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Alzheimer's disease biomarkers discovery using metabolomics approach

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Alzheimer's disease biomarkers discovery using metabolomics approach

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Abstract

Alzheimer's disease (AD) is a major debilitating disease with both cognition and independence gradually lost over time. AD can be diagnosed post-mortem when the accumulation of amyloid- β ($A\beta$) peptides and tau proteins in the brain are visible. The best diagnostic markers at present are cerebrospinal fluid (CSF) $A\beta$ and tau levels. However, the diagnostic accuracies of CSF markers are limited and all clinical trials targeting $A\beta$ and tau pathologies have failed to show promising results. Therefore, there is an urgent need for new molecular leads associated with AD which can potentially be utilised as disease-modifying therapy agents and as AD biomarkers. This thesis investigates the possibility of using metabolomics to discover AD associated metabolites.

Initially in chapter two, CSF samples (20 AD, 20 age-matched healthy controls) were analysed by Nuclear Magnetic Resonance (NMR). Results showed that metabolic fingerprints were non-differentiable between the two cohorts. One metabolite was found decreased, pyruvate, suggesting energetic hypometabolism in AD ($p < 0.05$). Acetate levels were increased in the AD group ($p < 0.05$), suggesting acetate being used as an alternative energy source to pyruvate.

In the next chapter, in order to scan for more metabolites, Liquid Chromatography-Mass Spectrometry (LC- MS) was applied on the same cohorts. The experiments allowed for the detection of 4426 metabolic features. Although the fingerprints were non-differentiable between the cohorts, 2 unidentified metabolic features were found to

be able to discriminate AD from age-matched controls with a Receiver Operating Characteristic (ROC) Area Under the Curve (AUC) value of 70%.

After this, work focused in blood metabolites because they are easily accessible. In chapter four we wanted to test whether lipids that had been previously implicated with AD would reproduce with similar trends. Semi-targeted analysis of 9 lipids was carried out (3 phosphatidylcholines (PC) and 6 ceramides) in AD (n=205) and Controls (n=207). Elevated levels of three ceramides and diminished levels of 2 PC molecules were found in AD blood ($p < 0.05$), one PC associated with hippocampal atrophy.

In the following chapter, these 9 lipids along with cholesterol and absolute cholesteryl esters levels were measured on pre-conversion serum samples (112 eventual AD participants and 113 Control participants at 3 time-points). At timepoints 1 and 2 (pre-clinical stage), no lipids showed significant differences between the pre-converters and the stable cohorts. One PC and total cholesteryl ester levels were diminished at the symptomatic stage, time point 3 ($p < 0.01$). When using lipidomics, the whole fingerprints and 1207 metabolic features, one feature (annotated as a PC) was found to be significantly different at symptomatic stage ($q < 0.05$).

Finally in chapter six, untargeted lipidomic analysis was performed on 148 AD and 152 control plasma samples in search of AD blood biomarkers. Application of random forest showed a combination of 24 lipid features consisting of cholesteryl ester, triglycerides and phosphatidylcholines being able to discriminate the cohorts with AD classification accuracy of $> 70\%$. In addition, other lipid signatures were found to predict disease progression ($R^2 = 0.10$) and brain atrophy in all brain regions except for left entorhinal cortex ($R^2 > 0.14$).

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Abbreviations

AA	Arachidonic Acid
ABC	Adenosine Triphosphate Binding Cassette
ACAT	Acyl-Cholesterol-Acyltransferase
acc	Accuracy
AD	Alzheimer's Disease
ADAD	Autosomal Dominant Alzheimer's Disease
ADAS-Cog	Alzheimer's Disease Assessment Scale-Cognitive
ADRDA	Alzheimer's Disease and Related Disorders Association
ALS	Amyotrophic Lateral Sclerosis
aMCI	amnesic Mild Cognitive Impairment
ANM	AddNeuroMed
APCI	Atmospheric Pressure Chemical Ionisation
ApoE	Apolipoprotein
APP	Amyloid Precursor Protein
APPI	Atmospheric Pressure Photo Ionisation
AUC	Area Under Curve
A β	Amyloid- β
BACE	β -secretase
BBB	Blood Brain Barrier
BEHAVE-AD	Behavioral Pathology in Alzheimer's Disease Rating Scale
BLSA	Baltimore Longitudinal Study of Aging
CBD	Corticobasal Degeneration
Cer	Ceramide
CERAD	Consortium to Establish a Registry for Alzheimer's Disease
ChoE	Cholesteryl Ester
ChoEI	Acetyl-Cholinesterase Inhibitor
CID	Collision Induced Dissociation
CLV	California Verbal Learning
CN	Cognitively Normal
CRP	C-Reactive Protein
CSF	Cerebrospinal Fluid
CT	Computed Tomography
CV	Coefficient Variation

CV-ANOVA	Cross-Validated Analysis of Variance
CVD	Cardiovascular Disease
CVLfrl	California Verbal Learning Free Recall Long-delay
CVLtca	California Verbal Learning Total Correct
DCR	Dementia Case Register
DESI	Desorption Electrospray Ionisation
DHA	Docosahexaenoic acid
DMT	Disease Modifying Therapy
DP	Dementia Pugilistica
DSM-III-R	Diagnostic and Statistical Manual of Mental Disorders, Third Edition
EDTA	Ethylenediaminetetraacetic
EESI	Extractive Electrospray Ionisation
EIC	Extracted Ion Chromatogram
EMA	European Medicines Agency
EOAD	Early-Onset Alzheimer's Disease
EPA	Eicosapentaenoic Acid
ER	Endoplasmic Reticulum
ER thickness	Entorhinal Cortex thickness
ERC	Entorhinal Cortex
ERC_L	Entorhinal Cortex Left
ERC_R	Entorhinal Cortex Right
ESI	Electrospray Ionisation
FA	Fatty Acid
FDA	Food and Drug Administration
FDG	Fluorodeoxyglucose
FDR	False Discovery Rate
FT-ICR-MS	Fourier Transform-Ion Cyclotron Resonance-Mass Spectrometry
FTD	Frontotemporal Dementia
GLM	Generalized Linear Model
GSK	Glycogen Synthase Kinase
HDL	High-Density Lipoprotein
HDL-c	High-Density Lipoprotein-Cholesterol
HILIC	Hydrophilic Interaction Liquid Chromatography
HDL-c	Low-Density Lipoprotein-Cholesterol
HMDB	Human Metabolome Database
KNN	K-Nearest Neighbours
LBD	Lewy Body Disease
LC-MS	Liquid Chromatography-Mass Spectroscopy
LCAT	Lecithin Cholesterol Acyl-Transferase
LCECA	Liquid Chromatography Electrochemistry Array
LCT	Low-Chain Triglyceride
LDL	Low-Density Lipoprotein
LOAD	Late-Onset Alzheimer's Disease

LOQ	Limit of Quantification
LysoPC	Lysophosphatidylcholine
m-In	Myo-Inositol
m/z	Mass Charge Ration
MAM	Mitochondrial-Associated ER Membranes
MAP	Microtubule Associated Protein
MCAT	Monocarboxylic Acid Transporter
MCI	Mild Cognitive Impairment
MCT	Medium-Chain Triglyceride
mDNA	Mitochondrial DNA
METLIN	Metabolite Link Database
MMSE	Mini-Mental State Examination
MoCA	Montreal Cognitive Assessment
MRI	Magnetic Resonance Imaging
MRS	Magnetic Resonance Spectroscopy
MS	Mass Spectrometry
MTBE	Methyl Tert-Butyl Ether
N-SMase	Neutral-Sphingomyelinase
NAA	N-Acetylasparate
NFD	Neurofibrillary Tangles
NIA	National Institute of Aging
NIH	National Institute of Health
NIH-AA	National Institute of Aging-Alzheimer's Association
	National Institute of Neurological and Communicative Disorders and
NINCDS	Stroke
NMDA	N-Methyl-D-Aspartate
NMR	Nuclear Magnetic Resonance
NP	Normal Phase
NPI	Neuropsychiatric Inventory
OHdG	Hydroxydeoxyguanosine
OPLS-DA	Orthogonal Partial Least Square-Discriminant Analysis
PC	Phosphatidylcholine
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PD	Parkinson's' Disease
PE	Phosphatidylethanolamine
PET	Positron Emission Tomography
PHF	Paired Helical Filaments
PI	Phosphatidylinositol
PiB	Pittsburgh Compound B
PiD	Pick Disease
PLA2	Phospholipase A2
PP	Protein Phosphatase

PSEN	Presenilin
PUFA	Poly-Unsaturated Fatty Acid
QC	Quality Control
RF	Random Forest
rfe	Recursive Feature Elimination
RFR	Random Forest Regression
RMSE	Root Mean Square Error
RNA	Ribonucleic Acid
ROC	Receiver Operation Characteristic
ROD	Rate of Cognitive Decline
ROS	Reactive Oxygen Species
RP	Reverse Phase
Rt	Retention Time
SD	Standard Deviation
SMase	Sphingomyelinase
sMRI	Structural Magnetic Resonance Imaging
SPECT	Single Photon Emission Computed Tomography
T2D	Type 2 Diabetes
TCA	Tricarboxylic Acid
Tg	Transgenic
TG	Triglyceride
TOF	Time of Flight
TSP	Trimethylsilylpropanoic acid
UPLC	Ultra Performance Liquid Chromatography
US	United States
UV	Unit Variance
VAD	Vascular Dementia
VI	Variable Importance
VIP	Variance Importance Plot
VLCT	Very Low-Chain Triglyceride
VLDL	Very Low-Density Lipoprotein

Introduction

1.1. Alzheimer's disease and its prevalence

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized with memory impairment and personality behavior changes, caused by gradual brain cell apoptosis. It is the most common form of dementia accounting for between 50% and 70% of cases. Other dementias include Lewy body disease (LBD), frontotemporal dementia (FTD), vascular dementia (VAD) and Parkinsons' disease (PD). In some cases, patients can be diagnosed with a combination of two or more dementias.

AD can present in two forms, early-onset AD (EOAD) and late-onset AD (LOAD), this thesis will mainly focus on LOAD. Incidence of LOAD normally occurs to people age of 65 or older, as it has no clear familial pattern thus it is considered sporadic (sporadic AD). EOAD is where its incidence occurs before age 65 or less (mean age of 46 years old, but can appear as early as 30s) [1], it accounts for less than 1% of all AD cases. EOAD is largely hereditary and genetic mutation is the biggest risk factor. EOAD has association with these deterministic variations and it can be called autosomal dominant AD (ADAD, also termed familial AD).

Currently AD is the 6th leading cause of death, 3rd leading cause of death among people aged 65 or older in the United States (US) [2]. Despite this, there is currently no treatment for the disease. The estimated incidence of AD is only likely to increase due to forecast of a rapid increase in numbers and proportions of elders. Current estimates suggest AD incidence will increase from the current 5.4 million (2016) to 13.7 million (2050) in the US [3, 4].

AD is also one of the most costly conditions, contributed by a number of factors. The progress of AD is relatively very slow, people often live on average 4 to 6 years after diagnosis, some can live as long as 20 years. People with AD require either partial or full assistance, meaning the disease has a very high care cost. Also AD patients lose ability to take care of their other chronic health problems, such as diabetes or heart disease. All of these factors contribute in making AD a very expensive disease. In the

US, the disease was reported to cost \$226 billion in 2012, and this cost is predicted to increase to \$1.2 trillion by 2050 [3].

Despite these facts (summarized in **Figure 1.1**), the reality is that very little is understood about AD. The disease mechanism is not comprehensively understood, definite AD diagnosis can only be made on post-mortem brains, and there is no AD prevention or treatment to reverse the disorder. Currently, just a few drugs are available for AD patients and they are only able to slow the progression of symptoms. Therefore it is vitally important to determine how AD affects brains, and develop tools for AD diagnosis, treatment and prevention.

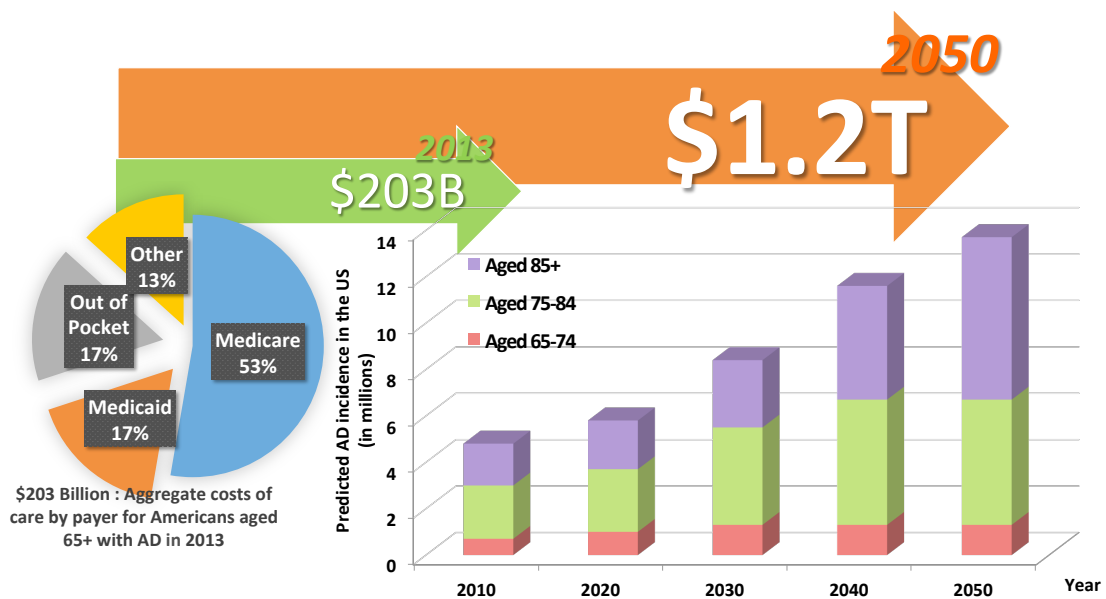


Figure 1.1. Alzheimer's projection on incidence cost of Alzheimer's care. Pie chart shows the breakdown of aggregate cost of care in the US. Data were drawn from [4-6].

1.2. Pathological hallmarks of AD

The first report on AD was in 1907 when Dr. Alois Alzheimer studied a woman with an unusual mental illness. Her symptoms included memory loss, communication problems and unpredictable behavior. After her death, he examined her brain and noticed brain shrinkage as well as abnormal ‘clumps’ and ‘tangled fibers’ deposition in and around the nerve cells. Later, these ‘clump’s and ‘tangled fibres’ were identified as neuritic plaques mainly consisting of amyloid- β ($A\beta$) peptides and intracellular neurofibrillary tangles of phosphorylated tau protein [3]. Many institutes define these features as two AD defining hallmarks and closely related with brain atrophy, which can be observed in AD brain (**Figure 1.2**).

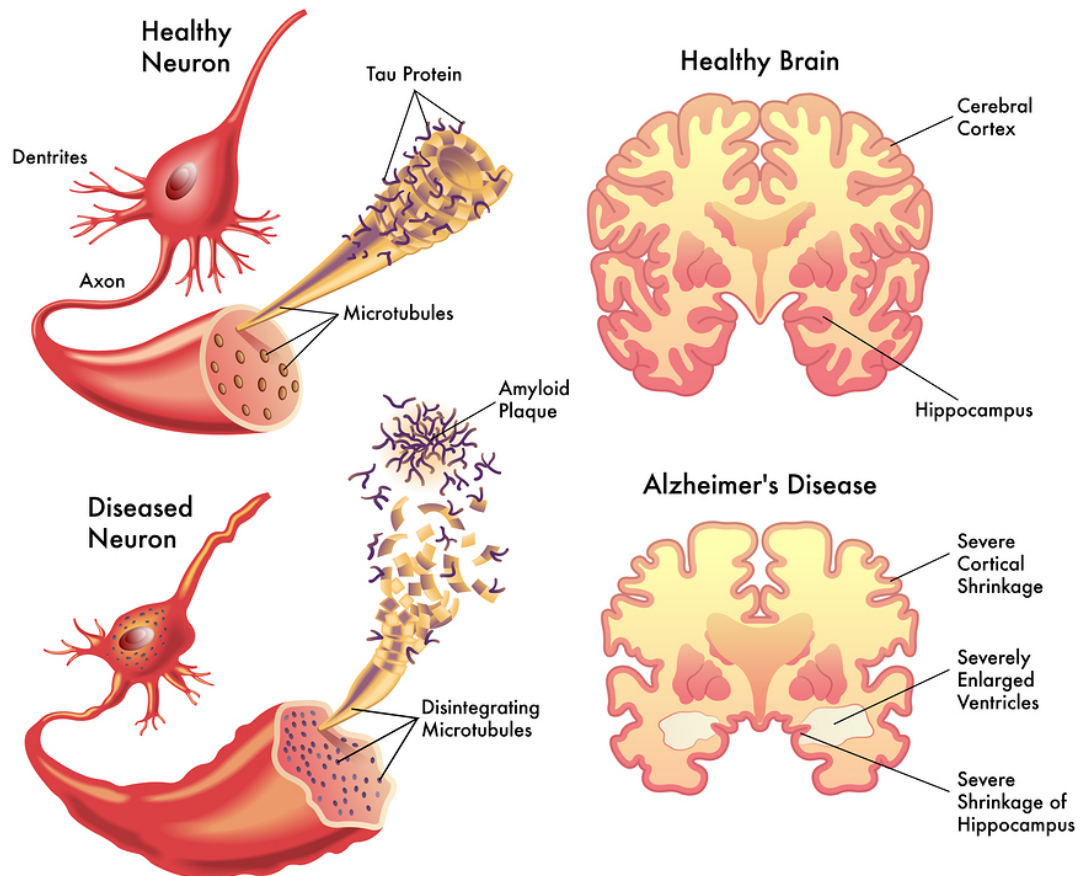


Figure 1.2. Normal (top) *vs.* AD (bottom) neuron and brain. In AD, a significant reduction in brain volume can be seen. Figure obtained from [7].

1.2.1. A β Plaques

One of the AD hallmarks is deposition of neurotic plaques, which are largely consisting of A β peptides, around nerve cells. A β peptide is 38-42 amino acids long and is formed when Amyloid Precursor Protein (APP), a cross-membrane protein, is cleaved in two successive steps, initially by β -secretase (BACE) [8], followed by γ -secretase [9]. First, β -secretase cleaves APP at the N-terminal end of the A β domain within APP, releasing sAPP β from the cell. The remaining fragment of APP, still tethered in the neuron's membrane, is then cleaved by γ -secretase at the C-terminal end of A β domain. Following the cleavages at both ends, A β is released into extracellular space. The cleavage processes in terms of visualisation can be seen in **Figure 1.3**. Freed A β peptides start to clump together to form A β oligomers in the extracellular space, and as further clumping (aggregation) continues, oligomers become insoluble entities, A β plaques.

During the second cleavage process by γ -secretase, two major isoforms of A β can be formed, one residue with 40 amino acids long (A β_{40}) and the other residue with 42 amino acids long (A β_{42}). A β_{42} is the lesser common isoform, but more susceptible to conformational changes leading to A β aggregation and accumulation [10]. The AD brain is known to produce higher proportion of A β_{42} , hence more susceptible to A β aggregation. It is unclear what triggers production of higher proportions of A β_{42} , reports suggest that accumulation of metal ions is an A β aggregation promoter [11, 12]. Also unclear is the role of A β_{42} , recent *in vitro* reports have suggested formation of plaques is a response of the immune system, to kill microbes in the brain [13, 14]. However it remains to be seen the role of plaques in study models involving normal infection.

One widely accepted role of A β_{42} is that its aggregated form (neuritic plaques) around neurons has an ability to induce neurotoxicity [15] and trigger synaptic degeneration [16], eventually leading to cell apoptosis. This hypothesis is often called amyloid cascade model. However its mechanistic pathways leading to neurodegeneration have

remained elusive and inconclusive [17, 18]. Recent reports have suggested that soluble oligomeric species, not insoluble aggregated species, to be the cytotoxic species [19-21]. Soluble oligomeric $A\beta$ species disrupt Ca^{2+} homeostasis in neurons and increase intracellular Ca^{2+} concentration, subsequently leading to neuronal apoptosis [22-24]. This hypothesis is often called “Ion Channel Hypothesis of AD”.

Mutations of three genes involved in the amyloid cascade model have strongly been linked with EOAD, and they are APP [25, 26], two γ -secretases, Presenilin-1 (PSEN1) and Presenilin-2 (PSEN2) [27]. Mutation of the APP gene is known to increase the production of APP by approximately 1.5 fold and subsequently leads to an increase in $A\beta$ production [28]. The two Presenilin proteins have been reported to increase the production of $A\beta_{42}$ which is more susceptible to $A\beta$ aggregation [29, 30].

Along with the formation of neurotic plaques in AD brain, a decrease in $A\beta_{42}$ concentration (to a half of the levels in controls) can also be observed in AD CSF [31-33]. Initially it was thought CSF $A\beta_{42}$ abnormality was caused by deposition of the plaques [31], but it was later found that the CSF $A\beta_{42}$ abnormality can develop before plaque formation in AD brain. Thus the $A\beta_{42}$ abnormality is often used as an AD predictor [33] while the ratio of $A\beta_{42}/A\beta_{40}$ slightly improves differentiation of AD from other dementias [34]

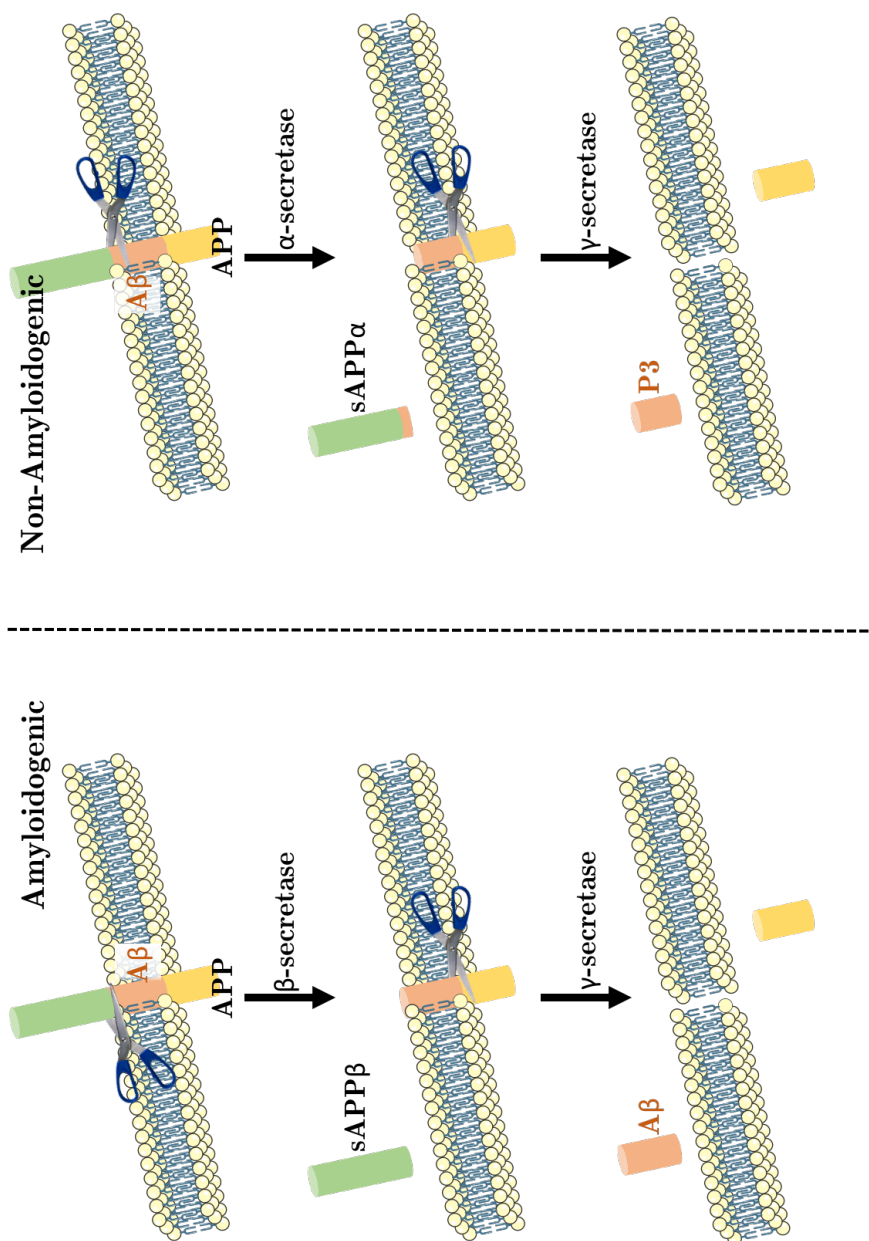


Figure 1.3. Amyloid Cascade model of Amyloid Precursor Protein Metabolism. Amyloidogenic pathway (left hand side) involves production of insoluble A β from APP by two successive cleavage steps, β -secretase followed by γ -secretase. Non-amyloidogenic pathway (right-hand side) involves production of P3 from APP by actions of α -secretase and by γ -secretase.

1.2.2. Neurofibrillary Tangles (NFT)

Tau pathology involving intracellular neurofibrillary tangles of hyper-phosphorylated tau protein is another hallmark of AD, but this pathology can also be observed in other neurodegenerative disease such as fronto-temporal dementia (FTD), Pick disease (PiD), corticobasal degeneration (CBD) and dementia pugilistica (DP)[35].

Tau is a major microtubule associated protein (MAP) and can be found predominantly in neurons. The protein can be present in 6 isoforms, these isoforms differ according to the contents of three or four tubulin binding domains (3R and 4R respectively) in the C-terminal and one (1N), two (2N), or no inserts (0N) of 29 amino acids each in the N-terminal portion of the molecule [36]. Therefore the 6 tau isoforms can be present in 0N3R, 1N3R, 2N3R, 0N4R, 1N4R or 2N4R (number of amino acids are 352, 381, 410, 383, 412, and 441 respectively). Tau's role involves promotion of axonal microtubules assembly and stabilization of their structure (**Figure 1.4**), this bioactivity being regulated by degree of phosphorylation present on tau [37-39]. Tau has 85 phosphorylation sites (80 serine or threonine and 5 tyrosine) [40], approximately 30 out of 85 being phosphorylated in a normal tau [41].

In AD brain, the phosphorylation level increases abnormally in all six isoforms of tau, which could be due to a decrease in dephosphorylation activity (via decreased phosphatase-2A activity) [42] or/and the presence of oligomeric A β species [43] which have been suggested as tau hyperphosphorylation promoters. The resulting hyperphosphorylated tau sequesters normal tau, and other MAPs (MAP1 and MAP2) from pre-assembled microtubules, consequently leading to breakdown and destabilization of microtubule networks and neuronal degeneration via axon deficits [39, 44, 45]. Additionally hyperphosphorylated tau has a tighter and more folded conformation, and this abnormal folding leads to co-aggregation between tau proteins and consequently into what is known as intracellular neurofibrillary tangles (NFT) of paired helical filaments (PHF) [35, 46]. NFT can block the intracellular trafficking of

the neurotrophins and other functional proteins and cause deficits of axonal and dendritic transport in the neurons [47].

Increased levels of total- and phospho- tau (2-3 fold increase) have also been reported in AD CSF [48-52], their levels also correlating with NFT in AD brain [53, 54]. The increase in CSF tau levels is believed to be due to cortical tangle formation and/or a result of cortical neuronal loss [55-60]. However, it is important to note that the formation of NFT in brain [35] and relatively high total and phosphorylated tau levels in CSF [61, 62] have been observed in other dementias. Therefore the tau pathology is associated with general neurodegeneration, and lacks AD specificity.

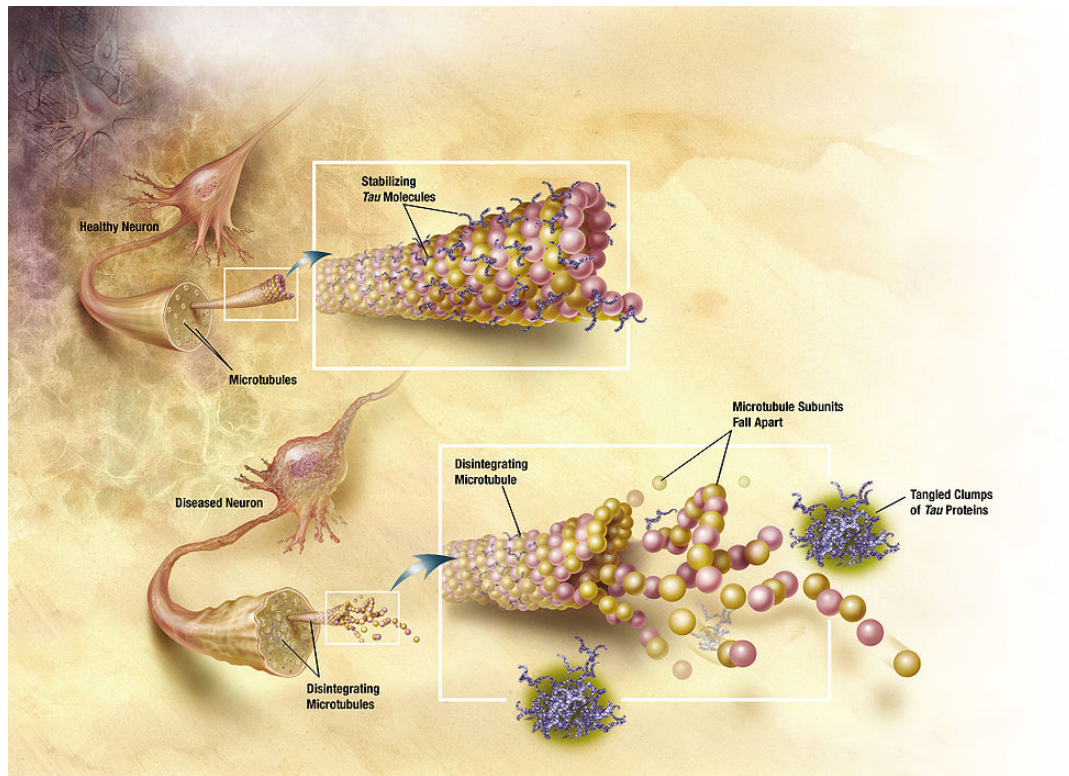


Figure 1.4. Microtubules act as information carriers in axon and dendrites. Tau proteins stabilize the structure of the microtubules via their tubulin binding domains in the C-terminal. In AD, tau is abnormally phosphorylated, sequestering normal tau from microtubules leading to destabilization of microtubules, and undergoes conformation changes resulting neurofibrillary tangles

Image obtained from National Institute on Aging/National Institute of Health.

1.2.3. Oxidative Stress & Mitochondrial Dysfunction

Presence of extensive oxidative stress is another characteristic of AD brains in addition to neuritic plaques and neurofibrillary tangles pathologies. Various markers of oxidative stress in AD brains have been reported and they include elevated levels of protein carbonyls and 3-nitrotyrosine (byproducts of protein oxidation) [63, 64], and 8-hydroxydeoxyguanosine (8-OHdG) and 8-hydroxyguanosine (markers of oxidative damage to DNA and RNA) [65, 66]. Lipid peroxidation has also been reported such as increased levels of malondialdehyde, 4-hydroxynonenal and F2-isoprostanes have been found in multiple brain regions [67-70]. These abnormalities are more localized in the synapses, suggesting a strong association between oxidative stress and synaptic loss in AD brain [71].

While it is clear oxidative stress is an important factor in AD pathogenesis, it remain unclear what initiates oxidative stress (or production of reactive oxygen species, ROS) and the mechanisms by which the redox is imbalanced. Numerous studies have shown accumulation of A β to be a triggering the production of hydrogen peroxide (H₂O₂), an oxidative stress marker [72-75]. Mechanism behind this theory is still an active area, but it is thought that A β deposits lead to mitochondrial dysfunction by inhibiting cytochrome oxidase activity, leading to disruption of the electron transport chain and increased production of ROS [76, 77]. Further, A β has been found in the mitochondria [77, 78] and disrupts its membrane permeability, consequently releasing cytochrome c, an intermediate in cell apoptosis [79, 80].

Pathology of tau hyperphosphorylation has also been frequently linked with oxidative stress [81-84]. Tau phosphorylation is regulated by tau protein kinase and phosphatase. Preliminary studies involving treatment with ROS have found increased tau hyperphosphorylation via upregulated activity of a tau kinase, glycogen synthase kinase-3 (GSK-3 β) [85] and downregulated activity of a phosphatase, protein phosphatase 2A (PP2A) [86]. However, the relationship between tau pathology and oxidative stress whether its via GSK3 and/or PPA2 remains to be further investigated.

A close relationship between oxidative stress and aberrant homeostasis of three transitional metal ions, Cu^{2+} , Zn^{2+} and Fe^{3+} has also recently been suggested to occur in AD brain [87-90]. Cu^{2+} and Zn^{2+} ions are normally involved in inhibition of N-methyl-D-aspartate (NMDA) receptors limiting Ca^{2+} entry into neurons. Fe^{3+} is involved in myelination, synaptogenesis and synaptic plasticity. In AD brain, aberrant accumulation of these three ions has been detected within $\text{A}\beta$ deposits suggesting their interaction with $\text{A}\beta$ [91, 92]. Cu^{2+} , Zn^{2+} have abilities to bind to histidine and tyrosine residues in $\text{A}\beta$, promoting aggregation of the peptide [11, 12, 93, 94]. Furthermore, these aberrant interactions between ions and $\text{A}\beta$ could be a source of ROS production. When Cu^{2+} binds with $\text{A}\beta$, $\text{A}\beta$ transfers an electron to the ion, reducing Cu^{2+} to Cu^{+} , and this Cu^{+} - $\text{A}\beta^{+}$ complex then donates electrons to O_2 to generate H_2O_2 , a ROS species [11, 95]. The binding affinity to Cu^{2+} is higher with $\text{A}\beta_{42}$ compared to $\text{A}\beta_{40}$, thus correlating with their cytotoxic activity seen in AD [96]. In a similar fashion to Cu^{2+} , the Fe^{3+} ion can also help generating H_2O_2 in its reduced form of Fe^{2+} [12]. These findings suggest transitional metal ions play vital roles in AD neuropathological changes by promoting $\text{A}\beta$ aggregation and generating ROS.

Aging itself is also another factor, maybe the greatest risk factor involved in neurodegenerative diseases, and mitochondria have been thought to play a vital role in it [97, 98]. During aging, the level of mitochondrial DNA (mtDNA) mutation is known to increase and this increase is strongly associated with mitochondrial dysfunction [99, 100]. Mitochondria's normal role includes regulating cell survival and death by balancing ROS metabolism. Dysfunction in mitochondria can attribute accumulation of ROS species which are certainly toxic and damage a variety of cellular component [101, 102].

Dysfunction in mitochondria can also contribute to impairment in glucose metabolism [103]. In the brain, the energy necessary to drive most cellular reactions is derived mostly from phosphorylation of ATP, and ATP is mostly produced in the mitochondria

via the oxidation of glucose. Faulty mitochondria therefore leads to reduced glucose metabolism (glucose hypometabolism) and studies looking at AD brain have been able to observe such abnormalities [[104-106](#)]. These studies were also able to report glucose hypometabolism in temporal cortex and posterior cingulate cortex to be AD characteristic [[104-106](#)], some even suggesting hypometabolism to be occurring before the onset of clinical symptoms [[104](#), [107](#), [108](#)]

This evidence demonstrates that oxidative stress is inextricably associated with several neuropathological processes in AD including $A\beta$ pathology, tau pathology, metal dyshomeostasis and mitochondria dysfunction.

1.2.3. Lipid Metabolism

Implication of altered lipid metabolisms in AD pathogenesis was first suggested in 1994 [109], but this hypothesis has been gaining acceptability only in recent years [110, 111] after discovery of $\epsilon 4$ allele of the apolipoprotein E (ApoE) gene being the strongest AD genetic risk factor [112, 113]. Implication of lipids in AD is perhaps logical considering all enzymes involved in amyloidogenic pathway (APP, β -secretase and γ -secretase) are transmembrane proteins. Perturbed lipid bilayer composition and organization will impact on trafficking and/or proteolytic activity of these transmembrane proteins and thus APP processing and A β production [114].

Of all lipid classes, high cholesterol levels in brain (cholesterol homeostasis) has been implicated with AD the most. Cholesterol has an essential role in the brain, helping to maintain neuronal functions such as neurotransmitter release and synaptic plasticity [115, 116]. However when cholesterol is in excess in the brain (*de novo*), it is esterified into cholesteryl esters by acyl-cholesterol-acyltransferase-1 (ACAT1), and accumulation of this intracellular cholesteryl ester can enhance A β release [117, 118]. Supporting this hypothesis, pharmacological inhibition of ACAT1 has been found to reduce both A β production and cholesteryl esters [119, 120]. Additionally, cholesterol is known to modulate β -secretase and γ -secretase activities directly, with a rich environment of cholesterol having been found to accelerate A β deposition [121-123].

Supporting these biochemical studies are case-control studies where statins, cholesterol-lowering drugs, have been found associating with lower risk of AD development [124, 125]. Statin has also shown to promote ectodomain (domain of a membrane protein that extends into extracellular space) shedding of APP through the α -secretase pathway (non-amyloidogenic pathway), inhibiting A β production in a cholesterol-independent manner [126]. However retrospective clinical trials involving prospective cohort have shown conflicting results, Wolozin *et al.* reporting lower incidence of the dementia [125] while Arvanitakis *et al.* showing no relationship between statins medication and AD incidence, cognitive decline and AD pathologies [127]. Additionally,

epidemiological studies testing associations between low-density lipoprotein (LDL) cholesterol (often referred as ‘bad’ cholesterol) and AD have also produced mixed results. While increased levels of total cholesterol levels has been found in post-mortem AD brains [128] and associated with higher AD risk [129, 130], no relationship [131] or opposite relationship [132-134] have also been reported.

Conflicting epidemiological evidence could have risen because of other factors such as cholesterol transportation between extracerebral (peripheral) and cerebral (*de novo*). Such a system is mediated by cholesterol acceptors (apolipoproteins), in brain ApoE is the predominant apolipoprotein [135]. Interestingly, ApoE is also involved in A β clearance [136]. The activity of ApoE is greatly dependent on its lipidation level, regulated by adenosine triphosphate binding cassette A1 (ABCA1). Immunochemical studies have demonstrated that the lack of ABCA1 results in deficient lipidation of ApoE and consequently leads to accumulation of amyloid plaques burden, indicating poorly lipidated ApoE to be associated with amyloidogenic pathway [137-139]. Additionally the effect of ApoE seems to be isoform-dependent, $\epsilon 4$ allele of ApoE results in the highest A β deposition ($\epsilon 4 > \epsilon 3 > \epsilon 2$) [140-142]. However the mechanism by which ApoE alleles differentially modulate the amyloidogenic pathway is less clear.

In addition to cholesterol, sphingolipids have been found to play a role in A β production. Sphingolipids include ceramide, sphingomyeline and glycosphingolipids, and are believed to play important roles in signal transmission and cell recognition [143, 144]. Ceramide, a central component in sphingolipid metabolism, has been suggested to facilitate the regulation of BACE-mediated processing of APP by prolonging the half-life of BACE [145]. Furthermore, these lipid species have been found to mediate oxidative stress-induced neuronal apoptosis, independently of its role in BACE activity regulation [146-149]. They have shown to activate enzymes involved in cell apoptosis ranging from protein kinase [150], cathepsin D [151] and serine/threonine protein phosphatase [152]. These findings are supported by retrospective epidemiologic studies where high ceramide levels have been found in AD blood [153], CSF [154] and brain

[155], while higher serum ceramide levels have also been associated with AD incidence [156]. These findings suggest potential of ceramide-based therapeutic treatments for delaying AD development. Inhibition of sphingomyelinase (SMase), a catalyst involved in sphingomyeline to ceramide conversion, has been suggested as a therapeutic agent. In addition neutral SMase (N-SMase) knockout mice has shown lower activation of pro-inflammatory enzymes and subsequently greater protection of neurons [157]. Dinkins *et al.* have also demonstrated that by inhibiting N-SMase activity they observed lower brain ceramide levels and A β ₄₂ burden [158].

Phospholipids are another family of lipid species to be associated with AD along with enzymes involved in phospholipid metabolism. A number of case-control studies have reported abnormal phospholipids levels in AD individuals, namely, lower levels for phosphatidylinositol (PI) [159-161], for phosphatidylethanolamine (PE) [159, 162, 163] and for phosphatidylcholine (PC) [159, 162-164] in AD brain and in AD blood [165, 166] while they were found increased in AD CSF [167, 168]. Phospholipids are a major component of the cell membrane and play important roles in cellular functions such as extracellular receptor signalling, intercellular second messengers and cellular pressure regulation [169]. However it is unclear how AD is associated with phospholipids and this is a subject that will be discussed in detail in the discussion sections of this thesis.

1.3. AD Clinical Symptoms

The most common first symptom of AD is memory impairment. Individuals who develop relatively worse memory will be diagnosed with a condition called ‘mild cognitive impairment’ (MCI). Over time, those with MCI can stay as stable MCI or even go back to normal cognition. However most will undergo further deterioration in memory and deficits in other cognitive domains such as executive dysfunction and visuospatial impairment, deficits in language and behavioural symptoms, which can be diagnosed as AD. The patterns (as well as their severity) at which these symptoms appear are often used to classify three different AD severity stages, mild AD, moderate AD, and severe AD [6].

1.3.1. Memory Impairment in AD

One of the earliest AD symptoms is impairment in episodic memory, memory of specific events occurring at a particular time and places. This is also the most central clinical feature of AD. This type of memory is served by the medial temporal lobe which includes the hippocampus and entorhinal cortex [170], the first brain structures damaged in AD [171]. Impairment in semantic memory, memory for facts such as vocabulary and concepts, often follows. This type of memory is governed by temporal lobes, particularly in the anterior temporal lobe [172]. Procedural memory (or motor learning memory) often becomes impaired during the latter stage of AD. It is a part of long-term memory, enabling learning skills that will become automatic and aiding the performance of particular types of tasks without conscious awareness, examples include typing and skiing. This memory is governed by prefrontal cortex, parietal cortex and cerebellum.

1.3.2. Executive dysfunction and Behavioural symptoms

As AD progresses, impairment in executive function begins to appear (early/ mild stages of AD). At this point, individuals are often diagnosed with AD. Executive function is controlled by frontal lobes and encompasses a number of cognitive abilities such as problem solving, organisation, initiation and decision-making [173, 174]. Although initially AD individuals will be able to carry out daily tasks, these tasks become harder to complete.

Neuropsychiatric (behavioural and psychological) symptoms often appear during moderate AD stage. These particular symptoms begin with relatively subtle symptoms including apathy, social disengagement, and irritability. In severe AD, other behavioural symptoms appear including agitation, aggression and psychosis (hallucinations, delusions). Also the severity of other cognitive functions reach their peak at this stage and individuals will be completely dependent on others.

1.3.3. Other symptoms

Olfactory dysfunction, namely odour discrimination and identification, is an inevitable consequence of the natural aging process, but it has a considerable prevalence in age-related dementias, with numbers estimated as high as 100% in AD [175], 90% in PD [176] and 96% in FTD [177]. It is related with the underlying neuropathological features of each dementia, and thus different profiles between AD and other dementias have been observed [178]. For example, severity has been found higher in AD individuals when compared to vascular dementia (VAD) individuals with similar cognitive impairment levels [178].

Sleep disturbances are common in individuals in mild and moderate AD stages, affecting 25~40% of the cases [179]. Such symptoms include insomnia at night, agitated behaviour at sunset and excessive sleeping during the daytime. In mouse and human based cross-sectional studies, brain A β level has been associated with severity in sleep disturbance [180-182]. Prospective studies have shown that such symptoms actually

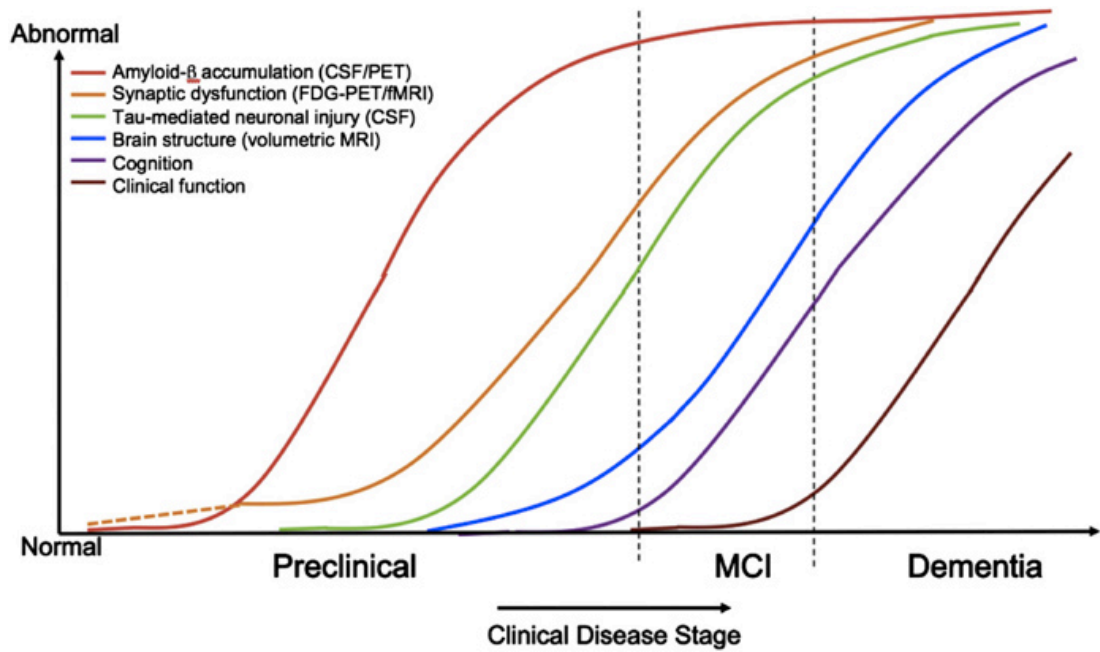
begin to occur in a pre-clinical stage of AD (before onset of cognitive impairment) and suggested poor quality of sleep may even be a risk factor for cognitive impairment [\[183, 184\]](#) and AD [\[185\]](#).

Occurring in 10~22% of AD cases, seizure can occasionally be presented, especially those in the later stages of AD [\[186, 187\]](#). Those with more severe AD symptoms and/or have autosomal dominant forms of AD (those with EOAD) are under higher risk of seizures [\[188\]](#).

1.4. Criteria and Guidelines for AD Diagnosis

The guideline for AD clinical diagnosis was first established in 1984 by National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA) [189]. The guideline was commonly referred to as the NINCDS-ADRDA criteria and diagnosis was based on symptoms, patient history, neuropsychological examinations and mental status evaluation. Histopathology examination would confirm the AD diagnosis. However it was later discovered that pathological changes occur many years (up to 20 years) before the first onset of clinical symptoms, and clinical dementia is just the end product of accumulated pathology [190, 191]. These pathological changes included abnormal levels of $A\beta_{42}$ and tau (total and phosphorylated-) in brain and cerebrospinal fluid (CSF), as well as brain atrophy (**Figure 1.5**). Therefore a new criteria was needed to address this pre-clinical stage as earlier AD detection can lead to earlier treatment of the disease and improve long-term quality for the AD individuals.

In 2011, newer criteria were established by the National Institute of Aging in collaboration with Alzheimer's Association (NIH-AA) [192]. This new criteria improved in 2 major ways; expansion of AD into 3 phases, and incorporation of biomarkers to provide information regarding pathophysiologic changes underline the disease state. Definition of biomarker and its importance will be discussed later.



Abbreviations: CSF, cerebrospinal fluid; PET, positron emission tomography; FDG-PET, fludeoxyglucose (F18) – PET; MRI, magnetic resonance imaging

Figure 1.5. Hypothetical model of biomarkers of the AD expanded to explicate the preclinical phase. The model shows pathological changes occurring well-before onset of clinical symptoms. Figure obtained from [193].

The 3 phases included 1) an asymptomatic, preclinical phase; 2) a symptomatic, pre-dementia phase; and 3) a dementia phase. The first phase, preclinical phase, is where pathological changes (abnormal $A\beta$ and tau levels) are detectable, yet individuals are asymptomatic or have a subtle symptoms. The pathological changes included decreased $A\beta_{42}$ levels and increased total and phosphorylated- tau proteins in CSF, and these are regarded as three core AD CSF biomarkers. However their uses are only recommended for use in research, in the context of clinical trials for early intervention strategies and longitudinal study of people at risk [194].

The second phase is also known as MCI phase and is when individuals exhibit mild decline in memory, attention, and thinking, but still able to carry out everyday tasks. There are four subtypes of MCI [195, 196]. “Amnestic MCI” is where memory impairment is primarily affected while “Non-amnestic MCI” is where cognitive domains but not memory are affected, and is less frequently associated with AD development. “Single-domain MCI” is where memory or one other cognitive domain is impaired while

“Multi-domain MCI” is where impairments in memory plus one or more other cognitive domains are present [195]. Amnesic, Single- or Multiple- domain MCI frequently represents an early stage of AD, while non-amnesic MCI is more related with other neurodegenerative diseases [196].

The third phase is the dementia phase where cognitive or behavioural problems develop to an extent that everyday activities and functioning are affected. The clinical criteria for this dementia phase are similar to those from 1984 and are sufficiently broad and flexible enough to be used as diagnostic tool without need of neuropsychological tastings and laboratory examinations. Individuals in this phase can be diagnosed either ‘Probable AD’ or ‘Possible AD’ depending on the level of diagnostic certainty. Probable AD is where other possible sources of cognitive impairments are ruled out and AD is the most likely cause of the dementia. Possible AD is where cognitive impairments are believed to be AD related, but also contributed by other dementias. More detailed guidelines can be found in [192].

1.5. AD Evaluation

The current diagnosis approaches mainly depend in clinical examination, neuropsychological assessment that may provide confirmatory information and aid in patient management, together with laboratory investigations that usually allow for exclusion of other diagnosis.

1.5.1. Clinical Assessment

Detailed clinical evaluation often provides reasonable diagnostic accuracy for AD, although its specificity can be limited because of other neurodegenerative causes [192]. The clinical criteria for AD are based on history of insidious onset and its progression, exclusion of other etiologies, and documentation of cognitive impairments in one or more domains.

Many clinicians measure severity and progression of cognitive impairments by a battery mental test. One of the most commonly used tests has been the mini-mental state examination (MMSE) [197, 198]. It is a short screening tool (30 point questionnaire, cut-off score for normal performance is 24) which is ideal for providing an overall measure of cognitive impairment. A meta-analysis looking at 108 studies has found MMSE having a diagnostic accuracy (sensitivity 81%, specificity 89%) [199]. However copyright issue has hindered its uses and many clinicians have now switched to other tests, including the Mini-Cog [200] and Montreal Cognitive Assessment (MoCA) [201]. Mini-Cog is a much shorter testing tool (<5 minutes), but still gives as high diagnostic accuracy as the MMSE [199]. MoCA is similar to MMSE, it is a 30-point screening tool where the typical cut-off score for normal performance is 26. While MMSE is better at differentiating cognitive impairment and cognitive normal at severe stages, MoCA is more sensitive in discriminating between the two groups at mild impairment stage [201-203]. A version of MoCA can be seen in **Figure 1.6**.

MONTREAL COGNITIVE ASSESSMENT (MOCA) Version 7.1 Original Version						NAME : Education : Sex :		Date of birth : DATE :		
VISUOSPATIAL / EXECUTIVE						Copy cube		Draw CLOCK (Ten past eleven) (3 points)		POINTS
										___/5
NAMING						Contour		Numbers		Hands
										___/3
MEMORY						Read list of words, subject must repeat them. Do 2 trials, even if 1st trial is successful. Do a recall after 5 minutes.				No points
						FACE		VELVET		
						1st trial				
						2nd trial				
ATTENTION						Read list of digits (1 digit/ sec.).		Subject has to repeat them in the forward order		___/2
								[] 2 1 8 5 4		
								Subject has to repeat them in the backward order		
								[] 7 4 2		
						Read list of letters. The subject must tap with his hand at each letter A. No points if ≥ 2 errors				___/1
								[] FBACMNAAJKLBAFAKDEAAAJAMOFAB		
						Serial 7 subtraction starting at 100		[] 93 [] 86 [] 79 [] 72 [] 65		___/3
								4 or 5 correct subtractions: 3 pts , 2 or 3 correct: 2 pts , 1 correct: 1 pt , 0 correct: 0 pt		
LANGUAGE						Repeat : I only know that John is the one to help today. []				___/2
								The cat always hid under the couch when dogs were in the room. []		
						Fluency / Name maximum number of words in one minute that begin with the letter F		[] _____ (N ≥ 11 words)		___/1
ABSTRACTION						Similarity between e.g. banana - orange = fruit		[] train - bicycle [] watch - ruler		___/2
DELAYED RECALL						Has to recall words		FACE		
						WITH NO CUE		[]		
								VELVET		
								[]		
								CHURCH		
								[]		
								DAISY		
								[]		
								RED		
								[]		
Optional						Category cue				
						Multiple choice cue				
ORIENTATION						[] Date		[] Month		
								[] Year		
								[] Day		
								[] Place		
								[] City		___/6
© Z.Nasreddine MD						www.mocatest.org		Normal $\geq 26 / 30$		TOTAL
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										Add 1 point if ≤ 12 yr edu

Figure 1.6. MoCA version 7.1.

1.5.2. Neuropsychological Testing

Neuropsychological examination measures a psychological function known to be linked to a particular brain structure or pathway. Examination involves an interview and a battery of tests, testing cognitive ability which includes memory, attention, processing speed, reasoning, judgement, problem solving and language function. Tests used for AD assessment include ADAS-Cog (AD Assessment Scale-Cognitive), Blessed Test, Clock Drawing Test, NPI (Neuropsychiatric Inventory) and BEHAVE-AD (Behavioral Pathology in Alzheimer's Disease Rating Scale) [\[204\]](#).

From the battery of tests, neuropsychologists can evaluate strengths and weakness among each cognitive and behavioural function and use them to;

1. Assist differentiating between different forms of neurodegenerative dementias or other etiologies of cognitive impairments such as depression, eventually leading to more accurate treatment.
2. Provide a baseline in order to follow individuals over time, and to help understand the progression of the disease.
3. Reveal daily functioning areas such as financial management and driving, which may require assistance or supervision.

1.5.3. Neuroimaging

One of the major AD hallmarks is brain atrophy, the hippocampus and entorhinal cortex being the first brain regions to be affected. A brain imaging technique, structural Magnetic Resonance Imaging (sMRI), can detect both generalized and focal atrophy of brain, as well as white matter lesions. The technique can allow documentation of potential alternative or additional diagnoses such as cerebrovascular disease and brain tumours. It focuses on the AD characteristic focal findings such as reduced hippocampal volume or medial temporal lobe atrophy [205-207], and hence individuals can be indicated to have suspected AD. Some studies have even suggested that these sMRI features can predict AD cognitive decline [208] and AD conversion from MCI [209], but these findings need to be validated. MRI images comparing brains from a CN individual and an AD individual can be seen in **Figure 1.7**.

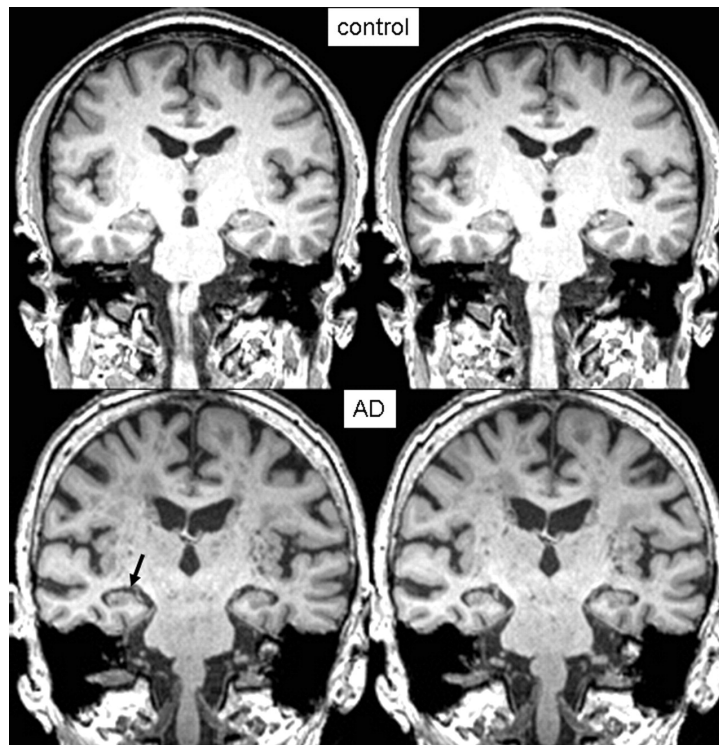


Figure 1.7. MRI images of normal control (top) and AD (bottom) brains. Volumetric differences in hippocampus (as pointed by the arrow in AD brain) can be seen. Image obtained from [210].

Neurofunctional imaging modalities such as FDG-PET (Fludeoxyglucose-positron emission tomography) and regional cerebral blood flow imaging with SPECT (single photon emission computed tomography) are often used to evaluate AD as they can provide, respectively, information about regional glucose hypometabolism and hypoperfusion. Hypometabolism and hypoperfusion in the temporoparietal cortex [211], inferior parietal lobules and precuneus [212] have been found to be AD characteristic. Moreover, Schroeter *et al.* [212] also demonstrated hypometabolism/hypoperfusion in the parietal lobe to be able to predict conversion from MCI to AD.

Accumulation of the A β in the brain is one of the major hallmarks of AD, and until recently only histopathological examination was able to confirm this [189, 213]. Recent developments in radiopharmaceutical agents aimed at identifying amyloid deposition have allowed *in vivo* amyloid deposition in human brains to be studied [214-217].

Briefly, radiopharmaceutical agents are injected and these agents (ligands) bind to amyloid aggregates, PET is applied to acquire brain images showing foci of abnormal amyloid accumulation [214]. PET scans comparing healthy and AD brains can be seen in **Figure 1.8**. Currently, three amyloid PET tracers (radiopharmaceutical agents) have been approved by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) as qualitative assessment tools of A β plaque density, and they are Florbetapir (^{18}F) [218], Flutemetamol (^{18}F) [219, 220] and Florbetaben (^{18}F) [221, 222]. Measurements of *in vivo* amyloid burden can help clinicians with AD diagnosis and prognosis, and differentiating AD from other causes of dementia [215, 217, 223, 224]. ^{11}C Carbon labelled Pittsburgh compound B (^{11}C -PiB) [225] has also been used extensively for PET imaging of cerebral A β pathology, but for research purpose only.

In addition to amyloid tracers, four tau-specific tracers for PET have recently been developed, they are THK5317 [226], THK5351 [227], AV-1451 [228] and PBB3 [229]. However these markers need to undergo extensive validation works before their use can be approved.

Overall, recent advancements in neuroimaging techniques has allowed to see whether brains from suspected AD individuals are experiencing AD pathology and help clinicians with diagnosis in cases of diagnostic uncertainty. However, they alone should not be used for AD diagnosis purpose due to their low specificity; “positive” scan will only indicate that the individuals have AD pathology while “negative” scan will just lower the likelihood that the individuals have AD.

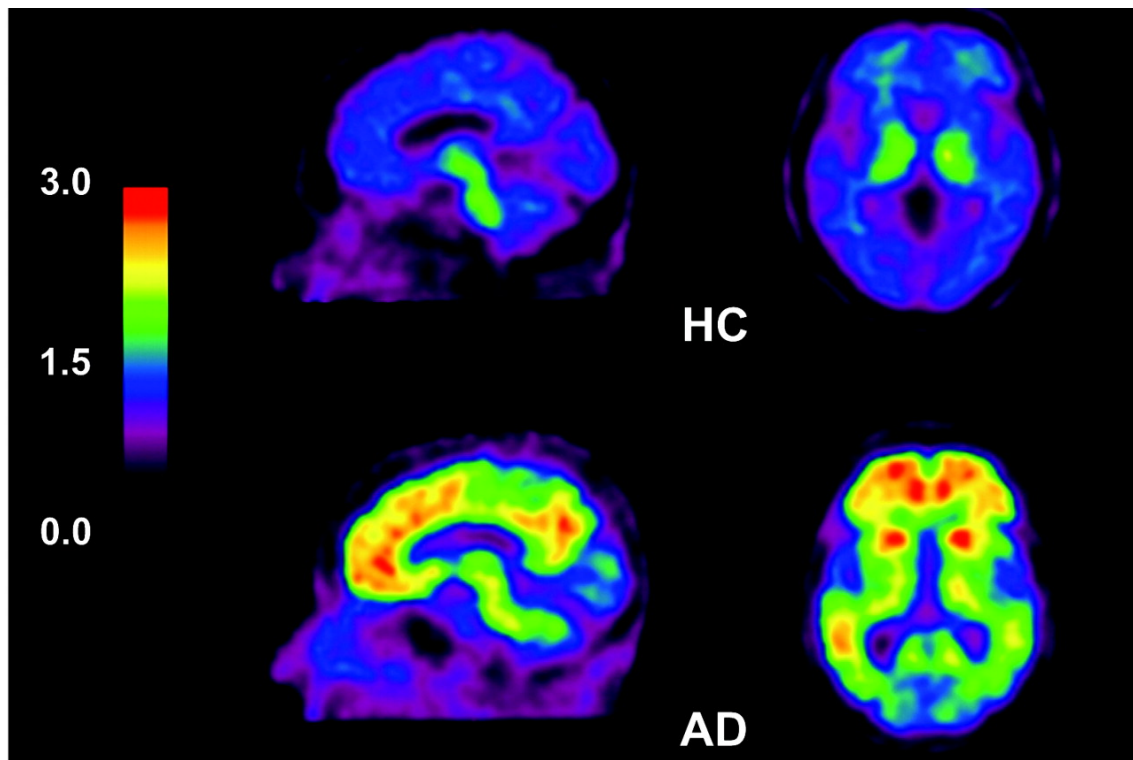


Figure 1.8. PET scans showing Sagittal (right) and transaxial (left) sections of healthy control (HC, top) and Alzheimer’s disease (AD, bottom) brains. PET scans show white matter uptake of $A\beta$ -ligand ^{11}C -PiB in HC brain, and extensive cortical and subcortical uptake of $A\beta$ -ligand ^{11}C -PiB in AD brain. Figure obtained from [230].

1.5.4. CSF A β and Tau

AD is characterized by abnormal patterns in structural and functional imaging, but as recently discovered, pathological CSF signatures can also characterize AD. These include lower A β_{42} peptide, higher total- and phosphor- tau proteins. Studies have shown an average decrease of 50% in A β_{42} [231], 300% increase in total-tau and 200% increase in phosphor-tau [232] in AD CSF when compared to healthy control CSF. Diagnostic accuracies of A β_{42} , total-tau and phosphor-tau have been found to range between 69% and 91% [233-237] while a combined assessment of A β_{42} and phosphor-tau has proven to be the best discriminator with accuracy ranging between 86% and 96% [234, 237, 238].

Abnormal levels of A β_{42} , higher total- and phosphor- tau in CSF have also been observed in MCI, their discrimination power against healthy controls being similar to those found in AD CSF [237, 239, 240], and suggest that they might help identifying individuals that in fact have incident AD. Findings from longitudinal studies support this assumption by showing that abnormal levels of the three molecules were present in asymptomatic elderlies who deteriorated to AD during follow-ups [241, 242].

However it worth noting that although the three CSF molecules reflect AD neuropathological changes with relatively high accuracy, like neuroimaging, they can only aid clinicians with decision-making, as a tool in the differential diagnosis and increase diagnostic confidence. They alone are not sufficiently specific to be used for diagnosis purposes [232, 243].

1.5.5. Genetic Testing

Currently 4 genes have been associated with increased incidence of AD, they are APP, PSEN1, PSEN2 and ApoE. Mutations of genes responsible for APP, PSEN1 and PSEN2 have been linked with EOAD [25-27], while the presence of ApoE ϵ 4 is a risk-factor gene for LOAD [244].

In EOAD, mutations in one of the three genes have been found to be more than 80% of the cases, PSEN1 mutation being the biggest attributor (estimated 66%) followed by APP mutation (16% of cases) [245, 246]. Genetic testing for APP, PSEN1, PSEN2 mutations is commercially available, but its use is only recommended when presenile dementia (early onset dementia) occurs in the setting of a family history. Also it should be emphasized that testing of asymptomatic at-risk individuals is predictive, not diagnostic, and results can potentially affect individual's personal relationships and may cause depression.

In LOAD, presence of ApoE ϵ 4 is the biggest genetic risk factor [247] as well as being the current best predictor of the disease [248]. Despite this, ApoE genotyping does not provide sufficient sensitivity and specificity to be used as an AD diagnostic test (65% and 68% respectively in a study looking at 2188 individuals [249]). Its use adds marginally to the predictive value of clinical criteria for AD and might stratify risk of conversion from MCI to AD. NIH only recommends ApoE genotyping to advance drug research in clinical trials [250].

1.6. AD Treatments and On-going Clinical Trials

Currently, there is no cure for AD while limited therapy is available to help with cognitive and behaviour symptoms. The FDA has approved five drugs for AD treatment, donepezil (Aricept[®]), rivastigmine (Exelon[®]), galantamine (Razadyne[®]), memantine (Namenda[®]) and Namzaric[®] and their primary goal is to slow down the clinical progression [6, 251, 252]. Donepezil, rivastigmine and galantamine are cholinesterase inhibitors and are prescribed to those with mild/moderate AD. They are used to treat symptoms related to memory, thinking, language, judgment and other thought process. Memantine is N-methyl-D-aspartic acid (NMDA) receptor antagonist; it regulates glutamate activity by protecting cells against excess glutamate. Its use is approved to only those in moderate/severe AD. Memantine may improve mental functions and daily activities for some, but some many not notice any effect. Namzaric[®] is a mix of donepezil and memantine and is prescribed for the treatment of moderate/severe AD.

Over the past decade, numerous candidate agents focusing on disease-modifying therapy (DMT) for AD have also been tested in clinical development. Their aim was to affect the underlying disease process by impacting brain changes characteristic of AD, namely A β . However, no agent with disease-modifying potential has been approved for marketing despite the fact that 221 agents have been assessed until 2014 [253]. As of early 2016, there were 17 agents in phase 3 of drug development for AD [254], two of them have now been completed but without positive results.

This 100% failure rate of DMT's could be, in part, because the drugs tested lacked sufficient target engagement, lack of AD pathogenesis understanding, complex pathophysiology of the disease and the slow progressive nature of AD [255, 256]. Also those enrolled into the clinical trials have considerable change within the brain already and even success in targeting AD pathology may not be enough to stop the disease progression.

A new paradigm shift in AD research has been toward an early intervention, by focusing on at-risk, asymptomatic individuals [\[257-259\]](#). Evidence supports this change in approach; solanezumab is an antibody targeting A β -pathology and its recent phase 3 clinical trial showed that although the agent failed to improve cognitive function for those in late-stages, it showed promise in delaying progression in early stage of AD [\[260\]](#).

1.7. Current AD Biomarkers and the Need for New Markers

Traditional AD diagnosis criteria set up by NINDS-ADRA in 1984 was reported to have average sensitivity and specificity of 81% and 70% respectively [261]. This meant that some individuals were identified to have AD when in fact they were non-AD dementias. A more recent guideline set up by NIA-AA in 2011 recommended measurements of A β and tau (brain and CSF) as diagnostic adjuncts for an increased AD diagnostic accuracy as well as identifying individuals in pre-clinical AD and/or MCI stages [192]. As the result, these abnormal observations (**Table 1.1**) are often termed AD biomarkers.

Biomarkers for AD	Pathophysiological correlate
Neuropsychological	
Tests for episodic memory / delayed recall	Hippocampal amnesia due to AD
Cerebrospinal fluid	
A β 42	Neuronal A β plaque accumulation
Total-tau	Neuronal degeneration
Phosphorylated tau	Intra neuronal NFT
Amyloid Imaging	
A β PET	Neuronal A β plaque accumulation
Structural neuroimaging	
Magnetic resonance imaging (MRI)	Neuronal injury marker
Computed tomography (CT)	Neuronal injury marker
Functional neuroimaging	
FDG-PET	Glucose hypometabolism

Table 1.1. Classification of biomarkers used in the differential diagnosis of AD. These markers are recommended by NIA-AA.

Biomarker (or biological marker) is defined by the U.S. National Institutes of Health Biomarkers Definitions Working Group as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic process, or pharmacologic responses to a therapeutic intervention” [262]. Biomarkers allow clinicians/researchers to evaluate the state of an individual’s health; confirm disease onset and progression; or gauge whether a treatment is working or not. They must display high sensitivity and specificity, while ease-of use is also an important factor to be defined as biomarkers. [263-265]. Examples include body temperature for fever, blood pressure for the risk of stroke, cholesterol values for coronary and vascular diseases, and C-reactive protein (CRP) for inflammation.

As shown in **Table 1.1**, AD biomarkers are mainly based on measurements of A β and tau and allow relatively high AD diagnosis accuracy [266]. In particular, CSF A β ₄₂, total- and phosphor- tau measurements can detect individuals in preclinical stage of AD, and therefore have an ability of predicting the risk of progression to AD. However, these markers face many issues and are subject of debate for a number of reasons. PET imaging is expensive while lumbar puncture for CSF collection is invasive. Most importantly, DMT drugs directed toward attenuating the A β and tau pathways have all failed to show positive results during clinical trials [267]. As a result, focus in AD research has shifted toward early intervention or prevention of AD. Also part of the scientific community believe that pathologies other than amyloid and tau need to be targeted [268, 269] as well as a need to discover new biomarkers which would recognise individuals in the asymptomatic (preclinical) stage.

Such a discovery would benefit AD research in a number of ways, it would distinguish AD from other types of dementia and allow more accurate treatment to be initiated [265]. It can potentially identify novel mechanistic pathways of the disease and consequently allow novel or repurposed therapeutic agents to finally achieve the disease-modifying status for AD. In short term, such biomarkers would allow the

correct selection of people in preclinical stage of AD for clinical trials and track both the disease progression and the response to treatment of the disease [270].

Consequently, “Biomarkers of Alzheimer’s disease” has become a very active topic in recent years with a push to identify a new biomarker for both preclinical and clinical stages of AD. This has involved assays of blood [271, 272], skin [273], urine [274], hair [275], odour [276], and olfactory deficits [277] whether the candidates are protein, gene, clinical symptoms, or small metabolites. Of these, peripheral blood has been the most extensively interrogated, as it is accessible by minimally invasive means and offers a relatively inexpensive substrate for analysis. Also crucially, blood is comprised of a liquid component (plasma or serum) and wide variety of cells (mononuclear leukocytes, erythrocytes and platelets). The liquid component is often used for proteins or small molecules profiling, Ribonucleic Acid (RNA) assay can be obtained from cells (also from serum and plasma exosomes). This particular nature of blood therefore represents a unique opportunity for biomarker discovery and development.

For discovery of novel blood-based biomarkers of AD, a wide array of technological platforms has been applied in recent years. For example, application of Polymerase Chain Reaction (PCR) has shown circulating microRNAs as potential biomarker candidates for AD [278, 279], the most promising result showing a panel of 12 microRNAs being able to differentiate AD from controls with accuracy of 93% [280]. Transcriptomic test has also been applied on blood; a signature consisting of 170 probesets has been identified to discriminate AD from controls with sensitivity and specificity of 81.3% and 67.1% respectively [281].

A wide number of studies have also focused on discerning blood-based proteomic biomarkers of AD, A β being the initial focus. Cross-sectional studies comparing levels of blood A β in AD have not shown consistent results, negative [282-285] as well as positive [286-288] test results have been reported. Several other proteins have also been looked at and suggested as potential biomarkers of AD. Ray *et al.* has described a

panel of 18 proteins being able to predict AD from controls with 90% accuracy [289] while glycan signatures have shown to discriminate with a sensitivity and specificity of 89.3% and 79.1% respectively [290]. More recently, O'Bryant *et al.* [291] demonstrated 21 proteins being a good AD discriminator in n=1329 study, the sensitivity and specificity were 63% and 98% respectively. Other groups have also looked at potential preclinical AD bio-signatures. A panel of 25 proteins has been found to predict conversion of AD from controls with sensitivity of 80% and specificity of 91% [292] while 10 plasma proteins were found to predict progression to AD from MCI (accuracy 87%) in a more recent study [272]. Another promising cross-sectional study showed 18 proteins being able to predict conversion from symptomatic MCI to AD with accuracy of 91% [293], but subsequent validation studies failed to replicate [294, 295].

1.8. Metabolomics and its analytical platforms

1.8.1. Metabolomics

Until recently, the focus in the AD biomarkers research field has mainly been identifying a panel of proteins or genes as possible candidates as AD biomarkers. However no candidate markers so far have been shown to meet the criteria to be defined as AD biomarkers and researchers have started approaching different angles. The last decade has seen advancements in analytical technologies such as nuclear magnetic resonance (NMR) and high-resolution tandem mass spectrometry (MS). These advancements have enabled accurate detection and quantification of small molecule metabolites (<1500Da), and such measurements of small molecules have become a new paradigm in biomarker studies. The field where small molecules are measured is often termed as ‘metabolomics’ or sometimes ‘metabonomics’.

Metabolomics involves comprehensive and simultaneous systematic profiling of metabolite concentrations and their fluctuation that reflects in response of living systems to pathophysiological stimuli and/or genetic modifications and the surrounding environment [296-298]. Metabonomics is another term often used interchangeably with metabolomics as the analytical and modeling procedures are the same. Metabolomics can be categorized into two distinct approaches, non-targeted (or untargeted) and targeted metabolomics. The non-targeted approach involves quantification/relative-quantification of as many as possible metabolites in a biological system. Targeted approach is where chemically characterized and biochemically annotated metabolites are quantified using specific methodologies designed with a target in mind [299].

Metabolomics has a number of inherent advantages compared to the use of other profiling techniques such as genomics and proteomics. Since metabolites are the terminal downstream product of the genome, monitoring the perturbations in a pool of metabolites could reflect underlying disease pathology and further, disease prognosis and diagnosis (**Figure 1.9**). Also metabolomics can react very fast to stimuli or to

change, therefore it is capable of viewing the most current view of the system's biology. Through evolution, metabolic pathways have been conserved which means metabolic pathways are similar in rodent and humans. Therefore metabolic signatures identified in mechanistic and therapeutic studies for animal models can in theory be translated into human studies [300]. Moreover, metabolomics is relatively inexpensive and time efficient when compared to genomics or proteomics and be applied to variety of easily accessible biofluids such as plasma, CSF, urine, peripheral tissues, thus highlighting the clinical utility of the approach [301].

The overall size of the metabolome remains elusive in metabolomics field, but it is estimated to range from a few thousand to tens of thousands of small molecules [302, 303]. Recent advancements in analytical technologies have allowed measurements of dynamic changes in many molecules in biological samples simultaneously. These technologies include high-resolution nuclear magnetic resonance (NMR) and mass spectrometry (MS) coupled with chromatographic techniques. However no analytical techniques is available for the analysis of the entire complex nature of the metabolome content. Often a compromise has to be considered depending upon a range of criteria such as speed, cost, properties of the analytes, sensitivity and the choice of biofluid as different analytical techniques have their own advantages and disadvantages.

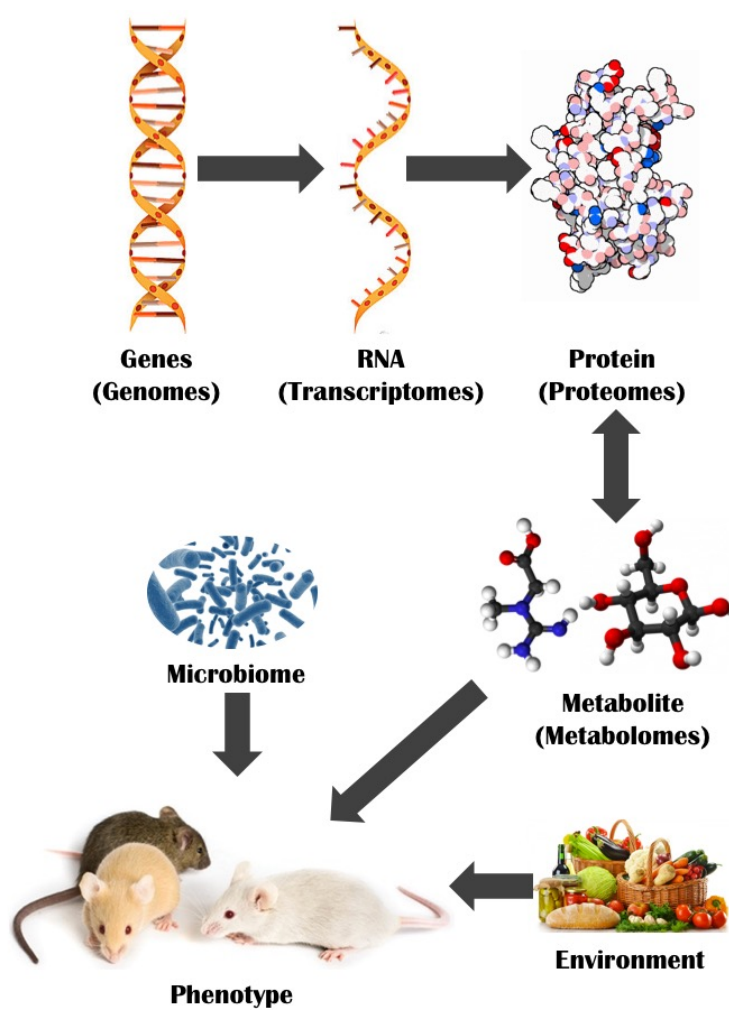


Figure 1.9. A schematic diagram of systems approach to biology. The diagram shows that metabolites are the terminal downstream products of genomes, transcriptomes, and proteomes in the whole organisms.

1.8.2. Nuclear Magnetic Resonance (NMR)

NMR in principle works by measuring energy differences between two spin states of certain nuclei when an external magnetic field is applied. The atomic nuclei include ^1H , ^{13}C , ^{15}N , ^{19}F , and ^{31}P , the most commonly studied nucleus in metabolomics is ^1H , followed by ^{13}C . More detailed principles of NMR and how the technique is used for application to metabolomics can be found in [304].

During the beginning of metabolomics, NMR was used exclusively. This was mainly due to its structural elucidation ability, the same reason that attracts structural biologist to NMR for the structural and dynamic analysis of proteins and nucleic acids. Application of various 1D and 2D NMR allowed this capability, but modern NMR now even allows rigorous structural analysis of many metabolites in crude extracts, cell suspensions, intact tissues, or whole organisms [305]. Further, de-novo structural analysis of unknown metabolites is now also feasible. Metabolite structural determination also allows detection of atomic positions of isotopic labels (for example, ^{13}C , ^{15}N , or ^2H) in different isotopomers (isotopic isomers) generated during stable isotope tracer studies [306-308]. As a result, detailed maps of biochemical pathways or networks can be constructed by using NMR with unprecedented speed [309, 310].

Other advantage of NMR, especially ^1H -NMR, is its capability of rapid detection of a large number of molecules with excellent quantitative precision, in a high throughput manner, ideal parameters for metabolomics application. Now with automatic sample preparation, analysis of more than 100 samples a day is attainable. A further advantage is that it is a non-destructive technique so that the same sample can be subsequently re-analysed by NMR or be re-used by other analytical platforms.

In summary, NMR is an ideal analytical platform for the metabolomics field for universal reasons. However NMR has a number of drawbacks, undoubtedly the largest disadvantage is its relatively poor sensitivity. This limits the observation to an estimated hundred or so metabolites per sample, accounting for less than 10% of an

organism's metabolome. Another disadvantage is its spectral complexity with superimposition of signals at certain spectral regions, compromising clear identification. A further weakness of NMR is high initial capital cost.

A similar analytical technique utilizing the theory of NMR is magnetic resonance spectroscopy (MRS). Its strengths are similar to those of NMR, but its able to detect metabolites in vivo without use of ionising radiation, therefore is a 100% non-invasive technique.

1.8.3. Mass spectrometry (MS)

Until recently, MS had been more widely used as a mainstay technique in the pharmaceutical industry for identification and quantification of drug metabolites. With recent MS technological advancement, this analytical platform is widely used in the metabolomics field, mainly because of its relatively high sensitivity when compared to NMR. The higher sensitivity allows detection and measurements of picomole to femtomole levels of many primary and secondary metabolites [311].

MS in principle works by transforming analytes into gaseous ions and then detecting the ionized ions according to their mass/charge (m/z) ratio. Modern MS offers an array of ionization and mass analyser technologies with different operational principle and performance. In the metabolomics field, the most common ionization techniques are electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo ionization (APPI) [312]. These techniques are especially popular in untargeted analysis due to their 'soft' ionization capability, generating little or no fragmentations which can aid identification of unknown metabolites, and also ionize metabolites at an extensive polarity range (**Figure 1.10**). Other ionization techniques are often used, and they included matrix-assisted laser desorption (MALDI) [313] and more recently, desorption ESI (DESI) [314] and extractive ESI (EESI) [315].

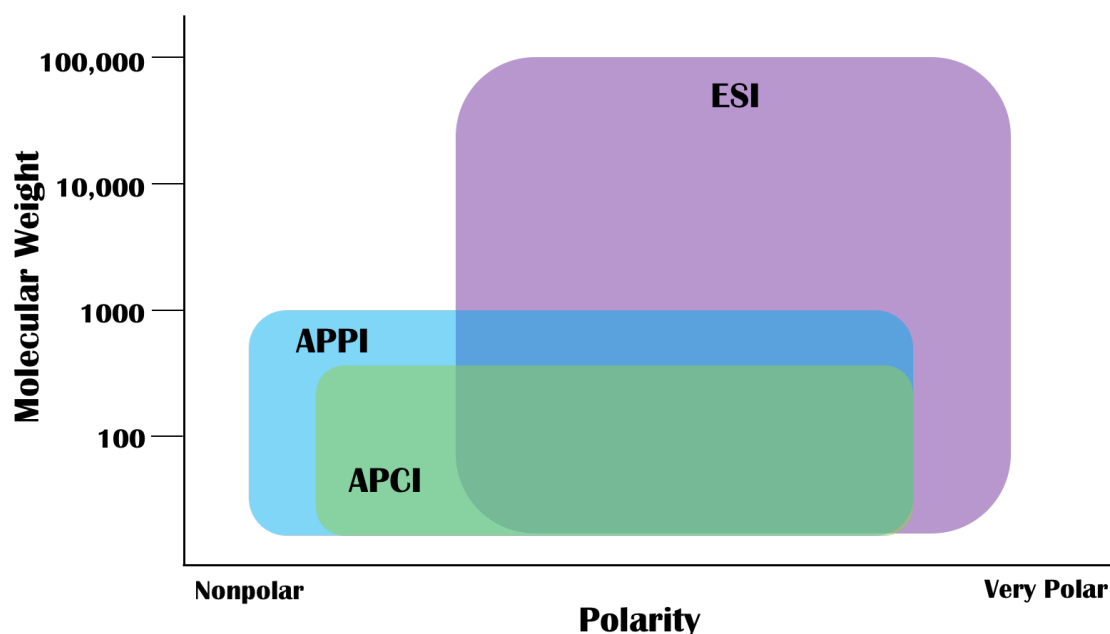


Figure 1.10. Application of soft ionization techniques, ESI, APPI and APCI, for compounds on the basis of polarity and molecular weight.

Mass analyzers can be categorized according to their different resolving powers. High/Ultrahigh resolution MS include Fourier transform-Ion cyclotron resonance-MS (FT-ICR-MS), orbitrap and time of flight (TOF). These high resolution MS instruments provide accurate mass measurements (which can aid metabolite identification) and accurate metabolite quantification. Low resolution MS such as ion trap and single quadrupoles are also utilized in metabolomics. Each of these mass analyzers has its own strengths and limitations, and selection of mass analyzers (and ionization techniques) in metabolomics depends on the goal of the metabolomics projects, throughput, and instrumental costs. Mass spectrometers can also be operated in tandem mode where two (MS/MS) or more (MS_n) spectrometers work in sequence, fragmenting selected ions from the first MS (MS¹) further by collision-induced dissociation (CID) in between mass spectrometers.

As mentioned earlier the main advantage of MS is sensitivity. However the spectrometer also has weaknesses, one being extensive sample preparation requirement in metabolomics. MS can only detect metabolites that can be transformed into gaseous

ions, and in order to increase the coverage of metabolites detected, biological samples often have to undergo different types of sample preparation. Another pitfall of MS is quantification. For accurate quantification for a specific metabolite, internal isotope-labeled standard with known amounts must be added during analysis. This strategy can be costly and is impractical for untargeted analyses. In untargeted metabolomics, although less accurate and precise, relative abundances of metabolites (peak area or intensity) are often used to compare between samples.

MS can be utilized in two ways, standalone (direct-infusion MS, DIMS) or coupled with chromatography. Direct-infusion MS approach is popular in metabolomics field because it provides a high throughput-screening tool and recent availability of advanced MS instruments (high resolution MS such as FT-ICR-MS and orbitrap) has further broadened DIMS applicability in metabolomics. Their high mass measurement accuracy provides elemental compositions of species while high resolution provide isotopic pattern information, helping in the unambiguous annotation of metabolites [\[316\]](#).

However, DIMS applicability is susceptible to ion-suppression where a large number of analytical species compete to be ionized. This limits the ionization efficiency and therefore affects both detection of metabolites as well as their reliable quantification. Also, DIMS approach is incapable of differentiating isomers. However these disadvantages can be surmounted/minimized by coupling MS to chromatographic separations.

1.8.4. MS coupled with Chromatography

Although DIMS method is applicable for metabolomics, separation techniques coupled to MS have been extensively used in metabolomics. This approach has a number of advantages; it reduces matrix effects and ionisation suppression, can potentially separates isomers, provides additional and orthogonal data (i.e. retention time), and allows for more accurate quantification. Currently, three predominant chromatographic

techniques have been incorporated in MS-based metabolomics and they are gas-chromatography (GC), liquid-chromatography (LC), and capillary electrophoresis (CE).

GC-MS is a relatively mature method, suited for the analyses of both volatile and non-volatile compounds (the latter has to follow a derivatization step, *i.e.* alkylation and silylation). Modern capillary GC offers high resolution and reproducible chromatographic separations while the standardised MS electron ionization energy of 70eV allows reproducible mass spectra. These allow more simplified compound identification by comparing retention time and mass spectra data to those of in reference library [317-319].

In metabolomics, GC coupled to single quadrupole MS is popularly used due to its reliability, effectiveness and affordable cost. Other MS such as TOF can be interfaced to GC to increase mass accuracy, mass resolution and fast scan speeds. The last of which is extremely valuable for the accurate deconvolution of overlapping high resolution or ultra-fast GC peaks, especially those encountered during complex metabolic mixture analyses. It is also worth noting a unique innovation of GC x GC, where two capillary columns with different stationary-phase selectivity are coupled in series through a flow modulator. This method offers increased separation efficiencies and peak capacities [320].

Unfortunately, GC-MS is less amenable to large highly polar metabolites due to their poor volatility even after following derivitization. Utilizing other chromatographic techniques such as LC can analyse such polar metabolites. LC-MS is an important tool (also the most popular tool) in metabolomics because of its flexibility. Normal-phase (NP) or reversed-phase (RP) columns can be employed for LC, although RP columns such as C₈ or C₁₈ are more utilized. Hydrophilic interaction liquid chromatography (HILIC) is also often employed to provide a complementary view metabolome along with RP-LC. HILIC is ideal for highly polar and ionic metabolites and therefore more suited for analysis of samples that contain predominantly polar metabolites. Examples

of such samples include urine and CSF. In addition to availability of different types of columns, different combinations of mobile phases conditions allow further tailored separations of compounds of interest, including chiral compounds. As a result of this flexibility of LC, most compounds can be analysed by LC-MS. In the last ten years development of fast and more efficient ultra-performance LC (UPLC) utilizing higher pressures has further increased chromatographic resolution and peak capacity.

These advantages have allowed LC-MS to be the most popular metabolomics analytical platform. However, it also suffers from a number of limitations, the major weakness being its difficulty in obtaining consistent quantitative precision caused by analytical variation. Different combinations of columns and mobile phases permits LC-MS to be flexible, but also contribute analytical variation such as drift of retention times, alteration of intensity values, and occasionally drift in m/z values. In order to evaluate these analytical variations, quality control (QC) samples are repeatedly analysed throughout the entire LC-MS experiment. QC samples are often aliquots of a sample pooled from multiple samples. By inspecting the QC runs, one can evaluate the presence and types analytical variation along the entire experiment.

CE-MS is also another MS-based analytical platform frequently used in metabolomics field, as it is fast, relatively inexpensive, and highly efficient. CE separates analytes based on charge and size, making the technique particular suited for separation of highly polar and ionic metabolites. Neutral analytes can also be separated by employing charged surfactants to form analytes-containing charged micelles. Although various MS techniques can be employed with CE, the most popular MS analyser is TOF due to its fast acquisition rates, while ESI is the most popular ionisation technique choice [321]. However use of CE-MS is still relatively low in metabolomics field, mainly because the analytical platform is often considered technically challenging and it suffers from relatively poor robustness and detection sensitivity [322]. More comprehensive discussion on various MS technologies used in metabolomics can be found in [321].

1.8.5. Liquid chromatography-electrochemistry array (LCECA)

Liquid chromatography-electrochemistry array (LCECA) is another analytical platform utilized for both targeted and non-targeted applications. The platform contains up to 16 coulometric electrodes in an array, allowing differential detection and quantification of small molecules on the basis of their oxidation-reduction potentials. This principle makes the technique ideal for detecting changes in neurotransmitter pathways and pathways involved in oxidative stress [323, 324]. This method has high sensitivity (2-3 orders of magnitude better than GC-MS) and reproducibility, but does not allow generation of structural information and suffers low throughput (12 samples per day using common metabolomics configuration methods) [305, 325].

1.8.6. Selecting a Platform

In an ideal setting, one analytical platform would be capable of accurate measurements of all compounds of interest in biological samples. However, metabolomics does not lend itself well to this as no single platform covers the entire complex nature of the metabolome content. This complexity is often overcome by employing multiple instruments, strengthening the data obtained by playing to the strengths of individual instruments and minimizing their weakness (**Table 1.2**). For example data for energy metabolism can be obtained by using NMR, lipids assay by a GC platform, neurotransmitter can be measured with an LCECA platform. This approach however, is impractical as it requires 1) access to multi-instrument platforms, 2) relatively larger amounts of biological specimens for multi uses, and 3) considerable skills for each instrument. As a result, it is critical to consider the logistics of analysis from the standpoint of what instrument would be the most practical. The criteria that need to be considered are speed, cost, properties of the analytes, sensitivity and the choice of biofluid.

Analytical platforms	Advantages	Disadvantages
NMR	Universal detection Precise quantification High throughput Structural elucidation and determination High reproducibility High reproducibility	Relatively insensitivity High instrument cost
DIMS	High sensitivity High throughput Fragmentation can provide structural data	Susceptible to ion-suppression Unable to differentiate isomers
GC-MS	Wide range of database Precise quantification High throughput Higher sensitivity compared to NMR Excellent tool for targeted studies	Derivatization required for non-volatile compounds Less structural information than NMR and LC-MS
LC-MS	High sensitivity High selectivity Derivatization not required Can be tailored for specific class of compounds Excellent tool for non-targeted studies Fragmentation provides structural data Detection of unidentified metabolites with the exact mass can be immediately known	Poor robustness Requires running QC samples Less structural data than NMR
LCECA	Detection of small metabolites based on oxidation/reduction High reproducibility High sensitivity	Lack of structural information Low throughput

Table 1.2. Strengths and weaknesses of analytical platforms utilized in metabolomics field

1.9. Application of Metabolomics in AD

Although metabolomics is a relatively new ‘omic’ field, in recent years, an extensive number of studies have applied this approach to establish differences in metabolites levels between cognitively normal (CN) individuals and AD patients. Various biological samples have been investigated, one of the most popular being CSF due to its composition directly reflecting the brain metabolic production. Non-invasive samples such as blood (serum or plasma), urine and saliva have also been analysed with the aim to identify simpler and cheaper biomarkers translatable to the clinical practice. Brain is also another source of AD studies, but the focus mainly has been the *in situ* characterization of neuropathological processes associated with AD. Previous animal model and human AD studies utilising metabolomics approach will be discussed in this section.

1.9.1. Metabolomics in animal models of AD

Metabolomic techniques for studying AD pathogenesis have also been utilized in animal models of AD. With regards to the animal models, it is important to note that the commonly employed transgenic (Tg) animal models are based on over-expression of APP (APP_{Tg2576}, APP_{V717F} and CRND8 transgenic lines). Some models also co-express the mutated human presenilin 1 (PSEN1) or presenilin 2 (PSEN2) allowing accelerated amyloid deposition (APP×PS1 or APP×PS2 double transgenic models respectively). TASTPM is another double transgenic mouse model carrying two mutations associated with APP and PSEN1. SAMP8 is another model frequently been used as the model displays a phenotype of accelerated aging and thus age-associated increase in hippocampal A β and age-associated behavioural impairments. These animal models are more closely associated with familial form of AD rather than LOAD, but they can still offer an opportunity to investigate the early pathological disease mechanisms and potentially identify novel therapeutic targets and relevant biomarkers.

The earliest studies on animal models were approached by MRS and investigated neurochemical profiles in cerebrum samples. Examination on cerebral cortex [326], cortex and hippocampus [327], and frontal cortex [328] showed a decrease in the levels of N-acetylaspartate (NAA) and glutamate in the brains where amyloid toxicity was wide spread. In addition, these metabolites have been found to correlate (negatively) with brain amyloid burden [328]. Studies utilizing different analytical platforms have also reported decreased NAA and glutamate levels, these include studies utilizing NMR approach on APP transgenic mouse hippocampus, cortex, frontal cortex, midbrain and cerebellum brain regions [329], and GC-MS metabolomics on PS1 transgenic hippocampus [330]. Glutamate is a primary excitatory neurotransmitter and its decreased levels may reflect a loss of glutamatergic neurons or decreased glutamate activity [331]. Biological function of NAA in the brain is not well understood, but it is commonly regarded as a marker of neuronal density and integrity [332]. A diagnostic test based on the decreased levels of NAA and glutamate in APP $\times$ PS2 mice brains was able to classify ‘AD like’ mice from normal mice with 92% sensitivity and 82% specificity [328]. In addition to decreased levels of NAA and glutamate, these studies have also reported myo-inositol (m-In) content elevated in APP and PS1 mouse cerebrums [327, 333, 334]. M-In is predominantly expressed in glial cells compared to neurons, its levels are considered to be indicative of osmotic stress and astrogliosis [335]. However other mouse model studies have shown contradicting m-In results [329, 330].

NMR platforms have also been applied on other sources of samples. Assays on plasma and liver samples from APP $\times$ PS1 transgenic mice showed significant changes in levels of lipids, nucleotides and energy-related metabolites, suggesting occurrence of oxidative stress and impaired energy metabolism [336]. The impaired energy metabolism was also visible when serum samples were subjected to NMR, glucose, citrate, 3-hydroxybutyrate, pyruvate were found to be decreased [337]. Moreover, analysis on urine samples from APP mice revealed significant metabolic alterations (increased levels of 3-hydroxykynurenine, homogentisate and allantoin) indicative of oxidative stress [338].

While NMR or MRS platforms are ideal for detecting highly abundant metabolites (particularly those involved in energy metabolism), MS based metabolomics allows to look at a wider range of the metabolome network. Direct MS analysis on APP transgenic mouse cortex and cerebellum showed lower sulfatide levels [339], while perturbations in homeostasis of lipids, energy management, and metabolism of amino acids and nucleotides were visible on APPxPS1 hippocampus and cortex [340]. Employing ultra high resolution MS instruments, Lin *et al.* has been able to observe over-production of eicosanoids indicating neuroinflammation on hippocampal tissues [341], and enhanced biosynthesis amino acid and amino acid derivatives on cerebellum [342]. Blood has also been assayed, direct MS methodology has identified a numerous disturbed pathways in serum samples from APPxPS1 mouse including perturbed phospholipid homeostasis, energy-related failures, hyperammonemia, inflammation (increased eicosanoids), among others [343]. Peripheral organs from APPxPS1 Tg mouse models have also been analysed, the organs included liver, kidneys, spleen and thymus [344]. The findings demonstrated that significant impairments in energy metabolism, lipid homeostasis, oxidative stress and amino acids metabolism. These findings were largely consistent with those found from Tg mouse brain tissue samples [340-342]. Impairments in such a wide range of the metabolome network indicate the systemic nature of AD is visible on multi-organs, not just brain.

Utilizing MS coupled with chromatographic science allows detection of broader network of metabolic pathways when compared to NMR or direct MS approaches. Whole brain metabolic profiles from APPxPS1 revealed perturbations related to amino acid metabolism, steroid biosynthesis [345]. Impairment in sterol metabolic pathway has also been visible in terms of increased cholesterol and cholesterol ester levels on whole brain [346, 347] and hippocampal tissues [348]. In addition to sterols, a close relationship between AD and the homeostasis of different lipid classes have also been decreased elsewhere. Lipid analysis on APPxPS1 Tg mouse whole brain samples showed elevated levels of lysophosphatidylcholines (LysoPC), free fatty acids, monoacylglycerols and phosphatidic acids [349]. The study was also able to identify significant

perturbations in the brain metabolism of eicosanoids [349], in line with another study [341]. Further inactivation of monoacylglycerol lipase has been found to reverse the perturbed metabolism of eicosanoids and reduce neuroinflammation that underlies AD pathogenesis, and thus suggesting monoacylglycerol lipase inhibitor as a potential therapeutic agent [349]. LC-MS techniques have also been applied for analysing hydrophilic metabolites in urine samples from CRND8 transgenic mice. Significant changes were observed in urinary levels of multiple families of metabolites, these included amino acids, fatty acids and neurotransmitter conjugates [350, 351]. Altered levels of these metabolites have been identified in brain and blood samples as well [326, 329, 337, 341], suggesting whole body response system to AD.

More recently, the use of metabolomics multi-platforms based on the combination of complementary tools has become increasingly popular as such methods allow more comprehensive characterization of metabolite alterations underlying to complex phenotypes to be achieved. González-Domínguez *et al.* has employed a combination of GC-/LC- MS platforms and investigation using APPxPS1 mouse model has shown numerous alterations in the homeostasis of lipids, amino acids, nucleotides, energy-related metabolites and some other compounds in the whole organism, including in serum [352], liver and kidney [353], spleen and thymus [354], and various regions of the brain [355]. Many of these impairments could also be detected in APP mouse hippocampus when the same methodology was applied [330].

Summary of metabolomics studies conducted in animal models of AD can be seen in **Table 1.3**.

Animal Model	Sample	Platforms	Findings	Ref.
APP _{Tg2576} (n=9) WT(n=10)	frontal cortex	NMR	↑taurine ↓N-acetyl-aspartate, glutamate, glutathione;	[326]
APPxPS1(n=7–8) WT(n=5–9)	whole brain	NMR	↑myo-inositol, choline, glycerophosphocholine; ↓succinate	[333]
CRND8 (n=5/5 young/old) WT (n=4/8 young/old)	cortex, frontal cortex, cerebellum, hippocampus, striatum, pons, midbrain, olfactory bulb	NMR	↑lactate, aspartate, glycine, alanine, leucine, isoleucine, valine, free fatty acids; ↓N-acetyl-aspartate, glutamate, glutamine, taurine, γ-aminobutyric acid, choline, phosphocholine, creatine, phosphocreatine, succinate;	[329]
APPxPS1(n=10) WT(n=10)	cortex(frontal, occipital, parietal, temporal), thalamus, hippocampus	NMR	↑myo-inositol, scyllo-inositol ↓N-acetyl-aspartate;	[334]
APP _{Tg2576} (n=39) WT(n=44)	frontal cortex, rhinal cortex, hippocampus, cerebellum, midbrain	NMR	↑creatine, taurine; ↓glutamate, N-acetyl-aspartate, myo-inositol, phosphocholine, γ-aminobutyric acid	[356]
APPxPS1(n=6) WT(n=6)	whole brain	NMR	↓N-acetyl-aspartate, γ-aminobutyric acid, creatine, ascorbic acid	[357]
APPxPS1(n=6) WT(n=6)	plasma	NMR	↓glutamate, glutamine, methionine, acetate, citrate	[357]
APPxPS1(n=27) WT(n=33)	plasma	NMR	↑lipoproteins, triglycerides	[336]
APPxPS1(n=27) WT(n=33)	liver	NMR	↑unsaturated fatty acids, 3-hydroxybutyrate, aspartate, glutamine, uridine diphosphate, uridine monophosphate, adenosine monophosphate; ↓alanine, lactate, betaine	[336]
SAMP8(n=5) SAMR1(n=5)	serum	NMR	↑lactate, saturated fatty acids, alanine, methionine, glutamine, VLDL; ↓inosine, glucose, polyunsaturated fatty acids, choline, phosphocholine, HDL, LDL, 3-hydroxy-butyrate, citrate, pyruvate;	[337]
APP _{Tg2576} (n=3–5) WT(n=3–5)	urine	NMR	↑3-hydroxykynurenine, homogentisate, allantoin, acetate, trans-aconitate, tyrosine, hippurate, citrate, urea; ↓trigonelline, 2-oxoglutarate, dimethylamine, trimethylamine, threonine, 1-methylnicotinamide ↓sulfatides	[338]
APP _{V717F} , APP _{V717F} ×APOE APP _{sw} APP _{sw} ×APOE WT	cortex, cerebellum	MS		[339]
APPxPS1(n=30) WT(n=30)	serum	MS	↑SFA-PLs, eicosanoids, triglycerides, diglycerides, choline, GPE, inosine; ↓PUFA-PLs, LPLs, cholesteryl esters, free fatty acids, urea, serine, valine, threonine, pyroglutamate, creatine, phosphoethanolamine, histidine, carnitine, glucose, tyrosine, tryptophan;	[343]

APP×PS1(n=30) WT(n=30)	hippocampus, cortex, cerebellum, olfactory bulb	MS	↑LPLs, PL(18:0/20:4), 22:5PLs, 22:4-PLs, propionyl-carnitine, free fatty acids, eicosanoids, uracil, hypoxanthine, xanthine, FAPy-adenine, adenosine, inosine, acetate, propionate, pyruvate, alanine, choline, valine, G3P, phosphocholine, N-acetyl-spermidine, GPC, GPI; ↓PUFA-PLs, acyl-carnitines, steroids, adenine, UMP, AMP, urea, aspartate, dopamine, tyrosine, homocarnosine;	[340]
APP×PS1(n=30) WT(n=30)	liver, kidney, spleen, thymus	MS	alterations in the metabolism of phospholipids, fatty acids, acyl-carnitines, eicosanoids, acyl-glycerols, steroids, amino acids, nucleotides, energy-related metabolites(changes depending on the tissue)	[344]
APP×PS1(n=8–9) WT(n=8–9)	brain, plasma	MS	longitudinal perturbations(6–18 months) in metabolism of phospholipids, amino acids, polyamines and acyl-carnitines	[358]
APP×PS1×IL4 (n=7) APP×PS1(n=7) WT(n=7)	serum	MS	↓urea, histamine, threonine, aspartate, urocanic acid, dopamine, citrulline, tyrosine;	[359]
CRND8(n=6) WT(n=6)	hippocampus	MS	↑1-methyl-histamine, eicosanoids alterations in the homeostasis of arachidonic acid, glucose metabolism and fatty acid β-oxidation	[341]
CRND8(n=6) WT(n=6)	cerebellum	MS	↑eicosanoids, amino acids and derivatives, xanthosine, xanthine, urate; ↓inosine, guanosine	[342]
SAMP8 n=5 (2 months) n=6 (7 months) n=7 (12 months)	hippocampus	GC-MS	↑cholesterol, oleic acid, phosphoglyceride, N-acetyl-aspartate; ↓alanine, serine, glycine, aspartate, glutamate, γ-aminobutyric acid, malic acid, butanedioic acid, fumaric acid, citric acid, pyroglutamic acid, urea, lactic acid	[348]
TASTPM(n=16) WT(n=5)	whole brain	GC-MS	↑d-fructose, valine, serine, threonine; ↑zymosterol	[345]
TASTPM(n=16) WT(n=5)	plasma	GC-MS	↑d-glucose, d-galactose, linoleic acid, arachidonic acid, palmitic acid, gluconic acid	[345]
APP _{Tg2576} (n=15) WT(n=17); CRND8(n=9) WT(n=9); APP _{V717I} (n=10) WT(n=12)	urine	GC-MS	↑phenylacetone; ↓3-methylcyclopentanone, 4-methyl-6-hepten-3-one, 6-hydroxy-6-methyl-3-heptanone	[360]
CRND8(n=12) WT(n=12)	urine	RP-MS	↑methionine, desaminotyrosine, phenylacetylglycine, dihydrouracil, ureidopropionic acid, thiocysteine; ↓choline, taurine, N1-acetyl-spermidine, 5-hydroxy-indoleacetic acid, hydroxyphenylglycine	[350]
CRND8 (n=18/12, 12/18 weeks) WT(n=12/12, 12/18 weeks)	urine	HILIC-MS	55 differential metabolites involved in the homeostasis of amino acids(e.g. tryptophan, tyrosine, phenylalanine, lysine), fatty acids, purines and pyrimidines, ascorbate, and others	[351]
APP×PS1(n=30) WT(n=30)	serum	RP-MS/ GC-MS	alterations in the metabolism of phospholipids, sphingolipids, fatty acids, cholesterol and bile acids, energy-related metabolites and amino acids	[352]

APP×PS1(n=30) WT(n=30)	hippocampus, cortex, striatum, cerebellum, olfactory bulb	RP-MS/ GC-MS	alterations in the metabolism of phospholipids, sphingolipids, energy- related metabolites, amino acids and nucleotides	[355]
APP×PS1(n=30) WT(n=30)	liver, kidney	RP-MS/ GC-MS	alterations in the metabolism of phospholipids, sphingolipids, fatty acids, acyl-carnitines, cholesterol and bile acids, energy-related metabolites and amino acids	[353]
APP×PS1(n=30) WT(n=30)	spleen, thymus	RP-MS/ GC-MS	alterations in the metabolism of phospholipids, sphingolipids, fatty acids, acyl-carnitines, energy-related metabolites, amino acids and purines	[354]
APP _{Td2576} (n=3) PS1(n=3) APP×PS1(n=6) WT(n=6)	hippocampus	RP-MS/ GC-MS	alterations in nucleotide, TCA cycle, energy transfer, carbohydrate, neurotransmitter and amino acid metabolic pathways	[330]

Table 1.3. Summary of metabolomics studies conducted in animal models of AD.

1.9.2. Metabolomics in AD patients

Compared to animal model studies, human studies allow more translational research into clinical practice, especially in the case of AD research. This is because AD mouse models do not present the extensive brain atrophy observed in AD individuals. Also animal models represent at best familial AD, not sporadic forms of AD which have multifactorial origins, contributed by many different risk factors. However unlike animal model studies, the direct comparisons are more challenging because of multifactorial issues such as age, disease severity and sex.

Brain

The earliest metabolomics studies on AD focused on post-mortem brain samples, utilizing MRS as an analytical platform. *in vitro* analyses were performed in order to quantify the content of some important neurochemicals in different brain regions. These studies commonly identified reduced levels of N-acetyl-aspartate, glutamate and glutamine, and increased myo-inositol content [361-365]. Shonk *et al.* evaluated these metabolites as putative neuronal markers, and found that the ratio of myo-inositol and N-acetyl-aspartate was able to distinguish AD from normality with 83% sensitivity and 98% specificity [365]. It is interesting to note that these changes were also identified in the brains of adult AD-like mouse in the presence of amyloid burden [326-329] as well as in the brains of young APPxPS1 Tg mouse in the absence of amyloid plaques [327, 366].

More recently, non-targeted approaches utilizing NMR technique have been employed. Graham *et al.* was able to identify increased alanine and taurine levels in the neocortex region of AD brains, ratio of these two metabolites was found to discriminate AD from controls with Receiver Operation Characteristic (ROC) area under curve (AUC) value of 76% [367]. Further when cortical profiles between AD and amyotrophic lateral sclerosis (ALS) were compared using the same methodology, alanine, glutamate and glutamine levels were found to be increased in AD, highlighting these abnormalities to be AD specific [368].

Numerous studies have also applied DIMS due to its high-throughput ability. In 2001 Han *et al.* reported a decrease in plasmalogen contents in cerebellar white matter at a very mild stage of AD [369], a trend which was also later seen in AD grey matter [370]. In the following year, Han *et al.* attempted to reproduce the finding of decreased plasmalogen levels in AD brain, but was unable to observe the change at a significant level [371]. Instead the group observed depletion in sulfatide levels in white matter and grey matter regions [371], a finding that was later seen in a mouse model study [339]. Sulfatide is known to mediate diverse biological processes including myelination, signal transduction, neuronal plasticity and cell morphogenesis [372, 373], suggesting sulfatide loss may lead to neuronal dysfunction and AD pathogenesis [371]. Interestingly, depletion of brain sulfatide level was also observed in pre-clinical AD [339] and mild AD [370], suggesting that sulfatide deficiency is possibly among the earliest events of AD development.

More hydrophilic brain metabolites have also been found to be associated AD pathology. Recently, Xu *et al.* employed GC-MS to compare profiles of 9 AD brains to 9 control brains, in 9 different brain regions [374]. The group detected 55 metabolites differentiating the two cohorts, these 55 metabolites were mainly amino acid and nucleoside, highlighting presence of widespread metabolic perturbations in AD brain [374]. In another study where LC-MS was employed, significant increases in the levels of spermine and spermidine were identified in AD brains, suggesting involvement of an abnormal brain expression of the polyamine pathway in AD pathogenesis [375].

CSF

There have been several studies analysing CSF in search of metabolites characterized by AD neuropathological processes because of its direct contact with extracellular space in the brain thus directly reflecting the brain metabolic production. Kaddurah-Daouk *et al.* has examined levels of metabolites involved in key neurotransmitter pathway and oxidative stress using 30 post-mortem ventricular CSF samples (15 AD *vs* 15 CN) [376].

For this purpose, a metabolic platform based on LCECA with pentane sulfonic acid as ion-pairing agent was employed. The study observed depletion of norepinephrine, methionine, α -tocopherol and 3-methoxytyramine levels and increased levels of 5-hydroxytryptophan in AD CSF [376]. However it is important to note that this study utilized post-mortem CSF samples [376]. Along with brain samples discussed in the earlier section, post-mortem samples generally imply that disease was at its severe-phase at the time of collection. This makes them less suitable source when investigating AD pathogenesis and progression for early stages. Additionally metabolite levels in post-mortem tissue/biofluid samples may be impacted by the death process (as well as post-mortem intervals) [377].

Applications of NMR-based metabolomics to CSF samples have demonstrated a great utility in differentiating AD from CN [378-380]. One of these studies quantified 31 metabolites from 76 AD *vs* 45 CN CSF samples and found these metabolites were able to classify the samples correctly with an accuracy of 85.5% [380]. LCECA has also been employed, Kaddurah-Daouk *et al* looked into metabolic profiles of 50 AD, 36 MCI and 15 CN CSF samples and was able to reveal elevated levels of methionine and 5-hydroxyindoleacetic acid in AD and MCI compared to CN samples [381]. These metabolites were also found to be related to CSF $A\beta_{42}$, indicating the perturbations in pathways involving methionine and 5-hydroxyindoleacetic acid are closely related with amyloidogenic pathway [381].

Multi-platforms based on the combination of complementary chromatographic science prior to MS detection have been also been employed for CSF analysis due to its ability of broader CSF profiling [382-384]. Utilising RP and HILIC coupled to MS, Trushina *et al.* has analysed 15 AD, 15 MCI and 15 CN CSF samples, and found approximately 30% of the metabolic pathways altered in MCI compared to CN, and 60% in AD compared to CN [382]. This finding showed the number of affected pathways increases with disease severity.

Another multiplatform available for CSF metabolic profiling is a combination of GC-MS and LC-MS methodology. Using this technique, Czech *et al.* has been able to establish differences in catecholamines and steroids in the CSF samples from 51 CN, 53 mild AD and 26 moderate AD participants [383]. The group also found increased cortisol levels associating with the AD progression, while combination of cysteine and various amino acids being able to provide AD (mild + moderate) predictive models with sensitivity and specificity above 80% [383].

In addition to examining changes in CSF metabolites for diagnostic purpose, there have been studies looking to identify metabolic changes with good prediction values of AD conversion. A recent study has employed CE-MS to monitor perturbations in the polar metabolome associated with AD progression by analysing CSF from subjects with different cognitive status, AD, MCI that progressed to AD within 2 years, MCI that remained stable after 2 years, and subjective cognitive impairment [385]. Initially ten metabolites including choline, valine, arginine, suberylglycerin, carnitine, creatine, serine and histidine were identified from 73 samples with classification accuracy of 90.1% for the four groups. The group then tested the classification model into 12 test samples, initially blinded to diagnosis, and 83% of the samples were correctly classified, indicating these polar CSF metabolites could potentially utilized as a tool for AD progression [385].

In the following year, the same group employed the combination of orthogonal RP-MS and HILIC-MS on the same sample cohorts [384]. From statistical modelling, 17 metabolites were identified showing strong association with cognitive status related to AD, and classification model based on these 17 metabolites was able to predict the development of AD with an accuracy of 98.7% and sensitivity and specificity values above 95%. The model included altered level of histidine [384] which was also present in a previous work by the same group [385].

Blood

The analysis of blood is an interesting alternative to the use of CSF samples, especially to find potential diagnosis biomarkers for their implementation in the clinical practice. Although CSF metabolites may be more reflective of brain changes, CSF collection is invasive, making it unsuitable for screening purposes. As a result, it is no surprise that most of the metabolic profiling studies have focused on blood-based biomarkers.

González-Domínguez *et al.* has looked into serum lipid profiles by utilizing various lipid extraction and DI-MS, each technique focusing on different classes of blood metabolites [386-389]. The group has been able to detect abnormal overproduction of several molecular species, 1) glycerides indicating membrane breakdown, 2) prostaglandins, imidazole and histidine indicating oxidative stress, and 3) glutamine, glutamate and dopamine indicating impaired neurotransmission systems. Other groups have also employed DIMS for profiling of blood metabolites and found altered levels of long-chain sphingomyelins and ceramides [390], plasmalogen and diglycerides [391], and phospholipids [392].

In addition to DIMS, RP-MS is also another popular tool for identification of blood metabolites (particularly more hydrophobic lipids) associated with the development of AD. Focusing on lipid profiles, Sato *et al.* has been able to find a considerable reduction in circulating cholesterol levels in AD plasma, both in untargeted and targeted analyses [393]. Other plasma lipid classes have been reported to have close relationships with AD, they included bile acids [394], LysoPCs and phytosphingosine (sphingolipid precursors) [395], long chain cholesteryl esters [396] among others [271]. Focusing on more hydrophilic blood metabolites, a study involving 75 AD, 17 MCI and 45 CN serum samples has revealed accumulation of acylcarnitine in relation to mitochondrial dysfunction, and lower levels of oleamide and monoglycerides caused by impaired endocannabinoid system [397]. A study involving much bigger cohorts of 660 AD and 583 MCI serum samples has found increased levels of sphinganine-1-phosphate and 7-ketocholesterol, the combination of these metabolites predicting AD conversion

with accuracy value of 95% [398]. These two metabolites have also been found to be strong AD discriminators from MCI and CN in saliva samples [399, 400].

Other chromatographic sciences coupled to MS have also been utilized, HILIC-MS approach has shown disturbance in 22 biochemical pathways including homeostasis of polyamines and arginine metabolisms [401], while CE-MS approach has revealed presence of oxidative stress and defects in energy metabolisms [402, 403]. Utilizing RP-MS/HILIC-MS multi-platform, Trushina *et al.* has identified 154 metabolites with significant changes in AD *vs* CN subjects [404] while Armirotti *et al.* has employed RP-MS/HILIC-MS multi-platform to find abnormally high levels of sphingomyelins in AD blood [405]. Combination of GC-MS and RP-MS is another multi-platform approach to have been used for blood metabolite assays. Wang *et al.* looked at profiles of 57 AD, 58 MCI and 57 CN plasma samples in 2014 and identified a panel of 6 metabolites discriminating AD from CN with ROC AUC of 1, and a panel of 5 metabolites discriminating MCI from CN with ROC AUC of 0.998 [406]. In a prospective study with aim to determine serum metabolomic profiles associating with progression and diagnosis of AD, Orešič *et al.* has utilized GC-MS/RP-MS to compare blood metabolic profiles of stable MCIs and converting MCIs [407]. A panel of 3 metabolites (lower levels of one PC, one carboxylic acid and 2-4-dihydroxybutanoic acid molecules) was identified to be associated with progressive MCI, its AD conversion predictive value (ROC AUC) was 0.77 [407].

Most recently, Toledo *et al.* employed a targeted approach and quantified 187 serum metabolites from 199 CN, 356 MCI and 175 AD participants. [408]. The study was able to reveal abnormal changes in sphingomyelins and PC levels related to early stages of AD, as well as abnormal changes in acylcarnitines and several amines levels related to later symptomatic stages of AD. The group hypothesized that dysregulation in lipid metabolism during early stages of AD leads to breakdown in lipid membranes which consequently results in disruption in energy metabolism seen during later stages of AD.

Saliva, Urine and Hair

Saliva, urine and hair metabolites have also been studied due to their ease-to-collect. Tsuruoka *et al.* has looked into saliva metabolic profiles to complement serum metabolic profiles by employing CE-MS [403]. Abnormal levels of arginine and tyrosine in AD saliva samples were found when compared to CN samples as well as FTD and LBD samples, highlighting the abnormal levels of arginine and tyrosine being specific to AD [403]. In a study involving urine samples, Cui *et al.* employed RP-MS and discovered impaired metabolic pathways involving L-glutamine and 5-L-glutamylglycine in AD [409]. The study then utilized these two metabolites to test their AD predictive abilities, and the corresponding ROC AUC values were 0.709 and 0.700 respectively [409].

In more recent years, sterol profiles of hair have been exploited as a possible source of AD markers due to hair being able to reflect biochemical stress at the local or at systemic levels [410, 411]. Son *et al.* analysed 31 AD, 15 MCI and 36 CN hair samples by employing GC-MS and discovered an increased metabolic rate of 7 β -hydroxycholesterol as a possible AD predictive biomarker [275]. Ratio of 7 β -hydroxycholesterol to cholesterol was found to be able to discriminate MCI *vs.* CN and AD *vs.* CN with ROC AUC values of 0.750 and 0.729 respectively [275].

Summary of metabolomics studies conducted on samples from AD participants can be seen in **Table 1.4**.

Sample Size	Sample Source	Platform	Findings	Ref.
AD(n=22) CN(n=18)	serum	DIMS	↓PUFA-PCs; ↑SFA-PCs	[412]
AD(n=22) CN(n=18)	serum	DIMS	↓PUFA-PLs, valine, glutamine, N-acetyl-glutamine, glutamate, histidine, arginine, dopamine, carnitine, creatine; ↑SFA-PLs, free fatty acids, eicosanoids, triglycerides, choline, glycerophosphocholine, glucose	[389]
AD(n=22) CN(n=18)	serum	DIMS	↓PUFA-PLs, oleamide, histidine, arginine, imidazole, taurine, guanidine, putrescine; ↑eicosanoids, diglycerides, kynurenine	[388]
AD(n=19) CN(n=17)	serum	DIMS	↓PUFA-PLs, LPLs, plasmalogens; ↑SFA-PLs	[387]
AD(n=30) CN(n=30)	serum	DIMS	↓fatty acid amides, urea, creatine, malate, taurine, dopamine, serotonin; ↑ceramides, diglycerides, free fatty acids, alanine, picolinic acid	[386]
AD(n=26) CN(n=26)	plasma	DIMS	↓long-chain sphingomyelins; ↑ceramides	[153]
AD(n=90) MCI(n=77) CN(n=51)	serum	DIMS	↓plasmalogens; ↑diglycerides	[391]
AD(n=43) MCI(n=33) CN(n=35)	plasma	DIMS	↓phosphatidylcholines; ↑lyso-phosphatidylcholines	[392]
AD(n=23) CN(n=21)	serum	GC-MS	↓valine, urea, aspartate, pyroglutamate, glutamine, phenylalanine, asparagine, ornithine, pipecolate, histidine, tyrosine, palmitic acid, urate, tryptophan, stearic acid, cysteine; ↑lactate, α-ketoglutarate, isocitrate, glucose, oleic acid, adenosine, cholesterol	[413]
AD(n=10) MCI(n=10) CN(n=10)	plasma	RP-MS	↓lysophospholipid (18:1)	[414]
AD(n=10) CN(n=10)	plasma	RP-MS	↓demosterol	[393]
AD(n=16) MCI(n=12) CN(n=10)	plasma	RP-MS	↑1-(9E-hexadecenoyl)-sn-glycero-3-phosphocholine, d-glucosaminide, glycocholic acid, glycodeoxycholic acid, glycochenodeoxycholic acid	[394]
AD(n=20) CN(n=20)	plasma	RP-MS	↓lysophosphatidylcholines, tryptophan, phytosphingosine, dihydrosphingosine, hexadecasphinganine	[415]
AD(n=36) MCI(n=48) CN(n=40)	plasma	RP-MS	↓long chain cholesteryl esters	[396]
AD/MCI(n=35) Converters(n=18) CN(n=53)	plasma	RP-MS	↓phosphatidylcholines, lyso-phospholipids, serotonin, phenylalanine, proline, lysine, taurine, acyl-carnitines, malate; ↑glycoursodeoxycholic acid, proline-asparagine	[271]
AD(n=75) MCI(n=17) CN(n=45)	serum	RP-MS	↓PUFA-PLs, PUFA-SMs, sulfatides, monoglycerides, oleamide, histidine pregnenolone sulfate; ↑SFA-PLs, SFA-SMs, ceramides, acyl-carnitines, phenyl-acetyl-glutamine	[416]
AD(n=148) CN(n=152)	plasma	RP-MS	↓cholesteryl esters, triglycerides ↑phosphatidylcholine	[417]
AD(n=175) MCI(n=356) CN(n=189)	serum	RP-MS	↓acyl-carnitines, phosphatidylcholines, sphingomyelins ↑valine, α-amino adipic acid	[408]
AD(n=660) MCI(n=583)	serum	RP-MS	↑sphinganine-1-phosphate, 7-ketocholesterol, 3-methoxytyrosine, deoxyribose-5-phosphate, p-phenyllactic acid, lyso-phosphatidylcholine; ↓phenylalanine, ornithine, glutamate	[398]
AD(n=46) CN(n=37)	serum	RP-MS	↓1-methylinosine, 16-bromo-9-hexadecenoic acid, lysophosphatidylcholines, dihydrosphingosine, N-acetyl-glutamine, 1α, 25-dihydroxy-2α-(3-hydroxypropoxy) vitamin D3; ↑palmitic amide, monoiodothyronine, antrimethyltridecanoic acid, PGE2α dimethyl amine, (6R)-vitamin D3 6, 19- (4-phenyl-1, 2, 4-triazoline-3, 5-dione)	[409]

AD_MCI(n=19) MCI(n=16) CN(n=37)	plasma	HILIC-MS	↓4-amino-butanal, γ-aminobutyric acid, ornithine, N-acetyl-putrescine; ↑creatinine, arginine, methylthioadenosine, N-acetyl-spermidine, N-diacetyl-spermine, putrescine, spermidine, spermine	[401]
AD(n=42) MCI(n=14) CN(n=37)	serum	CE-MS	↓creatinine, asparagine, methionine, histidine, carnitine, N-acetyl-spermidine, valeryl-carnitine; ↑choline, creatinine, dimethylarginine, homocysteinyl-cysteine, acyl-carnitines, peptides;	[402]
AD(n=47) MCI(n=143) CN(n=46)	serum	RP-MS/ GC-MS	baseline: ↓plasmalogens, phosphatidylcholines, sphingomyelins, sterols; ↑histamine progression: ↑2, 4-dihydroxybutanoic acid	[407]
AD(n=57) MCI(n=58) CN(n=57)	plasma	RP-MS/ GC-MS	↓fatty acids, dimethylglycine, glutamate, uridine, glyceraldehyde, butanedioic acid, 2-butenedioic acid, 5-oxoproline, taurine, hypotaurine, malate; ↑glutamine, 2-aminoadipic acid, proline, cysteine, cytidine, thymine, hypoxanthine, carbohydrates, citrate	[406]
AD(n=30) MCI(n=30) CN(n=30)	plasma	RP-MS/ HILIC-MS	↓sphingomyelins	[405]
AD(n=15) MCI(n=15) CN(n=15)	plasma	RP-MS/ HILIC-MS	changes in more than 150 metabolites (amino acids, energy-related metabolites, lipids, neurotransmitters)	[404]
AD(n=3) FTD(n=4) LBD(n=3) CN(n=9)	serum	CE-MS	↑β-alanine, creatinine, hydroxyproline, glutamine, isocitrate, cytidine	[403]
AD(n=12) CN(n=5)	whole brain	MRS	↓N-acetyl-aspartate, γ-aminobutyric acid; ↑ glutamate	[362]
AD(n=13) CN(n=4)	temporoparietal cortex	MRS	↓N-acetyl-aspartate, creatine, γ-aminobutyric acid	[361]
AD(n=13) CN(n=4)	hippocampus, cerebellum	MRS	↓N-acetyl-aspartate, γ-aminobutyric acid	[363]
AD(n=8) ALS(n=11)	frontal cortex	NMR	↑alanine, acetate, glutamate, glutamine; ↓lactate, creatine	[368]
AD(n=15) CN(n=15)	neocortex	NMR	↑alanine, taurine	[367]
AD(n=24) CN(n=6)	superior frontal cortex, superior temporal cortex, inferior parietal cortex, cerebellum	DIMS	↓plasmalogens	[369]
AD(n=17) CN(n=5)	middle frontal gyrus, superior temporal gyrus, inferior parietal lobule, hippocampus, subiculum, entorhinal cortex	DIMS	↓sulfatides; ↑ceramides	[371]
AD(n=6) CN(n=8)	superior frontal gyrus	DIMS	↓sulfatides	[418]
AD(n=34) MCI(n=19) CN(n=28)	frontal cortex grey matter, frontal cortex white matter	DIMS	↓plasmalogens, phosphatidylethanolamines, docosahexaenoic acid, phosphatidylethanolamines, sulfatides; ↑monoglycerides, diglycerides ; ↑monoglycerides, diglycerides, VLCFA 26:0	[370]
AD(n=9) CN(n=9)	hippocampus, entorhinal cortex, middle-temporal gyrus, sensory cortex, motor cortex, cingulate gyrus, cerebellum	GC-MS	changes in 55 metabolites, including energy related metabolites (glucose metabolism, TCA), amino acids, nucleosides, and others	[374]
AD(n=10) CN(n=10)	frontal lobe, parietal lobe, occipital lobe	RP-MS	↑spermidine, spermine, N-acetyl-spermidine, N-acetyl-spermine, putrescine	[375]
ADI-II(n=7) ADIII-IV(n=4) ADV-VI(n=5) CN(n=4)	entorhinal cortex	RP-MS	↓dGMP, glycine, xanthosine, inosine diphosphate, deoxyguanosine; ↑guanine	[419]
AD(n=15) CN(n=15)	neocortex	HILIC-MS	76 unidentified discriminant signals	[420]

AD(n=21) CN(n=19)	frontal cortex	RP-MS/ HILIC-MS	alterations in the metabolism of phospholipids and six metabolic pathways of the central metabolism: Alanine, Aspartate, and Glutamate Metabolism; Arginine and Proline Metabolism; Cysteine and Methionine Metabolism; Glycine, Serine, and Threonine Metabolism; Purine Metabolism; Pantothenate and CoA Biosynthesis	[421]
AD(n=15) CN(n=15)	post-mortem CSF	LCECA	↓norepinephrine, α-tocopherols, 3-methoxytyramine, ascorbate; ↑5-hydroxytryptophan, methoxy-hydroxyphenyl glycol	[376]
AD(n=34) MCI(n=19) CN(n=28)	CSF	MS	CSF: ↓docosahexaenoic	[370]
AD(n=20) CN(n=27)	CSF	NMR	specific resonances in AD (unidentified)	[378]
AD(n=10) CN(n=34)	CSF	NMR	↑creatinine	[379]
AD(n=76) early_AD(n=26) MCI(n=33) CN(n=45)	CSF	NMR	phenylalanine, glutamate, lactate, acetate, α-hydroxyisovalerate, α-hydroxybutyrate	[380]
AD(n=50) CN(n=50)	CSF	DIMS	↑sphingomyelins, phosphatidylcholines	[422]
AD(n=17) CN(n=17)	CSF	RP-MS	53 unidentified discriminant signals	[423]
AD(n=40) MCI(n=36) CN(n=38)	CSF	RP-MS	↑methionine, 5-hydroxyindoleacetic acid, vanillylmandelic acid, xanthosine, glutathione, hypoxanthine	[381]
AD(n=23) AD_MCI(n=9) sMCI(n=22) CN(n=19)	CSF	CE-MS	↓arginine, suberylglycine, carnitine, histidine; ↑choline, valine, tripeptide, dimethylarginine, creatine, serine	[385]
AD(n=79) CN(n=51)	CSF	RP-MS/ GC-MS	↓uridine; ↑cysteine, tyrosine, phenylalanine, methionine, serine, pyruvate, taurine, creatinine, cortisol, dopamine	[383]
AD(n=40) CN(n=38)	CSF	RP-MS/ GC-MS	2 unidentified discriminant signals	[424]
AD(n=21), cMCI(n=12) sMCI(n=21) CN(n=21)	CSF	RP-MS/ HILIC-MS	changes in levels of uracil, xanthine, uridine, tyrosyl-serine, methylsalsolinol, nonanoylglycine, dopamine-quinone, caproic acid, vanylglycol, histidine, pipercolic acid, hydroxyphosphinyl-pyruvate, creatinine, taurine, C16-sphingosine-1-phosphate, tryptophan, 5'-methylthioadenosine	[384]
AD(n=15) MCI(n=15) CN(n=15)	CSF	RP-MS/ HILIC-MS	changes in more than 150 metabolites (amino acids, energy-related metabolites, lipids, neurotransmitters)	[382]
AD(n=256) CN(n=218)	saliva	RP-MS	↓inosine, 3-dehydrocarnitine, hypoxanthine; ↑sphinganine-1-phosphate, ornithine, p-phenyllactic acid;	[400]
AD(n=660) MCI(n=583)	saliva	RP-MS	↓inosine, 3-dehydrocarnitine, hypoxanthine; ↑cytidine, sphinganine-1-phosphate, ornithine, p-phenyllactic acid, pyroglutamate, glutamate, tryptophan	[399]
MCI(n=20) CN(n=20)	saliva	RP-MS	↓taurine, peptides, 1, 8-diazacyclotetradecane-2, 9-dione, 4-(hydroxylamino)-N, N-dimethylaniline; ↑2-amino-heptanoic acid/l-alanine-n-butyl ester/N-methyl-isoleucine	[425]
AD(n=3) FTD(n=4) LBD(n=3) CN(n=9)	saliva	CE-MS	↓arginine, tyrosine	[403]
AD(n=46) CN(n=37)	urine	RP-MS	↓acetyl-carnitine, estra-1, 3, 5(10), 7-tetraene-3, 17α-diol, p-cresol glucuronide, etiocholanolone glucuronide ; ↑N-acryloylglycine, argininosuccinic acid, dethiobiotin, isobutyryl-l-carnitine, 2-hydroxy-N-(2-hydroxyethyl)-N, N-dimethyl-1-dodecanaminium, l-aspartyl-4-phosphate, l-glutamine, 5-l-glutamylglycine, azelaic acid, aminopterin, cytidine	[409]
AD(n=31) MCI(n=15) CN(n=36)	hair	GC-MS	↑7β-hydroxycholesterol	[275]

Table 1.4. Summary of metabolomics studies conducted in AD human samples.

1.9.3. Summary

Overall, an extensive number of studies have applied metabolomics in order to identify 1) perturbations in metabolic pathways that reflect changes associated with AD pathology, and 2) a panel of metabolites which would potentially be used as an AD diagnostic tool. Taking together the findings, it seems clear that multiple metabolic alterations could be occurring during progression to AD. These include lipids, metabolites involved in neurotransmitter systems and energy metabolism.

1.10. Aim

Alzheimer's disease is a devastating disease for patients and carers. At the moment there is no cure and diagnosis is confirmed too late when cognition is already lost. In order to find better diagnostics and a therapy, new approaches are urgently needed. The previous section has highlighted metabolomics as a suitable approach to identify alterations in multiple biochemical networks over the course of AD, however much more work is needed in order to be able to translate any findings.

In this report, initially, CSF metabolic profiles from four different clinical cohorts will be examined, AD, FTD, age-matched CN and relatively young CN. By including relatively young CN group, we will be able to determine whether changes in metabolite levels in AD CSF reflect an evolution of aging-related processes. Inclusion of the FTD group will allow to know whether changes in metabolite levels in AD CSF are specific to AD or FTD. Firstly NMR will be used for fingerprinting; secondly RP-MS and HILIC-MS will be employed, to allow acquisition of a comprehensive metabolic profile.

Then we will be utilizing blood samples and measure lipid levels using an in-house RP-MS method [426]. A number of lipid species which have previously been found to associate with AD will be quantified, first having a look at diagnosed AD and then having a look at the pre-clinical AD stage. This approach will help identifying when particular lipid dysregulation develops.

The final chapter in this thesis will aim to find lipid signatures associated with clinical AD diagnosis and AD endophenotypes, such as rate of cognitive decline and brain atrophy measures. By using a careful and systematic analysis pipeline, the metabolomics analysis in this chapter will be the most comprehensive.

***¹H-NMR based
Metabolic Phenotyping
of Cerebrospinal Fluid***

2.1. Introduction

The analysis of cerebrospinal fluid (CSF) is essential in understanding various neurodegenerative central nervous system (CNS) disorders such as Alzheimer's disease (AD), Parkinson's disease (PD) and Frontotemporal dementia (FTD). The fluid is in direct contact with the brain and spine, and is involved in transportation of biological substances from blood to brain and excretion of toxic waste substances from the brain. These transportation and excretion processes can be disturbed by neurological disorders and therefore analysis of CSF composition can be an excellent source for studying disordered biological processes of the brain [427, 428].

Studies on AD CSF in recent years have proposed a decrease in $A\beta_{42}$, increases in total and phosphorylated tau (t-tau and p-tau respectively) and their ratio as AD diagnosis. Furthermore, these abnormalities have been found well in advance of cognitive impairment [193, 429], therefore suggesting that they have a role in predicting the development of future cognitive decline at early and pre-dementia stages for those in high risks. Meanwhile PD CSF studies have shown a decrease in α -synuclein and a protein deglycase, DJ-1, as emerging candidate biomarkers for PD [430-432].

CSF protein markers are the best studied in the neurodegenerative disorders field, in more recent years, focus has shifted toward detecting changes in levels of relatively smaller metabolites during development of CNS disorders. Metabolomics data can be obtained by Nuclear Magnetic resonance (NMR) and/or Mass Spectrometry (MS) based analytical platforms.

In AD CSF metabolomics, NMR is often used due to it being high-throughput, quantitative and highly reproducible. A few earlier studies have showed a lower citrate level [433], higher inositol [434] and creatinine [435] in AD CSF compared to Controls. More recent studies have demonstrated that ^1H -NMR spectra of CSF from AD patients showed specific signals that would not be detected in the majority of CSF from cognitively healthy participants [436, 437]. The authors suggested the metabolite(s)

resulting the AD specific signals to have aromatic ring(s), but were unable to identify them [436, 437].

Similar to the previous studies [433-437], this study will also look at CSF metabolic profiles and try to identify CSF metabolite(s) that differentiate AD subjects from cognitively healthy subjects using ^1H -NMR. In our study another dementia group, FTD was included to increase the specificity of any metabolic differences. This is important considering the clinical presentations and neuropathological features in dementias overlap considerably. For examples, altered levels $\text{A}\beta_{42}$ has been reported in both AD and PD CSF [432, 438-442]. By comparing with FTD, this study will be able to test the discriminating ability of the newly found AD markers from FTD (and cognitively healthy), hence will potentially enhance the marker's clinical accuracy.

This study will also look at CSF metabolic profiles of Young Controls. This will allow us to observe whether AD-related pathological changes are associated with aging-related physiological processes, an important observation for both reasons of clinical utility as well as for greater specificity of AD biology.

Overall, by using CSF from young and elderly healthy Controls, and patients with a clinical diagnosis of AD or FTD, we will ask whether changes in small metabolite concentrations in AD CSF reflect an evolution of aging-related processes and/or specific AD-related pathological changes.

2.2. Methods

2.2.1. Patients

CSF samples were available from a total of 70 subjects who presented to the Johns Hopkins University School of Medicine’s Neurology clinics for evaluation of dementia, headache, seizures or demyelinating neurological disease. All patients provided written informed consent to lumbar puncture and use of CSF samples for research. The diagnoses of AD (NINCDS-ADRDA criteria) or FTD were made by an experienced neurologist (Professor Richard O’Brien). Demographic characteristics and clinical data of the participants are summarized in **Table 2.1**.

2.2.2. Immunoassays of CSF total-tau and A β ₄₂ concentrations

CSF immunoassays were performed for total-tau (t-tau) and A β ₄₂ concentrations. These measurements were performed using the xMAP-based AlzBio3 kits (Innogenetics, Ghent, Belgium) run on the Bioplex 200 system as reported previously [443]. These data were collected in the Johns Hopkins laboratory on all subjects except those in the ‘young-Control’ group.

2.2.3. Sample Preparation & NMR Data Acquisition

Prior to data acquisition, CSF samples were allowed to reach to room temperature. Based on a previously published protocol [444], 15 μ L of a standard solution (0.67% w/v NaN₃, 332.42mM imidazole in liquid chromatography-grade H₂O) and 185 μ L of deuterium oxide containing 0.05 wt. % 3-(trimethylsilyl)propanoic-2,2,3,3-d₄ acid, sodium salt (TSP-2,2,3,3-d₄) was mixed with 300 μ L CSF. Following 30 seconds of vortex, sample was then transferred to a 5mm NMR tube. Overall, prepared samples each containing 1.19mM TSP, 9.97mM imidazole and 0.02% w/v NaN₃ were analysed.

All ¹H-NMR spectra were collected on a Bruker Avance 400MHz spectrometer equipped with a 5mm QNP probe (Bruker UK Limited, Coventry, UK). ¹H- NMR

spectra were acquired at room temperature using zgesgp pulse sequence to suppress water signal using excitation sculpting with gradients [445]. The ^1H 90 degree pulse was 10.50 μs . Free induction decays were collected into 66k data points with 256 scan using a spectral width of 8250 Hz with an acquisition time of 3.97s between each scan. After Fourier transformation with 0.3 Hz line broadening, the spectra were calibrated to a TSP-2,2,3,3-d4 reference signal at 0 ppm followed by phase and baseline correction using TopSpin 3.0 (Bruker).

2.2.4. Data processing: Peak alignment and normalization

Pre-processing of spectra was carried out with in-house software using python programming language [446] and Matlab (version 7, Mathworks, Natick, USA). Spectral variables such as regions above 8.5 ppm and below 0.8 ppm as well as water and urea peaks were excluded. The spectra were aligned using the *icoshift* algorithm [447] to compensate for variation in resonance position according to pH. A binning algorithm was then incorporated by integrating within small spectral segments with a defined width of 0.01ppm. This approach allowed reduction of data variables from 16,812 to 453 for faster computation and further compensation of chemical shift variation by pH. The binned data then underwent probabilistic quotient normalization [448]. The processed data was exported as an ‘ASCII’ file.

2.2.5. Data analysis

For multivariate data analysis, the processed data underwent ‘pareto’ scaling and was subjected to principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) in SIMCA-P+ (Umertrics, Sweden). S-plots corresponding to each OPLS-DA models were used to select variables that discriminate different cohorts. For univariate data analysis, Mann-Whitney test was applied to compare metabolite levels between two groups. This was carried out in R (version 3.2).

2.2.6. Metabolite identification and quantification

Identification of metabolites was carried out using the 400 MHz library of metabolite NMR spectra from the Chenomx NMR Suite (version 7.6, Chenomx, Alberta, Canada) and also using the Human Metabolome Data Base (HMDB) [449] in conjunction with published literature [444, 450-452].

Quantification was carried out by Chenomx NMR Suite using the concentration of the known reference signal as calibration (TSP-2, 2, 3, 3-d4, 1.19 mM) by fitting peak shapes of compounds from an internal database of reference spectra to the experimental spectra. In contrast to a conventional method of quantification, i.e. peak integration, this approach is less sensitive to baseline distortion and overlapping resonances [453].

2.2.7. Association between CSF concentrations of selected metabolites, cohort status, MMSE scores and A β /tau concentrations

Metabolite levels were compared between the groups using boxplots in conjunction with Mann-Whitney test. Correlations between CSF metabolite concentrations and both MMSE scores and CSF tau/ A β concentrations were calculated. Diagnostic test evaluations for selected metabolites were carried out through Receiver Operating Characteristic (ROC) curve where area under the curve (AUC) represent how well the metabolites could distinguish between two diagnostic groups. All the univariate analyses were carried out in R.

2.3. Result

In this chapter, CSF metabolic fingerprints from 70 patients divided in 4 groups, were obtained using ^1H -NMR and were compared using multivariate data analysis approaches. A systematic workflow of this study can be seen next (**Figure 2.1**).

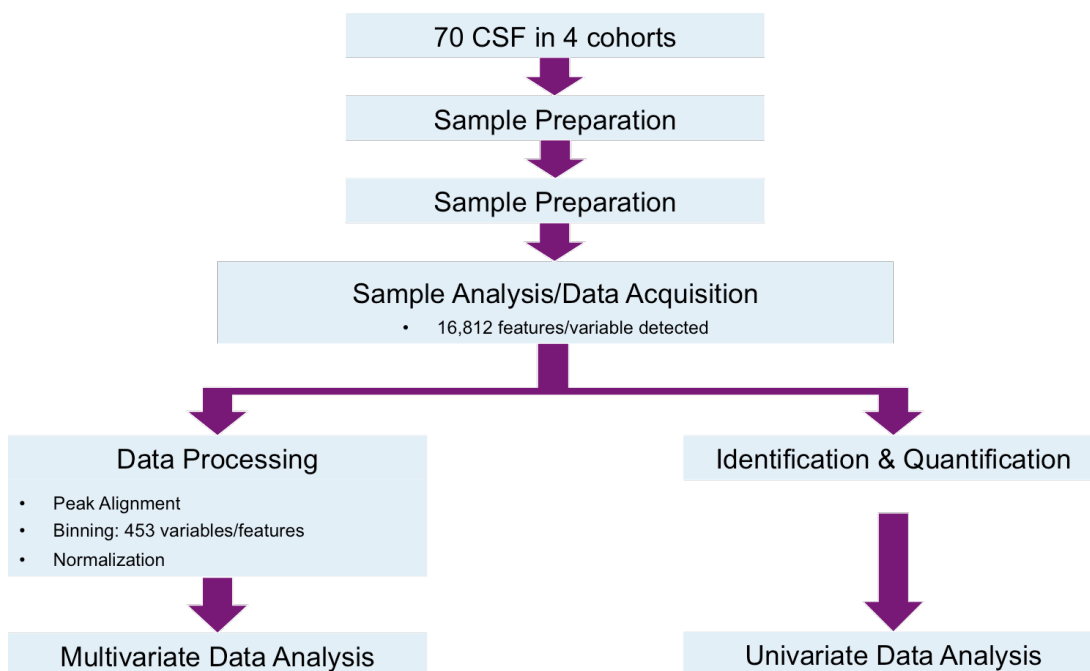


Figure 2.1. Sample preparation, data acquisition and analysis flow chart details for CSF fingerprinting by ^1H -NMR.

2.3.1. Clinical Outcomes

Clinical variables including age and sex were compared between AD and Old Controls which showed no significant differences. Then AD hallmarks, CSF $\text{A}\beta_{42}$ amounts, CSF total tau amounts, presence of ApoE4 gene and MMSE (Mini Mental State Examination) scores were compared between the two groups which showed significant differences. Summary of these results can be found in **Table 2.1**.

	Young Control (n=20)	Old Control (n=20)	AD (n=20)	FTD (n=10)	p-value AD <i>vs.</i> Old Control	p-value FTD <i>vs.</i> Old Control	Test
Mean age (s.d.)	30 (7)	77 (6)	77 (7)	73 (7)	0.968	0.242	MW
Male no. (%)	10 (50)	10 (50)	10 (50)	3 (30)	1	0.44	F
Mean A β ₄₂ (s.d.)	NA	834 (197)	380 (138)	854 (203)	<0.001	0.86	MW
Mean Total Tau (s.d.)	NA	59 (19)	163 (72)	85 (29)	<0.001	0.017	MW
With ApoE4, no. (%)	NA	3 (15)	16 (80)	1 (10)	<0.001	1	F
Median MMSE $\pm$ MAD	NA	29 $\pm$ 1	23 $\pm$ 4	NA	<0.001	NA	MW
Taking Aricept no. (%)	0 (0)	0 (0)	10 (50)	3 (30)	<0.001	0.03	F
Taking Namenda no. (%)	0 (0)	0 (0)	5 (25)	3 (30)	0.047	0.23	F
Taking Other ChEI no. (%)	0 (0)	0 (0)	3 (15)	1 (30)	0.23	1	F

Abbreviations: F, Fisher exact test (two sided); K, Kruskal-Wallis test; MAD, median absolute deviation; MW, Mann Whitney test; ChoEI, Cholinesterase Inhibitor

Table 2.1. Demographic characteristics and clinical data of the subjects involved in the study.

2.3.2. CSF Metabolomics profiling by ^1H -NMR in 4 cohorts

In order to determine changes in metabolites associated with AD, ^1H -NMR based metabolomics profiling was employed on CSF from 4 different cohorts, two Control, AD and FTD patients. Metabolic profiling initially produced more than 16,000 variables and these were reduced to 453 variables after undergoing data-processing.

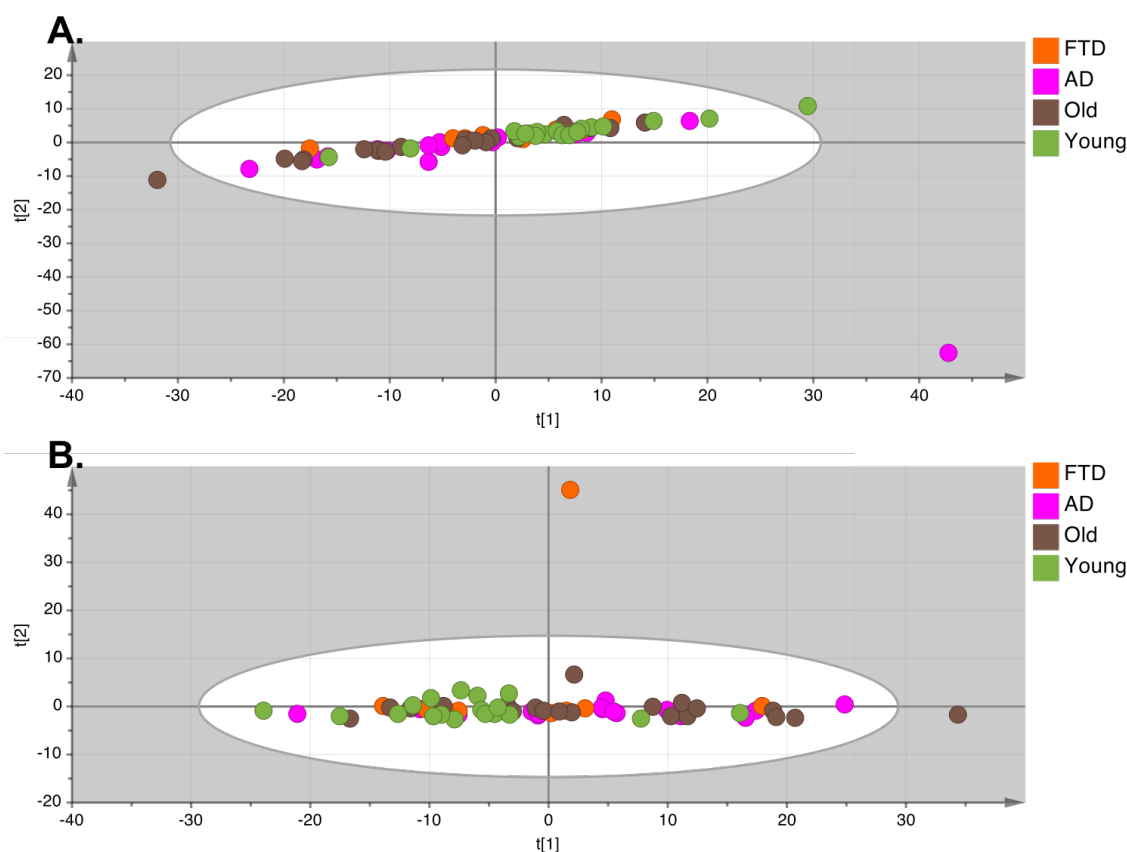


Figure 2.2. PCA score plot. (A) PCA score plot for all 70 CSF samples analysed by ^1H -NMR. One sample, AD (pink), was considered to be a strong outlier. **(B) PCA score plot after removing the outlying AD sample.** Two samples, one FTD (orange) and one Old Control (brown) samples were seen outside the Hotelling's T^2 tolerance ellipse that they were strong outliers.

For an overview of the dataset, a PCA model was built based on all 70 CSF samples (**Figure 2.2**). From the unsupervised models, three strong outliers were presented outside the Hotelling's T^2 tolerance ellipse in the score plot. These were samples from one FTD, one AD and one Old Control. NMR spectra of the three outliers were closely inspected and peaks for dimethyl sulfone, polyethylene glycol and possible ethanol were detected in the FTD, AD and Old Control samples respectively (**Figure 2.3**). These

three samples were considered to be outliers and were consequently removed from further analysis as they were shifting the principal components away from the mean. In terms of metabolic profile, PCA failed to separate each cohort suggesting subtle changes in total metabolite levels in CSF among these groups.

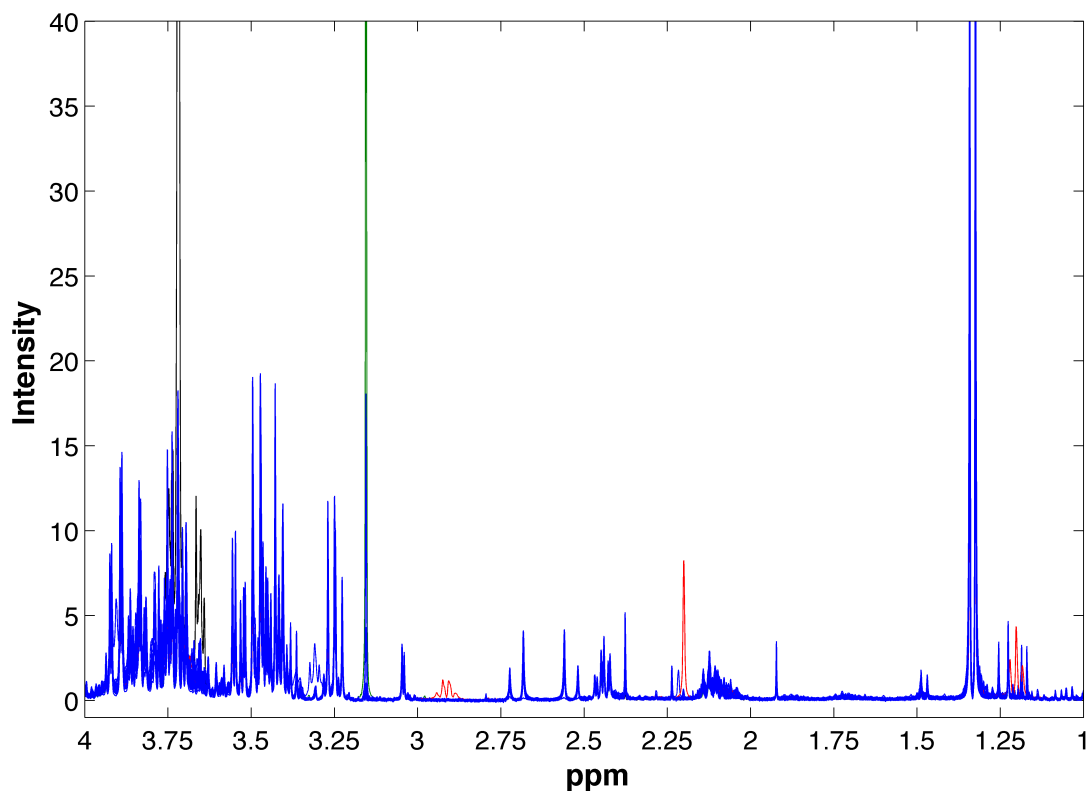


Figure 2.3. NMR spectra (between 1.00ppm and 4.00ppm) of all 70 CSF samples after data-processing steps. Spectra in black, green and red are AD, FTD and Old Control respectively which were found to be strong outliers from PCA models. Peaks at around 3.75ppm (red) are due to polyethylene glycol, peak at 3.20ppm (purple) is due to dimethyl sulfone and peaks at 2.90ppm, 2.45ppm and 1.20ppm (green) are possibly due to ethanol.

After excluding the outliers, the processed data from the NMR experiment underwent discriminant analysis by building an OPLS-DA model for all four groups (**Figure 2.4**). The biggest separation was observed between Young Control and all three old cohorts (AD, FTD and Old Control) suggesting the largest metabolic change between all groups was due to the effect of aging. Between the three old cohorts, moderate separations could be observed while AD and Old Control showing the weakest separation. In terms of metabolic profile changes from Old Control to neurodegenerative disorder disease, AD and FTD showed an opposite trend. However the score plot failed to produce distinct clusters from Old Control to the dementia groups. Subsequently pair-wise multivariate analysis were carried out in order to determine variables driving separations between the groups.

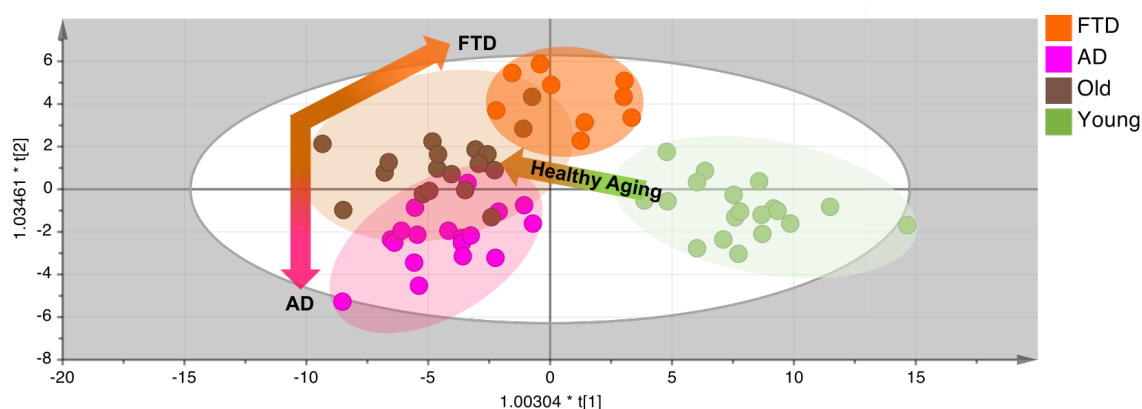


Figure 2.4. OPLS-DA score plot showing all CSF samples with disease class as a discriminating factor. Young Control group is well separated from the rest of the cohorts.

Figure 2.5 shows the OPLS-DA score plots in conjunction with the corresponding S-plots for each pair-wise comparison. The cross-validated predictive residuals (CV-ANOVA) from each OPLS-DA model were used to derive p-values for statistical significance of the detected group separations. When AD samples were compared to those from Young Control, a robust discrimination was observed with CV-ANOVA $p = 8.63 \times 10^{-4}$ (**Figure 2.5A**). The corresponding S-plot (**Figure 2.5B**) presented two variables showing high correlation (>0.6) and covariance (>0.1), one variable (1.18ppm) increased and the other variable (2.38ppm) decreased in AD.

Figure 2.5C shows the pair-wise group comparison between AD and Old Control groups which failed to reveal a significant separation (CV-ANOVA $p > 0.05$). Finally, the OPLS-DA model comparing AD to FTD revealed distinct clusters from each other with CV-ANOVA $p = 8.70 \times 10^{-3}$ (**Figure 2.5E**). Two variables with high correlation (>0.6), covariance (>0.1), both decreased in AD (1.25ppm, 2.38ppm), could be observed from the corresponding S-plot (**Figure 2.5F**).

Of the four metabolic variables with high correlation and covariance from the three pairwise models, two of them were found to be identical (2.38ppm), decreased in AD in both AD vs FTD and AD vs Young Control models.

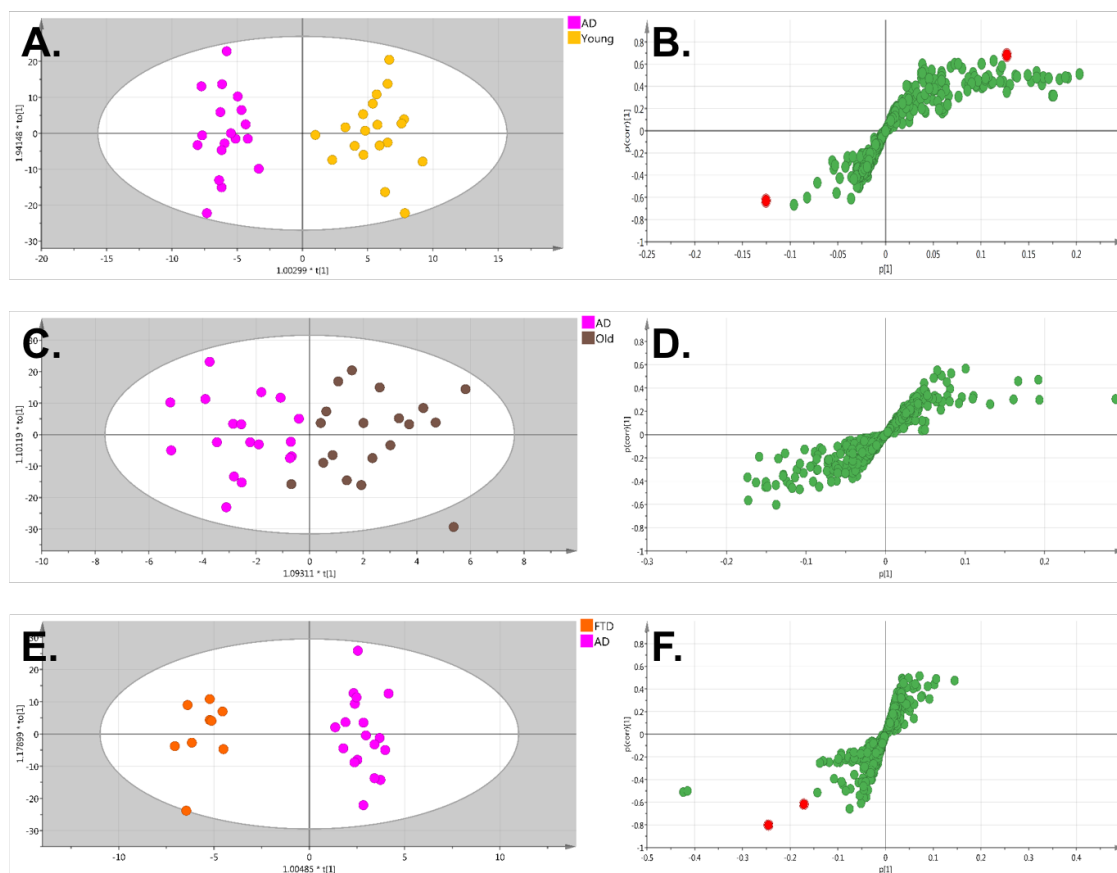


Figure 2.5. OPLS-DA of pair-wise comparisons between the four groups. (A,B) Score plot and corresponding S-plot comparing between AD and Young Control groups. The strength of the model was CV-ANOVA $p = 8.63 \times 10^{-4}$. (C,D) Score plot and corresponding S-plot comparing between AD and Old Control groups. There was no significant separation between the groups in its CV model. (E,F) Score plot and corresponding S-plot comparing between AD and FTD groups. The strength of the model was CV-ANOVA $p = 8.70 \times 10^{-3}$. Red dots in S-plots in B, D and F represent variables with high correlation (>0.6) and high covariance (>0.1) associated with AD.

2.3.3. Metabolite identification and quantification

The selected variables from the multivariate analysis underwent identification. Variable 2.38ppm was found to be pyruvate's resonance while we were unable to identify metabolites resulting from variables 1.25ppm (singlet) and 1.18ppm (doublet).

Along with pyruvate, 20 additional metabolites were further identified from a raw spectrum (**Figure 2.6**) using our in-house database and the literature [[444](#), [449-452](#)]. Concentrations of eleven of these 20 metabolites were found to be present above the limit of quantification (LOQ). These eleven included glucose and pyruvate and their levels were subsequently measured in mM amounts in all samples (summarised **Table 2.2**). Median levels were compared across the groups using a heat map which included clustering analysis (**Figure 2.7**). From the clustering analysis, 11 CSF metabolite concentrations in Young Control were found to be the most dissimilar and those in Old Control to be the most similar relative to the AD group.

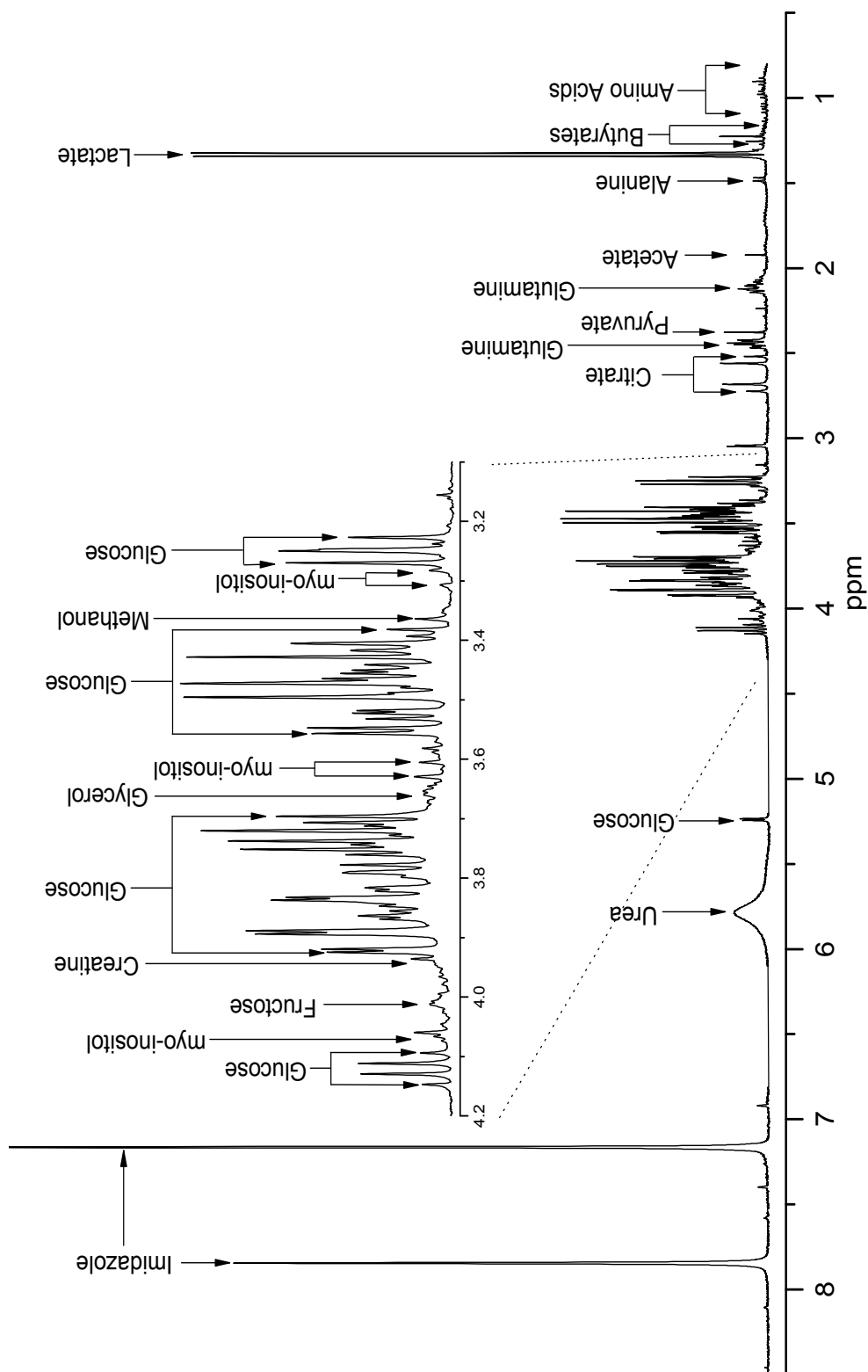


Figure 2.6. ^1H -NMR spectrum of CSF (an Old Control sample) with some resonances being identified and labeled.

Metabolites	ppm	Amount Average (mM)				p-value AD vs. Old
		AD (n=20)	FTD (n=10)	Old Control (n=20)	Young Control (n=20)	
Urea	5.78, s	2.9312	2.5505	3.1687	2.151	0.8331
Glucose	3.21, d	2.7309	3.0203	2.7399	2.5891	1
myo-inositol	4.05, t	0.0938	0.0941	0.0914	0.0799	0.5837
Creatinine	3.04, s	0.0425	0.0484	0.0477	0.0335	0.0274*
Creatine	3.04, s	0.03	0.0283	0.0284	0.0225	0.14
Citrate	2.67, d	0.1951	0.1936	0.1747	0.1461	0.2257
Pyruvate	2.38, s	0.0399	0.0596	0.0506	0.0475	0.0129* §
Acetate	1.91, s	0.0335	0.0238	0.0262	0.0199	0.0351*
Alanine	1.46, d	0.0319	0.0323	0.0342	0.0231	0.4314
Lactate	1.33, d	1.0491	1.247	1.071	0.9495	0.5273
Valine	1.03 ,d	0.017	0.0177	0.0187	0.0163	0.4823

Table 2.2. Concentrations in CSF in mM and p-values for AD vs Old Control (Mann-Whitney test). The test showed creatinine, pyruvate and acetate levels being significantly different between AD and Old Control (as denoted by *). FDR was then applied and showed pyruvate level being different at FDR $p < 0.05$ level (as denoted by §).

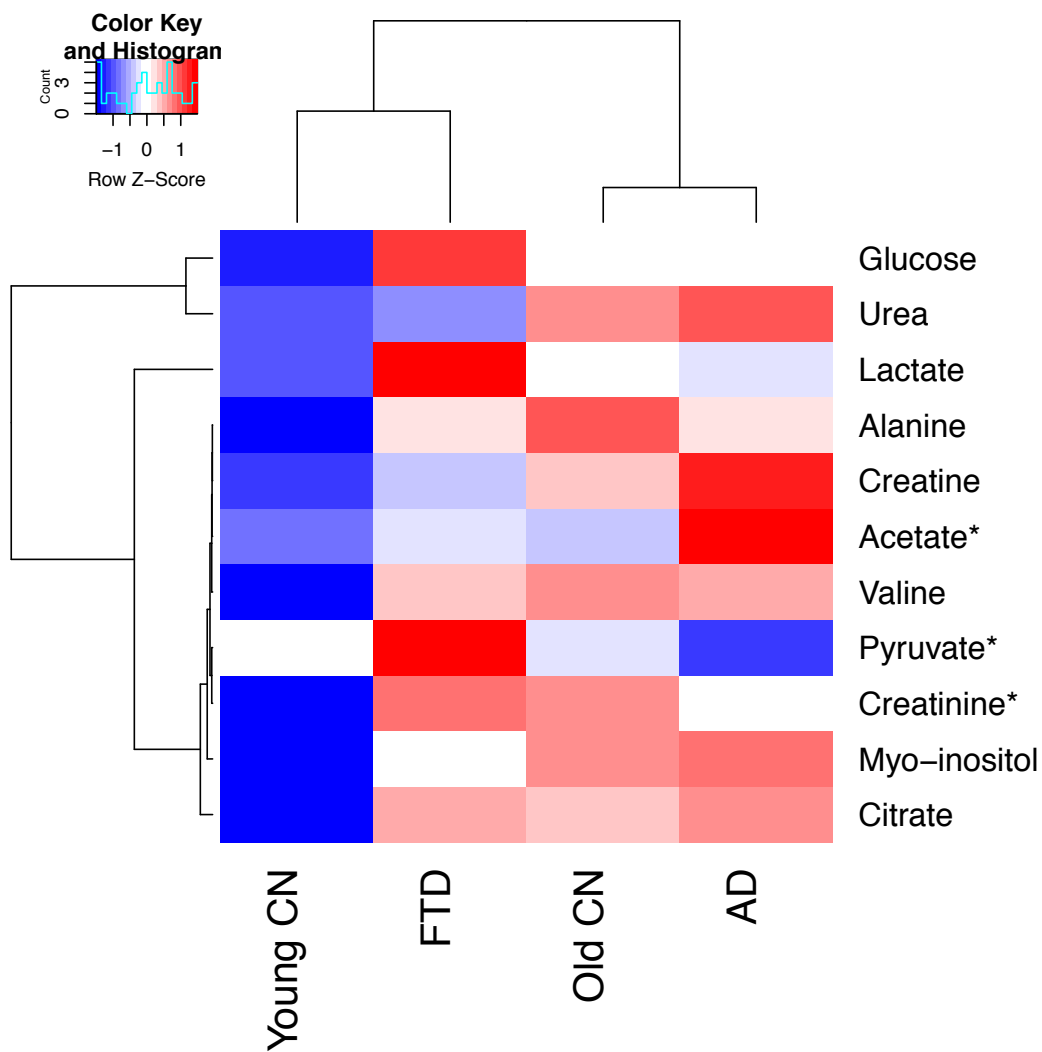


Figure 2.7. Heat map showing the median concentrations of eleven metabolites. The clustering analysis shows CSF concentrations of the 11 metabolites in Young-Control is the most dissimilar and those in Old Control to be the most similar relative to AD (*AD vs. Old Control shows pyruvate, creatinine and acetate $p < 0.05$, Mann-Whitney).

2.3.4. Pair-wise univariate analysis between groups

Univariate analyses of between-groups were then performed for the 11 metabolites using Mann-Whitney U tests. Of these, 3 metabolites in AD were found to be significantly different to those in the other groups (**Table 2.2**) with their statistical power <80%. CSF pyruvate and creatinine levels were found to be lower while acetate level was higher in AD compared to Old Control (Mann-Whitney $p < 0.05$). Subsequent box plots of these three metabolites were plotted to compare the levels between the four clinical groups (**Figure 2.8**).

The utility of the three selected metabolites was then tested as binary discriminators between AD and Old Control samples using AUC metric from ROC curves (**Figure 2.9**). AUC of 73.42%, 70.79%, 69.87% for pyruvate, creatinine and acetate CSF concentrations respectively were observed. On all three metabolites combined, the resulting AUC value was 74.47%.

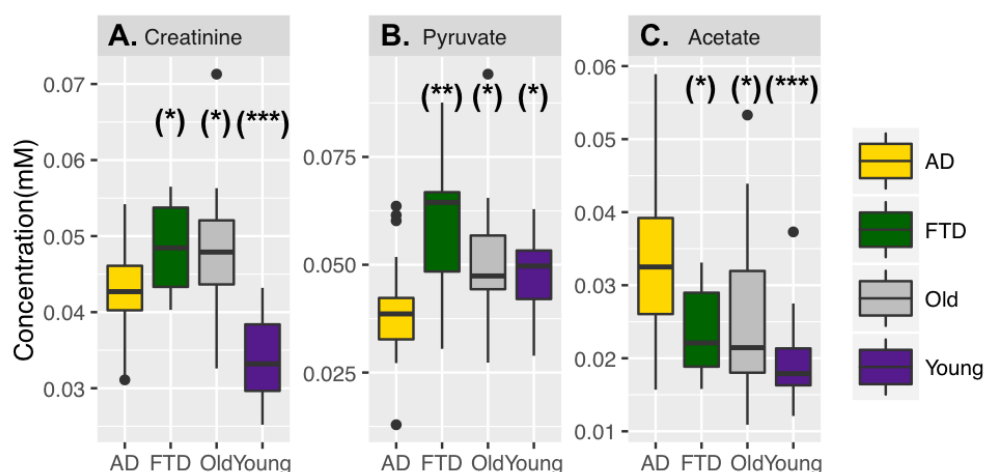


Figure 2.8. Box plots showing the levels of (A) Creatinine, (B) Pyruvate and (C) Acetate differentiating between AD and Old Control. Mann-Whitney p-values are calculated from the comparison between AD to the other groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

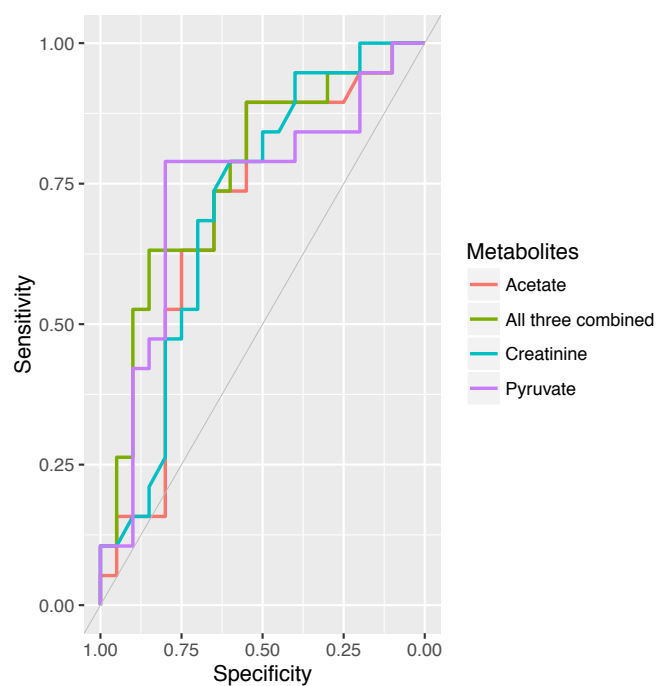


Figure 2.9. ROC curves for comparison between AD and Old Control for the three selected three markers and when all three are combined. Pyruvate, creatinine, acetate and all three together resulted in AUC of 73.42%, 70.79%, 69.87% and 74.47% respectively.

2.3.5. Association of pyruvate, acetate and creatinine with cognition, known CSF neuropathological features and aging

This study asked whether the candidate CSF metabolite biomarkers had any relationship with cognitive performance (MMSE) and well-established CSF measure of AD pathology i.e. high total tau and low $A\beta_{42}$ CSF concentrations (Table 2.3). After adjusting for age of participants at the time of CSF collection, the candidate markers were correlated with MMSE scores and $A\beta_{42}$ and total tau amounts in CSF (AD n=20, Old Control n=20). Pyruvate and acetate were found to be associated with $A\beta_{42}$ and total tau levels whereas creatinine showed no significant correlations. All three metabolites failed to show significant relationships with MMSE. Summary of these results can be found in **Table 2.3**.

The three candidate markers were also tested whether their levels were associated with natural healthy aging. Using Old Control and Young Control samples (n=40), Pearson correlations between the levels of three metabolites and participants' age at the time of CSF collection were carried out and showed acetate and creatinine associating with natural aging.

Metabolites	$^{\S}A\beta_{42}$	$^{\S}\text{Total Tau}$	$^{\S}\text{MMSE}$	$^{\perp}\text{Age}$
Pyruvate	0.4254**	-0.5281***	0.3082	0.1480
Acetate	-0.3441*	0.3395*	-0.3471	0.03360*
Creatinine	0.1948	-0.3202*	0.2219	0.7481***

Table 2.3. Correlation coefficient values derived from correlation analyses. Partial correlations (Pearson's) were carried out to CSF $A\beta_{42}$, CSF total tau and MMSE after adjusting for age. Pearson's correlations were carried out to participants' age.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

§ AD n=20, Old Control n=20

$^{\perp}$ Old Control n=20, Young Control n=20

2.3.6. CSF biomarkers for Alzheimer's disease versus FTD

Previously from AD versus FTD OPLS-DA model (Section 3.3.2), pyruvate was found to be one of the strongest discriminator between the groups. Box plots was employed and observed its level being significantly lower in AD when compared to FTD (**Figure 2.8**, Mann-Whitney p-value <0.01). When its utility as a binary discriminator was tested, the ROC AUC was 84.21% for the AD versus FTD, and 70.24% for the FTD versus Old Control (**Figure 2.10**).

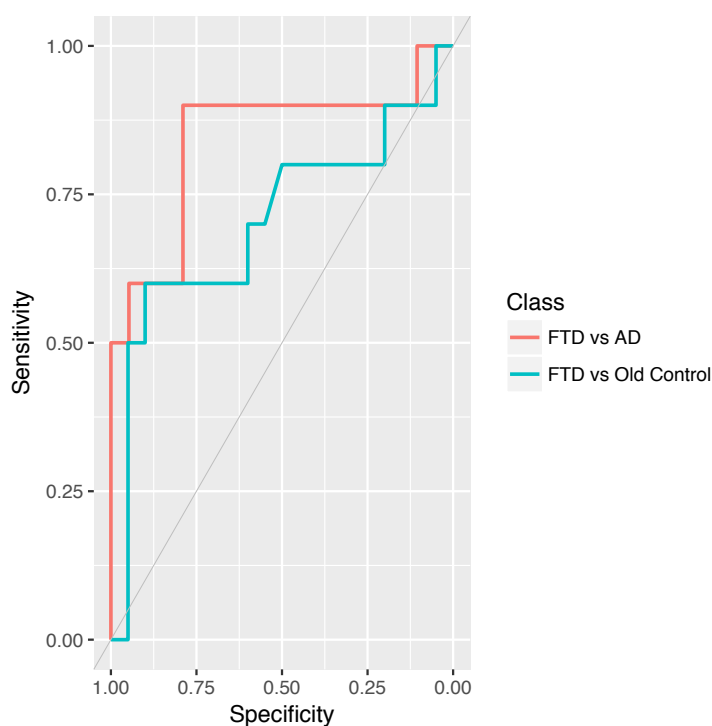


Figure 2.10. ROC curves for comparison for FTD vs AD (red) and FTD vs Old Control (green) in pyruvate concentration. The AUC values were 84.21% and 70.24% respectively.

2.4. Discussion

Here, we employed a metabolomics approach using ^1H -NMR to examine changes in the CSF concentrations of small metabolites across the spectrum of normal aging as well as in two neurodegenerative diseases, AD and FTD. A requirement of a AD diagnosis test is that the molecules differentiating AD from cognitively normal people must also differentiate from other neurodegenerative diseases. By including FTD in the study design, we were able to potentially enhance the specificity for Alzheimer's markers in CSF.

By applying multivariate analysis approach, 4 metabolic variables were mined which would discriminate AD from Old Control (2 features) and FTD (2 features). Variable 2.38ppm was present in both models and was later identified as pyruvate. We were unable to identify the other two variables, 1.18ppm and 2.25ppm.

Targeted analyses were subsequently carried out to find concentrations of pyruvate and 10 other metabolites. When their levels were compared across the four groups (**Figure 2.9**), Young Control was the most dissimilar, suggesting the natural ageing to be the biggest discriminator between the 4 clinical groups. Metabolite concentrations were relatively lower in Young Control, likely due to natural aging affecting choroid plexus. CSF production is lowered with aging and consequently CSF metabolites would be more concentrated in the elderly [[454](#), [455](#)].

When AD was compared to Old Control, pyruvate level was found decreased significantly. Previously pyruvate levels have been found increased in AD CSF [[456](#)], contradicting the trend found in this study. A possible explanation for the inconsistent result is that the published result were not validated and the criteria used for diagnosis of probable AD has now become obsolete and may be reclassified as having mild cognitive impairment (MCI) by recent guidelines. Yet again both studies had few patients and further work is needed to ascertain the different reported data.

In the central nervous system, energy metabolism, production of adenosine triphosphate (ATP), involves conversion of glucose to pyruvate (or lactose) by glycolysis, oxidation of pyruvate to CO_2 by the pyruvate dehydrogenase complex, and the Krebs tricarboxylic acid (TCA) cycle (**Figure 2.11**). Although the brain represents only 2% of the total body mass, it requires high amounts of energy, using glucose as its main energy substrate. About 20% of the oxygen and 25% of glucose consumed by the human body are dedicated to cerebral functions [457]. A number of AD studies have shown that glucose hypometabolism is known to occur in AD brain before the pathology and symptoms manifest [107, 458-460]. Although in our study did not show altered glucose level, the lower amounts of pyruvate could signify a disruption in energy metabolism as pyruvate is an end product of glycolysis and an essential component required for TCA cycle. This result can further be supported by a report which has shown a reduced level of succinate in AD CSF [461].

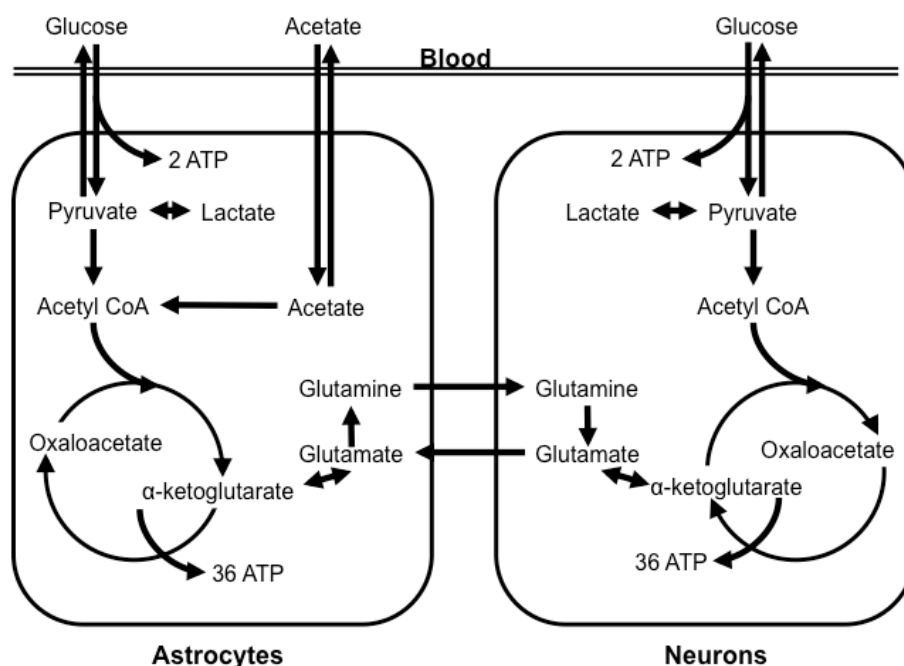


Figure 2.11. Metabolic pathways showing brain uptake of glucose and acetate in astrocytes and neurons. While Astrocytes can intake both glucose and acetate and consume them, neurons are known to consumes glucose only [462, 463].

We also found acetate concentration increased in AD CSF, a finding which has not been mentioned previously in AD CSF studies. In AD saliva, increased acetate has recently been reported [464]. Energetic hypometabolism in AD brain may lead to

increased energy requirements from additional energy sources in which acetate could be used as an alternative source of energy. This could be supported by the fact that the fold-changes in mean AD pyruvate and acetate levels compared to mean Old Control pyruvate and acetate levels were similar. Pyruvate decreased by 21.15% whereas acetate increased by 22.18% in AD. Acetate travels into the brain via monocarboxylic acid transporters (MCATs) from blood through the blood brain-barrier (BBB) which is then exclusively used in astrocytes [465, 466]. It could be hypothesised that in brain suffering from AD, high demand of this alternative energy source increases expression of MCATs and hence results in higher uptake of acetate in order to compensate energetic hypometabolism [467].

Creatinine, like pyruvate, was found significantly decreased in AD in contrast to previous studies where an increase was reported [468, 469]. Creatinine is a product of spontaneous non-enzymatic conversion from creatine phosphate, an energy storage source synthesized from creatine. Therefore the three nitrous compounds are closely related to each other and creatinine in CSF can be said to reflect creatine phosphate in brain [470]. Little is known on creatine/creatinine role in brain, but higher creatine level in AD from this study (although not significant) combined with lower creatinine level could represent dysfunction of creatine/creatine-kinase/phosphocreatine network suggesting further energy storage or energy production implication in brains with Alzheimer's disease pathogenesis [471].

Using a targeted approach, we have determined pyruvate, acetate and creatinine to be altered in AD, all involved in energetic metabolism. Altered energetic metabolism has also regularly been pointed out as a marker of natural aging [472-476]. So we asked whether changes in pyruvate, acetate and creatinine concentrations were a reflection of aging-related processes. Correlation analyses on two Control samples (Young and Old) showed acetate and creatinine levels increasing with healthy aging. When we looked at their trends from Young Control < Old Control < AD (**Figure 2.8**), acetate showed a

trend of increasing between the groups. This suggests altered acetate metabolism in AD may be a result of more advanced process of aging.

We then accessed their diagnostic ability when combined together, the ROC AUC value was 74.47% between AD and Old Control. This is interesting as it shows fair prediction accuracy. However this finding is needed to be tested on larger validation sample set since the small number of samples in this study did not permit a training and validation set partition. Of these three metabolites, pyruvate and acetate showed significant correlations to known AD pathological features, $A\beta_{42}$ and total tau. With CSF $A\beta_{42}$ and tau levels being known to be altered well in advance of cognitive impairment, the finding suggests a possible role of the two metabolites in pre-symptomatic diagnosis. Additionally pyruvate was also found to be the strongest discriminator in AD vs FTD (ROC AUC 84.21%). This suggests pyruvate has a fair AD discriminating abilities from both Old Control FTD, fitting two important criteria to be defined as an AD biomarker.

Overall findings from this study suggest a deranged energetic (pyruvate) metabolism in AD brain and acetate is used to compensate the hypometabolism. However it is important to note that these changes in CSF are not the direct consequence of altered metabolism in AD neurons and astrocytes. It is widely accepted changes in small metabolites' concentrations reflect in those brain [\[477, 478\]](#), but little is known about penetration abilities of metabolites between CSF and brain. Therefore a better understanding on the relative expression of small molecules transporters at the choroid plexus versus the brain endothelium is required before defining pathological profiles among AD brains.

2.5. Conclusion

The main finding to emerge from this study is that AD CSF showed an accentuation of age-related alterations, particularly those central to energy metabolism in the brain. Pyruvate and acetate showed significant correlations to two well-known AD pathological features, A β and tau, but not with cognitive performance. Additionally, we observed distinct profiles of CSF metabolites changes between FTD and AD, but no significant changes between AD and Old Control

***Mass-Spectrometry
based Metabolic
Phenotyping of
Cerebrospinal Fluid***

3.1. Introduction

The previous chapter (Chapter 2) attempted to find CSF (cerebrospinal fluid) metabolites which would discriminate AD from Old Control (age-matched to AD) using Nuclear Magnetic Resonance technique (NMR). Using a targeted approach, 11 metabolites were quantified and pyruvate, acetate and creatinine concentrations were found to be altered in AD CSF compared to Old Control. Combining these 3 metabolites provided predictive property through the estimation of Receiver Operating Characteristic (ROC) with Area Under the Curve (AUC) of 0.7447. Even though these were significant findings, identifying additional metabolic features could help with prediction values for AD classification. To achieve this goal, we decided to apply another analytical platform, Liquid Chromatography-Mass spectrometry (LC-MS) using the same set of samples as in the previous study.

LC-MS and NMR are the two most widely used techniques in metabolomics. Both are often used as complementary methods with each having their particular advantages and disadvantages. These techniques have been discussed in detail in Chapter 1. Briefly, NMR provides higher reproducibility, but only detects the more abundant metabolites (in mM concentration region). LC-MS has higher technical instability, but offers much higher sensitivity detecting metabolites in nM concentration regions. This advantage in terms of sensitivity in LC-MS technique would allow detection of more metabolic features from CSF and thus likely to find metabolic features with stronger discriminating ability between AD and Old Control. Wishart et al [444] showed that LC-MS detected in CSF more than 200 unique metabolic signatures/features while NMR was able to quantify 47 metabolites.

Additionally in this study, we decided to use two orthogonal Liquid Chromatography (LC) methods, Reversed Phase (RP)-based and Hydrophilic Interaction Chromatography (HILIC)-based, for metabolite separation. This would allow maximum coverage of CSF metabolites which are mainly present mid to high polarity. Reversed Phase method uses a column with a hydrophobic stationary phase, more suited for

separation of relative less polar metabolites such as lipids. HILIC uses a hydrophilic stationary phase, suited for separation of more polar metabolites such as lactate and urea.

Two AD CSF metabolomics studies have applied this particular ‘complementary RP and HILIC methods’. The first was by Trushina *et al* [382] which identified 150 metabolites with significant changes in their levels ($p < 0.05$). Of these 150, 4 metabolites were found to be 2-3 fold lower, 9 metabolites 2-3 fold higher in AD. The second study was by Ibanez *et al* [384] which was able to separate AD from controls with 97.6% prediction accuracy based on 17 metabolites. However, a key limitation of these two previous studies is a failure to show classification accuracy of AD from control samples on test samples, samples not used during discovery of AD discriminating metabolic features, a limitation that was also present in Chapter 2. In biomarker or/and informative studies, initial discovery of a predictive model would be a main objective, but it is equally important to assess the quality of the model using test samples. A poor performance of the model on test samples will suggest the model using training samples was over-fitted. Strong performance on test samples will point minimal over-fitting and more likely to produce better AD forecasting in general population.

In the present work, we will discuss metabolic differences in CSF samples between AD and Old Control groups using two LC-MS techniques, RP and HILIC. We will attempt to overcome the limitation seen in [382, 384] and Chapter 2 by initially identifying a panel of CSF metabolites with the most significant contribution toward AD classification using training samples (3/4) and assessing their predictive performances of the newly discovered panel using test samples (1/4). Additionally it is also important to note the inclusion of two additional clinical groups, Young Control and FTD in this cohort. This will allow us to discuss whether differences in AD CSF metabolites reflect on an evolution of aging-related process, normal healthy metabolite variation and/or AD-related pathological changes.

3.2. Methods

3.2.1. CSF sample collection and Immunoassays of CSF tau and A β_{42}

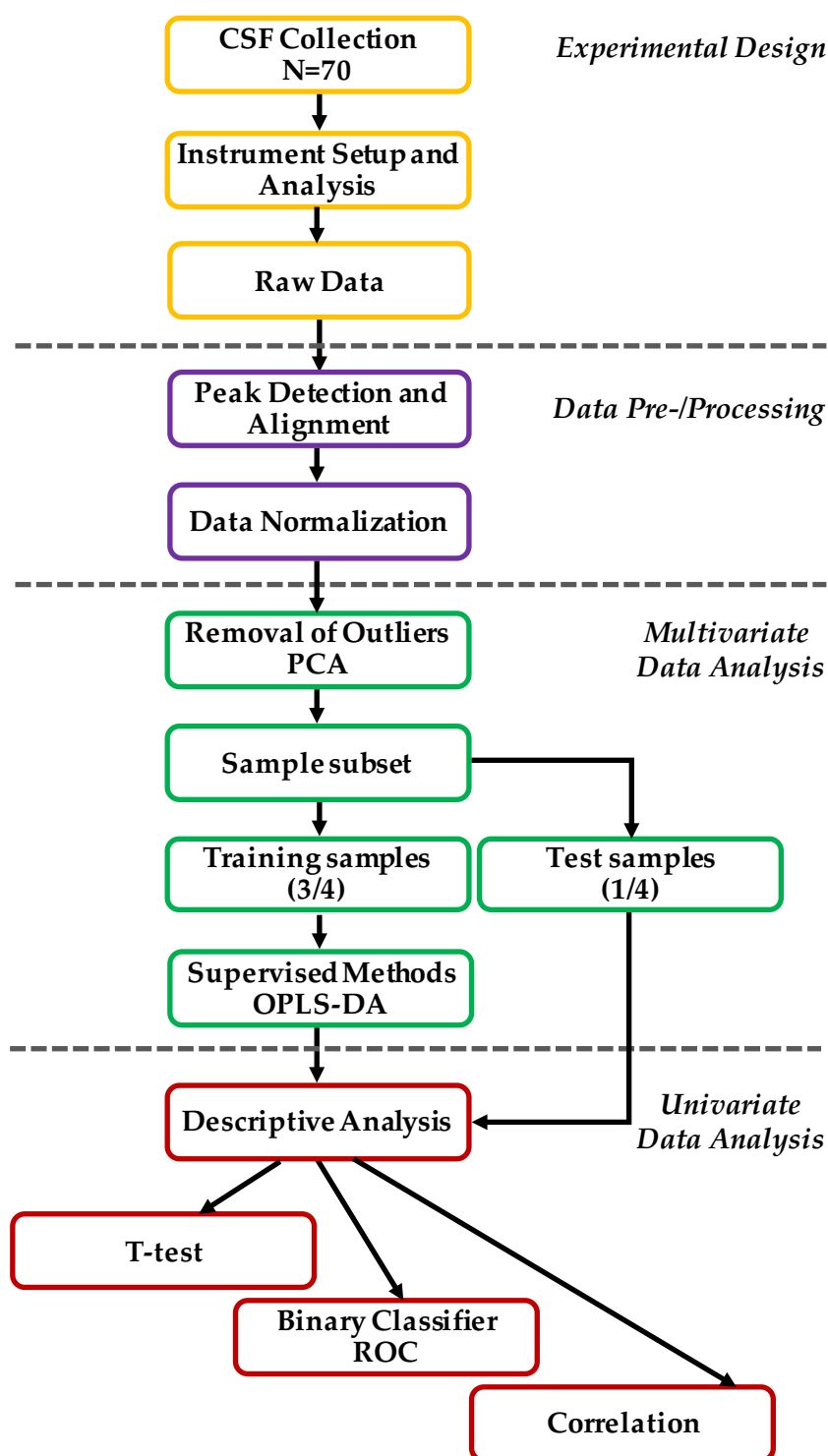
Same samples from the previous chapter were analysed in this investigation and thus the procedures for CSF collection and immunoassays of CSF tau and A β_{42} would be same (Section 2.2). Systematic workflow in which how sample analysis and data analysis were carried out in this study can be found in **Figure 3.1**.

3.2.2. Sample preparation

70 CSF samples were thawed on ice and 50 μ L of each CSF sample was added into a glass Liquid Chromatography vial with a 400 μ L insert (Chromacol, UK). One sample from AD group was found to have insufficient volume for analysis and consequently was not analyzed. 20 μ L from each of the samples were collected in an eppendorf tube in order to obtain a pooled sample for quality control (QC) analysis.

3.2.3. LC-MS data acquisition

LC-MS analysis was completed using a Waters ACQUITY UPLC® coupled to a Waters Xevo® QToF (Waters: Milford, USA). For RP, 10 μ L sample was injected onto C18 column (Supelco Discovery HS C18, 150 mm $\times$ 2.1 mm i.d., 3 μ m particle size). Separation was performed at 40 °C with a flow-rate of 0.1 mL/min. Mobile phases were composed of 0.1% w/v ammonium formate in water (A) and 0.1% w/v ammonium formate in methanol (B). Metabolites were eluted using the following gradient elution method: from 0% B at 0 min to 10% B in 4 min, then to 100% B in 6 min, maintaining this gradient 5 min. A 1 min re-equilibration was employed before injection of the next sample.



Abbreviation: PCA, Principal Component Analysis; OPLS-DA, Orthogonal Partial Least Square-Discriminant Analysis

Figure 3.1. Workflow of the study.

For HILIC, its method has been described elsewhere [479]. Briefly, 5 μ l of CSF was injected into on a Merck Sequant Zic-HILIC column (150 $\times$ 4.6 mm, 5 μ m particle size. Separation was performed at room temperature with flow rate of 0.3ml/min. Mobile phases were composed of 0.1% v/v formic acid in water (A) and 0.1% v/v formic acid in acetonitrile (B). Metabolites were eluted using the following gradient elution method: from 80% B at 0 min to 20% B in 30 minutes then 10 minutes of column re-equilibration.

The applied mass spectrometry conditions were the same for both the RP and HILIC methods. MS Data was collected in the centroid mode over the mass range m/z 50–1000 with an acquisition time of 0.2 second. The XEVO QToF was operated with a capillary voltage of 3.2kV in positive and -2.6kV in negative mode. Cone voltage was 35V in positive and 45V in negative modes. For both ionisation modes, the desolvation gas flow was 800L/hour and maintained at 400°C, the source temperature was 120 °C and the cone gas was set to 30 L/hour. All data were collected using Waters MS^e technology, enabling data collection at two “levels”. Level one (MS¹) is traditional MS raw data with parent molecular ionization. Level two (MS²) initiate high collision energies (10-50eV) within the MS source causing molecular fragmentation meaning fragmentation data can be collected simultaneously. Additionally LockSpray mode was used to ensure high accuracy is maintained; leucine-enkephalin was used as a lock mass (m/z 556.3 in positive and m/z 554.3 in negative) at a concentration of 200ng/mL and a flow rate of 10 μ L/min.

Samples were analyzed in a randomized order with QC samples being analyzed after every 10 CSF samples.

3.2.4. Data Processing and Treatment

The acquired data were converted from Waters ‘.raw’ file into ‘netCDF’ file in MassLynx (Waters, Milford, USA). In R (version 3.2), the ‘netCDF’ MS data (MS¹ level) underwent pre-processing steps including peak picking and alignment using the *xcms* software package [480]. For peak picking, the centWave algorithm was applied, its parameters were ppm=10 and peakwidth=5-60seconds. RP positive produced 2343 metabolic features, RP negative produced 621 features, HILIC positive produced 869 features, and HILIC negative produced 593 features.

The pre-processed datasets from the 4 methods were normalized separately which resulted in the best clustering of QC samples in their Principal Component Analysis (PCA) score-plots. Total normalization was applied for RP positive, quantile normalization for RP negative and HILIC positive [481], and linear baseline normalization for HILIC negative [482]. The processed datasets were then combined into one dataset (69 samples with 4426 features), and exported to SIMCA (MKS Umetrics AB, Sweden).

3.2.5. Data Analysis

Prior to data analysis, the unit variance (UV) scaled dataset was first inspected for detection of outlier(s). PCA models showed 3 AD samples outside the Hotelling’s T^2 tolerance ellipse and there were consequently removed (**Figure 3.2** and **Figure 3.3**).

As a first step of multivariate data analysis, an OPLS-DA model was built on all 4 groups to compare their metabolic profiles. Then the dataset containing 66 samples were divided into a training dataset (3/4) and test dataset (1/4), sex- and age-matched. The training dataset was subject to pairwise comparisons using OPLS-DA to detect variables driving group separations. Variance Important Plot (VIP) and coefficient plot from each OPLS-DA model were analysed and ‘top 50’ metabolic features from each plots were derived. Metabolic features present in both ‘top 50’s were eventually selected as the most important discriminating metabolic features.



3.2.6. Metabolic features Identification

Identification of the metabolic features discriminating between the groups was attempted by applying two procedures [483]. Firstly, we carried out “ion annotation” to recognize a group of metabolic features which are likely to originate from the same compound. For this, the discriminating metabolic features were correlated to other features with similar retention time (± 0.1 seconds), and extracted those with coefficients higher than 0.8 ($p < 0.05$). Then we compared their elution profiles, represented by their extracted ion chromatograms (EICs). If they shared similarly shaped elution profiles, they were considered to originate from the same compound. Differences in m/z values were calculated out to deduce their adducts and metabolite mass.

The second procedure, mass-based identification, involved using deduced metabolite mass to search against an in-house database and public metabolite databases (HMDB [449] and METLIN [484]). Similarly, their fragmentation patterns were also studied by comparing with database to aid their identifications.

3.3. Results

This chapter looks at CSF metabolic profiles of 66 samples using LC-MS as an analytical platform. Although 70 CSF samples were available initially, one sample had insufficient volume for analysis while three samples were classified as outliers and these 4 samples were therefore excluded from further analyses. Patient details on the remaining 66 samples including comparisons on clinical variables (age, sex, A β ₄₂ and tau) between the groups are represented in **Table 3.1**. Additionally, ROC analyses on CSF A β ₄₂, CSF total tau, ApoE4 and Mini Mental State Examination (MMSE) score were carried out to test their AD discrimination abilities from Old Control (**Table 3.2**). All 4 clinical variables showed strong discriminating abilities between AD and Old Control (all ROC AUC>0.80).

	AD (n=16)	FTD (n=10)	Old Control (n=20)	Young Control (n=20)	p-value			Test
					AD-Old Control	AD-FTD	AD- Young	
Mean age (s.d.)	78.50 (6.46)	72.90 (6.67)	76.80 (5.56)	30.10 (6.52)	0.3804	0.0539	<0.0001	MW
Male, no. (%)	8 (50)	3 (30)	10 (50)	10 (50)	1	0.428	1	F
Mean A β 42 (s.d.)	392.50 (1.47)	857.80 (201.71)	833.65 (196.93)	-	<0.0001	<0.0001	-	MW
Mean Total Tau (s.d.)	160.38 (73.79)	89.10 (32.77)	59.10 (18.58)	-	<0.0001	<0.0001	-	MW
With ApoE4, no. (%)	12 (75)	1 (10)	3 (15)	-	0.0005	0.0036	-	F
Mean MMSE (s.d)	22.25 (2.29)	-	28.65 (1.04)	-	<0.0001	-	-	MW
Taking Aricept, no. (%)	9 (56)	3 (30)	0 (0)	0 (0)	0.0001	0.2475	0.0001	F
Taking Namenda, no. (%)	4 (25)	3 (30)	0 (0)	0 (0)	0.0309	1	0.0309	F
Taking Other ChEI, no. (%)	2 (13)	1 (30)	0 (0)	0 (0)	0.1905	1	0.1905	F

Abbreviations: F, Fisher exact test, two sided; K, Kruskal-Wallis test; MW, Mann Whitney test.

Table 3.1. Demographic characteristics and clinical data of the 66 subjects involved in the study.

	ROC		
	AUC	Sensitivity	Specificity
CSF A β ₄₂	0.96	0.75	0.85
CSF Total Tau	0.99	0.94	0.95
ApoE4	0.80	0.75	0.85
MMSE	1.00	0.94	1.00

Table 3.2. Prediction abilities of AD clinical markers on AD (n=16) versus Old Control (n=20) using ROC analysis approach.

3.3.1. Metabolic profiling in all 4 cohorts

As a first step, a discriminant analysis was performed on all 4 groups by employing OPLS-DA. The score plot (**Figure 3.4**) shows the first latent component separating between young control and old participants (Old control, AD and FTD). The second latent component separated old participants, AD being further away from FTD than from Old Control. The total variance explained by these two components was 57.3%.

Using the training dataset, pairwise comparisons were carried out to test the magnitude of difference in CSF metabolic profiles between AD and three other groups using OPLS-DA method. All three OPLS-DA score plots showed clear separations between AD and other groups (**Figure 3.5**, **Figure 3.6**, **Figure 3.7**). We then tested reliabilities of these calculated models by performing 7-fold cross validation. CV-ANOVA showed AD *vs.* Old Control model not showing a reliable separation ($p > 0.05$) while AD *vs.* FTD and AD *vs.* Young Control models showing consistent separations ($p < 0.001$ for both).

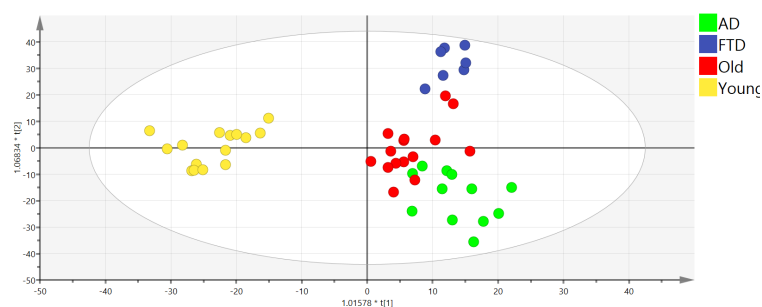


Figure 3.4. OPLS-DA score plot showing all CSF samples with disease class as a discriminating factor. Young control group is well separated from the rest of the cohorts.

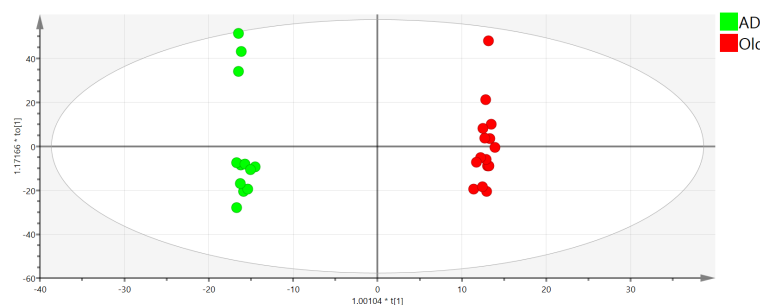


Figure 3.5. OPLS-DA score plot between AD and Old control. Strength of separation based on the cross-validation model is CV-ANOVA $p= 0.8066$.

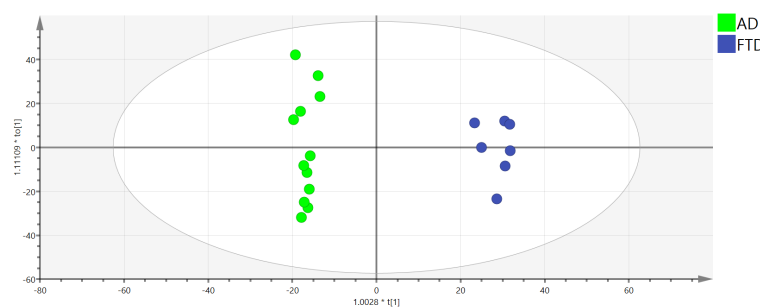


Figure 3.6. OPLS-DA score plot between AD and FTD. Strength of separation based on the cross-validation model is CV-ANOVA $p= 1.2337 \times 10^{-3}$.

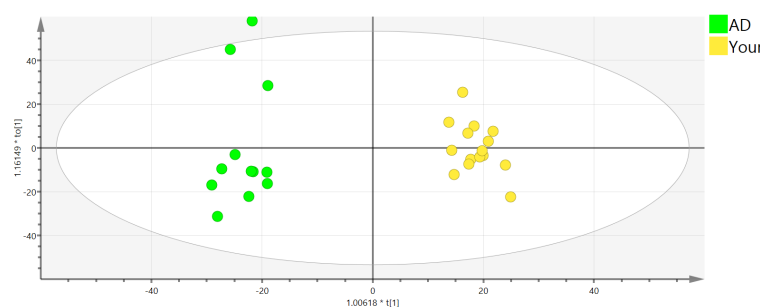


Figure 3.7. OPLS-DA score plot between AD and Young Control. Strength of separation based on the cross-validation model is CV-ANOVA $p= 3.54373 \times 10^{-6}$.

3.3.2. Pairwise comparison between AD and Old control using univariate approach

In order to identify which metabolic features are the strongest discriminators between AD and Old Control, VIP and coefficient plots were derived from its OPLS-DA model. From these two plots, top 50 metabolic features were derived and 9 features were found to be in both plots. These included 2 RP positive features, 2 RP negative features, 4 HILIC positive features and 1 HILIC negative features. A separate OPLS-DA model of AD *vs.* Old Control was built using these 9 features and CV-ANOVA p-value (<0.001) suggested the features to be good AD discriminators (**Figure 3.8**).

Univariate analyses were then followed on for these 9 features (**Table 3.3**). When student t-tests in conjunction with Bonferroni procedure were applied on training samples (12 AD and 15 Old Control), 8 features showed significant differences between the groups. T-value showed the relative amounts of 6 features being lower and 3 features being higher in AD when compared to Old Control.

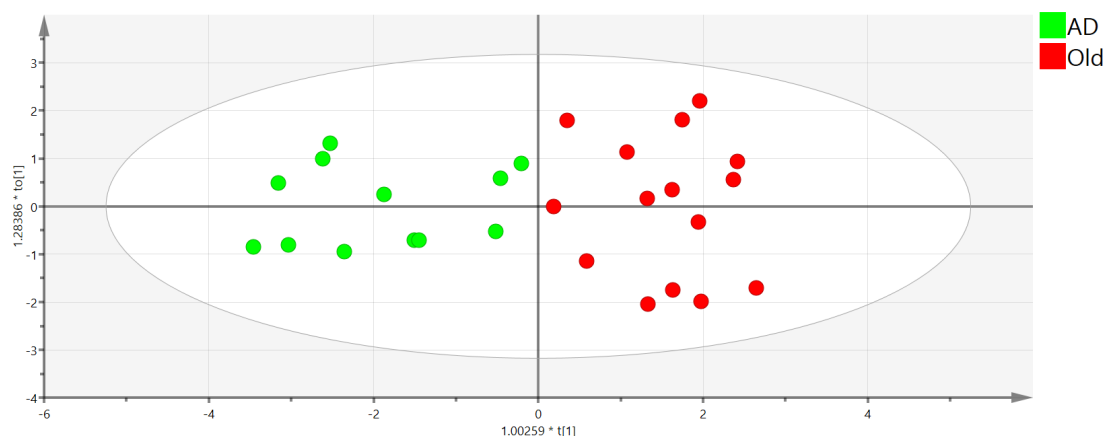


Figure 3.8. OPLS-DA score plot between AD and Old Control based on 9 AD discriminating metabolic features. Derived CV-ANOVA p-value of 9.58×10^{-6} suggests the features are reliable AD discriminator in the 7-fold cross-validated model.

Metabolic features	Training dataset (AD n=12, Old Control n= 15)							Test dataset (AD n=4, Old Control n=5)			
	AD Mean (S.D.)	Old Control Mean (S.D.)	T-Value	p-value	q-value	ROC AUC	ROC Sensitivity	ROC Specificity	ROC AUC	ROC Sensitivity	ROC Specificity
HP553.43_4.65	6.71 (0.43)	11.39 (3.62)	-4.71	***3.06×10 ⁻⁰⁴	**2.75×10 ⁻⁰³	0.91	0.75	0.87	0.75	0.75	0.40
RN682.52_3.24	-0.39 (0.54)	0.21 (0.21)	-3.65	**2.60×10 ⁻⁰³	*2.34×10 ⁻⁰²	0.89	0.67	0.80	0.60	0.25	0.60
HP531.44_4.67	9.32 (0.43)	11.58 (1.98)	-4.07	***9.30×10 ⁻⁰⁴	**8.37×10 ⁻⁰³	0.86	0.75	0.60	0.60	0.75	0.40
HN329.36_20.38	12.96 (0.91)	11.80 (0.89)	-3.55	**1.68×10 ⁻⁰³	*1.51×10 ⁻⁰²	0.84	0.75	0.87	0.75	0.50	0.80
HP267.29_4.79	10.88 (0.42)	10.25 (0.46)	3.7	***9.78×10 ⁻⁰⁴	**8.80×10 ⁻⁰³	0.84	0.67	0.87	0.70	0.25	0.20
HP211.16_5.78	10.59 (0.92)	11.55 (0.74)	-3.04	**6.23×10 ⁻⁰³	5.60×10 ⁻⁰²	0.82	0.58	0.87	0.60	0.50	0.20
RN342.23_13.50	0.43 (0.62)	-0.15 (0.30)	3.34	**4.87×10 ⁻⁰³	*4.38×10 ⁻⁰²	0.81	0.58	0.93	0.70	0.25	0.85
RP342.58_12.43	2.71×10 ⁻⁰⁴ (1.89×10 ⁻⁰⁴)	7.94×10 ⁻⁰⁵ (9.51×10 ⁻⁰⁵)	3.25	**5.12×10 ⁻⁰³	*4.60×10 ⁻⁰²	0.81	0.83	0.73	0.65	0.50	0.80
RP892.42_3.29	6.69×10 ⁻⁰⁵ (1.76×10 ⁻⁰⁴)	8.40×10 ⁻⁰⁵ (1.64×10 ⁻⁰⁴)	-3.1	**5.47×10 ⁻⁰³	*4.93×10 ⁻⁰²	0.79	0.58	0.80	0.50	0.50	0.40
All 9	NA	NA	NA	NA	NA	1.00	1.00	1.00	0.60	0.50	0.60
RP Positive	NA	NA	NA	NA	NA	0.87	0.75	0.93	0.65	0.50	0.80
RP Negative	NA	NA	NA	NA	NA	0.92	0.83	0.93	0.55	0.25	0.80
HILIC Positive	NA	NA	NA	NA	NA	1.00	1.00	1.00	0.65	1.00	0.40
HP553.43_4.65 + RP342.58_12.43	NA		NA	NA	NA	0.97	0.92	0.93	0.70	0.50	0.80

Table 3.3. Univariate analyses of 9 features selected from multivariate OPLS-DA model on AD vs. old control samples. Student t-test (with Bonferroni procedure) was first applied to test significance difference between the groups followed to ROC analysis to test how well AD can be discriminated from Old Control. Statistical Powers for student t-test were above 83.5%

On the same samples, ROC analyses based on generalized linear model (GLM) were carried out to test AD prediction abilities of the 9 metabolic features. Individually, they were able to classify AD samples from Old Control samples well (AUC 0.79-0.91), while combination of all 9 features returned an AUC of 1. Metabolic features in each method were then combined and their AD prognostic values were tested by ROC. 2 RP positive features combined produced AUC of 0.87, 2 RP negative features combined produced AUC of 0.92, and 4 HILIC positive features combined produced AUC of 1.

We then applied the same GLMs onto the test samples (4 AD *vs.* 5 Old control) to build new ROC models. Individually, these 9 features produced AUC values ranging from 0.5 to 0.75, when all 9 features were combined the AUC value was 0.60. On 2 RP positive features combined, 2 RP negative features combined, 4 HILIC positive features combined, the ROC AUC values were 0.65, 0.55 and 0.65 respectively. Results from this section are summarised in **Table 3.3**.

3.3.3. Relative amount of the selected 9 AD features across the 4 groups

From the previous section, we found 9 strongest discriminating features between AD and Old Control. We wanted to compare how the relative amounts of these 9 features vary across all four clinical groups. To achieve this, box-plots were produced in conjunction with ANOVA test, which showed 8 features being significantly different ($p < 0.05$) across the 4 groups (**Figure 3.9**).

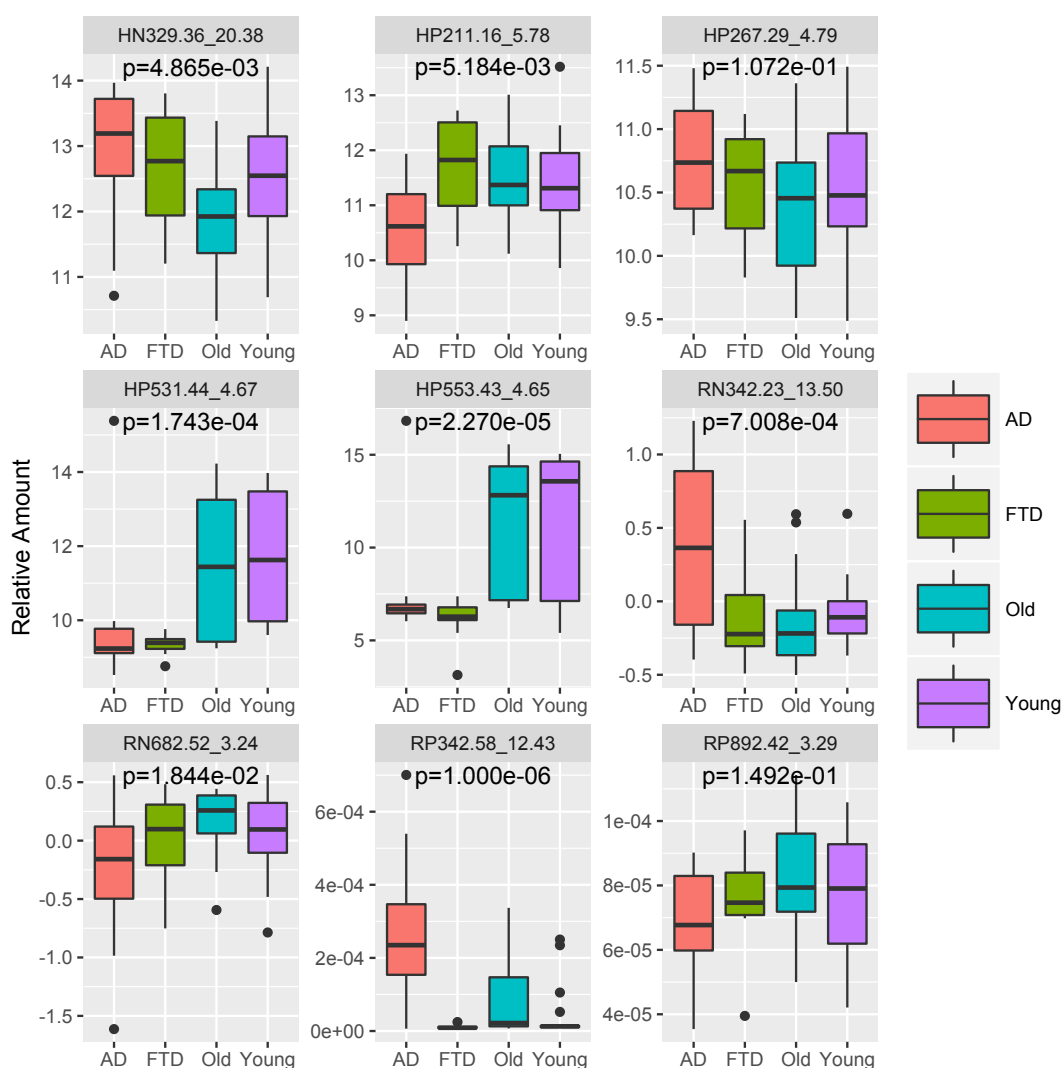


Figure 3.9. Box plots showing relative amounts of the 9 selected metabolic features across all 4 groups (n=66). p-values were derived from ANOVA test which compared the average levels between the 4 groups.

We then applied t-tests and ROC analyses to compare their levels in AD *vs.* FTD and AD *vs.* Young Control models (**Table 3.4**). From AD *vs.* FTD model, only 3 metabolic features were found to be different, HP211.16_5.78 ($p < 0.05$, AUC=0.79), RN342.23_13.50 ($p < 0.05$, AUC=0.75) and RP342.58_12.43 ($p < 0.001$, AUC=0.93). Adjusted p-values using Bonferroni procedure showed only RP342.58_12.43 being different between the groups. When 9 metabolic features were combined, they were able to predict all AD samples from FTD samples correctly (AUC=1).

Metabolic features	AD (n=16) vs. FTD (n=10)						AD (n=16) vs. Young control (n=20)					
	t-test p-value	t-test q-value	ROC AUC	ROC Sensitivity	ROC Specificity		t-test p-value	t-test q-value	ROC AUC	ROC Sensitivity	ROC Specificity	
HP553.43_4.65	1.24×10^{-01}	1	0.66	0.88	0.40		$**1.02 \times 10^{-03}$	$**9.21 \times 10^{-03}$	0.78	0.94	0.60	
RN682.52_3.24	1.77×10^{-01}	1	0.66	0.88	0.40		6.45×10^{-02}	5.80×10^{-01}	0.68	0.44	0.80	
HP531.44_4.67	4.03×10^{-01}	1	0.54	1.00	0.00		$**1.34 \times 10^{-03}$	$*1.21 \times 10^{-02}$	0.88	0.94	0.60	
HN329.36_20.38	5.22×10^{-01}	1	0.58	0.94	0.00		2.23×10^{-01}	1	0.64	0.50	0.750	
HP267.29_4.79	2.32×10^{-01}	1	0.63	0.94	0.30		1.72×10^{-01}	1	0.63	0.38	0.65	
HP211.16_5.78	$*1.06 \times 10^{-02}$	9.54×10^{-02}	0.79	0.88	0.50		$**7.42 \times 10^{-03}$	6.68×10^{-02}	0.75	0.56	0.80	
RN342.23_13.50	$*2.38 \times 10^{-02}$	2.14×10^{-01}	0.75	0.69	0.60		$**7.11 \times 10^{-03}$	6.64×10^{-02}	0.71	0.50	0.90	
RP342.58_12.43	$***1.37 \times 10^{-04}$	$**1.23 \times 10^{-03}$	0.93	0.88	0.90		$***5.03 \times 10^{-04}$	$**4.52 \times 10^{-03}$	0.85	0.75	0.90	
RP892.42_3.29	3.46×10^{-01}	1	0.62	0.94	0.10		1.99×10^{-01}	1	0.63	0.38	0.70	
All 9	NA	NA	1.00	1.00	1.00		NA	NA	0.95	0.81	0.95	
RP Positive	NA	NA	0.94	0.88	0.90		NA	NA	0.86	0.75	0.90	
RP Negative	NA	NA	0.77	0.75	0.70		NA	NA	0.75	0.50	0.90	
HILIC Positive	NA	NA	0.89	0.94	0.80		NA	NA	0.88	0.69	0.75	
HP553.43_4.65 + RP342.58_12.43	NA	NA	0.96	0.94	1.00		NA	NA	0.90	0.81	1.00	

Table 3.4. ROC analysis to test how well the 9 selected metabolic features can discriminate AD from FTD or Young controls. P-values were adjusted using Bonferroni procedure.

On AD *vs.* Young Control model, 5 features were found to be different, HP553.43_4.65 ($p < 0.01$, AUC=0.78), HP531.44_4.67 ($p < 0.01$, AUC=0.88), HP211.16_5.78 ($p < 0.01$, AUC=0.75), RN342.23_13.50 ($p < 0.01$, AUC=0.71) and RP342.58_12.43 ($p < 0.001$, AUC=0.85). RP342.58_12.43 was the only feature which differed in its amount between AD and Young Control when p-values were adjusted by Bonferroni procedure. When all 9 features were combined, most of the AD samples were classified accurately from Young Control samples (ROC AUC=0.95).

Additionally, we combined HP553.43_4.65 and RP342.58_12.43 and tested its AD discrimination ability from all three other clinical groups, Old Control (test samples), Young Control and FTD. The rationale behind the choice of this combination will be discussed later. The resulting AUC values were 0.70, 0.96 and 0.90 respectively. We also evaluated the robustness of acquisition of these two metabolic features by mass spectrometry and found their coefficient variation (CV) values $< 17\%$ (based on QC samples).

3.3.4. Association of 9 AD metabolic features with AD clinical markers

We then asked whether the 9 metabolic features have any relationship with the well-known clinical AD markers, CSF measures of $A\beta_{42}$ and tau as well as ApoE4 and MMSE score (**Figure 3.10**, **Table 3.5**). Partial correlation after adjusting for age of participants at the time of CSF collection revealed 4 features correlating with CSF $A\beta_{42}$ amount, 7 features correlating with CSF total tau amount, 3 features correlating with ApoE4, and 8 features correlating with MMSE. Two metabolic features, HP553.43_4.65 and RP342.58_12.43, moderately correlated with all 4 clinical AD markers (coefficient: > 0.37 , < 0.49 respectively).

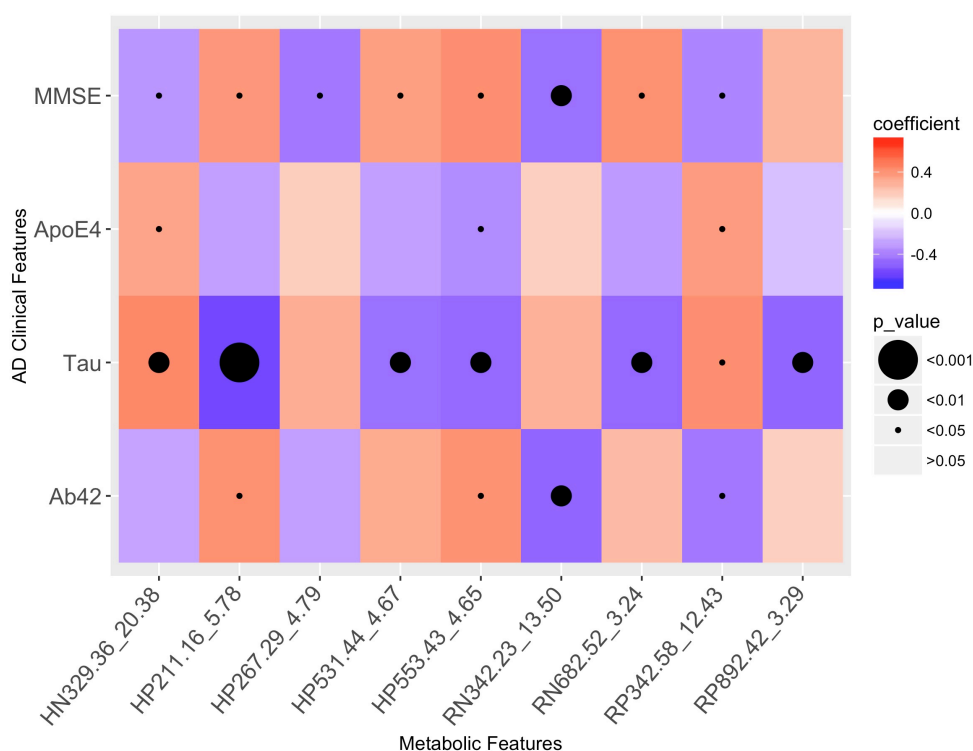


Figure 3.10. Summary plot showing results of partial correlation analyses (Person's) between 9 metabolic features (columns) and AD clinical variables (rows) after adjusting effect of age at the time of CSF collection. Colour and its intensity of each square represent correlation coefficient value while size of black circle represents p-value for each partial correlation. The analyses were based on 16 AD and 20 old control samples.

3.3.5. Association between 9 AD metabolic features AD drug medication

In order to investigate whether AD medications, acetyl-cholinesterase inhibitors (ChEI) and Namenda, had any effects on levels of these metabolic features, we employed regression models and found no relationships between them (**Table 3.6**).

Metabolic features	CSF A β 42			CSF total tau			ApoE4			MMSE		
	Coefficient	p-value	q-value	Coefficient	p-value	q-value	Coefficient	p-value	q-value	Coefficient	p-value	q-value
HP553.43_4.65	0.41	*1.43×10 ⁻⁰²	1.29×10 ⁻⁰¹	-0.48	**3.56×10 ⁻⁰³	*3.21×10 ⁻⁰²	-0.37	*2.97×10 ⁻⁰²	2.67×10 ⁻⁰¹	0.42	*1.13×10 ⁻⁰²	1.02×10 ⁻⁰¹
RN682.52_3.24	0.26	1.35×10 ⁻⁰¹	1	-0.48	**3.18×10 ⁻⁰³	*2.86×10 ⁻⁰²	-0.31	6.76×10 ⁻⁰²	6.08×10 ⁻⁰¹	0.42	*1.20×10 ⁻⁰²	1.08×10 ⁻⁰¹
HP531.44_4.67	0.32	5.68×10 ⁻⁰²	5.11×10 ⁻⁰¹	-0.46	**5.75×10 ⁻⁰³	5.18×10 ⁻⁰²	-0.3	7.94×10 ⁻⁰²	7.14×10 ⁻⁰¹	0.36	*3.19×10 ⁻⁰²	2.87×10 ⁻⁰¹
HN329.36_20.38	-0.29	9.69×10 ⁻⁰²	8.72×10 ⁻⁰¹	0.44	**7.67×10 ⁻⁰³	6.90×10 ⁻⁰²	0.34	*4.25×10 ⁻⁰²	3.83×10 ⁻⁰¹	-0.33	*4.94×10 ⁻⁰²	4.44×10 ⁻⁰¹
HP267.29_4.79	-0.31	6.87×10 ⁻⁰²	6.18×10 ⁻⁰¹	0.31	6.91×10 ⁻⁰²	6.22×10 ⁻⁰²	0.19	2.79×10 ⁻⁰¹	1	-0.43	*1.03×10 ⁻⁰²	9.26×10 ⁻⁰²
HP211.16_5.78	0.42	*1.31×10 ⁻⁰²	1.18×10 ⁻⁰¹	-0.59	***2.14×10 ⁻⁰⁴	**1.93×10 ⁻⁰³	-0.3	8.14×10 ⁻⁰²	7.32×10 ⁻⁰¹	0.39	*1.96×10 ⁻⁰²	1.76×10 ⁻⁰¹
RN342.23_13.50	-0.49	**2.56×10 ⁻⁰³	*2.31×10 ⁻⁰²	0.3	8.38×10 ⁻⁰²	7.54×10 ⁻⁰¹	0.18	3.07×10 ⁻⁰¹	1	-0.46	**5.68×10 ⁻⁰³	5.11×10 ⁻⁰²
RP342.58_12.43	-0.43	*1.01×10 ⁻⁰²	9.08×10 ⁻⁰²	0.42	*1.13×10 ⁻⁰²	1.01×10 ⁻⁰¹	0.38	*2.57×10 ⁻⁰²	2.31×10 ⁻⁰¹	-0.39	*2.19×10 ⁻⁰²	1.97×10 ⁻⁰¹
RP892.42_3.29	0.18	3.10×10 ⁻⁰¹	3.10×10 ⁻⁰¹	-0.49	**3.10×10 ⁻⁰³	*2.79×10 ⁻⁰²	-0.2	2.48×10 ⁻⁰¹	1	0.29	9.46×10 ⁻⁰²	8.51×10 ⁻⁰¹

Table 3.5. Partial correlation analysis (Pearson's after adjusting for patient age) between the 9 selected metabolic features and well-known AD markers. P-values were adjusted using Bonferroni procedure. 16 AD and 20 old control samples were used for the partial correlation analysis.

Features	GLM p-values		
	Aricept	Other CheI	Namenda
HP553.43_4.65	5.19×10^{-01}	8.53×10^{-01}	6.65×10^{-01}
RN682.52_3.24	8.75×10^{-01}	2.91×10^{-01}	1.04×10^{-01}
HP531.44_4.67	5.67×10^{-01}	8.61×10^{-01}	6.22×10^{-01}
HN329.36_20.38	2.75×10^{-01}	1.88×10^{-01}	4.05×10^{-01}
HP267.29_4.79	3.36×10^{-01}	3.15×10^{-01}	3.24×10^{-01}
HP211.16_5.78	1.15×10^{-01}	1.69×10^{-01}	5.38×10^{-01}
RN342.23_13.50	6.24×10^{-01}	8.38×10^{-01}	2.56×10^{-01}
RP342.58_12.43	9.60×10^{-01}	6.71×10^{-01}	7.73×10^{-01}
RP892.42_3.29	1.50×10^{-01}	6.20×10^{-01}	4.58×10^{-01}

Table 3.6. GLM analyses show no association between AD medications and the relative amounts of the 9 metabolic features.

3.3.6. Metabolic feature Identification

Prior to identification, we first carried out correlations between the 9 selected features to investigate whether any of them are of the same metabolites. **Figure 3.11** shows high correlation ($\text{corr} > 0.9$) between HP531.44_4.67 and HP553.43_4.65. During identification procedure, they were also found to share similar elution profiles (**Figure 3.12**), suggesting them to be a same metabolite with different adducts ($\text{M} + \text{H}^+$ and $\text{M} + \text{Na}^+$ respectively).

Then we attempted to identify HP553.43_4.65 and RP342.58_12.43 by first deriving metabolic features from the fingerprint datasets (MS^1 and MS^2 levels, pre-processed only) which share similar retention time. Correlation analyses showed HP553.43_4.65 correlating with HP531.44_4.67 only, as mentioned earlier. For RP342.58_12.43, three metabolic features from MS2 level correlated (**Figure 3.13**, $\text{corr} > 0.88$, $p < 0.01$) and they were RP58.07_12.44, RP123.12_12.44 and RP140.14_12.46. Subsequently their EICs were compared with that of RP342.58_12.43 (**Figure 3.14**) and found them sharing similar elution profiles. These findings suggested the three MS2 metabolic features (RP58.07_12.44, RP123.12_12.44 and RP140.14_12.46) to be fragment (or

daughter) ions of to be RP342.58_12.43. We then attempted to search against databases based on the obtained fragmentation patterns, but we were unable to annotate RP342.58_12.43. Database searching showed Epoxymurin and tocopheryl succinate to be possible candidate for feature HP553.43_4.65. Further searching on two candidates showed that Epoxymurin is exogenous to human while tocopherol has been found in CSF previously [485]. Taken together, we believe HP553.43_4.65 is tocopheryl succinate.

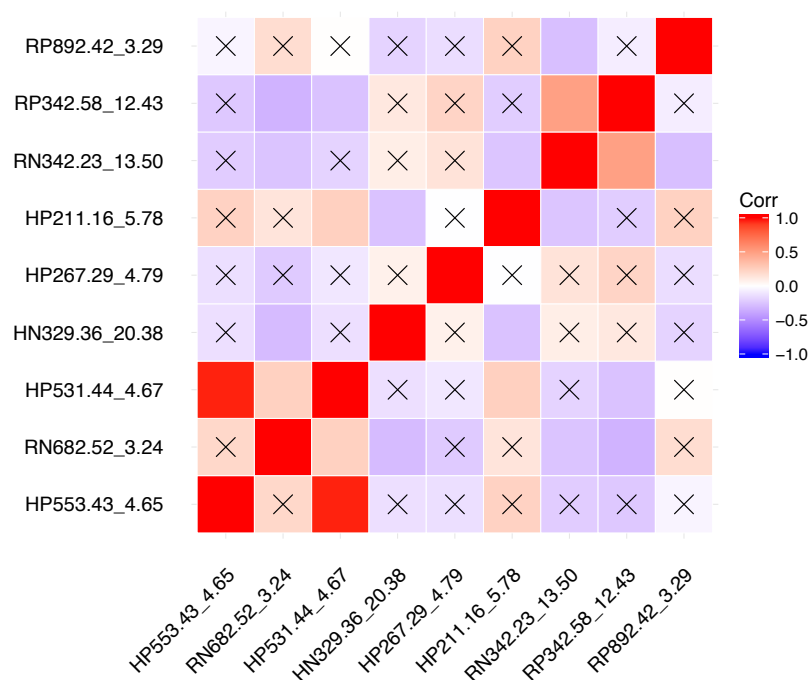


Figure 3.11. Plot of correlation matrix between 9 selected metabolic features. Red represent positive correlation while blue represent negative correlation. Intensity of 'redness' and 'blueness' represent correlation coefficient value. Cell with X shows correlation with no significance ($p > 0.01$).

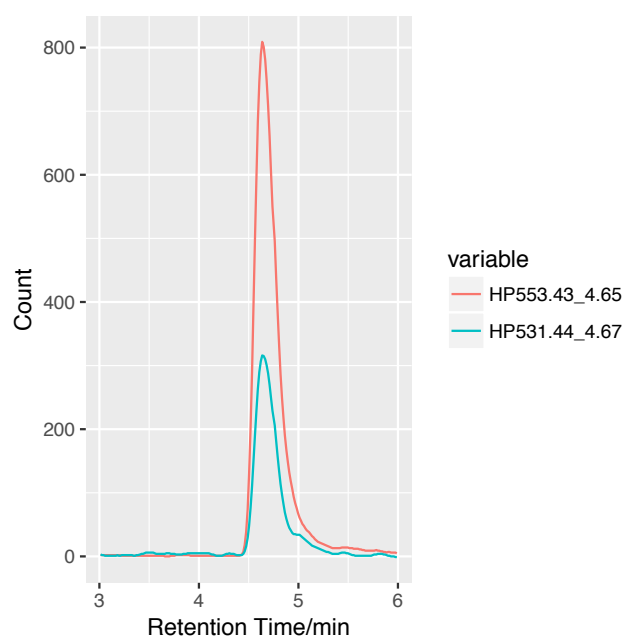


Figure 3.12. EICs of HP553.43_4.65 and HP531.44_4.65 from a QC sample between 3 and 6 minutes. Same elution profiles suggest these two features originate from a same compound.

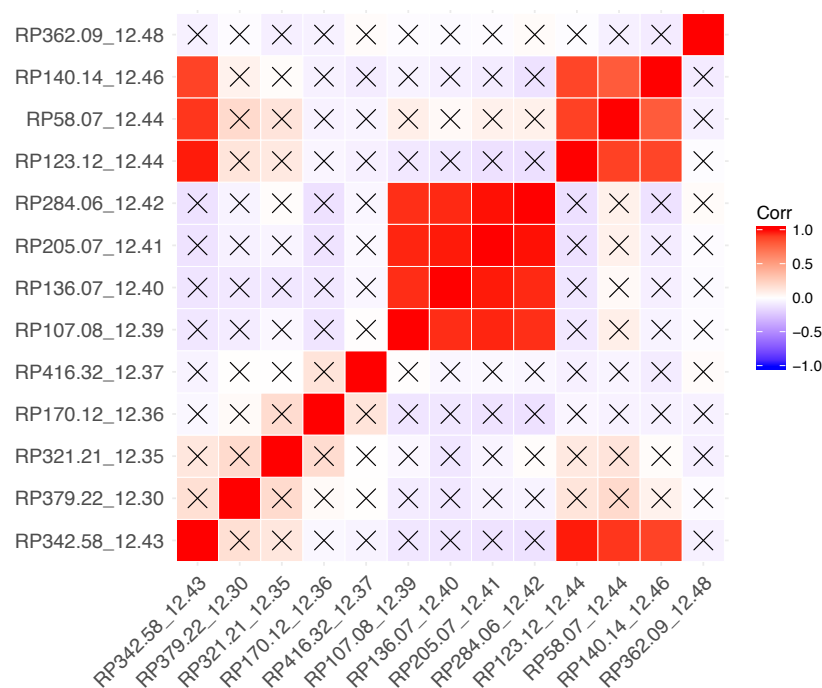


Figure 3.13. Plot of correlation matrix between RP342.58_12.46 and other metabolic features with similar retention time (from MS2). Red represent positive correlation while blue represent negative correlation. Intensity of 'redness' and 'blueness' represent correlation coefficient value. Cell with X shows correlation with no significance ($p > 0.01$).

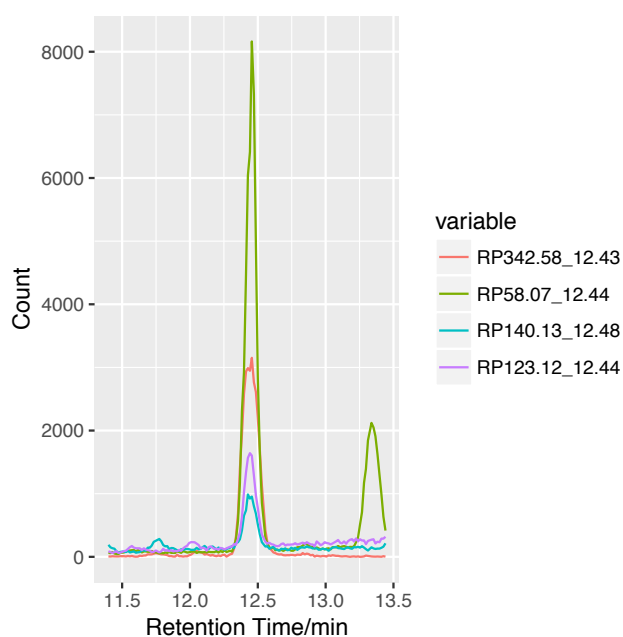


Figure 3.14. EICs of RP342.58_12.43, RP58.07_12.44, RP140.13_12.48 and RP123.12_12.44 from a QC sample between 11.5 and 13.5 minutes (MS2 level).

3.4. Discussion

In this report, we aimed to identify a panel of CSF metabolites that would discriminate AD from Old Control (age-matched) samples, then test how well this panel could discriminate AD from FTD and Young Control samples. We assayed 69 CSF samples (66 were used for statistical analysis) using a complementary RP and HILIC LC-MS techniques to allow for high coverage of CSF metabolites, acquiring relative amount of 4426 metabolic features which is significantly more than that from NMR experiment.

Using multivariate OPLS-DA, we first compared metabolic profiles between the 4 groups. The score plot resembled that from the NMR experiment (**Figure 2.4**), the biggest separation being between Young Control and all olds (AD, FTD and Old Control) suggesting natural aging is the biggest contributor for changes in CSF metabolic profile in this cohort. Within old groups, changes in metabolic profiles from Old Control to AD and FTD were opposite. This is interesting as it suggests that these two dementias are very ‘dissimilar’ even though they are both neurodegenerative disease. Single-photon emission computed tomography (SPECT) [486] and positron emission tomography (PET) [487] have been gaining significant clinical utility to differentiate AD and FTD, but both techniques require costly radio-ligands. The difference in CSF metabolic profiles between two clinical cohorts seen in this present study suggests difference in metabolite levels can also potentially aid clinicians to produce better diagnosis between AD and FTD. Further, this trend was also observed in a discriminant analysis carried out by Tsuruoka *et al.* [403] where 56 serum metabolites were looked at from 4 AD, 3 FTD and 9 Old Control samples. Such a distinctive global difference in CSF metabolic profiles is not an unexpected as changes occurring in AD and FTD brains and their clinical symptoms are dissimilar [488]. AD is characterized by hippocampal atrophy while FTD is associated with frontal and/or temporal lobes shrinkage, suggesting different metabolic pathways are affected in AD and FTD.

From pairwise-comparison between AD and Old Control, we mined 9 AD discriminating metabolic features and tested them on test samples, a blind test from training samples. This statistical approach prevented “overfitting” and allowed us to overcome a limitation present in the previous chapter (Chapter 2). On test samples, these features discriminated AD from Old Control with ROC AUC 50~75%, the strongest being HP553.43_4.65. In comparison, well-established AD neuropathological features, lower $A\beta_{42}$ and higher tau concentrations in CSF, are known to predict AD from cognitively normal with ROC AUC of >90% [232, 489]. In this study’s cohort, these two features also showed high AD prediction abilities (AUC>95%). The newly identified CSF metabolic features, even with its best combination, would therefore have lesser diagnostic potential, and also fall short of the minimum criteria required to be an AD biomarker candidate [490, 491].

Although their prognostic values were moderate, these metabolic features could still offer information on AD. Three features (HP211.16_5.78, HP533.43_4.65 and RP342.58_12.43) correlated with CSF $A\beta_{42}$, CSF tau and MMSE scores, suggesting an association with AD-pathology and may well have a potential applicability in monitoring disease progression. With abnormal CSF $A\beta_{42}$ and tau levels being known to occur before cognitive impairment, the three features could play a possible role in pre-symptomatic diagnosis. Furthermore, HP553.43_4.65 and RP342.58_12.43 were also found to correlate with ApoE4 status.

We then tried to establish whether changes in relative amount of these 9 features are age-related and the result of accelerated ‘normal’ cerebral aging. None showed such trend, suggesting abnormal levels of the features in AD CSF have no associations with natural aging.

We were then interested in asking whether they may represent exogenous entities such as medications used by AD patients. We therefore examined whether their amount in CSF differed between AD patients taking commonly used medications for AD,

Namenda and acetylcholinesterase inhibitors, and those who were not taking the drugs. Generalized linear models showed none of the 9 metabolic features associate with medications, suggesting an endogenous origin.

One of the requirements of AD diagnosis test is that it must be able to differentiate AD from other neurodegenerative disease. By comparing with FTD, we were able to test AD specificity of the selected 9 features. RP342.58_12.43 was found to be the best at differentiating the two dementia groups (ROC AUC=0.90). This feature also showed high discrimination ability from Young Control (AUC=0.85), suggesting it to be a good AD marker with high AD specificity. Meanwhile, the strongest AD discriminator from AD *vs.* Old Control model, HP553.43_4.65, lacked the ability to distinguish AD from FTD. Its level was depleted in both AD and FTD, signifying the particular feature is not AD specific and it could be a marker of neurodegeneration. This shows importance of inclusion of other neurodegenerative group(s) in AD biomarker studies as it allows for AD specificity to be tested.

Overall, we were able to determine HP553.43_4.65 to be a good neurodegeneration marker while RP342.58_12.43 to be the most AD specific marker. Combination of these two metabolic features was able to discriminate AD from Old Control fairly (test samples, AUC= 0.70). The reported value is lower than that from other CSF AD biomarker studies [383-385]. Ibanez *et al.* [384] proposed 17 metabolic features predicting the development of AD with an accuracy of 98.7%, yet the model was not tested on blind test samples. Two other AD CSF biomarker studies utilized test samples to examine their predictive models. While we utilized a stringent analysis pipeline where the exact predictive model (cut-off value) developed from training samples was applied on the test samples, a study by Czech *et al.* failed to mention how their predictive models were tested on blind test samples [383]. In our subsequent analysis, a panel of 9 features was able to predict AD from Old Control test samples with 100% accuracy if a separate classification model was built, highlighting importance of the way models are evaluated.

We then attempted to identify the features reported by studying their fragmentation pattern and database searching. We believe one feature (HP553.43_4.65) to be tocopheryl succinate. Tocopheryl succinate is a chemical compound of a vitamin E compound, Tocopherol, bonded with succinate via ester linkage. Vitamin E has long been associated with AD due to its role as an antioxidant, its major function involving neuronal cell protection against oxidative stress. Lower level of Vitamin E has been previously reported in plasma [492-495] and CSF [496, 497] of AD patients. Based on these finding, Vitamin E has been tested as both a means of prevention [498, 499] and treatment [500-502], but have shown inconclusive results. Benefit of Vitamin E supplementation on AD lack clinical evidence, but lower level in this study further demonstrates a possible link in AD (and FTD) development.

However it is to be noted that the proposed AD makers have to be demonstrated in a larger cohort. Furthermore, the present study did not contain an MCI (mild-cognitive impairment) clinical group. Inclusion of such group would give a clearer picture on whether the candidate markers are associated with AD initiation and/or its progression. Additionally, although our predictive models were evaluated using test samples, the partition of samples into training and test was carried out after data-processing and –treatment steps. Therefore it would be impractical to say our findings would be reflected on ‘real’ samples. This particular limitation can be overcome by normalising the relative amounts of the discriminating features using internal standard, but unfortunately it was not included during the sample preparation step. Also we were unable to identify the candidate markers, a limitation also seen in NMR experiment (Chapter 2). Taken together, the definitive structural identification of metabolites of interest represents a considerable challenge in metabolomics.

3.5. Conclusions

In this study using LC-MS, we were able to derive a panel of 2 metabolic features with stronger AD discriminating ability when compared with NMR (Chapter 2). One metabolic feature was initially annotated as tocopheryl-succinate and could be proposed as a neurodegeneration marker or symptom in AD and FTD. We were unable to annotate the other metabolic feature, but inclusion of this into the panel to enhanced AD specificity due to its strong discrimination ability between AD and FTD.

It should be noted, that these two metabolites we present here are by no means biomarkers to be used in clinical practice because they do not exceed the performance of the known protein markers of $A\beta_{42}$ and Tau in CSF. However, their correlations to these protein markers along with cognitive function suggest a role that should be studied further to be able to conclude any causative links.

***Altered
Phosphatidylcholine and
Ceramide metabolisms
in AD***

4.1. Introduction

Previous chapters looked at metabolic profiles in Cerebrospinal Fluid (CSF) as it is a great source for studying disordered biological processes of the brain [427, 428]. However collection of CSF from elderly patients is not practical as the technique itself is quite invasive. In turn, blood metabolites are easily accessible and minimally invasive, in addition they could represent a molecular fingerprint of disease progression, one that could theoretically cross the AD blood-brain barrier (BBB) [503].

Among blood metabolites, lipid-signaling pathways have attracted attention as an important and possibly critical factor in neurodegeneration [377, 390, 504, 505]. Non-targeted blood metabolomic studies have highlighted ceramides (Cer) to be associated with AD. Ceramides are core constituents of sphingolipids, they are composed of sphingosine and a fatty acid. These lipid species play a crucial role in various cell physiological functions and crucially in cell apoptosis [155]. Ceramides are often measured as markers of oxidative stress [146, 506, 507], and previous reports have found increased ceramide levels in various biofluid/tissues from AD patients. In a study involving 16 AD, 14 control and 13 amyotrophic lateral sclerosis (ALS) CSF samples, elevated level of ceramides were detected in AD relative to control and ALS ($p < 0.01$) [154]. Increased levels of ceramides in AD brains (7 vs 7) was also observed in a study by Cutler *et al.* [508], but only in vulnerable brain regions where extensive A β plaques and tau neurofibrillary were detected. A Cross-sectional analysis between 98 AD plasma and 92 control plasma samples found 6 ceramides that were significantly higher in AD, 2 of them were also found to associate with delusions and other 2 with depression.[509]. A study in 2012 by Mielke *et al.* proposed that higher serum ceramide levels to be linked with an increased risk of developing AD [510].

In addition to ceramides, Phosphatidylcholines (PCs) are another lipid family which has been linked to neurodegenerative disease. PC is the most abundant phospholipid, composed of two fatty acids; usually one is a saturated fatty acid while the other is an unsaturated fatty acid. In 2014, Whiley *et al.* identified a panel of three PCs,

PC(16:0/20:5), PC(16:0/22:6) and PC(18:0/22:6) that were decreased in the plasma of AD patients in comparison to cognitively normal controls and MCI patients [511]. This relatively underpowered study (n=91) showed that APOE's predictive performance of 67% was increased to 83% by including APOE and PCs as AD markers.

A longitudinal study (n=899) has also looked into PC DHA (docosahexaenoic acid) plasma levels at baseline (before pheno-conversion) [512]. The study was able to determine subjects in the upper quartile of plasma PC DHA levels having a AD risk ratio of 0.61 when compared to subjects in the three other quartiles, suggesting lower PC DHA associating with higher risk of AD development. Another longitudinal study measured concentrations of 180 plasma metabolites from 23 participants, and found a panel of ten lipids, which included seven PCs, to be predictive of AD phenoconversion within a 2-3 years timeframe with >90% accuracy [504].

Together these findings suggest that lipids play a crucial role in AD pathology and may represent a valuable clinical tool for diagnosis and prevention. However, it is equally important to replicate results in larger cohorts when possible [396, 513] and analyse how these effects depends on other major factors of AD; namely APOE4 status and age. Here we measured levels of 9 abundant plasma lipids previously linked to AD [508, 510, 511], in plasma from AD and elderly control volunteers in a relatively much larger dataset. The 9 lipids included 3 PC molecules and 6 ceramide molecules.

This study will utilize 412 plasma samples (205 AD and 207 controls) from the Dementia Case Register (DCR) at King's College London and the EU funded AddNeuroMed (ANM) study [511, 514]. DCR cohort (n=98) was analysed in 2012 by Whiley *et al* [511] where depletion of the three PCs was first reported. Using the same methodology, ANM cohort (n=314) samples were analyzed in 2014. Measurement of the levels of 9 lipids will be carried out using 'semi-quantification' where peak areas are acquired from extracted ion chromatograms (EICs). This semitargeted approach can allow for more accuracy when compared to a metabolic fingerprinting approach. We

will study whether any trends in the amounts of these 9 lipids would associate to AD variables (AD classification and brain atrophy). We will then study how associations between lipids levels and AD variables differ in 2 non-intersecting age bins.

4.2. Methods

4.2.1. Patient details and blood collection

This study utilized 412 plasma samples, obtained from DCR and AMN cohorts. Of these 412 participants, 122 also had structural magnetic resonance imaging (sMRI) data. Normal elderly control subjects were recruited from non-related family members of AD patients, care-givers' relatives, social centers for the elderly, or GP surgeries, and had no evidence of cognitive impairment. AD individuals were primarily recruited from local memory clinics. Relevant ethics boards approved the studies and informed consent was obtained for all subjects. Each patient was required to fast for two hours prior to sample collection and 10ml of blood was then collected in tubes coated with sodium ethylenediaminetetraacetic acid (EDTA) to prevent clotting. Whole blood was centrifuged to form a plasma supernatant, which in turn was removed and placed at -80°C until further use.

4.2.2. Measurements of lipid levels

Samples from the two clinical cohorts were prepared and analyzed separately. The first cohort (DCR, n=98) was analyzed in 2012 while AddNeuroMed (n=314) was in 2014, both using the identical methodology. Lipid extraction and analysis methods have been described elsewhere [396, 426]. Twenty microliters of plasma was added to a glass liquid chromatography vial with a 400µL insert (Chromacol, UK) followed by MS grade water (10µL). Forty microliters of MS grade methanol was added followed by 2 min vortex mix to precipitate proteins. 200µL of methyl tert-butyl ether (MTBE) containing 10µg/mL triptentadecanoin (Sigma-Aldrich, St. Louis, USA), an internal standard, was added which then underwent vortex mixing for 1hr. After adding an additional 50µL of water followed by a final sample mixing, the sample was centrifuged at 3000g for 10min.

The MTBE upper phase of the sample extract containing lipids was analysed by a liquid chromatography coupled to Xevo QToF MS (Waters, Milford, USA).

Chromatographic separation was achieved using an Agilent Poroshell 120 EC-C8 column (150mm $\times$ 2.1mm, 2.7 μ m) at 55°C. Mobile phases consisting 10mM ammonium formate in water (A) and 10mM ammonium formate in methanol (B) were employed, with flow-rate of 0.5mL/min. A gradient was established with 0min (75% B), 23min (96% B), 36min (100% B), 42min (100% B) followed by 9min column equilibration (75% B) prior to each injection.

The MS was operated in the positive ion mode with a capillary voltage of 3.2kV and a cone voltage of 35V. The desolvation gas flow was 800L/hour and the source temperature was 120°C. All analyses were acquired using the lock spray to ensure accuracy and reproducibility. Leucine enkephalin was used as lock mass (m/z 556.27 and 278.11) at a concentration of 200ng/mL and a flow rate of 10 μ L/min. Data were collected in the centroid mode over the mass range m/z 100–1000 with an acquisition time of 0.2 seconds per scan. Samples were analyzed in a randomized order with pooled plasma samples (Quality control (QC) samples) being analyzed after every 10 injections.

4.2.3. Lipid Identification and Quantification

Identification of the 9 lipids from the acquired data was carried out by searching against an in-house database and were verified by studying their MS2 fragmentation patterns and retention times with those from authentic compounds. Descriptive of the 9 lipids can be found in **Table 4.1** while EICs of a ceramide (Cer24:0 parent and daughter ions) can be seen in **Figure 4.1**. The single molecule integrated peak areas under the exact mass chromatographs of the nine lipids and the internal standard (Tripentadecanoin) were obtained by using QuanLynx (MassLynx 4.1, Waters) by setting up an integration parameter file using the average mass charge ratio (m/z) and retention time of the 9 lipids and internal standard. Tolerances for mass and retention time of 0.1Da and 5min respectively were typically applied. Lipids with the levels below the limit of quantification were not used for statistical analyses. After quantification, 3 ceramides, Cer16:0, Cer18:0 and Cer20:0, were found to be below limit

of quantification (signal:noise ratio of 10:1) in DCR cohort, subsequently the levels of these three lipids in DCR cohort was not used for further analyses.

Lipids PC(16:0/20:5), PC(16:0/22:6) and PC(18:0/22:6) were referred as (PC(36:5), PC(38:6), PC(40:6) and ceramides were referred as (Cer16:0, Cer18:0, Cer20:0, Cer22:0, Cer24:0 and Cer24:1) throughout the chapter.

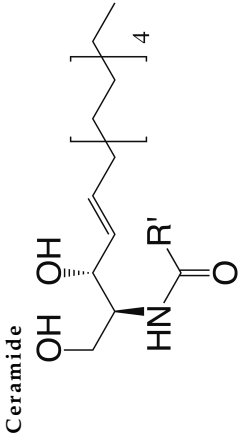
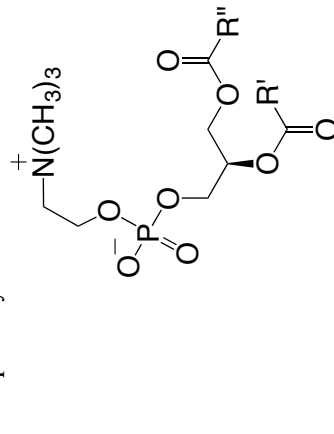
4.2.4. sMRI data acquisition and analysis

Volumes of hippocampi, white matter, entorhinal cortices (ERCs) and entorhinal cortex thickness (ER thickness), normalised by intracranial volume, were obtained from 122 subjects (ANM cohort, 52 AD patients and 70 controls) who had undergone sMRI. The volumetric data were not used to aid in the clinical diagnosis of AD. Detailed information regarding data acquisition, pre-processing, and quality control assessment have been described for this cohort elsewhere [515, 516].

4.2.5. Statistical Analysis

We applied generalized linear model (GLM) to compare lipid levels in AD relative to control group, and to study their relationship with brain atrophy. The GLM included covariates to control for centre of origin of each sample, gender, age, APOE status and technical variations (batch effect and sensitivity variations). When analysis was segregated according to age, the GLMs were separately applied to 2 non-intersecting age bins, corresponding to percentiles 0-50th, 50-100th. Each one of these bins therefore contained the same number of samples (ca. n=206 for AD status, n=61 for brain atrophy). All statistical analyses were carried out in R.3.2.3.

Figure 4.2 shows a schematic workflow of the data used in this study.

Lipid	Fatty Acid	m/z (Da)	Retention Time (min)
Ceramide 	16:0	-	520.50
	18:0	-	548.54
	20:0	-	576.57
	22:0	-	604.60
	24:0	-	632.62
Phosphatidylcholine 	20:5	16:0	780.56
	22:6	18:0	806.58
	22:6	18:0	834.61
	22:6	18:0	834.61
	22:6	18:0	834.61

Abbreviations: m/z, mass charge ratio; min, minutes

Table 4.1. Descriptive of 6 Ceramides and 3 PCs. Ceramide parent molecular ion is in a form of $[M-H_2O+H]^+$ while fragment ion 264.26Da corresponds to sphingosine base chain. Phosphatidylcholine parent molecular ion is in a form of $[M+H]^+$ while fragment ion 184.03Da corresponds to phosphocholine head group.

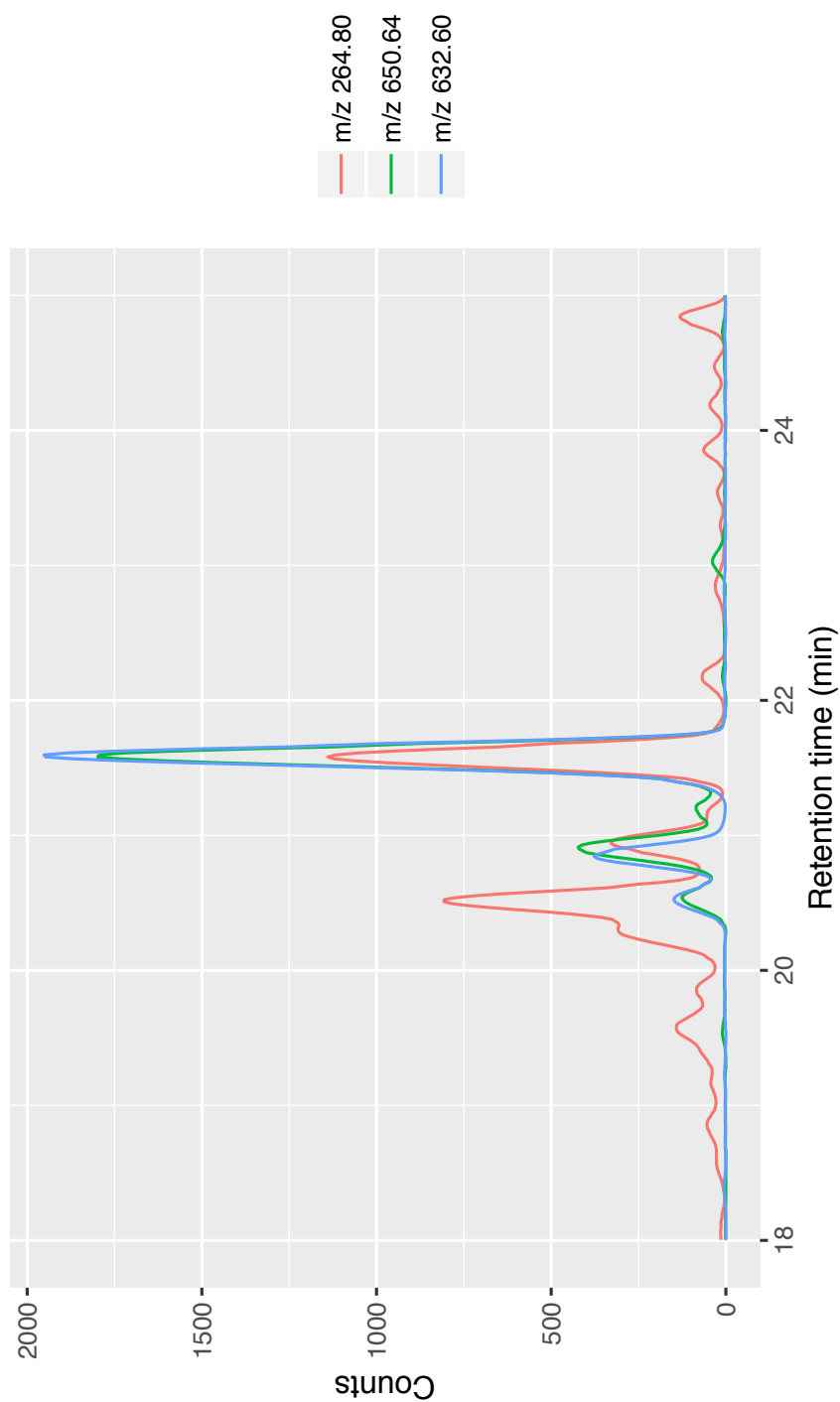
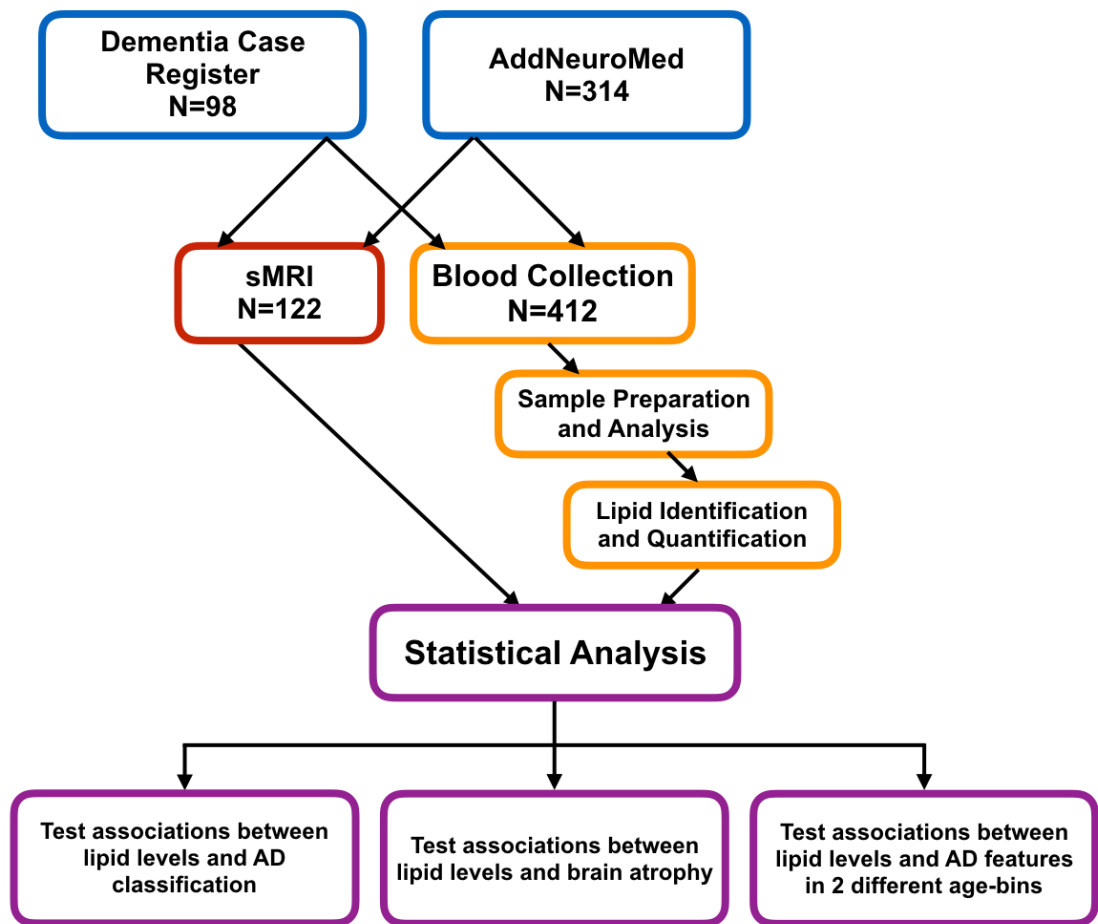


Figure 4.1. Extracted ion chromatograms of Cer24:0 parent and daughter ions from a QC sample. m/z 264.80 (red) corresponds a daughter ion of d18:1 sphingosine base chain. m/z 650.64 (green) and m/z 632.60 (blue) corresponds to parent molecular ions, $[M-H_2O+H]^+$ and $[M+H]^+$ respectively. All three ions share same elution profiles at retention time of 21.6 minutes which correspond to Cer24:0 retention time.



Abbreviation: sMRI, structural Magnetic Resonance Imaging.

Figure 4.2. Schematic workflow used in this study.

4.3. Results

4.3.1. Participant characteristics

The demographic characteristics (age, gender, APOE, volume in 4 different brain regions) of participants by diagnostic group are shown in **Table 4.2**. These characteristics were compared between the two clinical groups using either student t-test or chi-square test. AD participants were slightly older than controls; this is not unusual in AD studies with relatively larger sample size [517, 518]. The AD group had higher percentage of APOE carriers and presence of brain atrophy (p-values < 0.001). There were no significant differences between the groups on high-density lipoprotein, low-density lipoprotein, total cholesterol levels and total triglyceride levels.

4.3.2. Ceramides in AD plasma

Six ceramide levels from 202 plasma samples from the AD group and 207 plasma samples from the cognitively healthy group were compared using GLM. **Figure 4.3** shows the distribution of all ceramides, suggesting that some of them are elevated in the AD group (in red). **Table 4.3** confirms that single molecule plasma levels of Cer16:0 ($p < 0.01$), Cer18:0 ($p < 0.01$) and Cer24:1 ($p < 0.05$) in particular were elevated, while no significant differences were observed for Cer20:0, Cer22:0 and Cer24:0. The derived ‘z-values’ from GLM (**Table 4.3**) showed that the magnitude of the effect was the largest for Cer18:0 (-2.99), followed by Cer16:0 (-2.94) and Cer24:1 (-2.49). The ‘z-values’ for the non-significant ceramides were Cer24:0 (-1.89), Cer20:0 (-1.75) and Cer22:0 (-1.42).

Using the same GLM approach, we then tested if single ceramide levels would associate with atrophy in white matter, hippocampus, ERCs and ER thickness as measured in 122 participants. The statistical results tabulated in **Table 4.4** show that there was no association between the ceramides and atrophy in these 4 brain regions. **Figure 4.4** summarises these associations, including ceramides and PCs in the same figure for comparison.

	AD (n=205)	Control (n=207)	p-value
Average age (s.d)	77.35(6.88)	74.88(6.60)	^A 2.26×10 ⁻⁴
Gender (Male/Female)	81/123	77/130	^B 0.60
¹ APOE ε4, no. (%)	124(61.03)	60(29.13)	^B 5.85×10 ⁻¹¹
² Average White Matter (s.d)	0.6561(0.0733)	0.6985(0.0408)	^A 9.30×10 ⁻⁵
² Average Hippocampus Volume (s.d)	0.003678 (0.000757)	0.004969 (0.000628)	^A 4.84×10 ⁻¹⁸
² Average Entorhinal Volume (s.d)	0.001870 (0.000556)	0.002516 (0.000437)	^A 7.43×10 ⁻¹¹
² Average Entorhinal Thickness (s.d)	5.4709 (0.9866)	6.8090 (0.7207)	^A 1.62×10 ⁻¹⁴
³ Average HDL-c (s.d) mmolL ⁻¹	1.58 (0.37)	1.55 (0.38)	^C 0.068
³ Average LDL-c (s.d) mmolL ⁻¹	3.41 (1.01)	3.07 (0.82)	^C 0.529
³ Average TC (s.d) mmolL ⁻¹	5.69 (1.17)	5.29 (1.01)	^C 0.229
³ Average TG (s.d) mmolL ⁻¹	1.64 (1.04)	1.52 (0.67)	^C 0.885
⁴ Statins (Yes/No)	38/97	34/108	^B 0.509

¹ AD n=202, CTL n=206 ² AD n=52, CTL n= 70

³ AD n=102, CTL n= 106 ⁴ AD n=135, CTL n= 142

^A Student t-test ^B Chi-square test

^C Linear Regression after adjusting for age, gender, the APOE4 and the center of origin of each sample

Abbreviation: HDL-c, High Lipoprotein cholesterol; LDL-c, Low Lipoprotein cholesterol; TC, Total Cholesterol; TG, Total Triglyceride

Table 4.2. Demographic characteristics of participants in study samples from DCR and AddNeuroMed. p-values are obtained from the comparison between AD and control samples.

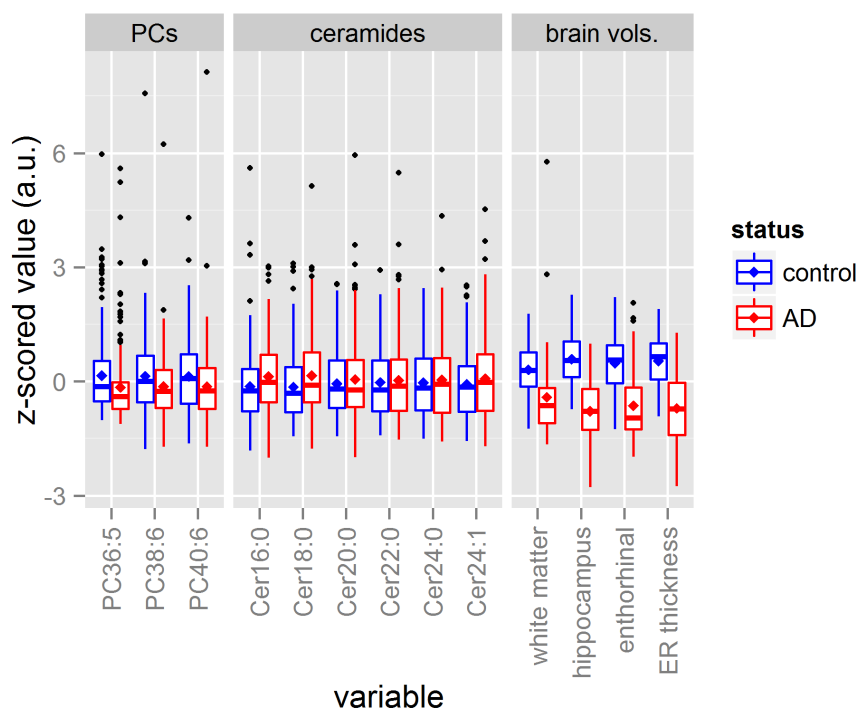


Figure 4.3. Data distributions. The figure shows the box plots corresponding to each phosphatidylcholine (PC), each ceramide, and each brain volume. All variables have been z-scored to ease visual comparison. The sizes of the boxes represent the 25th and 75th percentiles, while the central horizontal line dividing both boxes represents the median, and the mean is represented as a rhomboid. The length of each whisker extends to 1.5 times the length of the box of its corresponding side. Samples beyond the length of the whiskers are represented as individual black dots.

Lipid	Control (Mean± SD)	AD (Mean± SD)	GLM Result	
			z-value	p-value
Cer16:0¹	-0.13 ±0.07	0.10 ±0.06	-2.94	0.004
Cer18:0¹	-0.09 ±0.06	0.22 ±0.07	-2.99	0.003
Cer20:0¹	-0.10 ±0.06	0.01 ±0.08	-1.75	0.081
Cer22:0²	-0.06 ±0.05	0.00 ±0.06	-1.42	0.246
Cer24:0²	-0.05 ±0.05	0.03 ±0.06	-1.89	0.125
Cer24:1²	-0.06 ±0.05	0.09 ±0.06	-2.49	0.028
PC36:5¹	0.03 ±0.07	-0.30 ±0.06	2.35	0.005
PC38:6¹	0.01 ±0.06	-0.28 ±0.06	2.13	0.013
PC40:6¹	0.01 ±0.06	-0.27 ±0.06	1.83	0.023

¹ n=314 (ANM);

² n=412 (DCR+ANM)

Table 4.3. Group differences in lipid levels between AD and control groups. The table shows the mean and standard deviation for each one of the variables and derived factors used in this study. Second column and third column show mean and standard deviation (SD) values for the control and AD population respectively. The last two columns show the z-values and p-values derived from GLM for pairwise comparison between the groups.

Lipid	APOE ¹	White Matter ²	Hippocampus ²	Entorhinal ²	ER Thickness ²
Cer16:0	0.833	0.592	0.172	0.557	0.307
Cer18:0	0.530	0.470	0.064	0.527	0.230
Cer20:0	0.961	0.208	0.165	0.909	0.981
Cer22:0	0.639	0.179	0.457	0.876	0.692
Cer24:0	0.347	0.344	0.694	0.972	0.891
Cer24:1	0.549	0.669	0.420	0.413	0.740
PC36:5	0.469	0.588	0.009	0.761	0.640
PC38:6	0.164	0.599	0.398	0.794	0.561
PC40:6	0.100	0.951	0.348	0.481	0.488

¹ n=407 ; ² n=122

Table 4.4. p -values derived from GLM when testing for associations between lipid levels and atrophies of 4 brain regions and APOE status.

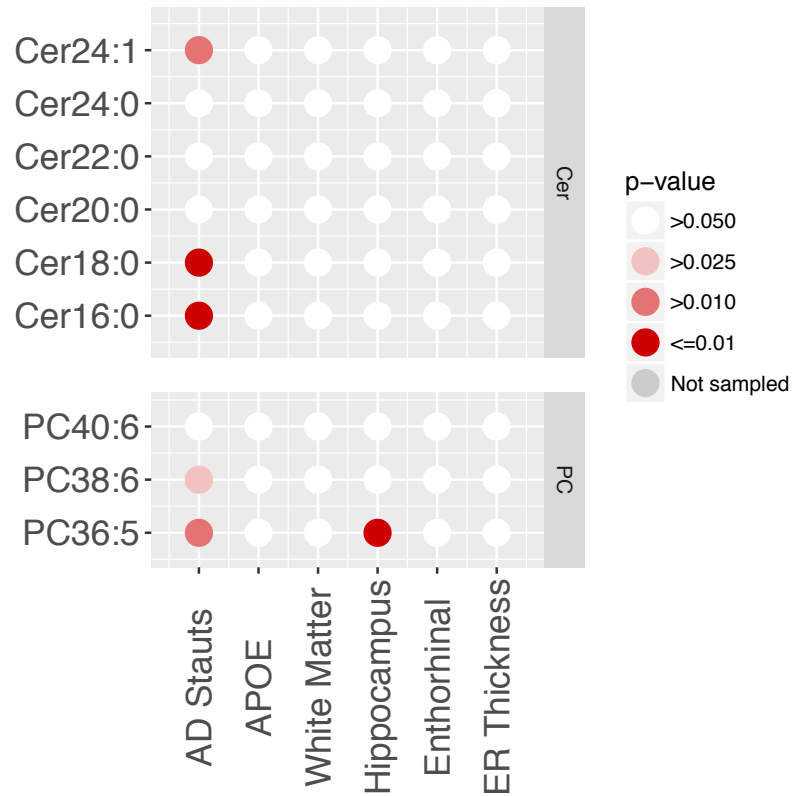


Figure 4.4. Effects found between the levels of 9 lipids and AD variables, using GLM. Columns represent AD variables while rows represent the lipids levels. Different shades of red indicate different standard significance thresholds.

4.3.3. Ceramides in age-bins

Previous GLM analyses (**Figure 4.4, Table 4.3**) showed Cer16:0, Cer18:0 and Cer24:1 associating with AD status but not with brain atrophy. However, this type of analysis (i.e. pooling all subjects together independently of age) may mask some age-specific associations (**Figure 4.5**). To investigate for this possibility, we further applied GLM on AD status, brain volume measurements and APOE4 status in two non-intersecting age-bins. The results of these analyses are summarized as line plots (**Figure 4.6**). The analyses showed no individual ceramides associating with AD classification and APOE4 status in any age-bins. When we tested the ceramide on brain atrophy in When we tested the ceramide levels on brain atrophy in these same age-bins, ceramide levels were associated with greater hippocampal volume loss in relatively younger participants (Cer16:0, Cer18:0, and Cer20:0; p -value <0.05 for age <75 ; see **Figure 4.6, Table 4.5**). In this age bin, associations were also found with ERC volume (Cer16:0, Cer18:0, Cer20:0, Cer22:0, and Cer24:0), ER thickness (Cer16:0 and Cer20:0), and white matter (Cer20:0; see **Figure 4.6, Table 4.5**). In other participants (age >75), only white matter atrophy was significantly associated with Cer18:0, Cer20:0, and Cer22:0. There were no associations between ceramides and ERC volume, ER thickness, or hippocampal volume for older participants. Cer20:0, and Cer22:0. There were no associations between ceramides and ERC volume, ER thickness, or hippocampal volume for older participants.

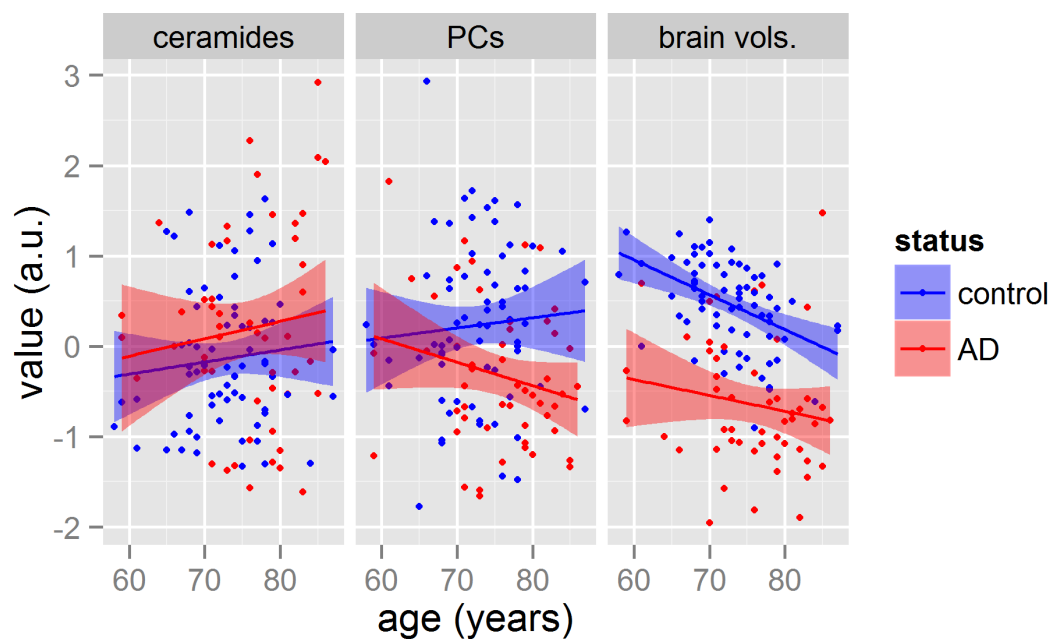


Figure 4.5. Comparisons on how variable groups (lipids and brain volume measurements) change with age in AD and control groups. The y-axis represents the averages of three groups of variables, namely: the six ceramides; the three phosphatidylcholines (PCs); and the four brain volumes (from left panel to right panel). The x-axis represents age. Each point presents a subject, while color represents the diagnosis of each patient. Solid line represents simple linear regression for each group, while the shaded area represents the 0.95 confidence interval.

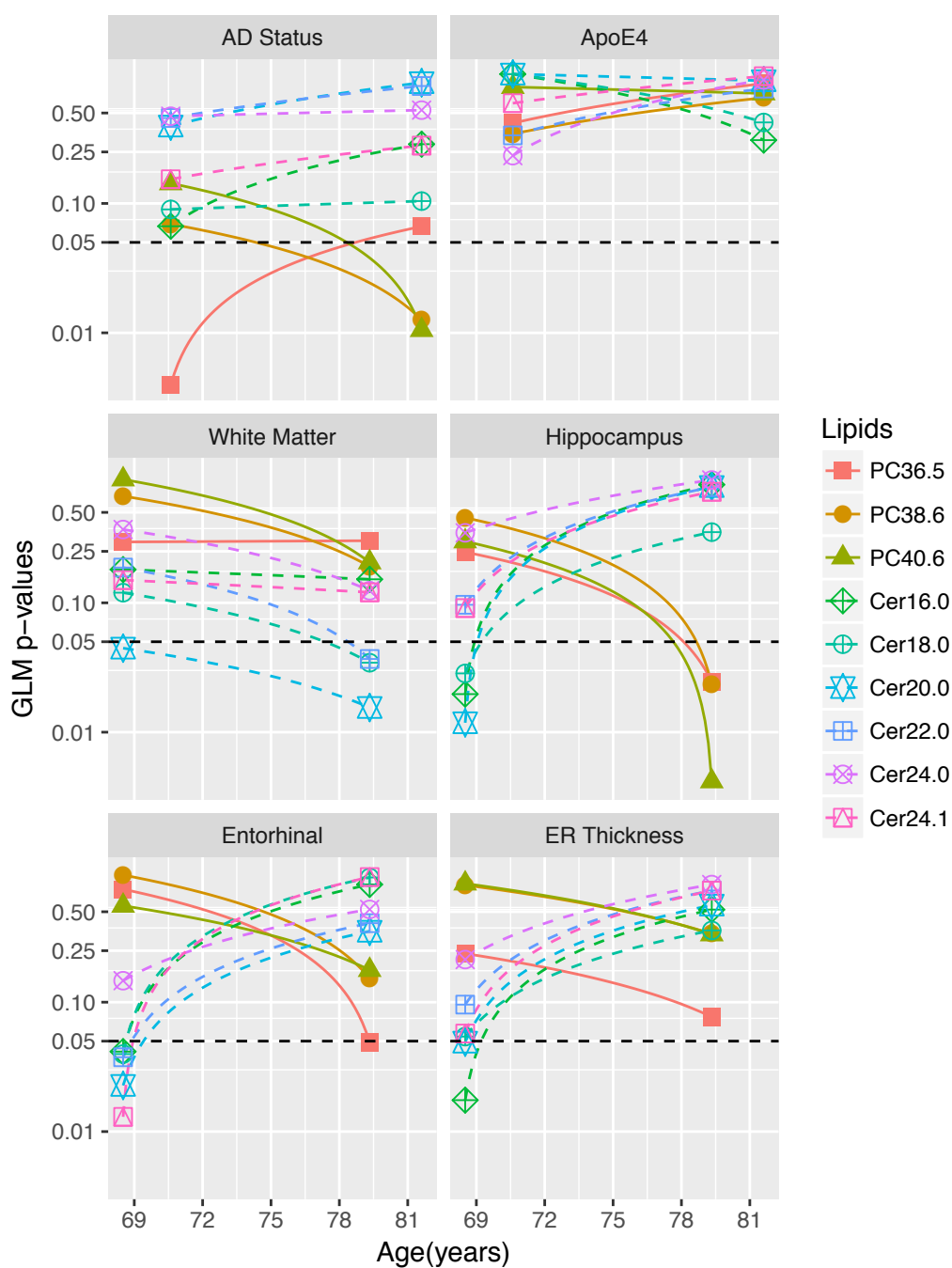


Figure 4.6. GLM were applied in 2 age bins to investigate statistical significance of effects between lipids and three target variables (APOE status, hippocampal volume and AD diagnosis) based on GLM models. Solid lines represent PC molecules while dotted lines represent ceramide molecules. P-values are shown in log scale, with black horizontal dotted lines representing the $p=0.05$ threshold.

4.3.4. Phosphatidylcholines in AD plasma

Three phosphatidylcholine levels from 156 plasma samples from the AD group and 158 from the cognitively healthy group (ANM cohort) were compared using GLM. **Figure 4.3** shows the distribution of all 3 PCs in both clinical groups. **Table 4.3** confirms that single molecule measurements plasma levels of PC(36:5) and PC(38:6) were significantly reduced in abundance in the AD group compared to healthy control group ($p < 0.05$ for both PCs), while PC(40:6) did not show a significant change between the groups.

In **Figure 4.4**, brain volume measurements in 122 participants showed PC(36:5) positively associated with hippocampal atrophy ($p < 0.01$). All other PC associations failed to show significances (white matter, hippocampus, ERC, ER thickness and white matter atrophy).

4.3.5 PCs in age-bins

We applied the same analysis in non-intersecting age-bins for PC species, where PC(36:5) revealed a strong association with AD status in younger participants ($\text{age} < 75$), while PC(38:6) and PC(40:6) did so in the older age bin ($\text{age} > 75$, all $p < 0.05$). Associations of all PCs with hippocampal volume, and of PC(36:5) with ERC volume, were found only in the older age bin. No other associations were found with PCs when segregating into older and younger participants (**Figure 4.6**, **Table 4.5**).

Lipids	AD Status ^A		APOE Status ^A		White Matter ^B		Hippocampus ^B		Entorhinal Cortex ^B		ER Thickness ^B	
	Young	Old	Young	Old	Young	Old	Young	Old	Young	Old	Young	Old
PC36:5	3.95×10^{-3}	6.64×10^{-2}	4.17×10^{-1}	8.44×10^{-1}	2.96×10^{-1}	3.02×10^{-1}	2.47×10^{-1}	2.46×10^{-2}	7.47×10^{-1}	4.89×10^{-2}	2.37×10^{-1}	7.69×10^{-2}
PC38:6	6.69×10^{-2}	1.26×10^{-2}	3.45×10^{-1}	6.56×10^{-1}	6.68×10^{-1}	1.91×10^{-1}	4.55×10^{-1}	2.34×10^{-2}	9.62×10^{-1}	1.53×10^{-1}	8.03×10^{-1}	3.38×10^{-1}
PC40:6	1.43×10^{-1}	1.05×10^{-2}	7.94×10^{-1}	7.08×10^{-1}	8.99×10^{-1}	2.05×10^{-1}	2.99×10^{-1}	4.17×10^{-3}	5.56×10^{-1}	1.77×10^{-1}	8.26×10^{-1}	3.34×10^{-2}
Cer16:0	6.66×10^{-2}	2.87×10^{-1}	9.99×10^{-1}	3.08×10^{-1}	1.80×10^{-1}	11.52×10^{-1}	1.97×10^{-2}	8.19×10^{-1}	4.15×10^{-2}	8.13×10^{-1}	1.75×10^{-2}	5.19×10^{-1}
Cer18:0	8.99×10^{-2}	1.04×10^{-1}	4.22×10^{-1}	4.23×10^{-1}	$.20 \times 10^{-1}$	3.45×10^{-2}	2.84×10^{-2}	3.52×10^{-1}	3.97×10^{-2}	9.20×10^{-1}	5.42×10^{-2}	3.59×10^{-1}
Cer20:0	3.93×10^{-1}	8.58×10^{-1}	8.88×10^{-1}	8.88×10^{-1}	4.46×10^{-2}	1.55×10^{-2}	1.19×10^{-3}	7.92×10^{-1}	2.27×10^{-2}	3.53×10^{-1}	4.91×10^{-2}	5.63×10^{-1}
Cer22:0	4.58×10^{-1}	8.08×10^{-1}	7.73×10^{-1}	7.73×10^{-1}	1.87×10^{-1}	3.68×10^{-2}	9.66×10^{-2}	7.85×10^{-1}	3.75×10^{-2}	4.08×10^{-1}	9.58×10^{-2}	7.30×10^{-1}
Cer24:0	4.72×10^{-1}	5.26×10^{-1}	9.02×10^{-1}	9.02×10^{-1}	3.69×10^{-1}	1.24×10^{-1}	3.48×10^{-1}	8.90×10^{-1}	1.47×10^{-1}	5.21×10^{-1}	2.14×10^{-1}	8.12×10^{-1}
Cer24:1	1.54×10^{-1}	2.80×10^{-1}	9.63×10^{-1}	9.63×10^{-1}	1.50×10^{-1}	1.20×10^{-1}	9.10×10^{-2}	7.22×10^{-1}	1.30×10^{-2}	9.28×10^{-1}	5.73×10^{-2}	7.28×10^{-1}

^A n=412^B n=122

Table 4.5. *p*-values derived from GLM when associations between lipids levels and AD variables (AD stats, APOE status and atrophies of 4 brain regions) in 2 non-intersecting age-bins. Age-bin ‘Young’ consists of 206 plasma samples (age 53-75) for AD status and APOE status while it consists of 61 plasma samples (age 58-75) for brain measurements. Age-bin ‘Old’ consists of 206 plasma samples (age 53-76) for AD and APOE status while it consists of 61 plasma samples (age 58-75) for brain measurements. *p*-values in this table represent those from Figure 4.6.

4.4. Discussion

A number of reports have implicated lipid dysregulation in AD pathology [508, 510, 511], and to explore these findings further, 6 ceramide and 3 phosphatidylcholine species were measured and compared between AD and cognitively healthy volunteers in this study. We tested whether lipid levels were associated with AD diagnosis, brain atrophy and after this, we divided the samples into two non-intersecting age-bins, each bin represented by the same number of participants, and tested whether the associations between lipid levels and AD would be significant in any of these age-bins.

This study found that from the six abundant ceramides measured in plasma and previously shown to be implicated in AD, 3 ceramides, Cer16:0, Cer18:0 and Cer24:1, were elevated in the plasma of AD patients compared to cognitively healthy group. As mentioned previously, there are several studies that have reported ceramide elevation in AD brain [155, 390, 508] and others reporting increased expression of sphingomyelinases (SMase), the enzyme that synthesizes ceramides [519, 520]. Ceramide involvement in neuronal death is likely to be related to its capability to activate caspase 3 [521, 522]. Another possible factor is that in neurons, oxidative stress induced by amyloid-beta ($A\beta$) accumulation can cause overexpression of SMase, this in turn causing elevated ceramides. Interestingly, ceramides have also been found to stimulate $A\beta$ production, which again would result in an increased rate of neuronal death [523].

With aging representing the greatest risk factor for late-onset AD [524], we explored how associations between ceramides and AD classification varied with age. In doing this we found no associations between ceramides and AD status in any of the two age-bins. This result contrasted to GLM models when calculated with all the participants in the study (**Table 4.3** and **Figure 4.4**) which showed three ceramides (Cer16:0, Cer18:0 and Cer 24:1) associating with AD status. However this difference in the results can be attributed to the smaller sample size used in the age-bins as the minimum effect size detectable (at 80% power) by a paired t-test when using the full

sample (n=412) would be 0.14 standard deviations, with age-bin segregation increasing this minimum by ca. 50% to 0.20.

Despite the power limitations of segregating into 2 bins, upon testing how relationships between ceramide levels and hippocampal volume loss varied with age, we observed levels of Cer16:0, Cer18:0 and Cer20:0 elevated in the plasma of younger participants (in ages below 75). Ceramides were also frequently associated with ERC volume and thickness in these younger participants. Hippocampal and ERC atrophy is known to occur before symptoms of cognitive decline during AD development [429] and accumulation of ceramides have been linked with a higher risk of developing AD [510, 525]. Our data suggests that higher levels are also detected in younger individuals with hippocampal atrophy, potentially adding evidence to a role in phenoconversion. This is very interesting since it could mean that ceramides have an important role in early diagnosis or as proposed by Mielke *et al* [525], in phenoconversion.

In addition to ceramides, we also measured PC(36:5), PC(38:6) and PC(40:6). These molecules were linked to AD when they were found associated with decline in AD cognitive function [511] and memory loss in healthy aging [526]. After comparing the PC levels in 314 plasma samples (AMN cohort), we observed diminished PC(36:5) and PC(38:6) levels in AD plasma, partially replicating our previous findings. The lower PC levels reported here, could be as a result of upregulation of PC hydrolysis induced by increased phospholipase A2 (PLA₂) activity and amyloid accumulation [527].

These PCs also contain omega-3 poly-unsaturated fatty acids (ω -3 PUFA). PC(36:5) contains an eicosapentaenoic acid (EPA) as part of its molecular structure, while PC(38:6) contains a docosahexaenoic acid (DHA) [511]. These fatty acids are thought to be absorbed into the brain when contained in molecules of another lipid family, lysophosphatidylcholines, which are direct metabolic products of phosphatidylcholines [528]. ω -3 PUFAs are well known for their neuroprotective properties in brain with higher omega-3 content being associated with lower incidence of dementia [529, 530].

Higher PCs in plasma (i.e. assuming higher ω -3 PUFA in plasma) could have neuroprotective properties by increasing ω -3 PUFA present in the brain. This hypothesis could be supported by a report which demonstrated that a ω -3 PUFA containing PC diet resulted in increased anti-inflammatory activity in the brain [531]. We also observed lower level of EPA containing PC(36:5) associating with hippocampal atrophy, this finding may be explained by EPA acting as a competitive inhibitor of arachidonic acid (AA). This particular role of EPA can thus results in down-regulation of pro-inflammatory AA-derived metabolites such as eicosanoids [532, 533].

When we assessed how associations between PCs and AD varied in different age-bins, we found that PCs showed no associations with white matter atrophy in the 2 age-bins. However, all 3 PCs were associated with hippocampal volume in older participants, while PC(36:5) was also marginally associated with ERC volume in this same age group. PC(36:5) was strongly associated with AD status in younger participants, while PC(38:6) and PC(40:6) were so in the older age bin, suggesting changes in peripheral PC levels may be also age-related or related to the AD severity stage.

Overall, we were able to partially replicate previous findings showing the presence of lipid dysregulation in AD [508, 510, 511]. However, there are some limitations in this study which need to be noted. First, although a more targeted approach was applied compared to a fingerprinting approach, measurements of lipids levels were not carried out under targeted analysis mode. Ideally, deuterated internal standards of ceramide and PC would be used to allow acquisition of quantitative measurements of the 9 lipids (in terms of concentration). These values would reflect a more accurate result. Secondly, potential confounding factors were controlled during analyses, however the influences of unknown or unmeasured factors cannot be excluded such as cholesterol and triglyceride levels [534-536] which are also known to be linked with AD. Thirdly, this study lacked a comparison between various disease states, including other forms of dementia. Without such comparisons, it would be too early to suggest that elevated ceramide and diminished PC levels are AD related or dementia/cognition related.

Therefore, more work is needed to understand the relationships between plasma lipids and AD progression, and importantly, to decipher if they should be considered as biomarkers for diagnosis or targets for AD therapies.

4.5. *Conclusions*

In this study, we attempted to see whether results from previous studies [508, 510, 511] could be reproduced using relatively much larger cohorts. We measured 9 lipids levels from 412 plasma samples and tested their associations with AD status and brain volume measurements. Additionally we also tested how their associations differ in two non-intersecting age bins. From the analyses, we found the levels of 2 PCs and 3 ceramides altered in AD plasma when compared to cognitively healthy plasma. Ceramides were found to be associated with atrophy of hippocampal, ERC and ER in younger participants (age<75). PCs were found decreased and associated with AD status, especially in older participants for PC(38:6) and PC(40:6) (age>75), but in younger participants for PC(36:5). These results contribute to the growing body of evidence that peripheral lipids are implicated in AD pathology. More work is needed to understand the relationships between plasma lipids and AD progression, and importantly, to decipher if they should be considered as biomarkers for diagnosis or targets for AD therapies.

***Altered
Phosphatidylcholine
metabolisms at
prodromal AD stage***

5.1. *Introduction*

Previous Alzheimer’s disease (AD) studies utilizing lipidomics, metabolomics and targeted approaches have identified some lipid pathways, particularly phosphatidylcholines (PCs) and ceramides, altered in the neurodegenerative disease [508, 510-512]. In Chapter 4, we explored these findings further by utilizing a relatively large cohort (AD N=205 and cognitively healthy controls N=207) and compared plasma levels of 3 PCs and 6 ceramides between the two clinical groups. Analyses showed increased ceramides levels and diminished PC levels in AD blood, partially replicating the previous findings [508, 510, 511]. One PC molecule was also found to associate with hippocampal atrophy.

Our focus then shifted toward determining if dysregulation of these particular lipid species develop before the expression of clinical AD symptoms. This is particularly interesting as AD, especially late-onset AD, is known to have a long pre-clinical stage (~20 years) that precedes prodromal amnesic mild cognitive impairment (aMCI). In the last few years, there has been a major push in neurological research concerning late-onset AD to better understand and define the preclinical pathological stage [537]. Identifying altered metabolic pathway at this stage would eventually lead to preclinical targeting treatments. Such treatments might be more effective as they may halt the progression of the disease before irreversible damages occur and help preserve higher levels of function. Similar approaches have been effective in other chronic disease, including diabetes and cardiovascular disease [538, 539].

Here, we test findings from Chapter 4 using a well-characterised and longitudinally followed cohort of older individuals from the U.S.A, the Baltimore Longitudinal Study of Aging (BLSA) [540]. Using the same analytical and statistical methods as in Chapter 4, we examined two or three serial serum lipid profiles from two clinical groups. The first group included “converters” who were cognitively normal at the first time points (or the first two time points). At the last time point, these participants developed AD

symptoms. The second group included “controls” who remained cognitively healthy throughout the study.

In addition to 3 PC and 6 ceramide molecules, we also semi-quantified total cholesterol and total cholesteryl ester (ChoE) levels. As discussed earlier in Chapter 1 (Section 1.2.3), sterol compounds have regularly been implicated with AD. Case-control studies have found high total cholesterol and ChoE levels in AD brain [[128-130](#), [541](#), [542](#)] while lower cholesterol and ChoE levels in AD blood [[118](#), [120](#), [396](#), [530](#), [543](#)]. Together these findings suggested that sterols play a crucial role in AD pathology and thus we decided to test whether metabolic pathways involving sterol compounds are altered at early stages of AD.

In addition to semi-quantification analysis, we also attempted to analyse the entire non-targeted lipidomics data to identify any other lipids which are dysregulated in converters. We first used lipidomics data from the last timepoint to identify a panel of lipids that accurately differentiated blood samples from converters and controls. We then asked whether the levels of the same lipids would differentiate the same cohorts at earlier timepoints. We also attempted to find how well the identified lipid signature could predict incident AD.

5.2. Methods

5.2.1. BLSA participants: demographic characteristics

This study utilized 616 serum samples from 225 BLSA participants collected at 2 or 3 different time points. BLSA cohort has been previously described elsewhere [544]. Blood serum samples from BLSA participants were collected at the National Institute of Aging (NIA) Clinical Research Unit in Harbor Hospital, Baltimore. Pre-analytical procedures including serum collection and sample storage can be found in [545]. Briefly, blood samples were collected between 6 and 7 AM following an overnight fast. Serum samples were aliquoted into 0.5mL volume in Nunc™ cryogenic tubes and stored at -80°C.

BLSA participants (N=225) were classified into two clinical cohorts, “converters” and “non-converters (or controls)”. Converters (N=112) were defined as participants who were cognitively normal at timepoint 1 and 2 (preclinical stages) and developed AD symptoms at timepoint 3 (prodromal stage, average 4 years before being diagnosed with AD). The other 113 participants were controls (non-converters or CTL), age- and sex-matched to converters. These participants remained cognitively normal throughout the timepoints (similar follow-up interval). Serial serum samples from the “converters” were obtained at each timepoint as follows:

Timepoint 1 : 3.90 (± 1.30) years **before** onset of cognitive impairment

Timepoint 2 : 2.27 (± 1.00) years **before** onset of cognitive impairment

Timepoint 3 : 0.18 (± 0.89) years **after** onset of cognitive impairment

More details on the demographic characteristics of BLSA participants by two clinical groups can be found in **Table 5.1**.

5.2.2. Cognitive status

Cognitive status was considered at consensus diagnosis conferences after each assessment/visit, using established procedures described previously [540, 544, 546]. Diagnosis of probable/possible AD was based on DSM-III-R (Diagnostic and Statistical Manual of Mental Disorders, Third Edition) and NINCDS-ADRDA (National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's Disease and Related Disorders Association) criteria. For converters, age at onset of initial AD symptoms was estimated at consensus case conferences using longitudinal cognitive performance data as well as information-based history.

5.2.3. Cognitive testing

All participants underwent detailed cognitive assessments using standardized protocols to assess memory and global mental status. California Verbal Learning (CVL) test [547] assessed memory and episodic verbal learning. In this study, two CVL tests were available, California Verbal Learning free recall long-delay (CVLfrl) and California Verbal Learning total correct (CVLtca). CVLfrl assessed number of words remembered after a long delay (scored 0-16 words). CVLtca assessed total number of words remembered across five learning trials (scored 0-96 words). Mini-Mental State Examination (MMSE, range 0 (worst) to 30 (best)) [548] was also available in this study which was administered to measure global cognitive function.

	Characteristics	Sample Size CTL/Converter	CTL	Converter	Difference p-value
Timepoint 1	Sex (F/M)	112/113	51/61	59/54	$§3.85 \times 10^{-1}$
	APOE4 (-/+)	97/98	74/23	64/34	$§1.26 \times 10^{-1}$
	Age	112/113	77.29 (6.83)	78.72 (6.48)	$†1.09 \times 10^{-1}$
	Years until onset			3.93(1.30)	NA
	Years until Diagnosis			6.35(2.22)	NA
	CVLfrl	82/54	10.99 (3.53)	9.31 (3.70)	$†9.86 \times 10^{-3}$
	CVLtca	83/54	53.02 (11.72)	46.63 (12.48)	$†3.34 \times 10^{-3}$
	MMSE	101/86	28.51 (1.71)	28.04 (1.56)	$†5.22 \times 10^{-2}$
	HDL-c(mg/dl)	108/109	52.29 (15.94)	55.62 (15.46)	$†1.20 \times 10^{-1}$
	LDL-c (mg/dl)	107/109	112.83 (34.15)	122.12 (29.70)	$†3.41 \times 10^{-2}$
	TG (mg/dl)	106/109	122.62 (91.71)	95.41 (43.05)	$†6.27 \times 10^{-3}$
	Statins (N/Y)	112/113	82/30	91/22	$§2.09 \times 10^{-1}$
Timepoint 2	Sex (F/M)	86/80	38/48	42/38	$§3.60 \times 10^{-1}$
	APOE4 (-/+)	75/71	57/18	50/21	$§5.66 \times 10^{-2}$
	Age	86/80	78.64 (6.57)	80.47 (6.20)	$†6.60 \times 10^{-2}$
	Yars from Timepoint 1		2.31 (0.74)	2.09 (0.61)	$†3.46 \times 10^{-2}$
	Years until onset		NA	2.27(1.00)	NA
	Years until Diagnosis		NA	4.66(2.07)	NA
	CVLfrl	66/39	10.58 (3.54)	8.69 (3.34)	$†7.73 \times 10^{-3}$
	CVLtca	66/39	50.45 (12.44)	44.23 (10.47)	$†7.37 \times 10^{-3}$
	MMSE	79/68	28.35 (2.15)	28.15 (1.47)	$†4.91 \times 10^{-1}$
	HDL-c (mg/dl)	83/77	52.28 (16.24)	54.56 (13.78)	$†3.38 \times 10^{-1}$
	LDL-c (mg/dl)	82/77	112.21 (36.65)	112.73 (29.12)	$†9.22 \times 10^{-1}$
	TG (mg/dl)	81/78	127.33 (105.80)	92.19 (43.90)	$†6.03 \times 10^{-3}$
Timepoint 3	Sex (F/M)	112/113	51/61	59/54	$§3.85 \times 10^{-1}$
	APOE4 (-/+)	97/98	74/23	64/34	$§1.26 \times 10^{-1}$
	Age	112/113	81.45 (6.34)	82.80 (6.33)	$†1.08 \times 10^{-2}$
	Years from Timepoint 1		4.16 (1.25)	4.11 (1.10)	$†7.48 \times 10^{-2}$
	Years after onset		NA	0.18(0.89)	NA
	Years until Diagnosis		NA	2.24(2.05)	NA
	CVLfrl	92/67	10.52 (3.63)	6.64 (3.74)	$†1.62 \times 10^{-9}$
	CVLtca	92/71	50.65 (12.22)	38.03 (11.51)	$†2.72 \times 10^{-10}$
	MMSE	108/104	28.41 (1.99)	27.26 (1.99)	$†3.91 \times 10^{-5}$
	HDL-c(mg/dl)	102/105	52.47 (15.58)	55.55 (15.18)	$†1.52 \times 10^{-1}$
	LDL-c(mg/dl)	101/104	106.83 (34.22)	117.59 (34.40)	$†2.58 \times 10^{-2}$
	TG (mg/dl)	102/105	113.38 (88.61)	92.09 (47.08)	$†3.31 \times 10^{-2}$
	Statins (N/Y)	112/113	82/30	91/22	$§2.09 \times 10^{-1}$

Abbreviation: CVLfrl: California verbal learning A long-delay free recall; CVLtca, California verbal learning Total correct A; MMSE, Mini mental state examination; HDL-c, High density lipoprotein cholesterol; LDL-c, Low density lipoprotein cholesterol; TG, Triglyceride

$§$ Chi-square test; $†$ Student t-test

Note: Otherwise stated, the values represented are average values while values in brackets represent standard deviation values.

Table 5.1. Demographic characteristics of BLSA participants whose serum samples were analysed in the current study.

5.2.4. Lipid levels acquisition

Samples were prepared and analysed in two separate batches. The first batch (Batch 1) was analysed in 2014 (N=534) while the second batch (Batch 2) was in 2016 (N=82), both using the identical methodology. Lipid extraction and analytic methods used are same as those in Chapter 4 (Section 4.2).

5.2.5. Lipid Identification and Quantification

Identification procedures for PC and Ceramide species has been described in Chapter 4 (Section 4.3) and their descriptive can be found in **Table 4.1**. Identification of cholesterol and ChoE was carried out by comparing retention time (Rt) of authentic compounds. Descriptive of these sterol compounds and their extracted ion chromatograms (EICs) can be found in **Table 5.2** and **Figure 5.1** respectively. The single molecule integrated peak areas under the exact mass chromatographs of the 11 lipids and the internal standard (Tripentadecanoin) were obtained by using QuanLynx (MassLynx 4.1, Waters) by setting up an integration parameter file using the average mass charge ration (m/z) and retention time of the 11 lipids and internal standard. Tolerances for mass and retention time of 0.1Da and 5min (10min for ChoE) respectively were typically applied. During quantification procedure, 4 ceramides, Cer16:0, Cer18:0, Cer20:0 and Cer22:0, were found to be below limit of quantification in >80% of the samples. These 4 ceramides were excluded from further analyses.

Untargeted lipidomics dataset was acquired by using the R package ‘*xcms*’ where feature detection and peak deconvolution across the samples were performed from netCDF files [549]. The dataset then underwent total area normalization. Using QC samples, CV (coefficient variation) values for each feature were derived, and features with CV values >20% were excluded from further analysis. Overall, 1208 features were available for further analyses.

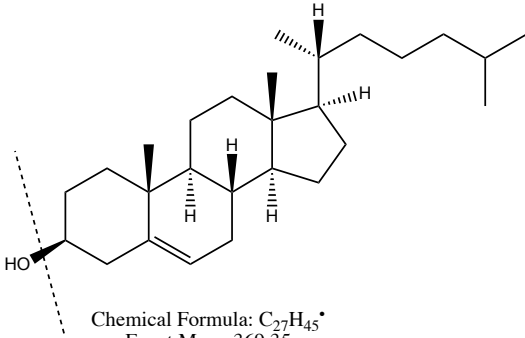
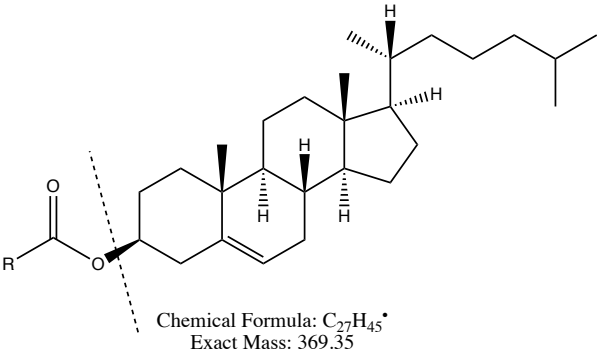
Cholesterol			
 Chemical Formula: $C_{27}H_{46}O$ Exact Mass: 386.35	Molecular Weight	386.65	
	Exact Weight	386.35	
	Base Ion [Cho-H ₂ O+H] ⁺	m/z 369.35	
	Retention Time	11.58 min	
Cholesteryl Ester			
 Chemical Formula: $C_{27}H_{46}O$ Exact Mass: 386.35	Molecular Weight	NA	
	Exact Weight	NA	
	Base Ion [Cho-RCOOH+H] ⁺	m/z 369.35	
	Retention Time	23~33 min	

Table 5.2. Descriptive of Cholesterol and Cholesteryl Ester. Both molecules produce ions with m/z 369.35 in positive mode which corresponds to cholesterol (or cholesterol head group) in a form of [M-H₂O+H]⁺.

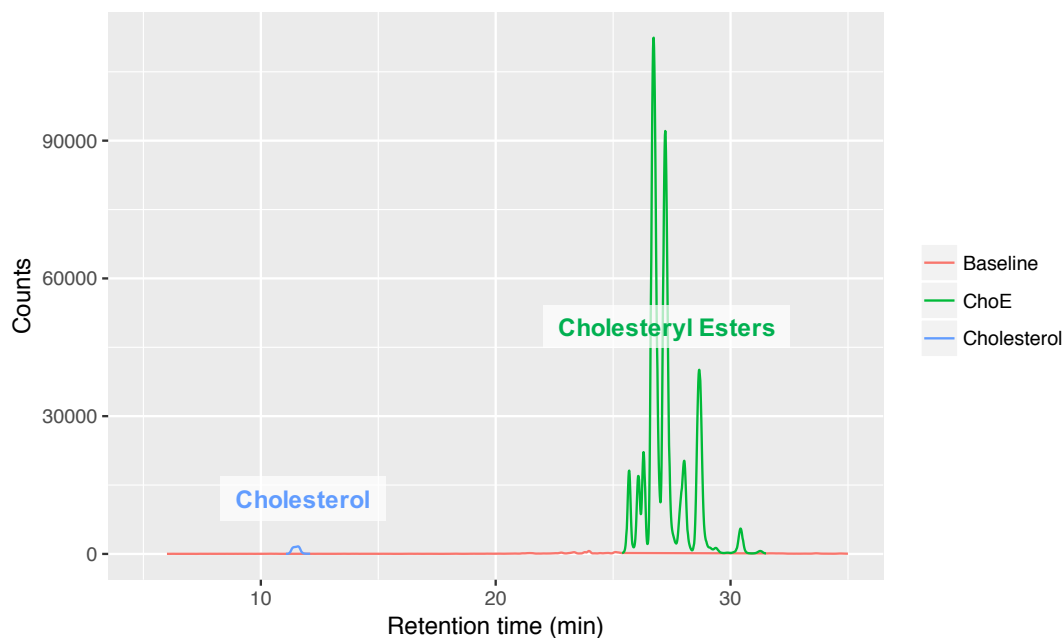


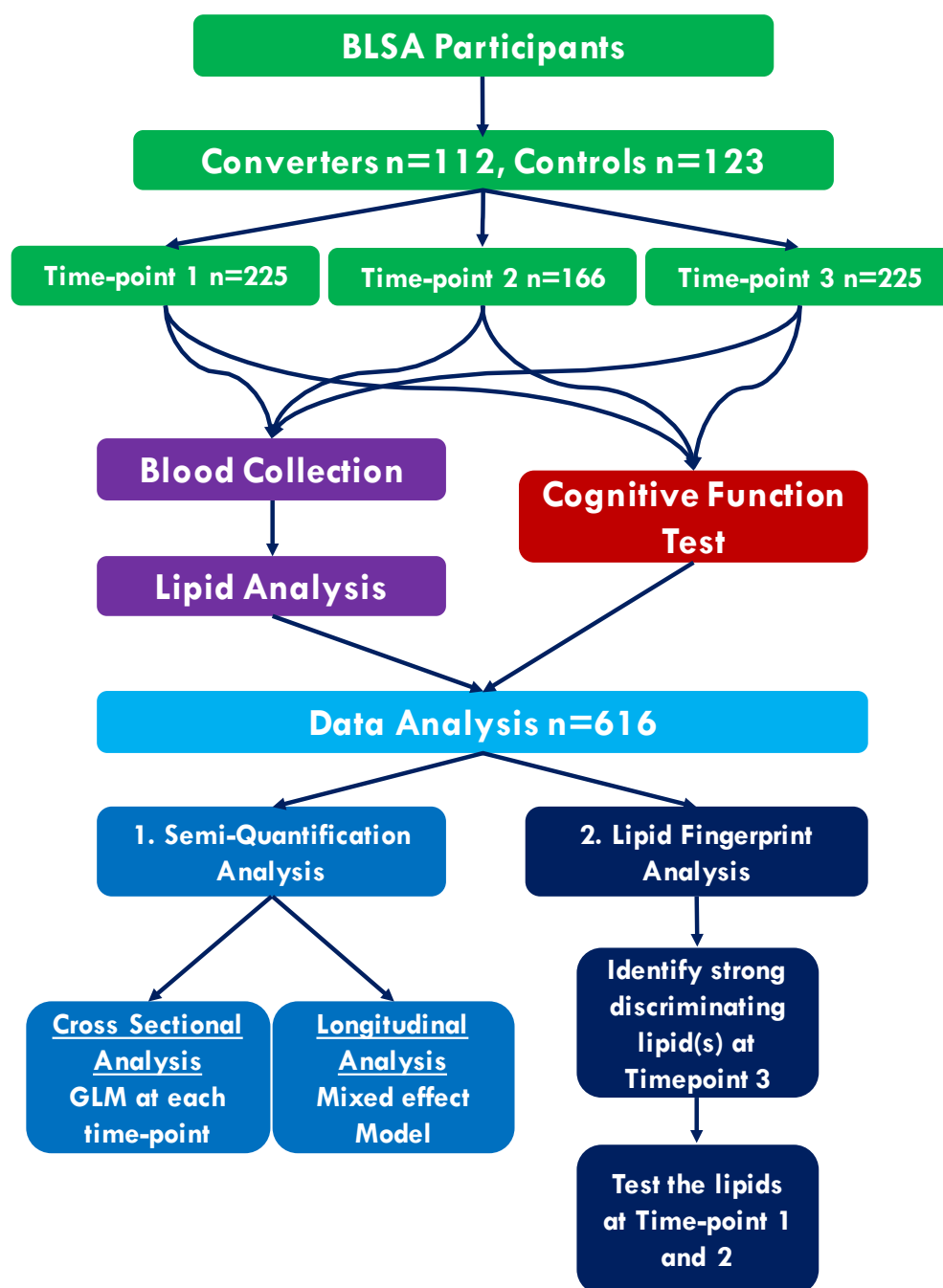
Figure 5.1. Extracted ion chromatogram of m/z 369.35 from a QC sample. A single peak at Rt 11.58 minutes (in blue) represents cholesterol. Multiple peaks at Rt 35~32 minutes (green) represent cholesterol ester molecules.

5.2.6. Statistical Analysis

Lipids from semi-quantification method first underwent cross-sectional analyses. Generalised linear model (GLM) were applied to compare lipid levels between two clinical groups at timepoint 1 and 3 (both N=225). The models were based on those used in Chapter 4 that they were adjusted for age at sampling, gender, presence of the apolipoprotein E 4 allele (APOE4) and technical variations (batch effect and sensitivity variations). GLM on timepoint 2 was not carried out because of the difference in sample size that would result in different statistical power on the resulting p-value. Then we carried out longitudinal analysis to determine whether changes in lipid levels over time differ between converters and controls. Mixed effect models were applied utilising all three timepoint samples (N=616) and interaction between time (number of years after timepoint 1 sampling) and clinical status (“converters” *vs* “controls”) was used as a fixed effect.

For analysis of untargeted lipid profile, multivariate and univariate data analyses were employed. Multivariate data analysis was carried out by employing Orthogonal Partial Least Square–Discriminating Analysis (OPLS-DA) on 184 samples (Batch 1 and timepoint 3) initially. From it, features strongly discriminating the two clinical groups were derived. These discriminating features were tested on 41 samples from Batch 2 & timepoint 3 for validation purpose. Then they were tested on timepoint 2 (N=166) and timepoint 1 (N=225) samples to test their discriminating abilities at pre-clinical stages. Multivariate data analyses were carried out in SIMCA software (MKS Umetrics AD, Sweden). For univariate data analyses, GLMs were employed. On timepoint 3 samples (N=225), features with $q < 0.05$ were derived. These features were then tested on timepoint 1 samples (N=225) as well as timepoint 2 samples (N=166).

Features derived from both multivariate and univariate analyses underwent mixed effect model to test how changes in their levels differ between “converters” and “controls” over time. All statistical analyses (except for OPLS-DA) were carried out in R.3.2.3. **Figure 5.2** shows a schematic workflow carried out in this study.



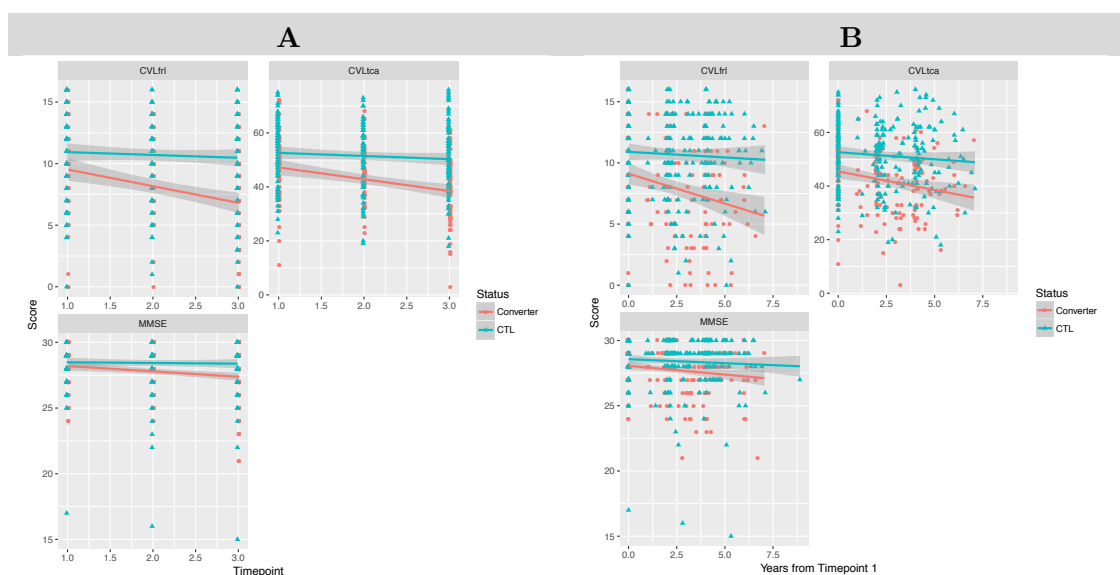
Abbreviation: BLSA, Baltimore Longitudinal Study of Aging; GLM, Generalised Linear Model

Figure 5.2. Schematic workflow used in this study.

5.3. Results

5.3.1. Sample Demographic

The demographic characteristics of participants from the BLSA included in this report are described in **Table 5.1**. Characteristics were compared between the two clinical groups at each timepoint. Chi-square test showed no difference in sex and presence of APOE4. Student t-test showed no difference in age at all three timepoints. Three different cognitive function test scores were also available. Mini mental state examination (MMSE) showed no differences at timepoint 1 and 2, while showed a difference at timepoint 3 (t-test $p < 0.05$). CVLfrl and CVLtca scores were different at all three timepoints ($p < 0.01$ for all). Linear regression models showed greater declines in all three cognitive test scores over time (timepoints) for the converter group (**Figure 5.3**). Total triglycerides (TG), high-density lipoprotein cholesterol (HDL-c) and low-density lipoprotein cholesterol (LDL-c) were also measured. LDL-c levels were differentiable between the groups at timepoint 1 and 3 while TG levels were at timepoint 3.



Abbreviation: CVLfrl: California verbal learning A long-delay free recall; CVLtca, California verbal learning Total correct A; MMSE, Mini mental state examination

Figure 5.3. Comparisons on cognitive test score changes with time in converter and control groups. (A) shows changes in test scores over three timepoints. (B) shows changes in test scores over time (number of years from timepoint 1). Each point presents a test score, while colour represents the clinical group. Solid line represents simple linear regression for each clinical group, while the shaded area represents a 0.95 confidence interval.

5.3.2. Semi-Quantification Results

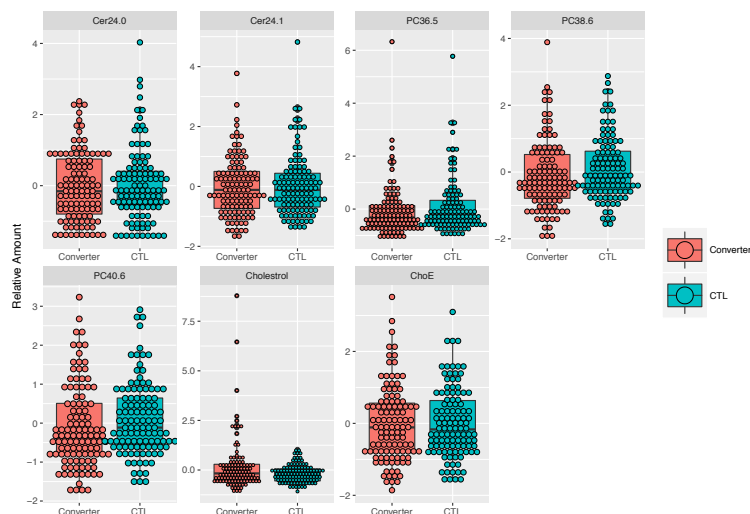
Logistic and Linear Regression Models (Cross-sectional analyses)

Initially after determining the levels of 2 ceramides, 3 phosphatidylcholines, cholesterol and ChoE using semi-quantification approach, boxplots were utilized to see the distribution of 7 lipid levels in converters and controls at all three timepoints (**Figure 5.4**). Logistic regression analyses (via GLM approach) were followed which investigated the associations between lipids levels and AD conversion at timepoint 1 and 3 (**Table 5.3**). At timepoint 1 (preclinical stage), no lipid levels were different between converters and controls (all $p > 0.05$). At timepoint 3 (prodromal stage), PC(36:5) and ChoE levels were found to be significantly different, both lipid species were lower in converters (all $p < 0.01$). These two lipids also passed correction for multiple testing at $q < 0.05$.

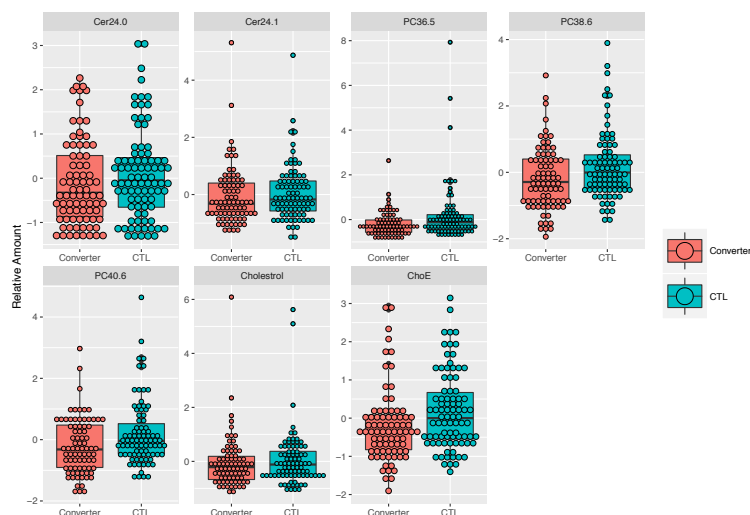
Then linear regression models were applied to investigate the association of each lipid with CVLfrl, CVLtca and MMSE test scores. At timepoint 1 and 3, no lipids associated with the three cognitive function tests (all $p > 0.05$).

For Cholesterol and ChoE, additional GLM models were built where the effect of statin usage were adjusted for in addition to age at sampling, gender, presence of the APOE4 and technical variations. **Table 5.4** shows that only subtle changes in p- and q- values can be observed between the two different types of GLM models suggesting little effect of statin usage on association between cholesterol/ChoE and AD.

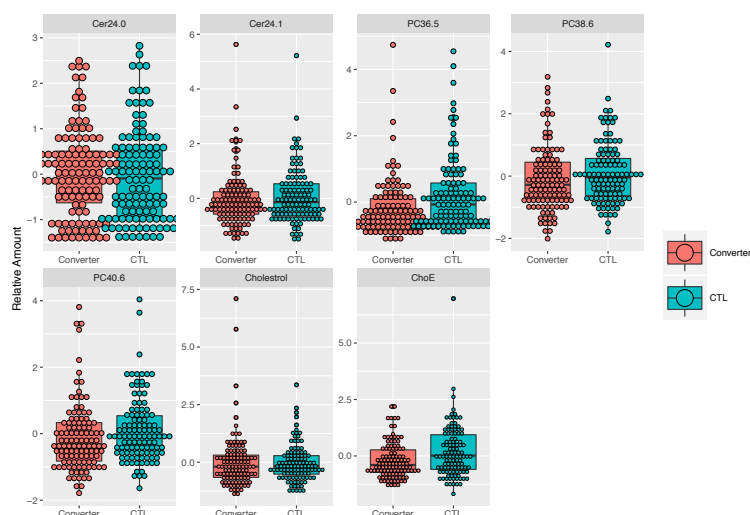
Timepoint 1



Timepoint 2



Timepoint 3



Abbreviation; Cer, Ceramide; PC, Phosphatidyl choline; ChoE, Cholesteryl Ester, CTL, Controls

Figure 5.4. Relative amount of 7 serum lipids levels in BLSA participants collected at three different timepoints. All variables (lipid levels) have been scaled to have mean of 0 and standard deviation of 1. The size of the boxes represent the 25th and 75th percentiles, the central horizontal line in the boxes represents the median.

Time-point		Cer24:0		Cer24:1		PC36:5		PC38:6		PC40:6		Cholesterol		ChoE	
		OR	p	OR	p	OR	p	OR	p	OR	p	OR	p	OR	p
Status	1	0.90	0.472	0.96	0.757	0.84	0.233	0.79	0.136	0.81	0.163	1.46	0.108	0.87	0.359
	3	1.00	0.992	0.89	0.422	0.64	0.007*	0.81	0.156	0.77	0.082	0.97	0.837	0.60	0.005*
CVLtca	1	0.33	0.318	1.27	0.832	1.76	0.576	1.43	0.753	0.97	0.981	0.18	0.507	0.81	0.846
	3	0.32	0.287	2.25	0.460	0.21	0.139	0.19	0.161	0.17	0.151	0.44	0.621	0.66	0.771
CVLfrl	1	0.67	0.236	0.96	0.912	1.28	0.425	1.01	0.973	0.90	0.768	0.79	0.760	0.90	0.749
	3	0.73	0.345	1.07	0.844	0.73	0.341	0.68	0.310	0.68	0.313	0.64	0.404	0.75	0.518
MMSE	1	0.77	0.454	1.10	0.492	1.14	0.328	1.03	0.835	0.99	0.949	0.66	0.145	1.04	0.791
	3	1.11	0.476	1.10	0.576	1.01	0.952	1.06	0.718	1.06	0.707	0.92	0.657	1.11	0.505

Abbreviation: OR, Odd Ratio; CVLfrl: California verbal learning A long-delay free recall; CVLtca, California verbal learning Total correct A; MMSE, Mini mental state examination.

* indicate $q < 0.05$ after applying False Discovery Rate multiple test correction procedure.

Note: Status is a comparison between converters and controls.

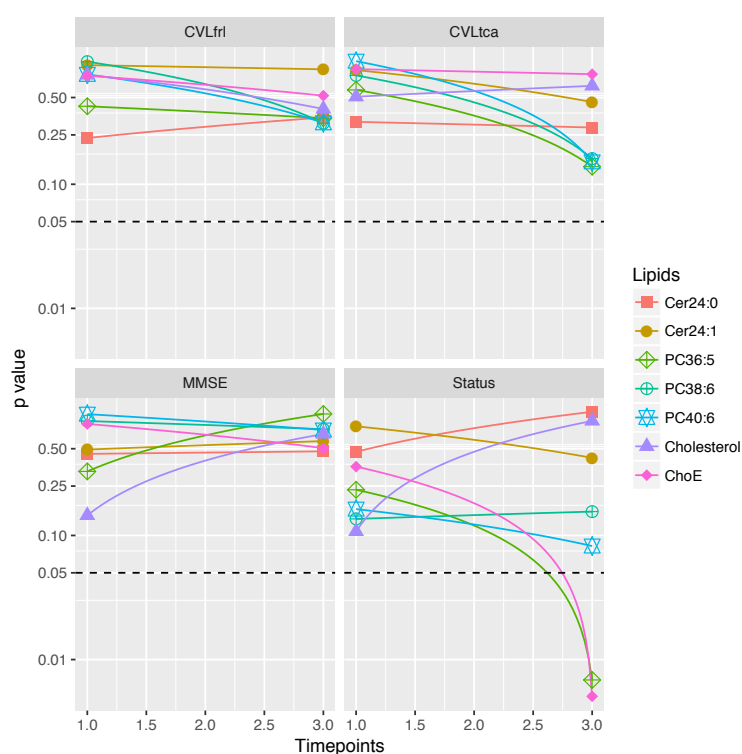
Table 5.3. This table shows correlations between each lipid and each AD phenotype. p- and q- values derived from regression models testing association between lipid levels and clinical status and three cognitive function test scores. Logistic regression models were applied to Status and linear models were applied for CVLtca, CVLfrl and MMSE. P-values were adjusted using False Discovery Rate correction procedures.

	Time-point	Cholesterol				ChoE			
		Exc. Statin		Inc. Statin		Exc. Statin		Inc. Statin	
		p	q	p	q	p	q	p	q
Status	1	1.08×10^{-1}	2.90×10^{-1}	1.21×10^{-1}	2.90×10^{-1}	3.59×10^{-1}	8.46×10^{-1}	4.79×10^{-1}	8.47×10^{-1}
	3	8.37×10^{-1}	8.37×10^{-1}	8.16×10^{-1}	8.16×10^{-1}	5.00×10^{-3}	2.02×10^{-2}	6.78×10^{-3}	2.71×10^{-2}
CVL-tca	1	5.07×10^{-1}	6.76×10^{-1}	5.09×10^{-1}	6.79×10^{-1}	8.46×10^{-1}	8.46×10^{-1}	8.47×10^{-1}	8.47×10^{-1}
	3	6.21×10^{-1}	8.37×10^{-1}	7.36×10^{-1}	8.16×10^{-1}	7.71×10^{-1}	7.71×10^{-1}	8.93×10^{-1}	8.93×10^{-1}
CVL-frl	1	7.60×10^{-1}	7.60×10^{-1}	7.62×10^{-1}	7.62×10^{-1}	7.49×10^{-1}	8.46×10^{-1}	7.50×10^{-1}	8.47×10^{-1}
	3	4.04×10^{-1}	8.37×10^{-1}	4.43×10^{-1}	8.16×10^{-1}	5.18×10^{-1}	6.90×10^{-1}	7.44×10^{-1}	8.93×10^{-1}
MMS-E	1	1.45×10^{-1}	2.90×10^{-1}	1.45×10^{-1}	2.90×10^{-1}	7.91×10^{-1}	8.46×10^{-1}	7.58×10^{-1}	8.47×10^{-1}
	3	6.57×10^{-1}	8.37×10^{-1}	5.90×10^{-1}	8.16×10^{-1}	5.05×10^{-1}	6.90×10^{-1}	3.21×10^{-1}	6.42×10^{-1}

Abbreviation: CVLfrl: California verbal learning A long-delay free recall; CVLtca, California verbal learning Total correct A; MMSE, Mini mental state examination.

Note: Status is a comparison between converters and controls

Table 5.4. Comparisons of p- and q- values for regression models excluding statins usages and including statins usages for cholesterol and ChoE levels.



Abbreviation: CVLfrl: California verbal learning A long-delay free recall; CVLlca, California verbal learning Total correct A; MMSE, Mini mental state examination.

Figure 5.5. Representation of p-values for each AD clinical variables derived from Table 5.3.

ROC models for converter *vs.* controls (Cross-sectional analyses)

ROC models were applied to investigate how well the 7 selected serum lipids could discriminate converters from controls (Table 5.5 and Figure 5.6). At timepoint 1 (preclinical stage), no single lipid showed accuracy (acc) and AUC values higher than 0.6. At timepoint 3, two lipids showed accuracy and AUC values higher than 0.6, and they were PC(36:5) (acc=0.604, AUC=0.626) and ChoE (acc=0.613, AUC=0.611). We then built another ROC model where PC(36:5) and ChoE were combined, the resulting classification values were acc=0.51, AUC=0.556 at timepoint 1, and acc=0.613, AUC=0.628 at timepoint 3. When all 7 lipids were combined, the resulting acc and AUC values were 0.556 and 0.598 respectively at timepoint 1, and 0.622 and 0.659 respectively at timepoint 3.

	Accuracy		Sensitivity		Specificity		AUC	
	T1	T3	T1	T3	T1	T3	T1	T3
Cer24:0	0.480	0.524	0.646	0.558	0.313	0.491	0.498	0.524
Cer24:1	0.476	0.533	0.602	0.690	0.348	0.375	0.503	0.542
PC36:5	0.516	0.604	0.690	0.726	0.339	0.482	0.552	0.626
PC38:6	0.551	0.538	0.646	0.637	0.455	0.438	0.577	0.563
PC40:6	0.560	0.547	0.664	0.646	0.455	0.446	0.577	0.587
Cholesterol	0.538	0.493	0.460	0.496	0.616	0.491	0.548	0.548
ChoE	0.493	0.613	0.628	0.708	0.357	0.518	0.482	0.611
APOE4	0.554	0.554	0.347	0.347	0.763	0.763	0.555	0.555
All_7_Lipids	0.556	0.622	0.469	0.690	0.643	0.554	0.598	0.659
PC36:5+ChoE	0.511	0.613	0.637	0.699	0.384	0.527	0.556	0.628
PC36:5+ChoE+APOE4	0.549	0.651	0.490	0.725	0.608	0.577	0.589	0.677

Abbreviation: T, Timepoint; AUC, Area Under the Curve; Cer, Ceramide; PC, Phosphatidyl cholines, ChoE, Cholesteryl Ester

Table 5.5. ROC model results (AD conversion) at timepoint 1 (preclinical stage) and at timepoint 3 (prodromal stage).

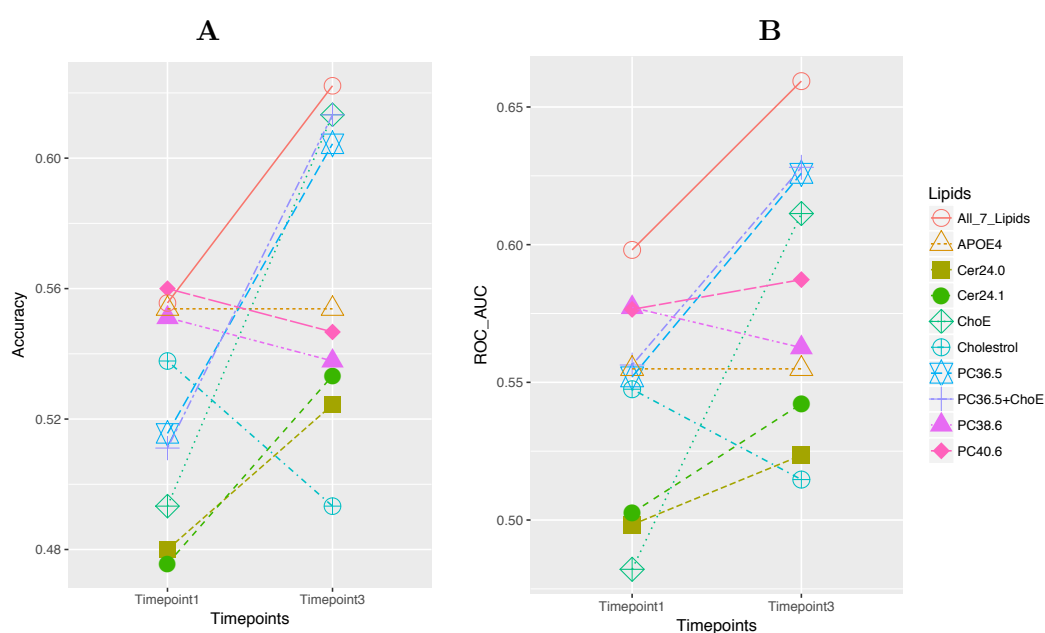


Figure 5.6. Figures showing how discriminating abilities between converters and controls changes from timepoint 1 to timepoint 3. Ten models were built, one for each lipid, APOE4, combination of PC36:5 and ChoE, and combination of all 7 lipids. (A) shows change in accuracy values, (B) shows change in ROC AUC values.

Linear mixed models (Longitudinal analyses)

Longitudinal associations between the 9 selected lipids and AD conversion were tested by employing mixed linear regression models (**Table 5.6** and **Figure 5.7**). For these analyses, serum samples from all three timepoints were utilized. After adjusting for age of BLSA participants at the initial blood draw (timepoint 1), longitudinal changes in Cholesterol ($\beta=-0.0985$) and ChoE ($\beta=-0.0944$) levels were found associate with AD conversion at FDR $q<0.05$ level.

Lipids	Beta (S.E.)	p- value	FDR q-value
Cer24:0	-0.0199 (0.0396)	6.15×10^{-1}	7.18×10^{-1}
Cer24:1	-0.0174 (0.0337)	6.06×10^{-1}	7.18×10^{-1}
PC36:5	-0.0601 (0.0334)	7.30×10^{-2}	1.70×10^{-1}
PC38:6	0.0033 (0.0257)	8.97×10^{-1}	8.97×10^{-1}
PC40:6	-0.0169 (0.0247)	5.17×10^{-1}	7.18×10^{-1}
Cholesterol	-0.0985 (0.0376)	9.16×10^{-3}	3.21×10^{-2}
ChoE	-0.0944 (0.0321)	4.14×10^{-3}	2.89×10^{-2}

Abbreviation: S.E., Standard error; FDR, False Discovery Rate

Table 5.6. Longitudinal association between changes in 7 selected serum lipid levels and AD conversion.

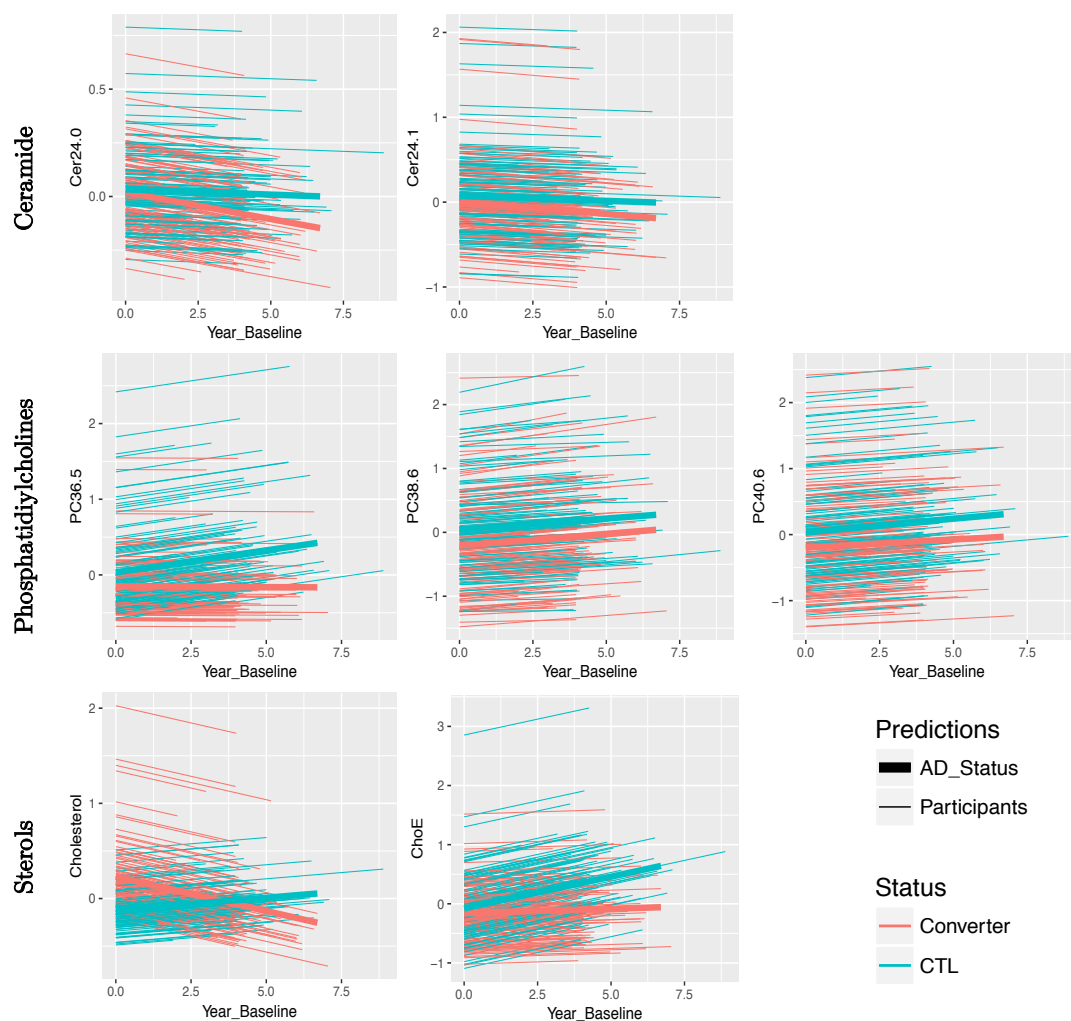


Figure 5.7. Longitudinal changes in lipid levels in converters and controls serum samples using mixed models. Y-axis represent predicted level of each lipid while x-axis represents number of years from the initial blood draw (timepoint 1). Relative thin line represent each participant while relatively thick line represent either converters as together (red) or controls as together (blue).

Correlation between the 7 lipids

We then asked whether the 7 selected lipids have any relationship with each other. Pearson's correlation analyses were carried out at timepoint 1 and 3 (**Figure 5.8**). At both timepoint, PC species can be seen to correlate with each other (all $r \geq 0.60$ and $p < 1.0 \times 10^{-15}$) as well as between Cholesterol and ChoE (timepoint 1 $r > 0.65$, and $p < 1.0 \times 10^{-15}$; timepoint 3 $r > 0.73$, $p < 1.0 \times 10^{-15}$). Although moderate, correlation analyses also showed ChoE correlating with three PC molecules (all $0.25 < r < 0.53$, $p < 1.0 \times 10^{-4}$).

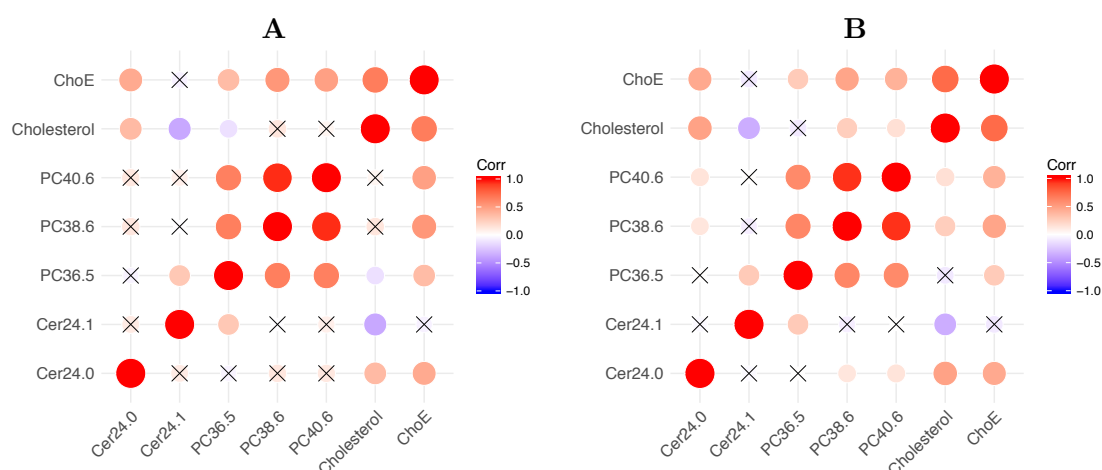


Figure 5.8. Plot of correlation matrix between the levels of 9 selected lipids. Red circle represents positive correlation while blue circle represents negative correlation. The size of circle and intensity of the colour represents correlation coefficient value. Cell with X represent correlation with no significance ($p < 0.05$). (A) is correlation plot at timepoint 1. (B) is correlation plot at timepoint 3.

5.3.3. Lipid fingerprint analyses

Next, we analysed the entire lipid dataset of 1208 lipid features in an attempt to find a panel of lipid features which would discriminate converter samples from non-converter (control) samples. Unlike semi-quantification approach, the analyses were carried out without priori knowledge on the nature and/or identity of the lipid features.

Multivariate Data Analysis

The first part of lipid fingerprint data analyses was to use multivariate data analysis approach. A first OPLS-DA model was built by utilizing Batch 1 & timepoint 3 samples (N=184). Although CV-ANOVA p value ($p=2.38\times 10^{-2}$) suggested the model to be reliable, the score-plot (**Figure 5.9A**) and $R^2Y=0.293$ showed a poor discrimination between converters and controls. From the model, the best discriminating features were extracted using thresholds of Variable Importance Score (VIP) >1 and coefficient value >0.01 . Thirteen features were extracted and we utilized these 13 features to build a second OPLS-DA model based on the same set of samples. The resulting OPLS-DA model had CV-ANOVA $p=1.65\times 10^{-5}$ suggesting the model being more reliable, but the score plot (**Figure 5.9B**) and its $R^2Y=0.185$ demonstrated segregation between the two clinical groups failed to improve. Finally, we built a third OPLS-DA model using the 13 features selected for the previous model and applied on Batch 2 & Timepoint 3 samples for testing purposes. The corresponding score-plot (**Figure 5.9C**) and its $R^2Y=0.132$ demonstrated no segregation between the group (CV-ANOVA $p=1$).

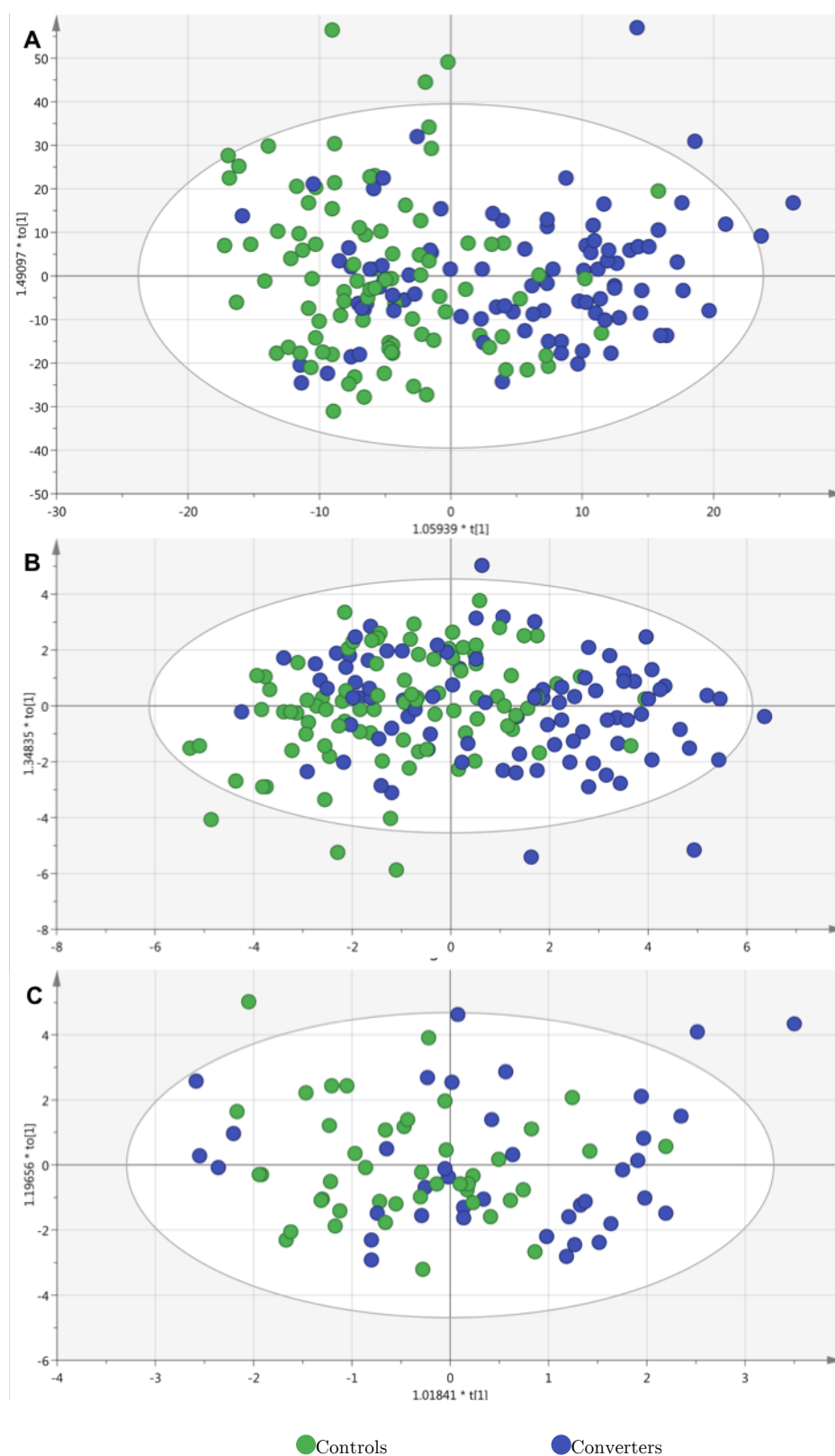


Figure 5.9. OPLS-DA score plots. (A) OPLS-DA model utilising all 1208 features on Timepoint 3 & Batch 1 samples. (B) OPLS-DA model utilising VIP > 1 and coefficient > 0.01 features on Timepoint 3 & Batch 1 samples. (C) OPLS-DA model utilising same features from (B) on Timepoint 3 & Batch 2 samples.

Univariate Data Analysis

Next, we utilised univariate data analysis approaches in order to identify lipid features discriminating converters from controls. Initially, GLMs were built for all 1208 features on Timepoint 3 & Batch 1+2 samples (N=225) (**Figure 5.10**). Unlike multivariate data analysis approach, GLM models allowed adjuncts for the effects of age, sex, presence of APOE4, statins usage and batch effect. From 1208 features, 249 features were found to have significant associations with AD conversion ($p < 0.05$). Of these 249 features, the serum levels of 151 features were lower and 98 features were higher in converters. When FDR procedure was applied, 1 feature passed the FDR $p < 0.05$ level (feature X805.56_16.52: positive ionisation, m/z 804.55 and Rt 16.52). We then inspected X805.56_16.52 feature from a QC sample MS spectrum, and determined X805.56_16.52 to be a C13 isotope of another feature X804.55_16.52 (**Figure 5.12**). Pearson correlation analysis showed these two features correlating strongly with each other ($r=0.78$, $p=2.26 \times 10^{-16}$), further highlighting these two features to originate from the same molecule. We then looked at the GLM for X804.55_16.52 and found the feature to be also associated with AD conversion at $p < 0.05$ level ($p=0.002$, FDR $p=0.736$).

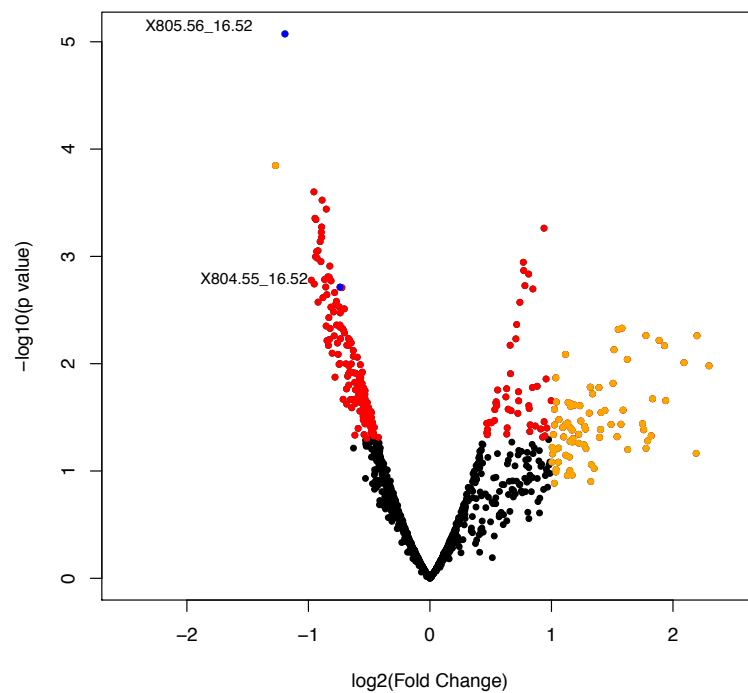


Figure 5.10. Volcano plot summarising results of GLM which investigated associations between 1208 features and AD conversion at timepoint 3. Red dots represent features with $p < 0.05$, yellow represent features with $\log_2(\text{Fold Change}) < 1$, blue dots represent X804.55_16.52 and X805.56_16.52.

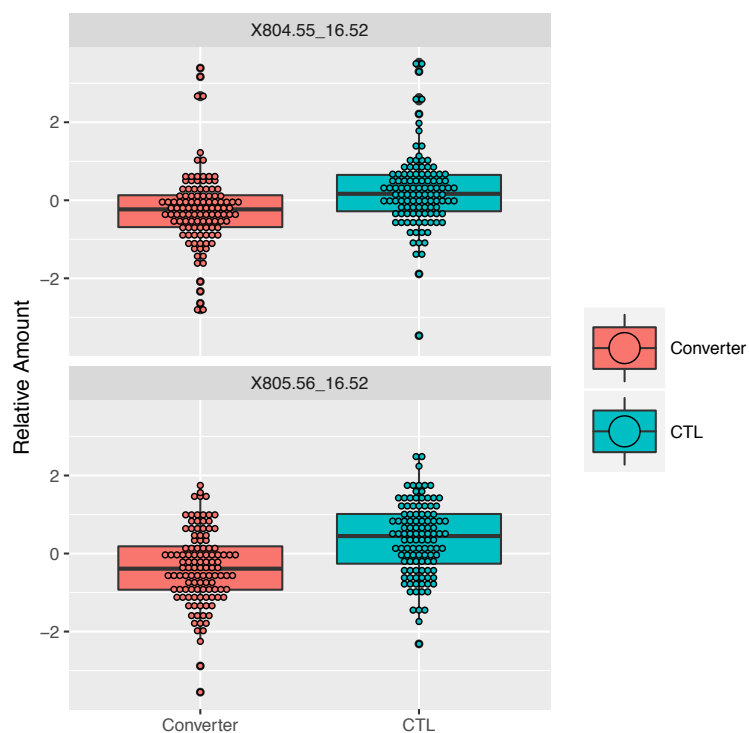


Figure 5.11. Relative amounts of X804.55_16.52 and X805.56_16.52 between two clinical groups at timepoint 3.

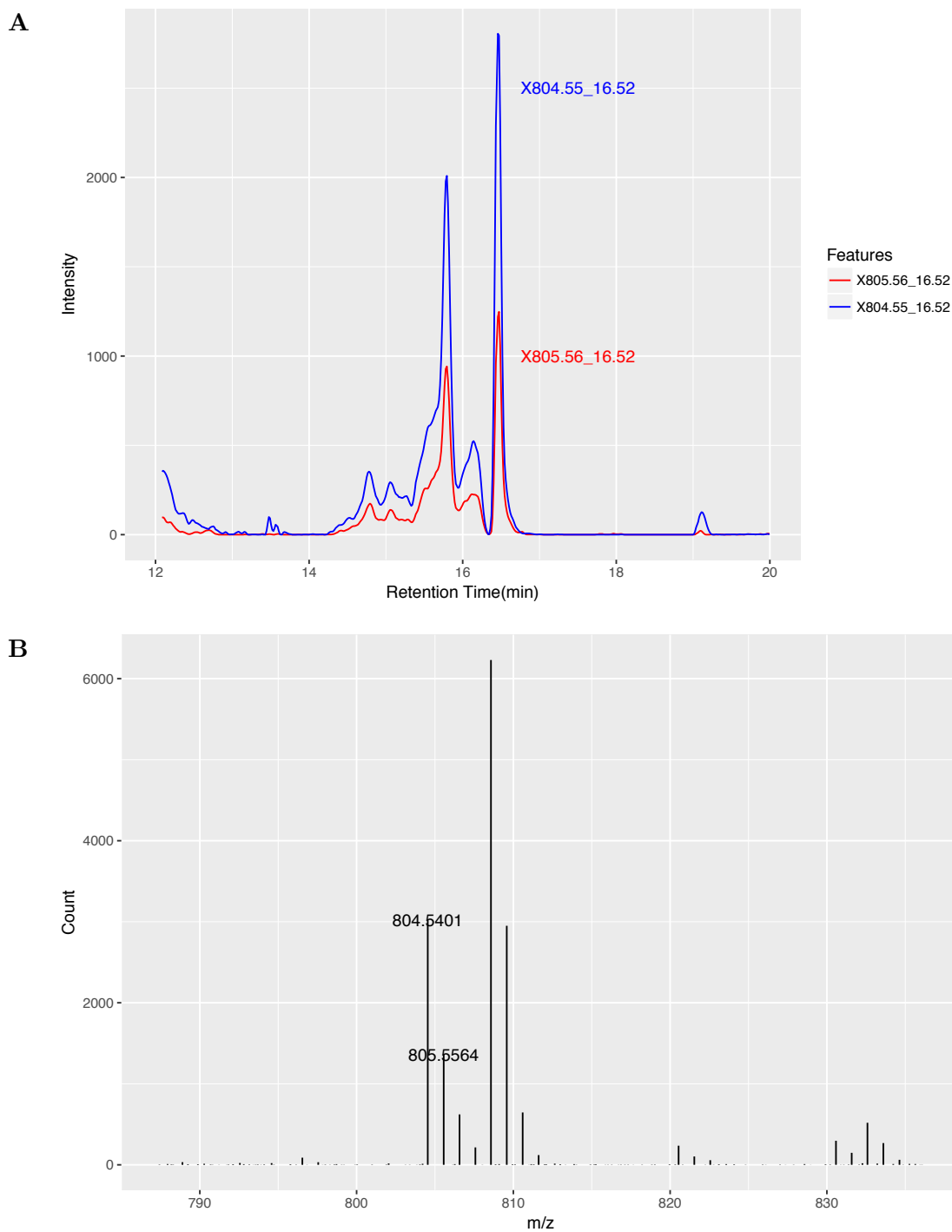


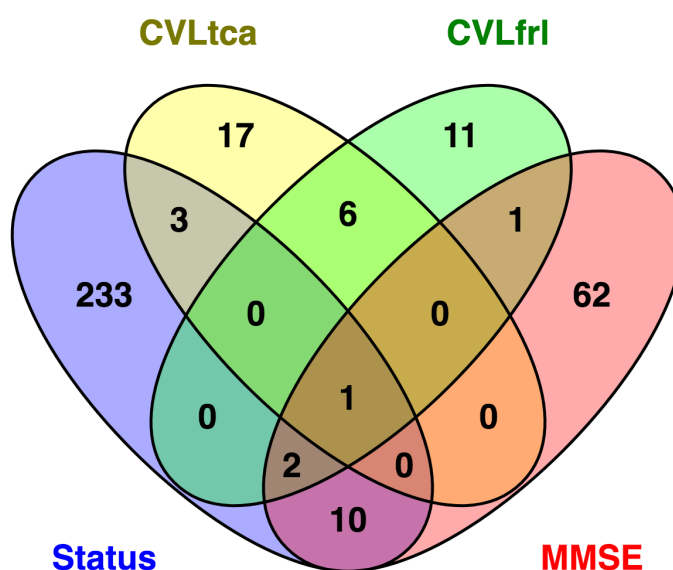
Figure 5.12. (A) Extracted ion chromatograms for m/z 804.56 (in blue) and m/z 805.56 (in red) between Rt 12 and 20 minutes. (B) MS^1 spectrum at Rt 16.48 minutes showing ion 805.56 to be C13 isotope of ion 804.55.

We then carried out further investigations using these two features on timepoint 3 samples. First, we tested how well the two features would associate with cognitive test scores from CVLtca, CVLfrl and MMSE (**Table 5.7**). Application of linear regression models showed feature X805.56_16.52 associating with none of the three phenotypes while feature X804.55_16.52 associating with all three at $p < 0.05$ level. In fact when all 1208 features were tested against all 4 AD phenotypes, AD conversion, CVLtca, CVLfrl and MMSE, X804.55_16.52 was the only feature associating with all four at $p < 0.05$ level (**Figure 5.13**).

	X804.55_16.52			X805.56_16.52	
	GLM	Odd Ratio (S.E)	p-value	Odd Ratio (S.E)	p-value
Timepoint 1	Status	1.21 (1.17)	2.19×10^{-1}	0.74 (1.17)	5.69×10^{-1}
	LM	β (S.E)	p-value	β (S.E)	p-value
	CVLtca	-0.55 (0.96)	5.63×10^{-1}	0.01 (1.12)	1
	CVLfrl	0.10 (0.29)	7.33×10^{-1}	0.17 (0.34)	6.29×10^{-1}
	MMSE	0.13 (0.13)	3.23×10^{-1}	0.10 (0.14)	4.92×10^{-1}
	GLM	Odd Ratio (S.E)	p-value	Odd Ratio (S.E)	p-value
Timepoint 2	Status	0.63 (1.25)	3.78×10^{-2}	0.59 (1.25)	1.78×10^{-2}
	LM	β (S.E)	p-value	β (S.E)	p-value
	CVLtca	-0.71 (1.14)	5.35×10^{-1}	-0.76 (1.13)	5.07×10^{-1}
	CVLfrl	-0.05 (0.35)	8.89×10^{-1}	-0.09 (0.35)	7.96×10^{-1}
	MMSE	-0.09 (0.17)	5.95×10^{-1}	-0.09 (0.17)	5.95×10^{-1}
	GLM	Odd Ratio (S.E)	p-value	Odd Ratio (S.E)	p-value
Timepoint 3	Status	0.60 (1.18)	1.93×10^{-3}	0.44 (1.20)	8.45×10^{-6}
	LM	β (S.E)	p-value	β (S.E)	p-value
	CVLtca	2.21 (1.07)	4.13×10^{-2}	2.10 (1.21)	8.54×10^{-2}
	CVLfrl	0.74 (0.32)	2.41×10^{-2}	0.52 (0.37)	1.61×10^{-1}
	MMSE	0.35 (0.15)	1.99×10^{-2}	0.30 (0.16)	6.57×10^{-2}
	GLM	Odd Ratio (S.E)	p-value	Odd Ratio (S.E)	p-value

Abbreviation: GLM, Generalized Linear Model; LM, Linear Model; CVLtca, California verbal learning Total correct A; CVLfrl, California verbal learning A long-delay free recall; MMSE, Mini mental state examination; S.E., Standard error

Table 5.7. Regression model analyses results showing associations between two lipid features (X804.55_16.52 and X805.56_16.52) and AD phenotypes (AD conversion, CVLtca, CVLfrl, MMSE). GLM was carried out to test association with AD conversion while LM was applied for CVLtca, CVLfrl and MMSE.



Abbreviation: California verbal learning Total correct A; CVLfrl, California verbal learning A long-delay free recall; MMSE, Mini mental state examination;

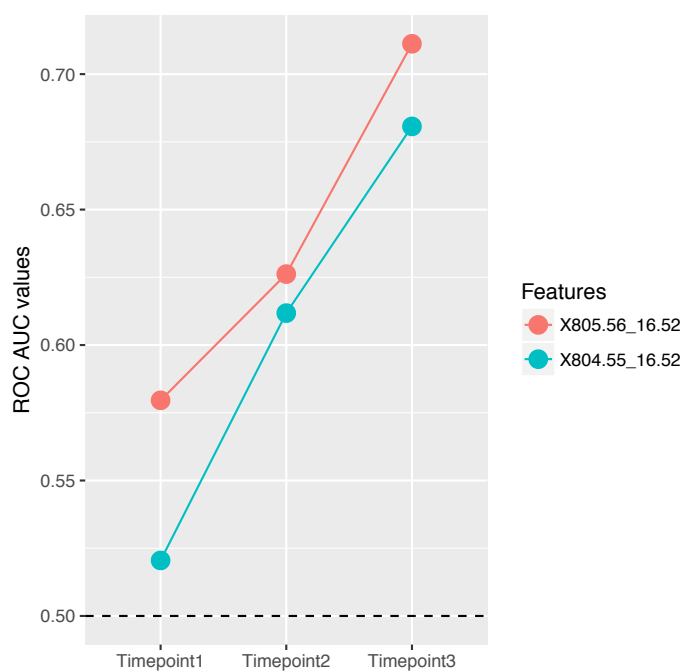
Figure 5.13. Venn Diagram showing overlap of the features associated with each AD phenotype, Status (AD conversion), CVLfrl, CVLtca and MMSE at $p < 0.05$ level. One feature (X804.55_16.52) can be seen to associate with all 4 phenotypes.

Next, we investigated how well X804.55_16.52 and X805.56_16.52 would associate with AD phenotypes at different timepoints. Summary of regression model results can be found in **Table 5.7**. At timepoint 1, the two features did not associate with any AD phenotypes ($p > 0.05$). At timepoint 2, the two features associated with AD conversion (X804.55_16.52, $p = 3.78 \times 10^{-2}$; X805.56_16.52 $p = 1.78 \times 10^{-2}$) but not with CVLtca, CVLfrl and MMSE ($p > 0.05$). Then we investigated how classification (converters *vs* controls) accuracies varied at different timepoints for these two features (**Figure 5.14** and **Table 5.8**). For X804.55_16.52, the ROC AUC values were 0.51 at timepoint 1, 0.61 at timepoint 2 and 0.68 at timepoint 3. For X805.56_16.52, the ROC AUC values were 0.58 at timepoint 1, 0.63 at timepoint 2 and 0.71 at timepoint 3.

X804.55_16.52	Timepoint	Accuracy	Sensitivity	Specificity	AUC
	1	0.47	0.47	0.46	0.51
	2	0.58	0.57	0.59	0.61
X805.56_16.52	Timepoint	Accuracy	Sensitivity	Specificity	AUC
	1	0.58	0.58	0.58	0.58
	2	0.59	0.58	0.60	0.63
	3	0.67	0.66	0.67	0.71

Abbreviation: AUC, Area Under the Curve

Table 5.8. ROC analyses summary on X804.55_16.52 and X805.56_16.52 at all three timepoints.



Abbreviation: ROC, Receiver Operation Characteristic; AUC, Area Under the Curve

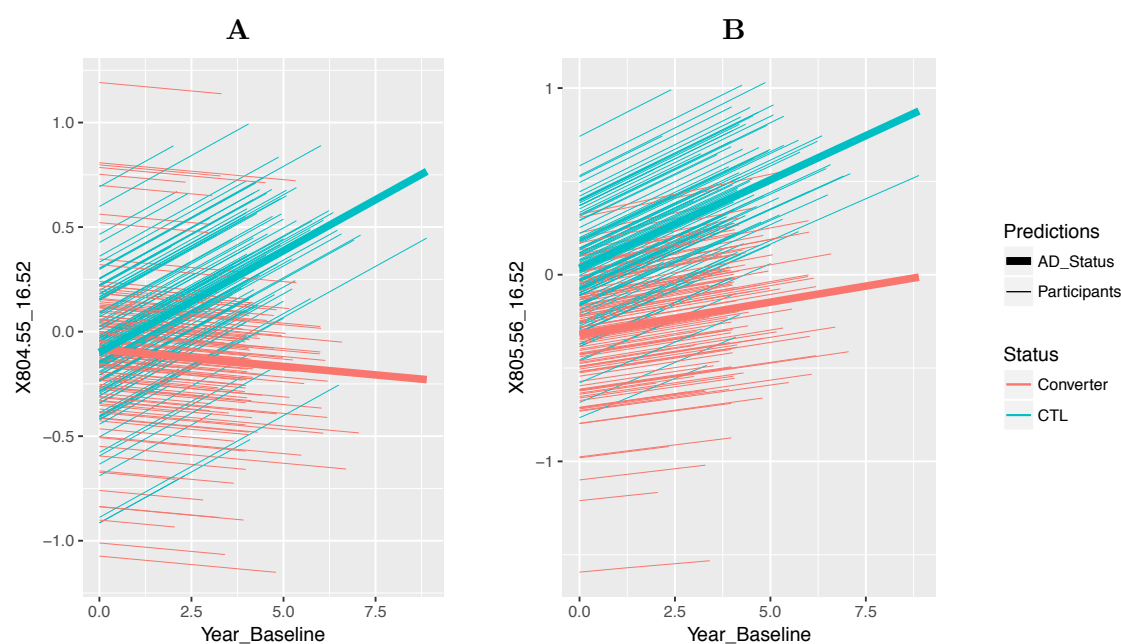
Figure 5.14. A Figure showing how discriminating abilities of X804.55_16.52 and X805.56_16.52 between converters and controls changes from over 3 timepoints.

Finally, we carried out mixed model analyses to test the associations between longitudinal changes in the levels of the two lipid features and AD conversion (**Figure 5.15** and **Table 5.9**). The analyses demonstrated longitudinal decline in X804.55_16.52 associating with AD conversion at $p < 0.05$ level, but not at FDR $q < 0.05$ level (when all 1208 features were tested). Longitudinal change in X805.56_16.52 was not found to associate with AD conversion at $p < 0.05$ level.

Feature	Beta (S.E.)	p- value
X804.55_16.52	-0.114 (0.037)	2.27×10^{-3}
X805.56_16.52	-0.060 (0.361)	9.60×10^{-2}

Abbreviation: S.E., Standard error; FDR, False Discovery Rate

Table 5.9. Mixed model results for X804.55_16.52 and X805.56_16.52



Abbreviation: CTL, Control

Figure 5.15. Longitudinal changes in levels of (A) X804.55_16.52 and (B) X805.56_16.52 in converters and controls using mixed models. Y-axis represents predicted level of each lipid while x-axis represents number of years from the initial blood draw (timepoint 1). Relatively thin lines represent participants while relatively thick lines represent either converters (red) or controls (blue).

Lipid annotation and putative identification

We opted to annotate the lipid corresponding to X804.55_16.52 (and/or X805.56_16.52) using our in-house database [426, 511, 550] and public metabolite database (HMDB [449] and METLIN [484]), and studying its MS² fragmentation patterns.

Databases were searched using a criterion where the difference between the reference m/z and the acquired m/z is within ± 0.1 Da, and one lipid was found to match this criterion which was PC38:7 (reference M+H⁺ ion m/z, 804.55). We then studied MS² spectrum and found a presence of a daughter ion with m/z 184.07. This daughter ion corresponds to phosphocholine, providing further evidence that X804.55_16.52 (and/or X805.56_16.52) originate from a possible PC molecule.

MS² fingerprint dataset was then utilized to find additional daughter ions corresponding features X804.55_16.52 and X805.56_16.52. Twenty seven MS² ions with similar retention time (± 0.1 minutes) were extracted and correlation analyses between them showed no MS² features correlating with X804.55_16.52 at $r > 0.8$ and $p < 0.01$ threshold.

5.4. Discussion

In Chapter 4, we reported dysregulation in ceramide and phosphatidylcholine metabolism in AD blood. Considering AD has a long preclinical phase in which the biological changes of the disease are already affecting the brain, we sought to further extend the work by examining whether blood levels of these particular lipid species would also differ in blood of cognitively normal people, before being diagnosed with AD in later dates. Utilizing blood from 225 BLSA participants, we semi-quantified the targeted lipid species and tested their associations with AD progression across both preclinical and prodromal stages of the disease using same GLM models as in the previous chapter. Additionally, our investigation arises from data developed from a longitudinal observation study and thus we ought to carry out longitudinal analyses. While case-control studies primarily infer these transition events by comparing health to disease, longitudinal studies allow direct assessment of pathobiology during times of transition.

This study found that from 3 PC molecules measured in serum, PC(36:5) level was found to be diminished in converters' blood at prodromal stage (timepoint 3). This is the same specie which was found to differentiate AD blood from Control blood at GLM $p=0.005$ level in Chapter 4 [551]. Lower level of this PC has also been reported to associate with lower levels of cognitive performance and neuronal activities in several brain regions related to higher order cognitive processing in older non-demented individuals [552]. Taking together with previous studies, our current finding suggests that the peripheral levels of PC(36:5) may start to be regulated differentially in AD at prodromal stage.

The specific roles of PC(36:5) in dementia is not well known, but PC(36:5) contains eicosapentaenoic acid (EPA) as a part of its molecular structure [511] and its has been reported that PC(36:5) acts as a major transporter of EPA [553]. EPA is an omega-3 poly-unsaturated fatty acid (ω -3 PUFA) and its neuroprotective role in dementia is well established [532, 533, 551, 554-556]. ω -3 PUFA is mainly present in either EPA or

docosahexaenoic acid (DHA) and when compared to DHA, EPA has additional role of inhibiting production of pro-inflammatory eicosanoids [532, 533, 556]. This particular role might explain why EPA containing PC(36:5) was strongly associated with AD conversion in our study, unlike DHA containing PC(38:6) or PC(40:6).

ω -3 PUFA levels have also been tested with dementia risk by a number of studies. In one study, free EPA and DHA blood levels were measured from 1213 non-demented participants (65 of whom later developed dementia) [557]. The group was able to observe lower EPA amount, but not DHA, associating with higher incidence of dementia, partially in line with our study. In another study, lower plasma EPA, again not DHA, was found in elderly participants with depressive symptomatology, a suspected predictor of dementia [558]. These evidences further provide for a greater role of altered EPA metabolism in AD pathogenesis from early stages when compared to DHA metabolism.

We also attempted to look at 2 ceramide species due to the findings of elevated ceramide levels in AD blood in the previous chapter and reports by other studies where ceramides have been proposed as an early AD markers [3, 37]. While we observed sparse associations between Cer24:0/Cer24:1 and preclinical/prodromal AD endophenotypes, similar findings have been reported elsewhere. Utilising a targeted approach, Mielke *et al* measured a number of blood ceramides including Cer24:0 and Cer24:1 [510]. While the highest tertile of Cer24:0 was found to associate with risk of AD, no association was observed with Cer24:1, partially in accord with our study. However the limitation of the study [510] included small sample size (the highest tertile of Cer24:0 only involved less than 10 ceramide measurements), examination of the ceramide-AD relationship among women only, and lack of information on APOE4 genotype. To overcome these limitations, the group later measured ceramide levels from larger samples of 992 cognitively normal individuals, 192 of whom later developed AD. They were unable to find associations between Cer24:0, Cer24:1 and AD risk [559].

Taken together, it can be suggested that alteration in ceramide metabolism is associated with AD, but have sparse associations with risk of progression to AD.

This study also looked at free cholesterol and ChoE levels in addition to PCs and ceramides as sterol compounds have become increasingly associated as a major risk factor for AD [129, 130, 133, 560]. While cross-sectional GLMs showed no difference in cholesterol levels at neither timepoints, its longitudinal changes were found to be associated with AD development. For ChoE level, both cross-sectional (at timepoint 3) and longitudinal models showed its association with AD development.

Many regard cholesterol as a major risk factor for AD although several observational epidemiological studies have yielded conflicting results [561-563]. However all these studies had looked from a cross-sectional point of view and to our best knowledge, there has been no studies examining associations between longitudinal changes in cholesterol levels and AD. Our longitudinal analyses showed rise in blood cholesterol levels with healthy aging (in control's blood), in agreement with other reports [564, 565], while very little changes with AD aging (in converter's blood). Considering high cholesterol content is associated as an AD risk factor, this was an interesting finding. However a longitudinal reduction on cholesterol levels has been proposed as a marker of occult disease or decline in overall health [566], and high fat and cholesterol diet is a new paradigm in AD prevention. Cholesterol plays a key role in neurotransmission by stabilising the receptor sites of the cells binding with the appropriate neurotransmitter, in particular acetylcholine and serotonin [567-569]. Depletion in cholesterol levels has been reported to contribute synaptic and dendritic spine degeneration, failed neurotransmission, and decreased synaptic plasticity [570]. Further, cholesterol-lowering statin drugs have been linked to serious impairments in memory and other cognitive functions [571-573]. Taken together, it can be suggested that with aging, more cholesterol is needed for its neuroprotective properties and insufficient supply of cholesterol may lead to greater exposure to incidence of AD.

While we observed longitudinal changes in cholesterol associating with AD conversion, both cross-sectional and longitudinal approaches showed associations between ChoE and AD. Compared to cholesterol, ChoE is less discussed as a potential AD risk factor or phenoconversion marker, but our findings suggest that altered ChoE metabolism may play a greater role in AD during its early stage. Similar findings to the present study have been reported elsewhere, a number of case vs controls epidemiological studies have found lower levels of ChoE in AD blood [118, 120, 396, 530, 543]. In addition, our previous study [396] and that by Tully et al [530] have shown blood ChoE levels being the lowest in AD followed by MCI and controls. These two studies also reported that the levels of blood ChoE to be associated with severity of cognitive impairment [396, 530], further highlighting implication of ChoE metabolism in early AD.

ChoE is a derivative of cholesterol, it is synthesized by esterification of cholesterol, and this can be taken via two pathways. The first is by the transfer of fatty acids to cholesterol from acetyl cholesterol acyltransferase (ACAT). ACAT has been the focus of intense research in AD because its activity has been found to regulate amyloid pathology in the AD brain [118, 120]. Further, high ChoE levels in AD brain [128-130, 541, 542] have been found associated with increased amyloid-beta ($A\beta$) production [117] while inhibition of ACAT has been found to contribute lowering $A\beta$ plaque burden [118, 543, 574].

In the blood circulation, the vast majority of ChoE is generated by another route, via transfer of fatty acids from PC to cholesterol, catalyzed by lecithin cholesterol acyl transferase (LCAT). While we have not carried out mechanistic studies to confirm this metabolic pathway, our subsequent correlation analyses between ChoE and PCs (all, $r > 0.6$, $p < 1 \times 10^{-15}$) showed very close relationships between these two lipid species. Although low ChoE levels in AD blood has been reported in a number of studies [117-120, 396, 530], the theory behind has remained largely unknown. Only recently has it been proposed that AD induced oxidative stress may play a role in impairing LCAT

activity [575, 576], and thus lowering ChoE levels. However it has to be noted that these studies observed lower LCAT activity in CSF and not in blood [575, 576].

After our primary analysis with the a priori selected 7 lipids using a semi-quantification approach, we examined the entire lipid fingerprint dataset from timepoint 3 using multivariate data analysis and univariate data analysis approaches. Multivariate data analyses did not reveal a high level of discrimination between groups based on serum lipid levels. Univariate data analyses revealed one lipid associating with all 4 AD phenotypes, AD classification, MMSE, CVLtca and CVLfrl, and we proposed this lipid to be PC38:7. We then tested how well this lipid discriminate converters from controls at all three timepoints and found the prediction accuracies increasing with time, suggesting the severity of PC38:7 metabolism dysregulation increasing with AD progression.

This particular lipid has previously been reported to be associated with AD [388]. Gonzalez-Dominguez et al [388] found lower levels for PC38:7 in 22 AD serum samples when compared to 18 control samples, and proposed the lipid to be a possible AD biomarker. Additionally, reductions in levels of PC species in AD blood have been reported by a majority of studies. In AD, abnormalities in phospholipids are mostly related to over-activation of an enzyme, phospholipase A2 (PLA2) [577, 578], which catalyzes the hydrolysis of the ester bonds liberating fatty acids from PCs. This over-activation of PLA2 leads to degradation of PC molecules and rise in of Lyso-PC levels which is known to be associated with neurodegeneration [161, 579].

Overall, the current findings extend our previous results and suggest that dysregulation in PC and sterol metabolisms are associated with initiation of AD. However, limitations of the study warrant consideration. First, the BLSA is a community-dwelling volunteer cohort that is predominantly white Caucasian samples of highly educated and relatively healthy older individuals. This hinders generalizability of the samples and therefore this study's findings merit confirmation in other cohorts with

higher prevalence of cardiovascular and cerebrovascular diseases associated with old age.

Secondly, we semi-quantified ChoE compounds in the serum and did not measure the composition of ChoEs by lipoprotein. Sterol compounds circulated in the blood are stored on multiple lipoproteins, and composition of the lipids on the specific lipoproteins may differ by age and with disease onset. For example, low-density lipoprotein cholesterol (LDL-c) is widely associated as an AD risk marker [580], and our clinical data showed higher level of LDL-c in converter's blood. Thus, the present work warrants further investigation on the association between AD and ChoE levels in specific lipoproteins.

Thirdly, although identical analytical methods to those in the previous chapter were employed, we were unable to measure some metabolites owing to lower MS sensitivity achieved in this study. These metabolites included 4 ceramides which have in the past been proposed as possible early AD markers [510, 525, 581]. In order to achieve more accurate quantitative measurements for ceramides (as well as others), more targeted analytical methods would be better suited due to its higher MS sensitivity.

Despite these limitations, there are also strengths to this study. Firstly, the study setting with large cohorts of carefully characterized and longitudinally followed is a major strength of the present study. Most AD metabolomics/lipidomics studies measure lipid levels from cross-sectional cohorts while only a handful studies have utilized longitudinal cohorts [504, 545, 559]. The present study measured lipid levels among 113 controls and 112 converters with at least 2 blood samples, making it one of the largest study looking to discover metabolites associated with AD conversion. To our best knowledge, only Casanova et al [545] has employed larger numbers of case samples (pre-conversion AD samples). However, unlike our study, longitudinal analyses were not carried out, failing to capture the true AD and age related phenomena. Secondly, the analyses in the present study involved controlling the effect of APOE4 in

the blood lipid levels. Studies of lipids and AD do not often control for this factor even though APOE is known to play a key role in the lipid metabolism. In the blood, APOE4 is associated with large, triglyceride-rich very low-density lipoprotein (VLDL) [582], an AD risk factor [583], while APOE4 allele is the biggest genetic risk factor for AD. Therefore findings of peripheral changes in lipid levels in AD are prone to the underlying effect of APOE4, in which the present study does not suffer from it.

5.5. Conclusions

In summary, we have extended our previous findings and have shown lower serum levels of PC(36:5) and ChoE associating with AD at prodromal stage. Further, longitudinal changes in levels of sterol compounds were found to be associated with AD progression during preclinical and prodromal AD. These lipids species have neuroprotective properties and perturbations involving metabolisms of these lipids are integral to the eventual expression of AD symptoms in cognitively normal older individuals. The findings from the present study have important implications both for early detection as well as effective interventions in AD.

***Association of blood
lipids with Alzheimer's
disease: A
comprehensive
lipidomics analysis***

6.1. *Introduction*

In Chapter 3, we reported two CSF metabolites predicting AD with Receiver Operating Characteristic (ROC) Area Under the Curve (AUC) value of 0.70. CSF has both advantages and disadvantages, an advantage is its proximity to brain cells and a disadvantage can be the invasive collection procedure especially for AD patients [263-265]. On the other hand, blood metabolites are easily accessible but don't necessarily reflect brain metabolism. However, metabolites could potentially cross an already compromised blood-brain barrier [584], thus changes in AD brain could be reflected in blood.

Among blood metabolites, the roles of lipid compounds have been highlighted (**Section 1.2.3** and **1.9**). In Chapter 4 and 5, we observed PC molecules being diminished in AD at both advanced and prodromal stages respectively. Therefore, we sought to further extend these previous results and find a panel of blood lipids which could potentially be utilized as an AD biomarker. In the previous chapters, Orthogonal Partial Least Square–Discriminant Analysis (OPSL-DA) was employed for the purpose, but this approach has a tendency of over-fitting data [585, 586]. To further explore the data and at the same time use methods that might improve over-fitting, we decided to use another classifier model, a machine learning method, random forest (RF).

Utilizing Dementia Case Register (DCR) cohort, we have previously reported ten lipids that predicted AD in a test dataset with 79% prediction accuracy [396]. Here in this chapter, we decided to employ the same RF model used in [396] on the lipid fingerprint dataset from AddNeuroMed (ANM) sample (n=277). This approach would allow us to achieve two objectives; (i) test if we can replicate the previous associations from [396]; (ii) discover new lipids associated with clinical AD diagnosis and AD phenotypes, (the rate of cognitive decline and brain atrophy measures). Compared to the previous chapters, this chapter will involve the most comprehensive profiling of blood lipids.

6.2. Methods

Systematic workflow of this study can be found in **Figure 6.1**.

6.2.1. Patient details and blood Collection

This study utilized same set of samples as those used in Chapter 4. The samples came from DCR at King’s College London and the EU funded ANM study [587]. All AD individuals met probable (NINCDS-ADRDA, DSM-IV) or definite Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) AD criteria. All cognitively healthy individuals (controls) were screened for dementia using the MMSE or ADAS-cog, or were determined to be free from dementia at neuropathological examination or had a Braak score ≤ 2.5 . Each individual was required to fast for two hours prior to sample collection and 10ml of blood was collected in tubes coated with sodium ethylenediaminetetraacetic acid (EDTA) to prevent clotting. Whole blood was centrifuged to form a plasma supernatant, which was removed and placed at -80°C .

6.2.2. Lipidomics

Lipid extraction and analysis methods have been described in Chapter 4 and elsewhere [396, 426]. DCR samples [396] were analysed in 2012 (only in positive ionisation mode). ANM samples were analysed in 2014 in both positive (in 4 batches) and negative ionisation modes. Samples were analysed in a randomised order with pooled plasma samples (Quality control (QC) samples) being analyzed at regular intervals. Lipid features were extracted from netCDF files using the R package ‘XCMS’ [480] which performed filtration, peak identification, matching of peaks across samples and retention time correction. In the previously published study utilizing DCR cohort [396], MassLynx^{TX} software (Waters, Milford, U.S.) was applied for feature detection. This approach restricted extraction of 576 features. In this chapter, XCMS was applied on both ANM and DCR cohorts, extracting a large number of previously unreported lipid features. Positive and negative datasets were extracted separately and quantile normalized.

6.2.3. Structural Magnetic Resonance Imaging (sMRI)

Volumes of whole brain and the hippocampi and entorhinal cortices were obtained using FreeSurfer 5.1.0 from 123 subjects (53 AD patients and 70 Controls) who had undergone structural Magnetic Resonance Imaging (sMRI). Regions were normalised by intracranial volume [588]. The volumetric data were not used to aid in the clinical diagnosis of AD. Detailed information regarding data acquisition, pre-processing, and quality control assessment have been described elsewhere [589, 590]. Prior to analyses sMRI measures were standardized to have a mean of 0 and a standard deviation (SD) of 1.

6.2.4. Calculation of Rate of Cognitive Decline (ROD)

The ROD was available for 118 AD patients and has been described elsewhere [591]. The ROD was based on longitudinal Mini Mental State Examination (MMSE) assessments [592] and only samples with at least three MMSE measures were included in the calculation using linear mixed effect models. After covariate adjustment [591], the slope coefficient for each sample was used as the ROD, defined as the change in MMSE per day.

6.2.5. Statistical analysis

Data Treatment

Data treatment procedure has been previously described [396] and included filtering of features and individuals, data transformation, batch effect correction, outlier detection and imputation. Briefly, lipid features detected in <80% of all samples were excluded from analyses. Normalities of Metabolic feature distributions were inspected using histograms and Shapiro–Wilks test. The distribution of a large number of lipid features was skewed and therefore the data were log2 transformed. The Empirical Bayes method ComBat [593] was used to correct for batch effects. Principal components analysis (PCA) was then used for the detection of outliers and to check whether the QC samples clustered together (**Figure 6.2**). Missing data points were imputed using

KNN (k-nearest neighbours, $k=10$), separately for each disease phenotype ('impute'). All analyses took place in R.3.01.

Univariate statistical analysis

Logistic regression (Generalized linear models (GLM)) investigated the association of each metabolite with clinical AD diagnosis. Linear regression investigated the association with cognitive decline and sMRI measures. Logistic regression and linear regression models for the sMRI measures were adjusted for age at sampling, gender, presence of the Apolipoprotein E (APOE) $\epsilon 4$ allele, batch and study site. For the ROD models, covariate adjustment was only applied for batch as the rest of the covariates were included in the ROD calculation [591]. sMRI measurements were not adjusted for diagnosis to allow identification of features associated with brain atrophy caused by AD. False Discovery Rate (FDR) correction (0.05) was applied to correct for multiple testing ('fdrtool'). Secondary models investigated whether any associations were modified by the APOE 4 allele or by gender.

Logistic regression results for the positive ionization metabolites were combined with the results from the Proitsi *et al* dataset (or DCR dataset) [396] using inverse variance weighted fixed effect meta-analysis.

Multivariate statistical analysis

A Random Forest (RF) classifier approach was employed to develop a clinical diagnosis classifier. From ANM dataset, AD cases and controls were divided into a training dataset (2/3 of the sample) matched for age, gender and site, and an independent (or test) dataset (the remaining 1/3 of the ANM sample). An RF model was built on the training dataset (100 Bootstraps) and in each iteration, each lipid feature was assigned a Variable Importance (VI) score. The summed VI ranks provided an indication of the predictive power for each lipid feature. The top 10% features were then selected for RF with recursive feature elimination (rfe) (100 Bootstraps) from 250 down to two features. For each subset of predictors, the mean bootstrap testing performance was

calculated and the optimal number of variables was identified using “sizeTolerance”, that picks a subset of variables that is small without sacrificing too much performance. Subset of variables within 2.5% and 5% of the optimum performance were examined and used to build final models in the complete training data. These models were then tested on the test dataset (the remaining 1/3 ANM dataset). The final models were also tested in the DCR dataset [396] which was used as an additional validation dataset (after excluding features in the negative ionization mode). The Area Under Curve (AUC) from Receiver operator characteristic (ROC) analysis was used to test the performance of each classifier. Additionally six discriminating features identified from [396] were also tested on ANM dataset.

For cognitive decline and sMRI measures, Random Forest Regression (RFR) models were built following the same strategy as for clinical diagnosis. The data set was split randomly into a training (2/3 of the data) and a test (1/3 of the data) datasets for each phenotype such that the training and test datasets were stratified for each phenotype and contained equal representation of each site. Age, gender and APOE ϵ 4 presence were included in the model development for the sMRI models and the Root Mean Squared Error (RMSE) was used to evaluate the performance of the models.

3.2.6. Lipid features Annotation

Annotation of the lipid features with top VI ranks was attempted. Firstly, lipid masses to charge ratios were searched against in-house database and public metabolite database (HMDB [449] and METLIN [484]). Then we utilized MS2 data (high level energy MS data for studying fragmentation patterns) and correlated the lipid features with other ions with similar retention time (± 0.1 seconds). From the correlation analysis, ions with coefficients higher than 0.8 and $p < 0.01$ were considered. Then we compared their elution profiles, represented by their extracted ion chromatograms (EICs). If they shared similarly shaped elution profiles, they were considered to originate from the same lipid. We then used m/z values to deduce the nature of the fragment ions.

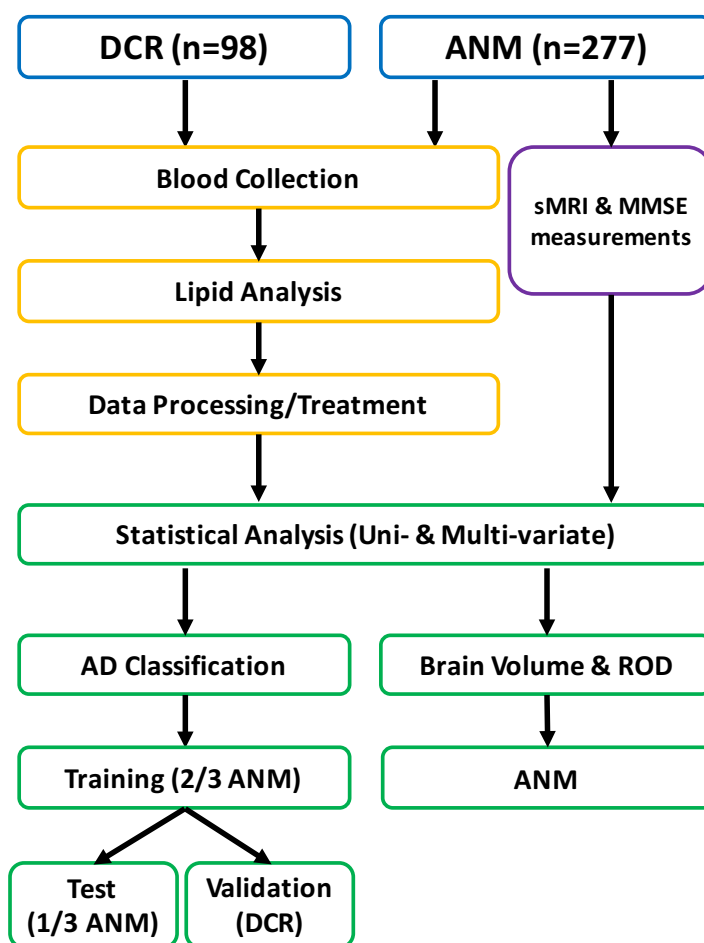


Figure 6.1. Schematic workflow used in this study.

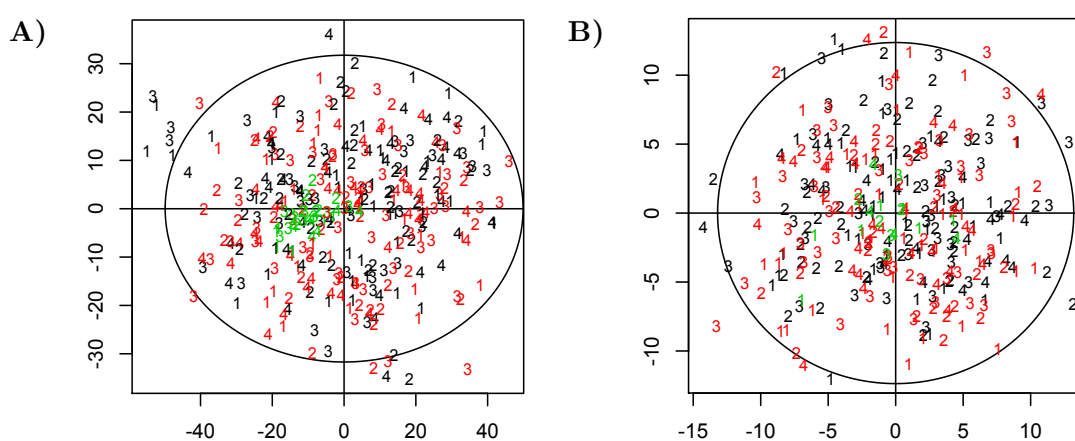


Figure 6.2. PCA plots of the normalized batch-corrected data (batch1-4) for the positive (A) and negative (B) ionisation modes. The PCA plots show the clustering of the QC samples from the four batches. Numbers (1-4) indicates experimental batches. Green: QCs; Red: AD participants; Black: Controls participants

6.3. Results

A total of 2539 and 358 lipid features were extracted from positive and negative ionization modes respectively. After data treatment, 2216 positive and 289 negative features from 277 individuals (142 AD cases and 135 controls) were used in subsequent analyses. Of these, 53 AD patients and 70 controls had sMRI data available, and 118 AD patients had ROD data available. Sample demographics for 277 individuals from ANM study are displayed in **Table 6.1**.

	AD (n=142)	Controls (n=135)	Difference between AD patients and controls*	
Age, mean (SD)	77 (6.5)	74 (5.9)	t=-4.8	p=2.62×10 ⁻⁶
Gender, (males/females)	48/87	47/95	χ ² =0.09	p=0.761
APOE ε4 allele, (absence/presence)	54/81	99/43	χ ² =23.53	p=1.23×10 ⁻⁶
MMSE, mean (SD; Range)	20.1 (4.6; 10–27)	29.2 (0.9; 27–30)	t =22.58	p<2.0×10 ⁻¹⁶
ROD (per year), mean (SD)†	-1.46 (1.26)	NA	NA	NA
Entorhinal cortex right, mean (SD)‡§	0.00092 (0.0003)	0.0013 (0.0003)	t=6.02	p=2.87×10 ⁻⁸
Entorhinal cortex left, mean (SD)‡§	0.00094 (0.0002)	0.0013 (0.0004)	t=5.26	p=1.58×10 ⁻⁶
Hippocampus right, mean (SD)‡§	0.0019 (0.0004)	0.0025 (0.0003)	t=9.06	p=1.3×10 ⁻¹⁴
Hippocampus left, mean (SD)‡§	0.0018 (0.0004)	0.0025 (0.003)	t=10.57	p<2.0×10 ⁻¹⁶
Mean HDL-c (SD), mmol/L	1.58(0.37)	1.55(0.38)	β=0.109 (SE=0.33)	p=0.068
Mean LDL-c (SD), mmol/L	3.42(1.01)	3.07(0.82)	β=0.092 (SE=0.15)	p=0.529
Mean TC (SD), mmol/L	5.69(1.17)	5.29(1.01)	β=0.209 (SE=0.173)	p=0.229
Mean TG (SD), mmol/L	1.64(1.04)	1.52(0.67)	β=0.021 (SE=0.146)	p=0.885
Statins (yes/no)	38/97	34/108	χ ² =0.436	p=0.509

Abbreviations: AD, Alzheimer's disease; MMSE, mini-mental state examination; ROD, rate of cognitive decline; SD, standard deviation; HDL-c, High Density Lipoprotein-cholesterol; LDL-c, Low Density Lipoprotein-cholesterol; TC, Total Cholesterol; TG, Triglyceride

* Differences were tested using t-test t, χ²(df)-test, or linear regression analyses after adjusting for age, gender, the APOE ε4 allele, and study site

†ROD was available for a subset of AD patients (n=118).

‡sMRI data were available for a subset of study participants (n=123).

§Normalized to intracranial volume.

||Serum HDL-c, LDL-c and TC, and TG levels were available for a subset of study participants (n=208).

Table 6.1. Sample Demographics.

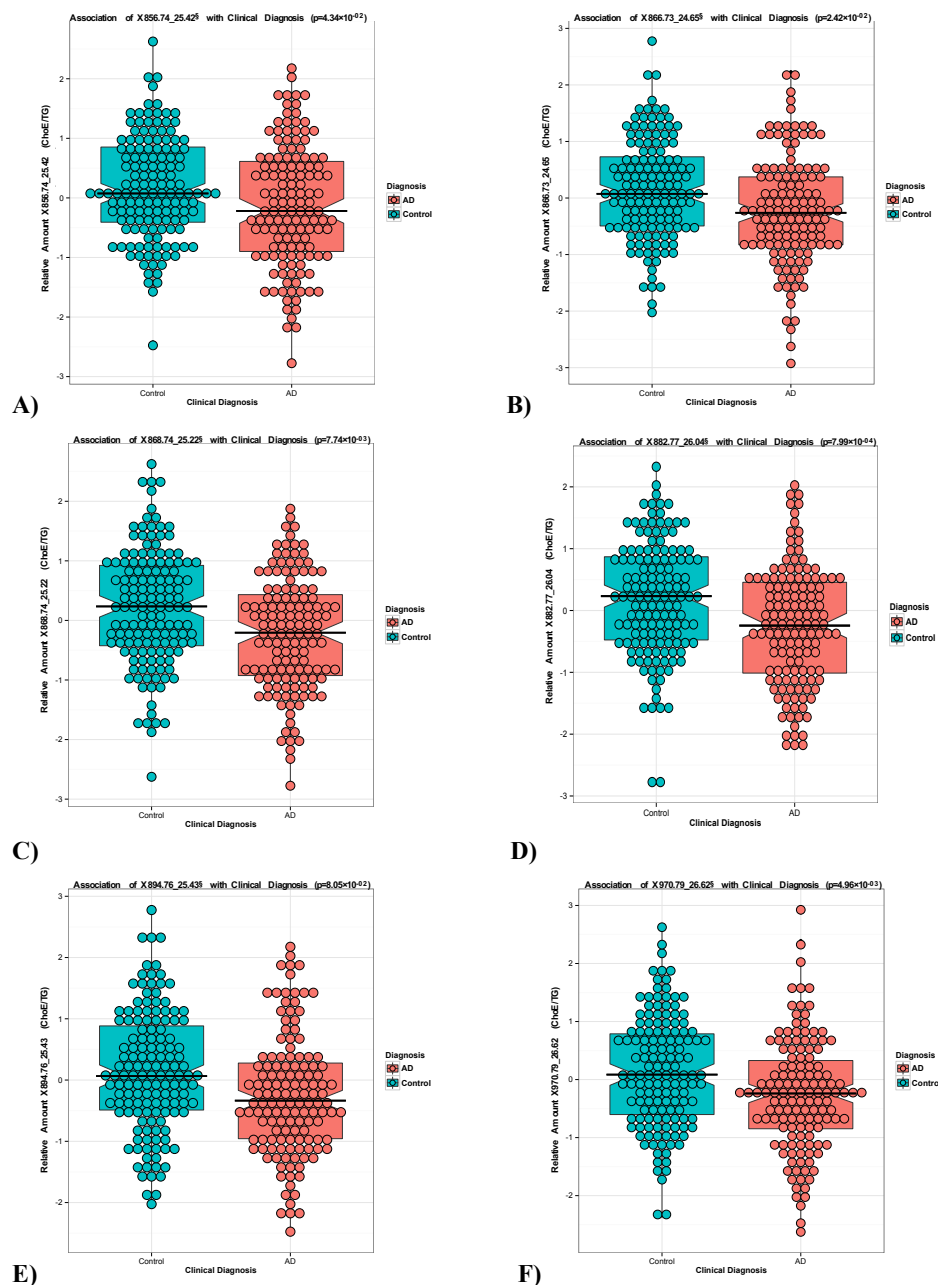
6.3.1. Univariate analyses results

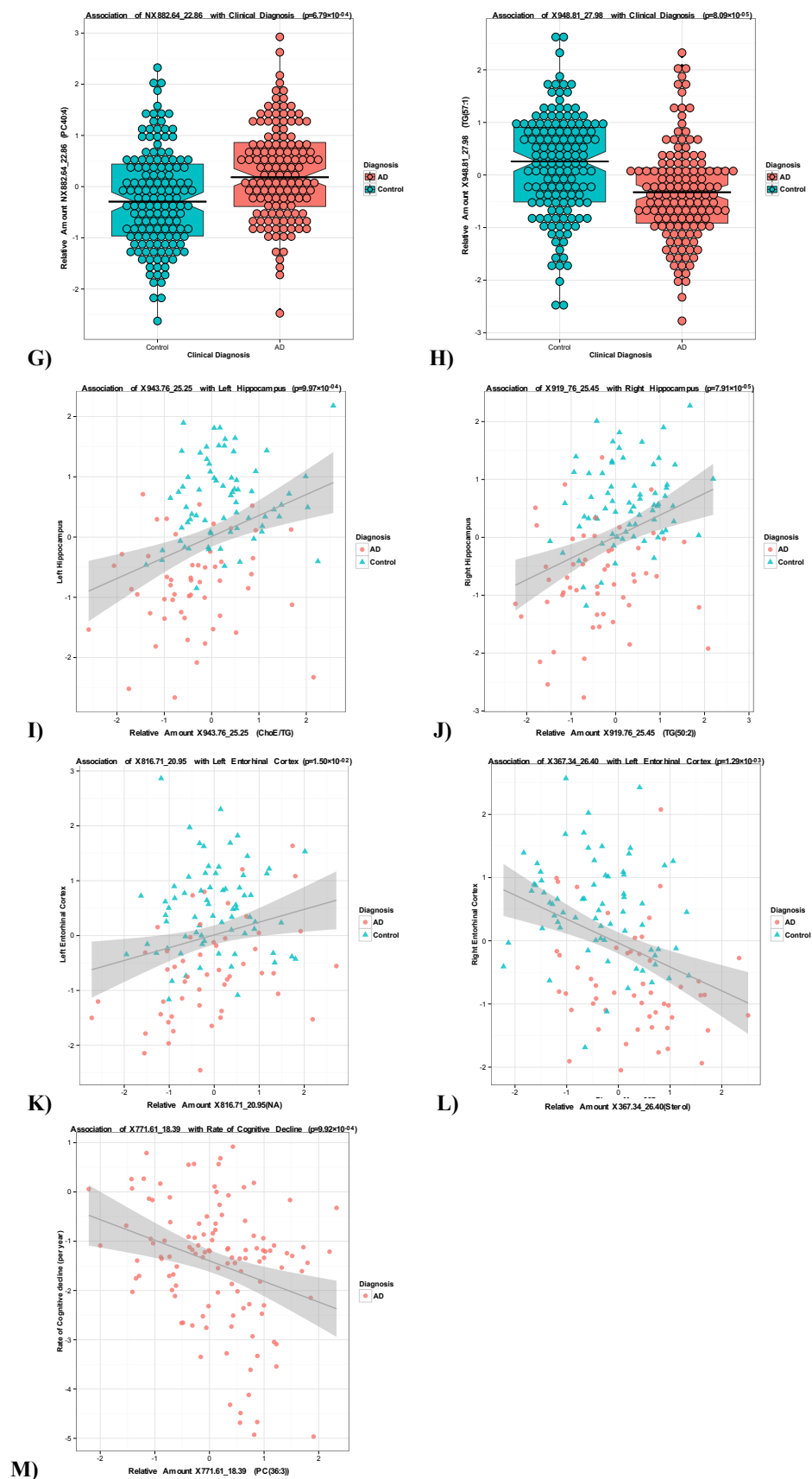
Logistic regression analyses were initially used to investigate the association of each lipid features with AD. In ANM dataset, 425 features were associated with AD at $p < 0.05$; of these, 87 features passed correction for multiple testing at $q < 0.05$. We then performed meta-analyses between the results of ANM dataset and DCR dataset [396]. From the meta-analysis, 377 features were associated with AD at $p < 0.05$ and 125 at $q < 0.05$. Six features from Proitsi et al (or DCR dataset) [396] were associated with AD at $q < 0.05$ in meta-analysis (**Figure 6.3(A-F)** and **Table 6.2**).

Linear regression models were applied to investigate the association of each lipid features with the rate of cognitive decline and brain atrophy. A total of 266 features were associated with the ROD at $p < 0.05$, but none passed multiple testing correction. A total of 181 features were associated with right hippocampus volume and 224 were associated with left hippocampus volume; only six features were associated with left hippocampus at $q < 0.05$. Finally, 156 and 124 features were associated with ERC volume (left and right, respectively) at $p < 0.05$, but none passed multiple testing corrections. All six features from Proitsi et al (or DCR dataset) [396] were associated with right hippocampus volume, five features were also associated with left hippocampus volume (**Figure 6.4**).

Overall, most lipid features were reduced in AD compared to controls (54 of the 87 features associated at $q < 0.05$ were reduced in AD). Additionally, we observed substantial overlap between features associated with clinical AD diagnosis and brain atrophy (**Figure 6.5**).

Figure 6.3. Associations of previously reported molecules from DCR dataset [396] with clinical AD diagnosis in the current (ANM) dataset (§A-F) and associations of putatively annotated molecules, selected through random forest analyses, with the respective phenotype (G-M). Association between clinical AD diagnosis and (A) X856.74_25.42, (B) X866.73_24.65, (C) X868.74_25.22, (D) X882.77_26.04, (E) X894.76_25.43, (F) X970.79_26.62, (G) NX882.64_22.86 (PC(40:4)), (H) X948.81_27.98 (TG (57:1)). (I) Association of X943.76_25.25 (ChoE/TG) with Hippocampus Left. (J) Association of X919.76_5.45 (TG(50:2)) and Hippocampus Right. (K) Association of X816.71_20.95 with Entorhinal Cortex Left. (L) Association of X367.34_26.40 (sterol) with Entorhinal Cortex Right. (M) Association of X771.61_18.39 (PC(36:3)) with the rate of cognitive decline (ROD). The P values displayed are for the univariate regressions after adjusting for covariates (age, sex and APOE ϵ 4). All molecules are scaled to have a mean of 0 and a standard deviation of 1.





Logistic Regression	Feature	Putative Annotation	ANM dataset		DCR dataset		Meta-analysis	
			OR(95% CI)	P value	OR(95% CI)	P value	OR (95% CI)	P value
Clinical AD diagnosis	NX882.64_22.86	PC40:4	1.996 (1.35~3.01)	6.79×10 ⁻⁴ *	NA	NA	NA	NA
	X948.81_27.98	TG(57:1)	0.514 (0.36~0.71)	8.09×10 ⁻⁵ *	0.522 (0.28~0.92)	3.01×10 ⁻²	0.516(0.39~0.69)	6.83×10 ⁻⁶ *
	X856.74_25.42†	ChoE/TG	0.711 (0.51~0.99)	4.34×10 ⁻²	0.141 (0.04~0.43)	1.75×10 ⁻³	0.632 (0.46~0.87)	4.94×10 ⁻³ *
	X866.73_24.65†	ChoE/TG	0.663 (0.46~0.94)	2.42×10 ⁻²	0.251 (0.10~0.52)	7.51×10 ⁻⁴ *	0.56 (0.41~0.79)	7.37×10 ⁻⁴ *
	X868.74_25.22†	ChoE/TG	0.65 (0.47~0.89)	7.74×10 ⁻³ *	0.218 (0.07~0.55)	3.15×10 ⁻³	0.561 (0.44~0.80)	7.16×10 ⁻⁴ *
	X882.77_26.04†	ChoE/TG	0.57 (0.41~0.79)	7.99×10 ⁻⁴ *	0.231 (0.08~0.53)	1.56×10 ⁻³	0.517 (0.38~0.71)	3.05×10 ⁻⁵ *
	X894.76_25.43†	ChoE/TG	0.732 (0.51~1.04)	8.05×10 ⁻²	0.151 (0.05~0.38)	3.14×10 ⁻⁴ *	0.615 (0.44~0.86)	4.65×10 ⁻³ *
	X970.79_26.62†	ChoE/TG	0.643 (0.47~0.87)	4.96×10 ⁻³ *	0.362 (0.18~0.67)	2.56×10 ⁻³	0.58 (0.44~0.77)	1.27×10 ⁻⁴ *
Linear regression			Beta(95% CI)	P value				
Hippocampus (R)	X919.76_25.45§	TG(50:2)	0.396 (0.20 0.59)	7.91×10 ⁻⁵ *	NA	NA	NA	NA
Hippocampus (L)	X943.76_25.25	ChoE/TG	0.32 (0.13~0.51)	9.97×10 ⁻⁴ *	NA	NA	NA	NA
Entorhinal Cortex (R)	X367.34_26.40	Sterol	-0.201 (-0.39~-0.01)	3.88×10 ⁻²	NA	NA	NA	NA
Entorhinal Cortex (L)	X816.71_20.95	NA	0.218 (0.03~0.41)	2.47×10 ⁻²	NA	NA	NA	NA
ROD	X771.61_18.39‡	PC(36:3)‡	-0.412 (-0.65~-0.17)	9.92×10 ⁻⁴	NA	NA	NA	NA

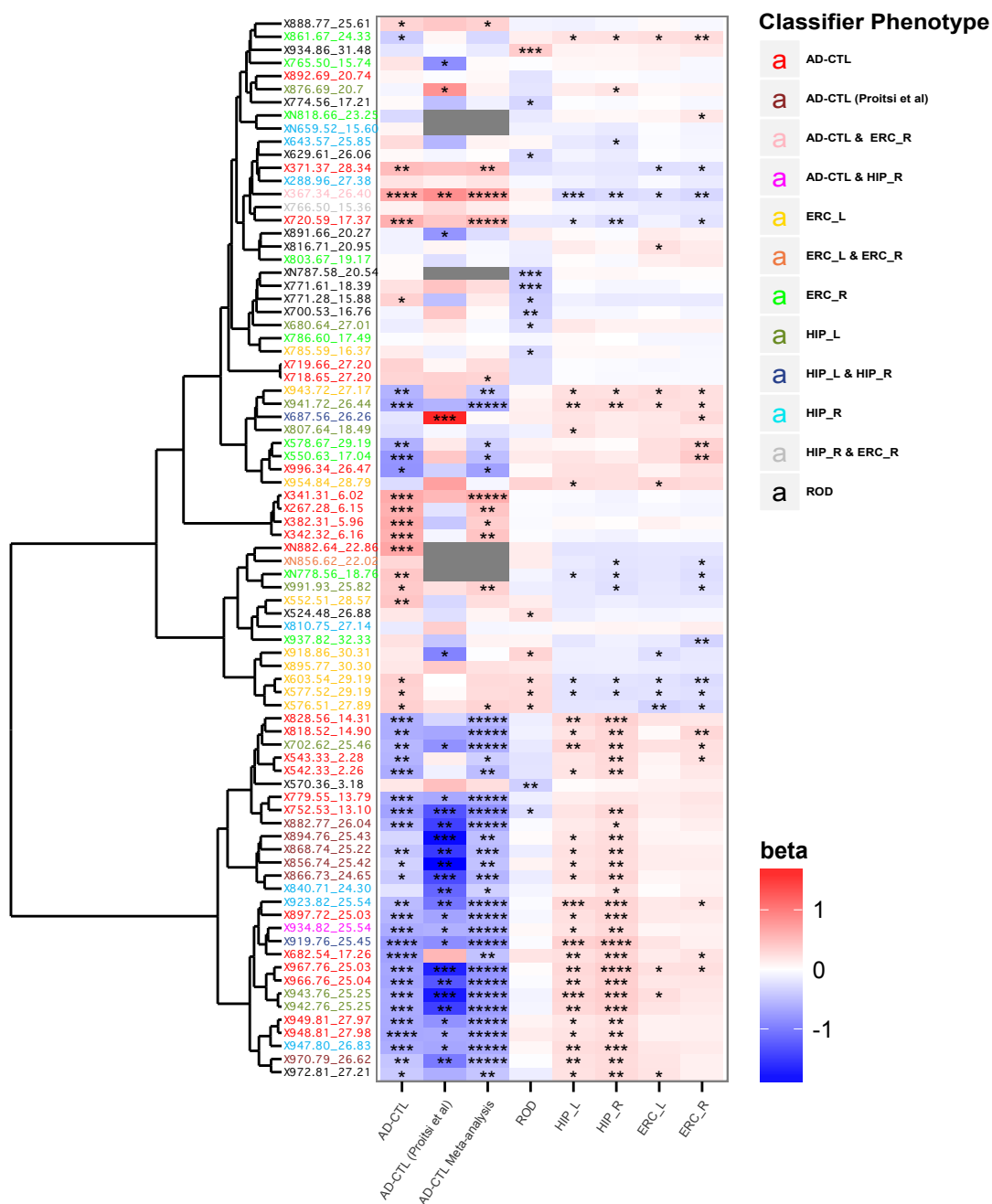
Abbreviations: AD, Alzheimer's disease; ChoE, cholesterol ester; CI, confidence interval; m/z, mass-to-charge ratio; OR, odds ratio; PC, phosphatidylcholine; ROD, rate of cognitive decline; TG, Triglyceride.

NOTE. The association of the six molecules previously reported by [396] is also presented.

*Q value <0.05; †Features from. [396]; §TG(50:2) has m/z 918.76 and m/z919.76 is its C13 isotope; ‡ PC(36:3) has m/z 770.61 and m/z 771.61 is its C13 isotope.

ChoE/TG indicates co-elution of ChoE and TG molecules

Table 6.2. List of putatively identified metabolite molecules selected by the six random forest models.



Abbreviations: AD-CTL, Clinical AD diagnosis; ROD, rate of cognitive decline; HIP_L, left hippocampus; HIP_R, right hippocampus; ERC_L, left entorhinal cortex; ERC_R, right entorhinal cortex.

Figure 6.4. Heatmap of the univariate associations between features selected during random forest analyses for each phenotype. The colour of each box represents the univariate logistic regression beta coefficient (log[OR]) for AD clinical diagnosis or the univariate scaled linear regression beta coefficients for the rate of cognitive decline and brain atrophy, after adjusting for covariates (age, sex & $\epsilon 4$). The stars on each box represent the strength of the association: *P-value<0.05; **P-value<0.01; ***P-value<0.001; ****P-value<0.0001; *****P-value<0.00001. The order of the metabolite molecules on the y-axis is based on a hierarchical clustering using the metabolites pairwise correlations. X denotes positive ionization mode features. XN denotes negative ionization mode feature.

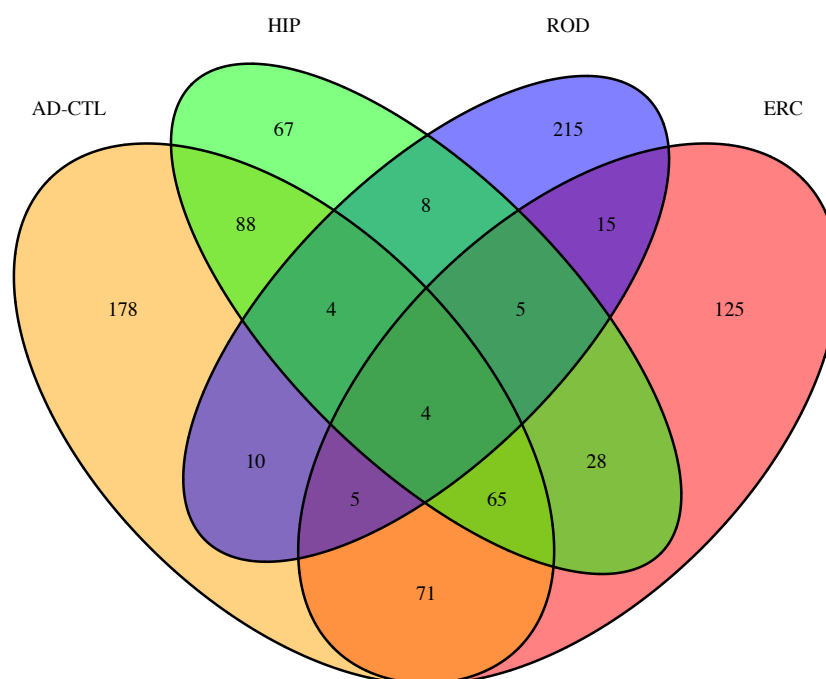


Figure 6.5. Overlap of features associated with each phenotype in univariate regression analyses ($p < 0.05$). AD-CTL: clinical diagnosis phenotype. ROD: rate of cognitive decline phenotype. ERC: Entorhinal Cortex phenotype. HIP: Hippocampus phenotype. To simplify the diagram features observed in either or both Left / Right hemispheres for the Hippocampus and the Entorhinal Cortex are included.

6.3.2. Multivariate analysis results

A RF approach was used to identify a panel of lipid features associated with clinical AD diagnosis. After an initial RF pre-selection step on the training data set, the top 10% lipids (250 features), in terms of their variable importance, were selected (after 100 Bootstraps). To choose a model with high accuracy while reducing the number of features as low as possible, a 5% tolerance RF-rfe model (25 features; 24 positive and 1 negative features) was fitted on the whole training dataset (ROC AUC=0.87). This model classified the test dataset with 73% prediction accuracy (ROC AUC=0.73) (**Table 6.3**).

Utilizing 24 positive features only (after excluding the one negative feature), the model classified the test dataset with 74% accuracy. On the validation dataset (DCR dataset), the classification accuracy was 71%. There was no increase in accuracy when covariates and the six AD predicting features from [396] were added to the models (**Table 6.3**).

RF regressions (RFR) using the same pipeline were applied to the ROD and brain atrophy measures. After RFR-rfe on the training dataset (100 Bootstrap), the lowest mean RMSE for ROD was for a model with 40 features. The 5% tolerance model of the lowest RMSE model (10 features) was fitted to the training dataset ($R^2=0.49$) and predicted the test dataset with $R^2=0.10$ (**Table 6.4**).

For right hippocampus, the lowest RMSE was with a model with 70 features that included age. A 5% tolerance model (12 features) was fitted to the training data set ($R^2=0.55$) and predicted the test data set with $R^2=0.15$ (**Table 6.4**). For left hippocampus, the lowest training RMSE was with 100 features that also included age. The 5% tolerance model (12 features) was fitted to the training data set ($R^2=0.59$) and predicted the test dataset with $R^2=0.15$ (**Table 6.4**). The performance of the models was almost identical when age was excluded.

Model (5% tolerance)	ANM Training dataset (n=179)		ANM Test dataset (n=98)				DCR Validation dataset (n=75)			
	Sens.	Spec.	AUC	Acc.	Sens.	Spec.	AUC	PPV	NPV	
Covariates only*	0.72	0.71	0.77	0.56	0.54	0.58	0.56	0.50	0.62	0.60 0.57 0.63
25 features††	0.82	0.82	0.87	0.73	0.77	0.69	0.73	0.66	0.79	NA NA NA NA NA
24 features‡‡	0.81	0.82	0.86	0.74	0.74	0.73	0.74	0.68	0.78	0.71 0.69 0.73 0.71 0.69 0.73
25 features†† + covariates*	0.83	0.83	0.88	0.75	0.79	0.71	0.75	0.68	0.81	NA NA NA NA NA
24 features‡‡ + covariates*	0.84	0.82	0.88	0.75	0.79	0.71	0.75	0.68	0.81	0.71 0.69 0.73 0.71 0.69 0.73
25 features†† + 6 ChoE/TG§	0.82	0.82	0.87	0.71	0.74	0.67	0.71	0.64	0.77	NA NA NA NA NA
24 features‡‡ + 6 ChoE/TG§	0.82	0.81	0.87	0.71	0.79	0.66	0.72	0.65	0.80	0.72 0.71 0.73 0.72 0.69 0.74
25 features‡‡ + covariates* + 6 ChoE/TG§	0.83	0.83	0.88	0.74	0.77	0.71	0.74	0.68	0.80	NA NA NA NA NA
24 features‡‡ + covariates* + 6 ChoE/TG§	0.83	0.82	0.88	0.74	0.79	0.69	0.74	0.67	0.81	0.71 0.69 0.73 0.71 0.69 0.73

Abbreviations: Acc, accuracy; AUC, area under the curve; ChoE, cholesteryl ester; NPV, negative predictive value; PPV, positive predictive value; Sens, sensitivity; Spec, specificity; TG, triglyceride.

*Age, sex, $\epsilon 4$.

†5% tolerance model including negative ionization molecule.

‡5% tolerance model excluding negative ionization molecule.

§Six features identified by [396].

Table 6.3. Random forest classifier model results (clinical AD diagnosis) for the training dataset and predictions on the test dataset and the DCR dataset.

Phenotype	Model (5% tolerance)	Train data set (n = 93)		Test data set (n = 28)	
		RMSE	R ²	RMSE	R ²
Hippocampus (R)	Covariates only*	0.92	0.28	1.09	0.02
	12 features††	0.58	0.55	0.9	0.15
Hippocampus (L)	Covariates only*	0.89	0.34	1.21	0.01
	12 features††	0.64	0.59	0.99	0.15
Entorhinal cortex (R)	Covariates only*	0.95	0.22	1.14	<0.01
	12 features	0.66	0.54	0.92	0.14
Entorhinal cortex (L)	Covariates only*	1.00	0.22	1.17	<0.01
	12 features	0.77	0.42	1.07	0.01
ROD	10 features‡‡	0.93	0.49	1.09	0.1

Abbreviations: RMSE, root mean squared error; ROD, rate of cognitive decline.

*Age, sex, $\epsilon 4$.

†Age was included in the final model. There was no difference in either train or test data set performance when age was excluded.

‡Covariates were already included in the calculation for the ROD.

Table 6.4. Random forest regression model results for the training data set and predictions on the test data set for each AD phenotype.

For the right EC, the lowest mean training RMSE was with 70 features; a 5% tolerance model (12 features) was fitted to the training data set ($R^2=0.54$) and predicted the test dataset with $R^2=0.14$. Finally, for left EC, a model with 90 features had the lowest RMSE, and a 5% tolerance model (12 features) was fitted to the training dataset ($R^2=0.42$) and predicted the test data set with $R^2=0.01$ (**Table 6.4**).

Results of 2.5% models are presented in **Table 6.5** for classification models and **Table 6.6** for regression models. The strength of association between selected features and each model is shown in **Figure 6.4**.

Model	Training dataset (n=179)			Test dataset (n=98)				DCR Validation dataset (n=75)			
	Sens.	Spec.	AUC	Acc.	Sens.	Spec.	AUC	Acc.	Sens.	Spec.	AUC
Covariates*	0.7	0.72	0.75	0.56	0.54	0.58	0.56	0.60	0.57	0.63	0.60
40 features	0.84	0.82	0.88	0.75	0.77	0.73	0.75	NA	NA	NA	NA
37 features†	0.84	0.82	0.88	0.71	0.72	0.71	0.72	0.68	0.66	0.7	0.68
40 features + covariates*	0.85	0.83	0.89	0.74	0.77	0.71	0.74	NA	NA	NA	NA
37 features† + covariates*	0.84	0.83	0.89	0.71	0.72	0.71	0.72	0.67	0.63	0.7	0.67
6 features‡‡	0.68	0.73	0.71	0.58	0.77	0.45	0.60	0.67	0.75	0.60	0.67
40 features + 6 features‡‡	0.84	0.82	0.88	0.72	0.77	0.68	0.72	NA	NA	NA	NA
37 features† + 6 features‡‡	0.82	0.82	0.87	0.71	0.74	0.68	0.71	0.67	0.60	0.73	0.66
40 features + 6 features‡‡ + covariates*	0.85	0.82	0.89	0.73	0.77	0.69	0.73	NA	NA	NA	NA
37 features† + 6 features‡‡ + covariates*	0.82	0.83	0.88	0.71	0.74	0.67	0.71	0.67	0.60	0.73	0.66

Abbreviation: Sens, Sensitivity; Spec, Specificity; AUC, Area Under the Curve; Acc, Accuracy; AUC, Area Under the Curve.

* Age, sex, ϵ_4

† Excluding 3 negative ionisation features in the 2.5% tolerance model

‡‡ Features from Proitsi et al [396]

Table 6.5. Random Forest classifier model results for clinical AD diagnosis (2.5% tolerance models).

Phenotype	Model (2.5% tolerance)	Train dataset (n=93)		Test dataset (n=28)	
		RMSE	R ²	RMSE	R ²
Hippocampus (Right)	Covariates only ¹	0.92	0.28	1.09	0.02
	19 features ²	0.64	0.63	0.87	0.20
Hippocampus (Left)	Covariates only ¹	0.89	0.34	1.21	0.01
	23 features ²	0.62	0.61	0.94	0.23
Entorhinal Cortex (Right)	Covariates only ¹	0.95	0.22	1.14	<0.01
	20 features	0.64	0.59	0.93	0.12
Entorhinal Cortex (Left)	Covariates only ¹	1.00	0.22	1.17	<0.01
	20 features	0.72	0.5	1.05	0.01
ROD	16 features ³	0.91	0.52	1.09	0.1

Abbreviation: RMSE, Root Mean Squared Error; ROD, Rate of Cognitive Decline

¹ Age, sex, ϵ_4

² Age excluded in the model. There no difference in either train or test dataset performance when age was excluded.

³ Covariates were already included in the calculation for the rate of cognitive decline

Table 6.6. Random Forest regression model results (2.5% tolerance model).

6.3.3. Lipid annotation and putative identification

We opted to annotate the top features, in terms of VI scores from each model and features selected in more than one model, using our in-house lipid database [426, 511, 550] and MS2 fragmentation patterns.

These features were annotated as triglycerides (TGs) and cholesteryl esters (ChoEs), some were PCs and a sterol. Features eluting after 25 minutes were annotated either as ChoEs or TGs, and lack of m/z 369.35 ion suggested the features to be TGs. m/z values were then used to determine size of TG molecules. In order to determine lengths and saturation levels of 3 fatty acids in each TG, MS2 ions with similar retention time (± 0.1 min) were correlated to the top VI features.

One clinical AD diagnosis feature X882.77_26.04 was found to be correlated with X621.49_26.02 at $r=0.84$ & 2.20×10^{-16} level (**Figure 6.6**). This MS2 X621.49_26.02 was interpreted as a loss of Fatty acid (FA) with 16 carbon length and 6 saturation levels (FA16:6). We were unable to decipher the other two FAs. For the other top features, no MS2 features showed correlation at $r>0.08$ and $p<0.01$ and matched elution profiles.

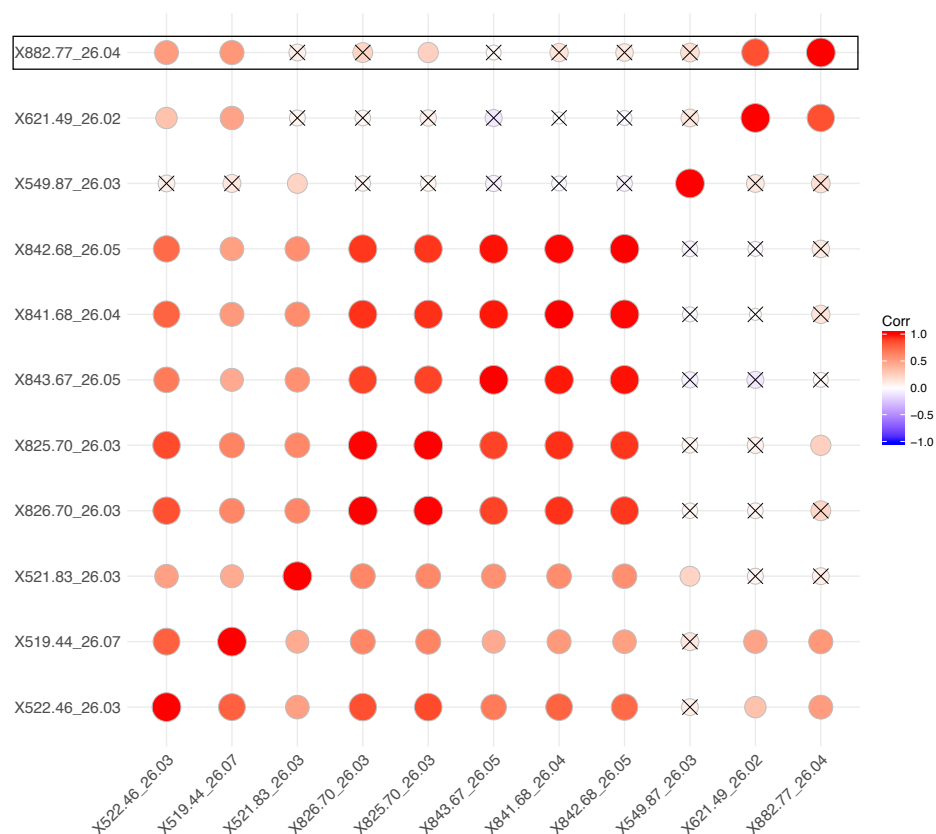


Figure 6.6. Plot of correlation matrix between 9 selected metabolic features. Red represent positive correlation while blue represent negative correlation. Intensity of 'redness' and 'blueness' represent correlation coefficient value. Cell with X shows correlation with no significance ($p > 0.01$).

6.4. Discussion

Here in this chapter, we expanded our group’s recent work [396]. Using univariate and multivariate approaches, we replicated the associations between six previously reported blood lipids and AD [396] and reported their association with brain atrophy. We further identified combinations of lipid features that classified AD participants with relatively good accuracy when tested in both test dataset and validation dataset ($>70\%$), and combinations of molecules that predicted changes in disease progression ($R^2=0.10$ for test dataset) and brain atrophy ($R^2\geq 0.14$ for all test datasets except for left EC). Overall, we observed substantial overlap between features associated with clinical AD diagnosis and brain atrophy. The associations of all molecules included in each model with all phenotypes is shown in **Figure 6.4**. Although these signatures cannot be used for diagnostic purposes yet, they suggest important biological mechanisms associated with AD.

We putatively identified two PC molecules; additionally, ChoEs and TGs were tentatively annotated due to chromatographic coelution, and finally we putatively annotated a molecule as a sterol. The higher MS sensitivity achieved here when compared to [396] enabled the detection of a number of additional lipids that co-eluted with ChoE; these were annotated as TGs (**Table 6.2**).

The association of PCs with AD and cognition has been extensively described elsewhere [511, 594]. Here, one of the lipid features most strongly associated with AD is a putative PC (PC(40:4)) and the top lipid feature in the ROD model is also a putative PC (PC(36:3)). In contrast to the same species of molecules we have previously reported as well as in Chapter 4 and 5, both PCs in this report are increased in AD, and PC (36:3) is associated with faster ROD. While the majority of studies to date have reported a reduction of PC levels in AD, an increase in CSF PCs has been observed in AD compared to control brains [595], and recently in “AD-like” patients based on their CSF $A\beta_{42}$, Tau and Phospho-Tau-181 levels [596]. A recent study also reported a parallel increase of PCs containing saturated and short chain fatty acids in

serum from AD patients [387]. These suggest deregulation in the biosynthesis, turnover and acyl chain remodelling of phospholipids, in accordance with increased phospholipid breakdown due to PLA2 [387] overactivation.

We have also reported associations with long-chain and very-long-chain triglycerides (LCTs/VLCTs; fatty acid chain length >16 carbons). One of the most interesting findings was that due to the higher MS sensitivity achieved in this chapter, we were able to observe putative VLCTs that were co-eluting with ChoEs. Synthesis of ChoE takes place by transfer of fatty acids from PC to cholesterol, a reaction catalysed by lecithin cholesterol acyl transferase (LCAT) in plasma and by acyl-coenzyme A: cholesterol acyl transferase 1 and 2 (ACAT1 and ACAT2) in other tissues, including the brain. The association of LCTs/VLCTs with AD is noteworthy. Although overall TGs are seen as risk factors for many disorders including cardiovascular disease (CVD) and type 2 diabetes (T2D), numerous investigations point to the diverse role of TGs with different chain lengths. It is known for example that medium-chain triglycerides (MCTs) and LCTs have different metabolic pathways in digestion and absorption [597]. Moreover, although TGs of lower carbon number and double bond content have been associated with increased CVD [598] and T2D [599], LCTs with higher carbon number and double bond content, like the ones here, have been associated with decreased risk of T2D [599], whereas no associations between T2D and total TG levels were observed in the same individuals [599]. Furthermore, decreased concentration of LCTs and an increased concentration of VLCTs have been associated with longevity [25]. These findings are particularly interesting as most vegetable oils are comprised of long-chain fatty acids; however, only MCTs have to our knowledge been implicated in AD, although findings are controversial [600]. When our group previously investigated the association of total cholesterol and TGs with AD in overlapping individuals using Mendelian randomization, no evidence for an association with AD was found [110]. Additionally, we observed no difference in serum Triglycerides, Total Cholesterol, LDL Cholesterol and HDL Cholesterol between AD patients and controls for a subset of study participants in this study, and no difference in the frequency of AD patients and

controls who were on statins. On the other hand, a recent study reported an overlap between genes involved in elevated plasma lipid levels and inflammation and the risk for AD [601]. All these highlight the relevance of investigating smaller lipid fractions as they highlight specific steps in their biosynthesis and metabolism that may be associated with AD.

Finally, we observed an association between most phenotypes and a feature of m/z 367. This lipid feature associated with an increased risk for AD in both ANM and DCR datasets and with reduced brain volume and was included in the ERC and clinical diagnosis models. We believe this feature is a fragment and a sterol, specifically an isomer of desmosterol. Desmosterol is a precursor of cholesterol and seladin (or 24-Dehydrocholesterol reductase, DHCR24), which governs the metabolism of desmosterol to cholesterol in specific brain areas. Desmosterol has been shown to inhibit β -secretase cleavage of APP and the formation of amyloid- β and lower desmosterol levels have been found in the plasma and brains of AD patients compared to controls [393, 602-604].

Although the association of aberrant lipid metabolism in AD pathogenesis is undisputed [605-607], at this stage, the mechanisms by which these changes in lipids might occur in AD are unclear. One possibility involves AD selective alterations to circulating lipid metabolism. However, another possibility relates to cellular lipid production. A number of phospholipids are synthesised within a specialised region of the endoplasmic reticulum (ER) that is closely associated with mitochondria, the mitochondria-associated ER membranes (MAM). The close association of MAM to mitochondria facilitates Ca^{2+} and phospholipid exchange between the two organelles [608-610]. Recent studies have shown that MAM contacts are damaged in AD [611-614]. Since ER-mitochondria contacts are required for the synthesis of certain lipids [608-610], such changes may affect lipid metabolism and lead to some of the changes described here. Indeed, different APOE alleles have been shown to influence MAM [615].

Overall in this study, a large well-characterized AD cohort and a careful and systematic analysis pipeline were used. Through bootstrapping, we have reduced over-fitting, and subsequently, we validated our results in an unseen test dataset for each phenotype and an additional validation dataset for clinical AD diagnosis. Our AD diagnostic classifier achieved 88% accuracy on the training dataset (summary of 100 bootstraps) and predicted the test and validation datasets with $>70\%$ accuracy. Our training dataset comprised of individuals matched on age and gender. On the other hand, the test dataset consisted primarily by females, and AD patients were significantly older than controls. Additionally, the validation dataset (DCR) included UK origin participants only, and they were older than ANM study participants. Findings based on the test and validation datasets therefore highlight robustness in the model. For the AD phenotypes, the Random Forest Regression models had a very good performance on all training datasets. Although the performance dropped significantly in the test datasets, we observed $R^2 > 0.10$ for all phenotypes except for Left EC ($R^2 = 0.01$). The drop in performance can be attributed to over-fitting of the training data sets and the smaller number of individuals with ROD/brain atrophy measures. The poor performance of the Left EC is in agreement with our univariate analyses that highlighted weaker associations with the EC for the whole sample; however, it is in contrast to the overall right-to-left asymmetry in AD [591].

A limitation of this study is that we were not able to decipher the exact fatty acid chain structure of some features. Owing to the higher MS sensitivity achieved here, we observed a number of putative ChoEs and TGs co-eluting, which is commonly observed in lipidomics studies due to hundreds of lipids detected in one analysis; to minimize co-elution problems, our chromatographic run is 2 hours long using ultra pressure chromatography [426, 616].

Additionally, this study is one of the largest non-targeted blood lipidomics study in AD to date, we acknowledge that the sample size is still modest and further replication is

required, especially for the ROD and brain atrophy phenotypes. Moreover, although we had information on the ROD, this calculation was based on the MMSE, which is a crude measurement of cognition. Furthermore, the present study did not contain an MCI cohort or information on conversion to MCI/AD, and therefore, we do not know whether these features are associated with initiation of AD. This study additionally suffers from limitations inherent to AD case-control studies, such as the large number of comorbidities in old age, the possibility that some of the elderly controls may already carry pathology, and that some of the clinically diagnosed AD may be pathologically non-AD dementias. Finally, this study lacks information on BMI and body fat distribution that could potentially explain some of the differences between AD patients and controls.

However, through the longitudinal nature of these cohorts, we know that all the AD patients used for our analysis maintained the diagnosis of AD as did all controls for at least 3 years from their baseline visit. Additionally, our information on disease progression and brain atrophy provide us with more precise phenotypes that capture different stages of disease pathology including the early preclinical stages. Given the good performance of these models, we believe that enrichment with additional individuals and pathology information would increase their performance. Finally, although we did not have BMI information for our cohort, we observed no difference in statin use or serum lipids between AD cases and controls.

6.5. Conclusion

In conclusion, the findings of this study deepen our knowledge of AD disease mechanisms and emphasize the importance of investigating in detail different lipid fractions in dementia research. As it is not known whether the observed changes in lipid levels are causally related to, or are just a marker of changes in lipoprotein dynamics and composition, studies that address causality are essential, as the success of targeting specific molecules and identifying potentially causal pathways amenable to intervention is predicated on these molecules being on the causal pathway. Finally, integrating additional types of biological modalities such as protein, gene expression and genotype information may increase the fit of these models and help us to understand more about the biological context in which these molecules operate.

***Conclusion & Future
Directions***

7.1. Conclusion

Alzheimer's disease (AD) is rapidly becoming a major global burden. However there is no treatment and definite diagnosis for AD, highlighting the absence of a comprehensive understanding of the biological mechanisms underlying the changes that occur during the process of neurodegeneration. This underscores the urgent need for markers associated with AD that would potentially be utilized as an AD diagnostic tool and/or AD progression marker. In recent years, the application of metabolomics technology for discovery of AD markers is receiving increasing attention. To this end, we decided to extend the work of AD metabolomics field further by examining metabolic profiles of AD cerebrospinal fluid (CSF) and blood and to identify metabolites associated with AD.

In the first study (Chapter 2), we employed ^1H -NMR (^1H -Nuclear Magnetic Resonance) to compare metabolic profiles between 20 AD, 10 FTD (Frontotemporal dementia), 20 age-matched CN (Cognitively Normal) and 20 relatively young CN CSF samples. Multivariate data analysis showed the metabolic fingerprints were non-differentiable between AD and age-matched CN. Univariate data analysis followed where levels of 11 metabolites were measured and compared between the clinical groups. In AD *vs* age-matched CN model, decreased pyruvate level (mean change 21.15%) and increased acetate levels (mean change 22.18%) in AD CSF were found. Decreased in pyruvate levels suggested energetic hypometabolism in AD brain leading to increased energy requirements from additional energy source in which acetate could be used as this alternative source of energy. This can be further supported by the fact that the fold-changes in mean pyruvate and acetate levels between AD and age-matched CN CSF were similar. Additionally these two metabolites were found to correlate with CSF $\text{A}\beta_{42}$ and total tau levels.

For the following chapter, Liquid Chromatography-Mass Spectrometry (LC-MS) was applied on the same cohorts to allow detection of more CSF metabolites. To maximize detection of metabolite numbers, two orthogonal LC methods, Reversed Phase (PR)

and Hydrophilic Interaction Chromatography (HILIC), were employed. This experimental design allowed detection of 4426 metabolic features. Fingerprints from four clinical groups were compared; its results were similar to those from NMR experiment. The AD CSF fingerprints were differentiable from those from FTD and young CN, and non-differentiable from those from age-matched CN. From the AD *vs.* age-matched CN model, two strong AD discriminating metabolic features were extracted and their AD prognostic value in terms of ROC AUC was 0.70 when tested on previously unseen test samples. Although their prognostic value was moderate, these two features were found to correlate with CSF A β_{42} , CSF tau and MMSE scores, suggesting their potential applicability in monitoring disease progression.

After the work on CSF, our focus shifted toward blood metabolites because their ease-to-use nature. In Chapter 4, we were interested in testing whether lipids that had previously been implicated with AD would be reproduced with similar trends, but using relatively larger sample cohorts. Semi-targeted analyses of 9 lipids, 3 phosphatidylcholine (PC) and 6 ceramide molecules were carried out in 205 AD and 207 CN plasma samples. Elevated levels of three ceramides and diminished levels of 2 PC molecules were observed in AD blood ($p < 0.05$), one PC was also found to associate with hippocampal atrophy. We then segregated the samples into 2 non-intersecting age-bins and tested associations between the 9 lipid levels and AD and its phenotypes at each age bin. Three ceramides were found to associate with hippocampal atrophy in younger participants, while three PCs were found to associate with hippocampal atrophy in older participants. The work in this chapter therefore contributed to the growing body of evidence highlighting peripheral lipids is implicated in AD pathology.

For Chapter 5, we ought to further extend the work of Chapter 4 by measuring the 9 lipids level (3 PCs, 4 ceramides plus cholesterol and absolute cholesterol esters levels) on pre-conversion serum samples. For this, we analysed serum samples from 112 eventual AD participants and 113 control participants at preclinical and prodromal stages. At preclinical stage, no lipids showed significant differences between the clinical

cohorts. At prodromal stage, one PC and cholesteryl ester levels were found diminished. Longitudinal analyses showed longitudinal changes in peripheral cholesterol and cholesteryl ester levels were implicated in AD development. We additionally studied the lipid fingerprint dataset, from 1208 metabolic features, one lipid was found to associate with AD conversion, memory impairment and cognitive function impairments. The findings showed implication of dysregulation in lipid metabolisms during early stages of AD.

For the final chapter, untargeted lipidomic analysis was performed on 148 AD and 152 control plasma samples in search of AD blood biomarkers. We utilized a careful and systematic analysis pipeline where random forest statistical method was applied on training dataset to identify lipid signature associated with AD. A combination of 24 lipid features consisting of cholesteryl ester, triglycerides and PCs was identified and this panel was tested on test and validation samples. The panel was able to discriminate the clinical cohorts with AD classification accuracy of $>70\%$ in both a test and a validation dataset. In addition, other lipid signatures were found to predict disease progression and brain atrophy in all brain regions except for left entorhinal cortex. Further, we observed substantial overlap between features associated with clinical AD diagnosis and brain atrophy.

Overall, this thesis has shown that, metabolic profiling techniques of various biological samples have a practical application in the discovery of metabolic signatures associated with AD.

7.2. *Future Directions*

7.2.1. Further validation of CSF markers

In Chapter 3, we were able to identify two CSF metabolic features associated with clinical AD diagnosis and AD pathologies. Their AD prognostic value was ROC AUC 0.70 on test samples. However the test samples consisted of small sized sample cohorts, 4 AD and 5 age-matched CN samples. Additionally these test samples were analysed by LC-MS and data processed together with the training samples. Therefore the primary interest for the future work involving CSF samples would be to validate AD prognostic value of these two metabolites in a separate large-scale multi-centered sample cohort.

Further, it would be a beneficial to measure the two selected CSF metabolite levels in a mild cognitive impairment (MCI) cohort which has information on conversion to AD. This approach would allow whether the two metabolites are associated with initiation of AD (CN to pre-clinical) and conversion from MCI to AD.

7.2.2. Metabolite Identification

One of the key challenges in metabolomics studies is identification of metabolites. Compared to peptides consisting of amino acids repeatedly arranged in linear orders, metabolites are random combinations of elements (e.g. C, H, O, S, N, and P). Further, the chemical and physical diversities of metabolites make them difficult to identify based on MS non-targeted approach. In Chapter 3, 5 and 6, we examined metabolic fingerprints obtained by LC-MS untargeted methods and found numerous metabolic features which have shown to be altered significantly between AD and controls. While we were able to annotate some of them, they need to undergo strict experimental verification procedures. Authentic standards need to be obtained and analyzed under the identical experimental conditions with the biological samples. For identification with high confidence level, mass, retention time and fragmentation (MS/MS) spectrum between the samples and the authentic standards need to be compared.

Identification of metabolites associated with AD would also allow us to carry out a secondary analysis allowing deeper analysis of metabolomics data. There are two main types of secondary analysis; enrichment analysis and metabolite mapping. During metabolomics analysis, statistical analysis would result in a list of metabolites where their abundances would differ between clinical cohorts. With this list, enrichment analysis computes which metabolic pathways have been significantly altered by using a collection of pre-defined metabolite pathways and disease states obtained from literatures. This analysis can aid identification and interpretation of patterns on metabolite concentration/level changes in a biologically meaningful context. For transcriptomics, a conceptually similar tool is available and it is Gene Set Enrichment Analysis. Metabolite mapping meanwhile provides a visual representation of metabolomics data by highlighting the identified metabolites (and their abundances) on a network graph, often obtained from a biochemical database. Therefore achieving identification of interest metabolites would allow these secondary analyses to be carried out, in turn providing good biological interpretations of the data.

7.2.3. Integration of metabolomics with other ‘omic’ data

Since metabolites are the terminal downstream products of genomes, transcriptomes and proteomes in the whole organisms, metabolic phenotypes can serve as good intermediate traits for a genome-wide association study (GWAS). Recently metabolite-based genome-wide association study (mGWAS) is being increasingly utilized where metabolomic and genetic data are combined and analysed together. This approach allows identification of a panel of single-nucleotide polymorphism affecting metabolite levels and associated with disease (or AD). It thereby can potentially provide access to the underlying molecular disease-causing mechanisms related to changes in metabolite levels. Further, proteomic data can be integrated with metabolomic and genomic data to explore metabolomic-proteomic associations and to establish links between genetic variants, metabolic traits, protein levels and AD. This would allow investigation into

how the interaction between multiple organizational layers of a biological system is associated with AD.

mGWAS was first utilized in 2008 [617]. However to our best knowledge, there has been no study in the field of AD where genetic, metabolomic and proteomic data are integrated. Therefore such analysis could allow deeper understanding in metabolic pathways involved in AD pathogenesis, and further facilitate development of approaches in the personalized medicine.

7.2.4. Future outlook of metabolomics

Metabolomics-based strategies have become a major part of model clinical research, allowing for a better understanding of pathophysiological conditions and disease mechanisms, as well as providing innovative tools for more adequate diagnostic and prognosis approaches. It has been paving the way for personalized health strategies for the development of novel and adequate therapies and the implementation of tailor-made and efficient diagnosis approaches. Nevertheless, despite its potential, application of metabolomics into personalized diagnostic and prognostic medicine is still at an infant stage. A big push has been put into the biomarkers discovery using metabolomics approaches and various candidates of biomarkers or metabolic patterns have now been highlighted in multiple diseases. Yet for large majority of them, they don't reach the validation phases and to be used in clinical applications so far. Therefore there is a great need to improve the validation of biomarker candidates. Most importantly, for metabolomics to be translated into precision medicine, there need to be cross-disciplinary efforts, translational collaborations between academic institutions, pharmaceutical companies and medicinal agencies and diagnostic companies, as well as standardization of procedures.

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Publications

Association between Plasma Ceramides and Phosphatidylcholines and Hippocampal Brain Volume in Late Onset Alzheimer's Disease

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Abstract. Lipids such as ceramides and phosphatidylcholines (PC) have been found altered in the plasma of Alzheimer's disease (AD) patients in a number of discovery studies. For this reason, the levels of 6 ceramides and 3 PCs, with different fatty acid length and saturation levels, were measured in the plasma from 412 participants (AD $n = 205$, Control $n = 207$) using mass spectrometry coupled with ultra-performance liquid chromatography. After this, associations with AD status, brain atrophy, and age-related effects were studied. In the plasma of AD participants, cross-sectional analysis revealed elevated levels of three ceramides (Cer16:0 $p < 0.01$, Cer18:0 $p < 0.01$, Cer24:1 $p < 0.05$). In addition, two PCs in AD plasma (PC36:5 $p < 0.05$, PC38:6 $p < 0.05$) were found to be depleted compared to the control group, with PC36:5 also associating with hippocampal atrophy ($p < 0.01$). Age-specific analysis further revealed that levels of Cer16:0, Cer18:0, and Cer20:0 were associated with

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hippocampal atrophy only in younger participants (age < 75, $p < 0.05$), while all 3 PCs did so in the older participants (age > 75, $p < 0.05$). PC36:5 was associated with AD status in the younger group ($p < 0.01$), while PC38:6 and 40:6 did so in the older group ($p < 0.05$). In this study, elevated ceramides and depleted PCs were found in the plasma from 205 AD volunteers. Our findings also suggest that dysregulation in PC and ceramide metabolism could be occurring in different stages of AD progression.

Keywords: Alzheimer's disease, brain atrophy, ceramide, phosphatidylcholine

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia accounting for 60–80% of all cases, representing 44 million cases globally with the number of diagnoses expected to triple by 2050 [1, 2]. The neurological disorder results from the cell loss triggered by a not well-understood metabolic cascade that involves numerous extrinsic and intrinsic pathways. Among these, lipid signaling pathways have attracted attention as an important and possibly critical factor in neurodegeneration [3–6].

Ceramides (Cer) have been linked to neurodegenerative disease by a growing body of evidence. Altered ceramide levels have been reported in AD cerebrospinal fluid (CSF) [7] and in AD brain [8]. More recently, Mielke et al. [9] found higher serum Cer16:0 and Cer24:0 levels to be linked with an increased risk of developing AD. In addition to ceramides, phosphatidylcholines (PCs) have been shown to be associated with AD. We previously identified a panel of three PCs, PC (16:0, 20:5), PC (16:0, 22:6), and PC (18:0, 22:6), that were decreased in the plasma of AD patients in comparison to cognitively normal controls and mild cognitive impairment patients [10]. This relatively underpowered study ($n = 35$ –40) showed that a predictive performance of APOE of 67% was increased to 83% by including APOE and PCs as markers [10]. A study by Mapstone et al. in plasma also reported a panel of ten lipids, which included seven PCs, to be predictive of AD phenocconversion [3], although this result was not replicated in serum in a recent follow-up study [11].

Together these findings suggest that lipids play a crucial role in AD pathology and may represent a valuable clinical tool for diagnosis and prevention. The untargeted approach is ideal in discovering new lipids associated with AD, as it scans for thousands of lipid molecules in each sample. More targeted analyses achieve better quality data for fewer lipid entities, usually up to 200 lipids. It is also essential to replicate results in larger cohorts when possible [12, 13]

and analyze how these effects depend on the other major factors of AD—namely APOE status and age. Here we measured levels of 9 abundant plasma lipids previously linked to AD [8–10], in plasma from AD and elderly control volunteers, and studied whether any trends in the amounts of these lipids were associated to AD, brain atrophy, APOE status and age of participants.

MATERIALS AND METHODS

Patient details and blood collection

This study utilized 412 plasma samples (205 AD and 207 controls) from the Dementia Case Register (DCR) at King's College London and the EU funded AddNeuroMed study [10, 14]. Of these 412 participants 122 also had structural magnetic resonance imaging (sMRI) data. Normal elderly control subjects were recruited from non-related family members of AD patients, care-givers' relatives, social centers for the elderly, or GP surgeries, and had no evidence of cognitive impairment. AD individuals were primarily recruited from local memory clinics. Relevant ethics boards approved the studies and informed consent was obtained for all subjects. Each patient was required to fast for two hours prior to sample collection and 10 ml of blood was then collected in tubes coated with sodium ethylenediaminetetraacetic acid (EDTA) to prevent clotting. Whole blood was centrifuged to form a plasma supernatant, which in turn was removed and placed at -80°C until further use. The sample demographics showing health and clinical characteristics of participants, by diagnostic group, are shown in Table 1.

Measurements of lipid levels

Samples from the two clinical cohorts were prepared and analyzed separately. The first cohort (DCR, $n = 98$) was analyzed in 2012 where depletion of the three PCs was first reported [10]. Meanwhile

Table 1

Demographic characteristics of participants in study samples from DCR and AddNeuroMed. *p*-values are obtained from the comparison between AD and control groups

	AD (<i>n</i> = 205)	Control (<i>n</i> = 207)	<i>p</i> -value
Average age (s.d) ¹	77.35 (6.88)	74.88 (6.60)	2.26×10^{-4A}
Gender (Male/Female) ¹	81/123	77/130	0.60 ^B
APOE ϵ 4, no. (%) ¹	124 (61.03)	60 (29.13)	5.85×10^{-11B}
Average white matter (s.d) ²	0.6561 (0.0733)	0.6985 (0.0408)	9.30×10^{-5A}
Average Hippocampus Volume (s.d) ²	0.003678 (0.000757)	0.004969 (0.000628)	4.84×10^{-18A}
Average Entorhinal Volume (s.d) ²	0.001870 (0.000556)	0.002516 (0.000437)	7.43×10^{-11A}
Average Entorhinal Thickness (s.d) ²	5.4709 (0.9866)	6.8090 (0.7207)	1.62×10^{-14A}
Average HDL-c (s.d) ³ /mmolL ⁻¹	1.58 (0.37)	1.55 (0.38)	0.068 ^C
Average LDL-c (s.d) ³ /mmolL ⁻¹	3.42 (1.01)	3.07 (0.82)	0.529 ^C
Average TC (s.d) ³ /mmolL ⁻¹	5.69 (1.17)	5.29 (1.01)	0.229 ^C
Average TG (s.d) ³ /mmolL ⁻¹	1.64 (1.04)	1.52 (0.67)	0.885 ^C
Statins (Yes/No) ⁴	38/97	34/108	0.509 ^B

HDL-c, high density lipoprotein-cholesterol; LDL-c, low density lipoprotein-cholesterol; TC, total cholesterol; TG, triglycerides. ¹AD *n* = 202, CTL *n* = 206. ²AD *n* = 52, CTL *n* = 70. ³AD *n* = 102, CTL *n* = 106. ⁴AD *n* = 135, CTL *n* = 142. ^AStudent *t*-test. ^BChi-square test. ^CLinear regression after adjusting for age, gender, the APOE ϵ 4 allele and the center of origin of each sample.

sample analysis from the second cohort (AddNeuroMed, *n* = 314) was carried out in 2014 using the identical methodology. Lipid extraction and analysis methods have been described elsewhere [12, 15] and can also be found in the Supplementary Material S1.

The single molecule integrated peak areas under the exact mass chromatographs of the nine lipids and the internal standard (Tripentadecanoin) were obtained by using QuanLynx (MassLynx 4.1, Waters) by setting up an integration parameter file using the average mass charge ration (*m/z*) and retention time of the 9 lipids and internal standard. The integration parameters used in lipid measurement can be found in the Supplementary Material S2. Tolerances for mass and retention time of 0.1 Da and 5 min, respectively, were typically applied. Lipids with the levels below the limit of quantification were not used for statistical analyses. Lipids PC (16:0, 20:5), PC (16:0, 22:6), and PC (18:0, 22:6) were referred as (PC36:5, PC38:6, PC40:6) and ceramides were referred as (Cer16:0, Cer18:0, Cer20:0, Cer22:0, Cer24:0, and Cer24:1) throughout the manuscript.

sMRI data acquisition and analysis

Volumes of hippocampi, white matter, entorhinal cortices, and entorhinal cortex thickness (ER thickness), normalized by intracranial volume, were obtained from 122 subjects (52 AD patients and 70 Controls) who had undergone sMRI. The volumetric data were not used to aid in the clinical diagnosis of AD. Detailed information regarding data acquisition, pre-processing, and quality control assessment have been described for this cohort elsewhere [16, 17].

Statistical analysis

We applied generalized linear models (GLM) to compare lipid levels in the AD group relative to the Control group, and to study their relationship with brain atrophy. The GLM included covariates to control for center of origin of each sample, gender, age, APOE status, batch effect, and internal standard. When the analysis was segregated according to age, the GLMs were separately applied to two non-intersecting age bins, corresponding to percentiles 0–50th and 50–100th. Each one of these bins therefore contained the same number of samples (ca. *n* = 170 for status, *n* = 60 for sMRI). More detailed methodology on the statistical analysis carried out in the Results section can be found in the Supplementary Material S3. Figure 1 shows a schematic workflow of the data used in this study.

RESULTS

Participant characteristics

The demographic characteristics (age, gender, APOE, cholesterol levels, triglyceride levels, and volume in four different brain regions) of participants by diagnostic group, are shown in Table 1. AD participants were slightly older than controls; this is usual in AD studies with a relatively larger sample size [18, 19]. The AD group had a higher percentage of APOE carriers and presence of brain atrophy (*p*-values < 0.001). There were no significant differences between the groups on high density lipoprotein,

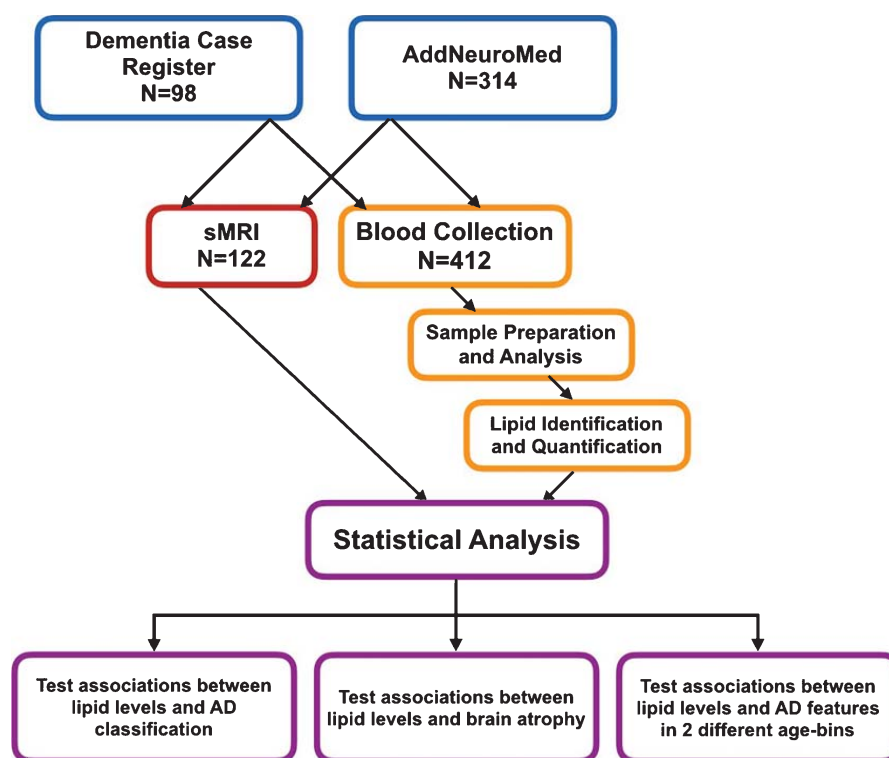


Fig. 1. Workflow of this study sMRI, structural magnetic resonance imaging.

low density lipoprotein, total cholesterol levels, and total triglyceride levels.

Ceramides

Elevated ceramide levels in AD plasma

Six ceramide levels from 202 plasma samples from the AD group and 207 plasma samples from the cognitively healthy group were compared using GLM. Figure 2 shows the distribution of all ceramides, suggesting that some of them are elevated in the AD group (in red). Table 2 confirms that single molecule plasma levels of Cer16:0 ($p < 0.01$), Cer18:0 ($p < 0.01$), and Cer24:1 ($p < 0.05$) in particular were elevated, while no significant differences were observed for Cer20:0, Cer22:0, and Cer24:0. T-values were derived from GLM (Table 2) and showed that the magnitude of the effect was the largest for Cer18:0 (−2.99), followed by Cer16:0 (−2.94) and Cer24:1 (−2.49). T-values for the non-significant ceramides were, following the same order, Cer24:0 (−1.89), Cer20:0 (−1.75), and Cer22:0 (−1.42).

Using the same GLM approach, we then tested if single ceramide levels would associate with atrophy in white matter, hippocampus, entorhinal, and ER thickness as measured in 122 participants. The statistical results tabulated in Table 3 show that there was no association between the ceramides and atrophy

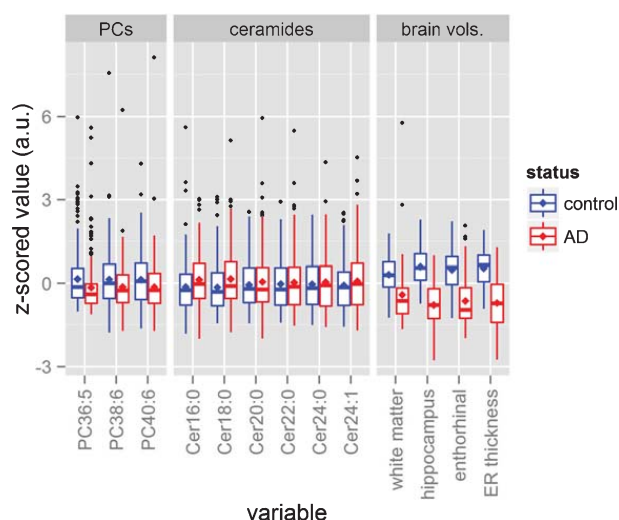


Fig. 2. Data distributions. The figure shows the box plots corresponding to each phosphatidylcholine (PC), each ceramide, and each brain volume. All variables have been z-scored to ease visual comparison. The sizes of the boxes represent the 25th and 75th percentiles, while the central horizontal line dividing both boxes represents the median, and the mean is represented as a rhomboid. The length of each whisker extends to 1.5 times the length of the box of its corresponding side. Samples beyond the length of the whiskers are represented as individual black dots.

in these four brain regions. Figure 3 summarizes these associations, including ceramides and PCs in the same figure for comparison.

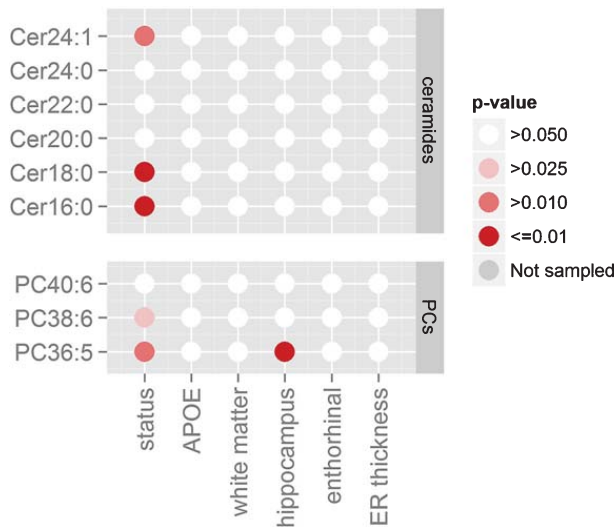


Fig. 3. Effects found with generalized linear models (GLM). Statistical results of the GLM on the combined AD cohorts. Columns represent AD demographics variables while rows represent the lipids levels. Different shades of red indicate different standard significance thresholds.

Table 2

Group differences in lipid levels between AD and control group. The table shows the mean and standard deviation for each one of the lipids measured in this study. Second column and third column show mean and standard deviation (SD) values for the control and AD population respectively. The last two columns show the *t*-values and *p*-values derived from GLM for pairwise comparison between the groups

Variables	Control (Mean $\pm$ SD)	AD (Mean $\pm$ SD)	GLM	
			<i>t</i> -value	<i>p</i> -value
PC36:5 ¹	0.03 $\pm$ 0.07	-0.30 $\pm$ 0.06	2.35	0.019
PC38:6 ¹	0.01 $\pm$ 0.06	-0.28 $\pm$ 0.06	2.13	0.034
PC40:6 ¹	0.01 $\pm$ 0.06	-0.27 $\pm$ 0.06	1.83	0.068
Cer16:0 ²	-0.13 $\pm$ 0.07	0.10 $\pm$ 0.06	-2.94	0.004
Cer18:0 ²	-0.09 $\pm$ 0.06	0.22 $\pm$ 0.07	-2.99	0.003
Cer20:0 ²	-0.10 $\pm$ 0.06	0.01 $\pm$ 0.08	-1.75	0.081
Cer22:0 ¹	-0.06 $\pm$ 0.05	0.00 $\pm$ 0.06	-1.42	0.156
Cer24:0 ¹	-0.05 $\pm$ 0.05	0.03 $\pm$ 0.06	-1.89	0.060
Cer24:1 ¹	-0.06 $\pm$ 0.05	0.09 $\pm$ 0.06	-2.49	0.013

¹*n* = 412; ²*n* = 314.

Table 3

p-values derived from GLM when testing for associations between lipid levels and atrophies of 4 brain regions and APOE status

Variables	APOE	White Matter	Hippocampus	Entorhinal	ER Thickness
PC36:5 ¹	0.469	0.588	0.009	0.761	0.640
PC38:6 ¹	0.164	0.599	0.398	0.794	0.561
PC40:6 ¹	0.100	0.951	0.348	0.481	0.488
Cer16:0 ²	0.833	0.592	0.172	0.557	0.307
Cer18:0 ²	0.530	0.470	0.064	0.527	0.230
Cer20:0 ²	0.961	0.208	0.165	0.909	0.981
Cer22:0 ¹	0.639	0.179	0.457	0.876	0.692
Cer24:0 ¹	0.347	0.344	0.694	0.972	0.891
Cer24:1 ¹	0.549	0.669	0.420	0.413	0.740

¹*n* = 412; ²*n* = 314.

Table 4

Statistical power associated with each analysis. In this study, different numbers of samples were available depending on the variables that were compared (metabolites and demographics). The temporal analysis of Fig. 5 further reduced the number of samples by a factor of 2 for each age bin. The table indicates what is the minimum effect size (in numbers of standard deviations) that could be detected with a *t*-test for the available number of samples corresponding to each comparison

Metabolites	Demographics	Age bins	N	Effect size*
PC36:5	AD status	None	412	0.14
PC38:6	APOE	2	206	0.20
PC40:6	White Matter	None	122	0.26
Cer22:0	Hippocampus			
Cer24:0	Entorhinal	2	61	0.36
Cer24:1	ER Thickness			
Cer22:0	AD status	None	314	0.16
	APOE	2	157	0.22
Cer24:0	White Matter	None	122	0.26
Cer24:1	Hippocampus			
	Entorhinal			
	ER Thickness	2	61	0.36

*Effect size corresponding to power 80%.

Ceramides in age-bins

Previous GLM analyses (Fig. 3, Table 2) showed Cer16:0, Cer18:0, and Cer24:1 associating with AD status but not with brain atrophy. However, this type of analysis (i.e., pooling all subjects together independently of age) may mask some age-specific associations (age trends of metabolite groups and brain volumes shown in Fig. 4). To investigate for this possibility, we further applied GLM on AD status, brain volume measurements, and APOE status in two non-intersecting age-bins. The results of these analyses are summarized as line plots (Fig. 5 and Supplementary Material S4). The analyses showed no individual ceramides associating with AD classification and APOE status in any age-bins. When we tested the ceramide levels on brain atrophy in these same age-bins, ceramide levels were associated with greater hippocampal volume loss in relatively younger participants (Cer16:0, Cer18:0, and Cer20:0; *p*-value < 0.05 for age < 75; see Fig. 5). In this age bin, associations were also found with entorhinal volume (Cer16:0, Cer18:0, Cer20:0, Cer22:0, and Cer24:0), ER thickness (Cer16:0 and Cer20:0), and white matter (Cer20:0; see Supplementary Material S4). In other participants (age > 75), only white matter atrophy was significantly associated with Cer18:0, Cer20:0, and Cer22:0. There were no associations between ceramides and entorhinal volume, ER thickness, or hippocampal volume for older participants.

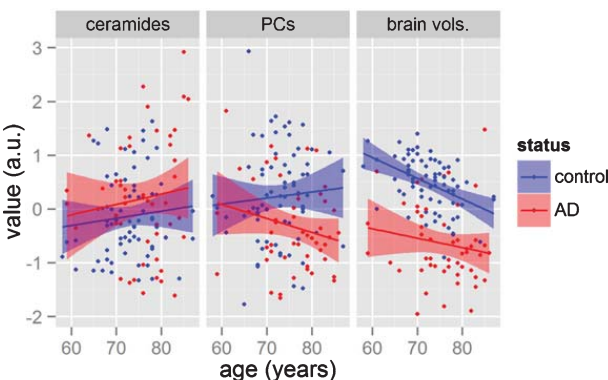


Fig. 4. Comparisons on how variable groups (lipids and brain volume measurements) change with age in AD and control groups. The y-axis represents the averages of three groups of variables, namely: the six ceramides; the three phosphatidylcholines (PCs); and the four brain volumes (from left panel to right panel). The x-axis represents age. Each point presents a subject, while color represents the diagnosis of each patient. Solid line represents simple linear regression for each group, while the shaded area represents the 0.95 confidence interval.

Phosphatidylcholines

Diminished phosphatidylcholine levels in AD plasma

Three PC levels from 202 plasma samples from the AD group and 207 plasma samples from the cognitively healthy group were compared using GLM. Figure 2 shows the distribution of all 3 PCs for ADs and controls. Table 2 confirms that single molecule measurements plasma levels of PC36:5 and PC38:6 were significantly reduced in abundance in the AD group compared to healthy control group ($p < 0.05$ for both PCs), while PC40:6 did not show a significant change between the groups.

In Fig. 3, brain volume measurements in 122 participants showed PC36:5 positively associated with hippocampal volume ($p < 0.01$). All other PC associations failed to show significance (hippocampus, entorhinal cortex thickness, and white matter atrophy).

PCs in age-bins

We applied the same analysis in non-intersecting age-bins, where PC36:5 revealed a strong association with AD status in younger participants (age < 75), while PC38:6 and PC40:6 did so in the older age bin (age > 75 , all $p < 0.05$). Associations of all PCs with hippocampal volume, and of PC36:5 with entorhinal volume, were found only in the older age bin. No other associations were found with PCs when segregating into older and younger participants (Fig. 5 and Supplementary Material S4).

DISCUSSION

A number of reports have implicated lipid dysregulation in AD pathology [8–10]; to explore these findings further, six ceramide and three phosphatidylcholine species were measured and compared between AD and a cognitively healthy volunteers. We tested whether lipid levels were associated with AD diagnosis and brain atrophy, and after this, we divided the samples into two non-intersecting age-bins, each bin represented by the same number of participants, and tested whether the associations between lipid levels and AD would be significant in any of these age-bins.

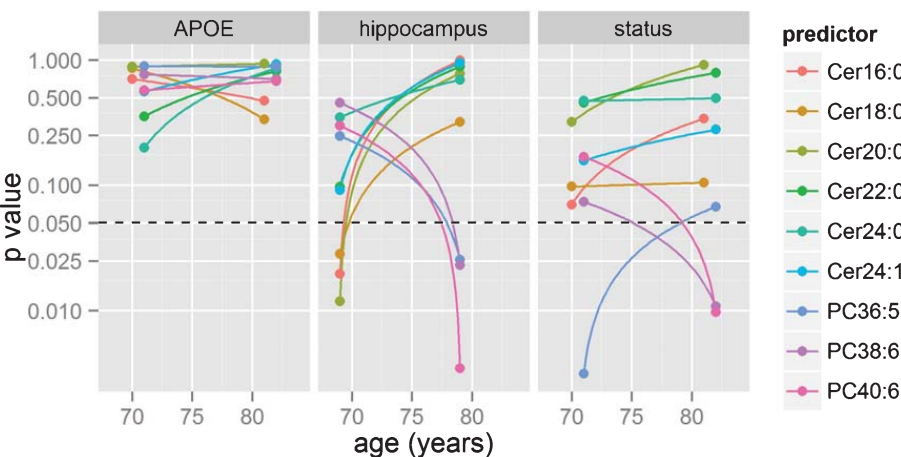


Fig. 5. Time or age-bin analyses. GLM were applied in two age bins to statistical significance of effects between lipids and three target variables (APOE status, hippocampal volume, and AD diagnosis) based on GLM models. p -values are shown in log scale, with dotted lines representing the $p = 0.05$ threshold.

This study found that from the six abundant ceramides measured in plasma and previously shown to be implicated in AD, three ceramides, Cer16:0, Cer18:0, and Cer24:1, were elevated in the plasma of AD patients compared to cognitively healthy group. As mentioned previously, there are several studies that have reported ceramide elevation in AD brain [5, 8, 20] and others, which report increased expression of sphingomyelinase, the enzyme that synthesizes ceramides [21, 22]. Ceramide involvement in neuronal death is likely to be related to its capability to activate caspase 3 [23, 24]. Another possible factor is that in neurons, oxidative stress induced by amyloid- β accumulation can cause overexpression of sphingomyelinase, this in turn causing elevated ceramides. Interestingly, ceramides have also been found to stimulate amyloid- β production, which again would result in an increased rate of neuronal death [25].

With aging representing the greatest risk factor for late-onset AD [26], we explored how associations between ceramides and AD classification varied with age. In doing this, we found no associations between ceramides and AD status in any of the two age-bins. This result contrasted with the GLM models when calculated with all the participants in the study (Table 2 and Fig. 3) that showed three ceramides associating with AD status. However this difference can be attributed to the smaller sample size used in the age-bins. As a reference, the minimum effect size detectable (at 80% power) by a paired *t*-test when using the full sample would vary between 0.14 and 0.26 standard deviations (depending on the number of patients where lipids and imaging were measured), with age-bin segregation increasing this minimum by ca. 50% (see Table 4).

Despite the power limitations of segregating into two bins, upon testing how relationships between ceramide levels and hippocampal volume loss varied with age, we observed levels of Cer16:0, Cer18:0, and Cer20:0 elevated in the plasma of younger participants (in ages below 75). Ceramides were also frequently associated with entorhinal volume and thickness in these younger participants (see Fig. 4 and Supplementary Material S4). Hippocampal and entorhinal atrophy is known to occur before symptoms of cognitive decline during AD development [27] and accumulation of ceramides have been linked with a higher risk of developing AD [9, 28]. Our data suggests that higher levels are also detected in younger individuals with hippocampal atrophy, potentially adding evidence to a role

in phenoconversion. This is very interesting since it could mean that ceramides have an important role in early diagnosis or as proposed by Mielke et al. [28], in phenoconversion.

In addition to ceramides, we also measured PC36:5, PC38:6, and PC40:6. These molecules were linked to AD when they were found associated with decline in AD cognitive function [10] and memory loss in healthy aging [29]. After comparing the PC levels in 412 plasma samples, we observed diminished PC36:5 and PC38:6 levels in AD plasma, partially replicating our previous findings. The lower PC levels reported here could be as a result of upregulation of PC hydrolysis induced by increased phospholipase A2 (PLA₂) activity and amyloid accumulation [30]. These PCs also contain omega-3 poly-unsaturated fatty acids (ω -3 PUFA). PC36:5 contains an eicosapentaenoic acid (EPA) as part of its molecular structure, while PC38:6 contains a docosahexaenoic acid (DHA) [10]. These fatty acids are thought to be absorbed into the brain when contained in molecules of another lipid family, lysophosphatidylcholines, which are direct metabolic products of PCs [31]. ω -3 PUFAs are well known for their neuroprotective properties in brain with higher omega-3 content being associated with lower incidence of dementia [32, 33]. Higher PCs in plasma (i.e., assuming higher ω -3 PUFA in plasma) could have neuroprotective properties by increasing ω -3 PUFA present in the brain. This hypothesis could be supported by a report which demonstrated that a ω -3 PUFA containing PC diet resulted in increased anti-inflammatory activity in the brain [34] and a recent study examining 2,000 healthy patients that found that during healthy-aging plasma shows an increasing trend for PC levels [35]. We also observed lower level of EPA containing PC36:5 associating with hippocampal atrophy, these initial finding may be explained by EPA acting as a competitive inhibitor of arachidonic acid (AA) thus resulting in down-regulation of pro-inflammatory AA-derived metabolites such as eicosanoids [36, 37].

When we assessed how associations between PCs and AD varied in different age-bins, we found that PCs showed no associations with APOE, white matter, or entorhinal cortex thickness atrophy in the two age-bins. However, all three PCs were associated with hippocampal volume in older participants, while PC36:5 was also marginally associated with entorhinal volume in this same age group. PC36:5 was strongly associated with AD in younger participants, while PC38:6 and 40:6 were so in the older

age bin, suggesting changes in peripheral PC levels may be also age-related or related to the AD severity stage.

In conclusion, we found the levels of two PCs and three ceramides altered in AD plasma when compared to cognitively healthy plasma. Ceramides were found to be increased in AD and associated with hippocampal and entorhinal atrophy in younger participants (age <75). PCs were found decreased and associated with status, especially in older participants for PC38:6 and 40:6 (age >75), but in younger participants for PC36:5. These results contribute to the growing body of evidence that peripheral lipids are implicated in AD pathology. More work is needed to understand the relationships between plasma lipids and AD progression, and importantly, to decipher if they should be considered as biomarkers for diagnosis or targets for AD therapies.

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SUPPLEMENTARY MATERIAL

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Featured Article

Association of blood lipids with Alzheimer's disease: A comprehensive lipidomics analysis

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Abstract

Introduction: The aim of this study was to (1) replicate previous associations between six blood lipids and Alzheimer's disease (AD) (Proitsi et al 2015) and (2) identify novel associations between lipids, clinical AD diagnosis, disease progression and brain atrophy (left/right hippocampus/entorhinal cortex).

Methods: We performed untargeted lipidomic analysis on 148 AD and 152 elderly control plasma samples and used univariate and multivariate analysis methods.

Results: We replicated our previous lipids associations and reported novel associations between lipids molecules and all phenotypes. A combination of 24 molecules classified AD patients with >70% accuracy in a test and a validation data set, and we identified lipid signatures that predicted disease progression ($R^2 = 0.10$, test data set) and brain atrophy ($R^2 \geq 0.14$, all test data sets except left entorhinal cortex). We putatively identified a number of metabolic features including cholesteryl esters/triglycerides and phosphatidylcholines.

Discussion: Blood lipids are promising AD biomarkers that may lead to new treatment strategies.

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Keywords:

Alzheimer's disease; Dementia; Brain atrophy; sMRI; Rate of cognitive decline; Lipidomics; Metabolomics; Biomarkers; Machine learning; Multivariate; Classification; Random forest

1. Introduction

Alzheimer's disease (AD) is a devastating illness and one of the major public health challenges of the 21st century. The lack of effective treatments and early diagnosis highlights the importance of the identification of noninvasive biomarkers, for early diagnosis and disease progression. Blood metabolites have recently emerged

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as promising AD biomarkers [1–4]. They are small molecules which could theoretically cross the already compromised AD blood-brain barrier [5]; they are easily accessible, and they represent an essential aspect of the phenotype of an organism and a molecular “fingerprint” of disease progression [6,7]. They can therefore aid early diagnosis, recruitment into trials and may help identify new therapeutic targets.

A number of blood metabolomic studies have highlighted the role of lipid compounds, such as phosphatidylcholines (PCs) in AD [1–4]. We previously identified three PCs that were diminished in mild cognitive impairment (MCI) individuals and AD patients [4] and were further associated with poorer memory performance and decreased brain function during aging [8]. We further performed lipidomics analysis and identified 10 metabolites that predicted AD in an unseen test data set with 79% accuracy [9]; six analytes were putatively identified as cholesteryl esters (ChoEs), molecules related to PCs, and were reduced in MCI and AD.

Here, we performed lipidomics analysis in a sample of 142 AD patients and 135 healthy controls aiming to (1) replicate our previous associations [9] and (2) discover new lipids and combinations of lipids associated with clinical AD diagnosis and AD endophenotypes, such as the rate of cognitive decline and brain atrophy measures. This is to our knowledge the most comprehensive blood lipidomics study to date to identify lipid signatures associated with AD and AD endophenotypes, improving our current knowledge of molecules associated with AD.

2. Methods

2.1. Patient sample collection

This study used 148 AD patients and 152 controls from the Dementia Case Register at King's College London and the EU-funded AddNeuroMed study [10]. All individuals with AD patients met criteria for either probable (NINCDS-ADRD, DSM-IV) or definite (CERAD) AD. All nonpopulation individuals who were controls were screened for dementia using the MMSE or ADAS-cog or were determined to be free from dementia at neuropathologic examination or had a Braak score ≤ 2.5 . Diagnosis was confirmed by pathologic examination for a proportion of cases and cognitively normal elderly controls. All AD cases had an age of onset ≥ 60 years, and controls were ≥ 60 years at examination. A total of 102 AD cases and 104 controls had HDL-c, LDL-c, TC, and TG serum levels (mmol/L) available. Nonoverlapping individuals from these cohorts have been previously reported [9]. Each individual was required to fast for 2 hours before sample collection, and 10 mL of blood was collected in tubes coated with sodium ethylenediaminetetraacetic acid to prevent clotting. Whole blood was centrifuged at 2000 g for 10 minutes at 4°C to separate plasma, which was removed and stored at

–80°C. All samples were centrifuged within approximately 2 hours of collection.

2.2. Lipidomics

Sample treatment has been described elsewhere [4,9,11] and is explained in detail in [Supplementary Methods 1](#). Briefly, 20 μ L of plasma was added to a glass HPLC vial containing a 400- μ L glass insert (Chromacol, UK). Ten microliters of high purity water and 40 μ L of MS grade methanol were added to each sample, followed by a 2-minute vortex mix to precipitate proteins; 200 μ L of Methyl tert-Butyl Ether (MTBE) containing 10 μ g/mL of internal standard Tripentadecanoin (TG45:0) was added, and the samples were mixed via vortex at room temperature for 1 hour. After addition of 50 μ L of high purity water, a final sample mixing was performed before centrifugation at 3000 g for 10 minutes. The upper, lipid-containing, MTBE phase was then injected onto the LC-MS system directly from the vial by adjustment of the instrument needle height (17.5 mm from bottom).

Lipidomics was performed by a Waters ACQUITY UPLC and XEVO QTOF system. The method has previously been published [4,12] and has been shown to quantitate >4500 metabolite species ([Supplementary Methods 1](#)). Samples were analyzed in a randomized order, in four batches, with pooled plasma sampled (QC) at regular intervals throughout the run ($n = 30$ for both positive and negative ionization). Features were extracted from netCDF files using the R package “XCMS” [13] which performed filtration, peak identification, matching of peaks across samples, and retention time correction. Positive and negative ionization mode data were extracted separately and quantile normalized.

2.3. Structural magnetic resonance imaging

Volumes of whole brain and the hippocampi and entorhinal cortices were obtained using FreeSurfer 5.1.0 from 123 subjects (53 AD patients and 70 Controls) who had undergone sMRI. Regions were normalized by intracranial volume [14]. The volumetric data were not used to aid in the clinical diagnosis of AD. Detailed information regarding data acquisition, pre-processing, and quality control assessment has been described elsewhere [15,16]. Before analyses, sMRI measures were standardized to have a mean of 0 and a standard deviation (SD) of 1.

2.4. Calculation of rate of cognitive decline

The ROD was available for 118 AD patients with analyte data and has been described elsewhere [17]. The ROD was based on longitudinal mini mental state examination (MMSE) assessments [18], and only samples with at least three MMSE measures were included in the calculation using linear mixed effect models. After covariate adjustment

[17], the slope coefficient for each sample was used as the ROD defined as the change in MMSE per day.

2.5. Statistical analysis

2.5.1. Quality control

Data QC has been previously described [9] and included filtering of features and individuals, data transformation, batch effect correction, outlier detection, and imputation (Supplementary Methods 2.1 and Supplementary Fig. 1). All analyses took place in R.3.01.

2.5.2. Single-analyte statistical analysis

Logistic regression investigated the association of each metabolite with clinical AD diagnosis and linear regression the association with cognitive decline and sMRI measures. Logistic regression and linear regression models for the sMRI measures were adjusted for age at sampling, gender, presence of the apolipoprotein E (*APOE*) $\epsilon 4$ allele, batch, and study site. For the ROD models, covariate adjustment was only applied for batch as the rest of the covariates were included in the ROD calculation [17]. sMRI measurements were not adjusted for diagnosis to allow identification of features associated with brain atrophy caused by AD. False discovery rate (FDR) correction (0.05) was applied to correct for multiple testing (“fdrtool”). Secondary models investigated whether any associations were modified by the *APOE* $\epsilon 4$ allele or by gender.

Logistic regression results (summary statistics) for the positive ionization metabolites were combined with the results (summary statistics) from the Proitsi et al data set [9] using in-

verse variance weighted fixed effect meta-analysis (“metafor”). The published data set [9] was restricted to 576 features extracted using Mass-Lynx, and therefore, the analysis presented here includes a large number of previously unreported molecules extracted using XCMS. All associations are reported as the change per one metabolite standard deviation (SD).

2.5.3. Multivariate statistical analysis

A random forest (RF) classifier approach (using “rf” and “rfe” in “CARET”) was used to develop a clinical diagnosis classifier as previously described [9] (Supplementary Methods 2.2). Briefly, AD cases and controls were divided into a training data set (2/3 of the sample) matched for age, gender, and site and an independent data set (rest 1/3 of the sample). An RF model was built on the training data set (100 bootstraps), and in each iteration, each variable was assigned a variable importance (VI) score. The summed VI ranks provided an indication of the predictive power for each variable, and the top 10% molecules were selected for RF with recursive feature elimination (rfe; 100 bootstraps) from 250 down to two features. For each subset of predictors, the mean bootstrap testing performance was calculated, and the optimal number of variables was identified using “sizeTolerance” that picks a subset of variables that is small without sacrificing too much performance. Subsets of variables within 2.5% and 5% of the optimum performance were examined and used to build final models in the complete training data, which were tested on the test set. The final model was also tested in the Proitsi et al data set [9] which was used as a validation data set, after excluding metabolites in the negative ionization mode. The area under curve (AUC) was used to test the performance of each classifier. Receiver operator curves (ROCs)

Table 1
Sample demographics

	AD (N = 142)	Controls (N = 135)	Difference between AD patients and controls*
Age, mean (SD)	77 (6.5)	74 (5.9)	$t = -4.8$ (270), P value = 2.62×10^{-6}
Gender (males/females)	48/87	47/95	$\chi^2 = 0.09$ (1), P value = .761
<i>APOE</i> $\epsilon 4$ allele (absence/presence)	54/81	99/43	$\chi^2 = 23.53$ (1), P value = 1.23×10^{-6}
MMSE, mean (SD; Range)	20.1 (4.6; 10–27)	29.2 (0.9; 27–30)	$t = 22.58$ (143), P value < 2.0×10^{-16}
ROD (per year), mean (SD) [†]	−1.46 (1.26)	NA	NA
Entorhinal cortex right, mean (SD) ^{‡§}	0.00092 (0.0003)	0.0013 (0.0003)	$t = 6.02$ (99), P value = 2.87×10^{-8}
Entorhinal cortex left, mean (SD) ^{‡§}	0.00094 (0.0002)	0.0013 (0.0004)	$t = 5.26$ (86), P value = 1.58×10^{-6}
Hippocampus right, mean (SD) ^{‡§}	0.0019 (0.0004)	0.0025 (0.0003)	$t = 9.06$ (100), P value = 1.3×10^{-14}
Hippocampus left, mean (SD) ^{‡§}	0.0018 (0.0004)	0.0025 (0.003)	$t = 10.57$ (104), P value < 2.0×10^{-16}
Mean HDL-c (SD), mmol/L	1.58 (0.37)	1.55 (0.38)	$\beta = 0.109$ (SE = 0.33), P value = .068
Mean LDL-c (SD), mmol/L	3.42 (1.01)	3.07 (0.82)	$\beta = 0.092$ (SE = 0.15), P value = .529
Mean TC (SD), mmol/L	5.69 (1.17)	5.29 (1.01)	$\beta = 0.209$ (SE = 0.173), P value = .229
Mean TG (SD), mmol/L	1.64 (1.04)	1.52 (0.67)	$\beta = 0.021$ (SE = 0.146), P value = .885
Statins (yes/no)	38/97	34/108	$\chi^2 = 0.436$ (1), P value = .509

Abbreviations: AD, Alzheimer's disease; MMSE, mini-mental state examination score; ROD, rate of cognitive decline; SD, standard deviation.

*Differences in the means/frequencies of clinical/demographic variables were tested using t test (df), χ^2 (df) test, or linear regression analyses after adjusting for age, gender, the *APOE* $\epsilon 4$ allele, and study site.

[†]Rate of decline data was available for a subset of AD patients (N = 118).

[‡]sMRI data were available for a subset of study participants (N = 123 [AD = 53, controls = 70]).

[§]Normalized to intracranial volume.

^{||}Serum HDL cholesterol, LDL cholesterol, total cholesterol, and triglyceride levels were available for a subset of study participants (N = 208 [AD = 102, Controls = 106]).

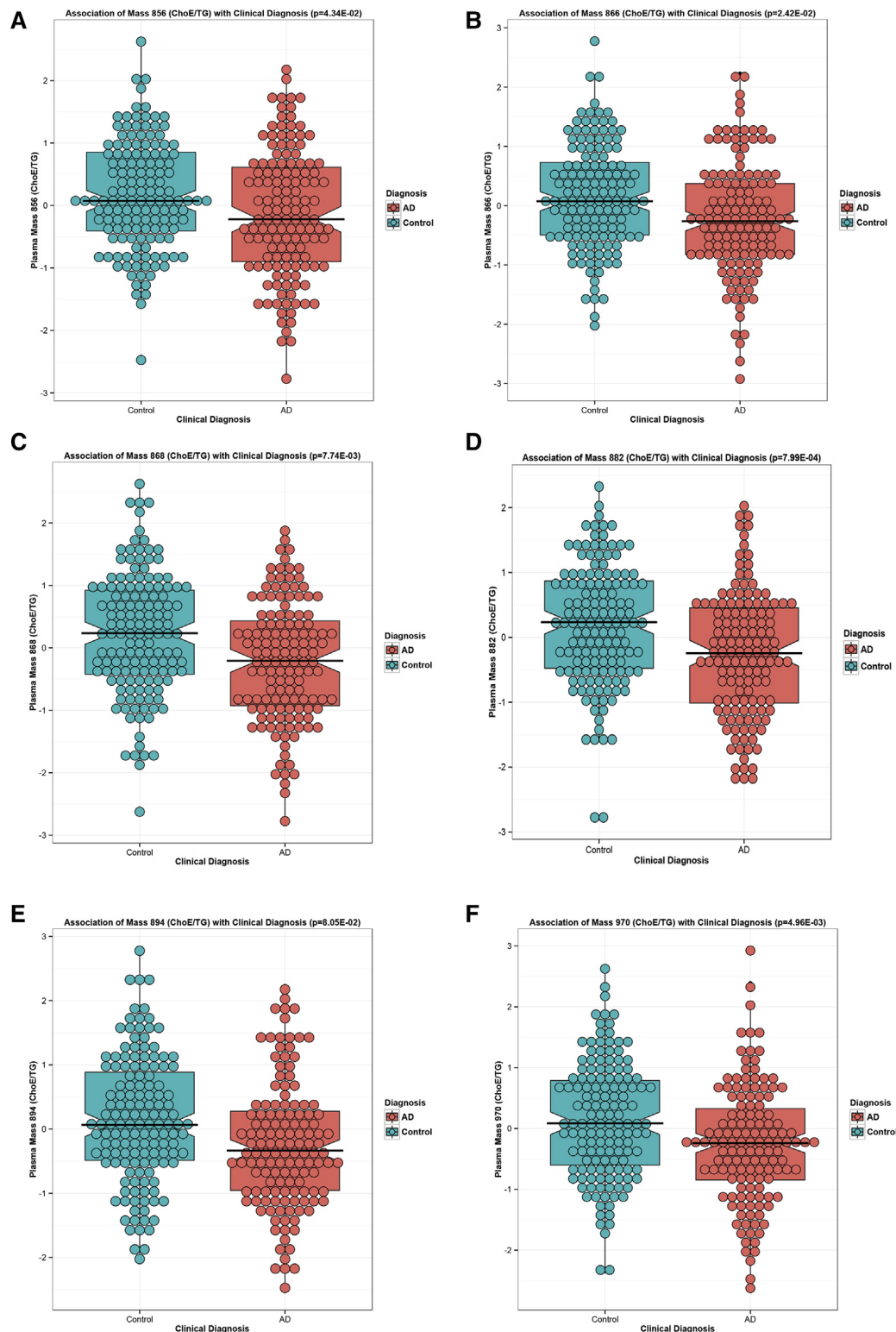


Fig. 1. Associations of previously reported molecules (Proitsi et al 2015) with clinical AD diagnosis in the current data set and associations of putatively annotated molecules, selected through random forest analyses, with the respective phenotype. (A) Association of Mass 856 with clinical AD diagnosis; (B) Association of Mass 866 with clinical AD diagnosis; (C) Association of Mass 868 with clinical AD diagnosis; (D) Association of Mass 882 with clinical AD diagnosis; (E) Association of Mass 894 with clinical AD diagnosis; (F) Association of Mass 970 with clinical AD diagnosis; (G) Association of Mass 882 (–) (PC 40:4) with clinical AD diagnosis; (H) Association of Mass 948 (+) TG (57:1) with clinical AD diagnosis; (I) Association of Mass 919 (+) TG 50:2 with Hippocampus (Right); (J) Association of Mass 943 (+) (ChoE/TG) with Hippocampus Left; (K) Association of Mass 367 (sterol) with Entorhinal Cortex (Right); (L) Association of Mass 816 (+) with Entorhinal Cortex (Left); (M) Association of Mass 771 (+) PC 36:3 with the rate of cognitive decline (ROD). The P values displayed are for the univariate regressions after adjusting for covariates. All molecules are scaled to have a mean of 0 and a standard deviation of 1.

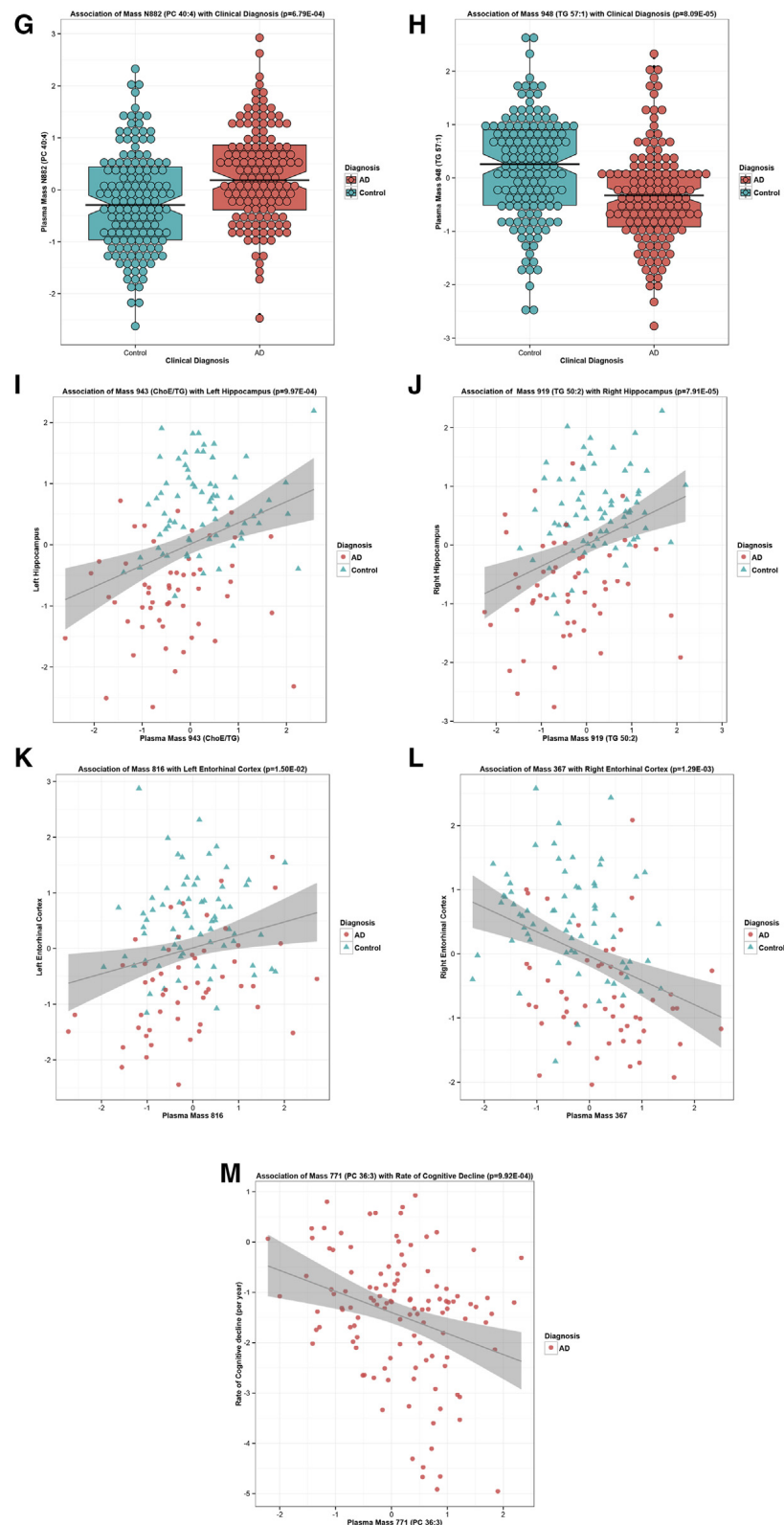


Fig. 1. (Continued)

were plotted using “ROCR”. Models including *APOE* $\epsilon 4$ and the six features in Proitsi et al [9] were also tested.

Random forest regression (RFR) models were built for cognitive decline and sMRI measures following the same strat-

egy as for clinical diagnosis. The data set was split randomly into a training (2/3 of the data) and test set (1/3 of the data) for each endophenotype such that the training and test data sets were stratified for each endophenotype and contained

equal representation of each site. Age, gender, and *APOE* $\epsilon 4$ presence were included in the model development for the sMRI models, and the root mean squared error (RMSE) was used to evaluate the performance of the models.

3. Results

A total of 2539 positive ionization and 358 negative ionization features were initially extracted from 300 individuals. After QC, 2216 positive and 289 negative ionization features from 277 individuals (142 AD cases and 135 controls) were used in subsequent analyses. Of these, 53 AD patients and 70 controls had sMRI data available, and 118 AD patients had ROD data available. Sample demographics are displayed in [Table 1](#).

3.1. Univariate analyses results

Logistic regression analyses were initially used to investigate the association of each lipid with AD. We then performed fixed-effects meta-analyses between the results of this data set and our previously published data set [9], using fixed-effects meta-analyses. Briefly, 425 features were associated with AD at P value $< .05$ in this data set; of these, 87 features passed correction for multiple testing at Q value < 0.05 . After meta-analysis, 377 features were associated with AD at P value $< .05$ and 125 at Q value < 0.05 . All six features from Proitsi et al [9] were associated with AD at Q value < 0.05 in meta-analysis ([Fig. 1](#) (A–F) and [Table 2](#)).

Linear regression investigated the association of each lipid with brain atrophy and the rate of cognitive decline. A total of 266 features were associated with the ROD at P value $< .05$, but none passed multiple testing correction. A total of 181 features were associated with right hippocampus volume and 224 were associated with left hippocampus volume; only six features were associated with left hippocampus at Q value < 0.05 . Finally, 156 and 124 features were associated with EC volume (left and right, respectively) at P value $< .05$, but no associations passed correction for multiple testing. Results for all logistic and linear regression analyses are provided in [Supplementary Table 1](#).

Overall, most lipids were reduced in AD compared to controls (54 of the 87 features associated at Q -value < 0.05 were reduced in AD). Additionally, we observed substantial overlap between features associated with clinical AD diagnosis and brain atrophy ([Supplementary Fig. 2](#)).

We further investigated whether the *APOE* $\epsilon 4$ and gender modified the associations between lipids and clinical AD diagnosis. *APOE* $\epsilon 4$ modified the association of 231 features with AD, and gender modified the association of 191 features with AD (P value $< .05$); none of these associations was significant at Q -value < 0.05 . There were only 3 individuals with the $\epsilon 2/\epsilon 4$ genotype, and there were therefore no differences in lipids levels between $\epsilon 4$ and non- $\epsilon 4$ carriers after excluding $\epsilon 2/\epsilon 4$ individuals.

3.2. Multivariate analysis results

A RF approach was used to identify a panel of molecules associated with clinical AD diagnosis. After an initial RF pre-selection step on the training data set, the top 10% lipids (250 features), in terms of their variable importance, were selected (after 100 Bootstraps). Furthermore, random forest with recursive feature elimination (RF-rfe) on the training data set showed that the best training performance was for a model with 240 features. To choose a model with high accuracy while reducing the number of features as low as possible, a 5% tolerance RF-rfe model (25 features) was fitted on the whole training data set (AUC, 0.87) and classified the test data set with 73% accuracy ([Supplementary Fig. 3](#) and [Table 3](#)). The model was then fitted on the training data set, excluding one negative ionization mode analyte and classified the test training data set with 74% accuracy and the Proitsi et al [9] validation data set with 71% accuracy. There was no increase in accuracy when covariates and the features from Proitsi et al [9] were added to the models ([Table 3](#)).

Random forest regressions using the same pipeline were applied to the ROD and brain atrophy measures. After RFR-rfe on the training data set, the lowest mean RMSE for ROD was for a model with 40 features. The 5% tolerance model of the lowest RMSE model (10 features) was fitted to the whole training data set ($R^2 = 0.49$) and predicted the test data set with $R^2 = 0.10$ ([Table 4](#)).

For right hippocampus, the lowest RMSE was with a model with 70 features that included age. A 5% tolerance model (12 features) was fitted to the training data set ($R^2 = 0.55$) and predicted the test data set with $R^2 = 0.15$ ([Table 3](#)). For left hippocampus, the lowest training RMSE was with 100 features that also included age. The 5% tolerance model (12 features) was fitted to the training data set ($R^2 = 0.59$) and predicted the test data set with $R^2 = 0.15$ ([Table 3](#)). The performance of the models was almost identical when age was excluded.

For the right EC, the lowest mean training RMSE was with 70 features; a 5% tolerance model (12 features) was fitted to the training data set ($R^2 = 0.54$) and predicted the test data set with $R^2 = 0.14$. Finally, for left EC, a model with 90 features had the lowest RMSE, and a 5% tolerance model (12 features) was fitted to the training data set ($R^2 = 0.42$) and predicted the test data set with $R^2 = 0.01$ ([Table 3](#)). Results of all 2.5% models are presented in [Supplementary Tables 2 and 3](#), and the list of molecules included in each classifier is found in [Supplementary Table 1](#). The strength of association between selected features and each model is shown in [Fig. 2](#), and the scaled VI of each lipid after RF-RFE/RFR-RFE for each phenotype is shown in [Supplementary Fig. 4](#).

3.3. Lipid annotation and putative identification

We opted to annotate the top features, in terms of VI from each model and features selected in more than one

Table 2

List of putatively identified metabolite molecules selected by the six random forest models

Logistic regression analysis	m/z (ionization mode)	Putative metabolite molecule	Present study data set			Proitsi et al 2015 data set			Meta-analysis		
			OR	95% CI	P value	OR	95% CI	P value	OR	95% CI	P value
Clinical AD diagnosis	882 (–)	PC 40:4	1.996	1.35–3.01	6.79E–04*	NA	NA	NA	NA	NA	NA
	948 (+)	TG 57:1	0.514	0.36–0.71	8.09E–05*	0.522	0.28–0.92	3.01E–02	0.516	0.39–0.69	6.83E–06*
	856 (+)	ChoE/TG [†]	0.711	0.51–0.99	4.34E–02	0.141	0.04–0.43	1.75E–03	0.632	0.46–0.87	4.94E–03*
	866 (+)	ChoE/TG [†]	0.663	0.46–0.94	2.42E–02	0.251	0.10–0.52	7.51E–04*	0.569	0.41–0.79	7.37E–04*
	868 (+)	ChoE/TG [†]	0.65	0.47–0.89	7.74E–03*	0.218	0.07–0.55	3.15E–03	0.591	0.44–0.80	7.16E–04*
	882 (+)	ChoE/TG [†]	0.57	0.41–0.79	7.99E–04*	0.231	0.08–0.53	1.56E–03	0.517	0.38–0.71	3.05E–05*
	894 (+)	ChoE/TG [†]	0.732	0.51–1.04	8.05E–02	0.151	0.05–0.38	3.14E–04*	0.615	0.44–0.86	4.65E–03*
	970 (+)	ChoE/TG [†]	0.643	0.47–0.87	4.96E–03*	0.362	0.18–0.67	2.56E–03	0.58	0.44–0.77	1.27E–04*
Linear regression analysis			Beta	95% CI	P value	NA	NA	NA	NA	NA	NA
Hippocampus (right)	919 (+)	TG 50:2	0.396	0.20–0.59	7.91E–05*	NA	NA	NA	NA	NA	NA
Hippocampus (Left)	943 (+)	ChoE/TG	0.320	0.13–0.51	9.97E–04*	NA	NA	NA	NA	NA	NA
Entorhinal Cortex (Right)	367 (+)	Sterol	–0.201	–0.39 to –0.01	3.88E–02	NA	NA	NA	NA	NA	NA
Entorhinal Cortex (Left)	816 (+)	NA	0.218	0.03–0.41	2.47E–02	NA	NA	NA	NA	NA	NA
ROD	771 (+)	PC 36:3 [‡]	–0.412	–0.65 to –0.17	9.92E–04	NA	NA	NA	NA	NA	NA

Abbreviations: AD, Alzheimer's disease; ChoE, cholesteryl ester; CI, confidence interval; m/z, mass-to-charge ratio; OR, odds ratio; PC, phosphatidylcholine; ROD, rate of cognitive decline; TG, Triglyceride.

NOTE. The six random forest models were for the clinical AD diagnosis, ROD, hippocampus (R/L), and entorhinal cortex (R/L) phenotypes. The association of each molecule is presented with the respective phenotype (i.e., primary phenotype of association). The association of the six molecules previously reported by Proitsi et al 2015 with AD is also presented.

*Q value <0.05.

[†]Features identified by Proitsi et al 2015 and for the Proitsi et al., data set semiquantified values are presented.

[‡]PC 36:3 has m/z 770, and m/z 771 is its C13 isotope. ChoE/TG indicates co-elution of ChoE and TG molecules.

model, using our in-house lipid database and MS/MS fragmentation patterns [4,11,12]. These features were annotated as mainly long-chain triglycerides (LCTs) and ChoEs, some were PCs and a sterol. Fig. 1 (G–M) and Table 2 present the univariate associations of these molecules with the respective phenotypes. The association of

the annotated molecules with all phenotypes is shown in Supplementary Fig. 5. The raw intensity counts for each AD associated lipid across AD and controls, along with the coefficients of variation (relative standard deviation [RSD]) of the pooled samples (QCs) are shown in Supplementary Table 4.

Table 3

Random forest classifier model results (clinical AD diagnosis) for the training data set and predictions on the test data set and the Proitsi et al data set

Model (5% tolerance)	Training data set (N = 179)			Test data set (N = 98)						Validation data set (N = 75)					
	Sens.	Spec.	AUC	Acc.	Sens.	Spec.	AUC	PPV	NPV	Acc.	Sens.	Spec.	AUC	PPV	NPV
Covariates only*	0.72	0.71	0.77	0.56	0.54	0.58	0.56	0.5	0.62	0.6	0.57	0.63	0.6	0.57	0.63
25 features [†]	0.82	0.82	0.87	0.73	0.77	0.69	0.73	0.66	0.79	NA	NA	NA	NA	NA	NA
24 features [‡]	0.81	0.82	0.86	0.74	0.74	0.73	0.74	0.68	0.78	0.71	0.69	0.73	0.71	0.69	0.73
25 features [†] + covariates*	0.83	0.83	0.88	0.75	0.79	0.71	0.75	0.68	0.81	NA	NA	NA	NA	NA	NA
24 features [‡] + covariates*	0.84	0.82	0.88	0.75	0.79	0.71	0.75	0.68	0.81	0.71	0.69	0.73	0.71	0.69	0.73
25 features [†] + 6 ChoE/TG [§]	0.82	0.82	0.87	0.71	0.74	0.67	0.71	0.64	0.77	NA	NA	NA	NA	NA	NA
24 features [‡] + 6 ChoE/TG [§]	0.82	0.81	0.87	0.71	0.79	0.66	0.72	0.65	0.80	0.72	0.71	0.73	0.72	0.69	0.74
25 features [†] + covariates* + 6 ChoE/TG [§]	0.83	0.83	0.88	0.74	0.77	0.71	0.74	0.68	0.80	NA	NA	NA	NA	NA	NA
24 features [‡] + covariates* + 6 ChoE/TG [§]	0.83	0.82	0.88	0.74	0.79	0.69	0.74	0.67	0.81	0.71	0.69	0.73	0.71	0.69	0.73

Abbreviations: Acc, accuracy; AUC, area under the curve; ChoE, cholesteryl ester; NPV, negative predictive value; PPV, positive predictive value; Sens, sensitivity; Spec, specificity; TG, triglyceride.

*Age, sex, ε4.

[†]5% tolerance model including negative ionization molecule.

[‡]5% tolerance model excluding negative ionization molecule.

[§]Six features identified by Proitsi et al 2015.

Table 4
Random forest regression model results for the training data set and predictions on the test data set for each AD endophenotype

Phenotype	Model (5% tolerance)	Train data set (n = 93)		Test data set (n = 28)	
		RMSE	R ²	RMSE	R ²
Hippocampus (right)	Covariates only*	0.92	0.28	1.09	0.02
	12 features [†]	0.58	0.55	0.9	0.15
Hippocampus (Left)	Covariates only*	0.89	0.34	1.21	0.01
	12 features [†]	0.64	0.59	0.99	0.15
Entorhinal cortex (right)	Covariates only*	0.95	0.22	1.14	<0.01
	12 features	0.66	0.54	0.92	0.14
Entorhinal cortex (left)	Covariates only*	1.00	0.22	1.17	<0.01
	12 features	0.77	0.42	1.07	0.01
ROD	10 features [‡]	0.93	0.49	1.09	0.10

Abbreviations: RMSE, root mean squared error; ROD, rate of cognitive decline.

*Age, sex, ε4.

[†]Age was included in the final model. There was no difference in either train or test data set performance when age was excluded.

[‡]Covariates were already included in the calculation for the ROD.

4. Discussion

This is to our knowledge, the largest nontargeted blood lipidomics study in AD to date. Here, we expanded our recent work [9], and using univariate and multivariate approaches, we replicated the associations between six previously reported blood lipids and AD [9] and reported their association with brain atrophy. We further identified combinations of lipids that classified AD patients with relatively good accuracy when tested in both a test and a validation data set (>70%), and combinations of molecules that predicted changes in disease progression ($R^2 = 0.10$ for test data set) and brain atrophy ($R^2 \geq 0.14$ for all test data sets except for left EC). Overall, we observed substantial overlap between features associated with clinical AD diagnosis and brain atrophy. The associations of all molecules included in each model with all phenotypes is shown in Fig. 2. Although these signatures cannot be used for diagnostic purposes yet, they suggest important biological mechanisms associated with AD.

4.1. Identification and role of lipids in AD

We putatively identified two PC molecules; additionally, ChoEs and triglycerides (TGs) were tentatively annotated due to chromatographic coelution, and finally, we putatively annotated a molecule as a sterol. The higher MS-MS sensitivity achieved here enabled the detection of a number of additional lipids that co-eluted with ChoE; these were annotated as TGs (Table 2).

The association of PCs with AD and cognition has been extensively described [4,8]. Here, one of the molecules

most strongly associated with AD is a putative PC (PC 40:4), and the top lipid in the ROD model is also a putative PC (PC 36:3). In contrast to the same species of molecules, we have previously identified, both PCs are increased in AD, and PC 36:3 is associated with faster ROD. Although most studies to date have reported a reduction of PC levels in AD, an increase in CSF PCs has been observed in AD compared to control brains [19] and recently in “AD-like” patients based on their CSF Amyloid-beta42, Tau, and Phospho-Tau-181 levels [20]. A recent study also reported a parallel increase of PCs containing saturated and short-chain fatty acids in serum from AD patients [21]. These suggest deregulation in the biosynthesis, turnover, and acyl chain remodeling of phospholipids, in accordance with increased phospholipid breakdown due to PLA2 [21] overactivation.

We have also reported associations with low-chain and very-low-chain triglycerides (LCTs/VLCTs; fatty acid chain length >16 carbons). One of the most interesting findings was that due to the higher MS-MS sensitivity achieved in this study, we were able to observe putative VLCTs that were coeluting with ChoEs (Table 2). We have previously reported on the synthesis of ChoEs [9]; briefly, it takes place by transfer of fatty acids from PC to cholesterol, a reaction catalyzed by lecithin cholesterol acyl transferase in plasma and by acyl-coenzyme A: cholesterol acyl transferase 1 and 2 (ACAT1 and ACAT2) in other tissues, including the brain. The association of LCTs/VLCTs with AD is noteworthy. Although overall TGs are seen as risk factors for many disorders including cardiovascular disease (CVD) and type 2 diabetes (T2D), numerous investigations point to the diverse role of TGs with different chain lengths. It is known for example that medium-chain triglycerides (MCTs) and LCTs have different metabolic pathways in digestion and absorption [22]. Moreover, although LCTs of lower carbon number and double bond content have been associated with increased CVD [23] and T2D risk [24], LCTs with higher carbon number and double bond content, like the ones here, have been associated with decreased risk of T2D [24], whereas no associations between T2D and total triglyceride levels were observed in the same individuals [24]. Furthermore, decreased concentration of LCTs and an increased concentration of VLCTs have been associated with longevity [25]. These findings are particularly interesting as most vegetable oils are comprised of long-chain fatty acids; however, only MCTs have to our knowledge been implicated in AD, although findings are controversial [26]. When we previously investigated the association of total cholesterol and TGs with AD in overlapping individuals using Mendelian randomization, we found no evidence for an association with AD [27]. Additionally, we observed no difference in serum triglycerides, total cholesterol, LDL cholesterol, and HDL cholesterol between AD patients and controls for a random subset of study participants in this study (AD = 102, controls = 106) that had serum lipid measures available for the same visit, as well as no difference in the

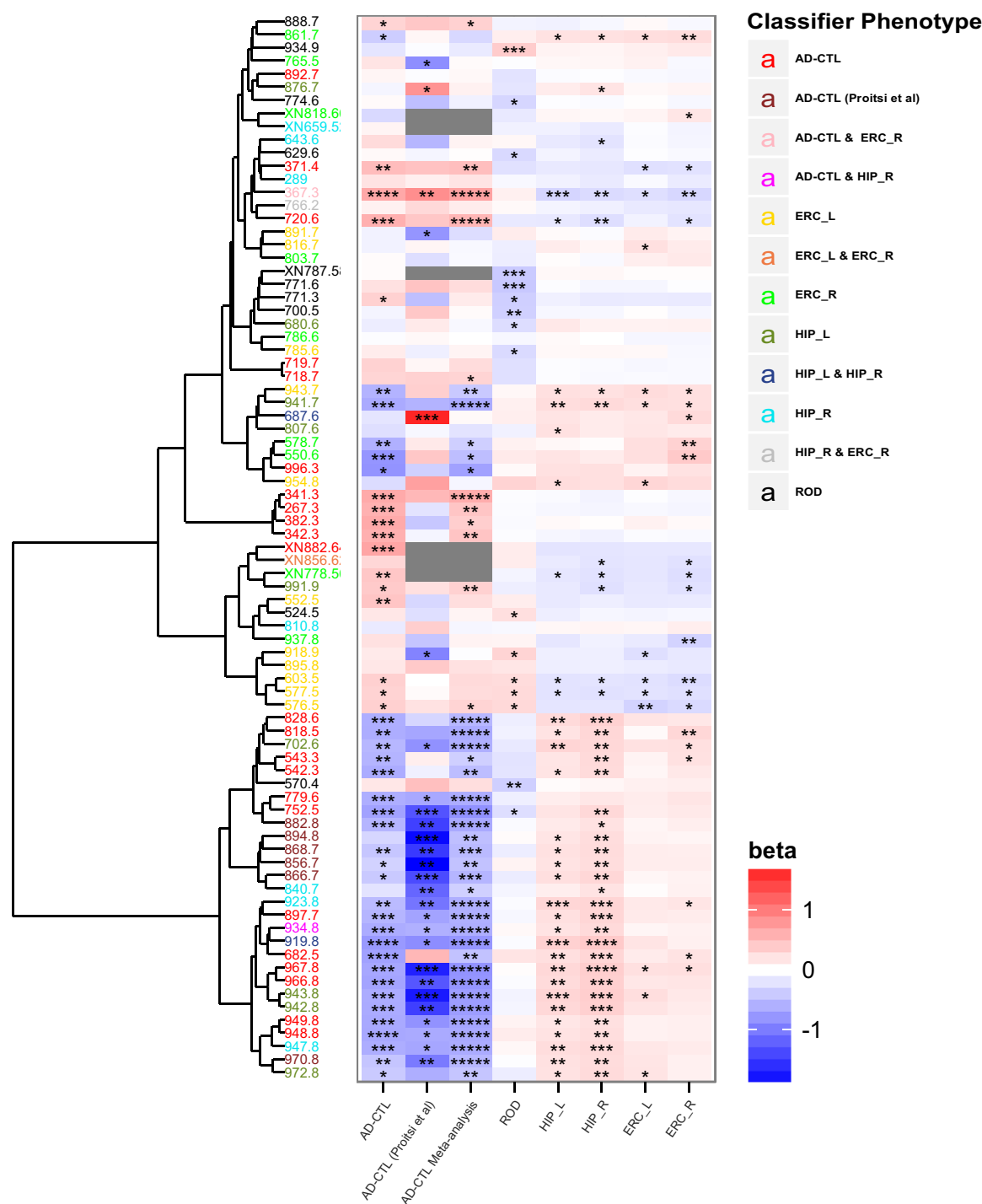


Fig. 2. Heatmap of the univariate associations between features selected during random forest analyses for each phenotype. The color of each box represents the univariate logistic regression beta coefficient (log[OR]) for clinical diagnosis or the univariate scaled linear regression beta coefficients for the rate of cognitive decline and brain atrophy, after adjusting for covariates. The stars on each box represent the strength of the association: **P* value <.05; ***P* value <.01; ****P* value <.001; *****P* value <.0001; ******P* value <.00001. The order of the metabolite molecules on the y-axis is based on a hierarchical clustering using the metabolites pairwise correlations. XN denotes negative ionization mode feature. Abbreviations: AD-CTL, Clinical AD diagnosis; ROD, rate of cognitive decline; HIP_L, left hippocampus; HIP_R, right hippocampus; ERC_L, left entorhinal cortex; ERC_R, right entorhinal cortex.

frequency of AD patients and controls who were taking statins. On the other hand, a recent study reported an overlap between genes involved in elevated plasma lipid levels and inflammation and the risk for AD [28]. All these highlight the relevance of investigating smaller lipid fractions as they

highlight specific steps in their biosynthesis and metabolism that may be associated with AD.

Finally, we observed an association between most phenotypes and a feature of *m/z* 367. We previously described a molecule with the same mass and similar retention time to

be reduced in AD [9]. The molecule discovered here was associated with an increased risk for AD in both data sets and with reduced brain volume and was included in the ERC and clinical diagnosis models. We believe this feature is a fragment and a sterol, specifically an isomer of desmosterol. Desmosterol is a precursor of cholesterol and seladin (*DHCR24*), which governs the metabolism of desmosterol to cholesterol in specific brain areas. Desmosterol has been shown to inhibit β -secretase cleavage of APP, and the formation of amyloid- β and lower desmosterol levels has been found in the plasma and brains of AD patients compared to controls [29–32].

Although the association of aberrant lipid metabolism in AD pathogenesis is undisputed [33–35]; at this stage, the mechanisms by which these changes in lipids might occur in AD are unclear. One possibility involves AD selective alterations to circulating lipid metabolism. However, another possibility relates to cellular lipid production. A number of phospholipids are synthesized within a specialized region of the endoplasmic reticulum (ER) that is closely associated with mitochondria, the mitochondria-associated ER membranes (MAM). The close association of MAM to mitochondria facilitates Ca^{2+} and phospholipid exchange between the two organelles [36–38]. Recent studies have shown that MAM contacts are damaged in AD [39–42]. Because ER-mitochondria contacts are required for the synthesis of certain lipids [36–38], such changes may affect lipid metabolism and lead to some of the changes described here. Indeed, different *APOE* alleles have been shown to influence MAM [43].

4.2. Strengths and limitations

Here, we have used a large well-characterized AD cohort and a careful and systematic analysis pipeline. Through bootstrapping, we have reduced over-fitting, and subsequently, we validated our results in an unseen data set for each phenotype and an additional validation data set for clinical AD diagnosis. Our AD diagnostic classifier achieved 88% accuracy on the training data set (summary of 100 bootstraps) and predicted the test and validation data sets with >70% accuracy. Our training data set comprised of individuals matched on age and gender. On the other hand, the test data set consisted primarily by females, and AD patients were significantly older than controls. Additionally, the validation data set [9] included AD patients and controls of UK origin only, older than the individuals of the current data set. These findings highlight robustness in the model. For the AD endophenotypes, the Random Forest Regression models had a very good performance on all training data sets. Although the performance dropped significantly in the test data sets, we observed $R^2 > 0.10$ for all phenotypes except for Left EC ($R^2=0.01$). The drop in performance can be attributed to over-fitting of the training data sets and the smaller number of individuals with ROD/brain atrophy measures. The poor

performance of the Left EC is in agreement with our univariate analyses that highlighted weaker associations with the EC for the whole sample; however, it is in contrast to the overall right-to-left asymmetry in AD [17].

A limitation of this study is that we were not able to decipher the exact fatty acid chain structure of some features. Owing to the higher MS-MS sensitivity, we observed a number of putative ChoEs and TGs co-eluting, which is commonly observed in lipidomics studies due to hundreds of lipids detected in one analysis; to minimize co-elution problems, our chromatographic run is 2 hours long using ultra pressure chromatography [11,44].

Additionally, although this is the largest AD lipidomics study to date, we acknowledge that the sample size is still modest and further replication is required, especially for the ROD and brain atrophy phenotypes. Moreover, although we had information on the ROD, this calculation was based on the MMSE, which is a crude measure of measurement of cognition. Furthermore, the present study did not contain an MCI cohort or information on conversion to MCI/AD, and therefore, we do not know whether these features are associated with initiation of AD. This study additionally suffers from limitations inherent to AD case-control studies, such as the large number of comorbidities in old age, the possibility that some of the elderly controls may already carry pathology, and that some of the clinically diagnosed AD may be pathologically non-AD dementias. Finally, this study lacks information on BMI and body fat distribution that could potentially explain some of the differences between AD patients and controls.

However, through the longitudinal nature of these cohorts, we know that all the AD patients used for our analysis maintained the diagnosis of AD as did all controls for at least 3 years from their baseline visit. Additionally, our information on disease progression and brain atrophy provide us with more precise phenotypes that capture different stages of disease pathology including the early preclinical stages. Given the good performance of these models, we believe that enrichment with additional individuals and pathology information would increase their performance. Finally, although we did not have BMI information for our cohort, we observed no difference in statin use or serum lipids between AD cases and controls.

5. Conclusion

In conclusion, the findings of this study deepen our knowledge of AD disease mechanisms and emphasize the importance of investigating in detail different lipid fractions in dementia research. As it is not known whether the observed changes in lipid levels are causally related to or are just a marker of changes in lipoprotein dynamics and composition, studies that address causality are essential, as the success of targeting specific molecules and

identifying potentially causal pathways amenable to intervention is predicated on these molecules being on the causal pathway. Finally, integrating additional types of biological modalities such as protein, gene expression, and genotype information may increase the fit of these models and help us to understand more about the biological context in which these molecules operate.

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Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jalz.2016.08.003>.

RESEARCH IN CONTEXT

1. Systematic review: The authors reviewed the literature using PubMed and reported key publications. There is a pressing need to identify noninvasive Alzheimer's disease (AD) biomarkers, and blood metabolites are promising biomarkers that could aid early diagnosis and ultimately lead to the development of more effective interventions. Recent blood metabolomic studies have highlighted the role of lipid compounds in AD. However, most studies are small and relatively heterogeneous.
2. Interpretation: This study replicated previous associations between blood lipids and AD and reported novel associations between blood lipids and clinical AD diagnosis, the rate of cognitive and brain atrophy. These findings deepen our knowledge of AD disease mechanisms and suggest novel targets for future work.
3. Future directions: Results of this study could be complemented with protein and genetic data. Future studies should address whether these changes are causally related to AD or are just a marker of changes in lipoprotein dynamics and composition.

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