven unsuccessful. As AD is a complex multifactorial disorder, there may also be multiple ways to prevent or delay the onset of AD (Galvin, 2017). It suggests that prevention studies focusing on risk reduction and lifestyle modification by diet and exercise may be an alternative approach offering additional benefits. In the modulation of lifestyle, diet plays a major role. The Mediterranean diet (MeDi) plays an important role to reduce the risk for AD (Scarmeas et al., 2006; Panza et al., 2018). MeDi is characterized by a high intake of vegetables, fruits, unsaturated fatty acids (in the form of olive oil), fish, a low-to-moderate intake of dairy products such as cheese or yogurt, a low intake of meat, and poultry and a regular but moderate amount of red wine (Scarmeas et al., 2006). These vital food items in a typical MeDi are rich in bioactive components that are reported as potentially beneficial for cognitive performance in AD (Cremonini et al., 2019; Grodzicki and Dziendzikowska, 2020).

One rich source of polyphenols is pomegranate, which possesses many polyphenolic compounds such as ellagitannins (ETs) and flavonoids (Sreekumar et al., 2014). Punicalagin is the most abundant ET in pomegranate juice with a very low bioavailability (Cerda et al., 2003). In the lower digestive tract, punicalagins are converted by the gut microbiota into urolithin A (UA), which has a relatively higher bioavailability (Seeram et al., 2006; Espin et al., 2013). According to Hartman et al. (2006), mice treated with pomegranate juice had significantly less (∼50%) soluble $A\beta_{42}$ and amyloid deposition in the hippocampus as compared to control mice (Hartman et al., 2006). However, the anti-AD effects of pomegranate are due to UA (Yuan et al., 2016; Gong et al., 2019). Luteolin (LUT) is a prominent flavone compound in pomegranate peel (Van Elswijk et al., 2004; Chaudhari et al., 2014; Liu et al., 2017). It shows potent anti-inflammatory and antioxidant activities (Xia et al., 2014). It also inhibits BACE1 by suppressing the BACE1 promoter by NF-κB signaling (Zheng et al., 2015). Moreover, LUT has been reported to reduce zinc-induced Tau hyperphosphorylation in SH-SY5Y Cells (Zhou et al., 2012). Luteolin has also been shown to ameliorate neurotoxicity in an Aβ toxicity model that used $A\beta_{25-35}$ peptide in murine cortical neurons (Choi et al., 2014). Overall, there are only a limited number of studies carried out on the activity of these pomegranate-related polyphenols on the inhibition of $A\beta_{1\text{-}42}$ induced toxicity.

Omega-3 polyunsaturated fatty acids including docosahexaenoic acid (DHA) naturally occur in marine food sources such as fish and algae (Tocher, 2015; Peltomaa et al., 2017). An *in vivo* experiment carried out in mouse expressing human APP K670N-M671L (APPsw) transgenic mouse model

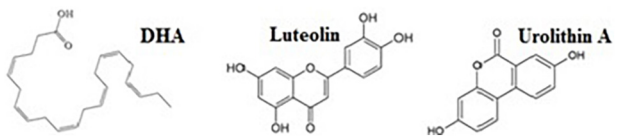


FIGURE 1 | The compounds of interest. The compounds investigated in the present study were, omega-3 polyunsaturated fatty acid, docosahexaenoic acid (DHA) and pomegranate-derived compounds: luteolin (LUT), urolithin A (UA).

(Tg2576) has shown that DHA treatment lowers $A\beta_{40}$ and $A\beta_{42}$ levels and Aβ plaque burden (Lim et al., 2005). Some *in vitro* experiments demonstrated that DHA decreases the BACE1 and γ-secretase activity and increases the α-secretase activity. It has been reported that DHA effectively reduced Aβ release by driving the amyloidogenic processing of APP toward non-amyloidogenic processing (Grimm et al., 2011). An *in vitro* study indicated that DHA reduced soluble Aβ oligomer levels and further inhibited formation of $A\beta_{1-42}$ fibrils (Hossain et al., 2009). Furthermore, another study showed that DHA reduced formation of Aβ oligomers and fibrils in the cerebral cortex of Aβ-infused rats (Hashimoto et al., 2009). However, the few studies that have investigated DHA on $A\beta_{1-42}$ induced toxicity need confirmation by a more thorough investigation.

Some reports have investigated the combined effect of multi-targeting nutraceutical compounds in AD *in vitro* models (Espargaró et al., 2017). It has recently been shown in Tg2576 transgenic mice that a combination of food-derived compounds, EGCG, DHA, and α-lipoic acid exerted potent anti-inflammatory and neuroprotective effects (Sharman et al., 2019). However, similar studies targeting bioactive compound combinations against AD are still limited in the literature.

The objective of the present study was to investigate the compounds, DHA, LUT, and UA (**Figure 1**) *in vitro* for any nutraceutical combinations potentially effective against AD. The compounds were initially screened for their activity to inhibit $A\beta_{1-42}$-induced toxicity and were subsequently used to determine synergistic combinations *in vitro* that may be more potent in action against $A\beta_{1-42}$ compared to single compounds. For drug combinations, quantifying synergism and antagonism through CI calculations was performed by the third-generation computer software, "CompuSyn" written by Ting-Chao Chou and Nick Martin (MIT, MA, United States) in 2005 (ComboSyn, Inc., MA, United States).

MATERIALS AND METHODS

Materials

cis-4,7,10,13,16,19-Docosahexaenoic acid (DHA: D2534), LUT (L9283), UA (SML1791), and dimethyl sulfoxide (DMSO) were obtained from Sigma Aldrich, United States. BE(2)-M17 cells (ATCC® CRL2267™) were purchased from American Type Cell Culture Collection (ATCC, Manassas, VA, United States). All cell culture reagents including Dulbecco's Modified Eagle Medium (DMEM), Ham's F12 medium, Hank's balanced salt solution (HBSS), fetal calf serum (FCS) and Trypsin-EDTA (0.5%) were purchased from GIBCO by Life Technologies (United States). Human $A\beta_{1-42}$ peptides were synthesized, purified and characterized by high pressure liquid chromatography (HPLC) and mass spectrometry (MS) by The ERI Amyloid Laboratories LLC, United States. Anhydrous DMSO was purchased from Molecular Probes by Life Technologies (United States). CellTiter 96® AQueous One Solution Cell Proliferation assay (MTS: 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and CytoTox-ONE™ Homogeneous Membrane Integrity assay kits (Lactate dehydrogenase: LDH assay), and CellTiter-Glo luminiscent cell viability assay kits were purchased from Promega (Madison, WI, United States).

Cell Culture

Human neuroblastoma BE(2)-M17 cells were maintained in T75 culture flasks containing 15 mL of DMEM/F12 (1:1 ratio) growth media supplemented with 10% (v/v) FCS and placed in a humidified incubator with 5% CO_2/95% air at 37°C. Upon reaching about 80% confluency, the cells were sub-cultured on to fresh cell culture flasks. For all cell culture experiments, passage number did not exceed 30.

Preparation of Oligomeric $A\beta_{1-42}$

The oligomeric $A\beta_{1-42}$ was prepared according to the method of Stine et al. (2011) with some modifications (Stine et al., 2011). The detailed method used for $A\beta_{1-42}$ preparation is explained in our previous work (Jayatunga et al., 2021).

$A\beta_{1-42}$ Induced Toxicity/Lactate Dehydrogenase Assay

For $A\beta_{1-42}$ toxicity experiments, cells were plated in 96-well tissue culture microplates at a density of 1.5×10^4 cells/well and incubated for 24 hours. The cell culture media was then replaced with treatment media (1% FCS) and the cells were pre-treated with different concentrations of the compounds, DHA, LUT, and UA (5 μM to 40 μM) for 24 hours. The cells were then treated with oligomeric 20 μM $A\beta_{1-42}$ with appropriate controls (vehicle-treatment: negative control; $A\beta_{1-42}$–treatement: positive control). The microplates were incubated in the humidified incubator with 5% CO_2/95% air for 72 h at 37°C. The percentage LDH release for all treatments were determined using LDH assay. LDH release results of $A\beta_{1-42}$-induced toxicity assays for the compounds DHA, LUT, and UA.

The% LDH release results of $A\beta_{1-42}$–induced toxicity assays for the compounds DHA, LUT, and UA were normalized according to the method used by Chumakov et al. (2015). As shown below, the vehicle and the $A\beta_{1-42}$ added treatments were considered as 1 and 0, respectively (Chumakov et al., 2015). The coded data were considered as the fractions affected (F_a) and used along with their respective concentrations (5 to 40 μM) as input for the computer program CompuSyn v.1.0.

Calculation of F_a - method adapted from Chumakov et al. (2015).

	Vehicle-treated control	$A\beta_{1-42}$-treated control	Test
% LDH release	X	y	z
% protection	(100-x)	(100-y)	(100-z)
Correction for the $A\beta_{1-42}$–induced toxicity	(100-x) - (100-y)	(100-y) - (100-y)	(100-z) - (100-y)
F_a (Fraction affected)	1	0	$\frac{(100-z) - (100-y)}{(100-x) - (100-y)}$

Preparation of Compound Solutions

The compounds DHA, LUT, and UA were dissolved in dimethylsulfoxide (DMSO) and 10 mM stock solutions were prepared from each. The stock solutions were frozen and working solutions were prepared using treatment media (DMEM/F12 supplemented with 1% FCS). Control solutions were used for all compounds at all concentrations.

Combination Studies

MTS Assay

Alternatively, all possible two-compound combinations (n = 75) for the compounds DHA, LUT, and UA (for the concentration range of 5 μM to 40 μM) were used for screening the combinations with the greatest efficacy to inhibit $A\beta_{1-42}$–induced toxicity. For that, BE(2)-M17 cells were maintained in DMEM/F12 medium supplemented with 10% FCS, in 5% CO_2 at 37°C. For $A\beta_{1-42}$ toxicity experiments, cells were plated in 96-well tissue culture microplates at a density of 1.5×10^4 cells/well and incubated for 24 h. After replacing the media with treatment media, the cells were pre-treated with each two-compound combinations for 24 h. The cells were then treated with oligomeric 20 μM Aβ including controls for vehicle (negative control) and $A\beta_{1-42}$ (positive control). The microplates were incubated in a humidified incubator with 5% CO_2/95% air for 72 h at 37°C. Percentage cell viability for each combination was determined (N = 4) using the MTS assay.

Determination of Best Combinations by LDH Assay

The combinations with higher% cell viabilities were re-screened with the LDH assay. Percentage protection was calculated from the% LDH release for all compound combinations. The experiments consisted of all 3 compounds combining with each other at 5, 10, 20, and 40 μM concentrations. The coded data were considered as data of F_a and used along with their respective concentrations (doses) as input in the computer program CompuSyn v.1.0 for calculating CI values. Thirteen synergistic combinations were recognized by the CI values less than 1.

Validation of the Synergistic Two-Compound Combinations Using CellTiter Glo Assay

The synergistic combinations were further validated and confirmed by cellular ATP levels using the CelltiterGlo assay. Briefly, the BE(2)-M17 cells in DMEM/F12 medium supplemented with 10% FCS were seeded in 96-well tissue culture microplates at a density of 1.5 × 104 cells/well and were incubated at 37°C for 24 h. After the respective treatment of compounds and incubation at 37°C, cellular ATP levels were measured using CellTiter Glo ATP detection kit as per the manufacturer's instructions (Promega). Briefly, cells were placed at RT for 30 min and then lysed by adding 100 μL of ATP-releasing reagent. The lysates were incubated with the luciferin substrate and luciferase enzyme in the dark for 10 min to stabilize the luminescence signal. The luminescence (RLU) was measured using a Perkin Elmer EnSpire multi-mode plate reader.

Prediction of a Potentially Synergistic Three-Compound Combination and Validation

Based on the validation data for the two-compound combinations, a new three-compound combination was predicted. This combination was repeated and confirmed as efficiently inhibiting $A\beta_{1-42}$-induced toxicity using MTS, LDH, and CellTiter Glo assays.

Statistical Analysis

All results were expressed as mean ± standard deviation (SD) from four (N = 4) independent experiments. Statistical significance was determined by one-way ANOVA and Tukey's *post hoc* test in SPSS v25. Significance was defined as $P < 0.05$.

RESULTS

Thirteen Synergistic Two-Compound Combinations Derived *in silico*

There were thirteen two-compound combinations that were determined to be synergistic based on their CI values, being less than 1 (**Table 1**). **Table 1** summarizes the thirteen synergistic combinations (numbered as combinations 1-13) that belong to UA-DHA, LUT-DHA, and UA-LUT. The inferences on synergy with subtle definitions were based on the work of Chou and Talalay (1984).

Two Best Synergistic Two-Compound Combinations Based on Validations for Relative ATP Levels

Based on the validation of results for all 13 synergistic combinations, the combination 2 (D_5U_5: DHA 5 μM and UA 5 μM) and 11 (L_5U_5: LUT 5 μM and UA 5 μM) were selected as the best combinations based on two reasons. First, their significantly higher relative ATP levels compared to both components in the combinations independently. Second, they both had the lowest possible concentrations used in this study.

TABLE 1 | *In silico*-derived synergistic two-compound combinations.

No.	combination	Dose of DHA (μM)	Dose of LUT (μM)	Dose of UA (μM)	Fraction affected (f_a)	Combination index (CI)	Inference
1	D_5U_{10}	5.0	-	10.0	0.96	0.00105	Very strongly synergistic
2	D_5U_5	5.0	-	5.0	0.52	0.32721	Synergistic
3	$D_{10}U_{10}$	10.0	-	10.0	0.66	0.19564	Strongly synergistic
4	$D_{10}U_5$	10.0	-	5.0	0.69	0.07573	Very strongly synergistic
5	$D_{20}L_5$	20.0	5.0	-	0.96	0.36279	Synergistic
6	$D_{10}L_{20}$	10.0	20.0	-	0.99	0.86996	Slightly synergistic
7	D_5L_{10}	5.0	10.0	-	0.95	0.78923	Moderately synergistic
8	$D_{10}L_{10}$	10.0	10.0	-	0.93	0.89802	Slightly synergistic
9	$D_{20}L_{10}$	20.0	10.0	-	0.92	0.94635	Nearly additive
10	$D_{10}L_5$	10.0	5.0	-	0.9	0.51704	Synergistic
11	L_5U_5	-	5.0	5.0	0.73	0.84538	Moderately synergistic
12	$L_{20}U_5$	-	20.0	5.0	0.99	0.86998	Slightly synergistic
13	$L_{10}U_5$	-	10.0	5.0	0.94	0.84733	Moderately synergistic

Relative ATP level of the cells was reduced to 51.9 + 7.0% of the control treatment after 72 h exposure to 20 μM $A\beta_{1-42}$ treatment. Pre-treatment with the combination 2 (D_5U_5) increased the cellular ATP levels to 71.3 + 7.9% ($P < 0.001$) (**Figure 2**).

Similarly, pre-treatment with the combination 11 (L_5U_5) increased the ATP levels to 99.6 ± 4.2% ($P < 0.001$) (**Figure 3**). In either case, the combinations gave significantly higher ATP levels against the $A\beta_{1-42}$-treated controls than the component compound concentrations (DHA, LUT, and UA 5 μM each). These results suggest that pre-treatment with these combinations effectively attenuated $A\beta_{1-42}$-induced toxicity.

Prediction and Validation of a New Three-Compound Combination-$D_5L_5U_5$ (DHA 5 μM, LUT 5 μM, UA 5 μM)

Based on the two best combinations identified, namely 2 and 11, a new combination was predicted. This composed of all the three compounds, DHA, LUT, and UA, each at a concentration of 5 μM and namely, $D_5L_5U_5$. The predicted three-compound combination, $D_5L_5U_5$ was analyzed for its ability to inhibit $A\beta_{1-42}$-induced toxicity using MTS and LDH assays. The analysis of LDH results by the program CompuSyn v.1.0 determined the three-compound combination, $D_5L_5U_5$ as synergistic with a CI value of 0.01 (**Table 2**). Percentage cell viability of BE(2)-M17 cells was decreased to 46.0 ± 3.7% of control ($P < 0.001$) after 72 h of 20 μM $A\beta_{1-42}$ treatment, while pre-treatment with $D_5L_5U_5$ improved the cell viability to 103.6 ± 8.7% ($P < 0.001$) (**Figure 4A**). Additionally, 20 μM $A\beta_{1-42}$ treatment increased the release of LDH in the cells from 7.39 ± 0.04% (vehicle-treated cells) to 25.4 ± 0.5% ($A\beta_{1-42}$-treated cells) ($P < 0.001$) and the $D_5L_5U_5$ pre-treatment significantly reduced the LDH release to 7.3 ± 1.4% ($P < 0.001$) **Figure 4B**. Cells after treating with $D_5L_5U_5$ showed an intact morphology with visually reduced toxic effects and increased proliferation compared to

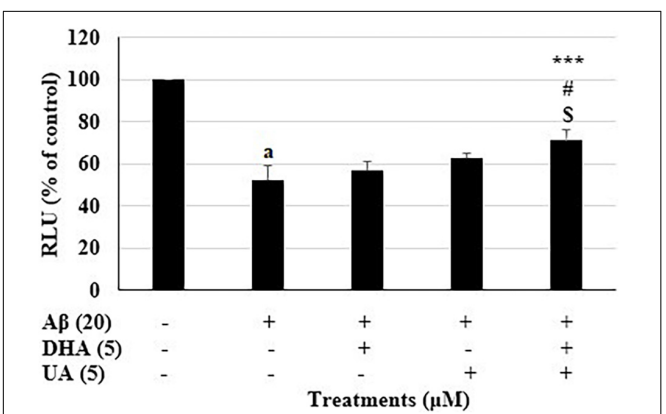


FIGURE 2 | Relative ATP levels for the combination 2 (D_5U_5) and its components. ATP levels for the combination 2 (D_5U_5) and its constituents were determined using CellTiter Glo assay. Data are expressed as mean ± SD from four ($N = 4$) independent experiments. Differences are significant at $^{a}P < 0.001$ vs. vehicle control, $^{***}P < 0.001$ vs. $A\beta_{1-42}$-treated control, $^{\#}P < 0.05$ vs. DHA 5 μM and $^{\$}P < 0.05$ vs. UA 5 μM.

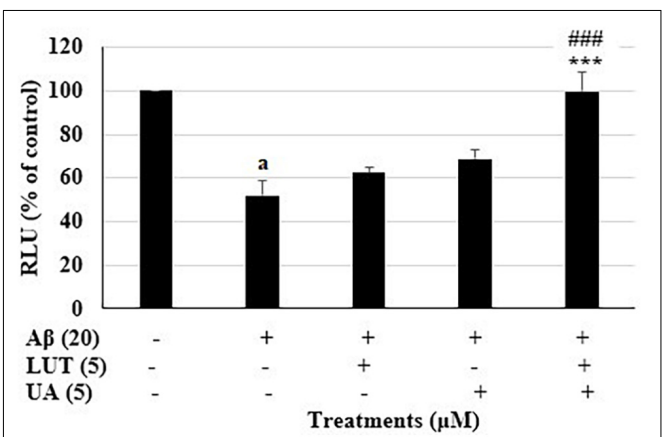


FIGURE 3 | Relative ATP levels for the combination 11 (L_5U_5) and its components. ATP levels for the combination 11 (L_5U_5) and its constituents were determined using CellTiter Glo assay. Data are expressed as mean ± SD from four ($N = 4$) independent experiments. Differences are significant at $^{a}P < 0.001$ vs vehicle control, $^{***}P < 0.001$ vs. $A\beta_{1-42}$-treated control, $^{\#\#\#}P < 0.001$ vs. LUT 5 μM and UA 5 μM.

$A\beta_{1-42}$-treated cells (**Figure 5**). These results together indicated that pre-treatment with the three-compound combination, $D_5L_5U_5$ attenuated $A\beta_{1-42}$-induced toxicity very effectively.

Validation of the combinations 2 and 11 and the three-compound combination along with their single components. ATP level of BE(2)-M17 cells was decreased to 51.9 ± 7.0% of control ($P < 0.001$) after 72 h of 20 μM $A\beta_{1-42}$ treatment. Pre-treatment with the combination 2 (D_5U_5) increased the cellular ATP levels to 71.3 ± 7.9% ($P < 0.001$)while the combination 11 (L_5U_5) increased the ATP levels to 99.6 ± 4.2% which is a significantly increased ATP level ($P < 0.001$) compared to the combination 2 (D_5U_5). However, pre-treatment with the three-compound combination ($D_5L_5U_5$) resulted in the highest most ATP levels which amounted to 110.8 ± 4.2%. This ATP level is significantly higher compared to that of both combination 2 (D_5U_5) ($P < 0.001$) and 11 (L_5U_5) ($P = 0.001$) (**Figure 6**). These results reflect the previous data that the three-compound combination, $D_5L_5U_5$ attenuates $A\beta_{1-42}$-induced toxicity better than its two-compound combination counterparts, D_5U_5 and L_5U_5.

DISCUSSION

Effective alternate approaches to AD drug development are critically needed as most of all clinical drug immunotherapy trials have failed to date (Anderson et al., 2017; Mehta et al., 2017). Thus, food-derived compounds warrant investigation being potential therapeutic agents (Thaipisuttikul and Galvin, 2012; Lange et al., 2019; Peng et al., 2021). These emerging alternative strategies using natural compounds hold promise for early intervention by targeting the prodromal phase of the disease (Lange et al., 2019). Considering the complexity and the multi-faceted nature of AD neuropathology, a combination of multiple therapeutic targets that can intervene several pathophysiological pathways is preferred. An advantage of combination therapy is where there is a disparity among the drugs of interest. For instance, if one drug has a desirable profile and the other gives undesirable side-effects at a selected dose, it may be possible to combine the two drugs by using different combination ratios, in obtaining a synergistic outcome (Chou, 2006, 2010).

In vitro studies are important as a starting point for drug combination studies. Even though *in vitro* and *in vivo* drug combination analyses follow the same principles, animal studies are more expensive, time consuming and often subjected to more variability of data. Although the latter is an essential next step in the evaluation process, initial investigations under *in vitro* conditions are a cost reduction and thereby, is the logical first step. Furthermore, *in vitro* studies are more flexible in liability considerations and in using death as an endpoint of toxicity (Chou, 2010). It is well known that *in vitro* data may not always predict *in vivo* results, and *in vivo* animal data may not always predict clinical results (Van Norman, 2020). However, drug combination studies strictly need an initial *in vitro* component as analyzing the effects of sub-optimal doses *in vivo* is not ethical. Therefore, it is recommended to initiate preclinical studies in cells before animal or human investigations (Chou, 2010). Reporting antagonistic drug combinations is equally important as it may hint on possible contraindications *in vivo* and thus avoid unnecessary preclinical and clinical trials (Chou, 2010). The current study used human neuroblastoma BE(2)-M17 cells for their relative convenience to use and ability to induce neuronal differentiation (Andres et al., 2013) that is required at next stages of this research work.

A synergistic three-compound combination ($D_5L_5U_5$) comprising of three nutraceutical compounds was identified *in vitro* from the present study. It was found to exert significantly higher ATP levels in the presence of $A\beta_{1-42}$ compared to the two two-compound combinations (D_5U_5 and L_5U_5) from which the three-compound combination was derived (**Figure 6**). This finding was aided by the Chou-Talalay method of drug combinations which is based on median-effect principle (Chou and Talalay, 1984). This method is widely used in drug combinations for cancer, where the goal is selective cytotoxicity. Opposingly, the context for AD is cytoprotection, which may be a reason for the sparse use of this method in the field of AD. The novelty of the present study lies on the fact that it adapted the Chou-Talalay method to screen nutraceutical combinations that inhibited $A\beta_{1-42}$–induced toxicity. The idea of prevention was explored in the current *in vitro* work by pre-treating with the compounds and the insult of $A\beta_{1-42}$ introduced secondarily. This implicates that the current results indicative of AD prevention rather than treatment. As mentioned earlier, combination index (CI) is a quantitative assessment of drug combinations which uses dose-effect data of single compounds and the combinations and statistically derived doses of single compounds that give the same effect as that of the combinations to calculate CI. Combination index equals 1 for additive effect, CI is less than 1 ($CI < 1$) for synergistic effects and higher than 1 ($CI > 1$) for antagonistic effects (Chou and Talalay, 1984). The predicted three-compound combination was shown to be synergistic based on its CI value calculated by CompuSyn v.1.0. Dose reduction is an important aspect in drug combinations. The validation studies on relative ATP levels confirmed that the combination itself significantly inhibited $A\beta_{1-42}$–induced toxicity compared to its constituents; DHA, LUT, and UA in equimolar doses (5 μM each) (**Figure 6**). The significance of the resulted combination is that it includes three neuroprotective compounds in relatively low concentrations (5 μM each) so that their multi-modes of actions are elicited without causing toxicity issues as observed for higher concentrations. Furthermore, dissolving these compounds in a single solvent (DMSO) was an added advantage that they could be combined within a single matrix without causing any solvent-based incompatibilities that may have resulted in cytotoxicity.

Polyphenolic conjugation is a novel strategy used to enhance the efficiency and biological activity of polyphenolic compounds (Cirillo et al., 2016). Similarly, fatty acid conjugation is reported to increase potency of therapeutic agents (Prakash et al., 2019). This technique is used in cancer drug therapy that anticancer drugs are conjugated with lipids such as DHA (lipid-drug conjugate) for targeted tumor therapy (Wang et al., 2012; Li et al., 2014; Irby et al., 2017). In fact, formation of fatty acid esters of polyphenols such as quercetin-3-O-glucoside have been

TABLE 2 | Predicted three-compound combination $D_5L_5U_5$ and its combination index derived by CompySyn v. 1.0.

Combination	Mean cell viability (%)	Mean LDH release (%)	Fraction affected (F_a)	CI
$D_5L_5U_5$	103.6 ± 8.7	7.3 ± 1.4	0.99	0.01
(Control)	46 ± 3.7	25.4 ± 0.5		

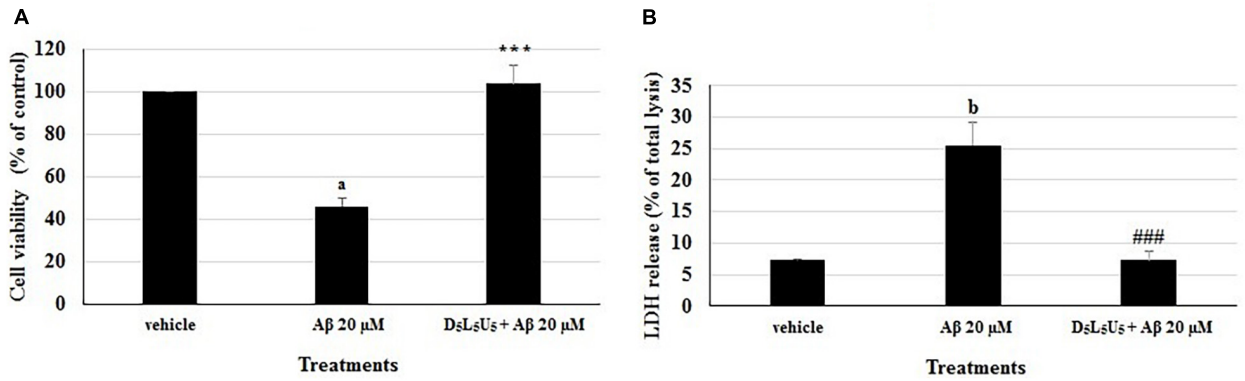


FIGURE 4 | The effect of $D_5L_5U_5$ on $A\beta_{1-42}$-induced toxicity. **(A)** % cell viability **(B)** % LDH release determined from MTS and LDH assays, respectively, with pre-treatment of $D_5L_5U_5$ in BE(2)-M17 cells for 24 h followed by incubation with 20 μM $A\beta_{1-42}$ for 72 h at 37°C. Data are expressed as mean ± SD from four ($N = 4$) independent experiments. Differences are significant at $^{a,b}P < 0.001$ vs vehicle control, $^{***}P < 0.001$, $^{\#\#\#}P < 0.001$ vs. $A\beta_{1-42}$-treated control.

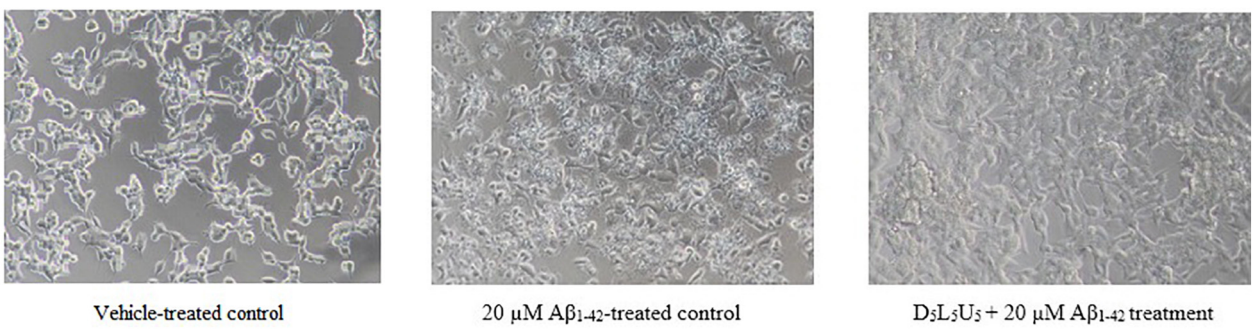


FIGURE 5 | BE(2)-M17 cell morphology for vehicle-treated control, $A\beta_{1-42}$-treated control and the $D_5L_5U_5$ treatment. Comparison of cells for 20 μM $A\beta_{1-42}$-treatment with and without $D_5L_5U_5$ pre-treatment. The BE(2)-M17 cells are pre-treated with $D_5L_5U_5$ followed by exposure of 20 μM $A\beta_{1-42}$ for 72 h. Cell morphology was imaged using a Nikon phase-contrast microscope (X40).

shown beneficial for cell viability and survival of both human lung fibroblasts and human primary hepatocytes against H_2O_2-induced cytotoxicity (Warnakulasuriya et al., 2016). As DHA is a constitutive fatty acid in cell membranes, it may facilitate the passage of the conjugated polyphenols into cells increasing their bioavailability. In a similar manner, DHA in the three-compound combination may potentially conjugate with LUT and UA, leading to their increased bioavailability and thereby resulting in increased cell viability. All possible structures of DHA ester derivatives of LUT and UA and polyphenolic associations that may form during the cellular pre-treatment of the three-compound combination are shown in the **Figure 7**.

Meanwhile, mechanisms of inhibiting $A\beta_{1-42}$-induced toxicity by $D_5L_5U_5$ are yet unknown. However, modified mitochondrial dehydrogenase activity which is indicated by the MTS results (**Figure 4A**) as well as the relatively higher ATP levels for $D_5L_5U_5$ (**Figure 6**), are suggestive of profound beneficial effects on mitochondria. It can be hypothesized, further, that the exact mechanisms are similar to that of individual drugs in a combination (Chou, 2010). Considering the components of $D_5L_5U_5$, DHA is thought to exert protection to neuronal mitochondria. Multiple lines of evidence show that dietary n-3 PUFA, specifically DHA gives beneficial effects on mitochondrial membrane organization (Fan et al., 2003; Khairallah et al., 2012) and mitochondrial function (Mayurasakorn et al., 2016). DHA has shown to reduce ROS production *in vitro* and Ca^{2+}-induced mitochondrial membrane permeabilization in neonatal C57BL/6J mice following hypoxia-ischemia-brain injury (Mayurasakorn et al., 2016). Manipulation of membrane phospholipids in the mitochondrial membrane such as increasing cardiolipin content is proposed to be the mechanism of many of the beneficiary effects of DHA (Pepe et al., 1999). Mitochondria

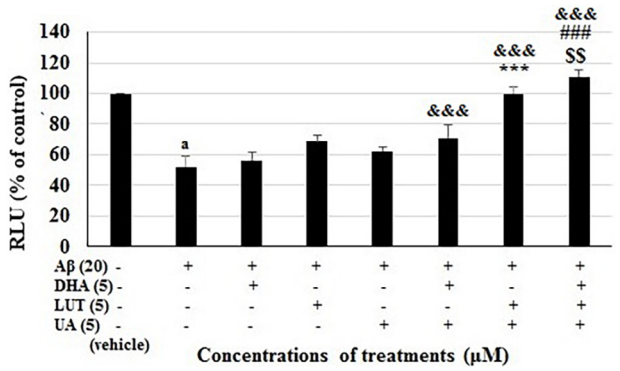


FIGURE 6 | Comparison of the two-compound combinations D_5U_5, L_5U_5 and the three-compound combination $D_5L_5U_5$. ATP levels for the D_5U_5, L_5U_5, $D_5L_5U_5$ and their single components were determined using CellTiter Glo assay. Data are expressed as mean ± SD from four ($N = 4$) independent experiments. Differences are significant at $^{a}P < 0.001$ vs. vehicle control, $^{\&\&\&}P < 0.001$ vs. $A\beta_{1-42}$-treated control, $^{***}P < 0.001$, $^{\#\#\#}P < 0.001$ vs. D_5U_5 and $^{\$\$}P = 0.001$ vs. L_5U_5.

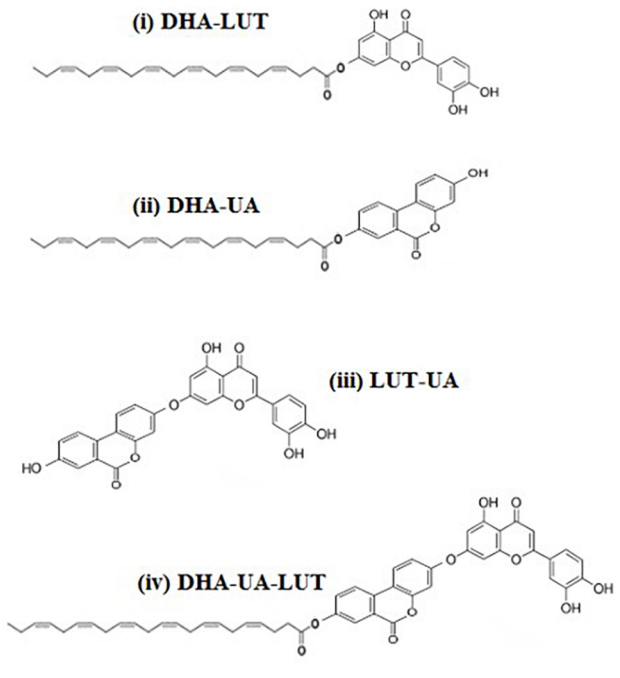


FIGURE 7 | Predicted chemical conjugations for the three-compound combination, $D_5L_5U_5$. For the DHA-LUT-UA combination, four chemical conjugations are predicted as (i) DHA-LUT (ii) DHA-UA (iii) LUT-UA (iv) DHA-UA-LUT.

determine cell survival through the opening of the mPTP, which occurs under conditions of cell stress, causing mitochondrial depolarization and triggering of cell death as well as mitophagy. It has recently been found that dietary supplementation with a mixture of DHA and EPA (70:30 ratio) increased DHA and EPA in cardiac mitochondrial phospholipids and the tolerance of isolated mitochondria to Ca^{2+}-induced mPTP opening (O'Shea et al., 2009). Moreover, it has been shown that supplementation with DHA *per se* also delayed Ca^{2+}-induced mPTP opening (Khairallah et al., 2010). Luteolin, the second component of $D_5L_5U_5$, is also widely appreciated in the literature for its mitoprotective activities. It has been shown to ameliorate mitochondrial damage in isoproterenol-induced myocardial infarction by maintaining lipid peroxidation metabolism due to its free radical scavenging, mitochondrial lipids, antioxidants and mitochondrial enzymes (Murugesan and Manju, 2013). It is thought to associate with up-regulation of autophagy (Hu et al., 2016; Cao et al., 2017) and improvement of mitochondrial biogenesis through inhibition of macrophage stimulating 1 protein (Hu et al., 2016). Interestingly, a growing body of evidence suggests that UA restores mitochondrial dysfunction by inducing mitophagy (Ryu et al., 2016; Andreux et al., 2019; Lin et al., 2020). Overall, further *in vitro* studies are warranted to identify the mechanisms of action of the synergistic three-compound nutraceutical combination for may be a steppingstone toward developing an advanced functional food for the prevention or co-treatment of AD.

CONCLUSION

The present study identified a synergistic three-compound combination, $D_5L_5U_5$ that inhibits $A\beta_{1-42}$-induced toxicity *in vitro*. This compound combination consisted of nutraceuticals: DHA, luteolin and Urolithin A each in 5 μM concentration, and Chou-Talalay method of drug combinations was used to derive it. Further *in vitro* and *in vivo* investigations are required to determine the mechanisms of action and validate this synergistic three-compound combination in the journey toward identifying an advanced functional food for the prevention or co-treatment of 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/s.

AUTHOR CONTRIBUTIONS

RM and DJ designed the study. DJ carried out all experiments and wrote the manuscript. EH closely supervised the experiments. RM, EH, GV, MG, and WF reviewed the manuscript intensively. EH, RM, MG, and WF edited the manuscript. All authors have read and agreed to the final version of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2022.780602/full#supplementary-material

Supplementary Figure 1 | Relative ATP levels for the combination 1 (D_5U_{10}) and its components. ATP levels for the combination 1 (D_5U_{10}) and its components were determined using the CellTiter Glo assay. Data are expressed as mean ± SD from four (N = 4) independent experiments. Differences are significant at $^{a}P < 0.001$ vs. vehicle control, $^{***}P < 0.001$ vs. $A\beta_{1-42}$-treated control, $^{\#}P < 0.05$ vs. DHA 5 μM and UA 5 μM.

Supplementary Figure 2 | Relative ATP levels for the combination 1 ($D_{10}U_{10}$) and its components. ATP levels for the combination 3 ($D_{10}U_{10}$) and its constituents were determined using CellTiter Glo assay. Data are expressed as mean ± SD from four (N = 4) independent experiments. Differences are significant at $^{a}P < 0.001$ vs. vehicle-treated control, $^{***}P < 0.001$ vs. $A\beta_{1-42}$-treated control, DHA 5 μM and UA 5 μM.

Supplementary Figure 3 | Relative ATP levels for the combination 1 ($D_{10}U_5$) and its components. ATP levels for the combination 3 ($D_{10}U_5$) and its constituents were determined using CellTiter Glo assay. Data are expressed as mean ± SD from four (N = 4) independent experiments. Differences are significant at $^{a}P < 0.001$ vs. vehicle control, $^{***}P < 0.001$ vs. $A\beta_{1-42}$-treated control, DHA 10 μM and UA 5 μM.

Supplementary Figure 4 | Relative ATP levels for the combination 1 ($D_{20}L_5$) and its components. ATP levels for the combination 4 ($D_{20}L_5$) and its constituents were determined using CellTiter Glo assay. Data are expressed as mean ± SD from four (N = 4) independent experiments. Differences are significant at $^{a}P < 0.001$ vs. vehicle-treated control, $^{***}P < 0.001$ vs. $A\beta_{1-42}$-treated control, DHA 10 μM.

Supplementary Figure 5 | Relative ATP levels for the combination 1 ($D_{10}L_{20}$) and its components. ATP levels for the combination 6 ($D_{10}U_5$) and its constituents were determined using CellTiter Glo assay. Data are expressed as mean ± SD from four (N = 4) independent experiments. Differences are significant at $^{a}P < 0.001$ vs. vehicle-treated control, $^{***}P < 0.001$ vs. $A\beta_{1-42}$-treated control, DHA 10 μM and UA 5 μM.

Supplementary Figure 6 | Relative ATP levels for the combination 1 (D_5L_{10}) and its components. ATP levels for the combination 6 (D_5L_{10}) and its constituents were determined using CellTiter Glo assay. Data are expressed as mean ± SD from four (N = 4) independent experiments. Differences are significant at $^{a}P < 0.001$ vs. vehicle control, $^{***}P < 0.001$ vs. $A\beta_{1-42}$-treated control, DHA 5 μM.

Supplementary Figure 7 | Relative ATP levels for the combination 7 ($D_{10}L_{10}$) and its components. ATP levels for the combination 7 ($D_{10}L_{10}$) and its constituents were determined using CellTiter Glo assay. Data are expressed as mean ± SD from four (N = 4) independent experiments. Differences are significant at $^{a}P < 0.001$ vs. vehicle control, $^{***}P < 0.001$ vs $A\beta_{1-42}$-treated control, DHA 10 μM.

Supplementary Figure 8 | Relative ATP levels for the combination 9 ($D_{20}L_{10}$) and its components. ATP levels for the combination 9 ($D_{20}L_{10}$) and its constituents were determined using CellTiter Glo assay. Data are expressed as mean ± SD from four (N = 4) independent experiments. Differences are significant at $^{a}P < 0.001$ vs. vehicle control, $^{***}P < 0.001$ vs. $A\beta_{1-42}$-treated control, DHA 20 μM.

Supplementary Figure 9 | Relative ATP levels for the combination 10 ($D_{10}L_5$) and its components. ATP levels for the combination 10 ($D_{10}L_5$) and its constituents were determined using CellTiter Glo assay. Data are expressed as mean ± SD from four (N = 4) independent experiments. Differences are significant at ^{a}P < 0.001 vs vehicle control, $^{***}P < 0.001$ vs. $A\beta_{1-42}$-treated control, DHA 10 μM.

Supplementary Figure 10 | Relative ATP levels for the combination 12 ($L_{20}U_5$) and its components. ATP levels for the combination 12 ($L_{20}U_5$) and its constituents were determined using CellTiter Glo assay. Data are expressed as mean ± SD from four (N = 4) independent experiments. Differences are significant at $^{a}P < 0.001$ vs. vehicle control, $^{***}P < 0.001$ vs. $A\beta_{1-42}$-treated control, LUT 20 μM and UA 5 μM.

Supplementary Figure 11 | Relative ATP levels for the combination 13 ($L_{10}U_5$) and its components. ATP levels for the combination 13 ($L_{10}U_5$) and its constituents were determined using CellTiter Glo assay. Data are expressed as mean ± SD from four (N = 4) independent experiments. Differences are significant at ^{a}P < 0.001 vs. vehicle control, $^{***}P < 0.001$ vs. $A\beta_{1-42}$-treated control, LUT 10 μM and UA 5 μM.

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Mitoprotective Effects of a Synergistic Nutraceutical Combination: Basis for a Prevention Strategy Against Alzheimer's Disease

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Evidence to date suggests the consumption of food rich in bioactive compounds, such as polyphenols, flavonoids, omega-3 fatty acids may potentially minimize age-related cognitive decline. For neurodegenerative diseases, such as Alzheimer's disease (AD), which do not yet have definitive treatments, the focus has shifted toward using alternative approaches, including prevention strategies rather than disease reversal. In this aspect, certain nutraceuticals have become promising compounds due to their neuroprotective properties. Moreover, the multifaceted AD pathophysiology encourages the use of multiple bioactive components that may be synergistic in their protective roles when combined. The objective of the present study was to determine mechanisms of action underlying the inhibition of $A\beta_{1-42}$-induced toxicity by a previously determined, three-compound nutraceutical combination $D_5L_5U_5$ for AD. *In vitro* experiments were carried out in human neuroblastoma BE(2)-M17 cells for levels of ROS, ATP mitophagy, and mitobiogenesis. The component compounds luteolin (LUT), DHA, and urolithin A (UA) were independently protective of mitochondria; however, the $D_5L_5U_5$ preceded its single constituents in all assays used. Overall, it indicated that $D_5L_5U_5$ had potent inhibitory effects against $A\beta_{1-42}$-induced toxicity through protecting mitochondria. These mitoprotective activities included minimizing oxidative stress, increasing ATP and inducing mitophagy and mitobiogenesis. However, this synergistic nutraceutical combination warrants further investigations in other *in vitro* and *in vivo* AD models to confirm its potential to be used as a preventative therapy for AD.

Keywords: mitobiogenesis, mitochondrial dysfunction, mitophagy, reactive oxygen species, synergistic nutraceutical combination, Alzheimer's disease

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia that constitutes approximately 60–80% of clinically diagnosed dementia cases, worldwide (Alzheimer's Association, 2020). The need for an effective treatment for AD is critical, especially considering the rapidly growing number of people living with the disease and the associated socioeconomic impact (Alzheimer's Association, 2020). However, there is currently limited success in clinical trials, with most ending in failure (Oxford et al., 2020). Most of the criticisms are attributed to the targeting of the clinical phenotype, where there is existing irreversible synaptic and neuronal degeneration and profound inflammatory changes (Huang et al., 2020). Other than the controversies related to very recently approved drug Aducanumab (Selkoe, 2021), the current situation where there is still no AD-targeted-therapy pending approval soon emphasizes the need for identification of AD preventive strategies. This approach targeting prevention is the most appealing, considering the long prodromal phase of AD, that can span more than 10 to 20 years before clinical signs of AD appear (Villemagne et al., 2013). As illustrated by Hodes et al. (2019), there is an ongoing debate over the use of the terms "prevention" and "risk reduction" in AD (Hodes et al., 2019). However, in either case of preventing or delaying the related cognitive decline, AD onset and progression requires a clinical intervention and alternative, preferably non-pharmacological, options have become increasingly attractive.

Lifestyle is thought to be one of the key modifiable risk factors for AD, with diet playing a major role (Hu et al., 2013). Thus, diet is important as a potential target for the development of preventive strategies for AD (Dadhania et al., 2016). The bioactive components of diets, the nutraceuticals (Kalra, 2003), are being investigated for their capabilities in preventing AD onset and progression (Farías et al., 2014). Accumulating evidence suggests that nutraceuticals, such as polyphenols and specific fatty acids found in fruits, vegetables, herbs, and nuts may slow neurodegeneration and improve memory and cognitive function (Joseph et al., 2009; Mecocci et al., 2014; Keservani et al., 2016). Furthermore, nutraceutical combinations can be the most effective, as they may act synergistically against the multiple aspects of AD pathogenesis. In general, the different phytochemicals in a nutraceutical combination can activate different cellular pathways by improving the bioavailability of active compounds. Thus, an advantage of a synergistic nutraceutical combinations is the possibility to reduce doses of individual compounds resulting in toxic effects at minimum (Santana-Gálvez et al., 2019). Therefore, synergistic nutraceutical combinations can be used to formulate novel and effective functional food products with anti-AD properties.

Recently, we identified a three-compound synergistic nutraceutical combination $D_5L_5U_5$ that exhibited synergistic activity against $A\beta_{1-42}$-induced toxicity in cell culture (Jayatunga et al., 2021). It consisted of three nutraceutical compounds docosahexaenoic acid (DHA), luteolin (LUT), and urolithin A (UA) each at 5 μM concentration. These constituents were considered in deciphering a probable mechanism of action for this nutraceutical combination. Docosahexaenoic acid is reported to modulate mitochondrial biogenesis, which is partially associated with increased mtDNA replication and PGC-1α gene expression in C2C12 muscle cells (Lee et al., 2016) and in C57BL/6J mice (Flachs et al., 2005). There is evidence that DHA promotes apoptosis and autophagy, however, there are no reports to date on its effect on mitophagy, which is the selective autophagy of superfluous mitochondria (Lemasters, 2005; Kubli and Gustafsson, 2012). The protective effects of LUT are associated with up-regulation of autophagy and improvement of mitochondrial biogenesis through Mst1 inhibition (Hu et al., 2016). Furthermore, LUT has exerted neuroprotection by enhancing autophagy and anti-oxidative processes in both *in vivo and in vitro* models of intracerebral hemorrhage (Tan et al., 2020). Luteolin has been recently reported to induce mitochondrial apoptosis in HT29 cells by inhibiting the Nrf2/ARE signaling pathway (Yang et al., 2020). Furthermore, LUT has protected endothelial cells against H_2O_2-induced oxidative stress via modulating ROS-mediated P38 MAPK/NF-κB and calcium-evoked mitochondrial apoptotic signaling pathways (Chen et al., 2020). However, evidence is scarce that LUT improves mitochondrial dynamics and mitophagy. It is increasingly identified that UA restores mitochondrial dysfunction in the aging skeletal muscle through up-regulation of genes associated with mitochondrial biogenesis, mitophagy and fatty acid oxidation (Yang, 2019). Recently, it has been proven in C57BL/6 male mice fed on high fat diet that UA resulted in elevated mitochondrial biogenesis in the liver (Toney et al., 2019) along with inducing mitophagy (Ryu et al., 2016). One study reported that mitophagy is not induced by UA in both ischemia/reperfusion−injured neuronal cells and mice (Ahsan et al., 2019). Therefore, further studies are still needed to confirm the mitophagy-inducing potential of UA. Taken collectively, these previous reports on mitoprotective effects of DHA, LUT, and UA provide further motivation to investigate if the beneficial effects still exist when the same are combined in low concentrations.

The present study was undertaken to evaluate the *in vitro* pathways $D_5L_5U_5$ take to protect against $A\beta_{1-42}$-induced toxicity. For comparison, the components of this combination, DHA, LUT, and UA were also assessed for their ability to protect from $A\beta_{1-42}$-induced toxicity.

MATERIALS AND METHODS

Materials

cis-4,7,10,13,16,19-Docosahexaenoic acid (DHA: D2534), LUT (L9283), UA (SML1791), N-acetyl L-Cysteine (NAC: A9165), Resveratrol (RES), Bafilomycin A (Baf A: B1793), sodium docecyl sulfate (SDS), Triton X-100, and dimethyl sulfoxide (DMSO) were obtained from Sigma Aldrich, United States. BE(2)-M17 cells (ATCC® CRL2267™) were purchased from American Type Cell Culture Collection (ATCC, Manassas, VA, United States). All cell culture reagents including Dulbecco's Modified Eagle Medium (DMEM), Ham's F12 medium, Hank's balanced salt solution (HBSS), fetal calf serum (FCS) and Trypsin-EDTA (0.5%) were purchased from GIBCO by Life

Technologies (United States). Human $A\beta_{1-42}$ peptides were synthesized, purified and characterized by high pressure liquid chromatography (HPLC) and mass spectrometry (MS) by The ERI Amyloid Laboratories LLC, United States. Anhydrous DMSO was purchased from Molecular Probes by Life Technologies (United States). CellTiter-Glo luminescent cell viability assays were purchased from Promega, Wisconsin, United States and 2′,7′-dichlorofluorescin diacetate (DCFDA) intracellular ROS assay kits (ab113851) were obtained from Abcam, Cambridge, United Kingdom. Bolt 4–12% Bis-Tris plus gels, gel running MES [2-(N-morpholino) ethanesulfonic acid] buffer, 4X Bolt Lithium dodecyl sulfate (LDS) sample buffer NuPAGE, 10X Bolt sample reducing agents, Novex Sharp pre-stained protein standards were purchased from Life Technologies, United States. Primary antibodies, p62, PINK1, TOMM20, TIMM23, FIS1, and PGC-1α and goat anti-rabbit Alexa Fluor488 secondary antibody were obtained from Abcam, Cambridge, United Kingdom. Primary antibodies, LC3B, MFN2, NDP52, OPTN and OPA1 were obtained from Cell Signaling. For Western blotting, trans blot turbo mini nitrocellulose transfer packs were purchased from Biorad, United States. Secondary antibodies, anti-mouse and anti-rabbit immunoglobulin G (IgG) horse radish peroxidase (HRP) linked whole antibodies and enhanced chemiluminescence (ECL) kits were obtained from GE Healthcare, United Kingdom.

Cell Culture

Human neuroblastoma BE(2)-M17 cells were maintained in T75 culture flasks containing 15 mL of DMEM/F12 (1:1 ratio) growth media supplemented with 10% (v/v) FCS and placed in a humidified incubator with 5% CO_2/95% air at 37°C. Upon reaching about 80% confluency, the cells were sub-cultured on to fresh cell culture flasks. For all cell culture experiments, passage number did not exceed 30.

$A\beta_{1-42}$ Peptide Preparation

Oligomeric $A\beta_{1-42}$ was prepared according to the method of Stine et al. (2011) with some modifications. Briefly, synthetic human $A\beta_{1-42}$ peptide of 4.5 mg was dissolved in 1000 μL HFIP solution and incubated for 30 minutes at room temperature (RT). The $A\beta_{1-42}$ dissolved HFIP solution was aliquoted using positive displacement pipetting, into 10 microcentrifuge tubes each containing 0.45 mg $A\beta_{1-42}$ peptide. The aliquots were then air-dried in a fume cupboard overnight at RT. The tubes containing the peptide films were further dried in a Savant vacuum concentrator (–80 °C). The $A\beta_{1-42}$ films were then stored at –30 °C in a sealed container with desiccant. All $A\beta_{1-42}$ peptide solutions were freshly prepared for each experiment. The 0.45 mg peptide film in the tube was dissolved in 20 μL of anhydrous DMSO (Molecular probes). The solution was briefly vortexed, then sonicated in a water bath for 10 minutes with temperature controlled by adding ice so as not to exceed RT. Then, 980 μL of ice-cold F12 media (without phenol red) was added. The solution was then briefly vortexed and incubated for 24 hours at 4 °C to be used in $A\beta_{1-42}$ toxicity experiments the next day.

Treatments

In all treatments, the cells were pre-treated with the compound combination $D_5L_5U_5$ (DHA 5 μM, LUT 5 μM, and UA 5 μM) and the individual compounds, DHA (30 μM), LUT (20 μM), and UA (30 μM) for 24 h. These concentrations of DHA, LUT, and UA were selected based on our previous studies (Jayatunga et al., 2021). Resveratrol (RES) was used as a positive control in all experiments as it is a known agent of inducing mitochondrial dynamics, mitophagy and mitobiogenesis. N-acetyl cysteine (NAC) is an antioxidant that was used as a reference compound for ROS analysis. The concentrations of RES and NAC used in the experiments were based on the available literature (Mitsopoulos and Suntres, 2011; Gueguen et al., 2015).

In all experiments, after pre-treatment with the compounds was followed by 20 μM oligomeric $A\beta_{1-42}$ treatment for 16 h unless otherwise specified. This time period was selected based on our previous findings that mitophagy is diminished after 16 h of 20 μM $A\beta_{1-42}$ treatment in BE(2)-M17 cells (Jayatunga et al., unpublished). All treatments were carried out with appropriate $A\beta_{1-42}$–treated and vehicle-treated controls.

DCFDA Assay

The BE(2) M17 cells seeded in 96 well plates at a density of 25×10^3 cells/well. After 24 h, the cells were pre-treated with the compounds for 24 h. The cells were then exposed to 20 μM $A\beta_{1-42}$ for 4 h in the incubator at 37°C and intracellular ROS levels were determined using the DCFDA assay. $A\beta_{1-42}$ incubation of 4 h was based on our previous work (Jayatunga et al., unpublished). Briefly, 45 min prior to completion of the treatment, the cells in color-free treatment media (100 μL per well) were overlaid with 100 μL of warm 50 μM DCFDA solution [diluted from the DCFDA stock (20 mM) using 1X wash buffer]. The microplate was incubated for 45 min at 37°C and fluorescence readings (Relative Fluorescence Units: RFU at excitation 485 nm/emission 535 nm) were obtained at excitation 485 and 535 nm using a Perkin Elmer EnSpire multi-mode plate reader.

CellTiter Glo Assay

The BE(2)-M17 cells were seeded in 96-well plates at a density of 15×10^3 cells/well. After 24 h, the cells were pre-treated with the compounds for 24 h. After exposure of 20 μM $A\beta_{1-42}$ for 4, 8, 16, 24, 48, and 72 h, the CellTiter Glo assay was used to determine relative ATP levels. Briefly, cells were placed at RT for 30 min and then lysed by adding 100 μL of ATP-releasing reagent. The lysates were incubated with the luciferin substrate and luciferase enzyme in the dark for 10 min to stabilize the luminescence signal. The luminescence (RLU) was measured using a Perkin Elmer EnSpire multi-mode plate reader.

Western Blot Analyses

The BE(2)-M17 cells were seeded in T25 cell culture flasks at a density of 1×10^6 cells per flask and grown for 24 h. Test samples were prepared by compound pre-treatments followed by 20 μM oligomeric $A\beta_{1-42}$ treatments for 16 h. Additionally, for p62, NDP52 and OPTN, Bafilomycin A (Baf A) was added to

the cell flasks after 15 h, i.e., 1 h prior to reaction completion. The cells were lysed in precooled lysis buffer (SDS: Triton X-100, 7:3, protease inhibitor, PBS) and protein concentrations were determined using a BCA protein assay kit. Protein samples were separated on 4–12% Bis-Tris gels by polyacrylamide gels electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. Then, membranes were blocked in 5% non-fat milk for 30 min at room temperature and incubated with primary antibodies (rabbit monoclonal MFN2 antibody; 1:1,000, rabbit monoclonal OPA1 antibody; 1:2,000, rabbit monoclonal FIS1 antibody; 1:5,000, mouse monoclonal p62 antibody; 1:2,000, rabbit polyclonal LC3B antibody; 1:1,000, rabbit polyclonal PINK1 antibody; 1:1,000, rabbit monoclonal NDP52 antibody; 1:1,000, rabbit monoclonal OPTN antibody; 1:500, rabbit polyclonal TIMM23 antibody; 1:200, mouse monoclonal PGC-1α antibody; 1:1,000) overnight at 4°C. Membranes were then washed and incubated with HRP-conjugated goat anti-rabbit and HRP-conjugated goat anti-mouse (1:5,000) secondary antibodies for 1 h at room temperature followed by development using ECL detection. The protein bands were normalized against GAPDH, using ImageLab program (Biorad; v. 6.0.1).

Statistical Analysis

All results were expressed as mean ± standard deviation (SD) from four ($N = 4$) independent experiments. Statistical significance was determined by one-way ANOVA and Tukey's *post hoc* test in SPSS v25. Significance was defined as $P < 0.05$.

RESULTS

$D_5L_5U_5$, DHA, Luteolin, and Urolithin A Reduce $A\beta_{1-42}$-Induced ROS Levels

In the DCFDA cellular ROS assay, the ROS level of vehicle control was considered as 100%. The treatment of 20 μM $A\beta_{1-42}$ significantly increased ROS levels at 4 h to 124.8 ± 4.0% ($P < 0.05$), however, $D_5L_5U_5$, DHA, LUT, and UA significantly decreased ROS levels to 101.6 ± 6.6% ($P < 0.001$), 95.7 ± 4.1% ($P < 0.001$) and 111.7 ± 5.9% ($P < 0.05$), respectively (**Figure 1**). Surprisingly however, RES resulted in further increased ROS level of 159.8 ± 1.4% ($P < 0.001$). As expected, NAC significantly reduced the ROS levels to 58.4 ± 6.4% ($P < 0.001$), while TBHP

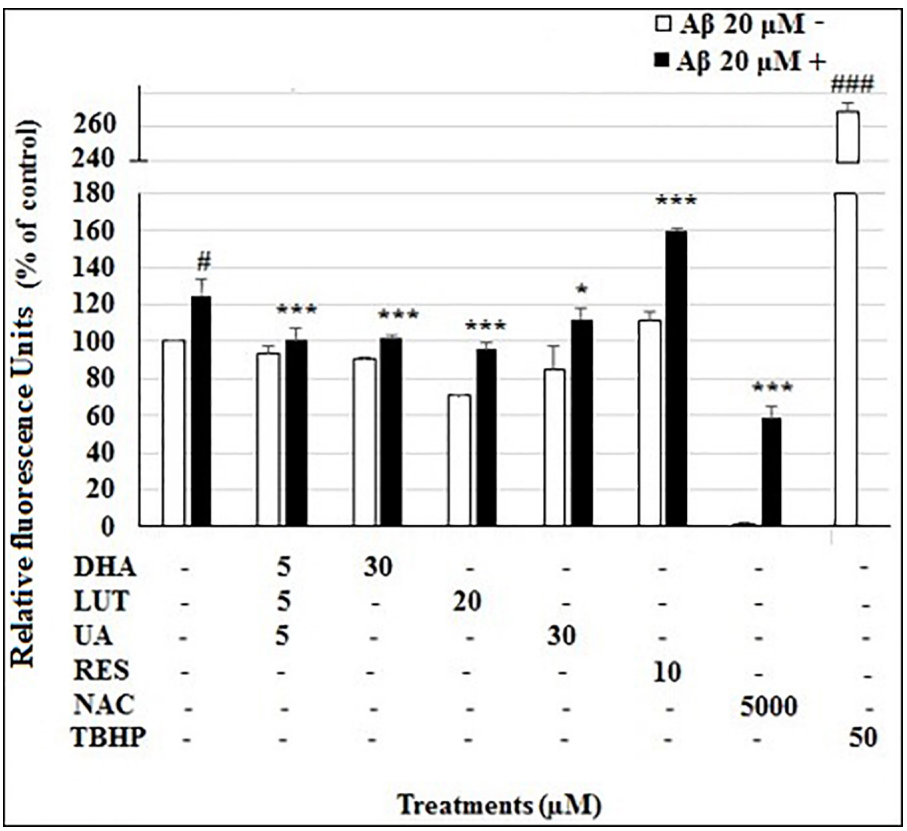


FIGURE 1 | The effect of the three-compound combination and the optimal concentrations of its components against $A\beta_{1-42}$-induced ROS levels. The BE(2)-M17 cells were pre-treated for 24 h with $D_5L_5U_5$, LUT, DHA, and UA. Resveratrol (RES) was used at a concentration of 10 μM. Tertbutyl hydrogen peroxide (TBHP) 50 μM and NAC 5 mM were used as positive and negative controls for the assay. The cells were then exposed to 20 μM of oligomeric $A\beta_{1-42}$ for 4 h to induce ROS production. Black bars indicate the ROS production in the presence of 20 μM $A\beta_{1-42}$ while the white bars indicate the ROS production in the presence of 20 μM $A\beta_{1-42}$. Cellular ROS levels were evaluated in relative fluorescence units (RFU) using the DCFDA assay. Data are expressed as mean ± SD from four ($N = 4$) independent experiments. Differences are significant at $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. vehicle control. $^{*}P < 0.05$, $^{***}P < 0.001$vs. $A\beta_{1-42}$-treated control.

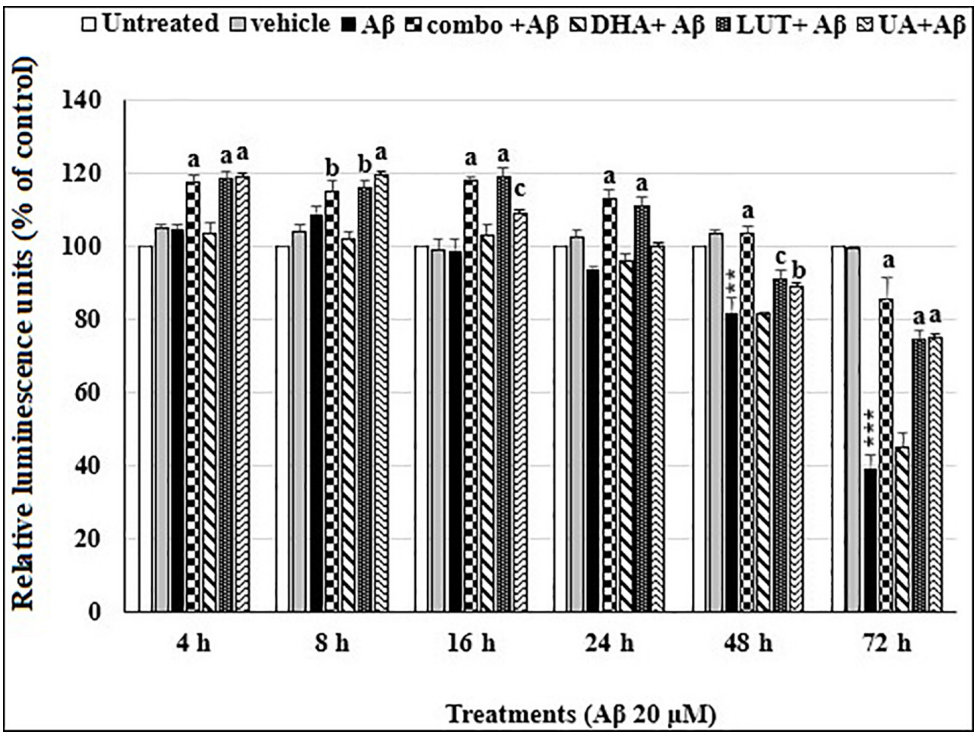


FIGURE 2 | The time-dependent effects of the three-compound combination and its components on oligomeric $A\beta_{1-42}$-affected ATP levels. The BE(2)-M17 cells were pre-treated for 24 h with $D_5L_5U_5$, LUT, DHA, and UA. Exposure of 20 μM oligomeric $A\beta_{1-42}$ was carried out for different time points (4–72 h) at 37∘C and relative ATP levels were detected as relative luminescence units (RLU) using CellTiter Glo assay. Data are expressed as mean ± SD from four ($N = 4$) independent experiments. Differences are significant at $^{**}P = 0.001$, $^{***}P < 0.001$ vs. untreated, $^{b}P < 0.05$, $^{c}P = 0.001$, $^{a}P < 0.001$ vs. $A\beta_{1-42}$-treated controls.

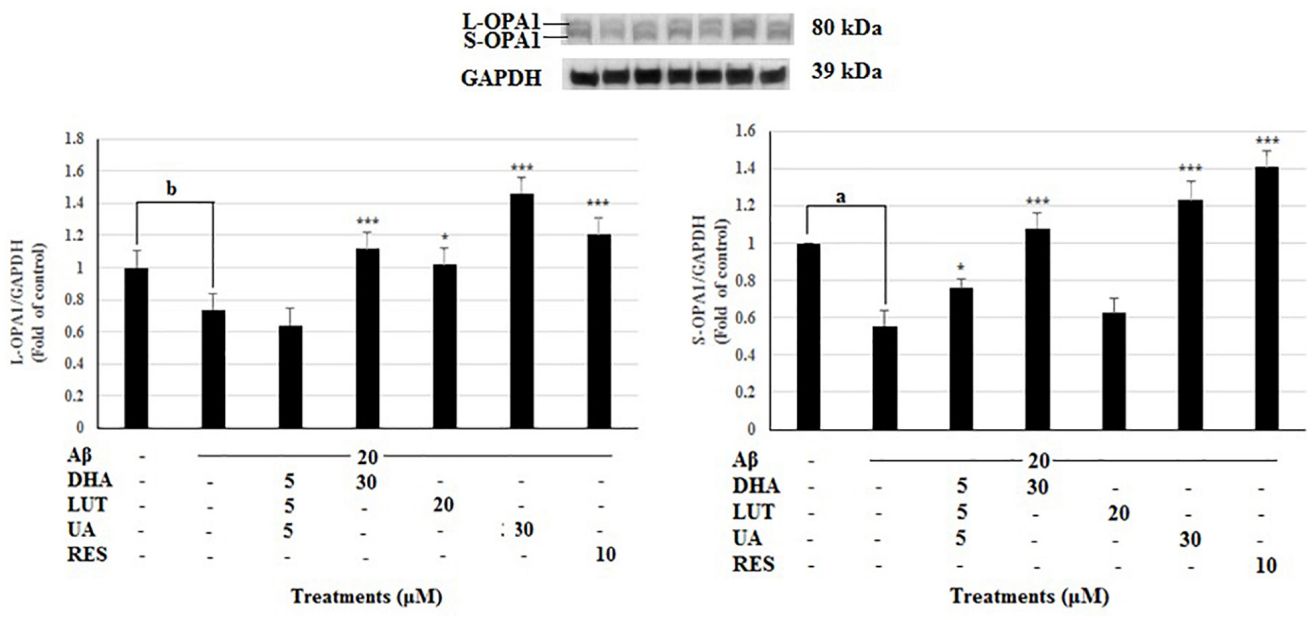


FIGURE 3 | The effect of the three-compound combination and its components on the levels of L-OPA1 and S-OPA1. The BE(2)-M17 cells were pre-treated for 24 h with the three-compound combination, $D_5L_5U_5$, DHA (30 μM), LUT (20 μM), UA (30 μM), and RES (10 μM). The cells were then exposed to 20 μM of oligomeric $A\beta_{1-42}$ for 16 h and subjected to Western blotting. The representative immunoblot and the graph indicate the levels of L-OPA1/S-OPA1 proteins for all treatments. The relative density of the bands was normalized against GAPDH. Fold values were calculated relative to vehicle control. Data are expressed as mean ± SD from four ($N = 4$) independent experiments. Differences are significant at $^{b}P < 0.05$ vs. vehicle control, $^{*}P < 0.05$, $^{***}P < 0.001$, $^{a}P < 0.001$ vs. $A\beta_{1-42}$-treated controls.

per se increased the ROS levels to 268.5 ± 10.6%, compared to vehicle control ($P < 0.001$).

$D_5L_5U_5$, Luteolin, and Urolithin A Restores $A\beta_{1-42}$-Induced Reduction of ATP Levels

As shown in **Figure 2**, the treatment of 20 μM $A\beta_{1-42}$ time-dependently decreased cellular ATP levels in cells at 48 h ($P = 0.001$) and 72 h ($P < 0.001$). However, pre-treatment with $D_5L_5U_5$, LUT, and UA caused increased ATP levels at each time point while DHA did not alter the decreased ATP levels. These results indicated that $D_5L_5U_5$, LUT, and UA increase the ATP levels of cells exposed to $A\beta_{1-42}$ (**Figure 2**).

$D_5L_5U_5$ Increases Mitochondrial Fusion Protein S-OPA1 and Decreases Fission Protein FIS1

Mitochondrial fusion proteins, OPA1 and MFN2 and the mitochondrial fission proteins FIS1 were evaluated in analyzing mitochondrial dynamics. The 20 μM $A\beta_{1-42}$ treatment significantly decreased L-OPA1 and S-OPA1 levels ($P < 0.05$ and $P < 0.001$, respectively). However, pre-treatments with DHA, LUT, UA and RES increased L-OPA1 levels ($P < 0.001$, $P < 0.05$, $P < 0.001$, and $P < 0.001$) while $D_5L_5U_5$, DHA, UA, and RES increased S-OPA1 levels ($P < 0.05$, $P < 0.001$, $P < 0.001$, and $P < 0.001$) (**Figure 3**). These results indicate that $D_5L_5U_5$, DHA, LUT, UA, and RES all increase OPA1 levels in general.

Treatment of 20 μM $A\beta_{1-42}$ markedly decreased the MFN2 levels ($P < 0.001$) indicating that $A\beta_{1-42}$ treatment inhibits the mitochondrial fusion process at 16 h, however, pre-treating with DHA and RES increased the MFN2 levels ($P < 0.001$, $P < 0.05$) while pre-treatment with $D_5L_5U_5$ did not give any change (**Figure 4**).

The treatment of 20 μM $A\beta_{1-42}$ caused a reduced trend in the FIS1 levels which is indicative of reduced mitochondrial fission process. However, all the compound treatments failed to restore the FIS1 levels, instead, $D_5L_5U_5$, and DHA resulted in significantly reduced FIS1 levels ($P < 0.001$ and $P < 0.05$) (**Figure 5**).

Urolithin A, Luteolin, and DHA Induces Mitophagy and $D_5L_5U_5$ Increases the Mitophagy Protein NDP52 but Not the Mitophagic Flux

The present study evaluated the impact of the test compounds on mitophagy to assess their activity on protecting against the $A\beta_{1-42}$-induced effect on mitophagy. To achieve this, the expression levels of a panel of mitophagy-related proteins including LC3-I/II, p62, NDP52, OPTN, PINK1, and IMM protein TIMM23 was assessed. For p62, NDP52, OPTN, additional treatments were carried out with Bafilomycin A (Baf A)-mediated lysosomal blockade to examine the transit of the mitophagy adaptors through the autophagy pathway. Under lysosomal blockade, it has been reported by Klionsky et al. (2016), that if a lysosomal blockade of a certain treatment (with Baf A) results in increased level of a degradable autophagosomal protein (e.g., p62, NDP52, OPTN) compared to that of the treatment and the Baf A-only group, it can be considered that the selective treatment enhances autophagic flux (Klionsky et al., 2016). Similarly, if the relevant protein level of lysosomal blockade of a certain treatment is higher than that of the Baf A-only group, it can be deemed that the treatment increases synthesis of autophagy-related membranes. On the other hand, if the treatment results in lower levels of the relevant protein compared to its lysosomal blockade (with Baf A), the treatment is considered to induce a partial block in autophagic flux. If these two entities are similar, a complete blockade of autophagy at the terminal stages is expected (Klionsky et al., 2016). These factors were made use in inferring mitophagy in the present study. However, consistent results could not be obtained for LC3I/II blots.

As shown in **Figure 6**, the insult of 20 μM $A\beta_{1-42}$ for 16 h significantly decreased p62 levels ($P < 0.05$), representing a decrease in mitophagy (Klionsky et al., 2016). Furthermore, decrease of intensity in Baf A-treated $A\beta_{1-42}$ control relative to Baf A-treated vehicle ($P < 0.001$) further confirmed that the exposure of 20 μM $A\beta_{1-42}$ caused the increased synthesis of autophagy-related membranes. Pre-treatments with DHA, LUT, UA, and RES significantly increased p62 levels ($P = 0.001$, $P < 0.001$, $P = 0.001$ and $P < 0.001$)

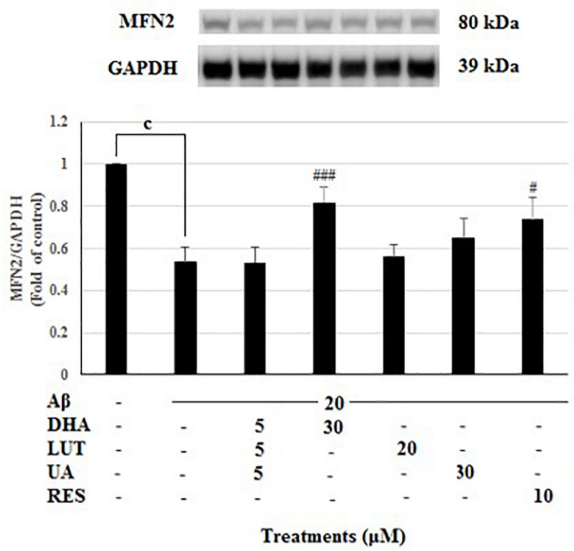


FIGURE 4 | The effect of three-compound combination and its components on the levels of MFN2. The BE(2)-M17 cells were pre-treated for 24 h with the three-compound combination, $D_5L_5U_5$, DHA (30 μM), LUT (20 μM), UA (30 μM), and RES (10 μM). The cells were then exposed to 20 μM of oligomeric $A\beta_{1-42}$ for 16 h and subjected to Western blotting. The representative immunoblot and the graph indicate the levels of MFN2 protein for all treatments. The relative density of the bands was normalized against GAPDH. Fold values were calculated relative to vehicle control. Data are expressed as mean ± SD from four ($N = 4$) independent experiments. Differences are significant at $^{c}P < 0.001$, $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. $A\beta_{1-42}$-treated control.

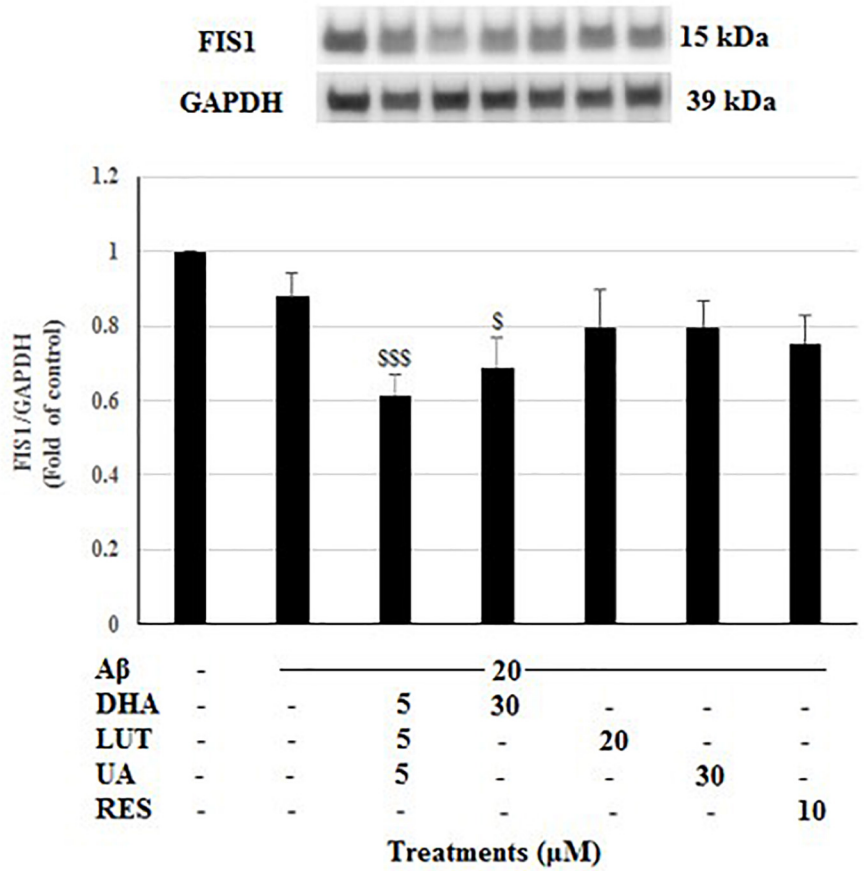


FIGURE 5 | The effect of three-compound combination and its components on the levels of FIS1. The BE(2)-M17 cells were pre-treated for 24 h with the three-compound combination, $D_5L_5U_5$, DHA (30 μM), LUT (20 μM), UA (30 μM), and RES (10 μM). The cells were then exposed to 20 μM of oligomeric $A\beta_{1-42}$ for 16 h and subjected to Western blotting. The representative immunoblot and the graph indicate the levels of FIS1 protein for all treatments. The relative density of the bands was normalized against GAPDH. Fold values were calculated relative to vehicle control. Data are expressed as mean ± SD from four ($N = 4$) independent experiments. Differences are significant at $^{\$}P < 0.05$, $^{\$\$\$}P < 0.001$ vs. $A\beta_{1-42}$-treated controls.

suggesting the induction of mitophagy. Similarly, considering the lysosomal blockade by Baf-A, p62 levels were increased by DHA, UA, and RES ($P < 0.001$ each) relative to Baf A-treated $A\beta_{1-42}$ treatment, suggesting that pre-treatment with these compounds increased the synthesis of autophagy-related membranes (Klionsky et al., 2016).

The 20 μM $A\beta_{1-42}$ treatment for 16 h significantly decreased the levels of mitophagy adaptor protein NDP52 ($P < 0.001$) indicating diminished mitophagy. However, pre-treatments with $D_5L_5U_5$, DHA, and LUT significantly increased NDP52 levels ($P < 0.001$ each) (**Figure 7**) reflecting increased mitophagy by these compounds. However, for $D_5L_5U_5$, DHA, and LUT, the NDP52 levels with the lysosomal blockade (with Baf A-treatment) were not significantly increased relative to Baf A-untreated for $D_5L_5U_5$, DHA and LUT suggesting a possible block in autophagy/mitophagy at the terminal stages. On the other hand, the snapshot NDP52 levels for UA showing a statistically significant increase and lysosomal blockade for UA (Baf A-treated UA) resulting in higher NDP52 levels suggest that mitophagic flux is increased for UA. On the other hand, NDP52 level for RES resulting in a comparable level to that of lysosomal blockade (Baf A-treated RES) is indicative of complete block in mitophagy at the terminal stages.

The treatment of 20 μM $A\beta_{1-42}$ for 16 h significantly decreased OPTN levels ($P < 0.001$) indicating decreased mitophagy. Furthermore, decreasing of $A\beta_{1-42}$ control with lysosomal blockade (Baf A-treated) relative to Baf A-treated vehicle ($P < 0.05$) further confirmed that exposure of 20 μM $A\beta_{1-42}$ decreased the synthesis of autophagy-related membranes. Pre-treatments with DHA, LUT, UA, and RES significantly increased OPTN levels ($P < 0.001$ each) reflecting an induction of mitophagy. Moreover, considering lysosomal blockade, increased OPTN levels for Baf A-treated UA ($P < 0.05$) suggested increased synthesis of autophagy-related membranes. However, RES and LUT resulting in unaltered OPTN levels for Baf A-treatments suggest a complete block of autophagy/mitophagy at the terminal stages. DHA resulting in decreased OPTN for Baf A-treatment ($P < 0.05$ each) implicated that a partial block in autophagic flux is induced by DHA (**Figure 8**).

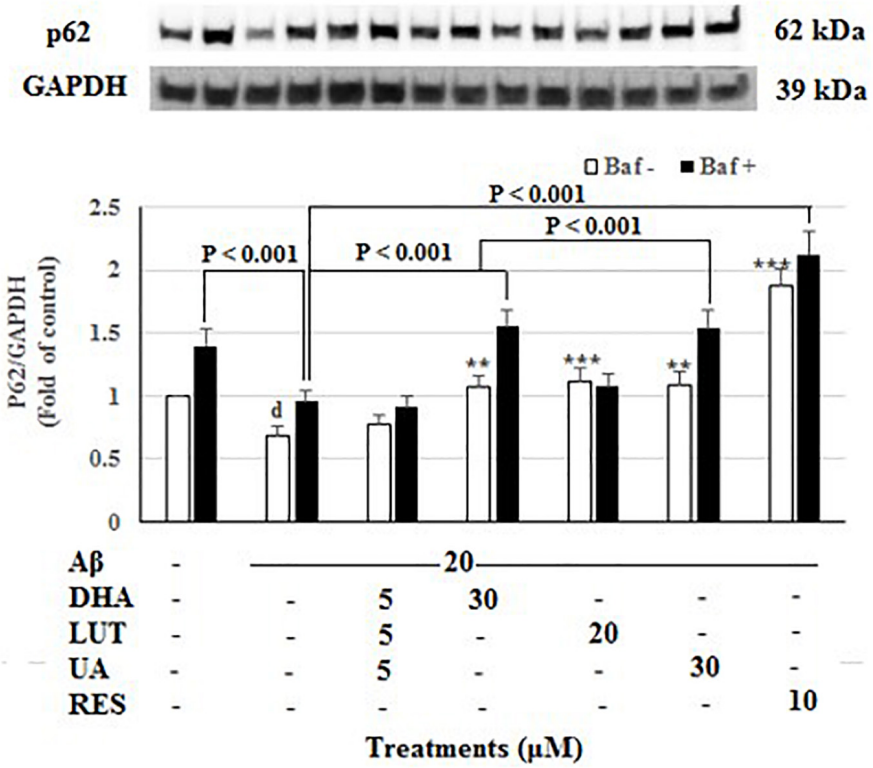


FIGURE 6 | The effect of the three-compound combination and its components on the levels of p62. The BE(2)-M17 cells were pre-treated for 24 h with the three-compound combination, $D_5L_5U_5$, DHA (30 μM), LUT (20 μM), UA (30 μM), and RES (10 μM). The cells were then exposed to 20 μM of oligomeric $A\beta_{1-42}$ for 16 h and subjected to Western blotting. The representative immunoblot and the graph indicate the levels of p62 protein for all treatments. Black bars indicate the p62 levels in the presence of bafilomycin A (Baf A) while the white bars indicate the p62 levels in the absence of Baf A. The relative density of the bands was normalized against GAPDH. Fold values were calculated relative to vehicle control. Data are expressed as mean ± SD from four ($N = 4$) independent experiments. Differences are significant at $^{d}P < 0.05$ vs. vehicle control, $^{**}P = 0.001$, $^{***}P < 0.001$ vs. $A\beta_{1-42}$-treated control.

As shown in **Figure 9**, 20 μM $A\beta_{1-42}$ treatment markedly decreased PINK1 levels ($P < 0.001$) suggesting that $A\beta_{1-42}$ causes a mitophagy deficit in PINK1/Parkin-mediated mitophagy pathway. However, pre-treatments with DHA, LUT, UA, and RES increased the PINK1 levels ($P < 0.05$, $P < 0.05$, $P < 0.05$, and $P < 0.001$). These results support the notion that DHA, LUT, UA, and RES may induce mitophagy possibly through PINK1/Parkin mediated pathway.

The treatment of 20 μM $A\beta_{1-42}$ for 16 h showed no significant difference in the TIMM23 levels compared to the untreated. Considering the TIMM23 levels as a marker of number of mitochondria, the stable TIMM23 levels suggest three possibilities. First, reduced mitophagy and mitobiogenesis. Second, an increase of both processes and thirdly, no change is both the processes. Similarly, the pre-treatments with $D_5L_5U_5$ and DHA did not alter the levels of TIMM23 (**Figure 10**). On the other hand, LUT, UA and RES markedly decreased the TIMM23 levels ($P < 0.001$ each) when compared to the $A\beta_{1-42}$-treated control. A reduction in TIMM23 levels can be a consequence of enhanced mitophagy or impairments in the mitochondrial biogenesis. Therefore, for further inference, the effects of the compounds on mitobiogenesis were evaluated next.

$D_5L_5U_5$ Increases Mitochondrial Biogenesis-Related Protein PGC1-α

Mitobiogenesis was evaluated in the present study by quantifying PGC1-α. As shown in **Figure 11**, exposure of 20 μM $A\beta_{1-42}$ for 16 h did not alter the levels of PGC1-α indicating that mitobiogenesis is not affected. However, pre-treatment with $D_5L_5U_5$ ($P < 0.05$) and DHA elevated PGC1-α levels with the latter resulting in a marked increase ($P < 0.001$) reflecting their increased ability to induce mitobiogenesis. These results taken together with the findings of altered TIMM23 levels (**Figure 10**) provide some insight of these compounds on mitophagy.

The results summarized in the **Table 1** shows that after 16 h of $A\beta_{1-42}$ exposure, the dynamics-related proteins L-OPA1, S-OPA1, MFN2, and FIS1 and the mitophagy proteins, p62, OPTN, and PINK1 all decreased. These results, along with the increased TIMM23 levels at 16 h indicate that there is a deficit in mitophagy in response to 16 h $A\beta_{1-42}$ exposure. However, the three-compound combination resulted in increased NDP52/PINK1 and PGC-1α levels indicating its ability to induce mitophagy and mitobiogenesis, respectively. Treatment with DHA increased OPA1 levels, p62/NDP52/OPTN/PINK1 levels and PGC-1α, indicating its activity in increasing mitochondrial

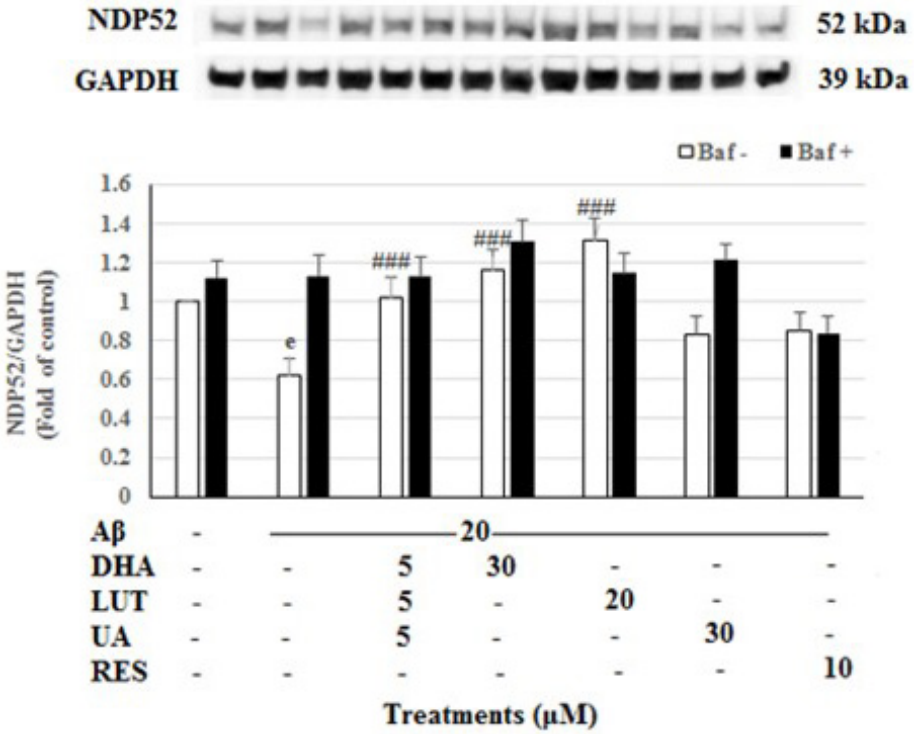


FIGURE 7 | The effect of the three-compound combination and its components on the levels of NDP52. The BE(2)-M17 cells were pre-treated for 24 h with the three-compound combination, $D_5L_5U_5$, DHA (30 μM), LUT (20 μM), UA (30 μM), and RES (10 μM). The cells were then exposed to 20 μM of oligomeric $A\beta_{1-42}$ for 16 h and subjected to Western blotting. The representative immunoblot and the graph indicate the levels of NDP52 protein for all treatments. Black bars indicate the NDP52 levels in the presence of bafilomycin A (Baf A) while the white bars indicate the NDP52 levels in the absence of Baf A. The relative density of the bands was normalized against GAPDH. Fold values were calculated relative to vehicle control. Data are expressed as mean ± SD from four ($N = 4$) independent experiments. Differences are significant at $^{e}P < 0.001$ vs. vehicle control, $^{###}P < 0.001$ vs. $A\beta_{1-42}$-treated controls.

fusion, mitophagy and mitobiogenesis. LUT resulted in increased L-OPA1 and p62/NDP52 levels reflecting its ability to induce mitochondrial fusion and mitophagy, respectively. Finally, UA was shown to induce mitochondrial fusion and mitophagy due to increased OPA1 levels and p62/OPTN levels, respectively.

DISCUSSION

Evidence is accumulating that a healthy diet enriched with bioactive components have a protective role in reducing the risk of AD. The current study presented a comprehensive cell-based investigation of the therapeutic effects of a previously determined nutraceutical combination, $D_5L_5U_5$ (Jayatunga et al., 2021) against $A\beta_{1-42}$-induced toxicity. As per the initial test results of MTS and ATP assays, the combination was anticipated to have beneficial effects on mitochondria. On the other hand, mitochondrial dysfunction is an important feature in $A\beta_{1-42}$-induced toxicity (Chen and Yan, 2007). Dysfunctional mitochondria are often portrayed by increased oxidative stress and deficits in mitochondrial dynamics, mitophagy and mitochondrial biogenesis. Therefore, inducing these processes are important therapeutic targets in AD (Su et al., 2010; Uittenbogaard and Chiaramello, 2014; Jayatunga et al., 2020). In this light, the present study explored the neuroprotective action of $D_5L_5U_5$.

In AD, excessive ROS is generated from impaired mitochondria as well as through Aβ interactions with transition metal ions. In fact, abnormal accumulation of Aβ and Tau proteins promote redox imbalance leading to oxidative stress. However, existing antioxidants have been shown to be relatively ineffective in combating AD (Lloret et al., 2011; Lloret et al., 2019). There may be several reasons for this. First, the antioxidants may not be able to cross the BBB and reach the specific sites of ROS release in the brain, which are suggested to be the hydrophobic domains within mitochondrial membranes. Second, the antioxidants may have a poor ability to scavenge ROS. The present study used the DCFDA assay to determine ROS in the cells. The DCFDA reagent diffuses into cells and gets deacetylated by cellular esterases to a non-fluorescent compound that gets oxidized to a fluorescent compound by cellular ROS. The results revealed that $D_5L_5U_5$ as well its components, DHA, LUT, and UA, decreased the cellular ROS levels, generated by 4 h of exposure of 20 μM $A\beta_{1-42}$. The potency of the antioxidant activity was, LUT > $D_5L_5U_5$ > DHA > UA (**Figure 1**).

Luteolin showed the highest antioxidant activity in the present study (**Figure 1**). It is consistent with previous reports indicating antioxidant effects of LUT (Xiao et al., 2019). Furthermore,

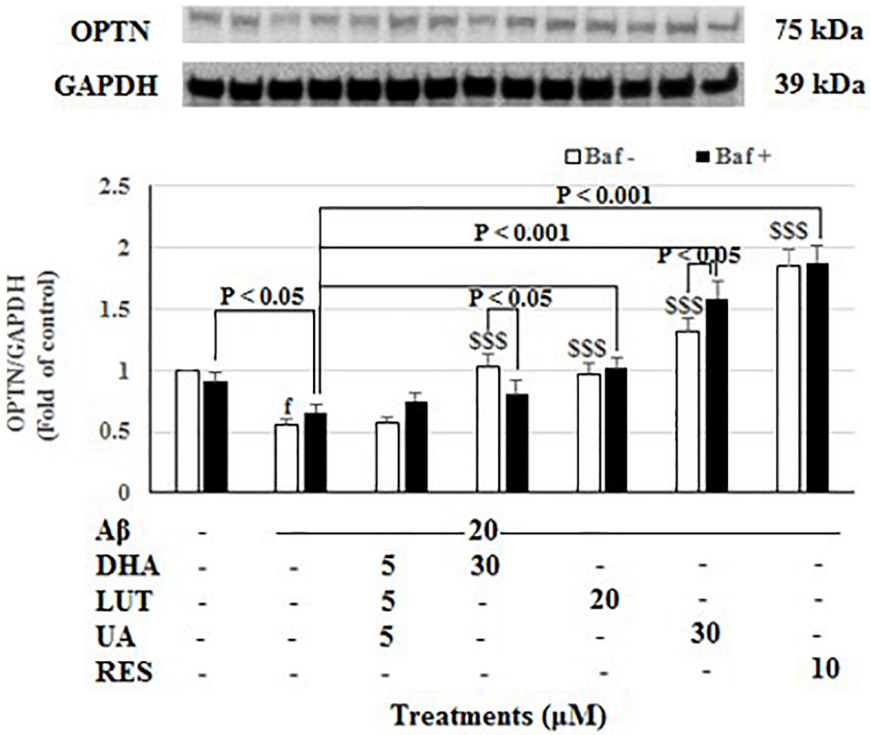


FIGURE 8 | The effect of the three-compound combination and its components on the levels of OPTN. The BE(2)-M17 cells were pre-treated for 24 h with the three-compound combination, $D_5L_5U_5$, DHA (30 μM), LUT (20 μM), UA (30 μM), and RES (10 μM). The cells were then exposed to 20 μM of oligomeric $A\beta_{1-42}$ for 16 h and subjected to Western blotting. The representative immunoblot and the graph indicate the levels of OPTN protein for all treatments Black bars indicate the OPTN levels in the presence of bafilomycin A (Baf A) while the white bars indicate the OPTN levels in the absence of Baf A. The relative density of the bands was normalized against GAPDH. Fold values were calculated relative to vehicle control. Data are expressed as mean ± SD from four ($N = 4$) independent experiments. Differences are significant at $^{f}P < 0.001$ vs. vehicle control, $^{\$\$\$}P < 0.001$ vs. $A\beta_{1-42}$-treated control.

it has been shown that LUT induces apoptosis by promoting antioxidant activity and activating MAPK signaling in human colon cancer cells (Kang et al., 2017). Being a flavone, the antioxidant activity of LUT is thought to possibly be exerted by its chemical structure consisting of four phenolic hydroxyl groups and C2=C3 double bond in conjugation with the C4-carbonyl group (Cai et al., 2006; Vo et al., 2019). Docosahexaenoic acid being a PUFA, showed a moderate ability reduce cellular ROS levels in the present study, and hence, DHA may act as a lipophilic antioxidant that can reach the mitochondrial hydrophobic domains of ROS production in the brain. Despite being a highly oxidizable polyunsaturated fatty acid, DHA is concentrated as a naturally occurring omega-3-PUFA in neuronal brain membranes where approximately 30–50% of total dry weight of the brain is lipid. In fact, there are reports of DHA on enzymatic antioxidant activities, such as catalase, glutathione peroxidase (GPx) and glutathione reductase (GR) (Hossain et al., 1999; Hashimoto et al., 2005).

The second highest potency of antioxidant activity was observed with the $D_5L_5U_5$ combination (**Figure 1**) accounting for its ability to inhibit $A\beta_{1-42}$-induced toxicity effectively (Jayatunga et al., 2021). The data showed that although UA could reduce ROS, it offered the least protection compared to other compounds. Previous work of UA has shown that it can decrease intracellular ROS levels and increase intracellular SOD and GPx activity *in vitro* (Wang et al., 2015). However, UA has been shown to exert pro-oxidant activities as well (Kallio et al., 2013). Moreover, at higher concentrations, a bi-phasic effect of DHA has also been reported in human blood platelets due to its pro-oxidant effects (Vericel et al., 2003). Luteolin is also reported to act as a pro-oxidant as part of its redox modulation activity (Lin et al., 2008). Thus, the three-compound combination, $D_5L_5U_5$ may also potentially have a pro-oxidant activity, however, it was not evaluated in the present study. It must be noted that the redox properties of compounds are highly reliant on the reaction conditions and employed assay system. Hence, it is important to study both the pro-oxidant and antioxidant activities simultaneously if possible (Kallio et al., 2013).

Apart from the above-mentioned ROS scavenging antioxidant strategies, the ability to directly block mitochondrial ROS production may be an effective way of inhibiting oxidative stress. Whist, it seems challenging to define both the normal and pathologically relevant ROS formation, lowering of ROS production by efficiently stabilizing mitochondrial energy production appears feasible. In healthy mitochondria, ATP, and ROS levels are coupled. However, it has been reported that a mild mitochondrial uncoupling (resulting in increased ATP levels and decreased ROS levels) due to controlled

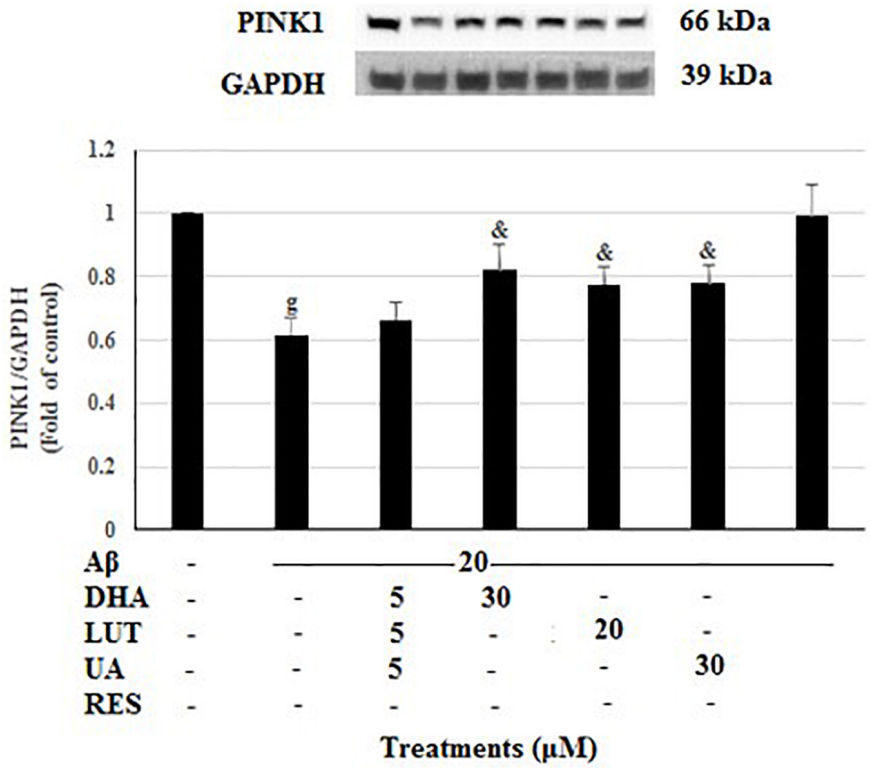


FIGURE 9 | The effect of the three-compound combination and its constituents on the levels of PINK1. The BE(2)-M17 cells were pre-treated for 24 h with the three-compound combination, $D_5L_5U_5$, DHA (30 μM), LUT (20 μM), UA (30 μM), and RES (10 μM). The cells were then exposed to 20 μM of oligomeric $A\beta_{1-42}$ for 16 h and subjected to Western blotting. The representative immunoblot and the graph indicate the levels of PINK1 protein for all treatments. The relative density of the bands was normalized against GAPDH. Fold values were calculated relative to vehicle control. Data are expressed as mean ± SD from four ($N = 4$) independent experiments. Differences are significant at $^{g}P < 0.001$ vs. vehicle control, $^{\&}P < 0.05$ vs. $A\beta_{1-42}$-treated control.

reduction of MMP, is a highly effective antioxidant strategy (Caldeira da Silva et al., 2008).

Therefore, the current study was designed to assess the ability of the compounds to restore the ATP levels affected by the administration of $A\beta_{1-42}$. As assessed at 4–72 h of $A\beta_{1-42}$ exposure, the reduced ATP levels are increased by pre-treatment with the compounds. Considering the activity of all the time points, the overall order of potency of increasing the ATP production by the compounds was, $D_5L_5U_5$ > LUT > UA (**Figure 2**). This order of potency was consistent over every time point. The mechanism by which ATP levels were increased is presently unclear, however the speculation is that the compounds could accept electrons from oxidative species and transfer them to cytochrome c, bypassing respiratory complexes I and III, at a level sufficient to compensate for reduced proton pumping (Komlódi and Tretter, 2017; Berry et al., 2018; Zhao et al., 2019).

Mitochondrial spare respiratory capacity is known to be an important aspect of mitochondrial function and is defined as the difference between basal ATP production and its maximal activity (Deng et al., 2015; Pfleger et al., 2015). It accounts for the extra ATP that can be produced by OXPHOS to overcome various cellular stressors. A cell with a larger spare respiratory capacity can produce more ATP and, in theory, overcome more stress. It has been reported that various polyphenolic compounds can augment the mitochondrial spare respiratory capacity by increasing mitochondrial biogenesis and antioxidant activities (Yamamoto et al., 2016). Although it was compelling to verify this effect for the compounds, in the present study itself, the requirement of bioenergetic flux analyses to confirm whether the compounds promoted enhanced oxygen consumption made it beyond the scope of the study.

It has been shown in our previous studies that the time of exposure of $A\beta_{1-42}$ to cells is a crucial determinant for inducing significant detrimental effects on mitochondrial dynamics, mitophagy and mitobiogenesis (Jayatunga et al., unpublished). Therefore, 20 μM $A\beta_{1-42}$ exposure time of 16 h was selected to determine the time-dependent marked deficits of mitochondrial dynamics, mitophagy and mitobiogenesis. Mitochondrial dynamics-related proteins (OPA1, MFN2, and FIS1), mitophagy-related proteins (p62, NDP52, OPTN, PINK1), IMM protein TIMM23 and mitochondrial biogenesis protein PGC-1α were analyzed using western blotting. Resveratrol was used as a reference compound for its ability to induce mitophagy (Kuno et al., 2018; Cao et al., 2019) and mitobiogenesis (Csiszar et al., 2009).

In the present study, DHA was shown to have increased L-OPA1, S-OPA1, and MFN2 levels implying its effects on increasing mitochondrial fusion. The increased TIMM23 levels

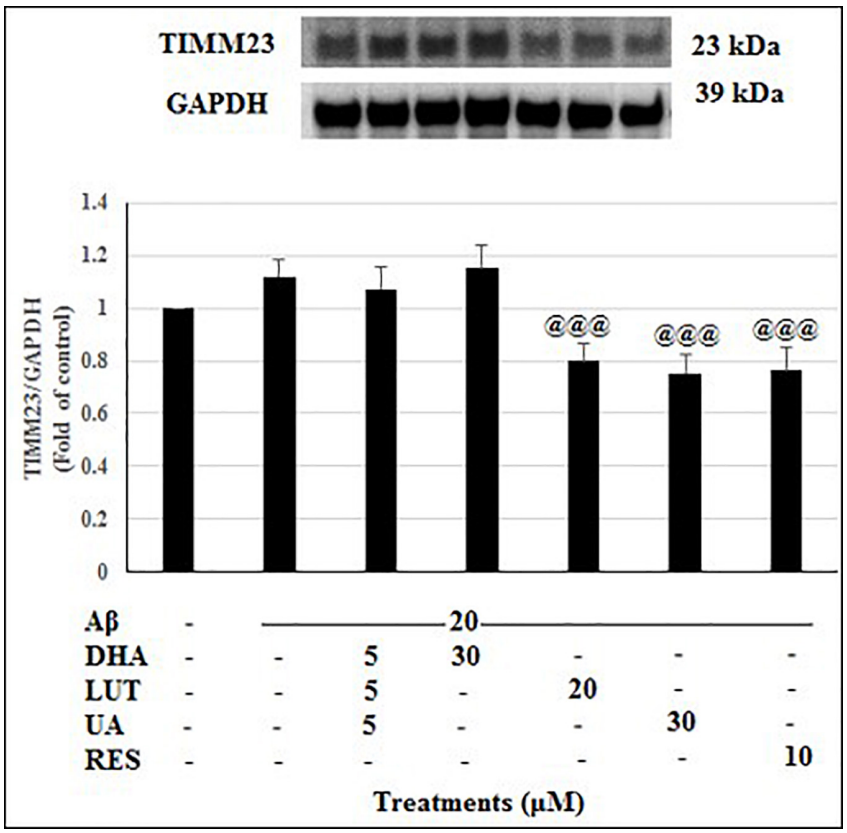


FIGURE 10 | The effect of the three-compound combination and its components on the levels of TIMM23. The BE(2)-M17 cells were pre-treated for 24 h with the three-compound combination, $D_5L_5U_5$, DHA (30 μM), LUT (20 μM), UA (30 μM), and RES (10 μM). The cells were then exposed to 20 μM of oligomeric $A\beta_{1-42}$ for 16 h and subjected to Western blotting. The representative immunoblot and the graph indicate the levels of TIMM23 protein for all treatments. Semi-quantitative analysis of TIMM23 protein levels. The relative density of the bands was normalized against GAPDH. Fold values were calculated relative to vehicle control. Data are expressed as mean ± SD from four ($N = 4$) independent experiments. Differences are significant at $^{@@@}P < 0.001$ vs. $A\beta_{1-42}$-treated control.

suggest that DHA may inhibit mitophagy or promote biogenesis. However, DHA induced mitophagy as well as mitobiogenesis due to its ability to increase of p62, OPTN, PINK1 and PGC-1α. Therefore, the increased TIMM23 levels denote the excess mitochondria biosynthesized after compensation with mitophagy. These findings are consistent with the previous studies that have identified the role of DHA as a potential mitophagy or autophagy inducer, or both (Jing et al., 2011; Shin et al., 2013).

It has been reported that mitobiogenesis is related to sterol regulatory element (SRE)-binding protein (SREBP-1c) as it is implicated in upregulation of PGC-1α (Kobayashi et al., 2018). However, it has been reported that DHA inhibits the promoter activity of SREBP-1c (Deng et al., 2015; Huang et al., 2017) suggesting that DHA may not promote mitobiogenesis in that pathway. However, the present study resulted in an outcome contrary to the above-mentioned studies by showing DHA induced mitobiogenesis. However, the current findings are consistent with several other reports that indicate mitobiogenesis is induced by DHA (Flachs et al., 2005; Lee et al., 2016).

Luteolin also appears to act as an autophagy/mitophagy inducer based on the present findings, due to increased p62, OPTN, PINK1 levels and decreased TIMM23 levels. It did not exert beneficial effects in inducing mitobiogenesis, despite inducing mitochondrial fusion due to increased L-OPA1 levels. These findings are in line with previous reports indicating that LUT is an autophagy or mitophagy inducing compound (Hu et al., 2016). In contrary to the present study, it has also been reported that LUT inhibited autophagy (Li et al., 2019) and induced mitobiogenesis through inhibition of the Mst-1 pathway (Hu et al., 2016).

The present study revealed that UA induced mitophagy as indicated by increased expression levels of p62, NDP52, OPTN, and PINK1. The OPA1 levels were also increased by UA treatment, indicating increased mitochondrial fusion. However, UA only exhibited a non-significant trend for raised PGC-1α levels, indicating that mitobiogenesis was not occurring at detectable levels. These findings of UA on inducing mitophagy are in line with previous reports (Ryu et al., 2016; Webb, 2017). Moreover, UA has shown to be beneficial in both Aβ and Tau based *Caenorhabditis elegans* models of AD (Fang et al., 2019). While one report states that UA stimulates autophagy but not mitophagy to inhibit ER stress in a model of ischemic neuronal

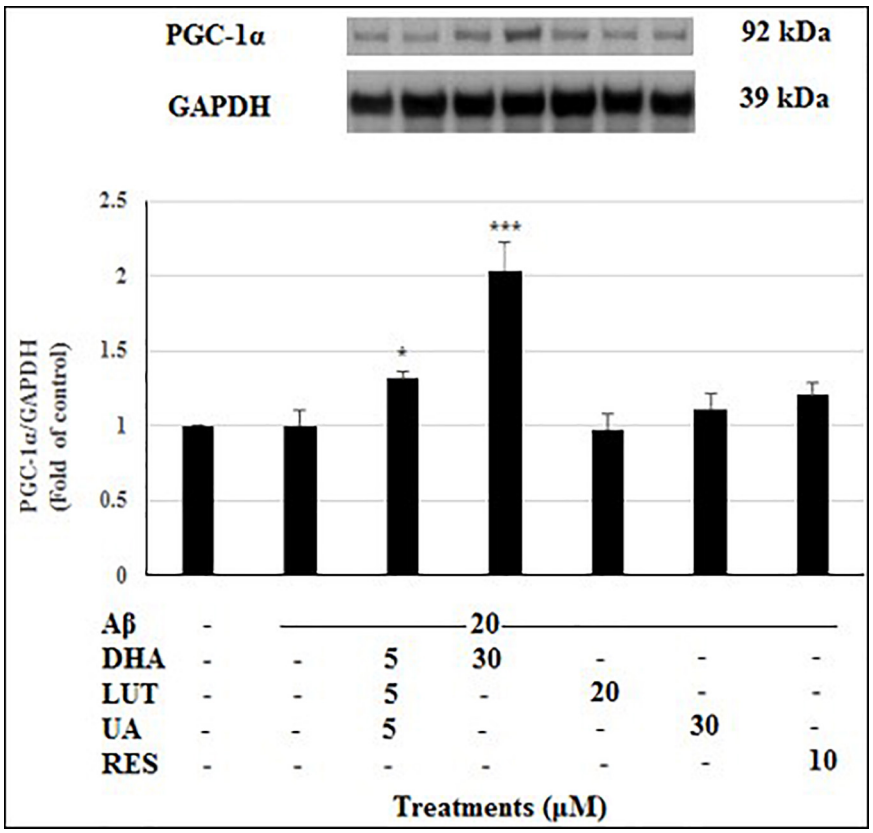


FIGURE 11 | The effect of three-compound combination and its components on the levels of PGC1-α. The BE(2)-M17 cells were pre-treated for 24 h with the three-compound combination, $D_5L_5U_5$, DHA (30 μM), LUT (20 μM), UA (30 μM), and RES (10 μM). The cells were then exposed to 20 μM of oligomeric $A\beta_{1-42}$ for 16 h and subjected to Western blotting. The representative immunoblot and the graph indicate the levels of PGC1-α protein for all treatments. The relative density of the bands was normalized against GAPDH. Fold values were calculated relative to vehicle control. Data are expressed as mean ± SD from four ($N = 4$) independent experiments. Differences are significant at $^*P < 0.05$, $^{***}P < 0.001$ vs. $A\beta_{1-42}$-treated control.

TABLE 1 | Changes in mitochondrial dynamics-related proteins, mitophagy-related proteins, and the mitobiogenesis-related protein with pre-treatment of compounds followed by $A\beta_{1-42}$ treatment.

Proteins	$A\beta_{1-42}$-control	$D_5L_5U_5$	DHA (30 μM)	LUT (20 μM)	UA (30 μM)	RES (10 μM)
For all proteins, vehicle-treated control was considered as 1						
Mitochondrial dynamics-related proteins						
L-OPA1	-	–	+	+	+++	+
S-OPA1	–	-	+	–	++	++
MFN2	–	–	-	–	–	-
FIS1	-	–	–	-	-	-
Mitophagy-related proteins						
p62	–	-	+	++	+	+++
NDP52	–	+	+	++	-	-
OPTN	–	–	+	-	++	+++
PINK1	–	–	-	-	-	-
TIMM23	+	+	+	-	-	-
Mitochondrial biogenesis-related protein						
PGC-1α	-	++	+++	-	+	++

Using Western blotting, protein fold changes were assessed in BE(2)-M17 cells pre-treated with $D_5L_5U_5$, DHA, LUT, UA, and RES for 24 h followed by 20 μM oligomeric $A\beta_{1-42}$ exposure for 16 h. ± values indicate the relative protein fold change compared to vehicle controls.
Protein fold changes at 0.50–0.70: –, 0.71–0.99: -, 1.01–1.20: +, 1.21–1.40: ++, 1.41 upwards: +++.
Significant changes vs. $A\beta_{1-42}$-treated control are indicated by ▭

injury (Ahsan et al., 2019), our findings indicated that UA induced both autophagy and mitophagy.

The present study revealed that pre-treatment with $D_5L_5U_5$ increased NDP52 ($P < 0.001$), an autophagy adaptor protein related to mitophagy, revealing its ability to trigger mitophagy. In fact, $D_5L_5U_5$ increased PGC-1α levels ($P < 0.05$) indicating induction of mitobiogenesis. The increased trend of TIMM23 was attributed to the mitochondrial mass balanced by mitobiogenesis and mitophagy processes. It was also indicated that $D_5L_5U_5$ increased S-OPA1 levels ($P < 0.05$). Apart from its action in mitochondrial fission, as a prerequisite to mitophagy, S-OPA1 has also been proposed to act as an enhancer of OPA1-Cardiolipin interaction that facilitates mitochondrial fusion (Rujiviphat et al., 2009). This may explain how the three-compound combination, $D_5L_5U_5$, increased mitochondrial fusion to result in increased mitobiogenesis.

The order of potency for mitophagy-inducing activity of the compounds is UA > DHA > LUT > $D_5L_5U_5$. However, the utility of inducing mitophagy is undoubtedly preferred only in a controlled manner without causing deterioration or toxicity in cellular respiration. In this regard, these compounds also have strong antioxidant properties. Hence, it is obvious that their mechanisms of activating mitophagy are not ROS-dependent or mitohormetic, thus positioning them as important candidates for potential AD therapy. Moreover, considering the antioxidant properties determined through the cellular ROS levels, the compounds can be positioned as UA > DHA > $D_5L_5U_5$ > LUT, suggesting a correlation between the level of ROS and mitophagy only at higher levels of ROS. It has been speculated that mitochondrial ROS (superoxide) involves in mitophagy, but this notion remains to be verified (Shefa et al., 2019; Schofield and Schafer, 2020). Further studies are needed to investigate the molecular mechanisms by which the three-compound combination reduces ROS levels and induces mitophagy and mitobiogenesis.

Compounds that activate mitophagy without inducing a respiration collapse are important as protectors of mitochondria. The three-compound combination, $D_5L_5U_5$ effectively attenuated $A\beta_{1-42}$-induced toxicity (Jayatunga et al., 2021), possibly due its ability to decrease ROS levels, induce mitobiogenesis and mitophagy, to some extent. In fact, $D_5L_5U_5$ was shown to have its protective ability on all aspects tested, namely, by reducing ROS levels, increasing ATP levels, and inducing mitophagy and mitobiogenesis. Taken together, having DHA, LUT, and UA in one single entity as $D_5L_5U_5$ immensely benefits and precedes the mitochondrial protective role as shown in the current study. It is of note that there may be other pathways that may account for the neuroprotection exerted by this synergistic nutraceutical combination. Further *in vitro* and *in vivo* studies are required to uncover the specific modes of mitophagy and mitobiogenesis elicited by the $D_5L_5U_5$.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

RM and DJ designed the study. DJ wrote the manuscript. RM, EH, GV, MG, and WF reviewed the manuscript intensively. MG and WF edited the manuscript. All authors have read and agreed to the final version of this manuscript.

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Role of BDNF/ProBDNF Imbalance in Postoperative Cognitive Dysfunction by Modulating Synaptic Plasticity in Aged Mice

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Postoperative cognitive dysfunction (POCD) is a disturbing neurological complication in patients undergoing anesthesia and surgical procedures. Brain-derived neurotrophic factor (BDNF) and its precursor proBDNF binding to their corresponding receptors tyrosine kinase (TrkB) and p75 neurotrophin receptor (p75NTR) exert quite an opposite biological function in neuron survival and synaptic function. This study aimed to demonstrate the critical role of the BDNF/proBDNF ratio in modulating synaptic plasticity, which further leads to anesthesia-/surgery-induced POCD. It also showed that the exogenous BDNF or p75NTR inhibitor could ameliorate cognitive dysfunction. In detail, 16-month-old C57BL/6 mice were subjected to a stabilized tibial fracture surgery with isoflurane anesthesia to establish the POCD animal model. The mice were then microinjected with either p75NTR inhibitor or exogenous BDNF into the dorsal hippocampus. Behavioral experiments were performed by open field and fear conditioning tests (FCTs). Western blotting was also used to measure the expression levels of BDNF, proBDNF, TrkB, p-TrkB, p75NTR, and synapse proteins. Golgi staining and electrophysiology were applied to evaluate the neuronal synaptic plasticity. Here, we demonstrated that anesthesia/surgery induced a reduction of BDNF/proBDNF, which negatively regulates the synaptic function in hippocampus, subsequently leading to cognitive impairment in aged mice. P75NTR inhibitor and exogenous BDNF could attenuate cognitive deficits by rescuing the dendritic spine loss and long-term potentiation (LTP) *via* altering the BDNF/proBDNF ratio. This study unveiled that the BDNF/proBDNF ratio in the hippocampus played a key role in anesthesia-/surgery-induced POCD. Thereby, tuning the ratio of BDNF/proBDNF is supposed to be a promising therapeutic target for POCD.

Keywords: postoperative cognitive dysfunction, BDNF, ProBDNF, synaptic plasticity, aged

INTRODUCTION

Postoperative cognitive dysfunction (POCD) is a common neurological complication in elderly surgical patients, which is mainly manifested clinically as declined learning, memory, concentration, and execution ability (Eckenhoff et al., 2020). Currently, clinical research revealed that POCD may contribute to adverse outcome, including increased morbidity and mortality, prolonged hospitalization, and reduced quality of life (Terrando et al., 2011). Since its mechanism stays unclear, whether anesthesia/surgery can be considered as etiologies of POCD remains controversial (Eckenhoff et al., 2020). At present, some acknowledged pathological mechanisms, including neuroinflammation (Safavynia and Goldstein, 2018), oxidative stress (Netto et al., 2018; Safavynia and Goldstein, 2018), Aβ protein metabolism (Shen et al., 2016), and neurotransmitter release disorders (Sarter et al., 2007), involved in POCD research were closely related to changes in synaptic plasticity. Synaptic plasticity, which can maintain neurons and neural circuits stability, plays an important role in learning, memory, cognitive function, and certain neurodegenerative diseases (Humeau and Choquet, 2019).

Brain-derived neurotrophic factor (BDNF) belongs to the neurotrophic family which is widely expressed in the central nervous system. BDNF exists in precursor and mature forms. ProBDNF can be secreted and proceeded extracellularly by plasmin or by matrix metalloproteases (MMPs) to produce mature BDNF (mBDNF) (Pang et al., 2004; Mizoguchi et al., 2011). Typically, tissue plasminogen activator (tPA) activated the extracellular protease plasmin to cleave proBDNF and produce mBDNF (also known as BDNF) (Pang et al., 2004). The main function of BDNF is to maintain neuronal survival and differentiation, facilitate synaptic transmission, enhance synaptic plasticity and strengthen synaptic growth through activation of TrkB receptor and ERK signaling cascade (Lu et al., 2013). However, its precursor, proBDNF, has quite the opposite biological properties, which is called a "Yin and Yang" model of neurotrophin action (Lu et al., 2005). ProBDNF causes cell death, dendritic arborization reduction, spine density loss (Yang et al., 2014), and long-term depression (LTD.) through its high-affinity p75 neurotrophin receptor (p75NTR) (Woo et al., 2005). Moreover, the combination of proBDNF binding with p75NTR will trigger the RhoGDI, which induces a reduction in spine density by modulating RhoA activity (Buhusi et al., 2017). TAT-Pep5, a peptide used to block the interaction between p75NTR and RhoGDI (Yamashita and Tohyama, 2003), has been verified to improve the behavior performance of elderly mice with memory impairment (Buhusi et al., 2017). Thus, regulating the ratio of extracellular proBDNF and BDNF seems to be crucial for synaptic function and memory consolidation.

Previous studies have shown that the reduction of BDNF is involved in the pathogenesis of surgery-induced POCD (Fan et al., 2016; Qiu et al., 2016). However, seldom research shows whether surgery can cause changes in proBDNF expression. Herein, we proposed a hypothesis that the ratio of BDNF/proBDNF by modulating synaptic plasticity plays a vital part in surgery-induced POCD in aged mice. Intrahippocampal infusions of TAT-Pep5 and exogenous administration of BDNF could attenuate surgery-induced cognitive deficits *via* tuning the ratio of BDNF/proBDNF and alleviate the deficiency of synaptic function, which is supposed to be a potential therapeutic target in future POCD prevention.

MATERIALS AND METHODS

Animals

All experiments were approved by the Institutional Animal Care and Use Committee at Capital Medical University (Beijing, China) and carried out under the regulations of Medical Research Center of Capital Medical University (protocol AEEI-2020-117). Wild-type male C57BL/6 mice (16-month- old, weighing 28–32 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Five mice per cage were housed (22–25°C) with access to food and water *ad libitum* under a 12-h light/dark cycle. All mice were adapted to the environment for 2 weeks and were randomly divided into treatment groups.

Experimental Protocol

The whole experiment was composed into two parts. In the experiment section A, the mice were randomly sorted into 2 groups: the control group (C) and the surgery group (S). The S group ($n = 24$) received intramedullary fixation of tibial fractures under isoflurane anesthesia, while the C group ($n = 24$) received 100% oxygen without surgery/anesthesia. In the experiment section B, the mice were randomly divided into 3 groups: the surgery + saline (Sur + S, $n = 8$), the surgery + TAT-pep5 (Sur + T, $n = 8$), and surgery + BDNF (Sur + B, $n = 8$), and all the mice underwent orthopedic surgery. According to the fear conditioning results in section A, we chose postoperative day 3 (POD 3) for the time point of Golgi staining and LTP in section A. All the experiments in section B were implemented on POD 3. A schematic diagram of the experimental procedure is summarized in **Figures 1A,B**.

Postoperative Cognitive Dysfunction Animal Model

Right tibial fracture with intramedullary fixation procedure was performed under anesthesia to establish the POCD animal model described in the previous study (Chen et al., 2019). Mice were subjected to 3% isoflurane for induction and 1.5% isoflurane for maintenance. Then an incision was made lateral to the right tibia to expose the bone and then a 0.38 mm intramedullary fixation needle was inserted into the tibial medullary canal for fixation. Finally, an osteotomy was performed in the middle and distal thirds of the tibia. The surgical incision was sutured with 4–0 silk non-absorbable suture (Mersilk; Ethicon, United States). The mice body temperature was adjusted at 36–37°C during the whole procedure by a heating pad. Ropivacaine (0.2%, Oxford; AstraZeneca) was applied locally to prevent postoperative pain.

Drug Delivery Regimen

Mice were anesthetized with isoflurane mentioned above. A sufficient amount of drugs were injected into the CA1 subfield

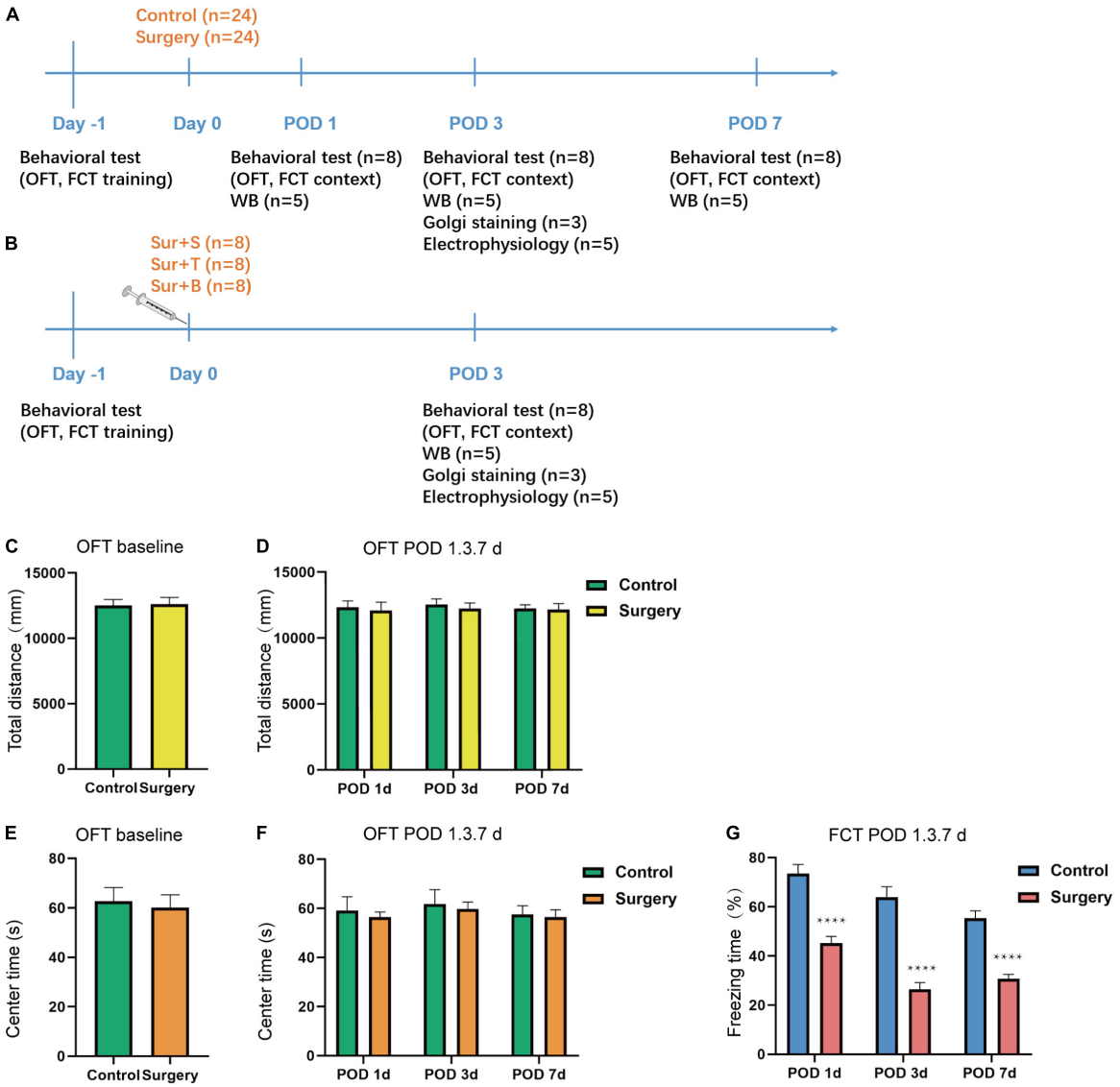


FIGURE 1 | Schematic timeline of the experimental design and results of behavioral tests. **(A)** The mice received OFT and fear conditioning training on day 1. The OFT and fear conditioning context tests were performed on postoperative days 1, 3, 7 (POD1, 3, 7). Orthopedic surgery was performed on day 0. Hippocampal tissues were harvested at the end of the behavioral tests on POD1, 3, 7. Western blotting analysis of BDNF,proBDNF, TrkB, p-TrkB, p75NTR, PSD-95, SYP expression was performed on POD1, 3, 7. Golgi staining and electrophysiology were performed on POD3. **(B)** Saline, TAT-pep5, and BDNF were perfused into the CA1 subfield of the dorsal hippocampus followed by the orthopedic surgery on day 0 after the OFT and fear conditioning training (on day −1) and the fear conditioning context tests were performed on POD3. Western blotting analysis of BDNF,proBDNF, TrkB, p-TrkB, PSD-95, and SYP expression was performed on POD3. Golgi staining and electrophysiology were performed on POD3. **(C–F)** The OFT was conducted to assess the total distance and time spent in the center arena on day −1 and on POD1, 3, 7. **(G)** The percent of freezing time in the fear conditioning context test were assessed on POD1, 3, 7. Data are presented as the mean ± SEM (n = 24, OFT baseline; n = 8 OFT/FCT POD1, 3, 7). ****$p < 0.0001$ compared to the control group.

under the mouse brain atlas (AP ± 2.1 mm, ML ± 1.5 mm, DV −1.5 mm) through a bilateral stereotactic instrument. Soon after drug delivery, an orthopedic procedure was performed immediately. Mice in the group Sur + S were injected with saline 0.8 μl/side. Mice in the group Sur + T were subjected to a solution of TAT-Pep5 (EMD Millipore, Billerica, MA, United States) 20 ng/μl, 0.8 μl/side (Buhusi et al., 2017). Mice in the group Sur + B were administered BDNF (recombinant human BDNF,248-BDB, R&D systems, Emeryville, CA, United States) 0.25 μg/μl, 0.8 μl/side (Kim et al., 2012). All drug solutions were infused at a speed of 0.1 μl/min. The needle was retained for 5 min after the injection for drug absorption and then slowly withdrawn.

Behavioral Tests

Open Field Test

We used the open field test (OFT) to assess the exploratory locomotor activity. According to the published protocol of

OFT (Kraeuter et al., 2019), a white opaque plastic chamber (50 × 50 × 40 cm) was used as the open-field arena. The center zone was one-half of the total area. The mice were placed individually in the center of the area to acclimate the environment for 5 min and allowed to move freely in the square field for 5 min. The movement traces and parameters of the mice were recorded by the video camera automatically. The data were analyzed by the supporting ANY-maze software system (ANY-maze, Stoelting Co., IL, United States). We used the total distance to determine the locomotor activity of the mice, while the time spent in the central zone was used to access the anxiety level of the mice. After each test, the arena was cleaned with 75% alcohol to avoid olfactory cues.

Fear Conditioning Test

Fear conditioning test (FCT) has been widely used to assess hippocampal-dependent memory in POCD. We employed the protocol described in the previous study (Xiong et al., 2018). The FCT consisted of a training phase and a test phase. During the training session, the mice were placed in the chamber to adapt to the environment for 180 s. Next, a 20 s, 70 dB tone (conditional stimulus, CS), and 2 s and 0.75 mA foot shock (unconditional stimulus, US) were applied in two pairs (60 s interval). There was a 10- s interval between CS and US. After 30 s recovery, the mice were placed back into the cages. For the test session, we were only concerned about the hippocampus-dependent memory, so we performed the context test solely (Qiu et al., 2020). Mice were returned into the same chamber for 5 min without any stimulus on POD1,3, and 7. Freezing behavior was recorded and analyzed by the ANY-maze (ANY-maze, Stoelting Co., IL, United States) animal tracking system software. Likewise, to avoid the olfactory cues, 75% ethanol was used to clean the chamber.

Western Blotting

Western blotting (WB) was performed as described before (Wang et al., 2020). Hippocampal total protein lysates were prepared by the RIPA lysis buffer (KeyGen Biotech, China) which contained the protease inhibitors and phosphatase inhibitors (Beyotime, China). Homogenized hippocampal tissues were quantified by Pierce BCA Protein Assay Kit (Thermo-Scientific, Waltham, MA, United States). Then, the denatured proteins were separated by 8 and 12% SDS-PAGE (Sigma-Aldrich) and transferred to 0.2 μm polyvinylidene difluoride (PVDF) membranes (Merck Millipore, ISEQ00010, United States). Membranes were blocked in a solution, including Tris-buffered saline with Tween (TBST), and 5% non-fat dry milk for 1 h at room temperature and incubated with primary antibodies overnight at 4°C. The primary antibodies were anti-TrkB (1:5,000, ab187041, United Kingdom), anti-phospho-TrkB (Y705) (anti-p-TrkB, 1:1,000, ab229908, United Kingdom), anti-p75NTR (1:1,000, 8238, Cell Signaling Technology, United States), anti-postsynaptic density protein 95 (anti-PSD95,1:1,000, 3450, Cell Signaling Technology, United States), anti-synaptophysin (anti-SYP, 1:1,000, 5461, Cell Signaling Technology, United States) and β-actin (1:5,000, 66009-1-Ig, proteintech, China). After evaluating several BDNF antibodies, anti-BDNF (1:1,000, ab108319, Abcam, United Kingdom) was chosen to reliably identify both BDNF (14 kDa) and proBDNF (34 kDa). After washing 3 times in TBST, the membranes were incubated for 1 h at room temperature with secondary antibodies (1:5,000, Gene-protein link, China). Blots were visualized using enhanced chemiluminescence (ECL) reagent (Millipore, Billerica, MA, United States) and imaged by VILBER Fusion FX6 Spectra (Vilber-Lourmat, Paris, France). For proteins with low expression and difficult to visualize, SuperSignal West Atto reagent (Thermo Fisher Scientific, Waltham, MA, United States) was used to detect them. The target protein bands were quantitated by Image J software (National Institutes of Health, Bethesda, MD, United States) and normalized to the density of β-actin in the same sample.

Golgi-Cox Staining

Golgi-Cox staining was used to reveal the structure of dendritic spines in the hippocampus by using the Golgi Stain Kit (#PK401, FD NeuroTechnologies, Columbia, MD, United States). Mice brains were harvested and rinsed in the milli-Q water before being immersed in a mixture of solutions A and B for 3 weeks at room temperature in darkness. Then the brains were transferred to solution C for at least 72 h (up to 1 week). Next, the brains were cut into slices of 100 μm thickness with cryotome (CM3050 S, Leica, Wetzlar, Germany)(chamber temperature—23°C) and mounted on gelatin-coated microscope slides (#PO101, FD NeuroTechnologies, Columbia, MD, United States). Finally, slides were stained in a mixture of solutions D and E, dehydrated with alcohol, and cleared in xylene before mounting with a coverslip. The CA1 region of hippocampal neurons was captured by a Panoramic scan digital slice scanner (3DHISTECH Ltd., Budapest, Hungary). The spine densities were calculated from 2 independent dendritic spines derived from 3 separate images of each animal. The counting was performed by two experimenters independently. We used the Neuron J plugin in Fiji software (Fiji-win64, NIH, United States) to track the branches of dendrites and calculate the total length of dendrite branches. Sholl analysis (Sholl, 1953) was used to measure the intersection of dendrites in every 10 μm from the cell soma to reflex the complexity of neuronal dendrites.

Electrophysiology

The 64-channel recording system (MED64, Panasonic Alpha-Med Sciences, Japan) was used to evaluate the function of synaptic plasticity by recording the long-term potentiation (LTP). By choosing one of the MED64 probes (Panasonic; MED-P515AP) as a stimulating electrode in the CA1 region of the hippocampus, the evoked field excitatory postsynaptic potentials (fEPSPs) were recorded by revised techniques described previously (Shimono et al., 2002) with a specially modified artificial cerebral spinal fluid (aCSF) formula for aging mice (Ting et al., 2014). Briefly, the mice were decapitated, and the brain tissue was placed in ice-cold NMDGaCSF filled with a gas mixture of 95% O_2 and 5% CO_2. Coronal slices (400 μm) were cut by a vibratome (Leica VT1200S) and incubated in NMDGaCSF at 32–34°C for no more than 12 min. Next, brain slices were transferred to a HEPES holding aCSF maintained at room temperature (22–25°C) for another 60 min prior to recording. Finally, the slices were placed in the MED probes and

perfused with recording aCSF at a rate of 4 ml/min. Above all the steps, aCSF was needed to be continuously perfused with the mixed gas. LTP was induced by five episodes of theta burst stimulation (TBS) in accordance with the previous protocols (Zhang et al., 2017). FEPSP slopes and amplitudes (peak to peak) for each slice were derived from the data which were averaged by 5 consecutive probes in the CA1 region. Figures were presented as the percentages of mean fEPSP slopes and mean amplitude of the baseline (before TBS) period. We used the fEPSP slopes and amplitude over the last 10 min of the recording for statistical analysis.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism8.0 (GraphPad, San Diego, CA, United States.). Data are presented as mean ± S.E.M. In experiment section A, the difference of behavioral test and WB results were analyzed by one-way ANOVA with a Tukey *post-hoc* test for multiple comparisons. Total dendritic length, dendritic intensity, fEPSP slope, and amplitude were compared by unpaired *t*-test. The dendritic intersections were analyzed by two-way ANOVA. In experiment section B, three groups were analyzed by one-way ANOVA with a Tukey *post-hoc* test for multiple comparisons. The dendritic intersections were analyzed by two-way ANOVA with a Tukey *post-hoc* test for multiple comparisons. A significant difference was defined as $p < 0.05$.

RESULTS

Anesthesia/Surgery Impaired Cognitive Function in Aged Mice

The OFT was applied to access the locomotor activity and anxiety-like behavior of aged mice. Stressful mice are showed less movement in the open area, while mice with lower anxiety are inclined to spend more time in the central area (Kraeuter et al., 2019). The result showed there was no significant difference in the total distance ($t = 0.1364$; $p = 0.8921$; **Figure 1C**) traveled by the mice and the time spent in the center arena ($t = 0.1980$; $p = 0.7377$; **Figure 1E**) between the C and S groups at baseline (Day −1). Additionally, there was no difference in the total distance ($t = 0.3103$, $p = 0.7609$, POD1; $t = 0.4785$, $p = 0.6397$, POD3; $t = 0.1476$, $p = 0.8848$, POD7; **Figure 1D**) and center time ($t = 0.4561$, $p = 0.6553$, POD1; $t = 0.3062$, $p = 0.7639$, POD3; $t = 0.2123$, $p = 0.8349$, POD7; **Figure 1F**) of the two groups on POD1, 3, 7. The fear conditioning contextual test was used to evaluate hippocampus-dependent long-term learning and memory. In the fear conditioning context test, the freezing time was significantly decreased on POD1, 3, and 7 in S group compared to the C group (POD1, $t = 6.160$; $p < 0.0001$; POD3, $t = 7.354$; $p < 0.0001$; POD7, $t = 6.989$; $p < 0.0001$; **Figure 1G**). Taken together, these results suggested that anesthesia/surgery-induced hippocampus-dependent memory declined while did not affect locomotor activity and anxiety-level in aged mice.

Anesthesia/Surgery-Induced Hippocampal Deteriorated Synaptic Plasticity in Aged Mice With Postoperative Cognitive Dysfunction

Previous studies have unveiled that hippocampal synaptic plasticity is a critical mechanism of memory formation, storage, and consolidation (Zhang et al., 2015). We used the Golgi-Cox staining to intuitively quantify the synaptic structure. The morphology of dorsal hippocampus and CA1 neurons are shown in **Figures 2A–C**. Sholl analysis was used to assess dendritic branching. The dendritic intersections at 50–140 μm from the soma were significantly declined in the S group [$F_{(24, 350)} = 4.151$; $p < 0.0001$; **Figure 2D**]. Similarly, the total dendritic length reduced in the S group ($t = 10.64$; $p < 0.0001$; **Figure 2E**). Consistent with the above results, the spine density also declined in the S group ($t = 13.60$; $p < 0.0001$; **Figure 2F**). Additionally, the function of synaptic plasticity was measured by electrophysiology. MED64 systems were used to record the LTP in the CA1 region of hippocampus on POD 3 (**Figure 2G**). Average fEPSP slope ($t = 7.073$; $p < 0.0001$) and amplitude ($t = 7.842$; $p < 0.0001$) decreased obviously in the S group (**Figures 2H,I**). In the hippocampal slices of C group, LTP was induced steadily by TBS stimulation and recorded for at least 60 min. The amplitude (peak to peak) showed a similar result (**Figures 2J,K**). These results suggested that anesthesia/surgery exacerbated both morphology and function of synaptic plasticity in the hippocampal CA1 region of aged mice, which might be implied as an underlying mechanism of POCD.

Anesthesia/Surgery-Induced Brain-Derived Neurotrophic Factor/Pro Brain-Derived Neurotrophic Factor Imbalance, Receptors, and Synaptic Proteins Changes in Aged Mice With Postoperative Cognitive Dysfunction

To determine whether anesthesia/surgery induced changes of BDNF/proBDNF expression in aged mice with POCD, we detected the proteins levels on POD 1, 3, and 7. Compared to C group, the proBDNF level was significantly increased [$F_{(3, 16)} = 12.96$; $p = 0.0001$; **Figures 3A,D**] in the S group. In contrast, the BDNF level was significantly decreased [$F_{(3, 16)} = 13.08$, $p = 0.0001$; **Figures 3A,E**] in S group. The ratio of BDNF and proBDNF was calculated to reflect the imbalance of the proteins intuitively. It turned out anesthesia/surgery significantly downregulate the BDNF/proBDNF levels [$F_{(3, 16)} = 93.88$; $p < 0.0001$; **Figure 3F**]. To evaluate the effects on synapse quantity, we measured the presynaptic protein SYP and postsynaptic protein PSD-95 level in the hippocampus. The level of PSD-95 declined compared to C group [$F_{(3, 16)} = 12.40$; $p = 0.0002$; **Figures 3B,G**]. Consistent with PSD-95, SYP expression declined simultaneously [$F_{(3, 16)} = 32.86$; $p < 0.0001$; **Figures 3C,H**].

To determine whether anesthesia/surgery impacts the corresponding receptors expression, we measured the expression level of p75NTR and TrkB. The p75NTR level was obviously

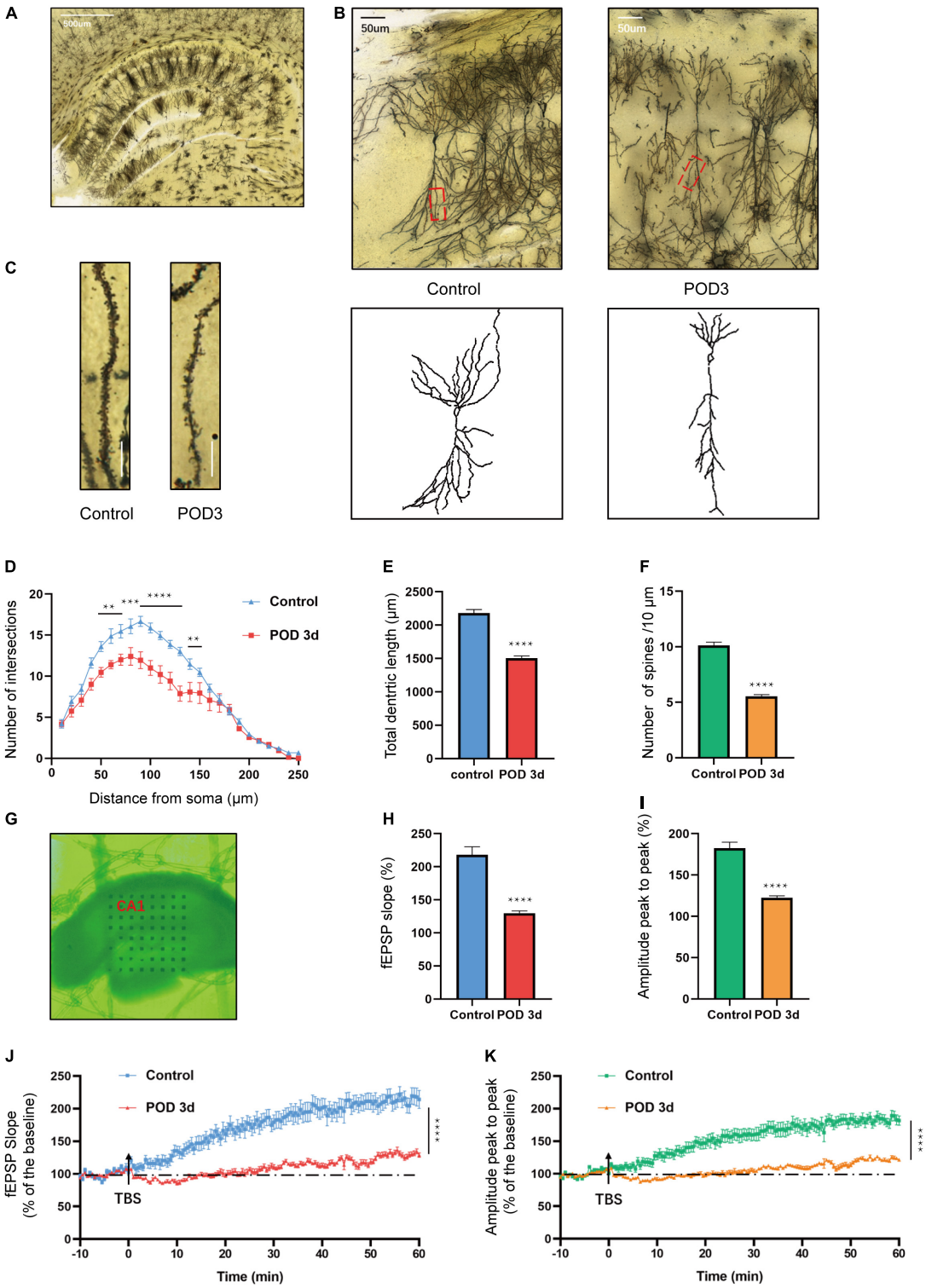


FIGURE 2 | Hippocampal synaptic plasticity changes on POD3 of aged mice with POCD. **(A)** A dorsal hippocampal profile image of Golgi-Cox staining. Scale bar = 500 μm. **(B)** Sample image of Golgi-Cox staining and camera tracings of hippocampal CA1 neurons. Scale bar = 50 μm. **(C)** Representative dendritic spine density of hippocampal CA1 neurons. Scale bar = 10 μm. **(D)** Quantification of dendritic intersections (n = 18 from 3 mice). **(E)** Quantification of the total dendritic length (n = 18 from 3 mice). **(F)** Quantification of the spine density (n = 18 from 3 mice). **(G)** Sample image of the MED64 probes location in the CA1 region of dorsal hippocampus. **(H,I)** Average fEPSP slope/amplitude during the last 10 min. **(J,K)** LTP/amplitude recording in the hippocampal CA1 region. Data are the percentage of mean fEPSP slopes/amplitude recorded during the baseline period (n = 15 slices from 5 mice). Arrows show the time point of TBS delivery. Data are presented as the means ± SEM for each group. $^{**}p < 0.01$ compared to the control group ($^{***}p < 0.001$, $^{****}p < 0.0001$).

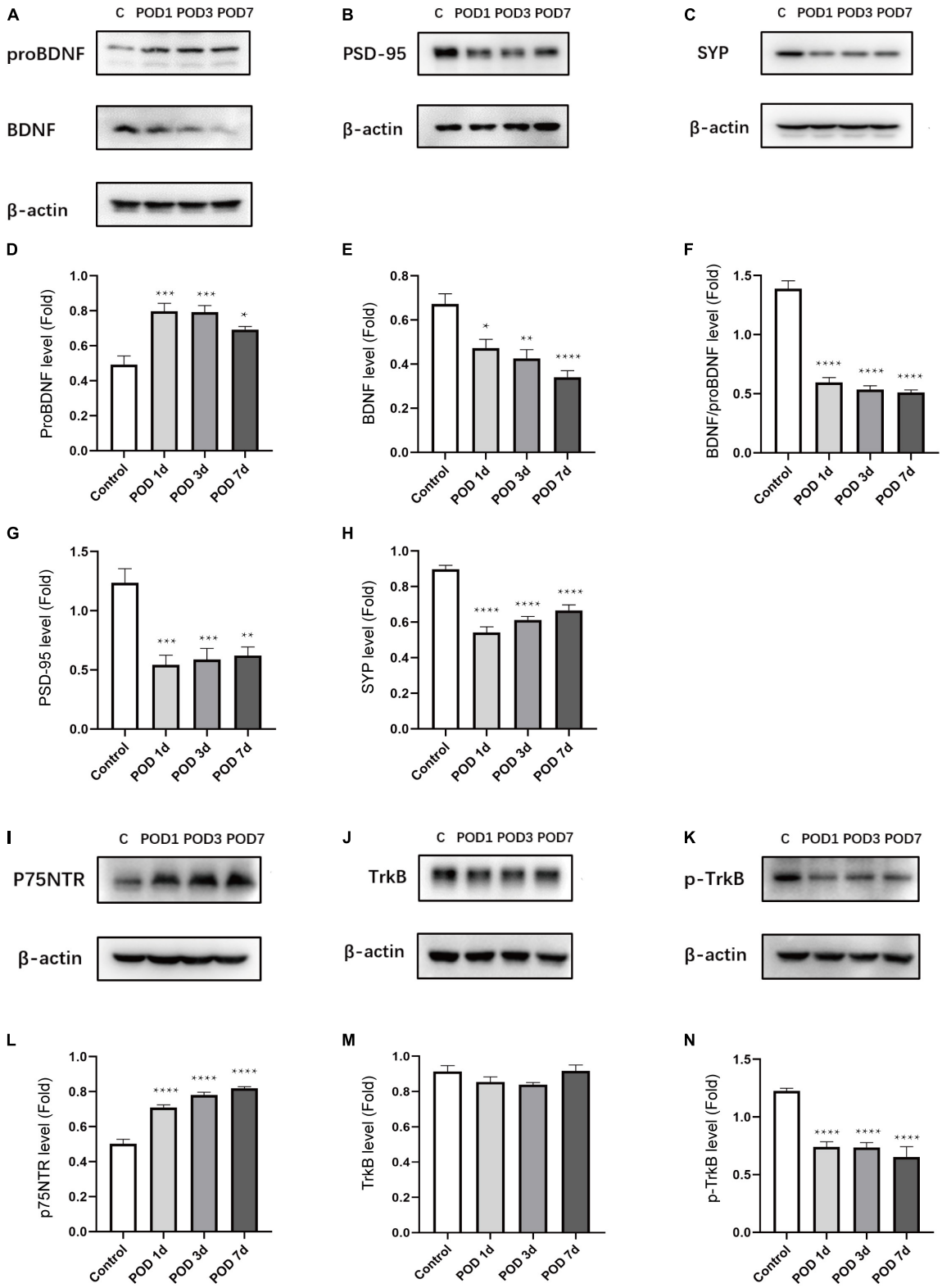


FIGURE 3 | Anesthesia/surgery induced BDNF/proBDNF imbalance and related proteins changes in the hippocampus of aged mice. **(A–C)** Representative western blots of proBDNF, BDNF, PSD-95, SYP in hippocampus on POD1, 3 and 7. **(D–H)** Quantitative analysis of proBDNF, BDNF, BDNF/proBDNF, PSD-95, and SYP levels. **(I–K)** Representative western blots of p75NTR, TrkB, p-TrkB in hippocampus on POD1,3 and 7. **(L–N)** Quantitative analysis of p75NTR, TrkB, p-TrkB levels. Data are presented as the mean ± SEM ($n = 5$). *$p < 0.05$ compared to the control group (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

upregulated in comparison with the C group [$F_{(3,\ 16)} = 64.73$; $p < 0.0001$; **Figures 3I,L**]. By binding with BDNF, the activation form of TrkB (p-TrkB) can lead to downstream signaling proteins activation. We found that there was no significant difference of TrkB between the C and S groups [$F_{(3,\ 16)} = 1.969$; $p = 0.1594$; **Figures 3J,M**]. However, the p-TrkB level was significantly downregulated in comparison with the C group [$F_{(3,\ 16)} = 22.29$; $p < 0.0001$; **Figures 3K,N**].

These results suggested that anesthesia/surgery downregulated BDNF and p-TrkB activation as well as synaptic protein (PSD-95, SYP) levels in the hippocampus. Correspondingly, the proBDNF and p75NTR were upregulated conversely. These indicated the role of BDNF/proBDNF imbalance played an important role in surgery-induced POCD in age mice.

P75NTR Inhibitor and Exogenous Brain-Derived Neurotrophic Factor Rescued Brain-Derived Neurotrophic Factor/Pro Brain-Derived Neurotrophic Factor Imbalance, Receptors, and Synaptic Proteins Expression in Aged Mice With Postoperative Cognitive Dysfunction

To determine the effect of p75NTR inhibitor and exogenous administration of BDNF on the imbalance of BDNF/proBDNF expression in aged mice with POCD, we detected the protein levels on POD3. The levels of proBDNF significantly decreased in Sur-B group, while there was no statistic difference between Sur-T group and Sur-S group [$F_{(2,\ 12)} = 55.37$, $p < 0.0001$; Sur-S vs. Sur-B, $p < 0.0001$; Sur-S vs. Sur-T, $p = 0.2542$; **Figures 4A,D**]. In stark contrast, the levels of BDNF significantly increased in Sur-T group and Sur-B group [$F_{(2,\ 12)} = 87.97$; $p < 0.0001$; **Figures 4A,E**]. We used the ratios to determine whether TAT-pep5 and exogenous BDNF rescued the imbalance of BDNF/proBDNF levels. It turned out these two interferences could upregulate the ratios of BDNF and proBDNF [$F_{(2,\ 12)} = 239.3$; $p < 0.0001$; **Figure 4F**].

Receptor activation and synaptic protein expression were consistent with the ratio alternation of BDNF/proBDNF. The PSD-95 level increased in the Sur-T group and Sur-B group [$F_{(2,\ 12)} = 26.61$, $p < 0.0001$; Sur-S vs. Sur-T, $p = 0.0004$; Sur-S vs. Sur-B, $p < 0.0001$; **Figures 4B,G**]. The SYP level also improved obviously in Sur-T group and Sur-B group [$F_{(2,\ 12)} = 29.42$, $p < 0.0001$; Sur-S vs. Sur-T, $p = 0.003$; Sur-S vs. Sur-B, $p < 0.0001$; **Figures 4C,H**]. We used the p-TrkB/TrkB ratio to determine the receptor activation (**Figures 4I–K**). P-TrkB/TrkB level increased in the Sur-T group and Sur-B group [$F_{(2,\ 12)} = 141.2$, $p < 0.0001$; Sur-S vs. Sur-T,$p = 0.0002$; Sur-S vs. Sur-B, $p < 0.0001$; **Figure 4L**].

These results indicated that p75NTR inhibitor (TAT-pep5) would not affect the expression level of proBDNF while its beneficial effect on POCD could somehow upregulate the expression level of BDNF. In the meantime, exogenous administration of BDNF could downregulate the proBDNF level accordingly. Both of them lead to ratio alternation, which ameliorates cognitive impairment in aged mice.

P75NTR Inhibitor and Exogenous Brain-Derived Neurotrophic Factor Improved Synaptic Plasticity and Alleviated Anesthesia/Surgery-Induced Memory Loss in Aged Mice With Postoperative Cognitive Dysfunction

The morphology of dorsal hippocampus and CA1 neurons among 3 groups is shown in **Figures 5A,B**. In terms of synaptic structural plasticity, TAT-pep5, and exogenous BDNF significantly upregulated the total dendritic length in Sur-T and Sur-B groups compared to the Sur-S group [$F_{(2,\ 51)} = 107.5$; $p < 0.0001$; **Figure 5C**]. Sholl analysis showed that the total number of dendritic intersections at 80—90,130 μm from the soma improved in Sur-T group [$F_{(24,\ 350)} = 3.015$; $p < 0.0001$; **Figure 5D**]. Likewise, the number of dendritic intersections at 50–130 μm from the soma were largely improved in the Sur-B group [$F_{(24,\ 350)} = 7.303$; $p < 0.0001$; **Figure 5D**]. Similarly, the spine density in Sur-T and Sur-B groups apparently increased in comparison to the Sur-S group [$F_{(2,\ 51)} = 132.7$; $p < 0.0001$; **Figure 5E**]. In terms of synaptic functional plasticity, hippocampal LTP in CA1 region was evaluated on POD3. As it turned out, p75 inhibitor and exogenous BDNF alleviated the LTP and amplitude loss in Sur-S group. Average fEPSP [$F_{(2,\ 42)} = 36.12$; $p < 0.0001$] and amplitude [$F_{(2,\ 42)} = 33.13$; $p < 0.0001$] improved largely compared to the Sur-S group (**Figures 5F,G**). In the hippocampal slices, LTP in Sur-T and Sur-B groups earned an obvious promotion compared to the Sur-S group, same as the amplitude (**Figures 5H,I**). These results suggested p75 inhibitor and extraneous BDNF may alleviate surgery-induced synaptic plasticity dysfunction, which attenuated cognitive impairment in aged mice.

In the OFT, the total distance [$F_{(2,\ 21)} = 0.2013$; $p = 0.8193$] and center time [$F_{(2,\ 21)} = 0.2439$; $p = 0.7858$] were not significantly different among the 3 groups at baseline (**Figures 5J,L**) or on POD3 [total distance $F_{(2,\ 21)} = 3.655$; $p = 0.0435$; center time $F_{(2,\ 21)} = 2.412$; $p = 0.1140$; **Figures 5K,M**]. These results showed that drug delivery regimen or the orthopedic operation did not affect the locomotor ability of mice. Moreover, p75 inhibitor and exogenous BDNF increased the freezing time in Sur-T and Sur-B groups compared to the Sur-S group in the context test [$F_{(2,\ 21)} = 68.75$; $p < 0.0001$; **Figure 5N**]. To sum up, these results indicated that inhibition of p75NTR and exogenous BDNF rescued hippocampus-dependent cognitive decline induced by anesthesia/surgery in aged mice.

DISCUSSION

The purpose of the current study showed that the balance of BDNF and proBDNF played a critical role in anesthesia/surgery-induced POCD in aged mice. We found that the expression of BDNF was largely decreased under surgical trauma with anesthesia in aged mice with POCD, while the proBDNF

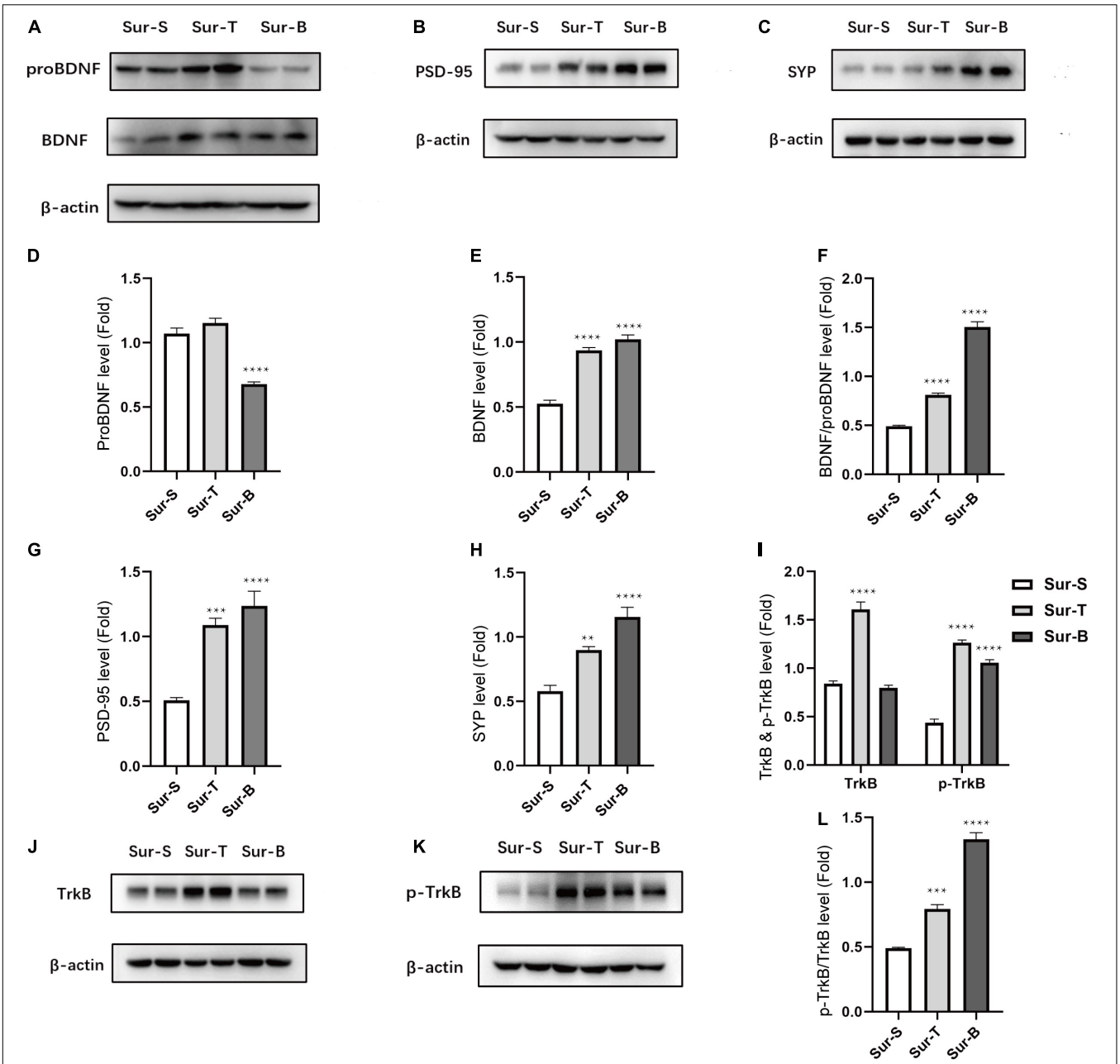


FIGURE 4 | Inhibition of p75NTR and exogenous BDNF rescued ratio imbalance and related proteins changes in hippocampus of aged mice with POCD. **(A–C)** Representative western blots of proBDNF, BDNF, PSD-95, and SYP in hippocampus on POD3. **(D–H)** Quantitative analysis of proBDNF, BDNF, BDNF/proBDNF, PSD-95, and SYP levels. **(J,K)** Representative western blots of TrkB, p-TrkB in hippocampus on POD3. **(I–L)** Quantitative analysis of p-TrkB/TrkB, TrkB, p-TrkB levels. Data are presented as the mean ± SEM ($n = 5$). $^{**}p < 0.01$ compared to the control group ($^{***}p < 0.001$, $^{****}p < 0.0001$).

level was increased. In addition, anesthesia/surgery exerted a detrimental effect on synaptic function which gives us a glimpse of the correlation between the imbalanced BDNF/proBDNF expression and synaptic plasticity in hippocampus of POCD mice. Moreover, the p75NTR inhibitor and the exogenous BDNF could rescue surgery-induced imbalance of BDNF and its precursor, and establish a relative new ratio homeostasis, which further ameliorates the synaptic dysfunction and cognitive impairment. Hence, these results indicated the vital role of BDNF/proBDNF in the therapeutic cognitive dysfunction induced by anesthesia/surgery in aged mice.

Although the pathological mechanism of POCD is complex and intricate, there is accumulating evidence suggesting the correlation between deficits of synaptic plasticity and POCD. For instance, surgical trauma could activate the hippocampal complement C3, causing a decrease in the expression of pre-

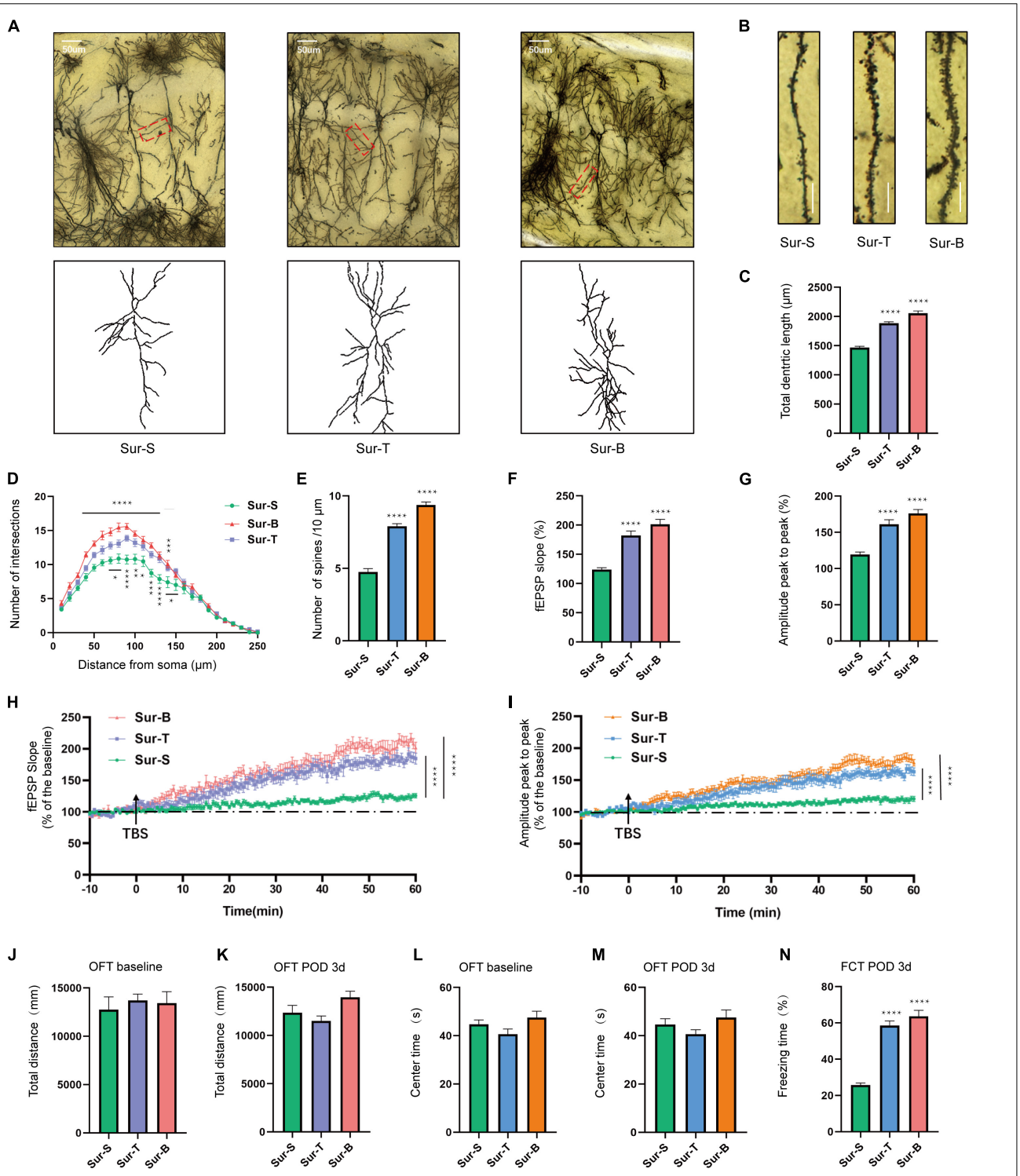


FIGURE 5 | Inhibition of p75NTR and exogenous BDNF ameliorated hippocampal synaptic plasticity impairment and facilitate memory consolidation of aged mice with POCD. **(A)** Sample image of Golgi-Cox staining and camera tracings of hippocampal CA1 neurons among the 3 groups. Scale bar = 50 μm. **(B)** Representative dendritic spine density of hippocampal CA1 neurons. Scale bar = 10 μm. **(C)** Quantification of the total dendritic length (n = 18 from 3 mice). **(D)** Quantification of dendritic intersections (n = 18 from 3 mice). **(E)** Quantification of the spine density (n = 18 from 3 mice). **(F,G)** Average fEPSP slope/amplitude during the last 10 min. **(H,I)** LTP/amplitude recording in the hippocampal CA1 region. Data are the percentage of mean fEPSP slopes/amplitude recorded during the baseline period (n = 15 slices from 5 mice). Arrows show the time point of TBS delivery. **(J–M)** The total distance and time spent in the center arena on day −1 and on POD1, 3, 7. **(N)** The percent of freezing time in the FCT context was assessed on POD3. Data are presented as the mean ± SEM (n = 8) $^{*}p < 0.05$ compared to the control group ($^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

and post-synaptic proteins SYP and PSD-95, which contributed to cognitive decline (Xiong et al., 2018). Moreover, surgical trauma could disrupt PGE2-EP3 signaling pathway by reducing hippocampal synaptic plasticity-related proteins, such as CREB, BDNF, and Arc, which participate in the formation of LTP (Xiao et al., 2018). Anesthesia/surgery led to mammalian target of rapamycin (mTOR) hyperactivity in the hippocampus and interfered with synaptic proteins homeostasis by inhibiting autophagy and resulted in cognitive impairment (Gao et al., 2021). Early pathological stage of POCD created an environment of inflammation and stimulated A1-specific astrocyte responses ultimately leading to sustained synaptic inhibition and cognitive deficiency (Li et al., 2020). In our study, we proved that anesthesia/surgery-induced an abnormal conversion of BDNF and its precursor (proBDNF), breaking the stable balance of the ratio in hippocampus of aged mice and consequently, leading to POCD *via* modulating the synaptic plasticity.

The ratio of BDNF/proBDNF varies between particular stages of brain development and regions. Pro-BDNF is highly concentrated in the postnatal period and drops gradually while BDNF expresses throughout postnatal development and prevails in adulthood (Yang et al., 2009, 2014). Efficient conversion to BDNF in adulthood is pivotal for its beneficial function in neuronal synaptic plasticity (Kowiański et al., 2018). As for the aging period, the study indicated that proBDNF and p75NTR increased largely in aged mice. Though the BDNF level has not shown any statistical difference, tPA, plasmin, and p-TrkB/TrkB declined accompanied with aging (Buhusi et al., 2017). These results fully illustrate in aged mice, proBDNF/BDNF increases (partly attributes to the reduction level of tPA and plasmin) and negatively regulates synaptic remodeling in coordinate with p75NTR. P75NTR inhibitor could rescue the synaptic deficits and memory consolidation. In our study, anesthesia/surgery not only induced reduction in BDNF level but also increment in proBDNF and p75NTR levels that have not been verified in other studies. The opposite changes down-regulated BDNF/proBDNF and led to dendritic spine shrinkage and declined in LTP. We also detected the rescue effect of the p75NTR inhibitor has not shown a significant impact on the proBDNF expression, while its beneficial impact on spine density, synapse proteins, and LTP promotion could feedback upregulate BDNF level and increase the BDNF/proBDNF ratio. We speculate that TAT-pep5 may influence the conversion of proBDNF to BDNF by reducing the formation of other fragments and facilitating more BDNF formation. It is known that the binding of proBDNF to p75NTR will trigger the association between p75NTR and RhoGDI, which eventually results in the reduction in spine density by modulating RhoA activity (Yamashita and Tohyama, 2003; Koshimizu et al., 2009), and RhoA activation is also essential in LTP induction by means of cofilin phosphorylation and inactivation (Rex et al., 2009). These results indicated that TAT-pep5, which blocked the interaction between p75NTR and RhoGDI, could largely improve synaptic plasticity by modulating BDNF/proBDNF. Thus, p75NTR could be considered as a potential therapeutic target for aging-related memory deficiency.

The binding of BDNF to TrkB will initiate a positive signal to enhance synaptic transmission and facilitate LTP induction and dendritic spine growth. However, proBDNF associated with p75NTR will act as a negative signal to accelerate neuron death, synaptic degeneration, and LTD (Barker, 2009). Previous studies suggested anesthesia/surgery-induced the activation of hippocampal microglia, the release of IL-1β, and the reduction of BDNF, which led to the cognitive deficiency in elderly mice (Qiu et al., 2016). Research also suggested BDNF expression and phosphorylation/activation of TrkB and ERK declined after surgery, while the expression of total TrkB was not significantly affected (Fan et al., 2016). The latest study also suggested that anesthesia/surgery-induced reduction of BDNF expression, and the study proposed that neuroinflammation and overactivated calcium signaling pathway may facilitate these changes. It was also suggested the expression of total TrkB was significantly decreased (Qiu et al., 2020) which contradicts with the study mentioned above. We reckon this discordance may attribute to the different surgical types, age of mice, and time point of tissue harvesting. In our study, we also found that the anesthesia/surgery reduced the expression of BDNF and p-TrkB, while the levels of total TrkB not statistically decreased. It is shown in other work that the anesthesia/surgery-induced spine density loss (Qiu et al., 2020; Liu et al., 2021), LTP decrease (Liu et al., 2021), and synapse proteins reduction (Xiong et al., 2018), which were consistent with previous research. To further understand the roles of BDNF and proBDNF in the development of POCD, exogenous BDNF and p75 inhibitor were microinjected into the mice hippocampus. The BDNF level increased as expected after being injected with exogenous BDNF, however, it is interesting that the proBDNF level decreased. We speculate the extra BDNF can suppress the proBDNF expression built on a negative feedback regulation mechanism. In this study, we also found that exogenous BDNF can promote the formation of dendritic spine arborization and LTP, which largely prospered the synaptic plasticity in the cognitively declined mice. The behavioral test shows that the anesthesia/surgery impaired hippocampus-dependent memory consolidation, which is consistent with the previous findings (Fan et al., 2016; Xiong et al., 2018; Qiu et al., 2020; Liu et al., 2021). In the FCT, context learning is associated with dorsal hippocampus, especially in the CA3 region. During the training session, we used the pattern of trace conditioning (interval or gap between US and CS) to isolate hippocampal involvement (Curzon et al., 2009). In accordance with the upregulation of BDNF/proBDNF by p75NTR inhibitor and exogenous BDNF, the memory decline has been largely improved. Taken together, these data indicated that alternation of BDNF/proBDNF may be an efficient method in the therapy of POCD.

The mechanism of how the imbalanced BDNF/proBDNF occurred in the current study deserved deeper thinking. Neuroinflammation is considered to be involved with several signaling pathways in correlation with BDNF. Nuclear factor-kappa B (NF-κB) is a transcription factor as well as the main factor of inflammatory activator, which can induce the expression of pro- and anti-apoptotic genes, including BDNF (Lima Giacobbo et al., 2019). Surgery-induced neuroinflammation triggered iron imbalance, microglia activation, and BDNF

impairments, while deferoxamine (a clinically used iron chelator) improved memory impairment by ameliorating inflammation and upregulating BDNF expression (Li et al., 2016). BDNF-pathway has been indicated as a mediator between neuroinflammation and cognitive impairment, and also IL-1β played a crucial role in regulation of BDNF levels (Hovens et al., 2014). So we suppose that the abnormal expression of BDNF/proBDNF level may be largely due to the inflammatory in anesthesia/surgery-induced brain disorders which remained further testified in our future study.

Accumulating evidence suggested that BDNF/proBDNF imbalance also impacts a variety of neurological diseases aside from cognitive decline. BDNF/proBDNF in hippocampus is critical in CUMS-induced depressive-like behaviors by alternating dendritic spines in the hippocampal CA1 pyramidal neurons (Qiao et al., 2017). BDNF/proBDNF is also crucial in the pathogenesis of post-stroke depression (PSD) in ischemic hippocampus. Aerobic exercise could relieve depression symptoms, promote neurogenesis, and increase the ratio of BDNF/proBDNF in the ischemic hippocampus of PSD rats (Luo et al., 2019). BDNF and proBDNF and their associated proteins participated in the pathogenesis and recovered from photothrombotic ischemia (Rahman et al., 2018). Our study indicates the ratio of BDNF/proBDNF is a key role in POCD in aged mice by modulating synaptic plasticity, which may open up new avenues in the research of neurocognitive diseases.

There are some limitations in this study. First, only short-term (7 days) cognitive performance by FCT after anesthesia and surgery was evaluated. It is expected that the detection of the effect on medium term (1–3 months), as well as the spatial learning and memory measured by Morris water maze or Barnes maze experiments should be investigated in a future study. Second, an anesthesia control group was not set up since we reckoned general anesthesia and surgical procedure were undividable in clinical practice. Studies revealed that inhaled anesthetic was also a key element involved in the POCD pathogenesis (Ni et al., 2015; Chen et al., 2020), so we would use sevoflurane as the inhaled anesthetic in the further study, which is consistent with the clinical procedures. Third, a C group was not established in experiment section B. since we considered the procedure of stereotactic brain injection itself as a minor craniocerebral surgery, so the Sur + S was used as the baseline. In the future study, a C + saline group (without orthopedic surgery) is expected to be set up for receiving a more convincing conclusion. Forth, there is a lack of explanation of the obvious upregulation of TrkB level in Sur-T. Alternatively, we used p-TrkB/TrkB to evaluate the signal activation degree in different groups. It is supposed such phenomenon might be derived from a feedback regulation intertwined with other receptors, though intensive research is needed to be further performed.

CONCLUSION

In summary, our study demonstrated that anesthesia/surgery-induced the imbalance of BDNF/proBDNF expression in the hippocampus and deteriorated neuronal synaptic plasticity. The broken homeostasis may ultimately cause cognitive impairment and memory decline. The result of this study suggests a novel hint in rescuing cognitive impairment by regulating the BDNF/proBDNF ratio. The TAT-pep5 (p75 inhibitor) and the exogenous BDNF can upregulate the ratio, which may be a potential therapeutic treatment of anesthesia/surgery-induced cognitive impairment.

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/s.

ETHICS STATEMENT

The animal study was reviewed and approved by all procedures were approved by the Institutional Animal Care and Use Committee at Capital Medical University (protocol AEEI-2020-117).

AUTHOR CONTRIBUTIONS

CW and ZX designed the research. ZX, MS, and XL performed the research. MS, YS, and JL analyzed the data. ZX contributed to writing-original draft preparation. CW, AW, and TL contributed to writing, review, and editing, supervision, funding acquisition. All authors approved the final manuscript.

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The Association Between Alzheimer's Disease-Related Markers and Physical Activity in Cognitively Normal Older Adults

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Previous studies have indicated that physical activity may be beneficial in reducing the risk for Alzheimer's disease (AD), although the underlying mechanisms are not fully understood. The goal of this study was to evaluate the relationship between habitual physical activity levels and brain amyloid deposition and AD-related blood biomarkers (i.e., measured using a novel high-performance mass spectrometry-based assay), in apolipoprotein E *(APOE)* ε4 carriers and noncarriers. We evaluated 143 cognitively normal older adults, all of whom had brain amyloid deposition assessed using positron emission tomography and had their physical activity levels measured using the International Physical Activity Questionnaire (IPAQ). We observed an inverse correlation between brain amyloidosis and plasma beta-amyloid $(A\beta)_{1-42}$ but found no association between brain amyloid and plasma $A\beta_{1-40}$ and amyloid precursor protein $(APP)_{669-711}$. Additionally, higher levels of physical activity were associated with lower plasma $A\beta_{1-40}$, $A\beta_{1-42}$, and $APP_{669-711}$ levels in *APOE* ε4 noncarriers. The ratios of $A\beta_{1-40}/A\beta_{1-42}$ and $APP_{669-711}/A\beta_{1-42}$, which have been associated with higher brain amyloidosis in previous studies, differed between *APOE* ε4 carriers and non-carriers. Taken together, these data indicate a complex relationship between physical activity and brain amyloid deposition and potential blood-based AD biomarkers in cognitively normal older adults. In addition, the role of *APOE* ε4 is still unclear, and more studies are necessary to bring further clarification.

Keywords: Alzheimer's disease, amyloid, physical activity, *APOE* genotype, biomarkers

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia in older adults, and the number of affected individuals is set to escalate in the coming decades. Currently, there is no cure for AD, and available pharmaceutical therapies are focused on relieving the severity of associated symptoms. Extracellular plaques primarily comprising beta-amyloid (Aβ) deposits are one of the major hallmark pathologies of AD. The deposition of Aβ in the brain has been demonstrated to occur many years before the onset of clinical symptoms and contributes to neuronal death and loss of cognitive abilities (Villemagne et al., 2013). Thus, this research is focused on understanding and identifying interventions that can slow amyloid deposition and improve cognition in cognitively normal (CN) older adults who are currently at-risk for AD (based on high brain amyloid deposition) and in individuals with an AD diagnosis.

High levels of physical activity (PA) have been reported as one lifestyle factor that may protect against the development of AD pathology and, in addition, can slow brain atrophy and the associated progressive cognitive decline (Schultz et al., 2015; Delli Pizzi et al., 2020; Dougherty et al., 2021). Substantial evidence exists from animal studies to support the role of exercise in reducing brain Aβ levels, likely through multiple mechanisms. These mechanisms affect the Aβ production, by shifting the processing of amyloid precursor protein (APP) toward the non-amyloidogenic pathway *via* increases in a disintegrin and metalloproteinase (ADAM10) and reductions in beta-site APP cleaving enzyme (BACE). In addition, exercise in animal models has also demonstrated increased Aβ catabolism by upregulating Aβ-degrading enzymes such as insulin-degrading enzyme and neprilysin, among others (Moore et al., 2016; Koo et al., 2017; Khodadadi et al., 2018; Brown et al., 2019; Zhang X. et al., 2019; Zhang X. L. et al., 2019). Building on this animal work, several reports from human studies have linked higher levels of PA with lower brain amyloid deposition, measured under positron emission tomography (PET) using amyloid-binding ligands (Liang et al., 2010; Brown et al., 2012, 2013; Okonkwo et al., 2014; Rabin et al., 2019). As expected, CSF-related biomarkers were also affected by PA in cognitively healthy adults (Law et al., 2018).

Based on the current literature, there appears to be individual variability in the relationship between PA and AD-related pathologies, with carriage of the apolipoprotein E (*APOE*) ε4 alleles, the greatest known genetic risk factor for sporadic (i.e., non-familial) AD. PA has been associated with lower brain amyloid (Head et al., 2012; Brown et al., 2013) and Tau burdens (Brown et al., 2018) and preserved cognitive functions (Jensen et al., 2019) to a greater extent in *APOE* ε4 carriers, compared with non-carriers in both healthy controls and patients with AD. However, studies that have reported higher PA levels to be associated with reduced dementia risk years later and have provided inconsistent results regarding whether ε4 carriers or non-carriers receive the greatest benefit (Podewils et al., 2005; Rovio et al., 2005).

In addition to amyloid burden measured *via* neuroimaging, PA is associated with AD-related blood biomarkers such as plasma Aβ measured by enzyme-linked immunosorbent assays (ELISA) (Baker et al., 2010; Brown et al., 2013; Stillman et al., 2017). More recently, a high-performance immunoprecipitation-mass spectrometry (IP-MS) assay quantifying plasma Aβ peptides was validated in two independent cohorts (Nakamura et al., 2018). This assay was able to differentiate between individuals with high brain amyloid deposition from those with low brain amyloid deposition. The high specificity and sensitivity are key features of this assay and may highlight differences in Aβ levels that could go undetected when using less sensitive assays. As a consequence, this more sensitive Aβ assessment could be used to more specifically evaluate the efficacy of medical/physical therapies with regard to blood-based biomarkers. As different techniques of Aβ measurement may yield different observations, the goal of this study was to assess the relationship between brain Aβ deposition, AD-related blood biomarkers (assessed with a high-performance mass spectrometry assay), and habitual PA levels. We also wanted to evaluate whether the association between PA and AD-related biomarkers was more marked in *APOE* ε4 allele carriers.

MATERIALS AND METHODS

The AIBL Cohort and Procedures

The Australian Imaging, Biomarkers and Lifestyle (AIBL) study of aging was approved by the Human Research Ethics Committees of St. Vincent's Health, Hollywood Private Hospital, and Austin Health and Edith Cowan University (Australia). All methods were performed in accordance with the relevant guidelines and regulations. AIBL is a longitudinal study comprising older adults (age range of 64–88 years) who are CN, have mild cognitive impairment (MCI) or AD, and are evaluated every 18 months. A more detailed description of the recruitment process has been previously described (Ellis et al., 2009). In the AIBL cohort, more than 2,350 individuals have been enrolled to date, and all participants gave written and informed consent before participation. Participants attended the study site in the morning, after an overnight fast. Several physical parameters, such as weight, blood pressure, and pulse rate, were recorded, after which a fasting blood sample was collected for subsequent processing and analysis (Ellis et al., 2009). Cognitive and lifestyle evaluations were then performed, and diagnostic classifications (i.e., CN, MCI, or AD) were performed in accordance with the National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria by an expert clinical panel (Folstein et al., 1975; Mckhann et al., 1984; Saxton et al., 2000; Winblad et al., 2004). For this study, a total of 143 CN participants (Mini-Mental State Examination [MMSE] ≥ 25) (Pangman et al., 2000) who were previously assessed for brain amyloidosis, PA, and blood biomarkers, assessed with the novel high-performance IP-MS assay, employed by Nakamura and colleagues (Nakamura et al., 2018), were included. We acknowledged that, however, the absence of brain Tau levels (either by imaging or biofluid) is a limitation of this study.

Blood Collection and APOE Genotype

Plasma was isolated from whole blood collected in ethylenediaminetetraacetic acid (EDTA) tubes by centrifugation, aliquoted, and stored at −80°C. The *APOE* ε4 status was determined by genotyping cells from whole blood as previously described (Gupta et al., 2011).

Measurement of Aβ Species

Plasma Aβ levels were measured using IP-MS to quantify Aβ-related peptides of different mass using matrix-associated laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry after isolation and enrichment by immunoprecipitation from plasma. Briefly, 250 μl of plasma was mixed with an equal volume of Tris buffer [10 pM stable-isotope-labeled (SIL) $A\beta_{1-38}$ peptide, 0.2% w/v *n*-dodecyl-β-D-maltoside (DDM), and 0.2% w/v *n*-nonyl-β-D-thiomaltoside (NTM)]. Normalization of the signal for all Aβ-related peptides was performed using the SIL-$A\beta_{1-38}$ peptide as internal standard, while DDM and NTM were used for reducing nonspecific binding. Antibody beads were prepared by coupling monoclonal antibody 6E10 (BioLegend) directly to Dynabeads M-270 Epoxy and then used to immunoprecipitate plasma Aβ-related peptides and the internal standard by incubating them with plasma samples for 1 h. Elution of the peptides was performed using glycine buffer (pH 2.8) containing 0.1% w/v DDM. Upon the adjustment of the pH to 7.4 with Tris buffer, the immunoprecipitation was repeated, and the peptides were eluted with 5 mM HCl in 70% acetonitrile and applied on four wells of a 900 μm μFocus MALDI plateTM (Hudson Surface Technology) prespotted with α-cyano-4-hydroxycinnamic acid (CHCA) and methanediphosphonic acid (MDPNA). MALDI-linear TOF mass spectrometer (AXIMA Performance, Shimadzu/KRATOS) equipped with a 337 nm nitrogen laser in the positive ion mode was used to acquire mass spectra. The levels of plasma Aβ-related peptides were normalized with SIL-$A\beta_{1-38}$ and used as plasma Aβ-related peptide levels. The reproducibility of the assay was verified using human EDTA plasma. The intra- and interday assay coefficients of variance (CVs) obtained for $A\beta_{1-40}$ were 4.2–4.7% ($n = 5$) and 3.2–6.8% ($n = 3$), respectively; for $A\beta_{1-42}$, the CVs were 6.8–7.8% and 1.6–7.7%, respectively, and for $APP_{669-711}$, the CVs were 2.9–8.2% and 4.7–10.7%, respectively, supporting the reliability of the measurements. A more detailed description of the mass spectrometry methods is reported elsewhere (Nakamura et al., 2018).

Imaging Data

All participants within the current study underwent a PET scan with either Pittsburgh compound B (^{11}C-PiB), flutemetamol (^{18}F-FLUTE), or florbetapir (^{18}F-FBP) to measure brain amyloid load (Pike et al., 2007). The PET methodology for each tracer has been described elsewhere (Rowe et al., 2010; Vandenberghe et al., 2010; Wong et al., 2010). For the semiquantitative analysis, a standardized uptake value (SUV) was obtained from cortical and subcortical brain regions and then related to the SUV of the recommended reference region of each tracer to generate a tissue ratio termed the SUV ratio (SUVR). For PiB, the SUVs were normalized to the cerebellar cortex; for flutemetamol, the SUVs were normalized to the whole cerebellum, and for florbetapir, the SUVs were normalized to the pons (Clark et al., 2011; Lundqvist et al., 2013). For the combination of data from different PET tracers, the Before the Centiloid Kernel Transformation (BeCKeT) values were used, which represent a linear transformed standardization of FLUTE and FBP SUVR onto "PiB-like" SUVR (Villemagne et al., 2014). The cutoff value used to define brain amyloidosis was 1.4, such that participants considered amyloid negative (Aβ-) had a BeCKeT SUVR score $<$ 1.4 and those considered amyloid positive (Aβ+) had a BeCKeT SUVR score $\geq$ 1.4.

Measurement of Physical Activity

Levels of PA were measured using the International Physical Activity Questionnaire (IPAQ) (Craig et al., 2003). The IPAQ is a subjective questionnaire that relies on participants to recall their PA from the previous 7 days. It is composed of 4 sections, namely, work activity, transportation activity, housework, and leisure-time activity. A metabolic equivalent score (MET) was associated with each question, and the total of the MET score was then assessed by multiplying the MET scores by the number of minutes per week spent participating in that activity to produce a 7-day activity score (i.e., METs min/week). We excluded questionnaires in which reported PA levels were two SDs above or below the mean, as well as incomplete questionnaires. The IPAQ has been validated in several studies indicating that the questionnaire is suitable for the measurement of PA (Craig et al., 2003; Hagstromer et al., 2006). Based on the standard IPAQ scoring instructions, participants within this study were divided into individuals with low-to-moderate level physical activity (LMPA; combined due to small number in low group) or high physical activity (HPA), using the same parameters described elsewhere (Brown et al., 2018). Our analysis used self-reported levels of PA, and although we acknowledged self-report can be erroneous, the IPAQ is a validated tool, and within the AIBL study cohort, we have reported associations between self-reported IPAQ data and measures of objective PA using actigraphy (Brown et al., 2012).

Statistical Analysis

Descriptive statistics including means and SDs or proportions were calculated for LMPA and HPA groups, with comparisons employing independent sample *t*-tests or χ^2 tests as appropriate. Linear models were employed to compare continuous variables (i.e., $A\beta_{1-40}$, $A\beta_{1-42}$, $APP_{669-711}$, $A\beta_{1-40}/A\beta_{1-42}$ ratio, and $APP_{669-711}/A\beta_{1-42}$ ratio) between categories, corrected for covariates age and sex. Response variables were log transformed as necessary to better approximate normality and variance homogeneity. The composite *z*-score was also calculated and used in linear model analyses (*z*-score: average of $A\beta_{1-40}/A\beta_{1-42}$ ratio and $APP_{669-711}/A\beta_{1-42}$ ratio individual *z*-scores [(*z*-score $A\beta_{1-40}/A\beta_{1-42}$ ratio + *z*-score $APP_{669-711}/A\beta_{1-42}$ ratio)/2]). Analyses were also run stratifying the cohort based on *APOE* ε4 carriage (ε4–/ε4+) and brain amyloid status (Aβ-/Aβ+). Associations between continuous variables were assessed using linear regression and partial correlation, with corrections for sex, age, and *APOE ε4* allele carriage status. A *p*-value $<$ 0.05 was

TABLE 1 | Demographic information of study participants based on self-reported physical activity groups.

	Total sample ($n = 143$)	LMPA ($n = 65$)	HPA ($n = 78$)	*p*
Age (years, mean ± SD)	74.0 ± 5.5	73.9 ± 5.5	74.0 ± 5.5	0.912
Sex (*n* M/F)	77/66	32/33	45/33	0.312
Brain Aβ status (*n* Aβ −/+)	81/62	36/29	45/33	0.782
APOE ε4 allele carriers (*n* −/+)	91/52	41/24	50/28	0.899
PiB/FLUTE/FBP (*n*)	60/39/44	26/19/20	34/20/24	0.871

Demographic data of study participants were stratified by physical activity levels. Low-to-moderate physical activity (LMPA) and high physical activity (HPA) levels were assessed using the International Physical Activity Questionnaire (IPAQ). Brain amyloid deposition was measured using positron emission tomography. Values are expressed as mean ± SD or as the number of cases overall. A univariate analysis or χ^2 analysis was used to assess differences between the LMPA and HPA groups.

regarded as significant. Analyses were carried out using the SPSS version 25 software (Chicago, IL, USA).

RESULTS

Demographic details of the study participants are summarized in **Table 1**. No significant differences in age, sex, brain amyloid deposition, *APOE* ε4 carriage, and tracer used were found between participants with LMPA and HPA levels.

We evaluated the differences in plasma AD-biomarker levels (i.e., $A\beta_{1-42}$, $A\beta_{1-40}$, and $APP_{669-711}$) between the PA groups in all participants and after stratifying by *APOE* ε4 carriage status or brain amyloid status (**Table 2**). Lower plasma $A\beta_{1-42}$ levels were observed in the HPA group compared with those in the LMPA group, in all participants ($p = 0.017$ and $p = 0.024$, unadjusted and adjusted, respectively). After stratifying by *APOE* ε4 status or brain amyloid status, this difference was evident only in non-ε4 carriers ($p = 0.003$ and $p = 0.004$, unadjusted and adjusted, respectively) and in the Aβ- group ($p = 0.014$ and $p = 0.012$, unadjusted and adjusted, respectively). A total of 60 individuals (out of 81) who are brain Aβ- were also *APOE* non-ε4 carriers (out of a total of 91). This may explain why brain Aβ- and *APOE* non-ε4 carrier groups appear to have similar results.

Similarly, lower plasma $A\beta_{1-40}$ levels were observed in the HPA group compared with the LMPA group, in all participants ($p = 0.043$, unadjusted and a trend toward statistical significance upon adjustment, $p = 0.057$). Further, after stratifying by *APOE* ε4 status or brain amyloid status, this difference was evident only in the non-ε4 carriers ($p = 0.020$ and $p = 0.019$, unadjusted and adjusted, respectively) and in the Aβ- group ($p = 0.043$ and $p = 0.031$, unadjusted and adjusted, respectively) (**Table 2**). Plasma $APP_{669-711}$ was observed to be lower in the HPA group compared with that in the LMPA group, in all participants ($p = 0.006$ and $p = 0.007$, unadjusted and adjusted, respectively). After stratifying by *APOE* ε4 status or brain amyloid status, this difference was significant only in the non-ε4 carriers ($p < 0.001$ for both unadjusted and adjusted) and in the Aβ- group ($p = 0.012$ for both unadjusted and adjusted) (**Table 2**).

TABLE 2 | Comparison of plasma biomarkers between low-to-moderate physical activity (LMPA) and high physical activity (HPA) groups.

	LMPA	HPA	p(F)	$p^a(F^a)$
$A\beta_{1-42}$				
(a) All	0.344 ± 0.087	0.312 ± 0.060	*0.017 (5.844)*	*0.024 (5.189)*
(b) ε4–	0.364 ± 0.090	0.316 ± 0.058	*0.003 (9.002)*	*0.004 (8.602)*
ε4+	0.311 ± 0.073	0.305 ± 0.064	0.772 (0.085)	0.986 (0.000)
(c) Aβ-	0.367 ± 0.091	0.324 ± 0.056	*0.014 (6.283)*	*0.012 (6.576)*
Aβ+	0.315 ± 0.074	0.295 ± 0.061	0.267 (1.257)	0.422 (0.655)
$A\beta_{1-40}$				
(a) All	8.731 ± 2.165	8.054 ± 1.686	*0.043 (4.184)*	0.057 (3.698)
(b) ε4–	8.948 ± 2.362	7.944 ± 1.537	*0.020 (5.655)*	*0.019 (5.745)*
ε4+	8.362 ± 1.766	8.251 ± 1.937	0.770 (0.086)	0.760 (0.094)
(c) Aβ-	8.849 ± 2.375	7.902 ± 1.631	*0.043 (4.220)*	*0.031 (4.817)*
Aβ+	8.586 ± 1.904	8.262 ± 1.761	0.483 (0.499)	0.867 (0.043)
$APP_{669-711}$				
(a) All	0.296 ± 0.061	0.270 ± 0.054	*0.006 (7.669)*	*0.007 (7.520)*
(b) ε4–	0.305 ± 0.064	0.259 ± 0.049	*<0.001 (15.512)*	*< 0.001 (14.810)*
ε4+	0.282 ± 0.054	0.289 ± 0.060	0.628 (0.237)	0.543 (0.375)
(c) Aβ-	0.294 ± 0.064	0.261 ± 0.050	*0.012 (6.612)*	*0.012 (6.580)*
Aβ+	0.299 ± 0.059	0.282 ± 0.059	0.227 (1.487)	0.260 (1.294)

Plasma $A\beta_{1-42}$, $A\beta_{1-40}$, and $APP_{669-711}$ levels were compared between low-to-moderate physical activity (LMPA) and high physical activity (HPA) groups in all (a) participants, (b) participants stratified by apolipoprotein E (APOE) ε4 genotype status (ε4–/ε4+), and (c) brain amyloid status (Aβ-/Aβ+), using general linear models. Physical activity was measured by the International Physical Activity Questionnaire (IPAQ), and brain amyloid deposition was measured using positron emission tomography. Plasma $A\beta_{1-42}$, $A\beta_{1-40}$, and $APP_{669-711}$ data were natural log transformed to better approximate normality and variance homogeneity. $p^a(F^a)$ represents p-values adjusted for age and sex. $P < 0.05$ (italic) was considered significant. Data are presented in mean ± SD.

We have also assessed the effect of PA with regard to brain amyloidosis, and we did not find any significant effect of PA before any stratification. The same results were observed after stratification for *APOE* ε4 status or brain Aβ status (**Supplementary Table 1**).

In **Table 3**, the ratios of plasma $APP_{669-711}/A\beta_{1-42}$ and $A\beta_{1-40}/A\beta_{1-42}$ were also assessed with regard to PA and *APOE* ε4 status or brain Aβ status, and no differences between the PA groups were observed in the whole cohort, nor after stratifying by *APOE* ε4 status or brain Aβ status. We also assessed the composite *z* score for plasma ratios of $APP_{669-711}/A\beta_{1-42}$ and $A\beta_{1-40}/A\beta_{1-42}$, and we did not observe any significant differences related to different intensities of PA. Stratifying by *APOE* ε4 status or brain amyloid status, like in our previous analyses, did not result in significant composite *z*-score differences in any of the subgroups (**Table 3**).

However, levels of plasma $A\beta_{1-42}$ were significantly lower ($p = 0.013$ and $p = 0.031$, unadjusted and adjusted, respectively), and the ratios $APP_{669-711}/A\beta_{1-42}$ and $A\beta_{1-40}/A\beta_{1-42}$ were significantly higher ($APP_{669-711}/A\beta_{1-42}$: $p < 0.001$ for both unadjusted and adjusted; $A\beta_{1-40}/A\beta_{1-42}$: $p = 0.001$ and $p < 0.001$, unadjusted and adjusted, respectively) in *APOE* ε4 carriers compared with the ε4 non-carriers

TABLE 3 | Comparison of plasma biomarkers ratios between low-to-moderate physical activity (LMPA) and high physical activity (HPA) groups.

	LMPA	HPA	p(F)	$p^a(F^a)$
$APP_{669-711}$/ $A\beta_{1-42}$				
(a) All	0.880 ± 0.148	0.874 ± 0.140	0.801 (0.064)	0.653 (0.203)
(b) ε4–	0.855 ± 0.140	0.826 ± 0.124	0.303 (1.073)	0.311 (1.039)
ε4+	0.924 ± 0.154	0.960 ± 0.127	0.355 (0.870)	0.556 (0.351)
(c) Aβ-	0.814 ± 0.130	0.809 ± 0.108	0.848 (0.037)	0.840 (0.041)
Aβ+	0.963 ± 0.128	0.963 ± 0.130	0.984 (0.000)	0.694 (0.157)
$A\beta_{1-40}/A\beta_{1-42}$				
(a) All	25.76 ± 4.16	25.97 ± 3.47	0.736 (0.114)	0.709 (0.140)
(b) ε4–	24.89 ± 4.07	25.28 ± 3.25	0.611 (0.261)	0.645 (0.124)
ε4+	27.24 ± 3.97	27.21 ± 3.58	0.977 (0.001)	0.659 (0.197)
(c) Aβ-	24.31 ± 3.90	24.43 ± 3.12	0.881 (0.022)	0.963 (0.002)
Aβ+	27.55 ± 3.81	28.08 ± 2.77	0.533 (0.394)	0.303 (1.079)
Composite z-score				
(a) All	−0.004 ± 0.882	0.003 ± 0.816	0.960 (0.003)	0.966 (0.002)
(b) ε4–	−0.208 ± 0.844	−0.256 ± 0.741	0.771 (0.085)	0.751 (0.101)
ε4+	0.344 ± 0.852	0.466 ± 0.744	0.582 (0.307)	0.534 (0.392)
(c) Aβ-	−0.427 ± 0.695	−0.429 ± 0.625	0.988 (<0.001)	0.927 (0.009)
Aβ+	0.522 ± 0.810	0.593 ± 0.665	0.703 (0.147)	0.720 (0.130)

$APP_{669-711}/A\beta_{1-42}$, and $A\beta_{1-40}/A\beta_{1-42}$ ratios and the composite z-score of the same ratios were compared between low-to-moderate physical activity (LMPA) and high physical activity (HPA) groups in all (a) participants, (b) participants stratified by apolipoprotein E (APOE) ε4 genotype status (ε4–/ε4+), and (c) brain amyloid status (Aβ-/Aβ+), using general linear models. Physical activity was measured by the International Physical Activity Questionnaire (IPAQ), and brain amyloid deposition was measured using positron emission tomography. $p^a(F^a)$ represents p-values adjusted for age and sex. P < 0.05 (italic) was considered significant. Data are presented in mean ± SD.

TABLE 4 | Comparison of the effect of APOE ε4 status and brain Aβ status on plasma $A\beta_{1-42}$ levels and blood biomarker ratios.

***APOE* ε4 status**				
$A\beta_{1-42}$	ε4–	ε4+	p(F)#	$p^a(F^a)$#
All	0.337 ± 0.077	0.308 ± 0.068	*0.013 (6.297)*	*0.031 (4.746)*
$APP_{669-711}/A\beta_{1-42}$	ε4–	ε4+	p(F)	$p^a(F^a)$
All	0.839 ± 0.131	0.943 ± 0.139	*< 0.001 (19.936)*	*< 0.001 (15.129)*
$A\beta_{1-40}/A\beta_{1-42}$	ε4–	ε4+	p(F)	$p^a(F^a)$
All	25.10 ± 3.62	27.22 ± 3.72	*0.001 (11.077)*	*< 0.001 (14.582)*
Brain Aβ status				
$A\beta_{1-42}$	Aβ-	Aβ+	p(F)#	$p^a(F^a)$#
All	0.343 ± 0.077	0.305 ± 0.068	*0.001 (11.733)*	*0.001 (11.876)*
$APP_{669-711}/A\beta_{1-42}$	Aβ-	Aβ+	p(F)	$p^a(F^a)$
All	0.811 ± 0.117	0.963 ± 0.128	*< 0.001 (54.898)*	*< 0.001 (54.199)*
$A\beta_{1-40}/A\beta_{1-42}$	Aβ-	Aβ+	p(F)	$p^a(F^a)$
All	24.38 ± 3.47	27.83 ± 3.28	*< 0.001 (36.509)*	*< 0.001 (36.304)*

Plasma $A\beta_{1-42}$ and the ratios $APP_{669-711}/A\beta_{1-42}$ and $A\beta_{1-40}/A\beta_{1-42}$ were compared with regards to APOE ε4 genotype status (ε4–/ε4+), and brain amyloid status (Aβ-/+) using general linear models. Brain amyloid deposition was measured using positron emission tomography. Plasma $A\beta_{1-42}$ data were natural log transformed to better approximate normality and variance homogeneity (#). $p^a(F^a)$ represents p-values adjusted for age and sex. P < 0.05 (italic) was considered significant. Data are presented in mean ± SD.

(**Table 4**). Similarly, the levels of $A\beta_{1-42}$ were significantly lower ($p = 0.001$ for both unadjusted and adjusted), and the ratios $APP_{669-711}/A\beta_{1-42}$ and $A\beta_{1-40}/A\beta_{1-42}$ were significantly higher ($APP_{669-711}/A\beta_{1-42}$: $p< 0.001$ for both unadjusted and adjusted; $A\beta_{1-40}/A\beta_{1-42}$: $p< 0.001$ for both unadjusted and adjusted) in the Aβ+ group compared with the Aβ- group (**Table 4**). More detailed analysis indicated that in most cases, the differences in $A\beta_{1-42}$ levels and $APP_{669-711}/A\beta_{1-42}$ and $A\beta_{1-40}/A\beta_{1-42}$ ratios are affected by *APOE* genotype and are irrespective of the intensity of the PA (**Supplementary Table 2**).

The linear regression analysis indicated that the levels of plasma $A\beta_{1-42}$ were significantly and negatively associated with brain amyloid deposition in the whole cohort and both PA groups ($p < 0.001$, $p = 0.010$ and $p < 0.001$ for the whole cohort, LMPA and HPA, respectively) (**Table 5**). These results were confirmed in partial correlation analyses upon correction for age, sex, and *APOE* ε4 status, ($p = 0.001$, $p = 0.027$, and $p = 0.001$ for the whole cohort, LMPA, and HPA, respectively) (**Table 5**). Conversely, no statistically significant associations were observed between brain amyloid and $A\beta_{1-40}$ or $APP_{669-711}$, regardless of whether the analysis was performed with or without adjustment for age, sex, and *APOE* ε4 status (**Table 5**).

DISCUSSION

In this report, we assessed the association between PA and blood biomarkers (plasma $A\beta_{1-40}$, $A\beta_{1-42}$, $APP_{669-711}$ levels) in CN older adults. Additionally, we examined how the carriage of the *APOE* ε4 allele (i.e., an important risk factor for sporadic AD) (Corder et al., 1993) affects the relationship between PA and AD biomarkers. We observed that (a) individuals reporting higher levels of PA had lower plasma AD biomarkers in *APOE ε4* noncarriers and brain Aβ deposition groups and (b) plasma biomarker ratios are associated with *APOE ε4* carrier status and with brain Aβ deposition status.

To date, the linkage between PA, risk of dementia, and *APOE* ε4 status are not clear. PA has shown to be inversely associated with brain amyloid deposition and to a greater extent in *APOE* ε4 carriers (Head et al., 2012; Brown et al., 2013); however, contradictory results have been reported when examining dementia risk as an outcome measure (Podewils et al., 2005; Rovio et al., 2005). Our results indicate that high PA has a trend-level association with lower brain amyloid levels only in individuals with high brain amyloidosis. Additionally, high PA is associated, albeit nonsignificantly, with lower SUVR in noncarriers of the *APOE* ε4 allele, while it was not a factor in *APOE* ε4 carriers. These data are in partial contradiction with previously published findings, also using AIBL study data (Brown

TABLE 5 | Correlation of brain amyloid load with blood biomarkers levels in the all-study participants and after stratification by PA levels.

Brain Aβ deposition (Ln Aβ)		All participants		LMPA		HPA	
		β	*p*	β	*p*	β	*p*
Plasma $A\beta_{1-42}$ (Ln $A\beta_{1-42}$)	Unadjusted	*−0.335*	*< 0.001*	*−0.318*	*0.010*	*−0.408*	*< 0.001*
	Adjusted*	*−0.290*	*0.001*	*−0.280*	*0.027*	*−0.389*	*0.001*
Plasma $A\beta_{1-40}$ (Ln $A\beta_{1-40}$)	Unadjusted	−0.016	0.850	−0.069	0.584	0.020	0.864
	Adjusted*	−0.007	0.935	−0.092	0.478	0.028	0.812
Plasma $APP_{669-711}$ (Ln $APP_{669-711}$)	Unadjusted	0.071	0.402	0.035	0.783	0.081	0.480
	Adjusted*	0.055	0.519	0.095	0.464	0.000	0.999

Linear regression was performed between brain Aβ deposition and plasma $A\beta_{1-42}$, $A\beta_{1-40}$, and $APP_{669-711}$ in all participants and after stratification based on physical activity levels. Data were natural log transformed to better approximate normality and variance homogeneity. Physical activity was measured by the International Physical Activity Questionnaire (IPAQ), and participants were stratified based on low-to-moderate-level physical activity (LMPA) and high physical activity (HPA). P < 0.05 (italic) was considered significant. Adjusted represents analyses adjusted for age, sex, and APOE ε4-carrier status.*

et al., 2013), in which the effect of PA on brain amyloid deposition was restricted to *APOE* ε4 carriers. Such discrepancies may come from the fact that these analyses were performed on different numbers of healthy controls, which may affect the final results. The main reason for using a different cohort was our interest in evaluating the effect of PA on AD biomarkers when these were assessed using a more specific assay. Such biomarkers were assessed using a high-performance mass spectrometry analysis (Nakamura et al., 2018), which has the advantage of having a higher specificity and sensitivity compared with commercial ELISAs, which have been widely used for the analysis of $A\beta_{1-40}$ and $A\beta_{1-42}$. Utilizing this more sensitive and more specific assay for the measurement of $A\beta_{1-42}$, $A\beta_{1-40}$, and $APP_{669-711}$, Nakamura et al. were able to identify individuals (i.e., CN, MCI, and AD) with aberrant brain amyloidosis. However, this study could only be performed using healthy controls from the AIBL cohort who had AD biomarkers assessed by this new technique, and while our results may suggest a novel approach, one of the limitations of this study is the size of the cohort used.

The analysis of our biomarkers indicated that higher PA was significantly associated with lower levels of $A\beta_{1-40}$, $A\beta_{1-42}$, and $APP_{669-711}$ in CN ε4 non-carriers, while no relationship was observed in those carrying at least one *APOE* ε4 allele. It must be noted, however, that while $A\beta_{1-42}$ levels were significantly higher in ε4 carriers, *APOE* ε4 status had no effect on $A\beta_{1-40}$ and $APP_{669-711}$ levels. These data are in accordance with other reports that show associations of higher PA with lower levels of plasma Aβ in humans and mouse models of AD (Baker et al., 2010; Stillman et al., 2017; Khodadadi et al., 2018), although our original report did not observe a similar association between plasma Aβ and PA (Brown et al., 2013). Our original report, however, indicated an effect of HPA on the $A\beta_{1-42}/A\beta_{1-40}$ ratio in *APOE ε4* noncarriers (Brown et al., 2013). While the size of the cohort may still be a limiting factor, the more sensitive Aβ assays may also play a role in justifying these discrepancies. Increased specificity and sensitivity may detect forms of Aβ that could go undetected in regular ELISA kits, and this could lead to assessing Aβ levels more accurately. This, in turn, would allow for more reliable analyses involving Aβ levels that could lead to a more appropriate assessment of biomarkers, as seen with the findings of Nakamura et al., where plasma Aβ and a related fragment reflected brain amyloidosis with high accuracy.

Additionally, an inverse correlation between brain amyloid deposition and Aβ plasma levels observed in AD participants within the AIBL cohort suggested that patients with AD with higher brain amyloid deposition had lower plasma $A\beta_{1-42}$ levels (Lui et al., 2010). These results indicate that in AD, $A\beta_{1-42}$ is sequestered in the brain in the form of amyloid plaques resulting in lower plasma $A\beta_{1-42}$ levels. As data from this study indicated that increased PA was associated with lower levels of brain amyloid and plasma levels of $A\beta_{1-40}$, $A\beta_{1-42}$, and $APP_{669-711}$, we then evaluated if such inverse correlation was retained. As shown, we have reported that there is a significant inverse correlation between brain amyloid and plasma $A\beta_{1-42}$, which was not affected by the level PA. One possible mechanism could be that the main effect of PA was associated with lower brain amyloid and plasma $A\beta_{1-40}$, $A\beta_{1-42}$, and $APP_{669-711}$ levels (in non-ε4 carriers only) and is more likely a consequence of a shift toward a non-amyloidogenic pathway, while it has no effect on the transport of Aβ through the blood-brain barrier into the circulation.

The ratio $A\beta_{1-40}/A\beta_{1-42}$ (or its inverse) and the ratio $APP_{669-711}/A\beta_{1-42}$ have also been indicated in previous reports as specific predictors of brain amyloidosis (Ovod et al., 2017; Nakamura et al., 2018; Chatterjee et al., 2019; Schindler et al., 2019) or AD (Lui et al., 2010). Our study, as all others, reported that these plasma biomarker ratios can significantly differentiate CN healthy controls with high brain amyloidosis vs. CN healthy controls with low brain amyloidosis; however, PA does not significantly alter these ratios. Similar results were obtained when stratified for *APOE* ε4 carrier status, where these ratios were significantly different in *APOE* ε4 carriers vs. noncarriers (due to the underlying *APOE* ε4 effect on plasma $A\beta_{1-42}$), but these results were not affected by PA. A schematic representation of how PA can affect plaque formation and $A\beta_{1-42}$ transport across blood-brain barrier (BBB) is illustrated in **Figure 1**.

To summarize, our data indicated that (a) PA was associated with lower levels of AD-related plasma biomarkers in healthy control *APOE* ε4 noncarriers and Aβ-individuals, (b) plasma

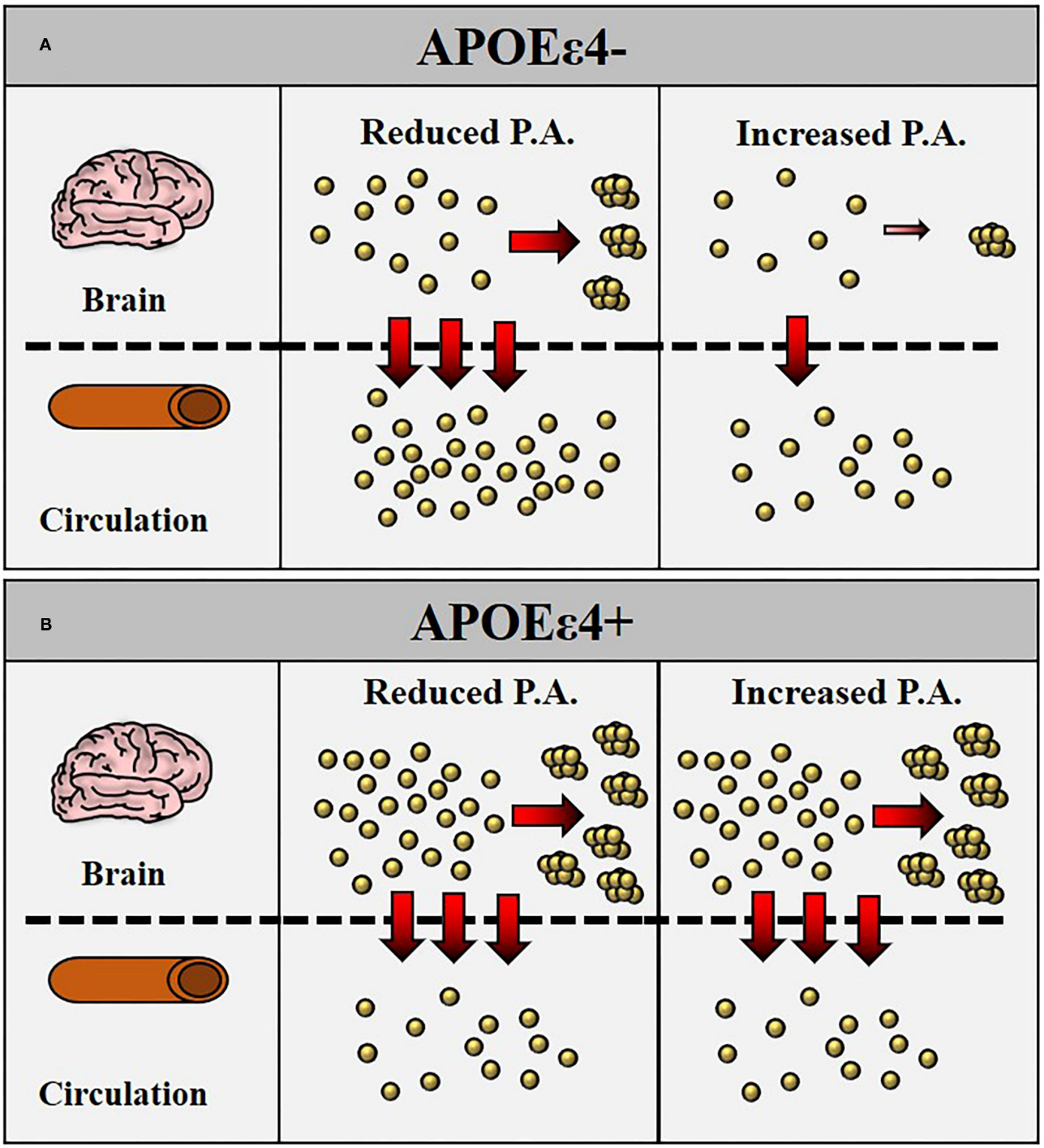


FIGURE 1 | Schematic representation of how physical activity (PA) may reduce Aβ generation with consequently reduced transport of $A\beta_{1-42}$ across the blood-brain barrier (BBB) into circulation. In apolipoprotein E *(APOE)* ε4– **(A)**, increased intensity of PA is followed by a reduced generation of $A\beta_{1-42}$ with consequently reduced plaque formation in the brain and reduced transport of $A\beta_{1-42}$ in the bloodstream. In *APOE* ε4+ **(B)**, the lower levels of $A\beta_{1-42}$ in the bloodstream are a consequence of increased $A\beta_{1-42}$ retention in the brain with greater plaque formation. The intensity of PA does not appear to affect $A\beta_{1-42}$ plasma levels.

levels of $A\beta_{1-42}$, but not $A\beta_{1-40}$ or $APP_{669-711}$, inversely correlated with brain amyloidosis, and (c) PA was associated with lower brain amyloidosis in healthy controls at risk of AD, although the analysis approaches statistical significance. Although we have indicated that our study has some limitations, we have reported that PA influences AD biomarker levels, likely affecting the process underlying the amyloidogenic pathway. Further studies are, therefore, necessary to confirm the validity of

our findings in a larger cohort and to determine the involvement of different levels of PA with regards to plasma AD biomarkers.

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/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Australian Imaging, Biomarkers and Lifestyle (AIBL) study of aging was approved by the Human Research Ethics Committees of St. Vincent's Health, Hollywood Private Hospital, Austin Health and Edith Cowan University (Australia). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SR-S, VD, NK, and BB carried out experiments and collected data. SP, PC, and BB analyzed data and wrote the manuscript. MT, EH, SG, KT, PB, AN, SR-S, VV, CR, DA, BF, IM, HS, CM, and RM provided scientific input. RM supervised the project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2022.771214/full#supplementary-material

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Physical Exercise-Induced Astrocytic Neuroprotection and Cognitive Improvement Through Primary Cilia and Mitogen-Activated Protein Kinases Pathway in Rats With Chronic Cerebral Hypoperfusion

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Chronic cerebral hypoperfusion (CCH) is closely related to vascular cognitive impairment and dementia (VCID) and Alzheimer's disease (AD). The neuroinflammation involving astrocytes is an important pathogenic mechanism. Along with the advancement of the concept and technology of astrocytic biology, the astrocytes have been increasingly regarded as the key contributors to neurological diseases. It is well known that physical exercise can improve cognitive function. As a safe and effective non-drug treatment, physical exercise has attracted continuous interests in neurological research. In this study, we explored the effects of physical exercise on the response of reactive astrocytes, and its role and mechanism in CCH-induced cognitive impairment. A rat CCH model was established by 2 vessel occlusion (2VO) and the wheel running exercise was used as the intervention. The cognitive function of rats was evaluated by morris water maze and novel object recognition test. The phenotypic polarization and the primary cilia expression of astrocytes were detected by immunofluorescence staining. The activation of MAPKs cascades, including ERK, JNK, and P38 signaling pathways, were detected by western blot. The results showed that physical exercise improved cognitive function of rats 2 months after 2VO, reduced the number of C3/GFAP-positive neurotoxic astrocytes, promoted the expression of S100A10/GFAP-positive neuroprotective astrocytes, and enhanced primary ciliogenesis. Additionally, physical exercise also alleviated the phosphorylation of ERK and JNK proteins induced by CCH. These results indicate that physical exercise can improve the cognitive function of rats with CCH possible by promoting primary ciliogenesis and neuroprotective function of astrocytes. The MAPKs signaling cascade, especially ERK and JNK signaling pathways may be involved in this process.

Keywords: physical exercise, primary cilia, astrocytes polarization, MAPKs pathway, cognition, chronic cerebral hypoperfusion

INTRODUCTION

Vascular diseases are always related to the cognitive impairment (Duncombe et al., 2017; Tang et al., 2021; Ungvari et al., 2021). More and more evidence show that vascular risk factors can lead to neurodegeneration, cognitive impairment and dementia (Duncombe et al., 2017). Certain areas of the brain responsible for the memory, cognition, and behavior are particularly vulnerable to insufficient blood supply (Thammisetty et al., 2021). Chronic cerebral hypoperfusion (CCH) often occurs due to diseases that affect the cerebral circulatory system, such as hypertension, diabetes, atherosclerosis and smoking, and is one of the prime factors leading to the development of vascular cognitive impairment and dementia (VCID) in the elderly (Thammisetty et al., 2021). CCH is also considered as a preclinical condition of mild cognitive impairment and precursor of dementia (Xu et al., 2020). In addition, CCH is a common and important cause of cognitive impairment associated with many neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease, and it plays an indispensable role in the development of these diseases (Tang et al., 2017; Xie et al., 2018; Feng et al., 2021). Recent studies conducted by the American AD Research Center revealed that up to 80% of the sporadic and late onset AD have some form of brain vascular pathology (Duncombe et al., 2017; Xu et al., 2020). The inflammation, oxidative stress, and amyloid-β (Aβ) accumulation commonly existed in AD pathology have been reported to be associated with CCH. In addition, CCH was found to appear in the "early" stage of AD. Before the obvious symptoms of AD appear, arterial spin-labeled magnetic resonance imaging has detected changes in cerebral blood flow, indicating that CCH may be a potential biomarker of AD (Duncombe et al., 2017; Xu et al., 2020). In general, it has been demonstrated that there is considerable overlap between the characteristics of VCID and AD. CCH seems to be the common underlying pathophysiological mechanisms, and it is the main contributor to cognitive decline and degenerative development of dementia (Duncombe et al., 2017; Xu et al., 2020).

It is well known that neuroinflammation is considered to be the important pathogenic mechanism of CCH-induced cognitive impairment (Du et al., 2020). Considerable evidence shows that CCH can promote the occurrence and progression of cognitive impairment and dementia through neuroinflammation (Kim et al., 2017). Astrocytes are the most abundant cells and play multiple roles in the central nervous system (CNS). It has been confirmed that astrocytes participate in the immune response of the CNS, and are potential sentinels in the brain parenchyma together with microglia (Leardini-Tristao et al., 2020). In addition, astrocytes are not only involved in the regulation of neurotransmission, neurodevelopment, cerebral blood flow, metabolism and neurogenesis, their coverage integrity but also related to the brain homeostasis and cognition (Leardini-Tristao et al., 2020; Miyamoto et al., 2020). Recent studies have confirmed that astrocytes have two phenotypes, the neurotoxic A1 and neuroprotective A2 phenotype (Liddelow et al., 2017). However, astrocytes activated after CCH mainly transform to A1 phenotype, which lose their normal function and aggravate the neuroinflammation progression (Miyamoto et al., 2020; Jiang et al., 2021). On the one hand, the loss of normal A1 astrocytes function and the interruption of their interaction with blood vessels may lead to homeostasis destruction of neurovascular units and cognitive impairment (Leardini-Tristao et al., 2020; Presa et al., 2020). On the other hand, it has been shown that the neuroinflammatory processes aggravated by the activated neurotoxic astrocytes are essential for the initiation and progression of cognitive disorders (Kim et al., 2017). Therefore, modulating the polarization of activated astrocytes from neurotoxicity to neuroprotective phenotype may be one of the potential therapeutic targets for CCH-induced cognitive impairment.

There is evidence that primary cilia play important roles in regulating inflammation, and the disturbance of primary cilia may lead to gliosis and neuroinflammation (Singh et al., 2019). Primary cilia are essential for the development and maintenance of neural homeostasis in the mammalian brain (Sipos et al., 2018). They act as the "sensing antenna" of cells and are the central hub for receiving and transducing extracellular signals to elaborate biological responses to a wide range of developmental and physiological processes. Primary cilia always play key roles in cell cycle control, cell proliferation, migration, and polarity (Alvarez-Satta et al., 2019; Alhassen et al., 2021). Additionally, large numbers of convincing studies have shown that there are strong correlations between primary cilia and cognition (Hu et al., 2017; Alhassen et al., 2021). Defects in primary cilia can lead to age-related cognitive decline, and primary cilia may be potential targets for AD and/or other dementia (Alvarez-Satta et al., 2019). Most mammalian cells have primary cilia, including astrocytes (Sipos et al., 2018). In response to various brain injuries, such as traumatic brain injury, ischemia, and neurodegenerative diseases, astrocytes proliferation is coordinated with the ciliogenesis and dismantling of astrocytes cilia (Sterpka and Chen, 2018). A study has reported that primary cilia dysfunction caused by congenital deletion of BBSome protein 8 can induce A1-like astrocyte activation, neuroinflammation, and changes in postsynaptic density (Singh et al., 2019). Primary cilia are an important component of the sonic hedgehog (Shh) signaling pathway, and the activation of the latter can promote astrocyte polarization toward to A2 phenotype (Payan-Gomez et al., 2018). These studies indicate that primary cilia are involved in the regulation of the A1/A2 phenotypic polarization of astrocytes. At present, little is known about the physiological and pathological functions of astrocytic primary cilia (Sterpka et al., 2020). Further exploration of the role of primary cilia in reactive astrocytes will not only help to better understand the regulatory mechanism of astrocytes polarization, but also to develop potential therapeutics for cognitive disorders.

Over the years, there have been many studies on the benefits of physical exercise. A lot of scientific evidences show that there is a positive correlation between active sports lifestyle and health benefits (De la Rosa et al., 2020; Marques-Aleixo et al., 2021; Padilha et al., 2021). Physical exercise is an effective non-toxic strategy to prevent and treat many chronic diseases. Its positive effects in reducing the risk of all-cause death and extending life span have been widely documented (Scheffer and Latini, 2020). More and more evidences showed

that physical exercise, including aerobic and resistance exercise training, can alleviate age-related cognitive impairment (Brach et al., 2021; Chow et al., 2021; Pronk, 2021). Physical exercise can promote different physiological phenomena including neurogenesis, synaptogenesis, angiogenesis, and neurotrophic factor stimulation by inducing a series of cellular and molecular processes, thereby causing changes in the brain at the cellular, molecular and anatomic level, and eventually enhancing learning, memory and brain plasticity (De la Rosa et al., 2020). Additionally, the beneficial effects of physical exercise on health outcomes also involve the regulation of the immune system. Regular physical exercise can prevent viral and bacterial infections and enhance the immune response to vaccines and pathogens. This makes physical exercise a valuable tool for preventing infectious diseases such as COVID-19 (Scheffer and Latini, 2020). Moreover, studies have shown that physical inactivity favors promoting the polarization of immune cells toward a pro-inflammatory phenotype. In contrast, regular moderate-intensity physical exercise can direct the immune response to an anti-inflammatory state, which is considered to be the main molecular mechanism required for the improvement of health outcomes (Scheffer and Latini, 2020). Our previous study also found that physical exercise can regulate the polarization of astrocytes from pro-inflammatory A1 to neuroprotective A2 phenotype, and improve the cognitive impairment of rats with CCH (Jiang et al., 2021).

In this study, we will investigate the effects of physical exercise on primary ciliogenesis and astrocyte polarization in rats with CCH. In addition, we will further investigate the role of MAPKs signaling cascades, including ERK, JNK, and P38 signaling pathways in this process.

MATERIALS AND METHODS

Experimental Animals and Groups

In this experiment, male Wistar rats 280–320 g were used and purchased from Unilever Laboratory Animal Company, Beijing. The rats were kept in cages with ambient temperature of 23 ± 1°C, humidity of 55 ± 5%, light/dark cycle of 12 h, and free access to food and water. All animal experiments were conducted in accordance with the Guide for the Care and Use of laboratory Animals of the National Institutes of Health (Publication No. 80-23, revised 1996) and approved by Institutional Animal Care and Use Committee of Wuhan University Center for Animal Experiment. The experimental rats were randomly divided into sham group and 2-vessel occlusion (2VO) group. The successful 2VO rats were further divided into control group and physical exercise group randomly. The experimental procedure is shown in **Figure 1A**.

Experimental Animal Model

Chronic cerebral hypoperfusion model was established by bilateral common carotid artery occlusion (2VO) in rats as previously described. For the 2VO operation, the rats were anesthetized with pentobarbital and the neck was incised longitudinally through the midline. Subsequently, the bilaterally common carotid arteries were exposed and carefully separated from the adjacent vagus nerve. The distal and proximal portions of the common carotid arteries were double permanently ligated with two 4-0 silk wires, respectively. Finally, the bilaterally common carotid arteries were cut between the middle of the two ligations. Rats in the sham group were subjected to the same procedure without ligation and cutting. The rats were placed on the heating pad and observed until they were fully awake before being brought back to the cage.

Physical Exercise Intervention

As described in our previous study (Jiang et al., 2017, 2021), rats in the physical exercise group were given electric wheel running training 48 h after 2VO. The initial speed was 5 rpm and gradually increased to 7 rpm in the first week after 2VO. It increased to 10 rpm in the second week, 15 rpm in the third week, 20 rpm in the fourth week after 2VO, and then maintained this speed until behavioral testing. The training time is 20 mins each time, two times a day, and six consecutive days a week. The animals in sham group and control group were kept in cages without exercise training intervention.

Morris Water Maze Test

The water maze test was used to evaluate the hippocampus-dependent spatial learning and memory of rats (Ghafarimoghadam et al., 2022). The experiment was carried out in a circular tank with a water depth of 30 cm, a diameter of 150 cm, and a height of 50 cm. The pool was divided into four quadrants, and a 1.5 cm underwater platform was placed at a fixed location in one of the quadrants. The place navigation test was designed for 5 days and four times a day. The animals were released into the water from a position in the middle of the outer edges of the four quadrants and facing the wall of the pool. During each trial, the escape latency was recorded as the time to find the platform within 60 s, and rats that could not find the platform within 60 s were guided to the platform. After the 5 day place navigation test, the underwater platform was taken away, and a spatial probe test was conducted on each animal to evaluate the reference memory. The data was recorded by Animal Video Tracking Analysis System (Anilab Scientific Instruments Co., Ltd., Ningbo, China).

Novel Object Recognition Test

The novel object recognition test was used to evaluate non-spatial memory between the prefrontal and subcortical circuits in rats. As described in previous studies (Jiang et al., 2017, 2021), the experiment was carried out in a transparent open box (72 cm^3 × 72 cm^3 × 35 cm^3). On the day before the test, rats were allowed to explore the box freely without any objects for 10 mins. The test on the second day was divided into two phases: familiarization and testing phase. In the familiarization phase, rats were placed in the box containing two identical objects and explored freely for 5 mins, and were considered to be exploring when they touched the objects with their noses or were within 1 cm of the objects. After the familiarization period, the box and objects were cleaned with 70% ethanol to eliminate residual odors. One hour later, during the testing phase, one of the

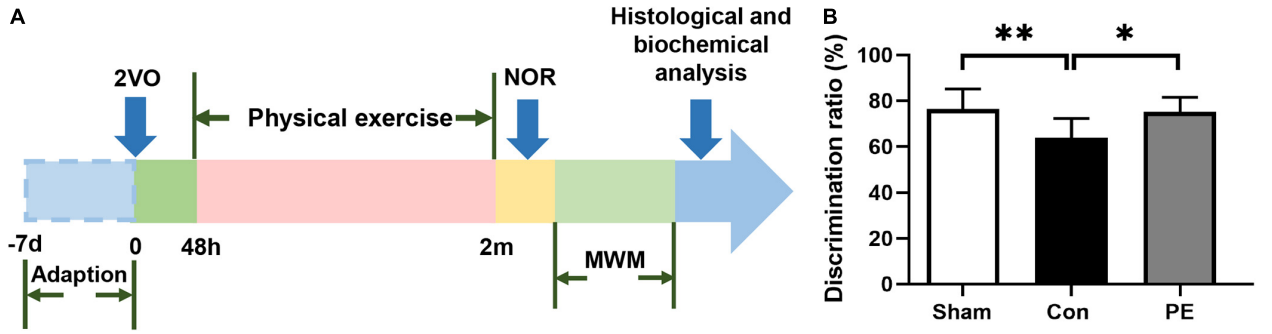


FIGURE 1 | Flow diagram of the experiment and the novel object discrimination ratio in rats with chronic cerebral hypoperfusion. **(A)** Flow chart of experimental design to explore the effect and mechanism of physical exercise on cognitive function in 2VO rats. 2VO, 2 vessel occlusion; NOR, novel object recognition; MWM, Morris water maze. **(B)** The novel object discrimination ratio of rats in each group. $n = 10$. Experimental data are expressed as the means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$. Con, control; PE, physical exercise.

objects was replaced with a new object of completely different size and shape, and the rats were allowed to explore the two different objects for 5 mins. The exploration time of familiar object (F) and new object (N) were recorded, respectively, and the discrimination ratio (DR) = N/(N + F) was calculated × 100%.

Tissue Preparation for Histochemistry

Two months after 2VO, rats in each group were sacrificed after behavioral testing. Rats were deeply anesthetized with pentobarbital and perfused transcardially with 0.9% saline at 4°C and 4% paraformaldehyde (PFA) successively. The brain tissues of rats were quickly separated and fixed in 4% PFA at 4°C for 24 h, and then dehydrated in 20% and 30% sucrose. Then, the brain tissues were embedded with OCT (American cherry blossom) and placed in a cryomicrotome (CM1900, Leica, Germany) for continuous coronal sectioning (10 μm) for subsequent histological experiments.

Immunofluorescence Staining

Immunofluorescence staining was as previously described (Jiang et al., 2017, 2021), the brain sections were pretreated in citrate buffer (85°C) for 5 mins for antigen retrieval, blocked with immunostaining blocking buffer (P0102, Beyotime, China) for 1 h at room temperature and then incubated overnight at 4°C with the following primary antibody: mixtures of mouse anti-GFAP antibody (1:200, Boster, China) and goat anti-C3d antibody (1:200, R&D, United States), mouse anti-GFAP (1:200, Boster, China) and chicken anti-S100A10 antibody (1:200, Abcam, United Kingdom), mouse anti-GFAP (1:200, Boster, China) and rabbit anti-ARL13B antibody (1:200, Proteintech, United States). The following day, brain sections were incubated with mixtures of goat anti-mouse IgG (DyLight 488 Conjugated; 1:200, Boster, China) and rabbit anti-goat IgG (CY3 Conjugated AffiniPure; 1:200, Boster, China), goat anti-mouse IgG (DyLight 488 Conjugated; 1:200, Boster, China) and goat anti-chicken IgG (Goat Anti-Chicken IgY; 1:400, Abcam, United Kingdom), and goat anti-mouse IgG (DyLight 488 Conjugated; 1:200, Boster, China) and goat anti-rabbit IgG (Alexa Fluor® 555 Conjugate; 1:500, Cell Signaling Technology, Boston, MA, United States) for 1 h at room temperature. Then, sections were washed with phosphate buffered solution (PBS) and sealed with a DAPI-containing antifade solution. The fluorescence signals were examined by fluorescence microscopy (BX53; Olympus).

Western Blot Analysis

The total protein was extracted from the corpus callosum of rats for western blot analysis. Briefly, rats were deeply anesthetized with pentobarbital and perfused transcardially with 4°C saline (50 ml). The corpus callosum was rapidly separated and removed on ice. After adding the lysis buffer, the brain tissue was fragmented using a tissue grinder. Protein concentrations of all extracted samples were measured using Bio-Rad Protein Assay (BioRad, Hercules, CA, United States) and bovine serum albumin (BSA) standards. A 30 μg protein sample was loaded and separated by sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane, which was then incubated overnight at 4°C with the following primary antibody: ERK (1:1,000, Cell Signaling Technology, Boston, MA, United States), P-ERK (1:1,000, Beyotime, China), JNK (1:1,000, Cell Signaling Technology, Boston, MA, United States), P-JNK (1:1,000, Beyotime, China), P38 (1:1,000, Cell Signaling Technology, Boston, MA, United States), P-P38 (1:1,000, Beyotime, China) and GAPDH (1:1,000, Servicebio, Wuhan, China). Next, the membrane was washed and incubated with horse-radish peroxidase-labeled goat anti-rabbit and goat anti-mouse secondary antibody (1:10,000, Boster, China) for 1 h at room temperature. Finally, the enhanced chemiluminescence system was used to observe protein bands.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 20.0 and GraphPad Prism 8.0. The data are expressed as mean ± SD. In the behavioral tests, the movement trajectories and data are recorded by behavioral recording software (Anilab Scientific Instruments Co., Ltd., Ningbo, China). For immunofluorescence, three slices of each brain were selected at the corpus callosum, and 3–6 regions of each slice were randomly selected to be

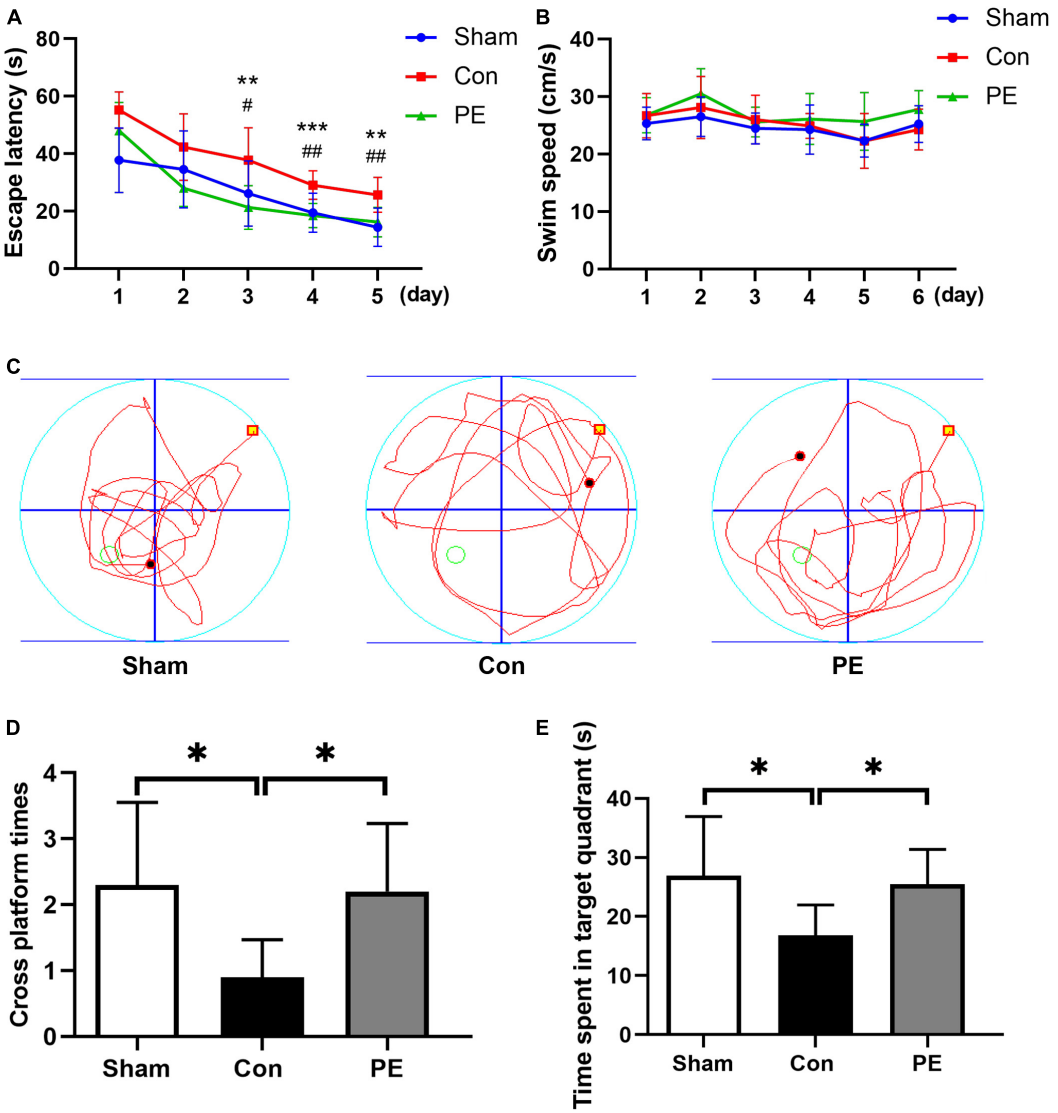


FIGURE 2 | Physical exercise improved spatial memory in rats with chronic cerebral hypoperfusion. In the place navigation test: **(A)** the escape latency of rats in each group. PE vs. Con: $^{**}P < 0.01$, $^{***}P < 0.001$; Sham vs. Con: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. **(B)** The swimming speed of rats in each group. There was significant difference in the escape latency ($P < 0.001$) but not the swimming speed ($P > 0.05$) between the different groups in the overall. In the spatial probe test: **(C)** Representative swimming trajectories of rats in each group. **(D)** Times of crossing the target platform of rats in each group. **(E)** Time spent in the target quadrant of rats in each group. $n = 10$. Experimental data are expressed as the means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$. Con, control; PE, physical exercise.

photographed for analysis. To measure the length of primary cilia, the standard scale bar was used as the reference and the length of primary cilia was measured from the base to the top by Image J software (National Institutes of Health, Bethesda, MD, United States). All immunofluorescence data were analyzed by Image J software. For western blot, the gray value of the western blot bands was measured by Image J software, and the gray value of the target protein was compared with the GADPH. The escape latency and swimming speed in the MWM test were analyzed by repeated measures analysis of variance (ANOVA). The platform crossings and dwell time in the MWM test, the discrimination index in the NOR test and the results of immunofluorescence and Western blotting were evaluated by one-way ANOVA followed by Tukey's *post hoc* test. $P < 0.05$ is considered to be statistically significant.

RESULTS

Physical Exercise Improved Cognitive Function in Rats With Chronic Cerebral Hypoperfusion

Spatial and non-spatial learning memory abilities of rats 2 months after 2VO were assessed by MWM and NOR test, respectively. The results of the NOR test showed that the ability

of novel object recognition in the control group was impaired compared to the sham group (63.91 ± 8.44 vs 76.29 ± 8.83%; $P < 0.01$). However, physical exercise alleviated this deficit as shown by the significantly improved discrimination index for novel objects (75.13 ± 6.44 vs 63.91 ± 8.44%; $P < 0.05$; **Figure 1B**). The results of the MWM test showed that in the place navigation test (**Figures 2A,B**), the control group had a longer escape latency to find the underwater platform on days 3–5 compared to the sham group (day 3: 37.74 ± 11.28 vs 26.17 ± 11.37 s, $P < 0.05$; day 4: 29.04 ± 4.96 vs 19.47 ± 6.79 s, $P < 0.01$; day 5: 25.67 ± 6.08 vs 14.38 ± 6.66 s, $P < 0.01$; respectively). However, the escape latency of the physical exercise group on days 3–5 was significantly shorter than the control group (day 3: 21.28 ± 7.56 vs 37.74 ± 11.28 s, $P < 0.01$; day 4: 18.46 ± 4.18 vs 29.04 ± 4.96 s, $P < 0.001$; day 5: 16.19 ± 5.11 vs 25.67 ± 6.08 s, $P < 0.01$; respectively). In the spatial probe test (**Figures 2C–E**), the control group had a reduced number of platform crossings (0.90 ± 0.57 vs 2.30 ± 1.25, $P < 0.05$) and decreased dwell time in the target quadrant (16.81 ± 5.17 vs 26.94 ± 10.00 s, $P < 0.05$) compared with the sham group. However, physical exercise increased the number of crossing the platform (2.20 ± 1.03 vs 0.90 ± 0.57, $P < 0.05$) and the time spent in the target quadrant (25.47 ± 5.91 vs 16.81 ± 5.17 s, $P < 0.05$) in rats relative to the control group. The above results indicate that physical exercise can improve not only the spatial memory but also the non-spatial memory between the prefrontal and subcortical circuits in rats with CCH.

Physical Exercise Promoted the Polarization of Astrocytes to Neuroprotective Phenotype

To investigate the effect of physical exercise on the phenotypic polarization of astrocytes in the brain of rats 2 months after 2VO, the C3/GFAP and the S100A10/GFAP immunofluorescent double-label staining were performed. In which C3 is considered as a marker for neurotoxic astrocytes of A1- phenotype and S100A10 is considered as a marker for neuroprotective astrocytes of A2- phenotype. The results showed an increased number of GFAP-positive astrocytes in the control group compared with the sham group ($P < 0.001$; **Figures 3A,B**, **4A,B**), indicating that there is astrocytes response and activation after CCH. At the same time, the number of C3/GFAP-positive astrocytes was also significantly higher in the control group (221.63 ± 7.14 vs 103.45 ± 13.02/mm^2; $P < 0.001$; **Figures 3A,C**), suggesting that activated astrocytes may shift primarily toward the A1 phenotype. However, physical exercise reduced the number of C3/GFAP-positive astrocytes (149.00 ± 8.41 vs 221.63 ± 7.14/mm^2; $P < 0.001$) and promoted the expression of S100A10/GFAP-positive astrocytes (132.13 ± 4.75 vs 38.41 ± 7.37/mm^2; $P < 0.001$) compared to the control group (**Figures 3A,C**, **4A,C**). These results suggest that physical exercise may create a favorable microenvironment for promoting the activated astrocytes polarization from A1 to A2 phenotype, and eventually exert neuroprotective functions.

Physical Exercise Enhanced Primary Cilia Expression and Promoted the Length Recovery of Primary Cilia in Astrocytes

The ARL13B/GFAP immunofluorescence double staining was performed to evaluate the effects of physical exercise on primary cilia expression in astrocytes of rats after 2VO. ARL13B is considered to be the main marker of primary cilia in astrocytes. The results showed that the average length of astrocytic primary cilia in the sham group was about 2.62 ± 0.33 μm (**Figure 5C**). Compared with the sham group, the expression (28.39 ± 3.17 vs 32.86 ± 8.76%; $P > 0.05$) and length of primary cilia (2.18 ± 0.27 vs 2.62 ± 0.33 μm; $P < 0.05$) in astrocytes were decreased in the control group (**Figures 5A–C**). The results indicate that the primary cilia of astrocytes have undergone adaptive expression and morphological changes to reduce the cilia signal transduction in astrocytes, in turn affecting astrocytes responses and functions to injury under CCH. However, physical exercise can promote not only astrocytic primary ciliogenesis (36.69 ± 5.59 vs 28.39 ± 3.17%; $P < 0.05$; **Figures 5A,B**), but also the restoration of primary ciliary length (2.81 ± 0.38 vs 2.18 ± 0.27 μm; $P < 0.05$; **Figures 5A,C**). These results suggest that physical exercise may affect the function of astrocytes by regulating the occurrence and length of astrocytic primary cilia.

Physical Exercise Reduced the Phosphorylation Levels of ERK and JNK Pathway

To assess the effects of physical exercise on the MAPKs signaling cascades, including ERK, JNK and P38 signaling pathways in rats 2 months after 2VO, Western blot analysis was performed to detect the protein expression of ERK1/2, P-ERK1/2, JNK, P-JNK, P38, and P-P38 in the corpus callosum. According to the results, the control group showed increased phosphorylation level of ERK1/2 and JNK compared with the sham group ($P < 0.05$ and $P < 0.001$). However, physical exercise ameliorated these negative effects, as evidenced by significantly lower phosphorylation levels of ERK and JNK ($P < 0.05$ and $P < 0.05$; **Figures 6A–D**). These findings showed that physical exercise can modulate the expression of MAPKs signaling cascades especially ERK1/2 and JNK pathways.

DISCUSSION

Physical exercise has beneficial effects on both peripheral tissues and CNS, and is a kind of safe and effective intervention to improve cognitive function (Maugeri et al., 2021). In human and animal models, the multiple benefits of regular physical exercise have been fully demonstrated. Whether aerobic exercise, anaerobic exercise or resistance exercise, are all considered to be beneficial to physical and mental health (Xu et al., 2021). More and more evidence showed that physical exercise can help maintain optimal cerebrovascular function, thereby preventing or slowing down the occurrence and development of cognitive impairment (Bliss et al., 2021). In this study, we found that

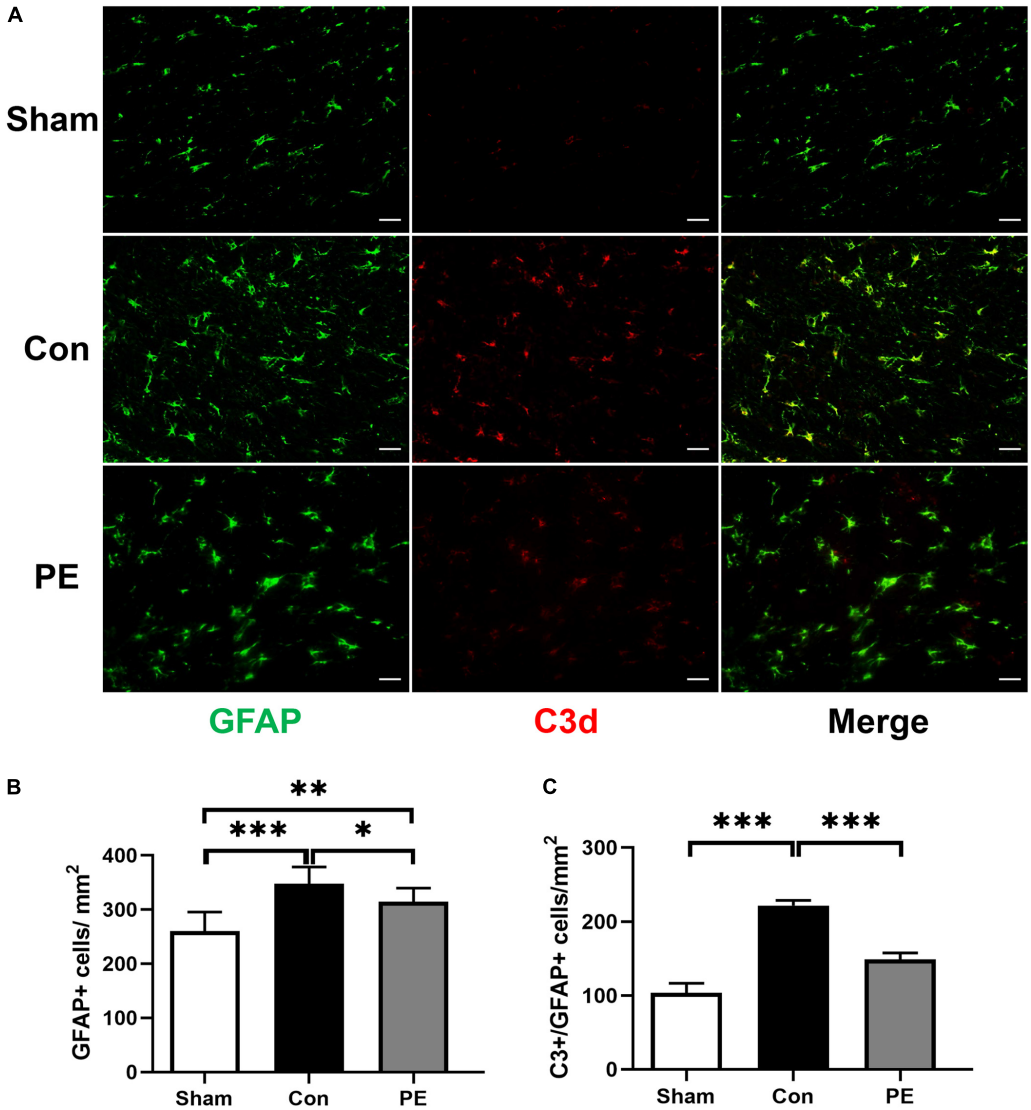


FIGURE 3 | Physical exercise decreased the number of A1 neurotoxic astrocytes. **(A)** Representative immunofluorescence images for GFAP (green) and C3 (red) double staining of rats in each group in the corpus callosum. Scale bar = 50 μm. **(B)** The quantification of GFAP positive astrocytes and **(C)** C3/GFAP positive A1 astrocytes. $n = 6$. Experimental data are expressed as the means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. GFAP, glial fibrillary acidic protein; C3, complement 3.

wheel running exercise for 2 months can improve the spatial memory and non-spatial memory of CCH rats (**Figures 1B**, **2A–E**). This is consistent with our previous study, which found that physical exercise alleviated the cognitive impairment of 2VO rats, and the effects tended to be more pronounced as the duration of exercise increased (Jiang et al., 2021). Similarly, some studies have shown that long-term physical exercise has positive effects through delaying the onset of physiological memory loss. However, it is worth mentioning that the late-onset exercise intervention has also shown positive impacts in delaying brain aging (De la Rosa et al., 2020). At present, the effects of pharmacological treatment for patients with cognitive impairment and dementia are limited, which not only brings a huge economic burden to society, but also seriously affects the quality of life of families facing such conditions. Since physical exercise has significant beneficial effects on the cognitive function of different diseases, it has aroused a lot of research interests as a kind of safe and effective non-drug treatment (Chan et al., 2021; Consorti et al., 2021). Physical exercise cannot only be used as a therapeutic protocol for neurological diseases, but also help to unlock the potential molecular targets of pharmacological methods (Consorti et al., 2021). Recently, exercise mimetics have been proposed as a class of therapeutics that can specifically mimic or enhance the therapeutic effects of physical exercise. Clarifying the mechanism mediating the beneficial impacts of exercise at the molecular, cellular and system levels is essential for the development of novel exercise mimetics (Gubert and Hannan, 2021). However, the

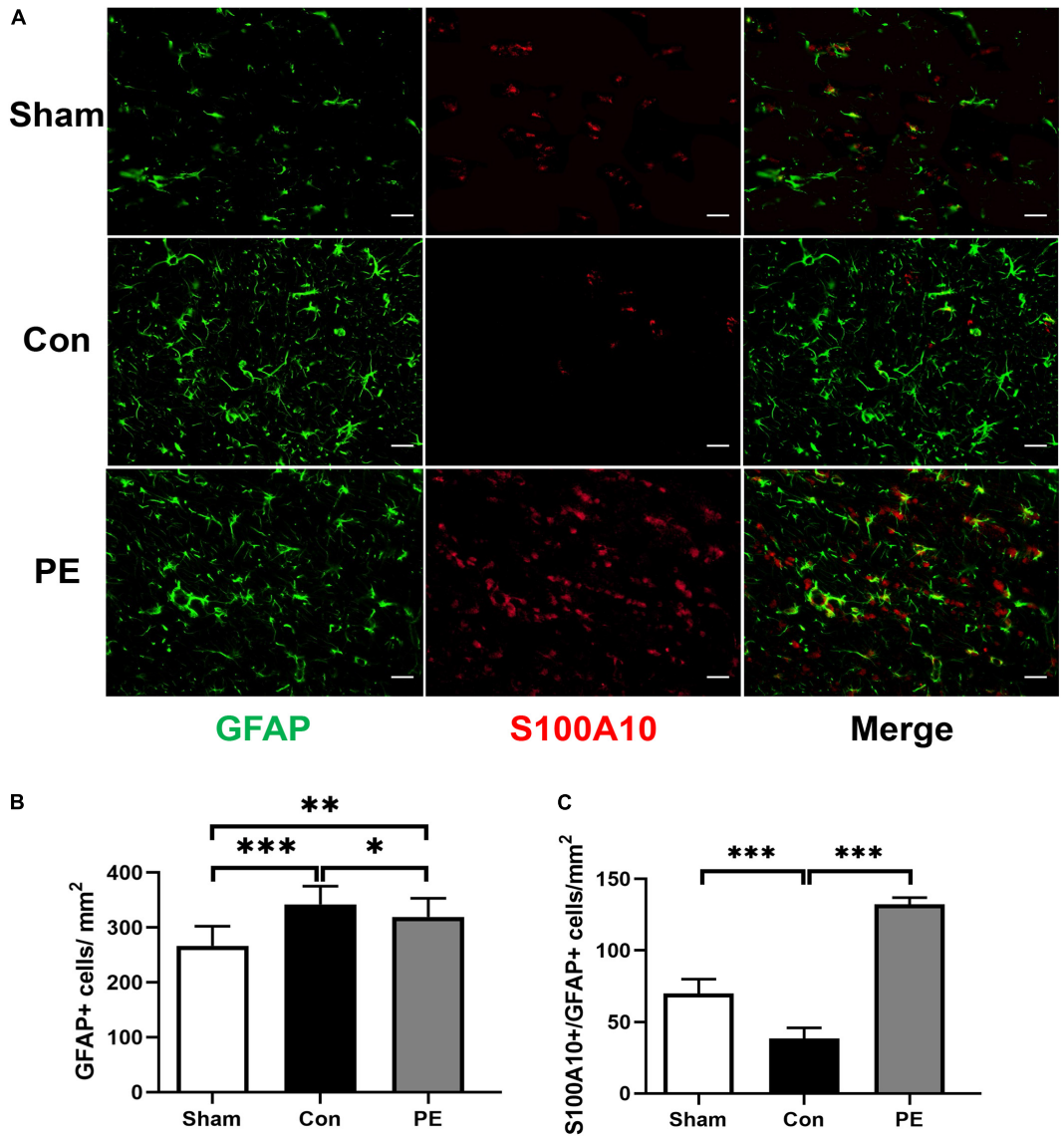


FIGURE 4 | Physical exercise enhanced the expression of A2 neuroprotective astrocytes. **(A)** Representative immunofluorescence images for GFAP (green) and S100A10 (red) double staining of rats in each group in the corpus callosum. Scale bar = 50 μm. **(B)** The quantification of GFAP positive astrocytes and **(C)** S100A10/GFAP positive A2 astrocytes. $n = 6$. Experimental data are expressed as the means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. S100A10, S100 calcium binding protein A10.

underlying mechanism by which exercise may improve cognition remains unclear.

Recently, more and more studies have shown that the changes in astrocytes may be a key mechanism accounting for the improvement of cognitive and executive functions related to exercise (Li et al., 2021; Maugeri et al., 2021). Astrocytes are the main homeostasis and defense elements of the brain, helping to maintain cognitive reserve through a variety of mechanisms (Verkhratsky et al., 2021). They can communicate with neurons and other astrocytes in a bidirectional manner by releasing transmitters, increasing synaptic activity and strength, and play an active role in storing and processing information in the brain (Maugeri et al., 2021). It has been shown that the multiple effects of physical exercise on astrocytes include the number of new astrocytes increase, the basal level of catecholamines maintenance, the glutamate uptake enhance, the trophic factors release, and the better astrocytic coverage of cerebral blood vessels (Maugeri et al., 2021). In this study, we found that the astrocytes were activated in the brain of rats 2 months after 2VO, and they were mainly polarized toward the neurotoxic A1 phenotype. While physical exercise can switch the polarization of astrocytes from neurotoxic A1 to neuroprotective A2 phenotype (**Figures 3A–C**, **4A–C**). Our previous study also reported that physical exercise cannot only

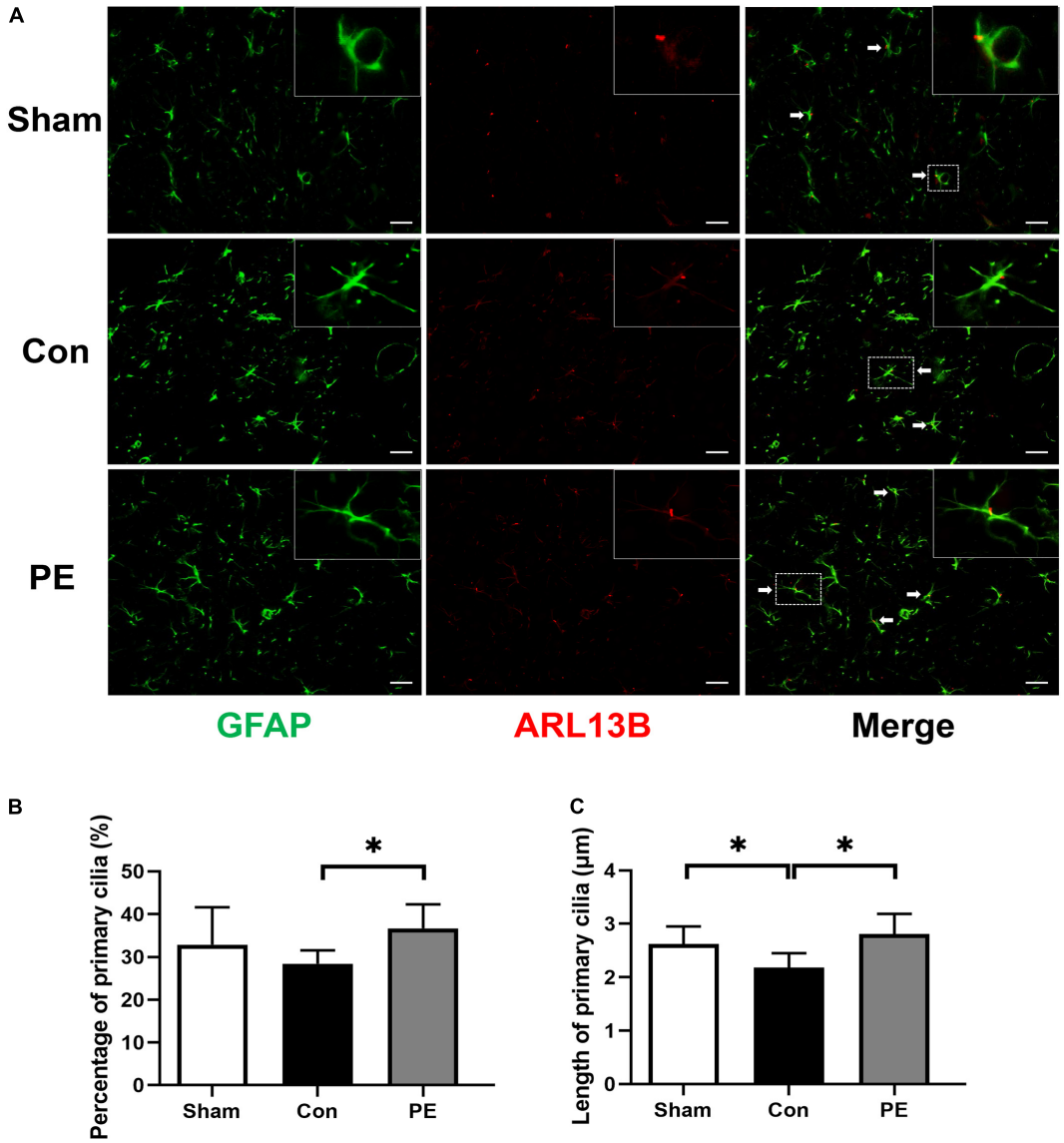


FIGURE 5 | Physical exercise augmented the expression and length of primary cilia in astrocytes. **(A)** Representative immunofluorescence images for GFAP (green) and ARL13B (red) double staining of rats in each group in the corpus callosum. Scale bar = 50 μm. **(B)** Quantification of the percentage of astrocytes containing primary cilia. **(C)** Quantification of length of astrocytic primary cilia. $n = 6$. Experimental data are expressed as the means ± SD. $^*P < 0.05$. ARL13B, ADP-ribosylation factor-like 13B.

modulate the transition of microglia from M1 to M2 phenotype, but also regulate the phenotypic polarization of astrocytes (Jiang et al., 2021). Consistently, similar studies have revealed that the low intensity motor balance and coordinated exercise can suppress the phenotypic polarization of M1 microglia and A1 astrocytes in the hippocampus of AD mice, contributing to the inhibition of Aβ accumulation and counteraction of behavioral and cognitive decline (Nakanishi et al., 2021). Recent studies have also emphasized the importance of physical activity as an intervention to prevent development of a severe form of COVID-19, as exercise can suppress excessive immune responses and trigger anti-inflammatory functions to promote psychological health (Maugeri et al., 2021). Interestingly, studies have shown that astrocytes may be the perspective targets of exercise mimetics (Lalo and Pankratov, 2021). In general, astrocytes are the central components for physical exercise-induced cognitive improvement in many neurodegenerative and neurovascular diseases (Li et al., 2021). The modulation of astrocytes polarization from neurotoxic A1 to neuroprotective A2 phenotypes may be an important mechanism involved.

As mentioned before, studies have indicated that primary cilia are involved in the regulation of the A1/A2 phenotypic polarization of astrocytes. It is well known that primary cilia can regulate astrocyte proliferation, polarization and tissue

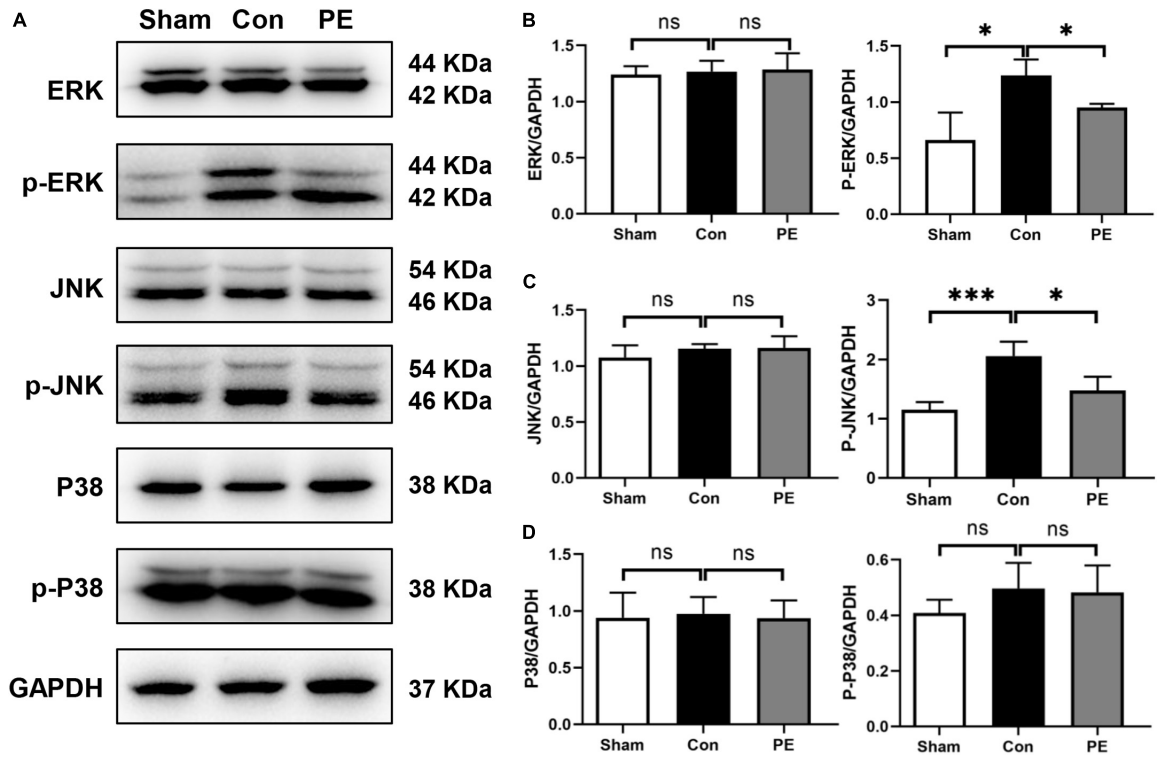


FIGURE 6 | Physical exercise inhibited ERK and JNK phosphorylation. **(A)** Representative images for western blotting of ERK, p-ERK, JNK, p-JNK, P38, and p-P38 expressions. **(B–D)** Densitometry analyses of the expression of ERK, p-ERK, JNK, p-JNK, P38, and p-P38 normalized to GAPDH. $n = 5$. Experimental data are expressed as the means ± SD. $^{*}P < 0.05$, $^{***}P < 0.001$.

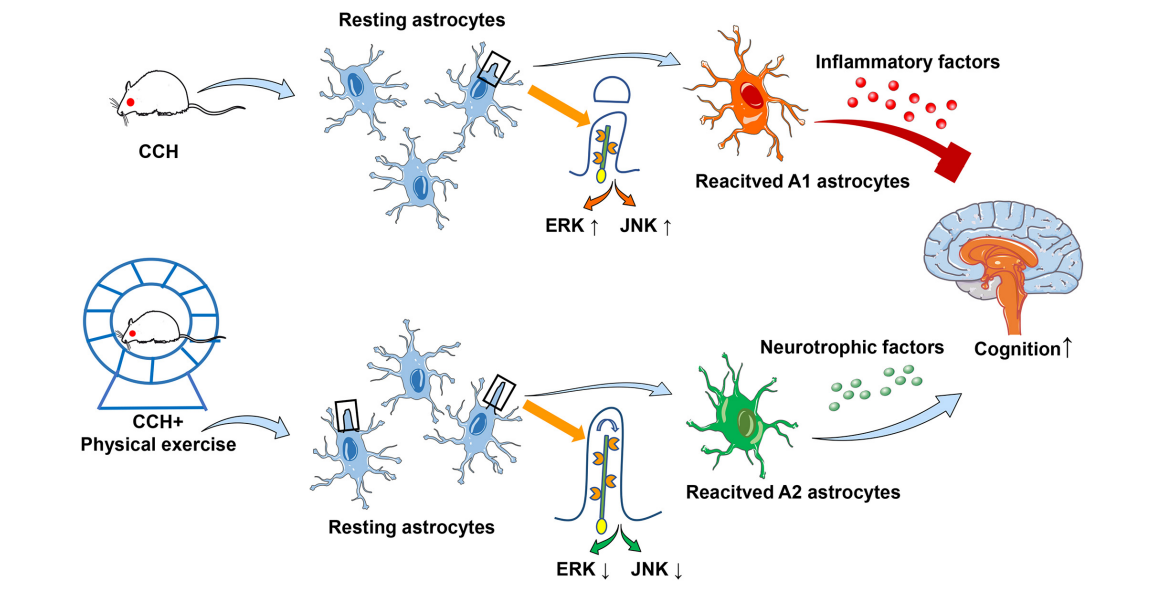


FIGURE 7 | Schematic diagram for the possible mechanism of primary cilia and astrocyte polarization involved in physical exercise induced cognitive improvement in rats with chronic cerebral perfusion.

regeneration (Sterpka et al., 2020). Since primary cilia originate from the maternal centrioles, astrocytes proliferation must be coordinated with the dismantling and ciliogenesis of astrocytic cilia (Sterpka and Chen, 2018). Studying astrocytic primary cilia will provide useful clues for intervention in reactive astrocytes to combat various neuropathologies such as brain injury and

ischemia (Sterpka and Chen, 2018). Primary cilia can be used as sensors for chemical and mechanical cues from cellular environment and play critical roles in transducing environmental stimuli and concentrating signal molecules to regulate cellular properties (Sun et al., 2021; Tereshko et al., 2021). However, current research about the effects of physical exercise on primary cilia is still limited. In this study, we found that the average length of primary cilia in the corpus callosum varied between 2.62 ± 0.33 μm in the sham group. Compared with the sham group, the length of astrocytic primary cilia in rats with CCH was shortened. In addition, the incidence of cilia also showed a decreasing trend, but there was no significant difference. However, physical exercise can significantly increase the expression and length of astrocytic primary cilia in the brain of rats 2 months after 2VO (**Figures 5A–C**). The length of primary cilia is closely related to their functions. Studies have found that primary cilia in hypocretin/orexin cells of obese mice were shorter, resulting in losing their ability to sense the local environment in the lateral/perifonical hypothalamus (Tan et al., 2020). Similarly, the shortened Arl13b-positive astrocytic cilia can be observed in the hippocampus of mice with spontaneous seizures (Sterpka et al., 2020). Whereas the ciliary length elongation has been proposed to fine-tune signaling activity of organelles in response to changes in the extracellular environment. The longer the cilia, the more functional they are and the greater capacity for signaling transduction in their internal receptor system (Brodsky et al., 2017; Sterpka et al., 2020). Therefore, astrocytic primary cilia are shortened after CCH, which may impair the ability of astrocytes to maintain normal function and perceive local damage environment, leading astrocytes polarization primarily toward the neurotoxic A1 phenotype. In contrast, physical exercise can rescue this negative impact and modulate astrocytes polarization to neuroprotective A2 phenotype by promoting primary cilia expression and elongation.

In order to clarify the possible mechanisms involved in the regulation of primary ciliogenesis and astrocyte polarization by physical exercise, we further observed the expression of mitogen-activated protein kinases (MAPKs) cascades. The MAPKs cascades mainly include JNK, P38, and ERK signaling pathways. They are involved in the regulation of cell proliferation, differentiation, migration, apoptosis and senescence, and are core components in signaling networks in various mammalian cells (Asih et al., 2020). Additionally, MAPKs are also the main effectors of inflammatory and mechanical stress (He et al., 2016). In this study, we found that compared with the sham group, the ERK and JNK signaling pathways in the brain of rats with CCH were activated, and physical exercise significantly reduced the expression of phosphorylated ERK and JNK, but without significant effects on the expression of the phosphorylated P38 (**Figures 6A–D**). These results are consistent with our previous study. We previously revealed that physical exercise can reduce ERK and JNK phosphorylation in the brain of rats 28 days after 2VO and promote microglia polarization from neurotoxic M1 to neuroprotective M2 phenotype, and finally promote remyelination and cognition improvement (Jiang et al., 2021). These findings suggest that the ERK and JNK signaling pathways may be the important effectors of exercise-induced anti neuroinflammatory responses. The inhibition of MAPKs activation, such as ERK, JNK, or P38, cannot only reduce inflammatory factors released from astrocytes and prevent astrocytes forming neurotoxic activity, but also increase neurotrophic factors release, maintain stable expression of glutamine synthetase from astrocytes, and promote neuroprotective effects of astrocytes (Efremova et al., 2017; Li et al., 2017; Liu et al., 2017; Wang et al., 2018, 2021; Zhou et al., 2019). Studies have also shown that mechanical signals sensed by primary cilia were transduced into biochemical signals involving pathways including ERK, JNK, and P38 (He et al., 2016). In some ciliopathy and cystic disease, MAPKs are important effector pathways closely associated with the process of ciliogenesis (Ma et al., 2013). Therefore, physical exercise may reduce the activation of ERK and JNK signaling pathways through regulating astrocytic primary cilia, and promote the activated astrocytes polarization from neurotoxic A1 to A2 phenotype to exert neuroprotective effects.

In summary, we have provided new evidence for clarifying the mechanism of physical exercise induced cognitive function recovery in CCH. Physical exercise can promote reactive astrocytes to exert neuroprotective roles by enhancing primary cilia length and ciliogenesis, and finally improve the cognitive function of rats with CCH. Moreover, the JNK and ERK signaling pathways are at least partly involved in this regulatory process (**Figure 7**).

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 authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Wuhan University Center for Animal Experiment.

AUTHOR CONTRIBUTIONS

TJ and WJL conceived and designed the experiments. WYC, JYL, and BRW performed research and data acquisition. JBL and WYC analyzed and interpreted data. TJ and WX wrote the manuscript. WJL, WX, and JBL reviewed and edited the manuscript. All authors read the submitted version of this article and approved it for publication.

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The Relationship Between Suboptimal Social Networks and Postoperative Delirium: The PNDABLE Study

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Background: Although it has been proven that social networks are related to cognition, studies are conducted to characterize the correlation between social networks and postoperative delirium (POD).

Objective: We investigated whether suboptimal social networks are a risk factor for POD, and to verify whether different levels of intimacy in the same social relationship can affect the concentration of cerebrospinal fluid (CSF) biomarkers, such as amyloid-β (Aβ42), total tau (T-tau), and phosphorylated tau (P-tau), and the mediating role of CSF biomarkers between social network and POD in middle-aged and elderly Han people.

Methods: Our study recruited 743 participants from The Perioperative Neurocognitive Disorder and Biomarker Lifestyle (PNDABLE) study. Confusion Assessment Method (CAM) was used to evaluate the incidence of POD and its severity was measured using the Memorial Delirium Assessment Scale (MDAS). The social networks were measured using self-reported questionnaires about social ties. Mann–Whitney *U* test, Logistic Regression and Independent-samples test were used for Statistical Analysis.

Results: The incidence of POD was 20.7%. Mann–Whitney *U* test showed that the total score of the social network was associated with POD ($P < 0.001$). Independent-samples test showed that different levels of intimacy in the same social relationship were significantly associated with CSF POD biomarkers, and mediation analyses revealed that the association between suboptimal social networks and POD was partially mediated by T-tau (proportion: 20%), P-tau (proportion: 33%), Aβ42/T-tau (proportion: 14%), and Aβ42/P-tau (proportion: 15%).

Conclusion: Having suboptimal social networks is a risk factor for POD in middle-aged and elderly Han people. CSF POD biomarkers can mediate the correlation between suboptimal social networks and POD, which is mainly mediated by tau protein.

Clinical Trial Registration: www.chictr.org.cn, identifier ChiCTR2000033439.

Keywords: social networks, lifestyle, cerebrospinal fluid, mediating effect, postoperative delirium

INTRODUCTION

Postoperative delirium (POD) is a state of acute delirium caused by dysregulation of basic neuronal activity secondary to systemic disorders, and is a type of delirium with typical characteristics of consciousness fluctuations, inattention, confusion and changes in consciousness level. It is a common complication of the central nervous system after surgery, most commonly seen 1 ∼ 3 days after surgery. POD can lead to more postoperative complications, higher mortality, longer hospital stay and higher hospitalization costs. Therefore, to uncover the risk factors of POD and accurately identify the high-risk population of POD has always been one of the hot topics in perioperative research.

Cerebrospinal fluid (CSF) biomarkers include β-amyloid 42 (Aβ42), total Tau (T-tau) and phosphorylated Tau (P-tau), which are the core biomarkers of postoperative delirium (POD). Associated with the development and progression of POD, reduced CSF Aβ42 levels were shown to be an independent predictor (Strozyk et al., 2003; Lin et al., 2021), and the deposition of amyloid Aβ42 in amyloid plaques was reciprocally elevated (Blennow et al., 2015; Cunningham et al., 2019). Increased T-tau levels reflect the intensity of axonal degeneration (Hesse et al., 2001; Bitsch et al., 2002; Riemenschneider et al., 2003; Hampel et al., 2010), while increased P-tau levels are associated with the amount of neurofibrillary tangles in the brain of AD patients (Tapiola et al., 2009). Therefore, high levels of T-tau or P-tau suggest neurodegeneration (Idland et al., 2017), and their peak changes are related to the severity of POD (Parker et al., 2021). In addition, decreased Aβ/tau ratio is associated with POD (Xie et al., 2014).

Social networks refer to a system of relatively stable relationship formed by interaction between individual members of the society. They focus on the interaction and connection between people, and the consequent impact on people's social behavior. A social network is essentially a social structure composed of many nodes, usually represented by individuals or organizations. Hence, social networks represent the social relationships ranging from casual acquaintances to tightly knit family among people and organizations.

Recently, the influence of social networks on cognitive status of middle-aged and elderly people has caught much attention. Maintaining an active social lifestyle in later life may enhance cognitive reserve and benefit cognitive function (Evans et al., 2018; Siette et al., 2020). Studies have proved that social relationship with friends or neighbors is associated with a lower risk of functional decline, and is not affected by family support (Murata et al., 2017). A rich social network was associated with a reduction in various adverse health outcomes and a lower risk of death in older adults (Oremus et al., 2019; Zahodne et al., 2021). In contrast, poor social networks may increase the risk of neurodegenerative diseases. For example, in a 2021 study, Akhter-Khan et al. (2021) reported that poor social networks (e.g., chronic loneliness) were independent predictors of AD. However, there are only a few studies on the correlation between social network and Postoperative cognitive dysfunction (POCD) and POD, and the pathological mechanism of neurodegenerative diseases caused by social network remains unclear.

Our study aims to explore whether suboptimal social networks are a risk factor for POD, and to characterize the mediating role of CSF core proteins between social networks and POD. It is of great clinical significance to explore the possibility of using social networks as an independent predictor to identify people at high risk of POD, and to validate the feasibility of using social network as an entry point to reduce the incidence of POD.

Abbreviations: AD, Alzheimer's disease; POCD, Postoperative cognitive dysfunction; POD, postoperative delirium; NPOD, no postoperative delirium; CSF, cerebrospinal fluid; Aβ, β-amyloid; T-tau, total tau; P-tau, phosphorylated tau; CAM, Confusion Assessment Method; MDAS, Memorial Delirium Assessment Scale; ELISA, enzyme linked immunosorbent assay; MMSE, Mini-Mental State Examination; PACU, post-anesthesia care unit; ASA, American Society of Anesthesiologist.

MATERIALS AND METHODS

Perioperative Neurocognitive Disorder and Biomarker Lifestyle Study

The Perioperative Neurocognitive Disorder and Biomarker Lifestyle study (PNDABLE) investigates the pathogenesis, risk factors, and biomarkers of perioperative neurocognitive disorders among the northern Chinese Han population. PNDABLE aims to identify lifestyle factors that may increase the risk of PND in the non-demented northern Chinese Han population and provide evidence for disease prevention and early diagnosis. This study has been registered in the Chinese Clinical Trial Registry (clinical registration number ChiCTR2000033439) and approved by the Ethics Committee of Qingdao Municipal Hospital. CSF samples from all participants were collected upon obtaining written informed consent from the patient or the legal representative.

Included Participants

The Han Chinese patients undergoing unilateral total knee arthroplasty [no gender limitations, aged 50 ∼ 90, American Society of Anesthesiologist (ASA) I ∼ II] combined with epidural anesthesia were enrolled in the PNDABLE study at

Qingdao Municipal Hospital. The exclusion criteria include: (1) preoperative MMSE score $\leq$ 23 points; (2) drug or psychotropic substance abuse, as well as long-term use of steroid drugs and hormone drugs; (3) pre-operative III–IV hepatic encephalopathy; (4) recent major surgery; (5) severe visual and hearing impairments; (6) abnormal coagulation function before surgery; (7) central nervous system infection, head trauma, multiple sclerosis, neurodegenerative diseases including AD (e.g., epilepsy, Parkinson's Disease), or other major neurological disorders; (8) major psychological disorders; (9) severe systemic diseases (e.g., malignant tumors) that may affect CSF or blood levels of AD biomarkers including Aβ and tau protein; (10) family history of genetic diseases.

A total of 743 cognitively normal participants from PNDABLE had available information on covariates. According to whether POD occurred or not, the participants were divided into POD group and no postoperative delirium (NPOD) group. The patient recruitment flowchart is shown in **Figure 1**.

Preoperative Assessment

We interviewed all the patients the day before surgery and collected their baseline data, including age, gender, body mass index (BMI), ASA physical status, years of education, smoking and drinking history and preoperative cognitive status. Other information including comorbidities and medical history was also collected according to the patients' medical records. All the history collection, physical examination and dementia-related cognitive assessment were performed by an anesthesiologist.

Anesthesia and Surgery

The participants did not receive preoperative medications, and they were instructed not to drink for 6 h and not to eat for 8 h before surgery. After entering the operating room, ECG, SpO2, and NBP were routinely monitored, venous access was opened, and 3 ml of whole venous blood was extracted. All patients underwent combined spinal-epidural block, with the lumbar spinous process space 3–4 (L3-4) being used as the puncture point. After successful puncture, 2 ml of CSF was extracted from the subarachnoid space, followed by injection of 2–2.5 ml Levobupivacaine (0.66%) for about 30 s. After anesthesia, the sensory level was controlled below the T8 level. During the surgery, oxygen was inhaled via mask at 5 L/min to maintain blood pressure within ±20% of the baseline value. If intraoperative NBP fell below 90 mmHg (1 mmHg = 0.133 kPa) or decreased by more than 20% of the baseline value, 5 mg ephedrine was injected intravenously. After the operation, the patient was sent to the post-anesthesia care unit (PACU).

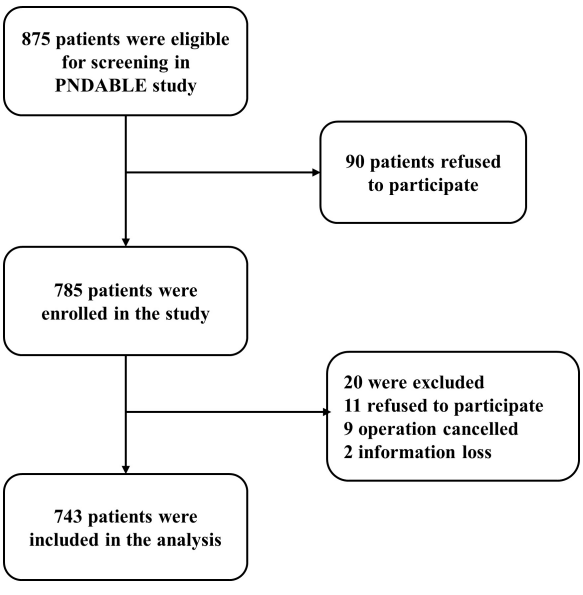


FIGURE 1 | Flow diagram of PNDABLE study.

Measurements of Cerebrospinal Fluid Sampling

Cerebrospinal fluid samples were processed immediately within 2 h after standard lumbar puncture. Each sample was centrifuged at 2,000 × *g* for 10 min, and CSF samples were separated and stored in an enzyme-free EP (Eppendorf) tube (AXYGEN; PCR-02-C) at −80°C under the international BIOMARKAPD project for further use in this study.

Cerebrospinal fluid biomarkers of POD were measured by enzyme linked immunosorbent assay (ELISA) using the microplate reader (Thermo Scientific Multiskan MK3). Aβ42 (BioVendor, Ghent, Belgium Lot: No. 296-64401), P-tau (BioVendor, Ghent, Belgium Lot: QY-PF9092) and T-tau (BioVendor, Ghent, Belgium Lot: No. EK-H12242). All ELISA measurements were performed in strict accordance with the manufacturer's instructions by experienced technicians who were blinded to the clinical information. The samples and standards were measured in duplicate, and the means of duplicates were used for the statistical analyses. All the antibodies and plates were from a single lot to exclude variability between batches. Moreover, the within-batch CV was <5% and the inter-batch CV was <15%.

The Assessment of Social Networks

A quantitative assessment of the social network was conducted through a self-administered questionnaire and confirmed by people close to the patient (e.g., family members) (Ma et al., 2021). Patients were asked about how they got along with neighbors, relatives and friends to assess the degree of intimacy of different social relationships. The subjective options of intimacy included estrangement, moderate and intimacy, which were scored 0, 1, and 2, respectively. The evaluation was conducted using blind method, regardless of the evaluation and measurement results of other items from the same subject. The total score of the final social network is the sum of the intimacy score of the three social relationships, ranging from 0 to 6 points. The higher the score is, the closer the social relationship of the subject is.

Neuropsychological Tests

The preoperative cognitive status was assessed by neurologists using the Mini-Mental State Examination (MMSE). Patients whose MMSE scores <23 points were excluded.

The delirium assessment was performed twice a day at 9:00–10:00 am and 2:00-3:00 pm on day 1–day 7 (or before discharge) post-operatively by an anesthesiologist. We used the visual analog scale (VAS) score of 0–10 (lower scores indicating lower levels of pain) to assess pain at the same time. POD was defined by the Confusion Assessment Method (CAM), and POD severity was measured using the Memorial Delirium Assessment Scale (MDAS). Thereafter, the post-operative CAM-positive and MDA-positive patients were recorded.

Statistical Analysis

Sample Size Calculation

The preliminary test in this study found that 7 covariates were expected to enter the Logistic regression. The POD incidence was 10%. And the loss of follow-up rate was assumed to be 20%. Therefore, the required sample size was calculated to be 875.

Outcome Analysis

Demographic characteristics were summarized as median (p25 and p75) for continuous variables and number (column percentage) for categorical variables. The Kolmogorov–Smirnov test was used to determine whether the measurement data conformed to the normal distribution. The *t*-test (normally distributed data) or Mann–Whitney *U* test (skewed distribution) was performed for continuous variables, while chi-square tests for categorical variables. *P*-values were calculated for comparing the between-group difference.

Firstly, we took the total score of the social networks as a comprehensive factor to explore its relationship with POD. Univariate binary logistic regression analysis of POD, social network score, Aβ42, T-tau, P-tau, Aβ42/T-tau, and Aβ42/P-tau was performed. Age, gender, years of education, and MMSE were chosen as covariates in multivariate logistic regression analysis.

Secondly, we built two models to explore the robustness of the main results. The co-variables of the first model include age (continuous variable), sex (female = 0, male = 1), years of education (continuous variable), MMSE (continuous variable), history of alcohol consumption (no = 0, yes = 1), history of smoking (no = 0, yes = 1), history of diabetes (no = 0, yes = 1), history of hypertension (no = 0, yes = 1), history of coronary heart disease (no = 0, yes = 1). The co-variables of the second model include age (65–90 years), sex (female = 0, male = 1), years of education (continuous variable), MMSE (continuous variable), history of alcohol consumption (no = 0, yes = 1), history of smoking (no = 0, yes = 1), history of diabetes (no = 0, yes = 1), history of hypertension (no = 0, yes = 1), history of coronary heart disease (no = 0, yes = 1). The multivariable logistic regression analysis was performed.

Thirdly, we divided social networks into three subgroups. Independent-samples test was used to examine the relationship between scores of the three subcomponents and CSF biomarkers of POD.

Finally, to examine whether the association between social network and POD was mediated by CSF POD biomarkers, logistic regression models were fitted based on the methods. The first equation regressed the mediator (CSF POD biomarkers) on the independent variable (social network). The second equation regressed the dependent variable (POD) on the independent variable (social network). The third equation regressed the dependent variable on both the independent variable and the mediator variable. Mediation effects were established if the following criteria were simultaneously met: (1) social network were significantly related to CSF POD biomarkers; (2) social network was significantly or not significantly related to POD; (3) CSF POD biomarkers were significantly related to POD; and (4) the association between social network and POD was attenuated when CSF POD biomarkers (the mediator) were added in the regression model. Furthermore, the attenuation or indirect effect was estimated, with the significance determined using 10,000 bootstrapped iterations, where each path of the model was controlled for age, gender, years of education, and MMSE.

The data were analyzed using SPSS version 23.0 (SPSS Inc., Chicago, IL, United States), R software version 4.4.1(R Foundation for Statistical Computing, Vienna, Austria) and Stata MP16.0 (Solvusoft Corporation Inc, Chicago, IL, United States). *P*-value < 0.05 was considered significant except where specifically noted.

RESULTS

Participant Characteristics

We recruited 875 participants, of which 743 met the requirements of our study and 132 were excluded. The reasons for dropping out were shown in **Figure 1**. Of the enrolled patients, 154 subjects experienced POD within 7 days after operation or before discharge, therefore, the incidence of POD was 20.7%. Participants were divided into two groups based on the presence or absence of POD (POD group and NPOD group). The demographic and clinical data of two groups were summarized in **Table 1** (Pinho et al., 2016; Wang et al., 2018).

Correlation Analysis of Social Network Score and Cerebrospinal Fluid Biomarkers With Postoperative Delirium

Mann–Whitney *U* test showed that social network score and CSF biomarkers (Aβ42, T-tau, P-tau, Aβ42/T-tau, and Aβ42/P-tau) were associated with POD ($P < 0.001$) (**Table 1**). The social network score and the CSF levels of Aβ42, Aβ42/T-tau, and Aβ42/P-tau in patients with delirium were significantly lower than in those without delirium. However, the CSF levels of T-tau and P-tau in POD patients were significantly lower than those in NPOD patients (**Figure 2**).

Univariate binary logistic regression analysis showed that increased CSF levels of T-tau (crude OR = 1.006, 95% CI 1.005–1.007, $P < 0.001$) and P-tau (crude OR = 1.062, 95% CI 1.051–1.074, $P < 0.001$) were risk factors for POD. However, increased levels of social network (crude OR = 0.723, 95% CI 0.642–0.814, $P < 0.001$), Aβ42 (OR = 0.997, 95% CI 0.996–0.998, $P < 0.001$), Aβ42/T-tau (crude OR = 0.407, 95% CI 0.325–0.510, $P < 0.001$), and Aβ42/P-tau (crude OR = 0.833, 95% CI 0.795–0.874, $P < 0.001$) were protective factors for POD (**Table 2**).

TABLE 1 | Characteristics of included participants in PNDABLE database.

Characteristics	All participants(n = 743)		P-value*
	POD(n = 154)	NPOD(n = 589)	
Age, year	74(71–78)	59(51–65)	<0.001***
Female, yes (%)	60(39.0)	236(40.0)	0.835
Education, year	9(6–12)	9(9–12)	0.072
MMSE	28(27–29)	29(28–30)	<0.001***
MDAS	12(3–22.25)	1(1–7)	<0.001***
Hypertension, yes, no. (%)	72(46.8)	190(32.2)	0.001**
Diabetes mellitus, yes, no. (%)	41(26.6)	82(13.9)	<0.001***
CHD, yes, no. (%)	35(22.7)	61(10.4)	<0.001***
Smoking history, yes, no. (%)	38(32.7)	175(29.7)	0.231
Drinking history, yes, no. (%)	42(37.5)	211(35.8)	0.056
CSF biomarkers, pg/ml			
Aβ42	245.0(142.8–405.3)	350.1(231.7–492.9)	<0.001***
T-tau	277.9(199.1–609.6)	190.7(144.8–260.1)	<0.001***
P-tau	63.2(40.2–83.0)	36.5(28.1–46.3)	<0.001***
Aβ42/T-tau	0.8(0.4–1.7)	1.8(1.1–2.7)	<0.001***
Aβ42/P-tau	4.4(2.0–8.2)	9.2(6.1–13.8)	<0.001***
Social network score	4(3–6)	6(4–6)	<0.001***

**P-value < 0.05, **p-value < 0.01, ***p-value < 0.001. CSF, cerebrospinal fluid; Aβ, amyloid-β; T-tau, total tau; P-tau, phosphorylated tau; CHD, Coronary Heart Disease; MMSE, Mini-Mental State Examination (Score range: 24–30); MDAS, Memorial Delirium Assessment Scale; Social network score (Score range: 0–6). Data are presented as median (IQR) unless otherwise indicated.*

After adjustment for the age, gender, years of education and MMSE, similar results were: T-tau (crude OR = 1.005, 95% CI 1.00–1.007, $P < 0.001$) and P-tau (crude OR = 1.063, 95% CI 1.045–1.081, $P < 0.001$) remained as risk factors for POD. Social network score (crude OR = 0.831, 95% CI 0.700–0.986, $P = 0.034$), Aβ42 (OR = 0.998, 95% CI 0.997–1.000, $P = 0.017$), Aβ42/T-tau (crude OR = 0.544, 95% CI 0.408–0.724, $P < 0.001$), and Aβ42/P-tau (crude OR = 0.866, 95% CI 0.825–0.9110, $P < 0.001$) were still protective factors for POD (**Table 2**).

Furthermore, sensitivity analysis was performed to verify the stability of the results. We first added more covariates including age, gender, years of education, MMSE, history of alcohol consumption, history of smoking, diabetes, hypertension, and coronary heart disease into the multivariate logistic regression analysis. The results showed that the social network score (crude OR = 0.812, 95% CI 0.689–0.979, $P = 0.028$) was still correlated with POD. Then we analyzed whether the association would change if only individuals aged over 65 at the baseline were selected, and found that the social network score (crude OR = 0.830, 95% CI 0.695–0.992, $P = 0.041$) and CSF biomarkers remained stable across two sensitivity analyses in our study (**Table 2**).

The Relationship Between Intimacy Degrees of Three Subcomponents and Cerebrospinal Fluid Postoperative Delirium Biomarkers

In the total population, social network subcomponents were significantly associated with CSF POD biomarkers. In the subcomponent of social relationships with friends, when intimacy degree changed from distant to close, the CSF levels of P-tau ($t = 2.678$, $P = 0.011$), Aβ42/T-tau ($t = -2.163$, $P = 0.031$), and Aβ42/P-tau ($t = -2.291$, $P = 0.028$) were also changed. In particular, when the intimacy degree changed from moderate to close, the CSF levels of P-tau ($t = 2.376$, $P = 0.018$) and Aβ42/P-tau ($t = -2.013$, $P = 0.044$) were significantly reduced.

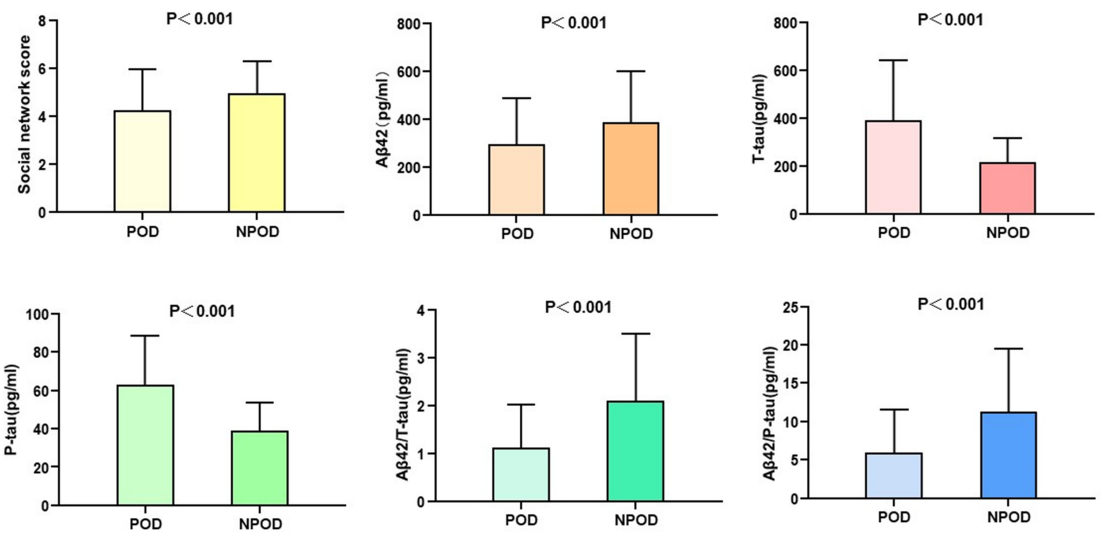


FIGURE 2 | Social network score and expression of biomarkers in CSF of POD patients and NPOD controls.

TABLE 2 | Logistic regression analysis and sensitivity analysis in PNDABLE study.

	Model 1		Model 2		Model 3		Model 4	
	OR (95% CI)	***P*-value**	**OR (95% CI)**	***P*-value**	**OR (95% CI)**	***P*-value**	**OR (95% CI)**	***P*-value**
Social network score	0.723(0.642–0.814)	<0.001***	0.831(0.700–0.986)	0.034*	0.821(0.689–0.979)	0.028*	0.830(0.695–0.992)	0.041*
Aβ42, pg/ml	0.997(0.996–0.998)	<0.001***	0.998(0.997–1.000)	0.017*	0.998(0.997–1.000)	0.025*	0.998(0.997–1.000)	0.028*
T-tau, pg/ml	1.006(1.005–1.007)	<0.001***	1.005(1.00–1.007)	<0.001***	1.005(1.004–1.007)	<0.001***	1.005(1.003–1.007)	<0.001***
P-tau, pg/ml	1.062(1.051–1.074)	<0.001***	1.063(1.045–1.081)	<0.001***	1.064(1.046–1.082)	<0.001***	1.067(1.048–1.087)	<0.001***
Aβ42/T-tau, pg/ml	0.407(0.325–0.510)	<0.001***	0.544(0.408–0.724)	<0.001***	0.548(0.409–0.733)	<0.001***	0.552(0.412–0.739)	<0.001***
Aβ42/P-tau, pg/ml	0.833(0.795–0.874)	<0.001***	0.866(0.825–0.910)	<0.001***	0.867(0.825–0.910)	<0.001***	0.870(0.828–0.915)	<0.001***

**P-value < 0.05, **P-value < 0.01, ***P-value < 0.001. Aβ, amyloid-β; T-tau, total tau; P-tau, phosphorylated tau. Model 1: The unadjusted logistic regression. Model 2: Adjusted logistic regression, the adjustment factors include age, gender, years of education, and MMSE. Model 3: First sensitivity analysis was based on more covariates including age, gender, years of education, MMSE, history of alcohol consumption and smoking, diabetes, hypertension, coronary heart disease. Model 4: Second sensitivity analysis was based on selecting only individuals older than 65 years.*

In the subcomponent of social relationships with relatives, when the degree of intimacy changed from distant to moderate, the CSF level of Aβ42/T-tau was significantly increased ($t = -3.226$, $P = 0.002$). When the intimacy degree changed from distant to close, the CSF levels of T-tau ($t = 2.078$, $P = 0.047$), P-tau ($t = 2.656$, $P = 0.008$), Aβ42/T-tau ($t = -4.386$, $P < 0.001$), and Aβ42/P-tau ($t = -2.253$, $P = 0.025$) were also changed. When the intimacy degree changed from moderate to close, Aβ42 ($t = -2.037$, $P = 0.042$) and Aβ42/P-tau ($t = -2.222$, $P = 0.027$) were significantly increased.

In the subcomponent of social relationships with neighbors, the intimacy degree mainly affects the CSF levels of P-tau [distant versus close ($t = 2.581$, $P = 0.010$), moderate versus close ($t = 2.282$, $P = 0.023$)] and Aβ42/P-tau [moderate versus close ($t = -2.008$, $P = 0.045$)] (**Table 3** and **Figure 3**).

Causal Mediation Analyses

We further explored the mediating effect of CSF biomarkers between suboptimal social networks and POD. The mediated effect analysis showed that CSF T-tau (proportion = 20%) and P-tau (proportion = 33%) contents as well as Aβ42/T-tau (proportion = 14%) and Aβ42/P-tau (proportion = 15%) had significant mediation effect on the correlation between suboptimal social networks and POD (**Figure 4**).

DISCUSSION

The results of this study support the association between suboptimal social networks and POD, with low social network score being an independent predictor. Social networks are founded upon interpersonal relationships, the establishment of which in turn may promote social participation and social support (Czaja et al., 2021). On the other hand, a low score for social networks may indicate relative alienation and even social isolation. In addition, animal models have proved that social isolation may cause or even aggravate memory loss (Leser and Wagner, 2015), while a rich social environment may reduce cognitive deficits in rats (Hsiao et al., 2012; Huang et al., 2015). A study conducted in 2020 demonstrated that social isolation is associated with cognitive decline in older adults (Yu et al., 2021), confirming the notion that high levels of social isolation may influence cognitive status in older adults by affecting episodic memory and mental states. Numerous studies have shown that a good social network can significantly improve the cognitive status of older adults (Fratiglioni et al., 2000, 2004; Balouch et al., 2019). People with rich social networks have more opportunities to communicate with the outside world, and are more likely to promote cognitive, physical and social participation, enhance physical and mental stimulation, and effectively reduce negative psychological states, thereby reducing the risk of cognitive dysfunction. Moreover, Preoperative cognitive status was related to POD (Choi et al., 2020), which is supported by our results. Besides, we could apply the results of this study to identify patients at a high risk for POD. Since POD, as a common perioperative complication in elderly patients, may affect the length of hospital stay and long-term prognosis, accurate preoperative identification of these patients and immediate intervention may reduce the incidence of POD, thereby shortening the length of hospital stay and improving long-term outcomes.

Based on the social network questionnaire, this study divided social networks into three subgroups. The scores of the three subcomponents reflected how close the patients were to different social relationships. Our study further examined the relationship between the scores of three subcomponents and CSF biomarkers of POD. The results confirmed that different social relationships are associated with different incidence of POD, and that the level of intimacy within the same social relationship affected CSF biomarkers.

Pathological changes of POD mainly include phosphorylation of tau protein and deposition of amyloid protein. In addition,

TABLE 3 | Three subgroups of social network and CSF biomarkers.

Subgroup	Aβ42 (63.5–998.0) pg/ml		T-tau (56.8–986.3) pg/ml		P-tau (10.1–130.4) pg/ml		Aβ42/T-tau (0.1–9.3)		Aβ42/P-tau (0.7–44.3)	
	t	*P*-value*	*t*	*P*-value*	*t*	*P*-value*	*t*	*P*-value*	*t*	*P*-value*
Social ties with friends										
Distant vs. Moderate	−0.841	0.401	1.789	0.082	1.765	0.085	−1.579	0.115	−1.202	0.230
Distant vs. Close	−1.473	0.142	2.002	0.053	2.678	0.011*	−2.163	0.031*	−2.291	0.028*
Moderate vs. Close	−1.396	0.163	0.668	0.504	2.376	0.018*	−1.816	0.070	−2.013	0.044*
Social ties with relatives										
Distant vs. Moderate	−0.822	0.412	1.897	0.067	1.745	0.082	−3.226	0.002**	−1.321	0.188
Distant vs. Close	−1.743	0.082	2.078	0.047*	2.656	0.008**	−4.386	<0.001***	−2.253	0.025*
Moderate vs. Close	−2.037	0.042*	0.537	0.591	1.845	0.066	−1.424	0.155	−2.222	0.027*
Social ties with neighbors										
Distant vs. Moderate	−0.517	0.606	1.566	0.119	1.131	0.259	−0.986	0.325	−0.767	0.444
Distant vs. Close	−1.426	0.155	2.009	0.051	2.581	0.010*	−1.784	0.075	−1.785	0.075
Moderate vs. Close	−1.740	0.082	0.861	0.390	2.282	0.023*	−1.699	0.090	−2.008	0.045*

**p-value < 0.05, **p-value < 0.01, ***p-value < 0.001. Aβ, amyloid-β; T-tau, total tau; P-tau, phosphorylated tau. Social ties between families, neighbors, and, relatives are not related.*

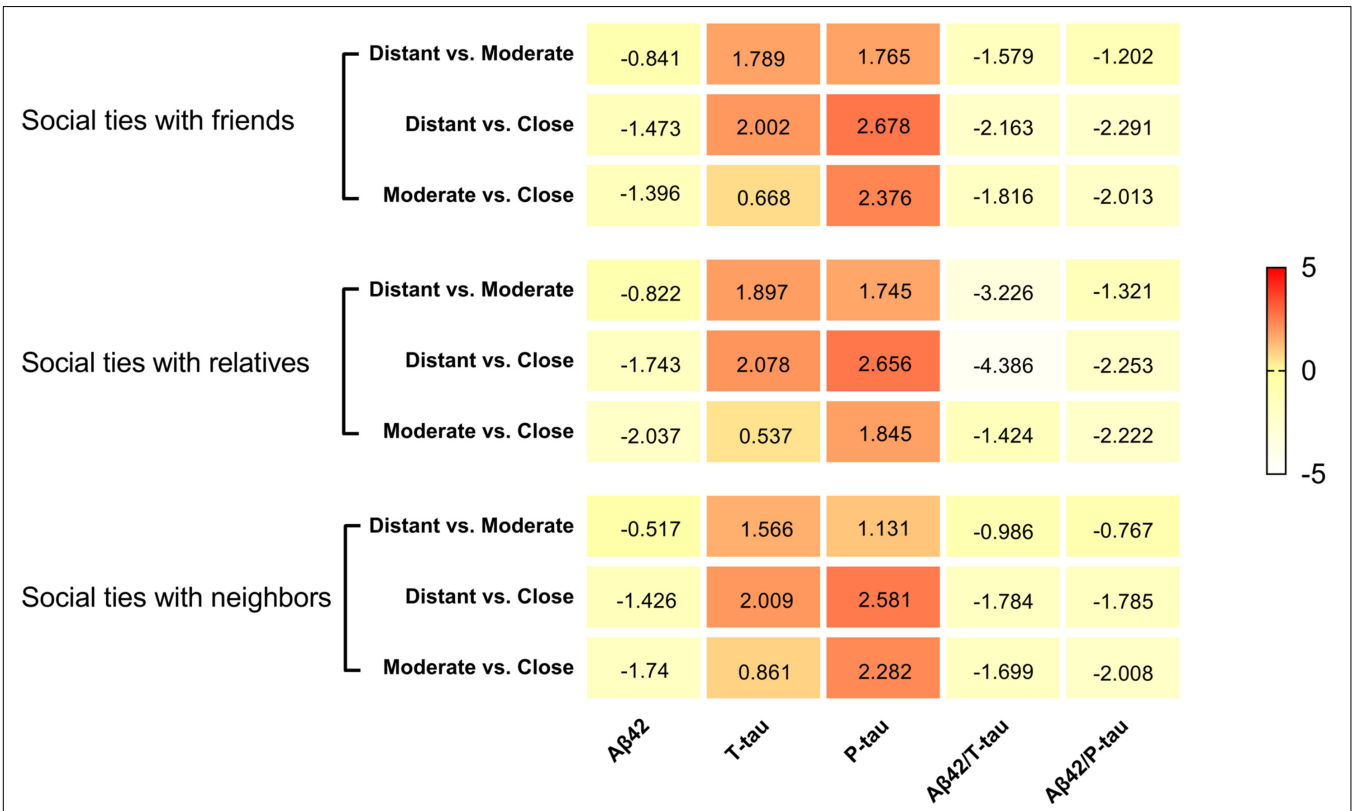


FIGURE 3 | Heat maps for social networks and CSF biomarkers of POD. The social networks showed several suggestive or significant associations with CSF indicators of multiple pathologies of POD in the whole participants. The color-coding and the positive or negative number in each cell represent a comparison of mean values between the two groups inferred from the Independent-Samples *T*-Test.

it has been reported that the ratio between CSF biomarkers is more sensitive to the identification of amyloid deposition than individual biomarkers (e.g., Aβ42/Aβ40 vs. Aβ42) (Janelidze et al., 2016). Therefore, the ratio was also used in this study to explore the relationship between social network and POD, and it was concluded that the intimacy of the three social relationships mainly affected the incidence of POD by modulating the level of T-tau and P-tau proteins, Furthermore, the degree of intimacy with friends mainly affected the value of P-tau, Aβ42/T-tau, and Aβ42/P-tau, the degree of intimacy with neighbors mainly

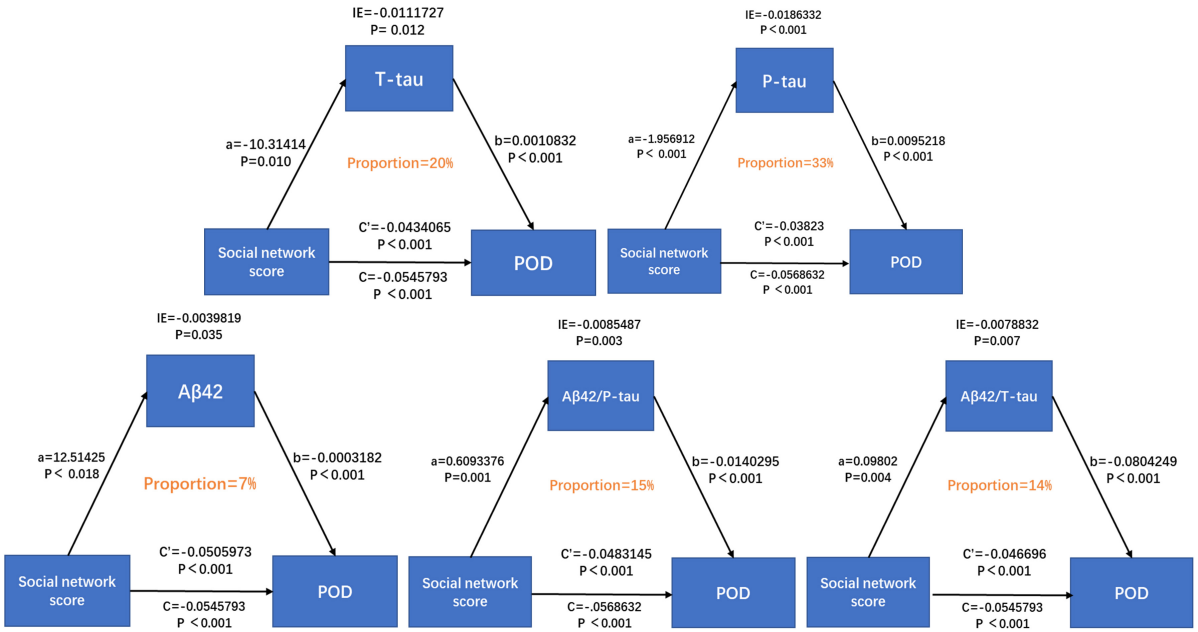


FIGURE 4 | Mediation analyses.

affected the value of P-tau, and, the degree of intimacy with relatives affected the value of Aβ42, T-tau, P-tau, Aβ42/T-tau, and Aβ42/P-tau.

In order to explore the mechanism of how social network relates to the incidence of POD, we explored the mediating effect of CSF biomarkers between social network and POD, and concluded that social network mainly affects T-tau protein, P-tau protein and the level of Aβ42/T-tau protein, Aβ42/P-tau affected the occurrence of POD. It has been found that rich social networks may reduce Aβ deposition and P-tau production (Huang et al., 2015; Donovan et al., 2016). In line with our study, previous research showed an elevated Aβ aggregation in isolated aged APP/PS1 mice, which was associated with increased secretase and decreased neprilysin expression (Hsiao et al., 2012; Huang et al., 2015). Besides, D'oleire Uquillas et al. (2018) found that after controlling for age, sex, and the Alzheimer's disease genetic risk factor, apolipoprotein E (APOE), a higher tau pathology in the right entorhinal cortex was associated with greater loneliness. This is consistent with our research.

In this study, we established three correction models, four variables in Model 2 (age, sex, years of education, and MMSE) and nine variables in Model 3 (age, sex, MMSE, years of education, history of alcohol consumption, history of smoking, history of diabetes, history of hypertension, and history of coronary heart disease) were used as co-variables for regression analysis. Suboptimal social networks are still significantly correlated with the occurrence of POD. In addition, patients older than 65 years old were included in the regression analysis as co-variable of model 4, and the conclusion has not changed, indicating that our conclusion is robust— Suboptimal social networks is a risk factor for the occurrence of POD.

In addition to confirming the correlation between social network and the incidence of POD in the elderly population, this study is also consistent with the recent research hotspot of "age-friendly environment." The concept of an age-friendly environment has attracted a lot of attention since its inception. How to integrate the advantages of medicine, environment, society and other disciplines and fully identify the environmental factors conducive to health and longevity is one of the cutting-edge research directions. Studies have proved that both natural and social environments have a significant impact on the physical and mental health and cognitive function of the elderly. For example, a study in 2021 confirmed that social isolation is harmful to the cognitive health of the elderly population (Li et al., 2021). Since the COVID-19 pandemic, home isolation has significantly increased the incidence of social isolation among the elderly, while unsurprisingly reducing their social support. Our result confirmed that the lack of social network is a risk factor for POD in aged population. Hence in future studies, through the preoperative score of social networks, we can predict or identify people at high risk of POD, make timely and necessary intervention, reduce the incidence of POD, so as to improve the long-term prognosis and promote the construction of healthy aging society.

There are some limitations to our study. Firstly, at the stage of data collection, we only counted three co-morbidities of patients, and the number of comorbid conditions was small. In subsequent studies, we will include as many comorbid conditions as possible to make the results more convincing. Secondly, this study is only a cross-sectional study which explores the relationship between preoperative total social network score and POD in middle-aged and elderly patients around the time of elective surgery, without following-up the incidence of delirium

and cognitive function status of patients for a long time after surgery. Hence we will further explore in future studies the influence of suboptimal social networks and other social factors on longitudinal changes of POD neuropathology through multi-center CSF and neuroimaging tests with a large sample size. Finally, this study only focused on the effect of social network on POD among Chinese Han population. As different groups and regions have different cultural backgrounds, the identified effect of social network on the cognitive function and mental health status of the elderly may change. Therefore, it is necessary to sort out the dimensions of social networks, develop social network measurement tools suitable for specific cultural backgrounds, and explore the correlation between the different groups and regions of China with different cultural backgrounds.

CONCLUSION

Our study confirms that suboptimal social networks are a risk factor for POD, and that different levels of intimacy in the same social relationship can affect the concentration of CSF POD biomarkers, mainly mediated by tau protein, hence influencing the incidence of POD. In other words, it can affect cognitive function by affecting the concentration of CSF biomarkers. This suggests that suboptimal social networks, as a risk factor for POD, can predict the population at high risk. This work will provide information for future social behavior research and age-friendly environment research, thus facilitating the development of intervention strategies for POD and promoting the construction of healthy aging environment.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Chinese Clinical Trial Registry (clinical registration number ChiCTR2000033439) and approved by the Ethics Committee of Qingdao Municipal Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YB conceived the current study. FW, JW, SL, and XW performed the experiments. RD, XL, HY, and BW analyzed the data. XT and BW performed the experiments and wrote and revised the manuscript. All authors have contributed to the manuscript revising and editing critically for important intellectual content and given final approval of the version and agreed to be accountable for all aspects of the work presented here and read and approved the final manuscript.

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The Relationship Between Cardiovascular Disease Risk Score and Postoperative Delirium: The PNDABLE Study

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Objective: We aimed to investigate the relationship between Framingham Heart Study general cardiovascular disease risk score (FHS–CVD risk score) and postoperative delirium (POD) among patients who had unilateral total knee arthroplasty performed under epidural anesthesia. Furthermore, we examined whether such a hypothesized relationship was mediated by the cerebrospinal fluid (CSF) biomarkers.

Methods: A total of 750 participants were included in the current study. And the data were drawn from the database obtained from the Perioperative Neurocognitive Disorder And Biomarker Lifestyle (PNDABLE) study. The preoperative cognitive function of participants was measured by using Mini-Mental State Examination (MMSE). The incidence of POD was assessed using the Confusion Assessment Method (CAM). The POD severity was measured using the Memorial Delirium Assessment Scale (MDAS). The POD CSF biomarkers included in the current study were: Aβ42, T-tau, P-tau, Aβ42/T-tau, and Aβ42/P-tau. The level of the CSF biomarkers was measured using the enzyme-linked immune-sorbent assay (ELISA) in the PNDABLE study. Linear regression analysis was performed to examine the relationship between the FHS–CVD risk score and the POD CSF biomarkers. Logistic regression was used to analyze the relationship between FHS–CVD risk score, POD CSF biomarkers, and POD incidence. The proposed mediating effect of CSF biomarkers was evaluated using Mediation Analysis with 10,000 bootstrapped iterations. The receiver operating characteristic (ROC) curve is chosen as the evaluation metric for assessing the efficacy of the FHS–CVD risk score in predicting POD.

Results: In the PNDABLE study, the overall incidence of POD was 22.9% with 37.2% in the higher vascular risk group and 7.9% in the lower vascular risk group. Multiple linear regression models showed that a higher preoperative FHS–CVD risk score was positively correlated with CSF T-tau (β = 0.218, $P = 0.015$) and P-tau level (β = 0.309, $P < 0.001$) in the higher vascular risk group. After adjusting for age (40–90 years), gender, education, MMSE, smoking history, drinking history, hypertension, diabetes, and the presence of CHD (cardiovascular heart disease), the results of the logistic

regression analysis demonstrated the effect of Aβ42 (OR = 0.994, 95% CI 0.992–0.996, $P < 0.001$), Aβ42/T-tau (OR = 0.353, 95% CI 0.254–0.491, $P < 0.001$), and Aβ42/P-tau (OR = 0.744, 95% CI 0.684–0.809, $P < 0.001$) in protecting patients against POD. However, the FHS–CVD risk score (OR = 1.142, 95% CI 1.017–1.282, $P = 0.025$) and the remaining two biomarkers: T-tau (OR = 1.005, 95% CI 1.004–1.007, $P < 0.001$) and P-tau (OR = 1.045, 95%CI 1.029–1.062, $P < 0.001$) were identified as the risk factors. Mediation analyses revealed that the association between FHS–CVD risk score and POD was partially mediated by T-tau (proportion: 31.6%) and P-tau (proportion: 23.6%). The predictive power of the FHS–CVD risk score was validated by the ROC curve with an AUC of 0.7364.

Conclusion: Higher vascular risk score is one of the preoperative risk factors for POD, which is partly mediated by CSF biomarker tau protein.

Clinical Trial Registration: [www.clinicaltrials.gov], identifier [ChiCTR2000033439].

Keywords: postoperative delirium, vascular risk score, cerebrospinal fluid, biomarkers, mediation analysis

INTRODUCTION

Postoperative delirium (POD), an acute or fluctuating mental status impairment, is one of the common adverse postoperative complications and the elderly are at higher risk because the predisposing risk factors are often associated with aging (Blazer and van Nieuwenhuizen, 2012). With the rapid aging of the population, the number of people with dementia is increasing (Young and Inouye, 2007; Aldecoa et al., 2017; Jia et al., 2020). The characteristics are perceptual, awareness, attention, and cognition dysfunction (Monk and Price, 2011; Blazer and van Nieuwenhuizen, 2012). POD starts during the recovery room and often occurs in the hospital up to 1-week post procedure or until discharge (Evered et al., 2018). The occurrence of delirium not only increases the patient's economic burden, prolonging the hospital stay, but also easily leads to the reduction of quality, leading to the inability to live normally (Witlox et al., 2010). However, there are no effective measures to prevent or cure dementia. Hence, it is crucial to identify the pathogenesis mechanisms and to find out the potential risk factors.

The cognitive impairment has been confirmed in connection with the multiple vascular and environmental risk factors. After fully validated and integrated multivariate variables, the Framingham Heart Study (FHS) was aimed at searching for the risk factors of cardiovascular disease (CVD) and predicting the 10-year risk of CVD to improve the prevention by formulating health guidelines (Vemuri et al., 2017). Over several decades, FHS has expanded to be an almost comprehensive study and grown into a three-generational prospective community-based cohort study of dementia and Alzheimer's disease (AD) and of the preclinical states preceding AD including various types of mild cognitive impairment (MCI) and pre-MCI (Dawber and Kannel, 1966; Feinleib et al., 1975; Splansky et al., 2007). Correlational studies have proven that FHS–CVD risk score is more applicable to assess the risk of cognitive decline.

Postoperative delirium is one of the most common postoperative neurodegenerative changes. Plenty of evidences suggest that cerebrospinal fluid (CSF) amyloid β 42 (Aβ42), total tau (T-tau), and phospho-tau (P-tau) are biomarkers of the POD process, reflecting plaque pathology, neurodegeneration, and neurofibrillary tangle pathology, respectively (Maldonado, 2013). The aforementioned biomarkers can be seen before the clinical emergence of dementia (Sperling et al., 2011). Lower preoperative CSF Aβ level and higher tau level are associated with both higher incidence and greater symptom severity of POD, which can distinguish patients with dementia from the healthy controls and predict the development of it. These results suggest that changes in the levels of Aβ and tau in CSF may be a common neuropathogenesis underlying both postoperative delirium and postoperative cognitive dysfunction.

To date, there has been no research demonstrating the overall impact of multiple vascular risk factors on cognitive function. Therefore, the purpose of this study is to explore the relationship between FHS–CVD risk score and POD, and to test whether the influences of FHS–CVD risk score on POD were mediated by the POD biomarkers.

MATERIALS AND METHODS

Perioperative Neurocognitive Disorder and Biomarker LifestylE Study

The Perioperative Neurocognitive Disorder and Biomarker LifestylE study (PNDABLE) aims at investigating the pathogenesis, risk factors, and biomarkers of perioperative neurocognitive disorders in the northern Chinese Han population. The purpose of PNDABLE is to identify lifestyle factors that may affect the risk of PND in the non-demented northern Chinese Han population to provide a foundation for disease prevention and early diagnosis. This study has been registered in the Chinese Clinical Trial Registry (clinical registration number ChiCTR2000033439) and approved by the Ethics Committee of Qingdao Municipal Hospital. CSF samples could be collected from all the participants after the written

informed consent was obtained from the patients or their legal representatives.

Participants

The Han Chinese patients undergoing unilateral total knee arthroplasty (no gender limitations, aged 40 ∼ 90, ASA I ∼ II) combined with epidural anesthesia were enrolled in the PNDABLE study Qingdao Municipal Hospital. The exclusion criteria include: (1) preoperative MMSE score < 24 points; (2) drug or psychotropic substance abuse, as well as long-term use of steroid drugs and hormone drugs; (3) pre-operative III-IV hepatic encephalopathy; (4) recent major surgery; (5) severe visual and hearing impairments; (6) abnormal coagulation function before surgery; (7) central nervous system infection, head trauma, multiple sclerosis, neurodegenerative diseases other than AD (e.g., epilepsy, Parkinson's Disease), or other major neurological disorders; (8) major psychological disorders; (9) severe systemic diseases (e.g., malignant tumors) that may affect CSF or blood levels of AD biomarkers including Aβ and tau; and (10) family history of genetic diseases.

We collected a total of 750 cognitively normal participants' available information on covariates from the PNDABLE study. According to whether POD occurred or not, all the participants were divided into two groups: POD group and NPOD group. A patient recruitment flowchart is shown in **Figure 1**.

The participants did not receive preoperative medications, and they were instructed not to drink for 6 h and not to eat for 8 h before surgery. After entering the operating room, we routinely monitored ECG, SpO_2, and NBP, opened vein access and extracted 3 ml of whole venous blood. All the patients underwent a combined spinal–epidural block, with the space between lumbar 3-4 spinous processes (L3-4) as the puncture site. After a successful puncture, 2 ml of cerebrospinal fluid was extracted from the subarachnoid space, followed by injection of 2–2.5 ml ropivacaine (0.66%) for about 30 s. After anesthesia, the sensory level was controlled below the T8 level. During the surgery, oxygen was inhaled *via* a mask at 5 L/min to maintain blood pressure within ± 20% of the baseline value. If intraoperative NBP < 90 mmHg (1 mmHg = 0.133 kPa) or it decreased by more than 20% of the baseline value, ephedrine 5 mg was injected intravenously. If HR < 50 beats/min, atropine 0.5 mg was injected intravenously. Intravenous patient-controlled analgesia (butorphanol 0.1 mg/ml + tropisetron 0.05 mg/ml, diluted with normal saline to a total volume of 100 ml) was used in acute postoperative pain management. After the operation, the patient was sent to the anesthesia resuscitation room (PACU).

We interviewed all the patients the day before surgery and collected their baseline data, including age, gender, body mass index (BMI), ASA physical status, and years of education. Other information including comorbidities, medical history, and fracture classifications were also collected according to the patients' medical records. The whole history collection, physical examination, and dementia-related cognitive assessment were conducted by an anesthesiologist.

Cerebrospinal Fluid Biomarkers of Postoperative Delirium Measurements

Cerebrospinal fluid samples were processed immediately within 2 h after standard lumbar puncture. Each sample was centrifuged at 2,000 × g for ten minutes, and CSF samples were separated and stored in an enzyme-free EP (Eppendorf) tube (AXYGEN; PCR-02-C) at −80°C under the international BIOMARKAPD project for further use in the subsequent steps of this study.

Cerebrospinal fluid biomarkers of POD were measured by ELISA using the microplate reader (Thermo Scientific Multiskan MK3). CSF biomarkers of POD measurements were done with other ELISA kits [Aβ42 (BioVendor, Ghent, Belgium Lot: No.296-64401), P-tau (BioVendor, Ghent, Belgium Lot: QY-PF9092) and T-tau (BioVendor, Ghent, Belgium Lot: No. EK-H12242)]. All the ELISA measurements were performed by experienced technicians in strict accordance with the manufacturer's instructions. They knew nothing about the clinical information. The samples and standards were measured in duplicates, and the means of duplicates were used for

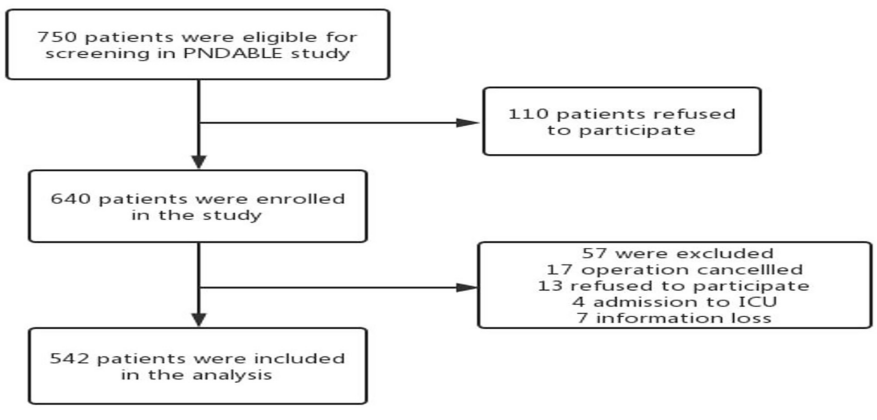


FIGURE 1 | The flow diagram showed that 750 participants were initially screened for the PNDABLE studies and finally 542 participants were included in the data analysis.

the statistical analyses. All the antibodies and plates were from a single lot to exclude variability between batches. Moreover, the within-batch CV was < 5% and the inter-batch CV was < 15%.

Assessment Criteria for Framingham Heart Study–Cardiovascular Disease Risk Score

The FHS–CVD risk score is a risk forecasting tool, which highlights gender specification and summarizes multiple factors to assess vascular accident within 10 years. The FHS–CVD risk score is calculated synthetically based on the participants' age, gender, antihypertensive therapy condition, systolic pressure, total cholesterol, high-density lipoprotein, smoking history, and diabetes history. The higher the patient's vascular risk score, the more severely the patient's vessels have aged, suggesting the higher risk of future vascular events. Previous studies have confirmed that individuals with a calculated 10-year risk of CAD-related death or non-fatal MI of 20% or greater are considered to be in the 'high-risk' category (McPherson et al., 2006).

Neuropsychological Tests

The preoperative cognitive status of participants was assessed by neurologists using the Mini-Mental State Examination (MMSE) assessed. Patients whose MMSE scores < 24 points were excluded.

The delirium assessment was performed at 9:00–10:00 a.m. and 2:00–3:00 p.m. twice a day on 1–7 days (or before discharge) by an anesthesiologist postoperatively. We used the visual analog scale (VAS) score of 0–10 (lower scores indicating lower levels of pain) to assess pain at the same time. POD was defined by the Confusion Assessment Method (CAM), and POD severity was measured by using the Memorial Delirium Assessment Scale (MDAS). Therefore, CAM-positive and MDAS-positive patients postoperatively were recorded.

Statistical Analysis

The preliminary test in this study found that 6 covariates were expected to enter the Logistic regression, the POD incidence was 10%, and the loss of follow-up rate was assumed to be 20%, so the required sample size was calculated to be 750 cases ($6 \times 10 \div 0.1 \div 0.8 = 750$).

The Kolmogorov–Smirnov test was used to determine whether the measurement data conformed to the normal distribution. The measurement data conformed to the normal distribution was expressed by mean ± standard deviation (SD), while the median (p25, p75) or a number (%) to express the data. We used × 2 tests (for categorical variables) and Mann–Whitney *U* test (for continuous variables) to test the difference of baseline characteristics between higher vascular risk group and lower vascular risk group. And the differences of CSF biomarkers levels between the higher and lower vascular risk groups were described in Box plots.

At first, we tested the relationships of the FHS–CVD risk scores with CSF POD biomarkers. Linear regression analysis of FHS–CVD risk score and Aβ42, T-tau, P-tau, Aβ42/T-tau, and Aβ42/P-tau was performed in the higher and lower vascular risk group, respectively. We also tested the relationships of POD with CSF POD biomarkers and FHS–CVD risk score. Binary logistic regression analysis of POD and FHS–CVD risk score, Aβ42, T-tau, P-tau, Aβ42/T-tau, and Aβ42/P-tau was performed.

Second, sensitivity analyses were performed by: (1) we tested the relationships of POD with FHS–CVD risk score and CSF POD biomarkers for patients adding four covariates, including age of 40∼90 age, gender, years of education and MMSE in multivariate logistic regression analysis; (2) we also tested the relationships of POD with FHS–CVD risk score and CSF POD biomarkers for patients over the age of 40∼90 age, gender, years of education and MMSE adding more covariates, including hypertension (yes or no), type 2 diabetes (yes or no), coronary heart disease (yes or no), smoking (yes or no), and alcohol intake (yes or no) were chosen as covariates in the multivariate logistic regression analysis.

Third, to examine whether the association between FHS–CVD risk score and POD was mediated by CSF POD biomarkers, linear regression models were fitted based on the methods proposed by Baron and Kenny (31). The first equation regressed the mediator (CSF POD biomarkers) on the independent variable (FHS–CVD risk score). The second equation regressed the dependent variable (POD) on the independent variable. The third equation regressed the dependent variable on both the independent variable and the mediator variable. Mediation effects were established if the following criteria were simultaneously reached: (1) FHS–CVD risk score was significantly related to CSF POD biomarkers; (2) FHS–CVD risk score was significantly related to POD; (3) CSF POD biomarkers were significantly related to POD; and (4) the association between FHS–CVD risk score and POD was attenuated when CSF POD biomarkers (the mediator) were added in the regression model. Furthermore, the attenuation or indirect effect was estimated, with the significance determined using 10,000 bootstrapped iterations, where each path of the model was controlled for age, sex, education, and MMSE.

Finally, the effectiveness of the FHS–CVD risk score in predicting POD was evaluated by introducing area under curve (AUC) of ROC curves.

The data were analyzed by using the Stata MP16.0 (Solvusoft Corporation, Inc., Chicago, IL, United States), GraphPad Prism version 8.0 (GraphPad Software, Inc., LaJolla, CA, United States), and R software version 4.4.1(R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Participant Characteristics

This study enrolled 750 participants. In total, five hundred forty-two (n = 542) were eligible for analysis, and 208 participants were excluded. The criteria for exclusion are shown in **Figure 1**. The demographical and clinical data are summarized in **Table 1**. The incidence of POD was observed at 22.9% (n = 124/542), with 37.2% (n = 103/277) in the higher vascular risk group and 7.9% (n = 21/265) in the lower vascular risk group. There was a

statistically significant difference in POD occurrence between two groups ($P < 0.05$). And the POD CSF T-tau and P-tau levels were higher in the higher vascular risk group (**Figure 2**).

The Relationship Between Framingham Heart Study–Cardiovascular Disease Risk Score and Cerebrospinal Fluid Postoperative Delirium Biomarkers

The concentrations of CSF biomarkers (Aβ42, T-tau, P-tau, Aβ42/T-tau, and Aβ42/P-tau) before operation in the higher and lower vascular risk group were compared with FHS–CVD risk score to investigate the relationship in linear regression analysis. Multiple linear regression models showed that higher preoperative FHS–CVD risk score were positively correlated with CSF T-tau (β = 0.218, $P = 0.015$) and P-tau level (β = 0.309, $P < 0.001$), while were not correlated with CSF Aβ42 (β = 0.004, $P = 0.961$), Aβ42/T-tau (β = −0.123, $P = 0.172$) and Aβ42/P-tau level (β = −0.135, $P = 0.135$) in the POD group. However, multiple linear regression models showed that higher preoperative FHS–CVD risk score were not correlated with CSF Aβ42 (β = −0.011, $P = 0.820$), T-tau (β = 0.070, $P = 0.153$), P-tau (β = 0.014, $P = 0.770$), Aβ42/T-tau (β = −0.058, $P = 0.236$), and Aβ42/P-tau level (β = −0.025, $P = 0.612$) in the NPOD group. And the linear regression results of the connection between FHS–CVD risk score and CSF POD biomarkers were shown in **Figure 3**.

The Relationship Among Framingham Heart Study–Cardiovascular Disease Risk Score, Cerebrospinal Fluid Biomarkers, and Postoperative Delirium

We compared the concentrations of CSF biomarkers (Aβ42, T-tau, P-tau, Aβ42/T-tau, and Aβ42/P-tau) and FHS–CVD risk score between patients with POD and non-POD before operation. Binary logistic regression analysis showed that the level of Aβ42(OR = 0.994, 95% CI = 0.992–0.995, $P < 0.001$), Aβ42/T-tau(OR = 0.285, 95% CI = 0.211–0.384, $P < 0.001$), and Aβ42/P-tau (OR = 0.714, 95% CI = 0.661–0.770, $P < 0.001$) maintained great significance and were protective factors of POD, while T-tau OR = 1.006, 95% CI = 1.005–1.008, $P < 0.001$), P-tau (OR = 1.059, 95% CI = 1.045–1.073, $P < 0.001$) and FHS–CVD risk score (OR = 1.231, 95% CI = 1.165–1.300, $P < 0.001$)were risk factors of POD (**Table 2**). To verify the stability of the results, we performed sensitivity analyses, respectively, using two models based on more covariables on studies. FHS–CVD risk score, Aβ42, T-tau, P-tau, Aβ42/T-tau, and Aβ42/P-tau remained stable across two sensitivity analyses (**Table 2**). To sum up, the sensitivity analysis has showed that the results were stable.

Causal Mediation Analyses

The multivariate regression in PNDABLE study showed that FHS–CVD risk score, T-tau, and P-tau were positively correlated with POD, while Aβ42, Aβ42/T-tau, and Aβ42/P-tau were negatively correlated with POD. Therefore, we speculated that FHS–CVD risk score is not only a risk factor of POD but may also regulate the occurrence of POD through Tau pathology. We further explored whether T-tau and P-tau could mediate the effect of FHS–CVD risk score on POD. The mediation analysis showed that the relationship between FHS–CVD risk score and POD was mediated by T-tau (the proportion of intermediaries is about 31.6%) and P-tau (the proportion of intermediaries is about 23.6%) (**Figure 4**). As the result showed, T-tau accounted for the largest proportion of the effect of vascular risk factors on the occurrence of POD among the CSF biomarkers.

Receiver Operating Characteristic Curve

The ROC curve showed that the FHS–CVD risk score (AUC = 0.7364) was effective in predicting POD occurrence (**Figure 5**).

DISCUSSION

After analyzing 542 participants under combined spinal–epidural anesthesia in this study, we found that FHS–CVD risk score is one of the preoperative risk factors for POD. The person with higher vascular risk scores had worse postoperative cognitive functioning overall. The underlying mechanism of such a relationship appears to be correlated with the significant association between the FHS–CVD risk score and tau protein deposition in cerebrospinal fluid. Our findings highlight the important role of adverse vascular risk in the pathogenesis of POD and postoperative cognitive decline.

Postoperative delirium is a syndrome associated with the interaction of patients' demographic factors, basic disease factors, and anesthesia and surgery factors (Aldecoa et al., 2017). As the most common postoperative neurodegenerative change, the incidence rate of POD is climbing with the arrival of global aging. However, there is currently a lack of effective treatments to treat POD, and it becomes a serious problem affecting postoperative prognosis. In recent years, epidemiological studies have played an important role in cognitive prevention of POD. A large number of studies have centralized and summarized potential controllable factors that can significantly reduce the prevalence of POD in asymptomatic preclinical stages. Due to its vital role in maintaining adequate blood supply and healthy substance exchange, vascular dysfunction is receiving increasing attention in the pathogenesis of POD (Iadecola, 2017).

Previous studies have confirmed that a variety of vascular risk factors will increase the risk of dementia (Hofman et al., 1997). Pathological injury and vascular injury coexist and then act together to promote the progression of POD (Regan et al., 2006; Tariq and Barber, 2018). After the well control of vascular risk factors, the prevalence of cognitive dysfunction has decreased in many developed countries (Hinterberger et al., 2016). However, there has no single vascular risk factor has been shown the associated with the incidence and development of the POD (Iadecola, 2013; He et al., 2020). Considering the interaction of the vascular risk factors, our study decides to summarize the influence of vascular risk factors to get the potential reliable conclusion.

TABLE 1 | Demographic and clinical characteristics of participants.

Variable	Higher vascular risk group	Lower vascular risk group	*P* value
Age, yr	68.0(63.0,74.0)	56.0(49.0,62.0)	< 0.001
Female	50(18.1)	156(59.1)	< 0.001
Education, yr	9.0(9.0,12.0)	12.0(9.0,12.0)	0.032
CM-MMSE	28.0(26.5,29.0)	29.0(27.0,30.0)	< 0.001
MDAS	3.0(1.0,9.0)	1.0(1.0,8.0)	< 0.001
Hypertension	109(39.4)	78(29.4)	0.015
Diabetes	48(17.3)	42(15.8)	0.644
CHD	34(12.3)	40(15.1)	0.339
Smoking history	124(44.8)	34(12.8)	< 0.001
Drinking history	97(35.0)	76(28.7)	0.114
FHS-CVD risk score	18.0(16.0,19.0)	14.0(12.0,19.0)	< 0.001
Aβ42,pg·ml^{-1}	281.6(174.3,435.1)	335.5(222.9,498.8)	0.009
T-tau,pg·ml^{-1}	211.1(159.6,296.0)	176.9(134.4,236.5)	< 0.001
P-tau,pg·ml^{-1}	42.5(34.7,58.0)	37.1(30.6,48.3)	< 0.001
Aβ42/T-tau	1.5(0.7,2.4)	1.9(1.0,2.9)	< 0.001
Aβ42/P-tau	6.8(3.9,11.3)	8.9(5.9,12.8)	< 0.001
POD occurrence	103 (37.2)	21(7.9)	< 0.001

Continuous variable use Student's t test or Mann-Whitney U, Categorical variable use Chi-square test.
The numerical variables of normal distribution are statistically described by Average Standard deviation.
Non-normally distributed numerical variables are statistically described by Interquartile range(IQR).
Categorical variables are statistically described by Sample size, Percent.

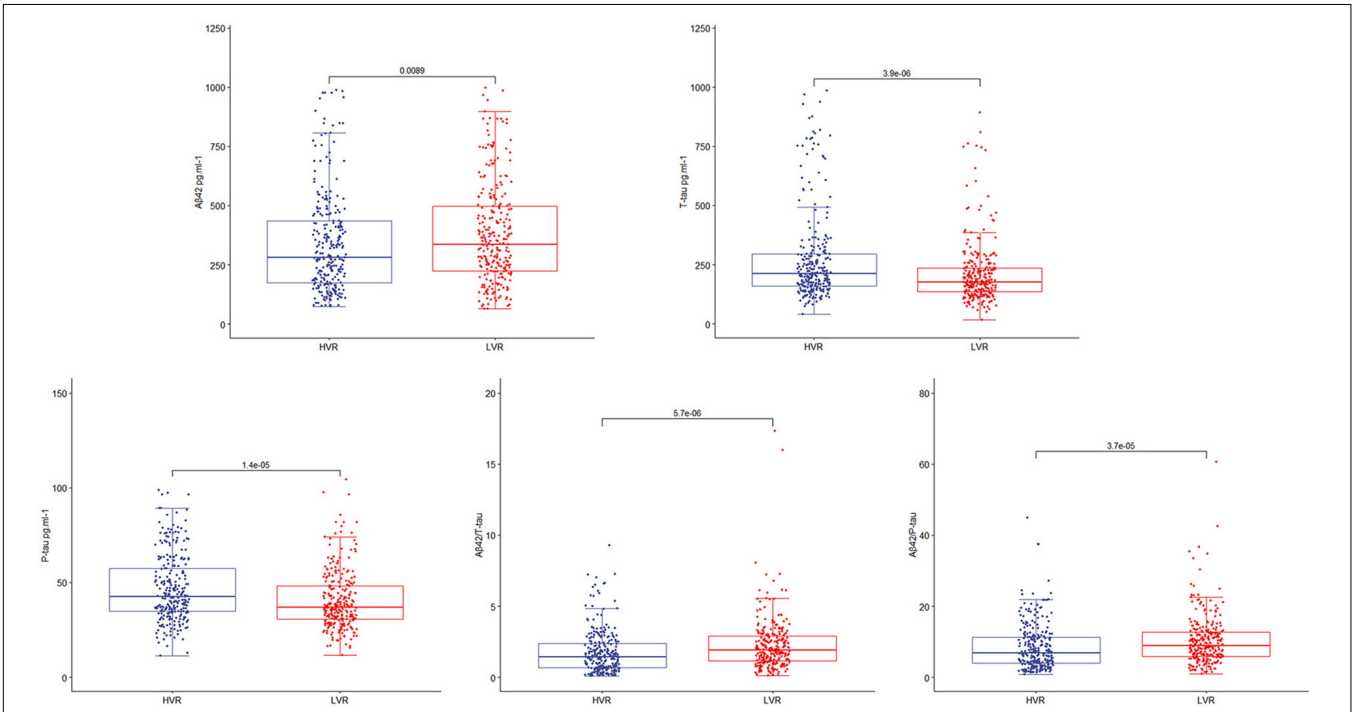


FIGURE 2 | The box-plots showed the level of CSF POD biomarkers in the higher vascular risk group (HVR) and the lower vascular risk group (LVR). The result showed that CSF T-tau and P-tau levels of the participants were higher in the higher vascular risk group.

The concentration of CSF biomarkers such as Aβ42, T-tau, P-tau, Aβ42/T-tau, and Aβ42/P-tau in patients have been used as a set of important indicators in previous clinical research and POD diagnosis (McKhann et al., 2011; Olsson et al., 2016; Cunningham et al., 2019). Specifically, increases in the T-tau level and P-tau level, respectively, reflect the intensity of axial degeneration and the amount of neurofibrillary tangles detected in patients with brain pathology. Regarding the amyloid

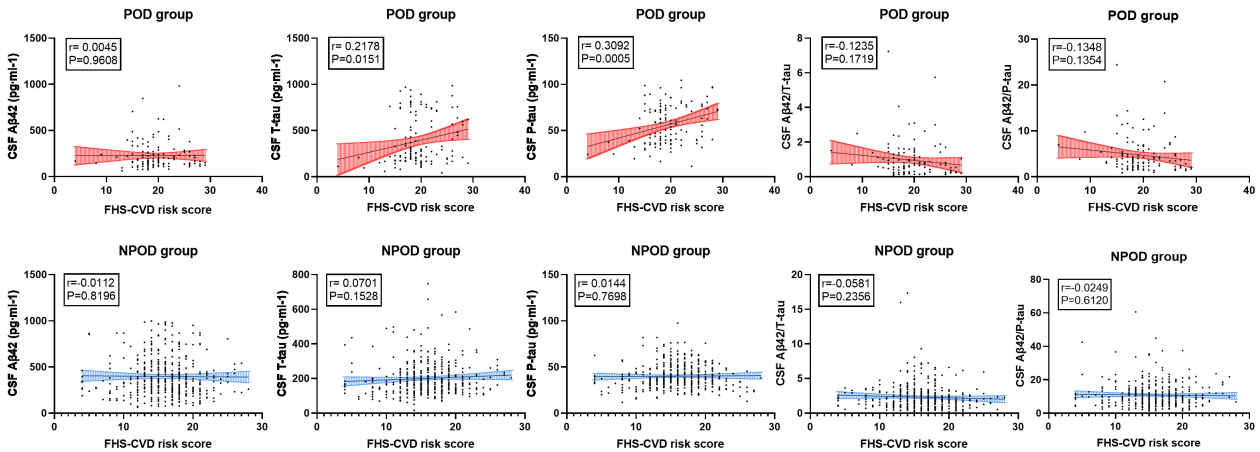


FIGURE 3 | The linear regression models showed the relationship between the CSF POD biomarkers and FHS–CVD risk score. The result showed that higher preoperative FHS–CVD risk score were positively correlated with CSF T-tau and P-tau level in the POD group.

TABLE 2 | The logistic regression analysis and sensitivity analysis among FHS-CVD risk score, CSF POD biomarkers and POD.

	Unadjusted		Adjusted1		Adjusted2	
	OR(95%CI)	***P* value**	**OR(95%CI)**	***P* value**	**OR(95%CI)**	***P* value**
Aβ42,pg·ml^{-1}	0.994(0.992−0.995)	< 0.001	0.994(0.992−0.996)	< 0.001	0.994(0.992−0.996)	< 0.001
T-tau,pg·ml^{-1}	1.006(1.005−1.008)	< 0.001	1.005(1.003−1.007)	< 0.001	1.005(1.004−1.007)	< 0.001
P-tau,pg·ml^{-1}	1.059(1.045−1.073)	< 0.001	1.045(1.029−1.061)	< 0.001	1.045(1.029−1.062)	< 0.001
Aβ42/T-tau	0.285(0.211−0.384)	< 0.001	0.362(0.262−0.501)	< 0.001	0.353(0.254−0.491)	< 0.001
Aβ42/P-tau	0.714(0.661−0.770)	< 0.001	0.746(0.687−0.811)	< 0.001	0.744(0.684−0.809)	< 0.001
FHS-CVD risk score	1.231(1.165−1.300)	< 0.001	1.148(1.026−1.283)	0.016	1.142(1.017−1.282)	0.025

Adjusted1: adjusted for age (40−90), gender, education and MMSE.
Adjusted2: adjusted for age (40−90), gender, education, MMSE, smoking history, drinking history, hypertension, diabetes and CHD.

protein Aβ42, its accumulation in amyloid plaques during POD pathology changes was shown to decrease the concentration of Aβ42 in CSF (Cunningham et al., 2019). Since the concentration of POD biomarkers have changed in the preclinical stage, there is an opportunity to investigate the relationship between the vascular risk factors and POD pathological changes.

According to our findings, we found that patients with higher vascular risk are often associated with higher CSF T-tau and P-tau levels, yet their CSF Aβ42, Aβ42/T-tau, and Aβ42/P-tau levels appeared to be lower. The results showed that there were significant differences in the expression of Aβ42, T-tau, P-tau, Aβ42/T-tau, and Aβ42/P-tau in CSF between higher vascular risk group and lower vascular risk group ($P < 0.05$), indicating an increased risk of POD in patients with higher vascular risk. Although there exist a few hypotheses about the underlying mechanism of the observed association between vascular risk factors and cognitive dysfunction, how one factor interacts with or perhaps causes the others remains unclear. So far, increasing evidence has been found for the cerebral hypoperfusion hypothesis, which postulates that the increased risk of vascular sclerosis and infraction could impair the cerebral vascular automatic regulation function and decrease intracranial blood supply. Chronic cerebral hypoperfusion leads to pathological changes such as abnormal protein synthesis, disturbance of energy metabolism, decreased glucose utilization, and loss of choline receptors in brain regions, and further leads to loss of neurons and impaired function (de la Torre, 2000, 2002). And similar pathological features can be found in brain ischemic diseases and POD like amyloid and tau protein changes, which is consistent with our findings.

As the mediation effect analysis showed, T-tau accounted for the largest proportion of the effect of vascular risk factors on the occurrence of POD among the POD CSF biomarkers. Previous studies have shown that vascular risk factors contribute to increased tau deposition (Vemuri et al., 2017). Studies have also found that cerebral ischemia itself can express hyperphosphorylated tau protein, which suggests that cerebral ischemia can promote POD pathological changes (Iadecola, 2013). The activation of the cell cycle signal transduction system was induced by cerebral ischemic injury, and cells enter into abnormal mitosis and apoptosis. This could lead to hyperphosphorylation of tau protein and formation of nerve fiber tangles, thus, participating in the pathological progress of POD (Stadelmann et al., 1999; Kang et al., 2013; Ye et al., 2020). So far, no studies have proven a correlation between FHS–CVD risk score and CSF Aβ42 level (Gupta et al., 2015). Nonetheless, there

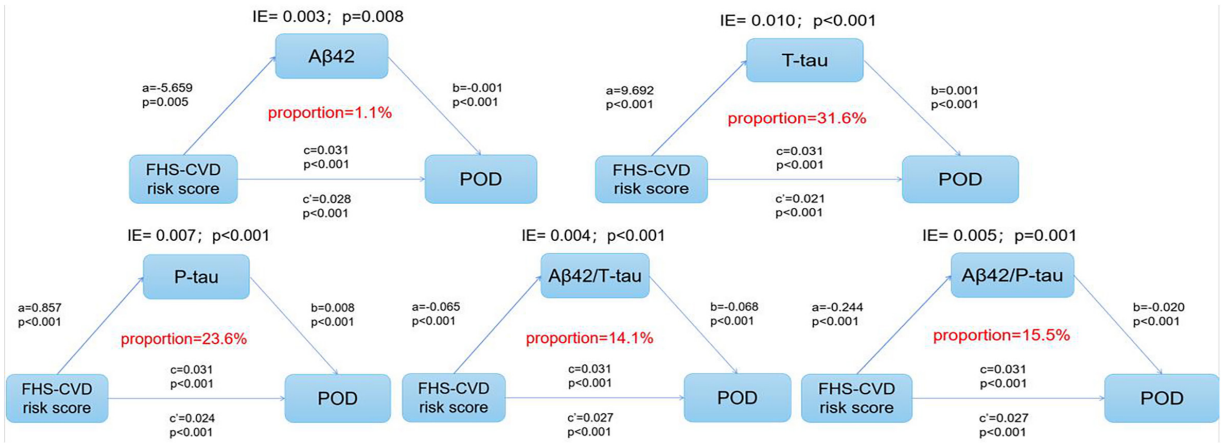


FIGURE 4 | The mediation analysis showed that the relationship between FHS–CVD risk score and POD was mediated by T-tau and P-tau protein.

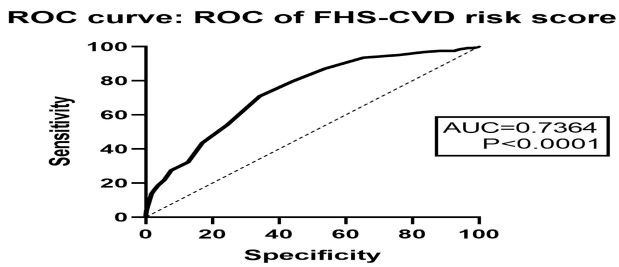


FIGURE 5 | The ROC curve showed that FHS–CVD risk score had effective diagnostic significance in predicting POD occurrence.

is evidence that brain ischemia induces ischemic generation of amyloid plaques, which can interact with vascular changes in the brain and progress to POD (Vemuri et al., 2017).

The clear and reliable relationship between the single vascular risk factor and POD incidence has not been shown in the previous studies. However, studies have shown that long-term hypertension causes changes in cerebral blood flow and brain atrophy, resulting in neuronal damage and other pathological changes, which can cause cognitive disorders (Whitmer et al., 2005). The relative or absolute lack of insulin in the early stage of insulin resistance and type II diabetes can cause cognitive decline, which share common neuropathological characteristics (Hughes and Craft, 2016). Smoking would exacerbate the risk of vascular disease, chronic inflammation, and oxidative stress, all of which are potential causes of cognitive dysfunction (Anstey et al., 2007). As an important agent of Aβ protein clearance through the blood–brain barrier, low-density lipoprotein receptor-related protein 1(LRP1) may be used as a therapeutic target for regulating the pathological changes of cognitive disorders (Sparks et al., 2006; Storck and Pietrzik, 2017).

It should also be emphasized that it is a group of vascular risk factors but not a single one, that may ultimately lead to vascular dysfunction. FHS–CVD risk score is a simple but reliable vascular risk assessment tool, which could be used by considering multiple vascular risk and disadvantages conditions simultaneously. Visual inspection of the ROC curve, our research finding indicated that FHS–CVD risk score was accurate in predicting POD. The items evaluated by the FHS–CVD risk score are mostly curable. Together with the comprehensive range of items included, such an assessment tool could be used as a valuable guidance for the clinicians to design their prevention and treatment plan. The study has potential implications for public health, and good use of medications such as antihypertensive, hypoglycemic, and lipid-lowering drugs may prevent, slow, or even reverse the progression of postoperative delirium (Sparks et al., 2006; Duron and Hanon, 2008; Vizcaychipi et al., 2014). The public is called on for active primary or secondary prevention.

The limitations of our study are as follows: first, according to the data from the PNDABLE, we could only assess the association among the FHS–CVD risk score, POD biomarkers, and POD incidence in the cross-sectional study. We are able to improve the longitudinal data on patients in the future study, and further clarify the association. Second, this study only focused on the relationship between vascular risk factors of the FHS–CVD risk score and the CSF biomarkers of POD and did not involve other related pathogenesis of POD, which may influence the POD incidence. AT third, this study was carried out among hospitalized patients, who are scheduled for surgery. The conclusion remains to be validated in animal research for further research.

CONCLUSION

To sum up, adverse vascular risk burden is one of the preoperative risk factors for promoting POD. The patient with higher FHS–CVD risk score has the higher occurrence of POD. And our study found the relationship between FHS–CVD risk score and POD might be partly mediated by CSF tau protein. Early prevention of POD can improve patient quality of life, shorten the hospital stays, and reduce the burden on family and society.

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 Chinese Clinical Trial Registry (clinical registration number ChiCTR2000033439) and approved by the Ethics Committee of Qingdao Municipal Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YB conceived the current study. XT, FW, SL, and XW performed the experiments. RD, XL, LW, and BW analyzed the data. JW and BW performed the experiments, wrote, and revised the manuscript. All authors have contributed to the manuscript revising and editing critically for important intellectual content and given final approval of the version and agreed to be accountable for all aspects of the work presented here.

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REVIEW
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Ellagic Acid: A Dietary-Derived Phenolic Compound for Drug Discovery in Mild Cognitive Impairment

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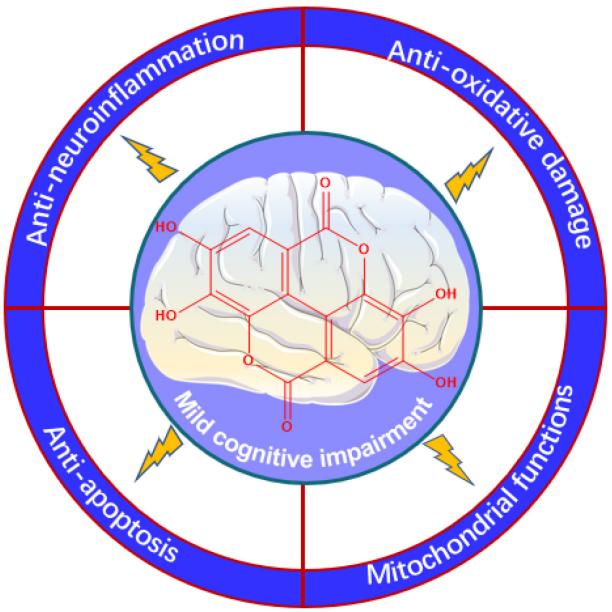
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Ellagic acid (EA), a naturally occurring polyphenolic compound, is detected in free form or linked to polyols or sugars, constituting hydrolyzable tannins or ellagitannins in distinct fruits, nuts, and herbs. Today, a considerable number of botanicals and enriched foods containing EA are commercially available as nutraceuticals and used to prevent mild cognitive impairment (MCI) due to the excellent neuroprotective capacity of EA. Here, this study aims to provide an overview of the physicochemical properties, source, and pharmacokinetics of EA and to emphasize the importance and mechanisms of EA in the prevention and management of MCI. To date, preclinical studies of EA and its derivatives in various cell lines and animal models have advanced the idea of dietary EA as a feasible agent capable of specifically targeting and improving MCI. The molecular mechanisms of EA and its derivatives to prevent or reduce MCI are mainly through reducing neuroinflammation, oxidative stress, neuronal apoptosis, synaptic dysfunction and loss, and defective mitochondrial functions. Nevertheless, well-designed and correctly large randomized controlled trials in the human population need to be performed to reinforce the scientific facticity of the beneficial effects of EA against MCI. Synchronously, the mechanism of EA against MCI is least provided cynosure and expects more attention from the emerging research community.

Keywords: ellagic acid (EA), mild cognitive impairment (MCI), sources, physicochemical properties, pharmacokinetics, mechanism

INTRODUCTION

The global rise in life expectancy is resulting in a dramatic increase in the size of the elderly population, with the number of people aged 60 years and over expected to rise from 600 million in 2000 to 2.1 billion globally by 2050 and this will have a major impact on the health, social, and economic sectors (Ellen et al., 2017). Mild cognitive impairment (MCI) is usually associated with cognitive impairment, which is seen with general age-related cognitive decline, but is not severe enough to lead to considerably impaired daily function (Stevens et al., 2022). Furthermore, the



GRAPHICAL ABSTRACT |

principal cognitive domain that could be afflicted refers to a decline in the capability to acquire raw information or recall stored information (Gatz et al., 2022; Kuroda et al., 2022). Clinically, age-related cognitive impairment represents the preclinical, transitional stage between healthy aging and dementia (Levey et al., 2021). In addition, other factors such as sleep deprivation, depression, or diabetes mellitus can also induce cognitive impairment (Guan et al., 2020; Yu et al., 2021). Since Petersen et al. (1997) first comprehensively described the characteristics of MCI in 1997, scholarly and clinical interests in MCI have grown exponentially. More than 55 million people all over the world are suffering from dementia (WHO, 2021). It is expected that the worldwide prevalence of dementia will nearly double every 20 years, with the number of patients expected to rise to 131 million by 2050 if breakthroughs are not made to prevent the disease or delay its onset (Martins et al., 2018). However, in the last 10 years, drug trials have failed 99.6% of the time because existent drug therapies for neurodegenerative diseases rarely curtail the underlying disease processes (Anderson, 2019).

Given this, researchers have shifted focus toward the early steps to counteract or, anyway, retard the progression from MCI to dementia, which would profoundly reduce the prevalence and costs of neurodegenerative diseases (Jongsiriyanyong and Limpawattana, 2018; Zhang et al., 2019). Therefore, it is mandatory to find more efficacious preventive strategies to straightly prevent, decelerate, and even discontinue this "dementia pandemic." Physical exercise and healthy dietary habits have been identified as some of the most efficient therapeutic approaches and are recommended from early life (Blumenthal et al., 2017). In addition, the connection between diet, health, and the presence of bioactive chemical compounds in food has gotten fantastic attention in recent years and consumers are progressively interested in food products that, apart from fulfilling nutritional demands, enhance physical performance, further wellbeing, and reduce the risk of producing diseases (Auxtero et al., 2021; Ozawa et al., 2021). Nutritional interventions or botanicals such as polyphenols can be a greatly accepted, inexpensive, and efficacious option to preserve against age-related cognitive decline and neurodegeneration, leading to important personal and societal benefits (Tewari et al., 2018; de Araujo et al., 2021). Synchronously, considering the transformation at the colonic level and absorption, the good bioavailability of some polyphenols owing to the ability to pass the blood–brain barrier, to a various extent, facilitated the interest in these molecules as fresh neuroprotective means against neurodegenerative disorders (Yan et al., 2022; Yuste et al., 2022).

Ellagic acid (EA), a polyphenol presents in fruits, nuts, and herbs, owns some salient pharmacological properties (Usta et al., 2013; Gupta et al., 2021). EA is also commercially available as nutraceuticals and used to protect against chronic diseases such as diabetes (Altindag et al., 2021) and cardiovascular (Mansouri et al., 2020) and neurodegenerative diseases (Kumar et al., 2021). In this circumstance, EA invited researchers' interest for its expected health benefits in pathological statuses of MCI and, currently, several literature data give support to such EA capability (Alfei et al., 2019).

This narrative review aims to summarize the physicochemical properties, source, and pharmacokinetics of EA and the most relevant evidence of the effects of EA on MCI. The potential mechanisms of action underlying the neuroprotective activity of EA, provided from animal or *ex-vivo* research, are discussed to consider knowing the therapeutic potential of EA and are expected to lay the solid foundation for the clinical application of EA in the prevention and management of MCI.

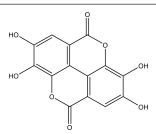
PHYSICOCHEMICAL PROPERTIES AND SOURCES OF ELLAGIC ACID

Ellagic acid, likewise recognized as a dimeric derivative of gallic acid, is a dietary-derived phenolic compound with a chemical formula of $C_{14}H_6O_8$ and from the physicochemical perspective, in the form of cream-colored needles or yellow powder with a melting point greater than 360°C, a boiling point of 796.50°C, a log P of 1.05, and a molecular weight of 302.194 g/mol (Garcia-Nino and Zazueta, 2015; Shakeri et al., 2018). EA is identified as 6,7,13,14-tetrahydroxy-2,9 dioxatetracyclo[6.6.2.$0^{4,16}$.$0^{11,15}$]hexadeca-1(15),4,6,8(16),11,13-hexaene-3,10-dione according to IUPAC nomenclature. The chemical structure of EA is composed of four free ·OH groups and two acyloxy groups standing for the hydrophilic moiety and a lipophilic planar moiety comprising two hydrocarbon phenyl rings (Sharifi-Rad et al., 2022). The structure of EA influences the solubility and, hence, the bioavailability. Indeed, EA is easily soluble in methanol (671 μg/ml, temperature at 37°C), polyethylene glycol 400, alkalies, dimethyl sulfoxide, triethanolamine, or pyridine, slightly

soluble in alcohol (200 μg/ml, temperature at 37°C) or water (about 9.3–9.7 μg/ml, temperature at 21°C, and pH 7.4), practically insoluble in ether, since from both its planar and symmetrical structure and a broad network of hydrogen bonds (Evtyugin et al., 2020). The detailed physicochemical properties of EA are shown in **Table 1**.

Our investigation shows that, so far, EA is isolated from the family Rosaceae, Saxifragaceae, Ericaceae, Combretaceae, Anacardiaceae, Vitaceae, Punicaceae, Juglandaceae, Fabaceae, Euphorbiaceae, Sapindaceae, and Simarubaceae. Especially, EA is primarily plentiful in berries of the family Rosaceae such as raspberry, cherry, and strawberry. Herein, **Figure 1** displays a comprehensive list of EA sources, arranged in fruits and derivatives, nuts, and herbs. Among these, this plant-based provision can embody EA in three distinct forms: free EA, ellagic acid glycosides, and polymeric ellagitannins even if in varying quantities (Lee et al., 2005). In fact, EA is predominately detected as ester-linked to sugars in the composition of hydrolyzable tannins that are called ellagitannins (Long et al., 2019). For instance, a strawberry yield 70 mg of ellagitannins, approximately 100 g of raspberry produces 300 mg of ellagitannins, and four walnuts produce 400 mg of ellagitannins (Aishwarya et al., 2021). Nevertheless, free EA is present in significant amounts in kakadu plums, raspberry fruit, and pomegranate. Among them, the content of EA in the kakadu plum is the highest, ranging from 8.26 to 14.7 mg/g of fresh fruit weight (**Table 2**).

TABLE 1 | Chemical and physical properties of ellagic acid (EA).

Chemical name	Ellagic acid
2D structure	
CAS number	476-66-4
Canonical SMILES	C1 = C2C3 = C(C(= C1O)O)OC(= O)C4 = CC(= C(C(= C43)OC2 = O)O)O
IUPAC name	6,7,13,14-tetrahydroxy-2,9 dioxatetracyclo[6.6.2.0 ^{4,16}.0^{11,15}]hexadeca-1(15),4,6,8(16),11,13-hexaene-3,10-dione
Molecular weight	302.194 g/mol
Molecular formula	$C_{14}H_6O_8$
Color/Form	Cream-colored needles or yellow powder
Melting point	Greater than 360°C
Solubility	Slightly soluble in alcohol or water, soluble in alkalies, in pyridine. Practically insoluble in ether
Density	1.667 at 64 °F
Hydrogen bond donor count	4
Hydrogen bond acceptor count	8
Covalently bonded unit count	1
Dissociation constants	pKa_1 = 6.69 (phenol); pKa_2 = 7.45 (phenol); pKa_3 = 9.61 (phenol); pKa_4 = 11.50 (phenol)
UV spectra	UV_{max} (ethanol): 366, 255 nm
ADMET BBB level	4
Druglikeness weight	0.327

PHARMACOKINETICS OF ELLAGIC ACID

An in-depth understanding of the pharmacokinetic properties of EA is helpful to understand its absorption, distribution, metabolism, and excretion processes in the body. Ellagitannins-rich foods are metabolically hydrolyzed in the gastrointestinal tract, releasing the amount of free EA, which is then absorbed in the body or undergoes a massive metabolism to yield a series of urolithins (dibenzopyran-6-one metabolites) by the gut microbiota, including urolithin M5, urolithin M6, urolithin M7, urolithin A, urolithin B, urolithin C, urolithin D, and urolithin E (**Figure 2**; Garcia-Villalba et al., 2013; Garcia-Nino and Zazueta, 2015; Watanabe et al., 2020). Among EA-derived metabolites, urolithin A is the major metabolite that is discovered in fecal matter in human beings (Gonzalez-Barrio et al., 2011; Jayatunga et al., 2021). The amount of EA in the peripheral tissues and systemic circulation is virtually paltry, while urolithins and their conjugates can extend to concentrations at the micromolar point (Cerda et al., 2005). Plasma levels of EA after the intake of vegetables and fruits (normal plasma concentrations of 0.1–0.4 μmol/l) are restricted by poor water solubility and bioavailability of EA (Seeram et al., 2004; Murugan et al., 2009). However, a growing number of studies are incessantly concentrated on arranging water-soluble and absorbable EA formulations, including drug delivery systems (i.e., nanoparticles, liposomes, microemulsions, and polymeric) and novel formulations (i.e., particle size reduction, amorphization, and lipid-based formulations), to ameliorate significantly the bioavailability and solubility of EA (Mady and Ibrahim, 2018; Nyamba et al., 2021). Furthermore, despite the therapeutic capability of available delivery systems to improve the bioavailability of EA, new and advanced formulations that can eliminate the hepatic first-pass pharmacokinetic impact and uphold superlative efficacious concentrations of EA in the systemic circulation are worthy.

THERAPEUTIC PROPERTIES OF ELLAGIC ACID IN MILD COGNITIVE IMPAIRMENT

Although a large number of environmental and genetic factors are associated with MCI, the pathological mechanism continues to be unclear. In recent years, numerous animal model studies have been used to examine the potential pharmacological effects of EA on MCI (**Table 3**). Thus, the evidence for the behavioral consequences of EA in animal models has improved considerably.

Behavioral Effects of Ellagic Acid in Models of Streptozotocin-Induced Mild Cognitive Impairment

Streptozotocin (STZ) is glucosamine-nitrosourea, which demonstrates insulin resistance-related brain abnormalities when administered intracerebroventricular (ICV) at a subdiabetogenic dose. These abnormalities include cognitive impairment,

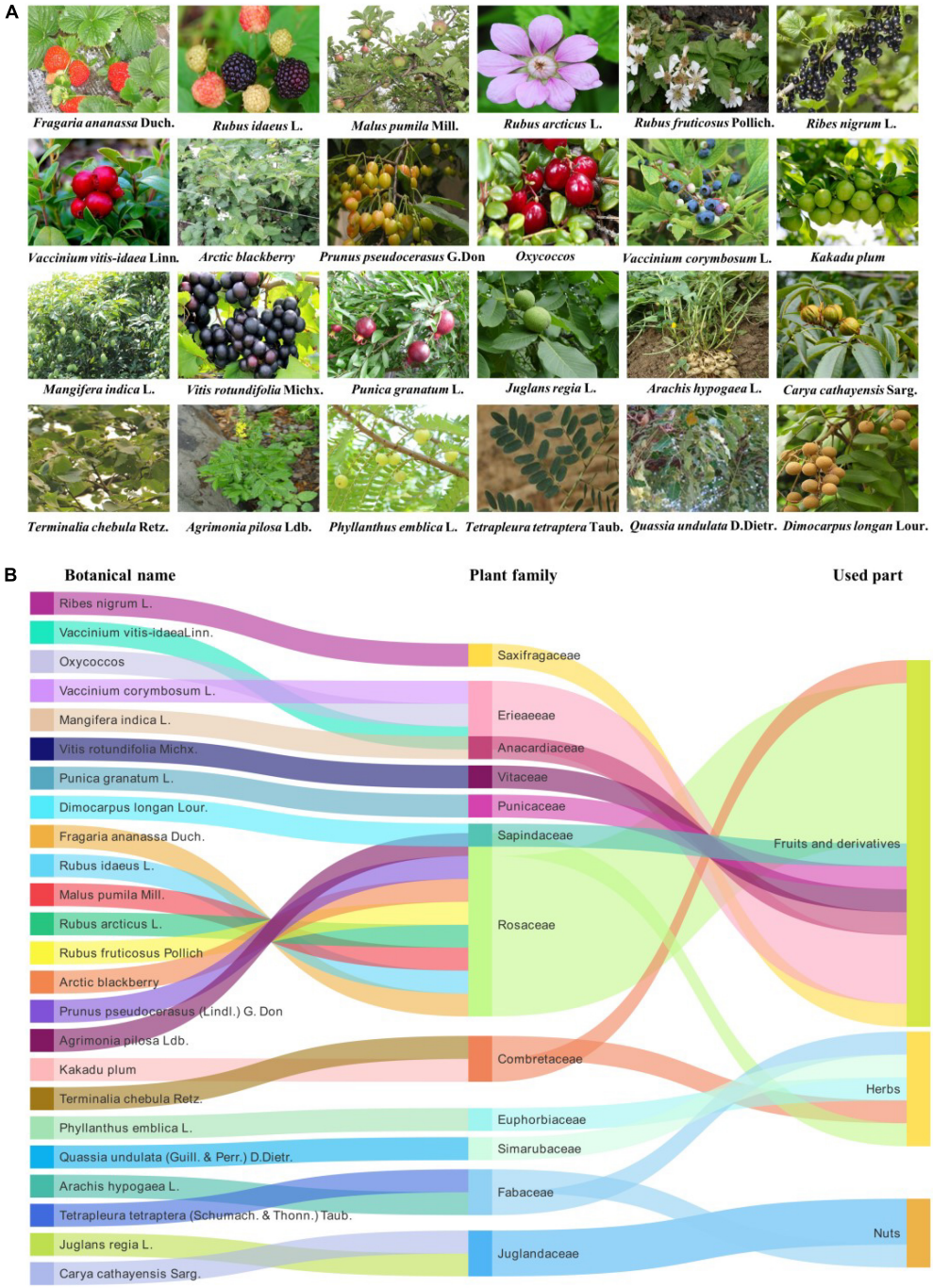


FIGURE 1 | Various sources of ellagic acid (EA). **(A)** The plant origins of EA. The pictures are from the website: https://www.gbif.org/; http://www.iplant.cn/frps. **(B)** EA as a major constituent of different fruits, nuts, and herbs.

impaired glucose and energy metabolism in the brain, amyloid β (Aβ) deposition, neuroinflammation, and other neurochemical and neuropathological changes that ultimately lead to memory and learning disabilities similar to those present in sporadic Alzheimer's disease (AD). Therefore, ICV-STZ can be used to establish experimental animal models of early pathological changes in sporadic AD (Noor et al., 2022). The insulin signaling cascade in the hippocampus is extremely significant for cellular metabolism and neuron survival and is associated with cognitive functions (Zeng et al., 2016). Furthermore, these involve defective mitochondrial functionalities, oxidative stress injury, and consequential cytoenergetic catastrophe, which are correlated with declined cognitive role through a modification in the architecture of the neural network. Administration with EA

TABLE 2 | Sources of ellagic acid (EA) and its content in different fruits, nuts, and herbs.

Plant family	Plant species	Botanical name	Used part	Amount isolated	References
Anacardiaceae	Mango	*Mangifera indica* L.	Fruits and derivatives	1.20 mg/g	Soong and Barlow, 2006
Combretaceae	Kakadu plum	*Kakadu plum*	Fruits and derivatives	Whole fruit: 8.26–14.7 mg/g; Puree: 6.15–13.31 mg/g	Williams et al., 2016
	Terminalia chebula	*Terminalia chebula* Retz.	Herbs	8.00 mg/g	Pellati et al., 2013
Erieaeeae	Bilberry	*Vaccinium vitis-idaea* Linn.	Fruits and derivatives		FooDB, 2020
	Cranberry	*Oxycoccos*	Fruits and derivatives	0.12 mg/g	Daniel et al., 1989
	Highbush blueberry	*Vaccinium corymbosum* L.	Fruits and derivatives	0.0014 mg/g	FooDB, 2020
Euphorbiaceae	Phyllanthus amarus	*Phyllanthus emblica* L.	Herbs		Alagan et al., 2019
Fabaceae	Tetrapleura tetraptera	*Tetrapleura tetraptera* Taub.	Herbs		Odubanjo et al., 2018
	Peanut	*Arachis hypogaea* L.	Nuts		FooDB, 2020
Juglandaceae	Walnut	*Juglans regia* L.	Nuts	0.59 mg/g	Daniel et al., 1989
	Pecans	*Carya cathayensis* Sarg.	Nuts	0.33 mg/g	Daniel et al., 1989
Punicaceae	Pomegranate	*Punica granatum* L.	Fruits and derivatives	8.61 mg/g External peels: 28.53 mg/g Juice: 0.0206 mg/mL	Masci et al., 2016
Rosaceae	Strawberry	*Fragaria ananassa* Duch.	Fruits and derivatives	0.683–0.853 mg/g	Koponen et al., 2007
	Raspberry	*Rubus idaeus* L.	Fruits and derivatives	2.637–3.309 mg/g Juice: 0.0084 mg/mL	Koponen et al., 2007; Neveu et al., 2010
	Apple	*Malus pumila* Mill.	Fruits and derivatives		FooDB, 2020
	Arctic bramble	*Rubus arcticus* L.	Fruits and derivatives	3.9 mg/g	Maatta-Riihinen et al., 2004
	Blackberry	*Rubus fruticosus* Pollich	Fruits and derivatives	1.5 mg/g	Daniel et al., 1989
	Arctic blackberry	*Arctic blackberry*	Fruits and derivatives	0.1715 mg/g	Neveu et al., 2010
	Cherry	*Prunus pseudocerasus* G. Don	Fruits and derivatives		FooDB, 2020
	Agrimonia pilosa	*Agrimonia pilosa* Ldb.	Herbs		Kashchenko and Olennikov, 2020
Sapindaceae	Dimocarpus longan	*Dimocarpus longan* Lour.	Fruits and derivatives		FooDB, 2020
Saxifragaceae	Blackcurrant	*Ribes nigrum* L.	Fruits and derivatives		FooDB, 2020
Simarubaceae	Quassia undulata	*Quassia undulata* D.Dietr.	Herbs		Odubanjo et al., 2018
Vitaceae	Muscadine grape	*Vitis rotundifolia* Michx.	Fruits and derivatives	Whole fruit: 0.0092 mg/g; Juice: 0.009–0.0093 mg/mL	Neveu et al., 2010; FooDB, 2020

(50 mg/kg, po) for 30 days can distinctly ameliorate MCI caused by streptozotocin (Jha et al., 2018). Meanwhile, administration of EA (35 mg/kg, po) for 4 weeks notably attenuated the neurotoxic influence of streptozotocin in the rat brain and thereby revealed memory-enhancing functions (Kumar and Bansal, 2018).

Behavioral Effects of Ellagic Acid in Models of Lipopolysaccharides-Induced Mild Cognitive Impairment

Neuroinflammation is a predisposing factor for the development of MCI (Merlini et al., 2019). Abnormal neuroimmune signaling is associated with cognitive dysfunction and systemic lipopolysaccharides (LPSs) administration has been identified as experimental model that simulates the pathological disorders of MCI. LPS can damage the consolidation of exact memory processes. Intraperitoneal (IP) injection of LPS causes cognitive impairment in experimental animals by activating microglia, which stimulates the production of proinflammatory mediators (Dornelles et al., 2020; Woodcock et al., 2021). On one hand, acute administration of LPS before practice harms the test of situational fear conditioning, a hippocampal-dependent learning paradigm, whereas chronic LPSs injection influences spatial memory and leads to impairment of memory and learning. On the other hand, systemic administration of LPS led to impairing the hippocampal-dependent object recognition memory, but did not affect spatial memory. Study employing this model has expected that IP application of LPS is able of inducing MCI in laboratory animals, while treatment with 100 mg/kg of EA for 8 days was capable of significantly preventing MCI, which was assessed through the open field and object recognition tests (Dornelles et al., 2020). In fact, geraniin, as a major ellagitannin, has the same effect on MCI as EA. This study showed that daily intragastrical administration with geraniin (20 mg/kg) for 14 days significantly mitigated LPS-elicited cognitive impairment in mice (Wang et al., 2019). Meanwhile, EA from *Phyllanthus amarus* Schum. and Thonn. that was given the dose of 200 and 400 mg/kg (for 14 and 28 days) effectively protected the rodents from LPS-induced MCI (Alagan et al., 2019).

Behavioral Effects of Ellagic Acid in Models of Transgenic Animals-Induced Mild Cognitive Impairment

A large number of studies disclosed that EA exerted neuroprotective properties on several neurodegenerative dysfunctions, including AD, Huntington's disease (HD), and Parkinson's disease (PD). R6/2 mice simulated human HD by

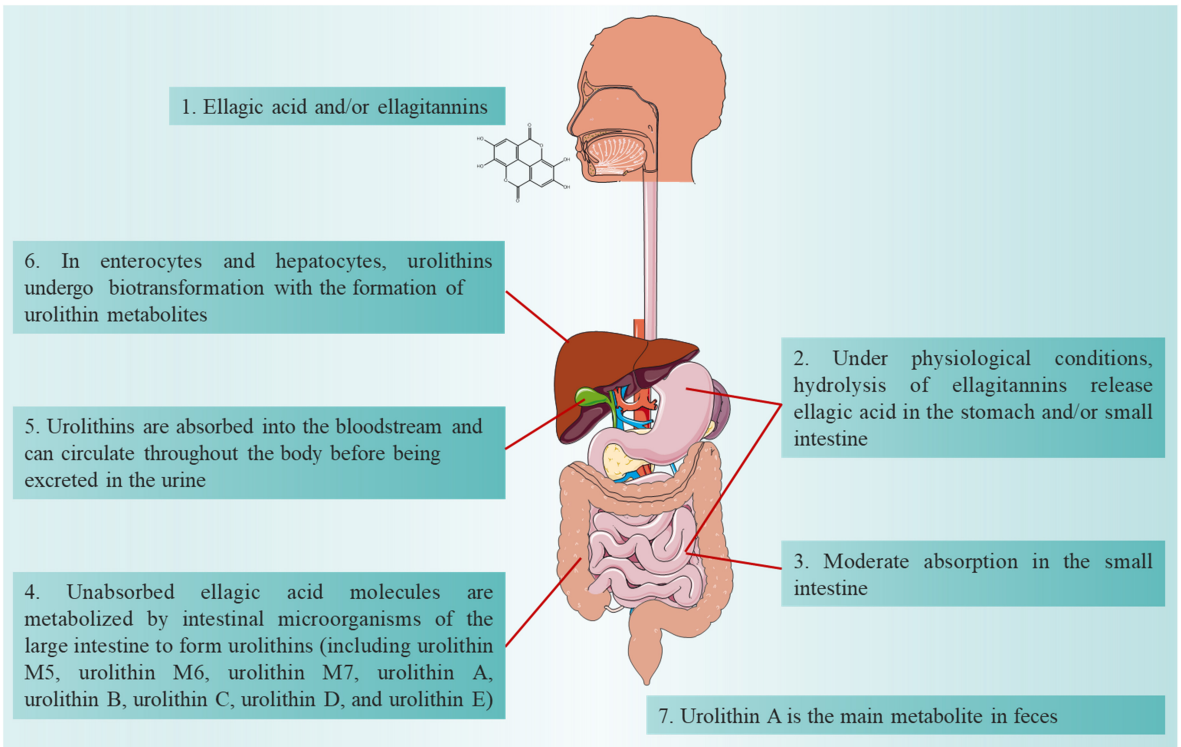


FIGURE 2 | Absorption, distribution, metabolism and excretion processes of ellagic acid (EA) in the body.

expressing part of the human HD gene [1 Kb 5′ untranslated region (UTR) sequence and 1 exon, approximately 120 CAG repeats] under the promoter element of the human gene. In short, a progressive neurological phenotype mimicking some features of HD in humans with early symptoms such as verbal fluency, mild word-finding difficulty, and cognitive impairment onset at 15–21 weeks is revealed by R6/2 mouse line. Sun et al. (2020) reported that both the high dose and low dose of EA (50 and 100 mg/kg, single oral gavage for 4 weeks) significantly attenuated MCI and ameliorated neuropathological features in R6/2 mice. Meanwhile, 3-nitropropionic acid is a natural systemic toxin, which is used to cause MCI in experimental animals with the HD model. Nevertheless, administration of EA (25, 50, and 100 mg/kg, po) for 21 days protected the rats against 3-nitropropionic acid-induced MCI, which was assessed by novel object recognition and elevated plus maze tests (Sharma et al., 2021). In addition, APP/PS1 double transgenic mouse model was crossbred between PRP-hAPPK595N/M596L single transgenic dementia mouse model and PrP-hPS1dE9 single transgenic dementia mouse model. An increasing number of witnesses have proven that APP/PS1 transgenic mice embody similar pathological changes to those of patients with AD and the memory impairment appears at 4–6 months of age and progresses in an age-dependent manner in APP/PS1 transgenic mice (Herran et al., 2015). Zhong et al. (2018) proved that treatment with EA (50 mg/kg for 60 consecutive days) ameliorated the MCI in APP/PS1 mice assessed by utilizing the Morris water maze test. As a major metabolite of EA, it is demonstrated that urolithin A also ameliorated MCI in APP/PS1 mice based on the Morris water maze test (Gong et al., 2019).

Behavioral Effects of Ellagic Acid in Models of Scopolamine- and Diazepam-Induced Mild Cognitive Impairment

The clinical features of neurodegenerative diseases such as AD and PD are a gradual loss of cognitive ability, affecting daily activities and learning and memory dysfunction. It is well known that myoglobin acetylcholinergic receptor antagonists impair learning and memory function and this impairment can be replaced by the non-selective antagonist scopolamine. Therefore, scopolamine can be used effectively in amnesic animal models while also inhibiting the activity of central cholinergic neurons (Balmus and Ciobica, 2017; Skalicka-Wozniak et al., 2018). Diazepam can also be used in the construction of this model. Up till now, the remedy for cognitive abnormalities such as attention deficit and amnesia is still far from being identified in medicine, while cognitive-enhancing drugs such as piracetam, memantine, aniracetam, and cholinesterase inhibitors are being applied for ameliorating memory, mood, and behavior; at the same time, the side effects of cognitive impairment have restricted these agents' usage. The animals administered EA at doses of 30 and 100 mg/kg for 10 days substantially antagonized the MCI induced by scopolamine or diazepam in the elevated plus-maze test in mice and rats, respectively (Mansouri et al., 2016). Also,

TABLE 3 | Animal behavioral tests representing the effects of ellagic acid (EA) against mild cognitive impairment (MCI).

Sl./No.	Animal model	EA dosage and treatment duration	Behavioral tests	References
I	Streptozotocin-induced MCI			
1	Wistar rats	EA (50 mg/kg, p.o.) for 30 days	Y-maze, Radial arm maze	Jha et al., 2018
2	Wistar rats	EA (35 mg/kg, p.o.) for 4 weeks	Morris water maze test, Elevated plus maze test	Kumar and Bansal, 2018
II	Lipopolysaccharides-induced MCI			
1	Wistar rats	EA (100 mg/kg, p.o.) for 8 days	Open field test, Novel object recognition test	Dornelles et al., 2020
2	Wistar rats	EA 218.06 μg/mL: quantitative analysis of *Phyllanthus amarus* (200 and 400 mg/kg, p.o.) for 14 and 28 days	Novel object recognition test	Alagan et al., 2019
3	C57BL/6 mice	Ellagitannin: geraniin (20 mg/kg, p.o.) for 14 days	Morris water maze test	Wang et al., 2019
III	Transgenic animals-induced MCI			
1	R6/2 mice	EA (50 and 100 mg/kg, p.o.) for 4 weeks	Novel object recognition test, Y-maze test, Open field test	Sun et al., 2020
2	APP/PS1 transgenic mice	EA (50 mg/kg, p.o.) for 60 days	Morris water maze test	Zhong et al., 2018
3	ICR mice	EA from persimmon (50 and 100 mg/kg, p.o.) for 3 weeks	Y-maze test, Passive avoidance test, Morris water maze test	Yoo et al., 2021
4	ICR mice	Urolithin A-the metabolite of EA (50, 100, and 150 mg/kg, p.o.) for 8 weeks	Morris water maze test, Novel object recognition test	Chen et al., 2019
5	APP/PS1 mice	Urolithin A-the metabolite of EA (300 mg/kg, p.o.) for 14 days	Morris water maze test	Gong et al., 2019
IV	Scopolamine- and diazepam-induced MCI			
1	Wistar rats	EA (30 and 100 mg/kg, i.p.) for 10 days	Elevated plus-maze test, Passive avoidance test, Open field test	Mansouri et al., 2016
2	Wistar rats	EA from *Tetrapleura tetraptera* and *Quassia undulata* (50 and 300 mg/kg, p.o.) for 14 days	Novel object recognition test, Y-maze test	Odubanjo et al., 2018
V	Other facts-induced MCI			
1	traumatic brain injury (TBI) in rat	EA (100 mg/kg, i.p.) for 3 days	Passive avoidance test	Mashhadizadeh et al., 2017
2	Symptoms of HD rats (3-nitropropionic acid)	EA (25, 50, and 100 mg/kg, p.o.) for 21 days	Novel object recognition test, Elevated plus maze test	Sharma et al., 2021

EA from *Tetrapleura tetraptera* and *Quassia undulata* (50 and 300 mg/kg) ameliorated the MCI induced by scopolamine in rats (Odubanjo et al., 2018).

MOLECULAR MECHANISM OF THERAPEUTIC PROPERTIES OF ELLAGIC ACID IN MILD COGNITIVE IMPAIRMENT

The molecular mechanisms by which EA prevented or reduced MCI vary and include antioxidative damage, modulation of synthesis of antineuroinflammation molecules, inhibition of apoptosis, preservation of mitochondrial functions, and synapse stabilization and plasticity relevant for MCI (**Figure 3**).

Antineuroinflammation

Neuroinflammation has been proven to be involved in the pathogenesis of aging and neurodegenerative diseases (Bradburn et al., 2019). Over the decades, a large number of studies on animal models and human beings implies that the immune system plays a fundamental part in learning, memory modulation, and neural plasticity (Yirmiya and Goshen, 2011). Under regular quiescent circumstances, immune mechanisms positively regulate neural circuit remodeling, the promotion of memory functions, and neurogenesis. Nevertheless, when the immune system is vigorously stimulated by infection, harm, or chronic stress state, the inflammatory mediators interrupt the sensitive balance required for neurophysiological actions and result in damaging effects on neural plasticity, neurogenesis, and memory. EA and its microbial metabolites can perform the role of the potential anti-inflammatory agent, mainly by reducing the production of microglial proinflammatory cytokines and chemokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-6, or interleukin-1β (IL-1β) in the brain tissue in the alleviation of neuroinflammatory responses and prevention of MCI. Alagan et al. (2019) showed that EA from *Phyllanthus amarus* can decrease the release of proteins such as IL-1β, TNF-α, and nitric oxide synthase (iNOS) in LPSs -induced MCI and neuroinflammation. Notably, in rat models of moderate traumatic brain injury, treatment of EA has significantly decreased the proinflammatory cytokine such as TNF-α in brain tissue (Mashhadizadeh et al., 2017). In addition,

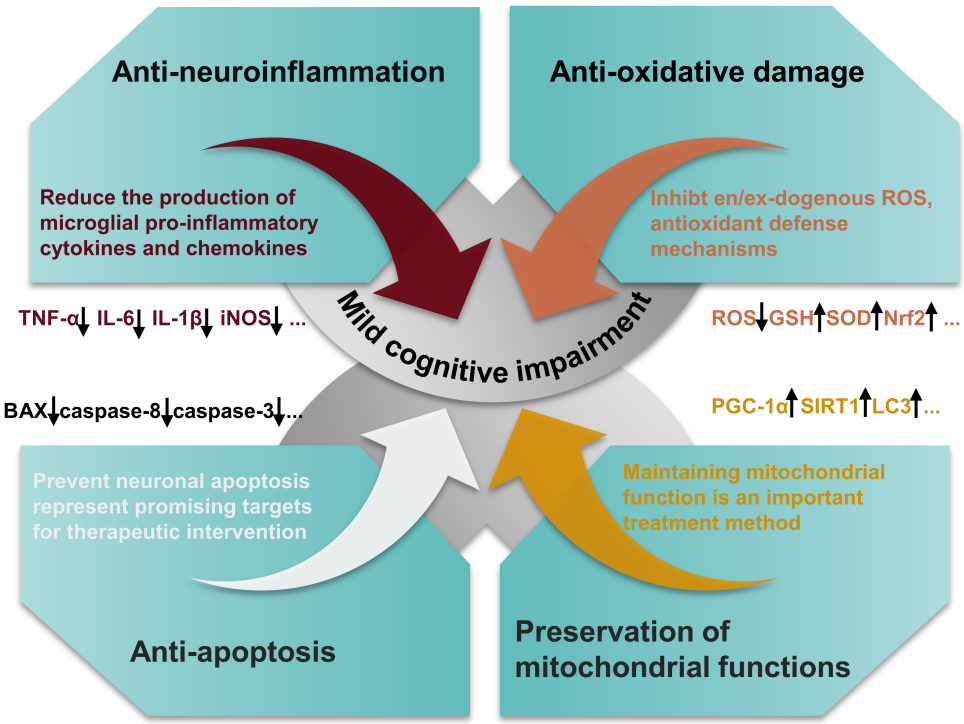


FIGURE 3 | Salient pharmacological features of ellagic acid (EA) elicited by involving anti-oxidant effects, modulation of synthesis of anti-inflammatory molecules, inhibition of neuronal apoptosis, and preservation of mitochondrial functions.

urolithin A, as the major gut-microbial metabolite of EA, has been revealed to have anti-inflammatory properties *in vitro* and *in vivo*. Gong et al. (2019) indicated that urolithin A not only reduced the levels of activated astrocytes and microglia in the mouse model of AD, but also involved in vital cell signaling pathways, explicitly by enhancing cerebral AMP-activated protein kinase (AMPK) activation, decreasing the activation of P65NF-κB and P38MAPK.

Antioxidative Damage

Free radical-mediated oxidative impairment of biological molecules has been connected with the pathogenesis of several neurodegenerative disorders (Kahroba et al., 2021). Particularly, a surfeit of lipid peroxidation and depletion of brain antioxidant defense ruin neuronal coherence and cognitive prowess. Antioxidants are proposed to modify or decelerate the progression of neurodegenerative disease pathogenesis and clinical symptoms such as MCI (Chauhan and Chauhan, 2020). EA possesses excellent antioxidant activities, as the two pairs of the hydroxyl group exist in the structure to enhance its antioxidant properties. In fact, EA comprises two lactone and four functional phenolic groups linked to involvement in redox reactions and thereby it can protect crucial molecules from hazardous free radicals (Alfei et al., 2020). Meanwhile, the gene expression of molecules that are engaged in antioxidant defense can be activated by EA (Zhang et al., 2022). In the same way, the expression of proteins, enzymes, and transcription factors that are participating in oxidative stress-producing pathways can be repressed by EA such as cytochrome P450-dependent phase-I enzymes and nicotinamide adenine dinucleotide phosphate oxidase. Previous findings confirmed that administration of EA prevents cognitive impairment through attenuation of the brain lipid peroxidation and prevented waning of the superoxide dismutase (SOD), glutathione (GSH), and catalase (CAT) activity in streptozotocin-induced rats (Kumar and Bansal, 2018). Cao et al. (2020) revealed that ellagitannin activates AMP-activated protein kinase (AMPK) to ameliorate oxidative stress by activation of NF-E2-related factor (Nrf2)-mediated phase II antioxidant enzymes. In such a manner, EA is involved not only in the management of endogenous and exogenous sources of reactive oxygen species (ROS), but also involved in antioxidant defense mechanisms.

Antiapoptosis

The internal signals of the mitochondria can exactly mediate apoptosis, a programmed cell death mechanism, or the extrinsic signals received to the cell surface (death domain triggering) receptors (Bedoui et al., 2020). Neuronal apoptosis is a vital attribute of neurodegenerative disease pathology. Lots of evidence indicated that preventing apoptosis also represents a promising target for therapeutic intervention. As an example, some recent studies suggested that markers for apoptotic cell death-like caspases are activated in striatal neurons from transgenic animals and patients with HD (Chongtham et al., 2021). An *in-vivo*-based study reported that treatment of EA of R6/2 mice significantly reduced the

apoptotic neurons by downregulating Bax and caspase-8 in the striatum and cortex (Sun et al., 2020). Notably, an *in-vivo* study of APP/PS1 transgenic mice and its amelioration by EA corroborated results on cleaved caspase-3 activation (Zhong et al., 2018). Interestingly, in a similar study, EA from immature persimmon (*Diospyros kaki*) ameliorated the cognitive dysfunction and downregulated the expression of Bax and caspase-3 in $(A\beta)_{1-42}$-induced Institute for Cancer Research (ICR) mice (Yoo et al., 2021). EA can prevent MCI by regulating Phosphoinositide 3 (PI3)-kinase-endothelial nitric oxide synthase (eNOS) signaling and thereby regulating cellular apoptosis in streptozotocin-treated rats (Kumar and Bansal, 2018). Meanwhile, urolithin A, the metabolite of EA, attenuated the D-galactose-induced neuronal apoptosis in aging mice by regulating the sirtuin 1/mammalian target of rapamycin (SIRT1/mTOR) signaling pathway (Chen et al., 2019).

Preservation of Mitochondrial Functions

Mitochondria generate the basic ATP for the optimal survival function of neurons and the accumulation of damaged mitochondria is a distinguishing feature of aging and age-related neurodegenerative disorders (Grimm and Eckert, 2017). Especially, mitochondrial dysfunction is a profound phenomenon in AD as it occurs in human samples of both the familial and sporadic types of the disease, as well as in transgenic AD mouse models' brain tissues (Fang et al., 2019). Mitochondrial quality control is regulated according to the procedures of mitophagy and mitochondrial biogenesis (Annesley and Fisher, 2019). Multiple evidence suggested that EA and its derivatives such as urolithin A restore defective mitochondria in a significant manner for MCI therapy by modulating mitochondrial biogenesis and rescuing dysfunctional autophagy (de la Torre et al., 2020; Jayatunga et al., 2021). A recent study showed that urolithin A inhibits high glucose-induced neuronal amyloidogenesis by regulating transglutaminase type 2-dependent endoplasmic reticulum-mitochondria contacts and calcium homeostasis (Lee et al., 2021). Meanwhile, urolithin A activates mitochondrial autophagy to exert neuroprotective effects through activation of the SIRT1/mTOR signaling pathway, which aids in preventing D-galactose-induced brain aging (Chen et al., 2019). Cao et al. (2020) found that punicalagin regulates mitochondrial biogenesis by activating AMPK/peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α) signaling, thereby preventing cognitive dysfunction. Additionally, mitochondrial respiratory dysfunction is also the main cause of ROS overproduction and treatment of EA can prevent the decrease of mitochondrial succinate dehydrogenase activity in the rat brain caused by

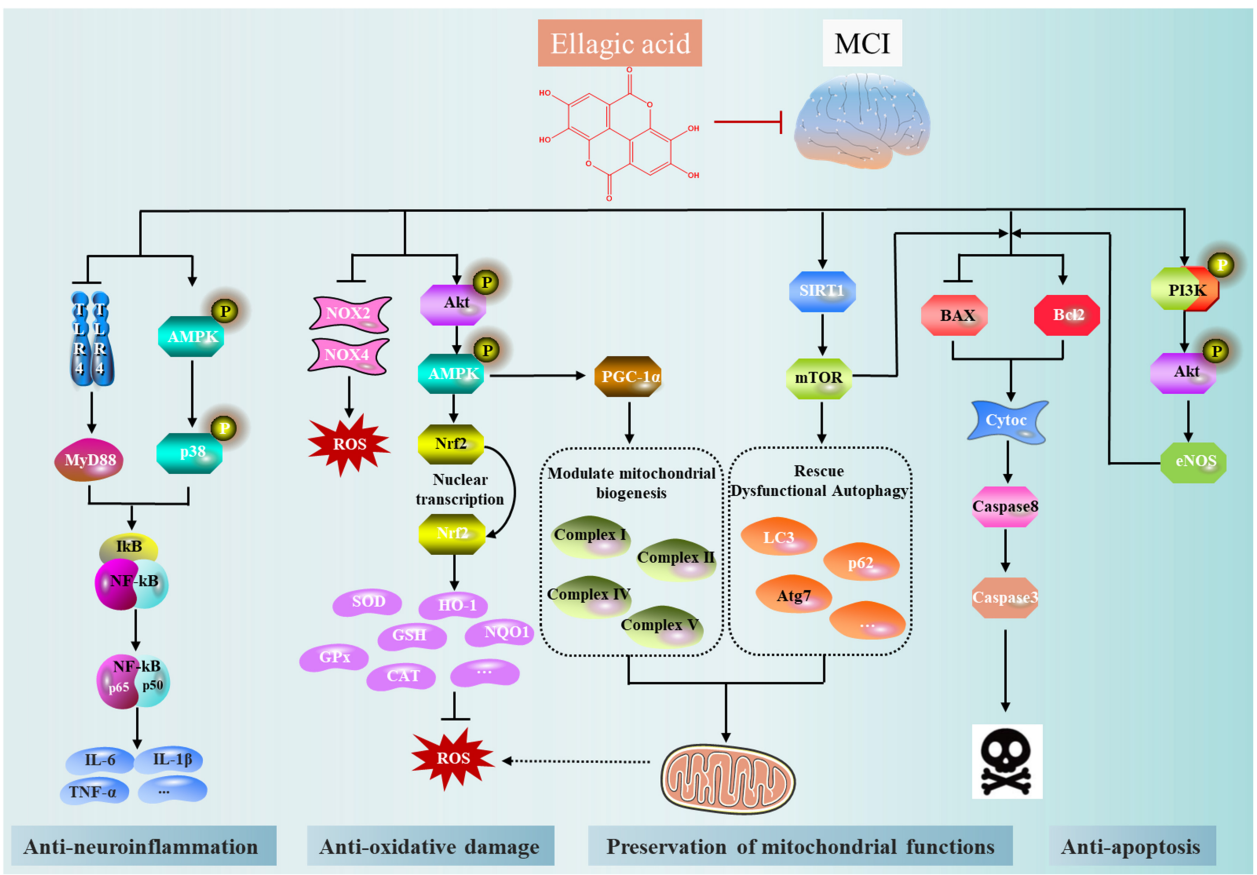


FIGURE 4 | The main mechanism network of anti-mild cognitive impairment (MCI) effect of ellagic acid (EA).

3-nitropropionic acid, thus protecting mitochondrial functions (Sharma et al., 2021).

CONCLUSION AND FUTURE PROSPECTS

Multiple evidences indicate that plant-derived EA and its derivatives existing in natural sources such as kakadu plum, pomegranate, and raspberry are natural bioactive compounds with considerable favorable health properties. Especially, EA has been accepted to be an existing food additive in Japan and is principally regarded as an antioxidant that can be utilized without any toxicological data or any limitations in respect of the concentration utilization limit. So far, the preclinical studies of EA and its derivatives conducted on various cell lines and animal models facilitated the idea of dietary EA as a feasible agent to be able to the specifical target and improve MCI. In addition, we found a wide range of doses of EA in these behavioral experiments, ranging from a minimum of 25 mg/kg to a maximum of 300 mg/kg. We speculated that this might be related to the strains of animals selected in the experiments and their physical conditions. But happily, all the witnesses mentioned above suggest that EA has little toxicity to tissue or normal cells. EA and its derivatives exert beneficial effects on MCI by reducing the ability of neuroinflammation, oxidative stress, neuronal apoptosis, synaptic dysfunction and loss, and defective mitochondrial functions (**Figure 4**). A randomized double-blind clinical trial found that EA could improve sleep quality and gastrointestinal symptoms in patients with irritable bowel syndrome and its antioxidant and anti-inflammatory properties may be the main reason for this effect (Mirzaie et al., 2021). In addition, another clinical trial study confirmed that supplements of EA can be used as dietary supplements in patients with type 2 diabetes by improving chronic adverse effects (Ghadimi et al., 2021). It has also been shown to reduce metabolic disorders in women (Kazemi et al., 2021). In conclusion, EA, as a dietary supplement, plays an important role in the treatment of many diseases. Nevertheless, there are no large randomized controlled clinical trials of EA for the treatment of MCI. Although a large amount of preclinical data has been accumulated on the use of EA for the treatment of MCI, the clinical research evidence, particularly the large randomized controlled trials, on the topic is still scarce. This emphasizes the well-designed and correctly powered randomized controlled trials in the human population that need to be performed to reinforce the scientific facticity of the beneficial effects of EA on MCI. Synchronously, the mechanism of EA against MCI is least provided cynosure and expects more attention from the emerging research community. In particular, the emergence of new technologies and methods, such as genomics, metabolomics, proteomics, and bioinformatics network analysis techniques (Hou et al., 2021; Wang et al., 2022), has provided support for the screening of potential targets and revealing the mechanism of action of EA in the treatment of MCI. All in all, drug development from these innate resources ought to be encouraged and upgraded, keeping in mind its effectiveness and capability to perform the role of a safe drug, thereby improving the quality of human life and promoting public health to manage MCI.

AUTHOR CONTRIBUTIONS

YZ, XM, and WW conceived and proposed the idea. WW designed the research and wrote the manuscript. SW revised and edited the illustrations and words in the manuscript. XW and YL revised the manuscript. All authors have read the final version of the manuscript.

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Fibrinogen in Alzheimer's Disease, Parkinson's Disease and Lewy Body Dementia: A Mendelian Randomization Study

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Fibrinogen is reportedly associated with neurodegenerative diseases (NDs), but the underlying causality remains controversial. Using Mendelian randomization (MR), this study aimed to assess the causal association between fibrinogen and Alzheimer's disease (AD), Parkinson's disease (PD), and Lewy body dementia (LBD). Genetic variants associated with fibrinogen and γ-fibrinogen were selected and used as instrumental variables. The effect estimates of the main analysis were obtained by inverse-variance weighting (IVW), complemented by sensitivity analyses to verify model assumptions, and multivariable MR was conducted to control for potential pleiotropic effect. Two-step MR was performed to assess the causal association through mediators. The main analysis suggested no causal association between genetically predicted plasma fibrinogen and γ-fibrinogen levels and the risk of AD, PD, and LBD. The effect estimates did not change in the follow-up sensitivity analyses and MVMR. However, the two-step MR analysis provides evidence that fibrinogen may contribute to the risk of AD via CRP levels. There was an inverse effect of adult height levels on the risk of AD. Our results support the effects of fibrinogen on the risk of AD through increasing plasma CRP levels. Our study found no evidence to support the effects of genetically determined fibrinogen and γ-fibrinogen levels on the risk of PD and LBD. Additionally, our findings suggested an inverse association between genetically determined adult height levels and the risk of AD. Future studies are needed to elucidate the underlying mechanisms and their clinical applications.

Keywords: fibrinogen, Alzheimer's disease, Parkinson's disease, Lewy body dementia, Mendelian randomization

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INTRODUCTION

Due to an aging population, neurodegenerative diseases (NDs) are one of the most serious public health burdens and causes of mortality and morbidity worldwide (GBD 2016 Neurology Collaborators, 2017). So far, there are few or no effective curative treatment options for NDs, although not for lack of effort. The processes of NDs begin long before clinical symptoms appear, but the lack of effective biomarkers prevents patients from being diagnosed in the early stages of these diseases. Hence, there is an urgent need to identify the risk factors that cause NDs and appraise patients at risk in order to allow for prevention and early intervention to slow down the course of these disorders. For many years, vascular factors and inflammatory responses were considered to be involved in the pathological process of some

NDs, such as Alzheimer's disease (AD), Parkinson's disease (PD), and Lewy body dementia (LBD) (Vemuri et al., 2015; Voet et al., 2019). Fibrinogen is a pleiotropic protein of the hemostatic system and inflammatory response (Sidelmann et al., 2000; Adams et al., 2007). The component is made up of three polypeptide chains named Aα, Bβ, and γ, which the γ chain accounting for around 8∼15% of total circulating fibrinogen (Cheung et al., 2009). Structurally, fibrinogen comprises receptors expressed by nervous system cells and binding sites for regulatory proteins of pivotal nervous system functions (Petersen et al., 2018). Recent studies have shown that fibrinogen plays a major role in NDs due to its involvement in the coagulation cascade (Sidelmann et al., 2000) and inflammatory system (Davalos and Akassoglou, 2012). As such, a growing number of studies have attempted to explore the potential role of fibrinogen in AD, PD and LBD (Chiam et al., 2015; Petersen et al., 2018; Zhou et al., 2020). However, the results of conventional observational studies may have been affected by residual confounding and reverse causation (Phillips and Smith, 1991), thus limiting our knowledge of the effect fibrinogen on the risk of NDs.

Mendelian randomization (MR) is a method that uses genetic variants, or single-nucleotide polymorphisms (SNPs), as instruments to infer causal effect of lifelong risk factors (exposure) on diseases (outcome). This method can reduce the impact of confounding and reverse causation (Smith and Ebrahim, 2003). In the current study, we performed the univariable and multivariable MR analyses to assess the associations between the genetically determined circulating fibrinogen and γ-fibrinogen levels and the risk of AD, PD and LBD (**Figure 1**). In addition, we extend our study using a two-step MR analysis to assess the causal effects of fibrinogen and γ-fibrinogen levels on AD through the potential mediators.

MATERIALS AND METHODS

Data Sources

Genetic Instruments Associations With Fibrinogen

We obtained the SNPs with fibrinogen-related phenotypes from the published genome-wide association study (GWAS) summary meta-analysis statistics (Sabater-Lleal et al., 2013). This study was comprised of 28 GWAS, including more than 90,000 individuals of European ancestry. Then, the SNPs with γ-fibrinogen levels were obtained from a recent GWAS of European ancestry from the Atherosclerosis Risk in Communities (ARIC) study (Maners et al., 2020). To validate and select the SNPs for plasma fibrinogen levels, we chose genetic instruments that were genome-wide significant ($5^{*}10^{-8}$). Additionally, the F statistics of each SNPs of at least 10 is considered as sufficient for performing the MR analysis, the calculation formula is as following: $F = R^{2*}(N-2)/(1-R^{2})$.

Outcome Datasets

Genetic variants associated with AD were obtained from the published genome-wide meta-analysis (Jansen et al., 2019), which includes data consisting of 71,880 cases of clinically diagnosed AD and AD-by-proxy, based on parental diagnoses, and 383,378 controls of European ancestry from four consortia: the Alzheimer's-disease working group of the Psychiatric Genomics Consortium (PGC-ALZ), the International Genomics of Alzheimer's Project (IGAP), the Alzheimer's Disease Sequencing Project (ADSP) and the UK Biobank. Summary statistics of PD risk for the selected instrument variables, which included 33,674 PD cases and 449,056 controls of European ancestry, were acquired from the published meta-analysis of PD GWAS from International Parkinson's Disease Genomics Consortium (IPDGC) (Nalls et al., 2019). Summary statistics with LBD were obtained from a recent published GWAS (Chia et al., 2021). That study recruited 2,591 individuals diagnosed with LBD and 4,027 neurologically healthy individuals across 44 institutions or consortia.

Details of the participant data for each dataset are described in the original publication.

Statistical Analysis

Mendelian Randomization

In the current study, we used several MR methods to estimate the causal associations between plasma fibrinogen levels and AD, PD, and LBD. The inverse variance-weighted (IVW) estimates were used for principal MR analysis, and the fixed-effects (FE) model was applied to homogeneous data, while the multiplicative random (RE) model was suitable for heterogeneous data (Burgess et al., 2019). Results are presented as odds ratios (OR) for each outcome risk per corresponding unit change in fibrinogen levels on natural logarithm transformation.

Sensitivity Analyses

For sensitivity analyses, we performed MR-Egger regression analysis (Bowden et al., 2015), maximum likelihood estimate (Pierce and Burgess, 2013), weighted-median estimator (Bowden et al., 2016) and MR Pleiotropy Residual Sum and Outlier (MR-PRESSO) methods (Verbanck et al., 2018) to verify the main MR assumptions. The MR-Egger regression, which requires the InSIDE (instrument strength independent of direct effects) assumption to be valid, performs a weighted linear regression of SNP outcome on the SNP fibrinogen effect estimates, which provides a valid effect estimate even when all the SNPs are invalid instruments. Then, the MR-Egger intercept estimates the overall unbalanced horizontal pleiotropy effect across the genetic variants. For the maximum likelihood estimate method, estimates of the probability distribution parameters can be obtained by maximizing of the likelihood. A weighted-median method can provide a consistent effect estimate if at least 50% of the genetic variants are valid. We then used the MR-PRESSO analysis to detect horizontal pleiotropy and outlier SNPs. Cochran's Q statistic for the IVW method was applied to test the heterogeneity of the genetic variants (Greco et al., 2015). The Radial MR method was performed to detect heterogeneity and outliers (Bowden et al., 2018). Additionally, the leave-1-out analysis were performed to determine whether the results were disproportionately affected by any single variants. The power calculations were estimated for the primary analyses using online tool (Brion et al., 2013; **Supplementary Table 5**). Finally, we

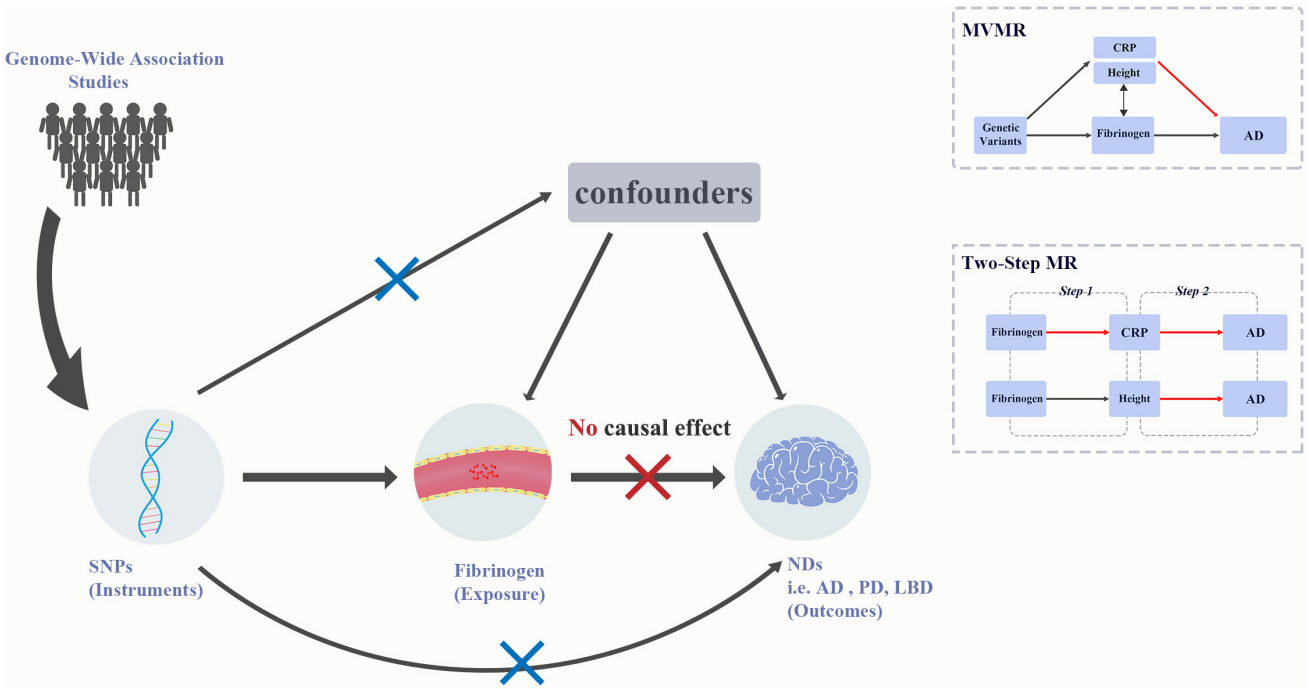


FIGURE 1 | Graphical abstract for current univariable MR and MVMR analyses. MR analysis is based on the three assumptions: (1) IVs should be associated with the exposure, (2) any association between the IVs and the outcome must come via the IVs association of IVs with the exposure, and (3) IVs should be independent of any measured and unmeasured confounders. The SNPs used in MR acted as IVs were obtained from large-scale genome-wide association studies. Results for univariable MR did not support the causal effect of fibrinogen and γ-fibrinogen on AD, PD, and LBD, whereas the results for MVMR showed the association between height and AD. AD, Alzheimer's disease; IVs, instrumental variables; LBD, dementia with Lewy bodies; MR, Mendelian randomization; MVMR, multivariable Mendelian randomization; NDs, Neurodegenerative diseases; PD, Parkinson's Disease.

conducted Bonferroni correction for multiple comparisons of 4 exposures and 3 outcomes (P-value threshold of 0.05/7 = 0.0071). Results that were significant before correction for multiple comparisons but not after correction were considered suggestive.

Multivariable Mendelian Randomization

To correct the effect of the potential confounders on fibrinogen levels, we performed the Multivariable MR (MVMR) analysis (Sanderson, 2021) based on the existing biological knowledge and potential pleiotropy on IVs of the exposure. Given the role of fibrinogen in inflammation (Davalos and Akassoglou, 2012), we performed the MVMR analysis adjusting the fibrinogen levels for CRP levels. Additionally, using the online database "PhenoScanner" (Staley et al., 2016) to detect potentially related phenotypes, we found that SNPs for fibrinogen (rs2706383) and γ-fibrinogen (rs59950280) levels are associated with height. Thus, we also performed MVMR analyses adjusting fibrinogen and γ-fibrinogen levels for height to adjust for potential genetic pleiotropy.

The MVMR analysis used summary level data from three GWAS. We used a GWAS of fibrinogen and γ-fibrinogen levels from population-based study of 10,708 European individuals (Pietzner et al., 2020). Genetic variants of CRP levels were obtained from a published GWAS involving 3,301 individuals of European descent (Sun et al., 2018). Then, we used GWAS summary data of adult height from a published study from the Genetic Investigation of ANthropometric Traits (GIANT) consortium (Berndt et al., 2013).

Two-Step Mendelian Randomization

Finally, we used a two-step MR analysis to assess the association of the exposure with the outcome, exposure with mediators, and mediators with the outcome. In the first step, we estimated the causal effect of fibrinogen and γ-fibrinogen levels on CRP levels and adult height. In the second step, we estimated the causal effect of CRP levels and adult height on the risk of AD.

All statistical analyses were performed with the "TwoSampleMR" and "MRPRESSO" and "MendelianRandomization" packages (Yavorska and Burgess, 2017; Hemani et al., 2018; Verbanck et al., 2018) in R, version 4.1.2.[1]

Data Availability

The datasets analyzed in this study are publicly available summary statistics. Summary statistics for PD, CRP, and height were obtained from MRC Integrative Epidemiology Unit (IEU) OpenGWAS database (Elsworth et al., 2020). Summary level data for fibrinogen, γ-fibrinogen, AD and LBD were downloaded from the NHGRI-EBI GWAS Catalog (Buniello et al., 2019).

[1]http://www.r-project.org

RESULTS

Selection of Genetic Instruments

We obtained the 23 SNPs associated with plasma fibrinogen levels at a genome-wide significance level. One SNP (rs16844401) was excluded because of F-statistics lower than 10. One of the instruments (rs1019670) was removed due to linkage disequilibrium clumping ($r^2 < 0.1$). After harmonizing the instruments and each outcome dataset, 18, 17, and 18 remaining SNPs were used as genetic instruments for causal estimation between fibrinogen levels with AD, PD, and LBD. Additionally, 16 SNPs were selected as genetic instruments for γ-fibrinogen. After harmonizing the instruments and the AD, PD, and LBD datasets, 14, 15, 14 SNPs remained for causal estimation between γ-fibrinogen and each outcome. Detailed information on the instruments associated with fibrinogen and each outcome is displayed in **Supplementary Tables 1A,B**.

Mendelian Randomization Analysis

Figures 2, 3 and **Supplementary Tables 2A,B** showed the causal effect on genetically elevated fibrinogen and γ-fibrinogen levels with each outcome. In the MR analysis using IVW, we found that genetically determined fibrinogen levels were not associated with AD [random effects OR = 1.075 (95%CI:0.977, 1.182), $P = 0.139$], PD [random effects OR = 1.187 (95%CI:0.647, 2.178), $P = 0.579$] and LBD [random effects OR = 1.298 (95%CI:0.294, 5.723), $P = 0.731$]. The same results were observed in the MR-Egger regression, weighted median method and maximum likelihood approach. Genetically determined γ-fibrinogen levels were not associated with AD [random effects OR = 0.984 (95%CI:0.959, 1.010), $P = 0.214$], PD [random effects OR = 1.015 (95%CI:0.885, 1.165), $P = 0.830$], and LBD [random effects OR = 1.032 (95%CI:0.651, 1.637), $P = 0.892$] using IVW analysis. The results were consistent across the MR-Egger regression, weighted median method and maximum likelihood approach.

Sensitivity Analysis

Figures 2, 3 and **Supplementary Tables 2A, 3B** show the sensitivity analysis for each outcome in this study. The *P*-values from the MR-Egger intercept test were not statistically significant therefore a strong pleiotropic bias was not found. The results of the Cochran's *Q*-test showed no apparent heterogeneity between individual SNP estimations. In addition, the analysis of MR-PRESSO test did not identify any outliers for any of the estimates. In the leave-one-out analysis, we did not find the risk estimates of genetically predicted fibrinogen levels on PD and LBD changed after excluding one SNP iteratively, indicating that there were no potentially influential SNPs could affect the causal association. However, the result of IVW-based leave-one-out analysis showed a significant association between fibrinogen and AD after the exclusion of rs4129267 ($P = 0.041$), but this association is not robust and stable.

Multivariable Mendelian Randomization

Using summary level data for each phenotype, univariable MR provided significant evidence that genetically determined fibrinogen [fixed effects OR = 1.030 (95%CI:1.003, 1.058), $P = 0.003$] and γ-fibrinogen [fixed effects OR = 1.027 (95%CI:1.001, 1.055), $P = 0.042$] levels were associated with AD. The results of univariable MR showed that fibrinogen and γ-fibrinogen were not associated with PD [IVW_{fib} fixed effects OR = 1.063 (95%CI:0.866, 1.305), $P = 0.560$] [IVW_{γ} fixed effects OR = 0.982 (95%CI:0.809, 1.190), $P = 0.850$] and LBD [IVW_{fib}

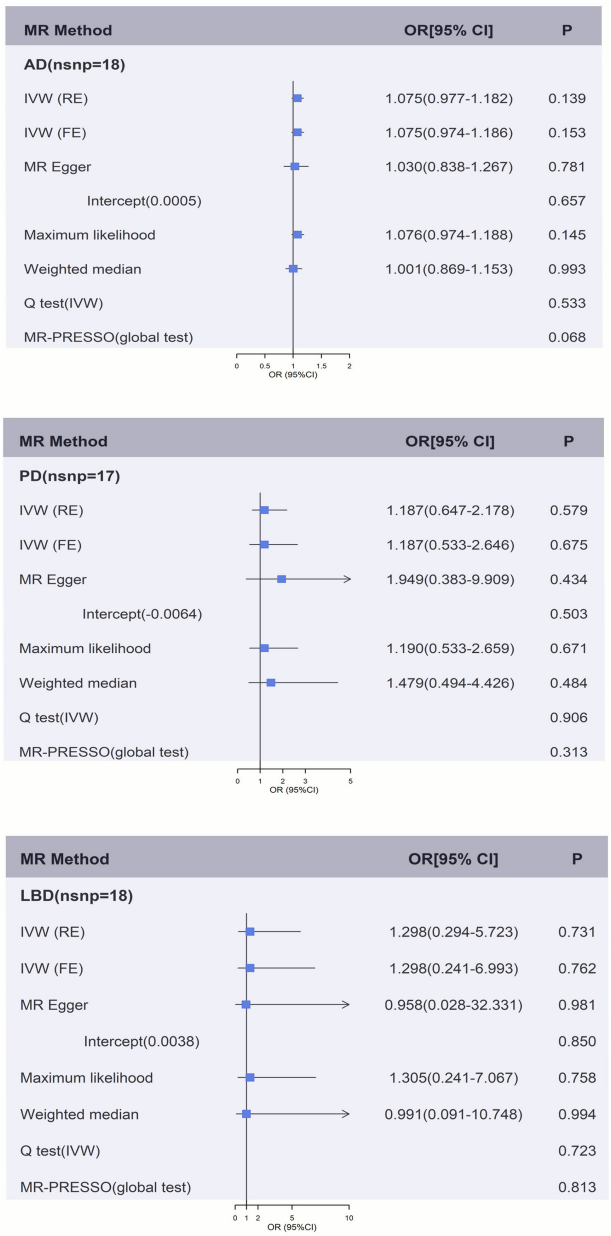


FIGURE 2 | Genetically predicted fibrinogen levels with risk of AD, PD, and LBD. Results of the primary MR and sensitivity analyses. Estimated ORs per unit increase in log-transformed fibrinogen for each outcome. AD, Alzheimer's disease; IVW, Inverse variance weighting; LBD, Lewy body dementia; FE, fixed effects; MR, Mendelian randomization; OR, odds ratio; PD, Parkinson's Disease; RE, random effects.

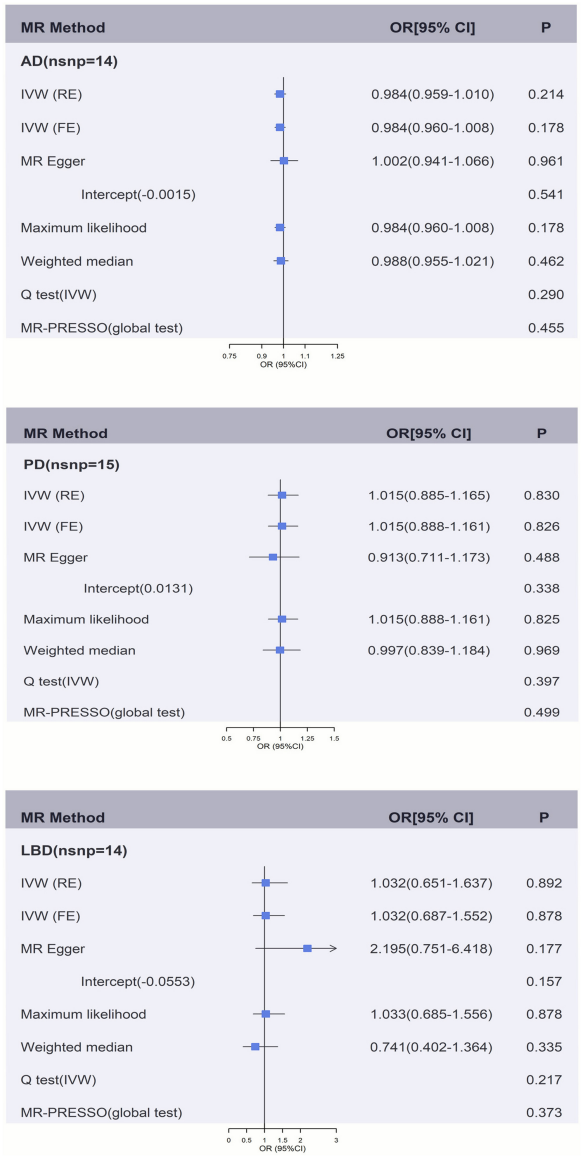


FIGURE 3 | Genetically predicted γ-fibrinogen levels with risk of AD, PD, and LBD. Results of the primary MR and sensitivity analyses. Estimated ORs per unit increase in log-transformed γ-fibrinogen for each outcome. AD, Alzheimer's disease; IVW, Inverse variance weighting; LBD, Lewy body dementia; FE, fixed effects; MR, Mendelian randomization; OR, odds ratio; PD, Parkinson's Disease; RE, random effects.

fixed effects OR = 0.912 (95%CI:0.581, 1.431), $P = 0.688$] [IVW_{γ} fixed effects OR = 0.837 (95%CI:0.558, 1.256), $P = 0.053$]. In the results of the fixed effects IVW-based MVMR, the effect of fibrinogen on AD did not change substantially after adjustment for height [fixed effects OR = 1.024 (95%CI:1.0005, 1.049), $P = 0.043$], whereas the results of random effects IVW showed a null effect [random effects OR = 1.024 (95%CI:0.999, 1.051), $P = 0.065$]. It suggests the results are not robust and stable. Results of MVMR showed that genetically determined fibrinogen levels were not associated with PD [random effects OR = 1.063 (95%CI:0.857, 1.319), $P = 0.581$] and LBD [random effects OR = 0.864 (95%CI:0.557, 1.340), $P = 0.513$] after adjustment for height. Then, after adjustment for height, genetically predicted γ-fibrinogen levels showed no association with AD [random effects OR = 1.018 (95%CI:0.993, 1.044), $P = 0.145$], PD [random effects OR = 0.992 (95%CI:0.797, 1.236), $P = 0.941$] and LBD [random effects OR = 0.821 (95%CI:0.535, 1.261), $P = 0.368$]. However, the results of MVMR indicated that adult height levels were inversely associated with the risk of AD [random effects OR = 0.996 (95%CI:0.992, 0.99992), $P = 0.023$]. In the results of IVW-based MVMR, there was no evidence of indirect effects through fibrinogen levels for AD, PD, and LBD after adjustment for CRP. Similarly, we did not observe a significant effect of γ-fibrinogen on each outcome after adjusting for CRP. The MVMR results are presented in **Table 1** and **Supplementary Tables 4A,B**.

Two-Step Mendelian Randomization

In the first step, genetically predicted fibrinogen levels were associated with the CRP levels [random effects OR = 5.952 (95%CI:2.212, 16.015), $P = 4.12E-04$]. However, there was no association between genetically predicated fibrinogen levels and taller adult height [random effects OR = 0.736 (95%CI:0.202, 2.687), $P = 0.643$]. In the second step, we found a suggestive association between genetically predicted CRP levels and the risk of AD [random effects OR = 1.015 (95%CI:1.002, 1.028), $P = 0.028$]. We also found a suggestive association between genetically predicted taller adult height and the risk of AD [random effects OR = 0.996 (95%CI:0.992, 0.999), $P = 0.024$]. The results of random effects IVW showed a causal effect of genetically predicted γ-fibrinogen levels on taller adult height [random effects OR = 1.693 (95%CI:1.346, 2.129), $P = 6.78E-06$]. However, the sensitivity analyses were inconsistent. There was no causal effect of genetically predicted γ-fibrinogen levels on CRP levels [random effects OR = 1.181 (95%CI:0.972, 1.436), $P = 0.094$]. The two-step MR results are presented in **Table 2**.

DISCUSSION

In this study, we performed univariable MR and MVMR analyses to evaluate the association between the genetically determined fibrinogen and γ-fibrinogen levels on NDs. The results of our principal analyses did not find evidence that plasma fibrinogen and γ-fibrinogen levels were associated with AD, PD and LBD, with consistent results in MVMR analyses after adjusting for CRP and height. For AD, these findings are generally consistent with previous MR analyses showing no effect of genetically determined fibrinogen levels on AD (Fani et al., 2021). Our findings are less consistent with a previous reviews and meta-analysis that reported on significant associations between fibrinogen levels and AD. This inconsistency could be explained by the confounding factors and selection bias. Given that AD primarily affects the elderly, using an older age group could lead to selection bias due to recruitment on surviving exposure and competing risk (Schooling et al., 2019). To avoid selection bias, we used both proxy-case and clinically diagnosed AD GWAS in the current

TABLE 1 | Results of multivariable MR adjusting for the effect of height and CRP.

Outcomes	Exposures	IVW (RE)		IVW (FE)		MR Egger	
		OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	*P*
AD							
	Fibrinogen	1.024 (0.999–1.051)	0.065	1.024 (1.000–1.049)	0.043*	1.019 (0.990–1.050)	0.219
	Height	0.996 (0.992–0.9999)	0.022*	0.996 (0.992–0.9999)	0.012*	0.995 (0.991–0.999)	**0.02***
	Fibrinogen	1.025 (0.990–1.062)	0.166	1.025 (0.998–1.054)	0.072	0.976 (0.919–1.374)	0.438
	CRP	1.013 (0.993–1.033)	0.197	1.013 (0.997–1.029)	0.094	0.964 (0.910–1.020)	0.197
	γ-fibrinogen	1.018 (0.993–1.044)	0.145	1.018 (0.996–1.040)	0.11	1.014 (0.985–1.044)	0.344
	Height	0.996 (0.992–0.9999)	0.023*	0.996 (0.992–0.9999)	0.012*	0.995 (0.991–0.999)	**0.021***
	γ-fibrinogen	1.023 (0.994–1.054)	0.134	1.023 (0.996–1.052)	0.096	0.991 (0.940–1.045)	0.734
	CRP	1.012 (0.996–1.028)	0.139	1.012 (0.996–1.028)	0.099	0.985 (0.945–1.027)	0.475
PD							
	Fibrinogen	1.063 (0.857–1.319)	0.581	1.063 (0.888–1.273)	0.508	1.068 (0.828–1.378)	0.611
	Height	1.004 (0.969–1.040)	0.81	1.004 (0.975–1.034)	0.773	1.004 (0.969–1.040)	0.807
	Fibrinogen	1.052 (0.852–1.300)	0.635	1.052 (0.852–1.300)	0.635	1.047 (0.659–1.663)	0.847
	CRP	1.035 (0.922–1.161)	0.568	1.035 (0.922–1.161)	0.568	1.028 (0.663–1.595)	0.901
	γ-Fibrinogen	0.992 (0.797–1.236)	0.941	0.992 (0.828–1.188)	0.928	1.039 (0.799–1.351)	0.773
	Height	1.005 (0.970–1.041)	0.775	1.005 (0.976–1.035)	0.727	1.006 (0.969–1.044)	0.741
	γ-Fibrinogen	0.972 (0.798–1.185)	0.781	0.972 (0.798–1.185)	0.781	1.147 (0.784–1.677)	0.48
	CRP	1.043 (0.929–1.171)	0.474	1.043 (0.929–1.171)	0.474	1.184 (0.898–1.561)	0.228
LBD							
	Fibrinogen	0.864 (0.557–1.340)	0.513	0.864 (0.577–1.294)	0.479	0.899 (0.542–1.491)	0.68
	Height	1.031 (0.963–1.105)	0.376	1.031 (0.967–1.100)	0.337	1.034 (0.963–1.109)	0.358
	Fibrinogen	0.868 (0.547–1.379)	0.552	0.868 (0.547–1.379)	0.552	0.847 (0.306–2.343)	0.75
	CRP	1.133 (0.877–1.465)	0.339	1.133 (0.877–1.465)	0.339	1.104 (0.418–2.913)	0.841
	γ-Fibrinogen	0.821 (0.535–1.261)	0.368	0.821 (0.553–1.220)	0.33	0.791 (0.478–1.310)	0.362
	Height	1.031 (0.963–1.105)	0.375	1.031 (0.969–1.098)	0.337	1.029 (0.959–1.105)	0.423
	γ-Fibrinogen	0.803 (0.530–1.216)	0.3	0.803 (0.530–1.216)	0.3	0.931 (0.412–2.103)	0.862
	CRP	1.149 (0.891–1.483)	0.286	1.149 (0.891–1.483)	0.286	1.276 (0.729–2.236)	0.392

AD, Alzheimer's disease; CRP, C-reactive protein; DLB, dementia with Lewy bodies; MR, Mendelian randomization; OR, odds ratio; PD, Parkinson's disease.
*$p < 0.05$.

study. Moreover, we used the MVMR analysis to eliminate confounding factors. Additionally, using two-step MR analysis, we found that higher fibrinogen levels could contribute to the risk of AD via increasing CRP levels. A recent MR analysis using larger numbers of IVs indicated that genetically predicted high CRP levels might increase the risk of AD, which could support the positive association in the current study (Zhang et al., 2022). To our knowledge, there has been no MR study performed on the casual effect of fibrinogen on PD and LBD. A previous observational study found that inverse associations with PD were observed for fibrinogen levels among women (Ton et al., 2012). However, observational studies may be subject to confounding factors and reverse causation.

Inflammation is a common feature of NDs. Sustained inflammatory responses may contribute to neuronal damage and disease progression in NDs. Previous research has proposed possible mechanisms for the association between inflammation and NDs (Glass et al., 2010; Amor et al., 2014). Fibrinogen is involved in the pro-inflammatory process by binding to a number of immune cell sites (Davalos and Akassoglou, 2012). Inflammation could cause blood-brain barrier (BBB) damage and leakage during the pathogenesis of NDs. In human tissue and relevant animal models, fibrinogen is deposited in the central nervous system (CNS) of NDs via a leaky (Petersen et al., 2018). Deposited fibrinogen may disrupt the BBB, potentially contributing to CNS pathologies and promoting NDs neuropathology. In this study, we found that higher fibrinogen levels may associated with the risk of AD via increasing CRP levels. This association indicated that fibrinogen might be involved in the pathophysiology of AD as a trigger of the inflammatory system. However, fibrinogen and γ-fibrinogen were not associated with PD and LBD. We speculate that fibrinogen may be a product of the process by which inflammation causes NDs rather than the cause of these diseases. During the slow progression of NDs, long-term accumulation of fibrinogen produced in the inflammatory response may promote and aggravate the neurodegenerative.

Surprisingly, our findings provide suggestive evidence that genetically determined taller adult height is inversely associated with the risk of AD. This finding is consistent with a previous

TABLE 2 | Results of two-step MR analysis.

Exposure	Outcome	MR Method	OR[95% CI]	*P*
Step 1				
Fibrinogen	**CRP**			
		IVW (RE)	5.952 (2.212, 16.015)	**4.12E-04***
		IVW (FE)	5.952 (1.897, 18.678)	**2.23E-03***
		MR Egger	2.530 (0.235, 27.239)	0.455
		Intercept		0.433
		Weighted median	4.942 (0.947, 25.786)	0.058
		Maximum likelihood	6.233 (1.974, 19.678)	**1.81E-03***
		Q-test		0.754
		MR-PRESSO (global test)		0.784
Fibrinogen	**Height**			
		IVW (RE)	0.736 (0.202, 2.687)	0.643
		IVW (FE)	0.736 (0.196, 2.760)	0.650
		MR Egger	5.332 (0.343, 82.771)	0.250
		Intercept		0.127
		Weighted median	1.256 (0.182, 8.674)	0.817
		Maximum likelihood	0.741 (0.196, 2.793)	0.658
		Q-test		0.498
		MR-PRESSO (global test)		0.49
γ-Fibrinogen	**CRP**			
		IVW (RE)	1.181 (0.972, 1.436)	0.094
		IVW (FE)	1.181 (0.986, 1.416)	0.071
		MR Egger	1.081 (0.740, 1.581)	0.694
		Intercept		0.599
		Weighted median	1.190 (0.938, 1.509)	0.151
		Maximum likelihood	1.185 (0.989, 1.420)	0.066
		Q-test		0.297
		MR-PRESSO (global test)		0.419
γ-Fibrinogen	**Height**			
		IVW (RE)	1.692 (1.346, 2.129)	**6.78E-06***
		IVW (FE)	1.692 (0.839, 3.414)	0.141
		MR Egger	0.683 (0.007, 63.199)	0.884
		Intercept		0.729
		Weighted median	1.692 (0.776, 3.687)	0.186
		Maximum likelihood	1.693 (0.837, 3.424)	0.143
		Q-test		0.956
		MR-PRESSO (global test)		0.960
Step 2				
CRP	**AD**			
		IVW (RE)	1.015 (1.002, 1.028)	**0.028***
		IVW (FE)	1.015 (1.0003, 1.030)	**0.046***
		MR Egger	1.062 (0.767, 1.469)	0.780
		Intercept		0.831
		Weighted median	1.010 (0.992, 1.028)	0.281
		Maximum likelihood	1.015 (1.0001, 1.030)	**0.048***
		Q test		0.439
Height	**AD**			
		IVW (RE)	0.996 (0.992, 0.999)	**0.024***
		IVW (FE)	0.996 (0.992, 0.999)	**0.016***
		MR Egger	0.977 (0.961, 0.995)	**0.014***
		Intercept		**0.039***
		Weighted median	0.995 (0.990, 1.0002)	0.059
		Maximum likelihood	0.996 (0.992, 0.999)	**0.017***
		Q-test		0.239
		MR-PRESSO (global test)		0.219

AD, Alzheimer's disease; CRP, C-reactive protein; DLB, dementia with Lewy bodies; MR, Mendelian randomization; OR, odds ratio; PD, Parkinson's disease.
**p < 0.05.*

MR analysis (Larsson et al., 2017) and a case-control study (Petot et al., 2007) showing an inverse association between adult height and AD. Our study extends previous MR findings by using the largest GWAS of AD, including clinically diagnosed AD and AD-by-proxy individuals, and applying additional sensitivity analyses, including the Cochran *Q*-test, maximum

likelihood estimate method and MR-PRESSO analysis. Previous studies speculated that childhood nutrition and height-related biological pathways could be associated with the risk of AD (Beeri et al., 2005; Wood et al., 2014). Although the underlying mechanisms of this association are unknown, we speculate that taller people secrete more growth hormone, which could stimulate cell division and growth, thus delaying the process of neurodegeneration. Moreover, recent studies suggest that adult height may be related to the risk of cancer (Choi et al., 2019), and cancer diagnosis has been proven to have an inverse effect on AD (Karanth et al., 2022). Its underlying pathway is still unclear. Further investigation may help explain the possible mechanism by which adult height is inversely associated with AD.

Study Strengths

Our study had several strengths. First, the design of MR analysis minimizes residual confounding and avoids bias from reverse causation. Second, we calculated and selected SNPs with F statistics greater than 10 as valid instrumental variables to exclude the effect of weak instrument bias. Then, considering AD typically occurs in old age, participants need to survive AD exposure and competing risk to be recruited, resulting in a selection bias (Gkatzionis and Burgess, 2019). We used the largest GWAS data, including clinically diagnosed AD and AD-by-proxy participants, to minimize the impact of selection bias. Finally, we used the MVMR method to control for confounder bias from biomarkers and height in order to form robust and reliable conclusions. We also performed substantial sensitivity analyses to validate the MR model assumptions.

Study Limitations

This study and MR analysis also had several limitations. First, only individuals of European descent were included which may restrict our ability to generalize the results. Second, genetic variables can affect an individual over the course of their lifetime. Thus, it is difficult to determine how short intervals of higher fibrinogen levels might influence the risk of developing AD, PD and LBD. Finally, pleiotropic effects amount the genetic instruments could result in deviated effect estimates; therefore, we performed a large quantity sensitivity analysis to minimize their impact.

In summary, the current MR study indicated that plasma fibrinogen levels might be associated with the risk of AD via increasing CRP levels. However, evidence from the current univariable MR and MVMR analysis did not support the presence of a causal association between plasma fibrinogen and γ-fibrinogen levels and PD and LBD. It suggests that fibrinogen may be involved in the inflammation pathway, which could relate to the pathogenesis of AD. Furthermore, our finding suggested that higher genetically determined height levels were inversely associated with an increased risk of AD. This signifies the possible role of height levels in the risk of developing AD under certain conditions. Further studies of underlying biological associations between fibrinogen and height with NDs would provide more insight into the etiology of NDs.

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

Our analysis is a secondary analysis of published study or publicly available GWAS summary data. Ethics approval and consent statement for each GWAS in this study can be found in the original publications.

AUTHOR CONTRIBUTIONS

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

ACKNOWLEDGMENTS

Data for plasma fibrinogen and γ-fibrinogen concentration were obtained from published studies by Sabater-Lleal et al. (2013) and Maners et al. (2020). Summary statistics for the genetic associations with AD, PD, LBD, fibrinogen, γ-fibrinogen, CRP and height were obtained from GWAS, respectively by Berndt et al. (2013), Petersen et al. (2018), Sun et al. (2018), Jansen et al. (2019), Nalls et al. (2019), and Chia et al. (2021). We thank all investigators for sharing the data.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2022.847583/full#supplementary-material

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The relationship between mild cognitive impairment and postoperative delirium undergoing total knee arthroplasty: The PNDABLE study

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Background: Patients undergoing surgery are at a higher risk of developing postoperative delirium (POD) as a result of anesthesia and surgical procedures. This study examined the association between POD and mild cognitive impairment (MCI) and whether MCI influences POD through the core pathology of POD.

Methods: We enrolled Chinese Han patients undergoing unilateral total knee arthroplasty (aged 50–90, weighing 50–80 kg, and using ASAI-II), combined with epidural anesthesia between October 2020 and June 2021. All the participants were assessed using Winblad's criteria for diagnosing MCI on pre-operation and using the Confusion Assessment Method (CAM) and the Memorial Delirium Assessment Scale (MDAS) postoperative 1–7 days (or before discharge) for diagnosing POD by an anesthesiologist. Cerebrospinal fluid (CSF) biomarkers of POD were measured by enzyme-linked immunosorbent assay (ELISA). To examine the mechanism by which POD pathologies affect cognition, causal mediation analyses were performed.

Results: POD incidence was 20.2%, including 32.5% in the MCI group and 12.4% in the non-mild cognitive impairment (NMCI) group. The MCI and CSF levels of T-tau and P-tau were risk factors, and the CSF levels of $A\beta_{42}$, $A\beta_{42}$/ T-tau, and $A\beta_{42}$/ P-tau were protective factors in POD ($p < 0.05$). Part of the effects of MCI on cognition can be attributed to amyloid pathology and tau.

Conclusion: MCI may be a reasonably good prognostic factor in POD development. Overall, amyloid pathology and tau protein might partially mediate the influence of MCI on POD.

Clinical trial registration: www.clinicaltrials.gov, identifier: ChiCTR2000033439.

KEYWORDS

mild cognitive impairment, biomarkers, neurodegeneration, geriatric, postoperative delirium

Background

Postoperative delirium (POD) is a serious complication resulting from anesthesia and surgery (Safavynia and Goldstein, 2018). Patients suffering from POD experience temporary or permanent cognitive decline, deterioration in verbal comprehension, and difficulties adapting to social situations. POD primarily affects the aged (> 65 years old) (Ramaiah and Lam, 2009), and it leads to increased mortality rates and hospitalization lengths, in addition, other complications, such as Alzheimer's disease (AD) and consequent higher treatment expenses (Steinmetz et al., 2009). POD is common and of great clinical significance. However, the mechanisms behind POD remain largely unexplained with no reliable biomarkers have been identified to date.

Amyloid beta (Aβ), including $A\beta_{40}$ and $A\beta_{42}$, is a component of AD senile plaques. Abnormal accumulation of Aβ initiates the cascade reactions within nerve cells, such as synaptic damage, excessive phosphorylation of Tau protein, formation of neurofibrillary tangles, and eventual damage on neurons, resulting in neuronal death and memory and cognitive dysfunction (Plotkin and Cashman, 2020). The microtubule-associated protein Tau plays an important role in the structure and function of microtubules in neurons (Guo et al., 2017). Phosphorylated Tau protein does not bind to tubulin but inhibits and destroys the formation of microtubules and finally results in cognitive dysfunction (Li et al., 2007). A recent study has revealed that preoperative positive cerebrospinal fluid (CSF) Aβ and Tau may be associated with an increased risk of POD (Fong et al., 2021). In several studies of senior adults without a previous diagnosis of dementia, preoperative positive CSF Aβ, Tau, and phosphorylated Tau (P-tau) were indicated as the strongest independent predictors of POD after elective arthroplasty (Planel et al., 2007; Nakajima et al., 2015; Cunningham et al., 2019; Dutkiewicz et al., 2020). In the present, Aβ and P-tau have been widely accepted as contributing factors in the development of POD.

In clinical and laboratory settings, mild cognitive impairment (MCI), the transitional state between normal aging and dementia, is an essential diagnostic indicator. MCI is associated with elevated prevalence of dementia, and the progression rate varies from 10 to 15% annually (Wang et al., 2015; Xue et al., 2017). MCI has three specificities: some cases get worse and develop into AD; some cases remain at this transitional stage for a considerable time, while a small number of cases could resume normal cognitive ability after active treatment. A previous study has exhibited a decrease in the level of $A\beta_{42}$ in CSF with the aggravation of patients with MCI (Fjell and Walhovd, 2012). Due to shared neuropathological mechanisms between AD and POD, MCI has been linked with neurodegeneration, suggesting a possible correlation between MCI and POD through changes in CSF Aβ.

Therefore, we planned to conduct a prospective, observational cohort study to investigate the relationship of MCI with POD and CSF POD biomarkers in addition to better understand the role of CSF POD biomarkers in mitigating the effects of MCI on POD. The aforementioned analyses were based on the PNDABLE (Perioperative Neurocognitive Disorder and Biomarker Lifestyle) study.

Materials and methods

PNDABLE study

The PNDABLE aims to investigate the pathogenesis, risk factors, and biomarkers of perioperative neurocognitive disorders (PND) in the Han Chinese population from northern China. It is deployed to identify lifestyle factors that may affect the risk of PND in the non-demented Han Chinese population from northern China to provide a foundation for disease prevention and early diagnosis. This study has been registered in the Chinese Clinical Trial Registry (clinical registration No. ChiCTR2000033439) and approved by the Ethics Committee of Qingdao Municipal Hospital. All the patients or their legal representatives signed written consent before CSF sample collections.

Participants

The Han Chinese patients underwent unilateral total knee arthroplasty (no gender limitations, aged, 50∼90; weight, 50–80 kg, ASA I∼II) combined with epidural anesthesia at Qingdao Municipal Hospital from October 2020 to June 2021 were enrolled to the PNDABLE study. The exclusion criteria include: (1) the preoperative MMSE score < 24 points; (2) Drug or psychotropic substance abuse, as well as long-term use of steroid drugs and hormone drugs; (3) preoperative III–IV hepatic

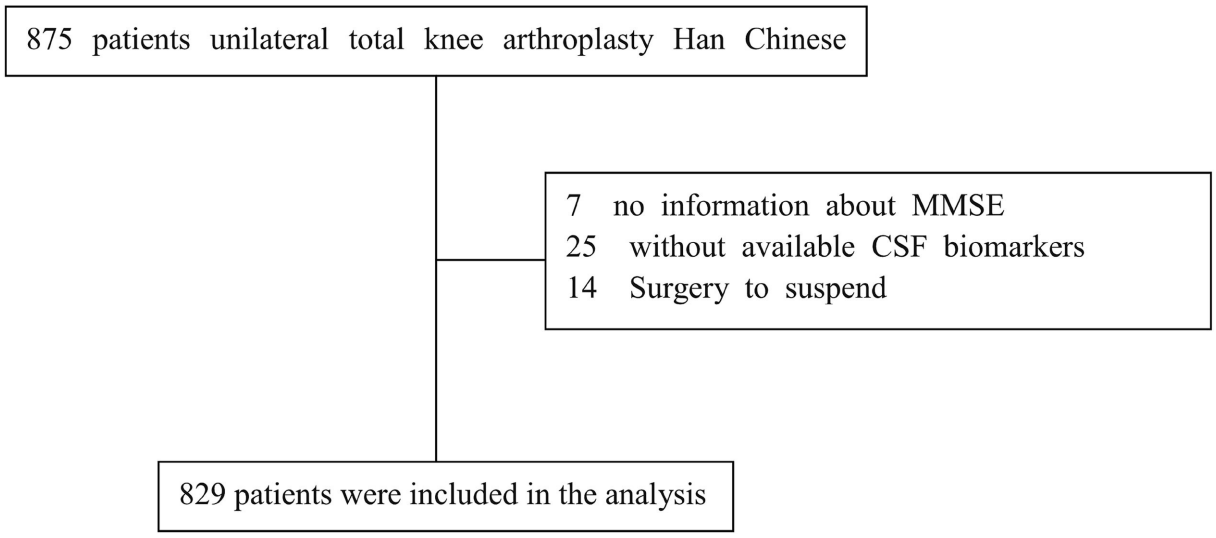


FIGURE 1
A flow diagram showed selection of eligible patients and the enrollment process.

encephalopathy; (4) Recent major surgery; (5) Severe visual and hearing impairments; (6) Abnormal coagulation function before surgery;(7) central nervous system infection, head trauma, multiple sclerosis, neurodegenerative diseases other than AD (e.g., epilepsy, Parkinson's disease), or other major neurological disorders; (8) major psychological disorders;(9) severe systemic diseases (e.g., malignant tumors) that may affect CSF or blood levels of AD biomarkers, including Aβ and tau; (10) family history of genetic diseases.

A total of 875 cognitively normal participants with available covariates from PNDABLE were included. The participants were divided into two groups based on MCI occurrence: the MCI group and the NMCI group. A patient recruitment flowchart is shown in Figure 1.

The participants were prohibited from drinking for 6 h and eating for 8 h before surgery. After entering the operating room, the participants underwent combined spinal-epidural block, AS-E/SIIepidural, and a lumbar puncture kit was used, with the space between lumbar 3–4 spinous processes (L_{3-4}) as the puncture point. With successful puncture, 2 ml of CSF was extracted from the subarachnoid space, followed by an injection of 2 to 2.5 ml of 0.66 percent ropivacaine (0.66%) in a duration of 30 s. During the surgery, to maintain blood pressure within +/− 20% of the baseline value, oxygen was inhaled by a mask at 5 L/min. A 5-mg injection of ephedrine was conducted intravenously in case intraoperative non-invasive blood pressure (NBP) was < 90 mmHg (1 mmHg = 0.133 kPa) or reduced by over 20% of the baseline value. If the heart rate (HR) < 50 beats/min, an injection of atropine 0.5 mg was given intravenously. Intravenous patient-controlled analgesia (butorphanol, 0.1 mg/ml + tropisetron, 50 g/ml, diluted with normal saline to a total volume of 100 ml) was used as acute postoperative pain management. The patients were sent to the anesthesia resuscitation room (PACU) after the operation.

The patients were interviewed the day before the surgery, in which their baseline information was collected, including age, gender, body mass index (BMI), ASA physical status, and educational background, etc. Other information about comorbidities and medical history was also gathered from the patients' medical records. As part of the procedure, the anesthesiologist compiled each patient's medical history, performed physical examination, and assessed each patient's cognitive abilities.

CSF biomarkers of POD measurements

CSF samples were processed immediately within 2 h of standard lumbar puncture. The samples were centrifuged at 2,000 × g for 10 min, and then separated and stored in an enzyme-free EP (Eppendorf) tube (AXYGEN; PCR-02-C) at −80°C under the international BIOMARKAPD project for further use in the subsequent steps of this study.

CSF biomarkers of POD were measured by ELISA using the microplate reader (Thermo Scientific Multiskan MK3). CSF biomarkers of POD measurements were done with other ELISA kits [$A\beta_{42}$ (BioVendor, Ghent, Belgium Lot: No. 296-64401), P-tau (BioVendor, Ghent, Belgium Lot: QY-PF9092), and T-tau (BioVendor, Ghent, Belgium Lot: No. EK-H12242)]. All ELISA measurements were performed by experienced technicians in strict accordance with the manufacturer's instructions.

Technicians were blinded to the clinical information. The samples and standards were measured in duplicate, which was also applied in the statistical analyses. In addition to the CSF sample, each plate also included blank and internal control samples. In case deviation occurred on the quality control sample, the plate would be reanalyzed. Standards and CSF samples were analyzed with duplicate specimens, and the mean value of both specimens was used for subsequent statistical analysis. All the antibodies and plates were from a single lot to exclude variability between batches. Moreover, the within-batch CV was <5%, and the inter-batch CV was <15%.

Diagnostic criteria for MCI

Winblad's criteria (Winblad et al., 2004) are applied to diagnose MCI. In this study, based on age and education-adjusted normative data, cognitive decline is defined as a deficit of minimum 1.5 SDs from premorbid performance on objective neuropsychological tests. The diagnosis requires both subjective and objective declines in cognition while retaining general functionality (i.e., only minimal change, if any, in basic and complex activities of daily living).

Neuropsychological tests

The preoperative cognitive status of the participants was assessed by neurologists using the Mini-Mental State Examination (MMSE). The participants with an MMSE score <24 points were excluded.

After the surgery, an anesthesiologist performed delirium assessments at 9:00–10:00 a.m. and 2:00–3:00 p.m. two times daily for 1–7 days (till the participant was discharged). Pain was assessed by the numerical rating scale (NRS) of 0–10 (lower scores indicate lower levels of pain) (Leung et al., 2008). POD definition is in accordance with the Confusion Assessment Method (CAM) (Chung et al., 2016), and severity of POD is measured by the Memorial Delirium Assessment Scale (MDAS) (Inouye et al., 1990). Modified Telephone Interview for Cognitive Status (TICS-m) was administered to assess cognitive function after 6 months. The quality of life was evaluated with the World Health Organization Quality of Life brief version (WHOQOLBREF).

Statistical analysis

The preliminary test in this study found that seven covariates were expected to enter the Logistic regression, the POD incidence was 10%, and the loss of follow-up rate was assumed to be 20%, so the required sample size was calculated to be 875 cases.

The Kolmogorov–Smirnov test is used to determine whether the measurement data conformed to normal distribution. Data conformed to normal distribution are expressed by mean ± standard deviation (SD), while the median (p25, p75) or a number (%) to express the data. The chi-square test (for categorical variables) and the Mann–Whitney U test (for continuous variables) are utilized to test the difference of baseline characteristics between the MCI and NMCI groups.

The first step was to evaluate the relationship between POD and MCI/CSF POD biomarkers. We used binary logistic regression analysis to analyze the association between POD and either MCI or CSF POD biomarkers. Additionally, we tested the relationships of MCI and CSF POD biomarkers, with consequent binary logistic regression analyses of MCI and CSF POD biomarkers performed. A two-factor ANOVA was deployed to determine whether gender affects the relationship between MCI and CSF POD biomarkers.

Second, sensitivity analyses were performed by adding more covariates, including BMI, type 2 diabetes (yes or no), hypertension (yes or no), smoking (yes or no), coronary heart disease (yes or no), and alcohol intake (yes or no) into multivariate logistic regression analysis.

Finally, an examination was conducted to determine whether the association between MCI and POD was mediated by CSF POD biomarkers by Stata MP 16.0 (Solvusoft Corporation, Inc., Chicago, Illinois, USA). Logistic regression models were fitted based on the methods. The first equation relates the mediator (CSF POD biomarkers) on the independent variable (MCI). The second equation relates the dependent variable (POD) on the independent variable (MCI). The third equation relates the dependent variable on both the independent variable and the mediator variable. Furthermore, the attenuation or indirect effect was also estimated, with the significance determined using 10,000 bootstrap iterations, where each path of the model was controlled for age, gender, years of education, and the MMSE score.

R software version 4.4.1 (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism version 7.00 (GraphPad Software, San Diego, CA) were used for statistical analyses and figure preparation. $P < 0.05$ was considered significant unless specifically noted.

Results

Comparison of demographic and clinical data of included participants

We found the incidence of POD is 20.2% ($n = 168$ of the 829 participants), respectively, 32.5% (105 of the 323 patients) in the

TABLE 1 Comparison of demographic and clinical data of unilateral total knee arthroplasty patients.

Variable	MCI ($N = 323$)	NMCI ($N = 506$)	*P*-value
Age (year)	65.98 ± 9.34	60.37 ± 9.02	0.001
Sex (female/male)	182/141	317/189	0.071
Body mass index (kg.m^{-2})	25.30 ± 4.17	25.35 ± 3.92	0.827
Education level (year)	10.19 ± 3.89	10.49 ± 3.72	0.258
ASA physical status (I/II)	222/101	341/165	0.687
Alcohol abuse, *n* (%)	105 (32.5)	175 (34.6)	0.537
Hypertension, *n* (%)	105(32.5)	184(36.4)	0.255
Dependence on smoking, *n* (%)	80 (24.8)	151 (29.8)	0.112
Coronary heart disease, *n* (%)	45 (13.9)	50 (9.88)	0.334
Diabetes, *n* (%)	55 (14.8)	82 (10.8)	0.755
Duration of anesthesia (min)	145.11 ± 14.56	144.57 ± 14.98	0.607
Duration of surgery (min)	121.28 ± 12.17	121.32 ± 12.08	0.964
Estimated volume of infusion (ml)	844.43 ± 92.07	849.51 ± 92.94	0.442
Estimated blood loss (ml)	121.16 ± 13.01	121.32 ± 13.41	0.827
Preoperative MMSE scores	28 (27, 29)	28 (27, 30)	0.300
Postoperative the highest MDAS score	9 (8, 16)	1 (1, 2)	<0.001
Postoperative the highest NRS score	3 (2–3)	3 (2–3)	0.482
Postoperative delirium, *n* (%)	105 (32.5)	63 (12.4)	<0.001
TICS-m score	36.84 ± 3.16	36.73 ± 3.11	0.619
WHOQOL-BREF score			
Physical domain	68.88 ± 3.56	69.08 ± 3.53	0.438
Psychological domain	75.45 ± 2.74	75.49 ± 2.75	0.830
Social relationships domain	67.41 ± 2.85	67.33 ± 2.89	0.727
Environment domain	83.20 ± 2.74	83.27 ± 2.76	0.705

POD, postoperative delirium; MMSE, mini-mental state examination; ASA, American Society of Anesthesiologists; MDAS, memorial delirium assessment scale; NRS, Numerical rating scale; SD, standard deviation; TICS-m, Telephone Interview for Cognitive Status; WHOQOL-BREF, World Health Organization Quality of Life -brief version.

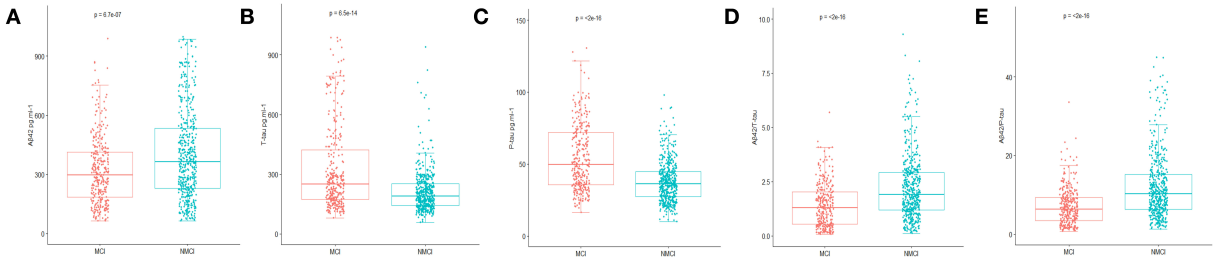


FIGURE 2
Distribution of CSF biomarkers levels for participants with and without MCI. **(A)** The concentrations of amyloid β_{42} ($A\beta_{42}$) decreased in MCI patients compared with NNMCI patients. **(B)** Total-tau (T-tau) increased in MCI patients compared with NNMCI patients. **(C)** Phosphorylated total-tau (P-tau) increased in MCI patients compared with NNMCI patients. **(D)** Amyloid β_{42}/ Total-tau ($A\beta_{42}$/ T-tau) decreased in MCI patients compared with NNMCI patients. **(E)** Amyloid β_{42}/ phosphorylated total-tau ($A\beta_{42}$/ P-tau) decreased in MCI patients compared with NNMCI patients. *P*-values were assessed by Student's *T*-test.

MCI group and 12.4% (63 of the 506 participants) in the NMCI group ($p < 0.05$).

In this study, the preoperative MMSE score showed no significant difference between the MCI group and the NMCI group. It is also unveiled that the participants in the MCI group demonstrate no cognitive function differences in the 6-month period. The demographic and clinical data of the participants are summarized, as shown in Table 1. The differences in

TABLE 2 Comparison of CSF biomarkers of unilateral total knee arthroplasty patients.

Variable	MCI ($N = 323$)	NMCI ($N = 506$)	*P*-value
Preoperative CSF$A\beta_{42}$ (pg/ml)	297.97 (183.75, 414.86)	364.53 (230.28, 534.03)	<0.001
Preoperative CSF T-tau (pg/ml)	250.73 (172.93, 423.06)	190.59 (143.04, 251.49)	<0.001
Preoperative CSF P-tau (pg/ml)	49.70 (35.75, 72.01)	36.30 (27.36, 44.69)	<0.001
Preoperative CSF$A\beta_{42}$/ T-tau	1.29 (0.54, 2.03)	1.90 (1.19, 2.93)	<0.001
Preoperative CSF$A\beta_{42}$/ P-tau	6.30 (3.45, 9.40)	10.22 (6.30, 15.15)	<0.001

TABLE 3.1 Logistic analysis for risk factors of POD patients.

	Unadjusted		Adjusted	
Factors of interest	**Odds ratio (95% CI)**	***P*-value**	**Odds ratio (95% CI)**	***P*-value**
MCI	3.387(2.382–4.815)	0.001	1.696(1.035–2.779)	0.036
Preoperative CSF$A\beta_{42}$	0.997(0.997–0.998)	0.001	0.998(0.997–0.999)	0.006
Preoperative CSF T-tau	1.006(1.005–1.007)	0.001	1.006(1.004–1.008)	0.001
Preoperative CSF P-tau	1.063(1.052–1.074)	0.001	1.060(1.044–1.075)	0.001
Preoperative CSF$A\beta_{42}$/ T-tau	0.393(0.316–0.489)	0.001	0.493(0.376–0.647)	0.001
Preoperative CSF$A\beta_{42}$/ P-tau	0.833(0.796–0.872)	0.001	0.867(0.827–0.910)	0.001

Adjusted by gender, age, years of education and MMSE for patients over the age of 50∼90.

CSF levels of $A\beta_{42}$, T-tau, P-tau $A\beta_{42}$/T-tau, and $A\beta_{42}$/P-tau of the MCI group are statistically significant compared with the NMCI group ($p < 0.05$), as shown in Figure 2 and Table 2.

Logistic regression analysis of the influencing factors of POD

In this study, logistic regression analysis results show that the participants with MCI are more prone to POD. MCI and CSF levels of T-tau and P-tau are risk factors of POD. However, the CSF levels of $A\beta_{42}$, $A\beta_{42}$/T-tau, and $A\beta_{42}$/P-tau are protective factors of POD by univariate analyses. Adding adjustments for age, gender, years of education, and MMSE scores, multivariate logistic regression analyses show results identical to the multivariate logistic regression analyses, as shown in Table 3.1. Sensitivity analyses also present the same results, as shown in Supplementary Table S1.

Logistic regression analysis of the influencing factors of MCI

With MCI as the dependent variable, logistic regression analyses were carried out to explore the influencing factors. The results show that the CSF levels of P-tau and T-tau are risk factors, and CSF levels of $A\beta_{42}$, $A\beta_{42}$/T-tau, and $A\beta_{42}$/P-tau are protective factors of MCI by univariate analyses. Adding adjustments for age, gender, years of education, and MMSE scores, multivariate logistic regression analyses demonstrate identical results, as shown in Table 3.2. Sensitivity analyses also show the same results, as in Supplementary Table S2. The relationship between MCI and biomarkers in CSF is not affected by gender, as shown in Supplementary Table S3.

Causal mediation analyses

It is discovered that the relationship between MCI and POD is mediated by $A\beta_{42}$, T-tau, P-tau $A\beta_{42}$/T-tau, and $A\beta_{42}$/P-tau. This effect is considered as partial mediation, with the mediation proportion varying from 10.77 to 78.45%, as shown in Figure 3.

Discussion

In the present study, the incidence of POD is 20.2%, which is consistent with the results in a previous study of 3.6–41% (Leung et al., 2013). Several studies have shown that 20% of patients experience POD after total knee and hip replacements under spinal anesthesia (Xie et al., 2014). Based on this study, MCI, CSF T-tau, and CSF P-tau are independently associated with POD. In addition, CSF $A\beta_{42}$, $A\beta_{42}$/T-tau, and $A\beta_{42}$/P-tau all act independently as protective factors of POD. In recent years, researchers have been focusing on finding ideal biological markers that indicate POD so as to reduce the occurrence of POD. The biochemical indicator, Aβ and Tau protein, all play an important role in the pathogenesis of POD (Li et al., 2014).

TABLE 3.2 Logistic analysis for risk factors of MCI patients.

Factors of interest	Unadjusted		Adjusted	
	Odds ratio (95% CI)	*P*-value	Odds ratio (95% CI)	*P*-value
Preoperative CSFAβ_{42}	0.998 (0.997–0.999)	0.001	0.998 (0.997–0.999)	0.001
Preoperative CSF T-tau	1.005 (1.004–1.006)	0.001	1.004 (1.003–1.005)	0.001
Preoperative CSF P-tau	1.051 (1.042–1.061)	0.001	1.046 (1.036–1.056)	0.001
Preoperative CSFAβ_{42}/ T-tau	0.572 (0.498–0.657)	0.001	0.609 (0.529–0.702)	0.001
Preoperative CSFAβ_{42}/ P-tau	0.873 (0.846–0.900)	0.001	0.885 (0.859–0.912)	0.001

Adjusted by gender, age, years of education and MMSE for patients over the age of 50∼90.

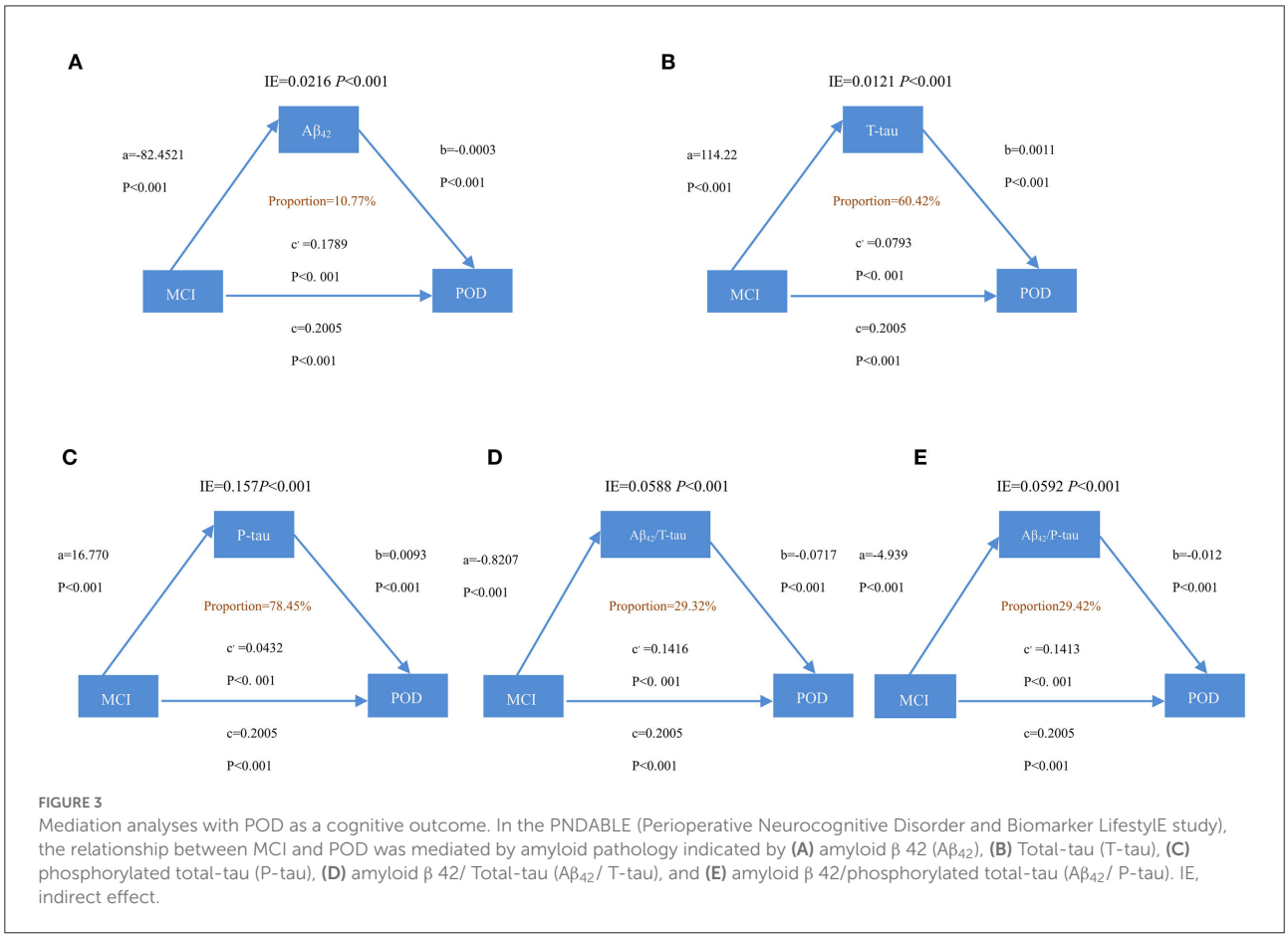


FIGURE 3
Mediation analyses with POD as a cognitive outcome. In the PNDABLE (Perioperative Neurocognitive Disorder and Biomarker LifestylE study), the relationship between MCI and POD was mediated by amyloid pathology indicated by **(A)** amyloid β 42 (Aβ_{42}), **(B)** Total-tau (T-tau), **(C)** phosphorylated total-tau (P-tau), **(D)** amyloid β 42/ Total-tau (Aβ_{42}/ T-tau), and **(E)** amyloid β 42/phosphorylated total-tau (Aβ_{42}/ P-tau). IE, indirect effect.

Evidence also suggests that the preoperative Aβ and Tau levels in cerebrospinal fluid are significantly related to the changes of postoperative cognitive dysfunction in elderly patients (Wu et al., 2018), which echoes with the present study. Results are not affected by adding more covariates or including patients of different ages (65–90).

As per the current study, the incidence of POD is 32.5% in the MCI group and 12.4% in the NMCI group. Also, changes in CSF Aβ_{42} and P-Tau may appear ahead of the symptoms of cognitive impairment on patients, and the above-mentioned changes in CSF biomarkers may also exist in patients with MCI (Sperling et al., 2011). Jack, Van et al. (Jack et al., 2013) conducted dynamic observation and found that the level of P-Tau in cerebrospinal fluid increases with the decline of cognitive function. These mentioned studies appear to support the assumption that P-Tau is associated with the severity of cognitive impairment, making it a valuable prognostic indicator. Two previous population-based studies examined risk of death among patients with MCI. Both reported that, in the follow-up period, MCI patients with CSF P-Tau have a mortality rate ∼1.7

times to that of those without cognitive impairment (Frisoni et al., 1999). Most studies were small scaled, with fewer than 100 subjects, each with mild cognitive impairment or no cognitive impairment, and did not adjust for variables associated with the development of cognitive dysfunction, e.g., age, gender, and educational background (Morris et al., 2001). To our knowledge, the current study is the first large sample report on the relative risk of POD and MCI. Our findings showed that CSF T-tau and CSF P-tau are independent risk factors of MCI. Moreover, CSF $A\beta_{42}$, $A\beta_{42}$/T-tau, and $A\beta_{42}$/P-tau are independent protective factors of MCI.

The study also discovered, for the first time, that the influence of MCI on POD is partially mediated by amyloid pathology and Tau. Based on the above findings, it could also be conceivably hypothesized that amyloid pathology and Tau could modulate the relationship of MCI and cognition impairment *via* mediation effects (the mediation proportion ranges from 10.77 to 78.45%). Normally, only Aβ can be detected in trace amounts in human brain tissue, yet, under specific pathological conditions, the clearance effect on Aβ by neurons to extracellular is reduced, and accumulated Aβ can stimulate nerve cells and glial cells (Salvadores et al., 2020). Accumulated Aβ also promotes the production of cytokines, which, in turn, transforms Aβ from soluble to insoluble and, eventually, into filaments deposited both intracellularly and extracellularly. Abnormal accumulation of Aβ initiates the cascade reactions within nerve cells, such as synaptic damage, excessive phosphorylation of Tau protein, formation of neurofibrillary tangles, and eventual damage on neurons, resulting in neuronal death and memory and cognitive dysfunction (Plotkin and Cashman, 2020). It has been noticed that accumulated Aβ protein in the brain could be high, while Aβ protein in CSF could be low. The possible mechanism behind this phenomenon remains unclear, and researchers have proposed that Aβ protein in the CSF is accumulated in the corresponding brain region, thereby reducing the amount of Aβ protein in the CSF (Zhang et al., 2018). Under normal physiological conditions, the human brain contains only 2–3 mol of phosphorylated Tau protein (P-Tau), and neuronal P-Tau is at a very low level. In case 3–4 times of P-Tau deposits exist in abnormal neurons around Aβ in the brain, it becomes abnormal phosphorylation of Tau protein (John and Reddy, 2021). Tau protein in normal human brains contains about two phosphoric acid molecules per molecule, but the number of phosphorylation sites of Tau protein in the brain of patients with cognitive impairment is increased, which is prone to induce phosphorylation (Didonna, 2020). Phosphorylated Tau protein does not bind to tubulin but inhibits and destroys the formation of microtubules (Li et al., 2007). Excessive Aβ causes a chain reaction, including increased production of phosphorylated Tau protein, which ultimately leads to decreased cognitive function (Ballatore et al., 2007). It has also been suggested that the aggregation of Aβ requires the presence of a certain microtubule-associated protein Tau (Roberson et al., 2007). To address that, a "two-channel-mechanism hypothesis" has been proposed (Small and Duff, 2008). According to this hypothesis, the same upstream mechanism triggers Aβ and Tau, respectively, and the developments of the two are independent and mutually promoting, and eventually lead to cognitive decline jointly. Studies have found that the content of $A\beta_{42}$ in cerebrospinal fluid decreases with the aggravation of MCI condition, showing a negative correlation between the severity of MCI and $A\beta_{42}$ levels (Jack et al., 2010). Phillip et al. found that P-tau in CSF is significantly associated with right hippocampus atrophy, indicating that P-tau protein may be involved in patients with MCI. The hippocampus is an organ related to human learning and memory, while the most common symptom of MCI is amnesia, which echoes the correlation between P-tau and MCI (Thomann et al., 2009). Therefore, one of the issues that emerge from these findings is that amyloid pathology and Tau may play important roles in the process of MCI-induced POD.

A number of limitations need to be noted regarding the present study. First, the number of the participants was limited. More eligible participants will be included in future studies. Second, this study is single-centered, which could be further validated by multicenter studies to come. Third, this study focused on the relationship between MCI and the biomarkers in patients' CSF with preliminarily discussions on the pathogenesis of POD caused by MCI, which depended on further research to prove the current findings *via* animal experiments and further probe into relevant mechanisms. Considerably, more work will need to be done to consolidate the results of the current study. With societies globally entering the state of population aging, despite the life quality at large of senior citizens has been improved greatly, MCI remains an ever-social issue. A reasonable approach to tackle this issue could be to enhance public knowledge of MCI and promote low cardiovascular and cerebrovascular risk life routines, especially among senior citizen groups. Proactive intervention on cardiovascular and cerebrovascular disease risk factors should also be implemented. Moreover, early detection and intervention are also vital as they can prevent or improve cognitive decline.

Conclusion

The evidence from this study suggests that MCI may be a sound prognostic factor of POD development. Additionally, in general, amyloid pathology and Tau protein might partially mediate the influence of MCI on POD.

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 Clinical Trial Ethics Committee of Qingdao Municipal Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

YB conceived the current study. JW, GZ, and CM performed the experiments. FW and XT analyzed data. XL, RD, and BW performed the experiments and wrote and revised the manuscript. All authors have contributed to the manuscript, revising and editing critically for important intellectual content and given final approval of the version and agreed to be accountable for all the aspects of the work presented here. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2022.959510/full#supplementary-material

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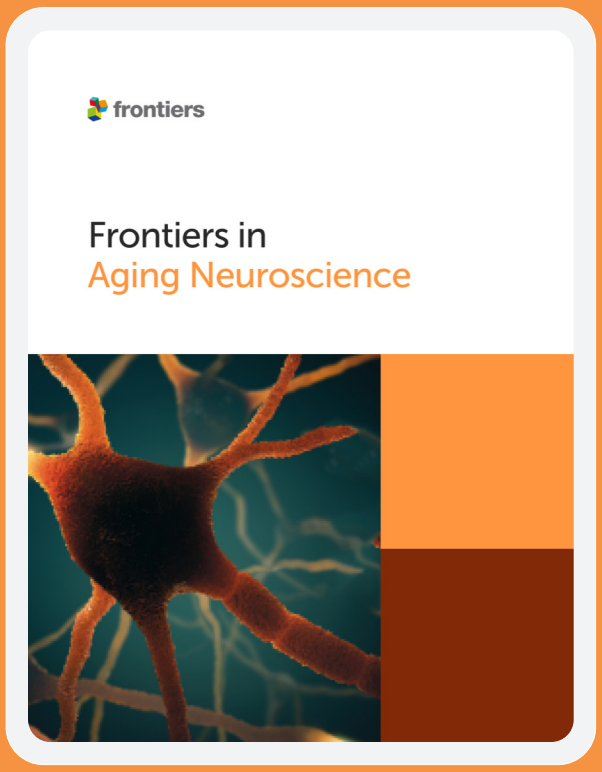
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