Human apolipoprotein E allele and docosahexaenoic acid intake modulate peripheral cholesterol homeostasis in mice

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Running title: Cholesterol metabolism in apolipoprotein E4 mice

Abstract

Carrying at least one apolipoprotein E ε4 allele (*E4+*) is the main genetic risk factor for Alzheimer's disease (AD). Epidemiological studies support that consuming fatty fish rich in docosahexaenoic acid (DHA: 22: 6 ω3) is protective against development of AD. However, this protective effect seems not to hold in *E4+*. The involvement of *APOE* genotype on the relationship between DHA intake and cognitive decline could be mediated through cholesterol. Many studies show a link between cholesterol metabolism and AD progression. In this study, we investigated whether cholesterol metabolism is improved in *E3+* and *E4+* mice consuming a diet rich in DHA. Plasma cholesterol was 36% lower in *E4+* mice compared to *E3+* mice fed the control diet ($p=0.02$) and in the liver there was a significant genotype effect where cholesterol levels were 18% lower in *E4+* mice than *E3+* mice. The low-density lipoprotein receptor was overexpressed in the liver of *E4+* mice. Plasma cholesterol levels were 33% lower after the DHA diet ($p=0.02$) in *E3+* mice only and there was a significant diet effect where cholesterol level was 67% lower in the liver of mice fed DHA. Mice fed the DHA diet also had 62% less lipolysis stimulated lipoprotein receptor expression in the liver compared to mice fed the control diet ($p<0.0001$) but there was no genotype effect. These findings suggest that plasma and liver cholesterol homeostasis and the receptors regulating uptake of cholesterol in the liver are modulated differently and independently by *APOE* allele and DHA intake.

Key words: Apolipoprotein E, docosahexaenoic acid, diet, cholesterol, metabolism, mice

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease modulated by several environmental, physiological and genetic risk factors. The main genetic risk of AD is carrying an ε4 allele of apolipoprotein E (*E4+*). Production of the apolipoprotein E (apoE) protein is controlled by the *APOE* gene for which three different alleles are recognized: ε2, ε3 and ε4 [1]. ApoE production occurs primarily in the liver and the brain and to a lesser extent, in macrophages [2-3]. ApoE plays a pivotal role in lipid homeostasis: it regulates cholesterol, triglyceride and phospholipid transport and metabolism via interactions with receptors of the LDL family [4]. The low-density lipoprotein receptor (LDLR) is the receptor responsible for the uptake of cholesterol-rich LDL particles [5]. However, LDLR is not the only apoE receptor involved in lipoprotein metabolism. The lipolysis-stimulated lipoprotein receptor (LSR) is a multimeric receptor in the liver that recognizes both apoB and apoE and plays a role in the clearance of both triglycerides-rich particles and LDL [6].

Cholesterol is a key structural molecule of cellular membranes and it is important for brain function because it is involved in synaptic plasticity, learning, memory and neuronal integrity during aging [7]. Molecular evidence points towards a link between peripheral cholesterol metabolism and AD since high levels of plasma cholesterol in mid-life has been associated with a higher risk of developing AD [8]. There is currently no drug to cure or delay cognitive deficits associated with late onset AD supporting that prevention strategies are urgently needed. A diet containing docosahexaenoic acid (DHA), a n-3 polyunsaturated fatty acid (PUFA) concentrated in fatty fish, have shown promising results in animals to prevent onset of cognitive decline but in humans, results are less consistent [9]. The mechanisms explaining why fortification of the diet with DHA might help to prevent cognitive decline might stand on its role in neuronal differentiation [10], neurogenesis [11] and protection against synaptic loss [12]. However, it seems that *E4+* are not protected against cognitive decline when eating DHA [13-14]. Human and animal studies suggest that higher plasma cholesterol levels are associated with higher risk of cognitive decline [8,15]. Since apoE protein plays a key role in plasma cholesterol homeostasis and since apoE genotype modulate plasma DHA response to a DHA diet [16], we hypothesize that apoE genotype modify plasma cholesterol levels under a DHA diet. In order to elucidate the multi-organ mechanisms linking APOE genotype with cholesterol metabolism, animal models are

required. To date, mice knocked-in for human APOE isoforms provide a unique and useful tool to characterize dysfunction in lipid metabolism according to APOE genotype [17]**.** Therefore, in this study, we sought to investigate in *E3+* and *E4+* mice whether there is an interaction between a diet rich in DHA and E4 allele on peripheral cholesterol level and on proteins involved in cholesterol metabolism.

2. Materials and Methods

2.1 Animals

APOE-targeted replacement mice expressing human *APOE* allele were purchase at Taconic (Hudson, NY, USA). From weaning to 4 months of age, mice were fed a regular chow diet containing 66% (w/w) carbohydrate, 5 % (w/w) fat, 20% (w/w) proteins (Teklad 2018, Harlan Laboratories, IN, USA). At 4 months, half of the mice were fed a diet containing 0.7% (w/w) DHA (DHA diet, Research Diets Inc New Brundswick, NJ, USA) while the other half remained on regular chow diet (n = 10-14/genotype). At 12 months of age, mice were anesthetised with ketamine/xylazine and 100 µL of blood was collected by cardiac puncture in a lithium heparin tube (Becton Dickinson, Franklin Lakes, NJ, USA) and centrifuged at 4°C for 5 min at 2000 g, and plasma was collected and frozen at -80°C. Mice were immediately perfused in the heart with 50 ml 0.1 M PBS buffer. Liver was fast frozen on dry ice. All experiments were performed in accordance with the Canadian Council on Animal Care and were approved by the Institutional Committee of the Centre Hospitalier de l'Université de Laval (CHUL).

2.2 Cholesterol analysis

Liver was pulverized in powder with a biopulverizer (Biospec products, Bartlesville, OK, USA). Total lipids were extracted using the Folch et al. method from a 50 mg sample of liver powder [18]. The liver total lipid extract was then saponified using 1 M KOH/methanol and heated at 90°C for 1 hour. To quantify cholesterol, 250 µg of 5 α-cholestane (10 mg/mL) was added to the samples before lipid extraction and area under the curve was used to quantify total cholesterol in the samples. Cholesterol was analysed by gas chromatography. Plasma cholesterol was measured by a commercially available kit (DIM chol cholesterol flex ; Siemens) on a clinical analyser.

106 **2.3 Western immunoblotting**

107 Total proteins were extracted from a sample of 50 mg of liver powder using 1 ml of
108 extraction buffer containing 50 mM Tris-HCL pH 7.4, 2.5 mM EDTA, 150 mM NaCL, 0.5%
109 (w/v) and protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). Tissue was
110 sonicated and centrifuged for 20 min at 100 000 g at 4°C. 20 µg of proteins were loaded on a
111 10% Mini-PROTEAN® TGX Stain-Free™ polyacrylamide gel (Bio-Rad, Laboratories,
112 Hercules, CA, USA). After electrophoresis, the proteins were transferred onto a polyvinylidene
113 difluoride membrane (Bio-Rad, Laboratories, Hercules, CA, USA). Membranes were blocked
114 with 5% (w/v) milk in 0.05% (v/v) TBS-tween for 60 min at room temperature, and thereafter
115 incubated overnight at 4°C with the following primary antibodies: LDLR (1:1000, Novus,
116 Vancouver, Canada), LSR (1:500, Sigma, Oakville, Canada), LRP1 (1:2000, Abcam, Cambridge,
117 UK), ApoE (1:500, Novus, Vancouver, Canada). Bands were revealed by chemiluminescence
118 with Luminata Crescendo HRP substrate (EMD Millipore, Billerica, MA, USA) using a
119 peroxidase-conjugated secondary antibody (1:2000, Cell Signaling Technology, Danvers, MA,
120 USA). Densitometry was assed using ChemiDoc™ MP System (Bio-Rad, Laboratories,
121 Hercules, CA, USA). Total proteins were quantified with the Stain-Free™ technology (Bio-Rad
122 Laboratories, Hercules, CA, USA) and used as loading control. This technology is a more robust
123 quantification technique compared to β-actin for Western immunoblotting [18-19]. Protein levels
124 of *E3*+ mice fed the control diet were standardised at 100%.
125
126 **2.4 Plasma apoE quantification**

127 ApoE levels were measured in plasma from mice expressing one of the two human *APOE*
128 alleles using a sandwich ELISA (Abcam Cambridge, UK). Briefly, plasma sample were diluted
129 1:200 into 1X Diluent N that was provided with the kit. 50 µL of sample or standard were loaded
130 into a 96-well plate that had been coated with an anti-apoE antibody. Levels of apoE were
131 performed in duplicate and quantification was performed using the standard curve. Absorbance
132 was measured at 450 nm using a VICTOR™ X Multilabel Plate Reader (PerkinElmer, Waltham,
133 MA, USA).
134
135 **2.5. Liver protein gene expression**

136 RNA in the liver powder was extracted using the RNeasy Minikit (Qiagen, Venlo,

Netherlands). RNA purity and integrity were assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Quantitative PCR was performed at the RNomics Platform, Laboratoire de Génomique Fonctionnelle, University of Sherbrooke, QC, Canada. cDNA synthesis was performed using 1.3 µg of RNA with Transcriptor reverse transcriptase, random hexamers and dNTPs (Roche Diagnostics, Basel, Switzerland). Quantitative PCR were conducted with 10 ng cDNA and 200 nM primer pair solution on a CFX-384 termocycler (Bio-Rad, Laboratories, Hercules, CA, USA). Relative expression calculations of the candidate genes were performed using the housekeeping genes Pum1, Sdha and Txnl4b for mouse cDNA.

2.6 Statistical analysis

Data are expressed as means ± SEM. Two-way ANOVA with genotype and diet as fixed factors were performed. When there was a significant genotype × diet interaction, subgroup analysis with t-test were performed to compare differences between genotypes in each dietary group separately and to compare differences between diets in each genotype group separately. Statistical significance was set at $p<0.05$.

3. Results

3.1 *E3*+ mice fed the DHA diet have lower plasma cholesterol levels compared to *E3*+ mice fed the control diet.

For plasma cholesterol, there was a trend towards a diet x genotype interaction (p=0.054). Plasma cholesterol was 36% lower in *E4*+ mice compared to *E3*+ mice fed the control diet (p=0.02, Fig 1). *E3*+ mice fed the DHA diet had 33% lower plasma cholesterol compared to *E3*+ mice fed the control diet (p=0.025, Fig 1). There was no such significant diet effect in *E4*+ mice (Fig 1). These results suggest that *E4*+ mice did not respond to the DHA diet in terms of plasma cholesterol lowering.

3.2 *E3*+ and *E4*+ fed the DHA diet have lower hepatic lipoprotein receptor protein and mRNA levels compared *E3*+ and *E4*+ mice fed the control diet.

There was no diet x genotype interaction on the protein levels and mRNA expression of the LDLR, LSR and low density lipoprotein receptor-related protein 1 (LRP1). There was a genotype effect on hepatic LDLR protein levels and its mRNA expression levels (p=0.004 and

p=0.026, Fig 2A and 2B). LDLR protein level was 60-66% higher in *E4+* mice than *E3+* mice whereas mRNA expression level was 23-33% higher in *E4+* mice than *E3+* mice and this effect was independent of the diet (Fig 2A and 2B). There was no diet effect on the protein level of LDLR but there was a 35-40% lower expression of LDLR mRNA in mice fed the DHA diet compared to the control diet ($p<0.0001$, Fig 2A and 2B). There was a diet effect for LSR protein level and its mRNA expression level ($p<0.0001$ and $p=0.002$ Fig 2C and 2D). LSR protein level was 62% lower in mice fed the DHA diet than mice fed the control diet whereas mRNA expression level was 23-33% lower in mice fed DHA than mice fed the control diet. The diet effects were independent from genotype (Fig 2C and 2D). There was no diet nor genotype effect for LRP1 protein levels (Fig 2E).

3.3 *E4+* mice have lower plasma apoE and higher liver apoE levels compared to *E3+* mice

There was no diet x genotype interaction on the plasma and liver levels of apoE. There was however an independent genotype effect on apoE levels in the plasma and the liver ($p<0.0001$ and $p=0.019$, Fig 3A and 3B). Plasma apoE level was ~ 35% lower in *E4+* mice than *E3+* mice (Fig 3A), whereas in the liver it was ~ 25% higher in *E4+* mice than *E3+* mice (Fig 3B).

3.4 *E3+* and *E4+* fed the DHA diet have lower liver cholesterol levels compared to *E3+* and *E4+* mice fed the control diet.

There was no diet x genotype interaction on the level of cholesterol in the liver. There was a diet effect and a genotype effect for cholesterol level in the liver ($p<0.0001$ and $p=0.015$, Fig 4). Cholesterol levels were ~ 67% lower in mice fed the DHA diet than mice fed the control diet (Fig 4). Moreover, cholesterol level in the liver was ~18% lower in *E4+* mice than *E3+* mice (Fig 4).

4. Discussion

Since apoE is a protein involved in cholesterol and fatty acid metabolism, we sought to evaluate whether there was an interaction between *E4+* genotype and a diet containing DHA. Our results support that plasma and liver cholesterol homeostasis and the receptors regulating uptake of cholesterol in the liver are differently and independently modulated by *APOE* allele and DHA intake.

Our results showed that plasma cholesterol levels were lower in *E4+* mice than *E3+* mice. Previous studies did not report difference in plasma cholesterol levels between *E4+* mice and *E3+* mice aged of 4 or 12 months [20-21] . One explanation as to why our results differ from the one published by other groups might rely on the diet composition: our diet had 5% fat and no extra added vitamins while the diet of the other investigators contained 21% fat [21] or vitamins [22]. Indeed, dietary fat composition and vitamin E supply affect hepatic lipogenesis and lipoprotein oxidation [22-23] Our results are also opposite to what is reported in humans where plasma total cholesterol levels were 2.3-6.5% higher in *E4+* than *E3+* [16, 24-25]. However, cholesterol metabolism differs between mice and humans since mice are deficient in cholesteryl ester transfer protein (CETP), which is involved in the transfer of cholesteryl ester from high-density lipoprotein (HDL) to other lipoproteins. Hence, in mice, cholesterol is mainly carried by HDL particles but in humans, LDL mainly carries cholesterol. In another study, introducing human CETP gene into mice reduced HDL levels while VLDL and LDL cholesterol were slightly increase [26-27]. Unfortunately, in this study, we did not perform a lipoprotein profile because we did not collect enough blood at sacrifice so we are not in a position to confirm this hypothesis.

Our results also showed that only *E3+* mice fed the DHA diet have lower plasma cholesterol levels. It has previously been reported that fish oil lowers the secretion and synthesis of lipoproteins in chick, rabbit and monkeys [30-32]. However, to our knowledge, there is currently no study reporting lipoproteins receptors in the liver of *E3+* and *E4+* mice fed a DHA diet. To understand why plasma cholesterol levels were not lowered in *E4+* mice fed the DHA diet, we investigated liver cholesterol receptors.

One key receptor to cholesterol homeostasis is the LDLR because it mediates removal of LDL, it is involved in chylomicron remnants uptake by binding with apolipoprotein B-100 and apoE and it plays a major role in regulating plasma cholesterol levels [33]. Here, we report for the first time that liver mRNA and protein LDLR expressions were higher in *E4+* mice than *E3+* mice. It is known that *E4+* mice have higher levels of LDL compared to *E3+* mice [34]. Hence, the overexpression of LDLR in the liver of *E4+* mice may be a compensatory mechanism to favor LDL removal from the plasma. Moreover, this process might be explained by a downregulation of the liver X receptor (LXR) pathway in *E4+* mice. LXR is a transcriptional

226 factor targeting many genes such as *APOE* and inducible degrader of the LDLR *(IDOL)* [35-36],
227 the latter mediates the ubiquitylation and degradation of LDLR. Mice infected with an adenoviral
228 vectors encoding the overexpression of mouse *IDOL* had lower LDLR protein and higher plasma
229 cholesterol levels [36]. In this study, *E4*+ mice had higher LDLR, lower cholesterol and lower
230 plasma apoE, all of which are in line with a downregulation of LXR pathway. Hence, to confirm
231 this hypothesis, further experiments using hepatocytes isolated from *E3*+ and *E4*+ mice should
232 be used to investigate the LXR pathway.

233 Another study using mice heterozygous for the human *LDLR* minigene were bred to mice
234 homozygous for either the human *E3*+ or *E4*+ allele. Mice were fed a diet with similar fat
235 content compared to our diet. The authors reported lower plasma cholesterol levels in *E4*+ mice
236 that were overexpressing LDLR compared to *E4*+ mice that were not overexpressing LDLR [37].
237 Hence, overexpression of LDLR in *E4*+ mice partially explains why plasma cholesterol levels
238 were lower than *E3*+ mice in our study.

239 Compared to mice fed the control diet, the ones consuming DHA had lower levels of
240 LDLR mRNA, but protein expression was unchanged. This result suggests that the diet effect
241 was more at the translational levels but posttranslational mechanisms might also be involved
242 since LDLR protein expression was unchanged. Mice consuming DHA also had lower LSR
243 receptor protein levels and mRNA expression compared to mice fed the control diet and this was
244 independent of *APOE* allele. This receptor is mainly involved in postprandial lipemia regulation
245 and its activity is regulated by plasma free fatty acids (FFA). When FFA interact with LSR, its
246 conformation is modify to expose a lipoprotein-binding site [38]. Oleate and palmitate
247 demonstrated the strongest response [38] whereas the response of DHA has never been
248 investigated. Here, we speculate that DHA could improve the activity of LSR, resulting in a
249 lower protein expression. The biochemical assay developed by Mann et al using purified plasma
250 membranes from rat hepatocytes should be used to investigate this hypothesis [38]. In $LSR^{+/-}$
251 mice, lipoprotein clearance was lower hence leading to higher plasma total cholesterol levels [6],
252 [39]. Since in this study, plasma cholesterol levels are lower in *E3*+ mice fed the DHA diet
253 compared to the *E3*+ mice fed the control diet, we expected higher levels of LSR in mice fed the
254 DHA diet but we report the opposite. However, mice were not fasted and LSR might not be the
255 key receptor to explain the diet effect in *E3*+ mice. Another hypothesis as to how DHA could

have lowered plasma cholesterol is with regards to lower intestinal absorption. Interestingly, two studies reported lower cholesterol absorption in rat and monkey after consuming a diet with n-3 PUFAs [40-41].

The mechanisms underlying the relationship between DHA intake, blood and liver cholesterol levels are not fully understood. One study in rats reported that dietary n-3 PUFA might improve LDL clearance by the liver without changing hepatic LDLR expression [42]. This is consistent with our results since the LDLR protein level was not modified by the diet while cholesterol levels was lower in the plasma. Our result might also be explained by higher hepatic LDLR activity since one study reported a higher hepatic LDLR activity in rats fed a DHA diet [42]. Another root of explanation for difference in response to the DHA diet in terms of plasma cholesterol levels between *E3*+ and *E4*+ mice involves the levels of apoE proteins in the plasma and in the liver of *E4*+ mice. Indeed, apoE levels were lower in the plasma and higher in the liver of *E4*+ mice than *E3*+ mice and apoE is a protein binding to LDLR and LSR so this could have changed the number of binding site for clearing lipids from the blood. Our results with regards to apoE levels in the plasma are similar to the one reported in *E4*+ mice from other investigators [43], [44] and parallel the results in humans [45]. However, in the liver, apoE levels were higher in *E4*+ mice than *E3*+ mice and this was independent of the diet. Liver LDLR overexpression in *E4*+ mice could increase apoE uptake and transport in the liver.

One intriguing result we obtained was the 67% lower liver cholesterol levels in *E3*+ and *E4*+ mice fed the DHA diet. There was also a genotype effect mainly driven by the 22% lower cholesterol levels in the liver of *E4*+ mice fed the control diet compared to *E3*+ mice fed the same diet. This range of lower liver cholesterol level was also reported by Vasandani et al. [46] in a LDLR knock out mouse model fed n-3 PUFA and in rats fed a DHA enriched diet [46-47]. Since LSR was originally identified as a liver receptor for the uptake of both trigycerides-rich particles and LDL, lower expression of LSR in the liver might contribute to explain why cholesterol levels were lower in the liver of mice fed DHA. Moreover, bile acid synthesis is a major pathway for hepatic cholesterol catabolism. In line with this, one study have shown that a diet enriched with DHA increases biliary secretion of cholesterol and other lipids in rats [47]. These speculations clearly need further investigations in *E3*+ and *E4*+ mice.

In conclusion, our results show that *E4*+ allele is associated with an overexpression of LDLR in the liver, lower plasma apoE levels, higher liver apoE levels and lower plasma cholesterol levels. Moreover, DHA intake has lowered cholesterol in the plasma of *E3*+ mice only and lowered cholesterol levels in the liver of *E3*+ and *E4*+ mice. mRNA or protein expression of lipoprotein receptors are lower with DHA intake. Taken together, our results showed that plasma and liver cholesterol homeostasis and the receptors regulating uptake of cholesterol in the liver are independently modulated by *APOE* allele and DHA intake. In light of these results, brain cholesterol homeostasis in response to a DHA supplement and in relation with cognition needs to be performed. This would be particularly relevant in *E4*+ since they are not protected against cognitive decline when eating DHA.

Conflict of Interest

The authors declare to have no conflict of interest.

Acknowledgements

This work was supported by the Canadian Institutes of Health Research (MOP119454), the Natural Sciences and Engineering Research Council of Canada, Institut de la Nutrition et des Aliments Fonctionnel (INAF) and centre de recherche sur le vieillissement du CIUSSS de l'Estrie CHUS for a postdoctoral scholarship to AP and Fonds de recherche du Québec – Santé for a Junior 2 salary award to MP.

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FIGURE LEGENDS

Fig 1: Apolipoprotein E ε4 (*APOE4*) allele and docosahexaenoic acid (DHA) diet are associated with decreased plasma cholesterol. Plasma cholesterol levels in mice knock-in for *APOE3* allele (E3, open bars) or in mice knock-in for *APOE4* allele (E4, solid bars) fed a control or a DHA diets were measured as described in materials and methods. Results are mean ± SEM (n=6/group) and P values are indicated when there was a significant result after comparing E4 to E3 groups. Two-way ANOVA were performed with apolipoprotein E genotype and diet as fixed factors. Subgroup analyses with t-test were performed.

Fig 2: Apolipoprotein E ε4 (*APOE4*) allele and docosahexaenoic acid (DHA) diet are associated with lower liver lipoprotein receptor expression. Membranes liver extracts from 12 months old mice knock-in for *APOE3* allele (E3, open bars, n=8-10) and mice knock-in for *APOE4* allele (E4, solid bars, n=8-10*)* fed a DHA or control diets were analyzed by western immunoblotting for protein levels of LDLR (A), LSR (C) and LRP1 (E). Top panels: representative blots for individual animals. Bottom panels: bar graphs of mean ± SEM values. Liver samples were analysed by quantitative RT-PCR for mRNA levels of LDLR (B) and LSR (D) in E3 and E4 mice on DHA or control diets. Two-way ANOVA were performed with apolipoprotein E genotype and diet as fixed factors.

Fig 3: Apolipoprotein E ε4 (*APOE4*) allele is associated with impaired liver and plasma apoE levels. Membranes liver extracts from 12 months old mice knock-in for *APOE3* allele (E3, open bars, n=8-10) and mice knock-in for *APOE4* allele (E4, solid bars, n=8-10) fed a docosahexaenoic acid (DHA) or control diets were analyzed by western immunoblotting for protein levels of apoE (B). Top panels: representative blots for individual animals. Bottom panels: bar graphs of mean ± SEM values. Plasma apoE levels were measured in 12 months old E3 mice and E4 mice (A) as described in materials and methods. Mean ± SEM values are shown. Two-way ANOVA were performed with apolipoprotein E genotype and diet as fixed factors.

Fig 4: Apolipoprotein E ε4 (*APOE4*) allele and docosahexaenoic acid (DHA) diet are associated with decreased liver cholesterol. Liver cholesterol levels were measured in 12 months old mice knock-in for *APOE3* allele (E3, open bars, n=8-10) and in mice knock-in for *APOE4* allele (E4,

491 solid bars, n=8-10) fed a DHA or control diets as described in materials and methods. Two-way
492 ANOVA were performed with apolipoprotein E genotype and diet as fixed factors.

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