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Mechanisms and pathways of action for nutritional effects on brain health

Edited by
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Mechanisms and pathways of action for nutritional effects on brain health

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ORIGINAL RESEARCH
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Association Between Wine Consumption and Cognitive Decline in Older People: A Systematic Review and Meta-Analysis of Longitudinal Studies

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Background: Low-to-moderate alcohol consumption appears to have potential health benefits. Existing evidence concludes that wine may be associated with a lower incidence of certain diseases. This systematic review and meta-analysis aim to examine evidence on the association between wine consumption and cognitive decline and to analyze whether this association varies depending on the wine consumption level or is affected by individual and study characteristics, including mean age, percentage of women participants, and follow-up time.

Methods: In this systematic review and meta-analysis, we undertook a search in MEDLINE (*via* PubMed), Scopus, Cochrane, and Web of Science databases for longitudinal studies measuring the association between wine consumption and cognitive decline from their inception to May 2021. Effect sizes were calculated using the DerSimonian and Laird and Hartung-Knapp-Sidik-Jonkman methods.

Results: The search retrieved 6,055 articles, 16 of which were included in this systematic review. In total, 12 studies were included in the meta-analysis. The studies were published between 1997 and 2019. They were conducted in nine different countries. The sample size of the included studies ranged from 360 to 10,308 with a mean age of 70 years old. Using the DerSimoniand and Laird method, the pooled RR for the effect of wine consumption on cognitive decline was 0.72 (95% CI 0.63–0.80; $I^2 = 82.4\%$; τ^2: 0.0154). Using the Hartung-Knapp-Sidik-Jonkman method, the RR was 0.65 (95% CI 0.52–0.79; $I^2 = 94{,}531\%$; τ^2: 0.057).

Conclusions: This study may show a protective effect of wine consumption against cognitive decline. However, it would be important for future research to differentiate the types of wine within consumption.

Keywords: cognitive decline, wine, older people, alcohol consumption, elderly

INTRODUCTION

The consumption of alcohol and tobacco are considered unhealthy habits, harmful to health, and are related to the development of pathologies such as cardiovascular diseases (CVD), digestive system diseases, hypertension, diabetes mellitus, or cognitive deterioration among others, representing a serious problem for public health (1–3). Indeed, alcohol consumption increases the risk of dementia, especially early-onset dementia (4–10), indicating a negative impact of alcohol consumption on different areas of familial, social, and cultural wellbeing (11–13).

The alcohol consumption recommendations established by the WHO are 30 g for men and 20 g for women, 3 standard drinking units (SBUs), and 2 SBUs, respectively, as 1 SBU corresponds to 10 g of pure alcohol (14). It has been suggested that low-to-moderate alcohol consumption could be beneficial to the health of middle-aged (15) and older subjects (16–18), leading to a J-shaped or inverse U-shaped association between alcohol and cognitive function, heart disease (19, 20) and all-cause mortality (21). Furthermore, and according to previous systematic reviews, moderate alcohol consumption appears to be associated with a lower risk of cognitive impairment, dementia, Alzheimer's disease, and better cognition (9, 22), which may be linked to its effects on cardiovascular disease (22). These effects of alcohol consumption are not observed for vascular dementia (9), neither for heavy, chronic, and irregular alcohol consumption which are associated with an increased risk of cognitive impairment or dementia (13).

Further analysis of the data indicates that the effect of alcohol depends on the type of alcoholic beverage analyzed (20), distinguishing between beer, white wine, red wine, fortified wine, and spirits (23). Although wine consumption has been associated with a reduced risk of cerebrovascular disease and Alzheimer's disease (24), there is controversy as to whether these benefits are also reported for beer and other spirits (4, 25). The specific characteristics of wine could be the reason for its benefits. Wine is produced from the fermentation of grapes, and yeast is added, causing the sugars present in the grapes to be converted into ethanol, endowing wine with different nutritional properties. It has been reported that some components of wine, such as resveratrol, phenolic acids, and flavonoids, may exert positive health effects (26). Previous research has shown that these components reduce free cholesterol (27), have a cardioprotective effect (28), induce endothelial relaxation (29), activate NO synthase (30), inhibit platelet aggregation, and (31) prevent oxidation of low-density lipoproteins (LDL) cholesterol (32).

Regardless of previous research, the evidence on the association between wine consumption and the risk of cognitive decline remains inconclusive (9, 14, 22, 33). This systematic review and meta-analysis aim to examine the strength of the association between wine consumption and cognitive decline and to analyze whether this association varies depending on the wine consumption level or is affected by individual and study characteristics, including the mean age, percentage of women participants, and follow-up time.

METHODS

Search Strategy and Study Selection

This systematic review and meta-analysis were performed according to the Cochrane Collaboration Handbook (34) and reported following the MOOSE guidelines (Meta-analysis of Observational Studies in Epidemiology) (35) this systematic review and meta-analysis were registered on PROSPERO (registration number CRD42021232060).

A systematic search was conducted in the MEDLINE (*via* PubMed), Scopus, Cochrane, and Web of Science databases from their inception to 25 May 2021. The literature search was updated on 15 February 2022. The search strategy included the following relevant terms: (1) "dementia," "mental deterioration," "Alzheimer's disease," "vascular dementia," "predementia syndromes," and "mild cognitive impairment"; (2) "alcohol," "wine," "alcohol consumption," and "wine consumption" and (3) "older," "elderly," "elderly people," and "older people." Finally, the reference list of the studies included in this systematic review was examined to identify relevant studies. **Supplementary Table 1** presents the complete search strategy for MEDLINE.

Eligibility

Eligible articles included longitudinal studies measuring the association between wine consumption and cognitive decline. The inclusion criteria were as follows: (i) subjects: general population without dementia at baseline aged 65 or over at the end of the study; (ii) outcomes: cognitive decline assessments using standardized and validated tests; (iii) study design: longitudinal studies; and (iv) studies reporting wine consumption separated from other alcoholic beverages. Studies were excluded if they: (i) were review articles, editorials, or patient case reports; or (ii) included patients with cognitive decline at baseline determined by a battery of psychometric tests or an examination by a neurologist. No language restrictions were applied to the search or study selection process.

Data Extraction and Quality Assessment

The following information was extracted from the included studies: study name; subject characteristics, including sample size, percentage of women and mean age at baseline; wine consumption; grams of ethanol consumed per day; type of wine consumed; test used to measure cognitive function and cognitive domains measured.

To evaluate the risk of bias of cohort studies, we used the Quality Assessment Tool for Observational Cohort and Cross-Sectional Studies from the United States National Institute of Health National Heart, Lung, and Blood Institute (36). This tool assesses 14 items for longitudinal studies in the following domains: (1) research question, (2) study population, (3) participation rate, (4) recruitment, (5) sample size, (6) timeframe for associations, (7) exposure levels, (8) exposure measures and (9) assessment, (10) repeated exposure assessment, (11) outcome measures, (12) blinding of exposure assessors, (13) loss to follow-up, and (14) statistical analyses.

For case-control studies, the Quality Assessment of Case-Control Studies (37) from the United States National Institute

of Health National Heart, Lung, and Blood Institute was used. This tool includes 12 domains, namely: (1) research question, (2) study population, (3) target population, (4) sample size, (5) recruitment, (6) inclusion and exclusion criteria, (7) case and control definitions, (8) random selection of study participants, (9) concurrent controls, (10) exposure measures and assessment, (11) blinding of exposure assessors, and (12) statistical analyses.

Finally, the overall risk of bias of each study was scored as "good" if most criteria were met; "fair" if some criteria were met; or "poor" if few criteria were met.

The search strategy, study selection, eligibility, data extraction, and quality assessment, were conducted by two independent reviewers (M.L.-L.-T and C.A.-B.). When the agreement was not reached, a third reviewer (I.C.-R.) was consulted.

Statistical Analysis and Data Synthesis

A meta-analysis was performed to determine the association between wine consumption and cognitive decline. The studies were classified according to wine consumption in the three subgroups, namely: (i) "within WHO recommendations" when studies reported wine consumption of <20 g in women and 30 g in men; (ii) "above the WHO recommendations" when studies reported higher wine consumption; and (iii) "unclassified" when studies did not report participants' wine consumption.

Some methodological considerations should be noted. When two studies reported data from the same population, we include in the meta-analysis the study with the largest sample size. The RR and odds ratio (OR) for the association between wine consumption and cognitive decline were jointly included in the meta-analysis (38). When studies reported the hazard ratio (HR), it was converted to RR using the following formula: $RR=(1 - e^{HR\ln(1 - r)})/r$ (38). In addition, the type and consumption of wine were collected as reported by the original studies and converted into grams using the equivalences of the SBU.

The DerSimonian and Lair and Hartung-Knapp-Sidik-Jonkman random-effects methods were used to compute the pooled estimate of the RR and their respective 95% Cis (39, 40). Following the Cochrane Handbook recommendations, the I^2 statistic was used to examine the inconsistency, which ranges between 0 and 100% (41). According to the I^2 values, inconsistency was considered not important (0–30%), moderate (≥30–50%), substantial (≥50–75%), or considerable (≥75–100%). The corresponding p-values were also considered. In addition, heterogeneity was evaluated using the τ^2 statistic, which was interpreted as low when τ^2 was lower than 0.04, moderate when τ^2 was from ≥0.04 to 0.14, and substantial when τ^2 was from ≥0.14 to 0.40 (42).

To assess the robustness of the summary estimates, sensitivity analyses were conducted by removing each study one at a time from the pooled estimations. Meta-regression analyses were performed to address whether mean age, percentage of women, and time of follow-up, as continuous variables, could modify the effect of the association between wine consumption and cognitive decline. Finally, publication bias was assessed through Egger's regression asymmetry test, where a p-value of <0.10 was used to determine if there was significant publication bias (43). Analyses were performed using Stata 15.0 (Stata, College Station, TX, USA).

RESULTS

Study Selection

The search retrieved 6,055 articles. From them, 101 studies were selected by reviewing the title and abstract, and 16 of which were included in this systematic review (25, 26, 44–58). Only 12 of these studies were included in the meta-analysis (**Figure 1**) (25, 26, 44–48, 50, 52, 53, 55, 58).

Study and Intervention Characteristics

The included longitudinal studies (25, 26, 44–58) were published between 1997 and 2019. They were conducted in ten different countries, including China (1 study), Canada (1 study), Germany (1 study), Sweden (2 studies), the United States (4 studies), the Netherlands (2 studies), France (2 studies), the United Kingdom (1 study), Italy (1 study), and Denmark (1 study).

The sample size of the included studies ranged from 360 to 10,308, with a total of 46,472 participants (60% women) aged 70 years old. Their follow-up periods ranged from 2 to 43 years. All the studies included the general population without dementia at baseline (25, 26, 44–58). One of the studies was conducted on twins born in Sweden between 1907 and 1925 (46), and another included only women (53). Only one study (54) did not report wine consumption and only three reported the type of wine consumed, distinguishing among white, red, and fortificated (**Table 1**) (41, 47, 51).

Finally, the most used tests to assess the risk of cognitive decline or dementia were the Mini-Mental State Examination (MMSE), the SIDAM test, the Global Deterioration Scale and the Blessed Dementia Rating Scale, and the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) neuropsychological battery. Other tests used were the Battery of psychometric test, the Diagnostic and Statistical Manual of Mental Disorders (DSM-III-R Criteria), the Modified Mini-Mental State (3MSE), the Health Study-Cognition (CHS), and the Informant Questionnaire on the Cognitive Decline of the Elderly (IQCODE) (**Table 1**).

Risk of Bias

For cohort studies, the overall risk of bias was low in all studies (100%). All the studies included information on the domains related to exposure levels and the time frame to observe an effect. Only two studies (45, 48) provided information on the blinding of the assessors (13.32%). In addition, the participation rate was not reported in 42.86% of studies, and the follow-up rate was not achieved in 35.71%. Finally, only one study (48) did not provide information on the exposure measures (6.66%) and only 28.6% of studies assessed the exposure on more than one occasion (**Supplementary Table 2**).

For case-control studies, the overall risk of bias was low in all studies (100%) (25, 50). The studies provided information on all the domains, except one study (50) that did not report information about blinding of exposure assessors (50%) (**Supplementary Table 3**).

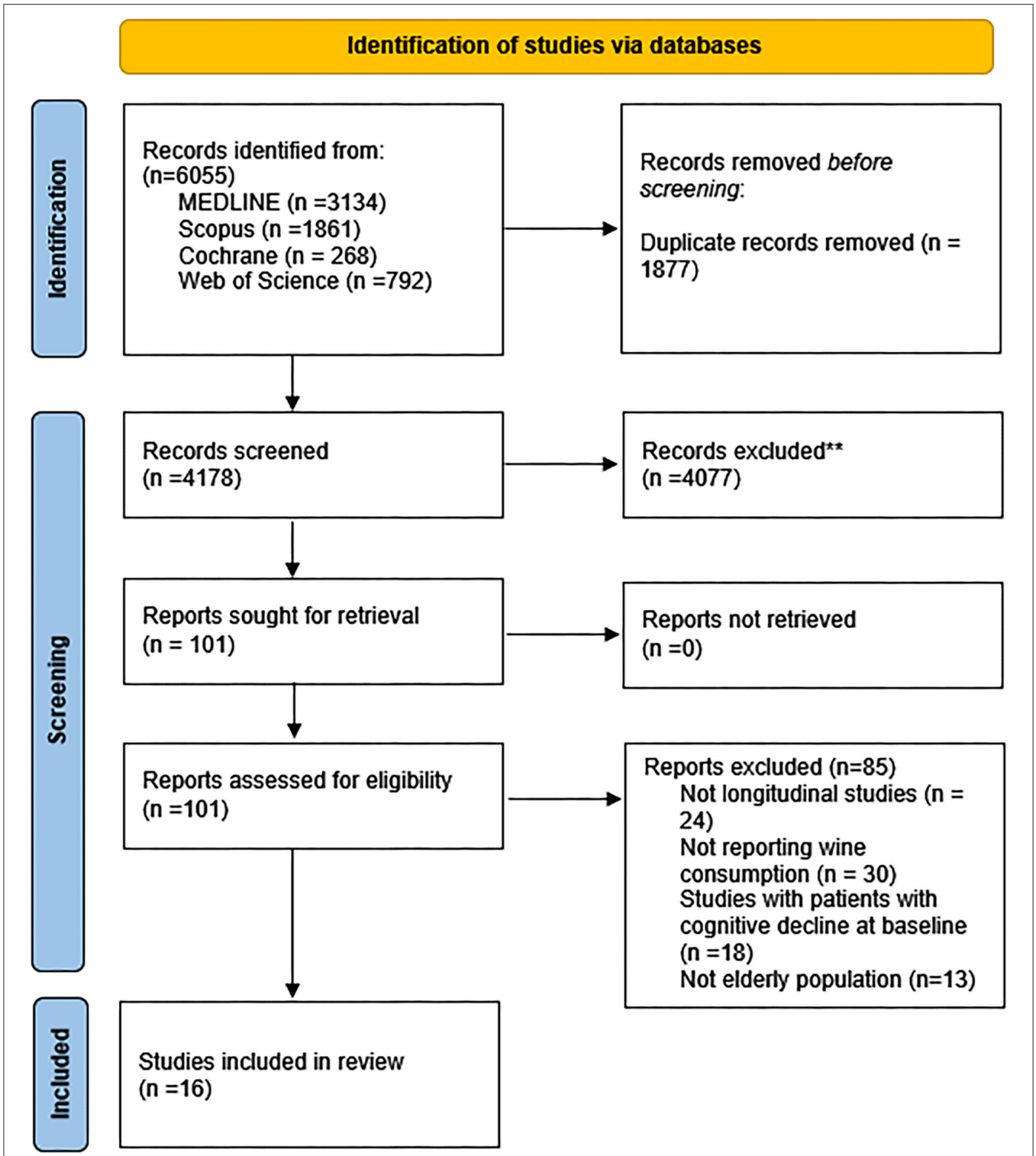

FIGURE 1 | PRISMA 2020 flow diagram for new systematic reviews which included searches of databases.

Meta-Analysis

Using the DerSimonian and Lair random effect models, the pooled RR for the association of wine consumption with cognitive decline was 0.72 (95% CI 0.63–0.80; $I^2 = 82.4\%$; τ^2: 0.0154). For the subgroup analysis, the pooled RR in those cohorts within the WHO recommendations was 0.59 (95% CI:

TABLE 1 | Main characteristics of the included studies.

References	Exposure			Outcomes				
	N, (women %); wine, (women %)	**Ages (DE)**	**Follow-up (years)**	**Wine consumption**	**Equivalent gr/day etanol in wine**	**Type of wine**	**Cognition test**	**Cognition domain**
Deng et al. (44) China	2,632 (56.38); 123	Non-drinker: 69.2 (8.8) Light-to-moderate drinker: 66.3 (6.3) Excessive drinker: 65.2 (7.7)	2	- Non-drinker (<1U/w) - Light-to-moderate drinker (1–21 U/w ♂, or 1–14 U/w ♀) - Excessive drinker (>21 U/w ♂, or >14 U/w ♀))	- Non-drinker: <1,43 gr - Light-to-moderate drinker: 1,43–30 gr ♂, 1,43–20 gr ♀) - Excessive drinker (>30 gr ♂, or >20 gr ♀)	Wine	MMSE Suspected dementia: - Examination by a neurologist - neuropsychological testing	- Dementia.
Fischer et al. (45) Germany	2,622 (65.3)	81.2 (3.4)	10	Red wine (%): - Never: 52.2 - <1 time/week: 20.4 - 1 time/week: 9.2 - Several times/week: 10.7 - Every day: 7.5 White wine (%): - Not at all :64.4 - <1 time/week:20.6 - 1 time/week: 6.1 - Several times/week: 7.0 - Every day: 1.9	NR	Red wine White wine	SIDAM Global Deterioration Scale Blessed Dementia Rating scale CERAD: - Word List Immediate Recall - Word List Delayed Recall - Word List Recognition subtest	- AD. - Dementia - Dementias. - Memory.
Handing et al. (46) Sweden	12,326 (55.5); 5,463	54.2 (5.9)	43	n (mean) - None (0): 31 (0.0) - Light (>0 to ≤5): 5,102 (0.5) - Moderate (>5 to ≤12): 255 (6.3) - Heavy (>12 to ≤24): 56 (18.9) - Very Heavy (>24): 19 (33.6)	- None: 0 gr - Light: 0–5 gr - Moderate: 5–12 gr - Heavy: 12 gr-24 gr - Very Heavy >24 gr	Wine	The National Patient Register and/or Cause of Death Register were used for diagnoses of dementia.	- Dementia.
Heymann et al. (47) EEUU	360 (58)	74.87 (8.897)	19.28	- Abstainers - Mild-moderate drinkers (1–7 D/w). - Heavy drinkers (8 or more D/w).	- Abstainers: 0 - Mild-moderate drinkers:11–77 gr - Heavy drinkers: >88 gr	Wine	MMSE.	- AD.

(Continued)

TABLE 1 | Continued

References	Exposure			Outcomes				
	N, (women %); wine, (women %)	**Ages (DE)**	**Follow-up (years)**	**Wine consumption**	**Equivalent gr/day etanol in wine**	**Type of wine**	**Cognition test**	**Cognition domain**
Leibovici et al. (48) France	833	>60	3	Below 75 years: - Low education: 26% - High education: 17% >75: - Low education: 47% - High education: 27%	NR	Wine	- Examen Cognitif par Ordinateur	- Working memory. - Language skills. - Visuospatial performance. - Focused and divided attention.
Lemeshow et al. (49) EEUU	3,777	≥65	3	Incident dementia. N (%) - None: 48 (4.9) - ≤1/4 liter/day: 47 (5.1) - >1/4 liter/day: 4 (1.1)	- ≤1/4 litter/day: 0–24 gr - >1/4 litter/day: >25 gr	Wine	Battery of psychometric test: - The Benton Visual Retention Test. - The Zazzo's Cancellation Test. - The Isaacs Stet Test for verbal fluency. - The Wechsler paired associate's test. - The Wechsler digit-symbol test. - MMSE. - DSM-III-R criteria.	- Cognitive functioning. - Global cognitive status. - Dementia. - AD.
Lindsay et al. (50) Canadá	Cases: 194 (67.5) Controls: 3894 (57.5)	Cases: 81 Controls: 72.9	5	- Cases exposed/total: 15/186 - Controls exposed/total: 668/3,789	NR	Wine	- 3MSE. - Neuropsychological tests	- AD
Low et al. (51) France	9,294 (66)	75.8 (4.35)	12	Wine (glasses/week) Cases: 8.4 (9.5) Controls: 9.3 (11.5)	NR	Wine Red wine	Mini-Mental State Examination. Benton Visual Retention Test. Isaac's Set Test. Trail-Making Test part A. Trail-Making Test part B.	Global cognition.
Luchsinger et al. (52) EEUU	980 (67); 162	73.3 (5.8) Age according to the frequency of consumption: None: 75.37 (5.78) Light to moderate: 75.11 (5.62) Heavy: 82.41 (0)	4	None:85,91% Light to moderate: 14,08% Heavy: 0,10%	None: 0 gr Light to moderate: 11–33 gr Heavy: >33 gr	Wine	Alzheimer's disease: - National Institute of Neurological and Cognitive Disorders and Stroke-Alzheimer's Disease and Related Disorders Association criteria. Dementia: - Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition criteria and required evidence of cognitive deficit on the neuropsychological test battery and evidence of impairment in social or occupational function; persons with a global summary score on the Clinical Dementia Rating (CDR).	- AD. - Dementia.

(Continued)

TABLE 1 | Continued

References	Exposure			Outcomes				
	N, (women %); wine, (women %)	**Ages (DE)**	**Follow-up (years)**	**Wine consumption**	**Equivalent gr/day etanol in wine**	**Type of wine**	**Cognition test**	**Cognition domain**
Mehlig et al. (53) Sweden	Four health examinations - 1968–1969: 1,458 - 1974–1975 (alive): 1,298 (1,426) - 1980–1981 (alive): 1,146 (1,366) - 1992–1993 (alive): 800 (1,118)	38–60 years	34	Four health examinations: - 1968–1969: 51% - 1974–1975: 59% - 1980–1981: 63% - 1992–1993: 64%	NR	Wine	Batteries of neuropsychiatric tests.	- Dementia.
Mukamal et al. (26) EEUU	746 (58,45)	77,65 (5.35)	6	- None - <1 - 1–6 - >o=7	- None: 0gr - <1: <2,35 gr - 1–6: 2,36gr-14,14 gr - >7: >14,14	Wine	- 3MSE - The CHS cognition study - IQCODE.	- Dementia.
Nooyens et al. (54) The Netherlands	2,613 (50.71);	55.78 (7)	7	NR	NR	White wine Red wine Fortified wine	The neuropsychological test battery included four tests: - The 15 Words Verbal Learning Test. - The Stroop Color-Word Test. - The Word Fluency Test. - The Letter Digit Substitution Test.	- Memory. - Speed and cognitive flexibility. - Semantic memory. - Speed.
Orgogozo et al. (55) France	3,777	73,6	3	- Non-drinkers: 0–<1 per week. - Mild drinkers: At least 2 drinks per week but not more than 250 ml per day. - Moderate drinkers: 250–500 ml per day. - Heavy drinkers: >500 ml per day.	- Non-drinkers: 0 mg. - Mild drinkers: no more than 25 gr. - Moderate drinkers: 25–50 gr. - Heavy drinkers: >50 gr.	Wine	Battery of psychometric test: - The Benton Visual Retention test. - The Zazzo's Cancellation Test. - The Isaacs Stet Test for verbal fluency. - The Wechsler paired associates test. - The Wechsler digit-symbol test. - MMSE. - DSM-III-R criteria.	- Cognitive functioning. - Global cognitive status. - Dementia. - AD.

(Continued)

TABLE 1 | Continued

References	Exposure			Outcomes				
	N, (women %); wine, (women %)	**Ages (DE)**	**Follow-up (years)**	**Wine consumption**	**Equivalent gr/day etanol in wine**	**Type of wine**	**Cognition test**	**Cognition domain**
Ruitenberg et al. (56) Netherlands	5,395 (59); 1,994 (42)	67,38 (7,48)	6	Median (IQR) drinks/day: -Total (n = 1,994): 0·14 (0·05–0·47) Men (n = 655): 0·24 (0·06–0·59) Women (n = 1,339): 0·12 (0·04–0·44)	NR	Wine	-MMSE	-Dementia.
Sabia et al. (57) UK	10,308 (33)	35-55	31	Units/week (%) - Abstinence: 0 (0) - 1–14 units/week: 2.8 (2.5) - >14 units/week7.9 (7.1)	NR	Wine	The national hospital episode statistics. The Mental Health Services Data Set. The mortality registers. Using ICD-10 codes F00-F03, F05.1, G30, and G31.	- Dementia.
Solfrizzi et al. (58) Italy	2,963; 1,131 (36.25)	71,69 (4.965) Alcohol consumption None: 71.09 (4.94) <1 drink/day: 72.12 (5.06) 1 or 2 drinks/day: 71.90 (5.06) >2 drinks/day: 71.67 (4.80)	3,5	Median (IQR) drinks/day: - Total: 1.69 (0.85–1.69) ♂: 1.69 (0.85–3.38) ♀: 0.85 (0.85–0.85)	- None - <1: <11,5 gr - 1–2: 12,5gr-24gr - >2: >25 gr	Wine	MMSE.	- Dementia. - Cognitive impairment.
Truelsen et al. (25) Denmark	Total: 1,709 (62.14) Cases: 83 (54.21) Controls: 1,626 (62.54)	Total: 75.8 (6) Cases: 73.3 (5.6) ♀: 78.9 (5.8) ♂: 77.6 (7.1) Controls: 78.3 (6.4) ♀: 73.3 (5.5) ♂: 73.2 (5.6)	3	% ♂; % ♀ - Never/hardly ever: 60.5; 62.2 - Monthly: 26.3; 26.7 - Weekly: 7.9; 8.9 - Daily: 5.3; 2.2	NR	Wine	MMSE.	- Dementia.

U/w, unit per week; D/w, drinks of alcohol per week; NR, not reported; MMSE, Mini-Mental State Examination; AD, Alzheimer's disease; DSM, Diagnostic and Statistical Manual of Mental Disorders; IQCODE, Informant Questionnaire on the Cognitive Decline of the Elderly; CHS, Health Study-Cognition; 3MSE, Modified Mini-Mental State; CERAD, the Consortium to Establish a Registry for Alzheimer's Disease neuropsychological battery.

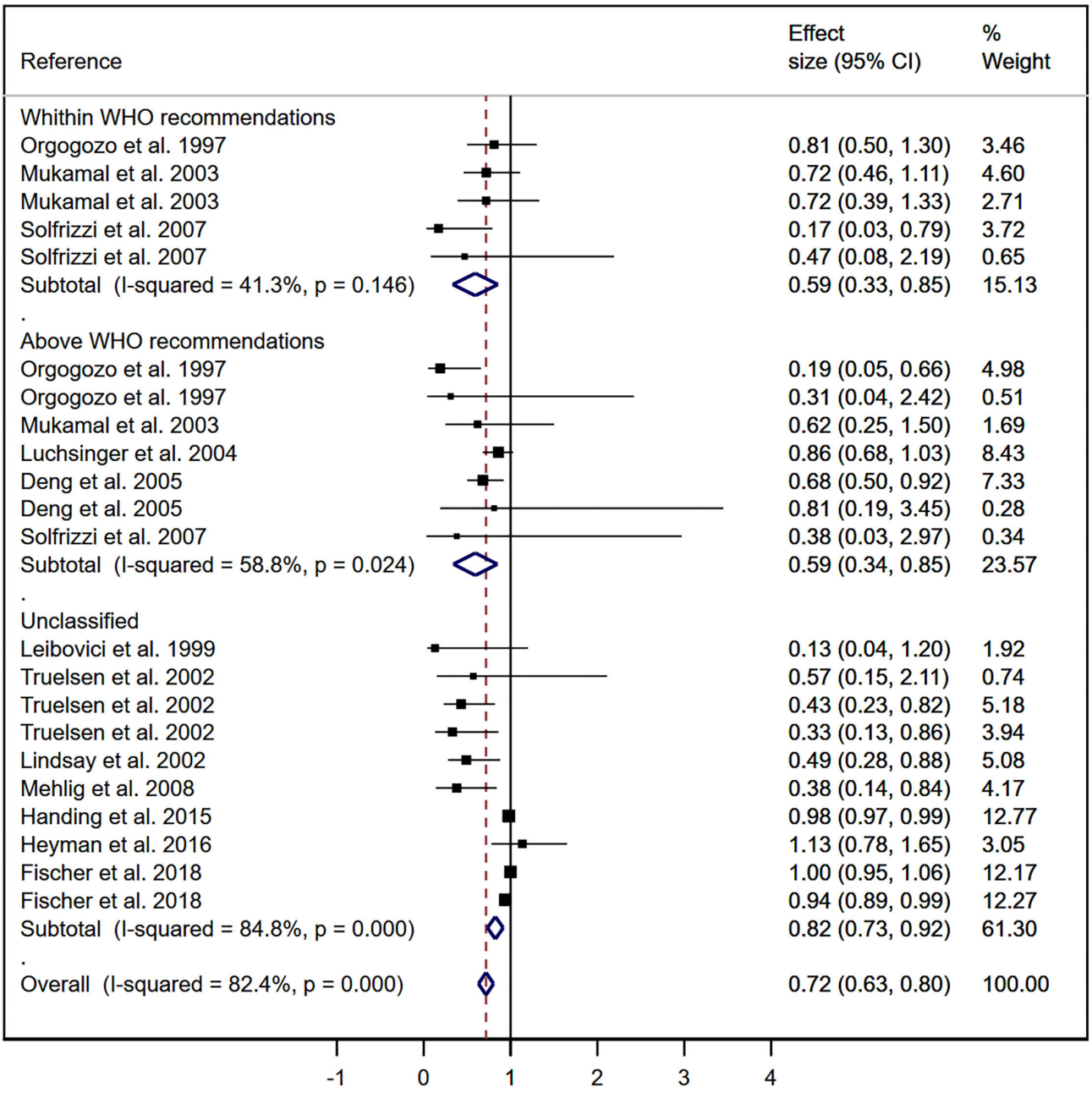

FIGURE 2 | Meta-analysis for the association of wine consumption and cognitive impairment.

0.33–0.85; I^2 = 41.3%; τ^2: 0.0348), the pooled RR for those cohorts above the WHO recommendations was 0.59 (95% CI: 0.34–0.85; I^2 = 58.8%; τ^2: 0.042), and the pooled RR for unclassified cohorts was 0.82 (95% CI: 0.73–0.92; I^2 = 84.8%; τ^2: 0.0088) (**Figure 2**).

Using the Hartung-Knapp-Sidik-Jonkman random effect models, the pooled RR for the association of wine consumption with cognitive decline was 0.65 (95% CI 0.52–0.79; I^2 = 94,531%; τ^2: 0.057). For the subgroup analysis, the pooled RR for those cohorts within the WHO recommendations was 0.59 (95% CI: 0.32–0.87; I^2 = 45.87%; τ^2: 0.042), the pooled RR for those cohorts above the WHO recommendations was 0.59 (95% CI: 0.32–0.86; I^2 = 63.9%; τ^2: 0.060), and the pooled RR for unclassified cohorts was 0.68 (95% CI: 0.47–0.90; I^2 = 98.27%; τ^2: 0.091).

Sensitivity and Meta-Regression Analysis

The pooled RR estimates were not modified after removing one study at a time from the analyses (**Supplementary Table 4**). In addition, random-effects meta-regression models for the

association between wine consumption and cognitive decline revealed that age, percentage of women, and duration of follow-up were not related to heterogeneity across studies (**Supplementary Table 5**).

Publication Bias

Publication bias was observed by Egger's test (43) for the overall effect of wine consumption on cognitive decline ($p < 0.01$). For subgroup analyses, publication bias was found for the unclassified cohorts ($p < 0.01$).

DISCUSSION

This systematic review and meta-analysis provide an overview of the evidence on the association of wine consumption with cognitive decline. Our data support the association between wine consumption and a lower risk of cognitive decline, with this evidence being stronger for those populations within WHO recommendations (30 g for men and 20 g for women, i.e., 3 SBUs and 2 SBUs, respectively) (14). Finally, this association did not seem to be modified by mean age, percentage of women, or follow-up time. Our results are in accordance with previous research (9, 22, 59) reporting the effect of alcohol consumption, including wine in sub-analyses, and add to the evidence that a protective effect could be exerted by low-to-moderate wine consumption.

Many lifestyle habits have been proposed to prevent or delay cognitive decline, including physical exercise, socialization, cognitive training, maintenance of good sleep hygiene, and adoption of the Mediterranean diet, among others. It has been reported that the Mediterranean diet could help to control cardiovascular parameters and oxidative stress. The Mediterranean diet contains a wide variety of foods rich in antioxidants, including a low-to-moderate wine consumption (60–62). This moderate alcohol consumption has been associated with better cognitive performance (63) and may contribute to maintaining cognition levels, although these results should be interpreted with caution since some types of alcohol-associated dementia have been identified (64, 65).

It is usually accepted that the limits for healthy alcohol consumption are 30 g for men and 20 g for women (14). Within these limits, most literature supports the possible protective effect of wine consumption against cognitive decline, even after controlling for covariates, such as age and percentage of women. However, some studies found no difference in the association of wine consumption and cognitive decline between women and men (25, 26, 44–58), Although, our data support no differences by sex, others have reported a higher association for women (45, 66) due to the differences in wine consumption between groups (45) and women's preference for white wine (33).

Wine consumption has been associated with a reduced risk of cognitive decline due to some of the components present in wine that may have an antioxidant function or that could inhibit the lethal events of oxidative stress produced by nitric oxide (24, 32). Grape skins are made up of different types of polyphenols, namely, quercetin, myricetin, catechin, and epicatechin (flavonols), gallic acid, and polymeric anthocyanins (24). The concentration of these components, which varies among types of wine, is responsible for the promising antioxidant potential of wine, with some wines being more protective than others. Flavonoids are components present primarily in red wine that might explain the reduction in the incidence of Alzheimer's disease and cerebrovascular disease (32, 66, 67). Resveratrol, a plant compound found in red wine grapes, might appear to have a neuroprotective effect by protecting neuronal cells from β-amyloid, a neurotoxin involved in the creation of senile plaques detected in neurodegenerative diseases, such as Alzheimer's disease. These senile plaques contribute to cell death (68), although removal of these plaques does not lead to improved cognition (69), resveratrol has anti-neuroinflammatory properties that protect against cognitive decline (57). This substance also has cancer-inhibiting effects (70) and reduces the incidence of coronary heart disease (71).

Moreover, there are also phenolic compounds in wine that contribute to the sensory properties of wine and protect it from oxidation (72, 73). These compounds also have antioxidant effects, reducing the risk of degenerative diseases such as osteoporosis, diabetes, and cancer (74). Various *in vitro* and animal studies have shown that polyphenols such as resveratrol could exert a number of health benefits, including anti-inflammatory and anti-atherogenic effects (27), and suggest a possible use of these polyphenols as therapeutic agents for ischemic and neurodegenerative events (75). In humans, a randomized clinical trial in women using a resveratrol supplement, concluded that a low-dose resveratrol could be a preventive strategy to counteract aging factors such as cognitive decline (76). Furthermore, it has been reported that resveratrol consumption needed for improvements in cognitive functions is far from the daily intake associated with food and wine consumption (77), A very high wine consumption, that would be detrimental to health, would be necessary to achieve the cognitive healthy resveratrol intake (78). In the case of observational studies with polyphenol supplementation through diet, including polyphenol-rich foods such as vegetables, fruit, or wine consumption (79), a low-dose wine consumption could produce benefits for some pathologies (53). In addition, evidence suggests that carrying the apolipoprotein E (*APOE*) epsilon 4 allele increases the likelihood of developing Alzheimer's disease (80). The risk of Alzheimer's disease in *APOE* ε4 carriers increased with white wine consumption vs. red wine consumption (41). Furthermore, the possible benefits of moderate wine consumption are increased in *APOE* ε4 noncarriers (6, 81). These possible benefits of wine components could explain the famous French paradox, where it was observed that mortality from CVD was much lower in France than in other industrialized countries (81). Further studies should consider additional confounding variables, namely, age, sex, race, body mass index, smoking, marital status, education, hormone treatment, and some pathologies such as diabetes (23).

Our systematic review and meta-analysis have some limitations that should be mentioned. First, studies included a wide variety of scales for measuring cognitive decline, which can lead to bias. Second, each study measured wine consumption differently, and some of the included studies that did not

quantify wine consumption. Third, there is no global consensus on maximum recommended intake or safe drinking limits, so the use of alcohol consumption limits, although recommended by the WHO, could represent a limit in addressing this issue. Fourth, most studies did not provide information on assessor blinding or whether exposure was assessed more than once over time. Fifth, we found evidence of publication bias using the Egger's test. Sixth, we only included studies in English and Spanish, in addition, gray literature was not included. Finally, due to the lack of data, we were unable to analyze the association between wine consumption and cognitive decline by type of wine.

In summary, this systematic review and meta-analysis identified a possible protective effect of wine consumption on the development of cognitive decline. This effect appears to be independent of age, the percentage of women, and follow-up time. These results do not suggest that the population should increase their wine consumption, as this could be harmful, especially in older persons who, with the loss of lean mass, polymedication and other factors, could suffer serious harm. However, considering our results, low wine consumption could be promoted within other lifestyles, including the Mediterranean diet, as an effective habit to prevent or delay cognitive deterioration in the healthy population. These results also support the international guidelines that suggest low-to-low-moderate alcohol consumption as the most acceptable level of consumption, both in the long and short term and for people who do not suffer any pathology such as liver cirrhosis or are not polymedicated or pregnant (82–84). Finally, it would be important for future research to differentiate between white and red wine consumption, which could allow to determine the association between wine consumption and cognitive decline by type of wine.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

ML-L-T and CÁ-B: conceptualization, investigation, and writing—original draft preparation. ML-L-T, CÁ-B, and IC-R: methodology. IC-R and CÁ-B: software. AS-L and CP-M: validation and visualization. ML-L-T and IC-R: formal analysis. ML-L-T, AS-L, and CP-M: resources. CÁ-B and VM-V: data curation. VM-V: writing—review and editing. CÁ-B: supervision. All authors revised and approved the final version of the articles.

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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.2022.863059/full#supplementary-material

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Chronic Consumption of Cranberries (*Vaccinium macrocarpon*) for 12 Weeks Improves Episodic Memory and Regional Brain Perfusion in Healthy Older Adults: A Randomised, Placebo-Controlled, Parallel-Groups Feasibility Study

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Background: Ageing is highly associated with cognitive decline and modifiable risk factors such as diet are believed to protect against this process. Specific dietary components and in particular, (poly)phenol-rich fruits such as berries have been increasingly recognised for their protection against age-related neurodegeneration. However, the impact of cranberries on cognitive function and neural functioning in older adults remains unclear.

Design: A 12-week parallel randomised placebo-controlled trial of freeze-dried cranberry powder was conducted in 60 older adults aged between 50 and 80 years. Cognitive assessment, including memory and executive function, neuroimaging and blood sample collection were conducted before and after the intervention to assess the impact of daily cranberry consumption on cognition, brain function and biomarkers of neuronal signalling.

Results: Cranberry supplementation for 12 weeks was associated with improvements in visual episodic memory in aged participants when compared to placebo. Mechanisms of action may include increased regional perfusion in the right entorhinal cortex, the accumbens area and the caudate in the cranberry group. Significant decrease in low-density lipoprotein (LDL) cholesterol during the course of the intervention was also observed. No significant differences were, however, detected for BDNF levels between groups.

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Conclusions: The results of this study indicate that daily cranberry supplementation (equivalent to 1 small cup of cranberries) over a 12-week period improves episodic memory performance and neural functioning, providing a basis for future investigations to determine efficacy in the context of neurological disease. This trial was registered at clinicaltrials.gov as NCT03679533 and at ISRCTN as ISRCTN76069316.

Keywords: brain, flavonoids, cognition, BDNF, cerebral blood flow (CBF), arterial spin labelling (ASL), MRI, LDL-cholesterol

INTRODUCTION

The WHO estimates that by 2050, 22% of the world's population will be aged over 60 years (1). This is a product of increasing life expectancy, which should be viewed as an exceptional achievement; yet the improvement remains somewhat overshadowed by the absence of preserved quality-of-life. With age, the predominant risk factor for numerous chronic and degenerative neurological conditions, maintaining quality-of-life represents a significant global challenge. Indeed, dementia incidence is projected to double every 20 years, affecting an estimated 152 million individuals by 2050 (2), placing considerable pressure upon already strained dementia care and disease management strategies. It is therefore imperative that proven effective solutions are developed to curb and manage current projections. The pathophysiological processes leading to the development of cognitive decline and dementias are complex and multifactorial, offering reasonable explanation for the failure of many pharmacological interventions to date (3). Future strategies may therefore benefit from a multifaceted approach. In addition to complex genetic predisposition, dementia risk is further influenced by numerous environmental factors such as diet, exercise and smoking status. Appealingly, these contributing environmental factors are often modifiable, and represent targets to address multiple underlying features of the disease process (4, 5). Therefore, achievable lifestyle interventions may represent an alternative approach to mitigate disease risk.

Epidemiological studies have reported that higher dietary intake of flavonoids is associated with slower rates of cognitive decline (6–8) and dementia (9). Foods rich in anthocyanins (responsible for imparting the red, purple and blue colour to several fruits and vegetables) and proanthocyanidins such as berries are consistently shown to improve cognition (10–13), and are further supported by a wealth of preclinical data (14–19), as well as emerging clinical evidence with fruit juices (20, 21) and freeze-dried fruit powder (22–24). Potential mechanisms include an enhancement of neuronal signalling and synaptic plasticity (17, 25), a modulation of glucose metabolism/insulin resistance (26, 27), a change in microbiota diversity and metabolism (28–30) along with regional increases in cerebral perfusion (31–34), however, further elucidation is required.

Cranberries (*Vaccinium macrocarpon*) are particularly rich in (poly)phenols such as anthocyanins, proanthocyanidins (both A- and B-type), flavonols and hydroxycinnamic acids (35), and are recognised for their antioxidant and anti-inflammatory effects (36–38) along with their capacity to modulate cardiometabolic endpoints (38–40). However, limited information is currently available regarding the evaluation of cranberries on cognitive performance, with the only previous study exploring the impact of cranberry intake on cognitive performance reporting no cognitive benefit of a cranberry juice consumption for 6 weeks in older adults with normal cognitive functions (41).

Here we report upon a single-centre, 12-week, double-blind, placebo-controlled parallel intervention study in which the impact of a freeze-dried cranberry powder intervention (equivalent of one small bowl) was examined in the context of cognitive health in healthy older adults (50–80 years). Cognitive health was determined using a battery of cognitive tests in combination with comprehensive biochemical and magnetic resonance imaging (MRI) assessments.

MATERIALS AND METHODS

Study Participants

Healthy male and female older adults were recruited in this study through online recruitment databases (Join Dementia Research)[1]; existing research databases within the Norwich Medical School, University of East Anglia, where participants had previously consented to be contacted about research studies; and community-based advertising (e.g., recruitment posters, leaflets, talks). Participants were first pre-screened over the telephone for eligibility for the study using a screening questionnaire and were invited if they were aged between 50 and 80 years and presented with no subjective memory complaints as assessed by the Cognitive Change Index (CCI) questionnaire (42). Married couples who lived together were particularly targetted to reduce the variability in background diet patterns; however, participants were permitted to take part in the study on their own.

Individuals were excluded if they had any of the following condition: a diagnosis of any form of dementia or significant neurological condition, significant memory complaints, uncontrolled blood pressure, currently smoking or ceased smoking less than 6 months prior to enrolment, clinically diagnosed with psychiatric disorder, or currently on antidepressant or antipsychotic medication, diagnosed with a gastrointestinal disorder, or currently on any medication that alters the function of the gastrointestinal tract, chronic fatigue syndrome, liver disease, diabetes mellitus, or gall bladder abnormalities including gall bladder removal, history or MRI evidence of brain damage, including significant trauma, stroke, learning difficulties or developmental disorders, or a

[1]https://www.joindementiaresearch.nihr.ac.uk/

previous loss of consciousness for more than 24 h. In addition, participants were not eligible for the study if they were prescribed anticoagulant medicine such as warfarin, due to potential interactions with the active cranberry powder. Other exclusion criteria were restrictive or unbalanced diet and excessive alcohol consumption (>15 units/week). Participants were also excluded if they were identified as having a high flavonoid intake defined as >15 portions of flavonoid rich foods (fruit, vegetables, tea and coffee, fruit juice, dark chocolate, and cocoa) per day during the telephone screening (43).

For MRI measures, participants were not eligible to undergo the neuroimaging component of the study if they had a cardiac pacemaker, any metal surgical implants that would not be safe within the MRI machine, or experienced claustrophobia in small spaces. If participants were unable to undergo the neuroimaging component of the study, they were still able to take part in the other components of the study.

Study Design

A single-centre, 12-week randomised, double-blind placebo-controlled parallel study design protocol was performed. Participants attended three visits in total: a screening visit (V0), a pre-intervention baseline visit (V1), and a follow-up visit at the end of the intervention (V2).

The screening visit (V0) involved obtaining informed consent, followed by collecting a fasted (>10 h) morning blood and urine sample, physical measurements (height, weight, and blood pressure). Basal blood pressure and heart rate was first collected after participants had been lying supine for 5 min, and then collected again upon standing. Participants were then provided with a standardised breakfast and underwent global cognitive screening using the Addenbrooke's Cognitive Examination III (ACE-III). Participants were excluded if they scored <88 on the ACE-III, or had abnormal blood biochemistry, blood pressure or urine results indicative of a potential exclusion condition (e.g., diabetes, uncontrolled hypertension).

If participants passed the screening visit, they were invited to a pre-intervention baseline visit (V1). Following a standardised breakfast and other measures, as described above, participants completed a longer cognitive battery (2.5 h) including measures of processing speed, working memory, episodic memory and spatial navigation, and other experimental and perception tests to be published elsewhere, with a half hour break partway through testing to avoid fatigue. A 30-min MRI scan was also conducted either during this visit or within the same week of this visit. At the end of the baseline V1 visit, participants were provided with sachets of study powder, assigned to them using a computer-generated algorithm. The intervention was provided in the form of sachets (4.5 g each) of freeze-dried cranberry powder (Cranberry Institute, United States) designed to be incorporated into food and beverages (see **Supplementary Table 1** for the product specifications for the freeze-dried cranberry powder). Participants were instructed to take two sachets per day, one in the morning and one in the evening, to maximise the physiological impact based on current understanding of bioavailability (44). The daily dosage of cranberry powder was roughly equivalent to consuming one cup or 100 g of fresh cranberries. This dosage was calculated to provide 281 mg proanthocyanidins, with increase of 20 mg flavonols and 59 mg anthocyanins per day (see **Supplementary Methods** and **Supplementary Table 2**). The placebo powder was designed to match the active cranberry powder for taste, colour, fructose, total sugar and calories and contained a blend of water, maltodextrin (CPC Maltrin M-180), citric acid, artificial cranberry flavour (Lorann oils), fructose, red colour (Lorann oils) and grape shade (Esco Foods) that had been freeze-dried. Participants were asked to return all remaining cranberry sachets at the end of the 12-week treatment period, with the number of leftover sachets being taken as one measure of compliance. Adherence to treatment was also determined by measuring total plasma (poly)phenol metabolites concentration as described previously thereafter.

Apart from the addition of the study powder, participants were asked not to modify their dietary intake in any further way, including any changes to their caloric intake. However, participants were asked to refrain from consuming any other non-essential supplements that could have a significant impact on the outcome measures for the duration of the study. Participants were also asked to fill in a validated, semi-quantitative SCG FFQ (version 6.6) (45) to account for their background diet.

The follow-up visit V2 was scheduled exactly 12 weeks following the baseline visit at the end of the intervention and was identical in procedures to the baseline visit. Fasted blood and urine samples were collected in addition to physical measurements, followed by the cognitive battery and MRI.

Cognitive Assessment

Participants completed all of the following cognitive tests at the baseline and follow-up visits. The only exception to this was the ACE-III, which was conducted at the screening visit in the first instance to assess eligibility for the study (i.e., total score >88) and then repeated at the follow-up visit.

Global cognition was assessed using the ACE-III questionnaire, which covers domains including attention and orientation, memory, fluency, language and visuospatial functions (46). Executive functions and working memory were measured by using the Trail Making Test (TMT) (47), a short test of processing speed, attention, and set-shifting, and the Digit Span (DS) test, a subtest from the Weschler Adult Intelligence Scale–third edition (WAIS III) that assesses attention and short-term memory. DS is composed of two tasks administered independently of each other: "digits forward" and digits backward. For each "digits forward" item, participants are presented with a series of digits in increasing length and must immediately repeat them to the examiner in the same order as presented. For digits backward, the participant is required to repeat the number sequence in the reverse order. A composite executive function score was also calculated out of the ACE-III Category Fluency score (/7), the DS Backwards Raw Score (/14), and the Scaled Trails B from the TMT based on previously published normative data (48).

Memory was evaluated by using the Rey Complex Figure Test (RCF), a short measure of visual memory and visuospatial

constructional ability (49). This study included the copy and 3-min recall trials of the test. A measure of verbal episodic memory was also measured using the delayed recall of the name and address on the ACE-III (score out of 7).

The Supermarket Test is a computer- and tablet-based assessment of spatial orientation that uses an ecological shopping environment (50). It includes a path integration test and measures (1) egocentric orientation, (2) short-term spatial memory, (3) heading direction, and (4) central (vs. boundary) based navigation preferences.

All tests were conducted using pen and paper, with the exception of the Supermarket Test which was administered using an Apple iPad.

Magnetic Resonance Imaging

Data Acquisition

Magnetic resonance imaging scans were conducted in all eligible and willing participants at baseline and end of intervention and took approximately 30 min. In order to monitor structural brain information across the study, a T_1-weighted 3D gradient-echo MR sequence was conducted at each testing visit. A T_2-weighted fluid attenuated inversion recovery (FLAIR) scan was also conducted during the study visits. Arterial spin labelling (ASL) has previously been used to monitor changes in cerebral blood flow (CBF) in Alzheimer's disease and mild cognitive impairment patients (51, 52).

All data were acquired on a 3 Tesla Discovery 750 w wide bore MR system (GE Healthcare, Milwaukee, WI, United States) with a 12-channel phased-array head coil for signal reception. After localisers, T_1-weighted structural data were acquired using a 3D inversion-recovery fast spoiled gradient recalled echo (IR-FSPGR) sequence with repetition time (TR) = 7.7 ms; echo time (TE) = 3.1 ms; inversion time = 400 ms; field-of-view = 256 mm × 256 mm; acquired matrix = 256 × 256; 200 sagittal sections of 1 mm thickness; flip angle = 11°; and ASSET acceleration factor = 2 in the phase-encoding direction. Furthermore, a 3D T_2-weighted FLAIR (T_2w FLAIR) sequence was prescribed as follows: TR = 4800 ms; TE = 129 ms; inversion time = 1462 ms; field-of-view = 256 mm × 256 mm; acquired matrix = 256 × 256; 182 sagittal sections of 1 mm thickness; flip angle = 90°; an ARC acceleration factor of 2 in the phase-encoding direction; and a "HyperSense" compressed sensing subsampling factor of 2. The ASL scan consisted of a 3D spiral pseudo-continuous ASL (pCASL) acquisition with the following parameters: TE = 10.7 ms, TR = 4854 ms, 8 spiral interleaves with 512 sample points, field-of-view = 240 mm × 240 mm × 128 mm with a reconstructed resolution of 1.9 mm × 1.9 mm × 4 mm; post-label delay = 1500 ms, number of excitations = 3. Before analyses, all participant scans were visually inspected for significant head movements and artefacts.

Image Analysis

Voxel-Based Morphometry (VBM) was used on whole-brain T_1-weighted scans using the VBM package in FSL (FMRIB Software Library, Oxford, United Kingdom) to confirm that there were no grey matter structural differences between the cranberry and placebo groups (53).

White matter hyperintensities (WMH) were rated using Multi-image Analysis GUI (Mango version 4.1, Research Imaging Institute, UTHSCSA, San Antonio, TX, United States) by one rater (EF). A well-established rating scale developed by Fazekas et al. (54) was used to qualitatively rate WMH in periventricular (PWMH) and deep (DWMH) regions using FLAIR images. WMH in the periventricular areas was rated as 0 = absent, 1 = "caps" or pencil-thin lining, 2 = smooth "halo," or 3 = irregular, whereas DWMH were rated as 0 = absent, 1 = punctate foci, 2 = beginning confluence of foci, or 3 = large confluent areas.

For regional perfusion (ASL), equilibrium magnetisation (M_0) and perfusion-weighted images were calculated in-line on the scanner workstation. All further analyses were performed using a processing pipeline written in bash and Python (v3.6, Python Software Foundation),[2] which was run on the ADA high-performance computing cluster at the University of East Anglia. The pipeline closely resembled that used for the Alzheimer's Disease Neuroimaging Initiative (ADNI)[3] ASL sub-study, substituting FastSurfer for brain segmentation instead of FreeSurfer (55). In brief, M_0 and perfusion-weighted images were scaled and used to calculate CBF maps in physical units of arterial water density (mL/min/100 g). T_1-weighted data were then segmented using FSL's FAST algorithm and the derived grey matter probability maps were used to register the ASL perfusion-weighted images to T_1 space—*via* FSL's FLIRT algorithm. ROIs from the FastSurfer segmentation were then used to determine ROI-wise CBF statistics: minimum maximum, mean, median, and standard deviation.

To visualise voxel-wise differences between groups, we performed higher-level general linear model analysis using FSL's "randomise" permutation-testing tool. To facilitate this, structural T1-weighted images for all participants were first non-linearly registered to the Montreal Neurological Institute standard brain using FNIRT and the resulting transformation was applied to the ASL data. Difference images were then generated for each individual through subtraction of the baseline ASL scan from the post-intervention scan. An unpaired *t*-test was then performed on these data using "randomise" with threshold-free cluster enhancement to compare changes in perfusion between the cranberry and placebo groups.

Biological Samples and (poly)Phenol Metabolites Analyses

A fasted blood sample was taken at the screening assessment visit and sent to the accredited pathology laboratories at the Norfolk & Norwich University Hospital (NNUH) for determination of markers of general health. Further blood samples were collected at baseline and follow-up in EDTA, SST and heparin vacutainer tubes (Becton-Dickinson, United Kingdom) for assessment of circulating metabolites, *APOE* genotype and blood biochemistry. Samples were immediately processed for serum/plasma, aliquoted and stored

[2]www.python.org

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at −80°C until analysis. BDNF levels in plasma were assessed by ELISA (R&D Systems, United Kingdom) following the manufacturers' instructions.

Plasma extraction of polyphenol metabolites was performed using microelution solid phase extraction (μSPE) according to validated protocols, with some modifications (56, 57). Briefly, plasma samples (350 μl) were diluted (1:1) with phosphoric acid 4% to reduce phenolic-protein interactions. Each sample (600 μl) was loaded on a 96 well μSPE plate, washed with water (200 μl) and 0.2% acetic acid (200 μl) and finally eluted with methanol (60 μl). The 96 well collection plates were directly put in the UHPLC autosampler for immediate analysis. Plasma samples were analysed through UHPLC DIONEX Ultimate 3000 fitted with a TSQ Vantage Triple Quadrupole Mass Spectrometer (Thermo Fisher Scientific Inc., San Jose, CA, United States) equipped with a heated-electrospray ionisation source (H-ESI-II; Thermo Fisher Scientific Inc.). Separations were performed with a Kinetex EVO C18 (100 mm × 2.1 mm), 2.6 μm particle size (Phenomenex). For UHPLC, mobile phase A was water containing 0.01% formic acid and mobile phase B was acetonitrile containing 0.01% formic acid. The gradient started with 5% B, keeping isocratic conditions for 0.5 min, reaching 95% B at 7 min, followed by 1 min at 95% B and then 4 min at the start conditions to re-equilibrate the column. The flow rate was set at 0.4 ml/min, the injection volume was 5 μl, and the column was thermostated at 40°C. The MS worked in negative ionisation mode with capillary temperature at 270°C, while the source was at 300°C. The sheath gas flow was 60 units, while auxiliary gas pressure was set to 10 units. The source voltage was 3 kV. Ultra-high-purity argon gas was used for collision-induced dissociation (CID). Compounds were monitored in selective reaction monitoring (SRM) mode, and characteristic MS conditions (S-lens RF amplitude voltage and collision energy) were optimised for each compound. Chromatograms, mass spectral data and data processing were performed using Xcalibur software 2.1 (Thermo Fisher Scientific Inc.). Quantification was performed with calibration curves of standards, when available; when not available, metabolites were quantified with the most structurally similar compound. Due to failure in collecting follow-up plasma, 14 volunteers were not considered for the calculations of plasma metabolite content.

Statistical Analyses

Data were analysed using the Statistical Package for the Social Sciences (SPSS; v28.0), applying standard statistical thresholds ($p < 0.05$) and were tested for normality using the Shapiro–Wilk test. Mann–Whitney U Independent-Samples tests were employed to detect differences between demographic, anthropometric and biochemical data at baseline. One-way ANCOVA's were used to detect baseline differences in cognition between groups controlling for age, education and gender. The impact of treatment on the cognitive outcomes of interest was established using mixed linear model with time and treatment as independent variables and with age, education and gender entered as covariates. Whole-brain differences in grey matter intensities were analysed between cranberry and placebo groups at baseline and follow-up with age added as a covariate. Periventricular and deep WMH were compared between cranberry and placebo groups at baseline and follow-up using ANCOVAs with age added as a covariate. Mean regional perfusion derived from ASL scans were analysed using mixed linear modelling with age entered as a covariate to determine and group × time interactions. Pearson correlations between significant cognition and regional perfusion at follow-up were also conducted.

Plasma metabolites, anthropometric and biochemical measures were compared between groups at baseline using non-parametric Mann-Whitney *U*-test analysis. Mixed linear modelling was used to detect within group differences between baseline and follow-up, as well as group × time interactions. Correlations analyses between plasma (poly)phenol metabolites and RCF delayed score along with regional blood perfusion from MRI ASL in regions found to be impacted by the cranberry intervention were conducted using non-parametric Spearman rank order. Unless otherwise stated, all results are presented as means (SD).

RESULTS

Study Participants

Figure 1 shows a Consolidated Standards of Reporting Trials (CONSORT) flow diagram. Seventy participants were consented into the study. Of these participants, seven of them did not pass screening and three declined further participation between the screening and baseline visits. The final study population consisted of 60 participants who attended the baseline visit and commenced the intervention. All participants reported being in good health, not consuming any food supplements or medications that would interfere with the tested product. No participants discontinued the intervention or were withdrawn after they had attended the baseline visit, resulting in 60 participants completing the follow-up visit. There were no serious adverse events or protocol deviations reported during the study; however, there were two cases of participants experiencing dental changes, which were documented. Of the participants who were eligible for the intervention, 29 participants were randomised into the active cranberry treatment group and 31 into the control groups. All baseline values were within the physiological range and groups did not differ in age, education, distribution of gender or global cognitive performance at screening (**Table 1**).

Overall, compliance was excellent based on returned empty sachets or unused study products and based on increased plasma total concentration of (poly)phenol metabolites. Specifically, plasma total concentration of (poly)phenol metabolites increased by 1.82 ± 0.57 μM in the cranberry group. No increase in plasma total (poly)phenol concentration was observed in the placebo group (**Supplementary Figure 1**).

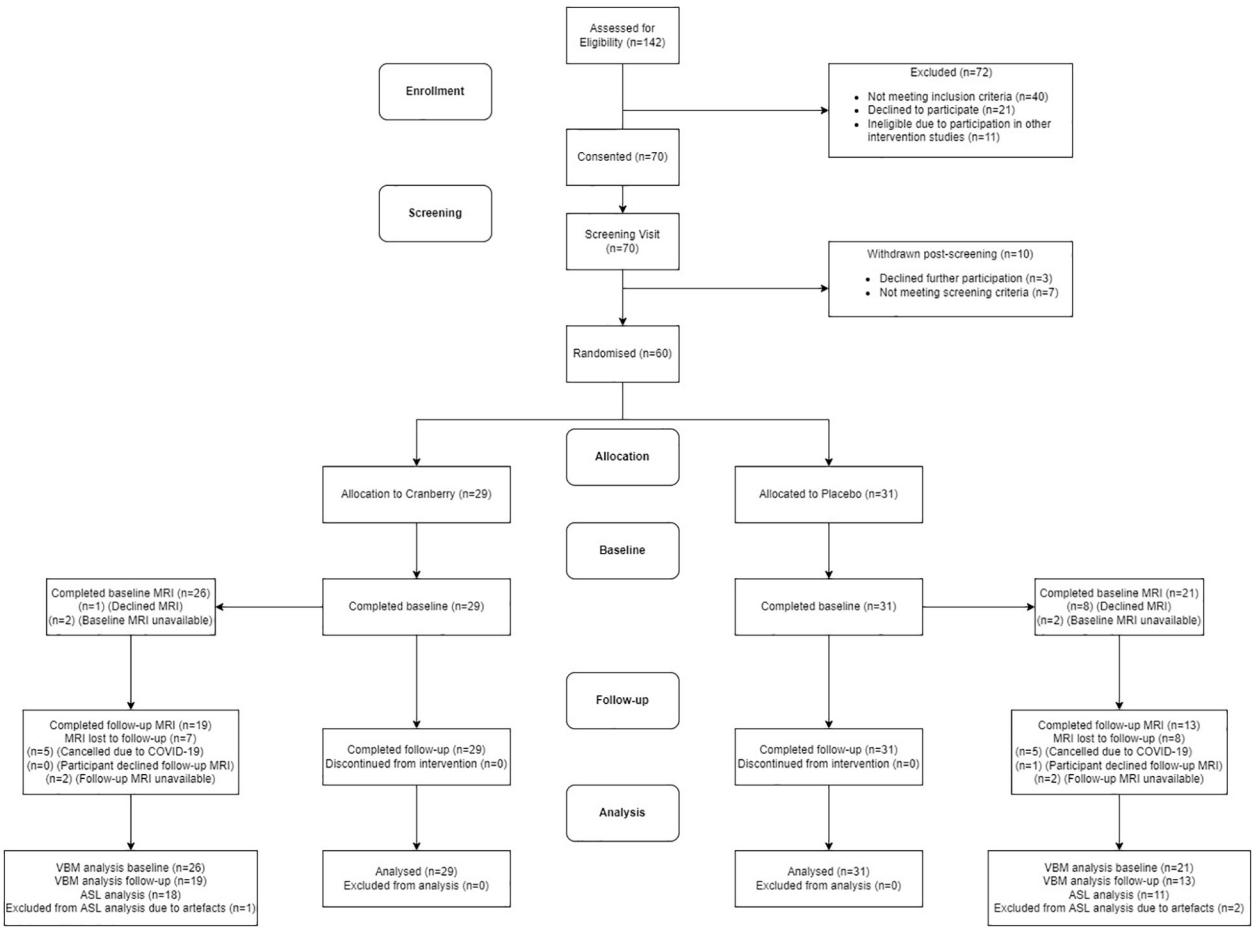

FIGURE 1 | Consolidated standards of reporting trials (CONSORT) flowchart diagram. ASL, arterial spin labelling; MRI, magnetic resonance imaging; VBM, voxel-based morphometry.

TABLE 1 | Demographic characteristics of the subjects at inclusion (n = 60).

Characteristics	Cranberry		Placebo		Sig. (p)
N	29		31		
Gender (M/F)	12/17		13/18		0.965
	Mean	**SD**	**Mean**	**SD**	
Age (years)	65.86	5.51	65.32	4.91	0.929
Education	14.38	2.60	14.61	3.01	0.610
ACE-III	96.58	2.39	96.10	2.80	0.644

(Chi-Squared and Mann–Whitney U Independent-Samples Test).

Food-frequency data indicated there was no difference between the placebo and the cranberry groups on the macronutrient content of their background diets. However, a significant difference was observed (p = 0.02) in vitamin D concentration between the two groups, with the placebo group having a lower concentration (**Supplementary Table 3**). Although not reaching significance, the cranberry group presented with increased concentration of caffeine intake (p = 0.09). Flavonoid content was similar between the two groups with an average intake of 228 mg/day in the placebo group and 217 mg/day in the cranberry group (**Supplementary Table 3**).

Cognitive Performance

There were no differences between cranberry and placebo groups at baseline on the ACE-III total score or on the sub-scores (Attention and Orientation, Memory, Fluency, Language, Address Delayed Recall and Category Fluency). No difference at baseline was also observed for RCF, Digits spans backward, Trail-making test A–B Scaled Score, or for the composite executive function score but there was a significant difference at baseline on visuospatial performance (p = 0.008, **Table 2**).

At follow-up, a significant group × time interaction [$F_{(1,55)}$ = 5.060; p = 0.028] was observed in performance of the RCF test delayed recall such that the cranberry group showed a significant improvement in performance between baseline and follow-up compared to the placebo group. Linear mixed modelling to detect group × time interactions between the groups between baseline and follow-up did not reveal any

TABLE 2 | Cognitive performance at baseline and follow-up, differences between groups at baseline (Mann–Whitney U Independent-Samples Test), and group × time interactions on linear mixed modelling.

			Baseline			Follow-up		Group × Time interaction
Measures		**Treatment**	**M**	**SD**	**Sig. (p)**	**M**	**SD**	**Sig. (p)**
ACE-III	Attention	Cranberry	17.65	0.72	0.219	17.66	0.55	0.467
		Placebo	17.35	1.11		17.60	0.62	
	Memory	Cranberry	24.97	1.18	0.726	25.10	1.05	0.498
		Placebo	24.84	1.70		25.67	1.95	
	Fluency	Cranberry	12.45	1.53	0.512	13.24	0.91	0.164
		Placebo	12.26	1.44		12.43	1.48	
	Language	Cranberry	25.79	0.49	0.305	25.76	0.51	0.401
		Placebo	25.58	0.54		25.83	0.46	
	Visuospatial	Cranberry	15.62	0.73	**0.008**	15.76	0.51	0.120
		Placebo	15.97	0.18		15.83	0.38	
	Address delayed recall	Cranberry	6.10	1.05	0.466	6.34	0.81	0.332
		Placebo	6.19	1.28		6.07	1.46	
	Category fluency	Cranberry	6.34	1.17	0.759	6.66	0.61	0.431
		Placebo	6.32	0.91		6.43	0.68	
RCF	Copy score	Cranberry	34.52	2.61	0.994	35.34	1.05	0.092
		Placebo	35.00	1.29		34.97	1.28	
	Delayed recall score	Cranberry	18.59	7.67	0.416	23.41	5.96	**0.028**
		Placebo	20.53	5.92		22.25	6.06	
Digit span	Forward raw score	Cranberry	11.28	2.28	0.437	11.41	2.01	0.309
		Placebo	10.84	2.28		11.39	2.50	
	Backward raw score	Cranberry	7.76	2.18	0.781	7.86	2.5	0.165
		Placebo	7.58	2.03		8.30	2.55	
TMT	A–B	Cranberry	37.41	21.84	0.416	35.93	16.22	0.639
		Placebo	33.29	16.89		34.14	13.73	
	B Scaled	Cranberry	14.83	1.79	0.756	15.03	1.66	0.127
		Placebo	15.13	1.73		15.37	1.97	
Executive composite score		Cranberry	28.93	3.50	0.964	29.34	3.73	0.430
		Placebo	29.03	3.41		29.93	3.45	

ACE III, Addenbrooke's cognitive examination III; RCF, Rey complex figure test; TMT, trail making test. Significant values p < 0.05 are in bold.

differential impact of the intervention on groups pre- to post-treatment for the copy score nor the other cognitive tests ($p > 0.05$) (**Table 2**).

No significant differences were detected between groups on the egocentric, allocentric error or allocentric heading subtotals and totals of the Supermarket Test (**Table 3**). When the linear mixed modelling was run on these subtotals and totals, no significant group × time interactions were found ($p > 0.05$ in all cases).

Magnetic Resonance Imaging

Among our study population, 47 participants were eligible and underwent the neuroimaging component of the study (26 in the cranberry group and 21 in the placebo group). Due to COVID-19 restrictions and reduced capacity of hospital facilities, 10 follow-up scans could not be conducted. An additional five follow-up scans could not be scheduled due to participants ($n = 1$) or scanning facilities ($n = 4$) being unavailable during the critical follow-up time window. Additionally, ASL data for 2 baseline scans and 1 follow-up scan were not usable due to severe motion artefacts.

There were no differences in whole brain grey matter intensity between cranberry and placebo groups found at either baseline or follow-up scans as assessed by the VBM package in FSL with a p threshold set at 0.05 with age added as a covariate. No statistically significant differences were observed between groups in periventricular WMH between the cranberry and placebo groups at baseline ($p = 0.688$) or follow-up ($p = 0.833$), or for deep WMH at baseline ($p = 0.693$) or follow-up ($p = 0.723$) in ANCOVA's with age added as a covariate. Two participants were rated as "3" for periventricular WMH (i.e., "irregular"), with the remaining participants being rated between 0–2.

Figures 2A–D depict representative magnetic resonance imaging data. Mean regional CBF from ASL for the cranberry and placebo groups at baseline and follow-up are summarised in **Supplementary Table 4**. No significant differences in regional perfusion between cranberry and placebo groups were detected at baseline ($p > 0.05$). ASL group analysis with FSL's "randomise" tool indicated voxels covering similar volumes to the ROI's indicated in the ROI-wise analyses (**Figure 2E**). Mixed linear modelling controlling for age and education

TABLE 3 | Spatial navigation performance at baseline and follow-up, differences between groups at baseline (Mann Whitney U independent-samples test), and group × time interactions on linear mixed modelling.

Measures			Baseline			Follow-up		Group × Time interaction
		Treatment	Mean	SD	Sig. (p)	Mean	SD	Sig. (p)
Supermarket test	Egocentric score section 1	Cranberry	3.32	1.84	0.383	3.76	1.96	0.906
		Placebo	3.67	1.76		3.92	1.91	
	Egocentric section 2	Cranberry	5.28	1.95	0.917	5.68	1.65	0.936
		Placebo	5.04	2.01		5.58	1.79	
	Egocentric total	Cranberry	8.60	3.29	0.656	9.44	3.29	0.885
		Placebo	8.71	3.36		9.50	3.27	
	Allocentric error section 1	Cranberry	11.72	4.35	0.324	12.54	8.31	0.267
		Placebo	13.00	4.94		11.96	5.81	
	Allocentric error section 2	Cranberry	14.28	3.00	0.164	15.47	10.54	0.481
		Placebo	14.11	4.55		13.27	4.76	
	Allocentric error total	Cranberry	13.00	3.14	0.964	13.98	8.88	0.325
		Placebo	13.55	4.47		12.70	4.70	
	Allocentric heading section 1	Cranberry	5.64	1.50	0.562	5.92	1.80	0.417
		Placebo	5.33	1.66		6.00	1.25	
	Allocentric heading section 2	Cranberry	5.60	1.50	0.489	5.84	1.34	0.697
		Placebo	5.38	1.41		5.79	1.29	
	Allocentric heading total	Cranberry	11.24	2.67	0.386	11.76	8.44	0.413
		Placebo	10.71	2.46		11.79	2.23	

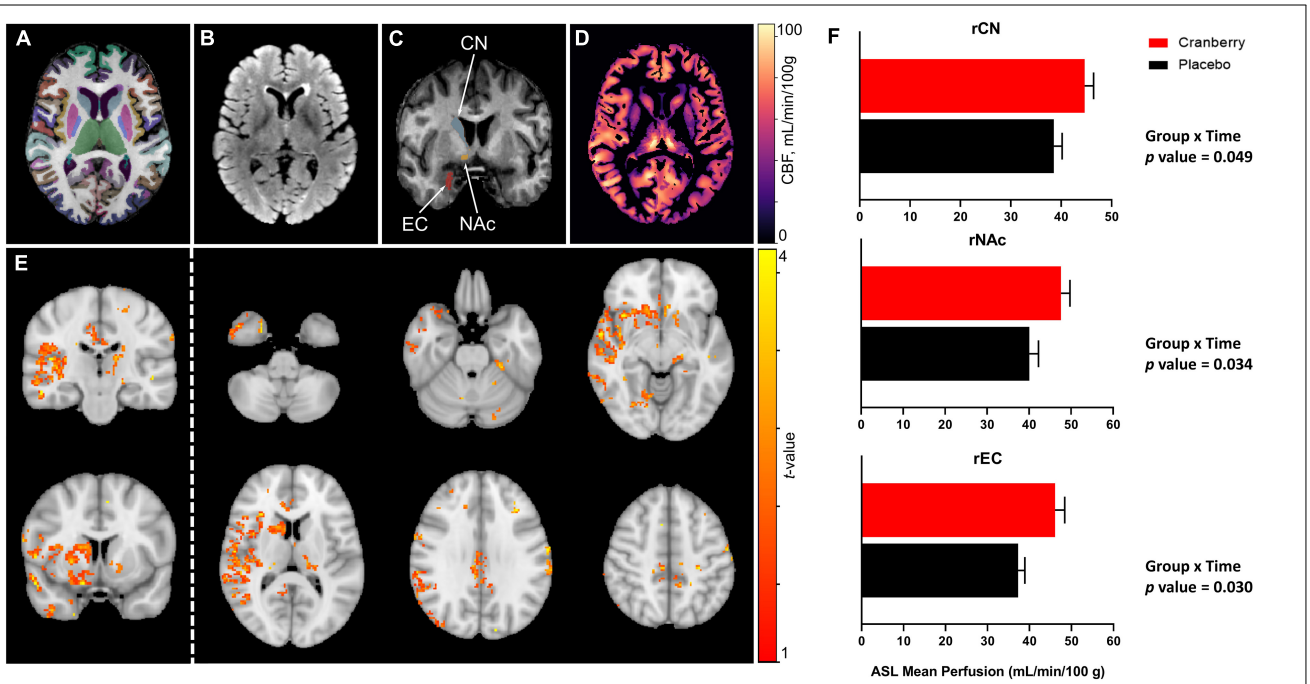

FIGURE 2 | Representative magnetic resonance imaging data following 12-week consumption of a cranberry extract or a placebo. **(A)** Axial view of FastSurfer cortical and subcortical segmentations superimposed on a T1-weighted image; **(B)** axial T2-weighted fluid attenuated inversion recovery (FLAIR) image; **(C)** a coronal view of the T1-weighted image indicating regions that showed significantly increased perfusion after 12 weeks consumption of a cranberry extract—namely, from superior to inferior, the right caudate nucleus, accumbens area, and entorhinal cortex; **(D)** an axial cerebral blood flow (CBF) map, in the T1 space, derived from arterial spin labelling data; **(E)** *t*-value maps overlaid on a standard brain show trends to increased perfusion in the cranberry group as compared to the placebo group, in similar regions to those indicated in the ROI-wise analyses; and **(F)** differences in mean blood perfusion for the right caudate nucleus (rCN), right nucleus accumbens (rNAc), and right entorhinal cortex (rEC), with *p*-values represented for group × time interaction effects between cranberry and placebo groups from baseline to follow-up.

detected significant group × time interactions for the right caudate [$F_{(1,29.275)}$ = 4.207, p = 0.049], right accumbens area [$F_{(1,31.744)}$ = 4.916, p = 0.034], and right entorhinal cortex [$F_{(1,30.558)}$ = 5.202, p = 0.030]. All models involved an increase in perfusion between baseline and follow-up in the cranberry group compared to a relative decrease in perfusion over time in the placebo group (**Figure 2F**).

As a significant group × time interaction was found for the delayed recall of the RCF, a correlation analysis was performed between the follow-up RCF delay scores and follow-up regional perfusion data, however, no significant correlation was found for either group ($p > 0.05$).

Biological Samples and Polyphenol Metabolites Analyses

There was a group × time effect on cholesterol, such that total, low-density lipoprotein (LDL) but not high-density lipoprotein (HDL) cholesterol decreased over the 12 weeks in cranberry but not in the placebo group [LDL; $F_{(1,40)}$ = 4.100; p = 0.048]. Although not reaching significance, a trend toward a decrease in total cholesterol was also observed in the cranberry group [$F_{(1,42)}$ = 4.073; p = 0.050] when compared to the placebo group (**Table 4**).

Investigating our data further, we observed a strong interindividual variability with male participants possibly experiencing a greater benefit from the cranberry intake. Indeed, a steep but not significant decrease in BMI (from 27 ± 4.2 to 24.5 ± 2.5 kg/m^2) in male participants was observed following 12 weeks of cranberry intake. This decrease also applied to fasting blood glucose and systolic blood pressure along with an increase in HDL cholesterol (**Supplementary Figure 2**).

There was no significant difference in BDNF concentration following cranberry intake for 12 weeks when compared to placebo (p = 0.7119) although concentrations were higher than in the placebo group at follow up (**Supplementary Figure 3**). High concentrations of BDNF in the cranberry group at baseline may be explained by higher concentration of caffeine intake in this group (**Supplementary Table 2**). BDNF at the follow-up visit did not correlate significantly with CBF in any treatment group ($p > 0.05$).

There were no significant differences in plasma total (poly)phenol metabolites at baseline, apart from the flavonol kaempferol-3-glucuronide which presented higher concentration in the placebo group [Mann Whitney-U (1, 43) = 351.00, p = 0.026; **Table 5**]. At follow-up, there were significant increases in 4-methylcatechol-sulphate, hippuric acid, caffeic acid and total (poly)phenol metabolites in the cranberry group when compared to the placebo (**Table 5**). No significant correlation between circulating (poly)phenol metabolites, RCF scores or regional blood perfusion at follow-up were observed ($p > 0.05$), indicating an indirect effect of the cranberry treatment on cognitive performance and brain perfusion.

No participants were found to carry two copies of the *APOE*-4 mutation. Furthermore, there were no differences between the cranberry and placebo groups for distribution of *APOE* genetic types, $\chi 2$ (3) = 2.60, p = 0.457 (**Table 6**).

DISCUSSION

This investigation reports the effect of long-term cranberry supplementation (12-week placebo-controlled intervention) upon cognitive performance and brain health. Daily supplementation with freeze-dried cranberry extract (equivalent to one cup of fresh cranberries) led to significant improvements in episodic memory performance, which coincided with increased perfusion of key neural areas which support cognition in older adults. Our results are in direct contrast to a previously conducted clinical investigation in which no significant change in memory performance was established following cranberry intake (41). The discordance between these results likely relates to experimental inconsistencies such as duration of the intake (6 vs. 12 weeks) or product formulation (cranberry juice vs. freeze-dried whole cranberry powder).

Surprisingly, the cranberry intervention had no further impact upon additional neurocognitive domains. Working memory and executive functioning (including the executive functioning composite score) remained unaltered despite the contrary being reported by others investigating flavonoid-rich juices e.g., blueberry, Concord grape juice and orange juice (21). This could in part relate to the distinct (poly)phenolic composition of each distinct intervention. Conversely, this may be a product of cognitive test choice, with tests such as the DS backward and trail making A and B believed to be less sensitive in detecting changes in executive functioning in non-cognitively impaired adults (58). In agreement with our results, a longer-term 12-week intervention with wild blueberry juice (20) led to similar improvements upon episodic memory performance in older adults. These two studies highlight that longer duration of supplementation is required to establish episodic memory enhancement associated with high anthocyanin and proanthocyanidin containing berries.

As anticipated, the cranberry intervention had no impact upon differences in structural grey matter between groups, nor did it influence differences in WMH over the 12-week period of investigation. However, in line with results suggesting that the cranberry intervention led to improved episodic memory performance, differences in perfusion in response to the intervention were detected between cranberry and placebo groups in key cerebral regions supporting memory consolidation and retrieval (59). A relative increase in perfusion was detected in the cranberry group between baseline and follow-up compared to the placebo group in medial temporal (entorhinal) and prefrontal (orbitofrontal) regions, as well as in the nucleus accumbens, which would provide optimal distribution of the essential nutrients for neuronal activity, such as oxygen and glucose (60).

The pathophysiological processes leading to neurodegeneration, like many other diseases, are proposed to involve the dysfunction of multiple systems within the body. Neurodegeneration is hypothesised to be characterised by progressive changes in several interlinked cellular and molecular mechanisms, including chronic neuroinflammation, oxidative stress and metabolic imbalances, as well as loss of vascular integrity and function, deposition of aggregated

TABLE 4 | Blood biochemistry, blood pressure, heart rate and anthropometry at baseline and follow-up, differences between groups at baseline (Mann-Whitney U Independent-Samples Test), and group × time interactions on linear mixed modelling.

			Baseline			Follow-up		Group × Time interaction
Measures		**Treatment**	**Mean**	**SD**	**Sig. (p)**	**Mean**	**SD**	**Sig. (p)**
Biochemistry	Cholesterol (mmol/L)	Cranberry	5.6	1.1	0.98	5.4	1.2	0.050
		Placebo	5.5	1.0		5.4	1.1	
	HDL cholesterol (mmol/L)	Cranberry	1.7	0.4	0.98	1.7	0.3	0.428
		Placebo	1.6	0.4		1.6	0.4	
	LDL cholesterol (mmol/L)	Cranberry	3.5	1.0	0.81	3.2	1.0	**0.048**
		Placebo	3.4	0.9		3.3	1.2	
	Triglyceride (mmol/L)	Cranberry	1.0	0.4	0.96	1.1	0.4	0.518
		Placebo	1.2	0.6		1.1	0.5	
	Glucose fasting (mmol/L)	Cranberry	4.7	0.4	0.98	4.6	0.4	0.963
		Placebo	4.8	0.5		4.7	0.5	
	ALT (U/L)	Cranberry	17.8	5.2	0.90	18.6	7.6	0.812
		Placebo	17.3	8.5		17.6	6.5	
	AST (U/L)	Cranberry	22.4	4.6	0.83	22.8	6.8	0.459
		Placebo	24.6	3.6		22.4	5.1	
	Alkaline phosphatase (U/L)	Cranberry	69.3	17.7	0.24	69.2	16.1	0.860
		Placebo	73.8	22.0		73.0	18.0	
	Creatinine (μmol/L)	Cranberry	72.9	12.4	0.96	73.4	10.5	0.609
		Placebo	73.1	14.0		71.3	11.6	
	Total bilirubin (μmol/L)	Cranberry	11.2	4.1	0.45	10.3	3.8	0.110
		Placebo	14.1	4.9		15.1	6.1	
	Total protein (g/L)	Cranberry	71.0	3.8	0.81	70.7	4.0	0.669
		Placebo	71.9	3.5		72.0	3.3	
	Albumin (g/L)	Cranberry	40.6	2.3	0.86	40.4	3.1	0.133
		Placebo	40.0	2.3		40.8	2.2	
	Globulin (g/L)	Cranberry	30.4	3.8	0.71	30.2	3.7	0.803
		Placebo	31.8	3.1		31.3	3.2	
	Urea (mmol/L)	Cranberry	5.1	1.0	0.96	5.0	1.1	0.862
		Placebo	4.9	1.0		4.7	1.2	
	Calcium (mmol/L)	Cranberry	2.3	0.1	0.98	2.3	0.1	0.198
		Placebo	2.4	0.1		2.4	0.1	
	Adjusted calcium (mmol/L)	Cranberry	2.4	0.1	>0.99	2.4	0.1	0.757
		Placebo	2.4	0.1		2.4	0.1	
	Phosphate (mmol/L)	Cranberry	1.0	0.2	>0.99	1.0	0.2	0.947
		Placebo	1.0	0.2		1.0	0.2	
	Bicarbonate (mmol/L)	Cranberry	27.0	3.2	0.77	26.7	2.2	0.341
		Placebo	25.9	2.2		26.1	1.9	
	Na (mmol/L)	Cranberry	139.5	2.3	0.19	139.7	2.4	0.496
		Placebo	134.5	2.9		138.0	2.2	
	K (mmol/L)	Cranberry	4.5	0.3	>0.99	4.4	0.2	0.996
		Placebo	4.5	0.3		4.5	0.4	
Blood pressure and heart rate	Diastolic (mm Hg)	Cranberry	79.3	9.9	0.60	81.2	10.7	0.786
		Placebo	81.3	11.2		82.9	10.5	
	Systolic (mm Hg)	Cranberry	139.0	17.4	0.85	134.9	18.5	0.521
		Placebo	139.7	18.3		132	13.6	
	Heart rate (bpm)	Cranberry	62.7	8.6	0.61	61.5	8.1	0.209
		Placebo	61.6	8.2		62.9	10.2	
Anthropometry	BMI (Kg/m^2)	Cranberry	24.9	4.0	0.93	24.1	3.0	0.305
		Placebo	25.0	5.9		25.7	3.9	
	Weight (Kg)	Cranberry	71.5	15.2	0.88	68.5	11.8	0.271
		Placebo	72.1	18.5		73.7	13.8	

ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; HDL, high-density lipoprotein; K, potassium; LDL, low-density lipoprotein; Na, sodium. Significant values $p < 0.05$ are in bold.

TABLE 5 | Plasma (poly)phenol metabolites at baseline and follow-up visits in μ mol/L.

Metabolite	Group	Baseline (μ mol/L)		Baseline difference	Follow-up (μ mol/L)		Group × Time
		M	SD	*p*	M	SD	*p*
Kaempferol-3-glucuronide (Flavonols)	Cranberry	0.017	0.018	**0.026**	0.019	0.020	0.274
	Placebo	0.033	0.034		0.028	0.022	
4-Methylcatechol-sulphate (Catechols)	Cranberry	0.033	0.016	0.617	0.042	0.015	**0.003**
	Placebo	0.032	0.012		0.024	0.010	
4-Hydroxybenzaldehyde (Benzaldehydes)	Cranberry	0.013	0.006	0.609	0.019	0.013	0.307
	Placebo	0.013	0.006		0.018	0.009	
Hippuric acid	Cranberry	2.82	1.58	0.892	4.29	1.94	**0.002**
	Placebo	2.76	1.12		2.53	1.78	
4-Hydroxyhippuric acid	Cranberry	0.039	0.020	0.540	0.043	0.025	0.508
	Placebo	0.043	0.023		0.041	0.025	
4-Hydroxybenzoic acid	Cranberry	0.024	0.022	0.056	0.027	0.012	0.358
	Placebo	0.032	0.079		0.038	0.054	
Benzoic acid-4-sulphate	Cranberry	0.023	0.023	0.107	0.028	0.024	0.192
	Placebo	0.015	0.009		0.014	0.009	
Benzoic acid-3-sulphate	Cranberry	0.026	0.038	0.609	0.020	0.021	0.588
	Placebo	0.015	0.014		0.012	0.011	
Caffeic acid (3′,4′-Dihydroxycinnamic acid)	Cranberry	0.021	0.012	0.856	0.032	0.014	**0.005**
	Placebo	0.020	0.008		0.019	0.013	
Ferulic acid-4-glucuronide	Cranberry	0.018	0.020	0.927	0.025	0.015	0.061
	Placebo	0.016	0.015		0.012	0.010	
3-Hydroxyphenylacetic acid (Phenylacetic acids)	Cranberry	0.078	0.041	0.496	0.087	0.042	0.811
	Placebo	0.068	0.033		0.074	0.028	
3-(3′-Hydroxyphenyl)propanoic acid (Phenylpropanoic acids)	Cranberry	0.051	0.043	0.115	0.039	0.036	0.166
	Placebo	0.077	0.054		0.041	0.049	
5-(Phenyl)-γ-valerolactone-methoxy-glucuronide (3′,4′) (Phenyl-γ-valerolactones)	Cranberry	0.018	0.011	0.751	0.017	0.010	0.190
	Placebo	0.017	0.009		0.013	0.008	
Total metabolites	Cranberry	3.18	1.68	0.820	4.69	2.01	**0.002**
	Placebo	3.14	1.20		2.87	0.321	

Significance of baseline group differences was determined by non-parametric Mann-Whitney U independent samples tests, and main effects of time within group between baseline and follow-up along with interactions between group and time was determined using linear mixed modelling. Significant values $p < 0.05$ are in bold.

TABLE 6 | *APOE* genetic status of participants in the cranberry and placebo groups, and overall totals.

APOE genotype	Cranberry	Placebo	Total
E2/E3	3	4	7 (11.67%)
E2/E4	0	2	2 (3.33%)
E3/E3	21	22	43 (71.67%)
E3/E4	5	3	8 (13.33%)

APOE, Apolipoprotein E.

proteins, mechanisms that underlie not only pathological but also normal brain ageing, resulting in loss of neural plasticity and neuronal death (61). However, the exact nature and results of these processes are still being elucidated. Treatments that can target and slow down these processes would be valuable in counteracting brain ageing and cognitive decline. Results from intervention studies involving humans remain less prevalent, and as such findings that lend support to the causal effects of berry (poly)phenols on the prevention of age-related cognitive decline and dementia from interventional studies remain sparse, and further attention needs to be dedicated to determining the causality of flavonoid consumption on improving cognition and preventing dementia.

Although it was anticipated that BDNF may increase in the cranberry group (in line with improved cognition) and as reported for other flavonoids (62), such results were not significant. In particular, a high concentration of BDNF was measured at baseline which masked the overall impact of cranberries at follow-up. Such increased concentration may be related to participants' higher consumption of caffeine in this group at baseline. During our analysis, we also observed a significant decrease in LDL cholesterol following cranberry intake, along with a trend in total cholesterol decreases. Such results are in agreement with a previous study demonstrating that cranberry supplements were effective in reducing atherosclerotic cholesterol profiles, including LDL cholesterol and total cholesterol levels (63). Interestingly, in our study, cranberry intake seemed to better benefit older male participants where a steep decrease in body weight and associated parameters [fasting glucose, high-density lipoprotein (HDL) cholesterol, blood pressure] were better controlled. Such results are in agreement with data demonstrating the anti-obesogenic impact of cranberries, although most of the information is derived from animal studies (64). Further studies would be necessary to confirm such findings in well controlled clinical studies.

In addition to biochemical measures, the circulating plasma (poly)phenol metabolites indicated that both groups were well matched at baseline, apart from flavanols which were higher in the placebo group before the intervention. The results of the background diet questionnaires also indicated that the placebo group had a higher average intake of flavonols, although this difference between groups was not significant. The high molecular weight fraction in our study represented 70.4%, so high concentrations of phenolic metabolites/catabolites were not expected after overnight fasting. There were, however, increases in total metabolites in the cranberry group as a result of the intervention, which appeared to be driven largely by significant increases in catechols and hippuric acid. Further and contrary to expectations, plasma (poly)phenol metabolite concentrations did not relate to either RCF delay score or regional blood perfusion within the regions found to be differentially changed between groups as a result of the intervention. It is therefore possible that the mechanisms underpinning the changes in cognition and regional blood perfusion in the brain were not the direct interaction between these metabolites and neural targets.

The relatively small sample size of this intervention may have been a limiting factor particularly with regards to having sufficient power to detect significant differences in both cognition and brain perfusion. Indeed, regarding the neuroimaging data, several regions showed a trend for perfusion differences such as the insula and the medial orbitofrontal cortex, which may have reached significance if more participant scans were available. Only a subsection of the study sample was able to have complete baseline and follow-up MRI scans due to practical constraints and the impact of COVID-19 lockdowns on hospital imaging facilities during the critical follow-up window for several (n = 14) patients. Furthermore, although it was not reported by any participants, other health conditions which may have influenced cognitive results such as sleep apnoea were not systematically excluded in this study. It is also important to note that the cranberries also contain other health-promoting compounds and nutrients, including fibre and other nutrients, making it sometimes difficult to determine whether it is in fact these specific polyphenols producing the health effects. For example, nutrients such as fermentable fibres can influence gut microbial metabolism of polyphenols (65). Furthermore, other mechanisms such as chronic neuroinflammation, mitochondrial function and compromised vascular integrity and function are increasingly becoming understood to be key mechanisms which also contribute to age-related cognitive decline and neurodegenerative conditions and provide targets for interventions to curtail the disease processes contributing to age-related neurodegeneration [for review, see Flanagan et al. (66)]. Indeed, these mechanisms are also among the targets of nutritional interventions including those involving high-polyphenol foods, particularly in light of their suggested bidirectional relationships with the function of gut microbiota. Similarly, markers of other factors that could be impacted by cranberry intake and could also relate to neurodegeneration such as chronic infection (67) were not measured. The focus of the intervention discussed in this study was the impact of a long-term cranberry intervention on cognition and brain function, and as such the investigation of the impact on these

additional mechanisms, although important, fell outside the scope of this study. Finally, as the physicochemical properties and dietary intake forms could impact the absorption and bioactivity of nutrients (68) contained in the cranberry powder, and as such could be controlled in future investigations to ensure that the effectiveness of the cranberry is not impacted by different methods of incorporating it into the diet.

These findings are, however, certainly encouraging that sustained intake of cranberry over a 12-week period produced significant improvements in memory and neural function in older adults who were cognitively healthy. Future studies investigating whether these changes translate to a clinical population of cognitively impaired adults in the context of neurodegenerative conditions such as mild cognitive impairment or dementia is warranted based on these results. Determining whether these changes are sustained following the cessation of intake, for how long and to what degree would also be of interest. Replication of this study in a larger sample size might also produce more robust results.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of East Anglia's Faculty of Medicine and Health Sciences Ethical Review Committee (Reference: 201819–039) and the Health Research Authority (IRAS number: 237251). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AN, MM, MH, and DV contributed to the conception and design of the study, funding acquisition, and wrote the manuscript. EF contributed to the day-to-day management of the study, data and sample acquisition, formal analysis and wrote the manuscript, and had primary responsibility for final content. MP analysed BDNF concentrations in biological samples. DC, CW, RS, and SS contributed to the MRI data analysis and statistical analyses. NT, PM, and DD analysed the (poly)phenol content in biological samples and extracts. All authors contributed to the manuscript revision, read, 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.2022.849902/full#supplementary-material

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Omega-3 Polyunsaturated Fatty Acids Supplementation Alleviate Anxiety Rather Than Depressive Symptoms Among First-Diagnosed, Drug-Naïve Major Depressive Disorder Patients: A Randomized Clinical Trial

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Background: Omega-3 polyunsaturated fatty acids (n-3 PUFAs) augmentation of antidepressants has shown great potential in the prevention and treatment of major depressive disorders (MDD).

Objective: To investigate the effect of n-3 PUFAs plus venlafaxine in patients with first-diagnosed, drug-naïve depression.

Method: A total of 72 outpatients with first-diagnosed depression were recruited. The daily dose of 2.4 g/day n-3 PUFAs or placebo plus venlafaxine was used for over 12 weeks. The outcomes were assessed by the Hamilton depression scale (HAMD), Hamilton anxiety scale (HAMA), Beck depression inventory (BDI), and Self-rating anxiety scale (SAS).

Results: Both groups exhibited improvement on clinical characteristics at week 4 and week 12 compared with baseline. The rate of responders for anxiety in n-3 PUFAs group (44.44%) was significantly higher than that in placebo group (21.21%) at week 4 (χ^2 = 4.182, p = 0.041), while week 12 did not show a difference (χ^2 = 0.900, p = 0.343).The rate of responders for depression at both week 4 (χ^2 = 0.261, p = 0.609) and week 12 (χ^2 = 1.443, p = 0.230) showed no significant difference between two groups. Further analysis found that Childhood Trauma Questionnaire (CTQ) had positive correlation with HAMA (r = 0.301, p = 0.012), SAS (r = 0.246, p = 0.015), HAMD (r = 0.252, p = 0.038) and BDI (r = 0.233, p = 0.022) with Pearson correlation analysis.

Social Support Rating Scale (SSRS) had negative correlation with SAS ($r = -0.244$, $p = 0.015$) and BDI ($r = -0.365$, $p = 0.000$).

Conclusion: This trial found that n-3 PUFAs supplementation in favor of venlafaxine alleviated the anxiety symptoms rather than depressive symptoms at the early stage of treatment (4 weeks) for first-diagnosed, drug-naïve depressed patients. However, the advantage disappeared in long-term treatment. Furthermore, childhood abuse and social support are closely related to the clinical and biological characteristics of depression. Both childhood trauma and lack of social support might be predictors of poor prognosis in depression.

Clinical Trial Registration: [clinicaltrials.gov], identifier [NCT03295708].

Keywords: nutrient intervention, antidepressant, omega-3 polyunsaturated fatty acids, major depressive disorders, childhood abuse, social support

INTRODUCTION

Depression is a common and serious mental disorder affecting more than 264 million people in the world (1). The World Health Organization reported that only 50% of severe cases have received effective treatment in developed countries, and less than 25% in some developing countries due to high prevalence and not recognized by professionals (2). Among them, only 50−70% of the patients respond to a single antidepressant medication (3, 4). These low treatment percentages and low therapeutic efficacy encourage other more acceptable alternatives or augmentation treatments for depression. Nutritional intervention is acceptable while the effective strategy has attracted interest in the prevention or treatment of depression (5, 6). Several clinical studies have shown that Omega-3 polyunsaturated fatty acids (n-3 PUFAs) could promise in alleviating anxiety, depression, and other related symptoms among those with depressive disorder (7, 8). The American Psychiatric Association (APA) practice guideline also recommended n-3 PUFAs as adjunctive therapy for mood disorders, such as depression (9).

N-3 PUFAs, mainly consisting of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (10, 11), are essential and critical for the maintenance of neuronal membrane structure, modulating inflammatory responses, mediating signal transduction, affecting the release and function of serotonin and dopamine in the nervous system (6, 12–15). Generally speaking, the physiological functions of EPA and DHA are different: EPA mainly plays an anti-inflammatory role in depression, while DHA mainly maintains cell membrane integrity and fluidity (16). These biological actions of n-3 PUFAs may influence the development of mental disorders, such as depression. Some clinical studies confirmed that a higher risk of mood disorder was associated with lower plasma levels of N-3 PUFA (17, 18), therefore, most studies have explored the efficacy of EPA, DHA, or their mixtures in MDD (19, 20). Due to the heterogeneity of these trials, the results are controversial and inconclusive (8, 21). The discrepant findings might be affected by the dosage and proportion of n-3 PUFAs, the treatment duration, and the enrolment of different types of participants (22, 23). EPA-major formulations and a daily dose of over 1.5 g of N-3 PUFA demonstrated clinical benefits in depression (24–27). Furthermore, the severity of clinical symptoms may affect the efficacy of N-3 PUFA. N-3 PUFA may have little or no effect on reducing the risk of depression or anxiety in mentally health participants at baseline (28). Appleton et al. also found that n-3 PUFAs may have a more pronounced effect in patients with more severe depressive symptoms (29). A growing number of clinical and, particularly, basic research has revealed the potential of N-3 PUFA in reducing depressive symptoms and the risk of onset or recurrence in MDD, yet the quality of evidence is too low to draw firm conclusions (28, 30). To summarize, there is insufficient high-quality evidence to assess the efficacy of N-3 PUFA as a treatment for depression, and more appropriate and well-designed studies will be required in the future to strengthen the clinical evidence.

Venlafaxine as the first-line drug for depression was introduced (31). Venlafaxine plus with n-3 PUFAs or placebo was investigated on the first-diagnosed, drug-naïve depressed patients for 12 weeks. Only first-diagnosed, drug-naïve depressed patients who never received any medications and psychotherapies before were recruited. It would be helpful of minimizing the heterogeneity of the participants. Interestingly, the baseline childhood trauma and social support may be the risk factors for depression and also could affect the efficacy of antidepressants during different treatment cycles (32–34). Therefore, the Childhood Trauma Questionnaire (CTQ) and Social Support Rating Scale (SSRS), which can be used for evaluation of childhood trauma and social support, respectively, were also collected. The purpose of this study was to investigate whether n-3 PUFAs could enhance the therapeutic efficacy of venlafaxine to attenuate depressive and anxiety symptoms.

MATERIALS AND METHODS

Subjects

A total of 72 first-diagnosed, drug-naïve depressed patients aged between 18 and 45 years were included in the study from March 1st, 2017, to January 20th, 2020 in the Second Xiangya Hospital of Central South University, China. The

participants must meet the Diagnostic and Statistical Manual of Mental Disorders-Fourth Edition (DSM-IV) criteria for current major depression by Structured Clinical Interview for DSM Disorders (SCID) and have a 24 item Hamilton Depression Rating Scale (HAMD) score greater than 20. The diagnoses of one patient were performed by two experienced psychiatrists. Venlafaxine was given as a therapeutic drug. The participants were excluded from the participants if they meet the following criteria: (1) suffering from other serious somatic diseases or comorbidities; (2) had serious nervous system disease; (3) in accordance with diagnostic standards of other mental illnesses; (4) were taking benzodiazepine every day, and need to be treated by electroconvulsive therapy currently or have received electroconvulsive therapy within the previous 6 months; (5) were pregnant women or lactating women; (6) had an apparent suicide attempt or suicidal behavior; (7) were taking omega-3 fatty acids supplements and eating more than 2 fish meals per week; and (8) any condition or medicines that may have an effect on biomarkers (within 1 week of the screening period or during the whole trial period) (35).

Study Design

The study was designed as a randomized, double-blind, placebo-controlled clinical trial to test the efficiency of n-3 PUFAs as an augmentation therapy to venlafaxine over 12 weeks. After meeting eligibility criteria, participants were randomized to receive either A or B drugs by an independent pharmacist who dispensed either active or placebo capsules according to the random number table generated randomization list. The information on the A and B drugs were sealed in an envelope which was made and kept by another independent pharmacist. The information inside the envelope was not made public until the end of the experiment. All participants, psychiatrists, and the researchers who met with the participants were blinded to group allocation. All participants needed to take venlafaxine (instruction recommend a dosage range of 75-225 mg/day) throughout the trial. The daily dose of n-3 PUFAs was 8 * 1 g capsules (EPA 1440 mg/day, DHA 960 mg/day) per day for 12 weeks based on the reported studies (12, 36, 37). The placebo group was given 8 * 1 g soybean oil placebo capsules daily. Soybean oil placebo capsules contained soybean oil with 1% fish oil so that it was flavored to taste and smell similar to the fish oil capsules. Both fish oil capsules and placebo capsules were supplied by Hunan Kang Q. Y. Bai Biological Technology Co., Ltd.

Following screening and baseline visits, study participants visit at week 4 and week 12. The 12 weeks of treatment length was determined based on previous trials of n-3 PUFAs for the treatment of depression (38–41). During the three follow-up visits (at baseline, week 4 and week 12) and two telephone contacts at the beginning of week 4 and week 12, the investigator encouraged medication adherence. The remaining medications were counted at each post-treatment follow-ups and subtracted from the number provided to determine adherence. Moreover, erythrocyte membrane EPA and DHA levels were obtained at baseline and post-treatment to confirm adherence to the fish oil capsules in the intervention arm. This study was approved by the ethics committee of Second Xiangya Hospital (MDD201610), and written informed consent was obtained from all participants.

Clinical Assessment

The baseline data included related demographics, clinical evaluations of psychiatric symptoms, a comprehensive medical history, a physical measurement of weight and height, and laboratory tests, including levels of EPA, DHA, and n-3 PUFAs. Follow-up visits were conducted at weeks 4 and 12 after the enrollment and drug treatment. The primary outcome was the change in 24 item Hamilton Depression Rating Scale (HAMD), and the secondary outcome was the change in the Hamilton anxiety scale (HAMA). Two self-reported rating scales were also introduced in this study, which was Beck depression inventory (BDI) and the Self-rating anxiety scale (SAS). All four questionnaires were completed by participants at each visit. The change in HAMD and HAMA total score from baseline at week 4 and week 12 were the response criterion. Responders were identified as those with a 50% or greater decrease in HAMD or HAMA score as previously described (42, 43). While remission was defined as the absolute value of HAMD or HAMA score less than 8.

The CTQ score was evaluated because of high internal consistency and strong retest reliability (44, 45). SSRS has good reliability and validity and is an important clinical assessment for depressed patients. CTQ and SSRS were completed at baseline by all participants. The receiver operating curve (ROC) was used to analyze the relationships between therapeutic effect and childhood trauma or social support. The sensitivity and specificity of clinical findings related to CTQ and SSRS were expressed by ROC analysis. The area under the curve (AUC) was calculated for each plot and used to evaluate the value of CTQ and SSRS in predicting the prognosis of depressed patients. Sensitivity and specificity were calculated by the following formulas (46):

$$\text{Sensitivity} = \text{True positives}/(\text{True positives} + \text{False negatives}) \times 100$$

$$\text{Specificity} = \text{True negatives}/(\text{True negatives} + \text{False positives}) \times 100$$

Fatty Acid Determination

Fatty acid determination followed the method from our previous report with a slight modification. Briefly, blood collection was performed in the early morning at baseline, 4 weeks and 12 weeks of antidepressant treatment for participants. The fasting blood samples were drawn into 5-ml vacutainer tubes containing EDTA, and then the blood was centrifuged for 5 min (3000 r/min). The red blood cells in the lower layer were transferred into Eppendorf tubes and stored at −80°C until lipid extraction and GC/MS analysis. To extract lipid, erythrocyte membranes were isolated from 150 μl of red blood cells vortexed with ice water for 3 min. Isopropanol (1.5 ml) and dichloromethane (1 ml) were successively added to the erythrocyte membranes. Then, 1 ml of 0.1 M potassium chloride solution was added to the

mixture. The mixture was blended for 3 min, and centrifuged for 5 min (2,000 rpm/min). The upper phase was removed, and we repeated the previous step on the lower layer twice. Subsequently, 10 μl of 100 μM butylated hydroxytoluene was added to the organic layer and the mixture was evaporated to dryness by nitrogen. The residue was taken through the same processing and analysis method as previously reported (11). The concentrations of fatty acids (ng/ml packed red blood cell) were expressed as the mean ± standard deviation (SD).

Statistical Analysis

Continuous variables were expressed as the mean ± standard deviation (SD) and categorical variables were presented as numbers. The difference between the mean values for each variable among the n-3 PUFAs augmentation group and placebo control group were tested for significance using an independent t test or paired t test (continuous variables). Categorical variables analysis was assessed using the χ^2 test. The Generalized Estimating Equation analysis was used for rating scales that repeatedly evaluated at each visit. Correlation of the DHA, EPA, total n-3 PUFAs level, and CTQ score with symptomatic index in the depression patients were calculated using Pearson's correlation, and Bonferroni corrections were utilized to adjust for multiple testing. All statistical analyses were carried out using SPSS statistical software version 24.0. The p-value of 0.05 was used to determine the statistical difference. We calculated the sample size by the non-inferiority test of the comparison of the two groups ($a = 0.05$, $\beta = 0.02$) in PASS 15, and 30% attrition was taken into account. With a sample size of 21 (treatment group) and 27 (control group), the trial will have more than 80% power to detect a difference.

RESULTS

Study Sample

A total of 72 first-diagnosed, drug-naïve depressed patients met all the study's eligibility criteria and randomly assigned to venlafaxine with n-3 PUFAs augmentation intervention group ($n = 36$; 50%) or venlafaxine with the placebo group ($n = 36$; 50%). The baseline characteristics of both groups were no statistical differences ($p \geq 0.05$). The detailed data were listed in **Table 1**. Of the 72 participants, 69 participants completed the 4-week follow-up: 36 participants in the n-3 PUFAs intervention group and 33 participants in the placebo group continued the 4-week trial. Finally, 49 individuals completed the trial at week 12: 21 participants in the n-3 PUFAs group and 27 participants in the placebo group finished the 12-week follow-up. The detailed flow of participants in the trial is shown in **Figure 1**.

Efficacy Outcomes

Results of changes in depression and anxiety questionnaire scores at baseline and after week 4 and week 12 are displayed in **Figure 2** and **Table 2**. The HAMD scores in n-3 PUFAs group and placebo group at week 4 and week 12 were reduced compared with baseline ($p < 0.01$). The patients who took n-3 PUFAs combined with venlafaxine had lower HAMD scores at week 12 than week 4 ($p < 0.01$). BDI scores both at week 4 and week 12 ($p < 0.01$) were lower than baseline in both the n-3 PUFAs group and the placebo group. Lower BDI scores were seen at week 12 than week 4 in the n-3 PUFAs group ($p < 0.01$) and placebo group ($p < 0.05$). HAMA anxiety questionnaire scores had a similar decreasing trend to BDI scores. The subjects in n-3 PUFAs and placebo groups get lower HAMA scores at week 4 and week 12 compared with baseline ($p < 0.01$). N-3 PUFAs group ($p < 0.01$) and placebo group ($p < 0.05$) had lower HAMA scores at week 12 than week 4. The patients in the n-3 PUFAs group had lower SAS scores at week 4 and week 12 than baseline ($p < 0.01$).

The rate of responders and the rate of full remission in n-3 PUFAs and placebo groups are presented in **Table 3**. The depression rate of responders between n-3 PUFAs and placebo group showed no significant difference at both week 4 ($\chi^2 = 0.261$, $p = 0.609$) and week 12 ($\chi^2 = 1.443$, $p = 0.230$). However, the anxiety rate of responders at week 4 in n-3 PUFAs was significantly higher than in the placebo group ($\chi^2 = 4.182$, $p = 0.041$). The anxiety rate of responders at week 12 had no significant difference between n-3 PUFAs group and the placebo group ($\chi^2 = 0.900$, $p = 0.343$). The depression rate of full remission at week 4 ($\chi^2 = 0.935$, $p = 0.416$), week 12 ($\chi^2 = 0.003$, $p = 1.000$) were no significantly different between n-3 PUFAs group and placebo group. Also, the anxiety rate of full remission at week 4 ($\chi^2 = 3.892$, $p = 0.115$), week 12 ($\chi^2 = 0.597$, $p = 0.683$) showed no significantly difference between groups.

Comparisons Between Depressed Patients With Childhood Trauma and Depressed Patients Without Childhood Trauma

The CTQ showed a good internal consistency (Cronbach's α coefficient = 0.792). The data in **Table 4** showed that the first-diagnosed, drug-naïve depressed patients with childhood trauma had higher baseline parameters. Depressed patients with childhood trauma had significantly higher SAS ($p < 0.05$), BDI ($p < 0.05$) scores and lower EPA ($p < 0.05$) levels than patients without childhood trauma.

Correlations Between Symptomatic Index, Omega-3 Levels, and Childhood Trauma Questionnaire, Social Support Rating Scale

The data in **Table 5** for first-diagnosed, drug-naïve depressed patients were analyzed separately to assess symptomatic index, n-3 PUFAs levels associated with CTQ or SSRS. Pearson correlation showed that CTQ had positive correlation with HAMA ($r = 0.301$, $p = 0.012$), SAS ($r = 0.246$, $p = 0.015$), HAMD ($r = 0.252$, $p = 0.038$) and BDI ($r = 0.233$, $p = 0.022$), and negative correlation with EPA level ($r = -0.238$, $p = 0.029$). SSRS had negative correlation with SAS ($r = -0.244$, $p = 0.015$) and BDI ($r = -0.365$, $p = 0.00$). Besides, the Pearson correlation analysis showed a symptomatic index that had no significant correlation with DHA, EPA, n-3 PUFAs levels, the detailed data were presented in **Supplementary Table 1**. The results indicated that some clinical symptomatic index and EPA levels would be affected by the CTQ score or SSRS score.

TABLE 1 | Baseline characteristics.

Parameter	n-3 PUFAs (*n* = 36)	Placebo (*n* = 36)	t/χ 2	*P*-value
Age (y), mean (SD)	26.33 (8.07)	27.11 (8.14)	−0.407	0.685
Males/females	15/21	11/25	0.963	0.326
BMI (kg/m^2)	20.58 (3.02)	21.83 (2.88)	0.006	0.937
Education (years)	14.42 (2.94)	13.26 (3.21)	1.566	0.122
Source of participants			0.229	0.633
Urban	22 (52.38%)	20 (47.62%)		
Rural	14 (46.67%)	16 (53.33%)		
Baseline DHA levels (umol/L), mean (SD)	0.76 (0.43)	0.81 (0.54)	−0.478	0.634
Baseline EPA levels (umol/L), mean (SD)	0.05 (0.03)	0.06 (0.03)	−0.506	0.615
Baseline Omega-3 fatty acids (umol/L), mean (SD)	1.29 (0.71)	1.41 (0.83)	−0.668	0.507
Baseline CTQ score, mean (SD)	52.61 (15.70)	52.17 (13.88)	0.125	0.901
Emotional abuse, mean (SD)	9.89 (4.52)	9.14 (3.77)	0.761	0.449
Physical abuse, mean (SD)	7.00 (3.14)	6.71 (2.47)	0.427	0.671
Sexual abuse, mean (SD)	6.05 (1.70)	6.29 (2.05)	−0.523	0.603
Emotional neglect, mean (SD)	13.65 (5.34)	12.80 (5.17)	−0.684	0.496
Physical neglect, mean (SD)	10.27 (3.13)	10.54 (4.08)	−0.319	0.751
SSRS, mean (SD)	28.11 (8.09)	28.83 (5.41)	−0.445	0.657
Subjective support	15.95 (4.95)	15.86 (4.29)	0.078	0.938
Objective support	7.22 (1.87)	7.22 (3.45)	−0.009	0.993

n-3 PUFAs, Omega-3 polyunsaturated fatty acids; BMI, Body Mass Index; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; CTQ, Childhood Trauma Questionnaire; SSRS, Social Support Rate Scale.

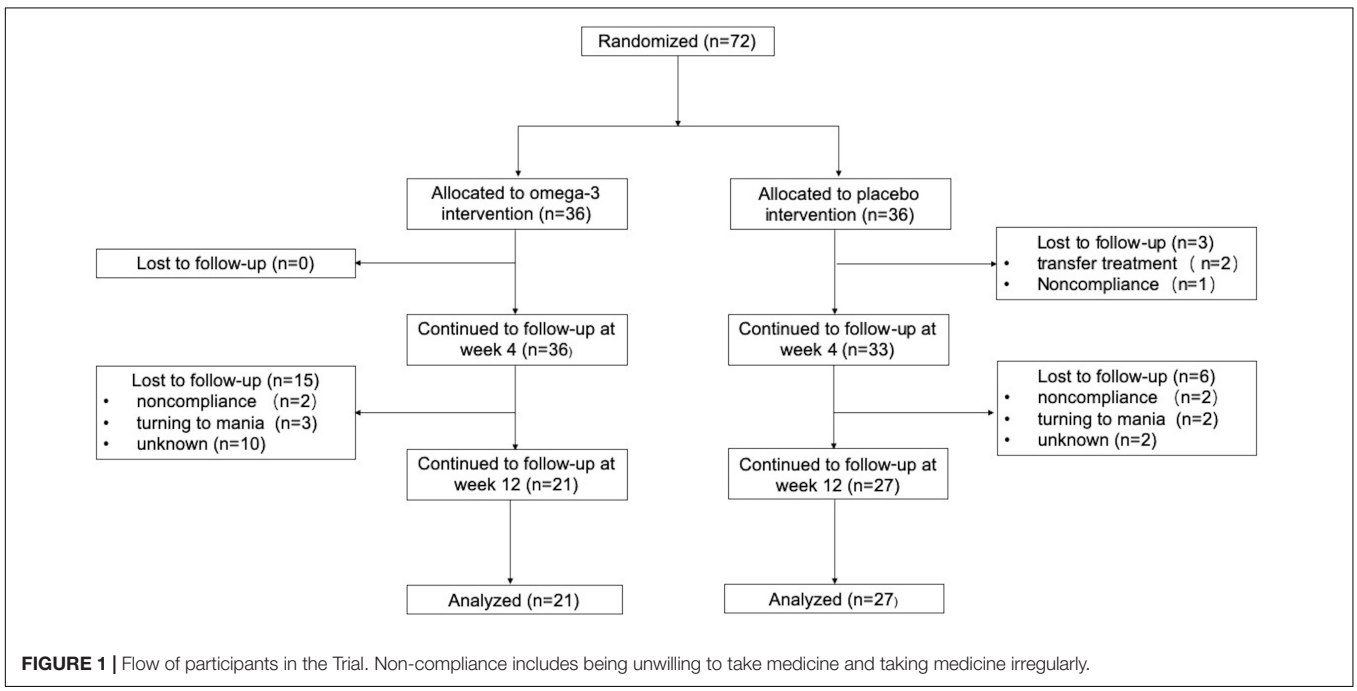


FIGURE 1 | Flow of participants in the Trial. Non-compliance includes being unwilling to take medicine and taking medicine irregularly.

The Receiver Operating Curve Curve Analysis of Childhood Trauma Questionnaire and Social Support Rating Scale

The ROC curve of CTQ, ROC curve of SSRS, and the combined ROC curve of CTQ and SSRS for the prognosis of depressed patients shown in **Figure 3** and the detailed data were provided in **Supplementary Tables 2–4**. About the SSRS ROC curve to evaluate the responders of HAMD: for n-3 PUFAs group at week 12 the AUC was 0.739 ± 0.107 with 95% credible interval (CI) was 0.530-0.949 (p = 0.062); for placebo group at week 12 the AUC was 0.789 ± 0.093 (95% CI: 0.606-0.971, p = 0.011). The SSRS ROC curve to evaluate the responders of HAMA for placebo subjects at week 4 the AUC was 0.764 ± 0.095 (95% CI: 0.577-0.951, p = 0.063). The CTQ AUC to evaluate the responders of HAMA for placebo subjects at week 12 was 0.714 ± 0.100 (95% CI: 0.518-0.909, p = 0.060).

In view of the combined ROC curve of CTQ and SSRS to evaluate the responders of depressive symptoms, the AUC was 0.726 ± 0.109 (95%CI: 0.514-0.939, p = 0.077) at week 12 in n-3 PUFAs group, 0.723 ± 0.098 (95%CI: 0.530-0.916, p = 0.046) at week 4 in placebo and 0.783 ± 0.092 (95%CI:

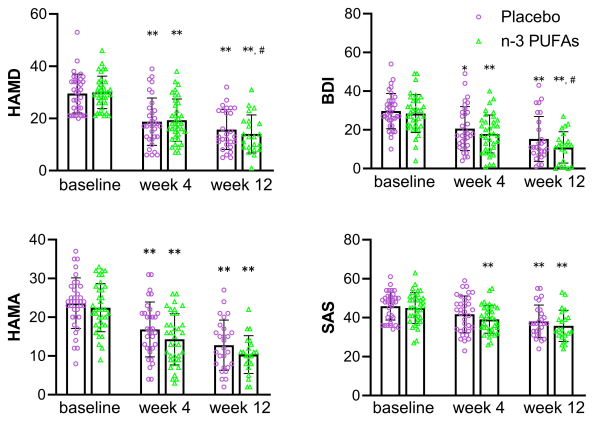


FIGURE 2 | The scores on depression and anxiety questionnaires in n-3 PUFAs and placebo group. *significantly different compared with baseline, # significantly different compared with week 4, *$p < 0.05$, **$p < 0.01$, # $p < 0.05$, ## $p < 0.01$. n-3 PUFAs, Omega-3 polyunsaturated fatty acids; HAMD, Hamilton Depression Scale; BDI, Beck Depression Inventory; HAMA, Hamilton anxiety scale; SAS, Self-rating Anxiety Scale. For n-3 PUFAs group, $n = 36$, after week 4, $n = 36$, and after week 12, $n = 21$. For the placebo group, $n = 36$, after week 4, $n = 33$, and after week 12, $n = 27$.

0.602-0.965, $p = 0.013$) at week 12 in placebo group, respectively. The AUC of combined CTQ and SSRS ROC curve to evaluate the responders of anxiety symptoms for placebo group at week 4 was 0.763 ± 0.100 (95%CI: 0.567-0.959, $p = 0.065$) and at week 12 was 0.728 ± 0.098 (95%CI: 0.535-0.920, $p = 0.045$).

Adverse Events

Adverse events were assessed at baseline, the 4-week and the 12-week follow-up visits. There were five participants withdrew from the trial because of turning from depression to mania. No adverse events were regarded as serious adverse events related to the study medication.

DISCUSSION

To our best knowledge, this is the first double-blind, placebo-controlled, randomized clinical trial to test the efficacy of n-3 PUFAs as adjunctive therapy combined with venlafaxine in the treatment of first-diagnosed, drug-naïve depressed patients. The results supported that n-3 PUFAs adjuvant therapy is more effective in improving anxiety symptoms than depressive symptoms at an early stage of treatment for depressed patients.

In this study, patients who took co-administration of venlafaxine at recommended dosage with n-3 PUFAs (EPA 1,440 mg/day, DHA 960 mg/day) or placebo had lower anxiety and depression questionnaires scores at each follow-up compared with baseline. These results were consistent with a previous study that reported that venlafaxine effectively reduced symptoms of depression in patients with MDD overall (47). We also observed that n-3 PUFAs supplementation increased the rate of responders for anxiety at week 4 and then the difference disappeared at week 12, which indicated that n-3 PUFAs supplementation could help to alleviate anxiety symptoms at an early stage of treatment for depressed patients. Considering the fact that conventional antidepressants take weeks or even months to have their full therapeutic effects (the effect varies from person to person) (48, 49), this rapid relief effect on anxiety may increase the confidence of patients and further increase treatment compliance. Furthermore, n-3 PUFAs are generally safe and well tolerated than other antipsychotics, and there were no significant differences in adverse events between the n-3 PUFAs and placebo groups in most studies (30, 50, 51). A subgroup meta-analysis based on ten studies revealed a significantly greater association of treatment with reduced anxiety symptoms in patients receiving

TABLE 2 | The scores on depression and anxiety questionnaires in n-3 PUFAs and placebo group.

	Baseline	Week 4	Week 12	Group Wald χ 2 (*P*-value)	Time Wald χ 2 (*P*-value)	Group * Time Wald χ 2 (*P*-value)
HAMD						
n-3 PUFAs	29.50 (27.11,31.89)	18.76 (15.71,21.81) ^{A}	15.70 (12.87,18.53) ^{B}	0.023 (0.880)	180.9 (0.000)	0.985 (0.611)
Placebo	29.97 (27.97,31.97)	19.31 (16.69,21.92) ^{A}	14.05 (11.07,17.02) ^{B,C}			
BDI						
n-3 PUFAs	29.67 (26.74,32.60)	20.64 (16.83,24.44) ^{A}	15.30 (10.99,19.60) ^{B,c}	2.182 (0.140)	110.4 (0.000)	0.992 (0.609)
Placebo	28.42 (25.27,31.56)	17.86 (14.70,21.02) ^{A}	10.91 (7.60,14.22) ^{B,C}			
HAMA						
n-3 PUFAs	23.61 (21.51,25.71)	17.70 (14.36,21.03) ^{A}	12.78 (10.38,15.18) ^{B,c}	3.406 (0.065)	156.6 (0.000)	1.282 (0.527)
Placebo	22.44 (20.44,24.45)	14.31 (12.17,16.44) ^{A}	10.36 (8.38,12.35) ^{B,C}			
SAS						
n-3 PUFAs	45.92 (43.60,48.23)	40.85 (37.79,43.91) ^{A}	38.15 (35.08,41.22) ^{B}	1.185 (0.276)	66.5 (0.000)	0.400 (0.819)
Placebo	44.92 (42.35,47.48)	39.11 (36.82,41.41) ^{A}	35.77 (32.53,39.02) ^{B}			

n-3 PUFAs, Omega-3 polyunsaturated fatty acids; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HAMA, Hamilton anxiety scale; SAS, Self-rating Anxiety Scale; HAMD, Hamilton Depression Scale; BDI, Beck Depression Inventory. The rating scale scores were expressed as Mean (95% Wald confidence interval).
**Significantly difference compared Placebo and n-3 PUFAs groups; *$p < 0.05$; **$p < 0.01$.*
A (a) Significantly difference compared baseline with week 4;a:$p < 0.05$;A:$p < 0.01$.
B (b) Significantly difference compared baseline with week 12;b:$p < 0.05$;B:$p < 0.01$.
C (c) Significantly difference compared week 4 with week 12;c:$p < 0.05$;C:$p < 0.01$.

TABLE 3 | The number of responders and the full remission in n-3 PUFAs and placebo group.

Parameter	n-3 PUFAs		Placebo		χ 2	*P*-value
	Responders	Non-responders	Responders	Non-responders		
The number of responders						
Depression						
Week 4	11	25	12	21	0.261	0.609
Week 12	13	8	12	15	1.443	0.230
Anxiety						
Week 4	16	20	7	26	4.182	**0.041**
Week 12	13	8	13	14	0.900	0.343
The number of participants who reach remission						
Depression						
Week 4	2	34	4	29	0.935	0.416
Week 12	3	18	4	23	0.003	1.000
Anxiety						
Week 4	6	30	3	30	0.871	0.351
Week 12	3	18	4	23	0.003	0.959

n-3 PUFAs, Omega-3 polyunsaturated fatty acids. The bold values represent the positive results.

at least 2,000 mg/d n-3 PUFAs treatment than in those not receiving it (52).

In our research, n-3 PUFAs supplementation with 2,400 mg/d improve anxiety symptoms faster and may have potential anxiolytic benefits. The potential anxiolytic benefits have been discovered over the past decade (53), and several possible explanations have been proposed. One of the explanations is that the deficiency of n-3 PUFAs and their derivatives in neuronal membranes might induce various behavioral and neuropsychiatric disorders, including anxiety-related behaviors (52). N-3 PUFAs supplementation will change the fatty acid composition of the cell membrane and increase the fluidity of the cell membrane. All of these changes would be beneficial for the relief of anxiety symptoms. Besides, inflammation was consistently found to induce psychotic symptoms, including anxiety (54). And n-3 PUFAs have anti-inflammatory effects which restrain the production of proinflammatory cytokine (55). N-3 PUFAs may help to improve anxiety by regulating the inflammatory factors. Furthermore, proinflammatory cytokines promote the secretion of corticotropin-releasing hormone (CRH), which is the main pathway of the hormonal stress response. CRH also stimulates the amygdala, which is a key brain area for fear and anxiety (56, 57).

An increasing number of dietary or nutrient-based interventions are investigated to be effective in preventing and managing psychiatric disorders (58). However, our research found that supplementation of n-3 PUFAs does not effectively improve depressive symptoms, which is consistent with previously reported studies (59, 60). One possible explanation is that the antidepressant effect of venlafaxine was too strong. So that the relatively weaker effect from n-3 PUFAs could hardly be discovered. Second, n-3 PUFAs supplementation might be much more effective among those with lower n-3 PUFAs levels. Thirdly, CTQ and SSRS might affect the prognostic outcomes of depression. Peng et.al found that mental disorders were associated with higher CTQ scores and lower SSRS scores (34). Childhood experiences, characters, and childhood trauma have all been shown to correlate with the occurrence of anxiety and

TABLE 4 | Comparisons between depressed patients with Childhood Trauma (MDD + CT) and depressed patients without Childhood Trauma (MDD).

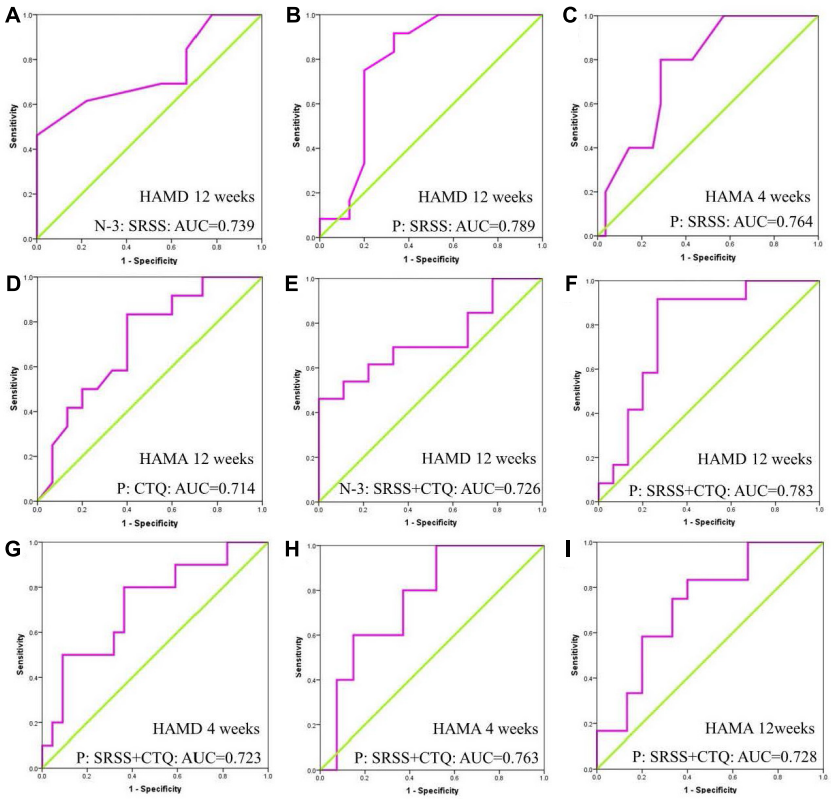
	MDD (*n* = 20)	MDD + CT (*n* = 50)	T	*P*-value
HAMA	21.12(6.09)	24.19(6.40)	1.87	0.07
SAS	42.82(9.79)	46.90(7.67)	2.11	**0.03**
HAMD	29.04(5.09)	29.34(7.49)	−0.18	0.86
BDI	24.38(8.85)	30.35(9.05)	2.49	**0.02**
EPA	0.07(0.04)	0.05(0.03)	2.26	**0.03**
DHA	0.79(0.53)	0.85(0.59)	−0.47	0.64
Total n-3 PUFAs	1.40(0.83)	1.43(0.90)	−0.15	0.88

Data were presented as MEAN(SD). HAMA, Hamilton anxiety scale; SAS, Self-rating Anxiety Scale; HAMD, Hamilton Depression Scale; BDI, Beck Depression Inventory; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; n-3 PUFAs, Omega-3 polyunsaturated fatty acids. The bold values represent the positive results.

TABLE 5 | Correlations between symptomatic index and n-3 PUFAs levels, CTQ, SSRS in patients with first-diagnosed drug-naïve depression.

	CTQ	SSRS
HAMA	**0.301 (0.012)**	0.043 (0.671)
SAS	**0.246 (0.015)**	**−0.244 (0.015)**
HAMD	**0.252 (0.038)**	−0.139 (0.169)
BDI	**0.233 (0.022)**	**−0.365 (0.000)**
EPA	**−0.238 (0.029)**	0.130 (0.232)
DHA	−0.114 (0.295)	0.037 (0.734)
Total n-3 PUFAs	−0.144 (0.185)	0.054 (0.620)

HAMA, Hamilton anxiety scale; SAS, Self-rating Anxiety Scale; HAMD, Hamilton Depression Scale; BDI, Beck Depression Inventory; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; n-3 PUFAs, Omega-3 polyunsaturated fatty acids. The bold values represent the positive results.



Parameter	Group	Graphic	AUC ± SD	P-value	95%CI	Cut-off	Sensitivity	Specificity	1-Specificity
SSRS	n-3 PUFAs -12 weeks (HAMD)	A	0.739±0.107	0.062	0.530-0.949	31.50	0.462	1.000	0
SSRS	Placebo-12 weeks (HAMD)	B	0.789±0.093	0.011	0.606-0.971	30.50	0.917	0.667	0.333
SSRS	Placebo-4 weeks (HAMA)	C	0.764±0.095	0.063	0.577-0.951	29.50	0.800	0.714	0.286
CTQ	Placebo-12 weeks (HAMA)	D	0.714±0.100	0.060	0.518-0.909	59.00	0.833	0.600	0.400
SSRS+ CTQ	n-3 PUFAs -12 weeks (HAMD)	E	0.726±0.109	0.077	0.514-0.939	0.748	0.462	1	0
SSRS+ CTQ	Placebo-12 weeks (HAMD)	F	0.783±0.092	0.013	0.602-0.965	0.394	0.917	0.733	0.267
SSRS+ CTQ	Placebo-4 weeks (HAMD)	G	0.723±0.098	0.046	0.530-0.916	0.262	0.800	0.636	0.364
SSRS+ CTQ	Placebo-4 weeks (HAMA)	H	0.763±0.100	0.065	0.567-0.959	0.107	1	0.481	0.519
SSRS+ CTQ	Placebo-12 weeks (HAMA)	I	0.728±0.098	0.045	0.535-0.920	0.369	0.600	0.400	0.385

FIGURE 3 | The ROC curve analysis of CTQ, SSRS, and combined effect for the prognosis of patients. ROC, receiver operating curve; AUC, area under the curve; SD, Standard deviation; CI, confidence interval; SSRS, Social Support Rate Scale; CTQ, Childhood Trauma Questionnaire; HAMA, Hamilton anxiety scale; HAMD, Hamilton Depression Scale; n-3 PUFAs, Omega-3 polyunsaturated fatty acids. The N-3 and P in images represent n-3 PUFAs and placebo group separately.

depressive disorder (61). Moreover, childhood trauma would increase the persistence of comorbidity and chronic disease in patients with anxiety and/or depressive disorders (62) and contribute to the severity of psychopathology (63, 64). The depressed patients who experienced childhood trauma had a poorer clinical course and therapeutic outcomes (65, 66). It was also reported that social support was associated with prognostic outcomes independent of treatment type (67). The interpersonal

behaviors of depressed patients usually cause rejection by others, and these rejection experiences in turn increase the severity of depression (68). Patients with mental disorders as lack of security are more likely to experience unsatisfactory social interactions and reduce perceived social support, which in turn will aggravate depression symptoms (68, 69).

Moreover, to confirm this possibility, we explored the relationships between these factors to understand their effect on depressive symptoms. The symptomatic index parameter like SAS, BDI, and EPA levels had significant differences between depressed patients with childhood trauma and patients without childhood trauma. Meanwhile, there were positive correlations between SAS and CTQ score, BDI and CTQ score, negative correlations between SAS and SSRS, BDI and SSRS. These results suggested that Childhood trauma and SSRS have an influence on anxiety, and depression. Besides, the results of ROC found that the combined ROC curve of CTQ and SSRS was more suitable for depressed patients without n-3 PUFAs supplementation to analyze prognostic outcomes, and suggested that the combined influence of CTQ and SSRS should be one of the factors affecting prognostic outcomes for depressed patients without n-3 PUFAs supplementation. These findings supported our previous hypothesis that the combined influence of CTQ and SSRS might be one of the reasons why supplementation with n-3 PUFAs is not effective in improving depressive symptoms. Based on the present results, it was more suitable for depressed patients without n-3 PUFAs supplementation to analyze prognostic outcomes with the combined effect of SSRS and CTQ.

Significantly negative correlations between CTQ score and EPA level at baseline, rather than DHA level, in first-diagnosed, drug-naïve depressed patients were discovered. Childhood trauma has a certain impact on the EPA level of depressed patients. There is growing evidence to support the link between depression and childhood trauma, like the existence of some biological markers that could explain the link including brain-derived neurotrophic factors and other inflammatory markers (70). Similarly, the connection between depression and EPA is also related to biomarkers (71, 72). Which suggested that EPA has a correlation with CTQ score and might be medicated through the effects of some biomarkers.

CONCLUSION

The study found that n-3 PUFAs supplementation in favor of venlafaxine alleviated the anxiety symptoms rather than depressive symptoms at the early stage of treatment for first-diagnosed, drug-naïve depressed patients. However, the advantage disappeared in the long-term treatment. Furthermore, childhood abuse and social support are closely related to the clinical and biological characteristics of depression. Both childhood trauma and lack of social support might be predictors of poor prognosis in depression.

LIMITATIONS

Due to withdrawal and non-compliance, and the influence of COVID-19, the follow-up rate was relatively low. The findings cannot be expanded to patients experiencing multiple depressive episodes or who have already received medication. While a placebo for n-3 PUFA was used, there was no placebo comparison for the antidepressant for both groups taking therapeutic doses of venlafaxine. For this case, it is impossible to distinguish the separate treatment efficacy from venlafaxine, placebo, and n-3 PUFAs alone. Furthermore, it is possible that the treatment efficacy of n-3 PUFAs on clinical characteristics may have been overshadowed by venlafaxine.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

MT and HT designed the experiments. RY, LW, and MT analyzed and interpreted the data and wrote the manuscript. MT, RY, LW, KJ, SC, and CW performed the experiments and collected the data. MT, JC, LW, and JG amended the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.876152/full#supplementary-material

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The role of vitamin D in the link between physical frailty and cognitive function: A mediation analysis in community-dwelling Chinese older adults

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Background: Physical frailty and cognitive aging have important influences on poor clinical outcomes in older adults. Many studies have investigated the association between frailty and cognitive function, but whether vitamin D mediates the association between frailty and cognitive function is unclear. We explored the mediating role of vitamin D on the cross-sectional association between physical frailty and cognitive function using data from the Chinese Longitudinal Healthy Longevity Survey (CLHLS).

Methods: We analyzed data from 1944 subjects aged 60 years and older from the 2011 CLHLS cohort. Frailty status was identified by the Osteoporotic Fracture Study (SOF) index. The Chinese version of the Mini-Mental State Examination (MMSE) was used to assess cognitive function. Linear regression models were used to examine the association between frailty, vitamin D, and cognition, adjusted for a range of covariates. Mediation analyses tested the indirect effects of vitamin D on physical frailty and cognitive function.

Result: Physical frailty was negatively associated with vitamin D levels and scores on the MMSE, and vitamin D levels were positively associated with scores on the MMSE. Linear regression analysis showed that physical frailty and serum vitamin D concentration were significant predictors of cognitive function. Importantly, mediation analysis showed that serum vitamin D concentration significantly mediated the relationship between physical frailty and cognitive function.

Conclusion: The association between physical frailty and cognitive function appears to be mediated by vitamin D. Future studies should explore whether serum vitamin D concentrations may mediate the association between physical frailty and cognitive decline and whether this mediating role is moderated by other factors.

KEYWORDS

vitamin D, physical frailty, cognitive function, mediation analysis, older adults

Introduction

Population aging continues to accelerate globally, especially in China, due to advances in health-care, public health, and social and economic development leading to increased survival rates. Between 2018 and 2050, China's population of people aged 60 and older will increase from 249 million to 478 million and from 17.9 to 35.1% of the total population (1). As the population of older adults increases, research hotspots and clinical practice are beginning to focus on physical frailty (PF), cognitive function, and their effects on functional independence in older adults (2). PF is a key intermediate state, which is a state of increased vulnerability and decreased responsiveness to stress in the aging process and is associated with multiple physiological systems gradually losing their intrinsic reserves (3–5). Although PF is an important predictor of some poor clinical outcomes, such as falls, dependence, hospitalization, disability, and death, (6) fortunately, it may be reversed or diminished by intervention (7). Another hallmark of neurological aging, cognitive aging, is characterized by deteriorating memory and reduced mental capacity and in turn leads to the development and progression of neuron-degenerative diseases (8). Although the age-related cognitive decline is a subtle and normative developmental process, it can damage many higher cortical functions, such as attention and executive function (9, 10), even increasing the risk of mild cognitive impairment and AD, and is becoming a public health concern (11, 12) Hence, PF and age-related cognitive decline directly affect physical health, increase disability, reduce the quality of life and lead to adverse consequences.

As aging is associated with physical frailty and cognitive decline, it is reasonable to understand the relationship between cognition and physical frailty. A large number of studies have verified that older adults with PF perform worse on global cognition (13, 14) and are more likely to experience cognitive decline(15–19) and cognitive impairment (20–22). In addition, there is also evidence that cognitive function can also have an impact on frailty in older people (23–25). However, few studies have explored the specific mechanisms of frailty and cognitive function. Therefore, to help maintain or improve the independent functioning and quality of life of older people, there is a need to further understand the possible mechanisms between physical frailty and cognitive function. Vitamin D, mainly synthesized in the skin during exposure to sunlight, also known as the "sunshine vitamin" and the "rickets vitamin," is a group of fat-soluble vitamins that have a role in bone and muscle health, cardiovascular disease, and even mortality (26). Although the results of some studies have not found a clear relationship between 25(OH)D and the risk of frailty (27, 28), in a large number of other studies, it can be found that older people with lower 25(OH)D levels are more likely to be frail than those with higher 25(OH)D levels (29–32). Additionally, the results of several meta-analyses also indicated that lower 25(OH)D levels were significantly associated with an increase in the severity of frailty (33–35). High levels of serum vitamin D may protect against physical frailty and reduce its occurrence (34). In addition, vitamin D (25(OH)D3), a neurosteroid hormone required for normal brain regulation and development, is strongly associated with cognitive decline and neuron-degenerative diseases (36, 37). Epidemiological evidence has identified vitamin D as a valid predictor of cognitive decline or dementia in older people (38–40). In a population-based longitudinal study, older adults with low baseline vitamin D levels were significantly associated with cognitive decline as assessed by the Brief Mental Status Examination at 2 years (36) and even with the risk of developing Alzheimer's disease (41). Previous studies have focused too much on the relationship between physical frailty and cognitive function and have rarely explored the underlying mechanisms or potential mediating factors of this association; additionally, it has seldom been explored in large epidemiological cohorts of older people. Furthermore, there is no consensus on the results of the studies available to determine the exact relationship between vitamin D and frailty and cognitive function.

Therefore, using nationally representative longitudinal survey data, the current study aimed to examine the relationship between vitamin levels, frailty status, and cognitive function among community-dwelling older adults, as well as to verify the mediating role of vitamin levels between PF and cognitive function.

Methods

Participants

Participants were recruited from the 6th (2011) wave of CLHLS, which was the first and largest national, community-based, longitudinal prospective cohort survey. The samples were randomly selected from half of the 22 counties and municipalities of the 31 provinces that make up approximately 85% of the population of China. The sampling characteristics were as follows: for each centenarian who voluntarily agreed to participate in the study, one octogenarian and one non-agenarian of predefined age and sex were randomly selected and interviewed by the CLHLS, and for every two centenarians, three nearby people aged approximately 65-79 were randomly selected. The CLHLS collected biomarkers in the longevity regions, including Xiayi County in Henan Province, Zhongxiang City in Hubei Province, Yongfu County in Guangxi Autonomous Area, Laizhou City in Shandong Province, Sanshui District in Guangdong Province, Mayang County in Hunan Province, Chengmai County in Hainan Province and Rudong County in Jiangsu Province. Therefore, it provides information on basic demographics, health status, socioeconomic characteristics, and lifestyle of the elderly (42), in addition to collecting biomarker datasets for 30 indicators

such as routine blood tests, blood biochemical tests, and urine tests (43).

More details of the CLHLS, such as the sampling design and assessment of data quality, are described at http://www.icpsr.umich.edu/icpsrweb/NACDA/studies/36179.

A total of 2,429 participants were initially enrolled in the study. In the analysis, younger ages (<60 years, $n = 16$) and missing data (cognitive function ($n = 143$), SOF index components ($n = 116$), Vitamin D3 ($n = 91$), and potential confounding variables ($n = 129$)) were excluded. Finally, we ultimately retained 1,944 older adults in this study. The study was approved by the Biomedical Ethics Committee of Peking University and Duke University, and all participants signed written informed consent forms.

Measurement

Assessment of physical frailty

The current study relied on the Study of Osteoporotic Fractures (SOF) frailty index to define physical frailty, which includes three simple self-reported components: underweight (defined as body mass index<18.5), low energy level (indicated by a positive response to the question "Over the last 6 months, have you been limited in activities because of a health problem?"), and muscle strength (inability to stand up from a chair without the assistance of arms) (44). The SOF frailty index is considered to be a useful tool in assessing the physical aspects of frailty at the population level (44–46), and frailty determined by this method is associated with falls, disability, fractures, and death (47, 48). As suggested, participants with two or more of the three components were defined as frail.

Assessment of cognitive function

Cognitive function of the CLHLS participants was measured using the Chinese version of the Mini-Mental State Examination (MMSE), which measures four aspects of cognitive function: orientation, short memory, attention and computation, recall, and language, with scores ranging from 0 to 30 (49). To truly reflect the cultural and socioeconomic conditions of the elderly in China, a Chinese version of the MMSE was created, which was adapted from the international MMSE questionnaire and has been verified in previous studies (50). The score on the MMSE is recognized as continuous data, with lower scores indicating poorer cognitive function (51).

Assessment of serum 25(OH)D

Because serum 25(OH)D reflects the source of vitamin D from sunlight exposure and diet, it is considered the best biomarker of vitamin D status. Therefore, we measured serum 25(OH)D concentrations to represent vitamin D levels(52). A detailed description of how to collect fasting venous blood and collect and transport blood samples has been published elsewhere (53). Plasma 25-hydroxyvitamin D [25(OH)D] levels were measured using an enzyme-linked immunosorbent assay (Immunodiagnostic Systems Limited, Bolton, UK), and the inter- and intra-assay coefficients of variation were <10 and 8%, respectively. The measured result was expressed in nmol/L.

Covariates

The covariates adjusted for in this study, including sociodemographic variables, health condition information, and confounding biomarkers, were obtained through structured questionnaires, physical examinations, and biomarker collections.

Sociodemographic variables included age, sex (female/male), marital status (married/other), place of residence (rural/other), and years of schooling.

Health status information is obtained through self-reporting and includes lifestyle and chronic disease status. The former included smoking (yes/no), alcohol consumption (yes/no), and exercise (yes/no), and the latter consisted of hypertension (yes/no), diabetes (yes/no), heart disease (yes/no), cerebrovascular disease (yes/no), and respiratory disease (yes/no). Hypertension was diagnosed by systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥90 mmHg (54). Diabetes mellitus was defined as fasting plasma glucose ≥7.0 mmol/L (36) Residual disease was identified by self-report.

The confounding biomarkers included indicators from both routine blood tests and blood biochemical tests and were performed by the central clinical laboratory at Capital Medical University in Beijing. The assessment of blood samples analysis was determined by using a commercial diagnostic kit (Roche Diagnostics, Germany) on an automated biochemistry machine (Hitachi 7180, Japan). Specifically, the Immunoturbidimetric assay method was used to measure C-reactive proteins (CRPS), the Cholesterol oxidase method was used to measure total cholesterol (CHO), and the picric acid method was used to test serum creatinine (CREA). The glycerol phosphate oxidase-peroxidase method was used to measure triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) by the direct method, and low-density lipoprotein cholesterol (LDL-C) by the Friedewald formula [LDL-C= TC-HDL-C-TG/2.17 (in mmol/L)]. Furthermore, the thiobarbituric acid method was used to measure malondialdehyde (MDA) and the xanthine/xanthine oxidase method was used to assay superoxide dismutase (SOD).

Statistical analysis

First, numbers and percentages were used to describe categorical variables, while means (standard deviation, SD)

or medians (interquartile range, IQR) were used to describe continuous data. ANOVA, Kruskal–Wallis test, or $\chi 2$ test were used to compare characteristics among groups.

Second, Spearman's coefficient was used to test the correlation between the variables.

Third, to determine the possible mediator role of vitamin D between physical frailty and cognitive function, we designed a mediation analysis (see Figure 1). We conducted the following analysis based on the multiple linear regression method technique proposed by Baron and Kenny (55): (1) exploring the relationship between cognitive function and physical frailty; (2) estimating the correlation between vitamin D and physical frailty; and (3) exploring the relationship between cognitive function and physical frailty following the incorporation of vitamin D. All analyses were adjusted for covariates. Finally, bootstrapping was used to assess the significance of the overall, indirect and direct utility of the mediation model. In the mediation analysis model, all paths were reported as unstandardized ordinary least squares regression coefficients, namely, total effect of X on Y (c) = indirect effect of X on Y through M (a × b) + direct effect of X on Y (c'). All statistical analyses were performed using SPSS version 21.0 (IBM, Armonk NY, USA) and the mediation effect was conducted in the Model 4 of PROCESS INDIRECT Macro 3.4. A p-value <0.05 was considered statistically significant. The 95% Cl for direct effects or indirect effects was based on a self-help sample of 5,000 and was considered statistically significant if the 95% Cl excluded zero.

Result

Participant characteristics

Table 1 shows the characteristics of the participants. Among 1,944 older adults, the mean age was 85.06 (standard deviation: 2.75) years, and 46.8% were male. Among these participants, the prevalence of PF was 22.8%. Those who were physically frail were more likely to be older and female than those who were not physically frail. The mean values of 25(OH)D3, total cholesterol, low-density lipoprotein cholesterol, and triglycerides and the number of years of education for subjects who were physically frail were significantly lower than those for non-physically frail subjects, while C-reactive proteins and superoxide dismutase were significantly higher. Smoking, drinking, and outdoor activities were more common in cognitively intact participants. The prevalence of at least one ADL limitation was higher in cognitively impaired subjects than in cognitively intact subjects. Being married, living in rural areas, smoking, drinking, exercising, diabetes, cerebrovascular diseases and respiratory diseases were more common among participants who were not physically frail. The mean score for MMSE of the frail subjects was significantly lower than that of the frail subjects.

Association between the level of 25(OH) D and the risk of frailty

Table 2 shows a significant negative association between the level of 25(OH) D and the risk of frailty. This association persisted even after adjusting for a range of covariates. In addition, in the final model, the level of 25(OH) D in the older adults without PF was 4.928 (2.777, 7.079) higher than that in those with PF.

Association between frailty and cognitive function

Table 3, the model without mediators (25(OH) D) showed that compared to non-PF subjects, frail subjects had significantly lower MMSE scores (−4.171, 95% CI = −5.013; −3.329). When including 25(OH) D in the model, even though the correlation was weakened, it was still statistically significant (−3.840, 95% CI = −4.675; −3.005).

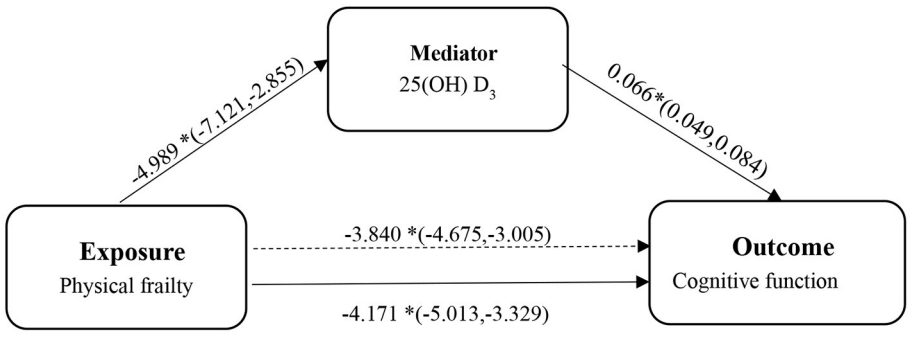

FIGURE 1
The diagram of the mediation analysis of physical frailty on cognitive function. *p < 0.05.

TABLE 1 Sample characteristics according to periodontal status.

Variables	Overall ($n = 1949$)	Physical frailty status		*P* value
		Non-PF ($n = 1500$)	PF ($n = 444$)	
Sociodemographics, n(%)				
Age,M (SD)	85.06 (12.75)	82.00 (12.04)	95.39 (9.14)	<0.001
Gender(male)	913 (46.8)	795 (52.9)	118 (26.5)	<0.001
Married	818 (42.0)	745 (49.6)	73 (16.4)	<0.001
Years of school	0 (0, 4)	0 (0, 5)	0 (0,0)	<0.001
Rural	1640 (84.4)	1266 (84.4)	374 (84.2)	0.933
Health characteristics, *n* (%)				
Current smoking	342 (17.5)	301 (20.0)	41 (9.2)	<0.001
Current drinking	327 (16.8)	295 (19.6)	32 (7.2)	<0.001
Current regular exercise	321 (16.5)	275 (18.3)	46 (10.3)	<0.001
Hypertension	518 (26.6)	419 (27.9)	99 (22.2)	0.058
Diabetes	43 (2.2)	35 (2.3)	8 (1.8)	0.028
Heart diseases	142 (7.3)	112 (7.5)	30 (6.7)	0.055
Cerebrovascular diseases	147 (7.5)	103 (6.9)	44 (9.9)	0.001
Respiratory diseases	172 (8.8)	128 (8.5)	44 (9.9)	0.012
Biomarkers, *M* (IQR)				
25 (OH) D (nmol/L)	39.55 (28.48, 54.10)	42.20 (30.89,56.47)	31.13 (23.30, 43.22)	<0.001
CRPS (mg/L)	0.89 (0.39, 2.45)	0.87 (0.39,2.25)	1.09 (0.41, 3.59)	0.004
Vitamin B_{12} (pmol/L)	346.50 (249.25, 500.00)	350.00 (256.00,503.00)	324.00 (229.50, 494.25)	0.237
CHO (mmol/L)	4.25 (3.60, 4.92)	4.27 (3.64,4.94)	4.10 (3.49, 4.76)	0.004
CREA (mmol/L)	77.00 (64.25, 92.00)	77.00 (65.25,92.00)	75.00 (61.00, 92.75)	0.739
HDLC (mmol/L)	1.24 (1.03, 1.50)	1.25 (1.03,1.50)	1.23 (1.04, 1.48)	0.900
LDLC (mmol/L)	2.5 (1.98, 3.04)	2.52 (2.00,3.06)	2.44 (1.89, 2.95)	0.039
TG (mmol/L)	0.82 (0.60, 1.17)	0.83 (0.60,1.21)	0.77 (0.59, 1.07)	<0.001
SOD (IU/mL)	57.77 (52.56, 62.56)	57.43 (52.11,62.22)	59.11 (53.96,63.54)	<0.001
MDA (umol/L)	4.80 (3.83, 5.86)	4.81 (3.87,5.89)	4.78 (3.68, 5.70)	0.757
Baseline MMSE score	28 (21,29)	28 (25, 29)	19.00 (6, 27)	<0.001

PF, physical frailty; CRPS, C-reactive proteins; CHO, total cholesterol; CREA=serum creatinine; HDLC, high-density lipoprotein cholesterol; LDLC, low-density lipoprotein cholesterol; TG, triglycerides, MDA, malondialdehyde, and SOD, superoxide dismutase;81CMMSE, the Mini Mental State Examination.

Mediating effect of 25(OH) D on the association between cognitive function and frailty

We subsequently examined whether 25(OH) D mediated the relationship between frailty and cognitive function using the PROCESS macro for SPSS. As shown in the mediation model (see Figure 1), we found that the independent variable (physical frailty) had an inverse relationship with cognitive function ($\beta = -4.171$, 95% CI $= -5.256; -3.087$). In addition, we found that physical frailty was inversely associated with (25(OH) D) ($\beta = -4.989$, 95% CI $= -7.121; -2.855$). The indirect effects showed that (25(OH) D) ($\beta = -0.331$, 95% CI $= -0.489; -0.195$) was an independent mediator of the detrimental effect of physical frailty on cognitive function.

Discussion

This is the first study to examine the possible mediating role of vitamin D on the relationship between physical frailty and cognitive function in a community elderly population in a long-lived region of China. We found a significant negative association between physical frailty and both vitamin D and cognitive function. In addition, our results suggest that the effect of frailty on cognitive function was partially mediated by vitamin D among the elderly community.

Although a number of studies have investigated the relationship between 25(OH) D levels and frailty, both cross-sectional surveys and longitudinal studies appear to have reached inconsistent conclusions. However, relevant meta-analyses have confirmed this association. One meta-analysis showed a significant association between low levels of 25(OH)

TABLE 2 The associations between physical frailty and serum levels of 25(OH) D_3 (nmol/L).

	Model 1[a**]	Model 2[b**]	Model 3[c**]	Model 4[d**]
PF	reference	reference	reference	reference
Non-PF	10.488 (8.477,12.499)	5.138 (2.988,7.287)	5.029 (2.878,7.179)	4.989 (2.855,7.122)

[a]Unadjusted model, B (95% CI);
[b]Adjusted for sociodemographics (age, sex, marital status, years of school, residence, B (95% CI));
[c]Adjusted for sociodemographics (age, sex, marital status, years of school, residence, health characteristics (smoking, drinking, regular exercise, and chronic diseases (e.g., hypertension, diabetes, heart diseases, stroke or cerebrovascular diseases, and respiratory diseases, B (95% CI));
[d]Adjusted for sociodemographics (age, sex, marital status, years of school, residence, health characteristics (smoking, drinking, regular exercise, and chronic diseases (e.g., diabetes, stroke or cerebrovascular diseases, and respiratory diseases) and confounding biomarkers (CRPS, CHO, LDLC, TG, SOD), B (95% CI))), **$P < 0.01$.

TABLE 3 The associations between physical frailty and cognitive function.

	Model 1[a**]	Model 2[b**]	Model 3[c**]	Model 4[d**]
PF	reference	reference	reference	reference
Non-PF	8.728 (7.867, 9.590)	4.270 (3.419, 5.120)	4.235 (3.387, 5.082)	4.171 (3.329, 5.013)

[a]Unadjusted model, B (95% CI);
[b]Adjusted for sociodemographics (age, sex, marital status, years of school, residence, B (95% CI));
[c]Adjusted for sociodemographics (age, sex, marital status, years of school, residence, health characteristics, (smoking, drinking, regular exercise, and chronic diseases) (e.g., hypertension, diabetes, heart diseases, stroke or cerebrovascular diseases, and respiratory diseases), B (95% CI));
[d]Adjusted for sociodemographics (age, sex, marital status, years of school, residence, health characteristics) (smoking, drinking, regular exercise, and chronic diseases) (e.g., diabetes, stroke or cerebrovascular diseases, and respiratory diseases) and confounding biomarkers (CRPS, CHO, LDLC, TG, SOD), B (95% CI), **$P < 0.010$.

D and the risk of frailty compared to high levels of 25(OH) D (33). Similarly, the results of another meta-analysis found that 25(OH) D concentrations were significantly lower in frail older people than in non-frail older people (35). As hypothesized by the study, the current findings suggest that even when frailty is identified using the SOF frailty index, levels of 25(OH) D concentrations are significantly lower in frail older adults than in non-frail older adults after adjusting for a range of covariates. Our findings are also consistent with previous studies involving older adults from Spain (56), the USA (32), Mexico (57), Italy (58), Germany (59), China (60), and the Netherlands (61). However, due to the lack of sufficient evidence to prove the effectiveness of vitamin D supplementation on elderly individuals with PF, it is necessary to further explore the role of vitamin D supplementation in the intervention treatment of elderly individuals with PF in the future.

In addition, certain factors have been identified in the literature as potential risk factors for frailty, such as chronic diseases, lifestyle, and some biomarkers (62–64).

However, after adjusting for these covariates in our study model, the association between 25(OH) D and frailty was not significantly confounded. This suggests that 25(OH) D is independently associated with the risk of frailty. Nevertheless, there are unknown factors that were not included in this study and whether these factors influence this relationship, so more comprehensive research is needed in the future.

The negative association between frailty and cognitive function has been well studied. A recent systematic review and meta-analysis of cross-sectional studies examining the relationship between physical frailty and cognitive function in older adults showed that frailty status had a significant negative effect on cognitive function, both in terms of overall cognitive function and in terms of individual cognitive domains. Furthermore, even after adjusting for age, the frailty assessment tool used and cognitive functioning status, the effect was not significantly reduced (65). Similarly, the results of this study indicate that frailty is significantly and negatively associated with cognitive function. Both physical frailty and cognitive decline are closely associated with aging, so both may have similar physiological mechanisms. Currently, available evidence suggests several mechanisms, such as increased proinflammatory states, mitochondrial dysfunction, epigenetic changes, hypothalamic-pituitary axis (HPA) dysfunction, AD pathology, hormones, nutrition, cardiovascular risk, mental health, and oxidative stress, that may be used to explain the link between physical frailty and cognitive function(24, 66, 67).

In this study, we found a positive association between the level of vitamin D and cognitive function, i.e., older adults with high levels of vitamin D concentrations in China were more likely to report higher MMSE scores than those with low levels of vitamin D concentrations. There is evidence of a protective effect of high levels of vitamin D concentrations on cognitive decline, dementia, and Alzheimer's disease in older adults (36, 41, 68, 69), which is consistent with our findings. The reason for this may be that vitamin D acts as a cognitive protector by controlling

oxidative stress, inflammation, and energy metabolism through its own receptor VDR (70, 71), but these findings were conducted in preclinical studies, therefore, the improvement effect of vitamin D supplementation is contradictory at this stage. As only a few randomized controlled trials (RCTs) have explored the effect of vitamin D supplementation on cognitive performance in older people, only two RCTs identified an improvement in cognitive function by vitamin D supplementation, and participants had cognitive impairment (69, 72). Additionally, we also found that vitamin D significantly mediated the association between physical frailty and cognitive decline. More specifically, a high level of serum vitamin D concentrations may partially counteract the effects of physical frailty on cognitive decline, a key finding in large epidemiological cohorts of older people. This study provides preliminary evidence that vitamin D supplementation appears to improve cognitive function performance in people with physical frailty. In summary, through our findings, we suggest that early monitoring of vitamin D levels in community-dwelling older adults may be warranted for better healthy aging. Second, the results of this mediated effects analysis highlight the importance of physically frail patients for the inclusion of vitamin D and cognitive performance in clinical practice. In response to the current findings, geriatrics, nutrition departments, and allied health professionals should appropriately increase their attention to vitamin D levels in the clinical practice of interventions for frail patients to intervene in cognitive decline.

The strength of the current study is that plasma blood samples were collected from participants from multiple communities in multiple regions of China, which allowed us to increase the credibility of the samples and enable our study to produce reliable results. In addition, to our knowledge, this is the first time that the relationship between vitamin D in physical frailty and cognitive function has been explored nationally in older Chinese adults.

This study also has limitations that may affect our interpretation of the findings. First, the cross-sectional study design did not allow for an examination of the causal relationship between physical frailty and cognitive decline because of the lack of temporality. Specifically, the exposure and the outcome are measured at the same point in time, rather than before the outcome occurs. Second, cognitive function was assessed *via* the MMSE scale. Because of the ceiling effect of MMSE scores, the MMSE is not a substitute for a complete final clinical diagnosis evaluation of any individual. In addition, potential confounders in this data release were collected through self-reported formats, potentially introducing bias in the analysis. Finally, although this study controlled for preexisting confounders to the extent possible, the possibility of other potential confounders cannot be ruled out.

Conclusions

In a cohort of older Chinese adults with representative longevity, frailty was found to be associated with significantly poorer cognitive performance, and frail older adults reported lower vitamin D levels. Furthermore, the association between frailty and impaired cognitive function appears to be mediated by vitamin D deficiency. Future studies should explore whether low vitamin D levels may mediate the association between physical frailty and cognitive decline and whether this mediating effect is regulated by oxidative stress, inflammation, and energy metabolism.

Data availability statement

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

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

Writing—original draft preparation and writing—review and editing: JX. Project administration: W-XX. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

Publisher's note

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Milk fat globule membrane supplementation to obese rats during pregnancy and lactation promotes neurodevelopment in offspring *via* modulating gut microbiota

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Pre-pregnancy obesity and high-fat diet (HFD) during pregnancy and lactation are associated with neurodevelopmental delay in offspring. This study aimed to investigate whether milk fat globule membrane (MFGM) supplementation in obese dams could promote neurodevelopment in offspring. Obese female rats induced by HFD were supplemented with MFGM during pregnancy and lactation. Maternal HFD exposure significantly delayed the maturation of neurological reflexes and inhibited neurogenesis in offspring, which were significantly recovered by maternal MFGM supplementation. Gut microbiota analysis revealed that MFGM supplementation modulated the diversity and composition of gut microbiota in offspring. The abundance of pro-inflammatory bacteria such as *Escherichia shigella* and *Enterococcus* were down-regulated, and the abundance of bacteria with anti-inflammatory and anti-obesity functions, such as *Akkermansia* and *Lactobacillus* were up-regulated. Furthermore, MFGM alleviated neuroinflammation by decreasing the levels of lipopolysaccharides (LPS) and pro-inflammatory cytokines in the circulation and brain, as well as inhibiting the activation of microglia. Spearman's correlation analysis suggested that there existed a correlation between gut microbiota and inflammation-related indexes. In conclusion, maternal MFGM supplementation promotes neurodevelopment partly *via* modulating gut microbiota in offspring.

KEYWORDS

milk fat globule membrane, maternal high-fat diet, neurodevelopment, gut microbiota, inflammation

Introduction

Maternal nutrition during the early life affects fetal growth and development, which programs the health status of offspring in later life (1). Maternal obesity and high-fat diet (HFD) intake increase the risk of metabolic syndromes such as obesity, insulin resistance, and hyperlipidemia in adulthood (2, 3). Maternal HFD from pre-pregnancy to lactation affects the neurodevelopment of offspring and exacerbates the occurrence of behavioral and emotional disorders (4).

The hippocampus is a key brain region responsible for learning and memory (5). The dentate gyrus (DG) of the hippocampus has a unique neurogenesis capacity, which generates new neurons *via* migration, proliferation and differentiation. Alterations in neurogenesis are associated with neurodevelopmental abnormalities and neurological disorders (6). The long-term and complex features of neurodevelopment make it highly sensitive to environmental factors, especially nutritional status in early life. Epidemiological studies revealed that pre-pregnancy overweight and obesity were correlated with lower scores for verbal recognition in children. Prenatal exposure to maternal obesity leads to lower intelligence quotients, delayed mental development and increased emotional and behavior problems (7–9). The maturation of neurological reflex behaviors such as free-fall righting and negative geotaxis in offspring were delayed by maternal HFD during lactation (10). Offspring of HFD dams were born obese, and presented impaired DG neurogenesis and hippocampal-dependent spatial cognitive function (6, 11). These studies show that maternal obesity and HFD could adversely affect the neurodevelopment of offspring.

Neurodevelopment can be substantially affected by the gut microbiota. Hippocampal neurogenesis was impaired at weaning in germ-free mice compared with normal mice, suggesting that neurogenesis could be regulated by gut microbiota (12). In the absence of gut microbiota, the main neuroimmune cells microglia were stunted and subsequently remained immature with limited immune responses to viruses and infections (13). Therefore, disturbance of the gut microbiota during development may affect neurodevelopment and adversely affect brain health in later life (14). The gut microbiota of infants is highly sensitive to disturbance of environmental factors such as dietary changes. In cohort studies, the diversity of gut microbiota altered and the abundance of *Bifidobacterium* and *Bacteroides* were decreased in the offspring exposed to maternal obesity or HFD, which had negative effects on energy acquisition and early immune development (15–17). In rodents, maternal HFD before and during pregnancy impaired the gut microbiota of both dams and offspring (18, 19). The α-diversity tended to decrease and the *Firmicutes*/*Bacteroidetes* ratio increased significantly in the gut microbiota of 2-week-old offspring from HFD dams (19). Maternal diet may affect the behavior of offspring *via* altering the gut microbiota. It was found that offspring exposed to maternal HFD exhibited severe social deficits, which was associated with changes in gut microbiota. However, postnatal supplementation with *Lactobacillus reuteri* (depleted due to maternal HFD) in offspring improved social behavior, indicating a causal link among maternal diet, gut microbiota, and neurodevelopment (20). Therefore, modulating the gut microbiota of offspring by intervention of maternal nutrition is an effective way to affect neurodevelopment of offspring.

Milk fat globule membrane (MFGM) composes of a three-layer membrane surrounding lipid droplets in milk, rich in glycoproteins and polar lipids. MFGM has shown the function of promoting infant growth and development, regulating immunity, and improving glycolipid metabolism. In a prospective, double-blind and randomized controlled trial, supplementation with MFGM narrowed the gap in neurodevelopment between infant formula-fed and breast-fed infants (21). Growth-restricted suckling mice supplemented with MFGM from birth to weaning increased cognitive scores (22). In our previous study, supplementation of milk polar lipids in obese dams enhanced offspring neurodevelopment *via* suppressing brain insulin resistance (23). These studies suggested that MFGM supplementation during the early life was beneficial to neurodevelopment. In addition, MFGM could alleviate endotoxemia by improving the gut microbiota of obese mice (24). MFGM supplementation during pregnancy and lactation ameliorated dysbiosis of obese rat dams (25), indicating that MFGM is favorable for obesity-related gut microbiota and inflammatory status. Based on these studies, the effects of MFGM supplementation to HFD-induced obese dams during pregnancy and lactation on the neurodevelopment of offspring were measured, and the corresponding changes in the gut microbiota and inflammatory responses were explored, which promoted neurodevelopment.

Materials and methods

Animals

Three to four-week-old female Sprague-Dawley rats were purchased from Beijing Vital River Laboratory Animal Technology Company Limited (Beijing, China), and were housed in the animal room of China Agricultural University under the environment of 22 ± 1°C with 12 h light-12 h dark cycle. After 1 week of acclimation, the rats were randomly divided into two groups: the control group was fed a control diet (n = 12) (10% calories from fat, D12450J, Research Diets), and the HFD group was fed a HFD (n = 12) (60% calories from fat, D12492, Research Diets) for 8 weeks. Body weight was weighed every week. Then female rats were caged with 10-week-old male rats at a ratio of 2:1 at 8:00 p.m. with free access to food and water, and the vaginal smears was examined

at 8:00 a.m. the next day. Once the vaginal smears were found, it was recorded as the first day of pregnancy. After mating, the control group of pregnant mice were randomly divided into 2 groups: one group was fed normal diet (CON, n = 6), and the other group was fed normal diet supplemented with 400 mg/kg BW MFGM (CON + MFGM, n = 6). Similarly, pregnant rats in the HFD group were randomly divided into 2 groups: one group was fed HFD (45% calories from fat, D12451, Research Diets) (HFD, n = 6), and the other group was fed with HFD supplemented with 400 mg/kg BW MFGM (HFD + MFGM, n = 6). The caloric information of diets was shown in **Supplementary Table 1**. MFGM was provided by Arla Co. (Sønderhøj, Viby J, Denmark). All groups maintained on the above diets until the end of lactation. At birth, pups were weighed and sex-determined, and litters were culled to 8 pups. Offspring were kept with their dams until postnatal day (PND) 21 (weaning). The animal study was approved by the Ethics Committee of China Agricultural University (License No. KY. 180026).

Reflex development

Righting reflex

Righting reflex was performed as previously described (26). On PND 3-7, the pups were placed on their backs on a rough wooden board. The day when pups turned over from the supine position to prone position within 5 s was recorded.

Cliff avoidance

Cliff avoidance was performed as previously reported (26). On PND 5-8, the pups were placed on the edge of a suspended plate with their nose and front paws over the edge. The day pups withdrew their nose and forepaws from the edge within 10 s was recorded.

Negative geotaxis

Negative geotaxis was performed as previously described (27). On PND 6-11, the pups were placed head down on a flat and rough wooden board with an inclination angle of 45°. The day the pups turned around and climbed up the board within 20 s was recorded.

Tissues collection and blood sampling

For offspring on PND 21, rats were fasted overnight and anesthetized with ethyl ether, and the trunk blood of the decapitated rats was rapidly collected. Blood samples were centrifuged at 1,000 g for 20 min at 4°C to obtain serum. Serum was stored at −80°C until needed. For immunohistochemical analysis, the brain was rapidly removed and fixed in 4% PFA. For western blot analysis, the brain was immediately frozen in liquid nitrogen, and stored at −80°C until needed.

Immunohistochemical examination

Immunohistochemical examination was performed as previously described (23). Coronal brain sections were sliced using a freezing microtome (Leica, Germany) and pretreated with 3% hydrogen peroxide to block endogenous peroxidase activity. The sections were incubated with 3% bovine serum albumin in PBS and were incubated overnight at 4°C with primary antibodies anti-doublecortin (Abcam, ab18723) and anti-Ki67 (Abcam, ab16667). After washing in PBS for three times, sections were incubated with a biotinylated secondary antibody (Abcam, ab205718) for 2 h at room temperature. Following another wash with PBS, sections were subjected to 3,3'-diaminobenzidine (DAB). Sections were counterstained with hematoxylin, dehydrated, and cleared in xylene. The sections were observed under the Olympus IX 73 microscope (Olympus Corporation Tokyo, Japan) and the average optical density were quantified using Image-Pro Plus 6.0 software.

16S rRNA gene sequence analysis

Fresh feces from weaned offspring were collected in dry sterile centrifuge tubes, and stored at −80°C. 16S rDNA high-throughput sequencing was conducted by Majorbio BioPharm Technology Co., Ltd. (Shanghai, China). Samples were thawed on ice, and total DNA was extracted with an E.Z.N.A. soil DNA kit (Omega Bio-Tek, Norcross, GA, United States). The DNA quality was detected by 1% agarose gel electrophoresis. The V3-V4 regions of the bacterial 16S rRNA gene was amplified with primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806 R (5'-GGACTACHVGGGTWTCTAAT-3'). PCR reactions were performed under the following program: pre-denaturation at 95°C for 3 min, 30 cycles (denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 45 s), extension at 72°C for 10 min, and then maintained at 10°C until use. The PCR products were detected by 2% agarose gel electrophoresis, and the purified and amplified fragments were used to construct a PE 2*300 library. After the library was verified, the Illumina MiSeq PE300 platform was used for sequencing according to the standard procedure. Raw fastq files were filtered and quality controlled by Trimmomatic, and spliced by FLASH. The sequences were clustered into OTUs according to the similarity of 97%, and the OTUs sequences were compared with the Silva database using RDP Classifier algorithm to analyze the taxonomic classification of gut microbiota.

Inflammatory factors analysis

Levels of LPS, IL-1β, IL-6, and TNF-α in the serum and brain were determined using ELISA assay kits according

to the manufacturer's instructions (Cusabio Life science, Wuhan, Hubei, China).

Western blot

Western blot was performed as previously described (28). Brains were weighed and homogenized in ice cold RIPA buffer (Beyotime, Shanghai, China) containing 1% protease phosphatase inhibitor (Beyotime, Shanghai, China), followed by centrifugation at 10,000 g for 15 min at 4°C to collect the supernatant. The protein concentration was determined by the BCA protein assay reagent (Tiangen Biotech, Beijing, China). The samples were stored at −20°C. After separation by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred to activated polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, United States). Membranes were blocked in 5% skim milk for 1 h at room temperature, and then incubated with primary antibodies targeting Iba1 (Abcam, ab178846, 1:1,000) and β-actin (Bioss, #bs-0061R, 1:1,000) overnight at 4°C. After being washed with TBST solution for 5 times, membranes were incubated with the horseradish-peroxidase-conjugated secondary antibody for 1 h at room temperature. The bands were visualized by enhanced chemiluminescence reagent (Millipore, Bedford, MA, United States) and quantified using ImageJ software.

Statistical analysis

Values are expressed as means ± standard error of the mean (SEM). Significant differences were determined using SPSS software (version 23.0, IBM Corp., United States). One-way analysis of variance (ANOVA) followed by *post hoc* Tukey's multiple comparison test was used for parametric analysis of variance between groups, and Student's *t*-test was used for comparing two groups. Mann-Whitney non-parametric tests were performed to determine the differences in reflex experiments. Significance was set at $P < 0.05$.

Results

Maternal milk fat globule membrane supplementation decreased body weight and promoted neurobehavioral development in high-fat diet offspring

Food intake of dams during pregnancy and lactation was shown in **Supplementary Figure 1**, and MFGM supplementation didn't influence food intake of HFD dams. The body weight of the offspring at birth and weaning was measured. As shown in **Figure 1A**, maternal HFD significantly increased the body weight of male offspring compared with that of CON offspring on PND 0 and 21. Maternal MFGM supplementation substantially decreased the body weight of offspring. Similar results were observed in the body weight of female offspring (**Figure 1B**). The body weight of female offspring born from HFD dams was higher than that of the CON offspring on PND 0 and 21 ($P < 0.01$), which was suppressed due to MFGM intervention in obese dams.

To evaluate the effect of MFGM on the development of neurological reflexes in offspring, righting reflex, cliff avoidance and negative geotaxis were performed. As shown in **Figures 1C,D**, there was no significant difference in the average age of finishing reflexes of the male and female offspring between the CON group and the CON + MFGM group, while HFD offspring had a significantly delayed time to finish righting reflex and cliff avoidance. Maternal MFGM intervention significantly promoted righting reflex development in female offspring, meanwhile there was no significant difference between the HFD + MFGM group and the CON group in male offspring. Besides, cliff avoidance occurred obviously earlier in HFD + MFGM male offspring than that in HFD male offspring, while no significant difference was observed in female offspring between HFD + MFGM group and CON group. The average age of finishing negative geotaxis displayed no differences among the four groups (**Figure 1E**). These results indicated that MFGM supplementation in obese dams during pregnancy and lactation could promote neurobehavioral development in offspring.

Maternal milk fat globule membrane supplementation promoted neurogenesis in high-fat diet offspring

To evaluate the effect of maternal MFGM supplementation on the hippocampal neurogenesis of the offspring at weaning, the positive cells of Ki-67, a marker of cell proliferation and doublecortin (DCX), a marker of newborn neurons, were determined. As shown in **Figure 2**, compared with the CON group, the optical density of hippocampal Ki-67 in the offspring of the HFD group was dramatically decreased ($P < 0.001$). MFGM supplementation in obese dams notably recovered the expression of Ki-67 ($P < 0.05$). Similarly, the optical density of the hippocampal DCX of the HFD offspring was notably decreased compared with the CON group, while this reduction was markedly prevented by maternal MFGM supplementation ($P < 0.01$). These results suggest that maternal MFGM supplementation attenuated the impairment of neurogenesis in weaned offspring from obese dams.

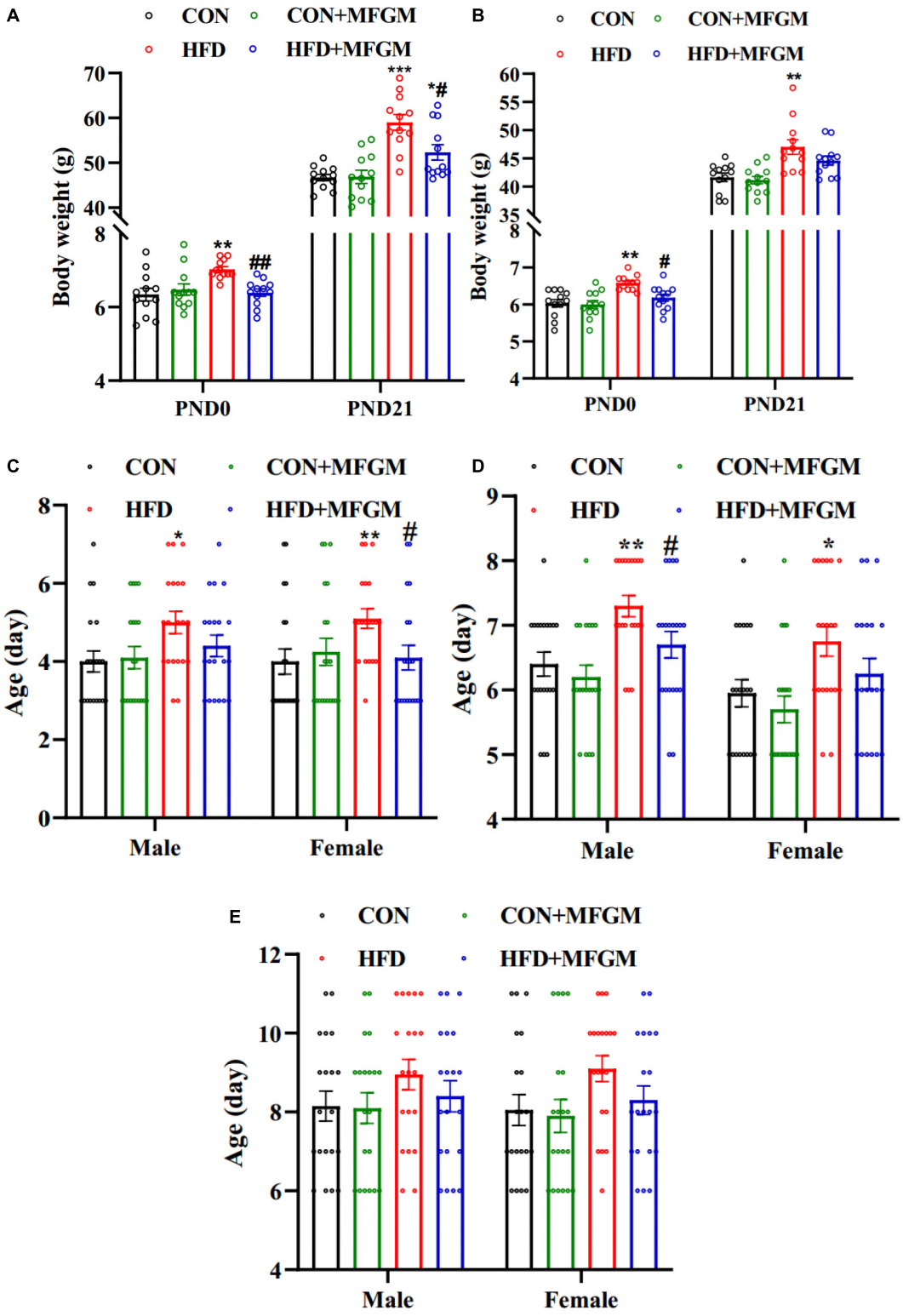

FIGURE 1
Maternal supplementation with milk fat globule membrane during pregnancy and lactation promoted neurobehavioral development in offspring from HFD dams before weaning. **(A)** Body weight of male offspring. **(B)** Body weight of female offspring, $n = 12$. The average age of reaching the criterion of right reflex **(C)** cliff avoidance **(D)** and negative geotaxis **(E)**, $n = 20$. Values are mean ± SEM. $^{*}P < 0.05$ vs. CON group. $^{\#}P < 0.05$ vs. HFD group. $^{**}P < 0.01$ vs. CON group. $^{\#\#}P < 0.01$ vs. HFD group. $^{***}P < 0.001$ vs. CON group.

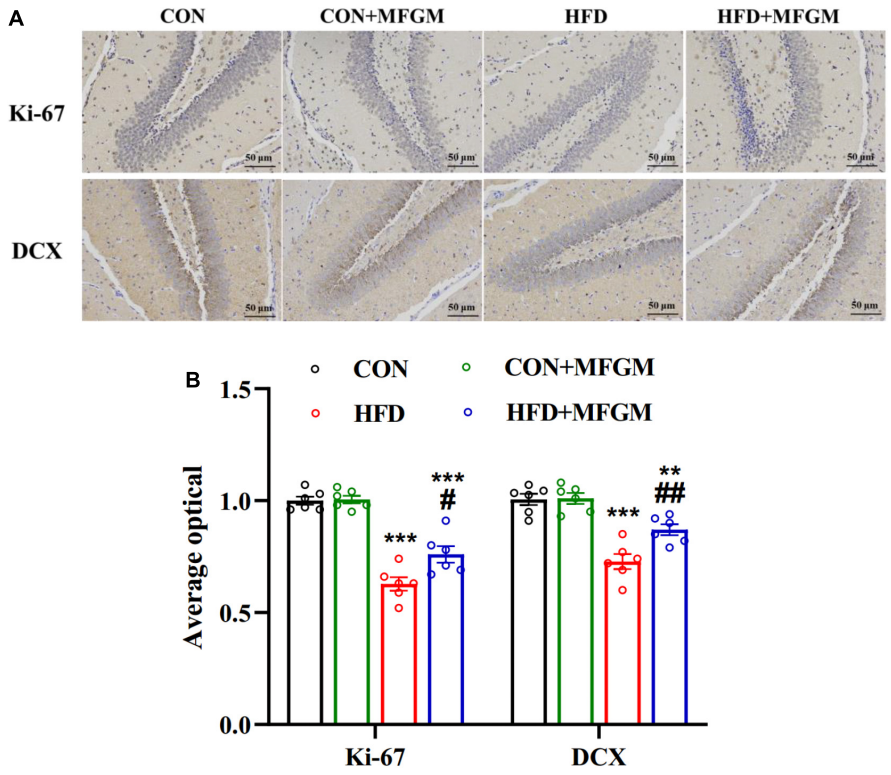

FIGURE 2
Maternal supplementation with milk fat globule membrane during pregnancy and lactation promoted neurogenesis in weaned offspring from HFD dams. **(A)** Immunohistochemical staining and average optical density value of Ki-67 and DCX in the dentate gyrus of the offspring. **(B)** Average optical density value of Ki-67 and DCX, $n = 6$. Values are mean ± SEM. $^{\#}P < 0.05$ vs. HFD group. $^{**}P < 0.01$ vs. CON group. $^{\#\#}P < 0.01$ vs. HFD group. $^{***}P < 0.001$ vs. CON group.

Maternal milk fat globule membrane supplementation modulated the diversity of gut microbiota in high-fat diet offspring

According to the PLS-DA (Figure 3A), there was a clear separation between the CON group and the HFD group, indicating that maternal HFD induced differences in the bacterial community structure of weaned offspring. Reduced gap and an overlap between HFD + MFGM and CON groups was observed, demonstrating that maternal MFGM supplementation restored the gut microbiota structure of offspring. The biological diversity within the sample was reflected by α-diversity, and the Ace index and the Simpson index were used to evaluate the community richness and diversity of the gut microbiota. Compared with the CON offspring, the Ace index of the gut microbiota in the HFD offspring did not change significantly but had a lower value, while HFD + MFGM offspring presented an increase in the Ace index ($P < 0.05$) (Figure 3B). The Simpson index in the HFD offspring was higher than that in the CON offspring ($P < 0.01$) (Figure 3C), indicating that maternal HFD reduced the species diversity of gut microbiota in weaned offspring. MFGM supplementation to HFD dams did not significantly change the Simpson index in offspring. These results indicate that maternal MFGM administration modulated the diversity of gut microbiota in HFD offspring.

Maternal milk fat globule membrane supplementation modulated the composition of gut microbiota in high-fat diet offspring

As shown in Figure 4A, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Verrucomicrobia* were the main dominant phyla in the gut microbiota of weaned offspring at the phylum level. Compared with the CON group, the relative abundance of *Proteobacteria* increased significantly in the HFD group ($P < 0.001$), while the HFD + MFGM offspring presented lower abundance of *Proteobacteria* (Figure 4B). At the genus level (Figures 4C,D), compared with the CON group, the relative abundance of *Lactobacillus* in the CON + MFGM group was significantly increased ($P < 0.05$). The relative abundance of

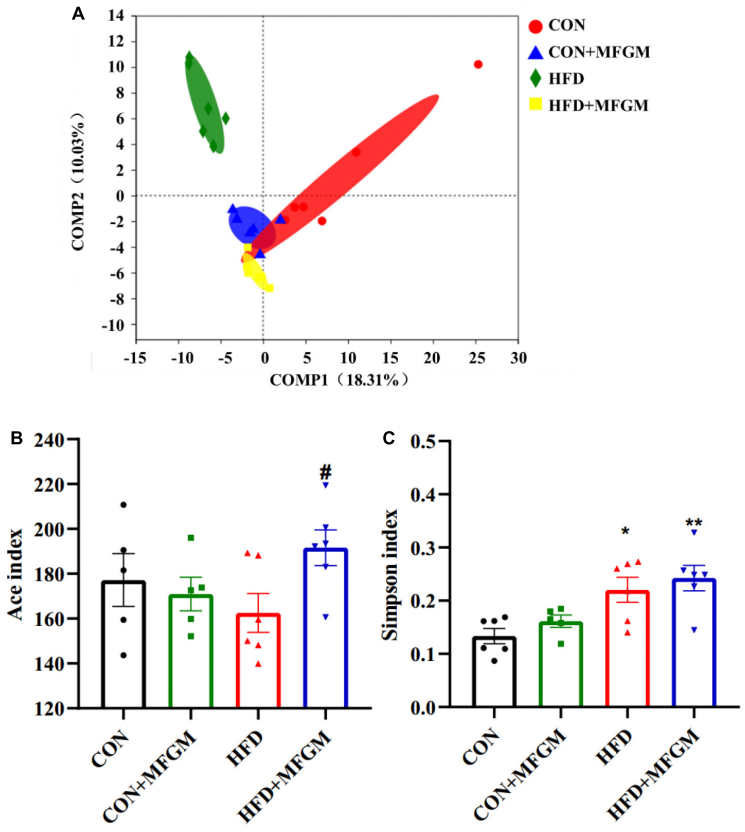

FIGURE 3
Maternal supplementation with milk fat globule membrane during pregnancy and lactation modulated the overall structure of gut microbiota in weaned offspring from HFD dams. **(A)** Principal component analysis plot of the gut microbiota at the OTU level. The α-diversity of gut microbiota depicted according to Ace index **(B)** and Simpson index **(C)** at the OTU level. Values are mean ± SEM, $n = 5–6$. $^{*}P < 0.05$ vs. CON group. $^{\#}P < 0.05$ vs. HFD group. $^{\#\#}P < 0.01$ vs. HFD group.

Lactobacillus was also higher in the HFD + MFGM offspring compared with HFD offspring ($P < 0.05$). Meanwhile, the relative abundance of *Akkermansia* was reduced by 78% in HFD offspring compared with CON offspring, while HFD + MFGM offspring presented an increase in the relative abundance of *Akkermansia*. Compared with the CON group, the relative abundance of *Escherichia shigella* and *Enterococcus* in the HFD offspring were upregulated by 181 and 154%, respectively, which was recovered by MFGM supplementation. These results suggest that maternal MFGM administration improved the composition of gut microbiota in offspring born to HFD dams.

Maternal milk fat globule membrane supplementation decreased serum pro-inflammatory factors in high-fat diet offspring

To determine whether the effects of MFGM on the gut microbiota could improve inflammation in HFD offspring, the levels of serum pro-inflammatory factors of the weaned offspring were measured firstly. As shown in Table 1, compared with the CON group, the serum levels of IL-1β, IL-6, TNF-α, and LPS were up-regulated by 50, 33, 11, and 46%, respectively, in HFD offspring, which were markedly reversed by MFGM intervention.

Correlation between gut microbiota and serum pro-inflammatory factors

To examine the correlation between gut microbiota and inflammatory responses, Spearman's correlation analysis was used to calculate the correlations of the top 30 most abundant bacteria at the genus level with serum inflammation-related parameters (Figure 5). The heatmap reflected significant negative correlations between serum pro-inflammatory factors (LPS, IL-1β, IL-6, and TNF-α) and *Lactobacillus*, *Blautia*, *Akkermansia*, and *norank_f_Muribaculaceae*, indicating that these bacteria may be beneficial for the alleviation of inflammatory response in offspring. Serum pro-inflammatory factors were positively correlated with *Escherichia-Shigella*, *Enterococcus*, *Parabacteroides*, *Tyzzerella_4*, *Ruminococcus_gnavus_group*,

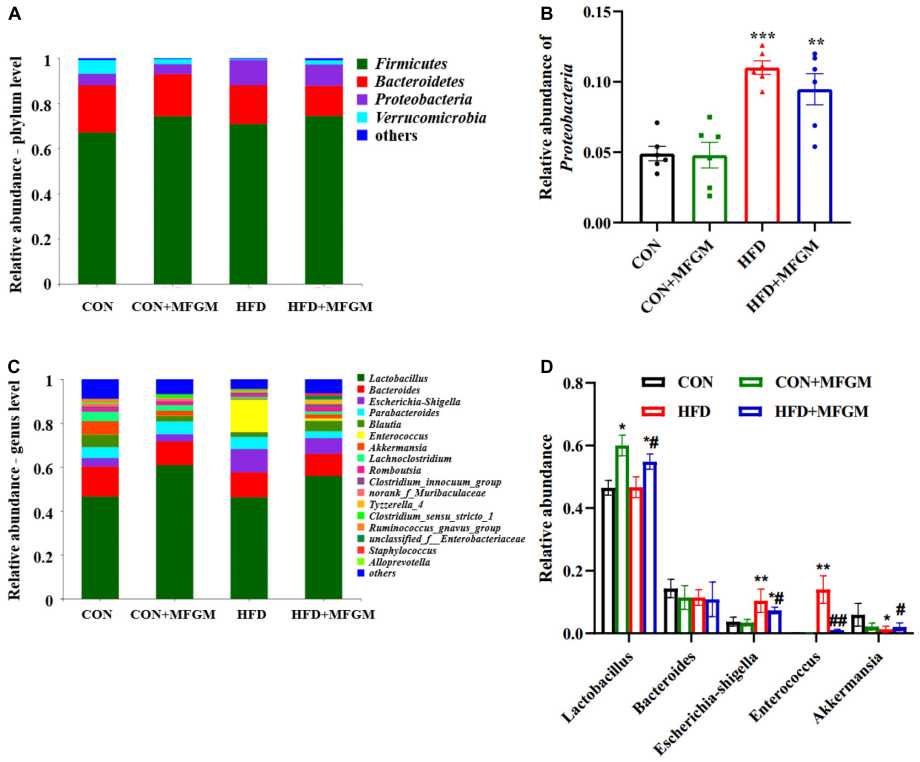

FIGURE 4
Maternal supplementation with milk fat globule membrane during pregnancy and lactation regulated the composition of gut microbiota in weaned offspring from HFD dams. **(A)** Relative abundance of gut microbiota at the phylum level. **(B)** Relative abundance of *Proteobacteria*. **(C,D)** Relative abundance of gut microbiota at the genus level. Values are mean ± SEM, n = 5–6. $^{*}P < 0.05$ vs. CON group. $^{\#}P < 0.05$ vs. HFD group. $^{**}P < 0.01$ vs. CON group. $^{\#\#}P < 0.01$ vs. HFD group. $^{***}P < 0.001$ vs. CON group.

TABLE 1 Maternal supplementation with milk fat globule membrane during pregnancy and lactation alleviated serum levels of inflammatory factors in weaned offspring from HFD dams.

	IL-1β (pg/mL)	IL-6 (pg/mL)	TNF-α (pg/mL)	LPS (EU/L)
CON	15.64 ± 1.54	79.86 ± 3.72	86.42 ± 3.62	355.45 ± 18.98
CON + MFGM	15.82 ± 1.70	82.33 ± 3.19	87.92 ± 2.27	328.69 ± 30.32
HFD	31.15 ± 0.95***	106.51 ± 2.31***	95.73 ± 2.84**	519.09 ± 40.99***
HFD + MFGM	23.06 ± 1.27***###	86.99 ± 3.58###	90.52 ± 1.55#	422.63 ± 12.89***##

Values are mean ± SEM, n = 6.
#P < 0.05 vs. HFD group. **P < 0.01 vs. CON group. ##P < 0.01 vs. HFD group. ***P < 0.001 vs. CON group. ###P < 0.001 vs. HFD group.

and *unclassified_f_Enterobacteriaceae*, indicating that these bacteria may be involved in the inflammatory state of HFD offspring.

Maternal milk fat globule membrane supplementation alleviated neuroinflammation in high-fat diet offspring

Circulating inflammatory factors could impair and cross the blood-brain barrier to induce neuroinflammation. To further investigate the effects of MFGM on the neuroinflammation in HFD offspring, the levels of brain pro-inflammatory factors and the protein expression of microglia marker Iba1 were analyzed. As shown in Figures 6A–D, compared with the CON offspring, the brain levels of IL-1β, IL-6, TNF-α, and LPS were notably increased in HFD offspring. The brain levels of IL-6, TNF-α and LPS were obviously down-regulated by MFGM intervention, and the value of IL-1β was lower than HFD group in HFD + MFGM group, suggesting that MFGM administration to HFD dams could attenuate cerebral inflammatory response. Furthermore, microglia activation is a marker of neuroinflammation. In order to

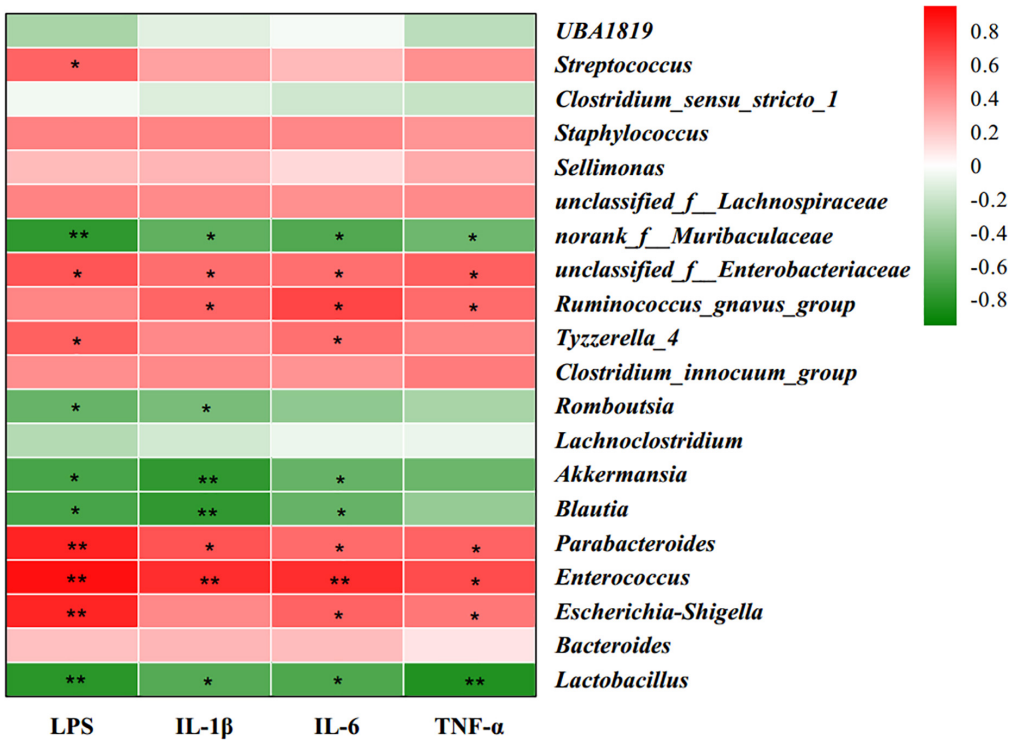

FIGURE 5
Heatmap of Spearman's correlation between gut microbiota (the 30 most abundant species at the genus level) and serum inflammatory factors. Red color represents a positive correlation, while green color represents a negative correlation. $n = 6$. *$0.01 < P \leq 0.05$, **$0.001 < P \leq 0.01$.

explore the effects of maternal MFGM supplementation on microglia activation of weaned offspring, the protein expression of Iba1 was measured (Figure 6E). Compared with the CON group, the protein content of Iba1 in the brain of HFD offspring was significantly increased, which was recovered by maternal MFGM intervention. These data showed that MFGM can alleviate neuroinflammation in HFD offspring. The correlation analysis identified significant negative correlations between *Lactobacillus*, *Blautia*, *Akkermansia*, and *norank_f_Muribaculaceae* and Iba1, and positive correlations between *Escherichia-Shigella*, *Enterococcus*, *Parabacteroides*, *Tyzzerella_4*, and *unclassified_f_Enterobacteriaceae* and Iba1, indicating that gut microbiota could modulate neuroinflammation of HFD offspring (Figure 6F).

Discussion

The development of neurological reflexes is an efficacious and reliable indicator of the neurodevelopment of pups. Different reflex behaviors during development can reflect the maturity of the nervous system and physical development. For example, righting reflex measures the development of muscle and motor function, cliff avoidance reflects the sensory-motor function, and negative geotaxis evaluates the maturation of cranio-caudal coordination (29, 30). Nutritional status in early life is a major determinant of neurodevelopment. Maternal HFD during pregnancy and lactation adversely affect the neurodevelopment of offspring, as evidenced by delayed maturation of physiological reflexes (31). Dietary supplementation of velvet antler in dams improved the acquisition of righting reflex, cliff avoidance and negative geotaxis in offspring (32). Supplementation with MFGM in infant formula-fed pups narrowed the gap in the maturation age of cliff avoidance and negative geotaxis compared with breast-fed pups (33). Consistent with these findings, the present study proved that exposure to maternal HFD delayed the maturation of righting reflex and cliff avoidance, while maternal MFGM administration during pregnancy and lactation could restore the development of neurological reflexes in the offspring, suggesting that MFGM is beneficial to neurodevelopment.

Neurogenesis in the hippocampus, including cell proliferation and cell survival, is critical for neurodevelopment. New neurons in the subgranular layer of the DG continuously generate, maturate, and integrate functionally into existing neural circuits, thereby promoting neurodevelopment and behavioral reflexes. Maternal nutrition affects hippocampal neurogenesis in offspring during early development. Maternal HFD impaired neurogenesis during offspring hippocampal development (6). Maternal choline supplementation partially

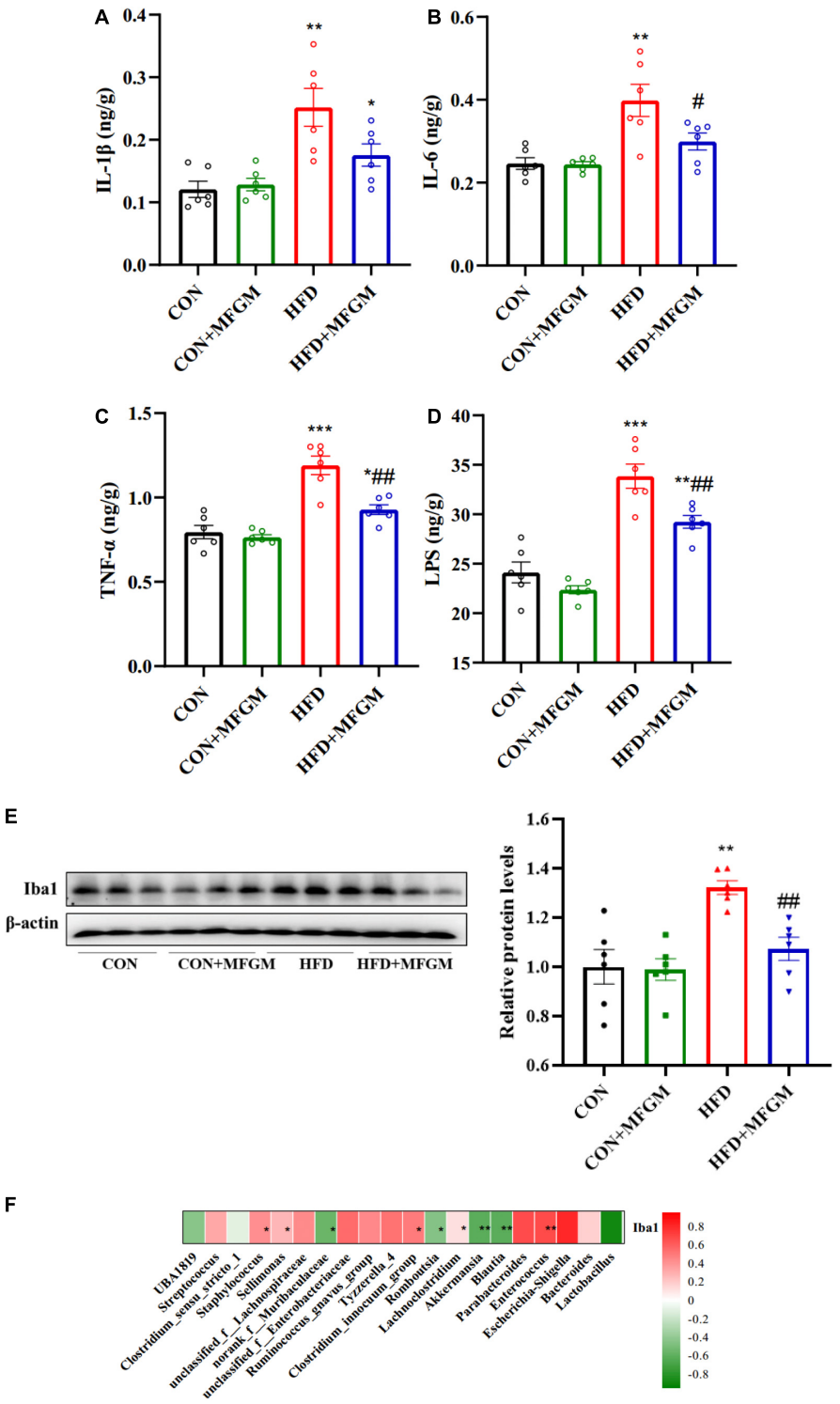

FIGURE 6
Maternal supplementation with milk fat globule membrane during pregnancy and lactation alleviated neuroinflammation in weaned offspring from HFD dams. IL-1β **(A)**, IL-6 **(B)**, TNF-α **(C)** and LPS **(D)** levels in the brain. **(E)** Representative image and relative quantitative analysis of Iba1 in the brain by Western blot. **(F)** Heatmap of Spearman's correlation between gut microbiota (the 30 most abundant species at the genus level) and Iba1. Values are mean ± SEM, $n = 6$. $^{*}P < 0.05$ vs. CON group. $^{\#}P < 0.05$ vs. HFD group. $^{**}P < 0.01$ vs. CON group. $^{\#\#}P < 0.01$ vs. HFD group. $^{***}P < 0.001$ vs. CON group.

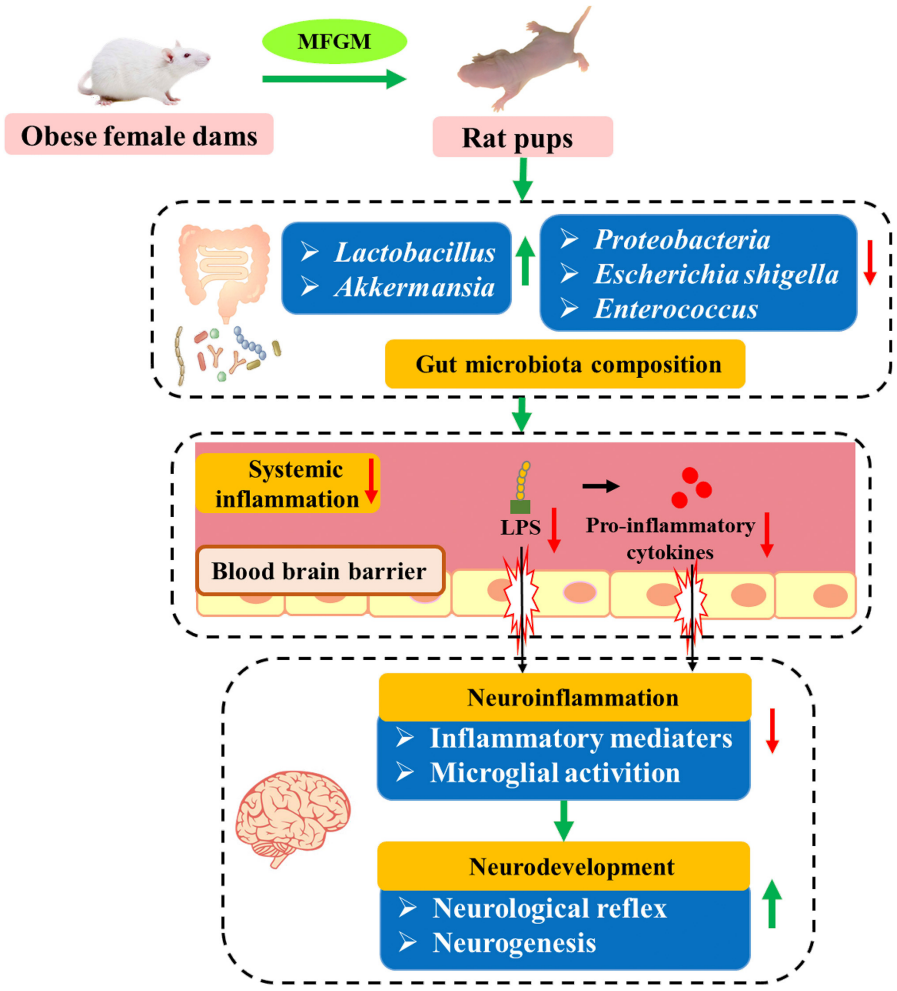

FIGURE 7
Possible mechanism for the beneficial effect of maternal MFGM supplementation on the neurodevelopment of offspring.

normalized neurogenesis in the offspring with Down syndrome (34). In the present study, maternal MFGM supplementation restored maternal HFD-induced reduction in the neurogenesis of weaned offspring, as evidenced by elevation of Ki-67 and DCX positive neurons, contributing to improved neurodevelopment. MFGM contains abundant polar lipids including phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), and gangliosides. Polar lipids and their metabolites such as long-chain unsaturated fatty acids and choline can be transmitted to offspring through placenta and breast milk and promote neurodevelopment (35–37). Maternal LC-PUFAs and choline supplementation were related to better memory and intelligence quotient in children (38, 39). Maternal supplementation with complex milk lipid during pregnancy and lactation increased ganglioside level in the brain of offspring, which was important for neurogenesis (40). Therefore, abundant polar lipids in MFGM contributed a lot to the beneficial effects of MFGM on neurodevelopment.

The mechanism of neurodevelopment is complex and can be regulated by multiple pathways. In recent years, communications between gut microbiota and brain have attracted a lot of attention. Based on the role of gut microbiota in neurogenesis and microglia maturation demonstrated in germ-free animals, disturbances of gut microbiota during development may affect neurodevelopment (14). Human studies showed that the composition of gut microbiota in infants was related to the diet and weight of mothers during pregnancy, and the β-diversity and α-diversity of gut microbiota in children were altered by maternal obesity (15). Meanwhile, the concentrations of pathogenic bacteria were elevated in infants of overweight mothers (16), which were associated with the occurrence of necrotizing enterocolitis in infants (41). Therefore, maternal obesity may have a negative

impact on early immune development. *Akkermansia*, novel beneficial bacteria, was higher in lean children compared with overweight/obese children. *Lactobacillus* abundance is positively correlated with memory (42), and supplementation of omega-3 fatty acids in pregnant mice increased fecal levels of *Lactobacillus*, thereby enhancing social and cognitive function (43). *Escherichia shigella* was a leading cause of diarrhea in children and was inversely associated with cognitive scores (44). Preterm newborns with gut microbiota dominated by *Enterococcus* were significantly associated with death after 4 weeks of age or the neurodevelopment at 2 years of age (45). In this study, the overall structure of gut microbiota in offspring was changed due to maternal HFD. The abundance of *Escherichia shigella* and *Enterococcus* were up-regulated, and *Akkermansia* was down-regulated in the weaned offspring of obese dams. However, maternal MFGM intervention could significantly reverse these changes. Of note, there was no significant change in the *Lactobacillus* abundance of HFD offspring, which was increased by MFGM intervention in both control and obese dams in the present study. Therefore, the modulation of MFGM on gut microbiota might promote the development of immune and cognition in offspring.

Maternal diet could affect the gut microbiota of offspring through vertically transmitted to the offspring during delivery, thus diet-induced changes in the gut microbiota of the mothers can directly affect the colonization of the gut microbiota of offspring (46). A previous study has found that administration of polar lipids-enriched MFGM in obese dams during pregnancy and lactation could restore the ratio of *Firmicutes* to *Bacteroides*, reduce the relative abundance of *Ruminococcaceae* and *Enterococcus*, and increase the relative abundance of *Akkermansia* (25), which were similar to the changes in the gut microbiota of offspring observed in the present study. Therefore, the beneficial effect of MFGM on the gut microbiota of offspring may be attributed to the improvement of the gut microbiota of dams. The benefits of MFGM in regulating gut microbiota may be attributed to its components and their metabolites. Dietary milk SM altered gut microbiota composition in HFD mice, with significantly reduced relative abundance of Gram-negative phyla, such as *Bacteroidetes* and *Tenericutes*, and the major digestion products of SM, sphingosine, exhibited strong antimicrobial properties against pathogenic bacteria *in vitro* (47, 48). Ethanolamine, which is the base constituent of PE, was found to be helpful for the development of infant intestine by improving intestinal antioxidant capacity, promoting intestinal cell differentiation and altering gut microbiota (49). Gangliosides reduced the relative content of *Escherichia coli* in preterm newborn infants and increased fecal *Bifidobacteria* counts (50). Therefore, the regulatory effect of MFGM on the gut microbiota of offspring may be due to simultaneous effects on both dams and offspring.

Microbiota shifts are well known to be associated with inflammation. LPS from the cell wall of Gram-negative bacteria in the gut microbiota is an important cause of systemic low-grade inflammation. LPS can activate the immune system through toll-like receptor 4 and downstream inflammatory signaling molecules, thereby promoting the production of inflammatory factors including IL-1β, IL-6, TNF-α (51). Exposure to maternal chronic low-grade inflammation induced by long-term HFD during pregnancy and lactation could also negatively affect the serum cytokine levels of offspring (52). In this study, offspring of obese dams had high levels of serum LPS and inflammatory factors at weaning, while MFGM intervention suppressed the inflammatory state of the offspring. Gut microbiome profile was linked to the inflammatory state of the host. For example, *Lactobacillus* could repair the intestinal mucosal barrier and prevent LPS and harmful bacteria from entering the circulation through the intestinal epithelium (53). *Muribaculaceae*, which produce butyrate, is associated with the degradation of complex carbohydrates, the formation of mucus layer in the colon and the improvement of barrier function (54). Moutan cortex polysaccharides up-regulated the abundance of *Lactobacillus* and *Muribaculaceae_unclassified*, thus improving intestinal barrier and inflammatory response in diabetic rats (55). In addition, overweight during pregnancy reduced the abundance of *Blautia* in the gut microbiota of newborns (56), which was associated with the deterioration of intestinal inflammation and metabolic phenotype in obese children (57). Except for the reduction in anti-inflammatory bacteria, the increase in pro-inflammatory bacteria also contributes to the inflammation. *Enterococcus* in infants fed with infant formula was significantly higher than that in breast-fed infants, which was a leading cause of sepsis in infants (58). Through correlation analysis in the present study, we found that *Lactobacillus*, *norank_f_Muribaculaceae*, *Akkermansia*, *Blautia*, *Escherichia-Shigella*, and *Enterococcus* were significantly correlated with the change of inflammation in offspring, demonstrating that maternal MFGM supplementation improved the inflammation of offspring *via* regulating gut microbiota.

Cytokines in circulation induced by LPS could damage the blood-brain barrier *via* binding to the endothelial receptors and releasing pro-inflammatory mediators (59). Subsequently, LPS and pro-inflammatory cytokines enter the brain, inhibit the phagocytosis of microglia and stimulate the production of pro-inflammatory cytokines, thereby contributing to microglia activation and the occurrence of neuroinflammation (60). In the present study, since the changes in inflammation-related bacteria and the improvement of gut microbiota in systemic inflammation have been verified, the effect of MFGM on neuroinflammation was further analyzed. Rats fed HFD from 4 weeks before mating to the end of lactation increased the expression of hippocampal microglial activation marker Iba1 in offspring at birth, and hippocampal IL-1β levels at weaning and adulthood were significantly higher than those in the control offspring (61). Consistent with this study, in our study, elevated inflammatory mediators

and microglial activation were observed in HFD offspring, which were alleviated by maternal MFGM supplementation. According to previous reports, gut microbiota could regulate the maturation and immune response of microglia through metabolites such as short-chain fatty acids, thus modulating neurodevelopment (62). Meanwhile, changes in some bacteria have been proved to be associated with neuroinflammation and cognition. *Lactobacillus plantarum* supplementation improved memory impairment in Alzheimer's disease mice *via* decreasing *Enterobacter* abundance and increasing *Lactobacillus* and *Bifidobacterium* abundance, reducing LPS levels in blood and feces, and inhibiting microglial activation (63). Early life HFD could damage neurodevelopment of mice and significantly reduce the abundance of *Akkermansia*, while supplementation of *Akkermansia* significantly reduced the activation of microglia and the expression of pro-inflammatory cytokines, thereby improving learning and memory ability (64). Increased *Escherichia Shigella* abundance was associated with peripheral inflammatory states in patients with cognitive impairment and brain amyloidosis (65). In the present study, the correlation between gut microbiota and neuroinflammation was further confirmed by Spearman's correlation analysis, and bacteria including *Lactobacillus*, *Akkermansia*, *Escherichia-Shigella*, and *Enterococcus* were significantly correlated with the change of microglial activation in offspring, suggesting that MFGM could improve neuroinflammation at least partially *via* modulating gut microbiota.

Conclusion

In summary, supplementation of MFGM to HFD-induced obese dams during pregnancy and lactation reduced postnatal body weight of offspring, promoted the maturation of neurological reflexes and hippocampal neurogenesis in the offspring. MFGM modulated the diversity of gut microbiota, downregulating the abundance of pro-inflammatory bacteria such as *Escherichia shigella* and *Enterococcus*, and upregulating the abundance of bacteria with anti-inflammatory and anti-obesity functions, such as *Akkermansia* and *Lactobacillus*. MFGM also reduced the levels of LPS and pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) in the serum and brain tissue of the offspring, and inhibited the expression of microglial activation marker Iba1, which were beneficial to reducing the neuroinflammation of offspring. The correlation between the changes in the gut microbiota and inflammation was further verified. Therefore, gut microbiota-mediated reduction of inflammatory response was the potential mechanism by which MFGM stimulated neurodevelopment (**Figure 7**). These findings provide new evidence of the MFGM as an effective functional component for neurodevelopment in early life.

Data availability statement

The raw data of 16S rRNA gene sequence analysis can be found online at: https://www.ncbi.nlm.nih.gov/sra/PRJNA847149.

Ethics statement

The animal study was reviewed and approved by the Ethics Committee of China Agricultural University.

Author contributions

QY, HG, and XM contributed to the conception and design of the study. QY and HG conducted experiments and analyzed the data. QY wrote the manuscript. MD, TL, and XM revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.945052/full#supplementary-material

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Kynurenic acid as a biochemical factor underlying the association between Western-style diet and depression: A cross-sectional study

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Consumption of a Western-style diet (WS-diet), high in saturated fat and added sugar, is associated with increased depression risk. However, the physiological mechanisms underlying the relationship requires elucidation. Diet can alter tryptophan metabolism along the kynurenine pathway (KP), potentially linking inflammation and depression. This study aimed to examine whether urinary inflammatory markers and KP metabolites differed according to WS-diet consumption and depression severity. Depression symptoms and habitual WS-diet consumption were assessed in 169 healthy adults aged 17–35 recruited from two experimental studies. Targeted metabolomics profiling of seven KP metabolites, ELISA-based assays of interleukin-6 (IL-6) and C-reactive protein (CRP) were performed using urine samples collected from the participants. Parametric tests were performed for group comparison and associations analysis. Multilevel mixed-effect modelling was applied to control for biases. Higher intake of WS-diet was associated with lower levels of neuroprotective kynurenic acid (KA; $R = -0.17$, $p = 0.0236$). There were no differences in IL-6 or CRP across diet groups ($p > 0.05$). Physical activity had negative associations with most KP metabolites. Mixed-effects regression analysis showed the glutamatergic inhibitor, KA, was the only biomarker to have a significant association with depression symptoms in a model adjusted for demographic and lifestyle variables: a unit increase in KA was associated with 0.21 unit decrease in Depression Anxiety and Stress Scale-21 depression score ($p = 0.009$). These findings suggest that urinary KA is associated with both habitual WS-diet intake, and levels of depression symptoms, independent of inflammation. Findings support the role of neuroprotection and glutamatergic modulation in depression. We propose that KA may act

as endogenous glutamatergic inhibition in regulating depression severity in the absence of inflammation. Further comparison with blood-based markers will assist in validating the utility of non-invasive urine samples for measuring KP metabolites.

KEYWORDS

Western-style diet (WSD), depression, kynurenic acid (KA), kynurenine pathway (KP), tryptophan metabolism, glutamatergic modulation, nutritional psychiatry

Introduction

Depression, a common mental disorder, is a leading cause of disability and major contributor to overall burden of disease globally (1). Its onset is typically around mid- to late- adolescence and it is a major risk factor for suicide, which is the second leading cause of death in young adults. Depression is commonly managed with psychological interventions, which can be costly, and although various medications are available, many individuals experience unpleasant side effects or are treatment-resistant (2). There is a need for low-cost, low-risk interventions that can target lifelong modifiable risk factors for depression (3).

Diet is a modifiable risk factor for depression, offering a promising target for treatment and prevention. Consumption of Western-style diet (WS-diet), high in processed foods, saturated fat, and added sugars, is associated with increased risk of depression, whereas conversely, a healthy diet pattern is associated with reduced risk of depression (4). Randomised controlled trials have demonstrated that improvement in diet reduces clinical depression (5) and depression risk (6). In young adults, we showed that a brief dietary intervention for 3 weeks, which involved increasing intake of fruit, vegetables, and complex carbohydrates, while reducing the consumption of processed foods, improved depression symptoms in young adults (7).

Although this accumulating evidence suggests dietary intervention may be an effective adjunct treatment for depression, there remains a need to understand the mechanisms underlying the relationship. There are several putative biochemical mechanisms that link diet quality and depression. A compelling theory is that WS-diet can alter the gut microbiota, influencing neurotransmitter metabolism, which can impact on brain function (8). This places tryptophan metabolism as an attractive target because of its multi-factorial role in diet, gut, and brain function. Tryptophan is an essential amino acid that is solely acquired through diet in vertebrates. The metabolism of tryptophan has been known for its important role in the regulation of many physiological functions, including immune activity, neurotransmission, mood, and behaviour (9). Catabolic routes of tryptophan *via* the serotonin-melatonin pathway and the kynurenine pathway (KP) produce neuroactive metabolites such as serotonin, kynurenic acid (KA), and quinolinic acid (QA) that alter serotonergic and glutamatergic neurotransmission, both of which are implicated in depression (**Figure 1**). Recent evidence suggests a strong interplay between tryptophan metabolism and the intestinal microbiota and host (10), and this relationship has been identified in people with depression and anxiety (11).

Another established theory, known as cytokine-induced sickness behaviour [see review (12)], involves inflammatory processes which can lead to increased production of pro-inflammatory mediators such as C-reactive protein (CRP) and interleukin (IL)−6. Again, the kynurenine-tryptophan pathway may be involved, as these pro-inflammatory mediators are strong inducers of the first enzyme in the KP, indoleamine 2,3 dioxygenase (IDO-1), which in turn activates the KP and alters tryptophan metabolism. Increased activation of IDO-1 can lead to decreased serotonin, thereby depleting the mood modulatory neurotransmitter [see serotonin hypothesis in review (13)], while increasing the *N*-methyl-D-aspartate (NMDA) agonist, QA, to exert an imbalance in (hypo)serotonergic and (hyper)glutamatergic neurotransmissions in depression. Moreover, WS-diet can increase pro-inflammatory cytokines, to drive this phenomenon (14). Taken together, it is possible that WS-diet can induce inflammatory processes that alter the host KP metabolism leading to the dysregulation of glutamatergic neurotransmission and depressive symptoms.

The above findings show it is important to gain greater understanding of the role of altered tryptophan metabolism down the KP in altering mood *via* dietary interventions (15, 16). Diet manipulations targeted at depression, including ketogenic and fasting diets, have been shown to modulate tryptophan-KP metabolism (17, 18). Further, individual diet components such as curcumin, resveratrol, black and green tea are associated with reduced depression symptoms and are also implicated in KP metabolism (19–22). High fat diets are associated with imbalanced intestinal flora, thereby affecting tryptophan metabolism (23), and supplementation with probiotics can modulate the KP activity (24, 25). Hence, tryptophan metabolism is a potential underlying physiological mechanism by which dietary intervention has the potential to improve mood.

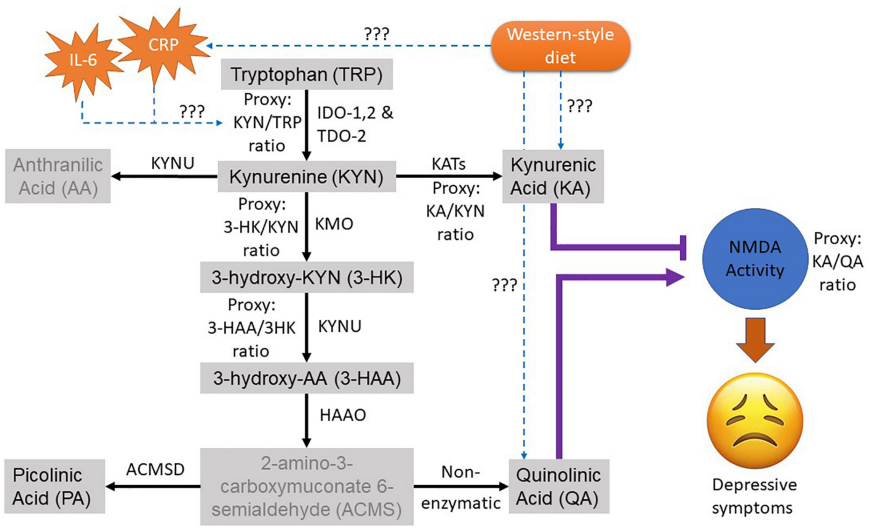

FIGURE 1
Overview of the kynurenine pathway (KP) of the tryptophan metabolism. The black arrow shows the natural route of the KP metabolism. Greyed metabolites indicate not measured in this study. The purple lines indicate the role of neuroactive KP metabolites on the glutamatergic NMDA activity where (T) indicates antagonistic and arrow indicate agonistic effect. The dotted blue arrow depicts the pathological interaction of interest with its effectors to be examined in this study. The ratio of the metabolites was used as proxies for their corresponding KP enzyme activities. CRP, C-Reactive protein; IL-6, interleukin-6; TRP, tryptophan; KYN, kynurenine; AA, anthranilic acid; KA, kynurenic acid; 3HK, 3-Hydroxykynurenine; 3HAA, 3-Hydroxyanthranilic acid; PA, Picolinic acid; QA, Quinolinic acid.

We have previously conducted two randomised controlled trials that examined the relationship between diet quality and severity of depression in young adults, where baseline measures included fat and sugar intake, depression symptoms and a urine sample (amongst other measures). This offered the opportunity to measure KP metabolites, and immune markers in the urinary samples collected at baseline. We hypothesised that WS-diet intake is associated with proinflammatory mediators, CRP and IL-6, which in turn is associated with aberrant KP metabolism. Specifically, with regard to altered KP metabolism, we predicted decreased levels of tryptophan and/or increased kynurenine with a downstream consequence of enhanced glutamatergic activity defined by increased NMDA agonist, QA, and/or decreased NMDA antagonist, KA. We further hypothesised that these changes in the biochemical measures would be associated with the severity of depression symptoms. Using statistical modelling, we were able to examine the relationship between WS-diet intake and individual differences in inflammation and KP metabolism relating to the severity of depression in our sample of healthy young adults.

Materials and methods

Participants and study design

Participant data were utilised from the baseline testing session of two separate studies where we obtained diet quality information using the Dietary Fat and Sugar Screener (DFS) and level of depression symptoms using the Depression, Anxiety and Stress Scale-21 (DASS-21). There were differences in the selection criteria for each study (outlined below), which reflected the overall aims. Study 1 (n = 100) aimed to recruit individuals who habitually consume a WS-diet and with elevated depression symptoms (7), whereas Study 2 (n = 73) aimed to recruit individuals with healthy diet and non-elevated levels of depression symptoms (26). By combining the baseline testing data from the two studies, we were able to produce an overall sample with variation in dietary fat and sugar intake and DASS-21-depression (DASS-21-D) symptoms. Participants from both studies were either recruited from an undergraduate psychology course, and participated for course credit, or *via* advertisement on campus and surrounds and participated for cash reimbursement.

Selection criteria

Study 1 inclusion criteria: aged 17–35 years, score of $\geq$ 7 on the DASS-21-D, which corresponds with moderate or higher depression symptoms (27), and a score of $>$ 57 on the Dietary Fat and Sugar Screener (DFS), with scores $>$ 57 representing higher intake of WS-diet that does not comply with the Australian Guide to Healthy Eating (28). If receiving antidepressant medication or psychological therapy, participants were required to be on the same treatment for at least 2 weeks before study participation. Study 2 inclusion criteria: aged 17–35 years, body mass index (BMI) between

17 and 26, DFS score < 57, and an overall depression score below 25 (27).

Exclusion criteria for both studies were similar but with slight differences. Study 1 exclusion criteria: pregnancy, currently dieting, history of eating disorders or metabolic disease(s), history of psychological illness other than depression or anxiety, medical condition that could be adversely affected by diet change, poor proficiency in English, recent illicit drug use, or sickness in the past week. Study 2 exclusion criteria: pregnancy, current/past metabolic, neurological or psychiatric illnesses, food allergies, vegan/vegetarian, non-pork eater, currently dieting, recent significant diet change, prescription medication use (other than the contraceptive pill and asthma medication), poor proficiency in English, illicit drug use, and current ill-health.

Ethics approval

Both studies were conducted according to the guidelines laid down by the Declaration of Helsinki, with written informed consent obtained for each participant. The studies protocols were approved by the Macquarie University Human Research Ethics Committee (5201822302603 and 5201600641).

Baseline measurement

Biographical and health data were collected during the baseline sessions of each study. Body measurements (height and weight) were taken to calculate BMI and a urine sample was provided. Current levels of physical activity were calculated using the International Physical Activity Questionnaire (29) and sleep quality using two items from the Pittsburgh Insomnia Rating Scale (30). Intake of saturated fats and added sugar was measured using the Dietary Fat and Sugar Screener (DFS) (28). In the DFS, participants were asked to rate how often they had consumed 26 food and drink items over the past year on a five-point category scale ranging from 1 = "Less than one time a month" to 5 = "Five or more times a week." Depression symptoms were assessed using the DASS-21-D, a self-report measure rated on a 4-point Likert scale regarding mood over the past week (27).

Biochemical measurement

Reagents

Analytical grade reagents and standards were purchased from Sigma-Aldrich (St Louis, MO, USA), unless otherwise stated. Deuterated internal standards were purchased from Medical Isotopes, Inc (Pelham, NH, USA).

Creatinine measurement

Mid-stream urine samples were collected for the determination of urinary creatinine concentrations using a Creatinine (urinary) Colorimetric Assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. The creatinine concentration was used as an index of normalisation to control for between-participant variability in excretion rate.

Immune marker profiling

Blinded urine samples were subjected to immunoassays to quantify for Interleukin-6 (IL-6) and C-reactive protein (CRP) using the Human IL-6 (ab178013, Abcam, Boston, MA, USA) and CRP (ab260058, Abcam, Boston, MA, USA) ELISA kits following the manufacturer's instructions.

Kynurenine pathway metabolites profiling

Before analysis, blinded urine samples were deproteinized with respective mobile phase of the KP assays at a dilution factor of 1:10. Samples were incubated for 5 min, vortexed and then centrifuged (4°C) for 10 min at 12,000 rpm. Supernatant was then extracted and filtered with syringe filters (0.22 μm) ready for injection into analysers.

Concurrent quantification of TRP, KYN, 3-HK, and 3HAA using an Agilent 1290 ultra-high performance liquid chromatography system while PA and QA were analysed using an Agilent 7890 gas chromatograph (GC) coupled with an Agilent 5975 mass spectrometer according to the method described in Lim et al. (31). KA quantification was conducted using an Agilent 1260 system as previously described in Jacobs et al. (32). Mixed standards of all metabolites were used for a six-point calibration curve to interpolate the quantity of the sample readout. The inter- and intra-assay coefficient of variation was within the acceptable range of 3–7% for the LC system and 5–10% for the GC system. Participant's urinary KP metabolites measurements were normalised with their corresponding creatinine levels. Five ratios were calculated based on the metabolite levels, namely, the kynurenine/tryptophan ratio, which depicts combined IDO and tryptophan dioxygenase activity, kynurenic acid/kynurenine ratio depicts kynurenine aminotransferase activity, 3-hydroxykynurenine/kynurenine ratio depicts kynurenine monooxygenase activity, 3-hydroxyanthranilic acid/3-hydroxykynurenine ratio depicts kynureninase activity, and kynurenic acid/quinolinic acid depicts KP-induced glutamatergic activity.

Statistical analysis

Prior to statistical analysis, all continuous variables were checked for normality. Data were log-transformed and scaled, where appropriate, to achieve a normal distribution for parametric analysis. The nature of the missing values was confirmed and treated as missing completely at random. The

outliers were examined based on DFBETA using bubble plots to look for overly influential observations.

Univariate analysis (unadjusted for covariates) was used for the descriptive statistics. For group comparisons between the three DFS groups, one-way ANOVA with Sidak multiple-comparisons *post-hoc* tests was used. Data are expressed as mean ± SD unless otherwise specified and $p < 0.05$ (two tailed) was considered statistically significant. Biochemical measures that showed differential expression were considered for further analysis. Apart from exploring associations among biochemical measures, potential confounders were identified using pairwise Pearson's correlations between biochemical measures and demographic factors. Any significant correlations were subsequently adjusted in the multiple regression models described below.

The univariate regression model was applied to tease out the association between each selected biochemical measure with the depression scores as outcomes, followed by a covariate regression analysis adjusting for potential confounding effects from the demographic factors. Models were checked for multicollinearity and variance inflation factors were < 3.

Two main models were created to examine the impact of both lifestyle factors and biochemical markers on the severity of depressive symptoms using multiple linear regression. The difference in the two models was the presence and absence of immune marker(s). The models comprised the DASS-21 depression score as dependent variable and three groups of predictors: (1) demographic factors, (2) lifestyle factors, and (3) urinary biochemical measures, as defined below:

Model 1 (only KP markers):

$$Depression\ score = Demographic\ factors + Lifestyle\ factors + KP\ markers$$

Model 2 (KP and immune markers):

$$Depression\ score = Demographic\ factors + Lifestyle\ factors + Immune\ markers + KP\ markers$$

The beta coefficients infer the unit change in (DASS-21) depression scores after adjusting for all other covariates in the model. The R^2 value depicts the goodness-of-fit of the regression model together with corresponding p values. Biochemical measures with $p < 0.05$ were considered important predictors of the outcome. To further control for the random effect from the two separate studies, we further modelled the outcome and selected predictors in Model 2 using mixed-effects multilevel linear regression. The intraclass correlation of the model was used to check for (in)dependency between the two RCTs.

All statistical analyses were performed in StataIC 16 (StataCorp LLC, CA, USA) and graphical illustrations were prepared with GraphPad Prism 9.0 (GraphPad Software, Inc, La Jolla, CA, USA).

Results

Group characteristics

Of the 173 participants recruited, four participants were removed due to lack of or inadeqaute sample volume for analysis and were treated as missing completely at random. A flow diagram outlining other missing values was presented in **Supplementary Figure 1**. Hence, a total of 169 samples were analysed that were classified into low (DFS score < 54), mid (DFS score ≥ 54 and < 66), and high (DFS score ≥ 66) DFS groups as outlined in **Table 1**. Comparison of demographic characteristics between the three DFS groups showed no differences in age or the frequencies of female-to-male ratio ($\chi^2 = 2.70$, $p = 0.758$) although there was a slight tendency for a higher proportion (52–67%) of female participants across the groups (**Table 1**). Other lifestyle-associated factors such as BMI and physical activity level were well-matched between the three DFS groups, showing no differences ($p > 0.05$) except for DFS itself [$F_{(2,\ 166)} = 310.11$, $p < 0.0001$]. There was no difference in the urinary creatinine level, suggesting that the diet did not affect the urinary excretion rate. The DASS-21-D score was significantly different between the DFS groups [$F_{(2,\ 166)} = 11.99$, $p < 0.0001$], with highest level of depression being observed in the high DFS group (**Table 1**).

Potential confounding effects between demographic factors and urinary biomarkers

We examined the immune markers and KP metabolites for any potential confounding effects with the cohort's demographic factors. Summarised in **Table 2**, we found that increased age was associated with an elevation in several KP metabolites and enzyme activities including KA, 3HAA, PA, KAT, KMO, KYNU, and KA/QA ratio. Our cohort showed a moderately strong effect size of sex differences in urinary QA level (Cohen's $D = 0.51$, $p = 0.0013$) and KMO activity ($D = 0.49$, $p = 0.0019$), being higher in males, while KA/QA ratio ($D = -0.36$, $p = 0.0235$) was higher in females.

For lifestyle factors, there was no association between BMI and any biochemical measure. The correlational analysis indicated that physical activity level was negatively correlated with all of the KP metabolites, except PA (**Table 2**). Among the KP metabolites, DFS scores were negatively associated with KA ($R = -0.17$, $p = 0.0236$) and positively associated with 3HAA ($R = 0.16$, $p = 0.0386$). Higher KMO activity ($R = 0.19$, $p = 0.016$), but lower KA/QA ratio ($R = -0.22$, $p = 0.0037$) were correlated with increasing DFS score. Taken together, our univariate correlational analysis indicated that subsequent regression modelling using the urinary KP biomarkers should be

TABLE 1 Demographic characteristics of cohort participants based on DFS score.

	Low DFS (n = 68)	Mid DFS (n = 55)	High DFS (n = 46)	F-statistic or χ^2 value	P-value
Age (years, mean ± SD)	20.97 ± 3.11	22.05 ± 4.48	20.80 ± 3.79	1.71[A]	0.185
Sex (F/M, % of F to total)	38/30 (55.88)	37/18 (67.27)	24/22 (52.17)	2.695[B]	0.260
BMI (mean ± SD)	22.14 ± 2.63	21.78 ± 2.82	22.19 ± 2.89	0.36[A]	0.701
Physical activity (mean ± SD)[¥]	12.09 ± 1.92	12.04 ± 1.65	11.84 ± 1.12	0.32[A]	0.725
DFS (mean ± SD)	45.44 ± 5.20	59.85 ± 3.22[$]	76.28 ± 11.58[$]	310.11[A]	**<0.0001**
Creatinine, mmol/L (mean ± SD)	15.25 ± 10.08	13.14 ± 7.08	10.89 ± 6.42	1.91[A]	0.151
DASS-21-D (mean ± SD)	2.87 ± 3.50	5.29 ± 4.71[^]	6.91 ± 5.65[$]	11.99[A]	**<0.0001**

DFS, dietary fat and free sugar screener score; DASS-21-D, Depression Anxiety Stress Scales 21 Depression score; BMI, Body Mass Index; F, Female; M, Male. [A]Denotes One-way ANOVA and [B]denotes Chi-square analysis. Significant p-value (< 0.05) are denoted in bold. [^]Denotes $p = 0.001$ and [$]denotes $p < 0.0001$ using Sidak *post-hoc* analysis in comparison to low DFS group. [¥]Denotes the mean of variable presented in $\log_2$-scale. All *post-hoc* between groups comparisons have $p < 0.0001$ in 1-way ANOVA for DFS.

TABLE 2 Univariate analysis of associations between demographic, lifestyle risk factors, and urinary biomarkers.

	Age	Sex[A]	BMI	Physical activity	DFS
Immune markers					
C-Reactive Protein (CRP)	−0.02	0.23	0.03	0.15*	0.07
Interleukin (IL)-6	0.03	0.29*	−0.01	0.08	0.00
KP metabolites					
Tryptophan (TRP)	−0.05	0.00	0.08	**−0.17***	0.08
Kynurenine (KYN)	−0.12	−0.16	−0.05	**−0.16***	−0.01
Kynurenic acid (KA)	**0.27^**	0.01	0.08	**−0.15***	**−0.17***
3-Hydroxykynurenine (3HK)	0.02	0.22	0.06	**−0.15***	0.13
3-Hydroxyanthranilic acid (3HAA)	**0.23#**	0.09	−0.01	**−0.16***	**0.16***
Picolinic acid (PA)	**0.24#**	−0.22	0.06	−0.10	0.11
Quinolinic acid (QA)	−0.01	**0.51***	−0.02	**−0.24#**	0.08
KP activity/ratio					
IDO/TDO activity	−0.06	−0.15	−0.12	0.02	0.08
KAT activity	**0.29$**	0.13	0.10	0.01	−0.13
KMO activity	**0.16***	**0.49***	0.13	−0.03	**0.19***
KYNU activity	**0.19***	−0.11	−0.07	−0.01	0.03
KA/QA ratio	**0.25^**	**−0.36***	0.09	0.03	**−0.22#**

IDO, Indoleamine 2,3-dioxygenase; TDO, Tryptophan dioxygenase; KYAT, Kynurenine aminotransferase; KMO, Kynurenine 3-monooxygenase; KYNU, Kynureninase; IDO/TDO activity is defined by KYN/TRP ratio; KAT activity is defined by KA/KYN ratio; KMO activity is defined by 3HK/KYN; KYNU activity is defined by 3HAA/3HK ratio. All reported values were derived from Pearson's correlation coefficient unless otherwise specified. [A]Denotes Cohen's D effect size (female to male). Significant correlations/effect sizes are denoted in bold *$p < 0.05$, #$p < 0.01$, ^$p < 0.001$, $$p < 0.0001$. *(unbold) indicate p-value close to 0.05.

adjusted for confounding effects with age, sex, physical activities, and DFS score.

Correlation analyses of immune and kynurenine pathway markers

We found that urinary CRP was correlated with IL-6 (R = 0.20, p = 0.008), although the effect size was small (Table 3). This is not surprising considering the relatively healthy population of our cohort with the expectation of more regulated inflammatory responses. The only KP metabolites that correlated positively with CRP levels were 3HK (R = 0.20, p = 0.011) and 3HAA (R = 0.28, p = 0.0002), showing a small effect size with statistical significance. We found that IL-6 correlated positively with the key KP metabolite involved in depression, KA, although the effect size was small (R = 0.20, p = 0.0079). Neither TRP nor KYN correlated with IL-6 despite the presence of depressive symptomology (>7 DASS-21-D scores) in ∼60% of participants.

As there is limited knowledge about KP homeostasis in adolescent/young adult cohorts, we used correlational analyses to study baseline KP metabolism in this age group. Our analysis indicated that in general, TRP positively correlates with its downstream KP metabolites suggesting a normal metabolic flux of more substrates, leading to more catabolites. We saw that the

TABLE 3 (Inter-)Relationship between immune markers and KP metabolites.

	IL-6	TRP	KYN	KA	3HK	3HAA	PA	QA
CRP	**0.20**$^{\#}$	0.08	0.06	−0.03	**0.20***	**0.28**$^{\wedge}$	0.07	0.10
IL-6								
TRP	0.07							
KYN	0.01	**0.45**$^{\$}$						
KA	**0.20**$^{\#}$	−0.02	0.14					
3HK	−0.03	**0.49**$^{\$}$	**0.70**$^{\$}$	0.09				
3HAA	0.04	0.08	**0.30**$^{\$}$	**0.21***	**0.40**$^{\$}$			
PA	0.03	0.14	**0.18***	**0.24**$^{\#}$	0.15	**0.37**$^{\$}$		
QA	0.09	0.13	**0.25**$^{\wedge}$	**0.29**$^{\wedge}$	**0.31**$^{\$}$	**0.20***	**0.29**$^{\wedge}$	

Significant correlations are denoted in bold. $^{*}p < 0.05$, $^{\#}p < 0.01$, $^{\wedge}p < 0.001$, $^{\$}p < 0.0001$. CRP, C-Reactive protein; IL-6, interleukin-6; TRP, tryptophan; KYN, kynurenine; KA, kynurenic acid; 3HK, 3-Hydroxykynurenine; 3HAA, 3-Hydroxyanthranilic acid; PA, Picolinic acid; QA, Quinolinic acid.

key branching metabolite of the KP, KYN, was highly associated (R = 0.70, $p < 0.0001$) with catabolites of the 3HK branch towards QA and not KA production. Other KP intermediates such as 3HK and PA, also showed moderate effects with QA. There was a moderate correlation between QA and KA, suggesting that increments in either one are likely to result in increases in its NMDA counterpart. Additionally, higher 3HAA, a precursor to PA and QA, was more likely to be associated with PA, rather than QA production.

Differential group expression of urinary kynurenine pathway metabolites

There were group differences [$F_{(2,\ 166)} = 3.86$, $p = 0.023$] in urinary KA levels, which were the highest in the Low DFS group compared to other groups, with significant differences in the High DFS group, as shown in Figure 2A. Next, we examined the overall kynurenine aminotransferase (KAT) activity defined by the ratio of KA to kynurenine. As expected, there were group differences [$F_{(2,\ 166)} = 4.10$, $p = 0.018$] in KAT activity as well. The high DFS group had the lowest KAT activity, and was significantly lower than the Mid DFS group and the Low DFS group, although the latter did not reach statistical significance (Figure 2B).

As QA and KA are the two key metabolites of the KP known to be associated with depression, with depressed individuals showing higher neurotoxic QA levels and lower neuroprotective KA levels. Considering this relationship with depression, we were interested in whether this ratio would differ across DFS group, representing a potential mechanism underlying the relationship between WS-diet intake and depression. We found significant differences in KA/QA ratio between the DFS groups [$F_{(2,\ 166)} = 5.70$, $p = 0.004$]. Although there was no difference between Low DFS and Mid DFS groups, the High DFS group had a significantly lower KA/QA ratio compared to the Low DFS group and Mid DFS group (Figure 2C). None of the urinary immune markers or other KP markers showed any group differences, as outlined in Supplementary Table 1.

Assessing depression with urinary biomarkers and demographic factors

Our results indicate that IL-6, but not CRP, was associated with the severity of depressive symptoms. A unit increase in urinary IL-6 was associated with a decrease of 0.041 units of depression score ($p = 0.013$), after adjusting for demographic factors (Table 4).

Among all KP markers, KA, KAT activity and KA/QA ratio had significant negative correlations with the severity of depression (Supplementary Table 2). However, only KA and KA/QA ratio remained to be significant biomarkers of depression after adjusting for demographic factors: one unit increase in KA levels and KA/QA ratio were associated with a decrease of 0.269 units ($p = 0.001$) and 0.220 units ($p = 0.005$) in depression score, as outlined in Table 4. Conversely, KMO activity showed a positive correlation with the severity of depression, but did not reach statistical significance when adjusted for confounding effects.

Relationship between depressive symptoms with diet, exercise, and kynurenic acid

We were interested to know whether lifestyle (i.e., diet and exercise) and intrinsic (i.e., host's biochemical measures) factors were related to severity of depressive symptoms defined by DASS-21-D scores. After a thorough interrogation of the dataset, we selected the important predictors in building our model using multivariate regression analysis. Two models were established, where in Model 1, only the KP metabolites but not immune mediator were used as the intrinsic biomarker and in

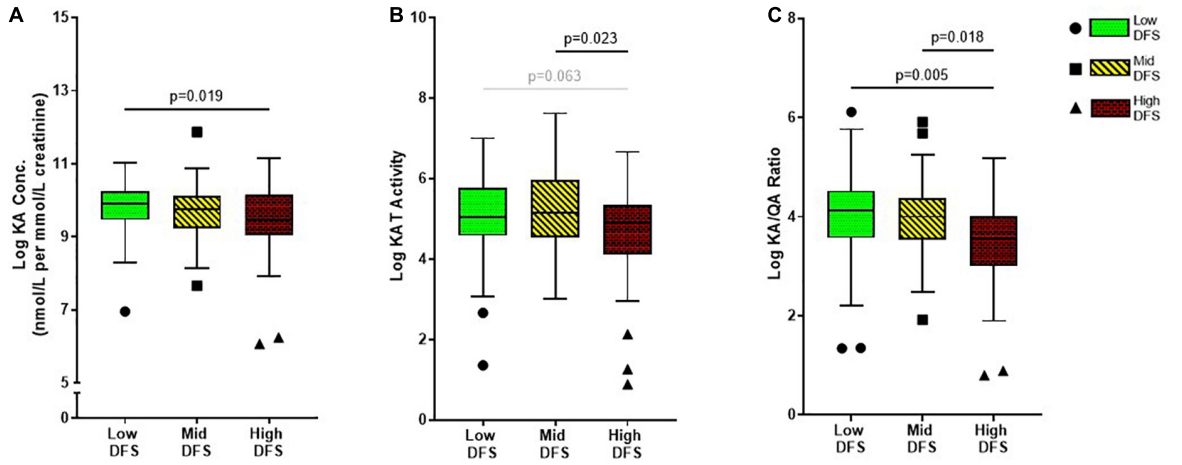

FIGURE 2
Boxplots showing the difference in **(A)** Kynurenic acid (KA), **(B)** Kynurenine aminotransferase (KAT) activity, and **(C)** KA/QA ratio across the low, mid, and high DFS groups. The low DFS participants are represented by green box-and-whisker and circle symbols, the mid DFS participants are represented by yellow box-and-whisker and square symbols, and the high DFS participants are represented by red box-and-whisker and triangle symbols. The symbols indicated participants that had > 2 standard deviations within the group. The median and interquartile range are displayed for each group.

TABLE 4 Associations between depression and biomarkers measures by univariate and covariate analyses.

	Univariate analysis			Covariate analysis*		
	β (95% CI)	R^2	*P*-value	Adjusted β (95% CI)	Adjusted R^2	*P*-value
Immune marker						
IL-6	−0.04 (−0.07 to −0.01)	0.04	**0.013**	−0.04 (−0.07 to −0.01)	0.15	**0.013**
KP markers						
KA	−0.30 (−0.46 to −0.14)	0.08	<**0.001**	−0.27 (−0.43 to −0.10)	0.17	**0.002**
KAT activity	−0.16 (−0.28 to −0.03)	0.04	**0.014**	−0.10 (−0.23 to 0.03)	0.13	0.123
KMO activity	0.18 (0.004–0.36)	0.02	**0.045**	0.14 (−0.04 to 0.33)	0.13	0.121
KA/QA ratio	−0.29 (−0.44 to −0.14)	0.08	<**0.001**	−0.22 (−0.37 to −0.06)	0.16	**0.006**

IL-6, Interleukin-6 (fg/mL per mmol/L creatinine); KA, Kynurenic acid (nmol/L per mmol/L creatinine); KAT, Kynurenine aminotransferase; KMO, Kynurenine 3-monooxygenase; KA/QA, Kynurenic acid/Quinolinic acid ratio; KAT activity is defined by KA/KYN ratio; KMO activity is defined by 3HK/KYN; *Multiple linear regression modelling was performed to adjust for demographic factors including age, sex, DFS, and physical activity. All variables were $\log_2$-transformed prior to regression analysis. The β denotes the beta coefficient of the regression model and R^2 refers to the coefficient of determinant indicating the goodness-of-fit of the regression analysis. Significant *p*-value (< 0.05) are denoted in bold. Only biomarkers that showed a significant relationship with depression score were presented in this table. For full analysis of the other biomarkers, refer to Supplementary Table 2.

Model 2, both immune and KP biomarkers were included. As the data were taken from the baseline session of two separate RCTs, there may be random effects (i.e., study factors) that were not taken into account in Models 1 and 2. Hence, a multilevel mixed-effect regression model was applied to adjust for random effects. Our analysis showed that there was no significant differences between the β-coefficients of the predictors when comparing Model 2 and 3 (Supplementary Table 3).

After adjusting for demographic factors (i.e., age, sex, and BMI) and random effects from the two separate RCTs, our final model (i.e., Model 3) showed that increasing physical activity was associated with reduced severity of depressive symptoms (Table 5). Every additional hour of physical activity is associated with a lower depressive symptom score of 5.4 units. Not surprisingly, both Mid and High DFS groups were associated with higher depression scores compared to the low DFS group, as shown in Table 1. In addition, every 1 unit increase in DFS score was associated with 0.69 units increase in depression score.

When accounting for the covariates, the immune marker IL-6 no longer became an important predictor of the model ($p > 0.05$). More importantly, only one biomarker, KA, showed a significant association with the severity of depressive symptoms, regardless of the presence or absence of IL-6. Our model indicates that with every decrease in 1 μM of the NMDA antagonist, KA level in urine, depression scores increased by 0.21. Our model could explain approximately 22% of the variance in relation to how diet, physical activity, and the

TABLE 5 Depression severity correlates with diet, physical activity, and the KP.

	Regression model β coefficient (95% CI) $n = 166$	*P*-value
Demographic factors		
Age (Years)	−0.25 (−0.80 to 0.30)	0.377
Sex	0.01 (−0.25 to 0.27)	0.929
BMI	−0.02 (−0.07 to 0.02)	0.341
Lifestyle factors		
Physical activity	**−0.09 (−0.16 to −0.01)**	**0.030**
DFS (as score)	**0.69 (0.32 to 1.05)**	**<0.001**
Low Group¤ (score < 54)	Reference	
Mid Group¤ (score 54–65)	**0.51 (0.22–0.80)**	**0.001**
High Group¤ (score ≥ 66)	**0.56 (0.26–0.87)**	**<0.001**
Immune marker		
IL-6, fg/mL per mmol/L Cr	−0.03 (−0.06 to 0.00)	0.077
KP marker		
KA, nmol/L per mmol/L Cr	**−0.21 (−0.37 to−0.05)**	**0.009**

The selected model was analysed using Mixed-effect multilevel regression to control for random effects from different studies. BMI, Body Mass Index; DFS, dietary fat and free sugar screener score; IL-6, interleukin-6; KA, kynurenic acid; Cr, creatinine. All variables except BMI were $\log_2$-transformed prior to analysis. ¤ denotes variable was chosen to be analysed primarily as a categorical variable rather than continuous variable. Only variables with *p*-values < 0.05 were considered important in predicting the severity of the depressive symptoms. Significant *p*-value (< 0.05) are denoted in bold.

KP impact on depression severity in an otherwise healthy young adult cohort.

Discussion

Depression is a common mental health disorder, with approximately 1 in 5 people experiencing at least one episode in their lifetime. An association between diet and depression has been demonstrated across numerous studies, and several RCTs have implied that eating a more healthful diet is a useful adjunct treatment for depression. However, the physiological mechanisms by which diet can impact on depression symptoms have yet to be established. This was the first study to examine and provide evidence to support the biochemical relationship between the urinary KP metabolites with WS-diet and depressive symptoms in otherwise healthy young adults. Overall, we showed that (a) WS-diet was associated with alterations to the KP profile, (b) the KP profile was associated with severity of depression symptoms, and (c) after adjusting for confounding effects, including diet, KA and KA/QA ratio remained strong predictors of level of depression symptoms. These findings are discussed in more depth below.

We found that higher saturated fat and sugar intake was associated with lower levels of neuroprotective KA. To understand the metabolic homeostasis associated with differing levels of saturated fat and sugar intake, we further examined the conversion of KYN to KA, operationalised as KAT activity, across the DFS groups. We found higher KAT activity in those who have healthier diet reflected by lower DFS score, whereas KYN levels remained consistent across groups (**Figure 2B**). This suggests that reduced consumption of WS-diet is linked to increased KAT activity, which may confer neuroprotection against depression. As hypothesised, higher WS-diet intake was also associated with a lower KA/QA ratio. Interrogating this relationship, we found that although levels of KA were correlated with that of QA, DFS score was only associated with KA (negatively) but not QA production, implying that WS-diet selectively alters the inhibitory glutamatergic activity by limiting the NMDA antagonist, KA. Hence, our study showed that, in otherwise healthy young adults, WS-diet was associated with lower levels of KA, KAT activity, and KA/QA ratio.

The glutamatergic modulators (i.e., KA and KA/QA), which were negatively correlated with WS-diet intake, were also negatively correlated with depressive symptoms. The role of QA and KA in depression, in the context of glutamatergic modulation, is well-known, though it is debatable whether QA or KA plays a more important role. A recent meta-analysis showed that reduced KA, but no change in QA was associated with MDD, which is consistent with our findings (33). It is possible that the diet plays a direct role in regulating KA levels, while QA levels are linked to inflammation in the pathology of depression; explaining why we did not observe a significant increase in QA levels. It is also possible that intestinal microbes can directly affect KA levels in the gut and influence the host's peripheral KA levels since microbes have aspartate aminotransferase, in addition to KAT (limited to vertebrates), which is an alternative to KA production *via* the transamination reaction. Hence, the diversity and capability of the host's gut microbiota to produce KA can contribute to differences in hosts' KA levels. However, our understanding of the crosstalk between luminal and peripheral KA levels is limited and warrants further investigation. Hence, our findings were consistent with the current literature supporting the dysregulation of the glutamatergic system and challenges the long-held belief that serotonin as the key neurotransmission system involved in depression.

Though the mechanisms of action underlying relationship between diet and depression have yet to be fully understood, tryptophan metabolism down the KP pathway has been of increasing interest. Tryptophan depletion by oral intervention in healthy male participants was shown to lead to depression (23). This was attributed to attenuation of the mood regulating neurotransmitter, serotonin and interpreted as support for the ongoing theory of tryptophan-serotonin depletion in depression (13). However, further studies showed the involvement of the glutamatergic-acting KP metabolites (as opposed to the other arm; the serotonin pathway) in people with severe depression (34). Our findings contribute to an emerging

literature demonstrating that the pathophysiology of depression extends beyond serotonergic modulation. Our study further suggests that in otherwise healthy young adults, KA may be an endogenous source of glutamatergic inhibition, acting to reduce depression. This notion is supported by recent studies showing glutamatergic inhibition by ketamine is an effective antidepressant (35–37).

One of the putative mechanisms for how diet can impact depression, which has garnered a lot of interest, is alterations to the gut-brain axis. The involvement of tryptophan-KP is congruent with this theory. WS-diet can directly alter the host's tryptophan (and therefore KP) metabolism through the interaction between diet composition and gut microbiota. Short-chain fatty acids (SCFAs), especially butyrate, are functional by-products from bacterial carbohydrate metabolism in the gut known to modulate the KP. Specifically, butyrate can inhibit IDO-1 activity in the gut environment to increase the bioavailability of luminal tryptophan in the host (38). This is consistent with a study by Gao et al. (39) which demonstrated increased luminal availability of carbohydrates by cecal starch infusion leads to suppression of tryptophan catabolism in the microbial environment, thereby resulting in greater bioavailability of tryptophan in the large intestine and subsequently higher level in hosts' serum (39). Similarly, another preclinical study showed that high-fat diet can attenuate microbial tryptophan degradation in the cecum of mice (40). This is in agreement with our results showing that participants with the highest levels of fat and sugar intake had the highest level of urinary tryptophan compared to Low and Mid DFS groups, although the group comparison was not statistically significant.

Given that WS-diets are known to induce systemic low-grade inflammation, we explored pro-inflammatory mediators (IL-6 and CRP) that are known to activate the KP *via* IDO-1 to establish the correlations between diet, inflammation and KP changes. There were no group differences in these pro-inflammatory cytokines across diet groups (**Supplementary Table 1**) and demographic factors did not correlate significantly with these proinflammatory cytokines in our cohort. As expected, IL-6 was a predictor of depression symptoms in our univariate regression model, although, to our surprise, urinary IL-6 had an inverse correlation with level of depression symptoms. This is inconsistent with the widely accepted role of IL-6 and inflammation in the pathogenesis of MDD [see review (41)]. However, this research is based on serum levels of IL-6, whereas in the current study we only examined urine levels. While we expected that peripheral markers would correlate positively with urine markers, most of the studies showing a positive correlation between serum and urine IL-6 levels have been conducted in patients with renal dysfunction. It may be the case that the relationship is different in otherwise healthy young adults. A possible explanation is that the immune markers in urine represent what is excreted from the host, as opposed to the blood-based profile, which directly reflects the host's systemic condition. Hence, the concentration of urinary markers would be inversely proportional to periphery markers. Our recent study comparing serum and matching urine immune markers between healthy controls and people with multiple sclerosis (MS) showed higher levels of immune markers (IP-10, IL-1ra, TNF-alpha, and RANTES) in the serum of people with MS compared to healthy controls, whereas, in urine, the result was the opposite to the matching serum (42). In our study, it is possible that healthy individuals who are less susceptible to depression may have greater efficiency in excreting immune metabolites. The lack of serum blood samples is thus a limitation of the current study, and future studies collecting both serum and urine samples may shed further light on this proposition. Furthermore, we are aware of only a few studies analysing urinary KP metabolites as a diagnostic biomarker in conditions such as attention-deficit hyperactivity disorder (43), cardiovascular events (44), heart failure (45), and breast cancer (46). That we could observe significant differences in KP metabolites according to both depression and WS-diet reveals further promise for urine based biomarkers as a less invasive and more affordable method of examining physiological mechanisms. For example, as a prognostic marker in predicting risk of a major depressive episode or suicidal ideation in younger people with depressive symptoms. However, this too requires replication in another cohort as well as validation against blood samples.

Though both inflammation and KP metabolism are implicated in the pathophysiology of depression, it is rather controversial as to whether inflammation is needed to drive the dysregulation of the KP activity (*via* IDO-1). For example, Öztürk et al. (47) showed that the KYN/TRP ratio and QA were significantly higher in MDD, but did not find any group differences in IL-6 and CRP levels compared to healthy controls (47). Contrary to this, Erhardt et al. (48) found higher IL-6 and QA in CSF of people with MDD compared to healthy controls (48). As such, KP related changes in depression may be independent of inflammation, and our findings support this notion based on the observations of: (a) an inverse relationship between IL-6 and depressive symptoms, (b) no inverse correlation between tryptophan and its downstream metabolites or differential in KYN/TRP ratio, an indicator of host IDO/TDO activity, (c) limited associations between the pro-inflammatory and KP markers, and (d) KA was the only biomarker to remain a significant predictor of depression severity in a model accounting for covariates including IL-6. Although the lack of proinflammatory status may imply that competition for tryptophan as substrate between the kynurenine and serotonin pathway may not be affected, however, future study should consider exploring serotonin and downstream metabolites such as 5HIAA in urine to delineate the metabolic flux of tryptophan metabolism in dietary intervention in the context of depression.

A number of expected findings emerged, which make us more confident in our data. Age was associated with an increase in several KP metabolites, which is in keeping with previous findings (49, 50). Age may be a confounder in prevalence and pathogenesis of depression, thereby limiting our findings to the young adult population. We also found some sex differences, such that QA levels and KMO activity were higher in males, whereas KA/QA ratio was higher in females. Similarly, higher urinary levels of KYN, 3-HAA, and TRP in males compared to females, in young adults aged 20–24 (51). Our results additionally demonstrated that increased physical activity was associated with decreased severity of depressive symptoms, as well as decreased levels of KP metabolites. Consistent with this, a recent study showed reduced urinary KP metabolites in those who exercise compared to those who do not exercise (46). Future clinical studies that examine KP metabolism should consider physical activity as a potential confounder.

In conclusion, this is the first study to examine the role of KP metabolism in the relationship between WS-diet and depression symptoms in an otherwise healthy young adult cohort. Our findings showed that higher WS-diet intake was related to reduced production of the neuroprotective KP metabolite, KA, as well as a reduced KA/QA ratio. These glutamatergic modulators, were also shown to be reduced in those with higher levels of depression symptoms, with further analysis showing KA was the only biomarker to have a significant association with depression symptoms in a model controlling for demographic and lifestyle variables. These findings appeared to be independent of inflammation, however, further comparisons between urine and blood based immune biomarkers are required. Animal studies may be helpful to investigate direction of causality in the relationship between depression symptoms and KP metabolism.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Macquarie University Human Research Ethics Committee (Ethics #5201822302603 and 5201600641). The patients/participants provided their written informed consent to participate in this study.

Author contributions

HF, RS, and CL contributed to the conception, design, supervision, and funding acquisition of the study. LE, TA, and DG were involved in the investigation and collection of the clinical data. LT and SB were involved in performing and collecting the biochemical data. CL and SB performed the statistical analysis. CL and HF wrote the first draft of the manuscript. LT, LE, and RS wrote sections of the manuscript. All authors contributed to manuscript revision, proofreading, and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.945538/full#supplementary-material

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Altered mannose metabolism in chronic stress and depression is rapidly reversed by vitamin B12

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GDP-Mannose Pyrophosphorylase B (GMPPB) is a key enzyme for glycosylation. Previous studies suggested a dysregulation of GMPBB and mannose in depression. Evidence, however, was sporadic and interventions to reverse these changes are unknown. Here, we show that GMPPB protein, but not RNA abundance is increased in the postmortem prefrontal cortex (PFC) of depressed patients and the chronic variable stress (CVS) mouse-model. This is accompanied by higher plasma mannose levels. Importantly, a single dose of intraperitoneally administered vitamin B12, which has previously been shown to rapidly reverse behavioral symptoms and molecular signatures of chronic stress in mice, normalized GMPPB plasma mannose levels and elevated GDP-mannose abundance. In summary, these data underline metabolic dysregulation in chronic stress and depression and provide further support for rapid effects of vitamin B12 on chronic stress.

KEYWORDS

stress, vitamin B12, depression, glycosylation, mannose

Introduction

GDP-Mannose Pyrophosphorylase B (GMPPB) is a key enzyme in the glycosylation pathway, which catalyzes the synthesis of GDP-mannose from mannose-1-phosphate and guanosine triphosphate (1). Its activity is regulated by its enzymatically inactive homologue GMPPA, which acts as an allosteric inhibitor of GMPPB. Mutations in GMPPB and GMPPA result in complex congenital disorders of glycosylation (2–4). Recently, a protein-wide association study on depressed patients found that GMPPB

protein levels are increased postmortem in the prefrontal cortex (PFC) of depressed patients (5). Accordingly, receptors for neurotransmitters that are essential to stress and depression in the PFC are regulated by glycosylation, including the NMDA receptor (6–8) and serotonin receptors (9, 10). In congenital disorders of glycosylation, depressive symptoms are relatively frequent (11, 12).

The substrate for GMPPB, mannose-1-phosphate, is reduced in the PFC in a rat model of chronic stress and depression (13). A high dose of intraperitoneally administered mannose may contribute to depression-like states in mice (14). However, it is, at least to our knowledge, unknown, whether mannose levels are intrinsically affected by depression and whether this correlates with GMPPB abundance.

We recently observed that vitamin B12 rapidly reverses depression-associated phenotypes in mice (15). Vitamin B12 (cobalamin) is an essential nutrient, which can only be synthesized by bacteria. Vitamin B12 is a cofactor in the one-carbon metabolism, which provides methyl donors, e.g., for the methylation of DNA, histones or other proteins. 15% of the human population suffers from vitamin B12 deficiency and affected individuals have an increased risk of suffering from depression (16–18). Importantly, there is also evidence that vitamin B12-supplementation in non-deficient populations may reduce depression risk (19, 20). We have previously shown that a single acute dose of vitamin B12 reduced depressive-like behavior and stress-linked biomarkers in mice in the chronic mild stress-model (15).

Here we confirm increased GMPPB protein levels in human PFC postmortem tissue of depressed patients. Furthermore, we observed increased mannose levels in plasma of depressed patients. Similar changes were found in the chronic variable stress (CVS) mouse-model, which is superior in modeling human molecular signatures of depression (21). Additionally, plasma GDP-mannose levels were increased by CVS in mice. Interestingly, an acute dose of vitamin B12 was sufficient to reverse GMPPB and plasma mannose levels. In summary, this study underlines an association between GMPPB and depression.

Methods

Animals and licenses

Mice were housed in accordance with the ethical guidelines of the Thüringer Landesamt für Verbraucherschutz (TLV). Experiments were conducted under Animal license UKJ-18-037 (Germany), which are complying to the EU Directive 2010/63/EU guidelines for animal experiments. C57Bl/6J mice were bred in the animal facility (FZL) of Jena University Hospital, Germany. Animals received standard chow (LASQCdiet Rod16-R, LASvendi GMBH, Soest, Germany), which contains 50 mg/kg chow vitamin B12. Mice were at least 10 weeks of age. Mice were housed in a 14L:10D light-cycle. For brain analysis, mice were sacrificed and PFC tissue was immediately frozen on dry ice and stored at −80°C until further use.

Drugs and chemicals

Mice were intraperitoneally (i.p.) injected with 2.7 mg/kg vitamin B12 (cyanocobalamin, #V6629, Sigma-Aldrich, Burlington, MA, USA) or saline at an injection volume of 10 ml/kg body weight and tested 24 h later as described in Engmann et al. (22).

RNA purification and quantification

RNA was purified by resuspension in Trizol and chloroform-precipitation. RNA was washed in isopropanol and 75% ethanol. After cDNA-conversion with a GoScript^{TM} Reverse Transcriptase kit (#A5001, Promega, Madison, WI, USA), quantitative realtime-PCR was performed on a Bio-rad CFX96 Real-time system. Quantitative PCR results were processed as described (23). Primer sequences were: *GMPPB*/*Gmppb*, Fvd: 5′-CCT CACTGGCATGTGC CTC-3′, Rev: 5′-GACTTGTGGGGC AGCACG-3′; *GAPDH* (22), Fvd: 5′-TGGGCAGCCGTTAGG AAAG-3′, Rev: 5′-AGTTAAAAGCAGCCCTGGTGA-3′; *Gapdh* (22), Fvd: 5′-AACTTTGGCATTGTGGAAGG-3′, Rev: 5′-ACACATTGGGGGTAGGAACA-3′.

Western blot

Tissue lysates were prepared with the Potter S tissue homogenizer (Sartorius, #S14492) in TBS-buffer (20 mM Tris, 150 mM NaCl, 1% (v/v) TritonX-100, complete protease inhibitor and complete phosphatase inhibitor (#04693124001, Sigma-Aldrich, Burlington, MA, USA). After sonication, homogenates were spun down at 16,900 g to remove nuclei and insoluble debris. The supernatant was stored at −80°C until further use. Proteins were denatured at 90°C for 5 min in Laemmli buffer (4X Laemmli buffer: 50% glycerol, 5% SDS, 0.25%1.5M Tris pH 6.8, 30% β-mercaptoethanol, 0.001% bromophenol blue, ddH_2O). After separation by SDS-PAGE (8% polyacrylamide glycine gels, run for 1.5 h at 80 V) proteins were transferred onto 0.45 μm PVDF membranes (#10600023, GE Healthcare) at 290 mA for 100 min. Membranes were blocked in 2% BSA for 1 h at RT and incubated with primary antibodies at appropriate dilutions in tris-buffered saline supplemented with 0.1% Tween-20 (TBS-T) overnight at 4°C. The following primary antibodies were used:

rabbit anti-GMPPA (#15517-1-AP, Proteintech, Rosemont, IL, USA) 1:500, rabbit anti-GMPPB (#15094-1-AP, Proteintech, Rosemont, IL, USA) 1:500, rabbit anti-GAPDH (#10494-1-AP, Proteintech, Rosemont, IL, USA) 1:1,000, self-made mouse anti-oligomannose antibody (6–9 terminal mannose residues) 1:50 [gift of Rüdiger Horstkorte, Halle (24)]. Membranes were washed in TBS-T and primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies in an appropriate dilution. Following secondary antibodies were used: donkey anti-rabbit IgG-HRP (#NA 9340V, Amersham, Buckinghamshire, UK) 1:4,000 and goat anti-mouse IgM # (31440, Thermo Fisher Scientific, Waltham, MA, USA) 1:4,000.

Measurement of plasma sugar concentrations

Human plasma samples were obtained in agreement with the Ethics committee of Magdeburg University Hospital, Germany (110/07). Patients did not receive psychopharmacological substances other than benzodiazepines for at least 6 weeks prior to testing. Blood plasma was collected by centrifuging blood immediately after collection for 10 min at 3,000 rpm (Hettich centrifuge EBA 2, type 2002). The supernatant consisting of plasma was aliquoted into DNA LoBind tubes (Eppendorf, Hamburg, Germany) and stored at −80°C. Samples from depressed patients and age-matched controls were collected at day 0 (T0) and 6 weeks after the beginning of treatment (T6). Treatment differed between patients and was individually tailored to their needs. As no difference was found in plasma sugar levels for patients between days T0 and T6 (data not shown), the average of both time points was calculated for each participant to reduce variability. Mouse blood was obtained from unfasted animals and incubated on ice for 15 min. Samples were centrifuged for 10 min at 4°C and 4,000 g. Sugars were measured in the supernatant with the D-mannose, D-fructose, D-glucose kit following manufacturer's instructions (#K-MANGL, Megazyme, Wicklow, Ireland).

GDP-mannose measurements

Plasma samples were obtained as described above and stored at −80°C. For sample preparations, 10 μL of plasma was added to 190 μL of Methanol/water/Chloroform mixture (8:1:1), followed by a vigorous shacking and then centrifuged at 13,200 rpm during 15 min at 4°C. Supernatant was dried by speed Vacuum and samples were re-suspended in 70 μL of methanol 50%, and finally were analyzed by LC/MS as described previously (2). Tissue quality control showed no differences in GMP, GDP, or GTP levels between treatments (data not shown).

Chronic variable stress induction

The CVS-protocol was performed as described (21). In brief, mice received 21 days of stress with one of three stressors presented in a semi-random order, where the same stressor does not occur on two consecutive days. The following stressors were used: 1 h of tube restraint, tail suspension or 100 mild electric random foot shocks. If only female experimenters were present, a used male t-shirt was wrapped in clean protective clothing from the animal unit and placed into the experimental room in order to avoid variability due to sex-specific scents of the scientists (3). All experiments were conducted in the light phase of the light-cycle to allow comparability with previous experiments (15). For CVS-groups, vitamin B12 was injected at the last day of CVS (day 21), just prior to the stressor.

Postmortem brain samples

Samples were generously provided by the Douglas-Bell Canada Brain Bank. Experiments were conducted in agreement with the Ethics committee of Jena University Hospital, Germany (Reg.-Nr. 2020-1862-Material) and Douglas Institute REB Approval #04/21.

Statistics

Statistical analysis was performed in GraphPrism. Two-tailed Student's *t*-test was used for comparison of two groups. Two-way ANOVA with Bonferroni *post-hoc* test was used, when two factors were varied. Outliers were removed when data points were more than two standard deviations away from the average.

Results

GDP-mannose pyrophosphorylase B and plasma mannose are increased in depressed patients and in a mouse model of chronic stress

We obtained PFC postmortem tissue from depressed patients to assess whether altered GMPPB levels observed by Wingo et al. (5) can be reproduced and whether this was associated with altered mannosylation (**Figure 1A**). Additionally, we obtained samples from the CVS mouse-model (**Figure 1B**). This model is the gold standard in mimicking molecular and behavioral changes linked to depression and is well suited for functional and interventional studies (21, 25).

Increased GMPPB protein levels in postmortem PFC-tissue from depressed patients were confirmed in our cohort

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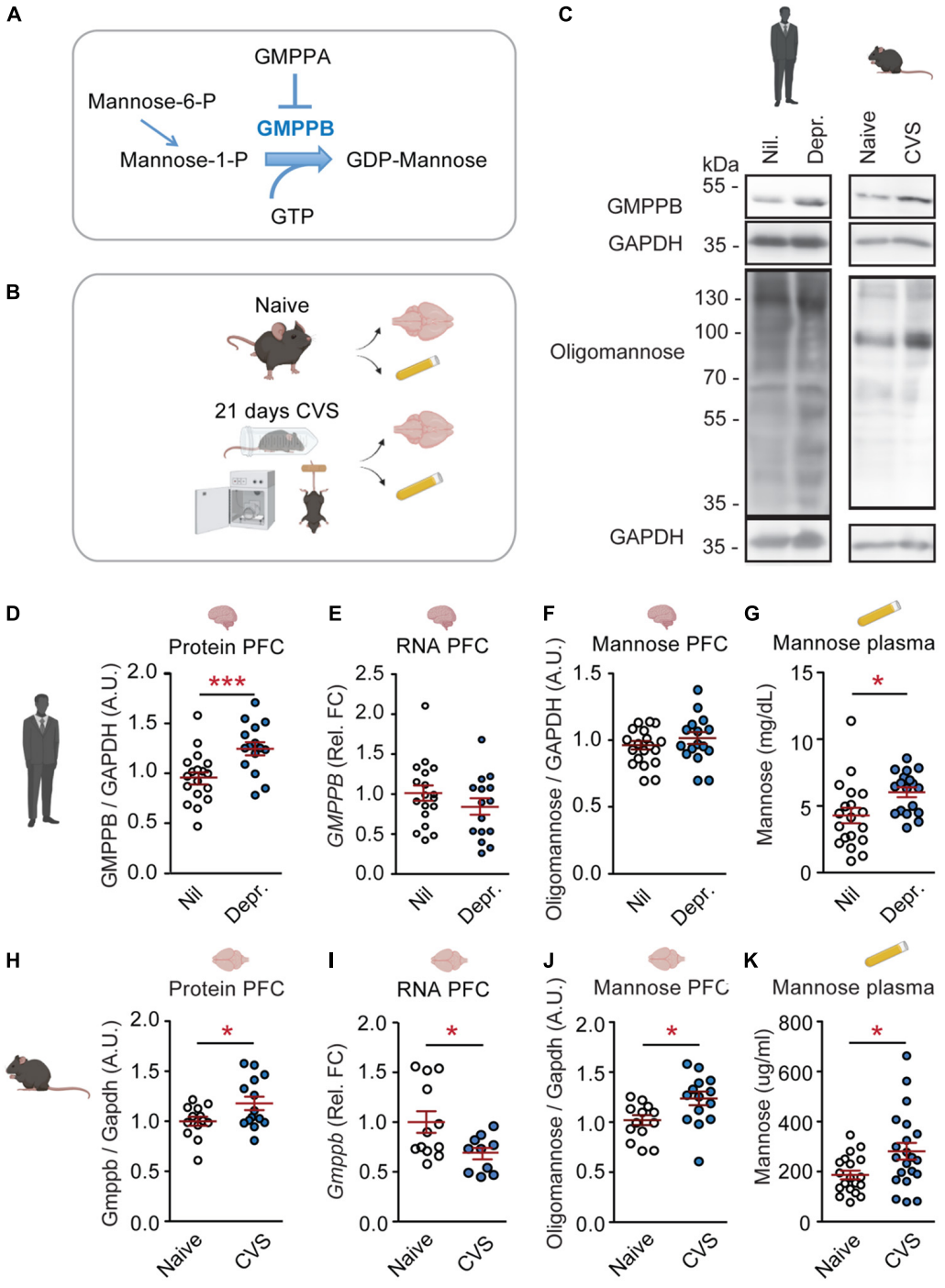

FIGURE 1
GMPPB and plasma mannose are increased by chronic stress and depression. **(A)** Cartoon illustrating the regulation of GDP-mannose production. **(B)** Experimental setup for CVS-experiment. **(C)** Representative western blots. **(D–K)** Statistics: Student's *t*-test. **(D–G)** Results from human cohorts. **(D–F,H–J)** Prefrontal cortex. **(G,K)** Plasma. **(D)** Increased GMPPB protein-levels in depressed patients (Depr.) vs. controls (Nil); $n = 15–18$ per group; $t_{31} = 3.32$, $^{**}P < 0.01$. **(E)** No association between depression and *GMPPB* RNA-levels; $n = 15–18$ per group; $t_{31} = 1.22$, $P = 0.23$. **(F)** No association between depression and oligomannose-levels. $n = 15–19$ per group; $t_{32} = 0.59$, $P = 0.56$. **(G)** Increased plasma mannose in depressed patients; $n = 18–19$ per group; $t_{35} = 2.51$, $^{*}P < 0.05$. **(H–K)** Results from the CVS mouse-model. **(H)** Increased GMPPB protein-levels in stressed mice; $n = 13–14$ per group; $t_{25} = 2.18$, $^{*}P < 0.05$. **(I)** Reduced *Gmppb*-RNA levels in CVS-group; $n = 10–12$ per group; $t_{20} = 2.13$, $^{*}P < 0.05$. **(J)** Increased oligomannose-levels in stressed mice; $n = 13–14$ per group; $t_{25} = 2.53$, $^{*}P < 0.05$. **(K)** Increased plasma mannose in CVS-group. $n = 19–21$ per group; $t_{38} = 2.44$, $^{*}P < 0.05$. **(D–K)** Individual data points are plotted and means ± s.e.m. are shown. A.U., Arbitrary units; Rel. FC, Relative fold change. Illustrations were generated with biorender.com.

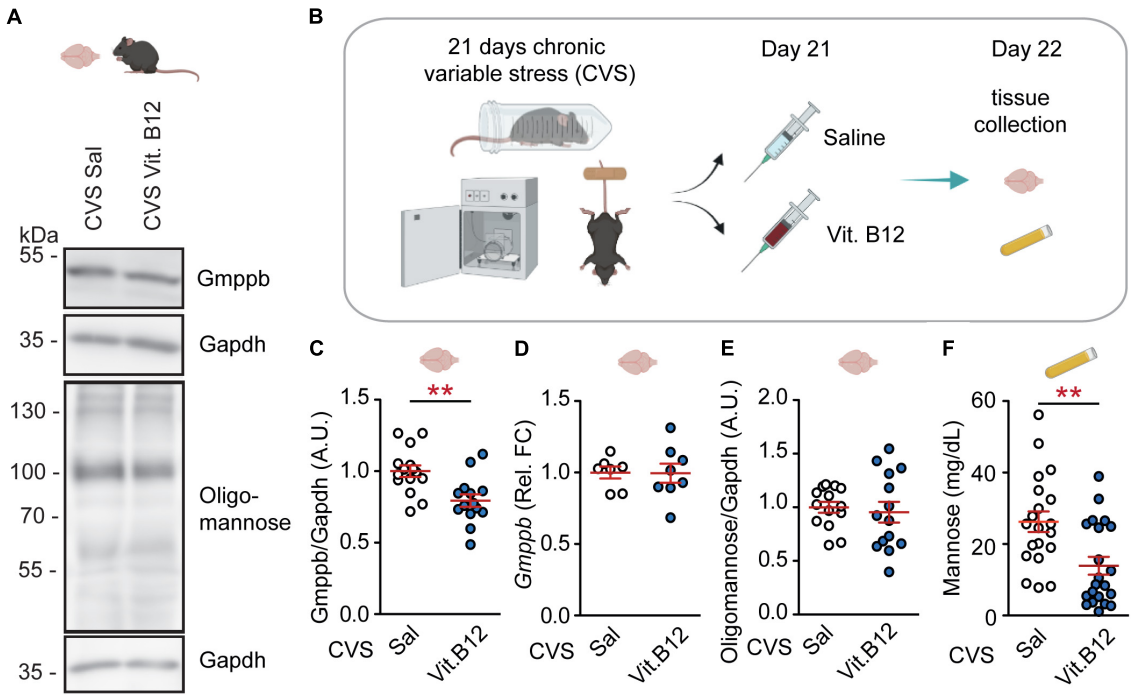

FIGURE 2
Vitamin B12 rapidly reverses GMPPB levels and plasma mannose in stressed mice. **(A)** Representative western blots. **(B)** Experimental setup for Vitamin B12-experiment. **(C–F)** Statistics: Student's *t*-test. **(C)** Vitamin B12 reduces GMPPB protein-levels in the PFC of stressed mice; $n = 14–15$ per group; $t_{27} = 3.43$, $^{**}P < 0.01$. **(D)** No effect of vitamin B12 on *Gmppb* transcription; $n = 7–8$ per group; $t_{13} = 0.05$, $P = 0.96$. **(E)** No effect of vitamin B12 on oligomannose abundance in the PFC; $n = 14$ per group; $t_{26} = 0.42$, $P = 0.68$. **(F)** Vitamin B12 reduces plasma mannose in CVS-treated mice; $n = 20–21$ per group; $t_{39} = 3.23$, $^{**}P < 0.01$. **(C–F)** Individual data points are plotted and means ± s.e.m. are shown. A.U., Arbitrary units. Illustrations were generated with biorender.com.

(Figures 1C,D). The change in GMPPB protein abundance was not accompanied by increased *GMPPB* transcripts, suggesting a regulation of the protein itself (Figure 1E). As GMPPB is a mediator of mannosylation, oligomannose levels in the PFC and plasma mannose levels were measured as well. While PFC oligomannose levels were not affected in depressed patients (Figure 1F), plasma mannose levels were increased (Figure 1G and Supplementary Figure 1).

In the CVS mouse-model, increased GMPPB was observed as well (Figure 1H). Here, too, *Gmppb* RNA-levels did not match the observed increase in protein abundance (Figure 1I). In the mouse model, both, PFC oligomannose and plasma mannose were increased (Figures 1J,K).

These data suggest that chronic stress and depression are indeed linked to altered PFC GMPPB and mannose metabolism. GMPPB and oligomannose changes in the CVS-model occurred in the PFC. No changes were observed in the hippocampus (Supplementary Figure 2). Moreover, GDP-mannose levels were significantly increased in murine plasma samples (Supplementary Figure 3). Mannose can be converted from other carbohydrates such as fructose and glucose (Supplementary Figure 4). Hence, plasma fructose and glucose levels were assessed in mice and human cohorts as well. While fructose was not affected in depressed patients or by CVS, glucose amounts were significantly reduced in depressed patients (Supplementary Figure 4).

Vitamin B12 rapidly reverses GDP-mannose pyrophosphorylase B- and plasma mannose-levels in chronically stressed mice

Previously, we have observed that a single dose of vitamin B12 at the end of a chronic stress paradigm can ameliorate symptoms associated with depression (15).

We observed that an acute dose of vitamin B12 reduced GMPPB protein-amounts in stressed mice (Figures 2A–C). *Gmppb* RNA-levels and PFC oligomannose were not affected (Figures 2D,E). Additionally, vitamin B12 decreased plasma mannose levels (Figure 2F) without affecting plasma fructose and glucose (Supplementary Figure 4). Moreover, plasma GDP-mannose levels were not altered by vitamin B12 (Supplementary Figure 3). These data suggest that in mice, a single dose of vitamin B12 can, at least in part, counteract chronic stress-induced changes in mannose metabolism.

GMPPA, an allosteric inhibitor of GDP-mannose pyrophosphorylase B, is affected by depression, but not chronic stress or vitamin B12

GMPPA is the allosteric feedback inhibitor of GMPPB (**Figure 1A**). GMPPA protein levels were increased in depressed patients (**Figures 3A,D**). However, they were not affected by chronic stress in untreated mice or by vitamin B12 in stressed mice (**Figures 3B–F** and **Supplementary Figure 5**). Hence, the observed GMPPB-changes appear to be selective in mice. In human depression cohorts, GMPPB-associated elevations in mannosylation might be counteracted by an increase in GMPPA abundance as a compensatory mechanism.

Discussion

Here we confirm a previous observation that altered GMPPB levels are increased in the postmortem PFC of depressed patients. We found similar changes in a mouse model of chronic stress and depression. Changes in mice were specific to the PFC and were not accompanied by altered GMPPA-levels. Notably, in a brain proteome-wide association study, Wingo et al. found not only GMPPB levels to be altered, but also beta 3-glucosyltransferase (B3GALTL). This indicates that various enzymes in the glycosylation process might be changed upon depression. We further observed increased plasma mannose-levels in patients suffering from depression. Unfortunately, it was not possible to obtain correlative data as the samples had to be taken from different alive and postmortem cohorts. Furthermore, we found that vitamin B12 can rapidly decrease GMPPB and plasma mannose in stressed mice.

While the CVS-model reflected main observations from human depressed patients, it differed in several details. For instance, *Gmppb* transcript-levels were reduced in stressed mice but not in depressed patients. Stressed mice, but not depressed patients, showed altered protein-bound oligo-mannose residues in the PFC. Plasma glucose-levels were decreased in depressed patients but not in stressed mice. In humans, but not mice, GMPPA levels were altered. These differences need to be considered when exploring functional links between GMPPB metabolism and depression. Causes may be species-differences or secondary biases due to life style-changes in depressed patients or sampling methods (e.g., altered dietary choices, fasting prior to testing in humans vs. mice, longer postmortem intervals in human samples). Furthermore, depressed patients were under medication (benzodiazepines for plasma cohort, various pharmaceuticals for the postmortem cohort), which may affect metabolic markers as well.

Based on the availability of samples, our data sets were skewed toward males and Caucasians. In order to ensure a wider applicability of findings, such biases should be avoided whenever possible in the future.

The discrepancy between regulation of *Gmppb* transcripts vs. proteins suggest a regulation on a protein level, e.g., via posttranslational modifications or proteasomal degradation. Furthermore, a clear link between vitamin B12 and carbohydrate mechanisms has not been explored. Being a regulator of methyl donors, it is conceivable that vitamin B12 affects the methylation of GMPPB or its' regulators, for instance on it's R357 residue (26). This possibility should be further investigated.

Currently it is unclear whether increased GMPPB abundance in the PFC will lead to increased plasma mannose levels, e.g., via degradation of glycoproteins. It is conceivable that, despite the observed brain-region specific alterations, GMPPB abundance may be altered in other tissues such as liver (27). We recently showed that hyperglycosylation of proteins is correlated with increased plasma mannose levels (2). Moreover, we showed that increased GMPPB levels correlate with protein hypermannosylation and enhanced plasma mannose levels (24). Altered glycosylation may affect protein stability and conformation, protein interactions and adhesion, as well as protein activity and localization (28).

In depressed patients and CVS mice, we found plasma mannose concentrations to be strongly increased, which may reflect increased release from glycans or increased generation of mannose from glucose or fructose. Although only approximately 2% of mannose entering the cell is used for glycosylation (29), the higher systemic mannose levels may contribute to the larger pool of GDP-mannose/hypermannosylated proteins in CVS mice and patients and thus hyperglycosylation.

In agreement with this, we detected increased plasma GDP-mannose levels in stressed mice. Plasma GDP-mannose levels are probably derived from blood cells or dead peripheral cells and not from an efflux from cells as reported for blood mannose (30). Plasma GDP-mannose levels were not affected by vitamin B12 in stressed mice. This may be due to previously observed tissue-specific effects of vitamin B12. Future studies might address blood glycoproteins and their regulation through vitamin B12 as well.

It has been shown that reduced GMPPB abundance leads to decreased GDP-mannose levels and thus affects neuronal and muscle development (4, 31, 32). For example, motor neurons were shortened (4), and an early marker of pan-neuronal cells was remarkably decreased in GMPPB knockdown zebrafish (33). Notably, GDP-mannose supplementation restored GDP-mannose levels, protein mannosylation and thus muscle and neuronal defects (4). Another study showed that GDP-fucose supplementation in a GDP-mannose 4,6 dehydratase mutant zebrafish where fucosylation was decreased restored protein fucosylation and the neuronal phenotype (34). However, it has been shown that extremely elevated GDP-mannose levels

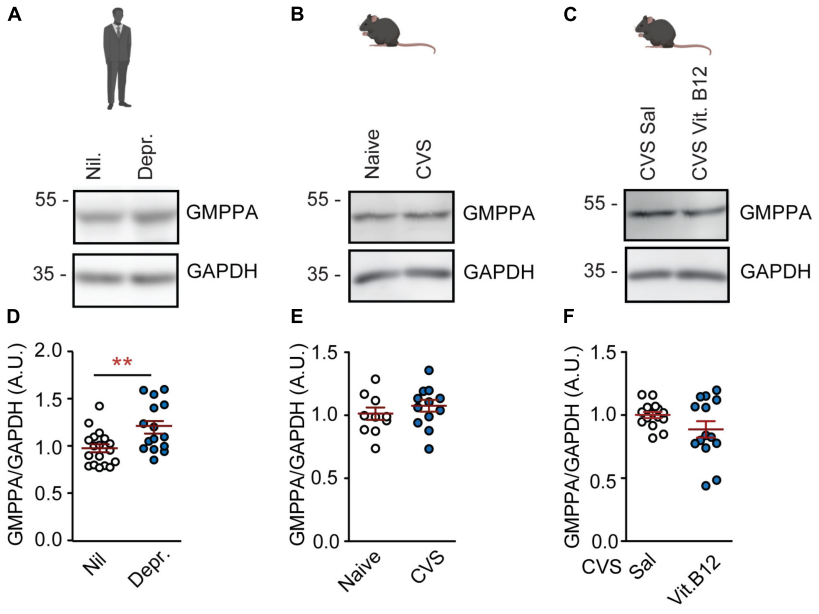

FIGURE 3
GMPPA levels are not associated with depression or chronic stress in the PFC. **(A)** Representative western blots for GMPPA and housekeeping gene, GAPDH on prefrontal cortex tissue. **(A)** Human cohorts. **(B)** Naïve mice and CVS model. **(C)** Vitamin B12 treatment in stressed mice. **(D–F)** Statistics: Student's *t*-test. **(D)** Increased GMPPA abundance in depression; $n = 15–18$ per group; $t_{31} = 2.91$, $^{**}P < 0.01$. **(E)** GMPPA is not altered by chronic stress; $n = 13–15$ per group; $t_{26} = 1.10$, $P = 0.28$. **(F)** Vitamin B12 does not affect GMPPA levels; $n = 13–15$ per group; $t_{26} = 1.95$, $P = 0.06$. **(D–F)** Individual data points are plotted and means ± s.e.m. are shown. A.U., Arbitrary units. Illustrations were generated with biorender.com.

affect neuron morphology and development (4). Thus, normal neuron function likely depends on a balanced GDP-mannose homeostatis mediated by GMPPB and GMPPA.

A possible link between GMPPB and plasma mannose levels may be addressed using GMPPB mutant mice. Furthermore, the impact of a mannose-enriched or mannose-depleted diet on symptoms of chronic stress and depression could be investigated.

This study further supports the rapid stress-ameliorating effects of vitamin B12. The current findings add a metabolic and possible protein-regulatory level to the previously observed changes in behavioral and transcriptional markers, highlighting a multidimensional impact of vitamin B12. To date, the vitamin B12 induced stress reversal has been observed in two different mouse models of chronic stress.

Hence, vitamin B12 should be tested as a rapid dietary intervention to treat symptoms associated with chronic stress and depression in human cohorts. Blood samples may be taken to investigate an impact on plasma mannose in addition to mood-related measures. This would allow in patient-correlations and may perhaps provide a therapeutic approach using a widely available, affordable, well-tolerated and rapid acting molecule to improve symptoms of stress and depression.

Despite the missing functional link between vitamin B12 and mannose metabolism, this study provides several novel insights: (1) Systemic mannose levels are altered in depressed patients and in a mouse model. The fact that vitamin B12 rapidly reverses plasma mannose changes in stressed mice demonstrates the dynamic nature of this marker. (2) Vitamin B12 rapidly normalizes metabolic correlates of depression in a mouse model. Together with previous behavioral and molecular data, this study further encourages testing of vitamin B12 as a fast-acting intervention to chronic stress.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. Raw data western blot images are shown in **Supplementary Figure 5**.

Ethics statement

The studies involving human participants were reviewed and approved by the 2020-1862-Material. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the UKJ-18-037. Human plasma samples were obtained in agreement with the Ethics committee of Magdeburg University Hospital, Germany (110/07).

Author contributions

OE provided the study design and ideas, conducted chronic-stress and RNA-experiments, and wrote the manuscript. PF performed the western blots and sugar measurements in plasma and performed the statistical analyses. SC performed the qPCR. GT and JS provided the human samples. TK analyzed the GDP-mannose levels. CH and MW provided the financial support and mentorship. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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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.2022.981511/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Demography of human cohorts. **(A)** Postmortem prefrontal cortex tissue. **(B)** Plasma samples. **(C)** Plasma-mannose levels (from **Figure 1**) analyzed by sex; $n = 9$ per group; 2-way ANOVA: effect of depr.: $F(1, 32) = 13.68$, $P = 0.0008$; effect of sex: $F(1, 32) = 0.02$, $P = 0.88$; no interaction: $F(1, 32) = 2.14$, $P = 0.15$; Bonferroni *post hoc* test: effect of depr. within females: $^{**}P < 0.01$; all other comparisons: $P > 0.05$. **(D)** Legend with abbreviations. **(C)** Individual data points are plotted and means ± s.e.m. are shown. Illustrations were generated with biorender.com.

SUPPLEMENTARY FIGURE 2
Hippocampal GMPPB and oligomannose are not affected by chronic stress or depression. **(A)** Representative western blots on hippocampal tissue. **(B–D)** Statistics: Student's *t*-test. **(B)** No difference in GMPPB-levels; $n = 9$ per group; $t_{16} = 1.10$, $P = 0.29$. **(C)** No difference in GMPPA-levels; $n = 10$ per group; $t_{18} = 0.69$, $P = 0.50$. **(D)** No difference in oligomannose-levels; $n = 9–10$ per group; $t_{17} = 0.18$, $P = 0.86$. **(B–D)** Individual data points are plotted and means ± s.e.m. are shown. A.U., Arbitrary units. Illustrations were generated with biorender.com.

SUPPLEMENTARY FIGURE 3
GDP-Mannose is increased in plasma of chronically stressed mice but not altered by vitamin B12. **(A,B)** Statistics: Student's *t*-test. **(A)** GDP-mannose is affected by CVS. $n = 9–10$ per group; $t_{17} = 2.95$, $^{**}P < 0.01$. **(A)** GDP-mannose is not affected by vitamin B12 in the CVS group. $n = 9–10$ per group; $t_{17} = 1.21$, $P = 0.24$. Individual data points are plotted and means ± s.e.m. are shown. A.U., Arbitrary units.

SUPPLEMENTARY FIGURE 4
Fructose and glucose levels in chronic stress and depression. **(A)** Overview of conversion pathway between mannose, fructose and glucose. **(B–G)** Statistics: Student's *t*-test. **(B,C)** Plasma sugar levels in human cohorts. **(B)** Fructose-levels are not significantly altered in depressed patients; $n = 19–20$ per group; $t_{37} = 1.86$, $P = 0.07$. **(C)** Glucose levels are reduced in depressed patients. $n = 19$ per group; $t_{36} = 3.80$, $^{***}P < 0.001$. **(D,E)** Plasma sugar levels in mice. **(D)** Fructose levels are not affected by CVS; $n = 19–20$ per group; $t_{37} = 1.15$, $P = 0.26$. **(E)** Glucose levels are not altered by CVS; $n = 19–21$ per group; $t_{38} = 1.47$, $P = 0.15$. **(F)** Vitamin B12 does not affect plasma fructose levels; $n = 20–21$ per group; $t_{39} = 0.13$, $P = 0.90$. **(G)** Plasma glucose levels are not affected by vitamin B12 in stressed mice; $n = 20$ per group; $t_{38} = 1.96$, $P = 0.06$. **(B–G)** Individual data points are plotted and means ± s.e.m. are shown. Illustrations were generated with biorender.com.

SUPPLEMENTARY FIGURE 5
Full length western blots.

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Effects of maternal iodine nutritional status on neurodevelopmental and cognitive function of rat offspring

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Objectives: This study aimed to explore the effect of maternal iodine status on the brain development of offspring in rats. Since in human studies, the interference of environmental factors and other nutrients cannot be removed.

Materials and methods: A total of 48 female Wistar rats were randomly divided into four groups: low iodine (LI), normal iodine (NI), 10-fold high iodine (10HI), and 50-fold high iodine (50HI). The rats were killed on the 15th day of pregnancy and lactation after collecting 24-h urine. The iodine concentration in 24-h urine, blood, and placenta of pregnant rats, and 24-h urine, milk, blood, and mammary glands of lactating rats was determined by inductively coupled plasma mass spectrometry. The thyroid hormone of pregnant and lactating rats was detected by chemiluminescence. The offspring were subjected to the Morris water maze on the 10th day after birth. Serum was collected to detect the thyroid hormone of offspring. The protein expression of neuroendocrine-specific protein (NSP)-A and brain-derived neurotrophic factor (BDNF) in the offspring brain were studied.

Results: Iodine storage in the placenta during pregnancy and mammary glands during lactation was positively correlated with iodine intake, and iodine storage in the placenta and mammary glands in the 50HI group was significantly higher than that in the NI group ($P = 0.045$ and $P = 0.040$). Compared with the NI group, the offspring thyroid-stimulating hormone (TSH) level was significantly higher in the 10HI group ($P = 0.046$), and the FT4 level was significantly lower in the 50HI group ($P = 0.032$). The Morris water maze showed that LI and 50HI groups required longer time and distance to find the platform than the NI group ($P < 0.001$). The platform crossing numbers in the LI and 50HI groups decreased significantly ($P < 0.001$). The expression of NSP-A in offspring brain was lower in the 10HI and 50HI groups than in the

NI group (P = 0.026 and P = 0,008). BDNF expression levels were significantly lower in the LI, 10HI, and 50HI groups than in the NI group ($P < 0.001$).

Conclusion: Maternal iodine intake affects iodine storage in the placenta and lactating mammary gland, which in turn affects thyroid function and BDNF and NSP-A expression in the offspring.

KEYWORDS

maternal iodine excess, maternal iodine deficiency, offspring neurodevelopment, placenta, mammary gland

Introduction

Infants are more sensitive to abnormal changes in iodine concentration than other age-groups as they have the highest iodine requirements per kilogram of body weight, but the thyroid stores a small amount of iodine with only about 300 μg even in iodine-rich areas (1). Inappropriate iodine nutrition leads to thyroid dysfunction. Thyroid hormone is particularly critical for fetal and infant neurodevelopment (2, 3). However, the effect of maternal iodine nutrition status in the first 1,000 days of life on the nerve and intelligence of offspring is controversial. A 9-year follow-up of the gestational iodine cohort revealed that even mild iodine deficiency during pregnancy could have long-term adverse impacts on fetal neurocognition, which were not ameliorated by iodine sufficiency during childhood (4). A U.K. study found that when the maternal urinary iodine-to-creatinine ratio was 50–150 μg/g (which indicates mild to moderate iodine deficiency), offspring had lower scores in the verbal intelligence quotient, reading accuracy, and reading comprehension (5). However, a randomized, double-blind, and placebo-controlled trial including 832 pregnant women with mild iodine deficiency (with a median urinary iodine concentration of 131 μg/L) has found that mild iodine deficiency in pregnancy has no effect on the development of offspring. This may be because pregnant women are able to physiologically adapt to mildly low iodine intakes during pregnancy, draw from intrathyroidal iodine stores, and maintain fetal euthyroidism, allowing for normal *in utero* development (6). In addition, iodine excess during pregnancy may lead to hypothyroidism in the fetus. Rapid normalization of fetal/newborn thyroid function is necessary to prevent neurological damage (7). An experiment of rats indicated that the excess iodine leads to the impairment of learning and memory, and it may be mediated *via* the mitochondrial apoptotic pathway. Long-term repetitive excess iodine exposure affects monoamine neurotransmitters in the hippocampus of rat offspring (8). But another study has shown that neurodevelopmental and cognitive deficits in pups were mild and temporary when maternal rats were given three times the normal amount of iodine (9). Therefore, the effects of iodine deficiency and excess on nerves and growth in infants need further study.

Maternal iodine is the only source of iodine for offspring during pregnancy and exclusive breastfeeding. During pregnancy, maternal iodine is passed through the placenta to the fetus (10). After parturition, breast milk is the only source of iodine for newborns (11, 12). Therefore, detecting the iodine content in the placenta during pregnancy and mammary gland during lactation more accurately reflects the iodine intake of offspring. During critical periods of brain development (from the beginning of pregnancy to early birth), the development of the nervous system is dependent on thyroid hormones (13). Tetraiodothyronine (T4) and triiodothyronine (T3) are the main hormones produced by the thyroid and are essential for growth, development, and metabolism in vertebrates (14). Maternal hypothyroidism affects the expression of fetal and newborn *brain-derived neurotrophic factor (BDNF)* (15, 16) and *neuroendocrine-specific protein (NSP)-A* (17), both of which are important mediators of thyroid hormone and have essential roles in brain development.

In this study, different concentrations of potassium iodide (KI) were added to the drinking water of female rats to construct pregnant and lactation rats with different iodine nutritional statuses. The diets and environments of female rats in each group were consistent. The Morris water maze (MWM) test was carried out for the offspring, and the expression of *BDNF* and *NSP-A* in the brain was detected to investigate the effect of maternal iodine status on the brain development of offspring.

Materials and methods

Animals and treatments

In total, 48 female and 24 male Wister rats aged 4 weeks after weaning were purchased from SBF Beijing Biotechnology Co.,

Ltd (Beijing, China). This study was approved by the Animal Research Committee of Tianjin Hospital of ITCWM Nankai Hospital (NKYY-DWLL-2021-048). The rats were maintained in standard cages at 22 ± 2°C, with a relative humidity of 40–80% under a 12-h light/12-h dark cycle and given free access to food and water.

The female rats were randomly divided into four groups (n = 12 rat/group): low iodine (LI, 1 μg/d), normal iodine (NI, 6 μg/d), 10-fold high iodine (10HI, 60 μg/d), and 50-fold high iodine (50HI, 300 μg/d). The rats in the LI group were provided with deionized water daily, and the other groups were given deionized water supplemented with different concentrations of KI. All rats were given a low iodine diet with plenty of other nutrients, and the average dietary iodine content was 50 mg/kg (Trophic Animal Free High-tech Co. Ltd., Jiangsu, China, TP016ID103). The male rats were fed the same as female rats in the NI group.

After 10 weeks of conditional intervention, the female rats cohabited with males (female:male = 1:1). A total of six maternal rats were selected randomly from each group to collect 24-hour (24-h) urine at the 15th day of pregnancy, whereafter, they were anesthetized with pentobarbital (40 mg/kg, intraperitoneally), and the blood and placenta were removed and weighed. To rule out other factors affecting the growth and development of the offspring and ensure consistent breastfeeding, each maternal rat kept only five pups after delivery. The day of birth was defined as postnatal day (PND) 0. On PND10, the Morris water maze (MWM) test lasted 6 days and was performed for pups to evaluate the ability of spatial learning and memory. Rat milk and 24-h urine were collected for 2 consecutive days begining on PND15 to determine iodine levels. Then, the rats were anesthetized, and the blood and mammary glands of the maternal rats were removed and weighed, as well as the blood and brain of offspring.

Urine

At 15 days of gestation and 15 days of lactation, six female rats in each group were placed in metabolism cages for 2 days to collect 24-h urine from the maternal rats, and then the urine weight was recorded.

Rat milk

The maternal rats were anesthetized with isoflurane and kept anesthetized during milk extraction. Then, the rats were intramuscularly injected with 0.5 mL of veterinary prolactin. After 5 min, the nipples were rubbed gently, and about 0.5 mL of milk was collected using Pasteur straws. Milk was randomly collected three times in 2 days to determine the iodine concentration in milk.

Measurement of iodine in tissue, urine, and serum

The iodine concentration of urine, milk, serum, placenta, and mammary glands was detected and analyzed by inductively coupled plasma mass spectrometry, ICP-MS (iCAP Q, Thermo Fisher Scientific, Frankfurt am Main, Germany) using Te for mass bias correction.

Thyroid function tests

Thyroid-stimulating hormone (TSH), free triiodothyronine (FT3), and free tetraiodothyronine (FT4) were measured using an automatic Immulite analyzer with a chemiluminescent kit (Sophonix, Beijing, China). Before testing the sample, each kit was controlled with a calibration solution and quality control product. Then 100 μL of calibration solution and quality control product were to each reagent. The samples were tested only after successful calibration and quality control, and each item requires 100 μL serum.

Morris water maze test

The MWM test was used to assess the ability of spatial learning and memory in pups about 10 days after birth. The MWM test was performed with the DMS-2 Morris water maze test system (Institute of Materia Medica at Chinese Academy of Medical Sciences, Beijing, China). The MWM test has two phases, namely, an acquisition test and a spatial probe trial. During the 5 consecutive days of the acquisition test, each pup was released from three random locations (except for the quadrant where the platform is). The platform was 2 cm underwater. The experiment was terminated when the pup reached the hiding platform. If the pup failed to locate the platform within the 60 s, it was gently guided onto the platform and allowed to stay there for 30 s, and the escape latency was recorded as 60 s. The spatial probe trial was tested on the 6th day of the MWM test, and the platform was removed. The test time was 60 s, and the crossing numbers on the location of the removed platform were recorded.

HE staining

HE staining of the brain sections was performed with an HE Staining Kit (Beyotime, Nantong, China) according to the manufacturer's instructions. Images (at × 20 magnification) were captured under an Olympus IX81 microscope (Olympus, Tokyo, Japan) in the bright-field mode.

Nissl staining

According to the manufacturer's instructions, the brain sections were stained with cresyl violet (Beyotime, Nantong, China). Neurons with discernable and rich Nissl staining were counted as viable neurons, whereas neurons with lost Nissl bodies and condensed cytoplasm were considered damaged neurons. Images from the cerebral cortex and hippocampal CA1 region (at × 200 magnification) were captured by an Olympus IX81 microscope (Olympus, Tokyo, Japan) in the bright-field mode, and the number of surviving neurons was analyzed by NIH ImageJ 1.61 software (National Institutes of Health, Bethesda, MD, USA).

Western blot analysis

Brains of offspring were homogenized in RIPA buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton-X100, 0.5% sodium deoxycholate, 1 mM PMSF, and 10 μg/ml leupeptin), incubated on ice for 15 min, and centrifuged at 14,000 g for 10 min at 4°C. Protein concentrations were determined by a Bicinchoninic Acid (BCA) Protein Assay Kit (Beyotime, Nantong, China). The protein samples were fractionated through SDS-PAGE and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The PVDF membranes were blocked with 5% BSA (Sigma, Darmstadt, Germany) in 1 × Tris-buffered saline Tween for 1 h at room temperature. The membranes were incubated with primary antibodies of mouse anti-NSP-A (1:500; Santa Cruz, CA, USA), rabbit anti-BDNF (1:1000; Bioss, Beijing, China), and rabbit anti-GAPDH (loading control, 1:5000; Bioss, Beijing, China) overnight at 4°C. After washing with TBST, they were incubated with relational horseradish peroxidase [HRP]-conjugated secondary antibodies for 1 h at room temperature. Then, the proteins were detected by chemiluminescence reagents (Sparkjade, Shandong, China) and observed using a ChemiDocTM XRS + Imaging System (Bio-Rad, Hercules, CA, USA). The protein levels were quantified by densitometry using NIH ImageJ 1.61 software (National Institutes of Health, Bethesda, MD, USA).

Immunohistochemistry

After the brains of the offspring were removed, they were immediately fixed with 4% paraformaldehyde and then embedded in paraffin. The sections were dewaxed and rehydrated, and the next steps were performed with rabbit-specific HRP/DAB (ABC) IHC Detection Kit ab64261(Abcam, Cambridgeshire, UK). The sections of brains were incubated with a primary antibody of rabbit mouse anti-NSP-A (1:100; Santa Cruz, CA, USA) and rabbit anti-BDNF (1:100; Bioss, Beijing, China) overnight at 4°C. Then, the biotinylated secondary antibody was bound to the primary antibody, and the HRP-labeled streptavidin was bound to the secondary antibody. The HRP produced a brown-colored substance at the site of primary antibody binding by reacting with DAB. The images were obtained with an inverted microscope (IX81; Olympus, Tokyo, Japan).

Statistical analysis

The statistical software package SPSS Statistics version 20.0 (Armonk, NY, USA) and GraphPad Prism v7 (GraphPad Software Inc., San Diego, CA, USA) were used for statistical analysis. The variables conforming to a normal distribution were expressed as means ± SD. Comparisons between different groups were performed by a one-way analysis of variance (ANOVA), followed by a Student–Newman–Keuls test for multiple comparisons. The variables without normal distributions were expressed as M (P25, P75), and comparisons between groups were performed by using a Kruskal–Wallis test. Repeated-measures data of escape latency in the MWM test were compared by ANOVA. $P < 0.05$ was considered statistically significant.

Result

Iodine nutritional status and thyroid function during pregnancy in rats

Serum was collected on the 15th day of pregnancy to detect serum iodine, and 24-h urine was collected from the metabolic cage for 2 days. The serum total iodine concentration (STIC) and 24-h urinary iodine concentration (UIC) of the 50HI group were significantly higher than those of the NI group ($P = 0.020$ and $P = 0.020$, Table 1).

Results of the thyroid function test during pregnancy are shown in Table 2. Compared with the NI group, serum FT4 levels were significantly increased in the 10HI and 50HI groups ($P = 0.010$ and $P = 0.012$).

Iodine stored in the placenta during pregnancy in rats

The placenta iodine concentration was positively correlated with iodine intake, and there was a significant change between the NI and 50HI groups ($P = 0.012$). With the increase in iodine intake, the placental iodine storage tended to increase, and the placental iodine storage in the 50HI group was significantly higher than that in the NI group ($P = 0.045$, Table 3).

Iodine nutritional status and thyroid function during lactation in rats

On the 15th day of lactation, we collected 24-h urine, serum, and milk from the maternal rats. As shown in Table 4, maternal 24h-UIC, STIC, and breast milk iodine concentration (BMIC) increased as iodine intake increased. The STIC, UIC, and BMIC of the 50HI groups were significantly higher than those of the NI group ($P = 0.020$, $P = 0.017$ and $P = 0.017$).

The thyroid function of the rats during lactation is shown in Table 5. Compared with the NI group, serum FT4 levels were significantly increased when exposed to 10HI and 50HI doses ($P = 0.037$ and $P < 0.001$).

TABLE 1 Serum iodine and 24-h urinary iodine concentration of pregnant rats.

Group	N	STIC (μg/l)	24-h UIC (μg/l)
LI	6	15.85 (10.31, 20.02)	87.45 (78.71, 95.64)
NI	6	42.28 (37.09, 49.17)	369.96 (312.10, 433.28)
10HI	6	144.99 (129.17, 164.23)	3569.18 (3287.96, 3793.56)
50HI	6	757.23 (703.17, 910.12)[a]	12286.04 (11470.80, 13362.59)[b]
P-value		<0.001	<0.001

STIC, serum total iodine concentration; UIC, urinary iodine concentration.
[a]Compared with the NI group, $P = 0.020$.
[b]Compared with the NI group, $P = 0.020$.

TABLE 2 TSH, FT3, and FT4 concentration of pregnant rats.

Group	N	TSH (mIU/L)	FT3 (pg/mL)	FT4 (pg/mL)
LI	6	0.02 ± 0.01	2.01 ± 0.28	6.00 ± 0.92
NI	6	0.02 ± 0.02	1.81 ± 0.13	7.11 ± 1.38
10HI	6	0.01 ± 0.01	1.85 ± 0.31	11.96 ± 2.95[a]
50HI	6	0.04 ± 0.04	2.02 ± 0.32	11.49 ± 4.30[b]
P-value		0.734	0.400	0.008

TSH, thyroid-stimulating hormone; FT3, free triiodothyronine; FT4, free tetraiodothyronine.
[a]Compared with the NI group, $P = 0.010$.
[b]Compared with the NI group, $P = 0.012$.

TABLE 3 Concentration and content of iodine in the placenta of pregnant rats.

Group	n	Placenta	
		Concentration (μg/g)	Storage (μg)
LI	6	0.02 (0.01, 0.08)	0.16 (0.04, 0.47)
NI	6	0.07 (0.04, 0.11)	0.59 (0.26, 0.87)
10HI	6	0.17 (0.11, 0.24)	0.32 (0.16, 0.90)
50HI	6	0.60 (0.37, 0.78)[a]	3.14 (0.65, 4.98)[b]
P-value		<0.001	0.022

[a]Compared with the NI group, $P = 0.012$.
[b]Compared with the NI group, $P = 0.045$.

TABLE 4 Iodine concentrations in 24-h urine, blood, and milk of maternal rats.

Group	N	STIC (μg/l)	24-h UIC[a] (μg/l)	BMIC[b] (μg/l)
LI	6	11.98 (79.51, 90.58)	85.56 (79.51, 90.58)	58.30 (48.26, 91.36)
NI	6	31.96 (28.61, 39.97)	206.61 (189.19, 298.43)	132.27 (115.34, 178.72)
10HI	6	210.09 (189.34, 235.65)	4143.90 (3333.18, 4880.84)	1057.15 (870.07, 1277.13)
50HI	6	654.93 (586.16, 684.29)[c]	13202.03 (12141.22, 15248.98)[d]	9801.73 (9296.99, 12279.88)[e]
P-value		<0.001	<0.001	<0.001

UIC, urinary iodine concentration; STIC, serum total iodine concentration; BMIC, breast milk iodine concentration.
[a]Urinary iodine concentration was average value over 2 days.
[b]Breast milk iodine concentration was the average value of three random samples.
[c]Compared with the NI group, $P = 0.020$.
[d]Compared with the NI group, $P = 0.017$.
[e]Compared with the NI group, $P = 0.017$.

TABLE 5 TSH, FT3, and FT4 concentration of lactating rats.

Group	N	TSH (mIU/L)	FT3 (pg/mL)	FT4 (pg/mL)
LI	6	0.01 ± 0.01	1.72 ± 0.45	8.45 ± 2.58
NI	6	0.05 ± 0.15	1.68 ± 0.45	9.76 ± 2.01
10HI	6	0.03 ± 0.05	1.60 ± 0.21	13.26 ± 3.78[a]
50HI	6	0.03 ± 0.02	1.98 ± 0.25	18.84 ± 5.26[b]
P-value		0.801	0.313	<0.001

TSH, thyroid-stimulating hormone; FT3, free triiodothyronine; FT4, free tetraiodothyronine.
[a]Compared with the NI group, $P = 0.037$.
[b]Compared with the NI group, $P < 0.001$.

Iodine stored in mammary glands during lactation in rats

During lactation, the main iodinated tissue is the mammary gland, in addition to the thyroid (18). The amount of iodine stored in the mammary gland more directly reflects the iodine intake of the newborn. Results of iodine concentration and storage in the mammary gland are shown in Table 6. With the increase in maternal iodine intake, the iodine concentration and storage in the mammary gland also increased, and the difference of the concentration ($P = 0.017$) and storage ($P = 0.040$) of iodine in the mammary gland between the 50HI group and the NI group was significant.

Iodine nutritional status and thyroid function of rat offspring

Our results show that the iodine nutrition status of the mother affected the iodine nutrition status and thyroid function

of the offspring. One pup per female was randomly selected for brachial artery blood sampling for serum iodine and thyroid function (Table 7). As iodine intake increased, the STIC gradually increased, and the difference in the STIC between the 50HI group and the NI group was significant (P = 0.003). Compared with the NI group, the TSH level was significantly higher in the 10HI group (P = 0.046), and the FT4 level was significantly lower in the 50HI group (P = 0.032).

Effects of maternal iodine malnutrition on the brain development of rat offspring

The MWM test was performed to evaluate spatial learning and memory ability. Representative swimming paths of rat offspring in the LI, NI, 10HI, 50HI groups on the 5th day (Figure 1A). Decreased values for escape latency and increased values for the platform crossing numbers, respectively, indicate better spatial learning ability and better memory ability. The MWM test was performed on five pups per female. With the increase in training time, escape latency decreased in all four groups (Figure 1B). Repeated-measures ANOVA showed that the LI and 50HI groups required longer time and distance to find the platform than the NI group ($P < 0.05$). The poor memory skill of LI and 50HI groups was also shown by the fewer platform crossing numbers measured on the 6th day (Figure 1C). The results of the MWM test showed that maternal iodine deficiency and excess affect the brain development of offspring.

TABLE 6 Iodine concentration and content in the mammary gland of lactating rats.

Group	N	Mammary gland	
		Concentration (μg/g)	Storage (μg)
LI	6	0.04 (0.02, 0.07)	0.75 (0.30, 1.16)
NI	6	0.06 (0.05, 0.10)	1.17 (0.66, 2.82)
10HI	6	0.48 (0.30, 0.80)	6.55 (2.86, 9.47)
50HI	6	5.22 (0.76, 8.50)[a]	90.37 (9.08, 158.53)[b]
P-value		<0.001	0.003

[a]Compared with the NI group, P = 0.017.
[b]Compared with the NI group, P = 0.040.

TABLE 7 Evaluation of iodine status and thyroid function of rat offspring.

Group	N	Iodine nutrition	Thyroid function		
		STIC (μg/L)	TSH (mIU/L)	FT3 (pg/mL)	FT4 (pg/mL)
LI	6	64.41 (51.66, 122.30)	0.005 ± 0.002	2.36 ± 0.48	23.41 ± 4.23
NI	6	105.79 (73.18, 126.78)	0.006 ± 0.001	1.91 ± 0.45	24.65 ± 9.51
10HI	6	635.36 (481.11, 665.84)	0.008 ± 0.03[b]	1.74 ± 0.44	21.77 ± 6.91
50HI	6	2956.58 (2130.11, 4486.79)[a]	0.007 ± 0.002	1.82 ± 0.43	15.70 ± 8.78[c]
P-value		<0.001	0.063	0.075	0.154

STIC, serum total iodine concentration; TSH, thyroid-stimulating hormone; FT3, free triiodothyronine; FT4, free tetraiodothyronine.
[a]Compared with the NI group, P = 0.003.
[b]Compared with the NI group, P = 0.046.
[c]Compared with the NI group, P = 0.032.

Effects of maternal iodine malnutrition on neuronal morphology in rat offspring

HE staining was performed to observe neuronal morphology in the cerebral cortex and hippocampal CA1 region. In the NI group, the majority of the neurons contained abundant cytoplasm with well-defined nuclei. Some of the neurons had disordered tissue arrangement in the LI, 10HI, and 50HI groups. Irregular cell morphology, pyknosis, unclear nucleolus, and deep staining of cytoplasm were found (Figures 2A,B). Nissl staining was performed to quantify neuronal survival. The neurons in the cerebral cortex and hippocampal CA1 region of the four groups were arranged densely and orderly, with abundant Nissl bodies in the cytoplasm. There is also no significant difference among the four groups (Figures 2C,D).

Effects of maternal iodine malnutrition on the expression of BDNF and NSP-A in the brain of the offspring

NSP-A and BDNF are affected by maternal thyroid hormones and have essential roles in brain development. Photomicrographs of the immunohistochemistry-stained brains of offspring on PND15 showed positive expression of NSP-A in the cerebral cortex (Figure 3A) and BDNF in the hippocampal CA1 region (Figure 3B) in all treatment groups. The general protein expressions of NSP-A and BDNF are shown in Figure 3C. The expression of NSP-A was lower in the 10HI and 50HI groups than in the NI group ($P < 0.05$, Figure 3D). The BDNF expression level was significantly lower in the LI, 10HI, and 50HI groups than in the NI group ($P < 0.05$, Figure 3E).

Discussion

In this study, we examined placenta iodine storage during pregnancy and mammary gland iodine storage during lactation

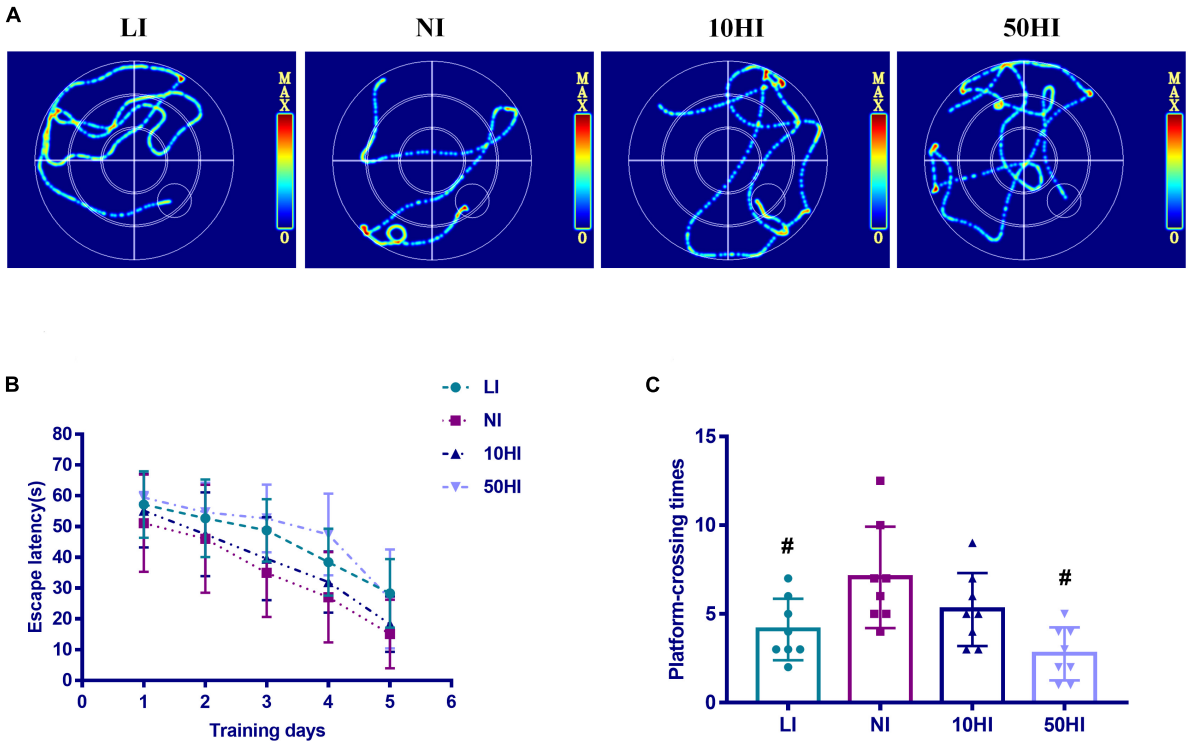

FIGURE 1
(A) Representative swimming paths. **(B)** Escape latency during the spatial acquisition phase of the MWM test. **(C)** Crossing numbers on the location of the removed platform during the spatial probe phase of the MWM test. (Values are shown as mean ± SD. *N* = 30 per group. Repeated-measures data of escape latency in the MWM test were compared using the repeated-measures ANOVA. One-way ANOVA and LSD tests were used to compare the number of crossing platforms between groups. $^{\#}P < 0.05$ compared with the NI group).

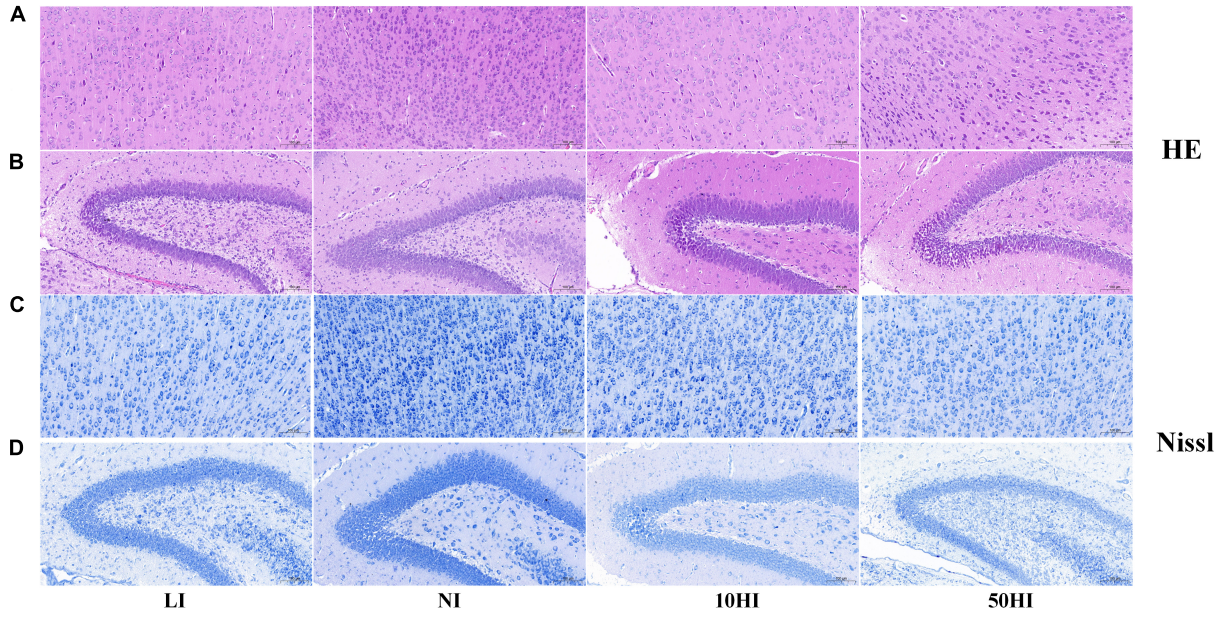

FIGURE 2
Representative micrographs of HE staining in the pup cerebral cortex **(A)** and hippocampal CA1 region **(B)**. Representative micrographs of Nissl staining in the pup cerebral cortex **(C)** and hippocampal CA1 region **(D)**. Scale bars: 50 mm.

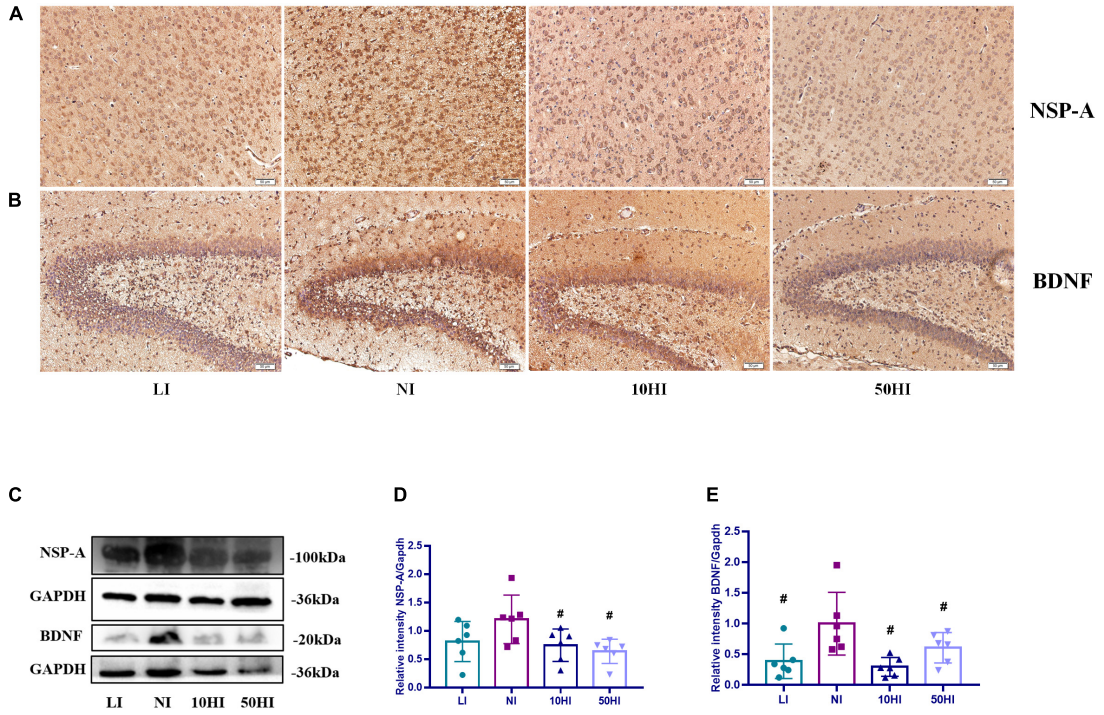

FIGURE 3
(A) Localization of NSP-A in the cerebral cortex. **(B)** Localization of BDNF in the hippocampal DG region. Scale bars: 50 μm. **(C)** Representative Western blot results of NSP-A and BDNF. Bar graphs show the semiquantitative levels of NSP-A **(D)** and BDNF **(E)** determined by band density analysis. (Values are shown as mean ± SD. $N = 6$ per group. Comparisons among different groups were performed by one-way ANOVA, followed by an LSD test for multiple comparisons.$^{\#}P < 0.05$ compared with the NI group).

in female rats, which more directly reflect iodine intake levels in the early life of offspring. Different concentrations of KI were added to the drinking water of female rats, and the diets and environments of maternal rats in each group were consistent. Our study clarifies the effects of maternal iodine nutrition levels during pregnancy and lactation on offspring brain development, overcoming the inability to control the effects of environmental and other nutrients on offspring brain development in human studies in addition to iodine.

Our findings showed that maternal iodine intake impacted the iodine content in the placenta. The placental iodine storage in the 50HI group was significantly higher than that in the NI group. Previous studies also support that the placenta stores iodine in a concentration-dependent manner and serves as long-term storage and supply (19–21). There was no statistically significant difference between the LI and 10HI groups and the NI group because the placenta is an important organ that forms a barrier where the maternal and fetal exchange occurs, which has a role in regulating abnormal maternal iodine status (22). Therefore, testing only maternal iodine intake and UIC in human studies does not accurately reflect the iodine received by the fetus.

Breast milk is currently considered the ideal natural food for the growth and development of infants. The energy and nutrients in breast milk meet the growth and development needs of infants up to 6 months of age (23). The calcium content in milk remains stable, regardless of whether the lactating mother has sufficient dietary calcium or not. When dietary calcium intake is insufficient, in order to maintain the milk calcium content constant, it is necessary to use the maternal bone calcium (24, 25). This explains mammary glands have certain regulation ability to the abnormal nutrients of the maternal body. During lactation, there is increased expression of NIS, which mediates iodine uptake into the mammary gland, and a portion of the body iodine is transferred to the mammary gland (26). It is uncertain whether breast NIS compensates for milk iodine. However, the results of this our study showed a positive correlation between the BMIC and iodine intake, but only the 50HI group was significantly higher than the NI group ($P = 0.017$). Therefore, the compensatory ability of the mammary gland for iodine deficiency and excess during lactation needs further study.

Maternal thyroid hormone plays an important role in fetal brain development. It regulates brain morphological and biochemical changes before the fetal thyroid is functioning, which occurs in the first trimester of pregnancy (27, 28). During pregnancy and early lactation, iodine of the offspring is derived completely from the mother. Excessive and insufficient iodine may cause hypothyroidism (29, 30). In the fetus and the infant, iodine deficiency affects brain development, leading to a decline

in mental activity and, in extreme cases, cretinism (31–33). Studies on the effects of iodine deficiency and excess on infant nerve and growth are scarce. In human studies, it is impossible to avoid the interference of nutrients other than iodine and the environment in the experimental results. For example, the energy required for neurodevelopment is maintained by glucose, iron, copper, zinc, and selenium. The structure and composition of the brain are influenced by protein, long-chain polyunsaturated fatty acid deposits, and folate *via* neurulation. The process of neural cell differentiation is supported by micronutrients, such as iodine and zinc (34). Perchlorate is an environmental contaminant that interferes with iodine uptake into the thyroid to affect brain development (35). The animal experiment we carried out fully screened for the effects of these confounding factors.

BNDF and NSP-A could be used to assess brain development of offspring at the molecular level. BDNF is a neurotrophic protein that plays a critical role in brain development and is involved in neurogenesis, neuronal differentiation, synaptogenesis, and memory formation and consolidation (36–39). NSP-A is believed to be an important mediator of thyroid hormone action during brain development and is involved in neuronal differentiation and axon guidance (40). A previous study has shown that on PND7, when iodine deficiency (iodine intake was 1.5 mg/d) and mild iodine overdose (iodine intake was 15–16 mg/d) occurred, BNDF protein expression was significantly reduced, while NSP-A protein expression was significantly increased. But on PND45, no significant increase in the NSP-A level was observed when iodine intake was 15–16 mg/d (9). On this basis, our study added to explore the situation of severe iodine excess for offspring on PND15. When iodine status was abnormal, the protein expression of BNDF was significantly lower than that of the NI group. However, our results showed that the expression of NSP-A in the 10HI and 50HI groups was lower than that in the NI group. Dowling et al. (17) showed that the expression of NSP-A was regulated by thyroid hormone, and the expression of NSP-A was positively correlated with T4 in the fetal rat brain cortex. According to the thyroid function of the maternal rats, as given in **Tables 2**, **5**, FT4 was significantly reduced in the 10 and 50HI groups. Therefore, when iodine is excessive, reduced FT4 may lead to reduced expression of NSP-A. Since there was no follow-up study of the offspring, we could not determine whether the brain damage in the offspring of the LI, 10HI, and 50HI groups was also temporary. This needs to be further discussed in future studies.

Conclusion

For maternal rats, iodine storage in the placenta during pregnancy and in the mammary gland during lactation is associated with iodine intake. However, the placenta and lactating mammary gland have compensatory regulatory effects on iodine deficiency and iodine excess. Maternal iodine nutritional status during pregnancy and lactation affects neurodevelopment and the cognitive level of offspring by affecting their thyroid function.

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 Research Committee of Tianjin Hospital of ITCWM Nankai Hospital.

Author contributions

WZ, MF, WW, and WG conceived and designed the experiments. MF, WW, WG, YG, RY, QJ, QM, YY, and ZW performed the experiments. MF, YG, and QJ analyzed the data. MF, WG, and YG wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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A multi-network comparative analysis of whole-transcriptome and translatome reveals the effect of high-fat diet on APP/PS1 mice and the intervention with Chinese medicine

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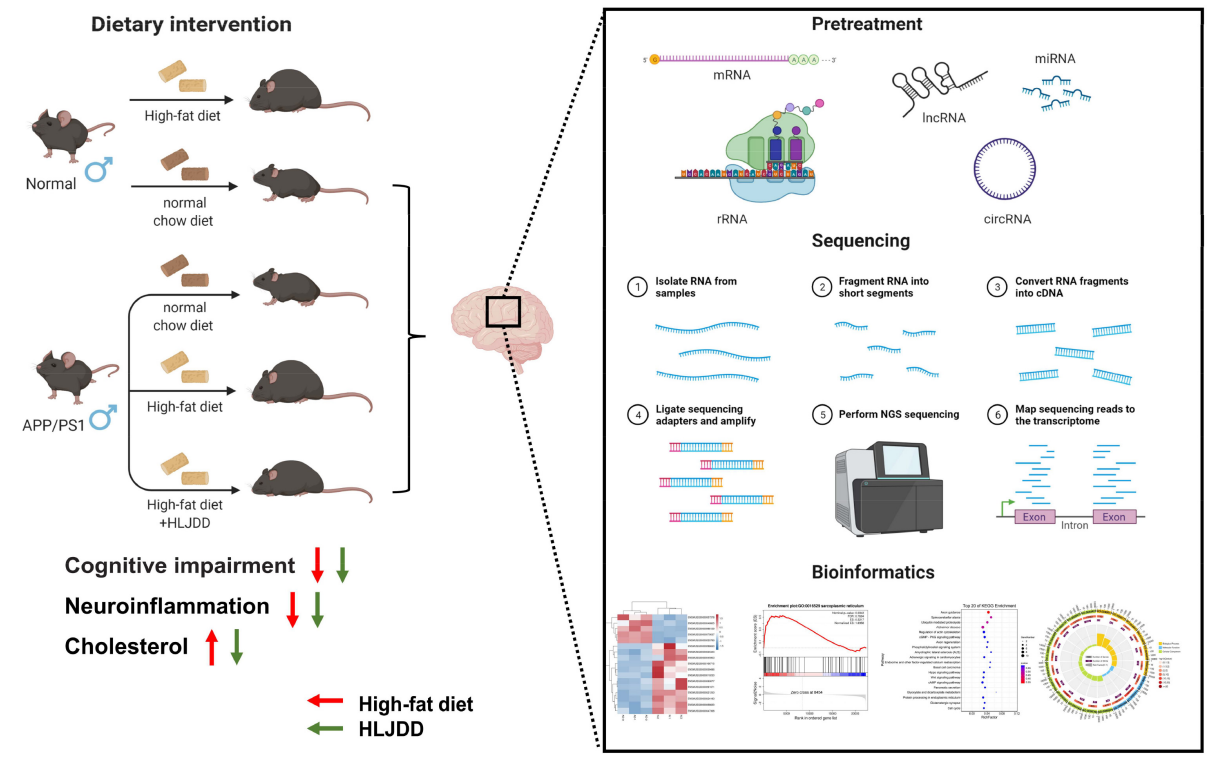
Different studies on the effects of high-fat diet (HFD) on Alzheimer's disease (AD) pathology have reported conflicting findings. Our previous studies showed HFD could moderate neuroinflammation and had no significant effect on amyloid-β levels or contextual memory on AD mice. To gain more insights into the involvement of HFD, we performed the whole-transcriptome sequencing and ribosome footprints profiling. Combined with competitive endogenous RNA analysis, the transcriptional regulation mechanism of HFD on AD mice was systematically revealed from RNA level. Mmu-miR-450b-3p and mmu-miR-6540-3p might be involved in regulating the expression of *Th* and *Ddc* expression. MiR-551b-5p regulated the expression of a variety of genes including *Slc18a2* and *Igfbp3*. The upregulation of *Pcsk9* expression in HFD intervention on AD mice might be closely related to the increase of cholesterol in brain tissues, while Huanglian Jiedu Decoction significantly downregulated the expression of *Pcsk9*. Our data showed the close connection between the alterations of transcriptome and translatome under the effect of HFD, which emphasized the roles of translational and transcriptional regulation were relatively independent. The profiled molecular responses in current study might be valuable resources for advanced understanding of the mechanisms underlying the effect of HFD on AD.

KEYWORDS

Alzheimer's disease, high-fat diet (HFD), Ribo-seq, RNA-seq, Huanglian Jiedu Decoction



GRAPHICAL ABSTRACT

Introduction

Alzheimer's disease (AD) was a progressive neurodegenerative disease related to aging, characterized by the pathological hallmarks of extracellular accumulation of amyloid-β (Aβ) plaques and intracellular accumulation of neurofibrillary tangles. AD was caused by the complex interaction of multiple mechanisms, and the etiology was still unclear. AD was generally considered to be related to genetic and environmental factors (1). Diet and nutrition displayed potential for non-pharmacological AD prevention. However, different studies on the effect of high-fat diet (HFD) on AD pathology in AD models reported conflicting conclusions. For instance, the HFD feeding induced Aβ accumulation and cognitive decline in APP/PSEN1 mice. Systemic inflammation and obesity could be reversed by a low-fat diet (2). Aβ and HFD had a synergic effect, leading to the impairment of endoplasmic reticulum and mitochondrial functions, glial reactivity status alteration and inhibition of insulin receptor signaling. These metabolic alterations would favor neuronal malfunction and eventually neuronal death by apoptosis, hence causing cognitive impairment (3). However, other studies found that HFD might promote better cognitive function by improving blood-brain barrier function and attenuating brain atrophy in AD, but it didn't seem to affect Aβ levels (4–6). Therefore, the influence of HFD on the progression of AD was controversial. A clear understanding of the HFD role in AD pathology would help improve the quality of life and relieve the demand pressure of aging population on the overall resources of society.

Huanglian Jiedu Decoction (HLJDD) was composed of Rhizoma coptidis, Radix scutellariae, Cortex phellodendri, and Fructus gardenia at a ratio of 3:2:2:3. HLJDD was a classic prescription for clearing away heat and toxic materials in past dynasties. Alkaloids, flavonoids and iridoid glycosides were the mainly active ingredients in the prescription (7). Modern research showed that HLJDD had many pharmacological effects, such as anti-inflammatory, antibacterial, antioxidant, lipid-lowering and hypoglycemic, antitumor, neuroprotection and so on (8). The literature researches and previous experiments of our research team showed HLJDD could reduce the accumulation of Aβ and Tau in central of APP/PS1 mice, improve cognitive ability, and ameliorate the lipids and inflammatory environment in the center and periphery (9). Furthermore, HLJDD could regulate the metabolism of central neurotransmitters, amino acids, peripheral bile acids, and relieve AD symptoms in combination with intestinal flora (10). In this study, we would continue to explore the curative effect and mechanism of HLJDD on HFD plus AD model mice.

Genome-wide association studies (GWAS) identified the following genes associated with AD risk: *ABCA7*, *BIN1*,

CASS4, CD33, *CD2AP*, *CELF1, CLU*, *CR1*, *DSG2, EPHA1*, *FERMT2, HLA-DRB5-DBR1, INPP5D, MS4A*, *MEF2C*, *NME8*, *PICALM*, *PTK2B, SLC24H4 RIN3*, *SORL1*, *ZCWPW1*, *PLD3, and TREM2* (11). The loci identified by large GWAS analysis for late-onset Alzheimer's disease (LOAD) were related to immune response, inflammation, lipid metabolism, endocytosis/intracellular trafficking, and cell migration (12). APP, PSEN1, and PSEN2 were associated with early onset AD (13), while APOE4 was considered as a risk factor for LOAD (14), in addition to genes related to cholesterol metabolism and immune response that could also increase the risk of LOAD (15). RNA sequencing (RNA-seq) provided an unbiased way to investigate the genome-wide transcriptome profiling, and it could help construct the complicated gene regulatory network in the dynamic progression of human diseases.

Competing endogenous RNAs (ceRNAs) were RNAs in the complex network of transcriptional regulation in organisms, including protein-coding mRNA, long non-coding RNA (lncRNA), pseudogene, and circular RNA (circRNA). The regions of these RNAs could be bound by systematically functionalizing microRNA (miRNA) response element (MRE)-harboring non-coding RNAs. Competing to bind common miRNAs through common MREs, the RNAs interacted and regulated the expression of target gene transcripts. Thus, through the miRNA, these RNAs could interact with each other to form complex miRNA-mediated ceRNA networks. The interaction relationship showed the possible functions of the lncRNAs and circRNAs. Significant changes in lncRNA were also observed in AD models, with studies reporting the upregulation of *MRAK088596, MRAK081790, and MAPK10* and downregulation of *BC092582, MRAK050857, and S100A8* in AD rats (16). CircRNA had been shown to play an important role in the development of AD by affecting neurogenesis and injury, Aβ deposition, neuroinflammation, autophagy and synaptic function through miRNA sponging. Large number of differentially expressed circRNAs were presented in the brains of AD patients (17). The association of various human miRNA with disease had been experimentally validated. A large set of miRNA-mRNA associations that were found in AD patients (18) and played important roles in the regulation of Aβ precursor protein expression, lytic enzyme activity and APP pathway-related signaling molecules. They also regulated tau protein expression, tau phosphorylation-related kinase and phosphatase function. The study showed that a decrease in miR-29a/b could contribute to increased BACE1 and Aβ levels in sporadic AD (19). MiRNAs also had the effect on learning and memory processes, regulating L-LTP, excitatory glutamatergic systems and other synaptic transport (20).

Gene expression in currently studies was mainly at the transcriptional level, largely ignoring translational regulation. However, translation regulation was accounted for more than half of all regulation in biological genetic information transfer and was the most important form of regulation in the cells. Ribosome profiling (Ribo-seq), in which next-generation sequencing used to identify ribosome-protected mRNA fragments, thereby revealing the positions of the full set of ribosomes engaged in translation, has emerged as a transformative technique for enabling global analyses of *in vivo* translation and coupled, translational events (21). Ribo-seq had been widely used in different species (22–25). The researchers analyzed gene expression in cerebral cortex of two AD model mouse strains, CVN ($APP_{Sw}DI/NOS2^{-/-}$) and Tg2576 (APP_{Sw}), by tandem RNA-seq and Ribo-seq. AD model mice had similar levels of transcriptome regulation, but differences in translatome regulation (26).

Previously, we detected that long-term HFD intervention altered the levels of cholesterol and polyunsaturated fatty acids in the brain tissue of APP/PS1 mice and influenced the secretion of peripheral bile acids (10). Translational regulation was considered to play a vital role in gene expression, but whether HFD functions through the regulation of gene translational level was still unclear. The mechanism linking HFD in the regulation of transcriptome and translatome in APP/PS1 mice had not yet been systematically elucidated. In order to analyze the overall effects of HFD on the AD mice, whole-transcriptome sequencing (mRNA-seq, lncRNA-seq, circRNA-seq, and miRNA-seq) and Ribo-seq were used to explore. In addition, the associations between transcriptional and translational levels corresponding to this phenotype further screened out some known target genes and new functional genes, followed by functional interaction prediction analysis. In summary, our analysis could reveal distinct roles of translational and transcriptional regulation in HFD intervention on AD mice. This study aimed to provide a new direction for the treatment of AD through the joint analysis of transcriptome and translatome.

Materials and methods

Animal and diet

Five-month-old SPF grade male C57BL/6J-TgN (APP/PS1) transgenic mice and C57BL/6J wild type mice (Shanghai Model Organisms Co., Ltd., Production license number SCXK 2014-004) were used in this study. All experiments and animal care in this study were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and the Provision and General Recommendation of Chinese Experimental Animals Administration Legislation. The study was approved by the Institutional Animal Care and Use Committee of the Beijing animal science Co., Ltd., and the animal ethics approval number was IACUC-2018100605.

Animals were housed in a single cage with chow and water *ad libitum* and a 12 h light-dark cycle and kept under a consistent temperature of 21°C. Wild type mice were assigned to the normal group (the Nor group, fed a normal chow diet) and the Nor_HFD group (fed a HFD diet). The HFD diet contained 63.6% basic feed, 15% lard, 20% sucrose, 1.2% cholesterol, and 0.2% cholate (Beijing Keao Xieli Feed Co., Ltd.). APP/PS1 mice were randomly allocated into 3 groups: one was fed a normal chow diet (the AD group), and one was fed a HFD diet (the AD_HFD group), and another was fed HFD diet and the powder of HLJDD (the H_H group). The HLJDD powder was prepared in our laboratory as previously described (7). Our research was a preventive protocol, and the gavage dose was 344 mg/kg/d (HLJDD) for 3 months. Animal weights were recorded every week.

Morris water maze test

The Morris water maze (MWM) test was performed to detect spatial memory as previously described with a slight modification (27). Mice participated in a navigation test for four consecutive days. Four sequential training trials began by placing the animals facing the wall of the pool but changing the drop position for each trial. If the mouse found the platform before the 90 s cut-off, allowing the mouse to stay on the platform for 10 s then return it to its home cage. Otherwise, we placed the mouse on the platform and allowed it to stay there for 20 s. The mouse was trained in different direction. We repeated the training for all mice in the trail in the next 4 days. In probe trial, we removed the platform from the pool and the test time was 60 s. Escape latencies, time spent or distance traveled in the target quadrant and platform-crossing times were recorded and analyzed using the analysis management system (Beijing Zhongshi Kechuang Co., Ltd.).

Brain sample collection

After the MWM test, all mice rested for 4 days under normal conditions. After anesthesia with 10% chloral hydrate, serum was collected from the heart, followed by removal of brain tissue on a sterile table, rinsing with pre-cooled RNAse-free saline at 4°C, blotting up. Then put the sample into 1.5 mL labeled RNase-free EP tubes, which were rapidly frozen in liquid nitrogen for 30 min and stored at −80°C in the refrigerator until use. Whole brain had been ground in liquid nitrogen.

Western blot assay

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

RT-PCR

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

RNA-seq and Ribo-seq

The experimental procedure and data analysis were listed in the **Supplementary material**.

Data availability

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found here: http://bigd.big.ac.cn/gsa/, CRA007307.

Statistical analysis

The results were expressed as the mean ± standard error of the mean (SEM). The significance of difference among the groups was assessed by Student's *t*-test for two groups and one-way ANOVA for more than two groups, followed by the LSD and Games-Howell post-test. Statistical calculations were performed using SPSS 20 software. Differences with statistical significance were denoted by *P*-value less than 0.05.

Results

Evaluation of high-fat diet intervention in the APP/PS1 mice

After 3 months of HFD administration, there was an obvious weight gain in AD_HFD mice at a rapid pace compared to normal chow diet mice (**Figure 1A**). HFD significantly accelerated the percentage of weight gain, and HLJDD could slow down the pace of weight gain caused by HFD. In the MWM test, the sequential changes in the average escape latency during spatial acquisition training were shown in **Figure 1B**. With the increase of training times, the incubation period of each group gradually was shortened. AD mice displayed longer average escape latency compared to Nor mice on day 2, 3, and 4. AD_HFD and H_H mice were not exhibited significantly different compared to AD mice. In the spatial probe test, the platform crossing number in AD mice was lower compared to the Nor group. Meanwhile, the percent distance and time spent in the target quadrant were significantly lower than those in the Nor group ($P < 0.05$). The platform crossing number, percent distance and time spent in the target quadrant in the AD_HFD group were higher than those in the AD group, indicating that it had improvement tendency toward the cognitive impairment with the HFD intervention. There is no significant difference between the H_H group and AD group (**Figure 1C**). The trajectory map of AD mice was disorganized and purposeless (**Figure 1D**). WB analysis revealed that the levels of $A\beta_{42}$ were increased in AD group compared to the Nor group and decreased in the AD_HFD and H_H groups compared with the AD group. The levels of PPAR-γ were decreased in AD mice compared to the Nor group, while increased in AD_HFD and H_H groups compared with AD group (**Figure 1E**). The mRNA expression of different proinflammatory cytokines, *IL-1β, IL-6, TNF-α, MCP-1*, *IL-12A, IL-12B,* and *IFN-γ*, were upregulated in the AD group compared to the Nor group. The mRNA levels of proinflammatory cytokines were slightly reduced in response to the HFD intervention. Inflammatory cytokine levels were also detected by enzyme-linked immunosorbent assay (ELISA). The expression of TNF-α and IL-1β was decreased in the AD_HFD and H_H groups compared to the Nor group, and IL-1β levels was significantly reduced ($P < 0.001$) (**Figure 1F**). Taken together, the HFD intervention might have the effects of relieving inflammation in AD model mice.

Overview data of mRNA-seq and Ribo-seq

At the mRNA sequence profiling, 18490 detected genes were identified. At the Ribo sequence profiling, 17433 detected genes were identified. The gene expression levels for both the transcriptome and the translatome were similar with normal distribution. The distribution of expression abundance among the samples was shown in **Supplementary Figures 1A,B**. The peaks of the samples were generally consistent, indicating that there was little difference in the overall expression of the genes at the transcriptional and translational levels among the samples. The heat maps were shown in **Figures 2A,B**. Pearson correlation coefficient (R) between Ribo sequence abundance and mRNA abundance was calculated, and the scatter plots (**Figure 2C**) were drawn to analyze the correlation at the translational and transcriptional levels. The *R*-values of Nor, AD, AD_HFD, and H_H groups were 0.62, 0.63, 0.65, and 0.7, indicating a moderate correlation between mRNA abundance and Ribo sequence abundance in four groups. Principal component analysis (PCA) of mRNA-seq and Ribo-seq were shown in **Supplementary Figures 1C,D**. The ribosome-protected fragments (RPFs) length distribution peaked at 28 nt in both groups (**Supplementary Figure 1E**). The mRNAs protein-coding sequences (CDS) contained the majority of RPFs in four groups, with an average distribution ratio of 89.26, 88.03, 88.02, and 87.95%, for the Nor, AD, AD_HFD and H_H groups separately. The 5′ UTR and 3′ UTR distribution ratio was less than 3%, respectively (**Supplementary Figure 1F**). These data demonstrated the reproducibility and reliability of this analysis. The identification and quantification information for the transcriptome and translatome were shown in **Supplementary Tables 2**, **3**.

Differential transcriptome analysis

Analysis of differently expressed genes in Alzheimer's disease mice with high-fat diet

Based on the HISAT2 comparison results, we reconstructed the transcripts using StringTie and calculated the expression of all genes in each sample. Using the reads count data of gene expression levels of each sample, we analyzed the difference between groups using DESeq2 software with $P < 0.05$ and $|\log_2 FC| \geq 0.585$ as significant differentially expressed genes (DEGs). 87 genes were up-regulated and 125 genes were down-regulated in the AD group compared to the Nor group. Compared to the AD group, 116 and 120 genes were up- and down-regulated in the AD_HFD group, respectively. 30 genes were significantly differentially expressed in both AD and AD_HFD groups compared to the Nor group. 13 of them were reduced and 16 were significantly increased in AD mice. In addition, *Gpr151* was reduced in the AD group but increased in the AD_HFD group. The volcano plot and Wayne plot of differentially expressed genes between the groups were shown in **Figures 3A,B**.

KEGG analysis (Nor vs. AD group) identified the significant enrichment pathways including apoptosis, MAPK signaling pathway, neuroactive ligand-receptor interaction, purine metabolism, dopaminergic synapse, serotonergic synapses, etc. The AD and AD_HFD groups were significantly enriched in

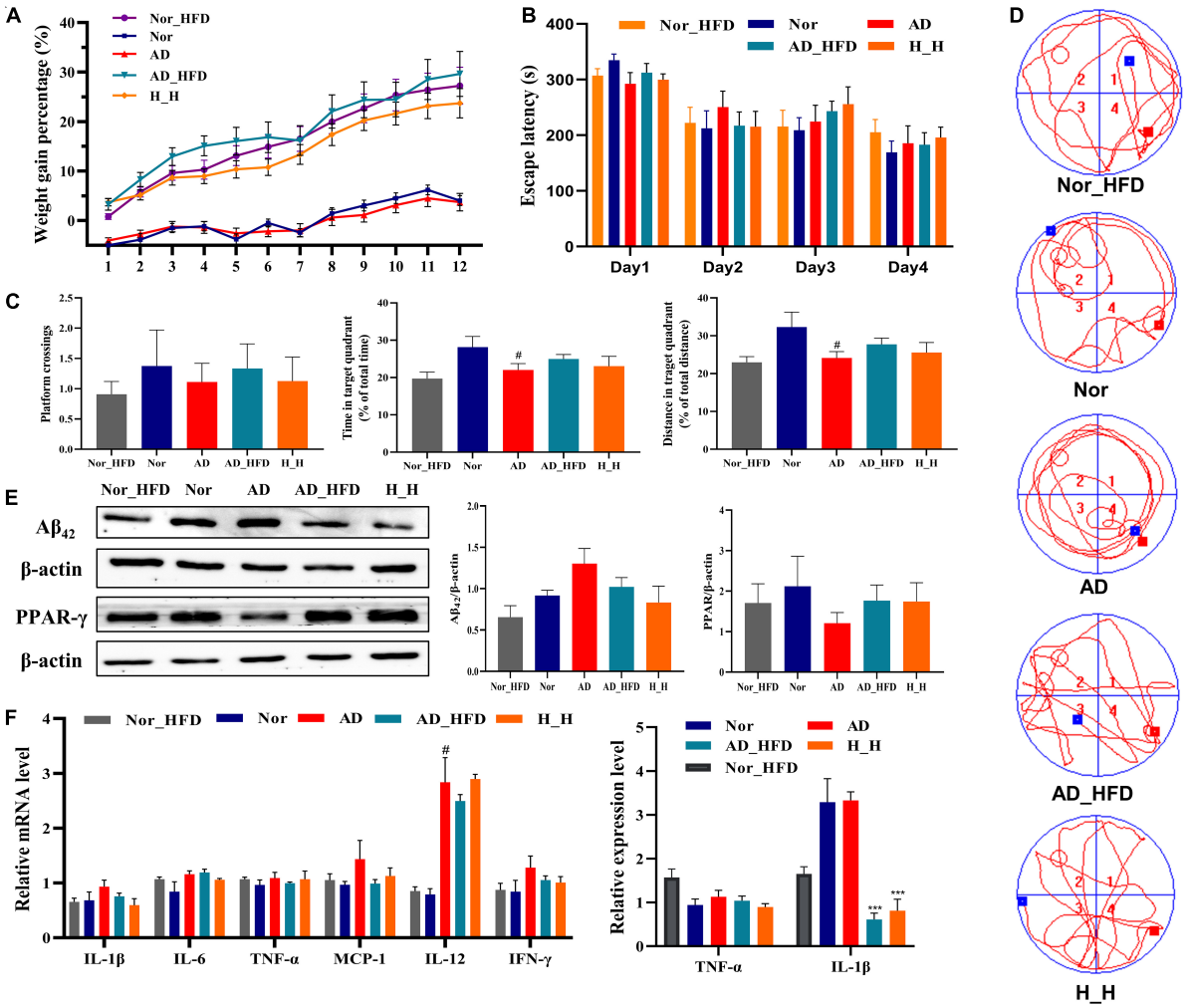


FIGURE 1
The effect of HFD on APP/PS1 mice cognitive impairment. **(A)** The weight gain percentage with normal diet or HFD for 3 months, respectively. **(B)** Escape latency during spatial acquisition training. **(C)** The platform crossing number, distance traveled percentage in the target quadrant and time spent percentage in the target quadrant in the spatial probe test (n = 10). **(D)** The real-time monitoring of mice motion track Morris Water Maze test experiment. **(E)** The relative protein concentration of $A\beta_{42}$ and PPAR-γ detected by WB (n = 3). **(F)** The expression of inflammatory cytokine detected by RT-PCR and Elisa method in brain tissue. All the results are expressed as the mean ± SEM; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ (compared to the Nor group); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (compared to the AD group).

the KEGG at dopaminergic synapse, synaptic vesicle cycle, cholinergic synapse, estrogen signaling pathway, neuroactive ligand-receptor interactions, MAPK signaling pathway, TNF signaling pathway, galactose metabolism, serotonergic synapse, starch and sucrose metabolism (Figures 3D,F). DEGs among Nor, AD and AD_HFD groups found by KEGG enrichment analysis were focused on the regulation of a variety of synapses, including dopamine, choline and serotonin, which indicated that the expression levels of regulatory neurotransmitter genes were altered in the brain tissue of AD mice and HFD intervention also had a greater effect on these genes.

The results of GO enrichment analysis results were shown in Figures 3C,E. The differential genes in the brain tissue of normal and AD mice were mainly enriched in behavioral, nervous system, neurotransmitter, immune and chemotactic terms in biological process ontology. The result suggested that neurotransmitter metabolism and inflammatory responses were disturbed in the brain tissue of AD mice compared to normal mice, and HFD intervention could affect neurotransmitter metabolism and inflammatory responses in AD mice.

Dusp1, *Gpr151*, *Th*, *Ddc*, and *Npas4* were upregulated, while *Ccl21b* and *Slc1a1* were downregulated in the AD_HFD group compared with the AD group. Dual specific phosphatase (DUSP) played an important immunomodulatory function through the DUSP-MAPK phosphatase pathway (28). DUSP1 played an important negative regulatory role in the inflammatory immune response of macrophages induced by Toll-like receptor ligand stimulation (29). Increased

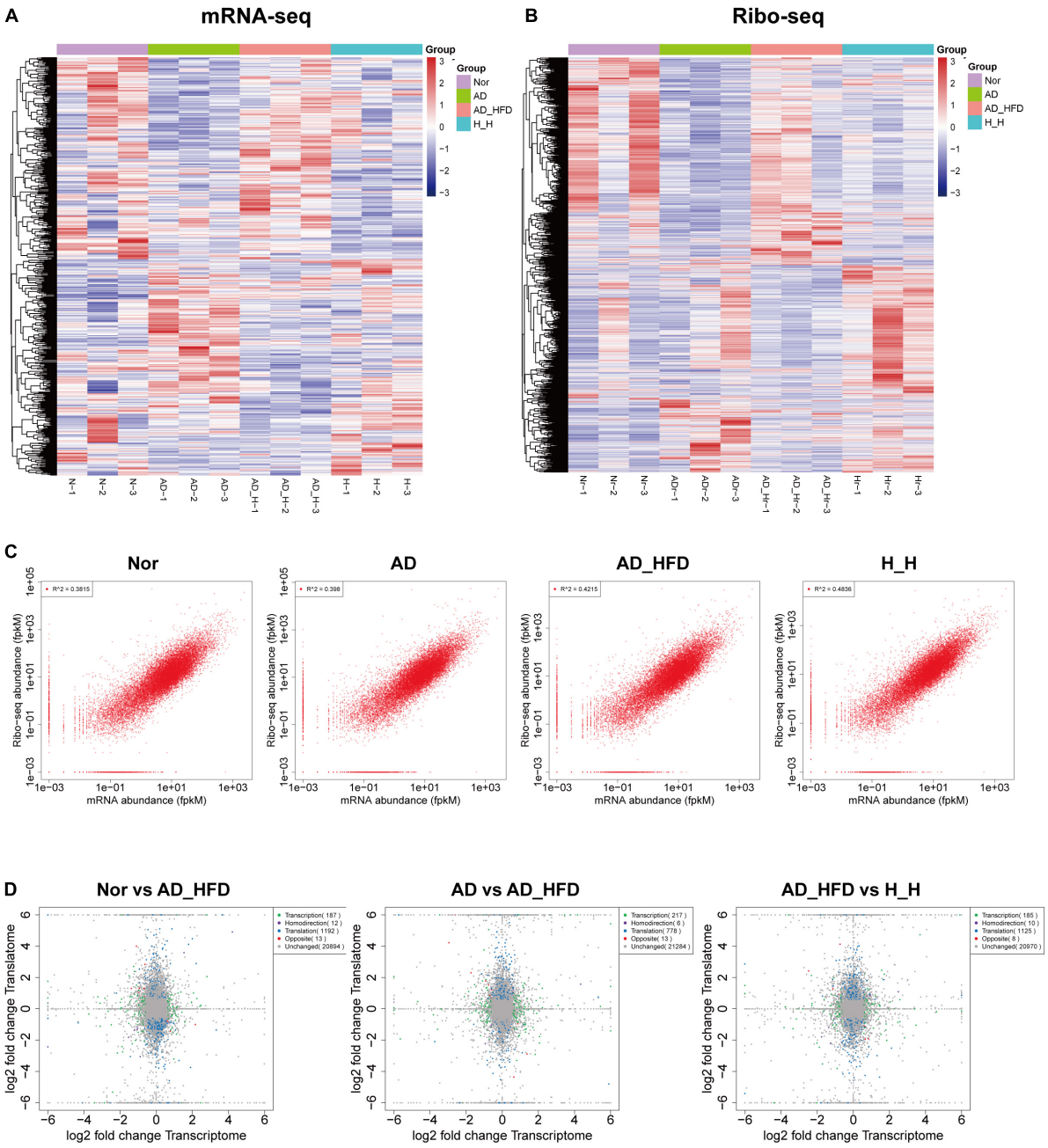


FIGURE 2
Overview of genes identified by transcriptome and translatome. Heatmap of cluster analysis of DEGs in the transcriptome **(A)** and translatome **(B)**. N-1, N-2, and N-3 represent the Nor group biological repetition. AD-1, AD-2, and AD-3 represent the AD group biological repetition. AD_H-1, AD_H-2, and AD_H-3 represent the AD_HFD group biological repetition. Blue represents the lowest and red represents the highest. **(C)** Scatter plot of transcriptome and translatome in four groups. **(D)** Quadrant diagram of the fold change at transcriptional and translational levels.

Dusp1 expression in the HFD group suggested a close association with partial remission of inflammation in brain tissue. Tyrosine hydroxylase (TH) was a catecholamine rate-limiting enzyme that catalyzed the conversion of tyrosine to dihydroxyphenylalanine and regulated the production of dopamine, noradrenaline and epinephrine neurotransmitters. Aromatic L amino acid decarboxylase (DDC) catalyzed the conversion of dopa to dopamine. Both were more highly expressed under HFD, indicating that HFD intervention would strengthen the dopamine neurotransmitter synthesis in the brain tissue of AD mice. Neuronal PAS domain binding protein 4 (NPAS4) mRNA was upregulated in the AD_HFD

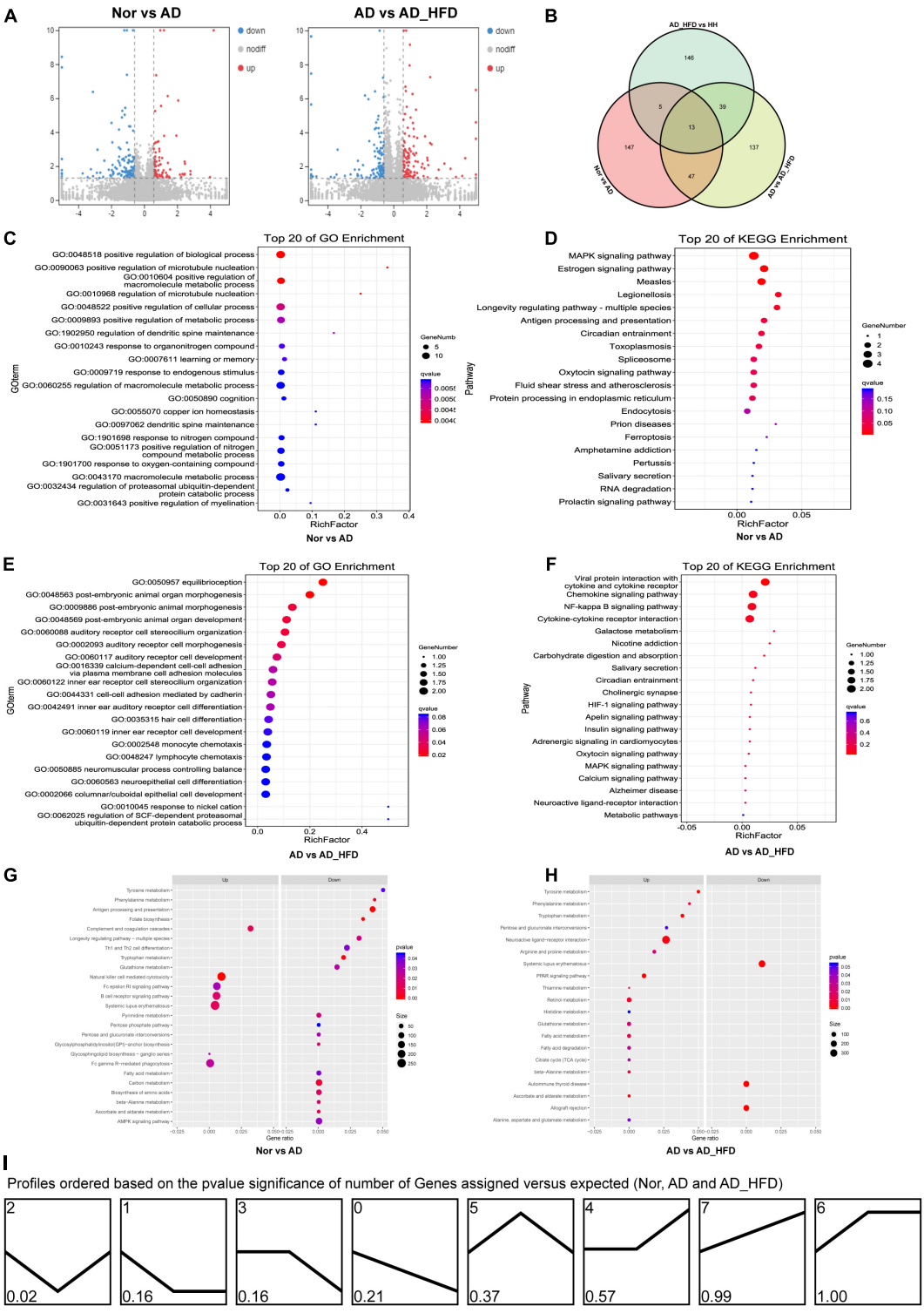


FIGURE 3
Transcriptome analysis. **(A)** Volcano plots of differentially expressed mRNA between groups in transcriptome. **(B)** Venn diagram showing the distinct and overlapping differential genes between groups of the transcriptome. **(C)** GO enrichment analysis of the transcriptome between the Nor and AD groups. **(D)** KEGG pathway analysis of the transcriptome between the Nor and AD groups. **(E)** GO enrichment analysis of the transcriptome between the AD and AD_HFD groups. **(F)** KEGG pathway analysis of the transcriptome between the AD and AD_HFD groups. **(G)** GSEA of the transcriptome between the AD and AD_HFD groups. **(H)** GSEA of the transcriptome between the AD and AD_HFD groups. **(I)** Trends in genetic variation among the Nor, AD and AD_HFD groups.

group, suggesting that HFD could enhance the regulation of glutamatergic and GABAergic synapses. In addition, GPR151 was associated with pineal synaptic function and nicotinic uptake.

GSEA analysis

In order to further understand the effect of HFD in AD, GSEA analysis was performed. GSEA analysis of KEGG revealed that 94 of the 324 gene sets were upregulated in the AD group compared to Nor group, including glycosphingolipid biosynthesis-ganglio series; 9 gene sets were downregulated in the AD group, including the specific pathways of folate biosynthesis, antigen processing and presentation, phenylalanine metabolism, tryptophan metabolism, alanine metabolism, ascorbate and aldarate metabolism, DNA replication, GPI-anchor biosynthesis, and carbon metabolism. 170 gene sets were upregulated in the AD_HFD group compared to AD group, while 154 gene sets were downregulated in the AD_HFD group. The results of the GSEA analysis with $P < 0.05$ were shown in **Figures 3G,H** and **Supplementary Figure 2**.

Differential genes in AD patients were mainly enriched in immune and metabolic pathways (30). Meta-analysis found the levels of acetylcholine and GABA were significantly lower and the levels of glycine were slightly higher in the cerebrospinal fluid of AD patients. Meanwhile, anaerobic glycolysis and the pentose phosphate pathway and the tricarboxylic acid cycle pathway were enhanced as well (31). Methionine, tryptophan and tyrosine purine metabolic pathways were altered in mild cognitive impairment (MCI) and AD patients (32). Phenylalanine, tyrosine and tryptophan levels were reduced in the serum of AD patients (33). Analysis of mRNA expression levels showed the downregulation of multiple metabolic pathways in AD mice, including phenylalanine, tryptophan and alanine metabolism. The effect of HFD on metabolism in AD mice was more extensive, with 18 of the 23 significantly upregulated gene sets being related to metabolic pathways, mainly involving the metabolism of amino acids and carbohydrates. Compared to normal mice, AD mice had metabolic abnormalities in lipid and amino acid metabolism. The metabolic pathways of phenylalanine, tryptophan and alanine were down-regulated in the AD group, while significantly up-regulated in the AD_HFD group, suggesting that HFD could regulate amino acid metabolism in the brain tissue of AD mice. The AD_HFD group could modulate the ascorbate and aldehyde metabolism pathways. In addition to substance metabolism, HFD also up-regulated PPAR signaling pathway and neuroactive ligand-receptor interactions pathway.

Differential gene expression trend analysis

A total of 8 patterns of gene trends among the Nor, AD and AD_HFD groups were plotted (**Figure 3I**), with profile 2 being significant and containing 72 genes. The analysis of the trends suggested that HFD intervention could callback profile2 genes, which might be associated with moderating the process in AD pathology. The profile2 genes were significantly enriched in KEGG pathways such as cholinergic synapses, dopaminergic synapses, MAPK signaling pathways, synaptic vesicle recycling, purine metabolism, serotonergic synapses, etc. The involved genes were *Slc6a3*, *Chrnb4*, *Fos*, *Hspa1b*, *Igfbp3*, *Gm45837*, *Dusp1*, *Slc18a2*, *Hspa1a*, *Itk*, *Gucy2c*, *Wnt9b*, and *Chrna6*.

Slc6a3 encoded the dopamine transporter and its variant carriers reduced cognitive performance and were at greater risk of developing dementia (34). In mouse models the activation of the endogenous *Nlrp3* promoter was catalyzed only by the dopaminergic neuron specific *Slc6a3* promoter. Dopaminergic neurons could accumulate NLRP3 inflammatory activators such as reactive oxygen species, dopamine metabolites, and misfolded proteins along with organismal aging. Activation of NLRP3 could induce inflammation and improve the cognitive impairment during normal aging and neuropathological processes (35). Heat shock protein (HSP) protected cells from oxidative stress, while HSP70 inhibited tau protein aggregation (36), effectively treating AD types with aging-related conditions (37). The expression of mRNA encoding HSP70 was increased in AD patients (38, 39), and APMAP levels were reduced. Nevertheless, HSPA1A and CD-M6PR levels, which controlled Aβ production, were increased (40). Proteomic studies found that HSPA1A levels in cerebrospinal fluid extracellular vesicles could monitor the course of AD (41). HSPA1B was associated with non-cognitive alterations in AD, and HSPA1B genes had significant AD non-cognitive symptoms (42). ITK regulated the signaling network downstream of T cell receptor signaling and influenced the differentiation of effector T cells. *Itk* could promote autoimmunity and central nervous system (CNS) inflammation (43). Suppression or deletion of *Itk* resulted in a decrease in Tr1 and TH17 cells and an increase in Treg cells (44).

Analysis of differently expressed genes in Alzheimer's disease mice combined with Huanglian Jiedu Decoction and high-fat diet

In comparison with the AD_HFD group, the level of 95 genes were up-regulated and 108 genes were down-regulated after HLJDD administration (**Supplementary Figure 3A**). 52 genes were significantly changed among the AD, AD_HFD and H_H groups, of which 26 were decreased and 26 were increased in the AD_HFD group, while in the H_H group the gene levels were back-regulated. A total of 27 genes were significantly altered among the Nor, AD_HFD and H_H groups, of which 17 were reduced and 9 were increased in the AD_HFD group, while the H_H group significantly modulated the changes of these genes.

The AD_HFD and H_H groups were significantly enriched in neuroactive ligand-receptor interaction, tyrosine metabolism, folate biosynthesis, galactose metabolism, Th1 and Th2 cell

differentiation, etc. Term was mainly enriched in the GO database for nervous system, behavior and neurotransmitters (**Supplementary Figures 3B,C**).

GSEA analysis of the KEGG pathway revealed that 136 of the 324 gene sets were upregulated in the H_H group compared to the AD_HFD group, involved the oxidative phosphorylation pathway. GSEA analysis of genes that changed between the two groups were shown in **Supplementary Figure 3D**. HLJDD up-regulated carbohydrate digestion and absorption, the phospholipase D signaling pathway, the longevity regulation pathway and axon regeneration, and down-regulated tyrosine metabolism, neuroactive ligand-receptor interactions, Th17 cell differentiation, the IL-17 signaling pathway, cholesterol metabolism and MAPK signaling pathway. The transcriptional level also indicated that HLJDD could inhibit the inflammatory response and regulate lipid metabolism, in addition to suggesting a regulatory effect on neuronal regeneration and neurotransmitter-like metabolism.

No significant changes were found in the trend analysis among the AD, AD_HFD and H_H groups. Profile 2 and 3 had significant changes in the Nor, AD_HFD and H_H groups (**Supplementary Figure 3E**), with 83 and 68 genes, respectively. Profile 2 genes were significantly enriched in homologous recombination, non-homologous end splicing, RIG-I-like receptor signaling pathway, NOD-like receptor signaling pathway and retinol metabolism, involving *Mre11a*, *Tbkbp1*, *Irf3*, *Gbp5*, *Rpe65*, *Gm5136*, and *Rdh16*. Genes in profile 3 were significantly enriched in neuroactive ligand-receptor interactions, folate biosynthesis, tyrosine metabolism, mitochondrial autophagy, cAMP signaling pathway, involving *Htr1f*, *Th*, *Alpl*, *Adh7*, *Prkn*, *Xrcc5*, *Nmu*, *Fosb*, *Atg9b*, *Drd1*, *Calca*, *Adora2a*, *Npffr2*, and *Trh*.

Analysis of genes related to cholesterol metabolism in different intervention methods

Using the transcriptome sequencing data as a benchmark, genes related to cholesterol transport, cholesterol biosynthesis, low density lipoprotein receptor (LDLR) gene family, bile acid biosynthesis, transport, secretion and metabolism were screened (**Figure 4**). Low expression genes were filtered out. The expression level between groups for the gene sets were compared, combined with the previous quantitative results, the effect of HFD on gene expression in the brain tissue of APP/PS1 mice was further analyzed.

Analysis of the above screened genes revealed that *Pcsk9*, a cholesterol transport-related gene, was significantly decreased in the H_H group. PCSK9 promoted low density lipoprotein (LDL) degradation. The upregulation of *Pcsk9* expression in the AD_HFD group might be closely related to the increase in cholesterol in brain tissues, while HLJDD significantly downregulated *Pcsk9* expression. The expression of *Slc10a4*, a bile acid transport-related gene, was significantly reduced in the AD group and significantly increased in the AD_HFD group and significantly reduced in the H_H group. SLC10A4 was a family of bile acid sodium cotransport proteins that were activated by proteases to transport bile acids (45) and could be involved in the transport of bile acids in brain tissue. SLC10A4 was significantly reduced in brain tissue at highly phosphorylated tau protein lesions, suggesting its close association with AD pathology (46). CYP27A1 regulated the synthesis of primary bile acids in the alternative pathway, and the results of our previous experiments on serum bile acids in mice also showed that HFD intervention increased the level of CDCA produced by the alternative pathway, once again confirming that HFD intervention could cause a significant increase in bile acid synthesis in AD mice.

Structural analysis of transcripts

The main variants type of single nucleotide polymorphism (SNP) was non-synonymous SNV, while the main variants location of SNP was in intronic. The SNP mutation types were transition (80.22%) and transversion (19.78%). A->G in transition accounted for the largest proportion. G->T in transversion accounted for the largest proportion. Among the analysis of alternative splicing, skipped exon accounted for the most in four groups (**Supplementary Figure 4**).

Differential translatome analysis

Differential translation genes (DTGs) between groups were performed using edgeR software. Compared to the Nor group, 336 DTGs were significantly up-regulated and 881 DTGs were down-regulated in the AD group; 603 and 194 DTGs were significantly up- and down-regulated in the AD_HFD group compared to the AD group, respectively; while H_H resulted in 851 and 292 DTGs being significantly up- and down-regulated, respectively.

A total of 382 differentially translated genes were co-varied among the Nor, AD and AD_HFD groups, of which 59 were up-regulated and 323 down-regulated in the AD group, while the HFD intervention significantly back-regulated changes in translated gene expression in the AD group. A total of 201 differentially translated genes were co-varied among AD, AD_HFD and H_H groups, 90 translated genes were down-regulated and 111 translated genes were up-regulated in the AD_HFD group, respectively. Except for *Zbtb16* and *Tmem121b*, all genes were significantly modulated by HLJDD.

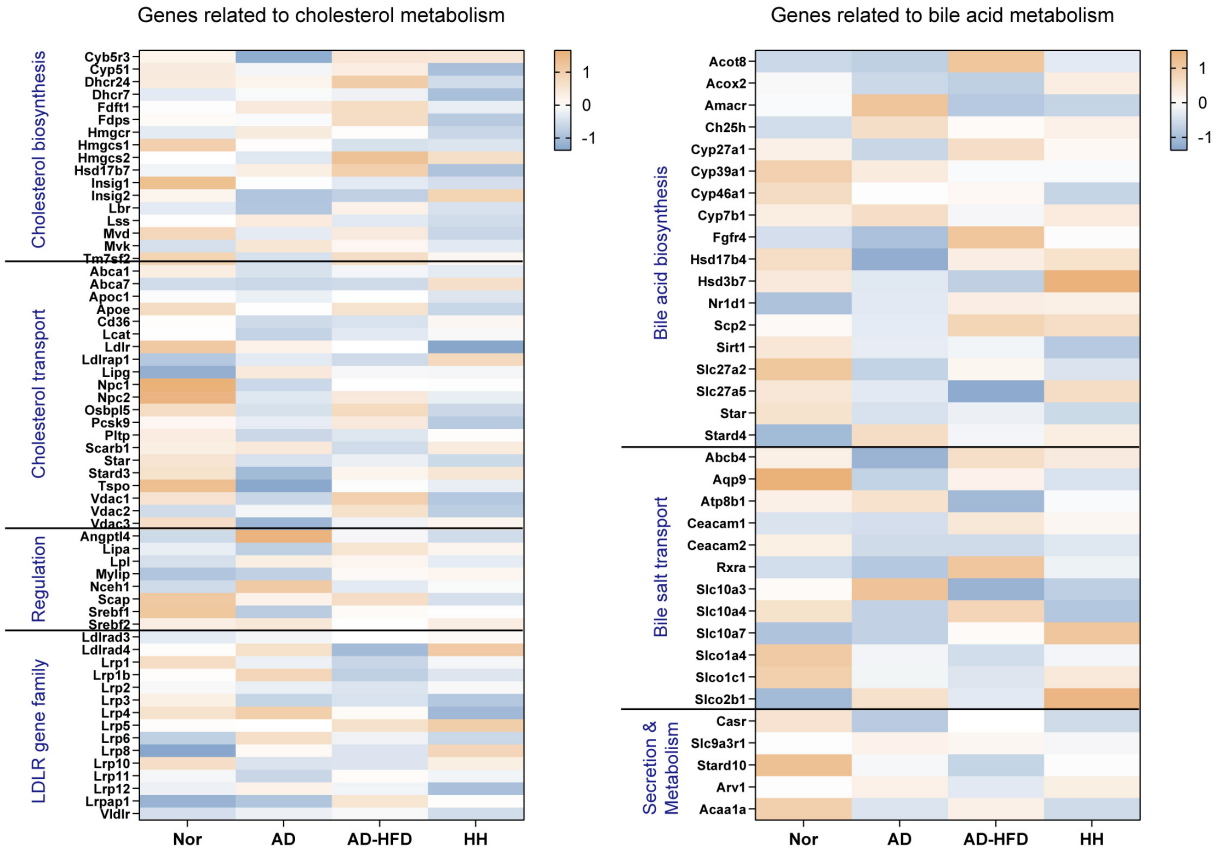


FIGURE 4
Heatmap of cholesterol and bile acid-related genes in the transcriptome. Blue represents the lowest while orange represents the highest.

Joint analysis of transcription and translation

Analysis of differentially expressed genes and differential translation genes

There were 212 DEGs and 1217 DTGs between the Nor and AD groups, and 25 genes that changed at both levels. The combination of transcriptional and translational analysis revealed that *Sgk1*, *Myo1f*, *Oip5*, and *Cst7* were both up-regulation; *Iqschfp*, *Gm45837*, *Itga2b*, *Alb*, *Npas4*, *Fos*, *Ccn1*, and *Dusp1* were both down-regulation; *Npy*, *Ptchd4*, *Clcc1*, *Thbs4*, and *Cdh12* were up-regulation in transcriptome and down-regulation in translatome; *Grid2ip*, *Gucy2c*, *Th*, *Eva1a*, *Ngb*, *Slc10a4*, *Hs3st3b1*, *and Hspb1* were down-regulation in transcriptome and up-regulation in translatome. Homodirectional genes were enriched in learning, memory, cognition, regulation of cell death, response to lipid, response to cAMP, negative regulation of p38MAPK cascade, negative regulation of microglial cell activation, nervous system development and regulation of neuroinflammatory response. The pathways of homodirectional genes were significantly enriched in fluid shear stress and atherosclerosis and MAPK signaling pathway. Gene ontology-biological process (GO-BP) of opposite genes were enriched in neuron development, negative regulation of response to oxidative stress, neuron differentiation and regulation of cellular response to oxidative stress. The pathways of opposite genes were enriched in tyrosine metabolism, VEGF signaling pathway, regulation of lipolysis in adipocyte, adipocytokine signaling pathway and dopaminergic synapse.

There were 236 DEGs and 797 DTGs between the AD and AD_HFD groups, and 19 genes that changed at both levels. The combination of differences based on transcriptional and translational analysis revealed that *Ecm1, Reep4, and Cmtm3* were both up-regulation; *Gbp5*, *H1f3*, and *H1f4* were both down-regulated; *Sspo*, *Hoxb5*, and *Ccm2* were up-regulation in transcriptome and down-regulated in translatome; *Slc1a1*, *Glt8d2*, *Serinc2*, *Cd34*, *C1ra*, *Thbs4*, *Lct*, *Gm45208*, *Ltf,* and *Cnpy1* were down-regulated in transcriptome and up-regulated in translatome. The GO-BP of homodirectional genes had function at cellular process and positive regulation of biological process. *H1f3*, *H1f4* were closely associated with histone modification. The pathways of homodirectional genes were significantly enriched in nucleotide-binding oligomerization

domain (NOD)-like receptor signaling pathway. The GO-BP of opposite genes had function at metabolic process, cellular process, biological regulation and developmental process. *Ccm2, Cd34, Thbs4, and Slc1a1* were closely associated with blood vessel development. Opposite genes were enriched in phagosome, galactose metabolism, carbohydrate digestion and absorption, synaptic vesicle cycle.

There were 203 DEGs and 1143 DTGs between the AD_HFD and H_H groups, and 18 genes that changed at both levels. The combination of differences based on transcriptional and translational analysis revealed that *Alms1, Lcmt2, Ryr3, and Ppp1r10* were both up-regulated; *Zfp968, Ccn1, Npas4, Fos, Dusp1, and Mpeg1* were both down-regulated; *C1ra, Mpp4, and Thbs4* were up-regulated in transcriptome and down-regulation in translatome; *Otof, Abl2, Glra1, Hoxb5,* and *Nrap* were down-regulated in transcriptome and up-regulated in translatome. The GO-BP of homodirectional genes were enriched in response to endogenous stimulus, learning, positive regulation of ceramide biosynthetic process, regulation of ceramide biosynthetic process, regulation of metabolic process, response to lipid and cognition. The pathways of homodirectional genes were significantly enriched in MAPK signaling pathway, Th1 and Th2 cell differentiation, IL-17 signaling pathway, TNF signaling pathway and dopaminergic synapse. The GO-BP of opposite genes were enriched in endothelial cell-cell adhesion, negative regulation of transmission of nerve impulse and behavior. The pathways of homodirectional genes were significantly enriched in ErbB signaling pathway and ECM-receptor interaction (**Figure 2D**).

Some of the genes were regulated differently in transcriptome and translatome. These outcomes suggested that regulation of translation had a relatively isolated role in regulating gene expression compared to regulation of transcription, and suggested sometimes translational regulation might completely reverse the effects of transcriptional regulation.

Analysis of differentially expressed genes and DTEGs

Using Ribo-seq and mRNA-seq data from the same sample, the translation efficiency (TE) of each gene was calculated. It exhibited a very weak correlation between TE and transcription abundance in four groups. 63 genes between the Nor and AD groups were significantly different at TE and transcription and had the opposite trends (opposite). The opposite genes were mainly enriched in the aspects of aging, neurotransmitter loading into synaptic vesicle, response to endogenous stimulus and dopamine metabolic process terms. The pathways were significantly enriched in cocaine addiction, amphetamine addiction, alcoholism, tyrosine metabolism, dopaminergic synapse and caffeine metabolism.

In total of 62 genes between the AD and AD_HFD groups were opposite. The genes were mainly enriched in neurotransmitter loading into synaptic vesicle, aminergic neurotransmitter loading into synaptic vesicle, response to nicotine, neurotransmitter transport, regulation of neurotransmitter levels terms in biological process ontology. The pathways were significantly enriched in neuroactive ligand-receptor interaction, dopaminergic synapse, synaptic vesicle cycle, alcoholism, and tyrosine metabolism.

Zfp968 and *Ccn1* between the AD_HFD and H_H groups were significantly different at both levels and had the same tendency. 60 genes between AD_HFD and H_H groups were opposite. The genes were mainly enriched in skeletal system morphogenesis, embryonic skeletal system morphogenesis and neuropeptide signaling pathway terms. The pathways were significantly enriched in neuroactive ligand-receptor interaction, galactose metabolism, carbohydrate digestion and absorption.

Differential expression analysis of no coding RNAs

The number of lncRNA transcripts reconstruction using StringTie was 7777. A total of 24717 circRNAs were identified in brain tissue samples, including 713 existing circRNAs and 24004 newly predicted circRNAs. A total of 1516 miRNAs were identified in mouse brain tissue samples. The length distribution obtained by miRNA sequencing of all samples was only one peak at 22bp. LncRNAs, circRNAs and miRNAs with $P < 0.05$ and $|\log_2 FC| \geq 0.585$ were screened as significant DEGs. According to the screening criteria, 114 or 137 were up- or down-regulated differentially expressed lncRNAs (dif-lncRNAs), 154 or 159 were up- or down-regulated differentially expressed circRNAs (dif-circRNAs), 11 or 5 were up- or down-regulated differentially expressed miRNAs (dif-miRNAs) in the AD group compared to the Nor group. There were 126 and 138 dif-lncRNAs, 193 and 202 dif-circRNAs, 17 and 4 dif-miRNAs up and down regulated in the AD_HFD group compared to the AD group. 150 and 129 dif-lncRNAs, 174 and 207 dif-circRNAs and 7 and 29 dif-miRNAs up and down regulated in the H_H group compared to the AD_HFD group (**Figures 5A–C**).

Long non-coding RNA analysis

Because of the complex origin of lncRNAs and the large variation in lncRNAs produced by different transcripts of the same gene, lncRNAs would be analyzed by transcript. The coding ability of new transcripts was predicted by CPC2 and CNCI software (**Supplementary Figure 5A**). The intersection of these non-coding potential transcripts was taken as a reliable predictor of the outcome. 734 transcripts with no coding ability were predicted. We performed *de novo* lncRNA prediction (**Supplementary Figure 5C**).

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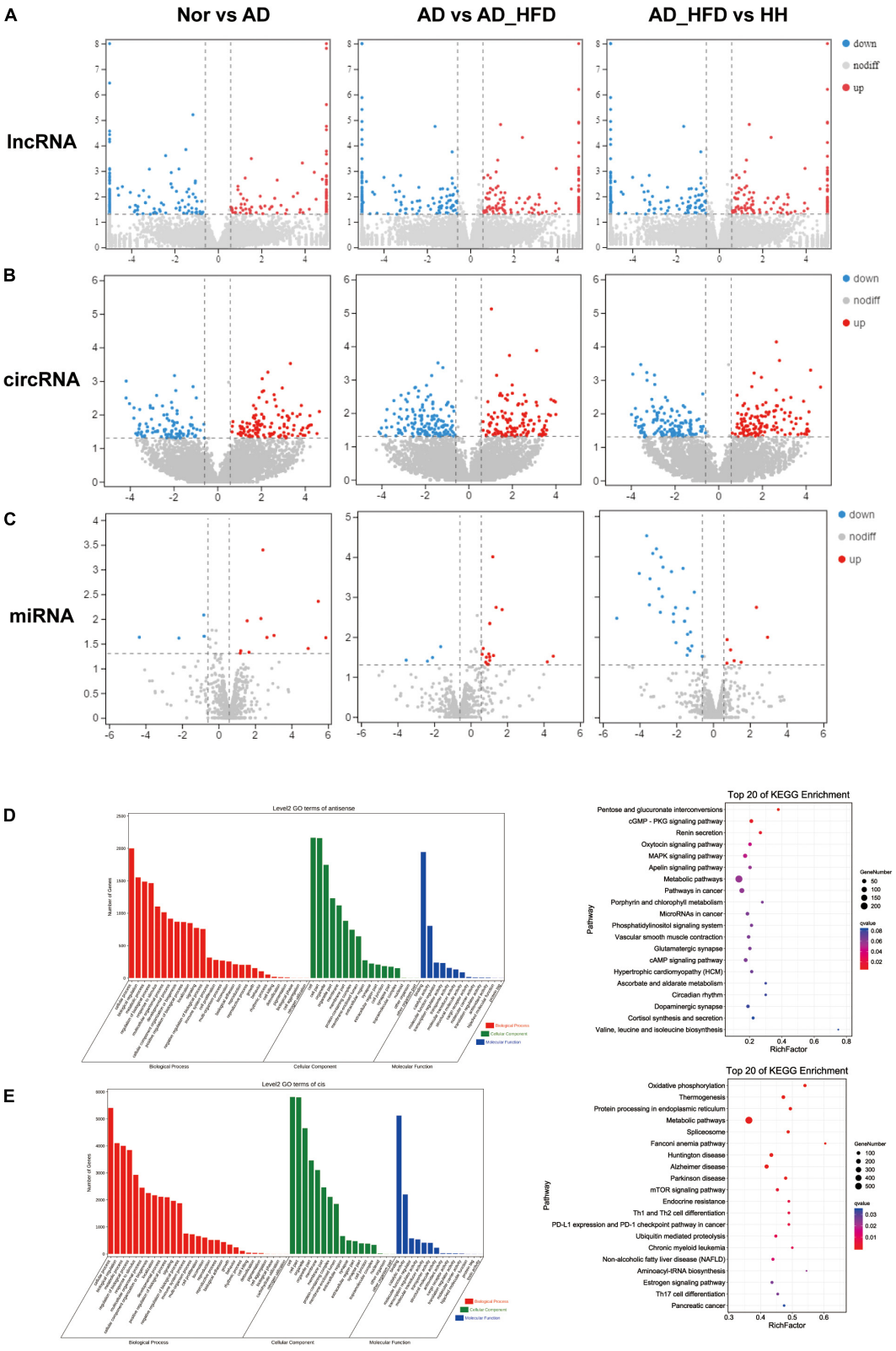

FIGURE 5
No coding RNAs sequence analysis. Differentially expressed genes heatmaps of lncRNA-seq **(A)**, circRNA-seq **(B)**, and miRNA-seq **(C)**. GO and KEGG enrichment analysis of antisense gene **(D)** and cis gene **(E)**.

Long non-coding RNA-mRNA association analysis

Long non-coding RNAs were involved in the regulation of many post-transcriptional processes, and were similar to small RNAs such as miRNAs and snoRNAs. These regulations were often associated with complementary pairing of bases. A fraction of antisense lncRNAs might regulate gene silencing, transcription and mRNA stability due to binding to mRNAs of the righteous strand. To reveal the interactions between antisense lncRNAs and mRNAs, we used RNAplex (47) to predict complementary binding between antisense lncRNAs and mRNAs.

We predicted antisense effects to obtain 3718 lncRNA-mRNA target gene pairs and cis effects to obtain 14398 lncRNA-mRNA target gene pairs. The pathways that were significantly enriched in KEGG of antisense effects were pentose and glucuronate interconversions, MAPK signaling pathway, apelin signaling pathway and metabolic pathways (**Figure 5D**). The pathways that were significantly enriched in KEGG of cis effects were oxidative phosphorylation, metabolic pathways, Alzheimer's disease, Parkinson's disease and mTOR signaling pathway. Cis effects of lncRNA-mRNA might be more involved in this AD experiment process (**Figure 5E**).

Circular RNA analysis

Trend analysis was used to observe the tendency in circRNA variation among the Nor, AD and AD_HFD groups. There were 820 dif-circRNAs in three groups, with significance in profile 2 and profile 5 (**Figures 6A,B**). These genes had a change trend of callback, suggesting that such circRNA source genes might be involved in the influence process of the HFD intervention on AD. KEGG enrichment analysis revealed that the pathways significantly enriched in genes of profile 2 were glutamatergic synapse, synaptic vesicle recycle, Rap1 signaling pathway, alanine metabolism, propanoate metabolism and GABAergic synapses (**Figure 6C**). Pathways significantly enriched in profile 5 were Rap1 signaling pathway, cholinergic synapse, cAMP signaling pathway, long-term depression, long-term potentiation and RAS signaling pathway (**Figure 6D**). The enrichment circle diagrams of GO enrichment analysis were shown in **Figures 6E,F**.

Competing endogenous RNA analysis

Screening of mRNAs, lncRNAs, miRNAs and circRNAs in the AD and AD_HFD groups yielded 164, 225, 50, and 309 differential genes, respectively. The miRNA-target gene pairs were predicted and screened for target gene pairs with Spearman's correlation coefficient less than or equal to 0.5, combined with ceRNA pairs with the positive expression correlation (Pearson's correlation coefficient more than 0.7) to obtain potential ceRNA pairs, and then screened for ceRNA pairs with *P*-value less than 0.05 as the final ceRNA pairs using hypergeometric distribution test.

Mmu-miR-551b-5p was competitively integrated by *Igfbp3* (ENSMUSG00000020427), *Slc18a2* (ENSMUSG00000025094), *Tmem265* (ENSMUSG00000106715), *Gbx2* (ENSMUSG00000034486), *Lhx9* (ENSMUSG00000019230), ENSMUST00000219444, and MSTRG.3992.1. Mmu-miR-211-5p was competitively integrated by *Lhx9* (ENSMUSG00000019230), *Tmem26* (ENSMUSG00000060044), *Tmem265* (ENSMUSG00000106715), *Gbx2* (ENSMUSG00000034486), ENSMUST00000219444, and MSTRG.3992.1. Mmu-miR-6540-3p was competitively integrated by *Slc18a2* (ENSMUSG00000025094), *Lhx9* (ENSMUSG00000019230), *Ddc* (ENSMUSG00000020182), novel_circ_014145, novel_circ_000302, ENSMUST00000129510, ENSMUST00000227816. Mmu-miR-221-3p was competitively integrated by *Chrna6* (ENSMUSG00000031491), *Shox2* (ENSMUSG00000027833). *Pou4f1* (ENSMUSG00000048349) was regulated by mmu-miR-200a-3p, mmu-miR-182-3p. Mmu-miR-450b-3p was competitively integrated by *Th* (ENSMUSG00000000214), *Lhx9* (ENSMUSG00000019230), *Abhd17b* (ENSMUSG00000047368), novel_circ_002080, ENSMUST00000129204, MSTRG.3992.1 (**Figure 6G**).

Discussion

Among all the relationship networks of ceRNAs, mmu-miR-450b-3p and mmu-miR-6540-3p regulated the expression of *Th* and *Ddc*, respectively. Both of them were closely related to the regulation of catecholamine neurotransmitters. A variety of lncRNAs and circRNAs were also involved in the regulation of their gene expression, and the specific mechanisms needed to be further validated and discovered. Insulin-like growth factor binding protein (IGFBP) was a family of proteins with high affinity for insulin-like growth factor (IGF). IGF-1 and IGFBP-3 were associated with oxidative stress and longevity (48). IGF-1 was thought to be a typical neuronal pro-survival factor in various brain injuries, promoting the clearance of Aβ and suppressing inflammatory responses. It could also affect cognitive performance by regulating synaptic plasticity, synaptic density and neurotransmission (49).

In addition to regulating IGF activity, IGFBP3 could also independently regulate cell growth and survival. IGFBP3 could bind and regulate retinoid X receptor α, upregulate pro-apoptotic signaling pathways such as TNFα and TGFβ (50). Current experimental studies and epidemiological findings on its relevance to AD were controversial, with some studies suggesting that higher serum total IGF-I levels and higher total IGF-I/IGFBP-3 ratios were associated with less cognitive decline (51). Low serum levels of IGF-1 and IGFBP-3 in male individuals were associated with AD (52). IGFBP-3 inhibited $A\beta_{42}$-induced apoptosis and long-term exposure to $A\beta_{42}$ could induce IGFBP-3 hypermethylation (53). In contrast, study suggested that $A\beta_{42}$ upregulate the expression of IGFBP3 (54), and the increased IGFBP3 expression was seen in senile plaques and

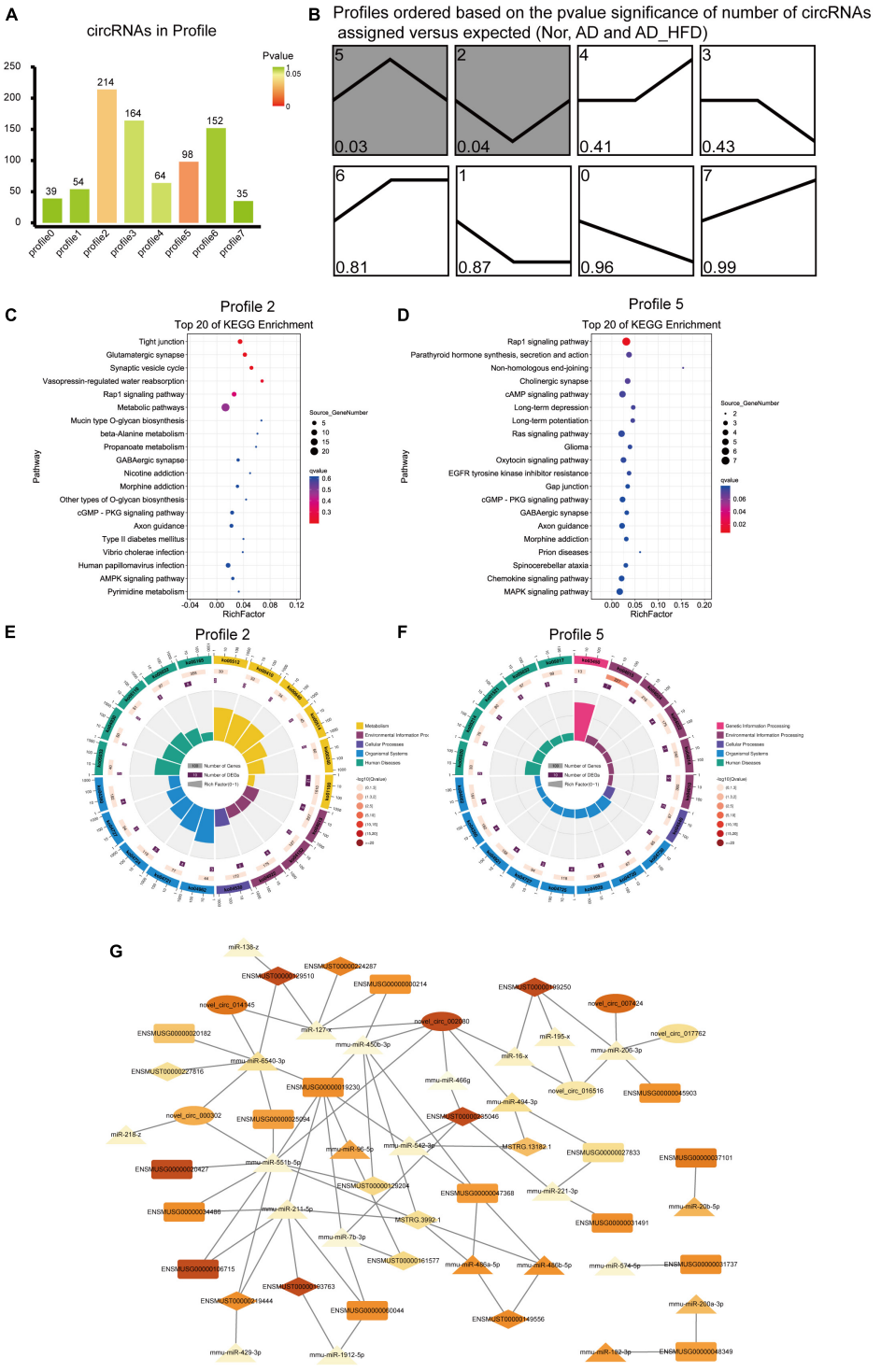

FIGURE 6
Trend analysis of circRNAs and ceRNA network. **(A)** The number of circRNAs in profile. Green represents *P*-value which is closed to 1 and red represents the *P*-value is closed to 0. **(B)** Trends analysis of circRNAs among the Nor, AD and AD_HFD groups. Pathways enriched by genes regulated by differentially expressed circRNAs in profile 2 **(C)** and profile 5 **(D)**. The enrichment circle diagrams of GO enrichment analysis in profile 2 **(E)** and profile 5 **(F)**. The first circle: enrichment of the first 20 GO term. Different colors represent different ontologies; the second circle: the number and *Q*-value of the GO term in each classification gene background. The more the number of gene background in each classification, the longer the bar, and the smaller the *Q*-value, the redder the color. The third circle: the number of genes in each category of the GO term; fourth circle: RichFactor value of each GO term. **(G)** The Competing Endogenous RNA Network. Rectangle indicates mRNA, ellipse indicates circRNA, and prism indicates lncRNA, triangle indicates miRNA. The larger the FC value, the darker the color.

neurofibrillary tangles (55). Aβ could activate calcium-regulated phosphatases in astrocytes, causing the release of IGFBP3, which in turn induced tau protein phosphorylation (56).

In this study, *Igfbp3* expression was reduced in the AD group, and its expression was significantly upregulated by HFD intervention, and its gene expression level was further increased by HLJDD administration. Further studies on the IGFBP3 were still needed to clarify its effect on the course of AD. LncRNA *Rmst-208* (ENSMUST00000219444), MSTRG.3992.1 in the ceRNA network were competed with *Igfbp3* to bind mmu-miR-551b-5p. In addition, *Pou4f1* in the competition network inhibited neuronal apoptosis, *Slc18a2* negatively regulated neurotransmitter transport, *Shox2* and *Irx5* were associated with neurodevelopment, and the transmembrane protein TMEM also played an important role in human immune-related diseases as well as tumor development (57, 58). *Slc18a* was associated with the regulation of neurotransmitter transport, which was regulated by mmu-miR-6540-3p and miR-551b-5p. ceRNA analysis revealed that *Lhx9*, lncRNA *Acbd5* competed with *Slc18a2* to bind mmu-miR-6540-3p. *Tmem265*, *Gbx2*, *Lhx9*, lncRNA *Rmst-208* and MSTRG.3992.1 competes with I*gfbp3* to bind mmu- miR-551b-5p. The prognostic value and underlying mechanisms of the miRNAs, lncRNAs and circRNAs that we identified needed to be further studied.

Interestingly, we also had a group of normal mice giving the HFD intervention in animal housing. However, this group was not performed the transcriptional and translational experiments. Compared to the normal diet, the HFD intervention resulted in a reduction in the number of platform penetrations, the percentage of platform quadrant distances and times in the normal mice. Whereas in the AD mice, on the contrary, the HFD intervention tended to ameliorate the cognitive impairment in the transgenic mice. This result meant that HFD had different effects on the animal. In the mRNA trend analysis and GSEA pathway enrichment results, most of the pathways enriched by DEGs in the AD group were related to the metabolism of neurotransmitter-like substances. Our laboratory examined the concentration of amino acids and neurotransmitters in mice brain tissue and found significant changes in acetylcholine, GABA, glutamine, phenylalanine, lysine, arginine, proline and alanine in the AD and AD_HFD groups (10). The result indicated the DEGs were involved in the metabolism of amino acids and neurotransmitters in the brain tissue of AD mice. We confirmed the HFD modulated brain tissue levels of serotonin, choline, tryptophan, GABA, glycine, phenylalanine, methionine, hypoxanthine and homovanillic acid in AD mice. In the present study, we also found that HFD could modulate the gene changes in profile 2 (transcriptome), and affect the metabolism of neurotransmitters in the brain tissue. In addition, IGFBP was associated with apoptosis and tau protein phosphorylation. The increased transcription of *Igfbp3* in the AD_HFD and H_H groups might be related to its cognitive impairment.

PCSK9 was found to promote LDL degradation. The upregulation of *Pcsk9* expression in the AD_HFD group might be closely related to the increase of cholesterol in their brain tissues, while HLJDD significantly downregulated the expression of *Pcsk9*. SLC10A4 was a family of sodium bile acid cotransport proteins that were activated by proteases to participate in the transport of bile acids in brain tissue. The expression of *Slc10a4* was significantly decreased in AD group and significantly increased in AD_HFD group. HLJDD could significantly reduce the expression of *Slc10a4*. CYP27A1 regulated the synthesis of primary bile acids in the alternative pathway. The results of the previous experiments on serum bile acids in mice also showed that HFD increased the level of CDCA produced by the alternative pathway in mice. This result once again confirmed that HFD intervention could cause the transformation in the bile acid synthesis pathway in AD mice.

During the imposed remodeling of gene expression, transcription level alterations of certain mRNA didn't closely correlate with those of the encoded proteins, which could partially depend on the differential recruitment of mRNAs to translate ribosomes. Translatome could provide vital information for the translational regulation, to study the process of protein production from mRNA translation. The translational response helped to establish complex genetic regulation that couldn't be achieved by controlling transcription alone. This suggested that the roles of translational and transcriptional regulation were relatively independent. A large amount of data still needed to be mined in depth to discover more valuable regulatory networks, which would provide a basis and direction for later studies on AD and HFD intervention mechanisms, thus providing a more comprehensive understanding of the occurrence and development of AD disease. In summary, our analysis revealed distinct and related roles for translational and transcriptional regulation in HFD on AD mice, highlighting a critical role of translational regulation on AD.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found here: http://bigd.big.ac.cn/gsa/, CRA007307.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the Beijing Animal Science Co., Ltd., and the animal ethics approval number was IACUC-2018100605.

Author contributions

WG, JZ, XF, LW, and XG performed the experiments. WG analyzed the data and wrote the original manuscript. YZ and HZ revised the manuscript. NS and HW contributed to the work. BB and HZ conceived and designed the experiments. All authors reviewed the manuscript and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.974333/full#supplementary-material

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Paternal preconceptional diet enriched with n-3 polyunsaturated fatty acids affects offspring brain function in mice

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Recent studies demonstrate that paternal nutrition prior to conception may determine offspring development and health through epigenetic modification. This study aims to investigate the effects of paternal supplementation of n-3 polyunsaturated fatty acids (n-3 PUFAs) on the brain development and function, and associated gene imprinting in the offspring. Three to four-week-old male C57BL/6J mice (founder) were fed with an n-3 PUFA-deficient diet (n-3 D), and two n-3 PUFA supplementation diets – a normal n-3 PUFA content diet (n-3 N) and a high n-3 PUFA content diet (n-3 H) for 12 weeks. Then they were mated to 10-week-old virgin female C57BL/6J mice to generate the offspring. The results showed that paternal n-3 PUFA supplementation in preconception reduced the anxiety- and depressive-like behavior, and improved sociability, learning and memory in the offspring, along with increased synaptic number, upregulated expressions of neuron specific enolase, myelin basic protein, glial fibrillary acidic protein, brain-derived neurotrophic factor in the hippocampus and cerebral cortex, and altered expressions of genes associated with mitochondria biogenesis, fusion, fission and autophagy. Furthermore, with paternal n-3 PUFA supplementation, the expression of imprinted gene Snrpn was downregulated both in testes of the founder mice and their offspring, but upregulated in the cerebral cortex and hippocampus, with altered DNA methylation in its differentially methylated region. The data suggest that higher paternal intake of n-3 PUFAs in preconception may help to maintain optimal brain development and function in the offspring, and further raise the possibility of paternal nutritional intervention for mental health issues in subsequent generations.

KEYWORDS

n-3 polyunsaturated fatty acids, paternal nutrition, offspring brain, gene imprinting, mouse

Introduction

N-3 polyunsaturated fatty acids (n-3 PUFAs) are paramount for human health, and their functional roles in cardiovascular system, brain development and function, immune response, allergy, and particular physiological states (e.g., pregnancy, prematurity, infancy), have been focused during the past decades (1, 2). N-3 PUFAs are components of cellular membranes and the precursors of several metabolites with different beneficial effects on cell membrane fluidity, neuronal growth and differentiation, intracellular signaling and gene expression, inflammation and oxidation (3, 4). However, the modern western diet has greatly reduced the intake of n-3 PUFAs with an increase of n-6 PUFAs, resulting in the ratio of n-6/n-3 PUFAs at 20–30:1 which is much higher than that at 1–2:1 in the Paleolithic diet (5, 6). Growing evidence suggests that this altered dietary n-6/n-3 PUFAs is closely associated with chronic non-communicable diseases, including cardiovascular diseases, diabetes, obesity, as well as neurodevelopmental diseases (e.g., autism, attention deficit and hyperactivity disorder, and schizophrenia) in children, and degenerative neurological diseases in the elderly, and that the ratio of n-6 to n-3 PUFAs in diets at 1–2:1 should be the target ratio for health (7, 8).

The fetal programming hypothesis and thereafter the development origins of health and disease hypothesis indicate that nutrition and other environmental factors in early life determine the offspring phenotype and health in later life (9, 10). Both animal and human studies have demonstrated that maternal dietary n-3 PUFA deficiency during pregnancy and lactation impairs learning and memory in adult offspring (11, 12), and a high level of seafood intake or supplementation of docosahexaenoic acid (DHA, C22:6n-3) during pregnancy and lactation can effectively improve the psychological, language learning and intellectual development levels in early childhood (13, 14), although some inconsistent findings exist owing to multiple factors, such as differences in body n-3 PUFA baseline, quantity and duration of supplementation, interference from harmful chemicals or substances in seafood, and/or micronutrient (iron, iodine, zinc, etc.) deficiency (15–20).

During the past decade, a substantial number of studies have strengthened the paternal origins of health and disease paradigm, which stresses the need for more research on the role of the father in the transmission of acquired environmental messages from his environment to his offspring (21, 22). Mammalian spermatozoa are rich in PUFAs, particularly DHA, which are important for spermatogenesis with higher sperm motility and concentration, and normal morphology (23, 24). The reduction of n-3 PUFA intake in modern population leads to obstacles to spermatogenesis and maturation, resulting in the decline of male fertility, which has become a global problem in reproduction (25, 26). However, there is lack of relevant research on whether paternal n-3 PUFA status in preconception impacts on offspring development and health, including brain development and function.

The mechanisms through which parental nutrition determines offspring health have been extensively investigated, but they are still not completely understood. The contribution of maternal n-3 PUFAs to offspring brain development has been considered to be associated with several pathways, including enhancement of prenatal and postnatal DHA accretion in offspring brain, and epigenetic and non-epigenetic regulation on the expression of genes associated with neuronal growth and differentiation, protection against neuroinflammation, oxidation and apoptosis, etc. (4, 27, 28). Additionally, maternal feeding of DHA exerts preventive effects on prenatal stress-induced brain dysfunction through modulating metabolism of mitochondria (29), which plays a decisive role in brain development by providing energy for cell proliferation and differentiation, and synaptogenesis (30, 31). Being different from the direct interaction between the mother and offspring by nutrient exchange during prenatal and postnatal periods, the sperm- and seminal plasma-specific mechanisms connect paternal nutrition with the offspring development and health, as well as the maternal health (32). Gene imprinting, one class of epigenetics, is particularly relevant to early life and transgenerational effects since imprints are established in the germline, maintained during the preimplantation reprogramming phase, and then passed on through the somatic cell lineages impacting on genome function and gene expression (33, 34). Imprints are particularly promising candidates in brain research as they are known to be important for neurogenesis, brain function and behavior (33–36).

Therefore, we hypothesized that paternal higher n-3 PUFA intake in preconception might produce positive influences on brain development and function in the offspring through altering mitochondria metabolism and associated gene imprinting. In this study, using a mouse model that was received feeding intervention, the impact of preconception n-3 PUFA status in the father on the brain function (anxiety-like behaviors, depression-like behaviors, and memory) and histological changes were determined in the offspring. Furthermore, changes in offspring brain mitochondria metabolism, and expressions of imprinted genes associated with brain development in the testis and brain were investigated.

Materials and methods

Diets

Three types of diets with n-3 PUFA deficiency (n-3 D), normal n-3 PUFA content (n-3 N), or high n-3 PUFA content (n-3 H) were designed and manufactured by modifying the oil type in the AIN-93G diet as our previously published

(37). The lard oil and sunflower oil were added in the n-3 D diet to produce n-3 PUFA deficiency with an n-6/n-3 PUFA ratio at 47.2:1; whereas the flaxseed oil and fish oil mixed with the lard oil and sunflower oil were added to the n-3 N and n-3 H diets to yield two different n-6/n-3 PUFA ratios at 4.3:1 and 1.5:1, respectively, which represent the current recommendation (4–10:1) and the dietary ratio for our ancestors, containing both very long-chain n-3 PUFAs, eicosapentaenoic acid (EPA; C20:5n-3), DHA and their precursor α-linolenic acid (ALA, C18:3n-3) (5–7). The AIN-93G growing diet and AIN-93M mature diet were used for maternal feeding during pregnancy and lactation, and offspring pup's feeding after weaning, respectively. Details for the diet formula and fatty acid compositions are shown in **Table 1**. All the diets were prepared by the Beijing Huafukang Bioscience Co. Inc. (Beijing, China) and were sterilized with γ-irradiation 25 kGy and stored at −20°C before use.

Animals

Three- to four-week-old male C57BL/6J mice were purchased from the Gempharmatech Co., Ltd (Nanjing, China) and were housed at the animal facilities with SPF-grade condition in the National Institute of Occupational Health and Poison Control, China CDC. Following one week of recovery from transportation, the mice were randomly classified into three groups (n = 12 in each group) and fed with one of the n-3 D, n-3 N and n-3 H diets, respectively. All the mice were free access to water and food under the condition of a 12-h light/12-h dark cycle and cycles of air ventilation. After 12 weeks of feeding intervention, the founder male mice were mated with 10-week-old virgin female mice (1 male for 2 females per cage) and fed the AIN-93G diet (H10293G), which lasted for the pregnancy and lactation of the mating female mice. A 12-week feeding intervention for the founder male mice was set up to ensure optimal models of n-3 PUFA deficiency and supplementation, owing to that 3–6 months are needed for tissue saturation of EPA and DHA concentrations with fish oil supplementation (38–40).

After weaning at 3 weeks of age, the offspring mice from the three groups were fed the AIN-93M diet which lasted for 6 weeks. At the end of experiments, examination of the brain function (anxiety-like behaviors, depression-like behaviors, and memory) was conducted in some offspring mice (n = 5 in each group for the same sex). To avoid bias due to behavior tests, the other offspring mice (n = 8 in each group for the same sex) in a fasted state were used for determination of gene expression and DNA methylation. Each offspring mouse selected in each paternal diet group was from separate litters to avoid being born from the same father. The mice were euthanized by intraperitoneal injection of an overdose of Avertin (2,2,2-tribromoethanol) (500 mg/kg) (T-4840-2, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) for anesthesia followed by decapitation. The testis, cerebral cortex and hippocampus were immediately dissected free of surrounding tissue, removed and frozen in liquid N_2 and then transferred to −80°C for gene expression analysis. Meanwhile, tissues of the cerebral cortex and hippocampus from mice for the brain function experiment were immediately fixed by immersion in 10% paraformaldehyde and 2.5% glutaraldehyde, respectively, for immunohistochemical determination and transmission electron microscopy analysis. After mating, the founder male mice were also euthanized and testes were collected, frozen in liquid N_2 and then transferred to −80°C for later use.

All experiments complied with the ARRIVE guidelines as well as the Guide for the Care and Use of Laboratory Animals in China. All procedures were conducted in accordance with the Animals (Scientific Procedures) 1986 Act (UK) (amended 2013) and approved by the Ethic Committee of the National Institute of Occupational Health and Poison Control, China CDC (No. EAWE-2021-06).

Fatty acid analysis

Fatty acid analysis in diets and tissues was conducted by gas chromatography on Agilent 6890N GC equipped with a flame ionization detector (FID) and injector, using the method of fatty acid methyl esters (FAMEs). Diets and tissues of testis and brain were homogenized using a tissue disrupter in 0.9% sodium chloride solution. Preparation of FAMEs from tissue homogenates was performed according to a modified Lepage method based on our previously published (37). The quantity of each fatty acid was expressed as the percent (%) (wt/wt) of total fatty acids.

Assessment of sperm counting and vitality

During the process of tissue collection, the cauda epididymidis of founder male mice was dissected, punctured and incubated in the prepared HEPES buffer for sperm to swim out. The supernatant was removed, centrifugated (3,000g for 5 min), washed twice in buffer PBS (41). The sperm preparations were assayed for sperm count and vitality assessment using the hemocytometer under the microscope.

Behavioral experiments

Offspring mice aged 9 weeks were subjected to a series of behavioral determination. Five days before the experiment conduction, all mice were kept in the specific room to adapt to the testing environment. All tests were performed between 9 am and 5 pm. Three tests were used to determine anxiety- and depressive-like behavior. The open-field test (OFT) was

TABLE 1 The ingredient compositions and fatty acid profiles in mouse diets.

	Founder diets			Growing diet	Mature diet
	n-3 D	n-3 N	n-3 H	AIN-93G	AIN-93M
Fat (g/kg)					
Lard oil	22	22	22	0	0
Sunflower oil	48	37	22	0	0
Flaxseed oil	0	7	17	0	0
Fish oil	0	4	9	0	0
Soybean oil	0	0	0	70	40
Other nutrients (g/kg)					
Casein	200	200	200	200	140
Corn starch	397	397	397	397	496
Maltodextrin	132	132	132	132	125
Sucrose	100	100	100	100	100
Mineral mix	35	35	35	35	35
Vitamin mix	10	10	10	10	10
Cellulose	50	50	50	50	50
Antioxidants	0.014	0.014	0.014	0.014	0.008
Choline	2.5	2.5	2.5	2.5	2.5
Fatty acids (%)					
ΣSFA	33.19	33.85	33.53	35.85	36.88
ΣMUFA	27.29	25.37	25.07	17.94	17.68
Σn-6 PUFAs	38.70	33.14	24.95	41.19	40.58
C18:2n-6 (LA)	38.04	32.29	24.23	41.18	40.46
C18:3n-6 (GLA)	0.41	0.47	0.34	–	0.12
C20:3n-6 (DGLA)	0.04	0.07	0.05	–	–
C20:4n-6 (AA)	0.14	0.16	0.19	–	–
C22:2n-6 (DDA)	0.05	0.09	0.07	–	–
C22:4n-6 (ADA)	0.02	0.06	0.06	–	–
C22:5n-6 (OA)	–	–	0.01	–	–
Σn-3 PUFAs	0.82	7.64	16.45	5.02	4.86
C18:3n-3 (ALA)	0.28	5.74	13.54	4.15	4.02
C18:4n-3 (STA)	0.32	0.46	0.32	0.08	0.06
C20:3n-3 (EA)	0.22	0.38	0.28	0.79	0.78
C20:5n-3 (EPA)	–	0.80	1.60	–	–
C22:5n-3 (DPA)	–	0.06	0.11	–	–
C22:6n-3 (DHA)	–	0.20	0.60	–	–
Ratio of n-6/n-3 PUFAs	47.2:1	4.3:1	1.5:1	8.2:1	8.4:1

ΣSFA, total saturated fatty acids; ΣMUFA, total monounsaturated fatty acids; Σn-6 PUFAs, total n-6 polyunsatuirated fatty acids; Σn-3 PUFAs, total n-3 polyunsaturated fatty acids; LA, linoleic acid; GLA, γ-linoleic acid; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; DDA, decanedicarboxylic acid; ADA, adrenic acid; OA, osbond acid; ALA, α-linolenic acid; STA, stearidonicacid; EA, eicosatrienoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoci acid; DHA, docosahexaenoic acid.

conducted by placing mice in the open field (L × W × H; 50 cm × 50 cm × 30 cm) individually and allowed 5 min of free movement, and the time spent in the center area was recorded. Light/dark test (LDT) was performed according to the procedure described by Heredia (42). The rectangular box (L × W × H; 50 cm × 30 cm × 30 cm) comprised two compartments, painted black (dark compartment) and another white (light compartment), which separated by a polymeric methyl methacrylate with a centrally-positioned 7.5 × 7.5 cm opening at floor level. Mice were individually placed in the center of the dark compartment and allowed 5 min to explore the apparatus. The inter-compartmental transitions and time spent in the dark compartment were evaluated. Sucrose preference test (SPT) was performed as the described method (43). Before the test, the mice were housed in the cage with two bottles of sucrose water [2% (w/v)] to acclimate for 24 h. Then, one bottle of sucrose water was replaced by tap water for 24 h, alternating the positions of two bottles every 6 h to eliminate the

possibility of side or position preference. The sucrose preference (SP) value was calculated as follows: SP (%) = sucrose intake (g)/[sucrose intake (g) + water intake (g)] × 100%.

The three-chamber test (TCT) was used for sociability assessment as described by Liu (44). Briefly, the mouse was first habituated to the empty box (L × W × H; 50 cm × 30 cm × 30 cm) with three equally sized, interconnected chambers (left, center, right) for 5 min. During the second 5-min, the tested mouse could interact either with an empty wire cup or the other wire cup contained a stranger. The time spent interacting (sniffing, crawling upon) with the two cups was recorded. Sociability index was calculated as follows: (time spent with stranger- time spent with empty cup)/(time spent with stranger + time spent with empty cup).

The novel object recognition (NOR) is an efficient method to test learning and memory in mice (45). The mouse was placed in the middle of the rectangular arena (L × W × H; 50 cm × 30 cm × 30 cm) and allowed to freely explore for 5 min and then removed out of the arena. Then two identical objects were placed in the central symmetrical positions of the arena. The mouse was again placed in the center of the arena, freely exploring the two objects for 5 min, the mouse was transported to the holding cage. One hour later, one of the training objects was replaced with a novel object, and the mouse was allowed to freely explore for 5 min. The discrimination index was expressed as the time spent exploring the novel object minus the time spent exploring the familiar object, divided by total exploration time, reflecting the preference for new objects.

Histological examination

Immunohistochemical analysis was performed on neuron specific enolase (NSE), myelin basic protein (MBP) and glial fibrillary acidic protein (GFAP), which are specific biomarkers for neuronal cell bodies, mature myelinated oligodendrocytes and astrocytes, respectively (46–48). Formaldehyde fixed brains were treated with 70% ethanol and xylene, embedded in paraffin, sectioned at 5 mm on the coronal plane, and air dried. At the level of anterior thalamus and hippocampus, samples were taken continuously through the cerebral hemisphere. The slices were dewaxed, rehydrated in ethanol, and then incubated in 0.01 mol/L sodium citrate (pH 6.0) at 98°C for 10 minutes. After cooling, the slices were washed with 0.3% PBS Triton, incubated with 3% hydrogen peroxide for 10 minutes at room temperature, and then washed with water. Sections were incubated overnight at 4°C against antibodies for NSE (1:500) (GB11376-1), GFAP (1:1200) (GB12096), or MBP (1:200) (GB11226). After the slides were washed in PBS, incubated with goat anti-rabbit IgG HRP, and finally stained with 3,3-diaminobenzidine (DAB) (DAB chromogenic kit, G1211). Slides incubated without the addition of primary antibody were used as negative control. All antibodies and reagents were purchased from Servicebio technology Co., Ltd. (Wuhan, China). Morphometric analysis was performed on Image Pro Plus 6.0 system (media cybernetics. USA) to measure the mean optical density (OD) of NSE positive neurons, GFAP positive astrocytes and MBP positive myelinated oligodendrocytes in the cerebral cortex and hippocampus.

Structure changes in mitochondria and synapses were determined by transmission electron microscopy according to description by Rybka (49). Briefly, fresh cerebral cortex and hippocampus were fixed in glutaraldehyde (2.5%). After washed by phosphate buffer (0.1 M, pH 7.4), the slices were postfixed in 1% osmium tetroxide for 2 h. Then, they were rinsed, dehydrated, saturated and embedded in mixtures of acetone and SPI-Pon 812 resin (SPI-Chem, USA). Ultrathin slices were sectioned and poststained with uranyl acetate and lead citrate in the avoidance of carbon dioxide, and then washed with ultrapure water and dried. Imaging was done with a HT7800/HT7700 transmission electron microscope (Hitachi, Tokyo, Japan).

Ribonucleic acid isolation and qRT-pCR

Total RNA in tissues was extracted using the RNAiso Plus (TaKaRa, Kusatsu, Japan) and complementary DNA was prepared from the total RNA using the All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (OneStep gDNA Removal) (TransGen Biotech, Beijing, China) according to the procedures provided by the manufacturer. The mRNA expression of targeted genes was measured by real-time qPCR with a CFX96 Touch^{TM} Real-Time PCR Detection System (Bio-Rad) using Top Green qPCR SuperMix (Trans Gen), with the thermocycle program consisting of an initial hot start cycle at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, 60°C for 15 s, and 72°C for 10 s. Based on their involvement in neural development, neuronal apoptosis, synaptic transmission, and neuropsychiartric disorders, a total of nine imprinted genes, Zac1, Ube3a, Peg1, Igf2 (Peg2), Peg3, Snrpn (Peg4), Ndn, Kcnk9 and RasGrf1 (34), and brain-derived neurotrophic factor (Bdnf), were included in the present study. The primer sequences can be found in **Supplementary Table 1**.

Analysis of mitochondria deoxyribonucleic acid copy number

Total DNA in the offspring brain was extracted with the Animal Tissue DNA Kit (catalogue no.3101250; Simegen Biotechnology Co., Ltd.). Mitochondria DNA (mtDNA) was amplified using primers specific for the mitochondrial cytochrome oxidase subunits I (CoxI) gene. Nuclear DNA was amplified using primers specific for the 18S rRNA gene.

Primer sequences can be found in **Supplementary Table 1**. The RT-PCR was performed on individual DNAs by using CFX96 Touch^{TM} Real-Time PCR Detection System (Bio-Rad). The relative number of mtDNA copies (mtDNA-CN) was calculated as the normalized ratio of CoxI/18S rRNA gene.

Deoxyribonucleic acid bisulphite conversion and sequencing

DNA methylation in differentially methylated region 1 (DMR1) of the Snrpn was determined by bisulphite sequencing. Briefly, bisulfite conversion of purified DNA of the testis and brain was treated with sodium bisulfite to convert the unmethylated cytosine into uracil using the EZ DNA Methylation Kit (catalogue no. D5002; Zymo Research). Converted DNA was amplified by nested PCR, and the PCR products were sequenced directly. The methylation fraction was calculated from the amplitude of cytosine and thymine within each CpG dinucleotide [C/(C + T)]. The assays were performed in triplicate. The primers used and annealing temperature are shown in **Supplementary Table 1**.

Statistical analysis

One-way analysis of variance (ANOVA) was used to compare means in different groups using SPSS 21.0, except for body weight analysis with repeated measures ANOVA. The Kolmogorov–Smirnov test was used to evaluate whether the data is normally distributed. Following ANOVA, a *post hoc* test was conducted using either Bonferroni test or Dunnett's T3 test for data lacking homogeneity of variance. For data with the non-normal distribution Kruskal Wallis test was used. $P < 0.05$ was considered be statistically significant in differences.

Results

Effects of paternal n-3 PUFA supplementation on testis fatty acid composition and sperm vitality in founder

As shown in **Table 2**, in founder male mice, testis DHA and total n-3 PUFAs were increased with both the n-3 N and n-3 H diet feeding, compared to the n-3 D diet feeding. Consistently, the sperm vitality was significantly increased by the n-3 N diet (84.10 ± 3.78%) and the n-3 H diet (82.02 ± 2.78%), compared with the n-3 D diet (66.62 ± 4.93%). Also, the sperm count was increased by the n-3 N and n-3 H diet (4.76×10^6/L and 4.78×10^6/L sperm preparations), compared to the n-3 D diet (3.51×10^6/L sperm preparations).

Effects of paternal n-3 PUFA supplementation on weight and fatty acid composition of the brain in offspring

As shown in **Figure 1**, The paternal n-3 N or n-3 H diet increased the hippocampus weight in the offspring, compared to the paternal n-3 D diet, with no effects on the body weight and the whole brain weight either in males or females. The n-3 PUFA content in the offspring brain was shown no differences among the three groups (**Table 2**).

Effects of paternal n-3 PUFA supplementation on behavior and cognition in offspring

The behavioral and cognitive experiments showed that offspring mice from the paternal n-3 H diet group had more time spent in central area in the OFT than those from the paternal n-3 D diet group. Also, they had shorter time spent in the dark section but higher number of inter-compartmental transitions in the LDT. The SPT indicated that offspring mice from the paternal n-3 N or n-3 H diet group had increased SP value. Results from the TCT exhibited that the sociability index, reflecting the length of interaction with social partner, was increased in offspring mice from the paternal n-3 N or n-3 H diet group, compared with the paternal n-3 D diet group. The changes in tests of the OFT, LDT, TCT and SPT were similar between offspring males and females. Furthermore, the NOR discrimination index was enhanced by both the paternal n-3 N diet and n-3 H diet in males instead of females in the offspring (**Figure 2**).

Effects of paternal n-3 PUFA supplementation on brain histology in offspring

Immunohistochemical analyses on brain NSE, GFAP and MBP in offspring were demonstrated in **Figure 3**. Compared to the paternal n-3 D diet group, males from the paternal n-3 H diet group had an increase in the average optical density (OD) on area of NSE-positive neurons in the cerebral cortex and hippocampus, GFAP-positive astrocytes in the hippocampus, and those from both the paternal n-3 N diet and n-3 H diet groups had more MBP-positive myelinated oligodendrocytes in the corpus callosum and hippocampus; whereas females had similar changes only in the OD on area of NSE positive neurons in the cerebral cortex, and MBP-positive myelinated oligodendrocytes in the corpus callosum and hippocampus.

TABLE 2 Fatty acid compositions of the founder testis and offspring brain.

Fatty acids (%)	Founder testis (n = 12)			Offspring brain (female) (n = 8)			Offspring brain (male) (n = 8)		
	n-3 D	n-3 N	n-3 H	n-3 D	n-3 N	n-3 H	n-3 D	n-3 N	n-3 H
ΣSFA	51.21 ± 7.34	45.15 ± 5.35[a]	46.00 ± 7.32[a]	56.88 ± 0.82	57.77 ± 0.81	59.47 ± 2.49	56.71 ± 2.64	55.46 ± 2.83	56.32 ± 0.99
ΣMUFA	17.27 ± 6.02	19.80 ± 6.40	18.06 ± 8.43	13.77 ± 0.50	13.60 ± 1.23	13.57 ± 0.96	13.94 ± 1.51	13.63 ± 0.91	14.21 ± 0.80
Σn-6 fatty acids	27.13 ± 3.36	24.24 ± 6.25	24.04 ± 6.18	15.52 ± 0.67	15.03 ± 1.02	14.07 ± 2.18	15.64 ± 0.78	17.48 ± 3.64	16.08 ± 0.57
C18:2n-6 (LA)	6.13 ± 3.73	6.36 ± 3.38	4.53 ± 2.80	0.94 ± 0.17	0.99 ± 0.13	0.85 ± 0.10	0.82 ± 0.07	0.91 ± 0.32	0.82 ± 0.09
C18:3n-6 (GLA)	–	–	–	0.37 ± 0.04	0.37 ± 0.15	0.37 ± 0.19	0.42 ± 0.13	0.36 ± 0.08	0.42 ± 0.08
C20:3n-6 (DGLA)	0.84 ± 0.13	1.13 ± 0.20	1.32 ± 0.34[a]	0.63 ± 0.07	0.60 ± 0.10	0.56 ± 0.15	0.64 ± 0.09	0.57 ± 0.06	0.61 ± 0.08
C20:4n-6 (AA)	9.90 ± 2.06	9.60 ± 1.90	9.91 ± 2.78	11.03 ± 0.55	10.57 ± 0.89	10.12 ± 1.38	11.11 ± 0.63	11.34 ± 0.76	11.41 ± 0.55
C22:4n-6 (ADA)	1.00 ± 0.24	0.93 ± 0.27	0.99 ± 0.28	2.13 ± 0.15	2.13 ± 0.11	2.03 ± 0.37	2.33 ± 0.14	3.58 ± 3.15	2.41 ± 0.16
C22:5n-6 (OA)	9.26 ± 2.20	7.81 ± 1.87	8.11 ± 2.23	0.44 ± 0.23	0.37 ± 0.36	0.13 ± 0.23	0.33 ± 0.34	0.71 ± 0.78	0.41 ± 0.21
Σn-3 fatty acids	4.40 ± 1.53	7.42 ± 1.54[a]	9.26 ± 1.47[a,b]	13.82 ± 0.51	13.63 ± 0.30	12.89 ± 1.02	13.71 ± 2.06	13.43 ± 1.36	13.38 ± 0.45
C18:3n-3 (ALA)	–	0.38 ± 0.28[a]	0.80 ± 0.92[a]	0.26 ± 0.04	0.25 ± 0.20	0.23 ± 0.19	0.23 ± 0.19	0.17 ± 0.12	0.30 ± 0.08
C18:4n-3 (STA)	0.05 ± 0.08	0.09 ± 0.09	0.05 ± 0.07	0.28 ± 0.03	0.24 ± 0.19	0.19 ± 0.18	0.30 ± 0.16	0.27 ± 0.07	0.31 ± 0.07
C20:3n-3 (EA)	1.74 ± 0.92	1.78 ± 0.94	1.70 ± 0.75	1.17 ± 0.22	1.49 ± 0.26	1.45 ± 0.35	1.80 ± 1.59	1.26 ± 0.62	1.16 ± 0.31
C20:5n-3 (EPA)	–	0.07 ± 0.11	0.07 ± 0.11[a]	0.22 ± 0.16	0.13 ± 0.22	0.20 ± 0.19	0.22 ± 0.22	0.21 ± 0.21	0.29 ± 0.16
C22:5n-3 (DPA)	–	–	0.04 ± 0.10[a,b]	–	0.13 ± 0.34	–	–	–	–
C22:6n-3 (DHA)	2.36 ± 0.60	4.77 ± 1.08[a]	6.16 ± 1.54[a,b]	11.89 ± 0.52	11.39 ± 0.86	10.82 ± 1.15	11.15 ± 1.09	11.52 ± 1.15	11.32 ± 0.50
C24:5n-3	0.22 ± 0.42	–	0.45 ± 0.46[ab]	–	–	–	–	–	–
Ratio of n-6/n-3 PUFAs	6.64 ± 1.63	3.26 ± 0.62[a]	2.60 ± 0.63[a,b]	1.12 ± 0.07	1.10 ± 0.07	1.10 ± 0.22	1.17 ± 0.22	1.32 ± 0.37	1.20 ± 0.06

Values are means ± SD.

[a]Compared to paternal n-3 D diet group, $P < 0.05$.

[b]Compared to paternal n-3 N diet group, $P < 0.05$.

ΣSFA, total saturated fatty acids; ΣMUFA, total monounsaturated fatty acids; Σn-6 PUFAs, total n-6 polyunsatuirated fatty acids; Σn-3 PUFAs, total n-3 polyunsaturated fatty acids; LA, linoleic acid; GLA, γ-linoleic acid; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; DDA, decanedicarboxylic acid; ADA, adrenic acid; OA, osbond acid; ALA, α-linolenic acid; STA, stearidonicacid; EA, eicosatrienoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoci acid; DHA, docosahexaenoic acid.

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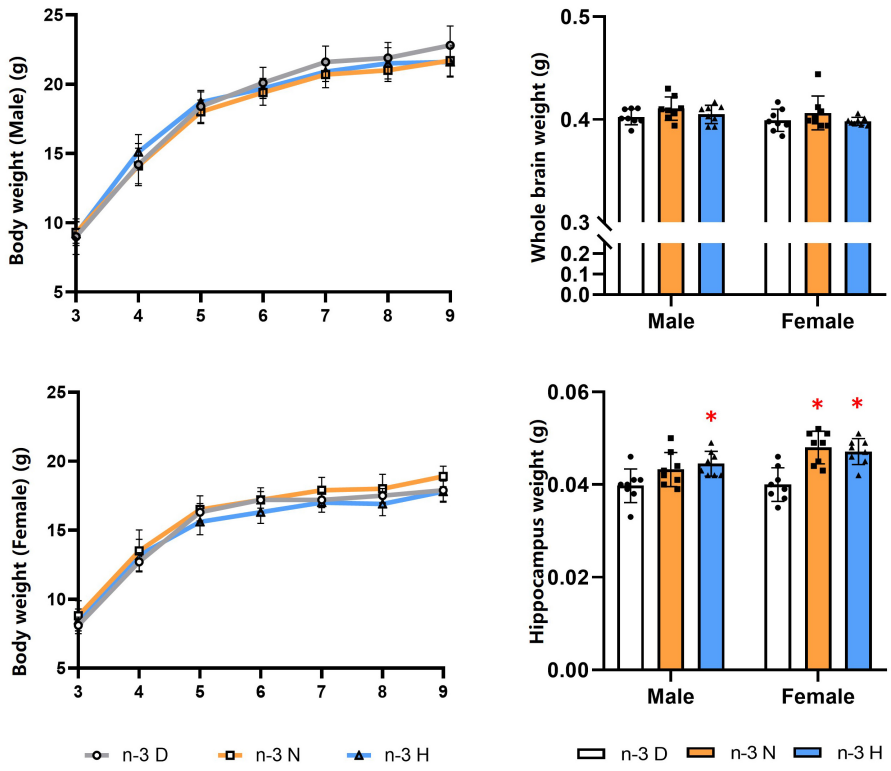

FIGURE 1
Effects of paternal n-3 PUFA supplementation on body and brain weight in offspring. Three to four-week-old male C57BL/6J mice were fed with a n-3 D, n-3 N and n-3 H diet for 12 weeks, and then mated to 10-week-old virgin female C57BL/6J mice to generate the offspring. Male and female offspring body weight were monitored weekly (n = 13 per diet intervention on each sex). After sacrificed at the end of experiment, brains were dissected and weighed (n = 8 per diet intervention on each sex). Values are means ± SD. *Compared to paternal n-3 D diet group within the same sex, $P < 0.05$; #Compared to paternal n-3 N diet group within the same sex, $P < 0.05$.

The transmission electron microscopy demonstrated that the number of synapses was significantly increased in the hippocampus but not cerebral cortex in males from both the paternal n-3 N diet and n-3 H diet groups, compared with the paternal n-3 D diet group. In females, the synaptic number was increased in the cerebral cortex but not hippocampus in both the paternal n-3 N diet and n-3 H diet groups. No differences were observed in the ultrastructure of mitochondria in offspring hippocampus and cerebral cortex between groups (Figure 4).

Effects of paternal n-3 PUFA supplementation on the expression of genes associated with brain function and mitochondria in offspring

As illustrated in Figure 5, the hippocampus mRNA expression of Gfap, Mbp, Nse, and Bdnf in the male offspring was upregulated by paternal n-3 N diet or n-3 H diet, but only the Mbp expression in female offspring was upregulated by paternal n-3 H diet. Examination on mitochondria showed similar changes between the male and the female offspring, in upregulated expression of genes associated with mitochondria biogenesis (Pgc-1α, CoxI), fusion and fission (Opa1, Drp1), and downregulated expression of genes related to mitochondria autophagy (Pink1) in the hippocampus or cerebral cortex by paternal n-3 N diet or n-3 H diet. Consistently, mtDNA-CN in the hippocampus and cerebral cortex was increased by paternal n-3 N diet or n-3 H diet both in the male and female offspring.

Effects of paternal n-3 PUFA supplementation on gene imprinting

A total of nine imprinted genes, which have been considered to be closely associated with brain development and function, were selected for expression analysis (Figure 6). The expression of Zac1, Ube3a, Peg1, Igf2, Peg3, Ndn, Kcnk9 and RasGrf1 showed no differences among the three groups, while the Snrpn was downregulated in mRNA expression by both paternal n-3 N diet and n-3 H diet in testes of the founder mice and their offspring. Therefore, the expression of Snrpn was examined in

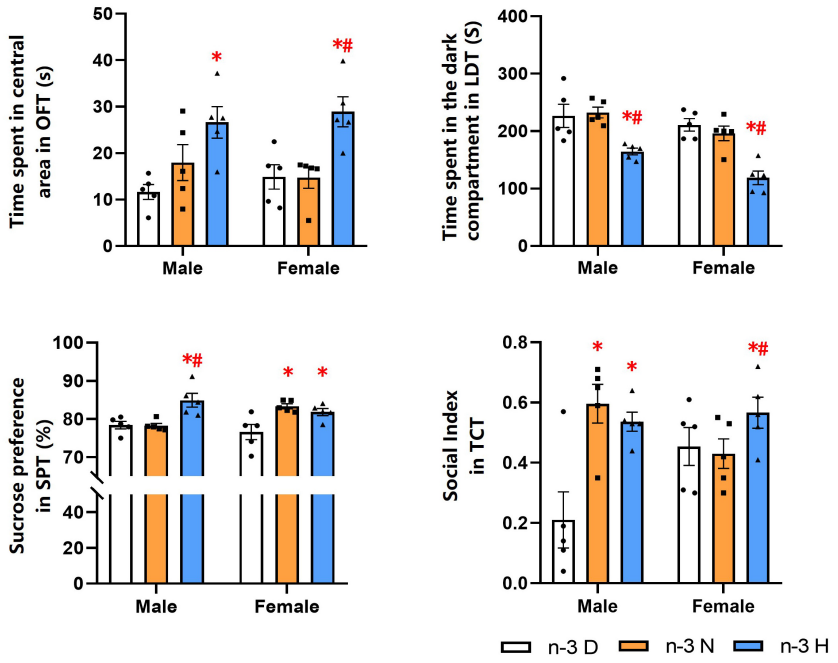

FIGURE 2
Paternal n-3 PUFA supplementation impacts behaviors and cognition in offspring. Offspring mice aged 9 weeks were subjected to a series of behavioral experiments. Time spent in central area in OFT was used to determine level of tension; Time spent in the dark compartment and the number transitions in LDT indicated anxiety behavior; Sucrose preference index in SPT was used to assess the core symptoms of depression (anhedonia); Social index in TCT represented sociability. Discrimination ratio in NOR was used to test learning and memory. Values are means ± SEM, n = 5 per diet intervention on each sex. *Compared to paternal n-3 D diet group within the same sex, $P < 0.05$; #Compared to paternal n-3 N diet group within the same sex, $P < 0.05$.

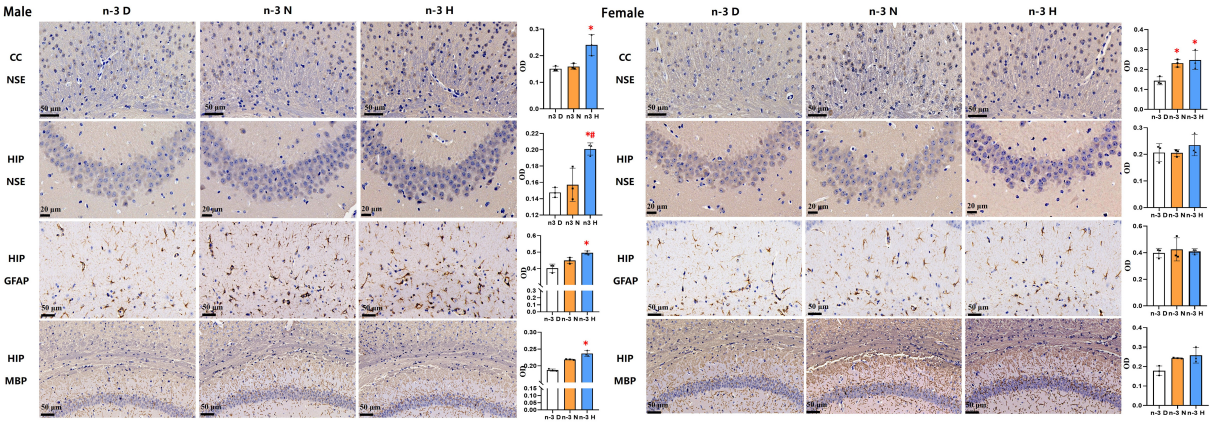

FIGURE 3
Immunohistochemical analysis in the offspring brains. The paraffin sections of the offspring cerebral cortex (CC) and hippocampus (HIP) were deparaffinized and hydrated, and specifically bound with antibodies for neuron specific enolase (NSE), myelin basic protein (MBP), glial fibrillary acidic protein (GFAP). 3,3-diaminobenzidine (DAB) staining was used to observe changes in neurons in the hippocampus (CA3 region) and cerebral cortex (cingulate), astrocytes in the hippocampus (CA1 region), and myelinated oligodendrocytes in the corpus callosum and hippocampus (CA1 region). Values are means ± SEM; n = 3 per diet intervention on each sex. *Compared to paternal n-3 D diet group, $P < 0.05$; #Compared to paternal n-3 N diet group, $P < 0.05$.

the offspring brain, and the results indicated that its expression was upregulated in the hippocampus by both paternal n-3 N diet and n-3 H diet either in males or females. In the offspring cerebral cortex, the Snrpn expression was upregulated by both paternal n-3 N diet and n-3 H diet in females, and by paternal n-3 H diet in males.

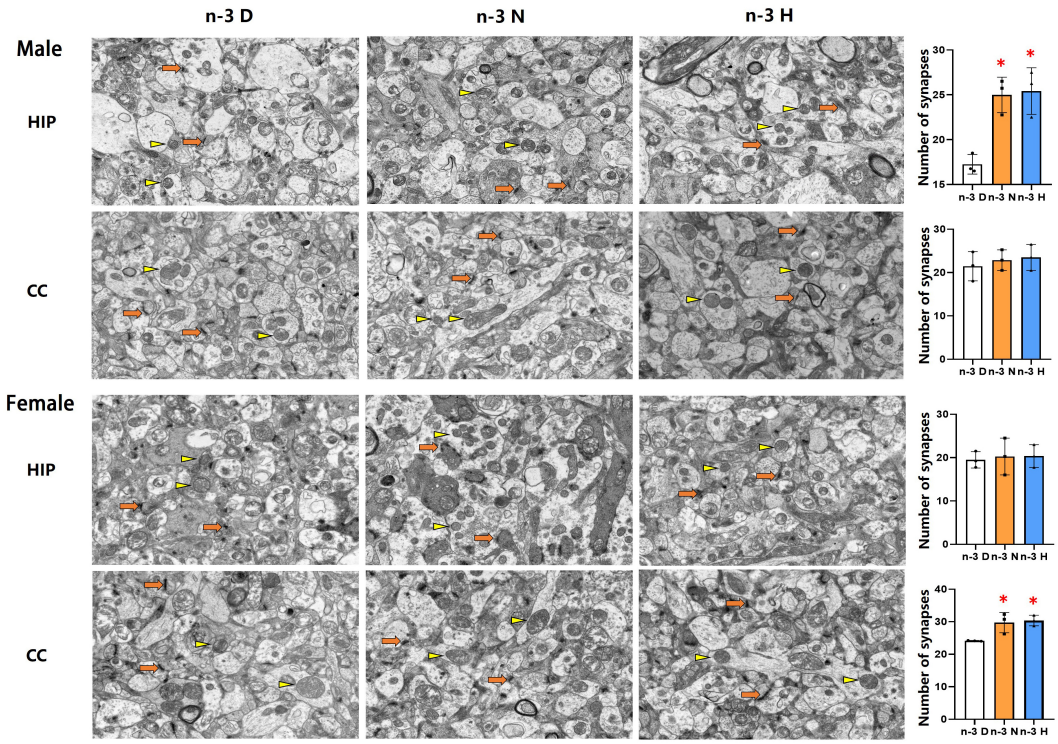

FIGURE 4
Ultrastructure changes in synapses and mitochondria in offspring cerebral cortex and hippocampus. Electron microscope photographs of the cerebral cortex (CC) and hippocampus (HIP) from offspring mice were taken at magnification (×5,000). Numbers of synapses were calculated based on electron microscope captured image. Arrows and triangles indicated synapses and mitochondria, respectively. Values are means ± SEM; n = 3 per diet intervention on each sex. *Compared to paternal n-3 D diet group, $P < 0.05$; #Compared to paternal n-3 N diet group, $P < 0.05$.

DNA methylation analysis showed that the methylation fractions of five CpG sites of the Snrpn DMR1 in testes of the founder were increased by paternal n-3 H diet, and those in testes of the offspring were increased by paternal n-3 N diet or n-3 H diet. In the hippocampus, the methylation fractions of the Snrpn DMR1 were decreased by paternal n-3 H diet in the male offspring, with no changes in the female offspring (**Figure 7**).

Discussion

A growing body of evidence suggests that the paternal diet plays a crucial role in health and disease in offspring's adult life through epigenetic modification on sperm (50). However, the effects of paternal nutrition on offspring brain development and function are scarcely reported. In the current study, we found that paternal n-3 PUFA supplementation in preconception reduced anxiety- and depressive-like behavior (OFT, LDT, SPT), improved sociability (TCT), learning and memory (NOR) in the offspring, along with increased synaptic number, upregulated expressions of NSE, GFAP, MBP, BDNF in the hippocampus and cerebral cortex, as well as altered expressions of genes associated with mitochondria biogenesis, fusion, fission and autophagy. Furthermore, the expression of imprinted gene Snrpn was consistently downregulated in testes of the father and their offspring, and was upregulated in the cerebral cortex and hippocampus by paternal n-3 PUFA supplementation, with altered DNA methylation in DMR1 of the Snrpn. Therefore, paternal n-3 PUFA status could impact offspring brain function and histology.

As well known, adequate nutrition in early life, particularly during pregnancy and infancy, is critical in supporting healthy brain development, with long-lasting effects on cognitive, and socio-emotional skills throughout childhood and adulthood (51). Recently, the impact of paternal nutrition prior to conception on offspring brain development and function has been reported in animal models (22). Paternal methyl donor deficiency or enrichment in diets lead to alterations in offspring brain function, including consolidation-conditioned fear memory and anxiety-like behaviors (52), and hippocampal-dependent learning and memory (53). Diet-induced paternal obesity or calorie restriction during pre-conceptional period has demonstrated impairment in hippocampus-dependent learning and memory function (54), and anxiety-like behaviors in the offspring (55). Herein, our results showed that paternal n-3

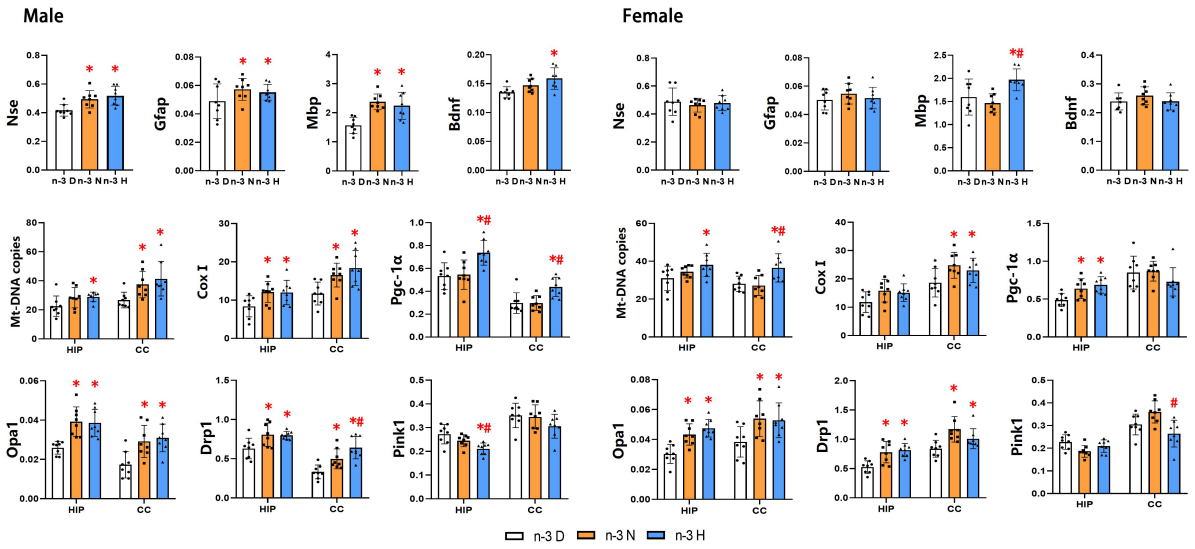

FIGURE 5
Effects of paternal n-3 PUFA supplementation on the expression of genes associated with brain function and mitochondria in offspring. RT-PCR was used to determine the mRNA expression of Nse, Gfap, Mbp and Bdnf in the hippocampus, and the mRNA expression of mitochondria associated genes (CoxI, Opa1, Drp1, Pink1, and Pgc-1α) and Mt-DNA copies both in the hippocampus (HIP) and cerebral cortex (CC) in offspring mice. The data were normalized to relative mRNA levels using the $2^{-\Delta CT}$ method. Values are means ± SD; n = 8 per diet intervention on each sex. *Compared to paternal n-3 D diet group (or with in the same tissue), $P < 0.05$; #Compared to paternal n-3 N diet group (or within the tissue), $P < 0.05$.

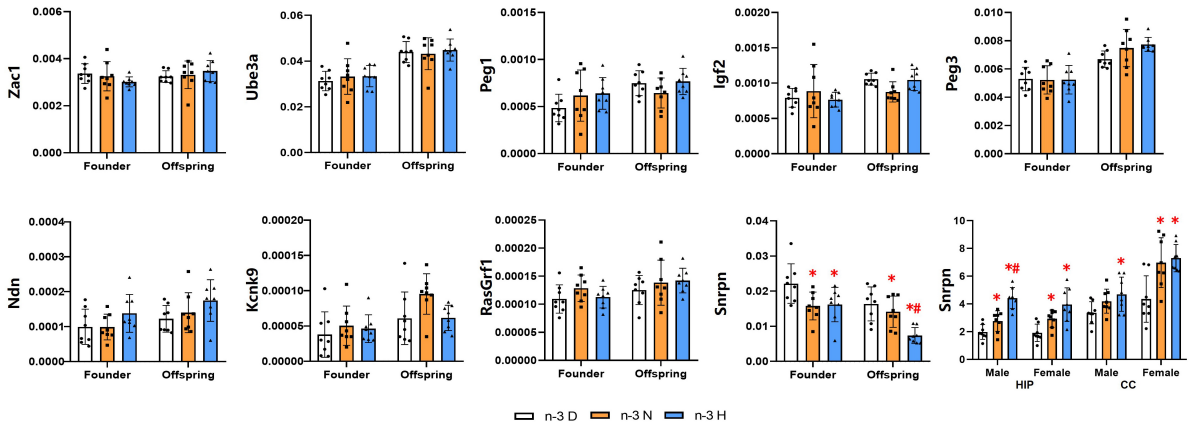

FIGURE 6
Effects of paternal n-3 PUFA supplementation on the expression of imprinted genes. RT-PCR was used to determine the mRNA expression of Zac1, Ube3a, Peg1, Igf2, Peg3, Ndn, Kcnk9, RasGrf1, and Snrpn in testes of the founders and their offspring, and Snrpn in offspring hippocampus and cerebral cortex. The data were normalized to relative mRNA levels using the $2^{-\Delta CT}$ method. Values are means ± SD; n = 8 per diet intervention on each sex. *Compared to paternal n-3 D diet group within the same generation or within the same sex and tissue, $P < 0.05$; #Compared to paternal n-3 N diet group within the same generation or within the same sex and tissue, $P < 0.05$.

PUFA supplementation reduced anxiety- and depressive-like behavior, and improved sociability, learning and memory in the offspring. Consistently, increased synaptic number and expressions of NSE, GFAP, MBP in the hippocampus and cerebral cortex in the paternal n-3 N diet and n-3 H diet groups indicated that, like maternal n-3 PUFAs (37), paternal n-3 PUFAs could promote the growth and maturation of neurons, astrocytes and myelin in the offspring.

The hippocampus is a primary region of the brain controlling the formation of memories, mood and learned behaviors. The ability to learn or form a memory requires a neuron to translate a transient signal into gene expression changes that have a long-lasting effect on synapse activity and connectivity (56). The behavioral and neurophysiological changes in offspring mice induced by paternal methyl-donor are associated with altered hippocampal expression of genes

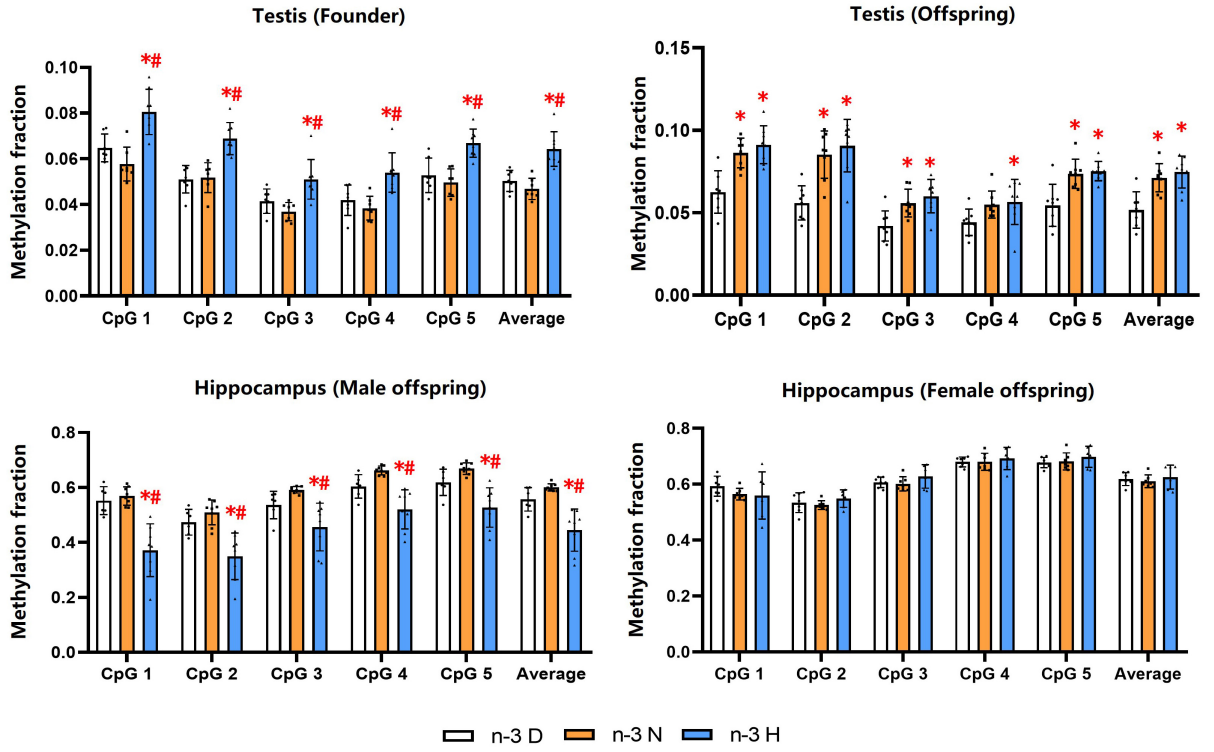

FIGURE 7
Effects of paternal n-3 PUFA supplementation on DNA methylation of imprinted gene Snrpn. Genomic DNA isolated from testes in both founders and their offspring, and the hippocampus in the offspring was given bisulfite conversion. Converted DNA was amplified by nested PCR, and the PCR products were sequenced directly for examination of CpG methylation in DMR1 of Snrpn. The methylation fraction was calculated from the cytosine and thymine within each CpG dinucleotide (C/C + T). Values are means ± SD; n = 6–7 per diet intervention on each sex. *Compared to paternal n-3 D diet group within the same CpG site, $P < 0.05$; #Compared to paternal n-3 N diet group within the same CpG site, $P < 0.05$.

including the potassium calcium-activated channel subfamily M regulatory beta subunit 2, methionine adenosyltransferase II alpha, calcium/calmodulin-dependent protein kinase II alpha and protein phosphatase 1 catalytic subunit (52, 53). The cognitive impairment in the offspring mice caused by paternal high fat diet is attributable to the reduced expression of Bdnf (54), which is a key regulator of neural circuit development and function, mediating neuronal differentiation and growth, synapse formation, and dendritic plasticity in the mammalian brain (57). In this study, the mRNA expression of Bdnf was upregulated by paternal n-3 PUFAs in the hippocampus of male offspring, which might contribute to the memory enhancement and the improvement in expressions of Nse, Gfap, Mbp (37). As well, our previous study demonstrated that the appropriate n-3 PUFA intake during pregnancy and lactation epigeneticly affects the expression of Bdnf, and thus is beneficial for neurogenesis and anti-apoptosis in adulthood of the offspring (58).

Mitochondria play a decisive role in brain development, not only providing energy for cell proliferation and differentiation, but also determining neural stem cell differentiation and synaptogenesis (30). The upregulated expression of Pgc-1α, Cox1, Opa1 and Drp1 with downregulated expression of Pink1 by paternal n-3 PUFA supplementation indicated the increased mitochondria biogenesis, dynamics and respiratory function, with reduced autophagy. The better mitochondria function is responsible for the memory enhancement and improvement in the expression of NSE, GFAP and MBP, and conversely the mitochondria dysfunction is a hallmark of many neurological diseases, including autism spectrum disorder, hyperactivity disorder, schizophrenia, Alzheimer's disease, Parkinson's disease, etc. (31). Studies on other nutrients demonstrate that paternal high fat intake has significant negative effects on the embryo at a variety of key early developmental stages, with reduced mitochondria membrane potential, resulting in delayed development, reduced placental size and smaller offspring (59).

To note, no differences in the fatty acid profile in offspring brains among the three groups implied that the altered behavior and cognition by paternal n-3 PUFAs are independent of their "direct" action, which is different from maternal n-3 PUFAs. During pregnancy and lactation, maternal n-3 PUFAs are transported to the fetus and infants via the placenta and breast milk and play a directable role in maintaining proper brain development and function (4).

The mechanisms by which the paternal nutrition influences the offspring's health are poorly understood, but emerging evidence suggests that it could be transmitted through the sperm epigenome (DNA methylation, histone modifications and sncRNAs) (22, 50, 60). Paternal lifestyle and exposures to environmental factors may alter the sperm DNA methylation including imprinted genes, and consequently affect both the embryonic developmental programing and the health of future generations (47). Several studies have found that paternal fast food intake and obesity can lead to changes in DNA methylation and expression of Meg3 and Nnat in sperm (61), and Igf2/H19 in the offspring (62, 63). In this study, among the nine imprinted genes selected, just Snrpn was affected by paternal n-3 PUFA supplementation, with increased expression in offspring brains and reduced expression in testes of fathers and their offspring. Correspondently, DNA methylation fractions of the Snrpn DMR1 were increased in testes of fathers and their offspring but reduced in the hippocampus of male offspring. These suggested that the impact of paternal n-3 PUFAs on the offspring brain might be mediated by the imprinting of Snrpn, which has been found to be associated with adult neural stem cell differentiation and positively correlated with cognitive abilities in childhood (64). One mechanistic pathway has recently been identified that proper Snrpn expression directly regulates the expression of nuclear receptor Nr4a1 which is critical for cortical neurodevelopment, and that a disruption in Snrpn expression is linked to developmental brain disorders (65). In addition, the differential expression of brain mitochondrial genes was found in mouse models with partial knockout of the Snrpn promoter, and abnormal mitochondrial number and structure were found in cardiac and skeletal muscle (66). PWS-IC del mice exhibit Prader-Willi syndrome, a neurodevelopmental multifactorial genetic disorder caused by lack of Snrpn expression, including deficits in energy metabolism, behavior, cognition, and structure (67). These findings indicate the regulatory role of Snrpn in mitochondria energy metabolism. Conversely, the prominent epigenetic process, methyl groups provided by S-adenosyl methionine, in mitochondria may affect the methylation of imprinted genes (68). Thus, how Snrpn and mitochondria interact to affect brain development and function needs to be explored.

Interestingly, we found that the effects of paternal n-3 PUFA supplementation on neurobehavioral outcomes and expression of associated genes are sex-specific in the offspring. Specifically, anxiety- and depressive-like behaviors (OFT, LDT, SPT) were reduced and sociability (TCT) was improved both in offspring males and females; whereas, learning and memory (NOR) were improved only in offspring males, with paternal n-3 PUFA supplementation in preconception. Histological findings showed that the number of synapses was increased in both the hippocampus and cerebral cortex in male offspring from the paternal n-3 N diet and n-3 H diet groups, but increased only in the cerebral cortex in female offspring. In keeping with other studies, it has been demonstrated that parental environmental factors including diet, metabolism, and stress, affect the behavior and cognition of offspring differently between males and females. For example, adult female but not male offspring of dams fed the low protein diet exhibited passive, and perhaps maladaptive coping strategies in response to stress, accompanied by a marked reduction in hippocampal 5-HT1A receptor function (69). Nutrient-restricted female offspring showed improved learning, while male offspring showed impaired learning and attentional set shifting and increased impulsivity (70). Chronic consumption of a high linoleic acid diet during pregnancy, lactation and post-weaning period increases depression-like behavior in male, but not female offspring (71). With respect to the pre-conceptional paternal nutrition, as early as 20 years ago, sex-specific, male-line transgenerational responses to paternal nutrition and environment have been found in humans. Early paternal smoking is associated with greater BMI at 9 years in sons, but not daughters, and paternal grandfather's and grandmother's food supply was linked to the mortality risk ratios of grandsons and granddaughters, respectively (72, 73). Thereafter, male but not female offspring of fathers fed with a high protein diet exhibited increased insulin sensitivity and decreased glucose induced insulin secretion, with preserved β cell mass and plasticity following metabolic challenge (74). Whereas, when fathers were fed a high fat diet for 10 weeks before mating, female (but not male) offspring had impaired pancreatic β-cell function, with increased bodyweight and glucose intolerance, and reduced insulin secretion (75).

Although the underlying mechanisms for gender differences in parental effects on offspring are not completely known, genetics, epigenetics and gene imprinting, together with the contribution of distinct gonadal steroid hormones and associated inflammatory responses and gene expression, may be involved in this sex dimorphism (76–80). It is acceptable that the sex-specific, male-line transmissions are mediated by the sex chromosomes, X and Y (73), and that paternally expressed genes are generally growth promoting, whereas maternally expressed genes are growth restricting (81). Also, the imprinted gene Dio3 in male pups, while H19 and Xist in female pups, were upregulated by high gestational folic acid supplementation, accompanied by different expressional changes in the candidate autism susceptible gene Auts2 between male and female pups (82). Estrogen has been demonstrated to produce beneficial effects in brain development and function as well as cardioprotective effects, and the advances in understanding the structural, epigenetic and transcriptional mechanisms mediating sexual differentiation of the brain have been reviewed (83–86). It is highlighted that a gene regulatory program activated by estrogen receptor α (Erα) following the perinatal hormone surge, and sustained sex-biased

gene expression and chromatin accessibility throughout the postnatal sensitive period, are of importance (86). Regarding estrogen cognitive protective effects, women with high estradiol (E2) show superior spatial reference memory (87). Female mice have a higher preference index in the NOR paradigm (62.3 ± 13.0%) than males (52.7 ± 5.9%), and are resistance to retroactive interference, which is mediated by estrogen signaling involving estrogen receptor α activation and extracellular signal-regulated kinase 1/2 in the dorsal hippocampus (88). In addition, E2 regulates hippocampus-dependent memory by promoting the synthesis of proteins and their degradation mediated by the ubiquitin proteasome system, that support structural changes at hippocampal synapses (89). As well, E2 treatment greatly upregulates the serum levels of Bdnf and transient receptor potential channels 6, the neuronal excitability indicated by an elevation in the thickness of postsynaptic density and the numbers of asymmetric synapses in rat (90). In the current study, compared to males, females in offspring from the paternal n-3 D diet group had higher NOR preference index (63.90 ± 4.30% vs 45.40 ± 3.30%), along with increased number of synapses (19.63 ± 3.42 vs 17.63 ± 3.02) and expression of Bdnf (0.24 ± 0.03 vs 0.14 ± 0.01) in the hippocampus, implying protective effects in females. Further findings showing no changes in these parameters in female offspring with paternal n-3 N diet and n-3 H diet, suggest that estrogen brain protective effects might override or mask any relationship between paternal n-3 PUFAs and offspring cognition.

The higher ratio of n-6/n-3 PUFAs (20–50:1) in modern diets, has been considered to be a risk factor for many chronic diseases (5). Individuals are required to take both series of PUFAs with the highly recommended n-6/n-3 ratio which is 4–5:1 (91). Considering the ratio of n-6/n-3 PUFAs at 1:1 in the Paleolithic diet, we previously investigated the effect of a higher intake of maternal dietary n-3 PUFAs during pregnancy and lactation on offspring and found that dietary n-6/n-3 PUFA ratio at 1–2:1 has optimal neurogenesis and maturation of neurons, astrocytes and myelin in the offspring brain (37, 58). As well, it is reported that diets with a ratio of n-6/n-3 PUFAs at 1:1 can improve the testicular development of boars and rats, and thus may more effectively reduce exogenous oxidative damage in sperm, providing a more favorable environment for sperm survival (92, 93). In the present study, the alteration in some parameters was different between the paternal n-3 N diet and n-3 H diet groups, indicating that paternal dietary n-6/n-3 PUFA ratio at 1:1 prior to conception might be more beneficial for the offspring brain development.

Our data indicate that paternal n-3 PUFAs may have an impact on offspring brain development. However, some limitations of our study should be addressed. In analyzing the impacts of paternal diet and other factors on the resultant offspring, the random effects of the mother and litter size using a random effects regression model were considered by some researchers (94–96). Unfortunately, in the current study, there exist the statistical limitations related to inability to analyze random effects form mothers and litter size due to the animal management practices in our institution, and this factor should be included in our future work. Therefore, it needs to be emphasized that the random effects statistical model is used in order to improve validity and reproducibility of research in developmental programming studies.

In conclusion, paternal pre-conceptional n-3 PUFA supplementation reduced anxiety- and depressive-like behaviors, and improved sociability, learning and memory in offspring, along with alterations in brain structural development and mitochondria, as well as the expression and DMR1 methylation of imprinted-gene Snrpn both in founder mice and their offspring. These data raise the possibility that paternal dietary factors may be relevant causal factors for mental health issues in the subsequent generation.

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 Ethics Committee of the National Institute of Occupational Health and Poison Control, China CDC (No. EAWE-2021-06).

Author contributions

ML performed the experiment, analyzed the data and prepared the manuscript. QS carried out mouse feeding. XJ and WH performed the experiment. PL participated in the statistical analysis. XL and XF participated in designing the research. KQ designed the research and revised the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.969848/full#supplementary-material

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Impact of feeding habits on the development of language-specific processing of phonemes in brain: An event-related potentials study

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Introduction: Infancy is a stage characterized by multiple brain and cognitive changes. In a short time, infants must consolidate a new brain network and develop two important properties for speech comprehension: phonemic normalization and categorical perception. Recent studies have described diet as an essential factor in normal language development, reporting that breastfed infants show an earlier brain maturity and thus a faster cognitive development. Few studies have described a long-term effect of diet on phonological perception.

Methods: To explore that effect, we compared the event-related potentials (ERPs) collected during an oddball paradigm (frequent /pa/80%, deviant/ba/20%) of infants fed with breast milk (BF), cow-milk-based formula (MF), and soy-based formula (SF), which were assessed at 3, 6, 9, 12, and 24months of age [Mean across all age groups: 127 BF infants, Mean (*M*) 39.6 gestation weeks; 121 MF infants, *M*=39.16 gestation weeks; 116 SF infants, *M*=39.16 gestation weeks].

Results: Behavioral differences between dietary groups in acoustic comprehension were observed at 24-months of age. The BF group displayed greater scores than the MF and SF groups. In phonological discrimination task, the ERPs analyses showed that SF group had an electrophysiological pattern associated with difficulties in phonological-stimulus awareness [mismatch negativity (MMN)-2 latency in frontal left regions of interest (ROI) and longer MMN-2 latency in temporal right ROI] and less brain maturity than BF and MF groups. The SF group displayed more right-lateralized brain recruitment in phonological processing at 12-months old.

Discussion: We conclude that using soy-based formula in a prolonged and frequent manner might trigger a language development different from that observed in the BF or MF groups. The soy-based formula's composition might affect frontal left-brain area development, which is a nodal brain region in phonological-stimuli awareness.

KEYWORDS

infancy, infant's diet, language development, stimuli awareness, MMN

1. Introduction

In infant development, the brain undergoes multiple changes, including increased myelination and configuration of synaptic connections needed to consolidate new brain networks. Volumetric brain growth, which proceeds through infancy, reaches adult levels at 3 years old. These changes are promoted by environmental stimuli (1), hormonal status and genetic factors (2). Moreover, infant

diet has been recently recognized as an important contributor to cognitive development, immune system development, and healthy physical growth (3–6). To support infant development, the diet should provide micro and macronutrients such as docosahexaenoic (DHA) and arachidonic (AA), long-chain fatty acids, lutein, choline, and hormones (7–9). Human milk provides these essential nutrients (5, 6) and promotes greater brain maturity characterized by healthier neuronal growth and myelination, and greater infant gray and white matter (8, 10–12).

Some studies report that breastfed infants show an earlier development of language perception (10, 13–15) and memory than those fed with nutrient-enriched formula (16). An explanation for this finding is that human milk changes in composition from colostrum to late lactation, and varies by the mother's biological condition, while milk-based formula maintains a stable composition (5, 9, 10). In particular, human milk seems to have a better nutritional composition than milk-based formula because: (1) complex oligosaccharides or lipid components such as gangliosides found in human milk are not available in milk-based formula composition or have not been clinically proven (17), and (2) the equilibrium in human milk's composition between DHA, lutein, choline (10, 16), and complex oligosaccharides seems to promote better cognition (18, 19). Therefore, differences in the proportions of formula components may negatively affect infant's nutrition, and consequently, the infant's cognitive development (20).

In the first year of life, phonological perception should be developed; otherwise, the infant will suffer delayed language development (21). This milestone entails the fast growth of multiple brain areas regulated by healthy nutritional habits, particularly microbiome is essential in synaptogenesis and metabolic brain requirements, affecting infant brain development and behavior (22, 23). Moreover, recent studies suggest that breastfeeding positively affects cognition and brain development compared with other feeding habits (24–28). They explain that this effect occurs because four reasons: (1) human milk might make a difference in brain structure and function *via* fatty acids, affecting cell membranes and influencing gene expression within these cells, (2) human milk contains a variety of constituents that promote optimal development, (3) the relationship between the immune system and breastfeeding might influence learning and memory, and (4) lactation affects mothers' way they teach the language (29).

Even *in utero*, infants are able to distinguish between sounds (30–32) and show habituation to repetitive stimuli (30). However, they must develop other abilities to reach adult levels of phonological perception. Within the first 2 months of life, normal infants show a precognitive detection of syllable length (33); at 4 months they begin to distinguish between tones and syllables (34). At 6 months old, they establish prototypes of vowels in their native language (31, 32), and around 10 months old, infants have prototypes of consonants (35). Between 9 and 10 months of age, infants can distinguish words (36), and preserve the detection of foreign-language contrast until 11 months old (37). By the end of the first year of life, they have access to phonological representations akin to those of adults, that is, the infant has developed two important properties for speech comprehension: (1) phonemic normalization and (2) categorical perception (30). These subtle behavioral changes are accompanied by the recruitment of frontal and temporal lobes responsible for phonological perception and semantic categorization, which are differentially developed in the first year of life (38, 39). In infants, brain maturity is reflected in the decrease of bilateral brain responses and increase in left lateralization of brain activity (40–42), culminating in the development of the adult pattern of dorsal and ventral pathways associated with language function (43–45).

Accordingly, brain-electrical activity associated with phonological perception also develops during infancy, reflecting the increasing ability to decode incoming speech supported by the accurate perception of rapid acoustic changes (21, 46, 47). The brain-electrical response to auditory tones [event-related potentials (ERPs)] in adults comprises the P1-N1-P2-N2 complex (48–50), and includes (1) a positive deflection at 150 ms on fronto-central sites (P150 or P1), which has been associated with features of acoustic stimulus (51–53) and modulated by an inter-stimulus interval (54); (2) a negative deflection at 250 ms (N250 or N1) and another at 450 ms (N450 or N2), which reflect the differences between acoustic stimulus (e.g., such as complexity and frequency) (54), and (3) a positive wave at 350 ms (P350 or P2) which has been associated with stimulus awareness and perceptual salience, and is commonly identified as an index of auditory recognition memory (51). However, these ERP components are not exhibited at birth, but develop gradually over the first year of life. At birth, infants display a large positive wave between 100 and 450 ms followed by the N2 component (49). At 3 months old, the positive wave is divided by the N1 component between 160 and 200 ms (49, 55), resulting in two ERP components: a P1 and P2, and the amplitude of these components seem to increase over the next month (49, 54). Between 3 and 6 months the amplitude of N1 and N2 components increase (56), and exhibit the P1-N1-P2-N2 complex. This ERP morphology is maintained until 2 years of age (49, 57), with an increase in component amplitude exhibited at 12-months old (49). Although few studies describe the functional significance of these ERP components in infancy, it has been speculated they have similar function to those observed in children and adults (53).

Development of phonemic perception requires infants to detect differences in acoustic features and phonological categories, leading to the use of experimental auditory oddball paradigms in which stimuli including differences between acoustic features, frequency or phonological categories are especially useful in assessing brain electrical activity associated with the acquisition of language (32–34, 58–61). From studies in children and adults, the expectation is that the amplitude of P1-N1-P2-N2 complex will be greater for uncommon than common repetitive stimuli (50), due to the fact that neuronal responses habituate to repeated presentation of the same stimulus, while a new, unusual stimulus will produce a large amplitude response (30, 62). The difference between the conditions is called mismatch negativity (MMN) (30, 63, 64). It has been reported that two MMNs which appear at 6 months (50, 54), correspond to the differences in P1 and P2 components (65). As described above, the MMN components undergo latency decreases with increasing age (50). In addition, the MMN components have been linked to the computation of acoustic features such as duration or intensity (66, 67), arbitrary rules (68), or lexical and grammatical status (58, 69), and their interpretation depends on the specific stimulus type presented.

While few studies have assessed how diet affects phonetic perception; those that did have shown variations on this cognitive process by diet. Li et al. (13) compared breastfed infants and infants fed with soy or cow-milk-based formula in their phonological perception at 3 and 6 months, using an oddball paradigm compromised of frequent and deviant syllables (/pa/standard and/ba/deviant). The authors found an advanced neural maturation in breastfed infants characterized by a greater P350/P2 amplitude in frontal regions at 3 months, and shorter P2 latency at 6 than 3 months old than the other dietary groups. Using the same paradigm, Pivik et al. (3) compared these same dietary groups and ages. However, they did not replicate the findings of Li et al. (13), reporting no age-related changes in ERP components. In this study,

differences were related only to diet group, with breastfed infants displaying shorter P1 latencies and smaller P1 amplitude for deviant rather than standard stimuli than infants fed with soy milk. The authors interpreted that to indicate that breast-fed infant show more rapid encoding of acoustic information than the other diet groups. The same diet groups were also studied at 4 and 5 months (14), where changes in P350/P2 amplitude across age for each syllable, depended on the diet. Infants fed with soy milk showed a decreased P2 amplitude for deviant stimuli than the other groups, while the breastfed infants displayed decreased amplitude for standard stimuli compared with other dietary groups. The authors concluded that diet affects attention and memory functions involved in the processing and discrimination of speech sounds.

The primary aim of the present study was to determine the differences in phonological perception assessed by electrophysiological response to frequent and deviant phonemes at 3, 6, 9, 12, and 24 months between three dietary groups: breast fed (BF), cow-milk-formula fed (MF), and soy-formula fed (SF) infants. As previous studies have reported evidence for earlier phonological perception in BF infants (13, 14, 65), we anticipated that the BF group would show (1) greater amplitude and shorter latency of MMN components than MF and SF groups, (2) greater amplitude and shorter latency of MMN components (13, 65) at 6 month-old when the P1-N1-P2-N2 complex reaches a stable morphology (49, 57), and at 12 months when ERP amplitudes have a stable morphology (49) and (3) greater hemispheric asymmetry of MMN components (40).

2. Materials and methods

2.1. Participants

The study included full-term infants between 3 and 24 months old. All of them had a birth weight of over 3 kg and were a product of uncomplicated pregnancies; the mothers reported no medical diagnoses during pregnancy or lactation. Mothers with alcohol, tobacco, or medication use were excluded. In this longitudinal study, 2-month-old infants were stabilized on one of three diets which were selected by parents: BF, MF, and SF, the two last fortified with DHA and AA. Each infant was provided the same diet until 12 months of age. The infants were assessed at 3, 6, 9, 12, and 24 months old, resulting in 15 groups of data (e.g., subjects aged at three-months-old distributed into three groups: BF, MF, and SF). Socioeconomic status [SES, measured by the Four-Factor Index of Social Positions (70)] of the infants' parents was collected at the beginning of this study. The infants' anthropometric measures (i.e., height, weight, and head circumference) and food intake history were collected at each visit. Infants and mothers underwent neuropsychological and psychophysiological testing, which was conducted by a certified examiner. The mother's assessment included Wechsler the Abbreviated Scale of Intelligence [WASI-II, (71)] and Symptoms Assessment-45 questionnaire [SA-45, (72)], while infants were evaluated using the Bayley Scales of Infant and Toddler Development [BSID-2, (73)], Preschool Language Scale [PLS-3, (74)] as well as the psychophysiological oddball paradigm to assess phonological-discrimination. Most of the parents reported English as their language at home (see Table 1). All mothers reached an Intelligence quotient (IQ) score higher than 70 on the WASI-II test. Participants were excluded from this study if they did not complete all assessments. The protocol was approved by the Institutional Review Board of the University of Arkansas for Medical Sciences. Informed consent was obtained from parents.

TABLE 1 Characteristics of dietary groups.

Age	Total (*n*)	Type of diet (*n*)	Mother IQ mean (SD)	Language in home
3 m	410	BF: 137	BF: 109.4 (10.2)	E (404)
		MF:138	MF: 105.8 (9.2)	S (1)
		SF: 135	SF: 102.9 (11.7)	E,S (4)
				E,O (1)
6 m	365	BF: 119	BF: 110.1 (10.1)	E (356)
		MF:126	MF: 105.7 (9.3)	E,S (7)
		SF: 120	SF: 103.3 (10.0)	E,O (2)
9 m	340	BF: 113	BF: 109.6 (10.5)	E (333)
		MF:114	MF: 105.4 (8.7)	S (1)
		SF: 113	SF: 103.8 (10.3)	E,S (5)
				E,O (1)
12 m	334	BF: 122	BF: 109.7 (10.3)	E (318)
		MF:112	MF: 105.0 (8.8)	S (2)
		SF: 100	SF: 103.2 (10.3)	E,S (10)
				E,O (4)
24 m	372	BF: 142	BF: 109.6 (10.4)	E (361)
		MF:117	MF: 105.8 (9.1)	S (1)
		SF: 113	SF: 104.5 (10.7)	E,S (7)
				E,O (3)

m, months; n, number of participants; BF, breast fed; MF, milk fed; SF, soy fed; IQ, intelligence quotient from Wechsler Abbreviated Scale of Intelligence (WASI-II); E, English; S, Spanish; O, other languages (i.e., Swedish, Arabic, and Chinese).

2.2. Experimental design

Phonological discrimination was assessed using an auditory-oddball paradigm while an electroencephalogram (EEG) was recorded. The infants were awake and seated in their parent's lap or infant chair in a sound-isolated, shielded recording chamber. Silent videos were played to engage the infant's attention. The paradigm was constituted of two types of stimuli, one of them was frequent (/pa/80%) and the other deviant (/ba/20%). Both stimuli were syllables with consonant-vowel structure, had the same intensity (72 dB SPL), and were pronounced by a native English speaker through speakers located at 5 ft. from the infant. The stimuli were designed and administered using E-Prime software (version 1). All stimuli appeared during 300 ms with a stimulus onset asynchrony (SOA) of 2,500 ms. The SOA was selected because longer intervals attenuate standard-deviant response differences (75) and exceeds the limits of sensory memory reported for infants (76). The task included three blocks of 90 trials for a total of 270 trials. The deviant stimuli (/ba/) randomly appeared in each block with a probability of 0.2. Each block lasted 4.2 min. The infants had two rest periods of 5 min between experimental blocks (see Figure 1).

2.3. Data acquisition/prep-processing

The EEG was acquired with a Geodesic Net Amps 200 system running Netstation 2 software using the 128-channel (Electrical Geodesics, Inc., Eugene OR, United States). Data were amplified with a bandpass of 0.1–100 Hz and a sampling rate of 250 Hz. Electrode impedances were

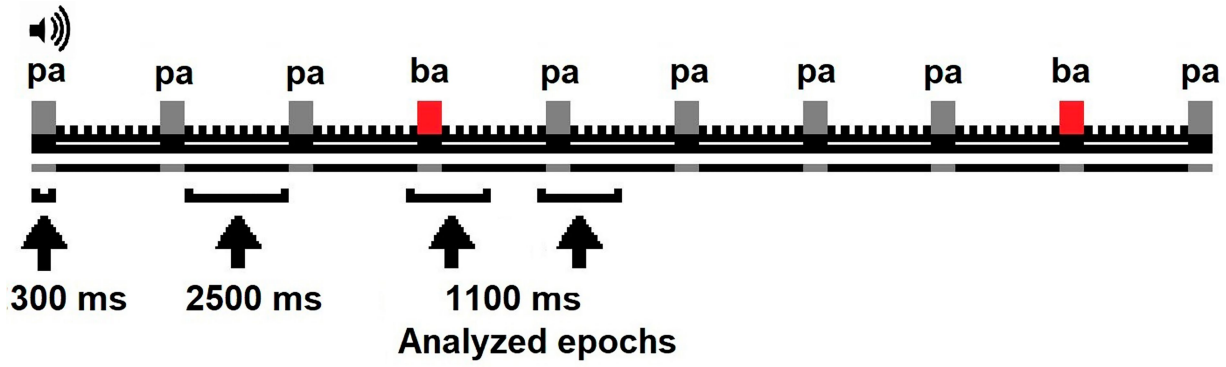

FIGURE 1
Oddball paradigm applied to infants.

kept below 40 kΩ. Eye movements and blinks were monitored. Data were analyzed offline using the Matlab toolbox (Matlab version R2020a). The EEG was segmented into epochs with a 100 ms pre-stimulus baseline and 1,000 ms stimulus/post-stimulus. The epochs were subjected to an automatic artifact detection algorithm. Bad channels (i.e., channels with fast average amplitude greater than 200 μV or/and differential average greater than 100 μV) were interpolated from nearby good channels using spherical splines. Trials with more than 10 bad channels were excluded. The accepted segments for each type of condition (/ba/or/pa/) were baseline corrected using a 100 ms pre-stimulus time window, re-referenced to the common mean, and averaged for each participant. The accepted segments were at least 35 per condition for each participant.

2.4. Event-related potentials

The average epoch for each condition per subject was obtained in four regions of interest (ROIs): Frontal Left (FL; sensors 28, 34, and 35) and Right (FR; sensors 117, 122, and 123), Temporal Left (TL; sensors 42, 47, and 48) and Right (TR; sensors 99, 103, and 104) (see Figure 2). Then, the difference wave was calculated in each ROI by subtracting the epoch associated with the frequent stimulus (/pa/) from that related to the deviant stimulus (/ba/). The grand average of difference wave was inspected in accordance with the ERP literature associated with phonological perception (30, 54, 63, 64). Two ERPs components were identified, two mismatch negativities; the first between 75 and 255 ms (MMN-1), and the second between 300 and 500 ms (MMN-2), the first functionally associated with the P1 component and the second with the P2 component.

2.5. Data analysis methods

2.5.1. Characteristics of dietary groups and behavioral data

2.5.1.1. Parental data

Parental SES in each age group (i.e., 3, 6, 9, 12, and 24-months old) was compared using one-way ANOVA. For both comparisons, dietary group (i.e., BF, MF, and SF) was included as a between-subjects factor, and total SES index was included as within-subject factors.

Maternal psychometric and psychiatric data: Psychometric and psychiatric test results were analyzed using two-way ANOVAs for each assessment (i.e., WASI-II and SA-45) and each age group. The dietary group was included as a between-subjects factor, and the within-subject factors were as follows:

- WASI-II is a test that estimates the general intellectual ability by measuring verbal, nonverbal, and general cognition of adults; this test consists of two indices: Perceptual reasoning index (PRI) and Verbal comprehension index (VCI). The indices were included as within-subjects factors.
- SA-45 is a questionnaire that is constituted by two indices designed to assess general psychiatric symptomatology. The indices are the Global severity index (GSI) and Positive symptom Index (PST); these were included as within-subjects factors.

2.5.1.2. Infants data

2.5.1.2.1. Infants' anthropometric and psychometric data

Birth data and anthropometric measures were compared at 3, 6, 9, 12, and 24-months old using one-way ANOVA. For both comparisons, the dietary group was included as a between-subjects factor, and gestational age, birth length, birth weight, height, weight, and head circumference were separately included as between-subjects factors. A chi-squared test was used to compare groups for infant's sex distribution.

Psychometric test results were analyzed using two-way ANOVAs for each neuropsychological assessment (i.e., BSID-2 and PLS-3 tests) and for each age group. The dietary group was included as a between-subjects factor, and within-subject factors are as follows:

- BSID-2 is a standard series of measurements used to assess the infant's development between one and 42 months, and it is constituted by Mental development index (MDI) and psychomotor development index (PDI). Both were included as within-subject factors.
- PLS-3 is a test used to assess receptive and expressive language skills in infants. This consists of two subscales: auditory comprehension (AC) and expressive communication (EC); these subscales were considered as a within-subject factor.

2.5.1.2.2. Infant' amplitude and latency analyses of ERPs

Comparisons between dietary groups for each age group: We considered MMN-1 and MMN-2 components for the statistical analyses. We calculated the mean amplitude and its latency (i.e., the

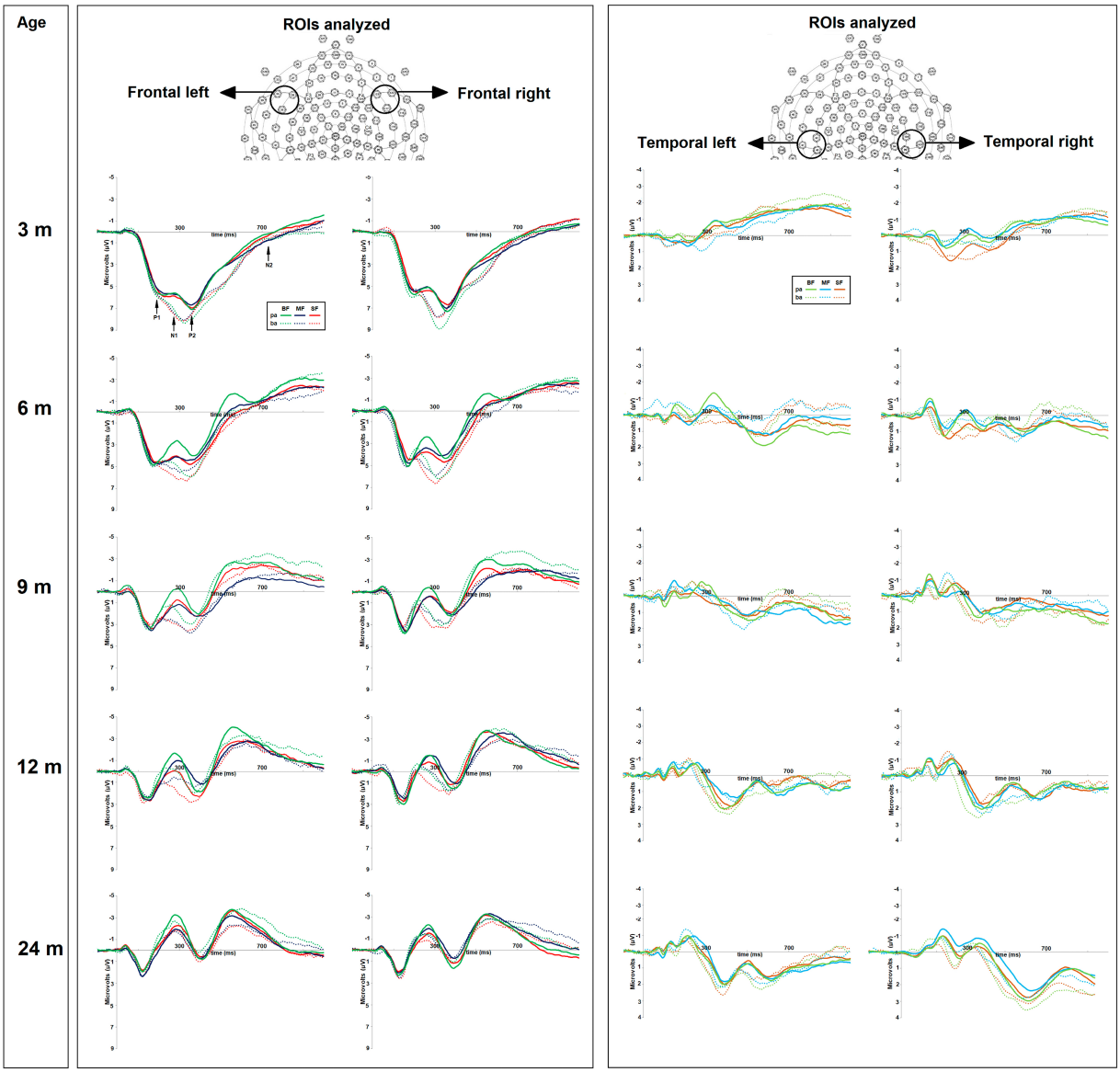

FIGURE 2
On the top, the regions of interest (ROIs) used for amplitude and latency analyses of ERP components. On the bottom, the grand average of ERPs of frequent "pa" and deviant "ba" conditions for each dietary group (BF, breast feed; MF, milk feed; SF, soy feed) at 3, 6, 9, 12, and 24-months old. The positive or negative event-related potentials (ERP) components were highlighted as follows: P1-N1, P2, and N2.

maximal peak of time window) for each ERP component. Then, we separately compared the amplitude and latency of each ERP component. ANCOVAs were also separately computed for each age group. The dietary group was the between-subject factor, FL, FR, TL, and TR ROIs were included as the within-subject factors, and gestational weeks and infant's sex as covariables.

We also assessed the hemispheric asymmetry of ERPs components, ANCOVAs were separately computed for the difference in amplitude or latency of ERPs between brain hemispheres in frontal or temporal regions (e.g., MMN-1 amplitude in frontal left ROI minus MMN-1 amplitude in frontal right ROI). The dietary group was the between-subject factor, frontal and temporal ROIs were included as the within-subject factors, and gestational weeks and infant's sex as covariables.

Comparisons between age groups for each dietary group: ANCOVAs were separately performed for the amplitude or latency of each ERP component and each dietary group. The age group (3, 6, 9, 12, and 24 months) was the between-subject factor, FL, FR, TL, and TR ROIs were included as the within-subject, and gestation weeks and infant's sex as covariables. Data were analyzed using SPSS Statistics 20 and Matlab (version R2020a). Greenhouse–Geisser corrections were made for violations of sphericity when the numerator was greater than 1. value of *p*s resulting from a set of comparisons were corrected by the FDR method. We report results surviving FDR correction (p-values <0.05).

2.5.1.3. Regression analyses

Regression analyses were performed to identify the association between amplitude and latency of ERP components in each ROI, that differed between dietary groups, and those factors that might explain the variability in the brain-electrical activity. The linear regression included amplitude or latency in FL, FR, TL or TR ROIs as the dependent variables, with dietary group (i.e., BF, MF, and SF), mom's cognitive and psychiatric status (WASI-II: PRI and VCI; SA-45: GSI and

PST), gestation weeks, infant's sex, PLS-3: AC and EC subscales as the independent variables. Linear regressions were performed by age group. The linear regression analyses included multiple-linear backward regressions to find a reduced model that best explains the data.

Regression analyses were also performed to identify the association between the hemispheric asymmetry of ERPs components and other variables. Hemispheric differences in frontal or temporal regions were included as dependent variables, and the independent variables were dietary group (i.e., BF, MF, and SF), mom's cognitive and psychiatric status (WASI-II: PRI and VCI; SA-45: GSI and PST), gestation weeks, infant's sex, PLS-3: AC and EC subscales. Linear regressions were performed by age group. The linear regression analyses included multiple-linear backward regressions to find a reduced model that best explains the data. Factors with the highest *value of p* were removed until all factors were statistically significant. A *value of* $p < 0.05$ was considered statistically significant in all analyses.

3. Results

3.1. Parental data

We observed a significant main effect of the dietary group at 3-months [$F(2,398)$ 3.5, $p = 0.03$], and 6-months of age [$F(2,361)$ 5.4, $p = 0.005$]. The *post hoc* tests showed that the BF group displayed a greater parental SES score than SF group at 3-months old [Mean difference (MD) = −2.7, $p = 0.03$; BF, Mean (M) 39.8; MF, $M = 38.4$; SF, $M = 37.0$], while at 6-months old, SF group was significantly different than BF and MF groups, displaying a lower parental SES score than the other groups (SF vs. BF, MD = −3.2, $p = 0.008$; SF vs. MF, MD = −2.8, $p = 0.02$; BF, $M = 40.0$; MF, $M = 39.6$; SF, $M = 36.8$).

The dietary groups differed in maternal WASI-II indices. In all comparisons, the *post hoc* tests showed that mothers from the BF group had greater WASI-II indices than mothers in the other dietary groups [3 months (m), BF vs. MF, MD = 3.1, $p = 0.02$; BF vs. SF, MD = 5.8, $p < 0.001$; 6 m, BF vs. MF, MD = 3.9, $p = 0.002$; BF vs. SF, MD = 6.1, $p < 0.001$; 9 m, BF vs. MF, MD = 3.7, $p = 0.007$; BF vs. SF, MD = 5.2, $p < 0.001$; 12 m, BF vs. MF, MD = 3.9, $p = 0.003$; BF vs. SF, MD = 5.7, $p < 0.001$; 24 m, BF vs. MF, MD = 3.3, $p = 0.01$; BF vs. SF, MD = 4.5, $p < 0.001$]. No significant dietary group by WASI-II indices interaction was found in any comparison (see Figure 3A).

Although no significant main effect of dietary group was observed in maternal SA-45 indices, a significant dietary group by SA-45 interaction was found at 12 months [$F(2,326)$ 6.3, $p = 0.002$, $\eta^2 = 0.04$, $\varepsilon = 1$]. However, the *post hoc* tests showed no significant differences between dietary groups in any SA-45 index (BF: GSI, $M = 45.6$; PST, $M = 45.1$; MF: GSI, $M = 45.6$; PST, $M = 44.3$; SF: GSI, $M = 45.7$; PST, $M = 45.4$).

3.2. Infant's data

3.2.1. Infant's anthropometric and psychometric data

3.2.1.1. Anthropometric data

As is shown in Table 2, gestational weeks differed between groups in all age groups, the *post hoc* tests evinced that the BF group had greater gestation weeks than the other dietary groups. The dietary groups also differed in birth weight at 6 months old. The *post hoc* test showed greater birth weight for BF than SF group (MD = 0.1, $p = 0.02$).

The height and weight differed between dietary groups at 9 and 12 months old, with *post hoc* tests showing lower height and weight for BF infants. The comparison also revealed differences between dietary groups in weight at 24 months old, with BF infants showing lower weight than SF group (MD = −0.5, $p = 0.001$). No differences between dietary groups were found in birth length, head circumference or infant's sex in any age group.

3.2.1.2. Psychometric data

Consistent with a previous behavioral study comparing these same dietary groups (4), no differences in MDI and PDI indexes of BSID-2 test were found at 3, 12, or 24 months old. The dietary groups only differed in BSID-2 indexes at 9-months old [$F(2,331)$ 3.6, $p = 0.03$, $\eta^2 = 0.02$] (see Figure 3B). The *post hoc* tests showed that the BF group displayed greater BSID-2 indexes than SF and MF groups (BF vs. MF, MD = 1.5, $p = 0.04$; BF vs. SF, MD = 1.8, $p = 0.01$). No significant dietary group by BSID-2 indexes interaction was found in any comparison. No significant main effect of group was observed in PLS-3 test in any age group. However, a significant dietary group by PLS-3 interaction was found at 24 months old [$F(2,336)$ 3.4, $p = 0.03$, $\eta^2 = 0.02$, $\varepsilon = 1$]. The *post hoc* tests revealed that the BF group displayed a greater AC score than the MF group (MD = 4.8, $p = 0.01$; see Figure 3C).

3.2.2. Infant's ERPs analysis

Comparisons between dietary groups for each age group.

3.2.2.1. Amplitude and latency of ERPs

3.2.2.1.1. MMN-1 component

The dietary groups did not differ in amplitude or latency of the MMN-1 component at any age group. No significant main effect of dietary group or dietary group by ROIs interactions were observed in any comparison (see Supplementary Table S1).

3.2.2.1.2. MMN-2 component

The dietary groups did not differ in MMN-2 amplitude. However, differences between dietary groups were observed in MMN-2 latency at 12 months old (see Supplementary Table S2). As shown in Figure 4, at 12 months of age a significant dietary group by ROI was found [$F(6,981)$ 3.1, $p = 0.006$, $\eta^2 = 0.02$, $\varepsilon = 0.9$]. The *post hoc* test showed that the SF group differed from the remaining groups in MMN-2 latency in frontal left and temporal right ROIs. The SF group displayed shorter MMN-2 latency than BF and MF groups in frontal left ROI (SF vs. BF, MD = −23.8, $p = 0.004$; SF vs. MF, MD = −27.7, $p = 0.001$), while in temporal right ROI, SF group showed longer MMN-2 latency than MF group (SF vs. MF, MD = 21.7, $p = 0.02$).

3.2.2.2. Hemispheric asymmetry of amplitude or latency of ERPs

3.2.2.2.1. MMN-1 component

The statistical analyses evinced no differences between dietary groups in hemispheric asymmetry of MMN-1 component.

3.2.2.2.2. MMN-2 component

Although the weight groups did not differ in hemispheric asymmetry of MMN-1 amplitude, they differed in MMN-2 latency at 12 months old (see Supplementary Table S3). The *post hoc* test showed

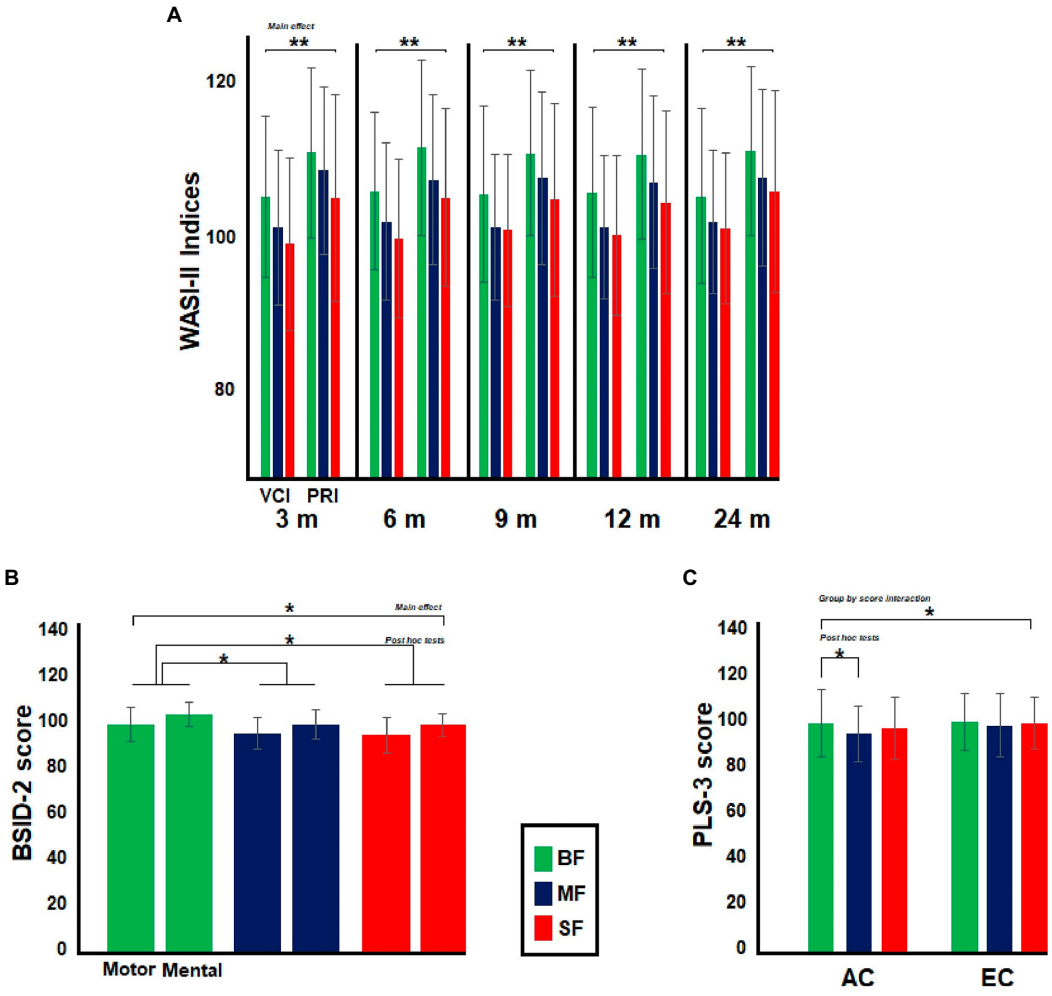

FIGURE 3
Differences between dietary groups in neuropsychological and psychophysiological assessment. In **(A)**, the bar graph illustrates differences between Infant's moms in Wechsler Abbreviated Scale of Intelligence (WASI-II). The moms from BF group showed greater WASI-II indices than the remaining dietary groups. In **(B)**, the bar graph shows differences between the dietary groups in Bayley Scales of Infant and Toddler Development (BSID-2) at 9-months old, BF groups displayed greater BSID-2 scores than MF and SF groups, while in **(C)**, the bar graph illustrates differences between dietary groups in Preschool Language Scale (PLS-3) at 24-months old. BF infants showed greater AC score than MF group. Significant value of *p*s has been represented as follows: *$p<0.05$, **$p<0.01$.

significant differences between MF and SF groups (MD = 27.19, $p = 0.002$; MF, $M = 13.72$; SF, $M = -13.56$; see Figure 5). The MF infants displayed greater MMN-2 latency in left than right hemisphere, while SF group displayed the inverse pattern (MF: left, $M = 416.78$ ms, right, $M = 404.59$ ms; SF: left, $M = 403.68$ ms, right, $M = 416.64$ ms). No significant dietary group by hemispheric asymmetry of ERP component was observed.

Comparisons between age groups for each dietary group.

3.2.2.3. Amplitude and latency of ERPs

3.2.2.3.1. BF group

3.2.2.3.1.1. MMN-1 component

Although age groups did not differ in MMN-1 amplitude, they did differ in MMN-1 latency, where a significant age group by ROI interaction was observed [$F(12,1792)$ 2.08, $p = 0.02$, $\eta^2 = 0.01$, $\varepsilon = 0.96$]. The *post hoc* test evinced differences between age groups in frontal right and temporal right ROIs. In frontal right ROI, the 3-month-old infants displayed shorter MMN-1 latency than 12-months-old participants, while in temporal right ROI, infants at 24 months of age had shorter MMN-1 latency than those participants at 3, 6, and 9 months old (see Supplementary Figure S1).

3.2.2.3.1.2. MMN-2 component

The age groups differed in both amplitude and latency of the MMN-2 component. A main effect of age was observed in MMN-2 amplitude [$F(4,623)$ 3.29, $p = 0.01$, $\eta^2 = 0.02$, $\varepsilon = 0.92$]. The *post hoc* test revealed smaller MMN-2 amplitude in infants at 3 compared to 24 months old. The 6-month-old infants also displayed smaller amplitude than the participants at 9, 12, and 24 months old (see Supplementary Table S4).

A significant age group by ROIs interaction was also seen [$F(12,1719)$ 2.86, $p = 0.001$, $\eta^2 = 0.02$, $\varepsilon = 0.92$]. The *post hoc* tests showed that age groups were different in frontal left, frontal right, and temporal right ROIs. In both frontal left and right ROIs, 3-month-old infants displayed smaller MMN-2 amplitude than infants at 12 and 24 months. We also observed that 6-month-old participants showed smaller MMN-2 amplitude than participants at 9, 12, and 24 months old in both

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TABLE 2 Infant's anthropometric measures by dietary group.

Age	Variables	Dietary group			Main effect of group	
		BF	MFMean (SD)	SF	*F*	*p*
3 m	Gestation (weeks)	39.5 (1.0)	39.1 (0.9)	39.1 (1.0)	6.9	0.001**
6 m		39.6 (1.0)	39.1 (0.9)	39.2 (1.0)	7.2	0.001**
9 m		39.6 (1.0)	39.2 (0.9)	39.2 (1.0)	6.3	0.002**
12 m		39.5 (1.0)	39.2 (0.9)	39.2 (1.1)	5.3	0.005**
24 m		39.6 (1.0)	39.2 (0.9)	39.1 (1.0)	8.8	0.000**
3 m	Birth weight (kg)	3.5 (0.3)	3.5 (0.4)	3.4 (0.4)	2.9	0.06
6 m		3.5 (0.3)	3.5 (0.4)	3.4 (0.4)	3.6	0.03*
9 m		3.5 (0.3)	3.5 (0.4)	3.5 (0.4)	1.4	0.2
12 m		3.5 (0.3)	3.5 (0.4)	3.4 (0.4)	1.8	0.2
24 m		3.5 (0.3)	3.5 (0.4)	3.4 (0.4)	2.1	0.1
3 m	Birth length (cm)	51.4 (2.2)	51.1 (2.5)	51.2 (2.2)	0.9	0.4
6 m		51.5 (2.3)	51.2 (2.4)	51.2 (2.1)	0.6	0.5
9 m		51.5 (2.1)	51.2 (2.7)	51.2 (2.0)	0.9	0.4
12 m		51.4 (2.1)	51.1 (2.5)	51.2 (2.1)	0.5	0.6
24 m		51.4 (2.2)	51.2 (2.4)	51.0 (2.10)	0.9	0.4
3 m	Height (cm)	60.1 (1.9)	58.9 (2.1)	59.7 (1.6)	1.5	0.2
6 m		66.0 (2.3)	66.4 (2.4)	66.4 (2.0)	1.5	0.2
9 m		69.9 (2.3)	70.7 (2.4)	70.8 (2.3)	5.2	0.006**
12 m		73.7 (2.3)	74.4 (2.3)	75.0 (2.3)	8.3	0.000**
24 m		86.2 (2.5)	86.3 (2.8)	86.7 (2.8)	1.3	0.3
3 m	Weight (kg)	6.1 (0.7)	6.1 (0.6)	5.9 (0.5)	2.2	0.8
6 m		7.6 (0.9)	7.9 (0.9)	7.8 (0.8)	2.8	0.06
9 m		8.6 (0.8)	9.0 (0.9)	9.1 (1.0)	10.8	0.000**
12 m		9.4 (1.0)	9.9 (0.9)	10.0 (1.0)	13.3	0.000**
24 m		12.2 (1.2)	12.4 (1.2)	12.7 (1.3)	6.3	0.002**
3 m	Head circ. (cm)	40.7 (1.1)	40.6 (1.1)	40.6 (1.1)	0.2	0.8
6 m		43.4 (1.3)	43.6 (1.1)	43.7 (1.3)	1.6	0.2
9 m		45.2 (1.2)	45.2 (1.2)	45.4 (1.3)	1.1	0.3
12 m		46.4 (1.3)	46.4 (1.2)	46.6 (1.4)	1.6	0.2
24 m		48.7 (1.3)	48.8 (1.3)	48.9 (1.4)	0.7	0.5
			F/M		χ^2 (2)	*p*
3 m	Sex	68/69	71/67	62/73	0.9	0.6
6 m		64/55	55/65	62/64	1.5	0.5
9 m		58/55	55/59	49/64	1.5	0.5
12 m		66/56	56/56	43/57	2.7	0.2
24 m		74/68	56/61	57/56	0.5	0.8

m, months; BF, breast fed; MF, milk fed; SF, soy fed; SD, standard deviation; F, female; M, male; Head circ., head circumference. *$p < 0.05$; **$p < 0.01$.

left and right frontal ROIs, while in temporal right ROI, a greater MMN-2 amplitude was observed in 3-month-old infants compared to participants at 24 months.

The differences between age groups in MMN-2 latency were observed regardless of ROI, a main effect of age group [$F(4,623)$ 3.87, $p = 0.004$, $\eta^2 = 0.02$, $\varepsilon = 0.94$] showed longer MMN-2 latency for 6-month than 3 months old participants. The 24-month-old infants also showed shorter MMN-2 latency than participants at 6, 9, and 12 months old. A significant age group by ROI interaction was also seen [$F(12,1759)$ 1.86, $p = 0.04$, $\eta^2 = 0.01$, $\varepsilon = 0.94$]. The age groups differed in MMN-2 latency in frontal left, frontal right, temporal right ROIs. The *post hoc* tests showed that infants at 3 months of age displayed longer MMN-2 latency than participants at 6, 9, 12, and 24 months in frontal left ROI, while in frontal right, 3-month-old infants displayed longer MMN-2 latency than infants at 9 months of age, and the participants at 9 months of age had longer MMN-2 latency compared to 24-month-old infants. In temporal right ROI, infants at 24 months of age displayed longer MMN-2 latency than participants at 3 and 6 months old (see Supplementary Figure S1).

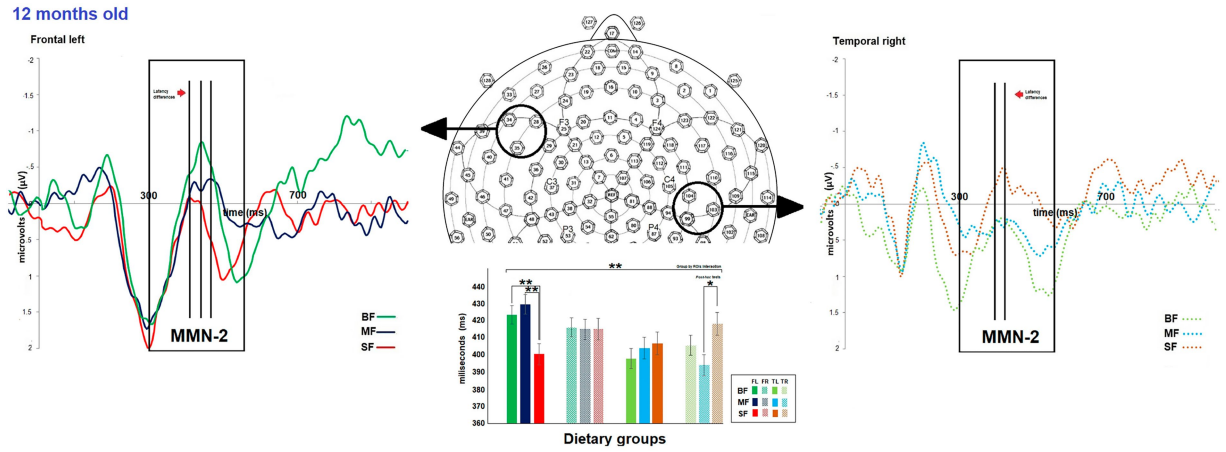

FIGURE 4
Differences between dietary groups in MMN-2 latency at 12-months old. On top, the grand average of difference wave of event-related potentials (ERPs) in the frontal left (FL) and temporal right (TR) regions of interest (ROIs) for each dietary group at 12-months old. The bar graph shows differences in MMN-2 latency between the dietary group FL and TR ROIs on the bottom. In FL ROI, the SF group displayed shorter MMN-2 latency than the other groups, while in TR ROI, the SF group showed longer MMN-2 latency than the MF group. Significant value of *p*s has been represented as follows: *$p<0.05$, **$p<0.01$.

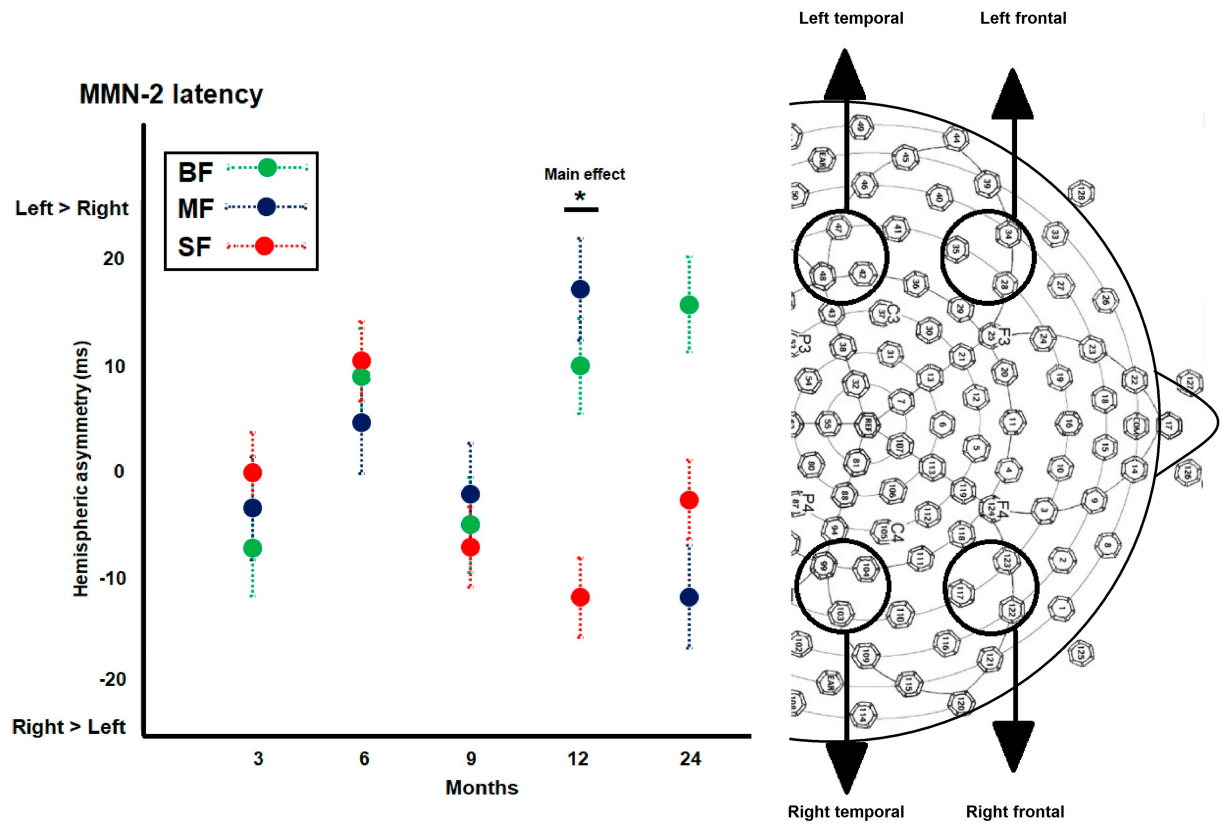

FIGURE 5
Differences between dietary groups in hemispheric asymmetry of event-related potentials (ERPs) components. The scatter plot illustrates differences between dietary groups in the hemispheric asymmetry of MMN-2 latency, which were observed at 12-months of age. Significant value of *p*s has been represented as follows: *$p<0.05$.

3.2.2.3.2. MF group

3.2.2.3.2.1. MMN-1 component

The age groups differed in amplitude and latency of MMN-1 component in the MF group. A significant age group by ROI interaction was seen for MMN-1 amplitude [$F(12,1,588)$ 2.39, $p=0.007$, $\eta^2=0.02$, $\varepsilon=0.89$]. The *post hoc* tests showed that age groups differed in MMN-1 amplitude all ROIs (i.e., frontal left, frontal right, temporal left, and temporal right). In the frontal left ROI, infants at 3 months of age displayed smaller MMN-1 amplitude than 24-month-old participants,

while in frontal right ROI, infants at 6 months of age displayed smaller MMN-1 amplitude compared to 9-and 12-month-old infants (See Supplementary Figure S2). In temporal left ROI, a smaller MMN-1 amplitude was observed in 3-month-old infants compared to 6-month-old participants. In addition, participants at 9 and 12 months of age displayed a smaller MMN-1 amplitude than 6-monts-old infants. In temporal right ROI, infants at 24 months of age displayed a smaller MMN-1 amplitude than those infants at 3 and 9 months old (see Supplementary Table S4).

In the comparisons between age groups in MMN-1 latency, we also found a significant age group by ROIs interaction [$F(12,1705)$ 2.05, $p = 0.02$, $\eta^2 = 0.01$, $\varepsilon = 0.96$]. The *post hoc* tests evidenced differences between groups in frontal left, frontal right, and temporal right ROIs. In frontal left ROI, shorter MMN-1 latency was seen in infants at 3 months of age compared to the participants at 6 and 12 months old, while in frontal right a similar pattern was observed, infants at 3 months of age displayed shorter MMN-1 latency than 24-month-old infants. In temporal right ROI, the 9-month-old infants displayed longer MMN-1 latency than infants at 3 months old, and shorter MMN-1 latency compared to 24-month-old infants.

3.2.2.3.2.2. MMN-2 component

Although the age groups did not differ in MMN-2 amplitude, they were different in MMN-2 latency. A significant age group by ROI interaction [$F(12,1,667)$ 2.93, $p = 0.001$, $\eta^2 = 0.02$, $\varepsilon = 0.94$] revealed that the groups differed in frontal left, frontal right, and temporal left ROIs. In frontal left ROI, infants at 3 months of age displayed shorter MMN-2 latency than participants at 9 and 12 months. The participants at 6 months of age also showed shorter MMN-2 latency compared to 12-month-old infants. However, at 24 months old, the infants displayed a shorter MMN-2 latency than the participants at 9 and 12 months. In frontal right ROI, longer MMN-2 latency was seen in 9-month-old infants compared to infants at 3 and 6 months old. In temporal left ROI, we found that infants at 6 months old displayed longer MMN-2 latency than 9-month-old infants (see Supplementary Figure S2).

3.2.2.3.3. SF group

3.2.2.3.3.1. MMN-1 component

There were no differences between age groups in amplitude or latency of MMN-1 component.

3.2.2.3.3.2. MMN-2 component

The age groups did not differ in MMN-2 amplitude, but they differed in MMN-2 latency. A significant main effect of group [$F(4,567)$ 2.57, $p = 0.04$, $\eta^2 = 0.02$, $\varepsilon = 0.96$] evinced that infants at 24 months of age displayed shorter MMN-2 latency compared to infants at 6, 9, and 12 months of age (see Supplementary Table S4).

A significant age group by ROI interaction was also seen [$F(12,1,635)$ 3.83, $p < 0.001$, $\eta^2 = 0.03$, $\varepsilon = 0.96$]. The *post hoc* tests showed that age groups differed in MMN-2 latency in frontal left, frontal right, temporal left, and temporal right ROIs. In frontal left ROI, infants at 3 months of age displayed shorter MMN-2 latency than participants at 6 and 9 months old. 12-months-old infants displayed shorter MMN-2 latency than participants at 6 months old. This same pattern was observed for 24-month-old infants, which displayed shorter MMN-2 latency compared to infants at 6 and 9 months old. In frontal right ROI, infants at 3 months of age also showed shorter MMN-2 latency than infants at 6, 9, and 12 months of age. However, at 24 months, infants displayed shorter MMN-2 latency than 9-month-old infants. In the temporal left ROI, the participants at 3 months of age displayed longer MMN-2 latency than 9-month-old infants, while in temporal right ROI, 3-month-old participants displayed longer MMN-2 latency compared to infants at 24 months of age and infants at 12 months old displayed longer MMN-2 latency than participants at 6 and 24 months old (see Supplementary Figure S3).

3.2.3. Regression results

As shown in Table 3, at 12-months old, only the infant's diet predicted MMN-2 latency in frontal left and temporal right ROIs. In this same age group, diet was also a predictor of hemispheric asymmetry in the MMN-2 latency.

4. Discussion

This study sought to identify electrophysiological differences between dietary groups at 3, 6, 9, 12, and 24 months of age. We expected to find an effect of diet on infant phonological processing, particularly at earlier developmental ages, which would be characterized by greater amplitude and shorter latency of MMN components and accompanied by a greater hemispheric asymmetry of MMN components for the BF group than MF and SF groups. Additionally, we expected greater amplitude and shorter latencies of MMN components for BF groups as age increased, which we expected to be less evident in the other dietary groups.

4.1. Phonological-perception development between dietary groups

Our findings partially matched our hypothesis with differences between dietary groups observed in only one of the MMN components. We did not find differences between dietary groups in amplitude or latency of MMN-1, which has been associated with the identification of acoustic features of a stimulus (i.e., MMN-1) (51–53). Although this finding is in line with findings of Li et al. (13), it did not match those reported by Pivik

TABLE 3 Regression models predicting latency or hemispheric asymmetry of MMN-2 component at 12-months old.

Age	Variables		Coefficient standardized			Model	ANOVA	
	ROI	Predictor	β	t	p-value	R^2	F	p-value
MMN-2 latency								
12 m	FL	Diet	0.1	2.8	0.005	0.02	8.0	0.005**
	TR	Diet	−0.1	−2.0	0.05	0.01	3.9	0.05*
Hemispheric asymmetry of MMN-2 latency								
12 m	Frontal	Diet	0.1	2.2	0.02	0.01	5.0	0.02*

m, months; FL, frontal left; FR, frontal right; TR, temporal right. *$p < 0.05$; **$p < 0.01$.

et al. (3) who reported differences in P1 amplitude between dietary groups, with greater amplitude in the deviant condition for soy milk fed infants than breastfed at 3 and 6-months. In our study we expected to find a greater MMN-1 amplitude for SF than BF groups, in keeping with the findings of Pivik et al. (3), but this was not the case. We suggest that our results could be explained by the type of ERP analyses performed. Pivik et al. (3) compared the amplitude and latency of the P1 component associated with frequent and deviant conditions, while in our study, we directly compared the differences between experimental conditions (i.e., MMN components), and included infant sex and gestation weeks as covariates.

We propose that our results might be explained by the suggestion of previous studies that identification of acoustic features is developed very early in infancy (30–32). Given that this precognitive process might not be under development during our evaluation period, the nutritional requirements to support brain networks need for efficient processing would be easily provided by each of the three diets evaluated.

We also hypothesized differences between dietary groups in the MMN-2 component at six and 12-months of age. Our results partially supported our hypotheses; dietary groups only differed at 12-months old, underpinning the idea that nutrient intake has a greater effect on an infant's cognition at a critical stage of language development. At this age, it is expected that infants show phonemic normalization and categorical perception (30]. Infants should recognize words (36) because they have already undergone extensive maturity changes in brain networks associated with production centers in the frontal region and the phonological store in the temporal region (38). Moreover, they already show a more mature hemispheric specialization associated with language processing (40, 77). As a consequence, phonological perception might require greater participation from neural networks that support attentional monitoring, inhibitory control, stimulus detection, and working memory (i.e., the dorsolateral prefrontal cortex, inferior frontal junction, inferior frontal gyrus, insula, presupplementary motor area, subthalamic nucleus, median cingulate, and striatum) (78) because they have attended syllables and inhibit their possible meaning in their native language, promoting greater participation from frontal brain areas related to attention-inhibition processing (38).

Our findings revealed that the SF group showed an inverse electrophysiological pattern to that of BF and MF infants; in which the SF group displayed shorter MMN-2 latency in frontal left ROI and longer MMN-2 latency in temporal right ROI. One explanation for the differences between dietary groups in MMN-2 latency in frontal left ROI is that the SF group exhibits a different attention-inhibition effort than the other groups, reflected in a reduced level of interference relative to the other groups. While shorter latencies might suggest more efficient processing, this finding might also indicate that SF infants have less linguistic information to inhibit or a weaker attention-inhibition brain network. This last explanation matches the findings of Li et al. (79) who reported lower executive function in children fed with soy formula in infancy than those fed with breast or cow-milk formula.

On the other hand, shorter MMN-2 latency for the SF group in temporal right ROI requires an additional explanation. Although how the hemispheric specialization in language processing develops during infancy is still debated, it has been hypothesized that the left hemisphere is specialized for speech stimuli, while the right hemisphere supports the auditory identification of non-speech stimuli (80, 81). In our study, the SF group displayed an enhanced response in the right hemisphere, suggesting that this group is attending the syllables as non-speech stimuli. This proposal is in accord with their brain response in frontal left ROI. They appear to expend less cognitive effort to attend syllables and inhibit linguistic context because they may be processing the syllables as tones (81). The SF group also exhibited a more right-lateralized MMN-2 asymmetry that has been suggested to be associated with a risk of delayed language development (40). Attenuation of left hemispheric ERPs (82, 83) or atypical enhanced responses in the right hemisphere (84, 85) have been to confer greater risk of poor language development. Moreover, given that regression analyses indicated that only infant diet predicted latency and hemispheric asymmetry of the MMN-2 component in frontal areas, the SF group's electrophysiological response might indicate a deviation from normal language development after a prolonged use of soy-based formula.

In addition, the electrophysiological pattern observed in SF groups does not match the temporal gradient in information processing (i.e., faster processing in temporal than frontal regions) observed in normal development (54). These findings addressed the speed at which information is processed between language areas, suggesting that the differences between dietary groups in frontal ROIs could be interpreted as modulations in brain networks to enhance the ability to distinguish between syllables and manage neural resources and cognitive effort.

Prior studies using animal models and humans have noted that soy food contains phytoestrogens such as isoflavones (86–88) that seem to have a negative effect on cognition, alter sexually dimorphic brain regions, learning, memory (89) and executive functions (79). We suggest that the deviation from normal language processing observed in the SF group may be attributable to the composition of soy formulas.

4.2. Phonological-perception development for each dietary group

Consistent with our hypothesis, dietary groups displayed changes in MMN components associated with age, and these changes were more evident in the BF group. The MMN-1 component appears to change with age only in the BF and MF groups. Both groups displayed an increase in MMN-1 latency in frontal ROIs, which may suggest greater participation of frontal areas supporting inhibitory control in order to better identify the features of acoustic stimuli (51–53). These dietary groups also displayed a decrease in MMN-latency in temporal ROIs, which might be explained as a reflection of a more available phonological store (38) as age increases. However, SF infants did not display these changes associated with age, suggestive of a less stable development of the ability to identify the features of acoustic stimuli. Another explanation for this result is that the SF group had high variability in their brain-electrical responses associated with identification of acoustic features at all ages, which would hamper the observation of differences between age groups and even more so between dietary groups.

Although the MMN-2 component changed with age in all dietary groups, only the BF group showed greater MMN-2 amplitude as age increased, as has been described in a previous study (50). This finding may be interpreted as greater availability of neural resources in older breastfed infants who seemed to show a greater stimulus awareness and perceptual salience, and thus a greater index of auditory recognition memory (51, 90) as age increased. This finding is consistent with behavioral results observed in 24-month-old infants on the PLS-3 test where breastfed infants showed greater auditory comprehension.

On the other hand, the electrophysiological pattern associated with age of MMN-2 latency also depended on regions of interest. BF and MF groups showed an increase in MMN-2 latency in the frontal left ROI from 3 to 12 months of age. This pattern was not observed in the SF group. Instead, that group displayed a concave-learning curve (91) characterized by a significant decrease of MMN-2 latency in frontal left ROI from

12 months of age. This finding may indicate reduced participation of the frontal left ROI in auditory recognition memory, consistent with a deviation from normal development in the recruitment of this brain area to process phonological awareness. In addition, an unexpected result was that SF infants displayed an increase in MMN-2 latency in temporal regions from this same age, which contrasted with the decreased age-associated finding in BF and MF groups. A previous study of language learning has described those greater fluctuations in learning curves as an indicator of slower learners, which may explain our findings in SF group (91). We add to this that SF infants may have a less available phonological store at 12 months of age. The unexpected electrophysiological pattern observed in SF infants temporally matches with a milestone in infant language development where they are expected to show greater stimulus awareness due to their ability to distinguish words, syllables, and tones. Therefore, we suggest that SF infants compensate for failures in the frontal left area by recruiting bilateral temporal ROI to distinguish between phonological features of words, syllables, and tones.

4.3. Limitations

There are inherent limitations in the present study. Although the longitudinal nature of this study may support interpretations of causality between diet and phonological processing, it is essential to highlight that the same subjects did not always constitute the sample at each moment evaluated. Some of them missed more than one measurement. Therefore, interpretations should be carried out carefully. In addition, given that our study implied infant nutrition, variables surrounding infant feeding were not wholly controlled, among them the mother's health or the amount of food provided to the infant, or complementary feeding habits. We did not explore why the parents choose one of the three diets offered. In this study, we used traditional anthropometric measures to assess the participants, while this is a common use of body composition measures (e.g., energy X-ray absorptiometry) or biochemical indices, these might provide more nuanced metrics for studies examining the impact of diet on neural maturation and cognitive function in infants.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Board of the University of Arkansas for Medical Sciences. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

GA-C, AA, SS, and LL-P contributed to the conception and design of the study. YG, DW, HD, and DH organized the database for the statistical analyses. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1032413/full#supplementary-material

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Challenging inhibitory control with high- and low-calorie food: A behavioural and TMS study

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Most people are often tempted by their impulses to "indulge" in high-calorie food, even if this behaviour is not consistent with their goal to control weight in the long term and might not be healthy. The outcome of this conflict is strongly dependent on inhibitory control. It has already been reported that individuals with weaker inhibitory control consume more high-calorie food, are more often unsuccessful dieters, overweight or obese compared to people with more effective inhibitory control. In the present study, we aimed at investigating inhibitory control in the context of human eating behaviour. A sample of 20 healthy normal-weight adults performed a 50% probability visual affective Go/NoGo task involving food (high- and low-calorie) and non-food images as stimuli. Single-pulse transcranial magnetic stimulation (TMS) was administered over the right primary motor cortex (M1) either 300ms after image presentation to measure corticospinal excitability during the different stimulus categories or 300ms after the appearance of a fixation point, as a control stimulation condition. The experimental session consisted of a food target and a non-food target block. Behavioural outcomes showed a natural implicit inclination towards high-calorie food in that participants were faster and more accurate compared to the other categories. This advantage was selectively deleted by TMS, which slowed down reaction times. MEPs did not differ according to the stimulus category, but, as expected, were bigger for Go compared to NoGo trials. Participants judged high-calorie food also as more appetising than low-calorie food images. Overall, our results point to a differential modulation when targeting inhibitory control, in favour of the more palatable food category (high-calorie). Present data suggest that the activity of the motor system is modulated by food nutritional value, being more engaged by appetising food. Future work should explore to what extent these processes are affected in patients with eating disorders and should aim to better characterise the related dynamics of cortical connectivity within the motor network.

KEYWORDS

food, high-calorie, low-calorie, Go/NoGo, inhibitory-control, transcranial magnetic stimulation (TMS), primary motor cortex (M1)

1. Introduction

Eating and managing caloric intake is essential for our survival. Food-related choices mediate a large part of a person's well-being from many points of view such as health, social life, and self-esteem. Since the dawn of neuroscience, the salience of food stimuli for the nervous system has been clearly recognised. The eminent Russian physiologist Pavlov has in fact demonstrated how food

generates an unconditioned response and how other stimuli become salient if repeatedly associated with it (1).

Most people, are often tempted by their impulses to "indulge" in high-calorie food, even if this behaviour is not consistent with their goal to control weight in the long term (e.g., 2). This conflict is exacerbated by our social environment, where the abundance of appetising high-calorie food can trigger overconsuming in individuals with enhanced food cue reactivity (3). The outcome of this conflict between short-term gratification and long-term goal is strongly dependent on inhibitory control, i.e., the ability to withhold pressing responses (4). In line with this view, previous studies have shown that individuals with weaker inhibitory control consume more high-calorie food, are more often unsuccessful dieters, and are more often overweight or obese compared to people with more effective inhibitory control (5–8). In contrast, individuals with abnormal inhibitory control can exhibit a dysfunctional restriction of food intake and weight loss (9, 10).

Previous work acknowledged that reactivity to food cues is part of a trait that combines increased appetitive drive and reduced inhibitory control, which in turn would explain why some individuals are more prone to uncontrolled eating or at the opposite restrictive eating behaviour (9, 11, 12). The ability to control impulses is challenged by appetizing stimuli. This does not seem to be related to the need to procure the food necessary for the sustenance of the organism (homeostatic drive), but rather resembles a mechanism similar to addiction (hedonic drive) (13, 14), which in some cases can become very harmful (15).

A large body of research showed that inhibitory control plays a crucial role in balancing food behaviour and in the psychopathology of eating disorders (16–21).

However, studies that have investigated food-related inhibitory control in healthy participants are few and have shown inconsistencies (22).

Inhibitory control can be assessed through the Go/NoGo task. Participants are instructed to respond to a target (Go trial) and withhold response to a non-target (NoGo trial), whilst response speed and accuracy are measured. Previous studies used a classic version of this task with abstract stimuli to investigate the relationship between inhibitory control and eating behaviour (23). However, both top-down inhibitory control and bottom-up drive to food stimuli interact to determine eating behaviour (24). Therefore, Go/NoGo tasks incorporating food stimuli are likely to be more informative.

Previous studies already used this task including food stimuli with promising results, but with some limitations. For instance, Batterink et al. (25) developed a Go/NoGo task using healthy food as Go stimuli and unhealthy food images as NoGo stimuli but lacked a control stimulus, such as non-food pictures, and the sample was limited to female participants. A following study (26), measured response inhibition in food and non-food trials in males and females, but the task used words rather than images, with the undesired involvement of reading ability and abstract thought.

Although inhibitory control has been traditionally considered to rely exclusively on the prefrontal cortex, recent findings using transcranial magnetic stimulation (TMS) have shown that other areas are involved. Not only does the prefrontal cortex send its inhibitory command to the primary motor cortex (M1) but other nodes of the motor system, such as the cerebellum, play an active role in inhibitory control (27, 28).

The aim of this study was twofold. First, we wanted to study inhibitory control when different food stimuli (high-calorie vs. low-calorie) are presented in a design that would overcome previous studies' limitations, by using images of food rather than words and by comparing the obtained response to a control stimulus (non-food). Second, we wanted to investigate the involvement of the motor system in inhibitory control and food-related environmental cues. Specifically, we used a Go/NoGo task with food and non-food images as Go and/or NoGo stimuli and concurrently investigate the excitability of the primary motor cortex (M1) collecting motor-evoked potentials (MEPs) elicited by Transcranial Magnetic Stimulation (TMS) in healthy eaters. We targeted M1 as a part of the inhibitory system because it can be easily accessed by TMS and because it provides a direct measure of the system excitability *via* MEP amplitude.

Since other factors such as current hunger or body weight may interfere with inhibitory mechanisms (22, 29), all participants were healthy eaters and they were tested under the same satiety state. Finally, we evaluated the relation between individual impulsivity traits and behavioural measures. We hypothesised that participants were faster and more accurate when presented with high-calorie, compared to low-calorie and non-food images, because they were adaptively and implicitly prompted to react at targets with good nutritive value.

2. Materials and methods

2.1. Participants

A gender-balance sample of 20 healthy participants (10 females: age 27.9 ± 3.8; years of education >13; body mass index – BMI 23.3 ± 2.9) was recruited. The sample size for the main mixed-design ANOVA (Stimulus type × TMS) was determined with G*power software (30). The effect size was estimated from a previous study (31). We set the expected effect size f(U) at 0.38, the α level at 0.05 and the desired power $(1-\beta)$ at 80%.

Inclusion criteria were the absence of any reported neurological or psychological disorders and the absence of eating disorders as measured by EDE-Q global score (32, 33). Moreover, vegetarian or vegan participants were excluded, as well as those who claimed to have particular food preferences or restrictions related to intolerances, allergies to foods or metabolic compromissions (e.g., diabetes and celiac disease). All participants were right-handed (Edinburgh Handedness Inventory, 34), reported normal or corrected-to-normal vision and were naïve about the aim of the study. Written informed consent was obtained from all participants according to the Declaration of Helsinki. The project was approved by the Santa Lucia Foundation IRCCS of Rome Ethical Committee.

2.2. Experimental procedure

Participants were seated in a comfortable armchair in a dimly illuminated, electrically shielded, and sound-proof room. Since fasting levels might have an impact on food related inhibitory performance (35), the experimental procedure consisted of a single session that was programmed at least 2 h after the last meal. Participants first performed the Go/NoGo task whilst TMS was delivered to the right primary motor cortex. At the end of the session, they were asked to rate the palatability of the food images presented during the task and to fill out questionnaires (see below for details).

2.3. Food Go/NoGo task

A 50% probability visual Go/NoGo task with food and non-food images was used. Images were selected from the extended food-pics database (36). Food stimuli included high-calorie and low-calorie food pictures whereas non-food images represented pleasant inedible objects. The experimental task consisted of a food-target and a non-food target block. In the food-target block, participants had to respond by pressing the space bar of a QWERTY keyboard with the right index finger when they recognised a food (Go trials) and refrain from responding when they saw a non-food picture (NoGo trials). In the non-food target block, the Go/NoGo categories were reversed (Figure 1A).

In each block, a total of 384 trials were presented, including 192 (50%) non-food images and 192 (50%) food images. Of the 192 food stimuli, 96 were high-calorie and 96 low-calorie. Each trial started with a fixation point (a yellow point at the centre of a white screen) presented for a variable interval between 500 ms and 1,500 ms (mean = 975 ms). The timing of the fixation point was varied pseudo-randomly to prevent the predictability of

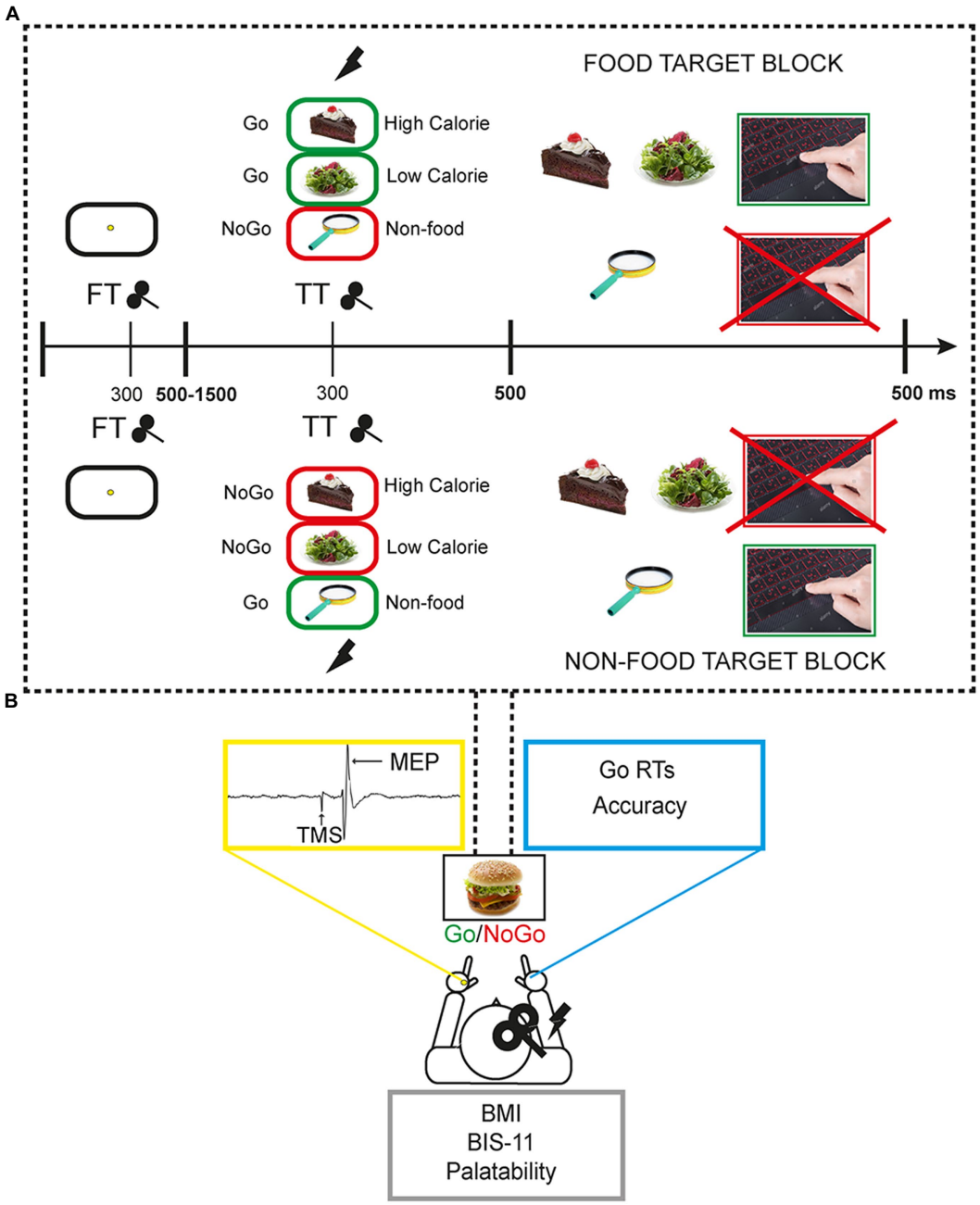

FIGURE 1
Experimental setting and behavioural task. **(A)** Each participant performed the task responding with the right hand whilst single pulse transcranial magnetic stimulation (spTMS) was applied over the right primary motor cortex (M1) and motor evoked potentials (MEP) were collected from the left hand. Each participant self-reported height and weight for BMI calculation, filled the Barratt-Impulsivity scale 11, and expressed its judgement about the palatability of task food images in a 5-point scale. **(B)** The Go/NoGo task was composed of two blocks in which participants had to alternatively respond to food or non-food stimuli.by pressing the spacebar. In FT trials spTMS was delivered 300ms following the fixation point onset, in TT trials spTMS was delivered 300ms following the target food/non-food target image onset. In NT trials spTMS was not delivered.

the stimulus. This was followed by a food or non-food image, presented for 500 ms. The duration of each trial ranged from 1,500 to 2000 ms. The duration of each block was approximately 10 min interspersed with two short breaks. Overall, the task lasted around 25 min. It is worth noting that having an equal probability of Go/NoGo trials could be less effective to elicit a clear inhibitory activity compared to Go/NoGo tasks with rare NoGo trials (37). However, the present choice was motivated by the need to have two equal blocks only differing in the instructions, and complicated by the existence of two (high-, low-calorie) food categories to be balanced with the non-food condition. Moreover, this choice was based on previous results by our group showing that a simple 50% probability Go/NoGo task can effectively modulate frontocentral cortical activity that is related to inhibitory control (Figure 1A; 28, 38).

The task was programmed and run through E-Prime 2.0 Professional software and stimuli were shown on a 23 inches monitor. For each task condition, reaction times (RTs) in ms and accuracy scores (the percentage of correct responses, i.e., button press for Go trials and no button press for NoGo trials) were collected. Both speed and accuracy were encouraged for task performance (Figure 1B).

2.4. Transcranial magnetic stimulation (TMS)

Single-pulse transcranial magnetic stimulation (TMS) was administered throughout the experiment over the right primary motor cortex (M1) to measure corticospinal excitability during the different task conditions (high-calorie, low-calorie, non-food). Namely, in a subset of 128 trials "TARGET TMS" (TT) [64 food (32 high-calorie, 32 low-calorie), 64 non-food stimuli] TMS was applied after 300 ms from the food/non-food picture appearance. In a subset of 128 trials "FIXATION TMS" (FT) [64 food (32 high-caloric, 32 low-caloric), 64 non-food stimuli] TMS was applied during the presentation of the fixation point, to have a control condition. TMS pulse was always released with a stimulus onset asynchrony (SOA) of 300 ms. This SOA has been previously used when a single TMS pulse was combined with the execution of a task in healthy participants (Figure 1; 39–42). Finally, in a subset of 128 "NO TMS" – NT – trials [64 food (32 high-calorie, 32 low-calorie) 64 non-food stimuli] TMS was not applied, to observe participants' behaviour in absence of any TMS interference.

TMS was performed using a MagStim Super Rapid magnetic stimulator (Magstim Company, Whitland, Wales, United Kingdom) connected to a figure-of-eight coil with a diameter of 70 mm. The magnetic stimulus had a biphasic waveform with a pulse width of about 300 µs. The coil over M1 was always placed tangentially to the scalp at the 45° angle from the midline of the central sulcus, inducing a posterior–anterior current flow. Electromyographic (EMG) traces were recorded from the left first dorsal interosseous (FDI) muscle by using 9-mm-in-diameter surface cup electrodes. The active electrode was placed over the muscle belly and the reference electrode over the metacarpophalangeal joint of the index finger. The ground electrode was placed over the left wrist. The TMS intensity was adjusted to evoke an MEP of ~1 mV peak to peak in the relaxed FDI (43). The average TMS intensity was 65 ± 12% of the maximum stimulator output.

Responses were amplified with a Digitimer D360 amplifier through filters set at 20 Hz and 2 kHz, with a sampling rate of 5 kHz and then recorded by a computer with the use of Signal software.

The average MEP peak-to-peak amplitude was calculated for each stimulus type (high-calorie, low-calorie, non-food) and TMS TT and FT conditions. MEPs above and below 2 standard deviations of the mean were removed from the analysis (44). The left FDI relaxation during the experiment was visually monitored by the experimenter who checked both the position of the hand and the EMG traces online. Participants responded to the task with their right hand, whilst the left hand, from which the MEPs were collected, was comfortably placed on an armrest. All participants were informed prior to the start of the task that their left hand could make small involuntary movements in response to the TMS. As specified above, MEPs above the 2 standard deviations were removed from the analysis to exclude trials where the muscle was not relaxed. The number of MEPs excluded for each condition was: 10.4 ± 6.5% of High-calorie Go; 12.5 ± 11.3% of Low-calorie Go; 7.3 ± 4.5% of Non-Food Go; 5.2 ± 4.8% of High-calorie NoGo; 10.4 ± 1.8% of Low-calorie NoGo; 12 ± 7.7% of Non-Food NoGo.

MEP amplitude for each stimulus type was then normalised using the MEP obtained for the FT condition, i.e., MEP amplitude obtained in the TT condition was expressed as a percentage of the amplitude recorded in FT trials.

2.5. Palatability of the images

After the Go/NoGo task, all high-and low-calorie food images were presented again in random order and participants were asked to score their palatability on a five-level Likert scale, from 1 (unappetising) to 5 (very appetising).

2.6. Impulsivity assessment

All participants filled out the Barratt Impulsiveness Scale-11 (BIS-11), a commonly used 30-item self-report questionnaire designed to assess impulsiveness (45). All items are measured on a four-point Likert-type scale. In the scoring procedure, the items are summed and the higher scores indicate greater impulsivity (ranging between 30 and 120). A summary of the BIS-11 results is reported in Table 1.

2.7. Eating behaviour assessment

The body Mass Index (BMI; kg/m^2) was calculated according to the self-reported weight and height values (46). All participants completed the latest edition of the Eating Disorder Examination Questionnaire (EDE-Q 6.0 – 32, 33). The questionnaire has been extensively studied, and its psychometric properties have been demonstrated to distinguish healthy participants from patients with eating disorders. Furthermore, the EDE-Q has shown high internal consistency in both nonclinical and clinical samples. The EDE-Q provides a global score based on four subscales (Restraint, Eating Concern,

TABLE 1 BIS-11 results.

Attention	Motor impulsiveness	Self-control	Cognitive complexity	Perseverance	Cognitive instability	Total score
9.55 ± 0.63	11.6 ± 0.61	11.95 ± 0.61	10.8 ± 0.37	6.8 ± 0.42	6.45 ± 0.39	57.15 ± 1.84

Mean ± standard deviation of BIS-11 subscale and total score.

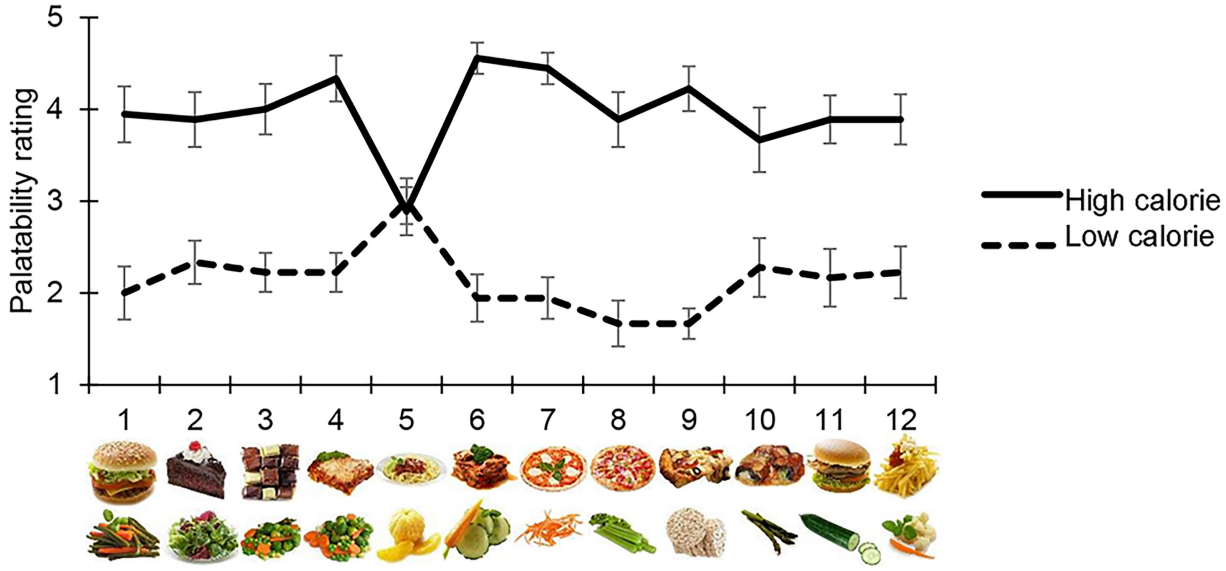

FIGURE 2
Palatability rating. Each participant judged the palatability of each food image used in the main task on a 5-point Likert's scale. The mean palatability score is shown on the y-axis, for each high/low-calorie stimuli used (*x*-axis). With the exception of high-calorie and low calorie item 5, which were excluded from the final analysis, participants considered high-calorie food images as being more appetising than low-calorie images.

Shape Concern and Weight Concern). Participants with clinical value in EDE-Q global score were excluded from the study.

2.8. Statistical analysis

Two-way 3 × 3 repeated measures ANOVAs were performed for each behavioural measure of interest (reaction times and accuracy scores) with factors *Stimulus type* (High-Calorie, Low-Calorie, Non-food), *TMS* (NT, FT, and TT). A two-way repeated measure ANOVA was performed on MEP amplitude with factors *Stimulus type* (High-Calorie, Low-Calorie, and Non-food), and *Trial type* (Go vs. NoGo). Statistical analyses were performed in STATISTICA 8.0 using two-tailed alpha levels of <0.05 for defining significance. *Post-hoc* comparisons were performed by paired *t*-tests (Bonferroni corrected). The effect size was indicated as partial eta square (η^{P}_{2}). The relationship between BMI and BIS-11 total scores was also investigated using Spearman's rho coefficient.

3. Results

3.1. Palatability

Participants judged high-calorie food as being the most appetising, with the exception of image 5 in the high-and low-calorie food categories, which were scored differently from other images of their same category, i.e., both were perceived as halfway between high-and low-palatable food (average score = 3). For this reason, all measurements collected for these images were removed from the final analysis (Figure 2).

3.2. Food Go/NoGo

The ANOVA on Go RTs showed significant main effects of *Stimulus type* ($F_{2,38} = 21.44$, $p < 0.001$, $\eta P_2 = 0.53$) and TMS ($F_{2,38} = 7.86$, $p = 0.00^1$ $\eta P_2 = 0.29$) and a significant *Stimulus type* × *TMS* interaction ($F_{4,76} = 8$, $p < 0.001$, $\eta P_2 = 0.3$). We first investigated the effect of *Stimulus type* in the NT condition. *Post-hoc* comparisons indicated that, in the absence of TMS, participants were faster in response to high-calorie (mean = 428 ms) compared to low-calorie (mean = 445 ms; $p < 0.002$) and non-food images (mean = 465 ms; $p < 0.001$). Participants were also faster to respond to low-calorie food images compared to non-food images ($p < 0.001$). *Post-hoc* comparisons on the FT condition revealed the same pattern, with faster RTs to high-calorie (mean = 422 ms) compared to low-calorie food (mean = 450 ms; $p < 0.001$) and non-food images (mean = 463; $p < 0.001$). Finally, when TMS was delivered during the image presentation (TT), we found an effect specific to high-calorie food with RTs significantly slower compared to the NT condition (high-calorie TT = 448 ms vs. high-calorie NT = 428 ms; $p < 0.001$). A summary of these results is shown in Figure 3A and Tables 2, 3.

The ANOVA performed on accuracy showed significant main effects of *Stimulus type* ($F_{2,38} = 7$, $p = 0.003$, $\eta P_2 = 0.27$) and *TMS* ($F_{2,38} = 4.4$, $p = 0.019$, $\eta P_2 = 0.19$) but not significant Interaction. *Post-hoc* comparisons indicated that participants were generally more accurate to respond to high-calorie (mean = 98.84%) than low-calorie (mean = 97.59%; $p = 0.002$) and non-food images (mean = 98.43%; $p < 0.05$). We also found that TMS caused a deterioration of the accuracy in TT compared to NT conditions ($p = 0.019$), regardless of *Stimulus type*. A summary of these results is shown in Figure 3B and Tables 2, 4. No significant correlation between BIS-11 total scores and self-reported BMI was found ($R = 0.197$; $p = 0.414$).

3.3. Motor-evoked potentials (MEPs)

The repeated measure ANOVA performed to investigate changes in MEP amplitude revealed no significant main effect of *Stimulus type* ($F_{2,38} = 0.21$, $p = 0.809$), *Trial type* ($F_{1,19} = 2.64$, $p = 0.121$) and no significant Interaction ($F_{2,38} = 0.11$, $p = 0.897$). To further investigate changes in M1 excitability due to the Go/NoGo condition, we decided to run an

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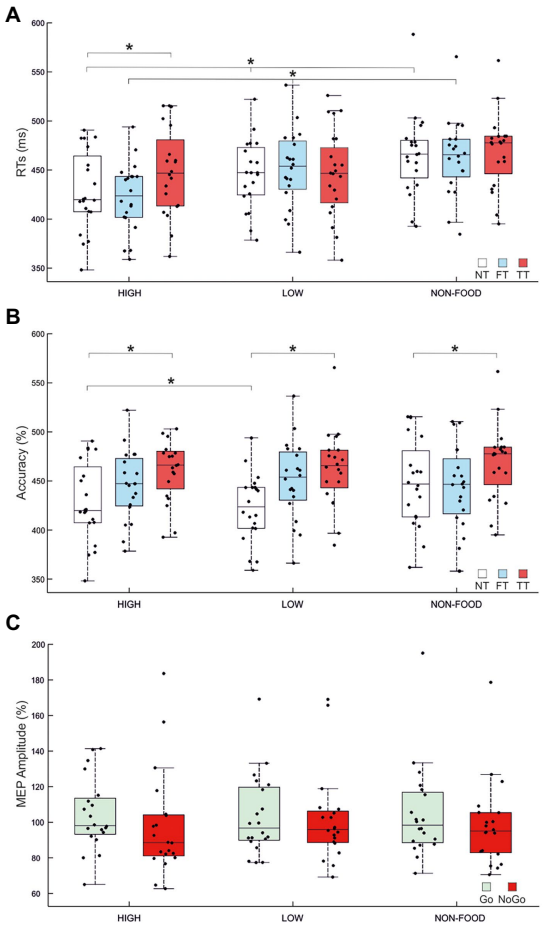

FIGURE 3
Effects of image type and TMS over MI. **(A)** Mean go reaction times (RTs) for High/Low-Calorie and Non-food trials for NT (No TMS), FT (Fixation TMS) and TT (Target TMS). In NT and FT, high-calorie RTs were faster than RTs collected for low-calorie and non-food images. Low-calorie RTs were also faster compared to non-food RTs. A specific increase of high-calorie RTs emerged between NT and TT conditions. **(B)** Mean accuracy for High/Low-Calorie and Non-food trials for NT, FT and TT. Participants were more accurate for high-calorie compared to low calorie NT trials. Accuracy decreased in TT conditions, regardless of stimulus type. **(C)** Mean normalised amplitude of motor evoked potentials (MEPs). MEP amplitude collected during the target presentation (TT) for High/Low-Calorie and Non-food trials in Go and NoGo conditions is expressed as percentage of the MEP amplitude collected at baseline (FT). As expected, corticospinal excitability is lower in NoGo conditions (p=0.036). In each panel, black dots represent individual data. The box and wiskers show the Median±1.5 interquartile range (IQR) and asterisks represent significance.

TABLE 2 Food Go/NoGo results.

	High	Low	Non-food
NT	428 ± 41 ms	446 ± 36 ms	466 ± 42 ms
	99.2 ± 1%	98.2 ± 1.9%	98.8 ± 0.9%
FT	422 ± 35 ms	451 ± 40 ms	463 ± 39 ms
	99 ± 1.3%	97.1 ± 1.9%	98.5 ± 1.4%
TT	448 ± 45 ms	444 ± 42 ms	469 ± 38 ms
	98.3 ± 1.7%	97.5 ± 2.7%	98 ± 2.2%

Mean ± standard deviation of RTs (ms) and accuracy scores (%) regarding *Stimulus type (High-, low-calorie, and non-food)* and *TMS (NT-NO TMS, FT-FIXATION TMS, and TT-TARGET TMS)* factors.

additional ANOVA, again with factors *Stimulus type* and *Trial type*, but only considering food vs. non-food stimuli, without distinguishing between high-and low-calorie images. We found a significant main effect of *Trial type* ($F_{1,19}$ = 5.129, p = 0.036, ηP_2 = 0.21), explained by a greater MEP amplitude in Go trials (mean Go amplitude = 104% vs. mean NoGo amplitude = 99%). No other significant effect was found. A summary of these results is shown in Figure 3C and Table 5.

4. Discussion

In the present study, we investigated whether there is a relationship between the visual appearance of food (high-calorie, low-calorie food) and inhibitory control in healthy individuals, in light of the growing interest for the topic in the eating-disorder literature (47). To this aim, we used an affective Go/NoGo task manipulating the stimulus category and coupled the behavioural measures with measures of corticospinal excitability (i.e., MEPs) to gain further insight on the contribution of the motor system. Overall, the designed task was innovative when compared to previous investigations because it used food stimuli rather than abstract stimuli (23), included a neutral condition rather than limiting the comparison to high-and low-calorie food (25) and prevented the undesired influence of reading ability or abstract thought on performance (26). Crucially, whilst previous investigations were limited to female participants (25) our sample included both normal-weighted male and female participants.

In line with a neuroimaging study using similar paradigms (48), we showed that RTs to high-calorie food were faster than RTs to low-calorie and non-food stimuli. High-calorie food images were considered also more appetising than low-calorie food images by our participants confirming the already reported correlation between the calorie content and the perceived palatability (49, 50).

This result suggests that the visual appearance of high-calorie food generates a state of heightened arousal in the observer, which in turn contributes to promptly responding to appetising food pictures and that we are naturally more inclined to respond to rewarding stimuli such as high-calorie food (48). Crucially, participants were not tested under conditions of starvation (i.e., they were invited to consume breakfast or lunch 2 or 3 h prior to the experimental session); therefore, the evidence of reduced RTs to high-calorie food was specifically powerful, even in absence of a starvation state. Collectively, these observations reinforce the view that high-calorie foods have high incentive value, prompt response (51) and increase arousal independently from satiety levels (52).

An interesting result is the increase of high-calorie RTs when a single TMS pulse was delivered over the primary motor cortex during the image presentation (TT condition). This effect is specific to high-calorie stimuli and therefore cannot be explained by a generic interference of TMS on task execution. The present result suggests that the motor system might be particularly engaged in movements aimed at approaching high-calorie and therefore high-nutritious/appetising food. In this sense, the response of the motor system, which we measure here by testing the excitability of the primary motor area, in reacting to high-calorie food compared to food with little (low-calorie) or no nutritional value (non-food) would be greater. The finding that the caloric content of food did shape task performance is particularly interesting because participants were unaware of the distinction between high-and low-calorie stimuli during the task. They were simply instructed to go or not to go in response to food or non-food images. The challenge

TABLE 3 RTs *post-hoc* results.

		High NT	Low NT	Non-food NT	High FT	Low FT	Non-food FT	High TT	Low TT	Non-food TT
High	NT		0.00259	0.00000	1	0.00003	0.00000	0.00029	0.00893	0.00000
Low	NT	0.00259		0.00029	0.00002	1	0.00328	1	1	0.00002
Non-food	NT	0.00000	0.00029		0.00000	0.02031	1	0.00259	0.00007	1
High	FT	1	0.00002	0.00000		0.00000	0.00000	0.00000	0.00007	0.00000
Low	FT	0.00003	1	0.02031	0.00000		0.15363	1	1	0.00191
Non-food	FT	0.00000	0.00328	1	0.00000	0.15363		0.02428	0.00091	1
High	TT	0.00029	1	0.00259	0.00000	1	0.02428		1	0.00020
Low	TT	0.00893	1	0.00007	0.00007	1	0.00091	1		0.00000
Non-food	TT	0.00000	0.00002	1	0.00000	0.00191	1	0.00020	0.00000	

p values regarding *Stimulus Type (High-, low-calorie, and non-food)* and *TMS (NT-NO TMS, FT-FIXATION TMS, and TT-TARGET TMS)* factors.

TABLE 4 Accuracy *post-hoc* results.

TMS	NT	FT	TT	Stimulus type	High	Low	Non-food
NT		0.16	0.02	High		0.002	0.702
FT	0.16		1	Low	0.002		0.056
TT	0.02	1		Non-food	0.702	0.056	

p values regarding *TMS (NT-NO TMS, FT-FIXATION TMS, and TT-TARGET TMS)* and *Stimulus type (High-, low-calorie, and non-food)* factors.

TABLE 5 MEP results.

	High	Low	Non-food
Go	104 ± 20	103 ± 23	105 ± 27
NoGo	98 ± 30	102 ± 25	99 ± 24

Mean ± standard deviation of MEP amplitude for each *Stimulus type (High, low-calorie, and non-food)* and *Trial type (Go vs. NoGo)*. MEP in the *TT (TARGET TMS)* condition were normalised using the *FT (FIXATION TMS)* condition and expressed as a percentage.

posed by appetising foods to inhibitory control mechanisms could explain why often during a diet we cannot stop right in front of high calorie foods. Most of the attempts so far have targeted the dorsolateral prefrontal cortex (47, 53). A different strategy could target and modulate the activity of other areas of the motor circuit, such as the primary motor cortex or the cerebellum.

We also found that accuracy was increased for high-compared to low-calorie food. This result corroborates previous studies (e.g., 54) suggesting a more efficient response to foods with greater salience. It is worth noting that the accuracy reflects the ability to effectively respond as well as refrain from when a stimulus is presented. Therefore, the selection of the appropriate response (go or no go, depending on the instructions) to palatable foods might reflect a fine-tuning of cognitive control processes as a result of the increased nutritive value and the appetising nature of high-calorie food. Accordingly, several studies have found that accuracy for high-calorie food during inhibitory control tasks is reduced in overweight population (55–57), suggesting a dysregulation of the inhibitory system that is specific for palatable foods. In addition, He et al. (48) showed that the ability to inhibit response to high-calorie foods is more difficult for individuals with higher BMI and who reported to consume more high-calorie foods. However, in the present study we did not find a direct correlation between response inhibition and individual BMI or impulsivity assessment. The lack of a correlation might be due to measurement errors, since weight and height were self-reported and not objectively measured in the laboratory. Furthermore, a sample size of 20 normal-weighted participants with very low variations in BMI might not be powerful enough to unveil a possible correlation. Future studies including participants with abnormal BMI (i.e., excessively high or low) are needed to clarify this relationship.

Last, neurophysiological results (MEPs) showed an effect of trial type, with higher corticospinal excitability for Go compared to NoGo trials, independently from the stimulus category. This is in line with previous studies (58, 59) showing that the decrease in MEP amplitude is due to the inhibition of the corticospinal pathway after the NoGo decision or to the increase of corticospinal excitability following Go stimuli, in line with premovement facilitation (60). However, the present MEP results are in contrast to what already reported in a preceding TMS study showing that the additional excitatory drive triggered by salient cues counteracts the presence of inhibitory influences to M1 (61).

One possible explanation of the null result regarding the modulation of MEP amplitude according to high, low-calorie, or non-food category could be the timing of the pulse delivery, i.e., 300 ms after the image presentation which could be too late to target the dynamics of corticospinal excitability. According to this view, in a previous study, the time course of corticospinal excitability changes during a similar task found effects on MEP amplitude up to 200 ms following the onset of a simple Go/NoGo visual cue (38). In the present study, we reasoned that more complex visual stimuli (images of food or objects instead of geometric shapes), would require a longer processing time and therefore we increased the cue to TMS interval to 300 ms. However, the average RTs to the food Go/NoGo task (high: 428 ms; low: 445 ms; non-food: 465 ms) are comparable to those of the simple Go/NoGo task used in Picazio et al. (38) (428 ms). It is therefore possible that using the same cue to TMS interval in the present study could have shown differences in the corticospinal activity depending on stimulus type. Therefore, we might have missed the relevant window for MEP modulation but were still able to interfere with RTs which are the final output of the motor process involved. Another explanation of this negative result for the MEPs could be the Go/NoGo trial ratio (50/50). This could be less effective in evoking a clear motor inhibition compared to Go/NoGo tasks with rarer NoGo trials (37).

The present data do not allow us to distinguish between externally-driven action inhibition, which is typically triggered by Go/NoGo tasks and internally-driven motivational factors that here could be elicited by the affective/nutritional component of the

task (62). This aspect should be explored in future studies in which participants could choose to respond or not when images are presented according to their preferences.

Finally, the present study has some limitations that could be addressed in future. First, larger sample sizes are needed to investigate any correlation between food-related inhibitory control and individual measures of body weight and impulsivity; second, subjective measures of weight and height should be replaced with objective measures; third, corticospinal excitability should be tested at different time points from stimulus presentation.

In the present study, we only tested healthy participants, but it has been previously reported that eating disorders are associated with altered responses in the control system. For example, in anorexia nervosa high-calorie food elicits an enhanced activation of cognitive control regions, explaining the persistent food avoidance and starvation (63). On the contrary, in obesity, high-calorie food is associated with abnormal activation of the impulsive system, which leads to excessive food consumption (64, 65). Therefore, in future studies should address the relationship between food-related inhibitory control mechanisms including also patients with eating disorders.

In conclusion, our findings show that the calorie content of food frequently corresponds to the perceived palatability in healthy participants and that the sight of high-calorie food triggers an implicit drive to approach and is characterised by a stronger activation of the primary motor cortex. This enhanced involvement of the motor circuit coupled with reduced reaction times and improved performance for high-calorie food might reflect the existence of adaptive mechanisms aimed to approach food with high nutritive value in healthy participants.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Fondazione Santa Lucia IRCCS. The patients/participants provided their written informed consent to participate in this study.

Author contributions

AD'A, SP, and VB collected the data. DV, SP, and VB analysed and interpreted the data. SP and VB wrote the paper. GK and SP designed the experiment. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

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Conflict of interest

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

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Associations between waist circumference and executive function among Chinese Tibetan adolescents living at high altitude

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Background: Associations between body composition and execution function (EF) were currently studied in low altitude (LA) areas. However, the research on the correlation between waist circumference (WC) and EF among adolescents living at high altitude (HA) was limited.

Objective: We sought to explore the association between WC and EF in Chinese Tibetan adolescents aged 13–18 years in HA areas.

Methods: After excluding invalid data and extreme values, 1,228 participants (583 boys and 645 girls) were eventually included. The areas of Lhasa (average elevation of 3650 m), Nagqu (4500 m), Qamdo (3500 m), and Nyingchi (3100 m) in China were chosen as study sites. Participants completed tasks to measure inhibitory control, working memory, and cognitive flexibility. The predictive association between WC and EF was explored by One-way ANOVA, Pearson correlation, and linear regression analysis.

Results: After controlling for concomitant variables, the reaction time (RT) of responding to inhibitory control (difference incongruent and congruent), working memory (1-back, 2-back), and cognitive flexibility (heterogeneous, difference in heterogeneous and homogeneous) stimuli in subjects with WC $\geq$ 85th percentile was longer than that in those with WC of the 15th percentile or below [by 1.785 ms (95% CI: 0.078, 3.491), 208.734 ms (95% CI: 96.886, 320.582), 106.679 ms (95% CI: 16.485, 196.873), 82.307 ms (95% CI: 19.171, 145.442), and 58.397 ms (95% CI: 0.343,116.452), respectively], ($P < 0.05$).

Conclusion: After adjustment for concomitant variables, WC was significantly positively associated with the RT of inhibitory control, working memory, and cognitive flexibility among Chinese Tibetan adolescents in HA areas.

KEYWORDS

waist circumference, executive function, Tibetan, high altitude, adolescents

1. Introduction

Executive function (EF) is an umbrella term for the related but distinct processes involved in the effortful control of goal-directed behavior (1), including three main factors, namely inhibitory control, working memory, and cognitive flexibility (2). Its purpose is to regulate and control the cognitive process (3). EF is known to be particularly sensitive to oxygen; studies have shown that the EF of people who live in high altitude (HA) areas with low temperatures and hypoxia may be impaired (4–6). Recently, the health question of humans in HA areas has become a topic under increasing discussion, and the impact of HA exposure on cognition has attracted the attention of scholars. Long-term exposure to HA areas may lead to cognitive deficits, such as in inhibitory control, working memory, and cognitive flexibility (7–9). Zhu et al. showed that the cognitive level of students in HA areas was relatively lower than that of students in low altitude (LA) areas, postulated to be mainly driven by chronic hypoxia in HA areas (10). Long-term exposure to HA areas has been shown to negatively affect individuals' spatial working memory and significantly reduce their inhibitory control (11, 12).

As one of the factors influencing EF and underlying brain developmental processes in adolescents, body composition may be statistically correlated with EF (13–15). Soaring obesity rates and declining physical health have become among the most serious public health concerns worldwide (16, 17), notably in places as diverse as the United States (18, 19), Serbia (20), and China (21). Meanwhile, recent evidence suggests that obesity may have adverse health effects. Obesity has also been associated with cognitive performance across the life cycle, particularly with negative associations with EF (22, 23). As an effective indicator of abdominal obesity mass, waist circumference (WC) is used to define abdominal obesity (24), reflecting the accumulation of fat in the abdomen and valid predictor of future cardio-metabolic and chronic diseases (25). Even after controlling for body mass index (BMI), high WC appeared to be particularly detrimental to metabolic regulation, affecting type 2 diabetes risk (26). Most studies have focused on the relationship between BMI and EF (27, 28), but, there have been few studies on the correlations between WC and EF in HA areas.

At present, the correlation between WC and EF was not very uniform. Some studies have confirmed that high WC was negatively associated with EF (29, 30). In structural equation modeling in adolescents, WC through a higher metabolic risk factor cluster score (MetS-cluster score) and through lower high-density lipid cholesterol (HDLc) displayed a statistically significant negative relationship with reaction time (RT) of incongruent stimuli (29). Research has also found that the combined adverse effects of high WC on academic performance were observed in both boys and girls for grade point average (GPA) indicator (30). However, some other studies found that high WC was positively correlated EF (31). The results were inconsistent with other research regarding the relationship between WC and EF, which found that higher WC predicted higher cognitive ability among Indian children aged 9–10 years (31). The relationship between WC and EF remains unclear.

Most previous studies have focused on the relationship between BMI and EF. However, the research on the relationship between WC and EF remains limited. In addition, previous studies have mainly focused on adolescents in LA areas (32), while there have been few studies on the relationship between WC and EF among adolescents in HA areas. As one of the highest-altitude populations in the world, making it more likely to observe differences among Chinese Tibetan adolescents in HA areas. Hence, this paper sought to clarify the independent and joint correlations among WC and EF of Tibetan adolescents living in HA areas.

2. Materials and methods

2.1. Data sources and participants recruitment

A stratified random cluster sampling method was adopted to identify test sites determined by surveys on the physical health of Tibetan students. Lhasa, Nagqu, Qamdo, and Nyingchi were chosen as test sites for this study (with average altitudes of 3650, 4500, 3500, and 3100 m, respectively). After performing the EF test, the valid data were the ones whose accuracy rate of each task reached over 80%. The specific inclusion criteria of the participants were people aged 13–18 years without color blindness or serious physical or mental illness. After excluding invalid data and extreme values, based on a gender ratio of approximately 1:1, data from 1,228 subjects (583 for boys and 645 for girls) were collected for this analysis in the Tibet Autonomous Region in China from August 2019 to December 2020 (**Figure 1**).

2.2. Waist circumference

Waist circumference was measured according to the specific requirements of the physical health survey of students in China (33). In the test, the participants were asked to stand upright and breathe gently. The investigators faced the participants and put the nylon tape 1 centimeter (cm) above the navel on the horizontal plane of the waist. The line of sight was on the same level as the nylon tape, and the reading was in cm (accuracy: two decimal place). The test error could not exceed 0.01 cm.

2.3. Body mass index

Body mass was measured to the nearest 0.1 kg on an electronic scale (Tanita BWB-800S, Tanita Corporation, Tokyo, Japan). Stature was measured to the nearest 0.5 cm using a portable stadiometer (SECA 214, Seca Corporation, Hamburg, Germany). BMI was calculated according to the results of the recorded height and weight test using the standard formula. BMI = weight (kg)/height (m^2) (33).

2.4. Executive function and related assessments

The EF task-cueing paradigm was that developed by Aiguo et al. (34), including inhibitory control test developed by Flanker (35),

working memory test (36), and cognitive flexibility test (37). The RT for a correct test was recorded as the final result, and a shorter RT reflected a better performance. The RTs of the congruent and incongruent exercise were used to show inhibitory control. The 1-back and 2-back tests were used to indicate working memory. The difference in RTs between heterogeneous and homogeneous blocks was used to estimate cognitive flexibility. The EF tests were conducted on a computer using a program created by the E-prime 1.1 software system (Psychology Software Tools Inc., Pittsburgh, PA, USA).

2.4.1. Inhibitory control

The Flanker task involved two types of tests, congruent and incongruent. Participants gaze at the center of the computer screen for 500 ms, and then a series of five capital letters appear on the screen for 1000 ms. One of two different situations appear randomly: one situation is congruent (LLLLL or FFFFF) and the other incongruent (LLFLL or FFLFF). The center of the screen alternates with a + sign, and each stimulus appears at an interval of 2 s. Participants press either the "F" or "L" key with their left or right index finger. The test was divided into pre-test practice, stage 1, and stage 2. The pre-test practice was 12 rounds of the judgment response task, and stage 1 and stage 2 included 48 rounds each. The inhibitory control was calculated as the difference in response time between incongruent and congruent stimuli.

2.4.2. Working memory

The 1-back task displayed five uppercase letters (A, S, P, G, T) in the center of the screen. The participants attempted to accurately remember the letters that appeared by pressing the "F" key if the letter appeared consistent with the previous letter, or the "J" key if it was inconsistent. The formal test was divided

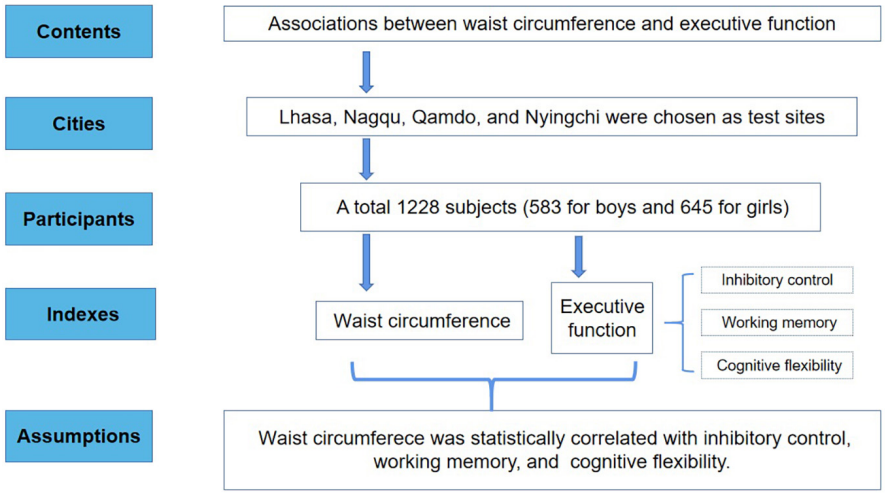

FIGURE 1
Flowchart of the study design.

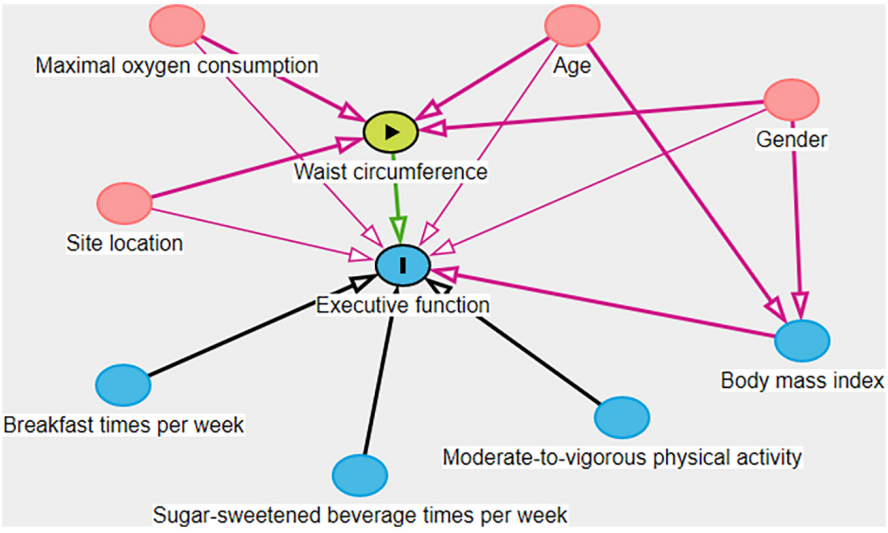

FIGURE 2
Directed acyclic graph of the associations between waist circumference and executive function.

into two stages of 25 repetitions each. The stimulus interval for each capital letter was 3 s, and the time for the letter to appear on the screen was 2000 ms. In this study, the test age of 1-back was 10–18 years.

The task if the 2-back exercise was to indicate whether the letter in the center of the screen was consistent with the one letter in front, pressing the "F" key quickly if it was, or pressing the "J" key if not. Other test requirements and methods were the same as with the 1-back test.

2.4.3. Cognitive flexibility

This experimental test was divided into three parts. Part 1: At the beginning of the task, a black Arabic number (1–4 and 6–9, in random order) continuously appeared in the center of the computer screen. Participants judged the size of a "5" by pressing the D key if the digit was smaller than the 5 and pressing the F key if the digit was larger than the 5. The final result for each participant was their average RT across all trials. Part 2: A green number (1–4 and 6–9, in random order) appeared in the center of the screen at the beginning of the task. Participants pressed the J key if the digit was odd and pressed the K key if it was even. The final result for each participant was his average RT across all tasks. The heterogeneous conditions comprised Parts 1 and 2. Part 3 was then conducted: This test randomly interspersed the tasks used in Part 1 and Part 2. That is, if the number was black, the participant judged the size of the 5 (by pressing the D key if it was smaller and the F key if it was larger, in both cases using the left hand). If the number was green, participants judged whether

TABLE 1 Characteristics and assessments of adolescent subjects.

Variable	Total sample	Standard error	IQR	Kurtosis	Skewness	Coefficient variable
N	1228					
Boys (%)	583 (47.48)					
Girls (%)	645 (52.52)					
Age	15.77 ± 1.68	0.048	3.00	−1.166	−0.215	10.64%
Waist circumference (cm)	68.21 ± 7.13	0.201	7.40	3.629	1.231	10.32%
WC < percentile 15 (%)	205 (16.69)					
WC ≥ percentile 15 and < percentile 85 (%)	870 (70.85)					
WC ≥ percentile 85 (%)	153 (12.46)					
BMI (kg/m^2)	20.38 ± 2.68	0.076	3.15	1.835	0.919	13.15%
Underweight (%)	63 (5.13)					
Normal weight (%)	1020 (83.06)					
Overweight (%)	115 (9.36)					
Obesity (%)	30 (2.45)					
VO_{2max} (mL/kg/min)	38.37 ± 7.82	0.232	12.01	−0.599	0.011	20.37%
Moderate-to-vigorous physical activity per week (h)	4.42 ± 0.85					
Breakfast times per week (times)	2.89 ± 0.40					
Sugar-sweetened beverage times per week (times)	1.41 ± 0.59					
Inhibitory control (ms)						
Congruent (ms)	774.94 ± 84.43	2.409	107.07	0.063	0.576	10.90%
Incongruent (ms)	794.51 ± 83.76	2.390	104.28	0.078	0.595	10.54%
Difference in incongruent and congruent (ms)	19.55 ± 6.52	0.186	11.03	−1.268	0.116	33.36%
Working memory (ms)						
1-back (ms)	965.67 ± 429.13	12.246	490.59	246.192	10.803	44.44%
2-back (ms)	1078.05 ± 377.96	10.786	621.04	−0.94	−0.092	35.06%
Cognitive flexibility (ms)						
Homogeneous (ms)	744.15 ± 123.04	3.511	198.67	−0.798	0.244	16.54%
Heterogeneous (ms)	1125.69 ± 266.37	7.601	307.08	−0.307	0.707	23.66%
Difference in heterogeneous and homogeneous (ms)	381.55 ± 231.02	6.592	272.66	0.099	0.437	60.55%

WC, waist circumference; BMI, body mass index; VO_{2max}, maximum oxygen uptake.

it was an odd or even number (by pressing the J key if it was odd and the K key if it was even, in both cases using the right hand). A participant's final result was their average RT across all trials (i.e., homogeneous RT). The whole test consisted of six stages, which were conducted in the order of A, B, C, C, B, A. A was Part 1 (i.e., the size judgment), and was performed 16 times in total. B was the parity judgment, Part 2, and was performed 16 times in total. C was the size/parity judgment, Part 3, which was performed 32 times in total. A practice round was not included in the test score; it was performed eight times before Part 1 and Part 2, and 16 times before Part 3.

2.5. Statistical analyses

Waist circumference was categorized according to percentile by the Lambda Mu Sigma (LMS): very low (WC < 15th percentile), normal (15th percentile ≤ WC < 85th percentile), high (WC ≥ 85th percentile) (38). Many studies have demonstrated that EF could be affected by obesity rate (22, 23), dietary intake (39), physical activity (40), and maximum oxygen uptake (VO_{2max}) (41). Therefore, sociodemographic and dietary intake information, BMI, WC, moderate-to-vigorous physical activity (MVPA), and VO_{2max} were used as covariates in this study. Based on previous studies and data in this study on the relationship between WC and EF in adolescents, directed acyclic graph (DAG) was used to identify possible covariates on exposure, and outcomes (**Figure 2**) (42). Three models were developed for this study: Model 1 was conducted after adjusting for demographic indicators (gender, age, BMI, and site location) by questionnaires. Based on Model 1, Model 2 controlled for MVPA, VO_{2max}. Physical activity status was obtained by the following two questions: "In the past 7 days, how many times did you have MVPA on school days and weekends, respectively?" If students answered more than 0 times, they were further asked about the duration each time, "On average, how long does each activity last?" The VO_{2max} was

TABLE 2 Status of executive function by WC percentile groups.

RT (ms)	Waist circumference	*N*	Mean	SD	Partial η^2	Cohen's^f	*F*	*P*
Inhibitory control								
Congruent	WC < percentile 15	153	763.85	78.34	0.006	0.075	3.402	0.034[a]
	WC ł percentile 15 and < percentile 85	870	774.1	82.05				
	WC ł percentile 85	205	786.81	96.92				
Incongruent	WC < percentile 15	153	782.42	78.35	0.007	0.083	4.199	0.015[a]
	WC ł percentile 15 and < percentile 85	870	793.53	81.27				
	WC ł percentile 85	205	807.65	95.88				
Difference in incongruent and congruent	WC < percentile 15	153	18.57	6.11	0.009	0.095	5.479	0.004[b]
	WC ł percentile 15 and < percentile 85	870	19.44	6.55				
	WC ł percentile 85	205	20.77	6.54				
Working memory								
1-back	WC < percentile 15	153	845.12	266.8	0.018	0.135	11.165	0.000[b]
	WC ł percentile 15 and < percentile 85	870	964.67	470.09				
	WC ł percentile 85	205	1059.91	305.65				
2-back	WC < percentile 15	153	972.55	368.13	0.014	0.119	8.63	0.000[b]
	WC ł percentile 15 and < percentile 85	870	1082.7	376.3				
	WC ł percentile 85	205	1137.05	378.32				
Cognitive flexibility								
Homogeneous	WC < percentile 15	153	727.69	120.54	0.006	0.076	3.51	0.030[b]
	WC ł percentile 15 and < percentile 85	870	742.9	123.79				
	WC ł percentile 85	205	761.69	120.14				
Heterogeneous	WC < percentile 15	153	1079.95	239.39	0.019	0.141	12.171	0.000[b]
	WC ł percentile 15 and < percentile 85	870	1115.13	266.12				
	WC ł percentile 85	205	1204.68	272.15				
Difference in heterogeneous and homogeneous	WC < percentile 15	153	352.26	220.33	0.015	0.123	9.315	0.000[b]
	WC ł percentile 15 and < percentile 85	870	372.22	233.67				
	WC ł percentile 85	205	442.99	217.76				

RT, reaction time.
[a] $P < 0.05$.
[b] $P < 0.01$.

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TABLE 3 Bivariate correlations between variables among Tibetan adolescents in China.

	1	2	3	4	5	6	7	8	9	10	11	12	13
Age (1)	1												
Gender (2)	0.031	1											
Site (3)	0.308[b]	0.070[a]	1										
BMI (4)	0.250[b]	0.239[b]	0.095[b]	1									
WC (5)	−0.018	−0.107[b]	−0.130[b]	0.590[b]	1								
VO_{2max} (6)	−0.211[b]	−0.448[b]	0.071[a]	−0.185[b]	−0.175[b]	1							
RT congruent (7)	−0.224[b]	0.036	−0.196[b]	−0.054	0.095[b]	−0.398[b]	1						
RT incongruent (8)	−0.237[b]	0.033	−0.203[b]	−0.052	0.102[b]	−0.398[b]	0.997[b]	1					
RT difference in incongruent and congruent (9)	−0.144[b]	−0.039	−0.071[a]	0.03	0.085[b]	0.041	−0.143[b]	−0.066[a]	1				
RT 1-back (10)	−0.124[b]	−0.031	−0.04	0.011	0.092[b]	−0.113[b]	0.206[b]	0.205[b]	−0.025	1			
RT 2-back (11)	−0.207[b]	−0.009	−0.166[b]	0.005	0.122[b]	−0.208[b]	0.249[b]	0.255[b]	0.05	0.273[b]	1		
RT homogeneous (12)	−0.125[b]	−0.007	−0.132[b]	−0.011	0.076[b]	−0.270[b]	0.393[b]	0.396[b]	−0.01	0.256[b]	0.304[b]	1	
RT heterogeneous (13)	−0.072[a]	−0.007	−0.088[b]	0.034	0.148[b]	−0.381[b]	0.404[b]	0.406[b]	−0.012	0.175[b]	0.290[b]	0.499[b]	1
RT difference in heterogeneous and homogeneous(14)	−0.017	−0.004	−0.032	0.045	0.130[b]	−0.296[b]	0.256[b]	0.258[b]	−0.008	0.066[a]	0.172[b]	0.043	0.887[b]

BMI, body mass index; WC, waist circumference; VO_{2max}, maximum oxygen uptake; RT, reaction time.

[a] $P < 0.05$.

[b] $P < 0.01$.

estimated by 20 m SRT and the details measurement of 20 m SRT was provided in our previous study (38). Based upon Model 2, Model 3 included breakfast, sugar-sweetened beverage intake as additional variables. The associations between WC and EF were evaluated using one-way ANOVA, *Pearson* correlation, and linear regression analysis. Data were analyzed using IBM SPSS 25.0 software (IBM Corp., Armonk, NY, USA); the test significance level was $\alpha = 0.05$. Results were considered statistically significant if $P < 0.05$.

3. Results

3.1. Descriptive characteristics for various variables

Among the 1,228 Tibetan adolescents studied, there were 583 boys (47.48%) and 645 girls (52.52%), with an average age of 15.77 ± 1.678 years. The average WC of children and adolescents aged 13–18 years was 68.21 ± 7.13 cm. Grouped by WC, the proportions of subjects in the <15th percentile WC group, the ≥ 15th and <85th percentile WC group, and the ≥ 85th percentile WC group were 16.69, 70.85, and 12.46, respectively. The average values of congruent, incongruent, 1-back, 2-back, heterogeneous, and homogeneous were 774.94, 794.51, 965.67, 1078.05, 744.15, and 1125.69 ms, respectively (Table 1).

3.2. Performance of different WC percentile groups in EF-measuring tasks

All measures of EF index [including inhibitory control RT, working memory RT (1-back, 2-back), and cognitive flexibility RT] differed significantly by WC percentile groups ($F = 5.479$, 11.165, 8.63, and 9.315, respectively, $P < 0.05$). The RTs of EF (inhibitory control, working memory, and cognitive flexibility) increased with WC percentile in different groups (Table 2).

3.3. Bivariate correlations between study variables

Table 3 showed the analysis of the double variable pearson correlations of the various variables of the subjects. There were no mean differences by gender in performance of executive function variables ($P > 0.05$). In general, the higher the WC, the longer the congruent RT, incongruent RT, difference in incongruent and congruent RT, 1-back RT, 2-back RT, homogeneous RT, heterogeneous RT, and difference in heterogeneous and homogeneous RT ($r = 0.095$, 0.102, 0.085, 0.092, 0.122, 0.076, 0.148, and 0.130, respectively, $P < 0.05$). In other words, there was a positive correlation between WC and inhibitory control, working memory, and cognitive flexibility RT, with shorter RT indicating better EF.

3.4. The multiple linear regression of executive function with different WC percentile groups

The correlation between WC and executive function is presented in Table 4. After controlling for concomitant variables, the RTs of inhibitory control (difference incongruent and congruent), working memory (1-back, 2-back), and cognitive flexibility (heterogeneous, difference in heterogeneous and homogeneous) stimuli in subjects with WC ≥ 85th percentile was longer than that in those with WC of the 15th percentile or below [by 1.785 ms (95% CI: 0.078, 3.491), 208.734 ms (95% CI: 96.886, 320.582), 106.679 ms (95% CI: 16.485, 196.873), 82.307 ms (95% CI: 19.171, 145.442), and 58.397 ms (95% CI: 0.343,116.452), respectively], ($P < 0.05$). In the RTs of inhibitory control evalution, the subjects in the WC ≥ 85th percentile rate significantly different in the inhibitory control compared to those in the WC ≥ 15th percentile and <85th percentile ($P < 0.05$).

4. Discussion

This study showed that WC was negatively associated with the inhibitory control, working memory, and cognitive flexibility among Chinese Tibetan adolescents in HA aeras. The RT of inhibitory control (difference incongruent and congruent), working memory (1-back, 2-back), and cognitive flexibility (heterogeneous, difference in heterogeneous and homogeneous) stimuli in subjects with WC ≥ 85th percentile was longer than that in those with WC of the 15th percentile or below, respectively. There were no mean differences by sex in performance EF variables, consistent with findings in prior research (43, 44).

TABLE 4 The multiple linear regression of executive function by WC percentile groups (n = 1228).

RT (ms)	WC	Model 1	Model 2	Model 3
Inhibitory control				
Congruent	WC < percentile 15	0 (Reference)	0 (Reference)	0 (Reference)
	WC ł percentile 15 and < percentile 85	14.098 (-0.433 28.629)	12.58 (-0.645 25.806)	13.088 (-0.156 26.331)
	WC ł percentile 85	31.854 (11.609 52.098)[b]	9.354 (-9.642 28.350)	9.467 (-9.525 28.460)
Incongruent	WC < percentile 15	0 (Reference)	0 (Reference)	0 (Reference)
	WC ł percentile 15 and < percentile 85	14.755 (0.401 29.110)[a]	13.434 (0.408 26.460)[a]	13.99 (0.949 27.030)[a]
	WC ł percentile 85	33.678 (13.679 53.678)[b]	11.14 (-7.570 29.849)	11.276 (-7.426 29.977)
Difference incongruent and congruent	WC < percentile 15	0 (Reference)	0 (Reference)	0 (Reference)
	WC ł percentile 15 and < percentile 85	0.654 (-0.496 1.804)	0.855 (-0.333 2.042)	0.904 (-0.286 2.094)
	WC ł percentile 85	1.745 (0.143 3.348)[a]	1.762 (0.056 3.467)[a]	1.785 (0.078 3.491)[a]
Working memory				
1-back	WC < percentile 15	0 (Reference)	0 (Reference)	0 (Reference)
	WC ł percentile 15 and < percentile 85	128.362 (52.815 203.908)[b]	123.963 (46.143 201.784)[b]	123.938 (45.947 201.929)[b]
	WC ł percentile 85	247.089 (141.834 352.343)[b]	209.68 (97.906 321.454)[b]	208.734 (96.886 320.582)[b]
2-back	WC < percentile 15	0 (Reference)	0 (Reference)	0 (Reference)
	WC ł percentile 15 and < percentile 85	105.681 (40.250 171.113)[b]	100.836 (38.022 163.650)[b]	100.895 (38.003 163.786)[b]
	WC ł percentile 85	151.559 (60.398 242.720)[b]	107.829 (17.608 198.049)[a]	106.679 (16.485 196.873)[a]
Cognitive flexibility				
Homogeneous	WC < percentile 15	0 (Reference)	0 (Reference)	0 (Reference)
	WC ł percentile 15 and < percentile 85	16.525 (-5.191 38.242)	16.517 (-4.676 37.709)	18.155 (-3.034 39.344)
	WC ł percentile 85	36.954 (6.698 67.210)[a]	23.146 (-7.293 53.585)	23.909 (-6.478 54.297)
Heterogeneous	WC < percentile 15	0 (Reference)	0 (Reference)	0 (Reference)
	WC ł percentile 15 and < percentile 85	39.242 (-7.810 86.295)	32.48 (-11.461 76.420)	34.468 (-9.556 78.493)
	WC ł percentile 85	135.369 (69.813 200.925)[b]	81.264 (18.152 144.377)[a]	82.307 (19.171 145.442)[a]
Difference in heterogeneous and homogeneous	WC < percentile 15	0 (Reference)	0 (Reference)	0 (Reference)
	WC ł percentile 15 and < percentile 85	22.717 (-18.362 63.796)	15.963 (-24.405 56.332)	16.313 (-24.168 56.794)
	WC ł percentile 85	98.415 (41.182 155.648)[b]	58.119 (0.137 116.100)[a]	58.397 (0.343 116.452)[a]

RT, reaction time; WC, waist circumference; Model 1, adjusting for gender, age, BMI, and site location; Model 2, adjusting for moderate-to-vigorous physical activity per week, maximum oxygen uptake; Model 3, adjusting for breakfast times per week, sugar-sweetened beverage times per week.
[a] $P < 0.05$.
[b] $P < 0.01$.

In terms of inhibitory control, after controlling for concomitant variables, WC was negatively correlated with inhibitory control among Tibetan adolescents in HA areas in China. The associations between WC and inhibitory control in the study were generally consistent with those of previous studies. A study found that WC was negatively correlated with inhibitory control ($P = 0.008$) in Danish adolescents (23). At the same time, in the structural equation model, WC was indirectly positively correlated with incongruent RT through higher metabolic risk factor cluster (MetS-cluster) score and lower HDLc. The only statistically significant direct relationship between WC and inhibitory control was for the incongruent RT in the model including HDLc as a mediator (29). Longitudinal associations between inhibitory control and body composition throughout childhood found that better inhibitory control predicted lower subsequent obesity at each measured time point (45). Recent prospective studies (46) reported evidence suggesting that low inhibitory control in adolescents increases the risk of gaining more weight and WC. Interestingly, this paper found that high WC values the factors that predisposes the development of low inhibitory control.

In this study, after adjustment in concomitant variables, the RT of working memory (1-back, 2-back) in the highest percentiles of WC was slower than among those in the lowest percentiles of WC. WC was negatively correlated with working memory of children and adolescents. There have been numerous studies on the associations between WC and working memory that were consistent with the results of this study. Khan et al. explored the associations between abdominal adipose and hippocampal memory forms among pre-pubertal children (7–9-year-olds, $n = 126$) and found that total abdominal adipose tissue had a significant negative association with hippocampal-dependent relational memory behavioral accuracy (47). Hassevoort et al. found that central adiposity was negatively correlated with hippocampal-dependent relational memory among 7–10-year-old children ($n = 40$) who completed a task designed to assess relevant memory performance ($P < 0.05$) (48). After adjusting for multiple factors, we observed lower visuospatial function, executive performance, and language scores in the abdominal obesity group (WC $\geq$ 90 cm for men and $\geq$85 cm for women) compared with those in the non-abdominal obesity group, which was supported by the negative correlation between WC and visuospatial function (49). Some studies have linked obesity to an increased risk of memory problems in children (50). Gonzales found that larger WC was associated with decreased associated with diminished working-memory-related blood oxygen level-dependent (BOLD) response in the right superior frontal gyrus ($\beta = -0.008$, $P = 0.001$, 95% CI: -0.012 to -0.004) (51).

However, the results were not in accord with a previous southern Indian study of the relationship between WC and working memory performance (31). This cross-sectional study of 540 children reported that after adjusting for age, sex, and socioeconomic factors, higher WC predicted greater cognitive ability (i.e., long-term retrieval/storage, memory, reasoning, verbal abilities, attention, and concentration) in South India (31). The reasons for the discrepancy between this study and previous results may be that the subjects lived in Tibetan HA areas and were therefore exposed to chronic hypoxia (4–6). A study found that acute short-term exposure to HA areas could cause significant WM deficits in healthy children; this effect was particularly significant and more severe in children who lived at HA for a long time (7). The response accuracy of the HA group was significantly reduced in the linguistic and spatial working memory tasks compared with that of the LA group (4). Furthermore, obese children did not demonstrate significant retrieval-induced forgetting (RIF), whereas RIF was present in children (8–12-year-olds) without obesity and in adolescents (13–18-year-olds) with and without obesity (44). One longitudinal study showed that better working memory in grade three predicted lower subsequent body composition in grade four (45).

After adjustment for concomitant variables, WC was negatively associated with cognitive flexibility (heterogeneous, difference in heterogeneous and homogeneous) among subjects living at HA in this study. To support this theory, cognitive flexibility was impaired in obese adolescents (52, 53). Cross-sectional and longitudinal studies involving performance-based cognitive tasks have shown that overweight children and adolescents exhibit poorer cognitive flexibility than do their healthy-weight peers. These findings demonstrated that selective alterations in specific components of cognitive flexibility of overweight adolescents (52, 53). The relationship between WC and cognitive flexibility remains to be confirmed by longitudinal studies. Currently, there is a lack of relevant longitudinal studies on this topic, but Tomaso et al. conducted a longitudinal study on the relationship between cognitive flexibility and body composition (45). A longitudinal study showed that better SF in third grade predicted lower subsequent BMI in fourth grade, but, BMI did not predict subsequent cognitive flexibility performance at any point in time (45).

The results in this study were basically consistent with those of previous studies that also reported a negative correlation between high WC and EF of adolescents. A relatively consistent finding was that obesity was associated with poor EF across the life cycle, particularly in children and adolescents (54). One investigation found that obese (high visceral adipose tissue, H-VAT) children performed significantly lower on cognitive function tests than did normal-weight children (13). Another found that obesity (in terms of waist-hip ratio) in children and adolescents was negatively correlated with all areas of cognitive control (27). However, these results were reversed in the southern Indian study of the relationship between WC and cognitive performance noted above (31). Other studies have differed as well. One study found that Raven colored progressive matrices (CPM) scores were poorer among students with either the lowest or highest body fat percentile (BF%) than among other BF% groups ($P < 0.05$) (55).

Therefore, there is no consensus about the correlation between WC and EF. Improvement in EF levels has been associated with weight loss among overweight and obese adolescents (56), suggesting that EF could be improved and have a positive impact on weight loss (57). EF may have a protective effect on weight problems, especially in young adolescents when these abilities have had more time to develop and children begin to gain more independence (45). The findings suggested that the adverse association between obesity and EF may be attributed to visceral fat (VF), rather than fat stored elsewhere in the body (28). Both obesity and cognitive function could have strong associations with environmental influencing factors (socioeconomic status), though those associations can vary considerably in different directions depending on the setting (58). The results of multiple studies

highlight the necessity of developing comprehensive adolescent health plans that promote both healthy body composition and brain function in the future.

4.1. Strengths and limitations

Many previous studies have compared WC and executive function in adolescents; nevertheless, their datasets have generally been small and unrepresentative. The strengths of this study include its large sample size (N = 1228) for WC and use of multiple-aspect executive function tasks among adolescents living at HA in China. Most previous studies have focused on the associations between BMI and EF, and without regard to elevation; few systematic studies have examined the relationship between WC and EF in HA areas.

This study has some limitations to note. First, this study did not include participants at LA, and we did not compare the differences between the LA and HA. Second, this was a cross-sectional investigation, and therefore could not discern a causal relationship between WC and EF. Third, the investigation of influencing factors in this study was largely based on the participants' recall ability and attitude, which inevitably led to certain deviations in the survey results. In the future, objective instruments could be used for accurate testing of physical activity parameters.

5. Conclusion

In general, after adjustment for concomitant variables, WC was significantly positively associated with the RT of inhibitory control, working memory, and cognitive flexibility tasks measuring executive function among Chinese Tibetan adolescents in HA areas. The reasons for these results should be further investigated by longitudinal studies. Furthermore, effective measures should be taken to reduce obesity and improve EF among adolescents in the HA areas of Chinese Tibet. Further investigation is needed to provide an evidence base for efforts to improve the physical health and EF of adolescents in these areas.

Data availability statement

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

Ethics statement

Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

Author contributions

YL and XY: conceptualization. YL and LG: methodology. XY: validation, supervision, and funding acquisition. YL: formal analysis, visualization, resources, writing—original draft preparation, and project administration. YL and LS: investigation. YG and PS: data curation. YL and FZ: writing—review and editing. All authors read and agreed to the published version of the manuscript.

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Conflict of interest

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

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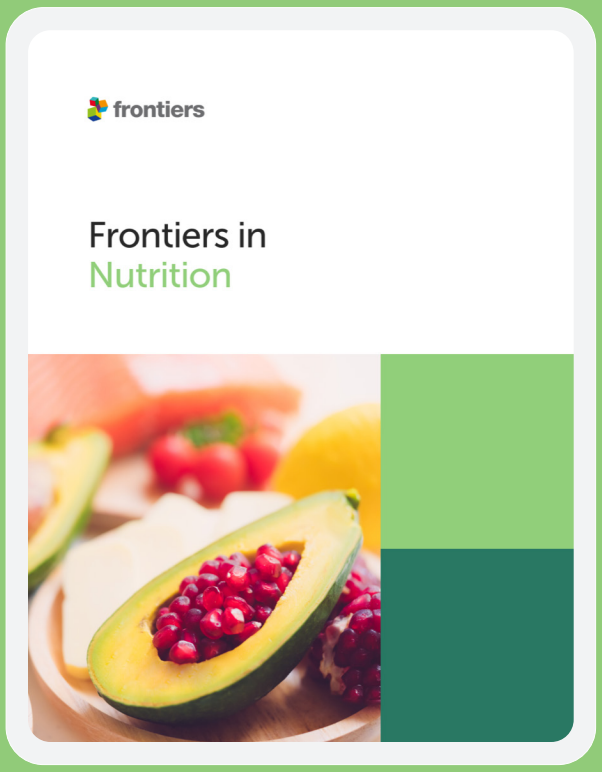

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