COGNITIVE AGEING: THE ROLE OF NUTRITION

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COGNITIVE AGEING: THE ROLE OF NUTRITION

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Evaluation of Effect of Ninjin'yoeito on Regional Brain Glucose Metabolism by ^{18}F-FDG Autoradiography With Insulin Loading in Aged Mice

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Introduction: A recent clinical study revealed that Ninjin'yoeito (NYT) may potentially improve cognitive outcome. However, the mechanism by which NYT exerts its effect on elderly patients remains unclear. The aim of this study is to evaluate the effect of Ninjin'yoeito on regional brain glucose metabolism by ^{18}F-FDG autoradiography with insulin loading in aged wild-type mice.

Materials and Methods: After 12 weeks of feeding NYT, mice were assigned to the control and insulin-loaded groups and received an intraperitoneal injection of human insulin (2 U/kg body weight) 30 min prior to ^{18}F-FDG injection. Ninety minutes after the injection, brain autoradiography was performed.

Results: After insulin loading, the ^{18}F-FDG accumulation showed negative changes in the cortex, striatum, thalamus, and hippocampus in the control group, whereas positive changes were observed in the NYT-treated group.

Conclusions: Ninjin'yoeito may potentially reduce insulin resistance in the brain regions in aged mice, thereby preventing age-related brain diseases.

Keywords: Ninjin'yoeito, glucose metabolism, 18F-FDG autoradiography, age, insulin loading

INTRODUCTION

With a global aging population, health issues caused by aging and age-related diseases have become inevitable challenges to all countries. Understanding the functional changes of organs that occur as a result of aging is essential to prevent age-related diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD) (1). In particular, the metabolism of glucose as an energy source has been regarded as a potential indicator for these body disorders.

Insulin resistance is one of the major underlying mechanisms in abnormal glucose tolerance (2). Accumulated evidence supports the idea that insulin resistance raises the risk of AD (3) and also correlates with the progression of PD (4). The aged brain has reduced insulin receptor expression levels, diminished insulin transport into the central nervous system, and may even experience insulin resistance (3–7). Therefore, reducing brain insulin resistance is one of the key points to prevent aging-related brain diseases.

Ninjin'yoeito (NYT, Ren-Shen-Yang-Rong-Tang) is a traditional Japanese medicine (Kampo medicine) and a multicomponent formulation composed of 12 crude drug extracts from ginseng, Japanese angelica roots, peony roots, *rehmannia* roots, *Atractylodes* rhizomes, *poria* sclerotium, cinnamon bark, *astragalus* root, *Citrus unshiu* peel, *polygala* roots, *schisandra* fruit, and *Glycyrrhiza*. NYT extract is approved by the Japanese Ministry of Health, Labor and Welfare as a Kampo medicine for the decline in physical strength, fatigue, anorexia, night sweats, cold extremities, and anemia (8). It is also used for individuals with deteriorating physical or psychiatric conditions, particularly among the elderly (9). Ninjin-to is composed of four medicinal herbs, *atractylodes* rhizome, ginseng, glycyrrhiza, and processed ginger, and has been reported to prevent the progression of diabetes mellitus in non-obese diabetic mice (10). Moreover, glucose intolerance in obese mice is alleviated by *astragalus* root through improvement of insulin resistance (11). Interestingly, NYT contains atractylodes rhizome, ginseng, and glycyrrhiza, which are components of Ninjin-to preventing the progression of diabetes (10). Furthermore, NYT also contains astragalus root, which improves insulin resistance (11). On the other hand, a recent clinical study revealed that NYT may potentially improve cognitive outcome and AD-related depression in patients with AD (8). Therefore, we hypothesize that NYT may potentially improve insulin resistance in the brain.

The glucose analog [^{18}F]-Fluoro-2-deoxy-2-D-glucose (^{18}F-FDG), a molecular imaging probe, is widely used in nuclear medicine for evaluating tissue glucose utilization and glucose metabolism (12–14). In this study, we attempted to clarify the effect of Ninjin'yoeito on regional brain glucose metabolism by ^{18}F-FDG autoradiography (ARG) with insulin loading in aged mice.

MATERIALS AND METHODS

Radiopharmaceutical and Reagent

NYT Extract

NYT is an herbal supplement composed of 12 crude drugs (15). The NYT extract we used was supplied by Tsumura & Co.

^{18}F-FDG

^{18}F-FDG supplied for clinical PET examinations, and synthesized by standard procedures was obtained from Fukushima Medical University Advanced Clinical Research Center Cyclotron Facility (Fukushima, Japan). The specific activity of ^{18}F-FDG was about 300 GBq/mmol.

Preparation of Animal Models

The entire experimental protocol was approved by the Laboratory Animal Care and Use Committee of Fukushima Medical University (Approval Number 30021) and performed in accordance with the Guidelines for Animal Experiments at Fukushima Medical University. Male C57BL/6J mice (72 weeks old) ($n = 24$) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). All mice were housed in a 12 h light/dark cycle at room temperature maintained at 23–25°C and relative humidity at 45–60%. Food and water were provided *ad libitum,* and the treatment and care of animals met all the criteria of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International (http://www.aaalac.org/) (16). The experimental protocol is shown in **Figure 1**. All mice were fed an original diet (CRF-6, ORIENTAL YEAST CO., LTD., Tokyo, Japan). After 12 weeks (84 weeks old), mice were randomly assigned to the control ($n = 12$) and treatment ($n = 12$) groups. The mice in the control group were continued on the original diet (100% CRF-6). The mice in the treatment group were fed the original diet mixed with NYT (97% CRF-6 mixed with 3% NYT, ORIENTAL YEAST CO., LTD., Tokyo, Japan). After 12 weeks (96 weeks old), all mice were fasted overnight and then both the control and treatment groups were further divided two subgroups without and with insulin loading (**Figure 1**).

Brain ^{18}F-FDG ARG Study

The four subgroups of mice ($n = 6$, each group) were the insulin- and non-insulin-loaded subgroups of the control and treatment groups. The mice in the insulin-loaded subgroups were intraperitoneally injected with human insulin (2 U/kg body weight, Eli Lilly & Co., Kobe) 30 min prior to ^{18}F-FDG injection. Each animal was initially anesthetized with 3–4% isoflurane in air and maintained *via* spontaneous ventilation with 2% isoflurane in air. ^{18}F-FDG (11.5 MBq/0.1 ml) was injected into the tail vein. Ninety minutes later, the animals were sacrificed; then their brains were rapidly removed, placed in Brain Matrix (Stoelting Co., USA) and cut into coronal slices (2 mm/slice), from which 9–10 coronal slices were obtained for exposure to a phosphor imaging plate (Fuji Imaging Plate BAS-SR 2025 for ^{18}F; Fuji Photo Film Co., Ltd., Tokyo, Japan) with a set of calibrated standards (17). This autoradiographic exposure was performed overnight to detect the distribution of ^{18}F-FDG. ARG images were analyzed using a computerized imaging analysis system (raytest, CR35, Version 2.1.0, Straubenhardt, Germany) with the image analysis software AIDA (Version 5.1 SP2, Straubenhardt, Germany). To determine brain radioactivity concentration, the cortex, striatum, thalamus, and hippocampus were defined using AIDA. The regions of interest (ROIs), namely, the cortex, striatum, thalamus, and hippocampus in the left and right hemispheres in all mice, were marked on the same anatomical plane with reference to the corresponding brain coronal slices (**Figure 2**). The radioactivity concentration in each ROI was determined per unit area, and the percentage of injected dose per pixel of the cortex, striatum, thalamus, and hippocampus was obtained and normalized to the animal weight [%ID/pixel/kg body weight (%ID/p/kg)]. Finally, the average of the left and

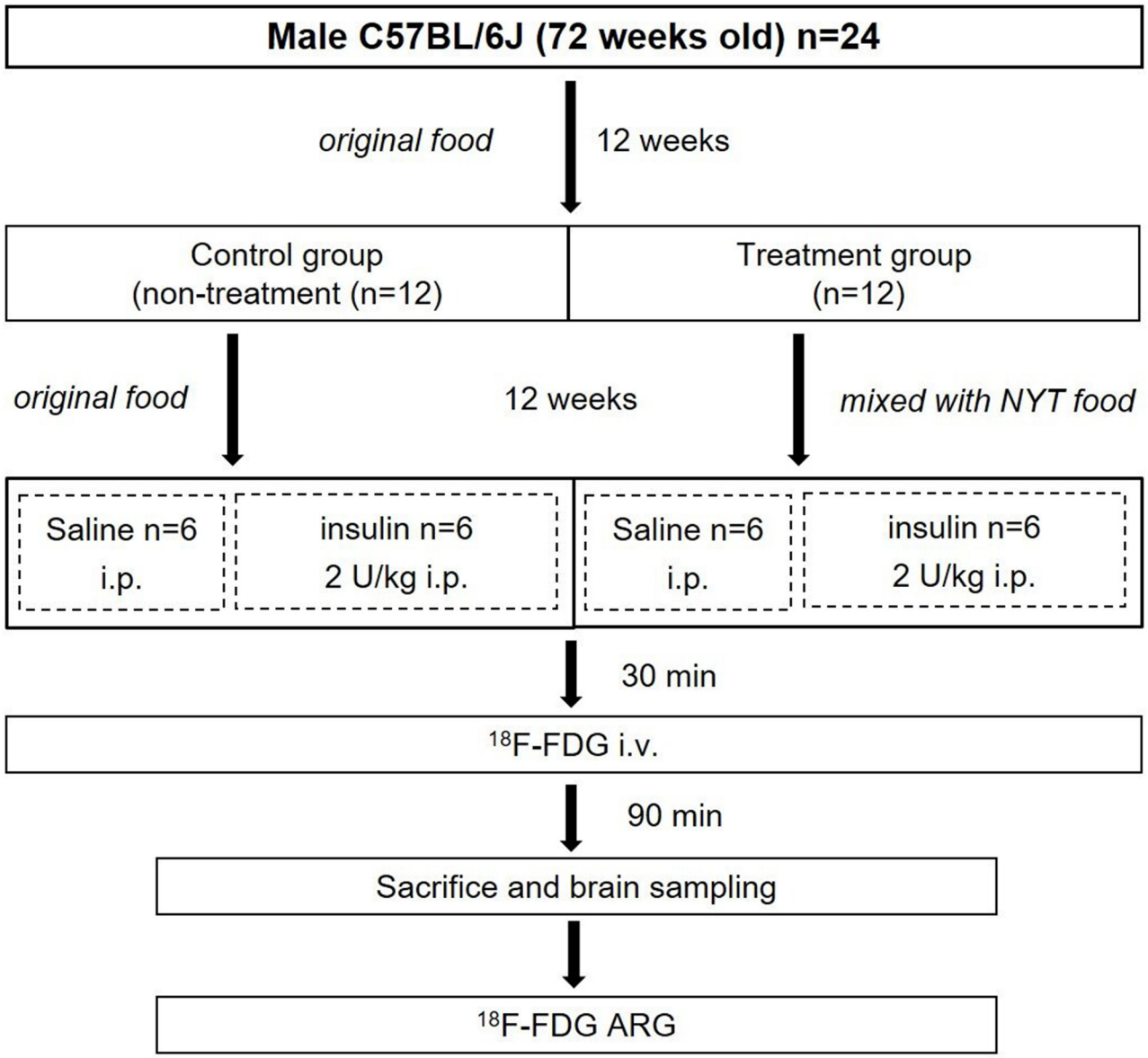

FIGURE 1 | Experimental protocol.

right values around each of the four regions was obtained. The rates of change in ^{18}F-FDG accumulation level before and after insulin loading were assessed using the following formula: [(^{18}F-FDG uptake level after insulin loading—^{18}F-FDG uptake level before insulin loading)/^{18}F-FDG uptake level before insulin loading] × 100%. Blood samples for glucose concentration measurement were obtained from all groups. When the blood glucose concentration decreased and displayed as "low" after insulin loading, we defined this blood glucose concentration as 20 mmol/dl which is the detection limit of blood glucose meter.

Statistical Analyses

All data are expressed as mean ± standard deviation. Statistical analyses were performed using the unpaired Student's *t*-test to evaluate the significance of differences between the control and treatment groups in body weight, blood glucose concentration, and ^{18}F-FDG distribution, as well as between non-insulin and insulin-loaded subgroups both in the control and treatment groups. Significance was assumed at $P < 0.05$.

RESULTS

Brain ^{18}F-FDG Autoradiographic Experiment in Control and Treatment Groups

The body weight and blood glucose concentration were determined in the control and treatment groups (**Table 1**).

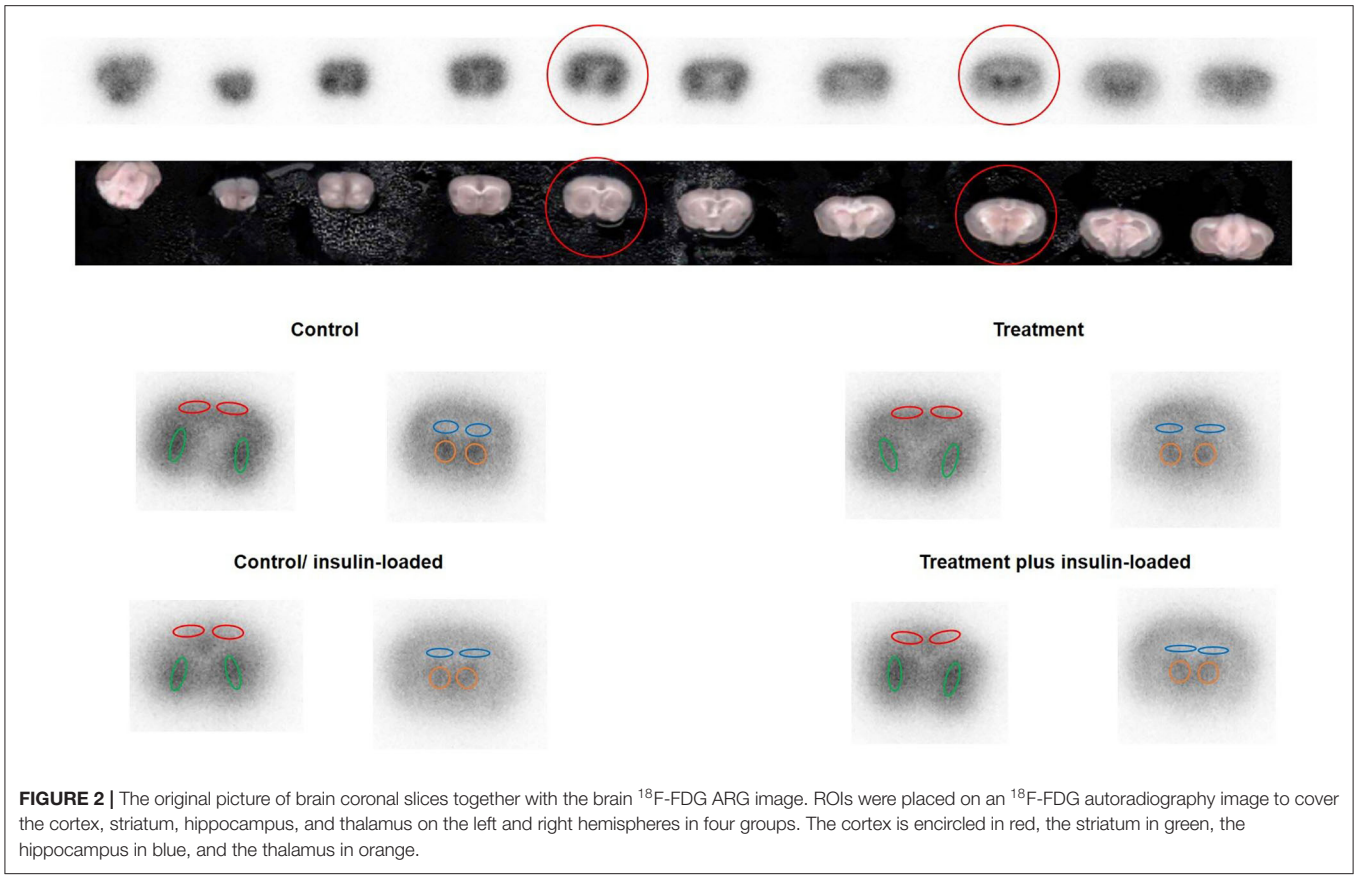

FIGURE 2 | The original picture of brain coronal slices together with the brain ^{18}F-FDG ARG image. ROIs were placed on an ^{18}F-FDG autoradiography image to cover the cortex, striatum, hippocampus, and thalamus on the left and right hemispheres in four groups. The cortex is encircled in red, the striatum in green, the hippocampus in blue, and the thalamus in orange.

TABLE 1 | Body weight (g) and blood glucose concentration (mg/dl) in brain ^{18}F-FDG autoradiography study.

	Control groups		Treatment groups	
	Without insulin ($n = 6$)	With insulin ($n = 6$)	Without insulin ($n = 6$)	With insulin ($n = 6$)
Body weight	35.1 ± 4.1	31.6 ± 3.5	32.4 ± 1.8	32.4 ± 3.0
Blood glucose	96.5 ± 9.5	27.2 ± 16.1****	90.8 ± 8.0	23.7 ± 6.2****

Data are shown in parentheses (mean ± SD).
Without insulin, no insulin-loaded groups; With insulin, insulin-loaded groups.
*****P < 0.0001 vs. value in with insulin-loaded group.*

TABLE 2 | ^{18}F-FDG accumulation in brain regions in mice (%ID/p/kg).

	Control groups		Treatment groups	
	Without insulin ($n = 6$)	With insulin ($n = 6$)	Without insulin ($n = 6$)	With insulin ($n = 6$)
Cortex	0.019 ± 0.002	0.016 ± 0.002*	0.017 ± 0.003	0.018 ± 0.002
Striatum	0.024 ± 0.004	0.024 ± 0.004	0.024 ± 0.004	0.028 ± 0.002#
Thalamus	0.023 ± 0.002	0.021 ± 0.005	0.021 ± 0.004	0.022 ± 0.003
Hippocampus	0.016 ± 0.002	0.016 ± 0.004	0.014 ± 0.002	0.017 ± 0.003

Data are shown in parentheses (mean ± SD).
Insulin, insulin-loaded group; Without insulin, no insulin-loaded groups.
**P < 0.05, vs. value in without insulin-loaded control group.*
#P < 0.05, vs. value in with insulin-loaded control group.

The levels of ^{18}F-FDG accumulation in brain regions were also determined in the control and treatment groups (**Table 2**). The body weight and blood glucose concentration were not significantly different between these two groups (**Table 1**). The levels of ^{18}F-FDG accumulation in the cortex, striatum, thalamus, and hippocampus were also not significantly different between these two groups (**Table 2**).

Changes in ^{18}F-FDG Accumulation Level Between Control and Control/Insulin-Loaded Groups

The body weight and blood glucose concentration were determined in the control and control/ insulin-loaded groups (**Table 1**). The levels of ^{18}F-FDG accumulation in brain regions were also determined in the control and control/insulin-loaded groups (**Table 2**). The body weight was not significantly different between these two groups (**Table 1**). After insulin loading, the blood glucose concentration significantly decreased ($P < 0.0001$) compared with that in the control group (**Table 1**). The level of ^{18}F-FDG accumulation in the cortex significantly decreased after insulin loading ($P < 0.05$; **Table 2**).

Changes in ^{18}F-FDG Accumulation Level Between Treatment and Treatment Plus Insulin-Loaded Groups

The body weight and blood glucose concentration were determined in the treatment and treatment plus insulin-loaded groups (**Table 1**). The levels of ^{18}F-FDG accumulation in brain

regions were determined in the treatment and treatment plus insulin-loaded groups (**Table 2**). The body weight was not significantly different between these two groups (**Table 1**). After insulin loading, the blood glucose concentration significantly decreased compared with that in the treatment group ($P < 0.0001$; **Table 1**). The levels of ^{18}F-FDG accumulation in the striatum and hippocampus tended to increase trend after insulin loading (**Table 2**).

Comparison of ^{18}F-FDG Accumulation Level Between Control and Treatment Groups After Insulin Loading

The body weight and blood glucose concentration were not significantly different between the control and treatment plus insulin-loaded groups (**Table 1**). The levels of ^{18}F-FDG accumulation in the cortex were higher in the treatment group than in the control group after insulin loading. The levels of ^{18}F-FDG accumulation in the striatum were significantly higher in the treatment group than in the control group after insulin loading ($P < 0.05$; **Table 2**).

Rates of Change in ^{18}F-FDG Accumulation Level Before and After Insulin Loading

The rates of change in ^{18}F-FDG accumulation level before and after insulin loading were assessed using the following formula: [(^{18}F-FDG uptake level after insulin loading—^{18}F-FDG uptake level before insulin loading)/^{18}F-FDG uptake level before insulin loading] × 100%. The ^{18}F-FDG accumulation showed negative changes in the cortex, striatum, thalamus, and hippocampus in the control group, whereas positive changes were observed in the treatment group (**Figure 3**).

DISCUSSION

To clarify the effect of Ninjin'yoeito on brain glucose metabolism, we examined and compared the ^{18}F-FDG accumulation in brain regions after NYT treatment with insulin loading in aged mice. The ARG method has also been widely used in the quantitative analysis of images of small animal models. ARG imaging plates have a much higher spatial resolution [the Fuji BAS-SR imaging plate is 25–100 μm (17)], than small-animal PET scanners and can visualize the distribution of the radiotracers, within small brain regions and improve the quantification of image analysis of the small brain regions. Therefore, we chose the ARG method to evaluate the effect of NYT on regional brain glucose metabolism in this study.

The highest concentration of insulin receptors was observed in neuronal cell bodies of the hippocampus and cerebral cortex (18, 19). Thalamic atrophy was found to start early in life and has a linear association with age; the same study showed that thalamic atrophy correlated with diminished performance on tests of processing speed (20). Effects of both deficient striatal neurogenesis and age-related neurodegeneration within the striatum accumulate, resulting in a progressive decline in the control functions of the basal ganglia, loss of dopaminergic neurons, and occurrence of PD clinical symptoms (21). On the basis of these studies, we chose the cortex, striatum, thalamus, and hippocampus in the aged brain for analysis in this study.

In this study, the blood glucose concentration and body weight were not significantly different between the control and treatment groups, and between the control and treatment plus insulin-loaded groups. The levels of ^{18}F-FDG accumulation in the cortex, striatum, thalamus, and hippocampus were not significantly different between the control and treatment groups. However, the level of ^{18}F-FDG accumulation in the cortex was higher in the treatment group than in the control group after insulin loading. Moreover, after insulin loading, the levels of ^{18}F-FDG accumulation in the striatum were significantly higher in the treatment group than in the control group. It can be seen from these data that after the insulin loading, the levels of ^{18}F-FDG accumulation were higher in the treatment group than in the control group, especially in the cortex and striatum.

On the other hand, in the control group, the level of ^{18}F-FDG accumulation in the cortex was significantly decreased after insulin loading, whereas no significant difference found in the treatment group. Insulin as a hormone secreted by pancreatic β-cells, which affects the peripheral system, has been well-characterized. Recent evidence has confirmed that active insulin could also be observed in the central nervous system. Despite the debate about whether insulin is synthesized in the adult brain, it is easily transported across the blood–brain barrier to the central nervous system through a process mediated by saturable receptors (22–24). Elevated peripheral insulin levels will sharply increase the brain and cerebrospinal fluid insulin levels, while prolonged peripheral hyperinsulinemia will downregulate the blood–brain barrier insulin receptors and inhibit the transport of insulin to the brain (25, 26). Insulin receptors are situated on the synapses of both neurons and astrocytes (27). Although insulin and insulin receptors are abundant in the brain, they are selectively distributed, with high concentrations in the cerebral cortex, hypothalamus, hippocampus, olfactory bulb, as well as in the amygdala and septum (23, 28–30). In the literature, it is widely accepted that aging is accompanied by an increase in insulin resistance (31, 32). Bingham et al. (13) demonstrated that glucose metabolism in the cerebral cortex increases significantly after treatment with low-dose insulin. The basis for insulin effects on glucose metabolism in reginal brains may be due to the distribution of glucose transporter isoforms (GLUTs) (33, 34). The insulin-sensitive GLUTs 4 and 8 are selectively distributed in the brain, and insulin enhances brain GLUT 4 expression and translocation (35). In rats, GLUT 4 is expressed in the cerebellum, sensorimotor cortex, hippocampus, pituitary, and hypothalamus (36–39) and GLUT 8 has been observed to be expressed in the hippocampus and hypothalamus (33).

In this study, after insulin loading, the brain ^{18}F-FDG uptake level in the cortex of aged mice did not increase but decreased in the control group. This result suggests that there was an obstacle in the cortical glucose metabolism in elderly mice. However, in the treatment group, although the ^{18}F-FDG uptake level in the cortex of aged mice did not increase, it did not decrease either. This may be explained by the fact that NYT can improve the insulin resistance of the cortex of aged mice. On the other hand, the ^{18}F-FDG uptake levels in the striatum

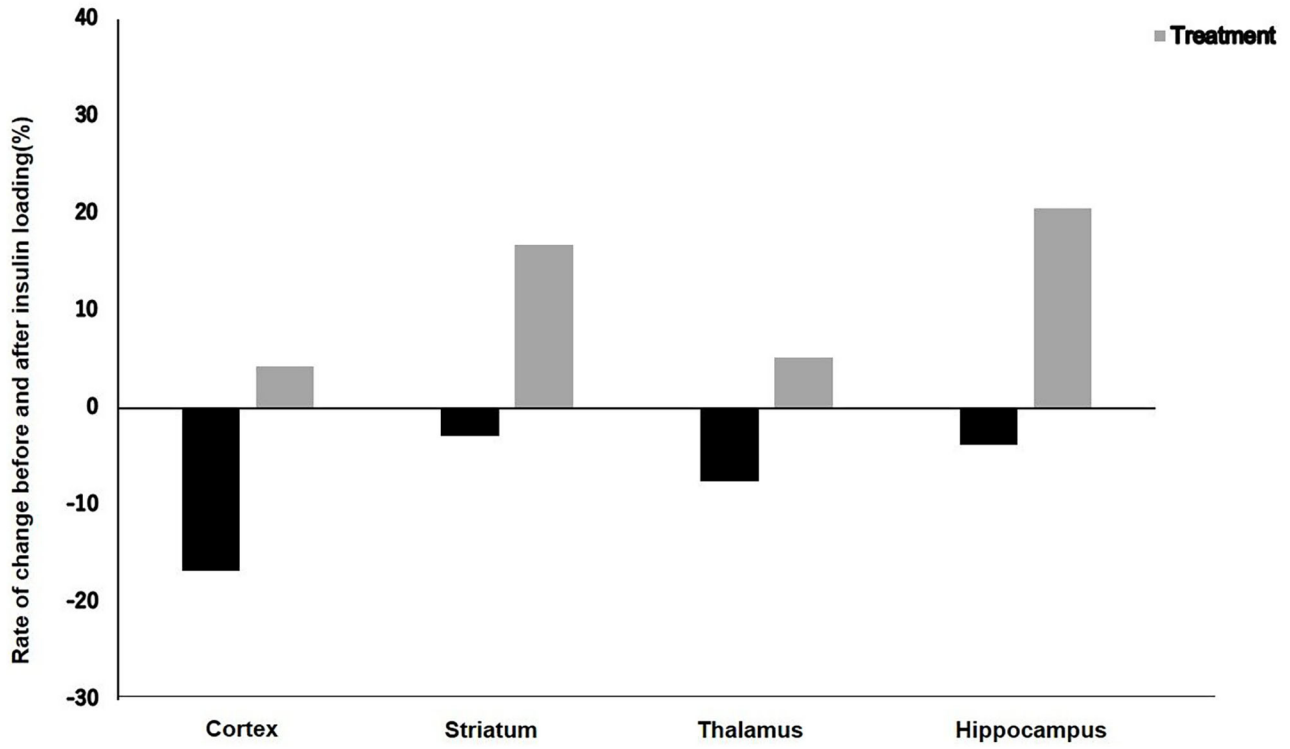

FIGURE 3 | Rate of change in ^{18}F-FDG uptake level before and after insulin loading in control and treatment groups.

and hippocampus of aged mice did not increase after insulin loading in the control group, whereas an increasing trend after insulin loading was observed in the treatment group. This result suggests that NYT may also improve the insulin resistance in the striatum and hippocampus. After insulin loading, the ^{18}F-FDG accumulation showed negative changes in the cortex, striatum, thalamus, and hippocampus in the control group. Previous studies ascribed the reduced ^{18}F-FDG uptake levels in tumors and inflammatory lesions with insulin-induced hypoglycemia to the effect of insulin, i.e., insulin shifts ^{18}F-FDG from the original area to insulin-sensitive organs (40, 41). This insulin effect may also explain the reduced ^{18}F-FDG accumulation level in the control group because of the decreased insulin sensitivity of the brain leading to the shift of ^{18}F-FDG to insulin-sensitive organs. However, in the treatment groups, the ^{18}F-FDG accumulation showed positive changes in the cortex, striatum, thalamus, and hippocampus after insulin loading. It may indicate that NYT may potentially reduce the insulin resistance in the brain regions of aged mice.

A study showed that insulin resistance in the periphery systems in patients with AD positively correlated with brain amyloid β-protein (Aβ) deposition in the frontal and temporal areas (42). Studies have shown that peripheral insulin resistance may precede the accumulation of Aβ in which midlife Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) predicted Aβ aggregation, as assessed by amyloid positron emission tomography 15 years after HOMA-IR was measured (43). In cognitively healthy adults, compared with Aβ-negative adults, peripheral insulin resistance is also associated with increased levels of Aβ accumulation within 2 years (44, 45). Brain insulin resistance impairs synaptic integrity, and tau and Aβ can also interfere with the actions of insulin at synapses (46). Insulin desensitization may be one of the base of PD disease progression. Clinical data demonstrate that around 8–30% of PD patients are diabetic, a significantly higher percentage than that of the age-matched control group (47–50). Previous studies have documented the importance of insulin signaling in the brain (51–53) and proved that insulin signaling is compromised in the brains of PD patients (54–56). Analogs of incretin hormones have been developed to improve insulin signaling in Type 2 diabetes (57, 58). These drugs enhance insulin release and insulin sensitivity. The antidiabetics in the class of incretin receptor agonists improve symptoms and brain pathology in AD and PD animal models, as well as glucose utilization in AD patients and clinical symptoms in PD patients after their systemic administration (59). The treatment to reduce brain insulin resistance is considered for the treatment of AD and PD.

Hosogi et al. found that NYT improved the serum glucose levels and insulin resistance in STZ-induced diabetic mice (60). This improvement by NYT might be due to the alleviation of

interstitial fluid acidification through the increased expression of SMCT1 in the proximal colon leading to the absorption of butyrate, a pH buffer, *via* epithelial cells of the proximal colon (60). The studies reported by Gonçalves and Martel (61) and Gao et al. (62) revealed the mechanism of NYT-induced improvement of insulin resistance: SMCT1 transports butyrate (61), and butyrate intake prevents insulin resistance in high-fat-diet-fed mice (62). These reports (61, 62) suggest that the elevation of SMCT1 expression prevents the occurrence of insulin resistance by increasing the intake of butyrate. The report by Gao et al. (62) indicates that butyrate treatment improves insulin sensitivity by decreasing the levels of blood lipids such as triglycerides, cholesterol, and total fatty acids, which are as critical factors causing insulin resistance. Therefore, the data showing that NYT reduces local brain insulin resistance in aged mice may provide new options for the treatment of AD and PD.

Small-animal PET as a non-invasive, *in vivo* molecular imaging modality has been widely used for preclinical animal models in research facilities, which provide longitudinal investigation of the same subject and voxel-wise analysis. However, most dedicated small-animal PET scanners are limited by their relatively coarse spatial resolution [typically 1 to 2 mm full width at half-maximum (FWHM)] and therefore cannot reliably define specific regions within the mouse brain (63).

The small-animal PET system (Inveon, PET/SPECT/CT) with a spatial resolution of 1.63 mm was installed in our preclinical facility (64). Prior to this study, we have also evaluated the quantitative analysis of ^{18}F-FDG PET images of the mouse brain. However, owing to the poor spatial resolution of ^{18}F-FDG PET images, it was impossible to accurately define specific regions and set the ROIs at specific regions within the mouse brain. Yang et al. indicated that high-resolution prototype small-animal ^{18}F-FDG PET scanner images showed a much higher spatial resolution and a more detailed structure of the mouse brain (63). However, the dedicated small-animal ^{18}F-FDG PET scanner (Inveon) could not.

The limitations of our study were as follows. The number of animals per group was small. We only performed image analysis of the coronal sections of the mouse brain, and we did not perform image analysis of the sagittal sections. We consider that more accurate quantitative image information from different sections can be obtained if ARG image analysis of the sagittal sections is performed in this study.

CONCLUSION

In summary, Ninjin'yoeito may potentially reduce insulin resistance in the brain regions in aged mice, thereby preventing age-related brain diseases.

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 entire experimental protocols were approved by the Laboratory Animal Care and Use Committee of Fukushima Medical University (Approval Number 30021) and performed in accordance with the Guidelines for Animal Experiments at Fukushima Medical University.

AUTHOR CONTRIBUTIONS

JZ designed the study and wrote the manuscript. RI, NU, CT, and SS performed animal studies. YO performed bait prescription. KT performed radiolabeling and QC examination. HI and YM contributed to the interpretation of the results. GN, SZ, and KS critically revised the manuscript for important intellectual content. All authors have reviewed the manuscript.

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REVIEW
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Therapeutic Opportunities for Food Supplements in Neurodegenerative Disease and Depression

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Emerging evidence is showing nutrition as a crucial factor in the high prevalence and incidence of neurodegenerative mental disorders. Preventive interventions on neuroinflammation seem to be able to interfere with neurodegeneration. Supplementation of essential nutrients, such as long-chain-polyunsaturated fatty acids, vitamin E and mineral elements, may minimize inflammation, enhancing antioxidative defense, and lowering the risk and incidence of age-related diseases, such as cardiovascular diseases and neurodegenerative diseases. This manuscript reviews the current evidence on the role of neuroinflammation in the pathophysiology of neurodegenerative and mental disorders, and preventive strategies for food supplementation in these neuropsychiatric diseases. Dietary supplementation-based strategies have been demonstrated to be effective in subjects with mild cognitive impairment, while weaker results have been obtained in patients with advance neurodegenerative disease. Adjunctive supplementation has also been demonstrated to improve depression, this being of marked benefit considering the comorbidity between cognitive impairment/dementia and depression. Further research is needed to improve the prescriptive precision of supplementation in patients, and to better understand potential interactions with clinical and pharmacokinetic factors.

Keywords: brain health, depression, neuroinflammation, diet, gut-brain axis

INTRODUCTION

Cognitive impairment is a relevant manifestation of neurodegenerative diseases, which are a heterogeneous group of nervous system conditions, including Alzheimer's disease (AD), Parkinson's disease, Lewy body dementia, and vascular dementia (1). These disorders are characterized pathologically by the abnormal deposition of proteins throughout the brain and spinal cord; glial activation; increased neuroinflammation and changes in metabolic functions of the central and peripheral nervous system (1). Clinical manifestations of these conditions, such

as depression and dementia, afflict a growing population worldwide and represent a significant healthcare, social and public policy burden. As an example, over 50 million people live with dementia in the world today and this number is projected to increase to 152 million by 2050 (2). In fact, dementia is considered a mental health issue, with neuropsychiatric symptoms, including depression. The actual prevalence of this latter manifestation can be high in AD patients, with great variability of estimates across studies according to criteria, ranging from 13% (3) to up to 97% (4). Moreover, depression occurring in mid-life or across the lifespan is associated with an increased risk of dementia (5).

Brain vulnerability is influenced by both non-modifiable and modifiable risk factors, including age, family history and genetics on one hand, and preventable cardiovascular risk, obesity, diabetes, sleep apnea, physical activity, tobacco/alcohol/drugs, and stress, on the other hand (6, 7). Diet is one of the most important lifestyle-related factors which may impact on brain vulnerability. Emerging evidence is showing nutrition as a crucial factor in the high prevalence and incidence of mental disorders suggesting that diet is important to brain health as much as to the health of other physiological systems, such as the cardiovascular, endocrine, and digestive systems (8). Micronutrients, such as vitamins and trace mineral elements, are involved in every cellular/biochemical process and play important roles in the brain and heart function, immunological responses, and antioxidant defense systems. Low levels of micronutrients reduce the activity of antioxidant enzymes, which may lead to DNA, protein, and fatty acid oxidation and crosslinking, along with mitochondrial ATP depletion, therefore contributing to cardiovascular or neurodegenerative disorders (9). n-3 Polyunsaturated fatty acids are involved in the maintenance of cognitive functions, promoting adult neurogenesis and neuronal plasticity through the modulation of membrane remodeling, inflammatory mediators and oxidative stress (10).

Growing evidence shows that the release of pro-inflammatory cytokines and mediators in the nervous system is associated with persistent stress stimuli and may lead to neuronal dysfunction and death (11–14). Recent studies also highlight the role of the gut microbiota in many neurodegenerative diseases, as a "microbiota–gut–brain axis" would synchronize the gut with the central nervous system (CNS) and modify the behavior and brain immune homeostasis (15). Modulation of the gut microbiota may be a tractable strategy for developing novel strategies for complex CNS disorders (16). Supplementation of essential nutrients, such as long-chain polyunsaturated fatty acids (LC-PUFAs), vitamin E and mineral elements, may minimize inflammation and enhance antioxidative defense. Consequently, it may lead to lowering the risk and incidence of age-related diseases, including neurodegenerative diseases.

This manuscript reviews the current evidence on the role neuroinflammation in the pathophysiology of neurodegenerative and mental disorders, and nutrient based preventive strategies for food supplementation in these neuro-psychiatric dysfunctions.

INFLAMMATORY MECHANISMS IN NEURODEGENERATION AND DEPRESSION

Functional and cognitive impairments featuring dementia, in particular AD, vascular dementia and Parkinson's disease, have been associated with depression (17, 18). Concurrent preclinical and clinical evidence has suggested in recent years that in a subset of patients, inflammatory processes and decreased cerebral levels of neurotrophic factors might play some role in the complex pathogenesis of depression (19). Volume reduction of the hippocampus was also observed in AD and depressed patients, and this morphological alteration was related to stress exposure, known to impair the dendritic complexity of the neurons in the CA3 subfield of the hippocampus and affects neurogenesis in the dentate gyrus (20–22). Oxidative stress plays a role in the pathophysiology of depression in bipolar disorder, and high levels of lipid peroxidation in the blood correlates with decreased dentate gyrus volume (23). The notion that depression, as well as infection, encompasses symptoms, such as malaise, anhedonia, decreased social behavior, decreased motor activity, sleep abnormalities and fatigue, which are collectively referred as "sickness behavior," also suggests the involvement of inflammatory processes in depression development. Such symptoms are confirmed by acute occurrence of depressive symptoms in healthy volunteers administered with lipopolysaccharide (24). Stress syndrome—that is, elevated cortisol levels—has been observed in up to 70% of patients with depression, and also in AD pathology (25).

The role of inflammation in the pathogenesis of depression is supported by studies showing increased levels of proinflammatory cytokines, such as IL-1β, IL-6, IL-12, TNF-α, and prostaglandin E2 (PGE2), in patients with depression (26, 27), and by some experiments in preclinical models in which the administration of inflammatory cytokines promoted a depressive-like syndrome (28, 29). Though it should be noted that inflammation occurs in a sub-set of people with depression and is not always apparent via cytokine blood assays (30). Both depression and heart disease have been linked to the impairment of the cholinergic anti-inflammatory pathway and have been proposed as clinical manifestations of one underlying mechanism (31, 32).

Persistent stress stimuli have, however, been observed to induce the synthesis and the release of pro-inflammatory cytokines and chemokines, such as IL-1β, mainly by neurons and the macrophage–monocyte line, including microglia, resulting in increased expression of inducible nitric oxide synthase and cyclooxygenase. Hydrogen peroxide acts as a cell-to-cell messenger, in the process of inflammatory responses induced by oxidative stress (33). In addition, neurons and glial cells can produce proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α (11, 14, 34, 35).

One further mechanism of depression is a deficit of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), which leads to altered synaptic plasticity and then to neuronal dysfunction and cell death (18, 36). Inadequate supply

of trophic factors also affects neurogenesis, reducing the number of neuronal stem cells able to multiply and differentiate. Several studies on stressed rodents and on patients with depression detected decreased levels of BDNF, together with abnormalities in the levels of neurotransmitters and neuroendocrine dysfunction (18). In humans, it was also reported that BDNF decreases with age and that higher levels of BDNF correlate with better cognitive performance in older adults (36).

The Role of Microglia

It has been observed that morphological and functional alterations of the microglia appear in adults suffering from depression. Such findings were also replicated in numerous experimental animal models subjected to chronic stress where high levels of proinflammatory cytokines were found to be secreted mainly by activated microglia within the brain. Moreover, an elevated level of microglia activation was detected in individuals with depression who had committed suicide (37).

A microglial signature dependent on TGF-β signaling was demonstrated and was found to be activated by any type of pathologic event or change in brain homeostasis (38). Microglia can strongly influence the pathologic outcome or response to a stressor due to the release of a plethora of substances, including cytokines, chemokines and growth factors. A diversity of microglia phenotypes has been described in response to health, aging, disease, and lifestyle (39). Consequently, the activation of microglia with release of cytokines and neuroinflammation takes place in some individuals and not in others (40). Briefly, catecholamines released during stress bind to receptors expressed by innate immune cells, this binding induces the synthesis and release of inflammatory cytokines that stimulate the production of corticotropin-releasing factor (CRF) by the hypothalamic paraventricular neurons. CRF transported by portal hypophyseal circulation targets the adenohypophysis leading to the release of adrenocorticotropic hormone (ACTH) that binds to adrenal glands to induce cortisol release. Cortisol has a double paradoxical effect: it has anti-inflammatory activity in the periphery and pro-inflammatory activity at the CNS level, where specific receptors are present within the hippocampus and amygdala. Stress conditions, as well as an exogenous application of glucocorticoids, can cause hippocampal neuronal damage and cognitive impairment (41–43).

RATIONALE FOR FOOD SUPPLEMENTATION TO CONTROL NEUROINFLAMMATION

Since food is one of the most important lifestyle factors accounting for mental health, one of the strategies currently being developed to treat depression, as well as AD, is to administer compounds with anti-inflammatory and antioxidant activity, capable of crossing the blood–brain barrier, and targeting cells and the molecular players of neuroinflammation. The relevance of this attempt is confirmed by evidence that treatment with anti-inflammatory drugs had beneficial effects on patients with major depression, although some studies failed to demonstrate that COX-2 inhibitors had an activity superior to placebo, most likely due to the fact that COX inhibitors do not affect the expression of pro-inflammatory cytokines and chemokines (44–47). As it is known that natural compounds present in plant-based foods, such as fruits (especially berries), display anti-inflammatory neuroprotective activity, nutritional protocols aiming at counteracting the progression of chronic diseases have been proposed (48–57). Interest has also focused on dietary patterns, such as feeding time and circadian rhythms, to increase availability of bioactive compounds capable of exerting both anti-inflammatory and antioxidant functions. The field of "nutritional psychiatry" aims to describe and understand the relationship between dietary factors and mental health disorders (10, 58).

n-3 LC-PUFAs (a.k.a. omega-3) are precursors of a series of lipid mediators, including resolvins, protectins and maresins, which are collectively termed "specialized pro-resolving mediators" (SPM), and which terminate inflammatory processes blocking polymorphonucleate migration and promoting macrophage M2 switch (59). Several preclinical and clinical studies have shown that n-3 LC-PUFAs are involved in the maintenance of cognitive functions acting on membrane remodeling, inflammation mediators and oxidative stress. They are considered beneficial for the CNS via the modulation of adult neurogenesis, synaptic and neuronal plasticity, and microglia activation (10). Docosahexaenoic acid (DHA) is a key structural component of membrane phospholipids in the brain and eicosapentaenoic acid (EPA) is a precursor of anti-inflammatory cytokines, an inhibitor of prostaglandins, thromboxanes and leukotrienes. In contrast, linoleic acid, a n-6 LC-PUFA (a.k.a. omega-6), is a precursor of the inflammatory mediator arachidonic acid (10). DHA is 250–300-times more abundant in brain tissue compared to EPA (60). It is known that EPA and DHA can cross the blood–brain barrier by diffusion and by blood-brain barrier transporters (61). The latter mechanisms could be defective in aged people or subjects with related genotypes, such as *APOE4*, suggesting higher requirements in population subgroups (62).

DHA intake has been related to lower rates of incident dementia. In populations with higher dietary intake of DHA and higher concentrations of plasma DHA there is a lower risk of cognitive impairment (61). In addition, peripheral blood macrophages obtained from patients with mild cognitive impairment (MCI) and AD, were unable to accomplish an effective phagocytosis for Aβ peptide (the main component of the amyloid plaques in AD), unlike macrophages obtained from healthy controls which could recover phagocytic functions via n-3-LC-PUFA. Recent studies of fish-derived omega-3 supplementation in patients with AD and MCI have shown polarization of *APOE3/E3* patients' macrophages to an intermediate M1–M2 phenotype that is optimal for Aβ peptide phagocytosis and the stabilization of cognitive decline (63, 64).

Moreover, DHA can increase neuronal membrane fluidity and decrease membrane peroxidation by reducing cholesterol levels in the cell membrane, which leads to reduced oxidative stress in the cerebral cortex and in the hippocampus (65, 66). DHA red blood cell content has been related with memory performance, as 2.4 g of EPA plus DHA/day significantly

improved red blood cell membrane EPA+DHA composition in older adults with memory impairment, and working memory and neuronal response were concomitantly improved (66). Other clinical trials showed different outcomes of the omega-3, such as EPA, docosapentaenoic acid (DPA), and DHA, supplementation, suggesting its effectiveness in the prevention of dementia although not in the treatment of overt dementia (65); this probably is in relation to the different genotypes of the subjects included in the trial (e.g., carriers of the *E4* allele) (67). Dietary supplementation of EPA, DPA, and DHA in aged rats restored reduced depolarization-induced transmitter release, by changing the membrane composition, while healthy human adults who received EPA supplementation have decreased serum proinflammatory cytokines levels (59, 68).

Great interest was also focused on the properties of some compounds to exert an antioxidant activity, such as vitamin E, and on the ability to interfere with one carbon metabolism by decreasing the quantities of homocysteine, such as the B vitamins (49). Administration of vitamin E at 2,000 IU/day was reported to slow functional decline in mild and mild to moderate AD, while it had no effect on subjects with MCI (69). Moreover, preclinical studies have reinforced the hypothesis of an antidepressant-like response of vitamin E, as the mechanisms underlying its effect seem to be related to the modulation of oxidative stress and neuroinflammation (70).

VARIABILITY IN RESPONSE TO NUTRACEUTICALS INTAKE: THE ROLE OF THE GUT–BRAIN AXIS AND THE MICROBIOME DURING AGING AND NEURODEGENERATIVE DISEASES

Although dietary supplements can be obtained and consumed without medical prescription, criteria should be necessary to identify patients affected with neurodegenerative diseases who would most benefit from such an approach. As an example, a meta-analysis of randomized controlled trials (RCT) found that higher intake of the omega-3 EPA and DHA improved specific cognitive domains in subjects with MCI without dementia but had no effects in healthy adults and those with AD (71). This suggests that people in the early stages of progression of cognitive decline and depression may benefit from treatment with omega-3, but that advanced disease is not susceptible to improvement. So, eligible patients need to be identified when such supplementation is proposed in a clinical setting. In this respect, a clinical trial carried out in elderly people with MCI demonstrated beneficial effect of B-vitamin treatment on brain atrophy rate of adults with MCI, only in subjects with high plasma n-3 LC-PUFAs (72). Of note, it has been observed that when the omega-3 fatty acids DHA and EPA concentrations are low, vitamin B treatment has no effect on cognitive decline in MCI, but when omega-3 levels are in the upper normal range, vitamin Bs interact to slow cognitive decline (73).

It is known that gut microbes are a relevant factor changing the effect of dietary supplements on the brain. The exposome represents all exogenous and endogenous environmental exposures of which microbiota, genes and lifestyle environment, may determine the metabolism associated with a specific phenotype. It is known that our microbiome is changing with growing age. Indeed, both cell culture-dependent and -independent studies show that the gut microbiota of older people differs from that of younger adults (74). There is no chronological threshold or age at which the composition of the microbiota suddenly alters; rather, changes occur gradually with time. O'Toole and Jeffery (75) showed that the gut microbiota of older people differs from that of younger adults and may modulate aging-related changes in innate immunity, sarcopenia and cognitive functions.

Accumulating evidence now indicates that the gut microbiota also communicates with the CNS, possibly through neural, endocrine and immune pathways, and thereby influences brain function and behavior (11). Thus, the emerging concept of a microbiota–gut–brain axis suggests that modulation of the gut microbiota may be a tractable strategy for developing novel therapeutics for complex CNS disorders (16).

The Human Microbiome Project and subsequent studies using next-generation sequencing technology have highlighted that thousands of different microbial species are present in the human gut, and that there has been a significant variability of taxa in the microbiota composition among people. Several factors (gestational age, mode of delivery, diet, sanitation, and antibiotic treatment) influence the bacterial community in the human gastrointestinal tract, and among these, dietary components, such as n-3 fatty acids, fibers and polyphenols, play a crucial role (76, 77).

A bidirectional communication between the gut and the brain occurs via the immune system, the vagus nerve, the enteric nervous system and microbial metabolites (78). Substances, including short-chain fatty acids (SCFAs), proteins and tryptophan metabolites, exchanged through the circulatory system, affect mood, cognition and other brain function parameters (15, 16).

It is important to consider that gut microbes can produce neurotransmitters, such as dopamine, 5-HT, GABA, and acetylcholine (16). These neurotransmitters may signal to the brain via the vagus nerve. In particular, gut microbes can stimulate immune cells to produce cytokines. These cytokines might target the brain via blood vessels of the circulatory system. In addition, gut microbes can produce metabolites, such as microbial fermentation end-products (SCFAs, e.g., butyrate) that can regulate the epigenetic synthesis of BDNF, for example (79, 80). These metabolites can potentially migrate to the brain via the blood vessels or may stimulate gut epithelial cells to produce neurotransmitters that activate the vagus nerve (16).

Activity of the gut–brain axis seems to be linked to certain genetic variations. One example is the apolipoprotein E4 (*APOE4*) genotype which has been associated with early cognitive decline and is the strongest prevalent genetic risk factor for AD. Apolipoprotein E is a transporter of cholesterol and it is present in the liver (80–90%), in the brain glia and macrophages. It was found to influence the structure and the function of the gut microbiome both in humans and mice (81, 82). This suggests that the gut–brain axis is a potential target to reduce

the impact of the *APOE4* allele on cognitive decline and for the prevention of AD (81). For example, fecal microbiota amplicon sequencing from age- and BMI-matched individuals revealed higher levels of *Prevotellaceae* in *APOE3/E3* carriers relative to other genotype subgroups, whereas higher levels of *Ruminococcaceae* were correlated with the *APOE2/E3* genotype relative to *APOE4* carriers. *Ruminococcaceae* are involved in the production of SCFAs, such that their depletion is causally linked to inflammation (83). These findings therefore suggest that these bacteria might contribute to the protective effects of *APOE2* and *APOE3* alleles against AD relative to the *APOE4* genotype (81).

As a confirmation of these concepts, it has been observed that fish oil intake works better in non-*APOE4* carriers. Huang et al. (84) highlighted that consumption of fatty fish was associated with a reduced risk of dementia and AD for those without the *APOE4* allele. Quinn et al. (85) performed a randomized, double-blind, placebo-controlled trial to determine that supplementation with DHA slows cognitive and functional decline in individuals with AD. Participants were randomly assigned to algal DHA at a dose of 2 g/day or to placebo for a duration of treatment of 18 months. Overall, supplementation with DHA compared with placebo did not slow the rate of cognitive and functional decline in patients with mild to moderate Alzheimer disease, but while there was no DHA treatment effect on any outcome measure in the *APOE4*-positive group, those receiving DHA in the *APOE4*-negative group had a significantly lower decline in mean change in Alzheimer's Disease Assessment Scale-Cognitive Subscale score over 18 months vs. placebo group.

More recently, Arellanes et al. (86) demonstrated that supplementation with DHA doses higher than 1 g per day may contribute to reducing certain biomarkers associated with dementia prevention. High doses of DHA are needed for adequate brain bioavailability and *APOE4* is associated with reduced delivery of DHA and EPA to the brain before the onset of cognitive impairment. A total of 33 individuals were provided with two vitamin B complex supplements per day (each containing 500 μg of vitamin B12, 50 mg of vitamin B6, and 400 μg of folic acid) and randomized to 2,152 mg of DHA per day or placebo over 6 months. There was a significant increase in cerebrospinal fluid (CSF) DHA in the DHA supplement arm compared with placebo. It was also observed that a trend for a greater increase in CSF DHA in *APOE4* non-carriers compared with carriers occurred (but the interaction between the treatment group and *APOE* group on the change of CSF DHA was not significant). CSF EPA levels were increased after DHA supplementation, with such results being in line with previous reports (87). However, change in CSF EPA levels in both groups was significantly greater in *APOE4* non-carriers compared to carriers.

As mentioned earlier, EPA and DHA are able to produce SPMs such as RvE1, RvE2, and RvD1, RvD2, respectively, that are involved in the resolution of inflammation (88). Martinsen et al. quantified cortical and hippocampal fatty acid, and phospholipid profiles along with select EPA- and DHA-derived SPMs in 2-, 9- and 18-month-old *APOE3* and *APOE4* male and female mice. A 10% lower cortical DHA was evident in *APOE4* females at 18 mo compared with 2 mo, with no significant decrease in *APOE3* or *APOE4* males. This decrease was associated with a reduction in DHA-phosphatidylethanolamine. In addition, although no sex***APOE* genotype interactions were observed for SPMs expressed as a ratio of their parent compound, higher cortical RvD3, neuroprotectin D1 (NPD1), maresin 1 (MaR1) were evident in females, and lower cortical 17R-resolvin D1, 10S, 17S-diHDHA, and 18-HEPE in *APOE4* (88).

Presented evidence suggests that precision medicine represents a future perspective for treatment and prevention of neurodegenerative diseases. Genetics, nutrigenetics, and pharmacogenetics have a major role in health maintenance and treatment of diseases. In particular, a genomics, transcriptomics, and epigenomics approach could be useful, and the potential advantages of a genotype-based personalized nutrition could facilitate early, personalized therapy, and improve motivation.

THERAPEUTIC OPPORTUNITIES FOR FOOD SUPPLEMENTS IN DEPRESSION: EVIDENCE, LIMITATIONS AND SUGGESTIONS FOR THE FUTURE

Evidence for the potential efficacy of several supplementation strategies in the prevention and treatment of neurodegenerative diseases has been obtained in clinical trials, although inconsistent results were produced. A systematic overview of all available top-tier meta-analyses of RCTs reported on the efficacy of nutrient supplements in patients with common and severe mental disorders (89). In particular, 33 unique meta-analyses with outcome data from placebo-controlled trials were included from a total of 10,951 participants. Results highlighted that omega-3 LC-PUFA, EPA at dose up to 4,400 mg/day (on average 1–2 g of EPA per day) in particular, is significantly effective in depression. Moreover, good evidence supported the effectiveness of high dose of methyl folate (15 mg/day) compared with folic acid and zinc as an adjunctive treatment in major depressive disorder (MDD) (89).

Nevertheless, some clinical trials failed to demonstrate the efficacy of LC-PUFA, either alone or associated with pharmacological or behavioral interventions, in patients with depression (90–93).

Unpromising results were also obtained by some authors researching the impact of the impact of LC-PUFA supplementation on cognitive functions (94–96). In some instances, clinical trials tested complex interventions and obtained data which may be difficult to interpret. As an example, Chew et al. evaluated the effects on cognitive function of oral supplementation with 1 g LC-PUFA and/or lutein 10 mg/zeaxanthin 2 mg in elderly subjects with age-related macular degeneration and failed to demonstrate efficacy of supplementation (97).

It is possible that multinutrient combinations may provide some advantage, with possible favorable synergistic interactions between components. For example, a recent 36-month, double-blind, placebo-controlled study in 311 participants with prodromal AD reported that a multinutrient intervention

containing DHA and EPA slowed decline of cognition, function, brain atrophy, and disease progression (98).

It has been observed that adjunctive use of nutraceuticals has the potential to modulate several key neurochemical pathways and can be used as an augmentation strategy to improve inadequate response to antidepressants. In this application, a systematic review and meta-analysis found primarily positive results for replicated studies testing adjunctive S-adenosyl methionine (SAMe), methyl folate, n-3 LC-PUFAs (EPA or ethyl-EPA), and vitamin D; positive isolated studies found efficacy of creatine and an amino acid formula; mixed results were found for zinc, folic acid, and vitamin C; and negative results were found for inositol (99).

Folate deficiency has been reported in approximately one-third of people suffering from depressive disorders (99). A combined folate, B12 and B6 dietary deficiency, induces hyperhomocysteinemia and imbalance of S-adenosylmethionine and S-adenosylhomocysteine, leading to an up-regulation of presenilin1 (PS1) and beta-secretase (BACE) and amyloid beta deposition, promoting progression to AD (100). Several studies tested folic acid adjunctively with antidepressants and most of these studies yielded positive results in regard to enhancing either antidepressant response rates or increasing response onset. The 5-methlytetrahydrofolate (dosage 400 μg to 15 mg) or folinic acid (400–800 μg) are considered safe and potentially effective forms (99).

Available data regarding the adjunctive use of n-3 LC-PUFAs in depression are in favor of n-3 compared with placebo. A recent network meta-analysis of 10 clinical trials including 910 patients, demonstrated that adjuvant supplementation with n-3 PUFAs was superior on MDD symptoms in comparison with placebo, and that high dose n-3 PUFAs (SMD: 0.908 ± 0.331; 95% CI: 0.262–1.581) were more effective than low dose n-3 PUFAs (SMD: 0.601 ± 0.286; 95% CI: 0.034–1.18) (101).

A recent key study, Mischoulon et al. (102), evaluated 196 patients with MDD who were administered with EPA 1 g/day or DHA 1 g/day or placebo for 8 weeks. Significant improvement in depression symptoms as measured by the 17 -item Hamilton Rating Scale (HAM-D-17), the Quick Inventory of Depressive Symptomatology–Self-report (QIDS-SR) and the Clinical Global Impression—Severity scale (CGI-S) was observed but neither active treatment reached statistical significance compared to placebo, with the response rates being 40–50% for each arm with a remission of ∼30%. The authors of the study concluded that neither EPA-enriched nor DHA-enriched n-3 was superior to placebo for the treatment of MDD, hypothesizing that baseline levels of inflammatory and metabolic markers, such as human C-reactive protein, IL-6, IL-1RA, leptin, and adiponectin could have an impact on response (102).

Rapaport et al. (103) explored in a *post-hoc* analysis of the above study, whether inflammatory biomarkers act as moderators of clinical response to omega-3 fatty acids in subjects with MDD. It has been observed that, overall, although treatment group differences were negligible (standardized treatment effect size, ES: −0.13–0.04), subjects with "high" inflammation improved more on EPA than placebo (ES: −0.39) or DHA (ES: −0.60) and less on DHA than placebo (ES: 0.21). Furthermore, difference between EPA and placebo effect increased with increasing numbers of markers of high inflammation. Therefore, the authors concluded that employing multiple markers of inflammation facilitated the identification of a more homogeneous cohort of subjects with MDD responding to EPA with an advantage over placebo.

A *post-hoc* analysis of an 8-week, double-blind, RCT ($n = 158$) investigated a combination of nutraceuticals comprising omega-3 (EPA 1 g/DHA 656 mg), SAMe, zinc, 5-hydroxytryptophan, folinic acid, and co-factors vs. placebo for the treatment of MDD. The study explored levels of PUFAs, folate, vitamin B12, zinc, homocysteine and BDNF as possible predictors and correlates of response to nutraceutical supplementation. It has been demonstrated that concentrations of EPA and DHA in red cell membranes increased in response to treatment and were significantly correlated with a decrease in depressive symptoms during active treatment ($p = 0.003$ and $p = 0.029$, respectively). Higher baseline levels of omega-3 fatty acid also correlated with depression reduction in the active treatment group ($p = 0.011$) (99). Therefore, changes in fatty acid levels resulting from a nutraceutical combination containing EPA and DHA provided a biomarker response in treating depression (99).

A subcommittee of the International Society for Nutritional Psychiatry Research organized an expert panel and developed a consensus-based practice guideline for clinical use of n-3 LC-PUFAs in MDD (104). Although a link between low omega-3 levels and depressive symptoms was acknowledged, evidence for supplementation as a prevention strategy was not sufficient. Stronger evidence was found for adjunctive supplementation with antidepressants, and acute use in the presence of obesity or inflammation was deemed as a potentially more effective treatment strategy. Evidence for efficacy of omega-3 supplementation in bipolar disorder was present, while evidence for use in children or elderly was not strong. Application in perinatal usage was based on weak evidence but omega-3 were indicated as safer than antidepressants (105). With respect to formulation and dosage, both pure EPA or an EPA/DHA combination of a ratio higher than 2 are considered effective, and the recommended dosages should be 1–2 g of net EPA daily, from either pure EPA or an EPA/DHA (>2:1) formula.

Current evidence most strongly supports a positive association between zinc deficiency and the risk of depression (106–108). Conversely, the relationship between magnesium and selenium deficiency and depression has not been fully understood. Several hypotheses have been advanced, and selenium and magnesium seem to be involved in the regulation of the hypothalamic–pituitary–adrenal axis, and of glutamate (109).

Owen et al. (110) measured plasma vitamin E (α-tocopherol) in 49 adults with major depression. It has been observed that subjects had significantly lower plasma vitamin E (4.71 ± 0.13 μmol/mmol cholesterol) than has previously been reported for healthy patients, and plasma vitamin E was inversely correlated to depression score. In addition, alpha tocopherol was found to be beneficial in mild to moderate AD by slowing decline of cognition (69).

In conclusion, good evidence supports the efficacy of adjunctive supplementation with EPA, vitamin D, and methyl

folate in patients with depression, and mixed results are available for zinc and vitamin C.

CONCLUSION

Neuroinflammatory mechanisms based on glial cell inflammatory processes and resulting neuronal dysfunction and impaired neurogenesis have been identified (106). Several neurodegenerative conditions have been linked to persistent oxidative stress, and pathological changes dependent on release of inflammatory mediators. In addition, the role of some nutrients, namely DHA and EPA among others we have not reviewed, in the regulation of microinflammation and central neurotransmission has been elucidated. Therefore, great interest has focused on diet and eating habits which may provide suitable levels of bioactive compounds capable of exerting both anti-inflammatory and antioxidant functions, and their therapeutic potential to prevent neurodegenerative diseases and one of their prodromes, clinical depression.

Dietary supplementation-based strategies have been demonstrated to be effective in people with depression and in those with MCI, while weaker results have been obtained in patients with advanced neurodegenerative disease.

When addressing prevention and treatment of neuroinflammation-dependent conditions by dietary supplementation, beyond the identification of required nutrients, it is necessary to evaluate substance metabolism, absorption and distribution, namely into the CNS, and the interaction with subject's feature including genetic variation and microbiota. The emerging concept of a microbiota–gut–brain axis suggests that modulation of the gut microbiota may be a tractable strategy for developing novel therapeutics for complex CNS disorders, and dietary habits play a crucial role in the selection of the bacterial community in the human gastrointestinal tract (77).

Future research ideally should focus on precision-based approaches tailoring supplementation based on nutrient deficiencies, neurochemical abnormalities or pharmacogenetic differences.

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

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Age-Dependent Association Between Elevated Homocysteine and Cognitive Impairment in a Post-stroke Population: A Prospective Study

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Background and Purpose: The results regarding the independent association between homocysteine (Hcy) levels and post-stroke cognitive impairment (PSCI) were inconsistent. The effect of age on this association has yet to be explored. This study aims to determine the relationship between Hcy levels, age, and cognitive impairment in a post-stroke population.

Methods: A total of 592 patients with acute ischemic stroke (AIS) completed follow-up. Serum Hcy levels were measured enzymatically by spectrophotometry within 24 h of admission. Cognitive function was evaluated by the Mini-Mental State Examination (MMSE) 1 month after stroke, and the scores $\leq$ 24 were considered as cognitive impairment. Our study was dichotomized into two groups by a cut-off of 65 years. Multivariate logistic regression models were used to determine the association between baseline Hcy levels and cognitive impairment.

Results: According to the MMSE score, 317 (53.5%) patients had cognitive impairment. Patients with higher levels of Hcy were more prone to have cognitive impairment 1 month after stroke than patients with lower levels of Hcy ($p < 0.001$). The optimal cut-off points of Hcy level (μmol/L) were (T1) $\leq$ 8, (T2) 8–12, and (T3) $\geq$ 12. After adjusting for confounding factors, the multivariate regression analysis showed that the third Hcy tertile was independently associated with cognitive impairment [odds ratio (OR) = 2.057, 95% confidence interval (CI) = 1.133–3.735, $p = 0.018$). A stronger association [T2 (OR = 2.266, 95% CI = 1.042–4.926, $p = 0.039$); T3 (OR =3.583, 95% CI = 1.456–8.818, $p = 0.005$)] was found in the younger group. However, the independent association was not confirmed in the older group.

Conclusions: Elevated Hcy levels in the acute phase of ischemic stroke were independently associated with cognitive impairment in a post-stroke population. Furthermore, the association was age-dependent and more meaningful in a younger population aged below 65. So, Hcy levels in patients with stroke should be well-monitored, especially in younger patients.

Keywords: homocysteine, age, ischemic stroke, cognitive impairment, Mini-Mental State Exam

INTRODUCTION

Currently, the prevalence of post-stroke cognitive impairment (PSCI) ranges from 20 to 80% among different countries, races, and diagnostic criteria (1), which is associated with functional outcomes and survival of stroke (2, 3) resulting in a tremendous clinical and economic burden on individuals and society. Moreover, cognitive impairment tends to progressively worsen following stroke (4), with 20–30% of the patients developing dementia (5). Hence, international guidelines recommend cognitive assessment as a routine neurological examination for all stroke survivors (6). Older age, poor educational status, female sex, and genetic factors are known conventional unamenable risk factors for PSCI (7). Hence, it is imperative to identify modifiable risk factors to guide medical treatments and reduce the prevalence of post-stroke dementia (PSD). Concentrations of homocysteine (Hcy) represent a modifiable risk factor that can be prevented and treated by vitamin supplementation. Therefore, understanding the prognostic impact of Hcy levels for PSCI is clinically relevant.

A growing body of evidence suggests that high Hcy levels may contribute to the pathogenesis of PSCI *via* oxidative stress (8), vascular inflammation (8), endothelial dysfunction (9, 10), and accelerate amyloid and tau protein accumulation (11). However, clinical studies investigating the association between Hcy levels and cognitive impairment in patients with stroke have yielded inconsistent results (12–16). The reasons for the contradictory results of previous studies remain equivocal. Age is one possible explanation for this discrepancy. Notably, Hcy concentrations were reported to increase with age (17, 18). Numerous studies have shown that age not only affects the relationship between Hcy and stroke (19–21) but also the relationship between Hcy and cognitive decline (22). For example, a nationwide study based on 12,683 patients with stroke suggested that the association between Hcy and stroke was strongest in younger individuals and declined linearly with increasing age (20). To the best of our knowledge, the effect of age on the association between Hcy and PSCI has yet to be explored. A traditional threshold of age at which people can be assumed to be "old," the age of 65 years, was linked with Alzheimer's disease (AD) (23) and PSD (4). To moderate the confounding effect of age, our study was dichotomized into two groups by a cut-off of 65 years.

Therefore, this study was designed to determine the relationship between Hcy levels, age, and cognitive impairment in a post-stroke population. In other words, we aimed to identify the association between Hcy levels and cognitive impairment in a post-stroke population and investigate the effect of age on the association.

METHOD

Study Design and Participants

From October 2013 to November 2019, patients with acute ischemic stroke (AIS) were consecutively recruited within 7 days of onset at the First Affiliated Hospital of Wenzhou Medical University. Eligible participants with AIS aged between 18 and 80 years were diagnosed using CT or MRI within 72 h of admission and were willing to support our work by completing the follow-up plans and cognitive assessments.

All the participants completed the detection of related indicators within 24 h of admissions, such as folate, vitamin B12, and Hcy. The Hcy levels were further divided into tertiles according to the number of patients and the distribution of the Hcy value, which makes the highest differences in this study. Furthermore, the Hcy tertiles were used to observe whether any enhanced performance could be quantified while maintaining the statistical efficacy in each category.

The exclusion criteria were as follows: (1) pre-existing transient ischemic attack or hemorrhagic stroke history, (2) intravenous thrombolytic therapy or interventional treatment received by the patient, (3) primary major cognitive impairment even dementia, (4) other central nervous system diseases, such as Parkinson disease and hydrocephalus, (5) serious mental illness, including pre-stroke depression and schizophrenia, (6) severe critical organ failure, especially severe kidney disease, (7) out of competence to complete cognitive assessments, such as coma, severe aphasia, dysarthria or hearing impairment, and (8) folate or vitamin B therapy within 2 weeks of admission or medication therapy that would affect Hcy levels. Ultimately, a total of 592 patients completed the follow-up and were included in this study.

This prospective cohort study was approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University and was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. All participants or their guardians understood our study well and signed written informed consent. For participants with less education or cognitive impairment, verbal informed consent was provided to them together with their guardians. Finally, with the full understanding and agreement of guardians, those participants who could not write signed a written informed consent using their thumbprints. Meanwhile, their guardians signed their names beside the thumbprints.

Data Collection

Baseline information was acquired through face-to-face interviews combined with electronic medical records and primary nursing records. A standardized questionnaire was designed to collect information on the demographic characteristics of trained neurological physicians. Demographic data included age, sex, body mass index (BMI), and years of education. Vascular risk factors included currently smoking, currently drinking, hypertension, diabetes mellitus, coronary artery disease (CAD), and atrial fibrillation (AF). Notably, blood pressure (BP) was recorded within 24 h after admission as systolic blood pressure (SBP) and diastolic blood pressure (DBP). Laboratory tests included Hcy, folate, vitamin B12, and estimated glomerular filtration rate (eGFR). The serum levels of Hcy, folate, and vitamin B12 were measured using an automatic biochemical analyzer (Beckman Olympus AU2700, USA) at the laboratory of our hospital. The concentrations were analyzed enzymatically using spectrophotometry with commercial reagents.

Other clinical data we collected included stroke severity, TOAST mechanism, and clinical depressive symptoms. However, this study lacked the Mini-Mental State Examination (MMSE) scores before the onset of stroke. The patients included in this study already had a stroke before admission to the Department of Neurology, so we were unable to collect the MMSE scores before the stroke. Although we excluded patients with mild cognitive impairment or dementia from this study, there were still potential confounders of pre-stroke cognitive impairment.

Stroke severity was assessed on admission by experienced neurologists using the National Institutes of Health Stroke Scale (NIHSS) (24). All the patients were investigated to clarify the stroke subtype according to the TOAST criteria (25). The severity of depression of patients was evaluated by Hamilton Depression Scale (HAMD-17) (26) within 7 days of onset, which was recorded as the HAMD score. According to the Diagnostic and Statistical Manual of Mental Disorders, Fifth, a HAMD score $\geq$ 7 combined with clinical manifestations indicated depressive symptoms.

A radiologist who was blinded to the clinical results performed the cranial CT or MRI on patients within 72 h after admission.

Outcome Assessment and Follow-Up

Experienced neurological physicians, blinded to the baseline characteristics of the patients, evaluated the cognitive function of the patients using the Chinese version of the MMSE (27) at 1 month after stroke. The MMSE has been translated into Chinese and was validated for reliability and validity as a screening tool for cognitive impairment in the Chinese stroke population (28). The MMSE scores range from 0 to 30, with higher scores indicating better performance. Considering the low education level of the patients, the MMSE scores $\leq$ 24 points indicated cognitive impairment (29–32).

Statistical Analyses

Categorical variables were shown as proportions, and group differences were analyzed using the chi-square test. According to the distribution of continuous data, mean $\pm$ SD was used to describe a normal distribution, and Student's *t*-test was used to assess the group differences. For skewed distribution, median (interquartile range, IQR) and Mann–Whitney *U*-test were implemented. According to the Hcy tertiles, all the patients were divided into three groups and the comparisons among the three tertiles were performed using the Kruskal–Wallis-test, one-way ANOVA, Pearson's chi-square-test, or Fisher's exact-test. Variables reflecting significant group differences ($p < 0.05$) were considered as confounding factors and were included in the univariate and multivariate logistic regression analyses. Model 1 included age, sex, and years of education. Model 2 was adjusted for age, sex, years of education, currently smoking, currently drinking, hypertension, AF, folate, eGFR, NIHSS score, TOAST mechanism, and HAMD score. Model 3 was adjusted for sex and years of education. Model 4 was adjusted for sex, years of education, currently smoking, currently drinking, hypertension, AF, folate, eGFR, NIHSS score, TOAST mechanism, and HAMD score. Two-tailed *p*-values < 0.05 were considered statistically significant, which were computed using IBM SPSS Statistics software Version 26 for Windows.

RESULT

Baseline Characteristics of Patients With AIS Grouped by Cognitive Impairment According to MMSE

During the research period, a total of 1,112 patients with AIS were enrolled in this prospective study, of which 705 patients were eligible for the study. Ultimately, 592 patients completed the 1-month follow-up and were included in this study. A total of 222 (37.5%) patients were female, and the median age of the enrolled patients was 64 years (range 30–80 years), and the median Hcy concentration was 10.0 μmol/L (range 3.1–38.0 μmol/L). In addition, 317 (53.5%) patients had cognitive impairment, which is similar to a number of previous research (1, 33).

Table 1 shows the baseline demographic, clinical, and laboratory characteristics of patients with and without cognitive impairment. Compared to patients without cognitive impairment, patients with cognitive impairment were more likely to be older and female. Also, they tended to have fewer years of education and higher NIHSS scores. Meanwhile, fewer cigarette smokers and alcohol drinkers were found in patients with cognitive impairment, but patients with a history of hypertension and AF were more likely to undergo cognitive impairment. Regarding laboratory parameters, patients with cognitive impairment had higher serum Hcy concentration, but lower serum folate concentrations and lower eGFR. Among patients with and without cognitive impairment, the median (IQR) values of Hcy were 10.9 μmol/L (8.0–13.0 μmol/L) and 9.5 μmol/L (4.0–12.0 μmol/L), respectively, and showed a significant difference ($p = 0.001$). Furthermore, patients with cognitive impairment had higher HAMD scores at baseline, reflecting a worse emotional status of these patients compared to those without cognitive impairment.

TABLE 1 | Baseline characteristics of patients with AIS grouped by cognitive impairment according to MMSE.

Variables	Total ($n = 592$)	MMSE > 24 ($n = 275$)	MMSE ≤ 24 ($n = 317$)	*P*-value
Age, year (median, IQR)	64.0 (57.0–70.0)	61.0 (51.0–67.0)	67.0 (60.0–73.0)	<0.001
Female, *n* (%)	222 (37.5%)	60 (21.8%)	162 (51.1%)	<0.001
BMI (median, IQR)	23.8 (22.0–26.1)	24.0 (22.1–26.3)	23.4 (21.6–26.0)	0.079
Education, year (median, IQR)	4.0 (0.0–6.0)	6.0 (4.0–9.0)	0.0 (0.0–5.0)	<0.001
Currently smoking, *n* (%)	274 (46.8%)	158 (58.3%)	116 (36.9%)	0.002
Currently drinking, *n* (%)	206 (36.1%)	111 (41.9%)	95 (31.0%)	0.007
Hypertension, *n* (%)	421 (71.1%)	184 (66.9%)	237 (74.8%)	0.035
Hyperlipidemia, *n* (%)	49 (8.3%)	27 (9.8%)	21 (6.7%)	0.170
Diabetes, *n* (%)	138 (23.5%)	63 (22.9%)	75 (24.0%)	0.764
CAD, *n* (%)	38 (6.5%)	18 (6.6%)	20 (6.4%)	0.902
AF, *n* (%)	47 (7.9%)	15 (5.5%)	32 (10.1%)	0.037
SBP, mmHg (mean ± SD)	150.0 ± 17.7	150.0 ± 17.9	149.9 ± 17.5	0.944
DBP, mmHg (mean ± SD)	82.7 ± 11.9	83.6 ± 11.0	81.9 ± 12.5	0.120
Hcy, μmol/L (median, IQR)	10.0 (4.7–12.6)	9.5 (4.0–12.0)	10.9 (8.0–13.0)	0.001
Folate, nmol/l (median, IQR)	5.8 (4.0–8.4)	6.6 (4.1–9.4)	5.2 (3.8–7.8)	0.002
B12, pmol/l (median, IQR)	352.0(253.0–488.0)	355.0 (265.3–495.3)	351.0 (244.5–478.0)	0.500
eGFR, ml/min/1.73 m^2 (mean ± SD)	92.7 (78.3–104.0)	95.0 (79.8–106.5)	90.1 (77.0–100.8)	0.001
NIHSS score (median, IQR)	1.0 (0.0–3.0)	1.0 (0.0–3.0)	1.5 (1.0–3.0)	0.001
TOAST mechanism				0.008
LAA, *n* (%)	474 (80.1%)	223 (81.1%)	251 (79.2%)	
CE, *n* (%)	58 (9.8%)	17 (6.2%)	41 (12.9%)	
SVO, *n* (%)	40 (6.8%)	21 (7.6%)	19 (6.0%)	
Others, *n* (%)	20 (3.4%)	14 (5.1%)	6 (1.9%)	
HAMD score (median, IQR)	4.0 (2.0–7.0)	4.0 (1.0–7.0)	4.0 (2.0–8.0)	0.016
Depressive symptoms, *n* (%)	139 (23.5%)	60 (21.8%)	79 (24.9%)	0.374

MMSE, Mini-Mental State Exam; BMI, body mass index; CAD, coronary artery disease; AF, atrial fibrillation; SBP, systolic blood pressure; DBP, diastolic blood pressure; HCY, Homocysteine; eGFR, estimated Glomerular Filtration Rate; NIHSS, National Institute of Health stroke scale; LAA, Large artery atherosclerosis; CE, Cardioaortic embolism; SVO, Small artery occlusion; Others, Other causes; HAMD, Hamilton Depression Scale.

Baseline Characteristics of Patients With AIS in Different Hcy Tertiles

Table 2 shows the baseline demographic, clinical, and laboratory characteristics of patients with AIS according to the Hcy tertiles. Levels of Hcy were ≤8 μmol/L in the tertile 1 and ≥12 μmol/L in the tertile 3. The incidence of cognitive impairment was significantly higher in the third Hcy tertile than in the first and second Hcy tertiles (29.7 vs. 52.0 and 62.2%, respectively, $p = 0.008$). The median (IQR) scores of the MMSE in the three groups were 25.0 (20.0–28.0), 24.0 (19.0–28.0), and 23.0 (18.0–27), respectively ($p = 0.005$). Patients with higher levels of Hcy had higher BMI, NIHSS scores, a higher percentage of smoking and AF and lower median age, percentage of females, levels of B12, levels of folate, and eGFR.

Association Between Hcy Levels and Cognitive Impairment

Table 3 shows the multivariate logistic analysis for the association between Hcy levels and cognitive impairment. In these regression models, the occurrence of cognitive impairment was considered as a dependent variable and the first Hcy tertile was used as a reference. After adjusting for potential confounders including age, sex, years of education, currently smoking, currently drinking, hypertension, AF, folate, eGFR, NIHSS score, TOAST mechanism, and HAMD score, the third Hcy tertile was independently associated with the prevalence of cognitive impairment [odds ratio (OR) = 2.057, 95% confidence interval (CI) =1.133–3.735, $p = 0.018$]. Moreover, age, years of education, NIHSS score, and cardioaortic embolism (CE) were significantly associated with cognitive impairment in the post-stroke population.

Baseline Characteristics of Patients With AIS Grouped by Age

Table 4 shows the baseline demographic, clinical, and laboratory characteristics of patients with AIS according to age. Compared with the older group (age ≥ 65 years), the younger age group (age < 65 years) had lower levels of Hcy [10.0 (5.0–13.0) vs. 11.0 (4.5–13.0), $p < 0.001$] and lower prevalence of cognitive impairment [126 (41.7%) vs. 191 (65.9%), $p < 0.001$]. The median (IQR) scores of the MMSE in the younger and older groups were 26.0 (21.0–28.0) and 22 (18.0–26.0), respectively ($p < 0.001$). Furthermore, the younger age group had a higher BMI, higher years of education, higher levels of B12, and higher eGFR but had lower DBP. As for the previous history of patients, patients in the older age group were more likely to have a history of

TABLE 2 | Baseline characteristics of patients with AIS in different HCY tertiles.

Variables	HCY tertiles			*P*-value
	Tertile 1 (*n* = 201)	Tertile 2 (*n* = 198)	Tertile 3 (*n* = 193)	
HCY, μmol/L	≤8	8–12	≥12	
MMSE ≤ 24, *n* (%)	94 (46.8%)	103 (52.0%)	120 (62.2%)	0.008
MMSE score (median, IQR)	25.0 (20.0–28.0)	24.0 (19.0–28.0)	23.0 (18.0–27.0)	0.005
Age, year (median, IQR)	63.0 (56.0–69.0)	63.0 (54.0–69.0)	67.0 (59.0–72.0)	0.001
Female, *n* (%)	83 (41.3%)	86 (43.4%)	53 (27.5%)	0.002
BMI (median, IQR)	23.8 (22.6–26.1)	24.2 (22.6–26.6)	23.4 (21.5–26.4)	0.040
Education, year (median, IQR)	4.0 (0.0–6.0)	4.0 (0.0–7.0)	4.0 (0.0–6.0)	0.869
Currently smoking, *n* (%)	92 (46.2%)	80 (40.8%)	102 (53.7%)	0.040
Currently drinking, *n* (%)	72 (36.9%)	66 (34.2%)	68 (37.2%)	0.799
Hypertension, *n* (%)	139 (69.2%)	142 (71.7%)	140 (72.5%)	0.740
Hyperlipidemia, *n* (%)	17 (8.5%)	18 (9.1%)	13 (6.8%)	0.705
Diabetes, *n* (%)	48 (24.0%)	51 (25.8%)	39 (20.5%)	0.467
CAD, *n* (%)	10 (5.1%)	13 (6.6%)	15 (7.9%)	0.515
AF, *n* (%)	8 (4.0%)	16 (8.1%)	23 (11.9%)	0.014
SBP, mmHg (mean ± SD)	149.8 ± 18.0	149.4 ± 17.6	150.8 ± 17.5	0.767
DBP, mmHg (mean ± SD)	81.9 ± 11.1	82.8 ± 12.1	83.4 ± 12.4	0.486
Hcy, μmol/L (median, IQR)	3.8 (3.4–6.2)	10.0 (9.0–11.0)	14.0 (12.1–16.2)	<0.001
Folate, nmol/l (median, IQR)	6.7 (4.6–9.2)	5.4 (3.4–8.2)	5.1 (3.8–8.0)	0.001
B12, pmol/l (median, IQR)	390.0 (265.0–528.0)	366.0(267.0–483.0)	309.0 (223.0–423.0)	0.003
eGFR, ml/min/1.73 m^2 (mean ± SD)	96.1 (87.0–104.9)	92.8 (80.4–105.3)	84.6 (71.2–99.5)	<0.001
NIHSS score (median, IQR)	1.0 (1.0–2.0)	1.0 (0.0–3.0)	2 (1.0–4.0)	<0.001
TOAST mechanism				0.341
LAA, *n* (%)	161 (80.1%)	160 (80.8%)	153 (79.3%)	
CE, *n* (%)	21 (10.4%)	16 (8.1%)	21 (10.9%)	
SVO, *n* (%)	9 (4.5%)	15 (7.6%)	16 (8.3%)	
Others, *n* (%)	10 (5.0%)	7 (3.5%)	3 (1.6%)	
HAMD score (median, IQR)	3.0 (1.0–7.0)	5.0 (2.0–7.8)	4.0 (2.0–7.0)	0.065
Depressive symptoms, *n* (%)	44 (21.9%)	50 (25.3%)	45 (23.3%)	0.689

MMSE, Mini-Mental State Exam; BMI, body mass index; CAD, coronary artery disease; AF, atrial fibrillation; SBP, systolic blood pressure; DBP, diastolic blood pressure; HCY, Homocysteine; eGFR, estimated Glomerular Filtration Rate; NIHSS, National Institute of Health stroke scale; LAA, Large artery atherosclerosis; CE, Cardioaortic embolism; SVO, Small artery occlusion; Others, Other causes; HAMD, Hamilton Depression Scale.

hypertension, CAD, and AF. Meanwhile, more cigarette smokers and alcohol drinkers were found in the younger age group.

Multivariate Adjusted OR for the Association Between Hcy Levels and Cognitive Impairment in the Subcategorized Groups of Age

Based on the Hcy tertiles, in the younger age group, 110 (36.4%) had lower Hcy levels and 78 (25.8%) had higher Hcy levels, while in the older age group, 91 (31.4%) had lower Hcy levels and 115 (39.7%) had higher Hcy levels (**Figure 1**).

Table 5 shows the association between Hcy levels and cognitive impairment in the different age groups. In univariate analyses, the third Hcy tertile was associated with cognitive impairment in the total group with an OR of 1.871 (95% CI = 1.252–2.796, p = 0.002) (**Table 3**). **Table 5** shows significant association between the third Hcy tertile and cognitive impairment with a higher risk of the younger age group (OR = 3.156, 95% CI = 1.506–6.613, p = 0.002) and the older age group (OR = 1.823, 95% CI = 1.161–3.504, p = 0.026). These differences remained significant after adjusting for sex and years of education. After further adjustments for the potential factors detected in the univariate analysis (Model 4: adjusting for sex, years of education, currently smoking, currently drinking, hypertension, AF, Folate, eGFR, NIHSS score, TOAST mechanism, and HAMD score), the association between the third Hcy tertile and cognitive impairment in the younger age group remained significant (Model 4: OR = 3.583, 95% CI = 1.456–8.818, p = 0.005). However, the association in the older age group disappeared (Model 4: OR = 1.273, 95% CI = 0.547–2.961, p = 0.576). Notably, in the younger age group, patients in the second Hcy tertile group were also associated with an increased risk of cognitive impairment with an OR of 2.625 (95% CI = 1.351–5.102, p = 0.004) in univariate analyses. These differences remained significant after adjusting for confounding factors and risk factors (Model 4: OR = 2.266, 95% CI = 1.042–4.926, p = 0.039).

TABLE 3 | Multivariate logistic analysis for the association between Hcy levels and cognitive impairment.

	Univariate analysis		Model 1[a]		Model 2[b]	
	OR (95%CI)	***p*-value**	**OR (95%CI)**	***p*-value**	**OR (95%CI)**	***p*-value**
Hcy tertiles						
Tertile 1	**Reference**		**Reference**		**Reference**	
Tertile 2	1.234 (0.833–1.828)	0.294	1.522 (0.928–2.495)	0.096	1.269 (0.720–2.234)	0.410
Tertile 3	1.871 (1.252–2.796)	0.002	2.633 (1.596–4.442)	<0.001	2.057 (1.133–3.735)	0.018
Age, years	1.073 (1.053–1.093)	<0.001	1.055 (1.032–1.079)	<0.001	1.057 (1.027–1.087)	<0.001
Gender, female	3.745 (2.610–5.374)	<0.001	2.224 (1.403–3.525)	0.001	2.092 (0.995–4.398)	0.052
Years of education	1.216 (1.110–1.333)	<0.001	0.720 (0.672–0.772)	<0.001	0.734 (0.677–0.797)	<0.001
Currently smoking	0.419 (0.300–0.585)	<0.001			0.835 (0.435–1.601)	0.587
Currently drinking	0.625 (0.443–0.881)	0.007			1.307 (0.755–2.264)	0.339
Hypertension	1.465 (1.025–2.093)	0.036			1.093 (0.649–1.841)	0.738
AF	1.946 (1.030–3.676)	0.040			0.885 (0.333–2.353)	0.807
Folate	0.514 (0.272–0.971)	0.040			0.978 (0.920–1.041)	0.484
eGFR	0.986 (0.977–0.994)	0.001			0.998 (0.985–1.012)	0.819
NIHSS score	1.142 (1.058–1.233)	0.001			1.160 (1.037–1.298)	0.009
TOAST mechanism						
LAA	**Reference**				**Reference**	
CE	2.143 (1.184–3.879)	0.012			2.378 (1.008–5.611)	0.048
SVO	0.804 (0.421–1.534)	0.508			1.165 (0.469–2.896)	0.742
Others	0.381 (0.144–1.008)	0.052			1.430 (0.363–5.636)	0.609
HAMD score	1.045 (1.005–1.086)	0.026			0.992 (0.937–1.049)	0.770

HCY, Homocysteine; AF, atrial fibrillation; eGFR, estimated Glomerular Filtration Rate; NIHSS, National Institute of Health stroke scale; LAA, Large artery atherosclerosis; CE, Cardioaortic embolism; SVO, Small artery occlusion; Others, Other causes; HAMD, Hamilton Depression Scale.

[a]*Model 1: adjusted for age, gender and years of education.*

[b]*Model 2: adjusted for variables in Model 1 plus currently smoking, currently drinking, hypertension, AF, folate, eGFR, NIHSS score, TOAST mechanism, and HAMD score.*

DISCUSSION

To our knowledge, this is the first study to discuss and analyze the relationship between Hcy levels, age, and cognitive impairment in a post-stroke population. Our study demonstrated that elevated Hcy levels in the acute phase of ischemic stroke were independently associated with cognitive impairment in the post-stroke population. In the comparison of different age groups (≥65 and <65 years), we found that elevated Hcy levels played a stronger role in the younger age group than in the older age group. After adjusting for the confounding factors, the association remained significant in the total group and the younger age group but disappeared in the older age group. The results indicated the age-dependent effect of Hcy on cognitive impairment in the post-stroke population. Of note, moderately raised serum Hcy (≥12 μmol/L in the total group and >8 μmol/L in the younger age group) increased the risk of 1-month cognitive impairment in the post-stroke population, as reflected by the MMSE scores.

In addition to age and years of education, the conventional factors of PSCI, we also found that higher NIHSS scores were independently associated with cognitive impairment in the post-stroke population, which has been proven in previous studies (34, 35). We also found that CE was associated with a higher prevalence of cognitive impairment in stroke survivors independently. In a previous study, PSCI was found to be common for all stroke subtypes (36), which was inconsistent with this study. The insufficient sample size of the CE might have imposed some biases in the results. In our study, large artery atherosclerosis accounted for a large proportion (80.1%), while CE accounted for only 9.8%. Therefore, further studies are needed to explore the relationship between the TOAST subtypes and PSCI. Moreover, fewer cigarette smokers were found in patients with cognitive impairment and moderately elevated Hcy levels (T2). However, previous studies showed that smoking was a risk factor for PSCI (4) and high Hcy levels (17, 18). The contrary results in this study may be attributed to the lower proportion of males among these patients.

This study found an independent association between Hcy levels and cognitive impairment in the post-stroke population. However, the results were contradictory, as mentioned earlier. One study conducted in 169 patients with stroke demonstrated that the linear relationship between Hcy levels and acute MMSE scores disappeared after adjusting for stroke subtypes (15). Another study failed to find a relationship between the plasma Hcy levels and the 27-month cognitive changes after stroke in 170 well-recovered elderly patients (16). The reasons for the contradictory results of previous studies remain unclear. One possible explanation for this discrepancy is age. The risk for AD increases rapidly with age, doubling every 5 years after the age of 65 (23). Similarly, a review of PSD revealed that age ≥ 65 years was a risk factor for PSD (4).To determine whether

TABLE 4 | Baseline characteristics of patients with AIS grouped by age.

Variables	Total ($n = 592$)	Younger age group (age < 65 y) ($n = 302$)	Older age group (age ≥ 65 y) ($n = 290$)	*P*-value
Age, year (median, IQR)	64 (57–70)	57 (50–61)	70 (67–74)	<0.001
Female, *n* (%)	222 (37.5%)	106 (35.1%)	116 (40.0%)	0.218
BMI (median, IQR)	23.8 (22.0–26.1)	24.2 (22.4–26.6)	23.1 (21.3–25.5)	<0.001
Education, year (median, IQR)	4.0 (0.0–6.0)	5.0 (0.0–8.0)	3.0 (0.0–6.0)	<0.001
Currently smoking, *n* (%)	274 (46.8%)	157 (53.0%)	117 (40.5%)	0.002
Currently drinking, *n* (%)	206 (36.1%)	119 (40.9%)	87 (31.1%)	0.015
Hypertension, *n* (%)	421 (71.1%)	203 (67.2%)	218 (75.2%)	0.033
Hyperlipidemia, *n* (%)	49 (8.3%)	25 (8.3%)	24 (8.4%)	0.980
Diabetes, *n* (%)	138 (23.5%)	69 (22.9%)	69 (24.0%)	0.749
CAD, *n* (%)	38 (6.5%)	12 (4.0%)	26 (9.1%)	0.013
AF, *n* (%)	47 (7.9%)	12 (4.0%)	35 (12.1%)	0.014
SBP, mmHg (mean ± SD)	150.0 ± 17.7	148.6 ± 18.6	151.4 ± 16.7	0.076
DBP, mmHg (mean ± SD)	82.7 ± 11.9	84.8 ± 12.1	80.5 ± 11.3	<0.001
Hcy, μmol/L (median, IQR)	10.0 (4.7–12.6)	10.0 (5.0–13.0)	11.0 (4.5–13.0)	<0.001
Folate, nmol/l (median, IQR)	5.8 (4.0–8.4)	6.0 (4.2–8.4)	5.2 (3.7–8.5)	0.133
B12, pmol/l (median, IQR)	352.0 (253.0–488.0)	371.0 (258.0–529.0)	337.5 (249.3–445.8)	0.024
eGFR, ml/min/1.73 m^2 (mean ± SD)	92.7 (78.3–104.0)	100.5 (85.6–109.4)	86.7 (73.1–95.3)	<0.001
NIHSS score (median, IQR)	1.0 (0.0–3.0)	1.0 (0.0–3.0)	1.0 (0.0–3.0)	0.816
TOAST mechanism				0.031
LAA, *n* (%)	474 (80.1%)	243 (80.5%)	231 (79.7%)	
CE, *n* (%)	58 (9.8%)	22 (7.3%)	36 (12.4%)	
SVO, *n* (%)	40 (6.8%)	22 (7.3%)	18 (6.2%)	
Others, *n* (%)	20 (3.4%)	15 (5.0%)	5 (1.7%)	
MMSE ≤ 24, *n* (%)	317 (53.5%)	126 (41.7%)	191 (65.9%)	<0.001
MMSE score (median, IQR)	24.0 (19.0–27.0)	26.0 (21.0–28.0)	22 (18.0–26.0)	<0.001
HAMD score (median, IQR)	4.0 (2.0–7.0)	4.0 (1.0–7.0)	4.0 (2.0–7.0)	0.531
Depressive symptoms, *n* (%)	139 (23.5%)	67 (22.2%)	72 (24.8%)	0.440

BMI, body mass index; CAD, coronary artery disease; AF, atrial fibrillation; SBP, systolic blood pressure; DBP, diastolic blood pressure; eGFR, estimated Glomerular Filtration Rate; NIHSS, National Institute of Health stroke scale; LAA, Large artery atherosclerosis; CE, Cardioaortic embolism; SVO, Small artery occlusion; Others, Other causes; MMSE, Mini-mental State Examination; HAMD, Hamilton Depression Scale.

there is an age-dependent association between Hcy and cognitive impairment in stroke survivors, we divided the total group into two subgroups, ≥65 and <65 years.

As mentioned earlier, an independent association has been reported between Hcy and PSCI with a variable time of outcome assessments from 2 months and up to 3 years after stroke (12–14). We observed an age difference in the relationship between Hcy levels and cognitive impairment in the post-stroke population. None of the aforementioned studies performed an age-specific analysis. Accumulating evidence indicated a relationship between Hcy levels, age, and stroke (19–21). For example, a prospective study revealed a strong, graded, and significant association between Hcy and stroke in young Asian patients with ischemic stroke (21). Moreover, Wang et al. (37) found no association between Hcy and functional outcome after stroke among elderly patients. In addition, a study based on cognitively healthy subjects discovered an age-dependent association between Hcy and cognitive decline and demonstrated that younger patients had a stronger association (22). Our findings regarding serum Hcy levels and cognitive impairment in the post-stroke population support and extend the previous studies. Indeed, we found that elevated Hcy could be an independent risk factor for cognitive impairment only in younger patients but not in older patients. Thus, the association between Hcy and cognitive impairment in the post-stroke population was age-dependent, suggesting that it could be useful in screening and targeting younger patients who need

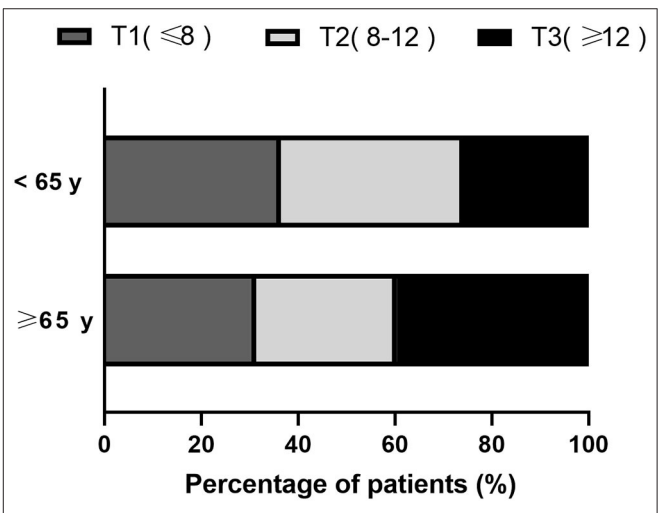

FIGURE 1 | Percentage in the subcategorized groups of age according to Hcy tertiles.

TABLE 5 | Multivariate adjusted odds ratios for the association between Hcy levels and cognitive impairment in the subcategorized groups of age.

	Univariate analysis		Model 3[a]		Model 4[b]	
	OR (95%CI)	***p*-value**	**OR (95%CI)**	***p*-value**	**OR (95%CI)**	***p*-value**
Younger age group (age < 65 y)						
Hcy tertiles						
Tertile 1	**Reference**		**Reference**		**Reference**	
Tertile 2	2.625 (1.351–5.102)	0.004	2.199 (1.120–4.317)	0.022	2.266 (1.042–4.926)	0.039
Tertile 3	3.156 (1.506–6.613)	0.002	3.767 (1.770–8.017)	0.001	3.583 (1.456–8.818)	0.005
Older age group (age ≥ 65 y)						
Hcy tertiles						
Tertile 1	**Reference**		**Reference**		**Reference**	
Tertile 2	1.291 (0.701–2.376)	0.413	0.941 (0.439–2.017)	0.875	0.661 (0.273–1.600)	0.358
Tertile 3	1.823 (1.161–3.504)	0.026	2.147 (1.035–4.456)	0.040	1.273 (0.547–2.961)	0.576

[a]*Model 3: adjusted for gender and years of education.*

[b]*Model 4: adjusted for variables in Model 1 plus currently smoking, currently drinking, hypertension, AF, folate, eGFR, NIHSS score, TOAST mechanism, and HAMD score.*

to lower their Hcy levels to improve the cognitive prognosis of ischemic stroke.

In our study, we also found that moderately raised Hcy (≥12 μmol/L in the total group and >8 μmol/L in the younger age group) increased the risk of cognitive impairment in the post-stroke population, which has not been studied yet. Previous studies only considered Hcy as a continuous variable in analysis and did not explore the graded association between Hcy and PSCI (12–14). The reference values for plasma Hcy ranged from 5 to 15 μmol/L. The latest guideline for the prevention and treatment of hypertension in China in 2010 has regarded an increase of Hcy ≥ 10 μmol/L as one of the important risk factors for cardiovascular and cerebrovascular diseases. Moreover, an International Consensus Statement in 2018 indicated that moderately elevated plasma total Hcy (>11 μmol/L) was one of the causes of age-related cognitive decline and dementia (38). Notably, we found a lower range of Hcy concentrations (>8 μmol/L), which would significantly increase the prevalence of cognitive impairment in younger stroke survivors. Therefore, Hcy levels in patients with stroke should be well-monitored, especially in younger patients. In other words, Hcy-lowering treatment should be administered once Hcy concentrations reach over 8 μmol/L in younger patients with stroke. Only one study has explored the association between Hcy-lowering therapies and cognitive impairment after stroke. Nevertheless, Hankey et al. (39) failed to discover an association between daily Hcy-lowering treatment and cognitive impairment in patients with previous stroke or transient ischemic attack. But in the aforementioned study, the Hcy-lowering treatment was administered without adjusting for age and Hcy levels. Interestingly, a *post-hoc* analysis from a clinical trial showed that younger patients may benefit the most from Hcy-lowering therapies (40). Despite the negative results in clinical studies using Hcy-lowering therapies to improve cognitive performance after stroke, the age-dependent effect on the association between Hcy and cognitive impairment in the post-stroke population is encouraging to pursue a feasible and effective treatment option in a specific subgroup of patients divided by age.

Homocysteine is postulated to cause PSCI *via* various mechanisms. Elevated Hcy is well-known to exert cytotoxic and pro-inflammatory effects, leading to vascular endothelial dysfunction and lipid metabolic disorders that cause thrombosis and arteriosclerosis (8–10, 41). All of these result in cerebrovascular disease, which indirectly causes vascular cognitive impairment (VCI). Moreover, Hcy neurotoxicity involves an increase in glutamate excitotoxicity (42), accelerating the development of oxidative stress in the hippocampal neurons (43), amyloid and tau protein accumulation (11), apoptosis (44), and neuronal death (45) contributing to the pathogenesis of AD. In short, elevated Hcy involves not only in VCI but also in the pathogenesis of AD, which has also been observed in the mechanisms of PSCI (1). However, these cannot explain why the association between Hcy and cognitive impairment exists only in younger patients. One possible explanation for this finding was that the association between Hcy levels and cognitive impairment in the post-stroke population was not causal among elderly patients. In other words, the harmful effects of elevated Hcy could be masked by other vascular risk factors, such as hypertension, diabetes, and AF. The prevalence rates of hypertension and diabetes were 75.2 and 24.0% in the older age group, respectively, which were much higher than the rate of the younger age group in the present study and the rates reported in other studies.

This study has certain limitations. First, this was a single-center and prospective study with a lack of baseline MMSE score. Therefore, we could not establish causality between Hcy and cognitive impairment in the post-stroke population. First, multicenter studies are needed to confirm these results and verify the justification of the Hcy tertiles. Second, cognitive function was merely measured by MMSE, other stroke-specific measures and more detailed neuropsychological assessments should be applied together with MMSE to evaluate the cognitive function accurately in the future study. Third, longer follow-up periods are needed to explore the effect of elevated Hcy on cognitive impairment in the post-stroke population. Fourth, we did not consider radiological parameters in the analysis. Fifth, the medications of the patients were not recorded on admission

and during the follow-up, which may cause some bias in this study. Finally, patients with aphasia or other serious conditions, and those aged > 80 years, were not included in our study. Meanwhile, patients enrolled in our sample analysis tended to have lower NIHSS scores and mostly minor strokes on admission. These may have underestimated the actual incidence of cognitive impairment after stroke and diminished the representativeness of the cohort, thus limiting the generalization of the results. Larger sample size and prolonged follow-up should be taken into account in the future.

CONCLUSION

In summary, our study suggested a graded association between elevated Hcy and cognitive impairment in the post-stroke population. We found that the association was stronger in younger patients but not in older patients. Therefore, Hcy levels in patients with stroke should be well-monitored, especially in younger patients. Further studies should be conducted among participants with different social and cultural backgrounds to replicate our findings.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

J-CH and S-NZ designed the study. S-NZ, J-HC, and LC wrote the manuscript. S-NZ did the statistical analyses. J-HC, LC, K-LF, W-WR, M-JX, Y-BC, D-DG, H-RC, X-QL, J-YS, and G-QL screened and extracted data. J-CH and G-QH supervised the study. All the authors have made an intellectual contribution to the manuscript and approved the submission.

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Carotenoid-Rich Brain Nutrient Pattern Is Positively Correlated With Higher Cognition and Lower Depression in the Oldest Old With No Dementia

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Background: Healthy dietary patterns are related to better cognitive health in aging populations. While levels of individual nutrients in neural tissues are individually associated with cognitive function, the investigation of nutrient patterns in human brain tissue has not been conducted.

Methods: Brain tissues were acquired from frontal and temporal cortices of 47 centenarians from the Georgia Centenarian Study. Fat-soluble nutrients (carotenoids, vitamins A, E, K, and fatty acids [FA]) were measured and averaged from the two brain regions. Nutrient patterns were constructed using principal component analysis. Cognitive composite scores were constructed from cognitive assessment from the time point closest to death. Dementia status was rated by Global Deterioration Scale (GDS). Pearson's correlation coefficients between NP scores and cognitive composite scores were calculated controlling for sex, education, hypertension, diabetes, and *APOE* ε4 allele.

Result: Among non-demented subjects (GDS = 1–3, $n = 23$), a nutrient pattern higher in carotenoids was consistently associated with better performance on global cognition ($r = 0.38$, $p = 0.070$), memory ($r = 0.38$, $p = 0.073$), language ($r = 0.42$, $p = 0.046$), and lower depression ($r = -0.40$, $p = 0.090$). The findings were confirmed with univariate analysis.

Conclusion: Both multivariate and univariate analyses demonstrate that brain nutrient pattern explained mainly by carotenoid concentrations is correlated with cognitive function among subjects who had no dementia. Investigation of their synergistic roles on the prevention of age-related cognitive impairment remains to be performed.

Keywords: dementia, cognition, carotenoids, vitamin A, vitamin E, vitamin K, fatty acids, centenarian adults

INTRODUCTION

Advancing age is the number one risk factor of age-related cognitive impairment and dementia, representing a major public health epidemic among older Americans (1). A systematic review of cross-sectional studies and longitudinal cohorts has identified a relation between diet quality and cognitive health in the aging population (2). A healthy diet covers a variety of dietary patterns such as Mediterranean diet (MeDi) (3, 4), Healthy Dietary Index (HDI) diet (5), Healthy Eating Index (HEI) diet (6), Dietary Approaches to Stop Hypertension (DASH) diet (7), and Mediterranean-DASH Intervention for Neurodegenerative Delay (MIND) diet (8). In some studies, dietary patterns are derived *a posteriori* with cluster analysis, factor analysis (e.g., principal component analysis [PCA]), or reduced rank regression (9–13). Though healthy dietary patterns have been diversely defined, they share common components of high intake of fruits, vegetables, whole grains, nuts, seeds, fish, and limited intake of added sugar, sodium, high-fat dairy products, red, and processed meat (2).

Traditionally, habitual dietary intake has been estimated using subjective recall data derived from food frequency questionnaires (FFQ). This approach, despite its direct translation for establishing dietary recommendations, does not account for recall bias, particularly among subjects with varied cognitive performance, and inter-individual variability in the nutrient absorption and metabolism (14). There has been an attempt to overcome these issues by constructing serum nutrient patterns (NP) as a marker of intake among subjects in the Oregon Brain Aging Study (15), and the Illinois Brain Aging Study (16–18). However, given that cognitive processes originate from the human brain, particularly in neocortices, and that nutrient uptake into the central nervous system (CNS) is strictly regulated at the blood-brain barrier (BBB) (19), we need to expand the scope of investigation further into the CNS for a greater apprehension of nutrition's roles in the aging brain.

While the evidence for relations between diet quality and cognitive function has been largely consistent among observational studies, the evidence from clinical trials of dietary supplements has been mixed (20). When investigating the relationship between nutrition and age-related diseases, the importance of examining nutrition as dietary patterns or NPs have been highlighted (21, 22), as most individuals acquire nutrients predominantly from foods, rather than supplements, throughout their lifespan. From a biochemical and molecular perspective, the etiology of age-related dementia, despite its heterogeneity, shares multiple mechanisms including cardiometabolic risk factors, elevated oxidative stress, neuroinflammation, and impaired AMP-activated kinase signaling (23). All of these factors can potentially be regulated by multiple dietary components. The pathology of age-related cognitive impairment is also different from cognitive symptoms caused by a deficiency of a single nutrient that may manifest during a shorter period of time and may be reversible—such as dementia caused by vitamin B12 or niacin deficiency, and Wernicke-Korsakoff syndrome caused by a genetic predisposition "to thiamin" deficiency (24, 25).

Therefore, the present research study was proposed based on the rationale that many fat-soluble nutrients (carotenoids, vitamins A, E, K, and fatty acids [FA]) are present in human brain (26–31), and that they are a part of dietary patterns and serum NPs previously reported to be associated with better cognitive function in multiple aging cohorts (2, 15, 26, 28, 32, 33). This was accomplished by constructing *a posteriori* NPs of fat-soluble nutrients measured in brain tissues acquired post-mortem from a subset of centenarians (defined as ≥98 years) who were enrolled in the Georgia Centenarian Study (GCS)—the longest running centenarian study in the U.S. to date (34, 35). Subsequently, the relationship between constructed NPs and cognitive performance at the time point closest to death was cross-sectionally investigated. Findings from this novel study may also provide insights into the role of nutrition in cognition in the oldest old, which may be similar or different from lesser aged older adults.

MATERIALS AND METHODS

Subject Recruitment and Brain Collection

The design of GCS, objectives, protocols of subject recruitment, and brain collection have been previously described in detail (34, 35). Briefly, the GCS was a population-based study conducted in 44 counties in northern Georgia. The GCS was primarily designed to identify biological, psychological, and social factors contributing to survivorship and successful aging. Brain tissues from frontal (FC) and temporal cortices (TC) were collected from 47 subjects who were a subset of centenarians enrolled in the phase III of the GCS (2001–2007) and gave consent to donate brain tissue upon death. After tissue collection, all samples were coded and stored at −80°C until the measures of nutrient concentration. All protocols were performed with an approval from the University of Georgia Institutional Review Board. Separate approval for using de-identified data for the present analyses was obtained from the Tufts University/Tufts Medical Center Institutional Review Board.

Nutrient Concentration Measures in Brain Tissues

Protocols for brain lipid extraction, separation, quantification, and concentrations have been previously and separately described for carotenoids, vitamin A (retinol), vitamin E (α- and γ-tocopherol [TP]), vitamin K (phylloquinone [PK] and menaquinone-4 [MK-4]), and individual FAs (27, 28, 36–39). In short, separation and quantification of five major dietary carotenoids (lutein, zeaxanthin, cryptoxanthin, β-carotene, and lycopene), retinol, and TPs were performed using high-performance liquid chromatography (HPLC) coupled with a photodiode array detector. The limit of detection (LOD) was 0.2 pmol for carotenoids, 2.0 pmol for retinol, 2.7 pmol for TPs per injection. Only levels of the all-*trans* isomer of each carotenoid, which is the most predominant isomer in human brain tissues, are reported (26, 40). Separation and detection of PK and MK-4 were performed using HPLC coupled with a fluorescence detector. The LOD was 0.03 pmol for both vitamin K vitamers. Separation and detection of individual FAs were

performed using a gas chromatography coupled with a flame ionization detector, and expressed as molar percentage (mol%). Total saturated FAs (SFAs) represent the sum of 10:0, 12:0, 14:0, 15:0, 16:0, 18:0, 20:0, 22:0, and 24:0. Total monounsaturated FAs (MUFAs) represent the sum of 16:1 (n-9), 16:1 (n-7), 18:1 (n-9), 18:1 (n-7), 20:1 (n-9), 22:1 (n-9), and 24:1 (n-9). Total omega-3 polyunsaturated FAs (n-3 PUFAs) represent the sum of 18:3, 18:4, 20:3, 20:5, 22:5, and 22:6. Total omega-6 polyunsaturated FAs (n-6 PUFAs) represent the sum of 18:2, 18:3, 20:2, 20:3, 20:4, 22:2, 22:4, and 22:5. Total *trans*-FAs represent the sum of 16:1 (n-9), 16:1 (n-7), trans-6-octadecenoic acid (18:1, n-10 to 12), 18:1 (n-9), 18:1 (n-7), *trans*-9, *trans*-12- octadecenoic acid (18:2 TT/TCTX), and conjugated linoleic acid (18:2, CLA).

Cognitive Assessment and Cognitive Domain Composite Scores

After enrollment in the GCS, cognitive assessment was performed every 6 months at the subject's residence as reported earlier (34). Cognitive data were obtained from the visit closest to death (<1 year for all subjects). Dementia status was assessed by geriatric psychiatrists using Global Deterioration Scale (GDS) and subjects were grouped based on GDS score. A score of 1–2 on GDS was clinically defined as no dementia; a score of 3 represented mild cognitive impairment; and a score of 4–7 represented increasing severity of dementia from mild to severe (41). Cognitive tests included Mini-Mental State Examination (MMSE, 24–30 = normal cognition; 19–23 = mild; 10–18 = moderate; or ≤ 9 = severe cognitive impairment) (42), Severe Impairment Battery (SIB, < 63 = very severely impaired cognition) (43), Fuld Object Memory Evaluation (FOME) (44), Controlled Oral Word Association Test (COWAT) (45), Wechsler Adult Intelligence Scale Third Edition (WAIS-III) Similarities (46), Behavioral Dyscontrol Scale (BDS) (47), and the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) battery which included Verbal Fluency (VF), Boston Naming Test (BNT), Constructional Praxis (CP), and Word List Memory Test (WLMT) (48, 49). Depression was assessed using Geriatric Depression Scale Short Form (GDSSF) (50), and activities of daily living were assessed using Direct Assessment of Function Status (DAFS) (51). All subtests have been validated and are considered reliable measures of cognition in normal aging and in AD (52).

To calculate cognitive domain composite scores, scores from each cognitive test were normalized using z-scoring as previously reported (53). Composite scores of five cognitive domains (memory, executive function, language, visuospatial function, attention), depression, and activities of daily living were then calculated by averaging the z-scores of tests based on the method adapted from Bowman et al. (15). The calculation method has also previously been reported and shown in **Supplementary Table 1**. Global cognition composite scores were also derived by combining total cognitive testing z-scores, MMSE, and SIB. Missing test scores were excluded and the denominator changed accordingly for the calculation of composite scores.

Statistical Analysis

Values are presented as mean (SD). All statistical tests were performed in R 3.5.1 with a significance level set at $\alpha = 0.05$. Findings with $p < 0.1$ but > 0.05 were reported as borderline significant. Comparisons of subject characteristics between non-demented (GDS 1–3, $n = 23$) and demented subjects (GDS 4–7, $n = 24$) were performed using Student's two-sample *t*-test and Fisher's exact test for continuous and categorical variables, respectively.

NPs were derived from concentrations of carotenoids, vitamins A, E, K, SFAs, MUFAs, n-3 PUFAs, n-6 PUFAs, and *trans*-FAs averaged from FC and TC (vitamin K only from FC due to limited brain sample availability) using PCA with a function pca in the R package "pcaMethods" (54). Concentrations of all nutrients were log transformed prior to PCA. Nutrient concentration matrix was unit-variance scaled and centered. We chose non-linear iterative partial least squares algorithm to calculate principal components, or NPs in our case, which is an iterative approach for estimating independent principal components by extracting them one at a time (55). This variation of PCA can handle small amount of missing values, which in our case were PK and MK-4 concentrations from two individuals due to insufficient brain tissues for vitamin K analysis. Only NPs with eigenvalue >1 were reported.

To investigate the relationship between brain nutrient concentrations or NPs and cognitive domain composite scores, Pearson's correlation test was performed with an adjustment for covariates sex, education, hypertension, diabetes, and presence of *APOE* ε4 allele. Additional adjustment for antithrombotic use was performed for vitamin K, and antidepressant use for depression score. Sub-analyses in non-demented (GDS 1–3) were also performed. Heat maps aided the visualization of correlations.

RESULTS

Subject Characteristics

Characteristics of all 47 subjects are reported in **Table 1**. By design, all subjects were ≥98 years old with an average age at death of 102.2 (2.5) years old. Eighty-nine percent were Caucasian and 89% were female. Subjects who did not finish high school accounted for 51%. Seventy percent were institutionalized at the visit closest to death. Body mass index (BMI) was 22.1 (3.9) kg/m^2 on average, excluding one double amputee whose BMI could not be calculated. Approximately half of the subjects had hypertension (53%) while only 3 subjects had diabetes (6%). In terms of medication and supplement uses, while no subjects took cholesterol-lowering medication, the majority of subjects used at least one form of dietary supplements (72%). Twelve subjects, ten of whom had dementia, could not provide data on history of smoking and alcohol use through recall. Among those with available data, 86% never smoked and 60% never used alcohol. Only one subject was an active smoker at death.

Subject characteristics between non-demented ($n = 23$) and demented ($n = 24$) subjects, as assessed by the GDS, were not statistically different, except that BMI in non-demented subjects was marginally higher than that in demented subjects [23.1 (3.5) vs. 21.0 (3.5) kg/m^2, $p = 0.067$] (**Table 1**). Although higher

TABLE 1 | Subject characteristics.

Characteristic	All subjects ($n = 47$)	GDS 1-3 ($n = 23$)	GDS 4–7 ($n = 24$)	*P*-value[a]
Age in years, mean (SD)	102.2 (2.5)	102.2 (2.3)	102.2 (2.8)	0.946
Female, *n* (%)	42 (89%)	19 (83%)	23 (96%)	0.188
Race, *n* (%)				0.348
Caucasian	42 (89%)	22 (96%)	20 (83%)	
Black	5 (11%)	1 (4%)	4 (17%)	
BMI in kg/m^2, mean (SD)[b]	22.1 (3.9)	23.1 (3.5)	21.0 (4.0)	0.067
Hypertension, *n* (%)	25 (53%)	12 (52%)	13 (54%)	1
Diabetes, *n* (%)	3 (6%)	2 (9%)	1 (4%)	0.609
Education, *n* (%)				0.204
< High school	23 (51%)	9 (39%)	14 (64%)	
High school	12 (27%)	7 (30%)	5 (23%)	
> High school	10 (22%)	7 (30%)	3 (14%)	
No data	2	0	2	
Living, *n* (%)				0.212
Community dwelling	14 (30%)	9 (39%)	5 (21%)	
Institutionalized	33 (70%)	14 (61%)	19 (79%)	
Dietary supplement use, *n* (%)	34 (72%)	14 (61%)	20 (83%)	0.111
Medications, *n* (%)				
Antidepressants	14 (30%)	4 (17%)	10 (42%)	0.111
Antipsychotics	5 (12%)	1 (4%)	4 (17%)	0.348
Anti-inflammatory medications	5 (12%)	4 (17%)	1 (4%)	0.188
Antithrombotics	10 (21%)	5 (22%)	5 (21%)	1
Antibiotics	7 (15%)	2 (9%)	5 (21%)	0.416
Smoking, *n* (%)				0.570
Never	30 (86%)	18 (86%)	12 (86%)	
Past	4 (11%)	3 (14%)	1 (7%)	
Present	1 (3%)	0 (0%)	1 (7%)	
No data	12	2	10	
Alcohol use, *n* (%)				0.041
Never	21 (60%)	9 (43%)	12 (86%)	
Past	6 (17%)	5 (24%)	1 (7%)	
Present	8 (23%)	7 (33%)	1 (7%)	
No data	12	2	10	
APOE, n (%)[c]				
ε2 allele carrier	8 (17%)	3 (13%)	5 (21%)	0.701
ε4 allele carrier	8 (17%)	3 (13%)	5 (21%)	0.701

[a] *Comparisons between subjects whose Global Deterioration Scale (GDS) = 1–3 (non-demented) and GDS = 4–7 (demented) using Student's t-test for continuous variables and Fisher's exact test for categorical variables.*

[b] *Body mass index (BMI) cannot be calculated for a double amputee whose GDS = 5.*

[c] *All subjects who carried an ε2 or ε4 allele were ε2/ε3 or ε3/ε4 except one subject with GDS = 4 who was an ε2/ε4.*

proportion of subjects without dementia reported a history of alcohol use ($p = 0.041$), the data were only available for only 58% of demented participants.

Nutrient Concentrations in FC and TC, and Establishing NPs

Concentrations of nutrients averaged from the FC and TC are reported in **Table 2** (vitamin K only from FC). These data have also been previously reported separately for FC and TC (27, 31, 40). Lutein was the most predominant carotenoid in all FC and TC tissues with a concentration of 79.50 (52.57) pmol/g. On the contrary, lycopene, at an average concentration of 20.41 (21.38) pmol/g, was not detected in both FC and TC in 20 subjects (43% of all subjects). Retinol, which included both free retinol, retinal, and retinyl esters before hydrolyzation during lipid extraction, had a concentration of 691.97 (305.56) pmol/g. Concentrations of α-TP and γ-TP were 66,917 (13,676) and 1,742 (1,018) pmol/g, respectively. While MK-4 was detectable in all brain tissues at 4.96 (2.32) pmol/g, PK was not detected in 17 subjects (38%). The predominant class of FA in the brain samples was SFA, which accounted for 15.36 (2.30) nmol/mg or 47.93 (1.68) mol%. Concentrations of individual FAs are shown in

TABLE 2 | Mean (SD) of nutrient concentrations averaged from the frontal and temporal cortices (vitamin K only in the frontal cortex).

Nutrient	All subjects ($n = 47$)	GDS 1–3 ($n = 23$)	GDS 4-7 ($n = 24$)	*P*-value[a]
Carotenoids (pmol/g)				
Lutein	79.50 (52.57)	76.34 (43.72)	82.52 (60.65)	0.828
Zeaxanthin	26.97 (12.73)	28.36 (14.06)	25.63 (11.44)	0.404
Cryptoxanthin (α + β)	62.06 (67.97)	57.15 (47.61)	66.76 (83.81)	0.787
β-Carotene	55.86 (35.56)	46.22 (24.20)	65.09 (42.27)	0.130
Lycopene	20.41 (21.38)	21.63 (21.57)	19.25 (21.59)	0.901
Retinol (pmol/g)	691.97 (305.56)	674.11 (286.94)	709.10 (327.65)	0.688
Vitamin E (pmol/g)				
α-Tocopherol	66,917 (13,676)	68,303 (13,732)	65,588 (13,782)	0.526
γ-Tocopherol	1,742 (1,018)	1,891 (1,049)	1,600 (989)	0.208
Vitamin K (pmol/g)[b]				
Phylloquinone	0.40 (0.39)	0.35 (0.42)	0.45 (0.36)	0.276
Menaquinone-4	4.96 (2.32)	5.05 (2.82)	4.88 (1.82)	0.772
Fatty acid (nmol/mg)				
Total SFA	15.36 (2.30)	15.04 (2.40)	15.66 (2.21)	0.333
Total MUFA	7.00 (2.41)	6.83 (1.93)	7.17 (2.83)	0.723
Total n-3 PUFA	4.20 (0.67)	4.11 (0.73)	4.29 (0.60)	0.321
Total n-6 PUFA	5.53 (1.26)	5.41 (1.04)	5.63 (1.45)	0.569
Total *trans*-FA	0.25 (0.09)	0.22 (0.06)	0.28 (0.10)	0.020

[a] *Comparisons between Global Deterioration Scale (GDS) 1–3 and GDS 4–7 using Student's t-test. Log (x+1) transformation was applied prior to comparisons.*
[b] *Brain tissue from two subjects was not available for vitamin K measures.*
SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; trans-FA, trans-fatty acid.

Supplementary Table 2. Docosahexaenoic acid (DHA, 22:6 n-3) and arachidonic acid (AA, 20:4 n-6) were the most predominant n-3 PUFA (11.90 (1.60) mol% or 90.22% of total n-3 PUFAs) and n-6 PUFA (8.54 (0.57) mol% or 66.36% of total n-6 PUFAs), respectively, in all brain tissues. Concentrations of each nutrient were not significantly different among demented and demented subjects. However, total *trans*-FA concentration was significantly higher among demented subjects ($p = 0.020$).

As shown in **Figure 1**, significant correlations were identified among concentrations of carotenoids, and among FAs (blue represents positive and red represents negative correlations). For others that also reached statistical significance, lycopene was also positively correlated with γ-TP ($r = 0.32$, $p = 0.027$) and negatively correlated with vitamin A ($r = -0.49$, $p < 0.001$), while α-TP was positively correlated with PK ($r = 0.46$, $p = 0.002$) and negatively associated with total *trans*-FAs ($r = -0.32$, $p = 0.030$). MK-4, but not PK, was also associated with FA concentrations including total SFA ($r = 0.45$, $p = 0.002$), n-3 PUFA ($r = 0.52$, $p < 0.001$), and n-6 PUFA ($r = 0.34$, $p = 0.023$). These correlations among nutrient concentrations further warranted the investigation of nutrients as NPs.

Next, PCA was used to derive NPs from brain nutrient concentrations. The first five NPs that explain the highest variance (each of which had an eigenvalue >1.0) among the 47 subjects are described in **Table 3**. No obvious outlier was detected in a PCA plot (data not shown). NP1 which has the highest variance accounted for 26.20% of the total variance. NP1 is described as higher concentrations of SFAs, MUFAs, and n-3 and n-6 PUFAs (all loading coefficients >0.40). NP2 is described by high concentrations of carotenoids (all loading coefficients ≥0.30), and NP3 is described by high concentrations of retinol, α-TP, and PK (all loading coefficients ≥0.40). The correlations of these nutrients in each NP are as depicted in **Figure 1**. The first five NPs accounted for 75.92% of the total variance of the original nutrient dataset.

Composite Scores on Cognitive Domains, Depression, and Activities of Daily Living

The time interval between the cognitive assessment at the time point closest to death and the autopsy was <1 year for all subjects with an average of 156 (93) days for those whose data could be accurately calculated (81%). Subjects with GDS 1–3 had significantly higher MMSE scores than those with GDS 4–7 ($p < 0.001$). Similarly, SIB score was higher in subjects with GDS 1–3 ($p < 0.001$). Therefore, GDS effectively separated subjects based on their performance on global cognition as also previously reported in the original GCS cohort ($n = 244$) (56). Further, the composite scores for cognitive domains, depression, and activities of daily living had been calculated (**Supplementary Table 3**). Subjects with GDS 1–3 had significantly higher composite scores on all six cognitive domains and activities of daily living, and significantly lower scores on depression (less depression). Of note, while composite scores for other cognitive domains were available for all subjects, visuospatial function score was available for 87% of participants without dementia and 50% of participants with dementia, and attention score was available for 70% of participants without demented and 46% of participants with dementia.

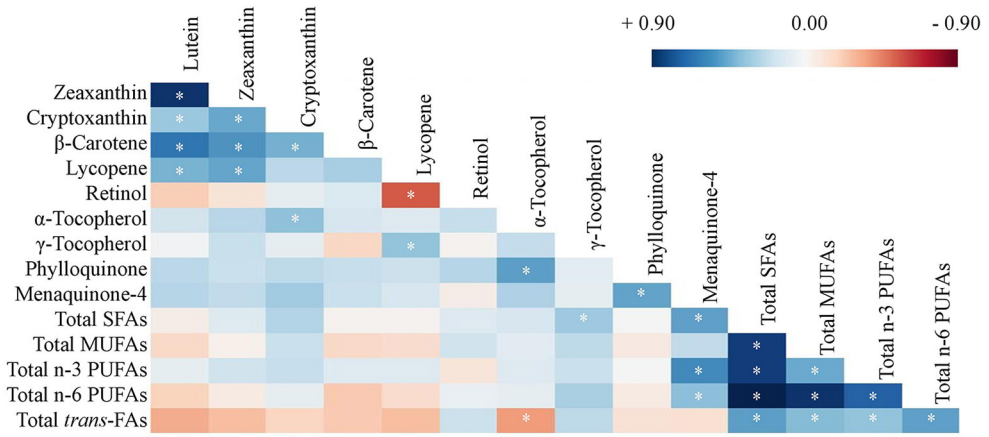

FIGURE 1 | Heat map of Pearson's correlation coefficients of concentrations of carotenoids, retinol, tocopherols, phylloquinone, menaquinone-4, and fatty acids averaged from frontal and temporal cortices (except vitamin K only in the frontal cortex, $n = 47$, $^*p < 0.05$). Log transformation has been applied to all nutrient concentrations. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; *trans*-FA, *trans*-fatty acid.

TABLE 3 | Construction of nutrient patterns (NPs), NP structure and variance explained ($n = 47$).

Nutrient	NP1	NP2	NP3	NP4	NP5
Carotenoids					
Lutein	0.02	0.45	−0.14	0.23	0.12
Zeaxanthin	0.09	0.44	−0.13	0.14	0.27
Cryptoxanthin	0.16	0.30	0.14	0.11	0.06
β-Carotene	0.01	0.37	0.08	0.43	0.11
Lycopene	0.02	0.32	−0.41	−0.31	0.03
Retinol (Vitamin A)	0.06	−0.09	0.61	0.18	0.40
Vitamin E					
α-TP	0.11	0.23	0.40	−0.42	−0.04
γ-TP	0.19	0.05	−0.16	−0.54	0.52
Vitamin K					
PK	0.07	0.23	0.41	−0.28	−0.11
MK-4	0.26	0.19	0.12	−0.04	−0.50
Fatty acids					
Total SFAs	0.50	−0.05	−0.05	0.07	−0.03
Total MUFAs	0.41	−0.13	0.02	0.04	0.08
Total n-3 PUFAs	0.41	0.03	−0.13	0.12	−0.23
Total n-6 PUFAs	0.46	−0.15	−0.06	0.01	−0.05
Total *trans*–FAs	0.22	−0.27	−0.08	0.15	0.37
Eigenvalue	3.89	3.39	1.68	1.27	1.06
% Variance	26.2	22.76	11.28	8.53	7.15
Cumulative % variance	26.2	48.96	60.24	68.77	75.92

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; trans-FA, trans-fatty acid; TP, tocopherol; PK, phylloquinone; MK-4, menaquinone-4.

Relationship Between Brain Nutrient Concentrations and Cognition

Scores of the first five NPs were not statistically different between demented and non-demented subjects (data not shown). However, among non-demented subjects, subjects who had mild cognitive impairment (GDS 3, $n = 11$) had significantly lower NP2 score than that of cognitively intact subjects (GDS 1–2, $n = 12$, $p = 0.002$), but not for other NPs. The difference remained statistically significant after an adjustment for sex, education, hypertension, diabetes, and presence of *APOE* ε4 allele ($p = 0.004$).

A heat map was constructed to provide Pearson's partial correlation coefficients between NP scores or nutrient concentrations and scores on cognitive domains, depression, and activities of daily living in all subjects (**Figure 2A**, *p*-values provided in **Supplementary Table 4A**). Pearson's partial correlations were adjusted for sex, education, diabetes, hypertension, and presence of *APOE* ε4 allele. No consistent relationship between NPs and cognitive domain scores that reached statistical significance was observed.

A subset analysis among non-demented subjects (GDS 1–3) was performed and Pearson's coefficients are illustrated in a heat map (**Figure 2B**, *p*-values provided in **Supplementary Table 4B**). In the models adjusted for covariates among the five NPs representing the highest variances, NP2 was consistently associated with higher scores on global cognition ($r = 0.38$, $p = 0.070$), memory ($r = 0.38$, $p = 0.073$), language ($r = 0.42$, $p = 0.046$), and lower depression score ($r = -0.40$, $p = 0.090$) (**Figure 3**). After additional adjustment for antidepressant use, the correlation with lower depression score remained borderline significant ($r = -0.35$, $p = 0.100$). Since NP2 is mainly described by carotenoids, significant correlations were also consistently observed between lutein, zeaxanthin, β-carotene and scores on global cognition, memory, language, and depression. Other notable associations included NP3 and NP5 and higher depression. Additional adjustment for antithrombotic use was performed for PK and MK-4 and their correlations with different cognitive domain composite scores remained statistically non-significant ($p > 0.05$) for all six cognitive

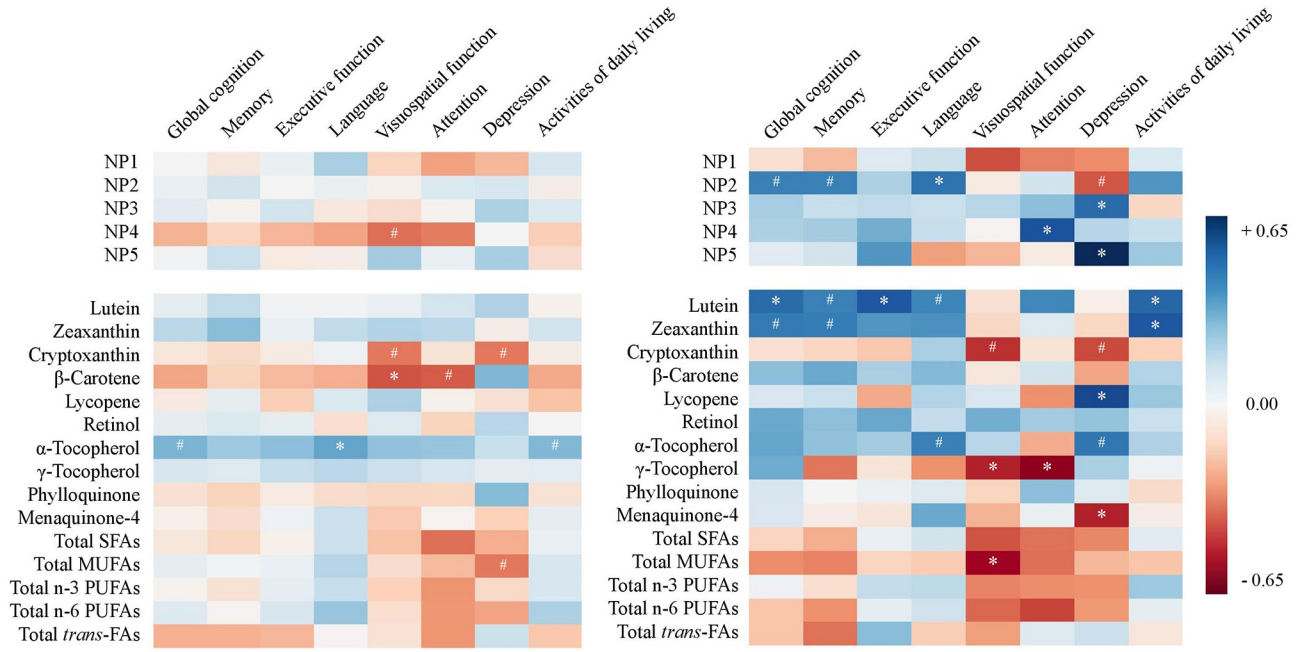

FIGURE 2 | Heat map of Pearson's correlation coefficients between nutrient pattern (NP) scores or nutrient concentrations and scores on cognitive domains, depression, and activities of daily livings in **(A)** all subjects ($n = 47$) and **(B)** non-demented subjects (Global Deterioration Scale = 1–3, $n = 23$). Correlations are adjusted for sex, education, diabetes, hypertension, and APOE ε4 allele ($^{\#}p < 0.10$, $^{*}p < 0.05$). SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; *trans*-FA, *trans*-fatty acid.

domains and activities of daily living, while the correlation between MK-4, but not PK, and depression remained statistically significant (**Supplementary Table 4B**).

DISCUSSION

This study documents that brain NP high in carotenoids was consistently associated with better performance on multiple cognitive domains, activities of daily living, and lower depression among non-demented older adults in the GCS. Results also confirm previously established positive relationships between serum and brain concentrations of carotenoids in this group of subjects independent of their cognitive status (27). Given that serum concentrations of carotenoids likely reflect their habitual intake in the oldest old as previously discussed (27), our findings in the present study underscore the timing of intervention with diet high in carotenoid content before the onset of age-related dementia. This is further supported by the fact that nutrient concentrations and NP scores were not different between demented and non-demented subjects. Our exploratory findings also corroborate previous findings where serum levels of carotenoids are positively associated with better cognition in aging subjects (26, 32, 57–60). Specifically, higher serum lutein concentration was reported to be associated with better performance on language (32), which is similar to the correlation between NP2 and language score in this study.

The present analysis investigated concentrations of nutrients in the brain, the organ most relevant to cognition, as compared to previous studies that have established dietary or serum NPs with similar exploratory approaches (10, 11, 13, 15). Although the relationship between better adherence to *a priori* hypothesized intake patterns (such as MeDi, HDI, HEI, DASH, MIND) and lower risk of cognitive decline have been established (2), *a priori* hypothesized NPs are difficult with brain concentrations since little is known regarding nutrient uptake across the BBB and nutrient metabolism in neural tissue. For instance, SFAs and MUFAs can be *de novo* synthesized in the liver and CNS and may not reflect intake levels (61), and among n-3 PUFAs, DHA preferentially accumulates in neural tissue (62, 63). While substitution of SFA and *trans*-FA intakes with MUFAs and PUFAs decreases risk of age-related cognitive impairment in many prospective cohorts (64), our findings with brain content cannot be directly compared with intake levels of SFAs and MUFAs. It has also been reported that higher SFA content in membranes is usually associated with higher PUFA content to maintain membrane stability (65, 66), which likely explains the high correlation between SFA and unsaturated FA observed in the GCS brain tissues. Similarly, retinol is thought to be either taken up into the brain by STRA6, a retinol-binding protein-receptor detected at the BBB or derived directly from the cleavage of provitamin A carotenoids (β-carotene and β-cryptoxanthin) by the enzyme BCO1 detected in human brain (67, 68). It

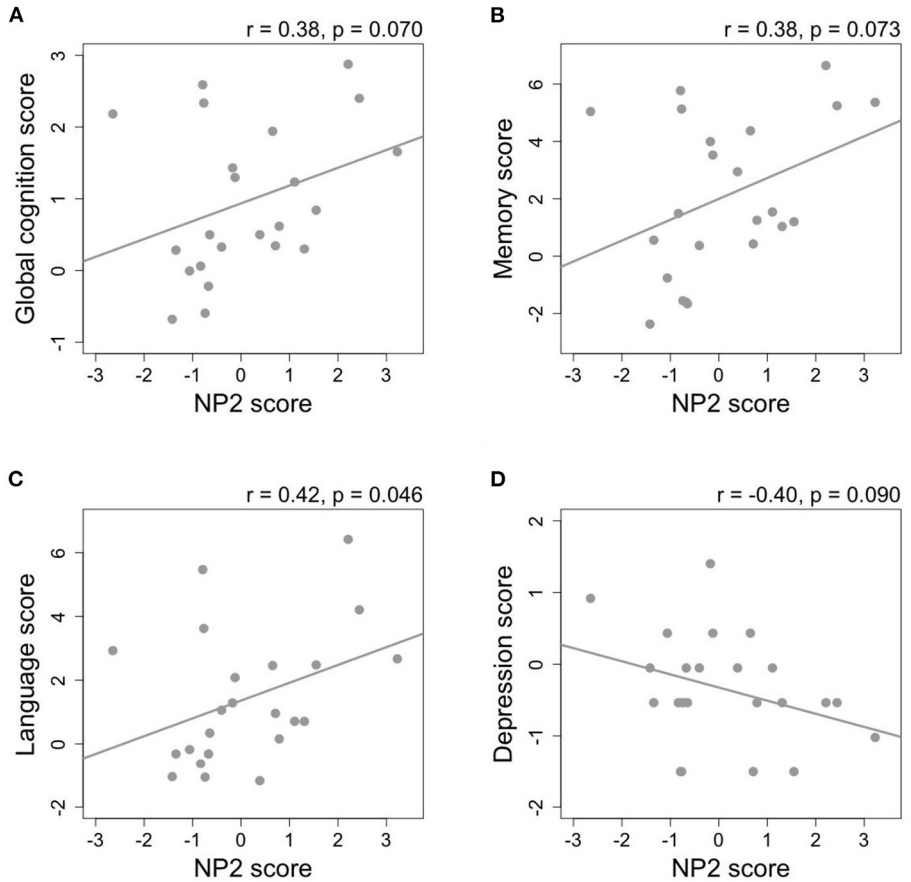

FIGURE 3 | The relationship between nutrient pattern 2 (NP2) score and composite scores of **(A)** global cognition, **(B)** memory, **(C)** language, and **(D)** depression among non-demented subjects (Global Deterioration Scale = 1–3, $n = 23$). Pearson's correlation coefficients are adjusted for sex, education, diabetes, hypertension, and APOE ε4 allele.

remains unknown how much each source contributes to vitamin A content in the brain. Moreover, a previous report on vitamin K metabolism in rat cerebellum also suggests that neural MK-4 content is regulated by the enzyme UBIAD1 (69). Overall, findings of nutrient levels in neural tissue need to be cautiously interpreted for dietary recommendations, especially for nutrients that can be derived from other substrates and nutrients whose levels are tightly regulated in the brain. Data on dietary intake in the GCS have been previously reported (70). However, the dietary assessment was subjective and might have overestimated or underestimated food intake, particularly in this population with varying degrees of cognitive performance. As a result, dietary intake data were not incorporated into the present analysis.

Age-related cognitive impairment, notwithstanding mixed clinical pathologies, shares molecular signatures of increased oxidative stress and neuroinflammation (71, 72). Both carotenoids and n-3 PUFAs, especially lutein and DHA both of which are selectively accumulated in the brain, have been proposed to interfere with the progression of cognitive impairment in aging, presumably owing to their antioxidative and anti-inflammatory properties (73, 74). Consistent with previous studies investigating neural concentrations of individual nutrients, a significant relationship was observed with lower carotenoids (mainly lutein and zeaxanthin) among cognitively impaired or demented subjects (26, 75–78). However, as previously discussed by Zamroziewicz and Barbey (22), univariate analytical approach with individual nutrients may be confounded by the effect of NPs and does not address the potentially interactive effects of multiple nutrients on cognitive health. An exploratory trial demonstrates that a combination of lutein and DHA supplements statistically improved performance on memory and learning in cognitively unimpaired elder women after 4 months whereas lutein or DHA supplement alone did not (79). Moreover, most individuals predominantly acquire nutrients from dietary sources that consist of a complex combination of nutrients. While a 6-month intervention with a lutein and zeaxanthin supplement failed to improve cognitive outcomes in subjects with or without Alzheimer's disease (80), a daily intervention with an avocado (a highly bioavailable source of 0.5 mg lutein and zeaxanthin, along with being a good source of potassium, B vitamins, vitamins C, E, K, MUFAs, and other non-essential phytochemicals) for 6 months has shown

to improve cognitive performance on the Spatial Working Memory and the Stockings of Cambridge in non-demented subjects with low baseline intake of lutein-rich foods (81, 82). In the present analysis, a multivariate analysis approach (PCA) has been adopted to address correlations among nutrients and inspect the nutrition variable as NPs which reflect how multiple nutrients may synergistically function in the context of cognitive functioning and age-related cognitive impairment. This is an important step in the field of nutritional cognitive neuroscience toward the application of emerging technologies (such as metabolomics and brain magnetic resonance imaging) to systematically identify underlying mechanisms that mediate the effect that a combination of nutrients have on clinical outcomes (22, 32).

We acknowledge that this exploratory study is limited by a relatively small sample size (which is reflected by borderline significant *p*-values in **Supplementary Table 4B**) of mostly Caucasian women, and the inability to control for other covariates that may affect cognitive function such as alcohol and smoking history, physical activity, social interactions, and genetics (83). However, nutrient profiles and concentrations in this current analysis are similar to those of other cohorts of older adults (29–31, 84–88). NP1, described mostly by high fat content, was not associated with cognition in this population. Previous studies have reported benefits of diets high in n-3 PUFAs, especially DHA, on cognitive health (33, 89). While it is more appropriate to use absolute concentrations of FAs in the PCA, relative concentrations of FAs (i.e., FA composition) may be more relevant to the biological function of the brain. Additionally, it is unclear if the FA compositions in the brain of this cohort of the oldest old were in the normal range, since altered fatty acid compositions among cognitively impaired or demented subjects were reported (29–31), but no difference were observed between those with and without dementia in this study.

Other nutrients and dietary compounds such as B vitamins, vitamin D, minerals, and polyphenols that may be beneficial to cognitive health were also not examined in this study (90), but propose the opportunity to expand the scope of investigation. Nutrients that are not present in the brain but sharing common dietary sources with carotenoids and n-3 PUFAs, such as fibers in fruits, vegetables, nuts and seeds, may provide additional benefits to the central nervous system by functioning systemically through the regulation of reverse cholesterol transport, gut microbiota, and gut-brain axis signaling (91, 92). Finally, a cross-sectional study does not address a causal and longitudinal relationship between nutrition and cognition. A reverse causation where cognitive impairment leads to changes in nutrient uptake and metabolism—for example through BBB breakdown—is possible (23). However, dietary intervention in human trials and animal studies have indicated a significant impact that nutrition has on cognitive health in aging (93–95).

In summary, this report is the first to adopt a multivariate analysis approach to address the co-existence of nutrients and dietary compounds in the brain when investigating the relationship between nutrition and cognitive function in an aging population. Our findings support beneficial effects of a NP higher in carotenoids potentially derived from a diet rich in fruits and vegetables similar to the MeDi and DASH diets, on lowering the risk of age-related cognitive impairment and dementia previously reported (2, 93, 94). As compared to symptoms of nutritional deficiency which could be caused by an inadequate intake of one single nutrient and manifest within a short period of time, we are aware of the need to assess diet as a dietary pattern or NP in a context of complex outcomes such as age-related cognitive impairment (20–22). The synergistic and cumulative effect of nutrients on a person's risk of chronic diseases have recently been highlighted in the Dietary Guidelines for Americans 2015–2020 and 2020–2025 (96, 97).

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: The data set is not publicly available but it is available on request from the corresponding author, Elizabeth J. Johnson. Requests to access these datasets should be directed to elizabeth.johnson@tufts.edu.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of Georgia Institutional Review Board. Separate approval for using de-identified data for the present analyses was obtained from the Tufts University/Tufts Medical Center Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JT, TMS, MAJ, LWP, and EJJ designed the study. MAJ and LWP contributed to the original design of the GCS and collection of biological samples. RV analyzed carotenoid, vitamin A, and vitamin E concentrations in all samples. NRM and AHL analyzed fatty acid concentration in all samples. GF analyzed vitamin K concentrations in all samples. JT performed statistical analysis, wrote the paper, and had primary responsibility for final content. All authors interpreted the data, 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/fnut.2021.704691/full#supplementary-material

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frontiers in Nutrition

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β-Lactolin Reduces Age-Related Inflammation and Cognitive Decline

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With the rapid increase in aging populations worldwide, there has been an increase in demand for preventive and therapeutic measures for age-related cognitive decline and dementia. Epidemiological studies show that consumption of dairy products reduces the risk for cognitive decline and dementia in the elderly. We have previously demonstrated in randomized trials that the consumption of β-lactolin, a whey-derived Gly-Thr-Trp-Tyr lactotetrapeptide, improves cognitive function in older adults. Orally administered β-lactolin is delivered to the brain and inhibits monoamine oxidase, resulting in alleviation of memory impairment. However, there is currently no evidence of the effects of long-term β-lactolin intake on aging. Here, we found that the discrimination index in the novel object recognition test for object recognition memory was reduced in mice aged 20 months compared with that in young mice, indicating that age-related cognitive decline was induced in the aged mice; in aged mice fed β-lactolin for 3 months, memory impairment was subsequently alleviated. In aged mice, impairment of light/dark activity cycles was found to be induced, which was subsequently alleviated by β-lactolin consumption. Additionally, the number of activated microglia in the hippocampus and cortex and the production of cytokines (tumor necrosis factor-α, macrophage inflammatory protein-1α, and macrophage chemoattractant protein-1) were increased in aged mice compared with those in young mice but were reduced in aged mice fed β-lactolin. The age-related hippocampal atrophy was improved in aged mice fed β-lactolin. Cytochrome c levels in the hippocampus and cortex were increased in aged mice compared with those in young mice but were also reduced by β-lactolin consumption. These results suggest that β-lactolin consumption prevents neural inflammation and alleviates aging-related cognitive decline.

Keywords: aging, cognitive decline, inflammation, β-lactoglobulin, β-lactolin, β-lactopeptide, memory, whey

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INTRODUCTION

Accompanying the rapid growth of aging populations worldwide, the increasing burden of age-related cognitive impairment falls not only on patients and their families but also on national healthcare systems. In normal aging, the size of the brain decreases with age (1, 2). MRI studies have shown that gray matter volume in the prefrontal cortex and medial temporal lobe, which includes the hippocampus, declines with aging (3, 4). It is suggested that this volume loss is attributed to neuronal loss associated with aging, which changes the structure and function of

synapses and neuronal networks (5). These changes in the brain are associated with age-related cognitive decline, such as memory and executive function impairment. Recent studies also show that age-related increases in inflammation are associated with cognitive decline (6). It is reported that age-related changed microglia in the aged brain shows the pro-inflammatory phenotype and reduce the protective function for neuron (7). It has been reported that the risk for cognitive decline and dementia in the elderly is associated with individual lifestyle (8); therefore, preventive approaches to cognitive decline, including dietary changes, have received increasing attention.

Recent investigations indicate that consumption of certain dairy products reduces the risk for dementia and for cognitive decline in the elderly. Ozawa et al. investigated dietary patterns and their potential association with reduced risk for dementia and cognitive decline in more than 1,000 dementia-free 60- to 79-year-old Japanese participants living in a local community (9, 10) and found that dietary patterns that included milk or dairy products reduced the risk for dementia in the general Japanese population. In addition, our previous study demonstrated that intake of Camembert cheese, a dairy product fermented by fungi, suppressed the activation of microglia and Alzheimer's disease pathologies in a 5 × FAD mouse model (11). These findings suggest that some ingredients, such as peptides, contribute to the prevention cognitive decline and dementia (12, 13).

A recent study identified β-lactolin, a β-lactoglobulin-derived Gly-Thr-Trp-Tyr tetrapeptide, which improves memory impairment in a pharmacologically-induced amnesia mouse model. β-lactolin belongs to the Trp-Tyr-related β-lactopeptide family, which also improve memory impairment and are abundant in Camembert and other types of cheeses fermented by *Penicillium* (14). Orally administered β-lactolin has been shown to be delivered to the brain, inhibit monoamine oxidase, and increase monoamine levels in the frontal cortex and hippocampus, resulting in improvements in spatial working memory and object recognition memory in mice (15–17). In addition to preclinical studies, previous clinical trials have demonstrated that supplementation with whey peptide rich in β-lactolin improves memory retrieval, attention, and executive function in the elderly (18, 19) and promotes neural activity in the cortex (20) and cerebral blood flow (21, 22). These findings suggest that consumption of β-lactolin is beneficial for the prevention of age-related cognitive decline. Furthermore, β-lactolin has been shown to suppress the activation of microglia and inflammation, which improves object recognition memory in 5 × FAD mice (23, 24). However, the effects of β-lactolin on age-related cognitive decline and age-related neuronal dysfunction have not yet been demonstrated. Most cognitive decline is associated with aging; thus, in the current study, we examined the effects of long-term consumption of β-lactolin on cognitive impairment using normally aged mice.

Abbreviations: NORT, novel object recognition test; DI, discrimination index; GFAP, glial fibrillary acidic protein.

MATERIALS AND METHODS

Animals

C57BL/6J male mice aged 8 and 68 weeks (Charles River Japan, Tokyo, Japan) were maintained at the Kirin Company Ltd. The Animal Experiment Committee of Kirin Company Ltd. approved all experiments, which were conducted from May 2017 to April 2019 in strict accordance with their guidelines (Approval No.; AN10607-Z00). The body weights did not differ between the aged mice groups. We made every possible effort to minimize suffering.

Mice were maintained at 23 ± 1°C under a constant 12-h light/dark cycle (light on from 8:00 a.m. to 8:00 p.m.). Behavioral pharmacological tests were performed in a sound-isolated room under the same conditions after 24 h habituation. Mice were fed a standard purified rodent diet (AIN-93M, Oriental Yeast, Tokyo, Japan) supplemented with 0, 0.05% (w/w) β-lactolin (GTWY peptide, Bachem, Bubendorf, Switzerland), or 5% (w/w) whey peptide rich in β-lactolin (whey peptide contains 1.6 mg of β-lactolin per gram and other components were amino acids and di, tripeptides Kirin Holdings Co. Ltd.), which was used in our previous clinical trials, for 3 months. Previously we showed that β-lactolin contributed to improve cognitive function as a responsible agent. Evaluation of the condition of skin and hair (glossiness, coarseness, hair loss, and ulcer) was performed using the senescence grading score system previously reported (25, 26). After each behavioral evaluation, samples taken from the brains of the mice were used for subsequent biochemical evaluations. Mice were euthanized in a chamber filled with 5% isoflurane vapor (Wako, Osaka, Japan). We tested 15 young and 15 aged mice with AIN-93M, 15 aged mice with AIN-93M containing 0.05% (w/w) β-lactolin, and 15 aged mice with 5% (w/w) whey peptide.

Novel Object Recognition Test

To evaluate episodic memory, we performed the NORT in accordance with the methods described in our previous study (14). The NORT was performed during the light period in a polyvinyl chloride box (25 × 40 × 20 cm) without a roof. For the acquisition trial, we used a pair of wooden triangle poles (4.5 × 4.5 × 4.5 cm) or wooden pyramids (4.5 × 4.5 × 4.5 cm); for the retention trial, we used a pair of poles or pyramids and a golf ball (4.5 cm diameter). In all trials, we placed the objects 7.5 cm from the corner of the box. In the acquisition trial, mice were allowed to explore the box with the two objects for 10 min at 1 h after oral administration of the test sample. Twenty-four hours after the acquisition trial and 1 h after oral administration, the mice were permitted to explore the box with the novel and familiar objects for 5 min. The discrimination index (DI) was calculated using the following equation:

$$\frac{\textit{novel object exploration time} - \textit{familiar object exploration time}}{\textit{total exploration time}}$$

Thus, equal exploration of both objects was indicated by a DI of 0.

Measurements of Activity, Food Intake, and Water Intake in Home Cages

We monitored the amount of food intake, water intake, and ambulatory activity in the home cages using a three-point meter (O'Hara & Co., Ltd., Tokyo, Japan) for 72 h according to a previously described method (27). Activity was monitored by infrared beams positioned on the X and Y axes around the home cages; the beams automatically measured the positions of the mice and their movement. In this experiment, we measured the moving distance of each mouse every 10 min for a total of 72 h.

Quantification of Cytokines and Chemokines

To measure cytokine levels, we homogenized the hippocampus and frontal cortex from the left hemisphere in Tris-buffered saline (Wako) with a multi-bead shocker (Yasui Kikai, Osaka, Japan). After centrifugation at 50,000 × *g* for 20 min at 4°C, we collected the supernatant. We measured the total protein concentration of each supernatant using a BCA protein assay kit (ThermoScientific, Yokohama, Japan). We assayed the supernatant to quantify cytokines and chemokines using a Bio-Plex assay system (Bio-Rad, Hercules, CA, USA).

Immunohistochemistry

The right-brain hemispheres were fixed in 10% formalin solution (Wako), paraffin-embedded, and cut into 5 μm serial sections to evaluate infiltration of activated microglia, astrocytes, and cytochrome C. The studied brain regions included the hippocampus and cerebral cortex (bregma 2.30 mm posterior). After the sections were dewaxed and rehydrated, those for ionized calcium-binding adaptor molecule 1 (Iba-1), glial fibrillary acidic protein (GFAP), and cytochrome C measurements were autoclaved at 121°C for 10 min in 0.2% citrate buffer (pH 6.0) for antigen retrieval. The sections were then incubated with blocking solution (8% w/v skimmed milk) for 30 min after inactivation of endogenous peroxidase with 3% H_2O_2 (Wako) in methanol for 5 min. Subsequently, the sections were incubated overnight at room temperature with primary antibodies: polyclonal anti-Iba-1 antibodies (Wako, 1: 500), polyclonal anti-GFAP antibodies (Dako, Glostrup, Denmark, 1: 500), or monoclonal anti-cytochrome C antibodies (Santa Cruz, CA, USA, 1: 100). After the sections were incubated with horseradish peroxidase-coupled goat anti-mouse or rabbit IgG antibodies (4 μg/mL, Nichirei, Tokyo, Japan) for 1 h at room temperature, they were visualized with 3,3'-diaminobenzidine (Wako) and counterstained with hematoxylin. The size of the positive region per area evaluated was measured using Image J image analysis software (National Institutes of Health, Bethesda, MD, USA).

Statistical Analyses

Data are presented as the mean and the error bars as the standard error of the mean. We analyzed data by one-way analysis of variance, followed by the Tukey–Kramer test or analyzed data by student's *t*-test. All statistical analyses were performed with the Ekuseru-Toukei 2012 software program (Social Survey Research Information, Tokyo, Japan). We considered a p-value < 0.05 to be statistically significant.

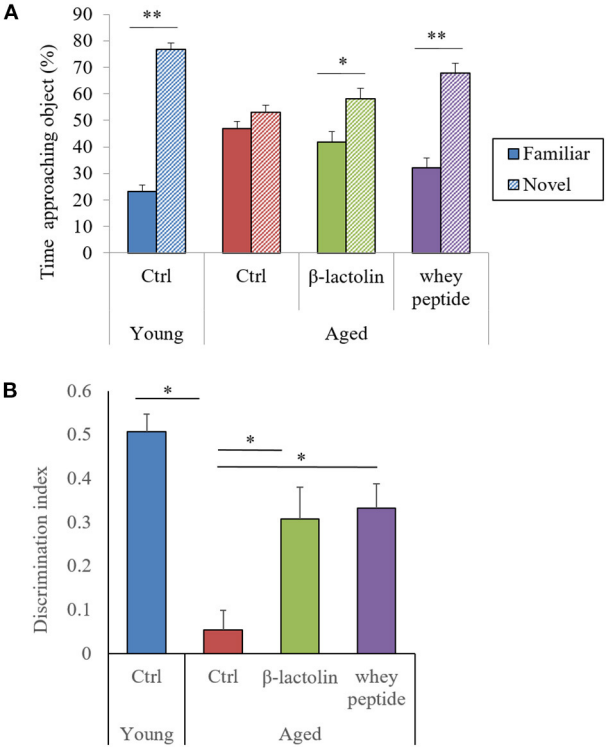

FIGURE 1 | Novel object recognition test. The time taken to approach a novel or familiar object was measured **(A)**, and the discrimination index **(B)** in the novel object recognition test was calculated. Data are represented as mean ± SE and were analyzed by Student *t*-test **(A)** and one-way ANOVA, followed by the Tukey–Kramer test **(B)**. *$p < 0.05$ and **$p < 0.01$.

RESULTS

Preventive Effects of β-Lactolin on Age-Related Memory Impairment in Aged Mice

To evaluate the effects of β-lactolin and whey peptide rich in β-lactolin on memory impairment in aged mice, we fed mice aged 8 weeks (young) and 65 weeks (aged) a diet containing β-lactolin or whey peptide for 3 months and subjected them to behavioral memory evaluations using the NORT. The time taken by young mice to approach a novel object was significantly longer than that to approach a familiar object, but this difference was not observed at a significant level in the control aged mice; however, it was observed in the aged mice fed β-lactolin or whey peptide (**Figure 1A**). This difference in approaching time between familiar object and novel object was greater in aged mice with whey peptide than those with β-lactolin. There was not significantly different in approaching time for novel object among the groups. The DI for control aged mice was significantly decreased compared with that for young mice, and for aged mice

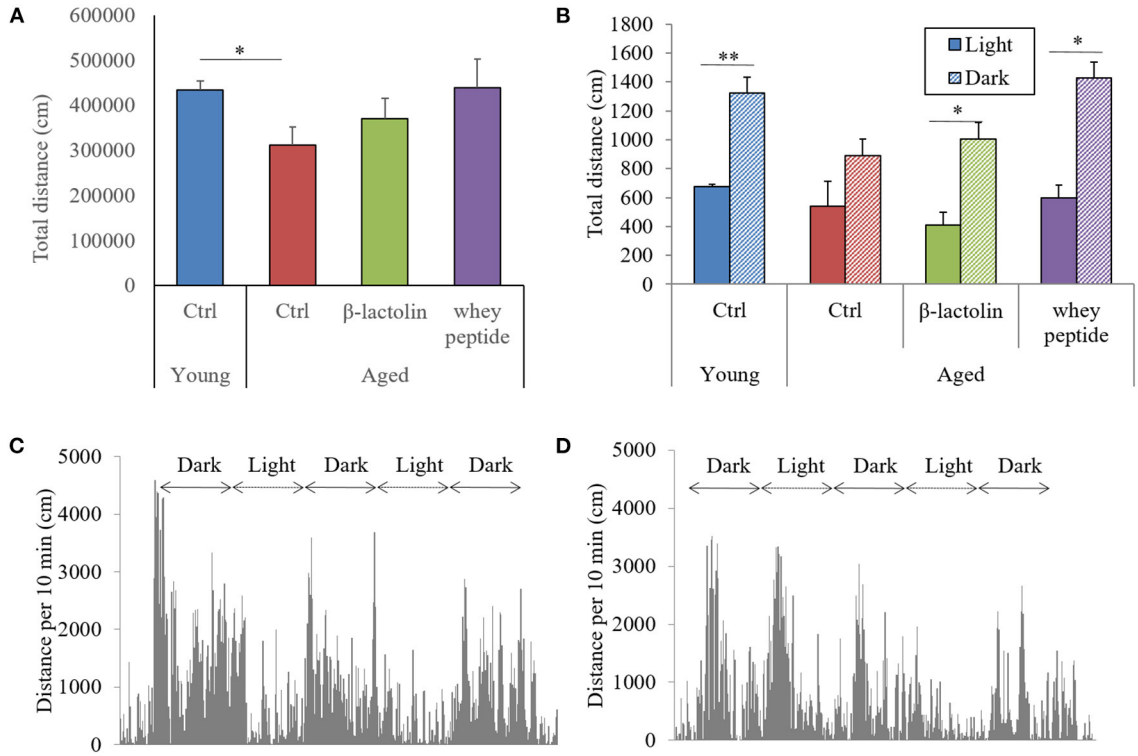

FIGURE 2 | Measurement of activity during light/dark cycles. Distance traveled every 10 min over 72 h. Total distance in the home cage over 72 h **(A)** and the distance over 10 min during light and dark periods **(B)**. Distance grap every 10 min over 72 h. by young mice **(C)** and aged control mice **(D)**. Data are represented as mean ± SE and were analyzed by Student *t*-test **(B)** and one-way ANOVA, followed by the Tukey–Kramer test **(A)**. $^{*}p < 0.05$ and $^{**}p < 0.01$.

fed with β-lactolin or whey peptide, the DIs were significantly increased compared with that for control aged mice (**Figure 1B**). The total time taken to approach each object did not differ among groups. These results indicate that object recognition memory in mice 80 weeks old had declined with age.

Effects of β-Lactolin on Locomotor Activity During Light/Dark Cycles in Aged Mice

To evaluate the effects of β-lactolin and whey peptide rich in β-lactolin on locomotor activity in aged mice, we monitored the activity of mice in their home cages for 72 h. The total distance traveled over 72 h by control aged mice was significantly decreased compared with that traveled by young mice, and this reduction was attenuated by β-lactolin and whey peptide (**Figure 2A**). Young mice showed a distinctive cycle of locomotor activity, which was significantly higher during dark periods than during light periods. This cycle was not observed in control aged mice (**Figure 2B**). Young mice showed a distinctive cycle of locomotor activity per 10-min period, which was higher during dark periods and lower during light periods (**Figure 2C**); the amplitude of this cycle was, however, diminished in aged mice (**Figure 2D**). On the other hand, this light-dark cycle was observed in aged mice fed β-lactolin or whey peptide (**Figure 2B**). However, there was no significant difference on total distance during light or dark periods among the group. These results indicate that activity and sleep cycles were impaired in aged mice; compared with control aged mice, aged mice fed β-lactolin or whey peptide showed improvements in these impairments. The amounts of food and water intake did not differ significantly among the groups (**Supplementary Figures 1A,B**). The body weight among the aged groups was not changed (**Supplementary Figure 1C**). Notably, the weight of the hippocampus was significantly reduced in aged mice compared with that in young mice, and in aged mice fed β-lactolin or whey peptide, it was increased compared with that in control aged mice (**Supplementary Figure 1D**).

Effects of β-Lactolin on Glial Activation and Cytochrome C in Aged Mice

To evaluate the age-induced effects of β-lactolin and whey peptide rich in β-lactolin on glial activation and cytochrome C, we performed immunohistochemical analysis. The positive areas of Iba-1 in the hippocampus and cerebral cortex of control aged mice were significantly increased compared with those in young mice (**Figures 3A,B**, respectively). The Iba-1 positive areas in the hippocampus and cerebral cortex of aged mice fed β-lactolin were significantly lower than those in control aged mice (**Figures 3A,B**, respectively), and in the

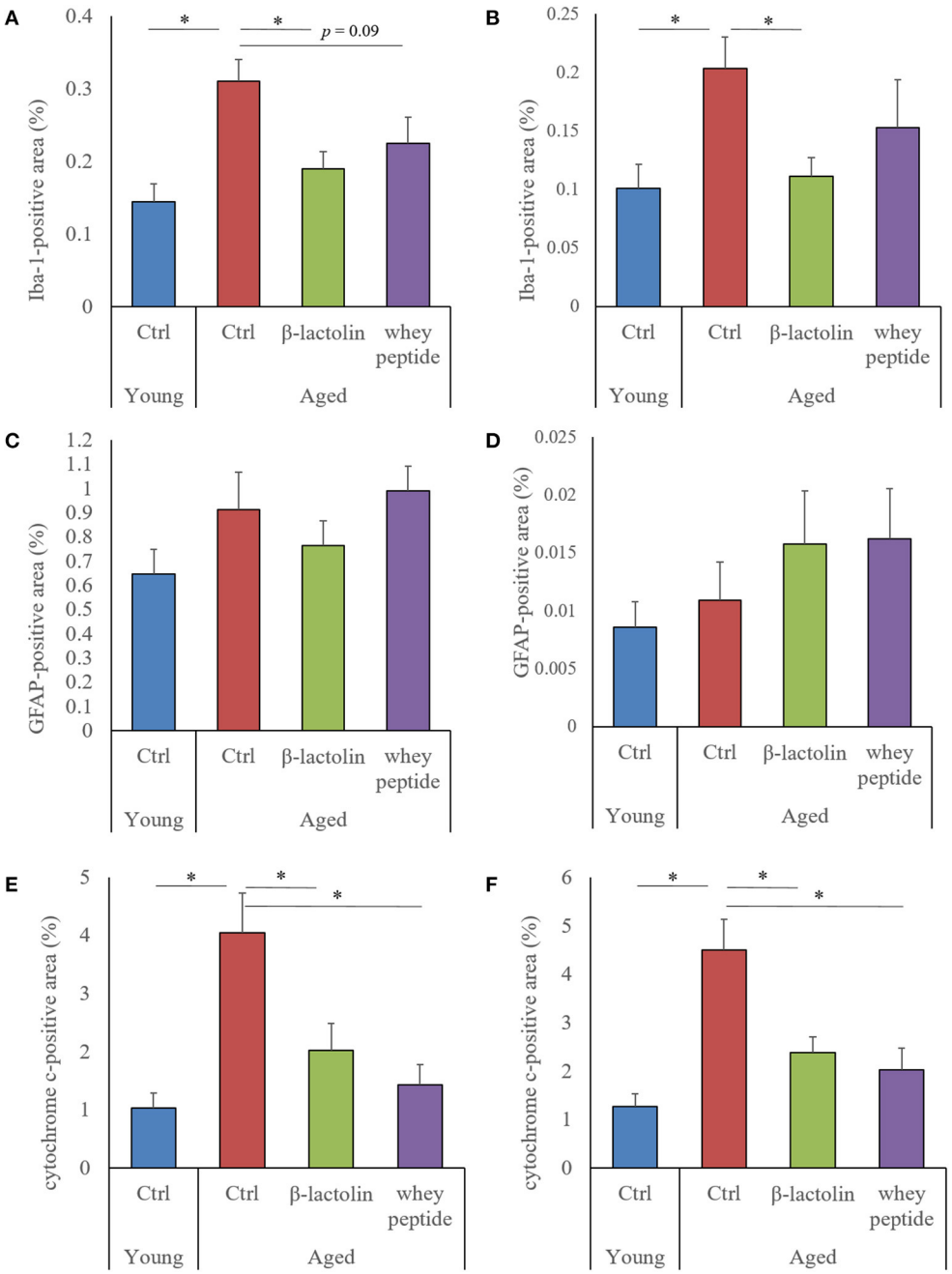

FIGURE 3 | Measurement of glial activation and cytochrome C in aged mice. In the hippocampus and cerebral cortex: percentage of the Iba-1-positive area (**A,B**, respectively); percentage of the GFAP-positive area (**C,D**, respectively); percentage of the cytochrome C-positive area (**E,F**, respectively). Areas were detected by immunohistochemistry. Data are represented as mean ± SE and were analyzed by one-way ANOVA, followed by the Tukey–Kramer test. *$p < 0.05$.

hippocampus of aged mice fed whey peptide, it tended to be significantly lower than that in control aged mice. The representative images in young mice, control aged mice, and aged mice fed with β-lactolin or whey peptide were shown in **Supplementary Figures 2A–D**, respectively. The positive areas of GFAP in the hippocampus and cerebral cortex did not show significant differences between young mice and aged mice (**Figures 3C,D**, respectively). The positive areas of cytochrome C in the hippocampus and cerebral cortex of the control aged mice were significantly increased compared with those of young mice and were significantly reduced in aged mice fed β-lactolin or whey peptide (**Figures 3E,F**, respectively).

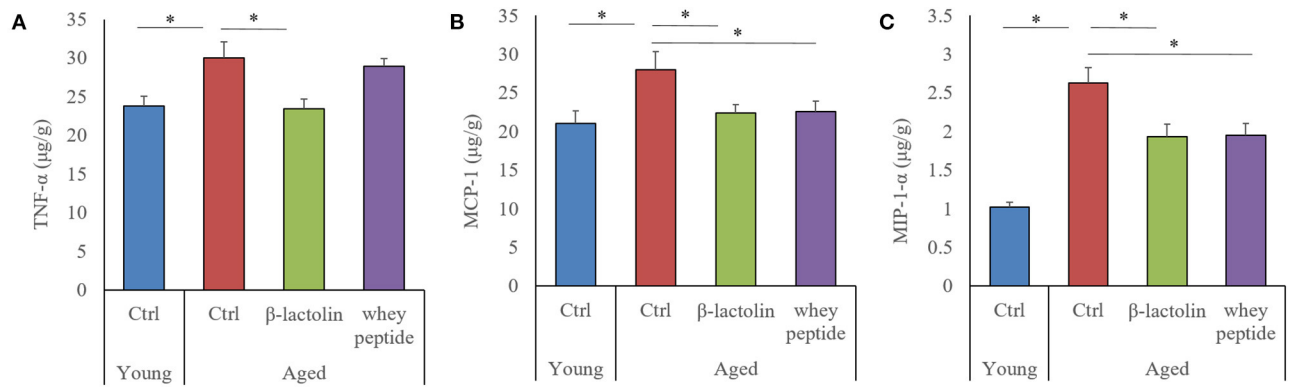

FIGURE 4 | Levels of cytokines and chemokines in the hippocampus of aged mice. The levels of TNF-α, MCP-1, and MIP-1α in the hippocampus (**A–C**, respectively). Data are represented as mean ± SE and were analyzed by one-way ANOVA, followed by the Tukey–Kramer test. *$p < 0.05$.

Effects of β-Lactolin on Cytokine and Chemokine Production in Aged Mice

The levels of tumor necrosis factor-α (TNF-α), macrophage chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-1α (MIP-1α) were measured to evaluate the age-induced effects of β-lactolin and whey peptide rich in β-lactolin on these proteins in the hippocampus of aged mice. The levels of TNF-α, MCP-1, and MIP-1α in the hippocampus of aged mice were significantly increased compared with those in the hippocampus of young mice (**Figures 4A–C**, respectively). The level of TNF-α in aged mice fed β-lactolin and the levels of MCP-1 and MIP-1α in aged mice fed β-lactolin or whey peptide were significantly reduced compared with those in control aged mice. These results indicate that age-related inflammation was induced in the hippocampus of aged mice, and those reactions were reduced by consumption of β-lactolin or whey peptide rich in β-lactolin.

DISCUSSION

Our study demonstrated that the consumption of β-lactolin or whey peptide rich in β-lactolin reduced age-related inflammation in the brain and improved memory impairment associated with aging. Aged mice displayed microglial activation, increases in levels of inflammatory molecules and apoptotic molecules of cytochrome C in the hippocampus and cerebral cortex, impairment of object recognition memory, and disruption of light/dark activity circadian cycles; these effects were improved by the administration of β-lactolin.

In aged mice, microglial inflammation has been reported to be induced by Aβ and/or other antigens; activated microglia produce pro-inflammatory cytokines and reactive oxygen species, both of which lead to cognitive impairment (28, 29). Our previous study showed that the levels of Aβ and glutamate in the hippocampus in aged mice were increased compared with young mice (27). Moreover, object recognition memory, assessed by the NORT in the present study, is a hippocampus-dependent type of memory (30–32). Inflammation in the hippocampus is reported to impair object recognition memory, and the suppression of inflammation subsequently improves these impairments (33, 34). These reports therefore suggest that β-lactolin and whey peptide rich in β-lactolin suppressed microglial inflammatory responses, including the production of the pro-inflammatory cytokine TNF-α and the chemokines MCP-1 and MIP-1α, resulting in the prevention of hippocampal cognitive decline associated with aging. In the current study, the preventive effects of whey peptide on microglia infiltration and TNF-α production was not observed, which was observed in the β-lactolin group. Recent study showed that BCAA (branched-chain amino acids) rich in whey peptide increase reactive oxygen and inflammation (35), which might affect the differences on the results between β-lactolin group and whey peptide group.

The current study also showed that cytochrome C is increased in the hippocampus and cerebral cortex and that the weight of the hippocampus was reduced in aged mice compared with these parameters in young mice. Cytochrome C is known as a mitochondria-related molecule that activates caspase signaling to induce apoptosis and is associated with inflammation (36). Mitochondrial dysfunction has been reported to be associated with hippocampal atrophy and memory impairment (37). Consequently, it is suggested that β-lactolin and whey peptide rich in β-lactolin may improve mitochondrial dysfunction, subsequently improving inflammatory responses and hippocampal atrophy and resulting in the improvement of cognitive function.

In our study, we further demonstrated the impairment of light/dark activity cycles in aged mice. Circadian rhythms are reportedly impaired in aged mice via sympathetic dysfunction in peripheral clock regulation (38). Neuroinflammation has also been associated with the dysfunction of circadian rhythms (39). The regulation of inflammation in the brain by β-lactolin and whey peptide rich in β-lactolin might improve age-related impairment of light/dark activity cycles. The current study also

showed that β-lactolin and whey peptide rich in β-lactolin suppressed the increases in senescence scores in aged mice (**Supplementary Figure 3**). β-lactolin may help prevent not only brain inflammation and cognitive decline but also other senescence phenotypes. Further study using aged mice is required to investigate these effects in detail.

Epidemiological studies have shown that regular intake of dairy products prevents cognitive decline in the elderly (9, 10), andour previous randomized clinical trials demonstrated that, compared with placebo, supplementation with whey peptide rich in β-lactolin for 6 or 12 weeks improves memory impairment (18, 19) and enhances cerebral blood flow (21, 22) and neural activity (20) in elderly with subjective cognitive decline. These reports expect that supplementations with β-lactolin for a long period is beneficial to prevent the age-related cognitive decline, on the other hand, the effects of long-term supplementation with β-lactolin, which is abundant in fermented dairy products, on age-related cognitive decline have not been investigated. As a mechanisms, in a pharmacokinetics study using radioisotope-labeled β-lactolin, orally administered β-lactolin was smoothly absorbed into the blood and delivered to each brain region (14). A previous study showed that β-lactolin inhibits the activity of monoamine oxidase B and increases the level of dopamine (14), neither of which is directly involved in inflammation. However, β-lactolin might be also associated with other molecular inflammation-related targets, such as mitochondria. High levels of some cytokine have been suggested to be associated with dementia and cognitive decline (40). The current study speculates that long-term supplementation with β-lactolin will be beneficial for the prevention of cognitive decline. Further clinical trials should investigate the effect of β-lactolin on inflammatory molecules and mitochondria-related molecules in the blood to elucidate the mechanism in humans.

The current study has some limitations. We performed novel object recognition test and Y-maze test to evaluate the memory function, whereas we could not detect the age-related deficit in Y-maze test. Further study need to evaluate another behavioral evaluation such as Morris water maze. We observed the difference of the activities during light and dark cycle between the young mice and aged mice but did not evaluate the circadian gene expressions including PERs and CRYs. To discuss the effects of β-lactolin on aged-related circadian rhythms, further study need to measure the gene expressions. We also observed β-lactolin suppressed the cytochrome c in aged mice, suggesting that β-lactolin improves the mitochondria related apoptosis, but did not evaluate the effects of β-lactolin on the activity of mitochondria. Further study need to evaluate the effects of β-lactolin on mitochondria.

In conclusion, our study demonstrated that β-lactolin, a β-lactoglobulin-derived lactopeptide, prevents age-related inflammation in the brain and cognitive decline in aged mice. The results of the current study support those of previous epidemiological studies as well as our previous reports. The consumption of β-lactolin and whey peptide rich in β-lactolin is a safe and easy practice to adopt in daily life and may be a beneficial approach to the prevention of aging-related brain disorders.

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 animal study was reviewed and approved by The Animal Experiment Committee of Kirin Company Ltd.

AUTHOR CONTRIBUTIONS

YA conducted the experiment and wrote the manuscript. RO did the experiment in the behavioral evaluations. AT, KU, and HN designed and conducted this research. 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/fnut.2021.724134/full#supplementary-material

Supplementary Figure 1 | Food and water consumption and body weight in aged mice. Food **(A)** and water **(B)** consumption over 72 h was monitored. Body weight and hippocampal weight **(C,D)**. Data are represented as mean ± SE and were analyzed by two-way ANOVA, followed by the Tukey–Kramer test.

Supplementary Figure 2 | Measurement of glial activation in aged mice. Iba-1-positive microglia and GFAP-positive astrocyte were detected by immunohistochemistry. **(A–D)** Representative immunohistochemistry images for Iba-1 in youg mice, control aged mice, and aged mice fed β-lactolin or whey peptide, respectively. **(E–H)** Representative immunohistochemistry images for GFAP in youg mice, control aged mice, and aged mice fed β-lactolin or whey peptide, respectively. Scale bars, 400 μm.

Supplementary Figure 3 | Senescence scores for aged mice. Senescence scores in the skin and hair category are shown. Data are represented as mean ± SE and were analyzed by one-way ANOVA, followed by the Tukey–Kramer test. $^{*}p < 0.05$ and $^{**}p < 0.01$.

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Metformin Use and Cognitive Function in Older Adults With Type 2 Diabetes Following a Mediterranean Diet Intervention

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Background and Purpose: Both adherence to the Mediterranean diet (MedDiet) and the use of metformin could benefit the cognitive performance of individuals with type 2 diabetes, but evidence is still controversial. We examined the association between metformin use and cognition in older adults with type 2 diabetes following a MedDiet intervention.

Methods: Prospective cohort study framed in the PREDIMED-Plus-*Cognition* sub-study. The PREDIMED-Plus clinical trial aims to compare the cardiovascular effect of two MedDiet interventions, with and without energy restriction, in individuals with overweight/obesity and metabolic syndrome. The present sub-study included 487 cognitively normal subjects (50.5% women, mean ± SD age of 65.2 ± 4.7 years), 30.4% of them ($N = 148$) with type 2 diabetes. A comprehensive battery of neurocognitive tests was administered at baseline and after 1 and 3 years. Individuals with type 2 diabetes that exhibited a good glycemic control trajectory, either using or not using metformin, were compared to one another and to individuals without diabetes using mixed-effects models with inverse probability of treatment weights.

Results: Most subjects with type 2 diabetes (83.1%) presented a good and stable glycemic control trajectory. Before engaging in the MedDiet intervention, subjects using

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metformin scored higher in executive functions (Cohen's $d = 0.51$), memory (Cohen's $d = 0.38$) and global cognition (Cohen's $d = 0.48$) than those not using metformin. However, these differences were not sustained during the 3 years of follow-up, as individuals not using metformin experienced greater improvements in memory ($\beta = 0.38$ vs. $\beta = 0.10$, $P = 0.036$), executive functions ($\beta = 0.36$ vs. $\beta = 0.02$, $P = 0.005$) and global cognition ($\beta = 0.29$ vs. $\beta = -0.02$, $P = 0.001$) that combined with a higher MedDiet adherence (12.6 vs. 11.5 points, $P = 0.031$). Finally, subjects without diabetes presented greater improvements in memory than subjects with diabetes irrespective of their exposure to metformin ($\beta = 0.55$ vs. $\beta = 0.10$, $P < 0.001$). However, subjects with diabetes not using metformin, compared to subjects without diabetes, presented greater improvements in executive functions ($\beta = 0.33$ vs. $\beta = 0.08$, $P = 0.032$) and displayed a higher MedDiet adherence (12.6 points vs. 11.6 points, $P = 0.046$).

Conclusions: Although both metformin and MedDiet interventions are good candidates for future cognitive decline preventive studies, a higher adherence to the MedDiet could even outweigh the potential neuroprotective effects of metformin in subjects with diabetes.

Keywords: cognition, Mediterranean diet, type 2 diabetes, metformin, metabolic syndrome, obesity, nutrition, overweight

INTRODUCTION

The prevalence of diabetes has reached epidemic proportions in the past few years. There are currently 463 million people living with diabetes, representing 8.5% of the world's adult population (1). It is estimated that diabetes affects 1 in 10 adults in Spain (1). Individuals with diabetes are at increased risk of blindness, lower limb amputation, and cardiovascular and kidney diseases, and have about 60% greater risk of developing dementia (2).

Diabetes consequences can be avoided or delayed with good glycemic control and the management of cardiovascular risk factors, which can be achieved by following a healthy diet, practicing regular physical activity, and smoking cessation (3). In individuals with type 2 diabetes, the traditional Mediterranean diet (MedDiet) has been shown to improve their glycemic control, various cardiovascular risk factors, and body weight (4, 5). In individuals without diabetes but with high cardiovascular risk, the MedDiet has also been shown to decrease the incidence of diabetes (6). Moreover, adherence to the MedDiet has been associated with improvements in some cognitive functions (7). Therefore, although type 2 diabetes has been associated with worse performance in executive functions (8), MedDiet interventions could be beneficial controlling glycemic levels and could prevent cognitive decline and even improve cognition in individuals with type 2 diabetes.

As lifestyle and weight management alone often fail to establish and sustain optimal glycemic control, glucose-lowering treatments are also an important component of diabetes management (3). Diabetes drugs may have indirect effects on the brain by affecting circulating concentrations of insulin and glucose (9, 10). However, although type 2 diabetes has been consistently associated with incident dementia, distinguishing between treated and untreated diabetes as a risk factor for dementia is challenging in most observational studies (11). Metformin has been used since the 1950s as first-line pharmacotherapy for treating patients with type 2 diabetes with good glycemic control because of its glucose-lowering effects, good safety profile and relatively low cost. However, for patients with poor glycemic control after this first-line therapy, alternative oral glucose-lowering medications and/or injectable insulin are preferable, in monotherapy or in combination with other therapy regimens (12).

Previous studies have shown that metformin use for more than 6 years was associated with lower risk of cognitive impairment (13) and with better performance in some cognitive domains over time in cognitively normal subjects with type 2 diabetes (14). However, there is currently considerable controversy about the effect of metformin on cognition (15). Given the varied response to glucose lowering medications and the heterogeneity in type 2 diabetes (16), the identification of different glycemic trajectory subgroups could help optimize the study of the effects of metformin on cognition. Thus, the analysis of trajectories of glycemic control is an important initial step toward the application of personalized medicine in the treatment of diabetes as it could help in the development of targeted strategies to improve the effectiveness of interventions (17).

The objective of this study is to characterize different glycemic trajectories subgroups and to examine the association between metformin use and cognition in subjects with type 2 diabetes that participated in the PREDIMED-Plus MedDiet intervention.

MATERIALS AND METHODS

Study Design and Participants

The present study is a prospective cohort study framed in the PREDIMED-Plus-*Cognition* sub-study, using a subset of

participants ($N = 487$) of the PREDIMED-Plus trial. Full details of the study design and procedures of the PREDIMED-Plus trial have been published elsewhere (5). Further details on the study inclusion/exclusion criteria as well as the study protocol are available at http://predimedplus.com/. Briefly, the PREDIMED-Plus study is an ongoing multi-center randomized parallel-group primary prevention trial ($N = 6{,}874$) designed to assess and compare the long-term effectiveness of an intensive lifestyle intervention with an energy-reduced Mediterranean diet (er-MedDiet, intervention group), physical activity (PA) promotion and behavioral support of weight loss goals, with a more common intervention featuring energy-unrestricted traditional MedDiet recommendations without any recommendations on PA and weight loss strategies (control group). In order to promote the adherence to the MedDiet both groups were free provided with an allotment of extra-virgin olive oil (1 L/month) and occasionally, tree nuts (125 g/month). Participant's recruitment took place between October 2013 and December 2016 across 23 Spanish hospitals, universities and research institutes. Participants were randomly assigned, in a 1:1 ratio, to intervention and control groups. The eligibility criteria for participants were community-dwelling adults with overweight grade II (18) or obesity [body mass index (BMI) between 27 and 40 kg/m^2] from Primary Care Health Centers of the Spanish National Health System aged between 55 and 75 years in the case of men and between 60 and 75 years in women who met at least three criteria for metabolic syndrome. The clinical trial is registered at the International Standard Randomized Controlled Trials database (ISRCTN; 89898870).

Four study sites participated in the PREDIMED-Plus-*Cognition* sub-study, with an in-depth assessment of the cognitive performance at baseline, 1 and 3 years after the initiation of the assigned PREDIMED-Plus intervention: Hospital del Mar Medical Research Institute (IMIM), Barcelona; Pere Virgili Institute for Health Research (IISPV), Reus; University of Valencia (UV), Valencia; Bellvitge University Hospital (HUB), Hospitalet de Llobregat. Exclusion criteria for the present sub-study are included in **Supplementary Table 1**. The data were analyzed using the PREDIMED-Plus-*Cognition* database dated 14th January 2021. All participants gave written informed consent. The protocol of the PREDIMED-Plus-*Cognition* sub-study was approved by the local Research Ethics Committees from the participating centers and adheres to the standards of the World Medical Association (WAMA) Declaration of Helsinki.

Outcomes and Assessments

Type 2 Diabetes

According to the American Diabetes Association criteria (19), type 2 diabetes was defined by previous clinical diagnosis of diabetes, HbA1c $\geq$ 6.5% (48 mmol/mol) or fasting plasma glucose >126 mg/dL at both the screening and baseline visit or use of oral anti-diabetic medication (metformin, dipeptidyl peptidase 4 inhibitors, sulfonylureas, insulin secretagogues, SLGT2 inhibitors or thiazolidinediones) or use of insulin.

Cognitive Performance

Cognitive function was assessed by trained neuropsychologists blinded to the participants' group assignment and included the following cognitive domains: (i) *Short-term and long-term auditory memory*, using the Rey's Auditory-Verbal Learning Test (RAVLT) (20); (ii) *Visuoconstructive praxis and attention*, *short- and long-term visuospatial memory and visual perception*, evaluated with the Rey–Osterrieth complex figure Test (RCFT) (21); (iii) *Processing speed*, evaluated with the Symbol Digit Modalities Test (SDMT) (22); (iv) *Inhibition and attention* (mental flexibility and interference resistance), evaluated with the Stroop Color-Word Interference Test (23); (v) *Decision-making abilities* (risk and reward and punishment values), evaluated with the Iowa Gambling Task (IGT) (24) (not administered to participants recruited at the UV site); (vi) *Inattentiveness, impulsivity, sustained attention and vigilance* evaluated with the Conners' Continuous Auditory Test of Attention (CPT) (25) (not administered to participants recruited at the UV site). Finally, a cognitive screening was also included at baseline using the Folstein Mini-Mental State Examination (MMSE) (26).

Composite scores of 3 cognitive domains, namely memory, executive function and global cognition, were calculated for each participant by standardizing raw test scores to z-scores using the mean and standard deviation of baseline data. The memory composite included the mean standardized individual scores of the RAVLT immediate and delayed scores and the RCFT immediate, delayed and recognition scores. The executive function composite included the RCFT copy score, the SDMT direct score, the Stroop interference score, the IGT total score and the CPT omission, commission and hit reaction time scores. Lastly, the global cognition composite included all the tests of memory and executive functions.

Depressive Symptomatology

The severity of depressive symptomatology was assessed using the Beck's Depression Inventory-II (BDI-II) (27) and was categorized according to general guidelines as no or minimal depression (0–9 points), mild-to-moderate depression (10–18 points), moderate-to-severe depression (19–29 points), and severe depression ($\geq$30 points).

Anthropometry and Cardiovascular Biomarkers

Weight and height were measured by nurses using standardized procedures. BMI (kg/m^2) was categorized as normo-weight (BMI 18.5–24.9 kg/m^2), overweight (BMI 25.0–29.9 kg/m^2), obesity I (BMI 30.0–34.9 kg/m^2), and obesity II (BMI 35.0–39.9 kg/m^2).

Fasting blood glucose, HbA1c and lipid levels (triglycerides, total cholesterol and HDL cholesterol) were determined using standard methodology after an overnight fast. LDL cholesterol concentrations were calculated using the Friedewald formula whenever triglycerides were lower than 300 mg/dL. Insulin was centrally measured by an electrochemiluminescence immunoassay using an Elecsys immunoanalyzer (Roche Diagnostics, Meylan, France). Insulin resistance was estimated at baseline using the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) index (28).

Intervention Adherence

Adherence to the er-MedDiet was evaluated with an adapted version of the validated 14-item PREDIMED questionnaire including 17-items, the energy-restricted Mediterranean Diet Adherence Screener (er-MEDAS) (29). Values ranged from 0 to 17 points and adherence was categorized as low (0–7 points), moderate (8–10 points), and high (11–17 points) using the cut-off values from previous studies based on approximate tertiles in the overall baseline PREDIMED-Plus sample (30). Physical activity categories (sedentary, under-active, moderately active, and active) were obtained from the Rapid Assessment of Physical Activity (RAPA) questionnaire (31).

Covariates

Covariates were evaluated at baseline through face-to-face interviews by trained staff using self-reported general questionnaires on socio-demographics (gender, age, years of education, employment status), lifestyle (smoking status), medication (use of treatment for high cholesterol, use of tranquilizers, or sedatives for anxiety or sleeping, use of medication for hypertension, use of medication for heart) and history of disease.

Statistical Analyses

Identification of Latent HbA1c Trajectories Subgroups

Longitudinal finite mixture modeling was applied to explain the between-subject heterogeneity in growth of HbA1c by identifying latent classes or subgroups with different growth trajectories (32). Thus, each latent class represents a group of subjects sharing a similar HbA1c trajectory. We first applied latent class growth analysis (LCGA), and two different types of growth mixture modeling (GMM): GMM with random intercept and GMM with random intercept and slope. In each type of model, we tested 1–5 latent classes, so 15 models were computed in total. In order to select the model with the best or most reasonable representation of the observed data, our model selection criteria were based on lowest fit information criteria statistics, including the Bayesian Information Criterion (BIC), the Akaike Information Criterion (AIC) and the sample-size adjusted BIC (SABIC), as well as at high entropy. Entropy is a measure of classification uncertainty in class assignment, with higher values indicating clearer delineation of classes. All these models did not include any covariates. Once the best model was selected among this first set of 15 models, we compared it with 3 additional models that included covariates (intervention group, diabetes medications or time-by-group interaction) as predictors of the growth factors and the class. Following the previously mentioned criteria for model selection, the best model was finally selected. The selected model presented a good discrimination index, since subjects classified in class 1, 2, and 3 had a mean probability of 98.4, 89.9, and 93.2% of belonging to their class, respectively. See details in **Supplementary Table 2**.

Descriptive and Inferential Statistics

Descriptive statistics of study variables stratified by diabetes status (yes/no) and by diabetes subgroups were obtained as mean and standard deviation (SD) or 95% confidence intervals (95%CI) for continuous variables and percentages for categorical variables. Univariate differences were estimated with the unpaired *t* test for continuous variables and chi-square test or Fisher's exact test, as deemed appropriate, for categorical variables. Additionally, standardized mean differences between groups were computed as Cohen's d (abbreviated as "d") with cut-offs for effect size interpretation of 0.2 (small), 0.5 (medium), 0.8 (large), and 1.2 (very large). Adjusted differences between diabetes subgroups at each time point (baseline, 1 and 3 years) were estimated with analysis of variance (ANOVA) from linear models adjusted by study site, years of education, use of treatment for high cholesterol, use of metformin and use of insulin. Correction for multiple comparisons was performed with the Tukey method when the explanatory variable was normally distributed and the Benjamini & Hochberg method otherwise. Finally, linear mixed effects models were used to test differences between groups in the mean rate of change in cognition from baseline, after 1 and 3 years.

Inverse Probability of Treatment Weights

Given that metformin was not randomly assigned, it was necessary to achieve comparability between groups with regard to pretreatment characteristics to reduce the potential confounding by indication bias and to get better estimates of the treatment effect. This was accomplished using inverse probability of treatment weights (IPTW) (33). IPTW are based on propensity scores estimated via generalized boosted models, a non-parametric machine-learning method that weights treated and control cases to estimate the population average treatment effect (ATE) weights. IPTW were used to generate a weighted "artificial" population (called "pseudo-population") with almost perfect covariate balance, in which treatment and measured pretreatment characteristics are independent. Three different IPTW were computed for each one of the following comparisons: (i) individuals with type 2 diabetes treated with metformin vs. individuals with type 2 diabetes not treated with metformin, (ii) individuals with type 2 diabetes treated with metformin vs. individuals without type 2 diabetes, and (iii) individuals with type 2 diabetes not treated with metformin vs. individuals without type 2 diabetes. Absolute standardized difference in means or proportions (abbreviated as "D"), was used to evaluate comparability between groups before and after IPTW matching. The relative influence of each variable in the models was also reported and expressed as a percentage. When there were residual differences in pretreatment characteristics between groups in the matched sample, regression adjustment was used to control for those unbalanced factors, which is known as a doubly robust approach (33). Accordingly, the comparison between individuals with diabetes exposed and not exposed to metformin was adjusted by sleep apnea. The comparison between subjects with and without diabetes and subjects taking metformin was adjusted by years of education, smoking status, total cholesterol, LDL-cholesterol, and use of treatment for high cholesterol. All the subsequent analyses were performed using weighted regression with robust standard errors.

Missing Data and Software

Missing data was reported as absolute and relative frequencies (*N*, %) and each specific analysis was performed on individuals with complete information on the variables involved. We used the R package "twang" to compute IPTW, the package "survey" to compute the weighted analysis and the package "nlme" to estimate linear mixed effects models.

RESULTS

Sample Characteristics

Baseline characteristics of study participants stratified by diabetes status are included in **Table 1**. Briefly, 50.5% were women, the mean (SD) age was 65.2 (4.7) years, 18.7% were employed and 62.1% were retired. Regarding their lifestyle, 12% were current smokers, most were underactive (66.9%) or sedentary (15.6%) and had a low or medium adherence to the er-MedDiet (45.4 and 41.5%, respectively). All participants were over-weight (27.3%) or obese (48.5% had obesity type I and 24.2% type II). Finally, 50.3% were taking medications for high cholesterol and 23.0% used tranquilizers or sedatives.

Compared to individuals without diabetes, individuals with type 2 diabetes were older (65.9 vs. 64.9 years), had less years of education (10.5 vs. 12.1 years) and took more treatments for high cholesterol (62.2 vs. 45.1%). Moreover, most individuals with diabetes were being treated with metformin (75.0%), only 6.7% were taking insulin and 34.5% were taking other oral medications for diabetes (alone or in combination with metformin or insulin). As expected, participants with diabetes had poorer glycemic profile than those without diabetes, with higher values of HbA1c (mean of 7.0 vs. 5.8%), fasting plasma glucose (mean of 146 mg/dL vs. 103 mg/dL) and HOMA-IR index (mean of 5.0 vs. 7.1).

Figure 1 includes a flow diagram of the follow-up of the present study.

Longitudinal HbA1c Trajectories Subgroups

First, participants with type 2 diabetes were classified into three distinct latent subgroups based on their HbA1c trajectory from baseline to 1 and 3 years of MedDiet intervention. Subgroup 1 (S1) contained most of the subjects with diabetes (83.1%, $N = 123$), and the remaining 11.5% ($N = 17$) and 5.4% ($N = 8$) were grouped into subgroups 2 (S2) and 3 (S3), respectively. **Figure 2A** shows the trajectories of HbA1c for the three different subgroups. At baseline, those in S1 presented good glycemic control (HbA1c $< 7\%$), with a mean (SD) of 6.6% (0.55), which decreased to 6.3% (0.6) after 1 year but following a return to 6.5% (0.7) after 3 years. This subgroup was termed "S1. Good-Stable glycemic control pattern." S2 individuals presented poor glycemic control at baseline (HbA1c $> 7\%$), with a mean (SD) of 9.0% (0.9), but it improved during the follow-up, with values of 7.9% (1.3) after 1 year and of 7.1% (1.1) after 3 years. This subgroup was termed "S2. Poor-Improved glycemic control pattern." Finally, those in S3 also presented poor glycemic control at baseline with high HbA1c levels (mean of 8.1%, $SD = 0.81$). Although after 1 year their glycemic control slightly improved (HbA1c declined to a mean of 7.5%, $SD = 0.70$), after 3 years it worsened and increased to 9.6% (0.7). This subgroup was termed "S3. Poor-Worsen glycemic control pattern." **Figure 2B** shows the HbA1c trajectory of individuals without diabetes.

As shown in **Figure 2C**, glucose trajectories in each subgroup mirrored HbA1c trajectories but with more variability, represented by a wider 95%CI. **Figure 2D** shows the mean glucose trajectory of individuals without diabetes. Regarding MedDiet adherence, at baseline, all subgroups scored a mean of 7.7–7.8 points, which is over the low adherence cut-off of 7 points and thus considered as moderate adherence. All subgroups presented mean improvements in their MedDiet adherence after 1 and 3 years. However, as shown in **Figure 2E**, S3 presented lower MedDiet adherence than S1 after 1 year ($d = -0.87$, 95% CI -1.59, -0.14) and especially after 3 years of follow-up ($d = -1.16$, 95% CI -1.89, -0.43). S2 also scored lower in MedDiet adherence than S1 after 1 year ($d = -0.57$, 95%CI -1.09, -0.04) but after 3 years they scored a high adherence for the MedDiet similar to the observed in S1 (11.5 vs. 11.8 points for S2 and S1, respectively). Despite of these moderate-to-large differences in MedDiet adherence between subgroups, results were not statistically significant, neither at baseline nor at the follow-up, probably due to the small subgroups sample size. See details in **Supplementary Table 3**. **Figure 2F** shows the MedDiet trajectory of individuals without diabetes.

Finally, metformin was used by 70.7% ($N = 87$) of participants from S1, and this treatment was maintained in 89.7% of them after 1 and 3 years of follow-up. From the remaining $N = 36$ participants from S1 that were not taking metformin at baseline, $N = 26$ (72.2%) continued without taking metformin throughout the 3 years of follow-up. Therefore, in S1 the metformin prescription did not change substantially throughout the follow-up (**Supplementary Figure 1**). Moreover, most of subjects from S1 who were not taking metformin did not take any treatment for type 2 diabetes (75%) or were taking other oral antidiabetic drugs (16.7%). The description and comparability between subgroups in terms of baseline characteristics and medications for diabetes is included in **Supplementary Tables 4**, **5**.

Metformin and Cognition in Subjects With Diabetes

Within the main subgroup of subjects with diabetes with a good-stable glycemic control (S1), those subjects using metformin ($N = 87$) were compared to those not using metformin ($N = 36$) (**Supplementary Table 6**). Before applying IPTW, those not using metformin presented higher total cholesterol (213 vs. 184 mg/dL) and higher LDL-cholesterol (131 vs. 108 mg/dL), but these differences vanished after matching with IPTW.

As shown in **Table 2** and **Figure 3**, at baseline, individuals with type 2 diabetes from S1 treated with metformin scored moderately higher in memory ($d = 0.38$, 95% CI -0.02, 0.79; $P = 0.115$), executive functions ($d = 0.51$, 95% CI -0.06, 1.08; $P = 0.086$) and global cognition ($d = 0.48$, 95% CI -0.01, 1.04; $P = 0.124$) than those not treated with metformin. Nonetheless, an effect difference ranging from $d = -0.06$, a very small negative association, to 1.08, a large positive association,

TABLE 1 | Baseline characteristics of study participants stratified by type 2 diabetes (T2D) status and univariate differences.

		All population	No-T2D	T2D	*P* *
Variable	**Category**	***N* (%)**	***N* (%)**	***N* (%)**	
N		487 (100)	339 (100)	148 (100)	
Study group	Intervention group	240 (49.3)	162 (47.8)	78 (52.7)	0.368
Study site	IMIM	116 (23.8)	65 (19.2)	51 (34.5)	**<0.001**
	IISPV	143 (29.4)	131 (38.6)	12 (8.1)	
	UV	70 (14.4)	34 (10.0)	36 (24.3)	
	HUB	158 (32.4)	109 (32.2)	49 (33.1)	
Sociodemographic characteristics					
Sex	Women	246 (50.5)	173 (51.0)	73 (49.3)	0.804
Age	*Mean (SD)*	65.2 (4.7)	64.9 (4.7)	65.9 (4.7)	**0.029**
Education (years)	*Mean (SD)*	11.7 (5.3)	12.1 (5.7)	10.5 (4.0)	**<0.001**
Employment status	Employed	91 (18.7)	68 (20.1)	23 (15.5)	0.611
	Unemployed	36 (7.4)	27 (8.0)	9 (6.1)	
	Housework	50 (10.3)	36 (10.7)	14 (9.5)	
	Retired	302 (62.1)	202 (59.8)	100 (67.6)	
	Missing	1	1		
Lifestyle, obesity and mental health					
Current smoker		59 (12.1)	47 (13.9)	12 (8.11)	0.101
Physical activity[a]	Sedentary	76 (15.6)	48 (14.2)	28 (18.9)	0.082
	Under-active	326 (66.9)	238 (70.2)	88 (59.5)	
	Moderately active	44 (9.03)	25 (7.37)	19 (12.8)	
	Active	41 (8.42)	28 (8.26)	13 (8.78)	
Er-MedDiet adherence[b]	Low	221 (45.4)	150 (44.2)	71 (48.0)	0.741
	Medium	202 (41.5)	144 (42.5)	58 (39.2)	
	High	64 (13.1)	45 (13.3)	19 (12.8)	
BMI category	Over-weight	133 (27.3)	99 (29.2)	34 (23.0)	0.156
	Obesity I	236 (48.5)	164 (48.4)	72 (48.6)	
	Obesity II	118 (24.2)	76 (22.4)	42 (28.4)	
Depressive symptomatology[c]	No or minimal	304 (62.4)	217 (64.0)	87 (58.8)	0.544
	Mild-to-moderate	140 (28.7)	93 (27.4)	47 (31.8)	
	Moderate-to-severe	43 (8.8)	29 (8.5)	14 (9.5)	
Medications					
Metformin		111 (22.7)	0 (0.00)	111 (75.0)	-
Insulin		10 (2.0)	0 (0.00)	10 (6.7)	-
Other treatments for diabetes[d]	51 (10.5)	0 (0.00)	51 (34.5)	-	
Tranquilizers/sedatives	112 (23.0)	72 (21.2)	40 (27.0)	0.201	
Cholesterol treatment	245 (50.3)	153 (45.1)	92 (62.2)	**0.001**	
***Intelligence Quotient (PD)*[e]**	*Mean (SD)*	92.0 (39.6)	88.7 (39.5)	99.5 (38.8)	0.006
Glycemic profile					
Hba1c (%)	*Mean (SD)*	6.1 (0.8)	5.8 (0.4)	7.0 (1.0)	**<0.001**
HbA1c (mmol/mol)	*Mean (SD)*	43.5 (9.2)	39.8 (4.2)	52.6 (11.2)	**<0.001**
Glucose (mg/dL)	*Mean (SD)*	116 (30.9)	103 (13.2)	146 (38.7)	**<0.001**
HOMA-IR index	*Mean (SD)*	5.6 (3.9)	5.0 (3.1)	7.1 (5.2)	**<0.001**

**T-test for continuous variables [presented as mean (SD)]; and chi-squared test (or Fisher's exact test when expected count in some cells is lower than 5) for categorical variables.*

[a]Obtained from the Rapid Assessment of Physical Activity (RAPA) questionnaire.

[b]Er-MedDiet, energy-restricted Mediterranean Diet adherence, from the 17-item er-MedDiet questionnaire.

[c]Obtained from the Beck's Depression Inventory-II (BDI).

[d]Includes: dipeptidyl peptidase 4 inhibitors (N = 35), sulfonylureas (N = 21), insulin secretagogues (N = 11), SLGT2 inhibitors (N = 6), thiazolidinediones (N = 1), and others (N = 0).

[e]Obtained from the WAIS-III Vocabulary Subtest.

HbA1c, glycosylated hemoglobin; IMIM, Hospital del Mar Medical Research Institute; IISPV, Pere Virgili Institute for Health Research; UV, University of Valencia; HUB, Bellvitge University Hospital. Bold values denote statistical significance at the $p < 0.05$ level.

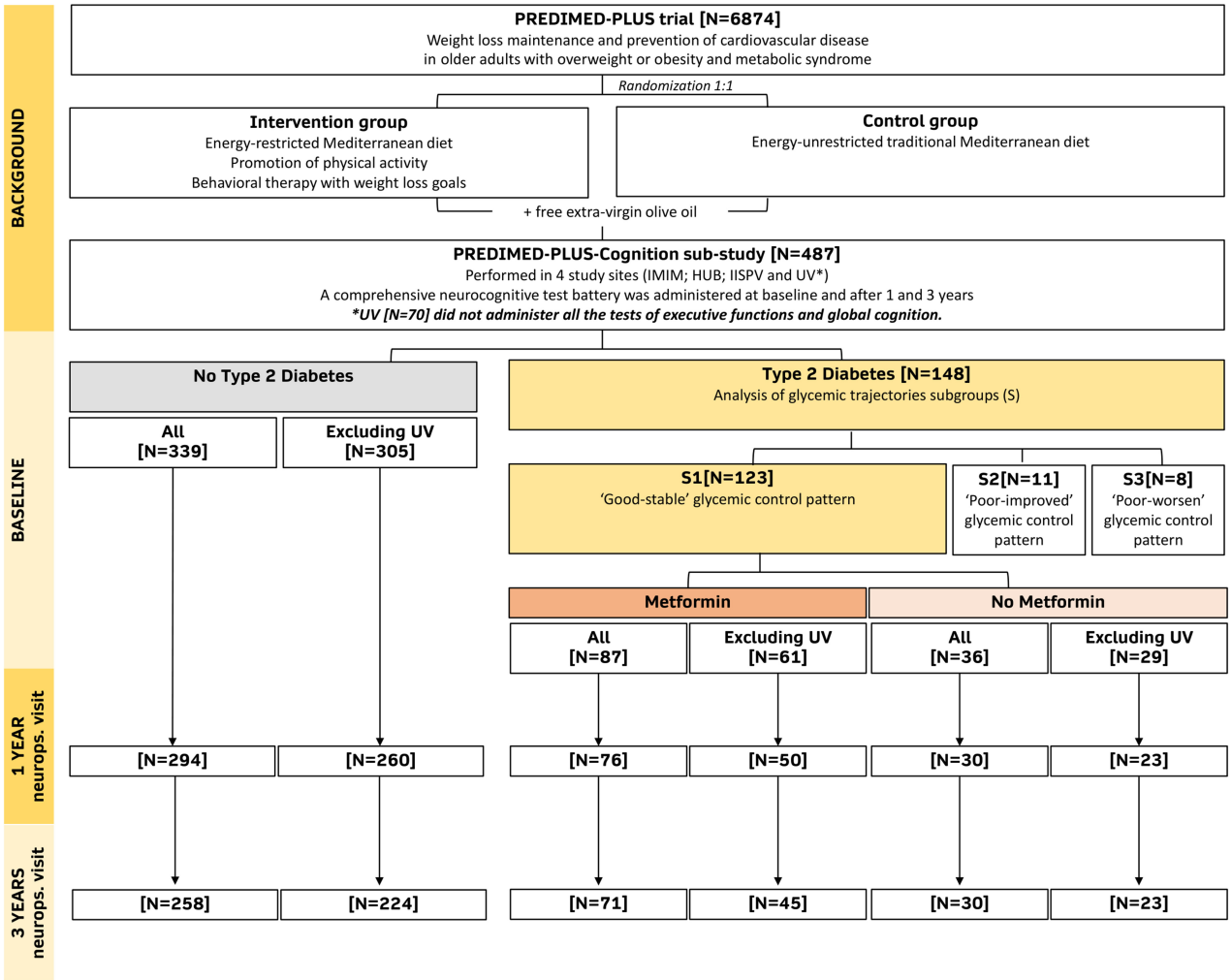

FIGURE 1 | Study flow diagram including the follow-up in the neuropsychological visits (neurops. visit) after 1 and 3 years of intervention. IMIM, Hospital del Mar Medical Research Institute. IISPV, Pere Virgili Institute for Health Research. UV, University of Valencia. HUB, Bellvitge University Hospital.

is also compatible with our data. However, no between-group differences in cognition were observed after 1 and 3 years of follow-up, given that those not treated with metformin compared to those treated with metformin experienced an improved performance from baseline to 3 years in memory (mean change of 0.38 vs. 0.10 SD from baseline mean [z-score], $P = 0.036$ for the between-group difference in mean change), executive functions (mean change of 0.36 vs. 0.02, $P = 0.005$) and global cognition (mean change of 0.29 vs. −0.02, $P = 0.001$).

Supplementary Table 7 includes the differences in each specific cognitive test between subjects with type 2 diabetes (S1) treated and not treated with metformin. At baseline, those treated with metformin scored moderately higher in decision-making abilities ($d = 0.60$, $P = 0.002$) and visuoconstructive praxis and attention ($d = 0.53$, $P = 0.018$), as well as slightly higher in short- and long-term visual memory ($d = 0.40$, $P = 0.050$; and $d = 0.45$, $P = 0.032$, respectively). However, after 3 years, those using metformin scored lower in short- and long-term verbal memory ($d = -0.42$, $P = 0.019$; and $d = -0.31$, $P = 0.051$, respectively) and presented lower improvements in decision-making abilities (mean change of −1.2 vs. 15.9 points, $P = 0.015$).

Finally, at baseline, both groups presented a moderate adherence to the MedDiet (mean score of 7.7). However, after 3 years of dietary intervention, MedDiet adherence increased to 12.6 points (95% CI 11.8, 13.4) in subjects with type 2 diabetes not treated with metformin and to 11.5 points (95% CI 10.9, 12.1) in subjects treated with metformin, a difference which was statistically significant ($d = -0.44$, 95% CI −0.84, −0.02; $P = 0.031$).

Diabetes vs. No Diabetes

Irrespective of metformin exposure, participants with type 2 diabetes from S1 were compared to participants without diabetes.

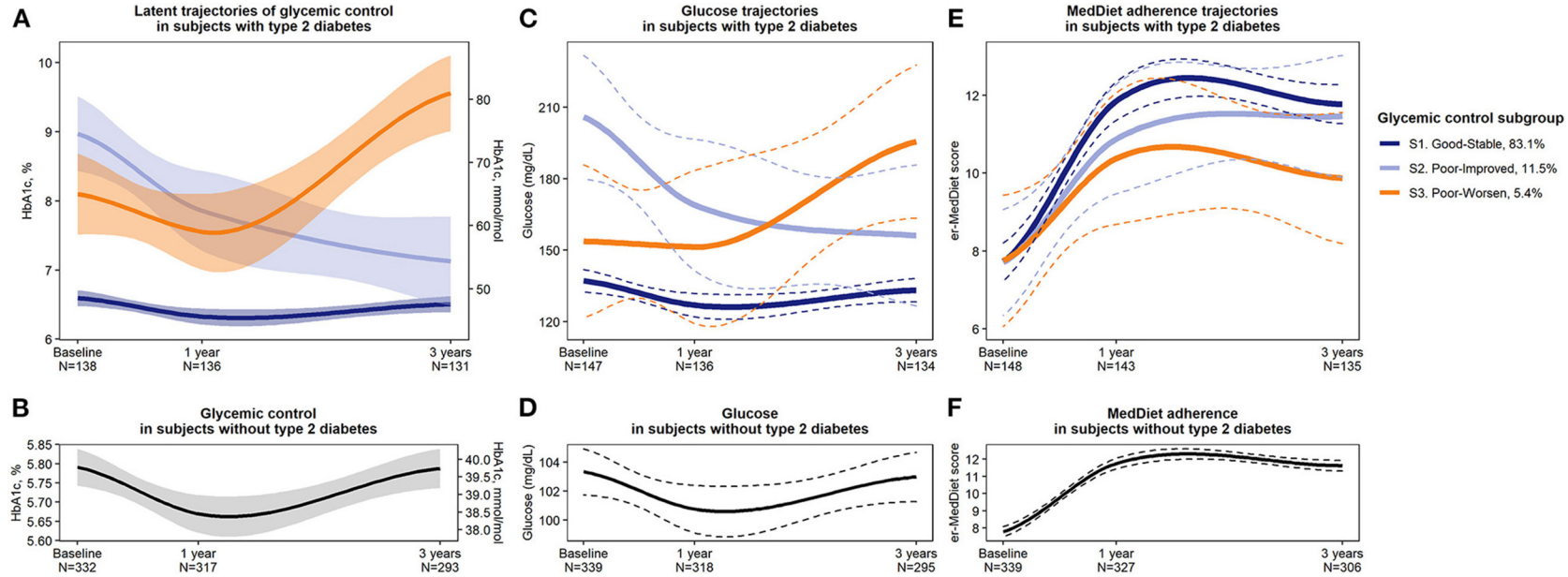

FIGURE 2 | Representation of **(A)** glycemic control (HbA1c) latent trajectories or subgroups in subjects with diabetes, **(B)** HbA1c trajectory in subjects without diabetes, **(C)** fasting plasma glucose levels in each subgroup of subjects with diabetes and **(D)** in individuals without diabetes, and **(E)** er-MedDiet adherence score in diabetes subgroups and **(F)** in subjects without diabetes. Solid lines represent mean values, and shaded areas or dashed lines represent 95% confidence intervals (95% CI). HbA1c, glycosylated hemoglobin. *N*, number. S, subgroup.

Participants with diabetes presented lower total cholesterol, LDL-cholesterol, lower HDL-cholesterol and were more physically active (**Supplementary Table 8**). Despite large differences in the glycemic profile (**Supplementary Table 9**), both groups did not differ in terms of MedDiet adherence and BMI, although subjects with diabetes scored higher in depressive symptomatology at baseline ($d = 0.39$, 95% CI 0.18, 0.60) and after 1 year ($d = 0.63$, 95% CI 0.41, 0.85).

There were no differences in baseline memory, executive functions and global cognition between subjects with and without type 2 diabetes (as shown in **Figures 4A–D** and **Supplementary Table 10**). However, from baseline to 3 years, those without diabetes exhibited a greater increase in their memory performance (mean change in memory z-score of 0.55 vs. 0.10, $P < 0.001$ for between group differences in mean change). This increase in the memory composite was mainly due to improvements in short- and long-term verbal memory. Subjects without diabetes also presented greater improvements in visuoconstructive praxis and attention (mean change of 2.8 vs. 0.9 points in RCFT figure copy task, $P = 0.009$) and did not present reductions in inhibition (mean change of −0.5 vs. −5.1 points in Stroop interference score), compared to subjects with diabetes. However, participants with diabetes presented greater reductions in the reaction time after 3 years (−48.5 ms vs. −0.5 ms in the CPT HRT, $P = 0.016$).

Diabetes Plus Metformin vs. No Diabetes

Subjects with type 2 diabetes from S1 treated with metformin were compared to subjects without diabetes (**Supplementary Table 11**). In the matched analysis (**Figures 4E–H** and **Supplementary Table 12**), baseline cognition did not differ between individuals with diabetes treated with metformin and individuals without diabetes. However, subjects without diabetes showed greater increases in memory after 1 and 3 years (mean change in z-score of 0.53 vs. 0.14, $P < 0.001$) and in global cognition after 3 years (mean change in z-score of 0.25 vs. −0.001, $P = 0.003$), compared to subjects with diabetes treated with metformin. These two groups did not differ in terms of executive functions and MedDiet adherence, neither at baseline nor after 1 and 3 years of follow-up.

Diabetes No-Metformin vs. No Diabetes

Participants without diabetes were compared to participants with type 2 diabetes from S1 who were not treated with metformin (**Supplementary Table 13**). Before matching these groups only differed in HDL-cholesterol (52.4 mg/dL in subjects without diabetes vs. 48.0 mg/dL in subjects with diabetes without metformin) but after matching this difference was balanced and no additional differences were observed. No differences in memory, executive functions and global cognition were observed between subjects with type 2 diabetes not treated with metformin and subjects without diabetes, except in the mean rate of change in executive functions, in which participants with diabetes not treated with metformin presented a greater improvement than participants without diabetes (mean change in z-score of 0.33 vs. 0.08, $P = 0.032$, as shown in **Figures 4I–L** and **Supplementary Table 14**). Moreover, participants with type 2 diabetes not treated with metformin also presented a greater adherence to the MedDiet after 3 years of follow-up ($d = 0.32$, 95%CI −0.03, 0.67; $P = 0.046$).

DISCUSSION

Main Findings

This is the first study to date to examine the effect of metformin on cognition in older adults with type 2 diabetes following a MedDiet intervention. We first examined the heterogeneity in HbA1c trajectories after 1 and 3 years of dietary intervention. We identified three different subgroups of individuals with diabetes irrespective of the intervention group. The largest group exhibited good glycemic control that remained stable during the follow-up, while the remaining two subgroups showed poor baseline glycemic control that improved or worsened during the follow-up. Among the group with good glycemic control, we observed that those treated with metformin presented a better baseline performance in memory, executive functions and global cognition than those not treated with metformin. However, those not treated with metformin presented higher adherence to the MedDiet over time as well as greater improvements in memory, executive functions and global cognition, so that baseline differences between individuals with type 2 diabetes treated and not treated with metformin vanished after 1 and 3 years of MedDiet intervention. These results suggest that adherence to a MedDiet intervention could be superior to the potential neuroprotective effects of metformin among older adults with overweight/obesity and metabolic syndrome who have good glycemic control of their type 2 diabetes.

Metformin Use and Cognition in Individuals With Diabetes

Our results suggest that metformin could have neuroprotective effects. Specifically, we observed that before starting the MedDiet intervention, individuals with type 2 diabetes from a group presenting good glycemic control (S1) treated with metformin presented a higher performance in memory, executive functions and global cognition than those not treated with metformin. These results agree with previous observational studies showing better memory performance (14) or greater maintenance of executive functions and global cognition (34) in cognitively normal subjects with diabetes type 2 treated with metformin, compared to those not treated with metformin. Metformin use has also been associated with lower dementia risk (35, 36) and better cognitive function (37), but results are still highly variable across studies (15). These inconsistencies could be explained by the fact that multiple neurocognitive pathways are affected by diabetes. Therefore, some pathways, but not all, may be improved with drug therapy (e.g., neurovascular alterations) (38). Moreover, metformin has the adverse effect of lowering

TABLE 2 | Differences in MedDiet adherence and in cognitive composites at each time point and in the mean change from baseline between individuals with type 2 diabetes from subgroup 1 (T2D-S1) treated with metformin and not treated with metformin (matched with inverse probability of treatment weights).

Variable	Time	T2D-S1 No-Metformin [*N* = 36][a]		T2D-S1 Metformin [*N* = 87][b]		Differences at each time point			Differences in the mean change (95%CI) from baseline		
		Missing [*N* (%)]	Mean (95% CI)	Missing [*N* (%)]	Mean (95% CI)	Cohen's D (95% CI)*	Effect Size[c]	*P*[d]	No-Metformin	Metformin	*P*[e]
er-MedDiet adherence score	Baseline	0 (0)	7.7 (6.8, 8.5)	0 (0)	7.7 (7.2, 8.3)	0.03 (−0.36, 0.41)	VS	0.752			
	1 year	1 (2.8)	12.1 (10.9, 13.3)	3 (3.4)	11.6 (11, 12.2)	−0.16 (−0.56, 0.23)	S	0.427	4.4 (3.4, 5.5)	3.9 (3.2, 4.6)	0.476
	3 years	1 (2.8)	12.6 (11.8, 13.4)	8 (9.2)	11.5 (10.9, 12.1)	−0.44 (−0.84, −0.02)	S	**0.031**	4.9 (3.9, 5.9)	3.9 (3.3, 4.4)	0.145
Memory composite (z-score)	Baseline	1 (2.8)	−0.17 (−0.46, 0.12)	3 (3.4)	0.1 (−0.03, 0.23)	0.38 (−0.02, 0.79)	S	0.115			
	1 year	7 (19.4)	0.1 (−0.21, 0.41)	13 (14.9)	0.18 (0.03, 0.33)	0.11 (−0.32, 0.54)	VS	0.795	0.2 (−0.03, 0.42)	0.01 (−0.11, 0.14)	0.307
	3 years	6 (16.7)	0.33 (0.04, 0.63)	16 (18.4)	0.29 (0.14, 0.44)	−0.06 (−0.49, 0.36)	VS	0.557	0.38 (0.15, 0.62)	0.1 (−0.05, 0.25)	**0.036**
Executive functions composite (z-score)[a,b]	Baseline	10 (34.5)	−0.14 (−0.42, 0.14)	21 (34.4)	0.13 (−0.02, 0.28)	0.51 (−0.06, 1.08)	M	0.086			
	1 year	10 (34.5)	−0.13 (−0.47, 0.21)	14 (23)	0.09 (−0.04, 0.21)	0.39 (−0.16, 0.93)	S	0.333	0.08 (0.00, 0.16)	−0.02 (−0.17, 0.13)	0.293
	3 years	14 (48.3)	0.23 (−0.14, 0.6)	28 (45.9)	0.14 (−0.01, 0.28)	−0.18 (−0.79, 0.44)	S	0.557	0.36 (0.13, 0.59)	0.02 (−0.09, 0.14)	**0.005**
Global cognition composite (z-score)[a,b]	Baseline	10 (34.5)	−0.1 (−0.35, 0.14)	22 (36.1)	0.13 (−0.02, 0.27)	0.48 (−0.1, 1.04)	M	0.124			
	1 year	10 (34.5)	0.02 (−0.34, 0.37)	15 (24.6)	0.15 (0.02, 0.29)	0.23 (−0.31, 0.77)	S	0.676	0.12 (−0.05, 0.29)	0.12 (0, 0.23)	0.511
	3 years	14 (48.3)	0.34 (−0.01, 0.69)	28 (45.9)	0.19 (0.03, 0.34)	−0.28 (−0.9, 0.34)	S	0.304	0.29 (0.10, 0.49)	−0.02 (−0.11, 0.07)	**0.001**

[a]N = 29 and [b]N = 61 when excluding participants from University of Valencia study site that did not receive all the tests from executive functions and global cognition.

[c]Effect Size: VS = very small (Cohen's d < 0.2); S = small [Cohen's d (0.2–0.5)]; M = medium [Cohen's d (0.5–0.8)]; L = large [Cohen's d (0.8–1.2)]; VL = very large (Cohen's d ≥ 1.2).

[d]ANOVA from multivariable-adjusted linear model.

[e]ANOVA from multivariable-adjusted linear mixed effects model.

Inverse probability of treatment weighting (IPTW) was applied to all analyses to weight each individual with his/her inverse probability of being treated with metformin, generating a pseudo-population with (almost) perfect covariate balance. All models were adjusted by diagnosis of sleep apnoea.

**Reference group= No-Metformin. Bold values denote statistical significance at the p < 0.05 level.*

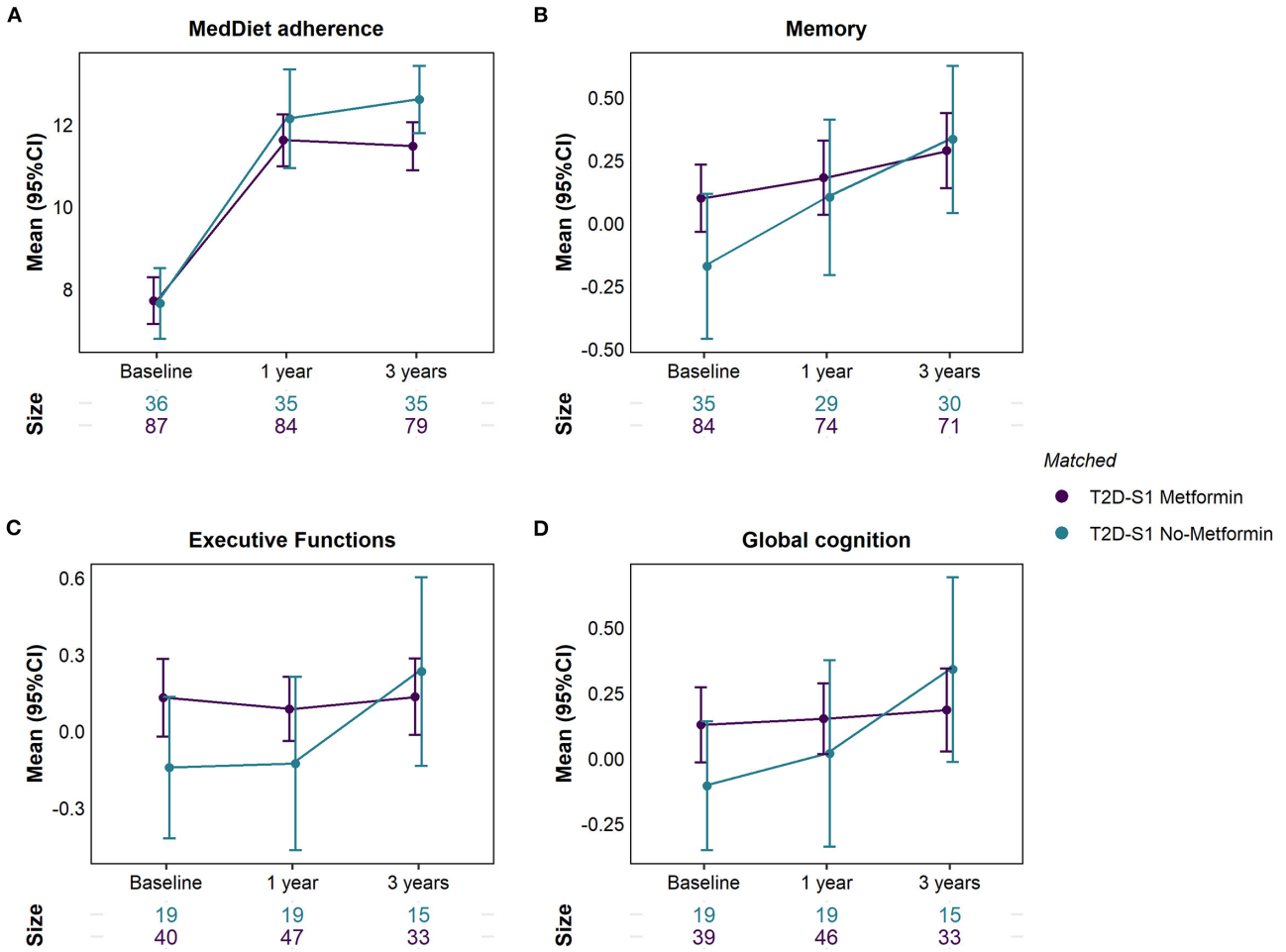

FIGURE 3 | Differences in **(A)** MedDiet adherence, **(B)** Memory, **(C)** Executive functions, and **(D)** Global cognition between participants with type 2 diabetes from subgroup 1 (T2D-S1) treated and not treated with metformin (individuals were matched with inverse probability of treatment weights).

serum vitamin B12 concentration, which can in turn, increase the risk of cognitive impairment (39).

Metformin mainly acts by reducing liver gluconeogenesis and inhibiting glucagon-mediated signaling in the liver, but it can also cross the blood brain barrier and thus affect the brain more directly (40). However, the potential neuroprotective effects of metformin have been mostly attributed to its anti-inflammatory and anti-coagulative properties, the prevention of metabolic syndrome (41) and the reduction of peripheral insulin levels that affect brain clearance of amyloid β-peptide (Aβ) (42). Several clinical trials have also tested the effects of metformin in subjects with mild cognitive impairment (MCI) (43) or Alzheimer's disease (AD) (40). In a pilot study in individuals with amnesic MCI ($N = 80$), metformin treatment for 12 months marginally improved the selective reminding score, but did not affect the global cognitive composite (ADAS-Cog) (43). However, in a cross-over RCT with 20 individuals with AD, metformin treatment for 8 weeks improved executive function and trends also suggested improved memory, learning and attention (40). These preliminary findings support the need for larger trials to evaluate the efficacy and cognitive safety of metformin in prodromal and dementia stages of AD, such as the one recently promoted by the University of Columbia (ClinicalTrials.gov Identifier: NCT04098666).

Mediterranean Diet Adherence and Cognition in Individuals With Diabetes

Our results also suggest that a higher adherence to the MedDiet could reverse the cognitive disadvantage of those subjects with diabetes that were not treated with metformin, since both groups with diabetes achieved similar cognitive scores along the follow-up. Subjects with diabetes from S1 not treated with metformin presented improvements in memory, executive functions and global cognition composites during the 3 years of follow-up, but cognition remained almost stable among those treated with metformin. Moreover, subjects with diabetes who were not exposed to metformin showed greater adherence to the MedDiet

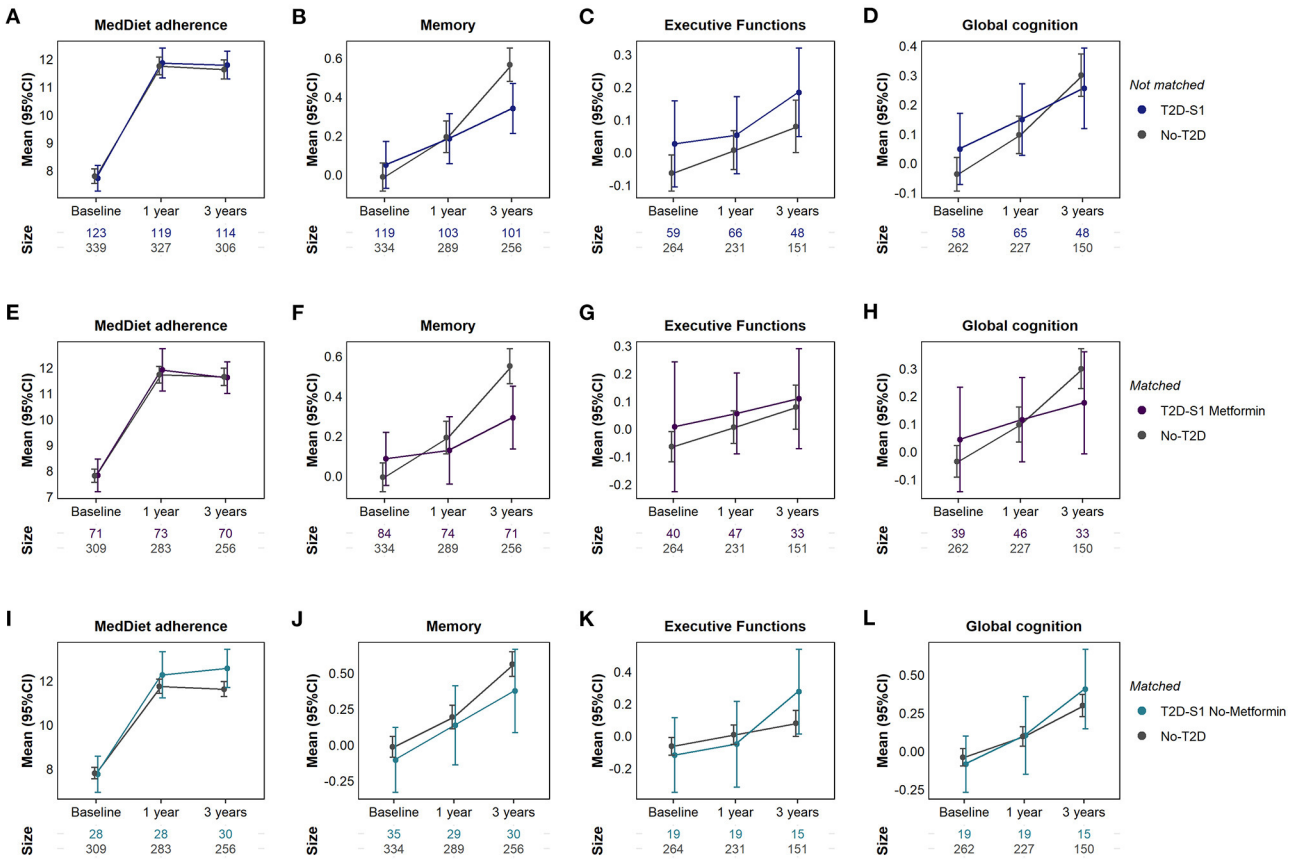

FIGURE 4 | Differences in MedDiet adherence, memory, executive functions and global cognition between subjects without type 2 diabetes (No-T2D) and all subjects with type 2 diabetes from subgroup 1 (T2D-S1) **(A–D)**; T2D-S1 treated with metformin **(E–H)** and T2D-S1 not treated with metformin **(I–L)**. In comparisons **(E-L)** subjects with and without type 2 diabetes were matched with inverse probability of treatment weights.

after 3 years of follow-up. The reason for this is unknown, but this indicates a group of subjects with a high capacity to make lifestyle changes. In fact, prior to participating in this study, most subjects from this group were able to control their blood glucose without medication and when offered a lifestyle intervention they adhered to it very faithfully, which probably translated into cognitive improvement. Another possibility is that those individuals with type 2 diabetes who take anti-diabetic drugs value lifestyle interventions less than those who do not take any drugs. Moreover, the greater compliance with the MedDiet among individuals with type 2 diabetes not taking metformin at baseline may also explain why they did not require metformin during the 3 years of follow-up. Previous studies have already reported the delayed need of medication for diabetes in patients with a newly diagnosed type 2 diabetes after a MedDiet intervention, compared to a low-fat diet (44, 45).

The favorable effect of the MedDiet intervention was likely due to the overall composition of the dietary pattern and not to a decreased caloric intake, weight loss or increased physical activity, because the allocation to the intervention or control group was balanced among subjects with diabetes either treated and not treated with metformin. In individuals with type 2 diabetes, the MedDiet has been consistently associated with better glycemic control (reduction of HbA1c by 0.32–0.53%) and a better profile of cardiovascular risk factors, compared to low-fat diets (4). These mechanisms could explain why adherence to the MedDiet might improve cognition in individuals with type 2 diabetes (46, 47). The high content in plant-based foods of the MedDiet (olive oil, legumes, vegetables, fruit, cereals, and nuts), along with fish and moderate red wine consumption during meals, make the MedDiet rich in phenolic compounds, n-3 polyunsaturated fatty acids and vitamins that, in conjunction, may contribute to a reduced oxidative stress and chronic inflammation and better neurovascular health (48, 49).

Differences Between Individuals With and Without Diabetes

When individuals with diabetes were compared to those without diabetes, we did not find baseline differences in memory, executive functions and global cognition composites. These results differ from previous cross-sectional studies in the overall PREDIMED-Plus population (N = 6,823) showing worse executive functioning (evaluated with different neuropsychological tests) at baseline among participants with

type 2 diabetes (8). However, we observed that participants with diabetes experienced fewer improvements in memory than participants without diabetes after 3 years of follow-up.

Nevertheless, in our study subjects with diabetes not treated with metformin experienced a greater increase in their executive functions than subjects without diabetes after 3 years of follow-up. Therefore, their greater adherence to the MedDiet could explain this difference in the rate of change in executive functions. In turn, MedDiet adherence did not differ between subjects with diabetes treated with metformin and subjects without diabetes. However, those using metformin experienced a lower improvement in their memory after 1 and 3 years, and in their global cognition after 3 years of follow-up, compared to subjects without diabetes. Thus, in the face of equivalent adherence to MedDiet, metformin was unable to neutralize the negative impact of type 2 diabetes on cognition.

Strengths

The strengths of this study include its longitudinal design with 3 years of follow-up and the large number of cognitive tests that are administered to participants, covering 12 different cognitive abilities that are grouped in memory, executive functions and global cognitive composites. Moreover, the methodology used in the analysis of results allowed us to minimize confounding by indication which is not frequently addressed in most studies of metformin and cognitive associations. Finally, we also described the heterogeneity in the response to a MedDiet intervention among individuals with diabetes type 2, which aligns with the current recommendations of more patient-centered research and care in the field of diabetes (3).

Limitations

However, this study has several limitations. First, the small sample size of the population with diabetes ($N = 148$) leads to a small number of participants in the subgroups with poor glycemic control ($N = 17$ in S2 and $N = 8$ in S3). This limits the capacity to detect statistically significant differences in their baseline characteristics. Moreover, when testing the associations between metformin use and cognition within the group presenting good glycemic control (S1), the small sample size of the untreated ($N = 36$) and treated ($N = 87$) groups also limited the study of gender effects, which should be addressed in future studies. Moreover, this sample size constraint also prevented the stratification of subjects exposed and not exposed to metformin according to their adherence to the MedDiet (high/medium/low). Consequently, it was not possible to study the simultaneous effect (interaction) of metformin use and high MedDiet adherence on cognition.

Second, there were losses in the evaluation of the cognitive function during the follow-up (within S1, 14% in the first year and 18% in the third year). They were not unexpected given the burden of neuropsychological visits and the fact that visits of this sub-study were performed on different days to those of the main trial. In addition, executive functions and global cognition composites excluded participants from the UV study site (representing 27% of subjects from S1) since not all the tests that made up the construct of executive functions were administered in this site. Therefore, selection bias cannot be completely excluded from this study.

Third, our methodology was not suitable for investigating causal effects since metformin administration was not randomized, and we did not collect data on the duration of metformin use, specific doses, or patients' adherence to their medication regimens. However, we noted that participants did not change their metformin treatment during the 3 years of follow-up. Moreover, we used IPTW to match treated and untreated subjects in each comparison. This approach allows to account for systematic differences in comorbidities between groups and is used to limit confounding by indication. We also had no information about the *APOE* genotype of participants, which could influence the association between metformin use and cognitive decline, as reported in previous studies (14).

Finally, this study does not have a control group since all subjects were exposed to a MedDiet intervention. However, without any intervention, individuals with metabolic syndrome would have probably presented a cognitive decline over time (50) and in this study their cognition improved independently of their underlying pathological condition.

CONCLUSIONS

In summary, both metformin and MedDiet seem to have neuroprotective effects in older adults at increased risk of pathological cognitive decline, presenting overweight/obesity, metabolic syndrome and type 2 diabetes. Given the heterogeneity in type 2 diabetes and in the response to lifestyle interventions and glucose-lowering medications, a group-based trajectory analysis was initially performed to stratify the population with diabetes. There were two minor subgroups with high HbA1c levels that did not achieve good glycemic control despite of the intensive MedDiet intervention. Future studies should consider applying more intensive and personalized dietary interventions to subjects with poor glycemic control of their type 2 diabetes. However, the majority subgroup of individuals with type 2 diabetes presented good glycemic control throughout the follow-up. In this subgroup, metformin treatment was associated with better memory, executive functions and global cognition at baseline. Nevertheless, after 1 and 3 years of MedDiet intervention, both metformin-treated and non-metformin-treated subjects achieved similar cognitive function. We postulate that increased adherence to the MedDiet explained the cognitive improvement observed in individuals with type 2 diabetes not treated with metformin. In conclusion, a high adherence to MedDiet seems to at least slow down cognitive decline in the elderly with metabolic syndrome and other chronic diseases. Our results support the hypothesis that both metformin and MedDiet interventions are good candidates for future cognitive decline preventive studies.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because there are restrictions on the availability of data

for the PREDIMED-Plus trial, due to the signed consent agreements around data sharing. Requestors wishing to access the PREDIMED-Plus dataset generated and/or analyzed during the current study can make a request to the PREDIMED-Plus trial Steering Committee chair. Requests to access the datasets should be directed to Jordi Salas-Salvadó, jordi.salas@urv.cat.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Parc de Salut Mar Drug Research Ethics Committee, Clinical Research Ethics Committee of Bellvitge University Hospital, Drug Research Ethics Committee of the Institut d'Investigació Sanitària Pere Virgili and Committee of Ethics and Research on Humans of Valencia University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RT, NS-D, AC-R, and LF contributed to the conception and design of the study, wrote the manuscript, and reviewed/edited the manuscript. NS-D performed the statistical analyses. AC-R, LF, NB, SN, CG-M, RF-C, AA-S, CV-A, SJ-M, and OC contributed to data acquisition. DC, SC, JD-E, OC, MG-G, XP, JS-S, and FF-A contributed to critical revision of the manuscript for key intellectual content. RT, JS-S, and FF-A obtained funding for the study. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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Bone and Lean Mass Loss and Cognitive Impairment for Healthy Elder Adults: Analysis of the Nutrition and Health Survey in Taiwan 2013–2016 and a Validation Study With Structural Equation Modeling

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Purpose: Bone and lean mass loss and cognitive impairment are prevalent in elder adults and have been hypothesized to share a potential link.

Methods: This nationwide cross-sectional study systemically sampled elder adults aged ≥65 years and conducted the door-to-door survey. The causal diagrams help to decide which covariates were included in the generalized linear mixed models (GLMMs). The structural equation modeling (SEM) was performed for the validation.

Results: A total of 535 participants were enrolled and categorized into the normal (67.3%), mild cognitive impairment (18.3%), and dementia groups (14.4%). With increasing in the severity of cognitive impairment, the bone marrow density and lean mass consistently showed the trend of decreasing values. In the GLMMs, a significant association existed between the decrease of the bone mineral density (BMD) and the Mini-Mental State Examination (MMSE) ($\beta = 5.819$ scores per g/cm^2 decrease, $p = 0.0305$) with adjustment of the age, sex, and physical activity. The SEM models confirmed that the MMSE was significantly and directly predicted by the age ($\beta = 0.1363$, $p = 0.0003$) and BMD ($\beta = 0.1251$, $p = 0.0006$) independently and indirectly predicted by lean mass ($\beta = 0.1138$, $p = 0.0003$) through the bone density path.

Conclusion: In conclusion, an independent association between bone loss and cognitive impairment was existed rather than the confounding effect and the decrease of lean mass indirectly contributed to cognitive impairment by influencing the bone density.

Keywords: bone loss, bone marrow density, cognitive impairment, dementia, osteoporosis

INTRODUCTION

Skeletal deficit and muscle loss have emerged as major issues for elder adults (1–3). Several reports have documented the comorbidity of osteopenia and osteoporosis in patients with dementia (4–6). Bone loss has also been reported for sharing a distinctive connection to cognitive impairment (7). The mechanical unloading of the skeleton has been proposed to cause loss of bone mass in patients with aging (8). In addition, decreased physical activity (9), fragility, and sarcopenia (10) may also directly or indirectly contribute to bone loss in elder patients with dementia. Collectively, the connection between the boss loss and cognitive impairment is not easily elucidated and possibly as a result of confounding by related various factors.

Physiologists proposed a paradigm that bone remodeling and energy metabolism are coregulated through the brain–bone axis (11–13). The skeleton is a metabolically active system and undergoes bone resorption and bone formation in whole life (14). Investigation into the brain–bone axis began with an emphasis on leptin (15), a hormone secreted by the adipose cells with remarkable effects in the brain for coregulation of the appetite and bone accrual (12, 16). Low levels of leptin have been reported in Alzheimer's disease (16, 17). Moreover, the lean mass has been found in a major source of neurotrophic factors for preventing cognitive impairment (18). Accordingly, two correlated factors of bone and lean mass are needed to be considered simultaneously to explore the relationship between body composition and cognitive impairment in a statistical model. However, most of the epidemiological studies (4, 19–27) only considered one of the two correlated factors at a time. An epidemiological study investigating the link between the bone and lean mass cognitive impairment in elder adults, while effectively controlling multiple contributing factors is warranted.

In statistical analysis, one of the major challenges to investigating the influential factors on cognitive impairment is that the numerous variables, including age, physical activity, bone mass, and lean mass, are correlated with each other. With respect to the methodological advances and software development, a structural equation modeling (SEM) permits the illustration of the relationship among many factors (28, 29). The flexibility of SEM allows its application in a cross-sectional study and other research designs (28). While the causal relationship cannot be obtained from a cross-sectional study, a directed acyclic graph (DAG) provides a simple way to demonstrate the relationships between the variables and to evaluate if confounding was present in the model (30).

The National Nutrition and Health Survey in Taiwan (NAHSIT) 2005–2008 described that osteoporosis was estimated to affect one-fourth of the general population in Taiwan (31). A recent 2018 report indicated that the incidence and prevalence of osteoporosis in Taiwan were similar to those in most of the Western countries with aging populations as well (32). In this study, we like to investigate whether an association exists between cognitive impairment and bone loss in our community-based elder participants by using the latest survey data NAHSIT 2013–2016. We adopted two novel approaches: (1) using DAGs to identify the confounding variables that are needed to be adjusted in the conventional regression analysis and (2) conducting an SEM to validify the best model constructed by a DAG.

METHODS

Study Design and Data Collection

The nationwide cross-sectional data were collected through the NAHSIT from January 1, 2013, to December 31, 2017, covering 359 townships or the city districts in Taiwan. The systematic sampling of the participants was classified into the eight strata by the characteristics of the population density, geographical area, and dietary habits. The door-to-door visits were carried out to obtain information of age, sex, body mass index (BMI), and physical activity by the trained interviewers. Mobile dual-energy X-ray absorptiometry (DXA) was performed to obtain the body composition parameters and the bone mineral density (BMD) in each specific body region. Based on the WHO definition, we defined osteoporosis as a BMD T-score (in g/cm^2) of $\leq$ 2.5 at the femoral neck or the lumbar spine in those aged 65 years and older. The DXA device (Prodigy, GE Healthcare Lunar, Wisconsin, USA) was used. Elder participants aged $\geq$ 65 years who agreed to complete the physical assessment were enrolled. Besides, the lipid profiles, vitamin A, and vitamin D were measured in the centralized laboratory. This study was approved by the Institutional Review Board on Biomedical Science Research, Academia Sinica, Taiwan (AS-IRB01-13067) and the Research Ethics Committee, National Health Research Institutes, Taiwan (EC1020110). Informed consent was acquired from the participants.

Cognitive Assessment

The diagnosis for dementia due to all the causes was following the guideline by the National Institute on Aging-Alzheimer's Association workgroups. Taiwanese version of the Mini-Mental State Examination (MMSE) (33) assessment was performed by the trained interviewers. The participants were categorized into the normal cognition, mild cognitive impairment (MCI), and dementia groups according to the previous literature. First, the participants with the MMSE scores of 27–30 ($\geq$9 education years) and of 26–30 (<9 education years) were classified into normal cognition. Second, the participants with the MMSE scores of 24–26 ($\geq$9 education years) and of 23–25 (<9 education years) were classified into MCI. Third, the participants with the MMSE scores of 14–23 ($\geq$9 education years) and of 11–22 (<9 education years) were classified into moderate dementia. Finally, the participants with the MMSE scores of 0–13 ($\geq$9 education years) and of 0–10 (<9 education years) were classified into severe dementia (33).

Causal Diagram

The DAGs were plotted by background knowledge (**Figure 1**). In Model 1, the unbiased causal path between bone loss and cognitive impairment was plotted with adjustment of age and sex. In Model 2, the causal path was plotted with adjustment of age, sex, and physical activity. In Model 3, the causal path was plotted with adjustment of the age, sex, physical activity, vitamin D, and total lean mass. The DAGs were produced by the DAGitty version 3.0 software (University of Lübeck, Germany).

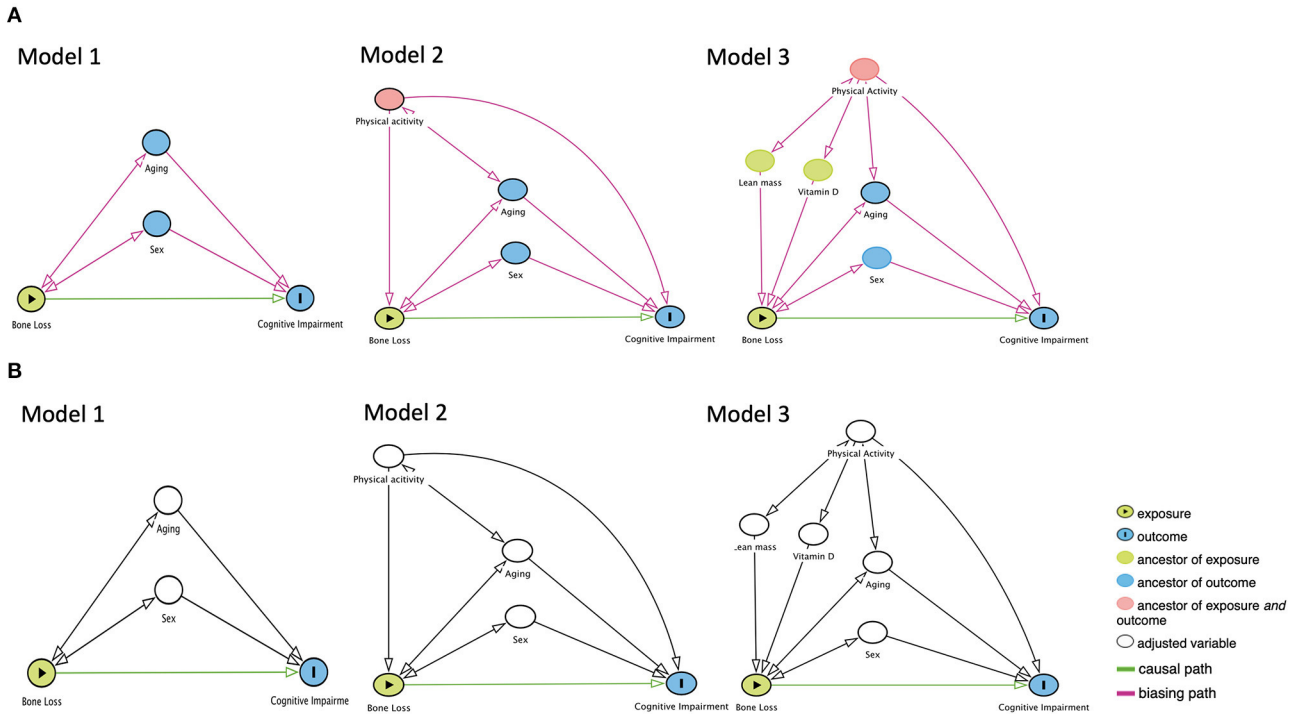

FIGURE 1 | The proposed directed acyclic graphs between bone loss and cognitive impairment. **(A)** Models of the no adjustment of confounders. **(B)** Models of the adjustment of confounders.

Statistical Analysis

The continuous and discrete variables were analyzed through the ANOVA and chi-squared test, respectively. The *p*-trend for the three cognitive groups was estimated by the generalized linear models for continuous variables and the Cochran–Armitage trend test for the discrete variable. We used the DAGs to decide which covariates were adjusted and put them into the multivariable model for obtaining the unbiasing results. The generalized linear mixed models (GLMMs) with the random intercept and unstructured covariance matrix were applied, with the MMSE scores being dependent variables and with total BMD (unadjusted model); with total BMD, age, and sex (in Model 1); with total BMD, age, sex, and physical activity (in Model 2); or with total BMD, age, sex, physical activity, vitamin D, and total lean mass (in Model 3) being independent variables. The model fit statistics of the Akaike information criterion (AIC) and the Schwarz information criterion (SIC) were used to select the best model. For the AIC and the SIC, a lower score indicates a better model (34). The statistical significance was defined as two-tailed $p < 0.05$. All the statistical analyses were performed by using SAS 9.4 (Cary, North Carolina, USA). In the subgroup analysis by gender, the GLMMs with the above settings were applied. Required sample sizes for the subgroup analysis were estimated by specifying an α error of 0.05, a power (1-β) of 0.80, the number of covariates of 5, and the R^2 values obtained in the unadjusted model.

Validation Study

To examine the relationship between bone loss and cognitive impairment in Models 1 to 3, we employed the SEM. For explaining the SEM fit, we focused on the chi-squared test, comparative fit index (CFI), and standardized root mean square residual (SRMR). The chi-squared test for the SEM with $p < 0.05$ was defined as a good model fit. The values of the CFI and SRMR ≥ 0.90 and the SEM < 0.80 were considered as acceptable levels of fit, respectively (35–37). For the assessment of the direct effects, β coefficient of < 0.05 was considered to be unmeaningful, β coefficient of 0.05–0.09 was small but meaningful, β coefficient of 0.10–0.24 was moderate, and β coefficient of ≥ 0.25 was large. For the assessment of the indirect effects, β coefficient of < 0.003 was unmeaningful, β coefficient of 0.003–0.010 was small but meaningful, β coefficient of 0.010–0.060 was moderate, and β coefficient of ≥ 0.060 was large (38).

RESULTS

Demographic Characteristics

The flowchart of enrolling the elder participants in this cross-sectional study is shown in **Figure 2**. A total of 535 elder participants who completed the assessment were enrolled (**Table 1**). The average MMSE scores for the normal cognition, MCI, and dementia groups were 28.5 ± 1.2, 24.7 ± 1.0, and 12.8 ± 9.6 ($p < 0.0001$, p-trend < 0.0001), respectively. In these

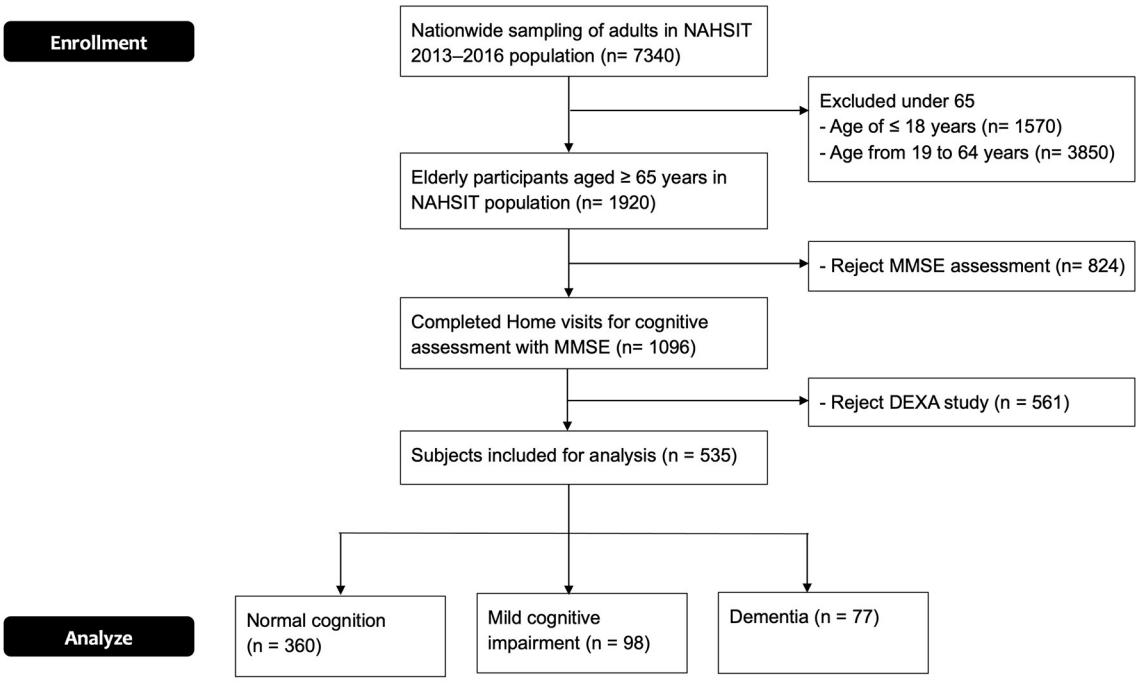

FIGURE 2 | Flowchart of the study.

three groups, the average ages were 71.3 ± 5.6, 73.2 ± 7.0, and 75.1 ± 6.8 ($p < 0.0001$, p-trend < 0.0001) and the education years were 9.5 ± 4.9, 8.2 ± 4.3, and 5.7 ± 4.2 ($p < 0.0001$, p-trend < 0.0001), respectively. Dementia and MCI groups had more female participants compared to the normal cognition. No significant difference in BMI was observed among these three groups.

Bone Marrow Density and Osteoporosis

The measurements of BMD in the whole body and specific body regions for the three cognitive groups are shown in **Table 2**. With increasing in the cognitive impairment, the whole-body BMD revealed the decreasing values (1.102 ± 0.136, 1.096 ± 0.136, 1.013 ± 0.139 g/cm^2, $p < 0.0001$, p-trend < 0.0001) among the normal cognition, MCI, dementia groups, respectively. Besides, BMD also showed the trend of decreasing values consistently in the upper extremities ($p = 0.0005$, p-trend $= 0.0016$), lower extremities ($p < 0.0001$, p-trend < 0.0001), spine ($p < 0.0001$, p trend < 0.0001), trunk area ($p < 0.0001$, p-trend < 0.0001), and femoral neck ($p < 0.0001$, p-trend < 0.0001), respectively. On the basis of the WHO definition, osteoporosis was more prevalent in the older adults with more severe cognitive impairment [normal cognition: 15.8%, MCI: 23.5%, and dementia groups: 32.5%, respectively ($p = 0.0022$, p-trend $= 0.0005$)]. The prevalence of osteoporosis in the overall participants was 19.6% (105/535).

Body Composition, Physical Activity, and Laboratory Tests

For body composition parameters (**Table 3**), the total lean mass was decreasing significantly with increasing in the cognitive impairment (40.79 ± 7.38, 39.14 ± 7.38, 37.21 ± 7.17 kg, $p = 0.0006$, p-trend < 0.0001). The lean mass in the arms ($p = 0.0047$, p-trend $= 0.0011$), trunk ($p = 0.0047$, p-trend $= 0.0011$), android ($p = 0.0045$, p-trend $= 0.0012$), gynoid ($p < 0.0001$, p-trend < 0.0001), and legs ($p = 0.0045$, p-trend $= 0.0012$) revealed as the same trend as lean mass in whole body. For fat mass, the three cognitive groups showed no significant differences among them. For physical activity (**Table 4**), the MCI group showed a little higher activity, but no significant differences were found in the three groups. The laboratory tests of the lipid profiles, ferritin, vitamin A, and vitamin D showed no significant differences among the three cognitive groups as well (**Table 4**).

Associations Between Bone Loss and Cognitive Impairment

The regression analysis of the GLMMs is presented in **Table 5**. In the unadjusted models, decrease of total BMD showed significantly decrease of the MMSE score ($\beta = 8.479$ per g/cm^2 decrease, SE $= 2.072$, $p < 0.0001$). In Model 1, the significant association between total BMD and the MMSE score remained with adjustment of the age and sex ($\beta = 5.792$ per g/cm^2 decrease, SE $= 2.681$, $p = 0.0312$). In Model 2, this significant association

TABLE 1 | Demographic characteristics of the three cognitive groups ($N = 535$).

Cognitive function	Normal cognition	Mild cognitive impairment	Dementia	*P* value	*P* trend
Number (%)	360/535 (67.3%)	98/535 (18.3%)	77/535 (14.4%)		
MMSE score	28.5 ± 1.2	24.7 ± 1.0	12.8 ± 9.6	<0.0001*	<0.0001*
Age (years)	71.3 ± 5.6	73.2 ± 7.0	75.1 ± 6.8	<0.0001*	<0.0001*
65–69 years	164/360 (45.6%)	38/98 (38.8%)	19/77 (24.7%)	<0.0001*	
70–74 years	101/360 (28.1%)	22/98 (22.5%)	19/77 (24.7%)		
75–79 years	62/360 (17.2%)	19/98 (19.4%)	11/77 (14.3%)		
≥80 years	33/360 (9.2%)	19/98 (19.4%)	28/77 (36.4%)		
Female Sex	138/360 (38.3%)	45/98 (45.9%)	50/77 (64.9%)	<0.0001*	<0.0001*
BMI (kg/m^2)	24.6 ± 3.5	24.9± 4.0	24.5 ± 3.8	0.6977	0.9175

**Statistical significance ($p < 0.05$).*
BMI, body mass index; MMSE, Mini-Mental State Examination.

TABLE 2 | Dual-energy X-ray absorptiometry (DXA) measurements for bone marrow density in the three cognitive groups.

Cognitive function	Normal cognition	Mild cognitive impairment	Dementia	*P* value	*P* trend
Bone marrow density (g/cm^2)					
Whole body	1.102 ± 0.136	1.096 ± 0.136	1.013 ± 0.139	<0.0001*	<0.0001*
Upper extremities	0.808 ± 0.157	0.813 ± 0.164	0.733 ± 0.136	0.0005*	0.0016*
Lower extremities	1.187 ± 0.178	1.170 ± 0.178	1.079 ± 0.191	<0.0001*	<0.0001*
Spine	1.064 ± 0.203	1.042 ± 0.214	0.947± 0.180	<0.0001*	<0.0001*
Trunk	0.880 ± 0.132	0.865 ± 0.143	0.797 ± 0.120	<0.0001*	<0.0001*
Bone marrow density (g/cm^2) by specific body region					
Femoral neck	0.812 ± 0.150	0.786 ± 0.177	0.728 ± 0.147	<0.0001*	<0.0001*
L-spine (L1 L4)	1.053 ± 0.217	1.020 ± 0.192	0.958 ± 0.235	0.0520	0.0162*
T-score					
Femoral neck	−1.134 ± 1.228	−1.235 ± 1.314	−1.642 ± 0.999	0.0053*	0.0022*
L-spine (L1 to L4)	−0.590 ± 1.760	−0.843 ± 1.561	−1.346 ± 1.934	0.0598	0.0193*
Osteopenia (%)	72/360 (20.0%)	20/98 (20.4%)	14/77 (18.2%)	0.9237	0.7752
Osteoporosis (%)	57/360 (15.8%)	23/98 (23.5%)	25/77 (32.5%)	0.0022*	0.0005*

**Statistical significance ($p < 0.05$).*
MMSE, Mini-Mental State Examination.

TABLE 3 | Dual-energy X-ray absorptiometry measurements for body composition in the three cognitive groups.

Cognitive function	Normal cognition	Mild cognitive impairment	Dementia	*P* value	*P* trend
Body composition of lean mass					
Total (kg)	40.79 ± 7.38	39.14 ± 7.38	37.21 ± 7.17	0.0006*	<0.0001*
Arms (kg)	4.58 ± 1.12	4.37 ± 1.03	4.13 ± 1.10	0.0047*	0.0011*
Trunk (kg)	19.76 ± 3.48	19.14 ± 3.63	18.22 ± 3.38	0.0030*	0.0007*
Android (kg)	2.97 ± 0.61	2.88 ± 0.68	2.71 ± 0.60	0.0045*	0.0012*
Gynoid (kg)	6.12 ± 1.22	5.79 ± 1.27	5.41 ± 1.23	<0.0001*	<0.0001*
Legs (g)	13.20 ± 2.73	12.48 ± 2.77	11.83 ± 2.69	0.0003*	<0.0001*
Body composition of fat mass					
Total (kg)	19.67 ± 6.94	20.06 ± 6.88	19.18 ± 6.24	0.7201	0.7650
Arms (kg)	1.98 ± 0.84	2.09 ± 0.84	2.11 ± 0.84	0.3712	0.1741
Trunk (kg)	11.44 ± 4.30	11.51 ± 4.26	10.91 ± 3.90	0.6137	0.4494
Android (kg)	2.09 ± 0.84	2.14± 0.87	1.97 ± 0.77	0.4291	0.4945
Gynoid (kg)	3.09 ± 1.08	3.13 ± 1.04	3.08 ± 0.98	0.9364	0.9541
Legs (kg)	5.48 ± 2.18	5.70 ± 2.11	5.42 ± 1.96	0.6411	0.9189

**Statistical significance ($p < 0.05$).*
MMSE, Mini-Mental State Examination.

TABLE 4 | Physical activity of the three cognitive groups.

Cognitive function	Normal cognition	Mild cognitive impairment	Dementia	*P* value	*P* trend
Physical activity (MET hours/week)	2.64 ± 11.01	3.19 ± 17.84	1.20 ± 5.94	0.7108	0.4941
Physical activity range				0.7815	
≥15.00 MET hours/week	17/360 (4.7%)	5/98 (5.1%)	3/77 (4.0%)		
7.50–14.9 MET hours/week	13/360 (3.6%)	2/98 (2.0%)	1/77 (2.0%)		
3.75–7.49 MET hours/week	6/360 (1.7%)	2/98 (2.0%)	0/77 (0%)		
<3.75MET hours/week	324/360 (90.0%)	89/98 (90.8%)	73/77 (94.0%)		
Lipid profile					
Total cholesterol (mg/dL)	187.9 ± 36.8	181.3 ± 29.9	179.4 ± 32.6	0.0739	0.0267*
LDL-C (mg/dL)	118.7 ± 33.1	115.6 ± 27.4	112.2 ± 28.6	0.2389	0.0905
HDL-C (mg/dL)	53.5 ± 15.8	51.3 ± 12.9	51.7 ± 14.0	0.3691	0.2088
Triglycerides (mg/dL)	122.6 ± 75.7	110.1 ± 45.3	113.2 ± 55.8	0.2214	0.1407
Ferritin (ng/mL)	235.8 ± 256.4	220.6 ± 161.3	202.9 ± 149.6	0.5086	0.2450
Vitamin A (μM)	2.3 ± 0.7	2.2± 0.8	2.3 ± 0.8	0.8295	0.7519
Vitamin D (μM)	34.3 ± 10.6	36.7 ± 11.7	32.9 ± 12.5	0.0821	0.7966

**Statistical significance ($p < 0.05$).*
HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; MET, metabolic equivalent of task.

TABLE 5 | Generalized linear mixed model estimates of the Mini-Mental State Examination (MMSE).

Scores of each item ($N = 535$)	Unadjusted Total MMSE Score†			Model 1 Total MMSE Score†			Model 2 Total MMSE Score†			Model 3 Total MMSE Score†		
	β	S.E.	*P* value	β	S.E.	*P* value	β	S.E.	*P* value	β	S.E.	*P* value
Total BMD (per g/cm^2)	−8.479	2.072	<0.0001*	−5.792	2.681	0.0312*	−5.819	2.682	0.0305*	−5.884	3.092	0.0576
Age (per 5 years)				−0.768	0.242	0.0016*	−0.764	0.242	0.0017*	−0.772	0.248	0.0020*
Sex (female vs male)				−0.826	0.754	0.2741	−0.786	0.756	0.2989	−1.825	1.000	0.0686
Physical Activity (per MET hours/week)							0.019	0.024	0.4278	0.020	0.023	0.3939
Vitamin D										−0.051	0.028	0.0772
Total lean mass (per kg)										−0.075	0.071	0.0391*
Model fit statistics												
AIC	3398.72			3391.61			3396.64			3014.11		
SIC	3394.72			3387.61			3392.64			3010.11		

**Variables with statistical significance ($p < 0.05$).*
† Regression analysis of the generalized linear mixed model with the random intercept (multilevel model) was employed.
AIC, Akaike information criterion; BMD, bone marrow density; MET, metabolic equivalent of the task; SIC, Schwarz information criterion.

was persisted even if adjustment of the age, sex, and physical activity ($\beta = 5.819$ per g/cm^2 decrease, SE = 2.682, $p = 0.0305$). In Model 3, no statistically significant association between the bone loss ($\beta = 5.884$ per g/cm^2 decrease, SE = 3.092, $p = 0.0576$) and cognitive impairment was found with adjustment of the age, sex, physical activity, vitamin D, and total lean mass. In comparison to Models 1 and 2, Model 3 with the lowest values of the AIC (3014.11) and the SIC (3010.11) was considered the best model, which was furtherly used for the validation with an SEM.

There was no sex difference observed in the GLMMs (**Supplementary Table S1**). In the unadjusted models, decrease of total BMD showed significantly decrease of the MMSE score in the males ($\beta = 6.926$ per g/cm^2 decrease, SE = 2.986, $p = 0.0211$) and in the females ($\beta = 9.857$ per g/cm^2 decrease, SE = 4.778, $p = 0.0403$), respectively. After adjustment for the age, physical activity, vitamin D, and total lean mass, a decrease of total BMD showed a reduction of the MMSE but without statistically significant. However, the sample size in the subgroup analysis by gender was inadequate (**Supplementary Table S2**). This study had 535 participants and out of them, 302 were males and 233 were females. The minimum sample size was 421 to attain an α error of 0.05 and apower (1-β) of 0.80 in the GLMMs with five covariates.

Validation With Structural Equation Modeling

In this study, the final SEM for the variables of age, bone mass, lean mass, vitamin D, physical activity, and the MMSE scores were plotted (**Figure 3**). This model also showed acceptable levels of fit [chi-squared test 22.75 with degrees of freedom

of 5 (p =0.0004), CFI: 0.97, adjusted CFI: 0.93, SRMR: 0.06]. The standardized effects for the direct, indirect, and total effects were listed (**Supplementary Tables S3–S5**). In the direct paths, the predictive model confirmed that the MMSE score was significantly and moderately predicted by age ($\beta = 0.1363$, $p = 0.0003$) and bone mass ($\beta = 0.1251$, $p = 0.0006$) by total BMD. In the indirect paths, the MMSE score was significantly and strongly predicted by lean mass ($\beta = 0.1138$, $p = 0.0003$).

DISCUSSION

This study examined the association between bone mass and cognitive function for ethnic Asian elder adults in Taiwan. We found that bone loss was independently associated with cognitive impairment in the primary analysis with the GLMMs. The association between bone mass and cognitive function was not simply the confounding effect of age or change of body composition. These findings were reproducible and confirmed by our separate validation study with SEM.

An earlier hospital-based study, using a nationwide health insurance database for the patients in Taiwan who were diagnosed as having osteoporosis and related fractures [the International Classification of Diseases-9-Clinical Modification (ICD-9-CM) codes 733.0 and 733.1], revealed a 1.4-fold relative risk of developing dementia (39). Another similar study conducted in Germany that analyzed patients who had osteoporosis-related fractures (the ICD-10 codes M80 and M81) from 1993 to 2012 revealed that the patients with osteoporosis had a 1.2–1.3-fold higher risk of developing dementia (6). This study extended and confirmed that the association between bone mass and cognitive impairment existed in both the hospital-based and community-based populations. In addition, the participants in this study were noninstitutionalized healthy elder adults. This indicated that bone health should be emphasized before elder adults developed obvious symptoms and signs of cognitive impairment.

Researchers have previously hypothesized that dementia and osteoporosis share mechanisms or are linked in other forms. In the SEM models, we found that the age and bone density individually directly predicted the MMSE score. This should be compatible with the unadjusted Model 1 and Model 2 in the regression analyses with the GLMMs. Therefore, we inferred that age and BMD were independently associated with cognitive impairment. On the other hand, BMD was not significantly associated with the MMSE score in Model 3 with adjustment of the vitamin D and lean mass. Additionally, lean mass was found significantly associated with the MMSE score in Model 3. In the SEM models, we found that the MMSE score was strongly and indirectly predicted by lean mass. These indirect paths from the lean mass to bone density, in the negative sense, should explain the attenuated association between BMD and the MMSE score by the GLMMs in Model 3. Additionally, we determined the SEM with acceptable fit with the three indicators of chi-squared test, CFI, and SRMR. Despite the root mean square error of approximation (RMSEA) was commonly used, we have the three main reasons that prefer SRMR to RMSEA: (1) SRMR is more accurate than RMSEA across small-to-large sample sizes (36, 40), (2) SRMR produces less type I error than RMSEA (36), and (3) RMSEA is more likely to overreject the true population models

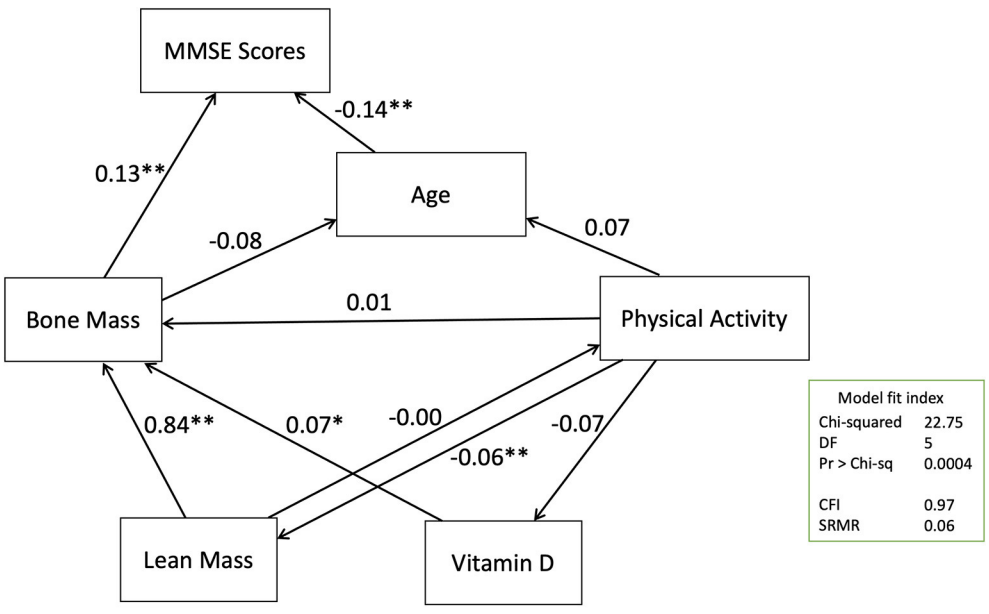

FIGURE 3 | Graphical presentation of the structural equation modeling. *$p < 0.05$, **$p < 0.01$.

by using the proposed cutoff criteria (41). Thus, we determined to use the three indicators, including the chi-squared test, CFI, and SRMR, to have an overall judgment of the good model fit.

The representative sampling of the community-based elder participants in this study should also be our strength. Proportions or prevalence of normal cognition, MCI, and dementia were similar to another nationwide population-based cross-sectional survey of cognitive impairment in Taiwan (42, 43). This study also had some limitations. First, owing to the cross-sectional design, we could not directly obtain the causal relationship between bone loss and cognitive impairment. However, with the SEM models, we may infer the most reasonable causal paths between bone loss and cognitive impairment. Second, the NAHSIT study focused on surveying the nutritional status of the elder participants and contained no more advanced genetic biomarkers. Third, this study had inadequate sample sizes in the subgroup analysis by gender. Though the overall analysis exhibited consistent results in both the males and females (**Supplementary Table S1**), larger sample size was required to confidently conclude no gender difference between the bone loss and cognitive impairment (**Supplementary Table S2**). Fourth, since this study aimed to survey the health status in the community population, the participants with higher health awareness were with higher willingness to complete all the assessments. A healthy volunteer bias possibly occurred and, therefore, the association between bone loss and cognitive impairment could be underestimated in this study. While the prevalence of osteoporosis in this study was 19.6%, the prevalence of osteoporosis by the National Health Insurance Research Database (NHIRD) of Taiwan, a real-world health database with coverage of > 99.9% residents in Taiwan (44), was from 17.4 to 25.0% (32). Our prevalence of MCI and dementia was similar to the prevalence by the NHIRD of Taiwan (42). Additionally, to ensure the representativeness for the total population in Taiwan, the NAHSIT 2013–2016 adopted the stratified sampling design by the characteristics of population density (with consideration of age and sex distribution) and geographical area. The enrollment protocol of the latest NAHSIT 2013–2016 (45) and the previous NAHSIT 2005–2008 (46) has been previously published. Consequently, we considered that the representative of the enrolled participants was not threatened.

In conclusion, these results support the association between bone loss and cognitive impairment for the older adults that were present and not simply a confounding effect from aging. The decrease of lean mass may indirectly affect cognitive impairment by influencing bone density. Further studies focus on exploring the biological plausibility that should be more convincing.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the NAHSIT 2013–2016 study was managed by the Health Promotion and Administration (HPA), Ministry of Health and Welfare, Taiwan. With legal restrictions imposed by the government of Taiwan on the distribution of the personal health data in relation to the Personal Information Protection Act, request for data needs a formal proposal to the Health and Welfare Data Science Center (HWDC), Ministry of Health and Welfare, Taiwan, and therefore the data was not publicly available. Requests to access the datasets should be directed to Ministry of Health and Welfare, Taiwan, https://www.mohw.gov.tw/np-126-2.html.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board on Biomedical Science Research, Academia Sinica, Taiwan; Research Ethics Committee, National Health Research Institutes, Taiwan. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

S-FL, Y-CF, W-HP, and C-HB contributed to the conception and design of the study, acquisition, analysis, and interpretation of the data. S-FL wrote the first draft of the article. 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/fnut.2021.747877/full#supplementary-material

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US Older Adults That Consume Avocado or Guacamole Have Better Cognition Than Non-consumers: National Health and Nutrition Examination Survey 2011–2014

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Purpose: The goal of this study is to examine how avocado relates to cognitive function among older adults.

Methods: A total of 2,886 National Health and Nutrition Examination Survey 2011–2014 participants aged 60 or older met the eligibility criteria and were included of our cross-sectional study. Participants were binarily classified as avocado consumers (i.e., reported consuming any avocado/guacamole in either 24-h dietary recalls) or non-consumers. Cognitive performance was evaluated with: Consortium to Establish a Registry for Alzheimer's disease (CERAD)—immediate and delayed recall (IWR/DWR), the Animal Fluency test, and the Digit Symbol Substitution Test. We calculated the education-dependent z-scores for each subject because education level can impact cognitive function. Global cognitive score, an average of the z-scores for each cognitive test, was calculated in participants who had completed all four tests. To account for relevant covariates, we tested for mean differences in cognition between consumers and non-consumers using independent sample *t*-tests and ANCOVA, special cases of ordinary least squares regression.

Results: Avocado consumers had significantly better cognitive scores across all cognitive tests and the global cognition score ($p < 0.05$) in the unadjusted model. Some mean differences attenuated after adjusting for potential confounders, but others remained significant. Compared to non-consumers, avocado consumers had significantly higher z-scores of 0.15, 0.15, and 0.11 for CERAD IWR and DWR, and global cognition score, respectively (all $p < 0.05$ in adjusted models).

Conclusion: Avocado consumption was associated with significantly better IWR, DWR, and the overall global cognition score, which remained significant when controlling for all relevant confounders.

Keywords: cognitive performance, older adults, avocado, brain health, NHANES

INTRODUCTION

Brain health is becoming an increasingly important research topic, especially in the older adult population, as it is estimated that diagnosis of Alzheimer's Disease (AD) will triple by 2060 from 2017 (1–3). Many of the known modifiable risk factors for cognitive decline and dementia are cardiovascular-related (1, 4). Some of those risk factors include high cholesterol, hypertension, obesity, diabetes, and tobacco use. Improved nutrition can help alleviate many of these modifiable risk factors and may directly impact the brain (1, 4).

There have been studies showing the potential benefits of single food items and dietary patterns on brain health. For instance, greater adherence to the Mediterranean diet has been associated with slower cognitive decline and cognitive impairment (5). In a cross-sectional analysis of the National Health and Nutrition Examination Survey (NHANES) survey (2011–2014), Taylor et al. found that greater Mediterranean Diet adherence was associated with better cognitive performance among individuals aged 60 years and older (6). Similarly, the German Longitudinal Cognitive Impairment and Dementia Study found that those with greater adherence to the Mediterranean Diet had better memory in older adults (7). Although avocados are not part of a traditional Mediterranean diet, consumption is dramatically increasing worldwide, and their nutrient profile aligns strikingly well with the Mediterranean dietary pattern.

A medium avocado fruit provides a lipid profile almost identical to olive oil plus 16% daily value (DV) niacin and riboflavin, 24% DV vitamin B6, 32% DV folate, 44% pantothenic acid, 408 ug of the carotenoid lutein, 36% of the DV for fiber, 16% DV vitamin C, and 20% DV vitamin E (8, 9). Single food components with similar fatty acid profiles to avocados, such as extra virgin olive oil and nuts, have been shown to correlate with cognition (10–12). Yet, few studies have investigated the association between avocados and cognition (13, 14). The two existing studies are limited in sample size and only one of them examined older adults, which may not be representative of the general older adult population. Both studies provided one whole avocado a day for 12 weeks or 6 months, which does not reflect habitual intake of avocado within the US population. Therefore, it's important to understand the role of this understudied but popularly consumed food on cognitive function using a large nationally representative health and nutrition survey. Specifically, this study aims to examine how avocado relates to cognitive function among individuals aged 60 or older from the Natonal Health and Nutrition Examination Survey (NHANES) 2011–2014.

MATERIALS AND METHODS

Study Population

This cross-sectional study was conducted using NHANES, a publicly available dataset. NHANES is a program within the National Center for Health Statistics, a part of the Centers for Disease Control and Prevention[1]. It used a multistage, probability sampling design to select participants representing the non-institutionalized US population (15). It also oversampled some subgroups to allow for a better estimate for those subgroups. All NHANES participants were provided informed consent, and the National Center of Health Statistics Research Ethics Review Board reviewed and approved NHANES protocols.

Cognitive tests were administered only in the 2011–2012 and 2013–2014 cycles to adults 60 years or older; thus we restricted our analysis to participants in this age group and survey waves (n = 3,472) (16). **Figure 1** displays the flow chart of eligible NHANES participants for this study. Subjects were included if they had at least one cognitive assessment and one 24-h food recall. Those with extreme total energy intake (e.g., women: <500 or >5000 kcal/day for women and <500 or >8,000 kcal/day for men) were further excluded (17). A total of 2,886 NHANES 2011–2014 participants were appropriate for this study.

Dietary Assessment

Trained interviewers collected 24-h dietary recalls. The first recall was conducted in the Mobile Examination Center (MEC), and the second recall was conducted over the phone 3–10 days later. More details of the interview process are described elsewhere (18).

Food codes from the USDA Food and Nutrient Database for Dietary Studies were used to identify avocado (63105010) and guacamole (63409010) consumers. Participants were binarily classified as consumers or non-consumers. Avocado consumers were defined as participants who reported consuming any avocado/guacamole in either 24-h dietary recalls. Avocado non-consumers were defined as participants who didn't report eating any avocado/guacamole during either 24-h dietary recalls.

Cognitive Function

NHANES participants aged 60 years or older not requiring a proxy informant in the 2011–2014 cohorts completed cognitive tests at home and in the MEC. The assessments were conducted in the following order: Consortium to Establish a Registry for Alzheimer's disease (CERAD)—immediate recall (IWR), the Animal Fluency test (AFT), the Digit Symbol Substitution Test (DSST), and CERAD—delayed recall (DWR). Cognitive tests were available in different languages, including English, Spanish, Korean, Chinese, and Vietnamese.

The CERAD assessment evaluates IWR and DWR of new verbal learning (19). Participants completed three learning trials where the trained interviewer showed words to the participants on flip cards. During each learning trial, the participants read the words out loud, one at a time. After flipping through all 10 cards, the participant had 90 s to remember as many words as possible. The CERAD score ranged from 0 to 10 for each learning trial; each correctly recalled word earned one point. The CERAD IWR score was an average of correctly recalled words from the three trials. After the AFT and DSST assessments, one delayed

[1]Centers for Disease Control and Prevention. *National Health and Nutrition Examination Survey*. Survey Methods and Analytic Guidelines. Available online at: https://www.cdc.gov/nchs/nhanes/index.htm (accessed April 25 2021).

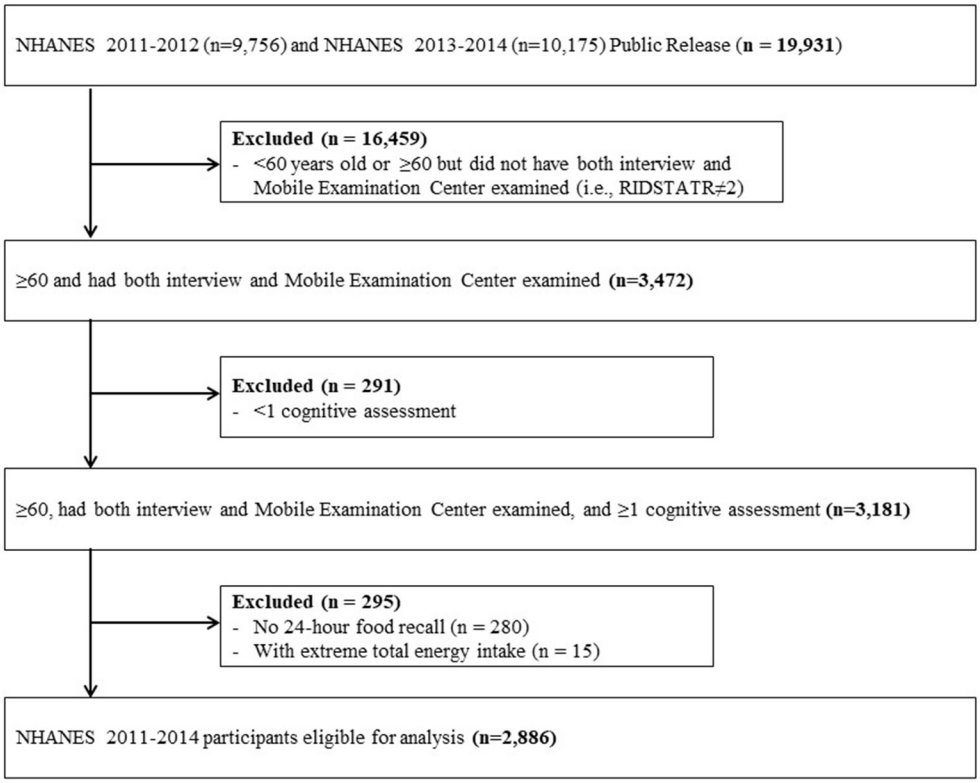

FIGURE 1 | Flow chart of eligible National Health and Nutrition Examination Survey (NHANES) participants included in this analysis.

recall was conducted, about 8–10 min after the initial word cards were presented.

The AFT test is a test of executive function, assessing verbal fluency by asking subjects to audibly list as many animals as possible within 1 min (20). As a practice trial, participants were asked to list aloud three clothing items. If they could not accomplish the test task, they did not continue with the AFT assessment. The calculated score equals the number of correct animals named.

The DSST is a subtest of the Wechsler Adult Intelligence Scale, Third Edition, which tests processing speed and attention using a paper form (21). The top of the page shows numbers one through nine with unique symbols associated with each number. Subjects were given 2 min to draw corresponding symbols in the 133 numbered boxes. The score ranged from 0 to 133; each point represents a correct match. Participants did a practice test, and those who were unable to complete it without the interviewer's help did not continue the DSST.

Because education level has been shown to impact cognitive function significantly, we calculated the education-dependent z-scores for each subject (22). Participants were first grouped by their educational levels (i.e., <9th grade, 9–11th grade, high school grad/GED or equivalent, some college or AA degree, and college graduate or above). We then calculated participants' education-dependent z-scores for each cognitive test with the mean and standard deviation derived from each education level group. Finally, a global cognitive score, taken as the average of the z-scores for each cognitive test, was calculated in participants who had completed all four tests.

Covariates

The following variables were assessed for confounding: age (continuous), gender (male and female), the ratio of family income to poverty (continuous), race (Mexican American, other Hispanic, non-Hispanic White, non-Hispanic Black, non-Hispanic Asian, and other race—including multi-racial), marital status (married, widowed, divorced, separated, never married, and living with a partner), smoked at least 100 cigarettes in life (yes and no), had at least 12 alcohol drinks per 1 year (yes and no), work activity (vigorous, moderate, and other), recreational activities (vigorous, moderate, and other), body mass index (continuous), Mediterranean Diet score (continuous), self-reported physician diagnosis of prediabetes or diabetes (yes and no), self-reported physician diagnosis of coronary heart disease (yes and no), self-reported physician diagnosis of high blood pressure (yes and no), and self-reported physician diagnosis of stroke (yes and no).

The work and recreational activities variables were calculated using the Global Physical Activity Questionnaire, which asks two sets of similar questions to characterize typical work activity and typical recreational activity. The first set asks whether the participant regularly engages in 10 min of continuous vigorous

activity and the other set determines whether the participant regularly engages in 10 min of continuous moderate activity. Subjects were classified as engaging in "vigorous work activity" if they answered "yes" to the vigorous work activity question, "moderate work activity" if they answered "no" to vigorous work activity and "yes" to moderate work activity, and "other" if they answered "no" for both questions. The same approach was used for questions relating to recreational activity.

Calculation of the Mediterranean Diet score in this sample has been previously described (6) using a modified version of Sofi, et al.'s 18-point Mediterranean Diet index (Sofi MedD Score) (23). Briefly, the Sofi MedD Score consists of 9 groups: fruit, vegetables, legumes, cereals, fish, meat, dairy, alcohol, and olive oils. Each group was assigned either 0, 1, or 2, depending on an individual's intake. Except for meat, dairy, and alcohol, a greater score represents a higher consumption of each food group.

Statistical Analysis

For all statistical analyses, we used sampling weights designed to account for multiple cycles of complex, multistage surveys (15). We performed descriptive statistics to show the baseline characteristics. Avocado consumers and non-consumers characteristics were compared using independent sample *t*-tests and chi-square tests. We compared means for education-dependent cognitive performance between the two avocado consumption groups using unadjusted and covariate-adjusted ordinary least squares (OLS) regression models (i.e., independent sample *t*-test and ANCOVA). Model 1 was unadjusted. Model 2 was adjusted for age, gender, the ratio of family income to poverty, race, and marital status. Model 3 further adjusted for smoking status, alcohol consumption, work activity, recreational activities, BMI, Mediterranean Diet score, self-reported physician diagnosis of prediabetes or diabetes, self-reported physician diagnosis of coronary heart disease, self-reported physician diagnosis of high blood pressure, and self-reported physician diagnosis of stroke. Covariates were selected based on previous research showing potential association between these covariates and exposure and/or outcomes (6, 16, 17, 24–27). There were no indications of multicollinearity in adjusted regression models (i.e., variance inflation factor $\leq$2.5 and tolerance >0.2), and model assumptions were tested using residual analyses (e.g., residual histograms and quantile-quantile plots). All analyses were performed with SAS 9.4 (SAS Institute Inc., Cary, NC), and the level of significance was considered at $p < 0.05$.

Protocol Registration and Checklist

We developed and registered our study protocol at: osf.io/yjbdn before data analysis (28). In addition, this study also followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) checklist to ensure the quality of the research.

RESULTS

Of 19,931 NHANES 2011–2014 participants, 2,886 individuals met the criteria for this study (**Figure 1**). Approximately 7% of them were avocado consumers with an average (standard error) avocado and guacamole consumption of 73.61 (7.25) and 53.37 (9.41) grams, respectively. In general, avocado consumers were younger, more likely to be married, had a higher family income to poverty ratio, and educational level (**Table 1**). They also had a lower BMI, engaged in more vigorous or moderate recreational activities, and had a higher diet quality, as measured by the Mediterranean Diet Score (**Table 1**).

Figure 2 illustrates the mean education-dependent z-scores, corresponding cognitive test 95% confidence intervals, and *p*-values for avocado consumers and non-consumers. In the unadjusted model, avocado consumers had significantly better cognitive performance across all cognitive tests and the global cognition score ($p < 0.05$) (**Figure 2** and **Supplementary Table 1**). Some mean differences attenuated after adjusting for potential confounders (models 2 and 3), but others remained significant. In the fully adjusted model 3, compared to non-consumers, avocado consumers had significantly higher *z*-scores of 0.15, 0.15, and 0.11 for CERAD IWR and DWR, and global cognition scores, respectively (all $p <$ 0.05).

Supplementary Table 1 presents complete regression statistics for all three of our OLS models using avocado consumer vs. non-consumer as the primary independent variable.

DISCUSSION

This study was the first to compare the differences in cognitive function between avocado consumers and non-consumers in an observational dataset of older Americans. Avocado consumers had significantly better scores for the CERAD IWR and DWR tests and the overall global cognition score and remained significant when controlling for relevant confounders (in all adjusted models). Our study found that avocados may have an impact independent of a number of covariates, including diet quality (per model 3), which is associated with slower cognitive decline and cognitive impairment (5). While the AFT and DSST scores were significantly better for avocado consumers in our unadjusted model, the significance was attenuated in the adjusted models. Given avocado consumption is growing dramatically worldwide, and research has shown the cardiometabolic benefits of eating avocados through the effects of various dietary components, phytochemical, and nutrients that avocados include as discussed by Silva Caldas et al. (29–34), it's relevant to study the cognitive benefits of avocado intake within a large population cohort.

We found that consuming avocado was most strongly related to better memory performance. Among all participants, avocado consumers recalled an average of 1.8 more words across the three learning trials for the IWR and 0.9 more words on the DWR. For cognitively normal individuals of this age group, the average performance by avocado consumers on these tasks was at the median for suggested normative performance while average performance by non-consumers was at the bottom of the suggested interquartile range (35). This is critical because memory is the most common complaint in older adults and likely the first cognitive domain to be affected in age-related neurodegenerative disease (i.e., AD and Mild cognitive impairment) (36, 37). Two previous studies investigated the

TABLE 1 | Baseline characteristics of NHANES participants by avocado/guacamole consumer vs. non-consumer (n = 2,886)*.

Avocado/guacamole consumer†	All (n = 2,886)	No (n = 2,693)	Yes (n = 193)	*P*
Age (yr)	69.3 (0.2)	69.4 (0.2)	67.9 (0.5)	0.03
Female, %	53.8	53.2	62.0	0.08
Family income to poverty ratio	3.1 (0.1)	3.1 (0.1)	3.7 (0.2)	0.003
Race/ethnicity, %				0.002
Mexican American	3.4	3.3	4.6	
Other hispanic	3.7	3.4	7.9	
Non-hispanic white	79.5	79.4	80.1	
Non-hispanic black	8.5	9.0	2.8	
Non-hispanic Asian	3.0	3.0	3.4	
Other race-including multi-racial	1.9	1.9	1.2	
Marital status, %				<0.001
Married	62.6	61.5	76.0	
Widowed	16.9	17.8	5.4	
Divorced	12.3	12.4	11.1	
Separated	1.2	1.3	0.4	
Never married	4.3	4.1	6.8	
Living with partner	2.6	2.8	0.3	
Education, %				<0.001
<9th grade	6.0	6.2	4.4	
9–11th grade	10.3	10.7	5.5	
High school grad/GED or equivalent	22.2	22.8	14.4	
Some college or AA degree	31.5	31.8	27.4	
College graduate or above	30.0	28.5	48.2	
BMI (kg/m^2)	29.1 (0.2)	29.3 (0.2)	27.5 (0.7)	0.02
Smoker, %‡	50.4	50.6	47.2	0.59
Alcohol drinker, %§	72.5	71.6	83.7	0.005
Work activity, %				0.99
Vigorous	12.9	12.8	13.4	
Moderate	21.9	21.9	22.1	
Other	65.2	65.2	64.5	
Recreation activities, %				0.0005
Vigorous	11.4	10.5	22.5	
Moderate	33.8	33.4	38.0	
Other	54.8	56.0	39.5	
Prediabetes or diabetes, %¶	30.2	30.6	25.7	0.34
Coronary heart disease, %¶	9.7	9.9	6.3	0.17
Stroke, %¶	6.6	6.8	4.5	0.47
Hypertension, %¶	59.4	59.7	55.7	0.42
Energy intake, kcals/day	1,870.7 (18.2)	1,862.5 (19)	1,973.0 (65)	0.12
Mediterranean Diet Score	5.3 (0.1)	5.2 (0.1)	6.2 (0.2)	0.0003
Raw cognitive scores				
CERAD immediate learning	6.5 (0.1)	6.5 (0.1)	7.1 (0.1)	<0.001
CERAD delayed recall	6.2 (0.1)	6.1 (0.1)	7.0 (0.1)	<0.001
Animal fluency test	18.1 (0.2)	17.9 (0.2)	20.1 (0.6)	0.0008
Digit symbol substitution test	52.4 (0.5)	51.8 (0.6)	58.9 (1.7)	0.0005

BMI, body mass index; CERAD, Consortium to Establish a Registry for Alzheimer's disease.

**Means (standard errors) for continuous variables and percentages for categorical variables.*

†*Avocado/guacamole consumers were identified as NHANES 2011–2014 participants who reported consuming any amount of avocado or guacamole during the 24-h dietary recall.*

‡*Smoker is defined as those who had at least 100 cigarettes in life.*

§*Alcohol drinker is defined as those who had at least 12 alcohol drinks/1 year.*

¶*Self-reported physician diagnosis.*

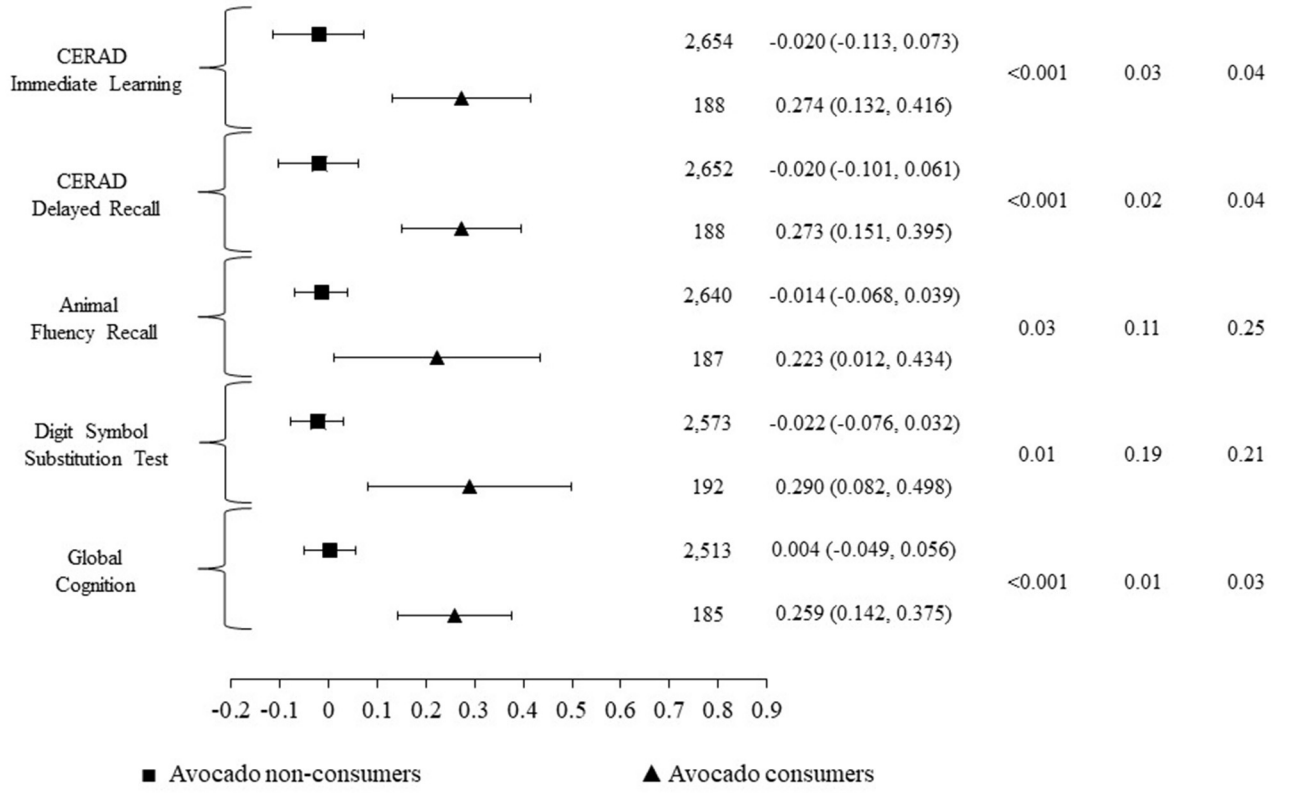

FIGURE 2 | Mean z-scores, corresponding cognitive test 95% confidence intervals, and p-values for each cognition test. CERAD, Consortium to Establish a Registry for Alzheimer's disease. *Model 1 is unadjusted. Sample sizes for each cognitive test were: Consortium to Establish a Registry for Alzheimer's Disease–Immediate Learning ($n = 2,842$); Consortium to Establish a Registry for Alzheimer's Disease-Delayed Recall ($n = 2,840$); Animal Fluency Test ($n = 2,827$); Digit Symbol Substitution Test ($n = 2,765$); Global cognition ($n = 2,698$). †Model 2 adjusted for age, gender, ratio of family income to poverty, race, and marital status. Sample sizes for each cognitive test were lower because of missing covariates: Consortium to Establish a Registry for Alzheimer's Disease-Immediate Learning ($n = 2,621$); Consortium to Establish a Registry for Alzheimer's Disease–Delayed Recall ($n = 2,619$); Animal Fluency Test ($n = 2,608$); Digit symbol substitution test ($n = 2,553$); Global cognition ($n = 2,491$). ‡Model 3 further included adjusted for smoking status, alcohol consumption, work activity, recreational activities, BMI, Mediterranean Diet score, self-reported physician diagnosis of prediabetes or diabetes, self-reported physician diagnosis of coronary heart disease, self-reported physician diagnosis of high blood pressure, and self-reported physician diagnosis of stroke. Sample sizes for each cognitive test were lower because of missing covariates: Consortium to Establish a Registry for Alzheimer's Disease-Immediate Learning ($n = 2,532$); Consortium to Establish a Registry for Alzheimer's Disease–Delayed Recall ($n = 2,531$); Animal Fluency Test ($n = 2,521$); Digit Symbol Substitution Test ($n = 2,477$); Global Cognition ($n = 2,417$).

effects of avocado on cognitive function and found some positive benefits, similar to our study. Scott et al. randomized healthy older adults into a 6-month trial where subjects consumed either one avocado or one cup of potato or chickpeas per day (13). The control foods were selected to limit the intake of lutein, a nutrient hypothesized to contribute to cognitive performance and present in the fresh avocado pulp. A battery of cognitive tests evaluated different cognitive domains, including attention (Choice Reaction Time and Rapid Visual Information Processing), visual memory (Delayed Match to Sample, Paired Associates Learning), and executive function, working memory, and planning tests (Spatial Span, Spatial Span Reverse, Spatial Working Memory, and Stockings of Cambridge). The avocado group had improvements in working memory. Additionally, as lutein accumulated in the eye's macula, working memory and efficiency of approaching a problem also improved. Similarly, we found that those consuming avocado scored higher on the CERAD IWR and DWR memory tests. Evidence suggests that avocados could benefit cognition across the lifespan, potentially influencing different cognitive domains at different life stages. The other randomized controlled trial conducted by Edwards et al. found that 84 overweight and obese adults performed better on a cognitive test of attentional inhibition when consuming fresh avocado daily for 12 weeks than an isocalorically controlled meal (14). To evaluate cognitive function, they used the Flanker task, oddball task, and Nogo task. They found that the avocado group improved accuracy in the Flanker task, even when accounting for numerous confounders. The other cognitive measures were not significantly changed. Although the tests used in this study differ from our current study, it is consistent that avocado consumption seems to benefit specific domains in cognitive function vs. all the domains. Our study suggests that additional clinical trial in older adults is necessary to elucidate effects of an avocado intervention.

There are several potential mechanisms to support the observed associations between avocado intake and cognitive function. One Hass avocado contains ∼369 micrograms of the carotenoid, lutein (8). Lutein preferentially accumulates in neural tissues and therefore is hypothesized to be involved in neurocognitive function across the lifespan, and numerous studies report lutein-related cognitive benefits (38). Kesse-Guyot et al. observed that a carotenoid-rich dietary pattern was associated with a better overall cognitive score after a 13-year follow-up (39). Two prospective studies reported reduced cognitive aging for individuals consuming higher amounts of lutein and zeaxanthin-containing vegetables (40–42). The relationship between brain lutein concentrations of decedents >98 y at death and premortem measures of cognitive function were assessed in a study by Johnson et al. Among the carotenoids, brain lutein content was reliably related to cognitive test scores (43). Although findings from the RCT (14) did not find a correlation between serum lutein levels and attentional inhibition, it is important to note that macular pigment measures of lutein were not significantly changed. Because it is assumed that lutein deposited in the macula correlates with brain lutein levels, the intervention likely did not signiticantly boost lutein levels in the brain and thus is not responsible for the positive cognitive findings in the study. As a whole food, it is inherently difficult to identify individual avocado nutrients or bioactive compounds that are responsible for the biological benefits observed. Instead, it's plausible that nutrients work additively or synergistically to benefit cognitive measures.

Avocados also contain 13.3 grams of monounsaturated fat and provide vitamins and minerals known to support brain health and cognitive function (5, 8). In addition, the anti-inflammatory and antioxidant effects of monounsaturated fat and its derivatives can help decrease chronic inflammation and oxidative stress, which have been observed in individuals with mild cognitive impairment and AD (44, 45). For example, in a prospective cohort study of women aged 60 years and older, Naqvi et al. found that monounsaturated fat intake was associated with a slower decline in cognitive function, as indicated by the overall cognitive function score (46). Interestingly, monosaturated fat intake was particularly beneficial to the memory and visual domains, similar to what we observed in our analysis.

Avocados contain B vitamins, which have been studied for their potential role in brain health because of their role in homocysteine metabolism (5, 8). Elevated homocysteine level is a risk factor for AD and dementia. B vitamins can help to lower homocysteine levels (47). For example, folate is needed for the remethylation of homocysteine to methionine, and vitamin B_6 is a cofactor for the enzyme which breaks down homocysteine to sulfate (48). Although results from B vitamin supplementation trials are mixed, observational studies that examined B-vitamins intake and cognitive function have shown some significant benefits (5, 49). For instance, several studies reported that a higher intake of B- vitamins (e.g., folate) was associated with less cognitive decline or less risk for AD and dementia (50–52).

As evidence linking cardiometabolic health with brain health and function continues to grow (53, 54), we also hypothesize that the relationship between avocado intake and better cardiometabolic health may provide explanation for our findings (29–34). For instance, avocados have micronutrients that may benefit blood pressure and avocado interventions have been shown to lower LDL and raise HDL in patients with hypercholesterolemia (30–32, 34) and improve peripheral blood flow acutely after consumption (29, 33).

Our study includes several strengths. First, we used a large nationally representative health and nutrition survey. Second, although we found that avocado consumers tended to be younger, more likely to be married, had a higher educational level and family income to poverty ratio, we adjusted for numerous characteristics in our final model to best understand the specific role of avocado on cognitive measures. One of those key characteristics was education level which is independently correlated with cognitive performance. Thus, we calculated education-dependent z-scores for each cognitive outcome.

Limitations of interpreting these findings include the study's cross-sectional nature, potential residual confounding of observational studies, and the limited number of dietary recalls used to assess avocado intake. For example, one or two 24-h dietary recall(s) may not accurately reflect usual intake. Thus, it would be important to replicate this study using a food frequency questionnaire. Also, since only 7% of the population studied were avocado consumers, we could not complete complementary sensitivity analyses in those with subjective memory complaints. However, avocado intake has dramatically increased from an annual 5 pounds per capita in 2011 to close to 9 pounds per capita in 2020, so the data analyzed in these NHANES cycles may underestimate current avocado consumption trends among older adults, and newer releases of NHANES data may capture a larger sample of avocado consumers.

Although our findings cannot be considered causal, and more studies are needed to confirm these findings, the data suggests a role for avocados in the cognitive function of older Americans. In addition, future studies are warranted to replicate these associations in other populations and examine the mechanism of action.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: The data that support the findings of this study are openly available in the National Health and Nutrition Examination Survey database from the Centers for Disease Control and Prevention at https://wwwn.cdc.gov/nchs/nhanes/Default.aspx.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Research Ethics Review Board at the National Center for Health Statistics. All NHANES participants provided their written informed consent to participate in this study. The

patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors conception and design, analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, and also have seen and agreed with the manuscript's contents and met the ICMJE requirements for authorship.

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REVIEW
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An Interactive Review on the Role of Tocotrienols in the Neurodegenerative Disorders

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Neurodegenerative disorders, such as Parkinson's and Alzheimer's disease, are claimed to be of major concern causing a significant disease burden worldwide. Oxidative stress, mitochondrial dysfunction and nerve damage are the main reasons for the emergence of these diseases. The formation of reactive oxygen species (ROS) is the common chemical molecule that is formed from all these three interdependent mechanisms which is highly reactive toward the neuronal cells. For these reasons, the administration of tocotrienols (T3s), which is a potent antioxidant, is proven to cater to this problem, through *in vitro* and *in vivo* investigations. Interestingly, their therapeutic potentials are not only limited to antioxidant property but also to being able to reverse the neuronal damage and act as a shield for mitochondria dysfunction. Thereby, T3s prevents the damage to the neurons. In regards to this statement, in this review, we focused on summarizing and discussing the potential therapeutic role of T3s on Alzheimer's and Parkinson's diseases, and their protective mechanisms based on evidence from the *in vitro* and *in vivo* studies. However, there is no clinical trial conducted to prove the efficacy of T3s for Alzheimer's and Parkinson's subjects. As such, the therapeutic role of T3s for these neurodegenerative disorders is still under debate.

Keywords: tocotrienols, Alzheimer's disease, Parkinson's disease, therapeutic potential, neuroprotective, reverse nerve damage, antioxidant, mitochondrial shield

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INTRODUCTION

Over the past 25 years, neurodegenerative disorders, such as Alzheimer's (AD), Parkinson's (PD), and Huntington (HD) diseases, are on the upsurge due to longer life expectancy, as age is one of the risk factors when contracting any one of these diseases (1). Due to the complex pathogenesis of neurodegenerative disorders, detecting and treating the disorders remain as a challenging task despite the existence of a wide range of modern technologies and medicine (2). With this in mind, there is a continuous interest among researchers to overcome such problems by using natural compounds as an alternative and complementary medicine (3). Besides consumed in their natural forms, these compounds can be modified to enhance or suppress certain properties such as stability, efficacy and bioavailability (4). Many of them serve as lead compounds for designing new drugs with desirable pharmacological activity.

The usage of natural compounds, such as tocotrienols (T3s) (3), curcumin (5), flavonoid apigenin (6), crocin (7), rosmarinic acid (8), green tea catechins (9), resveratrol (10), and hesperidin (11), has been reported to be effective against several types of neurodegenerative disorders such as

AD (3, 5–11), PD (3, 5–11), diabetic neuropathy (6, 11), stroke (6), epilepsy (7), cerebral ischemia (10), HD (10, 11), and multiple sclerosis (11). These compounds are capable of reducing the beta amyloid (Aβ) aggregation by increasing monoamine secretion in AD and PD (8), preventing abnormal accumulation of Aβ and α-synuclein in AD and PD (9), acting as an antioxidant against AD (3, 5–11), improving blood flow to the cerebral, and improving cognition and performance in AD, PD, HD, and multiple sclerosis (11). Interestingly, being a naturally existing substance, they have been reported to exhibit minimal adverse effects (3). Recent epidemiological data indicates that persons with high plasma levels or a high dietary intake of the combination of all natural vitamin E forms had a lower incidence of dementia or AD. In these, tocotrienols are more effective than tocopherol in neutralizing certain free radicals, given the fact that all vitamin E congeners show antioxidant activity (12, 13). Currently, more than 1/3 of the top selling pharmaceutical medicines are natural compound origin and labeled as traditional medicine. However, there are not many clinical trials that have proven the efficacy of these compounds. Yet, day by day, there are more and more natural product-based formulations being introduced to the market (14).

Neurodegenerative Disorders

The nervous system is made up of neurons comprising of nerves and specialized cells that transmit signals between various parts of the body. The transmission of signals greatly influences the normal coordination of body activity. A small alteration in the signal transmission may cause neurological changes that lead to neurodegenerative disorders. AD and PD are two of such diseases that have distinct pathologies yet share some similar features in pathogenesis. There are some common cascades of neuronal events such as protein toxicity and oxidative stress prior to progressive neurodegeneration in these patients. Toxicity of alpha-synuclein in PD and Aβ42 or tau proteins in AD are some of the common neuronal alternations detected among those patients (15). In terms of cell signaling, both show overlapping events in the activation of oxidative stress, glycogen synthase kinase-3 beta, mitogen-activated protein kinases (MAPK), and cell cycle re-entry (16).

In addition, the development of both the PD and AD occurs in the brain. Many factors, such as aging, exposure to environmental toxin and unhealthy lifestyle, attribute to the development of these diseases (17). Both of the diseases are linked with mitochondria dysfunction in association with increased oxidative stress. Exponential oxidative stress, together with DNA mutation, leads to mitochondria dysfunction which is much implicated in the aging process (18). In AD, oxidative stress leads to the activation of amyloidogenesis pathway which elevates the development and deposition of amyloid peptide (19). Equally in PD, oxidative stress leads to misfolding of α-synuclein protein that causes neurodegeneration; loss of dopaminergic neurons and loss of dopamine in the brain (20).

Alzheimer's Disease

AD is a form of dementia characterized by deposition of neurofibrillary tangles and amyloid plaques in the brain which resulted in loss of neurons and synapses (21). AD can also be defined by the deterioration in everyday activity due to progressive waning in two or more cognitive functions and verified by anomaly in clinical and neuropsychological testing (22). AD can be categorized into 4 stages; (1) preclinical stage of AD, (2) Mild Cognitive impairment due to AD and (3) moderate AD and (4) Severe AD (23). The pre-clinical stage of AD is characterized by slight memory loss, alterations in the cortex and hippocampus, but no significant hindrance in everyday life: the mild cognitive impairment due to AD displays early symptoms of AD such as memory loss, disorientation and mood swings with amyloid accumulation in the brain, moderate AD shows increase memory loss, failure to recognize friends and family members, with amyloid accumulation and loss of neurological function, and finally, the severe AD stage leads to complete failure recognizing in friend and family, loss of cognitive activities as the cortex area is fully affected (24). Collectively, the progression of AD can be monitored by determining the accretion neurochemical such as amyloid, tau proteins and neurofibrillary tangles (25).

Approximately 60–80% of dementia cases are due to AD (26). According to the world Alzheimer's report, it is expected that more than 130 million people tend to suffer from this condition by the year 2050 (27). Those who are diagnosed with AD tend to live up to 8 years. In the United States, AD has been recognized as the 6th leading cause of death (28). Increasing age is known to be a major risk factor for AD patients above 65 years old, but the exact cause of AD remains unknown. Current available treatment is said to be very limited and failed in reversing the progression of the disease, although some may improve the symptoms temporarily. AD development is closely related to two distinct mechanisms in the nervous system, namely deposition of extracellular Aβ and accumulation of intracellular tau protein in the brain (29).

Amyloid beta (Aβ) is part of a larger protein, the transmembrane protein, amyloid precursor protein (APP). Aβ peptide is comprised of up to 43 amino acids in length as a result of amyloid precursor protein (APP) cleavage via β- and γ-secretase. In normal condition, the monomers and oligomers of Aβ are degraded by protease and cleared in the brain (30). Defective clearance of Aβ from aberrant APP cleavage results in the accumulation of Aβ at the extraneural tissues. When this happens, the soluble Aβ monomers polymerize initially into soluble oligomers, and then into larger insoluble fragments such as Aβ42, which precipitated as amyloid fibrils (31). Elevated level of amyloid fibrils in extracellular brain region will suppress excitatory synaptic activity at the postsynaptic level, particularly those in the form of oligomers will impair the synapse transmission. This happens because oligomers will bind to the pre- and post-synaptic neurons. Thereafter, loss of long term potentiation, synapse damages, and neuronal apoptosis will occur (32).

Tau protein is found in the neurons and its primary role is to ensure the stabilization of the internal microtubules. Tau protein accumulation happens due to the degeneration of neurofibrils. Initially, tau is aggregated as a paired helical filament, twisted around each other, forming paired helical filaments. These

deposits interfere with cellular functions by displacing organelles. By altering the spacing of microtubules, axonal transport is impaired, thereby affecting the nutrition of dendrites and axon terminals. This will start at the entorhinal cortex, preceding to the hippocampus, and then to the connection cortex at later time (33). These will result in microtubules instability, mitochondrial damage, and pathogenic signaling that alter the molecular motor phosphorylation. Eventually, the associated motor functions are altered (34).

Parkinson's Disease

PD is a progressive neurodegenerative disorder that affects movements. It usually results in trembling, imbalance, and stiffness (35). Clinically, features such as rest tremor, bradykinesia, postural instability and akinesia are most well-identified symptoms of PD (36). Other non-motor symptoms include psychiatric impediments such as control disorders, anxiety, depression, dementia and sleep disorders (37). The clinical symptoms are prominent during the third stage of the disease where the death of dopaminergic neurons in the substantia nigra pars compacta and noradregenic neurons at the locus coeruleus occurs (38). This neurological abnormality is pathologically characterized by the accumulation of α-synuclein protein in the form of Lewy bodies and Lewy neurites (39). Meanwhile, in association with PD, the neurochemical metabolites, such as dopamine, 5-hydroxytryptamine (5-HT), gamma-aminobutyric acid (GABA), and glutamate, have been found to be lower in PD patients (40). These neurotransmitters can be used to track the development of PD.

Epidemiological data indicate that men are 50% more prone to get this disease in comparison to women (35). Although age is considered to be the primary risk factor for PD, <10% of patients develop the disease before the age of 50. In certain cases, the etiology of this disease is closely related to certain gene mutations which include synuclein, alpha (SNCA), Parkin RBR E3 ubiquitin protein ligase (PARK2), Parkinson's disease protein 7 precursor (PARK7), PTEN-induced putative kinase 1 (PINK1), ubiquitin carboxy-terminal hydrolase L1 (PARK5), and leucine-rich repeat kinase 2 (LRRK2) (41). There are a few known mechanisms that are related to the development of PD.

One of the mechanisms is due to the imbalance of ROS level which arises from the metabolism of dopamine. In the presence of iron, dopamine breakdown may occur spontaneously. Dopamine is an unstable compound which can form quinones and hydrogen peroxide (H_2O_2) by auto-oxidizing. H_20_2 may form more active hydroxyl radical (?OH) by reacting with irons or oxygen (O_2). Dopamine quinones can react with cysteine in sulfhydryl groups, especially reduced glutathione (GSH), a ROS scavenger, resulting in decreased GSH and increased ROS levels (42). On the other hand, monoamine oxidase (MAO) catalyzes the breakdown of dopamine in a reaction that generates H_2O_2. The H_2O_2level is considered as safe to cells until cytotoxic OH are produced excessively through Fenton reaction. ROS can lead to structural and functional alterations in proteins, DNA and lipids. Lipid damage causes a loss of membrane integrity while increasing permeability to ions such as calcium in substantia nigra that will trigger excitotoxicity (43).

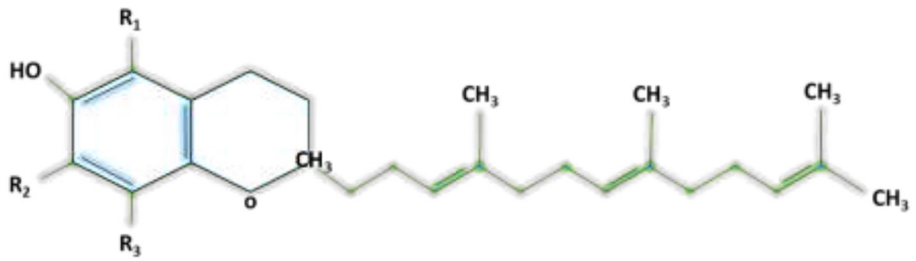
Another mechanism closely linked to PD is the loss of substantia nigra dopaminergic neurons associated with the involvement of intraneuronal inclusions known as Lewy bodies. The Lewy body is an unusual aggregation or clumping of the α-synuclein protein (44). In the brain, native α-synuclein is often deployed without a specified tertiary structure, although it can be present in stable tetramers that resist aggregation in aqueous solutions. Usually, α-synuclein folds through its N-terminal into α-helical structures upon interaction with negatively charged lipids, such as the phospholipids that make up cell membranes. In PD, α-synuclein adopts an amyloid-like structure rich in β-sheet that is vulnerable to aggregation. Several mechanisms, including serine 129 phosphorylation, ubiquitination, and C-terminal truncation, have been speculated for the conformational changes that lead to abnormal α-synuclein aggregation. α-synuclein can interact with the mitochondrial membrane and accumulate within the organelles. This contributes to the damage of complex I activity, eventually to mitochondrial dysfunction and increased oxidative stress. This is because the interaction between oligomeric α-synuclein and the translocase of outer membrane 20 (TOM20) mitochondrial receptor may lead to impairment of the machinery importing mitochondrial protein leading to decreased respiration which results in excessive formation of ROS (45).

VITAMIN E

Vitamin E is a lipid soluble compound that consists of eight members which are known for their antioxidant property. The members are divided into tocopherols and T3s such that each group is further divided into α, β, γ, and δ forms (46). Structurally, T3s and tocopherols share a chromanol head with an aliphatic side chain. Tocopherols have a phytyl tail (saturated) side chain, while T3s have an isoprenoid (unsaturated with three double bonds) side chain (47). The differences among α, β, γ, and δ forms are the position and number of the methyl groups on the chromanol head. **Figure 1** shows the different forms of T3s and tocopherols. Vitamin E can be found in nuts, vegetable oils and seeds, while palm oils, rice grain and annatto seed are a rich in T3s (47).

Biological Activities of Tocotrienols

The functional properties of tocopherols and T3s can be differed from one another: T3s have been reported to exhibit more potent activities than tocopherols. Apart from possessing neuroprotective effects, T3s have cholesterol-lowering capacity (48) and anti-tumor property (49). Despite the lower bioavailability, antioxidant activity of T3s outranges tocopherols. Previous study has shown that α-T3 has greater peroxyl radical scavenging capability in the liposomal membrane in comparison to the α-tocopherol due to the presence of unsaturated side chain which enhances the even distribution of T3 in the bilayer membrane. The even distribution facilitates the interaction between chromanol ring of α-T3 with lipid radicals (50). T3s also move much more rapidly than α-tocopherol within lipid vesicles (51).



Compound	R1	R2	R3	Phytyl chain
α-tocopherol	CH_3	CH_3	CH_3	Saturated
γ-tocopherol	H	CH_3	CH_3	Saturated
β-tocopherol	CH_3	H	CH_3	Saturated
δ-tocopherol	H	H	CH_3	Saturated
α-tocotrienol	CH_3	CH_3	CH_3	Unsaturated
γ-tocotrienol	H	CH_3	CH_3	Unsaturated
β-tocotrienol	CH_3	H	CH_3	Saturated
δ-tocotrienol	H	H	CH_3	Unsaturated

FIGURE 1 | Structures of tocotrienols and tocopherols.

THERAPEUTIC MECHANISMS OF TOCOTRIENOLS

AD and PD arise from distinct pathogenesis. Yet, they share some common molecular damages such as oxidative stress, mitochondrial dysfunction and neurodegeneration. In consideration to this, T3s have been shown to be effective in combating these damages. **Tables 1**, **2** summarizes the effect of T3s in AD and PD in regards to *in vitro* and *in vivo* trials.

Tocotrienols as an Antioxidant

The brain requires high level of oxygen for its optimal function. Due to this, it is the primary organ susceptible to oxidative insults by free radicals (45). The formation of Aβ hinders the activity of antioxidant enzymes, specifically glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) (52). Aβ binds to CAT, SOD and GPx with high affinity and inhibits H_2O_2 breakdown by these enzymes (52). Eventually, this will result in elevation of oxidative stress. As a consequence, proteins, lipids, and DNA will start to oxidize. Normally, SOD functions to dissociate excess superoxide anion into hydrogen peroxide (H_2O_2) and O_2, while glutathione peroxidase (GPx) and CAT will then reduce the H_2O_2 into water. In the absence of these enzymes, H_2O_2 will be transformed into hydroxide (^{-}OH) (53). In relation to this, α-T3 has been proven to increase the SOD activity in AD mice's brain (54). This clearly defines the reason behind the disruption of Aβ42 aggregation and reduction in Aβ deposition at the hippocampus of APPswe/PS1 AD mice (37, 39). This has been illustrated in **Figure 2** which refers to the mechanisms of action of T3s in AD. Similarly, Hamezah et al. (55) noticed a decrease of APP in the hippocampus of APPswe/PS1 mice treated with T3s-rich fraction (TRF). In addition, TRF hinders the formation of Aβ fibrils and oligomers in a cell-free assay, and decreases the accumulation of Aβ in the hippocampus and pre-frontal cortex of AβPP/PS1 mice (55).

Khor et al. (56) have shown that TRF has the capacity to upregulate gene expressions of GPx and CAT in senescent myoblasts. Furthermore, TRF decreases the activity of lipid peroxidase and inhibits its binding toward the DNA in neuronal cells of myoblasts, preventing oxidative damage to the DNA (56). T3s have the capacity to hinder the production of other reactive species such as nitric oxide molecule (3), particularly peroxynitrite (56) and nitrosamines (3). This mechanism is further supported by Mohamed et al. (57) who noticed a reduction in isoprostane F_2 by T3, a by-product of lipid peroxidation, in hippocampal cells prepared from 2-vessel occlusion neurodegenerative model rats. Damanhuri et al. (54) have shown that TRF increases CAT enzyme activity but has no effect on GPx activity in the hippocampus of AβPP/PS1 mice.

The existence of unsaturated side chain in γ-T3 eases the penetration of γ-T3 into tissues (58). This further ensures it is evenly distributed in the surface of the lipid layers of the cell membrane. With the aid of lipoprotein lipases or lipoprotein endocytosis mediated by receptors, tissue absorbs γ-T3 more effectively. Thus, peroxyl radicals will be removed rapidly as the even distribution most likely enhances the interaction of chromanols with lipid radicals (58). TRF is evidenced in protecting cytochrome P450 from oxidative damage (59). A study done by Tan et al. (60) proves that the location of α-T3, which is closer to the surface of the membrane, enhances the

TABLE 1 | Effects of T3s on AD in *in vitro* and *in vivo* studies.

References	Intervention	Gender and age	Model and strain	Findings
Yatin et al. (62)	T3&Tocopherol, dose not specified	18-day-old; gender not specified	Sprague Dawley rat's fetus; embryonic hippocampal neuronal cells of Aβ peptide-treated rat	• Reduced ornithine decarboxylase activity in the neuronal cells. • Decreased spermidine uptake by neuronal cells. • Increased polyamine metabolism in the neuronal cells.
Sung et al. (92)	T3&Tocopherol (member unspecified), 2 IU/g/d × 6 months, Regular chow	5 and 14 months; gender not specified	Tg2576 mice	• Reduced lipid peroxidation in hippocampus. • Reduced Aβ levels and amyloid deposition in hippocampus. • Reduced brain oxidative stress in hippocampus.
Conte et al. (93)	T3&Tocopherol, 2 IU/g/d × 8 weeks, Regular chow	11 months; female	Tg2576 mice	• Reduced lipid peroxidation in brain. • Reduced Aβ levels and amyloid deposition in brain. • Improved spatial learning ability. • Decreased ROS in brain.
Yao et al. (94)	T3&Tocopherol, 10 mg/ℓ/d × 7 months, Drinking water	8 months; female and male	Tg2576 mice	• Decreased Aβ1-40 and Aβ1-42 in hippocampus. • Suppressed brain inflammation and ROS in brain hippocampus. • Reduced isoprostane 8,12-iso-iPF2α (iPF$_{2\alpha}$-V) in brain hippocampus.
Grimm et al. (66)	10 μM α-T3, 24 h	Not applicable	SH-SY5Y wild type cells; APP695 transfected SH-SY5Y cells	• Reduced Aβ degradation in N2a cells. • Reduced ROS level. • Reduced cholesterol and cholesterol esters. • Reduced γ-secretase activity in neuro 2a (N2a) cells.
Damanhuri et al. (54)	TRF, 200 mg/kg/d × 6 months, Oral gavage	9 months; male	Wild type and double transgenic mouse B6C3-Tg (APPswe, PS1dE9) 85Dbo/Mmjax with C57BL/6J	• Increased superoxide dismutases (SOD) activity in hippocampus. • Increased catalase (CAT) activity in hippocampus. • Decreased DNA damage in the brain. • Increased in antioxidant effect.
Ibrahim et al. (95)	TRF, 1%, 0.01%, 0.001% v/v × 24 h	Not applicable	Cell free assay; Human neuroblastoma cell line SH-SY5Y	• Disrupted Aβ42 aggregation. • Reduced Aβ42 fibrils formation. • Inhibited Aβ oligomerization.
	TRF, 60 mg/kg/d × 15 months, Oral administration	5 months; female and male	APPswe/PS1dE9 double transgenic mice (APP/PS1)	• Absence of cytotoxic effect. • Reduced Aβ deposition in the hippocampus. • Reduced thioflavin-s-positive fibrillar type plaques in hippocampus and cortex.
Durani et al. (61)	TRF, 5 mL/kg/d × 15 months, Oral administration	5 months; female and male	APP/PS1 double transgenic mice	• Increased exploratory activity. • Enhanced spatial learning, memory and recognition memory. • Increased level of neurotransmitters such as (acetylcholine, L-tyrosine, L-glutamic acid, and L-aspartic acid) metabolism in medial pre-frontal cortex, striatum and hippocampus.
Mohamed et al. (57)	T3 (members unspecified), 100 mg/kg/d × 8 weeks, Oral administration	Male; age not specified	White albino rats (Sprague Dawley); 2-vessel occlusion rats	• Increased neurons with compact cellular structure of stratum pyramidale. • Increased well-demarcated cell membrane, clear cytoplasm, and distinct nucleus in brain. • Decreased Isoprostane F_2 (Iso-F_2) in brain.
Hamezah et al. (55)	TRF, 60 mg/kg/d × 10 months, Oral gavage	5 months; male and female	APPswe/PS1dE9 double transgenic (Tg) mice (AβPP/PS1)	• Inhibited ERK and c-Src in MAPK pathway. • Increased glial fibrillary acidic protein (GFAP) in hippocampus. • Decreased Interleukin enhancer-binding protein 2 (ILF-2), ACTR10 protein, APP and PTPRA protein expression in hippocampus.
Wan Nasri et al. (84)	TRF, 200 mg/kg/d × 6 months, Oral gavage	9 months; male	Double transgenic male mouse B6C3-Tg (APP swe, PS1dE9) 85Dbo/Mmjax with C57BL/6J	• Elevated gene expression of Ataxia telangiectasia mutated (ATM), and Calcium Voltage-Gated Channel Subunit Alpha1 B (CACNA1B). • Downregulated messenger RNA (mRNA) processing, focal adhesion-PI3K-Akt-mTOR signaling, epidermal growth factor receptor 1 (EGFR1) signaling, p53 signaling, T cell receptor signaling, Tumor necrosis factor-alpha (TNF-α) NF-kB signaling, and MAPK signaling.

TABLE 2 | Effects T3s on Parkinson's disease in *in vitro* and *in vivo* studies.

References	Intervention	Gender and age	Model and strain	Findings
Hagl et al. (96)	T3, tocopherol and γ-oryzanol, 150 mg/kg/d × 30 days, Oral gavage	Not applicable	PC12 cells; SNP-treated brain cells of guinea pigs fed	• Increased mitochondrial respiration in brain. • Increased dynamin-related protein 1 (Drp1) and fission 1 (fis1) proteins. • Increased mitochondrial mass.
Nakaso et al. (97)	10–1,000 nM γT3 and δT3 48 h	Not applicable	MPP^+-treated SH-SY5Y cells	• Cytoprotective against MPP+. • Activation of PI3K/Akt signaling pathway in cell line. • Formation of caveola. • Stimulation of translocation of estrogen receptor beta (ERβ) from cytosolic to the perinuclear space.
Hagl et al. (69)	T3, tocopherol and γ-oryzanol, 340 mg/kg/d × 3 weeks, Oral gavage	Female NMRI mice; 18 months	sodium nitroprusside (SNP)-treated mice	• Protected brain cells against nitrosative stress. • Increased mitochondrial content. • Increased mitochondrial membrane potential. • Increased adenosine triphosphate (ATP) production.
Nakaso et al. (98)	α-T3 and δ-T3, 100 μg/kg/d × 6 days, Oral gavage	Female and male; age not specified	MPTP induced C57BL/6 mice	• Increased expression of estrogen receptor-α in olfactory bulb and cerebellum. • Reduced estrogen receptor-β expression in the brain. • Preservation of neuron cells from oxidative damage. • Recovery of spontaneous activity.
Matsura (63)	γ-T3 and δ-T3, 48 h	Not applicable	MPP+-treated SH-SY5Y cells	• Cytoprotective against 1-methyl-4-phenylpyridinium (MPP+). • Activated MAPK, PI3K/Akt. • Translocation of Akt from cyctosolic to plasma membrane.
	γ-T3 and δ-T3, 6 days	Not specified	MPTP mouse PD model	• Recovered voluntary performance. • Reduced oxidative stress. • δ-T3 protection against MPTP-induced neurotoxicity.
Kumari (67)	α-T3 and γ-T3, 200 mg/kg/d × 28 days, Oral gavage	Male SD rats; 14 weeks old	6-OHDA-treated rats	• Delayed disease progression. • Diminished motor deficits. • Restored Parkinson's disease 6 (PARK 6) gene. • Restored gene expression of tyrosine hydroxylase (TH), Dopa-decarboxylase (DDC), Solute Carrier Family 18 Member A2 (SLC18A2), solute carrier family 6 member 3 (SLC6A3) and nuclear receptor related 1 (NURR1) genes. • Neuroprotective against dopaminergic neurons.

recycling process of chromanols. This serves as the main reason for continuous reduction of oxidative stress.

Most researchers agreed that T3s act as an antioxidant in AD mice (29, 31, 35–39). The protective mechanisms of T3s against AD has led to the reduction of Aβ, ROS, oxidative stress marker (Iso-F_2), or lipid peroxidation marker (iPF_{2a}-VI). In fact, the ability of T3s to act as an antioxidant is the primary reason to reverse certain age-associated symptoms such as memory. Durani et al. (61) noticed a drastic increase in spatial, exploratory, and recognition memory in AβPP/PS1 AD mice treated with TRF.

Yatin et al. (62) observed reduction in ornithine decarboxylase and spermidine by T3 in Aβ-treated embryonic hippocampal neuronal cultures of rat. The findings suggest that T3 acts as a free oxidative radical scavenger that enhances polyamine metabolism. The need for cellular polyamine biosynthesis and the ability to take up extracellular polyamines are increased when there is an increase demand for polyamines. A regulatory protein, an antizyme, is regulated by polyamine uptake and its biosynthetic rate. Antizyme synthesis is the cell's natural way to defend itself by slowing down transport of ornithine decarboxylase activity against over-accumulation of polyamines to toxic levels (62). This is another mechanism of T3 as an antioxidant agent.

Matsura (63) notices reduction of oxidative stress in 1-methyl-4-phenylpyridinium ion (MPP^+)-treated SH-SY5Y cells when the cells are supplemented with γ- and δ-T3 within 48 h. Comitato et al. (64) report that T3 is effective against PD-related toxicities such as MPP^+ through the binding of γ- and δ-T3 to ERβ which will lead to a marked activation of PI3K/Akt signaling pathway. Activation of PI3K/Akt signaling reduces ROS by negatively regulating expression of the downstream proteins such as Forkhead Box Protein O1 (FOXO1) and Caspase 3 (65). Comparably, Nakaso et al. (66) observe the activation of PI3K/Akt signaling via ERβ binding by γ- and δ-T3 in MPP^+ treated SH-SY5Y cells. The similar mechanism is believed to be underlaid in the study done by Kumari (67, 68) who notice a prevention in the loss of dopamine neurons in α- and γ-T3 treated groups. **Figure 3** summarizes the action of T3 in PD as an antioxidant agent.

Hagl et al. (69) observe the T3's ability to protect brain cells against sodium nitroprusside (SNP)-induced nitrosative stress in aged NMRI mice. Nitrosative stress is a common

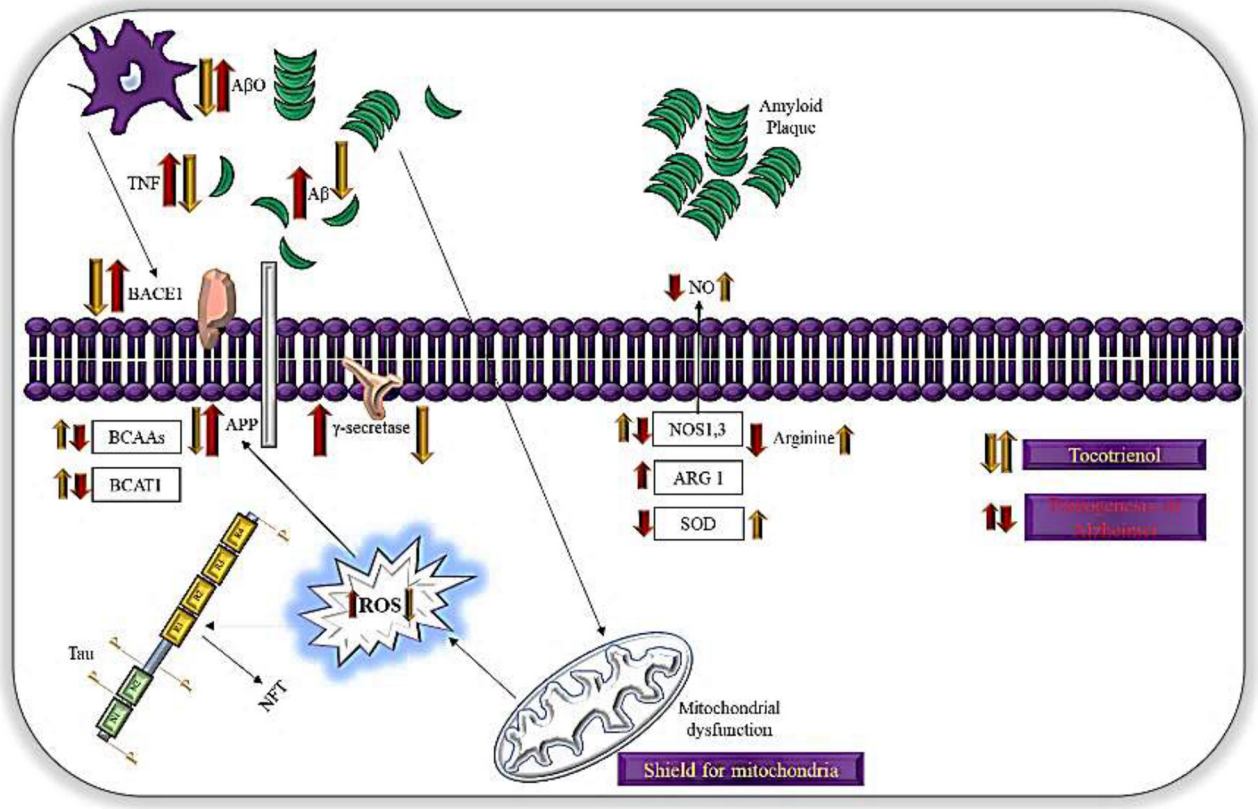


FIGURE 2 | Mechanisms of action of T3s in AD. T3possess antioxidant activities, which mitigate oxidative damage in neurons. T3 shown to increase the translocation of BACE1 to the cellular membrane leading to enhanced non-amyloidogenic APP processing and impaired γ-secretase dependent APP cleavage. This mechanism also due to lipid raft disruption. Mitochondria is a main generator for ROS while T3 reduces lipid peroxidation and act as shield for mitochondria, preventing formation of ROS.

pathology found in PD patients. It facilitates S-nitrosylation of neuroprotective proteins and compromises their function, thereby leading to cognitive impairments (70). This is because SNP is a potent neurotoxicant as it generates nitric oxide (NO), cyanides, and free irons. This could stimulate typical programmed cell death changes such as formation of the apoptotic nuclei, activation of caspase-3/7 and−9, decrease of the Bcl-2/Bax ratio, decrease level of matrix metalloproteinases (MMP), and release of cytochrome c in neuronal cells. In dopaminergic cells, this may trigger cytotoxicity that leads to dopaminergic cell death caused by SNP (71). At the same time, NO may combine with superoxide to form peroxynitrite, which rapidly causes protein nitration or nitrosylation, lipid peroxidation, DNA damage and neuronal cell death (72). In this scenario, γ- and δ-T3 reduce nitrosative stress by minimizing the activity of nitric oxide synthase and then leading to the reduced formation of nitric oxide (73). This is achieved by downregulating TNF-α, TNF-1β, NF-κB and caspase-3 activities (72).

Furthermore, in AD, an increase in 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase is a common pathology. Along with tocotrienol consumption, there are inverse associations that have been witnessed between the incidence of AD and blood levels of the HMG CoA reductase. T3s has the ability to inhibit the processing and nuclear localization of SREB-2, the transcriptional factor for HMG CoA reductase and farnesyl pyrophosphate (FPP) synthase, and further promotes HMG CoA reductase degradation. As a result, tocotrienols decrease the pool of FPP and geranylgeranyl pyrophosphate (GGPP), potentially slowing the progression of prenylation-dependent AD. T3s' anti-inflammatory properties contribute to their protection against Alzheimer's disease. In sum, T3s inhibit the processing and maturation of SREBP-2 and increase the breakdown of HMG CoA reductase, making them a preventive agent against AD (74).

Tocotrienols as a Cell Signaling Mediator

Neurotransmission of excitatory glutamatergic neurons is essential for the survival of neurons and synaptic plasticity. Normally, this neurotransmission mechanism takes place with N-methyl-d-aspartate receptor (NMDAR). However, small alteration in this activity, mainly elevation of NMDAR, could result in excitotoxicity of glutamatergic neurons, thereby leading to neuronal cell apoptosis in AD (75). In PD, slight increase in glutamate concentration due to loss of nigral dopaminergic neurons triggers the glutamate overactivity (76). In both

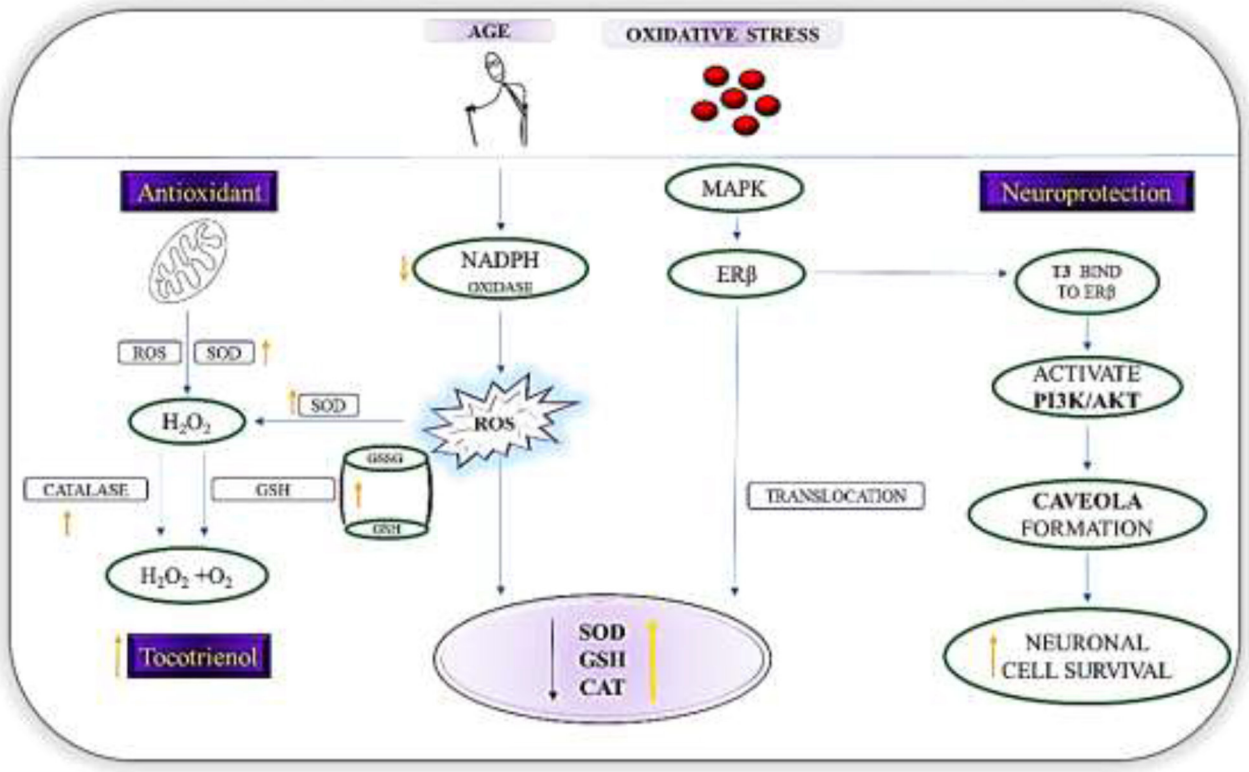


FIGURE 3 | Mechanisms of action of T3s in PD. Diagram shows the role of T3 as a first line defense antioxidants in PD by increasing the level of SOD, GSH, and catalase via the nuclear translocation of ERβ and breakdown of H_2O_2 to harmless molecules (H_2O_2/alcohol and O_2). T3, via the regulation of NADPH oxidase reduces reactive oxygen species formation. In addition, T3s binds directly ERβ, which then triggers the signal transduction pathway and activates PI3K/Akt pathway. Resultantly, caveola will be formed. These initial events constitute an estrogen dependent non-genomic pathway that is neuroprotective.

disorders, the increased level of glutamate eventually damages the neurons, especially the HT4 neural cell (77). Aside from this, in consequence of the glutamate rise, extracellular signal regulated kinase (ERK) (78) and 12-lipoxygenase (LOX) (79) will be activated. Hence, there will be a sudden rise in the level of calcium ion (Ca^{2+}), depletion of GSH, dysfunction of mitochondria and eventually neuronal death (79). T3s, especially α-T3, are found to provide neuroprotection in glutamate-induced death of neuron cells at nanomolar concentrations beyond its antioxidant nature (58).

Cellular antioxidant defense is compromised by a GSH-depleted state, accompanied by increased susceptibility of the cell to ROS. T3s, through an antioxidant-independent mechanism, are able to inhibit the activation of $pp60^{c-src}$ kinase. This happens as nanomolar level of α-T3 suppresses glutamate-induced early activation of c-Src kinase (58). Src family kinases are a group of genes that are involved in the regulating of cell growth. The Src family kinase product, c-Src, is strongly expressed in the brain. Overexpression of c-Src may stimulate the activation of glutamate leading to neurodegeneration caused by glutamate (80).

Apart from this, T3s enhance the re-entrance of injured cells into S phase (DNA synthesis) and G2/M phase (DNA repair and recovery) of cell cycle (81). This is to reverse the glutamate-induced apoptosis of the neuronal cell (58). Inversely, since brain contains a very high level of arachidonic acid (AA), a major component of polyunsaturated fatty acids (PUFAs), it is highly susceptible to oxidative metabolism. T3s have acted as mediators for AA, altering metabolism of AA (58). In this, α-T3 will interact directly with the enzyme that suppress AA anabolism (82). This happens as T3s are able to cleave the membrane phospholipid bilayer via cytosolic phospholipase A_2 (58). The carboxyl (COOH) terminal at the AA site will enter the 12-LOX solvent cavity while the catalytic site is accessed. It is therefore possible that the binding position of α-T3 prevents access of the AA substrate to the active 12-LOX site. As so, LOX-12 is inhibited. Thus, 12-LOX inhibition protects Aβ-induced toxicity in the corticol neurons (82). The reduction in receptor-type tyrosine-protein phosphatase alpha (PTPRA) protein expression by TRF, as shown by Hamezah et al. (55), further confirm the role of TRFs as a neuroprotective agent (55) via the inhibition of ERK and c-Src in MAPK pathway in APPswe/PS1 mice. PTPRA is perceived as a crucial mediator for synaptic plasticity and neuronal migration and association with memory. At the same time, PTPRA plays a central role as an activator of Src family kinases (83). In regards to this case, TRFs

have been proven to be effective in decreasing the excess level of PTPRA at the hippocampus of AβPP/PS1 mice, thereby exerting neuroprotective effect (55).

On the contrary, Wan Nasri et al. (84) observe a significant increase of *ATM* and *CACNA1B* expression in AβPP/PS1 mice treated with TRF. One of the primary functions of ATM is to provide sufficient duration for DNA repair in the cell cycle. This is a post mitotic process which prevents replicative stress on the neurons (85). Expression of ATM signaling is feasible for replication process of neurons, thereby reducing neuronal apoptosis. *CACNA1B* encodes the presynaptic neuronal voltage-gated calcium channel Cav2.2/N-type pore-forming subunit. As so, this *CACNA1B* is essential for neurotransmission (86). In other words, the expression of *CACNA1B* enhances excitatory transmitters (87). From here, we can speculate that in the AD mice, the action potential generated by excitatory neurotransmitters has been re-established upon being treated with T3s.

Tocotrienols as a Shield for Mitochondria Dysfunction

Mitochondrial dysfunction is an early hallmark of AD and PD. Extracellular or intracellular Aβs are imported into the inner membrane of mitochondria in the neuronal cell of AD brain. The progressive accumulation of mitochondrial Aβ leads to impaired energy metabolism, functional defects in key respiratory enzymes, increased mitochondrial ROS and altered mitochondrial biogenesis. This Aβ will bind to proteins in the mitochondria, such as cyclophilin D (CypD) and amyloid-binding alcohol dehydrogenase (ABAD), leading to mitochondrial dysfunction which will contribute to neuronal damage and cognitive impairment (88). Meanwhile, mutations in the mitochondrial DNA (mtDNA) are common feature of PD. The accumulation of mtDNA mutations will increase the relative levels of somatic mutations. mtDNA mutations lead to bioenergetic mitochondrial deficiency in the neurons of substantia nigra. With the accumulation of mtDNA mutations and increase in oxidative stress, reduction in mitochondrial function decreases cellular bioenergetics and favors α-synuclein aggregation, leading to the development of Parkinsonism (89).

T3s have been speculated as effective in shielding mitochondria and microsomes from oxidative stress. Mitochondria has been reported as the major target of oxidative stress due to its major role as an energy powerhouse which generates free radicals as by-products (90). Cytochrome c is generated excessively when ROS is produced inside the mitochondria (91). Cytochrome c will then come into contact with caspase-9 and Apaf-1. Thus, caspases-associated apoptosis is stimulated. Caspase-3 activation results in DNA-fragmentation and apoptosis (90).

T3s function not only limited to decreasing the activity of lipid peroxidation, but also act as a protective shield against mitochondrial dysfunction. γ-T3 exhibits positive effects such as improved mitochondrial function on aged mice through the elevation of ATP level and membrane potential. This is confirmed through the increase expression of mitochondrial transcription factor A which serves as an initiator of mtDNA duplication and further leads to increased mitochondrial function (81). Another study reveals significant reduction in mitochondrial levels of pro-apoptotic proteins such as Bid, Bax, and Bad upon being exposed to T3s in the hippocampus of AD mice (99). Concurrently, Park et al. (100) observe subsequent increase in mitochondrial levels of anti-apoptotic proteins such as Bcl-2 and Bcl-xL when α-T3s is used as a treatment in the glutamate-treated primary hippocampal neurons. Studies done by Hagl et al. (62, 92) have shown that T3s act as mitochondrial shield by increasing mitochondrial mass, content, membrane potential and respiration in the brain of APPswe/PS1 mice and APP695-treated SH-SY5Y cells. Therefore, it can be speculated that the stabilization and integrity of the mitochondrial membrane can be strengthened when a small amount of T3s is being used as treatment.

TOCOTRIENOLS-BASED SUPPLEMENTS IN THE MARKET

The beneficial effects of T3s on neurodegenerative disorders have been proven through *in vitro* and *in vivo* studies; and no adverse effect or dose toxicity of T3s has been reported in these studies. The positive outcomes have urged the pharmaceutical industries to formulate T3s into tablets, powder, or syrups for consumption as health supplement. Nevertheless, the efficacy of these products on human health remains to be tested. Most of these products are labeled as antioxidants, while others claimed to improve neurological function and prevent cognitive aging. With the evidence from cell and animal studies, more and new T3s-based products are expected to be released by the pharmaceutical companies, but clinical trials will be needed to convince the consumers in purchasing these products. **Table 3** shows the currently marketed T3 products which claimed to be neuroregenerative.

The bioavailability of T3 is the definition which incredibly impacts the degree of assimilation. The same group of analysts also demonstrate that self-emulsifying frameworks, that produce better beads of emulsion, noticeably upgrade the retention of T3 by ∼2–3-folds higher compared to non-emulsified definition. It is also observed that the slack time required for the emission of bile salts to emulsify the T3 in arrange to encourage their retention are moderately shorter with the nearness of self-emulsifying frameworks (13).

SAFETY, LIMITATION, AND FUTURE PROSPECTIVE OF TOCOTRIENOLS

The low bioavailability of T3s, particularly in the form of oral formulations, is one of the greatest challenges in the therapeutic field (101). Due to this, the recommended dose to consume is 50–100 mg of T3s in a day to experience the beneficial effect (103). In terms of cost, extraction of T3s can be very expensive. In relation to this issue, approximately more than US$8000 is needed to extract 1 g of γ-T3 from natural sources (46).

TABLE 3 | T3s-based products for neuroregeneration.

Formulation	Brand and company	Claim	Dosage	References
δ-T3	DeltaGold®, American River Nutrition	Prevent age-related cognitive decline	3.5 mg	(101)
α,β,δ, and γ-T3	Naturale, DavosLife	Cell wellness and protection	50 mg	(102)
Natural α,β,δ, and γ-T3	EVNol™, ExcelVite	Antioxidant in various system	30–50 mg/day	(103)
Bioenhanced natural α,β,δ, and γ-T3	EVNol Suprabio™, ExcelVite	Antioxidant in various system	30–50 mg/day	(103)
Water dispersible natural α,β,δ, and γ-T3	EVNolMax™, ExcelVite	Antioxidant in various system	30–50 mg/day	(103)
Water dispersible natural α,β,δ, and γ-T3	EVNolBeV™, ExcelVite	Antioxidant in various system	30–50 mg/day	(103)
Natural α,β,δ, and γ-T3	Tocobeads®, ExcelVite	Antioxidant in various system	30–50 mg/day	(103)
γ-T3	Tocotrol™ L50P, Fuji chemical industry	Improve neurological function	50–100 mg	(104)
30% Palm T3 Powder	TocoTab®, Fuji chemical industry	Improve neurological function	50–100 mg	(104)

This is the main reason why most of the study is limited to tocopherols or synthetic tocopherols, instead of focusing on T3s. Additionally, it is really hard to synthesize T3s due to the existence of three double bonds in the isoprenoid side chain (101). So far, there is no reported dose limiting toxicity that has been recorded for T3s consumption. However, it is advisable for those with blood disorders to avoid consuming T3s as they possess anticoagulant effect (81). There are limitations including the forms of vitamin E that are not clarified in some areas. As such, the composition of vitamin E supplement might not reflex the genuine composition within the diet. At present, there is no reliable evidence of adequacy of vitamin E within the avoidance or treatment of individuals with AD; in this way, more investigation is required. However, the preclinical evidence supporting the use of antioxidants to prevent or slow AD is strong. There is clear evidence for increased oxidative damage in the brain of AD patients and numerous potential sources of excess free radicals that may contribute to this damage. To add on, the market for T3s remains robust. By the year 2026, it is expected that the market value for T3s might reach up to USD522.0 million (105). In considering this, continuity in research, especially in bioavailability, is highly recommended to fully utilize T3s for the prevention of neurodegenerative disorders in the future.

CONCLUSIONS

In vitro and *in vivo* studies have confirmed the neuroprotective potential of T3s against neurodegenerative diseases such as AD and PD through the actions as antioxidant, shield of mitochondria damage, and neuroprotectant against neuronal cell death. These effects are expected to be validated by clinical trials. The market value for T3s as supplement for preventing neurodegenerative diseases is high, while approaches that lower their extraction cost and enhances bioavailability are anticipated as sensible focus of future research in T3s.

AUTHOR CONTRIBUTIONS

RN: conceptualization, software, data curation, and visualization. RN and NS: methodology and writing review and editing. HB and NS: validation and investigation. RN and HB: formal analysis and writing original draft preparation. PK: resources. HB: supervision and funding acquisition. NS: project administration. All authors have read and agreed to the published version of the manuscript.

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Dietary Fish Hydrolysate Improves Memory Performance Through Microglial Signature Remodeling During Aging

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Brain aging is characterized by a chronic low-grade inflammation, which significantly impairs cognitive function. Microglial cells, the immunocompetent cells of the brain, present a different phenotype, switching from a homeostatic signature (M0) to a more reactive phenotype called "MGnD" (microglial neurodegenerative phenotype), leading to a high production of pro-inflammatory cytokines. Furthermore, microglial cells can be activated by age-induced gut dysbiosis through the vagus nerve or the modulation of the peripheral immune system. Nutrients, in particular n-3 long chain polyunsaturated fatty acids (LC-PUFAs) and low molecular weight peptides, display powerful immunomodulatory properties, and can thus prevent age-related cognitive decline. The objective of this study was to investigate the effects of n-3 LC-PUFAs and low molecular weight peptides contained in a marine by-product-derived hydrolysate on microglial phenotypes and intestinal permeability and their consequences on cognition in mice. We demonstrated that the hydrolysate supplementation for 8 weeks prevented short- and long-term memory decline during aging. These observations were linked to the modulation of microglial signature. Indeed, the hydrolysate supplementation promoted homeostatic microglial phenotype by increasing TGF-β1 expression and stimulated phagocytosis by increasing Clec7a expression. Moreover, the hydrolysate supplementation promoted anti-inflammatory intestinal pathway and tended to prevent intestinal permeability alteration occurring during aging. Therefore, the fish hydrolysate appears as an interesting candidate to prevent cognitive decline during aging.

Keywords: n-3 long chain PUFA, low molecular weight peptides, microglia, memory, hydrolysate, cognitive decline, aging

INTRODUCTION

Brain aging has been associated with a chronic low-grade inflammation, in humans (1–3) and rodents (4–6). Neuroinflammation is finely orchestrated by microglial cells, the immunocompetent cells of the central nervous system (CNS). In the healthy brain, microglial cells exhibit a unique molecular homeostatic signature (M0) but with aging, these cells can display a novel

non-homeostatic signature called "MGnD" (microglial neurodegenerative phenotype) and become sensitized to inflammation and highly reactive, leading to an imbalance between pro- and anti-inflammatory cytokine production (7, 8). During aging, microglial cells express pro-inflammatory markers such as galectin 3 (Lgals3), the AXL receptor tyrosine kinase (Axl), c-type lectin domain family 7-member A (Clec7a), the major histocompatibility complex class II (MHCII) and the integrin subunit alpha X (Itgax also known as CD11c) (9–11). Moreover, transforming growth factor β (TGF-β), an important molecule in the maintaining of the M0 phenotype, is decreased in microglial cells of aged mice, contributing to the shift toward MGnD signature (7, 10). Microglia can also be activated by aged-induced gut dysbiosis. Indeed, aging has been linked to a decrease of gut microbiota diversity and an increase of intestinal permeability and inflammation, contributing to microglia activation *via* the vagus nerve or by direct modulation of the peripheral immune system (12–15).

This microglial dysfunction can lead to aged-related cognitive decline which is characterized by non-pathological, but significant alterations of memory in both humans and animals (16, 17). This cognitive decline can lead to alteration of well-being and quality of life (18, 19). Indeed, in humans, a mild stimulation of the host defense is associated with increased cytokine release and negative effects on emotional and memory functions (20). In rodents, interleukin (IL)-1β injection induces decreased memory performance as measured in an 8-arm radial maze or in the Morris water maze (21, 22). Moreover, spatial memory is altered in transgenic mice overexpressing tumor necrosis factor α (TNF-α) in the brain, while it is enhanced in TNF-α deficient mice (23, 24). Thus, targeting inflammation during aging constitutes a good strategy to delay or limit the development of age-related cognitive deficits (25).

Nutrition is an innovative strategy to prevent age-related cognitive impairments. Among all nutrients, n-3 long chain polyunsaturated fatty acids (LC-PUFAs) and low molecular weight peptides derived from proteins are good candidates for their immunomodulatory properties. n-3 LC-PUFAs, including docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), display powerful anti-inflammatory and pro-resolutive properties. Indeed, they regulate the release of pro-inflammatory mediators, as evidenced in clinical and pre-clinical *in vivo* studies, as well as in *in vitro* studies (26–28). In humans suffering from diseases associated with chronic low-grade inflammation, supplementations with EPA and/or DHA reduce circulating pro-inflammatory cytokines expression and increase the production of specialized pro-resolving mediators (SPM) (29–31). Supplementation with n-3 LC-PUFAs in adult rodents prevents the increase of the pro-inflammatory cytokine expression IL-1β, IL-6 and TNF-α induced by lipopolysaccharide (LPS) or IL-1β and increases hippocampal production of anti-inflammatory cytokines, such as IL-10 and IL-4 (32–38). Furthermore, numerous observational and interventional studies highlighted the positive association between the consumption of dietary n-3 LC-PUFAs and cognitive performance in the elderly (39–43). Similarly, beneficial effect of n-3 LC-PUFAs supplementations on cognition have also been shown in aged rodents (44–47). Aged mice supplemented with DHA and/or EPA are protected against neuroinflammation and cognitive impairment (46). *In vitro*, anti-inflammatory effects of n-3 LC-PUFAs have been demonstrated in microglial cells with the reduction of LPS-induced expression of pro-inflammatory cytokines as well as the polarization of microglial cells into an anti-inflammatory phenotype (48–54). Low molecular weight peptides (<1,000 Da) contained in protein hydrolysates are also nutrients of interest for their central and peripheral anti-inflammatory properties, demonstrated *in vivo* and *in vitro* (55–59). In a mouse model of Alzheimer's disease, peptides from milk reduced the expression of inflammatory factors such as TNF-α, monocyte chemoattractant protein-1 (MCP-1/CCL2) inducible nitric oxide synthase (iNOS) in the brain (60). *In vitro*, in human primary monocytes and murine macrophages, salmon- and lupine-derived peptides inhibited the production of nitric oxide (NO), prostaglandin (PG) E2 and pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β (61, 62). At the periphery, peptides from soy and milk reduced peripheral expression of pro-inflammatory factors such as TNF-α, IL-6, IL-1β, interferon-γ, or IL-17 in mice colon and abdominal aorta (56, 63). Furthermore, at the intestine level, an intake of marine n-3 PUFAs or bioactive peptides (from soy or oyster hydrolysate, for example) has been shown to decrease intestinal inflammation induced by inflammatory bowel diseases in humans and mice (64, 65). n-3 LC-PUFAs have been shown to influence the gut microbiota and improve intestinal immunity (66, 67). In rodents, supplementation with n-3 LC-PUFAs increases the number and abundance of beneficial bacteria, such as *Bifidobacterium* (68, 69). EPA and DHA have also been shown to prevent intestinal permeability changes induced *in vitro* and *in vivo* (70). Moreover, low molecular weight collagen peptides have been shown to protect the intestinal barrier function *in vitro via* the regulation of tight junction proteins zonula occludens 1 (ZO-1) and occludin (Ocln) expression and distribution and the myosin light chain kinase (MLCK) pathway (71, 72).

In this study we investigated the effects of n-3 LC-PUFAs and low molecular weight peptides contained in a marine by-product-derived hydrolysate on microglial signature, intestinal permeability, and cognition in mice.

MATERIALS AND METHODS

Animals

Fifteen-month old male C57Bl6/J mice (Janvier Labs, Le Genest-Saint-Isle, France) were housed under normal 12 h-12 h light/dark cycle on cellulose litter in a controlled environment (21–23°C, 40% of humidity), with *ad libitum* access to food and water. Animal husbandry and experimental procedures were done in accordance with the EU Directive 2010/63/EU for animal experiments and were approved by the local ethical committee (CE050 from Bordeaux) for the care and use of animals (approval ID APAFIS#14144-2018041213072383).

Diet

Mice were randomly assigned to different groups: one group ($n = 10$) fed a control diet (INRAE Jouy-en-Josas, France)

TABLE 1 | Composition of the control and the hydrolysate-enriched diets.

Components	Percent (%)	
	Control diet	Hydrolysate-enriched diet
Hydrochloric casein	18	18
Corn starch	45.9	45.7
Sucrose	24	24
Cellulose	2	2
Peanut oil	5	5
Mineral mix	4	4
Vitamin mix	1	1
+ DL methionine	0.1	0.1
+ Vitamin A 5 UI/g	5 UI/g	5 UI/g
Hydrolysate	0	0.29

and one group ($n = 11$) fed the hydrolysate-enriched diet (INRAE Jouy-en-Josas, France) containing 0.29% of the fish hydrolysate for 8 weeks (**Table 1**; **Figure 1**). The fish hydrolysate was provided by the BrainBooster Consortium. It was obtained from marine by-products and contained mostly low molecular weight peptides (<1,000 Da) and n-3 LC-PUFAs such as DHA and EPA. The specific composition of the fish hydrolysate is detailed in patent number B251427FR. The fish hydrolysate dose was determined as previously shown (73). The dose of low molecular weight peptides was 5.55 mg/mouse/day, and the dose of n-3 LC-PUFAs was 280 μg/mouse/day (of which 70 μg/mouse/day of DHA and 179 μg/mouse/day of EPA).

EchoMRI

Fat mass and lean mass were quantified at the beginning and at the end of the supplementation by magnetic resonance using minispec LF90 II (Bruker, Wissembourg, 67166).

Behavioral Tests

Y-Maze

Eight weeks after the beginning of the supplementation, short term spatial recognition memory was evaluated with the Y-maze test as described previously (74). The apparatus is a Y-shaped maze with 3 arms (35 cm long and 10 cm deep), illuminated at 15 lx. Extra-maze visual cues are placed on the walls, allowing mice to navigate in space. In the first trial, one arm of the Y-maze was closed, and mice were allowed to visit the two other arms for 5 min. Short term spatial memory was evaluated after a 1 h inter-trial interval (ITI), where mice were placed again in the start arm for the second trial and allowed to explore freely all three arms for 5 min. Start and closed arms were randomly assigned for each mouse. The animals were video-tracked (SMART system; Bioseb, Vitrolles, France) to analyze the distance traveled in the different arms.

Morris Water Maze

Spatial learning and memory were assessed in the Morris water maze as previously detailed (73, 75). Briefly, two familiarization days were designed. Mice had to find a submerged platform in a small pool (60 cm diameter) to familiarize with water and swimming (3 consecutive trials a day; 60 s cut-off). Then, visuomotor deficits were evaluated during a day of cued learning in the Morris water maze where mice had to find a submerged platform pointed out with a cue (6 trials a day; 90 s cut-off). During spatial learning, mice were trained during four consecutive days to learn the location of the submerged platform by using distal extra-maze cues (6 trials a day; 90 s cut-off). For each trial, the distance traveled to reach the platform was recorded by Imetronic videotracking system (Pessac, France). Spatial memory was assessed 72 h after the last day of training, during the probe test for 60 s in the maze without the platform. The distance traveled in the four quadrants was recorded using the SMART system (Bioseb, Vitrolles, France). The quadrant containing the platform is referred to as "target quadrant."

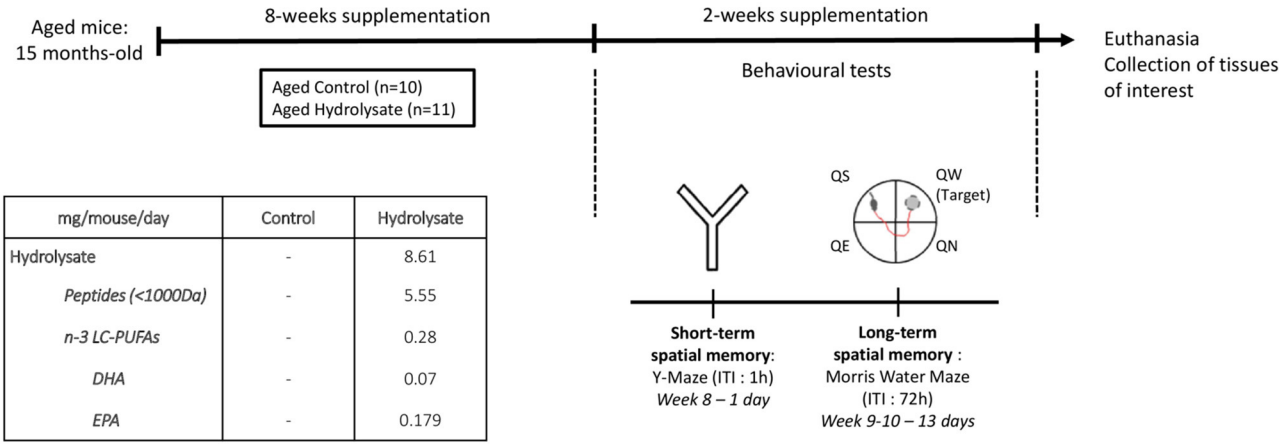
Tissue Preparation

Mice were euthanized by injection of a cocktail of ketamine/xylamin the day following the probe test. After transcardiac perfusion with phosphate buffered saline (PBS), brain structures and peripheral organs of interest were collected and frozen at −80°C until further analysis. For the immunohistochemistry analysis, hemispheres were post-fixed in 4% paraformaldehyde (PFA) overnight at 4°C, cryoprotected in 30% sucrose during 48 h at 4°C, rapidly frozen with isopentane and stored at −80°C.

Biochemical Measurements

Quantitative Real-Time PCR

The expression of the different genes of interest was evaluated by real time quantitative PCR as previously described previously (73). These analyses were performed on central (hippocampus) and peripheral structures (ileum and colon). Briefly, total RNAs were extracted from hippocampus, ileum and colon by TRIzol (Invitrogen, Life Technologies, Saint Aubin, France). Quantity and purity of RNA for each sample were measured by spectrophotometry (Nanodrop, Life technologies, Saint Aubin, France). Reverse transcription was performed on one or two micrograms of RNA by Superscript IV (Invitrogen, Life Technologies, Saint Aubin, France). TaqMan® specific primers were used to amplify genes of interest as previously described (73). We focused on IL-6 (Mm00446190_m1), IL-1β (Mm00434228_m1), TNF-α (Mm00443258_m1), TGF-β1 (Mm01178820_m1), transforming growth factor β receptor 2 (TGF-βr2; Mm03024091_m1), αM integrin (Itgam; Mm00434455_m1); transmembrane protein 119 (Tmem119; Mm00525305_m1), P2Y purinoceptor 12 (P2y12; Mm00446026_m1), colony-stimulating factor 1 receptor (CSF1r; Mm01266652_m1), MHCII (Mm00439216_m1), triggering receptor expressed on myeloid cells 2 (Trem2; Mm04209424_g1), Apolipoprotein E (ApoE; Mm01307193_g1), Lgals3 (Mm00802901_m1), Axl (Mm00437221_m1), Clec7a (Mm01183349_m1), Itgax (Mm00498708_g1), IL-10 (Mm01288386_m1), Ocln (Mm00500912_m1), ZO-1 (Mm00493699_m1), claudin 5 (Cldn5; Mm00727012_s1), and MLCK (Mm00653039_m1). The housekeeping gene was β-2-microglobulin (B2m; Mm00437762_m1). Fluorescence was determined on a LightCycler® 480 instrument II (Roche, La



mg/mouse/day	Control	Hydrolysate
Hydrolysate	-	8.61
Peptides (<1000Da)	-	5.55
n-3 LC-PUFAs	-	0.28
DHA	-	0.07
EPA	-	0.179

FIGURE 1 | Experimental design. Aged mice (15 months) were fed with the control diet or with the hydrolysate-enriched diet for 8 weeks. Behavioral tests were performed during the next 2 weeks. Total supplementation duration was 10 weeks. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ITI, inter-trial interval; LC-PUFAs, long chain polyunsaturated fatty acids; QE, quadrant east, QN, quadrant north; QS, quadrant south; QW, quadrant west.

Rochelle, France). Data were analyzed using the comparative threshold cycle (Ct) method and results were expressed as relative fold change (73, 76, 77) to control target mRNA expression.

Immunohistochemistry

Free-floating coronal sections of 40 µm through the hippocampus were collected on a cryostat (Leica Biosystems, Nanterre, France). After being washed for 10 min with PBS-Tween 0.01%, sections were blocked in a buffer containing 5% of donkey serum, 5% of bovine serum albumin (BSA), 0.3% Triton in PBS 1X for 1 h at room temperature (RT). Sections were then immunolabelled with a rabbit polyclonal antibody against Iba1 (1:1,000; Wako #019-19741, Plaisir, France) and a rat polyclonal antibody Clec7a (1:50, Invitrogen, Life Technologies # MABG-MDECT, Saint Aubin, France) in a staining buffer containing 5% of BSA, 0.1% of triton in PBS 1X over night at 4°C. After being washed in PBS-Tween 0.01%, slices were incubated with donkey anti-rabbit 488 (1:2,000; Invitrogen, Life Technologies #A-21206, Saint Aubin, France) and donkey anti-rat 594 (1:100, Invitrogen, Life Technologies #A-21209, Saint Aubin, France) secondary antibodies in a buffer containing 5% of BSA in PBS 1X for 2 h at RT. All sections were processed in parallel. Staining was visualized using DAPI (Santa Cruz Biotechnology, Heidelberg, Germany). Images were obtained with a 20× microscope objective and the software NIS-Elements AR3-2 (Nikon Eclipse 400, Nikon Corporation, Champigny-sur-Marne, France). The number of Iba1- and Clec7a-positive cells in the hippocampus was counted using Image J software (Image J, open source).

Western Blot

Proteins were extracted from the TRIzol fraction previously recovered from the RNA extraction step using the extraction protocol of Simões et al. (78). Protein concentration was determined by bicinchoninic acid protein assay (Interchim, Montlucon, France) according to the protocol. For analysis, proteins were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were incubated with different primary antibodies: a rabbit polyclonal anti-ZO-1 (1:500, #61-7300, Invitrogen, Life Technologies, Saint Aubin, France), a rabbit polyclonal anti-ocln (1:250, #40-4700, Invitrogen, Life Technologies, Saint Aubin, France) and a rabbit polyclonal anti-GAPDH as housekeeping protein (1:10,000; #51745, Cell Signaling, Leiden, Netherlands). These primary antibodies were detected with appropriated donkey horseradish peroxidase-conjugated secondary antibodies (1:5,000, #711-035-152, Jackson Immunoresearch, Westgrove, PA, USA). The membranes were incubated with a peroxidase revealing solution (SuperSignal West Dura, ThermoFisher, Waltham, MA, USA) and were revealed using ChemiDoc MP (Biorad, Hercules, CA, USA). Proteins of interest were normalized to GAPDH and results are expressed as relative expression.

Data Analysis

Hierarchical cluster analysis was performed using R free software (79), version 4.0.3. Forty variables were used (**Table 2**). Then, unsupervised hierarchical analysis was performed with *hclust* function (80) using Ward's linkage method (81). The resulting cluster dendrogram was then generated with the *plot* function. Correlation matrices were calculated and drawn in R with *heatmap.plus*, *gplots*, *psy*, *RcolorBrewer*, *corrplot*, *ggplot2*, *Hmisc* and *ggcorrplot* packages (cran.r-project.org). All aforementioned packages can be found on the CRAN repository (https://cran.r-project.org/).

Statistical analyses were conducted with GraphPad Prism 7 (GraphPadSotfware, San Diego, USA). Graphs are represented as mean ± standard error of the mean (SEM). A 2-way ANOVA with repeated measures was used to analyze body weight (factors: diet and time). The Y-Maze was analyzed using a 2-way ANOVA followed by a Tukey *post-hoc* test. Concerning the Morris water maze:

TABLE 2 | Variables used for hierarchical cluster analysis.

Family		Process	Variable	Full name
Central nervous system		Inflammation	IL6	Interleukin 6
			IL1b	Interleukin 1β
			TNFa	Tumor necrosis factor α
M0		Microglial phenotype	TGFb1	Transforming growth factor β1
			TGFbr2	Transforming growth factor β receptor 2
			Itgam	αM integrin
			Tmem119	Transmembrane protein 119
			P2Y12	P2Y purinoceptor 12
			CSF1r	Colony-stimulating factor 1 receptor
MGnD		Microglial phenotype	MHCII	Major histocompatibility complex class II
			Trem2	Triggering receptor expressed on myeloid cells 2
			ApoE	Apolipoprotein E
			Lgals3	Galectin 3
			Axl	Tyrosine-protein kinase receptor UFO
			Clec7a	C-type lectin domain containing 7A
			Itgax	αX integrin
			Clec7a^{+} Iba1^{+}/Iba1^{+}	C-type lectin domain containing 7A Ionized calcium binding adapter molecule1
Intestinal tract	Colon	Inflammation	IL6	Interleukin 6
			IL1b	Interleukin 1β
			TNFa	Tumor necrosis factor α
			IL10	Interleulin 10
		Permeability	Protein Ocln	Protein occludin
			Protein ZO-1	Protein ZO-1
			Ocln	Occludin
			ZO-1	Zonula occludens-1
			Cldn5	Claudin 5
			MLCK	Myosin light-chain kinase
	Ileum	Inflammation	IL6	Interleukin 6
			IL1b	Interleukin 1β
			TNFa	Tumor necrosis factor α
			IL10	Interleukin 10
		Permeability	Protein Ocln	Protein occludin
			Protein ZO-1	Protein ZO-1
			Ocln	Occludin
			ZO-1	Zonula occludens-1
			Cldn5	Claudin 5
			MLCK	Myosin light-chain kinase
Behavior		Cognition	Distance target	Distance in the target quadrant of the MWM
			Y.Maze. New arm	Distance in the new arm of the Y-Maze
			Y.Maze. Familiar arm	Distance in the familiar arm of the Y-Maze

M0, Microglial homeostatic signature; MGnD, Microglial neurodegenerative-associated disease phenotype.

- Cued learning was analyzed using an unpaired *t*-test.
- Spatial learning was analyzed using a 2-way ANOVA with repeated measures (factors: diet and days of learning).
- Probe test comparisons were performed for each group against chance level (25%) using a one sample *t*-test and a 1-way ANOVA (factor: quadrants) followed by a Dunnett's multiple comparisons *post-hoc* test. A 2-way ANOVA has also been performed (factors: quadrant and diet).

The other analyses were performed using unpaired *t*-tests (when variances were not different) or Welch-corrected *t*-tests (when variances were different) between groups. For the ANOVA analyses, the method of Geisser-Greenhouse was used to correct

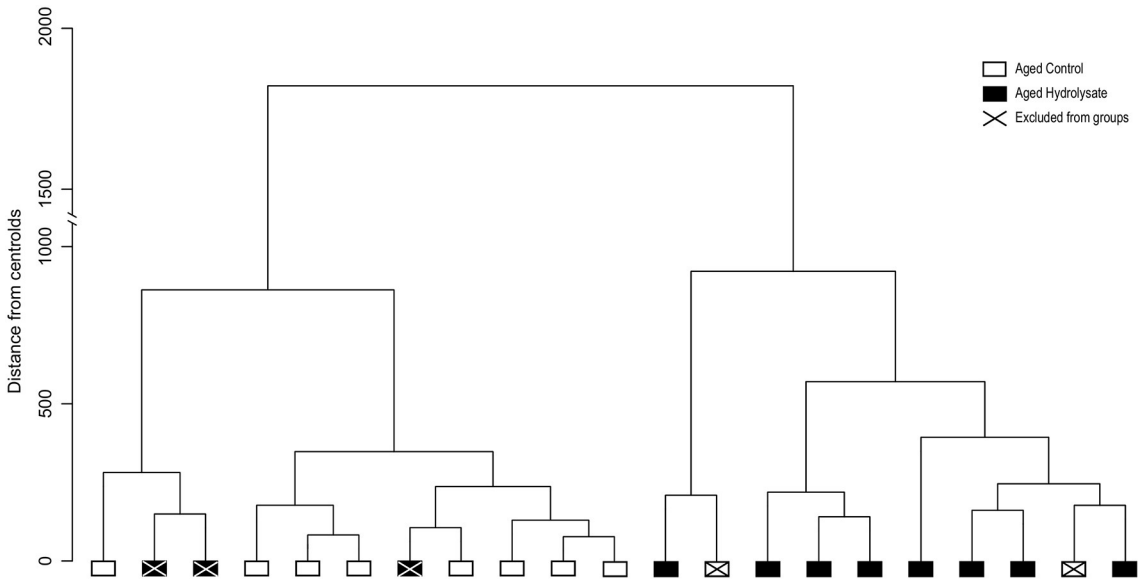


FIGURE 2 | Unsupervised hierarchical cluster dendrogram of individuals using Ward's linkage method. Dendrogram of the two clusters corresponding to control fed mice (white) and mice fed the hydrolysate-enriched diet (black). Mice removed from analyses are represented with a cross. Such analyses are based on cognitive behavior, central and intestinal inflammatory cytokines, microglial and intestinal permeability markers (**Table 2**).

the violation of the assumption of sphericity (82). Alpha has been set at 0.05 and all the *post-hoc* tests used in the present study (Tukey's and Dunnett's) are comparing multiple variables and are also correcting for family wise error rate.

RESULTS

Individual Heterogeneity in the Experimental Groups

Unsupervised hierarchical clustering analysis was performed using input from all the behavioral and biochemical parameters previously described (**Table 2**). Output clustered mice into two different clusters. The majority of mice that were given hydrolysate-enriched or control diets were segregated in two separate clusters (**Figure 2**). However, out of 11 mice fed with the hydrolysate enriched-diet, 3 mice did not behave as the majority of the group. Similarly, 2 out the 10 mice fed with the control diet did not behave as the majority of the group. Subsequently, these mice were considered as not homogenous within their respective groups and were therefore considered as outliers, and excluded from further analyses (**Figure 2**).

Weight and Body Composition

Weight, fat mass and lean mass were measured all along the 10 weeks of dietary supplementation. Body weight increased in both control and hydrolysate fed mice over the 10 weeks of diet (time effect [$F_{(9,\ 126)} = 25.93$, $p < 0.001$]), in a diet-independent manner (diet effect [$F_{(1,\ 14)} = 2.287$, $p = 0.153$]) (data not shown). Fat mass gain and lean mass reduction were also similar between mice fed either with the control diet or the hydrolysate enriched-diet [$t_{(14)} = 0.949$, $p = 0.359$ and $t_{(14)} = 0.688$, $p = 0.503$, respectively] (data not shown).

Short-Term and Long-Term Memory Evaluation

The effect of the hydrolysate supplementation on short-term spatial memory was assessed using a Y-maze test with a 1 h ITI. The 2-way ANOVA revealed an effect of arms [$F_{(1,\ 28)} = 25.62$, $p < 0.001$] but did not reveal any effect of the diet [$F_{(1,\ 28)} = 1.451$, $p = 0.239$]. However, the interaction between arms and diet was significant [$F_{(1,\ 28)} = 15.08$, $p < 0.001$]. The distance traveled in the familiar and in the new arm were not significantly different in control aged mice (Tukey *post-hoc* test: $p = 0.838$), characterizing short-term memory deficits. Furthermore, aged mice fed the hydrolysate diet traveled less distance in the familiar arm than control aged mice (Tukey *post-hoc* test: $p < 0.01$). These deficits were prevented in mice fed the hydrolysate diet, which traveled more distance in the new arm (Tukey *post-hoc* test: $p < 0.001$) (**Figure 3A**).

The effect of the hydrolysate supplementation on spatial learning and long-term memory was then assessed with the Morris water maze test. First, to evaluate their visuo-motor abilities, mice were trained to find a visible cued platform in the Morris water maze. Both groups traveled similar distances to reach the visible platform [$t_{(14)} = 1.515$, $p = 0.152$], meaning that they had similar visual abilities and did not display any impairment during the cued learning (**Figure 3B**). Mice were then submitted to the spatial learning. The 2-way ANOVA did not reveal any interaction [$F_{(3,\ 42)} = 1.119$, $p = 0.352$] or effect of the diet [$F_{(1,\ 14)} = 1.098$, $p = 0.313$]. However, both control and hydrolysate supplemented groups traveled significantly less

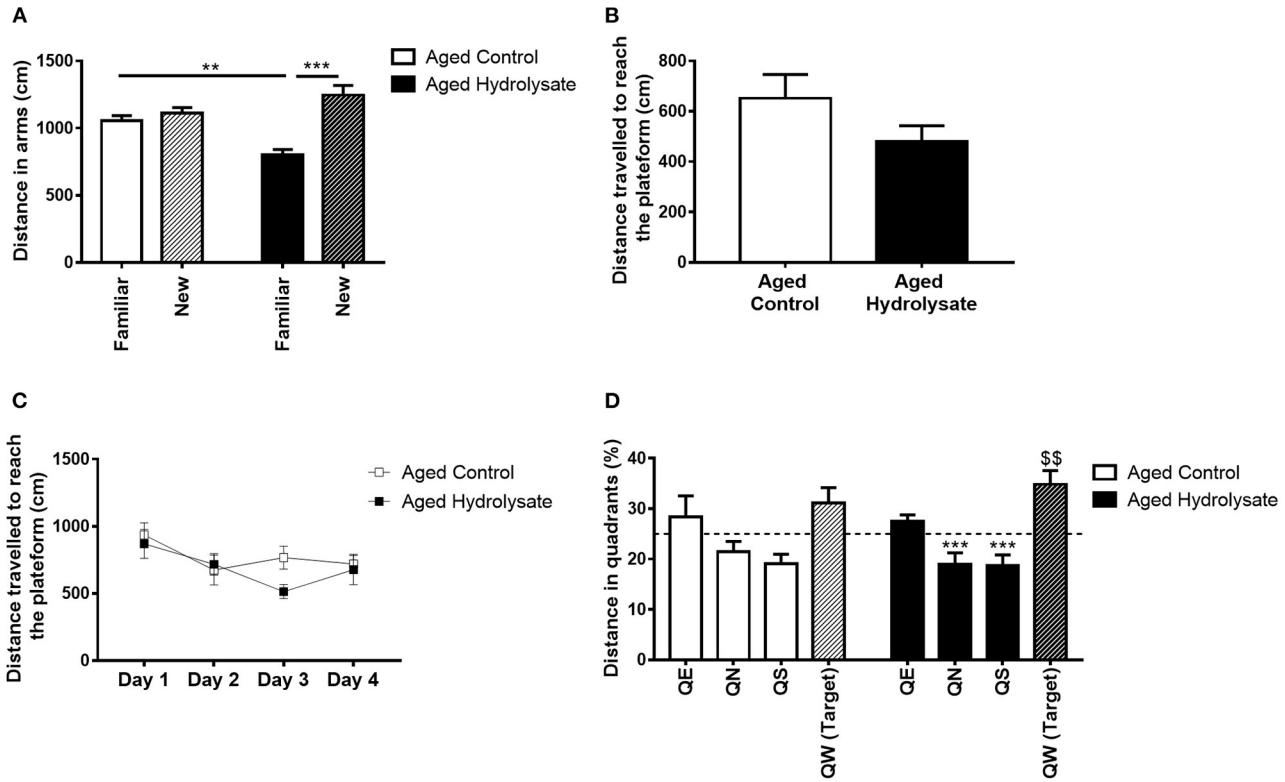


FIGURE 3 | Effects of the fish hydrolysate supplementation on short-term memory, spatial learning and long-term memory. **(A)** Recognition of the new arm after a 1 h ITI in aged mice fed the control diet or the hydrolysate-enriched diet (**$p < 0.01$, ***$p < 0.001$ by 2-way ANOVA followed by Tukey *post-hoc* test). **(B)** Distance traveled during the cued learning **(C)** Distance covered to reach the platform over the 4 consecutive days of spatial learning (day effect $p < 0.01$ by 2-way ANOVA with repeated measures). **(D)** Percentage of distance traveled in quadrants during the probe test. The dotted line represents chance level (25%) [$^{\$\$}p < 0.01$ vs. chance level by one-sample *t*-test. ***$p < 0.001$ compared to QW (Target) by One-way ANOVA and Dunnett's multiple comparison test; $n = 8$ per group]. Data are presented as mean ± SEM. QE, quadrant east; QN, quadrant north; QS, quadrant south; QW, quadrant west.

distance over the 4 days of training [day effect, $F_{(3, 42)} = 3.85$, $p < 0.05$] indicating that learning was achieved (**Figure 3C**). Mice fed the hydrolysate enriched-diet did not show better performance than control mice (diet effect [$F_{(1, 14)} = 1.098$, $p = 0.313$]). Spatial memory was evaluated 72 h after the last day of spatial learning, during the probe test. A 2-way ANOVA was performed for the probe test and did not reveal any interaction [$F_{(3, 42)} = 0.475$, $p = 0.702$] and no effect of the diet [$F_{(1, 14)} = 1.117$, $p = 0.308$]. However, the analysis revealed a significant quadrant effect [$F_{(3, 42)} = 10.47$, $p < 0.001$]. One sample *t*-test compared to the chance level (25%) showed that control mice didn't travel more distance in the target quadrant [$t_{(7)} = 1.533$ $p = 0.169$], revealing that 72 h after the last day of training, aged control mice presented memory alterations (**Figure 3D**). The hydrolysate supplementation prevented this memory long-term memory deficit as shown in **Figure 3D**. Indeed, supplemented mice significantly traveled more distance in the target quadrant [$t_{(7)} = 3.591$, $p < 0.01$]. Furthermore, aged control mice failed to discriminate the target quadrant [$F_{(3, 28)} = 2.164$, $p = 0.115$] to the contrary of aged mice supplemented with the hydrolysate enriched-diet [$F_{(3, 28)} = 12.61$, $p < 0.001$]. They significantly differentiated QN and QS from the target quadrant (QN vs. QW: $p < 0.001$; QS vs. QW: $p < 0.001$) and tended to differentiate QE from the target quadrant (QE vs. QW: $p = 0.061$). The absence of differences between QE and QW could be explained by the freezing of the mice for the 10 first seconds of the probe test, suggesting the presence of anxiety-like behavior, which were corrected by the hydrolysate supplementation. The freezing of the mice can be due to the cold water but we used the temperature used by Morris (75). Freezing can also be a temporary stress related to immobility. This behavior is commonly observed, especially in aged animals, while the Morris water maze is test known to induce stress (83).

Pro-inflammatory Cytokine Gene Expression in the Hippocampus

Gene expression of pro-inflammatory cytokines IL-6, IL-1β, and TNF-α was analyzed in the hippocampus of mice. The mRNA expression of IL-6, IL-1β, and TNF-α was not different between control and supplemented groups ([$t_{(14)} = 0.601$, $p = 0.557$]; [$t_{(14)} = 0.326$, $p = 0.749$], and [$t_{(14)} = 0.821$, $p = 0.426$], respectively) (**Figure 4**).

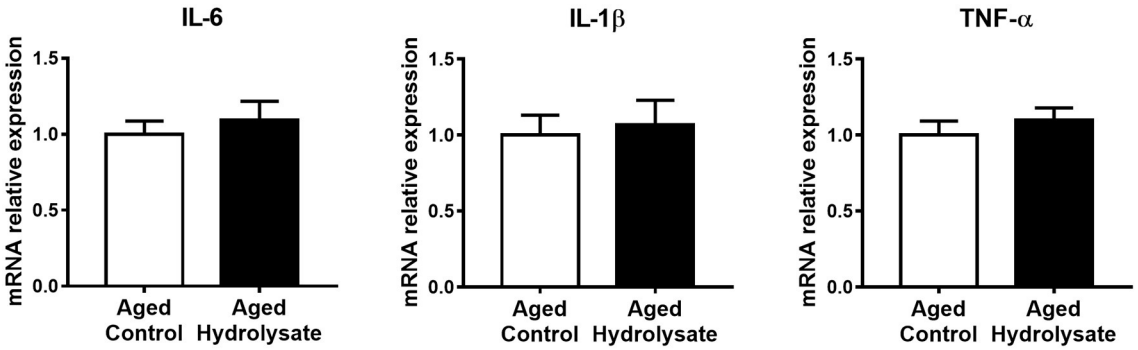


FIGURE 4 | Effects of the fish hydrolysate supplementation on pro-inflammatory cytokine expression in the hippocampus. Pro-inflammatory cytokines IL-6, IL-1β, and TNF-α mRNA expression in the hippocampus of aged mice fed with the control diet or the hydrolysate-enriched diet for 10 weeks. $n = 8$ per group. Data are presented as mean ± SEM.

Homeostatic and MGnD Microglial Signatures in the Hippocampus

The expression of genes that characterize the homeostatic microglial signature has been evaluated. Interestingly, the hydrolysate supplementation increased the expression of TGF-β1 compared to the control diet [$t_{(9.413)} = 2.34$, $p < 0.05$], which is essential for the maintenance of the homeostatic microglial signature (**Figure 5**). No differences were observed between mice fed with the control and the hydrolysate enriched-diet for TGF-βr2 [$t_{(14)} = 0.07$, $p = 0.945$], P2y12 [$t_{(14)} = 0.7$, $p = 0.496$], CSF1r [$t_{(14)} = 0.279$, $p = 0.784$], Itgam [$t_{(14)} = 0.301$, $p = 0.768$] and Tmem119 [$t_{(14)} = 0.012$, $p = 0.991$] (**Figure 5**).

The expression of genes that characterize the MGnD microglial signature, occurring during aging, has also been evaluated in the same cerebral structure. Mice fed the hydrolysate enriched-diet displayed higher expression of Clec7a, which is involved in phagocytosis, compared to mice fed the control diet [$t_{(14)} = 2.226$, $p < 0.05$] (**Figure 6**). Moreover, the hydrolysate supplementation tended to decrease the expression of Trem2 [$t_{(14)} = 1.84$, $p = 0.087$], which is involved in the shift toward MGnD phenotype (**Figure 6**). No differences were observed between both control and hydrolysate supplemented groups for ApoE [$t_{(14)} = 0.818$, $p = 0.427$], Axl [$t_{(14)} = 0.878$, $p = 0.395$], MHCII [$t_{(14)} = 0.987$, $p = 0.341$], Lgals3 [$t_{(14)} = 0.73$, $p = 0.478$] and Itgax [$t_{(14)} = 0.031$, $p = 0.976$] (**Figure 6**).

Hippocampal Clec7a-Positive Microglia

We wanted to go further with the increased mRNA expression of Clec7a in the hippocampus of the aged hydrolysate group. We then performed immunohistochemical analysis on the number of cells positive for Clec7a within Iba1-positive microglia in the hippocampus (**Figure 7A**). The analysis revealed no significant difference between the number of $Clec7a^+$ $Iba1^+$ cells between mice fed either the control or the hydrolysate-enriched diet in the whole hippocampus [$t_{(8)} = 1.265$, $p = 0.241$] (**Figure 7B**).

mRNA and Protein Expression of Intestinal Inflammation and Permeability Markers

Gut alterations related to aging lead to the production of inflammatory cytokines, thus contributing to chronic low-grade inflammation. Then, the effect of the hydrolysate supplementation was evaluated on inflammation in the ileum and the colon. In the ileum, gene expression of IL-6, IL-1β, TNF-α and IL-10 was not different between mice fed the control and the hydrolysate-enriched diet (IL-6 [$t_{(14)} = 0.115$, $p = 0.911$]; IL-1β [$t_{(14)} = 0.637$, $p = 535$]; TNF-α [$t_{(14)} = 0.061$, $p = 0.952$]; IL-10 [$t_{(14)} = 0.436$, $p = 0.67$]) (**Figure 8A**). In the colon, mRNA expression of IL-10 was significantly increased following the hydrolysate supplementation [$t_{(14)} = 2.27$, $p < 0.05$], suggesting an anti-inflammatory effect of the hydrolysate (**Figure 8B**). The mRNA expression of the other genes in the colon was comparable in the control and hydrolysate supplemented groups (IL-6 [$t_{(14)} = 0.899$, $p = 0.384$]; IL-1β [$t_{(14)} = 0.832$, $p = 0.42$]; TNF-α [$t_{(14)} = 1.046$, $p = 0.313$]) (**Figure 8B**).

Gut alterations related to aging, in addition to the production of inflammatory cytokines, lead to an increase of intestinal permeability. The effect of the hydrolysate supplementation was then evaluated on gene expression involved in ileum and colon permeability. In the ileum, gene expression of Ocln, ZO-1, Cldn5 and MLCK were not different between mice fed the control diet and mice fed the hydrolysate-enriched diet (Ocln [$t_{(14)} = 0.398$, $p = 0.697$]; ZO-1 [$t_{(14)} = 0.87$, $p = 0.399$]; Cldn5 [$t_{(14)} = 0.24$, $p = 0.814$]; MLCK [$t_{(14)} = 0.212$, $p = 0.835$]) (**Figure 9A**). In the colon, both control and supplemented groups displayed similar expression of Ocln [$t_{(14)} = 0.448$, $p = 0.661$], Cldn5 [$t_{(14)} = 0.203$, $p = 0.843$], and MLCK [$t_{(14)} = 0.641$, $p = 0.532$] (**Figure 9B**). The hydrolysate supplementation tended to increase the expression of ZO-1 [$t_{(14)} = 1.891$, $p = 0.08$] (**Figure 9B**).

To go further, protein expression of Ocln and ZO-1 were assessed in the ileum and the colon. As shown in **Figure 10** for Ocln, multiple reactive bands were observed at molecular weights of ∼62–65 kDa for the lower molecular weight and 71 kDa for the higher molecular weight, representing the hyperphosphorylated form of the lower molecular weight form. Relative protein expression is represented as the ratio of protein expression to

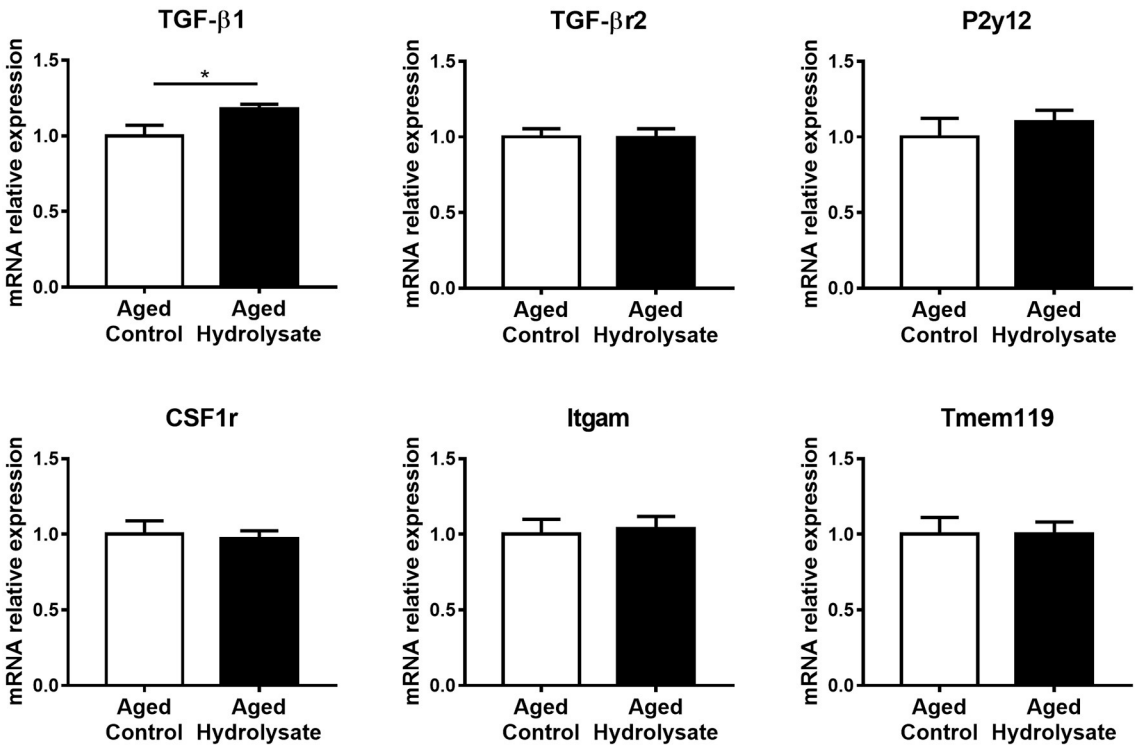


FIGURE 5 | Effects of the fish hydrolysate supplementation on homeostatic microglial signature in the hippocampus. mRNA expression of homeostatic microglial markers TGFβ1, TGFβr2, P2y12, CSF1r, Itgam, and Tmem119 in the hippocampus of aged mice fed with the control diet or the hydrolysate-enriched diet for 10 weeks (*$p < 0.05$ by unpaired *t*-test; $n = 8$ per group). Data are presented as mean ± SEM.

GAPDH. Total Ocln expression is represented as the ratio of the higher molecular weight form to the lower molecular weight form. No differences were observed between groups in the ileum (Ocln [$t_{(14)} = 0.191$; $p = 0.851$]; ZO-1 [$t_{(14)} = 0.497$; $p = 0.627$]) (**Figure 10A**) neither in the colon (Ocln [$t_{(14)} = 1.122$; $p = 0.281$]; ZO-1 [$t_{(8.81)} = 0.384$; $p = 0.71$]) (**Figure 10B**).

Shift in Inflammatory, Intestinal Permeability, and Behavioral Marker Profile

Correlation matrices were performed in aged control and aged hydrolysate groups. Overall, these correlation matrices revealed two different profiles based on the expression of hippocampal and intestinal inflammatory markers, intestinal permeability markers, behavioral assessment and immunological markers (**Figure 11**). Correlations between cognitive parameters and microglial markers were highlighted in both groups but to a lesser extent in the aged control group than in the aged hydrolysate group. In the aged hydrolysate group, the distance traveled in the new arm of the Y-Maze as well as in the target quadrant of the Morris water maze was positively correlated with genes involved in the homeostatic microglial signature, such as CSF1r, Tmem119, or P2y12, suggesting that the maintenance of this signature plays a role in cognitive function. In the aged control group, some markers of the MGnD microglial signature such as Trem2 or MHCII were negatively correlated with the distance traveled in the new arm of the Y-Maze. Likewise, the M0 marker P2y12 was positively correlated to the distance traveled in the target quadrant of the Morris water maze. Differences in the level of correlations between M0 and MGnD markers have been highlighted in the aged control group and the aged hydrolysate group. Indeed, in the aged hydrolysate group, the correlation matrix showed more significant positive than negative correlations, notably between the M0 markers TGF-βr2, Tmem119, P2y12, CSF1r and the MGnD markers MHCII, Trem2, ApoE, Lgals3, and Axl. In this group, negative correlations have also been highlighted between the M0 markers TGF-β1, Tmem119, P2y12, CSF1r and the MGnD markers Clec7a, Itgax, and $Clec7a^{+}Iba1^{+}$ cells. In comparison, the aged control group showed less significant correlations. Nevertheless, those correlations were similar to those observed in the aged hydrolysate group. The higher number of significant correlations in the aged hydrolysate group as compared to the aged control group suggested a higher microglial reactivity and switches between M0 and MGnD phenotype and a dysfunction in phenotype transition of microglial cells in the aged control group. Furthermore, in the aged hydrolysate group, markers of the MGnD microglial signature, such as Axl, Lgals3, ApoE, Trem2, and MHCII were negatively correlated with permeability markers, such as ZO-1, Cldn5, MLCK, Ocln (protein) in the ileum and Ocln, ZO-1, and Cldn5 in the colon, highlighting a link between microglial phenotypes and intestinal permeability. Moreover,

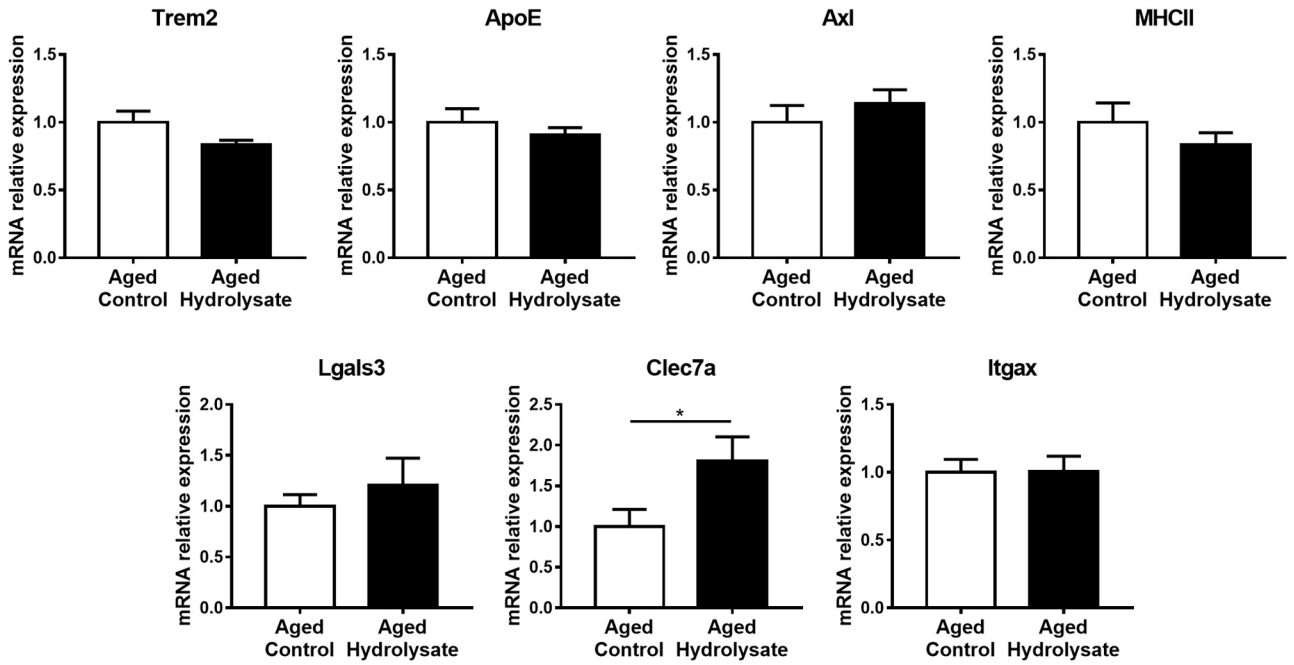


FIGURE 6 | Effects of the fish hydrolysate supplementation on MGnD microglial signature in the hippocampus. mRNA expression of MGnD microglial signature Trem2, ApoE, Axl, MHCII, Lgals3, Clec7a, and Itgax in the hippocampus of aged mice fed with the control diet or the hydrolysate-enriched diet for 10 weeks (*$p <$ 0.05 by unpaired *t*-test; $n = 8$ per group). Data are presented as mean ± SEM.

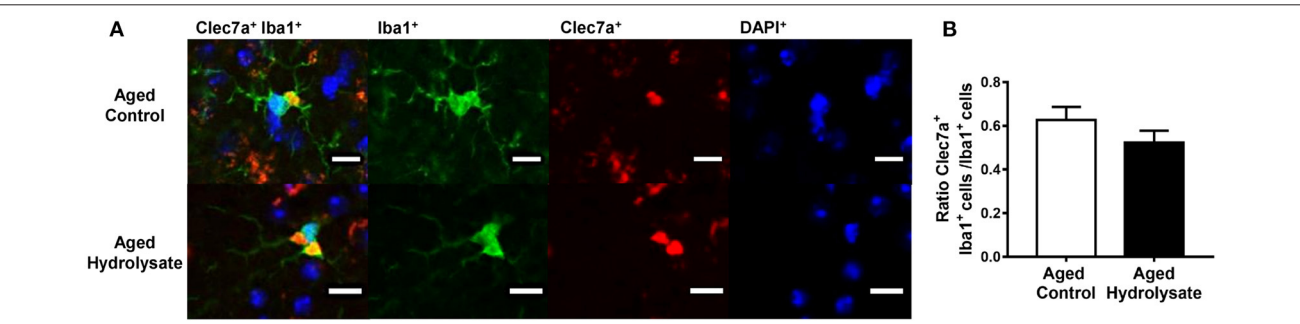


FIGURE 7 | Effects of the fish hydrolysate supplementation on Clec7a$^+$Iba1$^+$/Iba1$^+$ cells in the hippocampus. **(A)** Representative images of Clec7a$^+$Iba1$^+$/Iba1$^+$ cells (double staining), Iba1$^+$ cells (in green), Clec7a$^+$ cells (in red) and DAPI$^+$ cells (in blue) (scale bars: 10 μM) and **(B)** Number of positive cells for Clec7a and Iba1 staining in the hippocampus of aged mice fed with the control diet or the hydrolysate-enriched diet for 10 weeks ($n = 8$ per group). Data are presented as mean ± SEM.

different profiles were observed concerning correlations between intestinal inflammation and permeability. In the aged hydrolysate group, IL-10 in the ileum and the colon was positively correlated with ZO-1, Cldn5, and MLCK in the ileum and the colon whereas IL-10 in the ileum was negatively correlated to any variable in the aged control group. Concerning the pro-inflammatory cytokines, IL-6, IL-1β, and TNF-α in the ileum were positively correlated with Ocln, ZO-1, Cldn5, and MLCK in the ileum in the aged hydrolysate group. These correlations were not observed in the aged control group. We also noticed a positive correlation between colon inflammatory (IL-6, TNF-α) and permeability (Ocln, ZO-1, Cldn5) markers and brain IL-6. These results suggest a link between gut physiology and brain function.

DISCUSSION

Our results confirmed previous results obtained in our laboratory (73) and demonstrated that the hydrolysate supplementation prevents short- and long-term memory decline during aging. To better understand the mechanisms involved in the prevention of cognitive decline, we investigated the impact of the hydrolysate on microglial signature and on peripheral inflammation. We demonstrated that, without modulating pro-inflammatory cytokine expression, the fish hydrolysate supplementation modulated microglial signature. Indeed, mice supplemented with the fish hydrolysate displayed higher TGF-β1 expression, characteristic of the homeostatic microglial phenotype, higher

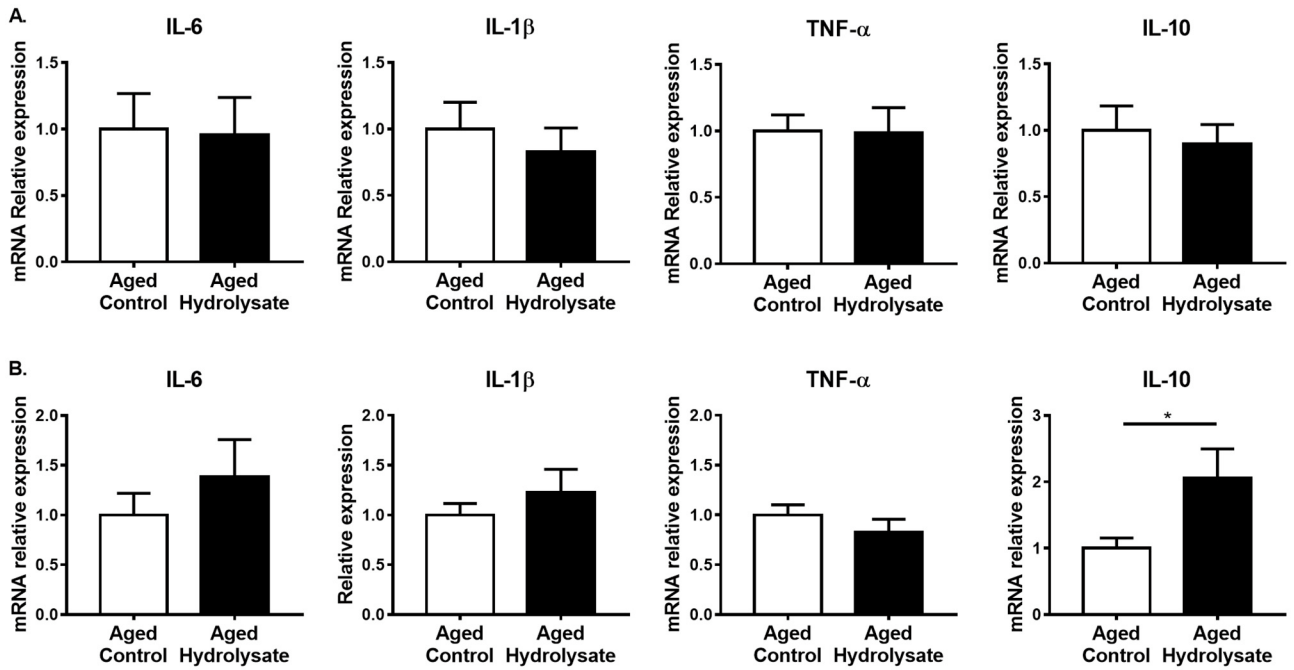


FIGURE 8 | Effects of the fish hydrolysate supplementation on intestinal inflammation. Pro-inflammatory cytokines IL-6, IL-1β, and TNF-α and anti-inflammatory cytokine IL-10 mRNA expressions in **(A)** the ileum and **(B)** the colon of aged mice fed with the control diet or the hydrolysate-enriched diet for 10 weeks (*$p < 0.05$ by unpaired *t*-test; $n = 8$ per group). Data are presented as mean ± SEM.

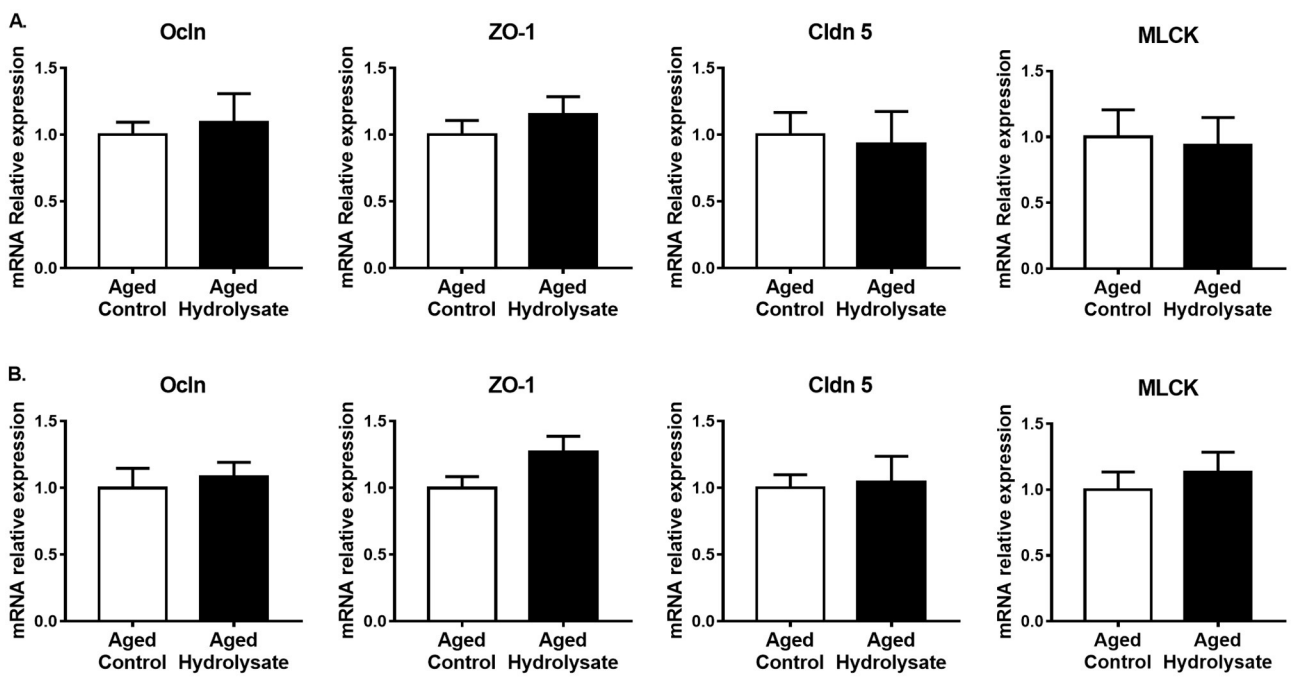


FIGURE 9 | Effects of the fish hydrolysate supplementation on gene expression of intestinal permeability markers. Intestinal permeability markers Ocln, ZO-1, Cldn 5 and MLCK mRNA expressions in **(A)** the ileum and **(B)** the colon of aged mice fed with the control diet or the hydrolysate-enriched diet for 10 weeks. $n = 8$ per group. Data are presented as mean ± SEM.

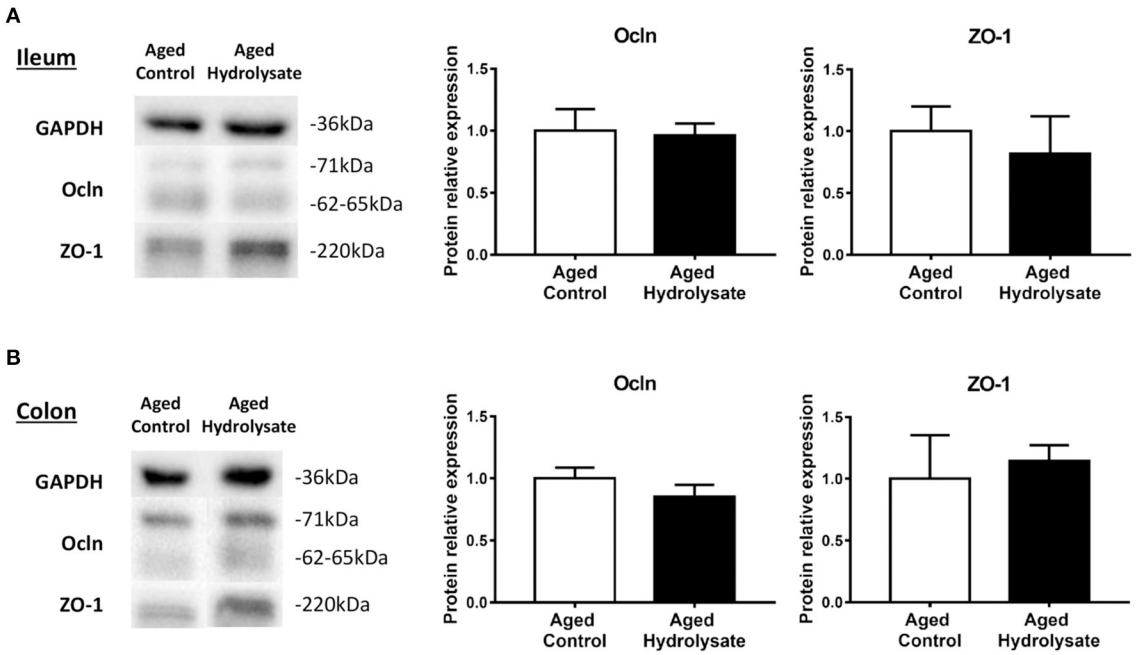


FIGURE 10 | Effects of the fish hydrolysate supplementation on protein expression of intestinal permeability markers. Intestinal permeability markers ocln and ZO-1 protein expressions in **(A)** the ileum and **(B)** the colon of aged mice fed with the control diet or the hydrolysate-enriched diet for 10 weeks. $n = 8$ per group. Data are presented as mean ± SEM.

expression of Clec7a, a marker of MGnD microglial signature involved in phagocytosis, and tended to express less Trem2. Our results represent a snapshot of the experimental conditions that we used during our study (i.e., population of microglia in the hippocampus of 17-months old mice supplemented or not). Although phenotypic changes can be transient and observed in a time-dependent manner, we can also suggest that these changes can be due to the fish hydrolysate supplementation that modulated microglia microenvironment. As shown by correlation matrices, aged mice supplemented with the hydrolysate enriched-diet displayed more significant and positive correlations between markers of the homeostatic microglial phenotype and markers of the MGnD phenotype, suggesting a higher reactivity of microglial cells as compared to the aged control group. The results are in accordance with a previous study that showed higher microglial reactivity in response to inflammation (84). Moreover, the hydrolysate supplementation promoted anti-inflammatory intestinal pathway and tended to prevent intestinal permeability alteration occurring during aging. The hydrolysate supplementation induced a shift in biochemical and behavioral marker profiles and appeared consequently as an interesting candidate to prevent cognitive decline during aging.

We highlighted a beneficial effect of the fish hydrolysate supplementation on TGF-β1 during aging, which is an anti-inflammatory cytokine largely involved in the regulation of inflammation, in cell proliferation, growth and differentiation as well as in neuroprotection (85). Moreover, protective effects against neuronal insults have also been observed before (10). We showed that TGF-β1 expression was higher in the hydrolysate supplementation group as compared to the control group. This effect could be linked to the enhancement of the cognitive performances in these mice. Indeed, defects in TGF-β1 have negative impact in physiological and pathological conditions. In normal aging in human, it was shown that a genetic variation within TGF-β1, leading to a lower production of TGF-β1, has a negative impact on functional and cognitive performance (85). In patients with Alzheimer's disease, impairment in TGF-β1 signaling is characterized by a reduction of TGF-β1 plasma levels and decreased receptor expression in neurons (85). In rodents, this cytokine seems to be involved in learning processes as demonstrated in mice and rats treated with a selective inhibitor of TGF-β1 signaling pathway (86, 87). These results suggest a possible role of TGF-β1 probably in the formation and remodeling of synapses (88).

In our study, aged mice fed the fish hydrolysate enriched-diet tended to express less Trem2 than aged control mice. This trend is interesting since this marker, which is highly expressed in glial cells, is involved in the switch from homeostatic microglial phenotype to MGnD phenotype (89, 90). Moreover, several studies have highlighted the beneficial effect of a Trem2 deficiency in aged mice on microglial activation, cognitive performance as well as hippocampal long-term potentiation, suggesting a potential detrimental role of Trem2 during physiological aging (89, 91). Further experimentations are needed to deepen the effect of the fish hydrolysate on Trem2 expression.

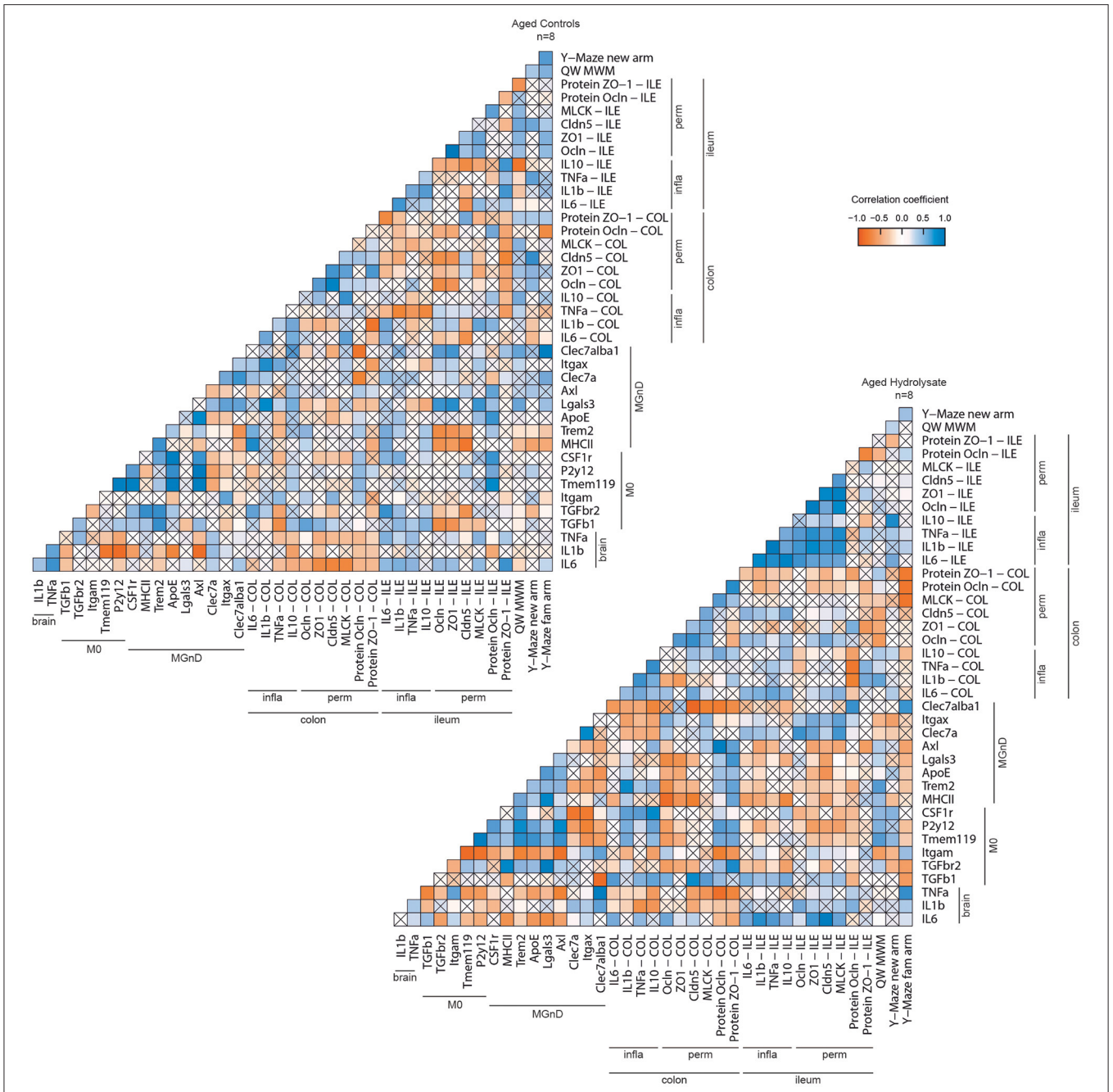


FIGURE 11 | Correlation matrix of behavioral and biochemical parameters of each group. Positive correlations are represented in blue, negative correlations in red. No significant correlations are represented by a cross.

The expression of another gene associated to the MGnD phenotype, (Clec7a), was higher following the fish hydrolysate supplementation, although the number of positive microglia for Clec7a was not different from the aged control group. This could be the result of infiltrating cells, other than microglia, since this marker is expressed by macrophages as well as dendritic cells (92). It promotes microglial phagocytosis but can also be involved in pathogen recognition and the regulation of autophagy (93). In fine, it enhances the removal of cellular debris or damaged cell accumulation. Clec7a has been reported to enhance neuroinflammation when acting in synergy with Toll-like receptor 2 (94, 95) but in the regeneration of damaged CNS (96), *via* Syk-dependent signaling pathway. Syk is activated by phosphorylation into p-Syk, which, in turn activates signaling molecules such as NF-κB (95). It would be interesting to evaluate Syk and p-Syk expression to demonstrate

the signaling pathways involved in the regulation of Clec7a by the hydrolysate.

Another possible mechanism of action of the fish hydrolysate during aging has been explored. We focused on the intestinal tract, which is involved in several physiological processes including nutrient intake as well as immune modulation (97). Indeed, a close link between gut inflammation, gut permeability and cognition has been demonstrated before (14). Age-related dysbiosis of the gut microbiota is known to be associated with aberrations of gut barrier integrity and enhanced pro-inflammatory cytokines. These changes impact the gut-brain axis thereby impairing neural, endocrine, and immunological signals between the gut and the brain *via* the enteric nervous system and could play a role in diseases of the CNS (98, 99). In aged rodents, several studies have reported an increased intestinal permeability to macromolecules and microbes, suggesting altered function and integrity of the intestinal barrier, leading to the leakage of microbial products in the circulation, thereby triggering systemic inflammation and contributing to cognitive impairments (12, 100–102). Recently, fecal microbiota transplant from aged mice to young mice has been shown to affect spatial learning and long-term memory, confirming the link between gut microbiota and cognitive function (103). It is also known that some nutrients can influence gut microbiota and functionality. In this study, aged mice fed the hydrolysate enriched-diet displayed higher colonic expression of IL-10 as compared to aged control mice. This is particularly interesting since IL-10 is a cytokine which plays a crucial role in the regulation of epithelial integrity as well as the regeneration of the colon (104, 105). In line with this, a positive correlation was also found between intestinal IL-10 and permeability markers ZO-1, Cldn5, and MLCK. Furthermore, IL-10 can interact with the intestinal microbiota to regulate epithelial function (106). The hydrolysate supplementation also tended to decrease intestinal permeability. The effects of n-3 LC-PUFAs on gut microbiota, intestinal permeability and immune function have recently been reviewed (66, 67, 70). EPA and DHA display significant beneficial effects on barrier integrity and intestinal inflammation as shown in *in vitro* and *in vivo* studies. Moreover, n-3 PUFAs can influence gut microbiota composition and, in turn, microbiota can impact the metabolism and absorption of n-3 PUFAs. However, less is known about the effects of low molecular weight peptides. Recently, the supplementation with small peptides from skipjack by-products have been shown to display anti-inflammatory effects in a mouse model of ulcerative colitis and to increase the diversity of the intestinal flora (107). These anti-inflammatory properties have also been observed in murine models of colitis supplemented with peptide derived from soy or oyster (63, 64). In addition, collagen peptides also protect the intestinal barrier function *in vitro*, *via* the regulation of ZO-1 and Ocln expression and distribution and the MLCK pathway (71, 72).

The effect of the fish hydrolysate on intestinal inflammation and permeability was highlighted in the colon but not in the ileum. This could be linked to differences in microbiota in these two intestinal tissues. Indeed, ileum and colon presented distinct microbiota suggesting different mechanisms of action (97, 108). Microbial signatures in colon and ileum are specific and may be differently modulated by the hydrolysate supplementation, as already shown in a study evaluating the effects of polyphenols on intestinal inflammation and gut microbiome signature (97). Moreover, aging induces change in microbiota diversity, which is linked to immune function and cognition (14). Comparisons between microbiota composition in the colon or the ileum would be interesting, as previous studies in humans have observed differences in composition and density between the microbiota of the distal ileum and the colon (109, 110). These microbiota compositions also changed with diet and we could speculate that it wasn't change similarly by the hydrolysate supplementation due to their basal composition.

This study has some limitations. A first limitation concerns the sample size, which could have been increased in order to increase the significance of the statistical tests. However, we had to comply with ethical regulations and our previous results have shown that the number of mice is sufficient to highlight beneficial effects of a hydrolysate supplementation on cognitive function as well as neuroinflammation (73, 84). We also acknowledge some potential bias for the multiple comparison analyses given the high risk of family-wise, thus given rise to potential false positives within the reported results. A second limitation concerns the lack of morphological analyses of microglia. Indeed, microglia morphology and function are closely related and morphological analyses would have given us information on their function. We chose to analyze the protein expression of Clec7a by immunofluorescence because it is involved in phagocytosis, which is enhanced during aging (10).

CONCLUSION

This study provides further evidence for the understanding of the mechanisms of action of the marine hydrolysate containing n-3 LC-PUFAs and low molecular weight peptides on inflammation and cognitive functions during aging. The beneficial effects induced by the hydrolysate supplementation on behavioral and biochemical markers reinforce the innovative character of this hydrolysate on the prevention of age-related cognitive decline.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by CE050 (Approval ID APAFIS#14144-2018041213072383).

AUTHOR CONTRIBUTIONS

VP, SL, AM, EB, A-LD, and CJ devised the project, the main conceptual ideas, and proof outline. MC, VP, SL, A-LD, and CJ conceived and designed experiments. MC and CL conducted research and analyzed data. MC and MDM performed statistical

analysis. MC wrote the manuscript with support of A-LD and CJ. All authors have read and agreed to the published version of the manuscript.

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