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Identification of plasma lipid biomarkers in Alzheimer's disease

Rhona Creegan
Edith Cowan University

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Identification of Plasma Lipid Biomarkers in Alzheimer's Disease

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MSc Nutrition Medicine

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This thesis is presented for the degree of Doctor of Philosophy at
Edith Cowan University

Faculty of Computing and Health Sciences

School of Medical Sciences

Abstract

Alzheimer's disease (AD), the commonest form of dementia, is a chronic, progressive neurodegenerative disease which manifests clinically as a slow global decline in cognitive function, including deterioration of memory, reasoning, abstraction, language and emotional stability, culminating in a patient with end-stage disease, totally dependent on custodial care. With an ageing population, there is predicted to be a marked increase in the number of people diagnosed with AD in the coming decades, making this a significant challenge to socio-economic policy and aged care. Currently there is no cure for AD and while current therapies may temporarily ameliorate symptoms, death usually occurs approximately 8 years after diagnosis. Attention is now being directed to the discovery of biomarkers that may not only facilitate pre-symptomatic diagnosis but provide an insight into aberrant biochemical pathways that may reveal potential therapeutic targets. AD pathogenesis develops over many years before clinical symptoms appear, providing the opportunity to develop therapy that could slow or stop disease progression well before any clinical manifestations develop.

Research and understanding of AD pathology has been driven in recent years by advances in technologies, enabling the precise investigation of the lipidome; the repertoire of lipid species present in cells and tissues that reflect the net effect of gene and protein expression, which in turn are influenced by the cellular environment. Lipidomic studies have identified abnormal lipid metabolism as a key component of the pathological processes which lead to the development of AD. Therefore, lipidomic studies are crucial for advancing the understanding of AD pathology and for identifying potential therapeutic targets; these studies may also facilitate biomarker discovery. Many studies have reported abnormal lipid profiles in both AD plasma and brain tissue.

This thesis investigated plasma lipid species using a "shotgun" lipidomics approach by electrospray ionisation tandem mass spectrometry (ESI/MS/MS). Additionally, Phospholipid Transfer Protein (PLTP); a protein involved in lipid metabolism was assayed using a commercial kit. The utility of these analytes as potential AD biomarkers was investigated by testing plasma samples from the

Abstract

highly characterised Australian Imaging, Biomarkers and Lifestyle (AIBL) study. The study cohort comprised over 1000 participants at inception who were classified as either healthy control (n=733), mild cognitive impairment (MCI, n=125) or AD (n=204): Samples from the baseline and 18 month follow-up time points were utilised. Plasma PLTP activity levels were measured in a subset of the baseline samples (n=259). Lipid and PLTP measurements were analysed in conjunction with supplementary neuroimaging and blood biomarker data collected as part of the AIBL study.

The thesis identified significant differences in several plasma lipids between clinical classification groups, including several ceramide, sphingomyelin (SM), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and plasmalogen species. Additionally, a panel of lipids was identified which could distinguish AD participants from healthy controls with a sensitivity and specificity of 80%. Plasma PLTP activity was significantly lower in AD and MCI groups compared to healthy controls, and levels correlated with plasma A β in all groups and cerebral A β in the healthy controls.

The results of this thesis validate and extend previous findings reported in the literature. The current findings provide evidence to indicate that several lipid species and PLTP show promise as potential blood biomarkers of AD. Further investigation using a targeted lipidomics platform and prospective longitudinal follow-up is warranted.

Declaration

I certify that this thesis does not, to the best of my knowledge and belief:

- i. Incorporate without acknowledgement any material previously submitted for a degree or diploma in any institution of higher education;
- ii. Contain any material previously published or written by another person except where due reference is made in the text of this thesis; or
- iii. Contain any defamatory material;

Date 10-03-14

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Firstly, I would like to thank my supervisor Ralph Martins for the opportunities and guidance you have provided me.

This research was supported by funding provided by the Cooperative Research Centres (CRC) for mental health.

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Thank you for the hard work and assistance provided by Dr Florence Lim and her collaborators whose expertise in the field of lipidomics was invaluable, with special thanks to Florence for the data extraction; a huge undertaking!

The statistical analysis of the data for this thesis was complex and time consuming and I am very grateful for the expertise and time provided by Dr James Doecke and Dr Samantha Burnham at CSIRO and was impressed by your efficiency and patience with my demands.

I would also like to thank everybody at the ECU laboratory for their assistance and support, with particular thanks to Rimi who was with me from the beginning and offered so many kind words.

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List of Abbreviations

| | |
|-------------------|---|
| α | Alpha-secretase |
| AA | Arachidonic acid |
| A β | Amyloid - beta |
| ABCA1 | ATP-binding cassette transporter A1 |
| ABCG1 | ATP-binding cassette transporter G1 |
| ACAT | AcylCoA-cholesterol acyl transferase |
| AD | Alzheimer's disease |
| ADAS-Cog | Alzheimer's disease assessment scale – cognitive subscale |
| ADDLs | Amyloid beta derived diffusible ligands |
| ADAM | A disintegrin and metalloproteinase |
| AGEs | Advanced glycation end products |
| AIBL | Australian Imaging, Biomarkers and Lifestyle study of Ageing |
| AIC | Akaike information criterion |
| AICD | APP intracellular domain |
| Akt/PKB | Serine/threonine-specific protein kinase B |
| ALA | Alpha-linolenic acid |
| AMPAR | α -amino-3-hydroxy-5-methyl-4-isoxazaolepropionic acid receptors |
| AMPK | Adenosine monophosphate kinase |
| ANOVA | Analysis of variance |
| Aph1 | Anterior pharynx defective -1 |
| ApoAI | Apolipoprotein AI |
| ApoB | Apolipoprotein B |
| ApoE | Apolipoprotein E |
| APOE | Apolipoprotein E gene |
| APOE ϵ 4 | Apolipoprotein E ϵ 4 gene |

Abbreviations

| | |
|------------------|---|
| ApoJ | Apolipoprotein J (clusterin) |
| APP | Amyloid precursor protein |
| APP _α | Soluble APP-alpha fragment |
| APP _β | Soluble APP-beta fragment |
| Arg | Arginine |
| ATP | Adenosine triphosphate |
| BACE or β | Beta site APP cleaving enzyme |
| BBB | Blood brain barrier |
| Bcl-2 | B-cell lymphoma-2 regulatory proteins |
| BDNF | Brain derived neurotrophic factor |
| C | Carbon |
| C | C-terminal |
| CAA | Cerebral amyloid angiopathy |
| CCT | CTP:phosphocholine cytidyltransferase |
| CDP | Cytidine diphosphate |
| CDR | Clinical dementia rating scale |
| CE | Cholesteryl ester |
| Cer | Ceramide |
| CETP | Cholesteryl ester transfer protein |
| Chol | Cholesterol |
| ChREBP | Carbohydrate responsive element binding protein |
| CID | Collision induced dissociation chamber |
| CLARA | Clustering for large applications |
| CM | Chylomicron |
| CNS | Central nervous system |
| CoA | Coenzyme A |
| CoQ10 | Coenzyme Q10 / Ubiquinone |

Abbreviations

| | |
|---------------------|--|
| COX | Cyclooxygenase |
| COX-2 | Cyclooxygenase-2 |
| cPLA ₂ | Cytosolic phospholipase A ₂ |
| CPT1 | Choline phosphotransferase 1 |
| CR | Chylomicron remnant |
| CRP | C-reactive protein |
| CSF | Cerebrospinal fluid |
| CSIRO | Commonwealth Scientific and Industrial Research Organisation |
| CT | Computed tomography |
| CTF | C-terminal fragment |
| CYP46A10 | Cytochrome P46A10 |
| Cys | Cysteine |
| C ₁₇ Cer | C ₁₇ -ceramide |
| DAG | Diacylglycerol |
| DALI | Diabetes Atorvastatin Lipid Intervention study |
| DES | Dihydroceramide desaturase |
| DHA | Docosahexaenoic acid |
| DHT | Dihydrotestosterone |
| diC ₈ PI | Diocanoyl-phosphatidylinositol |
| DM | Diabetes mellitus |
| DMPA | Dimyristoyl-phosphatidic acid |
| DMPC | Dimyristoyl-phosphatidylcholine |
| DMPE | 1,2-dimyristoyl-glycero-3-phosphoethanolamine |
| DMPG | Dimyristoyl-phosphatidylglycerol |
| DMPS | Dimyristoyl-phosphatidylserine |
| DNA | Deoxyribonucleic acid |

Abbreviations

| | |
|-----------|---|
| DSM-IV | Diagnostic and Statistical Manual of Mental Disorders, version IV |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme linked immunosorbent assay |
| EP4 | Prostaglandin E receptor 4 |
| EPA | Eicosapentaenoic acid |
| ER | Endoplasmic reticulum |
| ESI/MS/MS | Electrospray ionisation tandem mass spectrometry |
| F | Female |
| FA | Fatty acid |
| FAD | Flavin adenine dinucleotide |
| FAD | Familial Alzheimer's disease |
| FAS | Fatty acid synthase |
| FDG | Fluorodeoxyglucose |
| FFA | Free fatty acid |
| FMN | Flavin mononucleotide |
| FSH | Follicle stimulating hormone |
| FXR | Farnesoid X receptor |
| γ | Gamma secretase |
| GABA | Gamma amino butyric acid |
| Gal | Galactose |
| GALC | Galactosylceramidase |
| GDNF | Glial-derived neurotrophic factor |
| GDS | Geriatric depression scale |
| GLM | Generalised linear model |
| Glu | Glucose |
| GluCer | C8-Glucosylceramide |

Abbreviations

| | |
|--------------|---|
| GM1 | Ganglioside GM1 |
| GSK3 β | Glycogen synthase kinase 3 β |
| HC | Healthy control |
| HDL | High density lipoprotein |
| HFCS | High fructose corn syrup |
| HFS | High fat/high sugar |
| Hip Vol | Hippocampal volume |
| HMGCoAR | 3-hydroxy-3-methyl-glutaryl-CoA reductase |
| HPG | Hypothalamic pituitary gonadal axis |
| HPLC | High performance liquid chromatography |
| HRT | Hormone replacement therapy |
| ICD | International statistical classification of disease |
| IDE | Insulin degrading enzyme |
| IDL | Intermediate density lipoprotein |
| IL1 β | Interleukin 1 β |
| IL-6 | Interleukin-6 |
| iNOS | Inducible nitric oxide synthase |
| IR | Insulin resistance |
| IRS-1 | Insulin receptor substrate -1 |
| JAK2/STAT3 | Janus kinase 2 / Signal transducer and activator of transcription |
| LCAT | Lecithin-cholesterol acyl transferase |
| LC-FACS | Long chain fatty acyl CoA synthase |
| LDL | Low density lipoprotein |
| LDLR | Low density lipoprotein receptor |
| LH | Leutinisising hormone |
| LIMMA | Linear models for microarray |

Abbreviations

| | |
|----------------|--|
| LPL | Lipoprotein lipase |
| LPS | Lipopolysaccharide |
| LRP1 | Lipoprotein receptor-related protein 1 |
| LTP | Long term potentiation |
| LXR | Liver X receptor |
| LysoPA | Lysophosphatidic acid |
| LysoPC | Lysophosphatidylcholine |
| LysoPE | Lysophosphatidylethanolamine |
| LysoPI | Lysophosphatidylinositol |
| LysoPS | Lysophosphatidylserine |
| M | Male |
| MCI | Mild Cognitive Impairment |
| MHFTR | Methylene tetrahydrofolate reductase |
| MMSE | Mini mental state examination |
| MPRAGE | Magnetisation prepared rapid gradient echo |
| MRI | Magnetic resonance imaging |
| MRM | Multiple reaction monitoring |
| mRNA | Messenger ribonucleic acid |
| MUFA | Monounsaturated fatty acid |
| N | N-terminal |
| NAD | Nicotine adenine dinucleotide |
| NADP | Nicotine adenine dinucleotide phosphate |
| NADH | Nicotine adenine dinucleotide (reduced form) |
| NADPH | Nicotine adenine dinucleotide phosphate (reduced form) |
| NEFA | Non esterified fatty acid |
| NF- κ B | Nuclear factor kappa beta |
| NFT | Neurofibrillary tangles |

Abbreviations

| | |
|----------------|---|
| NGF | Neuronal growth factor |
| NIA/AA | National Institute on Aging and the Alzheimer's Association |
| NINCDS-ARDA | National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association |
| NMC | Non-memory complainer |
| NMDA | N-methyl D-aspartate |
| NMR | Nuclear magnetic resonance |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| NPD1 | Neuroprotectin D1 |
| P | Phosphate |
| PA | Phosphatidic acid |
| PAF | Platelet activating factor |
| PAM | Partitioning around medoids |
| PC | Phosphatidycholine |
| PDD | Parkinson's disease dementia |
| PE | Phosphatidylethanolamine |
| PEMT | Phosphatidylethanolamine–N-methyltransferase |
| PEN-2 | Presenilin enhancer-2 |
| PET | Positron emission tomography |
| PG | Phosphatidylglycerol |
| PGC-1 α | PPAR-gamma coactivator 1 α |
| PI | Phosphatidylinositol |
| PI3K | Phosphatidylinositol 3 kinase |
| PiB | Pittsburgh compound B |
| PiB-PET | Pittsburgh compound B- Positron emission tomography |
| PIP | Phosphatidylinositol phosphate |

Abbreviations

| | |
|------------------|--|
| PIP ₂ | Phosphatidylinositol 4,5-biphosphate |
| PKC-Delta | Protein kinase C delta |
| PL | Phospholipid |
| PLA ₂ | Phospholipase A ₂ |
| PLC | Phospholipase C |
| PLD | Phospholipase D |
| PLP | Pyridoxal-5-phosphate |
| PlsEtn | Plasmenylethanolamine (ethanolamine plasmalogen) |
| PLTP | Phospholipid transfer protein |
| PPAR | Peroxisome proliferator activated receptor |
| PS | Phosphatidylserine |
| PS1 | Presenilin 1 |
| PS2 | Presenilin 2 |
| PUFA | Polyunsaturated fatty acid |
| RBM | Rules based medicine |
| RCT | Reverse cholesterol transport |
| RDA | Recommended dietary allowance |
| ROC | Receiver operating characteristics |
| ROS | Reactive oxygen species |
| RXR | Retinoid X receptor |
| SAH | S-adenosylhomocysteine |
| SAMe | S-adenosyl methionine |
| SCAP | SREBP cleavage-activating protein |
| SCD | Stearoyl-CoA desaturase |
| SEM | Standard error of mean |
| SHBG | Sex hormone binding globulin |
| SIRT | Silent mating-type information regulation (sirtuin proteins) |

Abbreviations

| | |
|-------------------|---|
| SL | Sphingolipid |
| SM | Sphingomyelin |
| SMase | Sphingomyelinase |
| SMC | Subjective memory complainer |
| SNP | Single nucleotide polymorphism |
| SOD | Superoxide dismutase |
| SorLA/LR11 | Sortilin-related receptor |
| sPLA ₂ | Secretory phospholipase A ₂ |
| SPT | Serine-palmitoyl transferase |
| SR-B1 | Scavenger receptor B1 |
| SREBPs | Sterol regulatory element binding proteins |
| TACE | Tumour necrosis factor α -converting enzyme |
| TCA | Tricarboxylic acid |
| TNF- α | Tumour necrosis factor alpha |
| UDP | Uridine diphosphate |
| UGCG | UDP-glucose ceramide glucosyltransferase |
| VDR | Vitamin D receptor |
| Vit E | Vitamin E |
| VLDL | Very low density lipoprotein |
| WHASII | Women's Health and Aging study II of cognitive impairment |
| WHIMS | Women's Health Initiative Memory Study |
| WHIMSY | Women's Health Initiative Memory Study of Younger women |
| χ^2 | Chi squared |
| 4-HHE | 4-hydroxyhexanal |
| 4-HNE | 4-hydroxynonenal |
| 15-LO-1 | 15-lipoxygenase-1 |

Abbreviations

| | |
|-------|-------------------------|
| 24SHC | 24-S-hydroxycholesterol |
| 27HC | 27-hydroxycholesterol |

Publications and Conference Proceedings

Publications

IJ.Martins and **R.Creegan**. Links between insulin resistance, lipoprotein metabolism and amyloidosis in Alzheimer's disease. *Health- special issue on "Blood Lipids and Health"*. Submitted for online publication May 2014.

WLF.Lim, SM.Lam, G.Shui, A.Mondal, D.Ong, X.Duan, **R.Creegan**, IJ.Martins, M.Sharman, K.Taddei, G.Verdile, M.Wenk and RN.Martins. Effects of a high-fat, high cholesterol diet on brain lipid profiles in apolipoprotein E ϵ 3 and ϵ 4 knock-in mice. *Neurobiology of Aging*. 2013; 34 (9);2217-2224.

IJ.Martins, **R.Creegan**, WLF.Lim, RN.Martins. Molecular insights into appetite control and neuroendocrine disease as risk factors for chronic diseases in western countries. *Open Journal of Endocrine and Metabolic Diseases*. 2013;3;11-33.

Conference proceedings

Conference speaker – A5M 7th Annual Conference in Anti-Ageing & Aesthetic Medicine - Melbourne, Australia – August 2013.

Metabolic syndrome, obesity and Alzheimer's disease – abnormal lipid metabolism – a common link?

Rhona Creegan, Wei Ling Florence Lim, Ian J Martins, Simon M Laws ,Veer Bala Gupta, Stephanie Rainey-Smith, Guanghou Shui, Markus R Wenk, Ralph N Martins and the Australian Imaging, Biomarkers and lifestyle (AIBL) research group.

Poster presentation – 11th International Conference on Alzheimer's and Parkinson's diseases – Florence. Italy – March 6-10, 2013.

Plasma lipidomics in Alzheimer's disease (AD) – a potential biomarker: preliminary data from the Australian Imaging, Biomarkers and Lifestyle study (AIBL) of ageing.

Rhona Creegan, Wei Ling Florence Lim, Ian J Martins, Simon M. Laws, Veer Gupta, Stephanie Rainey-Smith, Guanghou Shui , Markus R. Wenk , Ralph N Martins and the AIBL research group.

Chapter 1

Literature Review

Lipids and Alzheimer's Disease

Chapter 1 – Review of the Literature – Lipids and AD

1.1 Alzheimer's Disease – Background

Alzheimer's disease (AD) is the commonest form of dementia, initially identified by Alois Alzheimer in 1906. It is a chronic, progressive neurodegenerative disease manifesting clinically as a disturbance in multiple higher functions of the brain, including memory, thinking, orientation, comprehension, calculation, learning capacity, language and judgement.

It can also be accompanied by deterioration in emotional control, motivation or social behaviour [1, 2]. In advanced stages, care by family members and carers is required to assist with daily living and ultimately an inability to recognise family members exacerbates the huge emotional burden. On average, the disease progresses from initial mild symptoms to severe dementia over a period of about eight years, with many sufferers requiring institutional care for several years.

The greatest risk factor for AD is age and so with an ageing population, there is predicted to be a marked increase in the number of AD cases in the coming decades; in both the developed and developing world [3, 4]. In Australia, approximately 300,000 people are suffering from dementia, with this figure expected to be close to 1 million by 2050 [5].

Current estimates put a direct cost of \$AUD3.2 billion to the healthcare system in Australia with a predicted increase to \$AUD6billion within the next five years [6]. This represents a major economic burden to an already stretched healthcare budget. According to the Alzheimer's Association in the US, unpaid care by family members and caregivers in 2009 represented \$144 billion. These alarming statistics emphasise the importance of gaining a greater understanding of the pathophysiology of AD with the aim of developing tests that will identify those at risk before the clinical hallmarks of AD manifest.

Currently, post mortem examination of the affected brain still provides the most affirmative diagnosis of AD, however with advances in brain imaging (amyloid ligand positron emission tomography (PET)), amyloid beta ($A\beta$) can now be visualised ante-mortem. This may assist in identifying pre-clinical stages of disease. Ultimately early diagnosis, identification of those at risk and the

development of intervention strategies offers the best chance of significantly reducing the number of AD cases.

1.1.1 AD Pathology – hallmarks of disease

In AD, the rate at which nerve cells deteriorate and die rapidly increases, greatly outstripping renewal and repair mechanisms resulting in shrinkage of several areas of the brain [7]. The causes and factors leading to disease progression are poorly understood but AD pathology is characterised by the presence of senile plaques, beta-amyloid ($A\beta$) deposits and neurofibrillary tangles in the cerebral cortex and sub-cortical grey matter.

An interesting and until recently often overlooked feature of AD pathology, originally described by Alois Alzheimer, is an increased number of “lipoid granules” in the AD brain [8]. Despite this observation, understanding the connection between abnormal lipid metabolism and AD has progressed slowly, although renewed interest in this area of AD pathology is gaining momentum due to the technological advances in lipid analyses.

There is much debate in the literature as to which of these hallmarks are causative and which result from other patho-physiological processes. Multiple factors have been implicated in the aetiology and pathogenesis of AD. These factors include genetic defects, abnormal lipid metabolism, energy metabolism deficits and mitochondrial defects, inflammation, abnormal amyloid precursor protein processing, deficiency of neurotrophic factors, glutamate excitotoxicity, free-radical induced neuron degeneration and trace element toxicity. It is unlikely that any of these factors are isolated, but when combined with imbalances in neurotransmitters and hormones and impaired hepatic metabolic and detoxification pathways, a complex web of dysfunctional biochemistry is established, resulting in the characteristic AD pathology [7].

1.1.2 Amyloid Plaques

Amyloid plaques are a characteristic feature of AD and contain aggregates of $A\beta$, a 4kDa peptide of 39-43 amino acids normally found in the brain, albeit at low concentrations [9]. The plaques when visualised at *post-mortem* are typically spherical and range from 10-150nm [10] (see Figure 1.1A). $A\beta$ is a

protein fragment cleaved by the action of secretase enzymes from its larger parent protein called amyloid precursor protein (APP). APP is located in the plasma (outer) membrane of brain cells, including neurons, glial cells and the endothelial cells of the blood brain barrier. APP is an abundant protein in the central nervous system but is also ubiquitously expressed in the peripheral tissues such as epithelium, muscle and circulating cells. Platelets are a rich source of APP and contain concentrations similar to that of brain cells [11].

The APP protein undergoes proteolytic cleavage by enzymes termed BACE (beta site APP cleaving enzyme) and the α - and γ -secretases which act via two competing pathways which depend on the localisation of APP to lipid rafts and feedback loops controlling gene transcription for the appropriate enzymes (see Section 1.5 and reviewed in [12]).

One product of APP cleavage is $A\beta$ of which two main forms of $A\beta$ are secreted; $A\beta_{40}$ and $A\beta_{42}$ consisting of 40 and 42 amino acids respectively. $A\beta_{42}$ accounts for approximately 10% of secreted $A\beta$, but is the main form found in amyloid plaques, suggesting a more pathological role for $A\beta_{42}$ due to its ability to aggregate and polymerise into amyloid fibrils more readily than $A\beta_{40}$ [9]. An overproduction or reduced clearance of $A\beta$ is considered a key component of AD and alterations in $A\beta$ kinetics may contribute to the induction of inflammation, oxidation and neurotoxicity, turning a physiologically relevant protein into a potentially toxic one [13].

Evidence also suggests that soluble oligomers of $A\beta$ known as amyloid beta derived diffusible ligands (ADDLs) accumulate and initiate a toxic cascade before plaque deposition and neuronal death occurs. This may explain the inconsistencies observed with plaque burden and cognitive deficits [14].

There is no doubt that the evidence suggests a key role for $A\beta$ in the neuropathology of AD, but the amyloid hypothesis as the sole driver has been challenged by recent studies using immunisation with pre-aggregated $A\beta_{1-42}$, demonstrating that in spite of almost complete elimination of senile plaques from the AD brain, cognitive decline still progressed [15]. This evidence has also been corroborated by the failure in clinical trials of the gamma-secretase inhibitor semagacestat, which reduced $A\beta$ production, but failed to retard

disease progression [16]. However, the patients used in these trials had advanced disease, where any increased clearance of A β may have occurred too late to influence cognitive decline. Further trials are required that target early stage disease. Moreover, it has been suggested that A β deposition merely represents a protective response to some other pathophysiological process or is involved with the chelation of potentially oxidative transition metals [17]. A more detailed section on APP processing, A β generation and lipids is presented later in the chapter (see Section 1.5.1).

1.1.3 Neurofibrillary tangles

Affected areas of the AD brain have significantly lower neuronal numbers and the remaining neurons possess reduced numbers of dendrite branches and synaptic densities. Neurofibrillary tangles (NFTs) arise within individual neurons as depositions of abnormally twisted filaments (see Figure 1B).

The tau protein in neurons is normally organised in a linear fashion into microtubules, which give structural integrity and are involved in intracellular transport [18]. Phosphorylation of tau is part of the normal process of assembly of microtubules, conferring stability. However, in AD the tau proteins become hyper-phosphorylated or glycosylated, causing them to twist and form tangles, which adversely affects neuronal function and results in loss of intracellular communication [19-21].

The phosphorylation and dephosphorylation of tau, as with other proteins, is tightly regulated by various kinases and phosphatases and dysregulation of these processes may contribute to the accumulation of NFTs.

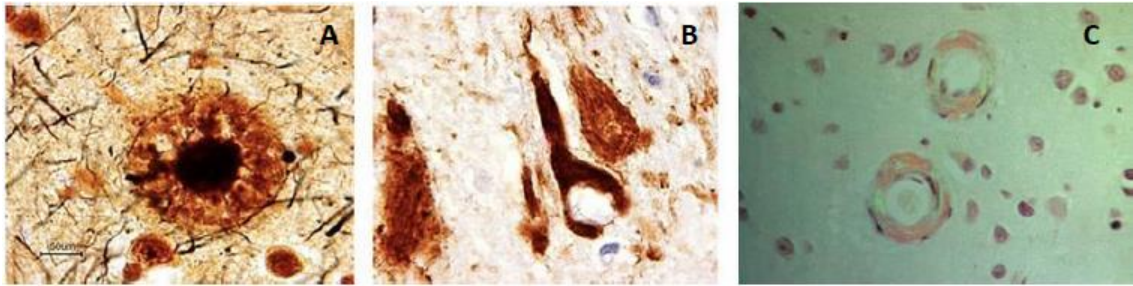


Figure 1.1: Pathological hallmarks of Alzheimer's disease. A) Amyloid plaques. B) Neurofibrillary tangles. C) Cerebral amyloid angiopathy (CAA). Amyloid plaques and NFTs are stained brown in 1A and 1B. Amyloid can also deposit in the cerebral and meningeal blood vessels as seen in 1C, where it appears to be associated with increased inflammation and risk of cerebral haemorrhage [22, 23]. However, both amyloid plaques and CAA can be seen in both cognitively normal individuals and in AD [24].

(Images adapted from coloradodementia.org and angiopathy.org)

1.1.4 Abnormal Lipid Metabolism

Abnormal lipid metabolism is emerging as a very important pathophysiological process in the development of AD. This is logical, as the adverse effects of abnormal lipid metabolism on neuronal biochemistry are numerous; affecting membrane lipid composition and a myriad of cellular signals generated by lipid mediators. The link between AD and lipid metabolism was firmly established when carriage of the apolipoprotein E ϵ 4 allele (APOE ϵ 4) was identified as a major risk factor for AD [25].

In recent years a greater understanding of the interaction between lipids, amyloidogenesis and tau pathology has emerged, with most classes of lipids being implicated. This makes sense as lipids are key components of membranes, providing an environment conducive for fluidity and ion permeability as well as regulating trafficking and proteolytic activity of key membrane-bound proteins (including APP, beta site APP cleaving enzyme, (BACE) and γ -secretase) involved in APP processing and AD [26].

Lipids may also affect the pathogenicity of A β by influencing its tendency to aggregate [27]. Lipid mediators are generated from phospholipids, sphingolipids and cholesterol by activation of phospholipases, sphingomyelinases and cytochrome p450-dependent oxygenases respectively, which exert numerous effects on cellular function. These effects involve modulation of enzyme activity,

neural cell differentiation, immune responses, inflammation, oxidation, cell migration, mitogenesis and apoptosis and therefore imbalances in any of these mediators would predictably contribute to AD pathology [28].

Later sections (Section 1.5) will outline the specifics of abnormal lipid metabolism and how this directly contributes to AD pathology when the homeostatic mechanisms become dysfunctional. This outline will be followed by individual discussions of the many integrated factors affecting lipid metabolism, bearing in mind the significant cross-talk and interaction of these regulatory factors. These sections have been separated in an attempt to simplify a highly complex and integrated system. As with other degenerative diseases, there are many feedback mechanisms and points of control required to maintain homeostasis, which when altered, synergistically combine to accelerate pathology.

1.2 Current diagnostic strategies

Current primary care approaches only detect AD once symptoms are already present, highlighting the need for a pre-symptomatic test. A primary care doctor relies upon determining a history of cognitive and behavioural change often with the help of an informant close to the patient.

Other causes of cognitive decline including head trauma, stroke, cancer, hormone, endocrine and nutrient abnormalities must be excluded by various testing. If a diagnosis of AD is suspected, the patient can then be referred to a specialist centre where various cognitive, physical and neurological tests can be performed alongside brain imaging.

Neuropsychological tests are used to assess memory, problem solving, communication and mood. Many population studies use a combination of these tests to improve sensitivity. In reality, no one test or imaging technique provides a definitive diagnosis and in practice combinations of assessment methods are used. The National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ARDA) established AD diagnostic criteria 27 years ago with a reported sensitivity and specificity of 81% and 70% respectively. To ensure flexibility in the criteria so that they may be used by both general healthcare providers and

specialist investigators, a recent revision of these criteria by the National Institute on Aging and the Alzheimer's Association (NIA/AA) now incorporates pre-clinical and mild cognitive impairment (MCI) stages [29].

Imaging and other biomarkers are also integrated into research settings to enhance specificity of diagnosis while key clinical criteria remain the main diagnostic tool in clinical practice. Emerging biomarker research and validation is expected to enhance the specificity of these diagnostic criteria. The following sections outline some of the tests commonly used in current diagnostic strategies, including those used in the well characterised Australian Imaging, Biomarkers and Lifestyle (AIBL) study of ageing; the cohort utilised in this study.

1.2.1 Neuropsychological Tests

A battery of neuropsychological tests can be employed to assess the level of cognitive impairment. These tests investigate the ability to acquire and remember new information, ability to plan and reason, visuospatial abilities and language functions. Testing can also help the doctor and family understand the impact of the disorder on the everyday functioning of the patient. There are several tests widely used, each having its own strengths and weaknesses. Relevant tests with respect to AIBL are briefly described below.

1.2.2 Mini Mental State Examination (MMSE)

The MMSE assesses global cognition and represents a commonly used test to evaluate memory problems as well as assisting with diagnosis of dementia and assessing progression and severity. The test involves a brief 30 point questionnaire probing memory, orientation and arithmetic functions [30]. MMSE scores range from 0 to 30 points: Table 1.1 facilitates interpretation.

Table 1.1: Mini-Mental State Examination (MMSE) scores and interpretation.

| MMSE Score | Interpretation |
|------------|---------------------------------|
| 0-9 | Severe AD |
| 10-19 | Moderate AD |
| 20-23 | Mild cognitive impairment (MCI) |
| 24-30 | Normal cognition |

Corrections to the score may have to be applied to account for age and education level, highlighting a limitation of this test, as a well educated person may score highly even when cognitive impairment is present. Equally a less educated, cognitively normal person may score in the lower range [31]. Consideration of an individual's hearing, reading, writing and drawing ability is also required when interpreting the scores, however despite these limitations, the MMSE is a useful tool. The MMSE is also capable of differentiating other types of dementia as AD patients typically score lower on orientation than Parkinson's, Lewy body and vascular dementia cases [32].

1.2.3 Clinical Dementia Rating (CDR) scale

The CDR is a scale used to assess the severity of dementia. Developed by John Morris in 1997 at the Washington University School of Medicine, a structured interview assesses the effects of cognitive loss on everyday activities [33]. Memory, orientation, judgement, problem solving, community affairs, personal care, home and hobbies are assessed and an algorithm applied to obtain a combined score of 0 to 3 as follows:

Table 1.2: Clinical Dementia Ratio (CDR) scale and interpretation.

| Total Score | Dementia |
|-------------|-----------|
| 0 | None |
| 0.5 | Very mild |
| 1 | Mild |
| 2 | Moderate |
| 3 | Severe |

The CDR is capable of detecting very mild cognitive deficits but it is however time consuming and a subjective measure. Despite its limitations it is widely accepted as a valid tool for assessing cognitive function.

1.2.4 Alzheimer's Disease Assessment Scale – Cognitive subscale (ADAS-Cog)

This test was specifically designed to evaluate the severity of cognitive decline and behavioural changes in AD [34]. This test is similar to the MMSE and uses a structured scale to probe executive functioning (the ability to carry out daily tasks, planning and decision making), memory, attention, language and orientation [35]. Executive functioning loss is an early event in AD.

1.2.5 Neuroimaging

Neuroimaging is an essential component of dementia diagnosis [35, 36]. Structural imaging with computed tomography (CT) or magnetic resonance imaging (MRI) is routinely used in the clinical evaluation of patients with AD. These techniques are used to exclude other causes of dementia such as normal-pressure hydrocephalus, vascular dementia, or intracranial mass. In addition, the degree of atrophy can be assessed [37].

Cortical atrophy is a consistent finding in AD, particularly within the medial temporal lobe structures including the hippocampus, amygdala and parahippocampal gyrus [38] (see Figure 1.2). Due to its high resolution (50-100 μ m), MRI can assist with diagnosis of AD by assessing specific brain region volumes, for example hippocampal atrophy.

Further, a recent study has shown good correlation between MRI determined grey matter volumes and total tau/A β levels in cerebrospinal fluid (CSF; a highly sensitive differential diagnostic test for AD) and explored the utility of MRI as a predictor of CSF total tau/A β levels. [39]. Structural changes are likely to be a feature of late stage disease and are predicted by functional changes seen with positron emission tomography (PET).

1.2.6 Positron Emission Tomography (PET)

PET is an imaging technique that enables three dimensional imaging and assessment of functional processes in the body by detecting gamma rays indirectly emitted by a positron emitting tracer such as ¹⁸F-fluorodeoxyglucose (FDG) or Pittsburgh compound B (PiB) [40, 41]. FDG is an analogue of glucose and is used to assess metabolic activity and regional glucose uptake, which is reduced in regions of the brain in those with AD [35].

The use of tracers has enabled a more robust differential diagnosis of AD from other dementias, due to the identification of the PiB compound ([N-methyl-¹¹C] 2-(4'-methylaminophenyl)-6-hydroxybenzothiazole). PiB directly binds to A β and can be used to assess brain A β burden [42]. However, individuals can accumulate A β and remain cognitively stable, and therefore PET imaging can facilitate interpretation of other measures but currently cannot be used alone as a predictor of cognitive decline (see Figure 1.2).

In addition, these imaging tests are currently limited to specialist or research centres and are expensive to perform, so the need for the development of other, cheaper and more accessible predictive tests is crucial.

This study aims to identify a blood lipid biomarker panel which can facilitate development of a definitive diagnostic test ante-mortem.

Volumetric Changes (MRI) and Amyloid Beta (PiB-PET) in the AD Brain

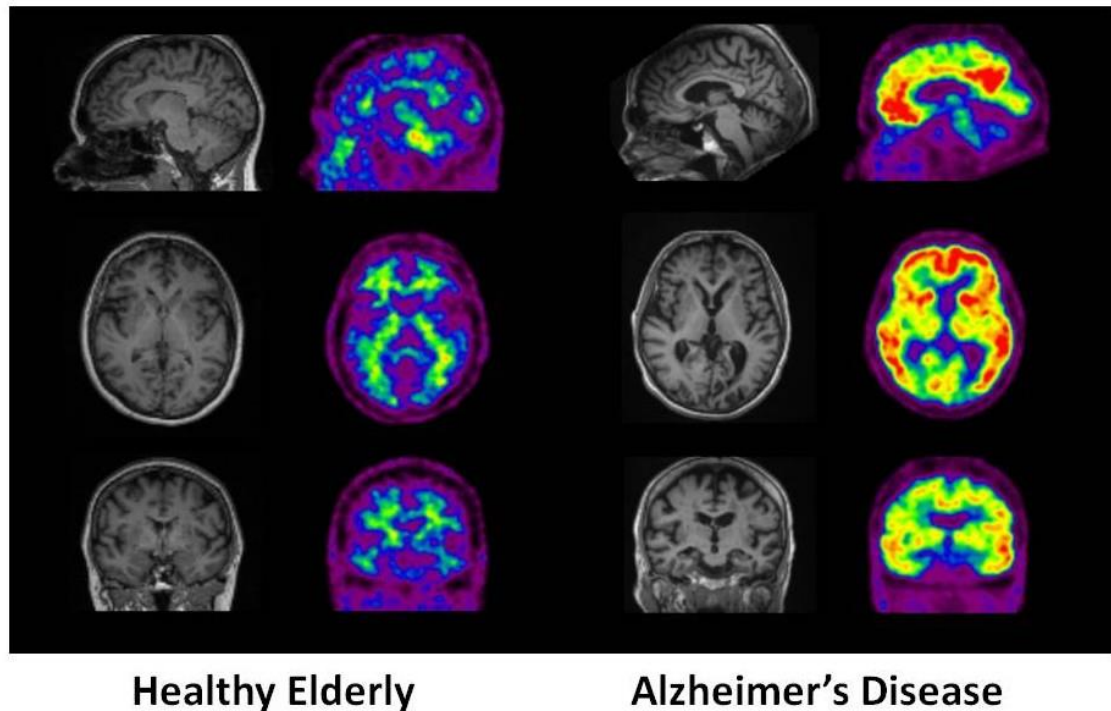


Figure 1.2: Age-matched AIBL Study participants. The AD brain on the right shows cortical atrophy and high concentrations of bound PiB (yellow/red), indicating a high amyloid load in these regions. The healthy brain on the left shows minimal shrinkage and a small amount of bound PiB.

Image courtesy of Professor Chris Rowe, Austin Health, Heidelberg, Victoria.

1.3 AD Risk factors

AD can be familial or sporadic. Familial Alzheimer's disease (FAD) is rare, accounting for less than 5% of all cases and symptoms usually manifest before age 65. In FAD, the major risk factor is possession of a mutant allele in one of three currently identified genes, which is inherited in an autosomal dominant fashion. However other as yet undiscovered genes may also be involved [43]. The genes currently known to carry mutations in FAD are those coding for APP, Presenilin 1 (PS1) and Presenilin 2 (PS2). Following APP cleavage by BACE, the membrane-bound complex γ -secretase generates $A\beta$ [9]. The presenilin genes code for the presenilin proteins that in association with nicastrin, Aph1 (anterior pharynx defective-1) and PEN-2 (presenilin enhancer-2) form the γ -secretase complex [44]. The many mutations identified in these three genes represent a fully penetrant risk factor for developing AD, and while rare compared to the more common sporadic form, provide a useful model for

studying patho-physiological processes. There are several known risk factors for sporadic AD; the more common form accounting for over 95% of all cases and typically affecting those over 65 years. These known risk factors will be discussed below.

1.3.1 Age

Advances in medical technology, control of infectious diseases and better living standards have resulted in an increase in the elderly population. This ensures an increase in age-related degenerative diseases, including age-related dementia; of which AD is the most common type. The following graph illustrates the advancing age of the Australian population since 1901.

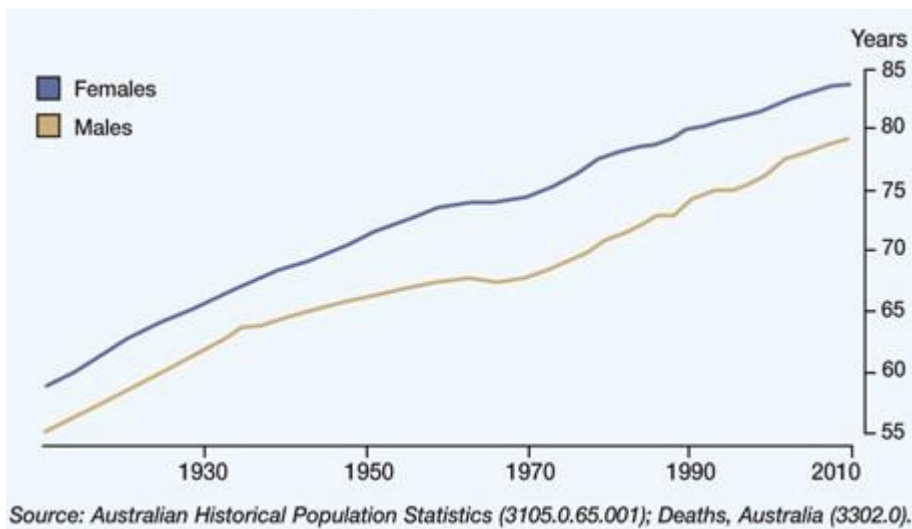


Figure 1.3: Life expectancy at birth, 1901-10 to 2008-10 in the Australian population.

The prevalence of AD at age 65 years is estimated to be approximately 5% and this rises dramatically to 20% for those over 80 years [3]. The multiple factors that are an inevitable part of the ageing process, such as abnormal lipid metabolism, mitochondrial defects, inflammation, oxidation, imbalances in neurotransmitters and hormones, compromised immune function, reduced hepatic detoxification capacity, digestive disorders and nutrient deficiencies, likely contribute to AD pathogenesis.

1.3.2 Apolipoprotein E ϵ 4 allele (APOE ϵ 4)

The late onset form of AD accounts for the majority of AD cases (as defined previously); with age and possession of the APOE ϵ 4 genotype being the predominant risk factors [45]. In humans the APOE gene is located on chromosome 19q13.2 [46] and encodes for a 299 amino acid protein of which there are three isoforms; ϵ 2, ϵ 3 and ϵ 4, resulting from the substitution of a cysteine with an arginine residue at codons 112 and 158 [47, 48]. Despite the amino acid difference at only one or two codons, the effect on risk of developing various diseases is quite marked due to conformational changes and the influence this has on binding capacity to lipids, proteins and receptors [48, 49]. The following table shows allele frequencies in caucasian and Australian populations and their influence on disease risk.

Table 1.3: Apolipoprotein E (APOE) gene frequency and disease risk [50-53].

| Genotype | Allele Frequency | Disease Association |
|--|------------------|---|
| APOE ϵ 2 (Cys ¹¹² Cys ¹⁵⁸) | 7% | ↑ and ↓ risk for atherosclerosis [54] and ↑ risk for Parkinson's Disease [55] |
| APOE ϵ 3 (Cys ¹¹² Arg ¹⁵⁸) | 79% | Considered neutral |
| APOE ϵ 4 (Arg ¹¹² Arg ¹⁵⁸) | 14% | ↑ risk for atherosclerosis, AD, cognitive impairment, ↓ hippocampal volume, faster progression of multiple sclerosis, cerebrovascular disease and sleep apnoea [25, 56-61] |

Cys: Cysteine, Arg: Arginine.

The locus within an apolipoprotein gene cluster on chromosome 19 was first shown to be an AD risk factor in 1993 [25]. ApoE was implicated, based on the facts that apoE is present in plaques and NFT, it binds to A β , and it is also the commonest brain apolipoprotein.

Further studies then demonstrated a strong allelic association between AD and the APOE ϵ 4 allele [56, 62]. It is estimated that being homozygous for APOE ϵ 4 increases the risk of developing AD by the age of 75 by ten to thirty times

compared to non-carriers of APOE ϵ 4 [53]. APOE ϵ 2 appears to be protective in terms of AD risk [53].

Apolipoprotein E (apoE) is the protein encoded by these genes and is an essential lipid transporter and component of triglyceride rich particles, such as chylomicrons and very low density lipoproteins (VLDL). ApoE is required for triglyceride catabolism, is involved with cholesterol metabolism in an isoform-dependent way and transports phospholipids and fat soluble vitamins (see Section 1.7 for more details). The importance of apoE was highlighted when it was demonstrated that familial dysbetalipoproteinaemia (type III hyperlipoproteinaemia) resulted from defects in the APOE gene affecting the function of the apoE protein. As clearance of chylomicrons and VLDL is impaired, elevated plasma cholesterol and triglycerides result, thereby increasing risk of cardiovascular and cerebrovascular disease, which are themselves risk factors for AD [63].

In the central nervous system, apoE is produced by astrocytes and microglia and neurons express apoE receptors, which are part of the low density lipoprotein receptor (LDLR) family, responsible for lipid transport among brain cells [64]. ApoE is a ligand for cell surface lipoprotein receptors, including those that chaperone APP and it has been shown to be directly involved in APP processing.

Studies have shown that when apoE is added to cell cultures a decrease in the secretion of A β is observed, with a rise in the APP C-terminal fragments; suggesting inhibition of γ -secretase and a subsequent reduction in A β generation [65]. There are several known mechanisms for clearing A β from the brain, and defects in any mechanism will contribute to an increase in brain A β load. The A β peptide can be cleaved directly by proteolytic enzymes, such as neprilysin and insulin degrading enzyme (IDE) [66]. Alternatively, A β can be internalised in apoE containing lipoprotein particles which can bind to low density lipoprotein receptor-related protein 1 (LRP1) receptors present on microglia, astrocytes and neurons – these particles are then endocytosed and transported to lysosomes for breakdown and recycling [67, 68].

A β can also be cleared in a similar manner by receptor-mediated transport into the periphery, following binding to LRP1-receptors on the blood brain barrier

(BBB). This results in efflux from the brain into the systemic circulation for processing by the liver and excretion via the bile [69]. A β ₄₀ is transported across the BBB via LRP1 much faster than A β ₄₂ [70].

The interaction of apoE-associated particles with A β peptides may facilitate the conversion of soluble to fibrillar A β when both apoE particles and A β are increased [71]. The APOE ϵ 4 isoform has the lowest affinity for A β binding and is the least effective in promoting both A β clearance and cholesterol efflux [72]. It is also the most immuno-reactive and least antioxidant form; all factors by which APOE ϵ 4 increases the risk for AD [73]. Recent studies also suggest that APOE ϵ 4 contributes to A β induced apoptosis in neuronal cells by forming a reactive intermediate that can insert into the lysosomal membrane causing destabilisation and lysosomal leakage [74, 75].

The APOE ϵ 4 allele is associated with high membrane cholesterol levels, which therefore promotes amyloidogenic APP processing. APOE ϵ 4 is also associated with a reduction in mRNA for the neuronal sortilin-related receptor (SorLA/LR11), a neuronal sorting protein that reduces APP trafficking to BACE and γ -secretase [76].

The amount of lipid carried by apoE appears to influence A β metabolism. Free A β is efficiently transported across the BBB but this transport is slowed in the presence of apoE. In addition, the reduced efflux of A β is influenced both by the degree of lipidation of the apoE and the isoform, with APOE ϵ 4 showing the greatest inhibition [77]. Apolipoprotein J, (ApoJ), the other main lipoprotein secreted by glial cells, promotes the transport of A β across the BBB [70].

Studies have shown that when A β binds to APOE ϵ 4 the transport of A β is redirected from LRP1 to the VLDL receptor (another member of the LDLR family) resulting in brain retention of A β as the binding affinity of the A β -apoE complexes is much lower for VLDL receptors compared to LRP1 [77]. Polymorphisms in the LRP1 gene itself have also been shown to increase risk of AD [78, 79].

Differences in apoE levels between healthy controls and AD patients have been reported in plasma and cerebrospinal fluid (CSF), although the results have been inconsistent [80-83]. Plasma apoE levels measured in the highly

characterised Australian Imaging, Biomarkers and Lifestyle (AIBL) study of ageing cohort have recently been shown to be lower in AD, which also correlated to brain A β load determined by PiB-PET imaging [84].

1.3.3 Diet and Nutrition

The type and amount of food eaten has a major influence on plasma lipid profiles and therefore any lipid biomarker panel. Dietary habits and nutritional status are also emerging as key components of chronic degenerative diseases, including AD. This is further compounded by the fact that AD is an age-related disorder with a general decline of digestive function, absorptive capacity and assimilation of nutrients.

Atrophic gastritis is common in older people and can affect absorption of key nutrients such as vitamin B12 and folate [85, 86], which have been associated with hyperhomocysteinaemia and increased risk of AD [87, 88]. This can also affect protein absorption and therefore the supply of essential amino acids, such as tryptophan and tyrosine, which are required for the synthesis of neurotransmitters such as serotonin and catecholamines [89].

There is an age related decline in metabolic rate [90] and coupled with a decrease in physical activity, elderly people tend to eat less and may consume inadequate micronutrients. Additionally, co-morbidities often exist, requiring pharmaceutical agents that may further compromise nutrient status. The well-established risk factors for developing AD are elevated cholesterol and dyslipidaemia, obesity, diabetes, hypertension, depression, cardiovascular and cerebrovascular disease (reviewed in [91, 92]), all of which are biochemically connected and are influenced by diet and possible nutrient deficiencies.

An increase in AD incidence is reported in populations who have a high intake of saturated fat, trans-fatty acids, refined sugar, processed foods, and total calories [93]. High fish consumption is inversely correlated with the development of dementia and moderate alcohol consumption appears to offer a protective effect [93-95].

Many studies have confirmed the link between high fat/high sugar (HFS) diets and declining cognitive function, strongly suggesting a role for insulin resistance and diet-induced endocrine abnormalities [96-99]. Diets that contain high

saturated fat, cholesterol, added sugar, including high fructose corn syrup (HFCS), and high glycaemic load foods contribute to dyslipidaemia [100, 101]. Animal studies have shown that a diet high in fat and refined sugar influences brain structure and function via regulation of neurotrophins [102]. Until recently it was assumed that the adverse effects of such diets on AD were a direct result of the negative influence on insulin sensitivity, metabolism and cardiovascular health. While these are major contributing factors, these studies have shown a direct effect of such diets on the brain.

Neuronal plasticity is the brain's ability to compensate for challenges by influencing synapse formation and neurite growth. A HFS diet has been shown in animals to decrease brain plasticity via regulation of brain-derived neurotrophic factor (BDNF) [102]; a major mediator of neuronal plasticity and contributor to learning and memory capabilities [103, 104]. Animals that learn a spatial memory task faster have been shown to have more BDNF mRNA and protein in the hippocampus, and after feeding a HFS for two months, the hippocampal levels of BDNF were reduced and the animals demonstrated reduced spacial learning performance [102].

In contrast to the standard western diet, comprising high fat, high refined sugar, low fibre and high salt, the Mediterranean diet has been shown to reduce the risk for AD [105, 106]. This eating style focuses on high intakes of vegetables, legumes, fruits, cereals and unsaturated fatty acids (mainly as oleic acid 18:1n-9, the monounsaturated fatty acid found in olive oil). The focus is also on high fibre, non-refined carbohydrates, making this a low glycaemic eating plan which helps prevent insulin resistance.

The intake of saturated fatty acids found in animal products is low, and fish intake is moderately high. Fish contains high levels of omega-3 fatty acids, docosahexaenoic acid (DHA 22:6n-3) and eicosapentaenoic acid (EPA 20:5n-3). Intake of dairy products is low to moderate, with those being consumed mainly in the form of cheese or yoghurt. Meat and poultry intake is also low and there is regular consumption of wine, usually with meals [105, 107].

The Mediterranean diet also contains numerous nutrients and plant phytochemicals that are anti-inflammatory, anti-oxidant and beneficial to health. For example low dietary intake of antioxidants may contribute to increased

oxidative stress, a feature of AD [93, 108]. Some of these nutrients and plant phytochemicals are now being shown to have a powerful influence on gene transcription factors that influence energy homeostasis, lipid metabolism and possibly longevity [109-113]. A meta-analysis involving twelve studies with a total of over 1.5 million people, followed for a period of 3 to 18 years showed a greater adherence to the Mediterranean eating style was associated with a reduced risk of mortality and morbidity, including AD [114]. The beneficial effects were seen in many markers of coagulation and inflammation, including homocysteine, C-reactive protein, interleukin-6, white cell count and fibrinogen. Blood lipids and blood pressure were also affected, all of which are risk factors for both cardiovascular disease and AD [115].

1.3.4 Specific nutrients and AD risk

The above section discussed general dietary considerations and risk for both metabolic disease and AD. Specific nutrient deficiencies in the elderly may exacerbate existing pathology in the brain, particularly in the presence of other risk factors [116].

The formation and maintenance of neurons relies on an adequate supply of the building blocks and cofactors required for normal functioning of biochemical and neurotransmitter pathways, all of which must be obtained from the diet [117]. In addition, mitochondrial decay due to oxidation is a feature of brain ageing and neurodegenerative disease and an adequate supply of nutrients that protect mitochondrial enzymes and mitochondrial membranes is crucial to support cellular energy generation and prevent neurological decline [118].

Vitamins, minerals and other metabolites act as critical cofactors for the synthesis of mitochondrial enzymes and other pathways and therefore diets that supply inadequate amounts of micronutrients can accelerate mitochondrial decay and neurodegeneration [119]. Nutrients supporting mitochondrial function include B vitamins, vitamin C, cysteine, ubiquinone (CoQ10), lipoic acid, sulphur, iron, copper, zinc, manganese and magnesium [119]. Deficiencies of several nutrients have been linked to dementia, including B vitamins, vitamin E, vitamin A, zinc, essential fatty acids, vitamin D and magnesium.

There have been several studies that have looked at various nutrient deficiencies in mild cognitive impairment (MCI). It is probably more 'useful' to study this group in terms of nutrient deficiencies because of the challenges faced in ensuring adequate dietary intakes associated with different stages of AD and possibly the increased nutrient requirement of a chronic disease process [120-123]. Table 1.4 summarises the effect of various nutrients on lipid metabolism and the known association with AD.

Various nutrients influence lipid metabolism and also have specific effects on lipid related AD pathology. The importance of methylation pathways in phospholipid, sphingolipid and sterol metabolism is discussed in Section 1.3.5, following Table 1.4, as dietary intakes of vitamins B6, B12 and folate influence these pathways. Additionally epigenetic modifications controlling gene expression can be adversely affected. Abnormal methylation pathways can elevate plasma homocysteine levels; a risk factor for developing AD. The interaction of dietary choline with phospholipid metabolism will also be discussed in Section 1.3.5..

Table 1.4: Summary of specific nutrients involved in lipid metabolism that also influence AD pathology.

| Nutrient | Influence on Lipid Metabolism | Influence on AD |
|---|---|---|
| Vitamin B1 (thiamine) | <p>Coenzyme for pentose phosphate pathway for production of pentose sugars and NADPH synthesis required for fatty acid synthesis [89].</p> <p>High fat diets increase thiamine requirement [89].</p> <p>Thiamine a cofactor for transketolase, pyruvate dehydrogenase, α-ketoglutarate dehydrogenase - involved in energy generation and lipid / glucose metabolism [124].</p> | <p>Thiamine dependent processes reduced in AD brain [125, 126].</p> <p>Recent metabolomic study showed up-regulation of pentose phosphate pathway in those who progressed to AD [127].</p> <p>Thiamine deficiency associated with dementia [128] and increased CSF phosphorylated tau [129].</p> <p>Nerve cell damage resulting from reduction in these enzymes [124].</p> <p>Thiamine dependent enzymes reduced in AD brain [130-132] causing increased oxidative stress. Thiamine deficiency induced oxidative stress promotes Aβ accumulation by regulating BACE maturation [133, 134].</p> |
| Vitamin B2 (riboflavin) | <p>Precursor of FMN and FAD, redox cofactors for mitochondrial ATP generation and therefore lipid metabolism [118].</p> <p>Riboflavin reduces oxidised glutathione, restoring antioxidant capacity and prevents lipid peroxidation [135]. FAD is cofactor for MTHFR which converts homocysteine to methionine in methylation pathways – involved with PL, SL metabolism [118, 136, 137].</p> | <p>Abnormal ATP production and hypometabolism [135, 138].</p> <p>Reduced glutathione and uric acid and increased homocysteine related to cognitive decline [139-141].</p> |
| Vitamin B3 (niacin) | <p>Required for formation of the ubiquitous redox coenzymes NAD and NADP, for mitochondrial ATP generation and numerous other biochemical processes involved with lipid metabolism [89].</p> | <p>Niacin deficiency and dementia are linked – important for DNA synthesis and repair, myelination, dendritic growth, cellular calcium signalling and anti-oxidation [142-145]. Trials using niacin have shown improvement in cognitive scores [145, 146].</p> |
| Vitamin B6 (pyridoxine), Vitamin B12 (cobalamin) Folate | <p>Coenzyme for several reactions in one carbon metabolism, including synthesis of PC [136]. SAH in methylation pathway is a potent inhibitor of PEMT, required for PC production [147].</p> <p>Adenosylcobalamin (Vit B12 metabolite) is a mitochondrial cofactor for the production of succinylCoA for preventing incorporation of odd chain fatty acids into PLs and SLs [148].</p> <p>Folate is associated with DHA release from the liver via PC and tissue concentrations of DHA are associated with folate status [149].</p> | <p>Nutrient insufficiency can increase plasma homocysteine, a risk factor for AD [139, 150, 151]. Abnormal methylation can increase gene expression of highly methylated APP, leading to increased Aβ production [152, 153].</p> <p>Important for preventing incorporation of odd chain fatty acids into myelin which can affect nerve conduction [148].</p> <p>In animals dietary folate deficiency causes depletion of DHA in neural tissue [154].</p> |

| | | |
|-------------------------|---|---|
| | | |
| Vitamin E (tocopherols) | <p>Prevents lipid peroxidation [155, 156]. Transported by PLTP to tissues; our studies show lower PLTP activity levels (unpublished data – see Chapter 5). Intracellular accumulation of ceramides and cholesterol can be blocked by vitamin E [157].</p> <p>Gene array studies in rats have associated vitamin E deficiency with down-regulation of genes involved with lipid metabolism and Aβ clearance, including growth hormone, thyroid hormones, melatonin and dopaminergic neurotransmission [158].</p> | <p>Lower levels associated with AD [159]. In neuronal cultures vitamin E inhibits Aβ-induced lipid peroxidation and cell death [160, 161].</p> <p>Elevated ceramides and cellular cholesterol abnormalities are a feature of AD [162-164].</p> <p>Genes affecting Aβ clearance and advanced glycation end products are down-regulated in vitamin E deficiency [165].</p> |
| Zinc | Cofactor for desaturase enzymes involved in PUFA metabolism [166-168]. | Insulin degrading enzyme is a zinc metallopeptidase, which breaks down A β [169]. |
| Vitamin D | Linked to insulin sensitivity [170] and sex steroid production [171, 172]. | Vitamin D up-regulates NPD1 production from DHA and GDNF, promoting neuronal survival [173]. |
| Vitamin A | <p>Studies in rats have shown vit A deficiency decreases liver content of PLs [174], probably due to lower PC synthesis and reduced availability of fatty acids. This may be explained by low activation by the Retinoid X receptor (RXR) of peroxisome proliferator-activated receptor α (PPARα) which regulates lipid metabolism [175]. Affects delivery of PLs to other tissues.</p> <p>Essential cofactor for protein kinase C-delta (PKC-delta), a nutritional sensor regulating energy homeostasis [176].</p> | <p>Vitamin A involved in production of α-secretase and acetylcholine reduces excessive microglial activation [177, 178]. Retinoic acid is a powerful modulator of excessive inflammation via suppression of IL-6 and TNFα, both of which are induced by Aβ [178].</p> <p>Vitamin A insufficiency may contribute to the hypometabolism seen in AD [176, 179].</p> |

Abbreviations: ATP: Adenosine Triphosphate, APP: Amyloid Precursor Protein, BACE: Beta site APP Cleaving Enzyme, CSF: Cerebrospinal Fluid, DHA: Docosahexaenoic Acid, DNA: Deoxyribonucleic Acid, FAD: Flavin Adenine Dinucleotide, FMN: Flavin Mononucleotide, GDNF: Glial Derived Neurotrophic Factor, IL-6: Interleukin 6, MTHFR: Methylene tetrahydrofolate Reductase, NAD: Nicotinamide Adenine Dinucleotide, NADP: Nicotinamide Adenine Dinucleotide Phosphate, NPD1: Neuroprotectin D1, PC: Phosphatidylcholine, PEMT: Phosphatidylethanolamine Methyl Transferase, PL: Phospholipid, PLTP: Phospholipid Transfer Protein, PUFA: Polyunsaturated Fatty Acid, NADPH: Reduced Nicotinamide Adenine Dinucleotide Phosphate, SAH: S-adenosyl Homocysteine, SL: Sphingolipid, TNF- α : Tumour Necrosis Factor alpha.

1.3.5 Homocysteine, choline and methylation pathways

Homocysteine is a sulphur containing amino acid that exists at a critical biochemical intersection in the ubiquitous methionine cycle, whose function is to generate one carbon methyl groups for transmethylation reactions and synthesise cysteine and taurine [155] (see Figure 1.4). Methionine is converted to S-adenosyl-methionine (SAME), which is the most important methyl donor in the body and is critical for stabilising many macromolecules such as myelin and DNA. Methylation of DNA is an epigenetic modification that controls gene expression, including the highly methylated APP gene [152]. Methylation via SAME is involved with synthesis of numerous compounds including phosphatidylcholine, melatonin, serotonin, nor-adrenalin, Coenzyme Q10 and carnitine [136]. SAME is a crucial component of both phase I and phase II detoxification pathways in the liver, providing the methyl group directly. In addition a balanced methionine cycle is required to supply taurine for bile acid synthesis and cysteine for both sulphur conjugation and for the formation of glutathione, which is both an antioxidant and a vital phase I and II component [136]. All these processes are crucial for both normal neurological and normal metabolic function.

The link between elevated homocysteine and AD risk has been firmly established in several studies [139, 150, 151] and homocysteine levels are also correlated to vitamin B6, B12 and folate status [180-182], but the exact mechanisms behind the elevated homocysteine and connected metabolites remain to be fully elucidated. It is probable that elevated homocysteine promotes AD by more than one mechanism and that while homocysteine serves as a surrogate marker for nutrient status; which when deficient can promote neurological damage in its own right, evidence suggests that homocysteine has direct actions on the brain. Cerebral microangiopathy has been demonstrated in stroke associated hyperhomocysteinaemia [183] and endothelial dysfunction, oxidative damage and neuronal DNA damage are all reported consequences of elevated homocysteine [184-186]. There also appears to be an enhancement of A β mediated neurotoxicity and apoptosis where homocysteine levels are elevated [187, 188] and homocysteic acid, which is a metabolite of

homocysteine, is possibly an NMDA agonist itself, causing excitotoxicity and apoptosis [189, 190].

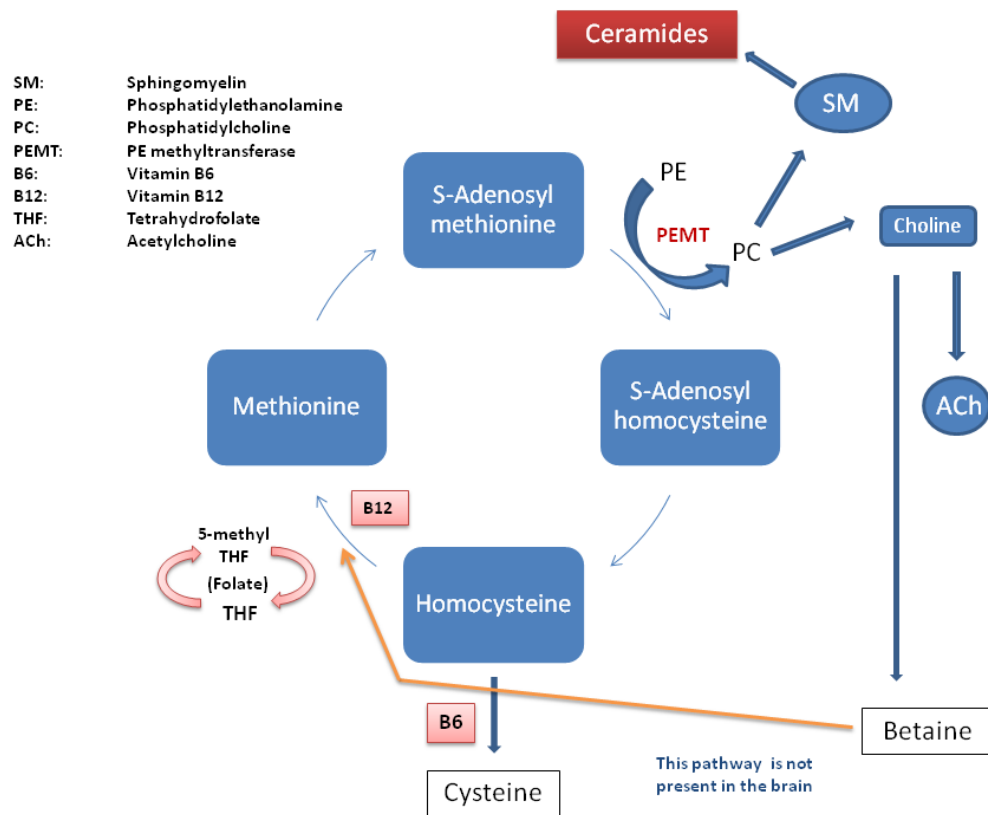


Figure 1.4: Methylation pathway depicting interaction with phospholipids and sphingolipids. After donating the methyl group SAME is converted into homocysteine via S-adenosylhomocysteine (SAH). The homocysteine is then broken down by one of three pathways. Firstly it can be converted back to methionine by accepting a methyl group from methylcobalamin (vitamin B12), secondly, it can be converted to methionine by accepting a methyl group from trimethylglycine (betaine) or thirdly it can be converted to cysteine and taurine via serine and activated vitamin B6 [155]. The catabolism of homocysteine depends on an adequate supply of vitamin B6, folate and vitamin B12. The majority of the essential nutrient choline is present in phosphatidylcholine (PC) and sphingomyelin (SM), major components of all cell membranes. Additionally, PC and SM are precursors for the signalling molecules ceramide, platelet activating factor and sphingophosphorylcholine [137]. Choline is required for synthesis of the neurotransmitter, acetylcholine and as it is oxidised to betaine (trimethylglycine), plays a crucial role as a methyl donor in the methionine/homocysteine pathway.

The conversion of S-adenosylhomocysteine (SAH) to homocysteine is a reversible reaction and so elevated homocysteine can also lead to an increase in SAH which is a potent inhibitor of methyltransferase enzymes (including

phosphatidylethanolamine methyltransferase (PEMT) required for phosphatidylcholine (PC) production), further reducing the capacity of SAMe to participate in methylation reactions: This adversely affects numerous biochemical processes [147]. The APP gene is highly methylated and decreased methylation may increase expression leading to increased A β production [152, 153]. A link between elevated SAH and polyunsaturated fatty acid metabolism has also been established in AD [191]. The connection between homocysteine and lipid metabolism has been further highlighted as homocysteine-induced endoplasmic reticulum stress interferes with phospholipid metabolism. This has the effect of activating sterol regulatory element binding proteins (SREBPs) associated with increased expression of genes involved with cholesterol and triglyceride uptake and intracellular accumulation of cholesterol (see Section 1.5) [192]. A regulatory feedback circuit has been identified where SREBP-1 controls the production of SAMe and therefore PC production, which is dependent on methylation reactions, and giving merit to the notion that nutritional or genetic factors limiting the production of SAMe or PC, may activate SREBP-1 and contribute to metabolic dysfunction [193]. The inhibition of methyltransferase enzymes by SAH is very important in the brain as the alternate pathway for homocysteine catabolism via trimethylglycine (betaine) has no activity [87] (see Figure 1.4). Central nervous system cells can't export SAH and therefore conversion to homocysteine and extracellular transport is the only way to remove SAH, causing a subsequent rise in plasma homocysteine. This raises the possibility that SAH could be the toxic metabolite, at least in some tissues [147] and that the question of elevated homocysteine and increased disease risk has been difficult to answer with certainty.

Dietary fat and cholesterol are transported to the liver via chylomicrons and then packaged into VLDL in the liver for delivery to other tissues. PC is an essential component of these lipoproteins and deficiency of choline and PC results in fat accumulation in the liver. Requirement for choline depends on the status of other methyl group donors such as folate and SAMe. When dietary choline is inadequate, the liver has a back-up pathway to provide choline from PE via a three step methylation pathway by SAMe; a reaction catalysed by

Phosphatidylethanolamine-N-methyl transferase (PEMT) [194]. Deficiencies of nutrients involved in this pathway, could reduce the availability of choline. Interestingly, oestrogen induces endogenous synthesis of choline by upregulating PEMT, which may put postmenopausal women at risk of choline deficiency, when dietary intakes are inadequate [195]. Additionally disturbed choline transport is suggested to play a role in various neurological disorders, including AD [196]. Loss of cholinergic neurons is a feature of AD and various choline transporters have been investigated as potential targets for increased choline delivery to the neurons for acetylcholine synthesis [196-198]. Studies have shown that one of the choline transport systems in erythrocytes is abnormal in patients with AD [199].

1.3.6 Dietary fatty acids

Apart from dietary fatty acids being an energy generating nutrient, the amount and type of fat is important for determining disease risk due to the diverse function of lipids in general; the structure and function of which is influenced by the fatty acids they contain. It has already been stated that the Mediterranean style diet, which is low in saturated fat and cholesterol, high in monounsaturated and polyunsaturated fats and low glycaemic load is beneficial. High saturated fat intake is strongly associated with the development of dyslipidaemia, insulin resistance, obesity, diabetes, vascular disease and metabolic abnormalities; all risk factors for AD [200-202]. Saturated and trans fatty acid intakes are positively correlated with increased cholesterol levels and unfavourable shifts in low density lipoprotein: high density lipoprotein (LDL:HDL) ratios [203-205]. Excess palmitic acid can also upregulate ceramide production, which is considered a major contributor to dyslipidaemia and insulin resistance [206, 207] (see Figure 1.15).

The most commonly consumed fatty acids are myristic (14:0 – 14 carbon chain length with no unsaturated/double bonds), palmitic (16:0), stearic (18:0), oleic (18:1) and linoleic (18:2). Each of these fatty acids affects plasma cholesterol levels differently due to their impact on the LDL receptor, the activity of which is regulated by the sterol content of the cell via SREBP [89]. When the sterol content of the cell is low, a fragment of SREBP is cleaved by a protease which

acts as a transcription factor to activate the LDL receptor gene. Dietary fatty acids and cholesterol can increase the sterol regulatory pool by their effects on the enzyme acylCoA-cholesterol acyl transferase (ACAT) [202, 208]. The activity of this enzyme is determined in part by the amount and type of dietary fatty acid, which therefore affects plasma cholesterol levels [89]. The saturated fatty acid, palmitic acid partially inhibits cholesteryl ester (CE) formation by ACAT, therefore increasing the amount of free cholesterol. As the sterol regulatory pool increases, SREBP cleavage is inhibited leading to the reduced expression and activity of the LDL receptor. In contrast, if the liver cells are enriched with monounsaturated fats (MUFA) and polyunsaturated fats (PUFA), ACAT activity is increased as it is a preferred substrate and CE formation is promoted [209]. Short and medium chain saturated fatty acids (4:0, 6:0, 8:0 and 10:0), are rapidly oxidised to acetyl CoA in the liver and do not alter the concentration of free cholesterol or cholesteryl esters and therefore have no effect on the LDL receptor. Stearic acid (18:0) has been shown to be neutral in respect to its effect on plasma cholesterol levels and saturated fatty acids in general inhibit CE formation and reduce LDL receptor number and activity, therefore increasing plasma cholesterol levels [202].

Dietary *trans* fatty acids are known to contribute to dyslipidaemia and associated increased disease risk [210] and have been linked to brain ageing and impaired cognition [211, 212]. Most naturally occurring fatty acids have double bonds in a *cis* configuration which allows the chain to bend. The *trans* bond imparts a rigid structure which is similar to saturated fatty acids and increased consumption from processed and fast foods can lead to dyslipidaemia [210] [213]. *Trans* fatty acids alter membrane fluidity and responses of various membrane receptors through their incorporation into membrane phospholipids. As fatty acids are ligands for nuclear receptors, such as peroxisome proliferator activated receptors (PPARs), liver X receptor (LXR) and SREBP, regulation of gene transcription can be altered [214-216], directly modulating metabolic and inflammatory responses in an adverse way. The effects on lipid metabolism are due to several mechanisms. Firstly, *trans* fatty acids alter the secretion, composition and size of apolipoprotein B-100 produced in the liver. This decreases the rate of LDL catabolism. The

catabolism of ApoA1 is increased, which reduces plasma HDL cholesterol. *Trans* fatty acids increase the cellular accumulation and secretion of free cholesterol and cholesterol esters by the liver [217]. The effects on reducing HDL are thought to be due to an increase in cholesteryl ester transfer protein (CETP) which transfers cholesterol esters from HDL to LDL and VLDL [218]. Additionally the fluidity, determined by the fatty acid at the sn-2 of PC is a major regulator of lecithin:cholesterol acyltransferase (LCAT), which is required for the formation of mature HDL. *Trans* fatty acids reduce fluidity and can therefore reduce activity of LCAT [219].

Lipids form the membrane bilayer and are important signalling molecules via the generation of second messengers [63, 89]. The diet provides fatty acids, or the precursors for their synthesis, and therefore influences structure and function of all lipids. Of particular importance are the long chain polyunsaturated fatty acids (PUFAs) and the ratio of the omega-3 and omega-6 series, which have opposing effects in terms of the signalling molecules generated. They are competitive substrates for the enzymes involved in PUFA metabolism [89], which makes the relative amounts important in terms of the signals generated. The PUFAs are converted via the action of cyclooxygenase and lipoxygenase enzymes to prostaglandins, leukotrienes, thromboxanes and other metabolites which are important mediators of cellular function and therefore the signalling molecules generated are partly influenced by dietary intakes [220]. The omega-6 fatty acids are more inflammatory, atherogenic and pro-thrombotic than the omega-3 series [89]. There are two essential fatty acids that have to be obtained from the diet as humans lack the delta-12 and delta-15 desaturase enzymes to produce them from other fatty acids [221, 222]. Linoleic acid (18:2n-6) is abundant in plants, seeds and nuts and is the precursor for the omega-6 series of eicosanoids. Alpha-linolenic acid (ALA) (18:3n-3) is the precursor for the important eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which is the most abundant PUFA in the central nervous system (CNS), and is particularly associated with phosphatidylethanolamine species [223].

Deficiency of the essential fatty acids is rare, but conversion of ALA by the action of elongase and desaturase enzymes to the longer chain PUFAs is limited and also affected by hormone imbalances, diet and nutrient deficiencies

[224-226]. High intakes of omega-6 fatty acids and arachidonic acid (AA) from meat can elevate pro-inflammatory eicosanoids and up-regulate pro-inflammatory cytokines. Levels of the non-enzymatically derived isoprostanes, which are vasoconstrictive, are also elevated in AA enriched diets [227]. Conversely, diets enriched in DHA from fish and fish oil are more anti-inflammatory, anti-thrombotic and vasodilatory and have neuroprotective effects in terms of synaptic function and plasticity via generation of docosanoids [220, 228]. The ratio of dietary AA to DHA would appear to influence several diseases, including AD, and may be important in designing nutrition-based strategies for disease prevention [229]. The brain relies on a supply of AA and DHA from the periphery, which are delivered via plasma lipoproteins and lysophospholipids. Unesterified AA and DHA can also enter the brain where they are esterified into the sn-2 position of phospholipids following activation by long chain fatty acyl CoA synthase (LC-FACS) [89].

DHA and EPA are important in reducing plasma triglyceride levels as they regulate the activity of various nuclear receptors resulting in a repartitioning of fatty acids away from storage as triglycerides and towards oxidation. These receptors include LXR, hepatocyte nuclear factor 4 α , farnesoid X-activated receptor (FXR), and PPARs. Each of these receptors is in turn regulated by SREBP-1c [230]. EPA and DHA reduce SREBP-1c which is the main genetic switch controlling lipogenesis. This reduces the amount of free fatty acids available for VLDL synthesis. EPA and DHA are highly unsaturated and are prone to peroxidation which stimulates the degradation of apolipoprotein B (ApoB), required for VLDL synthesis and their presence in lipoproteins may also enhance postprandial chylomicron clearance by stimulating lipoprotein lipase activity [230].

The carbohydrate responsive element binding protein (ChREBP) is a transcription factor that responds to glucose levels and is involved in hepatic lipid synthesis by the transcription of genes involved in lipogenesis, such as fatty acid synthase and acetylCoA carboxylase [231]. ChREBP activity can be inhibited or normalised by omega-3 fatty acids [230, 231].

1.3.7 Dietary Carbohydrates

The amount and type of dietary carbohydrate has a significant impact on lipid profiles and the risk of developing AD risk factors such as cardiovascular disease and type 2 diabetes. Long term consumption of high glycaemic load, refined carbohydrates and simple sugars can lead to insulin resistance and metabolic syndrome [101, 232]. Insulin resistance and metabolic syndrome are also known risk factors for dyslipidaemia and AD. Insulin resistance results in an atherogenic lipid profile which also includes a decrease in LDL particle size and a postprandial accumulation of triglyceride rich remnant lipoproteins. Adipose tissue becomes resistant to the anti-lipolytic effects of insulin which increases the concentration of circulating free fatty acids. The combination of high insulin and free fatty acids acts on the liver to increase the synthesis and secretion of VLDL causing hypertriglyceridaemia. Once the plasma triglyceride pool is increased the transfer rate of cholesterol ester by CETP from HDL to VLDL increases. This then reduces the HDL levels. The fractional catabolic rate of apolipoprotein A-1 is also increased with high triglycerides [233-235]. The composition of the LDL particles is also abnormal in insulin resistance as cholesterol ester depletion results in a small dense LDL [236]. This is due to the action of CETP which mediates the exchange of VLDL triglyceride for cholesterol ester in LDL. This triglyceride rich, cholesterol ester depleted LDL interacts with lipoprotein or hepatic lipase which hydrolyses the triglyceride to produce small, dense LDL [236]. Excess consumption of simple sugars, including fructose, can also have adverse effects on lipid metabolism. The liver rapidly absorbs and metabolises fructose and exposure to large amounts of fructose leads to lipogenesis and triglyceride accumulation. The accumulation of triglycerides contributes to reduced insulin sensitivity, hepatic insulin resistance and impaired glucose intolerance [237] .

From Section 1.3.3 it is clear that diet and specific nutrients contribute to AD risk indirectly by increasing AD related risk factors such as cardiovascular disease and type 2 diabetes, but also contributing directly to many lipid-related processes involved in AD pathology.

1.3.8 Lifestyle

Apart from dietary considerations, data from observational studies indicate that several lifestyle factors can reduce the risk of developing AD (reviewed in [238, 239], including physical activity, mental stimulation, being a non-smoker, and social interaction. Intensity of physical activity has been investigated and positively associated with improved cognition [240] whilst higher levels of total physical activity have been shown to be linked to biological and imaging markers of AD [241]. Being physically active has known beneficial effects on body composition, insulin sensitivity and lipid profiles. Although a topic of intense scientific debate, the beneficial effects of physical activity on metabolic disease and ageing are thought to be mediated via sirtuins (SIRTs) [242]. SIRTs are NAD-dependent histone deacetylases which are activated by cellular stresses to enhance cellular defense and repair pathways and to mediate adaptive responses to changing energy requirements and physical activity. This is achieved by deacetylation and transcriptional control of numerous genes involved in lipid and glucose metabolism [243]. Additionally Sirt1, has been shown to attenuate amyloidogenic processing of APP both in cell culture models and in transgenic mice [244, 245], suggesting a possible mechanistic link between physical activity and improved markers of AD pathology.

1.3.9 Hormones

As balance and levels of hormones inevitably changes with ageing, many studies have examined the role of hormones in the pathogenesis of AD. Due to the highly integrated nature of the endocrine system, the metabolic, reproductive, adrenal, thyroid, gut-derived, adipocyte-derived and appetite regulating hormones all have a role to play in terms of their direct effects on AD pathology, but also their indirect effects due to altered metabolism. Specifically, all these hormones have a marked effect on lipid homeostasis.

1.3.10 Sex Steroids and Gonadotropins

The first clue as to hormonal involvement in AD is the reported gender differences in AD risk, with a higher prevalence reported in women, although in general women have a greater life expectancy [246, 247]. This observation also

appears to be study group specific with gender differences being reported in Swedish, Chinese, French and European populations but not within several large American studies, including the Framingham and Rochester studies [248]. This highlights the complex nature and long duration of the neurodegeneration seen in AD, with associated co-morbidities and hormonal influences complicating the picture. It has also been reported that AD pathology and cognitive deficits are more severe in women, which has prompted the investigation of sex steroids and risk of AD [249, 250]. The results of studies into the use of hormone replacement therapy in women to reduce the risk of AD have been conflicting. The Women's Health Initiative Memory Study (WHIMS) showed an almost two-fold increased risk of AD and deficits in cognitive functioning in older women (over 65 years), but with no effect in younger women [251, 252]. The Women's Health Initiative Memory Study of Younger Women (WHIMSY), included 1326 participants, and concluded that hormone therapy given to postmenopausal women at ages 50-55 years resulted in no differences in global cognitive function scores or individual cognitive domains [252]. Some studies of women who had been on hormone replacement (HRT) in their 50s found that the HRT did reduce the risk of dementia (whereas there was no effect if the women in the studies were in their 70s), suggesting that oestrogen needs to be taken early, perhaps to reduce early pathogenic mechanisms. Several reasons for these conflicting results include the type of hormone used, form of delivery and the combination with progesterone. Many past studies have used conjugated equine oestrogens and/or synthetic progestins. Studies are required that use bio-identical forms of these hormones. A recent systematic review of data recommended that hormone therapy should not be used for dementia prevention [253]. Animal studies into the non-reproductive roles of oestrogen have highlighted neuroprotective and neurotrophic/synaptic plasticity roles and effects on attenuating A β accumulation [249]. In premenopausal women, oestrogen may protect against dietary choline deficiency because oestrogen induces the endogenous synthesis of choline from PE via the enzyme PE methyl transferase (PEMT) [195].

Testosterone has also been shown to have similar neuroprotective properties, which are likely mediated by the direct action of dihydrotestosterone (DHT) or via aromatisation to oestrogen [250]. Further, an age related decline in testosterone production occurs normally after about the third decade which correlates with cognitive decline. Sex steroid production is regulated by the complex feedback loops of the hypothalamic-pituitary-gonadal axis (HPG), involving the pituitary gonadotrophins, luteinising hormone (LH) and follicle stimulating hormone (FSH). Increasing gonadotrophin levels, as occurs with age, are now being shown to contribute to AD, with APP processing and A β production, and neuronal cell cycle abnormalities reported [254-256]. A recent study investigating the association between gonadotrophins, testosterone, plasma A β levels and brain amyloid burden (as determined by PiB retention) in the AIBL cohort, demonstrated involvement of testosterone and LH at different stages of disease in men and recommended consideration of their effects when investigating other potential biomarkers for AD [257].

1.3.11 Metabolic Hormones

In recent years numerous reports have highlighted the strong relationship between dementia and metabolic disorders that include dyslipidaemia, obesity, diabetes, cardiovascular disease and hypertension (reviewed in [179, 258-262]). While the mechanisms that underpin this association are beyond the scope of this chapter, it is important to emphasise that the above conditions rarely occur in isolation, and the complex network of metabolic dysfunction which occurs in these conditions has been shown to influence many aspects of AD pathogenesis and neurodegeneration. For example, insulin is required for blood glucose regulation in the periphery and the brain. When peripheral insulin resistance (IR) develops metabolic dysfunction results and brain insulin signalling is altered affecting glucose utilisation, A β and tau pathology, vasculature, mitochondrial function, inflammation, oxidation, neuronal maintenance and plasticity [98, 179, 263, 264]. A neurodegenerative cycle consisting of AD pathogenesis, insulin resistance and inflammation has often been described. The components of this cycle have elevated ceramide levels as a common factor, suggesting these toxic lipids may represent the link between all these pieces of the AD puzzle [207, 265]. The literature is teeming

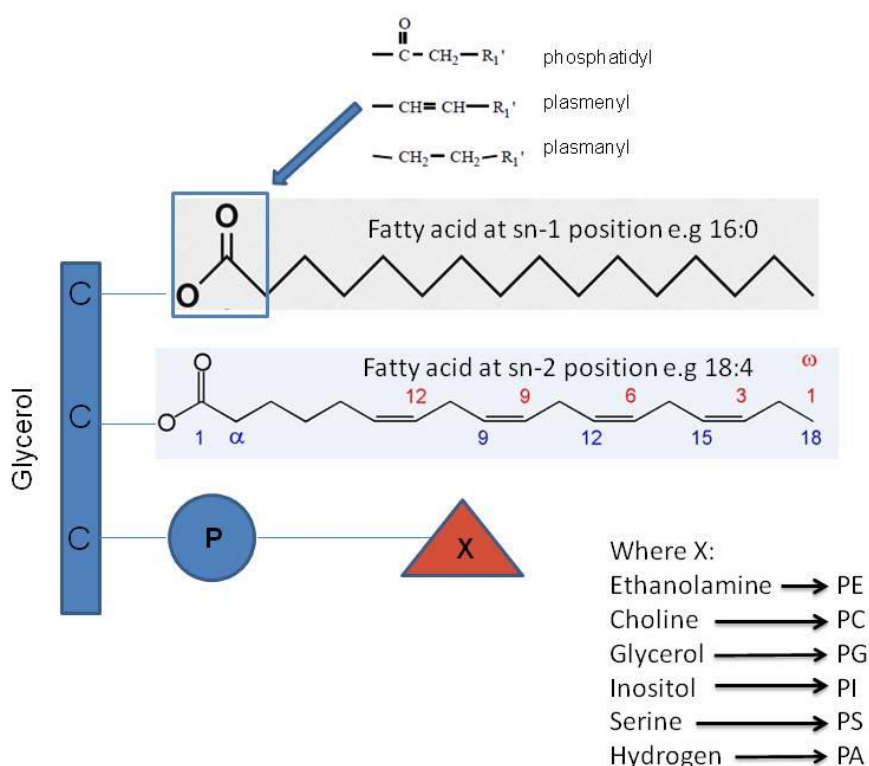
with reports and studies into the effects of hormones on AD, with virtually all known metabolic hormones being involved, including insulin, thyroid, cortisol (and other non-sex steroids), growth hormone, leptin, ghrelin, adiponectin and vitamin D to name a few. For example, thyroid hormones have a profound effect on lipid profiles [266] and direct effects on cognition [267] and patients with AD and other neurodegenerative diseases have chronically elevated cortisol levels, suggesting dysregulation of the hypothalamic-pituitary-adrenal axis [268]. Furthermore, leptin is an adipose tissue-derived hormone, involved in energy storage and availability, and the hormone orexin (also called hypocretin) is a neurotransmitter that regulates arousal, wakefulness, and appetite; and orexin dysregulation, insulin resistance and leptin resistance, which can all occur with obesity, have all been linked to neurodegeneration [269].

1.4 Lipids and Lipidomics

Lipidomic approaches have greatly improved understanding of the mechanisms of disease and have lead to the development of biomarkers and therapeutic strategies for a wide range of conditions including cardiovascular disease, metabolic disease and cancer [270-272]. Altered lipid metabolism has clearly been established as a contributor to neurodegenerative disease. The nervous system has the second highest concentration of lipids, exceeded only by adipose tissue. Lipids in the nervous system also show enormous structural diversity and by studying lipid profiles, insight into the biochemical pathways that alter levels of individual species is obtained, leading to a greater understanding of pathology. The lipid profiling part of this project for the identification of a lipid blood biomarker focuses on analysing phospholipid and sphingolipid species.

Cell membranes are dynamic and require continuous remodelling of phospholipids for function. A major factor determining the shape of phospholipids is the nature of the side group and the fatty acids linked to sn-1 and sn-2 positions of the glycerol backbone (see Figures 1.5 and 1.6). The length of the carbon chain and number of double bonds of the fatty acid determines the length and shape. The molecular shape of the phospholipid in the membrane bilayer is also influenced by the compatibility between the size of

its polar head group and the hydrophobic tail [273]. A huge variety of lipid species arise from the various combinations of fatty acids with backbone structures such as glycerol and sphingoid bases [274] (see Figures 1.5 and 1.6). The most common class of lipids consist of fatty acids linked by either an ester bond to glycerol (a trihydric alcohol) or other alcohols such as cholesterol or by amide bonds to sphingoid bases. In addition they may contain alkyl moieties other than fatty acids, phosphoric acid, organic bases, carbohydrates and various other components, which can be released by various hydrolytic processes [275].



PE:Phosphatidylethanolamine, PC: Phosphatidylcholine, PG: Phosphatidylglycerol, PI: Phosphatidylinositol, PS: Phosphatidylserine, PA: Phosphatidic Acid.

Fig 1.5: General structure of phospholipids. The fatty acids at positions sn-1 and sn-2 can vary in chain length and number of double bonds. In this example, the fatty acid at the sn-1 position contains 16 carbon atoms and has no double/unsaturated bonds (palmitic acid). The example at the sn-2 position contains 18 carbon atoms and has 4 unsaturated/double bonds (stearidonic acid). The linkage of the sn-1 acyl group determines the subclass. i.e. ester linkage forms phosphatidyl species, where a vinyl ether linkage forms the plasmeryl or plasmalogen species. The linkage of the side group to the phosphate (P) group at the sn-3 position determines the phospholipid species. If X=ethanolamine, phosphatidylethanolamine (PE) or ethanolamine plasmalogen will be formed.

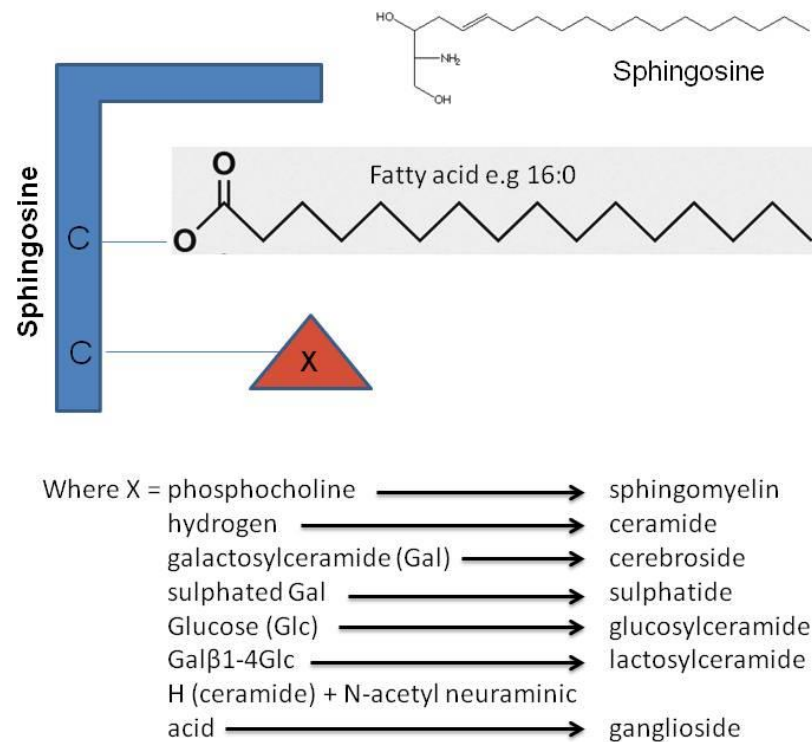


Fig 1.6: General structure of sphingolipids. The fatty acid can vary in chain length and number of double bonds. In this example the fatty acid has 16 carbon atoms (C) and no unsaturated/double bonds (palmitic acid). The group attached at X determines the sphingolipid species. For example if X is hydrogen, ceramide is formed.

It is evident that many neurological disorders involve complex interactions between proteins and lipids, and in AD, lead to the increased formation of protein deposits [276]. A key development in the study of lipids and neurodegenerative disease has been in the advancement of technologies to determine profiles of lipids and their interacting partners. This is of major importance, as it is known that cellular signalling and membrane trafficking are often regulated by peripheral proteins, which reversibly interact with membrane lipid molecules in highly regulated spatial and temporal fashions. Lipidomic research has particular relevance to biomarker development. In the case where there is great overlap with AD and other conditions involving altered lipid metabolism, such as cardiovascular disease and diabetes, there are new technologies that may help distinguish between them, including computational biology, cellular lipid mapping, and single molecule imaging [270, 277]. This will be of value as specific lipid classes and sub-classes are believed to play an

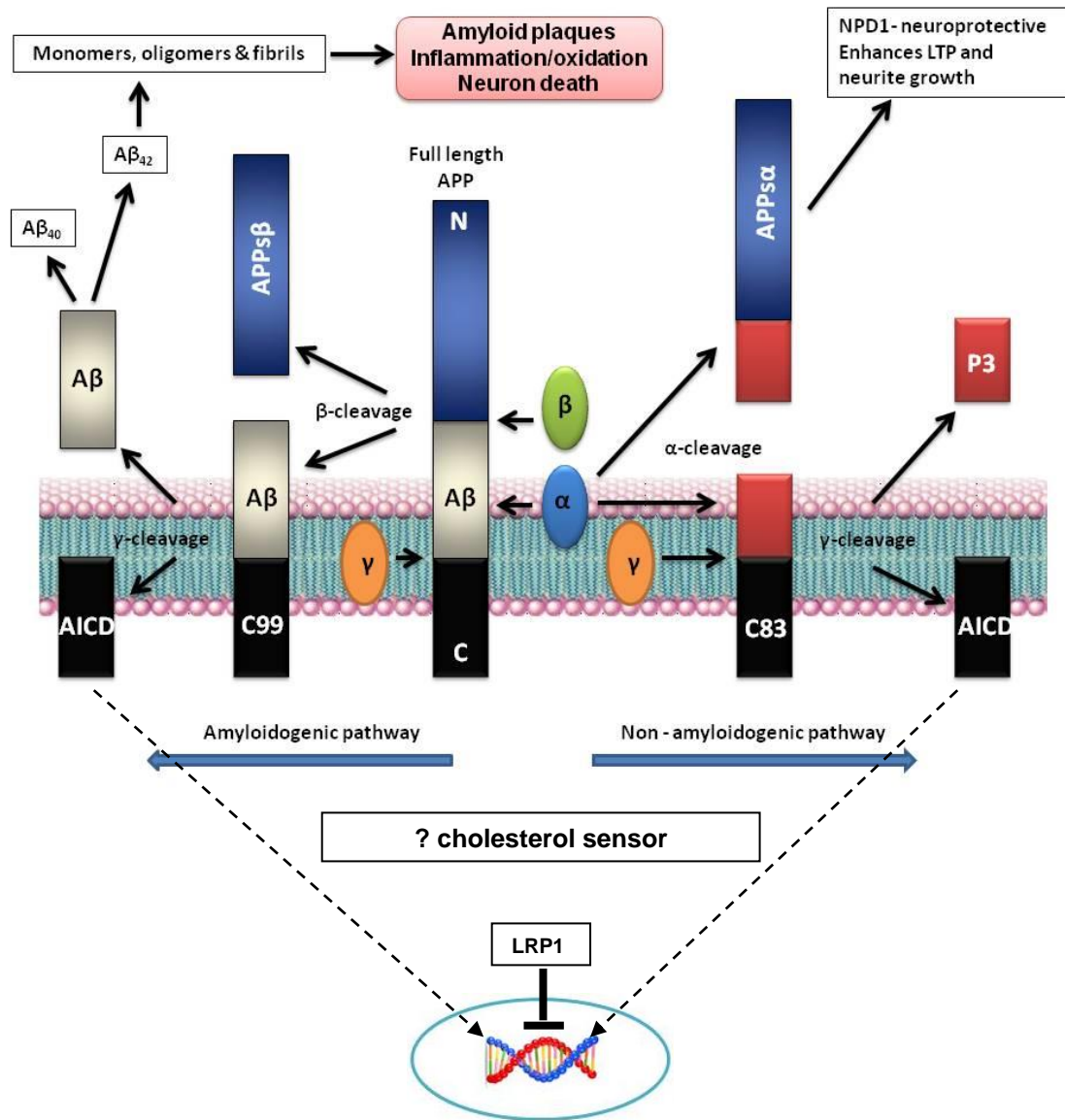
important role in AD, and many changes in brain lipid composition have been reported [273].

1.5 Lipidomics and AD

The specific relevance of each lipid class and their contribution to known pathological processes in AD will be described in the following sections. It is apparent that disturbances in lipid metabolism in general have an effect on AD pathology and the discovery of blood lipid biomarkers may help predict some of these pathological changes at an early stage of disease or even pre-clinically.

1.5.1 Lipids, APP processing and A β

Amyloid precursor protein (APP) is a transmembrane protein expressed by many tissues and is concentrated in the synapses of neurons [278]. While its primary function remains to be elucidated it is thought to be involved with synapse formation, neural plasticity and iron export [279, 280]. APP expression is up-regulated during neuronal differentiation, following neural injury and this protein is involved in cell surface protein signalling [278]. APP has also been shown to have ferroxidase activity which catalyses the oxidation of iron during iron transport. Accumulated A β may trap zinc, which inhibits this enzyme and subsequent transport of iron. Imbalances in redox metals are a contributor to increased oxidative stress and AD pathology [280]. As previously mentioned in Section 1.1.2, APP can be processed by two different pathways, depending on the localisation of APP to lipid rafts and feedback loops controlling gene transcription for the appropriate enzymes (see Figure 1.7); processes which are influenced by membrane composition.



α : α -secretase, β : Beta site APP cleaving enzyme, γ : γ -secretase, N and C: N and C terminals of APP, AICD: APP intracellular domain, C99: carboxyterminal fragment (CTF) 99, C83: CTF 83, APPs β and APPs α : secreted APP β and α respectively, NPD1: neuroprotectin D1, LTP: long term potentiation, LRP1: LDL-receptor related protein 1.

Figure 1.7: APP processing - The non-amyloidogenic and amyloidogenic pathways and relationship to cholesterol. The non-amyloidogenic processing of APP involves cleavage by α and γ secretases resulting in generation of a secreted form of APP (APPs α), CTF83, AICD and P3. APPs α has neuroprotective properties via its effect on NPD1 production, enhancing LTP and neurite outgrowth. The amyloidogenic processing of APP involves cleavage by β and γ resulting in generation of secreted APP β (APPs β), CTF99 and A β . Under certain conditions the A β_{42} can be overproduced or inefficiently cleared. A toxic cascade of neuro-inflammation and oxidation ensues resulting in neuronal death. The AICD can translocate to the nucleus to suppress or transcribe several genes. By suppression of LRP1 it may act as a cellular cholesterol sensor. The biological relevance of the CTFs and P3 remain to be determined.

The APP protein undergoes proteolytic cleavage (see Figure 1.7) in the non-amyloidogenic pathway by α -secretase or in the amyloidogenic pathway by beta site APP cleaving enzyme (BACE), formerly known as β -secretase [281]. In both cases, initial cleavage is followed by an additional cleavage by γ -secretase [282]. Several enzymes including ADAM 10, ADAM 17 (a disintegrin and metalloproteinase 10 or 17) and TACE (tumour necrosis factor α -converting enzyme) show α -secretase activity [9]. α -secretase cleaves within the A β domain of APP liberating a soluble APP fragment (APP_{sa}), which precludes the formation of A β and has neurotrophic and neuroprotective properties [282]. The soluble APP fragment (APP_{sa}) is thought to be neuro-protective by promoting neuronal cell survival and repair following cellular injury. An up-regulation of the enzymes phospholipase A₂ (PLA₂) and/or 15-lipoxygenase, required for the biosynthesis of DHA-derived neuroprotectin D1 (NPD1), may elicit this neurotrophic activity and represent a complimentary cell survival mechanism activated early in AD pathogenesis [283]. APP is processed by BACE and γ -secretase in the amyloidogenic pathway to release the A β peptide. Under certain conditions these peptides can become potent, self aggregating neurotoxins when they accumulate as a result of increased production or poor clearance [13]. The fate of APP in terms of processing is determined by its location within the cell membrane and cellular compartments and the up-regulation of enzymes in response to various signals, which in turn appear to be influenced by factors including genetic make-up, diet, nutritional status, lifestyle and hormonal status.

APP clusters in cholesterol-rich lipid rafts in the membranes of neurons, astrocytes and microglia [284]. Lipid rafts are domains within the plasma membrane bilayer consisting of cholesterol, glycosphingolipids and protein receptors. These specialised regions compartmentalise cellular processes and act as organising centres for assembly of signalling molecules. Rafts also influence membrane fluidity and are involved with membrane protein and receptor trafficking and the regulation of neurotransmitters [285]. When the cholesterol content of the rafts increases, the activity of α -secretase decreases and the activity of BACE increases, thereby promoting amyloidogenic processing of APP [284]. The immediate precursor to A β is the C-terminal

domain of APP called C99 (see Figure 1.7 – APP processing), the product of BACE cleavage of APP and has an affinity for cholesterol. The localisation of APP/C99 is therefore directed to the cholesterol-rich rafts, where the BACE and γ -secretase are concentrated, thereby increasing amyloidogenic processing of APP [284]. The α -secretase and BACE are concentrated in different areas of the cell [286]. Optimal α -secretase activity is found in cell surface regions that contain few lipid rafts, in particular fluid regions with a high DHA content [286]. When APP accumulates at the cell surface, it is more likely to be cleaved by α -secretase. When APP is shifted inside the cell to the Golgi apparatus, endosome or lysosome, BACE activity predominates and $A\beta$ is more likely to be produced. Evidence is emerging which suggests cholesterol and low density lipoprotein receptors (LDLR) influence where APP accumulates [287]. Dysregulation of APP processing occurs early in the disease process, possibly as a result of cardiovascular risk factors such as insulin resistance, lipid dysregulation, inflammation, hormonal imbalances and oxidative stress, resulting in overproduction of $A\beta_{42}$, $A\beta$ aggregation (causing toxicity and further oxidative stress) and eventually fibril and plaque deposition, as well as NFT formation [9]. This is the basis of the “amyloid hypothesis”, which states that $A\beta$ accumulation is central in the aetiology of AD [288]. Cholesterol and plasma membrane lipids have roles in regulating APP metabolism [45] and although $A\beta$ is proposed as a main driver for AD, the BACE and γ -secretases that generate it are up-regulated by high membrane cholesterol levels. This suggests that a possible physiological role for $A\beta$ is to recycle brain cholesterol and promote its efflux into the systemic circulation and thus protect against elevated brain cholesterol [289].

$A\beta$ increases cholesterol efflux from cells by assembling high-density lipoprotein-like particles during secretion [290]; this assembly being mediated by the ATP-binding cassette transporter 1 (ABCA1) [289]. Animal studies have reported that $A\beta$ containing lipoproteins are excreted much more efficiently than those without $A\beta$ [69, 289]. Evidence supports the notion that the products of the amyloidogenic processing of APP ($A\beta$ and AICD) act to lower cellular cholesterol levels [284]. $A\beta$ stimulates the release of cholesterol and other lipids from cells via lipoproteins [289]. $A\beta$ may also reduce biosynthesis of cholesterol

and other lipids by inhibition of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase); the rate-limiting enzyme in the cholesterol biosynthesis pathway [291]. Additionally, AICD can translocate to the nucleus where it suppresses transcription of the gene encoding LDL-receptor related protein (LRP1); a major apoE receptor in the brain mediating cellular cholesterol uptake via endocytosis [292]. In this way APP may act as a cellular cholesterol sensor by creating a negative-feedback loop (see Figure 1.7) as it has been shown that the C-terminal (mostly intracellular) domain of APP specifically binds membrane cholesterol, thus favouring lipid raft localisation and promoting amyloidogenic processing, the products of which (in particular AICD, as mentioned) reduce apoE-mediated cholesterol uptake and synthesis [284]. In normal neurons the optimal amount of cholesterol present in the membrane confers “stiffness” which influences raft structure and the interaction between BACE and APP and thus influences A β generation [293]. Statins, inhibitors of hydroxymethyl co-enzyme A-reductase, are widely prescribed for their cholesterol lowering ability. Statins reduce the production of cholesterol and isoprenoid intermediates including geranylgeranyl and farnesyl pyrophosphate. In experimental models of AD, statins also reduce the production of A β by reducing membrane cholesterol, disrupting secretase enzyme function and by reducing neuroinflammation. However these results have been questioned as some of the studies used levels of statins that resulted in unnaturally low levels of membrane cholesterol, thus disrupting normal membrane function.

In 2000, the protein Seladin 1 was found to be down-regulated in AD-affected brain regions, this protein was later found to be an enzyme (DHCR24) that converts desmosterol into cholesterol. Interestingly, 17 β -oestradiol increases Seladin 1 levels as well as membrane cholesterol levels, and is known to protect cells against A β aggregate-induced toxicity [294]. In support of this, other cell culture studies have shown that a reduction in membrane cholesterol and associated reduction in rigidity facilitates the interaction of exogenous A β with the membrane, allowing the opening of Ca²⁺ pores and subsequent excitotoxicity [295], demonstrating a derailment of normal physiological control. However more recently, another study has found the levels of the cholesterol precursor desmosterol are lower in AD-affected brain regions compared to age-

matched controls, suggesting Seladin 1 activity may not be the limiting step or factor [296]. It is likely that there is an optimum level for membrane cholesterol, above or below which membrane protein metabolism is disrupted, and further studies are needed to clarify all these aspects of brain cholesterol metabolism.

1.5.2 Cholesterol and metabolites

The brain contains almost 25% of total body cholesterol, the majority of which is unesterified and is present in myelin and the plasma membranes of neurons and astrocytes [297]. Unlike fatty acids which are also synthesised by the liver and delivered to the CNS [298], brain cholesterol is mainly synthesised *de novo* by glial cells, reflecting the importance of adequate amounts and lack of reliance on peripheral supply for maintenance of brain function [297]. When released from degenerating neurons and glial cells, cholesterol is actively recycled by astrocytes. Cholesterol dysregulation in the brain is suggested as an important factor for A β overload [45, 284], with both increased and reduced levels influencing neurodegeneration [162, 299, 300]. Cholesterol homeostasis is maintained by a complex interplay of synthesis, transport and metabolic pathways. Cholesterol and other lipids are transported in the CNS as lipoproteins. Lipoproteins in the CNS resemble plasma HDL in terms of size, density and composition; the major difference being that CNS HDL contains apoE as its predominant protein compared to apoAI in plasma HDL. ApoE is abundantly synthesised by microglia and astrocytes [301]. Normally small amounts of cholesterol can be delivered from the periphery through the small HDL, which can cross the blood brain barrier (BBB). ApoAI is not synthesised in the brain, but low levels are found in the CSF, supporting the fact that peripheral HDL, or at least apoAI can cross the BBB [302]. The larger peripheral LDL and VLDL are thought to be excluded by the BBB, however recent evidence challenges this concept as it has been shown that frequent, persistent, post-prandial elevated plasma cholesterol can result in significant amounts of cholesterol and therefore the A β they contain being delivered to the CNS. This is suggested to be a consequence of vascular injury, inflammation and subsequent impairment of BBB function [179, 303], thereby contributing to increased A β load. The cholesteryl esters released from degenerating nerve cells are hydrolysed by cholesterol esterase. The free cholesterol is cleared by

binding, mainly to apoE, which delivers cholesterol back to neurons [63]. Excess free cellular cholesterol is converted into cholesteryl esters by acyl CoA:cholesterol acyl transferase 1 (ACAT1) [304].

Cholesterol equilibrium is maintained by a negative feedback loop between free cholesterol, A β and HMG-CoA reductase. Inhibition of HMG-CoA reductase by statins has been shown in animal models to reduce A β [305] but clinical trial results in terms of reducing AD risk have been inconsistent [306]. ACAT inhibition is also a therapeutic target to reduce A β as increasing levels of cholesteryl ester enhances A β release in cultured cells [307].

Cholesterol homeostasis is maintained in the brain by a balance between synthesis and removal. Brain cholesterol is metabolised by conversion to 24-S-hydroxycholesterol (24SHC) by 24-cholesterol hydroxylase (CYP46A10), an enzyme found almost exclusively in the brain [45]. 24SHC readily crosses the blood brain barrier and is excreted by the liver [45]. Since most of the circulating 24SHC originates from the brain, plasma levels may reflect cholesterol turnover in the CNS [303]. In a mouse model of AD, genetic ablation of ACAT1 also increases 24SHC, suggesting a potential role for this oxysterol in decreasing amyloidogenesis [308]: The increased free cholesterol being metabolised into 24SHC, which crosses the BBB and is removed from the CNS, may explain this. 27-hydroxycholesterol (27HC) is a peripheral cholesterol metabolite that can also cross the BBB [309]. Brain levels of oxysterols are key regulators of brain cholesterol via their influence on gene expression, in particular sterol regulatory element binding protein (SREBP), LDL receptor and HMGCoA reductase [310]. Recent studies have also suggested that α -secretase and BACE activity are regulated by 24HSC and 27HC. In cultured neuroblastoma cell lines 24HSC was shown to decrease BACE and increase α -secretase activity, thereby reducing amyloidogenic APP processing [311]. This would suggest that the balance between free cholesterol and cholesteryl esters might represent a key factor in regulating amyloidogenesis [307]. Cholesterol efflux may also control generation of A β via the ATP-binding cassette transporter (ABCA1) [310, 312].

The cross regulation of lipid pathways is also highlighted by studies showing that oxysterols activate glycerophospholipid and sphingomyelin synthesis by

activating CTP:phosphocholine cytidyltransferase (CCT); the rate limiting step in the Kennedy pathway for PC synthesis [313], possibly to protect cells from excess sterol accumulation (see Figure 1.9). Membrane composition is determined by the co-ordinated synthesis, breakdown and efflux of cholesterol, phospholipids and sphingolipids, ensuring membrane integrity and preventing the accumulation of bioactive lipids, such as ceramides and diacylglycerol (DAG), which can be detrimental to the cell [313]. An inability of PC synthesis to proceed at the same pace as cholesterol accumulation can lead to activation of the endoplasmic reticulum (ER) unfolded protein response and apoptosis [314].

Cellular cholesterol homeostasis is regulated by two main nuclear ligand-activated transcription factors; liver-X-receptors (LXR) and sterol regulatory element binding proteins (SREBPs), which activate over thirty genes involved in synthesis, uptake and processing of cholesterol, fatty acids, triglycerides and phospholipids [64, 315, 316]. SREBP-2 functions to increase cellular cholesterol whereas LXRs (LXR α /LXR β) facilitate reverse cholesterol transport (RCT) leading to cholesterol removal via the liver. SREBP-2 is activated when the cellular sterol pool is decreased. SREBP-2 is present as an inactive precursor in the ER bound to SREBP cleavage-activating protein (SCAP). SCAP is retained in the ER when bound to Insigs (insulin induced gene) and prevents transport of the SCAP/SREBP complex to the Golgi apparatus. Sterols mediate feedback inhibition of SREBP-2 via Insigs and when cholesterol levels decrease the SCAP/SREBP-2 complex moves to the Golgi apparatus for proteolytic cleavage. An active fragment is then released which enters the nucleus and binds to sterol response elements on the promoter region of target genes such as HMGCoAR and the LDL receptor gene, leading to increased cholesterol synthesis and uptake [312, 317]. Insigs also inactivate HMGCoAR within the ER by promoting ubiquitination and degradation when sterols accumulate [317]. Diabetes and the associated impairment of insulin signaling have been shown to suppress key enzymes in the cholesterol biosynthesis pathway by inhibition of SREBP-2 in the brain. Reduced synaptosomal membrane cholesterol synthesis results, with associated defects in neuronal function [318]. LXRs form dimers with the retinoid-X-receptor (RXR) which result in gene transcription [319]. In the CNS LXR β is more abundant [320] and acts as a cholesterol

sensor to prevent cellular cholesterol overload. LXRs stimulate cholesterol efflux by promoting gene expression to increase transporters (ABCA1 and ABCG) and apoE in the CNS and apoAI in the periphery for transport back to the liver. The main physiological LXR activators are oxysterols, which can be intermediates in the cholesterol synthesis pathway, intermediates of the steroid hormone synthesis pathway or cholesterol breakdown pathways [321]. LXRs also suppress several inflammatory genes coding for cytokines such as tumour necrosis factor (TNF α), interleukins IL-6 and IL-1 β and enzymes such as cyclooxygenase-2 and inducible nitric oxide synthase, suggesting reciprocal regulation of lipid metabolism and inflammation [322]. LXR agonists could potentially alleviate AD pathology by simultaneously acting on brain lipid metabolism and therefore A β deposition, while reducing inflammation; both being pieces of the AD puzzle [323].

ABCA1 regulates both the levels and amount of lipid associated with apoE by promoting the efflux of excess intracellular cholesterol and its subsequent binding to lipoproteins, including unlipidated apoE [324]. Studies using cultured cells have shown that increasing levels of ABCA1 lowers A β , and in a mouse model of AD, deletion of the ABCA1 gene leads to a substantial reduction in both brain and peripheral apoE, leading to increased A β deposits [325, 326]. This increase in A β deposition could be mediated by an increase in the degradation of poorly lipidated apoE; a process paralleled in the periphery by increased renal catabolism of poorly lipidated plasma apoAI [327]. The degree of lipidation of apoE may be more important for A β metabolism and amyloidogenesis than total brain levels of apoE. ABCA1 mediated lipidation of apoE is reported to assist with degradation of A β by both the extracellular insulin degrading enzyme (IDE) secreted by microglia and astrocytes, and the intracellular neprilysin in microglia [328]. *In vitro* studies have shown that APOE ϵ 4 is the least efficient at promoting A β degradation and microglial A β catabolism is impaired in the absence of both ABCA1 and apoE [328]. Phospholipid transfer protein (PLTP) plays a major role in lipid metabolism and A β clearance by modulating apoE secretion by astrocytes and through its influence on ABC transporters, reverse cholesterol transport (RCT) and lipoprotein modelling (see Section 1.7 and Chapter 5).

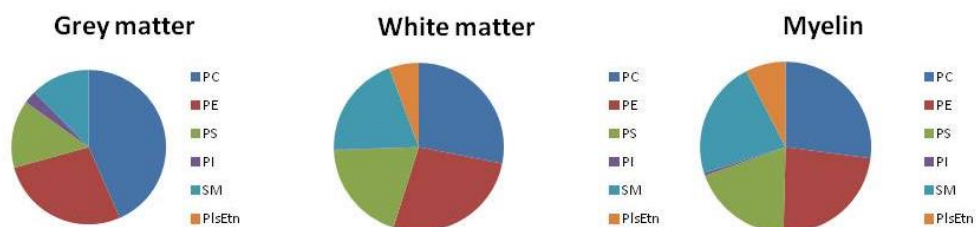
1.5.3 Phospholipids

All human cells are contained by a plasma membrane which is composed of a lipid bilayer with embedded proteins. This lipid bilayer is fluid and selectively restricts the exchange of compounds between the extracellular fluid and the intracellular compartments. The main lipids in the bilayer are phospholipids (phosphatidylcholine, PC; phosphatidylethanolamine, PE; and phosphatidylserine, PS) with the polar head groups facing the aqueous media and the fatty acyl chains forming the hydrophobic core. Another major component is the sphingolipid, sphingomyelin (SM). PC is the most abundant in most cell types and the sphingolipids are generally the most variable [63, 89, 275]. The bilayer composition is also asymmetric with PC and SM being more abundant in the outer leaflet and PS and PE higher in the inner leaflet. PS has a net negative charge which contributes to membrane potential and binds positively charged molecules. Phosphatidylinositol (PI) is only found in the inner membrane and is a signalling intermediate for hormones and neurotransmitters [275].

The fluidity of the membrane is highly dependent on cholesterol, which is interspersed between the phospholipids. The unsaturated fatty acid chains of the phospholipids are in the *cis* conformation which forms a pocket for cholesterol by binding its hydrophilic OH group and therefore the hydrophobic steroid nucleus is anchored in the membrane core. This interaction prevents the hydrophobic chains from packing too closely together and allows protein and lipids that are not bound to structural proteins to move laterally and rotate within the membrane [275]. The fluidity of the membrane is partially determined by the fatty acid composition of the diet, particularly the unsaturated fatty acids. This maybe one of the reasons why high consumption of *trans* fatty acids is detrimental to one's health as it reduces fluidity of the membranes [210, 213]. Membrane composition is non-uniform, and is dynamic, such that sections of the membrane can bud off into vesicles to remove membrane components to other parts of the cell. The membrane also accepts vesicles formed in the Golgi apparatus and other parts of the cell to enable delivery of new and recycled components back to the membrane. Individual fatty acids can be hydrolysed from the membrane lipids by various enzymes and replaced as required [329].

Flippase enzymes (P4 ATPases) transfer lipids between membrane leaflets and are required for maintenance of membrane asymmetry [330]. Loss of asymmetry on the extracellular surface of a cell is proposed to be an early marker of apoptosis, such as the appearance of PS on the outer leaflet where it is not normally present [331]. A β is capable of initiating this response in neurons and this mechanism has been proposed to contribute to A β toxicity [332]. Additionally, genome wide association studies in AD have identified a SNP in ATP8B4 (cation transport ATPase) as a potential AD risk factor, resulting in P4 ATPase deficiency [333].

PLs make up more than 60% of the total brain lipids, with the majority being incorporated into cell membranes. The six most abundant phospholipids in the brain are PC, PE, PS, PI, SM and ethanolamine plasmalogen. Studies have shown that the phospholipid profile of the brain is consistent: it is not significantly altered by different metabolic conditions. Thus, alterations in the profile may reflect changes in membrane biology [334]. Using ^{31}P NMR spectroscopy, the following phospholipid composition was determined in various fractions of the healthy brain [334].



PC: phosphatidylcholine, PE: phosphatidylethanolamine, PS: phosphatidylserine, PI: phosphatidylinositol, SM: sphingomyelin, PlsEtn: plasmalogen ethanolamine

Figure 1.8: Phospholipid distribution in healthy brain. This figure shows the predominance of PE and PC in all three regions, with SM also being a major component of white matter and myelin. Plasmalogen species are notably abundant in white matter and myelin.

Changes in composition and metabolism of several phospholipids have been described in brain tissue from AD patients when compared to age-matched control brains, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and plasmalogens [270, 335, 336]. Various groups have reported differences in lipid biochemistry in both brain tissue [337-339] and plasma [120, 127, 272]. A recent lipidomic study of the metabolic syndrome and obesity showed these conditions were associated with elevated plasma LysoPC, PC and PE species as well as reduced ether phospholipids (plasmalogens) [340]. In AD, lower peripheral ethanolamine plasmalogen (PlsEtn) has been reported, and these levels not only appear to correlate with disease severity, but also linear regression models suggest that serum PlsEtn levels decrease years before clinical symptoms appear, thus providing a potential early disease marker [341].

Glycerophospholipids are a source of lipid mediators, including arachidonic acid (AA) and docosahexaenoic acid (DHA), which are released from membrane phospholipids by the action of phospholipases. Variations in phospholipid metabolising enzymes, such as phospholipase A₂, phospholipase C, phospholipase D and phosphoinositidases have also been reported in AD [342, 343]. Initially most PLs have a saturated fatty acid at the sn-1 position and an unsaturated fatty acid at the sn-2 position of the glycerol backbone. However due to cellular requirements, the fatty acid distribution is constantly in flux by remodelling that occurs through the action of various phospholipases. Once synthesised, the PLs bud off from the ER in vesicles and are then orientated in the various cellular membranes by the action of flippase and floppase enzymes [344, 345]. All nucleated cells are capable of *de novo* synthesis of PLs by two pathways. The predominant “Kennedy” pathway occurs in the endoplasmic reticulum (ER) membrane from cytosolic precursors in most cells or alternatively synthesis occurs via activated diacylglycerol (DAG) described in Figure 1.9.

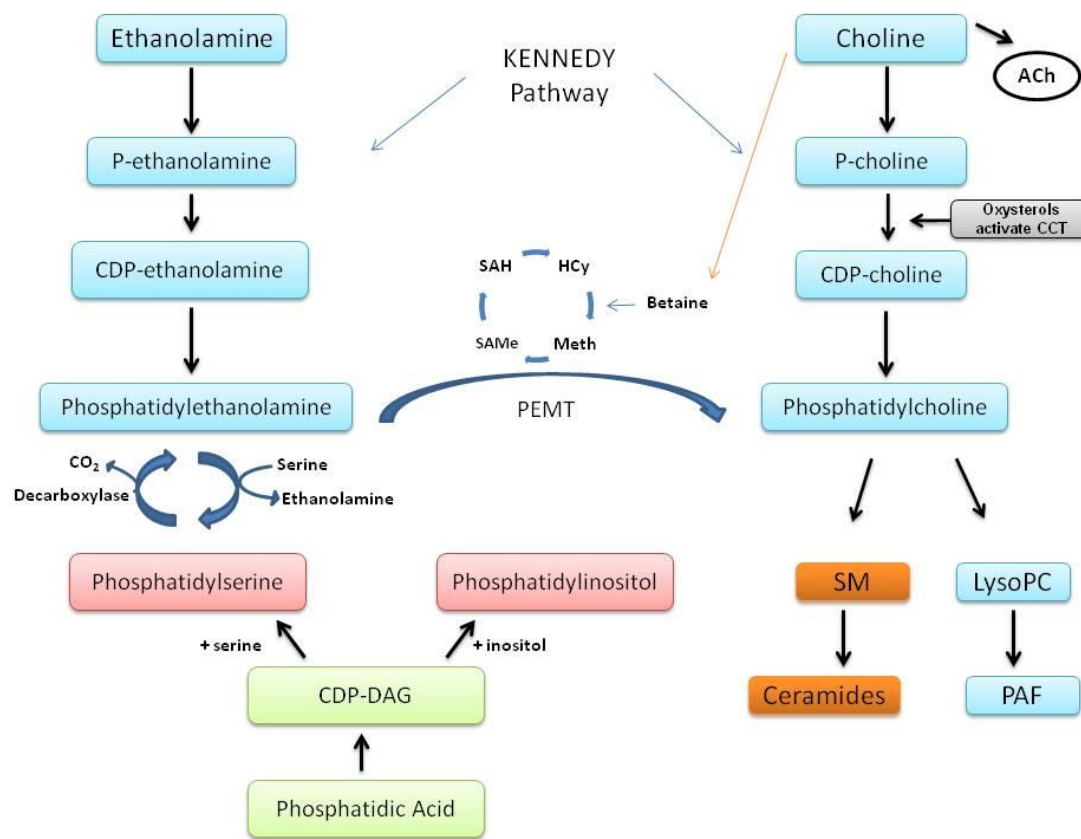


Figure 1.9: Phospholipid synthesis and connection with sphingolipid and cholesterol metabolism. ACh: Acetylcholine, CDP:cytidine diphosphate, CCT:CTP-phosphocholine cytidyltransferase, SAH: S-adenosyl homocysteine, HCy: Homocysteine, Meth: Methionine, SAmE: S-adenosyl methionine, PEMT: phosphatidylethanolamine methyltransferase, SM: sphingomyelin, PAF: platelet activating factor, DAG: diacylglycerol

All nucleated cells are capable of *de novo* synthesis of PLs by two pathways. The predominant “Kennedy” pathway occurs in the endoplasmic reticulum (ER) membrane from cytosolic precursors in most cells and utilises a cytidine-diphosphate (CDP)-activated polar head group (choline or ethanolamine) for attachment to the phosphate of phosphatidic acid. Alternatively a CDP-activated diacylglycerol is attached to an inactivated polar head group (glycerol, serine or inositol) [63, 275, 346]. The importance of the methylation pathway is highlighted to provide methyl groups for PC production from PE when dietary sources are inadequate to meet demand. The cross regulation of steroid, PL and SL pathways is shown by oxysterol activation of CCT, the rate limiting step in PC synthesis to maintain optimal steroid to PL ratios in the membrane.

1.5.4 Phosphatidylcholine (PC)

Choline is phosphorylated by choline kinase to phosphocholine. Cytidine di phosphate (CDP) is then added to form CDP-choline. CDP-choline then reacts with diacylglycerol to form PC (catalysed by choline phosphotransferase-CPT1). This process is referred to as the Kennedy pathway for *de novo* synthesis of PC (see Figure 1.9). The liver has a back up pathway via the second most abundant PL, PE to provide choline when dietary sources of this essential compound are low. This pathway involves a three step methylation by SAMe, catalysed by PEMT [194, 196, 347] (see Figure 1.9). PC is required for the secretion of VLDL from hepatocytes and is a major source of the second messengers DAG, PA, LysoPA, LysoPC, platelet activating factor and AA for eicosanoid synthesis. PC is also a direct substrate for the formation of SM via sphingomyelin synthase which transfers the choline-phosphate group to ceramide [348].

1.5.5 Phosphatidylethanolamine (PE)

Ethanolamine cannot be synthesised and therefore must be obtained from dietary lipids. PE is synthesised via the Kennedy pathway described above and by the carboxylation of PS [346] (see Figure 1.9). Additionally LysoPE can be acylated by a LysoPE-acyltransferase [349]. PE species make up 45% of the total brain PLs (see Figure 1.8), where its conical shape and ability to form reversed non-lamellar structures, facilitate membrane fusion and fission; processes that are particularly important in the brain [349, 350]. PE also confers curvature to the cell membrane which influences structure, folding and activity of membrane proteins [346]. As the nitrogen group on ethanolamine can be positively charged, PE is a zwitterion, enabling establishment of hydrogen bonds with various amino acid residues and thereby anchoring proteins. Like PC, PE is a precursor to many signalling molecules, such as DAG, FAs and PA. Ethanolamine is also a precursor for anandamide (an N-acyl ethanolamine), which is a ligand for cannabinoid receptors in the brain [351].

1.5.6 Phosphatidylserine (PS)

The brain is enriched with PS compared to other tissues where the brain PS acyl chains are highly enriched with DHA [352, 353]. As shown in Figure 1.9, PS is an important precursor for mitochondrial PE by conversion via PS carboxylase. PS is mainly located on the inner leaflet of the membrane bilayer and is thought to become an apoptotic signalling molecule when relocated to the outer leaflet. This surface exposure is a recognition signal for phagocytic removal [354, 355].

1.5.7 Plasmalogens

Plasmalogens are a sub-class of phospholipid characterised by a vinyl-ether linkage at the sn-1 position and an acyl linkage at sn-2 of the glycerol phospholipid backbone. Plasmalogens contribute to membrane integrity and are involved in many cellular processes such as vesicle formation and membrane fusion, ion transport and the generation of secondary signal mediators such as platelet activating factor [63]. The presence of the vinyl ether bond imparts antioxidant properties which reduces free radical based cellular damage as the products of enol ether oxidation do not propagate the oxidation of polyunsaturated fatty acids (PUFA); a benefit in the CNS with PUFAs in abundance and a high metabolic rate. Studies have shown a significant loss of plasmalogen in post mortem AD brains [337, 356, 357]. Ethanolamine plasmalogen (PlsEtn) is a subclass of PE and plays important roles as a reservoir for AA release, in the facilitation of membrane fusion and as an antioxidant [337]. PlsEtn is a major component of neuronal membranes, accounting for approximately 30% of the total phospholipids and 90% of the PE species [339]. Plasmalogens and their precursors are synthesised in the liver and transported to the brain bound to transport proteins. The PlsEtn species in grey matter contain mainly arachidonate and docosahexaenoate, whereas PlsEtn in the white matter contain mainly monounsaturated acyl chains at the sn-2 position. Plasmalogen deficiency in the grey matter may be directly related to neurodegeneration as deficiency leads to membrane instability [358]. Plasmalogens are key regulators of cholesterol and studies have shown that plasmalogen deficient cells exhibit lower HDL-mediated cholesterol efflux and

impaired LDL-mediated transport [359-361]. This results in an increase in free cellular cholesterol and reduced levels of cholesteryl esters via suppression of sterol-O-acyltransferase gene expression and therefore reduced levels of the enzyme it encodes: acyl-coenzyme A:cholesterol acyl transferase (ACAT). ACAT is a critical membrane bound cholesterol processing enzyme which promotes the formation of cholesteryl esters and increases LDL receptor expression [356]. The synthesis of plasmalogens is dependent on peroxisomes to generate 1-alkyl-glycerol ether lipid precursors and peroxisomal function decreases with age. A recent study showed that decreased plasmalogen precursor levels in the CNS correlate with functional decline in AD [357].

1.5.8 Phosphatidylinositol (PI)

Phosphatidylinositol (PI) is an important membrane constituent and participates in essential metabolic processes via a number of phosphorylated metabolites. It is abundant in brain tissue, comprising 10% of phospholipid species. PI has a characteristic high content of stearic acid (C18:0) linked to sn-1, and arachidonic acid (C20:4) linked to sn-2 [274]. This makes PI a primary source of arachidonic acid required for eicosanoid synthesis via the action of phospholipase A₂ [274]. Phosphoinositol phosphates (PIPs) have roles in both signal transduction, by generation of second messengers (diacylglycerol (DAG) and inositol triphosphate) and as membrane docking sites for protein signalling, such as the insulin receptor [63]. PI changes have been linked to several diseases, including diabetes and cancer [362]. Evidence suggests that alterations in PI metabolism may play a role in AD too as PI kinases play a key role in the transport and sorting of newly synthesised proteins, thus affecting the intracellular trafficking of APP [363]. Phosphatidylinositol 4,5-bisphosphate (PIP₂) is important in the regulation of ion channels and membrane trafficking. In familial forms of AD, presenilin mutations cause an imbalance in PIP₂ metabolism, with studies suggesting that this imbalance may contribute to Aβ₄₂ generation by modulating the trafficking of γ-secretase [364]. It is suggested that PIP₂ competitively interferes with γ-secretase cleavage of APP [365] and in fact an inverse correlation between cellular PIP₂ and secreted Aβ₄₂ has been demonstrated in cultured fibroblasts [364]. An increased turnover of PIP₂ by increased phospholipase C (PLC) activity has also been shown in familial AD

resulting from presenilin mutations, as PLC inhibitors cause a reduction in $A\beta_{42}$ secretion [364]. On the other hand, PLC hydrolysis of PIP_2 produces diacylglycerol (DAG) which has been shown to stimulate α -secretase and therefore promote the non-amyloidogenic processing of APP [366]. It would appear therefore that in familial AD at least, that PI metabolism is disturbed when presenilin mutations are present. Phosphatidylinositol 3-kinase (PI3K) is a key enzyme involved in PI metabolism and *in vitro* and *in vivo* studies using wortmannin, an inhibitor of phosphatidylinositol 3-kinase, have reported the inhibitor reduces levels of $A\beta$: This provides further evidence for a role for PI in AD [367, 368]. Epidemiological studies have shown an increased risk of dementia in patients with diabetes mellitus (DM) and the role of insulin receptor dysfunction in AD is an active area of AD research [369]. Hyperphosphorylation of the tau protein is a key step in the formation of neurofibrillary tangles. This phosphorylation is catalysed by various protein kinases, including glycogen synthase kinase 3 β (GSK3 β) [369]. This enzyme is negatively controlled by the PI-3-kinase/phospho-Akt pathway, which is stimulated by insulin. This suggests one possible mechanism by which impaired insulin signalling contributes to AD pathology. A recent study found reduced PI3K activity in peripheral blood mononuclear cells in AD compared to age matched controls [369], supporting a role for this enzyme in contributing to the pathogenesis of AD. Identification and quantification of phosphatidylinositol phosphate (PIP) and phosphatidylinositol biphosphate (PIP_2) with different fatty acid compositions may uncover abnormalities in PI metabolism in AD [370].

1.5.9 Phospholipases

Phospholipids are constantly being remodelled in terms of their fatty acyl chains and polar head groups and also in the generation of second messengers. This occurs through the action of various phospholipase enzymes as illustrated in the following figure.

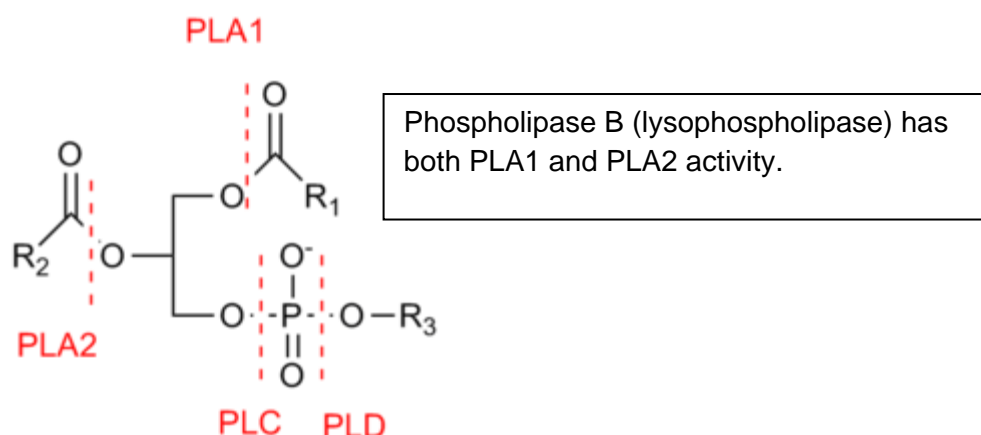


Fig 1.10: Phospholipase enzymes and site of action.

PLA1: phospholipase A1, PLA2: phospholipase A2, PLC: phospholipase C, PLD: phospholipase D, R_1 and R_2 : fatty acyl chains, R_3 : phospholipid head group

Phospholipase D (PLD), which hydrolyses PC to produce phosphatidic acid (PA) and choline, has also been implicated in AD pathology [343]. With the creation of animal models to study PLD, this enzyme is emerging as a key player in regulating phospholipid metabolism and is now a therapeutic target for several brain disorders, including AD, due to its multiple roles in signal transduction pathways [343]. PLD1 has been shown to be involved with APP and presenilin intracellular trafficking and PLD2 involved with $A\beta$ signalling. Ablation of PLD2 in a transgenic model of AD, has been shown to be neuroprotective [343]. In addition, elevated levels of PL degradation products have also been described [371] suggesting that the delicate equilibrium of PL metabolism is disturbed in AD. PLD is also involved in the generation of N-acyl ethanolamines (endocannabinoids) from their precursors, N-acyl PEs. Endocannabinoids are lipid signalling molecules that are implicated in a diverse range of processes including pain, cognition, appetite, inflammation, memory and hippocampal neurogenesis [372-374].

Increased expression or activation of PLA2 in AD has recently been measured in CSF and was shown to correspond to an increase in PL breakdown products, particularly PE [375]. The resulting defects in membrane structure can expose membrane bound proteins, such as APP to abnormal processing, and the production of $A\beta$ and is consistent with the reported co-localisation of PLA₂ with amyloid plaques [376]. Activation of various phospholipases can lead to

alterations in membrane fluidity, permeability and ion balance resulting in oxidative stress [377]. The release of AA and DHA is highly regulated and the fate of both is determined by numerous signals. These polyunsaturated fatty acids (PUFAs) can undergo oxidation by both enzymatic and non-enzymatic pathways, producing an array of interconnected lipid mediators that influence oxidative stress and neuro-inflammation; key components of AD pathology [28]. Glycerophospholipid degradation products generated enzymatically include eicosanoids, lysophospholipids, docosanoids, platelet activating factor (PAF), and endocannabinoids [28]. Many of these mediators are pro-inflammatory and their generation activates astrocytes and microglia to release inflammatory cytokines such as tumour necrosis factor ($\text{TNF}\alpha$), interleukin 1β ($\text{IL}1\beta$) and interleukin 6 ($\text{IL}6$). These cytokines in turn promote and intensify further oxidative stress and neuroinflammation, by up-regulating various isoforms of PLA_2 , cyclooxygenases (COX) and nitric oxide synthases (NOS) [378]. It is suggested that $\text{A}\beta$ accumulation itself may increase cytosolic PLA_2 (cPLA_2) activity [379]. Ceramide and ceramide-1-phosphate, generated from sphingolipids, have also been shown to stimulate cPLA_2 [380]. The Ca^{2+} -independent PE- PLA_2 isoform may contribute to neural damage by decreasing plasmalogen levels causing increased membrane permeability, Ca^{2+} flux and excitotoxicity [377]. The increased Ca^{2+} flux also promotes the relocation of cPLA_2 from the cytosol to the plasma and nuclear membranes where it accelerates the breakdown of PC [28]. As Ca^{2+} levels increase further, the secretory isoform of PLA_2 is activated (sPLA_2), exacerbating neural cell injury [28]. Increased levels of lipid mediators derived from AA and DHA by the action of PLA_2 can result in abnormal and imbalanced signalling leading to neurodegeneration. The proportion of AA and DHA in PLs varies in different regions of the brain, with AA being more evenly distributed throughout grey and white matter and DHA more concentrated at synaptic membranes [381]. PL balance is maintained through regulated hydrolysis by phospholipases and synthesis by reacylation and deacylation. There is also some *de novo* synthesis in the brain [28].

1.5.10 Phospholipid derived metabolites

The following figure shows the different actions of the PL derived metabolites involving arachidonic acid and docosahexaenoic acid.

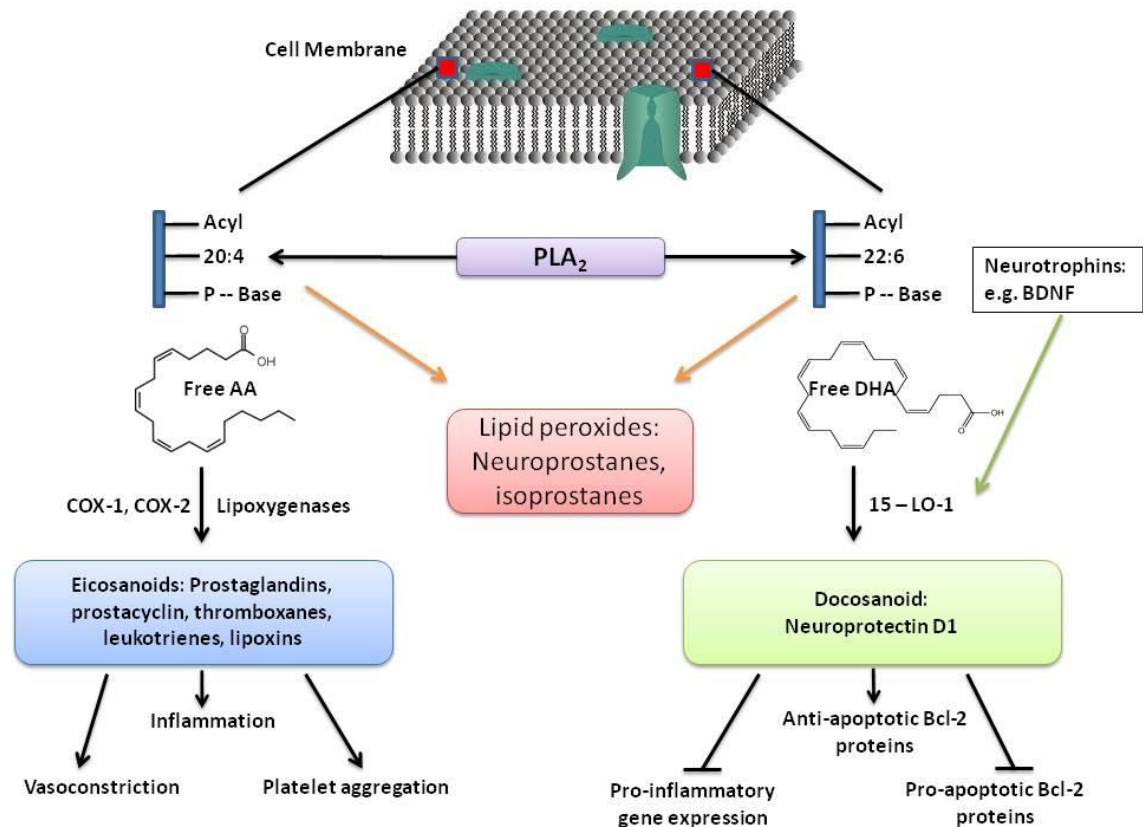


Figure 1.11: Phospholipid derived metabolites from the action of PLA_2 .

PLA_2 : phospholipase A_2 , AA: arachidonic acid (20:4), DHA: docosahexaenoic acid (22:6), BDNF: brain-derived neurotrophic factor, $COX-1$ and 2 : cyclooxygenase-1 and 2, $15-LO-1$: 15-lipoxygenase-1. Bcl-2: B cell lymphoma-2 regulatory proteins.

The free AA and DHA are released from membrane phospholipids. Cyclooxygenases or lipoxygenases transform these into eicosanoids from AA and docosanoids from DHA. The eicosanoids are potent inflammatory, vasoconstricting and platelet aggregating compounds important in immunity and wound healing. The docosanoids, including neuroprotectin D1 suppress inflammation and promote neuronal cell survival via production of anti-apoptotic proteins and suppression of pro-apoptotic proteins, such as those of the Bcl-2 family. Docosanoid production is promoted by neurotrophins, such as BDNF. Non-enzymatic transformation of AA and DHA also occurs (depending on the redox state of the cell) to produce neuroprostanes and isoprostanes respectively: These can exacerbate oxidative stress and damage to cell membranes.

1.5.11 AA and metabolites

AA is the most important omega-6 fatty acid in the brain, present as free fatty acid and enriched in phospholipids [382]. The brain relies on a steady supply of AA from the plasma via lipoproteins and LysoPLs as very little linoleic acid (LA), the essential fatty acid precursor of AA, is present in brain and even when present, it is not readily converted to AA [383]. AA has several important physiological effects as it can increase neuronal firing and enhance synaptic transmission and long term potentiation (LTP), thus influencing learning and memory. It is still not clear how AA does this, but one suggestion has been that AA rapidly increases the level of α -amino-3-hydroxy-5-methyl-4-isoxazaolepropionic acid receptors (AMPA) at the neuronal surface, which might increase neuronal activity by increasing currents through Ca^{2+} -permeable AMPAR [384]. AA may also influence brain function indirectly via the generation of eicosanoids (such as prostaglandins), which regulate inflammation, blood clotting and vascular tone (see Figure 1.11) [385]. Increases in neuronal activity must be matched by increased delivery of oxygen and nutrients through the neurovascular unit of the BBB, and PLA_2 - and AA- derived mediators play an important role in this regulation [386, 387]. AA also regulates the activity of many enzymes, including choline transferase and protein kinases A and C, involved with acetylcholine production and glucose/lipid metabolism respectively [388]. Glutamate excitotoxicity can be enhanced by AA and its inflammatory mediators, as activated microglia impair the astrocytic ability to sequester glutamate and therefore imbalances in AA or its metabolites contribute to neuronal damage [389, 390].

1.5.12 DHA and metabolites

DHA is a major component of neural membrane lipids where it has both a structural and functional role. The fluidity of the membrane is crucial for membrane protein interaction, speed of signal transduction, neurotransmission and the formation of lipid rafts. DHA has the unique ability to shift its shape millions of times per second, which provides membrane fluidity [286]. DHA is mainly obtained from the diet, although small amounts can be made by a series elongation and desaturation reactions of its precursor α -linolenic acid (18:3n-3):

This process occurs in the liver and astrocytes with the final elongation and saturation reaction occurring in peroxisomes; function of which is known to decline with age. However endogenous production of DHA is not sufficient to meet the brain's neuroprotective needs. Regardless of the source, DHA is rapidly internalised by neurons and incorporated mainly into PS and PE [391]. Evidence from animal studies demonstrates that DHA exerts a protective effect against several pathological signs of AD, including A β accumulation and hyperphosphorylation of tau. DHA has been shown to attenuate A β generation and increase synthesis of the DHA derived messenger, neuroprotectin D1 (NPD1) [392].

DHA is a direct signalling molecule as it binds and activates various nuclear receptors, including peroxisome proliferator-activated receptors (PPARs), retinoid X receptors (RXR) and sterol regulatory-element binding protein (SREBP). These nuclear receptors activate genes involved in anti-inflammatory processes, lipid metabolism and other brain functions. DHA can specifically bind RXR, regulating pathways involved in synaptic plasticity and memory [393]. PUFAs bind to PPARs more readily than saturated or monounsaturated fatty acids. The RXR receptor, normally regulated by retinoic acid, can also be activated by DHA. Retinoic acid signalling is involved in synaptic plasticity and memory, and abnormal retinoid metabolism has recently been suggested as a possible contributor to AD pathology [176, 394, 395].

Total DHA levels are decreased in the AD brain [396]. This deficiency may be due to enhanced free radical – mediated lipid peroxidation, decreased dietary intakes and/or impaired liver DHA shuttling to the brain [283]. It is generally assumed that fatty acid transport into the brain occurs from the non-esterified pool (NEFA), bound to albumin. Albumin also binds lysophospholipids, mainly lysophosphatidylcholine (LysoPC). In addition to the LysoPC generated by the action of phospholipase enzymes, it is also produced when lecithin cholesterol acyl transferase (LCAT) transfers the acyl chain from the sn-2 position of HDL-phosphatidylcholine to free cholesterol to form cholesteryl ester in HDL. Studies have shown that DHA uptake by the brain is around 10 times more efficient when incorporated into LysoPC compared to the NEFA form. LysoPC-DHA may

therefore represent a privileged form of DHA transport into the brain and may have implications for abnormal lipid metabolism and reduced HDL [397].

Studies with primary neurons in culture, stressed by A β and interleukin-1 β (IL-1 β) have shown up-regulation of PLA₂ [286] and may explain the increase in unesterified DHA seen in AD. The bioavailability of unesterified DHA is a highly regulated event. Once DHA is freed from membrane phospholipids it can go down two different pathways – one protective and one damaging (see Figure 1.11). The pathway taken appears to be influenced by the redox balance of cells [398]. DHA is the most unsaturated fatty acid, making it a target for primary lipid peroxidation. During periods of excessive oxidative stress, DHA may be non-enzymatically oxidised into neuroprostanes [352]. Generation of docosanoids from DHA is an emerging area of research. Resolvins and neuroprotectins are derived from DHA by the action of 15-lipoxygenase and maresins are formed by the action of 14-lipoxygenase and are so called because they “resolve” inflammation and promote “protection” of cells against injury [274]. It would therefore appear that the beneficial actions of DHA in the brain are a result of direct maintenance of neuronal plasma membrane fluidity and through the generation of docosanoids. The docosanoids are similar in structure to eicosanoids and appear to antagonise the potent inflammatory and immunoregulatory functions of the omega-6 generated counterpart [399]. Therefore deficits in DHA or its metabolites may contribute to the inflammatory signalling, apoptosis and neuronal dysfunction seen in AD [286]. Resolvins have been shown to block TNF α induced cytokine release from microglial cells and regulate leucocyte infiltration in the brain [400].

A major DHA derived mediator is neuroprotectin D1 (NPD1) or 10R,17S-dihydroxy-docosa-4Z,7Z,11E,15E,19Z hexaenoic acid. NPD1 is synthesised as an alarm response to oxidative stress and by neurotrophic activation in an attempt to maintain cellular integrity and promote brain cell survival and repair, through anti-apoptotic and anti-inflammatory signalling [286, 401]. Recent evidence suggests that synthesis of NPD1 is activated by growth factors and neurotrophins. NPD1 is also being shown to interact with the mechanisms associated with APP and A β peptide [286]. Both DHA and NPD1 promote non-amyloidogenic processing of APP and decrease the rate of A β generation.

NPD1 has been shown to specifically down regulate A β ₁₋₄₂-induced expression of the pro-inflammatory cyclooxygenase-2 (COX-2) and BACE1, while activating ADAM10 and increasing production of the neuro-protective sAPP α [402]: This diminishes A β -mediated pro-inflammatory, pro-apoptotic and pathogenic consequences. NPD1 also reduces brain cell damage by shifting the balance from expression of apoptotic proteins such as caspase-3, to expression of the anti-apoptotic, survival promoting members of the Bcl-2 gene family during oxidative stress [403] (see Figure 1.11). Animal and *in vitro* studies have helped to clarify the mechanisms of NPD1 protection and have shown that along with DHA it also interacts with PPAR γ , a transcription factor regulating lipid metabolism, inflammation and insulin sensitivity [402]. Studies using the PPAR γ agonist rosiglitazone (Avandia™), the irreversible antagonist GW9662 and overexpression of PPAR γ demonstrated that NPD1-mediated down regulation of BACE and A β ₄₂ peptide release are PPAR γ activation dependent [402]. PPAR γ activation leads to anti-inflammatory, anti-amyloidogenic and anti-apoptotic actions, as does NPD1. The molecular mechanisms of the A β -lowering effects by PPAR γ remain unclear, although several mechanisms have been proposed: Induction of A β ubiquitination, leading to enhanced degradation, is one possible mechanism. Alternatively, A β clearance may be enhanced by improved insulin sensitivity and increased insulin degrading enzyme activity in the brain, which plays a major role in degrading A β [404]. Additional studies have also shown that NPD1's stimulation of non-amyloidogenic pathways can also be PPAR γ -independent.

1.5.13 Lysophospholipids and Platelet Activating Factor (PAF)

Lysophospholipids are transiently produced during phospholipid metabolism by the action of PLA₂ and include LysoPC, LysoPE, LysoPS, LysoPI, LysoPA and Lysoplasmalogens [63]. Apart from being intermediates of PL metabolism, at high concentrations they influence membrane permeability and interact with membrane bound enzymes and other membrane lipids, affecting various functions and growth factors [28]. LysoPLs can be reacylated to restore PLs to membranes or acetylated to form platelet activating factor (PAF); a potent inflammatory lipid with multiple signalling roles [405]. LysoPC is involved in

microglial and immune activation in the brain, where activated microglia help clear cellular debris in areas where senile plaques and neurofibrillary tangles are accumulating [28]. Analysis of CSF in AD patients has shown LysoPC to be significantly reduced compared to controls, indicating abnormal PC metabolism with resulting alterations in membrane fluidity, permeability, ion homeostasis and promotion of oxidative stress [371]. However, a recent study contradicted these findings by reporting an increase in the LysoPC:PC ratio in both nanometer particle fractions (more representative of brain tissue) and supernatant fractions of CSF in AD [375], which was accompanied by increased PLA₂ activity. LysoPA is involved in neurotransmitter action, including stimulation of noradrenaline and dopamine release [406].

1.5.14 Non-enzymatic derived lipid mediators

Non-enzymatic degradation of glycerophospholipids results in the formation of isoprostanes, neuroprostanes, isofurans, neurofurans, isoketals, neuroketals, 4-hydroxynonenal (4-HNE), 4-hydroxyhexanal (4-HHE) and malondialdehyde [407]. These prostaglandin-like compounds trigger further reactive oxygen species (ROS) generation, exacerbating damage to cellular membranes. Neuroprostanol-containing phospholipids adversely affect neuronal function by interfering with membrane function [408]. AA peroxidation results in the formation of 4-HNE, which is highly reactive and increases the expression and activity of BACE1 [28]. Measurement of F4-neuroprostanes (derived from DHA) and hydroxynonenal (HNE) reflect the general state of oxidative stress and lipid peroxidation in 'stressed' brain cells and these molecules may be useful biomarkers for neurodegeneration [409]. Notably F4-neuroprostanes are elevated in the cortex of AD brains [410] and may therefore serve as a potential biomarker for the specific oxidation of DHA [352].

1.5.15 Sphingolipids

Sphingolipids, including sphingomyelin, ceramides, sulphatides and glycosphingolipids are key components of lipid rafts and therefore affect APP processing and A β binding and aggregation. Ceramides are central components of sphingolipid metabolism and are core constituents of most sphingolipids, which are either synthesised from fatty acyl CoA and serine,

sphingosine or produced by enzymatic hydrolysis of other sphingolipids [63]. The following figure depicts sphingolipid metabolism.

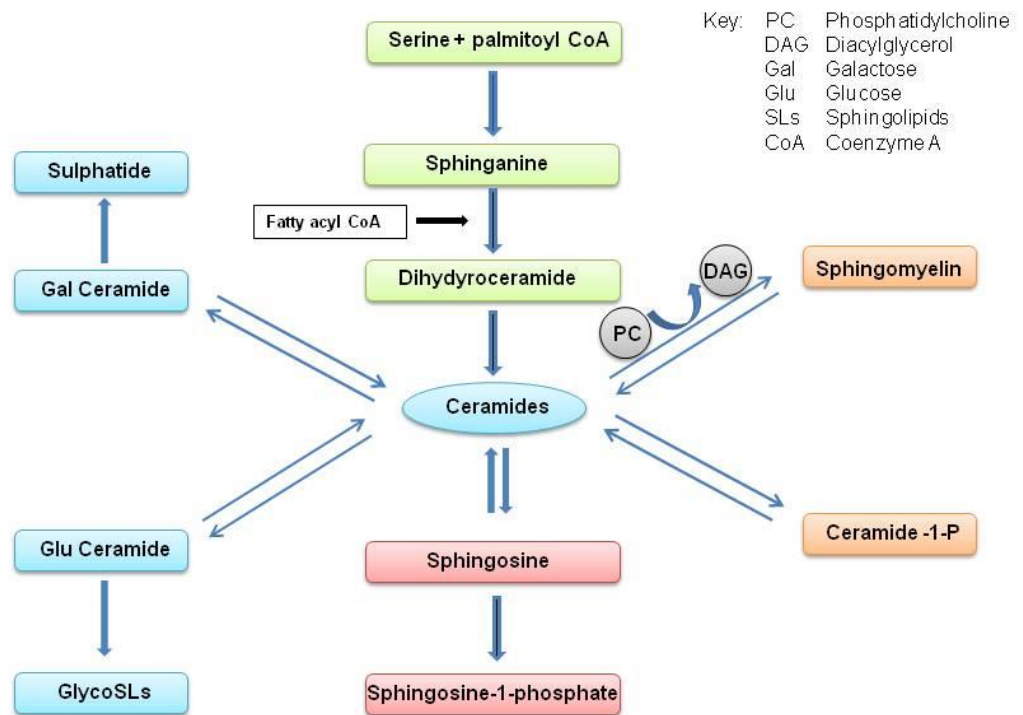


Figure 1.12: Sphingolipid metabolism.

Sphingolipid synthesis begins with ceramide which can be formed *de novo* from palmitoyl-CoA and serine or be produced from other sphingolipids by the action of various enzymes. Ceramide reacts with phosphatidylcholine (PC) to form sphingomyelin (SM) and can react with uridine diphosphate (UDP) sugars to form cerebrosides (usually galactose or glucose). Galactoceramide (cerobroside) can react with 3'-phosphoadenosine 5'-phosphosulphate (PAPS, an active sulphur donor) to form sulphatides. Additional sugars may be added to ceramide to form globosides (glycoSLs). Gangliosides are produced by the addition of *N*-acetylneuraminic acid. (Figure adapted from [411]).

Sphingomyelin (SM) and glycosphingolipids, in particular gangliosides, are abundant in the brain and are major components of neuronal membranes. Sphingolipids are also directly involved with APP processing, which in turn affects cholesterol and sphingomyelin metabolism. This suggests a cross regulation of both processes and a link between A β -regulated cholesterol and sphingomyelin metabolism, with γ -secretase activity controlling various enzymes involved in lipid metabolism [291]. Inhibition of sphingomyelinase, the enzyme that converts sphingomyelin to ceramide, results in SM accumulation, which in turn reduces A β secretion due to inhibition of γ -secretase. Studies have shown that activation of neutral sphingomyelinase (SMase) by A β_{42} reduces SM and increases ceramide. However, A β_{40} lowers cholesterol by inhibition of HMGCoA reductase and reducing *de novo* cholesterol synthesis. The reduced membrane cholesterol and SM produced by A β affects γ -secretase activity and therefore normally provides a feedback mechanism [291] (see Figure 1.13): This suggests a role for sphingolipids as γ -secretase modulators. Additionally the presence of a sphingolipid binding sequence on A β has been identified that increases affinity of A β to bind lipid rafts rich in cholesterol and sphingolipids [412]. This binding sequence may have implications for aggregation and processing of A β , which can influence pathogenicity as studies have shown that A β aggregation requires an environment rich in SM [291].

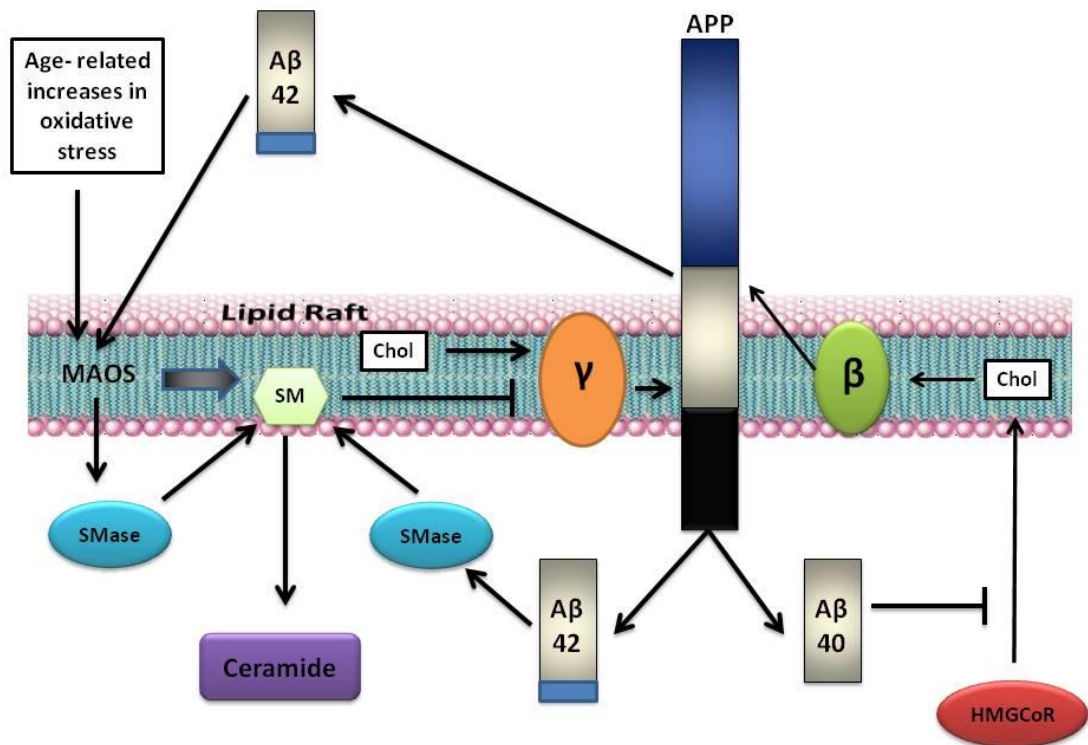


Figure 1.13: Interactions between Cholesterol, Sphingolipids and APP processing.

APP: Amyloid Precursor Protein, γ: Gamma secretase complex, β: Beta site APP cleaving enzyme (BACE), Chol: Cholesterol, SM: Sphingomyelin, MAOS: Membrane Associated Oxidative Stress, SMase: Sphingomyelinase, HMGCoR: 3-hydroxy-3-methyl-glutaryl-CoA reductase.

APP, BACE and γ-secretase are concentrated in lipid rafts which are rich in Cholesterol and SM. Cholesterol increases the activities of BACE and γ-secretase, increasing production of Aβ₄₂, which can directly activate SMase resulting in increased ceramide production. Additionally, age-related increases in oxidative stress and Aβ₄₂ induce membrane-associated oxidative stress which also activates SMase. An increase in ceramides can impair synaptic function and damage neurons. Aβ₄₀ inhibits HMGCoAR, the rate limiting step in the cholesterol biosynthesis pathway. An increase in the Aβ₄₂/Aβ₄₀ ratio will therefore increase levels of cholesterol and decrease SM in membranes.

SM is a major source of ceramides and sphingomyelinases which hydrolyse phosphocholine from sphingomyelin to produce ceramides, are activated by inflammatory cytokines and oxidative stress [157]. Ceramides are potent second messengers that regulate diverse cellular processes. They activate enzymes involved in stress signalling and are linked to caspase activation and pro-apoptotic signalling via reactive oxygen intermediates. In early stages of AD, marked decreases in sulphatides have been described, apparently not due

to deficiencies in production, but rather due to greater conversion to ceramides, which are a class of potential degradation products of sulphatides [413]. Accumulation of ceramide, with increased brain and CSF levels has been reported at the earliest clinically recognisable stages of AD [273, 337]. Ceramide levels have been found to be more than three-fold higher in white matter of the AD brain compared to normal control brain. It appears that gene expression changes in the early stages of AD shift sphingolipid metabolism towards accumulation of ceramide, depletion of glycosphingolipids and a reduction in the anti-apoptotic signalling molecule, sphingosine-1-phosphate [335]. Lipid metabolism is tightly connected to APP processing, and A β ₄₂ may activate sphingomyelin catabolism causing accumulation of ceramide which exacerbates neurodegeneration [414]. Adverse consequences result from ceramide accumulation, including up-regulation of cytokines, reactive oxygen species generation, mitochondrial dysfunction and apoptosis [415]. Ceramides act directly on mitochondria to induce membrane permeability transition and the release of cytochrome c, thereby compromising ATP generation and promoting cell death [157]. Ceramides have also been shown to increase APP processing by BACE, by enhancing BACE stability through the formation of ceramide-enriched platforms in the lipid rafts [416] (see Figure 1.14). The substantial elevation of ceramide in the AD brain might also be a consequence of sulphatide degradation and an increase in synthesis or catabolism of SM and/or cerebroside (see Figure 1.12) [337].

Sialylation of glycoceramides produces gangliosides, which are currently receiving considerable attention as accelerators of AD pathology [27, 411, 417]. A β is thought to exert its toxic effects by interacting with cell membranes and membrane proteins, which alters the folding of the peptide. The abnormal A β then interferes with cell signalling and membrane integrity and reacts with other components, which promote aggregation [418]. A possible mechanism by which A β assembles into insoluble fibrils is through binding to the ganglioside GM1. The conformation of A β from random coils to structures with increased β -sheet content results when A β is bound to GM1 [27]. In this way, the membrane bound GM1 acts as a seed for A β aggregation. This process is increased with ageing and appears to be facilitated by cholesterol rich environments, such as

lipid rafts [419]. A β bound to gangliosides in plaques may also escape immune surveillance and indirectly promote further plaque formation by decreasing microglial clearance of A β [420].

Sulphatides are a class of myelin-specialised glycosphingolipids, involved in various cellular processes, including cell growth, protein trafficking, signal transduction, cell-cell recognition, neuronal plasticity and cellular integrity [421]. Studies have shown that apoE is associated with sulphatide transport and mediates sulphatide homeostasis in the CNS through lipoprotein metabolism pathways. Alterations in apoE mediated sulphatide trafficking have been shown to deplete sulphatide in the brain, and CNS levels of sulphatide have been shown to be apoE isoform dependent, with the lowest levels observed with APOE ϵ 4 [71]. An increased rate of apoE/sulphatide containing particle uptake and increased expression of lipoprotein receptors can lead to increased breakdown of sulphatide, depleting sulphatide from the myelin sheath. Sulphatide containing apoE-associated particles facilitate A β clearance. This may represent a further mechanism through which APOE ϵ 4 increases the risk for AD [71]. Studies with APP transgenic mice have demonstrated that clearance of increased A β production requires increased apoE, which collectively increases sulphatide trafficking and degradation [71].

Serum SM and ceramide levels were measured in women as part of the longitudinal WHASII study of cognitive impairment and the results suggest that these lipids may be used as biomarkers of memory impairment and disease progression [163, 422]. However, this study gave conflicting results between cross sectional and longitudinal data. Lower levels of serum ceramide and SM were found to be associated with memory impairment cross-sectionally, but higher ceramide levels were found to be associated in the longitudinal data [163, 423]. The potential reasons for these differences will be explored in Chapters 3 and 4. The potential link between ceramides and AD pathology can be summarised in the following figure:

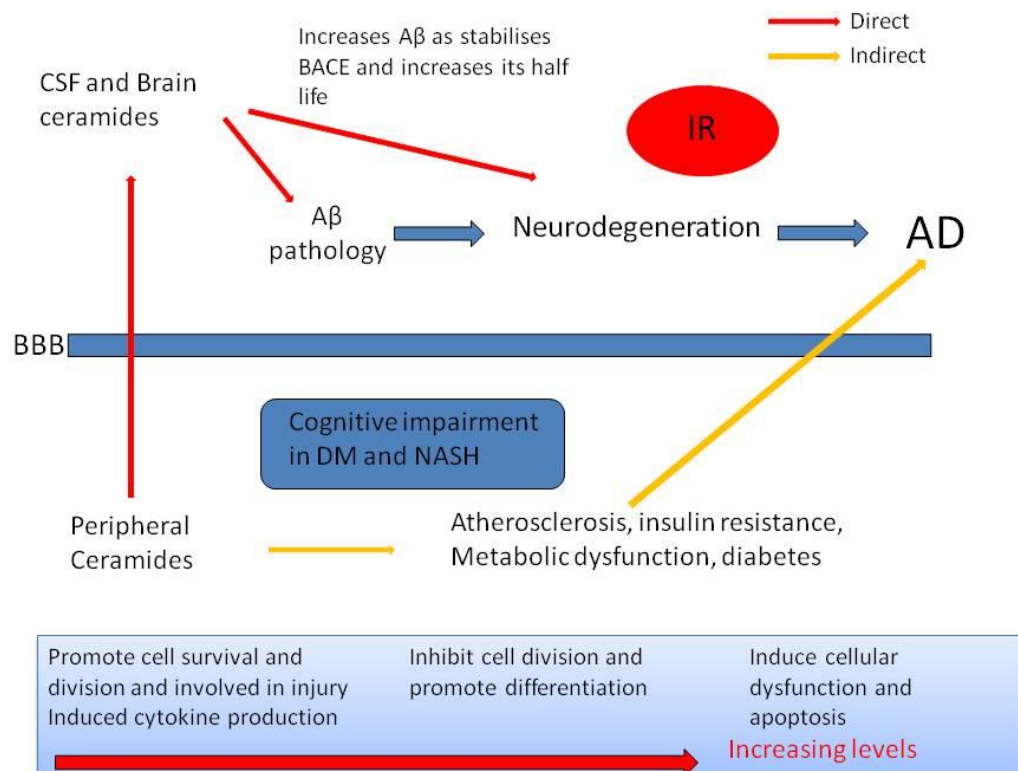


Figure 1.14: The potential mechanisms for both direct and indirect effects of increased ceramides influencing AD risk.

CSF: Cerebrospinal Fluid.

Elevated plasma ceramides are a feature of diabetes mellitus (DM), atherosclerosis, insulin resistance (IR) and metabolic dysfunction; all risk factors for developing Alzheimer's disease (AD). (reviewed in [207, 265]). Additionally it is hypothesised that the existence of a liver/peripheral-brain axis of neurodegeneration results in the increased production and delivery of ceramides across the blood brain barrier (BBB): This provides a possible explanation for the cognitive impairment seen in DM and non-alcoholic steatohepatitis (NASH) [165]. In the central nervous system, the elevated ceramides have direct effects on AD pathology by stabilising beta site APP cleaving enzyme (BACE) and increasing A β generation [416]. Ceramides may also induce brain insulin resistance, which alters brain glucose metabolism and interferes with numerous signalling pathways involved with synapse maintenance and brain plasticity [263, 265]. (Figure adapted from [424]).

A recent probe into the plasma lipidome of individuals with metabolic syndrome and obesity showed various changes including increased ceramide species [340].

1.6 Control of lipid metabolism

Section 1.3.3 discussed diet and nutrition as risk factors for developing AD. The composition of the diet and nutrient status has profound effects on lipid metabolism through their influence on the endocrine system and energy homeostasis via transcription factors regulating metabolic gene expression, including SIRT1, adenosine monophosphate kinase (AMPK) and PPAR-gamma coactivator 1- α (PGC1 α) (reviewed in [425-427]). A recent publication has also shown that metabolic stress modulates BACE gene transcription via effects on SIRT1, PPAR γ and PGC1 α in neurons [428]. Due to the integrated nature of the endocrine system, virtually all hormones will have some influence on lipid metabolism and therefore influence a myriad of cellular functions. These hormones therefore, may need consideration when interpreting blood lipid profiles. Insulin is a master hormone regulating glucose uptake and utilisation, but it also has many other effects on metabolism in general and acts as a signalling molecule for a vast array of cellular processes. As previously described, it has wide ranging effects on the CNS and is considered a key player in the AD picture [98, 179]. The effects of insulin on cholesterol metabolism via gene expression changes in SREBP and HMGCoAR have also been described in previous sections. Insulin has a major influence on peripheral plasma lipid profiles due to its affect on directing metabolism towards energy storage. When insulin resistance is present increased circulating free fatty acids (FFA) result from increased adipose tissue lipolysis as insulin normally inhibits this process. The increased FFAs eventually lead to increased VLDL secretion, increased cholesterol and an associated increased amyloid burden [260]. Insulin has also been shown to induce fatty acid desaturase expression in monocytes and may provide an explanation of the increased inflammation promoted by post-prandial hyperinsulinaemia when the dietary omega-6 to omega-3 ratio is high [429]. Of particular relevance to lipidomics is the emerging evidence to suggest an association between insulin resistance and ceramides; where toxic ceramides may be the intermediate that links excess dietary saturated fatty acids and inflammatory cytokines with insulin resistance [265]: This is depicted in the following diagram.

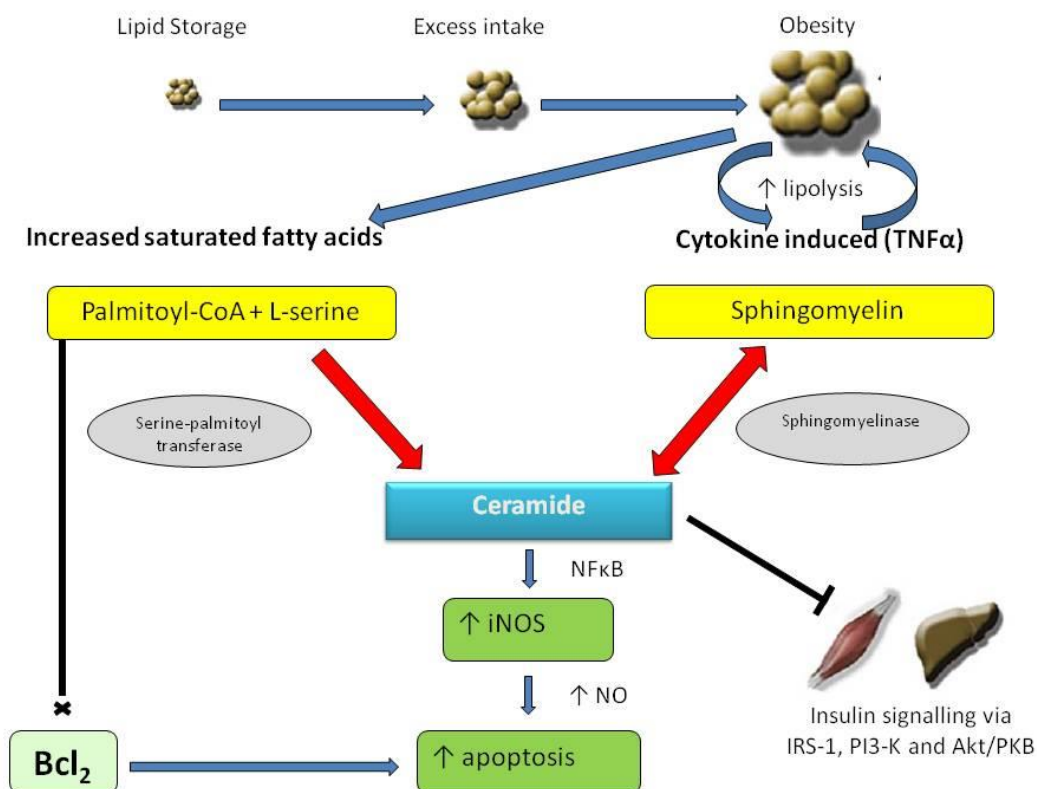


Fig 1.15: Ceramides – the toxic intermediate linking metabolic dysfunction, inflammatory cytokines and insulin resistance? TNF α : tumour necrosis factor alpha, CoA: Coenzyme A. NF κ B: nuclear factor kappa B, iNOS: inducible nitric oxide synthase, NO: nitric oxide, Bcl₂: B cell lymphoma 2 anti-apoptotic proteins, IRS-1: insulin receptor substrate-1, PI3-K: phosphatidylinositol-3 kinase, Akt/PKB: serine/threonine-specific protein kinase.

When adipose tissue exceeds its storage capacity, adipokines increase inflammation which increases ceramides. This inhibits insulin signalling, further increasing lipolysis and increasing the release of fatty acids for ceramide synthesis. Ceramide promotes apoptosis and the elevated saturated fatty acids inhibit the Bcl₂ family of anti-apoptotic proteins.

1.7 Lipoprotein metabolism and phospholipid transfer protein (PLTP)

Lipoproteins are complexes of protein and lipids that solubilise lipids so they can be transported in the circulation between tissues. Lipoproteins are synthesised in the intestines from dietary lipids or in the liver from endogenous lipids and can also be remodelled from precursor lipoproteins under the influence of various metabolic signals. Lipoproteins are generally spherical structures, typically consisting of a neutral lipid core of cholesteryl esters and triglycerides and a surface layer of phospholipids, unesterified cholesterol and

various apolipoproteins. These apolipoproteins not only solubilise the lipids but also act as ligands for cell surface receptors and cofactors for plasma enzymes. High LDL, VLDL and low HDL are known risk factors for vascular disease, diabetes and AD [430-432] and dyslipidaemia has been shown to be more prevalent in AD patients with BBB impairment [433]. Lipoproteins are very dynamic and are constantly being modified by the action of enzymes, lipid transfers and apolipoprotein exchange. This ensures specific lipid delivery to different tissues [63, 89]. Lipoprotein metabolism and the associated lipid transfer proteins are shown in the following figure:

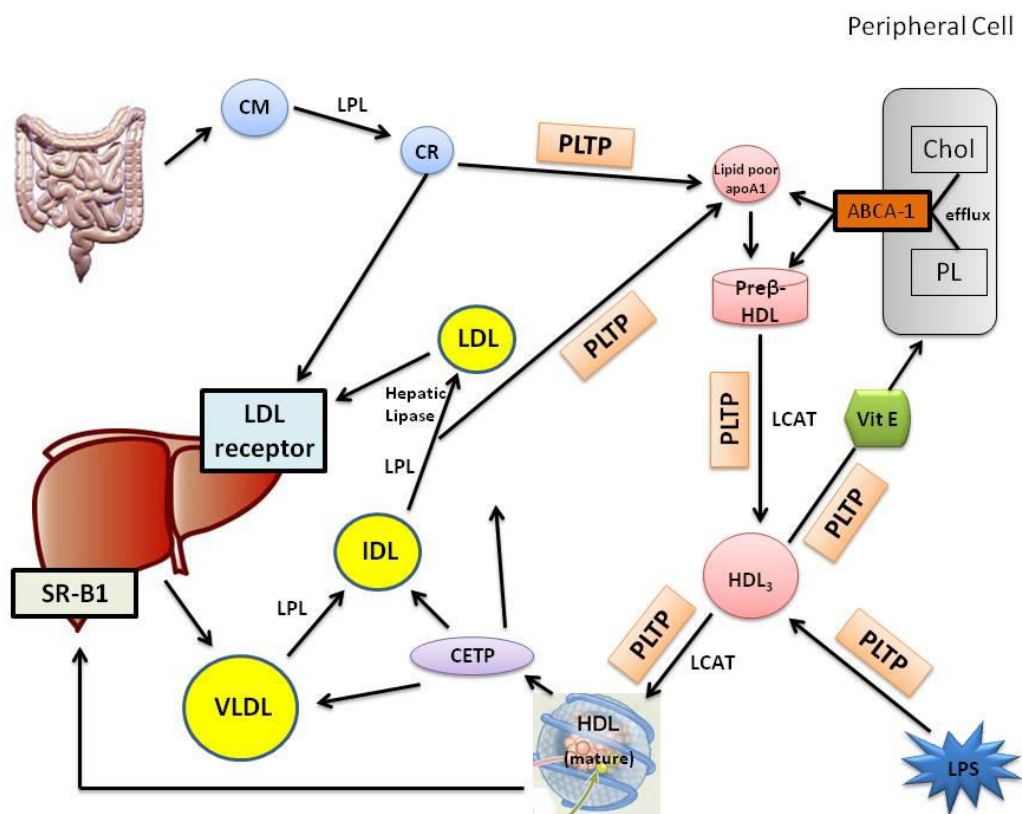


Figure 1.16: Lipoprotein metabolism and lipid transfer proteins.

CM: Chylomicrons, LPL: Lipoprotein lipase, CR: Chylomicron remnants, PLTP: Phospholipid transfer protein, Chol: Cholesterol, PL: Phospholipid, ABC-1: ATP binding Cassette Transporter 1, HDL: High density lipoprotein, LDL: Low density lipoprotein, VLDL: Very low density lipoprotein, IDL: Intermediate density lipoprotein, SR-B1: Scavenger receptor B1, CETP: Cholesteryl ester transfer protein, LCAT: Lecithin-Cholesterol acyl transferase, VitE: Vitamin E, LPS: Lipopolysaccharide

CM are assembled from dietary fat in the intestine and enter the circulation. At the same time lipid poor apolipoprotein AI (apoAI) is secreted from the intestine and reaches the circulation. The triglycerides of CM undergo lipolysis by lipoprotein lipase and the surface remnants (phospholipids and apolipoproteins) of the CR are transferred via PLTP to apoAI. The CRs are then taken up by the LDL/remnant receptor in the liver. Triglyceride rich lipoproteins (VLDL) are secreted by the liver. VLDLs also contain a large amount of cholesteryl ester and are enriched with apolipoprotein B100 (apoB100) and phosphatidylethanolamine (PE). The CE is transferred from HDL by CETP and moves most of the CEs to apoB containing lipoproteins which can be cleared by the liver. LPL hydrolyses the TG of VLDL to form IDL, which are further modified by hepatic lipase to form LDL. The surface PLs and protein remnants at each stage are transferred to HDL via PLTP. Lipid poor apoAI acquire free cholesterol and phospholipids via ABCA-1 mediated efflux from the liver and peripheral cells (and from VLDL and CR via PLTP) to form nascent pre- β HDL, which progressively acquire additional PL and cholesterol via ABCA-1 efflux from tissues. The cholesterol acquired by these discoid nascent HDL is then esterified by LCAT (synthesised and secreted by the liver), which the HDL obtains from the circulation. If the cholesterol is not modified within the HDL particle it can move out again so conversion to CEs ensures that cholesterol is trapped within HDL as CEs move to the centre of the HDL, producing the more spherical α -HDL. The activity of LCAT is crucial for maintaining a steady gradient of free cholesterol allowing the HDL to accept additional cholesterol from other sources. CETP also enriches HDL with TG which enhances lipolysis of PLs and TG by hepatic lipase leading to smaller HDL₃ and pre β -HDL. Large mature HDL particles enriched with CE are removed from the circulation by the liver scavenger receptor-B1 (SR-B1). PLTP, by transferring PLs and apolipoproteins is constantly remodelling HDL for reverse cholesterol transport. It also transfers vitamin E and lipopolysaccharides from and to HDL₃ respectively.

In plasma there are two main lipid transfer proteins; cholesterol ester transfer protein (CETP) and phospholipid transfer protein (PLTP). CETP is secreted mainly by the liver and circulates bound to HDL. It redistributes cholesteryl

esters from HDL to TG rich lipoproteins (CM and VLDL) and transfers TGs from VLDL and CM to LDL and HDL [434]. PLTP facilitates the transfer of phospholipids from TG rich lipoproteins to HDL and together with CETP is important in reverse cholesterol transport by modifying the size and function of HDL. The physiological relevance of PLTP is highlighted in PLTP-deficient mice, which demonstrate a total absence of PL transfer from VLDL to HDL and markedly reduced PLTP and HDL levels [435, 436]. The lack of substrate specificity of PLTP is also highlighted by its ability to transfer DAG, which are released by lipoprotein lipase in capillary endothelium and during lipolysis in adipose tissue [437]. DAG is also transported by HDL. PLTP also facilitates the transport of other lipophilic substances such as α -tocopherol [438]. Dietary α -tocopherol (Vitamin E) is incorporated in CM and transported to the liver where it is packaged into VLDL. Vitamin E is important in preventing LDL oxidation and subsequent uptake of oxidised LDL by vascular endothelium [439]. PLTP deficiency in the brain has been shown to result in reduced α -tocopherol and an increase in oxidative injury, leading to neurological dysfunction [440]. This suggests that PLTP is a local transporter of α -tocopherol and may transport α -tocopherol across the BBB. A major function of PLTP is to maintain the plasma sub species of HDL [441, 442] and the CNS HDL like lipoproteins [443]. The literature regarding the role of PLTP in disease is not straightforward and conflicting. Most studies have been conducted in relation to atherosclerosis and metabolic disease and are contradictory with some finding higher PLTP activity levels and increased risk of disease [444, 445] and others finding lower levels associated with disease [446, 447]. These conflicting results may be due to study design as some were prospective and others cross-sectional. Assay methodology may also contribute to the disparate results as there are notable differences in the assays used. The protective or disease promoting effects of PLTP remain unclear as HDL cholesterol levels vary in response to PLTP gene overexpression or deficiency [448, 449]. It may be that the effect of PLTP on disease depends on the background lipid profile, metabolic landscape, co-morbidities, medication use and dietary considerations. Research into PLTP and disease is gaining momentum. The emerging diverse roles and involvement

in both lipid metabolism and AD pathology certainly make this a protein of enormous interest and an intriguing addition to the lipidomic part of this study.

1.8 Lipidomics and AD biomarkers

The remarkable diversity of plasma lipids was recently highlighted by a study which measured over 500 distinct lipid species in a pooled human plasma sample [450]. The greatest challenge when studying the CNS is to ascertain direct links between blood-based analytes and neuronal biochemistry. The BBB separates the CNS from the periphery but in terms of lipids, some are excluded and others are not. In addition, ageing and inflammation can disrupt the integrity of the BBB and complicate the picture. Metabolic dysfunction, which is common in ageing and present as a risk factor for AD, can also disturb normal lipoprotein metabolism resulting in abnormal lipid transfer between the periphery and the CNS [302, 443]. CSF is thought to be the most relevant sample for studying the CNS but its collection, however, is an invasive procedure, requiring specialised clinicians and is therefore not appropriate for large population screening and follow-up. Conversely, blood is an easily obtainable and convenient sample to use for this study with the additional benefit of accessing samples from a highly characterised cohort of ageing where lipid profiles can be assessed in the context of other AD-related biomarkers such as cerebral amyloid load.

Diagnostic strategies were discussed in detail earlier in this chapter (see Section 1.2). Ante-mortem diagnosis of AD is currently made using neuropsychological assessments and neuroimaging in some cases. Imaging of the brain for amyloid burden enables AD to be identified pre-clinically, but is not feasible for large population screening and some individuals can accumulate A β but remain cognitively stable. [36, 41, 42]. Definitive diagnosis can only be made by examination of the brain post-mortem and therefore the need for the development of biomarkers to enable early diagnosis and monitoring of disease progression ante-mortem is a priority. Such biomarkers will enable therapeutic and intervention strategies to be implemented prior to manifestation of irreversible neurodegeneration.

A biomarker can be defined as:

“A characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (an indicator of disease status, both clinical and sub-clinical)”. [451].

In addition an appropriate AD biomarker should be able to detect neuropathology, should be accurate, non-invasive, reliable, reproducible and inexpensive. It should also be validated in confirmed AD and non-AD cases [452]. The development of a blood-based biomarker would represent a huge leap forward in terms of meeting the non-invasive, simple to perform and inexpensive criteria. However, due to the complex nature of AD and presence of co-morbidities, it is unlikely that a single biomarker will be identified offering sensitivity and specificity of greater than 80%. It is more likely that a panel of biomarkers will be identified comprising lipids, proteins and gene variants. This study aims to contribute towards the identification of such a panel.

1.9 Hypotheses

1. AD patients have abnormal plasma lipid profiles.
2. Altered plasma lipid profiles will reflect altered cerebral A β deposits.
3. Lipid profiling of plasma will yield sensitive and specific markers of AD.
4. Plasma PLTP activity levels are different between AD patients and healthy controls.

1.10 Aims of the study

These hypotheses will be addressed by the following specific aims:

1. To determine plasma lipid profiles of baseline and 18 month follow up AIBL samples using electrospray ionisation mass spectrometry (ESI-MS/MS).
2. To determine differences in plasma lipid profiles between healthy controls and AD, by using a shotgun lipidomics approach. In particular plasma levels of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), sphingomyelin (SM), lysophospholipids, ceramides and plasmalogens will be measured.
3. In addition, lipoprotein metabolism will be investigated using the measurement of phospholipid transfer protein (PLTP) activity.
4. To correlate candidate lipid biomarkers to disease progression, using standard uptake value ratios (SUVR).

Chapter 2

Materials and Methods

Chapter 2 - Materials and Methods

This chapter describes the ethics, research design, the study cohort, biomarkers (blood and imaging) and genotypes that have been used for the work described in this thesis. The overall aim of the methods used was to evaluate associations between blood lipid biomarkers and other Alzheimer disease (AD)-related variables of interest and to investigate the potential of a biomarker panel as an early predictive test for AD.

2.1 Ethics

The Australian Imaging, Biomarkers and Lifestyle Study of Ageing (AIBL) is approved by research ethics committees from the major participating institutions: St Vincent's Health, Austin Health, Hollywood Private Hospital and Edith Cowan University. All participants were provided with information and consent documentation outlining all procedures which included information on potential risks and benefits associated with participation in the study. After being provided with this documentation, a signed consent form was obtained from each participant before commencement of data collection. This project involved utilisation of previously collected blood samples and analysis of supplementary AIBL data (neuroimaging, genotypes, clinical classifications and blood analyte measurements) and therefore no participant interaction was involved.

2.2 Study Population

The AIBL volunteer database was created as a joint venture between leading Australian researchers in Alzheimer's disease and the Australian Commonwealth Scientific Industrial and Research Organisation (CSIRO) in 2005 [453]. The aims of this collaboration were to recruit a cohort of Australian subjects aged 60 years and over ($n = 1112$) and to collect comprehensive information including cognitive function status, amyloid brain load, blood biomarkers and lifestyle habits. This information has been collected every 18 months since study inception. Only baseline and 18 month time point data and blood samples were used for this study. The cohort consisted of three main groups:

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- Cognitively healthy controls (HC) which can further be divided into non-memory complainers (NMC) and subjective memory complainers (SMC)
- Individuals with mild cognitive impairment (MCI)
- Individuals with early-stage AD

Figure 2.1 illustrates the cohort breakdown by clinical classification at baseline and 18 month follow-up.

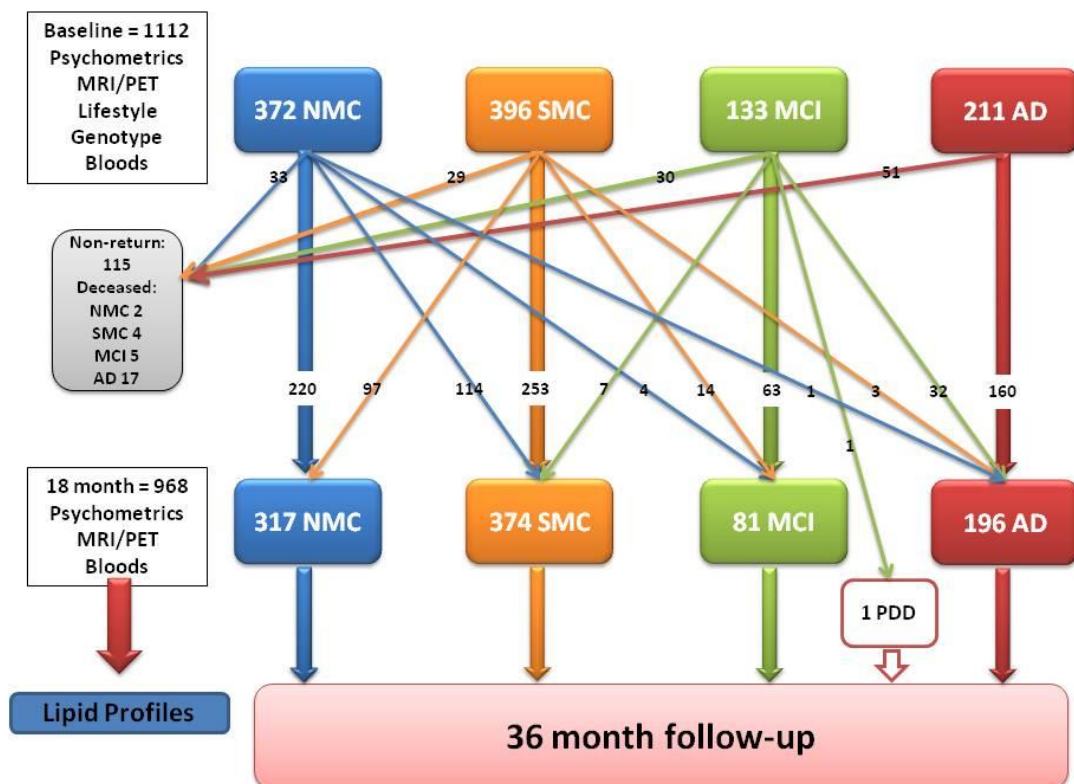


Figure 2.1: AIBL cohort at baseline and 18 months. There were 1,112 baseline samples. These were classified as 211 AD, 133 MCI and 768 healthy controls (372 non-memory complainers (NMC) + 396 subjective memory complainers (SMC)). 115 of the baseline participants did not return for 18 month follow-up and of these 28 were deceased. There were 968 18 month follow-up samples which were classified as 196 AD, 81 MCI and 691 healthy controls (317 NMC + 374 SMC). One participant in the MCI group was reclassified as non-AD dementia due to Parkinson's Disease (PDD). The arrows and associated numbers reflect the individuals moving between groups.

Schematic adapted from an image provided by the AIBL Study Scientific Management Committee.

Several high impact studies investigating cognitive function in the aged, lifestyle, neuroimaging and blood biomarkers of AD have been published

utilising data from the AIBL study: A list of these publications can be found at www.aibl.csiro.au.

2.2.1 Recruitment, eligibility screening and exclusion criteria

Volunteers for this study were recruited in Melbourne and Perth, Australia, via media advertisements (television, radio and newspapers). Over 4000 individuals responded who then underwent eligibility screening by telephone to obtain basic demographics (contact details, sex and age). In addition, information regarding medical history, with specific information related to eligibility was obtained. Information on alcohol intake was also collected. All AIBL volunteers were aged 60 years and above at baseline, and excluded if they had a history of non-AD dementia, schizophrenia, bipolar disorder, significant current depression (geriatric depression scale score or “GDS” > 5), Parkinson’s disease, cancer within the last two years (except basal cell skin carcinoma), symptomatic stroke, insulin-dependent diabetes, uncontrolled diabetes mellitus or current regular alcohol consumption exceeding four standard drinks per day for men or two per day for women. There was no maximum age for inclusion.

2.2.2 Classification of Study Population

Classification of individuals into AD, MCI or cognitively healthy groups was made by utilising all available information, including medical history, memory complaints, information from family and neuropsychological test data. A consensus diagnosis was assigned for each participant by a clinical review panel, which included consideration of diagnostic criteria, DSM-IV diagnosis (Diagnostic and Statistical Manual of Mental Disorders, version IV) and ICD-10 diagnosis (International Statistical Classification of Disease) [454]. Where appropriate, ICD-10 dementia severity rating, NINCDS-ADRDA (National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer’s Disease and Related Disorders Association) AD diagnosis (probable or possible) and MCI classifications were applied. Diagnosis of individuals with MCI was made according to the internationally agreed criteria [455, 456] and these participants had also reported memory difficulties either personally or through an informant. The clinical review panel comprised a neurologist, a

geriatrician, neuropsychologists and geriatric psychiatrists to ensure consistency of diagnoses.

2.2.3 Study visit

All participants who met the inclusion criteria were scheduled for a study visit at one of three locations in Melbourne and one location in Perth. On the morning of the appointment, measurements of pulse, blood pressure, height, weight and abdominal circumference were obtained. Following these measurements, an 80mL blood sample (following an overnight fast) was collected from the antecubital vein using standard venipuncture technique. Following blood collection, participants were given a light breakfast and then required to complete a medical history questionnaire. A series of cognitive and mood assessments were then performed. Separate appointments were made for the purpose of undertaking brain scans. All procedures were repeated at the 18 month follow up assessment.

2.2.4 Demographics, medical history and medication use

A detailed questionnaire regarding demographic information, medical history, medication use and lifestyle (alcohol consumption and smoking) was completed by each participant. Demographic information included marital status, country of birth, primary language spoken, living arrangements, handedness, occupation (past/present), primary source of income, years of education, pet ownership and participation in community activities. Medical questions included current or past history of cardiovascular disease, hypertension, stroke, diabetes, visual defects (including colour deficiency), cancer, history of falls, thyroid/parathyroid disease, gastrointestinal complaints, arthritis, kidney disease, liver disease, joint replacement, epilepsy, serious head injury, neurological disorders, depression, anxiety and psychiatric disorders. Current medication use including name, dose and frequency was also recorded.

2.3 Blood Biomarkers

2.3.1 Sample collection [453]

At baseline and 18 month follow-up, 80mL of fasted blood was collected. From each participant, three different blood collection tubes were used:

- Lithium heparin
- EDTA with added prostaglandin E1 (Sapphire Biosciences, 33.3ng/mL)
- Serum tubes

27mL of the 80mL collected was sent to either PathWest Laboratory Medicine, Nedlands, Western Australia or Melbourne Health, Victoria for analysis of full blood count, erythrocyte sedimentation rate, urea and electrolytes, creatinine, androgen levels, globulin levels, sex hormone binding globulin (SHBG), glomerular filtration rate, calcium, liver function tests, serum lipids, homocysteine, serum and red cell folate, B12, glucose, insulin, caeruloplasmin, ferritin/transferrin/iron, oestradiol, luteinising hormone, thyroid function tests (thyroid stimulating hormone, free thyroxine, free triiodothyronine) and prostate specific antigen (males only). 0.5mL of whole blood was sent for apolipoprotein E (APOE) genotyping (see Section 2.3.2). The remaining blood was fractionated into the following components:

- Serum
- Plasma
- Platelets
- Red blood cells
- White blood cells (in distilled water)
- White blood cells in RNA later, (Ambion, Thermo Fisher Scientific, Waltham, MA, USA).

These blood fractions were stored in NUNC cryovials (Nalge Nunc International, Rochester, NY, USA) in liquid nitrogen in aliquots which ranged in size from 0.25mL to 1.0mL.

The EDTA plasma samples were used for lipid extraction and profiling.

2.3.2 DNA extraction and APOE genotyping

DNA was extracted from 200µL of whole blood using a Qiagen Blood Mini-Kit (Qiagen, Hilden, Germany). 200µL of whole blood was added to 200µL of buffer containing proteinase K, pulse vortexed for 15 seconds and then incubated for 10 minutes at 56°C. After centrifugation, 200µL of absolute ethanol was added to the tube. The mixture was then applied to the QIAamp Mini Spin Column and centrifuged for 1 minute at 6,000g. The filtrate was discarded and the QIAamp Mini Spin Column placed into a clean tube, followed by the addition of 500µL of buffer and centrifugation for 1 minute at 6,000g. Again the filtrate was discarded and the QIAamp Mini Spin Column placed into a clean tube with the addition of 500µL of buffer and centrifugation for 3 minutes at 20,000g. The column was placed into a clean tube with the addition of 200µL distilled water and incubated at room temperature for 1 minute before centrifugation at 6,000g for 1 minute. The extracted DNA was genotyped by the following published method [457]:

PCR reaction:

200nM primer Forward: 5' GCCTACAAATCGGAACTGGA 3'

Reverse: 5' ACCTGCTCCTTCACCTCGT 3'

1 unit Platinum Taq Polymerase (Invitrogen, Carlsbad, CA, USA)

1.5mM Magnesium chloride

200µM deoxyribonucleotide triphosphate

94°C 7 secs denaturation

(94°C for 30 secs, 55°C for 30 secs, 72°C for 30 secs) x 30 cycles

Sequencing with the forward primer was carried out with BDV3.1 on a 3130-xl genetic analyser (Applied Biosystems, Foster City, CA, USA).

The APOE genotype was then determined using the following fragments:

Table 2.1: APOE genotype and fragment size as determined by DNA sequencing.

| Genotype | Fragment Size (bp) |
|-------------------------|------------------------|
| $\epsilon 2/\epsilon 2$ | 91 + 83 |
| $\epsilon 2/\epsilon 3$ | 91 + 83 + 48 + 35 |
| $\epsilon 2/\epsilon 4$ | 91 + 83 + 72 + 48 + 35 |
| $\epsilon 3/\epsilon 3$ | 91 + 48 + 35 |
| $\epsilon 3/\epsilon 4$ | 91 + 72 + 48 + 35 |
| $\epsilon 4/\epsilon 4$ | 72 + 48 + 35 |

bp: base pairs.

APOE genotyping was undertaken and the protocol provided by Dr Simon Laws.

2.3.3 Sample extraction for lipid profiling

A modified Bligh and Dyer method was used [458].

900 μ L of ice-cold organic solvent mixture (chloroform/methanol 1:2 v/v) was added to 150 μ L of plasma (in duplicate) and vortexed for 15 seconds. The samples were then incubated in a vacuum (Desi-Vac Container) on ice inside a 4°C dark room with agitation at 180rpm for one hour. After incubation, 250 μ L of ice-cold water and 300 μ L of ice-cold chloroform were added to the sample to break the phase. The samples were then vortexed for 15 seconds and incubated on ice for one minute and then vortexed again for 15 seconds. The samples were subsequently centrifuged at 9,000rpm for 2 minutes at 4°C. The lower organic phase was transferred into a clean eppendorf tube and 500 μ L of ice cold chloroform added to the original tube for a second extraction. After vortexing for 15 seconds this sample was placed in a vacuum on ice inside a 4°C dark room with agitation at 180rpm for 30-60 minutes. Following centrifugation the lower organic phase was removed and added to the original extracted lower phase. The pooled organic phases from both extractions were dried in a Savant SpeedVac® (Thermo Fisher Scientific, Scoresby, Victoria, Australia). The dried, extracted lipids were stored at -80°C until required for analysis.

2.3.4 Sample preparation for shotgun lipidomics (direct-flow injection)

For analysis the dried samples were reconstituted in 100 μ L of chloroform/methanol (1:1 v/v). The sample was vortexed and centrifuged at 12,000 rpm for 2 minutes. Following centrifugation 80 μ L was added to 120 μ L of chloroform/methanol (1:1 v/v). The sample was again centrifuged at 12,000 for 2 minutes. 80 μ L was then transferred into a sample vial with 80 μ L of the working internal standard mix (shown in Table 2.2) and loaded on to the analyser. For the positive mode samples a further dilution was required. 10 μ L of the prepared sample (already spiked with internal standard) was removed from the vial and mixed in a separate vial with 80 μ L of chloroform/methanol (1:1 v/v).

The internal standard mix was prepared as outlined in Table 2.2.

Table 2.2: Internal Standards used to prepare mix for lipid profiling.

| Standard | [Stock mg/mL] | [Working μ g/mL] |
|---------------------|---------------|----------------------|
| DMPE | 0.2 | 5 |
| DMPG | 0.2 | 2 |
| DMPS | 0.5 | 2 |
| DMPA | 0.1 | 1 |
| diC ₈ PI | 0.25 | 2 |
| DMPC | 0.2 | 10 |
| GluCer | 0.1 | 2 |
| SM | 0.5 | 6 |
| C ₁₇ Cer | 0.1 | 2 |
| LysoPE | 0.1 | 0.5 |
| LysoPG | 0.1 | 0.5 |
| LysoPI | 0.1 | 0.5 |

DMPE: 1,2-dimyristoyl-glycero-3-phosphoethanolamine, DMPG: dimyristoyl-phosphatidylglycerol, DMPS: dimyristoyl-phosphatidylserine, DMPA: dimyristoyl-phosphatidic acid, diC₈PI: dioctanoyl-phosphatidylinositol, DMPC: dimyristoyl-phosphatidylcholine, GluCer: C8-Glucosylceramide, SM: C-12 sphingomyelin, C₁₇Cer: C₁₇-ceramide, LysoPE: Lysophosphatidylethanolamine, LysoPG: Lysophosphatidylglycerol, LysoPI: Lysophosphatidylinositol.

The diC₈-PI was supplied by Echelon Bioscience Inc, USA. All other lipids were supplied by Avanti Polar Lipids, Alabaster, Alabama, USA.

The working mix was stored wrapped in foil at -80°C

2.3.5 Shotgun lipid profiling by mass spectrometry

Electrospray ionisation mass spectrometry (ESI-MS) was performed on an Applied Biosystems (Foster City, CA, USA) high performance FlashQuant™ workstation that incorporates a 4000Q Trap Instrument (a triple quadrupole/linear ion trap liquid chromatography-tandem mass spectrometer (MS/MS) and a Shimadzu High Performance Liquid Chromatography (HPLC) (Shimadzu, Kyoto, KYT, Japan) autosampler to quantify individual polar lipids [370]. This method combines triple quadrupole scan modes with linear ion trap scans, therefore allowing multiple analyses. The sample was mixed with chloroform/methanol (1:1 v/v) in the autosampler and then injected into the mass spectrometer via loop injections with the mobile phase which was chloroform/methanol (1:1 v/v), at a flow rate of 0.25mL/min. In the ESI chamber, the analyte is volatilised by the tiny charged capillary and the sample propelled out of the capillary in the form of an aerosol. Nitrogen gas was used as the carrier gas. The sample then passes through to the second chamber (MS1) and then through the collision induced dissociation (CID) chamber where further fragmentation of the analyte occurs (see Figure 2.2). The MS2 chamber is a quadrupole ion trap, which acts as a mass-selective filter, trapping and sequentially ejecting the ions.

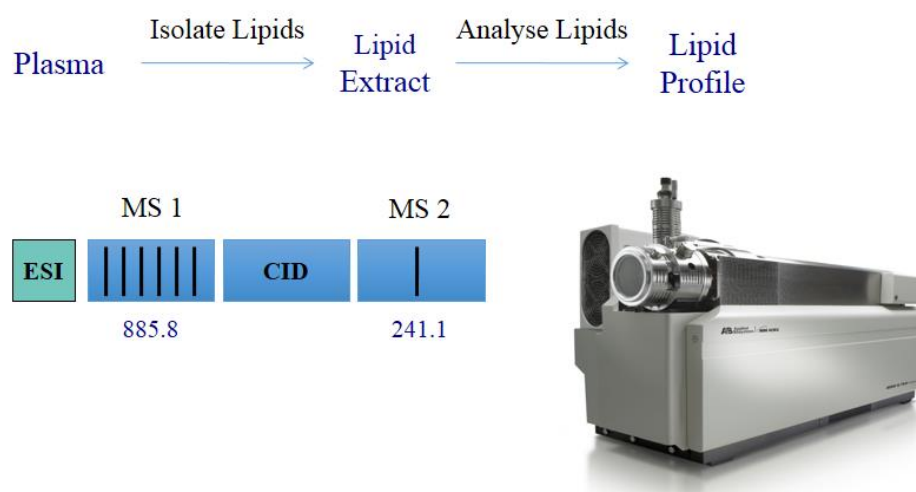


Figure 2.2: Schematic of method used for lipid profiling.

ESI: Electrospray ionisation, MS1: mass spectrometer chamber 1, CID: Collision induced dissociation chamber, MS2: Quadrupole ion trap.

In the example used in the figure, the parent lipid is detected at 885.8 m/z (mass to charge) and after passing through the CID a unique fragment at 241.1 m/z is detected. The resultant mass to charge ratio of 885.8/241.1 identifies this as phosphatidylinositol (PI 38:4), where the sn-1 and sn-2 side chains contain fatty acids with a sum of 38 carbons and 4 double bonds.

Each individual sample is spiked with a known quantity of commercially available internal standard as described in Table 2.2. Multiple reaction monitoring (MRM) transitions were used for quantitative analyses and were set up based on product and precursor ion analysis of various lipid head groups. The signal intensity of each MRM value was normalised to the signal intensity of the spiked internal standards and the sum of the individual lipid species before being corrected for molecular weight.

2.3.6 Phospholipid Transfer Protein (PLTP) Activity Assay

A commercially available PLTP activity assay kit was purchased from Roar Biomedical, Inc. Audubon Biomedical Centre, 3960 Broadway, New York, USA and was utilised according to the manufacturer's protocol. PLTP mediates the transfer of an exogenous fluorescent substrate in this assay. Briefly, frozen plasma samples were thawed on ice and then centrifuged at 12,000rpm for 10 minutes at 4°C. A 96 well black microtitre plate (Nalge Nunc International, Rochester, NY, USA) was placed on wet ice to cool. A grand mix was prepared containing 4200µL of 1 x assay buffer and 300µL of donor particle. 45µL of this grand mix was added to each well of the microtitre plate. 5µL of sample or an assay blank (5µL of 1 x assay buffer) was added to the wells. All plasma samples, control samples and blanks were run in duplicate. 50µL of the acceptor particle was then added using an 8 tip multichannel pipette. The plate was covered and incubated in a 37°C water bath for 20 minutes. Following incubation, the plate cover was removed and the fluorescence read at 37°C using an Enspire Multimode Plate Reader (Perkin Elmer, Waltham, MA, USA) with the excitation and emission wavelengths set at 465nm and 535nm respectively.

While the plate containing the samples, controls and blanks was incubating a standard curve was prepared for a concentration range of 0-80 picomoles. Six

tubes were labelled S0 to S5, each containing 1mL of isopropanol, with tube S5 containing an additional 1mL. 5µL of PLTP donor particle was added to tube S5 which was vortexed to adequately disperse the donor particle in the isopropanol. 1mL of S5 was transferred to S4, 1mL of S4 transferred to S3, 1mL of S3 transferred to S2, 1mL of S2 transferred to S1, with vortex mixing after each addition. S0 is the isopropanol blank. 100µL of each standard was pipetted, in duplicate, into two rows of a black microtitre plate (separate to the sample plate) and fluorescence read (Ex:465 nm; Em:535 nm) at 37°C. A standard curve was created by plotting the fluorescence intensity versus the picomole concentrations of the standards read. The fluorescence intensity units transferred during the incubation time were determined by subtracting the assay blank (assays performed without plasma, but using 5µL 1x assay buffer) from the fluorescence values of the samples and then applied to the standard curve to obtain a PLTP activity level.

2.3.7 Plasma beta amyloid

Plasma A β ₁₋₄₀ and A β ₁₋₄₂ were measured according to the manufacturer's protocol by a commercial kit (INNO_BIA plasma A β assay, Innogenetics, Inc., Gent, Belgium). Briefly, the thawed plasma samples were diluted 1:3 using the supplied buffer. Coated beads were vortexed and sonicated for 3 minutes, diluted 100 times in diluent and protected from light. The pre-made standards and controls were thawed at room temperature and loaded onto the plate after vortexing. All standards, controls and plasma samples were run in duplicate. For the measurement of A β ₁₋₄₀ and A β ₁₋₄₂, Conjugate 1A was diluted 100 times with Conjugate Diluent. A 96-well polyvinylidene fluoride microfilter plate was washed once with Wash Solution, followed by addition of 100µL of the diluted bead mix to each well. The contents of the plate were aspirated by vacuum and 25µL of diluted Conjugate 1A was then added to each well. 75µL of standards, controls and diluted plasma was then added to the appropriate well. Assay blanks used Diluent only in place of sample. The plate was covered with foil and incubated at 4°C overnight on an orbital plate shaker. Following overnight incubation, the Detection Conjugate was diluted 100x in Diluent and protected from light. The plate was aspirated by vacuum and washed three times with wash solution. 100µL of diluted Detection Conjugate was then added to each

well. The plate was covered with foil and incubated at room temperature for 1 hour on an orbital plate shaker. Following incubation, the wells were aspirated and washed 3 times with Wash Solution. 100µL of Reading Solution was added to each well and the plate covered with foil and placed on an orbital plate shaker for at least 2 minutes to a maximum of 5 minutes. The plates were read using a Luminex xMAP reader system (Bio-Plex 200 System, Bio-Rad). A minimum of 100 beads per region were read for each sample. Data analysis was performed using a 5-PL logarithmic equation built into the instrument software, which automatically calculated for dilution factors. The plasma A β ₁₋₄₂:A β ₁₋₄₀ ratio was calculated. The ratio is preferred to measurement of A β ₁₋₄₂ alone as A β ₁₋₄₀ is taken into account as an internal reference. Dr Alan Rembach provided the protocol and performed the plasma A β assay.

2.3.8 Plasma apolipoprotein E (apoE)

Plasma apoE was measured according to the manufacturer's protocol by a commercial kit (ApoE4/Pan ApoE ELISA, MBL co., Ltd., Woburn, USA). This sandwich enzyme-linked immunosorbent assay (ELISA) kit measures the amount of total ApoE using purified polyclonal antibodies against ApoE. Briefly, thawed plasma samples were diluted 1:500 using the Assay Diluent. Working concentrations of the standards were prepared from solutions of the provided calibrator, which was reconstituted 1:10 with the assay diluent. All standards, controls and plasma samples were run in duplicate. Standards or thawed, diluted plasma samples were loaded into microwell strips that were coated with anti-Human Pan-ApoE antibody. The plates were covered and incubated at 37°C for 1 hour. Following incubation the plate was washed four times with wash solution. 100µL of a peroxidase conjugated anti-ApoE polyclonal antibody was then added and incubated at 37°C for 1 hour. The plates were again washed before the addition of 100µL of the peroxide substrate, followed by further incubation at 37°C for 30 minutes. To terminate the enzyme reaction and stabilise the developed colour, 100µL of an acid solution was added to each well. The optical density of each well was measured at 450nm using a BMG microplate reader. Concentrations of Pan-ApoE were determined from a standard curve based on reference standards. The sensitivity of the assay is

8ng/mL. The ApoE assay was performed and the protocol provided by Dr Veer Gupta.

2.3.9 High Density Lipoprotein (HDL) cholesterol - subfractions

Plasma HDL cholesterol sub-fractions were analysed using the Lipoprint system (Quantimetrix, Redondo Beach, CA, USA), according to the manufacturer's protocol. Briefly, 25µL of plasma was added to loading buffer in glass gel tubes and left for 30 minutes to polymerise. Samples were then separated in the electrophoretic chamber at 3mA per tube for 1-1.5 hours. Following separation the gels were scanned and the sub-fraction band intensities were obtained using the supplied software. Ten subclasses of HDL were measured, which were divided into three groups; HDL large (subclasses 1-3), HDL intermediate (subclasses 4-6) and HDL small (subclasses 7-10). Dr Steve Pedrini and Dr Eugene Hone provided the protocol and performed the HDL subfraction analyses.

2.3.10 Plasma clusterin

Plasma clusterin was measured according to the manufacturer's protocol using a commercial kit (Quantikine® ELISA, Human Clusterin Immunoassay, R&D Systems, Inc., Minneapolis, USA). This sandwich immunoassay kit measures the amount of clusterin using a specific monoclonal antibody. Briefly, thawed plasma samples were diluted 1:2000 using Calibrator Diluent RD5T. Working concentrations of the clusterin standard were prepared from a series of dilutions with calibrator diluents to a range of 0-200ng/mL. All standards, controls and plasma samples were run in duplicate. 100µL of RD1-19 assay diluent was added to the wells of the microplate that were coated with anti-human clusterin antibody. 50µL of standard, control or sample were loaded into the appropriate wells. The plates were covered and incubated for two hours at room temperature on a horizontal orbital microplate shaker. Following incubation each well was aspirated and washed with wash buffer four times. 200µL of Clusterin Conjugate was then added and the covered plate incubated at room temperature for two hours on the shaker. The plates were again aspirated / washed four times before the addition of 200µL of substrate solution, followed by further incubation at room temperature for 30 minutes. To terminate the

enzyme reaction, 50µL of stop solution was added to each well. The optical density of each well was measured at 450nm using a BMG microplate reader. Concentrations of Clusterin were determined from a standard curve based on the supplied reference standards. The sensitivity of the assay is 0.189nM/mL. The Clusterin assay was performed by Dr Veer Gupta.

2.3.11 Apolipoprotein AI (ApoAI)

Plasma ApoAI was measured as part of the Rules Based Medicine (RBM, Austin, TX, USA) analysis and biomarker panel used as part of the AIBL study. Plasma samples were analysed with a 151-analyte multiplex Human Discovery Map® v1.0 panel. Only ApoAI was selected for use in investigating PLTP activity levels (see Chapter 5).

2.4 Neuroimaging Biomarkers

2.4.1 Image acquisition

2.4.1.1 Magnetic resonance imaging

Protocol provided by Dr Pierrick Bourgeat of CSIRO, Brisbane for the AIBL study: *“Participants underwent T1 weighted magnetic resonance imaging (MRI) using the ADNI 3-dimensional (3D) Magnetization Prepared Rapid Gradient Echo (MPRAGE) sequence, with a 1 x 1 mm in-plane resolution and 1.2 mm slice thickness, repetition time/echo time/inversion time = 2300 ms/2.98 ms/900 ms, flip angle 9°, and field of view 240 x 256 and 160 slices.”*

2.4.1.2 [¹¹C] Pittsburgh compound-B positron emission tomography

Protocol provided by Dr Victor Villemagne of Austin Health, Heidelberg, Victoria for the AIBL study: *“Participants underwent a [¹¹C] Pittsburgh compound-B positron emission tomography (PiB PET) scan at Austin Health Centre for PET, Melbourne or Western Australia PET and cyclotron service, Sir Charles Gairdner Hospital and Oceanic Medical Imaging, Perth. [¹¹C] PiB production was performed using a one-step [¹¹C] methyl triflate approach. The average radiochemical yield was 30% after a synthesis time of 45 minutes with a purity of > 98% and a specific activity of 30 ± 7.5 GBq/µmol. Each subject received an intravenous injection of ~370 MBq [¹¹C] PiB over one minute. PET images were*

acquired on a Phillips Allegro™ (Phillips Medical Systems, Eindhoven, The Netherlands) PET camera, 40-70 minutes post-PiB injection. A transmission scan was performed for attenuation correction. PET images were reconstructed using a 3D RAMLA algorithm using a voxel size of $2.0 \times 2.0 \times 2.0 \text{ mm}^3$ (x y z). Summed images for the 40-70 minute time frame were used.”

2.4.2 Image processing

2.4.2.1 Magnetic resonance imaging tissue segmentation

Protocol provided by Dr Pierrick Bourgeat of CSIRO, Brisbane for the AIBL study: *“For each subject, the T1 weighted images were classified into grey matter, white matter and cerebrospinal fluid using an implementation of the expectation maximization segmentation algorithm [459]. Briefly, using a mix of Gaussian distributions, this algorithm models image intensity histograms. The parameters of the Gaussian mixture are iteratively updated using an expectation maximisation approach. In order to provide initialization and spatial consistency to the expectation maximisation algorithm, an atlas and associated probability maps of grey matter, white matter and cerebrospinal fluid were spatially normalized to each individual scan, first through an affine registration, and then using a diffeomorphic non-rigid registration [460]. The regions of interest (ROI; hippocampus, anterior cingulate gyrus, posterior cingulate gyrus, temporal lobes and others) were propagated from the atlas to the T1W to extract the various regional volumes. To improve the robustness of the approach, it was repeated nine times, with different elderly atlases, resulting in nine segmentations and nine ROIs. The segmentations and the ROIs were voted to generate a consensus grey matter, white matter and cerebrospinal fluid segmentation as well as a consensus ROI for the investigated brain regions. The grey matter segmentation was then used to mask out non grey matter voxels from the ROI. Region of interest volumes were averaged over both hemispheres. The processing of MRIs was performed by Dr Pierrick Bourgeat of CSIRO, Brisbane. All volumetric measures of brain regions were corrected for intracranial volume (ICV) using the following equation:*

Adjusted volume = raw volume – b x (ICV – mean ICV); where b is the slope of the regression of an ROI on ICV.”

2.4.2.2 Pittsburgh compound-B positron emission tomography image analysis

Protocol provided by Dr Victor Villemagne of Austin Health, Heidelberg, Victoria for the AIBL study: *“Acquired [^{11}C] PiB PET images were processed using a semi-automatic ROI method. The semiautomatic method used a preset template of narrow cortical ROIs that were applied to the PiB scan via placement on the subject’s co-registered MRI by an operator who was blind to the subject’s clinical status. Co-registration of each individual’s MRI with the PET images was performed with SPM5 [Statistical Parametric Mapping 5, University College London, UK; 461]. The region of interest template was placed on the co-registered MRI and then transferred to the co-registered PET images. Follow-up PiB images were co-registered with the baseline PiB images and the same ROI templates were applied to baseline and follow-up scans. Standardised uptake values (SUV) for PiB were calculated for all brain regions examined and standard uptake value ratios (SUVR) were generated by dividing all regional SUV by the cerebellar cortex SUV. The cerebellar cortex was used as a reference region as it is relatively devoid of senile plaques and shows no PiB binding in controls or AD [462, 463]. Region of interest measurements were averaged across both hemispheres. Neocortical A β burden was expressed as the average SUVR of the area-weighted mean for the following ROIs: frontal (consisting of dorsolateral prefrontal, ventrolateral prefrontal and orbitofrontal regions), superior parietal, lateral temporal, lateral occipital and anterior and posterior cingulate. The image analysis of PiB PET scans was performed by Dr Victor Villemagne and colleagues of Austin Health, Heidelberg, Victoria.”*

2.5 Statistical Analysis

Statistical analysis of the data will be described in detail in the methods section of each chapter. All assumptions for statistical tests were considered and met before detailed analysis was undertaken. All analyses were performed using R version 3.0.2 (R Development Core team, 2009, Vienna, Austria). Statistical analyses were performed by Biostatisticians Dr James Doecke and Dr Samantha Burnham using statistical models previously used for the AIBL study.

Statistical methods utilised for this thesis are summarised in Table 2.3.

Table 2.3: Summary of statistical methods.

| Data to be analysed | Statistical Method Used |
|--|--|
| Descriptive statistics: Categorical variables Continuous variables MMSE | χ^2 ANOVA Kruskal-Wallace |
| Mean lipid levels differences | ANOVA |
| Significant differences in lipid levels between clinical classifications pre adjustment for co-variables | LIMMA |
| Significant differences in lipid levels between clinical classifications including adjustment for co-variables | GLM |
| Those lipids which are able to predict clinical classification with the greatest certainty. | Variable selection |
| Relationship between lipids selected by variable selection | Partitioning Around Medoid Clustering (Kaufman and Rousseeuw, 1990) and Pearson's correlations |
| Sensitivity/Specificity of predictive lipid panel | ROC curve |
| Longitudinal lipid data comparing baseline to 18 month data | LIMMA, GLM, Multiple regression analysis |
| Correlations between PLTP and other blood and neuroimaging biomarkers | Pearson's correlations |

χ^2 : Chi squared, ANOVA: Analysis of variance, MMSE: Mini-mental state examination, LIMMA: Linear Models for Microarray Data, GLM: Generalised Linear Model, ROC: Receiver Operating Characteristics.

Chapter 3

Plasma Lipid Profiles in AIBL Baseline Samples

Chapter 3 – Plasma Lipid Profiles In AIBL Baseline Samples

3.1 Introduction

A huge research effort in the past few decades has led to a greater understanding of the patho-physiology of Alzheimer's disease. However, despite these great advances many of the fundamental questions regarding AD remain unanswered. These questions include: what risk factors contribute to disease and can or how may these risk factors be modified to reduce disease susceptibility? Can we identify those at risk by investigating biomarkers that reveal increased susceptibility to disease or indicate accelerated disease progression? With the identification of lipid biomarkers, can we explain the aberrant biochemical pathways underlying these findings and use this information to design prevention strategies or implement therapeutic interventions? This study aims to contribute to the existing body of literature to help address these questions.

The nervous system has the second highest concentration of lipids, exceeded only by adipose tissue. Lipids in the nervous system also show enormous structural diversity and lipidomic studies will assist in understanding the mechanistic significance of lipid diversity and the biology and disease of neurons. Neurological disorders involve a complex interaction of proteins and lipids, which lead to the increased formation of protein deposits such as A β ; a major component of AD pathology [276].

A key development in the study of lipids and neurodegenerative disease has been in the advancement of technologies to determine profiles of lipids and their interacting partners. The improved technology in the field of mass spectrometry has enabled more specific characterisation of lipid species in larger cohorts. Three approaches are generally used: "Shotgun lipidomics" uses direct infusion of the sample with multiple scan modes to provide broad coverage of lipid species, which are quantitated with the use of internal standards and allows high sample throughput [381]. A more targeted approach combines liquid chromatography with multiple reaction monitoring, also allowing precise quantitation with the use of internal standards [450, 464]. Finally a non-targeted

approach combines liquid chromatography with high mass accuracy analysis to profile as many groups of lipids as possible, some sub-species of which may be unidentified [465]. The method is usually selected after consideration of factors such as type of sample, sample number to be analysed and scope of the project. The direct infusion method was chosen for this project due to the high sample number (>1000) and requirement for broad coverage of lipid species.

Lipidomic research has particular relevance to biomarker development and therefore this study, especially when there is great overlap with AD and other conditions involving altered lipid metabolism, such as cardiovascular disease and diabetes, which are themselves risk factors for AD [270]. Specific lipid classes and sub-classes are thought to play an important role in AD, where changes in brain lipid composition have been reported [273]. Lipids are now considered to play other important roles besides merely serving as stored energy or the matrix of membranes in which proteins are embedded. The majority of cellular lipids form the membrane bilayer, whose integrity and physical characteristics are vital for cellular processes. Membrane lipids are involved in vesicle fusion, regulation of ion fluxes and create specialised regions within cells that are involved with cellular communication. They are also very important signalling molecules via generation of second messengers created by the actions of a variety of intracellular enzymes. The diversity of lipids that participate in signalling is highlighted by the multiple integrated roles of eicosanoids, docosanoids, lysolipids, diacylglycerols, ceramides, phosphatidic acids and other cell signalling lipids. Neural lipids alter the geometric properties of membranes and act as ligands for proteins.

As mentioned above, specific lipid classes and sub-classes play an important role in AD, where changes in brain lipid composition have been reported involving all classes (cholesterol, sphingolipids, phospholipids and their metabolites) [273]. Changes in composition and metabolism of several phospholipids have been described in brain tissue from AD patients, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphoinositol (PI) and plasmalogens [270, 335, 336] and loss of neuronal membrane phospholipids and associated fatty acids is suggested to be an early event in

the development of AD [466-468]. Glycerophospholipids are a source of lipid mediators, including arachidonic acid (AA) and docosahexaenoic acid (DHA), which are released from membrane phospholipids by the action of phospholipases. Variations in phospholipid metabolizing enzymes, such as phospholipase A₂ (PLA₂), phospholipase C, phospholipase D and phosphoinositidases have also been reported in AD [342, 343]. Phospholipase D, which hydrolyses phosphatidylcholine to produce phosphatidic acid (PA) and choline has been implicated in AD pathology [343]. Post mortem studies of AD brains compared to control brains have shown elevated levels of phospholipid degradation products such as LysoPC, glycerophosphocholine, and glycerophosphoethanolamine [371, 469, 470]. Brain sphingolipid metabolism has also been shown to be altered in AD [291, 335, 471].

Despite many reports in the literature of animal and tissue studies linking lipids to AD, few studies have examined cerebrospinal fluid (CSF) or blood for changes in lipid species. Higher CSF glycerophosphocholine levels were reported in AD patients compared to age-matched controls [472]. Some studies have reported a lower LysoPC to PC ratio in the CSF of AD patients compared to normal controls [371], while a more recent study reported a higher ratio of LysoPC to PC in AD [375]. These studies were cross-sectional and had low participant numbers (n=19/19 healthy controls/AD and n=70/40/29 healthy controls/MCI/AD respectively). Several reports have examined the association of levels of plasma plasmalogens. A pilot study involving 40 participants evaluated the relationship between serum total ethanolamine plasmalogen levels and Alzheimer Disease Assessment Scale–Cognitive (ADAS-Cog) scores. This study found that ADAS-Cog scores increased significantly in AD patients with circulating plasmalogen levels that were less than 75% of that of the age-matched controls at baseline [357]. Further, serum plasmalogen ethanolamine levels were measured in five independent populations comprising clinically demented and non-demented subjects. This study showed reduced serum plasmalogen levels at all stages of dementia [341].

Serum sphingomyelin (SM) and ceramides have been measured in several studies as part of the longitudinal Women's Health and Aging study II of

cognitive impairment (WHASII) [163, 422, 423, 473, 474]. These studies showed that high ceramides were associated with memory impairment and hippocampal volume loss and associated with incident dementia when measured as an outcome. The studies found lower levels of serum ceramides and SM associated with memory impairment in the cross-sectional data but higher levels in the longitudinal data, suggesting that serum ceramides and SM vary according to time of onset of memory impairment. However, the number of AD cases in this study was low, limited to women and the samples were collected from individuals in a non-fasted state; a factor that can significantly influence plasma lipid profiles.

A recent probe into the plasma lipidome of individuals with metabolic syndrome and obesity; risk factors for AD, showed various changes including increased LysoPC, decreased ether PLs and significant increases in some PC and PE species. Additionally several ceramide species were increased [340]. Therefore the importance of having the neuroimaging data collected as part of the study utilised in this thesis cannot be overstated as the SUVR shows that abnormal lipid profiles are likely to be AD related and facilitates delineation of abnormal lipid profiles associated other age-related co-morbidities that often exist with AD.

Several reasons can be suggested for the discrepancies reported between various studies. These include study design, disease classification, analytical factors, sample type and sample number. However the weight of evidence is compelling enough to suggest that the delicate equilibrium of phospholipid and sphingolipid metabolism is disturbed in AD.

The separation of the brain from the periphery presents a particular challenge when studying the relevance of plasma lipids to neurological disease as some lipids are transported across the blood brain barrier, while others appear to be excluded. This separation can be compromised by age-related changes in the integrity of the blood brain barrier, thereby altering lipid and A β kinetics. Chapter 1 described in detail how changes in membrane composition in the brain can affect A β production and promote aggregation and toxicity. Chapter 1 also described how peripheral lipids and lipoproteins can influence pathological

processes in the AD brain. High plasma cholesterol has been associated with increased delivery of peripheral lipoprotein associated A β [475]. Additionally, peripheral lipids, influenced by diet and metabolic hormones will have an effect on brain biochemistry and vasculature and on increased risk of developing cardiovascular and metabolic disease with associated abnormal plasma lipid profiles; all risk factors for AD.

The results from previous studies provide enough optimism to warrant further investigation of lipid profiles in the highly characterised AIBL cohort. Many previous studies have targeted specific lipid groups, have not had the benefit of neuroimaging biomarkers such as MRI and PiB-PET as indicators of AD pathology, and have been cross sectional in design. The AIBL cohort has neuroimaging and blood biomarker data and is a longitudinal prospective study. In addition, the cohort is made up of a large number ($n=1112$) of well characterised participants, the blood has been collected in a fasted state and is enriched with those carrying the APOE ϵ 4 allele; a genetic risk factor for AD. In contrast to other studies, a broad range of lipid species will be measured in plasma to facilitate identification of a sensitive and specific biomarker panel for AD. To my knowledge, a study of a large, highly characterised cohort with biomarker and lifestyle data has not been pursued with respect to biomarker discovery using such a broad range of lipids.

3.2 Aims

This chapter aims to investigate plasma lipid profiles via a direct infusion with a multiple scans technique in the highly characterised Australian Imaging, Biomarkers and Lifestyle study of ageing (AIBL). Differences in lipid profiles between clinical classifications will be compared to supplementary data collected as part of this study, including brain amyloid burden and APOE ϵ 4 allele carriage. The results will also be assessed for their ability to distinguish AD patients from cognitively normal individuals and those with mild cognitive impairment (MCI).

3.3 Methods

Details of the study population and the methods for sample collection, lipid extraction, and analysis by electrospray ionisation tandem mass spectrometry (ESI-MS/MS) have been described in detail in Chapter 2, Sections 2.2 and 2.3. A total of 189 lipids were measured including sub-species of PE, LysoPE, PC, Lyso PC, PI, LysoPI, PA, PG, PS, ether PLs, SM and ceramides. This chapter describes cross sectional results where baseline samples only were used.

The following figure provides a detailed breakdown of the clinical classifications of participants in the AIBL cohort at baseline.

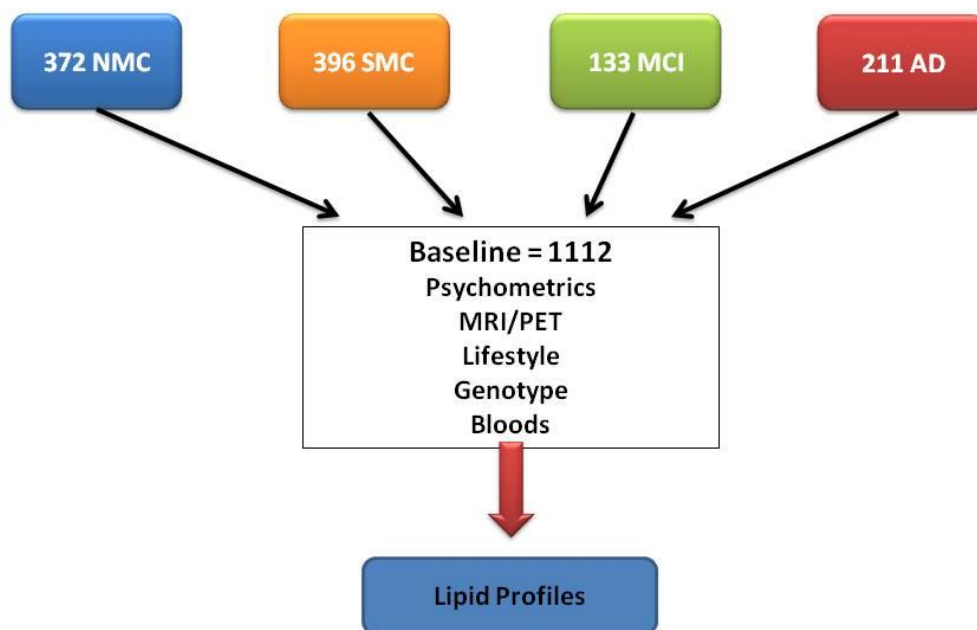


Figure 3.1: The AIBL cohort at baseline. There were 1,112 baseline samples. These were classified as 211 AD, 133 MCI and 768 healthy controls. Healthy controls can be further divided into 372 non-memory complainers (NMC) + 396 subjective memory complainers (SMC). Psychometric testing, neuroimaging, lifestyle data collection and APOEε4 genotyping were carried out on the participants and the blood collected was used for lipid profiling.

Schematic adapted from an image provided by the AIBL Study Scientific Management Committee.

3.3.1 Statistical analysis

To assess the study population demographics between the healthy controls, MCI and AD groups, χ^2 analyses was used to test the effect of age, gender and APOE ϵ 4 allele status. A Kruskal-Wallis formula was applied to MMSE scores. Marginalised means and p-values were obtained for the lipid levels using a t-test for two classifications (i.e. HC and AD or HC and MCI) or ANOVA for three classifications (HC, MCI and AD). Additionally ANOVA was used to test inter group differences in SUVR and hippocampal volume.

The heatmaps were generated using Matlab version R2006a (Massachusetts, USA) and R version 3.0.2 was used to calculate the p-values. LIMMA (Linear Models for Microarray Data) analysis was used to obtain a Q value (false discovery adjusted value). The fold lipid change was calculated using unadjusted raw expression values to provide a relative lipid difference between clinical classifications. A generalised linear model (GLM) was used for those lipids selected by LIMMA which adjusted for age, gender, APOE ϵ 4 allele carrier status and site of collection (Melbourne or Perth). This GLM determines the pairwise differences in lipid species between the healthy control, MCI and AD groups. Using the Bonferroni multiple adjustment approach, the α value for p-value comparison was 2.65E-4 (0.05/189), where 189 is the number of lipid species.

Variable selection and prediction of classification analyses were conducted to choose a short list of lipid biomarkers capable of predicting a diagnosis of AD. These analyses utilised all 189 lipid species and not those identified by LIMMA and GLM. The variable selection pipeline utilised creates an importance ranking for each lipid species by applying four different statistical methods; random forest, boosted trees, regression trees and LIMMA, while using clinical classification as a binary outcome factor. The statistical pipeline was run 1000 times and each time a lipid was selected, a frequency score was assigned if it was chosen from all four or three out of four statistical methods. Those lipids with the highest frequencies of selection were clustered using Partitioning Around Medoids (Kaufman and Rousseeuw, 1990) to find a central lipid and cluster other correlated lipids (from the variable selection) around it: This

correlates lipids that are related to each other without consideration of clinical classification. A CLARA (Clustering for Large Applications) algorithm was applied to create a clusplot of correlated lipids. Standard Pearson's correlations were applied to the lipids chosen in the variable selection pathway and these lipids and R-values were subjected to further statistical assessment using a stepwise generalised linear model (GLM) to reduce the number of lipids in the final predictive model. This stepwise GLM algorithm removes lipids from the predictive model until the lipids left are those giving the best Akaike Information Criterion (AIC) report, which is a measure of the relative quality of a statistical model for a given set of data and therefore provides a means for selecting the best model. Once lipids were selected using the stepwise GLM, a reduced set of lipids were run using the class prediction statistical pathway. This pathway includes taking a random sample of the data (comprising 70%) as the training set, performing the GLM model, and obtaining predicted probabilities for each participant for the model fit. These predicted probabilities are then used to predict the remaining 30% of the data as a test set. This class prediction pathway was iterated through 100 times and then repeated after each individual lipid removal following assessment of model prediction accuracy. A final panel is selected that contains the least number of lipids with maximal sensitivity and specificity for predicting disease classification. Figure 3.2 summarises the statistical analyses described and used for the study of baseline lipid profiles in this chapter.

Chapter 3: Plasma Lipid Profiles in AIBL Baseline Samples

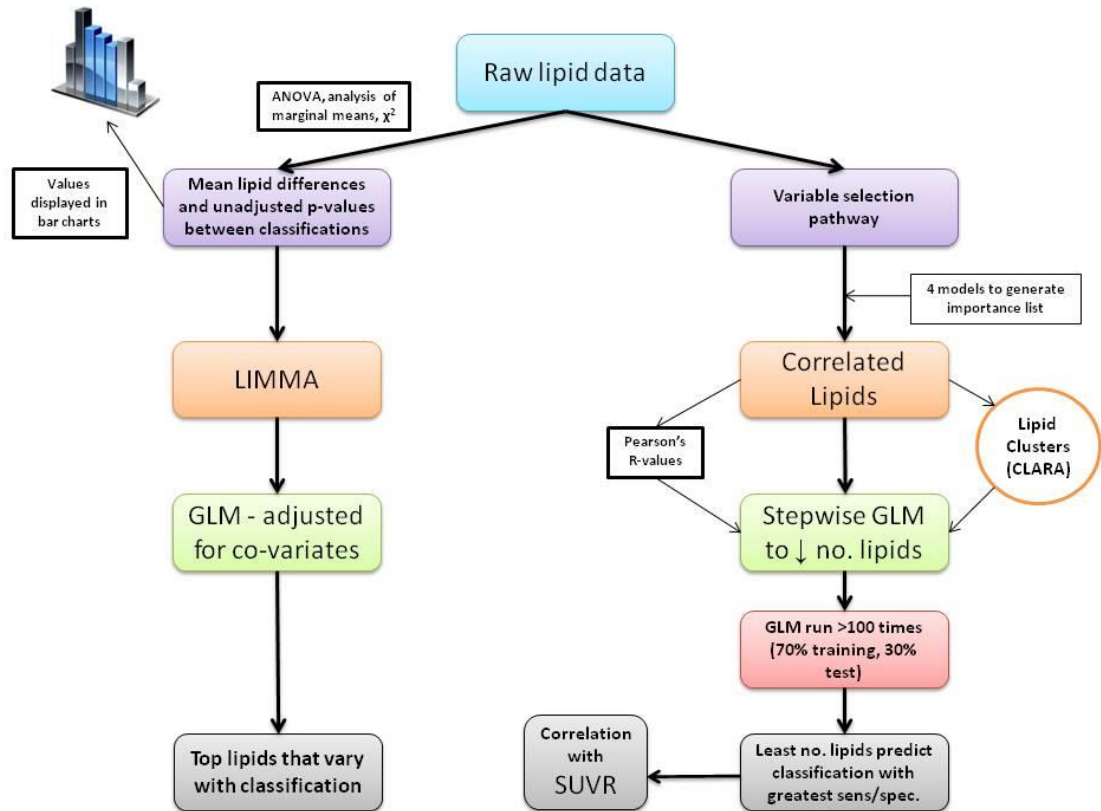


Figure 3.2: Summary of statistical methods used for analysis of AIBL baseline samples.

ANOVA: Analysis Of Variance, LIMMA: Linear Models for Microarray Data, GLM: Generalised Linear Model, CLARA: Clustering for Large Applications, sens/spec: Sensitivity and Specificity, SUVR: Standardised uptake value ratio.

The R statistical software environment, version 3.0.2, was used for all statistical analysis (R Development Core Team, 2009).

3.4 Results

The following table describes the demographics of the baseline cohort divided according to clinical classification.

Table 3.1: AIBL baseline demographics.

| Characteristic | HC | MCI | AD | p-value |
|------------------------------------|---------------------|---------------------|---------------------|---------|
| Number (n) | 733 | 125 | 204 | |
| Age, years (Mean \pm SD) | 70.59 (\pm 7.02) | 76.09 (\pm 7.58) | 78.83 (\pm 8.52) | <0.0001 |
| Gender (F/M) | 425/308 | 71/54 | 127/77 | 0.495 |
| ApoE4 (-ve/+ve) | 531/202 | 62/63 | 76/128 | <0.0001 |
| MMSE, score (Mean \pm SD) | 29 (\pm 1.2) | 26 (\pm 2.65) | 20 (\pm 5.25) | <0.0001 |
| SUVr (n) | 163 | 52 | 49 | |
| SUVr (Mean \pm SD) | 1.42 (\pm 0.4) | 1.88 (\pm 0.58) | 2.33 (\pm 0.37) | <0.0001 |
| Hippocampal Volume (n) | 152 | 43 | 36 | |
| Hippocampal Volume (Mean \pm SD) | 3.15 (\pm 0.33) | 2.93 (\pm 0.38) | 2.80 (\pm 0.49) | <0.0001 |

HC: Healthy Controls, MCI: Mild Cognitive Impairment, AD: Alzheimer's Disease, F: Female, M: Male, APOE ϵ 4: negative or positive for the APOE ϵ 4 allele, MMSE: Mini Mental State Examination, SUVr: Standard Uptake Value Ratio. Hippocampal volume is measured mL. The characteristics were composed using analysis of variance (ANOVA) to calculate differences for the continuous variables and χ^2 for categorical variables. A Kruskal-Wallis formula was applied to the MMSE scores.

The cohort consisted of 733 healthy controls (HC), 125 MCI and 204 AD participants. Whilst the number of men and women in each classification did not significantly differ (p-value = 0.495), significant differences in mean age were seen in the AD group (p=<0.0001). The AD group are older, which is expected as AD is an age related disease. It is worthy of note that there are more APOE ϵ 4 +ve individuals in the AIBL cohort compared to epidemiological allele frequencies reported in other studies (see Table 1.3, Chapter 1). This is because the AIBL cohort was enriched with APOE ϵ 4 allele carriers. As expected, the mean MMSE score is lower in the MCI and AD groups compared to healthy controls and cerebral amyloid burden as depicted by mean SUVr is significantly higher in the MCI and AD groups compared to healthy controls and significantly higher in the AD group compared to MCI. Predictably, hippocampal volume is reduced in MCI and AD compared to the HC group.

Of the original 1112 baseline samples a total of 1062 plasma samples were analysed to profile the lipids by direct flow injection mass spectrometry (ESI MS/MS), using the ABSciex QTrap 4000 instrument and multi reaction monitoring (MRM) which fragments the headgroups from their precursor lipids.

Chapter 3: Plasma Lipid Profiles in AIBL Baseline Samples

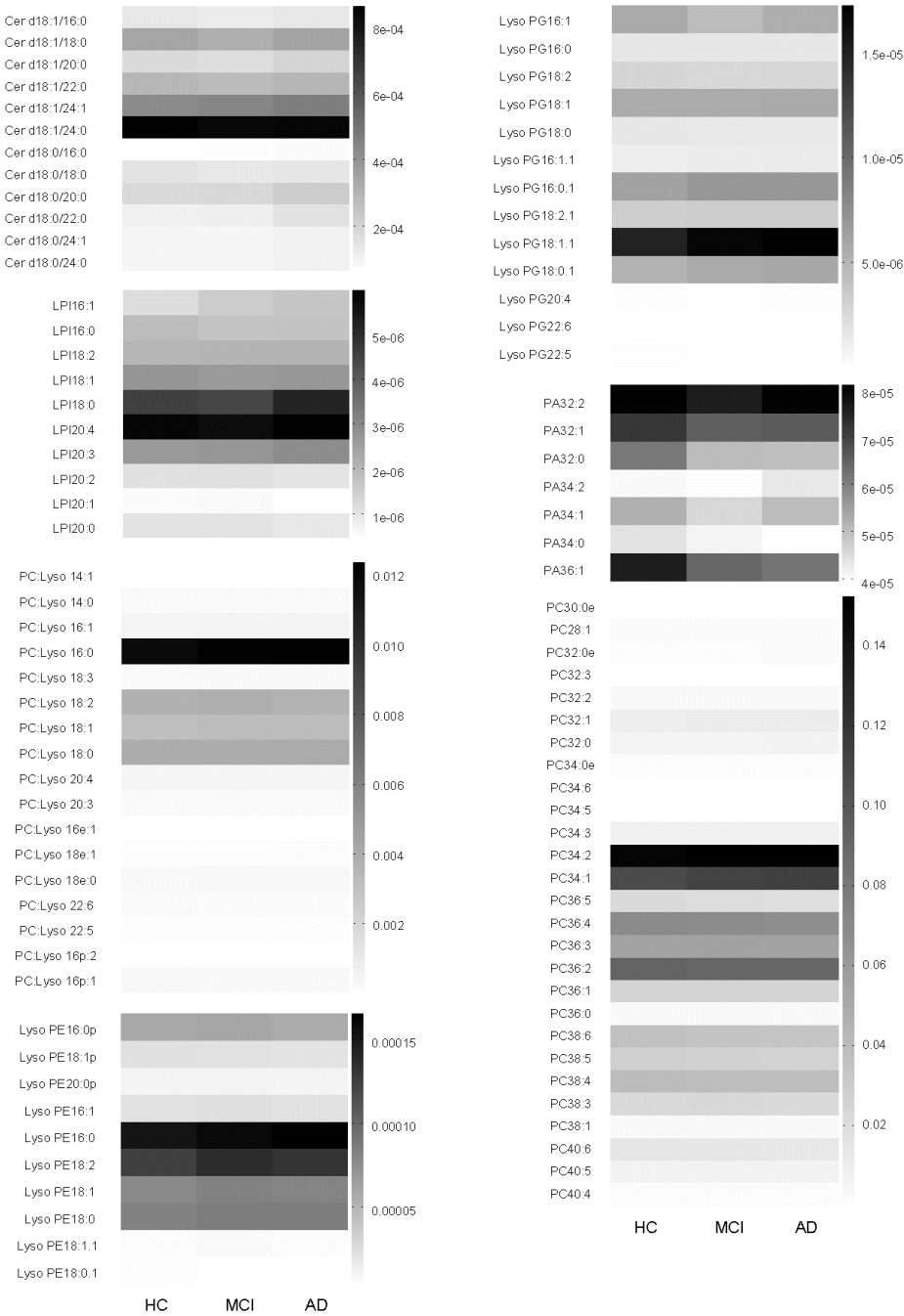
Method details are described in Chapter 2, Sections 2.3.3-2.3.5. The missing samples are accounted for by failure to extract lipids at the extraction stage or failure of analysis at the mass spectrometry stage due to poor sample quality. Table 3.2 lists the 189 lipid species that were measured.

| Lipid | | | | | | | | | | | | |
|---------|-------------|--------|-----------------|-------------|--------|--|--------|-------------|--------|--------|-----------|----------------|
| PE | LysoPE | PC | PCp or ether | LysoPC | PI | LysoPI | PG | LysoPG | PS | PA | SM | Cer |
| PE34.2 | LysoPE16.1 | PC28.1 | PCp32.0 | LysoPC14.1 | PI32.2 | LysoPI16.1 | PG34.2 | LysoPG16.1 | PS36.2 | PA32.2 | SM18.16.1 | Cer.d18.1.16.0 |
| PE34.1 | LysoPE16.0 | PC32.3 | PCp34.2..34.3e | LysoPC14.0 | PI32.1 | LysoPI16.0 | PG34.1 | LysoPG16.0 | PS36.1 | PA32.1 | SM18.16.0 | Cer.d18.1.18.0 |
| PE36.5 | LysoPE18.2 | PC32.2 | PCp34.1..34.2e | LysoPC16.1 | PI34.3 | LysoPI18.2 | PG34.0 | LysoPG18.2 | PS38.6 | PA32.0 | SM18.18.1 | Cer.d18.1.20.0 |
| PE36.4 | LysoPE18.1 | PC32.1 | PCp34.0..34.1e | LysoPC16.0 | PI34.2 | LysoPI18.1 | PG36.4 | LysoPG18.1 | PS38.5 | PA34.2 | SM18.18.0 | Cer.d18.1.22.0 |
| PE36.3 | LysoPE18.0 | PC32.0 | PCp36.4/etherPC | LysoPC18.3 | PI34.1 | LysoPI18.0 | PG36.3 | LysoPG18.0 | PS38.4 | PA34.1 | SM18.20.1 | Cer.d18.1.24.1 |
| PE36.2 | LysoPE20.1 | PC34.6 | PCp36.3/etherPC | LysoPC18.2 | PI34.0 | LysoPI20.4 | | LysoPG16.1f | PS40.6 | PA34.0 | SM18.20.0 | Cer.d18.1.24.0 |
| PE36.1 | LysoPE20.0 | PC34.5 | PCp36.2/etherPC | LysoPC18.1 | PI36.4 | LysoPI20.3 | | LysoPG16.0f | PS40.5 | PA36.1 | SM18.22.1 | Cer.d18.0.16.0 |
| PE36.0 | LysoPEp16.0 | PC34.3 | PCp36.1/etherPC | LysoPC18.0 | PI36.3 | LysoPI20.2 | | LysoPG18.2f | | | SM18.22.0 | Cer.d18.0.18.0 |
| PE38.7 | LysoPEp18.1 | PC34.2 | PCp36.0/etherPC | LysoPC20.4 | PI36.2 | LysoPI20.1 | | LysoPG18.1f | | | SM18.24.1 | Cer.d18.0.20.0 |
| PE38.6 | LysoPEp20.0 | PC34.1 | PCp38.5..38.6e | LysoPC20.3 | PI36.1 | LysoPI20.0 | | LysoPG18.0f | | | SM18.24.0 | Cer.d18.0.22.0 |
| PE38.5 | | PC36.5 | PCp38.4..38.5e | LysoPC16e.1 | PI38.6 | | | LysoPG20.4 | | | | Cer.d18.0.24.1 |
| PE38.4 | | PC36.4 | PCp38.3..38.4e | LysoPC18e.1 | PI38.5 | | | LysoPG22.6 | | | | Cer.d18.0.24.0 |
| PE40.6 | | PC36.3 | PCp38.2..38.3e | LysoPC18e.0 | PI38.4 | | | LysoPG22.5 | | | | |
| PE40.5 | | PC36.2 | PCp38.1..38.2e | LysoPC22.6 | PI38.3 | Table 3.2: List of plasma lipid species measured in the AIBL baseline samples. PE: Phosphatidylethanolamine, PC: Phosphatidylcholine, PI: Phosphatidylinositol, PG: Phosphatidylglycerol, PS: Phosphatidylserine, PA: Phosphatidic acid, SM: Sphingomyelin, Cer: Ceramide. p: vinyl ether bond or plasmalogen, e: ether linkage – can be plasmaynyl (vinyl ether) or plasmenyl (alkyl ether)- (see Figure 1.5), etherPC: where fatty acyl groups cannot be determined, f: An alternate mass is also detected in Q3 of the mass spectrometer Numbers represent carbon atoms in acyl chain and number of unsaturated/double bonds. For example PE38.5 is phosphatidylethanolamine with two acyl chains totalling 38 carbons and 5 unsaturated bonds. | | | | | | |
| PE40.4 | | PC36.1 | PCp40.6..40.7e | LysoPC22.5 | PI38.2 | | | | | | | |
| PE34p.3 | | PC36.0 | PCp40.5..40.6e | LysoPCp16.2 | PI40.6 | | | | | | | |
| PE34p.2 | | PC38.6 | PCp40.4..40.5e | LysoPCp16.1 | PI40.5 | | | | | | | |
| PE36p.4 | | PC38.5 | PCp40.3..40.4e | | PI40.4 | | | | | | | |
| PE36p.3 | | PC38.4 | PCp40.2..40.3e | | PI40.3 | | | | | | | |
| PE36p.2 | | PC38.3 | PCp40.1..40.2e | | PI40.2 | | | | | | | |
| PE36p.1 | | PC38.1 | PC30.0e | | | | | | | | | |
| PE38p.6 | | PC40.6 | PC32.0e | | | | | | | | | |
| PE38p.5 | | PC40.5 | PC34.0e | | | | | | | | | |
| PE38p.1 | | PC40.4 | | | | | | | | | | |
| PE40p.7 | | | | | | | | | | | | |
| PE40p.6 | | | | | | | | | | | | |
| PE40p.5 | | | | | | | | | | | | |
| PE40p.4 | | | | | | | | | | | | |
| PE42p.6 | | | | | | | | | | | | |
| PE42p.5 | | | | | | | | | | | | |
| PE42p.4 | | | | | | | | | | | | |

Chapter 3: Plasma Lipid Profiles in AIBL baseline samples

The mass spectrometer generates electrical signals of each of the lipid species detected. The signal intensities of the individual lipids are normalised against the signal intensities of the lipid internal standards for each respective lipid group, corrected for their respective molecular weights before normalisation to the sum of all lipid species measured. The lipid levels are expressed as molar fractions. A heatmap of the baseline results was plotted in Figure 3.3 to enable visualisation of differences in lipid species between the clinical classification groups.

Chapter 3: Plasma Lipid Profiles in AIBL baseline samples



Chapter 3: Plasma Lipid Profiles in AIBL baseline samples

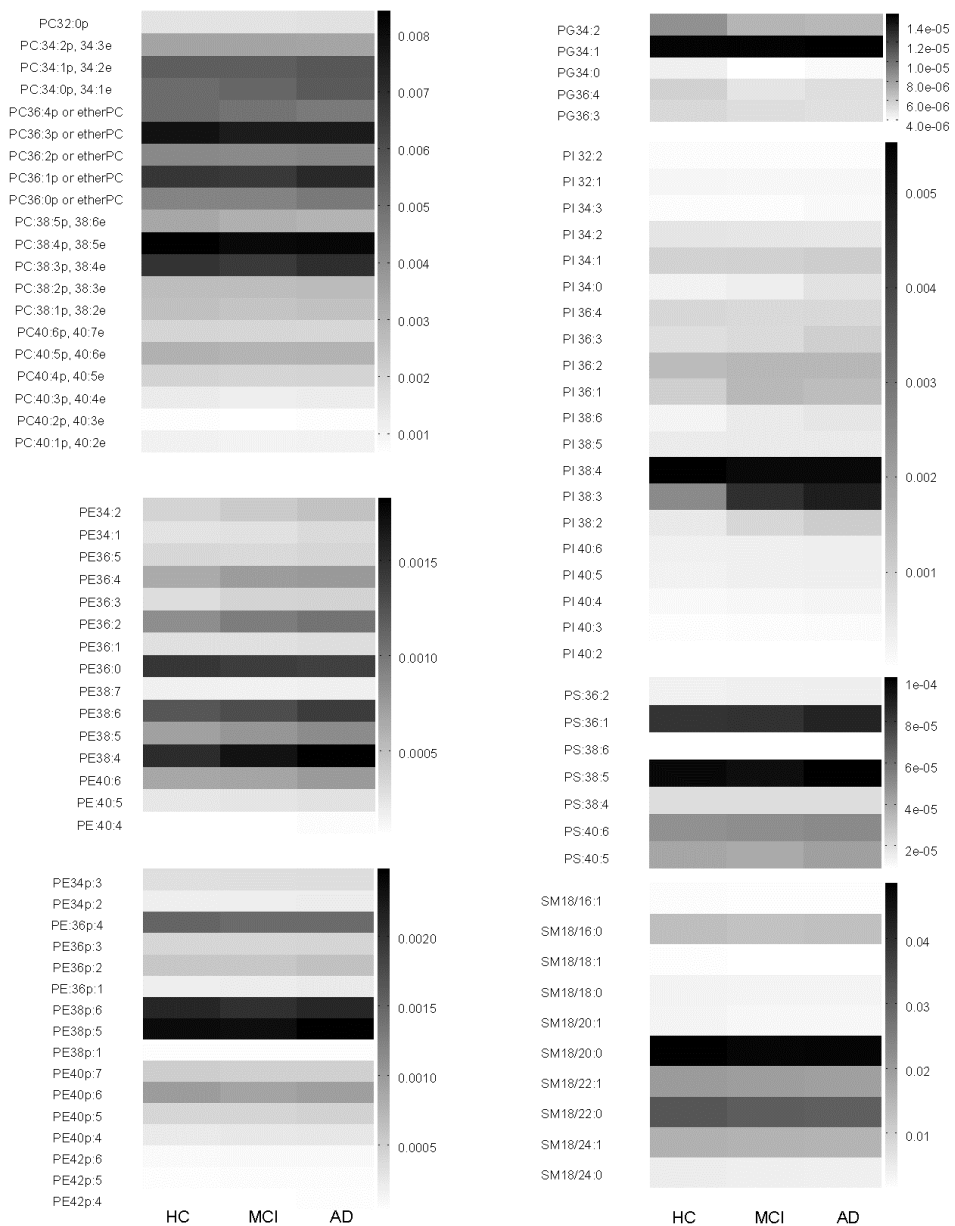
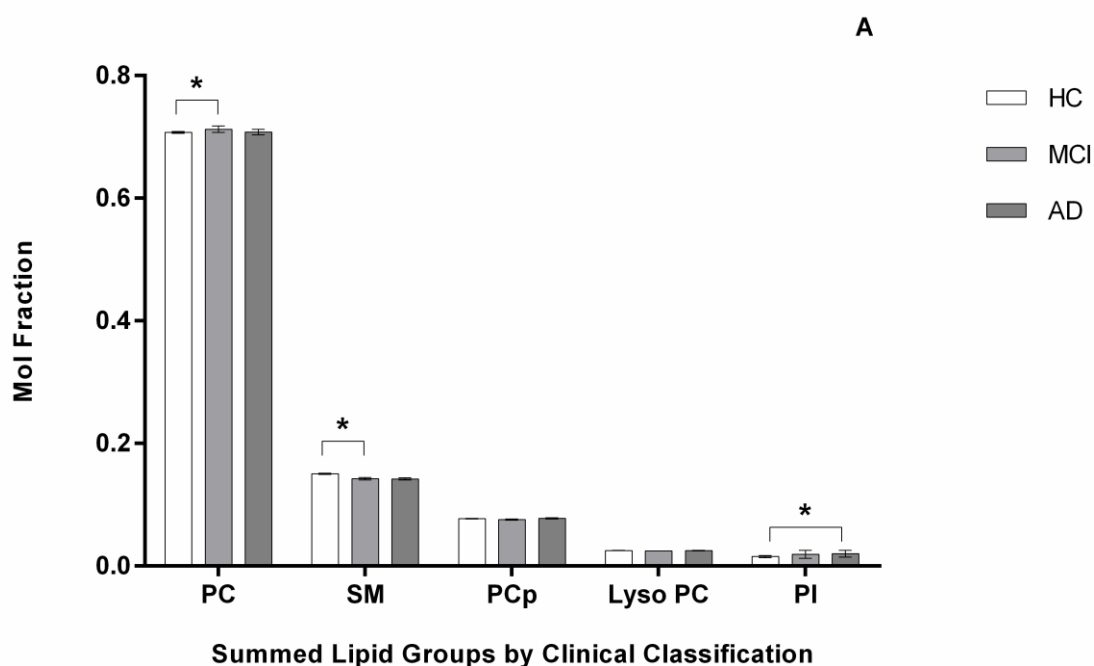


Figure 3.3: Heatmaps of lipid profiles of 1062 samples between clinical classification groups.

The scale on right side of the charts displays the mean lipid levels expressed as molar fractions. HC: Healthy Control, MCI: Mild Cognitive Impairment, AD: Alzheimer's Disease, PA: phosphatidic acid, PE: phosphatidylethanolamine, PC: phosphatidylcholine, PS: phosphatidylserine, PI: phosphatidylinositol, SM: sphingomyelin, Cer: ceramide. White colour reflects low levels, while black colour reflects higher levels.

Chapter 3: Plasma Lipid Profiles in AIBL baseline samples

The heatmaps depicted in Figure 3.3 showed all the individual lipid species measured in the HC, MCI and AD groups. The heatmaps show that many of the PE and PEp species were higher in AD compared to the healthy control group. Ceramides appear to be higher and SMs lower in the AD group compared to healthy controls. The abundance of the various lipid species in plasma is quite different with PC and SM being present in the greatest amounts. To allow this data to be displayed graphically three separate bar charts, to account for abundance differences, are depicted in Figure 3.4, showing the non-adjusted marginalised means of the summed lipid species groups and their significance, as represented by ANOVA generated p-values, with respect to clinical classification.



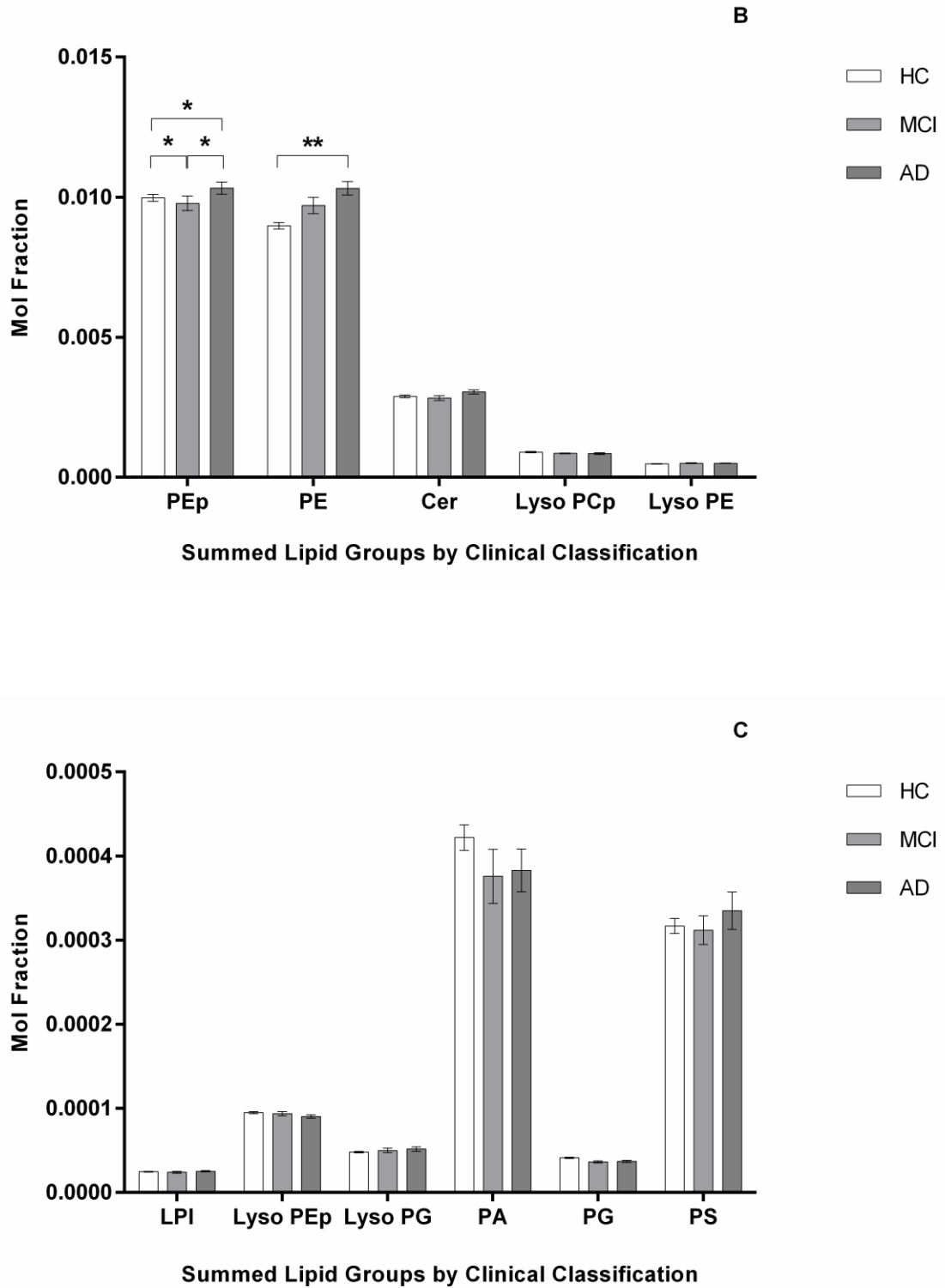
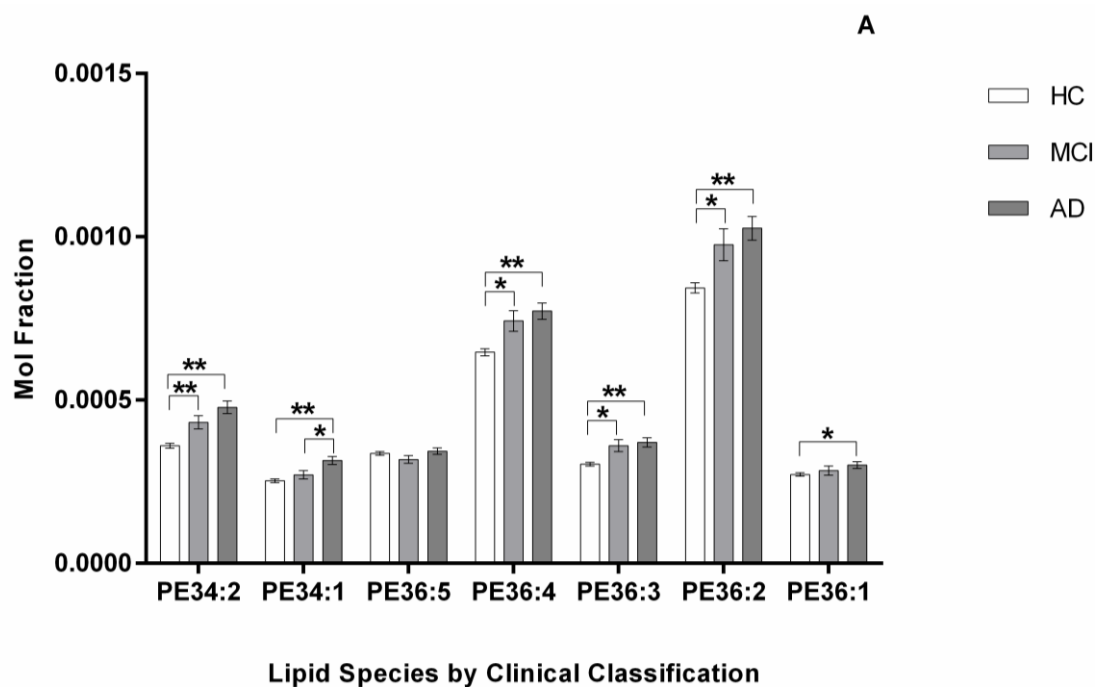


Figure 3.4: Grouped mean lipid levels differences between clinical classifications. A: PC: Phosphatidylcholine, SM: Sphingomyelin, PCp: Choline plasmalogen, LysoPC, PI: Phosphatidylinositol. **B:** PEP: Ethanolamine plasmalogen, PE: Phosphatidylethanolamine, Cer: Ceramide, LysoPCp, LysoPE. **C:** LPI: LysoPI, LysoPEp, LysoPG, PA: Phosphatidic acid, PG: Phosphatidylglycerol, PS: Phosphatidylserine.

Mean \pm SEM. Analysis of Variance (ANOVA): * $p < 0.05$ ** $p < 0.001$

When lipids are grouped into their respective classes, significant changes were observed for total PI, total PE, total PC and total SM. Whilst Figure 3.4 shows the mean total levels for each lipid group, it does not provide information in relation to sub-species and any significant differences in these lipid sub-species between clinical classifications. ANOVA analysis of the individual lipid groups are represented in the following graphs showing how these species levels differ between clinical classifications. A summary of these results is presented in Table 3.3 immediately following the graphs in Figures 3.5 to 3.16.



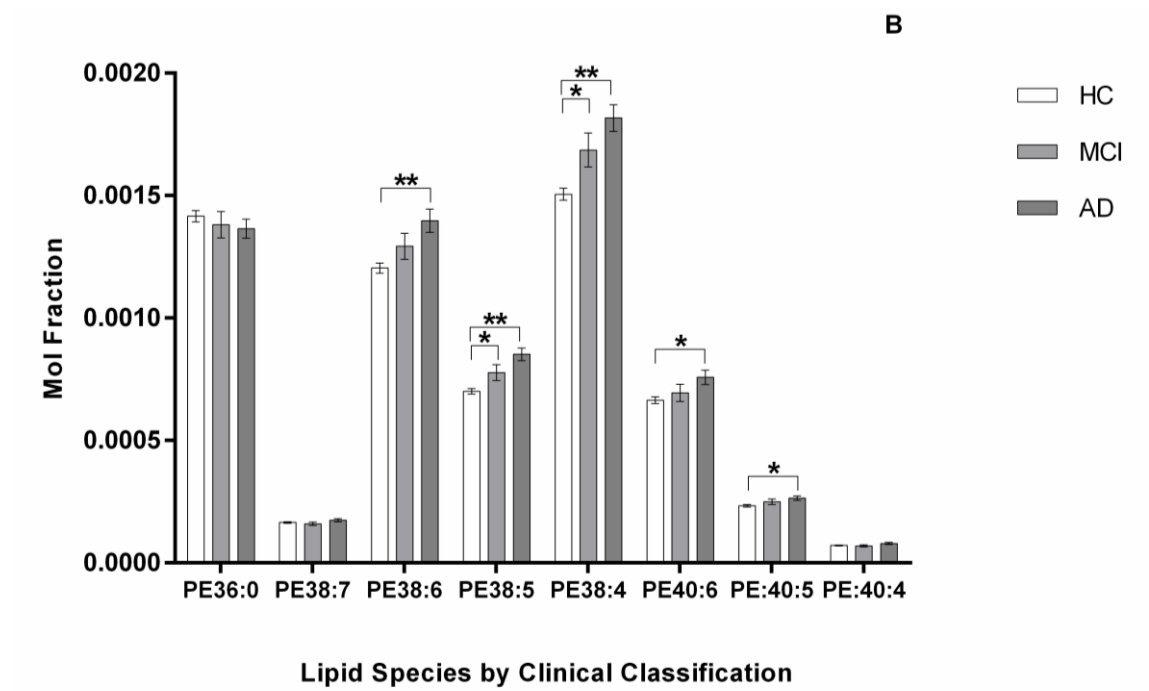
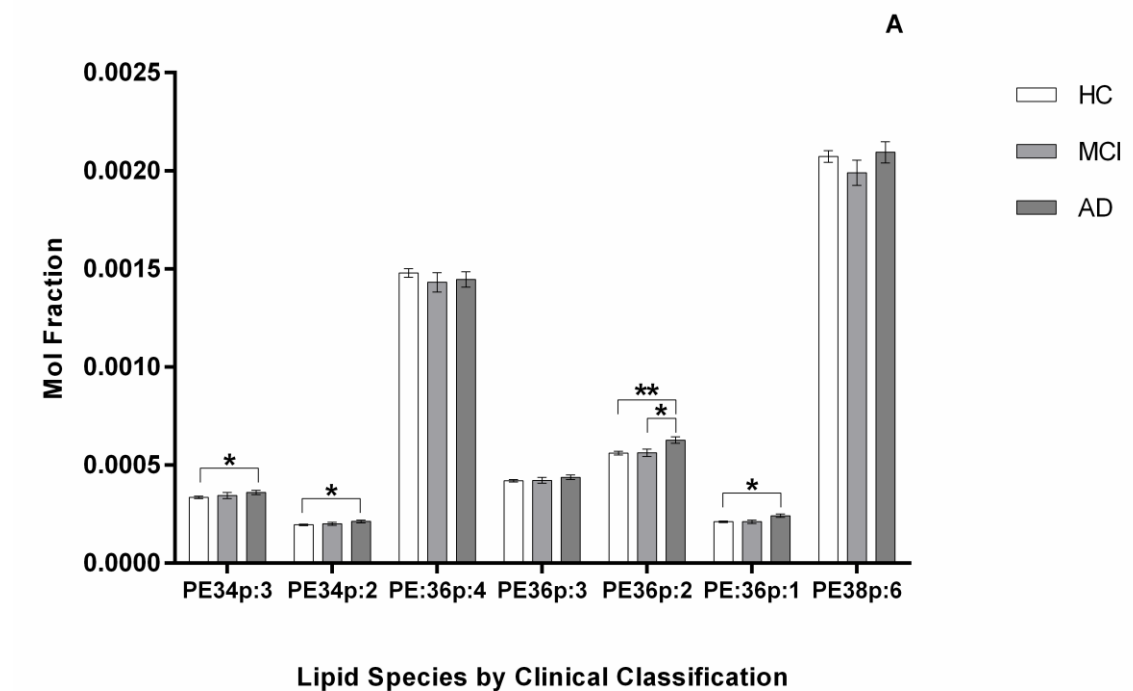


Figure 3.5A and B: Mean PE levels between clinical classifications.

PE: Phosphatidylethanolamine, HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer's disease. Mean \pm SEM. Analysis of Variance (ANOVA): * $p < 0.05$ ** $p < 0.001$



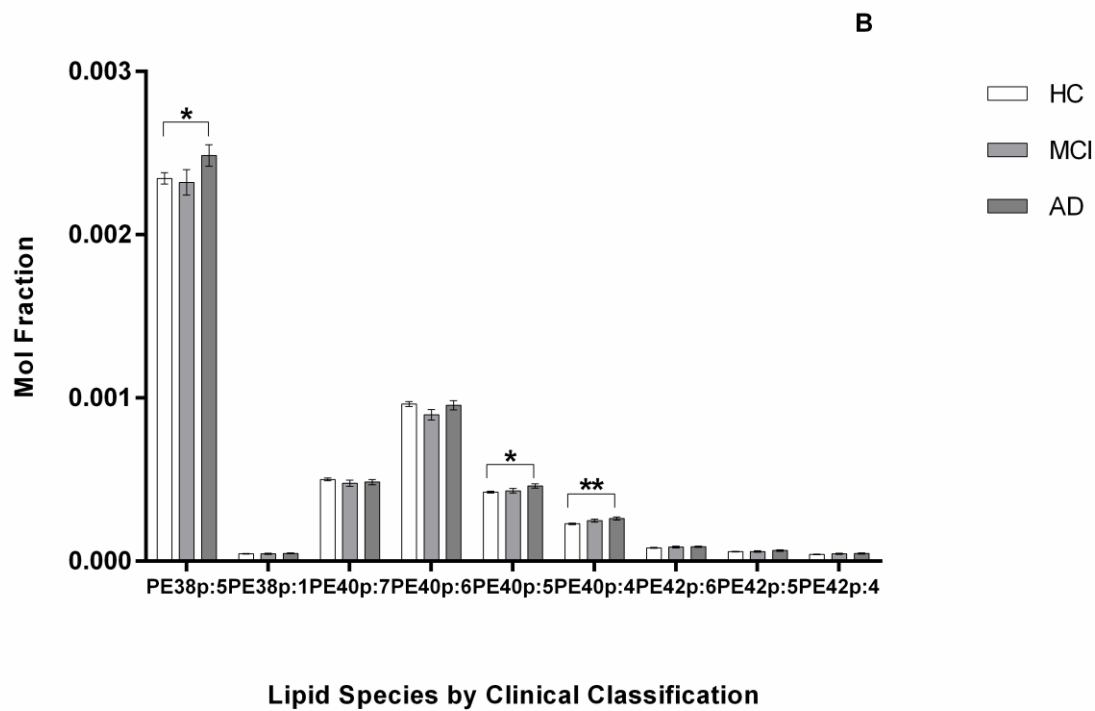
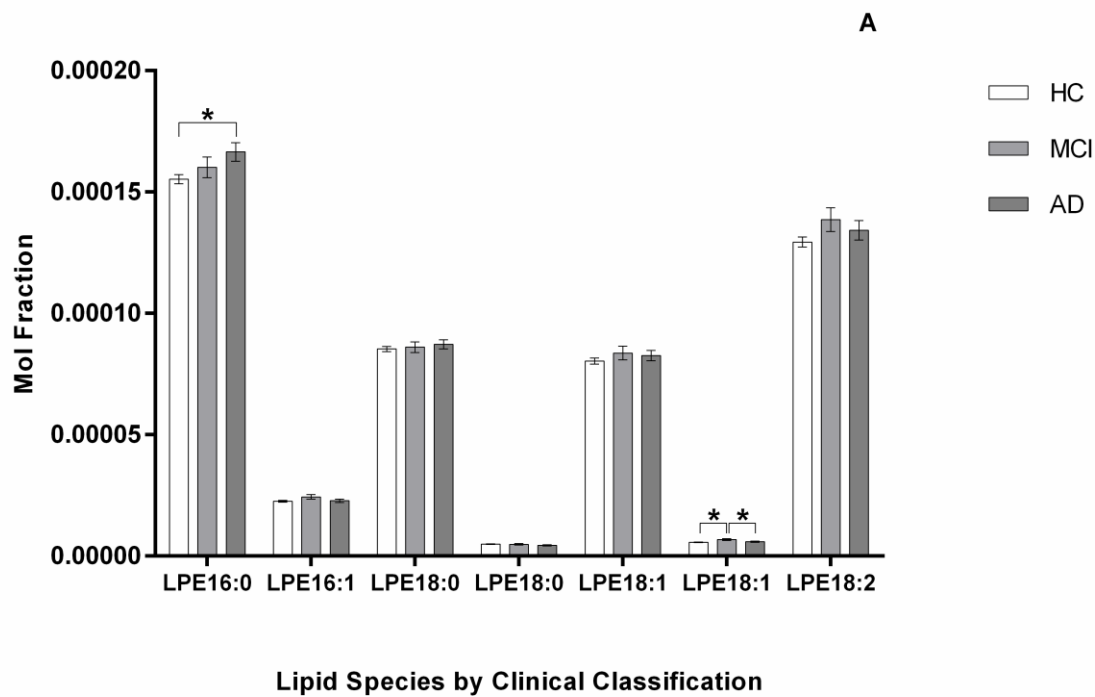


Figure 3.6A and B: Mean Ethanolamine plasmalogen levels between clinical classifications.

PE: Phosphatidylethanolamine, HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer's disease. Mean \pm SEM. Analysis of Variance (ANOVA): * $p < 0.05$ ** $p < 0.001$



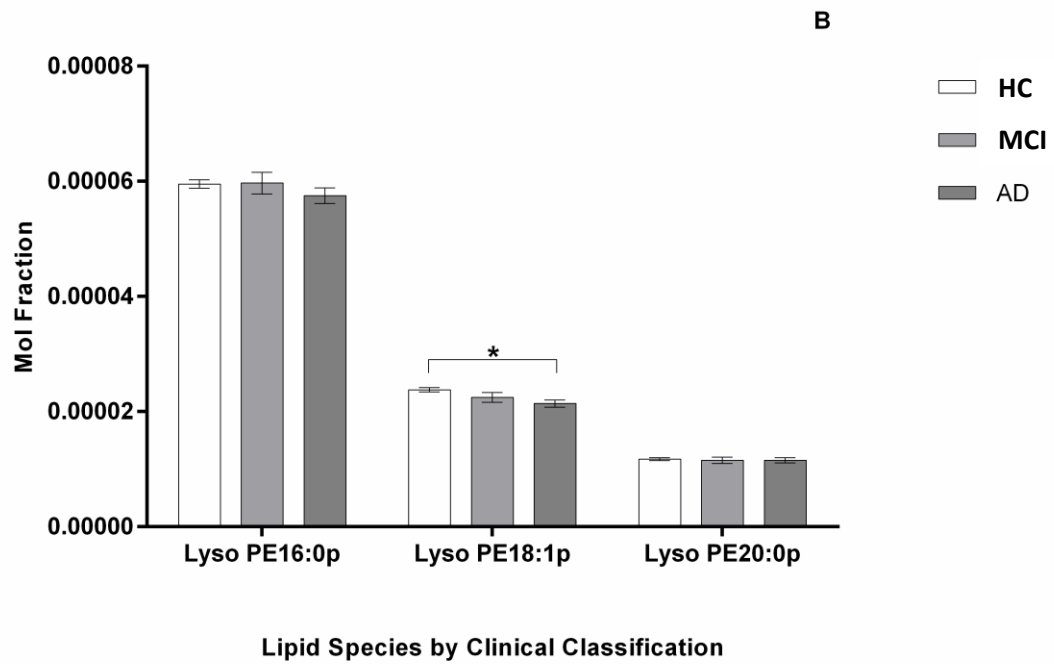


Figure 3.7: A: Mean Lyso-Phosphatidylethanolamine (LysoPE) and B: Mean Lyso-ethanolamine plasmalogen levels between clinical classifications.

HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer's disease.
Mean \pm SEM. Analysis of Variance (ANOVA): * $p < 0.05$ ** $p < 0.001$

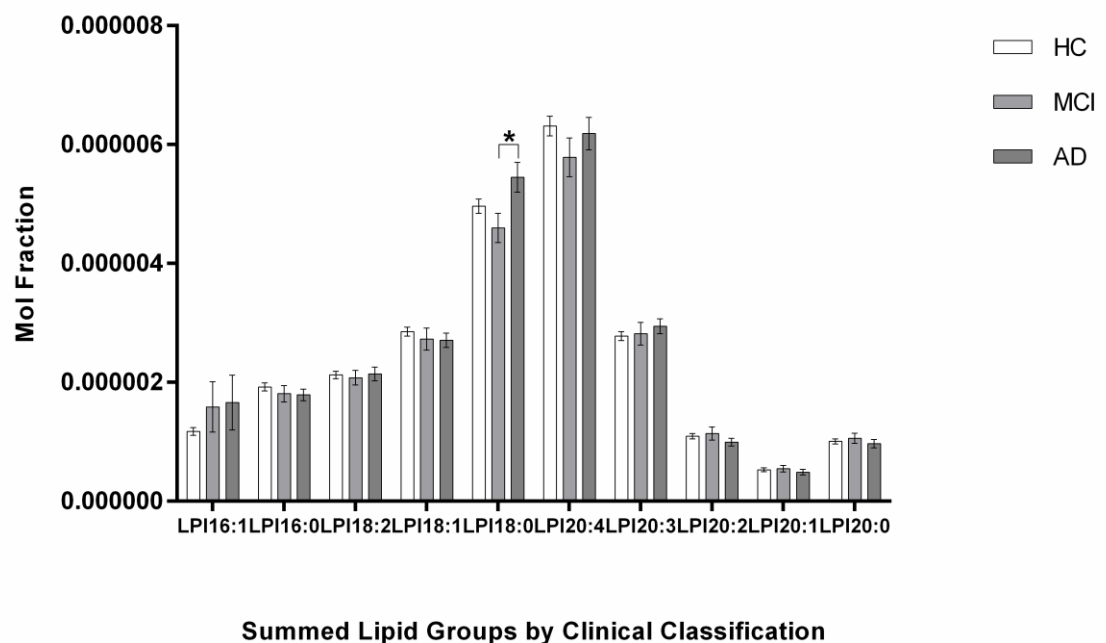


Figure 3.8: Mean Lyso- Phosphatidylinositol (LPI) levels between clinical classifications.

HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer's disease.
Mean \pm SEM. Analysis of Variance (ANOVA): * $p < 0.05$

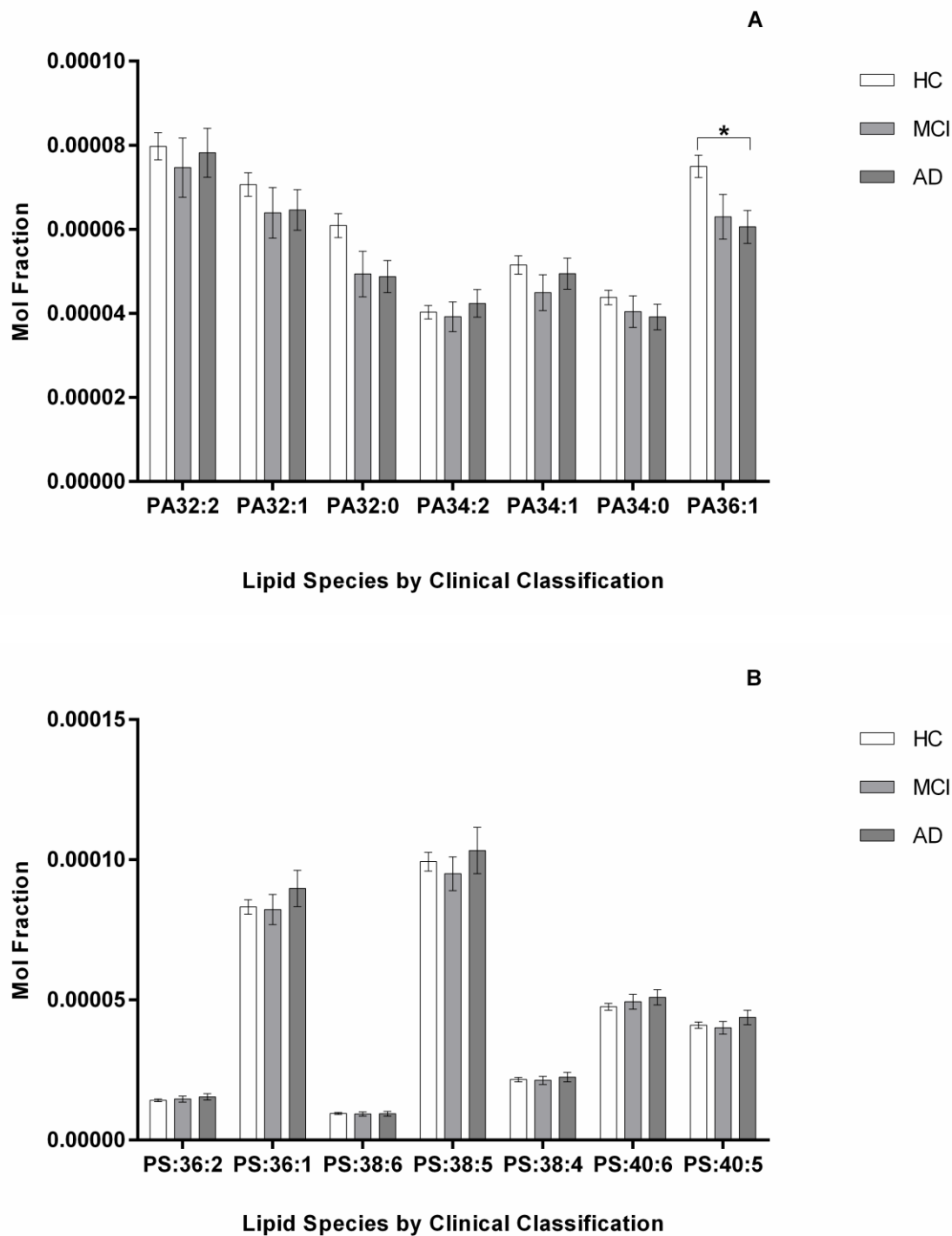


Figure 3.9: A: Mean Phosphatidic acid and B: Mean Phosphatidylserine (PS) levels between clinical classifications.

HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer's disease.
Mean \pm SEM. Analysis of Variance (ANOVA): * $p < 0.05$ ** $p < 0.001$

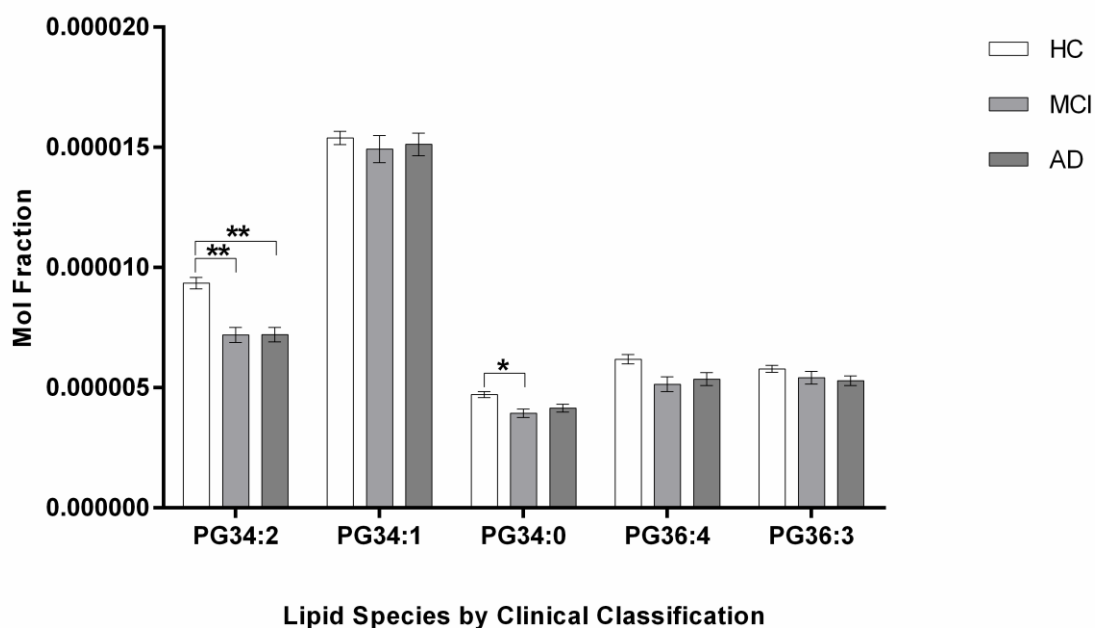
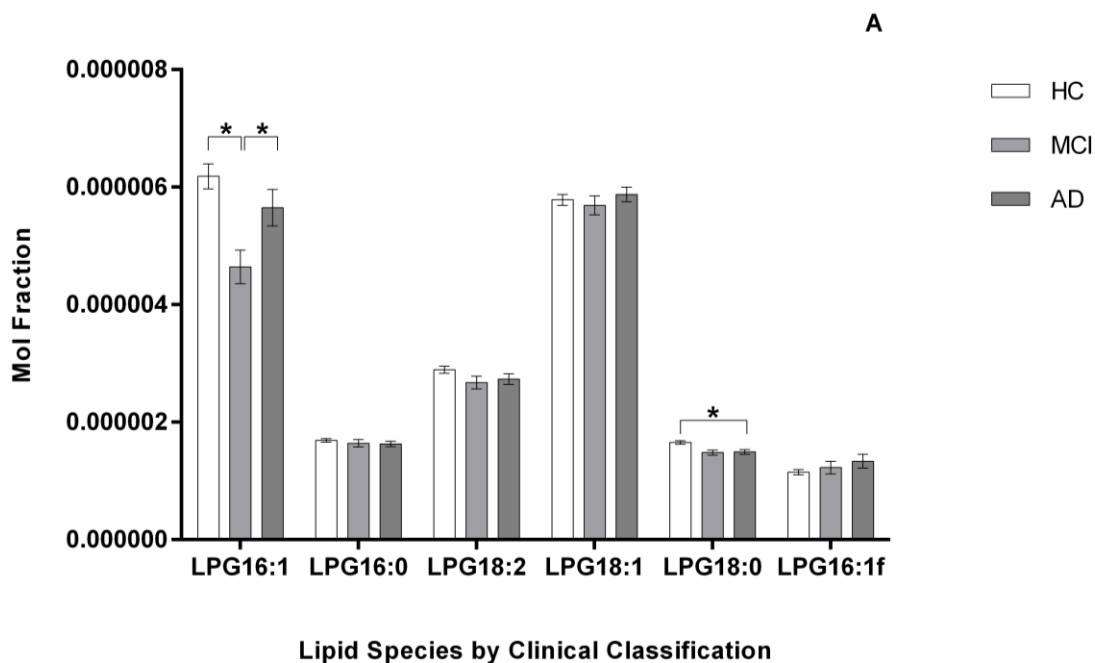


Figure 3.10: Mean Phosphatidylglycerol (PG) levels between clinical classifications.

HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer's disease.

Mean \pm SEM. Analysis of Variance (ANOVA): * $p < 0.05$ ** $p < 0.001$



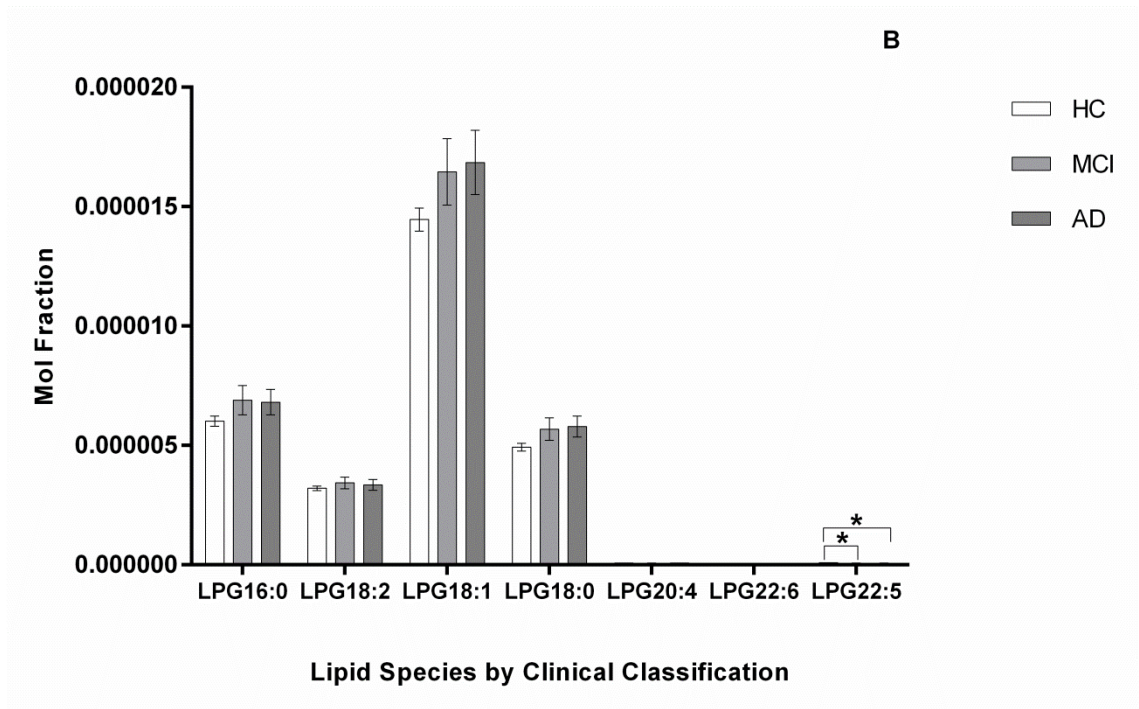
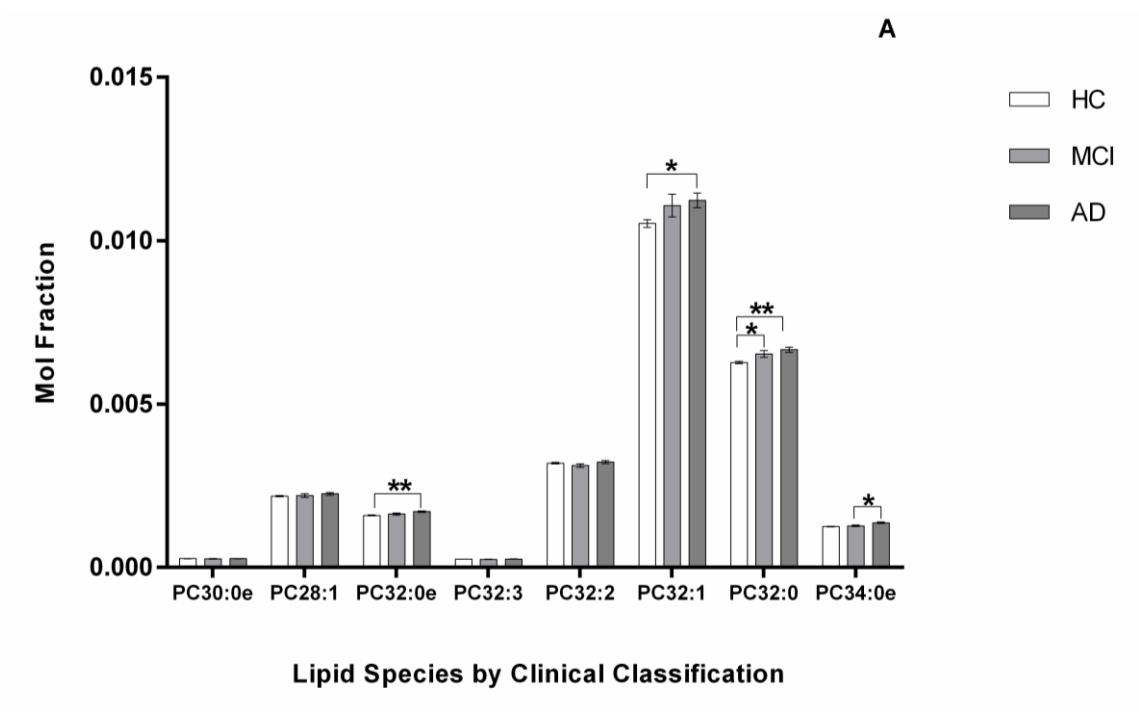


Figure 3.11 A and B: Mean Lysophosphatidylglycerol (LPG) levels between clinical classifications.

LPG16.0, LPG18.2, LPG18.1 and LPG18.0 are the “f” species in Figure 3.11B (see Table 3.2).
HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer’s disease.
Mean \pm SEM. Analysis of Variance (ANOVA): * $p < 0.05$ ** $p < 0.001$



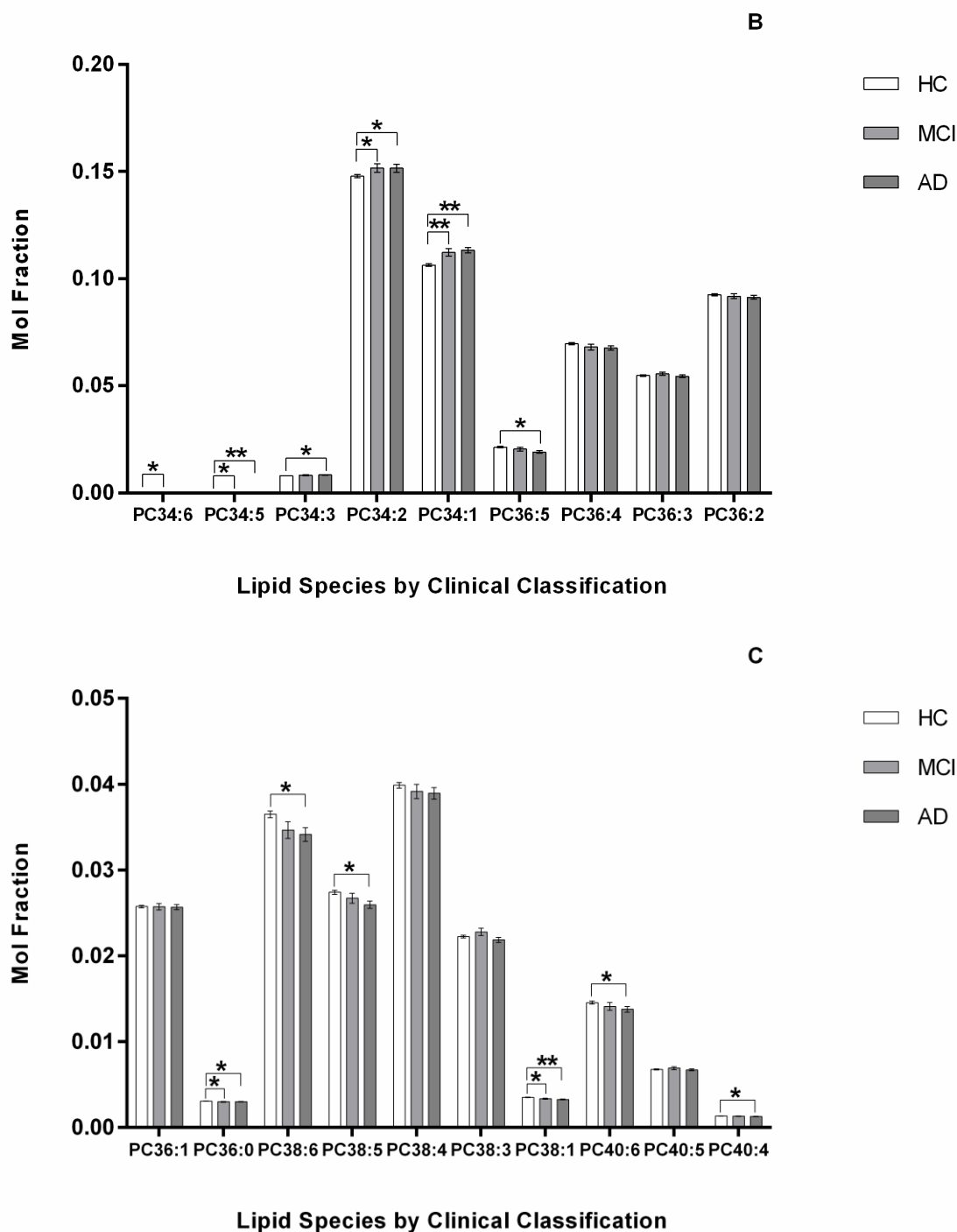
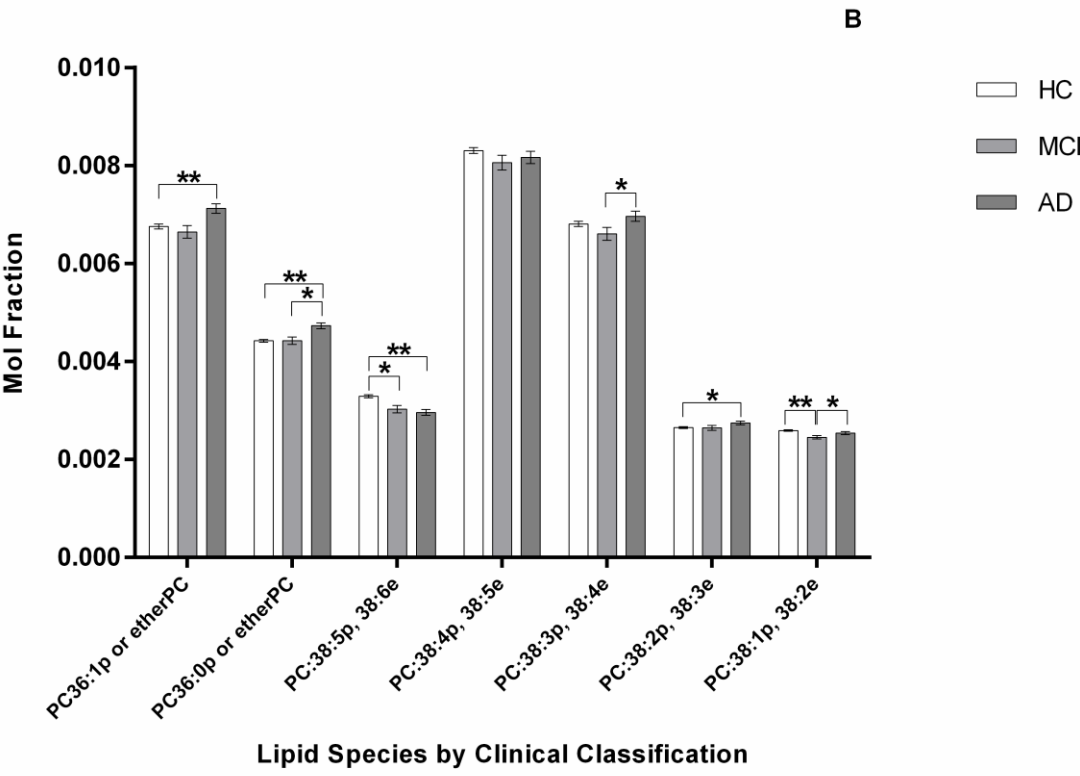
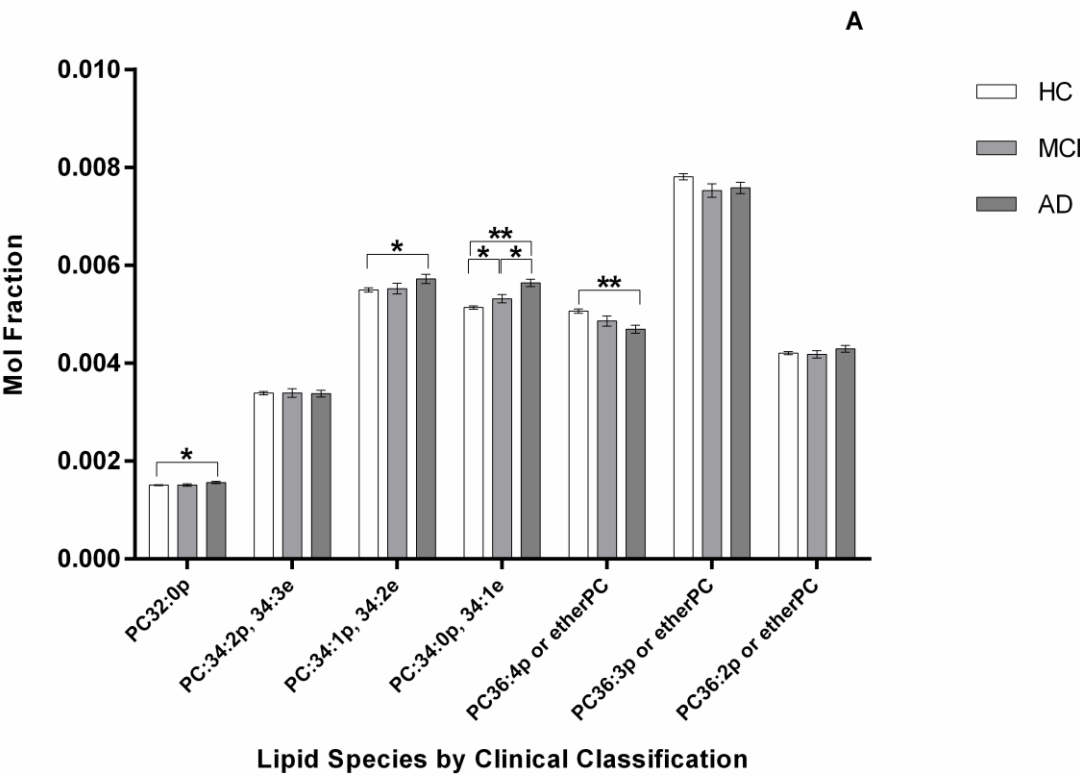


Figure 3.12 A, B and C: Mean Phosphatidylcholine (PC) levels between clinical classifications.

HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer's disease.
Mean \pm SEM. Analysis of Variance (ANOVA): * $p < 0.05$ ** $p < 0.001$



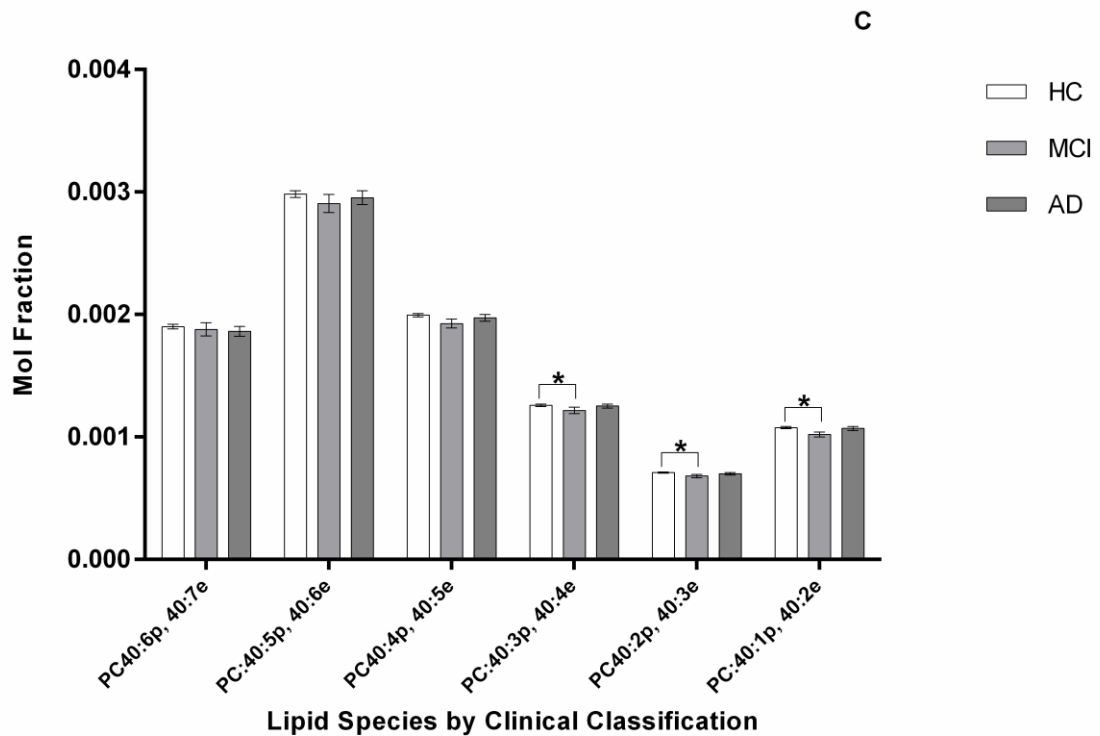
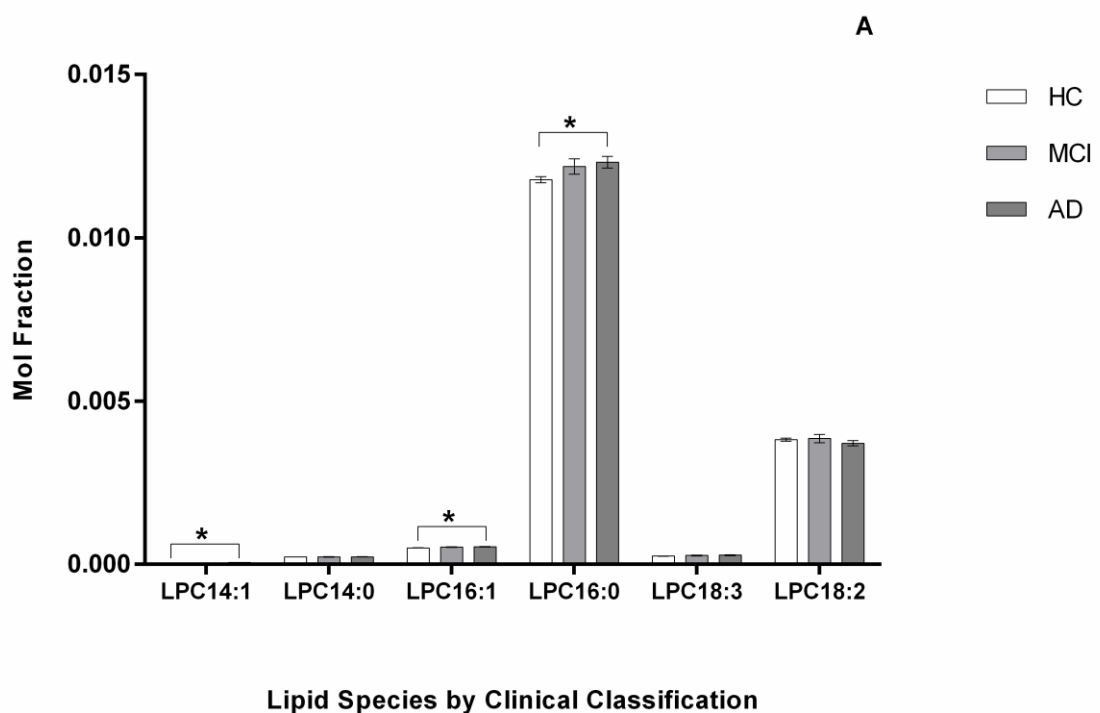


Figure 3.13 A, B and C: Mean choline plasmalogen (PCp) levels between clinical classifications.

HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer's disease.
 Mean \pm SEM. Analysis of Variance (ANOVA): * $p < 0.05$ ** $p < 0.001$



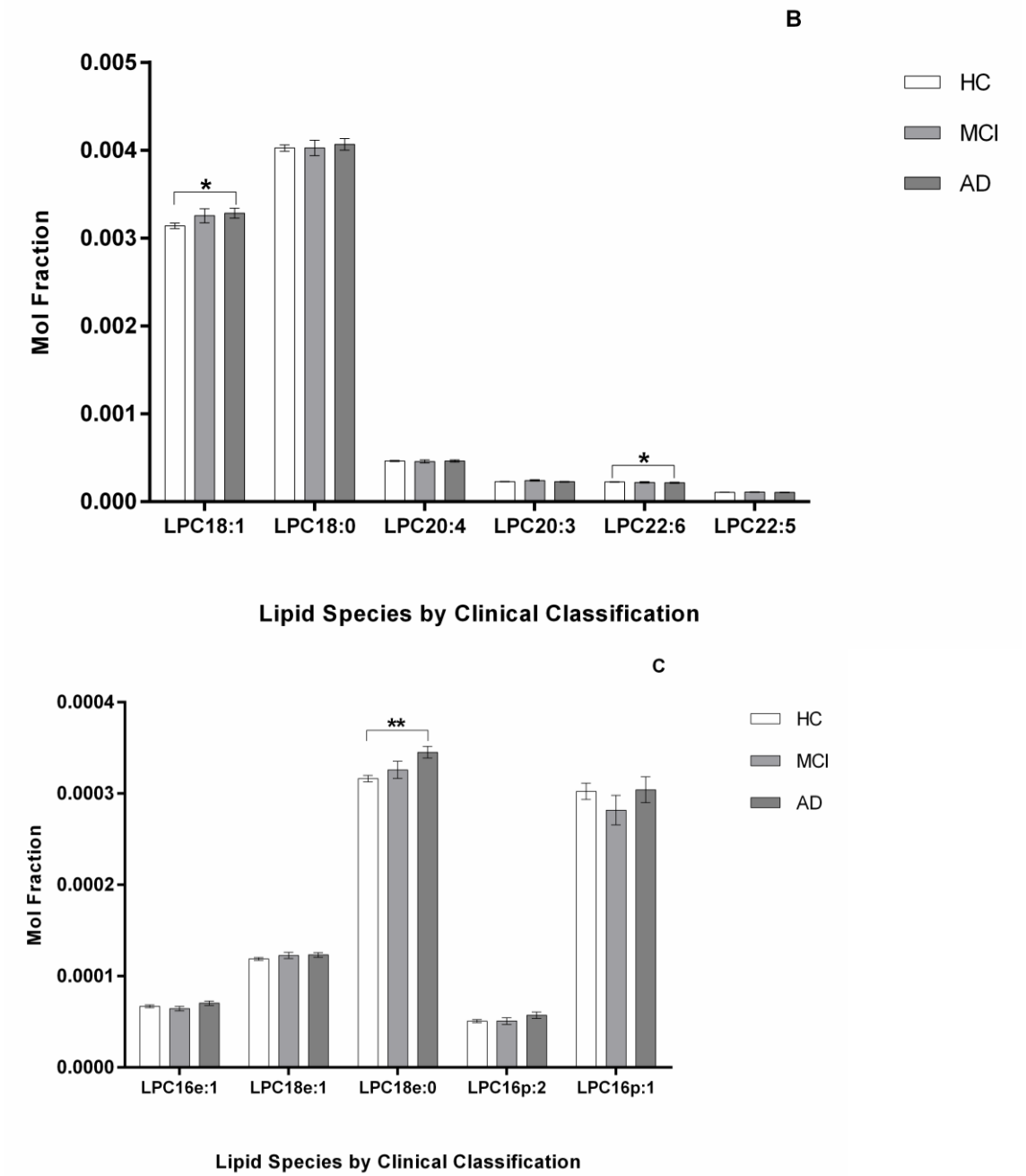


Figure 3.14 A and B: Mean Lysophosphatidylcholine (LPC) levels and C: Mean Lysocholine plasmalogen levels between clinical classifications.
HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer’s disease.
Mean \pm SEM. Analysis of Variance (ANOVA): * $p < 0.05$ ** $p < 0.001$

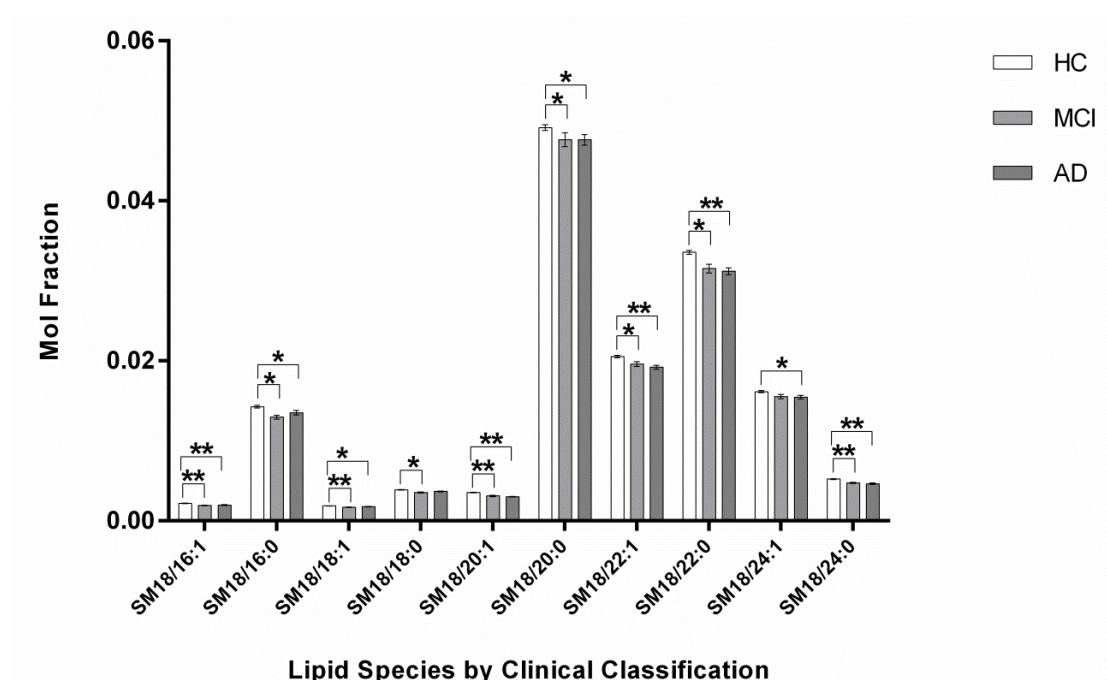


Figure 3.15: Mean Sphingomyelin (SM) levels between clinical classifications.

HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer's disease.

Mean \pm SEM. Analysis of Variance (ANOVA): * $p < 0.05$ ** $p < 0.001$

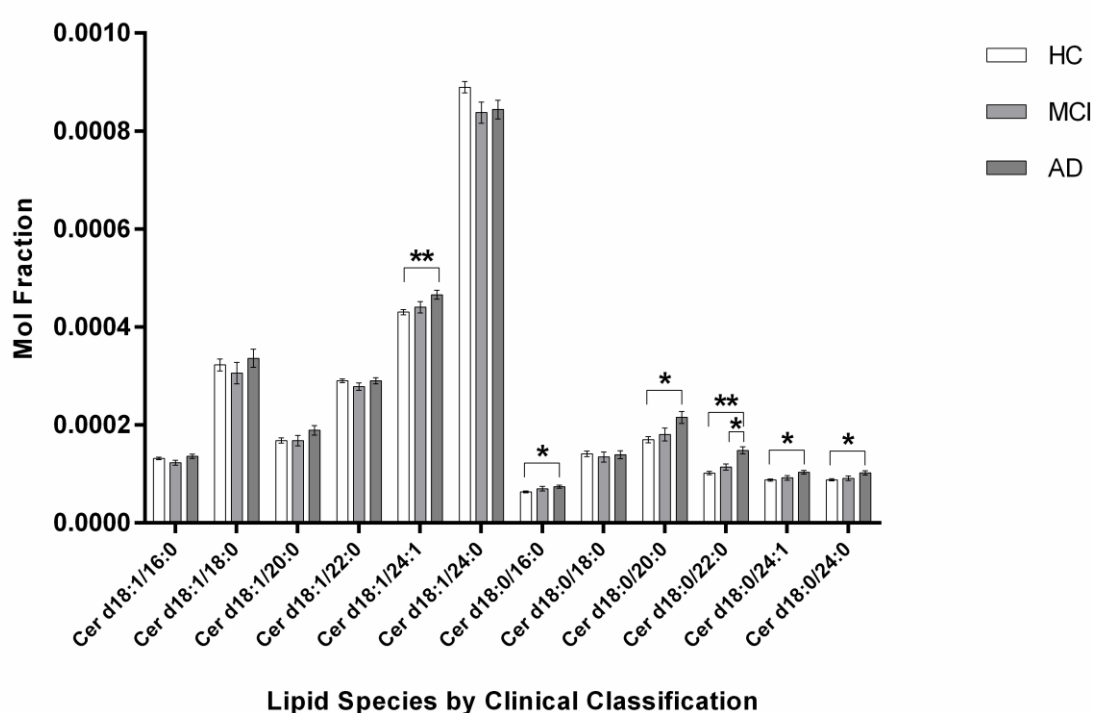


Figure 3.16: Mean Ceramide levels between clinical classifications.

HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer's disease.

Mean \pm SEM. Analysis of Variance (ANOVA): * $p < 0.05$ ** $p < 0.001$

The bar charts in Figures 3.5 to 3.16 show how the non-adjusted marginalised means of each individual lipid sub-species vary between the healthy controls, MCI and AD groups. Significant differences, as represented by the p-values between the lipids sub-species and the clinical classifications, were seen for many of the lipids, including PE, PC, PI, SM and Ceramide species. A total of 86 out of 189 lipid species measured showed a significant difference with a p-value of < 0.05 or < 0.001 between HC and AD, HC and MCI or MCI and AD groups. Table 3.3 lists those lipids that were significantly different between clinical classifications.

Table 3.3: List of lipids that were significantly different between clinical classifications.

| Lipid – HC vs. AD | | Lipid – HC vs. MCI | | Lipid – MCI vs. AD | |
|---|--|--|--|---|-----------|
| p < 0.05 | p < 0.001 | p < 0.05 | p < 0.001 | p < 0.05 | p < 0.001 |
| Total PI | Total PE | Total PC, Total SM | | | |
| PE36.1, PE40.6, PE40.5, PE34p.3, PE34p.2, PE36p.1, PE38p.5, PE40p.5, LysoPE16.0, LysoPE18.1p | PE34.2, PE34.1, PE36.4, PE36.3, PE36.2, PE38.6, PE38.5, PE38.4, PE36p.2, PE40p.4 | PE36.4, PE36.3, PE36.2, PE38.5, PE38.4, LysoPE18.1 | PE34.2 | PE34.1, PE36p.2, LysoPE18.1 | |
| PC32.1, PC34.2, PC34.3, PC36.5, PC36.0, PC38.6, PC38.5, PC40.4, PC 40.6, PC32.0p, PC34.1p..34.2e, PC38.2p..38.3e, PCLyso14.1, PCLyso16.1, PCLyso16.0, PCLyso18.1, PCLyso22.6, | PC32.0, PC34.1, PC34.5, PC34.0p..34.1e, PC34.0e PC36.4p/etherPC, PC38.1, PC36.1p/etherPC, PC36.0p/etherPC, PC38.5p..38.6e, PC32.0e PCLyso18e.0 | PC32.0, PC34.6, PC34.2, PC34.5, PC36.0, PC38.1, PC34.0p..34.1e, PC38.5p..38.6e, PC40.1p..40.2e, PC40.3p..40.4e, PC40.2p..40.3e | PC34.1, PC38.1p..38.2e | PC34.0e, PC36.0p/etherPC, PC36.1p/etherPC, PC34.0p..34.1e, PC38.3p..38.4e, PC38.1p..38.2e | |
| PI32.1, PI34.1, PI34.2, PI 36.1, PI36.3, PI36.4, PI38.4, PI40.3, | PI34.0 | PI32.2, PI34.0 | | LysoPI18.0 | |
| LysoPG18.0, LysoPG22.5 | PG34.2 | PG34.0, LysoPG22.5, LysoPG16.1, LysoPG18.0 | PG34.2 | LysoPG16.1 | |
| PA36.1 | | | | | |
| SM18.16.0, SM18.18.0, SM18.18.1, SM18.20.0, SM18.24.1 | SM18.16.1, SM18.20.1, SM18.22.1, SM18.22.0, SM18.24.0 | SM18.16.0, SM18.18.0, SM18.20.0, SM18.22.0, SM18.22.1 | SM18.16.1, SM18.18.1, SM18.20.1, SM18.24.0 | | |
| Cer.d18.0.16.0, Cer.d18.0.24.1, Cer.d18.0.24.0 | Cer.d18.1.24.1, Cer.d18.0.22.0, Cer.d18.0.20.0 | | | Cer.d18.0.22.0 | |

The red text indicates lower mean levels in AD compared to HC, MCI compared to HC and AD compared to MCI. The black text indicates higher mean levels AD compared to HC, MCI compared to HC and AD compared to MCI.

PI: Phosphatidylinositol, PE: Phosphatidylethanolamine, PC: Phosphatidylcholine, SM: Sphingomyelin, PA: Phosphatidic acid, PG: Phosphatidylglycerol, Cer: Ceramide.

To further examine the significance of differences in levels observed between lipid species and clinical classification, a LIMMA model (see Section 3.4.1) was applied to obtain a false discovery adjusted value for each lipid to exclude any false associations that may have been included following simple ANOVA.

3.4.1 Linear Models for Microarray Data (LIMMA)

LIMMA (Linear Models for Microarray Data) analysis was used to obtain Q values (false discovery adjusted values) and Bonferroni correction were used to generate stricter p-values to exclude some of the 86 lipid species level differences selected and displayed graphically in Figures 3.5 - 3.16 and in Table 3.3. Using the LIMMA model, 27 out of 86 lipid species levels were significantly different between healthy controls and AD participants and these lipids are listed in Table 3.4.

Table 3.4: Linear Models for Microarray Data (LIMMA), listed in order of most to least significant for Healthy Controls compared to AD.

| Lipid | p.value | Lipid | p.value |
|-------------------|----------|--------------------|----------|
| PC.34.0p..34.1e | 4.68E-11 | PE36.4 | 2.30E-06 |
| SM18.20.1 | 3.62E-10 | PE34.1 | 2.31E-06 |
| PE34.2 | 4.23E-10 | PE36.3 | 3.16E-06 |
| Cer.d18.0.22.0 | 7.92E-10 | PC32.0 | 4.73E-06 |
| SM18.24.0 | 1.34E-09 | PC34.5 | 1.09E-05 |
| PE38.5 | 3.59E-09 | PG34.2 | 2.20E-05 |
| PE38.4 | 5.70E-09 | SM18.16.1 | 3.64E-05 |
| PC34.1 | 9.44E-09 | PE38.6 | 4.12E-05 |
| PC34.0e | 3.07E-08 | SM18.22.1 | 5.95E-05 |
| SM18.22.0 | 4.92E-07 | PC.Lyso.18e.0 | 9.64E-05 |
| PC38.1 | 6.92E-07 | PC36.4p or etherPC | 1.34E-04 |
| PC.38.5p..38.6e | 9.58E-07 | PC32.0e | 1.36E-04 |
| PE36.2 | 1.35E-06 | PI34.0 | 1.98E-04 |
| PC36.0por etherPC | 1.39E-06 | | |

p-values less than the Bonferroni cut off (0.05/189).

PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine, Cer: Ceramide, PG: Phosphatidylglycerol, PI: Phosphatidylinositol.

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A fold lipid change was subsequently calculated using unadjusted raw expression values to provide a relative lipid difference between clinical classifications. The mean levels, difference in levels between groups, the directional change i.e. increased or decreased in AD compared to healthy controls and fold change is shown in Table 3.5.

Table 3.5: Mean value difference in lipid species levels between groups, direction of change in lipid levels between HC and AD participants and fold change.

| Lipid | Mean HC | Mean AD | Difference | Direction of change | Fold Δ |
|--------------------|---------|---------|------------|---------------------|---------------|
| PE34.2 | 0.00036 | 0.00046 | 0.00010 | ↑ in AD | 1.288 |
| PE34.1 | 0.00025 | 0.00031 | 0.00006 | ↑ in AD | 1.228 |
| PE36.4 | 0.00065 | 0.00076 | 0.00012 | ↑ in AD | 1.180 |
| PE36.3 | 0.00030 | 0.00036 | 0.00006 | ↑ in AD | 1.200 |
| PE36.2 | 0.00084 | 0.00100 | 0.00016 | ↑ in AD | 1.193 |
| PE38.6 | 0.00120 | 0.00140 | 0.00019 | ↑ in AD | 1.161 |
| PE38.5 | 0.00070 | 0.00084 | 0.00014 | ↑ in AD | 1.207 |
| PE38.4 | 0.00150 | 0.00182 | 0.00032 | ↑ in AD | 1.211 |
| PC.Lyso.18e.0 | 0.00032 | 0.00035 | 0.00003 | ↑ in AD | 1.091 |
| PC.34.0p..34.1e | 0.00515 | 0.00564 | 0.00050 | ↑ in AD | 1.096 |
| PC36.0p or etherPC | 0.00443 | 0.00473 | 0.00030 | ↑ in AD | 1.068 |
| PC32.0e | 0.00160 | 0.00170 | 0.00010 | ↑ in AD | 1.066 |
| PC32.0 | 0.00629 | 0.00666 | 0.00038 | ↑ in AD | 1.060 |
| PC34.0e | 0.00126 | 0.00137 | 0.00011 | ↑ in AD | 1.091 |
| PC34.1 | 0.10666 | 0.11382 | 0.00715 | ↑ in AD | 1.067 |
| Cer.d18.0.22.0 | 0.00010 | 0.00015 | 0.00005 | ↑ in AD | 1.435 |
| PI34.0 | 0.00012 | 0.00010 | -0.00002 | ↓ in AD | 0.858 |
| PG34.2 | 9.02E-6 | 7.21E-6 | -1.81E-6 | ↓ in AD | 0.799 |
| PC36.4p or etherPC | 0.00506 | 0.00470 | -0.00037 | ↓ in AD | 0.927 |
| PC38.5p..38.6e | 0.00329 | 0.00296 | -0.00033 | ↓ in AD | 0.900 |
| PC34.5 | 0.00026 | 0.00023 | -0.00003 | ↓ in AD | 0.878 |
| PC38.1 | 0.00352 | 0.00329 | -0.00024 | ↓ in AD | 0.933 |
| SM18.16.1 | 0.00217 | 0.00196 | -0.00021 | ↓ in AD | 0.904 |
| SM18.20.1 | 0.00355 | 0.00302 | -0.00053 | ↓ in AD | 0.852 |
| SM18.22.1 | 0.02032 | 0.01922 | -0.00110 | ↓ in AD | 0.946 |
| SM18.22.0 | 0.03332 | 0.03102 | -0.00229 | ↓ in AD | 0.931 |
| SM18.24.0 | 0.00515 | 0.00462 | -0.00053 | ↓ in AD | 0.897 |

Those lipids in the blue section indicate an increase in the mean lipid levels and those in the grey section indicate a decrease in mean lipid levels in AD compared to healthy controls.

PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine, Cer: Ceramide, PG: Phosphatidylglycerol, PI: Phosphatidylinositol.

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Table 3.5 shows the mean value, the difference between the mean lipid profile and the fold change for each of the 27 lipids identified as being significantly different between HC and AD by LIMMA. 16 lipid species had mean levels higher and 11 had mean levels lower in AD compared to healthy controls. Whilst Tables 3.4 and 3.5 compared healthy controls to AD, the following two tables show the repeat LIMMA analysis for the healthy controls compared to MCI groups.

Table 3.6: Linear Models for Microarray Data (LIMMA), listed in order of most to least significant for Healthy Controls compared to MCI.

| Lipid | p.value |
|-----------|----------|
| PC34.1 | 3.48E-05 |
| SM18.20.1 | 9.25E-05 |
| SM18.16.1 | 1.55E-04 |
| SM18.24.0 | 2.20E-04 |
| PE34.2 | 2.56E-04 |

p-values less than the Bonferroni cut off (0.05/189).

PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine.

Table 3.6 lists the five lipid species levels that were significantly different between healthy controls and MCI participants. The mean level, difference in level between groups, the directional change i.e. increased or decreased in MCI compared to healthy controls and fold change of these five lipid species is shown in Table 3.7.

Table 3.7: Mean value difference in lipid species levels between groups, direction of change in lipid levels between HC and MCI participants and fold change.

| Lipid | Mean HC | Mean MCI | Difference | Direction of change | Fold Δ |
|-----------|---------|----------|------------|---------------------|---------------|
| PE34.2 | 0.00036 | 0.00043 | 0.00007 | ↑ in MCI | 1.198 |
| PC34.1 | 0.10666 | 0.11295 | 0.00628 | ↑ in MCI | 1.059 |
| SM18.16.1 | 0.00217 | 0.00193 | -0.00024 | ↓ in MCI | 0.892 |
| SM18.20.1 | 0.00355 | 0.00313 | -0.00042 | ↓ in MCI | 0.882 |
| SM18.24.0 | 0.00515 | 0.00475 | -0.00040 | ↓ in MCI | 0.923 |

Those lipids in the blue section indicate an increase in the mean lipid levels and those in the grey section indicate a decrease in mean lipid levels in MCI compared to healthy controls.

PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine.

The above table shows the mean value, the difference between the mean lipid levels and the fold change for each of the five lipids identified as being significantly different between HC and MCI by LIMMA. Two lipid species had mean levels higher and three had mean levels lower in MCI compared to healthy controls.

The LIMMA analysis was repeated comparing lipid species levels between MCI and AD classifications. Interestingly no significant difference was observed in the mean levels of lipid species.

3.4.2 Generalised Linear Model (GLM)

The LIMMA analyses, while identifying those lipids species levels that are significantly different between clinical classifications, do not take into consideration factors which may influence the significance of the observed differences. Consequently a generalised linear model (GLM) was created to control for age, APOE ϵ 4 allele carriage status, gender and collection site. The results from the GLM comparing HC to AD are displayed in Table 3.8.

Table 3.8: Generalised Linear Modelling (GLM) adjusted for age, sex, APOEε4 allele status (i.e. carriage or non-carriage of an APOEε4 allele) and sample collection site (Perth or Melbourne) in the healthy control compared to the AD group.

| Lipid | p-value | Age | APOEε4 | Sex | Site |
|---------------------|----------|----------|----------|-------|-------|
| SM18.20.1 | 2.65E-06 | 1.52E-42 | 1.04E-21 | 0.061 | 0.406 |
| Cer.d18.0.22.0 | 2.69E-06 | 1.37E-40 | 3.81E-22 | 0.221 | 0.010 |
| PE38.4 | 3.33E-06 | 8.60E-41 | 1.97E-22 | 0.457 | 0.011 |
| PE34.2 | 8.98E-06 | 6.42E-40 | 8.18E-23 | 0.345 | 0.047 |
| PC.34.0p..34.1e | 2.23E-05 | 3.61E-36 | 7.04E-24 | 0.311 | 0.041 |
| PE38.5 | 3.56E-05 | 5.22E-40 | 3.67E-22 | 0.533 | 0.015 |
| PC34.1 | 5.84E-05 | 9.46E-40 | 2.49E-22 | 0.123 | 0.015 |
| PE36.2 | 0.00014 | 7.38E-42 | 9.27E-23 | 0.342 | 0.025 |
| PC34.0e | 0.00016 | 1.61E-37 | 7.96E-24 | 0.229 | 0.026 |
| SM18.16.1 | 0.00022 | 1.88E-42 | 1.07E-22 | 0.146 | 0.007 |
| PE34.1 | 0.00025 | 3.86E-41 | 5.74E-23 | 0.231 | 0.028 |
| PI34.0 | 0.00032 | 1.44E-43 | 4.77E-23 | 0.158 | 0.090 |
| PC32.0 | 0.00038 | 9.00E-40 | 9.13E-24 | 0.143 | 0.027 |
| PG34.2 | 0.00080 | 4.96E-43 | 5.62E-23 | 0.344 | 0.188 |
| PE36.4 | 0.00092 | 2.55E-41 | 9.50E-23 | 0.421 | 0.020 |
| PC38.5p..38.6e | 0.00105 | 8.52E-41 | 4.55E-22 | 0.177 | 0.007 |
| PC32.0e | 0.00107 | 1.26E-40 | 1.11E-23 | 0.120 | 0.022 |
| SM18.24.0 | 0.00134 | 5.16E-39 | 3.18E-22 | 0.387 | 0.014 |
| PC36.0p or ether PC | 0.00240 | 2.21E-39 | 3.96E-23 | 0.295 | 0.018 |
| PE36.3 | 0.00364 | 2.11E-41 | 1.73E-22 | 0.328 | 0.019 |
| SM18.22.1 | 0.00394 | 1.50E-41 | 8.31E-23 | 0.212 | 0.020 |
| SM18.22.0 | 0.00411 | 2.51E-40 | 2.26E-22 | 0.239 | 0.022 |
| PC34.5 | 0.00523 | 2.34E-41 | 2.37E-22 | 0.151 | 0.014 |
| PC.Lyso.18e.0 | 0.00661 | 2.25E-41 | 1.17E-22 | 0.104 | 0.027 |
| PC38.1 | 0.02124 | 1.02E-39 | 3.47E-22 | 0.317 | 0.007 |
| PE38.6 | 0.02217 | 1.44E-41 | 2.42E-22 | 0.467 | 0.022 |
| PC36.4p or ether PC | 0.08371 | 3.55E-41 | 2.38E-22 | 0.298 | 0.012 |

Those lipids above the solid line remain significant ($p < 0.000265$) after adjustment.

PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine, Cer: Ceramide, PG: Phosphatidylglycerol, PI: Phosphatidylinositol.

Table 3.8 shows the p-values for each of the 27 lipids identified by LIMMA as being significantly different between HC and AD, adjusted through GLM for the following covariates; age, sex, APOEε4 allele status and collection site. The p-values for each of the covariates are also shown in the table. The data are sorted by the strongest p-value for the lipid level post adjustment i.e. where the levels are most different between the clinical classification groups. The top 11 lipids (above the solid line) in the table remain statistically significant (less than the Bonferroni cut off value of $p < 0.000265$ ($0.05/189$)) after adjustment for age, sex, APOEε4 allele status and collection site. The p-values indicate that neither site of collection or gender significantly influence the results, but as expected age and APOEε4 allele carriage do significantly influence the results.

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The GLM was repeated to compare the healthy control and MCI groups following adjustment of the LIMMA results to control for age, gender, APOE ϵ 4 allele status and collection site.

Table 3.9: Generalised Linear Modelling (GLM) adjusted for age, sex, APOE ϵ 4 allele status (i.e. carriage or non-carriage of an APOE ϵ 4 allele) and collection site (Perth or Melbourne) in the healthy control compared to the MCI group.

| Lipid | p-value | Age | APOE ϵ 4 | Sex | Site |
|-----------|---------|----------|-------------------|-------|-------|
| SM18.16.1 | 0.0001 | 4.20E-15 | 2.10E-08 | 0.875 | 0.216 |
| SM18.20.1 | 0.0007 | 6.82E-15 | 3.98E-08 | 0.682 | 0.803 |
| PC34.1 | 0.0009 | 3.01E-14 | 8.72E-08 | 0.790 | 0.286 |
| PE34.2 | 0.0045 | 1.48E-14 | 3.13E-08 | 0.809 | 0.472 |
| SM18.24.0 | 0.0269 | 1.86E-13 | 2.54E-08 | 0.761 | 0.305 |

Those lipids above the solid line remain significant ($p < 0.000265$) after adjustment.

PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine.

Table 3.9 shows the p-values for each of the five lipids identified by LIMMA as being significantly different between HC and MCI, adjusted through GLM for the same covariates (age, gender, APOE ϵ 4 allele status and collection site). The p-values for each of the covariates are also shown in the table. The data are again sorted by the strongest p-value for the lipid level post adjustment. Only one lipid in the table (above the solid line) remained statistically significant (less than the Bonferroni cut off value of $p < 0.000265$ ($0.05/189$)) after adjustment for age, sex, APOE ϵ 4 allele status and collection site. The p-values again indicate that neither site of collection or gender significantly influence the results, but as expected age and APOE ϵ 4 allele carriage do significantly influence the results.

No GLM was created for the MCI compared to AD groups as no lipid species were found to be significantly different between groups after LIMMA analysis.

3.4.3.1 Variable selection for HC vs. AD groups

The LIMMA and GLM models assess associations between lipids and clinical classification, i.e. levels of lipid species that are significantly different between clinical classifications. Variable selection analysis attempts to identify lipid species which when considered collectively are able to predict clinical classification with greatest certainty, i.e. if you measure levels of lipid species x, y, z you can determine whether an individual would be classified as healthy control, MCI or AD. As such the level at which clinical classification can be predicted, is determined by modelling the selected lipids and covariates using GLM and Receiver Operating Characteristics (ROC) models.

Previous analysis of biomarkers in the AIBL cohort utilised a variable selection pathway to create lists of biomarkers that were chosen from four separate selection methods; random forest, boosted trees, regression trees and LIMMA and used clinical classification as a binary outcome, i.e. HC or AD [476] . In this study, a variable selection pathway was applied to the 189 lipid species to create an importance ranking (in addition to generation of p-values), where 1000 iterations were performed to define a list of those lipid species that are selected most often as differing in levels when comparing clinical classifications. Each time a lipid was selected a frequency score was assigned if it was chosen from all four statistical methods or from three out of the four statistical methods. Table 3.10 lists the lipid species selected most often when comparing the HC and AD groups. The lipid species that are selected in at least 10% of the 1000 iterations from all four statistical methods are listed in column A and the lipid species that were selected in at least 10% of the 1000 iterations from three of the four statistical methods are shown in column B.

Table 3.10: Lipid Markers selected by the Variable Selection Pathway for HC vs. AD.

| List A (4/4) | | List B (3/4) | |
|----------------|-------------------|--------------------|-------------------|
| Lipid | Frequency (/1000) | Lipid | Frequency (/1000) |
| SM18.20.1 | 610 | PC34.1 | 583 |
| Cer.d18.0.22.0 | 419 | Cer.d18.0.22.0 | 541 |
| PC34.0e | 173 | PC34.0e | 539 |
| PC32.0 | 108 | PC32.0 | 485 |
| | | PC38.5p...38.6e | 446 |
| | | PC34.0p...34.1e | 432 |
| | | PE34.2 | 421 |
| | | SM18.20.1 | 385 |
| | | PE38.4 | 375 |
| | | PG34.2 | 331 |
| | | SM18.24.0 | 291 |
| | | PE34.1 | 285 |
| | | PC32.0e | 273 |
| | | PE38.5 | 233 |
| | | PC36.0p.or.etherPC | 231 |
| | | SM18.16.1 | 205 |
| | | PI34.0 | 181 |
| | | PE36.4 | 173 |
| | | PE36.3 | 157 |
| | | Cer.d18.0.20.0 | 156 |
| | | PE36.2 | 144 |
| | | Cer.d18.0.24.1 | 120 |

PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine, Cer: Ceramide, PG: Phosphatidylglycerol, PI: Phosphatidylinositol. The lipids highlighted in grey appeared in both list A and list B.

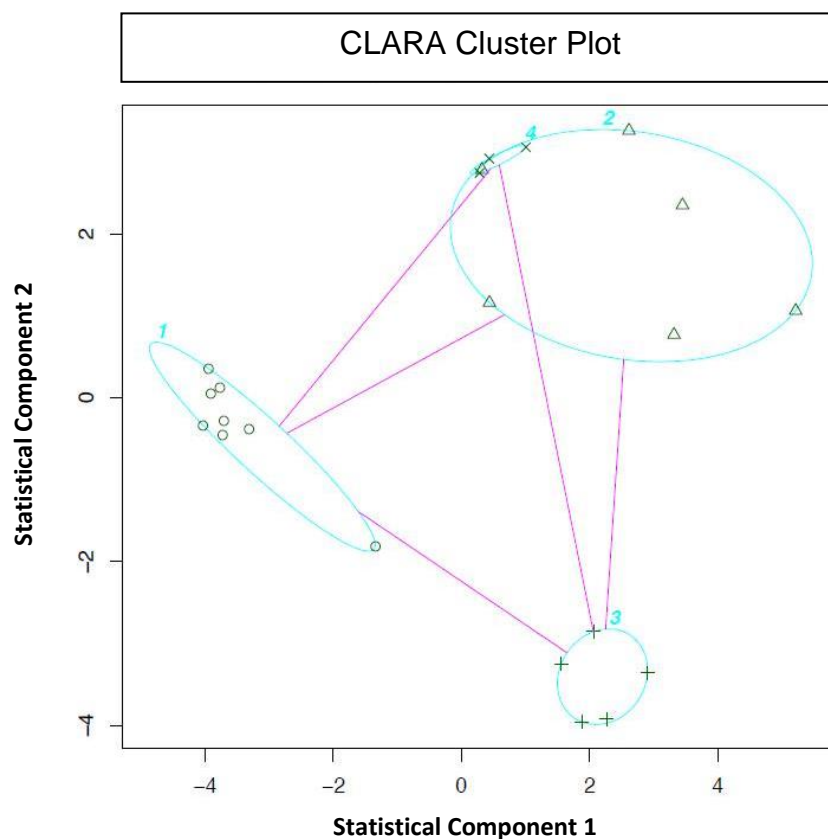
Those lipids with the highest frequencies of selection from both lists A and B of Table 3.10 were clustered using Partitioning Around Medoids (PAM) to find a central lipid and cluster other correlated lipids around it. This statistical method correlates lipids that are related to each other without consideration of clinical classification and is performed to see if lipids can be eliminated from a final model if they behave in the same way as other lipids in their respective clusters in terms of predicting clinical classification. A CLARA (Clustering for Large Applications) algorithm was applied to create a clusplot of correlated lipids which resulted in the formation of four lipid clusters, listed in Table 3.11 and represented graphically in Figure 3.17.

Table 3.11: Four groups of clustered lipids were determined using medoid clustering in HC vs. AD.

| Cluster 1 | Cluster 2 | Cluster 3 | Cluster 4 |
|-----------|----------------|-----------------|----------------|
| PE34.2 | PI34.0 | PC34.0p..34.1e | Cer.d18.0.20.0 |
| PE34.1 | PG34.2 | PC36.0p/etherPC | Cer.d18.0.22.0 |
| PE36.4 | PC38.5p..38.6e | PC32.0e | Cer.d18.0.24.1 |
| PE36.3 | SM18.16.1 | PC32.0 | |
| PE36.2 | SM18.20.1 | PC34.0e | |
| PE38.5 | SM18.24.0 | | |
| PE38.4 | | | |
| PE34.1 | | | |

PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine, Cer: Ceramide, PG: Phosphatidylglycerol, PI: Phosphatidylinositol.

Of the 22 lipids selected by Variable Selection Pathways for HC vs. AD, cluster 1 has eight lipids, cluster 2 has six lipids, cluster 3 has five lipids and cluster 4 has three lipids.

**Figure 3.17: Clusplot of correlated lipids showing the 8, 6, 5 and 3 lipids from clusters 1 to 4 respectively.**

The clusplot shows a stronger relationship for the lipids within each of clusters 1, 3 and 4. The lipids in cluster 2 are by contrast more diffuse, suggesting a weaker intracluster relationship of those lipids. This may be explained by the fact that clusters 1, 3 and 4 all contain lipids of the same class (PE, PC and ceramides respectively), whilst cluster 2 is heterogeneous containing one PI species, one PG species, one ether PC species and three SM species.

Standard Pearson's correlations were applied to the lipids in each cluster to explore the directional grouping of the relationship between correlated lipids and clinical classification. These relationships between the scaled mean expression for HC and AD groups are shown in Figure 3.18 with the R-values for the lipid correlations shown in Table 3.12.

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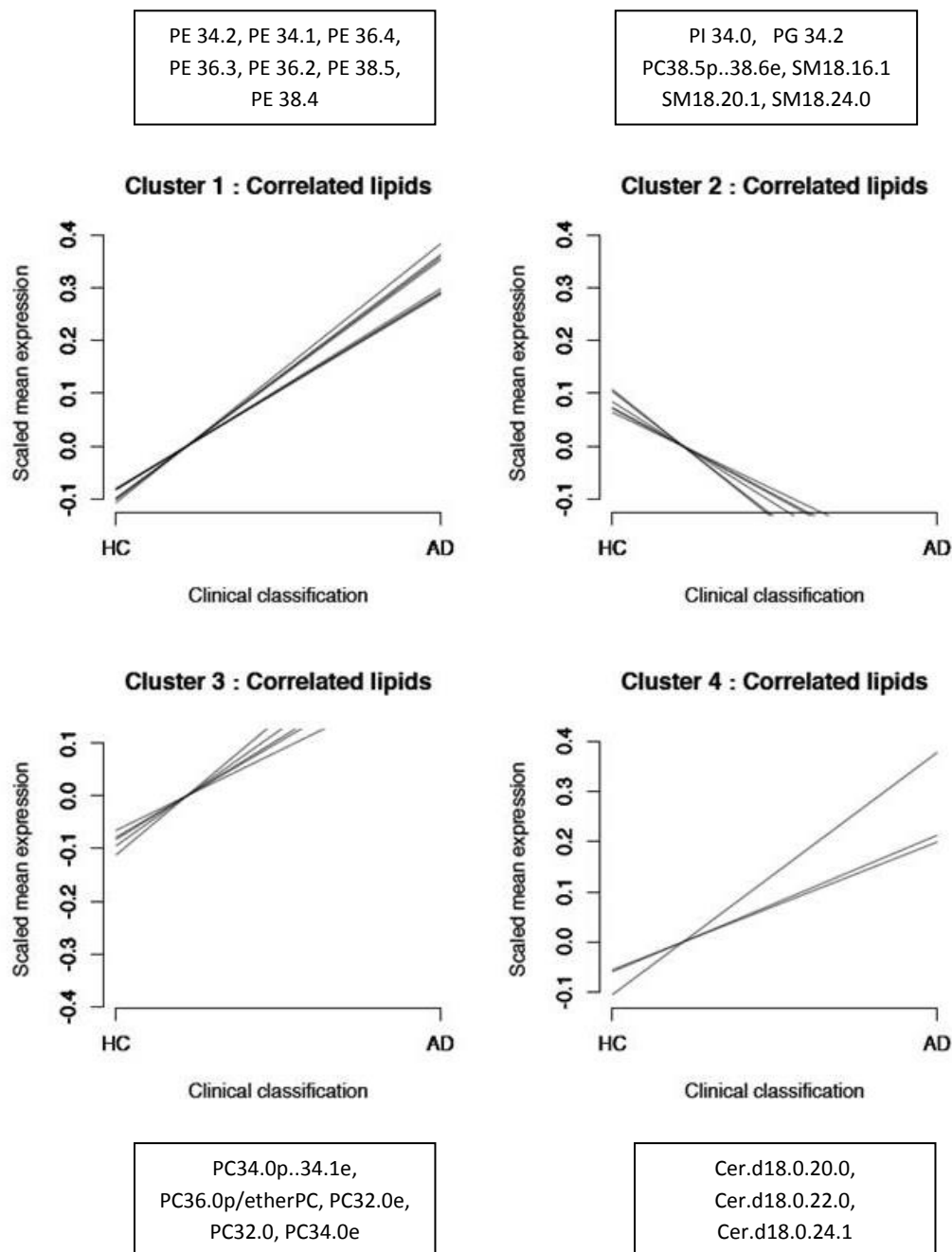


Figure 3.18: Correlated lipids between clinical classifications.

PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine, Cer: Ceramide, PG: Phosphatidylglycerol, PI: Phosphatidylinositol. Scaled mean expression is used to enable graphical representation of lipids that differ in abundance.

| | PE34.2 | PE34.1 | PE36.4 | PE36.3 | PE36.2 | PE38.5 | PE38.4 | PI.34.0 | PG34.2 | PC.34.0p..3 | PC36.0p.or | PC.38.5p..3 | PC32.0 | PC32.0 | PC34.0e | PC34.1 | SM18.16.1 | SM18.20.1 | SM18.24.0 | Cer.d18.0 | Cer.d18.0.2 | Cer.d18.0.24.1 | |
|--------|--------|--------|--------|--------|--------|--------|--------|---------|--------|-------------|------------|-------------|--------|--------|---------|--------|-----------|-----------|-----------|-----------|-------------|----------------|--------------------|
| | 1.000 | 0.625 | 0.640 | 0.643 | 0.707 | 0.483 | 0.538 | 0.131 | 0.178 | 0.162 | 0.067 | -0.397 | 0.062 | 0.122 | 0.109 | 0.264 | -0.040 | -0.050 | -0.069 | 0.070 | 0.048 | -0.020 | PE34.2 |
| | | 1.000 | 0.672 | 0.589 | 0.621 | 0.535 | 0.607 | 0.151 | 0.198 | 0.153 | 0.086 | -0.331 | 0.044 | 0.171 | 0.124 | 0.336 | -0.025 | -0.087 | -0.068 | 0.068 | 0.046 | 0.009 | PE34.1 |
| | | | 1.000 | 0.645 | 0.626 | 0.646 | 0.763 | 0.189 | 0.194 | 0.123 | 0.085 | -0.329 | 0.017 | 0.153 | 0.127 | 0.318 | -0.012 | -0.011 | -0.082 | 0.065 | 0.035 | 0.001 | PE36.4 |
| | | | | 1.000 | 0.692 | 0.554 | 0.583 | 0.069 | 0.206 | 0.118 | 0.086 | -0.368 | 0.006 | 0.041 | 0.072 | 0.202 | -0.043 | -0.020 | -0.078 | 0.098 | 0.056 | 0.016 | PE36.3 |
| | | | | | 1.000 | 0.516 | 0.597 | 0.168 | 0.198 | 0.070 | 0.017 | -0.428 | -0.010 | 0.040 | 0.026 | 0.221 | -0.048 | -0.008 | -0.047 | 0.088 | 0.051 | 0.002 | PE36.2 |
| | | | | | | 1.000 | 0.718 | 0.049 | 0.087 | 0.113 | 0.120 | -0.105 | 0.019 | 0.133 | 0.122 | 0.226 | -0.081 | -0.110 | -0.135 | 0.098 | 0.075 | 0.006 | PE38.5 |
| | | | | | | | 1.000 | 0.050 | 0.166 | 0.058 | 0.071 | -0.330 | -0.026 | 0.070 | 0.079 | 0.226 | -0.042 | -0.081 | -0.093 | 0.084 | 0.084 | 0.031 | PE38.4 |
| | | | | | | | | 1.000 | 0.258 | 0.088 | -0.013 | -0.137 | 0.096 | 0.174 | 0.098 | 0.215 | -0.017 | 0.290 | 0.056 | 0.082 | 0.008 | 0.008 | PI.34.0 |
| | | | | | | | | | 1.000 | -0.040 | 0.028 | -0.150 | -0.008 | -0.052 | 0.008 | -0.057 | 0.219 | 0.380 | 0.301 | 0.111 | 0.047 | 0.028 | PG34.2 |
| .1e | | | | | | | | | | 1.000 | 0.696 | 0.017 | 0.750 | 0.558 | 0.862 | 0.351 | 0.073 | 0.026 | -0.026 | 0.077 | 0.037 | 0.004 | PC.34.0p..34.1e |
| therPC | | | | | | | | | | | 1.000 | 0.033 | 0.488 | 0.172 | 0.744 | 0.204 | 0.147 | 0.052 | -0.033 | -0.025 | -0.025 | -0.015 | PC36.0p.or.etherPC |
| .6e | | | | | | | | | | | | 1.000 | 0.171 | 0.115 | 0.107 | -0.327 | 0.100 | -0.083 | 0.105 | -0.076 | -0.045 | 0.037 | PC.38.5p..38.6e |
| | | | | | | | | | | | | | 1.000 | 0.619 | 0.773 | 0.150 | 0.123 | -0.012 | 0.104 | 0.022 | -0.004 | 0.014 | PC32.0e |
| | | | | | | | | | | | | | | 1.000 | 0.574 | 0.423 | 0.073 | -0.129 | 0.029 | -0.030 | -0.076 | -0.110 | PC32.0 |
| | | | | | | | | | | | | | | | 1.000 | 0.272 | 0.125 | 0.050 | 0.016 | 0.066 | 0.006 | -0.008 | PC34.0e |
| | | | | | | | | | | | | | | | | 1.000 | -0.300 | -0.174 | -0.303 | 0.140 | 0.119 | 0.056 | PC34.1 |
| | | | | | | | | | | | | | | | | | 1.000 | 0.305 | 0.525 | -0.436 | -0.419 | -0.352 | SM18.16.1 |
| | | | | | | | | | | | | | | | | | | 1.000 | 0.348 | 0.164 | 0.104 | 0.108 | SM18.20.1 |
| | | | | | | | | | | | | | | | | | | | 1.000 | -0.111 | -0.141 | -0.093 | SM18.24.0 |
| .0 | | | | | | | | | | | | | | | | | | | | 1.000 | 0.804 | 0.694 | Cer.d18.0.20.0 |
| .0 | | | | | | | | | | | | | | | | | | | | | 1.000 | 0.786 | Cer.d18.0.22.0 |
| .1 | | | | | | | | | | | | | | | | | | | | | | 1.000 | Cer.d18.0.24.1 |

Table 3.12: Standard Pearson's Correlations between 22 lipid species chosen from the variable selection pathway for the HC vs. AD groups. Correlation coefficients (R) for each pairwise correlation are shown. Correlations of greater than 0.5 or less than -0.5 are highlighted in red.

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The strongest correlations, as highlighted in red in Table 3.12, are seen between lipids of the same group. i.e. PEs, PCs or ceramides.

Looking at the directional grouping of the relationship between correlated lipids and clinical classification as depicted in Figure 3.18, it can be seen that each of the lipids in each cluster have very similar relationships with each other.

Taking the 22 lipids chosen from the variable selection pathway and factoring in their correlations as determined by the Pearson's correlations R-values (see Table 3.12), the lipids were subjected to further statistical assessment using a stepwise generalised linear model (GLM) to reduce the number of lipids in the final predictive model. This GLM algorithm was run repeatedly to remove lipids from the predictive model until the lipids left were those giving the best Akaike Information Criterion (AIC) report, which is a measure of the relative quality of a statistical model for a given set of data and therefore provides a means for selecting the best model. This stepwise model also factors in covariates of age, gender, APOE ϵ 4 allele status and collection site, as described in the previous GLM (Table 3.8). This prediction model split the analysis of the 1060 study participant plasma lipid profiles into a run comprising 70% training (that includes clinical classification) and 30% test data (blind to clinical classification) and was repeated over 100 times until the greatest sensitivity and specificity was achieved. A final panel was selected that contained the least number of lipids with maximal sensitivity and specificity for predicting clinical classification.

Sensitivity is the ability of a biomarker to correctly identify AD and specificity is the ability of a biomarker to correctly identify a healthy control. Maximal sensitivity and specificity (80%) for predicting participants with AD as compared to healthy controls was attained by variable selection and stepwise GLM with the following six lipids.

Table 3.13: List of six lipids yielding maximal sensitivity and specificity between HC and AD groups.

| Lipid |
|--------------------|
| PI.34.0 |
| PC36.0p.or.etherPC |
| PC38.5p..38.6e |
| PC32.0 |
| SM18.20.1 |
| Cer.d18.0.22.0 |

PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine, Cer: Ceramide, PI: Phosphatidylinositol.

All of these lipid species were also identified as being significantly different when assessed by the standard LIMMA analyses.

Figure 3.19 shows a Receiver Operating Characteristic (ROC) curve from the GLM of the top six lipids including age, gender, APOE ϵ 4 allele status and collection site as covariates.

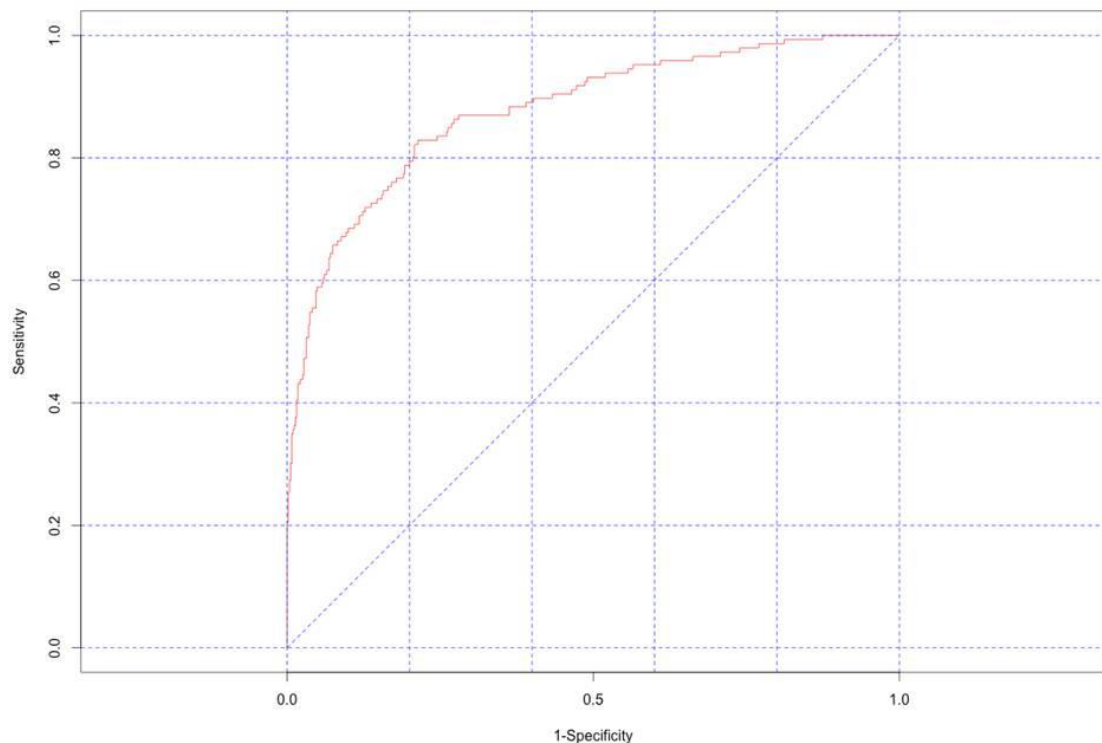


Figure 3.19: ROC curve of the top six lipids from stepwise GLM showing sensitivity and specificity of 80% for the HC vs. AD group.

The ROC curve is a plot of the true positive rate (sensitivity) against the false positive rate (specificity) of a diagnostic test. The closer the curve follows the left hand border and then the top border, the more accurate the test. The accuracy depends on how well the panel of six lipids separates those participants with AD from healthy controls and is measured by the area under the ROC curve, which in this case indicates 80%.

3.4.3.2 Variable selection for HC vs. MCI groups

As for the healthy controls vs. AD groups, the variable selection pathway was repeated for the healthy control vs. MCI groups. This generated a list of lipids that were chosen from four separate selection methods as previously described for the HC vs. AD groups. The variable selection pathway was again applied to the 189 lipid species to create an importance ranking (in addition to generation of p-values), where 1000 iterations were performed to define a list of those lipid species that are selected most often as differing in levels when comparing HC vs. MCI. Each time a lipid was selected a frequency score was assigned if it was chosen from all four statistical methods or from three out of the four statistical methods. Table 3.14 lists the lipid species selected most often when comparing the HC and MCI groups. As before, the lipid species that were selected in at least 10% of the 1000 iterations from all four statistical methods are listed in column A and the lipid species that were selected in at least 10% of the 1000 iterations from three of the four statistical methods are shown in column B.

Table 3.14: Lipid Markers selected by the Variable Selection Pathway for HC vs. MCI

| List A (4/4) | | List B (3/4) | | | |
|----------------|--------------|-----------------|--------------|--------------------|--------------|
| Lipid | Freq (/1000) | Lipid | Freq (/1000) | Lipid | Freq (/1000) |
| SM18.20.1 | 609 | PC34.1 | 998 | SM18.18.1 | 274 |
| Cer.d18.0.22.0 | 418 | PC.38.5p..38.6e | 944 | SM18.22.0 | 271 |
| PC34.0e | 173 | SM18.24.0 | 863 | PC38.1 | 249 |
| PC32.0 | 108 | SM18.20.1 | 841 | LysoPE18.1 | 235 |
| | | PE34.2 | 739 | PC36.0p.or.etherPC | 231 |
| | | Cer.d18.0.22.0 | 708 | PI34.0 | 224 |
| | | PC32.0 | 641 | PE36.2 | 201 |
| | | PE38.4 | 628 | PC38.1p..38.2e | 190 |
| | | SM18.16.1 | 612 | PE40.4p | 177 |
| | | PC34.0e | 539 | SM18.18.0 | 174 |
| | | PG34.2 | 476 | Cer.d18.0.20.0 | 157 |
| | | PE36.4 | 467 | SM18.24.1 | 149 |
| | | PC34.0p..34.1e | 452 | Cer.d18.0.24.1 | 120 |
| | | PE36.3 | 316 | LysoPE18.2 | 114 |
| | | PE34.1 | 291 | LysoPC18.0e | 108 |
| | | PC32.0e | 277 | PC34.2 | 101 |
| | | PE38.5 | 277 | | |

SM: Sphingomyelin, Cer: Ceramide, PC: Phosphatidylcholine, PE: Phosphatidylethanolamine, PG: Phosphatidylglycerol, PI: Phosphatidylinositol. The lipids highlighted in grey appeared in both list A and list B.

Those lipids with the highest frequencies of selection from both lists A and B of Table 3.14 were clustered as done previously for the HC vs. AD groups. Clustering of the lipids for the HC vs. MCI groups resulted in the formation of four lipid clusters listed in Table 3.15.

Table 3.15: Four groups of clustered lipids were determined using medoid clustering in HC vs. MCI.

| Cluster 1 | Cluster 2 | Cluster 3 | Cluster 4 |
|------------|----------------|-----------------|----------------|
| LysoPE18.2 | PG34.2 | LysoPC18.0e | PC34.2 |
| LysoPE18.1 | PC38.5p..38.6e | PC34.0p..34.1e | Cer.d18.0.20.0 |
| PE40p.4 | PC38.1 | PC36.0p/etherPC | Cer.d18.0.22.0 |
| PE34.2 | SM18.16.1 | PC38.1p..38.2e | Cer.d18.0.24.1 |
| PE34.1 | SM18.18.1 | PC32.0e | |
| PE36.4 | SM18.18.0 | PC32.0 | |
| PE36.3 | SM18.20.1 | PC34.0e | |
| PE36.2 | SM18.22.0 | | |
| PE38.5 | SM18.24.1 | | |
| PE38.4 | SM18.24.0 | | |
| PI34.0 | | | |
| PC34.1 | | | |

PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine, Cer: Ceramide, PG: Phosphatidylglycerol, PI: Phosphatidylinositol.

Of the 33 lipids selected by Variable selection Pathways for HC vs. MCI, cluster 1 has twelve lipids, cluster 2 has ten lipids, cluster 3 has seven lipids and cluster 4 has three lipids. A clusplot was generated (Figure 3.20) by plotting the lipids as clusters of correlations, which revealed that certain lipids fall into groups.

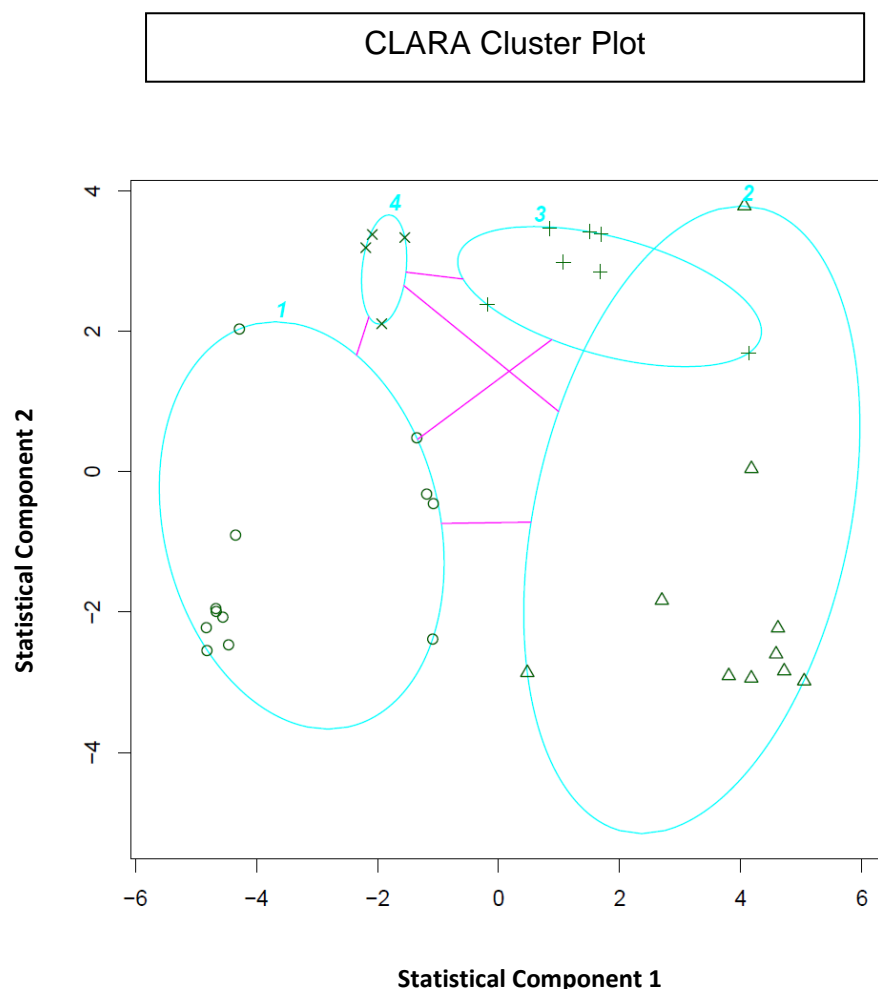


Figure 3.20: Clusplot of correlated lipids showing the 12, 10, 7 and 4 lipids from clusters 1 to 4 respectively.

The clusplot shows a stronger relationship for the lipids within each of clusters 3 and 4. The lipids in clusters 1 and 2 are by contrast more diffuse, suggesting a weaker intracluster relationship of those lipids. This may be explained by the fact that cluster 3 contains all PC species and cluster 4 contains mainly ceramides. Clusters 1 and 2 are more heterogeneous in terms of the lipid species they contain.

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Standard Pearson's correlations were applied to the lipids in each cluster to explore the directional grouping of the relationship between correlated lipids and clinical classification. These relationships between the scaled mean expression for HC and MCI groups are shown in Figure 3.21 with the R-values for the lipid correlations shown in Table 3.16.

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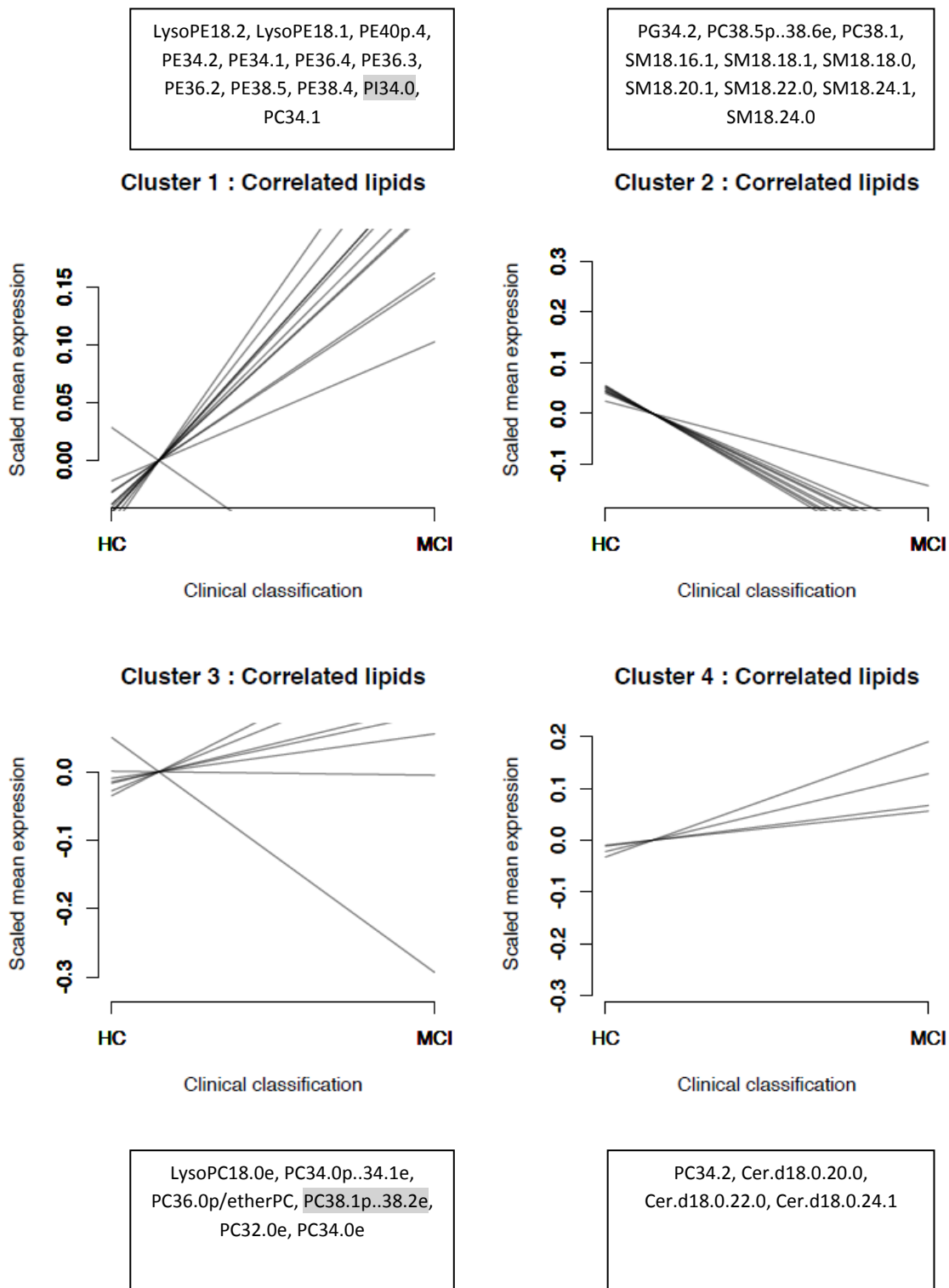


Figure 3.21: Correlated lipids between clinical classifications.

PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine, Cer: Ceramide, PG: Phosphatidylglycerol, PI: Phosphatidylinositol. Scaled mean expression is used to enable graphical representation of lipids that differ in abundance. The lipids species highlighted in grey show a different directional change than the other lipids in the cluster.

| Lyso.PE18.2 | Lyso.PE18.1.1 | PE34.2 | PE34.1 | PE36.4 | PE36.3 | PE36.2 | PE38.5 | PE38.4 | PI.34.0 | PG34.2 | PC.Lyso.18e.0 | PC.34.0p..34.1e | PC36.0p.or.etherPC | PC.38.5p..38.6e | PC.38.1p..38.2e | PC32.0e | PC32.0 | PC34.0e | PC34.2 | PC34.1 | PC38.1 | SM18.16.1 | SM18.18.1 | SM18.18.0 | SM18.20.1 | SM18.22.0 | SM18.24.1 | SM18.24.0 | Cer.d18.0.20.0 | Cer.d18.0.22.0 | Cer.d18.0.24.1 | | |
|-------------|---------------|--------|--------|--------|--------|--------|--------|--------|---------|--------|---------------|-----------------|--------------------|-----------------|-----------------|---------|--------|---------|--------|--------|--------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|----------------|----------------|----------------|----------------|--------------------|
| 1.000 | 0.222 | 0.035 | 0.383 | 0.253 | 0.188 | 0.362 | 0.364 | 0.040 | 0.112 | 0.172 | 0.364 | 0.169 | 0.141 | 0.070 | -0.406 | 0.053 | 0.114 | 0.012 | 0.078 | 0.192 | 0.043 | -0.024 | 0.166 | 0.080 | 0.068 | 0.212 | 0.363 | 0.140 | 0.176 | 0.020 | -0.007 | -0.019 | Lyso.PE18.2 |
| | 1.000 | 0.141 | 0.160 | 0.164 | 0.118 | 0.114 | 0.117 | 0.109 | 0.135 | -0.050 | 0.118 | 0.148 | 0.032 | 0.023 | -0.069 | -0.064 | 0.025 | 0.034 | 0.026 | -0.016 | 0.048 | -0.041 | 0.002 | 0.034 | 0.034 | -0.089 | 0.011 | 0.064 | 0.031 | -0.006 | 0.016 | 0.012 | Lyso.PE18.1.1 |
| | | 1.000 | 0.153 | 0.193 | 0.257 | 0.166 | 0.140 | 0.289 | 0.388 | -0.073 | 0.032 | 0.177 | 0.134 | 0.109 | -0.027 | 0.078 | 0.083 | 0.058 | 0.156 | -0.149 | 0.007 | 0.089 | -0.016 | 0.009 | 0.025 | -0.061 | -0.055 | 0.054 | 0.042 | 0.062 | 0.051 | 0.077 | PE40p.4 |
| | | | 1.000 | 0.613 | 0.638 | 0.630 | 0.693 | 0.489 | 0.552 | 0.153 | 0.184 | -0.018 | 0.119 | 0.033 | -0.413 | -0.073 | 0.058 | 0.105 | 0.058 | 0.118 | 0.248 | -0.130 | 0.001 | 0.016 | 0.010 | -0.034 | 0.048 | 0.040 | -0.020 | 0.013 | -0.011 | -0.051 | PE34.2 |
| | | | | 1.000 | 0.664 | 0.568 | 0.604 | 0.531 | 0.588 | 0.176 | 0.176 | -0.017 | 0.107 | 0.049 | -0.358 | -0.126 | 0.018 | 0.157 | 0.079 | -0.046 | 0.341 | -0.144 | -0.008 | -0.008 | -0.028 | -0.076 | -0.073 | 0.009 | -0.030 | 0.035 | 0.014 | -0.008 | PE34.1 |
| | | | | | 1.000 | 0.612 | 0.618 | 0.653 | 0.759 | 0.210 | 0.173 | -0.033 | 0.095 | 0.052 | -0.352 | -0.108 | -0.003 | 0.145 | 0.087 | -0.148 | 0.322 | -0.163 | -0.006 | 0.020 | 0.015 | -0.005 | -0.146 | 0.033 | -0.075 | 0.031 | -0.005 | -0.028 | PE36.4 |
| | | | | | | 1.000 | 0.680 | 0.552 | 0.566 | 0.098 | 0.189 | 0.041 | 0.103 | 0.070 | -0.376 | -0.033 | 0.018 | 0.032 | 0.056 | 0.097 | 0.194 | -0.137 | -0.054 | -0.063 | -0.089 | -0.031 | -0.016 | -0.030 | -0.077 | 0.077 | 0.038 | -0.003 | PE36.3 |
| | | | | | | | 1.000 | 0.530 | 0.609 | 0.154 | 0.187 | -0.025 | 0.038 | 0.001 | -0.448 | -0.102 | -0.026 | 0.027 | -0.001 | 0.141 | 0.203 | -0.124 | -0.033 | -0.048 | -0.049 | -0.027 | 0.055 | -0.019 | -0.047 | 0.047 | 0.020 | -0.015 | PE36.2 |
| | | | | | | | | 1.000 | 0.715 | 0.053 | 0.078 | 0.086 | 0.067 | 0.084 | -0.113 | -0.040 | -0.016 | 0.088 | 0.079 | -0.191 | 0.191 | -0.089 | -0.094 | -0.054 | -0.045 | -0.122 | -0.227 | -0.036 | -0.119 | 0.080 | 0.053 | 0.034 | PE38.5 |
| | | | | | | | | | 1.000 | 0.050 | 0.144 | 0.062 | 0.016 | 0.024 | -0.341 | -0.103 | -0.059 | 0.040 | 0.043 | -0.188 | 0.180 | -0.110 | -0.038 | 0.025 | 0.030 | -0.081 | -0.126 | 0.072 | -0.057 | 0.057 | 0.040 | 0.012 | PE38.4 |
| | | | | | | | | | | 1.000 | 0.248 | -0.192 | 0.106 | 0.002 | -0.145 | -0.004 | 0.113 | 0.221 | 0.119 | -0.049 | 0.259 | -0.065 | -0.020 | -0.050 | -0.007 | 0.289 | 0.006 | 0.048 | 0.053 | 0.073 | 0.026 | 0.012 | PI.34.0 |
| | | | | | | | | | | | 1.000 | 0.049 | -0.011 | 0.078 | -0.176 | 0.085 | 0.015 | -0.060 | 0.035 | -0.172 | -0.037 | 0.026 | 0.209 | 0.204 | 0.160 | 0.386 | 0.266 | 0.290 | 0.322 | 0.104 | 0.052 | 0.048 | PG34.2 |
| | | | | | | | | | | | | 1.000 | 0.293 | 0.530 | 0.147 | 0.339 | 0.289 | -0.064 | 0.393 | -0.101 | -0.178 | 0.058 | 0.051 | 0.044 | 0.036 | -0.169 | -0.003 | -0.023 | -0.040 | 0.089 | 0.132 | 0.172 | PC.Lyso.18e.0 |
| | | | | | | | | | | | | | 1.000 | 0.675 | 0.023 | 0.447 | 0.758 | 0.505 | 0.848 | -0.009 | 0.302 | 0.002 | 0.074 | 0.081 | 0.137 | 0.037 | -0.019 | 0.033 | -0.028 | 0.052 | -0.011 | -0.046 | PC.34.0p..34.1e |
| | | | | | | | | | | | | | | 1.000 | 0.026 | 0.558 | 0.445 | 0.080 | 0.733 | -0.162 | 0.137 | 0.009 | 0.153 | 0.209 | 0.201 | 0.063 | 0.008 | -0.001 | -0.048 | -0.022 | -0.044 | -0.039 | PC36.0p.or.etherPC |
| | | | | | | | | | | | | | | | 1.000 | 0.193 | 0.153 | 0.099 | 0.094 | -0.256 | -0.338 | 0.290 | 0.073 | 0.026 | 0.044 | -0.106 | -0.104 | 0.012 | 0.061 | -0.064 | -0.020 | 0.052 | PC.38.5p..38.6e |
| | | | | | | | | | | | | | | | | 1.000 | 0.312 | 0.021 | 0.456 | -0.141 | -0.329 | 0.355 | 0.332 | 0.321 | 0.276 | 0.235 | 0.162 | 0.269 | 0.247 | 0.072 | 0.032 | 0.003 | PC.38.1p..38.2e |
| | | | | | | | | | | | | | | | | | 1.000 | 0.592 | 0.767 | 0.070 | 0.116 | 0.110 | 0.111 | 0.087 | 0.192 | -0.016 | 0.036 | 0.092 | 0.107 | 0.017 | -0.021 | -0.005 | PC32.0e |
| | | | | | | | | | | | | | | | | | 1.000 | 0.510 | -0.105 | 0.417 | 0.050 | 0.052 | -0.001 | 0.124 | -0.145 | -0.231 | 0.010 | 0.033 | -0.065 | -0.130 | -0.153 | PC32.0 | |
| | | | | | | | | | | | | | | | | | | 1.000 | -0.146 | 0.227 | 0.031 | 0.133 | 0.163 | 0.232 | 0.072 | -0.031 | 0.046 | 0.026 | 0.047 | -0.032 | -0.044 | PC34.0e | |
| | | | | | | | | | | | | | | | | | | | 1.000 | 0.060 | -0.149 | -0.319 | -0.329 | -0.287 | -0.009 | 0.155 | -0.336 | -0.179 | 0.200 | 0.199 | 0.147 | PC34.2 | |
| | | | | | | | | | | | | | | | | | | | | 1.000 | -0.306 | -0.308 | -0.243 | -0.186 | -0.175 | -0.416 | -0.252 | -0.298 | 0.126 | 0.077 | 0.012 | PC34.1 | |
| | | | | | | | | | | | | | | | | | | | | | 1.000 | 0.299 | 0.101 | 0.224 | -0.013 | 0.163 | 0.412 | 0.635 | -0.104 | -0.096 | -0.051 | PC38.1 | |
| | | | | | | | | | | | | | | | | | | | | | | 1.000 | 0.739 | 0.293 | 0.486 | 0.509 | 0.534 | -0.448 | -0.429 | -0.350 | SM18.16.1 | | |
| | | | | | | | | | | | | | | | | | | | | | | | 1.000 | 0.813 | 0.379 | 0.400 | 0.553 | 0.414 | -0.241 | -0.246 | -0.208 | SM18.18.1 | |
| | | | | | | | | | | | | | | | | | | | | | | | | 1.000 | 0.306 | 0.358 | 0.548 | 0.488 | -0.262 | -0.292 | -0.256 | SM18.18.0 | |
| | | | | | | | | | | | | | | | | | | | | | | | | | 1.000 | 0.468 | 0.342 | 0.333 | 0.184 | 0.134 | 0.113 | SM18.20.1 | |
| | | | | | | | | | | | | | | | | | | | | | | | | | | 1.000 | 0.469 | 0.563 | -0.120 | -0.118 | -0.126 | SM18.22.0 | |
| | | | | | | | | | | | | | | | | | | | | | | | | | | | 1.000 | 0.723 | -0.059 | -0.064 | -0.042 | SM18.24.1 | |
| | | | | | | | | | | | | | | | | | | | | | | | | | | | | 1.000 | -0.104 | -0.127 | -0.094 | SM18.24.0 | |
| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 1.000 | 0.814 | 0.712 | Cer.d18.0.20.0 | |
| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 1.000 | 0.802 | Cer.d18.0.22.0 | |
| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 1.000 | | Cer.d18.0.24.1 |

Table 3.16: Standard Pearson's Correlations between 33 lipid species chosen from the variable selection pathway for the HC vs. MCI groups.
Correlation coefficients (R) for each pairwise correlation are shown. Correlations of greater than 0.5 or less than -0.5 are highlighted in red.

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The strongest correlations, as highlighted in red in Table 3.16, are seen between lipids of the same group. i.e. PEs, PCs or ceramides. A statistical correlation between the biologically related SM18.24.0 and PC38.1 is also shown. SM can be formed by the transfer of phosphocholine to ceramide.

Looking at the directional grouping of the relationship between correlated lipids and clinical classification as depicted in Figure 3.21, it can be seen that each of the lipids in clusters 2 and 4 have very similar relationships with each other. Clusters 1 and 3 have one lipid in each cluster that behaves differently to the other lipids (highlighted in grey in Figure 3.21). PI34.0 and PC38.1..38.2e levels are decreased in MCI compared to healthy controls in clusters 1 and 3 respectively.

As per the statistical methodology employed for the HC vs. AD groups, taking the 33 lipids chosen from the variable selection pathway for HC vs. MCI and factoring in their correlations as determined by the Pearson's correlations R-values (see Table 3.16), the lipids were subjected to further statistical assessment using a stepwise generalised linear model (GLM) to reduce the number of lipids in the final predictive model. The GLM algorithm was run repeatedly to remove lipids from the predictive model until the lipids left were those giving the best AIC report, as previously described for the healthy controls and AD groups. This stepwise model again factors in covariates of age, gender, APOEε4 allele status and collection site, as described in the previous GLM (Table 3.9). The prediction model once again split the analysis of the 1060 study participant plasma lipid profiles into a run comprising 70% training and 30% test data and was repeated over 100 times until the greatest sensitivity and specificity was achieved.

A final panel was selected that contained the least number of lipids with maximal sensitivity and specificity for predicting clinical classification. In this case sensitivity represents the ability of a biomarker to correctly identify MCI and specificity is the ability of a biomarker to correctly identify a healthy control. Maximal sensitivity and specificity (71%) for predicting participants with MCI as compared to healthy controls was attained by variable selection and stepwise GLM with the following four lipids listed in Table 3.17.

Table 3.17: List of four lipids giving maximal sensitivity and specificity between the HC and MCI groups.

| Lipid |
|---------------|
| PE34.1 |
| PE34.2 |
| LysoPE18.1 |
| PG34.2 |

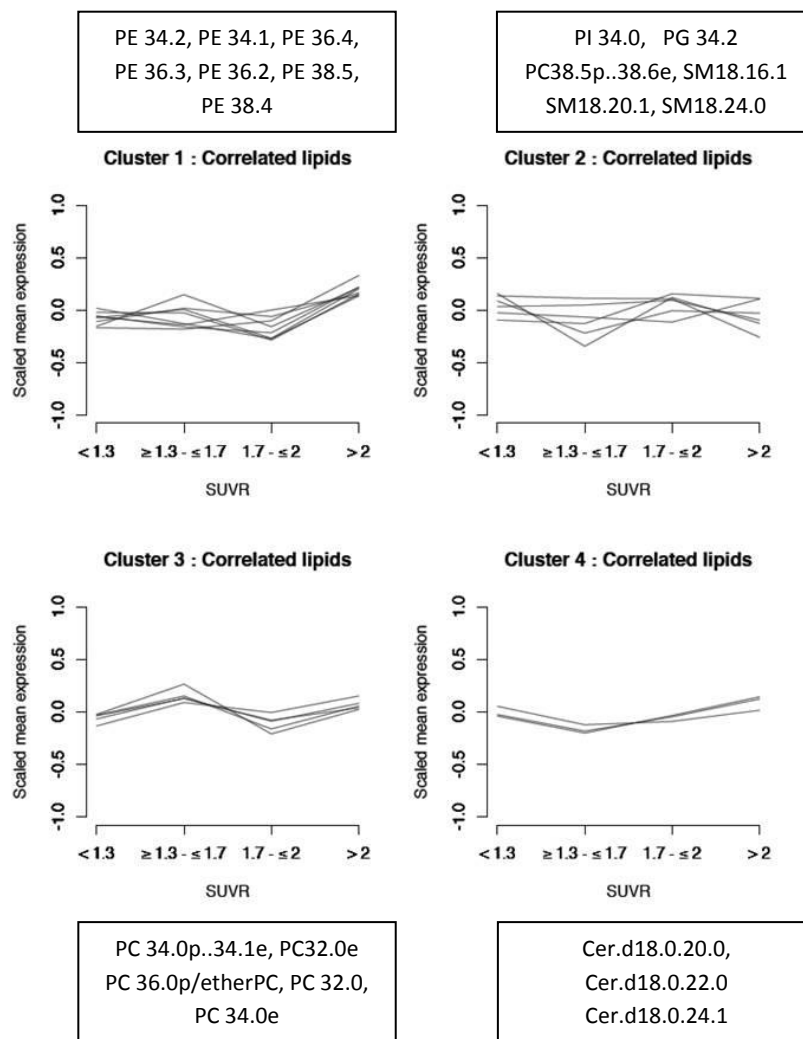
The bolded lipid, PE34.1, was also identified in the standard LIMMA analyses.

Lower sensitivity and specificity is observed for the 4 lipids predicting MCI as compared to the 6 lipids capable of predicting AD (Table 3.13). This may be due to the fact that the HC vs. MCI group is less discrete than the HC vs. AD group with more overlap between profiles. The MCI group is clinically more heterogeneous and often but not always represents a prodromal stage of AD. This may explain the sensitivity and specificity of 71% for the HC vs. MCI group as compared to 80% for the HC vs. AD group.

3.4.4 Correlation with Standardised Uptake Value Ratio (SUVR)

The previous section assessed the ability of a panel of lipids to predict clinical classification with the aim of identifying a sensitive and specific diagnostic blood lipid biomarker panel. Cerebral amyloid burden as determined by PiB-PET neuroimaging, whilst representing a useful AD biomarker is expensive to perform, requires access to specialist facilities, and is associated with radiation exposure. Therefore development of a blood based biomarker test to replace neuroimaging is highly desirable. Consequently, the relationship of the panel of lipids determined in section 3.4.3 by variable selection analysis was assessed against neocortical amyloid burden represented as SUVR; an established neuroimaging biomarker of AD. Each of the 22 lipids (as they sit in the four clusters) identified in the variable selection pathway (Section 3.4.3) for healthy controls compared to AD were plotted against categories of baseline SUVR. Figure 3.22 shows the clusters of correlated lipids compared to baseline SUVR categories for the healthy controls and AD groups.

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| SUVR category | Interpretation |
|-------------------|---|
| < 1.3 | Individuals with minimal accumulation of A β |
| > 1.3 to < 1.7 | Includes individuals with borderline transitions from amyloid -ve to amyloid +ve |
| 1.7 to \leq 2.0 | Individuals who are definite accumulators, with increased rate of A β accumulation |
| > 2.0 | Individuals with the highest A β accumulation but rate plateaus with saturation between 2-3 |

Figure 3.22: Clustered and correlated lipids compared with baseline SUVR categories. The SUVR categories used in this table are those described by Villemagne *et al* [477].

It is interesting to see that many of the lipids in cluster 1 have a similar profile, with increased means for those participants with an SUVR of greater than 2. A standard Pearson's Correlation test was performed with each of the 22 lipids that were selected from the variable selection pathway. This analysis shows

that the strongest correlation with SUVR was with PE34.2 ($R=0.22$), which belongs to cluster 1. However, when the panel of six lipids was assessed for its relationship with SUVR in the healthy control vs. AD groups no significant correlation was observed (data not presented). Additionally no figure is presented for the comparison of the HC vs. MCI groups with SUVR as this also did not show any significant correlation.

3.5 Discussion

Alterations in lipid metabolism have been demonstrated in a wide variety of neurological disorders, including Alzheimer's disease and Parkinson's disease [270, 478]. The amyloid precursor protein (APP) and the secretase enzymes associated with its processing are integral membrane proteins. Therefore the lipid environment in which these proteins are placed predictably regulate the processing of APP and formation of A β ; ultimately influencing AD pathogenesis. Numerous studies have established and verified this lipid-AD connection, with cholesterol, phospholipids and sphingolipids all playing a role (described in detail in Chapter 1). These studies have also given insight into the possible mechanisms by which lipids influence AD pathology [291, 414, 479, 480]. Changes in lipid species profiles have been reported with significant decreases in sulphatides in the brain and CSF of those individuals in the early stages of AD [413, 481]. Significantly increased CSF ceramides were also reported in AD [413]. Various groups have investigated specific classes of plasma or serum lipids. Studies demonstrated lower serum total ethanolamine plasmalogen levels as a predictor of cognitive decline [357] and another study showed reduced serum plasmalogen levels at all stages of dementia [341]. There have been inconsistencies in results from studies investigating LysoPC and PC ratios with some showing elevated ratios [375] and others showing decreased ratios [371]. Clear outcomes have also not resulted from various sphingolipidomic studies when serum ceramides and sphingomyelin have been measured for use as potential biomarkers [163, 422].

The advancement in technologies for lipidomic research has undoubtedly gained momentum in the past decade and made many of these studies possible. The current challenge, highlighted by a lack of consistency in previous

studies, is in the confident characterisation of the various lipid species to help establish a robust panel of lipid biomarkers that is specific and sensitive enough to predict clinical classification and possesses sufficient accuracy and precision in a laboratory setting to be feasible for routine use. The complexity of method choice used in lipidomic studies and the challenges associated with establishing large scale highly characterised cohorts can account for many of these inconsistencies in previous studies. A panel consisting of the least number of lipid species that achieves maximal sensitivity and specificity is sought as routine assay feasibility is a consideration, particularly as the increasing incidence of AD will predictably increase sample numbers requiring analysis. Therefore the purpose of the study presented in this chapter was to add to the existing literature by investigating plasma lipid profiles in a highly characterised cohort, where changes in lipid profiles were compared to supplementary data collected as part of this study, including brain amyloid burden. The highly characterised AIBL cohort provides a statistically relevant sample number of individuals whose clinical classification can be as accurate as possible with the aid of neuroimaging; helping to address some of the limitations of sample number with previous studies. The results were assessed for their ability to distinguish AD from cognitively normal individuals and those with mild cognitive impairment. The major findings from the study presented in this chapter were:

- 86 out of 189 plasma lipid species were significantly different when comparing the unadjusted mean values of the lipid species between clinical classifications.
- Twenty seven lipids were shown to be significantly different between the healthy control and AD groups by LIMMA analysis when applying a Bonferroni correction.
- 11 of these lipids remained significant after adjustment for age, gender, APOE ϵ 4 allele status and collection site when comparing the HC and AD groups.
- Using a variable selection pathway, a panel of six lipids was able to distinguish healthy controls from AD patients with a sensitivity and specificity of 80%.

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- Five lipid species were shown to be significantly different between the healthy control and MCI groups by LIMMA analysis when applying a Bonferroni correction.
- One lipid remained significant after adjustment for age, gender, APOE ϵ 4 allele status and collection site.
- Using a variable selection pathway, a panel of four lipids was able to distinguish healthy controls from those with MCI with a sensitivity and specificity of 71%.
- The LIMMA and GLM models did not identify any lipids that were significantly different between the MCI and AD groups.
- No significant correlation could be found when comparing SUVR categories and the results from the panel of six lipids identified by variable selection for the healthy vs. AD groups.

The heatmaps shown in Figure 3.3, the barcharts in Figures 3.4 - 3.16 and Table 3.3 showed significant differences between the unadjusted mean lipid species levels and clinical classification for 86 out of the 189 lipids measured. This significance was strongest for the greatest number of lipids when comparing the healthy control and AD groups. When comparing mean levels of summed lipid groups, statistical significance was only shown for total Phosphatidylethanolamine (PE) and Phosphatidylinositol (PI) species ($p < 0.001$ and $p < 0.05$ respectively) between the healthy control and AD groups. Between the healthy control and MCI classifications, statistical significance was only shown for total Sphingomyelin (SM) and Phosphatidylcholine (PC) species ($p < 0.05$). Interestingly, no significant summed lipids groups were identified when comparing the MCI and AD groups. When investigating changes in summed lipid groups, changes in individual species within a group may be masked due to the directional change being opposite in effect i.e. some increased and some decreased, which would neutralise any significance when looking at the sum of the lipid species within a group. Therefore considering the individual lipid species within a group will likely provide more meaningful results in terms of significant different in lipid species levels between clinical classification.

Chapter 3: Plasma Lipid Profiles in AIBL baseline samples

When considering the individual lipid species the most significant differences in levels between clinical classifications were seen with Phosphatidylethanolamine (PE), Phosphatidylinositol (PI), Phosphatidylcholine (PC), Sphingomyelin (SM) and ceramide species. Many of the PE species were elevated in AD compared to healthy controls. Similarly, increases in LysoPC's and ceramides were seen in the AD groups compared to healthy controls. The significant PI and SM species were lower in the AD compared to healthy controls. When comparing the healthy control and MCI groups, the predominant species that were significantly different were in the PE and SM groups, with higher and lower levels respectively in the MCI group. PC species were higher or lower in the MCI compared to the healthy control groups with the plasmalogen PC species being lower.

Changes in brain and plasma lipid composition and metabolism have been previously reported [127, 270, 335, 336, 371, 472] and while direct comparisons of the results presented here with other studies cannot be made due to study populations, analytical methodology and sample differences, the changes described in other studies show some similarity with respect to specific lipid groups. In agreement with other plasma studies we report reduced sphingomyelins, increased ceramides and increased LysoPCs in AD. A recent plasma lipidome study in metabolic syndrome identified increases in PC, PE, LysoPC and ceramides, with reduced plasmalogen species, suggesting a link between these lipids in both metabolic syndrome and AD [340]. Cardiovascular disease, diabetes and metabolic syndrome have all been linked to an increased risk of AD, and these changes in lipid metabolism which are common to AD and metabolic syndrome may represent causality or a similarity in metabolic changes that occur in the different conditions, or a combination of these possibilities. Longitudinal studies will help characterise more clearly the relationship between these conditions.

Our study has measured 189 lipids in plasma and has revealed changes in individual plasma lipid species that have not been simultaneously measured in previous studies. For example most studies investigating plasmalogen species have used a more targeted analytical approach which have focused on

ethanolamine plasmalogens only and have described reduced levels in AD [341, 357]. In contrast our study has measured PC and PE plasmalogen and shows that some are elevated (PE36p.2, PE40p.4, PE34p.3, PE34p.2, PE36p.1, PE38p.5, PE40p.5, PC34.0p..34.1e, PC36.1p/etherPC, PC36.0p/etherPC, PC32.0p, PC34.1p..34.2e) while others are reduced (LysoPE18.1p, PC36.4p/etherPC, PC38.5p..38.6e, PC32.0e). Interestingly, none of the species that are increased between the healthy control and AD groups contain DHA (22.6n-3), while one species that is reduced possibly does as indicated by an un-saturated bond count of at least 6 (PC38.5p..38.6e). This may reflect inadequate dietary intakes of polyunsaturated fatty acids or represent defects in the desaturase or elongase enzymes that produce the long chain polyunsaturated fatty acids from the essential fatty acids in AD patients. A more targeted analysis using liquid chromatography coupled with mass spectrometry would further separate the lipid subspecies and specifically identify the acyl chains of each species, including those with DHA.

Plasmalogens are abundant in the CNS and ethanolamine plasmalogens make up 30% of the total brain phospholipids and 90% of the ethanolamine glycerophospholipid species [339]. Plasmalogens and their precursors are supplied to the CNS by the liver. Decreased plasmalogen species in neuronal cell membranes can lead to increased membrane cholesterol as plasmalogen deficient cells exhibit lower HDL-mediated cholesterol efflux [356, 359]. This may therefore promote amyloidogenic processing of APP and increase A β generation (described in detail in Chapter 1, Section 1.5.1 and 1.5.2). An increase in A β_{42} can directly activate sphingomyelinases thereby reducing SM and elevating ceramide levels (see Chapter 1, Figure 1.13), consistent with the plasma levels reported in this chapter. The synthesis of plasmalogens is dependent on peroxisomes to generate the 1-alkyl-glycerol ether lipid precursors. The final elongation and desaturation step in the synthesis of DHA also occurs in peroxisomes and therefore reduced peroxisomal function can be suggested as a potential reason for reductions in both amongst the AD group.

The significant differences in individual lipid species between clinical classifications just described were identified from non-adjusted marginalised

means with an alpha level set at $p < 0.05$. Stricter criteria for determining any significant differences were applied using LIMMA analysis to identify those lipid species with p-values less than the Bonferroni cut off between clinical classifications. For the healthy control compared to AD group, 27 lipids were identified and for the healthy control compared to MCI group five lipids were identified. No lipids were significantly different between the MCI and AD groups. The lipids identified by LIMMA as being significant between clinical classifications were subjected to a Generalised Linear Model to control for age, APOE ϵ 4 genotype, gender and collection site. This model selected 11 of the 27 lipids for the healthy control vs. AD groups and one lipid for the healthy control vs. MCI group, following adjustment. The 11 lipids consisted of five PE species (PE38.4, PE34.2, PE38.5, PE36.2 and PE34.1), three PC species (PC34.0p..34.1e, PC34.1, PC34.0e), two SM species (SM18.20.1 and SM18.16.1) and one ceramide species (Cer.d18.0.22.0). SM18.16.1, the lipid identified in the HC vs. MCI groups was also selected in the HC vs. AD analysis post adjustment for age, gender, APOE ϵ 4 allele status and collection site. All eleven lipids, except the SM species were elevated in the AD group compared to healthy controls. The SM species were lower in the AD and MCI groups compared to healthy controls.

The four lipid groups (PE, PC, SM and Ceramide) that showed differences between HC and AD and the one species that was different between the HC and MCI groups have a strong biological relationship with each other and the directional change of these lipids with respect to clinical classification may be predicted. The interconnection of PE, PC, SM, Ceramide and sterol metabolism has been depicted in Chapter 1, Figure 1.9. The relative level of the lipid groups represents a balance between *de novo* synthesis, remodelling from other lipids, production of metabolites and breakdown. Production may be increased or breakdown enhanced by the action of various enzymes upregulated in response to a range of signals such as inflammatory cytokines, which have been shown to be upregulated in AD [482, 483]. We have shown that many PE and a few PC species are increased in AD, which could be an adaptive response in an attempt to increase precursors for neuronal repair. The elevated PE species may also be a result of reduced activity of PE methyltransferase

which converts PE to PC, especially when dietary choline intake is inadequate. The activity of this enzyme can be affected by nutrient status of the B group vitamins (see Chapter 1, Section 1.3.5), which may alter PC production and may reduce the delivery of polyunsaturated fatty acids from the liver to the peripheral tissues, including the CNS [149, 191]. The phosphocholine group of PC is transferred to ceramide for SM synthesis [348]. The increased PC, increased ceramide and reduced sphingomyelin may represent alterations in this pathway with reduced sphingomyelin synthase activity. Additionally, sphingomyelinase activity is increased in AD [157] and activated by inflammatory cytokines and oxidative stress, another likely contributor to AD pathogenesis [484], which can increase SM breakdown and further increase the ceramide pool.

From Table 3.8 it can be seen that only one lipid species (PC38.5p..38.6e) identified as being significantly different by LIMMA and GLM may contain docosahexaenoic acid (DHA) where levels are reduced in AD compared to healthy controls. However, PE38.4 and PE38.5 may contain arachidonic acid (AA, 20.4n-6) and these are increased in the AD group compared to healthy controls. The balance of omega-3 to omega-6 fatty acid containing phospholipids may be influenced by factors such as dietary intakes of fatty acids and other nutrients but also metabolic factors affecting the elongation and desaturation steps in polyunsaturated fatty acid pathways (see Chapter 1, Section 1.3.6). Increases in AA containing phospholipids may have implications in AD pathology (see Chapter 1, Section 1.5.10). Studies investigating lipid metabolism in the AD brain have confirmed increased membrane turnover and remodelling with increased expression of enzymes such as cPLA₂ (cytosolic phospholipase A₂) and sPLA₂ (secretory phospholipase A₂) involved with lipid remodelling [485, 486] and cyclooxygenase-2 (COX-2) associated with neuroinflammation [487]. Ante-mortem PET imaging in AD patients has also demonstrated increased brain AA metabolism which may increase production of prostaglandin E₂ [488]; a potent inflammatory eicosanoid which also stimulates A β formation through internalisation of the prostaglandin E receptor 4 (EP4) [489].

Chapter 3: Plasma Lipid Profiles in AIBL baseline samples

The associations between clinical classification and individual lipid species explored using LIMMA and GLM models in this chapter are useful in terms of speculating aberrant pathways that may be present in disease, but in terms of biomarker discovery, a different statistical approach was required to identify a panel of lipids that can predict clinical classification with the greatest certainty. The variable selection pathways, stepwise GLM model and ROC curves identified a panel of six lipids that gave a sensitivity and specificity of 80% when comparing healthy controls and AD. These lipids (also identified by the LIMMA analyses) were PI34.0, PC36.0p/etherPC, PC38.5p..38.6e, PC32.0, SM18.20.1 and Cer.d18.0.22.0. The levels of PC36.0p/etherPC, PC32.0 and Cer.d18.0.22.0 were increased and PI34.0, PC38.5p..38.6e and SM18.20.1 were decreased in the AD group compared to healthy controls. These six lipids were selected by statistical models for their ability to predict clinical classification rather than their ability to explain aberrant pathways which may explain why none of the PE species were selected when this statistical modelling was used.

Lower sensitivity and specificity is observed for the 4 lipids predicting MCI as compared to the 6 lipids capable of predicting AD (Table 3.13). As previously mentioned, this may be due to the fact that the HC vs. MCI group is less discrete than the HC vs. AD group with more overlap between profiles. The MCI group is clinically more heterogeneous and often but not always represents a prodromal stage of AD. This may explain the sensitivity and specificity of 71% for the HC vs. MCI group as compared to 80% for the HC vs. AD group.

The correlation between SUVR, a measure of brain amyloid burden and the lipid panel failed to show any significance in either the HC vs. AD or the HC vs. MCI groups. This may reflect a limitation of SUVR to distinguish between clinical classifications when used in isolation i.e. it is more helpful when SUVR is combined with other measures such as neuropsychological test data. In fact published data from the AIBL study has shown that approximately 30% of healthy controls and approximately 60% of MCI participants have a significant cerebral amyloid burden with a SUVR value of >1.5 . This may account for a lack of correlation. Amyloid deposition is known to start occurring many years

before symptoms appear, and it has been known for many years that amyloid burden is a poor indicator of disease severity, even though amyloid deposits do indicate the presence of AD (symptomatic or pre-clinical stages). The inclusion of neuropsychological data in future analyses may also have limitations however. For example, factors known as brain reserve and cognitive reserve have been shown to influence the appearance or severity of symptoms [490]. In simple terms, brain reserve can be related to a number of functioning neurons and healthy brain tissue, whereas cognitive reserve is related to a person's resilience and adaptability to change, and may be associated with the number of synaptic or inter-neuronal connections. These two factors are influenced by education level and mental activity, and determine the amount of AD-related neuropathology that can be tolerated before symptoms of AD appear. Neuropsychological test data were used to categorise the cohort subjects for this study, and as mentioned above, the SUVR values for many of the subjects, even in the healthy group, indicate AD pathology is already present. Cognitive reserve is likely to have influenced the neuropsychological testing. In fact cognitive reserve is thought to play such an important role in determining disease severity that it has been suggested that some sort of quantitative measure of cognitive reserve will help dementia research considerably [491]. Therefore, in future studies, the best approach might be to include all three factors: SUVR, neuropsychological test results, as well as some measure of cognitive reserve.

The next step is to analyse the 18 month time point to determine whether the same lipid species or lipid groups are identified. As the AIBL study is a prospective longitudinal study, analysis of the 18 month follow up samples will also enable analysis from a longitudinal perspective to facilitate identification of plasma lipid species that differ in those participants whose clinical classification changes during the study and facilitate identification of those lipids that may contribute to a biomarker panel capable of predicting both clinical classification and disease progression. This data is presented in Chapter 4.

Chapter 4

Plasma Lipid Profiles in AIBL 18 Month Samples

Chapter 4 – Plasma Lipid Profiles In AIBL 18 month samples

4.1 Introduction

The previous chapter investigated plasma lipid profiles in baseline samples from the AIBL cohort. This chapter investigates plasma lipid profiles in samples collected from AIBL participants at the 18 month follow up time point. The aim of this chapter is to determine whether lipid species identified as being significantly different in levels between clinical classifications at baseline are also different at the 18 month time point, thus corroborating the results reported in Chapter 3. This chapter will also provide a longitudinal perspective to the data through examination of which lipid species differ in levels between the transition and non-transition groups i.e. comparing those participants whose clinical classification changes versus those who remain stable over the 18 month time frame. Many studies investigating plasma lipid profiles in AD have been cross sectional in nature. A notable exception is the longitudinal Women's Health and Aging study II of cognitive impairment (WHASII), where elevated ceramides were associated with memory impairment, hippocampal volume loss and incident dementia [163, 422, 474]. There is a critical need for additional longitudinal data collected from well-characterised ageing cohorts; the results reported in this chapter will help address this deficit.

Longitudinal MRI and PiB-PET neuroimaging data is collected as part of the AIBL study. Whilst this data provides pathological insight, routine use as a biomarker of AD is unlikely due to the associated costs, radiation exposure and low accessibility. Furthermore, PiB-PET determined SUVR as an indicator of brain amyloid burden is limited in its ability to distinguish between clinical classifications when used in isolation. For example, approximately 30% of healthy controls and approximately 60% of MCI participants in the AIBL study have a significant cerebral amyloid burden with a SUVR value of greater than 1.5; therefore combining neuroimaging results with neuropsychological test data is required to determine clinical status [477]. This highlights the importance of identifying blood based biomarkers of AD which may supplement or potentially replace neuroimaging.

The lipidomic study of the baseline AIBL samples presented in Chapter 3 showed that the mean lipid species levels differed when comparing clinical classifications for 86 of the 189 lipid species measured. Following adjustment for age, gender, APOEε4 allele status and collection site, 11 of these lipid species remained significant when comparing the healthy control and AD groups, yet only 1 lipid species remained significant when comparing the healthy control and MCI groups. A variable selection pathway was applied to choose the least number of lipids which predict clinical classification with the greatest sensitivity and specificity. A panel of six lipids was selected for the HC vs. AD groups and a panel of four lipids were selected for the HC vs. MCI groups, with a sensitivity and specificity of 80% and 71% respectively. When the panel of six lipids was compared to SUVR, no significant correlation was identified.

4.2 Aims

The aim of the work in this chapter is to investigate plasma lipid profiles via a direct infusion with multiple scans technique, using plasma samples obtained as part of the highly characterised Australian Imaging, Biomarkers and Lifestyle Study of Ageing (AIBL). This study will analyse samples collected at the 18 month follow up time point. Changes in lipid profiles will be compared to supplementary data collected as part of the AIBL study: including brain amyloid burden as measured by PiB-PET determined SUVR. The results will be assessed for their ability to distinguish AD patients from cognitively normal individuals and those with mild cognitive impairment (MCI), and will be compared to the results obtained from the baseline samples (described in Chapter 3). Additionally, the data will be examined longitudinally to determine if changes in lipid profiles were reflected by transition to a different clinical classification within the 18 month follow up period.

4.3 Methods

Details of the study population and the methods for sample collection, lipid extraction, and analysis by electrospray ionisation tandem mass spectrometry (ESI-MS/MS) have been described in detail in Chapter 2, Sections 2.2 and 2.3.

Chapter 4: Plasma Lipid Profiles in AIBL 18 Month Samples

A total of 189 lipids were measured including sub-species of PE, LysoPE, PC, Lyso PC, PI, LysoPI, PA, PG, PS, ether PLs, SM and ceramides. This chapter describes results generated following analysis of 18 month follow up AIBL study samples.

Figure 4.1 provides a detailed breakdown of the clinical classifications of participants in the AIBL cohort at 18 months.

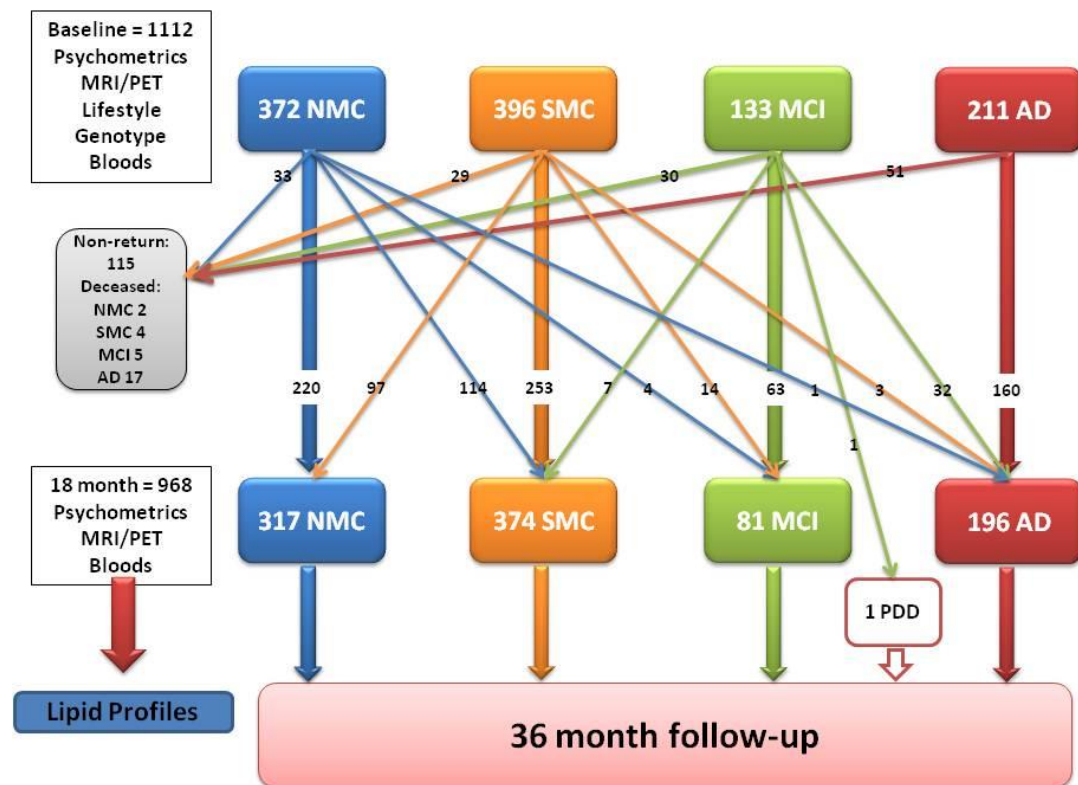


Figure 4.1: The AIBL cohort at 18 months. There were 968 participants at 18 month follow up. These participants were classified as 196 AD, 81 MCI and 691 healthy controls (317 non-memory complainers (NMC) + 374 subjective memory complainers (SMC)). From baseline 115 participants did not return for follow-up: Of these, 28 were deceased and one participant in the MCI group developed dementia associated with Parkinson's disease and was therefore reclassified as non-AD dementia (PDD). Psychometric testing and neuroimaging was performed on participants, and blood samples collected at 18 months were used for lipid profiling. The numbers of non-transition and transition participants are indicated on the arrows.

Schematic adapted from an image provided by the AIBL Study Scientific Management Committee.

At baseline and the 18 month time points the participants were classed as healthy control, MCI or AD. Clinical classification of participants at each time point was determined by a panel of clinicians with experience in diagnosing MCI and AD. MCI was assigned using the Petersen criteria [456] and AD was defined using the NINCDS-ARDA criteria [454] as outlined in Chapter 2.

4.3.1 Statistical analysis

As previously described in Chapter 3, the study population demographics were assessed to compare the healthy controls, MCI and AD groups. χ^2 analyses were used to test the effect of age, gender and APOE ϵ 4 allele status. A Kruskal-Wallis formula was applied to MMSE scores. ANOVA was used to test inter-group differences in SUVR and hippocampal volume.

Marginalised means and p-values were obtained for the lipid levels using a t-test for two classifications (i.e. HC and AD, HC and MCI or MCI and AD) or ANOVA for three classifications (HC, MCI and AD).

The heatmaps were generated and LIMMA and GLM analyses performed as outlined in Chapter 3. Variable selection with clustering and prediction of classification analyses were also conducted as described in Chapter 3. Additionally, LIMMA and GLM analysis were performed to assess differences in plasma lipid species between the transition groups. Figure 4.2 summarises the statistical analyses used for the study of the 18 month lipid profiles in this chapter.

Chapter 4: Plasma Lipid Profiles in AIBL 18 Month Samples

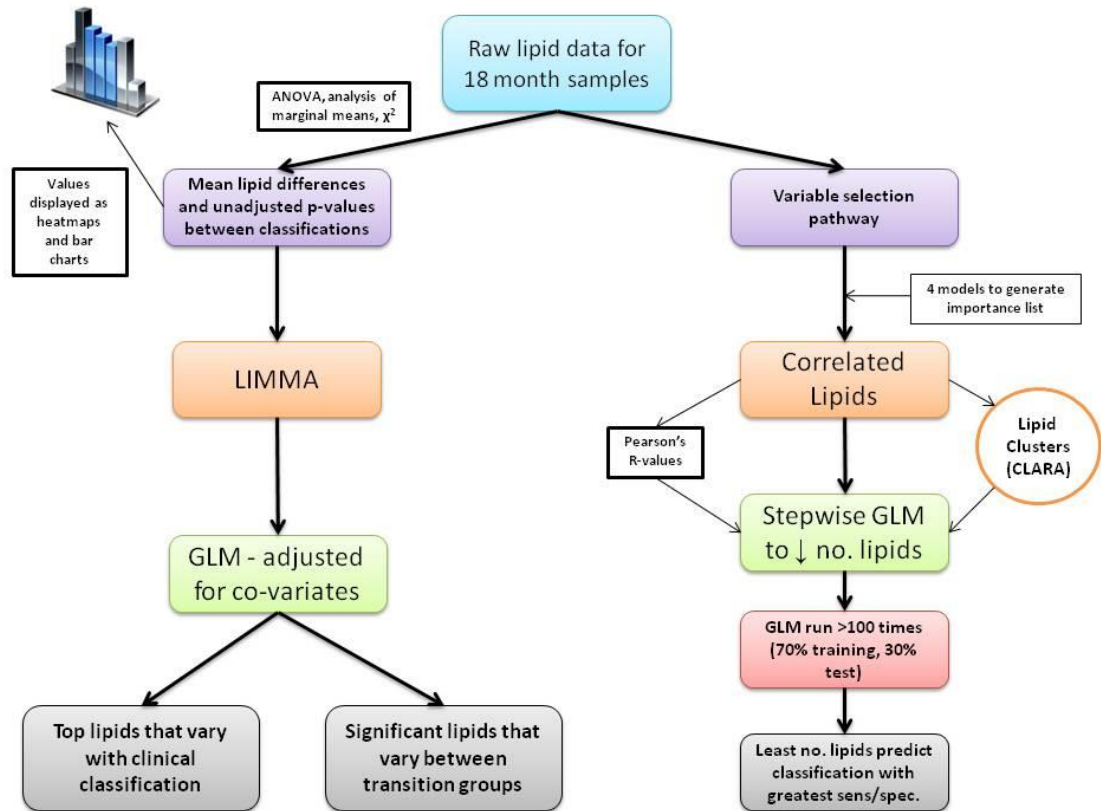


Figure 4.2: Summary of the statistical methods used for analysis of AIBL 18 month plasma samples.

ANOVA: Analysis Of Variance, LIMMA: Linear Models for Microarray Data, GLM: Generalised Linear Model, CLARA: Clustering for Large Applications, Sens: Sensitivity, Spec: Specificity.

The R statistical software environment, version 3.0.2, was used for all statistical analysis (R Development Core Team, 2009).

4.4 Results

The following table describes the demographics of the 18 month cohort divided according to clinical classification.

Table 4.1: AIBL 18 month time point demographics.

| | HC | MCI | AD | p-value |
|-----------------------------|---------------------|---------------------|---------------------|---------|
| Number (n) | 686 | 74 | 188 | |
| Age, years (Mean \pm SD) | 71.78 (\pm 6.74) | 76.72 (\pm 7.55) | 79.22 (\pm 7.93) | <0.0001 |
| Gender (F/M) | 403/283 | 35/39 | 111/77 | 0.156 |
| ApoE ϵ 4 (-ve/+ve) | 502/184 | 44/30 | 57/131 | <0.0001 |
| MMSE score (Mean \pm SD) | 29 (\pm 1.33) | 27 (\pm 2.29) | 18 (\pm 7.1) | <0.0001 |
| SUVr (n) | 152 | 26 | 32 | |
| SUVr (Mean \pm SD) | 1.38 (\pm 0.39) | 1.82 (\pm 0.64) | 2.37 (\pm 0.44) | <0.0001 |
| Hip Vol (n) | 143 | 21 | 29 | |
| Hip Vol (Mean \pm SD) | 3.15 (\pm 0.33) | 2.93 (\pm 0.38) | 2.80 (\pm 0.49) | <0.0001 |

HC: Healthy Controls, MCI: Mild Cognitive Impairment, AD: Alzheimer's disease, F: Female, M: Male, APOE ϵ 4 (-ve/+ve): negative or positive for the APOE ϵ 4 allele, MMSE: Mini Mental State Examination, SUVr: Standard Uptake Value Ratio, Hip Vol: Hippocampal volume, measured in mL. SD: Standard deviation. The characteristics were composed using analysis of variance (ANOVA) to calculate differences for the continuous variables and χ^2 for categorical variables. A Kruskal-Wallis formula was applied to the MMSE scores.

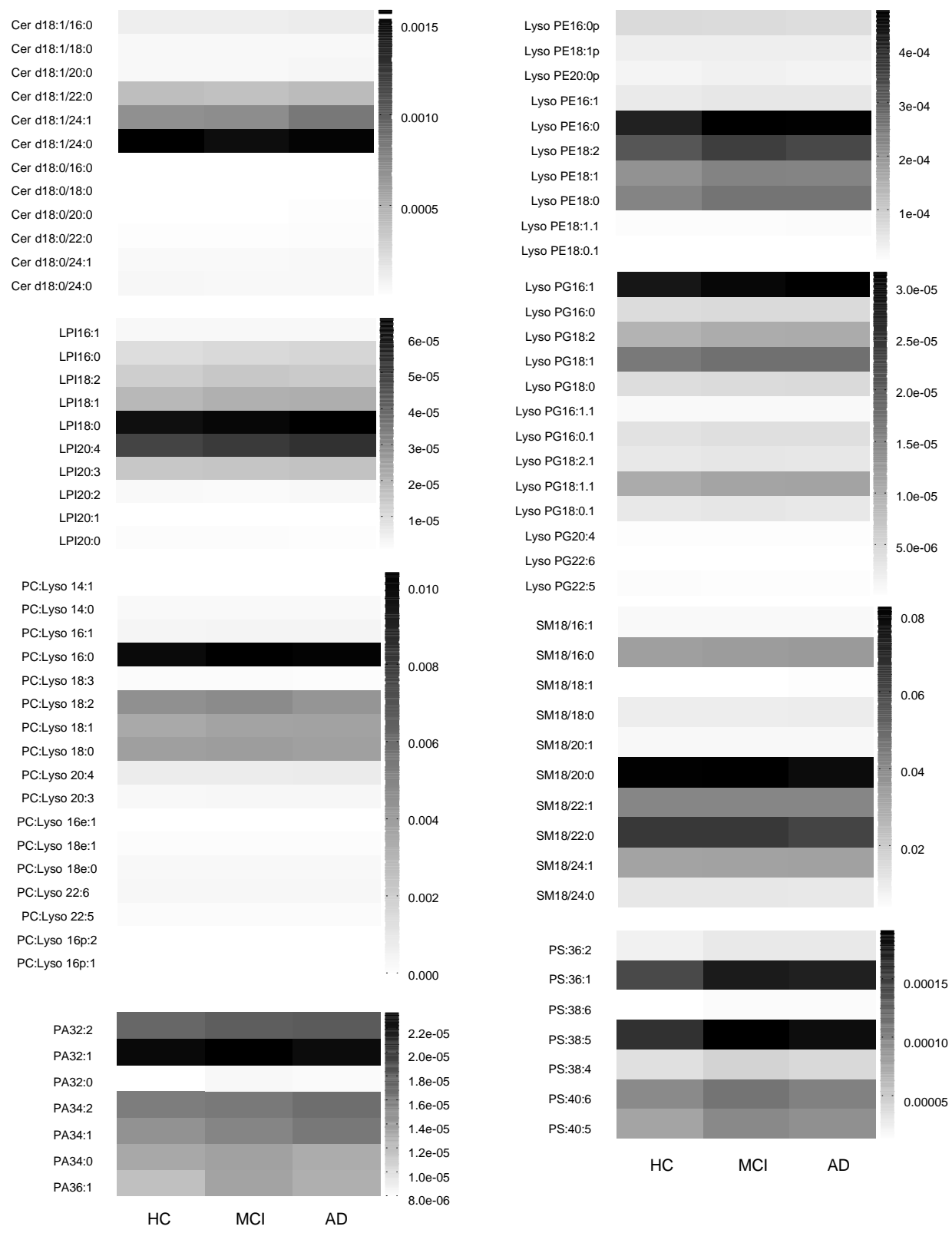
The cohort at 18 month follow up consisted of 686 healthy controls (HC), 74 MCI and 188 AD participants. As was reported for the baseline demographics in Chapter 3, the number of men and women in each classification did not differ significantly ($p=0.156$), but significant differences in mean age were seen ($p<0.001$); the AD group is older, which is expected as AD is an age-related disease. The AIBL cohort was enriched with APOE ϵ 4 allele carriers, consequently there are more APOE ϵ 4 allele carriers compared to epidemiological allele frequencies previously reported (see Table 1.3, Chapter 1). As expected, and consistent with the baseline cohort demographics described in Chapter 3, the mean MMSE score is lower in the MCI and AD groups compared to healthy controls, and cerebral amyloid burden as depicted by mean SUVr is significantly higher in the AD group compared to the MCI and healthy control groups. Further, hippocampal volume is reduced in MCI and AD compared to the healthy control group.

Chapter 4: Plasma Lipid Profiles in AIBL 18 Month Samples

Of the original 968 follow-up samples, a total of 948 plasma samples were analysed to profile the lipids by direct flow injection mass spectrometry (ESI-MS/MS), using the ABSciex QTrap 4000 instrument and multi reaction monitoring (MRM) which fragments the headgroups from their precursor lipids. (Details of methods are outlined in Chapter 2, Sections 2.3.3-2.3.5). The 20 samples that were not successfully analysed failed at either the extraction or the MS analysis stage. The 189 lipid species measured are outlined in Table 3.2, Chapter 3.

As with analysis of the baseline samples, the signal intensities of the individual lipids were normalised against the signal intensities of the lipid internal standards for each respective lipid group, corrected for their respective molecular weights before normalisation to the sum of all lipid species measured. The lipid levels were expressed as molar fractions. A heatmap of the 18 month lipid profiles was plotted (Figure 4.3) to enable visualisation of differences in lipid species levels between the healthy control, MCI and AD groups.

Chapter 4: Plasma Lipid Profiles in AIBL 18 Month Samples



Chapter 4: Plasma Lipid Profiles in AIBL 18 Month Samples

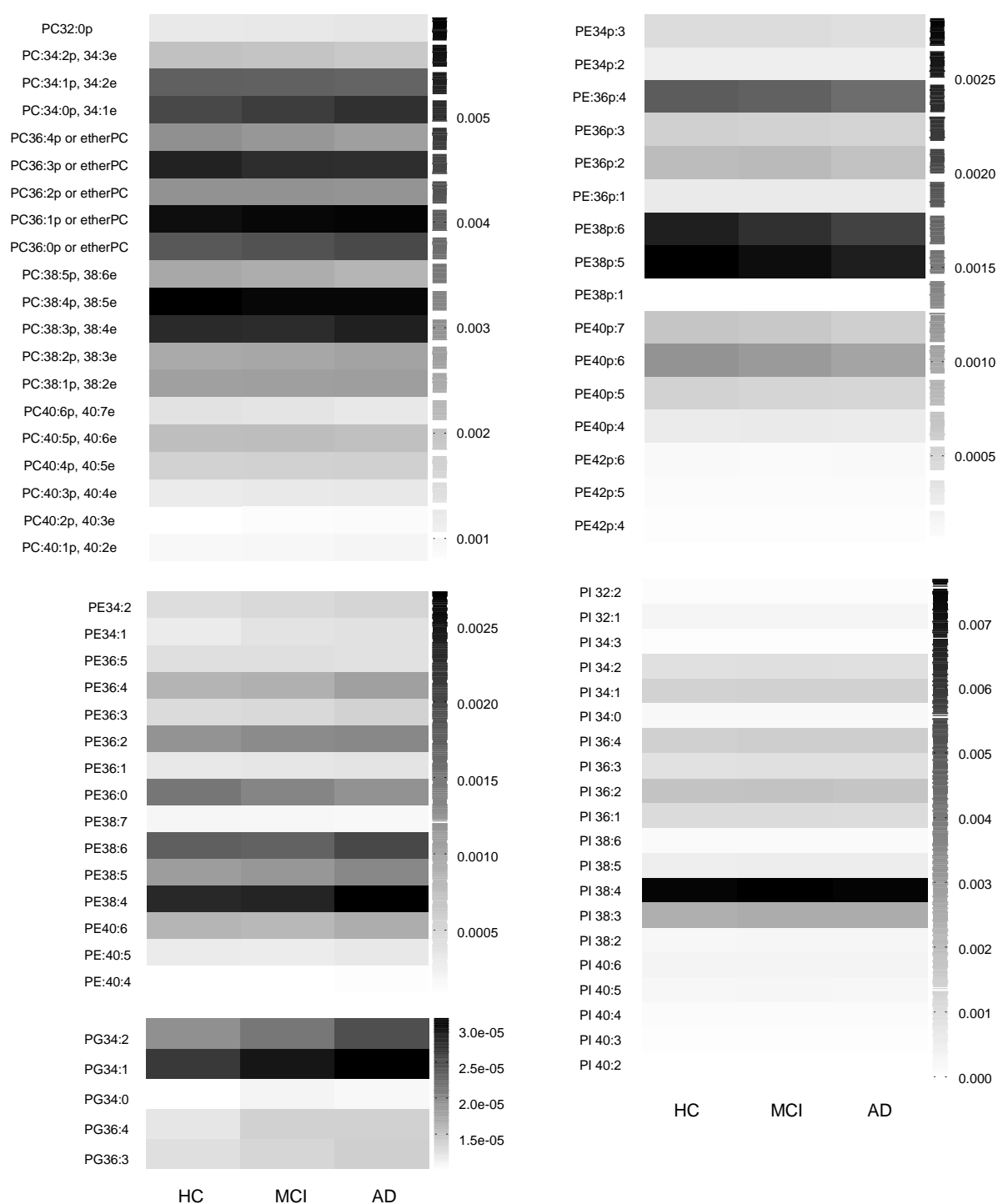
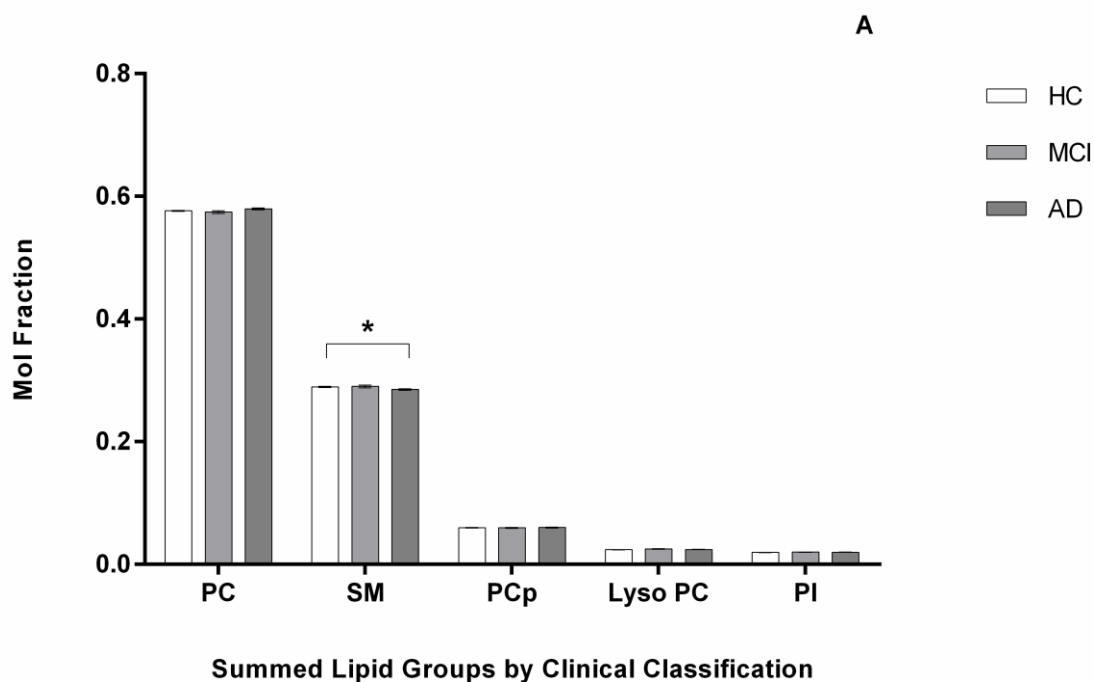


Figure 4.3: Heatmaps of lipid profiles of 948 AIBL study plasma samples collected at the 18 month time point, illustrating differences in lipid species levels between clinical classification groups. The scale on the right side of the charts displays the mean lipid levels expressed as molar fractions.

HC: Healthy Control, MCI: Mild Cognitive Impairment, AD: Alzheimer's Disease, PA: Phosphatidic acid, PE: Phosphatidylethanolamine, PC: Phosphatidylcholine, PS: Phosphatidylserine, PI: Phosphatidylinositol, SM: Sphingomyelin, Cer: Ceramide. White colour reflects low levels, while black colour reflects higher levels.

Chapter 4: Plasma Lipid Profiles in AIBL 18 Month Samples

The heatmaps depicted in Figure 4.3 allow comparison of each lipid species between the healthy control, MCI and AD groups. However, as previously mentioned in Chapter 3, they do not allow comparison across lipid species, as the relative abundance of lipids is only shown within each lipid group. The heatmaps show differences in many of the PE, PC, SM and ceramide species between clinical classifications. The abundance of the various lipid species in plasma is quite different with PC and SM being the most abundant. To allow this data to be displayed graphically three separate bar charts to account for abundance differences were created (Figure 4.4), showing the non-adjusted marginalised means of the summed lipid species groups and their inter-clinical classification differences, as determined by ANOVA.



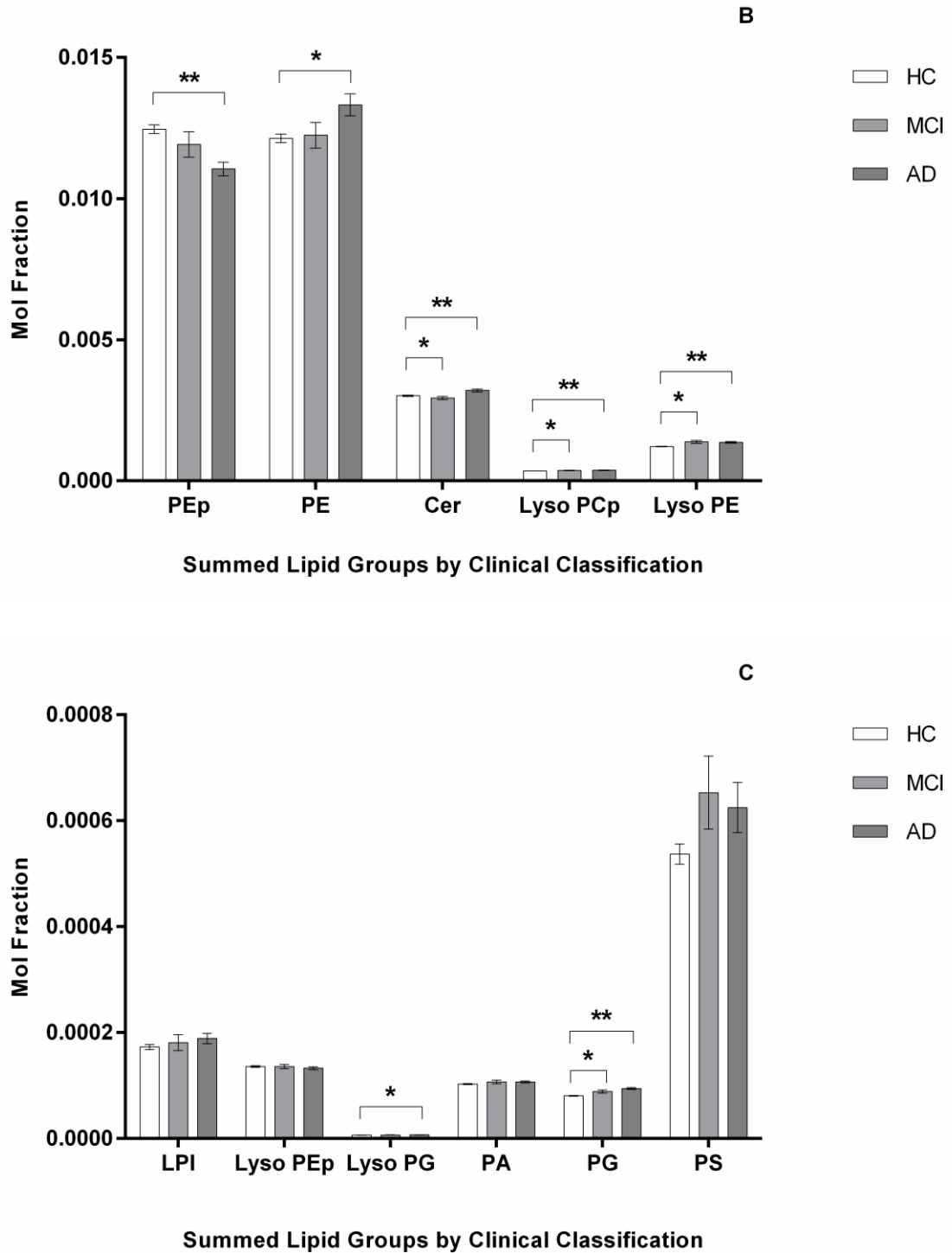
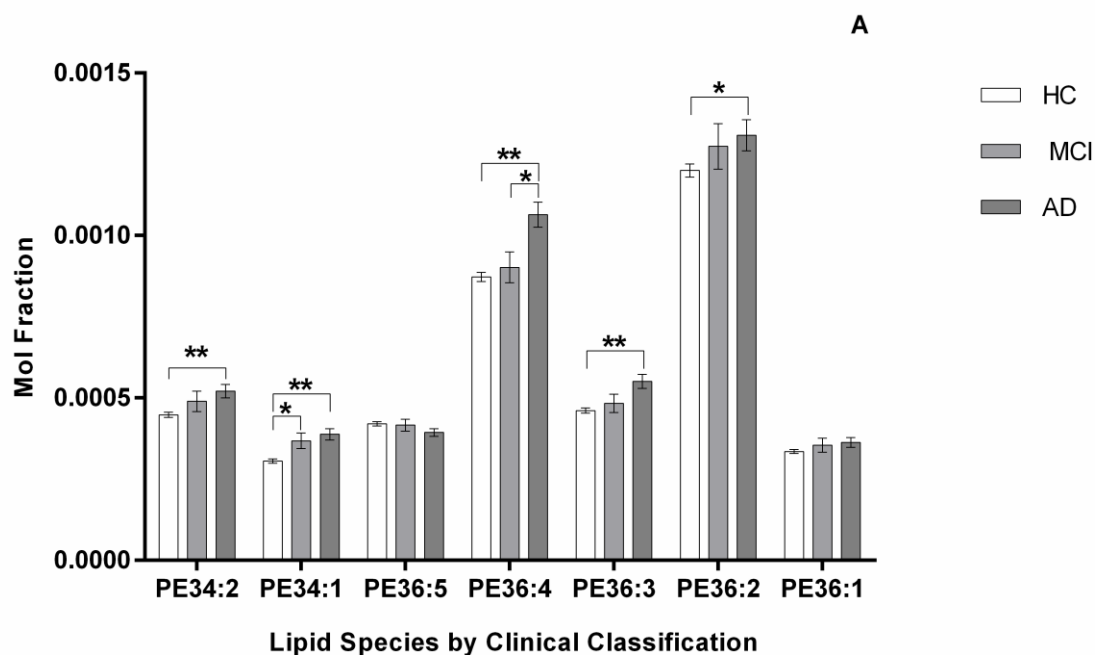


Figure 4.4: Grouped mean lipid levels between clinical classifications. A: PC: Phosphatidylcholine, SM: Sphingomyelin, PCp: Choline plasmalogen, LysoPC, PI: Phosphatidylinositol. **B:** PEp: Ethanolamine plasmalogen, PE: Phosphatidylethanolamine, Cer: Ceramide, LysoPCp, LysoPE. **C:** LPI: LysoPI, LysoPEp, LysoPG, PA: Phosphatidic acid, PG: Phosphatidylglycerol, PS: Phosphatidylserine.

Mean \pm SEM. Analysis of Variance (ANOVA): * $p = <0.05$ ** $p = <0.001$

When lipids are grouped into their respective classes, significant differences in levels were observed for SM, PE_p, PE, Ceramide, LysoPC_p, LysoPE, LysoPG and PG between the healthy control and AD groups. Ceramide, LysoPC_p, LysoPE and PG were also significantly different between the healthy and MCI groups. Whilst Figure 4.4 shows the mean total levels for each lipid group, it does not provide information in relation to sub-species or any significant differences between these sub-species and clinical classification. ANOVA analysis of the individual lipid groups are represented in the following graphs (Figures 4.5 to 4.17), and these show how sub-species levels differ between clinical classifications. A summary of these results is presented in Table 4.2 immediately following Figure 4.17.



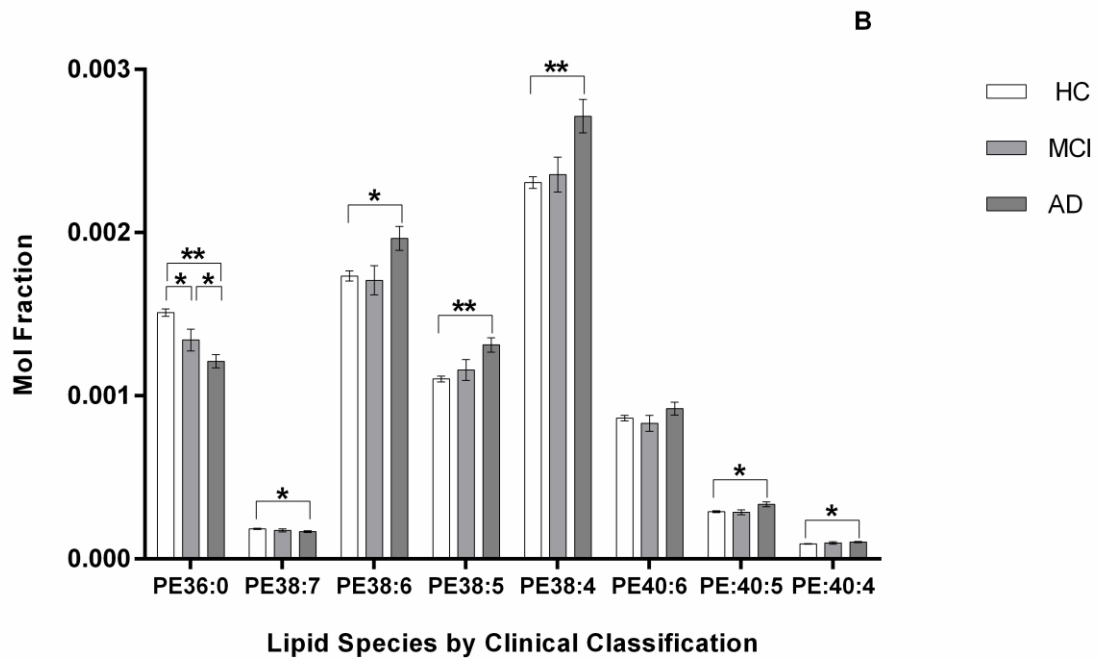
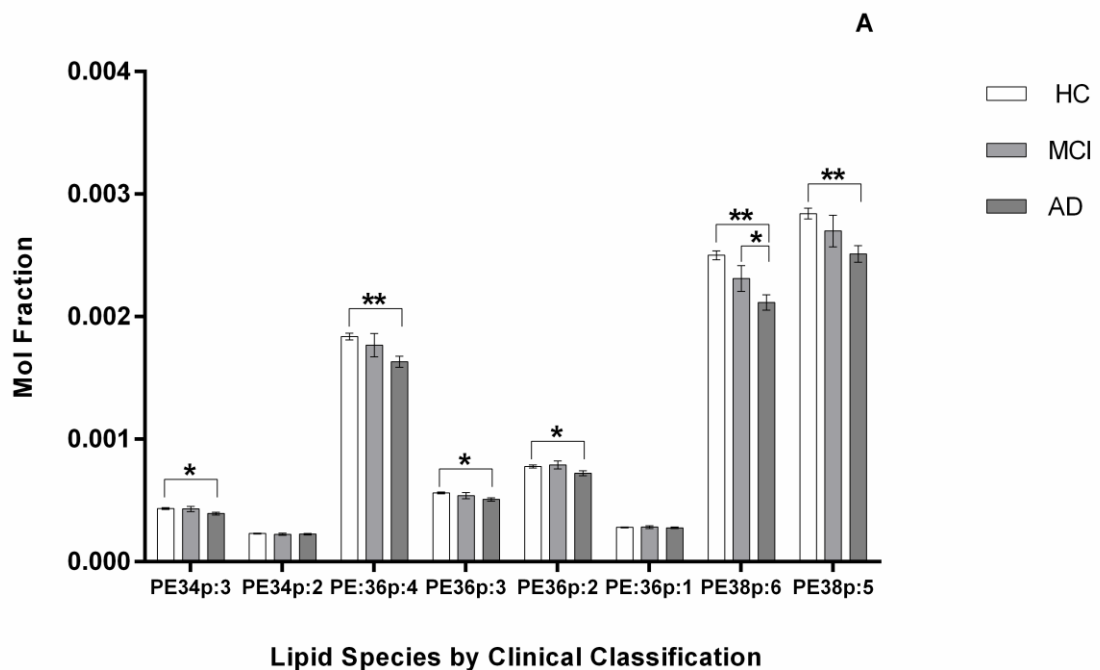


Figure 4.5A and B: Mean PE levels between clinical classifications.

PE: Phosphatidylethanolamine, HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer's disease. Mean \pm SEM. Analysis of Variance (ANOVA): * $p < 0.05$ ** $p < 0.001$



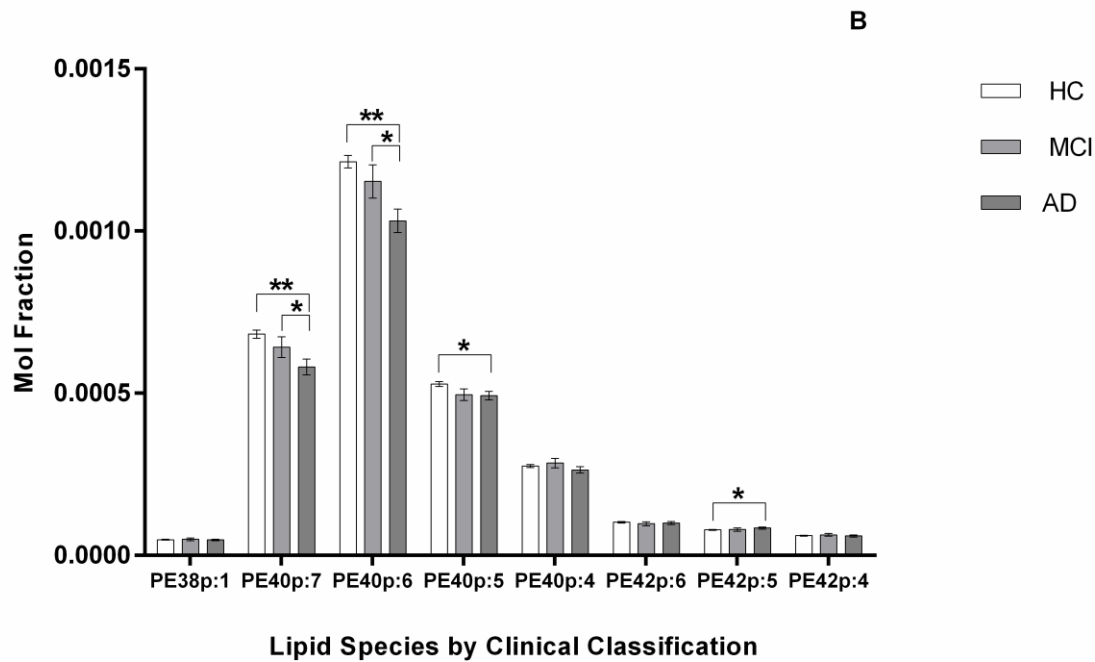
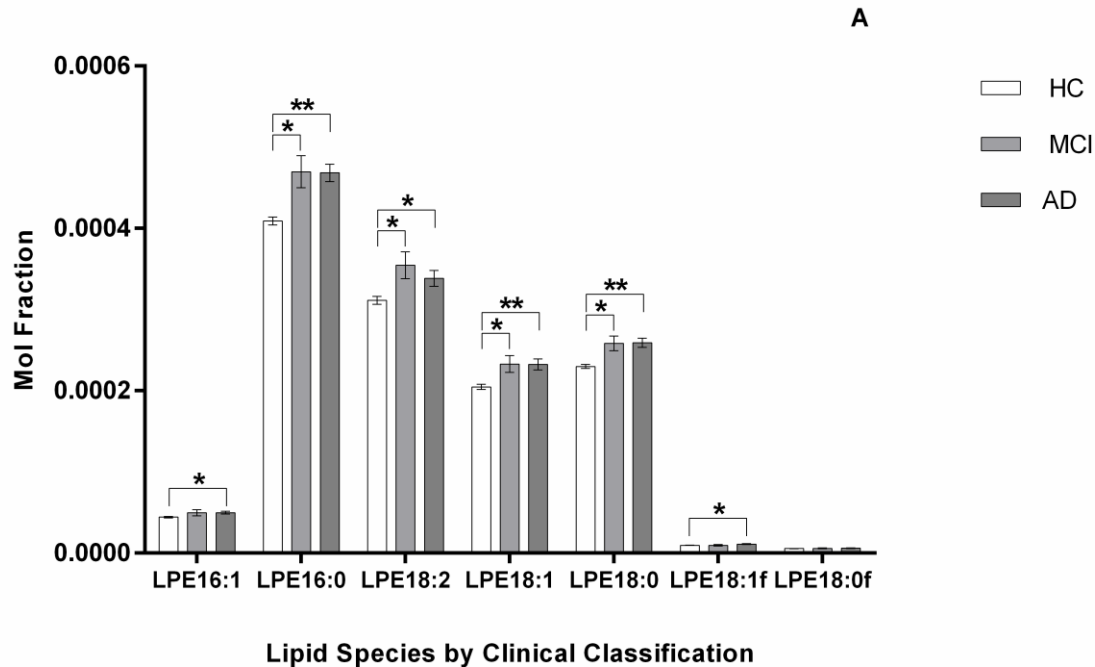


Figure 4.6A and B: Mean Ethanolamine plasmalogen levels between clinical classifications.

PE: Phosphatidylethanolamine, HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer's disease. Mean \pm SEM. Analysis of Variance (ANOVA): * $p < 0.05$ ** $p < 0.001$



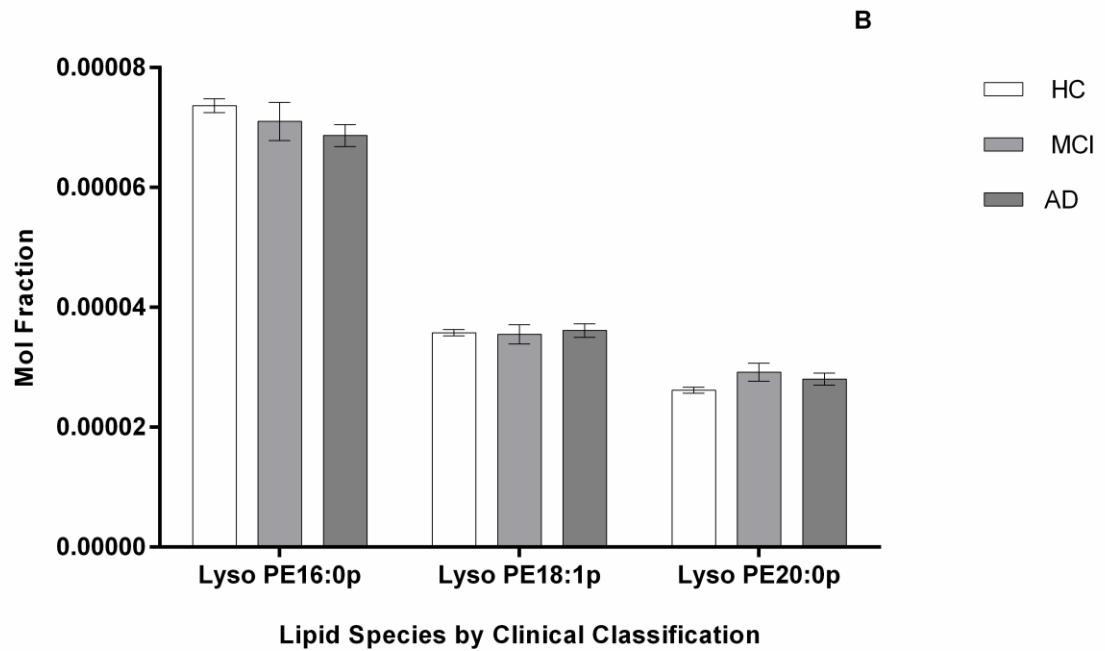
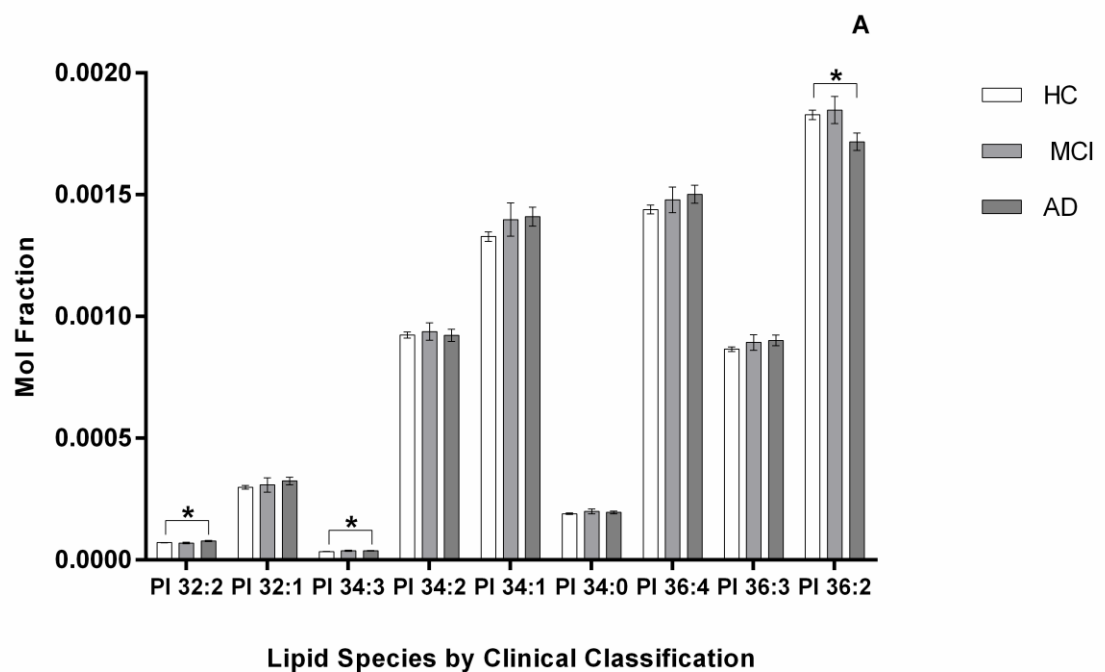


Figure 4.7: A: Mean Lyso-Phosphatidylethanolamine (LPE) and B: Mean Lyso-ethanolamine plasmalogen (LysoPEp) levels between clinical classifications.

HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer's disease.

Mean \pm SEM. Analysis of Variance (ANOVA): * $p < 0.05$ ** $p < 0.001$



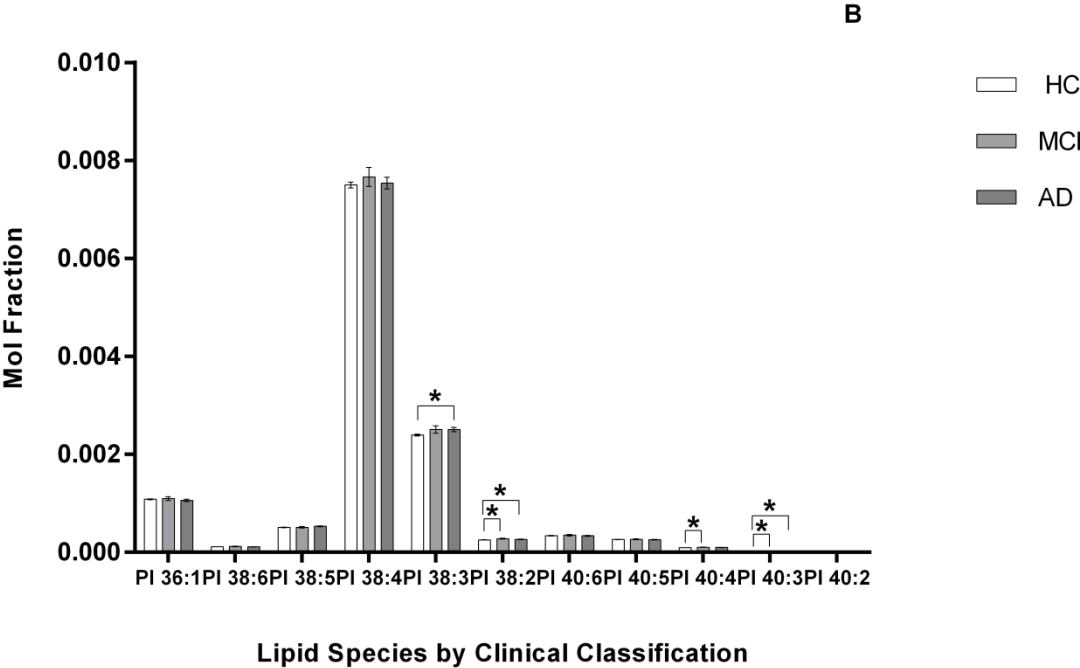


Figure 4.8 A and B: Mean Phosphatidylinositol (PI) levels between clinical classifications.
HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer’s disease.
Mean \pm SEM. Analysis of Variance (ANOVA): * $p = <0.05$ ** $p = < 0.001$

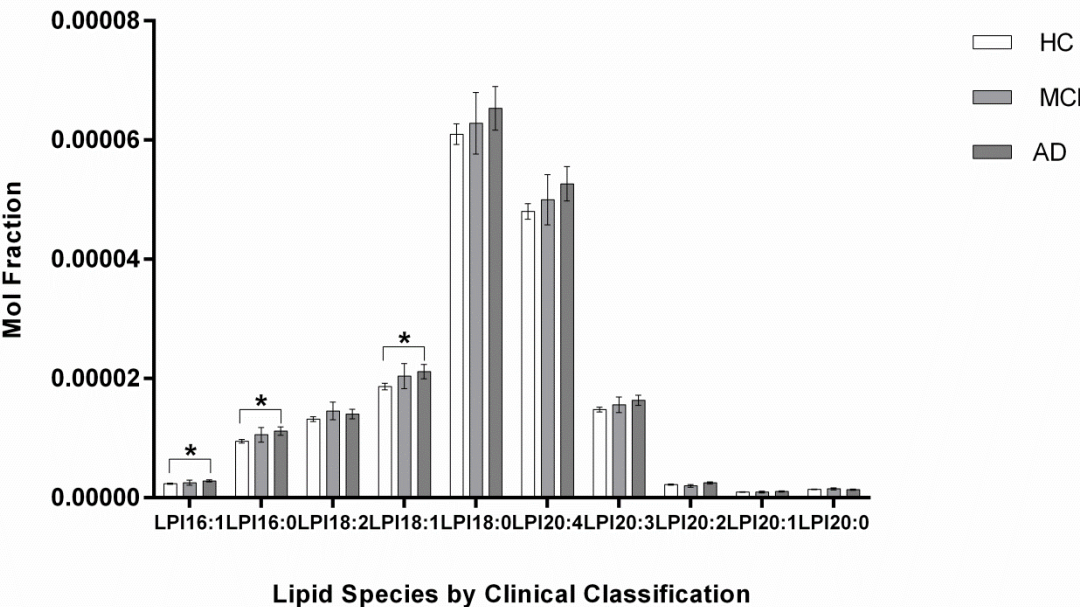


Figure 4.9: Mean Lyso- Phosphatidylinositol (LPI) levels between clinical classifications.
HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer’s disease.
Mean \pm SEM. Analysis of Variance (ANOVA): * $p = <0.05$ ** $p = < 0.001$

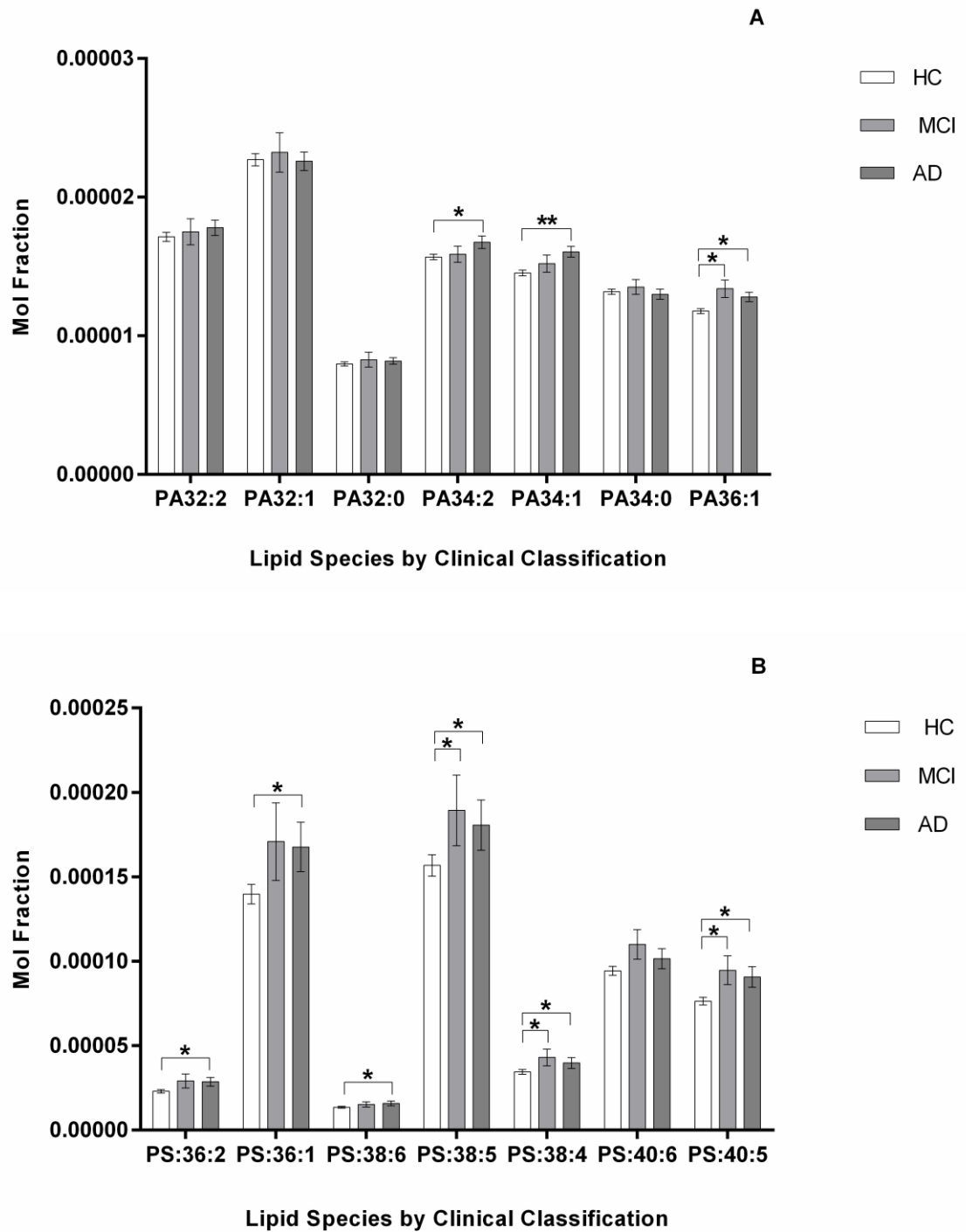


Figure 4.10: A: Mean Phosphatidic acid (PA) and B: Mean Phosphatidylserine (PS) levels between clinical classifications.

HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer's disease.

Mean \pm SEM. Analysis of Variance (ANOVA): * $p < 0.05$ ** $p < 0.001$

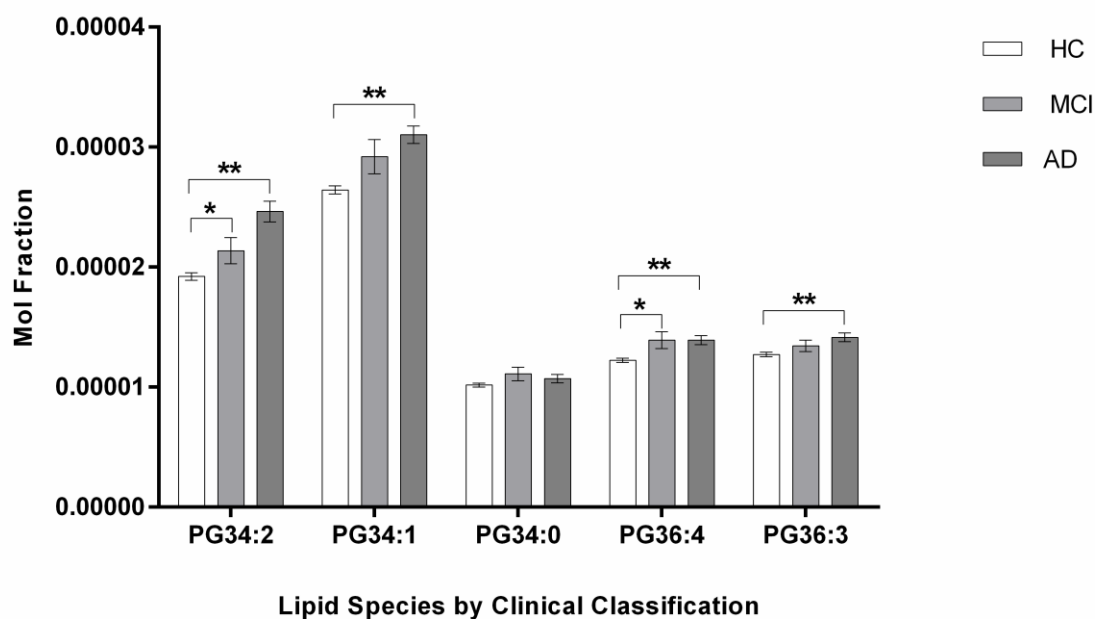
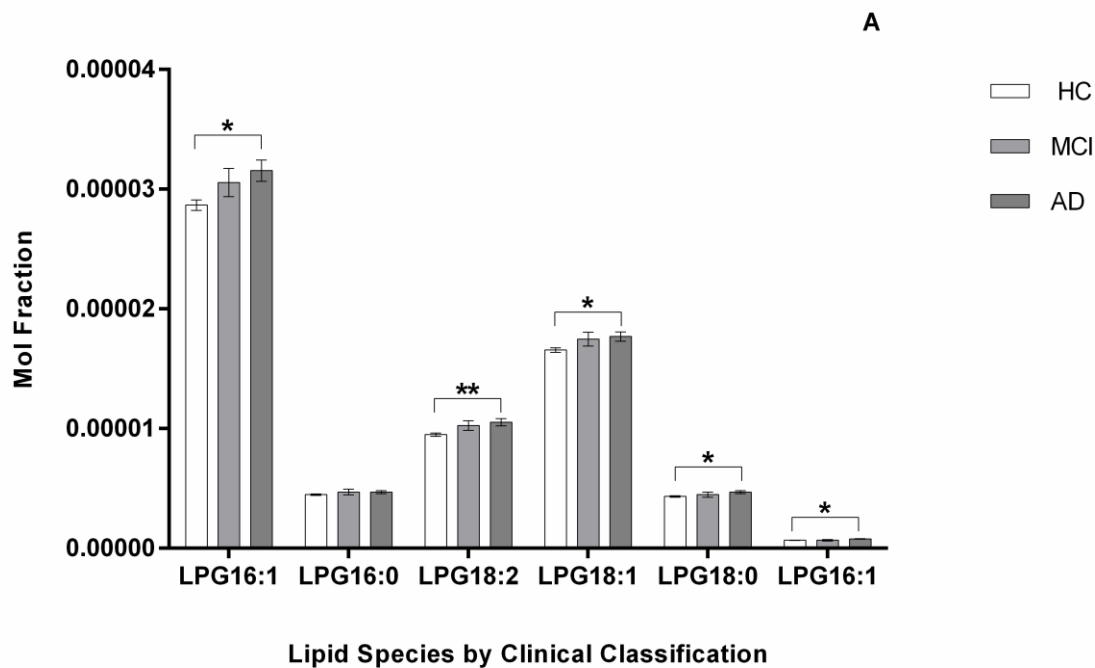


Figure 4.11: Mean Phosphatidylglycerol (PG) levels between clinical classifications.

HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer’s disease.

Mean \pm SEM. Analysis of Variance (ANOVA): * $p < 0.05$ ** $p < 0.001$



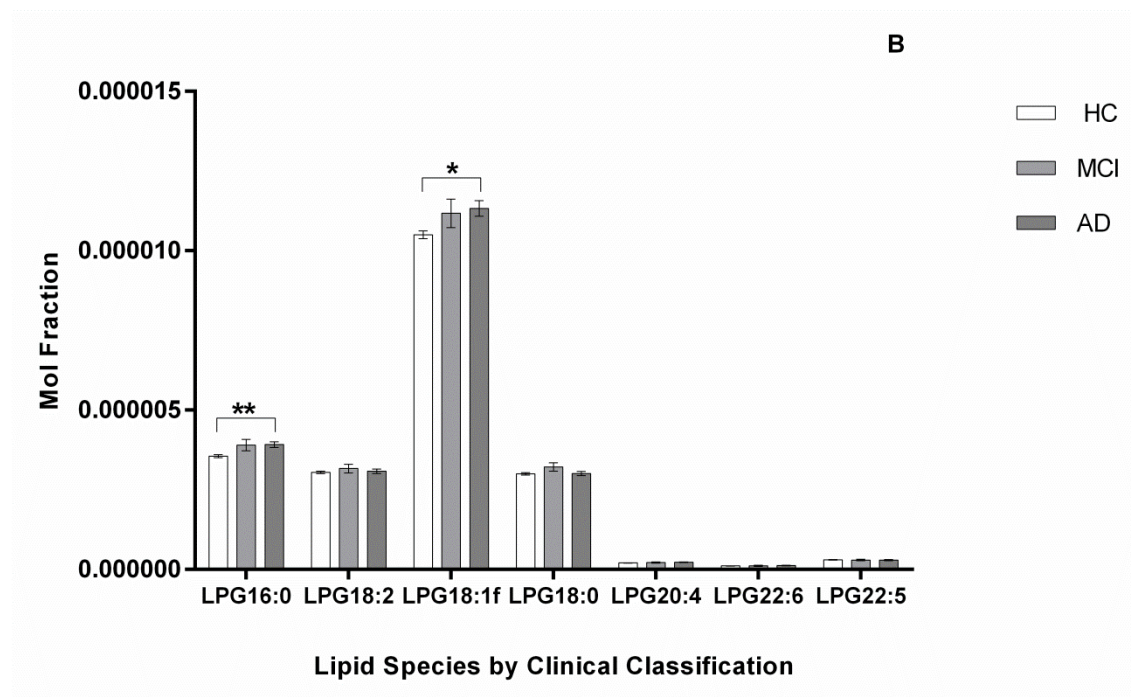
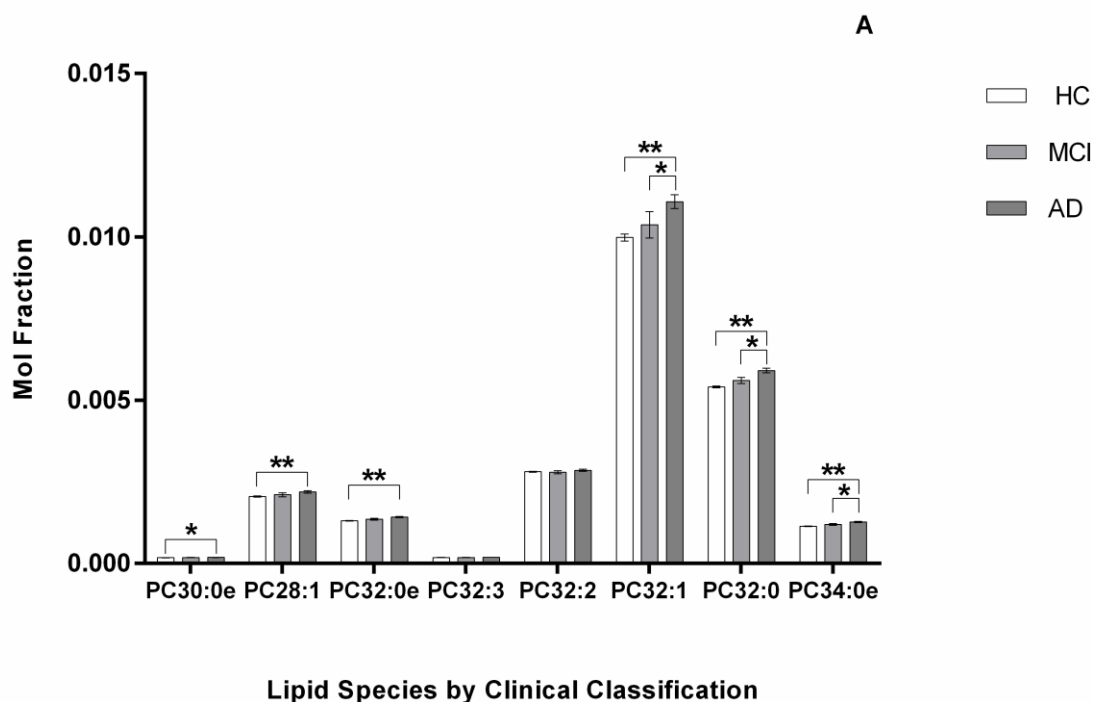


Figure 4.12 A and B: Mean Lysophosphatidylglycerol (LPG) levels between clinical classifications.

The second LPG 16.1 from Figure 4.12A and LPG16.0, LPG18.2, LPG18.1 and LPG18.0 in Figure 4.12B (see Table 3.2). are the “f” species. HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer’s disease.

Mean \pm SEM. Analysis of Variance (ANOVA): * $p < 0.05$ ** $p < 0.001$



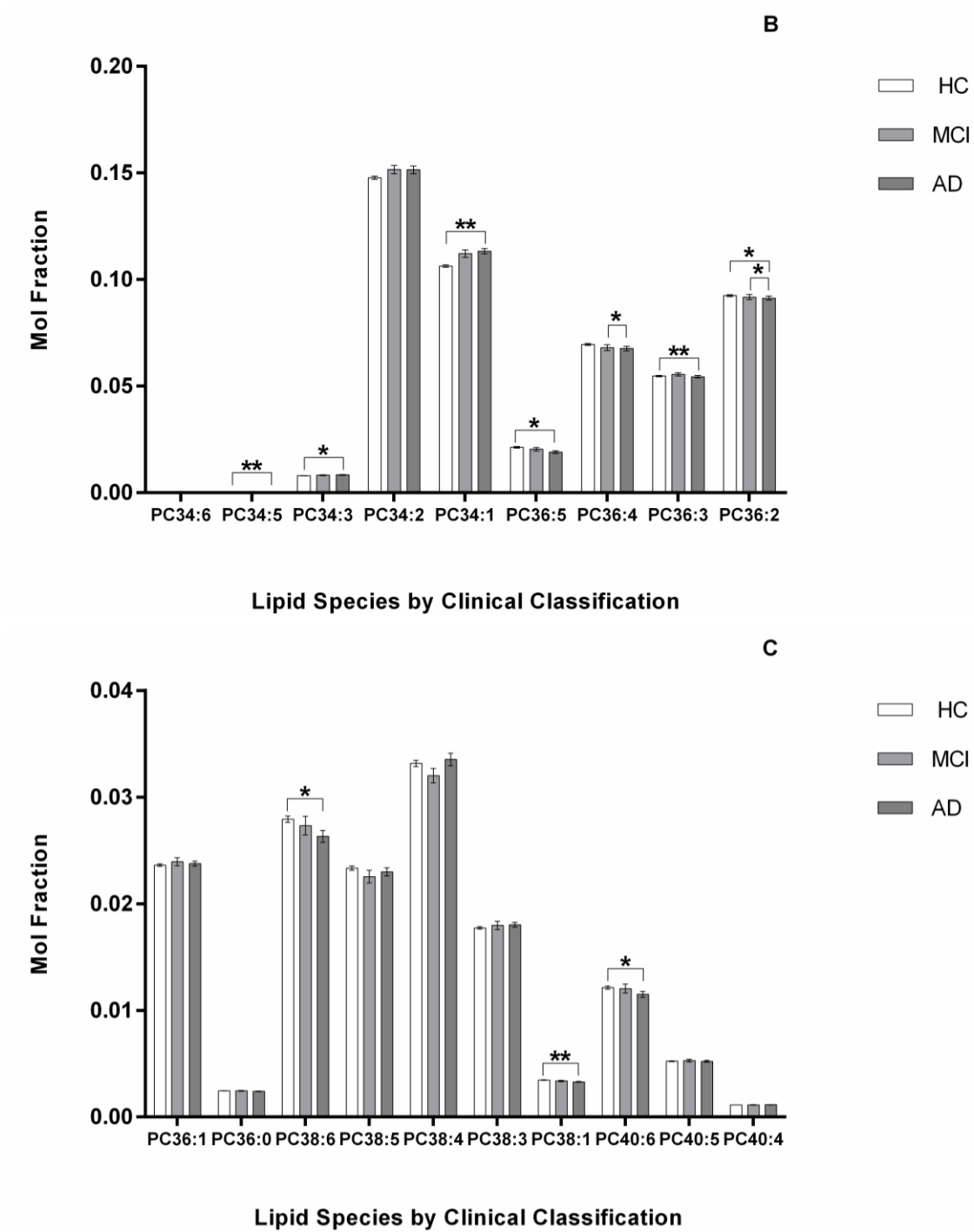
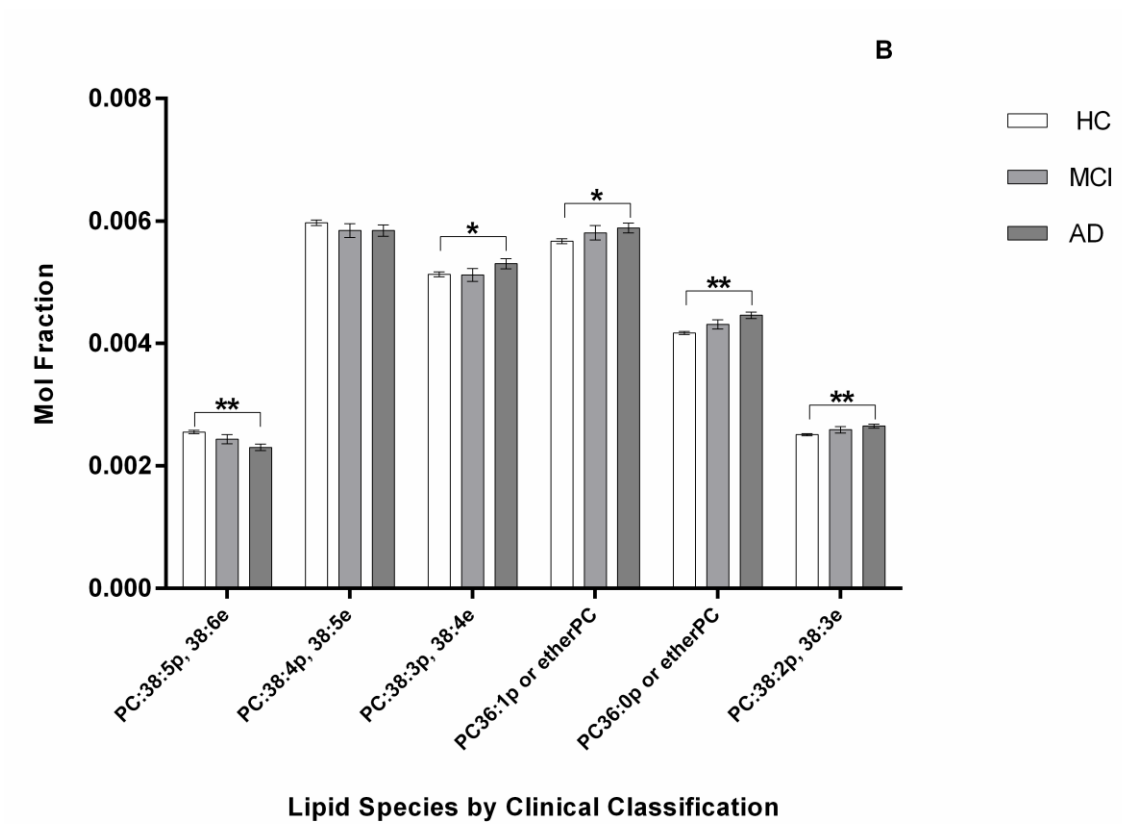
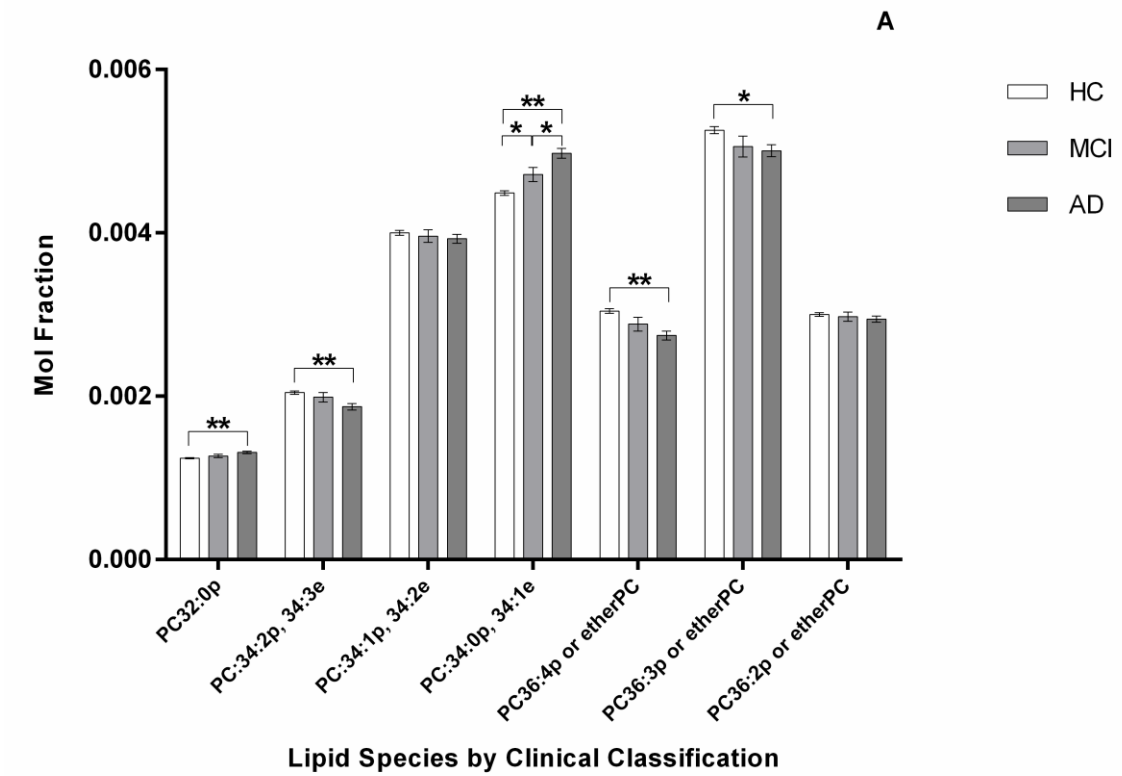


Figure 4.13 A, B and C: Mean Phosphatidylcholine (PC) levels between clinical classifications.

HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer's disease.

Mean \pm SEM. Analysis of Variance (ANOVA): * $p < 0.05$ ** $p < 0.001$



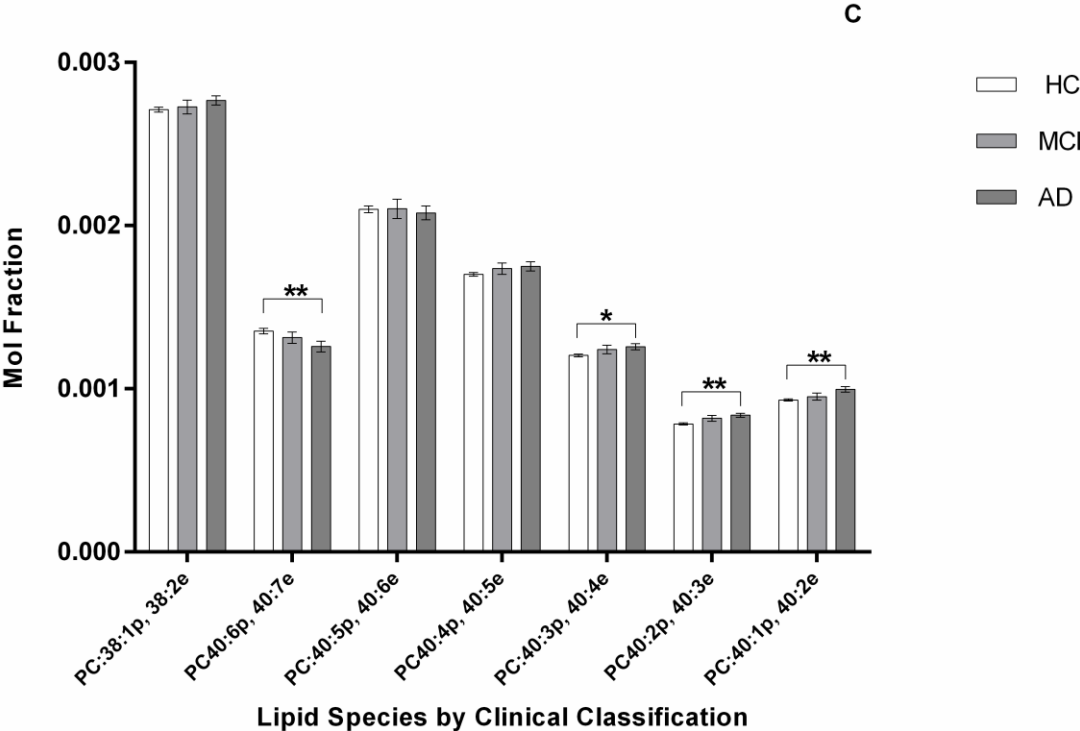
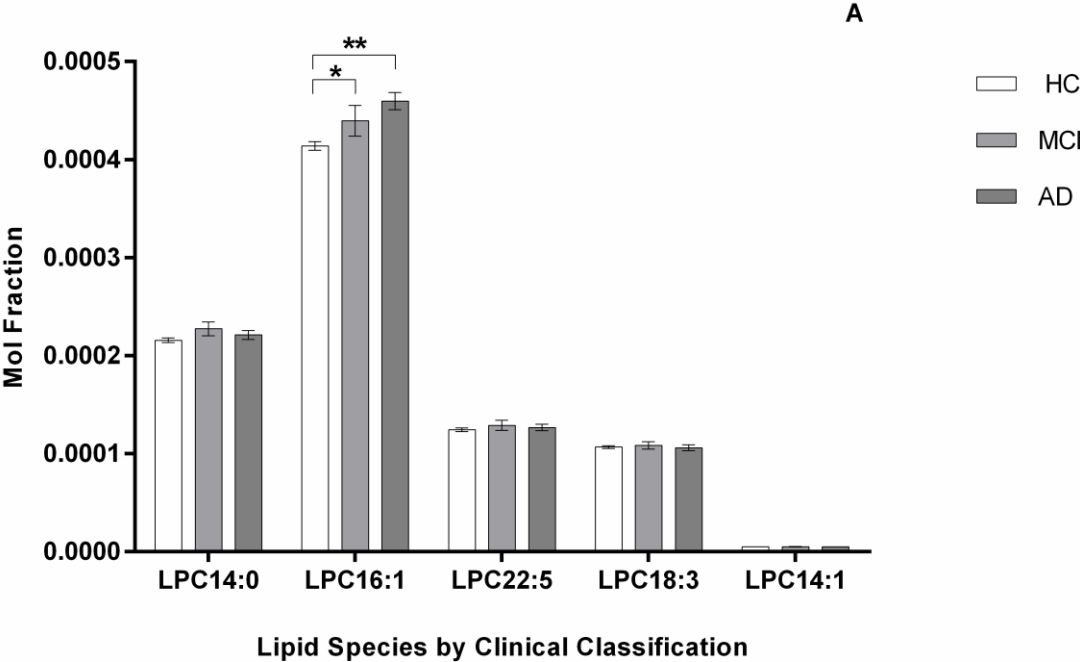


Figure 4.14 A, B and C: Mean choline plasmalogen (PCp) levels between clinical classifications.

HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer's disease.

Mean \pm SEM. Analysis of Variance (ANOVA): * p = <0.05 ** p = < 0.001



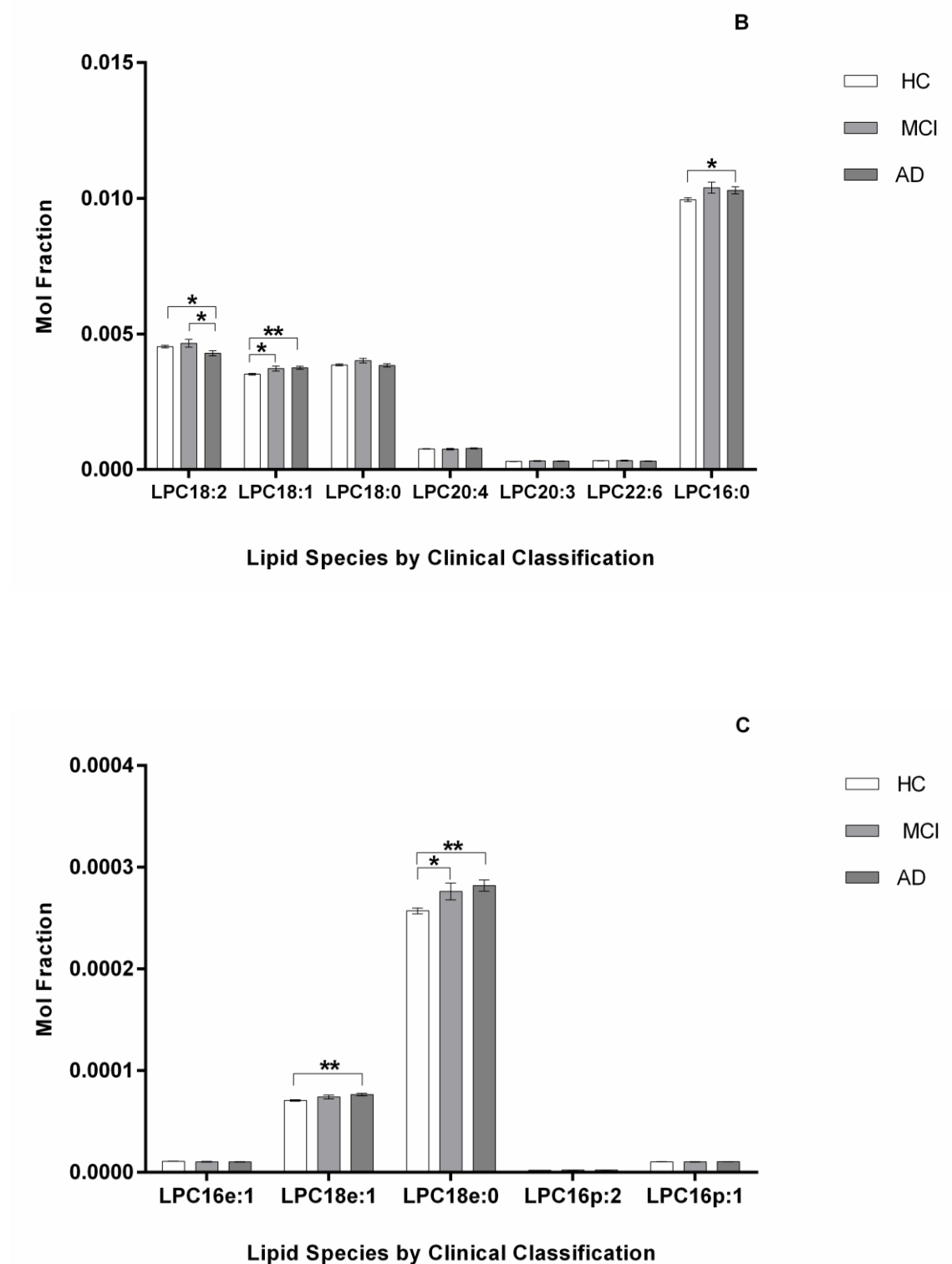


Figure 4.15 A and B: Mean Lysophosphatidylcholine (LPC) levels and C: Mean Lysophosphatidylcholine plasmalogen levels between clinical classifications.

HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer's disease.

Mean \pm SEM. Analysis of Variance (ANOVA): * $p < 0.05$ ** $p < 0.001$

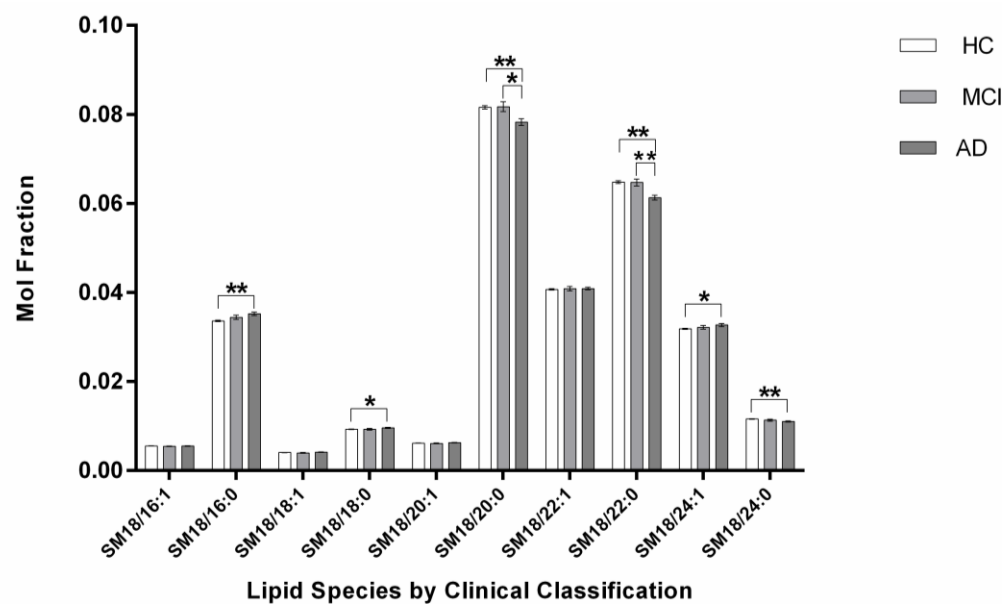
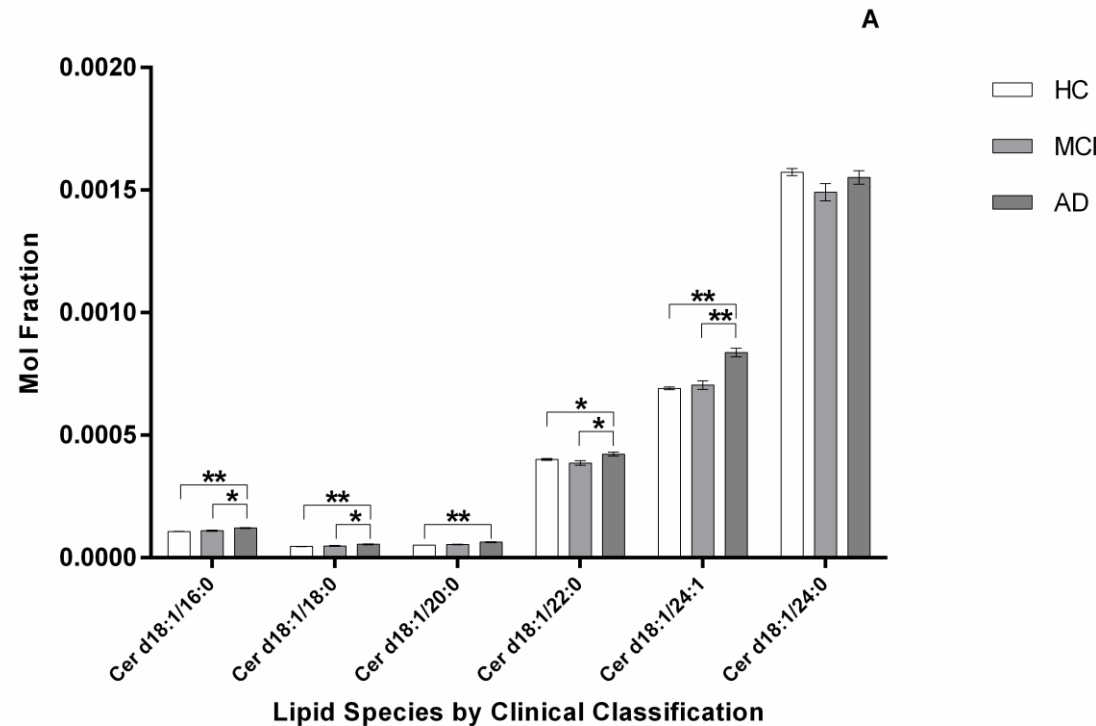


Figure 4.16: Mean Sphingomyelin (SM) levels between clinical classifications.

HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer's disease.

Mean \pm SEM. Analysis of Variance (ANOVA): * $p < 0.05$ ** $p < 0.001$



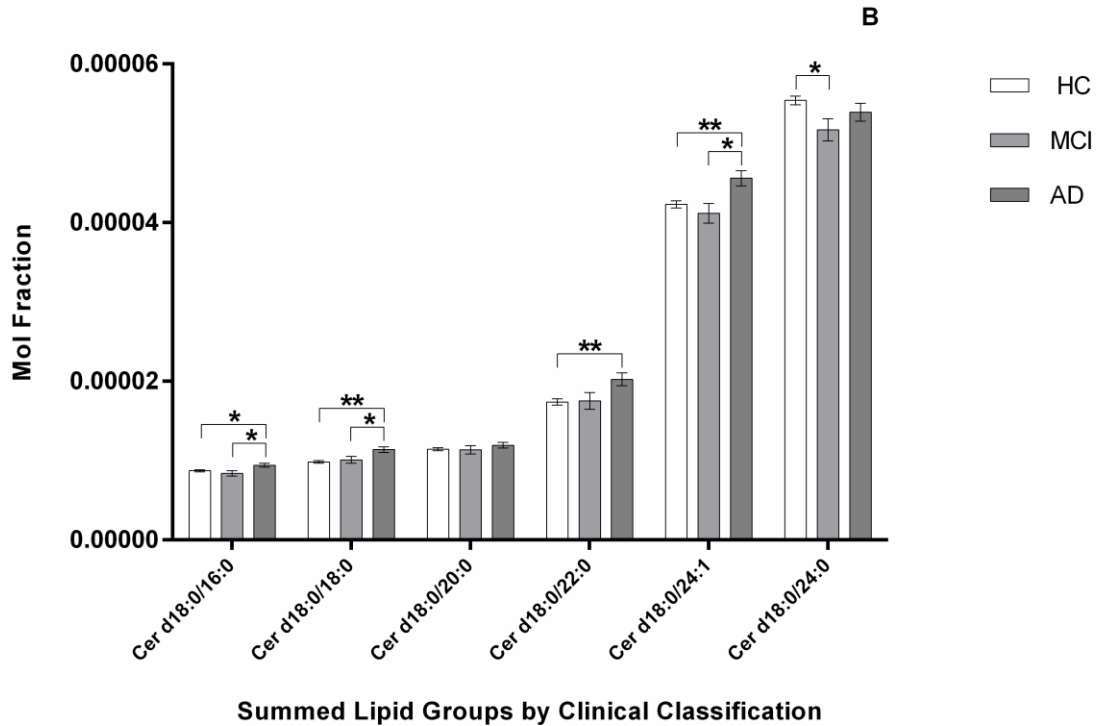


Figure 4.17A and B: Mean Ceramide (Cer) levels between clinical classifications. HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer's disease.

Mean \pm SEM. Analysis of Variance (ANOVA): * $p < 0.05$ ** $p < 0.001$

The bar charts in Figures 4.5 to 4.17 show how the non-adjusted marginalised means of each individual lipid species vary between the healthy control, MCI and AD groups. Significant differences between the lipid species and clinical classifications were seen for many of the lipids including PE, PC, SM and ceramide species. A total of 110 out of 189 lipid species measured showed a significant difference (as indicated by a p-value of <0.05 or <0.001) between the HC and AD, HC and MCI or MCI and AD groups: These results are summarised in Table 4.2.

Table 4.2: List of lipids that were significantly different between clinical classifications. (Table legend on following page)

| Lipid – HC vs. AD | | Lipid – HC vs. MCI | | Lipid – MCI vs. AD | |
|---|---|--|-----------|--|----------------|
| p < 0.05 | p < 0.001 | p < 0.05 | p < 0.001 | p < 0.05 | p < 0.001 |
| Total SM, Total PE, Total LysoPG | Total PEp, Total Cer, Total LysoPCp, Total LysoPE, Total PG | Total Cer, Total LysoPCp, Total LysoPE, Total PG | | | |
| PE36.2, PE38.7, PE38.6, PE40.5, PE40.4, PE34p.3, PE36p.3, PE36p.2, PE40p.5, PE42p.5, LysoPE16.1, LysoPE18.2, LysoPE18.1f | PE34.2, PE34.1, PE36.4, PE36.3, PE36.0, PE38.5, PE38.4, PE36p.4, PE38p.6, PE38p.5, PE40p.7, PE40p.6, LysoPE16.0, LysoPE18.1, LysoPE18.0 | PE34.1, PE36.0, LysoPE16.0, LysoPE18.2, LysoPE18.1, LysoPE18.0 | | PE36.4, PE36.0, PE38p.6, PE40p.7, PE40p.6 | |
| PC30.0e, PC34.3, PC36.5, PC36.2, PC38.6, PC40.6, PC36.3p/etherPC, PC36.1p/etherPC, PC38.3p..38.4e, PC40.3p..40.4e, PCLyso16.0, PCLyso18.2 | PC28.1, PC32.0e, PC32.1, PC32.0, PC34.0e, PC34.5, PC34.1, PC36.3, PC38.1, PC32.0p, PC34.2p..34.3e, PC34.0p..34.1e, PC36.4p/etherPC, PC36.0p/etherPC, PC38.5p..38.6e, PC38.2p..38.3e, PC40.6p..40.7e, PC40.2p..40.3e, PC40.1p..40.2e, PCLyso16.1, PCLyso18.1, PCLyso18e.1, PCLyso18e.0 | PC34.0p..34.1e, PCLyso16.1, PCLyso18.1, PCLyso18e.0 | | PC32.1, PC32.0, PC34.0e, PC36.4, PC36.2, PC34.0p..34.1e, PCLyso18.2 | |
| PI32.2, PI34.3, PI36.2, PI38.3, PI38.2, PI40.3, LysoPI16.1, LysoPI16.0, LysoPI18.1 | | PI38.2, PI40.4, PI40.3 | | | |
| PS36.2, PS36.1, PS38.6, PS38.5, PS38.4, PS40.5 | | PS38.5, PS38.4, PS40.5 | | | |
| LysoPG16.1, LysoPG18.1, LysoPG18.0, LysoPG16.1f, LysoPG18.1f | PG34.2, PG34.1, PG36.4, PG36.3, LysoPG18.2, LysoPG16.0f | PG34.2, PG36.4 | | | |
| PA34.2, PA36.1 | PA34.1 | PA36.1 | | | |
| SM18.18.0, SM18.24.1 | SM18.16.0, SM18.20.0, SM18.22.0, SM18.24.0 | | | SM18.20.0 | SM18.22.0 |
| Cer.d18.1.22.0, Cer.d18.0.16.0 | Cer.d18.1.16.0, Cer.d18.1.18.0, Cer.d18.1.20.0, Cer.d18.1.24.1, Cer.d18.0.18.0, Cer.d18.0.22.0, Cer.d18.0.24.1 | Cer.d18.0.24.0 | | Cer.d18.1.16.0, Cer.d18.1.18.0, Cer.d18.1.22.0, Cer.d18.0.16.0, Cer.d18.0.18.0, Cer.d18.0.24.1 | Cer.d18.1.24.1 |

Chapter 4: Plasma Lipid Profiles in AIBL 18 month samples

Table 4.2 legend: The red text indicates lower mean levels in AD compared to HC, MCI compared to HC and AD compared to MCI. The black text indicates higher mean levels in AD compared to HC, MCI compared to HC and AD compared to MCI.

PI: Phosphatidylinositol, PE: Phosphatidylethanolamine, PC: Phosphatidylcholine, SM: Sphingomyelin, PA: Phosphatidic acid, PG: Phosphatidylglycerol, Cer: Ceramide.

To test further for significant differences in lipid species levels between clinical classifications using stricter criteria, a linear Models for Microarray Data (LIMMA) model was applied to obtain a false discovery adjusted p-value for each lipid to exclude any false associations identified by simple ANOVA.

4.4.1 Linear Models for Microarray Data (LIMMA) comparing clinical classifications

LIMMA analysis was used to obtain Q values (false discovery adjusted values), and Bonferroni comparisons were used to generate p-values to reduce the number of lipids identified by ANOVA. This model selected 46 out of 189 lipid species levels that were significantly different between healthy controls and AD participants and these lipids are listed in Table 4.3.

Table 4.3: Linear Models for Microarray Data (LIMMA) for 18 month samples for HC vs. AD.

| Lipid | p-value | Lipid | p-value |
|-----------------|----------|-----------------|----------|
| Cer.d18.1.24.1 | 2.22E-21 | PC36.4p/etherPC | 2.46E-06 |
| Cer.d18.1.20.0 | 4.86E-17 | PE38.5 | 2.98E-06 |
| PC34.0p..34.1e | 1.19E-15 | PC32.0p | 8.48E-06 |
| PC32.0 | 2.85E-13 | PG36.4 | 1.03E-05 |
| PC34.0e | 6.16E-13 | PE34.1 | 1.12E-05 |
| PG34.2 | 1.28E-11 | PE36.3 | 1.62E-05 |
| PE36.0 | 1.66E-11 | Cer.d18.0.18.0 | 2.00E-05 |
| Cer.d18.1.16.0 | 1.20E-10 | PC34.5 | 2.42E-05 |
| PG34.1 | 1.18E-09 | PC40.2p..40.3e | 2.53E-05 |
| Cer.d18.1.18.0 | 1.54E-09 | PCLyso18e.1 | 2.74E-05 |
| PC32.0e | 8.60E-09 | SM18.16.0 | 3.66E-05 |
| PC34.1 | 1.11E-08 | PCLyso18e.0 | 4.13E-05 |
| PE38p.6 | 1.37E-08 | PC40.6p..40.7e | 4.94E-05 |
| SM18.22.0 | 3.21E-08 | SM18.20.0 | 7.04E-05 |
| PE40p.6 | 4.63E-08 | PC38.1 | 8.26E-05 |
| LysoPE16.0 | 7.89E-08 | SM18.24.0 | 9.81E-05 |
| PE36.4 | 2.47E-07 | PC40.1p..40.2e | 9.94E-05 |
| PC36.0p/etherPC | 2.63E-07 | LysoPE18.1 | 1.00E-04 |
| PE40p.7 | 3.15E-07 | PG36.3 | 1.50E-04 |
| LysoPE18.0 | 4.25E-07 | PA34.1 | 1.72E-04 |
| PCLyso16.1 | 5.15E-07 | PE38.4 | 2.08E-04 |
| PC38.5p..38.6e | 5.47E-07 | PC38.2p..38.3e | 2.40E-04 |
| PC32.1 | 1.84E-06 | PC34.2p..34.3e | 2.58E-04 |

Listed in order of most to least significant for p-values less than the Bonferroni cut off (0.05/189).

PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine, Cer: Ceramide, PG: Phosphatidylglycerol, PI: Phosphatidylinositol, PA: Phosphatidic acid.

A fold lipid change was subsequently calculated using unadjusted raw expression values to provide a relative lipid difference between clinical classifications. The mean levels, difference in levels between groups, the directional change i.e. increased or decreased in AD compared to healthy controls and fold change is shown in Table 4.4.

Table 4.4: Mean value difference in lipid species levels between groups, direction of change in lipid levels between HC and AD participants and fold change.

| Lipid | Mean HC | Mean AD | Difference | Direction of change | Fold Δ |
|-----------------|-----------|-----------|------------|---------------------|---------------|
| LysoPE16.0 | 4.093E-04 | 4.685E-04 | 5.923E-05 | ↑ in AD | 1.145 |
| LysoPE18.1 | 2.047E-04 | 2.325E-04 | 2.781E-05 | ↑ in AD | 1.136 |
| LysoPE18.0 | 2.304E-04 | 2.593E-04 | 2.893E-05 | ↑ in AD | 1.126 |
| PE34.1 | 3.056E-04 | 3.680E-04 | 6.247E-05 | ↑ in AD | 1.204 |
| PE36.4 | 8.717E-04 | 1.038E-03 | 1.666E-04 | ↑ in AD | 1.191 |
| PE36.3 | 4.615E-04 | 5.443E-04 | 8.284E-05 | ↑ in AD | 1.180 |
| PE38.5 | 1.105E-03 | 1.295E-03 | 1.906E-04 | ↑ in AD | 1.173 |
| PE38.4 | 2.305E-03 | 2.617E-03 | 3.117E-04 | ↑ in AD | 1.135 |
| PA34.1 | 1.44eE-05 | 1.607E-05 | 1.603E-06 | ↑ in AD | 1.111 |
| PG34.2 | 1.913E-05 | 2.393E-05 | 4.792E-06 | ↑ in AD | 1.250 |
| PG34.1 | 2.639E-05 | 3.104E-05 | 4.645E-06 | ↑ in AD | 1.176 |
| PG36.4 | 1.219E-05 | 1.393E-05 | 1.737E-06 | ↑ in AD | 1.142 |
| PG36.3 | 1.264E-05 | 1.146E-05 | 1.515E-06 | ↑ in AD | 1.120 |
| PCLyso16.1 | 4.122E-04 | 4.564E-04 | 4.427E-05 | ↑ in AD | 1.107 |
| PCLyso18e.1 | 7.051E-05 | 7.656E-05 | 6.057E-06 | ↑ in AD | 1.086 |
| PCLyso18e.0 | 2.568E-04 | 2.821E-04 | 2.526E-05 | ↑ in AD | 1.098 |
| PC32.0p | 1.240E-03 | 1.313E-03 | 7.320E-05 | ↑ in AD | 1.059 |
| PC34.0p..34.1e | 4.479E-03 | 4.953E-03 | 4.739E-04 | ↑ in AD | 1.106 |
| PC36.0p/etherPC | 4.169E-03 | 4.463E-03 | 2.938E-04 | ↑ in AD | 1.070 |
| PC38.2p..38.3e | 2.509E-03 | 2.652E-03 | 1.429E-04 | ↑ in AD | 1.057 |
| PC40.2p..40.3e | 7.853E-04 | 8.387E-04 | 5.343E-05 | ↑ in AD | 1.068 |
| PC40.1p..40.2e | 9.318E-04 | 9.924E-04 | 6.061E-05 | ↑ in AD | 1.065 |
| PC32.0e | 1.302E-03 | 1.421E-03 | 1.189E-04 | ↑ in AD | 1.091 |
| PC32.1 | 9.965E-03 | 1.108E-02 | 1.113E-03 | ↑ in AD | 1.112 |
| PC32.0 | 5.407E-03 | 5.910E-03 | 5.029E-04 | ↑ in AD | 1.093 |
| PC34.0e | 1.142E-03 | 1.272E-03 | 1.305E-04 | ↑ in AD | 1.114 |
| PC34.1 | 8.348E-02 | 8.897E-02 | 5.494E-03 | ↑ in AD | 1.066 |
| SM18.16.0 | 3.361E-02 | 3.512E-02 | 1.509E-03 | ↑ in AD | 1.045 |
| Cer.d18.1.16.0 | 1.076E-04 | 1.202E-04 | 1.263E-05 | ↑ in AD | 1.117 |
| Cer.d18.1.18.0 | 4.676E-05 | 5.421E-05 | 7.450E-06 | ↑ in AD | 1.159 |
| Cer.d18.1.20.0 | 5.192E-05 | 6.258E-05 | 1.066E-05 | ↑ in AD | 1.205 |
| Cer.d18.1.24.1 | 6.915E-04 | 8.276E-04 | 1.362E-04 | ↑ in AD | 1.197 |
| Cer.d18.0.18.0 | 9.786E-06 | 1.139E-05 | 1.605E-06 | ↑ in AD | 1.164 |
| PE38p.6 | 2.498E-03 | 2.080E-03 | -4.188E-04 | ↓ in AD | 0.832 |
| PE40p.7 | 6.750E-04 | 5.631E-04 | -1.118E-04 | ↓ in AD | 0.834 |
| PE40p.6 | 1.211E-03 | 1.008E-03 | -2.032E-04 | ↓ in AD | 0.832 |
| PE36.0 | 1.514E-03 | 1.193E-03 | -3.215E-04 | ↓ in AD | 0.788 |
| PC34.2p..34.3e | 2.040E-03 | 1.873E-03 | -1.673E-04 | ↓ in AD | 0.918 |
| PC36.4p/etherPC | 3.046E-03 | 2.745E-03 | -3.017E-04 | ↓ in AD | 0.901 |
| PC38.5p..38.6e | 2.551E-03 | 2.274E-03 | -2.771E-04 | ↓ in AD | 0.891 |
| PC40.6p..40.7e | 1.344E-03 | 1.237E-03 | -1.077E-04 | ↓ in AD | 0.920 |
| PC34.5 | 1.828E-04 | 1.591E-04 | -2.376E-05 | ↓ in AD | 0.870 |
| PC38.1 | 3.476E-03 | 3.300E-03 | -1.760E-04 | ↓ in AD | 0.949 |
| SM18.20.0 | 8.164E-02 | 7.831E-02 | -3.330E-03 | ↓ in AD | 0.959 |
| SM18.22.0 | 6.482E-02 | 6.134E-02 | -3.482E-03 | ↓ in AD | 0.946 |
| SM18.24.0 | 1.164E-02 | 1.105E-02 | -5.871E-04 | ↓ in AD | 0.950 |

Those lipids in the blue section indicate an increase in the mean lipid levels and those in the grey section indicate a decrease in mean lipid levels in AD compared to healthy controls.

HC: Healthy Control, AD: Alzheimer's disease, Δ : Change, PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine, Cer: Ceramide, PG: Phosphatidylglycerol, PI: Phosphatidylinositol. PA: Phosphatidic acid.

Table 4.4 shows the mean value, the difference between the mean lipid species between clinical classifications and the fold change for each of the 46 lipids identified as being significantly different between HC and AD by LIMMA. 33 lipid species had mean levels higher and 13 had mean levels lower in AD compared to healthy controls. Whilst Tables 4.3 and 4.4 compare healthy controls to AD, LIMMA analysis was also repeated for the healthy controls compared to MCI and the MCI compared to AD groups. No lipids were identified as being significantly different following Bonferroni correction between the HC and MCI groups and only one lipid (Cer.d18.1.24.1) was different ($p=6.473E-6$) between the MCI and AD groups: levels of this lipid were higher in AD compared with MCI.

4.4.2 Generalised Linear Model (GLM) comparing clinical classification

The LIMMA analyses, while identifying those lipid species levels that are significantly different between clinical classifications, do not take into consideration factors which may influence the significance of the observed differences between mean lipid levels. Consequently a generalised linear model (GLM) was created to control for age, APOE ϵ 4 allele carriage status, gender and sample collection site (which was either Melbourne or Perth). The results from the GLM comparing the HC and AD groups are displayed in Table 4.5.

Table 4.5: Generalised Linear Modelling (GLM) for the 18 month samples for HC vs. AD

| Lipid | p-value | Age | APOEε4 | Sex | Site |
|-----------------|----------|----------|----------|-------|-------|
| Cer.d18.1.24.1 | 9.47E-14 | 2.63E-23 | 1.03E-26 | 0.180 | 0.013 |
| Cer.d18.1.20.0 | 1.72E-12 | 7.77E-28 | 2.24E-25 | 0.304 | 0.018 |
| PC34.0p..34.1e | 1.28E-07 | 1.20E-23 | 8.18E-28 | 0.505 | 0.200 |
| PC32.0 | 1.34E-07 | 1.57E-25 | 1.40E-27 | 0.303 | 0.148 |
| Cer.d18.1.18.0 | 4.40E-07 | 7.56E-30 | 4.39E-26 | 0.617 | 0.067 |
| Cer.d18.1.16.0 | 1.01E-06 | 1.32E-26 | 9.33E-27 | 0.267 | 0.027 |
| PG34.2 | 2.55E-06 | 7.73E-29 | 2.21E-26 | 0.513 | 0.906 |
| PC34.0e | 4.43E-06 | 3.99E-24 | 4.96E-28 | 0.498 | 0.194 |
| PC32.0e | 2.31E-05 | 1.06E-26 | 5.05E-28 | 0.175 | 0.172 |
| PE36.0 | 4.57E-05 | 3.07E-29 | 2.14E-25 | 0.501 | 0.611 |
| PG34.1 | 8.19E-05 | 1.72E-28 | 3.26E-26 | 0.593 | 0.244 |
| PE38p.6 | 1.12E-04 | 5.60E-31 | 1.51E-25 | 0.466 | 0.714 |
| PCLyso16.1 | 2.08E-04 | 4.70E-31 | 5.57E-26 | 0.518 | 0.277 |
| SM18.16.0 | 2.09E-04 | 4.34E-30 | 5.58E-27 | 0.412 | 0.062 |
| PA34.1 | 2.18E-04 | 5.77E-32 | 2.51E-26 | 0.237 | 0.103 |
| PC32.0p | 2.40E-04 | 1.15E-29 | 3.20E-27 | 0.383 | 0.113 |
| Cer.d18.0.18.0 | 2.44E-04 | 2.44E-31 | 3.47E-26 | 0.419 | 0.137 |
| PE40p.6 | 2.49E-04 | 5.75E-31 | 1.63E-25 | 0.377 | 0.486 |
| PG36.4 | 0.0003 | 1.84E-31 | 3.80E-26 | 0.353 | 0.241 |
| LysoPE18.0 | 0.0004 | 7.81E-31 | 2.80E-25 | 0.472 | 0.145 |
| LysoPE16.0 | 0.0005 | 2.09E-29 | 1.65E-25 | 0.397 | 0.079 |
| PC40.2p..40.3e | 0.0007 | 6.55E-31 | 4.36E-27 | 0.484 | 0.334 |
| PE40p.7 | 0.0008 | 8.10E-31 | 1.17E-25 | 0.343 | 0.366 |
| PC40.1p..40.2e | 0.0011 | 1.89E-30 | 1.99E-27 | 0.457 | 0.215 |
| PC36.4p/etherPC | 0.0012 | 1.12E-30 | 2.92E-26 | 0.865 | 0.273 |
| PC32.1 | 0.0013 | 7.65E-30 | 7.88E-27 | 0.999 | 0.301 |
| PG36.3 | 0.0031 | 2.23E-31 | 2.32E-26 | 0.317 | 0.218 |
| PE36.4 | 0.0037 | 7.20E-29 | 4.86E-26 | 0.833 | 0.100 |
| SM18.22.0 | 0.0046 | 1.17E-28 | 8.37E-27 | 0.578 | 0.616 |
| PC34.1 | 0.0049 | 2.92E-28 | 1.06E-26 | 0.488 | 0.498 |
| PCLyso18.1e | 0.0056 | 4.26E-30 | 5.34E-27 | 0.231 | 0.308 |
| SM18.20.0 | 0.0060 | 5.61E-31 | 1.97E-26 | 0.831 | 0.209 |
| PC40.6p..40.7e | 0.0085 | 1.28E-31 | 1.06E-25 | 0.560 | 0.248 |
| PC36.0p/etherPC | 0.0092 | 7.60E-29 | 4.30E-27 | 0.634 | 0.594 |
| PE36.3 | 0.0095 | 8.68E-29 | 7.21E-27 | 0.787 | 0.106 |
| PC34.2p..34.3e | 0.0098 | 7.69E-31 | 3.71E-27 | 0.710 | 0.387 |
| PC38.5p..38.6e | 0.0106 | 9.41E-31 | 1.32E-25 | 0.629 | 0.447 |
| PCLyso18e.0 | 0.0109 | 1.28E-30 | 1.47E-26 | 0.307 | 0.272 |
| PE34.1 | 0.0118 | 1.49E-29 | 3.25E-26 | 0.570 | 0.106 |
| PE38.5 | 0.0183 | 2.89E-29 | 5.31E-26 | 0.934 | 0.114 |
| PC34.5 | 0.0242 | 1.48E-30 | 1.94E-26 | 0.441 | 0.323 |
| LysoPE18.1 | 0.0351 | 9.05E-31 | 1.12E-25 | 0.271 | 0.149 |
| PC38.2p..38.3e | 0.0653 | 7.40E-31 | 6.96E-27 | 0.519 | 0.448 |
| PE38.4 | 0.0796 | 4.15E-30 | 2.94E-26 | 0.767 | 0.123 |
| PC38.1 | 0.3542 | 1.55E-29 | 1.00E-26 | 0.670 | 0.214 |
| SM18.24.0 | 0.5028 | 1.35E-29 | 7.46E-27 | 0.633 | 0.241 |

Adjusted for age, sex, APOEε4 allele status (i.e. carriage or non-carriage of an APOEε4 allele) and sample collection site (Perth or Melbourne). Lipids with a statistically significant p-value less than the Bonferroni cut off (0.05/189) are above the solid black line.

PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine, Cer: Ceramide, PG: Phosphatidylglycerol, PI: Phosphatidylinositol, PA: Phosphatidic acid, APOEε4: Apolipoprotein E ε4 allele.

Table 4.5 shows the p-values for each of the 46 lipids identified by LIMMA as being significantly different between HC and AD, adjusted through GLM for the following covariates; age, sex, APOEε4 allele status and sample collection site. The p-values for each of the covariates are also shown in the table. The data are sorted by the strongest p-value for the lipid level post adjustment i.e. where the levels are most different between the clinical classification groups. The top 18 lipids (above the solid line) in the table remain statistically significant (less than the Bonferroni cut off value of $p < 0.0003$ (α of 0.05 / n of 189)) after adjustment for age, gender, APOEε4 allele status and collection site. The p-values indicate that neither site of collection or gender significantly influence the results, but as expected, age and APOEε4 allele carriage do significantly influence the results.

No GLM was performed to compare the healthy control and MCI groups as no lipids were identified as being significantly different between these clinical classifications by LIMMA.

The GLM of the MCI compared to AD groups showed that the Cer.d18.1.24.1 species identified as being significantly different by LIMMA between these groups, also remained significant ($p < 0.0003$) following adjustment for age, gender, APOEε4 allele status and sample collection site.

4.4.3. Variable selection for predictive lipid panel

As previously described for the baseline data in Chapter 3, the LIMMA and GLM models assess associations between lipids and clinical classification, i.e. levels of lipid species that are significantly different between clinical classifications. Variable selection analysis attempts to identify lipid species which when considered collectively are able to predict clinical classification with greatest certainty. As such, the level at which clinical classification can be predicted, is determined by modelling the selected lipids and covariates using GLM and Receiver Operating Characteristics (ROC) models.

As described in Chapter 3, previous analysis of biomarkers in the AIBL cohort utilised a variable selection pathway to create lists of biomarkers that were chosen from four separate selection methods; random forest, boosted trees,

regression trees and LIMMA and used clinical classification as a binary outcome, i.e. HC or AD [476]. In this study, a variable selection pathway was applied to the 189 lipid species to create an importance ranking (in addition to generation of p-values), where 1000 iterations were performed to define a list of those lipid species that are selected most often as differing in levels when comparing clinical classifications. Each time a lipid was selected, a frequency score was assigned if it was chosen from all four statistical methods, or from three out of the four statistical methods.

4.4.3.1 Variable selection for HC vs. AD groups

Table 4.6 lists the lipid species selected most often when comparing the HC and AD groups. The lipid species that are selected in at least 10% of the 1000 iterations from all four statistical methods are listed in column A and the lipid species that were selected in at least 10% of the 1000 iterations from three of the four statistical methods are shown in column B.

Table 4.6: Lipid Markers selected by the Variable Selection Pathway for HC vs. AD in the 18 month samples.

| List A (4/4) | | List B (3/4) | |
|----------------|-------------------|-----------------|-------------------|
| Lipid | Frequency (/1000) | Lipid | Frequency (/1000) |
| Cer.d18.1.24.1 | 766 | Cer.d18.1.20.0 | 626 |
| Cer.d18.1.20.0 | 362 | PG34.1 | 466 |
| PC34.0p..34.1e | 163 | PE36.0 | 451 |
| PG34.2 | 127 | PC34.0p..34.1e | 444 |
| | | PC32.0 | 444 |
| | | PC34.0e | 345 |
| | | PG34.2 | 343 |
| | | Cer.d18.1.18.0 | 334 |
| | | SM18.22.0 | 283 |
| | | Cer.d18.1.16.0 | 237 |
| | | Cer.d18.1.24.1 | 230 |
| | | PC32.0e | 164 |
| | | PC38.5p..38.6e | 155 |
| | | PC36.4p/etherPC | 140 |
| | | PE38p.6 | 138 |
| | | PC34.1 | 133 |
| | | LysoPE16.0 | 125 |

PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine, Cer: Ceramide, PG: Phosphatidylglycerol, PI: Phosphatidylinositol. The lipids highlighted in grey appeared in both list A and list B.

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Those lipids with the highest frequencies of selection from both lists A and B of Table 4.6 were clustered using Partitioning Around Medoids (PAM) to find a central lipid, and then cluster other correlated lipids around it. As described in Chapter 3, this statistical method correlates lipids that are related to each other without consideration of clinical classification, and is performed to see if lipids can be eliminated from a final model if they behave in the same way as other lipids in their respective clusters in terms of predicting clinical classification. A CLARA (Clustering for Large Applications) algorithm was applied to create a clusplot of correlated lipids, which resulted in the formation of four lipid clusters, listed in Table 4.7 and represented graphically in Figure 4.18.

Table 4.7: Four groups of clustered lipids were determined using medoid clustering when comparing the HC and AD groups.

| Cluster 1 | Cluster 2 | Cluster 3 | Cluster 4 |
|------------|-----------------|----------------|----------------|
| LysoPE16.0 | PE38p.6 | PC34.0p..34.1e | Cer.d18.1.16.0 |
| PG34.2 | PE36.0 | PC32.0e | Cer.d18.1.18.0 |
| PG34.1 | PC36.4p/etherPC | PC32.0 | Cer.d18.1.20.0 |
| PC34.1 | PC38.5p..38.6e | PC34.0e | Cer.d18.1.24.1 |
| | SM18.22.0 | | |
| | | | |
| | | | |
| | | | |

PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine, Cer: Ceramide, PG: Phosphatidylglycerol, PI: Phosphatidylinositol.

Of the 17 lipids selected by Variable Selection Pathways for HC vs. AD, cluster 1 has 4 lipids, cluster 2 has 5 lipids, cluster 3 has 4 lipids and cluster 4 has 4 lipids.

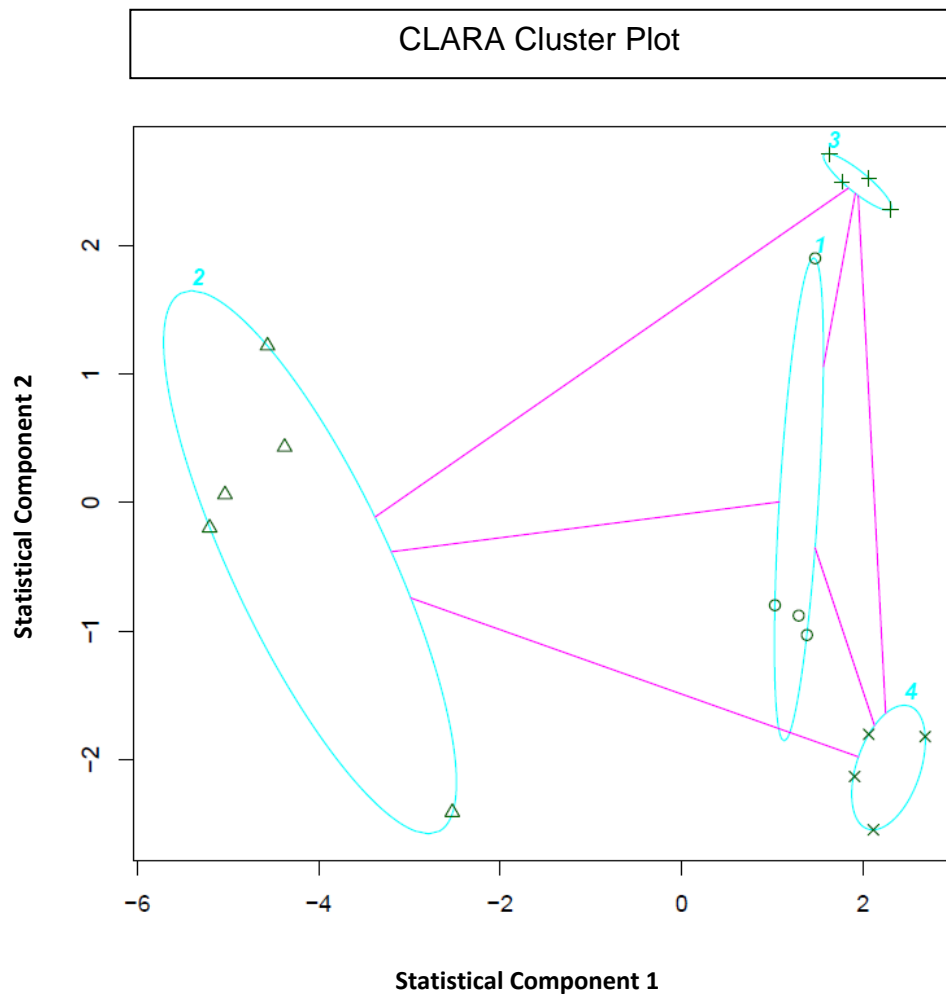


Figure 4.18: Clusplot of correlated lipids showing the 4, 5, 4 and 4 lipids from clusters 1 to 4 respectively.

The clusplot shows a stronger relationship for the lipids within clusters 3 and 4. The lipids in clusters 1 and 2 are by contrast more diffuse, suggesting a weaker intracluster relationship between those lipids. The strength of these relationships may be explained by the fact that clusters 3 and 4 both contain lipids of the same class (PC and ceramides respectively), whilst clusters 1 and 2 are more heterogeneous containing a mixture of PG, PE, PC and SM species.

Standard Pearson's correlations were applied to the lipids in each cluster to explore the directional relationship between correlated lipids and clinical classification. These relationships between the scaled mean expression for HC and AD groups are shown in Figure 4.19 with the r-values for the lipid correlations shown in Table 4.8.

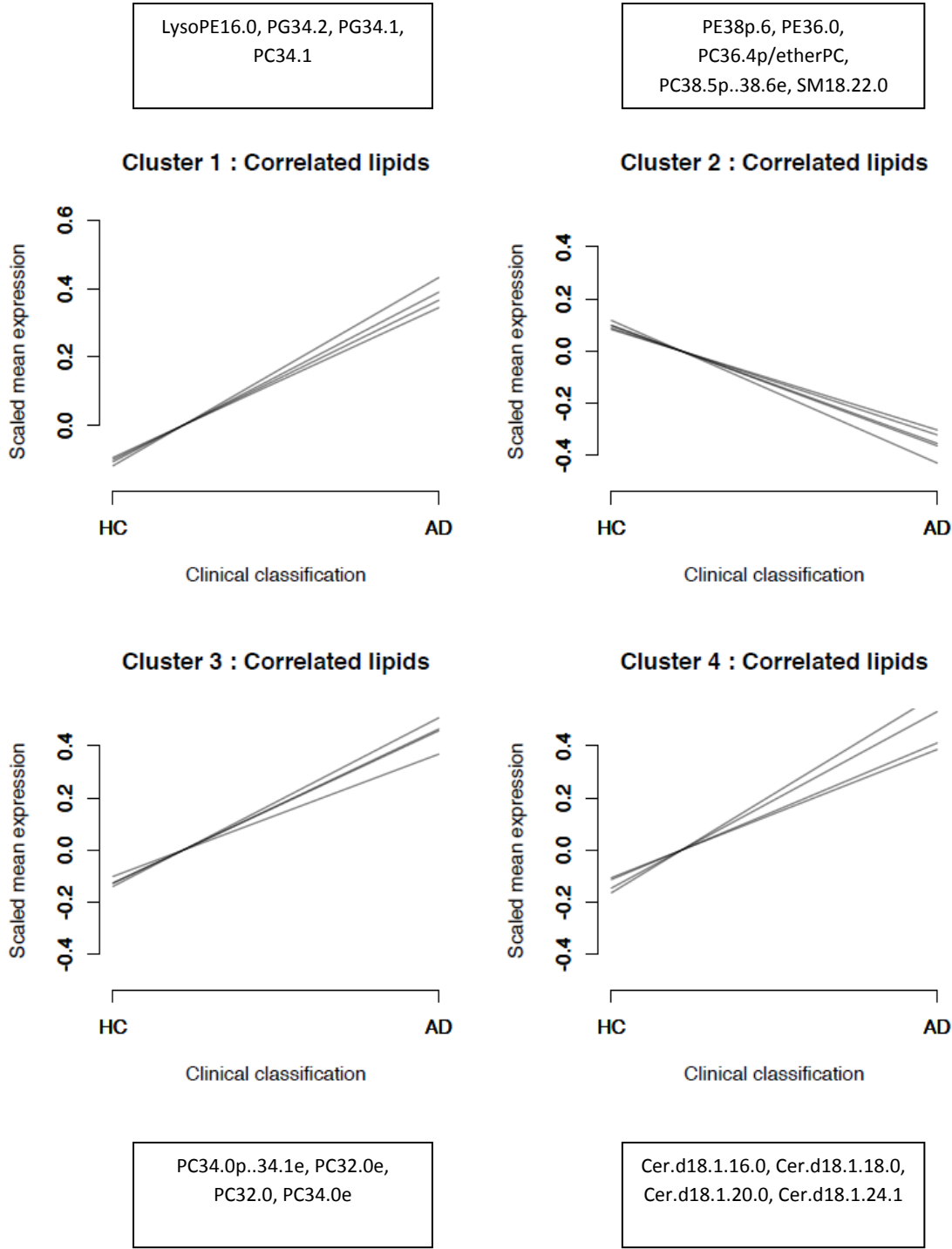


Figure 4.19: Correlated lipids between clinical classifications.

PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine, Cer: Ceramide, PG: Phosphatidylglycerol. Scaled mean expression is used to enable graphical representation of lipids that differ in abundance.

Table 4.8: Standard Pearson's Correlations between 17 lipid species chosen from the variable selection pathway for the HC vs. AD groups.

| A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | |
|---|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|--------------------|
| 1 | -0.113130 | -0.092540 | 0.024544 | 0.291661 | 0.217801 | -0.171040 | 0.009912 | 0.182172 | 0.179702 | 0.204909 | 0.043866 | -0.005970 | 0.238777 | 0.205044 | 0.293567 | 0.303768 | A Lyso.PE16.0 |
| | 1 | 0.769412 | -0.298200 | -0.125650 | -0.155970 | 0.534321 | 0.530812 | -0.144360 | -0.145240 | -0.066340 | -0.232740 | -0.030280 | -0.127930 | -0.070670 | -0.106140 | -0.150560 | B PE38p.6 |
| | | 1 | -0.286610 | -0.105350 | -0.246010 | 0.415336 | 0.646169 | -0.168570 | -0.102940 | -0.155530 | -0.275870 | 0.038010 | -0.128800 | -0.094130 | -0.140680 | -0.159100 | C PE36.0 |
| | | | 1 | 0.362691 | 0.061308 | -0.152030 | -0.194020 | 0.003428 | 0.059416 | 0.031222 | 0.225135 | -0.112510 | 0.090714 | 0.108925 | 0.123625 | 0.193643 | D PG34.2 |
| | | | | 1 | 0.047099 | -0.192260 | -0.144210 | -0.025630 | 0.145080 | 0.084246 | 0.267404 | -0.243100 | 0.128726 | 0.227648 | 0.227120 | 0.303871 | E PG34.1 |
| | | | | | 1 | -0.042850 | -0.014050 | 0.788231 | 0.475870 | 0.855177 | 0.302029 | -0.079970 | 0.313523 | 0.170491 | 0.204510 | 0.318756 | F PC.34.0p..34.1e |
| | | | | | | 1 | 0.693248 | 0.013221 | 0.011570 | -0.006280 | -0.177810 | -0.234220 | -0.167150 | -0.104370 | -0.188880 | -0.169820 | G PC36.4p.or.ethPC |
| | | | | | | | 1 | 0.067121 | 0.040785 | 0.031442 | -0.375050 | 0.032488 | -0.015490 | -0.046800 | -0.079600 | -0.052150 | H PC.38.5p..38.6e |
| | | | | | | | | 1 | 0.614448 | 0.783217 | 0.131912 | -0.049800 | 0.338028 | 0.128871 | 0.108996 | 0.266251 | I PC32.0e |
| | | | | | | | | | 1 | 0.547570 | 0.457070 | -0.347740 | 0.190831 | 0.128015 | 0.056191 | 0.204358 | J PC32.0 |
| | | | | | | | | | | 1 | 0.275150 | -0.166950 | 0.263676 | 0.203571 | 0.195802 | 0.274928 | K PC34.0e |
| | | | | | | | | | | | 1 | -0.528100 | -0.066970 | 0.057543 | 0.003977 | 0.058526 | L PC34.1 |
| | | | | | | | | | | | | 1 | 0.175049 | -0.063300 | 0.046131 | -0.053410 | M SM18.22.0 |
| | | | | | | | | | | | | | 1 | 0.528673 | 0.545311 | 0.626519 | N Cer.d18.1.16.0 |
| | | | | | | | | | | | | | | 1 | 0.666193 | 0.595002 | O Cer.d18.1.18.0 |
| | | | | | | | | | | | | | | | 1 | 0.667528 | P Cer.d18.1.20.0 |
| | | | | | | | | | | | | | | | | 1 | Q Cer.d18.1.24.1 |

Correlation coefficients (R) for each pairwise correlation are shown. Correlations of greater than 0.5 or less than -0.5 are highlighted in red.

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The strongest correlations, as highlighted in red in Table 4.8 were seen between lipids of the same class. i.e. PCs, PEs and ceramides. A correlation was also shown between PC34.1 and SM18.22.0. It is important to note that sphingomyelin can be formed by the transfer of phosphocholine to ceramide.

Looking at the directional grouping (increased or decreased in AD compared to HC) of the relationship between correlated lipids and clinical classification as depicted in Figure 4.19, it can be seen that the lipids in each cluster have a very similar relationship.

Taking the 17 lipids chosen from the variable selection pathway, and factoring in their correlations as determined by the Pearson's correlation R-values (see Table 4.8), the lipids were subjected to further statistical assessment using a stepwise generalised linear model (GLM) to reduce the number of lipids in the predictive model. This methodology was also utilised for the baseline data in Chapter 3. The GLM algorithm was run repeatedly to remove lipids from the predictive model until the lipids left were those giving the best Akaike Information Criterion (AIC) report, which is a measure of the relative quality of a statistical model for a given set of data, and therefore provides a means for selecting the best model, as previously described in Chapter 3. This stepwise model also factors in covariates of age, gender, APOE ϵ 4 allele status and collection site, as described for the previous GLM. This prediction model split the analysis of the 948 study participant plasma lipid profiles into a run comprising 70% training (that includes clinical classification) and 30% test data (blind to clinical classification), and was repeated over 100 times until the greatest sensitivity and specificity was achieved. A final panel of 9 lipids was selected that contained the least number of lipids with maximal sensitivity and specificity for predicting disease classification: these 9 lipids are listed in Table 4.9.

Table 4.9: List of the nine lipids that yielded maximal sensitivity and specificity between HC and AD groups at the 18-month follow-up time point.

| Lipid |
|----------------|
| LysoPE16.0 |
| PG34.2 |
| PC34.0p..34.1e |
| PC38.5p..38.6e |
| PC32.0e |
| PC32.0 |
| SM18.22.0 |
| Cer.d18.1.20.0 |
| Cer.d18.1.24.1 |

PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine, Cer: Ceramide, PG: Phosphatidylglycerol. Those lipids highlighted in grey were also selected from the baseline data.

All 9 lipid species were also identified as being significantly different when assessed by the standard LIMMA analyses (Tables 4.3 and 4.4). PC38.5p..38.6e and PC32.0 were also selected in the variable selection pipeline for the baseline data in Chapter 3.

Maximal sensitivity and specificity (80%) for predicting participants with AD as compared to healthy controls is shown in the Receiver Operating Characteristic (ROC) curve in Figure 4.20, with respect to the GLM of the top nine lipids adjusted for age, gender, APOE ϵ 4 allele status and collection site.

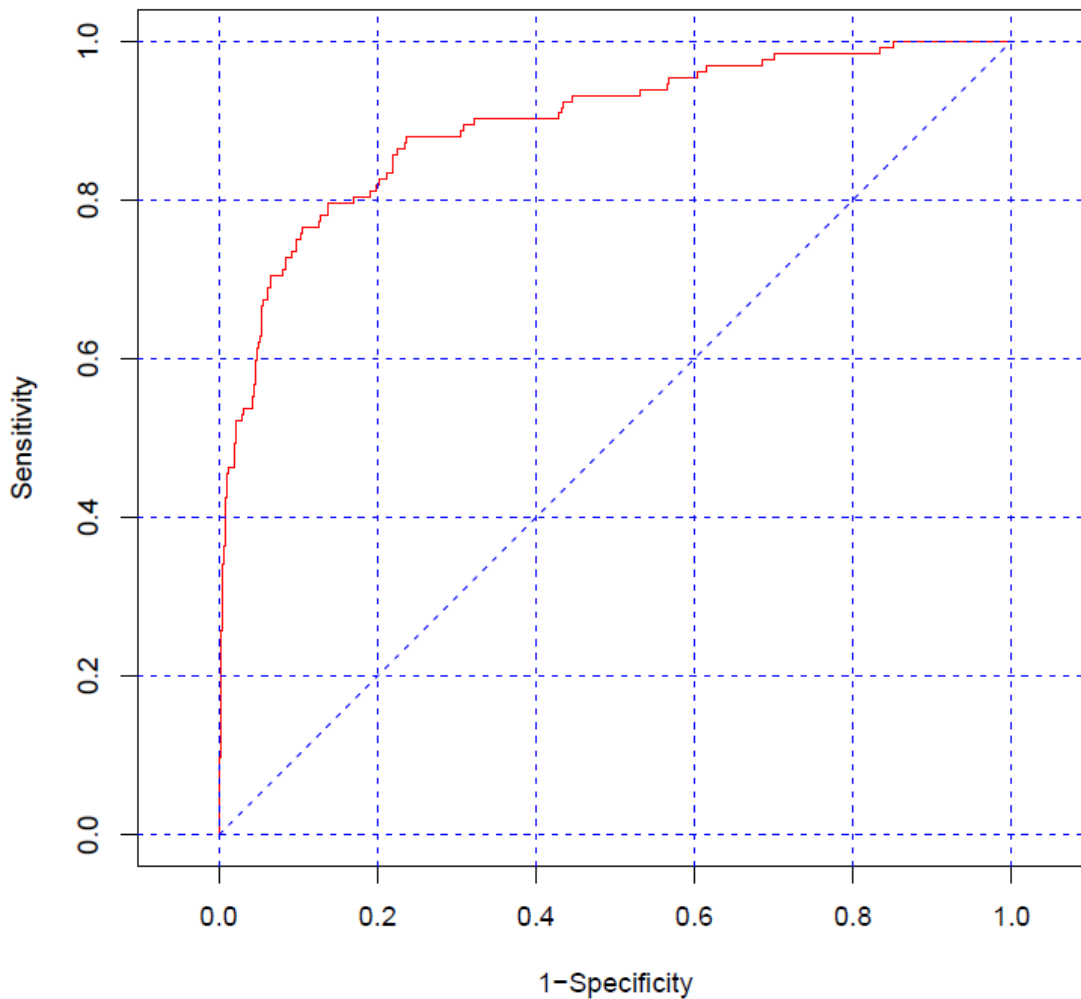


Figure 4.20: Receiver Operating Characteristics (ROC) curve of the top nine lipids from stepwise Generalised Linear Modelling (GLM) showing sensitivity and specificity of 80% for the HC vs. AD group.

As previously described in Chapter 3, the ROC curve is a plot of the true positive rate (sensitivity) against the true negative rate (specificity) and is therefore a measure of the accuracy of a diagnostic test, which in this case is the ability of the panel of nine lipids to distinguish those participants with AD from healthy controls. A sensitivity and specificity of 80% for the panel of nine lipids is indicated by the area under the ROC curve. Although there were only two lipids (PC38.5p..38.6e and PC32.0) in the predictive panel for the 18 month data that were also selected in the baseline predictive panel, the remainder were common to the same classes yet were different sub-species at both time points. Despite slight differences in the lipids selected at 18 months compared

to baseline, the sensitivity and specificity of the predictive panel remained unchanged at 80%.

4.4.3.2 Variable Selection for HC vs. MCI groups

As carried out for the healthy controls vs. AD groups, the variable selection pathway was repeated for the healthy control vs. MCI groups. This generated a list of lipids that were chosen from four separate selection methods as previously described for the HC vs. AD groups. Table 4.10 lists the lipid species selected most often when comparing the HC and MCI groups. As before, the lipid species that were selected in at least 10% of the 1000 iterations from all four statistical methods are listed in column A and the lipid species that were selected in at least 10% of the 1000 iterations from three of the four statistical methods are shown in column B.

Table 4.10: Lipid Markers selected by the Variable Selection Pathway for HC vs. MCI in the 18 month samples.

| List A (4/4) | | List B (3/4) | |
|--------------|-------------------|--------------|-------------------|
| Lipid | Frequency (/1000) | Lipid | Frequency (/1000) |
| | | LysoPG16.1 | 376 |
| | | PI38.2 | 205 |
| | | PS38.5 | 173 |
| | | PCLyso16.1 | 167 |
| | | PS36.2 | 125 |
| | | | |
| | | | |

PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine, Cer: Ceramide, PG: Phosphatidylglycerol, PI: Phosphatidylinositol.

The variable selection pipeline for the HC vs. MCI groups selected seven lipids (PI38.2, PS38.5, PA36.1, LysoPE18.1, LysoPE18.0, LysoPE18.2, PG36.4) from all four statistical methods: the maximum frequency however was 1.5%, thus too low to be on the list above. The five lipids with the highest frequencies of selection from list B of Table 4.10 were clustered as previously described: As too few lipids were selected however, a CLARA (Clustering for Large Applications) was not generated. Adding the five lipids listed in Table 4.10B to the predictive GLM/ROC models gave a sensitivity and specificity of just 69% for distinguishing those participants with MCI from healthy controls following adjustment for age, gender, APOEε4 allele status and collection site.

4.4.3.3 Variable selection MCI vs. AD groups

As described previously, the variable selection pathway was repeated for the MCI vs. AD groups. Table 4.11 lists the lipid species selected most often when comparing the MCI and AD groups. As before, the lipid species that were selected in at least 10% of the 1000 iterations from all four statistical methods are listed in column A and the lipid species that were selected in at least 10% of the 1000 iterations from three of the four statistical methods are shown in column B.

Table 4.11: Lipid Markers selected by the Variable Selection Pathway for MCI vs. AD in the 18 month samples.

| List A (4/4) | | List B (3/4) | |
|----------------|-------------------|----------------|-------------------|
| Lipid | Frequency (/1000) | Lipid | Frequency (/1000) |
| Cer.d18.1.24.1 | 377 | Cer.d18.1.20.0 | 325 |
| | | Cer.d18.1.24.1 | 299 |
| | | SM18.22.0 | 203 |
| | | Lyso.PG16.1f | 112 |
| | | PE40p.4 | 107 |

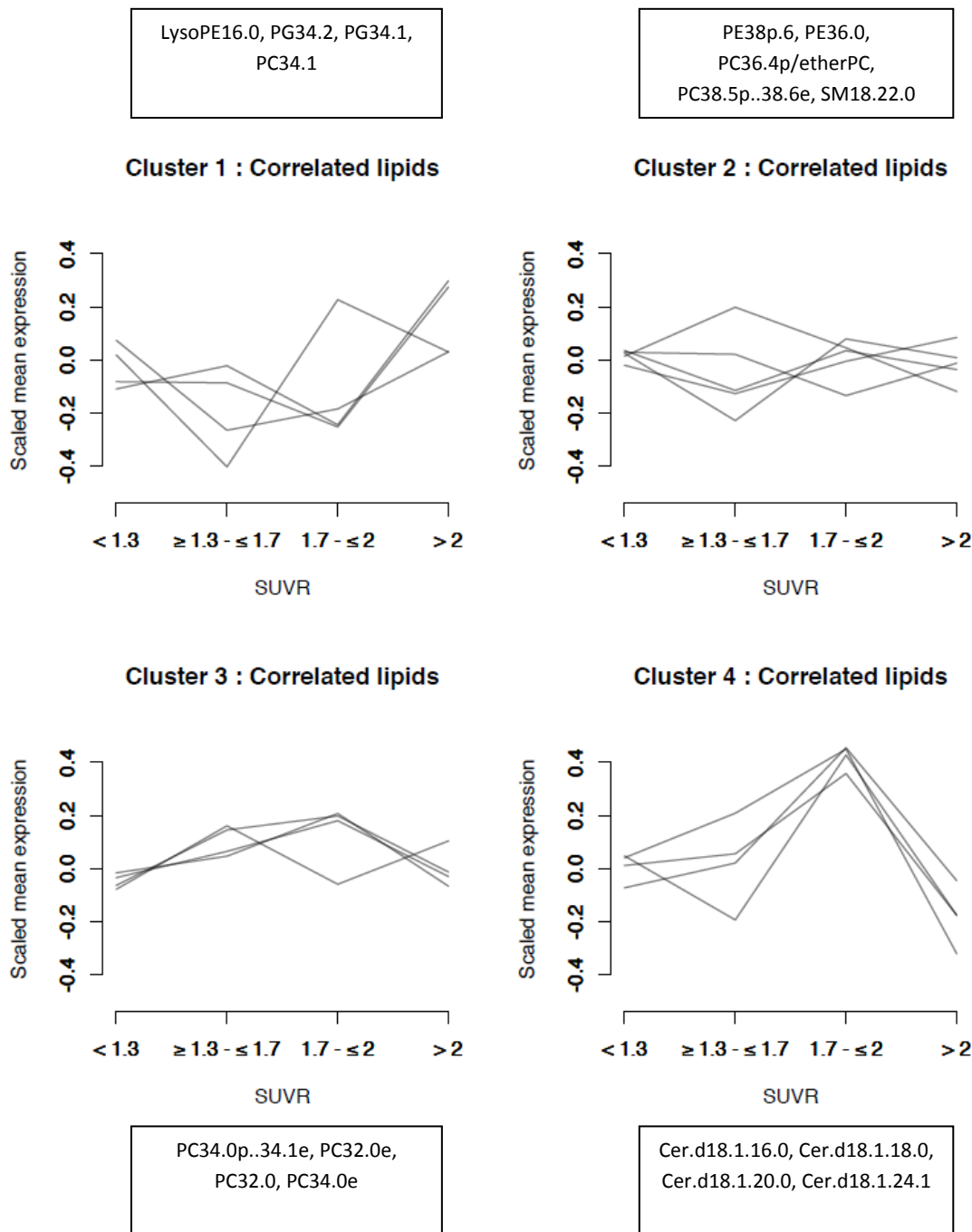
Cer: Ceramide, SM: Sphingomyelin, PE: Phosphatidylethanolamine, LysoPG: Lysophosphatidylglycerol. The lipid highlighted in grey appeared in both list A and list B.

The variable selection pipeline for the MCI vs. AD groups selected only one lipid (Cer.d18.1.24.1) from all four statistical methods in at least 10% of the 1000 iterations. Those lipids with the highest frequencies of selection from both lists A and B of Table 4.11 were clustered as previously described. However, as too few lipids were once again selected, a CLARA (Clustering for Large Applications) was not generated. Adding the five lipids listed in Table 4.11 to the predictive GLM/ROC models also gave a sensitivity and specificity of just 69% for distinguishing those participants with AD from MCI following adjustment for age, gender, APOEε4 allele status and collection site.

4.4.4 Correlation with Standardised Uptake Value Ratio (SUVR)

The previous sections assessed the ability of a panel of lipids to predict clinical classification as the aim of the study was to develop a sensitive and specific diagnostic blood biomarker lipid panel. In this section, as with the baseline data, the relationship of the lipids determined (Section 4.4.4) by variable selection analysis was assessed against neocortical amyloid burden represented by SUVR; an established neuroimaging biomarker of AD. Each of the 17 lipids identified by the variable selection pathway for the healthy control vs. AD groups (Table 4.6) was plotted by cluster (Table 4.7) against categories of SUVR measured at the 18 month time point; see Figure 4.21 below.

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| SUVR category | Interpretation |
|-------------------|---|
| < 1.3 | Individuals with minimal accumulation of A β |
| > 1.3 to < 1.7 | Includes individuals with borderline transitions from amyloid -ve to amyloid +ve |
| 1.7 to \leq 2.0 | Individuals who are definite accumulators, with increased rate of A β accumulation |
| > 2.0 | Individuals who have the highest accumulation but rate plateaus with saturation between 2-3 |

Figure 4.21: Clustered and correlated lipids identified by the variable selection pathway for the healthy control vs. AD groups compared with 18 month SUVR categories.

AD: Alzheimer's disease, SUVR: Standard uptake value ratio. A β : Amyloid Beta. The SUVR categories used in this table are those described by Villemagne *et al.* [477].

Figure 4.21 shows that some of the lipids in each cluster behave in a similar way when compared to SUVR categories. The lipids levels in clusters 2 and 3 are approximately the same at SUVR of <1.3 and >2 . Cluster 4 contains four ceramide species which show very similar relationships with SUVR, rising between a SUVR of $(> 1.3 \text{ to } < 1.7)$ and $(1.7 \leq 2.0)$, then a sudden decline between a SUVR of $(1.7 \leq 2.0)$ and (> 2) , the point at which A β accumulation plateaus.

4.4.5 LIMMA and GLM comparing the transition groups

To assess the lipid profile data longitudinally rather than cross-sectionally, a LIMMA with Bonferroni comparisons was applied to participants following classification as either non-transition (classification remained the same: excepting stable AD), transition (classification changed to a different group) or stable AD (remained in the AD group) over the 18 month follow up period. The transition group also included those participants with MCI: “transition” in the AIBL cohort is defined as transition towards AD or declining mental health as determined by neuropsychological testing [492]. Table 4.12 shows the demographics of each classification group for the 948 individuals assessed longitudinally, i.e. for whom lipid profiles were measured at both baseline and 18 month follow up.

Table 4.12: Demographics of AIBL participants classified as either non-transition, transition or stable AD over the 18 month follow up period.

| | Non-transition | Stable AD | Transition | p-value |
|-----------------------------|---------------------|---------------------|---------------------|---------|
| Count (n) | 679 | 155 | 114 | |
| Age, years (Mean \pm SD) | 71.76 (\pm 6.75) | 79.25 (\pm 7.96) | 77.22 (\pm 7.63) | <0.0001 |
| Gender (F/M) | 400/279 | 94/61 | 55/59 | 0.077 |
| APOE ϵ 4 (-ve/+ve) | 498/181 | 50/105 | 55/59 | <0.0001 |
| MMSE (Mean \pm SD) | 29 (\pm 1.33) | 18 (\pm 7.05) | 26 (\pm 3.06) | <0.0001 |
| SUVr (n) | 149 | 23 | 38 | |
| SUVr (Mean \pm SD) | 1.38 (\pm 0.39) | 2.37 (\pm 0.48) | 1.93 (\pm 0.63) | <0.0001 |
| Hip Vol (n) | 140 | 22 | 31 | |
| Hip Vol (Mean \pm SD) | 3.15 (\pm 0.33) | 2.80 (\pm 0.49) | 2.85 (\pm 0.42) | <0.0001 |

Non-transition: Classification remained the same (excepting stable AD), Stable AD: Remained in the AD group, Transition: Classification changed to a different group over the 18 month follow up period, AD: Alzheimer’s Disease, F: Female, M: Male, APOE ϵ 4 (-ve/+ve): Negative or positive for the Apolipoprotein (APOE) ϵ 4 allele, MMSE: Mini Mental State Examination, SUVr: Standard Uptake Value Ratio, Hip Vol: Hippocampal volume (measured in mL), SD: Standard deviation. The characteristics were composed using analysis of variance (ANOVA) to calculate differences for the continuous variables and χ^2 for categorical variables. A Kruskal-Wallis formula was applied to the MMSE scores.

The 948 individuals in whom lipid profiles were measured at both baseline and 18 month follow up consisted of: 679 non-transition, 114 transition and 155 stable AD participants. The number of men and women in each classification did not significantly differ, but significant differences in mean age, APOE ϵ 4

allele status, MMSE, SUVR and hippocampal volume were observed between groups.

LIMMA analysis of the non-transition vs. stable AD groups identified 44 out of the 189 lipid species measured that were significantly different in levels between groups. Table 4.13 lists these lipids.

Table 4.13: Linear Models for Microarray Data (LIMMA) for 18 month samples comparing the non-transition vs. stable AD groups.

| Lipid | p-value | Lipid | p-value |
|-----------------|----------|-----------------|----------|
| Cer.d18.1.20.0 | 3.71E-18 | PC32.1 | 2.40E-06 |
| Cer.d18.1.24.1 | 4.43E-18 | PC36.4p/etherPC | 5.03E-06 |
| PC34.0p..34.1e | 1.16E-15 | PE40p.7 | 5.11E-06 |
| PC34.0e | 5.11E-13 | PE38.5 | 5.46E-06 |
| PC32.0 | 7.53E-12 | PC40.2p..40.3e | 6.30E-06 |
| PE36.0 | 1.35E-10 | SM18.20.0 | 8.65E-06 |
| Cer.d18.1.16.0 | 2.88E-10 | PCLyso18.1 | 1.41E-05 |
| PG34.2 | 4.02E-10 | SM18.24.0 | 1.56E-05 |
| PC34.1 | 9.12E-09 | PCLyso18e.0 | 1.93E-05 |
| Cer.d18.1.18.0 | 9.35E-09 | PC38.1 | 2.12E-05 |
| PG34.1 | 1.43E-08 | PG36.4 | 2.96E-05 |
| PC32.0e | 6.33E-08 | PC34.5 | 3.86E-05 |
| LysoPE16.0 | 6.38E-08 | LysoPE18.1 | 4.78E-05 |
| PE38p.6 | 6.53E-08 | PE34.1 | 5.30E-05 |
| SM18.22.0 | 7.52E-08 | PC34.2p..34.3e | 6.14E-05 |
| PC36.0p/etherPC | 7.68E-08 | PC38.2p..38.3e | 6.53E-05 |
| PE36.4 | 1.61E-07 | PC32.0p | 6.68E-05 |
| PCLyso16.1 | 3.10E-07 | SM18.16.0 | 1.21E-04 |
| LysoPE18.0 | 4.19E-07 | PA34.1 | 1.85E-04 |
| PE40p.6 | 5.37E-07 | Cer.d18.0.22.0 | 1.86E-04 |
| PCLyso18e.1 | 1.61E-06 | Cer.d18.0.18.0 | 2.20E-04 |
| PC38.5p..38.6e | 1.96E-06 | PE36.3 | 2.47E-04 |

p-values less than the Bonferroni cut off (0.05/189).

PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine, Cer: Ceramide, PG: Phosphatidylglycerol, PI: Phosphatidylinositol, PA: Phosphatidic acid.

A fold lipid change was subsequently calculated using unadjusted raw expression values to provide a relative lipid difference between transition classifications. The mean levels, difference in levels between groups, the directional change i.e. increased or decreased in stable AD compared to the non-transition group and fold change is shown in Table 4.14.

Chapter 4: Plasma Lipid Profiles in AIBL 18 month samples

Table 4.14: Mean value difference in lipid species levels between groups, direction of change in lipid levels between the non-transition and stable AD and groups and fold change.

| Lipid | Mean Non-trans | Mean Stable AD | Difference | Direction of change | Fold Δ |
|-----------------|----------------|----------------|------------|---------------------|---------------|
| LysoPE16.0 | 4.086E-04 | 4.734E-04 | -6.480E-05 | ↑ in stable AD | 1.159 |
| LysoPE18.1 | 2.041E-04 | 2.355E-04 | -3.148E-05 | ↑ in stable AD | 1.154 |
| LysoPE18.0 | 2.298E-04 | 2.610E-04 | -3.123E-05 | ↑ in stable AD | 1.136 |
| PE34.1 | 3.025E-04 | 3.672E-04 | -6.193E-05 | ↑ in stable AD | 1.203 |
| PE36.4 | 8.704E-04 | 1.053E-03 | -1.822E-04 | ↑ in stable AD | 1.209 |
| PE36.3 | 4.614E-04 | 5.374E-04 | -7.599E-05 | ↑ in stable AD | 1.165 |
| PE38.5 | 1.101E-03 | 1.300E-03 | -1.992E-04 | ↑ in stable AD | 1.181 |
| PA34.1 | 1.444E-05 | 1.616E-05 | -1.720E-06 | ↑ in stable AD | 1.119 |
| PG34.2 | 1.908E-05 | 2.376E-05 | -4.685E-06 | ↑ in stable AD | 1.246 |
| PG34.1 | 2.639E-05 | 3.106E-05 | -4.673E-06 | ↑ in stable AD | 1.177 |
| PG36.4 | 1.219E-05 | 1.395E-05 | -1.769E-06 | ↑ in stable AD | 1.145 |
| PCLyso16.1 | 4.119E-04 | 4.610E-04 | -4.907E-05 | ↑ in stable AD | 1.119 |
| PCLyso18.1 | 3.508E-03 | 3.824E-03 | -3.157E-04 | ↑ in stable AD | 1.090 |
| PCLyso18e.1 | 7.030E-05 | 7.777E-05 | -7.467E-06 | ↑ in stable AD | 1.106 |
| PCLyso18e.0 | 2.562E-04 | 2.845E-04 | -2.835E-05 | ↑ in stable AD | 1.111 |
| PC32.0p | 1.239E-03 | 1.310E-03 | -7.121E-05 | ↑ in stable AD | 1.057 |
| PC34.0p..34.1e | 4.473E-03 | 4.988E-03 | -5.147E-04 | ↑ in stable AD | 1.115 |
| PC36.0p/etherPC | 4.163E-03 | 4.496E-03 | -3.335E-04 | ↑ in stable AD | 1.080 |
| PC38.2p..38.3e | 2.507E-03 | 2.676E-03 | -1.689E-04 | ↑ in stable AD | 1.067 |
| PC40.2p..40.3e | 7.842E-04 | 8.462E-04 | -6.197E-05 | ↑ in stable AD | 1.079 |
| PC32.0e | 1.301E-03 | 1.422E-03 | -1.211E-04 | ↑ in stable AD | 1.093 |
| PC32.1 | 9.970E-03 | 1.116E-02 | -1.190E-03 | ↑ in stable AD | 1.119 |
| PC32.0 | 5.406E-03 | 5.914E-03 | -5.080E-04 | ↑ in stable AD | 1.094 |
| PC34.0e | 1.140E-03 | 1.282E-03 | -1.423E-04 | ↑ in stable AD | 1.125 |
| PC34.1 | 8.346E-02 | 8.943E-02 | -5.967E-03 | ↑ in stable AD | 1.071 |
| SM18.16.0 | 3.359E-02 | 3.510E-02 | -1.512E-03 | ↑ in stable AD | 1.045 |
| Cer.d18.1.16.0 | 1.075E-04 | 1.208E-04 | -1.325E-05 | ↑ in stable AD | 1.123 |
| Cer.d18.1.18.0 | 4.674E-05 | 5.437E-05 | -7.639E-06 | ↑ in stable AD | 1.163 |
| Cer.d18.1.20.0 | 5.182E-05 | 6.367E-05 | -1.185E-05 | ↑ in stable AD | 1.229 |
| Cer.d18.1.24.1 | 6.909E-04 | 8.250E-04 | -1.342E-04 | ↑ in stable AD | 1.194 |
| Cer.d18.0.18.0 | 9.779E-06 | 1.128E-05 | -1.505E-06 | ↑ in stable AD | 1.154 |
| Cer.d18.0.22.0 | 1.732E-05 | 2.074E-05 | -3.417E-06 | ↑ in stable AD | 1.197 |
| PE38p.6 | 2.499E-03 | 2.065E-03 | 4.341E-04 | ↓ in stable AD | 0.826 |
| PE40p.7 | 6.751E-04 | 5.663E-04 | 1.088E-04 | ↓ in stable AD | 0.839 |
| PE40p.6 | 1.212E-03 | 1.010E-03 | 2.028E-04 | ↓ in stable AD | 0.833 |
| PE36.0 | 1.514E-03 | 1.179E-03 | 3.347E-04 | ↓ in stable AD | 0.779 |
| PC34.2p..34.3e | 2.040E-03 | 1.842E-03 | 1.976E-04 | ↓ in stable AD | 0.903 |
| PC36.4p/etherPC | 3.047E-03 | 2.729E-03 | 3.187E-04 | ↓ in stable AD | 0.895 |
| PC38.5p..38.6e | 2.549E-03 | 2.263E-03 | 2.863E-04 | ↓ in stable AD | 0.888 |
| PC34.5 | 1.828E-04 | 1.577E-04 | 2.508E-05 | ↓ in stable AD | 0.863 |
| PC38.1 | 3.472E-03 | 3.267E-03 | 2.055E-04 | ↓ in stable AD | 0.941 |
| SM18.20.0 | 8.169E-02 | 7.767E-02 | 4.015E-03 | ↓ in stable AD | 0.951 |
| SM18.22.0 | 6.479E-02 | 6.111E-02 | 3.676E-03 | ↓ in stable AD | 0.943 |
| SM18.24.0 | 1.163E-02 | 1.092E-02 | 7.038E-04 | ↓ in stable AD | 0.939 |

Those lipids in the blue section indicate an increase in the mean lipid levels and those in the grey section indicate a decrease in mean lipid levels in stable AD compared to the non-transition groups.

Non-trans: Non-transition (classification stayed the same (excepting stable AD) from baseline to 18 month follow up), AD: Alzheimer's disease, Δ : Change, PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine, Cer: Ceramide, PG: Phosphatidylglycerol, PI: Phosphatidylinositol. PA: Phosphatidic acid.

The above table shows the mean levels for the groups, the difference between the group mean lipid species levels, and the fold change for each of the 44 lipids identified as being significantly different between the non-transition and stable AD groups by LIMMA. For 32 of the lipid species, mean levels were higher and for 12 of the lipid species, mean levels lower, when comparing the stable AD group with the non-transition group. The LIMMA analysis, while identifying 44 lipid species levels that are significantly different between the non-transition and stable AD groups, does not take into consideration factors which may influence the significance of the observed differences in mean lipid levels. Consequently a generalised linear model (GLM) was created to control for age, APOE ϵ 4 allele carriage status, gender and collection site. The results of the GLM are displayed in Table 4.15.

Table 4.15: Generalised Linear Modelling (GLM) for the 18 month samples in the non-transition compared to the stable AD group.

| Lipid | p-value | Age | APOEε4 | Sex | Site |
|-----------------|----------|----------|----------|-------|-------|
| Cer.d18.1.20.0 | 5.29E-14 | 4.20E-25 | 6.31E-21 | 0.246 | 0.003 |
| Cer.d18.1.24.1 | 7.08E-12 | 1.08E-21 | 1.69E-22 | 0.187 | 0.005 |
| PC34.0p..34.1e | 8.18E-08 | 3.45E-21 | 2.17E-23 | 0.465 | 0.084 |
| PC32.0 | 2.07E-07 | 1.98E-23 | 1.98E-23 | 0.269 | 0.052 |
| Cer.d18.1.16.0 | 3.80E-07 | 2.44E-24 | 2.33E-22 | 0.239 | 0.006 |
| Cer.d18.1.18.0 | 4.57E-07 | 1.26E-27 | 6.54E-22 | 0.581 | 0.023 |
| PC34.0e | 1.93E-06 | 1.14E-21 | 1.13E-23 | 0.456 | 0.077 |
| PG34.2 | 1.48E-05 | 9.94E-27 | 2.43E-22 | 0.473 | 0.633 |
| PC32.0e | 3.10E-05 | 1.35E-24 | 1.16E-23 | 0.161 | 0.065 |
| PE36.0 | 8.48E-05 | 7.21E-27 | 3.02E-21 | 0.446 | 0.319 |
| LysoPE18.0 | 8.83E-05 | 2.31E-28 | 5.45E-21 | 0.411 | 0.046 |
| LysoPE16.0 | 9.86E-05 | 8.56E-27 | 3.30E-21 | 0.331 | 0.021 |
| PCLyso16.1 | 1.31E-04 | 2.52E-28 | 1.36E-21 | 0.463 | 0.113 |
| PG34.1 | 1.91E-04 | 4.18E-26 | 4.83E-22 | 0.533 | 0.095 |
| PE38p.6 | 2.08E-04 | 2.00E-28 | 2.10E-21 | 0.422 | 0.381 |
| PA34.1 | 2.26E-04 | 3.44E-29 | 6.13E-22 | 0.213 | 0.036 |
| PG36.4 | 2.29E-04 | 5.54E-29 | 5.10E-22 | 0.319 | 0.098 |
| SM18.16.0 | 2.40E-04 | 1.46E-27 | 8.28E-23 | 0.401 | 0.021 |
| PC40.2p..40.3e | 0.0003 | 2.89E-28 | 1.33E-22 | 0.429 | 0.170 |
| PE40p.6 | 0.0005 | 1.49E-28 | 1.76E-21 | 0.337 | 0.236 |
| PCLyso18e.1 | 0.0007 | 5.07E-27 | 1.39E-22 | 0.157 | 0.137 |
| Cer.d18.0.22.0 | 0.0008 | 5.69E-29 | 1.68E-22 | 0.355 | 0.133 |
| PC32.0p | 0.0008 | 3.76E-27 | 5.74E-23 | 0.382 | 0.044 |
| PE36.4 | 0.0012 | 4.06E-26 | 8.71E-22 | 0.780 | 0.029 |
| PCLyso18.1 | 0.0014 | 2.22E-28 | 7.42E-22 | 0.128 | 0.162 |
| PC32.1 | 0.0017 | 3.63E-27 | 1.59E-22 | 0.911 | 0.123 |
| Cer.d18.0.18.0 | 0.0024 | 1.46E-28 | 5.49E-22 | 0.391 | 0.058 |
| SM18.20.0 | 0.0026 | 5.73E-28 | 5.77E-22 | 0.800 | 0.080 |
| PC36.4p/etherPC | 0.0026 | 4.87E-28 | 6.04E-22 | 0.768 | 0.119 |
| PE40p.7 | 0.0027 | 2.16E-28 | 1.54E-21 | 0.320 | 0.157 |
| PC34.1 | 0.0040 | 2.16E-25 | 1.95E-22 | 0.423 | 0.242 |
| PCLyso18e.0 | 0.0043 | 7.17E-28 | 3.32E-22 | 0.255 | 0.115 |
| PC34.2p..34.3e | 0.0053 | 4.90E-28 | 9.64E-23 | 0.662 | 0.181 |
| SM18.22.0 | 0.0055 | 3.23E-26 | 1.72E-22 | 0.504 | 0.312 |
| PC36.0p/etherPC | 0.0068 | 5.98E-26 | 9.37E-23 | 0.592 | 0.332 |
| LysoPE18.1 | 0.0082 | 7.06E-28 | 2.71E-21 | 0.195 | 0.044 |
| PE34.1 | 0.0133 | 4.50E-27 | 4.88E-22 | 0.512 | 0.037 |
| PC38.5p..38.6e | 0.0134 | 3.17E-28 | 1.91E-21 | 0.552 | 0.209 |
| PE38.5 | 0.0159 | 1.14E-26 | 9.53E-22 | 0.872 | 0.038 |
| PE36.5 | 0.0230 | 9.95E-27 | 1.18E-22 | 0.692 | 0.042 |
| PC34.5 | 0.0238 | 5.23E-28 | 4.15E-22 | 0.405 | 0.147 |
| PC38.2p..38.3e | 0.0301 | 3.59E-28 | 1.81E-22 | 0.461 | 0.249 |
| PC38.1 | 0.1620 | 1.75E-26 | 2.67E-22 | 0.670 | 0.074 |
| SM18.24.0 | 0.2443 | 1.80E-26 | 1.95E-22 | 0.634 | 0.094 |

Adjusted for age, sex, APOEε4 allele status (i.e. carriage or non-carriage of an APOEε4 allele) and sample collection site (Perth or Melbourne). Lipids with a statistically significant p-value less than the Bonferroni cut off (0.05/189) are above the solid black line.

PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine, Cer: Ceramide, PG: Phosphatidylglycerol, PI: Phosphatidylinositol, PA: Phosphatidic acid. APOEε4: Apolipoprotein E ε4 allele.

Table 4.15 shows the p-values for each of the 44 lipids identified by LIMMA as being significantly different between the non-transition and stable AD groups, adjusted through GLM for the following covariates; age, sex, APOE ϵ 4 allele status and collection site. The p-values for each of the covariates are also shown in the table. The data are sorted by the strongest p-value for the lipid level post adjustment, i.e. where the levels are most different between the classification groups. The top 18 lipids (above the solid line) in the table remain statistically significant (less than the Bonferroni cut off value of $p < 0.0003$ ($0.05/189$)) after adjustment for age, gender, APOE ϵ 4 allele status and collection site. The p-values indicate that neither site of collection or gender significantly influence the results, but as expected age and APOE ϵ 4 allele carriage do significantly influence the results.

Whilst Tables 4.13 and 4.14 compare the non-transition to stable AD groups, LIMMA analysis was also repeated for the non-transition compared to transition groups and the transition compared to the stable AD groups. Three lipids were identified as being significantly different following Bonferroni correction between the non-transition and transition groups (PG34.2, PC34.0p..34.1e and PA36.1) but these did not remain significant following adjustment for age, gender, APOE ϵ 4 allele status and collection site. One lipid (Cer.d18.1.20.0) was identified by LIMMA as being significantly different between the transition and stable AD groups, where levels of this lipid were higher in stable AD compared to the transition group. This difference remained significant after adjusting for age, gender, APOE ϵ 4 allele status and collection site following assessment by GLM.

The results reported in Tables 4.13, 4.14 and 4.15 and in the above text examined the relationship between the non-transition, transition and stable AD groups. These classification groups were defined as described for previous analysis of data from the AIBL cohort [492]. Using this definition, the transition group also included those participants with MCI, where “transition” in the AIBL cohort represents transition towards AD, or declining mental health as determined by neuropsychological testing. A different analysis was then undertaken to explore the relationship between lipid profiles and ‘clinical

classification transition', i.e. analysis included those individuals classified at baseline as HC or MCI who had *transitioned* to MCI classification or AD diagnosis by the time of the 18 month follow up, yet excluded those participants who were classified as MCI at both baseline and the 18 month follow up. This reduced the sample number to n=40. LIMMA analysis identified Cer.d.18.1.24.1 and PG34.2 as being significantly different in those participants who transitioned by the 18 month follow up. These two lipids remained significant following adjustment for age, gender, APOE ϵ 4 allele status and collection site. Taking the top markers from the non-transition vs. the transition groups, and after removing those that were highly correlated the best sensitivity and specificity (78%) was achieved using Cer.d18.1.24.1, PG34.2, PC32.0, Cer.d18.0.18.0.

A recent report by Mapstone *et al* published in Nature Medicine [493] identified a panel of 10 lipid biomarkers that were able to predict phenoconversion to either amnesic MCI or AD within 2-3 years with over 90% accuracy. This lipid panel comprised of 8 PC species (LysoPC18.2, PC36.6, PC38.0, PC38.6, PC40.1, PC40.2, PC40.6, PCp40.6) and 2 acyl carnitine species (Propionyl AC and C16.1-OH). Out of the ten lipid species identified in this lipid panel, only four were measured in our study (PC 38.6, PC40.6, PC40.6p and LysoPC18.2). Using these four lipids in a model to predict those participants whose clinical classification transitioned within the 18 month follow up period gave an average sensitivity of 73.5%.

4.4.6 Comparison of baseline and 18 month data

To enable comparisons between the results obtained in Chapters 3 and 4, Table 4.16 displays significant differences in lipid species levels and the predictive lipid panels between clinical classifications for both the baseline and 18 month data. The lipid species which showed different levels when comparing the original classification groups, as well as transition groups at baseline and at the 18-month follow-up are also displayed.

Table 4.16: Summary of the significant differences in lipid species levels following adjustment for age, gender, APOEε4 allele status and collection site assessed by GLM, and for the predictive lipid panel generated by the variable selection pathways at both baseline and 18 month follow up.

| Significant Lipids identified by GLM | | | | | | | | | | | |
|---|--|------------|---|---|--|---|-----|---------------|-----|----------------|-----|
| Baseline | | | 18 month | | | Transition groups | | | | | |
| HC vs. AD | HC vs. MCI | MCI vs. AD | HC vs. AD | HC vs. MCI | MCI vs. AD | NonT vs. St AD | | NonT vs. Tran | | Tran vs. St AD | |
| SM18.20.1, Cer.d18.0.22.0, PE38.4, PE34.2, PC34.0p..34.1e, PE38.5, PC34.1, PE36.2, PC34.0e, SM18.16.1, PE34.1 | SM18.16.1 | | Cer.d18.1.24.1, Cer.d18.1.20.0, PC34.0p..34.1e, PC32.0, Cer.d18.1.18.0 Cer.d18.1.16.0, PG34.2, PC34.0e, PC32.0e. PE36.0, PG34.1, PE38p.6, PCLyso16.1, SM18.16.0, PA34.1, PC32.0p, Cer.d18.0.18.0, PE40p.6 | | Cer.d18.1.24.1 | Cer.d18.1.20.0, Cer.d18.1.24.1, PC34.0p..34.1e, PC32.0, Cer.d18.1.16.0, Cer.d18.1.18.0, PC34.0e, PG34.2, PC32.0e, PE36.0, LysoPE18.0, LysoPE16.1, PCLyso16.1, PG34.1, PE38p.6, PA34.1, PG36.4, SM18.16.0 | | | | Cer.d18.1.20.0 | |
| Variable Selection Pathways For Predictive Model | | | | | | | | | | | |
| Baseline | | | 18 month | | | Sensitivity and Specificity | | | | | |
| HC vs. AD | HC vs. MCI | MCI vs. AD | HC vs. AD | HC vs. MCI | MCI vs. AD | HC vs. AD | | HC vs. MCI | | MCI vs. AD | |
| PI34.0, PC36.0p/etherPC, PC38.5p..38.6e, PC32.0, SM18.20.1, Cer.d18.0.22.0 | PE34.1, PE34.2, LysoPE18.1, PG34.2 | Not done | LysoPE16.0, PG34.2, PC34.0p..34.1e, PC38.5p..38.6e, PC32.0e, PC32.0, SM18.22.0, Cer.d18.1.20.0, Cer.d18.1.24.1 | Lyso.PG16.1f, PI.38.2, PS.38.5, PC.Lyso.16.1, PS.36.2 | Cer.d18.1.20.0, Cer.d18.1.24.1, SM18.22.0, Lyso.PG16.1f, PE40p.4 | B/L | 18 | B/L | 18 | B/L | 18 |
| | | | | | | 80% | 80% | 71% | 69% | n/d | 69% |

HC: Healthy control, AD: Alzheimer's disease, MCI: Mild cognitive impairment, NonT: Non-transition, Tran: Transition, St AD: Stable AD, PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine, Cer: Ceramide, PG: Phosphatidylglycerol, PI: Phosphatidylinositol, B/L: Baseline, 18: 18 months, GLM: Generalised Linear model, APOEε4: Apolipoprotein E ε4 allele, n/d: Not done.

Table 4.16 summarises the results from analysis of the baseline and 18 month samples. Lipid sub-species of the same lipid groups were consistently identified in all comparisons and in the predictive modelling. Of particular note are the ceramides, sphingomyelins and several ether PC species. There were no consistent differences in PI, PA, PG or PS species. The only lipids that were different when considering the HC vs. MCI and MCI vs. AD groups were one SM species and one ceramide species respectively, probably due to the fact that there is considerable overlap between these classification groups in terms of stages of AD pathology. The ability of the predictive model to predict clinical classifications reliably gave the highest sensitivity and specificity (80%) for the HC vs. AD groups in both the baseline and 18 month samples, which might be expected as these groups are much more clinically and pathologically discrete. However, the lipids that were chosen for the baseline and 18 month panels differed, with the exception of PC38.5p..38.6e and PC 32.0.

Many lipids identified as being different when considering any change of clinical classification (transition groups) over the 18 month follow up period were also identified in the 18 month samples when comparing clinical classifications (see Table 4.16). There were a few notable differences; e.g. two extra LysoPE species were identified as differing between the non-transition and stable AD groups. The greatest differences were observed between the non-transition and stable AD groups, which again reflects the greater clinical and pathological separation between these two groups. The only lipid that was different between the transition and stable AD groups was Cer.d18.1.20.0.

4.5 Discussion

The aim of the work in this chapter was to investigate plasma lipid profiles in the 18 month time point samples from the AIBL cohort, and to compare the results with those obtained from analysis of the baseline samples presented in Chapter 3. In addition, plasma lipid profiles were investigated after the cohort was classified differently, into non-transition, transition and stable AD groups. The major findings reported in this chapter are as follows:

- 110 out of 189 plasma lipid species were significantly different when comparing the unadjusted mean values of the lipid species between clinical classifications.
- 46 lipids were significantly different between the healthy control and AD groups by LIMMA analysis when applying a Bonferroni correction.
- 18 of these lipids remained significant after adjustment for age, gender, APOE ϵ 4 allele status and collection site when comparing the HC and AD groups using a GLM.
- Using a variable selection pathway, a panel of nine lipids was able to distinguish healthy controls from AD patients with a sensitivity and specificity of 80%.
- No lipids were identified by LIMMA as being significantly different between the HC and MCI groups.
- Cer.d18.1.24.1 plasma levels were identified by LIMMA as being significantly different between the MCI and AD groups, and remained significant following adjustment for age, gender, APOE ϵ 4 allele status and collection site using a GLM.
- The variable selection pathways identified a panel of five lipids with a sensitivity and specificity of 69% for predicting clinical classification when comparing the HC and MCI groups.
- The variable selection pathways identified a panel of five lipids with a sensitivity and specificity of 69% for predicting clinical classification when comparing the MCI and AD groups.
- No significant correlation could be found when comparing SUVR categories and the results from the predictive panel identified by variable

selection for the HC vs. AD groups, although the levels of four ceramide species showed a distinct relationship with SUVR categories.

- After re-classifying at 18 months into non-transition, transition and stable AD, 44 lipids were significantly different between the non-transition and stable AD groups by LIMMA analysis, when applying a Bonferroni correction.
- 18 of the 44 lipids remained significant after adjustment for age, gender, APOE ϵ 4 allele status and collection site when comparing the non-transition and stable AD groups using a GLM.
- No lipids were significantly different between the non-transition and transition groups following adjustment for age, gender, APOE ϵ 4 allele status and collection site using a GLM.
- The plasma levels of Cer.d18.1.20.0 was identified by LIMMA as being significantly different between the transition and stable AD groups, and remained significant following adjustment for age, gender, APOE ϵ 4 allele status and collection site using a GLM.

The heatmaps shown in Figure 4.3, the bar charts in Figures 4.4 – 4.17 and Table 4.2 showed significant differences between the unadjusted mean lipid species levels and clinical classification for 110 out of the 189 lipids measured in the 18 month follow up samples. This was more than the 86 species identified in the baseline samples, although there was considerable overlap between the species identified at both time points. The differences in levels were greatest when comparing the healthy control and AD groups. When comparing mean levels of summed lipids groups, statistical significance was shown for total SM, PE, LysoPG, PEp, Ceramides, LysoPCp, LysoPE and PG at the 18 month time point (see Figure 4.4). While differences in total SM and PE were also observed in the baseline data, the other summed groups identified in the 18 month samples were not identified as being significantly different between clinical classifications at baseline. This variation between the baseline and 18 month results highlights the fact that measuring summed lipid groups has limited ability to show differences between clinical classifications and its use may be

restricted to identifying lipid groups that warrant further investigation through measurement of the individual sub-species within the lipid group.

There were noticeable similarities to the baseline results when considering the individual lipid species measured at the 18 month time point: the most significant differences in levels between clinical classifications were seen with PE, PC, SM, PI and Ceramides. Despite the similarities, a few notable directional differences between the baseline and 18 month results were observed in those lipids identified as differing significantly between clinical classifications. The mean plasma level of PE36.2p was shown to be higher in AD when compared to HC at the baseline time point, yet at 18 months, PE36.2p levels were lower in AD when compared to HC. Further, mean plasma levels of PG34.2, SM18.16.0, SM18.18.0 and SM18.24.1 were lower in AD compared to HC at baseline, yet were found to be higher in AD compared to HC at 18 months.

In agreement with other studies, some of the similarities observed between the baseline and 18 month samples involved sub-species of ceramides, choline and ethanolamine plasmalogens [163, 341, 357, 422]. Despite the similarities there were differences in some ethanolamine species levels in the 18 month samples that were not found in the baseline samples; at 18 months, plasma mean levels of PE38.7, PE36p.4, PE38p.6, PE40p.6 and PE40.7p were shown to be significantly lower in AD compared to healthy controls. With respect to the choline plasmalogen species, the baseline and 18 month data showed general agreement in terms of species that were identified as being significantly different between clinical classifications. Of note however, in the 18 month samples, the mean plasma level of PC40.6p..40.7e was identified as being significantly lower in AD compared to the HC group. Therefore, all of these PE and PC species which showed reduced mean plasma levels in AD (PE38.7, PE38p.6, PE40p.6, PE40p.7 and PC40.6p..40.7e) could contain DHA (22:6n-3) as one of the acyl chains (represented by an unsaturated bond count of 6 or greater). As previously described for the baseline data, this could reflect inadequate dietary intakes of omega-3 polyunsaturated fatty acids, or defects in the desaturase or elongase enzymes required to produce long chain fatty acids

from their precursor essential fatty acids. Additionally, lower levels of the ethanolamine plasmalogen species may be a result of elevated ceramides, as it is also suggested that ceramides can induce a plasmalogen-selective phospholipase A₂ [494], although no significant change in LysoPEp species (a product of the action of plasmalogen-selective phospholipase A₂ on PEP) were observed.

Furthermore, several of the LysoPE and LysoPC species were elevated in the AD compared to the healthy control group, which may suggest increased PLA₂ activity associated with inflammatory processes [28, 405].

Overall when comparing the baseline and 18 month samples there are many similarities. Any differences may potentially be explained by analytical factors and by the fact that the characteristics of the cohort at each time point will differ slightly: for example, each participant is 18 months older and for those in the MCI or AD groups, pathological processes have advanced. Cross sectional analysis of plasma lipid profiles at the 36 month time point may assist in determining whether these differences are due to changes in lipid profiles within an individual or due to analytical and sample handling factors.

The significant differences in individual lipid species between clinical classifications just described were identified from non-adjusted marginalised means with an alpha level set at 0.05. Stricter criteria for determining any significant differences were applied using LIMMA analysis to identify those lipids species with p-values less than the Bonferroni cut off of 0.05/189 (α/n) between clinical classifications. LIMMA analysis identified 46 differences in the lipids when comparing HC vs. AD, yet only one significant lipid difference when comparing MCI vs. AD. No lipids were identified for the HC vs. MCI group. These lipids identified by LIMMA were then controlled in a GLM for age, gender, APOE ϵ 4 allele status and collection site. A comparison of the adjusted significant lipids identified in the baseline and 18 month samples is presented in Table 4.16.

The GLM identified 18 lipid species that remained significant following adjustment when comparing the HC and AD groups. These 18 lipids consisted

of five ceramide species (Cer.d18.1.24.1, Cer.d18.1.20, Cer.d18.1.18.0, Cer.d18.1.16.0 and Cer.d18.0.18.0), six PC species (PC34.0p..34.1e, PC32.0, PC34.0e, PC32.0e, PCLyso16.1 and PC32.0p), two PG species (PG34.2 and PG34.1), three PE species (PE36.0, PE38p.6 and PE40p.6) one PA and one SM species (PA34.1 and SM18.16.0). All mean plasma levels were higher in AD compared to healthy controls except for PE36.0, PE38p.6 and PE40p.6, which were lower in AD compared to healthy controls. Two of these species (PE38p.6 and PE40p.6) may contain DHA as indicated by an unsaturated bond count of 6. PE38p.6 and PE40p.6 were not significantly different between the HC and AD groups at baseline. However, the significantly lower plasma levels of PE38p.6 and PE40p.6 shown in the AD compared to healthy control group observed at 18 months is in agreement with previous studies showing disease severity is associated with decreased levels of circulating ethanolamine plasmalogens [341, 357]. This association with disease severity could also provide an explanation as to why these lipids were not identified at baseline: pathology may have progressed sufficiently at the 18 month time point to enable differences between the AD and HC groups to be determined.

As previously mentioned, several species that were lower in AD compared to healthy controls possibly contain DHA. This may be explained by inadequate intakes and/or the presence of abnormal biochemical pathways involving DHA. While DHA can be obtained from dietary sources, an adequate supply to the brain also relies on peroxisomal production. The elongation and desaturation of fatty acids occurs in the endoplasmic reticulum (ER) or microsomes, but the conversion of eicosapentaenoic acid (EPA, 20:5n-3) to DHA requires a final additional step that only occurs in peroxisomes [495]. EPA undergoes two further elongation steps and a final delta-6 desaturation occurs in the ER to yield tetracosahexaenoic acid (24:6n-3). This very long chain fatty acid is transported to the peroxisome for a final β -oxidation step to remove 2 carbons to yield DHA (22:6n-3)[496]. This additional step is suggested as a reason for inefficient conversion of the essential fatty acid, α -linolenic acid (18:3n-3) to DHA and why additional dietary intakes of DHA may be required to meet the needs of the brain. A recent study has shown deficient liver biosynthesis of DHA in AD patients, as higher levels of the DHA precursor tetracosahexaenoic acid

(24:6n-3), and lower expression of peroxisomal Δ -bifunctional protein (required for the conversion of 24:6n-3 to 22:6n-3) were detected in AD patients [497]. Any accumulation of very long chain fatty acids can adversely affect mitochondrial function [498], and it is important to remember that mitochondrial dysfunction and oxidative stress are thought to be central to AD pathogenesis. Additionally, as previously mentioned in Chapter 3, the plasmalogen precursors (1-alkyl-glycerol ether lipids) are also synthesised in peroxisomes and peroxisomal dysfunction in the liver and brain has been reported as a feature of AD [498, 499].

It is also worthy of note that all of the elevated PC and PE species seen in the AD group compared to the HC group at both time points contain few unsaturated bonds and have smaller acyl chain lengths: inferring the intriguing possibility that abnormal elongase and desaturase enzyme activity could be present in AD, resulting in a lower than normal production of longer chain polyunsaturated fatty acids (PUFAs). This may then have multiple consequences including reduced membrane fluidity and an imbalance in PUFA-derived signalling molecules such as eicosanoids and docosanoids. Delta 6-desaturase acts as a gateway for the flow of fatty acids into the elongation and desaturation pathways; and the activity of this enzyme may be influenced by dietary intakes of fatty acids, various nutrients such as zinc, magnesium and vitamin B6 [224] and metabolic hormones [500, 501]. Insulin is known to affect activity of both delta 5 and delta 6 desaturases [225] and by reducing the supply of long chain PUFA to the brain, may represent an additional mechanism by which metabolic dysfunction and associated insulin resistance could contribute to AD.

Significant elevations were seen in five species of ceramide in AD compared to healthy controls and additionally Cer.d18.1.24.1 was also significantly elevated in AD compared to MCI. The results presented in this chapter are in general agreement with other reports showing elevated ceramides and reduced SM in AD compared to HC and MCI [163, 422, 502], although some variation in the individual sub-species is noted, which may reflect the different methodologies used in these studies. Sulphatide depletion in the AD cortex has been reported

[338, 413], and studies have shown that when individual sulphatide species are measured, the major depleted species contain d18.1.24.1 (nervonic acid), d18.1.24.1h (α -hydroxynervonic acid) and d18.1.26.1 (hexacosanoic acid) [503]. Sulphatides are synthesised from cerebroside (galactosylceramide), with the reaction being catalysed by galactocerebroside sulphotransferase. Importantly, activities of this enzyme or the levels of precursor cerebroside do not appear to be altered in the AD cortex [335], strongly suggesting that sulphatide breakdown is the major reason for the observed sulphatide depletion. As ceramides are a product of sulphatide catabolism, this could explain the increase in nervonic acid containing Cer.d18.1.24.1. Decreases in sulphatides in the AD cortex are reflected by large increases in ceramides in both the cortex and CSF [413, 471, 504]. If increased plasma ceramide levels parallel those changes observed in the brain and CSF, plasma levels of Cer.d18.1.24.1 could possibly represent a surrogate measure of sulphatide status; plasma levels of which are very low and therefore difficult to measure. It also appears that brain sphingolipid levels vary depending on extent of neurodegeneration, with the greatest changes occurring in early disease [413]. Figure 4.21 shows the scaled mean expression levels of four ceramides (Cer.d18.1.16.0, Cer.d18.1.18.0, Cer.d18.1.20.0 and Cer.d18.1.24.1) in the 18 month plasma samples and how the levels relate to SUVR categories, which are representative of cerebral amyloid burden. There is a clear increase in levels of all these ceramides between the SUVR categories of (> 1.3 to < 1.7) and (1.7 to ≤ 2) with 1.5 being considered the cut off between PiB-PET negative and positive. Interestingly, a dramatic drop in the plasma levels of these ceramides follows as SUVR reaches a value of > 2 . At this more advanced stage, cerebral amyloid burden is severe and the rate at which further amyloid accumulation occurs plateaus. The results provide compelling evidence of abnormal sphingolipid metabolism which warrants further investigation, and the inclusion of ceramides and sphingomyelins in a potential diagnostic blood biomarker panel seems justified.

The endogenous ceramide pool consists of production from three main pathways. The *de novo* pathway, the sphingomyelinase pathway which catabolises SM and the salvage pathway which breaks down complex

sphingolipids such as sulphatides, gangliosides and cerebroside [505]. In the *de novo* pathway ceramides are produced from palmitoyl CoA and serine by the coordinated action of four enzyme groups; Serine palmitoyltransferase (SPT), 3-ketodihydrosphingosine reductase, dihydroceramide synthases (CerS1-6) and finally dihydroceramide desaturase (DES). The dihydroceramide synthases (CerS1-6) are specific for fatty acyl chain lengths (CerS1: C18, CerS2: C20-26, CerS3: C18 and 24, CerS4: C18 and C20, CerS5: C16, and CerS6: C14 and C16) [506]. The various fatty acyl chains introduced by these specific CerS are thought to alter membrane dynamics but the exact biological function of the different species remains to be elucidated. However, the existence of 6 specific individual CerS enzymes implies the different species are likely to have different roles [507]. The final step in the synthesis of ceramide is the desaturation of dihydroceramide by DES. The formation of SM via incorporation of phosphocholine and complex sphingolipids is then facilitated by the action of SM synthase and glucosyl or galactosyl ceramide synthases respectively [505]. Ceramides can also be produced from the catabolism of SM via sphingomyelinase enzymes (SMases). It would appear that sphingolipid metabolism is regulated by a complex interplay of synthesis from basic precursors and catabolism of both SM and more complex sphingolipids. Altered activities of the many enzymes involved in these pathways are emerging as not only contributors to AD pathology but also potential targets for therapeutic intervention.

Studies have shown elevated acid SMase activity is associated with an increase in ceramides, A β and phosphorylated tau, as well as an associated reduction in SM in the AD brain [471]. Serine palmitoyltransferase (SPT), which is the first enzyme and likely the rate limiting step in the *de novo* ceramide synthesis pathway [508] has also recently been shown to be regulated by the APP intracellular domain (AICD). Increased SPT activity has been demonstrated in APP knock-in mice missing a functional AICD domain, and increased SPT activity has also been reported in both familial and sporadic *post mortem* AD brains [509]. Inhibition of SPT with L-cycloserine was recently shown to down regulate production of cortical A β ₄₂ and hyperphosphorylated tau in a mouse AD model [510]. Dihydroceramide desaturase (DES), the final enzyme in the *de*

novo ceramide synthesis pathway that converts dihydroceramide to ceramide is receiving attention for its role in sphingolipid metabolism [511]. Dihydroceramide and dihydrosphingomyelin may interfere with lipid raft processes by increasing rigidity within these microdomains [512]. APP processing by secretases is fundamental to AD pathology and inhibition of DES1 has been proposed as a potential target to reduce secretase activity and therefore A β production. Interestingly, DES regulation has been shown to be influenced by fatty acids in a cell culture model, with palmitate increasing levels of DES1 mRNA and therefore increasing ceramide levels, whereas oleate did not induce this increase [513]: This could represent another link between the protective effects of the Mediterranean diet, rich in olive oil, on AD and metabolic disease. The plasma levels of the dihydroceramide species in our study (Cer.d18.0.18.0) were significantly different between healthy controls and the AD group. The plasma levels of dihydroceramides or ratio of dihydroceramides to ceramides may become an important tool for monitoring response to therapy should DES inhibitors be introduced into any clinical trials.

Another recent study compared the brain levels of six ceramide subspecies, differing in the length of their acyl moieties, and found elevations in Cer16, Cer18, Cer20 and Cer24 in AD [514], consistent with our plasma findings. These authors then investigated the possible mechanisms underlying the increase in levels of these ceramides by evaluating the expression of acid SMase, neutral SMase2 (which breaks down SM), UDP-glucose ceramide glucosyltransferase (UGCG, which catalyses the conversion of ceramides into glycosphingolipids) and galactosylceramidase (GALC, which produces ceramide from galactosylceramide, a major SL of myelin and precursor for sulphatides). The results suggested abnormal expression patterns in AD, with increased expression of GALC, acid SMase and neutral SMase and reduced expression of UGCG; all of which may increase ceramides [514].

As ceramides can easily cross the blood brain barrier [515], it is possible that plasma ceramides can influence gene expression changes in the brain, and elevated ceramides in the brain can influence plasma levels. All these studies

present intriguing avenues for future research, and the measurement of plasma ceramide levels will be an important inclusion.

As described in Chapter 3 for the baseline data, a variable selection pathway was also used in the analysis of the 18 month data, to identify a panel of lipid biomarkers capable of predicting clinical classification with the greatest certainty. The variable selection pathways, stepwise GLM and ROC curves identified a panel of nine lipids that gave a sensitivity and specificity of 80% when analysing the healthy control and AD groups. These lipids (also identified by LIMMA analyses) were LysoPE16.0, PG34.2, PC34.0p..34.1e, PC38.5p..38.6ePC32.0e, PC32.0, SM18.22.0, Cer.d18.1.20.0 and Cer.d18.1.24.1. All of these lipids, except for PC38.5p..38.6e and SM18.22.0 were elevated in AD compared to healthy controls. The sensitivity and specificity of the separate lipid panels chosen by variable selection for the HC and MCI and MCI and AD groups was only 69% however; this may reflect the fact that MCI is a more clinically heterogeneous group which can often overlap both the HC and AD groups and often, but not always represents a prodromal stage of AD.

Whilst the lipids chosen by the statistical pipeline were able to differentiate between the healthy control and AD groups with a sensitivity and specificity of 80% at both baseline and 18 months, the lipid panels selected at the two time points were different. Table 4.16 lists the predictive lipid panels for both baseline and the 18 month time points. Only two of the predictive lipids (PC38.5p..38.6e and PC32.0) were common to both the baseline and 18 month time points, however the subclasses identified were very similar, excepting PI34.0 at baseline and LysoPE at 18 months. The observed differences between time points may once again be due to analytical and sample handling factors, or may reflect a difference in the characteristics of the cohort over time, as previously described. Analysis of samples collected at the 36 month time point may help to obtain a clearer picture of lipids that are able to predict clinical classification with the greatest certainty.

The analytical methods chosen for this study were based on the need for simultaneous quantification of a broad range of lipids in a large number of

samples. The analytical techniques that were employed have been useful in identifying which lipids are significantly different between clinical classifications and to speculate on aberrant pathways that may be present in AD. Further studies using a more targeted approach with LC separation is warranted, particularly with the PC, PE, sphingolipids and plasmalogen species, which appear to offer the best prospect of forming a lipid biomarker panel.

As in the previous chapter, the possibility of correlations between SUVR, a measure of brain amyloid burden, and lipid species was investigated. When comparing the predictive lipid panels identified by variable selection, no significant correlations were identified. However as described earlier, an intriguing pattern emerged when assessing the relationship of mean ceramide levels with SUVR (see Figure 4.21). This analysis showed the levels of four ceramide species (Cer.d18.1.16.0, Cer.d18.1.18.0, Cer.d18.1.20.0 and Cer.d18.1.24.1) were relatively low if the SUVR was below 1.7, high at a SUVR between 1.7 and 2 and yet markedly lower again at a SUVR above 2, at which stage amyloid accumulation plateaus according to PiB-PET, with saturation occurring between 2-3 [477]. Statistical significance may not be revealed when looking at the means of the lipid levels for each SUVR category; however, it may be useful to investigate how an individual's lipid levels vary with SUVR, as this may reveal a significant correlation between these ceramide species and SUVR.

For the longitudinal study, the diagnostic classifications were revised at the 18 month time point and participants were classified into three groups: non-transition, transition or stable AD (remained in the AD group). The non-transition group included those participants whose clinical classification was the same at inception and 18 months (excepting stable AD). The transition group included those participants whose clinical classification was different at inception and 18 months as well as including the MCI group, who are considered to be in transition towards AD or declining mental health [492]. The stable AD group included individuals who were classified as AD at both baseline and 18 month follow up. The greatest difference in mean lipid species levels was observed between the non-transition and stable AD groups; these groups are the most clinically and pathologically discrete. Between the non-transition and stable AD

groups, 44 lipid species were identified as being significantly different. Following adjustment for age, gender, APOE ϵ 4 allele status and collection site, 18 of these 44 lipid species remained significant. Most of these lipids were the same as those identified as being significantly different between the healthy control and AD group, except PG36.4, LysoPE16.0 and LysoPE18.0, which were identified as being significantly different between the non-transition and stable AD groups only.

The ceramide species Cer.d18.1.20.0 was the only lipid to have significantly different mean plasma levels between the transition and stable AD groups. Interestingly, a previous study identified elevated plasma levels of very long chain ceramides as being predictive of memory loss and right hippocampal volume loss amongst people with MCI [423]. While any association of the very long chain ceramides with hippocampal volume and cognitive scores was not assessed in this study, it clearly warrants further investigation. Correlations between the transition groups and SUVR were not assessed here, as intra-individual levels of cerebral A β do not differ significantly between baseline and 18 month time points: This requires evaluation at subsequent time points, which is beyond the scope of this thesis. As described earlier, however, an intriguing pattern was uncovered when assessing the relationship between mean ceramide levels and SUVR cross-sectionally at the 18 month follow up (see Figure 4.21).

When the MCI group was excluded from the analysis and only those participants whose clinical classification ‘transitioned’ from HC to MCI, HC to AD or MCI to AD were included, only Cer.d18.1.24.1 and PG34.2 were identified as being significantly different. Taking the top markers from the non-transition vs. the transition groups, and after removing those that were highly correlated the best sensitivity and specificity (78%) was achieved using Cer.d18.1.24.1, PG34.2, PC32.0, Cer.d18.0.18.0. However the number of participants that were re-classified at the 18 month follow up (after removing the MCI group) was only 40 and therefore analysis of the samples collected at the 36 month time point may improve the statistical power of the longitudinal analysis.

As previously described, a recent report by Mapstone *et al* published in Nature Medicine [493] identified a panel of 10 lipid biomarkers that were able to predict phenoconversion to either amnestic MCI or AD within 2-3 years with over 90% accuracy. This lipid panel comprised of 8 PC species and 2 acyl carnitine species; the levels of which were reduced in the plasma of those study participants who phenoconverted compared to the control group. The levels of these lipids also remained lower in the converters and in MCI and AD groups compared to controls. The analytical approach used in this reported study combined untargeted and targeted analyses facilitating the measurement of a large number of lipid species. Out of the ten lipid species identified in the lipid panel, only four were measured in our study (PC 38.6, PC40.6, PC40.6p and LysoPC18.2); all levels of which were lower in the MCI and AD groups compared to healthy controls. When these four lipids were used in a prediction model, a sensitivity and specificity of only 73.5% was achieved. The predictive ten lipid panel identified in the study by Mapstone *et al*, predicted conversion between 2-3 years and therefore analysis of the transition group samples with the inclusion of the other six lipids measured in their study may become more relevant in the AIBL 36 month follow up time point.

Plasma lipid profiles are influenced by many factors such as diet, nutrient status, hormone balance, medication use and the existence of co-morbidities such as diabetes and cardiovascular disease. The plasma levels of individual lipid species are also the net result of a complex set of synthesis, metabolic and catabolic pathways influenced by a myriad of signals and catalytic enzymes. In addition, lipoprotein metabolism and lipid species are regulated by a number of proteins including phospholipid transfer protein (PLTP), cholesterylester transfer protein (CEPT) and lecithin-cholesterol acyl transferase (LCAT). One of these proteins, PLTP, will be investigated in Chapter 5 for both its relationship to plasma lipid species levels and its association with clinical classification. PLTP has been shown to be associated with some features of AD pathology and has been studied for its role in metabolic and cardiovascular disease: consequently, correlations between plasma PLTP activity and other AD biomarkers will also be investigated.

Chapter 5

Plasma PLTP Activity Levels

Chapter 5 - Plasma PLTP Activity

5.1 Introduction

The previous two chapters investigated plasma lipid profiles, their association with clinical classification and the ability of a panel of lipid species to predict clinical classification. Plasma lipids are influenced by many factors such as diet, nutrient status, hormone balance, medication use and the existence of co-morbidities such as diabetes and cardiovascular disease; which also happen to be risk factors for AD.

Phospholipid transfer protein (PLTP) is one of two or more lipid transfer proteins that are found in plasma, and one of its major roles involves the transfer of phospholipids from triglyceride-rich lipoproteins to high density lipoprotein (HDL), thus regulating the size and composition of HDL. Due to its role in peripheral lipid and lipoprotein metabolism, and its role in other processes that may influence AD pathology, PLTP may represent a mechanistic link between abnormal lipid metabolism in the periphery and that of the brain. This chapter will investigate whether there is any association between plasma PLTP activity levels and the lipid profiles measured in the previous chapters, whether PLTP activity levels differ with clinical classification, and whether levels correlate with those of other biomarkers measured in the AIBL cohort.

PLTP is a ubiquitously expressed protein belonging to the family of lipopolysaccharide (LPS) binding and lipid transfer genes that includes LPS binding protein (LBP), neutrophil bactericidal permeability increasing protein (BPI) and cholesteryl ester transfer protein (CETP) [516-518]. Despite its name, PLTP is emerging as a multifaceted protein involved in lipoprotein remodelling, reverse cholesterol transport, phospholipid transfer and immunity. In plasma, PLTP is mainly associated with high-density lipoproteins (HDL) as mentioned above, yet in addition to phospholipids, it also efficiently transfers α -tocopherol (a form of vitamin E), diacylglycerol, lipopolysaccharides, cerebrosides (monoglycosylceramides; important components of nerve cell membranes) and other amphiphilic molecules such as A β [519, 520]. This multi-functionality suggests important roles not only in lipoprotein metabolism but also

inflammation and oxidative stress, which are thought to be involved in AD pathogenesis [482, 484].

Dietary α -tocopherol is incorporated in chylomicrons and transported to the liver where it is packaged into very low density lipoproteins (VLDL) for release into the circulation [521]. The tocopherols are important in preventing low density lipoprotein (LDL) oxidation and subsequent uptake of oxidised LDL by vascular endothelium [439]. A deficiency of PLTP in PLTP (-/-) transgenic mice has been shown to result in a reduced delivery of α -tocopherol to the brain and an associated increase in oxidative injury, which may contribute to neurological dysfunction. This suggests that PLTP is a local transporter of α -tocopherol and that it may transport this antioxidant vitamin across the blood brain barrier (BBB) [440]. More recent studies of PLTP knockout mice demonstrated that intracerebral injection of A β 25-35 oligomers caused memory deficits and oxidative stress (effects not seen in wild-type mice), and that chronic dietary supplementation with vitamin E could reduce these symptoms. These studies demonstrate the importance of PLTP in vitamin E transport and/or delivery to the brain, and how a PLTP deficiency can lead to an inability to prevent oxidative damage [522].

PLTP transfers phospholipids from triglyceride-rich and apoB-containing lipoproteins (chylomicrons and VLDL) to HDL during lipolysis by lipoprotein lipase. It also facilitates the formation of LDL and maturation of HDL by the transfer of surface lipids between lipoproteins [523, 524]. Studies have shown that PLTP transfers all major phospholipid classes although phosphatidylethanolamine (PE) is transferred more slowly. Transfer is not influenced by chain length of the acyl groups or their sn-1 or sn-2 positions on the phospholipid molecule [437, 525]. Studies into cellular ceramide homeostasis, have shown that PLTP can also transfer sphingomyelin (SM) from vesicles to HDL [164]. Evidence suggests that newly synthesised hepatic ceramides are secreted via VLDL, and PLTP may be one of the proteins that facilitates the transfer of sphingolipids onto nascent lipoproteins [164, 526, 527]. The physiological relevance of PLTP is highlighted in PLTP-deficient mice, which demonstrate a total absence of phospholipid transfer from VLDL to HDL

and subsequently markedly reduced HDL levels [436]. The PLTP gene promoter region contains response elements for farnesoid X-activated receptor (FXR), peroxisome proliferator-activated receptor α (PPAR α) and the liver X-activated receptor (LXR); involved in bile acid, fatty acid, glucose and cholesterol metabolism respectively [528, 529]. A major function of PLTP is in maintaining the heterogeneity of plasma HDL which therefore facilitates reverse cholesterol transport due to its ability to generate nascent pre β -HDL. Pre β -HDL particles are primary acceptors of cell derived cholesterol, and facilitate cholesterol and phospholipid efflux from peripheral cells via the ATP-binding cassette transporters ABCA1 and ABCG [442, 530, 531].

Two different forms of PLTP exist in plasma: An active form, measured by its ability to transfer phosphatidylcholine (PC) to HDL and an inactive form that lacks this capability [532, 533]. Plasma contains varying amounts of the active and inactive PLTP forms [518, 534]. Active and inactive PLTP levels also appear to be tissue-dependent, with no inactive form being detected in CSF or tear fluid and the majority of the inactive form being present in the vascular compartment [535-537]. Additionally, plasma levels of the inactive form have been shown to decrease, and the active form increase in correlation with acute phase response inflammatory markers, such as C-reactive protein [538, 539]; suggesting that one function of the inactive form is to act as a reserve that may be converted to the active form in response to changes in the physiological or pathological landscape. The active form may then transfer more phospholipids to cells for membrane repair and maintenance of integrity during inflammatory stress [540, 541]. The complete repertoire of inactive PLTP function is currently unknown but while it is unable to transfer lipids from PC-containing vesicles to HDL *in vitro* or mediate other activities involved with lipoprotein metabolism of its active counterpart, it can bind lipids with equal capacity and has been shown to attach strongly to surface phospholipids [542]. It would therefore appear prudent to suggest that inactive PLTP may activate signal transduction pathways. In plasma, both forms of PLTP are present in complexes, some of which are lipid-protein complexes whilst others contain protein only. Studies using anti-PLTP antibodies and proteomics have identified several proteins in these complexes, including clusterin (apoJ), fibrinogen, transthyretin,

plasminogen and apoAI [518, 538]. Some of these proteins are thought to stabilise or determine lipid transfer activity of PLTP within these complexes and a large number of these proteins are actually acute phase proteins involved in inflammatory pathways and compliment activation; suggesting a major role for PLTP in immunity and inflammation, in addition to its role in lipid transfer [541, 543].

As PLTP is now being shown to play a role in processes other than lipid transfer, research efforts are changing direction, and the idea that PLTP acts as a regulator of signal transduction is being actively explored [544, 545]. Initial studies have shown that both apoAI and PLTP participate in lipid efflux via ABCA1, but in slightly different ways. The ApoAI pathway utilises lipid-poor HDL particles whereas PLTP requires the presence of mature, lipidated HDL [544, 546]. PLTP has been shown to bind to and stabilise ABCA1 and at the same time competitively inhibit apoAI binding to ABCA1, suggesting PLTP mimics apolipoproteins with respect to the transfer of cellular lipids, particularly cholesterol. More recent studies have expanded this work and have shown that like apoAI, PLTP is involved in signal transduction pathways - binding of PLTP to ABCA1 activates the Janus kinase 2 / Signal Transducer and Activator of Transcription (JAK2/STAT3) pathway in macrophages, which significantly reduces secretion of pro-inflammatory markers [518, 547]. Recent evidence has also demonstrated that in macrophages, PLTP inhibits nuclear factor kappa B (NFkB); a transcription factor involved in cellular responses to stimuli such as stress, cytokines, oxidative stress, oxidised-LDL and antigens [548, 549]. These authors have also shown that PLTP modulates two other ABCA1-independent, signal transduction pathways in human neuronal cells; the PI3K/Akt/GSK3 β pathway via the insulin receptor and the Dab1-dependent (reelin) pathway [550]. Collectively, these findings suggest PLTP has the ability to modulate a selection of cell signalling pathways (irrespective of its lipid transfer properties) that are linked to many functions in the brain that are adversely affected in AD, such as plasticity, insulin signalling, tau phosphorylation and neurogenesis.

Neuronal cells require a constant supply of cholesterol and other lipids for membrane and acetylcholine synthesis and while *de novo* synthesis of a wide

range of lipids occurs in neurons there is still a reliance on exogenous sources contained in lipoproteins, which are bound and internalised from the extracellular fluid [302, 551]. Excess lipids also need to be eliminated, which occurs via a bidirectional lipoprotein mediated transport involving apoE [443]. ApoE, the major apolipoprotein synthesised by astrocytes, is usually present in the CSF in a lipidated state [552], where it exists as cholesterol rich, discoidal complexes, resembling plasma HDL. These complexes readily target LDL-receptor related protein (LRP), which is abundantly expressed on the surface of neurons [302]. As in plasma, CSF lipoproteins can also be remodelled by lecithin-cholesterol acyltransferase (LCAT) and PLTP; reflecting the key role of HDL like particles in cholesterol homeostasis within the central nervous system (CNS) [443]. ApoE secretion by astrocytes is modulated by PLTP and a correlation between CSF levels of apoE and PLTP has been shown [553]. HDL and the metabolism of A β , the accumulation of which is a key feature of AD pathology, are also intimately connected [554].

An increase in the production of A β occurs in response to higher cellular cholesterol levels, most likely due to the influence of membrane cholesterol on lipid rafts and on the activity of APP cleavage enzymes [26] (see Chapter 1, Section 1.5.1). It has also been suggested that PLTP and apoE-containing HDL in the brain may exert several neuroprotective effects via suppression of A β production, by activation of reverse cholesterol transport, mediated by ATP-binding cassette (ABC) transporters [555]. Studies have shown that the apoE in HDL can bind to A β and prevent its oligomerisation [554], and that HDL transports A β in both the CSF and plasma and represents a method of elimination from the brain. PLTP may have a crucial role in this process as it also transports amphiphilic molecules, such as A β .

ApoAI has been shown to be present in the CNS and as this molecule is not thought to be locally synthesised, it is probably of plasma origin [302]. In addition to mediating cholesterol removal, ApoAI is considered to be anti-inflammatory [556], and may represent the mechanistic link between low plasma HDL and memory deficits [557].

Cells of the BBB express several ABC transporters, of which ABCA1 and ABCB1 play an important role in A β removal from the CNS. ABCB1 (also known as P-glycoprotein 1 and multidrug resistant protein 1) is highly expressed on the luminal side of endothelial cells within the BBB. A β can be transported into the periphery via A β binding to ABCB1 or via LRP-1 (which can bind A β directly or via A β ligands, such as apoE) [558, 559]. ABCA1 is expressed in astrocytes and microglia and at the abluminal side of the endothelium where PLTP-mediated lipidation of apoE is enhanced; facilitating A β trafficking to the BBB for elimination via ABCB1 [555].

The widespread distribution of PLTP in the human CNS has been demonstrated, with synthesis by glia and neurons having been confirmed; furthermore, a study has reported a significant increase in PLTP in the AD brain compared to control brain [535]. The authors of this study suggest that an increase in PLTP levels in brain tissue may represent a compensatory response to an underlying aberrant metabolic process present in AD [535], and the accumulation of PLTP in myelin fibres, as shown by immunohistochemistry, suggest a role for PLTP in preserving the functional and structural integrity of myelin [535].

Sulphatides are a class of myelin-specialised glycosphingolipids involved in various cellular processes, including cell growth, protein trafficking, signal transduction, cell-cell recognition, neuronal plasticity and cellular integrity [270, 421]. Sulphatide levels in the AD brain are markedly reduced [413]. Studies have shown that apoE is associated with sulphatide transport and mediates sulphatide homeostasis in the central nervous system through lipoprotein metabolism pathways. Alterations in apoE mediated sulphatide trafficking have been shown to deplete sulphatide in the brain [337]. An increased rate of apoE/sulphatide particle uptake and an increased expression of lipoprotein receptors can lead to increased degradation of sulphatide-containing apoE particles, resulting in accelerated sulphatide depletion from the myelin sheath. [71]. CNS levels of sulphatide have been shown to be apoE isoform-dependent, the lowest levels being seen with the APOE ϵ 4 genotype [71]. Sulphatide containing apoE-associated particles facilitate A β clearance, thus APOE ϵ 4

carriers having the lowest sulphatide levels represent a further mechanism for APOE ϵ 4 increasing the risk for AD [71]. A role for PLTP in A β clearance could be strongly suggested due to its importance in lipid transfer, lipoprotein metabolism, transport of amphiphilic molecules, and its association with apoE, in addition to its widespread CNS distribution.

A study of PLTP activity, serum apoE concentration and APOE genotype in a Finnish population, showed apoE levels to be highest in APOE ϵ 2 carriers and lowest in APOE ϵ 4 carriers, yet with no difference observed between the groups in terms of PLTP activity [534]. However, this study utilised a cognitively normal population. In another study, CSF PLTP activity levels were shown to be significantly lower in AD compared to healthy controls, suggesting sequestration of PLTP in brain tissue [536]. In cognitively normal individuals, a significant physiological link between CSF levels of PLTP, apoE, APP and tau has also been demonstrated [553].

The importance of PLTP in lipoprotein metabolism and the discovery of new roles for this protein have understandably driven research into its association with cardiovascular, metabolic, inflammatory and neoplastic diseases. However, due to the complexity of function, tissue specific distribution, genetic polymorphisms, the regulatory proteins and lipids, and different isoforms, it is not surprising that determining possible associations with disease has not been straightforward. A recent comparison of relationships between cardiovascular disease and plasma PLTP has highlighted inconsistencies, due to conflicting results [449]. PLTP and cholesteryl ester transfer protein (CETP) are proteins involved in the exchange of phospholipids and cholesteryl esters (respectively) between HDL and other lipoproteins, and low CETP and high PLTP activity both result in higher plasma HDL-cholesterol levels. Activities of both these enzymes were recently measured in the Framingham Heart Study cohort, which showed a lower plasma CETP and higher PLTP activity was associated with an increased risk of cardiovascular disease, but in men only [444]: A gender difference has not previously been reported in other studies. The relevance of plasma PLTP activity levels in AD is currently unknown but despite the obvious caveats, and due to its wide ranging functions involving lipid metabolism,

inflammation, oxidation and possible removal of A β , it is highly likely to play a significant role and therefore warrants further investigation.

It is known from the literature that PLTP activity levels both influence and are associated with plasma lipoprotein levels. What is less clear is any association between plasma lipid species levels (e.g. such as those measured in Chapters 3 and 4) and plasma PLTP activity levels. To our knowledge this is the first study to investigate any association between plasma lipidomics and PLTP activity in a highly characterised ageing cohort.

5.2 Aims

This chapter aims to investigate any association of the lipid species levels measured in Chapter 3 with plasma PLTP activity levels determined using a commercial assay kit. Differences in PLTP activity levels between clinical classifications will be compared to supplementary data collected as part of the AIBL study, including brain amyloid burden as measured by PiB-PET determined SUVR, and APOE ϵ 4 allele status. The results will also be assessed for correlation with other AD-related blood biomarkers measured in the cohort including plasma A β , ApoE and clusterin levels.

5.3 Methods

Details of the study population and the methods for sample collection, neuroimaging, lipid extraction, analysis by electrospray ionisation tandem mass spectrometry (ESI/MS/MS), and determination of plasma PLTP activity levels, plasma A β levels, plasma ApoE levels, clusterin levels and HDL-cholesterol subfraction levels have been described in detail in Chapter 2, Sections 2.2, 2.3 and 2.4. Plasma PLTP activity levels were measured in a subset of the AIBL study baseline samples (n=259).

5.4 Statistical analysis

Pearson's correlations and logistic regression were used to assess possible linear relationships between plasma PLTP activity levels and plasma levels of each of the 189 lipids species measured in Chapter 3. To assess the study population demographics between the healthy controls, MCI and AD groups, χ^2 analyses were used to test the effect of age, gender and APOE ϵ 4 allele status. A Kruskal-Wallis formula was applied to MMSE scores. Marginalised means and p-values were obtained for the lipid levels using a student's unpaired t-test for two classifications (i.e. HC and AD, or HC and MCI) or ANOVA for three classifications (HC, MCI and AD). Additionally ANOVA was used to test inter group differences in SUVR and hippocampal volume.

The statistical analysis was performed using R version 3.0.2 (R Development Core team, 2009, Vienna, Austria) and IBM SPSS Statistics version 22 (SPSS Inc., Chicago, IL, USA).

5.5 Results

5.5.1 Correlation of plasma PLTP activity levels with lipid species

Plasma PLTP activity levels were measured in a subset of AIBL study baseline samples and the activity levels were correlated (independent of clinical classification) with the mean plasma lipid species levels of 189 lipid species measured in Chapter 3 (see Table 3.2). Figure 5.1 shows the correlation of the summed lipid groups with plasma PLTP activity levels; the strongest association is indicated by a higher adjusted R squared value.

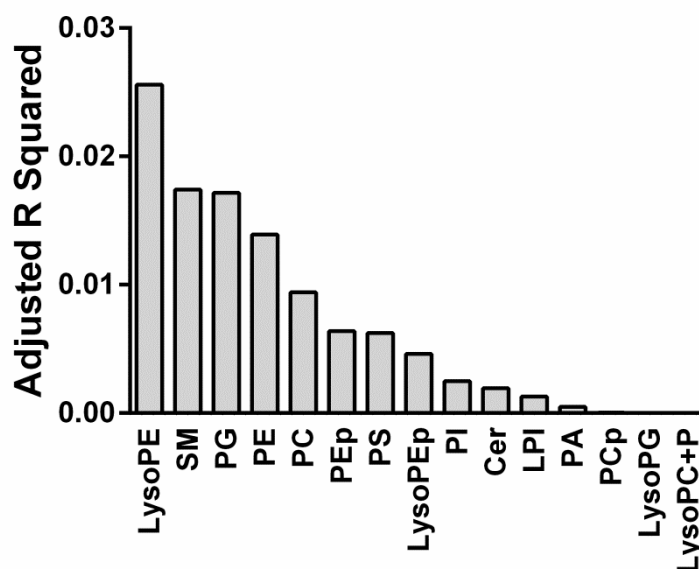


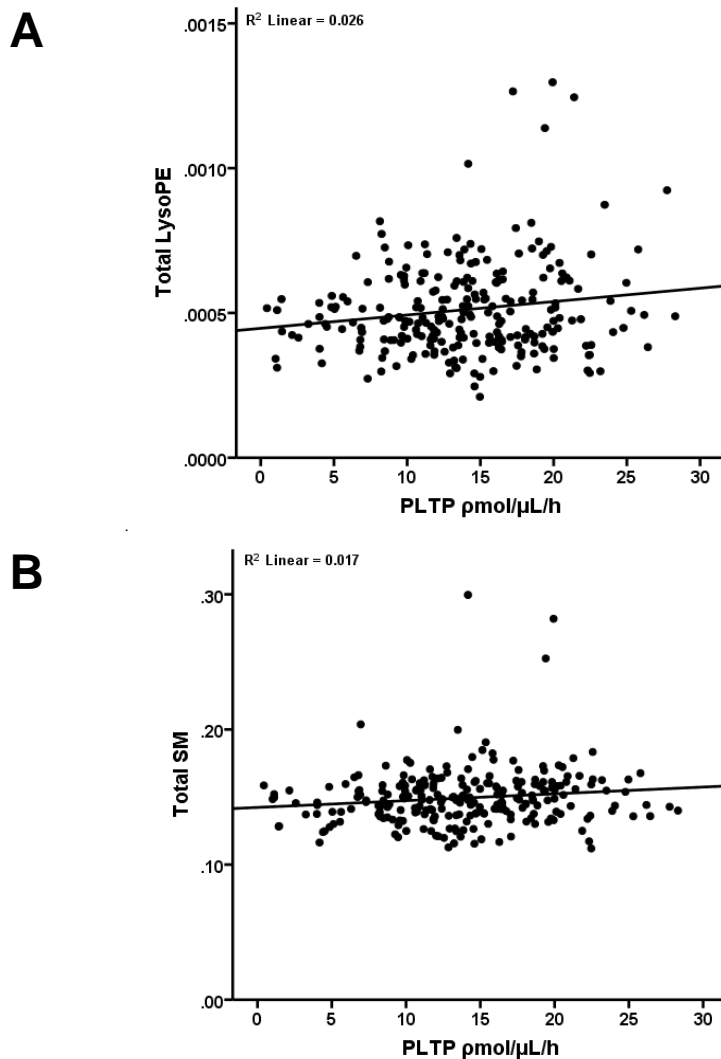
Figure 5.1: Correlation between plasma PLTP activity levels and summed lipid groups.

Pearson's correlations adjusted for age, gender and APOEε4 allele status.

PLTP: Phospholipid transfer protein, LysoPE: Lysophosphatidylethanolamine, SM: Sphingomyelin, PG: Phosphatidylglycerol, PE: Phosphatidylethanolamine, PC: Phosphatidylcholine, PEP: Ethanolamine plasmalogen, PS: Phosphatidylserine, LysoPEp: Lyso-ethanolamine plasmalogen, PI: Phosphatidylinositol, Cer: Ceramide, LPI: Lysophosphatidylinositol, PA: Phosphatidic acid, PCp: Choline plasmalogen, LysoPG: Lysophosphatidylglycerol, LysoPC+P: Lysophosphatidylcholine plus Lyso-choline plasmalogen.

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Plasma PLTP activity levels showed the strongest correlation with the levels of LysoPE, SM and PG groups; these relationships are statistically significant (Figure 5.1) ($p < 0.05$; Pearson's correlations adjusted for age, gender and APOE $\epsilon 4$ allele carriage). None of the other lipid groups significantly correlated with plasma PLTP activity levels. The correlation between plasma PLTP activity levels and plasma total LysoPE, total SM and total PG levels are shown in Figure 5.2.



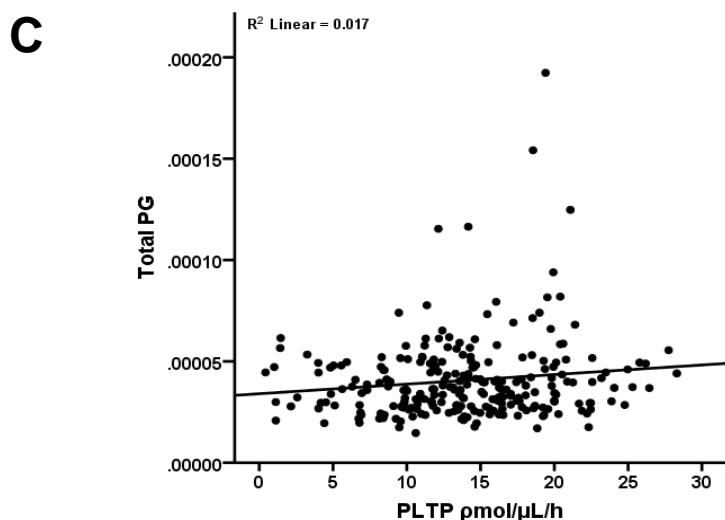


Figure 5.2: Correlation between plasma PLTP activity and plasma lipid levels of (A) Total LysoPE, (B) Total SM, (C) Total PG. Lipid levels expressed as molar fractions. Pearson's correlations, adjusted for age, gender and APOEε4 allele status. p value < 0.05.

PLTP: Phospholipid transfer protein, pmol/μL/h: Picomole per microlitre per hour, LysoPE: Lyso-phosphatidylethanolamine, SM: Sphingomyelin. PG: Phosphatidylglycerol.

Figure 5.2A shows a positive correlation between the mean plasma total LysoPE levels and plasma PLTP activity levels. When mean plasma total LysoPE levels were measured in the baseline cohort, no significant difference was observed between clinical classifications (see Figure 3.4B).

Figure 5.2B shows a positive correlation between the mean plasma total SM levels and plasma PLTP activity levels. When mean plasma total SM levels were measured in the baseline cohort, a significant difference was observed between the HC and MCI groups (see Figure 3.4A), where levels were lower in the MCI group compared to the HC group. Significant differences in the mean plasma levels of many sub-species of SM were also observed between clinical classifications with SM species levels lower in the MCI and AD groups compared to healthy controls (see Figure 3.15).

Figure 5.2C shows a positive correlation between the mean plasma total PG levels and plasma PLTP activity levels. When mean plasma total PG levels were measured in the baseline cohort, no significant differences were observed between clinical classifications (see Figure 3.4C). However, a significant

Chapter 5 – Plasma PLTP Activity Levels

difference in the mean plasma level of the PG sub-species PG34.2 was shown between clinical classifications with PG34.2 lower in the MCI and AD groups compared to healthy controls (see Figure 3.10).

The association between plasma PLTP activity levels and mean plasma levels of the individual lipid species was then assessed. Figures 5.3 and 5.4 show the correlation of the individual 189 lipid species with plasma PLTP activity levels; the strongest association is indicated by a higher adjusted R squared value.

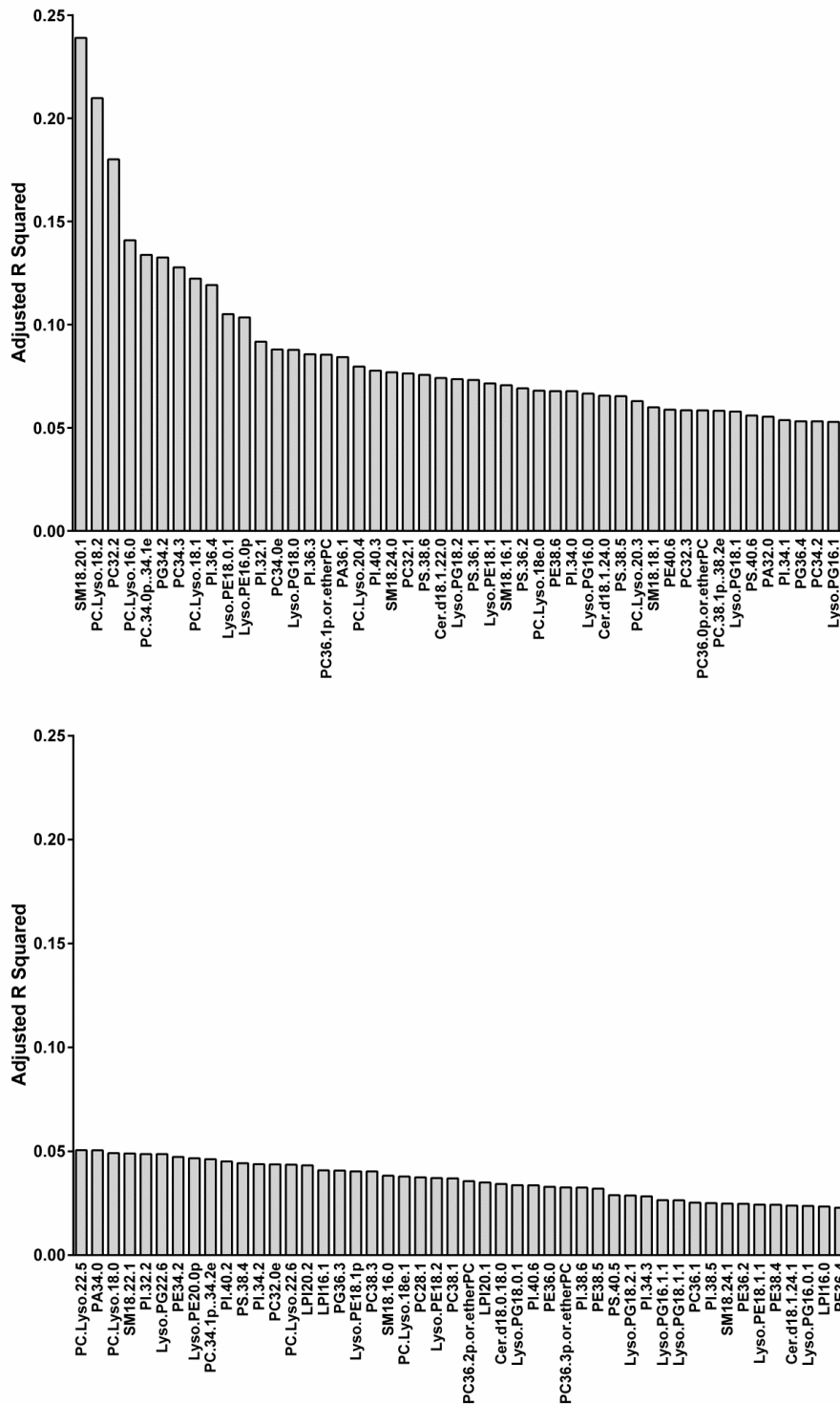


Figure 5.3: Correlation between plasma PLTP activity levels and individual lipid species.

Pearson's correlations adjusted for age, gender, APOE ε4 allele status.

PLTP: Phospholipid transfer protein, LysoPE: Lysophosphatidylethanolamine, SM: Sphingomyelin, PG: Phosphatidylglycerol, PE: Phosphatidylethanolamine, PC: Phosphatidylcholine, PEP: ethanolamine plasmalogen, PS: Phosphatidylserine, LysoPEp: Lyso-ethanolamine plasmalogen, PI: Phosphatidylinositol, Cer: Ceramide, LPI: Lysophosphatidylinositol, PA: Phosphatidic acid, PCp: Choline plasmalogen, LysoPG: Lysophosphatidylglycerol.

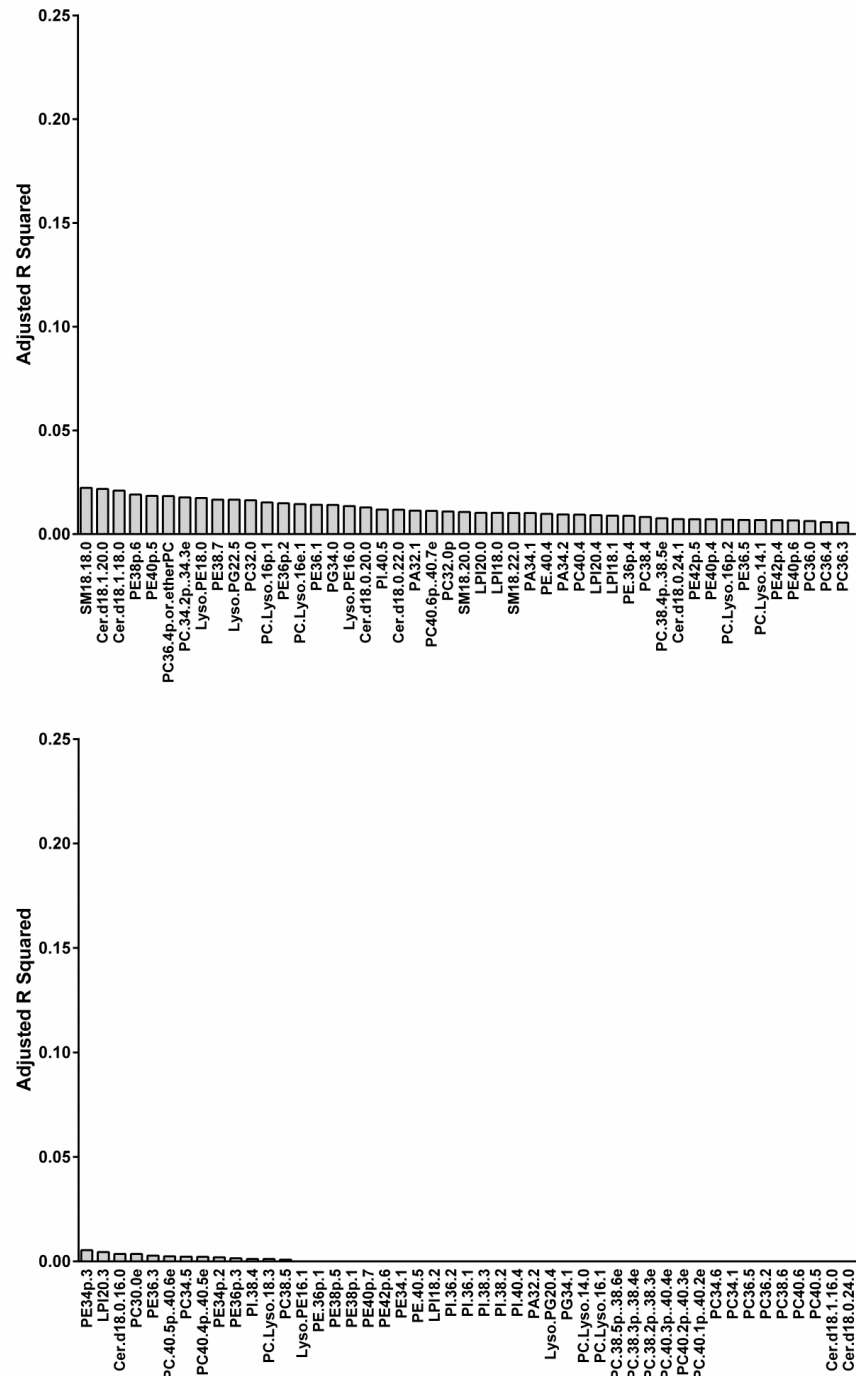


Figure 5.4: Correlation between plasma PLTP activity levels and individual lipid species.

Pearson's correlations adjusted for age, gender, APOE ε4 allele status.

PLTP: Phospholipid transfer protein, LysoPE: Lysophosphatidylethanolamine, SM: Sphingomyelin, PG: Phosphatidylglycerol, PE: Phosphatidylethanolamine, PC: Phosphatidylcholine, PEp: ethanolamine plasmalogen, PS: Phosphatidylserine, LysoPEp: Lyso-ethanolamine plasmalogen, PI: Phosphatidylinositol, Cer: Ceramide, LPI: Lysophosphatidylinositol, PA: Phosphatidic acid, PCp: Choline plasmalogen, LysoPG: Lysophosphatidylglycerol.

From the overall model assessing association of the levels of the 189 lipids against plasma PLTP activity levels (shown in Figures 5.3 and 5.4), the top 5 lipids (SM18.20.1, PCLyso18.2, PC32.2, PCLyso16.0 and PC34.0p..34.1e) were individually assessed for significant relationships with plasma PLTP activity using partial Pearson's correlations.

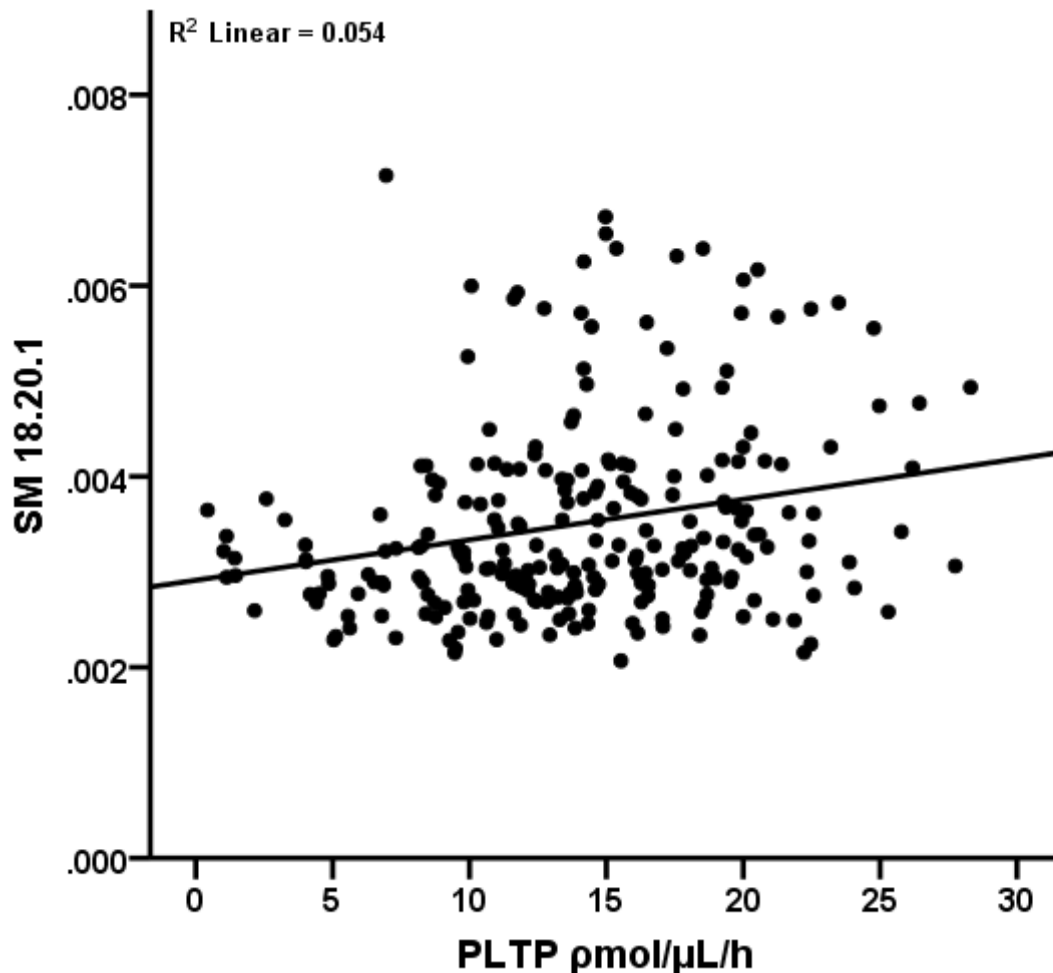


Figure 5.5: Correlation of SM18.20.1 with plasma PLTP activity levels.

Levels of SM18.20.1 expressed as molar fraction. SM: Sphingomyelin, PLTP: Phospholipid transfer protein, pmol/μL/h: Picomole per microlitre per hour.

Pearson's correlation: *** $p < 0.001$, adjusted for age, gender, APOE $\epsilon 4$ allele status.

Figure 5.5 shows a significant positive correlation between plasma mean levels of SM18.20.1 and plasma PLTP activity levels. Interestingly, the mean level of this sphingomyelin species was also the most significantly different between the healthy control and AD groups after adjustment for age, gender, APOE $\epsilon 4$ allele status and collection site when assessed by general linear model (GLM) in

Chapter 5 – Plasma PLTP Activity Levels

Chapter 3 (see Table 3.8). Additionally, it was one of the lipid species chosen by the variable selection pipeline for inclusion in the panel for predicting clinical classification (see Chapter 3, Section 3.4.3.1).

Whilst a positive association between SM18.20.1 and plasma PLTP activity levels was seen, the other 4 of the top 5 lipids from Figure 5.3 (PCLyso18.2, PC32.2, PCLyso16.0 and PC34.0p..34.1e) failed to show any statistical significance (data not presented).

The data presented thus far in this chapter show that plasma PLTP activity levels are significantly associated with the levels of several lipid groups (total LysoPE, total SM and total PG) and the levels of a sphingomyelin sub-species (SM18.20.1), which has also been shown in Chapter 3 to differ in mean plasma levels between clinical classifications. In the next section, the association between plasma PLTP activity and clinical classification will be investigated.

5.5.2 Plasma PLTP activity levels and clinical classification

The PLTP activity data was analysed to determine whether there was any relationship between activity levels and clinical classification. Table 5.1 describes the demographics of the study population stratified by clinical classification.

Table 5.1: Demographics of the AIBL baseline sub cohort in which PLTP activity levels were determined.

| Characteristic | HC | MCI | AD | p-value |
|-------------------------------------|---------------------|---------------------|---------------------|----------|
| Number (n) | 158 | 38 | 63 | |
| Age, years (Mean \pm SD) | 71.58 (\pm 7.39) | 74.71 (\pm 7.16) | 77.03 (\pm 9.70) | < 0.0001 |
| Gender (F/M) | 80/78 | 16/22 | 41/22 | 0.0536 |
| APOE ϵ 4 (-ve/+ve) | 91/67 | 18/20 | 23/40 | < 0.05 |
| MMSE, score (Mean \pm SD) | 29 (\pm 1.27) | 26 (\pm 3.04) | 20 (\pm 4.84) | < 0.0001 |
| SUVr (n) | 158 | 38 | 26 | |
| SUVr (Mean \pm SD) | 1.42 (\pm 0.41) | 1.97 (\pm 0.59) | 2.28 (\pm 0.52) | < 0.0001 |
| PiB status (-ve/+ve) | 108/50 | 10/28 | 2/24 | < 0.0001 |
| Hippocampal Volume (n) | 145 | 32 | 17 | |
| Hippocampal Volume, (Mean \pm SD) | 3.15 (\pm 0.33) | 2.93 (\pm 0.38) | 2.80 (\pm 0.49) | < 0.0001 |

HC: Healthy Controls, MCI: Mild Cognitive Impairment, AD: Alzheimer's disease, F: Female, M: Male, APOE ϵ 4 (-ve/+ve): Negative or positive for the Apolipoprotein E ϵ 4 allele, MMSE: Mini Mental State Examination, SUVr: Standard Uptake Value Ratio, PiB status (-ve/+ve): Pittsburgh Compound B negative (SUVr < 1.5), or positive (SUVr \geq 1.5), SD: Standard deviation. Analysis of variance (ANOVA) was used to calculate differences for the continuous variables and χ^2 for categorical variables. A Kruskal-Wallis formula was applied to the MMSE scores. Hippocampal volume measured in mL.

The AIBL study baseline sub cohort (n=259) in which plasma PLTP activity levels were determined comprised of 158 healthy controls (HC), 38 MCI and 63 AD participants. Whilst the number of men and women in each classification group did not significantly differ (p=0.0536), the AD group are significantly older (p < 0.0001), which is expected as AD is an age related disease. The percentage of APOE ϵ 4 allele carriers was significantly different between clinical classifications (42%, 53% and 63% for the HC, MCI and AD groups respectively) and is higher than reported epidemiological allele frequencies (see Table 1.3, Chapter 1) due to deliberate enrichment of the AIBL cohort with APOE ϵ 4 allele carriers. As expected (and partly by definition), the mean MMSE score is lower in the MCI and AD groups compared to healthy controls and the cerebral amyloid burden as measured by mean SUVr is significantly higher in the MCI and AD groups compared to healthy controls and significantly higher in

the AD group compared to MCI. Predictably, hippocampal volume is reduced in MCI and AD compared to the HC group.

Plasma PLTP activity levels were measured in the samples obtained from 259 AIBL subjects described above, and the activity means for each of the clinical classifications are displayed graphically in Figure 5.6.

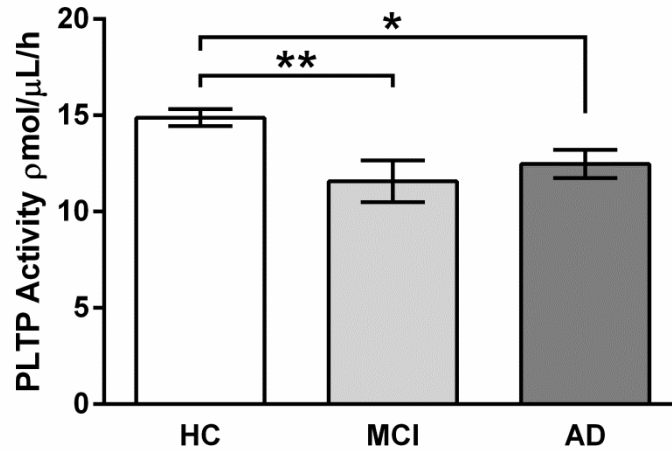


Figure 5.6: Mean differences in plasma PLTP activity levels between clinical classifications.

PLTP: Phospholipid transfer protein, pmol/μL/h: Picomole per microlitre per hour, HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer's disease. One way ANOVA: * $p < 0.05$, ** $p < 0.01$ with Bonferroni post hoc test.

Figure 5.6 shows that mean plasma PLTP activity levels are lower in MCI and AD groups compared to HC ($p < 0.01$, $p < 0.05$ respectively, using one way ANOVA with Bonferroni post hoc test). The data was then assessed to determine whether PLTP activity levels are influenced by gender or APOEε4 allele status. No significant differences were shown between males and females (data not shown), which is in contrast to the gender difference reported in the Framingham study [444], where higher plasma PLTP activity was associated with an increased risk of cardiovascular disease in men only. The relationship between APOEε4 allele carriage and PLTP activity is explored further in the following section.

5.5.3 Plasma PLTP activity levels and APOE ϵ 4 allele status

Figure 5.7 shows plasma PLTP activity levels APOE ϵ 4 allele and non-allele carriers.

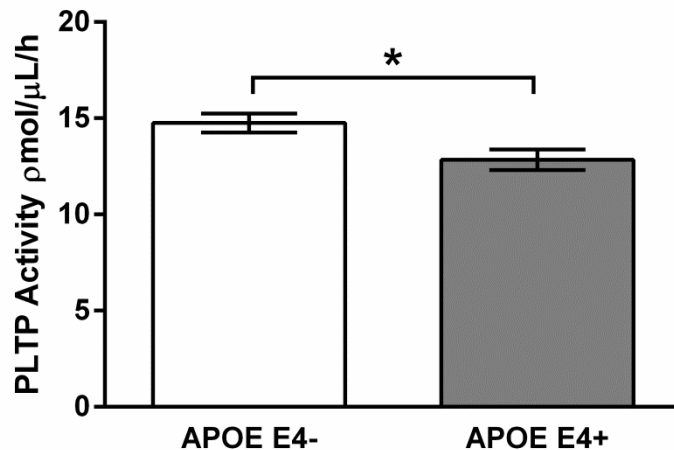


Figure 5.7: Mean plasma PLTP activity levels in APOE ϵ 4 allele carriers compared to APOE ϵ 4 non-carriers.

PLTP: Phospholipid transfer protein, pmol/μL/h: Picomole per microlitre per hour, APOE E4-: Apolipoprotein E ϵ 4 allele non-carriers, APOE E4+: Apolipoprotein E ϵ 4 allele carriers. * $p < 0.05$; student's unpaired t-test.

Figure 5.7 shows that plasma PLTP activity levels are lower in APOE ϵ 4 allele carriers compared to non-carriers whilst Figure 5.6 demonstrates that plasma PLTP activity levels are reduced in MCI and AD groups compared to HC. As observed in Table 5.1, more individuals in the MCI and AD groups carry an APOE ϵ 4 allele, which may account for the observed results in Figure 5.7. Consequently, the mean plasma PLTP activity level in APOE ϵ 4 allele carriers was assessed when the cohort was stratified by clinical classification.

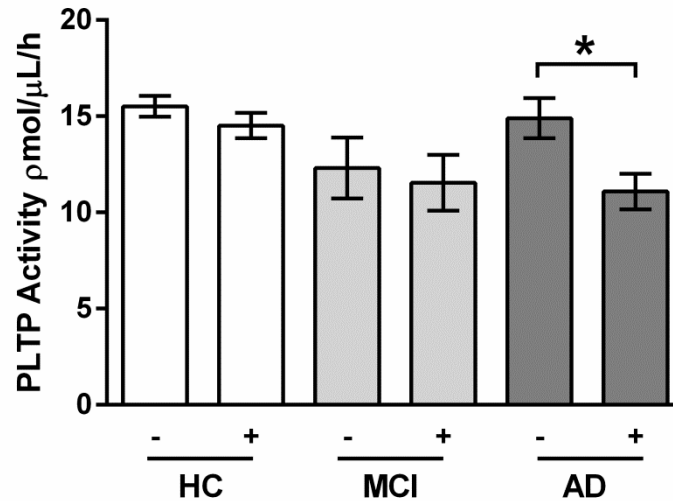


Figure 5.8: Mean plasma PLTP activity levels in APOEε4 allele carriers and non-carriers in each clinical classification group.

PLTP: Phospholipid transfer protein, pmol/μL/h: Picomole per microlitre per hour, HC: Healthy control, MCI: Mild cognitive impairment, AD: Alzheimer's disease, -: APOEε4 allele non-carriers, +: APOEε4 allele carriers, * $p < 0.05$; One way ANOVA with Bonferroni post hoc test.

Figure 5.8 illustrates that plasma PLTP activity levels are significantly different between APOEε4 allele carriers compared to non-carriers within the AD group, with levels lowest amongst APOEε4 positive individuals. A trend towards lower plasma PLTP activity levels is also seen amongst APOEε4 allele carriers in the HC and MCI groups. Having considered the relationship between plasma PLTP activity levels and APOEε4 allele status, a known genetic risk factor for AD, the relationship between PLTP activity levels and neuroimaging biomarkers of AD, such as MRI determined hippocampal volume and PiB-PET determined cerebral amyloid burden was explored.

5.5.4 Plasma PLTP activity levels and hippocampal volume

Hippocampal atrophy is an end stage feature of AD [560]. To investigate any association between PLTP activity and hippocampal volume, plasma PLTP activity levels were compared to MRI-determined hippocampal volume. The results of this analysis are shown in Figure 5.9.

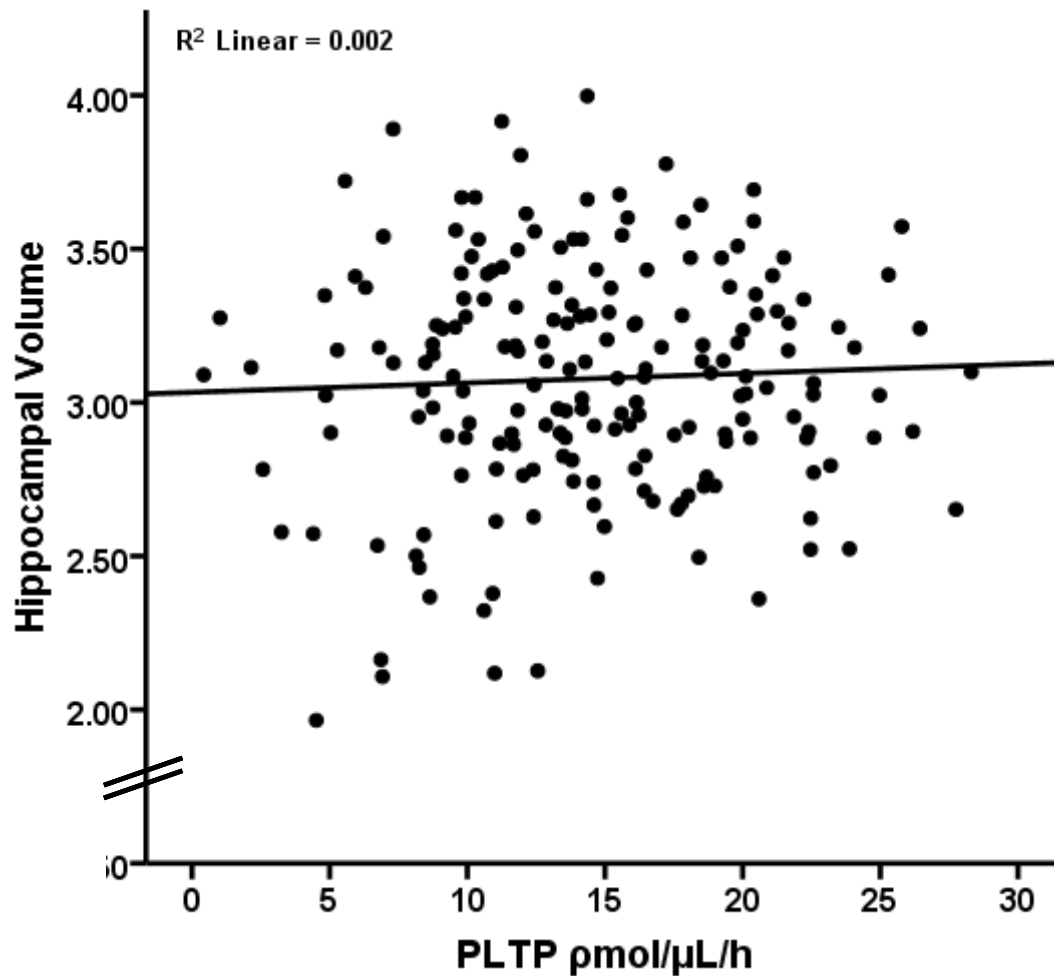


Figure 5.9: Correlation of hippocampal volume with plasma PLTP activity levels.

PLTP: Phospholipid transfer protein, pmol/μL/h: Picomole per microlitre per hour. Pearson's correlation controlled for age, gender, APOEε4 allele status, years of education and intracranial volume (factors which significantly influence hippocampal volume).

No significant association was seen between plasma PLTP activity levels and hippocampal volume when all clinical classifications were considered together (Figure 5.9), or when the PLTP cohort was stratified by APOEε4 allele carriage or by amyloid status, i.e. PiB-positive or PiB-negative. In each case the following factors known to significantly influence hippocampal volume were controlled for; age, gender, APOEε4 allele carriage (except during stratification by APOEε4 carriage), years of education and intracranial volume. To ensure that the established relationship between decreased hippocampal volume and AD was not 'masking' any potential relationship between hippocampal volume

and PLTP activity amongst the HC group, the analysis was repeated for the HC group alone and for the HC group following stratification by APOE ϵ 4 allele carriage and amyloid status. Once again, no significant association was observed between hippocampal volume and PLTP activity (data not shown).

It is possible that reduced statistical power due to small sample size may account for the lack of significance observed (MRI + PLTP cohort; HC n=145, MCI n=32, AD n=17). However, it is also important to note that hippocampal atrophy is one of the last clinical manifestations of AD and the significant reduction in plasma PLTP activity levels observed in the AD and MCI groups compared to the healthy controls (see Figure 5.6) may be representative of an ongoing pathological process affecting PLTP levels that precedes, or is unrelated to end-stage hippocampal atrophy.

As mentioned previously, structural changes in the AD brain such as loss of hippocampal volume are end stages of the disease spectrum. Functional changes in the AD brain such as cerebral amyloid deposition predate structural changes with amyloid deposition, represented by PiB-PET determined SUVR, commencing approximately 20 years before clinical diagnosis is possible. Consequently, the relationship between plasma PLTP activity levels and SUVR was investigated.

5.5.5 Plasma PLTP activity levels and cerebral amyloid burden

The relationship between plasma PLTP activity levels and SUVR, a measure of PiB-PET determined cerebral amyloid burden was assessed. Figure 5.10 shows the correlation between plasma PLTP activity levels and SUVR amongst the PiB-negative (SUVR < 1.5) and PiB-positive (SUVR \geq 1.5) groups when controlling for age, gender and APOE ϵ 4 allele status.

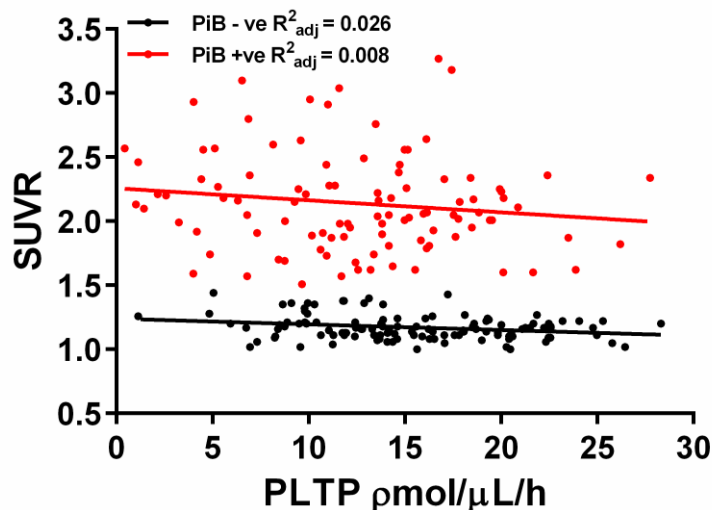


Figure 5.10: Correlation of plasma PLTP activity levels with brain amyloid burden represented by SUVR.

SUVR: Standardised Uptake Value Ratio, PiB: Pittsburgh Compound B, PiB –ve: Individuals with a SUVR value of < 1.5 , PiB +ve: Individuals with a SUVR value of ≥ 1.5 , PLTP: Phospholipid transfer protein, pmol/μL/h: Picomole per microlitre per hour. Pearson's correlations adjusted for age, gender and APOEε4 allele status.

No statistical significance was observed when PiB-PET determined SUVR was plotted as a continuous variable against PLTP activity with the PLTP cohort stratified by PiB-PET status (independent of clinical classification). Previously published AIBL data indicate that 98% of the AD group are PiB-positive (mean SUVR amongst PLTP sub-cohort is 2.28) whilst approximately 30% of healthy controls have a significant cerebral Aβ burden with an SUVR of > 1.5 [477]. Therefore to investigate the potential of PLTP to distinguish PiB-negative from PiB-positive 'cognitively normal' individuals (i.e. individuals at increased risk of developing AD) we considered the HC group separately. First, the mean plasma PLTP activity levels were plotted against SUVR as a categorical variable where individuals are classified as either PiB-positive or PiB-negative. Figure 5.11 shows the mean difference in plasma PLTP activity levels between the PiB-negative and PiB-positive HCs.

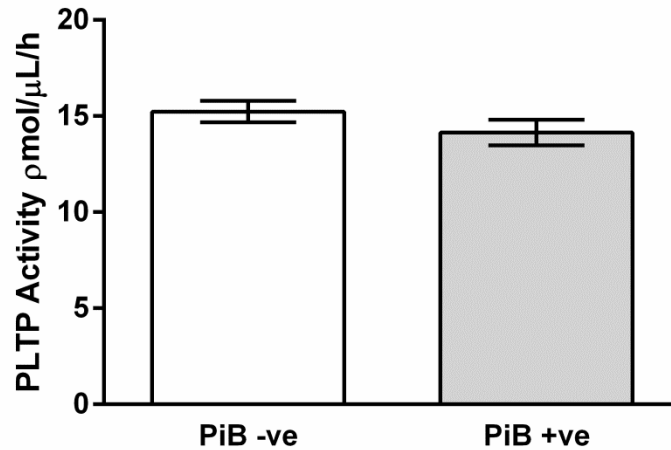


Figure 5.11: Mean difference in plasma PLTP activity between healthy controls classified as either PiB-negative or PiB-positive.

PLTP: Phospholipid transfer protein, pmol/μL/h: Picomole per microlitre per hour, PiB: Pittsburgh Compound B, SUVR: Standardised Uptake Value Ratio, PiB -ve: Individuals with a SUVR value of < 1.5, PiB +ve: Individuals with a SUVR value of ≥ 1.5. $p > 0.05$; student's unpaired t-test.

Figure 5.11 shows no significant difference in PLTP activity levels between the PiB-positive and PiB-negative healthy controls. However, there is a trend towards lower plasma PLTP activity levels amongst PiB-positive individuals which may become statistically significant with a larger sample size. To increase statistical power, we assessed the relationship between PLTP activity levels and SUVR plotted as a continuous variable among the healthy controls: The relationship was not significant (data not shown). However, it can be argued that the PiB-positive individuals within the healthy control group represent a biologically distinct sub-group within whom the processes associated with AD pathogenesis are more advanced, and these individuals are at increased risk of transitioning to AD. Consequently, we further investigated the association between PLTP activity and SUVR following stratification of the healthy control cohort by amyloid status i.e. PiB-positive or PiB-negative.

Figure 5.12 shows the correlation of plasma PLTP activity against SUVR plotted as a continuous variable, following stratification of the HC group into PiB-positive and PiB-negative status.

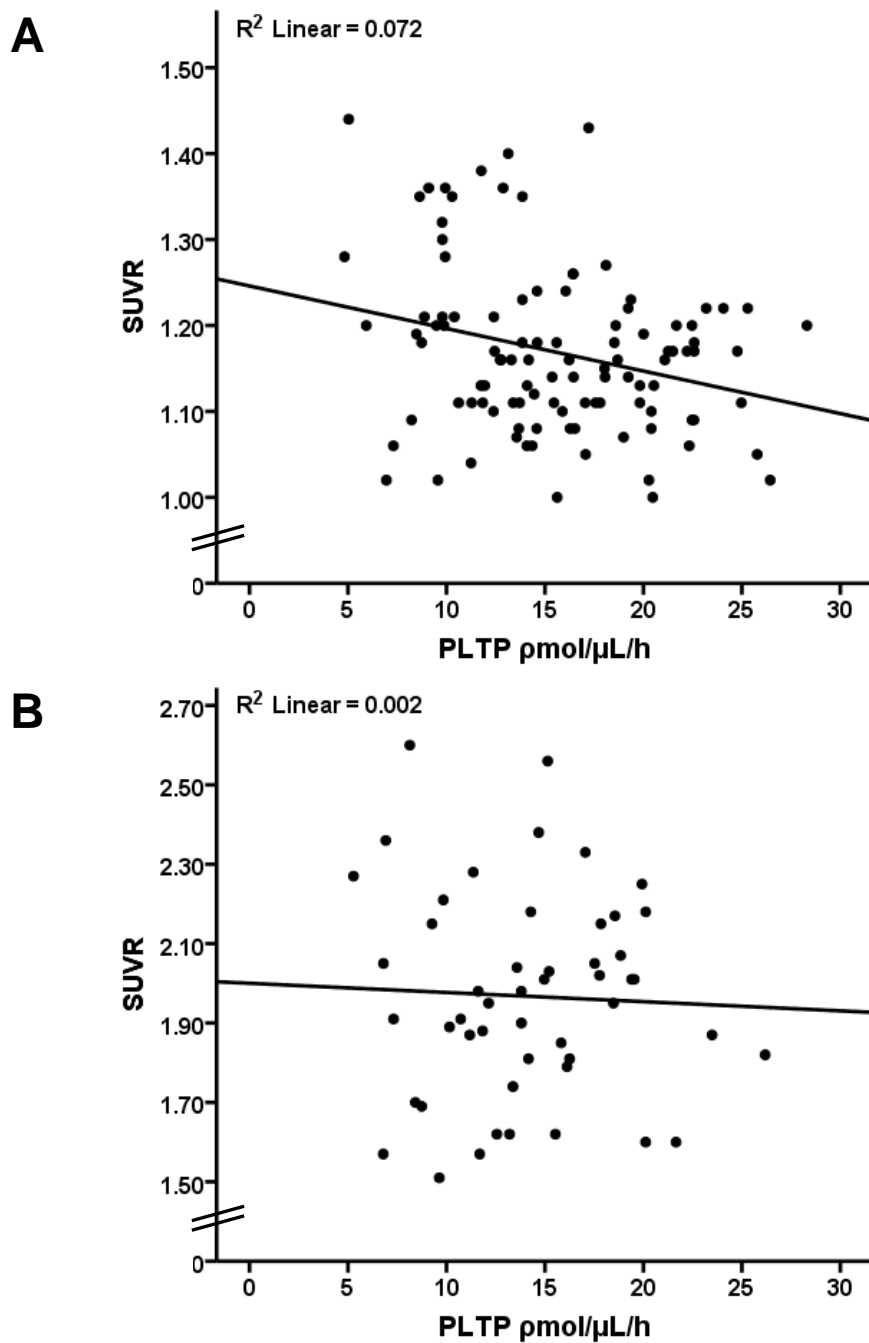


Figure 5.12: Correlation of plasma PLTP activity levels with SUVR in the healthy control group following stratification into (A) PiB-negative individuals or (B) PiB-positive individuals. PiB: Pittsburgh Compound B, SUVR: Standardised Uptake Value Ratio, PiB-negative: Individuals with a SUVR value of < 1.5, PiB-positive: Individuals with a SUVR value of ≥ 1.5 . PLTP: Phospholipid transfer protein, $\mu\text{mol}/\mu\text{L}/\text{h}$: Picomole per microlitre per hour. Pearson's correlations adjusted for age, gender and APOE ϵ 4 allele status.

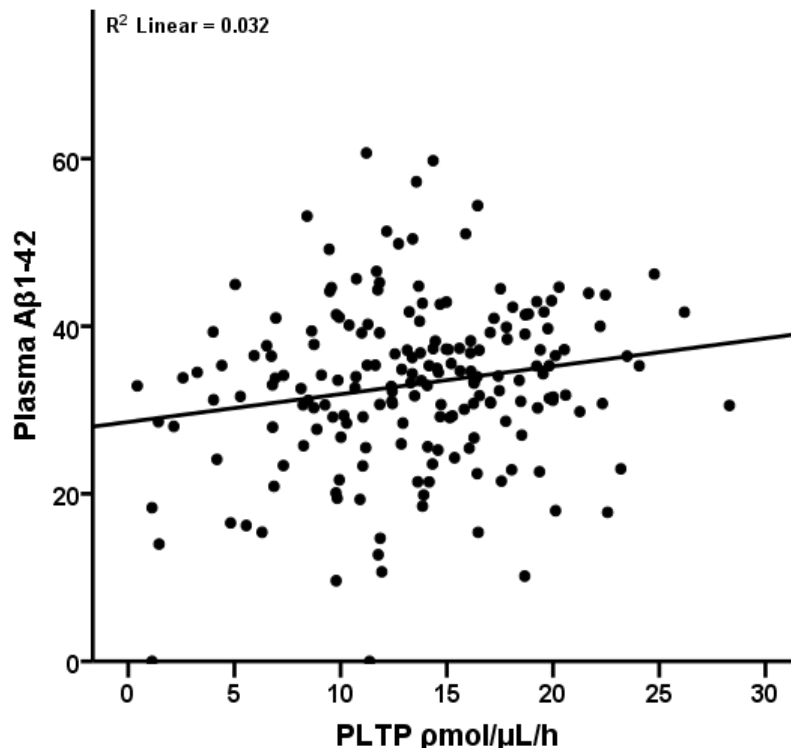
Figure 5.12A shows a significant negative correlation between plasma PLTP activity and SUVR amongst PiB-negative HC individuals ($p < 0.01$), where higher SUVR is associated with lower plasma PLTP activity, whilst figure 5.12B shows no significant correlation in the PiB-positive HC group ($p = 0.748$).

As SUVR is indicative of cerebral A β burden and PLTP is suggested to have a role in peripheral A β metabolism the relationship between plasma PLTP activity levels and plasma A β was also investigated.

5.5.6 Plasma PLTP activity levels and potential blood biomarkers of AD

A recent publication from the AIBL study showed a decrease in the plasma ratio of A β_{1-42} :A β_{1-40} and an inverse correlation with SUVR in AD patients compared to healthy controls. Additionally a reduction in plasma A β_{1-42} was reported in MCI and those participants who transitioned from the healthy control to MCI group [561]. Lower plasma A β_{1-42} levels and plasma ratios of A β_{1-42} :A β_{1-40} in AD compared to healthy controls have also been reported in other studies [562]. A relationship between lipoprotein metabolism, A β and PLTP was described at the beginning of this chapter and therefore the association between plasma PLTP activity levels and plasma A β_{1-42} as well as the ratio of A β_{1-42} :A β_{1-40} in plasma was investigated. The results are shown in Figure 5.13.

A



B

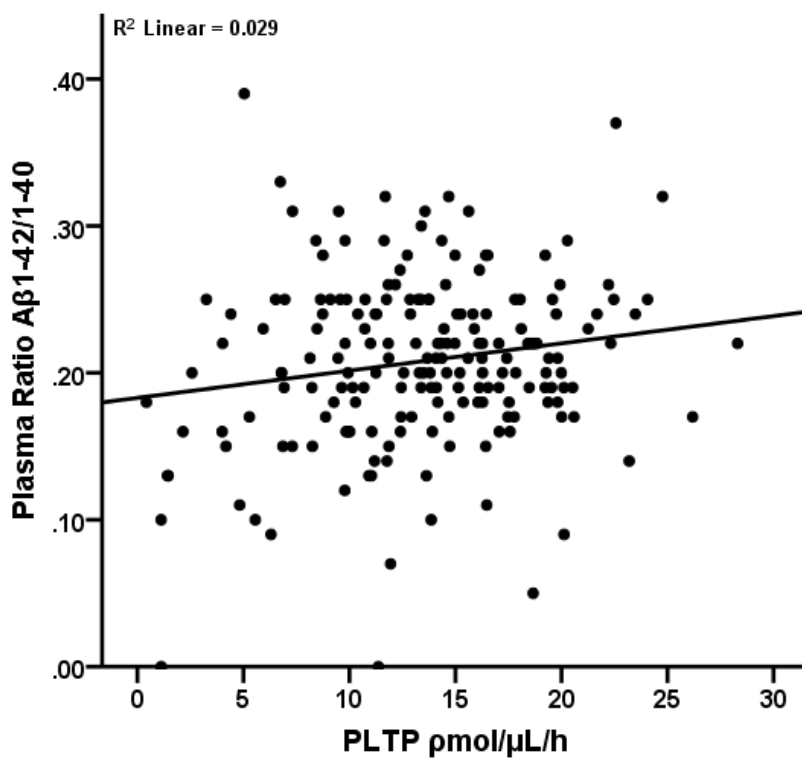


Figure 5.13: Correlation of (A) plasma $A\beta_{1-42}$ and (B) plasma ratio of $A\beta_{1-42}:A\beta_{1-40}$ with plasma PLTP activity levels.

$A\beta$: Amyloid beta, PLTP: Phospholipid transfer protein, pmol/ μ L/h: Picomole per microlitre per hour. Pearson's correlations adjusted for age, gender and APOE ϵ 4 allele status; $p < 0.05$.

Figures 5.13A and 5.13B show significant positive correlations between plasma $A\beta_{1-42}$ and the ratio of $A\beta_{1-42}:A\beta_{1-40}$ with plasma PLTP activity levels respectively, i.e. lower plasma PLTP activity levels are associated with lower plasma $A\beta_{1-42}$ and a lower $A\beta_{1-42}:A\beta_{1-40}$ ratio. This is consistent with Figure 5.6 where plasma PLTP activity levels were shown to be significantly lower in AD patients compared to healthy controls.

A weakly statistically significant ($p=0.049$) positive correlation was also observed between $A\beta_{1-42}$ and plasma PLTP activity levels (data not presented).

Additionally, plasma PLTP activity levels were also compared to plasma apoE, clusterin (apoJ) and apoAI levels, with no significant correlation observed for any of these blood biomarkers (data not shown). Plasma HDL-cholesterol subfraction levels were also compared to plasma PLTP activity levels, yet no significant relationship observed (data not shown). In the case of plasma HDL-cholesterol subfractions, the results are likely to be influenced by medication use (e.g. statins); a factor that was not controlled for during this analysis.

5.6 Discussion

Differences in plasma lipid species between clinical classifications were investigated in Chapters 3 and 4 for their potential use as blood biomarkers to reliably differentiate those with AD and MCI from healthy controls. By measuring the plasma levels of many different lipid species, some insight into AD-associated aberrant biochemical pathways can also be obtained. However, when using blood to investigate processes that are brain-related, a major assumption is that processes in the brain correlate with those in the periphery, and while this has been shown for some markers, it has also been shown not to apply to others. AD is a chronic age related progressive disease in which co-existence of other age related factors, impacting lipid metabolism, is inevitable: These factors include dietary intakes, digestive capacity, nutrient status, hormone balance, the existence of co-morbidities and medication use. These factors present a major challenge when studying blood lipid biomarkers as considerable interference from systemic metabolic dysfunction may be present.

The purpose of the study presented in this chapter was to measure the activity levels of a protein (PLTP) that potentially connects plasma lipids to some features of AD pathology, to determine if there is any correlation between PLTP and established blood and neuroimaging markers of AD. Research investigating the relationship between AD and PLTP activity is relatively new and so a pilot study was undertaken on a subset of the baseline AIBL cohort to determine whether the results justified analysis in a larger sample size. According to a recent report, the sensitivity and specificity of clinically diagnosing AD is 80%, with neuroimaging biomarkers not improving this accuracy [563]. The lipid panels identified in Chapters 3 and 4 were also able to distinguish AD from healthy controls with a sensitivity and specificity of 80%. Plasma PLTP activity was investigated to determine whether there was any merit in its measurement in the AIBL cohort and whether it may show potential in terms of improving the sensitivity and specificity of a diagnostic biomarker panel. The major findings from the study presented in this chapter were:

- A significant positive correlation was seen between plasma PLTP activity levels and the plasma mean total LysoPE, total SM and total PG species.
- A significant positive correlation was seen between plasma PLTP activity levels and the lipid SM18.20.1, the levels of which were shown in Chapter 3 to differ significantly between clinical classifications; this lipid was also later chosen in the variable selection pathway for predicting clinical classification (Figure 3.15 and Section 3.4.3.1).
- Plasma PLTP activity levels were significantly lower in the MCI and AD groups compared to healthy controls.
- When comparing the cohort as a whole, APOE ϵ 4 allele carriers had significantly lower mean plasma PLTP activity levels than non-carriers.
- When stratified by clinical classification, only in the AD group did the APOE ϵ 4 allele carriers have significantly lower plasma PLTP activity levels than non-carriers.
- No significant correlation was seen between plasma PLTP activity levels and hippocampal volume.
- When investigating the cohort as a whole, no significant correlation was seen between plasma PLTP activity levels and cerebral amyloid burden

amongst PiB-positive or PiB-negative individuals. However, when the healthy control group was stratified into PiB-positive and PiB-negative, a significant negative correlation was observed between SUVR and PLTP activity levels amongst the PiB-negative group.

- Significant positive correlations were observed between PLTP activity levels and $A\beta_{1-42}$ levels in plasma, as well as between PLTP activity levels and $A\beta_{1-42}:A\beta_{1-40}$ ratios in plasma.

Statistically significant positive correlations were seen between plasma PLTP activity levels and the mean total levels of LysoPE, SM and PG. The total levels of LysoPE did not significantly differ between clinical classifications when assessed in Chapter 3. However, the mean plasma level of LysoPE16.0, the most abundant LysoPE species (see Chapter 3), was higher in AD compared to healthy controls. On the other hand, no correlation was observed between LysoPE16.0 and plasma PLTP activity levels in this chapter.

A statistically significant association was seen between plasma PLTP activity levels and plasma mean total SM as well as levels of the SM species SM18.20.1. Figures 5.2B and 5.5 show that lower plasma PLTP activity levels are associated with lower total SM, and lower SM18.20.1 respectively. When mean plasma SM species levels were assessed in Chapter 3 all sub-species measured were significantly different between clinical classifications with mean plasma levels lower in the AD and MCI groups compared to the HC group.

Studies into cellular ceramide homeostasis have shown that PLTP can transfer sphingomyelin (SM) from vesicles to HDL [164]. There is also evidence to suggest that newly synthesised hepatic ceramides are secreted via VLDL, and PLTP is believed to be one of the proteins that facilitates the transfer of sphingolipids onto nascent lipoproteins [164, 526, 527]. A reduction in this PLTP mediated secretion may adversely affect SM levels as SM are synthesised from newly synthesised ceramides. Other evidence that PLTP influences ceramide/VLDL production comes from studies of PLTP-deficient mice, which show an impairment in hepatic secretion of VLDL, whereas an overproduction of PLTP in mice has been shown to lead to excessive hepatic VLDL production [564, 565]. All these studies indicate that changes in PLTP

levels may influence several aspects of sphingolipid metabolism, and thus lower PLTP activity may be reducing the transfer of sphingomyelin (SM) from vesicles to lipoproteins such as HDL, leading to reduced plasma SM levels seen in this study. Conversely, the ceramide pool can be elevated by the breakdown of other sphingolipids and therefore ceramide levels may be unaffected by a reduction in PLTP activity (see Chapter 4, Section 4.5). Further analysis of plasma PLTP activity levels and plasma sphingolipid levels in a larger sample number is required to investigate the potential of PLTP and sphingolipids as a combined blood biomarker panel, for the purpose of distinguishing between clinical classifications.

Plasma PLTP activity levels were significantly lower in the MCI and AD groups compared to the healthy control group. In other studies, AD patients have been shown to have lower CSF PLTP activity [535, 536]; showing a level of agreement between studies, if one assumes plasma PLTP activity levels parallel CSF levels. Due to the role of PLTP in lipoprotein metabolism and reverse cholesterol transport, PLTP has been extensively studied for its role in cardiovascular disease (CVD) and other metabolic diseases, such as diabetes [444, 445, 520, 566], where plasma PLTP activity levels have been reported to be elevated. The AIBL recruitment process excludes potential participants with a history of insulin dependent or uncontrolled diabetes, or a history of CVD or recent cardiovascular event and therefore the potential influence of these co-morbidities on PLTP activity levels in our study should have been minimised.

The link between atherosclerosis and PLTP levels was suggested by studies of various mouse models which reported a marked reduction in HDL as a result of increased PLTP expression [567]. On the other hand, PLTP expression in macrophages has been shown in animal studies to reduce atherosclerosis [568], suggesting that protective or disease-promoting effects may be tissue specific. Nevertheless, most but not all studies suggest elevated plasma PLTP activity levels are associated with an increased risk of CVD. The results presented in this chapter show reduced plasma PLTP activity levels in AD compared to healthy controls which may appear counter-intuitive given that CVD is proposed to contribute to the development of AD. There are, however,

several potential explanations for our findings: Firstly, the cohorts are significantly different in terms of age (cohorts investigating CVD are usually comprised of younger participants compared to studies of AD), although PLTP activity is also suggested to increase with age [518]. Secondly, there is a suggested correlation between plasma PLTP activity levels and the acute phase response protein, C-reactive protein (CRP) [539], which is reportedly elevated in CVD. CVD may involve a more acute type of inflammatory response, reflected in an elevated CRP and PLTP, whereas in AD more chronic inflammatory processes are present which are not reflected by a peripheral increase in acute inflammatory markers. Therefore, it may be that different factors in AD and CVD are influencing plasma PLTP activity levels. It would be interesting to investigate plasma PLTP activity in the context of other forms of dementia, specifically vascular dementia, to determine whether the differences shown in this study between clinical classifications are driven by specific features of AD pathology. As mentioned previously, the relationship between PLTP activity and vascular disease is neither predictable nor straightforward: Some studies have shown an association between elevated PLTP activity and coronary artery disease as well as left ventricular dysfunction [445, 569], while others have shown reduced PLTP activity is associated with peripheral vascular disease [447].

Significantly lower plasma PLTP activity levels were found in APOE ϵ 4 allele carriers compared to non-carriers, in the cohort as a whole. When the cohort was stratified according to clinical classification, the significance between carriers and non-carriers remained in the AD group (see Figure 5.8). A study of PLTP activity, serum apoE concentration and APOE ϵ 4 in a Finnish population showed serum apoE levels to be highest in those carrying an APOE ϵ 2 allele and lowest in those carrying an APOE ϵ 4 allele but failed to show a difference in PLTP activity [534], however this study utilised a 'cognitively healthy' population with an age range of 30-94. Our study reports lower PLTP activity levels in APOE ϵ 4 allele carriers, and a published study from the AIBL cohort has described significantly lower plasma total apoE levels in patients with AD and specifically those carrying the APOE ϵ 4 allele. This study also demonstrated an APOE ϵ 4 gene dose effect in further reducing plasma apoE levels [84]. ApoE

complexes with, and stabilises its activity, and therefore a role for the regulation of plasma PLTP activity by apoE is suggested [570]; the results presented in this chapter are consistent with this suggestion.

As mentioned previously, pathological changes in the AD brain such as cerebral amyloid deposition (which can be quantitated by SUVR) predate the onset of clinical symptoms. Brain atrophy in selected regions such as the hippocampus has also been shown, mostly by structural magnetic resonance (MRI), to predate symptom onset [560]. The entorhinal cortex is the first region to display atrophy in AD, this is followed by atrophy in the hippocampus, amygdala and parahippocampal gyrus [571]. The next region to display atrophy includes the posterior cingulate cortex; eventually the atrophy becomes generalised to the temporal neocortex and neocortical association areas [572]. Thus, the neuroimaging findings on MRI closely mirror the progression of neurodegenerative pathology, suggesting that regional brain volume assessment using MRI could be used for early diagnosis and disease monitoring. However, the MRI technique is time-consuming and requires expensive technology, and a blood biomarker test would clearly be preferable.

In our study, no significant correlation was found between hippocampal volume and plasma PLTP activity levels. Unfortunately, hippocampal volume data was only available for 17 out of 63 AD participants in whom PLTP activity levels were measured; an increased sample size is required to confirm any lack of correlation.

When the whole cohort was divided into two groups - the PiB-negative and PiB-positive groups - no relationship could be observed between plasma PLTP activity levels and cerebral amyloid burden as measured by SUVR. However when the HC group was stratified into PiB-positive or PiB-negative, a significant negative correlation was observed between SUVR and PLTP activity levels amongst the PiB-negative group. This correlation was 'masked' when the PiB-negative and PiB-positive groups were considered collectively, and as referred to in earlier text, may be due to different biochemical processes occurring in the pathologically distinct groups. This result could reflect the suggested roles of PLTP in A β metabolism, as lower plasma PLTP activity may reduce A β removal

from the brain to the periphery for degradation. A significant positive correlation was observed between plasma PLTP activity levels and plasma $A\beta_{1-42}$, as well as the ratio of plasma $A\beta_{1-42}:A\beta_{1-40}$, which is consistent with a role for PLTP in the transfer and metabolism of $A\beta$. Plasma levels of $A\beta_{1-42}$ and the plasma ratio of $A\beta_{1-42}:A\beta_{1-40}$ were recently shown in the AIBL study to be lower in AD; these $A\beta$ measures also showed a negative correlation with SUVR. The correlation reported in this chapter between plasma PLTP activity levels and both plasma (positive correlation) and cerebral $A\beta$ (negative correlation) strengthens the hypothesis that PLTP may be involved in $A\beta$ transport from the brain. The “peripheral sink” hypothesis assumes that a balance exists between peripheral and brain levels of soluble $A\beta$, and posits that efficient removal of $A\beta$ from the periphery (by enzymatic breakdown or tissue uptake followed by breakdown) should help decrease brain levels of $A\beta$. According to the hypothesis, a disruption or decrease in clearance from the brain contributes to cerebral retention of $A\beta$, $A\beta$ aggregation and amyloid formation [573, 574]. This hypothesis remains controversial, as pharmacological intervention efforts to reduce peripheral and cerebral $A\beta$ have sometimes reduced $A\beta$ in one or both compartments. However, the animal models or people in most of these pharmacological trials have not shown significant cognitive improvement [16, 575, 576]. A recent animal study using the $A\beta$ degrading enzyme neprilysin, showed an effective degradation of $A\beta$ in the periphery but no alteration in brain or CSF $A\beta$ levels [577]. Some studies have shown promise however, for example intravenous application of a form of low-density lipoprotein receptor-related protein-1 (LRP) in an AD mouse model was found to reduce $A\beta$ accumulation and aggregation in the brains of the mice, and also was shown to improve their learning and memory [578]. The concept of improving clearance of brain $A\beta$ via increasing $A\beta$ removal from the periphery is a relatively new idea, and although many trials have not yet shown symptom improvement, as one review has suggested - it may take longer than expected in ongoing trials for such neurobiological effects to translate into measurable clinical improvements [579]. However, whilst such studies are still debating whether the “peripheral sink” hypothesis offers an effective therapy for AD, the results do not dispute suggested roles of PLTP in $A\beta$ metabolism, transport and removal from the brain, or its potential use as a biomarker of AD. A very recent study of

PLTP-deficient mice has found that a lack of PLTP causes an increase in cerebral and hippocampal A β ₁₋₄₂ levels and a decrease in cerebral A β ₁₋₄₀ levels. The PLTP deficiency also increased brain expression of γ -secretase catalytic units and decreased the content of apo E, and was found to impair learning and memory in the mice [580]. Collectively, studies such as these together with the findings reported in this chapter suggest that the relationship between PLTP, peripheral and cerebral A β warrants further investigation.

No significant correlations were seen when plasma PLTP activity levels were compared with plasma apoE, clusterin (apoJ) or apoAI levels. The plasma HDL-cholesterol subfractions also failed to show significance when compared with PLTP activity levels. It should be noted however, that the use of medications such as statins and fibrates which would have been likely to impact on such an association was not included as a covariate during analysis, and thereby represents a limitation and significant caveat when interpreting the results of this aspect of the study. Indeed, a recent investigation, as part of the Diabetes Atorvastatin Lipid Intervention study (DALI), showed that PLTP activity and plasma apoE were both lowered by treatment with Atorvastatin over a 30 week period [581]. Furthermore, fibrates (PPAR α activators) also lower plasma triglyceride and can increase HDL-cholesterol levels. In PLTP studies utilising 'healthy' cohorts, the level of triglycerides has been the parameter that has consistently contributed to variations in plasma PLTP activity levels, which is likely due to its role in transferring lipids from apoB containing lipoproteins [518, 534]. Thus, a logical recommendation for future investigation is controlling for medications that can potentially affect biomarker levels.

A genetic polymorphism has been identified in the PLTP gene (rs7679) and interestingly this has been shown to predict both serum triglyceride levels as well as mRNA PLTP expression in the liver [582]. The rs7679 T allele was shown to be associated with increased PLTP expression, increased plasma HDL and lower plasma triglycerides. Another study showed higher PLTP activity, higher plasma HDL and lower plasma triglycerides were associated with the T allele, suggesting a cardioprotective effect of increased PLTP activity [583]; adding to the confusion surrounding the influence of PLTP on

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cardiovascular disease. The association of AD with this rs7679 polymorphism and other variants of the PLTP gene should be investigated in future studies; if any of these genetic variants can predict PLTP activity, this may circumvent methodological disparities associated with measurement of PLTP and thereby enhance comparability of results between cohorts.

The results presented in this chapter strongly suggest a relationship between PLTP activity levels, certain plasma lipid species levels, cerebral and plasma A β levels. In the quest to identify a sensitive and specific blood biomarker for AD, plasma PLTP activity levels may prove to be an important additional measurement, and PLTP warrants further assessment both cross-sectionally and longitudinally.

Chapter 6

General Discussion and Future Directions

Chapter 6 – General Discussion and Future Directions

6.1 Introduction

Over the past few decades the role of lipids in brain biochemistry and the involvement of particular lipid species in the pathological processes associated with AD have been more clearly defined. A key protein central to AD pathology is the transmembrane protein APP. Processing of this protein, which appears to be highly regulated by the membrane lipid environment, leads to the production of the A β peptide which is neurotoxic when its levels are elevated. Undoubtedly, progress in lipid research and our understanding of AD pathology has been driven by advances in technologies enabling the more precise investigation of the lipidome; the repertoire of lipid species present in cells and tissues. The specific type, concentration and flux of these lipid species present at a given time or associated with a pathological state are the net result of gene and protein expression, which are influenced by the cellular environment. Abnormal lipid metabolism, involving cholesterol, phospholipids and sphingolipids has emerged as a key component of the pathological processes leading to the development of AD. While selected factors, including abnormal lipid metabolism have been identified as key features of AD pathology, it is unlikely that any of these factors is the sole disease causing culprit. It is more reasonable to assume, due to the highly integrated nature of metabolic pathways, that these multiple factors work together in response to perhaps a series of insults that eventually lead to AD. Lipidomic studies, therefore, are vital for gaining a greater understanding of disease mechanisms, aberrant biochemical pathways, and importantly for biomarker discovery. The identification of sensitive and specific biomarkers is a priority not only in terms of early AD diagnosis, but to assist in the validation and monitoring of clinical interventions that target these aberrant pathways and their associated lipids and proteins. The results presented in this thesis add to the existing body of knowledge and open new avenues of exploration in the pursuit of diagnostic tests and therapeutic strategies for AD.

The challenge for future research is to answer the following questions with more certainty:

- What factors control lipid metabolism, how are they integrated, and which signals go wrong in AD?
- How does this lead to amyloid deposition, tau pathology and neurodegeneration; the hallmarks of AD?
- Is abnormal lipid metabolism a driver or is it influenced by aberrant upstream signals that effect cell metabolism, cell survival and DNA repair?
- How can this information be used to diagnose, prevent and treat AD?

When considering the measurement of blood lipids, the influence of dietary and lifestyle factors cannot be ignored. Diet and lifestyle factors have an effect on the complex network of hormones and transcription factors that orchestrate metabolism and influence anti-ageing and pro-survival proteins. Such proteins include the sirtuins and other metabolic sensors such as PPAR-gamma coactivator 1 α (PGC1 α) and adenosine monophosphate kinase (AMPK), which allow proper adaptation to energy requirements and environmental challenges. The existence of these factors may be a major reason why the identification of highly specific and sensitive blood lipid biomarkers has been difficult in AD due to the interference of plasma abnormal lipid profiles associated with metabolic dysfunction and other co-morbidities.

A highly complex relationship between AD pathology and lipid metabolism exists and this association was investigated by measuring plasma lipid species to identify which lipids groups were significantly different between clinical classifications and if these lipids correlated with other AD biomarkers. The ability of certain plasma lipids species to predict clinical classification and disease progression was also assessed. Additionally, the activity of the lipid transfer protein PLTP was measured in plasma and assessed for its association with plasma lipid profiles, clinical classification and other AD biomarkers.

6.2 Plasma lipid profiles and their association with clinical classification

In Chapters 3 and 4, 189 plasma lipid species were investigated to determine their feasibility as blood biomarkers for AD. Following complex statistical modelling, several lipids were identified as differing significantly in levels between clinical classifications, after adjustment for age, gender, APOE ϵ 4 allele status and collection site in samples collected at both baseline and the 18 month follow up. Further, lipid panels were determined at baseline and the 18-month follow up which could distinguish between healthy control and AD participants with a sensitivity and specificity of 80%, which compares to the current sensitivity and specificity of both clinical diagnosis and neuroimaging biomarkers for diagnosing AD [563]. Lipid panels identified at both time points for their ability to distinguish MCI from healthy controls, and AD patients from MCI, demonstrated reduced sensitivity (69% and 71% respectively), possibly due to the heterogeneity of the MCI group. The lipids selected at baseline and 18 months for inclusion in the HC vs. AD panel were not the same (excepting 2 lipids), and although the change in characteristics of the cohort between baseline and 18 months may explain the differences, further work is recommended to improve the diagnostic utility of the blood lipid biomarker panels presented in this thesis. The analytical platform chosen for this study simultaneously identified a broad range of lipids (189 species) in a large cohort (n=1112) at two time points. While this has been extremely useful in identifying lipids that differ with clinical classification, and providing information that can contribute to an understanding of altered lipid metabolism in AD, the lipid panels as they stand cannot be used in a routine setting for the diagnosis of AD. However these results have generated many future research avenues to pursue, for example they have provided a selection of lipid groups which should be investigated for biomarker discovery by a more targeted analytical approach.

A recent study by Mapstone *et al.* [493] has just reported the identification of a panel of ten lipid biomarkers that predicted conversion to either amnesic mild cognitive impairment or AD within a 2-3 year timeframe, with greater than 90% accuracy. Using a combination of untargeted and targeted lipidomic methodologies, eight PC species and two acyl carnitine species were identified

for inclusion in the panel. As part of our study acyl carnitines were not measured, and only four of the PC species identified by Mapstone *et al.* were measured. Additionally, analysis of the transition group data reported in this thesis was undertaken at 18 months, whilst according to the report by Mapstone *et al.*, phenoconversion occurred on average at 2.1 years. The results presented in this thesis agree with the report by Mapstone *et al.* showing lower mean plasma levels of the four PC species measured (PC38.6, PC40.6, PC40.6p and LysoPC18.2) between the HC and AD groups and analysis of AIBL samples at additional time points may identify important lipids able to predict transition groups.

The lipid differences that were noted between clinical classifications were not identical at the two time points; however similarities exist in terms of sub-class, acyl chain length and number of unsaturated bonds. In addition, the relative levels of some species compared to others enabled plausible explanations of the biological relationships and allowed speculation of possible interference in the lipid pathways by dietary and metabolic factors.

In general agreement with other studies, the major lipids that were shown to differ between clinical classifications at either or both time points were several PE, PC, plasmalogen, sphingomyelin and ceramide species. Of note was the directional change (increased or decreased) with clinical classifications of the various species differing in acyl chain length and degree of saturation in the PE and PC species. The majority of species levels that were increased in AD and MCI compared to healthy controls in the PE and PC groups had shorter chain lengths and less saturated bonds: this may suggest abnormalities with the desaturase and elongation enzymes required to produce long chain unsaturated fatty acids from shorter omega-3 or omega-6 precursors, including the essential fatty acids, linoleic and α -linolenic acids. It may also suggest essential fatty acid deficiency (and therefore reduced production of AA (20.4n-6) and DHA (22.6n-3)) or a combination of fatty acid deficiency and reduced enzyme activity. If abnormal desaturase or elongase activity exists, shorter chain fatty acids with few saturated bonds would accumulate. However, if the desaturase and elongation activities were normal yet there was a deficiency of

essential fatty acids, the unsaturated mead acid (22.3n-9) would accumulate in plasma. The preferred substrates of the delta 6 and delta 5 desaturase enzymes are omega-3 and omega-6 fatty acids which produce the long chain polyunsaturated fatty acids. If there is a deficiency in the essential fatty acids (18.2n-6 and 18.3n-3), the desaturase and elongase enzymes are then free to act on their less preferred fatty acid substrates, omega-9 series (18.1n-9). Consequently, mead acid accumulates which acts as a marker for essential fatty acid deficiency.

While inferences can be made from the lipid species identified in Chapters 3 and 4, the analytical platform used does not allow precise determination of the fatty acid composition of all species when two fatty acids are present in a particular phospholipid species. For example, PE38.4 indicates two fatty acids with a combined total of 38 carbon chains and 4 unsaturated bonds, which is likely but not certain to be 18.0 at sn-1 and 20.4 at sn-2. Further studies are needed to identify the individual acyl groups in the lipid species, which will also determine whether abnormal desaturase and elongase activities or essential fatty acid deficiencies are the cause of reduced acyl chain length and desaturation in the PE and PC species. A more targeted analysis coupling chromatography to mass spectrometry and the measurement of plasma fatty acids will meet this need. Such information would possibly lead to increased sensitivity and specificity of a plasma lipid biomarker panel when added to those lipid subspecies identified in Chapters 3 and 4.

Interestingly a recent study has shown an upregulation of stearoyl-CoA desaturase (SCD) in the AD brain, and increased nervonic (24.1n-9) acid; resulting in an increased ratio of monounsaturated fatty acid to saturated fatty acid [584]. This is interesting for several reasons. The first step in the synthesis of nervonic acid (22.1n-9), a crucial component of myelin sphingolipids including sulphatide, is the desaturation of stearic (18.0) to produce oleic acid (18.1n-9). Oleic acid is then elongated to form nervonic acid. The authors found no increase in nervonic acid containing sphingolipids, and subsequently concluded that the reported increase in nervonic acid was unlikely to result from sphingolipid breakdown and was more likely due to upregulation of SCD,

although they were unable to exclude sampling effects. Chapter 4 of this thesis showed plasma ceramide containing nervonic acid (C.d18.1.24.1) to be one of the lipids with the greatest difference in levels between the healthy control and AD groups and was later selected in the statistical model for predicting AD. It is certainly feasible that the increase in C.d18.1.24.1 seen in our AD patients results from both breakdown of sulphatide and increased *de novo* synthesis of nervonic acid containing ceramide. Sulphatide is a crucial component of myelin and its degradation appears to be accelerated in AD, thus it is also possible that there is a compensatory increase in nervonic acid production to supply precursors for synthesis. The authors reporting upregulation of SCD in the AD brain also reported elevated mead acid (22.3n-9). Mead acid is the only polyunsaturated fatty acid that can be synthesised *de novo*, with SCD being the rate limiting enzyme. When DHA deficiency is present in the brain, mead acid production may be increased as a compensatory mechanism to maintain a minimum unsaturation level of fatty acids in the membrane to preserve function. Assuming that these changes are related to plasma lipid species, their measurement may contribute to a biomarker panel that reflects an aberrant brain pathway. As previously mentioned, a future addition to the lipid biomarkers measured in Chapters 3 and 4 will be the measurement of both mead and nervonic acids as components of plasma fatty acid profiling.

The results of Chapters 3 and 4 also provide several indicators of peroxisomal dysfunction, a reported feature of AD [498]. These indicators include the decrease in plasma ether lipid species containing greater than six unsaturated bonds (DHA, 22.6n-3) in the AD group compared to healthy controls; the supply of both the plasmalogen precursors (1-alkyl-glycerol ether lipids) and the final desaturation step in DHA production which is carried out in peroxisomes. Again, plasma fatty acid profiling, including measurement of the peroxisomal DHA precursor tetracosahexaenoic acid (24.6n-3) and targeted lipidomics for plasmalogen species, will assist in determining the utility of peroxisomal dysfunction and specific plasmalogen species as sensitive and specific AD biomarkers.

In agreement with other studies, the plasma lipid profiles reported in Chapters 3 and 4 showed significant changes in sphingolipids between clinical classifications [163, 422, 423, 474]. Generally, the sphingomyelin species levels were decreased and the ceramide species were elevated in AD. Ceramide is the central sphingolipid from which other complex sphingolipids (including sphingomyelin (SM), cerebrosides and gangliosides) are derived. The reduced sphingomyelin species seen in AD may result from decreased synthesis via SM synthase or increased catabolism via SMases or a combination of both. As described in detail in Chapter 1 (Section 1.5.4), a tight connection exists between cholesterol and sphingomyelin, and a reduction in sphingomyelin has consequences for membrane biology and APP processing. Furthermore, reduced sulphatides have been reported at an early stage of disease in the AD brain [413]. The increase in ceramide species in AD may be a net result of increased *de novo* synthesis from serine and palmitoyl CoA and increased catabolism of sphingomyelin and sulphatide. In agreement with previous reports [163, 502], this study reports a reduction in plasma sphingomyelin levels in AD patients. As previously mentioned, the ceramide containing nervonic acid C.d18.1.24.1 was one of the lipids with the greatest difference in levels between clinical classifications and was also selected as part of the predictive panel in Chapter 4: this data may represent an intriguing link to brain sulphatide degradation.

When the ceramide containing nervonic acid C.d18.1.24.1 and three other ceramides chosen in the HC vs. AD predictive panel were compared with SUVR categories, used as a measure of cerebral amyloid burden (see Chapter 4, Figure 4.21), mean plasma levels of all four lipid species rose with the SUVR up to a value of 2, this was followed by a marked decline in levels when the SUVR levels were greater than 2. This striking relationship may suggest that plasma ceramides increase as the rate of A β accumulation increases in the brain and then decline as the A β accumulation rate plateaus. This warrants further investigation both through validation in other cohorts and through studying the correlation between intra-individual rates of A β accumulation and ceramide species in this cohort.

The results presented in this thesis show that markers of sphingolipid metabolism have the potential to be sensitive and specific biomarkers for AD. Studies have shown that there are several enzymes involved in sphingolipid metabolism which have altered activities in AD resulting in accumulation of ceramides and alterations in other specific sphingolipids [471, 511, 514]. Potential therapeutic targets for AD are emerging from these studies and measurement of plasma sphingolipids will provide a valuable tool to assess efficacy and monitor progress of therapeutic interventions. A more targeted approach coupling chromatography with mass spectrometry for sphingolipidomic studies would enable identification of specific species and their ratios which may facilitate discovery of more sensitive and specific biomarkers. For example the measurement of sphinganine and dihydroceramide may provide information on the *de novo* ceramide synthesis pathway and the relative activity of the enzymes serine-palmitoyl transferase (SPT) and dihydroceramide desaturase (DES); the activities of which are reportedly altered in AD, thereby identifying them as potential therapeutic targets for reducing ceramides.

6.3 Plasma PLTP activity levels and their association with plasma lipid species and clinical classification

Phospholipid Transfer Protein (PLTP) is involved in lipoprotein metabolism and lipid transfer and has suggested roles in processes involved with AD pathology, including A β metabolism. Therefore, any association of PLTP with the lipid species measured in Chapters 3 and 4, clinical classification and other AD biomarkers was investigated, to determine whether PLTP shows promise as a potential biomarker of AD. Plasma PLTP activity, measured in a subset of the AIBL baseline samples, correlated with several of the lipid species measured in Chapters 3 and 4 as well as with other AD biomarkers. Plasma PLTP activity levels showed the strongest correlation with mean plasma levels of SM18.20.1; this lipid species was also shown in Chapter 3 to be lower in the AD group compared to healthy controls. The relationship of PLTP with individual lipid species should be reassessed when PLTP activity measurement is undertaken in the entire baseline cohort and when the 18 month samples are assessed.

Plasma PLTP activity levels were measured and found to be lower in the MCI and AD groups compared to the healthy controls. Additionally, PLTP activity levels were lower in APOE ϵ 4 allele carriers compared to non-carriers. However, no correlation was observed between plasma apoE levels and PLTP activity. When comparing PLTP activity levels with neuroimaging results, no significant correlation was observed with hippocampal volume or PiB-PET determined SUVR as a marker of cerebral amyloid burden, when assessing all clinical classifications together. To investigate the potential of PLTP to distinguish PiB-negative from PiB-positive ‘cognitively normal’ individuals (i.e. individuals at increased risk of developing AD, or in the early pre-symptomatic stages of AD) the HC group was investigated further. Following stratification into PiB-positive and PiB-negative groups, a significant negative correlation was observed between PLTP activity and SUVR in the PiB-negative HC group, suggesting that PLTP activity may be indicative of cerebral amyloid load in the early stages of A β deposition; an effect which diminishes as SUVR increases beyond a certain point.

Interestingly, of all the blood biomarkers assessed for their relationship with plasma PLTP activity levels (A β , apoE, clusterin and HDL subfractions) only plasma A β showed a significant positive correlation, with lower plasma PLTP levels associated with lower plasma A β ₁₋₄₂ and the plasma ratio of A β ₁₋₄₂:A β ₁₋₄₀. Lower levels of both these A β measurements have been reported in AD in the AIBL study [561], which is in agreement with a suggested role for PLTP in A β metabolism.

Collectively, the results of PLTP activity measurement in the healthy control group of the baseline AIBL samples clearly suggest further investigation of this protein is warranted. Assessing a larger sample number longitudinally will assist with determining PLTP’s potential as a biomarker of AD. How plasma PLTP activity levels and SUVR vary for a particular individual over time should also be investigated.

6.4 Future directions

Plasma lipid profiles show great potential as blood biomarkers of AD, with the lipid panels identified in Chapters 3 and 4 distinguishing AD patients from healthy controls with a sensitivity and specificity of 80%. The analytical platform used in this study enabled the simultaneous measurement of 189 plasma lipid species in each sample. The method confers the advantages of facilitating the identification of both differences in individual lipid species between clinical classifications, and also patterns within related groups, such as the sphingolipids; therefore directing future investigations to specific lipid targets. The shotgun lipidomics method allows high sample throughput but suffers from a compromise in the resolution, which may be addressed by a more targeted approach utilising chromatographic separation prior to MS analysis. However, before any further analyses are undertaken, the AIBL study has a wealth of existing biomarker and lifestyle data that will continue to accumulate and assessment of this data in the context of the plasma lipid profiles measured in this study is required.

The results presented in this thesis have also opened up many areas of potential future investigation, which may contribute towards the identification of a sensitive and specific plasma AD biomarker panel that can be used in a routine diagnostic laboratory setting; a necessity given the predicted marked increase in the number of AD cases. Such lipidomic studies will likely lead to a greater understanding of the pathological processes involved in AD and may identify novel targets for which therapeutic interventions are directed. The following summarises suggested areas for further investigation:

- To improve the diagnostic sensitivity and specificity of the existing lipid profiles, assessing ratios of lipid species may provide additional information. The ratio of ceramides to sphingomyelin with same acyl chain and the ratio of dihydroceramides to ceramides, may provide information regarding SMases and dihydroceramide desaturase (DES) respectively. The ratio of LysoPLs with their corresponding PL may be altered by abnormal phospholipase activity, which is also reported in AD. Additionally, the saturated to unsaturated ratio of the acyl species of the

phospholipids may indicate problems with fatty acid metabolism. The ratio of PE to PC species may indicate abnormal methylation patterns and reduced choline availability, which is required for the synthesis of both SM and acetylcholine.

- Plasma fatty acid analysis will enable identification of important polyunsaturated fatty acids such as DHA and also reveal whether there is an essential fatty acid deficiency or abnormal metabolism involving desaturase and elongase enzymes. Additionally measurement of nervonic (24.1n-9), mead (22.3n-9) and tetracosahexaenoic (24.6n-3) acids, which have been shown to be altered in AD, may be useful additions to existing lipid profiles.
- LC-MS/MS analysis of sphingolipids is justified as many ceramide and sphingomyelin species were significantly different between clinical classifications and changes in sphingolipids have been reported to predict memory impairment [163]. Additionally in light of the recent report by Mapstone *et al*, more targeted analysis of PC species with the addition of acyl carnitines may identify an accurate panel for predicting phenocconversion.
- Chapter 1 described the interconnection of cholesterol, sphingolipid and phospholipid metabolism, which suggest that the measurement of cholesterol metabolites such as oxysterols may improve the sensitivity and specificity of a lipid biomarker panel.
- Determining PLTP activity levels within the entire baseline cohort is recommended, as the pilot study reported in Chapter 5 showed promising results. PLTP activity should also be assessed longitudinally and compared to intra-individual SUVR. Additionally, the potential of PLTP as an AD biomarker should be explored, both in combination with plasma A β , and also as a component of a potential biomarker panel including inflammatory markers. Ceramides are connected to

inflammatory cytokines and insulin resistance [207, 265] and as a role for PLTP in inflammatory signalling is suggested; an intriguing relationship may be uncovered. The combined measurement of specific ceramide species, inflammatory markers and PLTP could form an additional biomarker panel.

6.4 Limitations

This thesis had the aim of identifying a sensitive and specific blood biomarker panel for AD and reports the association of plasma lipid profiles and a lipid transfer protein with clinical classification and various other AD biomarkers. However, several limitations should be considered when interpreting the findings.

Although samples were analysed at two time points in the AIBL cohort, further longitudinal analysis is required. Whilst the baseline and 18 month lipid species results were compared, the characteristics of the cohort changed over the 18 month period in terms of age, disease progression and non-returning participants: Factors which must be considered when comparing the cross sectional results at both baseline and 18 months. When analysing the data longitudinally over 18 months, statistical power was limited in the context of clinical classification transition, as only 54 of the participants who returned at 18 months transitioned to MCI or AD. It is likely that analysis of samples collected at additional time points (i.e. 36 and 54 months) will reveal greater diversity in the lipid profiles of individuals transitioning between clinical classifications, compared to those who remain within their original classification group, thereby facilitating the identification of a more sensitive and specific biomarker panel for predicting clinical classification transition.

‘True comparison’ of the data collected at each of the two time points may also have been affected by sample handling, as storage of the two sample sets differed. The 18 month samples were stored in liquid nitrogen until the lipids were extracted whereas the baseline samples, while initially stored in liquid nitrogen, were stored for several years at -80°C prior to lipid extraction. The effect of -80°C versus liquid nitrogen storage on lipid profiles was not tested.

A “shotgun” lipidomics method was used for this study which simultaneously measured a large number of lipid species with high throughput. This method may have had lower resolution compared to a more targeted approach, and therefore the measurement of some less abundant lipid species may have been compromised. However, this analytical platform forms a solid foundation to indicate which lipid species may need further investigation for their association with AD.

When investigating plasma PLTP activity, only a sub-set of the cohort was assessed. This analysis should be extended to include the whole cohort to improve the power of the study.

6.5 Conclusion

The results presented in this thesis indicate that levels of several plasma lipids significantly differ between healthy control, MCI and AD individuals and that further investigation using complimentary lipidomic analytical approaches may reveal highly sensitive and specific blood biomarkers for predicting AD. The analytical platform chosen for this study was based on simultaneously identifying a broad range of lipids (189 species) in a large cohort ($n > 1000$). Several lipid species were identified in both the baseline and 18 month samples as being different between clinical classifications and a panel of lipids was identified at each time point that was able to distinguish participants with AD from healthy controls with a sensitivity and specificity of 80%. While this is extremely useful in identifying lipids that differ with clinical classification and providing information that can contribute to an understanding of altered lipid metabolism in AD, the lipid panels as they stand cannot be used in a routine setting for the diagnosis of AD. However several specific lipid species, in particular ceramides and sphingomyelins showed great potential as blood AD biomarkers; therefore further, more targeted lipidomic studies are recommended. In addition the results from the measurement of plasma PLTP activity revealed some important associations with other AD biomarkers which warrant further investigation.

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