2015

Investigating changes in human brain phospholipids during normal ageing

Sarah E. Hancock
University of Wollongong

UNIVERSITY OF WOLLONGONG

Investigating changes in human brain phospholipids during normal ageing

A thesis submitted in fulfilment of
the requirements for the award of the degree

Doctor of Philosophy

from

UNIVERSITY OF WOLLONGONG

by

Sarah E. Hancock

Bachelor of Science (Honours Class I)

School of Medicine

Faculty of Science, Medicine and Health

2015

THESIS CERTIFICATION

CERTIFICATION

I, Sarah E. Hancock, declare that this thesis, submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Medicine, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

Sarah E. Hancock

31st March 2015

DEDICATION

I dedicate this thesis to my grandparents, Meg and Bryan Weaver.

Thank you for always believing in me.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PUBLICATIONS

Chapter 4 Norris SE, Friedrich MG, Mitchell TW, Truscott, RJW, Else PL, (2015) Human prefrontal cortex phospholipids containing docosahexaenoic acid increase during normal adult aging, whereas those containing arachidonic acid decrease, *Neurobiology of Aging*, 36(4);1659-69

Chapter 5 Hancock SE, Friedrich MG, Mitchell TW, Truscott, RJW, Else PL, (2015) Decreases in the phospholipids containing adrenic and arachidonic acids occurring in the human hippocampus over the adult lifespan, *Lipids*, 50(9);861-872

Manuscripts submitted for publication

Chapter 6 Hancock SE, Friedrich MG, Mitchell TW, Truscott RJW, Else PL (2015), "The phospholipid composition of the human entorhinal cortex remains relatively stable over 80 years of adult aging"

Conference Proceedings and Presentations

Norris SE, Skora A, Mitchell TW, Truscott RJW, Else PL, (May 2012) Investigating cellular membrane changes in brain tissue during ageing using shotgun tandem mass spectrometry and 2D gel electrophoresis. *American Society for Mass Spectrometry, Vancouver, Canada.*

Norris SE, Skora A, Mitchell TW, Truscott RJW, Else PL, (May 2012) Investigating cellular membrane changes in brain tissue during ageing using shotgun tandem mass spectrometry and 2D gel electrophoresis. *International Society for the Study of Fatty Acids and Lipids, Vancouver, Canada.*

Norris SE, Mitchell TW, Truscott RJW, Else PL, (January 2013) Investigating changes to the human brain lipidome during ageing. *Australian Lipidomics Meeting, Melbourne, Australia.*

Norris SE, Mitchell TW, Truscott RJW, Else PL, (January 2013) Investigating changes to the human brain lipidome during ageing. *The Australian and New Zealand Society for Mass Spectrometry, Melbourne, Australia.*

Norris SE, Friedrich MG, Mitchell TW, Truscott RJW, Else PL, (May 2014) Phospholipid molecular species of the human dorsolateral prefrontal cortex: increases seen in phospholipids containing 22:6 fatty acids during normal aging. *Australian Society for Medical Research NSW Scientific Meeting, Sydney, Australia*

Norris SE, Friedrich MG, Mitchell TW, Truscott RJW, Else PL, (September 2014) Phosphatidylcholines are elevated in the mitochondrial and microsomal membranes of the human hippocampus in Alzheimer's disease, while phosphatidylethanolamines are reduced *APD/CADD Symposium, Sydney, Australia*

Norris SE, Friedrich MG, Mitchell TW, Truscott RJW, Else PL, (December 2014) Investigating changes to phospholipids of the human brain during normal ageing. *Australian Lipids Meeting, Wollongong, Australia*

ACKNOWLEDGEMENTS

There are so many people that without whose assistance this thesis would not have been possible.

Firstly, I'd like to thank my three supervisors, Paul Else, Todd Mitchell, and Roger Truscott, for their support and contributions over the past few years. Thank you for the red pen, the left-handed compliments and the assistance in obtaining income scholarships to support my lavish lifestyle.

Secondly, I thank the people that provided me with training and/or assistance in the laboratory throughout my candidature, including Jennifer Saville, Sarah Abbott, Amanda Skora, Michael Friedrich, Kalani Ruberu and Surabhi Bhatia. Also a big thank you to the remainder of the Bl-itchell group for your input and comments on countless group meeting presentations. And for generally being a huge amount of fun to hang out with at conferences.

Thirdly are my friends and colleagues at the University, who provided caffeine and moral support during my candidature. Colin (my PhD brother), Megan Kelly, Adam Zieba, as well as Sarah Abbott and Michael Friedrich. Thanks for remembering to text me most mornings!

Last, but by no means least, I have to thank my family for their support during my studies: my husband Paul, for all your love, support and help over the past five years, my in-laws for their words of encouragement, and finally my mother, sisters, brother and grandparents for not asking me too many times about when my thesis will be finished.

ABBREVIATIONS

4-HNE	4-hydroxy-2-nonenal
Aβ	Amyloid-beta
AA	Arachidonic acid
ALA	α-linolenic acid; 18:3n-3
AD	Alzheimer's disease
BA	Brodmann area
BHT	Butylated hydroxytoluene
COX-1/2	Cyclooxygenase 1/2
CPT	CDP-choline:1,2-diacylglycerol cholinephosphotransferase
CJD	Creutzfeld-Jakob disease
DHA	Docosahexaenoic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid; 20:5n-3
EPT	CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase
HNE	4-hydroxy-2-nonenal
IsoPs	Isoprostanes
LA	Linoleic acid; 18:2n-6
LC-MS	Liquid chromatography mass spectrometry
MAM	Mitochondria-associated membrane
MDA	malondialdehyde
mtDNA	Mitochondrial DNA
n-3	Omega-3 fatty acid
n-6	Omega-6 fatty acid
nanoESI	Nanoelectrospray ionisation
NFTs	Neurofibrillary tangles
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine *N*-methyl transferase
PS	Phosphatidylserine
PSD	Phosphatidylserine decarboxylase
PSS1/2	Phosphatidylserine serine synthase 1/2

PUFA	Polyunsaturated fatty acid
PVDF	Polyvinylidene fluoride
ROS	Reactive oxygen species
sER	Smooth endoplasmic reticulum
TBST	Tris-buffered saline with Tween®-20

ABSTRACT

The world's population is rapidly ageing, and with that has come a corresponding increase in the number of people suffering age-related diseases. Dementia is a group of age-related neurocognitive disorders, with the most common of these being Alzheimer's disease (AD). The number one risk factor for developing AD is advanced age, with the incidence rising from 1 in 10,000 at age 60 to 1 in 3 by age 85. AD is characterised by the deposition of extracellular amyloid-β aggregates (Aβ) and intracellular neurofibrillary tangles (NFTs) formed by hyperphosphorylated tau. Both Aβ and NFTs spread throughout the brain by separate but predictable pathways during the progression of the disease. Many studies have shown an involvement of membrane lipids, including phospholipids, in the pathogenesis of AD. Less is known about the changes occurring in membrane lipids such as phospholipids within the brain over the normal adult lifespan. Lipids are major components of the brain comprising 40-55% of the dry matter present, with phospholipids making up half of total brain lipid. The human brain undergoes a number of structural changes during the course of ageing that could theoretically lead to alterations in brain phospholipids. Changes to phospholipids with age within the human brain could be driven by two current theories of ageing: i) the mitochondrial free radical oxidative stress theory of ageing, which proposes that ageing is driven by damage to macromolecules such as lipids by reactive oxygen species produced by the mitochondria; or ii) the "inflammageing" theory, which suggests that ageing is driven by chronic, low-grade inflammation over the lifetime of an organism. Several studies conducted over twenty years ago attempted to characterise any changes occurring to brain phospholipids with age, but newer methods utilising mass spectrometry to identify and quantify lipids have become available since then. Understanding the changes occurring to phospholipids during normal ageing may lead to a better interpretation of the changes occurring during AD while also clarifying what fundamental mechanism underpins ageing within the human brain.

Therefore, the aim of this thesis was to characterize any age-related changes occurring to the three dominant phospholipid classes present in the human brain: phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS). To accomplish this, post-mortem tissue (n = 36, age range 18-104 years) from five different regions of the brain was obtained from neurologically normal donors via the

New South Wales Tissue Resource Centre at the University of Sydney. These five areas of the brain were chosen based on the spread of Aβ and NFTs during the progression of AD. The dorsolateral prefrontal cortex, entorhinal cortex and hippocampus all develop Aβ and NFTs during the progression of AD while the motor cortex and cerebellum remain relatively unaffected. Tissue from each region was homogenised, fractionated into a mitochondrial and microsomal component, and lipids were extracted. Molecular phospholipids were identified by complementary positive and negative ion scans using tandem mass spectrometry, and linear regression was performed to determine any age-related changes. The fractionation of the tissue into mitochondrial and microsomal membranes allowed the characterisation of any observed age-related changes to phospholipids to a particular theory of ageing.

A variety of phospholipids underwent changes with age, with no specific trend identified for one membrane fraction over another. No changes with age in total quantified phospholipid levels were seen in either membrane fraction across the five regions. Only the entorhinal cortex experienced age-related changes in a phospholipid class, with increases in mitochondrial PC with age found alongside decreases in mitochondrial PE. Several key age-related changes were seen in phospholipids across the five brain regions. The phospholipid with the largest increase with age was PS 18:0_22:6 within the mitochondrial membranes of the hippocampus and both the mitochondrial and microsomal membranes of the prefrontal cortex and cerebellum. PE 18:0_22:6 also underwent sizeable increases in both membrane fractions of the cerebellum. The largest decrease with age was seen in PS 18:0_18:1 in both membrane fractions of the cerebellum. This was followed by PE 18:0_20:4 in the mitochondria of the prefrontal cortex, the microsomes of the entorhinal cortex, and in both membrane fractions within the cerebellum. The cerebellum showed the largest number of changes to its phospholipids with age in both the mitochondrial and microsomal membranes while the entorhinal and motor cortices experienced the fewest. The only phospholipid to show consistent age-related changes across all five regions was an increase in mitochondrial PC 16:0_18:2. A trend was identified across the five brain regions for increases in phospholipids containing a 22:6 fatty acid (docosahexaenoic acid, DHA) with age, while those containing 20:4 (arachidonic acid, AA) or 22:4 (adrenic acid) tended to decrease. All three fatty acids are polyunsaturated fatty acids that can be classified into omega-3 (DHA) and omega-6 (AA and adrenic acid). Further regressions

with age were conducted to confirm these findings across all five regions of the brain. Total DHA levels increased significantly with age within the mitochondrial and microsomal membranes of the prefrontal cortex and cerebellum. These increases with age were driven by rises in PS-DHA in both of these regions, and by PE-DHA in the cerebellum. Likewise, decreases were seen with age for AA and adrenic acid across both membrane fractions in nearly all regions of the brain examined. This finding suggests that there is a loss of long-chain omega-6 fatty acids within the brain over the adult lifespan, while the omega-3 fatty acids increase.

Compared to the previous literature, the novel finding of this thesis is that the level of DHA increases within specific regions of the brain in neurologically normal subjects over the course of the adult lifespan. This differs drastically from what is seen in the brain during the progression of AD, in which levels of DHA within the affected brain regions are severely reduced. Due to this increase in DHA within both membrane fractions of the brain with age, it was determined that the findings of this thesis aligned more closely with the "inflammageing" theory of ageing than the mitochondrial free radical oxidative theory. Several key pathways involved in the production of phospholipids primarily containing DHA were identified as being useful targets for future research. Future research should also focus on eicosanoid production pathways in order to confirm the involvement of phospholipids in low-grade, chronic inflammation, and the role that this may play in healthy ageing in the human brain.

SECTION 1

INTRODUCTION

Chapter 1 Thesis Overview

1.1 Introduction

An increase in lifespan has led to a dramatic shift in the age demographic of the population worldwide, with the number of people over the age of 65 years projected to triple to 1.5 billion by 2050 [1]. Within Australia, our total population has increased fivefold over the 20th century, but the number of people aged 65 years and over increased more than fifteen-fold in the same period [2]. This aged population is also expected to continue to rise from 13% in 2007 to 23-25% of the population by 2056 [3].

Alongside this increasingly aged population is a corresponding increase in the number of people suffering from age-related disease. Dementia is a group of neurocognitive disorders resulting in the disturbance of higher cortical functions, including memory, thinking, comprehension, language and learning ability. An estimated 35.6 million people were living with dementia worldwide in 2011, with that number predicted to double every 20 years [4]. The economic burden of dementia is staggering, with the global societal costs of the disease estimated at around US$604 billion in 2010 [4]. In Australia alone there were an estimated 298,000 people suffering from dementia nationally in 2011, which resulted in direct and indirect costs of around $4.9 billion [5]. With an increasingly older population and a rising rate of dementia incidence worldwide, research into effective prevention and treatment strategies for such age-related diseases are becoming more and more important.

Alzheimer's disease (AD), the most common form of dementia, is a neurodegenerative disease typified by synaptic loss and increased neuron apoptosis that leads to the extreme atrophy of several brain regions, particularly those involved in memory, speech and behaviour. AD is characterised by the presence of intracellular protein aggregates formed from amyloid-β (Aβ) peptides and extracellular neurofibrillary tangles (NFTs) of hyperphosphorylated Tau. The deposition of Aβ plaques and NFTs follow a different progression to each other during the pathogenesis of AD, but both pathways follow predictable patterns of spread within the brain with little variation between individuals [6,7]. Aβ accumulation can be divided into three stages (Figure 1-1) [7]. Aβ plaques are

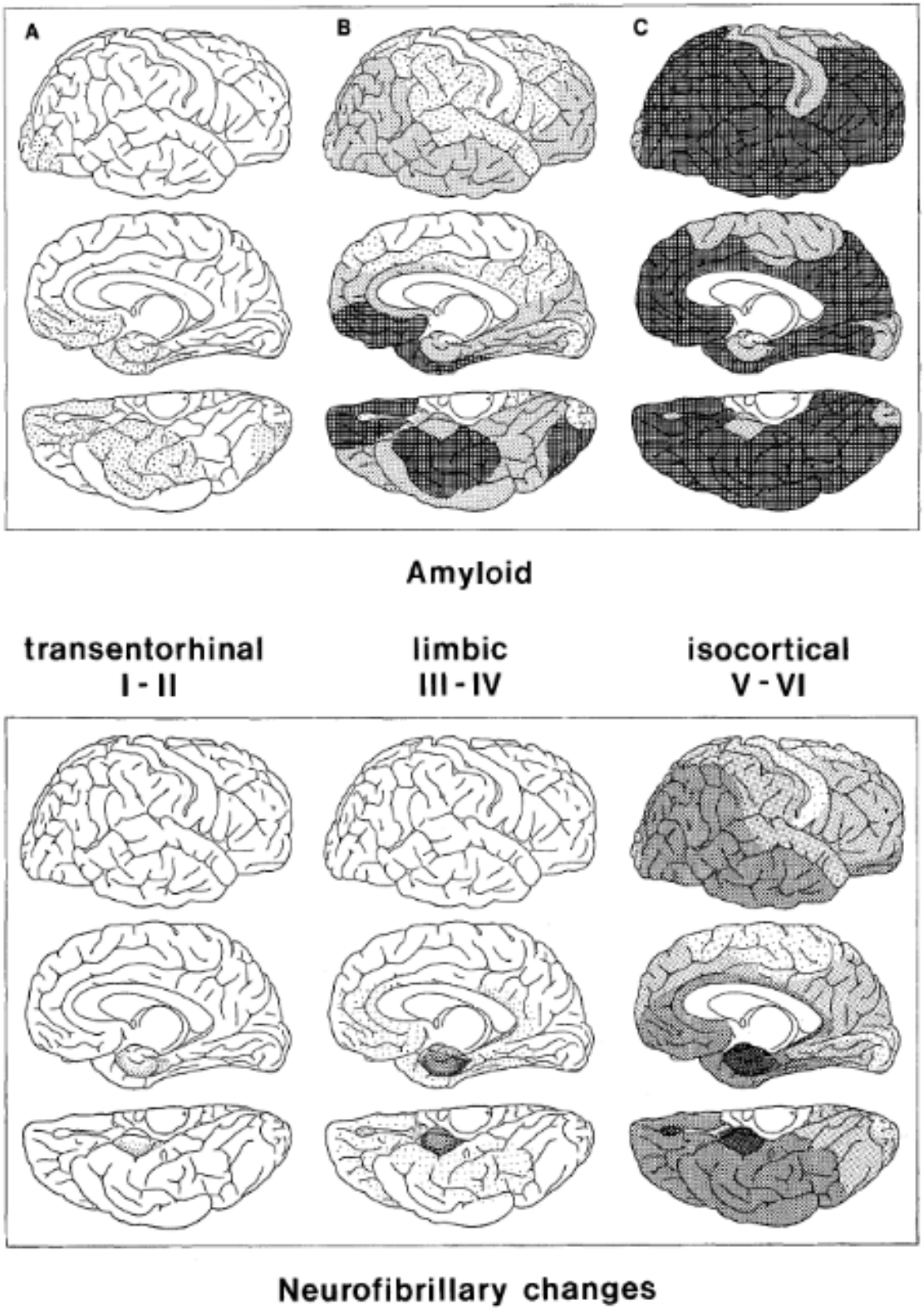


Figure 1-1: The progression of deposition of amyloid-β plaques (upper, stages A through C) and neurofibrillary tangles formed from hyperphosphorylated tau (lower, stages I-VI) in the human brain during the pathogenesis of Alzheimer's disease. From Braak and Braak [7].

initially found within the isocortex (particularly in the basal regions of the frontal, temporal and occipital lobes), and during the progression of the disease spread to almost all of the isocortical association areas. Only the primary sensory and motor cortices remain free of Aβ in the late stages of AD. Aβ accumulation occurs in the hippocampus and entorhinal cortex from the middle stages onwards. Levels of Aβ do not necessarily correlate with cognitive performance, with Aβ plaques commonly seen in the brain of non-demented elderly on autopsy [8] and by imaging techniques *in vivo* [9]. Conversely, NFTs are first present in the region bordering the entorhinal cortex and temporal cortex. In the middle stages, NFTs are found in the limbic system, particularly the hippocampus, which produces mild cognitive impairment and personality changes [6]. In the final stages, large numbers of NFTs are displayed throughout virtually all subdivisions of the neocortex, alongside severe destruction of neocortical association areas. The highest risk factor for developing AD is advanced age with incidence rising sharply from 1 in 10,000 at age to 60 to 1 in 3 by age 85 [10]. Most cases of AD are sporadic in nature with the genetic causes being largely unknown; however, the few genetic associations that are known point towards an involvement of lipid metabolism in the pathogenesis of AD. The strongest known genetic risk factor for AD is homozygosity of the APOE-ε4 allele [11,12]. The APOE gene encodes for apolipoprotein E, which is involved in the trafficking of lipids in the brain. Recent genome-wide association studies have also unearthed several other genetic markers of late-onset AD, several loci of which have roles directly or indirectly in lipid metabolism within the brain [13–15]. Alongside these genetic risk factors, there is increasing evidence for a cell membrane defect in AD, with alterations in phospholipids and other membrane lipids being observed in the disease (reviewed in Chapter 2).

Given that the principal risk factor for AD is advanced age, there is surprisingly little information available on the changes occurring in membrane lipids such as phospholipids during normal ageing. The few studies that have been conducted on normal ageing in the brain were performed over twenty years ago (reviewed in Chapter 2). The human brain undergoes a number of structural changes during ageing, including decreases in cortical thickness [16–21], volume [16,19,22–26], density [27], surface area [16,18], and white matter volume [21,23,26]. Declines in brain metabolic parameters such as oxygen consumption and blood flow [28] with age are also seen. All of these recorded age-related changes could feasibly lead to alterations in brain

phospholipid composition. Since the publication of these past studies on age-related changes in human brain phospholipids, newer methods that use mass spectrometry to examine phospholipids at a molecular level have been developed [29]. Filling this gap in the literature would lead to a better understanding of the changes occurring in phospholipids as a result of neurodegenerative diseases such as AD, and potentially a better understanding of the cellular mechanisms of ageing.

1.2 Statement of the problem

The overall aim of this thesis is to address the current deficit in knowledge regarding the changes occurring in the phospholipid composition of the human brain during normal ageing. A secondary aim is to compare and contrast these age-related changes in the phospholipids of the human brain to those previously reported in the literature for AD. AD was chosen specifically due to the established involvement of phospholipids and other membrane lipids in the pathogenesis of the disease compared to other types of dementia (reviewed in Chapter 2). To achieve the aims of this thesis, the phospholipids of five regions of the brain were systematically analysed using state-of-the-art mass spectrometry techniques. To aid in pinpointing the specific subcellular location where age-related changes occur, the brain tissue from each region was fractionated to give a mitochondrial-enriched and microsomal (mixed cellular membrane) fraction. This fractionation method required substantial method development (Chapter 3). The five brain regions examined in this thesis are shown in Figure 1-2. Three of these five regions were chosen based on their involvement in the pathogenesis of AD (Figure 1-1). They consisted of the dorsolateral prefrontal cortex (Chapter 4), hippocampus (Chapter 5), and entorhinal cortex (Chapter 6) (Figure 1-2). The remaining two brain regions, the cerebellum (Chapter 7), and the motor cortex (Chapter 8) undergo few changes during AD. Due to this they are often used as control brain regions when studying the disease (Figure 1-2). Comparison of the five regions was also conducted to establish any region-specific changes in phospholipid during normal ageing, as well as recommendations for future research (Chapter 9).

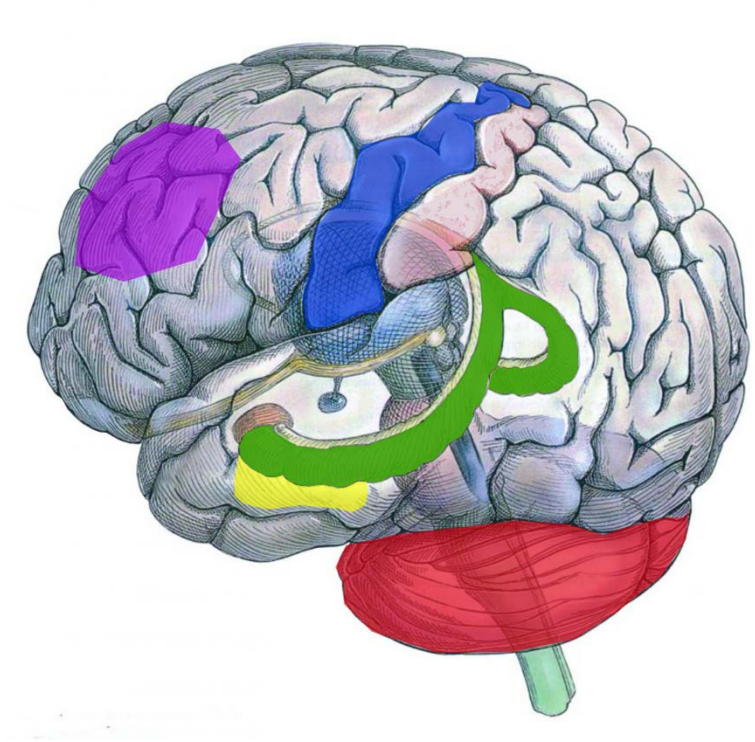


Figure 1-2: Model of the human brain highlighting the five regions analysed within this thesis : dorsolateral prefrontal cortex (purple), hippocampus (green), entorhinal cortex (yellow), cerebellum (red) and motor cortex (blue). The dorsolateral prefrontal cortex, hippocampus, and entorhinal cortex all undergo substantial changes during the pathogenesis of AD, while the cerebellum and motor cortex remain relatively spared (as shown in Figure 1-1).

Chapter 2 Literature review

2.1 The importance of phospholipids in the brain

2.1.1 Phospholipids

The bilayer present in cellular membranes is formed by a range of lipid molecules, with the primary type of lipid present being phospholipids. Glycerophospholipids, more commonly known as phospholipids, are amphipathic molecules consisting of a polar head group and two fatty acids attached to a glycerol backbone (Figure 2-1). Different combinations of head group and fatty acids in a phospholipid can produce up to 10 000 different molecular species [30]. Such variation in their structure can in turn affect membrane properties including membrane fluidity and the modulation of membrane-bound protein activity [31–33].

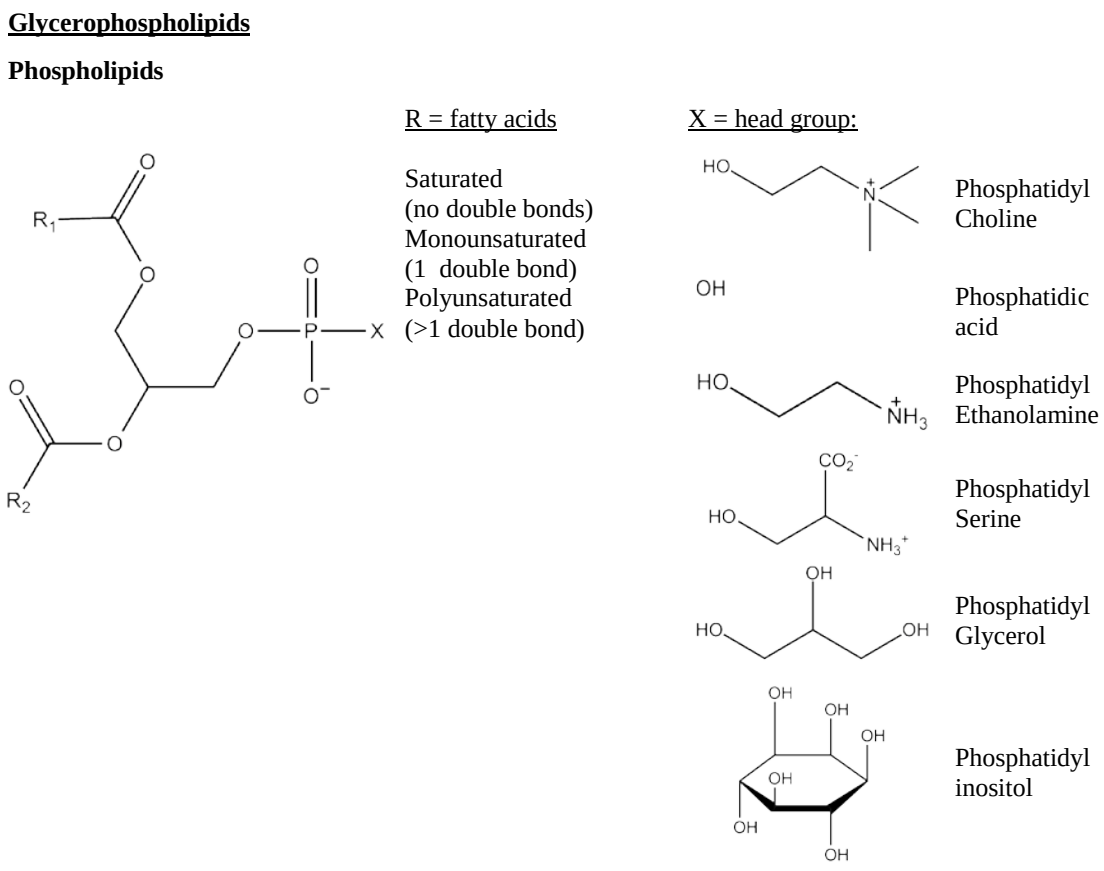


Figure 2-1: Glycerophospholipids. The structural diagrams show possible head group (X) and fatty acid placement (R; see Figure 2-2).

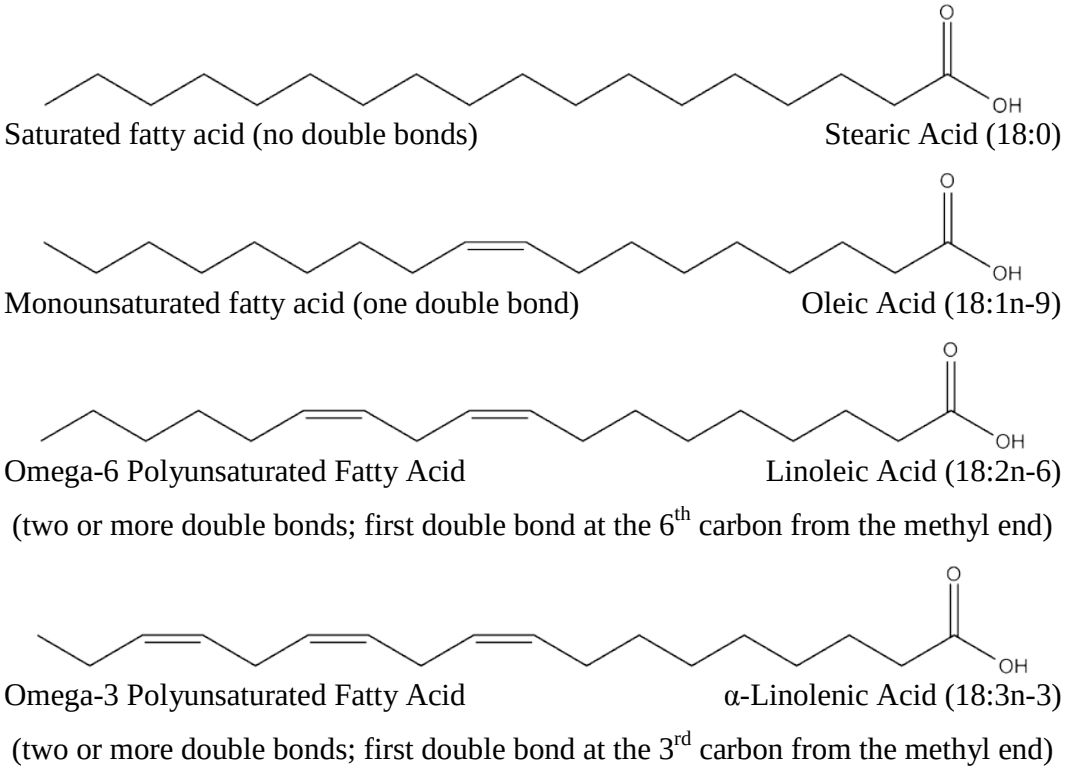


Figure 2-2: Examples of different fatty acids from the 18-carbon series.

The three most common phospholipid classes found in mammals are phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS). The head groups are linked to the glycerol backbone by phosphate in the *sn*-3 position (Figure 2-1). Other less common phospholipid classes include phosphatidic acid, phosphatidylglyercol and phosphatidylinositol. The fatty acids of phospholipids are esterified to the *sn*-1 and *sn*-2 positions of glycerol, and can be classified by their chain length (number of carbons). Fatty acids can also be classified by the number of double bonds present: saturated (no double bonds), monounsaturated (one double bond) and polyunsaturated (more than one double bond) (Figure 2-2). Polyunsaturated fatty acids (PUFA) can be further classified by the position of the first double bond from their methyl end into omega-3 (n-3) and omega-6 (n-6) fatty acids. Phospholipids can also contain ether-linked fatty acids, which are joined to the glycerol backbone by either an alkyl or alkenyl ether bond. In this literature review, the focus will be mainly on the three predominant phospholipids present in mammalian cellular membranes, PC, PE and PS.

2.1.2 Phospholipids in the human brain

Lipids are found in high amounts in the human brain, making up around 40-55% of the dry matter present, but this can be considerably higher in myelinated neurons, rising to around 80% of total dry matter [34]. Phospholipids are the dominant lipid found in cellular membranes and comprise around half of the lipids present in the dry matter of the human brain [34]. The overall phospholipid composition of the top three phospholipids found in the adult brain is approximately 35-40% PC, 35-40% PE and 20% PS [35]. Compared with other tissues, the brain is highly enriched in phospholipids containing PUFA, particularly docosahexaenoic acid (DHA, 22:6n-3), which is found mostly in the PE and PS classes of phospholipids. Within the grey matter of the human cerebral cortex, both PE and PS phospholipids consist of approximately 20-25% DHA [35]. Other PUFA, such as arachidonic acid (AA, 20:4n-6), are also present in high levels in the human brain, particularly in PC and PE, making up approximately 15% of the fatty acid present in each class [35]. PUFA have many important biological roles within cell membranes, including the modulation of membrane fluidity, influencing the activity of membrane-bound proteins, and acting as secondary messengers in inflammatory pathways [31,36–38]. Described below are the ways in which phospholipids and their fatty acids are synthesised within mammalian tissues.

2.1.3 Synthesis of phospholipids

Phospholipids present in cellular membranes can be obtained directly from the diet, synthesised *de novo* or produced via deacylation and reacylation pathways which remodel the fatty acids present in membranes. Only a few phospholipid molecular species can be synthesised *de novo*, with the vast array of structural diversity seen in phospholipids being the result of the remodelling pathways [39]. Little is known about specific pathways of phospholipid synthesis in the human brain, and as such this section will focus on general phospholipid biosynthesis pathways in mammalian tissues.

Both PC and PE follow a parallel pathway during *de novo* synthesis (Figure 2-3) [40]. The first step in PC synthesis is the phosphorylation of choline by choline kinase.

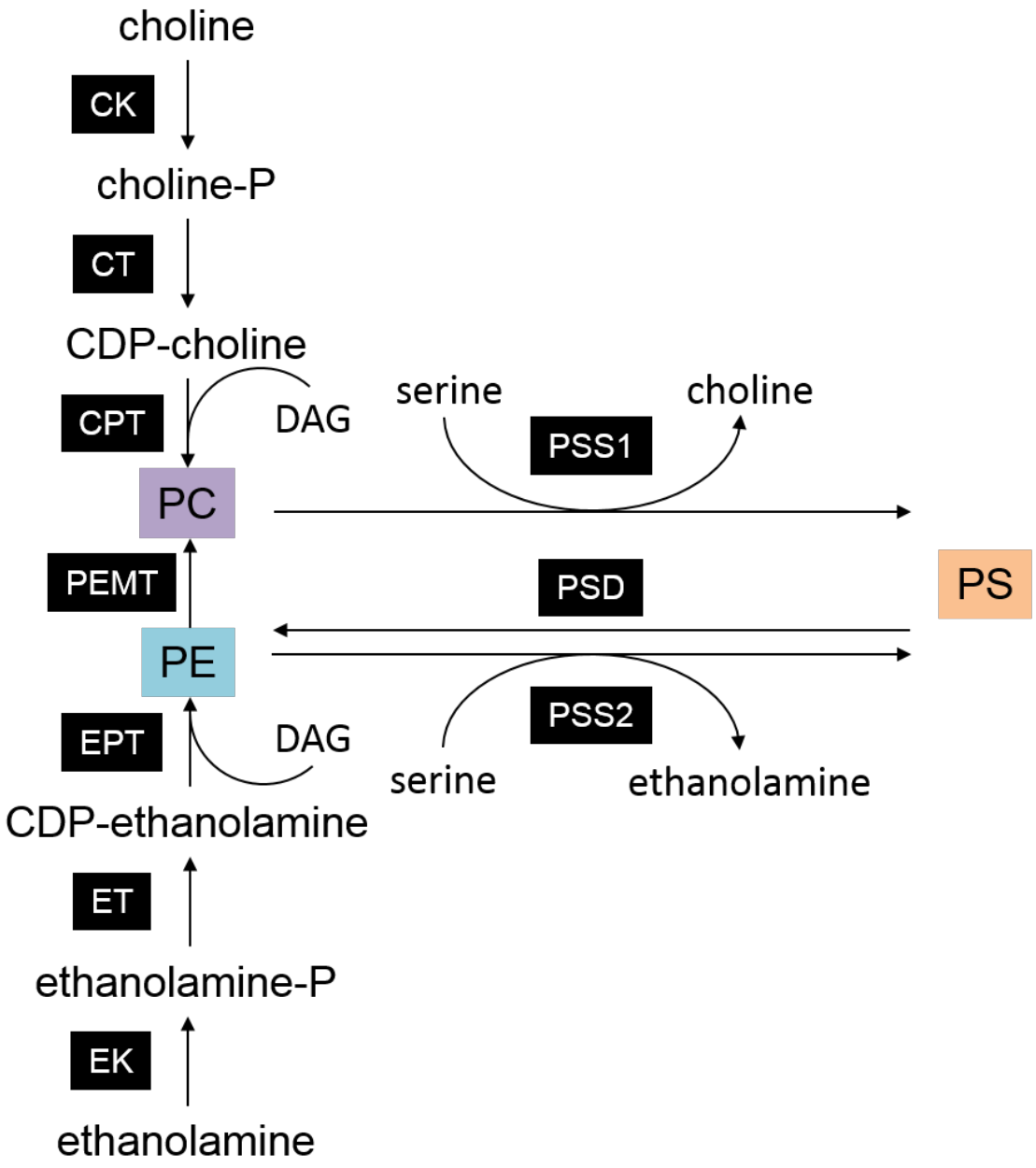


Figure 2-3: *Synthesis of the three most abundant phospholipids present in mammalian cellular membranes . CK* choline kinase; *CPT* CDP-choline:1,2-diacylglycerol cholinephosphotransferase; *CT* CTP:phosphocholine cytidylyltransferase; *DAG* diacylglycerol; *EK* ethanolamine kinase; *EPT* CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase; *ET* CTP:phosphoethanolamine cytidylyltransferase; *PC* phosphatidylcholine; *PE* phosphatidylethanolamine; *PEMT* phosphatidylethanolamine *N*-methyl transferase; *PS* phosphatidylserine; *PSD* phosphatidylserine decarboxylase; *PSS1* phosphatidylserine synthase 1; *PSS2* phosphatidylserine synthase 2.

Next, CDP is added to phosphocholine by CTP:phosphocholine cytidylyltransferase. Finally, CDP-choline is transferred to diacylglycerol by CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT) to form phosphatidylcholine. Phosphatidyl-ethanolamines are synthesised *de novo* following the same steps as phosphatidylcholine, but by ethanolamine-specific enzymes (Figure 2-3). The synthesis of PC and PE phospholipids takes place in the smooth endoplasmic reticulum (sER), but both CPT and CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (EPT) can also be localised to a specific portion of the sER known as the mitochondria-associated membranes (MAM) [41]. CPT is also present in the outer mitochondrial membrane, allowing the mitochondria to synthesise PC phospholipids *de novo*, but EPT is only present on the sER. There are four primary PC and PE molecular phospholipids that are known to be synthesised *de novo* via these pathways: 16:0/18:2n-6, 16:0/18:1, 16:0/22:6n-3 and 18:1/18:2n-6 [42]. The vast bulk of structural diversity seen within phospholipids is not the result of *de novo* synthesis, but arises from deacylation and reacylation pathways that remodel the fatty acids present in phospholipids. Phospholipase A_1 and A_2 cleave fatty acids from the *sn*-1 and *sn*-2 positions of phospholipids respectively to form lysophospholipids. Acyl-CoA:lysophospholipid acyltransferases specific to both phospholipid head group and *sn* position esterify fatty acids onto lysophospholipids to reform diacyl phospholipids. The net result of these processes are phospholipids predominately with a saturated fatty acid present in the *sn*-1 position and an unsaturated fatty acid in the *sn*-2 position (reviewed by Yamashita et al. [43]).

In contrast to PC and PE, there are no *de novo* synthesis pathways for the synthesis of PS in mammalian membranes. Rather, PS is synthesised through a calcium-dependent serine base-exchange reaction from PC by phosphatidylserine synthase 1 (PSS1) and from PE by phosphatidylserine synthase 2 (PSS2, Figure 2-3). Both PSS1 and PSS2 are located in the sER, primarily within the MAM [44], with the close proximity of the two membranes allowing the transfer of PS into the mitochondrial membranes after synthesis [40]. PSS2, in particular, is highly expressed in the brain of mammals, yet mice with PSS2-deficiency have normal membrane phospholipid content [45]. PSS2 displays a preference for PE phospholipids containing DHA in the *sn*-2 position over those containing other fatty acids such as AA or oleic acid (18:1) [46]. This preference leads to the high level of polyunsaturation seen in PS phospholipids in the brain.

Increases in PS synthesis via serine-base exchanges have been reported in aged rats in the cerebellum and cerebral cortex, both with and without the addition of exogenous calcium [47].

PC and PE phospholipid can also be synthesised via remodelling pathways. Within the inner mitochondrial membrane, the synthesis of PE phospholipids occur by decarboxylation of PS by phosphatidylserine decarboxylase (PSD, Figure 2-3). This pathway displays a preference for generating phospholipids containing PUFA in the *sn*-2 position [48] but is thought to only account for 7% of total PE production within the brain [49]. Deletion of PSD in mice causes embryonic death at day 9.5, with morphologically aberrant, fragmented mitochondria present [50,51]. No changes in the activity of PSD have been observed in the cerebral cortex or cerebellum of aged rats [47]. PC phospholipids can also be generated by the methylation of PE via phosphatidylethanolamine *N*-methyl transferase (PEMT, Figure 2-3). PEMT activity is present in the sER, and immunoreactive PEMT has also been identified in the MAM [52]. Although a seemingly redundant pathway, mice with homozygous deletion of PEMT show liver failure within three days when choline is eliminated from the diet, with no overt changes in the phospholipid composition of any other tissue [53]. PEMT activity has been found to increase with age in the cerebral cortex of rats [47]. Alterations to PEMT structure and activity may also be involved in AD, with a single nucleotide polymorphism in PEMT associated with sporadic AD in a Han Chinese population [54]. Decreased levels of PEMT activity have also been seen in the cerebral cortex of people with AD [55].

2.1.4 Synthesis of polyunsaturated fatty acids

Unlike saturated and monounsaturated fatty acids, n-3 and n-6 PUFA are considered to be essential in the diet because they cannot be synthesised *de novo* by mammals. However, long-chain PUFA of 20 carbons or longer can be synthesised through elongation and desaturation of shorter-chain precursors via a pathway common to both n-3 and n-6 fatty acids (Figure 2-4). This pathway begins with either linoleic (LA, 18:2n-6) or α-linolenic (ALA, 18:3n-3) acid in the endoplasmic reticulum. Both of these fatty acids undergo a series of alternating double bond insertions (desaturation) by Δ6 and Δ5 desaturases, followed by the addition of two carbons (elongation) via elongases.

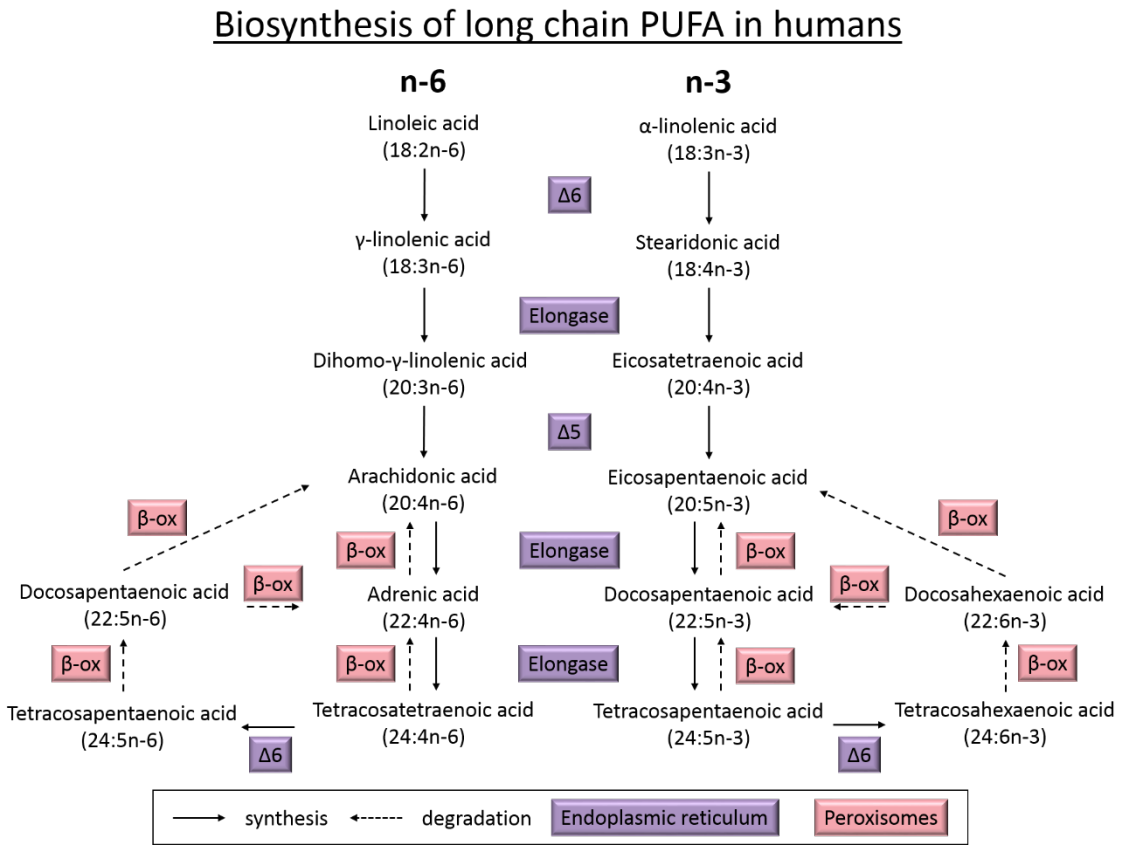


Figure 2-4: Biosynthesis of long-chain polyunsaturated fatty acids (PUFA) in humans from precursor omega-3 (n-3) and omega-6 (n-6) PUFA, which occurs via a common desaturase/elongase system. In the endoplasmic reticulum Δ6- and Δ5-desaturases introduce double bonds at the sixth and fifth carbons from the carboxyl end respectively, while several elongase isoforms increase the fatty acid chain length by two carbons at the carboxyl end. Long chain PUFAs containing 22 carbons are synthesised via additional β-oxidation steps in the peroxisomes Adapted from [56].

Initially, it was assumed that the 22 carbon series PUFA such as docosapentaenoic acid (22:5n-6) and DHA were converted from AA and eicosapentaenoic acid (EPA, 20:5n-3) respectively in humans and mammals by elongation and subsequent desaturation by a Δ4 desaturase within the endoplasmic reticulum. However, experiments by Voss et al. [57] in rat microsomes using either ^{14}C labelled docosapentaenoic acid (22:5n-3) or tetracosapentaenoic acid (24:5n-3) and tetracosahexaenoic acid (24:6n-3) resulted in the production of radiolabelled DHA. This finding indicated that at least one round of β-oxidation of 24-carbon series PUFA was required for DHA synthesis. Unknown at this time was whether this β-oxidation step was occurring in the mitochondria or the peroxisomes. Martinez and colleagues [58,59] had noted that people suffering from the peroxisomal disorder Zellweger syndrome were massively deficient in long chain

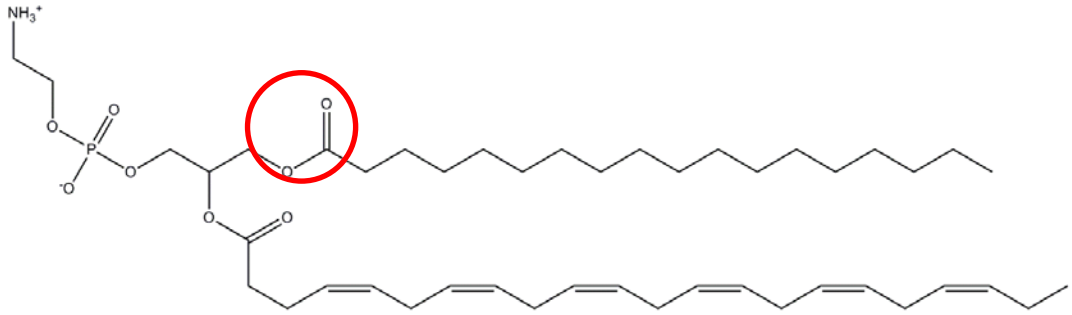
PUFA such as docosapentaenoic acid (both n-3 and n-6) and DHA, particularly in the brain, hinting that the peroxisomes are required for the synthesis of long chain PUFA. This deficiency in DHA is so severe that children with Zellweger syndrome suffer acute neurological impairment and rarely survive past their first year of life. To test this, Christensen et al. [60] incubated ^{14}C radiolabelled adrenic acid (22:4n-6) and docosapentaenoic acid (22:5n-3) in both normal and Zellweger fibroblasts. They found that the Zellweger fibroblasts were unable to retroconvert these fatty acids to AA and EPA respectively, demonstrating that the peroxisomes were required for the β-oxidation step needed to synthesise long-chain PUFA. This finding was confirmed by Moore et al., [61] who found an inability of Zellweger fibroblasts to produce DHA from ^{14}C radiolabelled ALA, docosapentaenoic, tetracosapentaenoic or tetracosahexaenoic acid.

There are three possible ways to meet the brain's high requirement for DHA: local synthesis from short chain precursors within the brain, synthesis by other organs such as the liver, or incorporation from the diet. Whether the human brain itself can synthesise adequate levels of DHA from ALA has not been well studied. Cho et al. [62] described abundant amounts of Δ6 desaturase mRNA within male adult human brain from post-mortem tissue. However, experiments in rats have shown that the use of DHA by the brain vastly outweighs its ability to synthesise it from ALA, with a 10-fold increase in the rate of liver DHA synthesis compensating for this deficiency [63]. Experiments *in vitro* have found a low capacity for cultured neurons to make their own DHA from ALA or EPA [64], but marked increases in the synthesis of DHA and AA from ALA or LA respectively have been reported in cocultures of astrocytes with brain endothelial cells [65,66]. Regardless, the production of DHA from either ALA or EPA is known to be quite inefficient overall in adult humans [67,68]. Both n-3 and n-6 PUFA use the same enzymatic pathway for synthesis and compete for use of this pathway (Figure 2-4). Under normal conditions desaturases have a preference for n-3 over n-6 PUFA, but LA is so highly abundant in the average Western diet that levels of long-chain n-6 PUFA such as AA never fall below optimum levels (except in cases of severe dietary fatty acid deprivation) [69]. By comparison, the levels of ALA in the diet are much lower and combined with an overall inefficient conversion to long chain PUFA in humans this results in a lower available pool of EPA and DHA. Indeed, most of the DHA present within the brain accumulates very early on in development [70], either prior to or in the first few months after birth [71–73]. However, newer research suggests some capacity

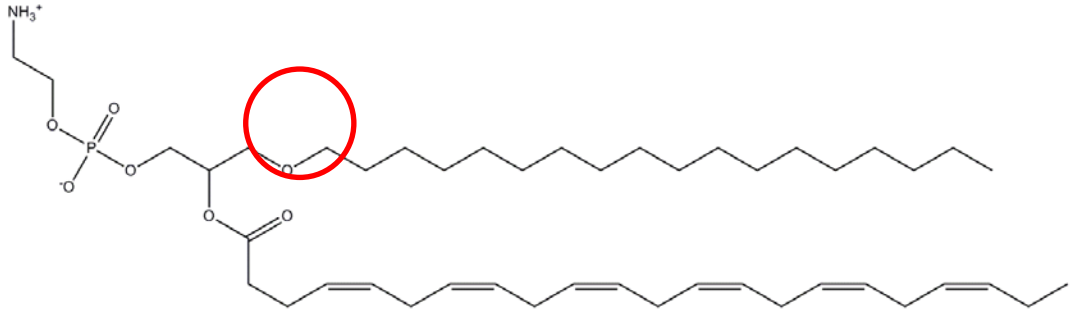
for transport of long-chain n-3 PUFA in the brain of adults [74,75], which likely serves to maintain levels of PUFA accrued during infant brain development [76]. Due to the brain's lifelong need for long-chain n-3 PUFA, this has led to the belief that both EPA and DHA should be considered essential dietary fatty acids [69,77].

2.1.5 Ether-linked phospholipids

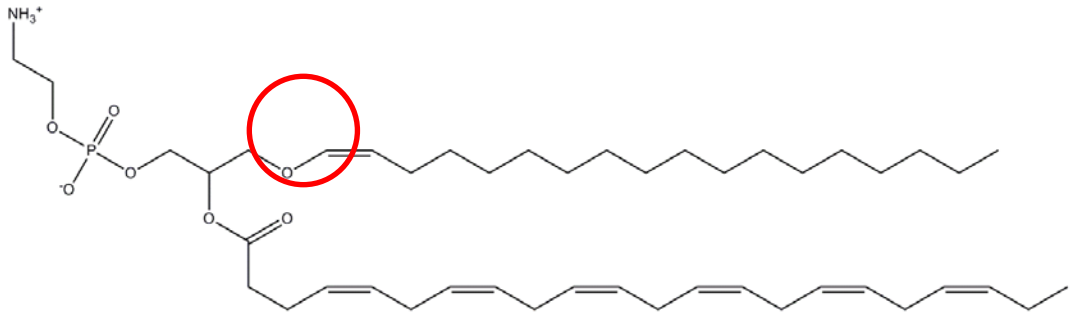
The human brain is also highly enriched in phospholipids that contain ether-linked fatty acids; mainly the vinyl-ether phospholipids known as plasmalogens. Two types of ether-linked phospholipids exist: 1-O-alkyl-2-acyl phospholipids, which contain an alkyl ether- linked fatty acid in the *sn*-1 position and an esterified fatty acid in the second; and 1-O-alkenyl-2-acyl phospholipids, which have an alkenyl (or vinyl) ether bond linking the fatty acid at the *sn*-1 position (Figure 2-5). 1-O-alkenyl-2-acyl phospholipids are better known by their common name, plasmalogens, and are particularly abundant within the PE class of phospholipids in the human brain, comprising one-half to two-thirds of this phospholipid class [78]. These PE plasmalogens contain PUFA such as DHA and AA in the sn-2 position mostly. Ether-linked phospholipids are synthesised *de novo* in the peroxisomes and endoplasmic reticulum (reviewed by [79,80], Figure 2-6). Biosynthesis of alkyl-ether phospholipids and plasmalogens begins in the peroxisomes, where 1-alkyl-dihydroxyacetone phosphate is formed from dihydroxyacetone phosphate via a series of steps. 1-alkyl-dihydroxyacetone phosphate can then be reduced to 1-alkyl-2-hydroxy-sn-glycerophosphate by an acyl/alkyl reductase located on the outside of both peroxisomal and endoplasmic reticulum membranes. Within the endoplasmic reticulum, the phosphate group is removed by phosphatidic acid phosphatase and an acyl group is placed in the *sn*-2 position to form 1-alkyl-2-acyl-sn-glycerol. From here it is converted to either a PC or PE either by the same CPT and EPT that add CDP-choline and CDP-ethanolamine on diacylglycerol to produce diacyl PC or PE (discussed in section 2.1.3), forming an alkyl-ether containing PC and PE phospholipids. The vinyl-ether bond seen in plasmalogens is introduced by Δ-1'-desaturase, with 1-alkyl-2-acyl-PE being the preferred substrate. Choline plasmalogens are primarily formed from hydrolysis of ethanolamine plasmalogens followed by the addition of choline via choline-transferase [81]. The final fatty acid composition of both alkyl-ether phospholipids and



1-octadecanoyl-2-docosahexaenoyl-phosphatidylethanolamine PE 18:0/22:6



1-O-octadecyl-2-docosahexaenoyl-phosphatidylethanolamine PE O-18:0/22:6



1-O-octadecenyl-2-docosahexaenoyl-phosphatidylethanolamine PE P-18:0/22:6

Figure 2-5: Three subclasses of phospholipids bearing either 1,2-acyl (top), 1-O-alky-2-acyl (middle) or 1-O-alkenyl-2-acyl (bottom) fatty acids. 1-O-alkenyl-2-acyl phospholipids are more commonly known as plasmalogens. Shorthand notation for each type of phospholipid is indicated on the right. The different sn-1 linkages are highlighted in red.

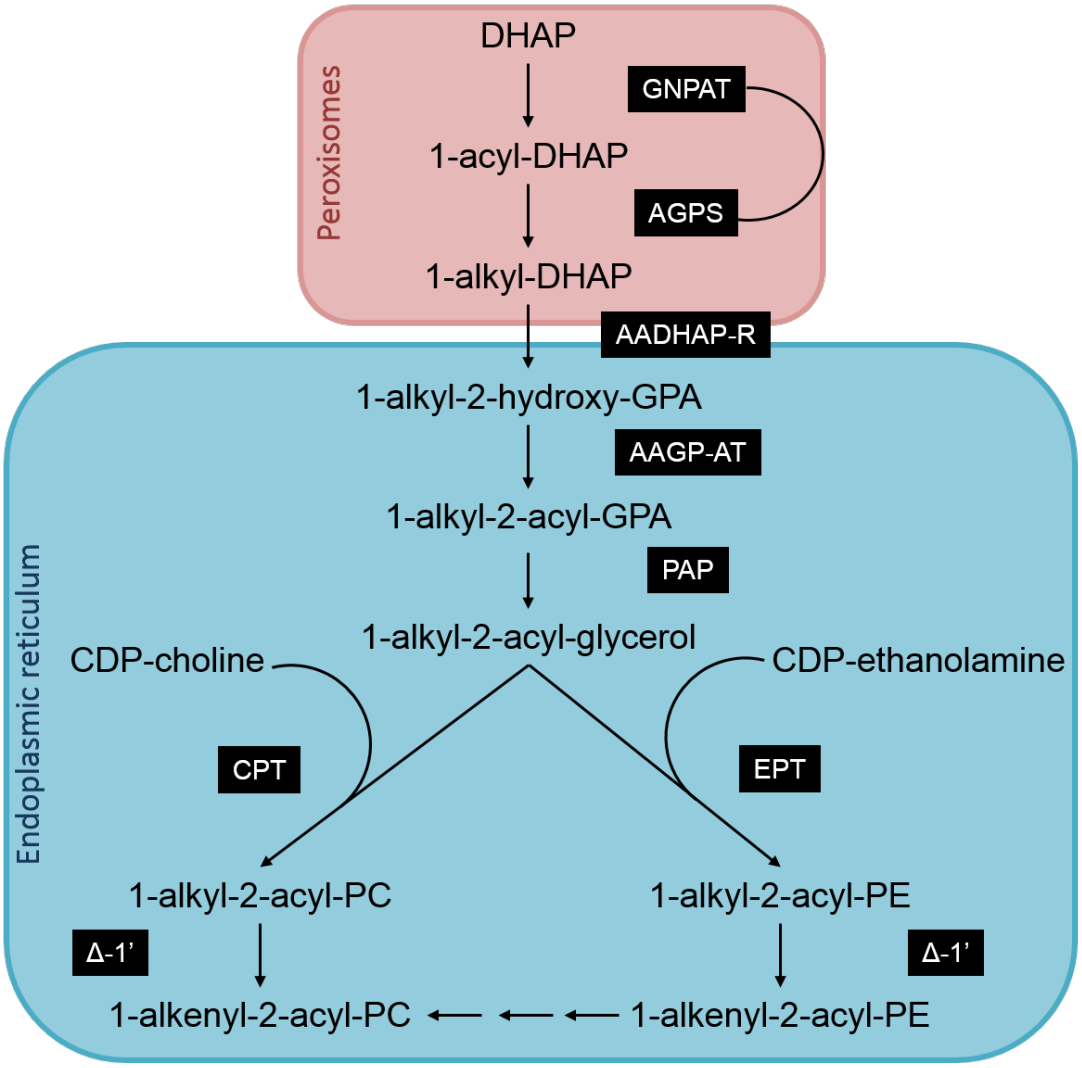


Figure 2-6: Synthesis of ether-linked phospholipids within mammalian cells. *Δ-1'* Δ-1' desaturase; *AADHAP-R* alkyl/acyl DHAP reductase; *AAGP-AT* acyl/alkyl glycerophosphate acyltransferase; *AGPS* alkylglycerone phosphate synthase; *CPT* CDP-choline:1,2-diacylglycerol cholinephosphotransferase; *DHAP* dihydroxyacetone phosphate; *EPT* CDP-ethanolamine:1,2-diacylglycerol ethanolamine-phosphotransferase; *GPA* glycerophosphate; *GNPAT* glyceronephosphate-O-acyltransferase; *PAP* phosphatidic acid phosphatase; *PC* phosphatidylcholine; *PE* phosphatidylethanolamine. Adapted from [79,80].

plasmalogens is mostly attained via the fatty acid remodelling process described above in section 2.1.3.

Plasmalogens and alkyl-ether lipids have several distinct biological roles. Firstly, their presence can alter membrane properties by decreasing fluidity, increasing order, and promoting the formation of non-bilayer phases at lower temperatures, a process needed for membrane fission and fusion events [79]. Plasmalogens are highly enriched in lipid raft domains of the plasma membrane [82,83]. The vinyl ether bond present on plasmalogens is also thought to be preferentially oxidised by free radicals and singlet oxygen, protecting nearby unsaturated fatty acids [79,80,84]. A deficiency of plasmalogens within cell membranes has been observed in several diseases, including AD [85–87]. The changes that occur to plasmalogens in AD will be discussed in more detail below (section 2.3).

2.2 Changes to phospholipids in the brain during normal ageing

It is known that throughout infancy and childhood development DHA accumulates in the brain within PE and PC phospholipids [70]. Surprisingly little is known about the changes occurring in the composition of phospholipids in the human brain over the adult lifespan. The current knowledge regarding age-related changes in phospholipids of the human brain and animal models (predominately rat) is reviewed below.

2.2.1 Human studies

2.2.1.1 Total phospholipids

The level of total phospholipid within the different regions of the human brain either decreases or remains unchanged with advanced age (Table 2-1). Decreases with age in total phospholipids have been noted in whole cerebral cortex [88] frontal cortex (including grey matter) [89–91], temporal cortex (including grey matter) [89,91], and hippocampus [90]. No changes in total phospholipids with age have been reported in the cerebellum [88,90] or temporal white matter [91].

2.2.1.2 Phospholipid classes

Only three studies have examined the changes occurring in individual phospholipids classes during normal ageing (Table 2-2). The combined weight of evidence suggests that there are no changes with age to total PC, PE or PS within the frontal cortex or cerebellum [90,92]. However, there is some disagreement within the literature as to whether total PC and PE change with age within the hippocampus, with an equal

Table 2-1: Summary of the available literature on changes in total brain phospholipids within different regions of the human brain during normal ageing.

Total phospholipids	**Change with age**	
Whole cerebral cortex		Decreased[88]
Frontal Cortex		Decreased[89]
- **Grey**		Decreased[90,91,93]
- **White**	No change[91]	Decreased[90,93]
Temporal Cortex		Decreased[89]
- **Grey**		Decreased[91]
- **White**	No change[91]	
Hippocampus		Decreased[90,93]
Cerebellum	No change[88,90]	

Phospholipids determined by phosphorous assay in all studies.

Table 2-2: Summary of the available literature on changes in phospholipid classes within different regions of the human brain during normal ageing.

Phospholipid class	Change with age	Frontal Cortex	Cerebellum	Hippocampus
PC	No change	[90,92]	[90]	[90]
	Decrease	[93]		[93]
PE	No change	[90,92,93]	[90]	[90]
	Decrease			[93]
PS	No change	[90,92]	[90]	[90]

Phospholipid classes separated by thin-layer chromatography and determined by phosphorous assay[90,93] or densitometry [92].

number of studies finding either no change [90] or decreases with age [93] in both PC and PE.

2.2.1.3 Total phospholipid-fatty acids

Five studies have previously examined the human brain for any age-related changes in the total phospholipid fatty acids (Table 2-3). The frontal cortex is the most highly studied brain region for changes to phospholipid-fatty acids with age, with all four studies finding few changes to the fatty acids of total phospholipids with age. Increases have been reported with age in myristic acid (14:0) within the frontal cortex [94,95], as well as in the level of arachidic acid (20:0) [94]. Many of the results within this region were conflicting though, with an equal number of studies reporting either no change [94,96] or a decrease [92,95] in 22:5n-6 with age, as well as age-related increases [94] or no change [96] in 22:5n-3. Carver et al. [94] observed several decreases with age within the frontal cortex in long-chain n-6 PUFA, including dihomo-γ-linolenic acid (20:3n-6) and adrenic acid, but neither finding was replicated by other studies of this brain region [95,96]. The other regions examined,were the temporal and parietal cortices, as well as the hippocampus, showed no changes to any phospholipid-fatty acids with age [93,96], but these regions have each only been examined in a single study and more work is needed to confirm these findings.

2.2.1.4 Phospholipid fatty acids by class

Only a single study has looked for any age-related changes in fatty acids within separate phospholipid classes in the human brain. No changes were found in either PC or PE fatty acids during normal ageing in either the frontal cortex, pons or hippocampus [93].

Table 2-3: Summary of the available literature showing changes in total phospholipid fatty acids within different regions of the human brain during normal ageing

Fatty acid	Change with age	Frontal Cortex	Temporal Cortex	Parietal Cortex	Hippocampus
14:0	Increased	[94,95]			
	No change	[96]	[96]	[96]	
15:0	No change	[92]			
16:0	Increased	[94]			
	No change	[92,93,96]	[96]	[96]	[93]
	Decreased	[95]			
16:1	Increased	[95]			
	No change	[92,96]	[96]	[96]	
18:0	No change	[92–94,96]	[96]	[96]	[93]
	Decreased	[95]			
18:1	No change	[93]			[93]
18:1(n-7)	Increased	[95]			
	No change	[92,96]	[96]	[96]	
18:1(n-9)	Increased	[95]			
	No change	[92,94,96]	[96]	[96]	
18:2(n-6)	Increased	[94]			
	No change	[92,95,96]	[96]	[96]	
20:0	Increased	[94]			
20:1	Increased	[95]			
	No change	[92–94,96]	[96]	[96]	[93]
20:2(n-6)	No change	[94]			
20:3(n-6)	No change	[95,96]	[96]	[96]	
	Decreased	[94]			
20:4(n-6)	No change	[92–94,96]	[96]	[96]	[93]
	Decreased	[95]			
22:0	No change	[94]			
22:2	No change	[92]			
22:4(n-6)	No change	[93,95,96]	[96]	[96]	[93]
	Decreased	[94]			
22:5					
22:5(n-3)	Increased	[94]			
	No change	[96]	[96]	[96]	
22:5(n-6)	No change	[94,96]	[96]	[96]	
	Decreased	[92,95]			
22:6(n-3)	No change	[92–94,96]	[96]	[96]	[93]
	Decreased	[95]			
24:0	No change	[94]			
24:1	No changes	[94]			

Total phospholipid fatty acids were determined by the following methods: phospholipids transmethylated [92–94] or saponified [95,96], and fatty acid methyl esters quantified by gas chromatography.

2.2.2 Animal models

Changes in the phospholipid composition of the brain during normal ageing have also been studied using animal models, most notably in the rat. One of the most common findings across studies of the rat brain during normal ageing is a reduction in the amount of PUFA present in phospholipids, particularly in DHA [97–103]. These age-related losses of DHA have been reported in PC [100], PE [97,99], and PS [100], as well as in total phospholipids fatty acids [98,99,101,103]. DHA is also reported to decrease with age in the mouse brain [104]. Other losses of PUFA with age in the rat brain included AA in PC [100], PS [102] and in total phospholipids [101,103]; LA in PS and PC [102] and in total phospholipids [101,103]; and in adrenic acid in PS [100]. Increases with age have been seen consistently in the rat brain monounsaturated fatty acids, principally in PE [98,100] and total phospholipids [103]. Changes in total amount of phospholipid within the classes have also been reported with age in rat and mouse brain, with decreases seen in PC [105,106] , PE [98,105,106], PS [106] and total phospholipids [99] with age. Conversely, Modi, Katyare and Patel [105] reported increases in PS phospholipids with age in the rat brain, while Fabelo et al. [107] found increases in both PE and total phospholipids with age.

2.3 Changes to phospholipids in the brain during dementia

Compared to normal ageing, the changes occurring to phospholipids in the human brain as a result of dementia have been better studied. AD, the most common form of dementia, has been particularly well studied for such changes. A summary of what is currently known about the changes occurring within the human brain during different dementias is detailed below.

2.3.1 Alzheimer's disease

AD is not only the most common type of dementia, but also the best-studied dementia in terms of phospholipid changes to the human brain. As stated in Chapter 1 (section 1.1), there is strong evidence for an involvement of membrane lipids in the pathogenesis of AD. The strongest known genetic risk factor for AD is homozygosity of the APOE-ε4 allele, with apolipoprotein E being involved in lipid trafficking and metabolism within the brain [11,12]. Several genome-wide association studies have also unearthed other genetic markers of AD, with many loci being either indirectly or directly involved in lipid metabolism [13–15]. Over twenty studies to date have looked for any

relationship between brain phospholipids and Alzheimer's disease, and typically included subjects aged between 60 and 100 years of age. However, two studies also included subjects less than 60 years old in both control and Alzheimer's disease groups [96,108].

Decreased amounts of total phospholipid have been reported in the hippocampus in AD [86,109,110], with possible increases seen in the temporal cortex [109,111]. No changes to total phospholipid amount have been seen in AD in the frontal cortex [87,108], occipital cortex or cerebellum [111]. Within the individual phospholipid classes, the combined available evidence suggests no changes have been reported in AD in the total amount of PC in the frontal [87,92,108,111–116], entorhinal [116], occipital [111–113], temporal [111–115,117] or parietal cortices [111–114], or in the cerebellum [111,112,115,116]. Similarly, no changes have been observed in total PE in AD within the frontal [87,92,108,111–114], parietal [111–114], entorhinal [116] or occipital cortices [111–113], or in the cerebellum [85,111,112,116]. However, decreases in total PE in AD have been observed in the hippocampus [86,111,113], while an equal number of studies have reported either no change [111,114,117] or decreases [85,112,113] in total PE within the temporal cortex. Interestingly, there were two studies that reported decreases in total PE in AD within the frontal cortex that used mass spectrometry techniques [85,116]. Within the PE class, there is conflicting information available on what occurs to the levels of plasmalogens in AD. Decreases in PE plasmalogen have been observed in the hippocampus in AD [86]. No changes have been observed in PE-plasmalogen levels in the occipital [112] or entorhinal cortices [116], as well as in the cerebellum [85,112,116] in AD. In the frontal cortex, one study reported increases in PE-plasmalogens in AD [112] while others reported no change [87,116] or decreases in PE-plasmalogens in AD [85,86]. Similarly, either increases [112] or decreases [85] in PE-plasmalogens have been reported in AD in the temporal cortex, as well as either no change [112] or decreases [85] in the parietal cortex. Levels of total PS have also been found to not change in AD within the frontal [86,87,92,108,111–114,116,117], temporal [111,112,114,117], parietal [111,113,114,117], occipital [111,113], or entorhinal cortices [116], or in the cerebellum [111,112] and hippocampus [86,111].

In terms of fatty acids present in phospholipids, several literature reviews have stated that there is a distinct loss of PUFA in AD [38,118–120]. However, examination of the

available literature suggests that this conclusion is far from clear. In the frontal cortex, the brain region best studied for changes to phospholipids in AD, a number of n-6 and n-3 phospholipid-fatty acids have shown no changes in total amount in AD, including: LA [87,92,96], dihomo-γ-linolenic acid [87,96], AA [87,92,96,121], EPA [87], adrenic acid [87,96], docosapentaenoic acid (both 22:5n-3 [87,96] and 22:5 n-6 [87,92,96]), and DHA [87,96,121]. Only a single study has looked at the total phospholipid fatty acids in either the temporal cortex [96] or parahippocampus [121], with both cited studies also examining the parietal cortex. Correspondingly, these two studies also reported a lack of change to many PUFA in AD, with only decreases in adrenic acid observed in the temporal cortex [96]. When the fatty acids of the three phospholipid classes PC, PE and PS are analysed separately, changes to PUFA in AD have been noted. For most PC-fatty acids in the frontal cortex, it appears that there are no changes in their amount in AD in the frontal cortex [86,93,108]; however, an equal number of studies found either no changes [86,108] or decreases [93,122] in the level of PC-AA. Similar results were seen for PC-DHA in the frontal cortex, with both no changes [86,108] or decreases [87,93] reported in AD. No changes in AD have been observed in PC fatty acids within the parahippocampus/hippocampus [93,122,123]. Only a single study has looked at PC-fatty acid changes in AD in the pons [93], as well as the temporal and parietal cortices and cerebellum [123], finding decreases in cerebellar DHA only. Similarly, few changes have been reported for PE-fatty acids within the brain in AD. Of note are possible increases in PE-myristic acid [93] and decreases in PE-EPA [87] in the frontal cortex, decreases in PE-AA [93,122,123], PE-adrenic acid [93,122] and PE-DHA [93,123] in the parahippocampus/hippocampus, decreases in PE-stearic acid (18:0), PE-oleic acid and PE-AA in the parietal cortex [123]. In the pons increases in PE-myristic acid, PE-palmitic acid (16:0) and PE-stearic acid have been reported in AD while decreases have been seen in PE-AA, PE-adrenic acid and PE-DHA [93]. PS is the least well-studied phospholipid class for changes to its fatty acids in AD, but similar to the other two phospholipids few changes have been reported in AD when all the current literature is compared. Decreases in PS-EPA [87], PS-docosapentaenoic acid (n-3 [87] and n-6 [87,108]) have been reported in the frontal cortex, with no change to PS-DHA in AD [87,108]. Within the parahippocampus/hippocampus, decreases in AD have been observed in PS-adrenic acid only [122], as well as in PS-DHA in both the temporal and parietal cortices [114].

There are several problems that are driving the ambiguity surrounding many of the changes observed in phospholipids in AD. The largest problem is that there are several different methods that have been used to analyse phospholipids extracted from tissue. Historically, phospholipid classes have been separated and quantified by thin-layer chromatography, either by densitometry or by scraping off the various separated classes and quantifying by the phosphorous assay. Phospholipid fatty acids have been typically analysed by gas chromatography after transmethylation or saponification of the phospholipids, sometimes in conjunction with thin-layer chromatography for class separation. Newer techniques have been developed for the accurate identification and quantification of phospholipids from tissue extracts; however, such instruments are costly to purchase and maintain. This idea will be further explored in Chapter 3. Only a few studies so far have applied mass spectrometry to the study of phospholipids changes in the human brain during AD [85,115]. It is expected that as more studies are conducted using such techniques that the changes occurring to phospholipids in the human brain as a result of AD will become clearer.

2.3.2 Other dementias

2.3.2.1 Parkinson's disease

Only a single study has examined changes in phospholipid composition in Parkinson's disease (age range of controls: 79-102 years, Parkinson's: 66-91 years) [124]. This study used liquid chromatography mass spectrometry (LC-MS) to identify a number of changes to phospholipids of the visual cortex, amygdala and anterior cingulate cortex. A number of phospholipid *bruto* species (phospholipid class and total fatty acids carbon-chain length and unsaturation) decreased in the brains of those with Parkinson's disease, particularly in the PE-class within the visual cortex. No losses of any particular type of unsaturated fatty acid were reported; however, it is unclear if the authors performed such an analysis.

2.3.2.2 Vascular/multi-infarct dementia

Vascular dementia (also known as multi-infarct dementia) is caused by problems in blood supply to the brain, typically by a minor stroke. Occasionally vascular dementia can overlap with the presence of lesions typical of AD, leading to the classification of mixed dementia.

Only a single study has examined the changes occurring to phospholipids in vascular and mixed dementia, which used LC-MS to identify and quantify phospholipids of the temporal lobe (average ages of controls 79.9±2 years, vascular dementia 85.3±3 years, and mixed dementia 83.4±3 years) [125]. Total PC and PS were observed to increase in the white matter in vascular dementia, with no change in grey matter for PC, PE or PS. Conversely, levels of both total PC and PE decreased in the grey matter of those afflicted with mixed dementia. *Bruto* phospholipids were also analysed in the grey and white matter of the temporal cortex in both vascular and mixed dementia. Increased amounts of PC and PS phospholipids were seen in the white matter in vascular dementia while decreases in PC phospholipid were mostly observed in the grey matter of mixed dementia patients. Given the different aetiologies of vascular dementia and AD, it is not surprising that such opposing differences were found between vascular and mixed dementia. Further research is needed to understand why these differences occur, and if the same pattern is repeated in other parts of the brain affected in vascular and mixed dementia.

2.3.2.3 Huntington's disease

The only study of changes in phospholipids in Huntington's disease to date found no differences in the ratio of PE-plasmalogen to diacyl PE within the caudate nucleus in Huntington's disease (average age of controls 77±4 years, Huntington's 55±5 years) [126].

2.3.2.4 Creutzfeld-Jakob disease

Two studies have been performed to ascertain any changes in phospholipid content in Creutzfeld-Jakob disease (CJD) [127,128]. In a case-control study (n=1 per group) Federicco, Annunziata and Malentacchi [128] observed decreases in total phospholipids in CJD, with increases found in PE in the white matter and PS in grey matter. Corresponding reductions in PS and PC were observed in the white matter in CJD, alongside decreases in PC and PE in the grey matter (age of control and CJD subject both 67 years). Conversely, Tamai et al. [127] found no changes to any phospholipid class in CJD, nor within the fatty acids of PC and PS phospholipids (age of subjects not stated). However, they did observe marked increases in PE-oleic acid and decreases in PE-adrenic acid and PE-DHA in the brain tissue of humans with CJD. Although CJD can be transmitted to humans and from animals to humans via ingestion of or exposure

to infected tissues (variant CJD), CJD can also be sporadic or familial in nature. Further research is needed to ascertain whether there is a membrane lipid involvement in the pathogenesis of CJD, particularly in familial cases.

2.3.2.5 Frontotemporal dementia

No studies currently exist that have examined changes in phospholipid composition in frontotemporal dementia.

2.4 The involvement of phospholipids in the current theories of ageing

2.4.1 Why do we age?

There are many proposed theories that attempt to explain the mechanism behind the ageing process. Many such theories fall into two broad categories: programmed or error theories [129]. According to the programmed theory of ageing, ageing depends upon the regulation of the body's internal "biological clock" through the different stages of growth, development, maturity and old age via the switching on and off of certain genes [129]. Alternatively, the error theory of ageing proposes that ageing is a result of continual environmental stress at the molecular, cellular and whole-organism level, resulting in cumulative damage and dysfunction over the lifespan and, eventually, death. There are two main error theories of ageing that could apply to changes in phospholipids during normal ageing within the human brain, and these theories will be explored in the final part of this literature review: the mitochondrial free radical oxidative theory of ageing and the "inflammageing" theory.

2.4.2 Mitochondrial free radical oxidative theory of ageing

Over the lifetime of an organism, many mutations occur within mitochondrial DNA (mtDNA) leading to the progressive loss of mitochondrial function with age. This age-dependent accumulation of mtDNA mutations can be a result of i) the inherent error rate of the mtDNA polymerase γ [130]; or ii) due to the accumulation of reactive oxygen species (ROS) generated as byproducts of oxidative phosphorylation over time which overwhelm the few existing mtDNA repair mechanisms [131]. mtDNA is thought to be especially vulnerable to damage by ROS due to its proximity to the electron transport chain, a lack of repair mechanisms and an absence of protective histones [132,133]. ROS produced by the mitochondria have the capability to damage macromolecules such as DNA, proteins and lipids. Over time, these macromolecules become progressively more damaged, leading to the production of more ROS, which leads to a vicious cycle of ROS production and macromolecule damage; the net result of which is thought to be ageing [134]. The principle ROS generated by oxidative phosphorylation is superoxide, but other ROS of significance include hydroxyl radicals, hydrogen peroxide and singlet oxygen [135].

Harman first proposed the free radical theory of ageing in the 1950s [136], and this theory was further strengthened by the discovery of superoxide dismutase in the late

1960s [137]. Superoxide dismutase is an essential antioxidant defence enzyme that catalyses the ROS superoxide into hydrogen peroxide and oxygen. Hydrogen peroxide can then be transformed into water by catalase or glutathione peroxidase. Nonenzymatic ROS scavengers (also known as antioxidants) also exist within cells, including ascorbate, flavonoids, carotenoids and glutathione. In regards to phospholipids, it is the fatty acids that are susceptible to oxidative damage by ROS such as superoxide. The susceptibility of fatty acids to oxidative damage by ROS is known to be dependent upon the degree of unsaturation (i.e. number of double bonds present). Bisallylic methylene groups are particularly susceptible to oxidation as the presence of the double bonds weakens the bond energy of this group, allowing the extraction of a proton by ROS to form a lipid peroxyl radical (Figure 2-7). Due to this, PUFAs with a large number of methylene-interrupted double bonds such as DHA are particularly susceptible to oxidative damage [138]. A number of oxidative molecules can be derived from lipid peroxyl radicals, including lipid peroxides, aldehydes such as malondialdehyde and

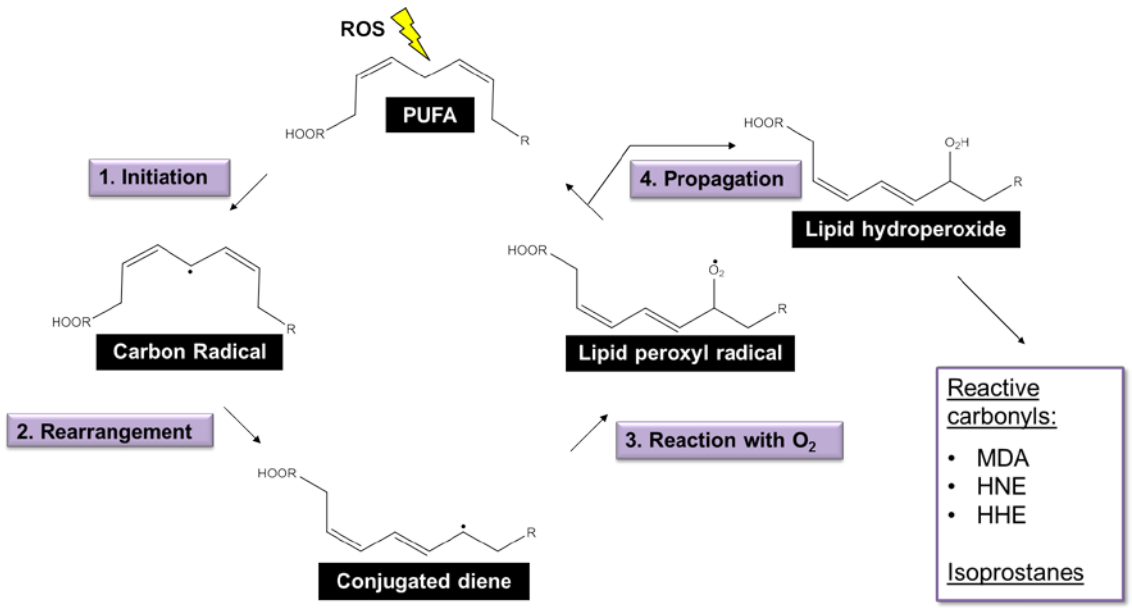


Figure 2-7: Lipid peroxidation initiation and propagation : 1. a hydrogen is removed from a bisallylic group on a PUFA, forming a carbon radical. 2. molecular rearrangement occurs to form a more stable conjugated diene. 3. the carbon radical reacts with molecular oxygen (O_2) to form a lipid peroxyl radical. 4. A second hydrogen is then abstracted from a neighbouring PUFA, forming a lipid hydroperoxide and propagating the peroxidation cycle. Both lipid hydroperoxides and peroxyl radicals then undergo further reactions to form an array of biologically active carbonyls. *HHE* 4-hydroxy-2-hexanal,, *HNE* 4-hydroxy-2-nonenal, *MDA* malondialdehyde, *PUFA* polyunsaturated fatty acid, *ROS* reactive oxygen species.

4-hydroxy-2-nonenal (4-HNE), and isoprostanes such as F2-isoprostanes and F4-neuroprostanes. Elevated levels of malondialdehyde have been observed in the brain tissue of aged rats [139–143] and humans [144]. Increases in 4-HNE have also been reported in the brains of aged mice [145], senescence-accelerated mice [146], rats [147], and dogs [148]. Changes with isoprostanes during normal ageing have been better studied in humans using cerebrospinal fluid, with increases reported in F2-isoprostanes with age [149,150]. However, in the brain tissue of rats there has been no reported changes in F2-isoprostanes or F4-neuroprostanes with age [151]. Elevated amounts of lipid oxidation products have also been reported in age-related neurodegenerative diseases such as AD [152].

If the mitochondrial free radical oxidative theory of ageing is one of the main driving forces behind ageing, then from a phospholipid perspective it is likely that decreases in phospholipids containing PUFA within the human brain would occur with age. Theoretically, long chain PUFA, particularly DHA, would be most susceptible to damage by ROS due to the high number of methylene-interrupted double bonds present in this fatty acid. The n-6 fatty acids AA and adrenic acid are also expected to be significantly reduced with age according to the free radical oxidative theory of ageing. Due to the high proportion of PUFA present in the PE and PS phospholipids of the human brain we would also expect to see a corresponding reduction in these phospholipid classes with age or a corresponding increase in saturated or monounsaturated fatty acids. On a subcellular level, as superoxide is unable to cross cellular membranes [153,154] and is primarily generated within the mitochondria, we would expect to see a greater loss of phospholipids containing PUFA in the mitochondrial membranes with age over other cellular membranes.

2.4.3 “Inflammageing” theory of ageing

The term “inflammageing” was first coined by Franceshci and colleagues in 2000 [155] and represents the idea that ageing is driven by the presence of low-grade, chronic inflammation throughout the lifespan. Franceshci and colleagues have pointed to increases in inflammatory markers such as interleukins and tumour necrosis factors with age [155–157]; however, it is theoretically possible that phospholipids may be involved in this process too. AA is the precursor for a number of pro-inflammatory molecules

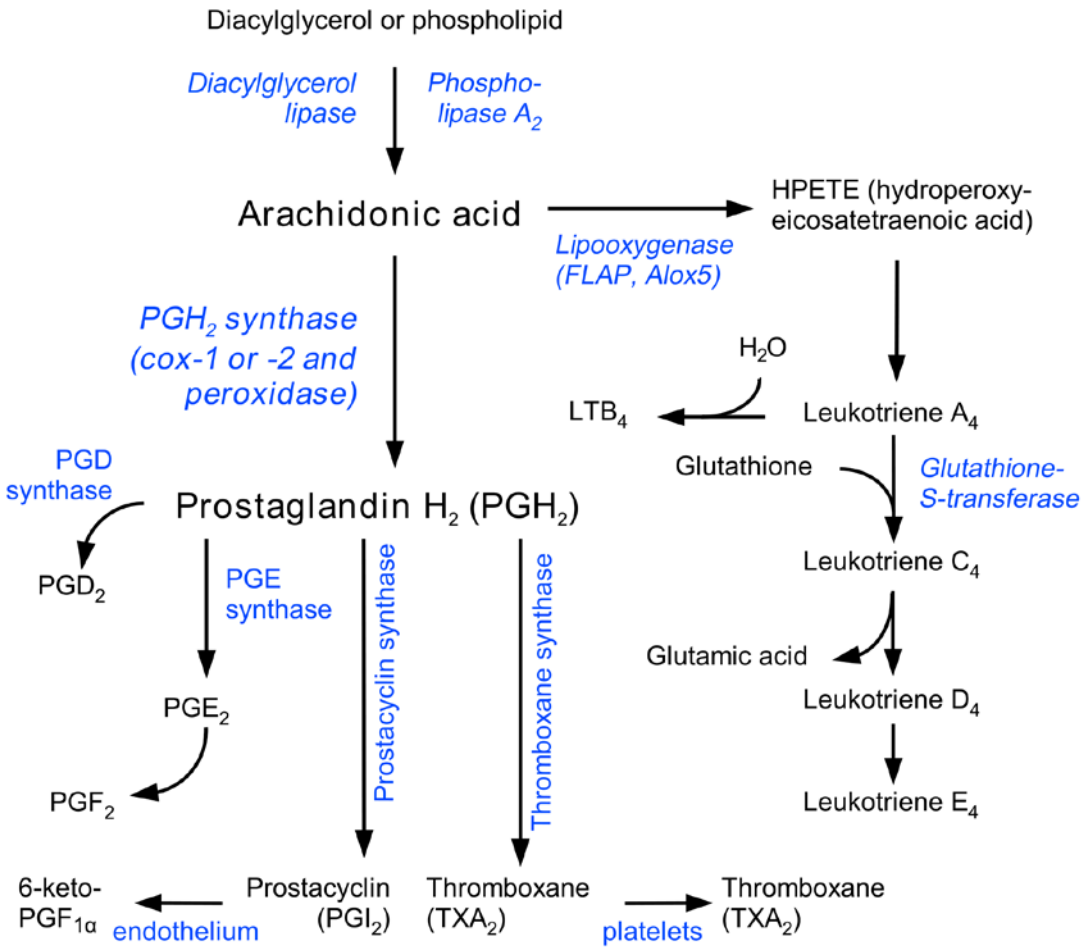


Figure 2-8: Synthesis of inflammatory eicosanoids from arachidonic acid after cleavage from phospholipids by phospholipase A_2. Image adapted from [158]

including prostaglandins, leukotrienes and thromboxanes (Figure 2-8). AA is liberated from phospholipids within cellular membranes by phospholipase A_2 [159], and this free AA can then be converted to leukotrienes via the lipoxygenase pathway, or prostaglandins and thromboxanes via the cyclooxygenase (COX) pathway. The cytosolic phospholipid A2 isoforms primarily present within the brain [160] have a high specificity for AA in the *sn*-2 position [161]. Within mammalian tissues, there are two COX present: COX-1 and COX-2. COX-1 is expressed ubiquitously in many tissues and cell types, and has a role in maintaining general cell physiology and homeostasis while COX-2 shows greater restriction and is known for its role as a respondent to stress and insult [162–164]. COX-2 is also known to be expressed throughout the hippocampus and cortex [165]. Both COX enzymes convert AA into prostaglandin H_2, which can then be selectively converted into a number of prostanoids by specific

synthases. COX-2 expression within the brain is normally very low but is markedly increased in age-related neurodegenerative diseases such as AD [166,167]. There is also some evidence implicating changes during ageing to prostaglandin levels and to COX-2 expression and activity levels in animal models [168]. Both COX-1 and COX-2 can also utilise other PUFA such as EPA to produce a range of anti-inflammatory eicosanoids including resolvin E1 in non-neural cells [169]. Such metabolites of EPA are yet to be detected within brain tissue, possibly due to the low level of PL-EPA present within neural membranes. COX-2 can also utilise DHA as a substrate to produce various docosanoids [170–172], but a rate approximately 7-fold lower than AA [171].

If the "inflammageing" theory of ageing is one of the main driving forces behind normal ageing in the human brain, then we would expect to see substantial decreases in n-6 fatty acids such as AA and adrenic acid over the adult lifespan. Inflammatory eicosanoids are known to be produced from adrenic acid in kidney [175], but it is also likely that adrenic will undergo β-oxidation in the peroxisomes to replenish the pool of available AA (section 2.1.4, Figure 2-4). Assuming no change to PUFA synthesis rates in humans with age, if the pool of available AA decreases within the membrane then we would expect to see a corresponding age-related decrease in adrenic acid in order to maintain membrane homeostasis. Levels of long chain n-3 fatty acids such as DHA within the cellular membranes would theoretically be minimally impacted in advanced age under this theory due to the preference for AA exhibited by inflammatory enzymes [159,161,170–172]. Unlike the mitochondrial free radical theory of ageing, where the loss of n-6 PUFA with age would theoretically be confined to the mitochondria, in the inflammageing theory of ageing it is hypothesised that all of the membranes making up the various organelles of the cell would be affected.

2.5 Summary and conclusion

In this review the importance of phospholipids in the human brain was established, particularly those that contain long-chain PUFA such as DHA and AA. The known changes to phospholipids within the human brain during normal ageing were detailed, with very few studies having been performed in this area. To make up for this deficit in the literature, normal ageing in the brains of animal models were also discussed. Between animals and human, there appears to be some loss of total PC and PE phospholipids with age while animal studies alone showed significant decreases in PUFA within the brain over the lifespan. The changes in phospholipid composition in the brain as a result of AD have not only been better studied than normal ageing, but also more thoroughly than other types of dementia. However, the abundance of literature available on the topic (particularly within the frontal cortex), compounded with the range of different methods used have produced conflicting results. The sum of the available literature suggests that there are perturbations in the total amounts of each phospholipid class in several regions of the brain in AD, with possible changes in the fatty acid composition. Finally, the current theories of ageing in which phospholipids could be involved were explored. In particular, the mitochondrial free radical theory of ageing was discussed, as well as the inflammation theory of ageing.

In order to understand how any changes to the phospholipids of the human brain fit into either theory of ageing, there is a requirement to isolate particular organelles by subcellular fractionation in order to examine their membranes separately. The next chapter will detail the method development of such a technique, as well as refinements to the shotgun lipidomic method used to profile membrane composition.

SECTION 2

METHOD

Chapter 3 Method development

3.1 Introduction

There are many methods described for the fractionation of subcellular components from cell culture and fresh tissues. These methods often rely on differences in sedimentation rates and buoyant density between different subcellular organelles and components. Subcellular fractionation of frozen tissue, however, requires more consideration. The use of frozen tissue samples are necessary for the study of the human brain, as fresh tissue collection is unfeasible, and human tissue banks store their archived samples snap-frozen at -80°C. Frozen tissue that has undergone multiple freeze/thaw cycles becomes unsuitable for subcellular fractionation, as freezing and thawing can lead to large ice crystal formation which causes lysis of organelles [176,177]. Therefore, the first step was to develop a suitable method for the fractionation of frozen human brain tissue.

The method developed for the subcellular fractionation of frozen tissue also had to be suitable for the extraction and analysis of both membrane lipids and proteins. While the focus of this thesis was age-related changes in membrane lipids in the human brain, this work formed part of a larger study also examining age-related changes to membrane proteins. The most commonly used method for membrane lipid extraction is a biphasic organic solvent system that requires very little starting sample, but this method is incompatible with samples containing a large amount of salt or water. Conversely, the conventional methods used for protein extraction and analysis require large amounts of starting material, plus buffered salt and detergent solutions that would interfere with lipid extraction and analysis. Thus, several potential methods were investigated in order to achieve a compatible method for both membrane lipid and protein analysis from the same tissue specimens.

In this chapter, method development will be presented and discussed, including the final method used for subcellular fractionation and analysis of membrane lipids from frozen post-mortem human brain tissue.

3.2 Results/Discussion

3.2.1 Subcellular fractionation of frozen tissue

The first step in method development was to find a subcellular fractionation method for use with frozen tissue. Method development was conducted using lamb brain sourced from a local butcher. This tissue was purchased for use directly after delivery from the abattoir, which ensured that the tissue had not been previously frozen. Approximately 10 mm sections of frontal lobe (anterior to ansate sulcus) from both hemispheres were dissected from the lamb brain and snap frozen in liquid nitrogen. The samples were then stored at -80°C until required.

3.2.1.1 Subcellular fractionation: method one

The first fractionation method assessed followed the procedure outlined by Bourova et al. [178]. Frozen lamb brain tissue (250 mg, n = 6) was homogenised using a glass dounce homogeniser in an ice-cold buffer consisting of: 250 mM sucrose, 20 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), and complete protease inhibitor cocktail. After centrifugation to remove large cellular debris (1000 x *g*, 10 min), the homogenate was filtered (80 µm pore) and applied to the top of a 30% Percoll solution. Ultracentrifugation (65 000 x *g*, 30 min) was then used to separate mitochondria from the other cellular membranes. Two visible layers were expected after the 30 min centrifugation; however, this did not occur. Accordingly, the filtered homogenate was subjected to a second centrifugation (65 000 x *g*, 30 min). After the second centrifugation two layers were present: a thick, viscous upper layer identified by Bourova et al. [178] as the "plasma membrane layer", and a second mitochondrial layer under a compacted layer of Percoll sediment. The upper plasma membrane layer was collected; however the mitochondrial layer was small and unable to be separated from the compacted Percoll. The collected plasma membrane layer was resuspended in the same buffer, and any remaining Percoll was removed by ultracentrifugation (175 000 x *g* 90 min). Following this, the plasma membrane fraction (n = 3) was suspended in a second buffer containing: 150 mM NaCl, 20 mM Tris-HCl pH (7.4), 3 mM $MgCl_2$, and 1 mM EDTA, and applied to a density gradient consisting of 1.5 mL layers of 15%, 20%, 25%, 30%, 35% sucrose. This gradient was ultracentrifuged (187 000 x *g* 24 hours) to separate the plasma membrane into two

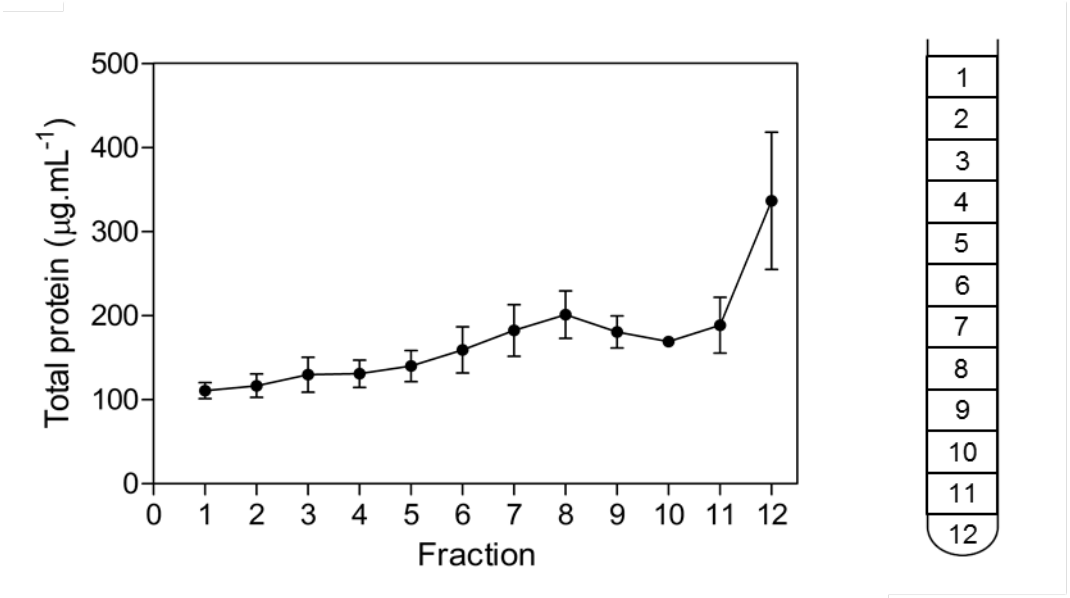


Figure 3-1: Sucrose density gradient profile of total protein as a plasma membrane marker. Total protein was measured from *n*=3 samples, and results reported as mean ± S.E.M

layers. These layers were identified by Bourova et al. [178] as being "low density (myelin)" and "bulk plasma membrane". As neither of these fractions were clearly visible, the gradient was collected from the top to the bottom in 1 mL aliquots and subjected to a protein assay for determination of membrane location (Figure 3-1).

According to the results of Bourova et al. [178], the bulk plasma membrane fraction should have been easily located in fractions 7-8 by the highest levels of total protein. However, our results showed that fraction 12 contained the largest amount of total protein (Figure 3-1). Examination of the literature revealed that the subcellular fractionation of frozen tissue is difficult to perform successfully as freeze-thaw cycles tend to lyse the cells and their subcellular compartments [176,177]. Since our experiment used frozen tissue this would explain the abundance of protein present in the lowest fraction: the lamb brain tissue may have been exposed to multiple freeze-thaw cycles, damaging membrane integrity and resulting in fragmentation which prevented the cellular membranes from fractionating completely. Bourova et al. [178] used fresh rat brain tissue in their study, which meant that they would not suffer the same problems. Therefore, an alternative method was needed for use in this study.

3.2.1.2 Subcellular fractionation: method two

With that in mind, a simpler method using differential centrifugation was trialled. The advantage of subcellular fractionation by differential centrifugation without a gradient such as sucrose or Percoll is that it requires fewer purification steps, which can increase the yield of the available substrate [179]. This method also had the advantage of having been successfully applied to frozen human brain tissue with a sufficient degree of specificity for the isolation of the intended subcellular compartment [177]. To explore the suitability of this method, approximately 100 mg of frozen lamb brain was homogenised using a bead homogeniser (FastPrep®-24 Instrument, MP Biomedicals, NSW, Australia) with 1.4 mm zirconium oxide beads at a speed of 6.0 m/s, in 1 mL of an ice-cold 20 mM Tris buffer (pH 7.4) containing: 250 mM sucrose, 2 mM of EDTA, 2 mM of dithiothreitol (DTT) and complete protease inhibitor. The homogenate was centrifuged (1000 x *g*, 10 min) to give a pellet containing nuclei and large cellular debris, which was discarded. The supernatant was collected and centrifuged (35 min, 10 000 x *g)* to produce a pellet enriched in mitochondria. The supernatant was centrifuged again (40 min, 100 000 x *g*) to produce a microsomal pellet (endomembranous system comprised of Golgi, endoplasmic reticulum and plasma membrane) and cytosolic supernatant. Both the mitochondrial and microsomal pellets were resuspended in milliQ H_2O (4°C) prior to being prepared for lipid and protein analysis.

3.2.1.3 Western blotting

To test whether this subcellular fractionation method isolated the correct fraction, a series of Western blots were performed looking for specific subcellular markers. Both the microsomal and mitochondrial fractions were obtained from lamb brain using the second fractionation method (section 3.2.1.2). Whole mitochondrial and microsomal pellets were solubilised in 500 µL and 100 µL respectively of a solution consisting of 7 M Urea, 10 mM Tris buffer (pH 8.0) and 5 µL of 1 mM DTT. Both 1x and 2x dilutions were made for both membrane fractions, and these were incubated with cracking buffer (100°C, 10 min). The dilutions of both fractions were separated by electrophoresis on a 12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis gel and transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was probed for 2 hours at 4°C with a primary antibody corresponding to a marker of a particular subcellular organelle. Sodium-potassium-ATPase were used as a plasma membrane marker (1:5000, Sapphire Biosciences, Australia), cytochrome c for mitochondria

(1:1000, Sigma-Aldrich, Australia) and β-actin as a control (1:4000, Sigma-Aldrich, Australia). An additional marker for endoplasmic reticulum was also trialed (ERP29, Sigma-Aldrich, Australia), but this antibody was found to be unsuitable for use with lamb brain. After incubation with the primary antibodies the membrane was washed in TBST (3 times, 5 mins each), and incubated with horseradish peroxidase-conjugated IgGs (anti-rabbit or anti-mouse depending on primary antibodies, 1:2000, Sapphire Biosciences, Australia) at room temperature for 1 hour. Following this the membrane was washed again in TBST (3 times, 5 mins each) and developed colourmetrically using 0.05% 3,3'-diaminobenzidine and 30% hydrogen peroxide. After 2-5 mins of incubation with 0.05% 3,3'-diaminobenzidine, the membrane was washed in milliQ H_2O and stored in PBS. The bands typically developed their maximum colour overnight while stored in PBS, after which they were recorded using a densitometer (GS-800™, BioRad, USA). The results of the Western blotting for the subcellular markers can be seen in Figure 3-2.

Immunoblotting of the mitochondrial and microsomal subcellular fractions showed that the sodium-potassium-ATPase was primarily located in the microsomal fraction. Lower amounts sodium-potassium-ATPase were also seen in the mitochondrial

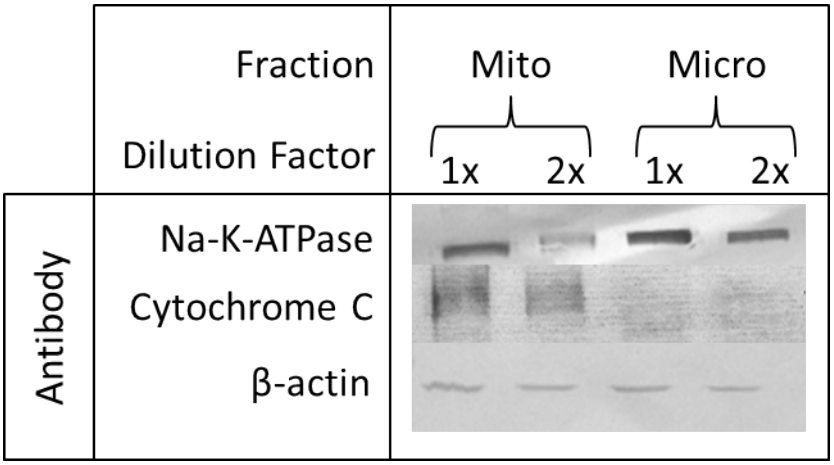


Figure 3-2: Western blot of mitochondrial (Mito) and microsomal (Micro) subcellular fractions obtained from frozen lamb brain. Fractions were run either undiluted (1x) or at 1:1 dilution (2x) in buffer containing 7M urea and 10mM Tris (pH 8.0). Primary antibodies used included sodium-potassium-ATPase (Na-K-ATPase) as a plasma membrane marker, and cytochrome c as the mitochondrial marker, with β-actin run as a control. The labelled bands were visualised colourmetrically by developing with 3,3'-diaminobenzidine and hydrogen peroxide after application of secondary antibodies.

fraction as well, but this was expected with this crude method of fractionation. Cytochrome c appeared in the mitochondrial fraction as a tetramer at around 50-60 kDa, but most importantly it was not present in the microsomes, indicating that there was no mitochondria present in the microsomal fraction. These results show that while crude, this fractionation method was suitable for use with frozen brain tissue in this study.

3.2.2 Membrane lipid extraction and analysis

Recent advances in mass spectrometry techniques such as electrospray ionisation have enabled increased sensitivity in the detection and accurate characterization, identification, and quantitation of membrane lipid species from biological samples. A shotgun lipidomic approach involves direct infusion of extracted lipid mixtures from biological samples into the mass spectrometer without any prior chromatographic separation. The most commonly reported method for extraction of membrane lipids from biological samples is the Folch method [180]. The Folch method is a biphasic solvent system consisting of a 2:1 v/v mixture of chloroform:methanol which separates into an organic phase and an aqueous phase upon the addition of water and salt, allowing the partitioning of polar and non-polar lipids from the tissue homogenate. This method also results in the precipitation of any proteins in the extract, which appear as a layer at the interface between the organic and aqueous phases. In the present study, 2 mL of the chloroform:methanol solvent mixture was added directly to the mitochondrial and microsomal pellets obtained from lamb brain tissue, along with butylated hydroxytoluene (BHT; 0.01% w/v) to prevent oxidation of the lipids during extraction.

3.2.2.1 Membrane lipid extraction: method one

An internal standard solution was employed during initial experiments for use in lipid quantification and consisted of the following lipids stored in chloroform:methanol (2:1, v/v): 250 µM PC 19:0/19:0, 188 µM PE 17:0/17:0, 188 µM PS 17:0/17:0, 25 µM phosphatidylglycerol 17:0/17:0, 25 µM phosphatidic acid 17:0/17:0, 25 µM cardiolipin 14:0/14:0/14:0/14:0, 250 µM dihydrosphingomyelin 12:0, 25 µM lyso-PC 17:0, 25 µM lyso-PE 14:0 and 13 µM ceramide 17:0. Internal standards were added to the whole fractionated mitochondrial and microsomal pellets at a volume of 20 µL and 10 µL respectively. After the addition of the internal standards, all samples were allowed to rotate overnight at 4°C. Lipid extraction then followed a modified Folch extraction method reported by Deeley et al. [181]. Firstly, 500µL of 0.15 M ammonium acetate

was added to each sample. Samples were vortexed vigorously and centrifuged (1000 x *g*, 10 min) in order to separate the aqueous (upper) and organic (lower) phases. The organic (lipid-containing) phase was then removed and a second 2 mL volume of chloroform:methanol (2:1 v/v with 0.01% BHT) was added. Following a second round of vortexing and centrifugation the lower phase was again removed and combined with the first organic phase. A second 500 µL of 0.15 M ammonium acetate was added to the combined organic phases, and the samples were vortexed and centrifuged a final time. The upper aqueous phase was removed, and the lower phase dried at 37°C under nitrogen. The dried lipids were then reconstituted in 1 – 1.5 mL of chloroform:methanol (1:2 v/v with 0.01% BHT) and stored at -20°C until mass spectrometry was performed.

3.2.2.2 Membrane lipid analysis by mass spectrometry

Nano-electrospray ionization (nano-ESI) mass spectrometry of lipid extracts was performed using a hybrid triple quadrupole linear ion trap mass spectrometer (QTRAP® 5500 AB Sciex, USA) equipped with an automated chip-based nano-ESI source (TriVersa Nanomate™, Advion Biosciences, USA). Samples were diluted to approximately 10 µM for total phospholipids, spiked with 5 mM of ammonium acetate, and loaded onto a 96-well plate. The plate was centrifuged (2200 x *g*, 10 min) prior to direct infusion. Spray parameters were set at a gas pressure of 0.4 psi and a voltage of 1.2 and 1.1 kV for positive and negative ion mode respectively for all acquisitions. Phospholipid data was acquired by targeted ion scans using multi-channel acquisition (Table 3-1). A faster scan rate (1000 Da/s) was used for negative ion fatty acid scans to ensure spectra could be obtained within one hour (average maximum stability of nanospray for 10 µL of sample).

Lipidview™ software (version 1.2, AB Sciex, USA) was used to quantify phospholipids from internal standards present in positive ion head group scans. Processing settings in LipidView™ were set with a mass tolerance of 0.5 Da, with a minimum intensity of 0.1% and a minimum signal/noise of 10. Isotopic correction was also used. Any phospholipids comprising less than 0.5% of each class across the cohort were removed from the final analysis.

Table 3-1: nanoESI-MS settings for targeted ion scans used to acquire phospholipid data in human brain mitochondria and microsomes

Lipid	Ion Mode	Scan	CE	Mass range (Da)
Head group scans				
PC	+ve	PI 184.1	47	640-1000
PE	+ve	NL 141.0	30	685-950
PS	+ve	NL 185.0	30	755-965
Lyso-PC	+ve	PI 184.1	47	450-600
Lyso-PE	+ve	NL 141.0	30	400-600
Fatty acyl chain scans				
14:0	-ve	PI 227.2	-55	580-900
16:1	-ve	PI 253.2	-55	600-900
16:0	-ve	PI 255.2	-55	600-900
17:0	-ve	PI 269.3	-55	560-900
18:2	-ve	PI 279.2	-40	600-900
18:1	-ve	PI 281.3	-55	600-900
18:0	-ve	PI 283.3	-55	600-900
19:0	-ve	PI 297.3	-55	600-900
20:5	-ve	PI 301.2	-40	500-1000
20:4	-ve	PI 303.2	-40	600-1000
20:3	-ve	PI 305.2	-40	600-1000
20:2	-ve	PI 307.2	-40	600-1000
20:1	-ve	PI 309.2	-55	600-1000
20:0	-ve	PI 311.2	-55	600-1000
22:6	-ve	PI 327.2	-40	700-1000
22:5	-ve	PI 329.2	-40	700-1000
22:4	-ve	PI 331.2	-40	700-1000
22:3	-ve	PI 333.3	-40	600-1000

Scan rate for positive ion mode was 200 Da/s, for negative ion mode 1000 Da/s. Mass shifting was prevented in negative ion mode by increasing the number of summed scans. *PI* Precursor ion, *NL* neutral loss, *CE* collision energy.

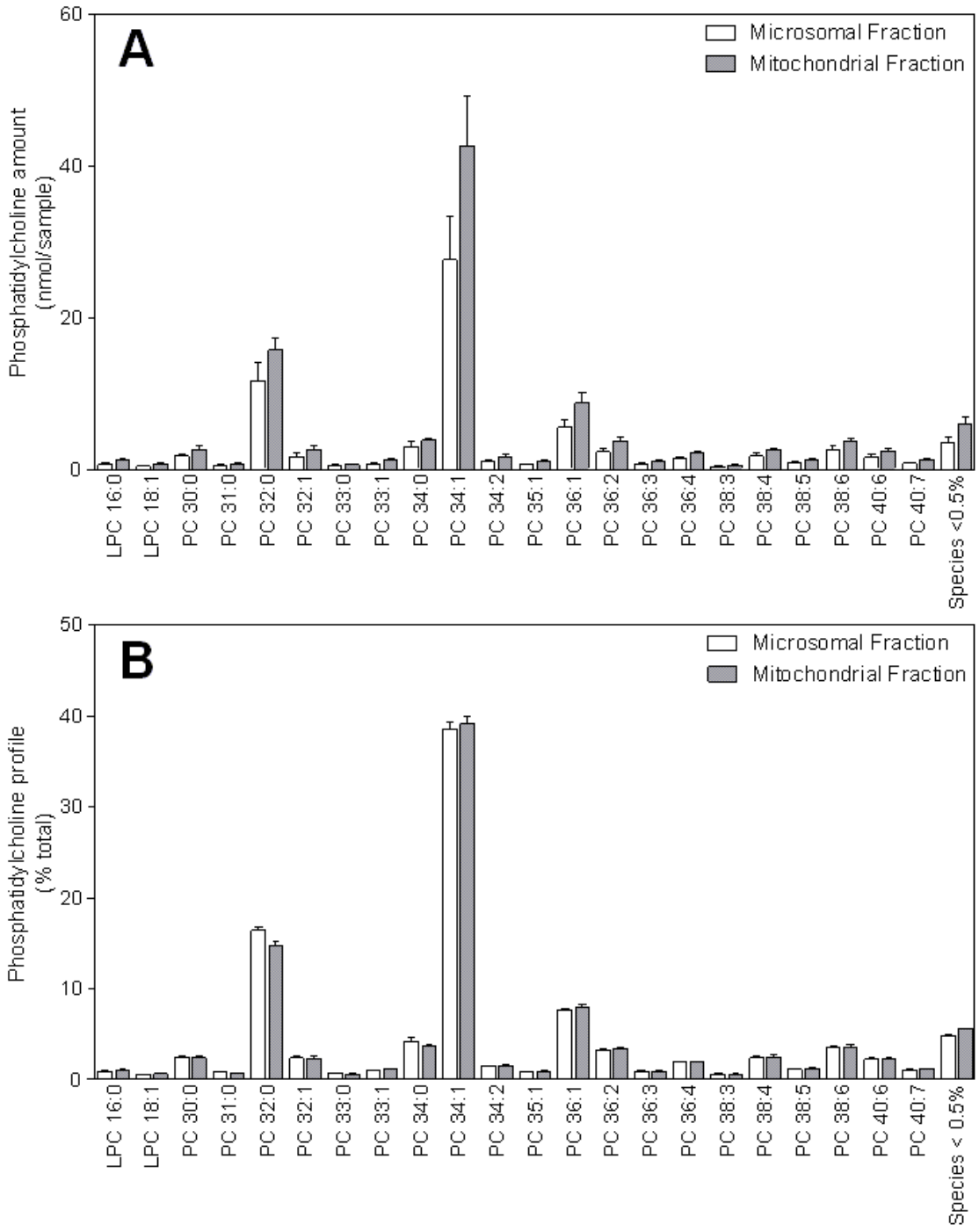


Figure 3-3: Phosphatidylcholines (PC) obtained from microsomal and mitochondrial fractions of lamb brain (n = 4, error bars show S.E.M.). PC was detected from both fractions by tandem mass spectrometry from a precursor ion scan of 184.1 m/z. PCs are shown as: A) amount of each phospholipid detected (nmol/sample), and B) as a percentage of total PC detected.

3.2.2.3 Analysis of results for membrane lipids

Due to the inability to obtain an accurate weight for the mitochondrial and microsomal fractions, the internal standards were initially added to the fractions in a specific amount (20 µL and 10 µL respectively). However, this became problematic during quantification as the differences seen in the amount of each molecular phospholipid between the two fractions was based on the quantity of total lipid present in the pellet of each membrane fraction without considering individual pellet yield of each fraction (Figure 3-3A). The mitochondrial fraction produced a much larger pellet during fractionation than the microsomes, and when both fractions were plotted against each other all phospholipids detected in the mitochondria were higher in abundance than those in the microsomes. Plotting the phospholipids as a proportion of total PC (Figure 3-3B) shows the profile of the two species to be almost equal, however. Therefore it was determined that addition of the internal standards should be on the basis of a common factor between the two fractions, such as total protein amount, to better show any differences in each phospholipid independent of pellet mass per membrane fraction.

3.2.2.4 Membrane lipid extraction: method two

To that end, the internal standards were adjusted to 20 µM each of: PC 19:0/19:0, PE 17:0/17:0, PS 17:0/17:0, phosphatidylglycerol 17:0/17:0, phosphatidic acid 17:0/17:0, cardiolipin 14:0/14:0/14:0/14:0 and dihydrosphingomyelin 12:0; along with 10 µM of lyso-PC 17:0, lyso-PE 14:0 and ceramide (17:0). Aliquots (75 µg of total protein) from the mitochondrial and microsomal fractions were added to chloroform:methanol (2 mL, 2:1 v/v with 0.01% BHT) with 50 µL of the adjusted internal standard mixture. Lipid extraction then followed the method previously outlined above in section 3.2.2.1.

Results from the analysis of PC from both the microsomal and mitochondrial fractions of lamb brain with the new internal standard formulation can be seen in Figure 3-4. In Figure 3-4A it can be seen that the quantified values of the microsomal fraction are no longer consistently lower than the mitochondrial fraction, and variation in the amount of individual phospholipids are seen between the two membrane fractions. The standard error of each quantified phospholipid is also significantly reduced. This result demonstrates that this second method of quantification using total protein as a denominator is superior to the first method trialled, and is more suitable for use in the extraction and analysis of lipids from fractionated human brain issue.

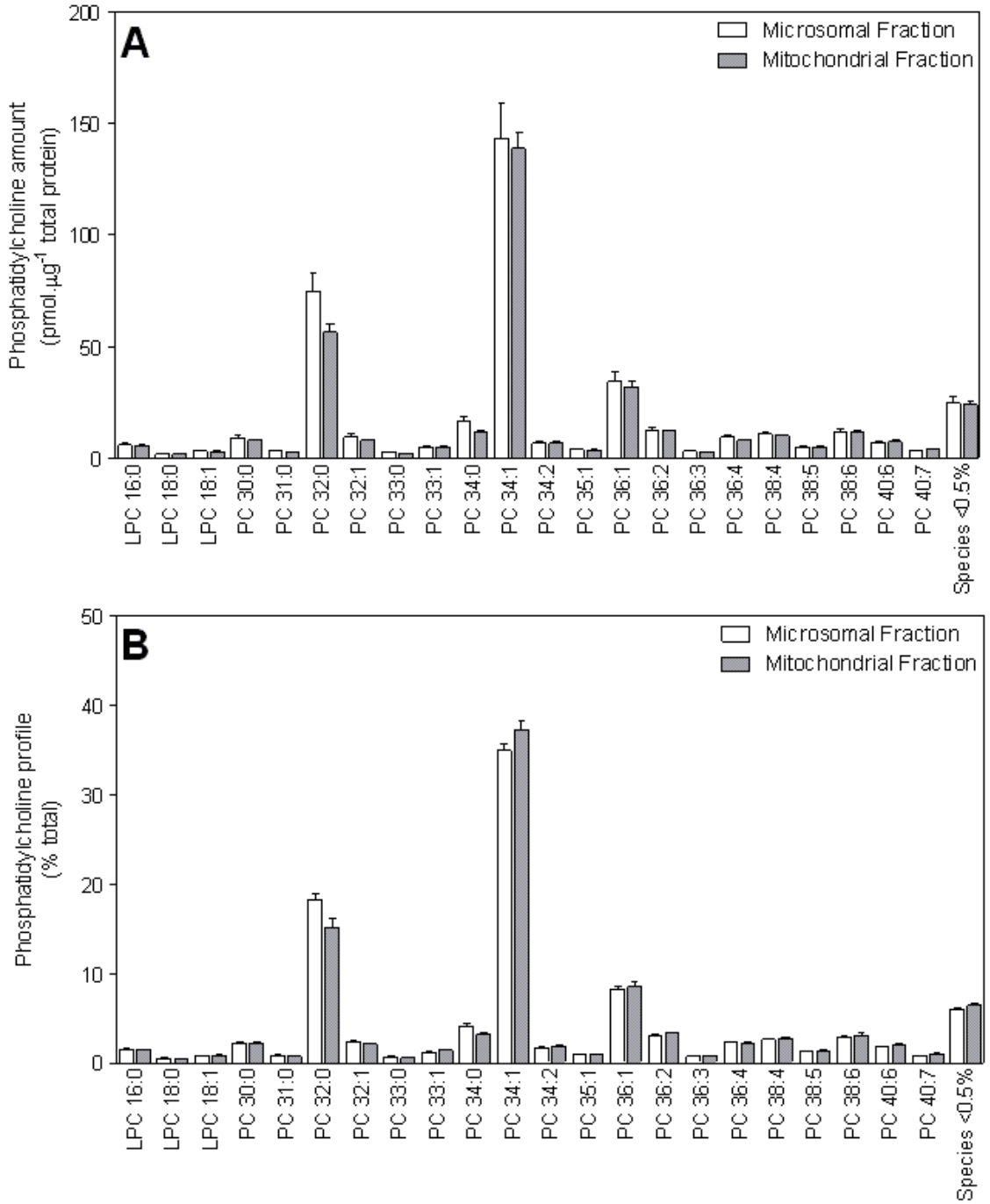


Figure 3-4: Phosphatidylcholines (PC) obtained from microsomal (n = 6) and mitochondrial fractions of lamb brain (n = 5), with error bars showing S.E.M. PC was detected from both fractions by tandem mass spectrometry from a precursor ion scan of 184.1 m/z. PCs are shown as: **A** amount of each phospholipid detected (pmol/µg total protein) and **B** as a percentage of total PC detected.

3.2.2.5 Development of a method for analysing complete phospholipids

The final hurdle in the development of a method for the extraction, detection, and analysis of membrane lipids from the mitochondrial and microsomal fractions of human brain tissue was to produce an automated method to characterise all phospholipids within a sample complete with fatty acyl chain pairing. The analysis of phospholipids is conducted using both positive and negative ion scans by tandem mass spectrometry (Table 3-1). These positive precursor ion and neutral loss scans are used to detect the head groups for PC, PE and PS, and with the use of Lipidview™ (version 1.2) the *bruto* structure of the phospholipids can be obtained, including the total number of carbons in both fatty acids and the presence of any double bonds (i.e PC 34:1). This result does not give any specific information on the identity of each of the individual fatty acids present on the phospholipid though (i.e. PC 16:0_18:1). The fatty acid data is detected in the negative precursor ion scans, but the version of Lipidview™ available at the time of this analysis did not allow the concurrent analysis of both positive and negative ion spectra to obtain complete molecular phospholipid structure. This procedure has been previously performed manually in our laboratory by inspecting the spectra and looking for complementary positive and negative ions. An example of how such an analysis can be achieved is shown in Figure 3-5. Completing the analysis of the molecular phospholipids manually for this study was unfeasible though, due to the high number of samples. Therefore, there was a need to develop an automated method for the quantification of complete phospholipids with fatty acid pairs.

The first step in developing this method was to quantify all detected phospholipids from the positive ion precursor ion and neutral loss scans using Lipidview™ as described previously (section 3.2.2.2). Next, each phospholipid quantified by Lipidview™ was assessed for possible isobaric molecular phospholipids (i.e. phospholipid species of the same mass but with a different fatty acid combination) using the lipid catalogue tool in Lipidview™, and a target list of all possible fatty acid pairs for each phospholipid detected in positive ion mode was generated. An example of the target list entry for PC 34:1 can be seen in Table 3-2. Acetate adducts were used in negative precursor ion scans for PC fatty acids, while deprotonated ions were used for the fatty acids of PE and PS. Once generated, this target list was then applied to the negative precursor ion scans for fatty acids using Lipidview™ with the same settings as described above (section

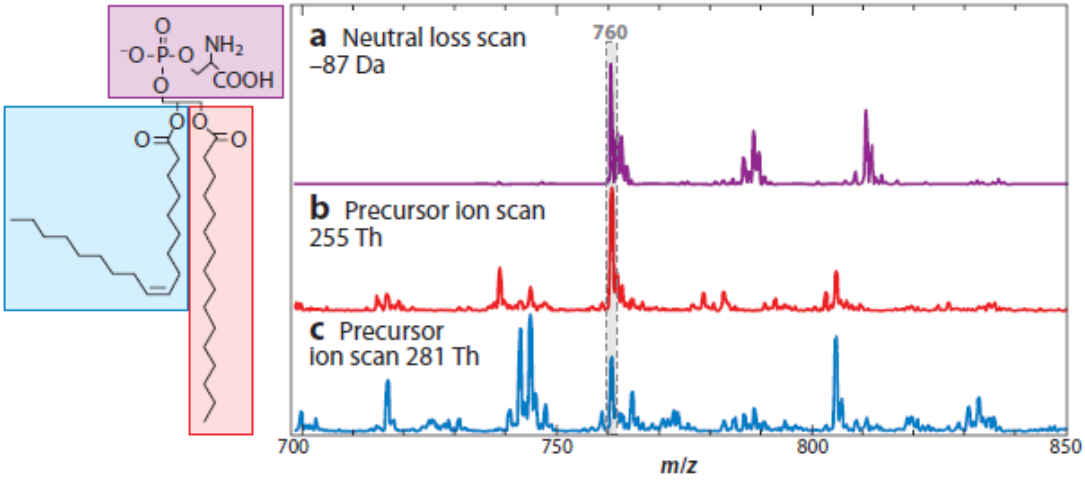


Figure 3-5: Example of how phospholipid composition can be determined using negative-ion mass spectra. Neutral loss scan with an offset of -87 Da identifies phospholipids with a phosphatidylserine head group. (b) Precursor ion scan for m/z 255 identifies phospholipids with a palmitate (16:0) acyl chain. (c) Precursor ion scan for m/z 281 identifies phospholipids with an oleate (18:1) acyl chain. Observation of m/z 760 in all three scans identifies the presence of glycerophosphatidyl serine (16:0_18:1) in the extract. Figure from Blanskby and Mitchell[29]

3.2.2.2). After analysis by Lipidview™ the peak areas of each fatty acid detected were grouped by molecular phospholipid and exported for further analysis in Microsoft® Excel (2010).

A spreadsheet was developed using Microsoft® Excel (2010) to quantify complete molecular species of phospholipids by combining the results of the negative ion fatty acid peak areas with the gross phospholipid structure quantified from the positive ions. This was achieved for diacyl phospholipids (phospholipids with two esterified fatty acids) by several steps. An example of how this spreadsheet quantifies complete phospholipid molecular species is shown in Figure 3-6 for six samples of the prefrontal cortex using PC 34:1. The target list generated from the negative ion Lipidview™ analysis contained fatty acid pairs arranged by mass (see Table 3-2 for example), and from this peak areas could be generated and exported directly as a spreadsheet in Microsoft® Excel. The next step adds the peak areas for corresponding fatty acids together to represent the total peak area for a given molecular phospholipid. Both fatty acids within the phospholipid had to have a peak area greater than zero for it to be included in the analysis. The following step determines the proportion of a given

Table 3-2: Example of target list entry used for phospholipid molecular species determination of PC 34:1 in negative ion mode.

PL molecular species	Mass (*m/z*)	Precursor ion scan	Ion Mode	Fatty acid	PL Class	PL species	Isotope correction factor
PC 14:0_20:1+AcO	818.6	227.20	Negative	14:0	PC	PC 34:1+AcO	1.6420
PC 14:0_20:1+AcO	818.6	309.20	Negative	20:1	PC	PC 34:1+AcO	1.6420
PC 16:0_18:1+AcO	818.6	281.30	Negative	18:1	PC	PC 34:1+AcO	1.6420
PC 16:0_18:1+AcO	818.6	255.20	Negative	16:0	PC	PC 34:1+AcO	1.6420
PC 16:1_18:0+AcO	818.6	253.20	Negative	16:1	PC	PC 34:1+AcO	1.6420
PC 16:1_18:0+AcO	818.6	283.30	Negative	18:0	PC	PC 34:1+AcO	1.6420

Phospholipid *PL* Phosphatidylcholine *PC* Acetate adduct *+AcO*

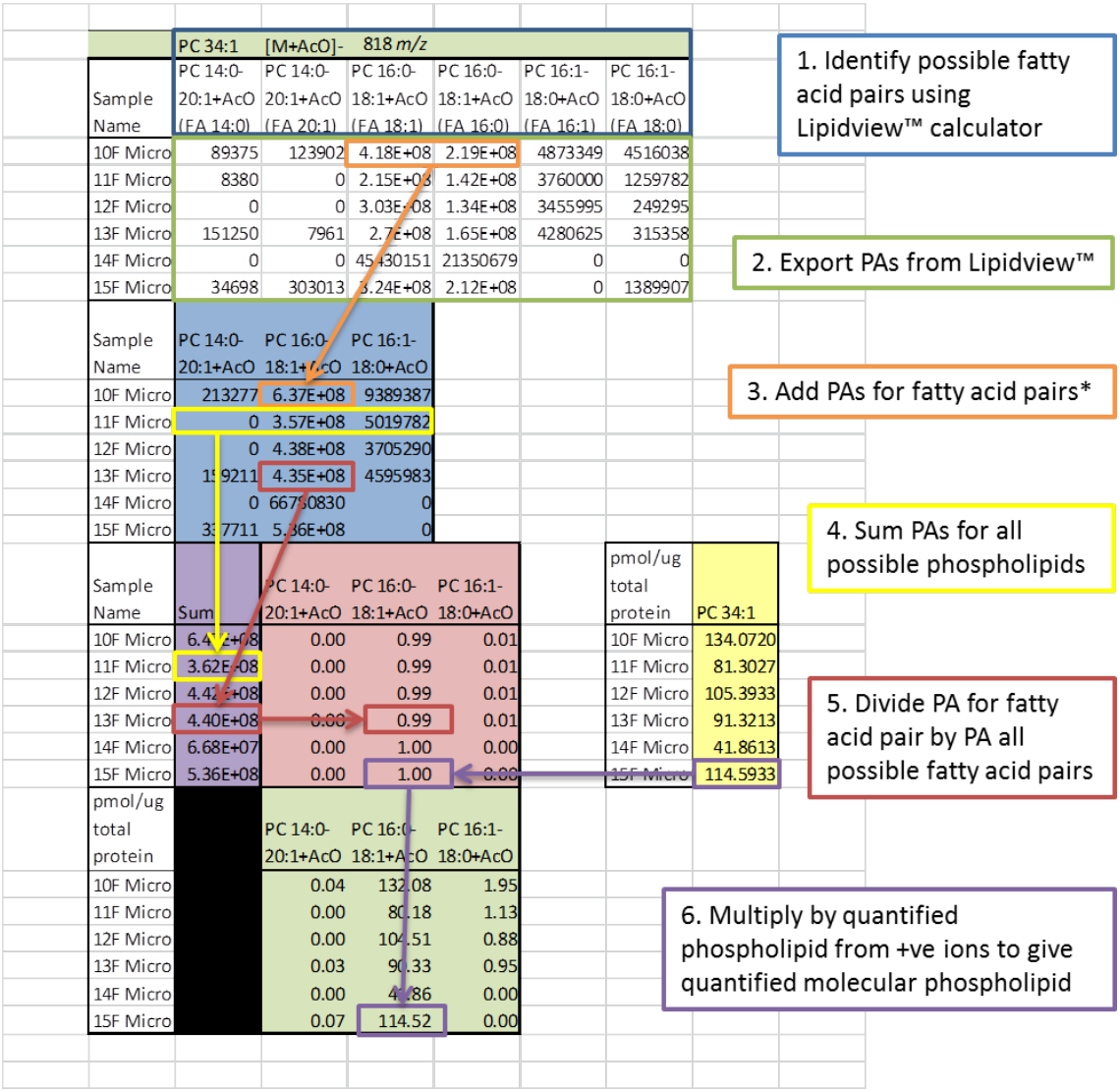


Figure 3-6: Example of the spreadsheet used for identification and quantification of phospholipids with isobaric fatty acids. Peak areas used in this example come from 6 microsomal fraction samples taken from the human prefrontal cortex. Peak area *PA.*
* Only added if the peak area from both fatty acids is present

phospholipid within the total isobaric molecular phospholipids. Using the example of PC 34:1 from Figure 3-6, the peak area of PC 16:0_18:1 is divided by the sum of the peak areas from PC 14:0_20:1, PC 16:0_18:1 and PC 16:1_18:0 to obtain PC 16:0_18:1 as a proportion of total PC 34:1. This ratio could then be multiplied by the amount quantified for PC 34:1, giving the total quantified amount of PC 16:0_18:1 in the sample. Using a worked example from Figure 3-6, in sample 10F Micro PC 16:0_18:1 made up 99% of all isobaric phospholipids detected for PC 34:1. Total PC 34:1 was quantified as being134.07 pmol/µg membrane protein in the same sample from the positive ion spectra. Therefore the quantified amount of PC 16:0_18:1 in sample 10F Micro is 99% of 134.07 pmol/µg membrane protein, which is equal to 132.08 pmol/µg membrane protein. This same process is used for every sample in all regions examined in this study, with the spreadsheet designed to handle up to 36 samples. Isobaric phospholipids containing either an odd-chain or ether-linked fatty acids in the *sn*-1 position required a different approach to that used for to diacyl phospholipids. The odd-chain fatty acids, being esterified to glycerol, can be detected using the negative precursor ion scans. The ether-linked fatty acids, however, are not able to be detected as the energy required to break the ether bond is not accessible in a triple quadrupole mass spectrometer [182]. The *sn*-2 esterified fatty acid can also be detected, but the peak area measured for this fatty acid will be the total peak area for both isobaric species. For example, PE 15:0_22:6 is isobaric with PE O-16:0_22:6, and in negative ion mode both will have a deprotonated mass of 748.6 *m/z*. We can detect the 15:0 and 22:6 fatty acids for both isobaric species with our precursor ion scans, but not O-16:0. We cannot determine the proportion of PE O-16:0_22:6 from this data by subtracting the peak area measured for 15:0 from that of 22:6 either due to differences in fragmentation efficiency between *sn*-1 and *sn*-2 positional fatty acids [183]. Therefore we can only say that either isobaric phospholipid may be present at this mass, and report it as being either phospholipid (i.e. PE 15:0_22:6/O-16:0_22:6).

However, there are some limitations to this method of determining complete phospholipids, including the previously mentioned inability to separate isobaric species with either odd-chain or ether-linked fatty acids. The second limitation is that we cannot definitively distinguish between phospholipids containing an alkenyl ether versus phospholipids with an alkyl ether-linked fatty acid containing a double bond. Alkenyl ethers (also known as plasmalogens) are fatty acids that contain a vinyl ether bond (i.e. a double bond on the first carbon from the ether end). With the mass spectrometry

technique used in this study, it is impossible to tell the position of that double bond along the fatty acid chain. Therefore, we can only say that the double bond is present in an ether-linked fatty acid, rather than identifying it specifically as a plasmalogen. Due to the presence of the vinyl ether bond in the *sn*-1 position, plasmalogen PEs do not show abundant peaks when detected by the neutral loss of 141 Da compared to diacyl PE phospholipids [184,185], and a correction factor is commonly applied to account for this [186]. Because we could not definitively distinguish plasmalogens in this study no such correction factor was used, and due to this plasmalogen levels will likely be underestimated. Likewise we were unable to determine double bond position for PUFA, and thus could not distinguish between n-3 and n-6 fatty acids. Putative classification of n-3 and n-6 fatty acids can be made, however, based on their synthesis as this pathway produces specific fatty acid isomers. Identification of the fatty acids can also be made from previous studies of human brain lipids that used analysis techniques with the ability separate n-3/n-6 isomers (such as gas chromatography). The final limitation is that we cannot tell which *sn* position the fatty acids are in on the glycerol backbone using this mass spectrometry technique; we can only assume *sn* position for fatty acids based on the previous literature. Hence, the nomenclature employed in this thesis follows that set out by Liebisch et al.[187] for fatty acids with unknown positional information, with the two fatty acids identified being separated by an underscore.

3.3 Conclusions

Described in this chapter are the preliminary stages of this thesis, which investigated methods for the subcellular fractionation of frozen post-mortem human brain tissue. Also developed were methods for the extraction and analysis of membrane lipids from the fractionated tissue.

The fractionation of frozen human brain tissue into its subcellular components required consideration. Only a small amount of tissue could be obtained, and the method needed to overcome the problems associated with frozen tissue such as multiple freeze/thaw cycles leading to damage of organelles. The methods used were also required to be compatible with the downstream analysis of membrane lipids and protein, both of which require very different conditions. To that end, a simple but crude fractionation method was developed which relied on the differences in sedimentation rates between the various fractions.

While crude, this method was specific for the organelles being examined (section 3.2.1.3) and gave a sufficient yield of fraction for analysis of lipids.

After the fractionation method had been developed, procedures for the analysis of membrane lipids were examined. Methods for the extraction and analysis of membrane lipids had previously been developed in our laboratory, and these methods were tested for compatibility with this study. An adjustment to how the internal standards were added to the subcellular fractions was required to improve quantification. Additionally, a spreadsheet to quantify phospholipids complete with fatty acid data from both the positive and negative ion mass spectrometry data was developed for this study.

The details of the final methods for membrane lipid extraction and analysis as determined by these investigations are given in detail in section 3.4 below, as well as the final demographics of the cohort from which the brain tissue for all regions studied was obtained. These methods for subcellular fractionation and membrane lipid extraction and analysis were then applied to the studies described in the following chapters.

3.4 Method

3.4.1 Post-mortem human brain tissue

Neurologically normal frozen post mortem human brain tissue from five different regions was obtained from the New South Wales Tissue Resource Centre at the University of Sydney. These regions included the dorsolateral prefrontal cortex, hippocampus, entorhinal cortex, primary motor cortex, and cerebellum. The complete demographics for these samples are listed in Table 3-3. No differences between the two sexes were observed in post-mortem interval and brain pH, but females were significantly older than males (71.4±7.0 years versus 52.7±3.9 respectively, $p < 0.05$). The samples were shipped on dry ice and stored at -80°C prior to analysis. The brain tissue was pulverised on dry ice prior to being accurately weighed for subcellular fractionation. All experiments were approved by and conducted in accordance with the Human Research Ethics Committee of the University of Wollongong (HE11/267).

3.4.2 Subcellular Fractionation

Approximately 100 mg of pulverised brain tissue from each brain region was homogenised with a bead homogeniser (FastPrep®-24 instrument, MP Biomedicals, NSW, Australia) set at a speed of 6.0 m/s for 40 seconds, using 1.4mm zirconium oxide

beads in 1 mL of an ice-cold 20 mM Tris buffer (pH 7.4) containing 250 mM sucrose, 2 mM EDTA, 2 mM DTT and complete protease inhibitor. The homogenate was then centrifuged (1000 x *g*, 10 min) to give a pellet containing nuclei and large cellular debris. The supernatant was collected and centrifuged (10 000 x *g*, 35 min*)* to produce a pellet enriched in mitochondria. The supernatant was centrifuged again (100 000 x *g*, 40 min) to produce a microsomal pellet and cytosolic supernatant. All centrifugation steps were performed at 4°C. Both the mitochondrial and microsomal pellets were resuspended in milliQ H_2O, and total protein content was determined using the BCA assay (ThermoFisher, USA).

3.4.3 Lipid extraction

Aliquots (75 µg of total protein) from the mitochondrial and microsomal fractions were added to chloroform:methanol (2 mL, 2:1 v/v with 0.01% BHT) with 50 µL of internal standard mixture (as described in 3.2.2.4). Lipids were extracted using a modified Folch method as previously outlined in this chapter (section 3.2.2.1). Extracted lipids were reconstituted in chloroform:methanol (1 mL, 1:2 v/v with 0.01% BHT), and stored at -20°C until analysed. All solvents used were of HPLC-grade or higher.

3.4.4 Mass spectrometry and lipid analysis

NanoESI mass spectrometry of lipid extracts was performed using a hybrid triple quadrupole linear ion trap mass spectrometer (QTRAP® 5500 AB Sciex, MA, USA) equipped with an automated chip-based nanoESI source (TriVersa Nanomate™, Advion Biosciences, NY, USA). Samples were diluted to approximately 10 µM for total phospholipids, spiked with 5 mM of ammonium acetate, and loaded onto a 96-well plate. The plate was then centrifuged (10 min, 2200 x *g)* prior to direct infusion. Spray parameters were set at a gas pressure of 0.4 psi and a voltage of 1.2 kV and 1.1 kV for positive and negative ion mode respectively for all acquisitions. Phospholipid data was acquired by targeted ion scans using multi-channel acquisition (Table 3-1).

Table 3-3: Demographics and cause of death of donors from which all brain tissue was obtained.

Age (Years)	Gender	PMI (hours)	Brain pH	Cause of death category	Cause of death clinical
18	Male	28.5	6.7	Cardiac	Primary cardiac arrhythmia
21	Female	39.5	6.83	Cardiac	Primary cardiac arrhythmia
22	Male	50	6.86	Trauma	Blunt trauma
24	Male	43	6.27	Cardiac	Undetermined (but consistent with idiopathic cardiac arrhythmia).
33	Female	24	6.77	Cardiac	Cardiac arrhythmia; myocardial fibrosis
36	Male	34	6.67	Cardiac	Acute chronic cardiac failure
37	Male	14.5	6.46	Cardiac	Presumed Cardiac Dysrhythmia due to natural cause
37	Male	24	6.7		Unascertained.
40	Male	27	6.79	Vascular	1.a) Pulmonary thromboemboli b) deep venous thrombosis
47	Male	27	6.66	Cardiac	Ischaemic heart disease.
48	Male	17	6.62	Cardiac	Ischaemic heart disease. Coronary artery atheroma
49	Male	38	6.92	Cardiac	Coronary Artery Disease
50	Male	40	6.87	Cardiac	Haemopericardium
50	Male	34	6.77	Cardiac	Acute Myocardial infarction
51	Male	35	7	Cardiac	cardiomegaly
52	Male	36	6.82		Undetermined.
57	Male	18	6.39	Cardiac	Myocardial Infarction
58	Male	39	6.49	Cardiac	Ischaemic heart disease.
59	Male	15	6.54	Cardiac	Hypertensive and atherosclerotic heart disease
62	Female	35	6.06	Cardiac	Hypertensive and atherosclerotic heart disease
64	Male	17	6.55	Cardiac	Haemopericardium
66	Male	32	6.66	Cardiac	cardiomegaly
67	Male	25	6.7	Cardiac	Hypertensive Heart disease
69	Female	39	6.72	Cardiac	Coronary Artery Disease and asthma
72	Female	25	7	Cardiac	Atherosclerotic cardiovascular disease
73	Female	45	6.86	Cardiac	Atherosclerotic cardiovascular disease.
74	Female	20	6.59	Cancer	Cancer of breast, liver and bone metastases
78	Female	45	6.05	Toxcity	Multiple drug toxicity (7-amino nitrazepam, nitrazepam and dextropropoxyphene)
80	Male	12	6.5	Respiratory	Emphysema
81	Male	29	6.57	Cardiac	Heart Failure
83	Male	10	6.67	Respiratory	Pulmonary embolus
86	Female	14.5	6.36	Infection	Septicaemia; gangrenous foot; peripheral vascular disease
87	Female	5	6.38	Cancer	Metastatic breast cancer
88	Male	9	6.36	Respiratory	Pneumonia; Debility; Chronic obstructive airways disease
98	Female	6	6.7	Respiratory	Pneumonia; congestive cardiac failure
104	Female	27	5.89	Respiratory	Bilateral bronchopneumonia

Lipidview™ software (version 1.2, AB Sciex, MA, USA) was used to quantify phospholipid species against internal standards using positive ion head group scans. Processing settings in LipidView™ were set at a mass tolerance of 0.5 Da, with a minimum intensity of 0.1% and a minimum signal-to-noise ratio of 10. Any phospholipid species comprising less than 0.5% of each phospholipid class across the cohort were removed from the analysis. Each phospholipid quantified was then assessed for possible isobaric molecular phospholipids using the lipid catalogue tool from Lipidview™, and confirmatory ions from the negative ion fatty acid precursor scans were used to identify the amount of each molecular phospholipid present in each sample. Isobaric phospholipids containing either an odd-chain or ether-linked fatty acid were unable to be separated using this method and were reported as being either species. Due to this no correction factor was applied to ether-linked PEs (see section 3.2.2.5). Molecular phospholipids that appeared in less than 75% of samples were removed prior to statistical analysis. Phospholipids are reported using nomenclature outlined by Liebisch et al. [187], as normalised values relative to total phospholipid detected in each phospholipid class to reduce variation between the samples. Regression parameters for phospholipids showing significant age-related changes in quantified amount (pmol of phospholipid/µg total membrane protein) have been included for comparison.

3.4.5 Statistical analysis

All statistical analysis in the following chapters was performed using SPSS Statistics (version 19, IBM Corp., NY, USA). The Wilcoxon signed-rank test was used for comparisons of phospholipid classes between the mitochondrial and microsomal fractions. To examine the relationship between age and phospholipids, or age and total protein content, linear regression was used with sex as a second independent variable, with significance set a level of $p < 0.05$. Normality of the dependent variable was assessed by examining the histograms of the residuals, and non-normal data was transformed where required. Positively skewed residuals were transformed using logarithmic or reciprocal transformations depending on the level of skew present while negatively skewed data used the reflection of these transformations. Outliers were identified as being greater than three standard deviations from the mean of the standardised residuals, and were dealt with by either transformation of the dependent variable or removal from the analysis. Normality of the dependent variable was assessed by examining the histograms of the standardised residuals, and non-normal data was

transformed where required. Influential data points were identified by a cook's distance of greater than one and were removed from the analysis. Where the dependent variable was transformed, data is displayed in figures as untransformed scatterplots with transformed beta-coefficients and *p* value to allow direct comparison between phospholipids. As this was an exploratory study a correction for multiple comparisons was not applied.

SECTION 3

RESULTS

Chapter 4 The Dorsolateral Prefrontal Cortex

This chapter is an amended version of the published manuscript: Norris SE, Friedrich MG, Mitchell TW, Truscott, RJW, Else PL, (2015) Human prefrontal cortex phospholipids containing docosahexaenoic acid increase during normal adult aging, whereas those containing arachidonic acid decrease. *Neurobiology of Aging*, 36(4)1659-69.

4.1 Introduction

This chapter will examine the changes seen during normal ageing in PC, PE and PS phospholipids present in the mitochondrial and microsomal membranes of the dorsolateral prefrontal cortex.

The dorsolateral prefrontal cortex (henceforth referred to as the prefrontal cortex) is located in the middle frontal gyrus (see Chapter 1 Figure 1-2), and has connections to many brain regions including the orbitofrontal cortex, the thalamus, the hippocampus, and the primary and secondary association areas of the neocortex [188]. The functions of this region include executive functions such as working memory, cognitive flexibility, as well as planning and inhibition [188].

Changes to phospholipid composition have been particularly well-studied in different parts of the frontal and prefrontal cortex in AD [85–87,92,93,111,113,115,116,189]. Both Svennerholm [88] and Söderberg et al. [90,93] have reported losses of total phospholipid with age in whole human neocortex and frontal cortex respectively over the adult lifespan. Losses of PC and PE within the frontal cortex with advanced age have also been reported by Söderberg et al. [93].

Thus, the aim of this chapter was to expand on the limited knowledge available on age-related changes in phospholipids of the mitochondrial and microsomal membranes of the human prefrontal cortex.

4.2 Results

4.2.1 Age-related changes to total protein content in subcellular fractions

No changes with age in total protein concentration (mg/g tissue) were seen in the whole tissue homogenate, or in the mitochondrial and microsomal membranes.

4.2.2 Major phospholipid classes of mitochondrial and microsomal fractions

The three major phospholipid classes PC, PE and PS showed similar abundances in the mitochondrial and microsomal membranes when expressed as a percentage of total phospholipid (Figure 4-1). Overall, PC represented approximately 56%, PE 29% and PS 15% of total phospholipid. Small differences in abundance were seen between the membrane fractions, including a slightly higher level of PE in the mitochondria ($p < 0.001$) and a marginally higher level of PC in the microsomal membranes ($p < 0.01$). No significant differences were seen in the abundance of PS between the two membrane fractions.

No differences were observed between the mitochondrial and microsomal fractions for any phospholipid class or in total phospholipid content when analysed as a quantified amount (i.e. pmol phospholipid/µg of membrane protein). Additionally, no age-related changes were seen for any of the three phospholipid classes or total phospholipids in either the mitochondrial or microsomal fraction when expressed as either percent of total phospholipid class or as quantified amounts.

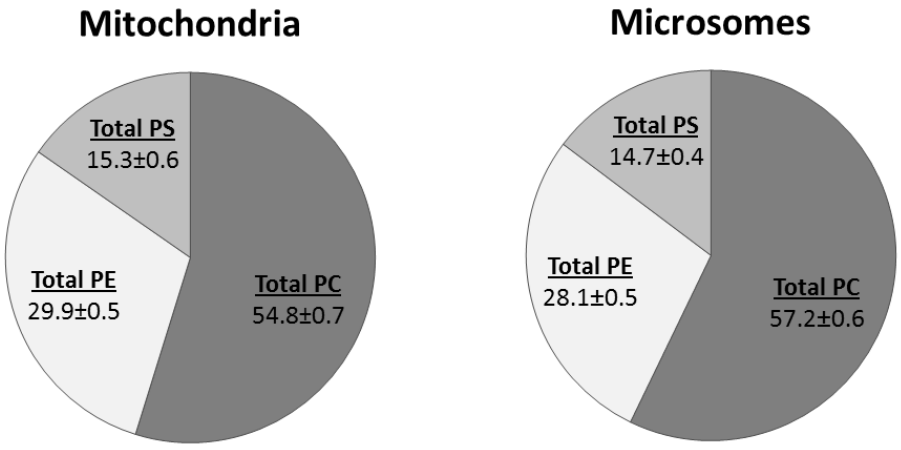


Figure 4-1: Percent composition of the three major phospholipid classes within the mitochondrial (left) and microsomal (right) fractions in normal human prefrontal cortex. Values are the mean ± SEM for the entire cohort. The microsomal membranes showed significantly higher amounts of total phosphatidylcholine (PC) compared to mitochondria ($p < 0.01$, Wilcoxon signed-rank test), while the mitochondria contained more phosphatidylethanolamine (PE) ($p < 0.001$, Wilcoxon signed-rank test). There were no differences between membrane fractions for phosphatidylserine (PS).

4.2.3 Changes in mitochondrial phospholipids with age

4.2.3.1 Phosphatidylcholines

The composition of PC, PE and PS phospholipids detected in the mitochondrial membranes of the entorhinal cortex can be seen in (Figure 4-2). The most abundant phospholipid found in the mitochondrial membranes of the prefrontal cortex was PC 16:0_18:1, representing approximately 25% of all phospholipids measured. From ages 20 to 100 years, PC 16:0_18:1 increased from 41% to 44% of all PC molecules analysed, representing a 7% increase from its initial abundance over the 80 year period (Figure 4-3, Table 4-1). Six other PCs also changed with age in abundance when expressed as percent composition (percent of total PC), including PC 16:0_22:6, PC 18:0_18:2, PC 16:0_18:2, PC 14:0_16:0, lyso-PC 16:0, and PC 15:0_16:0/O-16:0_16:0. Of these six phospholipids only lyso-PC 16:0 decreased in abundance over the adult lifespan.

Analysis of quantified phospholipids (as pmol/µg of membrane protein) found that PC 16:0_18:0 and lyso-PC 16:0 decreased with age, while PC O-16:1_18:0 increased in quantified amount with age (Table 4-2).

4.2.3.2 Phosphatidylethanolamines

Ten mitochondrial PEs changed in their percent composition with age in the human prefrontal cortex (Figure 4-4 & Table 4-1). Decreases with age were seen in the second-most abundant mitochondrial PE, PE 18:0_20:4, which declined from approximately 19% of total mitochondrial PE to 15% from ages 20 to 100. Considering the abundance of this phospholipid, this represents the largest change with age seen in any mitochondrial PE. A second highly abundant mitochondrial PE, PE 18:0_22:4, also decreased by 24% from ages 20 to 100. Decreases with age were also seen in PE 18:0_18:1 and PE 16:0_18:1, two moderately abundant phospholipids that together comprise approximately 6.7% of total mitochondrial PEs (Figure 4-2). PE 16:0_20:4, PE 17:0_22:6/O-18:0_22:6 and PE O-16:1_22:4 also decreased across the adult lifespan in the prefrontal cortex, but these phospholipids are only of low abundance in the mitochondria.

Increases with age were reported for three mitochondrial PEs, PE 18:1_22:6, PE O-18:1_22:6 and PE O-18:1_22:5, which increased in abundance by 42%, 60%, and 133%

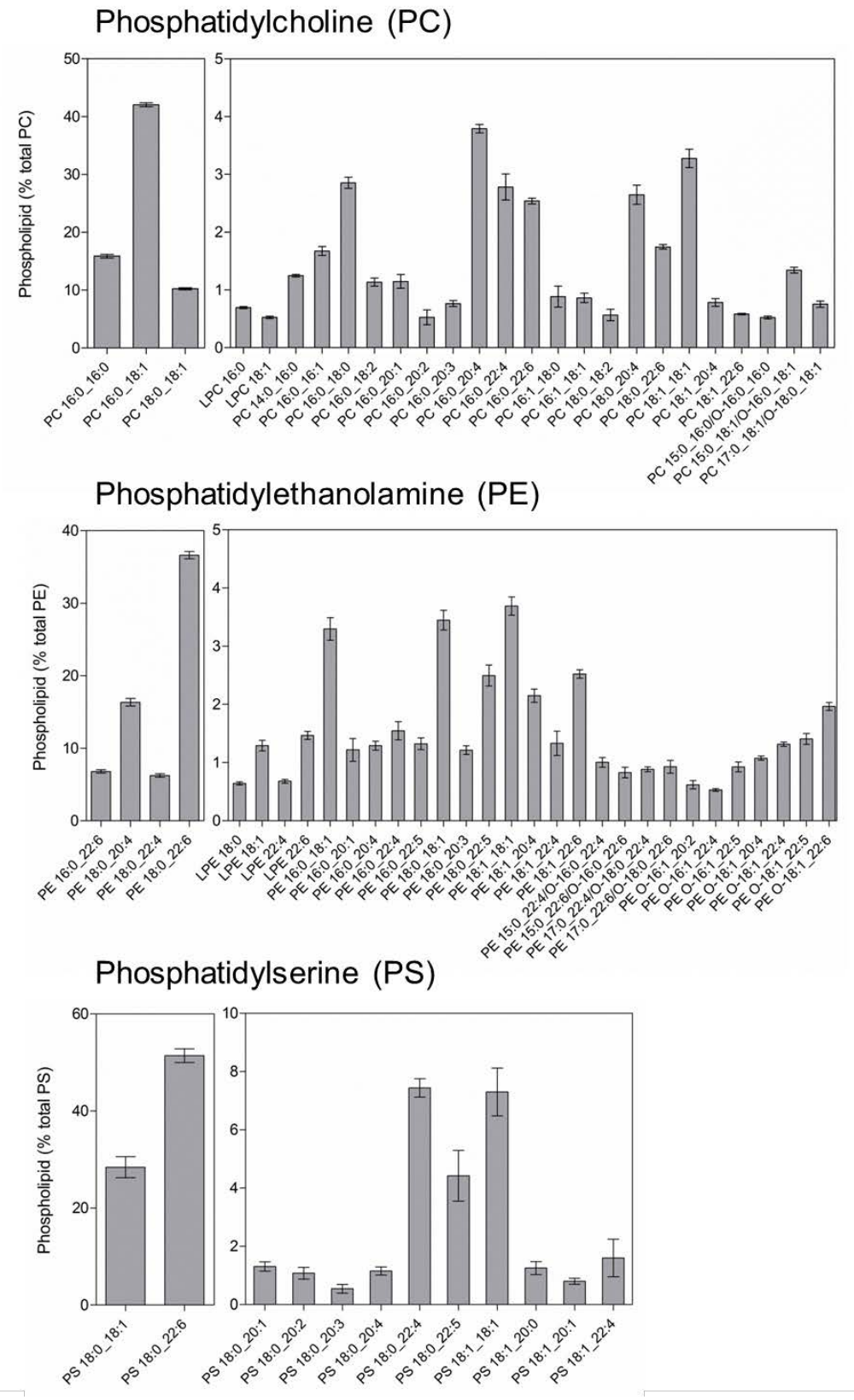


Figure 4-2: Phospholipids detected within PC, PE and PS in the mitochondrial fraction of human prefrontal cortex (as a percent of total phospholipid within each class). Phospholipids were quantified as described in materials and methods. Values are mean across the cohort ± SEM.

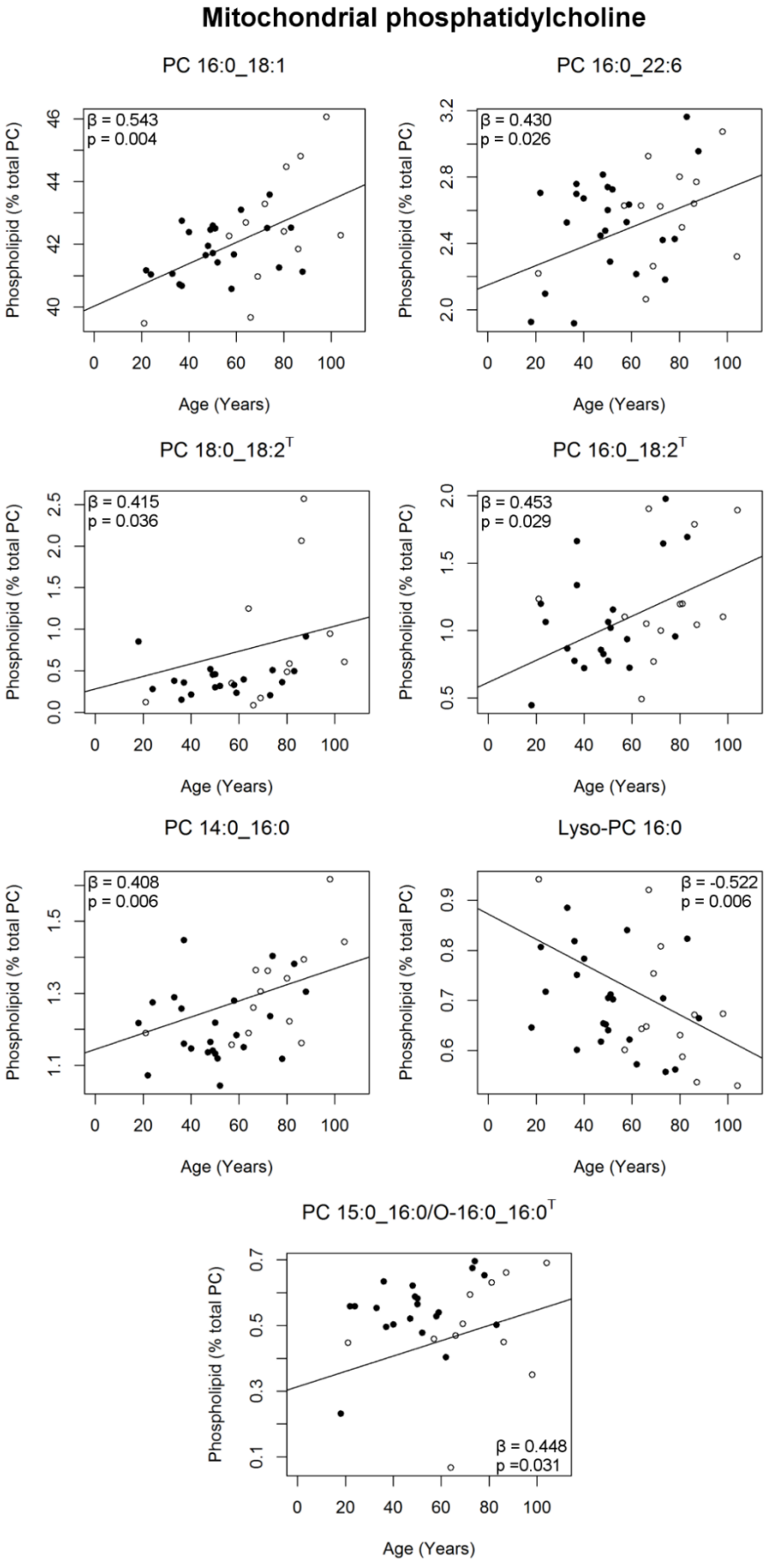

Mitochondrial phosphatidylcholine
PC 16:0_18:1
β = 0.543
p = 0.004
PC 16:0_22:6
β = 0.430
p = 0.026
PC 18:0_18:2T
β = 0.415
p = 0.036
PC 16:0_18:2T
β = 0.453
p = 0.029
PC 14:0_16:0
β = 0.408
p = 0.006
Lyso-PC 16:0
β = -0.522
p = 0.006
PC 15:0_16:0/O-16:0_16:0T
β = 0.448
p =0.031
Phospholipid (% total PC)
Age (Years)

Figure 4-3: Mitochondrial PCs changing significantly with age (as a percent of total PC) in normal human prefrontal cortex (*n* = 30-36). Regression model was adjusted for sex: males (●), females (○). ^{T} indicates dependent variable transformed for linear regression, with transformed beta-coefficient and p-value reported on original scatterplot for comparison. Regression parameters are shown in Table 4-1.

Table 4-1: Mitochondrial phospholipids (percent of phospholipid within phospholipid class) changing significantly with age in the human prefrontal cortex.

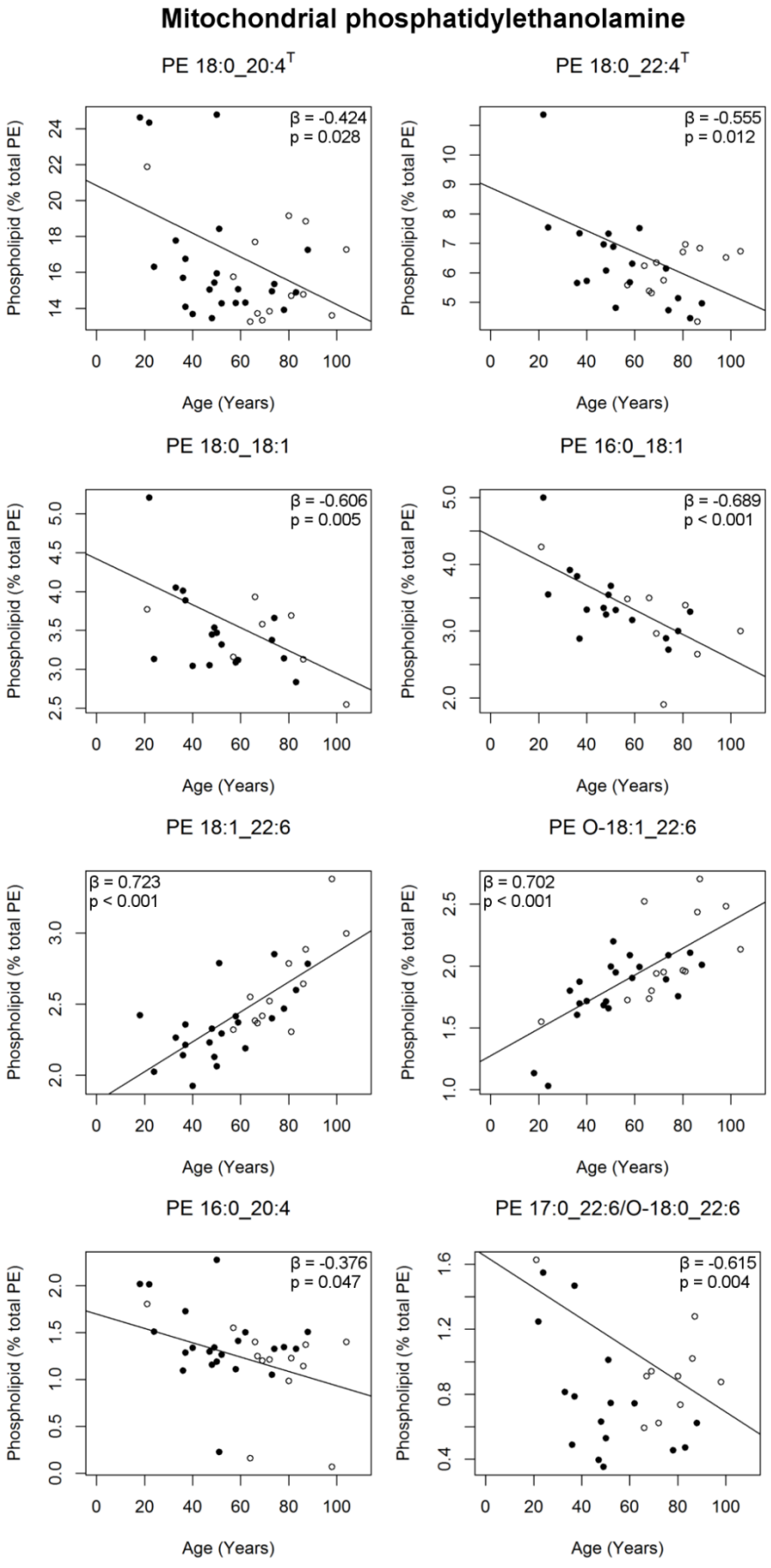
Phospholipid	**Slope**	**95% CI**		**β**	**df**	**t**	***P* value**
		Lower	**Upper**				
Phosphatidylcholine							
Lyso-PC 16:0	-0.0025	-0.0043	-0.0008	-0.522	2,33	-2.946	0.006**
PC 14:0_16:0	0.0022	0.0003	0.0042	0.408	2,33	2.397	0.022*
PC 16:0_18:1	0.0339	0.0119	0.0559	0.543	2,31	3.148	0.0036**
PC 16:0_18:2^{L}	0.0016	0.0002	0.0030	0.453	2,30	2.295	0.029*
PC 16:0_22:6	0.0058	0.0008	0.0108	0.430	2,33	2.338	0.026*
PC 18:0_18:2^{I}	0.0028	0.0002	0.0054	0.415	2,27	2.207	0.036*
PC 15:0_16:0 /O-16:0_16:0^{IR}	0.0017	0.0002	0.0032	0.448	2,28	2.274	0.031*
Phosphatidylethanolamine							
PE 16:0_18:1	-0.0184	-0.0284	-0.0084	-0.689	2,21	-3.842	0.0009***
PE 16:0_20:4	-0.0077	-0.0152	-0.0001	-0.376	2,32	-2.066	0.047*
PE 18:0_18:1	-0.0147	-0.0246	-0.0049	-0.606	2,21	-3.103	0.005**
PE 18:0_20:4^{I}	-0.0002	-0.0003	-0.00002	-0.424	2,33	-2.299	0.028*
PE 18:0_22:4^{I}	-0.0006	-0.0011	-0.0001	-0.555	2,27	-2.705	0.012**
PE 18:1_22:6	0.0105	0.0060	0.0150	0.723	2,30	4.773	0.00004***
PE 17:0_22:6 /O-18:0_22:6	-0.0096	-0.0158	-0.0033	-0.615	2,23	-3.167	0.004**
PE O-16:1_22:4	-0.0026	-0.0052	-0.0001	-0.390	2,33	-2.089	0.045*
PE O-18:1_22:5	0.0147	0.0039	0.0256	0.548	2,31	2.763	0.0095**
PE O-18:1_22:6	0.0109	0.0065	0.0153	0.702	2,31	5.041	0.00002***
Phosphatidylserine							
PS 18:0_20:4	-0.0198	-0.0327	-0.0069	-0.588	2,25	-3.163	0.004**
PS 18:0_22:6	0.1605	0.0229	0.2981	0.425	2,30	2.383	0.024*

Phospholipid molecular species were detected using nanoelectrospray ionisation mass spectrometry and quantified as described in materials and methods. Regression was adjusted for gender. Superscript indicates dependent variable transformed for linear regression, with transformed regression output reported: L logarithm, I reciprocal, IR reflect and reciprocal. * P <0.05 ** P <0.01 *** P <0.001

Table 4-2: Quantified mitochondrial phospholipids (pmol/µg total membrane protein) changing significantly with age in the human prefrontal cortex.

Phospholipid	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Phosphatidylcholine							
Lyso-PC 16:0	-0.0070	-0.0120	-0.0020	-0.512	2,33	-2.871	0.007**
PC 16:0_18:0	-0.0216	-0.0428	-0.0003	-0.384	2,31	-2.073	0.047*
PC O-16:1_18:0	0.0118	0.0024	0.0211	0.538	2,20	2.627	0.016*
Phosphatidylethanolamine							
PE 18:0_22:4	-0.0404	-0.0750	-0.0057	-0.503	2,27	-2.391	0.024*
PE 18:1_22:6[IR]	0.0005	0.0001	0.0009	0.456	2,32	2.563	0.015*
PE 17:0_22:6 /O-18:0_22:6	-0.0106	-0.0194	-0.0018	-0.516	2,23	-2.494	0.020*
PE O-18:1_22:5	0.0174	0.0040	0.0309	0.526	2,31	2.646	0.013*
PE O-18:1_22:6	0.0122	0.0047	0.0197	0.540	2,33	3.323	0.002**
Phosphatidylserine							
PS 18:0_20:4	-0.0091	-0.0181	-0.00002	-0.427	2,25	-2.065	0.049*
PS 18:0_22:4	-0.0017	-0.0031	-0.0002	-0.440	2,30	-2.337	0.026*
PS 18:1_20:1[I]	-0.0037	-0.0071	-0.0002	-0.424	2,28	-2.188	0.037*

Phospholipid molecular species were detected using nanoelectrospray ionisation mass spectrometry and quantified as described in materials and methods. Regression was adjusted for gender. Superscript indicates dependent variable transformed for linear regression, with transformed regression output reported: [I] reciprocal, [IR] reflect and recipr ocal. * *P* <0.05 ** *P* <0.01 *** *P* <0.001


Mitochondrial phosphatidylethanolamine
PE 18:0_20:4T
β = -0.424
p = 0.028
PE 18:0_22:4T
β = -0.555
p = 0.012
PE 18:0_18:1
β = -0.606
p = 0.005
PE 16:0_18:1
β = -0.689
p < 0.001
PE 18:1_22:6
β = 0.723
p < 0.001
PE O-18:1_22:6
β = 0.702
p < 0.001
PE 16:0_20:4
β = -0.376
p = 0.047
PE 17:0_22:6/O-18:0_22:6
β = -0.615
p = 0.004
Phospholipid (% total PE)
Age (Years)

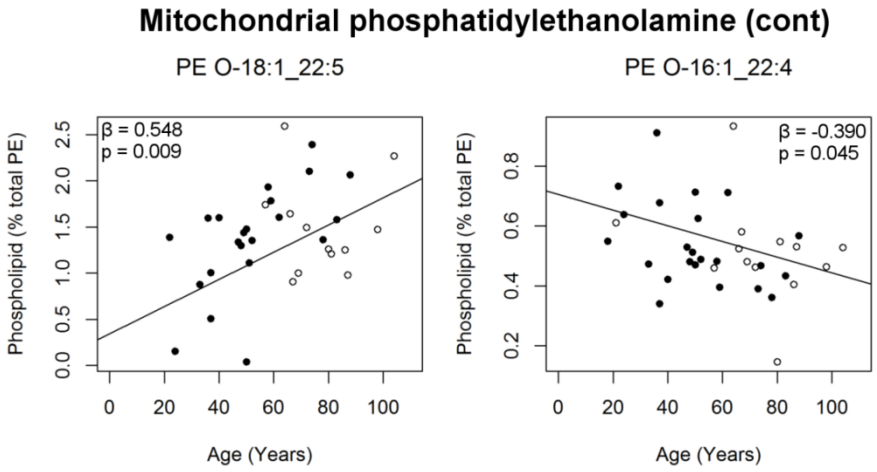


Figure 4-4: Mitochondrial PEs changing significantly with age (as a percent of total PE) in normal human prefrontal cortex (*n* = 24-36). Regression model was adjusted for sex: males (●), females (○). ^{T} indicates dependent variable transformed for linear regression, with transformed beta-coefficient and p-value reported on original scatterplot for comparison. Regression parameters are shown in Table 4-1.

respectively across the 80 year period. While seemingly substantial increases were seen in PE O-18:1_22:5 with age, this phospholipid is only of low abundance and comprises less than 2% of total mitochondrial PE (Figure 4-2) and 0.4% of total mitochondrial phospholipid.

Analysis of the quantified data (pmol/µg membrane protein) showed similar trends for all PE phospholipids measured. PE 18:0_22:4 and PE 17:0_22:6/O-18:0_22:6 continued to decrease significantly with age, while PE 18:1_22:6, PE O-18:1_22:6 and PE O-18:1_22:6 all maintained age-related increases (Table 4-2).

4.2.3.3 Phosphatidylserines

Two PS phospholipids, PS 18:0_22:6 and PS 18:0_20:4, changed with age in their percent composition in the mitochondrial membranes of the human prefrontal cortex (Figure 4-5, Table 4-1). PS 18:0_22:6 is the principal PS found in mitochondrial membranes, representing just over half of all mitochondrial PS (Figure 4-2) and 8.3% of total mitochondrial phospholipids. PS 18:0_22:6 increased by 34% in abundance over 80 years, rising from approximately 44% of total PS phospholipids at age 20 to 57% at 100 years of age. This represents the largest change with age in abundance for any mitochondrial phospholipid.

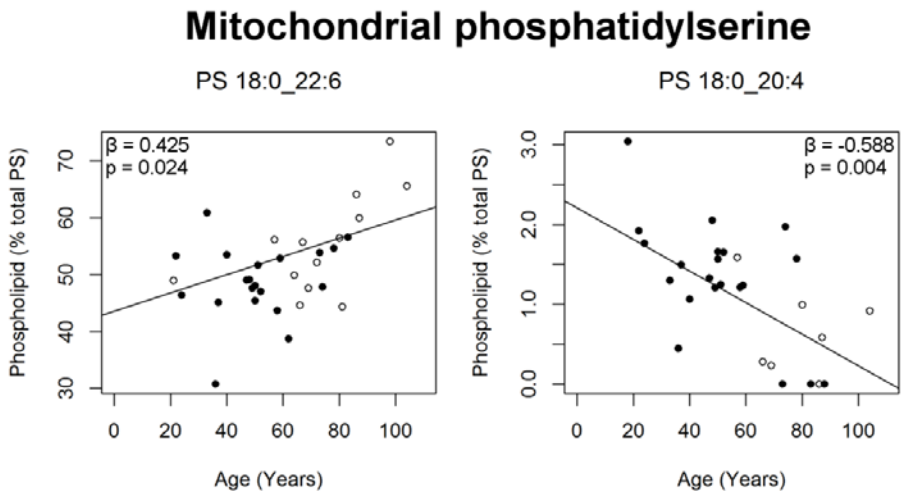


Figure 4-5: Mitochondrial PSs changing significantly with age (as a percent of total PC) in normal human prefrontal cortex (*n* = 28-33). Regression model was adjusted for sex: males (●), females (○). Regression parameters are shown in Table 4-1.

In contrast, PS 18:0_20:4 decreased with age by 85% when expressed as a percent of total PS (Figure 4-5, Table 4-1) and by 70% quantitatively (pmol/µg membrane protein, Table 4-2) over the age range studied. Two other mitochondrial PSs changed across the 80 year period in their quantitative amount, with PS 18:0_22:4 decreasing and PS 18:1_20:1 increasing with age (Table 4-2).

4.2.4 Changes in microsomal phospholipids with age

4.2.4.1 Phosphatidylcholines

The composition of PC, PE and PS phospholipids detected in the microsomal membranes of the prefrontal cortex can be seen in (Figure 4-6). Age-related changes in PC phospholipids in microsomal membranes of the prefrontal cortex were small, with only three relatively minor phospholipids changing significantly with age in their percent composition (Figure 4-7, Table 4-3). These included PC 16:0_20:4, PC 16:0_18:2, and PC 14:0_16:0. PC 16:0_20:4 decreased in abundance with age by 14% from age 20 to 100, while PC 16:0_18:2 and PC 14:0_16:0 increased by 32% and 14% respectively. These three phospholipids are of low to moderate abundance, however, comprising only 3.3% of the total microsomal phospholipid when combined.

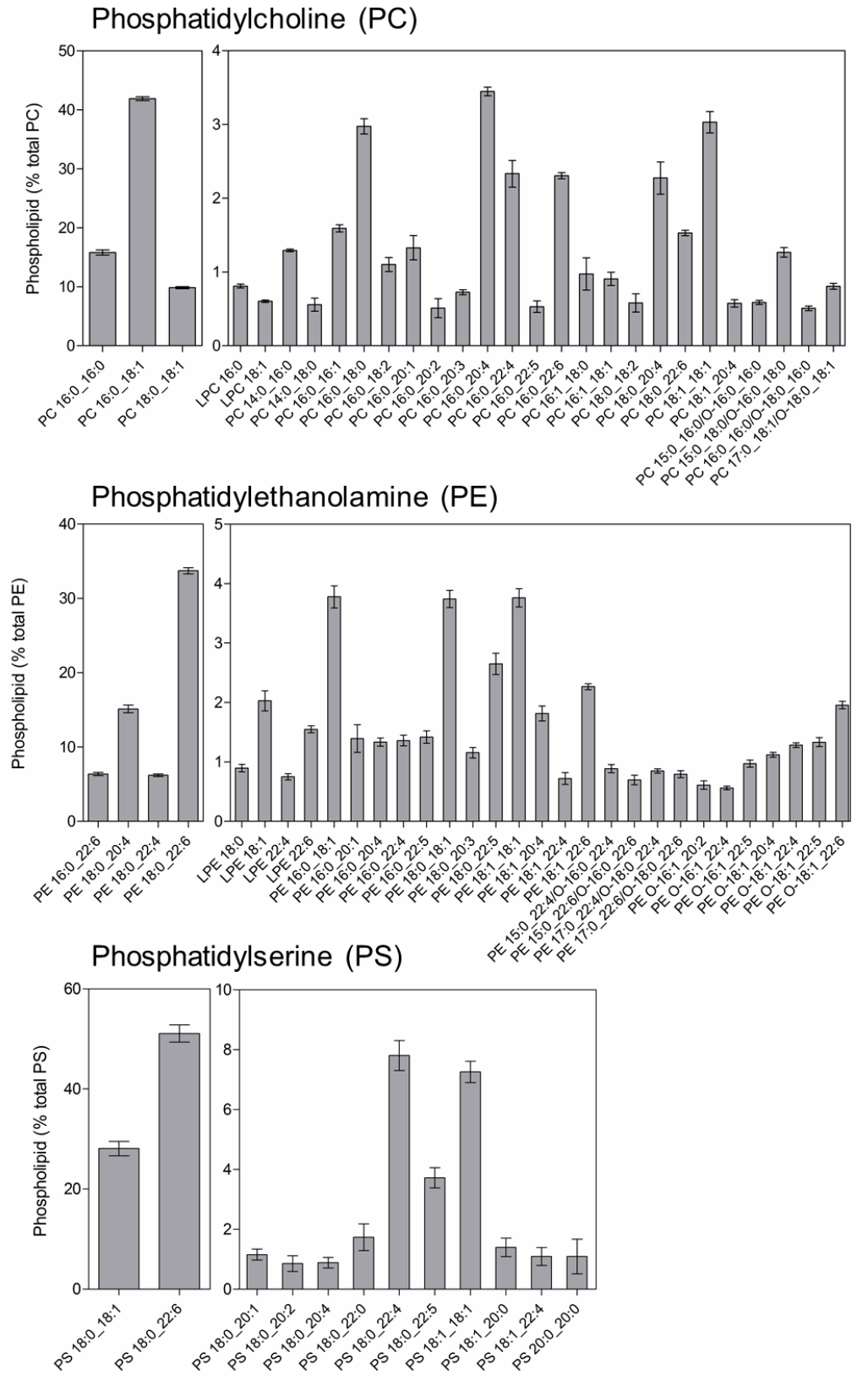


Figure 4-6: Phospholipids detected within PC, PE and PS in the microsomal fraction of human prefrontal cortex (as a percent of total phospholipid within each class). Phospholipids were quantified as described in materials and methods. Values are mean across the cohort ± SEM.

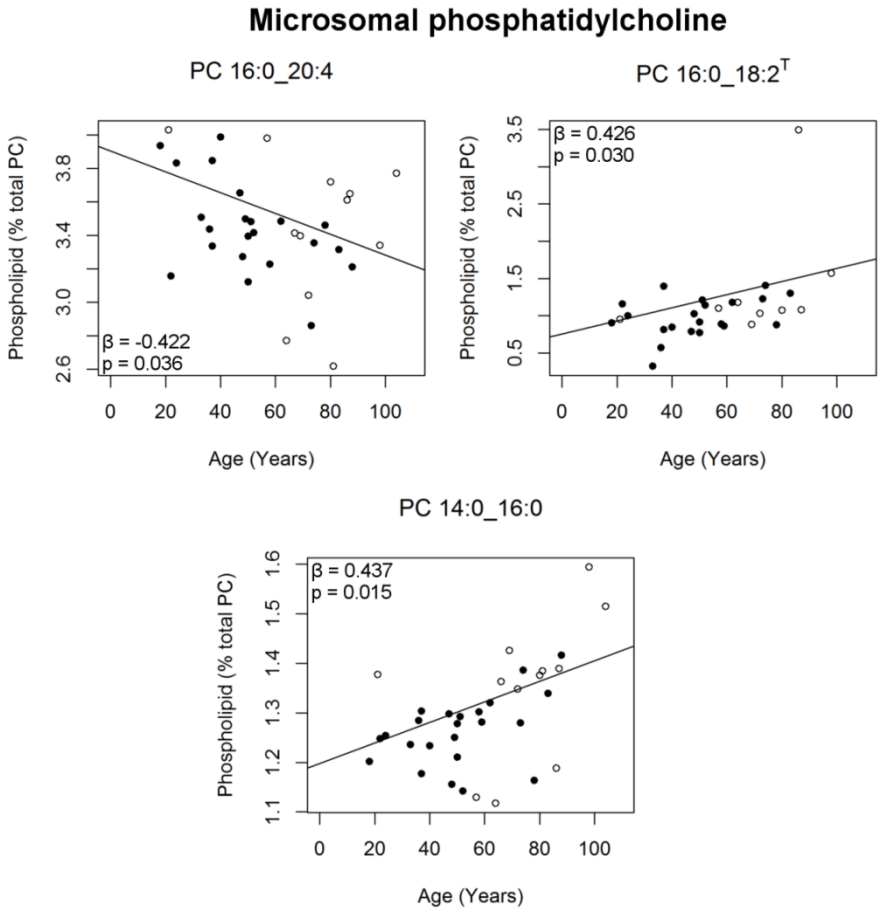


Figure 4-7: Microsomal PCs changing significantly with age (as a percent of total PC) in normal human prefrontal cortex (*n* = 30-34). Males (●), females (○).Regression model was adjusted for sex: males (●), females (○). ^{T} indicates dependent variable transformed for linear regression, with transformed beta-coefficient and p-value reported on original scatterplot for comparison. Regression parameters are shown in Table 4-3.

Table 4-3: Microsomal phospholipids (percent of phospholipid within phospholipid class) changing significantly with age in the human prefrontal cortex.

Phospholipid	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Phosphatidylcholine							
PC 14:0_16:0	0.0021	0.0004	0.0037	0.437	2,32	2.575	0.015*
PC 16:0_18:2^{I}	0.0017	0.0002	0.0032	0.426	2,27	2.295	0.030*
PC 16:0_20:4	-0.0062	-0.0120	-0.0004	-0.422	2,31	-2.198	0.036*
Phosphatidylethanolamine							
Lyso-PE 22:4	-0.0055	-0.0091	-0.0018	-0.493	2,32	-3.032	0.005**
Lyso-PE 22:6	-0.0079	-0.0132	-0.0026	-0.515	2,33	-3.015	0.005**
PE 16:0_20:4	-0.0047	-0.0083	-0.0011	-0.539	2,29	-2.644	0.013*
PE 18:0_18:1^{L}	-0.0020	-0.0031	-0.0009	-0.693	2,26	-3.830	0.0007***
PE 18:0_22:5	-0.0255	-0.0422	-0.0087	-0.561	2,29	-3.107	0.004**
PE 18:1_18:1^{L}	-0.0019	-0.0030	-0.0007	-0.588	2,30	-3.294	0.003**
PE 18:1_22:6	0.0069	0.0014	0.0122	0.494	2,32	2.581	0.015*
PE 17:0_22:4 /O-18:0_22:4	-0.0040	-0.0068	-0.0011	-0.498	2,30	-2.821	0.008**
PE O-16:1_22:4^{I}	-0.0009	-0.0014	-0.0004	-0.599	2,33	-3.558	0.001**
PE O-18:1_20:4^{I}	-0.0007	-0.0014	-0.0001	-0.491	2,31	-2.444	0.020*
Phosphatidylserine							
PS 18:0_22:6	0.1915	0.0217	0.3614	0.424	2,32	2.297	0.028*

Phospholipid molecular species were detected using nanoelectrospray ionisation mass spectrometry and quantified as described in materials and methods. Regression was adjusted for gender. Superscript indicates dependent variable transformed for linear regression, with transformed regression output reported: ^{L} logarithm, ^{I} reciprocal. * P <0.05 ** P <0.01 *** P <0.001

Table 4-4: Quantified microsomal phospholipids (pmol/µg total membrane protein) changing significantly with age in the human prefrontal cortex.

Phospholipid	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Phosphatidylethanolamine							
PE 15:0_22:6 /O-16:0_22:6	0.0070	0.0007	0.0133	0.395	1,32	2.275	0.030*

Phospholipid molecular species were detected using nanoelectrospray ionisation mass spectrometry and quantified as described in materials and methods. Regression was adjusted for gender. * P <0.05

Analysis of the quantified data for PC phospholipids (as pmol/μg of membrane protein) showed similar trends to those reported for the percent data, but no PC reached statistical significance in the microsomal fraction.

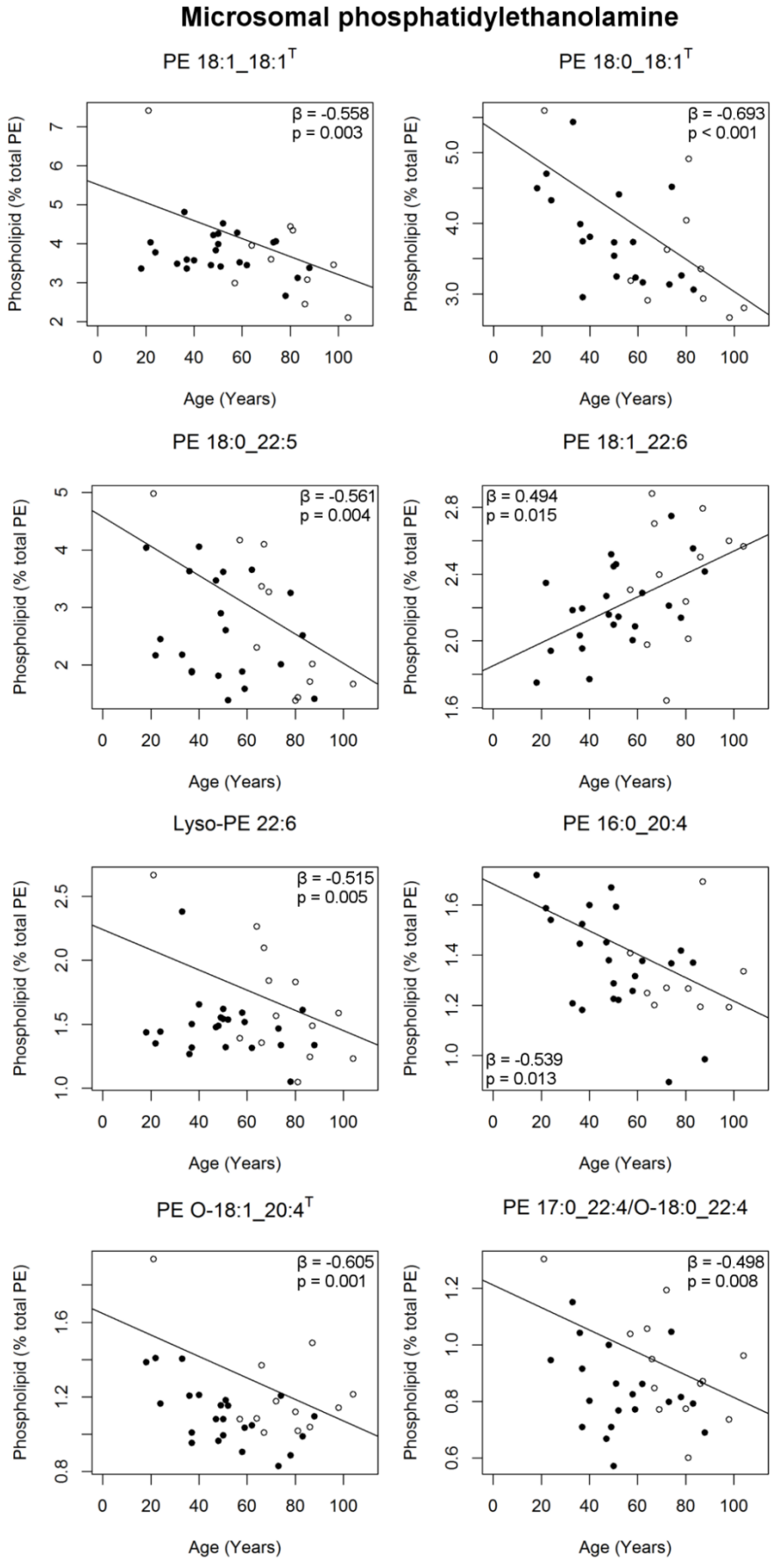
4.2.4.2 Phosphatidylethanolamines

Ten microsomal PEs showed significant age-related changes in the prefrontal cortex (Figure 4-8, Table 4-3). PE 18:1_18:1, PE 18:0_18:1 and PE 18:0_22:5 are all moderately abundant PEs present in the microsomes, and these phospholipids decreased in abundance by 30%, 31% and 61% respectively from ages 20 to 100. The remaining six microsomal PEs that decreased with age were of low abundance, and included lyso-PE 22:6, PE 16:0_20:4, PE O-18:1_20:4, PE 17:0_22:4/O-18:0_22:4, lyso-PE 22:4 and PE O-16:1_22:4. The only microsomal PE to increase in abundance with age was PE 18:1_22:6, a phospholipid which is also of low abundance in the microsomal membranes (0.6% of all microsomal phospholipids). All of the major microsomal PEs, including PE 16:0_22:6, PE 18:0_22:6, PE 18:0_20:4 and PE 18:0_22:4 (Figure 4-6), showed no changes with age in the human prefrontal cortex. PE 15:0_22:6/O-16:0_22:6 was the only microsomal phospholipid that increased in quantitative amount (pmol of phospholipid/μg of membrane protein) in the prefrontal cortex (Table 4-4). The quantified PE data showed similar trends to those reported for the percent data, but none of these phospholipids reached statistical significance.

4.2.4.3 Phosphatidylserines

Only a single microsomal PS phospholipid, PS 18:0_22:6, changed in its percent composition with age in the human prefrontal cortex (Figure 4-9, Table 4-3). This phospholipid is the primary PS found in microsomal membranes representing approximately 50% of all microsomal PS (Figure 4-6) and 7.5% of all phospholipids. PS 18:0_22:6 increased by 35% in abundance from ages 20 to 100 (Figure 4-9, Table 4-3).

No age-related changes were found for any PS phospholipids in the quantified microsomal data (pmol/μg of membrane protein).


Microsomal phosphatidylethanolamine
PE 18:1_18:1T
β = -0.558
p = 0.003
PE 18:0_18:1T
β = -0.693
p < 0.001
PE 18:0_22:5
β = -0.561
p = 0.004
PE 18:1_22:6
β = 0.494
p = 0.015
Lyso-PE 22:6
β = -0.515
p = 0.005
PE 16:0_20:4
β = -0.539
p = 0.013
PE O-18:1_20:4T
β = -0.605
p = 0.001
PE 17:0_22:4/O-18:0_22:4
β = -0.498
p = 0.008
Phospholipid (% total PE)
Age (Years)

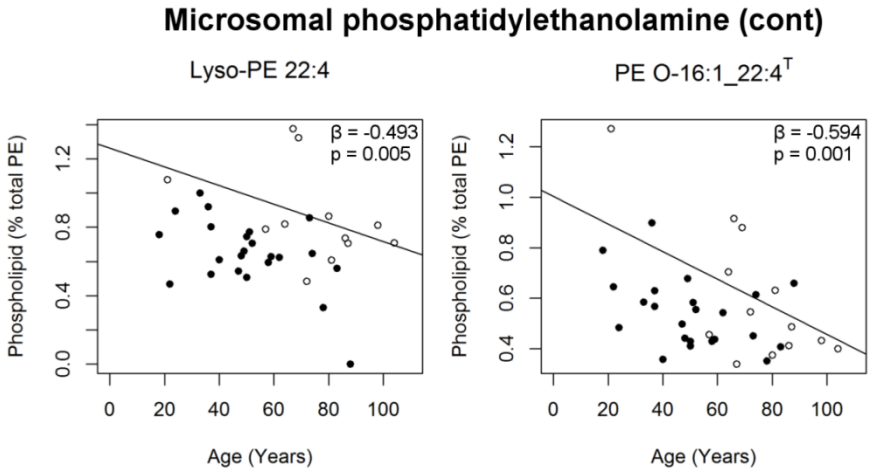


Figure 4-8: Microsomal PEs changing significantly with age (as a percent of total PC) in normal human prefrontal cortex (n = 29-36). Regression model was adjusted for sex: males (●), females (○). T indicates dependent variable transformed for linear regression, with transformed beta-coefficient and p-value reported on original scatterplot for comparison. Regression parameters are shown in Table 4-3.

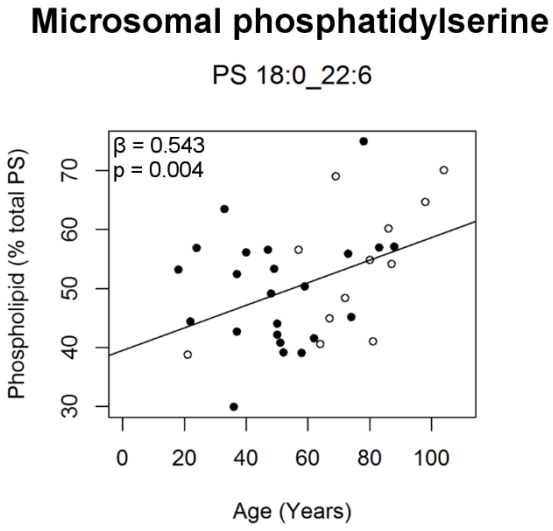


Figure 4-9: Microsomal PSs changing significantly with age (as a percent of total PC) in normal human prefrontal cortex (*n* = 35). Regression model was adjusted for sex: males (●), females (○). Regression parameters are shown in Table 4-3.

4.3 Discussion

This chapter examined age-related changes in the major phospholipids present in both the mitochondrial and microsomal membranes of normal human prefrontal cortex over the adult lifespan. A diverse range of molecular phospholipids were found to undergo changes with age in both membrane fractions, with none of these changes being limited to a particular class of phospholipid.

The largest age-related change for a single molecular phospholipid was seen in PS 18:0_22:6 in both the mitochondrial and microsomal membranes (Figure 4-5 & Figure 4-9). This one phospholipid makes up around 8.3% and 7.5% of the total phospholipid present in mitochondrial and microsomal membranes respectively, and a substantial age-related increase of approximately one third in abundance was found in both membrane fractions. Several other phospholipids containing 22:6 (DHA) also increased with age in both membrane fractions, including mitochondrial PC 16:0_22:6 (Figure 4-3, Table S3) and PE O-18:1_22:6 (Figure 4-4, Table S3), and both mitochondrial and microsomal PE 18:1_22:6 (Figure 4-4 & Figure 4-8; Table 4-1 & Table 4-3).

Significant increases in DHA have been reported previously in early childhood development [70]. DHA is known to incorporate itself preferentially into amino phospholipids such as PE or PS in the brain [190]. The importance of PS containing DHA in neuronal survival has been shown *in vitro* with decreased cell death reported in neuro-2A cells supplemented with both DHA and serine together over DHA alone, indicating that accumulation of DHA in PS is responsible for this protective effect [191]. Further evidence for the importance of DHA in the human brain has been seen in patients with Zellweger syndrome, a peroxisomal disorder where depletion of long chain PUFA such as DHA results in severe brain abnormalities and death within the first year of life [58]. Depletion of DHA in the human frontal cortex has been reported in AD [92,93], but several other studies have also reported no changes in DHA levels with the disease [86,87,108,121].

In the present chapter many phospholipids containing a 20:4 fatty acid decreased significantly in abundance with age in both the mitochondrial and microsomal fractions. Although double bond position cannot be definitively determined by the mass spectrometry technique used in this thesis, 20:4 can be putatively classified as the n-6 PUFA AA as the n-3 isomer has not been detected in any previous studies of the human

brain. The primary AA-containing phospholipid decreasing with age in the mitochondrial membranes was PE 18:0_20:4, a phospholipid which comprises 16% of total PE (Figure 4-2). Mitochondrial PE 18:0_20:4 declined by 25% in abundance across the 80 year period studied (Figure 4-4, Table 4-1). Other less abundant mitochondrial phospholipids containing a AA fatty acid that also declined in abundance with age included PE 16:0_20:4, which showed a 20% loss from ages 20 to 100 (Figure 4-4, Table 4-1), and PS 18:0_20:4 which declined by 82% (Figure 4-5, Table 4-1). Microsomal phospholipids containing AA that decreased with age included PE 16:0_20:4 and PE O-18:1_20:4 (Figure 4-8, Table 4-3). PE 18:0_20:4 showed a trend towards decreasing with age within the microsomal membranes but failed to reach statistical significance (β=-0.416, p=0.051). Interestingly several 22:4-containing phospholipids also decreased in abundance with age, including mitochondrial PE O-16:1_22:4 (34% loss, Figure 4-4, Table 4-1), and microsomal PE O-16:1_22:4 (46% loss), PE 17:0_22:4/O-18:0_22:4 (33% loss), and lyso-PE 22:4 (54% loss, Figure 4-8, Table 4-3). 22:4 can be putatively classified as an n-6 fatty acid (adrenic acid) based on its synthesis via the elongation and desaturation pathway (see Chapter 2 section 2.1.4) [56].

Because of the age-related changes identified for phospholipids containing either DHA, AA or adrenic acid further regressions with age were carried out for both the mitochondrial and microsomal membranes (Table 4-5 & Table 4-6 respectively). Increases with age were seen in the abundance of DHA in the combined phospholipids, which was driven by a rise in PS-DHA in both membrane fractions. In contrast, AA decreased in both mitochondrial and microsomal combined phospholipids, with decreases seen in mitochondrial PE-AA and microsomal PE-AA and PS-AA. Adrenic acid did not show many statistically significant decreases with age in any of the three phospholipid classes in either membrane fraction aside from a reduction in mitochondrial PC. Interestingly, when the two n-6 fatty acids were combined with mitochondrial PC one of the most statistically significant changes in this study was seen (Table 4-5). Previous studies have also reported significant decreases with age in n-6 fatty acids in the human frontal cortex across the adult lifespan [94,95]. Contrary to the present study, Carver et al. [94] reported no changes for DHA with age while McNamara [95] described significant decreases. Other age-related changes reported in

Table 4-5: Linear regression of the most abundant polyunsaturated fatty acids (percent of total fatty acids within phospholipid class) in the mitochondrial membranes with age in human prefrontal cortex

Mitochondrial fatty acids	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Combined phospholipids							
20:4^{IR}	-0.0007	-0.0011	-0.0002	-0.515	2,33	-2.913	0.006**
22:4	-0.0057	-0.0197	0.0083	-0.159	2,33	-0.827	0.414
20:4 + 22:4	-0.0189	-0.0347	-0.0030	-0.424	2,33	-2.425	0.021*
22:6	0.0369	0.0075	0.0663	0.431	2,33	2.554	0.015*
Phosphatidylcholine							
20:4	-0.0077	-0.0227	0.0073	-0.205	2,33	-1.047	0.303
22:4^{L}	-0.0045	-0.0075	-0.0015	-0.518	2,33	-3.049	0.004**
20:4 + 22:4^{LR}	0.0023	0.0013	0.0034	0.676	2,33	4.444	0.00009***
22:6	0.0023	-0.0026	0.0072	0.185	2,33	0.939	0.354
Phosphatidylethanolamine							
20:4^{I}	-0.0003	-0.0005	-0.00002	-0.411	2,33	-2.215	0.034*
22:4	0.0046	-0.0299	0.0391	0.054	2,33	0.274	0.786
20:4 + 22:4	0.00009	-0.00006	0.0002	0.241	2,33	1.237	0.225
22:6	0.0345	-0.0205	0.0895	0.246	2,33	1.279	0.210
Phosphatidylserine							
20:4	-0.0059	-0.0119	0.0002	-0.322	2,33	-1.980	0.056
22:4	0.0261	-0.0070	0.0591	0.301	2,33	1.604	0.118
20:4 + 22:4	-0.0146	-0.0308	0.0015	-0.345	2,30	-1.848	0.074
22:6	0.0803	0.0115	0.1490	0.425	2,30	2.383	0.024*

Fatty acids calculated from phospholipid molecular species data. Regression was adjusted for gender. Superscript indicates dependent variable transformed for linear regression: ^{L} logarithm, ^{LR} reflect and logarithm, ^{I} reciprocal, ^{IR} reflect and reciprocal. * P <0.05 ** P <0.01 *** P <0.001

Table 4-6: Linear regression of most abundant polyunsaturated fatty acids (percent of total fatty acids within phospholipid class) in the microsomal membranes with age in human prefrontal cortex

Microsomal fatty acids	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Combined phospholipids							
20:4	-0.0151	-0.0256	-0.0046	-0.507	2,33	-2.931	0.006**
22:4	0.0071	-0.0026	0.0168	0.285	2,33	1.484	0.147
20:4 + 22:4	-0.0080	-0.0209	0.0049	-0.244	2,33	-1.266	0.215
22:6	0.0464	0.0224	0.0703	0.645	2,33	3.941	0.0004***
Phosphatidylcholine							
20:4	-0.0122	-0.0256	0.0013	-0.350	2,33	-1.844	0.074
22:4	0.0013	-0.0104	0.0131	0.046	2,33	0.233	0.817
20:4 + 22:4^{IR}	-0.0006	-0.0011	-0.0002	-0.492	2,33	-2.760	0.009**
22:6	0.0023	-0.0022	0.0067	0.201	2,33	1.028	0.312
Phosphatidylethanolamine							
20:4^{I}	-0.0003	-0.0005	-0.0002	-0.631	2,32	-3.972	0.0004***
22:4^{IR}	0.0004	0.00004	0.0008	0.348	2,33	1.834	0.076
20:4 + 22:4	-0.0059	-0.0371	0.0252	-0.084	2,31	-0.390	0.699
22:6	0.0294	-0.0037	0.0626	0.375	2,31	1.809	0.080
Phosphatidylserine							
20:4^{I}	-0.0042	-0.0079	-0.0004	-0.413	2,32	-2.239	0.032*
22:4	-0.0096	-0.0350	0.0158	-0.155	2,31	-0.722	0.445
20:4 + 22:4	-0.0181	-0.0445	0.0083	-0.275	2,31	-1.398	0.172
22:6	0.0958	0.0108	0.1807	0.424	2,31	2.297	0.028*

Fatty acids calculated from phospholipid molecular species data. Regression was adjusted for gender. Superscript indicates dependent variable transformed for linear regression: ^{I} reciprocal, ^{IR} reflect and reciprocal. * P <0.05 ** P <0.01 *** P <0.001

the literature includes a loss of total phospholipid in human frontal cortex [88,90,93], a result which was not seen in this study in either the mitochondrial or microsomal membranes. In a later study, Söderberg et al. [93] reported a significant age-related decline in total PC phospholipids in frontal grey matter, a finding which was also not replicated in the present study.

Several significant age-related changes also occurred in highly abundant phospholipids containing saturated and/or monounsaturated fatty acids. The most abundant phospholipid of this type was mitochondrial PC 16:0_18:1, which increased from approximately 41% of total mitochondrial PC phospholipids at age 20 to 44% by 100 years of age (Figure 4-3, Table 4-1). The next most abundant mitochondrial phospholipid showing significant age-related changes was PE 18:0_18:1, which declined by 30% in abundance over the 80 year period studied, followed by PE 16:0_18:1 with a 37% loss. In the microsomal membranes the most abundant phospholipids of this type showing significant changes with age were PE's 18:0_18:1 and 18:1_18:1, both of which contribute approximately 1% each to total phospholipids detected (Figure 4-8, Table 4-3). Both of these microsomal PE phospholipids declined in abundance by 35% and 32% respectively from ages 20 to 100. While these findings are noteworthy, the specific functions of phospholipids containing saturated and/or monounsaturated fatty acids in the human brain are mostly unknown. Current *in vitro* evidence suggests a potential role for such phospholipids as modulators of membrane bilayer fluidity [192,193].

In summary, many different phospholipids changed significantly with age in the mitochondrial and microsomal membranes of normal human prefrontal cortex, and these changes were not confined to a particular class of phospholipid in either membrane fraction. Notable phospholipids increasing over the adult lifespan included mitochondrial and microsomal PS 18:0_22:6 and other phospholipids containing DHA. Decreases were seen in phospholipids containing AA or adrenic acid, including the highly abundant mitochondrial PE 18:0_20:4. While increases in DHA have previously been reported in early childhood, to our knowledge this is first time age-related changes have been reported for individual phospholipids during normal adult ageing in the human brain, as well as being the first report of increases in phospholipids containing DHA over the adult lifespan.

Chapter 5 The Hippocampus

This chapter is an amended version of the published manuscript: Hancock SE, Friedrich MG, Mitchell TW, Truscott, RJW, Else PL, (2015) Decreases are seen in the human hippocampus over the adult lifespan in phospholipids containing adrenic and arachidonic acids, *Lipids*, 50(9);861-872

5.1 Introduction

This chapter will examine the changes seen during normal ageing in PC, PE and PS phospholipids present in the mitochondrial and microsomal membranes of the hippocampus.

The hippocampus is located in the medial temporal lobe of the brain and belongs to the limbic system (see Chapter 1, Figure 1-2). It has important roles in the consolidation of short-term memory to long-term [194,195], as well as spatial navigation [196]. The hippocampus is one of the earliest sites where atrophy is exhibited in the development of AD [197,198]. Less severe atrophy of this region is also seen during normal ageing [199]. The presence of Aβ deposits characteristic of AD within the medial temporal region is thought to accelerate atrophy within the hippocampus and other brain structures of the medial temporal region [200]. Additionally, the hippocampus is believed to be one of the initial sites of the development of NFTs [7].

The phospholipids of the hippocampus have been examined for their role in the pathogenesis of AD in several studies over the past few decades [86,111,113,121–123], but very few studies have examined the changes occurring in hippocampal phospholipids during normal ageing [90,93]. Decreases in total phospholipids with age have been reported after age 60 in the hippocampus [90], alongside reductions in total PC and PE [93]. No age-related changes have previously been reported for the fatty acids of either PC or PE phospholipids in the human hippocampus [93].

Therefore, the aim of this chapter was to expand on the current knowledge of the changes occurring to the PC, PE and PS phospholipids of the human hippocampus during normal ageing.

5.2 Results

5.2.1 Age-related changes to total protein content in subcellular fractions

Statistically significant age-related declines were seen in the total protein content of the microsomal fraction of the hippocampus, but no changes with age were found for either the whole tissue homogenate or mitochondrial fraction (Figure 5-1, Table 5-1). Total protein decreased in the microsomal membranes from 2.5 µg/mg wet weight of tissue to 1.9 µg/mg wet weight of tissue from ages 20 to 100, representing a decrease of 48%. A trend was seen for a decline in total protein with age within the whole tissue homogenate, but this failed to reach statistical significance ($\beta = -0.356$, $p = 0.055$).

5.2.2 Major phospholipid classes of mitochondrial and microsomal fractions

The ratio of the three major phospholipid classes PC, PE and PS showed a similar distribution between the mitochondrial and microsomal membranes (Figure 5-2). Overall, PC comprised 43%, PE 36% and PS 21% of total phospholipids measured in each fraction with some small differences in phospholipid content between the two membrane fractions. A slightly higher percentage of PE was present in the mitochondria ($p < 0.001$) while the microsomes contained more PC ($p < 0.001$). There were no differences in the percent abundance of PS between the two membrane fractions. The percent contribution of total PC, PE, PS, and total phospholipids were also examined for any age-related changes in the mitochondrial and microsomal fractions, with no significant age-related changes seen.

The quantified amount of each phospholipid class (as pmol/µg membrane protein) between the two membrane fractions was also analysed, with the microsomal fraction found to contain higher amounts of PC ($p < 0.001$) and PS ($p = 0.005$). Total quantified phospholipids were also greater in the microsomes than in the mitochondria ($p = 0.001$). Regression of quantified phospholipids (pmol/µg membrane protein) with age showed no statistically significant age-related changes for any phospholipid class in either membrane fraction, including total quantified phospholipids.

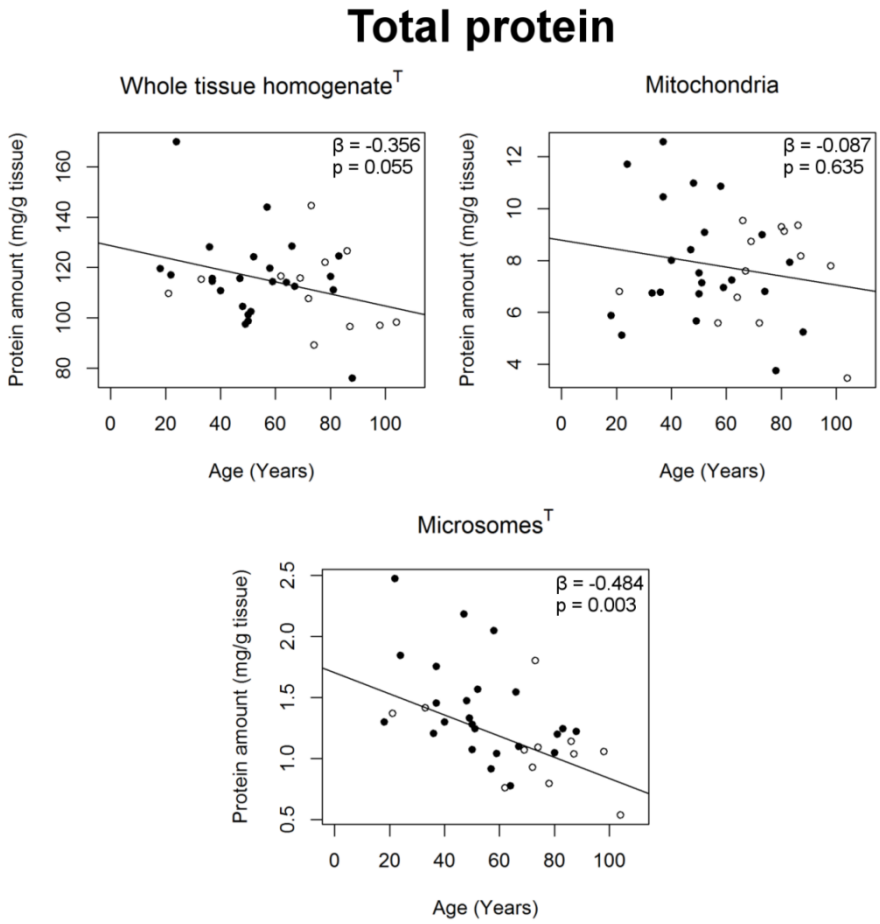


Figure 5-1: Linear regression of age against total protein amount (mg/g wet tissue) measured in whole tissue homogenate and subcellular fractions derived from normal human hippocampus. Total protein was measured using BCA assay as described in methods and materials. Regression model was adjusted for sex: males (●), females (○). ^{T} indicates dependent variable transformed for linear regression, with transformed beta-coefficient and p-value reported on original scatterplot for comparison. Regression parameters are shown in Table 5-1.

Table 5-1: Linear regression of total protein (mg/g of tissue) with age in the whole tissue homogenate and subcellular fractions of normal human hippocampus

Phospholipid	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Whole tissue homogenate[I]	-0.00002	-0.00004	-0.0000004	-0.356	2,33	-1.994	0.055
Mitochondria	-0.0082	-0.0430	0.0267	-0.087	2,33	-0.479	0.635
Microsomes[L]	-0.0016	-0.0027	-0.0006	-0.484	2,33	-3.164	0.003**

Total protein amount measured using BCA Assay. Regression was adjusted for gender. Superscript indicates dependent variable transformed for linear regression, with transformed regression output reported: [L] logarithm ** P <0.01

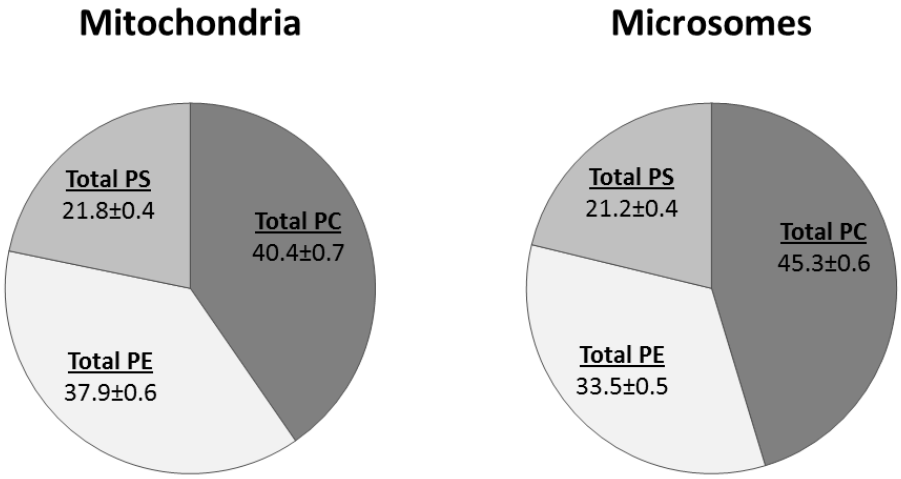


Figure 5-2: Percent composition of the three major phospholipid classes within the mitochondrial (left) and microsomal (right) fractions in normal human hippocampus. Values are the mean ± SEM for the entire cohort. The microsomal membranes showed significantly higher amounts of total phosphatidylcholine (PC) compared to mitochondria ($p < 0.001$, Wilcoxon signed-rank test), while the mitochondria contained more phosphatidylethanolamine (PE) ($p < 0.001$, Wilcoxon signed-rank test). There were no differences between fractions for phosphatidylserine (PS).

5.2.3 Changes in mitochondrial phospholipids with age

5.2.3.1 Phosphatidylcholines

The composition of PC, PE and PS phospholipids detected in the mitochondrial membranes of the hippocampus can be seen in (Figure 5-3).Three mitochondrial PCs changed with age in the human hippocampus (Figure 5-4, Table 5-2). PC 16:0_18:1, the most abundant phospholipid present in mitochondrial membranes, increased from 40.8% of total mitochondrial PC at age 20 to 42.5% at age 100, representing a 4% increase from its initial abundance. PC 16:0_18:2 also increased in abundance with age, but this phospholipid is only present in low-to-moderate amounts within mitochondrial membranes (Figure 5-3). The only mitochondrial PC to decrease with age was PC 18:0_20:4, which declined in abundance by 24% from ages 20 to 100 (Figure 5-4, Table 5-2).

Regression of quantified phospholipids (pmol phospholipid/µg total protein) with age was also performed, with no mitochondrial PCs showing any statistically significant age-related changes (Table 5-3).

5.2.3.2 Phosphatidylethanolamines

Age-related changes were seen in several mitochondrial PEs (Figure 5-5, Table 5-2). PE 18:0_22:4 was the most abundant mitochondrial PE to decrease with age, declining by 13% in abundance over the 80 year period. This phospholipid is the third-most abundant mitochondrial PE, comprising approximately 8% of total mitochondrial PE (Figure 5-3) and 3% of total mitochondrial phospholipid. Three other mitochondrial PEs decreased with age: PE 16:0_22:4, PE O-16:1_20:2 and PE O-18:1_22:3 (Figure 5-5, Table 5-2). PE 16:0_22:4 is a moderately abundant mitochondrial PE, which contributes up to 1.5% of mitochondrial PE (Figure 5-3) and 0.6% of total mitochondrial phospholipid. This phospholipid decreased by 19% in abundance from ages 20 to 100 (Figure 5-5, Table 5-2). The remaining phospholipid to decline with age, PE O-16:1_20:2, is only of low abundance in the mitochondrial membranes, comprising just over 0.4% of total mitochondrial phospholipid.

Three mitochondrial PEs increased in percent abundance over the 80 year period: PE 18:1_22:6 lyso-PE 22:6 and lyso-PE 18:1. PE 18:1_22:6 is a moderately abundant

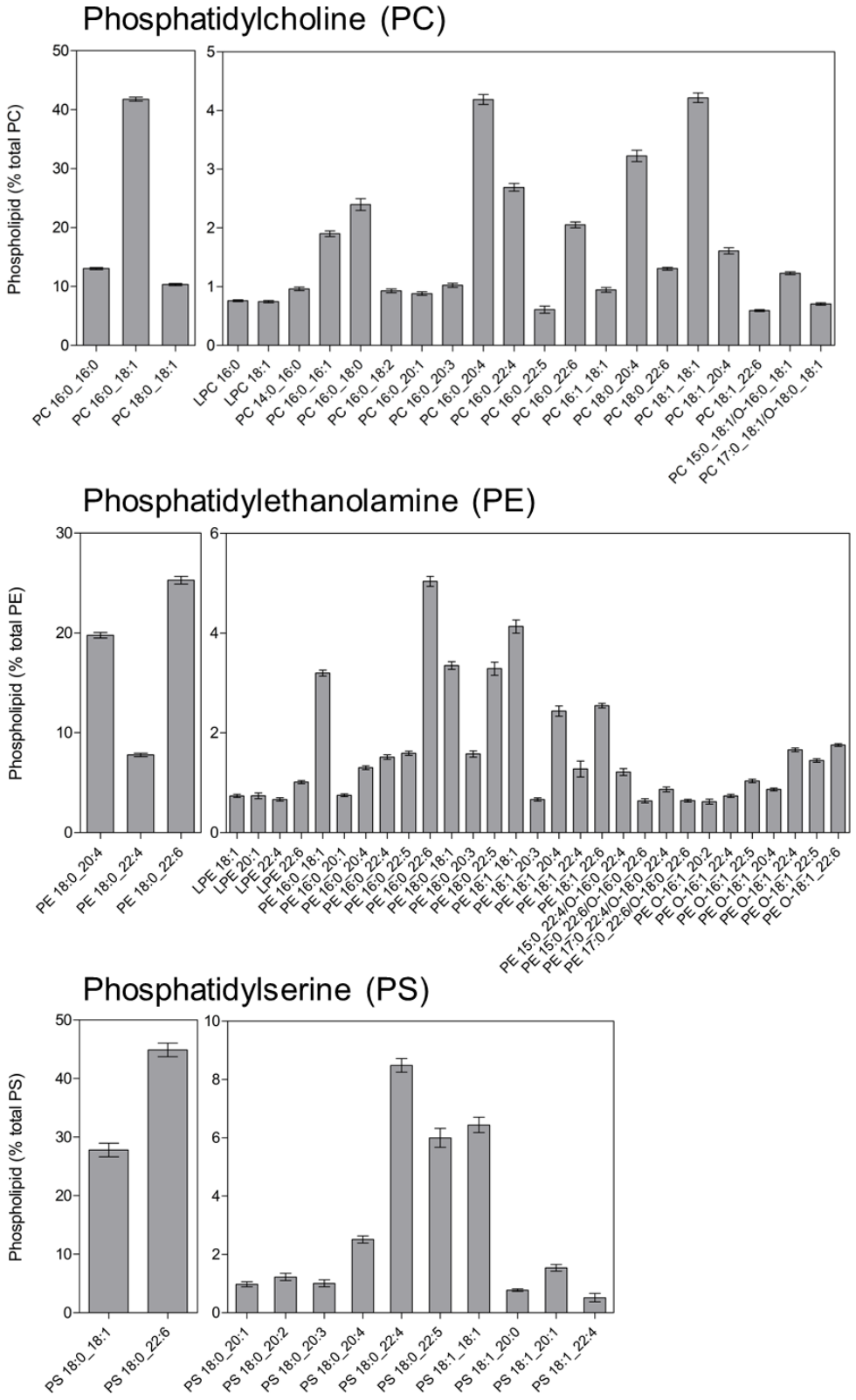


Figure 5-3: Phospholipids detected within PC, PE and PS in the mitochondrial fraction of human hippocampus (as a percent of total phospholipid within each class). Phospholipids were quantified as described in materials and methods. Values are mean across the cohort ± SEM.

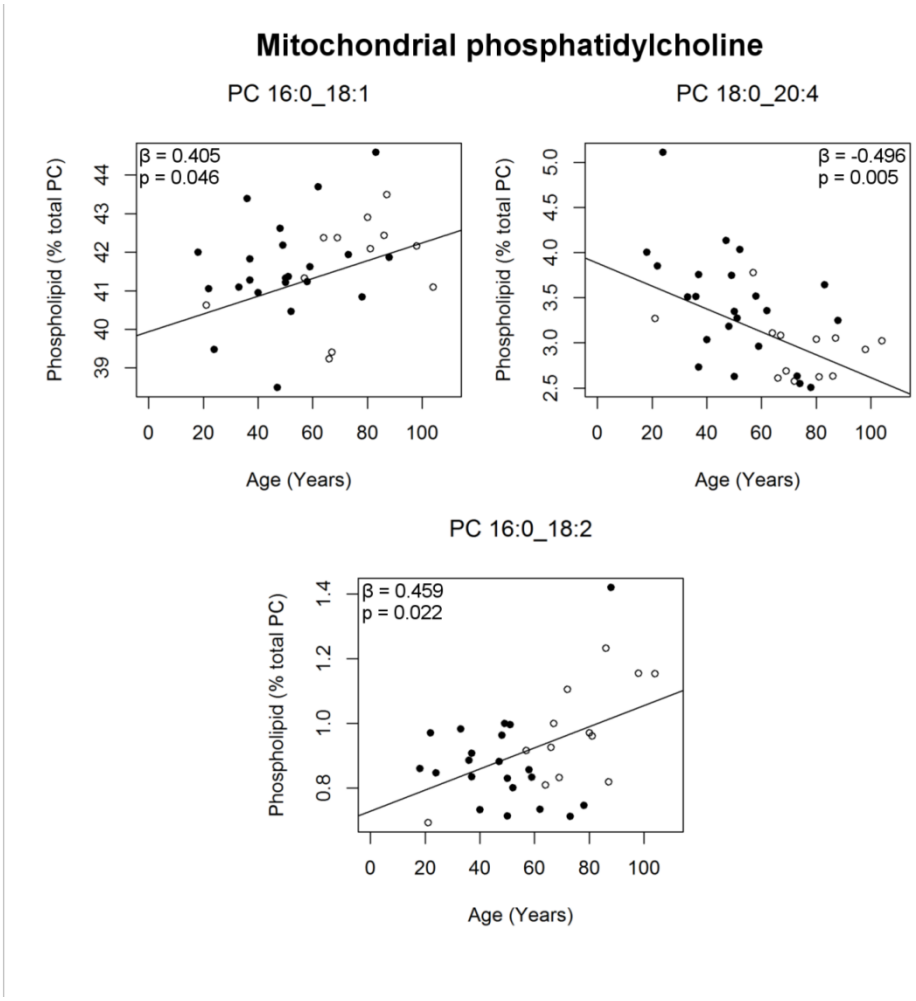


Figure 5-4: Mitochondrial PCs changing significantly with age (as a percent of total PC) in normal human hippocampus (n = 33-36). Regression model was adjusted for gender: males (●), females (○). Regression parameters are shown in Table 5-2.

Table 5-2: Mitochondrial phospholipids (percent of phospholipid within phospholipid class) changing significantly with age in the human hippocampus.

Phospholipid	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Phosphatidylcholine							
PC 16:0_18:1	0.0230	0.0005	0.0456	0.405	2,31	2.083	0.046*
PC 16:0_18:2	0.0033	0.0005	0.0060	0.459	2,31	2.416	0.022*
PC 18:0_20:4	-0.0127	-0.0211	-0.0042	-0.496	2,33	-3.049	0.005**
Phosphatidylethanolamine							
Lyso-PE 18:1	0.0034	0.0005	0.0062	0.438	2,33	2.411	0.022*
Lyso-PE 22:6	0.0037	0.0005	0.0069	0.431	2,33	2.338	0.026*
PE 16:0_22:4	-0.0039	-0.0071	-0.0007	-0.453	2,30	-2.510	0.018*
PE 18:0_22:4^{I}	-0.0002	-0.0004	-0.00002	-0.371	2,33	-2.221	0.033*
PE 18:1_22:6	0.0074	0.0040	0.0108	0.668	2,32	4.442	0.0001***
PE O-16:1_20:2	-0.0055	-0.0098	-0.0013	-0.502	2,29	-2.654	0.013*
Phosphatidylserine							
PS 18:0_20:1^{L}	-0.0019	-0.0039	-0.00001	-0.383	2,32	-2.048	0.0489*
PS 18:0_20:4	-0.0161	-0.0253	-0.0070	-0.614	2,30	-3.589	0.0016**
PS 18:0_22:4	-0.0186	-0.0362	-0.0009	-0.381	2,32	-2.138	0.040*
PS 18:0_22:6	0.1167	0.0014	0.2320	0.384	2,32	2.062	0.047*
PS 18:1_20:0	-0.0049	-0.0094	-0.0005	-0.416	2,32	-2.259	0.031*

Phospholipid molecular species were detected using nanoelectrospray ionisation mass spectrometry and quantified as described in materials and methods. Regression was adjusted for gender. Superscript indicates dependent variable transformed for linear regression, with transformed regression output reported: ^{L} logarithm, ^{I} reciprocal. * P <0.05 ** P <0.01 *** P <0.001

Table 5-3: Quantified mitochondrial phospholipids (pmol/µg total membrane protein) changing significantly with age in the human hippocampus.

Phospholipid	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Phosphatidylethanolamine							
PE O-16:1_20:2	-0.0097	-0.0158	-0.0035	-0.588	2,29	-3.200	0.003**
Phosphatidylserine							
PS 18:0_18:1^{L}	-0.0024	-0.0045	-0.0002	-0.427	2,32	-2.266	0.030*
PS 18:0_20:1	-0.0111	-0.0170	-0.0053	-0.654	2,31	-3.864	0.0005***
PS 18:0_20:2	-0.0116	-0.0206	-0.0025	-0.497	2,24	-2.630	0.015*
PS 18:0_20:4	-0.0278	-0.0407	-0.0149	-0.709	2,30	-4.403	0.0001***
PS 18:1_20:0	-0.0052	-0.0103	-0.0001	-0.394	2,32	-2.097	0.044*

Phospholipid molecular species were detected using nanoelectrospray ionisation mass spectrometry and quantified as described in materials and methods. Regression was adjusted for gender. Superscript indicates dependent variable transformed for linear regression, with transformed regression output reported: ^{L} logarithm.
* P <0.05 ** P <0.01 *** P <0.001

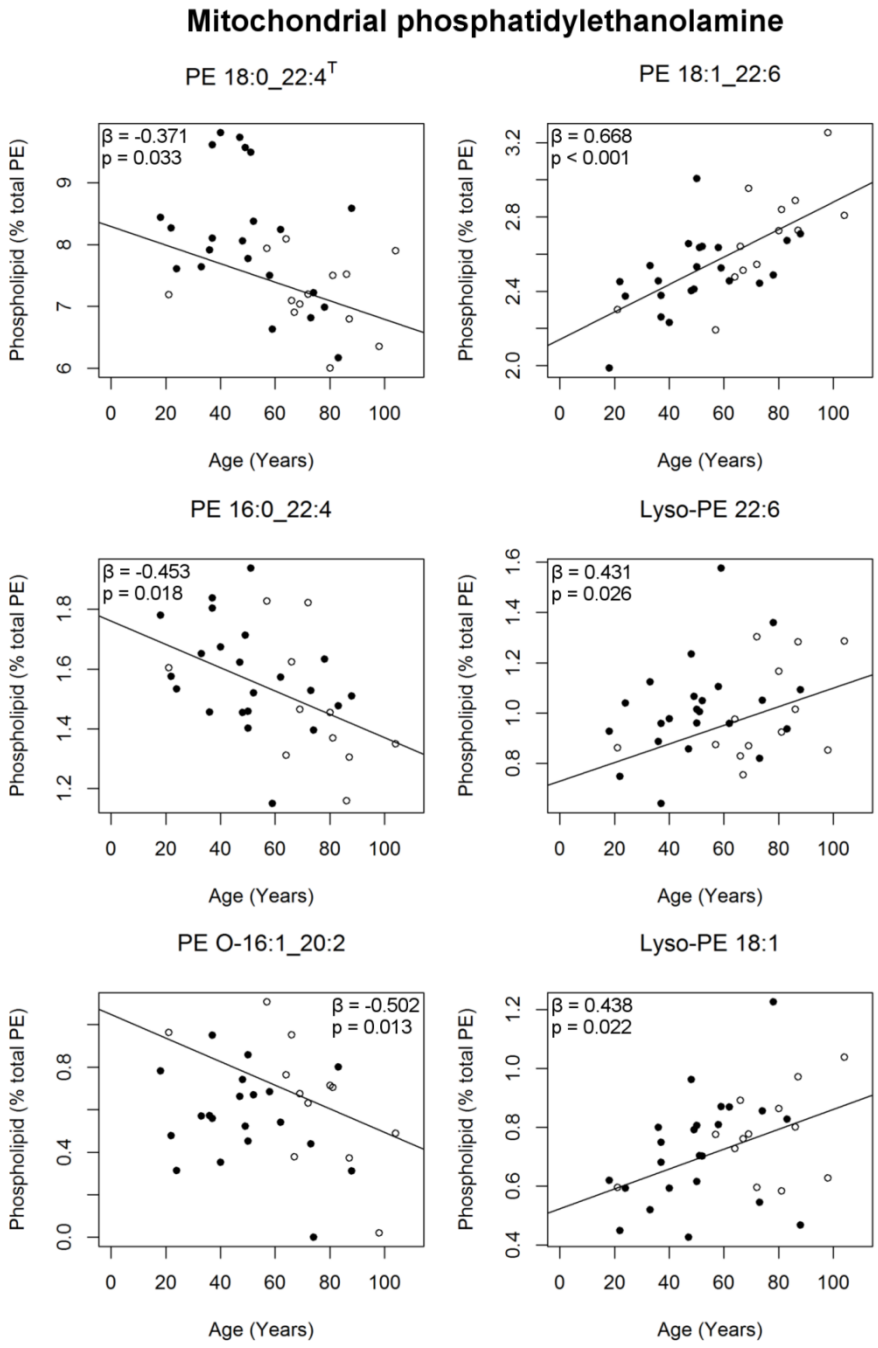


Figure 5-5: Mitochondrial PEs changing significantly with age (as a percent of total PE) in normal human hippocampus (n = 32-36). Regression model was adjusted for sex: males (●), females (○). ^{T} indicates dependent variable transformed for linear regression, with transformed beta-coefficient and p-value reported on original scatterplot for comparison. Regression parameters are shown in Table 5-2.

phospholipid in the mitochondrial membranes, comprising approximately 1% of total mitochondrial phospholipids. PE 18:1_22:6 increased from 2.3% of total mitochondrial PE to 2.9% from ages 20 to 100, representing an increase in abundance of 26%. The two other lyso-PEs showing increases with age, lyso-PE 22:6 and lyso-PE 18:1, comprised less than 1.7% of total mitochondrial PE when combined (Figure 5-3).

Analysis of the quantified phospholipids (pmol phospholipid/µg membrane protein) showed similar trends for all mitochondrial PEs identified as changing significantly in their percent abundance with age, but only a single mitochondrial PE reached statistical significance: PE O-16:1_20:2 (Table 5-3). PE O-16:1_20:2 decreased with age from 1.150 pmol/µg membrane protein to 0.374 pmol/µg membrane protein from ages 20 to 100.

5.2.3.3 Phosphatidylserines

Within the mitochondrial membranes, five PS phospholipids changed in percent abundance with age (Figure 5-6, Table 5-2). The most abundant phospholipid to show an age-related change was PS 18:0_22:6, which increased from 37.0% of total mitochondrial PS to 46.3% from ages 20 to 100. The remaining four phospholipids all decreased with age in the mitochondrial membranes. PS 18:0_22:4, a moderately abundant mitochondrial phospholipid, declined by 17% over the 80 year period (Figure 5-6, Table 5-2). PS 18:0_20:4, PS 18:0_20:1 and PS 18:1_20:0 are all low abundance mitochondrial phospholipids, and decreased respectively by 39%, 30% and 35% from age 20 to 100.

Three of these mitochondrial PSs also declined significantly in quantified amount (pmol phospholipid/µg membrane protein) with age: PS 18:0_20:4, PS 18:0_20:1 and PS 18:1_20:0 (Table 5-3). A further two mitochondrial PS also decreased significantly in quantified amount with age, PS 18:0_18:1 and PS 18:0_20:2.

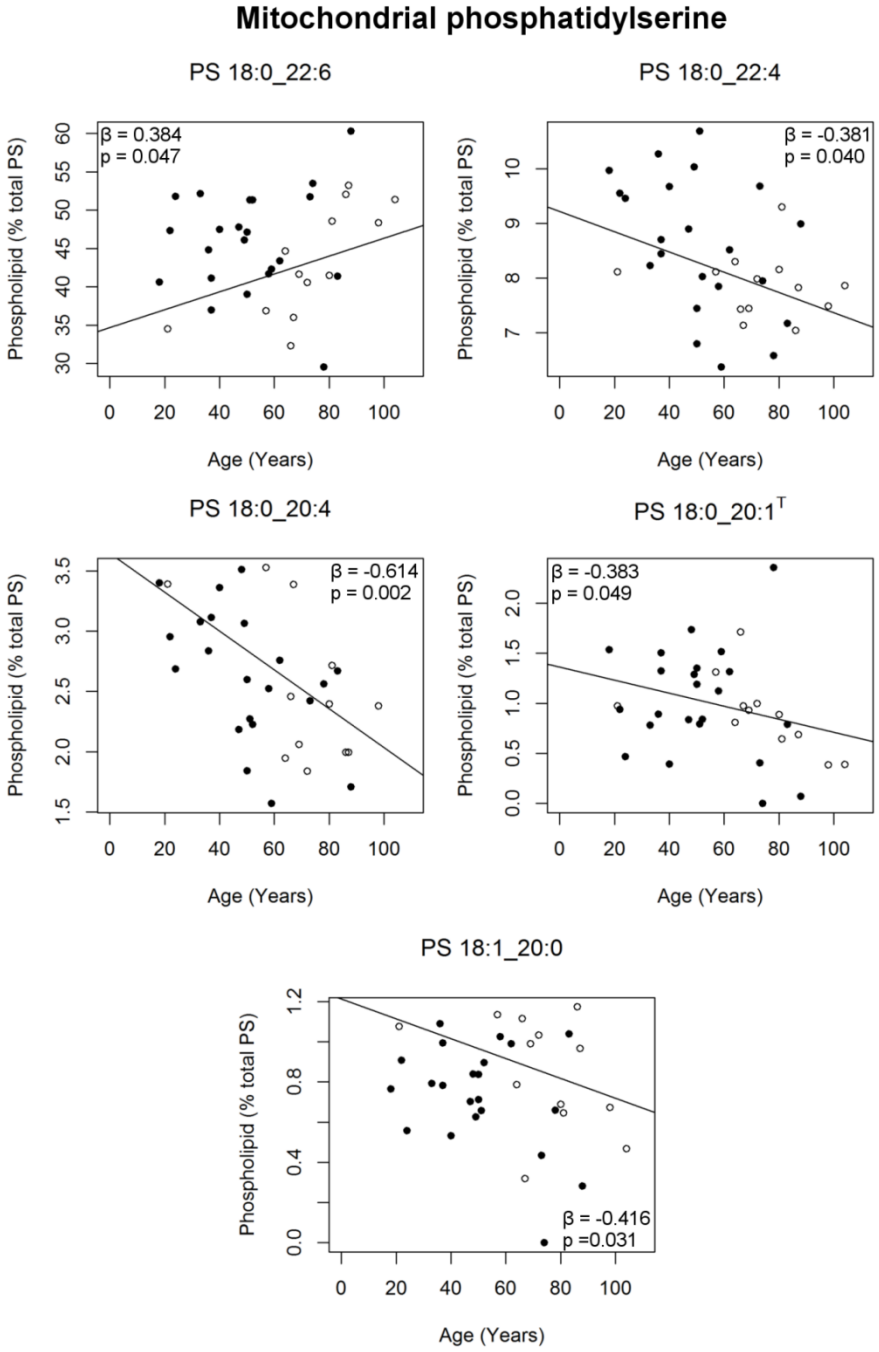


Figure 5-6: Mitochondrial PSs changing significantly with age (as a percent of total PS) in normal human hippocampus (n = 33-35). Regression model was adjusted for sex: males (●), females (○). ^{T} indicates dependent variable transformed for linear regression, with transformed beta-coefficient and p-value reported on original scatterplot for comparison. Regression parameters are shown in Table 5-2.

5.2.4 Changes in microsomal phospholipids with age

5.2.4.1 Phosphatidylcholines

The composition of PC, PE and PS phospholipids detected in the microsomal membranes of the hippocampus can be seen in (Figure 5-7).Two microsomal PCs changed with age (Figure 5-8, Table 5-4). Decreases with age were observed in PC 18:0_18:1, the third most abundant microsomal PC. PC 18:0_18:1 comprises 10% of total microsomal PC (Figure 5-7) and 4% of total microsomal phospholipids, and declined by 15% from ages 20 to 100 (Figure 5-8, Table 5-4). An age-related increase of 35% in abundance was found for PC 15:0_16:0/O-16:0_16:0 (Figure 5-8, Table 5-4), but this phospholipid comprises only 0.2% of total microsomal phospholipid.

PC 15:0_16:0/O-16:0_16:0 also increased in quantified amount with age within the microsomal membranes, doubling from 0.78 pmol/µg membrane protein at age 20 to 1.6 pmol/µg membrane protein by age 100 (Table 5-5).

5.2.4.2 Phosphatidylethanolamines

Three microsomal PEs changed with age in their percent composition (percent of total microsomal PE) in the hippocampus (Figure 5-9, Table 5-4). The most abundant microsomal PE showing age-related changes was PE 18:0_22:4, which decreased by 18% in abundance across the 80 year period. PE 18:0_22:4 is the third most abundant microsomal PE, comprising 8% of total microsomal PE (Figure 5-7). The two remaining microsomal PEs increased significantly in percent abundance with age. PE 18:1_22:6, a moderately abundant microsomal PE, increased from 1.9% of microsomal PE to 2.5% from ages 20 to 100, representing an increase of 27% in abundance (Figure 5-9, Table 5-4). PE 15:0_22:6/O-16:0_22:6 rose by a little over 70% across the 80 year period, but this phospholipid is only of low abundance in microsomal membranes (Figure 5-7). PE 15:0_22:6/O-16:0_22:6 also increased with age in quantified amount, rising from 0.8 pmol/µg membrane protein at age 20 to 1.5 pmol/µg membrane protein at age 100 (Table 5-5).

5.2.4.3 Phosphatidylserines

Only a single PS phospholipid changed in its percent composition with age in the microsomal membranes: PS 18:0_20:4 (Figure 5-10, Table S3). PS 18:0_20:4 decreased from 3.3% of microsomal PS to 2.3% over the 80 year period studied, representing a loss of one-third in abundance.

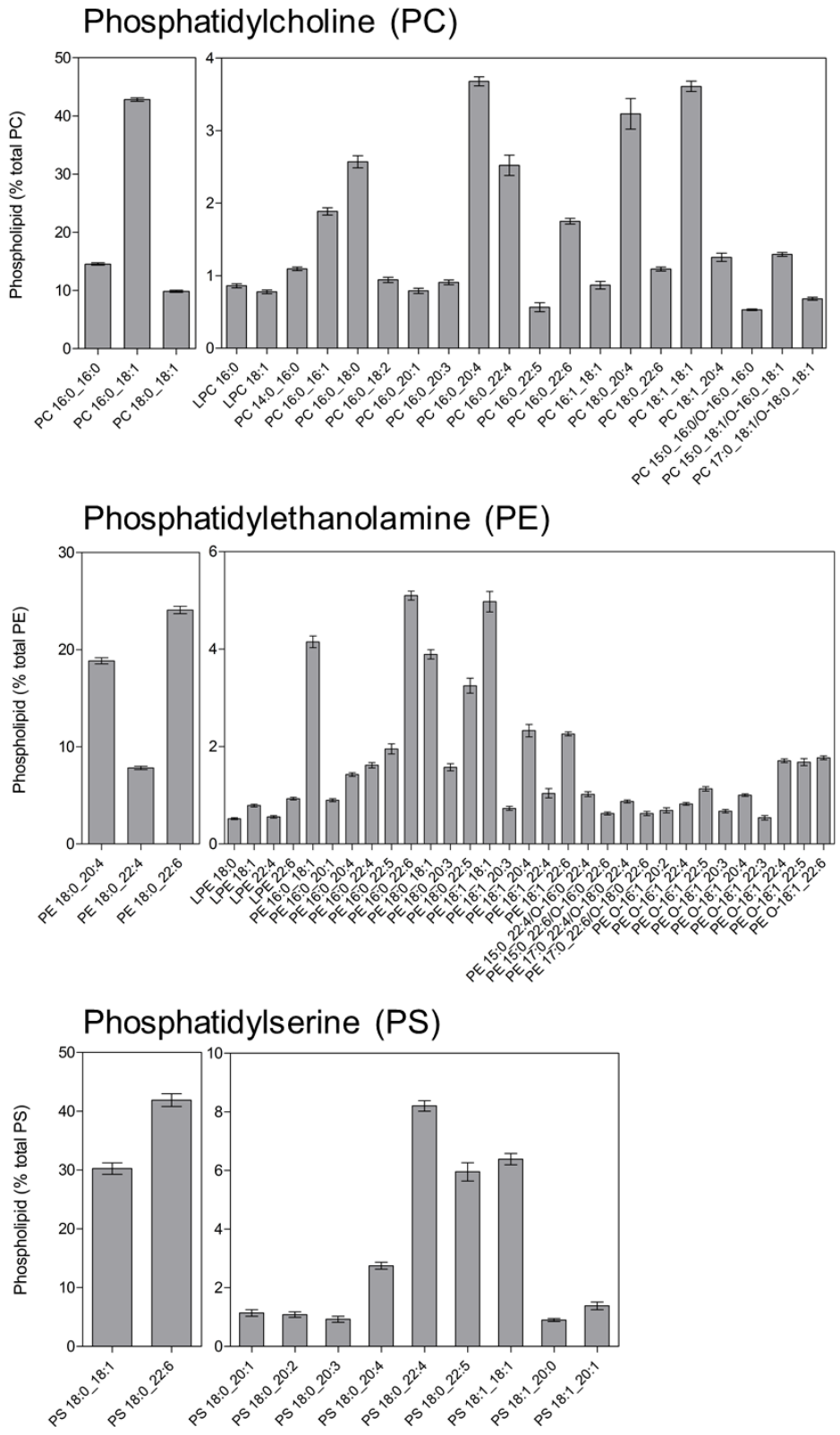


Figure 5-7: Phospholipids detected within PC, PE and PS in the microsomal fraction of human hippocampus (as a percent of total phospholipid with each class). Phospholipids were quantified as described in materials and methods. Values are mean across the cohort ± SEM.

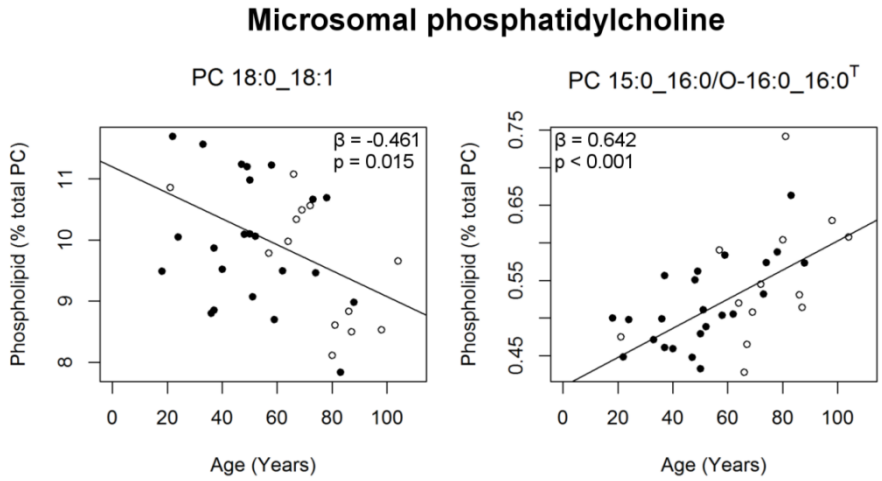


Figure 5-8: Microsomal PCs changing significantly with age (as a percent of total PC) in normal human hippocampus (n = 36). Regression model was adjusted for sex: males (●), females (○). ^{T} indicates dependent variable transformed for linear regression, with transformed beta-coefficient and p-value reported on original scatterplot for comparison. Regression parameters are shown in Table 5-4.

Table 5-4: Microsomal phospholipids (percent of phospholipid within phospholipid class) changing significantly with age in the human hippocampus.

Phospholipid	**Slope**	**95% CI**		**β**	**df**	**t**	***P* value**
		Lower	**Upper**				
Phosphatidylcholine							
PC 18:0_18:1	-0.0212	-0.0380	-0.0044	-0.461	2,33	-2.566	0.015*
PC 15:0_16:0 /O-16:0_16:0^{I}	0.0008	0.0004	0.0012	0.642	2,33	4.059	0.0003***
Phosphatidylethanolamine							
PE 18:0_22:4	-0.0190	-0.0360	-0.0020	-0.396	2,33	-2.275	0.030*
PE 18:1_22:6	0.0065	0.0027	0.0104	0.588	2,32	3.443	0.0016**
PE 15:0_22:6 /O-16:0_22:6	0.0036	0.0006	0.0067	0.460	2,31	2.460	0.020*
Phosphatidylserine							
PS 18:0_20:4	-0.0123	-0.0239	-0.0008	-0.426	2,29	-2.184	0.037*

Phospholipid molecular species were detected using nanoelectrospray ionisation mass spectrometry and quantified as described in materials and methods. Regression was adjusted for gender. Superscript indicates dependent variable transformed for linear regression, with transformed regression output reported: ^{I} reciprocal. * P <0.05 ** P <0.01 *** P <0.001

Table 5-5: Quantified microsomal phospholipids (pmol/µg total membrane protein) changing significantly with age in the human hippocampus.

Phospholipid	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Phosphatidylcholine							
PC 14:0_16:0^{L}	0.0019	0.00006	0.0038	0.382	2,33	2.102	0.043*
PC 16:0_18:2	0.0195	0.0057	0.0332	0.491	2,30	2.889	0.006**
PC 18:1_18:1	0.0017	0.00005	0.0034	0.363	2,33	2.098	0.044*
PC 15:0_16:0 /O-16:0_16:0	0.0107	0.0030	0.0183	0.483	2,33	2.835	0.003**
PC 15:0_18:1 /O-16:0_18:1^{I}	0.0013	0.0002	0.0025	0.439	2,31	2.324	0.027*
Phosphatidylethanolamine							
PE 18:1_20:3^{I}	0.0021	-0.0006	0.0037	0.522	2,29	2.922	0.007**
PE 15:0_22:6 /O-16:0_22:6	0.0090	0.0005	0.0174	0.405	2,31	2.151	0.039*
PE O-18:1_22:5	0.0228	0.0010	0.0446	0.398	2,33	2.128	0.041*

Phospholipid molecular species were detected using nanoelectrospray ionisation mass spectrometry and quantified as described in materials and methods. Regression was adjusted for gender. Superscript indicates dependent variable transformed for linear regression, with transformed regression output reported: ^{L} logarithm, ^{I} reciprocal. * *P* <0.05 ** *P* <0.01 *** *P* <0.001

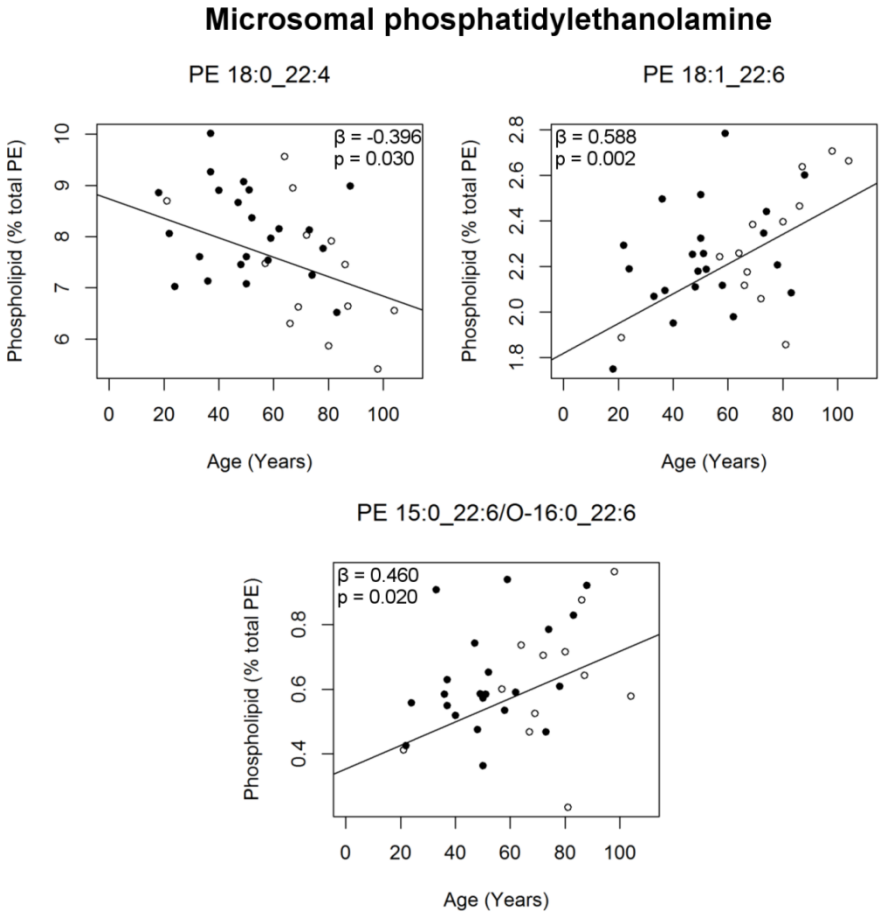


Figure 5-9: Microsomal PEs changing significantly with age (as a percent of total PE) in normal human hippocampus (n = 34-36). Regression model was adjusted for sex: males (●), females (○). Regression parameters are shown in Table 5-4.

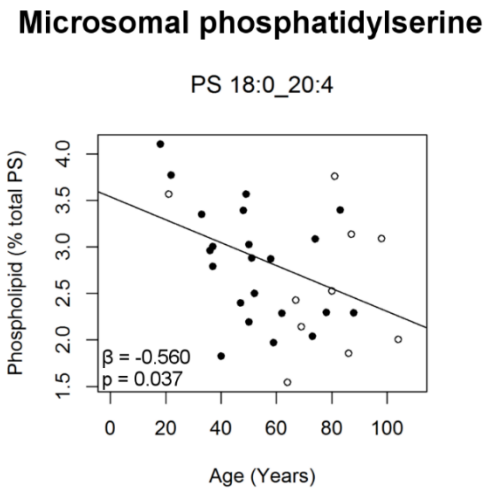


Figure 5-10: Microsomal PSs changing significantly with age (as a percent of total PS) in normal human hippocampus (n = 32). Regression model was adjusted for sex: males (●), females (○). Regression parameters are shown in Table 5-4.

There were no microsomal PSs that changed significantly in quantified amount over the 80 year period (pmol phospholipid/µg membrane protein, Table 5-5).

5.3 Discussion

Examination of the mitochondrial and microsomal membranes of the human hippocampus showed that many phospholipids underwent significant changes during normal ageing. These changes were not limited to any single class of phospholipid or a single membrane fraction. Rather, the age-related changes in the hippocampus occurred in a broad range of phospholipids in both the mitochondrial and microsomal membranes, with many of these changes occurring in low-to-moderately abundant phospholipids. In particular, phospholipids containing a 22:4 fatty acid (adrenic acid) underwent consistent age-related decreases in both membrane fractions across the 80 year period studied, while those containing a 22:6 fatty acid (DHA) increased across the adult lifespan.

PE 18:0_22:4 is the third most abundant PE in both the mitochondria and microsomes (Figure 5-3 & Figure 5-7), and a decrease of approximately 13% and 18% in percent abundance with age was found in both membranes fractions respectively (Figure 5-5 & Figure 5-9, Table 5-2 & Table 5-4). Several other phospholipids with a 22:4 fatty acid also declined in percent abundance with age in the mitochondrial membranes: PE 16:0_22:4 and PS 18:0_22:4 declined by 19% and 17% respectively from ages 20 to

100 (Figure 5-5 & Figure 5-6, Table 5-2). The 22:4 fatty acid can be putatively assigned as being adrenic acid, an n-6 PUFA, based on its synthesis via the elongation and desaturation pathway (see Chapter 2, Figure 2-4) [56]. Adrenic acid is the product of elongation of the n-6 PUFA 20:4 (AA) by two carbons. Two isomers of 20:4 can exist: an n-3 and an n-6 isomer (AA) which differ only by the position of their double bonds. As discussed in Chapter 4 (section 4.3), the mass spectrometry method used in this thesis cannot determine double bond position. However, previous studies of fatty acids within the phospholipids of human hippocampus that used gas chromatography have not detected any n-3 20:4, so we can assume that the 20:4 detected is primarily AA [90,93,122,123]. Many phospholipids containing AA also decreased with age in the mitochondrial and microsomal membranes. Common between both membrane fractions was a decline in the percent abundance of PS 18:0_20:4 with age, by 39% and 30% in mitochondrial and microsomal membranes respectively (Figure 5-6 & Figure 5-10, Table 5-2 & Table 5-4). Age-related decreases were also seen in mitochondrial PC 18:0_20:4, which declined by 24% over the 80 year period (Figure 5-4, Table 5-2).

Due to these age-related declines in mitochondrial and microsomal phospholipids containing the n-6 fatty acids adrenic acid or AA, additional regressions were conducted to check for changes in fatty acid content with age in the hippocampus (Table 5-6 & Table 5-7). Decreases with age in AA were seen in the combined phospholipids of the mitochondrial membranes (Table 5-6). When phospholipids containing either AA or adrenic acid were pooled together, an even greater loss with age was revealed in the combined mitochondrial phospholipids. In the microsomal membranes, only the pooled AA and adrenic acid declined with age in the combined phospholipids (Table 5-7). Together, these results indicate that there is a significant loss of long-chain n-6 fatty acids in both membrane fractions within the hippocampus over the adult lifespan. Despite significant age-related losses of PE 18:0_22:4 in both membrane fractions (Figure 5-5 & Figure 5-9) there were no changes seen with age to either PE-adrenic acid or to pooled PE-AA and PE-adrenic acid in either the mitochondrial or microsomal membranes (Table 5-6 & Table 5-7). Instead most of the age-related declines in adrenic

Table 5-6: Linear regression of most abundant polyunsaturated fatty acids (percent of total fatty acids within phospholipid class) in the mitochondrial membranes with age in human hippocampus

Mitochondrial fatty acids	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Combined phospholipids							
20:4^{LR}	-0.0022	-0.0044	-0.00003	-0.385	2,33	-2.065	0.047*
22:4	-0.0084	-0.0178	-0.0009	-0.324	2,33	-1.835	0.076
20:4 + 22:4 ^{LR}	-0.0038	-0.0070	-0.0006	-0.429	2,33	-2.443	0.020*
22:6	0.0211	-0.0065	0.0486	0.299	2,33	1.556	0.129
Phosphatidylcholine							
20:4	-0.0077	-0.0165	0.0010	-0.320	2,32	-1.794	0.082
22:4	0.0003	-0.0033	0.0038	0.028	2,33	0.146	0.885
20:4 + 22:4	-0.0071	-0.0178	0.0037	-0.243	2,32	-1.341	0.189
22:6	0.0006	-0.0036	0.0047	0.057	2,33	0.289	0.775
Phosphatidylethanolamine							
20:4	-0.0068	-0.0247	0.0110	-0.151	2,33	0.781	0.440
22:4	-0.0127	-0.0298	0.0044	-0.285	2,33	0.140	0.140
20:4 + 22:4	-0.0195	-0.0437	0.0047	-0.315	2,33	-1.642	0.110
22:6	0.0245	-0.0021	0.0512	0.356	2,33	1.877	0.069
Phosphatidylserine							
20:4^{LR}	-0.0081	-0.0127	-0.0035	-0.614	2,30	-3.589	0.001**
22:4^{I}	-0.0004	0.0008	-0.00001	-0.352	2,33	-1.969	0.057
20:4 + 22:4^{L}	-0.0015	-0.0025	-0.0004	-0.494	2,33	-2.869	0.007**
22:6	0.0804	0.0302	0.1305	0.568	2,31	3.265	0.003**

Fatty acids calculated from phospholipid molecular species data. Regression was adjusted for gender. Superscript indicates dependent variable transformed for linear regression: ^{L} logarithm, ^{LR} reflect and logarithm, ^{I} reciprocal. * P <0.05 ** P <0.01

Table 5-7: Linear regression of most abundant polyunsaturated fatty acids (percent of total fatty acids within phospholipid class) in the microsomal membranes with age in human hippocampus

Microsomal fatty acids	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Combined phospholipids							
20:4	-0.0060	-0.0169	0.0049	-0.201	2,33	-1.125	0.269
22:4	-0.0103	-0.0208	-0.0003	-0.363	2,33	-1.986	0.055
20:4 + 22:4	-0.0163	-0.0300	-0.0026	-0.395	2,33	-2.249	0.021*
22:6	-0.0014	-0.0250	0.0223	-0.023	2,32	-0.118	0.907
Phosphatidylcholine							
20:4	-0.0012	-0.0165	0.0141	-0.031	2,33	-0.164	0.871
22:4	-0.0035	-0.0147	-0.0078	-0.124	2,33	-0.626	0.536
20:4 + 22:4	-0.0047	-0.0131	0.0037	-0.195	2,33	-1.134	0.265
22:6	-0.0004	-0.0042	0.0034	-0.046	2,33	-0.232	0.818
Phosphatidylethanolamine							
20:4	-0.0071	-0.0255	0.0113	-0.154	2,32	-0.783	0.440
22:4	-0.0131	-0.0309	0.0047	-0.287	2,33	-1.150	0.143
20:4 + 22:4	-0.0173	-0.0407	0.0061	-0.292	2,32	-1.504	0.142
22:6	0.0025	-0.0254	0.0305	0.037	2,32	0.186	0.854
Phosphatidylserine							
20:4	-0.0004	-0.0097	0.0089	-0.016	2,33	-0.087	0.931
22:4	-0.0095	-0.0189	-0.0001	-0.373	2,30	-2.072	0.047*
20:4 + 22:4	-0.0112	-0.0265	-0.0041	-0.260	2,31	-1.492	0.146
22:6	-0.0394	-0.0173	0.0961	0.274	2,33	1.413	0.167

Fatty acids calculated from phospholipid molecular species data. Regression was adjusted for gender. * P <0.05

acid and AA were seen in PS, with mitochondrial and microsomal PS-adrenic acids declining significantly over the 80 year period, alongside considerable losses of mitochondrial PS-AA. In the mitochondria this loss of PS-adrenic acid and PS-AA were driven predominantly by declines in PS 18:0_22:4 and PS 18:0_20:4 respectively (Figure 5-6, Table 5-2), while in the microsomal membranes there were losses with age of PS 18:0_22:4 (Figure 5-10, Table 5-4). These results emphasise that age-related changes in individual phospholipids are not necessarily reflected in the total contribution of a given fatty acid within a class of phospholipid. Previous studies using combinations of thin-layer chromatography and gas chromatography to examine the changes with age to phospholipids and their fatty acids as separate entities would not have seen these age-related changes.

Interestingly we saw substantial increases in mitochondrial PS 18:0_22:6 over the adult lifespan in the human hippocampus (Figure 5-6, Table 5-2). This finding is similar to what was reported in Chapter 4 (section 4.2) for the mitochondrial and microsomal fractions of the human prefrontal cortex. In Chapter 4 (section 4.3) the importance of DHA within PS phospholipids of the human brain was discussed. Several other low-abundance phospholipids containing DHA also increased with age in both membrane fractions of the hippocampus, including mitochondrial and microsomal PE 18:1_22:6 (Figure 5-5 & Figure 5-9, Table 5-2 & Table 5-4), mitochondrial lyso-PE 22:6 (Figure 5-4, Table 5-2) and microsomal PE 15:0_22:6/O-16:0_22:6 (Figure 5-9, Table 5-4). Increases in mitochondrial PS-DHA were also seen with age in the human hippocampus (Table 5-6), but this age-related increase in DHA was not reflected in combined mitochondrial phospholipids. This suggests that the increase in DHA across the adult lifespan within the hippocampus is not as extensive as that seen in the human prefrontal cortex, but that this smaller increase in mitochondrial PS-DHA still may exhibit a neuroprotective effect leading to increased longevity.

The largest change in any single phospholipid of the human hippocampus occurred in mitochondrial PC 16:0_18:1, the predominant phospholipid present in the mitochondrial membranes (Figure 5-7). Mitochondrial PC 16:0_18:1 increased from 40.8% of total mitochondrial PC to 42.5% from ages 20 to 100, representing a 4% increase in initial abundance over the 80 year period (Figure 5-4, Table 5-2). In the previous chapter, we also saw age-related increases in mitochondrial PC 16:0_18:1 within the prefrontal

cortex. As discussed in Chapter 4 section 4.3, the role of this particular molecular phospholipid is largely unknown; however, its high abundance could suggest a structural or scaffolding role in cellular membranes. Evidence is also available that suggests a role for PC 16:0_18:1 in modulating membrane fluidity [192,193], as well as participating in the formation of lipid raft domains [201].

Two previous studies have examined changes in the phospholipids of the human hippocampus during normal ageing. Similar to the present study, Söderberg et al. [90] reported decreases in total protein concentration (mg/g of wet tissue) in the human hippocampus with age. However, unlike this thesis Söderberg et al. [90] did not perform a fractionation step and so was unable to pinpoint the loss of protein with age to a particular subcellular fraction (Figure 5-2). Both studies by Söderberg et al. [90,93] reported decreases with age in the concentration of total phospholipids (mg/g wet tissue) in the hippocampus from ages 50-60 years, a finding that was not seen in this region in the present thesis. Between the two studies by Söderberg et al. there were conflicting results for age-related changes to phospholipid classes. Söderberg et al. [90] reported no changes in concentration to either total PC or PE with age in the hippocampus, while Söderberg et al. [93] found age-related decreases in both total PC and PE. In the present chapter, no changes with age were seen for any phospholipid class, either in percent abundance or quantified amount. Söderberg et al. [93] also examined changes in hippocampal PC and PE fatty acids with no age-related changes with age being found.

In summary, there were several phospholipids containing either adrenic acid or AA that decreased with age in the mitochondrial and microsomal membranes of the human hippocampus. The most abundant phospholipid of this type was PE 18:0_22:4 in both membrane fractions, which decreased by approximately 13-18% in abundance from ages 20 to 100. Analysis of total fatty acids found that both adrenic acid and AA decreased with age in the combined phospholipids in both membrane fractions, but no age-related changes were seen specifically in the PE class for either fatty acid. Similar to our previous finding for the human prefrontal cortex in Chapter 4, significant age-related increases were seen in mitochondrial PS 18:0_22:6 within the hippocampus.

Chapter 6 The Entorhinal Cortex

The following chapter is an amended version of the manuscript submitted for publication: Hancock SE, Friedrich MG, Mitchell TW, Truscott RJW, Else PL (2015), "The phospholipid composition of the human entorhinal cortex remains relatively stable over 80 years of adult aging".

6.1 Introduction

This chapter will examine the changes seen during normal ageing in PC, PE and PS phospholipids present in the mitochondrial and microsomal membranes of the entorhinal cortex.

The entorhinal cortex is located in the medial temporal lobe in the anterior portion of the parahippocampal gyrus, and is considered to be the interface between the hippocampal formation and the neocortex (see Chapter 1, Figure 1-2). Within the hippocampal formation, there are circuits of interconnected neurons between the layers of the entorhinal cortex and regions of the hippocampus that form the perforant pathway involved in encoding episodic memory [202]. There is some disagreement in the literature as to whether this region is subject to significant age-related changes during normal ageing, with some studies finding significant declines in volume with age [17,19] while others have reported no age-related changes [203]. The entorhinal cortex also undergoes a loss in volume during the earliest stages of AD [203–205]. Both the Aβ plaques and tau NFTs characteristic of AD appear in this region in early stages of the disease [7]. Despite the significant changes occurring in the entorhinal cortex in AD surprisingly few studies have looked at alterations to phospholipid composition specifically in the entorhinal cortex [116], although others have examined the entire parahippocampal gyrus [121,123]. To date, there have been no studies conducted that test if any changes occur in the phospholipids of this region during normal ageing.

Therefore, the aim of this chapter was to characterise the age-related changes occurring to the phospholipid composition of the mitochondrial and microsomal membranes of the human entorhinal cortex.

6.2 Results

6.2.1 Age-related changes to total protein content in subcellular fractions

No changes with age in total protein concentration (mg/g tissue) were seen in the whole tissue homogenate, or in the mitochondrial and microsomal membranes.

6.2.2 Major phospholipid classes of mitochondrial and microsomal fractions

Each of the three phospholipids classes measured in this study (PC, PE and PS) reported as a fraction of total phospholipid detected in the mitochondrial and microsomal membranes are shown in Figure 6-1. The most abundant of the three phospholipid classes in both membrane fractions was PC, making up around 50% of the total phospholipids detected, followed by PE at 30% and PS at just under 20%.

When the percent composition of each phospholipid class was compared with each fraction, the mitochondria contained slightly less PC compared to the microsomes ($p < 0.05$), and slightly more PE ($p < 0.001$). No differences were seen in PS content between the fractions. The quantified amounts of each phospholipid class (as pmol of phospholipid/µg membrane protein) were also analysed, with approximately 20% more

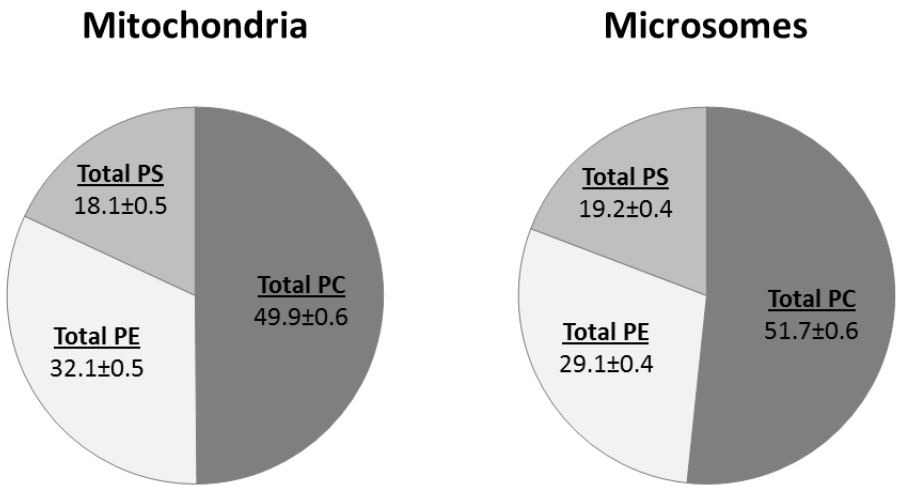


Figure 6-1: Percent composition of the three major phospholipid classes within the mitochondrial (left) and microsomal (right) fractions in normal human entorhinal cortex. Values are the mean ± SEM for the entire cohort. The microsomal membranes showed significantly higher amounts of total phosphatidylcholine (PC) compared to mitochondria ($p < 0.05$, Wilcoxon signed-rank test), while the mitochondria contained more phosphatidylethanolamine (PE) ($p < 0.001$, Wilcoxon signed-rank test). There were no differences between fractions for phosphatidylserine (PS).

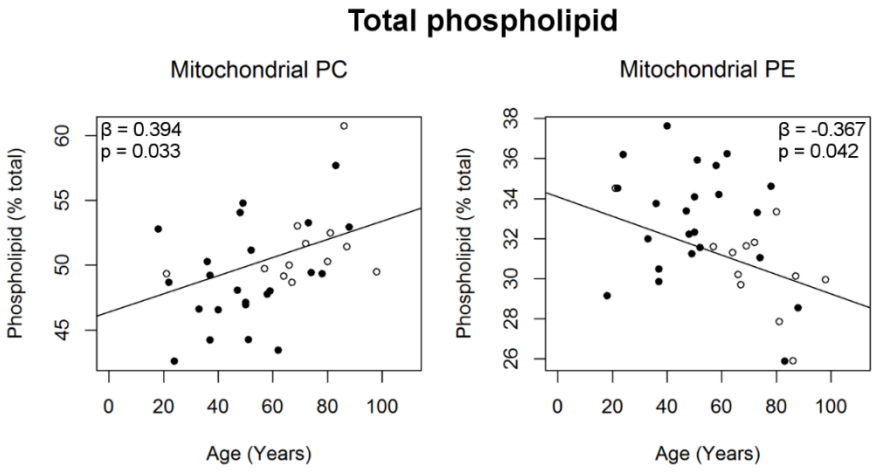


Figure 6-2: Phospholipid classes changing significantly with age (as a percent of total phospholipid) in normal human entorhinal cortex (*n* = 36). Regression model was adjusted for sex: males (●), females (○). Regression parameters are shown in Table 6-1.

Table 6-1: Phospholipid classes changing significantly with age in the normal human entorhinal cortex

Phospholipid	**Slope**	**95% CI**		**β**	**df**	**t**	***P* value**
		Lower	**Upper**				
Percent composition (% total phospholipid)							
Mitochondrial PC	0.0700	0.0061	0.1339	0.394	2,33	2.232	0.033*
Mitochondrial PE	-0.0487	-0.0953	-0.0019	-0.367	2,33	-2.122	0.042*

Phospholipid molecular species were detected using nanoelectrospray ionisation mass spectrometry and quantified as described in materials and methods. Regression was adjusted for gender. * P <0.05

PC, PS and total phospholipid being present in the microsomal fraction ($p < 0.01$, data not shown).

Regressions with age were carried out for each of the phospholipids classes in both the mitochondrial and microsomal membranes. Mitochondrial PC increased by 12% in abundance over the 80 years (Figure 6-2, Table 6-1). Conversely, mitochondrial PE declined by 11% in abundance from ages 20 to 100. No changes with age were reported for any of the quantified phospholipid classes (as pmol of phospholipid/µg membrane protein) in either membrane fraction, including total mitochondrial and microsomal phospholipid.

6.2.3 Changes in mitochondrial phospholipids with age

6.2.3.1 Phosphatidylcholines

The composition of PC, PE and PS phospholipids detected in the mitochondrial membranes of the entorhinal cortex can be seen in Figure 6-3. A significant age-related increase was found for the most abundant phospholipid of the mitochondrial fraction: PC 16:0_18:1 (Figure 6-4, Table 6-2). PC 16:0_18:1 increased from 42% of total mitochondrial PC at age 20 to 44% at age 100. Due to the high abundance of this phospholipid, this represents one of the largest age-related changes for any single mitochondrial phospholipid in the human entorhinal cortex.

Only one other mitochondrial PC increased with age in its percent composition, PC 16:0_18:2, which increased in abundance by 73% across the 80 years. However, this phospholipid is relatively low in abundance, making up only 0.5% of total mitochondrial PC. Only PC 16:0_18:2 maintained its age-related increase when analysed as quantified data (pmol of phospholipid/µg membrane protein, Table 6-3).

6.2.3.2 Phosphatidylethanolamines

A total of six mitochondrial PEs increased in abundance over the 80 year period studied when expressed as a percent of total PE (Figure 6-5, Table 6-2). Four of these phospholipids contained a 22:6 fatty acid: PE 16:0_22:6, PE 18:1_22:6, PE O-18:1_22:6, and PE 17:0_22:6/O-18:0_22:6. Only three mitochondrial PEs containing a

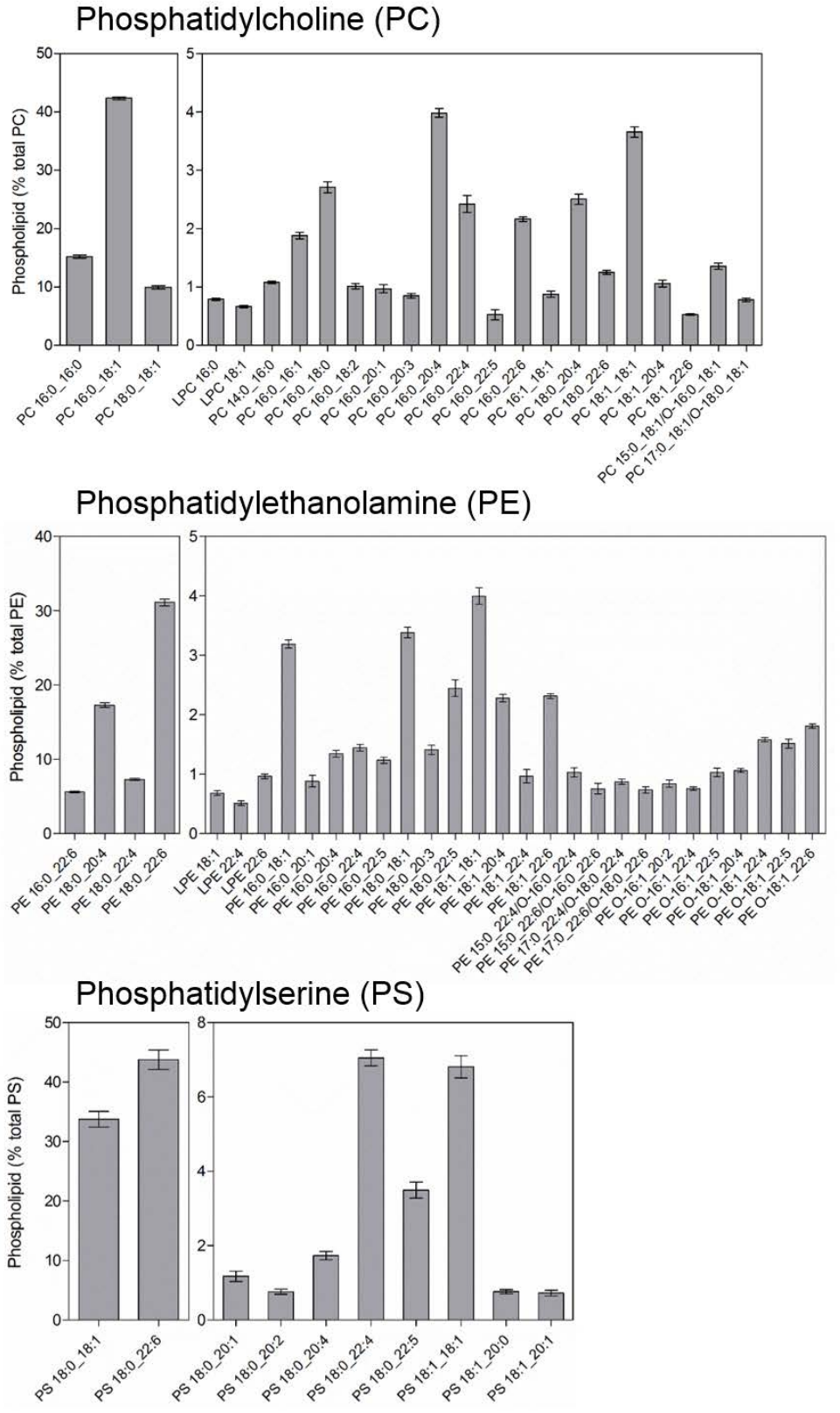


Figure 6-3: Phospholipids detected within PC, PE and PS in the mitochondrial fraction of human entorhinal cortex (as a percent of total phospholipid within each class). Phospholipids were quantified as described in materials and methods. Values are mean across the cohort ± SEM.

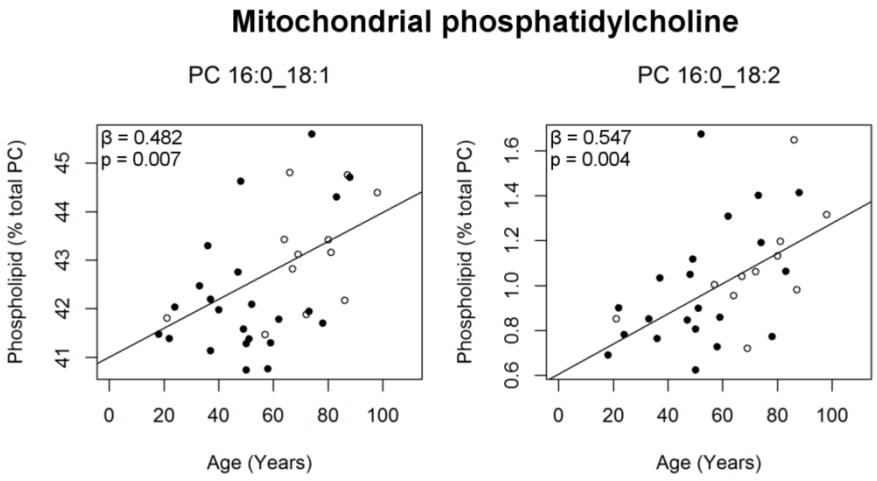


Figure 6-4: Mitochondrial PCs changing significantly with age (as a percent of total PC) in normal human entorhinal cortex (*n* = 30-36). Regression model was adjusted for sex: males (●), females (○). Regression parameters are shown in Table 6-2.

22:6 fatty acid did not show any age-related increases. These were lyso-PE 22:6, PE 15:0_22:6/O-16:0_22:6 and PE 18:0_22:6, the most abundant PE present in the mitochondria (Figure 6-3).

PE 16:0_22:6 is the fourth-most abundant mitochondrial PE, comprising just under 6% of total mitochondrial PE (Figure 6-3) and 1.8% of total mitochondrial phospholipid. An increase of 17% in abundance was observed for this phospholipid over the 80 year period (Figure 6-5, Table 6-2). Of the remaining 22:6-containing mitochondrial PEs, PE 18:1_22:6 increased by 18%, PE O-18:1_22:6 by 15%, and PE 17:0_22:6/O-18:0_22:6 by 251% from ages 20 to 100. Though seemingly large increases in abundance were seen in PE 17:0_22:6/O-18:0_22:6 with age, this phospholipid is of low abundance in the mitochondria, making up only 0.7% of total mitochondrial PE (Figure 6-3). Aside from these age-related increases seen in 22:6-containing PEs, two other mitochondrial PEs increased with age in their percent composition: PEs 16:0_18:1 (25%), 16:0_20:1 (30%) (Figure 6-5, Table 6-2).

Only PE 17:0_22:6/O-18:0_22:6 maintained an increase with age when analysed as a quantified amount (pmol of phospholipid/µg membrane protein, Table 6-3). Two other mitochondrial PEs decreased in quantified amount with age in the human entorhinal cortex, PE 18:0_22:4 and PE O-18:1_22:4.

Table 6-2: Mitochondrial phospholipids (percent of phospholipid within phospholipid class) changing significantly with age in the human entorhinal cortex.

Phospholipid	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Phosphatidylcholine							
PC 16:0_18:1	0.0298	0.0086	0.0511	0.482	2,32	2.861	0.007**
PC 16:0_18:2	0.0067	0.0023	0.0111	0.547	2,29	3.131	0.004**
Phosphatidylethanolamine							
PE 16:0_18:1	0.0091	0.0022	0.0161	0.470	2,31	2.690	0.011*
PE 16:0_20:1	0.0026	0.000003	0.0053	0.386	2,27	2.054	0.0498*
PE 16:0_22:6	0.0109	0.0020	0.0197	0.450	2,31	2.505	0.018*
PE 18:1_22:6	0.0049	0.0009	0.0089	0.452	2,30	2.511	0.018*
PE 17:0_22:6 /O-18:0_22:6	0.0085	0.0029	0.0141	0.612	2,26	3.116	0.004**
PE O-18:1_22:6	0.0033	0.00002	0.0067	0.328	2,32	2.051	0.0485*

Phospholipid molecular species were detected using nanoelectrospray ionisation mass spectrometry and quantified as described in materials and methods. Regression was adjusted for gender. * *P* <0.05 ** *P* <0.01

Table 6-3: Quantified mitochondrial phospholipids (pmol/µg total membrane protein) changing significantly with age in the human entorhinal cortex.

Phospholipid	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Phosphatidylcholine							
PC 16:0_18:2	0.0138	0.0030	0.0246	0.483	2,32	2.614	0.014*
Phosphatidylethanolamine							
PE 18:0_22:4	-0.0502	-0.0882	-0.0123	-0.488	2,33	-2.702	0.011*
PE 17:0_22:6 /O-18:0_22:6	0.0099	0.0010	0.0188	0.482	2,30	2.297	0.030*
PE O-18:1_22:4	-0.0085	-0.0165	-0.0005	-0.384	2,33	-2.160	0.038*
Phosphatidylserine							
PS 18:0_22:4	-0.0253	-0.0484	-0.0022	-0.404	2,33	-2.226	0.033*

Phospholipid molecular species were detected using nanoelectrospray ionisation mass spectrometry and quantified as described in materials and methods. Regression was adjusted for gender. * *P* <0.05

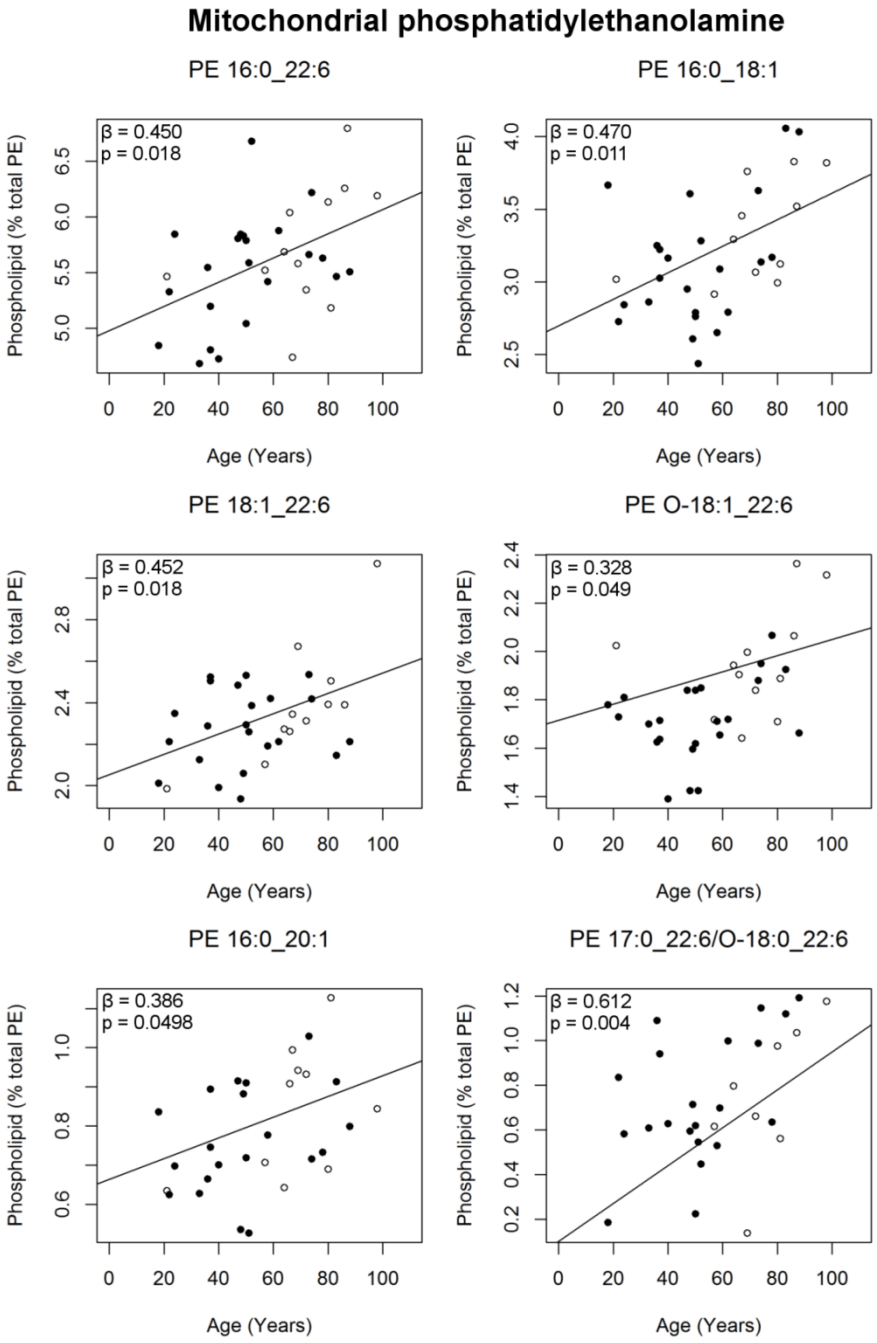


Figure 6-5: Mitochondrial PEs changing significantly with age (as a percent of total PE) in normal human entorhinal cortex (n = 30-36). Regression model was adjusted for sex: males (●), females (○). Regression parameters are shown in Table 6-2.

6.2.3.3 Phosphatidylserines

No PS phospholipids changed with age in either percent abundance (percent of total PS) or quantified amount (pmol phospholipid/µg membrane protein) in the mitochondrial fraction.

6.2.4 Changes in microsomal phospholipids with age

6.2.4.1 Phosphatidylcholines

The percent composition of each microsomal phospholipid within PC, PE and PS can be seen in Figure 6-6. There were three microsomal PCs observed to undergo age-related changes, all of which increased in abundance (Figure 6-7, Table 6-4). The most abundant of these was PC 18:1_18:1, a phospholipid that is found in moderate amounts in the microsomal membranes of the human entorhinal cortex (Figure 6-6). PC 18:1_18:1 increased from 2.7% of microsomal PC to 3.6% from ages 20 to 100 (Figure 6-7, Table 6-4). The remaining two microsomal PCs increasing in abundance with age were PC 14:0_16:0 and PC 15:0_16:0/O-16:0_16:0, which rose by 23% and 38% in abundance over the 80 year period. However, both of these phospholipids are only of low abundance making up less than 1% of the total microsomal phospholipid when combined.

No age-related changes were observed for the quantified microsomal PC phospholipids (pmol phospholipid/µg membrane protein) in the entorhinal cortex.

6.2.4.2 Phosphatidylethanolamines

Analysis of the microsomal PEs of the entorhinal cortex found three phospholipids that changed with age (Figure 6-8, Table 6-4). Two of these phospholipids increased with age, PE 18:1_22:6 and PE 16:0_22:5, by 28% and 25% in abundance respectively over the 80 years. PE 16:0_20:1 decreased by 22% in abundance over the same period. All three phospholipids are of low abundance in the microsomal membranes, however, making up less than 1.4% of the total microsomal phospholipid when combined (Figure 6-6).

Only a single microsomal PE changed with age when quantified phospholipids (pmol phospholipid/µg membrane protein) were analysed (Table 6-5). PE 18:1_22:4 increased from 2.0 pmol/µg membrane protein at age 20 to 2.6 pmol phospholipid/µg membrane protein at age 100.

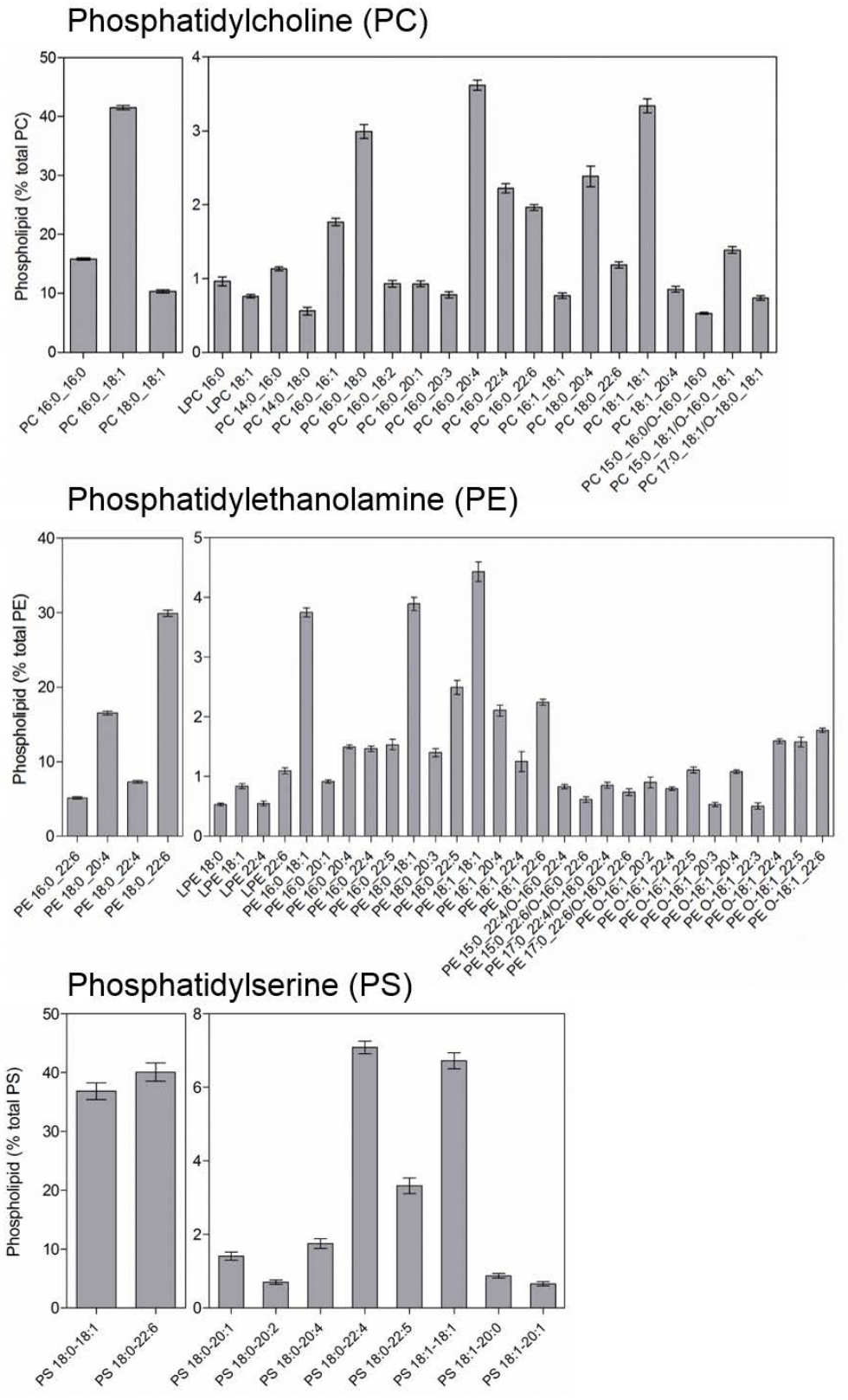


Figure 6-6: Phospholipids detected within PC, PE and PS in the microsomal fraction of human entorhinal cortex (as a percent of total phospholipid within each class). Phospholipids were quantified as described in materials and methods. Values are mean across the cohort ± SEM.

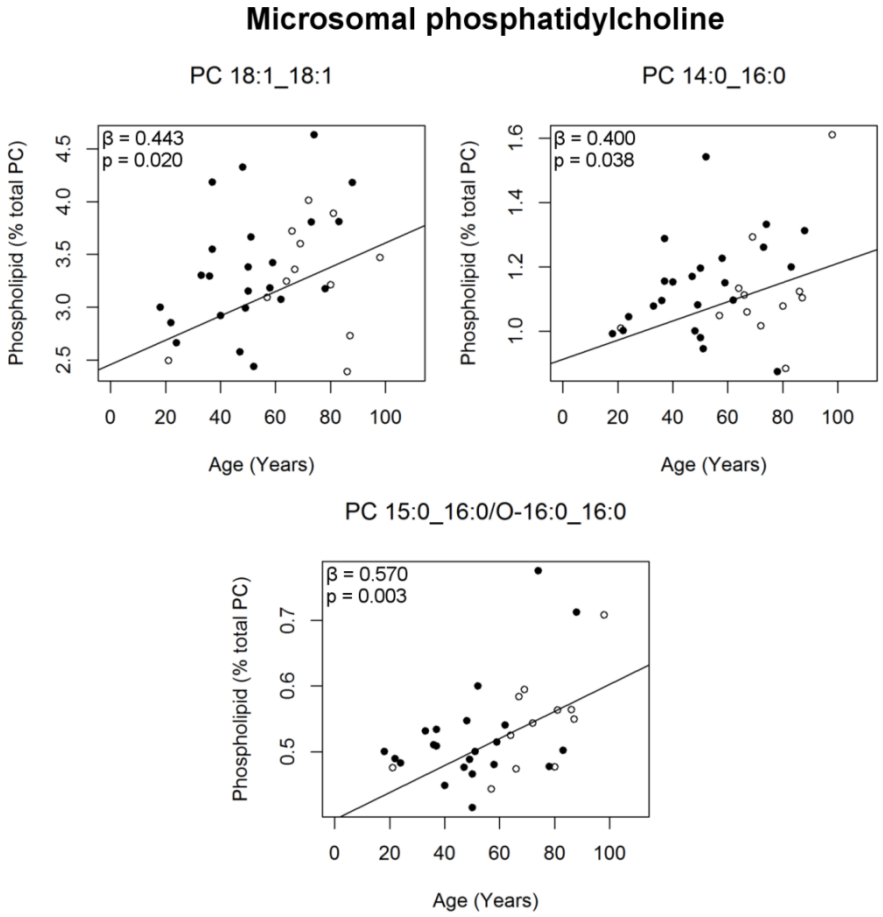


Figure 6-7: Microsomal PCs changing significantly with age (as a percent of total PC) in normal human entorhinal cortex (n = 30-36). Regression model was adjusted for sex: males (●), females (○). Regression parameters are shown in Table 6-4.

Table 6-4: Microsomal phospholipids (percent of phospholipid within phospholipid class) changing significantly with age in the human entorhinal cortex.

Phospholipid	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Phosphatidylcholine							
PC 14:0_16:0	0.0030	0.0002	0.0058	0.400	2,32	2.168	0.038*
PC 18:1_18:1	0.0115	0.0020	0.0211	0.443	2,32	2.452	0.020*
PC 15:0_16:0 /O-16:0_16:0	0.0021	0.0008	0.0033	0.570	2,31	3.271	0.003**
Phosphatidylethanolamine							
PE 16:0_20:1	-0.0029	-0.0055	-0.0003	-0.456	2,24	-2.273	0.032*
PE 16:0_22:5	0.0043	0.0006	0.0080	0.409	2,28	2.395	0.024*
PE 18:1_22:6	0.0069	0.0024	0.0115	0.517	2,31	3.096	0.004**
Phosphatidylserine							
PS 18:0_22:4	-0.0203	-0.0382	-0.0023	-0.418	2,32	-2.301	0.028*
PS 18:1_20:1	0.0071	0.0011	0.0131	0.440	2,32	2.421	0.021*

Phospholipid molecular species were detected using nanoelectrospray ionisation mass spectrometry and quantified as described in materials and methods. Regression was adjusted for gender. * P <0.05 ** P <0.01

Table 6-5: Quantified microsomal phospholipids (pmol/µg total membrane protein) changing significantly with age in the human entorhinal cortex.

Phospholipid	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Phosphatidylethanolamine							
PE 18:1_22:4	0.0264	0.0020	0.0508	0.413	2,29	2.216	0.035*

Phospholipid molecular species were detected using nanoelectrospray ionisation mass spectrometry and quantified as described in materials and methods. Regression was adjusted for gender. * P <0.05

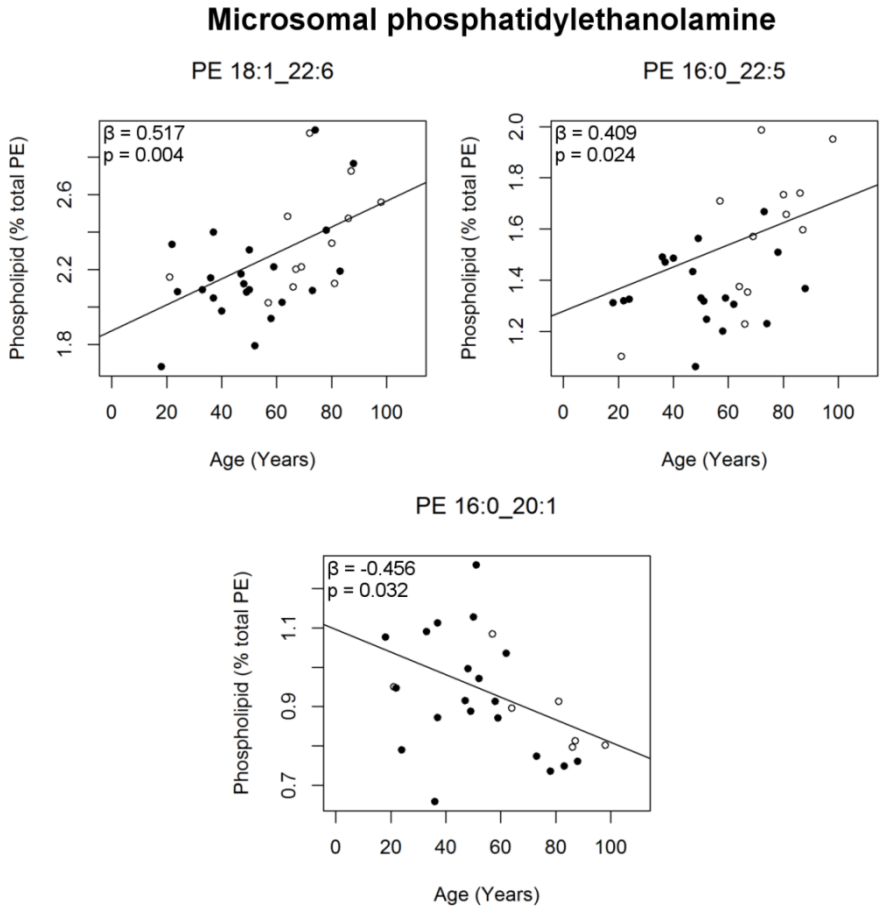


Figure 6-8: Microsomal PEs changing significantly with age (as a percent of total PE) in normal human entorhinal cortex (n = 30-36). Regression model was adjusted for sex: males (●), females (○). Regression parameters are shown in Table 6-4.

6.2.4.3 Phosphatidylserines

Only two PS phospholipids changed with age in the microsomal fraction: PS 18:0_22:4 and PS 18:1_20:1 (Figure 6-9, Table 6-4). PS 18:0_22:4, a moderately abundant phospholipid making up 7% of microsomal PS (Figure 6-6) and 1.4% of total microsomal phospholipids, decreased in percent composition by 20% from ages 20 to 100 (Figure 6-8, Table 6-4). PS 18:1_20:1 increased its percent composition, by 180% over the 80 year period. While a seemingly large increase with age, PS 18:1_20:1 is only found in low amounts in microsomal membranes, making up only 0.7% of microsomal PS (Figure 6-6).

No microsomal PS changed in quantified amount (pmol phospholipid/µg membrane protein) with age in the human entorhinal cortex.

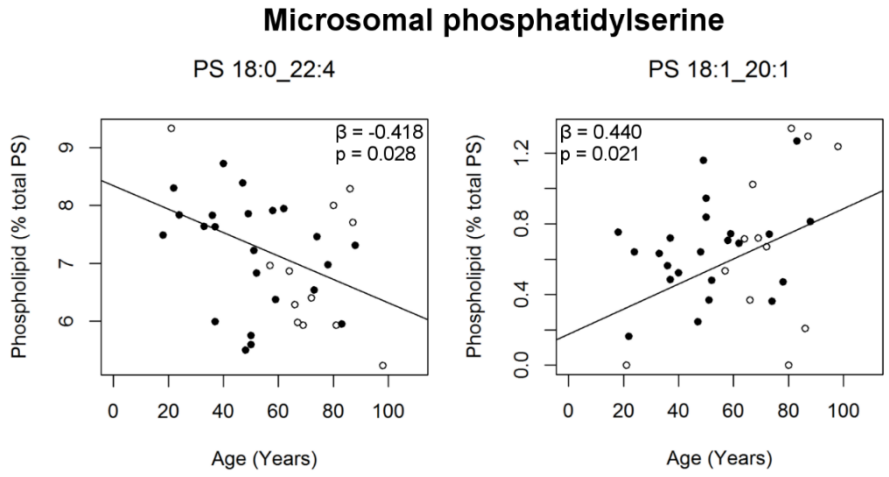


Figure 6-9: Microsomal PSs changing significantly with age (as a percent of total PS) in normal human entorhinal cortex (*n* = 30-36). Regression model was adjusted for sex: males (●), females (○). Regression equations are presented in Table 6-4.

6.3 Discussion

In the present chapter, a number of phospholipids underwent changes in the mitochondrial and microsomal membranes of the human entorhinal cortex during normal ageing. Similar to what was reported for the prefrontal cortex (Chapter 4) and hippocampus (Chapter 5), the changes observed in the entorhinal cortex were not limited to a particular class of phospholipid, nor were they limited to either the mitochondrial or microsomal fraction. Many age-related increases were seen specifically in mitochondrial PE phospholipids containing a 22:6 fatty acid (DHA), a finding similar to that previously reported in the prefrontal cortex (Chapter 4) and, to a lesser extent, the hippocampus (Chapter 5).

The most interesting age-related change in the phospholipids of the entorhinal cortex was found within the mitochondrial PE phospholipids. Four of the six mitochondrial PEs that changed significantly in percent composition with age contained a DHA: PE 16:0_22:6, PE 18:1_22:6, PE O-18:1_22:6, and PE 17:0_22:6/O-18:0_22:6 (Figure 6-5, Table 6-2). There were only three PE phospholipids containing a 22:6 fatty acid that did not change with age in the mitochondrial fraction: lyso-PE 22:6, PE 15:0_22:6/O-16:0_22:6 and the most abundant mitochondrial PE, PE 18:0_22:6. As discussed in previous chapters, the 22:6 fatty acid can be putatively classified as the n-3 fatty acid DHA based on its synthesis via the elongation and desaturation pathway (see Chapter 2, Figure 2-4) [56]. DHA is the most abundant n-3 fatty acid present in the brain; other n-3 fatty acids such as 20:5 (EPA) are typically only found in trace amounts in the human brain. Indeed, no phospholipids with this fatty acid were found in any of the three regions examined so far. DHA has a number of important biological roles in neuronal cellular membranes, including neurite growth, membrane fluidity, synaptic transmission and the production of anti-inflammatory eicosanoids [77]. The importance of phospholipids containing DHA with a PS head group in neuron survival and longevity was discussed previously in Chapter 4, but no changes with age to PS 18:0_22:6 were observed in the entorhinal cortex in either membrane fraction. Conversely, the specific cellular role of PE phospholipids containing DHA is widely unknown. The only available evidence suggests a role for some PE-DHA fatty acids as preferential precursors for the synthesis of PS-DHA phospholipids via PSS2 [46]. PSS2 is located in the mitochondria-region of the endoplasmic reticulum and is highly expressed in both

brain and testes in mammals. Increases in PS synthesis via serine-base exchanges have been reported in aged rats in the cerebellum and cerebral cortex, both with and without the addition of exogenous calcium [47]; however, if this also occurs in humans then we would expect levels of PE-DHA to decrease with age with a corresponding increase in PS-DHA. More research is needed to elucidate the mechanism and significance of PE-DHA phospholipids increasing with age in this region of the brain.

Due to the number of mitochondrial PEs containing DHA increasing in their percent composition with age, additional analysis was carried out to examine if any changes with age occurred to the most abundant PUFA across both the mitochondrial (Table 6-6) and microsomal membranes (Table 6-7). Surprisingly no age-related changes were seen in DHA in any of the phospholipid classes in the mitochondrial membranes despite the finding that many mitochondrial PEs containing DHA increased with age (Table 6-6). Examination of individual mitochondrial PE phospholipids showed that the lack of age-related changes to total PE-DHA could be attributed to a lack of age-related changes in the most abundant mitochondrial PE phospholipid, PE 18:0_22:6. This PE makes up 31% of total mitochondrial PE (Figure 6-3), which is nearly three times the combined amount of the four mitochondrial PE-DHA phospholipids that increased with age. Interestingly there were also decreases reported for total mitochondrial PC-22:4 fatty acids with age despite no single mitochondrial PC with a 22:4 fatty acid changing significantly with age (Figure 6-4). These results emphasise that significant age-related changes in specific phospholipids may be masked by examining fatty acids in isolation using techniques such as GC.

The phospholipid that underwent one of the largest age-related changes in the entorhinal cortex was mitochondrial PC 16:0_18:1, the most abundant phospholipid present in this fraction (Figure 6-3). While only a 6% increase in abundance was found for PC 16:0_18:1 over the 80 year period (Figure 6-4, Table 6-2), this phospholipid comprises approximately 42% of total mitochondrial PC (Figure 6-3) and 21% of total mitochondrial phospholipids, making this one of the largest changes in membrane composition reported in this region. A similar age-related change in the abundance of PC 16:0_18:1 was reported in the mitochondria of the prefrontal cortex (Chapter 4)

Table 6-6: Linear regression of most abundant polyunsaturated fatty acids (percent of total fatty acids within phospholipid class) in the mitochondrial membranes with age in human entorhinal cortex

Mitochondrial fatty acids	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Combined phospholipids							
20:4	-0.0039	-0.0148	0.0069	-0.144	2,32	-0.739	0.465
22:4	-0.0145	-0.0256	-0.0034	-0.441	2,32	-2.653	0.012*
20:4 + 22:4	-0.0184	-0.0338	-0.0031	-0.417	2,32	-2.447	0.020*
22:6	-0.0011	-0.0250	0.0228	-0.019	2,32	-0.096	0.924
Phosphatidylcholine							
20:4	-0.0007	-0.0108	0.0094	-0.028	2,31	-0.140	0.889
22:4	-0.0098	-0.0191	-0.0004	-0.387	2,32	-2.129	0.041*
20:4 + 22:4	-0.0105	-0.0200	-0.0010	-0.400	2,31	-2.264	0.031*
22:6	-0.0013	-0.0051	0.0026	-0.130	2,32	-0.665	0.511
Phosphatidylethanolamine							
20:4	-0.0029	-0.0219	0.0161	-0.061	2,32	-0.310	0.758
22:4	-0.0134	-0.0369	0.0101	-0.216	2,32	-1.162	0.254
20:4 + 22:4	-0.0163	-0.0448	0.0121	-0.222	2,32	-1.168	0.252
22:6	0.0202	-0.0080	0.0482	0.278	2,32	1.460	0.154
Phosphatidylserine							
20:4	0.0036	-0.0025	0.0097	0.231	2,31	1.197	0.240
22:4	-0.0095	-0.0206	0.0017	-0.321	2,31	-1.722	0.094
20:4 + 22:4	-0.0059	-0.0160	0.0042	-0.219	2,31	-1.183	0.245
22:6	0.0432	-0.0418	0.0128	0.196	2,30	1.034	0.308

Fatty acids calculated from phospholipid molecular species data. Regression was adjusted for gender. * P <0.05 ** P <0.01 *** P <0.001

Table 6-7: Linear regression of most abundant polyunsaturated fatty acids (percent of total fatty acids within phospholipid class) in the microsomal membranes with age in human entorhinal cortex

Mitochondrial fatty acids	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Combined phospholipids							
20:4	-0.0056	-0.0149	0.0037	-0.239	2,32	-1.235	0.226
22:4	-0.0056	-0.0138	0.0026	-0.260	2,32	-1.383	0.176
20:4 + 22:4	-0.0112	-0.0252	0.0028	-0.307	2,32	-1.632	0.113
22:6	-0.0008	-0.0262	0.0247	-0.012	2,32	-0.060	0.952
Phosphatidylcholine							
20:4	-0.0021	-0.0114	0.0072	-0.092	2,31	-0.470	0.641
22:4	-0.0027	-0.0062	0.0008	-0.314	2,30	-1.586	0.123
20:4 + 22:4	-0.0027	-0.0128	0.0075	-0.104	2,32	-0.537	0.595
22:6	-0.00003	-0.0046	0.0045	-0.002	2,32	-0.012	0.990
Phosphatidylethanolamine							
20:4	-0.0145	-0.0341	0.0051	-0.270	2,32	-1.506	0.142
22:4	-0.0051	-0.0190	0.0088	-0.136	2,30	-0.750	0.459
20:4 + 22:4	-0.0089	-0.0354	0.0177	-0.134	2,32	-0.680	0.501
22:6	0.0152	-0.0118	0.0422	0.220	2,32	1.148	0.259
Phosphatidylserine							
20:4	-0.0007	-0.0080	0.0065	-0.041	2,32	-0.207	0.837
22:4	-0.0125	-0.0230	-0.0020	-0.440	2,32	-2.417	0.022*
20:4 + 22:4	-0.0132	-0.0233	-0.0031	-0.476	2,32	-2.660	0.012*
22:6	0.0319	-0.0520	0.1157	0.148	2,32	0.774	0.445

Fatty acids calculated from phospholipid molecular species data. Regression was adjusted for gender. * P <0.05 ** P <0.01 *** P <0.001

and the hippocampus (Chapter 5). As discussed previously in Chapter 4 and Chapter 5, the function of this particular phospholipid is currently unknown, but its high abundance in both the mitochondrial and microsomal membranes suggests a structural role. However, it has been hypothesised that this particular phospholipid could play a role in modulating the fluidity of the membrane bilayer [192,193] as well as participate in the formation of lipid rafts [201].

Despite reports of decreases in the volume of the entorhinal cortex during normal ageing [17,19] there have been no published studies to date that have examined age-related changes to the phospholipids of this region, which prevent any comparisons being made with this study. The entorhinal cortex has also not been extensively studied for changes in membrane phospholipids in AD despite this region displaying losses in volume and significant tau and Aβ deposition in the early to mid-stages of the disease [203–205]. Only one study has specifically examined the entorhinal cortex for any changes its phospholipids in AD, finding no changes in either total PC, PE or PS phospholipid [116]. However, a further two other studies have examined the entire parahippocampal gyrus, looking at total phospholipid-fatty acids [121] and fatty acids separated by phospholipid class [123]. Skinner et al. [121] reported increases in total 18:1 and 22:4 in AD, with no changes being reported for DHA. Conversely, Prasad et al. [123] observed decreases in several PE-fatty acids in the hippocampus/parahippocampal gyrus, including both AA and DHA. Although the current evidence is relatively sparse, if the loss of PE-DHA in the entorhinal cortex and greater parahippocampal gyrus is indeed indicative of AD then the rise in several PE phospholipids containing DHA seen in the present chapter during normal ageing in the entorhinal cortex could be protective against the disease. More extensive research will be needed to see if there is a substantial loss of DHA in AD specifically in the entorhinal cortex to confirm this hypothesis.

In summary, there were many age-related increases seen in the entorhinal cortex in both the mitochondrial and microsomal membranes, including the rise of several mitochondrial PE phospholipids containing DHA. These PE-DHA phospholipids included PE 16:0_22:6, PE 18:1_22:6, PE O-18:1_22:6, and PE 17:0_22:6/O-18:0_22:6; however, the most abundant DHA-containing mitochondrial PE, PE 18:0_22:6, did not change with age. These contralateral changes in mitochondrial PEs

containing DHA led to no significant age-related changes being observed overall for total mitochondrial PE-DHA. Out of the three regions analysed so far, the entorhinal cortex has displayed the fewest number of changes to phospholipids during normal, adult ageing in both the mitochondrial and microsomal membranes.

Chapter 7 The Cerebellum

7.1 Introduction

This chapter will examine age-related changes in PC, PE and PS phospholipids in the mitochondrial and microsomal membranes of the human cerebellum.

Latin for the “the little brain”, the cerebellum is located at the bottom of the brain (see Chapter 1, Figure 1-2) and has a primary role in the coordination and precision of motor activity The cytoarchitecture of the cerebellum consists of three layers: a superficial molecular layer, a layer of Purkinje cells and a granular layer comprised of small, densely-packed granular cells. The cerebellum is thought to be largely spared during the pathogenesis of AD, with only small amounts of Aβ plaques characteristic of the disease being found in the molecular layer of the cerebellum [206,207]. The NFTs also characteristic of AD are largely absent in the cerebellum in affected individuals [206,207]. Likewise, many studies examining changes in phospholipids within the cerebellum during the pathogenesis of AD have found few to no changes as a result of the disease [85,111,112,115,116,123]. A recent stereological study found no changes in either Purkinje or granule cell number or density in AD, but a significant reduction in total cerebellar volume was seen in the AD group [208]. Due to the small amount of reported changes in the cerebellum in AD described above this region is often used as a control brain region.

Significant losses in cerebellar volume have also been reported during normal ageing, particularly in advanced age, using both stereological and *in vivo* imaging techniques [209]. Despite these reported changes, only a single study has looked at any changes to membrane lipids with age [90]. Söderberg et al. [90] found no changes during normal ageing in the levels of total phospholipid, PC or PE. Therefore, the aim of this chapter was to identify if any changes are occurring to the molecular phospholipids of the mitochondrial and microsomal membranes of the human cerebellum during normal ageing.

7.2 Results

7.2.1 Changes in total protein concentration during normal ageing

The total protein content (mg/g wet tissue) of both the mitochondrial and microsomal fractions declined significantly with age (Figure 7-1, Table 7-1). Mitochondrial protein concentration fell by 54%, decreasing from 7.9 to 3.7 mg protein/g wet tissue from ages 20 to 100, while microsomal protein decreased from 1.5 to 0.9 mg/g wet tissue, a loss of 38%. The concentration of total protein within the whole tissue showed a trend towards decreased abundance with advanced age, but this failed to reach statistical significance ($p = 0.076$).

7.2.2 Major phospholipid classes of mitochondrial and microsomal fractions

The proportion of PC, PE and PS detected in the mitochondrial and microsomal fractions (as a percent of total phospholipid) is shown in Figure 7-2. The most abundant phospholipid class present in the two membrane fractions was PC, which comprised around 45% of total phospholipids in the two fractions, followed by PE at approximately 35% of total phospholipids and PS at 20%. The microsomal membranes contained more PC than the mitochondrial ($p < 0.001$) while the mitochondrial membranes contained more PE and PS ($p < 0.001$). The quantified amount (pmol/µg membrane protein) of each phospholipid in all three classes was also analysed, with higher levels of total PC ($p < 0.01$) , PE and PS ($p < 0.001$) found in the mitochondria, as well as higher levels of total quantified phospholipid ($p < 0.001$). Each phospholipid class was also assessed for any changes with age in both the mitochondrial and microsomal membranes, with no age-related effect found in either membrane fraction for any phospholipid class measured as percent abundance (% total phospholipid) or as quantified phospholipids (pmol phospholipid/µg membrane protein). Likewise, no statistically significant changes with age were found in either membrane fraction for total quantified phospholipid.

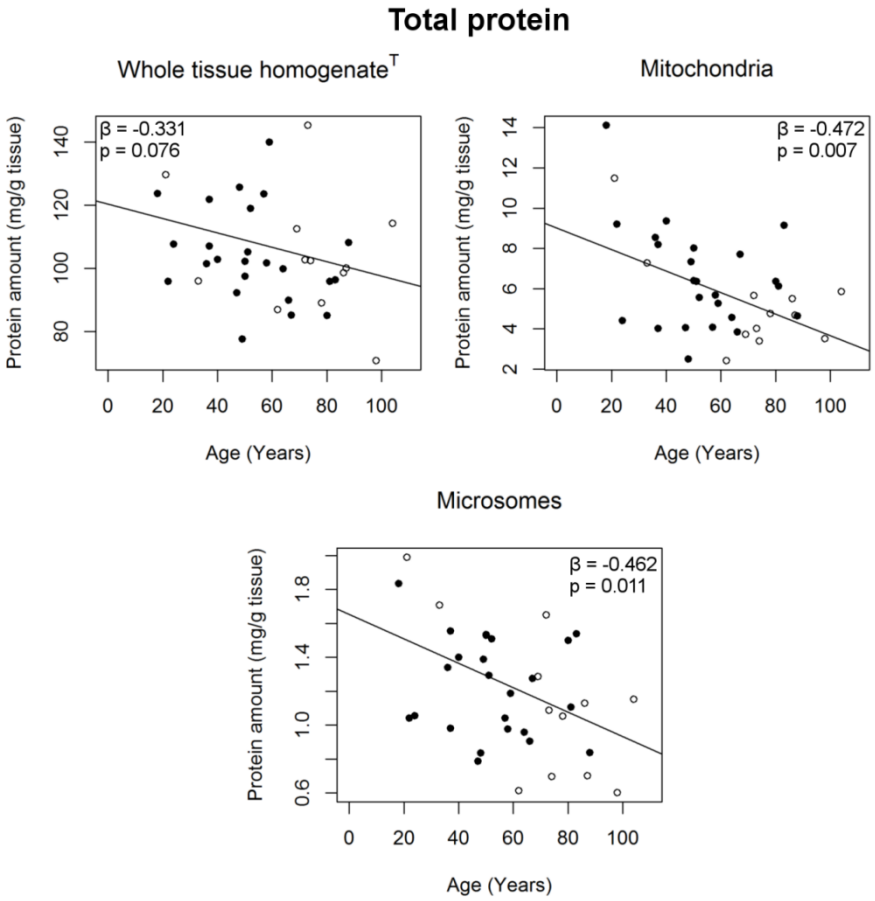


Figure 7-1: Linear regression of total protein amount (mg/g wet tissue) measured in whole tissue homogenate and subcellular fractions derived from normal human cerebellum against age. Total protein was measured using BCA assay as described in methods and materials. Regression model was adjusted for sex: males (●), females (○). ^{T} indicates dependent variable transformed for linear regression, with transformed beta-coefficient and p-value reported on original scatterplot for comparison. Regression parameters are shown in Table 7-1.

Table 7-1: Significant linear regression results of total protein (mg/g of tissue) with age in the subcellular fractions of neurologically normal human cerebellum

Phospholipid	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Whole tissue homogenate[I]	-0.00002	-0.00005	-0.0000002	-0.331	2,33	-1.833	0.076
Mitochondria	-0.0534	-0.0913	-0.0155	-0.472	2,33	-2.865	0.007**
Microsomes	-0.0072	-0.0126	-0.0018	-0.462	2,33	-2.708	0.011*

Total protein amount measured using BCA Assay. Regression was adjusted for gender. Superscript indicates dependent variable transformed for linear regression, with transformed regression output reported: [I] reciprocal. * P <0.05 ** P <0.01

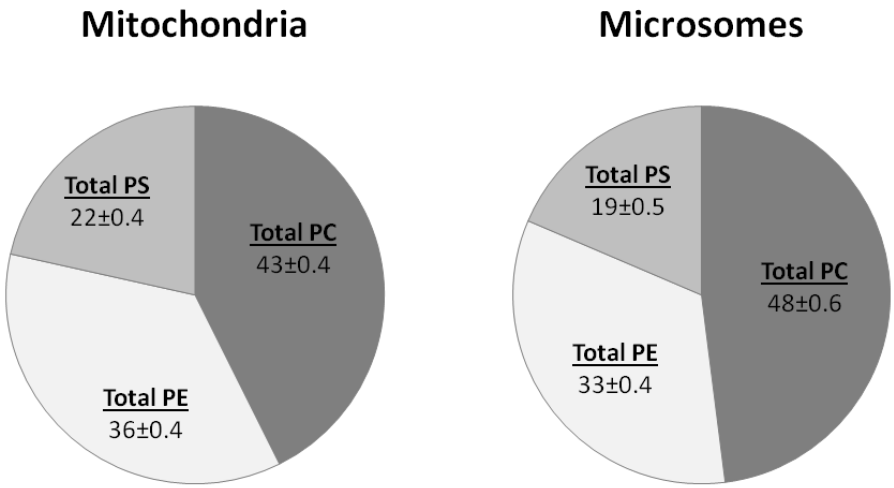


Figure 7-2: Relative percentage contribution of the three major phospholipid classes within the mitochondrial (left) and microsomal (right) fractions in neurologically normal cerebellum. Values are the mean ± SEM for the entire cohort. The microsomal membranes showed significantly higher amounts of total phosphatidylcholine (PC) compared to mitochondria ($p < 0.001$, Wilcoxon signed-rank test), while the mitochondria contained more phosphatidylethanolamine (PE) ($p < 0.001$, Wilcoxon signed-rank test) and phosphatidylserine (PS) ($p<0.001$, Wilcoxon signed-rank test).

7.2.3 Changes in mitochondrial phospholipids with age

7.2.3.1 Phosphatidylcholines

The percent composition of all phospholipids detected in the mitochondrial membranes is shown in Figure 7-3. Four mitochondrial PCs underwent statistically significant changes with age in the human cerebellum (Figure 7-4, Table 7-2). The most abundant mitochondrial PC to change with age was PC 16:0_22:6, which increased from 3.6% of total mitochondrial PC at age 20 to 4.3% at 100 years of age. This represents an increase of 20% from its initial abundance. Only one other mitochondrial PC showed an age-related increase, PC 16:0_18:2, which rose by 39% in abundance over the 80 year period. However, PC 16:0_18:2 is only of low abundance in the mitochondrial membranes, comprising only 1.1% of mitochondrial PC (Figure 7-3) and 0.5% of total mitochondrial phospholipid.

The remaining statistically significant mitochondrial PCs decreased with age as follows: PC 16:0_22:4 by 25%, and PC 16:0_20:1 by 27% over the 80 year period (Figure 7-4, Table 7-2). PC 16:0_22:4 is a moderately abundant phospholipid, comprising 2.9% of mitochondrial PC, while PC 16:0_20:1 is of lower abundance making up less than 1.5% of mitochondrial PC (Figure 7-3). Of these four mitochondrial PCs only PC 16:0_18:2 maintained its increase with age when analysed as quantified amount (pmol phospholipid/µg membrane protein; Table 7-3).

7.2.3.2 Phosphatidylethanolamines

Within the mitochondria, eleven PEs changed in percent composition with age (Figure 7-5, Table 7-2). The mitochondrial PE with the largest age-related change was PE 18:0_22:6, the most abundant PE present in the mitochondria (Figure 7-3). Mitochondrial PE 18:0_22:6 increased from 26.0% of total mitochondrial PE to 31.5% over the 80-year period. Four other mitochondrial PEs containing a 22:6 fatty acid also increased with age: PE 16:0_22:6, PE 18:1_22:6, PE O-18:1_22:6 and PE 15:0_22:6/O-16:0_22:6 (Figure 7-5, Table 7-3). PE 16:0_22:6, the third-most abundant mitochondrial PE, increased by 34% in percent composition from ages 20 to 100. Both PE 18:1_22:6 and O-18:1_22:6 are moderately abundant phospholipids (Figure 7-3) that increased by 33% and 22% in abundance respectively over the 80 year period (Figure 7-5, Table 7-2). PE 15:0_22:6/O-16:0_22:6 more than tripled in abundance from ages 20 to 100,

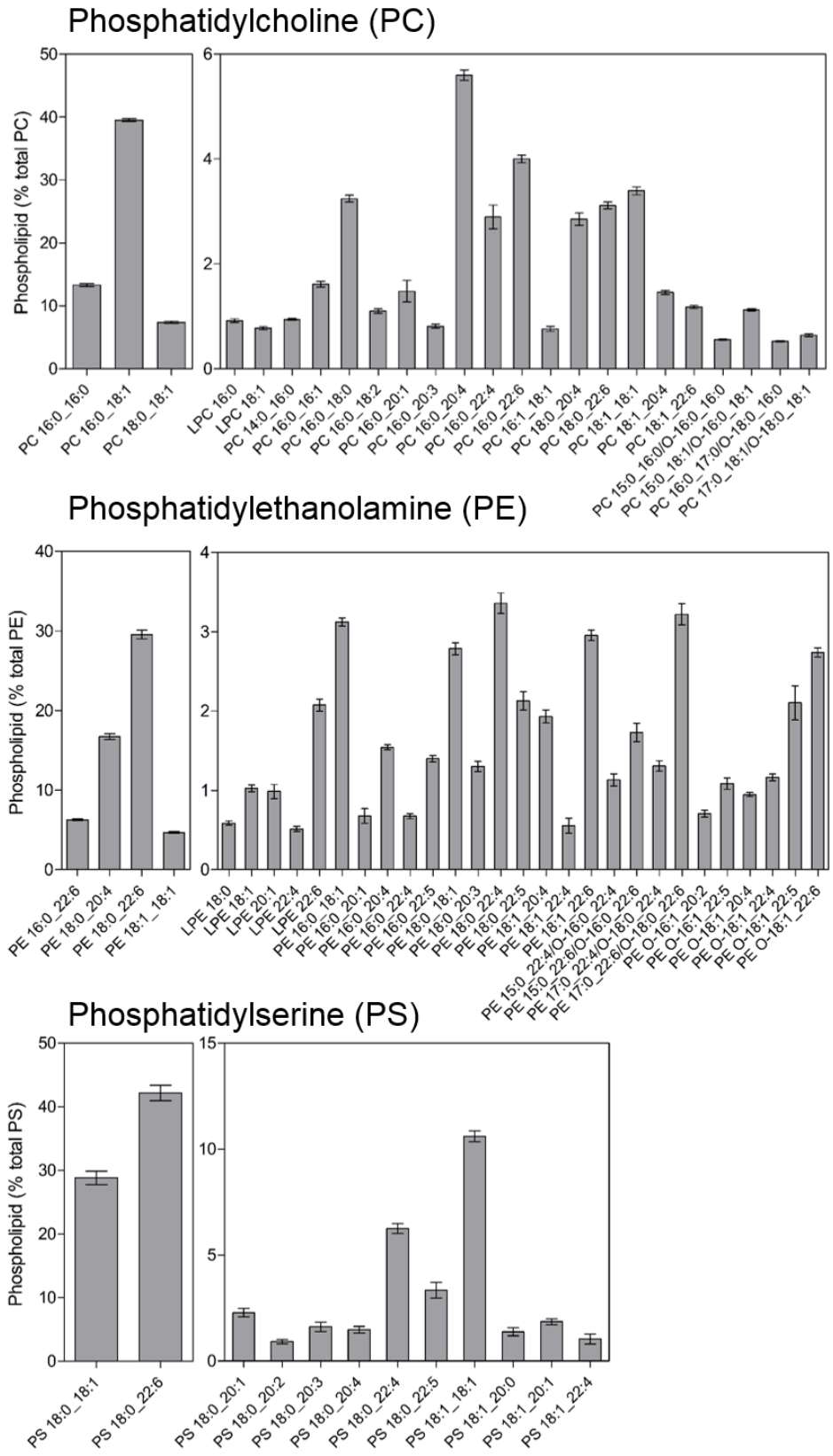


Figure 7-3: Phospholipids detected within PC, PE and PS in the mitochondrial fraction of human cerebellum (as a percent of total phospholipid within each class). Phospholipids were quantified as described in materials and methods. Values are mean across the cohort ± SEM.

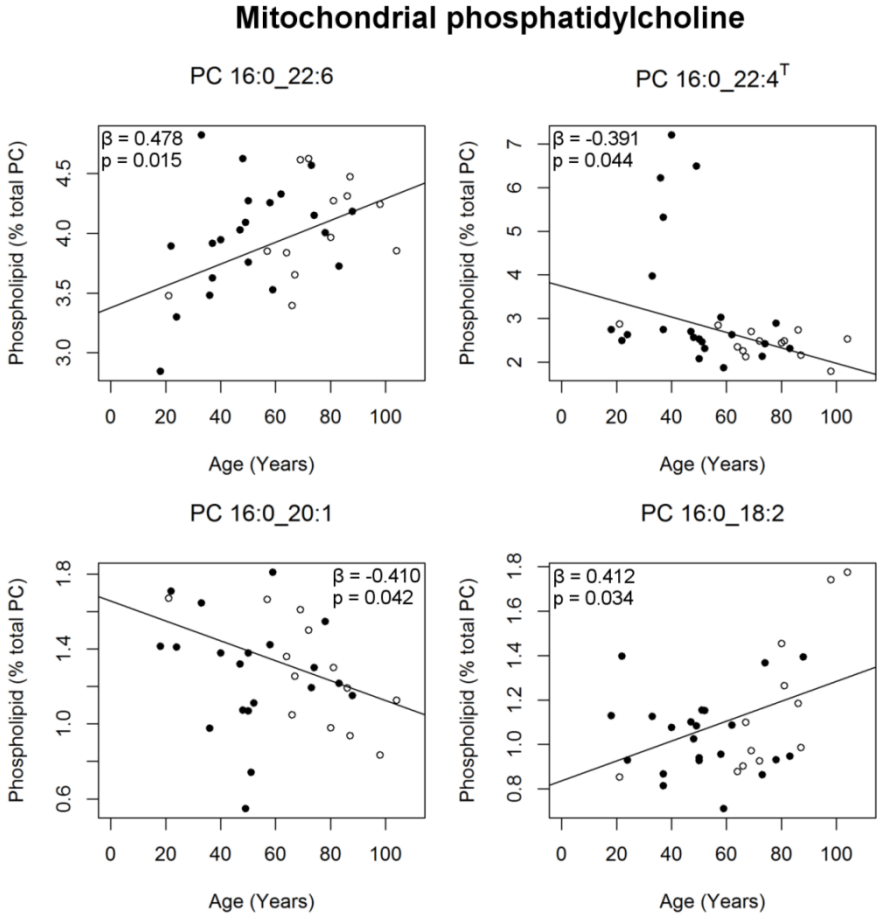


Figure 7-4: Mitochondrial PCs changing significantly with age (as a percent of total PC) in normal human cerebellum (n = 33-35). Regression model was adjusted for sex: males (●), females (○). T indicates dependent variable transformed for linear regression, with transformed beta-coefficient and p-value reported on original scatterplot for comparison. Regression parameters are shown in Table 7-2.

Table 7-2: Mitochondrial phospholipids (percent of phospholipid within phospholipid class) changing significantly with age in the human cerebellum.

Phospholipid	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Phosphatidylcholine							
PC 16:0_18:2	0.0045	0.0004	0.0086	0.412	2,31	2.218	0.034*
PC 16:0_20:1	-0.0053	-0.0104	-0.0002	-0.410	2,30	-2.126	0.042*
PC 16:0_22:4^{I}	-0.0010	-0.0019	-0.00003	-0.391	2,32	-2.102	0.044*
PC 16:0_22:6	0.0091	0.0019	0.0164	0.478	2,31	2.575	0.015*
Phosphatidylethanolamine							
Lyso-PE 22:4	-0.0035	-0.0069	-0.0001	-0.394	2,33	-2.116	0.042*
PE 16:0_22:6	0.0197	0.0114	0.0279	0.708	2,30	4.883	0.00003***
PE 18:0_18:1	-0.0114	-0.0186	-0.0041	-0.539	2,32	-3.205	0.003**
PE 18:0_20:4	-0.0606	-0.0931	-0.0281	-0.600	2,32	-3.799	0.0006***
PE 18:0_22:4	-0.0214	-0.0332	-0.0096	-0.616	2,33	-3.694	0.0008***
PE 18:0_22:6	0.0688	0.0132	0.1244	0.460	2,33	2.518	0.017*
PE 18:1_22:6	0.0106	0.0054	0.0158	0.606	2,33	4.122	0.0002***
PE 15:0_22:6 /O-16:0_22:6	0.0210	0.0097	0.0323	0.669	2,32	3.785	0.0006***
PE 17:0_22:4 /O-18:0_22:4	-0.0095	-0.0157	-0.0034	-0.542	2,33	-3.171	0.003**
PE O-16:1_22:5	-0.0124	-0.0188	-0.0060	-0.647	2,33	-3.936	0.0004***
PE O-18:1_22:6	0.0065	0.0007	0.0123	0.421	2,33	2.268	0.030*
Phosphatidylserine							
PS 18:0_18:1	-0.1269	-0.2293	-0.0246	-0.445	2,33	-2.523	0.017*
PS 18:0_22:4^{L}	-0.0025	-0.0036	-0.0015	-0.735	2,32	-4.856	0.00003***
PS 18:0_22:6^{L}	0.0022	0.0012	0.0032	0.681	2,33	4.451	0.00009***

Phospholipid molecular species were detected using nanoelectrospray ionisation mass spectrometry and quantified as described in materials and methods. Regression was adjusted for gender. Superscript indicates dependent variable transformed for linear regression, with transformed regression output reported: ^{L} logarithm, ^{I} reciprocal. * *P* <0.05 ** *P* <0.01 *** *P* <0.001

Table 7-3: Quantified mitochondrial phospholipids (pmol/µg membrane protein) changing significantly with age in the human cerebellum.

Phospholipid	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Phosphatidylcholine							
PC 16:0_18:2	0.0092	0.0024	0.0160	0.494	2,31	2.768	0.009**
Phosphatidylethanolamine							
PE 16:0_22:6[I]	0.0004	0.00004	0.0008	0.422	2,31	2.243	0.032*
PE 18:0_22:4	-0.0230	-0.0451	-0.0007	-0.380	2,32	-2.101	0.044*
PE 18:1_22:6[I]	0.0008	0.0002	0.0015	0.473	2,33	2.642	0.013*
PE 15:0_22:6 /O-16:0_22:6	0.0260	0.0095	0.0425	0.610	2,32	3.219	0.003**
PE 17:0_22:4 /O-18:0_22:4	-0.0106	-0.0197	-0.0015	-0.434	2,33	-2.363	0.024*
PE O-16:1_22:5	-0.0122	-0.0203	-0.0042	-0.544	2,33	-3.098	0.004**
PE O-18:1_22:6[I]	0.0006	0.00001	0.0012	0.388	2,33	2.079	0.046*
Phosphatidylserine							
PS 18:0_22:4	-0.0237	-0.0448	-0.0026	-2.284	2,32	-2.284	0.029*
PS 18:0_22:6	0.1998	0.0585	0.3410	2.877	2,33	2.877	0.007**

Phospholipid molecular species were detected using nanoelectrospray ionisation mass spectrometry and quantified as described in materials and methods. Regression was adjusted for gender. Superscript indicates dependent variable transformed for linear regression, with transformed regression output reported: [I] reciprocal.
* $P < 0.05$ ** $P < 0.01$

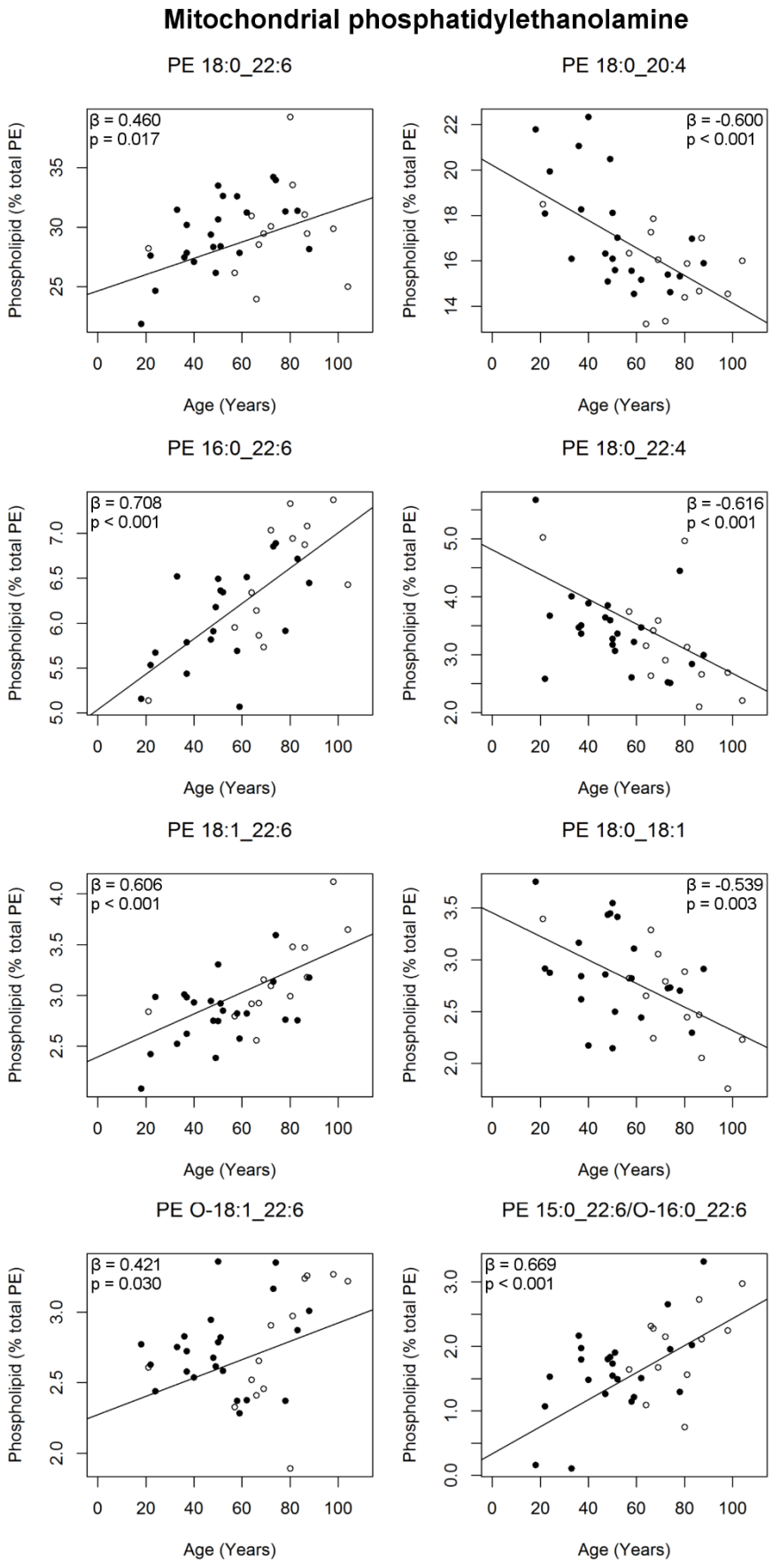
but this phospholipid is only present in low amounts in the mitochondria (Figure 7-3). The remaining statistically significant mitochondrial PEs all decreased in abundance over the 80 year period. The second-most abundant mitochondrial PE, PE 18:0_20:4, fell from 19.0% of mitochondrial PE to 14.2% from ages 20 to 100 (Figure 7-5, Table 7-2). Two moderately abundant mitochondrial PEs decreased in abundance over the 80 year period: PE 18:0_22:4 by 39% and PE 18:0_18:1 by 28%. The remaining three mitochondrial PEs decreasing with age, PE 17:0_22:4/O-18:0_22:4, PE O-16:1_22:5 and lyso-PE 22:4 are all low abundance phospholipids, making up just over 1.0% of total mitochondrial phospholipid when combined.

Seven of these mitochondrial PEs maintained age-related changes when examined as quantified amount (pmol/µg membrane protein, Table 7-3). Increases with age were seen in quantified PE 16:0_22:6, PE 18:1_22:6, PE O-18:1_226, and PE 15:0_22:6/O-16:0_22:6. Phospholipids decreasing in both percent abundance and quantified amount with age included PE 18:0_22:4, PE 17:0_22:4/O-18:0_22:4 and PE O-16:1_22:5

7.2.3.3 Phosphatidylserines

There were three mitochondrial PSs that changed significantly with age in the cerebellum (Figure 7-6, Table 7-2). PS 18:0_22:6, the most abundant mitochondrial PS (Figure 7-3), increased considerably from 34.3% of total mitochondrial PS to 51.4% from ages 20 to 100 (Figure 7-6, Table 7-2). The remaining two PS phospholipids, PS 18:0_18:1 and PS 18:0_22:4 both decreased in abundance with age in the mitochondria, by 30% and 37% respectively over the 80 year period. PS 18:0_18:1 is the second most abundant PS in the mitochondria, making up 28.9% of total mitochondrial PS (Figure 7-3) and 6.3% of total mitochondrial phospholipid. PS 18:0_22:4 is a moderately abundant phospholipid, comprising approximately 6.3% of total mitochondrial PS.

Two of these three mitochondrial PSs also showed changes with age in quantified amount (pmol/µg membrane protein; Table 7-3). PS 18:0_22:6 retained its increase with age in the mitochondria, rising from 20.5 pmol/µg membrane protein to 36.5 pmol/µg membrane protein from ages 20 to 100, representing a 78% increase from its initial abundance. PS 18:0_22:4 also maintained a loss with advanced age, decreasing by 35% in abundance over the 80 year period.


Mitochondrial phosphatidylethanolamine
PE 18:0_22:6
β = 0.460
p = 0.017
PE 18:0_20:4
β = -0.600
p < 0.001
PE 16:0_22:6
β = 0.708
p < 0.001
PE 18:0_22:4
β = -0.616
p < 0.001
PE 18:1_22:6
β = 0.606
p < 0.001
PE 18:0_18:1
β = -0.539
p = 0.003
PE O-18:1_22:6
β = 0.421
p = 0.030
PE 15:0_22:6/O-16:0_22:6
β = 0.669
p < 0.001
Phospholipid (% total PE)
Age (Years)

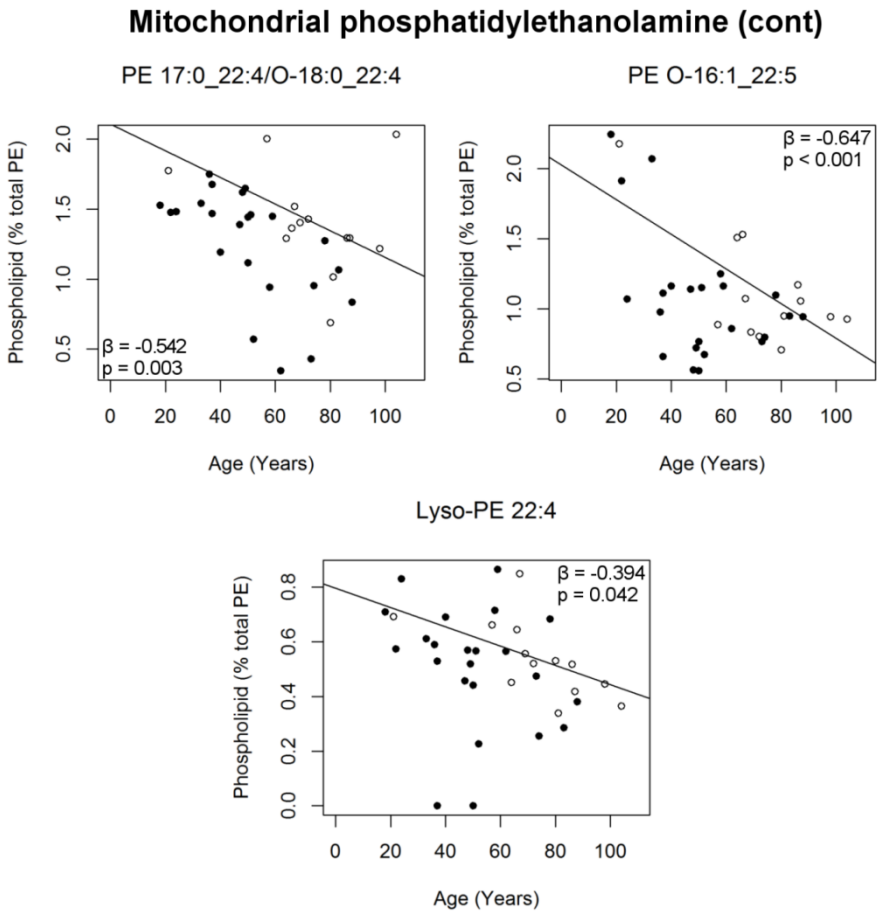


Figure 7-5: Mitochondrial PEs changing significantly with age (as a percent of total PE) in normal human cerebellum (*n* = 33-36). Regression model was adjusted for sex: males (●), females (○). Regression parameters are shown in Table 7-2

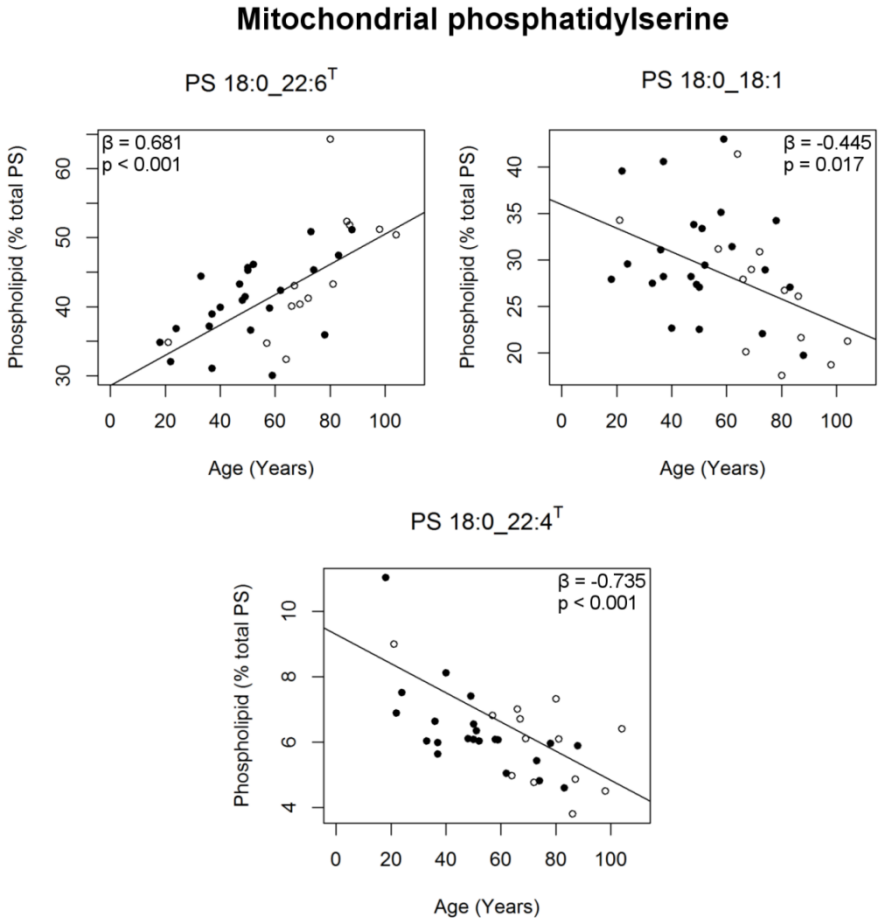


Figure 7-6: Mitochondrial PSs changing significantly with age (as a percent of total PS) in normal human cerebellum (n = 35-36). Regression model was adjusted for sex: males (●), females (○). ^{T} indicates dependent variable transformed for linear regression, with transformed beta-coefficient and p-value reported on original scatterplot for comparison. Regression parameters are shown in Table 7-2.

7.2.4 Changes in microsomal phospholipids with age

7.2.4.1 Phosphatidylcholines

All phospholipids detected in the microsomal fraction are presented as a percent of phospholipid within each phospholipid class in Figure 7-7. There were seven microsomal PCs that changed with age in the cerebellum, with two of these phospholipids increasing with age (Figure 7-8, Table 7-4). The second-most abundant phospholipid present in microsomal membranes, PC 16:0_16:0, increased from 11.8% to 15.3% of total microsomal PC from ages 20 to 100. Only one other microsomal PC increased with age in the cerebellum: PC 16:0_18:2. However, this phospholipid is only of low abundance in the microsomes, making up less than 1.2% of microsomal PC (Figure 7-7) and 0.6% of total microsomal phospholipids. PC 16:0_18:2 increased by 54% in abundance over the 80 year period studied (Figure 7-8, Table 7-4).

The remaining five phospholipids, PC 18:0_18:1, PC 16:0_22:4, PC 16:0_20:1, PC 18:1_22:6, and lyso-PC 18:1, all decreased in abundance with age (Figure 7-8, Table 7-4). PC 18:0_18:1 is the third-most abundant microsomal PC (Figure 7-7), and it decreased by 18% from ages 20 to 100 (Figure 7-8, Table 7-4). PC 16:0_22:4, a moderately abundant microsomal phospholipid, decreased from 2.7% of microsomal PC to 2.1% from ages 20 to 100. The remaining three significant microsomal PCs, PC 16:0_20:1, PC 18:1_22:6 and lyso-PC 18:1 are all of low abundance within microsomal membranes, and they decreased in abundance by 23%, 59% and 10% respectively over the 80 year period. There were no microsomal PCs that changed significantly with age when analysed as quantified mount (pmol phospholipid/µg membrane protein).

7.2.4.2 Phosphatidylethanolamines

There were eight microsomal PEs that changed in their percent composition with age (Figure 7-9, Table 7-4). The most abundant PE in microsomes, PE 18:0_22:6, increased with age, rising from 26.0% of total microsomal PE to 31.1% from ages 20 to 100. Three other microsomal PEs containing a 22:6 fatty acid also increased with age, including PE 16:0_22:6, PE 18:1_22:6 and PE 15:0_22:6/O-16:0_22:6. PE 16:0_22:6 is the third-most abundant microsomal PE, making up 6.2% of microsomal PE (Figure 7-7) and 2.1% of the total microsomal phospholipid. PE 16:0_22:6 increased by 25% in percent abundance over the 80 year period (Figure 7-9, Table 7-4). PE

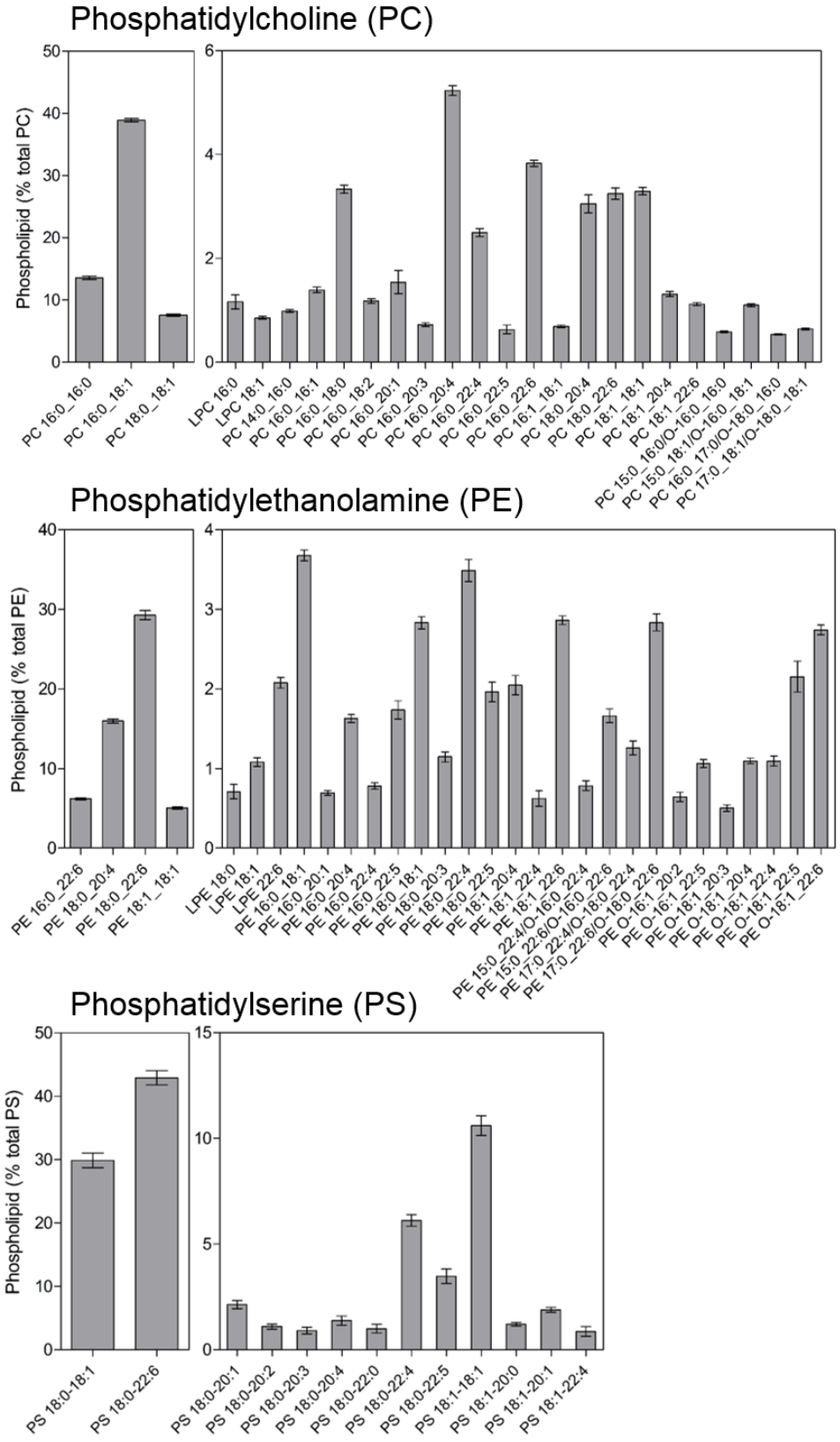


Figure 7-7: Phospholipids detected within PC, PE and PS in the microsomal fraction of human cerebellum (as a percent of total phospholipid within each class). Phospholipids were quantified as described in materials and methods. Values are mean across the cohort ± SEM.

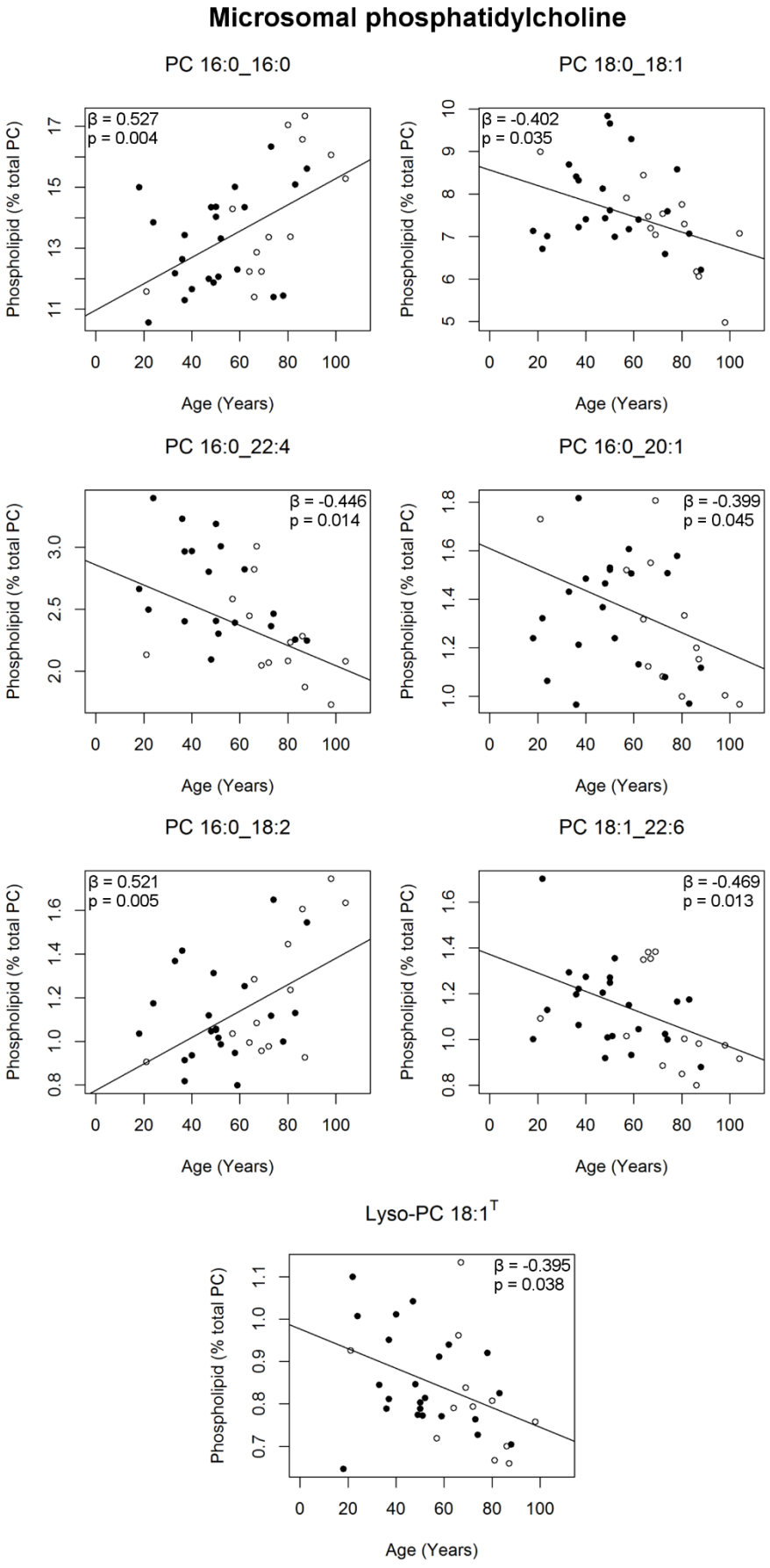

Microsomal phosphatidylcholine
PC 16:0_16:0
β = 0.527
p = 0.004
PC 18:0_18:1
β = -0.402
p = 0.035
PC 16:0_22:4
β = -0.446
p = 0.014
PC 16:0_20:1
β = -0.399
p = 0.045
PC 16:0_18:2
β = 0.521
p = 0.005
PC 18:1_22:6
β = -0.469
p = 0.013
Lyso-PC 18:1T
β = -0.395
p = 0.038
Phospholipid (% total PC)
Age (Years)

Figure 7-8: Microsomal PCs changing significantly with age (as a percent of total PC) in normal human cerebellum (n = 32-36). Regression model was adjusted for sex: males (●), females (○). [T] indicates dependent variable transformed for linear regression, with transformed beta-coefficient and p-value reported on original scatterplot for comparison. Regression parameters are shown in Table 7-4

Table 7-4: Microsomal phospholipids (percent of phospholipid within phospholipid class) changing significantly with age in the human cerebellum.

Phospholipid	**Slope**	**95% CI**		**β**	**df**	**t**	***P* value**
		Lower	**Upper**				
Phosphatidylcholine							
Lyso-PC 18:1[I]	-0.0007	-0.0013	-0.00004	-0.395	2,32	-2.166	0.038*
PC 16:0_16:0	0.0431	0.0147	0.0715	0.527	2,33	3.088	0.004**
PC 16:0_18:2	0.0061	0.0019	0.0102	0.521	2,32	2.980	0.005**
PC 16:0_20:1	-0.0043	-0.0086	-0.0001	-0.399	2,31	-2.089	0.045*
PC 16:0_22:4	-0.0081	-0.0145	-0.0018	-0.446	2,29	-2.608	0.014*
PC 18:0_18:1	-0.0182	-0.0351	-0.0014	-0.402	2,32	-2.203	0.035*
PC 18:1_22:6	-0.0040	-0.0072	-0.0009	-0.469	2,33	-2.620	0.013*
Phosphatidylethanolamine							
PE 16:0_22:4	-0.0052	-0.0088	-0.0017	-0.534	2,32	-3.023	0.005**
PE 16:0_22:6	0.0171	0.0072	0.0269	0.597	2,30	3.550	0.001**
PE 18:0_18:1	-0.0110	-0.0181	-0.0039	-0.530	2,33	-3.125	0.004**
PE 18:0_20:4	-0.0348	-0.0595	-0.0102	-0.502	2,33	-2.872	0.007**
PE 18:0_22:4	-0.0202	-0.0335	-0.0069	-0.548	2,32	-3.094	0.004**
PE 18:0_22:6	0.0641	0.0075	0.1206	0.427	2,33	2.305	0.028*
PE 18:1_22:6	0.0084	0.0039	0.0130	0.589	2,33	3.757	0.0007***
PE 15:0_22:6 /O-16:0_22:6	0.0099	0.0015	0.0183	0.428	2,33	2.403	0.022*
Phosphatidylserine							
PS 18:0_18:1	-0.1699	-0.2468	-0.0929	-0.662	2,32	-4.498	0.00008***
PS 18:0_20:1	-0.0206	-0.0403	-0.0009	-0.396	2,31	-2.136	0.041*
PS 18:0_20:2[L]	-0.0026	-0.0047	-0.0004	-0.472	2,27	-2.412	0.023*
PS 18:0_22:4[L]	-0.0018	-0.0033	-0.0003	-0.445	2,33	-2.428	0.021*
PS 18:0_22:6	0.2064	0.1160	0.2968	0.692	2,32	4.652	0.00005***
PS 18:1_20:1	0.0179	0.0077	0.0281	0.570	2,32	3.574	0.001**

Phospholipid molecular species were detected using nanoelectrospray ionisation mass spectrometry and quantified as described in materials and methods. Regression was adjusted for gender. Superscript indicates dependent variable transformed for linear regression, with transformed regression output reported: [L] logarithm, [I] reciprocal. * P <0.05 ** P <0.01 *** P <0.001

Table 7-5: Quantified microsomal phospholipids (pmol/µg membrane protein) changing significantly with age in the human cerebellum.

Phospholipid	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Phosphatidylethanolamine							
PE 16:0_22:4	-0.0044	-0.0087	-0.0002	-0.398	2,30	-2.139	0.041*
Phosphatidylserine							
PS 18:0_18:1^{L}	-0.0031	-0.0055	-0.0006	-0.454	2,33	-2.568	0.015*
PS 18:0_20:1	-0.0142	-0.0251	-0.0033	-0.464	2,31	-2.658	0.012*
PS 18:0_20:2^{I}	-0.0030	-0.0051	-0.0008	-0.536	2,27	-2.834	0.009**
PS 18:0_22:4^{L}	-0.0024	-0.0045	-0.0002	-0.419	2,33	-2.263	0.030*
PS 18:0_22:5	-0.0212	-0.0389	-0.0036	-0.548	2,22	-2.493	0.021*
PS 18:1_20:1	0.0017	0.0002	0.0033	0.413	2,32	2.241	0.032*

Phospholipid molecular species were detected using nanoelectrospray ionisation mass spectrometry and quantified as described in materials and methods. Regression was adjusted for gender. Superscript indicates dependent variable transformed for linear regression, with transformed regression output reported: ^{L} logarithm, ^{I} reciprocal. * P <0.05 ** P <0.01

18:1_22:6 is a moderately abundant phospholipid comprising 2.9% of microsomal PE (Figure 7-7), and it increased in abundance by 26% from ages 20 to 100 (Figure 7-9, Table 7-4). The final microsomal PE containing a 22:6 fatty acid that increased with age, PE 15:0_22:6/O-16:0_22:6, showed a large increase in abundance with age of 61%. However, this phospholipid is only present in low amounts in the microsomes comprising less than 1.7% of microsomal PE (Figure 7-7) and 0.6% of total microsomal phospholipids.

Four microsomal PEs decreased in abundance with age in the cerebellum (Figure 7-9, Table 7-4). The most abundant microsomal PE to decline with age in was PE 18:0_20:4, the second-most abundant PE in the microsomes (Figure 7-7). This phospholipid comprised 16.0% of microsomal PE and 5.3% of the total microsomal phospholipid and declined by 16% in abundance over the 80 year period studied (Figure 7-9, Table 7-4). Two moderately abundant microsomal PEs decreased with age, PE 18:0_22:4 and PE 18:0_18:1, decreasing by 36% and 27% respectively over the 80 year period. The final microsomal PE to decline with age was PE 16:0_22:4, a low abundance phospholipid which fell by 38% from ages 20 to 100.

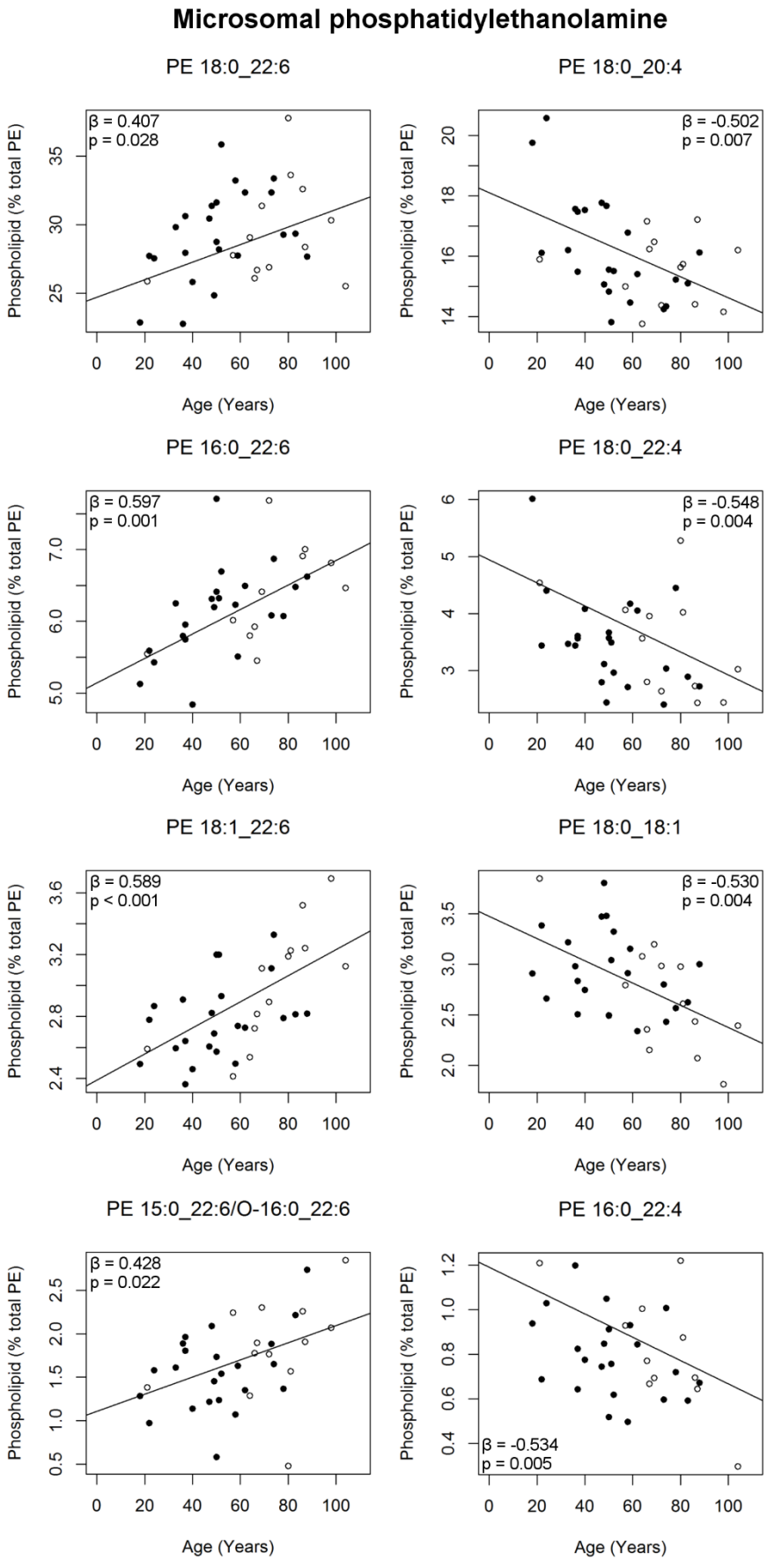

Microsomal phosphatidylethanolamine
PE 18:0_22:6
β = 0.407
p = 0.028
PE 18:0_20:4
β = -0.502
p = 0.007
PE 16:0_22:6
β = 0.597
p = 0.001
PE 18:0_22:4
β = -0.548
p = 0.004
PE 18:1_22:6
β = 0.589
p < 0.001
PE 18:0_18:1
β = -0.530
p = 0.004
PE 15:0_22:6/O-16:0_22:6
β = 0.428
p = 0.022
PE 16:0_22:4
β = -0.534
p = 0.005
Phospholipid (% total PE)
Age (Years)

Figure 7-9: Microsomal PEs changing significantly with age (as a percent of total PE) in normal human cerebellum (n = 33-36). Regression model was adjusted for sex: males (●), females (○). Regression parameters are shown in Table 7-4.

Only a single microsomal PE maintained its decrease with age when analysed as quantified amount (pmol/µg membrane protein), PE 16:0_22:4 (Table 7-5). This phospholipid decreased from 0.9 pmol/µg membrane protein at age 20 to 0.5 pmol/µg membrane protein at 100 years of age.

7.2.4.3 Phosphatidylserines

Six microsomal PSs changed with age in the cerebellum (Figure 7-10, Table 7-4). The most abundant PS in the microsomes, PS 18:0_22:6, increased from 34.5% of microsomal PS to 51.0%. Only one other microsomal PS increased significantly with age, PS 18:1_20:1, which doubled in abundance over the 80 year period studied. PS 18:1_20:1 is a low abundance phospholipid, however, making up only 1.9% of microsomal PS (Figure 7-7) and 0.3% of the total microsomal phospholipid.

Four microsomal PS phospholipids decreased with age (Figure 7-10, Table 7-4). The second-most abundant microsomal PS, PS 18:0_18:1, shrank by 38% from ages 20 to 100. PS 18:0_22:4 and PS 18:0_20:1, both moderately abundant microsomal PS phospholipids, decreased by 28% and 56% in abundance over the 80 year period respectively. The final PS phospholipid to change with age, PS 18:0_20:2, is of low abundance in microsomal membranes, comprising just about 1% of microsomal PS (Figure 7-7) and 0.2% of total microsomal phospholipid. PS 18:0_20:1 decreased from its initial abundance by 38% from ages 20 to 100.

Five of these microsomal PS phospholipids also changed with age in their quantified amount (pmol/µg membrane protein; Table 7-5). These included PS 18:0_18:1, PS 18:0_20:1, PS 18:0_20:2, PS 18:0_22:4 and PS 18:1_20:1. A sixth microsomal PS was also found to decline in quantified amount with age, PS 18:0_22:5, which decreased from 2.9 pmol/µg membrane protein to 1.2 pmol/µg membrane protein from ages 20 to 100.

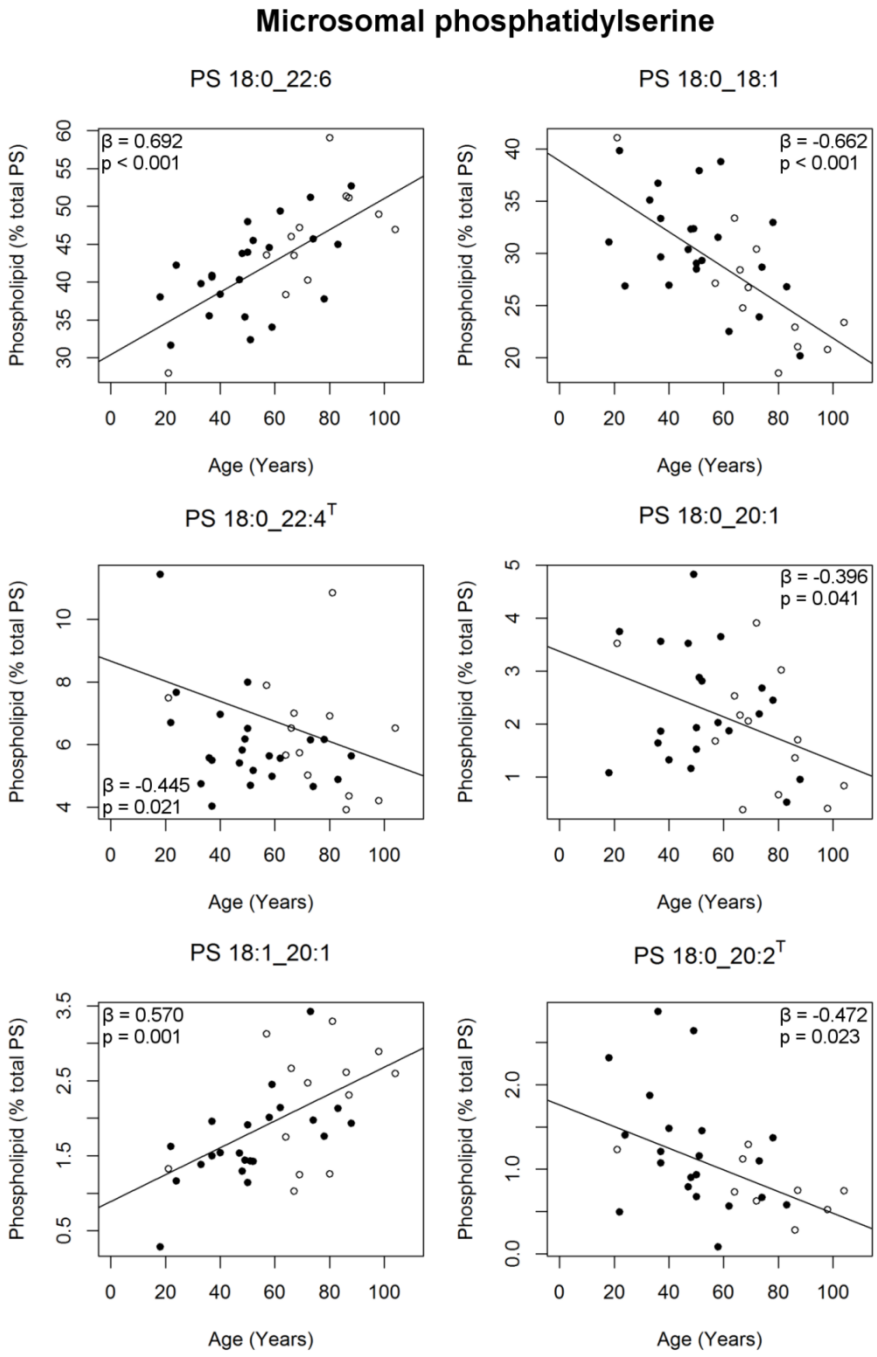


Figure 7-10: Microsomal PSs changing significantly with age (as a percent of total PS) in normal human cerebellum (n = 30-36). Regression model was adjusted for sex: males (●), females (○). ^{T} indicates dependent variable transformed for linear regression, with transformed beta-coefficient and p-value reported on original scatterplot for comparison. Regression parameters are shown in Table 7-4.

7.3 Discussion

There were many changes in PC, PE and PS phospholipids in the mitochondrial and microsomal membranes of the human cerebellum during normal ageing. In effect, the cerebellum experienced the highest number of changes to its phospholipids with age compared to any other region of the brain studied so far in this thesis. Of particular note were age-related increases in both mitochondrial and microsomal PS 18:0_22:6 and PE 18:0_22:6 over the 80 year period, as well as age-related increases in many other phospholipids containing DHA in both membrane fractions.

Both mitochondrial and microsomal PS 18:0_22:6 increased by approximately half their initial abundance from ages 20 to 100 (Figure 7-6, Figure 7-10). PS 18:0_22:6 is the most abundant PS present in both the mitochondrial and microsomal membranes, making up approximately 42% of mitochondrial and microsomal PS (Figure 7-3, Figure 7-7), and approximately 8-9% of total phospholipid in each fraction. Due to the high abundance of PS 18:0_22:6, these age-related increases in mitochondrial and microsomal PS 18:0_22:6 represent a substantial shift in the phospholipid composition of both membrane fractions in the cerebellum during normal ageing. Increases of a similar magnitude in PS 18:0_22:6 with age were also previously seen in the mitochondrial and microsomal membranes of the prefrontal cortex (Chapter 4), and in the mitochondria of the human hippocampus (Chapter 5). Other highly abundant phospholipids containing DHA also increased with age, including both mitochondrial and microsomal PE 18:0_22:6, a phospholipid that is equally as abundant as PS 18:0_22:6 (Figure 7-3, Figure 7-7). Both mitochondrial and microsomal PE 18:0_22:6 increased by one-fifth in abundance from ages 20 to 100 (Figure 7-5, Figure 7-9). This is the first instance of increasing amounts of mitochondrial and microsomal PE 18:0_22:6 with age in any brain region studied so far. Other phospholipids with DHA showing age-related increases included both mitochondrial and microsomal PE 16:0_22:6, PE 18:1_22:6, and PE 15:0_22:6/O-16:0_22, and microsomal PE O-18:1_22:6. The essentiality of DHA within the brain was established in Chapter 2 (section 2.1.4). As discussed in Chapter 4, DHA preferentially incorporates itself into phospholipids with a PS head group during biosynthesis in the brain [210], and increased PS 18:0_22:6 within neural cells is thought to promote longevity and decrease cellular apoptosis [191]. Unlike PS 18:0_22:6 there is little information available on the

possible role of PE 18:0_22:6 within cellular membranes, aside from its preference as a substrate for the synthesis of PS phospholipids with DHA by PSS2 (Chapter 6). Increases in PS synthesis by both PSS1 and PSS2 have been reported in the cerebellum of aged rats [47]; however, if this were also true for humans then we would expect to see a corresponding decrease in PE 18:0_22:6 and/or PC 16:0_22:6 with age and not the age-related increases observed for these phospholipids in this chapter

Due to the increased levels of phospholipids containing DHA in the mitochondrial and microsomal membranes of the cerebellum in older individuals, additional regressions with age were carried on the dominate PUFA in this region for both mitochondrial (Table 7-6) and microsomal (Table 7-7) membranes. Increases in total DHA were seen in both the PE and PS classes, as well as in the combined phospholipids of both the mitochondrial and microsomal membranes (Table 7-6 & Table 7-7). The increases in DHA with age in the combined phospholipids of the mitochondria occur at nearly twice the rate of that seen in the corresponding microsomal membranes. Looking at individual phospholipid classes, this disparity in the rate of increases in DHA between the two membrane fractions arises from PE-DHA phospholipids and not from PS-DHA. Both mitochondrial and microsomal PS phospholipids have a similar rate of increase with age, with both mitochondrial and microsomal PS-DHA increasing in amount within the PS class by 0.10% per year. Mitochondrial PE-DHA increased within the PE class by 0.09% per year (Table 7-6), however, a rate that is one-third higher than that seen for microsomal PE-DHA (0.06% per year, Table 7-7). Between the two membrane fractions a similar number of phospholipids increased with age, with the mitochondrial fraction only experiencing one more significant PE-DHA phospholipid: PE O-18:1_22:6 (Figure 7-5, Table 7-2). PE O-18:1_22:6 is not of high abundance within either the mitochondrial membranes, however, so it is unlikely that this single phospholipid is responsible for the large differences seen in the rate of increase between the mitochondrial and microsomal PE-DHA. Rather, each PE-DHA phospholipid in the mitochondrial fraction increased at a slightly higher rate per year than its microsomal counterpart (Table 7-2, Table 7-4). The only phospholipid class to not show any age-related changes in DHA in both the mitochondrial and microsomal fractions was PC, a phospholipid class that contains negligible amounts of DHA.

Table 7-6: Linear regression of most abundant polyunsaturated fatty acids (percent of total fatty acids within phospholipid class) in the mitochondrial membranes with age in human cerebellum

Mitochondrial fatty acids	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Combined phospholipids							
20:4	-0.0114	-0.0227	0.00001	-0.379	2,32	-2.034	0.050
22:4	-0.0185	-0.0276	-0.0094	-0.666	2,33	-4.146	0.0002***
20:4 + 22:4	-0.0304	-0.0471	-0.0137	-0.615	2,32	-3.717	0.0008***
22:6	0.0567	0.0337	0.0797	0.749	2,33	5.023	0.00002***
Phosphatidylcholine							
20:4	0.0049	-0.0050	0.0147	0.196	2,33	1.002	0.324
22:4	-0.0137	-0.0252	-0.00210	-0.423	2,33	-2.403	0.022*
20:4 + 22:4	-0.0088	-0.0180	0.0004	-0.327	2,33	-1.939	0.061
22:6	0.0061	-0.0011	0.0133	0.320	2,33	1.711	0.096
Phosphatidylethanolamine							
20:4	-0.0359	-0.0566	-0.0151	-0.589	2,32	-3.523	0.001**
$22:4^{I}$	-0.0006	-0.0010	-0.0003	0.594	2,33	3.512	0.001**
20:4 + 22:4	-0.0582	-0.0865	-0.0300	-0.675	2,32	-4.194	0.0002***
22:6	0.0930	0.0545	0.1315	0.734	2,33	4.916	0.00002***
Phosphatidylserine							
20:4	-0.0034	-0.0120	0.0052	-0.158	2,33	-0.802	0.429
22:4	-0.0229	-0.0394	-0.0064	-0.506	2,33	-2.825	0.008**
20:4 + 22:4	-0.0263	-0.0458	-0.0068	-0.494	2,33	-2.744	0.0097**
22:6	0.1050	0.0625	0.1474	0.742	2,32	5.036	0.00002***

Fatty acids calculated from phospholipid molecular species data. Regression was adjusted for gender. Superscript indicates dependent variable transformed for linear regression: ^{I}reciprocal. * P <0.05 ** P <0.01 *** P <0.001

Table 7-7: Linear regression of most abundant polyunsaturated fatty acids (percent of total fatty acids within phospholipid class) in the microsomal membranes with age in human cerebellum

Microsomal fatty acids	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Combined phospholipids							
20:4	-0.0039	-0.0132	0.0054	-0.166	2,33	-0.853	0.400
22:4	-0.0142	-0.0242	-0.0041	-0.511	2,33	-2.872	0.007**
20:4 + 22:4	-0.0181	-0.0332	-0.0029	-0.422	2,33	-2.428	0.021*
22:6	0.0319	0.0073	0.0564	0.477	2,33	2.637	0.013*
Phosphatidylcholine							
20:4	-0.0023	-0.0135	0.0089	-0.079	2,33	-0.417	0.679
22:4	-0.0055	-0.0135	0.0025	-0.270	2,33	-1.392	0.173
20:4 + 22:4	-0.0078	-0.0180	0.0024	-0.288	2,33	-1.559	0.129
22:6	-0.0033	-0.0117	-0.0050	-0.154	2,33	-0.812	0.423
Phosphatidylethanolamine							
20:4	-0.0151	-0.0339	0.0038	-0.311	2,33	-1.625	0.114
22:4	-0.0223	-0.0422	-0.0024	-0.419	2,33	-2.281	0.029*
20:4 + 22:4	-0.0374	-0.0660	-0.0088	-0.478	2,33	-2.657	0.012*
22:6	0.0642	0.0308	0.0976	0.644	2,33	3.910	0.0004***
Phosphatidylserine							
20:4	-0.0011	-0.0104	0.0082	-0.046	2,32	-0.238	0.814
22:4	-0.0193	-0.0341	-0.0046	-0.481	2,33	-2.664	0.012*
20:4 + 22:4	-0.0207	-0.0406	-0.0009	-0.394	2,33	-2.126	0.041*
22:6	0.1032	0.0580	0.1484	0.692	2,33	4.652	0.00005***

Fatty acids calculated from phospholipid molecular species data. Regression was adjusted for gender. * P <0.05 ** P <0.01 *** P <0.001

In the brain regions previously analysed in this thesis decreases with age were commonly seen in phospholipids containing either 20:4 or 22:4 fatty acids. Both 22:4 and 20:4 fatty acids can be putatively classified as the n-6 fatty acids adrenic acid and AA (Chapter 4, section 4.3). Decreases in adrenic acid and, less frequently, in AA with age were also seen across all three classes of phospholipid in both the mitochondrial and microsomal fractions of the cerebellum, (Table 7-6, Table 7-7). Losses of adrenic acid with age were seen in all three phospholipid classes individually, as well as in the combined phospholipids classes, in both membrane fractions with the exception of microsomal PC. Only mitochondrial PE reported losses of AA with age, however, despite the significant losses seen in both mitochondrial and microsomal PE 18:0_20:4 (Figure 7-5, Figure 7-9). Combining the two n-6 fatty acids together resulted in losses in both membrane fractions, with only mitochondrial and microsomal PC remaining unaffected (Table 7-6, Table 7-7).

The cerebellum is often used as a control tissue in the study of changes in membrane lipids during AD, a finding presumed to be due largely to the absence of any significant burden of intra- and extracellular protein aggregates compared with other brain regions [206,207]. Indeed, very few changes have been seen in the membrane lipids of cerebellum during the pathogenesis of AD compared with other regions [85,111,112,115,116,123]. Only in the white matter of the cerebellum have significant losses of phospholipids been reported in AD [85]. Only a single study has examined changes in cerebellar phospholipids during normal ageing, finding no changes to total PC or PE with advanced age [90]. No changes with age to either total PC or PE phospholipids in either the mitochondrial or microsomal membranes were observed in the current chapter, a finding that agrees with the previous study. However, numerous changes to molecular phospholipids were seen in all three phospholipids classes both membrane fractions. Indeed, the cerebellum underwent the most numerous changes to its molecular phospholipids during ageing compared to any region analysed so far in this thesis. The sheer number of changes to phospholipids of the cerebellum during normal ageing combined with its substantial cytoarchitectural differences to the rest of the brain suggest that the cerebellum may not be the ideal choice as a control tissue for studying the effects of age-related neurodegenerative diseases such as AD.

In summary, there were many changes seen in the PC, PE and PS phospholipids of the mitochondrial and microsomal membranes of the human cerebellum during normal ageing. Significant age-related increases were seen in mitochondrial and microsomal PS 18:0_22:6 and mitochondrial and microsomal PE 18:0_22:6, as well as many other phospholipids containing DHA. This led to increases with age in total DHA in PE, PS and combined phospholipids in both the mitochondria and microsomes. Age-related decreases were reported for total adrenic acid in all classes and membrane fraction except for microsomal PC. Despite significant losses of mitochondrial and microsomal PE 18:0_20:4 with age, no age-related changes were seen in either mitochondrial or microsomal PE-AA. The multitude of changes to the phospholipids of the human cerebellum during normal ageing compared with the other three brain regions studied suggests that the cerebellum may not be a good choice for examining the changes occurring to phospholipids in age-related neurodegenerative diseases such as AD.

Chapter 8 The Motor Cortex

8.1 Introduction

This chapter will investigate age-related changes to the PC, PE and PS of the mitochondrial and microsomal membranes in the human motor cortex.

The motor cortex is a brain region classified by closely related anatomical and functional areas. It is located in the posterior region of the frontal lobe anterior to the central sulcus (see Chapter 1, Figure 1-2). This area of the brain is also known as the agranular frontal cortex because it lacks an internal granular layer (layer IV). Another cytoarchitectural marker of the motor cortex is the presence of large neurons known as Betz cells that, along with other cortical neurons, extend long axons down the corticospinal tract. Many neurons from the motor cortex terminate in the brain stem, while others form reciprocal circuits between the motor cortex and somatosensory cortex that allow the translation of sensory information into motor action (reviewed by Rizzolatti et al. [211]).

There is some disagreement within the literature as to whether this region undergoes changes in grey matter volume and/or cortical thickness during normal ageing. Some studies have reported age-related changes in grey matter volume and/or cortical thickness within this region [16,18–23,212], while others have found the motor cortex to be relatively spared of these changes during normal ageing [24–26,213]. As discussed by Lemaître et al. [16], studies which have reported age-related declines in grey matter volume and/or cortical thickness within the motor cortex have contained large numbers of participants over the age of 60 years, suggesting that any changes occurring in this region happen late in life. The motor cortex also experiences very few changes in grey matter volume and/or cortical thickness until the later stages of AD [214–216], which corresponds to a later appearance of Aβ aggregates and tau NFTs within this region [7]. For these reasons, the motor cortex is often used as a control region when studying the pathogenesis of AD, although not as commonly as the cerebellum.

Currently, there are no published studies on whether the phospholipids of the primary motor cortex undergo significant changes during normal ageing. A single study has

examined the total phospholipid-fatty acids of an anatomically and functionally related brain region, the premotor area (BA 4), finding no changes with age [96]. Considering the relative stability of this region during both normal ageing and the pathogenesis of AD, it was hypothesised that very few changes would be detected in this region. Therefore, the aim of this chapter was to examine whether any changes occur to the phospholipids of the motor cortex with age in both the mitochondrial and microsomal membranes.

8.2 Results

8.2.1 Changes in total protein concentration during normal ageing

No age-related effects were observed for total protein concentration in the whole tissue homogenate, or in either the mitochondrial or microsomal fractions.

8.2.2 Major phospholipid classes of mitochondrial and microsomal fractions

Each of the three phospholipid classes measured in the mitochondrial and microsomal membranes of the motor cortex expressed as a percent of total detected phospholipid is shown in Figure 8-1. Approximately 56% of detected phospholipids in both membrane fractions were PC phospholipids, while PE and PS made up 27% & 17% respectively. The microsomal membranes contained slightly higher amounts of total PS compared to the mitochondrial membranes ($p < 0.01$). No statistically significant differences were seen between the membrane fractions for either PC or PE. Analysis of the quantified phospholipid classes (as pmol phospholipid/µg membrane lipid) showed similar results to the percent composition data. There was more PS in the microsomal fraction ($p < 0.05$), with no significant differences seen in PC or PE between the two membrane fractions. Total quantified phospholipids were also increased in the microsomal fraction ($p < 0.05$).

Total PC, PE, PS were also assessed for any age-related changes in both mitochondrial and microsomal membranes. No age-related changes to any class in either membrane fraction was observed when analysed as a percent of total detected phospholipid or as quantified phospholipids. Likewise, there were no changes with age total quantified phospholipids in either membrane fraction.

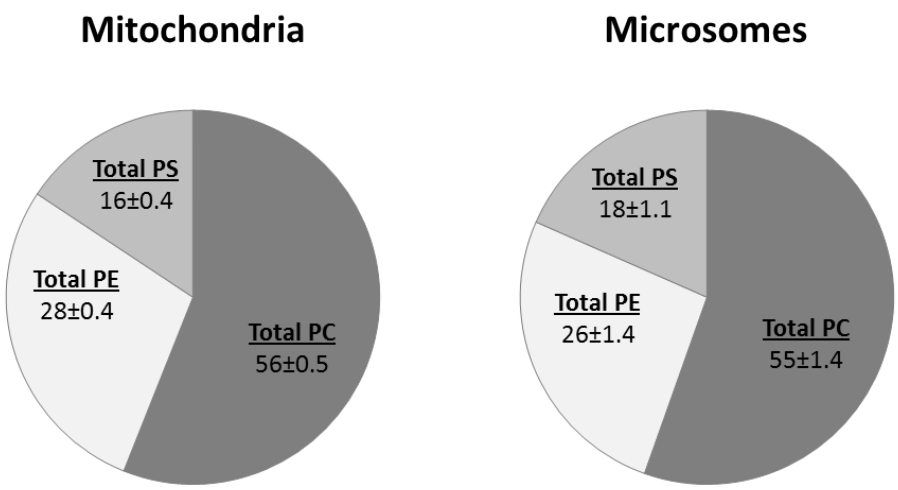


Figure 8-1: Percent composition of the three major phospholipid classes within the mitochondrial (left) and microsomal (right) fractions in neurologically normal motor cortex. Values are the mean ± SEM for the entire cohort. The microsomal membranes showed significantly higher amounts of total phosphatidylserine (PS) compared to mitochondria ($p < 0.01$, Wilcoxon signed-rank test). No statistically significant difference was seen for phosphatidylcholine (PC) or phosphatidylethanolamine (PE) between the mitochondria and microsomes.

8.2.3 Changes in mitochondrial phospholipids with age

8.2.3.1 Phosphatidylcholine

The composition of the PC, PE and PS phospholipids detected in the mitochondrial fraction can be seen in Figure 8-2. Three PCs changed with age when analysed as a percent of total mitochondrial PC: PC 16:0_20:4, PC 16:0_22:4 and PC 16:0_18:2 (Figure 8-3, Table 8-1). All three phospholipids are moderately abundant within mitochondrial membranes, comprising 3.6%, 2.0% and 1.3% of total mitochondrial PC respectively (Figure 8-2). Both PC 16:0_20:4 and PC 16:0_22:4 declined with age within mitochondrial membranes, with decreases of 17% and 41% in abundance seen respectively (Figure 8-3, Table 8-1). PC 16:0_18:2 increased with age, rising from 0.9% of total mitochondrial PC at age 20 to 1.5% at age 100, representing an increase of 59% from its initial abundance.

Only PC 16:0_22:4 retained its decrease with age within the mitochondria when analysed as quantified phospholipid (pmol of phospholipid/µg membrane protein). PC 16:0_22:4 decreased from 5.3 to 2.3 pmol/µg membrane protein over the 80 year period (Table 8-2)

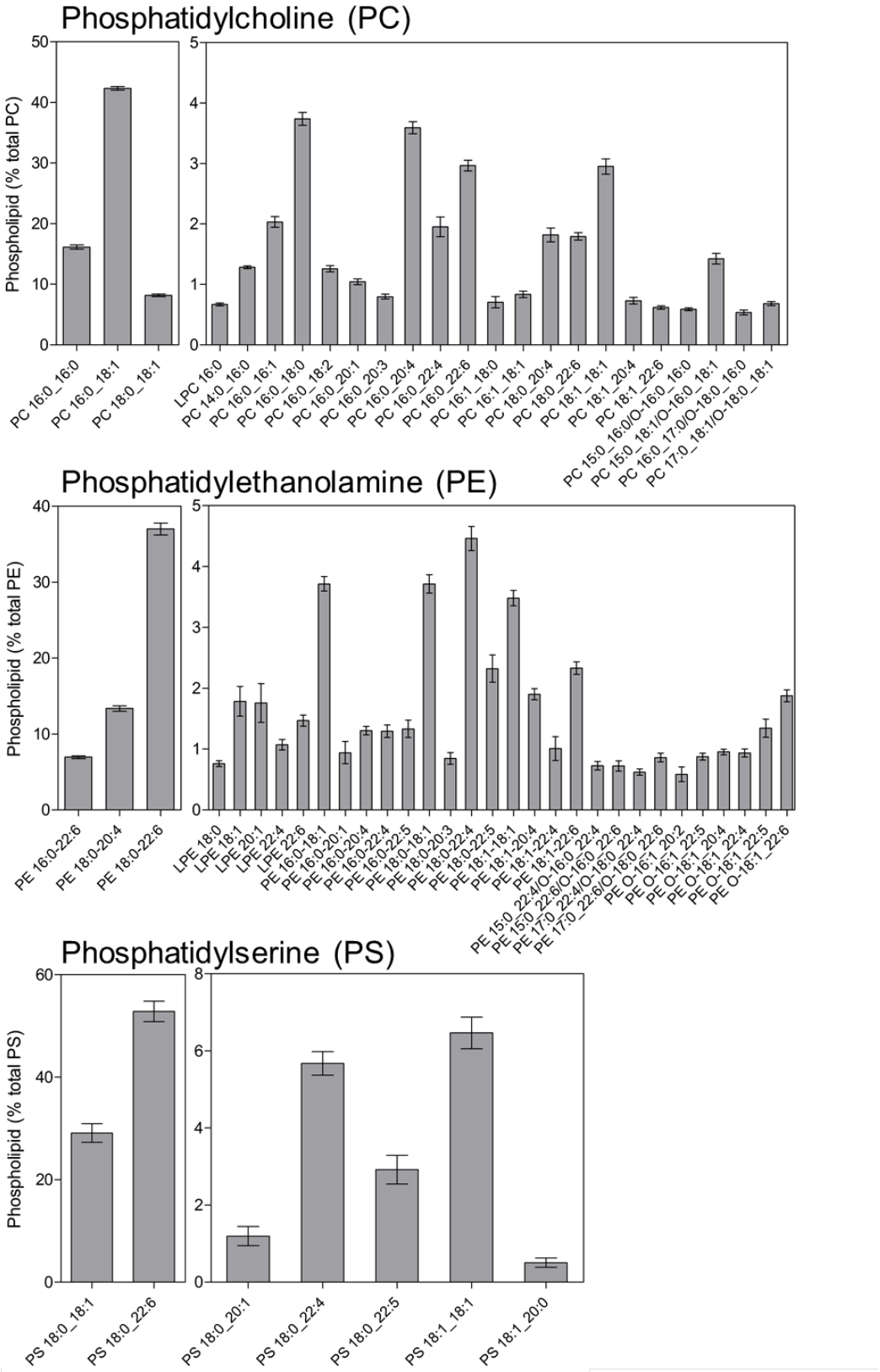


Figure 8-2: Phospholipids detected within PC, PE and PS in the mitochondrial fraction of human motor cortex (as a percent of total phospholipid within each class). Phospholipids were quantified as described in materials and methods. Values are mean across the cohort ± SEM.

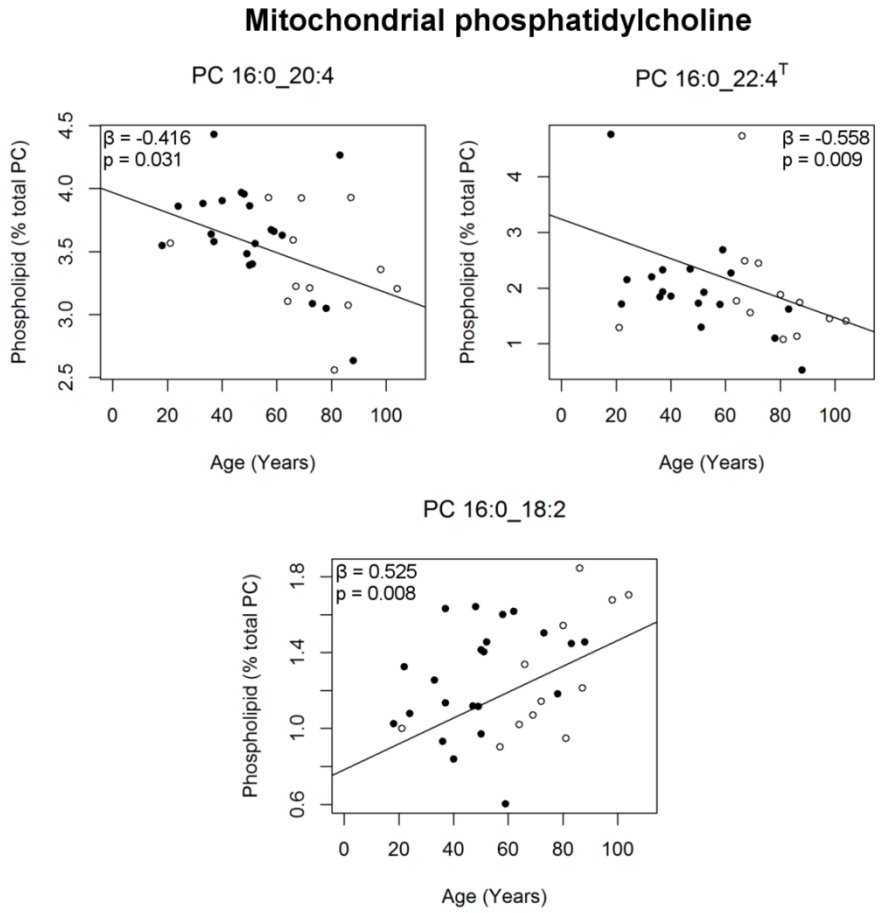


Figure 8-3: Mitochondrial PCs changing significantly with age (as a percent of total PC) in normal human motor cortex (*n* = 30-34). Regression model was adjusted for sex: males (●), females (○). ^{T} indicates dependent variable transformed for linear regression, with transformed beta-coefficient and p-value reported on original scatterplot for comparison. Regression parameters are shown in Table 8-1

Table 8-1: Mitochondrial phospholipids (percent of phospholipid within phospholipid class) changing significantly with age in the human motor cortex.

Phospholipid	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Phosphatidylcholine							
PC 16:0_18:2	0.0068	0.0019	0.0117	0.525	2,31	2.824	0.008**
PC 16:0_20:4	-0.0080	-0.0151	-0.0008	-0.416	2,30	-2.263	0.031*
PC 16:0_22:4[I]	-0.0021	-0.0038	-0.0006	-0.558	2,27	-2.779	0.0098**

Phospholipid molecular species were detected using nanoelectrospray ionisation mass spectrometry and quantified as described in materials and methods. Regression was adjusted for gender. Superscript indicates dependent variable transformed for linear regression, with transformed regression output reported: [I] reciprocal.
* *P* <0.05 ** *P* <0.01

Table 8-2: Quantified mitochondrial phospholipids (pmol/µg total membrane protein) changing significantly with age in the human motor cortex.

Phospholipid	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Phosphatidylcholine							
PC 16:0_22:4	-0.0370	-0.0707	-0.0034	-0.460	2,27	-2.260	0.032*
Phosphatidylethanolamine							
PE O-18:1_20:4	-0.0048	-0.0096	-0.00003	-0.394	2,31	-2.052	0.049*

Phospholipid molecular species were detected using nanoelectrospray ionisation mass spectrometry and quantified as described in materials and methods. Regression was adjusted for gender. ** *P* <0.01

8.2.3.2 Phosphatidylethanolamines

No mitochondrial PEs changed with age within the motor cortex when analysed as a percent of total PE, or when analysed as quantified mitochondrial PE (pmol of phospholipid/µg membrane protein)

8.2.3.3 Phosphatidylserines

Similarly to mitochondrial PE, no mitochondrial PS phospholipids changed with age in either their percent composition (percent of total mitochondrial PS) or quantified amount (pmol/µg membrane protein) in the motor cortex.

8.2.4 Changes in microsomal phospholipids with age

8.2.4.1 Phosphatidylcholines

The composition of PC, PE and PS phospholipids within the microsomal membranes can be seen in Figure 8-4. Within microsomal PC there were three phospholipids that changed with age in the motor cortex (Figure 8-5, Table 8-3). PC 18:0_20:4, PC 14:0_16:0 and PC 16:0_18:2 are all moderately abundant phospholipids within microsomal membranes. PC 18:0_20:4 comprises 1.7% of total microsomal PC (Figure 8-4), and it decreased by 28% in abundance from ages 20 to 100 (Figure 8-5, Table 8-3). Levels of both PC 14:0_16:0 and PC 16:0_18:2 rose with age within the microsomes, with increases of 15% and 51% seen respectively over the 80 year period.

Only PC 18:0_20:4 maintained its decrease with age within the microsomal membranes of the motor cortex when analysed as quantified phospholipid (pmol/µg membrane protein; Table 8-4). PC 18:0_20:4 decreased by 39% in quantified amount from ages 20 to 100 years of age.

8.2.4.2 Phosphatidylethanolamines

Five microsomal PEs changed with age within the motor cortex, with four of these phospholipids increasing in abundance with age (Figure 8-6, Table 8-3). PE O-18:1_22:6 is a moderately abundant microsomal PE present at 2.0% of total microsomal PE (Figure 8-4) and 0.5% of the total microsomal phospholipid. PE O-18:1_22:6 increased from its initial abundance by 16% from ages 20 to 100 (Figure 8-6, Table 8-3). PE O-18:1_22:5, PE 15:0_22:6/O-16:0_22:6 and PE 15:0_22:4/O-16:0_22:4 also increased in abundance with age, by 75%, 83% and 86% respectively over the 80 year

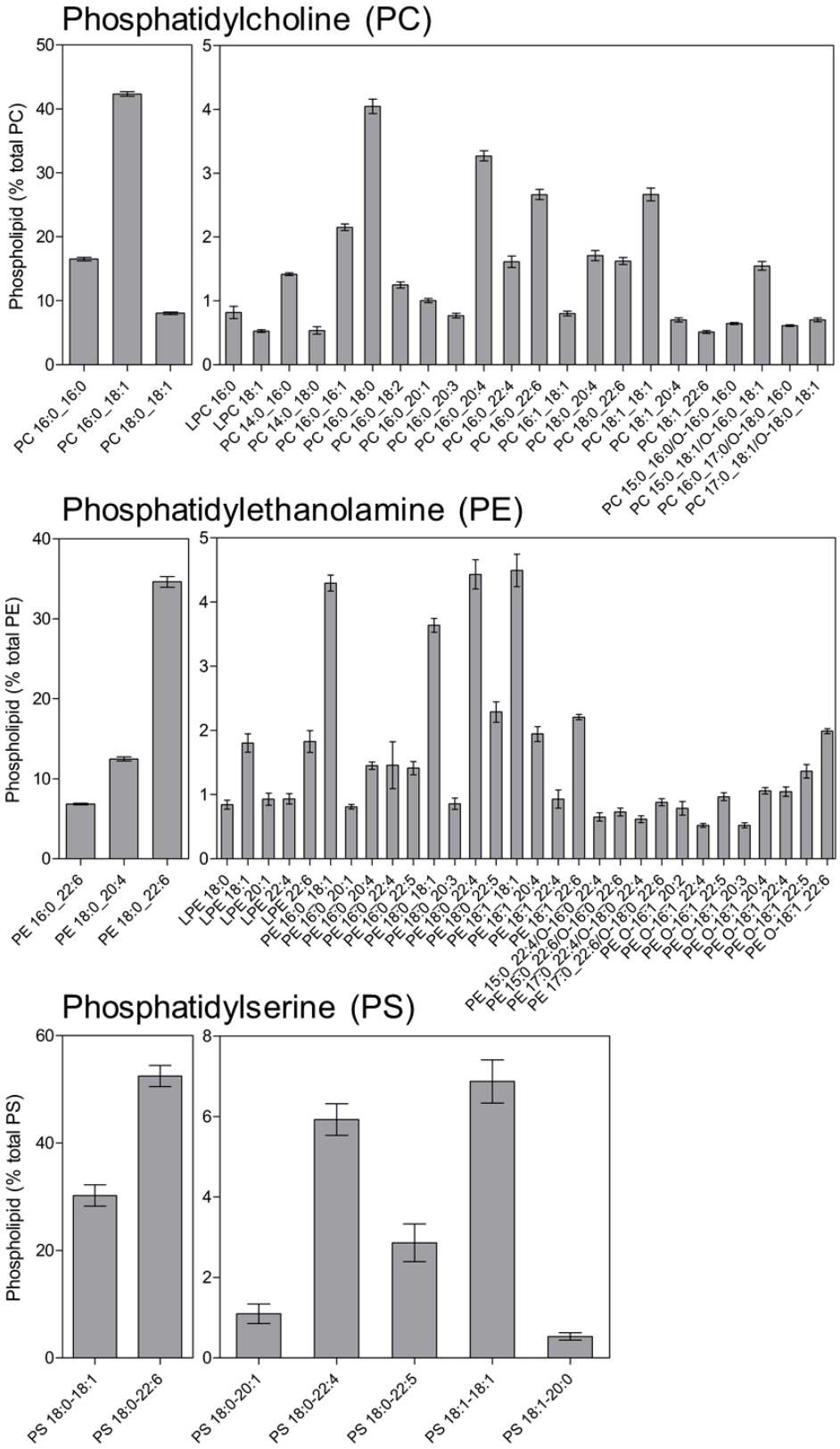


Figure 8-4: Phospholipids detected within PC, PE and PS in the microsomal fraction of human motor cortex (as a percent of total phospholipid with each class). Phospholipids were quantified as described in materials and methods. Values are mean across the cohort ± SEM.

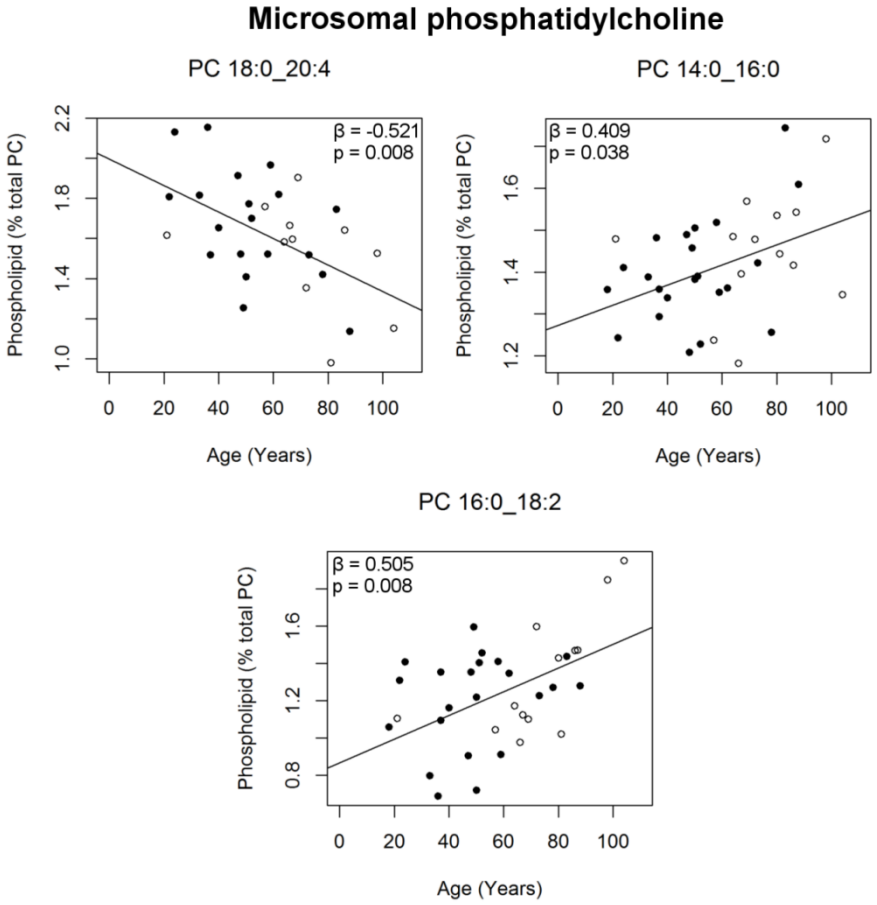


Figure 8-5: Microsomal PCs changing significantly with age (as a percent of total PC) in normal human m motor cortex (n = 30-35). Regression model was adjusted for sex: males (●), females (○). Regression parameters are shown in Table 8-3.

Table 8-3: Microsomal phospholipids (percent of phospholipid within phospholipid class) changing significantly with age in the human motor cortex.

Phospholipid	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Phosphatidylcholine							
PC 14:0_16:0	0.0024	0.0001	0.0047	0.409	2,32	2.168	0.038*
PC 16:0_18:2	0.0064	0.0018	0.0110	0.505	2,32	2.818	0.008**
PC 18:0_20:4	-0.0066	-0.0113	-0.0019	-0.521	2,27	-2.892	0.007**
Phosphatidylserine							
PE 15:0_22:4 /O-16:0_22:4^{IR}	0.0020	0.0002	0.0039	0.424	2,29	2.300	0.029*
PE 15:0_22:6 /O-16:0_22:6	0.0053	0.00008	0.0106	0.415	2,27	2.083	0.047*
PE O-16:1_22:4	-0.0039	-0.0068	-0.0011	-0.518	2,32	-2.854	0.008**
PE O-18:1_22:5	0.0104	0.0004	0.0205	0.378	2,32	2.111	0.043*
PE O-18:1_22:6	0.0038	0.0001	0.0076	0.377	2,32	2.097	0.044*

Phospholipid molecular species were detected using nanoelectrospray ionisation mass spectrometry and quantified as described in materials and methods. Regression was adjusted for gender. Superscript indicates dependent variable transformed for linear regression, with transformed regression output reported: ^{IR} reflect and reciprocal.
* P <0.05 ** P <0.01

Table 8-4: Quantified microsomal phospholipids (pmol/µg total membrane protein) changing significantly with age in the human motor cortex.

Phospholipid	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Phosphatidylcholine							
PC 18:0_20:4	-0.0197	-0.0332	-0.0061	-0.504	2,27	-2.974	0.006**

Phospholipid molecular species were detected using nanoelectrospray ionisation mass spectrometry and quantified as described in materials and methods. Regression was adjusted for gender. ** P <0.01

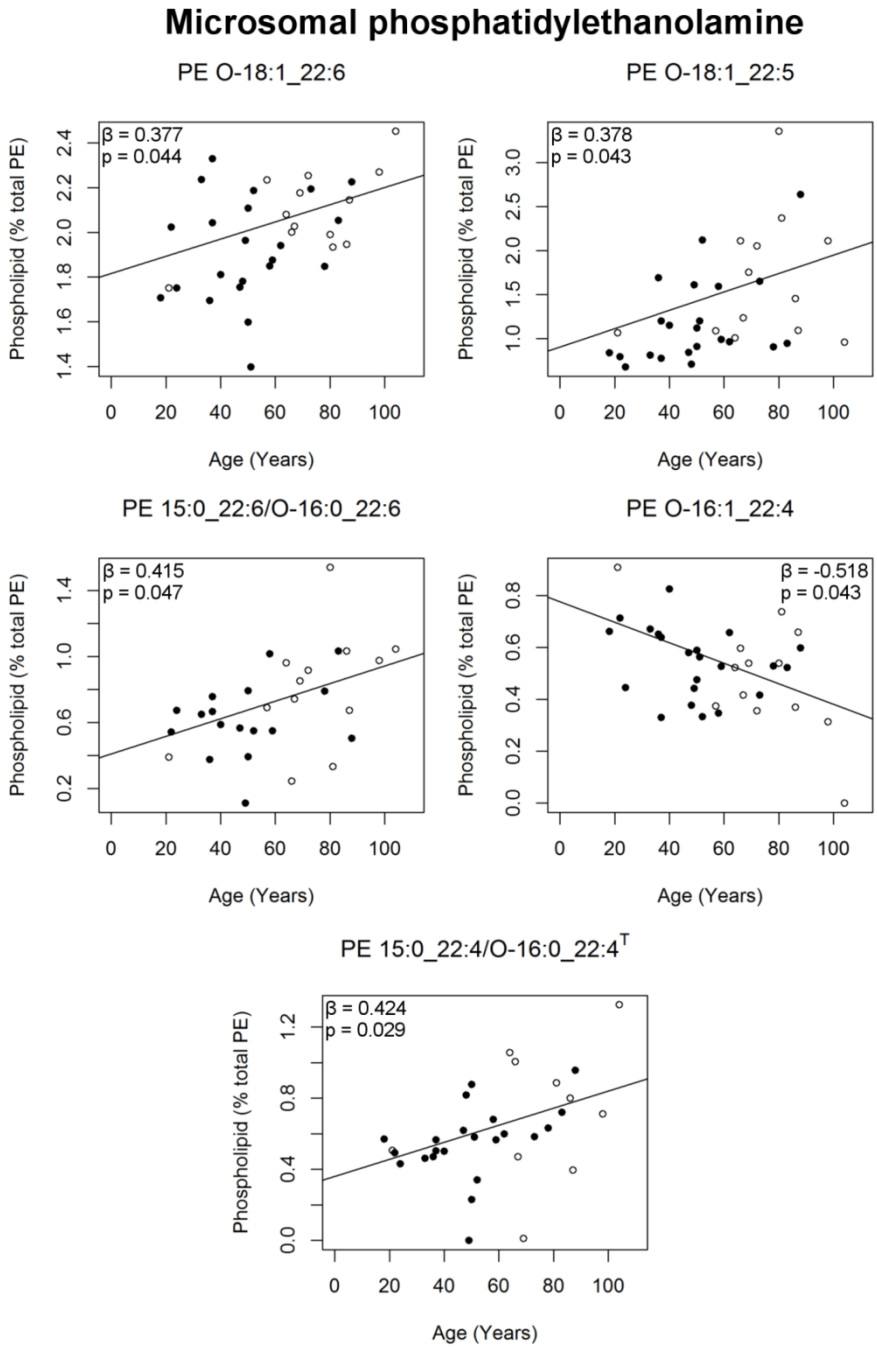


Figure 8-6: Microsomal PEs changing significantly with age (as a percent of total PC) in normal human m motor cortex (n = 30-35). Regression model was adjusted for sex: males (●), females (○). ^{T} indicates dependent variable transformed for linear regression, with transformed beta-coefficient and p-value reported on original scatterplot for comparison. Regression parameters are shown in Table 8-3

period. However these three phospholipids are only of low abundance in microsomal membranes, comprising only 2.7% of total microsomal PE when combined (Figure 8-4). The final microsomal PE to show a change with age, PE O-16:1_22:4, decreased in abundance from ages 20 to 100 by 45%. Similarly, PE O-16:1_22:4 is only of low abundance within microsomal membranes, comprising only 0.5% of total microsomal PE (Figure 8-4)

No microsomal PEs showed statistically significant changes with age when analysed as quantified phospholipid (pmol/µg membrane protein).

8.2.4.3 Phosphatidylserines

No microsomal PS phospholipids changed with age in the motor cortex in either percent composition (percent of total microsomal PS) or quantified amount (pmol/µg membrane protein).

8.3 Discussion

There were very few changes seen with age in the phospholipids that make up the mitochondrial and microsomal membranes of the human motor cortex compared to the previously four brain regions examined. All of the changes that occurred in the mitochondrial and microsomal membranes of the motor cortex took place in phospholipids that were of low-to-moderate abundance.

Notably, there were no age-related changes reported for any PS phospholipids in the motor cortex in either the mitochondrial or microsomal membranes. A similar trend was seen in the entorhinal cortex (Chapter 6), where no mitochondrial PS phospholipids changed with age, but the motor cortex is the only brain region examined to show no changes to both mitochondrial and microsomal PS. PS is synthesised in the ER, primarily within the MAM [217]. The MAM is commonly found in the mitochondrial fraction using the subcellular fractionation method employed in this study [41,218]. Phospholipids synthesised within the MAM are sent to the mitochondrial membranes by means of the close association between the two membranes [219]. As outlined in Chapter 2 (2.1.3), PS is produced in the MAM by serine-exchange with PC and PE by PSS1 and PSS2 respectively. Mitochondrial membranes also lack the ability to synthesise PE phospholipids *de novo*, and only a small amount of *de novo* synthesis of PE is conducted within the MAM. Instead, PSD converts PS into PE for use in the inner

and outer mitochondrial membranes [219]. Taken together, this relative lack of change in PS phospholipids within either the mitochondrial or microsomal membranes as well as no changes to PE phospholipids within the mitochondria suggests that homeostasis of PS metabolism is unperturbed in both mitochondrial and microsomal membranes within the motor cortex of the human brain over the adult lifespan. This combined with the lack of changes with age to the grey matter volume and/or cortical thickness of the motor cortex described in the introduction of this chapter (section 8.1) could also suggest that maintenance and regulation of these PS synthesis and degradation pathways could be crucial in preserving grey matter in healthy brain ageing.

Unlike the previous four regions analysed there did not seem to be any definite trend towards either losses with age of phospholipids containing adrenic acid (22:4) or AA (20:4), or increases with age in phospholipids containing DHA (22:6) in either membrane fraction within the motor cortex. Similar to the previously analysed brain regions, further linear regression of the most abundant PUFA with age was conducted to confirm these results for both mitochondrial (Table 8-5) and microsomal (Table 8-6) membranes. No changes with age were observed for either AA, adrenic acid, AA combined with adrenic or DHA in any phospholipid class or combined phospholipids in the mitochondrial fraction (Table 8-5). However, in the microsomes losses with age were found for AA in both PC and PE (Table 8-6). While this age-related loss of microsomal PC-AA could be explained by the loss of microsomal PC 18:0_20:4 seen with age (Figure 8-5, Table 8-3), no single PE phospholipid containing AA was identified as changing significantly with age in the microsomal fraction (Figure 8-6, Table 8-3). This finding implies that the loss of PE-AA with age seen in the microsomal fraction is likely the net result of small losses of many microsomal PE phospholipids containing AA with age, rather than being limited to a single, dominant phospholipid molecular species. Both AA and adrenic acid are long-chain n-6 PUFA, and AA combined with adrenic acid within microsomal PC and PE also declined with age (Table 8-6). This suggests that there is a loss of long chain n-6 PUFA with age within PC and PE in the microsomal membranes of the motor cortex, but the lack of change in AA and adrenic acid within the combined phospholipids suggests that there is no decline overall in total membrane long-chain n-6 PUFA with age

Table 8-5 Linear regression of the most abundant polyunsaturated fatty acids (percent of total fatty acids within phospholipid class) in the mitochondrial membranes with age in human motor cortex

Mitochondrial fatty acids	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Combined phospholipids							
20:4	-0.0023	-0.0138	0.0092	-0.080	2,32	-0.405	0.688
22:4	-0.0078	-0.0188	0.0031	-0.290	2,32	-1.458	0.155
20:4 + 22:4	-0.0101	-0.0242	0.0039	-0.280	2,32	-1.466	0.152
22:6	-0.00005	-0.0351	0.0350	-0.001	2,32	-0.003	0.998
Phosphatidylcholine							
20:4	0.0032	-0.0086	0.0150	0.111	2,32	-1.047	0.303
22:4	-0.0089	-0.0186	0.0008	-0.363	2,32	-1.860	0.072
20:4 + 22:4	-0.0057	-0.0172	0.0059	-0.201	2,32	-1.000	0.325
22:6	0.0012	-0.0076	0.0100	0.056	2,32	0.275	0.785
Phosphatidylethanolamine							
20:4	-0.0137	-0.0318	0.0044	-0.291	2,31	-1.540	0.134
22:4	-0.0085	-0.0358	0.0189	-0.129	2,32	-0.632	0.532
20:4 + 22:4	-0.0192	-0.0485	0.0102	-0.261	2,31	-1.332	0.193
22:6	0.0035	-0.0381	0.0451	0.035	2,31	0.171	0.865
Phosphatidylserine							
20:4	-0.0005	-0.0043	0.0032	-0.058	2,32	-0.283	0.779
22:4	-0.0017	-0.0213	0.0180	-0.036	2,32	-0.175	0.862
20:4 + 22:4	-0.0022	-0.0223	0.0179	-0.046	2,32	-0.224	0.825
22:6	0.0111	-0.0981	0.1203	0.042	2,32	0.207	0.837

Fatty acids calculated from phospholipid molecular species data. Regression was adjusted for gender.

Table 8-6 Linear regression of the most abundant polyunsaturated fatty acids (percent of total fatty acids within phospholipid class) in the microsomal membranes with age in human motor cortex

Microsomal fatty acids	Slope	95% CI		β	df	t	*P* value
		Lower	Upper				
Combined phospholipids							
20:4	-0.0081	-0.0196	0.0033	-0.278	2,32	-1.447	0.158
22:4	-0.0042	-0.0141	0.0057	-0.169	2,32	-0.867	0.392
20:4 + 22:4	-0.0012	-0.0281	0.0039	-0.276	2,32	-1.542	0.133
22:6	0.0050	-0.0507	0.0607	0.037	2,32	0.184	0.855
Phosphatidylcholine							
20:4	-0.0081	-0.0158	-0.0003	-0.400	2,32	-2.108	0.043*
22:4	-0.0007	-0.0066	0.0051	-0.050	2,32	0.233	0.817
20:4 + 22:4	-0.0088	-0.0160	-0.0017	-0.426	2,32	-2.509	0.017*
22:6	-0.0014	-0.0095	0.0066	-0.075	2,32	-0.365	0.717
Phosphatidylethanolamine							
20:4	-0.0166	-0.0315	-0.0017	-0.400	2,31	-2.271	0.030*
22:4	-0.0120	-0.0275	0.0036	-0.314	2,31	-1.569	0.127
20:4 + 22:4	-0.0284	-0.0524	-0.0043	-0.431	2,32	-2.405	0.022*
22:6	0.0268	-0.0152	0.0688	-0.259	2,32	1.300	0.203
Phosphatidylserine							
20:4	-0.0012	-0.0047	0.0024	-0.135	2,32	-0.660	0.514
22:4	0.0009	-0.0218	0.0236	0.016	2,32	0.080	0.937
20:4 + 22:4	0.0003	-0.0231	0.0226	-0.005	2,32	-0.023	0.982
22:6	0.0083	-0.0983	0.1150	0.032	2,32	0.159	0.875

Fatty acids calculated from phospholipid molecular species data. Regression was adjusted for gender. * $P < 0.05$

This relative lack of change in either the PUFA component of phospholipids combined with the few age-related changes seen in both mitochondrial and microsomal phospholipids suggests that the membrane composition of the motor cortex is the most stable during normal ageing compared to the other four brain regions examined in this thesis. As discussed in the introduction to this chapter, the greater motor area is thought to remain relatively unchanged in grey matter volume and thickness throughout ageing, and only exhibits losses in these structures with advanced age [16]. The motor cortex also remains relatively unchanged during the pathogenesis of AD, with losses of grey matter thickness and/or volume during the pathogenesis of AD not seen in the motor cortex until the very late stages of the diseases [214–216]. Likewise, the characteristic Aβ aggregates and tau NFTs also fail to appear in the motor cortex until the later stages of the disease [7]. The only study to examine changes in phospholipids with age in the motor cortex looked at total phospholipid fatty acids in a closely related region, the premotor area (BA 4) [96], finding no changes with age to any fatty acids in this region. The results of the present study combined with the relative lack of changes to grey matter thickness and/or volume reported in the motor cortex during both normal ageing and in diseases of ageing such as AD suggest that this region may be a good control for comparing and contrasting both age-related and AD-related changes to phospholipids. Indeed, the lack of age-related changes in the motor cortex make this region more suitable than the cerebellum, the other commonly used control region, which was found to undergo numerous changes in its phospholipids during normal ageing (Chapter 7).

In summary, there were very few changes occurring to the mitochondrial and microsomal phospholipids of the human motor cortex during normal ageing. All phospholipids that changed in composition with age were of low-to-moderate abundance within both the mitochondrial and microsomal fractions. No changes with age were seen in any PS phospholipid in either the mitochondrial or microsomal membranes in the motor cortex. Likewise, no age-related changes were observed for any mitochondrial PE phospholipids. Compared to previously studied regions, the motor cortex showed fewer changes in phospholipids containing PUFA, but some decreases with age in phospholipids containing AA and adrenic acid were seen in microsomal PC and PE. Considering these and previous findings, the motor cortex may be a better region to use as a control tissue than the cerebellum both when studying

changes in phospholipids during normal ageing and during the pathogenesis of age-related diseases such as AD.

SECTION 4

DISCUSSION & CONCLUSION

Chapter 9 Discussion and Conclusion

9.1 Introduction

The primary aim of this thesis was to determine if any age-related changes occur in phospholipids across five regions of the human brain. This chapter analyses the changes occurring across those brain regions in both the mitochondrial and microsomal membranes, and compares these results to that reported previously in the literature for both normal ageing and during the pathogenesis of the different dementias, specifically within AD. The findings from this thesis will then be examined to see where they fit into the two theories of ageing outlined in Chapter 2 (section 2.4); chiefly, either the mitochondrial free radical theory of ageing or the "inflammageing" theory of ageing. Finally, future recommendations for this research will be suggested

9.2 Regional differences in phospholipid composition during normal ageing

9.2.1 Method

To facilitate discussion of the results from the five different regions of the human brain examined within this thesis, the slope of the regression line for all of the statistically significant changes with age in the PC, PE and PS phospholipids of the prefrontal cortex, hippocampus, entorhinal cortex, cerebellum and motor cortex were collated and converted into heat maps for both the mitochondrial (Figure 9-1) and microsomal fractions (Figure 9-2). These heat maps were created in R (v. 3.1.1) using the "gplots" package. An example of the script used in R to create these heat maps is included in Appendix 1. Hierarchical clustering was used to look for any similarities in age-related phospholipid changes across the five regions examined within the two membrane fractions. The normalised slope of phospholipids transformed for linear regression was calculated from the back-transformed amounts present at 20 and 100 years of age to allow for comparison with non-transformed regressions. Phospholipids that underwent the largest age-related changes are present at the top of the heatmap, while those that experienced biggest decreases with age are at the bottom.

9.2.2 Results and discussion

9.2.2.1 Age-related changes in mitochondrial phospholipids

Hierarchical clustering analysis of the mitochondrial membranes of the five brain regions examined in this thesis found three distinct groups displaying similar changes with age: the cerebellum alone, the hippocampus and prefrontal cortex, and the motor and entorhinal cortices (Figure 9-1). The phospholipid with the largest age-related increase was PS 18:0_22:6 in the hippocampus, prefrontal cortex and cerebellum, followed by PE 18:0_22:6 in the cerebellum only. The largest decreases with age were seen in PS 18:0_18:1 within the cerebellum, PE 18:0_20:4 in the cerebellum and prefrontal cortex, and PS 18:0_22:4 in the cerebellum and hippocampus. Only a single phospholipid molecular species showed statistically significant age-related changes across all five regions of the brain: PC 16:0_18:2, which increases with age. Surprisingly, there were two mitochondrial phospholipids that showed an opposite trend with age between two different regions. In the entorhinal cortex PE 16:0_18:1 and PE 17:0_22:6/O-18:0_22:6 increased with age, whereas both phospholipids decreased with age in the prefrontal cortex.

The mitochondrial phospholipids of the cerebellum appeared to share many similar phospholipids undergoing age-related changes like those in the prefrontal cortex and hippocampus. All three regions experienced increases with age in PS 18:0_22:6, PE 18:1_22:6, and PC 16:0_18:2, and age-related decreases in PE 18:0_22:4. The cerebellum and prefrontal cortex share common increases with age in PC 16:0_22:6 and PE O-18:1_22:6 and common decreases in PE 18:0_18:1 and PE 18:0_20:4. However, in the cerebellum and hippocampus only decreases in PS 18:0_20:4 were found in common. The main difference separating the cerebellum from the prefrontal cortex and hippocampus is the magnitude of the age-related changes occurring. The cerebellum had many large changes with age clustered at the top and bottom of the heat map, indicating that these are comparatively large changes in phospholipids with age. However, both the hippocampus and prefrontal cortex had more phospholipids present in the middle of the heat map, which implied smaller but significant changes with age within these regions. The hippocampus in particular had many such small but statistically significant age-related changes, while the prefrontal cortex occupied the middle ground between the hippocampus and cerebellum.

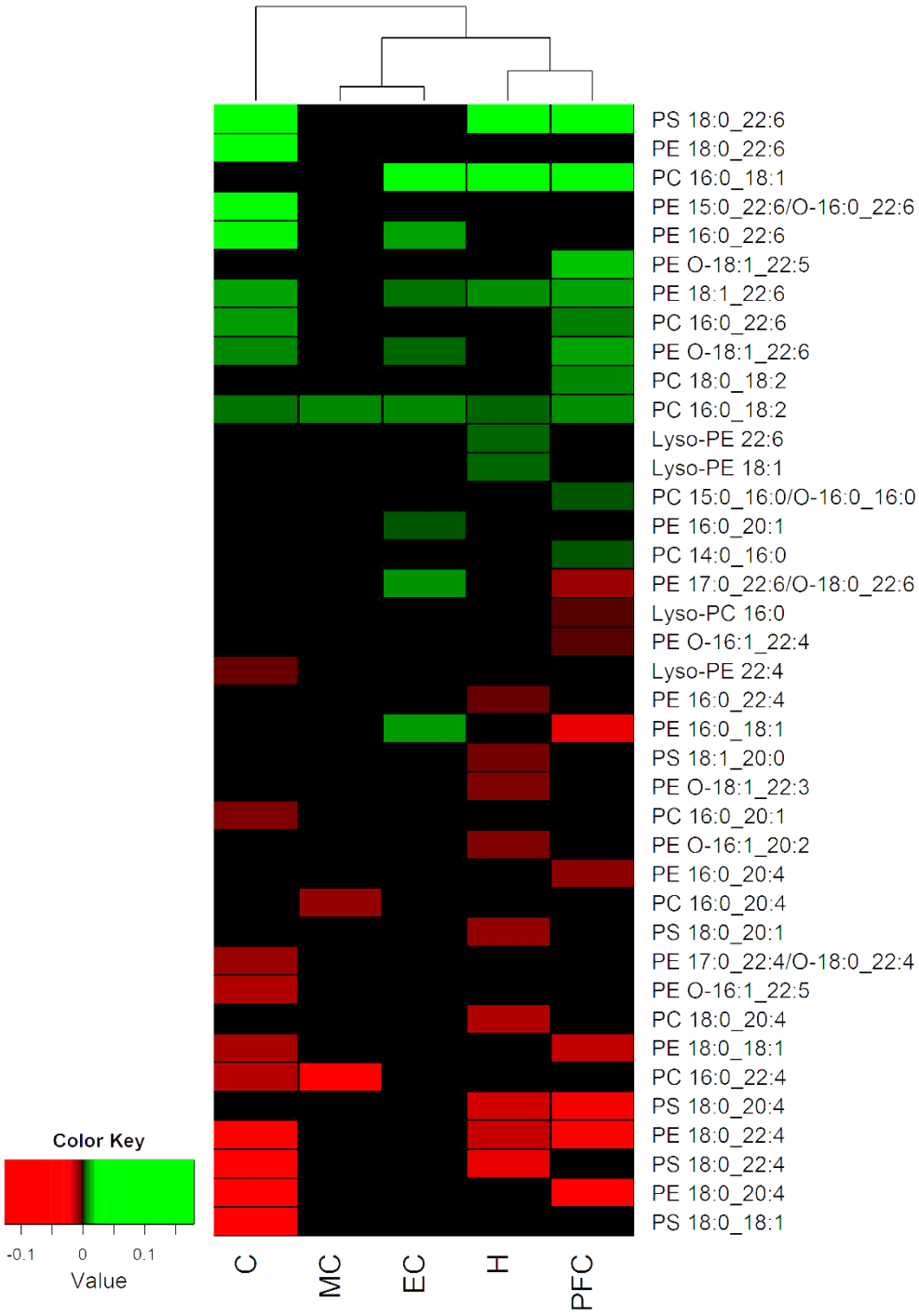


Figure 9-1: Summary of significant changes seen during normal ageing in the phospholipid composition (% of phospholipid class) in the mitochondrial fraction of the human prefrontal cortex (PFC), hippocampus (H), entorhinal cortex (EC), cerebellum (C), and motor cortex (MC). Increases in phospholipids with age are shown in green and decreases in red. Hierarchical clustering of the five brain regions is shown by the dendrogram at the top. Heat map was generated in R (v 3.1.1) using the gplots package.

The entorhinal and motor cortices are grouped together due to a lack of age-related changes in phospholipids within the mitochondrial membranes. Only three age-related changes in mitochondrial phospholipids were seen in the motor cortex: increases in PC 16:0_18:2 and decreases in PE 16:0_20:4 and PS 18:0_20:1. In contrast, the entorhinal cortex only exhibited increases with age within the mitochondrial membranes: in two PC phospholipids and six PEs. Common between the mitochondrial membranes of the two regions is a lack of age-related changes within the PS class. As discussed in Chapter 8 (section 8.1), the motor cortex, in particular, is known to exhibit few changes in grey matter volume and/or cortical thickness with age until advanced age, and so it is unsurprising that few age-related changes were seen to phospholipids in either membrane fraction within in this region.

9.2.2.2 Age-related changes in microsomal phospholipids

Analysis of all the statistically significant age-related changes seen in the microsomal membranes of the five brain regions by hierarchical clustering found both similarities and differences to that observed in the mitochondrial membranes. In the microsomes the five regions were classified into two groups: the first group consisting of the cerebellum only, and the second group containing the hippocampus, frontal, entorhinal and motor cortices (Figure 9-2). Within the second group, several sub-groups were also identified, with the entorhinal cortex and motor cortex being grouped together.

There were many similarities seen between the microsomal and mitochondrial membranes. The largest increase with age was in microsomal PS 18:0_22:6 in the cerebellum and prefrontal cortex, while the largest age-related decreases were seen in cerebellar PS 18:0_18:1 and PE 18:0_20:4. Again, there were several phospholipids showing changes with age in common between the cerebellum and prefrontal cortex. This included increases in PS 18:0_22:6, PE 18:1_22:6 and PC 16:0_18:2, and decreases in PE 18:0_18:1. Similar to the mitochondrial fraction, the main difference between the cerebellum and the other four brain regions appears to be due to the vast changes with age seen in several phospholipids of the cerebellum. Compared to the other four regions, many large age-related changes were observed in microsomal phospholipids of the cerebellum, as shown by the clustering of the data in the extreme ends of the heat map. A final similarity between both membrane fractions was the

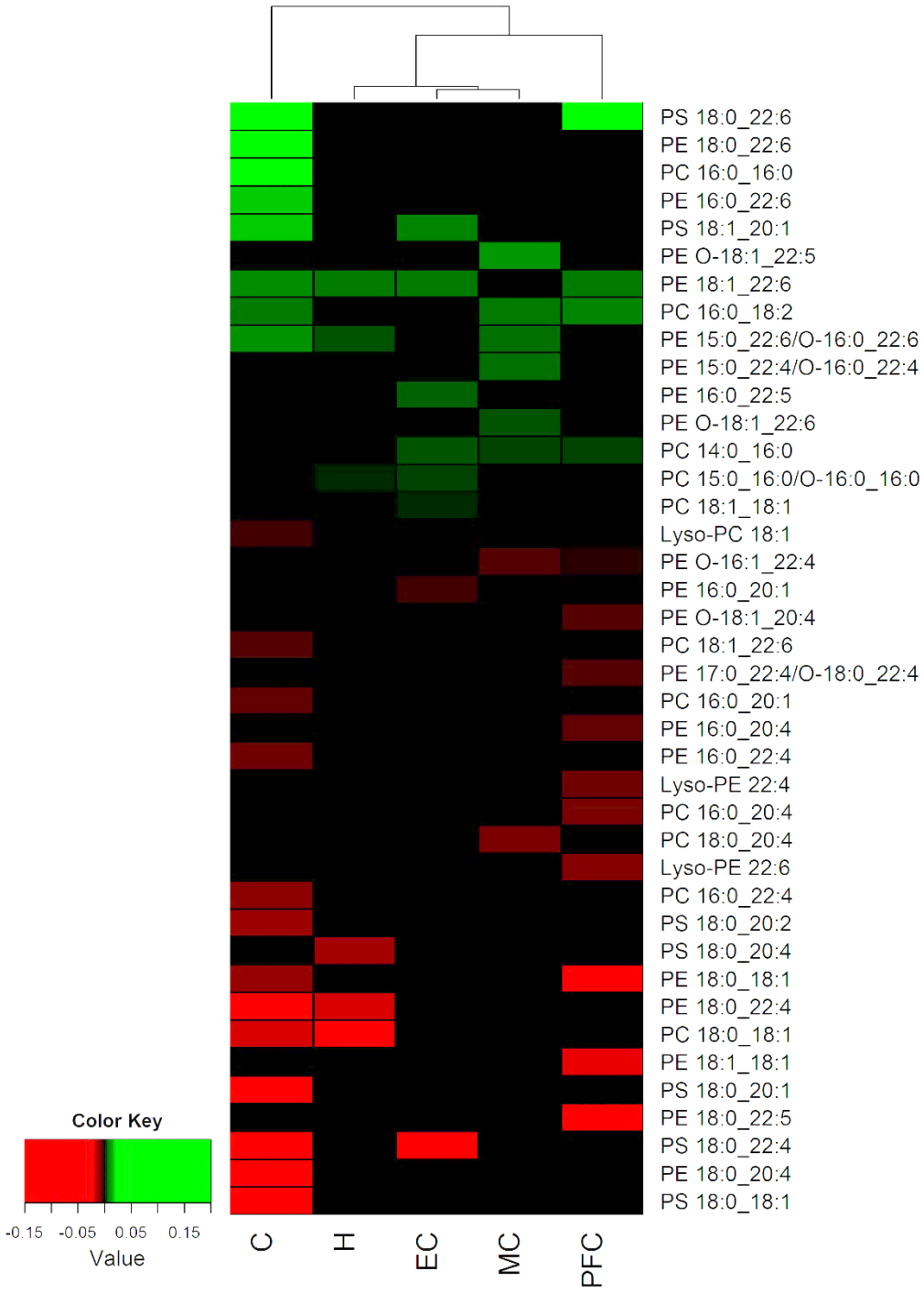


Figure 9-2: Summary of significant changes seen during normal ageing in the phospholipid composition (% of phospholipid class) in the microsomal fraction of the human prefrontal cortex (PFC), hippocampus (H), entorhinal cortex (EC), cerebellum (C), and motor cortex (MC). Increases in phospholipids with age are shown in green while decreases are in red. Hierarchical clustering of the five brain regions is shown by the dendrogram at the top. Heat map was generated in R (v 3.1.1) using the gplots package.

grouping of the entorhinal and motor cortices together on the basis of having the fewest changes with age to their phospholipids. However, there were a higher number phospholipids changing with age identified within the microsomal membranes of the motor cortex compared to the mitochondria. Unlike the mitochondrial membranes, the prefrontal cortex and hippocampus were not grouped together via hierarchical clustering within the microsomal membranes. Only a single phospholipid had age-related changes common to these two regions within the microsomal membranes: increases in PE 18:1_22:6. Indeed, more age-related changes were found in phospholipids common between the cerebellum and hippocampus than prefrontal cortex and hippocampus, including increases in PE 18:1_22:6 and PE 15:0_22:4/O-16:0_22:4, and decreases in PE 18:0_22:4 and PC 18:0_18:1. Additionally, the hippocampus displayed fewer overall changes with age in microsomal phospholipids compared to those seen in the mitochondrial fraction. Finally, unlike the mitochondrial fraction there was no single microsomal phospholipid that changed with age in all five regions with age. PC 16:0_18:2, which increased with age in the mitochondrial fraction of all five brain regions, only increased with age in the microsomal membranes of the cerebellum, motor and prefrontal cortices. The microsomal phospholipid with the highest number of changes across the five regions was PE 18:1_22:6, which increased with age in the cerebellum, hippocampus, and in the entorhinal and prefrontal cortices.

9.3 Comparison to current literature

In Chapter 2 (section 2.2) the current knowledge on changes in phospholipid composition in the human brain during normal ageing was reviewed. There are currently no studies available that examine changes in molecular phospholipids with age, but others have reported analysis of total phospholipids, phospholipid classes and phospholipid-fatty acids. Below is a brief analysis of how the results from this thesis compare to that reported in the literature for total phospholipids, phospholipids classes, and phospholipid-fatty acids.

9.3.1 Changes to phospholipids during normal ageing

9.3.1.1 Total phospholipids

No age-related changes were found in total quantified phospholipids within any of the five regions examined in the present study for either the mitochondrial or microsomal

fractions. This finding agrees with that found previously in the cerebellum [88,90], but disagrees with that of the frontal cortex [89–91,93] and hippocampus [90,93] (Chapter 2, Table 2-1). These discrepancies could be due to the differences in methods between the current and previous studies. All previous studies used the phosphorous assay to determine total phospholipid content, which measures all lipids with phosphorous present within the brain including phosphatidylglycerol, phosphatidylinositol, phosphatidic acid, cardiolipin and sphingomyelin. While phosphatidylglycerol and phosphatidic acid are only minor membrane constituents, phosphatidylinositol and sphingomyelin comprise a comparatively larger proportion of the cellular membranes within the brain [35] and significant age-related changes to either phospholipid class could feasibly influence changes in total phospholipids with age. Similarly, age-related changes to cardiolipin levels within the brain could affect the level of total phospholipid measured by the phosphorous assay. While the precise amount of cardiolipin present within mitochondria of the human brain is currently unknown, studies using rodents have estimated it at 9-10% of total mitochondrial phospholipid [220,221].

9.3.1.2 Phospholipid classes

Generally, the findings of the present thesis agree with the previous literature in respect to changes with age in total PC, PE and PS within the different regions of the human brain (Chapter 2, Table 2-2). Overall, no changes were seen with age to total PC, PE or PS within the frontal cortex, cerebellum and hippocampus, which agrees with the weight of evidence available for these three brain regions [90,91,93]. Only the entorhinal cortex changed with age in the amount of phospholipid classes present, including increases in mitochondrial PC and decreases in mitochondrial PE (Chapter 6), but no previous studies of age-related changes in phospholipids of this region are available for comparison.

9.3.1.3 Total phospholipid fatty acids

Within the five regions examined in this study analysis was only performed for changes with age to the predominant PUFA, and so only the current literature on these fatty acids have been included for analysis (Chapter 2, Table 2-3). Three of the five regions examined in this thesis have been previously studied for changes in total phospholipid fatty acids during normal ageing: the frontal and premotor cortices, as well as the hippocampus [92–96]. The present study agrees with previous findings of changes to

AA, adrenic acid and DHA with age within the human brain; however, some differences between the current and previous studies occurred. In the hippocampus, decreases in mitochondrial AA were seen with age while no changes in AA levels had been reported in this region previously [93]. Within the frontal cortex the present work found decreases in mitochondrial and microsomal AA with age, a finding which agrees that of McNamara et.al. [95]. However, this disagrees with three other studies that have reported no change in AA levels within the frontal cortex with age [92–94]. Likewise, increases with age in mitochondrial DHA were reported in the prefrontal cortex in the present study, which conflicts with previous studies that found no change in DHA with age in the frontal cortex [92–94]. The discrepancies between the current and previous findings within the frontal cortex could be explained by differences in methods used to identify and quantify phospholipids between the current and present studies. However, these dissimilarities might also be explained by differences between the anatomical and functional area of the frontal cortex examined by each study. The frontal cortex consists of a number of cyto- and myeloarchitectural regions divided on function, histology and white matter connections [222], all of which may have differing phospholipid compositions. The present study examined two regions of the frontal cortex: the dorsolateral prefrontal cortex (BA 9/46), and the primary motor cortex (BA 4). Other studies examined the frontal eye fields (BA 8) [92], the premotor cortex (BA 6) [96], the orbitofrontal cortex (BA 10) [95] or an undisclosed section of the frontal cortex [93,94]. For the purposes of comparison, the previous study of the premotor cortex [96] was grouped in with the results of this thesis for the primary motor cortex as they share similar histological features (i.e. lack of a granular layer). Likewise, the hippocampus proper can be divided into various subfields with different histology and connections to other regions of the brain, and feasibly each subfield could also have differences in phospholipid composition. This possibility was not explored in this thesis, however, due to the limited amount of tissue available for use.

9.3.1.4 Phospholipid fatty acids by class

Only a single study has examined the changes occurring in PC and PE fatty acids by class during normal ageing within the human brain, finding no changes with age to any PC or PE fatty acids within the frontal cortex or hippocampus [93]. The present results generally agree with those findings, with a few exceptions. Within the hippocampus no changes with age were seen in any of the three PUFA examined within the PC and PE

classes within both the mitochondria and microsomes, which agrees with the previous findings of Söderberg et al. [93]. However, there were several changes recorded within the PS class in the hippocampus, including decreases in mitochondrial PS-AA, decreases in mitochondrial and microsomal PS-adrenic acid, and increases in mitochondrial PS-DHA. Likewise, the present thesis also generally agrees with that reported previously for PC- and PE-PUFA in the frontal cortex, with the exception of decreases with age mitochondrial PC-adrenic acid, as well as in mitochondrial and microsomal PE-AA. Several changes with age were also seen for the first time in the frontal cortex in PS-fatty acids, including decreases in microsomal PS-AA and increases in mitochondrial and microsomal PS-DHA.

9.3.2 Changes to phospholipids in dementia compared to healthy ageing

As reviewed in Chapter 2 (section 2.3), many changes to phospholipids have been reported in the literature during the pathogenesis of the different dementias, with AD having the most evidence for an involvement of phospholipids. The changes observed to phospholipids in AD in previous studies could be classified into three broad categories: changes to the amount of total phospholipid present, changes in the amount of particular phospholipid classes, and changes to total or class-specific phospholipid-fatty acids.

The first characteristic separating the changes to phospholipids during normal ageing versus that seen in AD is the lack of changes in total quantified phospholipids in normal ageing. A number of changes to total phospholipids have been reported in several brain regions in AD, including decreases in frontal grey [86,109] and white matter [109,111], as well as in the hippocampus [86,109,110]. No changes to total phospholipid amount were observed in either the mitochondrial or microsomal membranes for any brain region studied in this thesis. The second factor separating the changes seen during normal ageing and those found in AD is the lack of changes to levels of phospholipid classes. The present work showed that the only region to exhibit any age-related changes to phospholipid classes was the entorhinal cortex, in which increases in mitochondrial PC and decreases in mitochondrial PE were observed (Chapter 6, Figure 6-2). Finally, and perhaps most importantly, a critical defining change was seen for the fatty acids present in phospholipids during normal ageing versus those reported previously for AD; namely that increases in DHA were observed during normal ageing. While many studies provided conflicting evidence on changes to DHA in AD (Chapter

2, section 2.3.1), most reported either a decrease [87,93,123] or no change [86,87,96,108,121] in DHA levels in either total phospholipid or within the phospholipid classes across various brain regions with the disease. However, in the present study increases were noted in many molecular phospholipids containing DHA in both membranes fraction and in nearly all of the regions studied. As stated in Chapter 2 (section 2.1.2), PUFA such as DHA have a number of important roles in cellular membranes. An absence of DHA from cellular membranes, as seen in the peroxisomal disorder Zellweger syndrome, results in acute neurological impairment and death within the first year of life (Chapter 2, section 2.1.4). We know that DHA is preferentially incorporated into PS phospholipids during biosynthesis in the brain and that an increased level of PS-DHA leads to decreased apoptosis within neuro-2A cells (Chapter 4, section 4.3). Therefore, it is feasible that increased levels of DHA with the cellular membranes of the human brain could be both promoting longevity and protecting against age-related neurodegenerative disease. So far clinical trials of DHA supplementation in the healthy elderly, elderly with mild cognitive impairment and elderly with neurodegenerative disease such as AD have shown mixed outcomes, with those with mild cognitive impairment showing the most consistent improvement across studies (reviewed by [73]). Understanding how this accretion of DHA within neural cellular membranes occurs, and how this may be influenced in order to promote healthy ageing and prevent the development of neurodegenerative disease such as AD is likely to be an area of interest in the future.

9.4 Theories of ageing

In Chapter 2 (section 2.4), the current theories of ageing pertaining to phospholipids were reviewed, including the mitochondrial and free radical oxidative theory of ageing and the "inflammageing" theory of ageing. The results of this thesis in relation to the tenets of these two theories will be outlined below

9.4.1 Mitochondrial free radical oxidative theory of ageing

The mitochondrial free radical oxidative theory of ageing was described in detail in Chapter 2 (section 2.4.2). Briefly, this theory proposes that ageing is the net result of damage to macromolecules such as protein, DNA and lipids over the lifetime of an organism by ROS produced by mitochondrial dysfunction. Within phospholipids, it is the fatty acids that are most susceptible to damage by ROS, chiefly PUFA that contain

more than one double bond (Chapter 2, Figure 2-7). Theoretically, the greater the number of double bonds the more susceptible the fatty acid is to oxidative damage. Accordingly, DHA, a PUFA with six double bonds, would be most susceptible to oxidative damage by ROS, alongside other PUFA such as docosapentaenoic acid (n-3 and n-6), and the n-6 fatty acids AA and adrenic acid. Because ROS are primarily generated in mitochondria, we would expect that there would be a greater loss of PUFA-containing phospholipids within this subcellular fraction.

Within this thesis, numerous changes occurred to PUFA-containing phospholipid molecular species in all regions of the brain. The findings generally followed the same trend across the different regions: decreases with age were observed in long chain n-6 PUFA such as AA and/or adrenic acid, whereas age-related increases with age were seen in phospholipids containing DHA. DHA is the only n-3 fatty acid found in measurable amounts in phospholipids of the human brain, with only traces of EPA (20:5n-3) known to be present. Indeed, no EPA was observed in this thesis in any region of the brain examined. No particular preferences for age-related changes were observed in the mitochondrial membranes versus microsomal. While the decreases in AA and adrenic acid found in this thesis were expected under the mitochondrial free radical oxidative theory of ageing, the increase in phospholipids containing DHA in many regions of the brain directly contradicts this theory. Therefore, the results of the thesis do not align with the mitochondrial free radical oxidative theory of ageing as being the driving force behind normal ageing in the human brain. However, these findings are far from conclusive, and require the further analysis of other molecules involved in this pathway.

9.4.2 “Inflammageing”

The “inflammageing theory of ageing was reviewed in Chapter 2 (section 2.4.3) and is the idea that ageing is driven by chronic, low-grade inflammation over the lifetime of an organism. Specifically, increased levels of inflammatory markers such as interleukins and tumour necrosis factors have been observed with advanced age [155–157]. Theoretically, phospholipids could play a role in the inflammageing theory, as AA preferentially cleaved from phospholipids is a precursor in several pro-inflammatory enzymatic pathways. Due to this, it is predicted by this theory that there will be a decline in phospholipids containing AA during ageing. Age-related decreases in

phospholipids containing other long-chain n-6 fatty acids such as adrenic and docosapentaenoic acid are also expected under this theory, as both can undergo β-oxidation within the peroxisomes to form AA (Chapter 2, Figure 2-4). Minimal changes with age in levels of phospholipids with DHA are expected under this theory due to the comparably low affinity of both cytosolic phospholipid A2 and COX-1/2 for DHA (Chapter 2, section 2.4.3). Additionally, the changes with age due to chronic, low-grade inflammation are not expected to be confined to a single membrane fraction.

Within the five regions of the human brain examined in this thesis decreases occurred with age in numerous phospholipids containing long-chain n-6 fatty acids, particularly AA and adrenic acid. n-6 docosapentaenoic acid was not able to be separated from its n-3 isomer by the mass spectrometry method used, and so any isomer-specific age-related changes to this fatty acid remain undiscovered. Increases with age in molecular phospholipids containing DHA were observed in both the mitochondrial and microsomal fractions of most of the regions of the brain examined in this thesis. The findings of the present work correspond to that predicted by the inflammageing theory, indicating that chronic, low-grade inflammation may by be a considerable driving force behind normal ageing within the human brain. However, more work is needed to confirm this finding, and any recommendations for future research to corroborate this involvement of phospholipids in the inflammageing theory of ageing will be discussed below.

9.5 Summary and future recommendations

The primary aim of this thesis was to quantify for the first time the changes occurring to molecular phospholipids within the three major classes during normal ageing across five regions of the human brain. There were three main findings that could be observed across the five regions. Firstly, there were no changes to total quantified phospholipids with ageing uncovered within this thesis. Secondly, few changes with age were observed in the total amount of PC, PE or PS phospholipids within the mitochondrial or microsomal membranes in most brain regions. The only region to experience such age-related changes was the entorhinal cortex, in which decreases in total mitochondrial PC and increases in total mitochondrial PE with age were observed (Chapter 6, section 6.2.2). Finally, many age-related changes were reported in a diverse range phospholipid molecular species within both the mitochondrial and microsomal membranes, with no

preference for changes in either membrane fraction over the other. Many phospholipids with n-6 fatty acids decreased with age in most of the examined brain regions while increases were seen in many phospholipids containing the n-3 fatty acid DHA. This age-related increase in DHA within the brain was a key feature that distinguished the changes to phospholipids during normal ageing from those reported in the literature for dementia such as AD (section 9.3.2). Alongside the lack of change to one specific membrane system over another (i.e. mitochondrial versus microsomal), the results of this thesis were determined to support the inflammageing theory of ageing rather than the mitochondrial free radical oxidative theory of ageing.

Since this is the first report of changes occurring to molecular phospholipids with age in the human brain, the findings of this thesis should be confirmed by future studies utilising a different, larger, cohort of subjects. The finding that DHA gradually increases within the phospholipids of the human brain with age in particular warrants further analysis to confirm and to establish the mechanism driving such changes. It will be difficult, if not impossible to test if diet drives this increase in DHA with age with a human post-mortem cohort. However, examining the changes with age in the activity of enzymes involved in the formation of DHA from short chain precursors in post-mortem tissue is a possibility, as well as the exploration of age-related changes in the deacylation and reacylation pathways that remodel phospholipids to contain predominately PUFA in the *sn*-2 position. All three phospholipid classes can be produced by remodelling pathways, some of which are known to have specificity for phospholipids containing DHA. PSS2 is such an enzyme, which synthesises PS from PE and is known to have a preference for phospholipids with DHA in the *sn*-2 position [46]. PSD, which synthesises PE preferentially from PS phospholipids with PUFA in the *sn*-2 position within the inner mitochondrial membrane [48], is another phospholipid synthesis pathway that should be targeted for future research. There is little information overall available on the activity of enzymes involved in the synthesis of molecular phospholipids within the human brain, and so investigating this and any age-related changes would fill a considerable gap in the current literature.

Due to the mass spectrometry methods used in this study, the changes with age within the brain to alkyl- and alkenyl-ether (plasmalogens) were not able to be separated from those of their odd-chain diacyl counterparts and so any age-related changes in these

phospholipids within the human brain remain to be discovered. Shotgun methods are available to separate such phospholipids from each other, either by derivatisation or by exploiting the lability of the ether-bond present. One example of such a method is the hydrolysis of the vinyl ether bond present in plasmalogens by acid vapour, a technique that separates plasmalogens from both isobaric alkyl-ether and odd-chain diacyl phospholipids [184]. Another involves selective derivitisation of plasmalogens within a lipid mixture, and the resulting mass-shift being used to identify plasmalogens preferentially from alkyl-ether and odd-chain diacyl phospholipids [223]. Alternatively, age-related changes to ether-phospholipids could be explored using liquid chromatography mass spectrometry. However, such techniques would require repeating the entire cohort and was unfeasible for the present thesis due to time and financial constraints. All of the samples extracted for this study have been stored, and so such a study could be conducted in the future.

The data collected within this thesis supported the predictions of the inflammageing theory of ageing over that of the mitochondrial free radical oxidative theory of ageing. However, to definitively determine what is driving age-related changes within the human brain more study need to be performed. Enzymatic (inflammation) and non-enzymatic (oxidative) pathways produce different molecules which can be measured in post-mortem tissue and quantified. Previous studies have found increases in several lipid products of oxidative stress with age, including MDA in the brain tissue of aged rats [139–143] and humans [144], 4-HNE in the brains of aged mice [145], senescence-accelerated mice [146], rats [147], and dogs [148], and F2-isoprostanes in human cerebrospinal fluid [149,150]. However, these studies are far from providing the complete assessment of age-related changes to the levels of oxidative products with the human brain, and much work remains to confirm these findings. A thorough literature search for any reported age-related changes in expression or activity levels of enzymes involved in the production of inflammatory eicosanoids from AA within any human tissue failed to find any such data. Therefore, it remains to be confirmed if the changes seen during normal ageing within this thesis can be directly attributed to low-grade chronic inflammation.

Bibliography

[1] WHO, "Global health and ageing," Oct-2011. [Online]. Available: http://www.who.int/ageing/publications/global_health/en/index.html. [Accessed: 12-Feb-2014].

[2] Australian Bureau of Statistics, *Census of Population and Housing: Ageing in Australia 2001*. Canberra, Australia, 2003.

[3] Australian Bureau of Statistics, *Population Projections Australia: 2006 to 2101*. Canberra, Australia, 2008.

[4] World Health Organisation and Alzheimer's Disease International, "Dementia: a public health priority," *WHO*, 2012. [Online]. Available: http://www.who.int/mental_health/publications/dementia_report_2012/en/. [Accessed: 12-Feb-2014].

[5] Australian Institute of Health and Welfare, *Dementia in Australia*. Canberra, Australia, 2012.

[6] H. Braak and E. Braak, "Staging of alzheimer's disease-related neurofibrillary changes," *Neurobiol. Aging*, vol. 16, no. 3, pp. 271–278, May 1995.

[7] H. Braak and E. Braak, "Neuropathological stageing of Alzheimer-related changes," *Acta Neuropathol. (Berl.)*, vol. 82, no. 4, pp. 239–259, 1991.

[8] R. H. Swerdlow, "Brain aging, Alzheimer's disease, and mitochondria," *Biochim. Biophys. Acta BBA - Mol. Basis Dis.*, vol. 1812, no. 12, pp. 1630–1639, Dec. 2011.

[9] M. N. Braskie and P. M. Thompson, "Understanding cognitive deficits in Alzheimer's disease based on neuroimaging findings," *Trends Cogn. Sci.*, vol. 17, no. 10, pp. 510–516, Oct. 2013.

[10] K. Blennow, M. J. de Leon, and H. Zetterberg, "Alzheimer's disease," *Lancet*, vol. 368, no. 9533, pp. 387–403, Jul. 2006.

[11] W. J. Lukiw, M. Pappolla, R. P. Pelaez, and N. G. Bazan, "Alzheimer's disease--a dysfunction in cholesterol and lipid metabolism," *Cell. Mol. Neurobiol.*, vol. 25, no. 3–4, pp. 475–483, Jun. 2005.

[12] M. M. Mielke and C. G. Lyketsos, "Lipids and the pathogenesis of Alzheimer's disease: is there a link?," *Int. Rev. Psychiatry Abingdon Engl.*, vol. 18, no. 2, pp. 173–186, Apr. 2006.

[13] L. Bertram and R. E. Tanzi, "Genome-wide association studies in Alzheimer's disease," *Hum. Mol. Genet.*, vol. 18, no. R2, pp. R137–R145, Oct. 2009.

[14] K. Bettens, K. Sleegers, and C. Van Broeckhoven, "Genetic insights in Alzheimer's disease," *Lancet Neurol.*, vol. 12, no. 1, pp. 92–104, Jan. 2013.

[15] D. Harold, R. Abraham, P. Hollingworth, R. Sims, A. Gerrish, M. L. Hamshere, J. S. Pahwa, V. Moskvina, K. Dowzell, A. Williams, N. Jones, C. Thomas, A. Stretton, A. R. Morgan, S. Lovestone, J. Powell, P. Proitsi, M. K. Lupton, C. Brayne, D. C. Rubinsztein, M. Gill, B. Lawlor, A. Lynch, K. Morgan, K. S. Brown, P. A. Passmore, D. Craig, B. McGuinness, S. Todd, C. Holmes, D. Mann, A. D. Smith, S. Love, P. G. Kehoe, J. Hardy, S. Mead, N. Fox, M. Rossor, J. Collinge, W. Maier, F. Jessen, B. Schürmann, R. Heun, H. van den Bussche, I. Heuser, J. Kornhuber, J. Wiltfang, M. Dichgans, L. Frölich, H. Hampel, M. Hüll, D. Rujescu, A. M. Goate, J. S. K. Kauwe, C. Cruchaga, P. Nowotny, J. C. Morris, K. Mayo, K. Sleegers, K. Bettens, S. Engelborghs, P. P. De Deyn, C. Van Broeckhoven, G. Livingston, N. J. Bass, H. Gurling, A. McQuillin, R. Gwilliam, P. Deloukas, A. Al-Chalabi, C. E. Shaw, M. Tsolaki, A. B. Singleton, R.

Guerreiro, T. W. Mühleisen, M. M. Nöthen, S. Moebus, K.-H. Jöckel, N. Klopp, H.-E. Wichmann, M. M. Carrasquillo, V. S. Pankratz, S. G. Younkin, P. A. Holmans, M. O'Donovan, M. J. Owen, and J. Williams, "Genome-wide association study identifies variants at CLU and PICALM associated with Alzheimer's disease," *Nat. Genet.*, vol. 41, no. 10, pp. 1088–1093, Oct. 2009.

[16] H. Lemaître, A. L. Goldman, F. Sambataro, B. A. Verchinski, A. Meyer-Lindenberg, D. R. Weinberger, and V. S. Mattay, "Normal age-related brain morphometric changes: nonuniformity across cortical thickness, surface area and gray matter volume?," *Neurobiol. Aging*, vol. 33, no. 3, pp. 617.e1–617.e9, Mar. 2012.

[17] A. M. Fjell, L. T. Westlye, H. Grydeland, I. Amlien, T. Espeseth, I. Reinvang, N. Raz, A. M. Dale, and K. B. Walhovd, "Accelerating Cortical Thinning: Unique to Dementia or Universal in Aging?," *Cereb. Cortex*, vol. 24, no. 4, pp. 919–934, Apr. 2014.

[18] L. J. Hogstrom, L. T. Westlye, K. B. Walhovd, and A. M. Fjell, "The Structure of the Cerebral Cortex Across Adult Life: Age-Related Patterns of Surface Area, Thickness, and Gyrification," *Cereb. Cortex*, vol. 23, no. 11, pp. 2521–2530, Nov. 2013.

[19] J. Jiang, P. Sachdev, D. M. Lipnicki, H. Zhang, T. Liu, W. Zhu, C. Suo, L. Zhuang, J. Crawford, S. Reppermund, J. Trollor, H. Brodaty, and W. Wen, "A longitudinal study of brain atrophy over two years in community-dwelling older individuals," *NeuroImage*, vol. 86, pp. 203–211, Feb. 2014.

[20] D. H. Salat, R. L. Buckner, A. Z. Snyder, D. N. Greve, R. S. R. Desikan, E. Busa, J. C. Morris, A. M. Dale, and B. Fischl, "Thinning of the Cerebral Cortex in Aging," *Cereb. Cortex*, vol. 14, no. 7, pp. 721–730, Jul. 2004.

[21] D. A. Ziegler, O. Piguet, D. H. Salat, K. Prince, E. Connally, and S. Corkin, "Cognition in healthy aging is related to regional white matter integrity, but not cortical thickness," *Neurobiol. Aging*, vol. 31, no. 11, pp. 1912–1926, Nov. 2010.

[22] C. D. Good, I. S. Johnsrude, J. Ashburner, R. N. A. Henson, K. J. Friston, and R. S. J. Frackowiak, "A Voxel-Based Morphometric Study of Ageing in 465 Normal Adult Human Brains," *NeuroImage*, vol. 14, no. 1, pp. 21–36, Jul. 2001.

[23] H. Lemaître, F. Crivello, B. Grassiot, A. Alpérovitch, C. Tzourio, and B. Mazoyer, "Age- and sex-related effects on the neuroanatomy of healthy elderly," *NeuroImage*, vol. 26, no. 3, pp. 900–911, Jul. 2005.

[24] H. Oh, C. Madison, S. Villeneuve, C. Markley, and W. J. Jagust, "Association of Gray Matter Atrophy with Age, β-Amyloid, and Cognition in Aging," *Cereb. Cortex*, vol. 24, no. 6, pp. 1609–1618, Jun. 2014.

[25] N. Raz, F. M. Gunning, D. Head, J. H. Dupuis, J. McQuain, S. D. Briggs, W. J. Loken, A. E. Thornton, and J. D. Acker, "Selective aging of the human cerebral cortex observed in vivo: differential vulnerability of the prefrontal gray matter.," *Cereb. Cortex*, vol. 7, no. 3, pp. 268–282, Apr. 1997.

[26] S. M. Resnick, D. L. Pham, M. A. Kraut, A. B. Zonderman, and C. Davatzikos, "Longitudinal Magnetic Resonance Imaging Studies of Older Adults: A Shrinking Brain," *J. Neurosci.*, vol. 23, no. 8, pp. 3295–3301, Apr. 2003.

[27] E. R. Sowell, B. S. Peterson, P. M. Thompson, S. E. Welcome, A. L. Henkenius, and A. W. Toga, "Mapping cortical change across the human life span," *Nat. Neurosci.*, vol. 6, no. 3, pp. 309–315, Mar. 2003.

[28] J. Aanerud, P. Borghammer, M. M. Chakravarty, K. Vang, A. B. Rodell, K. Y. Jónsdottir, A. Møller, M. Ashkanian, M. S. Vafaee, P. Iversen, P. Johannsen, and

A. Gjedde, “Brain energy metabolism and blood flow differences in healthy aging,” *J. Cereb. Blood Flow Metab.*, vol. 32, no. 7, pp. 1177–1187, Jul. 2012.

[29] S. J. Blanksby and T. W. Mitchell, “Advances in mass spectrometry for lipidomics,” *Annu. Rev. Anal. Chem. Palo Alto Calif*, vol. 3, pp. 433–465, 2010.

[30] L. Yetukuri, K. Ekroos, A. Vidal-Puig, and M. Orešič, “Informatics and computational strategies for the study of lipids,” *Mol. Biosyst.*, vol. 4, no. 2, pp. 121–127, Jan. 2008.

[31] A. A. Farooqui, L. A. Horrocks, and T. Farooqui, “Glycerophospholipids in brain: their metabolism, incorporation into membranes, functions, and involvement in neurological disorders,” *Chem. Phys. Lipids*, vol. 106, no. 1, pp. 1–29, Jun. 2000.

[32] G. van Meer, D. R. Voelker, and G. W. Feigenson, “Membrane lipids: where they are and how they behave,” *Nat. Rev. Mol. Cell Biol.*, vol. 9, no. 2, pp. 112–124, Feb. 2008.

[33] V. Frisardi, F. Panza, D. Seripa, T. Farooqui, and A. A. Farooqui, “Glycerophospholipids and glycerophospholipid-derived lipid mediators: A complex meshwork in Alzheimer’s disease pathology,” *Prog. Lipid Res.*, vol. 50, no. 4, pp. 313–330, Oct. 2011.

[34] J. S. O’Brien and E. L. Sampson, “Lipid composition of the normal human brain: gray matter, white matter, and myelin,” *J. Lipid Res.*, vol. 6, no. 4, pp. 537–544, Oct. 1965.

[35] L. Svennerholm, “Distribution and fatty acid composition of phosphoglycerides in normal human brain,” *J. Lipid Res.*, vol. 9, no. 5, pp. 570–579, Sep. 1968.

[36] A. A. Farooqui, L. A. Horrocks, and T. Farooqui, “Modulation of inflammation in brain: a matter of fat,” *J. Neurochem.*, vol. 101, no. 3, pp. 577–599, May 2007.

[37] K. A. Youdim, A. Martin, and J. A. Joseph, “Essential fatty acids and the brain: possible health implications,” *Int. J. Dev. Neurosci.*, vol. 18, no. 4–5, pp. 383–399, Jul. 2000.

[38] L. A. Horrocks and A. A. Farooqui, “Docosahexaenoic acid in the diet: its importance in maintenance and restoration of neural membrane function,” *Prostaglandins Leukot. Essent. Fatty Acids*, vol. 70, no. 4, pp. 361–372, Apr. 2004.

[39] P. C. Schmid, I. Spimrova, and H. H. O. Schmid, “Generation and remodeling of highly polyunsaturated molecular species of rat hepatocyte phospholipids,” *Lipids*, vol. 32, no. 11, pp. 1181–1187, Nov. 1997.

[40] J. E. Vance and D. E. Vance, “Phospholipid biosynthesis in mammalian cells,” *Biochem. Cell Biol. Biochim. Biol. Cell.*, vol. 82, no. 1, pp. 113–128, Feb. 2004.

[41] J. E. Vance, “Phospholipid synthesis in a membrane fraction associated with mitochondria.,” *J. Biol. Chem.*, vol. 265, no. 13, pp. 7248–7256, May 1990.

[42] P. C. Schmid, E. Deli, and H. H. O. Schmid, “Generation and Remodeling of Phospholipid Molecular Species in Rat Hepatocytes,” *Arch. Biochem. Biophys.*, vol. 319, no. 1, pp. 168–176, May 1995.

[43] A. Yamashita, Y. Hayashi, Y. Nemoto-Sasaki, M. Ito, S. Oka, T. Tanikawa, K. Waku, and T. Sugiura, “Acyltransferases and transacylases that determine the fatty acid composition of glycerolipids and the metabolism of bioactive lipid mediators in mammalian cells and model organisms,” *Prog. Lipid Res.*, vol. 53, pp. 18–81, Jan. 2014.

[44] S. J. Stone and J. E. Vance, “Phosphatidylserine Synthase-1 and -2 Are Localized to Mitochondria-associated Membranes,” *J. Biol. Chem.*, vol. 275, no. 44, pp. 34534–34540, Nov. 2000.

[45] M. O. Bergo, B. J. Gavino, R. Steenbergen, B. Sturbois, A. F. Parlow, D. A. Sanan, W. C. Skarnes, J. E. Vance, and S. G. Young, "Defining the Importance of Phosphatidylserine Synthase 2 in Mice," *J. Biol. Chem.*, vol. 277, no. 49, pp. 47701–47708, Dec. 2002.

[46] A. K. Kimura and H.-Y. Kim, "Phosphatidylserine synthase 2: high efficiency for synthesizing phosphatidylserine containing docosahexaenoic acid," *J. Lipid Res.*, vol. 54, no. 1, pp. 214–222, Jan. 2013.

[47] M. . Ilincheta de Boschero, M. . Roque, G. . Salvador, and N. . Giusto, "Alternative pathways for phospholipid synthesis in different brain areas during aging," *Exp. Gerontol.*, vol. 35, no. 5, pp. 653–668, Aug. 2000.

[48] O. B. Bleijerveld, J. F. H. M. Brouwers, A. B. Vaandrager, J. B. Helms, and M. Houweling, "The CDP-ethanolamine Pathway and Phosphatidylserine Decarboxylation Generate Different Phosphatidylethanolamine Molecular Species," *J. Biol. Chem.*, vol. 282, no. 39, pp. 28362–28372, Sep. 2007.

[49] D. H. Kim, S. H. Yeo, J.-M. Park, J. Y. Choi, T.-H. Lee, S. Y. Park, M. S. Ock, J. Eo, H.-S. Kim, and H.-J. Cha, "Genetic markers for diagnosis and pathogenesis of Alzheimer's disease," *Gene*, vol. 545, no. 2, pp. 185–193, Jul. 2014.

[50] D. E. Vance and J. E. Vance, "Physiological consequences of disruption of mammalian phospholipid biosynthetic genes," *J. Lipid Res.*, vol. 50, no. Supplement, pp. S132–S137, Dec. 2008.

[51] J. E. Vance, "Thematic Review Series: Glycerolipids. Phosphatidylserine and phosphatidylethanolamine in mammalian cells: two metabolically related aminophospholipids," *J. Lipid Res.*, vol. 49, no. 7, pp. 1377–1387, Jul. 2008.

[52] Z. Cui, J. E. Vance, M. H. Chen, D. R. Voelker, and D. E. Vance, "Cloning and expression of a novel phosphatidylethanolamine N-methyltransferase. A specific biochemical and cytological marker for a unique membrane fraction in rat liver.," *J. Biol. Chem.*, vol. 268, no. 22, pp. 16655–16663, Aug. 1993.

[53] C. J. Walkey, L. Yu, L. B. Agellon, and D. E. Vance, "Biochemical and Evolutionary Significance of Phospholipid Methylation," *J. Biol. Chem.*, vol. 273, no. 42, pp. 27043–27046, Oct. 1998.

[54] X.-H. Bi, H.-L. Zhao, Z.-X. Zhang, and J.-W. Zhang, "PEMT G523A (V175M) is associated with sporadic Alzheimer's disease in a Chinese population," *J. Mol. Neurosci. MN*, vol. 46, no. 3, pp. 505–508, Mar. 2012.

[55] Z.-Z. Guan, Y.-N. Wang, K.-Q. Xiao, P.-S. Hu, and J.-L. Liu, "Activity of phosphatidylethanolamine-N-methyltransferase in brain affected by Alzheimers disease," *Neurochem. Int.*, vol. 34, no. 1, pp. 41–47, Jan. 1999.

[56] H. Sprecher, "Metabolism of highly unsaturated n-3 and n-6 fatty acids," *Biochim. Biophys. Acta BBA - Mol. Cell Biol. Lipids*, vol. 1486, no. 2–3, pp. 219–231, Jul. 2000.

[57] A. Voss, M. Reinhart, S. Sankarappa, and H. Sprecher, "The metabolism of 7,10,13,16,19-docosapentaenoic acid to 4,7,10,13,16,19-docosahexaenoic acid in rat liver is independent of a 4-desaturase.," *J. Biol. Chem.*, vol. 266, no. 30, pp. 19995–20000, Oct. 1991.

[58] M. Martinez, "Abnormal profiles of polyunsaturated fatty acids in the brain, liver, kidney and retina of patients with peroxisomal disorders," *Brain Res.*, vol. 583, no. 1–2, pp. 171–182, Jun. 1992.

[59] M. Martinez and I. Mougan, "Fatty acid composition of brain glycerophospholipids in peroxisomal disorders," *Lipids*, vol. 34, no. 7, pp. 733–740, Jul. 1999.

[60] E. Christensen, B. Woldseth, T. A. Hagve, B. T. Poll-The, R. J. Wanders, H. Sprecher, O. Stokke, and B. O. Christophersen, "Peroxisomal β-oxidation of polyunsaturated long chain fatty acids in human fibroblasts. The polyunsaturated and the saturated long chain fatty acids are retroconverted by the same acyl-CoA oxidase," *Scand. J. Clin. Lab. Investig. Suppl.*, vol. 215, pp. 61–74, 1993.

[61] S. A. Moore, E. Hurt, E. Yoder, H. Sprecher, and A. A. Spector, "Docosahexaenoic acid synthesis in human skin fibroblasts involves peroxisomal retroconversion of tetracosahexaenoic acid.," *J. Lipid Res.*, vol. 36, no. 11, pp. 2433–2443, Nov. 1995.

[62] H. P. Cho, M. T. Nakamura, and S. D. Clarke, "Cloning, Expression, and Nutritional Regulation of the Mammalian Δ-6 Desaturase," *J. Biol. Chem.*, vol. 274, no. 1, pp. 471–477, Jan. 1999.

[63] S. I. Rapoport and M. Igarashi, "Can the rat liver maintain normal brain DHA metabolism in the absence of dietary DHA?," *Prostaglandins Leukot. Essent. Fatty Acids*, vol. 81, no. 2–3, pp. 119–123, Aug. 2009.

[64] S. A. Moore, E. Yoder, S. Murphy, G. R. Dutton, and A. A. Spector, "Astrocytes, not neurons, produce docosahexaenoic acid (22:6 omega-3) and arachidonic acid (20:4 omega-6)," *J. Neurochem.*, vol. 56, no. 2, pp. 518–524, Feb. 1991.

[65] C. Bénistant, M. P. Dehouck, J. C. Fruchart, R. Cecchelli, and M. Lagarde, "Fatty acid composition of brain capillary endothelial cells: effect of the coculture with astrocytes.," *J. Lipid Res.*, vol. 36, no. 11, pp. 2311–2319, Nov. 1995.

[66] N. Bernoud, L. Fenart, C. Bénistant, J. F. Pageaux, M. P. Dehouck, P. Molière, M. Lagarde, R. Cecchelli, and J. Lecerf, "Astrocytes are mainly responsible for the polyunsaturated fatty acid enrichment in blood–brain barrier endothelial cells in vitro," *J. Lipid Res.*, vol. 39, no. 9, pp. 1816–1824, Sep. 1998.

[67] J. T. Brenna, N. Salem Jr., A. J. Sinclair, and S. C. Cunnane, "α-Linolenic acid supplementation and conversion to n-3 long-chain polyunsaturated fatty acids in humans," *Prostaglandins Leukot. Essent. Fatty Acids*, vol. 80, no. 2–3, pp. 85–91, Feb. 2009.

[68] G. C. Burdge, Y. E. Finnegan, A. M. Minihane, C. M. Williams, and S. A. Wootton, "Effect of altered dietary n-3 fatty acid intake upon plasma lipid fatty acid composition, conversion of [13C]α-linolenic acid to longer-chain fatty acids and partitioning towards β-oxidation in older men," *Br. J. Nutr.*, vol. 90, no. 02, pp. 311–321, 2003.

[69] H. D. Le, J. A. Meisel, V. E. de Meijer, K. M. Gura, and M. Puder, "The essentiality of arachidonic acid and docosahexaenoic acid," *Prostaglandins Leukot. Essent. Fatty Acids*, vol. 81, no. 2–3, pp. 165–170, Aug. 2009.

[70] M. Martínez and I. Mougan, "Fatty Acid Composition of Human Brain Phospholipids During Normal Development," *J. Neurochem.*, vol. 71, no. 6, pp. 2528–2533, Dec. 1998.

[71] S. M. Innis, "Dietary (n-3) Fatty Acids and Brain Development1,2," *J. Nutr.*, vol. 137, no. 4, pp. 855–9, Apr. 2007.

[72] R. S. Kuipers, M. F. Luxwolda, P. J. Offringa, E. Rudi Boersma, D. A. J. Dijck-Brouwer, and F. A. J. Muskiet, "Fetal intrauterine whole body linoleic, arachidonic and docosahexaenoic acid contents and accretion rates," *Prostaglandins Leukot. Essent. Fatty Acids*, vol. 86, no. 1–2, pp. 13–20, Jan. 2012.

[73] C. I. F. Janssen and A. J. Kiliaan, "Long-chain polyunsaturated fatty acids (LCPUFA) from genesis to senescence: The influence of LCPUFA on neural

development, aging, and neurodegeneration," *Prog. Lipid Res.*, vol. 53, pp. 1–17, Jan. 2014.

[74] L. N. Nguyen, D. Ma, G. Shui, P. Wong, A. Cazenave-Gassiot, X. Zhang, M. R. Wenk, E. L. K. Goh, and D. L. Silver, "Mfsd2a is a transporter for the essential omega-3 fatty acid docosahexaenoic acid," *Nature*, vol. 509, no. 7501, pp. 503–506, May 2014.

[75] J. A. Hamilton and K. Brunaldi, "A Model for Fatty Acid Transport into the Brain," *J. Mol. Neurosci.*, vol. 33, no. 1, pp. 12–17, Jul. 2007.

[76] R. P. Bazinet and S. Layé, "Polyunsaturated fatty acids and their metabolites in brain function and disease," *Nat. Rev. Neurosci.*, vol. 15, no. 12, pp. 771–85, Dec. 2014.

[77] N. Parletta, C. M. Milte, and B. J. Meyer, "Nutritional modulation of cognitive function and mental health," *J. Nutr. Biochem.*, vol. 24, no. 5, pp. 725–743, May 2013.

[78] N. Nagan and R. A. Zoeller, "Plasmalogens: biosynthesis and functions," *Prog. Lipid Res.*, vol. 40, no. 3, pp. 199–229, May 2001.

[79] N. E. Braverman and A. B. Moser, "Functions of plasmalogen lipids in health and disease," *Biochim. Biophys. Acta BBA - Mol. Basis Dis.*, vol. 1822, no. 9, pp. 1442–1452, Sep. 2012.

[80] P. Brites, H. R. Waterham, and R. J. A. Wanders, "Functions and biosynthesis of plasmalogens in health and disease," *Biochim. Biophys. Acta BBA - Mol. Cell Biol. Lipids*, vol. 1636, no. 2–3, pp. 219–231, Mar. 2004.

[81] T. Lee, "Biosynthesis and possible biological functions of plasmalogens," *Biochim. Biophys. Acta BBA - Lipids Lipid Metab.*, vol. 1394, no. 2–3, pp. 129–145, Nov. 1998.

[82] M. Honsho, Y. Yagita, N. Kinoshita, and Y. Fujiki, "Isolation and characterization of mutant animal cell line defective in alkyl-dihydroxyacetonephosphate synthase: Localization and transport of plasmalogens to post-Golgi compartments," *Biochim. Biophys. Acta BBA - Mol. Cell Res.*, vol. 1783, no. 10, pp. 1857–1865, Oct. 2008.

[83] L. J. Pike, X. Han, K.-N. Chung, and R. W. Gross, "Lipid Rafts Are Enriched in Arachidonic Acid and Plasmenylethanolamine and Their Composition Is Independent of Caveolin-1 Expression: A Quantitative Electrospray Ionization/Mass Spectrometric Analysis†," *Biochemistry (Mosc.)*, vol. 41, no. 6, pp. 2075–2088, Feb. 2002.

[84] A. Broniec, R. Klosinski, A. Pawlak, M. Wrona-Krol, D. Thompson, and T. Sarna, "Interactions of plasmalogens and their diacyl analogs with singlet oxygen in selected model systems," *Free Radic. Biol. Med.*, vol. 50, no. 7, pp. 892–898, Apr. 2011.

[85] X. Han, D. M. Holtzman, and D. W. McKeel Jr, "Plasmalogen deficiency in early Alzheimer's disease subjects and in animal models: molecular characterization using electrospray ionization mass spectrometry," *J. Neurochem.*, vol. 77, no. 4, pp. 1168–1180, May 2001.

[86] Z. Guan, Y. Wang, N. J. Cairns, P. L. Lantos, G. Dallner, and P. J. Sindelar, "Decrease and structural modifications of phosphatidylethanolamine plasmalogen in the brain with Alzheimer disease," *J. Neuropathol. Exp. Neurol.*, vol. 58, no. 7, pp. 740–747, Jul. 1999.

[87] M. Igarashi, K. Ma, F. Gao, H.-W. Kim, S. I. Rapoport, and J. S. Rao, "Disturbed choline plasmalogen and phospholipid fatty acid concentrations in Alzheimer's

disease prefrontal cortex," *J. Alzheimers Dis. JAD*, vol. 24, no. 3, pp. 507–517, 2011.

[88] L. Svennerholm, K. Boström, and B. Jungbjer, "Changes in weight and compositions of major membrane components of human brain during the span of adult human life of Swedes," *Acta Neuropathol. (Berl.)*, vol. 94, no. 4, pp. 345–352, Oct. 1997.

[89] L. Svennerholm, K. Boström, C. G. Helander, and B. Jungbjer, "Membrane lipids in the aging human brain," *J. Neurochem.*, vol. 56, no. 6, pp. 2051–2059, Jun. 1991.

[90] M. Söderberg, C. Edlund, K. Kristensson, and G. Dallner, "Lipid compositions of different regions of the human brain during aging," *J. Neurochem.*, vol. 54, no. 2, pp. 415–423, Feb. 1990.

[91] L. Svennerholm, K. Boström, B. Jungbjer, and L. Olsson, "Membrane Lipids of Adult Human Brain: Lipid Composition of Frontal and Temporal Lobe in Subjects of Age 20 to 100 Years," *J. Neurochem.*, vol. 63, no. 5, pp. 1802–1811, 1994.

[92] V. Martín, N. Fabelo, G. Santpere, B. Puig, R. Marín, I. Ferrer, and M. Díaz, "Lipid alterations in lipid rafts from Alzheimer's disease human brain cortex," *J. Alzheimers Dis. JAD*, vol. 19, no. 2, pp. 489–502, 2010.

[93] M. Söderberg, C. Edlund, K. Kristensson, and G. Dallner, "Fatty acid composition of brain phospholipids in aging and in Alzheimer's disease," *Lipids*, vol. 26, no. 6, pp. 421–425, Jun. 1991.

[94] J. D. Carver, V. J. Benford, B. Han, and A. B. Cantor, "The relationship between age and the fatty acid composition of cerebral cortex and erythrocytes in human subjects," *Brain Res. Bull.*, vol. 56, no. 2, pp. 79–85, Sep. 2001.

[95] R. K. McNamara, Y. Liu, R. Jandacek, T. Rider, and P. Tso, "The aging human orbitofrontal cortex: Decreasing polyunsaturated fatty acid composition and associated increases in lipogenic gene expression and stearoyl-CoA desaturase activity," *Prostaglandins Leukot. Essent. Fatty Acids*, vol. 78, no. 4–5, pp. 293–304, Apr. 2008.

[96] T. Fraser, H. Tayler, and S. Love, "Fatty acid composition of frontal, temporal and parietal neocortex in the normal human brain and in Alzheimer's disease," *Neurochem. Res.*, vol. 35, no. 3, pp. 503–513, Mar. 2010.

[97] G. Barceló-Coblijn, E. Hőgyes, K. Kitajka, L. G. Puskás, Á. Zvara, L. Hackler, C. Nyakas, Z. Penke, and T. Farkas, "Modification by docosahexaenoic acid of age-induced alterations in gene expression and molecular composition of rat brain phospholipids," *Proc. Natl. Acad. Sci.*, vol. 100, no. 20, pp. 11321–11326, Sep. 2003.

[98] S. Favrielère, S. Stadelmann-Ingrand, F. Huguet, D. De Javel, A. Piriou, C. Tallineau, and G. Durand, "Age-related changes in ethanolamine glycerophospholipid fatty acid levels in rat frontal cortex and hippocampus," *Neurobiol. Aging*, vol. 21, no. 5, pp. 653–660, Oct. 2000.

[99] S. J. Little, M. A. Lynch, M. Manku, and A. Nicolaou, "Docosahexaenoic acid-induced changes in phospholipids in cortex of young and aged rats: A lipidomic analysis," *Prostaglandins Leukot. Essent. Fatty Acids*, vol. 77, no. 3–4, pp. 155–162, Oct. 2007.

[100] G. H. López, M. G. Ilincheta de Boschero, P. I. Castagnet, and N. M. Giusto, "Age-associated changes in the content and fatty acid composition of brain glycerophospholipids," *Comp. Biochem. Physiol. B Biochem. Mol. Biol.*, vol. 112, no. 2, pp. 331–343, Oct. 1995.

[101] B. M. McGahon, D. S. D. Martin, D. F. Horrobin, and M. A. Lynch, "Age-related changes in synaptic function: analysis of the effect of dietary supplementation with ω-3 fatty acids," *Neuroscience*, vol. 94, no. 1, pp. 305–314, Sep. 1999.
[102] L. Ulmann, V. Mimouni, S. Roux, R. Porsolt, and J.-P. Poisson, "Brain and hippocampus fatty acid composition in phospholipid classes of aged-relative cognitive deficit rats," *Prostaglandins Leukot. Essent. Fatty Acids*, vol. 64, no. 3, pp. 189–195, Mar. 2001.
[103] L. Yang, Y. Zhang, S. Wang, W. Zhang, and R. Shi, "Decreased liver peroxisomal β-oxidation accompanied by changes in brain fatty acid composition in aged rats," *Neurol. Sci.*, vol. 35, no. 2, pp. 289–293, Feb. 2014.
[104] Lihe Jiang, Yan Shi, Yin Long, and Zhirong Yang, "The influence of orally administered docosahexaenoic acid on monoamine neurotransmitter, nerve growth factor, and brain-derived neurotrophic factor in aged mice," *Pharm. Biol.*, vol. 47, no. 7, pp. 584–591, Jul. 2009.
[105] H. R. Modi, S. S. Katyare, and M. A. Patel, "Ageing-Induced Alterations in Lipid/Phospholipid Profiles of Rat Brain and Liver Mitochondria: Implications for Mitochondrial Energy-Linked Functions," *J. Membr. Biol.*, vol. 221, no. 1, pp. 51–60, Jan. 2008.
[106] I. Rappley, D. S. Myers, S. B. Milne, P. T. Ivanova, M. J. LaVoie, H. A. Brown, and D. J. Selkoe, "Lipidomic profiling in mouse brain reveals differences between ages and genders, with smaller changes associated with α-synuclein genotype," *J. Neurochem.*, vol. 111, no. 1, pp. 15–25, 2009.
[107] N. Fabelo, V. Martín, R. Marín, G. Santpere, E. Aso, I. Ferrer, and M. Díaz, "Evidence for Premature Lipid Raft Aging in APP/PS1 Double-Transgenic Mice, a Model of Familial Alzheimer Disease," *J. Neuropathol. Exp. Neurol.*, vol. 71, no. 10, pp. 868–81, Oct. 2012.
[108] B. W. Brooksbank and M. Martinez, "Lipid abnormalities in the brain in adult Down's syndrome and Alzheimer's disease," *Mol. Chem. Neuropathol. Spons. Int. Soc. Neurochem. World Fed. Neurol. Res. Groups Neurochem. Cerebrospinal Fluid*, vol. 11, no. 3, pp. 157–185, Dec. 1989.
[109] L. Svennerholm and C. G. Gottfries, "Membrane lipids, selectively diminished in Alzheimer brains, suggest synapse loss as a primary event in early-onset form (type I) and demyelination in late-onset form (type II)," *J. Neurochem.*, vol. 62, no. 3, pp. 1039–1047, Mar. 1994.
[110] C. G. Gottfries, I. Karlsson, and L. Svennerholm, "Membrane components separate early-onset Alzheimer's disease from senile dementia of the Alzheimer type," *Int. Psychogeriatrics IPA*, vol. 8, no. 3, pp. 365–372, 1996.
[111] M. Söderberg, C. Edlund, I. Alafuzoff, K. Kristensson, and G. Dallner, "Lipid composition in different regions of the brain in Alzheimer's disease/senile dementia of Alzheimer's type," *J. Neurochem.*, vol. 59, no. 5, pp. 1646–1653, Nov. 1992.
[112] J. W. Pettegrew, K. Panchalingam, R. L. Hamilton, and R. J. McClure, "Brain membrane phospholipid alterations in Alzheimer's disease," *Neurochem. Res.*, vol. 26, no. 7, pp. 771–782, Jul. 2001.
[113] K. Wells, A. A. Farooqui, L. Liss, and L. A. Horrocks, "Neural membrane phospholipids in alzheimer disease," *Neurochem. Res.*, vol. 20, no. 11, pp. 1329–1333, Nov. 1995.
[114] S. C. Cunnane, J. A. Schneider, C. Tangney, J. Tremblay-Mercier, M. Fortier, D. A. Bennett, and M. C. Morris, "Plasma and brain fatty acid profiles in mild

cognitive impairment and Alzheimer's disease," *J. Alzheimers Dis. JAD*, vol. 29, no. 3, pp. 691–697, 2012.

[115] M. O. W. Grimm, S. Grösgen, M. Riemenschneider, H. Tanila, H. S. Grimm, and T. Hartmann, "From brain to food: Analysis of phosphatidylcholins, lyso-phosphatidylcholins and phosphatidylcholin–plasmalogens derivates in Alzheimer's disease human post mortem brains and mice model via mass spectrometry," *J. Chromatogr. A*, vol. 1218, no. 42, pp. 7713–7722, Oct. 2011.

[116] R. B. Chan, T. G. Oliveira, E. P. Cortes, L. S. Honig, K. E. Duff, S. A. Small, M. R. Wenk, G. Shui, and G. Di Paolo, "Comparative lipidomic analysis of mouse and human brain with Alzheimer disease," *J. Biol. Chem.*, vol. 287, no. 4, pp. 2678–2688, Jan. 2012.

[117] R. M. Nitsch, J. K. Blusztajn, A. G. Pittas, B. E. Slack, J. H. Growdon, and R. J. Wurtman, "Evidence for a membrane defect in Alzheimer disease brain.," *Proc. Natl. Acad. Sci.*, vol. 89, no. 5, pp. 1671–1675, Mar. 1992.

[118] D. A. Butterfield and C. M. Lauderback, "Lipid peroxidation and protein oxidation in Alzheimer's disease brain: potential causes and consequences involving amyloid β-peptide-associated free radical oxidative stress," *Free Radic. Biol. Med.*, vol. 32, no. 11, pp. 1050–1060, Jun. 2002.

[119] C. M. Milte, N. Sinn, and P. R. Howe, "Polyunsaturated fatty acid status in attention deficit hyperactivity disorder, depression, and Alzheimer's disease: towards an omega-3 index for mental health?," *Nutr. Rev.*, vol. 67, no. 10, pp. 573–590, Oct. 2009.

[120] P. L. Wood, "Lipidomics of Alzheimer's disease: current status," *Alzheimers Res. Ther.*, vol. 4, no. 1, p. 5, Feb. 2012.

[121] E. R. Skinner, C. Watt, J. a. O. Besson, and P. V. Best, "Differences in the fatty acid composition of the grey and white matter of different regions of the brains of patients with Alzheimer's disease and control subjects," *Brain*, vol. 116, no. 3, pp. 717–725, Jun. 1993.

[122] F. M. Corrigan, D. F. Horrobin, E. R. Skinner, J. A. Besson, and M. B. Cooper, "Abnormal content of n-6 and n-3 long-chain unsaturated fatty acids in the phosphoglycerides and cholesterol esters of parahippocampal cortex from Alzheimer's disease patients and its relationship to acetyl CoA content," *Int. J. Biochem. Cell Biol.*, vol. 30, no. 2, pp. 197–207, Feb. 1998.

[123] M. R. Prasad, M. A. Lovell, M. Yatin, H. Dhillon, and W. R. Markesbery, "Regional membrane phospholipid alterations in Alzheimer's disease," *Neurochem. Res.*, vol. 23, no. 1, pp. 81–88, Jan. 1998.

[124] D. Cheng, A. M. Jenner, G. Shui, W. F. Cheong, T. W. Mitchell, J. R. Nealon, W. S. Kim, H. McCann, M. R. Wenk, G. M. Halliday, and B. Garner, "Lipid Pathway Alterations in Parkinson's Disease Primary Visual Cortex," *PLoS ONE*, vol. 6, no. 2, p. e17299, Feb. 2011.

[125] S. M. Lam, Y. Wang, X. Duan, M. R. Wenk, R. N. Kalaria, C. P. Chen, M. K. P. Lai, and G. Shui, "The brain lipidomes of subcortical ischemic vascular dementia and mixed dementia," *Neurobiol. Aging*, vol. 35, no. 10, pp. 2369–2381, Oct. 2014.

[126] L. Ginsberg, S. Rafique, J. H. Xuereb, S. I. Rapoport, and N. L. Gershfeld, "Disease and anatomic specificity of ethanolamine plasmalogen deficiency in Alzheimer's disease brain," *Brain Res.*, vol. 698, no. 1–2, pp. 223–226, Nov. 1995.

[127] Y. Tamai, H. Kojima, F. Ikuta, and T. Kumanishi, “Alterations in the composition of brain lipids in patients with Creutzfeldt-Jakob disease,” *J. Neurol. Sci.*, vol. 35, no. 1, pp. 59–76, Jan. 1978.
[128] A. Federico, P. Annunziata, and G. Malentacchi, “Neurochemical changes in Creutzfeldt-Jakob disease,” *J. Neurol.*, vol. 223, no. 2, pp. 135–146, 1980.
[129] B. T. Weinert and P. S. Timiras, “Invited Review: Theories of aging,” *J. Appl. Physiol.*, vol. 95, no. 4, pp. 1706–1716, Oct. 2003.
[130] N.-G. Larsson, “Somatic Mitochondrial DNA Mutations in Mammalian Aging,” *Annu. Rev. Biochem.*, vol. 79, no. 1, pp. 683–706, 2010.
[131] M. Lagouge and N.-G. Larsson, “The role of mitochondrial DNA mutations and free radicals in disease and ageing,” *J. Intern. Med.*, vol. 273, no. 6, pp. 529–543, Jun. 2013.
[132] P. Mecocci, U. MacGarvey, A. E. Kaufman, D. Koontz, J. M. Shoffner, D. C. Wallace, and M. F. Beal, “Oxidative damage to mitochondrial DNA shows marked age-dependent increases in human brain,” *Ann. Neurol.*, vol. 34, no. 4, pp. 609–616, 1993.
[133] T. Finkel and N. J. Holbrook, “Oxidants, oxidative stress and the biology of ageing,” *Nature*, vol. 408, no. 6809, pp. 239–247, Nov. 2000.
[134] S. Hekimi, J. Lapointe, and Y. Wen, “Taking a ‘good’ look at free radicals in the aging process,” *Trends Cell Biol.*, vol. 21, no. 10, pp. 569–576, Oct. 2011.
[135] S. I. Liochev, “Reactive oxygen species and the free radical theory of aging,” *Free Radic. Biol. Med.*, vol. 60, pp. 1–4, Jul. 2013.
[136] D. Harman, “Aging: A Theory Based on Free Radical and Radiation Chemistry,” *J. Gerontol.*, vol. 11, no. 3, pp. 298–300, Jul. 1956.
[137] J. M. McCord and I. Fridovich, “Superoxide Dismutase AN ENZYMIC FUNCTION FOR ERYTHROCUPREIN (HEMOCUPREIN),” *J. Biol. Chem.*, vol. 244, no. 22, pp. 6049–6055, Nov. 1969.
[138] B. Halliwell and J. Gutteridge, *Free Radicals in Biology and Medicine*, 4th edition. Oxford ; New York: Oxford University Press, 2007.
[139] E. Babusikova, J. Hatok, D. Dobrota, and P. Kaplan, “Age-related Oxidative Modifications of Proteins and Lipids in Rat Brain,” *Neurochem. Res.*, vol. 32, no. 8, pp. 1351–1356, Aug. 2007.
[140] B. S. Baek, H. J. Kwon, K. H. Lee, M. A. Yoo, K. W. Kim, Y. Ikeno, B. P. Yu, and H. Y. Chung, “Regional difference of ROS generation, lipid peroxidaton, and antioxidant enzyme activity in rat brain and their dietary modulation,” *Arch. Pharm. Res.*, vol. 22, no. 4, pp. 361–366, Aug. 1999.
[141] E. M. Kawamoto, A. R. Vasconcelos, S. Degaspari, A. E. Böhmer, C. Scavone, and T. Marcourakis, “Age-related changes in nitric oxide activity, cyclic GMP, and TBARS levels in platelets and erythrocytes reflect the oxidative status in central nervous system,” *AGE*, vol. 35, no. 2, pp. 331–342, Apr. 2013.
[142] I. R. Siqueira, C. Fochesatto, A. de Andrade, M. Santos, M. Hagen, A. Bello-Klein, and C. A. Netto, “Total antioxidant capacity is impaired in different structures from aged rat brain,” *Int. J. Dev. Neurosci.*, vol. 23, no. 8, pp. 663–671, Dec. 2005.
[143] P. D. A. Woźniak, P. D. G. Drewa, P. D. B. Woźniak, and P. D. D. O. Schachtschabel, “Activity of antioxidant enzymes and concentration of lipid peroxidation products in selected tissues of mice of different ages, both healthy and melanoma-bearing,” *Z. Für Gerontol. Geriatr.*, vol. 37, no. 3, pp. 184–189, Jun. 2004.

[144] R. Dei, A. Takeda, H. Niwa, M. Li, Y. Nakagomi, M. Watanabe, T. Inagaki, Y. Washimi, Y. Yasuda, K. Horie, T. Miyata, and G. Sobue, "Lipid peroxidation and advanced glycation end products in the brain in normal aging and in Alzheimer's disease," *Acta Neuropathol. (Berl.)*, vol. 104, no. 2, pp. 113–122, Aug. 2002.

[145] H. M. Abdul, R. Sultana, D. K. St. Clair, W. R. Markesbery, and D. A. Butterfield, "Oxidative damage in brain from human mutant APP/PS-1 double knock-in mice as a function of age," *Free Radic. Biol. Med.*, vol. 45, no. 10, pp. 1420–1425, Nov. 2008.

[146] H. F. Poon, G. Joshi, R. Sultana, S. A. Farr, W. A. Banks, J. E. Morley, V. Calabrese, and D. A. Butterfield, "Antisense directed at the Aβ region of APP decreases brain oxidative markers in aged senescence accelerated mice," *Brain Res.*, vol. 1018, no. 1, pp. 86–96, Aug. 2004.

[147] L. K. Gilmer, M. A. Ansari, K. N. Roberts, and S. W. Scheff, "Age-related changes in mitochondrial respiration and oxidative damage in the cerebral cortex of the Fischer 344 rat," *Mech. Ageing Dev.*, vol. 131, no. 2, pp. 133–143, Feb. 2010.

[148] I. K. Hwang, Y. S. Yoon, K.-Y. Yoo, H. Li, J. H. Choi, D. W. Kim, S. S. Yi, J. K. Seong, I. S. Lee, and M.-H. Won, "Differences in Lipid Peroxidation and Cu,Zn-Superoxide Dismutase in the Hippocampal CA1 Region Between Adult and Aged Dogs," *J. Vet. Med. Sci.*, vol. 70, no. 3, pp. 273–277, 2008.

[149] J. Guest, R. Grant, T. A. Mori, and K. D. Croft, "Changes in Oxidative Damage, Inflammation and [NAD(H)] with Age in Cerebrospinal Fluid," *PLoS ONE*, vol. 9, no. 1, p. e85335, Jan. 2014.

[150] T. J. Montine, M. D. Neely, J. F. Quinn, M. F. Beal, W. R. Markesbery, L. J. Roberts, and J. D. Morrow, "Lipid peroxidation in aging brain and Alzheimer's disease," *Free Radic. Biol. Med.*, vol. 33, no. 5, pp. 620–626, Sep. 2002.

[151] J. A. Youssef, L. S. Birnbaum, L. L. Swift, J. D. Morrow, and M. Z. Badr, "Age-independent, gray matter-localized, brain-enhanced oxidative stress in male fischer 344 rats: brain levels of F2-isoprostanes and F4-neuroprostanes," *Free Radic. Biol. Med.*, vol. 34, no. 12, pp. 1631–1635, Jun. 2003.

[152] X. Wang, W. Wang, L. Li, G. Perry, H. Lee, and X. Zhu, "Oxidative stress and mitochondrial dysfunction in Alzheimer's disease," *Biochim. Biophys. Acta BBA - Mol. Basis Dis.*, vol. 1842, no. 8, pp. 1240–1247, Aug. 2014.

[153] F. Missirlis, J. Hu, K. Kirby, A. J. Hilliker, T. A. Rouault, and J. P. Phillips, "Compartment-specific Protection of Iron-Sulfur Proteins by Superoxide Dismutase," *J. Biol. Chem.*, vol. 278, no. 48, pp. 47365–47369, Nov. 2003.

[154] F. L. Muller, Y. Liu, and H. V. Remmen, "Complex III Releases Superoxide to Both Sides of the Inner Mitochondrial Membrane," *J. Biol. Chem.*, vol. 279, no. 47, pp. 49064–49073, Nov. 2004.

[155] C. Franceschi, M. Bonafè, S. Valensin, F. Olivieri, M. De Luca, E. Ottaviani, and G. De Benedictis, "Inflamm-aging: An Evolutionary Perspective on Immunosenescence," *Ann. N. Y. Acad. Sci.*, vol. 908, no. 1, pp. 244–254, Jun. 2000.

[156] C. Franceschi, M. Capri, D. Monti, S. Giunta, F. Olivieri, F. Sevini, M. P. Panourgia, L. Invidia, L. Celani, M. Scurti, E. Cevenini, G. C. Castellani, and S. Salvioli, "Inflammaging and anti-inflammaging: A systemic perspective on aging and longevity emerged from studies in humans," *Mech. Ageing Dev.*, vol. 128, no. 1, pp. 92–105, Jan. 2007.

[157] C. Franceschi and J. Campisi, “Chronic Inflammation (Inflammaging) and Its Potential Contribution to Age-Associated Diseases,” *J. Gerontol. A. Biol. Sci. Med. Sci.*, vol. 69, no. Suppl 1, pp. S4–S9, Jun. 2014.
[158] Quibik, *Pathways in biosynthesis of eicosanoids from arachidonic acid: there are parallel paths from EPA & DGLA*. 2007.
[159] E. A. Dennis, “The growing phospholipase A2 superfamily of signal transduction enzymes,” *Trends Biochem. Sci.*, vol. 22, no. 1, pp. 1–2, Jan. 1997.
[160] M. A. Balboa, I. Varela-Nieto, K. Killermann Lucas, and E. A. Dennis, “Expression and function of phospholipase A2 in brain,” *FEBS Lett.*, vol. 531, no. 1, pp. 12–17, Oct. 2002.
[161] E. A. Dennis, J. Cao, Y.-H. Hsu, V. Magrioti, and G. Kokotos, “Phospholipase A2 Enzymes: Physical Structure, Biological Function, Disease Implication, Chemical Inhibition, and Therapeutic Intervention,” *Chem. Rev.*, vol. 111, no. 10, pp. 6130–6185, Oct. 2011.
[162] E. Ricciotti and G. A. FitzGerald, “Prostaglandins and Inflammation,” *Arterioscler. Thromb. Vasc. Biol.*, vol. 31, no. 5, pp. 986–1000, May 2011.
[163] J.-H. Won, “Cyclooxygenase-2: A Potential Target in Human Cancer,” *Cancer Res. Treat. Off. J. Korean Cancer Assoc.*, vol. 36, no. 3, pp. 161–162, Jun. 2004.
[164] J. R. Vane, Y. S. Bakhle, and R. M. Botting, “Cyclooxygenases 1 and 2,” *Annu. Rev. Pharmacol. Toxicol.*, vol. 38, no. 1, pp. 97–120, 1998.
[165] K. Yamagata, K. I. Andreasson, W. E. Kaufmann, C. A. Barnes, and P. F. Worley, “Expression of a mitogen-inducible cyclooxygenase in brain neurons: Regulation by synaptic activity and glucocorticoids,” *Neuron*, vol. 11, no. 2, pp. 371–386, Aug. 1993.
[166] Y. Kitamura, S. Shimohama, H. Koike, J. Kakimura, Y. Matsuoka, Y. Nomura, P. J. Gebicke-Haerter, and T. Taniguchi, “Increased Expression of Cyclooxygenases and Peroxisome Proliferator-Activated Receptor-γ in Alzheimer’s Disease Brains,” *Biochem. Biophys. Res. Commun.*, vol. 254, no. 3, pp. 582–586, Jan. 1999.
[167] L. Minghetti, “Cyclooxygenase-2 (COX-2) in Inflammatory and Degenerative Brain Diseases,” *J. Neuropathol.*, vol. 63, no. 9, pp. 901–910, 2004.
[168] P. Casolini, A. Catalani, A. R. Zuena, and L. Angelucci, “Inhibition of COX-2 reduces the age-dependent increase of hippocampal inflammatory markers, corticosterone secretion, and behavioral impairments in the rat,” *J. Neurosci. Res.*, vol. 68, no. 3, pp. 337–343, May 2002.
[169] A. A. Farooqui and T. Farooqui, Eds., *Molecular Aspects of Neurodegeneration and Neuroprotection*. Bentham Science Publishers, 2012.
[170] A. J. Vecchio, B. J. Orlando, R. Nandagiri, and M. G. Malkowski, “Investigating Substrate Promiscuity in Cyclooxygenase-2 THE ROLE OF ARG-120 AND RESIDUES LINING THE HYDROPHOBIC GROOVE,” *J. Biol. Chem.*, vol. 287, no. 29, pp. 24619–24630, Jul. 2012.
[171] A. J. Vecchio, D. M. Simmons, and M. G. Malkowski, “Structural Basis of Fatty Acid Substrate Binding to Cyclooxygenase-2,” *J. Biol. Chem.*, vol. 285, no. 29, pp. 22152–22163, Jul. 2010.
[172] S. C. Dyall, “Long-chain omega-3 fatty acids and the brain: a review of the independent and shared effects of EPA, DPA and DHA,” *Front. Aging Neurosci.*, vol. 7, Apr. 2015.
[173] S. Aïd and F. Bosetti, “Gene expression of cyclooxygenase-1 and Ca2+-independent phospholipase A2 is altered in rat hippocampus during normal aging,” *Brain Res. Bull.*, vol. 73, no. 1–3, pp. 108–113, Jun. 2007.

[174] G. R. Weerasinghe, S. L. Coon, A. K. Bhattacharjee, G. J. Harry, and F. Bosetti, "Regional protein levels of cytosolic phospholipase A2 and cyclooxygenase-2 in Rhesus monkey brain as a function of age," *Brain Res. Bull.*, vol. 69, no. 6, pp. 614–621, May 2006.

[175] P. G. Kopf, D. X. Zhang, K. M. Gauthier, K. Nithipatikom, X.-Y. Yi, J. R. Falck, and W. B. Campbell, "Adrenic Acid Metabolites as Endogenous Endothelium-Derived and Zona Glomerulosa-Derived Hyperpolarizing Factors," *Hypertension*, vol. 55, no. 2, pp. 547–554, Feb. 2010.

[176] B. Cox and A. Emili, "Tissue subcellular fractionation and protein extraction for use in mass-spectrometry-based proteomics," *Nat. Protoc.*, vol. 1, no. 4, pp. 1872–1878, 2006.

[177] S. Gandhi, M. M. K. Muqit, L. Stanyer, D. G. Healy, P. M. Abou-Sleiman, I. Hargreaves, S. Heales, M. Ganguly, L. Parsons, a J. Lees, D. S. Latchman, J. L. Holton, N. W. Wood, and T. Revesz, "PINK1 protein in normal human brain and Parkinson's disease.," *Brain J. Neurol.*, vol. 129, no. Pt 7, pp. 1720–31, Jul. 2006.

[178] L. Bourova, J. Stohr, V. Lisy, V. Rudajev, J. Novotny, and P. Svoboda, "Isolation of plasma membrane compartments from rat brain cortex; detection of agonist-stimulated G protein activity," *Med. Sci. Monit.*, vol. 15, no. 4, pp. BR111–BR122, Mar. 2009.

[179] R. Lund, R. Leth-Larsen, O. N. Jensen, and H. J. Ditzel, "Efficient Isolation and Quantitative Proteomic Analysis of Cancer Cell Plasma Membrane Proteins for Identification of Metastasis-Associated Cell Surface Markers research articles," *J. Proteome Res.*, vol. 8, no. 6, pp. 3078–3090, Jun. 2009.

[180] J. Folch, M. Lees, and G. H. Sloane Stanley, "A simple method for the isolation and purification of total lipides from animal tissues," *J. Biol. Chem.*, vol. 226, no. 1, pp. 497–509, May 1957.

[181] J. M. Deeley, T. W. Mitchell, X. Wei, J. Korth, J. R. Nealon, S. J. Blanksby, and R. J. W. Truscott, "Human lens lipids differ markedly from those of commonly used experimental animals," *Biochim. Biophys. Acta*, vol. 1781, no. 6–7, pp. 288–298, Jul. 2008.

[182] K. A. Kayganich and R. C. Murphy, "Fast atom bombardment tandem mass spectrometric identification of diacyl, alkylacyl, and alk-1-enylacyl molecular species of glycerophosphoethanolamine in human polymorphonuclear leukocytes," *Anal. Chem.*, vol. 64, no. 23, pp. 2965–2971, Dec. 1992.

[183] K. Ekroos, C. S. Ejsing, U. Bahr, M. Karas, K. Simons, and A. Shevchenko, "Charting molecular composition of phosphatidylcholines by fatty acid scanning and ion trap MS3 fragmentation," *J. Lipid Res.*, vol. 44, no. 11, pp. 2181–2192, Nov. 2003.

[184] K. A. Z. Berry and R. C. Murphy, "Electrospray ionization tandem mass spectrometry of glycerophosphoethanolamine plasmalogen phospholipids," *J. Am. Soc. Mass Spectrom.*, vol. 15, no. 10, pp. 1499–1508, Oct. 2004.

[185] J. M. Deeley, M. C. Thomas, R. J. W. Truscott, T. W. Mitchell, and S. J. Blanksby, "Identification of Abundant Alkyl Ether Glycerophospholipids in the Human Lens by Tandem Mass Spectrometry Techniques," *Anal. Chem.*, vol. 81, no. 5, pp. 1920–1930, Mar. 2009.

[186] T. W. Mitchell, R. Buffenstein, and A. J. Hulbert, "Membrane phospholipid composition may contribute to exceptional longevity of the naked mole-rat (Heterocephalus glaber): A comparative study using shotgun lipidomics," *Exp. Gerontol.*, vol. 42, no. 11, pp. 1053–1062, Nov. 2007.

[187] G. Liebisch, J. A. Vizcaíno, H. Köfeler, M. Trötzmüller, W. J. Griffiths, G. Schmitz, F. Spener, and M. J. O. Wakelam, "Shorthand notation for lipid structures derived from mass spectrometry," *J. Lipid Res.*, vol. 54, no. 6, pp. 1523–1530, Jun. 2013.

[188] B. L. Miller and J. L. Cummings, *Human Frontal Lobes : Functions and Disorders (2nd Edition)*. New York, NY, USA: Guilford Press, 2006.

[189] S. C. Cunnane, R. Chouinard-Watkins, C. A. Castellano, and P. Barberger-Gateau, "Docosahexaenoic acid homeostasis, brain aging and Alzheimer's disease: Can we reconcile the evidence?," *Prostaglandins Leukot. Essent. Fatty Acids*, vol. 88, no. 1, pp. 61–70, Jan. 2013.

[190] H.-Y. Kim, B. X. Huang, and A. A. Spector, "Phosphatidylserine in the brain: Metabolism and function," *Prog. Lipid Res.*, vol. 56, pp. 1–18, Oct. 2014.

[191] H.-Y. Kim, M. Akbar, and Y.-S. Kim, "Phosphatidylserine-dependent neuroprotective signaling promoted by docosahexaenoic acid," *Prostaglandins Leukot. Essent. Fat. Acids PLEFA*, vol. 82, no. 4–6, pp. 165–172, Apr. 2010.

[192] E. Hassan-Zadeh, E. Baykal-Caglar, M. Alwarawrah, and J. Huang, "Complex Roles of Hybrid Lipids in the Composition, Order, and Size of Lipid Membrane Domains," *Langmuir*, vol. 30, no. 5, pp. 1361–1369, Feb. 2014.

[193] C. Klose, M. A. Surma, and K. Simons, "Organellar lipidomics — background and perspectives," *Curr. Opin. Cell Biol.*, vol. 25, no. 4, pp. 406–413, Aug. 2013.

[194] L. Nadel, A. Hupbach, R. Gomez, and K. Newman-Smith, "Memory formation, consolidation and transformation," *Neurosci. Biobehav. Rev.*, vol. 36, no. 7, pp. 1640–1645, Aug. 2012.

[195] T. Kitamura and K. Inokuchi, "Role of adult neurogenesis in hippocampal-cortical memory consolidation," *Mol. Brain*, vol. 7, p. 13, Feb. 2014.

[196] E. I. Moser, E. Kropff, and M.-B. Moser, "Place Cells, Grid Cells, and the Brain's Spatial Representation System," *Annu. Rev. Neurosci.*, vol. 31, no. 1, pp. 69–89, 2008.

[197] N. C. Fox, W. R. Crum, R. I. Scahill, J. M. Stevens, J. C. Janssen, and M. N. Rossor, "Imaging of onset and progression of Alzheimer's disease with voxel-compression mapping of serial magnetic resonance images," *The Lancet*, vol. 358, no. 9277, pp. 201–205, Jul. 2001.

[198] R. I. Scahill, J. M. Schott, J. M. Stevens, M. N. Rossor, and N. C. Fox, "Mapping the evolution of regional atrophy in Alzheimer's disease: Unbiased analysis of fluid-registered serial MRI," *Proc. Natl. Acad. Sci.*, vol. 99, no. 7, pp. 4703–4707, Apr. 2002.

[199] R. I. Scahill, C. Frost, R. Jenkins, J. L. Whitwell, M. N. Rossor, and N. C. Fox, "A longitudinal study of brain volume changes in normal aging using serial registered magnetic resonance imaging," *Arch. Neurol.*, vol. 60, no. 7, pp. 989–994, Jul. 2003.

[200] M. Ewers, P. Insel, W. J. Jagust, L. Shaw, J. Q. T. J, P. Aisen, R. C. Petersen, N. Schuff, and M. W. Weiner, "CSF Biomarker and PIB-PET–Derived Beta-Amyloid Signature Predicts Metabolic, Gray Matter, and Cognitive Changes in Nondemented Subjects," *Cereb. Cortex*, vol. 22, no. 9, pp. 1993–2004, Sep. 2012.

[201] O. Szekely, Y. Schilt, A. Steiner, and U. Raviv, "Regulating the Size and Stabilization of Lipid Raft-like Domains and Using Calcium Ions as Their Probe," *Langmuir*, vol. 27, no. 24, pp. 14767–14775, Dec. 2011.

[202] M. Gallagher and M. T. Koh, "Episodic memory on the path to Alzheimer's disease," *Curr. Opin. Neurobiol.*, vol. 21, no. 6, pp. 929–934, Dec. 2011.

[203] J. L. Price, A. Ko I., M. J. Wade, S. K. Tsou, D. W. McKeel, and J. C. Morris, "Neuron number in the entorhinal cortex and ca1 in preclinical alzheimer disease," *Arch. Neurol.*, vol. 58, no. 9, pp. 1395–1402, Sep. 2001.
[204] J. L. Whitwell, S. A. Przybelski, S. D. Weigand, D. S. Knopman, B. F. Boeve, R. C. Petersen, and C. R. Jack, "3D maps from multiple MRI illustrate changing atrophy patterns as subjects progress from mild cognitive impairment to Alzheimer's disease," *Brain*, vol. 130, no. 7, pp. 1777–1786, Jul. 2007.
[205] L. E. M. Wisse, G. J. Biessels, S. M. Heringa, H. J. Kuijf, D. (H.) L. Koek, P. R. Luijten, and M. I. Geerlings, "Hippocampal subfield volumes at 7T in early Alzheimer's disease and normal aging," *Neurobiol. Aging*, vol. Article in Press, 2014.
[206] S. E. Arnold, J. B. Toledo, D. H. Appleby, S. X. Xie, L.-S. Wang, Y. Baek, D. A. Wolk, E. B. Lee, B. L. Miller, V. M.-Y. Lee, and J. Q. Trojanowski, "Comparative survey of the topographical distribution of signature molecular lesions in major neurodegenerative diseases," *J. Comp. Neurol.*, vol. 521, no. 18, pp. 4339–4355, Dec. 2013.
[207] H. Braak, E. Braak, J. Bohl, and W. Lang, "Alzheimer's disease: amyloid plaques in the cerebellum," *J. Neurol. Sci.*, vol. 93, no. 2–3, pp. 277–287, Nov. 1989.
[208] K. Andersen, B. B. Andersen, and B. Pakkenberg, "Stereological quantification of the cerebellum in patients with Alzheimer's disease," *Neurobiol. Aging*, vol. 33, no. 1, pp. 197.e11–197.e20, Jan. 2012.
[209] J. A. Bernard and R. D. Seidler, "Moving forward: Age effects on the cerebellum underlie cognitive and motor declines," *Neurosci. Biobehav. Rev.*, vol. 42, pp. 193–207, May 2014.
[210] H.-Y. Kim, J. Bigelow, and J. H. Kevala, "Substrate Preference in Phosphatidylserine Biosynthesis for Docosahexaenoic Acid Containing Species," *Biochemistry (Mosc.)*, vol. 43, no. 4, pp. 1030–1036, Feb. 2004.
[211] G. Rizzolatti, G. Luppino, and M. Matelli, "The organization of the cortical motor system: new concepts," *Electroencephalogr. Clin. Neurophysiol.*, vol. 106, no. 4, pp. 283–296, Apr. 1998.
[212] L. Su, L. Wang, F. Chen, H. Shen, B. Li, and D. Hu, "Sparse Representation of Brain Aging: Extracting Covariance Patterns from Structural MRI," *PLoS One*, vol. 7, no. 5, May 2012.
[213] A. M. Fjell, L. T. Westlye, I. Amlien, T. Espeseth, I. Reinvang, N. Raz, I. Agartz, D. H. Salat, D. N. Greve, B. Fischl, A. M. Dale, and K. B. Walhovd, "High Consistency of Regional Cortical Thinning in Aging across Multiple Samples," *Cereb. Cortex*, vol. 19, no. 9, pp. 2001–2012, Sep. 2009.
[214] J. C. Baron, G. Chételat, B. Desgranges, G. Perchey, B. Landeau, V. de la Sayette, and F. Eustache, "In Vivo Mapping of Gray Matter Loss with Voxel-Based Morphometry in Mild Alzheimer's Disease," *NeuroImage*, vol. 14, no. 2, pp. 298–309, Aug. 2001.
[215] X. Guo, K. Chen, Y. Zhang, Y. Wang, and L. Yao, "Regional covariance patterns of gray matter alterations in Alzheimer's disease and its replicability evaluation," *J. Magn. Reson. Imaging*, vol. 39, no. 1, pp. 143–149, Jan. 2014.
[216] P. M. Thompson, M. S. Mega, R. P. Woods, C. I. Zoumalan, C. J. Lindshield, R. E. Blanton, J. Moussai, C. J. Holmes, J. L. Cummings, and A. W. Toga, "Cortical Change in Alzheimer's Disease Detected with a Disease-specific Population-based Brain Atlas," *Cereb. Cortex*, vol. 11, no. 1, pp. 1–16, Jan. 2001.
[217] J. E. Vance and R. Steenbergen, "Metabolism and functions of phosphatidylserine," *Prog. Lipid Res.*, vol. 44, no. 4, pp. 207–234, Jul. 2005.

[218] A. E. Rusiñol, Z. Cui, M. H. Chen, and J. E. Vance, "A unique mitochondria-associated membrane fraction from rat liver has a high capacity for lipid synthesis and contains pre-Golgi secretory proteins including nascent lipoproteins.," *J. Biol. Chem.*, vol. 269, no. 44, pp. 27494–27502, Nov. 1994.

[219] Y. J. Shiao, G. Lupo, and J. E. Vance, "Evidence that phosphatidylserine is imported into mitochondria via a mitochondria-associated membrane and that the majority of mitochondrial phosphatidylethanolamine is derived from decarboxylation of phosphatidylserine," *J. Biol. Chem.*, vol. 270, no. 19, pp. 11190–11198, May 1995.

[220] T. Melo, R. A. Videira, S. André, E. Maciel, C. S. Francisco, A. M. Oliveira-Campos, L. M. Rodrigues, M. R. M. Domingues, F. Peixoto, and M. Manuel Oliveira, "Tacrine and its analogues impair mitochondrial function and bioenergetics: a lipidomic analysis in rat brain," *J. Neurochem.*, vol. 120, no. 6, pp. 998–1013, Mar. 2012.

[221] M. A. Kiebish, X. Han, and T. N. Seyfried, "Examination of the Brain Mitochondrial Lipidome Using Shotgun Lipidomics," *Methods Mol. Biol. Clifton NJ*, vol. 579, pp. 3–18, 2009.

[222] K. Zilles and K. Amunts, "Centenary of Brodmann's map — conception and fate," *Nat. Rev. Neurosci.*, vol. 11, no. 2, pp. 139–145, Feb. 2010.

[223] C. J. Fhaner, S. Liu, X. Zhou, and G. E. Reid, "Functional Group Selective Derivatization and Gas-Phase Fragmentation Reactions of Plasmalogen Glycerophospholipids," *Mass Spectrom.*, vol. 2, no. Spec Iss, 2013.

Appendices

Appendix 1 R Script for generation of heatmaps

heatmap2_noscale.R*

Source on Save | Run | Source

```
#Load packages for Heatmap into library
library(gplots)
library(RColorBrewer)


#Import data from csv
heatmap.data <- read.csv("~/R/heatmap/slope/heatmap_micro_new.csv")
#Define row names, and remove as seperate column in table
row.names(heatmap.data) <- heatmap.data$Phospholipid
heatmap.data$Phospholipid <- NULL

#Create heatmap from data file
heatmap.matrix <- data.matrix(heatmap.data)

#Add in color breaks to account for outliers
breaks=seq(-0.05, 0.05, by=0.001)
breaks=append(breaks,.2)
breaks=append(breaks, -.15, 0)
mycol <- colorpanel(102, low="red",mid="black", high="green")

#Export heatmap as compressed TIFF file
#Set dendrogram (i.e. hierarchical clustering) for colmuns
tiff(filename="Microslope new.tif",width=8, height=10,
     units="in", res=300, bg="white",
     compression = "lzw")
heatmap <- heatmap.2(heatmap.matrix, dendrogram="col",
                     distfun = dist, hclustfun = hclust,
                     trace="none", density.info="none",
                   Rowv=F, Colv=TRUE,
                   col=mycol, breaks=breaks,
                   symm=F,symkey=F,symbreaks=T,
                   scale="none",
                   margins=c(5,20), key=T, keysize = 1,
                   lmat=rbind(c(0,3),c(2,1),c(4,1)),
                   lwid=c(1.5,4), lhei=c(1.5,15,3),
                   cexRow=1.5,cexCol=2,
                   colsep=1:5,
                   rowsep=1:40,
                   sepcolor="black",
                   sepwidth=c(0.01,0.01))
dev.off()
#if having trouble with graphics, run
#dev.off()
```

27:22 (Top Level) R Script