UNIVERSITAT ROVIRA i VIRGILI

DIFFERENCES IN THE CEREBRAL LIPID PROFILE IN MICE DEPENDING ON THE APOE GENOTYPE AND THE POSTNATAL EXPOSURE TO CHLORPYRIFOS

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ABBREVIATIONS

AChE	Acetylcholinesterase
ANOVA	Analysis of variance
APOE/ApoE	Apolipoprotein E
ApoE-TR	Apolipoprotein E targeted replacement
BBB	Blood-brain barrier
ChoE	Cholesteryl esters
CNS	Central nervous system
CPF	Chlorpyrifos
DG	Diglycerides
HDL	High-density lipoprotein
LCAT	Lecithin-cholesterol acyltransferase
LPC	Lysophosphocholines
PC	Phosphatidylcholines
PE	Phosphatidylethanolamines
PND	Postnatal day
SM	Sphingomyelin
TG	Triglycerides

ABSTRACT

The brain is a lipid-rich organ. Several lipid molecules from different families, including glycerophospholipids and sphingolipids, play crucial roles in brain development as well as signal transduction during early stages of life. Lipid content and distribution is highly dependent on the activity of apolipoprotein E (ApoE). The different APOE polymorphisms are associated with different lipid patterns. Moreover, the different ApoE isoforms confer distinguishable vulnerabilities to pesticides exposure, such as chlorpyrifos (CPF), whose mechanism of action is the inhibition of the acetylcholinesterase enzyme. The aim of the present work was to assess the differences in the cerebral lipid profile depending on the APOE genotype and the postnatal CPF exposure during neurodevelopmental stages. For this, preweaning C57BL/6, ApoE3-TR and ApoE4-TR male mice were orally exposed to 1 mg/kg/day of CPF from postnatal day 10 to 15, whereas the control group was exposed to the vehicle (corn oil). Sacrifice took place four hours after the last CPF dose administration. Whole brain samples underwent a UHPLC-MS analysis for lipid identification and quantification. The results show clear differences in the brain lipid profile provided by the APOE genotype contribution. Statistical differences were found for several lipid groups, including cholesteryl esters, diglycerides, lysophosphocholines, phosphatidylcholines, sphingomyelin, and triglycerides. General treatment effects were observed in a lesser extent. Some genotype x treatment interactions were also present. Considering the role of lipids during brain development, the differences in the brain lipid profile point to a cognitive advantage depending on the APOE genotype during early stages of life. Overall, this study gives insight into the influence of the APOE genotype on the brain lipid composition.

Keywords: APOE · Lipid profile · Neurodevelopment · Chlorpyrifos · Postnatal exposure

1. INTRODUCTION

The brain is a lipid-rich organ whose maturation takes place during neurodevelopment in early stages of life. Lipid molecules play a crucial role in brain development processes, such as axon and dendrites outgrowth, synaptogenesis, and myelination process, as well as signal transduction (Ledo *et al*., 2019). Lipid content and distribution can be affected by the different apolipoprotein E (ApoE) polymorphisms (Phillips *et al*., 2014). Moreover, the genetic background is a pivotal element when determining the vulnerability of an individual against an environmental factor, as the exposure to toxic compounds can be (Tratnik *et al*., 2017). Interestingly, pesticide exposure, and especially the organophosphate pesticide chlorpyrifos (CPF), has been shown to exert its toxic impact in the brain at different stages of life, including neurodevelopmental stages (Guardia-Escote *et al*., 2019). Given that the CPF targets and inhibits the acetylcholinesterase (AChE) enzyme as well as several lipoprotein lipases (Greer *et al*., 2019), and considering the implication of the *APOE* gene in the cholinergic system and the lipid transport in the central nervous system (CNS), this suggests that CPF could also interfere with lipid metabolism in early stages of neurodevelopment.

1.1. Lipids in the brain

The brain is a highly lipid-rich organ, in which the lipid content represents the 50% of its dry weight. Thousands of different lipid metabolites exist in the brain and are differentially distributed depending on the brain region. Their chemical properties are diverse and lead to different functions in the brain. Moreover, lipids create a hydrophobic atmosphere, allowing several chemical reactions, which could not occur in aqueous medium (Ledo *et al*., 2019).

The brain lipidome is formed by different lipid families and each of them has a specific neurological function. Cholesterol, sphingolipids, and phospholipids are the ones that are found in higher proportions. Some of the lipidome constituents, such as cholesterol, are synthetised *de novo* in the brain, whereas some plasma lipids have the ability to cross the blood-brain barrier (BBB) from the circulation to reach brain (Ledo *et al*., 2019).

1.1.1. Cholesteryl esters

Cholesterol is synthetised *de novo* from acetyl-CoA in the CNS mainly by astrocytes, oligodendrocytes, and microglia, whereas neurons produce it in a lesser extent. Approximately 20% of the body total cholesterol levels are contained in the brain, which

represents only a 2% of the body mass. Oligodendrocytes produce large amounts of cholesterol during early stages of development, as 70% of the cerebral cholesterol content is used for myelination and the rest acts as structural component in glial cells and neuronal membranes (Mahley, 2016). In the latter case, cholesterol acts as an ion homeostasis and endocytosis regulator, as well as controlling the formation of synapses and dendrites (Ledo *et al*., 2019). Once the developmental myelination process is completed, cholesterol production in these cell types proceeds to a steady state (Mahley, 2016).

Cholesterol is unable to exit the CNS and, instead, a turnover process takes place via ApoE pathway. One of the recycling processes is the formation of cholesteryl esters (ChoE) by the lecithin-cholesterol acyltransferase (LCAT), which is an important process for an optimal cholesterol uptake and transport by high-density lipoproteins (HDL) from cells. The ApoE4 isoform has been shown to have a decreased efficiency in activating LCAT. Consequently, lower levels of ChoE production can lead to an impairment of the CNS HDL maturation by limiting the amount of available cholesterol and other essential lipids for cells (Mahley, 2016). Indeed, depletion of cholesterol is a limiting factor for neuronal activity, including alterations in the synaptic vesicle endocytosis and neurotransmission (Ledo *et al*., 2019).

Nonetheless, a presence of excessive cholesterol levels could lead to an intracellular accumulation of ChoE in lipid droplets. This internal storage could also be indicative of myelin structural alterations which prevent a correct cholesterol uptake and usage (Mutka *et al*., 2010).

1.1.2. Diglycerides or diacylglycerols

Diacylglycerol (DG) is an important second messenger in cells. Apart from being itself a direct activating effector, DG can act as a precursor of other signalling lipids. This pathway is mediated by DG lipases (DAGL), enzymes responsible for the conversion of DG to 2-arachidonoylglycerol. The latter is an endocannabinoid molecule and a lipid transmitter that plays a pivotal role in the CNS by regulating pain, emotional and addiction behaviours. Furthermore, it has been demonstrated that sharp changes in the cerebral lipid signalling network lead to an altered synaptic plasticity by inhibiting DAGL (Reisenberg *et al*., 2012). Indeed, decreased amounts of the lipid transmitter 2-arachidonoylglycerol are shown to follow brain trauma, convulsion, and stroke outcomes. On the other side, excessively increased levels of DG are also shown to be detrimental, as they point to degradation processes of cell membrane constituents which ultimately leads to brain damage (Ledo *et al*., 2019).

1.1.3. Lysophosphocholines

Lysophosphocholine (LPC) has been shown to act as an endogenous non-proton ligand for the activation of acid-sensing ion channels. These channels are a member of the degenerin/epithelial sodium channels and have a role in mechanotransduction. Thus, they function as indicators of the homeostatic status of different physiological features, such as muscle contraction, blood volume and pressure, and pain stimuli (Cheng *et al*., 2018). Nonetheless, the upregulation of LPC in the brain may lead to the activation of apoptotic pathways (Smith *et al*., 2015).

1.1.4. Phosphatidylcholines

Phosphatidylcholine (PC) biosynthesis in tissues implicates the intermediate metabolite phosphocholine. It has been considered a structural lipid since its discovery due to its implication as a as a major structural component of biological membranes Besides, it can have potential benefits in foetal neuroprotection. However, some studies carried out for myelin damage assessment have found increased concentrations of PC in cerebral lipid quantification. Thus, high levels of PC could be an indicator of disrupted myelination patterns (Xu *et al*., 2007).

1.1.5. Phosphatidylethanolamines

Phosphatidylethanolamine (PE) is a bioactive lipid which constitutes the second most abundant phospholipid in mammals. It is a non-bilayer phospholipid, as it is part of the inner cell membranes, corresponding approximately to the 15-25% of the total lipid content in cells. The functions of PE are diverse, and include membrane stability by conferring lateral pressure, participation in membrane fusion phenomena, membrane protein chaperoning, and initiation of cell death and autophagy by oxidation of its acyl side chains, among others (Patel and Witt, 2017). Phosphoethanolamine is the anabolic precursor molecule of PE, which plays a crucial role in the hippocampal differentiation of neural progenitor cells to astrocytes. Considering the importance of astrocytes functionality in the neurite growth and synaptogenesis, PE is a potentiator of a proper neuron maintenance in adulthood (Donoso *et al*., 2020). Nonetheless, PE has been also shown to play a role in lipid-induced endoplasmic reticulum stress, which ultimately leads to neurodegeneration (Patel and Witt, 2017).

1.1.6. Sphingomyelin

Sphingomyelin (SM) is one of the most important cell membrane constituents as it is the most abundant sphingolipid in eukaryotic organisms. It is especially present in the CNS, where it is necessary for the myelin sheath formation. This function is crucial for neuronal axon maturation and for maintenance of myelin integrity. Owing to its implication in the brain myelination process, SM is particularly relevant during neurodevelopment, especially during the period that covers mid-gestation and the first postnatal year. An important cognitive role is also attributed to SM as it promotes brain networking due to its functional involvement in allowing a proper transmission of nerve impulses (Schneider *et al*., 2019).

1.1.7. Triglycerides or triacylglycerols

Triglycerides (TG) in the circulation are able to cross the brain blood barrier and enter the CNS. Once they reach the brain, their function is to exert an effect on the hypothalamus, which is associated with the regulation of energy expenditure and controls body weight and metabolism. In this sense, TG interfere with insulin and leptin receptors. The first ones trigger a response towards blood sugar levels and determines a lipid or glucose metabolization as an energy source, whereas the second ones are responsible for satiety control. The interaction between TG and these receptors results in a peripheral insulin and leptin resistance, which represents a hunger stimulus and a need of fat storage (Banks *et al*., 2018).

1.2. Apolipoprotein E

1.2.1. Gene polymorphisms, functions and derived susceptibilities

Apolipoprotein E is a member of a protein family found in vertebrate species and consists of a 299-residue glycoprotein. ApoE can be synthetised by different cell types, including hepatocytes, adipocytes, astrocytes, and macrophages. Furthermore, neurons produce a splice variant which is not translated to protein and its processing is induced by injury. Specifically, astrocytes are the main ApoE producers in the CNS, whereas neurons and microglia synthetise it in a lesser extent. ApoE secretion is increased during brain development and as a response to brain injury. However, ApoE physiological functions in the CNS may differ depending on its origin (Kockx *et al*., 2017).

Human ApoE is found in three different isoforms, which are ApoE2, ApoE3 and ApoE4. The genetic difference among them is a single nucleotide polymorphism at two positions within the gene. ApoE3 is considered to be the parent form of apolipoprotein E is the most frequently found allele among humans. The genetic polymorphism leads to amino acid substitutions at positions 112 and 158 of the protein. The parent form ApoE3 contains a cysteine at position 112 and an arginine at position 158. The ApoE2 form differs from the ApoE3 form in the amino acid at position 158, presenting a cysteine instead of an arginine, while the ApoE4 form contains an arginine at position 112 instead of a cysteine (Phillips, 2014). The ApoE3 isoform is found in approximately 78% of the human population, whereas the frequencies of the ApoE2 and ApoE4 isoforms are 7 and 14%, respectively, being APOE3 the most common polymorphism among the population (Phillips, 2014).

The main function of ApoE is the regulation of lipid levels in plasma and it promotes it by joining plasmatic lipoprotein particles. The ApoE molecule has the ability to exchange between lipid-rich very low-density lipoprotein particles and protein-rich HDL particles (Yassine and Finch, 2020). ApoE3 is the most efficient form to assist in the TG-rich lipoprotein removal. Consequently, there is a cholesterol reduction in plasma which results in an atheroprotective effect of ApoE. However, ApoE2 and ApoE4 isoforms are linked to dyslipidaemias owing to their altered structure regarding to the ApoE3 isoform. Indeed, APOE2 and APOE4 carriers present a major risk of developing a cardiovascular disease (Phillips *et al*., 2014).

Besides, ApoE is functionally involved in the brain by regulating the lipid transport and maintaining the cholesterol homeostasis. The possession of the APOE4 allele is associated with greater risk of Alzheimer's disease onset (Phillips, 2014). Despite the risk of a neurodegenerative outcome in elder ages, APOE4 allele in pregnant women has been shown to have a beneficial role during foetal neurodevelopment due to a higher level of cholesterol content, which is important for synaptogenesis and neurite outgrowth.

Nonetheless, ApoE4-carrier children which are exposed to pollutants, such as metal elements (including mercury (Hg) and lead (Pb), among others) exhibit a poorer cognitive function and learning ability regarding to APOE2 and APOE3 alleles (Tratnik *et al*., 2017). This susceptibility to metal toxicity can be explained by the structure of ApoE isoforms. The ApoE4 form does not contain cysteine residues, which contain a thiol group in their structure. This thiol group can act as a scavenger by binding the metal ions and promote their removal (Tratnik *et al*., 2017). Such susceptibilities have been described for pesticides as well. Considering that the ApoE4 isoform is associated with a decreased cholinergic function and the impact of organophosphate pesticides on the cholinergic

system, there is evidence pointing to an interaction between the APOE genotype and pesticide exposure (Guardia-Escote *et al*., 2019).

The APOE genotype has also shown to have a relationship with dietary response. It has been demonstrated that elder individuals with cognitive impairment respond positively to an acute high-fat diet only in the case of APOE4 carriers. These individuals experience an increase of mitochondrial oxidation of poly-unsaturated fatty acids from diet intake for brain fuel (Yassine and Finch, 2020). This association may come from the need of higher cerebral lipid supply for brain remodelling and synaptic plasticity maintenance.

1.2.2. Apolipoprotein E and central nervous system

The lipid transport protein ApoE is responsible for the extracellular cholesterol uptake and delivery to cells. Particularly in the CNS, ApoE plays a pivotal role in providing necessary lipid species for neurodevelopment. Cholesterol and cerebral lipids are especially important for neurite outgrowth, myelin formation and synaptogenesis. The ApoE3 isoform is associated with normal lipid levels, whereas the APOE4 carriers are more prone to increased cholesterol levels. This fact could be important in early developmental stages of life, where maturation of the brain structure takes place. In this sense, carrying APOE4 alleles is suggested to be beneficial for CNS maturation and a greater neurodevelopment rate (Wright *et al*., 2003).

At the same time, ApoE is one of the major genetic determinants for neuropathological outcomes, such as Alzheimer's disease, with a possible accelerating effect by interacting with environmental and metabolic stress factors (Mahley *et al*., 2008). There are several metabolic pathways which explain the involvement of ApoE4 in promoting a higher risk for Alzheimer's disease developing. On the one hand, ApoE4 has been proved to undergo a greater proteolytic cleavage in neurons, resulting in the production of neurotoxic fragments which promote tau-hyperphosphorylation in the cytosol. This fact ultimately leads to neurodegeneration onset. On the other hand, ApoE4 exerts a less efficient cholesterol transport in the brain owing to a low affinity for lipid-binding. Moreover, a higher level of ApoE4 degradation is produced in astrocytes and neurons compared to the other isoforms. Thus, a decreased amount and lipid affinity of ApoE4 leads to a lower lipid supply enters the CNS for neuronal maintenance functions, such as repair and remodeling (Mahley, 2016).

1.3. Chlorpyrifos

1.3.1. Properties, ecotoxicity and human exposure

Chlorpyrifos is a chlorinated organophosphate compound widely used as a pesticide for agricultural uses. It is extensively used to control foliar and soil insects and mites, in both larval and adult forms, from causing crop damage throughout the whole year (Solomon *et al*., 2014). Chlorpyrifos was first designed as a gas agent and used as a weapon during World War II. Afterwards, the substance was adapted for agricultural uses in lower concentrations due to its effectivity against insects (Trasande, 2017).

Both chemical and physical properties of CPF determine its accumulation and fate in the ecosystem, which includes movement, adsorption, degradation, and catabolism (Solomon *et al*., 2014). Chlorpyrifos is distributed whether as a granular or a spray formulation and it is water-soluble in a concentration lower than 1 mg/L. Chlorpyrifos enters the ecosystem after treatment of crops. A volatilization process takes place during the following 12 hours (Solomon *et al*, 2014). As a result, a gaseous form of the pesticide enters the air, where it initiates a windborne long-range transport process and reaches distant regions from the site of application (Mackay *et al*., 2014). An adsorption process to foliage and soil prevails over volatilization during the days after agricultural distribution. Chlorpyrifos can rapidly be dissipated from leaves by photolysis, whereas dissipation from soil strongly depends on its characteristics. These include biological and microbial activity, pH, presence of catalysts and the oxygen availability, among others. Half-life of CPF has been determined to range between 2 and 335 days (Solomon *et al*, 2014). Degradation products of CPF are detailed in Figure 1S (Annex 1).

Although a strong adsorption to the soil matrix prevents the pesticide uptake by the roots, decreasing the risk of intoxication by consumption of treated plants (Solomon *et al*, 2014), it cannot be guaranteed the absence of CPF in the food. Indeed, residual amounts of CPF in food should be continuously monitored for bioaccumulation to avoid health hazard (Mojsak *et al*., 2018). Occupational exposure to CPF represents a greater risk for workers who manufacture and apply it. Dermal uptake and inhalation are the commonest ways of exposure to CPF among applicators who do not wear personal protective equipment (PPE) (Atabila *et al*., 2017; Gao *et al*., 2013). A method for CPF levels quantification is through a urine analysis. Chlorpyrifos absorption after application can reach a 30-fold increase compared to the background absorption levels, which result from contaminated food and water intake. This data suggests a potential risk of chronic and acute health issues (Atabila *et al*., 2018).

Owing to the potential toxicity that CPF exerts on the neurodevelopment, its domestic use has been limited in the United States since 2000 (Greer *et al*., 2019). Despite the scientific evidence of health hazard, CPF is currently used over the world. The US Environmental Protection Agency has set a threshold dose of 0.0012-0.002 μg/kg body weight/day, whereas the European Food Safety Agency has established an acceptable daily intake of 1 μg/kg body weight/day. This bias needs to be evaluated more accurately in order to assure global safety (Mie *et al*., 2018).

1.3.2. Toxicity in the nervous system

The mechanism of action of CPF is the inhibition of the AChE enzymatic activity, which catalyses the acetylcholine neurotransmitter breakdown (Greer *et al*., 2019). It has been shown that the cytochrome P450 mono-oxygenases are capable to use CPF as a substrate and convert it to CPF-oxon. This derived metabolite inhibits AchE irreversibly. Consequently, a neuronal hyperactivity is produced. The lessen AChE activity leads to an uncontrolled overstimulation of the cholinergic postsynaptic axons (Greer *et al*., 2019).

Although the precise mechanism by which CPF affects neurodevelopment are not fully understood, AChE inhibition is one of the most potential explanatory reasons. It has been demonstrated that CPF exerts a neurotoxic effect on brain modelling even at an exposure level which is below the safety threshold (Slotkin *et al*., 2019). However, alternative pathways of CPF-induced neurodevelopmental impairments have been described lately. Interestingly, administration of such low doses of CPF that are insufficient to inhibit the AChE activity during developmental periods of mammals has still shown to entail altered neurological outcomes, especially the motor condition (Perez-Fernandez *et al*., 2020). The possible molecular mechanism involved is the alteration of some neurotransmitter system or their constituents. Moreover, the brain-derived neurotrophic factor appears to be downregulated, indicating a poorer neural cell differentiation and brain plasticity in treated individuals (Perez-Fernandez *et al*., 2020).

A study carried out in humans showed an inverse relationship between the CPF metabolites concentration in urine and motor and social developmental quotients in children. It is suggested that an altered transcription of paraoxonase-1, an antioxidant enzyme involved in the metabolization of CPF-oxon, beside a disruption in the cell differentiation regulation and the formation of synapses could also play an important role in cognitive impairment (Guo *et al*., 2019).

1.3.3. Chlorpyrifos and lipid modulation

Besides the AChE inhibiting effect of CPF, the pesticide is also involved in the inhibition of the serine lipase activity by the oxon form in a similar way to the AChE as well as the alteration of cerebral lipid metabolites (Greer *et al*., 2019). Fatty acids usually undergo a hydrolytic process by serine lipases which leads to free fatty acids formation. Inhibition of these enzymes alters the composition and distribution of free fatty acids (palmitic acid, linolenic acid, eicosadienoic acid, arachidonic acid, and docosahexaenoic acid) and other lipid metabolites (such as lysophosphatidylethanolamines and TG) by decreasing their concentration in the brain. This lipidic alteration causes changes in transcription pathways which are induced by free fatty acids. The disruption of the normal signalling pathways in the brain results in a potential impact on the brain development (Greer *et al*., 2019).

A study carried out in preweaning mice showed CPF-induced changes in some short-chain fatty acids in the brain. Isovaleric acid and 4-methylvaleric acid were the two species which were altered by CPF exposure. Both short-chain fatty acids were found to exhibit higher concentrations in the brain of treated animals than the control group. Apparently, these changes in the short-chain fatty acids concentration is due to the dysbiosis which chlorpyrifos induces on gut microbiota. These findings support the importance of the gut microbiota influence on the cerebral lipid modulation (Guardia-Escote *et al*., 2020). These effects may imply an impact on cognitive function of exposed individuals.

Besides the increase in the concentration of cerebral lipid metabolites, preadipocytes also seem to have a potentiating impact derived from CPF exposure. An increase in size and number of preadipocytes as well as their capacity of lipid droplet storage is enhanced by CPF treatment, suggesting an obesogenic effect of the pesticide (Blanco *et al*., 2020).

Moreover, CPF exerts dyslipidaemic effects in the matter of serum. it has been shown that the pesticide is involved in an increase in the serum levels of total cholesterol, TG and low-density lipoprotein when administered chronically. However, serum HDL levels are significantly decreased after CPF exposure (Uchendu *et al*., 2018).

2. HYPOTHESIS AND OBJECTIVES

2.1. Hypothesis

The clear evidence of the differences in the lipid composition in the CNS which exists between adults depending on whether they carry the APOE3 or the APOE4 allele suggests differences in early developmental stages are also present. Moreover, organophosphate pesticides have been related with the inhibition of the cholinesterase enzymes and several lipases. We hypothesise that CNS maturation and function, as well as the lipid content in the brain are affected by the *APOE* polymorphism, which in turn confers different vulnerability to neurotoxic agents, including CPF. Many studies have been conducted to assess the impact of CPF, and pesticides in general, on the adult population. However, there is a considerable lack of studies on young population. For this reason, we hypothesise that exposure to CPF during developmental periods could have a neurotoxic impact on the proper maturation of the CNS.

2.2. Objectives

The main objective of this study is to evaluate the brain lipid composition of genetically modified mice during a neurodevelopmental period with and without a postnatal pesticide exposure.

2.2.1. Specific objectives

- To determine differences between murine or human APOE3 or APOE4 genotypes in the lipid content in the brain of 15-day-old mice.
- To assess general effects of treatment with CPF on postnatal day (PND) 15 on the lipid content in the brain of 15-day-old mice.
- To establish interactions between CPF exposures and the murine or human APOE3 or APOE4 genotypes on the lipid content in the brain of 15-day-old mice.

3. MATERIALS AND METHODS

3.1. Animal characterization and care

In this study, C57BL/6 mice from Charles River Laboratories (Charles River, Barcelona, Spain) and homozygous ApoE3 and ApoE4 mice from Taconic Biosciences (Taconic Europe, Lille Skensved, Denmark) were used. ApoE targeted replacement (ApoE-TR) mice were the research model, whereas C57BL/6 mice were used as the genetic background. The animals were kept under standard conditions, where the temperature was 22 ± 2 ºC and the relative humidity was 50 ± 10%. The light cycle was automatically kept assuring 12h of light and 12h of darkness. Water and a regular chow diet were guaranteed *ad libitum*. Offspring of each type of mouse was obtained locally in the own animal facilities in order to obtain an appropriate number (*n*) of animals for the study. An identical genotypic males and females crossbreed was carried out for one week and females were weighted two times for pregnancy control. Successfully pregnant females were isolated in individual cages until parturition, considering that moment as PND 0. In total, 36 male mice were the object of study, which were divided into 6 experimental groups. The groups were classified depending on their genotype (C57BL/6, APOE3 or APOE4) and each of them was subdivided in control group and treated group. The experimental design obtained a favourable report by the Ethics Committee on Animal Experimentation of Rovira i Virgili University (Reus, Spain). All the procedures on live animals were conducted in accordance with the Spanish Royal Decree 53/2013, which guides for the protection of research animals, as well as the European Communities Council Directive (2010/63/EU).

3.2. Treatment with the toxic compound

The organophosphate pesticide CPF, formulated as O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate, was purchased from Sigma-Aldrich® (Madrid, Spain) with a purity of the 99,5%. The compound was dissolved in corn oil adjusting a concentration of 1 mg/kg. Chlorpyrifos was administered to the treated group in a liquid form in a dose of 1µL/g of body weight, whereas the control group received only the vehicle (corn oil). Both were orally administered with a micropipette from PND 10 to PND 15, both included.

3.3. Sacrifice and sample management

The general anaesthetic isoflurane was used prior sacrifice of all the 36 animals of study due to its minor aggressivity compared to other inhalation methods, such as CO_2, and its null interference in the further biochemical procedures of the tissue samples. Isoflurane 2% was supplied for inhalation until death. The euthanasic procedure was carried out 4 hours after the administration of the last treatment dose, corresponding to the PND 15. Decapitation was followed by whole brain samples collection. Several organs, including plasma, liver and intestinal content were collected besides the brain for further analysis. All samples were maintained in liquid nitrogen during the sacrifice period and stored at -80ºC until analysis.

3.4. Lipidomic profile analysis

The sample analysis was performed with the equipment of the Centre for Omic Sciences (COS), Joint Unit of the Universitat Rovira i Virgili and Eurecat (Reus, Spain). Brain tissue homogenate was subjected to a chloroform:methanol extraction. After centrifugation with water and 0,9% of NaCl at 15.000 rpm and 4ºC for 10 minutes, the lower phase was extracted and analysed by UHPLC-qTOF (model 6550 of Agilent, USA) in positive electrospray ionization mode, consisting of approximately 5 mg of homogenized tissue.

Sequential elution of the different lipid types was achieved by chromatography in gradient elution using a ternary mobile phase composed by water, methanol, 2-propanol, ammonium formate and formic acid. The stationary phase consisted of a C18 column (Kinetex EVO C18 Column).

The different lipid species contained in the brain samples were putatively identified by mass and tandem mass spectrum match with Metlin-PCDL from Agilent as well as chromatographic behaviour of pure standards for each lipidic family. Once identified, every lipid type was semi-quantified using internal chemical standard calibration curves. Quantification results are given as pmol/mg of tissue after concentration normalization with the original tissue weight of each brain sample.

3.5. Statistics

The results from lipid concentration determination of each mouse sample were plotted as heatmaps for each lipidic family with the metabolomic platform MetaboAnalyst in order to obtain a general screening of the changes in expression of each lipid depending on

the factors that could have an influence on it (APOE genotype and treatment with CPF). Herein, an outlier corresponding to a control ApoE4 sample was detected and eliminated from the database for the following statistical analysis. The sample size for the statistical analysis of the lipidomic results was $n = 35$. Animal weight differences were assessed with $n = 36$.

Data corresponding to lipid concentration were statistically analysed with the IBM SPSS Statistics 22 software. Levene's test was first performed for the homogeneity of variance assessment. Each identified lipid in the samples was classified either as parametric or nonparametric, depending on the equality of the variance between groups.

A general multivariate regression model was performed for a screening of general significant differences given either by genotype, treatment, or genotype-treatment interaction. A two-way analysis of variance (ANOVA) and a Tukey's test for *post-hoc* analysis were carried out to determine significant differences between genotypes, whereas effects of the treatment conditions were indicated by the two-way ANOVA in parametric lipids. A Kruskal-Wallis test was carried out to determine significant differences between groups in the case of nonparametric lipids, considering both genotype and treatment for each group. Additionally, animal body weight was analysed with a general multivariate regression model to detect possible differences between genotypes and treatment condition. P values < 0.05 were considered statistically significant. The results are represented as mean values ± SEM.

4. RESULTS

4.1. Animal weight

Mice weight was statistically analysed to assess differences among genotypes or as a treatment result (Figure 1). A significant difference [$F_{1,35}$ = 12.923; p = 0.001] was obtained between control and treated groups. Differences in weight are not statistically significant regarding to the genotype.

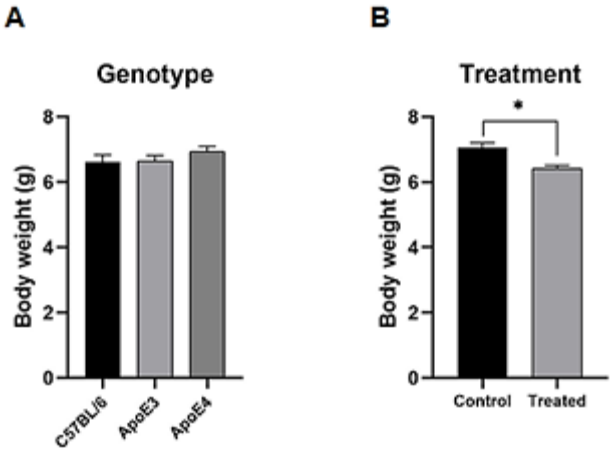

Figure 1. Representation of the body weight (g) on PND 15 depending on (A) the genotype (C57BL/6, APOE3 or APOE4) (n = 12) and (B) the treatment condition (control or treated with chlorpyrifos) (n = 18).
The data indicate the mean ± SEM; *p<0.05; multivariate regression analysis.

4.2. Cerebral lipid families

4.2.1. Cholesteryl esters

An overall screening of ChoE concentration in brain was performed through a heatmap, where a general overview of group behaviour can be seen (Figure 1A). In general, the APOE4 genotype presents the highest levels of cerebral ChoE. Both control and treated APOE3 groups express the lowest ChoE levels. While the treatment effect on C57BL/6 clearly lowers the ChoE concentrations, the opposite effect is seen in APOE4, where ChoE are highly expressed in the treated group. A more specific representation of each individual brain sample behaviour on ChoE levels can be seen in Figure 2S (Annex 2).

Genotype influence on ChoE concentration in brain (Figure 2B) was assessed by a two-way ANOVA, showing statistical differences in several ChoE species, including ChoE 20:2 [$F_{2,34}$ = 4.069; p = 0.027], ChoE 20:3 [$F_{2,34}$ = 3.280; p = 0.050], ChoE 20:4 [$F_{2,34}$ =

5.226; p = 0.011], ChoE 20:5 [$F_{2,34}$ = 6.188; p = 0.005], and ChoE 22:4 [$F_{2,34}$ = 17.875; p = 0.000]. A further *post-hoc* analysis was carried out to determine which genotypes manifest these differences, showing that C57BL/6 and APOE4 differ in ChoE 20:2 levels, APOE3 and APOE4 differ in ChoE 20:3 levels, C57BL/6 and APOE3 differ in both ChoE 20:4 and ChoE 20:5 levels, and all three genotypes differ in the case of ChoE 22:4. The APOE4 genotype presents the highest ChoE levels in brain, except for ChoE 20:4 and ChoE 20:5. Moreover, general treatment effects were observed for one lipid species, corresponding to ChoE 18:3, which undergoes a lowering response compared to the control group [$F_{1,34}$ = 4.533; p = 0.042] (Figure 2C).

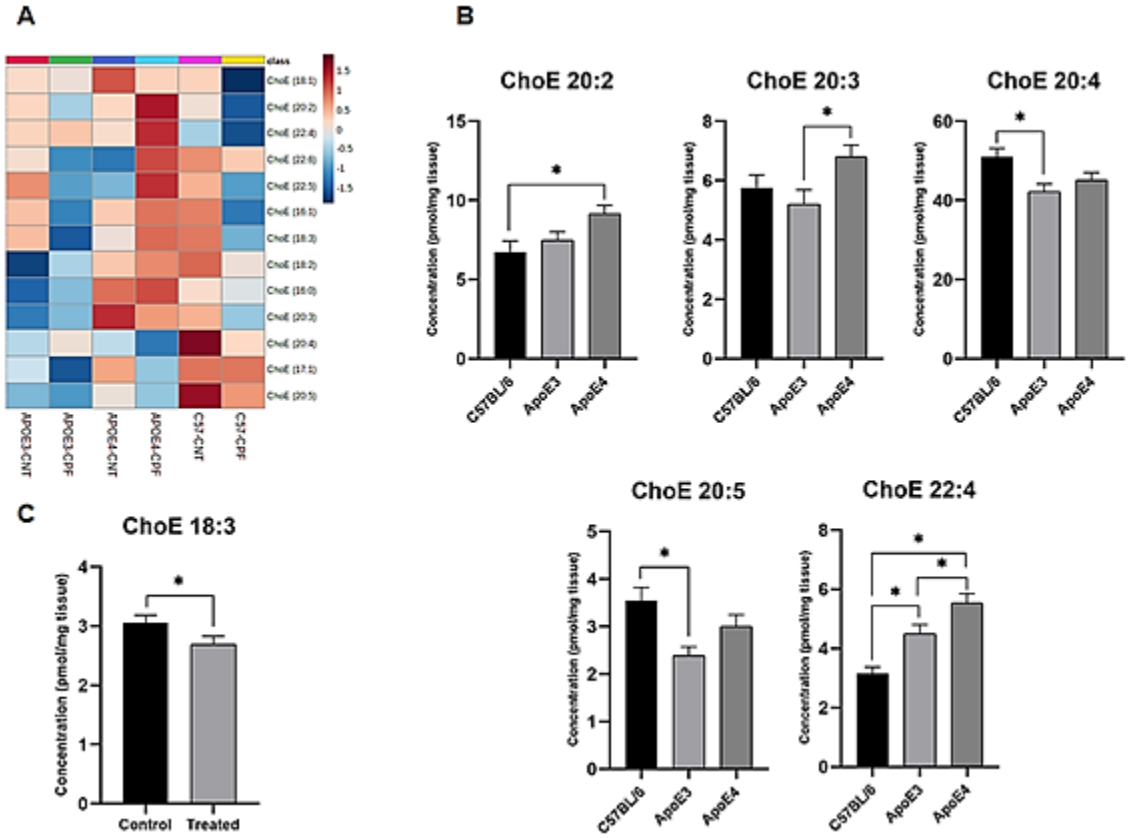

Figure 2. Cholesteryl esters (ChoE) profile in mouse brain. (A) The heatmap shows the concentration (pmol/mg tissue) of all the ChoE species found in the mice brain samples. Each column represents a group (CNT, control; CPF, CPF-treated; n = 5-6). (B) Bar graphs corresponding to genotype effect on the concentration (pmol/mg tissue) of ChoE species in the brain. Both control and treated groups are integrated as one for each genotype (n = 11-12). (C) Bar graph showing the effect of the treatment on ChoE concentration (pmol/mg tissue) in the brain. All three genotypes were included in one group for each treatment condition (control or treated) (n = 17-18).
The data in figures B and C indicate the mean ± SEM; *p<0.05; two-way ANOVA and Tukey's test for *post-hoc* analysis.

Additionally, an interaction between the APOE genotype and the CPF treatment is observed for ChoE 20:2 [$F_{2,34}$ = 3.640; p = 0.039] and ChoE 22:6 [$F_{2,34}$ = 4.506; p = 0.020] species (Figure 3). The difference is evident between APOE3 and APOE4 genotypes in both ChoE species. The treated C57BL/6 group is also significantly different from APOE4 in the case of ChoE 20:2.

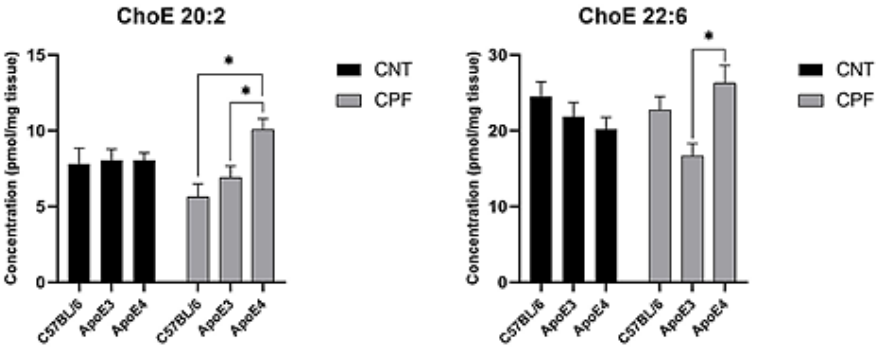

Figure 3. Representation of the concentration (pmol/mg tissue) of cholesteryl ester (ChoE) species in the brain depending on a genotype x treatment interaction effect (CNT, control; CPF, CPF-treated; n = 5-6).
The data indicate the mean ± SEM; *p<0.05; two-way ANOVA and Tukey's test for *post-hoc* analysis.

4.2.2. Diglycerides

The heatmap (Figure 4A) shows a general screening of DG levels in brain. The C57BL/6 genotype presents the lowest DG levels, whereas APOE4 appears to have the highest ones. Generally, postnatal exposure to CPF leads to a decrease in DG concentration in both C57BL/6 and APOE3. The opposite effect is seen in the case of APOE4, where the treatment apparently causes an increase in cerebral DG levels. A detailed representation of each individual brain sample behaviour on DG levels is shown in Figure 3S (Annex 2).

A two-way ANOVA test revealed significant differences in several DG species in the brain, including DG 36:3 [$F_{2,34}$ = 8.124; p = 0.001], DG 36:4 [$F_{2,34}$ = 23.200; p = 0.000] and DG 40:4 [$F_{2,34}$ = 5.932; p = 0.006] (Figure 4B). The statistical analysis was led to a greater extent with a *post-hoc* analysis, which showed that DG 36:3 in APOE4 is differently expressed than in C57BL/6 and APOE3, presenting higher levels in brain. For DG 36:4, all three genotypes behave differently, being APOE4 the one presenting the highest levels. In the case of DG 40:4, significant differences are found between C57BL/6 and APOE4.

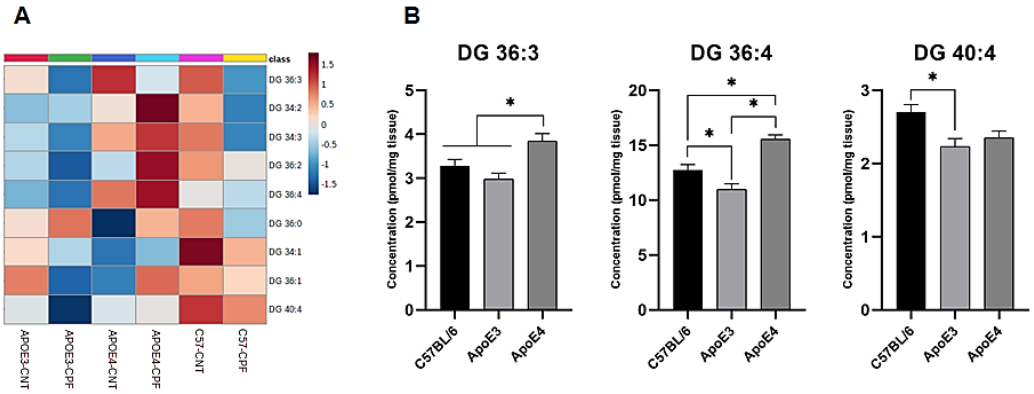

Figure 4. Diglycerides (DG) profile in mouse brain. (A) The heatmap shows the concentration (pmol/mg tissue) of all the DG species found in the mice brain samples. Each column represents a group (CNT, control; CPF, CPF-treated; n = 5-6). (B) Bar graphs corresponding to the genotype effect on DG concentration (pmol/mg tissue) in the brain. Both control and treated groups are integrated as one for each genotype (n = 11-12.
The data in figure B indicate the mean ± SEM; *p<0.05; two-way ANOVA and Tukey's test for *post-hoc* analysis.

4.2.3. Lysophosphocholines

A general overview of all LPC species levels in brain is represented in a heatmap (Figure 5A). The APOE3 genotype exhibits the most different LPC profile compared to the other genotypes, presenting the lowest LPC levels. Interestingly, the APOE4 and C57BL/6 control groups seem to exhibit a complementary LPC species distribution. The diminished LPC species levels in APOE4 are the ones most highly expressed in C57BL/6, and vice versa. In terms of treatment effects, a decrease in LPC concentration can be observed for all three genotypes. A complete representation of each individual brain sample behaviour on LPC levels is shown in Figure 4S (Annex 2).

Genotype effects (Figure 5B) were assessed by a two-way ANOVA test, showing statistical differences among LPC 15:0 [$F_{2,34}$ = 3.596; p = 0.039], LPC o-16:1 [$F_{2,34}$ = 6.195; p = 0.005] and LPC 18:2 [$F_{2,34}$ = 5.930; p = 0.006] species. A general genotype effect is observed for LPC o-16:0 [$F_{2,34}$ = 3.355; p = 0.048]. A *post-hoc* analysis demonstrated that differences in LPC 15:0 exist between C57BL/6 and APOE4 genotypes. C57BL/6 and APOE4 are different from APOE3 in the case of LPC o-16:1, whereas APOE4 is different from both C57BL/6 and APOE3 for LPC 18:2 levels. In overall terms, C57BL/6 and APOE3 exhibit a similar behaviour in terms of LPC concentration, whereas APOE4 is the most different, presenting the highest levels of LPC.

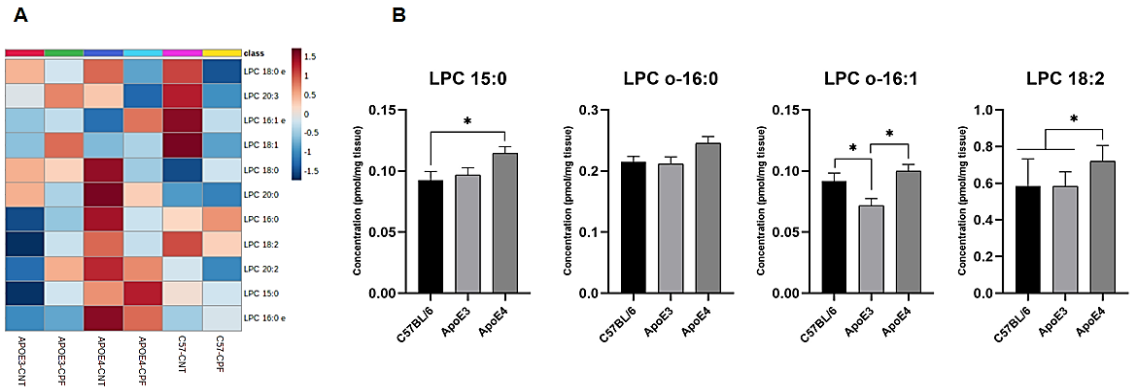

Figure 5. Lysophosphocholines (LPC) profile in mouse brain. (A) The heatmap shows the concentration (pmol/mg tissue) of all the LPC species found in the mice brain samples. Each column represents a group (CNT, control; CPF, CPF-treated; n = 5-6). (B) Bar graphs corresponding to the LPC concentration (pmol/mg tissue) in the brain depending on the genotype effect. Both control and treated groups are integrated as one for each genotype (n = 11-12).
The data in figure B indicate the mean ± SEM; *p<0.05; two-way ANOVA and Tukey's test for *post-hoc* analysis.

4.2.4. Phosphatidylcholines

An overall screening of PC concentration is represented in Figure 6A. The heatmap shows that the APOE4 genotype, in both control and treated groups, presents the highest

levels of PC in the brain regarding to C57BL/6 and APOE3, which seem to have a similar behaviour. The treatment effects on PC levels are disperse, exhibiting a different pattern in each genotype. Postnatal CPF exposure generally exerts a diminishing effect on C57BL/6 genotype, a rising effect on most PC species concentration in the case of APOE4, and an increasing or decreasing effect depending on the PC species in APOE3. An itemised heatmap of each individual brain sample behaviour on PC levels is shown in Figure 5S (Annex 2).

Statistical differences were obtained as a genotype effect for ten of the PC species after the performance of a two-way ANOVA test (Figure 6B). These species include PC 30:0 [$F_{2,34}$ = 5.284; p = 0.010], PC 31:0 [$F_{2,34}$ = 5.286; p = 0.010], PC 32:1 [$F_{2,34}$ = 3.823; p = 0.032], PC 34:0 [$F_{2,34}$ = 4.281; p = 0.023], PC 34:4 [$F_{2,34}$ = 5.029; p = 0.013], PC o-36:4 [$F_{2,34}$ = 4.261; p = 0.023], PC 36:5 [$F_{2,34}$ = 4.268; p = 0.023], PC o-38:3 [$F_{2,34}$ = 9.685; p = 0.001], PC o-38:4 [$F_{2,34}$ = 3.417; p = 0.045] and PC o-38:6 [$F_{2,34}$ = 4.420; p = 0.020]. Moreover, a general genotype effect was assessed for PC 32:2 [$F_{2,34}$ = 3.494; p = 0.042] and PC 37:6 [$F_{2,34}$ = 2.808; p = 0.049]. Species showing significant differences among genotypes were further examined with a *post-hoc* analysis. Differences are mainly found between C57BL/6 and APOE4 genotypes, pattern in which PC 30:0, PC 31:0, PC 32:0, PC 34:4, PC o-36:4 and PC 36:5 are reflected. However, differences between APOE3 and APOE4 are also observed in the case of PC 34:0, PC o-38:4 and PC o-38:6. In addition, APOE4 genotype differs from both C57BL/6 and APOE3 in the matter of the PC o-38:3 species. APOE4 mostly presents the highest levels for all species, except for PC 37:6, where APOE3 seems to predominate in terms of concentration.

Moreover, a genotype x treatment interaction is observed for eight of the PC species (Figure 7), including PC 30:0 [$F_{2,34}$ = 3.385; p = 0.048], PC 31:0 [$F_{2,34}$ = 5.203; p = 0.012], PC 32:2 [$F_{2,34}$ = 3.967; p = 0.030], PC 33:0 [$F_{2,34}$ = 3.418; p = 0.046], PC 36:5 [$F_{2,34}$ = 4.322; p = 0.023], PC 37:6 [$F_{2,34}$ = 6.565; p = 0.004], PC 40:4 [$F_{2,34}$ = 4.010; p = 0.029], and PC 40:5 [$F_{2,34}$ = 3.872; p = 0.032]. Similar to the genotype differences, in this case the C57BL/6 and APOE4 treated groups are the statistical different groups in terms of PC 30:0, PC 40:4, and PC 40:5 concentration. In contrast, the C57BL/6 and APOE3 treated groups are the differentiated group with reference to PC 33:0. Both APOE3 and APOE4 treated groups behave differently regarding PC 31:0, PC32:2, and PC 37:6.

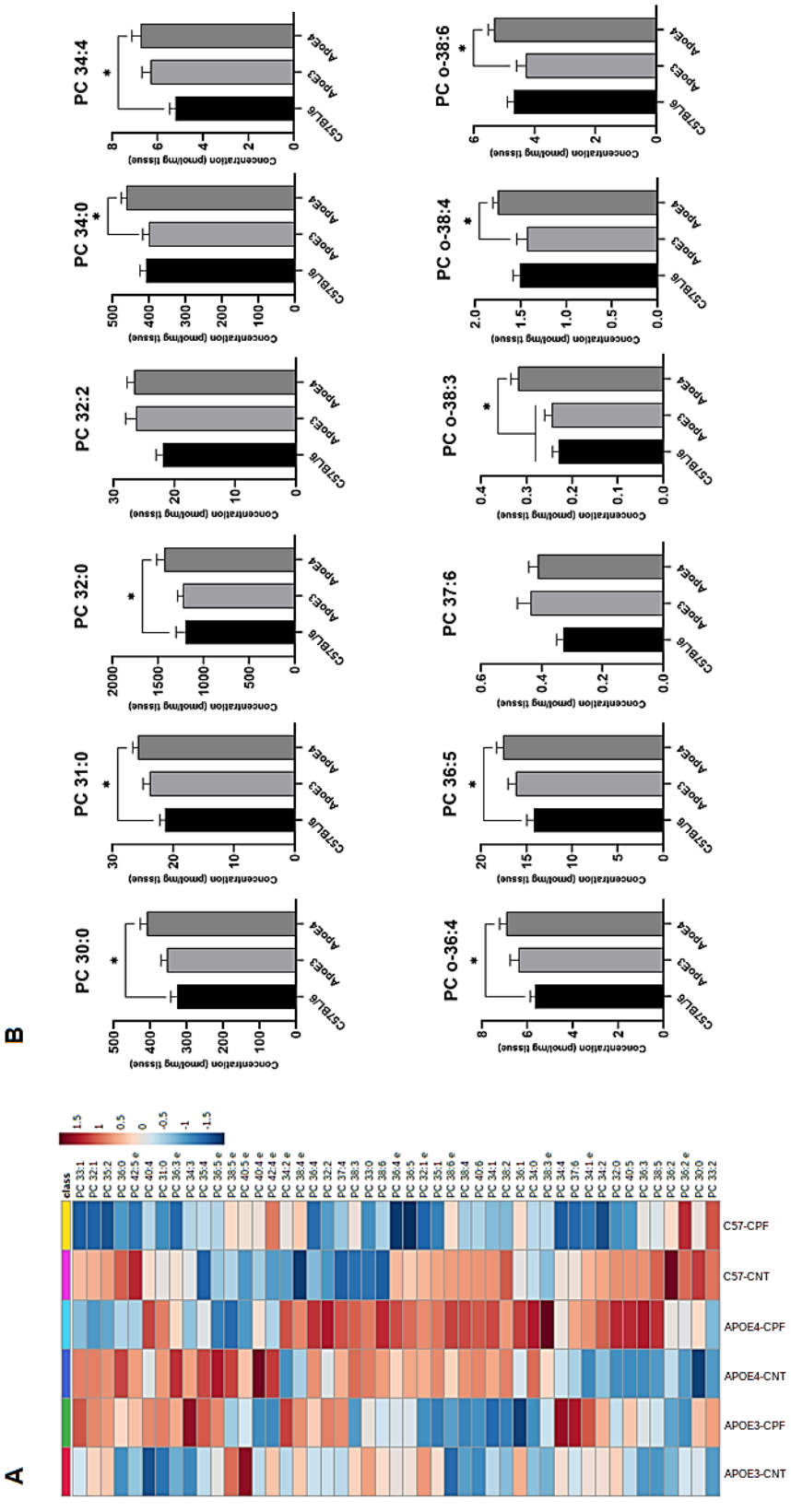

Figure 6. Phosphatidylcholines (PC) profile in mouse brain. (A) The heatmap shows the concentration (pmol/mg tissue) of all the PC species found in the mice brain samples. Each column represents a group (CNT, control; CPF, CPF-treated; n = 5-6). (B) Bar graphs corresponding to the difference in PC concentration (pmol/mg tissue) in the brain depending on the genotype. Both control and treated groups are integrated as one for each genotype (n = 11-12). The data in figure B indicate the mean ± SEM; *p<0.05; two-way ANOVA and Tukey's test for *post-hoc* analysis.

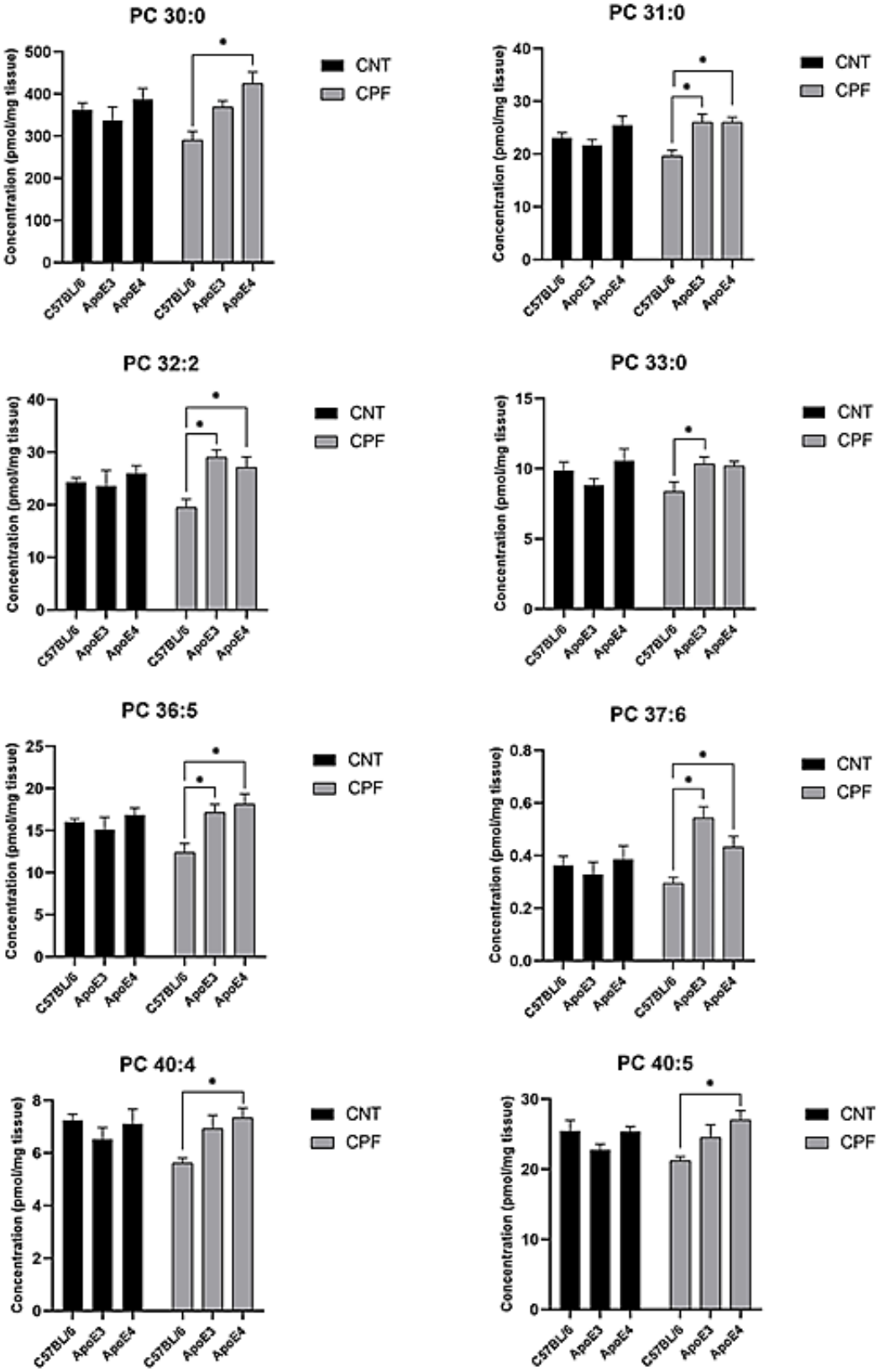

Figure 7. Representation of the concentration (pmol/mg tissue) of phosphatidylcholine (PC) species in the brain depending on a genotype x treatment interaction effect (CNT, control; CPF, CPF-treated; n = 5-6).
The data indicate the mean ± SEM; *p<0.05; two-way ANOVA and Tukey's test for *post-hoc* analysis.

4.2.5. Phosphatidylethanolamines

A global overview of PE concentration in the brain is shown in Figure 8. The highest levels for PE attributed to the APOE4 control group can be deduced from the heatmap. The C57BL/6 control groups seems to be an intermediate stage between both APOE genotypes, whereas the APOE3 control group presents the lowest PE levels. In overall, treatment with CPF seems to influence PE levels by decreasing them in both APOE4 and C57BL/6, whereas it has an opposite increasing effect on PE concentration in APOE3. Further qualitative information about PE levels of each individual brain sample behaviour on PE levels can be seen in Figure 6S (Annex 2).

However, no statistical differences were found for genotype, treatment, not either for genotype x treatment effect in the case of PE concentration in the brain (data not shown).

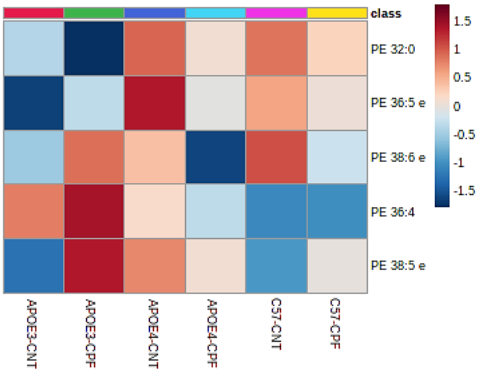

Figure 8. General screening on phosphatidylethanolamines (PE) in mouse brain. The heatmap shows the concentration (pmol/mg tissue) of all the PE species found in the mice brain samples. Each column represents a group (CNT, control; CPF, CPF-treated; n = 5-6).

4.2.6. Sphingomyelin

A general overview of SM levels in brain is represented in Figure 9A. The heatmap indicates disperse results, showing high concentrations of some SM species and low to very low levels of other SM species for all three genotypes and the treatment conditions. However, a greater amount of SM species with high concentrations in brain are exhibited for the APOE4 genotype, in both control and treated groups. Concurrently, the APOE3 genotype seems to have the lowest SM concentrations for most of the species regarding to the other genotypes. It is not clear whether the postnatal exposure to CPF induces an increase or a decrease in SM levels in brain, as some of the species seem to be more abundant after treatment and others have poorer levels depending on each individual case. A detailed heatmap of each individual brain sample behaviour on SM levels is shown in Figure 7S (Annex 2).

In order to determine existing significant differences between genotypes on SM levels (Figure 9B), a two-way ANOVA was carried out. The results demonstrated statistical

differences for two SM species, which are SM 35:1 [$F_{2,34}$ = 4.195; p = 0.024] and SM 42:3 [$F_{2,34}$ = 5.408; p = 0.009]. The *post-hoc* analysis revealed that C57BL/6 and APOE4 were the genotypes which presented differences in SM 35:1, whereas APOE4 had different SM 42:3 concentrations from both C57BL/6 and APOE3. In overall, APOE4 is the genotype which expressed higher levels of cerebral SM.

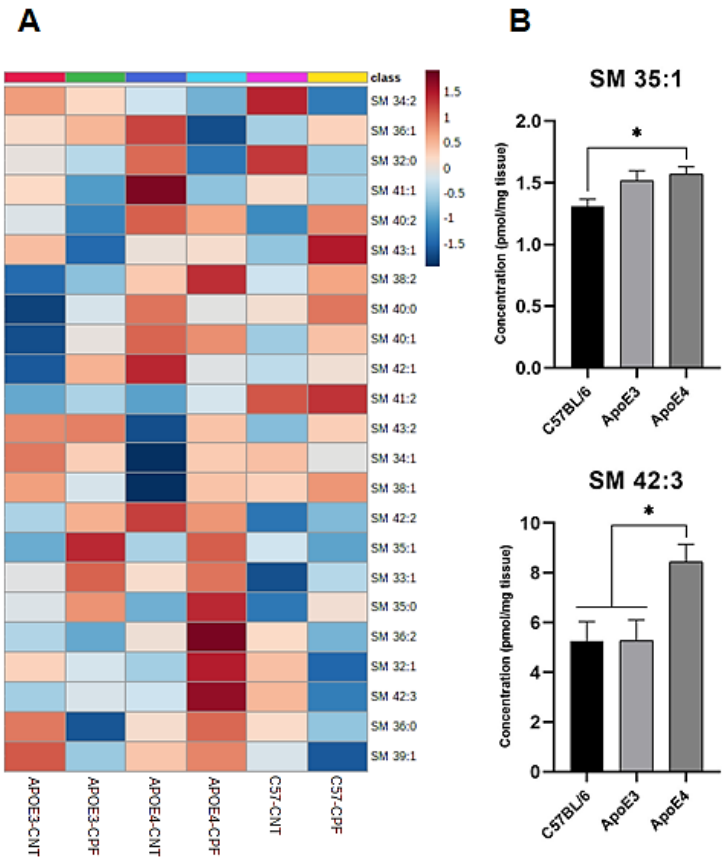

Figure 9. Sphingomyelin (SM) profile in mouse brain. (A) The heatmap shows the concentration (pmol/mg tissue) of all the SM species found in the mice brain samples. Each column represents a group (CNT, control; CPF, CPF-treated; n = 5-6). (B) Bar graphs corresponding to the differences in the SM concentration (pmol/mg tissue) in the brain depending on the genotype effect. Both control and treated groups are integrated as one for each genotype (n = 11-12).
The data in figure B indicate the mean ± SEM; *p<0.05; two-way ANOVA and Tukey's test for *post-hoc* analysis.

4.2.7. Triglycerides

A general screening of TG concentration in the brain is shown in Figure 10A. APOE4 is clearly the genotype which presents the highest levels of TG in both control and treated groups, comparing to C57BL/6 and APOE3. The postnatal treatment with CPF exerts an evident lowering effect on TG concentration in the case of both C57BL/6 and APOE3 genotypes. However, this treatment effect is not that obvious in the case of APOE4, where some TG species experience an increase in concentrations, whereas others seem to decrease their levels after CPF exposure. An itemised heatmap of each individual brain sample behaviour on TG levels is shown in Figure 8S (Annex 2).

Significant differences among genotypes (Figure 10B) were studied in detail through a two-way ANOVA test. The analysis indicated statistical differences in concentration of

several TG species, including TG 48:0 [$F_{2,34}$ = 4.545; p = 0.018], TG 50:0 [$F_{2,34}$ = 4.973; p = 0.013] and TG 50:4 [$F_{2,34}$ = 3.623; p = 0.038]. A *post-hoc* analysis revealed that the APOE4 TG profile is different from both C57BL/6 and APOE3 profiles in the case of TG 48:0 and TG 50:0, whereas C57BL/6 and APOE4 are the genotypes with differences in TG 50:4 levels. Moreover, a general genotype effect was detected for TG 50:1 [$F_{2,34}$ = 3.357; p = 0.047], TG 52:1 [$F_{2,34}$ = 3.630; p = 0.038] and TG 54:2 [$F_{2,34}$ = 3.070; p = 0.042].

Additionally, treatment effects on TG levels in the brain were also found (Figure 10C). Significant differences were obtained for TG 48:0 [$D_{1,34}$ = 4.963; p = 0.034], TG 50:0 [$F_{1,34}$ = 4.897; p = 0.035] and TG 52:4 [$F_{1,34}$ = 5.443; p = 0.027],

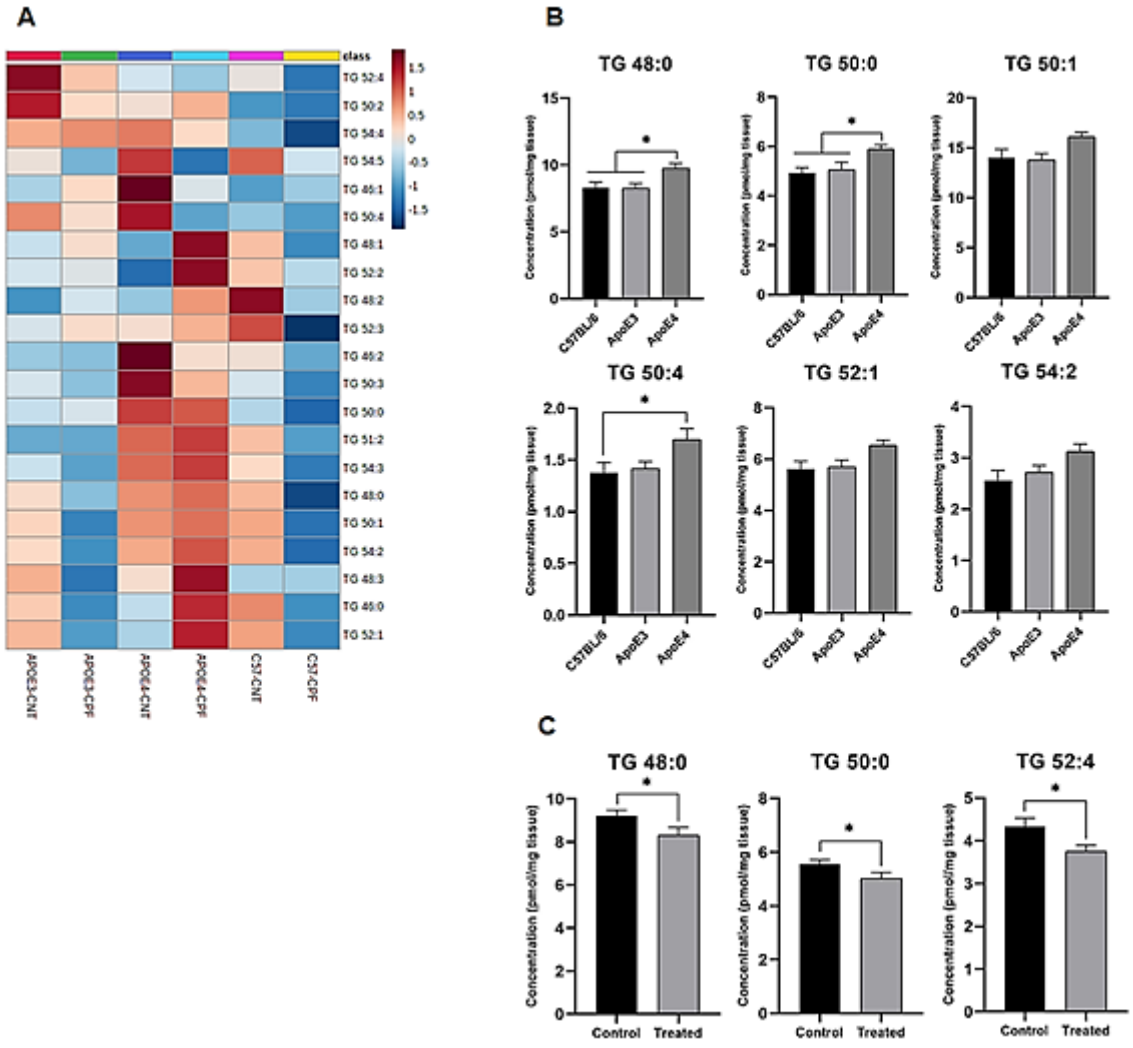

Figure 10. Triglycerides (TG) profile in mouse brain. (A) The heatmap shows the concentration (pmol/mg tissue) of all the TG species found in the mice brain samples. Each column represents a group (CNT, control; CPF, CPF-treated; n = 5-6). (B) Bar graphs corresponding to the TG concentration (pmol/mg tissue) in the brain depending on the genotype. Both control and treated groups are integrated as one for each genotype (n = 11-12). (C) Bar graph showing the treatment effect on TG concentration (pmol/mg tissue) in the brain (two-way ANOVA). All three genotypes were included in one group for each treatment condition (control or treated) (n = 17-18).
The data in figures B and C indicate the mean ± SEM; *p<0.05; two-way ANOVA and Tukey's test for *post-hoc* analysis.

5. DISCUSSION

The aim of the present work was to assess the differences in the cerebral lipid profile in mice depending on the APOE genotype and the treatment with CPF. A lipidomic analysis was performed to determine different lipid concentrations in mice brain samples with and without exposure to CPF.

Clear differences are manifested as a result of the APOE genotype contribution to the lipid profile modulation in the brain during neurodevelopment. The general screening of the different lipid families' concentration showed higher levels in the case of the APOE4 genotype in most of the lipid species. Statistical analysis corroborated this observation, certainly indicating that the behaviour of ApoE4 individuals is the most different among the analysed genotypes regarding the cerebral lipid content. Considering the pivotal role of lipids in the brain, especially during the neurodevelopmental period, increased cerebral lipid levels at PND 15 indicate an enhanced level of CNS maturation in ApoE4 individuals. Several studies carried out in young individuals support these findings. Wright *et al*. (2003) executed a measurement of the mental development index of 24-month-old children taking into account their APOE genotype. The results showed a difference of 4.4 points towards an enhanced performance in children carrying the APOE4 allele comparing to APOE2 and APOE3 carriers. In turn, Remer *et al*. (2020) evaluated the white matter myelin by magnetic resonance imaging of infants aged from 2 to 68 months old and associated it with their APOE genotype and cognitive outcome. The findings pointed to the existence of myelination differences among ApoE variants and a greater learning development in ApoE4 individuals. Interestingly, APOE4 carriers exhibited a slower myelination rate in different neuroanatomical regions at early developmental stages, compared to non-carriers. This event could be explained by the distribution of the different ApoE isoforms, which varies depending on the allele. It has been demonstrated that the APOE3 genotype expresses higher ApoE levels than the APOE4 genotype in both human and mouse species. Specifically, ApoE4-TR mice exhibit a 28% lower ApoE amount in the brain than ApoE3-TR, in which ApoE appears to be more abundant (Mahley, 2016). However, the initial developmental onset in ApoE4 was evidently starting earlier, which suggests a cognitive advantage for APOE4 carriers regarding to non-carriers (Remer *et al*., 2020).

Specifically, ChoE are expressed in a higher proportion in ApoE4 as well as C57BL/6 individuals compared to the levels found in the case of ApoE3. They constitute an esterificated form of cholesterol, which is mandatorily *de novo* synthesised in glial and neuronal cells for the brain developmental processes, even though in early stages of life

(Dietschy, 2009). Provided that ChoE are derived from cholesterol esterification by LCAT, the C57BL/6 genotype may be beneficiary by high LCAT activity. In turn, higher levels of ChoE in ApoE4 may be due to higher basal levels of cholesterol, owing to the lower levels of LCAT activation which the APOE4 genotype presents, as stated by Mahley (2016). Thus, the high ChoE content in brain represents an evidence for an enhanced neurodevelopment in ApoE4 individuals.

Particularly for PC and SM, ApoE4 mice unmistakable present the highest levels of these lipid families in the brain. As it is well known, a prevailing amount of choline content is used as a substrate for PC and SM synthesis, which are destined for cell membrane components (Amenta and Tayebati, 2008). At the same time, Basaure *et al*. (2018) found that ApoE4 mice manifest higher rates of AChE activity in the brain compared to ApoE3 mice in a study assessed at the age comprised between PND 10 and PND 15. Since greater levels of PC and SM are synthesised in ApoE4 individuals, it can be deduced that higher levels of choline are present in the organism, including the brain, which uptakes choline from the bloodstream (Amenta and Tayebati, 2008). Based on these premises, ApoE4 can be suggested to present an enhanced cholinergic system activity, which results in a better cognitive outcome.

By the same token, TG concentration in ApoE4 mice are clearly higher in both control and treated groups than in APOE3 and C57BL/6 mice. Little is known about the presence and function of TG in the CNS and their ability to cross the BBB. Provided that TG basically exert a metabolic and not structural function in the organism, TG are suggested to have a role in the energy expenditure and homeostasis. High circulating levels of TG in plasma have been shown to induce peripheral leptin and insulin resistance, leading to uncontrolled starvation signals and ultimately to obesity development (Banks *et al*., 2018). High cerebral TG could lead to the thought of high plasma TG as well, suggesting an increase of the body mass. However, no significant differences in mice weight have been demonstrated herein regarding to the genotype. It could be possible that the early age range studied herein was insufficient to exhibit leptin or insulin resistance.

Despite the clear evidence of the APOE4 genotype presenting the highest levels of the analysed lipid families in brain, such differences are not obvious for the CPF treatment effects. Solely a few ChoE and TG species are shown to experience a decrease in their cerebral levels as a result of the treatment with CPF. On the one hand, cholesterol is a structural molecule with considerable importance for brain developing, as stated before (Dietschy, 2009). A decrease in ChoE levels in brain could be indicative of diminished cerebral cholesterol levels. Thus, chlorpyrifos could be interfering in the integrity of cell membranes. On the other hand, TG also appear to be affected by CPF. Although the

mechanism by which TG enter the CNS is not clear, Banks *et al*. (2018) are pioneer in demonstrating the presence of plasma TG in the brain, suggesting their ability to cross the BBB. In turn, Etschmaier *et al*. (2011) have identified the expression of adipose triglyceride lipase in some regions of the brain, where it plays a crucial role in the cerebral TG metabolism, concretely at the interface between the brain and the cerebrospinal fluid, and the BBB. Once TG reach the CNS, they may act as starvation signals after a fasting period, leading to the propensity of a higher food intake. As shown in the results, CPF tends to cause a decrease in the TG levels, indicating that such signals are less powerful. Consequently, this assumption correlates with the fact that, in general, treated group mice weigh less than the ones from the control group. Nonetheless, these findings are not consistent with the results obtained by Blanco *et al*. (2020) where they demonstrate an obesogenic effect of CPF on 3T3-L1 mouse preadipocytes *in vitro*. Similarly, Peris-Sampedro *et al*. (2015) found CPF as a risk agent towards obesity development in ApoE3 mice. However, obesogenic effects are not elucidated in this study. Further research should be carried out for plasmatic TG assessment and correlation with TG levels in brain in order to unravel the current incongruence.

However, a tendency for the CPF effects can be deduced from the general screening. The heatmaps reveal a decreasing effect of CPF on lipid concentration in overall, although some lipid species undergo an increase. Interestingly, the APOE4 genotype presents higher levels of most ChoE and DG species as well as some PC, SM and TG species after treatment. A plausible explanation for the lipid profile alteration could be due to the considerably low activity of paraoxonase-1 at birth, which is a key enzyme for the detoxification of CPF-oxon (Marsillach *et al*., 2016). Several studies point to the alteration of several metabolic and cognitive aspects, such as serum lipids (Uchendu *et al*., 2018), learning and memory (Basaure *et al*., 2019), gut microbiota and cerebral short-chain fatty acids (Guardia-Escote *et al*., 2020) after CPF exposure in murine models. Notwithstanding, significant differences in the analysed cerebral lipid families derived from CPF effects are not conclusive. The administered dose of CPF and the short-term exposure should be considered as determinant factors for the inability to see significant changes in the lipid profile in the brain.

Finally, the presence of genotype x treatment interaction effect demonstrated that the association of toxic exposure and a specific APOE genotype can be determinant for CPF-induced changes. However, the results do not show a clear association with a particular genotype, as the distribution is not equal. This means that treatment influences the lipid concentration in a non-homogeneous manner, thus, it does not indicate an APOE genotype which is clearly more vulnerable to CPF than the other ones.

6. CONCLUSIONS

In conclusion, the findings of the current study demonstrate that differences in the brain lipidic profile in male mice are already observed in early stages of life depending on the APOE genotype. Generally, ApoE4 individuals have shown greater levels of all the analysed lipid families in comparison with the APOE3 and the C57BL/6 genotypes, which exhibit a similar behaviour in terms of cerebral lipids. Taking into account that most of these lipid families play a crucial role during neurodevelopment, the evidence suggests that APOE4 allele carrying individuals develop earlier in time than APOE3 carriers. Moreover, regarding the treatment effects, CPF has been shown to exert a decreasing effect on lipid concentration. The existence of genotype x treatment effects on some lipid species gives insight into the vulnerability of the APOE genotype towards pesticide exposure, despite not being clear which allele confers a higher susceptibility to CPF. However, more research is needed to elucidate genotype and, particularly, CPF effects on the brain lipid profile. In order to assess the cerebral lipid differences to a greater extent, these evaluations should also be carried out on female mice, which could provide different lipid patterns in the brain. It is of special interest as well to test if effects of CPF are maintained in time after exposure cessation or if they aggravate when the postnatal CPF exposure period is prolonged during the developmental stage.

7. REFERENCES

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ANNEXES

Annex 1. Chlorpyrifos metabolites

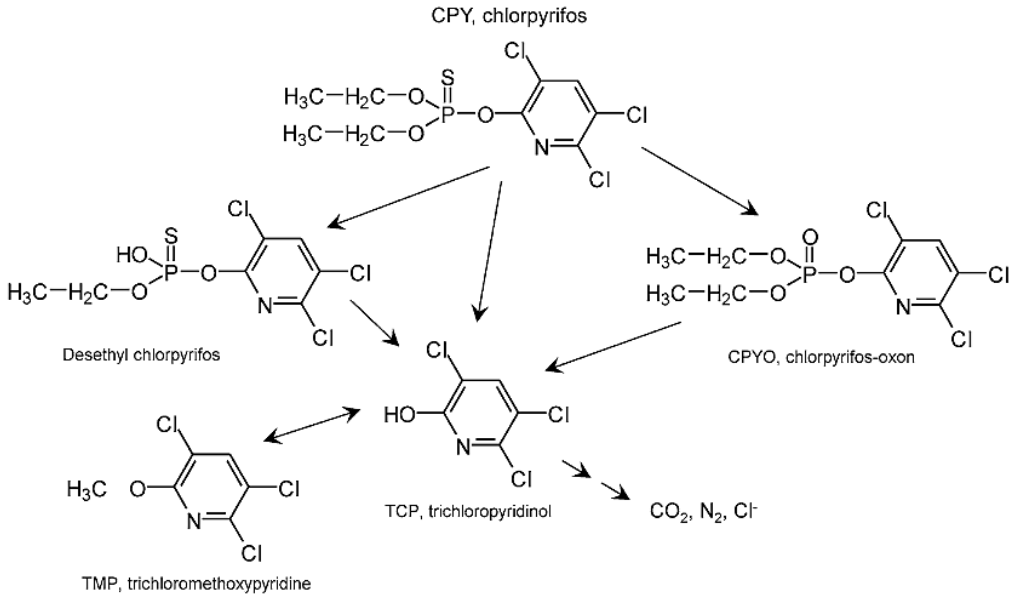

Figure 1S. Chlorpyrifos-derived products after degradation processes in the environment. [Extracted from: Solomon *et al*. (2014)].

Annex 2. Lipid concentration heatmaps for each brain sample

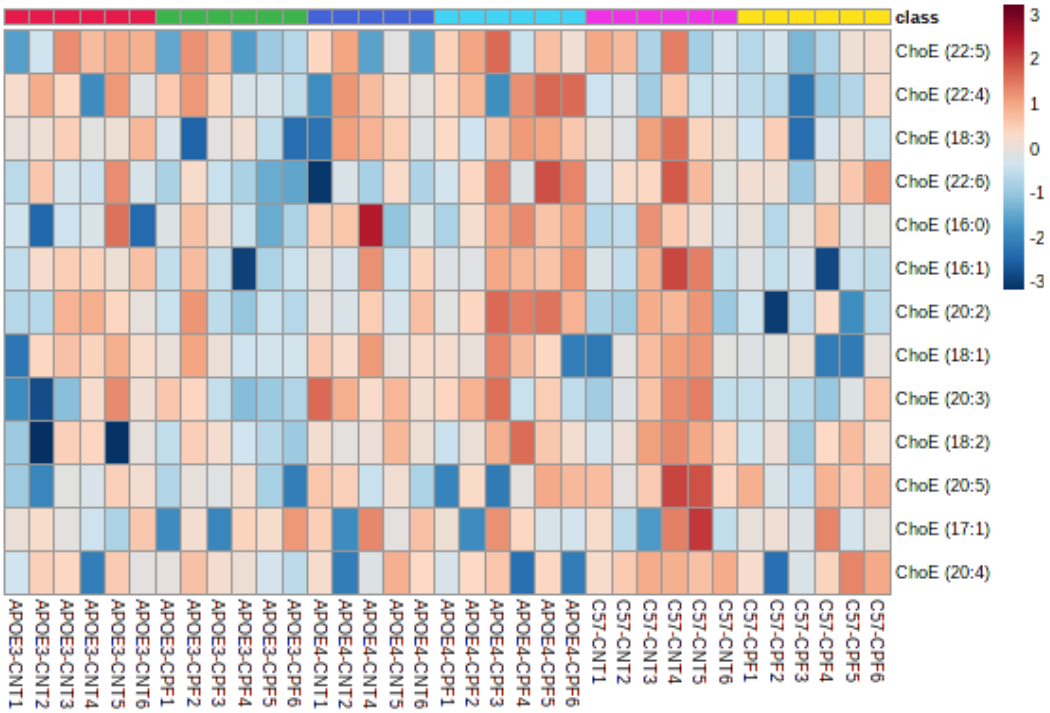

Figure 2S. The heatmap shows the concentration (pmol/mg tissue) of all the cholesteryl ester species found in each mouse brain sample individually. Each column represents an individual.

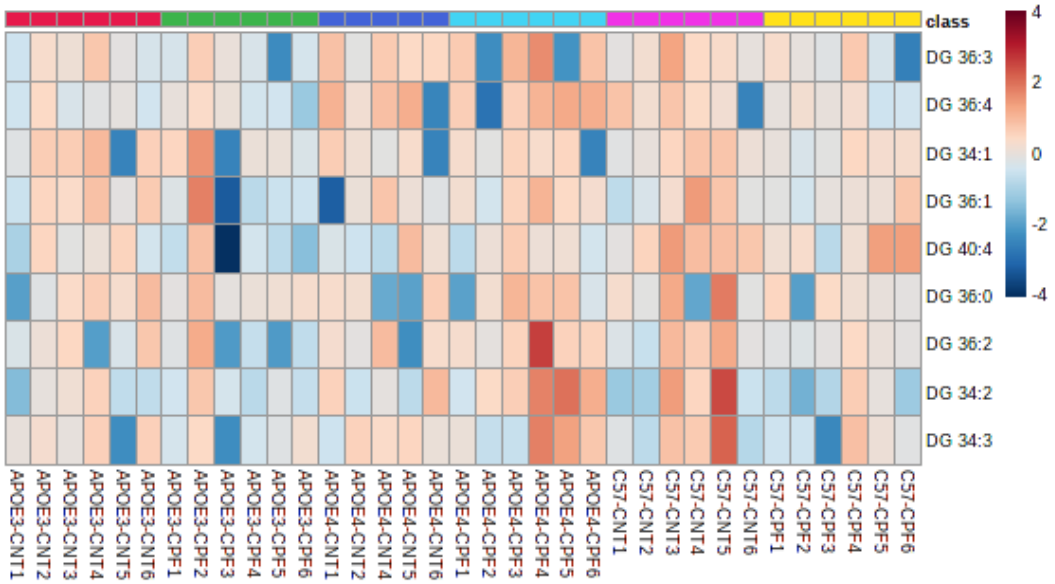

Figure 3S. The heatmap shows the concentration (pmol/mg tissue) of all the diglyceride species found in each mouse brain sample individually. Each column represents an individual.

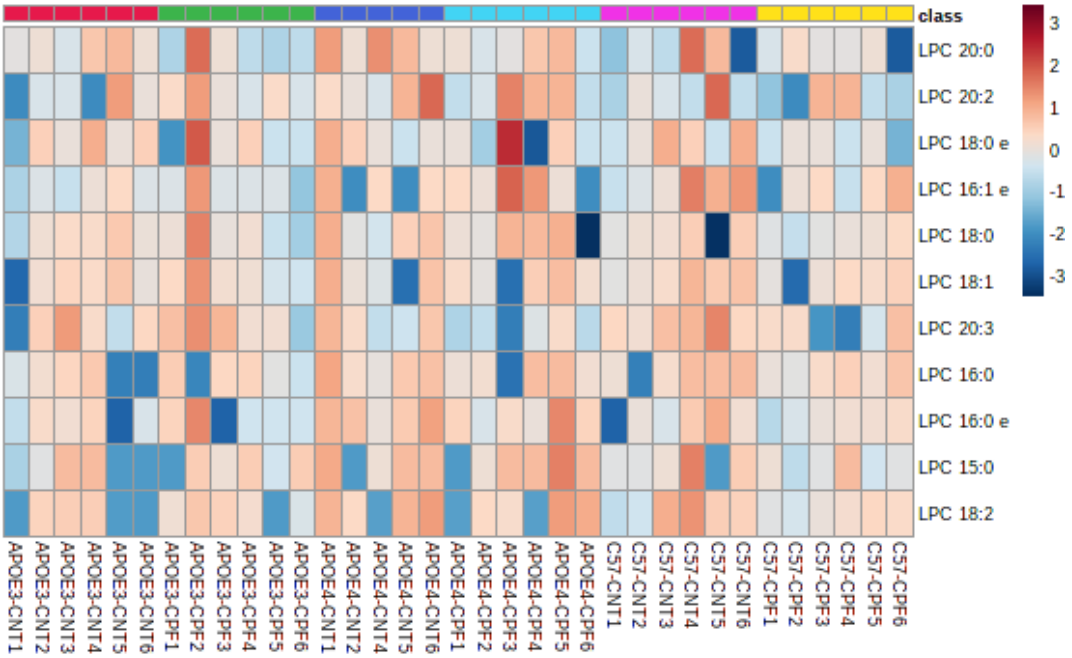

Figure 4S. The heatmap shows the concentration (pmol/mg tissue) of all the lysophosphocholine species found in each mouse brain sample individually. Each column represents an individual.

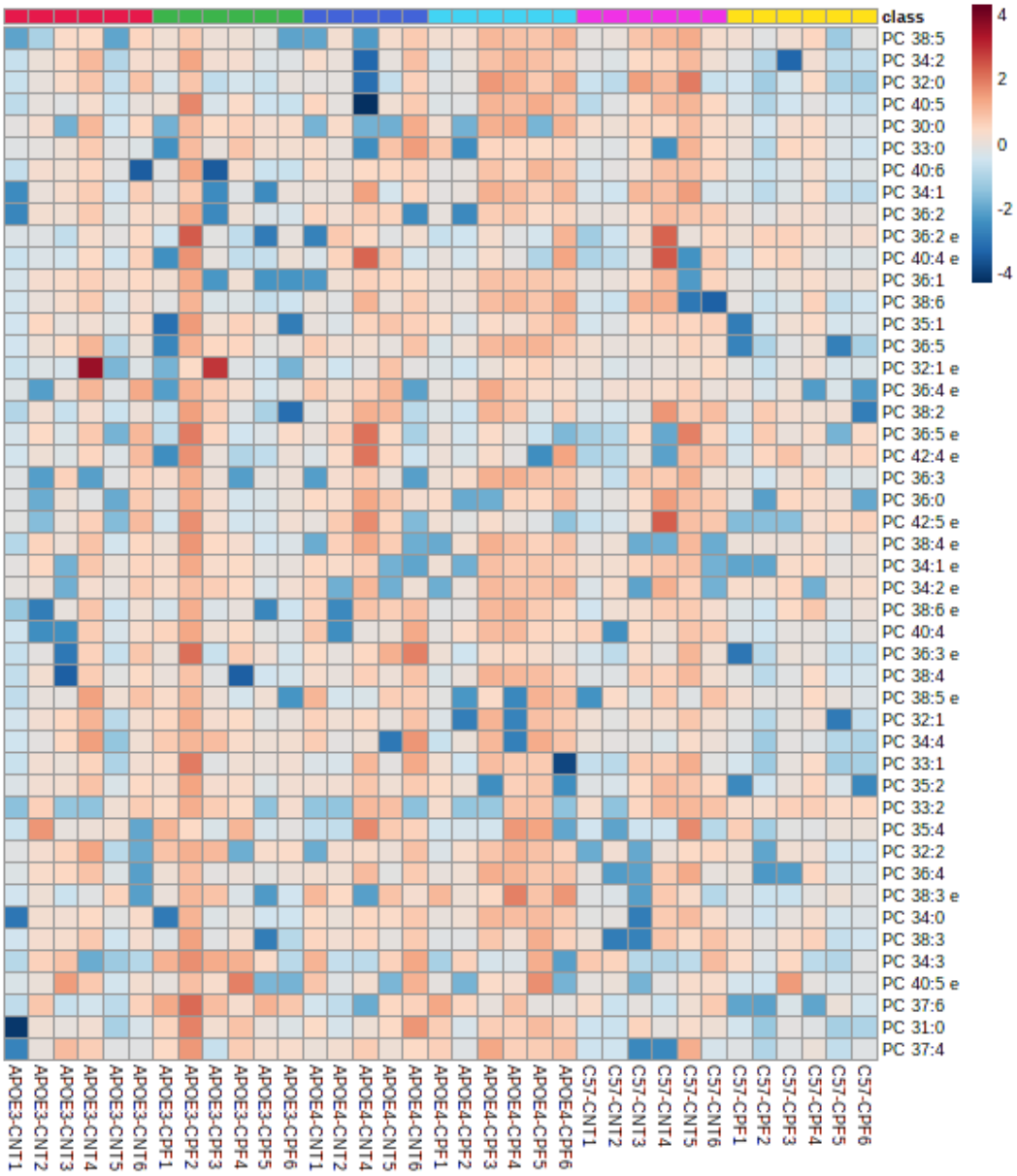

Figure 5S. The heatmap shows the concentration (pmol/mg tissue) of all the phosphatidylcholine species found in each mouse brain sample individually. Each column represents an individual.

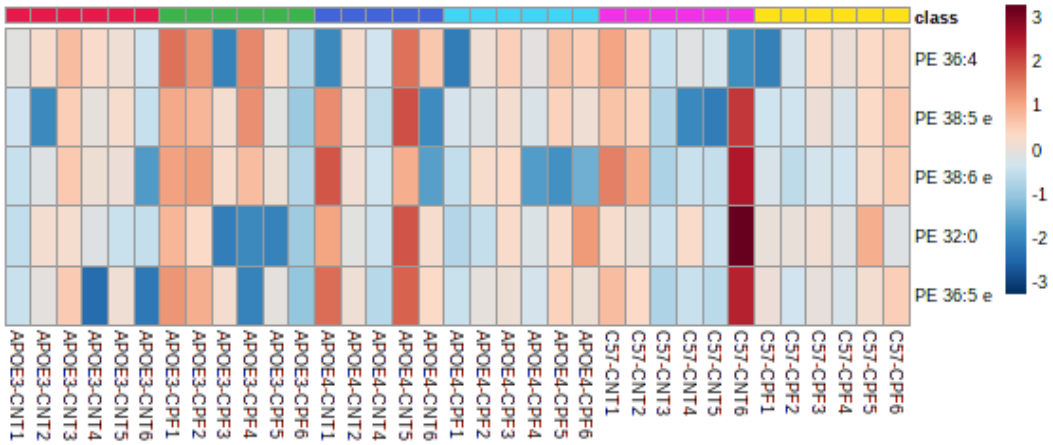

Figure 6S. The heatmap shows the concentration (pmol/mg tissue) of all the phosphatidylethanolamine species found in each mouse brain sample individually. Each column represents an individual.

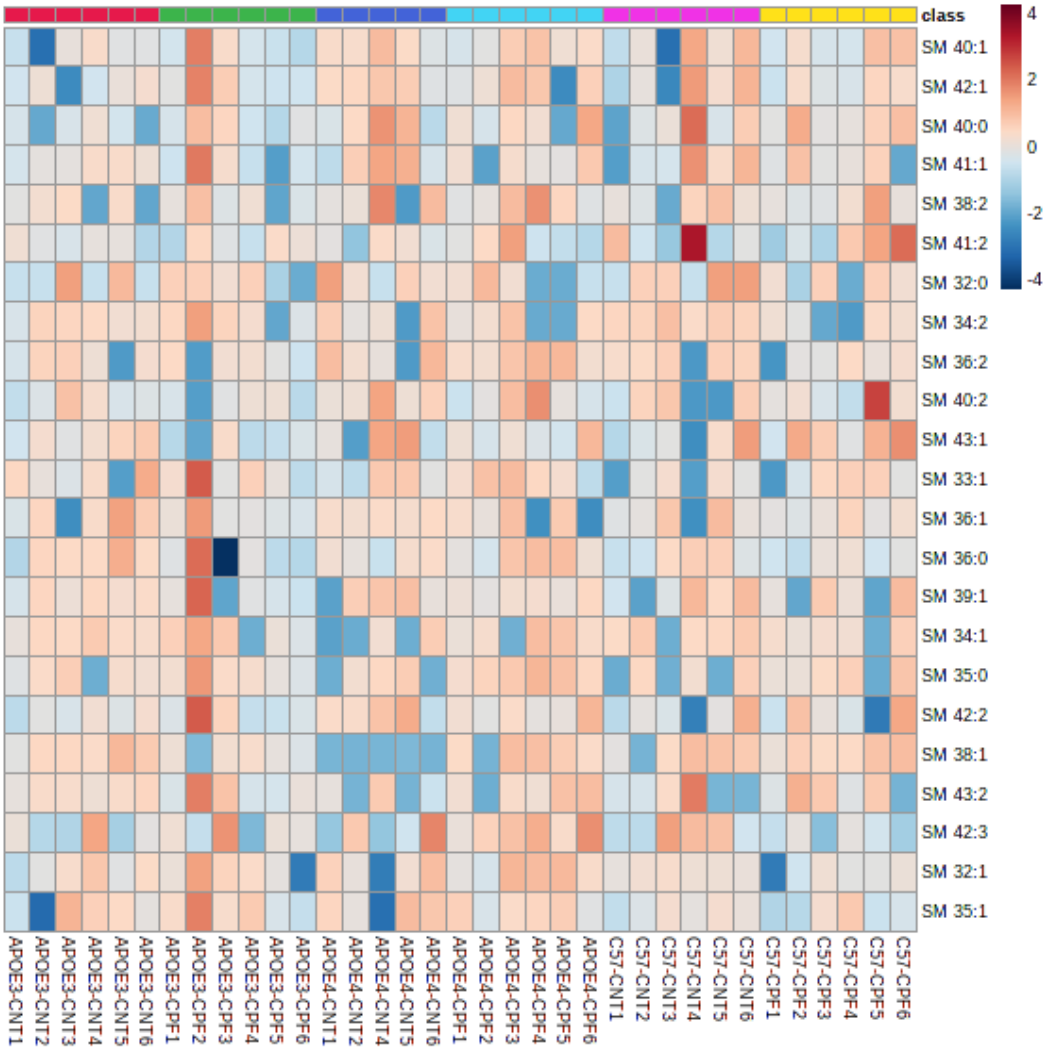

Figure 7S. The heatmap shows the concentration (pmol/mg tissue) of all the sphingomyelin species found in each mouse brain sample individually. Each column represents an individual.

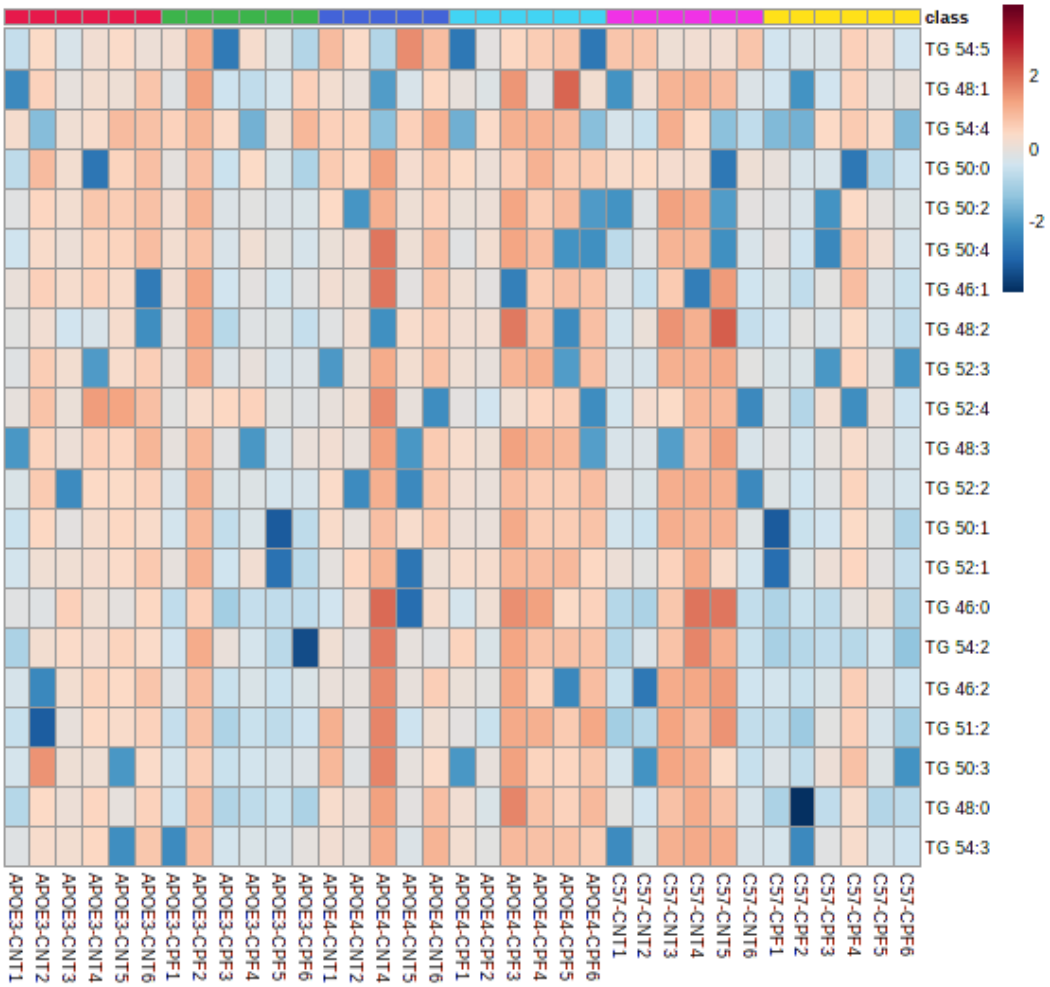

Figure 8S. The heatmap shows the concentration (pmol/mg tissue) of all the triglyceride species found in each mouse brain sample individually. Each column represents an individual.