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Discrepant Modulating Effects of Dietary Docosahexaenoic Acid on Cerebral Lipids, Fatty Acid Transporter Expression and Soluble Beta-Amyloid Levels in ApoE$^{-/-}$ and C57BL/6J mice

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Key Points:

- The level of cortical TC and LDL-C decreased but the ratio of HDL-C/LDL-C increased in ApoE$^{-/-}$ mice after DHA treatment.
- The expression of cortical *Fabp5* mRNA decreased but *Cd36* and *Scarb1* mRNA increased in ApoE$^{-/-}$ mice following DHA treatment.
- Higher cortical Aβ_{1-42} was shown in ApoE$^{-/-}$ mice but not in C57 wt mice.
- DHA treatment did not affect cortical Aβ_{1-40} levels in ApoE$^{-/-}$ mice but it was significantly increased in C57 wt mice.

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Abstract:

Aims: The study was designed to explore the role of ApoE deficiency concomitant with dietary DHA treatment on brain β-amyloid (Aβ) and lipid levels.

Method: A 5-month dietary DHA intervention was conducted in ApoE-deficient mice ($ApoE^{-/-}$) and wild type C57BL/6J (C57wt) mice. The Morris water maze test was performed to assess the behaviour of the animals. The cortical contents of soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ were detected by ELISA. Cortical fatty acid levels were detected by gas chromatography. Gene and protein expression of molecules associated with cerebral Aβ and lipid metabolism were measured using real-time PCR, Western blot and histological methods.

Results: DHA treatment increased the content of cortical DHA and n-3 PUFAs, but decreased the ratio of n-6/n-3 PUFAs in $ApoE^{-/-}$ mice; while the content of cortical DHA, n-3 PUFAs in C57wt mice remained unchanged after DHA treatment. *Fabp5* and *Cd36* gene expression were significantly down-regulated in DHA-fed C57wt mice; cerebral *Cd36* and *Scarb1* gene expression were significantly up-regulated, while *Fabp5* gene expression was down-regulated in DHA-fed $ApoE^{-/-}$ mice. In comparison with C57wt mice, the content of cortical soluble $A\beta_{1-42}$, TC, and LDL-C increased, while the level of HDL-C decreased in $ApoE^{-/-}$ mice. Interestingly, these differences were significantly reversed by DHA dietary treatment.

Conclusion: DHA intervention has discrepant impacts on cerebral lipids, fatty acid transporter expression and soluble Aβ levels in ApoE-/- and C57wt mice, suggesting the modifying role of ApoE status on the responses of cerebral lipids and Aβ metabolism to DHA treatment.

Keywords: $ApoE^{-/-}$ Mice; DHA; Lipids; Cholesterol; β-Amyloid

1. INTRODUCTION

Approximately 50 million people were diagnosed with dementia globally in 2015. 60-70% of them are Alzheimer's disease (AD), the most common type of dementia. With the population ageing, AD is expected to affect about 135 million people by 2050[1]. However, it is frustrating that there is no definitive approach available for clinical treatment of AD currently. Thus, it is urgent to establish an effective preventive strategy to mitigate the social impact and public health burden of AD.

Previous studies have demonstrated dose-response protective effect(s) of long-chain n-3 polyunsaturated fatty acids (n-3 PUFAs) rich diet on cognitive function[2]. *In vivo* and *in vitro* studies have consistently shown the neuroprotective effect of n-3 PUFAs[3, 4]. However, data from randomized controlled trials have failed to provide convincing evidence of the cognitive-based preventive effect(s) of the n-3 PUFAs supplementation in mild-to-moderate AD patients[5]. Discrepancies in the experimental designs, the populations studied, the dosages of n-3 PUFAs used, as well as the pathophysiological status of participants might be the reasons accounting for these contradictions and inconclusive outcomes. Additionally, the genetic variations of individuals may potentially account for the diversified responses to n-3 PUFAs intervention.

Apolipoprotein E (ApoE) acts as a lipid carrier to mediate lipolysis and lipid endocytosis in peripheral tissues and central nervous systems (CNS). Genetic variations have been found in the ApoE gene, resulting in three different protein isoforms (ApoE *ε2*, ApoE *ε3,* and ApoE *ε4*), and the alteration in ApoE function has also been identified with their specific isoforms[6]. ApoE *ε4* has consistently been demonstrated to be an independent genetic risk factor for AD[7,

[8]. A strong correlation between ApoE *ε4*-associated neuro-inflammation and tauopathy has been reported[9]. ApoE *ε4* is associated with earlier age of dementia diagnosis in human studies[10], and ApoE *ε4* has been implicated in AD pathology through the potential regulatory effect(s) on promoting extracellular Aβ aggregation in the brains of carriers[11]. Indeed, in ApoE *ε4* carriers, impaired blood-brain barrier (BBB) integrity coupled with decreased capacity of lipid-transport apolipoprotein E was observed. Different ApoE isoforms affect brain PUFAs transport and uptake. Compared with ApoE *ε2* and ApoE *ε3* isoforms, the ApoE *ε4* isoform is hyperlipidaemic, which is associated with increased serum lipids and decreased efficiency of docosahexaenoic acid (DHA) transport across the BBB[12, 13]. Current evidence indicates that dietary DHA supplementation can attenuate the effects associated with ApoE *ε4*'s role in the Aβ-related AD pathological process via anti-inflammatory and anti-oxidative mechanisms[14]. Further evidence from the published literature also hints at the modulatory effects of ApoE genotype status on the efficacy outcomes of DHA treatment. This observation may partly be attributed to the fact that ApoE *ε4* is associated with decreased efficiency in brain DHA transport[12, 13]. However, the neuro-specific effect and precise mechanism(s) of ApoE status associated with DHA treatment in AD remain to be clarified.

It is known that the amyloidogenic activity in the CNS is linked to lipids levels and mobilization (especially the cholesterols), and ApoE plays a critical role in lipid metabolism. Nevertheless, it remains unclear how ApoE gene function affects lipids and Aβ levels in the CNS based on DHA treatment. In the current study, we conducted a 5-month dietary DHA intervention in ApoE-deficient ($ApoE^{-/-}$) and wild type C57 BL/6J (C57 wt) mice, aiming to explore the role of ApoE deficiency and the outcome of DHA treatment on brain Aβ

generation and lipid levels. This study may assist in uncovering the underlying mechanism through which DHA exhibits AD preventive effects when the ApoE gene is dysfunctional and potentially bridge the knowledge gap existing between dietary DHA intervention and efficient AD prevention.

2. METHODS & MATERIAL

2.1 Animals

A total of 24 C57 wt (12 males and 12 females) and 24 $ApoE^{-/-}$ mice (12 males and 12 females) aged 12 weeks were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). They were given 2 weeks to adapt to the environment with normal growth and breeding feeds (Beijing Keao Xieli Feed Co., Ltd.). The mice were housed at room temperature with a 12-hour light-dark cycle and were given free access to water and food. This study was approved by the Animal Experiment and Laboratory Animal Welfare Committee of Capital Medical University, Beijing, China (AEEI-2015-040).

2.2 Treatment

Based on the baseline results of behavioural testing with a Morris water maze, the animals were randomly divided into four groups with 12 mice in each group (6 males and 6 females). The mice were fed with a control diet or a diet fortified with DHA for 5 months. The experiments were performed according to the guide for the care and use of laboratory animals (AEEI-2015-040). The composition of experimental diets is shown in Table 1. The interventional feeds were purchased from Changzhou SYSE Bio-Tec. Co., Ltd. (Changzhou, China). The supplemented DHA was obtained through fish oil powder, which was provided by Royal DSM Company (DSM Nutritional Products, Ltd., product code: 50-1526-9, Heerlen,

Holland). The ingredients of the fish oil powder are shown in supporting information 1.

2.3 Behavioural Testing

After 5 months of dietary intervention, the Morris water maze tests[15] were performed to assess whether ApoE status and diets had effects on the learning and cognitive function of mice. The escape latency time was recorded, and the average swim speed was determined by using a computerized tracking system (Water Maze 2.6 Institute of Materia, Chinese Academy of Medical Sciences DMS-2, Beijing, China).

2.4 Tissue Preparation

Following behavioural testing, all mice were euthanatized and sacrificed. Blood samples were taken through the neck artery, and they were collected in an anticoagulant vacutainer containing lithium heparin. After centrifuging at 1500 rpm for 10 minutes, the plasma at the upper layer was separated and then stored at -80℃ for biochemical analysis and testing. The brains were removed and separated along the middle sagittal sulcus. Half of the left-brain hemisphere was put in 4% paraformaldehyde and used for immunohistochemistry. The remaining half was immediately placed in a cold saline solution and then the cortical and hippocampal regions were dissected. The separated cortex and hippocampus were stored at -80 ℃ for real-time PCR, western blotting, and biochemical testing.

2.5 Biochemical Analysis

Plasma parameters, including total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) levels and cortical lipids levels were measured using the assay kits from NanJingJianCheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instruction. Briefly,

cortical tissues were weighed accurately, and then 0.9% sodium chloride solution was added [weight (g): volume (mL), 1: 9], then homogenized mechanically in an ice water bath, and centrifuged at 2500 rpm for 10 minutes. After that, the supernatant was collected for biochemical measurement.

The enzyme-linked immunosorbent assay (ELISA) method was applied for detecting the content of cortical soluble $A\beta_{1-40}$ and $A\beta_{1-42}$. Briefly, the cortex was homogenized in RIPA buffer (250 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris HCl, pH = 8.0) with protease inhibitors, and the homogenates were then centrifuged for 20 minutes at 100,000×g, 4℃ to get the supernatant for soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ measurement according to the manufacturer's instruction. Protein concentrations were determined by bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA).

2.6 Measurement of the Content of Cortical Fatty Acids

The content of cortical fatty acids was determined according to the method described previously[16]. Briefly, total lipid was extracted from brain homogenates by methanol and chloroform (2:1, v/v). Samples were centrifuged at 3000 rpm for 10 minutes and the lower phase (chloroform and lipids) was removed. Chloroform was added to the upper phase, the samples were centrifuged again at 3000 rpm for 10 minutes and the lower phase was combined with the initial one. The chloroform fractions were dried, and the fatty acids were derivatized to their methyl ester with methanol sodium hydroxide (NaOH) and boron trifluoride. The collected fatty acid methyl ester was dissolved in isooctane and analysed by gas chromatography (Shimadzu GC-2010, Japan) with a flame ionization detector. Separations were performed using a capillary column (SP2560, Supelco, Bellefonte, PA,

USA) according to Connor's description[17]. Fatty acid compositions of 6 brains per group were reported, and the fatty acid content was expressed as g/100 g of total fatty acids.

2.7 Histological measurement

Briefly, deparaffinized and hydrated tissue sections were incubated in 3% H_2O_2 for 10 minutes. After washing with distilled water, epitope retrieval was performed via digesting with gastric enzymes for 20 minutes at 37°C. The sections were then washed with phosphate-buffered saline (PBS) and incubated in 10% goat serum for 15 minutes for reducing non-specific binding. After incubating with anti-ATP-binding cassette transporter A1 (ABCA1, 1:100 dilution, Abcam, Cambridge, UK), anti-liver X receptor α and β (LXRα/β, 1:100 dilution, Abcam, Cambridge, UK), anti-angiotensin-converting enzyme 1 (ACE1, 1:100 dilution, Servicebio, Wuhan, China), anti-angiotensin-converting enzyme 2 (ACE2, 1:100 dilution, Servicebio, Wuhan, China) primary antibody, the tissue sections were washed in PBS and incubated with horseradish peroxidase-conjugated secondary antibody (Promega, Madison, WI, USA) for 15 minutes at 25 ℃. Finally, after washing in PBS, the sections were dehydrated in an alcohol gradient and mounted using neutral gums for scanning.

2.8 Real-Time PCR

RNA was isolated using TRIzol reagent (Invitrogen, USA) as per the manufacturer's instructions. The quality of RNA was assessed by using a microplate reader (Epoch, BioTek, USA). Three micrograms of RNA were reverse transcribed using a reverse transcription system kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Real-time PCR was performed in a 20 μl reaction system containing 10 μl SYBR Green Mix (SYBR Green, Kapa Biosystems, USA), 1 μl of cDNA, 8.2 μl ddH_2O, and 0.4 μl 500 nM of

each specific primer. The cycling parameters were as follows: 95 ℃, 30 s; 95 ℃, 30 s and 62 ℃, 30 s; 65 ℃ and 95 s. The primers used for PCR were as follows: glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), forward: 5'GGTTGTCTCCTGCGACTTCA3', reverse: 5'GCTCTTCCATTTCCGAGTCACTG3'; fatty acid-binding protein 5 (*Fabp5*), forward: 5'TTACCCTCGACAACAACAACC3', reverse: 5'CTTCCCGTCCTTCAGTTTTCT3'; cluster of differentiation 36 (*Cd36*), forward: 5'GCGACATGATTAATGGCACA3', reverse: 5'CGTTGGCTGGAAGAACAAAT3'; scavenger receptor, class B type 1 (*Scarb1*), forward: 5'GTTCGTTGGGATGAACGACT3', reverse: 5'ATTCGGGTGTCATGAATGGT3'; amyloid precursor protein (*App*), forward: 5'TGAATGTGCAGAATGGAAAGTG3', reverse: 5'AACTAGGCAACGGTAAGG AATC3'; insulin degrading enzyme (*Ide*), forward: 5'GCCAGATTATCTCGGAGCCTTGC3', reverse: 5'ACGCCTCCTCTGTCATGTCCTC3'; LDL Receptor Related Protein 1 (*Lrp1*), forward: 5'GCGACGGCAAGGATGACTGTG3', reverse: 5'CGTGGCGGTTGGAGAAGATGATG3'; sortilin-related receptor, LDLR class A repeats-containing (*Sorl1*), forward: 5'GCAGCAACGGCAACTGTATCAAC3', reverse: 5' CGCTACGACACTGGTGCATCTC3'.

2.9 Western Blotting

Tissue samples were homogenized in chilled TBS supplemented with protease and phosphatase inhibitor cocktail. The protein concentration of the homogenate was determined by the BCA protein assay. A total of 30 μg of protein lysis extracts were resolved on polyacrylamide gels (Bio-Rad, USA) and transferred to nitrocellulose membranes. Membranes were blocked in Tris-buffered saline (TBS), 0.1% Tween 20, and 5% non-fat dry

milk for 1 hour, followed by overnight incubation with primary antibody. After washing with 0.1% Tween in TBS, the membrane was incubated with a peroxidase-conjugated secondary antibody for 1 hour. The following primary antibodies were used: rabbit monoclonal anti-β-actin (1:1000), anti-3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMGCR, 1:5000), anti-acetyl coenzyme A acetyltransferase 1 (ACAT1, 1:1000), anti-fatty acid binding protein 5 (FABP5, 1:1000), rabbit polyclonal anti-cluster of differentiation 36 (CD36, 1:1000), anti-scavenging receptor B1 (SRB1, 1:1000), anti-beta-secretase 1 (BACE1, 1:1000), anti-amyloid precursor protein (APP, 1:10000), anti-insulin degrading enzyme (IDE, 1:5000), anti-low-density lipoprotein receptor-related protein 1 (LRP1, 1:20000), and anti-sortilin-related receptor, LDLR class A repeats-containing (SorLA, 1:1000). Goat anti-rabbit IgG secondary antibody horseradish peroxidase (HRP) conjugated (1:5000) was used for western blotting. Protein bands were visualized by chemiluminescence (ECL western blotting substrate, Thermo Scientific, USA) using a FUSION-FX imaging system (Vilber Lourmat, France). For quantitative analysis, bands from the raw 16-bit TIFF images were integrated using FusionCapt software (Vilber Lourmat, France).

2.10 Statistical Analysis

Data are expressed as means and standard deviations (mean ± SD) or means and standard error of the mean (mean ± SEM) and analysed with SPSS software (SPSS Inc., Chicago, IL, USA). Comparisons between different groups were determined using one-way analysis of variance (ANOVA) followed by post-hoc multiple comparison tests. Statistical significance was set at $P < 0.05$.

3. RESULTS

3.1 Dietary Consumption and Body Weight

The daily food consumption and changes in body weight of experimental animals are shown in Figure 1. There was no significant difference in the amount of daily food consumption between mice with different ApoE statuses. DHA treatment did not affect daily food intake in both C57 wt and ApoE$^{-/-}$ mice (Figure 1A). ApoE status and DHA treatment had no impact on body weight. C57 wt and ApoE$^{-/-}$ mice from both control and DHA-fortified diet groups displayed similar body weights at the end of the experimental period (Figure 1B).

3.2 Behaviour Performance

As indicated in Table 2, after five days of training, a significant decrease in escape latency time was found in both C57 wt and ApoE$^{-/-}$ mice between Day 1 and Day 6 ($P < 0.05$). On the testing day (Day 6), the control diet-fed ApoE$^{-/-}$ mice displayed a shorter escape latency time than C57 wt mice ($P < 0.05$), but the difference was not found in DHA-treated mice ($P > 0.05$). According to the escape latency time on the testing day (Day 6), DHA treatment showed no effect on improving learning and memory ability in C57 wt and ApoE$^{-/-}$ mice ($P > 0.05$).

3.3 Plasma Biochemical Parameters

As shown in Table 3, the depletion of ApoE in the mice caused a dramatic increase in the level of plasma TC, TG, HDL-C, and LDL-C as compared with C57 wt mice ($P < 0.05$). DHA treatment had no effect on plasma lipid levels in C57 wt mice ($P > 0.05$), but higher plasma TG levels were found in DHA-fed ApoE$^{-/-}$ mice than in control diet-fed ApoE$^{-/-}$ mice ($P < 0.05$), while, for other lipid parameters, no significant difference was found between the

two groups ($P > 0.05$).

3.4 Cortical Lipids and Expression of Lipid Metabolism Molecules

As shown in Figure 2A, in comparison with C57 wt mice, ApoE$^{-/-}$ mice exhibited an elevated cortical TC level ($P < 0.05$). Compared to the control diet-fed animals, the level of cortical TC decreased significantly in both DHA-treated C57 wt and ApoE$^{-/-}$ mice ($P < 0.05$). But DHA treatment caused a greater decrease in cortical TC level in C57 wt mice than that in ApoE$^{-/-}$ mice ($P < 0.05$).

ApoE$^{-/-}$ mice displayed a lower level of cortical HDL-C, but higher LDL-C than C57 wt mice ($P < 0.05$). DHA intervention remarkably increased the level of cortical HDL-C in the C57 wt mice ($P < 0.05$), but did not affect that in ApoE$^{-/-}$ mice ($P > 0.05$). The level of cortical LDL-C was increased significantly in DHA-treated C57 wt mice ($P < 0.05$), but showed a decreased trend in DHA-treated ApoE$^{-/-}$ mice.

The ratio of cortical HDL-C/LDL-C was significantly lower in ApoE$^{-/-}$ mice than in C57 wt mice ($P < 0.05$). While this ratio was significantly increased in both C57 wt and ApoE$^{-/-}$ mice after DHA treatment ($P < 0.05$). There was no difference in the level of cortical TG and ApoB (apolipoprotein B) between ApoE$^{-/-}$ mice and C57 wt mice fed with control diet ($P > 0.05$). After DHA treatment, the level of cortical TG was elevated significantly in C57 wt mice ($P < 0.05$), but it showed a decreased trend in ApoE$^{-/-}$ mice even though there was no statistical difference ($P > 0.05$). DHA treatment did not affect the level of cortical ApoB in both C57 wt and ApoE$^{-/-}$ mice, although there was a slightly increasing trend in ApoE$^{-/-}$ mice ($P > 0.05$).

As shown in Figure 2B, in comparison with C57 wt mice, ApoE$^{-/-}$ mice showed slightly higher expression of cortical HMGCR protein, but no significant difference was observed.

The DHA treatment had no effect on the expression of cortical HMGCR protein in both C57 wt and ApoE$^{-/-}$ mice. There was no significant difference in cortical ACAT1 protein expression between C57 wt and ApoE$^{-/-}$ mice, and its expression was not affected by DHA treatment.

In both cortex and hippocampus, ABCA1 protein expression in ApoE$^{-/-}$ mice was significantly higher than in C57 wt mice ($P < 0.05$). Due to DHA treatment, cortical and hippocampal ABCA1 protein expression was up-regulated in C57 wt mice, but down-regulated in ApoE$^{-/-}$ mice ($P < 0.05$) (Figure 2C). As shown in Figure 2D, lower expression of cortical and hippocampal LXRα/β protein was found in ApoE$^{-/-}$ mice compared to that in C57 wt mice ($P < 0.05$). Its expression in both cortex and hippocampus was reduced after DHA treatment in C57 wt mice ($P < 0.05$), especially in the male mice; but increased in DHA-fed ApoE$^{-/-}$ mice ($P < 0.05$).

Altogether, our data showed that abnormalities in cerebral lipid metabolism occurred spontaneously in ApoE$^{-/-}$ mice. Dietary DHA treatment could modulate cerebral lipid levels in both C57 wt and ApoE$^{-/-}$ mice by modulating the expression of lipid metabolism-related molecules.

3.5 The Content of Cortical Fatty Acid and the Expression of Fatty Acid Transporters

Compared with C57 wt mice, higher content of cortical n-6 PUFAs and the ratio of n-6 to n-3 PUFAs (n-6/n-3 ratio) were found in ApoE$^{-/-}$ mice, but only the difference in the ratio of n-6/n-3 reached statistical significance ($P < 0.05$). DHA-fortified diet significantly increased the levels of cortical DHA and n-3 PUFAs in ApoE$^{-/-}$ mice ($P < 0.05$). Compared with control diet-fed ApoE$^{-/-}$ mice, the ratio of cortical n-6/n-3 PUFAs was decreased significantly in

DHA-treated ApoE$^{-/-}$ mice ($P < 0.05$). The content of cortical n-6 PUFAs remained unchanged in both DHA-fed C57 wt and ApoE$^{-/-}$ mice ($P > 0.05$) (Figure 3A).

ApoE$^{-/-}$ mice showed lower expression of cortical *Fabp5* mRNA than C57 wt mice ($P < 0.05$). DHA treatment decreased the expression of cortical *Fabp5* mRNA in both C57 wt and ApoE$^{-/-}$ mice ($P < 0.05$), and the lowest expression was found in ApoE$^{-/-}$ mice. ApoE$^{-/-}$ mice also exhibited lower expression of cortical *Cd36* mRNA than C57 wt mice ($P < 0.05$). DHA treatment decreased the expression of cortical *Cd36* mRNA in C57 mice ($P < 0.05$), but increased its expression in ApoE$^{-/-}$ mice. ApoE$^{-/-}$ mice showed higher expression of cortical *Scarb1* mRNA than C57 wt mice ($P < 0.05$). DHA treatment significantly induced its expression in ApoE$^{-/-}$ mice ($P < 0.05$). The expression of FABP5 protein showed an increased trend in DHA-fed C57 wt and ApoE$^{-/-}$ mice, although there was no statistical significance ($P > 0.05$). In comparison with C57 wt mice, ApoE$^{-/-}$ mice demonstrated a slightly higher expression of cortical SRB1 protein, but no statistical significance was observed between groups ($P > 0.05$) (Figure 3B).

In conclusion, despite no differences found in protein expression of these fatty acid transporters in our study, differences in gene expression were found based on apolipoprotein status and dietary intervention. Moreover, the treatment of DHA caused a change of cortical fatty acids, especially a significant increase of cortical DHA content in DHA-fed ApoE$^{-/-}$ mice.

3.6 The Level of Cerebral Soluble Aβ and the Expression of Aβ Metabolism Molecules

ApoE$^{-/-}$ mice displayed a higher level of soluble cortical $A\beta_{1\text{-}42}$ than C57 wt mice ($P < 0.05$). DHA treatment did not affect the level of cortical soluble $A\beta_{1\text{-}40}$ in both C57 wt and ApoE$^{-/-}$

mice, but increased the level of cortical soluble $A\beta_{1-42}$ in C57 wt mice, accompanied by a decreased ratio of cortical $A\beta_{1-40}/A\beta_{1-42}$ ($P < 0.05$). There was no change in the level of cortical $A\beta_{1-42}$ and $A\beta_{1-40}$ in control diet-fed and DHA-fed ApoE$^{-/-}$ mice. ApoE$^{-/-}$ mice demonstrated a lower expression of cortical BACE1 protein than C57 wt mice ($P < 0.05$), and the treatment of DHA had no effect on the expression of cortical BACE1 protein in both C57 wt and ApoE$^{-/-}$ mice ($P > 0.05$) (Figure 4A).

Brain Aβ metabolism-related molecular gene and protein expressions are illustrated in Figure 4B. ApoE$^{-/-}$ mice displayed higher expression of cortical *App* mRNA than C57 wt mice ($P < 0.05$). DHA treatment further induced the expression of *App* mRNA in both C57 wt and ApoE$^{-/-}$ mice ($P < 0.05$), especially in C57 wt mice, and the expression of cortical APP protein was also enhanced ($P < 0.05$). After DHA treatment, cortical *Ide* mRNA expression was up-regulated in ApoE$^{-/-}$ mice but not in C57 wt mice. There was a lower expression of cortical IDE protein in ApoE$^{-/-}$ mice than in C57 wt mice ($P > 0.05$), and the expression of cortical IDE protein showed a decreased trend after DHA treatment in both C57 wt and ApoE$^{-/-}$ mice. DHA treatment significantly downregulated the gene expression of *Lrp1* in the cortex of ApoE$^{-/-}$ mice ($P < 0.05$). The expression of cortical LRP1 protein remained unchanged in both mice. The expression of cortical *Sorl1* mRNA was lower in ApoE$^{-/-}$ mice than in C57 wt mice ($P < 0.05$). DHA treatment had no effect on the expression of cortical *Sorl1* mRNA in both C57 wt and ApoE$^{-/-}$ mice, but the expression of cortical SorLA protein showed a decreased trend in C57 wt mice, but no statistical significance was observed.

As shown in Figure 4C, ApoE$^{-/-}$ mice displayed slightly lower expression of cortical ACE1 than C57 wt mice. DHA treatment decreased the expression of cortical ACE1 in both mice,

but no statistical significance was detected. In comparison with C57 wt mice, ApoE$^{-/-}$ mice showed a significantly higher expression of ACE1 in the CA1 area of the hippocampus ($P < 0.05$). The treatment of DHA significantly decreased the expression of hippocampal ACE1 in the CA1 area in ApoE$^{-/-}$mice ($P < 0.05$), especially in female ApoE$^{-/-}$ mice, but no change was found in C57 wt mice. Both DHA-fed C57 wt and ApoE$^{-/-}$ mice showed an increased trend of ACE1 expression in the hippocampus CA3 area, but the difference did not reach statistical significance ($P > 0.05$). ApoE$^{-/-}$ mice showed relatively lower expression of cortical ACE2 protein than C57 mice, but no statistical significance was observed ($P > 0.05$) (Figure 4D). DHA treatment had no effect on the expression of cortical and hippocampal ACE2 protein in both C57 wt and ApoE$^{-/-}$ mice.

In summary, we found that the status of ApoE might affect the content of cortical soluble Aβ in mice; and the change of soluble Aβ content to DHA treatment was also ApoE status-dependent. We also observed different Aβ metabolism-related molecular gene and protein expressions in C57 wt and ApoE$^{-/-}$ mice, as well as discrepant expression responses of these molecules to DHA treatment. We speculate that the net effect of these Aβ metabolism-related molecular gene and protein expressions after DHA treatment might ultimately determine the soluble Aβ level in the brain of mice.

4. DISCUSSION

The present study explored the impact of dietary DHA treatment on cerebral lipids and soluble Aβ levels in ApoE$^{-/-}$ and C57 wt mice. Our data showed that the absence of ApoE significantly affected lipid profiles and Aβ generation in the brain of mice. The treatment of DHA was efficient in regulating brain lipid and Aβ levels in an ApoE-dependent way.

The associations between n-3 PUFAs and circulating lipid profiles have been reported inconsistently. Some studies have demonstrated the serum TG-lowering effects and LDL-C-increasing effects of n-3 PUFAs, while others reported a positive association between the levels of plasma total TC and LDL-C and the dietary n-3 PUFAs intake[18,19]. A study conducted in C57BL/6J male mice showed that the serum TC, LDL-C, and HDL-C levels in high-fat and high-sucrose diet-treated mice was higher than that in low-fat diet-fed mice. In addition, fish oil supplementation significantly decreased the serum TC level [20]. In another study, after treating male ApoE$^{-/-}$ mice with n-3 PUFA-rich diet (a diet with different ratios of DHA and EPA) for 12 weeks, the serum TC level of the ApoE$^{-/-}$ mice in the DHA/EPA=2:1 group decreased significantly as compared with the ApoE$^{-/-}$ mice from control diet-fed; the decrease of serum TG level was observed in the mice treated with DHA/EPA=2:1 and DHA/EPA=1:1 diets; the decrease of serum HDL-C level was found in the mice from DHA/EPA=1:2 group; while, the serum LDL-C level remained unchanged after n-3 PUFA intervention [21]. These data indicated that the ratio of n-3 PUFAs in diet might have a different impact on serum lipid parameters. In the current study, we failed to observe any significant changes in plasma lipids in DHA-treated C57 wt and ApoE$^{-/-}$ mice. The reason for these inconsistencies might be attributable to the differences in study design, species difference of the experimental animals, the ratio of n-3 PUFAs in diet, and the duration of intervention. In contrast, ApoE status-specific regulatory effect of DHA on cerebral lipids was observed, which was demonstrated by a dramatically decreased level of cortical TC, but an increased level of cortical TG, HDL-C, LDL-C, and the ratio of HDL-C/LDL-C in DHA-treated C57 wt mice. In ApoE$^{-/-}$ mice, DHA treatment resulted in a significantly decreased level of cortical

TC, TG, and LDL-C, but had no effect on cortical HDL-C. Moreover, ApoE$^{-/-}$ mice displayed slightly higher expression of cortical HMGCR protein and higher expression of both cortical and hippocampus ABCA1 protein than C57 wt mice, which indicated a compensatory inhibition of cholesterol synthesis, and an enhanced cellular efflux of cholesterol in the brain of ApoE$^{-/-}$ mice. The difference in cortical lipid profile in DHA-fed C57 wt mice and ApoE$^{-/-}$ mice highlights the modifying impact of ApoE on the outcomes of DHA treatment. These outcomes critically imply that dysfunction of the ApoE gene might affect central lipid mobilization and homeostasis, accounting for the discrepant and inconclusive outcomes of human-based studies regarding the efficacy of DHA treatment in neurodegenerative diseases.

A study conducted in mice indicated that deletion of the ABCA1 could reduce content and lipidation of ApoE in the brain and cerebrospinal fluid (CSF) [22]. Results from *in vivo* studies also indicated that a defect of ABCA1 would result in a decrease of 65% and 75-80% of ApoE levels in the whole brain and the hippocampus respectively[23]. These results suggested that there was a linkage between ABCA1 and ApoE in regulating lipid metabolism in the brain. In our study, ApoE$^{-/-}$ mice displayed a dramatically increased expression of cortical and hippocampal ABCA1 than that in C57 wt mice. Since the key function of ABCA1 is to transport cholesterol from cell membrane to ApoE to form HDL, we thus speculate that the damaged cholesterol transport in ApoE$^{-/-}$ mice induced a compensatory up-regulation of ABCA1 expression in the brain. As a key participant in ApoE metabolism, ABCA1 is required to maintain the normal level of ApoE in the brain. Our observations further provide a link between ABCA1 and apoE metabolism in the brain.

The liver X receptors (LXRs) belong to the nuclear receptor superfamily. Alteration of lipid

homeostasis has been reported in LXR-deficient mice, and LXRs were reported to play an essential physiological role in cholesterol and lipid metabolism by regulating the gene expression of ABCA1 and ApoE. Both LXRα and LXRβ are present in the brain, therefore, the protein expression of these two isoforms was evaluated. We found that the defect of ApoE significantly decreased the expression of LXRα/β protein in mice brains. Activation of LXR could induce the expression of ABCA1 in rat brain[24], indicating that ABCA1 was the target gene of LXR. Moreover, LXRs are activated by certain cholesterol derivatives such as several oxidized cholesterol metabolites or oxysterols. Inconsistent with these findings, we found that the ApoE$^{-/-}$ mice displayed a dramatically increased level of cerebral cholesterol and the expression of ABCA1, but a significantly inhibited expression of LXRα/β compared with those in C57 wt mice. These results suggested a LXR-independent regulating way of lipid and cholesterol metabolism caused by ApoE deficient. LXRα/β was an efficient regulator of both ABCA1 and ApoE gene expression, the abnormal expression of ABCA1 in ApoE$^{-/-}$ mice thus caused a negative feedback effect on the expression of LXRα/β to counteract the over-expression of ABCA1.

Previous studies indicated that DHA might enhance the expression of reverse cholesterol transport gene ABCA1 and ApoE due to its action on nuclear receptors in the brain. Casali's study also found that DHA could enhance the expression of LXR target gene ABCA1 and ApoE, and reduce the soluble forms of Aβ in an AD model mouse[25]. Our study demonstrated an ApoE-dependent modifying impact of DHA on cerebral LXRα/β expression, which was indicated by the increase of cortical and hippocampal LXRα/β expression in ApoE$^{-/-}$ mice, but the decrease of this protein expression in C57 wt mice. All these results suggest that the

abnormal cholesterol metabolism in the brain of ApoE$^{-/-}$ mice could lead to discrepant responses of brain cholesterol metabolism to DHA treatment. In this study, increased LXRα/β and decreased ABCA1 expression were simultaneously observed in DHA-fed ApoE$^{-/-}$ mice. As one important function of ABCA1 is to transfer phospholipid and cholesterol to ApoE, these results hint that the defect of ApoE potentially attenuated LXRα/β-mediated ABCA1 expression in ApoE$^{-/-}$ mice, therefore resulting in discrepancy response of brain lipid profile to DHA treatment.

Furthermore, another study has also reported that centrally and peripherally-derived ApoE exists in distinct pools and they are independent of each other[26]. This finding may partially explain the differences in the response of central and peripheral lipids to DHA treatment. Altogether, our study provides additional evidence that DHA ameliorates lipid disorders in the brain of ApoE$^{-/-}$ mice. Well-designed studies are needed to explore the molecular mechanism of whether the n-3 PUFAs regulate central and peripheral lipid metabolism in order to ascertain these findings.

ApoE receptor-mediated uptake of PUFAs in CNS has been reported[27]. ApoE deficiency is suspected to affect the formation of HDL-C particles and the exchange of lipids between glial cells and neurons[28]. It has also been found that, when ApoE is absent, the availability of PUFAs in the CNS is enhanced[29]. Consistent with this report, we found that the ApoE$^{-/-}$ mice showed a higher level of cortical DHA, n-3 PUFAs, and n-6 PUFAs than those in C57 wt mice. Our data confirmed the participation of ApoE in regulating the level of cerebral PUFAs. The uptake of fatty acids in CNS is through multiple transit-pathway-dependent complex processes, including passive diffusion of non-esterified PUFAs and fatty acid

transporter-mediated fatty acid uptake. The deletion of ApoE showed extensive regulatory effects on the expression of PUFAs transporter genes in the cortex, such as *Fabp5*, *Cd36*, and *Scarb1*, suggesting that except for lipoprotein receptor-mediated cerebral PUFAs uptake, some other genes or compensatory molecular mechanisms might play key roles in increasing PUFAs level in ApoE$^{-/-}$ mice[30].

Consistent with the previous study[31], we found that DHA treatment effectively increased the content of cortical n-3 PUFAs in ApoE$^{-/-}$ mice. These results demonstrate that the deletion of ApoE enhanced the uptake of n-3 PUFAs by CNS or mitigated the metabolism of PUFAs in the CNS. Further studies are encouraged to explore the underlying mechanism for this finding. In C57 wt mice, DHA treatment significantly inhibited the gene expression of both *Fabp5* and *Cd36* in the cortex, implying that, under normal ApoE status, the increase of cortical n-3 PUFAs in DHA-treated C57 wt mice could not be mediated by these fatty acid transporters. These results suggest that other transporters, such as major facilitator superfamily domain containing 2A (Mfsd2a) or fatty acid transport protein 4 (FATP4), may mediate brain n-3 PUFAs uptakes in these wild type animals. Contrary to the findings in the C57 wt mice, DHA treatment in ApoE$^{-/-}$ mice caused a dramatically increased expression of both *Cd36* and *Scarb1* mRNA in the cortex but a decrease of the *Fabp5* gene expression in the cortex. Clearly, oxidative-related cortical lipids (e.g., TC and LDL-C) increased in ApoE$^{-/-}$ mice compared to those in C57 wt mice. The decreased expression of *Fabp5* and increased expressions of both *Cd36* and *Scarb1* genes following DHA treatment could possibly be indicative of a negative feedback loop to protectively curb further uptake of fatty acids (FAs) into the cortical cells, attempting to ultimately stabilize oxidative stress and inflammation.

Moreover, the extensively induced fatty acid transporter gene expressions might contribute to the ApoE-specific outcomes of an increased level of cortical n-3 PUFAs in response to DHA treatment. The net effect of ApoE deletion on different fatty acid transporters gene expressions promotes the delivery of PUFAs to the brain, resulting in a significant increase in the level of cortical n-3 PUFAs. Altogether, our data indicated that there is an ApoE-dependent response of brain n-3 PUFAs to DHA treatment. The mechanisms behind the various responses remain unclear. Further studies need to be done to uncover the relationship between ApoE, fatty acids transporter genes, and n-3 PUFAs uptake in the brain.

Excessive accumulation and deposit of Aβ in the brain have been proved to be associated with impaired cognition and memory decline[32]. The reported correlation between cholesterol and AD pathology directs us to further explore how ApoE deletion affects the amyloidogenic process in the brain. In the current study, insoluble Aβ plaque was not found in the brains of $ApoE^{-/-}$ mice (Supporting information 2). These results were in line with the unchanged behavioural performance in $ApoE^{-/-}$ mice. Liao et al's study also found that anti-ApoE antibodies could inhibit amyloid accumulation, supporting a hypothesis that aggregation of ApoE might facilitate Aβ plaque formation in the brain[11]. Based on these results, the ApoE deficient phenotype of $ApoE^{-/-}$ mice might explain the undetectable Aβ plaque in the brain. Nevertheless, an increased cortical soluble $A\beta_{1-42}$ content was observed in $ApoE^{-/-}$ mice. It is reported that the increase of cholesterol in the brain could modify both the composition and function of lipid raft in neuronal membranes and enhance β-secretase-mediated APP processing, ultimately promoting over-generation of β-amyloid[33]. Additionally, the BACE1 enzyme-dependent processing of APP has been documented as a primary source of Aβin the

brain[34]. IDE-catalysed degradation and clearance of Aβ have also been demonstrated in the literature[35]. In the current study, significantly increased level of cortical TC as well as the expression of APP gene and protein, while decreased cortical BACE1 and IDE protein expression were found in ApoE$^{-/-}$ mice, indicating that the disturbance of cerebral cholesterol homeostasis in ApoE$^{-/-}$ animals might contribute to abnormal Aβ metabolism.

An increase of cortical soluble Aβ_{1-42} content was found in DHA-treated C57 wt and ApoE$^{-/-}$ mice. As a typical AD pathological biomarker[36], the definitive role of Aβ in the brain is uncertain. Aβ-mediated neuron protective effects have been extensively reported[37, 38]. A close relationship between Aβ and intracellular cholesterol transport has been established by previous studies, stating that newly synthesized Aβ_{1-42} could facilitate cholesterol trafficking from the neuronal membrane to the Golgi apparatus, while on the contrary, aged Aβ_{1-42} showed decreased cholesterol trafficking capacity to the Golgi apparatus[39, 40]. In the present study, we detected an ApoE phenotype-dependent impact of DHA supplement on cerebral cholesterol levels. Compared to ApoE$^{-/-}$ mice, a dramatic decrease in cortical total cholesterol was found in C57 wt mice following DHA treatment, suggesting that an amyloidogenic process was induced to facilitate intracellular cholesterol transportation, finally resulting in cholesterol homeostasis .

The observation of increased total cholesterol in ApoE$^{-/-}$ mice may also imply that under the dysfunctional ApoE condition, the cellular integrity, which is required for neuronal organelles to execute intact cholesterol trafficking, may be impaired. This was further proven by the increased expression of cortical APP protein, but lower expression of SorLA protein in DHA-treated C57 wt mice. As a sorting receptor, SorLA could prevent APP from processing

into Aβ[41], thereby reducing Aβ generation in cells to express endogenous APP[42]. The down-regulation of IDE and SorLA protein expression might further contribute to the increased cortical $A\beta_{1-42}$ status in DHA-treated C57 wt mice. On the contrary, the mRNA expression of the *Ide* gene was induced by DHA treatment in $ApoE^{-/-}$ mice, while the mRNA expression of *Lrp1* was inhibited. Given the reported role of LRP1 in promoting APP processing to Aβ[43, 44], we speculated that the diversified expression of molecules involved in amyloidogenesis might explain the discrepancy of cortical $A\beta_{1-42}$ status in DHA-treated C57 wt and $ApoE^{-/-}$ mice. Since the newly synthesized $A\beta_{1-42}$ is crucial for the trafficking of neuronal cholesterol from the membrane to the Golgi apparatus[37-40], our data suggested that higher levels of cortical soluble $A\beta_{1-42}$ in the $ApoE^{-/-}$ mice might be a compensatory response to their significantly increased cortical TC and LDL-C levels. However, we observed that in C57 wt mice, DHA treatment increased the level of cortical soluble $A\beta_{1-42}$ and subsequently decreased the ratio of $A\beta_{1-40}/A\beta_{1-42}$. This implies that, in normally functional ApoE status, $A\beta_{1-42}$ is essentially needed for cholesterol transport in the neuron, and the ApoE gene may play a pivotal role in this process by first modulating the new synthesis of $A\beta_{1-42}$ and later spontaneously converting aged $A\beta_{1-42}$ (a toxic form) to $A\beta_{1-40}$ (a less toxic form) for neuronal clearance. Future studies are required to explore the potential basis for the interactions of the ApoE gene and cholesterol in Aβ synthesis and mobilization.

Angiotensin-converting enzyme 1/2 (ACE1/2) plays a prominent role in AD pathogenesis[45]. Elevated ACE1 levels and activity were detected in the frontal cortex of post-mortem AD patients[46]. In ACE2 gene defect mice, ACE2 deficiency resulted in impaired cognitive function[47]. ACE2 activity was inversely correlated with β-secretase activity and parenchymal

Aβ load in the post-mortem-brain tissue of AD patients. $A\beta_{43}$ is an early deposited and highly amyloidogenic form of Aβ and it seeds plaque formation[48]. Studies reported that ACE2 could convert $A\beta_{43}$ to $A\beta_{42}$, which in turn is cleaved by ACE1 to less toxic $A\beta_{40}$ and $A\beta_{41}$ species[49], this indicates that ACE1/2 may contribute in some way to the regulation of APP amyloidogenic processing[50]. In our study, $ApoE^{-/-}$ mice showed a higher level of cortical $A\beta_{42}$ than that in C57 wt mice, suggesting this abnormal increase in $A\beta_{42}$ leads to negative regulation of ACE2 expression to antagonize the over-generation of $A\beta_{42}$ in the brain. The significant increase of ACE1 expression in the hippocampus of $ApoE^{-/-}$ mice further hints that there is a potential relation between ApoE and ACE1/2 in regulating the metabolism of Aβ. Given the participation of ACE1/2 in the metabolism of Aβ, the increased expression of hippocampal ACE1 protein in $ApoE^{-/-}$ mice might be a compensated negative response to the increased $A\beta_{42}$ level to reduce and slow down the accumulation of Aβ in this ApoE defect animals. These findings demonstrated the participation of ACE1/2 in ApoE defect-mediated dysregulation of Aβ metabolism in the brain.

Moreover, we also detected obvious gender differences in the regulation of DHA on ACE1 expression in $ApoE^{-/-}$ mice, as indicated by a much more significant decreased expression of both cortical and hippocampal ACE1 in female mice than those in the male ones. These data demonstrate the potential interaction of ApoE and gender in affecting the outcome of DHA intervention on brain ACE1 expression. Inconsistent results were documented regarding the impact of DHA on the ACE2 expression. Tseng's study found that DHA significantly decreased ACE2 mRNA expression in porcine adipocyte[51]. While Ulu and co-workers reported that n-3 PUFAs rich diet up-regulated renal ACE2 expression in a murine model of

angiotensin-II dependent hypertension[52]. In our study, DHA had no effect on the expression of ACE2 in C57 wt and ApoE$^{-/-}$ mice. It is well known that DHA is a potential regulator of phospholipid fatty acid composition[53], and the defect of ApoE in ApoE$^{-/-}$mice might disturb DHA's regulating effect, which may be responsible for the differential effect on ACE2 expression in the brain.

5. Conclusion

ApoE deletion might trigger compensatory changes in an array of signalling molecules that participate in cerebral cholesterol homeostasis, fatty acids uptake, and amyloid generation. The findings from this study indicated the important role of ApoE in modifying the responses of cerebral fatty acids and cholesterols status and β-amyloid generation to DHA treatment. DHA treatment was effective in affecting lipid and cholesterol reverse transport molecules expression to regulate β-amyloid generation in the brain, and this process is modifiable by ApoE gene deficit. Further studies are needed to evaluate the ApoE-genotype-associated efficacy and effects associated with DHA treatment on lipids and amyloidosis in the brain.

Author Contributions

Linhong Yuan designed the work; Jingjing Xu, Xiaochen Huang, Yujie Guo, Xiaojun Ma, and Pengfei Li performed lab works; Linhong Yuan, Xiaochen Huang, and Jingjing Xu contributed to the data interpretation; Linhong Yuan, Xiaochen Huan, Jingjing Xu, Shaobo Zhou, Chi Zhang, Rui Chen, and Nicholas Van Halm-Lutterodt contributed to drafting the manuscript. All authors approved the final version of the manuscript.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Abbreviations:

ABCA1	ATP-binding cassette transporter A1
ACAT1	acetyl coenzyme A acetyltransferase 1
ACE1	angiotensin-converting enzyme 1
ACE2	angiotensin-converting enzyme 2
AD	Alzheimer's disease
ALA	α-linolenic acid
ANOVA	one-way analysis of variance
ApoB	apolipoprotein B
ApoE	apolipoprotein E
$ApoE^{-/-}$	ApoE-deficient
APP	amyloid precursor protein
Aβ	β-amyloid
BACE1	beta-secretase 1
BBB	blood-brain barrier
BCA	bicinchoninic acid
C57 wt	wild type C57BL/6J
CD36	cluster of differentiation 36
CNS	central nervous systems
CSF	cerebrospinal fluid
DHA	docosahexaenoic acid
ELISA	enzyme-linked immunosorbent assay
EPA	eicosapentaenoic acid
FABP5	fatty acid binding protein 5
FAs	fatty acids
FATP4	fatty acid transport protein 4
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GLU	glucose
HDL-C	high-density lipoprotein cholesterol

HMGCR	3-hydroxy-3-methyl glutaryl coenzyme A reductase
HRP	horseradish peroxidase
IDE	insulin degrading enzyme
LDL-C	low-density lipoprotein cholesterol
LRP1	LDL receptor related protein 1
LXRα/β	liver X receptor α and β
Mfsd2a	major facilitator superfamily domain containing 2A
n-3 PUFAs	n-3 polyunsaturated fatty acids
PBS	phosphate buffered saline
SD	standard deviations
SorLA	sortilin-related receptor, LDLR class A repeats-containing
SRB1	scavenger receptor, class B type 1
TBS	Tris-buffered saline
TC	total cholesterol
TG	triglyceride

Table 1. Composition of experimental diets

Nutrients in diet	Groups	
	Control diet	DHA fortified diet
Ingredient (g/kg)		
Casein	140	140
L-Cystine	1.8	1.8
Corn starch	495.692	482.392
Maltodextrin	125	125
Sucrose	100	100
Cellulose	50	50
Choline bitartrate	2.5	2.5
Fish oil powder	0	33.3
Lard	15	15
Corn oil	25	5
Retinol	0.008	0.008
α-Tocopherol	0.100	0.103
DHA	0	5.5
kcal %		
Protein	14.7	14.7
Carbohydrate	75.9	75.9
Fat	9.4	9.4

Table 2. Escape latency time (second) of experimental animals in the water maze test

Group	C57 wt		ApoE$^{-/-}$	
	Day1	Day6	Day1	Day6
Control diet	74.26 ± 5.46	36.71 ± 6.84*	73.73 ± 3.30	20.04 ± 4.84$^{\#,a}$
DHA-fortified diet	56.61 ± 7.35	35.93 ± 6.96*	65.72 ± 4.71	27.22 ± 3.56$^{\#}$

Data are shown as mean ± SEM, $n = 12$ for each group. *: $P < 0.05$ compared with day 1 in C57 wt mice; #: $P < 0.05$ compared with day 1 in ApoE$^{-/-}$ mice; a: $P < 0.05$ compared with day 6 in C57 wt mice.

Table 3. Plasma lipid levels in experimental animals after DHA treatment for 5 months

Parameters	C57 wt		ApoE$^{-/-}$	
(mmol/l)	Control diet	DHA-fortified diet	Control diet	DHA-fortified diet
TC	2.81 ± 0.57	2.18 ± 0.66	14.87 ± 4.6^{ab}	14.47 ± 2.88^{ab}
TG	0.45 ± 0.25	0.33 ± 0.11	0.81 ± 0.19^{ab}	1.07 ± 0.38^{abc}
HDL-C	2.09 ± 0.37	1.69 ± 0.58	5.82 ± 1.74^{ab}	4.77 ± 0.80^{ab}
LDL-C	0.19 ± 0.09	0.15 ± 0.08	3.43 ± 1.02^{ab}	3.70 ± 0.91^{ab}

Data are shown as mean ± SD, *n = 12* for each group (6 males and 6 females). TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. a: $P < 0.05$ compared with control diet-fed C57 wt mice; b: $P < 0.05$ compared with DHA-fortified diet-fed C57 wt mice; c: $P < 0.05$ compared with the control diet-fed ApoE$^{-/-}$ mice.

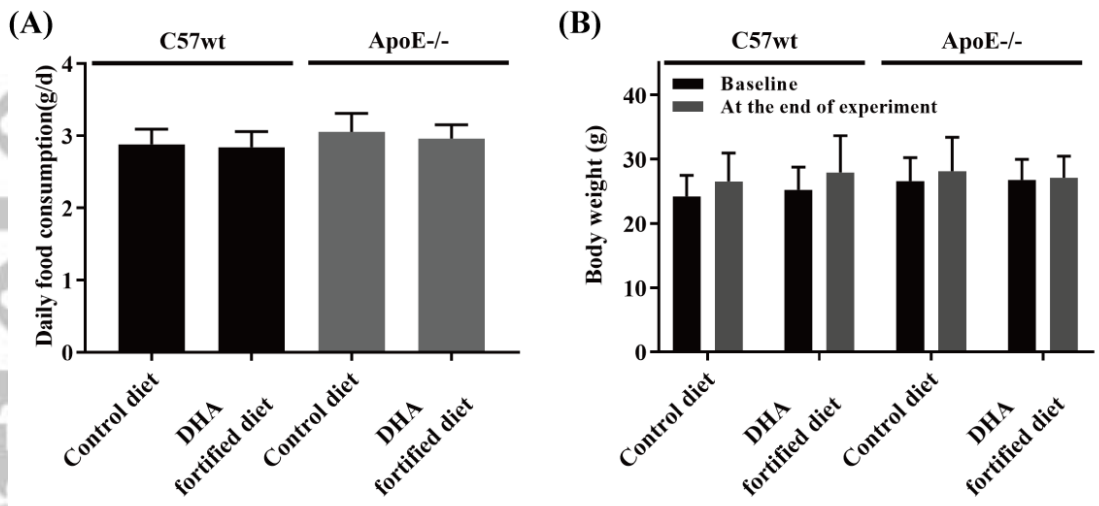
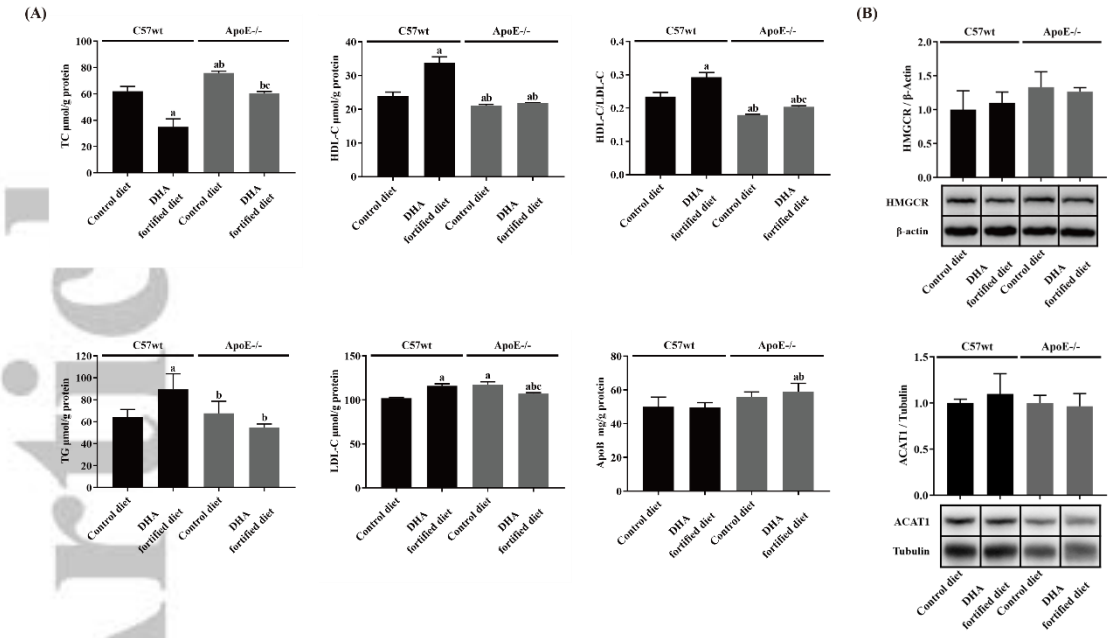
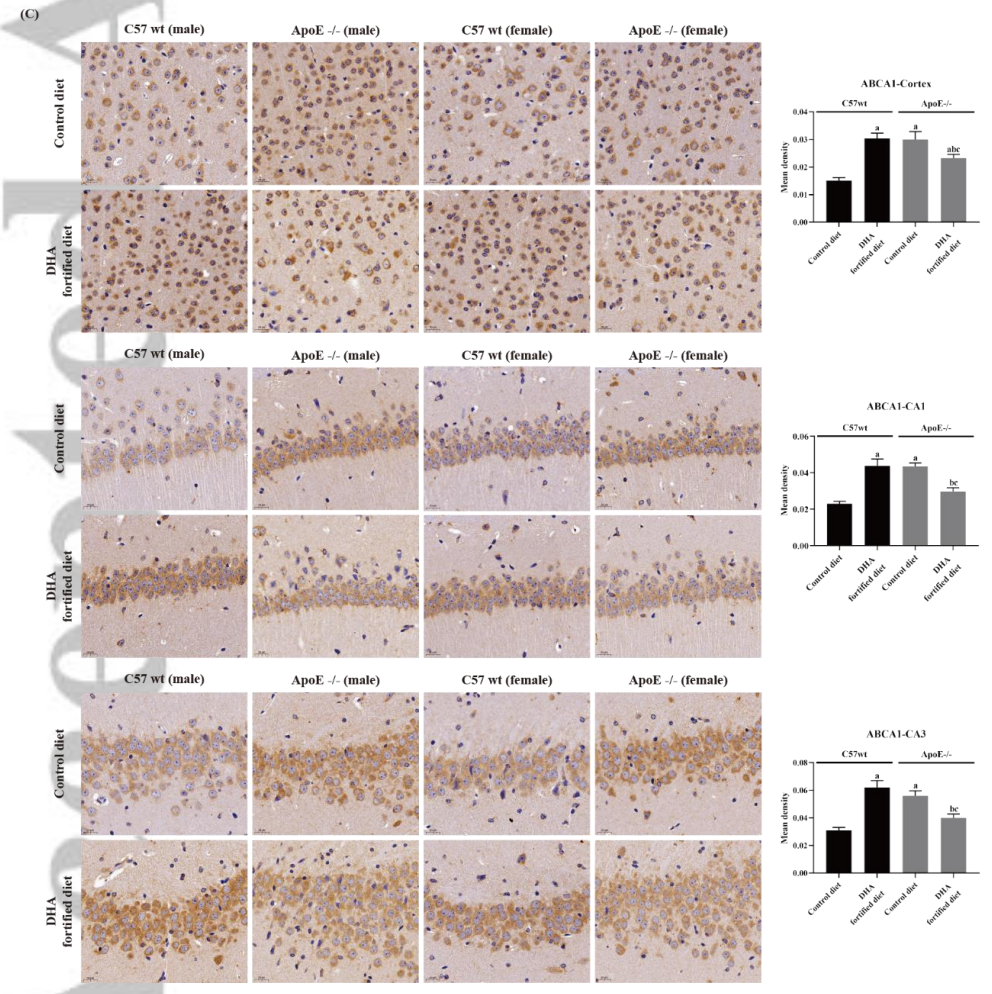


Figure 1. The daily food consumption and the average weight of the animals. C57 wt and ApoE$^{-/-}$ mice were given a control diet or DHA-fortified diet respectively for 5 months. Data are shown as mean ± SD. (A) The daily food consumption of experimental animals; (B) The changes in body weight of experimental animals at baseline and at the end of the experiment (*n = 12* for each group, 6 males and 6 females).





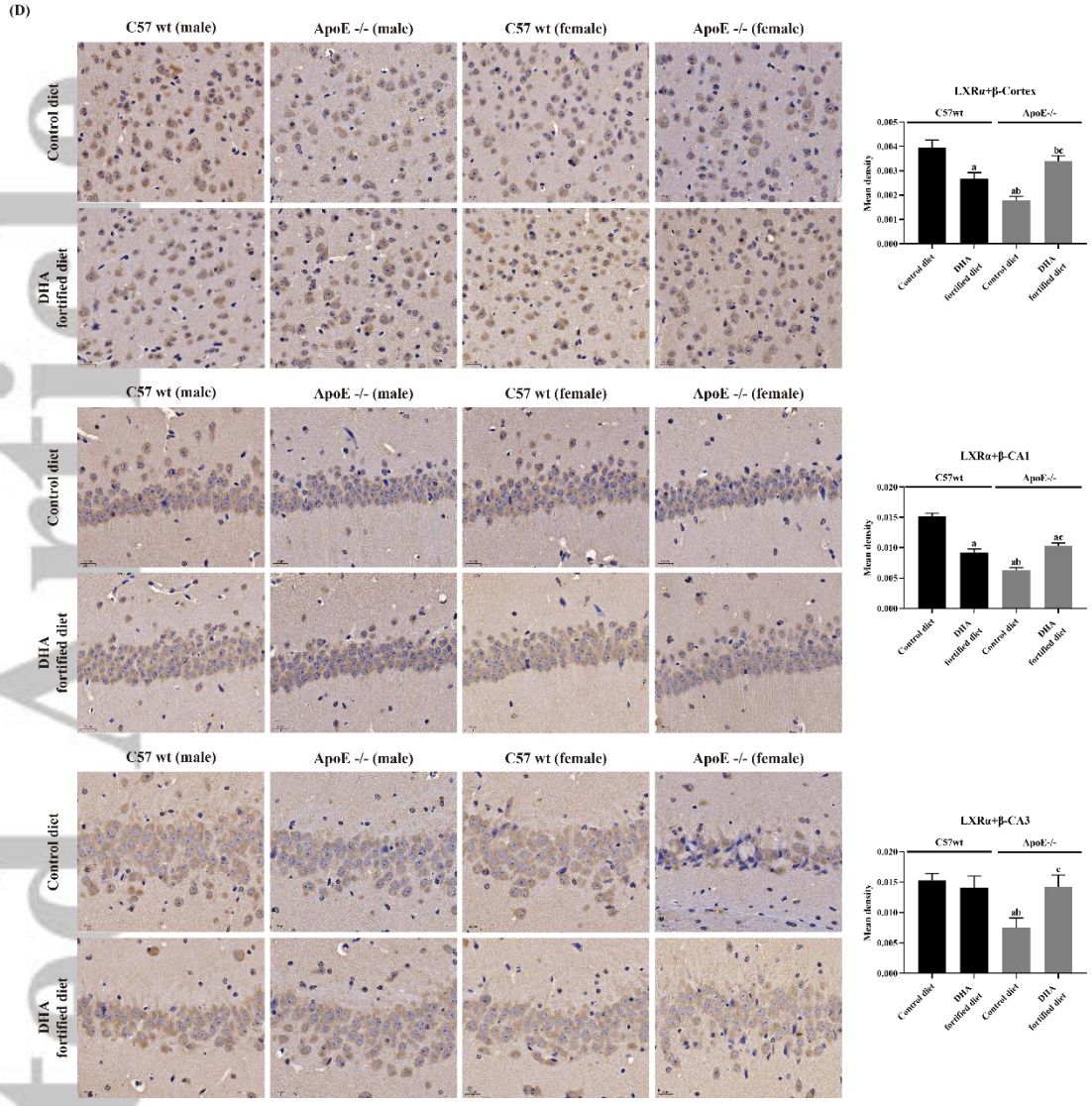


Figure 2. Cortical lipid parameters and the expression of lipid metabolism-related molecular protein in C57 wt and ApoE$^{-/-}$ mice. C57 wt and ApoE$^{-/-}$ mice were given a control diet or a DHA-fortified diet for 5 months. Cortical lipids levels and the expression of lipid metabolism-related molecules were measured (*n = 12* for each group, 6 males and 6 females). Data are expressed as mean ± SD. (A) Lipid parameters in cortex from C57 wt and ApoE$^{-/-}$ mice treated with different diets. (B) HMGCR and ACAT1 protein expressions in cortex from C57 wt and ApoE$^{-/-}$ mice treated with different diets. (C) & (D) The expression of ABCA1 and LXRα/β protein in cortex and hippocampus from C57 wt and ApoE$^{-/-}$ mice treated with different diets. Scale bar: 20μm. The bar charts are quantitative results for the representative images on the left, respectively. Data are expressed as mean ± SD. a: $P < 0.05$ compared with the control diet-fed C57 wt mice; b: $P < 0.05$ compared with the DHA-fortified diet-fed C57 wt mice; c: $P < 0.05$ compared with the control diet-fed ApoE$^{-/-}$ mice.

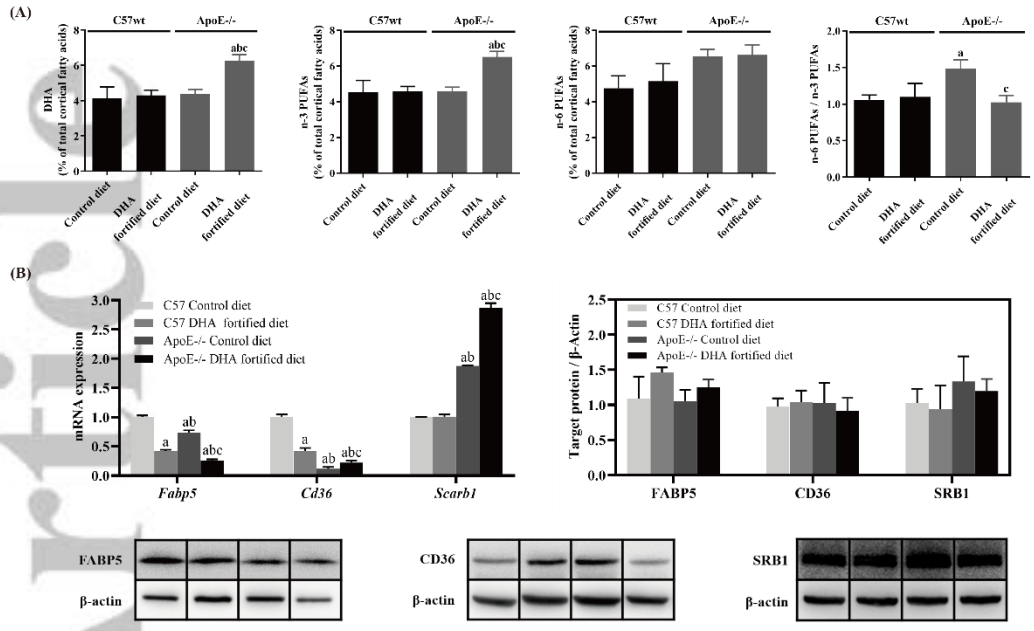
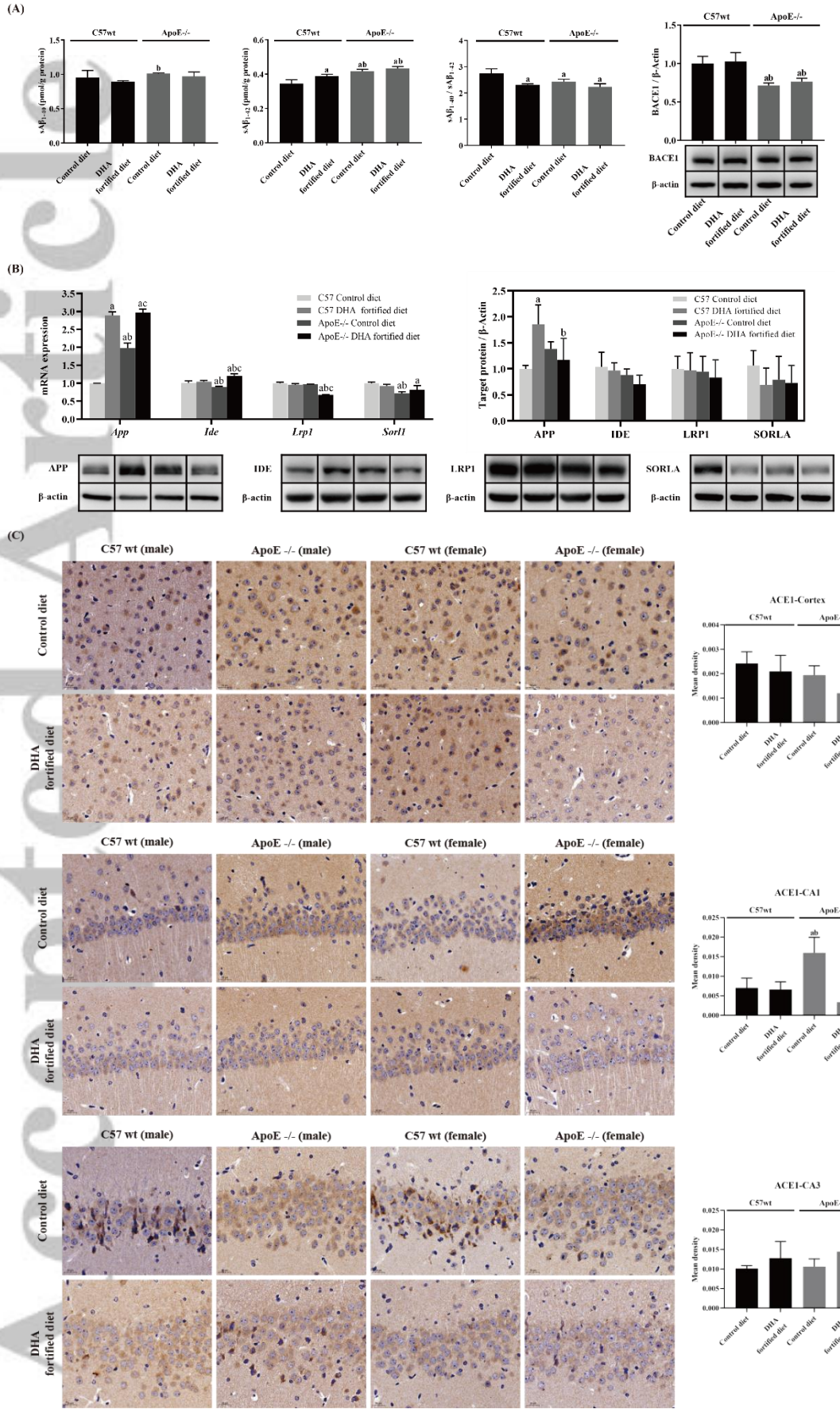


Figure 3. Cortical fatty acids and fatty acid transport-related molecular gene and protein expression in C57 wt and ApoE$^{-/-}$ mice. C57 wt and ApoE$^{-/-}$ mice were given a control diet or a DHA-fortified diet for 5 months. Cortical n-3 and n-6 PUFAs levels and fatty acid transportation molecular mRNA and protein expression were measured (*n = 12* for each group, 6 males and 6 females). (A) Cortical DHA, n-3, and n-6 PUFAs levels; (B) Cortical fatty acid transportation molecular mRNA and protein expression. Data are expressed as mean ± SD. a: $P < 0.05$ compared with the control diet-fed C57 wt mice; b: $P < 0.05$ compared with the DHA-fortified diet-fed C57 wt mice; c: $P < 0.05$ compared with the control diet-fed ApoE$^{-/-}$ mice.



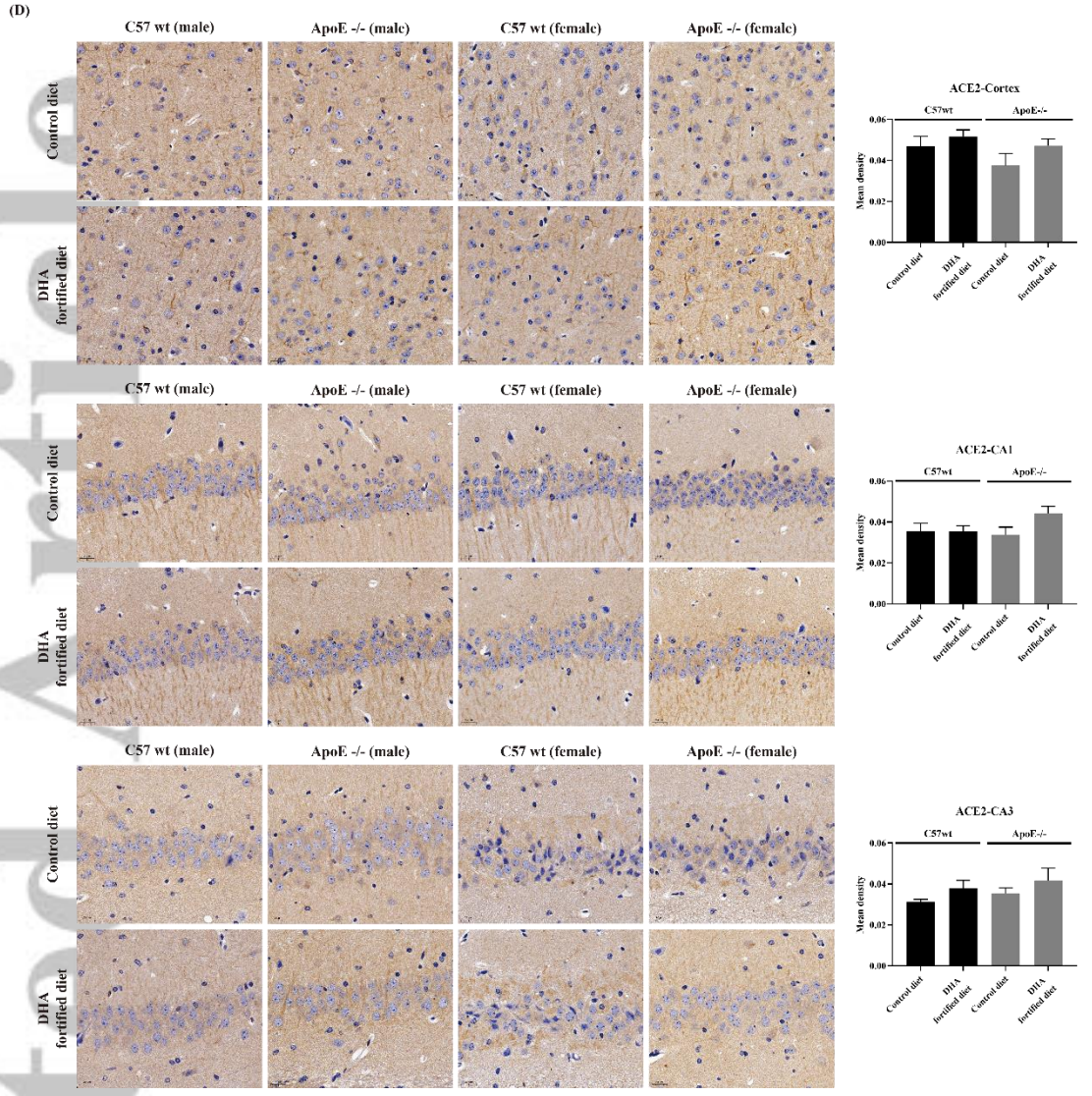


Figure 4. Cortical soluble Aβ (sAβ) content and Aβ metabolism-related molecular gene and protein expression in C57 wt and ApoE$^{-/-}$ mice. C57 wt and ApoE$^{-/-}$ mice were treated with a control diet or a DHA-fortified diet for 5 months. Soluble $A\beta_{1\text{-}40}$, $A\beta_{1\text{-}42}$ content, and BACE1 protein expression in the cortex were measured (*n = 12* for each group, 6 males and 6 females). (A) Soluble $A\beta_{1\text{-}40}$, $A\beta_{1\text{-}42}$ content, and BACE1 protein expression in the cortex. (B) Aβ metabolism-related molecular mRNA and protein expression in the cortex. (C) & (D) ACE1 and ACE2 protein expression in cortex from C57 wt and ApoE$^{-/-}$ mice treated with different diets. Scale bar: 20μm. The bar charts are quantitative results for (C) and (D) representative images. Data are expressed as mean ± SD. a: $P < 0.05$ compared with the control diet-fed C57 wt mice; b: $P < 0.05$ compared with the DHA-fortified diet-fed C57 wt mice; c: $P < 0.05$ compared with the control diet-fed ApoE$^{-/-}$ mice.

Graphical Abstract