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Human milk phospholipid substitutes improve mice's cognitive behavior and brain lipid profile

Teng Wei[a,b], Yanping Wu[a], Jiaxin Shen[a], Manman Zhang[a], Yong Sun[a,c], Zeyuan Deng[a,c,d], Jing Li[a,c,d,*]

[a] State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang 330047, China
[b] College of Food Science and Engineering, Jiangxi Agricultural University, Nanchang 330045, China
[c] International Institute of Food Innovation, Nanchang University, Nanchang 330031, China
[d] National Center of Technology Innovation for Dairy, Hohhot 010110, China

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ABSTRACT

Although human milk fat substitute (HMFS) has been wildly applied in infant formula (IF), there are still some differences in fat composition between human milk and IF, which is extremely attributed to the phospholipid composition. We therefore designed a human milk phospholipid substitute (HMPS), and investigated its nutritional outcomes on brain development using C57BL/6J mice. Results showed that HMPS improved mice's cognitive behavior and dendritic development compared to soybean phospholipids and no-phospholipid diets. Based on the lipidomics, we revealed that these beneficial outcomes were potentially associated with the increased biosynthesis of *N*-acylethanolamines, phosphatidylethanolamine (PE), and ether PE, and the decreased metabolism of diacylglycerol and hexosylceramide in brain. It was further found in Kyoto Encyclopedia of Genes and Genomes analysis that glycerophospholipid metabolic pathway participated in the improvement of HMPS on mice's neurodevelopment. In conclusion, this study demonstrated that HMPS improves mice's neurodevelopment, and the glycerophospholipid metabolic pathway plays a major role in this beneficial result, and provided evidence for future application of HMPS in commercial IF.

1. Introduction

Human milk is recommended as the optimal food for infants, but breastfeeding is not always available for a variety of reasons[1]. In several cases, commercial infant formulas (IF) are the best substitutes to meet the nutritional needs of infants. Researchers have tried to replicate human breast milk by developing various products to meet the needs of normal infant growth. Currently, although 1,3-dioleoyl-2-palmitoyl-glycerol (OPO) has been legally supplemented in Chinese infant formulas to mimic the fatty acid composition and positional distribution of breast milk fat, especially to replicate the final nutritional outcomes of human milk fat on infants, there is still a significant difference in fat composition between infant formula and human milk fat, which is reflected in phospholipid composition. Indeed, although phospholipids account for less than 2% of human milk fat, they play a key role in regulating lipid digestion and absorption and promoting the neurodevelopment of infants[2-4]. A certain amount of phospholipid (commonly from soybean lecithin) has therefore been added to the current IF[3]. As the composition of milk fat globule membrane (MFGM) is revealed, scholars have recognized that vegetable phospholipid is not a perfect substitute for human milk phospholipid due to its failure to simulate the phospholipid composition of MFGM, especially vegetable phospholipid barely providing sphingomyelin (SM). Indeed, MFGM is a component of a unique biological assembly. MFGM is composed of not only glycerophospholipids but also sphingolipids, which are mostly in the form of SM[5] and contribute to the gap in phospholipid composition between vegetables and animals. Hence,

* Corresponding author at: State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang 330047, China.
E-mail address: lijing66@ncu.edu.cn (J. Li)
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MFGM fractions rich in SM extracted from cow milk have been commercially available and added to some IF on the market. However, the SM level in IF is still lower than that in human milk fat. Our group therefore designs a human milk phospholipid substitute (HMPS) according to the human milk phospholipid composition using purified milk phospholipid concentrate[6-8].

In recent years, the number of reports describing MFGM's metabolic effects has increased dramatically. The reported benefits include impacts on lipid metabolism, gut microbiota, and neurodevelopment[5,9]. The present study therefore investigates the effects of HMPS on mice's cognitive behavior, which is very important for infants at an early stage of life, and attempts to clarify how HMPS affects the neurodevelopment of mice using the lipidomics platform. We hope to provide evidence for the final application of HMPS in the future.

2. Materials and methods

2.1 Chemicals and reagents

SM-rich milk phospholipid concentrate (SMPL) was prepared from milk phospholipid concentrate (Ausnutria, Changsha, China). Soybean phospholipids (SPL) and sunflower seed phospholipids (SSPL) were purchased from Anqing Zhongchuang Engineering Technology Co., Ltd. (Anqing, China). Acetonitrile and isopropanol (Honeywell, Muskegon, MI, USA), *N*,*N*-dimethylformamide, isopropanol, acetone, ethanol, and ethyl ether (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) were of chromatographic reagent grade, and other solvents were analytical reagent grade (Xilong Chemical Co., Ltd., Shanghai, China).

2.2 Preparation of phospholipids

2.2.1 SPL and SSPL preparation

SPL and SSPL were purified according to their insolubility in acetone[10]. In brief, crude SPL and SSPL were mixed with cold acetone at 1:3 (*m*/*V*), then agitated on a magnetic stirrer (DF101B, Jintan Jingke Essence Technology Co., Ltd., Changzhou, China) for 30 min. After centrifugation, the precipitate was collected, and the above steps were repeated until no neutral lipid remained using TLC analysis. The precipitate was dried to produce SPL and SSPL in a vacuum oven at 60 ℃.

2.2.2 SMPL preparation

SMPL was prepared as follows: cow milk phospholipid (MPL) concentrate was dispersed in ethanol at 1:5 (*m*/*V*), then agitated on a magnetic stirrer for 2 h and stood overnight. The ethanol phase was dried using a rotary evaporator (RE201D, Shanghai Precision Instrumentation Co., Shanghai, China) to prepare crude milk phospholipid. Crude milk phospholipid was further treated as described in Section 2.2.1 to obtain purified MPL. Subsequently, MPL was mixed with ethyl ether at 1:5 (*m*/*V*) and stood at 30 ℃ for 30 min to separate SM and glycerophospholipids. After centrifugation, the precipitate was collected, and the ethyl ether extraction step was repeated until no glycerophospholipids remained using TLC analysis. The precipitate was dried to produce the SMPL in a vacuum oven at 60 ℃.

2.2.3 Head space gas chromatography

2.2.3.1 Chromatographic conditions

GB 1886.358–2022 provides standards for phospholipids as food additives. Therefore, we analyzed the residual solvents of SPL and HMPS, such as acetone, ethanol, and ethyl ether, using an Agilent 6890N equipped with an DB-624 capillary column (30.00 m × 0.25 mm, 1.4 μm). The initial column temperature was 30 ℃ and held for 1 min, then increased to 60 ℃ at a rate of 5 ℃/min and held for 2 min, and finally programmed at 40 ℃/min to 220 ℃ and held for 2 min. The temperature of the flame ionization detector (FID) was 250 ℃, inlet temperature was 200 ℃. The carrier gas was high-purity nitrogen at a 2 mL/min constant flow rate and a 10:1 split ratio[11].

Headspace injection conditions: incubation at 85 ℃ for 30 min, injection needle temperature of 85 ℃, and injection volume of 1 mL.

2.2.3.2 Sample preparation

The internal standard solution was prepared by diluting 100 mg of isopropanol to 25 mL with the *N*,*N*-dimethylformamide. This gave an internal standard of approximately 4 mg/mL for isopropanol.

Approximately 100 mg of acetone, ethanol, and ethyl ether were accurately weighted into a 25 mL calibrated flask and diluted to volume with internal standard solution to give a stock solution of approximately 4 mg/mL of each solvent.

Apporximatedly 1 g of SPL and HMPS were accurately weighted into a 20 mL headspace injection bottle and dissolved in 5 mL of internal standard solution by shaking.

2.3 HMPS design and preparation

HMPS was designed according to the human milk phospholipid composition[6-8], which has been described by patent ZL202211288479.4. In brief, we first analyzed the phospholipids and major fatty acid compositions of MPL, SMPL, SPL, and SSPL. Then, the results were uploaded into Lingo 18.0 (Lingo System Inc., USA), which further generated all possible formulas (69 kinds of formulas) that simulate the human milk phospholipid profile. The similarity of all possible formulas to human milk phospholipids was assessed in Matlab 16.0 (MathWorks Inc., Massachusetts, USA) using Mahalanobis distance. The HMPS was prepared by mixing MPL, SMPL, SPL, and SSPL according to the best formula.

2.4 Animal and experimental protocol

A total of 36 male C57BL/6J mice (3 weeks old) were provided by Junke Biological Co., Ltd. (Nanjing, China). The mice were housed individually at the Laboratory Animal Facility in the Nanchang University of China in metal cages under a 12-h light/dark cycle, a constant temperature of (24 ± 1) ℃, and a relative humidity of (65 ± 15)% with free access to water and food for 7 days. All

aspects of the experiment were conducted according to guidelines provided by the ethical committee of experimental animal care at Nanchang University of China (Permit Number: 86 SYXK (GAN) 2015-0001). The mice were randomly assigned to the following 3 groups and were fed with different diets for 4 weeks: the control group, the soybean phospholipid-feeding (SPL) group, and the HMPS group. The mice in the control group received only corn oil, while the mice in the SPL and HMPS groups received 98.8% corn oil combined with 1.2% phospholipids. The diets were modified according to the recommendation of the AIN-93G, as shown in Table S1. After the 4-week feeding trial, the mice were transferred to the behavior test room for 1 day of adaptation before the Morris water maze test. After behavioral tests, mice were killed, and then the brain samples were collected by snap-frozen liquid nitrogen and stored at –80 °C for lipidomics analysis.

2.5 Morris water maze (MWM) test

The MWM test was commonly used to measure the spatial learning and memory of mice as described previously[12]. The apparatus consists of a large circular pool, 1.2 m diameter and 40 cm high, containing water at around 25 °C. The mice received four habituation trainings on day 0. The platform was visible (2 cm above the water surface), and the water was un-dyed. The water was then made opaque by adding white dye (food-grade titanium dioxide) that helped to hide the submerged platform (days 1–6). Test trials were conducted for 5 consecutive days (days 1–5). On day 6, a probe trial was conducted in which mice were placed in the pool for 60 s without the platform, and the time that was spent in the target quadrant (TQRT) and the latency to the platform (the latency to find the platform) were measured. All the data was recorded automatically using a video tracking system (Samsung 3000 PH).

2.6 Lipid metabolites composition in brain

2.6.1 Tissue lipid extraction

Each 30 mg brain sample was added to 0.4 mL of Milli-Q water and ground with a high-speed tissue grinder (Servicebio, Wuhan) to obtain a homogenate. Then the lipid in the homogenate was extracted with a 3.4 mL two-phase system (CH_2Cl_2:MeOH:H_2O = 1.0:2.0:0.4). After a 2-min vortex extraction, 1 mL of CH_2Cl_2 and 1 mL of H_2O were added for secondary extraction. Then the lower chloroform layer was collected after 15 min of centrifugation at 4 200 r/min and dried under nitrogen. The extracts were redissolved in 600 μL of isopropanol/acetonitrile solution (9:1) before LC-MS was run[13]. Quality control (QC) samples for method validation were prepared by mixing equivalent aliquots of all the samples, respectively.

2.6.2 Lipidomics analysis

The UPLC analysis was performed using an UHPLC (Vanquish, Thermo Fisher Scientific, USA) equipped with a Phenomen Kinetex C_{18} column (2.1 mm × 100 mm, 1.7 μm, Torrance, CA, USA). The UPLC conditions were as follows: the column temperature was 45 °C, and the flow rate was 300 μL/min. Mobile phase A consisted of acetonitrile/water (6:4, *V*/*V*, containing 10 mmol/L ammonium formate), and mobile phase B consisted of acetonitrile/isopropanol (1:9, *V*/*V*) containing 10 mmol/L ammonium formate. The injection volume was 2 μL. For gradient elution, the concentration of mobile phase B was set to 30% for the first 2 min and then increased linearly to 100% at 25 min, after which it was reduced to 30% at 35 min[14].

MS/MS analysis was acquired on Q-Exactive Focus (Thermo Fisher Scientific, San Jose, CA, USA) using electrospray ionization (ESI) in both positive and negative ion modes. The conditions of ion mode are: heater temperature, 300 °C; sheath gas flow rate, 45 arb; aux gas flow rate, 15 arb; sweep gas flow rate, 1 arb; capillary temperature, 350 °C; the spray voltages of positive and negative ion modes were 3.0 and 2.5 kV; S-Lens RF levels were 50% and 60%; the scan range was *m*/*z* 200–1 800 and 250–1 800, respectively. Finally, MS-DIAL 4.60 was used to preprocess the raw data obtained from UHPLC-MS analysis. The main settings were as follows: precursor tolerance, 5×10^{-6}; product tolerance, 5×10^{-6}; product ion threshold, 5%.

2.7 Statistical analysis

Data were expressed as the mean ± standard deviation (SD) and were analyzed with SPSS 22.0 (IBM, Armonk, NY, USA). Figure drawing was achieved using GraphPad Prism 8.0 and Hiplot (https://hiplot.com.cn/home/index.html). Differences among groups were analyzed using a one-way ANOVA. Tukey's procedure was used for post-hoc testing. Differences between groups were considered significant at *P*-values < 0.05. The data matrix for brain lipidomics was imported into SIMCA 14.1 (MKS Umetrics, Malmö, Sweden) for further analysis, including principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). A volcano plot was also drawn using SIMCA under the following criteria: fold change (FC) > 1.2 and < 0.5, $P < 0.05$.

3. Results

3.1 The design of HMPS formula

In this study, Lingo generated a total of 69 possible formulas simulating the human milk phospholipids, shown in Fig. 1. Subsequently, the similarity of these formulas to human milk phospholipids was assessed using Mahalanobis distance, and the formula with the minimum distance was recognized as the HMPS formula that was closest to human milk phospholipid composition. Based on this, F8 was selected to be the HMPS formula and consisted of 15.82% SPL, 1.45% SSPL, 39.17% MPL, and 43.55% SMPL, and contained 26.48% PE, 24.64% PC, 36.19% SM, 6.35% PI, 6.32% PS, and 40.51% palmitic acid (PA), 17.02% stearic acid (SA), 29.19% oleic acid (OA), and 13.26% linoleic acid (LA) (Tables 1 and S2). Meanwhile, SPL, the standard formula, contained 29.45% PE, 35.82% PC, 0% SM, 22.17% PI, 12.57% PS, 24.94% PA, 8.04% SA, 12.29% OA, and 54.77% LA (Table 1).

We further analyzed the solvent residues of HMPS and SPL. Results showed that HMPS contained 0.005 7 mg/g of solvent residues including acetone, ethanol, and ethyl ether, while SPL had 0.003 9 mg/g of acetone (Table 1 and Fig. S1), which met the standard of solvent residues in phospholipids provided by GB 1886.358–2022 (0.05 mg/g).

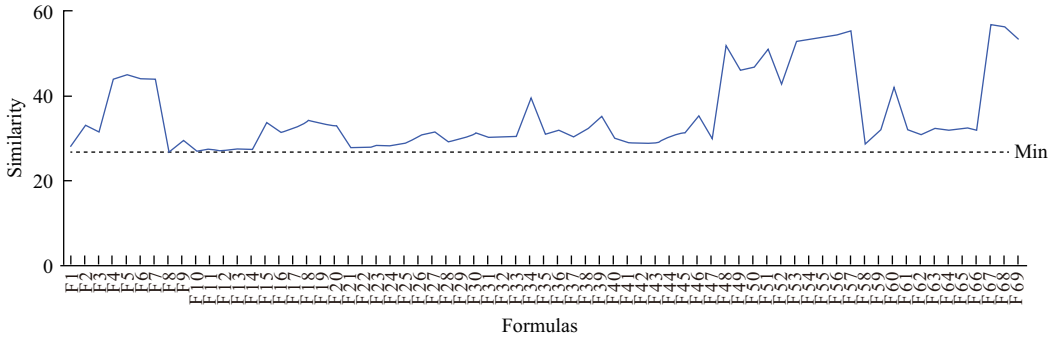

Fig. 1 Similarity of formulas to human milk phospholipids.

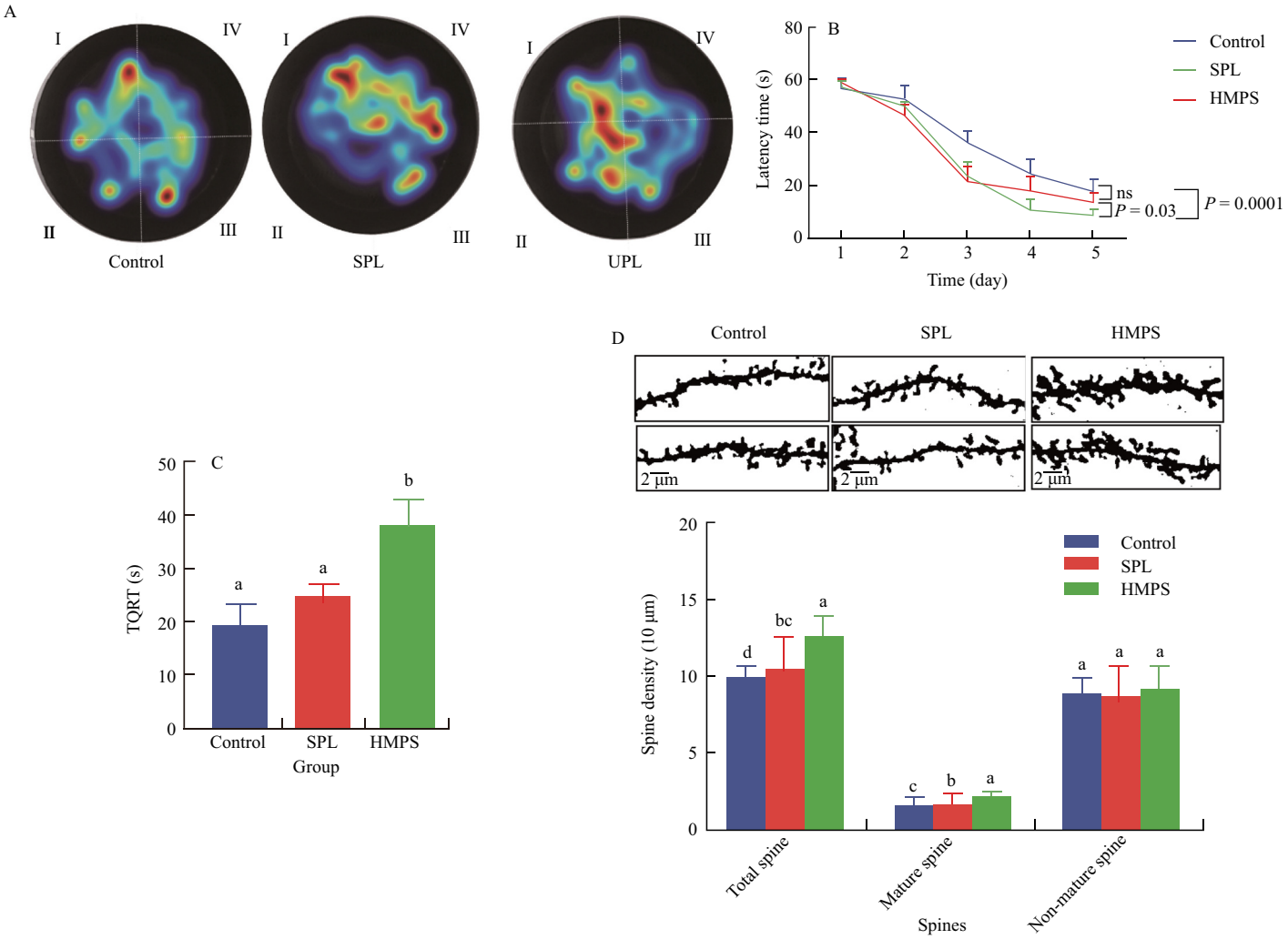

Fig. 2 Cognitive behavior and spines development. (A) Representative track image of mice in a different group in the 6th day of MWM test; (B) the escape latency of mice in the different group over the 5 days of training in the MWM test (n = 8); (C) target quadrant residence time of mice for each group in the MWM test; (D) spines images and number. Data were expressed as mean ± SD. Different letters indicate a significant difference at the $P < 0.05$; ns, no significant difference ($P > 0.05$).

3.2 Effects of HMPS on mice's memory and learningm

Mice's memory and learning were evaluated using the MWM test. On the first day of the MWM test, an insignificant difference in the escape latency among the groups was observed (Figs. 2B and C). With the progress of the test, the escape latency of mice in each group gradually decreased and showed differences (Fig. 2B). After 3 days of training, HMPS feeding significantly lowered the escape latency compared to the control group ($P < 0.05$, Fig. 2B). On the test day, mice in the HMPS group showed significantly higher TQRT as compared to the control and SPL groups (Fig. 2C). Meanwhile, compared with the control group, the trajectories of mice in the HMPS group were mainly concentrated in quadrant III rather than swimming around the wall (Fig. 2A). In short, these data suggested that HMPS improved mice's spatial learning and memory.

Table 1
Relevant researches on the environmental factors of Chinese Baijiu fermentation in recent years.

Components	SPL	HMPS
Phospholipids composition (%)		
PE	29.45 ± 0.83	26.48
PC	35.82 ± 0.58	24.64
SM	nd	36.19
PI	22.17 ± 1.69	6.35
PS	12.57 ± 2.32	6.32
Fatty acid composition (%)		
PA	24.94 ± 2.46	40.51
SA	8.04 ± 0.35	17.02
OA	12.29 ± 0.21	29.19
LA	54.77 ± 2.56	13.26
Solvent residues (mg/g)	0.003 9 ± 0.000 9	0.005 7 ± 0.001 1

Note: nd, not detected; HMPS was generated by mixing 15.82% SPL, 1.45% SSPL, 39.17% MPL, and 43.55% SMPL. The fatty acid composition and phospholipid composition of these phospholipid materials are shown in Table S2.

Phospholipids reportedly participated in the construction of the neuronal cytoskeleton and signal transduction[15] and played a critical role in dendritic spine development and maturation[16]. Spine was the main target of excitatory input, played a crucial role in synaptic plasticity, and was reportedly associated with memory and learning[17]. We therefore analyzed the spine's development here. This study showed that both SPL and HMPS improved the growth of spines, which was evidenced by the significant increase observed in mature spine number (Fig. 2D).

3.3 *Effects of HMPS on mice's brain lipid metabolic profile*

A total of 284 lipid species were identified in the brains of mice in the control, SPL, and HMPS groups. As shown in Fig. 3A, PE, PC, and ether PE were the major lipid classes in the mice brain and accounted for 40% of total lipids. Of which, the relative abundance of PE was increased in mice fed phospholipid diets, and the highest proportion of PE was observed in mice in the HMPS group. On the contrary, PC showed an opposite trend to PE in mice's brains among the 3 groups.

In order to understand how these lipids change with the intervention of HMPS, we conducted a trend clustering analysis of lipids among these mice from the 3 groups. As shown in Fig. 3B, it was found that lipid trends are divided into 4 clusters. Interestingly, cluster 2 and cluster 3 showed an opposite trend in the HMPS group when compared to the control and SPL groups, making them important. These 2 clusters were therefore analyzed and shown in Figs. 4A and B. By comparing the lipid composition in cluster 2 and cluster 3, we found some significant lipids, such as DGs and NAEs. Of which, DGs were decreased in mice in HMPS groups, while NAEs were increased.

Subsequent PCA analysis further showed that there are indeed some differences in lipid profiles among these 3 groups, as depicted in Fig. 4C.

3.4 *Differentially regulated lipids in mice in HMPS group*

To identify the differentially regulated lipids associated with the intervention of HMPS, we generated OPLS-DA models of HMPS *vs*. control, HMPS *vs*. SPL, and SPL *vs*. control (Figs. 5A, C and E). Their permutation tests were shown in Figs. S2A-C, respectively. Volcano plots were further used to visualize the differentially regulated lipids obtained from OPLS-DA models (Figs. 5B, D and F).

As illustrated in a volcano plot based on the HMPS *vs*. control model, 23 decreased and 36 increased lipids were identified using the following criterion: $P < 0.05$ and FC ≥ 1.2 and ≤ 0.5 (Fig. 5B). Under the same criterion, it was observed in the HMPS *vs*. SPL model that 49 lipids were decreased in the HMPS group while 86 lipids were increased (Fig. 5D). Moreover, as illustrated in the third volcano plot, SPL significantly increased 17 lipids and decreased 8 lipids when compared with the control group ($P < 0.05$, and FC ≥ 1.2 and ≤ 0.5) (Fig. 5F).

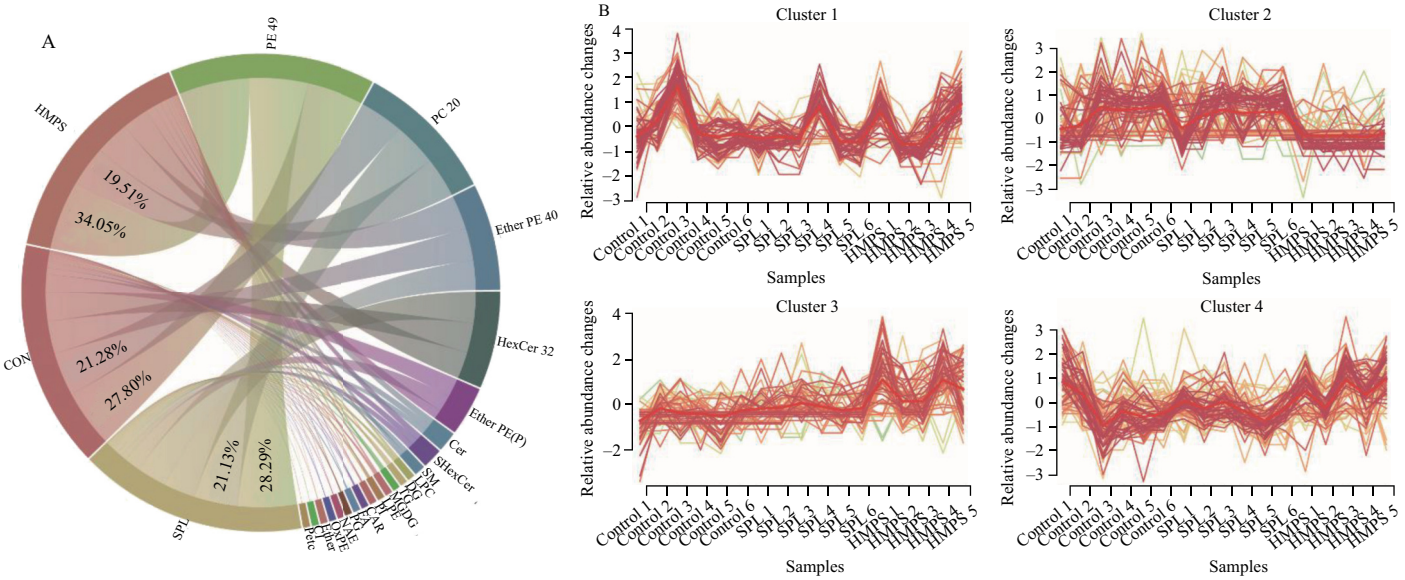

Fig. 3 Lipidomics of mice's brain. (A) Lipid profile in mice's brain from 3 groups; (B) trend clustering analysis of lipid profile.

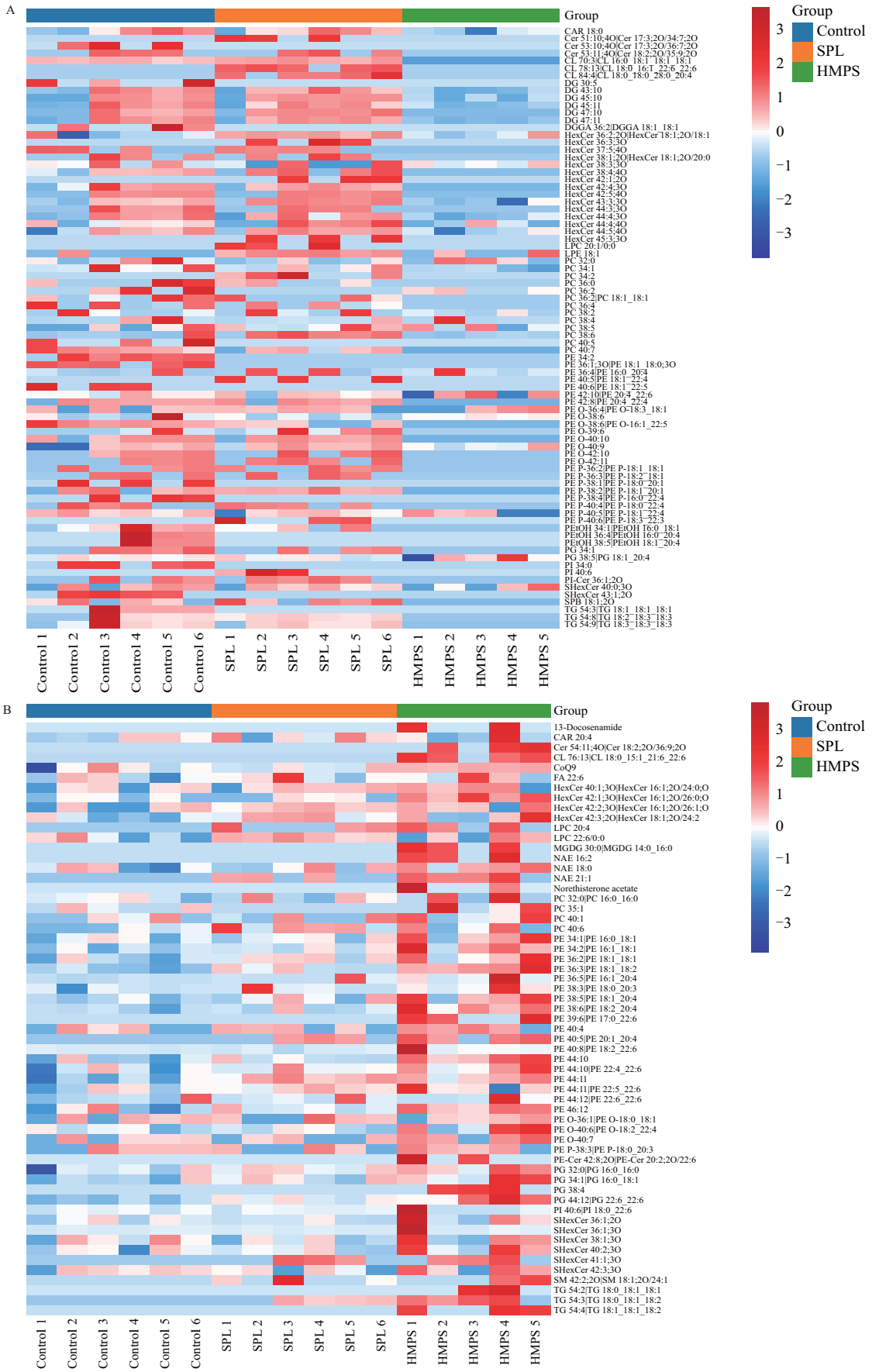

Fig. 4 Lipid profile of mice's brain. (A) Heatmap for relative abundance of lipids in cluster 2; (B) heatmap for relative abundance of lipids in cluster 3; (C) PCA analysis of lipid profile.

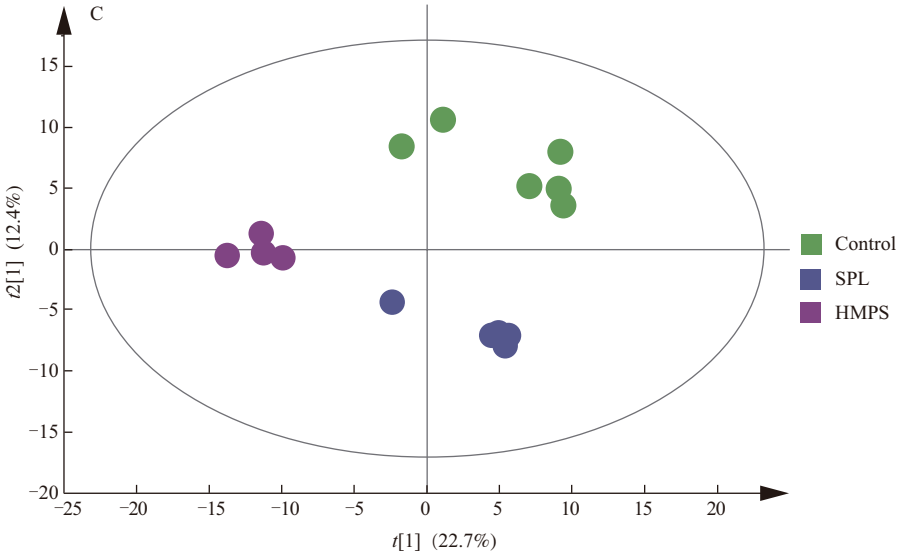

Fig. 4 (*Continued*)

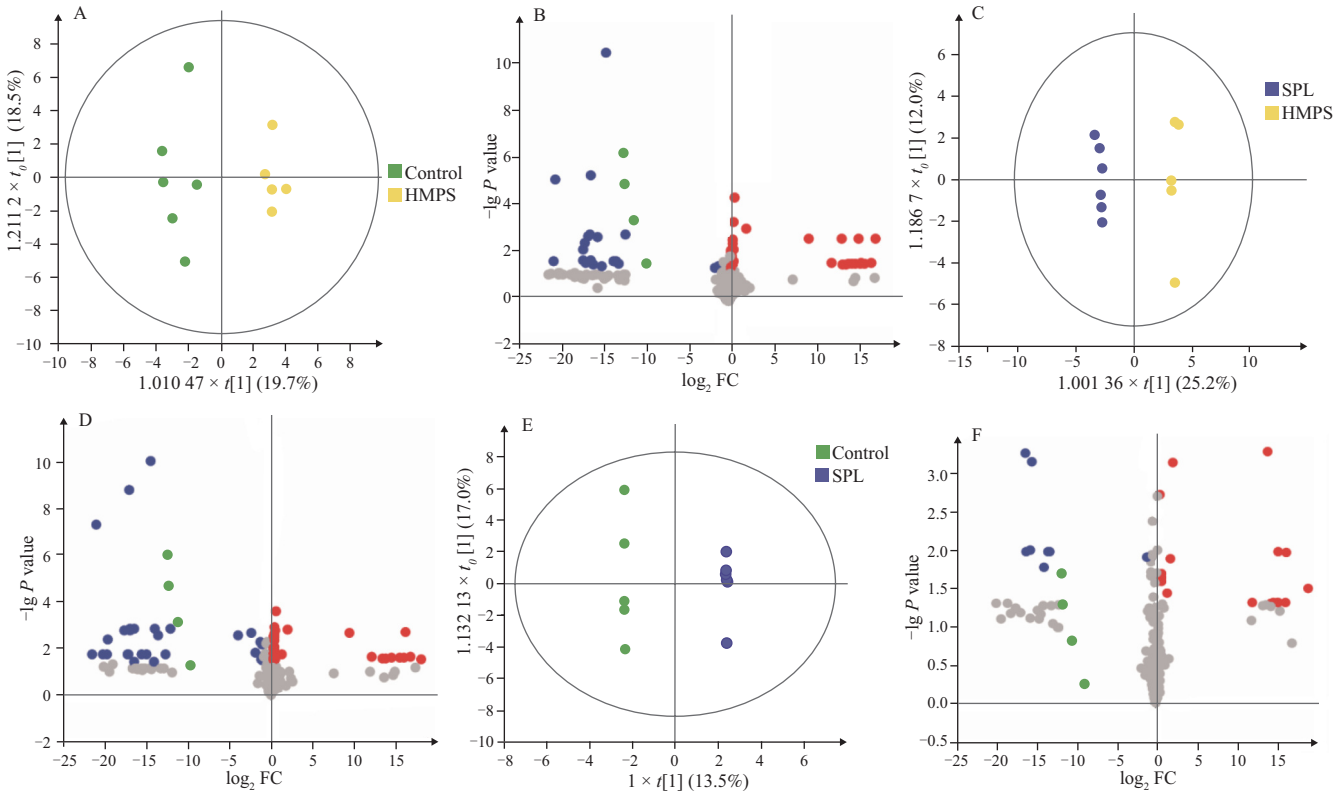

Fig. 5 Multivariate statistical analysis of brain lipids among 4 groups. (A, C and E) OPLS-DA models for control *vs*. HMPS, SPL *vs*. HMPS and control *vs*. SPL; (B, D and F) volcano plot of brain lipid profile ($P < 0.05$, FC ≥ 1.2 and ≤ 0.5): control *vs*. HMPS, SPL *vs*. HMPS and control *vs*. SPL.

Differentially regulated lipid species were selected as potential lipid biomarkers based on more strict criteria (variable important for the projection (VIP) > 1, FC ≥ 1.2, and ≤ 0.5). HMPS significantly elevated the PE and ether PE levels compared with both the control and SPL groups (Figs. 6A and B). In addition, several lipids, such as PE 40:7, PE 38:6, PE 38:5, and PE O-18:1_22:6, were both identified as potential biomarkers in the 2 models and were elevated in the HMPS group. Moreover, it was found that dietary HMPS decreased the biosynthesis of HexCers and DGs when compared with both the control and SPL groups (Figs. 6A and B). However, similar results were not found in the control *vs*. SPL model (Fig. 6C).

3.5 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis

The obtained differentially regulated lipids were introduced in MetaboAnalyst 5.0 for pathway analysis (Fig. 7). The results were shown in the form of a bubble chart, and each bubble represented a metabolic pathway. The abscissa value and bubble size represented the degree of impact in topology analysis, and the larger the abscissa value and bubble size, the higher the enrichment degree. The ordinate value and bubble color represented the *P*-value of enrichment

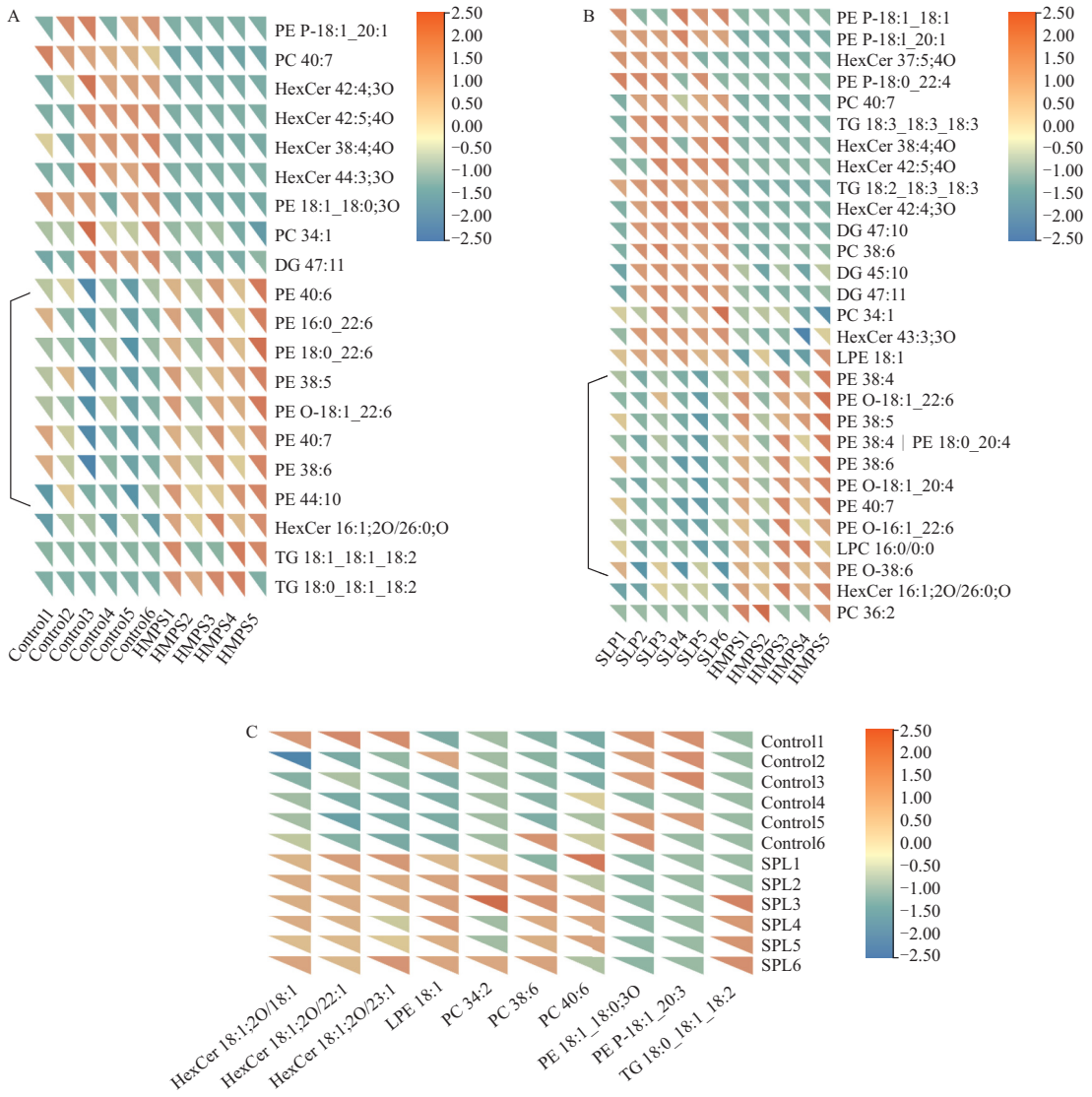

Fig. 6 Heatmap of brain differentially regulated lipids (VIP > 1, $P < 0.05$, FC > 1.2 and < 0.5). (A) Control *vs*. HMPS; (B) SPL *vs*. HMPS; (C) Control *vs*. SPL.

analysis, and the darker the bubble color, the smaller the *P*-value, the more significant the degree of enrichment. Interestingly, distinct results were documented in the HMPS *vs*. control and HMPS *vs*. SPL models. In HMPS *vs*. control, it was observed that glycerophospholipid metabolism and *α*-linolenic acid (ALA) metabolism were significantly altered, the former of which performed a higher value of impact, indicating that glycerophospholipid metabolism is the most significant channel in pathway analysis (Fig. 7A). Two compounds, C00350 (PE, ether PE) and C00157 (PC), were involved in the glycerophospholipid metabolism (Fig. 7B). Interestingly, glycerophospholipid metabolism was not important in the HMPS *vs*. SPL model due to its lower lg *P*, despite the fact that its impact value was the highest. The glycerolipid metabolism and glycosylphosphatidylinositol (GPI)-anchor biosynthesis were found to be the significant metabolic pathways in the HMPS *vs*. SPL model (Fig. 7C), of which glycerolipid metabolism has the highest lg *P*. In addition, TAG (C00422) was found in the glycerolipid metabolism (Fig. 7D).

4. Discussion

Phospholipid is an important component in human milk fat, whereas there is a substantial gap in phospholipid composition between human milk fat and current IF. This study therefore designed a HMPS that mimics the phospholipid composition of human milk fat and investigated its nutritional outcomes on cognitive behavior using C57BL/6J mice. This study showed that the HMPS diet indeed improved mice's neurodevelopment.

The MWM test is commonly used to test the spatial memory and learning of mice. In this study, HMPS significantly improved the escape latency during the training phase, which was recorded as a significant lower escape latency observed in the HMPS group. Moreover, at the testing stage, mice in the HMPS group showed a significantly higher TQRT compared with those in the control and SPL groups. Taken together, HMPS significantly improved the spatial memory and learning of mice.

Phospholipid played a critical role in dendritic spine development and maturation[16]. In addition, correlations between spine number and

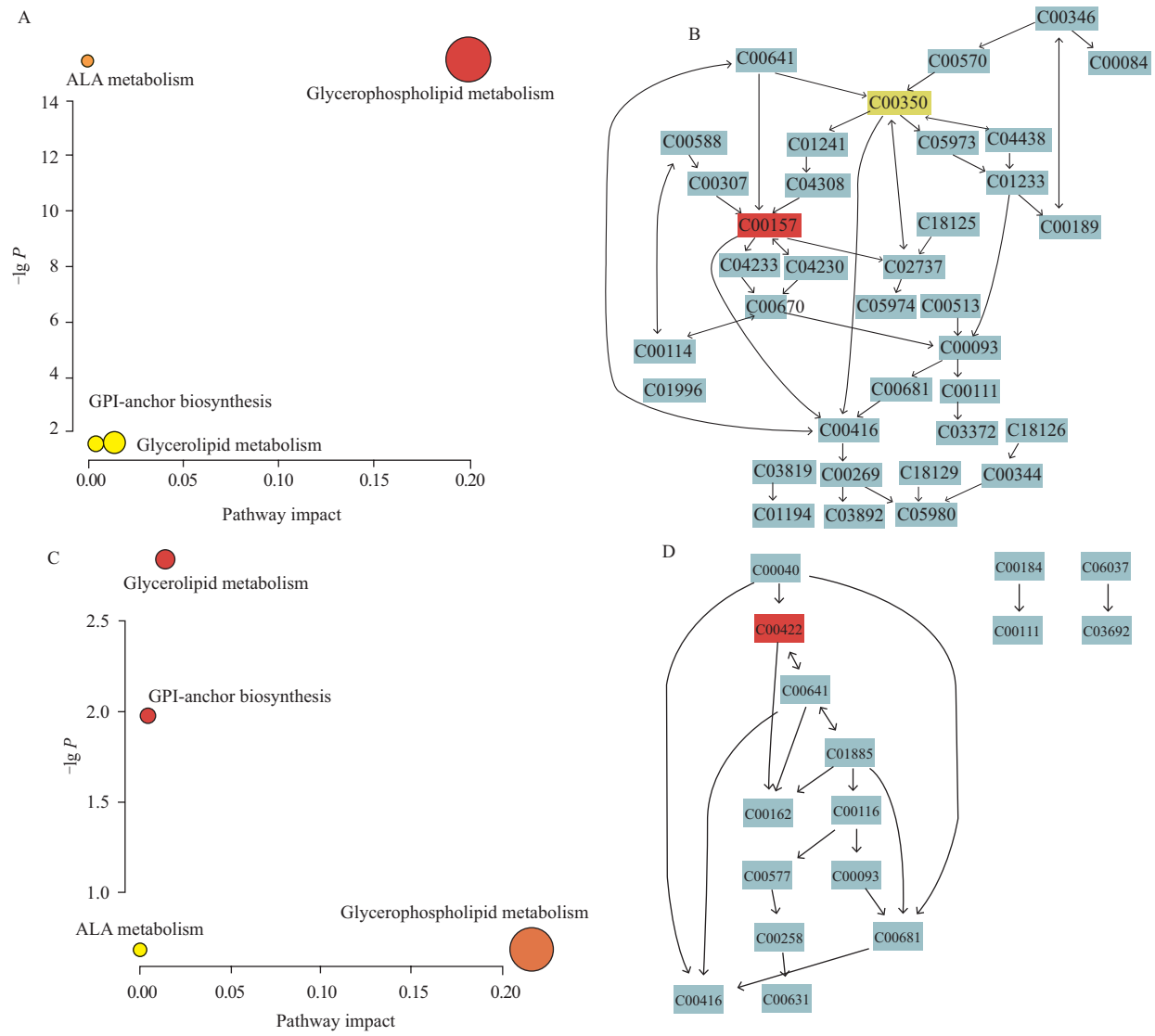

Fig. 7 KEGG analysis of differentially regulated lipids. (A) KEGG pathway in control *vs*. HMPS; (B) glycerophospholipid metabolism; (C) KEGG pathway in SPL *vs*. HMPS; (D) glycerolipid metabolism.

memory behavior have been observed in various disease models[18-19]. Lee et al.[19] found that administration of ibrutinib increased the number of spines in the hippocampus in 5XFAD mice and improved the mice's long-term memory. In this study, the results of the dendritic spine image showed that SPL and HMPS significantly increased the number of mature spines while having no significant influence on non-mature spines. Generally, the AMPA receptor was distributed on the heads of mature spines, while the NMDA receptor was located on non-mature spines[20]. This suggested that the improvement of HMPS on mice's learning and memory might be mediated by the AMPA receptor. Moreover, the improvement of diet on spine development has been widely reported. For example, dietary essential precursors of phosphatidylcholine (uridine and choline) increased the number of spines on hippocampal neurons[21]. SPL and HMPS in this study could provide these 2 substances for mice's spine development. In addition, when compared to the SPL group, more spines were observed in the HMPS group.

Overall, these results showed that dietary HMPS was more favorable to neurodevelopment than SPL feeding, which might be attributed to the difference in fatty acid composition and phospholipid composition (Table 1). In terms of fatty acid composition (Table 1), there was a significant difference in LA between SPL and HMPS, and its relative percentage was extremely lower in HMPS (13.26%) than in SPL (54.77%). Interestingly, an increasing body of evidence has proven that decreasing the intake of LA benefits the infant's neurodevelopment[22-24]. In fact, dietary LA influenced brain functions by decreasing the brain docosahexaenoic acid (DHA) concentration (DHA reportedly benefits the formation of spines[21,25]). The biosynthesis of long-chain polyunsaturated fatty acids (LCPUFAs) from LA and ALA in the liver and other tissues involved a series of elongation and desaturation steps. LA and ALA used the same set of enzymes for conversion to ARA and DHA and therefore competed with each other[26]. Therefore, increasing LA in diet inhibited endogenous DHA synthesis and resulted in higher ω6 LCPUFAs in the circulation, which further limited DHA incorporation in the developing brain as circulating ω3 and ω6 LCPUFA competed for uptake by the brain as well[27]. In animal models, lowering the LA supply early in life positively influenced the structural development of the brain and improved neurocognitive function later

in life[28-29]. In addition, changes in phospholipid composition also contributed to regulating mice's brain functions. In this study, HMPS contained 36.19% SM, while there was no SM in SPL (Table 1). The effect of dietary SM on brain functions has been widely discussed[25,30-31]. Tanaka et al.[31] found that dietary SM increased the DHA concentration in the red cell membrane[31]. Reportedly, DHA promoted myelin formation in the central nervous system and brain neuroplasticity[25], which might suggest that dietary SM improved brain functions *via* increasing endogenous DHA biosynthesis. Moreover, different from glycerophospholipid, the digestion of SM in the intestinal tract was slow and incomplete, which suggested that dietary SM might be utilized by the microbiota to improve brain functions *via* the "gut-brain" axis. However, although the benefits of SM on the intestinal environment have been reported[32-34], the evidence on the "gut-brain" axis associated with the influence of dietary SM on neurodevelopment is still limited, and further investigation is warranted in the future. All in all, previous studies showed that both decreased LA and increased SM might improve neurodevelopment by enhancing the biosynthesis of endogenous DHA, which was supported by the elevated DHA-enriched lipids in the HMPS group in the present study (Fig. 4B), including LPC 22:6, PE17:0_22:6, PE 18:2_22:6, PE 22:4_22:6, PE 22:5_22:6, PE 22:6_22:6, PG 22:6_22:6, and PI 18:0_22:6.

To further clarify how dietary HMPS influences the neurodevelopment of mice, we conducted lipidomics analysis on the brain lipid profile using the Q-Exactive platform. Further trend clustering analysis showed that HMPS changed the abundance of lipids belonging to clusters 2 and 3. DGs and NAEs were the 2 important lipid classes observed in cluster 2 and cluster 3, respectively. In fact, DGs have been wildly associated with neurodevelopment. Additionally, it was found that the most unique and consistent observation of the lipidomics studies on Alzheimer's disease is the elevation of DAG levels in the frontal cortex[35]. Generally, DAGs are essential second messengers in the nuclear lipid signaling pathway, with levels being tightly controlled by DAG kinase, which terminates DAG signaling *via* the conversion of DAGs to phosphatidic acids[36]. Our observations of decreased DAGs in the HMPS group suggested that there may be an improvement in the DAG kinase regulation of DAG steady-state levels in mice in the HMPS group.

In addition, it was observed that NAEs (in cluster 3) were elevated in mice in the HMPS group. NAEs are endogenous bioactive lipids consisting of an acyl chain linked by an amide bond to ethanolamine and are the endogenous ligand of the cannabinoid receptors CB1 and CB2, on which they act as a partial agonist. Although the functions of NAE 16:2, NAE 18:0, and NAE 21:1 are not clear (Fig. 4B), the NAE family is generally known to be anti-inflammatory, analgesic, and neuroprotective[37-38]. Taken together, this study showed that elevated NAEs and decreased DGs might contribute to the improvement of HMPS on mice's cognitive behavior.

Based on more strict criteria, we further identified some lipids in the brain that were significantly regulated by HMPS. As shown in Fig. 6, HMPS elevated the PE and ether PE levels in the brain when compared with both the control and SPL groups. Interestingly, PE and ether PE were strongly associated with neuronal development. Previous studies have shown that *n*-3 fatty acid supplementation increased PE levels, neurite outgrowth, neuronal differentiation, and synaptogenesis during neuronal development[39-40], potentially indicating a positive relationship between PE metabolism and dendritic growth. Furthermore, ether PE was a unique type of GP in which the *sn*-1 chain was connected *via* an ether or vinyl ether linkage to the glycerol backbone[41]. In the nervous system, ether lipids are major components of myelin sheaths and play an essential role in modulating neural synaptic vesicle trafficking[42]. Previous studies have evidenced that the presence of ether PE in the neuronal culture medium increased total and matured spines significantly[43]. In conclusion, the enrichment of PE and ether PE observed in the HMPS group might contribute to the improvement of spatial memory and learning in mice.

In addition, we also observed a decrease in HexCer in the HMPS group when compared with both the control and SPL groups. Although it is not clear how endogenously produced HexCer influences the mice's neurodevelopment, HexCer was notorious because of its potential link with neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease, and Parkinson's disease[44-46]. In humans, the brain levels of HexCer are also elevated during brain aging[45,47], which could be reversed by anti-inflammatory means[47].

The KEGG pathway generated from differentially regulated lipids revealed that different pathways were identified in the HMPS *vs*. control model and the HMPS *vs*. SPL model. Compared with the control group, HMPS significantly regulated the glycerophospholipid metabolic channel. In fact, glycerophospholipids consist of neuron membranes and play both structural and functional roles[48]. Moreover, the stage of well-pronounced Alzheimer's disease-like pathology in OXYS rats was also related to abnormalities in glycerophospholipid metabolism[49]. In this pathway, the main transformation between C00350 (PE, ether PE) and C00157 (PC) was shown in Fig. 5B. Phosphatidyl-*N*-methylethanolamine (C01241) and phosphatidyl-*N*-dimethylethanolamine (C04306) were found to be the 2 important intermediates that were involved in the transformation of PE and ether PE to PC, which was catalyzed by the phosphatidylethanolamine-*N*-methyltransferase and functions in regulating neurodevelopment[50].

In contrast, although 3 compounds (C00350 (ether PE), C00157 (PC) and C04230 (LPC)) were observed in the glycerophospholipid metabolism in the SPL *vs*. HMPS model, the $-\lg P$ of glycerophospholipid metabolism was not significant. Glycerolipid metabolism and GPI-anchor biosynthesis were the 2 significant metabolic channels in the SPL *vs*. HMPS model. C00422 (TAG) and C00350 (ether PE) were found in the above 2 pathways, respectively. In general, glycerolipid metabolism is associated with neuronal axon regeneration, which has been reported by Yang et al.[51]. In addition, GPI is used for anchoring many cell surface proteins to the plasma membrane[52], a deficiency of which generally leads to cognitive impairment[53].

Overall, this study showed that the beneficial outcome of HMPS on mice's cognitive behaviors was associated with the improvement of lipid profiles, including elevated PEs, ether PEs, and NAEs, and decreased DGs, and HexCers. Moreover, glycerophospholipid metabolism and glycerolipid metabolism were in response to the models of HMPS *vs*. control and HMPS *vs*. SPL, respectively.

One possible limitation of our study was that the mice involved in the current study were weaned, which might not exactly mimic the growth and metabolism in an infant's body. In fact, we attempted to use unweaned mice in pre-experiments. However, during the adaptive feeding period, mice subjected to neonatal maternal separation exhibited unhealthy outcomes and even mortality. Furthermore, some

studies have employed neonatal maternal separation to establish an irritable bowel syndrome model that has been used to establish a cognitive impairment model[54]. Future work is required to determine how to utilize infant mice while avoiding adverse outcomes.

In conclusion, HMPS is beneficial to mice's neurodevelopment compared with SPL and no-phospholipid diets, which shows a tendency to decrease escape latency, increase TQRT in MWM testing, and promote the growth of mature spines. Lipidomics results reveal that this beneficial outcome of dietary HMPS might be attributed to the enrichment of NAE, PE, and ether PE and the decrease of DG and HexCer in the brain. Further KEGG analysis suggests that glycerophospholipid metabolism and glycerolipid metabolism may play an important role in improving mice's neurodevelopment through the intervention of HMPS. This information supports the application of HMPS in future IFs.

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Declaration of competing interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2024.9250343.

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