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*Article*

# Lipid Dys-Homeostasis Contributes to APOE4 Associated AD Pathology

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**Abstract:** The association of the APOE4 (vs APOE3) isoform with an increased risk of Alzheimer's Disease (AD) is unequivocal, but the underlying mechanisms remain incompletely elucidated. A prevailing hypothesis incriminates the impaired ability of APOE4 to clear neurotoxic amyloid-β peptides (Aβ) from the brain as the main mechanism linking apolipoprotein isoform to disease aetiology. APOE protein mediates lipid transport both within the brain and from the brain to the periphery, suggesting that lipids may be potential co-factors in APOE4-associated physiopathology. The present study reveals several alterations in pathways of lipid homeostasis in the brains of mice expressing the human APOE4 *versus* APOE3 isoform. Carriers of APOE4 had deficient cholesterol turnover, an imbalance in the ratio of specific classes of phospholipids, lower levels of phosphatidylethanolamines bearing poly-unsaturated fatty acids and an overall elevation in levels of monounsaturated fatty acids. These modifications in lipid homeostasis were related with increased production of Aβ peptides as well as augmented levels of tau and phosphorylated tau in primary neuronal cultures. This suite of APOE4-associated anomalies in lipid homeostasis and neurotoxic protein levels may be related to the accrued risk for AD in APOE4 carriers and provides novel insights into potential strategies for therapeutic intervention.

**Keywords:** lipid homeostasis; APOE4; Alzheimer's Disease; Aβ peptide; tau

## 1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia and a major public health concern, with >130 million cases worldwide anticipated by 2050. Late-onset sporadic AD (sAD) represents more than 90% of AD cases, and is associated with age-related cellular and molecular alterations in the brain. Two main neuropathological features characterize AD: the accumulation of aggregates of amyloid β (Aβ) peptides in extracellular brain parenchyma and in the perivascular areas (senile plaques and amyloid angiopathy respectively) and intracellular fibrils of hyper-phosphorylated tau (the neurofibrillary tangles) [1]. Besides these two defining hallmarks, numerous studies indicate an implication of lipid metabolism in the development and the progression of the disease. The presence

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of abnormal lipid granules in neuroglia was noticed from the time of Alois Alzheimer [2–4]. Later on, lipidomic approaches revealed alterations in the level of several specific lipids in AD brains, from cholesterol and ceramides to plasmogens [5–7]. Genome-Wide Association Studies (GWAS) and Transcriptome-Wide Association Studies (TWAS) [8,9] later identified several lipid-related genes associated with sAD pathology. Among them, APOE, low-density lipoprotein receptors LRP1, acyltransferases ACAT1, ABCA1 and ABCA7 transporters, and the enzyme CYP46A1 [10–16] may be highlighted. In fact, *APOE ε4* allele – encoding the lipid binding apoliprotein E - is the strongest genetic risk factor for AD [17], and *APOEε4* carriers are more susceptible to brain amyloid burden, even at asymptomatic stages [18]. The APOE4 isoform is less efficient in clearing Aβ from the brain to periphery [19–21], enhancing the conversion of the peptide into toxic species and increasing plaque load in brain parenchyma [22,23]. The APOE4 isoform was also associated with an exacerbation of tau pathology in a mouse model of taupathy [24] and altered blood brain barrier integrity [25]. Furthermore, astrocytes and neurons carrying the *APOEε4* allele have decreased lipid-binding capacity [26]. Taken together, these findings point to a cumulative effect of APOE genotype combined with altered lipid homeostasis in AD pathology.

Prevailing evidence points, thus, to a strong link between *APOE* genotype and lipid metabolism in the progression of AD but this relationship is not yet fully understood. Studies on human *APOE3/4* knock-in (KI) mice, revealed no effect of the genotype on brain levels of cholesterol and intermediates/metabolites (lathosterol, desmosterol and oxidized metabolites) [27,28]. The global level of phospholipids in the brain of APOE3 and APOE4 KI-mice was examined in a separate study that found no major effect of the genotype on the abundance of different classes of phospholipids [29]. Another study focused on the analysis of fatty acids in the hippocampus and cortex of male and female APOE3 and APOE4 KI-mice, yet the analysis revealed only age and gender - associated alterations [30]. These investigations have not, then, proven sufficient to elucidate the cellular substrates of the interplay between APOE genotye, lipid homeostasis and AD pathology.

In order to further clarify the link between *APOE* genotype and lipid metabolism and to elucidate their joint impact on AD pathology, we characterized the brain lipidomic profile of KI mice expressing human APOE3 or APOE4 and related it to potential alterations in levels of the neurotoxic proteins, Aβ and tau. We exploited a suite of highly sensitive mass spectrometry (MS)-methodologies for broad-based lipidomic analysis: *first*, gas chromatography coupled with triple quad mass spectrometry (CG-MS/MS) for determination of cholesterol and its derivatives; *second*, Liquid-Chromatography coupled with tandem mass spectrometry (LC-MS/MS) for characterisation of the species of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), and *third GC* for fatty acid quantification. The expression of the genes involved in the metabolism of the dysregulated lipids was also analysed employing a recently published RNAseq data set that use the same mouse models [31]. Finally, in order to evaluate whether the identified perturbations in lipid metabolism were associated with prototypical cellular signals of AD pathology, we measured the levels of Aβ, tau and hyper-phosphorylated tau (p-tau) in primary neurons derived from the APOE3 and APOE4 KI mice.

APOE4 mice revealed several alterations in lipid homeostasis, including an increase in the ratios of cholesterol/desmosterol and cholesterol/β-cholestanol, an aberrant PC/PE ratio, and a reduction in PE- bearing polyunsaturated fatty acids (PUFA), particularly ω3-fatty acids in APOE4 mice. We also observed that changes in lipid homeostasis in the brain of APOE4 mice were accompanied by higher production of Aβ peptides and increased levels of tau and p-tau, unveiling

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the interrelationship between anomalous lipid metabolism dysregulation and core AD pathology.

## 2. Materials and Methods

### *2.1 Sample preparation and lipid extraction*

Half forbrain tissues from adult B6.129P2-*Apoe*$^{tm3(APOE*4)Mae}$ N8 and B6.129P2-Apoetm3(APOE34)Mae N8 12 months old male mice were collected and snapped frozen at -80°C, homogenized by cryogenic grinding of frozen samples, using a mortar and pestle cooled up with liquid nitrogen. Mice were ordered from TACONIC (www.taconic.com).

Total brain homogenates (approx. 130mg) were extracted according to Folch procedure (FOLCH et al., 1957): 2ml of cold methanol-BHT (butyl-hydroxytoluene) spiked with several standards (50µg or PC di-17:0, PE di-17:0 and TG tri-17:0, 5µg of cholesterol, 10ng of PGE2, LTB4 and HETEd8) were added to the homogenate and stirred for 10 min, at 4°C. The homogenates were further incubated with 4 ml of chloroform for 2h at 4°C and the mixture was adjusted with 1.5ml of water. It was further vortexed for 30s and centrifuged for 5 min at 2000 rpm. The lower (organic) phase was removed and a second extraction was performed, by adding 4 ml of chloroform to the remaining aqueous phase. The two extracts were combined and dried under nitrogen (protocol adapted from Sharman et al, 2010 [29]*).* Lipid extracts resolubilized in chloroform/methanol 2:1 (v/v) were dispatched in three loads for targeted lipidomics (cholesterol and derivatives, phospholipids and fatty acids).

### *2.2 Lipid analysis*

Lipid analysis were performed on the Functional Lipidomics platform acknowledged by IBiSA (Infrastructure in Biology, Health and Agronomy).

*Analysis of cholesterol and derivatives by GC*

Cholesterol was isolated from the total lipid extract by Thin Layer Chromatography as follows: 50µl of total lipid in chloroform were deposited on a silica plate, along with several standards: cholesterol, triglycerides, phospholipids, diglycerides and cholesteryl esters. A mixture of Hexan:Diethylether:Glacial Acetic Acid (80:20:1, v/v/v) was used as eluent. The different classes of lipids were revealed by spraying the plate with 0.02% dichlorofluorescein in ethanol. The band corresponding to free cholesterol was retrieved by scratching with a cutter and cholesterol and its derivatives were extracted from the silica gel using chloroform. The dry residue was derivatized with BSTFA (*N, O-bis (trimethylsilyl)trifluoroacetamide*) and then analysed by gas chromatography coupled with a triple quad mass spectrometry (GC-MS/MS) using electron impact ionization (EI) mode (Agilent, 7000C).

*Phospholipids analysis by electrospray mass spectrometry (ESI-MS)*

The phospholipids (PL) were isolated from the total lipid extract, in parallel to cholesterol, by Thin Layer Chromatography. The band corresponding to PL was retrieved by scratching and the lipids were extracted from the silica gel using toluene-methanol (60:40). They were dried under $N_2$ atmosphere and resolubilized in methanol for LC-MS analysis.

*Analysis of FA composition by GC*

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A total volume of 50µl of total lipid extract was evaporated and resolubilized in 500µl toluen-methanol (1:1, v/v) for methylation. The methylation reaction was performed in the presence of 500µl BF3/methanol (14%) used as catalyser, under $N_2$ atmosphere, by heating at 100°C for 90 min. The reaction was stopped by fast cooling of the samples and the addition of $K_2CO_3$ (10% in water). Isooctane was further added to extract the methylated fatty acids– the upper organic phase; the extraction is performed 2 times. The samples were dried under $N_2$ and re-solubilized isooctane for GC analysis.

*2.3 Cultures of primary neurons*

Primary cortical neurons cultures were performed by dissecting P0-P1 B6.129P2-*Apoe*$^{tm3(APOE*4)Mae}$ N8 and B6.129P2-*Apoe*$^{tm3(APOE34)Mae}$ N8 new born mice as described before (Boussicault et al., 2018). Cortices were incubated in L15 medium (Invitrogen) containing 0.05% trypsin/EDTA (Gibco) for 8 min at 37°C. Mechanical dissociation was applied to the cells kept in Neurobasal (without glutamine, Gibco) with 2% B27 (Gibco), 1% N2-supplement (Thermofisher scientific), 1% Glutamax (Gibco) and 1% penicillin/streptomycin (Gibco, and DNAse I (10 000U/mL, Serlabo) and 30% FCS. Cells were centrifuged 5 min at 152g at 4°C, plated in 6 wells microtiter plates coated with poly-D-lysine (0.1mg/mL, Sigma Aldrich) and grown in 2% B27, 2 mM glutamax, 1% penicillin/streptomycin (all from Life Technologies) at 37°C 5% $CO_2$. Half of the medium was changed at DIV7. After 5, 9 and 12 days in vitro (DIV5, DIV9 and DIV12), supernatants were aliquoted in 1.5mL polypropylene tubes (Corning, Corning, NY, USA) containing a protease inhibitor cocktail (Roche, Penzberg, Germany) and were then stored at −80°C.

*2.4 Aβ 38, 40 and 42 measurements*

Concentrations of the Aβ38, Aβ40 and Aβ42 species of β-amyloid peptide were measured by multiplex Electro-Chemiluminescence Immuno-Assay (ECLIA). Assays were performed according to the manufacturer's instructions. Briefly, samples were analysed using Meso Scale Discovery (MSD) SECTOR™ Imager 2400 (Meso Scale Discovery, Gaithersburg, MD, USA), with the Rodent Aβ triplex kit (also from MSD); carbon 96-well plates contained in each well four capture spots, one of which was blocked with BSA (as standard curve control), and the three others coated with isoform specific anti-Aβ antibodies specific for Aβ38, Aβ40, Aβ42, respectively. 100 µl of blocking buffer solution were added to all wells to avoid non-specific binding. The plates were then sealed, wrapped in tin foil, and incubated at room temperature on a plate shaker (600 rpm) for 1 h. Wells were then washed three times with washing buffer, and 25 µl of the standards (Aβ38, Aβ40, Aβ42) and samples were then added to the wells, followed by an Aβ-detecting antibody at 1 µg/ml (MSD) labelled with a Ruthenium (II) trisbipyridine N-hydroxysuccinimide ester; this detection antibody was 4G8 (which recognizes the epitope Aβ18-22 of the human and rodent peptide). Plates were then aspirated and washed 3 times. MSD read buffer (containing TPA) was added to wells before reading on the Sector Imager. A small electric current passed through a microelectrode present in each well producing a redox reaction of the Ru2+ cation, emitting 620 nm red light. The concentration of each Aβ isoform was calculated for each sample, using dose–response curves, the blank being cell-less culture medium.

*2.5 Western blotting*

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Primary neurons were washed with 1X PBS and then lysed on ice using RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) (Sigma Aldrich), to which were added phenylmethylsulfonyl fluoride 100X (Sigma Aldrich) and a cocktail of inhibitors of proteases (Complete Mini, Roche). Lysates were sonicated 3 times for 5 min then stored at -80 °C. Protein concentration of the lysates was quantified by Bradford assay (Biorad) according to the manufacturer's instructions. Western blots were made from the cell lysates. Proteins from cultured lysates were separated in 16.5% Tris-Tricine (Biorad) polyacrylamide gels. Proteins were transferred to a polyvinylidene difluoride membrane (Biorad) at 150 V for three hours at 4 °C. After 1 hour of saturation with 10 % milk, membranes were incubated overnight at 4 °C with primary antibodies directed against APP (APPCter-C17 [34]), actin β (Sigma Aldrich), total tau (B19[35]) diluted 1/2000 and Phospho-tau (AT8 Thermofisher) diluted 1/1000. Membranes were incubated with fluorescent goat secondary antibodies (diluted to 1/10,000 in 0.05 % TBS-tween solution) anti-mouse (LI-COR) for anti-actin and AT8 and anti-rabbit (LI-COR) for anti-APP, total tau and APOE, for 1 hour under agitation at room temperature and away from direct light. The revelation and quantification of fluorescence were carried out by Odyssey's analysis software (Set up ImageStudio CLx) (Odyssey Clx LI-COR).

*2.6 Data processing*

Lipid analysis was performed with conventional methods. Average and standard error were estimated over 8 samples/phenotype. For each class of phospholipid their species were expressed as a percentage of total content. Only the species with a relative abundance greater than 1% were considered. Statistical analysis was performed using multiple paired student test and ANOVA. Statistical significance was defined as: * for $p < 0.05$, ** for $p<0.005$ and ***: $p <0.001$.

## 3. Results

*3.1. APOE genotype disturbs cholesterol turnover*

The main role of APOE in the brain is to transport cholesterol. To determine if there is a direct link between APOE genotype and cholesterol production and transport, we investigated the levels of cholesterol in the brain of human APOE3 and APOE4 KI mice. The abundance of free cholesterol in the two isogenic APOE mice was similar (Figure 1A), as previously described in the literature [36]. We also inspected the level of cholesterol precursors and derivatives. Besides cholesterol, we detected two of its derivatives: desmosterol, a precursor of cholesterol and *beta*-cholestanol, a metabolite of cholesterol. Significantly lower levels of desmosterol and β-cholestanol were found in APOE4 mice (Fig 1A). These findings suggested impaired cholesterol homeostasis associated with *ε4* allele. In the brain, cholesterol level is regulated by synthesis, metabolism (degradation) and transport within a cell and from cell to cell. Thus, in order to evaluate cholesterol turnover, we measured the ratios cholesterol-to-desmosterol and cholesterol-to-β-cholestanol (Figure 1B). The ratio cholesterol/desmosterol as well as cholesterol/β-cholestanol were significantly increased in APOE4 mice suggesting an imbalance between cholesterol and the other sterols.

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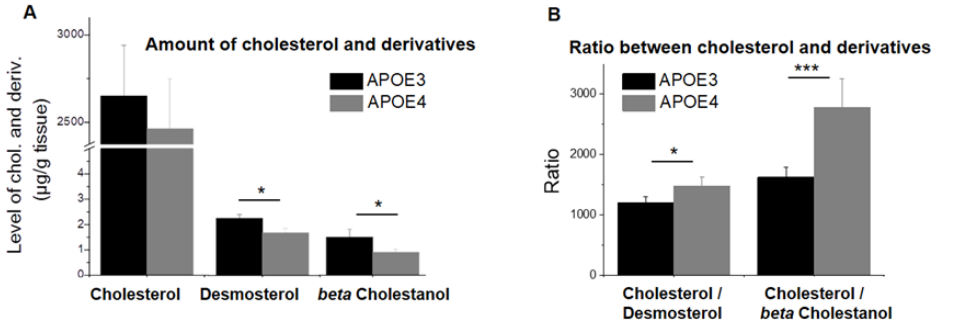


**Figure 1**. Level of cholesterol and other sterols in the brain of APOE3 and APOE4 KI mice: A- amount of different sterols detected; B – ratio between cholesterol and derivatives; the results are means of 8 samples/ APOE genotype; Statistics: Multiple paired t-test *: p <0.05, **: p <0.01, ***: p <0.001; n= 8 brains per genotype

*3.2 Levels of PC and PE and changes in their composition in APOE4 KI mice*

APOE is not only a transporter of cholesterol but also of phospholipids in the brain. We investigated the impact of *APOE* genotype on the main classes of phospholipids in the brain: phosphatidylcholines (PC) and phosphatidylethanolamines (PE). Both classes showed a similar profile in the brain of APOE3 mice (Figure 2A). In APOE4 mice the proportion of PC is considerably increased with respect to PE, the ratio PC/PE being significantly higher (Figure 2B). We further investigated potential alterations in the composition of PC and PE for the two *APOE*

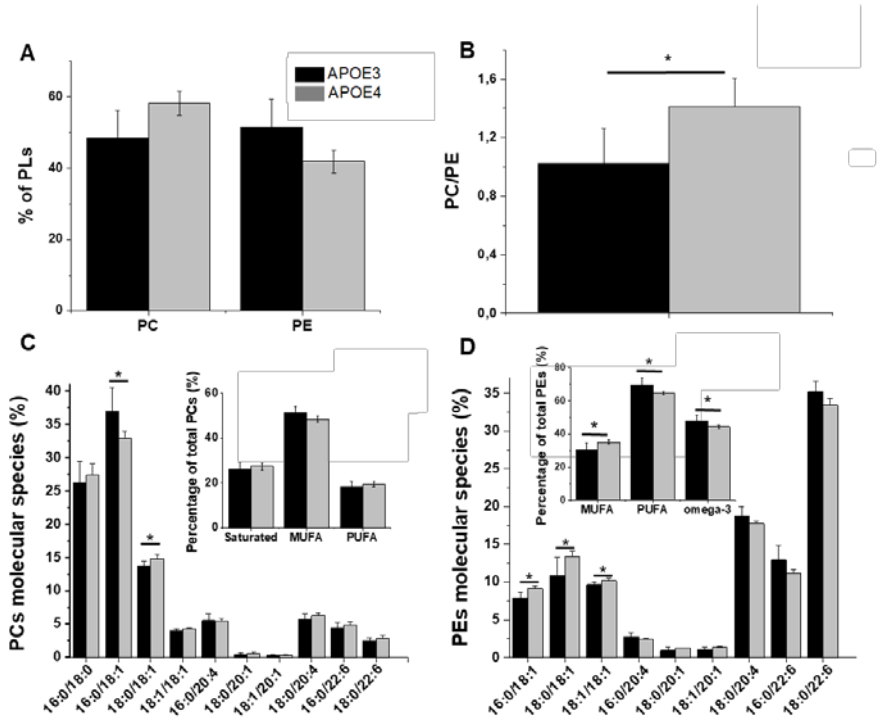


**Figure 2.** Distribution of the two major classes of phospholipids detected in APOE3 and APOE4 mice: A: percentage of PCs and PEs in the brain of APOE3 and APOE4 mice; B: the ratio PC/PE; C: molecular species of PCs and percentage of PCs bearing saturated, monounsaturated (MUFA) or polyunsaturated fatty acids (PUFA); D: molecular species of PEs and percentage of PEs bearing monounsaturated (MUFA), polyunsaturated fatty acids (PUFA) or omega-3 fatty acids; Statistics: Multiple paired t-test *: p <0.05, **: p <0.01, ***: p <0.001; n= 8 brains per genotype

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genotypes. The most abundant molecular species of PC is PC (16:0/18:1) followed by PC (16:0/18:0) and PC (18:0/18:1). In ApoE4 mice PC (16:0/18:1) was depleted while PC (18:0/18:1) was enriched (Figure 2C). Still, the level of the other phosphatidylcholine species bearing long chain fatty acids or the degree of unsaturation was similar for APOE3 and APOE4 mice. *APOE* genotype had a strong effect on PE species containing oleic acid (18:1), namely PE (16:0/18:1), PE (18:0/18:1) and PE (18:1/18:1), that were upregulated in APOE4 genotype (Figure 2D). In addition, significantly higher levels of PE species bearing monounsaturated fatty acids (MUFA) and lower levels of PE species with polyunsaturated fatty acids (PUFA) were observed, particularly omega-3 fatty acids. Actually, information concerning changes in the degree of unsaturation of PE fatty acids in *APOE4* allele is limited although it is clear that PUFA are critical for normal neuronal functioning and neuroprotection [37–39].

### *3.3 APOE genotype and fatty acid profile and metabolism*

In order to determine whether *APOE* genotype alters the global balance of fatty acids in brain lipids or only in phospholipids, we have profiled their extent in APOE3 and APOE4 KI mice. The level of several fatty acids was dependent on APOE genotype: palmitic acid (16:0) was significantly decreased in APOE4 mice while eicosenoic acid (20:1n-9), lignoceric acid (24:0) and the nevronic acid (24:1n-9) were enriched (Fig 3A). This enrichment was associated with a significant increase in the level of MUFA, especially omega-9 fatty acids, counterbalanced by a decrease (although non-significant) in PUFA (Fig. 3B and 3C). There was no interaction between *APOE* genotype and the global level of omega-3 and omega-6 fatty acids. These unsaturated fatty acids are important components of cell membrane and involved in complex metabolic pathway.

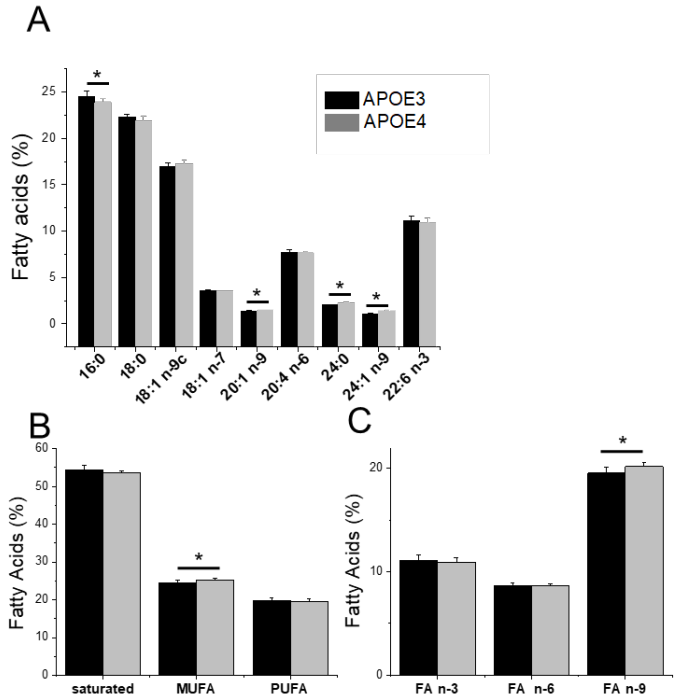


**Figure 3.** Fatty acids of the total lipid extract of APOE3 and APOE4 mice: A – overview of the fatty acid composition; B – level of fatty acids classified as a function of their degree of unsaturation; C – proportion of PUFA; Statistics: Multiple paired t-test *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$; n= 8 brains per genotype

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*3.4 Lipid dysregulations and gene expression in APOE genotypes*

The imbalance in cholesterol turnover and lipid homeostasis was linked to dysfunctions in the production or the metabolic pathway, as revealed by the expression levels of genes encoding several key enzymes from a recently published set of RNAseq data [31] (Table S1). Several of the genes involved in cholesterol synthesis and metabolism showed a reduced expression: DHCR7 - the reductase involved in the final steps of cholesterol synthesis, HSD3b - that converts cholesterol to *beta*-cholestanol, and CYP46A1 - that controls cholesterol removal from the brain (Figure 4). The expression level of different genes involved in phospholipid production and processing was also altered in APOE4 genotypes. Pcyt2 gene, belonging to cytidylyltransferase family, a key regulator of PE synthesis was underexpressed while Pcty1b gene, related to PC synthesis was overexpressed in APOE4 mice males. Moreover, two of the genes involved in the rate-controlling of FA metabolism, from the family of elogases (ELOVL – ELongation of Very Long fatty acids) were also altered; the level of expression of ELOVL5, (known to have a certain preference for PUFA) [40] was lower, while the one of ELOVL7, (particularly selective for saturated or MUFA) [41] was significantly increased in APOE4 males.

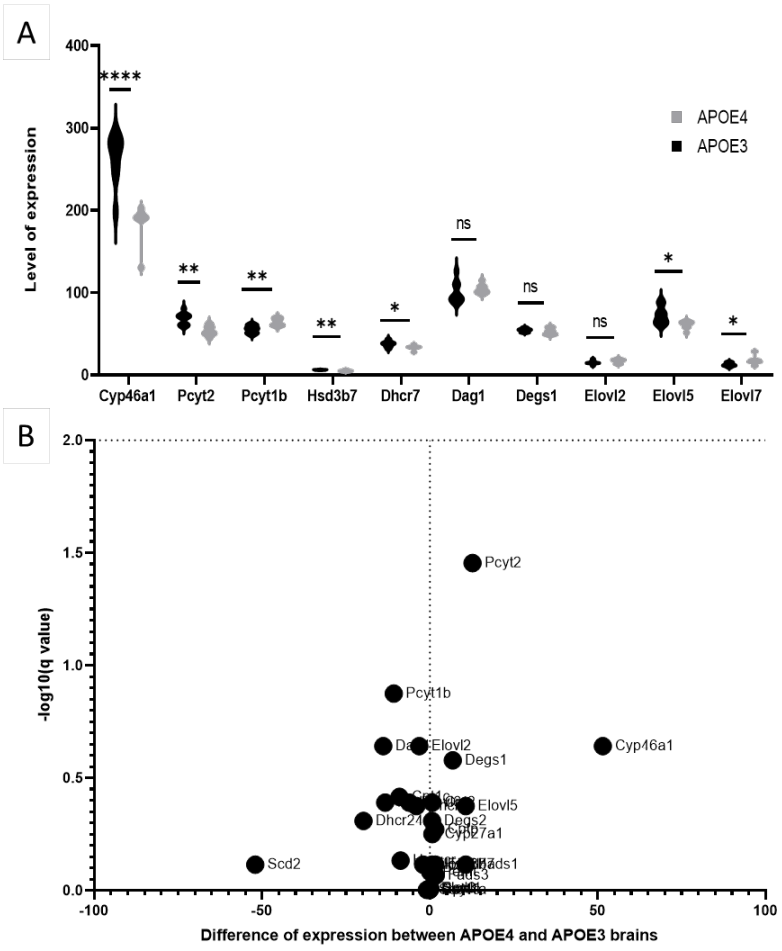


**Figure 4**. Gene expression of enzymes form the cholesterol pathway in the brain of KI mice carrying the human APOE3 or APOE4 alleles (PMID32703241). **A** Level of expression analysed with multiple unpaired t-test *: p <0.05, **: p <0.01, ***: p <0.001; n=6 to 8 brains per genotype. **B** Volcano plots

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*3.5 Primary neurons from APOE4 KI mice produce higher amount of Aβ peptide*

The effect of *APOE* genotype on the capacity of neurons to produce Aβ peptides was investigated on primary cortical neurons of APOE3 and APOE4 KI mice. We observed a significant overproduction of the three main Aβ peptides: 38, 40 and 42 in APOE4- as compared to APOE3-derived neurons (Figure 5). Moreover, the over-accumulation of the peptides in the culture media in APOE4 neurons was heightened overtime. Aβ38 was undetectable at div 5 (5 days in vitro).

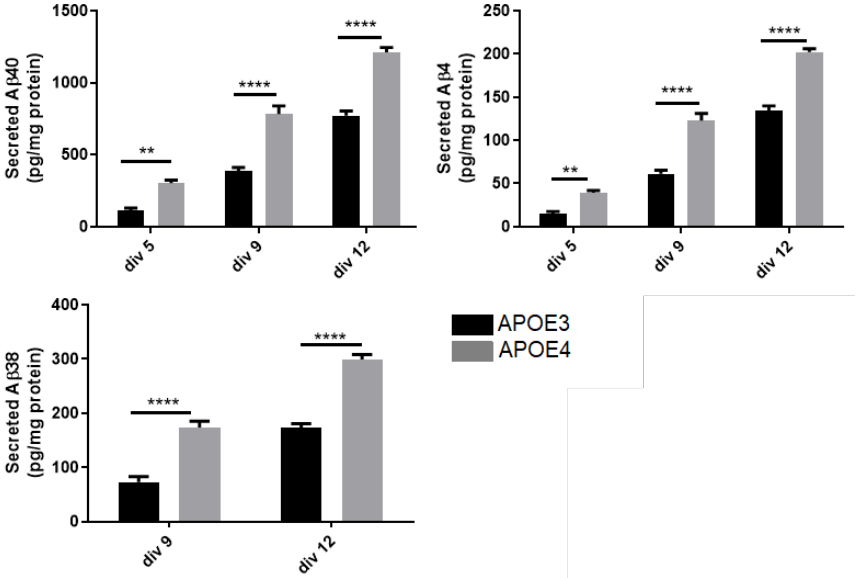


**Figure 5.** Effects of APOE genotype on Aβ secreted by primary neurons. Primary cortical neurons cultures were obtained from P0-P1newborn KI mice carrying the human APOE3 or APOE4 alleles. After 5, 9 or 12 days in culture (DIV5, DIV9 and DIV12), secreted Aβ38, Aβ40 and Aβ42 were assessed in the culture medium using the MSD multiplex ELISA (pg/mg of proteins measured in cells lysates). Statistics: two way ANOVA mean +/- SEM, *: p <0.05, **: p <0.01, ***: p <0.001, comparison with APOE3, 3 independent cultures with 2 to 3 replicates per culture.

We verified that overproduction of Aβ peptides in APOE4 mice was not due to an overexpression of APP. Indeed, levels of APP protein measured by western blotting were similar in APOE3 and APOE4 derived neurons (Figure 6A). Since astrocytes are the main source of APOE production in the brain (Liu et al., 2017), we also investigated the levels of intracellular APOE. APOE was significantly reduced in APOE4-derived neurons, and thus its turnover probably increased, suggesting a dependence of Aβ production on APOE trafficking from the environment (Figure 6B).

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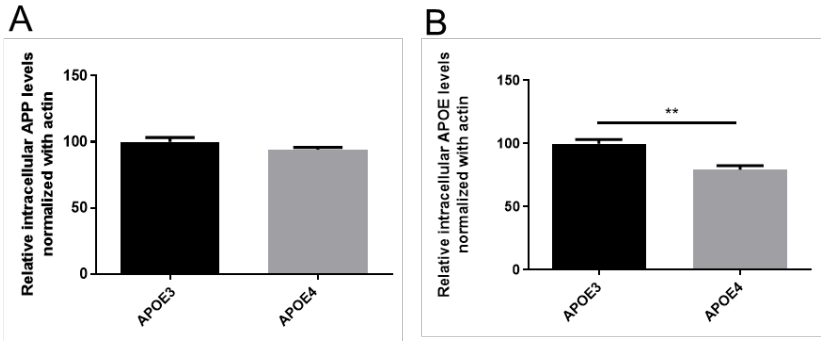


**Figure 6.** Effects of APOE genotype on intracellular levels of APP (A) and APOE (B). Primary cortical neurons cultures were obtained from P0-P1 newborn KI mice carrying the human APOE3 or APOE4 alleles. After 12 days in culture, APOE and APP levels were assessed in cell lysates using western blot and normalization with actin levels. Statistics: Mann and Whitney test *: p <0.05, **: p <0.01, ***: p <0.001, comparison with APOE3, 3 independent cultures with 2 to 3 replicates per culture.

We also questioned the involvement of APOE on tau pathology in our model. Levels of total tau were largely increased in APOE4- compared to APOE3-neurons. Similarly, levels of pathological phosphorylated tau were higher in APOE4-derived neurons (Figure 7). Thus, APOE4 was also able to heighten tau pathology as well.

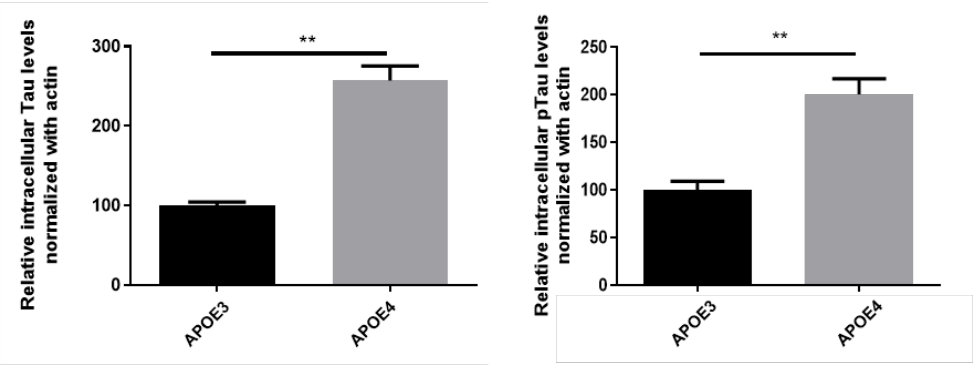


**Figure 7.** Effects of APOE genotype on intracellular levels of Tau and pTau. Primary cortical neurons cultures were obtained from P0-P1newborn KI mice carrying the human APOE3 or APOE4 alleles. After 12 days in culture, tau and p-tau levels were assessed in cell lysates using western blot and normalization with actin levels. Statistics: Mann and Whitney test *: p <0.05, **: p <0.01, ***: p <0.001, comparison with APOE3, 3 independent cultures with 2 to 3 replicates per culture.

## 4. Discussion

The *APOEε4* allele is the most prominent genetic risk factor for AD. Heterozygous carriers have a three-fold higher AD risk, while homozygous individuals have a 15-fold higher AD risk [43] corresponding to a prevalence 30% of AD cases at the age of 75 and over 50% by the age of 85 [44]. Many hypotheses have been proposed to explain the relationship between APOE4 and the pathogenesis of AD, most of them pointing to Aβ transport and clearance, that culminates with increased accumulation, spread and deposition in the brain [45,46]. Nonetheless, APOE may play a role in pathology not only downstream of Aβ production but also up-stream by influencing APP processing. Since evidence for a connection between lipid homeostasis, APOE, Aβ and tau pathology remains sparse [36,47],

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in this study, we questioned to what extent APOE variants may influence brain lipid homeostasis, Aβ peptide production and tau hyper-phosphorylation. In contrast to previous lipidomic studies that focused only on one particular lipid class – either cholesterol or fatty acids, the present approach comprises a comprehensive quantitative investigation of the main lipids that have been previously linked to AD pathology: cholesterol, phosphatidylcholines and phosphatidylethanolamines, and the related fatty acids. We have, furthermore, correlated the lipid dysregulations with the level of expression of several genes involved in lipid metabolism in addition to the pathological markers of AD like Aβ and tau / phospho-tau.

**Altered cholesterol turnover in APOE4-KI mice**

We first evaluated the homeostasis of cholesterol in the brains of APOE4- compared to APOE3-KI mice. We mainly detected cholesterol and two other sterols (desmosterol and *beta*-cholestanol). Desmosterol is a precursor of cholesterol while *beta*-cholestanol is a metabolite of cholesterol degradation. Though levels of lathosterol and 7-dehydrocholesterol, the alternative precursors of cholesterol were probably too low to be detected, in APOE KI mice. These data confirm that synthesis of cholesterol mainly uses the desmosterol pathway also known as the Bloch pathway (Figure 9A) [48,49]. The amount of cholesterol in brains of APOE4 mice was slightly diminished with respect to isogenic APOE3 mice (although not statistically significant), with desmosterol and *beta*-cholestanol showing significantly lower levels. Therefore, the ratios between cholesterol/desmosterol and cholesterol/*beta*-cholestanol were higher in APOE4 mice compared to APOE3. The decreased levels of desmosterol in APOE4 brains may correspond to reduced de novo cholesterol synthesis, mirroring observations in AD brains [50,51]. Indeed, the gene encoding DHCR7, an enzyme involved in down-stream cholesterol synthesis (Figure 9A), was under-expressed. On the other hand, lower levels of *beta*-cholestanol could be explained by restricted degradation of cholesterol in order to ensure its required efflux to neurons [52,53]. The reduced expression of the gene encoding HSD3β7 and CYP46A1 enzymes (both involved down-stream to cholesterol synthesis, Figure 9A) suggests the necessity to maintain a certain level of cholesterol for transport to neurons, as previously established [54–56].

The present study suggests that cerebral cholesterol synthesis is impaired in APOE4-KI mice and that cholesterol turnover is altered. Such an effect of *APOEε4* allele on brain desmosterol and *beta*-cholestanol levels has not been reported previously.

**APOE4 genotype is associated to lower levels of phosphatidylethanolamines bearing poly-unsaturated fatty acids**

We further investigated alterations in the composition of the two major phospholipids. We observed only modest modifications in the levels of PCs and PEs in APOE4 mice but a significant increase of the PC-to-PE ratio. These two phospholipids share similar but parallel steps in their bio-synthesis involving two rate limiting enzymes (Figure 9B): phosphoethanolamine cytidylyltransferase (PECT) – encoded by Pcyt2 and phosphocholine cytidylyltransferase (PCCT) - encoded by Pcyt1b, to generate CDP-ethanolamine and CDP-choline intermediates [57]. The enhancement of PC/PE ratio observed in APOE4 mice was related to these key enzymes, as transcripts of Pcyt2 and Pcyt1b showed altered expression in APOE4 genotypes: Pcyt1b -encoding PCCT was upregulated while Pcyt2, encoding PECT was underexpressed. In line with these observations, increased PCCT

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levels in AD brain (not linked to APOE phenotype) have previously been documented [58] as well as an elevated PC-to-PE ratio in AD mice, particularly in the hippocampus [59].

Besides alterations in phospholipids composition, the APOE4 isoform was also associated with changes in the array of fatty acids. The precise balance between saturation and unsaturation/poly-unsaturation determines the bio-mechanical properties of membranes and, consequently the handling of membrane-associated proteins. Both PC and PE had altered levels of long chain fatty acids (from 16 to 24 C atoms), either monounsaturated or polyunsaturated. MUFA-PE and the oleic acid-PE were elevated in APOE4. PUFA-PE and particularly PE containing omega-3 fatty acids were, on the contrary, significantly depleted. It is known that plasma saturated FA and MUFA levels increase with aging, while PUFA are decreased [60,61]. Saturated fatty acids provide a certain rigidity to membrane and MUFA have a low capacity to fluidify plasma membrane [62,63]. This is interesting since PUFA and especially omega-3 are the essential FA able to fluidify plasma membranes and to diminish lipid raft clustering [64,65].

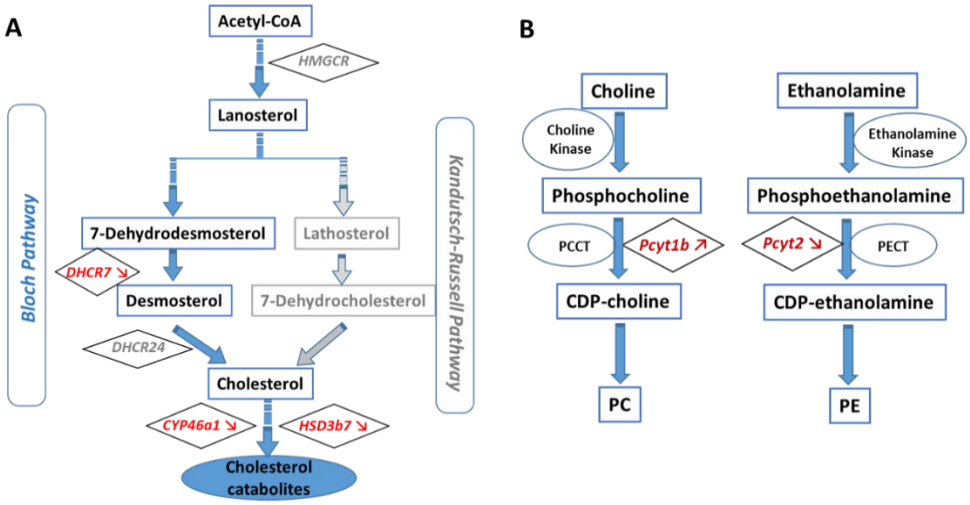


**Figure 8.** Pathway of cholesterol synthesis in the brain fromAcetylCoA reduction by HMGC reductase, through the action of 7-dehydrocholesterol reductase (DHCR7) and 3β-hydroxysterol 24-reductase (DHCR24); its catabolism involves the formations of various catabolites by the actions of several enzymes like cytochrome P450 family 46 subfamily A member 1 (CYP46a1) and of hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7 (HSD3b7); B: Pathway of PC and PE synthesis [66]; abreviations: PCCT - phosphocholine cytidylyltransferase, ecoded by Pcyt1b; PECT - phosphoethanolamine cytidylyltransferase, ecoded by Pcyt2; CDP-choline – cytidil-5-diphosphocholine; CDP-ethanolamine - cytidil-5-diphosphoethanolamine; PC – phosphatidylcholine; PE - phosphatirylethanolamine

**Elevated levels of MUFA, particularly omega-9 FA are found in APOE4-KI mice**

The global levels of several other fatty acids were also altered in APOE4 KI mice. We observed elevated levels of two MUFA - eicosenoic acid (20:1) and nevronic acid (24:1). A similar link between APOE4 and MUFA overload was previously described [67] as well as an increase of MUFA with age [68]. Omega-9 FA and particularly nevronic acid have been previously associated with AD pathology [69]. Interestingly levels of nevronic acid were significantly elevated in mid-frontal cortex, temporal cortex and hippocampus of AD patients [70]. We found that the APOE4 genotype was correlated with overexpression of ELOVL7 gene,

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encoding the enzymes involved in fatty acids elongation (particularly MUFA), while ELOVL5 – involved in the elongation of PUFA - was downregulated [71]. These alterations in gene expression were consistent with the amount of available FA substrates (significant enrichment in MUFA and reduction in PUFA) [71]. Lipid metabolism involving synthesis, elongation, desaturation, oxidation and degradation of fatty acids is a complex processing involving a series of redundant enzymes with preferences for one particular lipid substrate. Thus, variations in the expression levels of these enzymes do not impact one lipid species in particular, but generate interconnected modifications of the abundance of FA, the precise quantification of which is technically challenging and will require additional study.

**Interrelationship between lipid dys-homeostasis and AD pathological markers**

In order to link these dysregulations in lipid homeostasis with the pathophysiology of AD, we investigated the levels of different pathological markers in primary neurons of APOE3/APOE4 KI mice. The broad-based lipidomic approach would have been inconceivable on primary neurons (due to insufficient quantity of lipids for a proper quantification). Still, neurons are the main source of Aβ and tau production in the brain [72,73]. Thus primary neuron cultures represented a suitable source for protein investigations.

We observed a marked increase in Aβ production in primary neurons derived from APOE4 brains as compared to APOE3. As the level of APP was unchanged, the overproduction of Aβ peptides was not apparently caused by overexpression of their substrate. The levels of tau and hyper-phosphorylated tau in neurons derived from APOE4-KI mice was also doubled with respect to the APOE3 genotype. Moreover, we observed lower levels of APOE in primary neurons of APOE4 mice, similar to findings reported by Mann et al. [36].

APOE4 is known to be less efficient in shuttling cholesterol to neurons than its isoform APOE3 [26]. In view of the high requirement of neurons for cholesterol, the rate of cholesterol shuttling via lipoproteins from the astrocytes could be expanded, as already demonstrated [74]. This augmented frequency in cholesterol loaded-lipoproteins uptake in APOE4 neurons may increase APP internalization and recycling, and as a consequence, Aβ production [75]. This possibility is supported by several studies showing that lipid-poor APOE can increased Aβ production by augmenting APP internalization and recycling [76,77]. Our observations also confirm and amplify a previous report that a deficiency in cholesterol turnover and transport correlates with increased tau phosphorylation and microtubule depolymerisation in axons [78]. These findings suggest that cholesterol dyshomeostasis in APOE4 genotype impacts AD pathological markers via its trafficking frequency and turnover and not by directly interfering with APP processing. Abnormally low content of PUFA-PE in APOE4 mice leads to the formation of rigid lipid platforms [79,80] that favour the encounter between APP and its processing enzyme BACE and as a consequence Aβ production [81–83]. Lipid rafts can also promote accumulation of Aβ oligomers and of hyperphosphorylated [84,85]. Moreover, lower levels of PUFA, particularly omega-3 PUFA, impair the counter-regulation of neuro-inflammation. Indeed, resolvins, protectins and other lipoxines are derived from PUFA and are critical in modulating inflammation and oxidative stress [86], important for alleviating Aβ and tau induced toxicity [87]. Likewise, lower levels of PE with respect to PC, as observed in the APOE4 genotype may induce a dysfunction in autophagy, and thus compromise

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the ability to degrade and clear neuro-toxic proteins [86,87]. Finally, the dysregulations observed in the global composition of fatty acids aggravates the detrimental effects discussed above: that is, the increase in oleic acid load stimulates activity of gamma-secretase (the second cleaving enzyme involved in APP processing), augmenting Aβ production in mice [88] and promotes pathological actions of tau, as observed in vitro [89].

## 5. Conclusions

In conclusion, this study reveals a diverse pattern of dysregulation of lipid homeostasis in APOE4 mice: notably, impaired cholesterol turnover, an imbalanced PC-to-PE ratio, lower levels of omega-3 bearing PE and disrupted levels of several fatty acids. These alterations were associated with changes in the expression of several genes linked to lipid metabolism. They were also related to increased levels of Aβ, tau and hyper-phosphorylated tau protein. In view of these observations, it is possible that readjusting lipid homeostasis may represent a novel direction for opposing and/or delaying the pathological events triggering AD. Future studies should examine more closely the precise molecular mechanisms interwinning lipid dysregulations with the APOE4 genotype, in particular, the altered generation and processing of cholesterol and phospholipids. Pathways linking lipid dys-homeostasis to the anomalous generation of neurotoxic peptides and other pivotal cellular substrates of AD also merit further characterisation.

**Supplementary Materials:** The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Expression of genes involved in cholesterol synthesis and metabolism in APOE4 and APOE3 KI mice extracted from the published RNAseq study [31].

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