DOCTORAL THESIS

Aston University
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Polyunsaturated fatty acid oxidation in Alzheimer's disease.

Simon Smith

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Simon Andrew Smith

Doctor of Philosophy

ASTON UNIVERSITY

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Thesis Summary

Alzheimer's disease is a neurodegenerative disorder which has been characterised with genetic (apolipoproteins), protein (β-amyloid and tau) and lipid oxidation/metabolism alterations in its pathogenesis. In conjunction with the Dementia Research Group, Bristol University, investigation into genetic, protein and lipid oxidation in Alzheimer's disease was conducted. A large sample cohort using the double-blind criteria, along with various clinical and chemical data sets were used to improve the statistical analysis and therefore the strength of this particular study. Bristol University completed genetic and protein analysis with lipid oxidation assays performed at Aston University. Lipid oxidation is a complex process that creates various biomarkers, from transient intermediates, to short carbon chain products and cyclic ring structures. Quantification of these products was performed on lipid extracts of donated clinical diseased and non-diseased frontal and temporal brain regions, from the Brain Bank within Frenchay Hospital. The initial unoxidised fatty acids, first transient oxidation intermediates the conjugated dienes and lipid hydroperoxides, the endpoint aldehyde biomarkers and finally the cyclic isoprostanes and neuroprostanes were determined to investigate lipid oxidation in Alzheimer's. Antioxidant levels were also investigated to observe the effect of oxidation on the defence pathways. Assays utilised in this analysis included; fatty acid composition by GC-FID, conjugated diene levels by HPLC-UV and UV-spec, lipid hydroperoxide levels by FOX, aldehyde content by TBARs, antioxidant status by TEAC and finally isoprostane and neuroprostane quantification using a newly developed EI-MS method. This method involved the SIM of specific ions from F-ring isoprostane and neuroprostane fragmentation, which enabled EI-MS to be used for their quantification. Analyses demonstrated that there was no significant difference between control and Alzheimer samples across all the oxidation biomarkers for both brain regions. Antioxidants were the only marker that showed a clear variance; with Alzheimer samples having higher levels than the age matched controls. This unique finding is supported with the observed lower levels of lipid oxidation biomarkers in Alzheimer brain region samples. The increased antioxidant levels indicate protection against oxidation which may be a host response to counteract the oxidative pathways, but this requires further investigation. In terms of lipid oxidation, no definitive markers or target site for therapeutic intervention have been revealed. This study concludes that dietary supplementation of omega-3 fatty acids or antioxidants would most likely be ineffective against Alzheimer disease, although it may support improvement in other areas of general health.

Dedication

This thesis is dedicated to all my family and close friends who have supported me not only in this project but throughout my university life. Without these people I would not be where I am today.

Acknowledgements

Throughout my university life I have had the pleasure of meeting some great people but I have also made friends with a lot of different personalities who have provided a wide range of opinions and characteristics. In the outset I would like to acknowledge the role of Anuj Chander, Johnny Zhou, Tulpesh Patel and Stephen Farmer as the backbone of the research laboratory as they provided strength, knowledge and more importantly a working environment based on outstanding work ethics but with moments of great humour. Gratitude is also extended to my supervisor Dr Gareth Griffiths for allowing me to work on a very important project and being able to work with such amazing people.

My family play a huge role in the success of this thesis, by enabling me to pursue this qualification and being the foundation and emotional support required to conduct this work. There are so many people that deserve acknowledgement for either contribution to this project or just being someone for me to sound off at. For all these people I have one comment for you, if you can read this thesis and feel a sense of pride and satisfaction then I thank you and I owe you a great deed of gratitude and wish you every success in everything you do.

There is a moment in PhD projects where you begin to question everything, at this moment take stock and remember the destination to where you are travelling to which is the acquisition of your doctorate.

Contents

Tables and Figures

Abbreviations

AA – arachidonic acid (all *cis* $\Delta^{5, 8, 11, 14}$)

ABTS – 2,2'azino*bis*-3-ethylbenzothiazoline-6-sulphonic acid

AD – alzheimer's disease

AdA – adrenic acid (all *cis* $\Delta^{7, 10, 13, 16}$)

APOE – apolipoprotein E epilson

APP – amyloid precursor protein

Aβ – β-amyloid

BBB – blood brain barrier

BHT – butylated hydroxytoluene

CD – conjugated diene

CHP – cumene hydroperoxide

CK – choline kinase

CNS – central nervous system

CoA – coenzyme A

COX-2 – cyclooxygenase-2

CPT – choline phosphotransferase

CSF – cerebrospinal fluid

CT – cytidylytransferase

DAG – diacylglycerol

DHA – docosahexaenoic acid (all *cis* $\Delta^{4, 7, 10, 13, 16, 19}$)

diDHA – didocosahexaenoyl

ω-3 DPA – docosapentaenoic acid (all *cis* $\Delta^{7, 10, 13, 16, 19}$)

ω-6 DPA – docosapentaenoic acid (all *cis* $\Delta^{4, 7, 10, 13, 16}$)

EI-MS – electron ionisation mass spectrometry

ω-3 EPA – eicosapentaenoic acid (all *cis* $\Delta^{5, 8, 11, 14, 17}$)

ER – endoplasmic reticulum

FAME – fatty acid methyl ester

FOX – ferrous orange xylenoyl

FR – frontal

GC-MS – gas chromatography mass spectrometry

GC-NICI-MS – gas chromatography negative ion chemical ionisation mass spectrometry

HHE – hydroxyhexenal

HNE – hydroxynonenal

HNF-4α – hepatocyte nuclear factor 4 alpha

HPETE – hydroperoxyeicosatetraenoic acid

IL-1 – interleukin-1

IsoPs – isoprostanes

LA – linoleic acid (all *cis* $\Delta^{9, 12}$)

LC-MS – liquid chromatography mass spectrometry

ω-3 LNA – linolenic acid (all *cis* $\Delta^{9, 12, 15}$)

ω-6 LNA – linolenic acid (all *cis* $\Delta^{6, 9, 12}$)

LOOH – lipid hydroperoxide

LOX - lipoxygenase

LTs - leukotrienes

LxR – liver X receptor

LXs – lipoxins

LysoPC – lysophosphatidylcholine

MANOVA – multiple analysis of variance

MDA – malondialdehyde

NFTs – neurofibrillary tangles

NFκB – nuclear factor κB

NMDA – *N*-methyl-D-aspartate

NO – nitrous oxide

NPs – neuroprostanes

ONE – 4-oxo-2-nonenal

PAF – platelet activating factor

PC – phosphatidylcholine

PCOOH – phosphatidylcholine hydroperoxide

PDME - phosphatidyldimethylethanolamine

PE – phosphatidylethanolamine

PEOOH – phosphatidylethanolamine hydroperoxide

PFB-Br – pentafluorobenzyl bromide

PGs – prostaglandins

PI – phosphatidylinositol

PKC – protein kinase C

PLA_2 – phospholipase A_2

PMME – phosphatidylmonomethylethanolamine

PP – phytoprostane

PPAR – peroxisome proliferator activated receptor

PS – phosphatidylserine

PSD – phosphatidylserine decarboxylase

PUFAs – polyunsaturated fatty acids

ROS – reactive oxygen species

RP – reverse phase

RT – room temperature

RTP – room temperature and pressure

RxR – retinoid X receptor

SIM – selective ion monitoring

SPE – solid phase extraction

SREBP – sterol regulatory element binding proteins

TBARs – thiobarbituric acid residues

TEAC – total equivalent antioxidant capacity

TLC – thin layer chromatography

TMS – trimethylsilyl

TNF – tumour necrosis factor

TP – temporal

TxBs – thromboxanes

1 Introduction

1.1 - Alzheimer's disease

"To achieve this goal, all the choir need to sing from the same hymn sheet [1]"

In 1901, the German psychiatrist and neuropathologist Aloysius "Alois" Alzheimer first observed a 51-year-old patient, Auguste D., who was plagued by symptoms that did not fit any existing diagnosis. These symptoms included rapid failing memory, confusion, disorientation, trouble expressing her thoughts, progressive cognitive impairment, hallucinations, delusions and unfounded suspicions about her family and the hospital staff. His patient deterioated inexorably, stating to Dr. Alzheimer, "I have lost myself." Her subsequent death on April 8, 1906, allowed Alzheimer to identify β-amyloid (Aβ) plaques, neurofibrillary tangles (NFTs), and arteriosclerotic changes after *post-mortem* in her brain [2, 3]. At the 37th Assembly of the Society of Southwest German Psychiatrists in Tubingen 1906, Dr Alzheimer presented for the first time these observations about amyloid plaques, NFTs, and arteriosclerotic changes. The neuropathological hallmarks of what later was termed AD [4]. Alzheimer's disease (AD) is a neurodegenerative disorder that is normally diagnosed in the elderly. Due to longer life expectancy in these modern times, the older population is growing but this means that the risk of AD increases also. Recently there has been significant media and research attention, from the open talks by Sir Terry Pratchett regarding his personal management of the disorder to current news and radio broadcasts discussing a potential drug cure [5] and vitamin B supplementation [6]. However, to date, no definitive cure or medical intervention has been able to reduce significantly the effects of this mental disorder.

Figure 1 – Depiction of tissue damage observed in AD [2].

Healthy Brain

Advanced Alzheimer's Disease
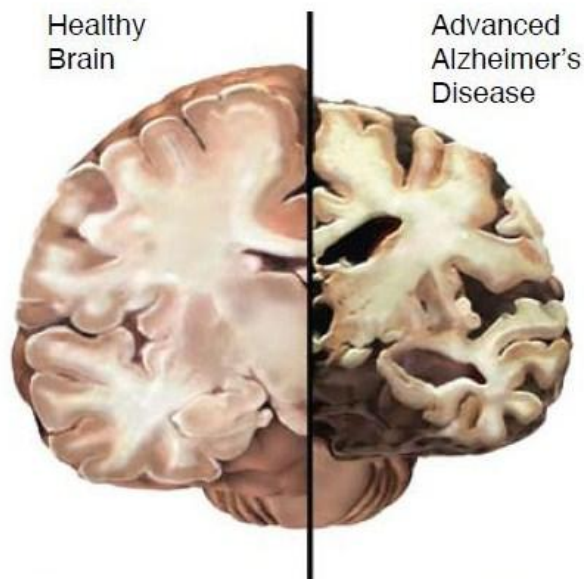
AD is a destructive neurodegenerative disease that causes trauma to the patient but also for the patient's friends and family. The image above shows a dramatic representation of the damage AD can inflict on brain size in comparison to a healthy, non-AD, brain.

Figure 2 – Timeline of AD pathogenesis [7].
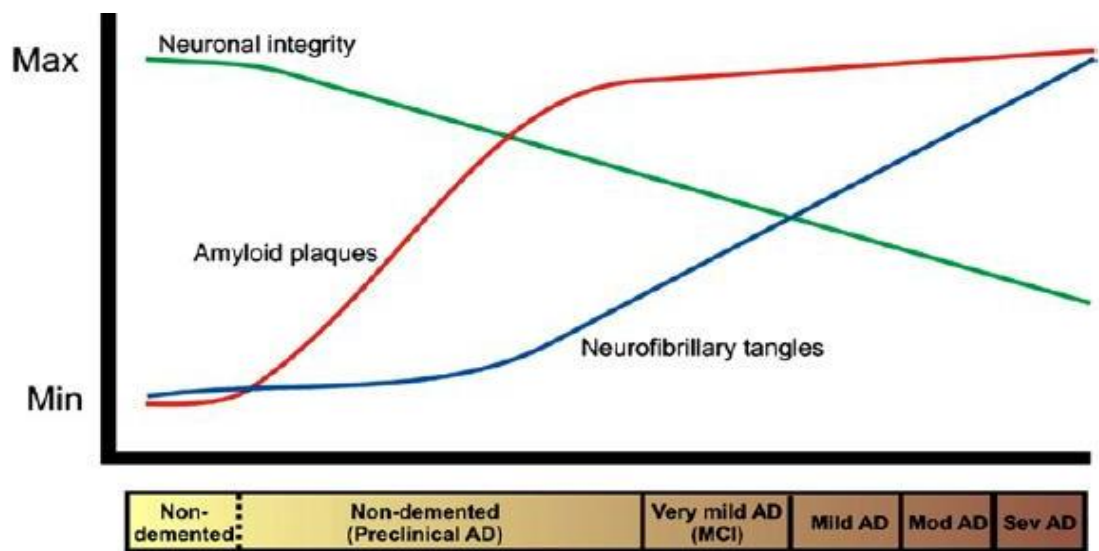
In the early stages of AD there are two main increases in amyloid plaques and NFTs as the process of AD evolves. Entwined with this is a marked reduction in neuronal integrity which is a consequence of Aβ and NFT formation.

1.1a - Symptoms and Statistical Prevalence

AD symptoms often appear as subtle problems in remembering minor events of everyday life, such as forgetfulness and difficulties recalling new names or recent conversations. However, these particulars are synonymous with other neurological disorders [8]. Early in the disease process, depression and anxiety occur with psychiatric symptoms which increase as the disease progresses [9]. Delusions, hallucinations and agitation occur within the middle to late stages [9] and during this later stage; profound dementia develops affecting multiple cognitive abilities and behavioural aspects. This involves memory loss, disorientation and impairment of judgement and reasoning [10], resulting in the patient being unaware of time and place, but more importantly at times being unable to identify close family members [8]. Death occurs, on average, 9 years after the initial clinical diagnosis and is usually caused by respiratory complications, such as aspiration pneumonia [8].

There are two general types of AD. Familial AD is an autosomal dominant disease associated with genes and their mutation. This type of AD is defined as being early-onset, linked to incorrect amyloid precursor protein (APP) production and presenilin-1 and 2 genes. The sporadic form of AD occurs in the majority of cases, termed late-onset which affects people over the age of 65 years. Risk factors for late-onset include the presence of apolipoprotein E Ɛ 4 isoform (ApoE Ɛ 4) and other environmental factors [detailed in section 1.1d (iii)] [11]. AD is the most prevalent form of dementia and significantly impacts patients, families, caregivers, communities, and society as a whole [2].

According to the 2003 World Health Report, dementia contributed to the most years lived with a disability in people aged 60 years and older, more than stroke, musculoskeletal disorders, cardiovascular disease, and all forms of cancer. In 2006, there were 26.6 million cases of AD in the world. By the year 2050, the worldwide prevalence of Alzheimer's will grow fourfold, to 106.8 million, with 1 in 85 persons living with AD [12]. Furthermore, it is estimated that at least 4 million people in the United States have been diagnosed with AD [13]. Approximately 200,000 diagnosed with early-onset AD (familial 4%), with the remaining cases being late-onset AD (sporadic 96%) [2]. In Europe the number of people with dementia may increase from 7.6 million prevalent cases in 2000 to 16.2 million in 2050 [14]. Finanically, in the United Kingdom (UK),

224 000 of the 461 000 elderly people with cognitive impairment live in institutions at a cost of £4.6 billion [3]. In the US, 5.1 million people were estimated to be living with AD in 2007 and $148 billion is spent each year to provide health care for these individuals [11]. Delaying disease onset by an average of 2 years would decrease the worldwide prevalence of AD by 22.8 million cases. Even a modest 1-year delay in disease onset would result in 11.8 million fewer cases worldwide [12]. Until methods of preventing AD are discovered, it is not only the tens of millions of disease victims and their families that will suffer, but also our nation, our healthcare system, and our economy as a whole [15].

1.1b - *Post-mortem* classification of disease state (Figure 3)

During *post-mortem*, AD brain samples are classified using a definitive scale known as Braak staging. There are six stages which are distinguishable from each other allowing for determination of disease progression, the higher the stage, the greater the AD severity.

Stage I. The most mildly affected cases show an involvement of the cortex confined to the transentorhinal region, which is involved in memory. The transentorhinal neurons generally are the first nerve cells in the brain to develop NFTs. Stage I is characterised by a modest number of these changes [16].

Stage II. The next stage is an aggravation of stage I and exhibits numerous NFTs in the transentorhinal region. Their density decreases slightly when approaching the proper entorhinal region. Stages I and II are characterised by the transentorhinal region being preferentially affected with only mild involvement of the hippocampus [16].

Stage III The characteristic feature of the third stage is a severe involvement of both the transentorhinal and entorhinal region. The isocortex remains virtually devoid of changes or is only mildly affected [16].

Stage IV. The fourth stage exhibits large numbers of tangles which are present in both the transentorhinal and entorhinal region with the subiculum, found in the hippocampus, only mildly involved. The key feature of stages III and IV is that both the entorhinal and transentorhinal regions are conspicuously affected and this is supplemented by a mild to

moderate hippocampal and low isocortical damage. Consequently being summarised as the "limbic stages" [16].

Stage V. The main feature of stage V is the damage to the isocortex region. In cases where the isocortex is severely affected, these areas tend to show the highest packing density of NFTs with virtually all isocortical areas being affected. A new component appearing in stage V is a brushwork of argyrophilic cellular processes covering the anteroventral nucleus [16].

Stage VI. In stage VI all the previous changes are even more pronounced. The large number of NFTs-bearing granule cells in the fascia dentata facilitates differentiation of stage V from stage VI. The obvious hallmark of stages V and VI is that the isocortex has been devastatingly affected by NFTs and plaques. The pathology includes, of course, all the changes noted in earlier stages. Stages V and VI are, therefore, denoted as "isocortical stages" [16].

1.1c - Hypotheses in relation to Alzheimer's disease

Many questions about pathogenesis of this disease still remain unanswered and therapeutic options are few. Therefore research and development of different novel strategies which target the mechanisms leading to dementia are constantly being investigated. In this context, it becomes essential to identify the molecular constituents and pathways involved in AD pathogenesis [17]. Generally, there are three common areas under investigation in AD which include oxidation, genetic control of protein synthesis and dietary risk factors.

Oxidative stress is caused by a relative increase in the ratio of free radicals to antioxidants [2] and has been implicated in multiple diseases, but importantly in the development of neurodegenerative diseases' including AD [18]. Oxidative stress results in the destruction and death of neurons in the brain, contributing to the progression of the disease [13, 19]. The brain areas demonstrating the highest levels of oxidative stress are typically the hippocampus, amygdala and parietal cortex [2]. Measuring this stress involves analytical methods determining the damage to nucleic acids, proteins, DNA and RNA, monitoring the levels of reactive oxygen species (ROS) [20], biomarkers [21] and finally compounds of lipid peroxidation [19].

Figure 3 – Schematic representation of Braak staging [22, 23]
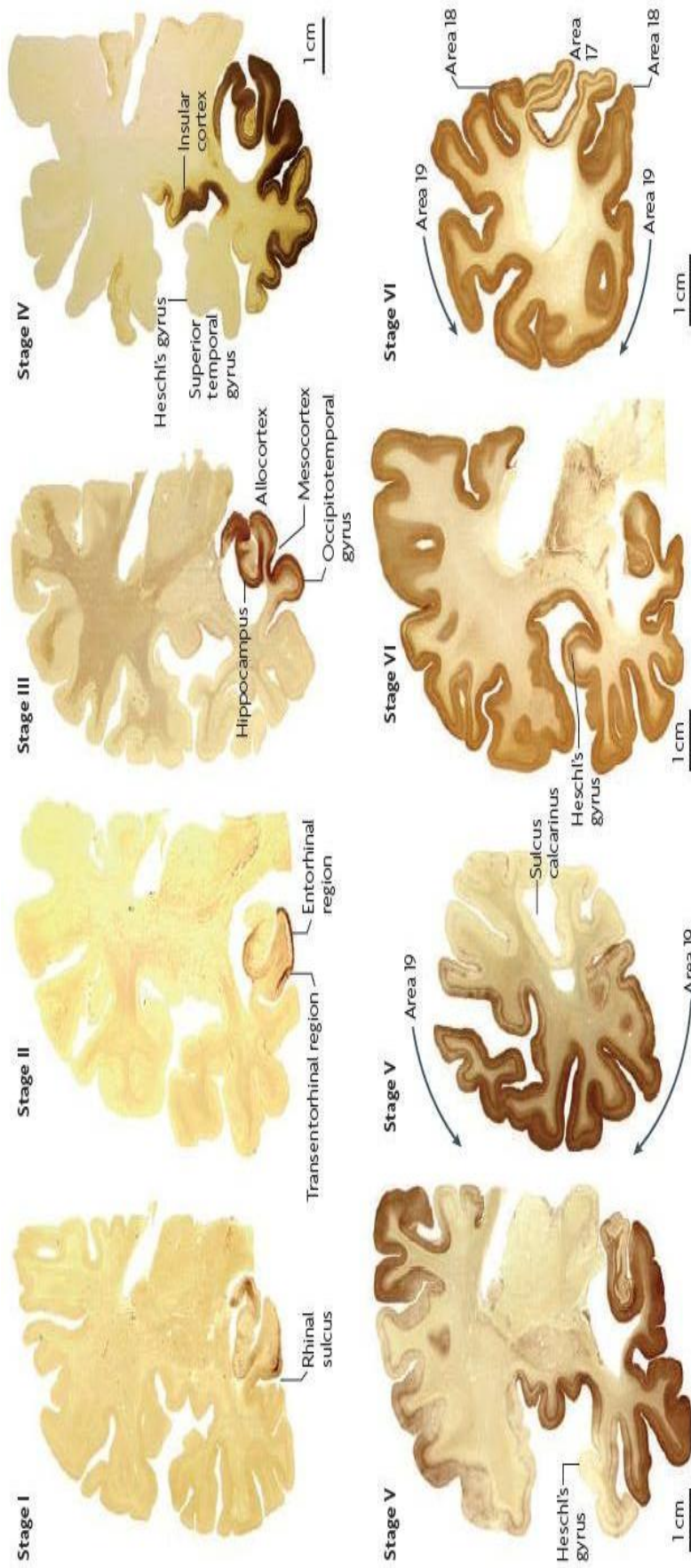
Braak staging is used to designate the extent of AD pathology in *post-mortem* brain samples. The image above demonstrates the differences between the stages using a immunostain for abnormal tau on 100μm polyethylene glycol-embedded hemisphere sections. In the early stages I and II damage to the transentorhinal region denotes the initiation of the disease state. Stages III and IV show severe damage to these regions and the beginning of further brain area damage, primarily to the isocortex. The last stages start to show devastating damage to many brain regions in association with severe AD, as seen by the darker staining and reduction in size.

AD is also associated with two major protein synthesis pathological traits. First, there is an overproduction and accumulation of Aβ peptide produced by the proteolytic processing of APP. Aβ then folds incorrectly and aggregates to form senile plaques. The biological role of APP is undefined; however this protein has been linked with cell growth, cell adhesion and metal ion homeostasis but in AD the processing of APP becomes disregulated and produces Aβ [24]. Secondly, there is the formation of NFTs caused by the aggregation of phosphorylated tau protein within neurons resulting in neuronal cell loss [11, 25]. Tau proteins provide the scaffolding matrix for neurite growth and the conversion to phosphorylated tangles begins to twist the neurite structure affecting neuronal activity. Individuals with genetic alterations in the genes that code for three transmembrane proteins; APP, presenilin-1 and presenilin-2 all deposit large amounts of the amyloid fragment Aβ in the brain and develop AD [26-28]. Numerous studies and theories are covered in the literature regarding Aβ and tau in the pathogenesis of AD [15, 17, 29-32].

Inheritance of APOE Ɛ 4 allele is a major risk factor for late-onset AD. APOE proteins belong to the family of plasma lipid-binding proteins involved in triacylglycerol and cholesterol transport and delivery, but also contribute to the clearance of Aβ through binding to lipoprotein receptors [17, 33, 34]. APOE is a 299-amino acid protein that exists as three common isoforms. APOE Ɛ 2, APOE Ɛ 3, and APOE Ɛ 4 are encoded by alleles, which are a form of gene (similar to blood typing where the alleles are defined as whether you are AO, AA etc) and the more alleles present, the greater the risk of AD onset [35]. In addition to the genetic association between APOE and susceptibility to AD, immunoreactive APOE can be observed within the NFTs and Aβ proteins in the senile plaques in the brains of AD patients [35].

Besides age, gender, education level and social activities, dietary parameters represent common risk factors for neurodegenerative and cardiovascular diseases. This leads to the hypothesis that nutrition could delay the onset of AD or at least slow its progression [2, 17, 36, 37]. Higher intake of vitamin C, vitamin E, flavonoids, unsaturated fatty acids, fish oil, vitamin B12 and folate; along with lower saturated fat intake have been related to a lower risk for AD or slower cognitive decline [34, 38]. While genetics play a major role in the risk of familial AD, the majority of AD is late onset and appears to involve the interplay of multiple risk factors. The Mediterranean diet which is high in vegetables, fruits, nuts, cereals, fish, and olive oil are low in saturated fats and is linked to reducing

the risk of AD onset [39]. Most observational studies report an association of high fish intake, rich in omega-3 fatty acids, with better cognition or lower risk of dementia [40]. People who consume 1 or more fish meals per week have 60% less risk of AD than people who reported eating fish rarely or never [41].

1.2 – Fatty acids

"For one simple molecule (DHA) to affect so many seemingly unrelated processes, it must function at a fundamental level common to most cells, such as transcription events, membrane structure and functions, and/or signal transduction [42]"

A simple description of a fatty acid is a hydrocarbon chain with a carboxyl group at one end which provides a means of attachment and polarity, whilst at the other a methyl group creates a hydrophobic environment. PUFAs are fatty acids which contain two or more double bonds within their hydrocarbon chain [43, 44]. Polyunsaturates have two naming systems which are used to differentiate between multiple bond systems. One is the delta and the other omega. The delta nomemclature is used to attribute the position of the double bond from the carboxyl end group. The omega classification system uses the position of the double bond from the methyl end to denote the class. For example, α-linolenic acid (LNA; eighteen carbons with three double bonds) using the delta system is presented as $\Delta^{9, 12, 15}$ as the double bonds are positioned on C-9, 12, 15 respectively. Using the omega system the same α-linolenic acid is called omega-3 as the last double bond in the fatty acid is three carbons from the terminal methyl end.

1.2a – Fatty acid synthesis

Synthesis of fatty acids occurs in mammals through systematic addition of smaller chain building blocks to an intermediate in order to form longer chains. The intermediate required is malonyl-CoA, a three carbon chain, which is made from acetyl-CoA (a two carbon fatty acid) in an enzymatic reaction by acetyl-CoA carboxylase [45]. Both acetyl and malonyl groups need to be activated by coenzyme A (CoA) so that these components can bind with the fatty acid synthase enzyme where fatty acid synthesis occurs. Acetyl-CoA and malonyl-CoA are transferred to the ACP and ACP track sites of the synthase enzyme by transacylase activity. These two esterified ACP components on the fatty acid synthesis enzyme enables for the initial stage of fatty acid synthesis to start, a process involving addition and condensation. Malonyl-ACP and acetyl-ACP groups combine forming a four carbon chain carbonyl, accompanied with the release of carbon dioxide from the reaction [45].

Figure 4 – Fatty acid structures
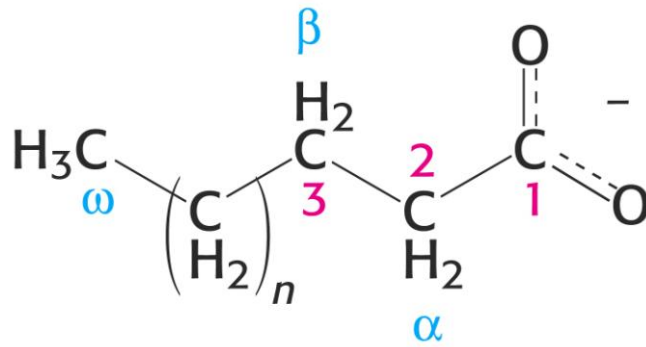
Linoleic acid (LA; $\Delta^{9, 12}$ or $\omega^{6, 9}$)
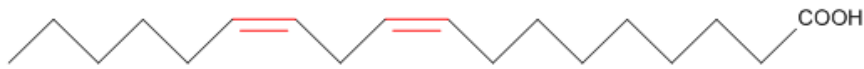
α-Linolenic acid (α–LNA; $\Delta^{9, 12, 15}$ or $\omega^{3, 6, 9}$)

γ-Linolenic acid (γ–LNA; $\Delta^{6, 9, 12}$ or $\omega^{6, 9, 12}$)
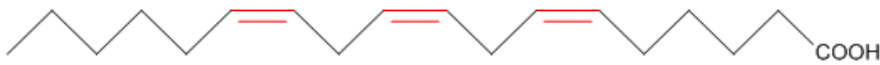
Arachidonic acid (AA; $\Delta^{5, 8, 11, 14}$ or $\omega^{6, 9, 12, 15}$)

Eicosapentaenoic acid (EPA; $\Delta^{5, 8, 11, 14, 17}$ or $\omega^{3, 6, 9, 12, 15}$)

Docosahexaenoic acid (DHA; $\Delta^{4, 7, 10, 13, 16, 19}$ or $\omega^{3, 6, 9, 12, 15, 18}$)

These three images above are all examples of fatty acids. The first image shows a simple saturated fatty acid with no double bonds in its structure and a methyl and carboxyl group on either end, where n can represent any amount of carbon chain units. As stated in the text, unsaturated fatty acids are defined as being omega-3 or omega-6, denoted by the position of the nearest double bond to the terminal methyl group in the second picture. The final picture shows the structure of some PUFAs, linoleic acid (18:2), α- and γ-linolenic acids (LNA), arachidonic acid (AA), eicosapentaenoic acid (EPA) and finally docosahexaenoic acid (DHA).

Figure 5 – *De novo* fatty acid synthesis [45].
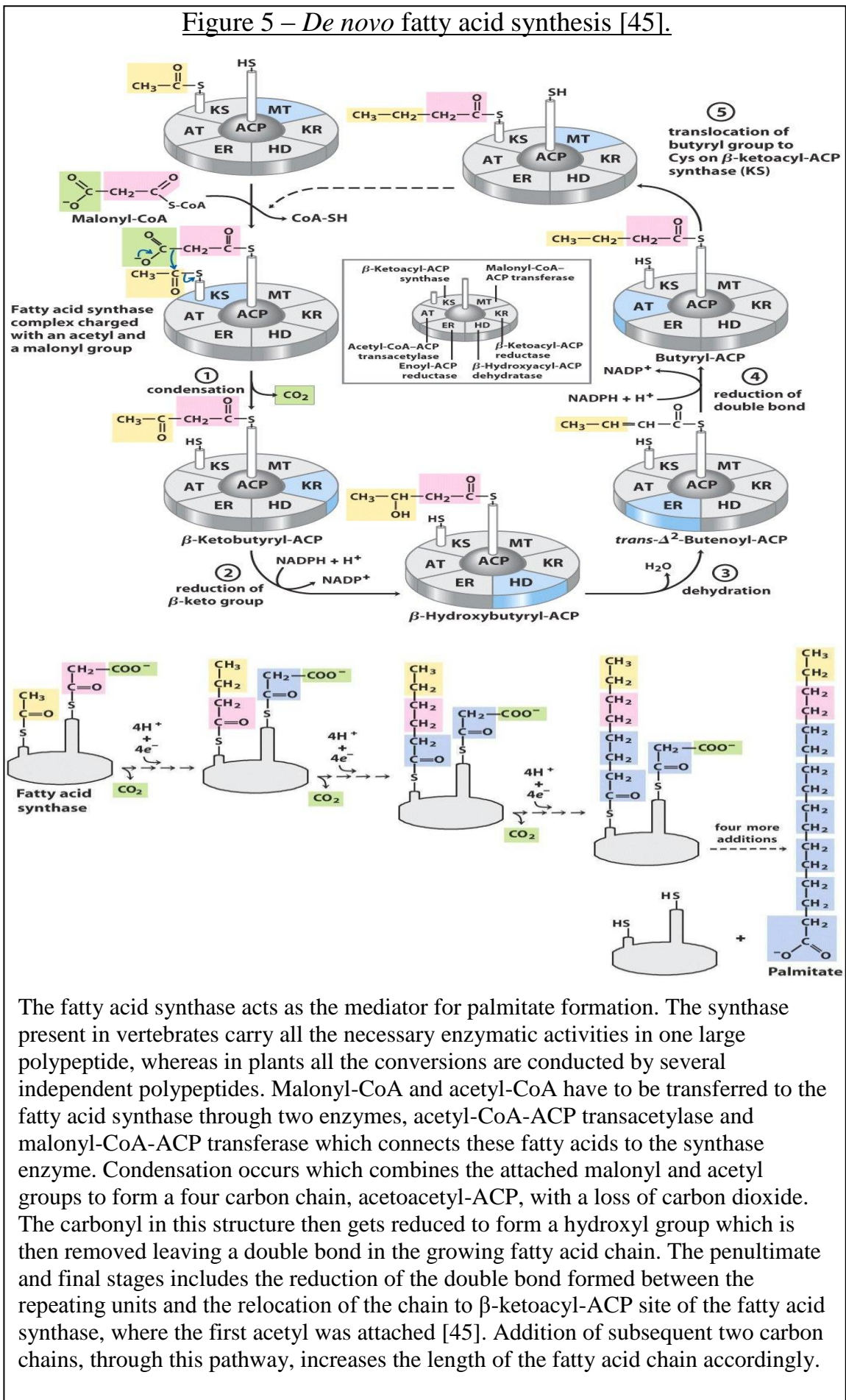
The fatty acid synthase acts as the mediator for palmitate formation. The synthase present in vertebrates carry all the necessary enzymatic activities in one large polypeptide, whereas in plants all the conversions are conducted by several independent polypeptides. Malonyl-CoA and acetyl-CoA have to be transferred to the fatty acid synthase through two enzymes, acetyl-CoA-ACP transacetylase and malonyl-CoA-ACP transferase which connects these fatty acids to the synthase enzyme. Condensation occurs which combines the attached malonyl and acetyl groups to form a four carbon chain, acetoacetyl-ACP, with a loss of carbon dioxide. The carbonyl in this structure then gets reduced to form a hydroxyl group which is then removed leaving a double bond in the growing fatty acid chain. The penultimate and final stages includes the reduction of the double bond formed between the repeating units and the relocation of the chain to β-ketoacyl-ACP site of the fatty acid synthase, where the first acetyl was attached [45]. Addition of subsequent two carbon chains, through this pathway, increases the length of the fatty acid chain accordingly.

The synthesised carbonyl is then reduced to form a hydroxyl group, which in turn is then dehydrated to form a four chain fatty acid with a double bond. The double bond is then reduced to create a saturated four carbon activated fatty acid. The newly synthesised four carbon fatty acid is on one site of ACP and addition of another malonyl-CoA occurs at the second position; thus initiating the above process which continues to add two carbon units. This process of *de novo* fatty acid synthesis is depicted in figure 5. When this growing chain reaches sixteen carbons long (palmitic acid), no more carbons can be added by this mammalian system. Longer chains have to be created by elongase enzymatic activity, which is separate to the fatty acid synthase, in order to generate stearic acid (18:0) from palmitic acid (16:0).

Double bonds can be introduced by another set of enzymes which are called desaturases, generating unsaturated fatty acids. Desaturases insert double bonds into fatty acids by removal of protons from the acyl chain to molecular oxygen. This process requires NADH as a co-reductant in which electrons are shuttled through cytochrome b5 and the b5 reductase onto the terminal desaturase which ultimately converts molecular oxygen to water. Both CoA and phospholipid linked fatty acids can act as substrates for destaurases [46] .It must be noted that humans do not possess all types of desaturases, PUFAs like linoleic acid (LA), (omega-6) and α-LNA (omega-3) are classes as essential fatty acids. These are obtained through diet as they cannot be synthesised in mammals by the *de novo* pathway detailed above. This is because mammals lack the desaturase enzymes that insert double bonds after C-9; and consequently these lipids are defined as essential fatty acids. Major sources of omega-6 fatty acids include vegetable oils such as corn, sunflower and soybean oil, whereas omega-3 fatty acid sources are for example fish, such as salmon, trout and tuna [17, 42, 47, 48].

1.2b – Metabolism of essential fatty acids

DHA and eicosapentaenoic acid (EPA) are the most important products of omega-3 PUFA metabolism, with AA and docosapentaenoic acid (ω-6 DPA) the equivalent omega-6 products [11, 49, 50]. Neurons in the brain lack the enzymes necessary for *de novo* PUFA synthesis, of DHA and AA. These fatty acids are derived either from the diet, or synthesised from two dietary precursors. The liver is considered to be the primary site for biosynthesis of these fatty acids, which are delivered to the brain through subsequent secretion into the circulating blood stream. To synthesise AA, the initial

precursor is ω-6 LA. This fatty acid is desaturated, introducing another double bond at Δ6 enzymatically, to form γ-LNA ($\Delta^{6, 9, 12}$ – ω-6, 18:3). A elongase enzyme converts ω-6 γ-LNA to dihomo-γ-linolenic acid ($\Delta^{8, 11, 14}$ – ω-6, 20:3) and then a Δ5-desaturase adds another double bond creating AA ($\Delta^{5, 8, 11, 14}$ – ω-6, 20:4). This key intermediate can be enzymatically converted into prostaglandins (PGs), thromboxanes (TxBs), leukotrienes (LTs) and lipoxins (LXs) which all have inherent physiological, pharmacological and biological activities [51-53]. Elongation converts AA to adrenic acid (AdA; $\Delta^{7, 10, 13, 16}$ – ω-6, 22:4), then another elongase adds two more carbons making tetracosatetraenoic acid ($\Delta^{9, 12, 15, 18}$ – ω-6, 24:4). Insertion of a double bond at C-6 by a Δ6-desaturase synthesises tetracosapentaenoic acid ($\Delta^{6, 9, 12, 15, 18}$ – ω-6, 24:5), then β-oxidation removes two carbons forming DPA ($\Delta^{4, 7, 11, 13, 16}$ – ω-6, 22:5) [48, 54-56].

It is well established that DHA can be biosynthesised from ω-3 α-LNA, through the same processes detailed above. Starting with ω-3 α-LNA, it is desaturated to 18:4 ($\Delta^{6, 9, 12, 15}$- ω-3, 18:4) by a Δ6-desaturase. Chain elongation creates 20:4 ($\Delta^{8, 11, 14, 17}$- ω-3, 20:4), which is then converted to ω-3 EPA by a Δ5-desaturase in the endoplasmic reticulum (ER). In mammals, ω-3 EPA is twice elongated to 24:5 ($\Delta^{9, 12, 15, 18, 21}$- ω-3, 24:5) *via* ω-3 DPA. A double bond is introduced by a Δ6-desaturase to produce 24:6 ($\Delta^{6, 9, 12, 15, 18, 21}$- ω-3, 24:6). Subsequently, ω-3 24:6 is transferred to peroxisomes and converted to DHA by removal of two carbon atoms by β-oxidation. The synthesised DHA is transferred back to the ER and quickly incorporated into phospholipids [46, 57-60]. It has been suggested that the rate limiting step in the formation of DHA from α-LNA is the final step, involving peroxisomal oxidation of 24:6 omega-3 to DHA [61].

Due to its degree of unsaturation, DHA can undergo rapid inter-conversions between many torsional states. These conversions happen because of the rotation of the methylene carbon groups situated between the double bonds [42]. This chain flexibility allows for changes to the bilayer properties and also the short-range interactions with other membrane components, which can subsequently affect the function of integral membrane proteins [62]. The apparent structural simplicity of DHA belies its biological versatility. DHA membranes are actually quite thin and leaky [63]. Data from a series of model membranes with increasing levels of lipids containing unsaturated fatty acids, predicted that DHA behaves as if it is actually shorter than the 18-carbon fatty acid oleic acid (18:1) [63].

Figure 6 – Sprecker pathway [48]

Linoleic acid
18:2 n-6   α-linolenic acid
18:3 n-3

← **delta-6-desaturase** →

γ-linoleic acid
18:3 n-6   Stearidonic acid
18:4 n-3

← **Elongase** →

Eicosanoids:
Prostaglandins
(2-series)
Thromboxanes
(2-series)
Leukotrienes
(4-series)
Epoxyeicosatri-
enoic derivates
Hydroxyleicosatetra-
enoic derivates
Lipoxins

Dihomo–γ-linoleic acid
20:3 n-6   Eicosatetraenoic acid
20:4 n-3

← **delta-5-desaturase** →

Arachidonic acid
20:4 n-6   Eicosapentaenoic acid
20:5 n-3

Eicosanoids:
Prostaglandins
(3-series)
Thromboxanes
(3-series)
Leukotrienes
(5-series)
Resolvins

← **Elongase** →

Docosatetraenoic acid
22:4 n-6   Docosapentaenoic acid
22:5 n-3

← **Elongase** →

Tetracosatetraenoic acid
24:4 n-6   Tetracosapentaenoic acid
24:5 n-3

← **delta-6-desaturase** →

Tetracosapentaenoic acid
24:5 n-6   Tetracosahexaenoic acid
24:6 n-3

← **β-oxidation** →

Docosapentaenoic acid
22:5 n-6   Docosahexaenoic acid
22:6 n-3

The Sprecker pathway denotes the synthesis longer chain PUFAs from two essential fatty acids, ω-6 LA and ω-3 α-LNA from dietary intake. Both precursors are converted following the same enzymatic processes, yet yield different end products. Starting with ω-6 LA, $\Delta6$ desaturase activity introduces a double bond at $\Delta6$ of ω-6 LA, creating ω-6 γ-LNA ($\Delta^{6, 9, 12}$). Elongase activity adds two carbon atoms to ω-6 γ-LNA, making ω-6 dihomo-γ-LNA ($\Delta^{8, 11, 14}$), which has a double bond inserted at $\Delta5$ by a $\Delta5$-desaturase resulting in the formation of AA. This fatty acid can be converted to potent lipid mediators by cyclooxygenase and lipoxygenase activity after phospholipase release, when required. Continuing with the pathway, AA is elongated twice more, first to form ω-6 AdA ($\Delta^{7, 10, 13, 16}$ – 22:4) then onto tetracosatetraenoic acid ($\Delta^{9, 12, 15, 18}$ – ω-6 24:4). Introduction of another double bond at $\Delta6$ by $\Delta6$ desaturase activity yields tetracosapentaenoic acid ($\Delta^{6, 9, 12, 15, 18}$ – ω-6 24:5) before a final β-oxidation step removes two carbons creating ω-6 DPA ($\Delta^{4, 7, 10, 13, 16}$). In the ω-3 pathway, the precursor is ω-3 α-LNA. A double bond is inserted at $\Delta6$ by a desaturase forming stearidonic acid ($\Delta^{6, 9, 12, 15}$ – ω-3 18:4), then an elongase enzyme adds two carbons creating eicosatetraenoic acid ($\Delta^{8, 11, 14, 17}$ – ω-3). $\Delta5$ desaturase activity puts a double bond at $\Delta5$ creating ω-3 EPA, which can be converted to metabolites but are less potent than the previous AA metabolites. Double elongation steps on ω-3 EPA produce ω-3 DPA ($\Delta^{7, 10, 13, 16, 19}$) first, which has different bond positions to ω-6 DPA, then into tetracosapentaenoic acid ($\Delta^{9, 12, 15, 18, 21}$ – ω-3 24:5). A final double bond is inserted at $\Delta6$ by a desaturase to make tetracosahexaenoic acid ($\Delta^{6, 9, 12, 15, 18, 21}$ – ω-3 24:6) before β-oxidation removes two carbons yielding the main product ω-3 synthesis product, DHA.

The flexible chain of DHA results in rapid inter-conversion between conformational states that range from extended towards the inner layer of the membrane, to bent conformers where the terminal methyl approaches the outer layer. The fluctuating surface of a PUFA chain compared to the rigid moiety of a neighbouring cholesterol molecule deters close approach and creates lipid rafts within the membrane. These rafts comprise of saturated lipid classes including cholesterol, sphingolipids and ceramides which are depicted as isolated elements, or rafts in the membrane and are not as dynamic as PUFAs. The flexibility of the PUFA chain accounts for the high "fluidity", permeability, elasticity, fusion, enhanced flip-flop and preference for non-lamellar phases observed in specialised neural membranes [63].

To maintain basal DHA levels in the brain, $\Delta 5$ and $\Delta 6$ desaturase activities are only induced in the absence of the precursors (LA and α-LNA). Despite a strong capacity of the central nervous system (CNS) to retain its DHA, an animal study revealed that the dietary intake of DHA can significantly alter its levels in the brain [64]. Generally, it is difficult to deplete DHA from the neural membranes of adult mammals even with a diet low in this fatty acid, presumably because of preferential uptake of it to support the basal turnover in the brain [57]. Unlike other tissues, the brain uptake needs to overcome the blood–brain barrier (BBB), and the transport mechanisms involved for DHA uptake through this barrier are not understood [42]. Studies with whole animals as well as with *in vitro* systems, revealed that DHA and other long chain PUFAs are transported across the BBB more efficiently when they are esterified to lysophosphatidylcholine (lysoPC) [65]. Upon its entry, DHA is subsequently released and then activated by an acyl-CoA synthetase. This newly formed docosahexaenoyl-CoA can be esterified *via* an acyl-CoA transferase to the stereospecifically numbered *sn*-2 position of phospholipids [66]. However, as humans age, the level of PUFAs in the brain decreases and this observation may be attributed to (a) poor passage rate *via* the BBB, or (b) decreased rate of incorporation into the membrane, or (c) a decrease in the activities of $\Delta 6$ and $\Delta 9$ desaturase enzymes [67].

1.2c – Putative functions of DHA within the brain

The fatty acid composition of biological membranes significantly influences physical properties of biological membranes. This results in higher or lower membrane fluidity and modulated activity of membrane-associated proteins, as well as vesicle formation

and fusion [17, 48]. DHA can also significantly alter phase behaviour, elastic compressibility, ion permeability and rapid flip-flop of cell membranes. The degree of unsaturation, makes DHA sterically incompatible with saturated sphingolipid and cholesterol classes and, therefore, is capable of altering lipid raft behaviour where these components reside [68]. DHA and AA containing phospholipids have been proposed to play a special role in biomembranes including neurons. This fact is due to their favourable biophysical properties such as high motility and motional freedom [59, 69-71].

(i) Effect on membrane bound enzyme activity and ion channels; DHA can modulate cyclooxygenase-2 (COX-2) activity and eicosanoid production, by replacing AA in the phospholipids, reducing the availability of this precursor for these metabolites. DHA can exhibit a neuroprotective effect. This is supported by evidence that calcium independent phospholipase A_2 (PLA_2) inhibition, increased neuronal death by reactive oxygen species (ROS) [72]. Through *N*-methyl-D-aspartate (NMDA) responses and blocking K^+ channels, DHA can increase the excitability of neurons; but can also stabilise neuronal membranes by suppressing voltage-gated Ca^{2+} currents and Na^+ channels [50, 65, 70, 73, 74].

(ii) Modifications to receptors and neurotransmitters; Reduction in DHA increases dopamine concentrations and reduce its binding to D_2-receptors, which can be reversed through DHA supplementation [73]. Within the brain, DHA acts as a secondary messenger *via* coupling to neuroreceptors. It is also converted, by enzymatic lipoxygenation, to a variety of signalling molecules known as neuroprotectins (dihydroxyl docosanoid) and resolvins (trihydroxyl docosanoid), which have potent anti-inflammatory properties and are involved in the resolution of inflammation [66, 70].

(iii) Signal transduction; By changing the organisation and/or composition of the lipid rafts, DHA can modify many signalling pathways [42, 67]. Omega-3 FAs decrease fatty acid synthesis *via* inhibition of sterol regulatory element-binding proteins (SREBP) [75], which are involved in cholesterol and fatty acid synthesis. They can also increase lipid oxidation through activation of peroxisome proliferator-activated receptor (PPAR) [48]. DHA is a physiological inhibitor of glutamate transport at the synapse, playing a major role in the regulation of synaptic efficacy. This action of DHA in glutamate synaptic transmission may explain, in part, the memory deficit associated with omega-3 PUFA

deficiency [76]. In a series of seminal findings, it was demonstrated that DHA acts as an inhibitor of various receptors, suppressing nuclear factor-κB (NF-κB) related cell death signal transduction [48, 68]. Phosphatidylethanolamine (PE) containing DHA has the propensity to form nonbilayer phases, which allow binding of G proteins to occur, suggesting a role in G protein-coupled signaling pathways [59, 69].

(iv) Gene modification; DHA alters the transcription of the gene encoding the Aβ scavenger, transthyretin, which could reduce Aβ plaque formation [17, 42, 50]. Docosahexaenoyl-CoA is a hepatocyte nuclear factor (HNF-4α) ligand, and HNF-4α is involved with the transcription of DNA homodimers. Free unesterified DHA is a ligand for PPARs, a gene linked to lipid metabolism [66, 77]. It can also directly bind to lipid-related transcription factors including forms of liver X receptor and retinoid X receptor (RxR) [15, 50].

(v) Promotion of nonapoptotic pathways; DHA enrichment has been shown to promote phosphatidylserine (PS) accumulation in membranes, which enhances Raf-1, a kinase involved in multiple signal transduction pathways [78], and through the modulation of the phosphatidylinositol (PI) 3-kinase/Akt signalling cascade. This leads to a suppression of caspase-3 activation and cell death. In neuronal cells DHA can be replaced by ω-6 DPA in omega-3 deficiency. This replacement results in lower promotion of PS clustering and Akt translocation and does not aid cell survival [15, 48, 66, 79-81]. The enrichment of membranes in DHA has thus proven crucial in maintaining sufficient rates of these phosphorylated/active proteins, whose associated survival pathways are promoted in neurons [42].

(vi) Neurite growth; DHA can increase both maximum neurite length and the total number of neurites through activating syntaxin-3, a synaptic membrane protein that promotes neurite outgrowth [59, 70, 81, 82], which may be associated with a determinant neurotrophic role of this PUFA in the maturation of the CNS [42].

(vii) Inflammatory processes; Omega-3 fatty acid supplementation leads to a decrease in lymphocyte proliferation in humans and rats, a decrease in interleukin-1 (IL-1) production, and a decrease in IL-2 production [67, 68]. In addition, omega-3 decreases proinflammatory tumour necrosis factor (TNF) alpha production in humans [67, 68]. Bioactive DHA products, such as neuroprotectins and resolvins, possess potent anti-inflammatory, immunoregulatory, and neuroprotective actions by negating the pro-

inflammatory mediators formed from omega-6 PUFAs especially AA, defined as the resolution of inflammation [72].

1.3 - Phospholipids

Cell membranes are composed of a wide range of molecular species of phospholipids determined by the acyl composition and distribution at the sn-1 and sn-2 positions. Membranes vary widely in their phosphoipid composition and show an asymmetric distribution even within a single memebrane [48, 67, 83]. Within biological membranes phospholipids tend to form bilayer structures and are highly laterally mobile, while flip-flopping i.e. movement from one side to the other is rare. The fluidity of membranes is dependent, in part atleast, to the acyl compositionin which highly unsaturated fatty acids tend to lead to increased fluidity and high levels of saturation favour rigidity due to closer packing of the chains. Bilayers are held together by various hydrophobic, coulombic, van der Waal and hydrogen bond interactions between lipids and lipid – protein interactions. Membrane fluidity can be regulated by activation of desaturases which are often temperature dependent [75].

Phospholipids are made up of several distinct classes with different polar head groups, including phosphatidylcholine (PC), PE, and PS. Each phospholipid class consists of a different combination of fatty acids, that can vary in chain length and degree of unsaturation at the *sn*-1 and *sn*-2 positions on the glycerol backbone, figures 7 and 8 [84]. In adult brain, phospholipids account for 20–25% of the dry weight and 50-60% of neural membranes [85-88]. The two major phospholipid components in the brain are PE and PC [89]. The distribution of phospholipids is normally asymmetric across the plane of the membrane, with PE and PS concentrated in the inner layer and PC and sphingomyelin concentrated in the outer layer of the bilayer. When PC, PE, and PS are mixed, the order parameters of *sn*-1 hydrocarbon chains of all three lipids are generally similar, indicating that the lipids mix more or less homogeneously [90]. PC tends to form bilayers with little curvature, while PE imposes a negative curvature on these lipid bilayers [60]. These bilayers are penetrated to varying degree by receptors, enzymes, and ion channels. These proteins can protrude differentially through the membrane or are localised predominantly on the intracellular or extracellular membrane surface [83, 86, 91].

Figure 7 – General depiction of a phospholipid [45]

Glycerophospholipid (general structure)

Saturated fatty acid (e.g., palmitic acid)

$^1CH_2-O-C$

$^2CH-O-C$

$^3CH_2-O-P-O-$ X

Head-group substituent

Unsaturated fatty acid (e.g., oleic acid)
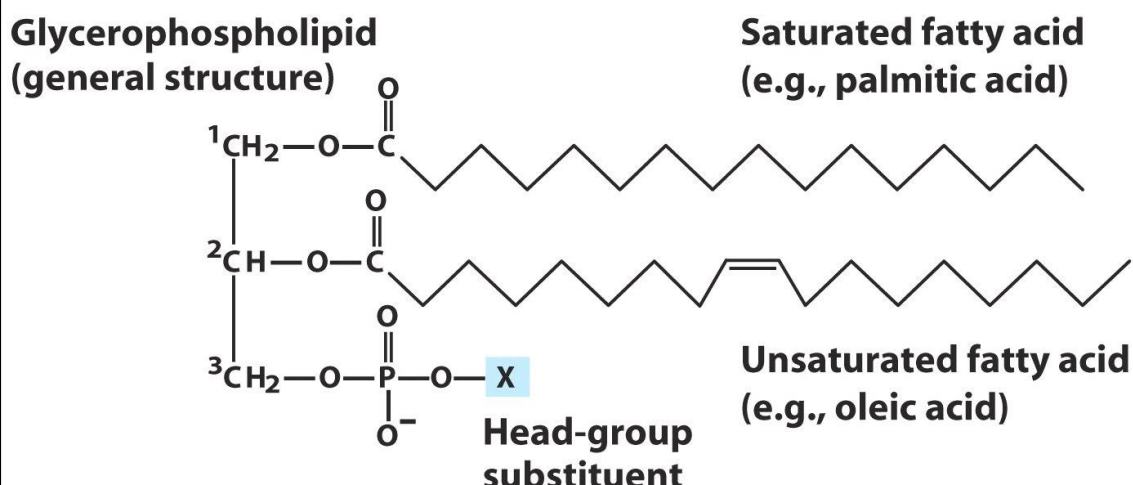
Phospholipids consist of a glycerol backbone with two fatty acids and a phosphate group where various headgroups can be attached. These positions are labelled sn-1, sn-2 and sn-3.

Figure 8 – Several different phospholipids [92]

Phosphatidyl serine

Phosphatidyl choline

Phosphatidyl ethanolamine

Phosphatidyl inositol
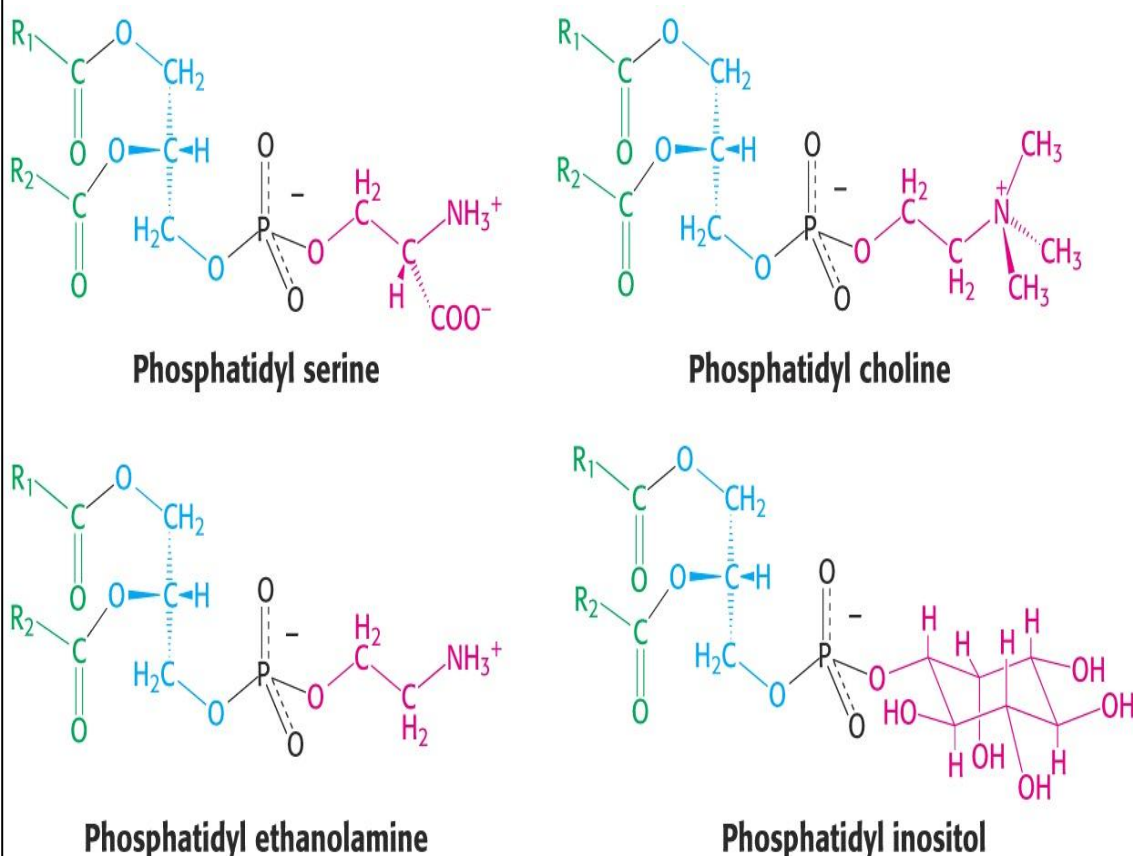
These are the major phospholipids observed across biological samples. The choline and ethanolamine headgroups are similar in structure with the only difference being the three methyl groups on the nitrogen in the choline moiety. The inositol head group comprises a sugar unit whilst the amino acid serine is the head group in PS.

In phospholipids saturated fatty acids preferentially located at the *sn-1* and have a role in neuroplasticity, while unsaturated fatty acids are located at the *sn-2* are associated with the generation of second messengers [43, 49, 63, 89, 93]. Such *sn-2* PUFAs are also prone to oxidation which can modify the properties of biological membranes since they behave more like saturated fatty acids. Removal of these oxidised fatty acids and replacing them with non-oxidised PUFAs is important to maintain membrane function and a suite of enzymes is involved in this process which includes phospholipase A_2 and an acyltransferase. in a mechanism known as the Lands cycle. [94].

1.3a - Synthesis of phospholipids

Synthesis of phospholipids occurs at the ER. The newly synthesised phospholipids self-assemble into thermodynamically stable bilayers. These layers form vesicles that detach from the ER and travel to other sites to resupply the membranous structures [86]. The pathways of phospholipid synthesis and metabolism are highly interrelated, and one phospholipid can be converted into another relatively simply [91]. Phospholipids can be made by two strategic approaches, one by CDP-diacylglycerol and the other CDP-headgroup. The CDP-diacylglycerol mechanism involves an activated fatty acid esterfied glycerol backbone, which is converted to a phospholipid by reaction with a headgroup. The CDP-headgroup is the opposite of the previous pathway; an activated headgroup binds with a diacylglycerol molecule, but still yields phospholipids as products. The biosyntheses of phospholipids involve multiple enzymatic steps and variations in the levels of the substrates and the activities of the rate-limiting enzymes themselves, are generally the main factors controlling the rates at which phospholipids are synthesised.

1.4 - Oxidation

"While the use of more than one measurement of lipid peroxidation is important, since no single lipid peroxidation index is completely satisfactory, caution must be taken in interpreting the results [95]"

Oxidative stress occurs due to an imbalance between the pro-oxidant and antioxidant systems. Among these targets, the breakdown of lipids is damaging because the formation of lipid peroxidation products leads to an uncontrollable spread of free radical reactions [60]. Oxidative damage to phospholipid acyl chains causes rearrangement of double bonds, chain shortening, and the introduction of a variety of polar group moieties into the hydrophobic core of the bilayer. As a consequence, structural characteristics and

dynamics of lipid bilayers can be affected, leading to impairment of integral enzyme functions [60, 96-98]. Many studies have shown that free radical damage and lipid peroxidation increase as a function of the degree of unsaturation of the fatty acids present. In this regard it has been demonstrated that the number of *bis*-allylic positions contained in the cellular lipids of intact cells, determines their susceptibility to free radical-mediated peroxidative events. Therefore, a high content of highly unsaturated fatty acid such as AA or DHA in the cell membranes, may increase the oxidative stress of biological systems [42, 60, 99-103]. Free radicals or ROS are defined as an atom or group of atoms, which have one or more unpaired electron(s), that are capable of reacting with lipids and initiating the peroxidation process and abstracting hydrogen from the PUFA [51, 84, 101, 104]. However autoxidation of PUFAs may also be triggered by low-valent transition-metal ions such as Cu^+ or Fe^{2+} in the presence of ascorbate or heme iron complexes under aerobic conditions [51, 105].

1.4a – Formation of conjugated lipid hydroperoxides

Oxidation of fatty acyl residues proceeds by a series of reactions to give phospholipid hydroperoxides, epoxides, and hydroxides [103]. Several species can abstract the first hydrogen atom and these include the radicals: hydroxyl (•OH), alkoxyl (RO•) and peroxyl (ROO•). The membrane lipids, mainly phospholipids containing PUFAs, are susceptible to peroxidation because hydrogen abstraction from a methylene ($-CH_2-$) group occurs, removing one electron from the bond, leaving behind an unpaired electron on the carbon, $-•CH-$. The presence of a double bond in the fatty acid weakens the C–H bonds on the carbon atom and thus facilitates H• abstraction. For example, the initial reaction of •OH with PUFAs produces a lipid radical (L•), which in turn reacts with molecular oxygen to form a lipid peroxyl radical (LOO•). The LOO• can abstract hydrogen from an adjacent fatty acid to produce a LOOH and a second lipid radical, perpetuating the chain reaction. The LOOH formed can undergo reductive cleavage by reduced metals, such as Fe^{2+}, producing lipid alkoxyl radical (LO•) [32, 60, 98, 104, 106-108]. Lipid peroxidation is terminated by two of the peroxyl radical quenching each other to form a non-radical and oxygen [21]. The radicals produced in the initiation process undergo rearrangements from 1, 4-pentadiene (methylene-interrupted) structures into 1, 3-pentadiene [figure 9] systems [109].

Figure 9 – Formation of lipid hydroperoxides and conjugated dienes

R = various carbon chain lengths

L-H = lipid non-radical

L• = lipid radical
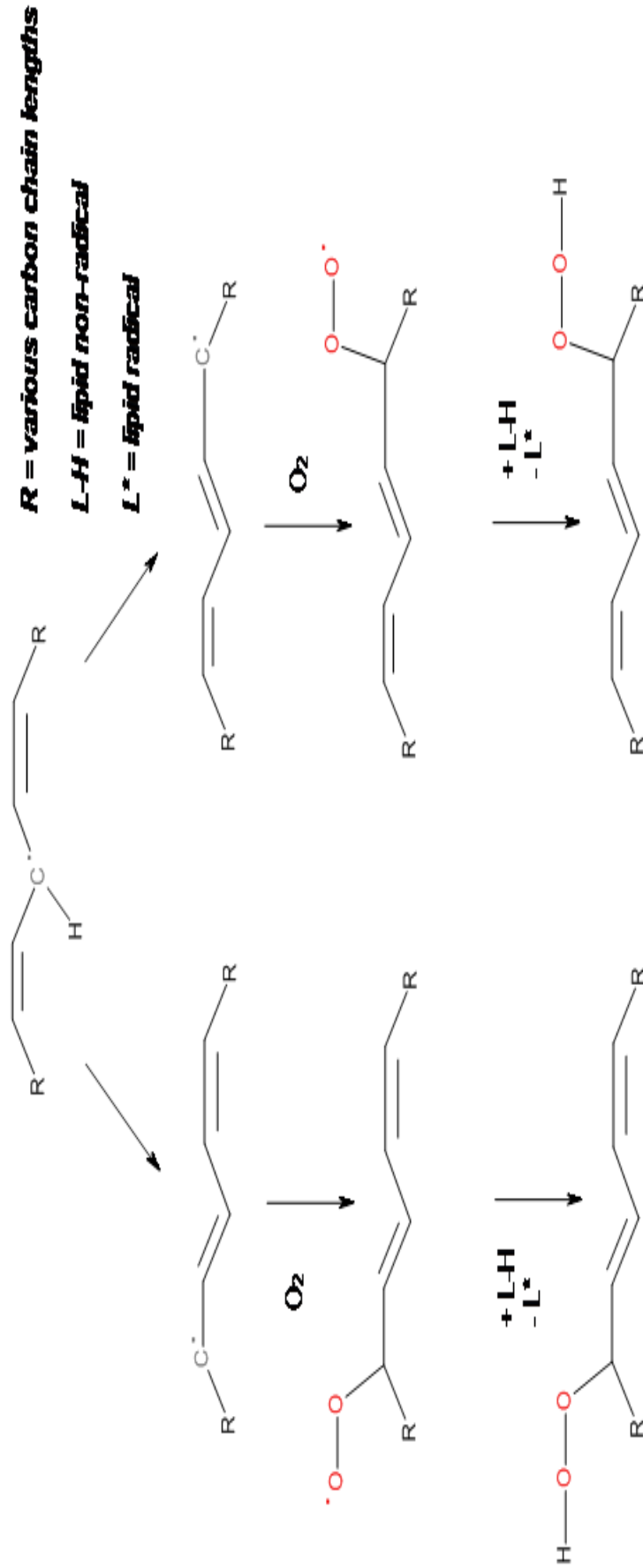
Methylene carbons in PUFA backbones are highly susceptible to hydrogen abstraction by free radicals. When a hydrogen atom is abstracted, one of the neighbouring double bonds flips creating a conjugated diene system with the other double bond. The site of hydrogen abstraction is not where oxygen attaches. Oxygen addition occurs two carbons away from the abstraction site, creating the peroxyl group. For example, abstraction at C-11 of ω-6 LA, results in the formation of C-9 and C-13 peroxyl radicals. It can then be stabilised by abstracting hydrogen from another lipid molecule or from an antioxidant. This then forms the conjugated lipid hydroperoxide.

Rearrangement or β-scission of the generated peroxy fatty acid radical, yields a variety of end products such as aldehydes, short chain alkanes and hydroxylated fatty acids [110]. Cyclisation of the peroxyl radical gives rise to bicyclic endoperoxides that can be reduced to form prostanoids [84]. This results in the formation of two structurally different biomarkers, aldehydes that can be formed from the chain scission of fatty acids and cyclic rings that are created from PUFAs containing three or more double bonds [Isoprostanes (IsoPs) from AA, neuroprostanes (NPs) from DHA]. Therefore these two need to be monitored in neural tissue because of the high PUFA content, to understand whether ring formation of chain breakdown is occurring when the LOOHs breakdown.

1.4b - Aldehydes

Breakdown of LOOHs into smaller fragments, generates a wide variety of water-soluble carbonyl compounds, such as aldehydes, 2-alkenals, and 4-hydroxyalkenals *via* β-scission [111]. These aldehydes show different reactivity compared with free radicals because of their longer half-lives, which allows diffusion of the aldehyde-based species to sites distant from their formation [26]. Examples of aldehydes formed from lipid peroxidation include 4-hydroxynonenal (HNE), 4-hydroxyhexenal (HHE) and malondialdehyde (MDA). HNE is one of the main aldehydes formed during lipid peroxidation of omega-6 PUFAs, such as LA and AA [60]. The lipophilicity of HNE, means that phospholipids and proteins, which are embedded in biomembranes (such as transporters, ion channels and receptors), become especially susceptible to attack by HNE [26, 112]. HNE causes degeneration and death of cultured hippocampal neurons, by impairing ion-motive adenosine triphosphatase activity, disrupting calcium homeostasis and impairing glucose and glutamate transport [101]. It can also react with PE, yielding a Michael adduct and a Schiff-base adduct which can be present as pyrrole derivative. Interestingly, PS, which has the same potential as PE, reacts weakly with HNE [111]. HNE is also known to make adducts with primary amine and thiol groups in proteins and can also inhibit NF-κB activation [113, 114].

Lipid peroxidation of omega-3 PUFAs such as LNA and DHA generates a closely related compound, HHE [60, 115, 116]. Despite the structural similarity between HNE and HHE, the biological actions and efficacies of these aldehydes differ significantly. For example, HHE has a more pronounced effect on mitochondrial permeability transition than HNE. HHE modulates endothelial nitric oxide synthase through NF-κB activation

[114] and is a potent inducer of permeability transition in hepatic mitochondria, but not in mitochondria isolated from brain [116].

MDA is a three-carbon, low-molecular weight aldehyde that can be produced from trienes *via* K- or L-unsaturated peroxides of PUFAs regardless if they are omega-3 or omega-6 [96]. MDA concentration is not a good index of lipid peroxidation. MDA is labile; *in vivo*, it is metabolised or is used in lipid cross-linking reactions and its concentration reflects unsaturated fatty acid composition as much as the extent of lipid peroxidation [109]. The assessment of TBARs provides a crude measure of aldehydes given the high reactivity of thiobarbituric acid with lipid peroxidation products [21, 26].

1.5 - Isoprostanes and neuroprostanes

Conjugated lipid hydroperoxides of PUFAs containing three or more double bonds can undergo cyclic ring formation, instead of aldehyde formation generating another endpoint oxidation biomarker. The notion that IsoPs could be generated *in vitro* non-enzymatically as products of autoxidation of fatty acids was actually first demonstrated over 20 years ago [117, 118]. IsoPs are believed to be formed *via* a unified free radical mechanism [84, 101, 119, 120], which are independent of the enzymatic COX pathway for PG synthesis [121-123]. IsoPs were shown to have structures similar to the PGs, but with *cis*-dialkyl stereochemistry on the cyclopentane rings as a consequence of hex-5-enyl radical cyclisation. PGs are synthesised from free fatty acids whereas IsoPs are formed *in situ* on phospholipid moieties before being released by PLA_2 activity [51], [124].

The NPs were later identified. They are structurally similar to the IsoPs, but are derived from DHA [26, 105, 110, 124-131]. Since DHA is highly concentrated in neuronal membranes, NPs offer a unique window into free radical damage to neuronal membranes *in vivo* [132-134]. While there are only four regioisomeric families of the IsoPs creating 64 compounds [122, 135-138], there are eight regioisomeric families of the NPs [126]. NPs have eight regioisomers, each consisting of eight racemic diastereomers for a theoretical total of 128 different compounds [139]. The regioisomeric series of prostanoids are distinguished by the position of the hydroxy group in the side chain [51]. Quantification of NPs formed from DHA oxidation were observed to exceed the levels of IsoPs generated from AA, which may provide a unique marker of oxidative injury to the

brain [140-142]. The highly stable, chemically inert F ring IsoPs have been quantified as markers of oxidative damage, and have been shown to be elevated in brain tissue affected by numerous neurodegenerative conditions [13, 21, 140, 143, 144]. F-ring compounds are not the only autoxidation product, many other different ring structures can be formed, detailed in figure 10. Formation of these rings depends upon the reduction conditions on the H_x-ring intermediate; however it is widely accepted that quantification of the F-ring compounds is the most effective biomarker in quantification of oxidation [134, 145-147].

1.5a - Formation of the cyclic prostanoids

Non-enzymatic oxidative formation of prostanoids is a facile process which occurs very readily in biological tissues. The process of cyclic prostanoid formation involves free radical attack on fatty acids esterified to phospholipids, the creation of the cyclic ring, followed by subsequent phospholipase hydrolysis to release the prostanoid [125, 148]. The formation of these cyclic compounds requires a fatty acid that has at least three double bonds in its structure, as the creation of the carbon ring is at the expense of two double bonds. For example, when AA (four double bonds) oxidises in this process it creates F-ring IsoPs, which lose two double bonds and the resultant IsoP is referred to as a F_2-IsoP because there are only two double bonds left. The mechanism of formation shares the same initiation reaction observed in LOOH creation, instead of the peroxyl radical stabilising to form a hydroperoxy group, it attacks the fatty acid chain resulting in the formation of the cyclic ring.

This pathway starts with free radicals initiating the peroxidation of PUFAs by abstracting a *bis*-allylic hydrogen to form a lipid radical. Molecular oxygen adds extremely rapidly to this lipid radical and then this peroxyl radical attacks an adjacent double bond to form an endoperoxide radical. The endoperoxide radical can undergo internal cyclisation to form a bicyclic endoperoxide radical which will eventually add a second molecule of oxygen to form a bicylic endoperoxide-peroxyl radical that can be reduced to form the H_x-ring isomers. Different regioisomers of H_x-ring can form, depending on which of the *bis*-allylic hydrogens is extracted by the radical of one of the methylene groups. The entire reaction scheme occurs *via* radical reactions so that each of the stereocenters in H_x-ring is racemic [figure 10] [134, 149]. The endoperoxide unit in H_x-ring is reduced easily to generate F-ring IsoPs. Several sulfur species are capable of reducing H_x-ringto F_x-ring and antioxidants such as α-tocopherol, are also effective in reducing H_x-rings [51].

The mechanism of formation of the IsoPs, from AA, involves initial hydrogen abstraction from one of the three bisallylic methylene groups of AA at carbon atoms 7, 10, and 13 [figure 10] [150]. Molecular oxygen adds, exactly the same as in LOOH formation, creating a peroxyl group at position C-11 for example, if hydrogen abstraction occurs at C-13. The C-11 peroxyl then attacks the C-9 double bond, forming the endoperoxide cyclic ring and a lipid radical at C-8. This radical at C-8 then attacks the double bond at C-12 creating the bicyclic endoperoxide, but also facilitates molecular oxygen addition at C-15 making another peroxyl group. The bicyclic endoperoxide and peroxyl groups then get reduced to form the F-ring IsoP, which contains two hydroxyl groups on the cyclopentane ring and another hydroxyl on the methyl terminus side chain. This particular IsoP forme is known as the 15-series, as the hydroxyl group on the methyl terminus chain is fifteen carbons from the carboxyl end. Removal of hydrogen through abstraction at C-10 gives rise to the formation of two regioisomers the 8-series and 12-series, whereas at C-7, it leads to the formation of 5-series [120, 135].

The free radical-initiated peroxidation of α-LNA and its esters generates mixtures of primary hydroperoxides and cyclic peroxides. Two regioisomeric series are formed from the oxidation of linolenate depending on the site of initial hydrogen abstraction and subsequent oxygen insertion and are called phytoprostanes (PPs). Hydrogen atom abstraction at C-14 generates type I PP whereas hydrogen abstraction at C-11 yields type II PP [figure 11]. Each series is composed of 16 isomers and 32 PPH_1 can be formed in total [151]. The autoxidation of DHA derivatives proceeds very similarly to that of the IsoPs and leads to NPs. The higher number of *bis*-allylic positions results in the formation of eight classes of NPs by a radical peroxidation, bicyclisation, oxygenation and final reduction. The assignment of the individual regioisomers of NPs was based on the cyclisations of the individual DHA-derived hydroperoxides [figure 12].

The releasing of esterified prostanoids follows a rapid de-esterification phase, which is regarded as one of the rate limiting steps for their release. This reaction is the first step of enzymatic degradation of esterified prostanoids, which later secrete effectively into the peripheral circulation [51, 119, 152]. One important aspect of prostanoid formation is that, while they result from non-enzymatic rearrangement of lipid peroxyl radicals, the initial source of the free radicals or the lipid peroxyl radicals is often enzymatic [134].

40

Figure 10 – Isoprostane ring structures and their mechanism of formation [51, 153]
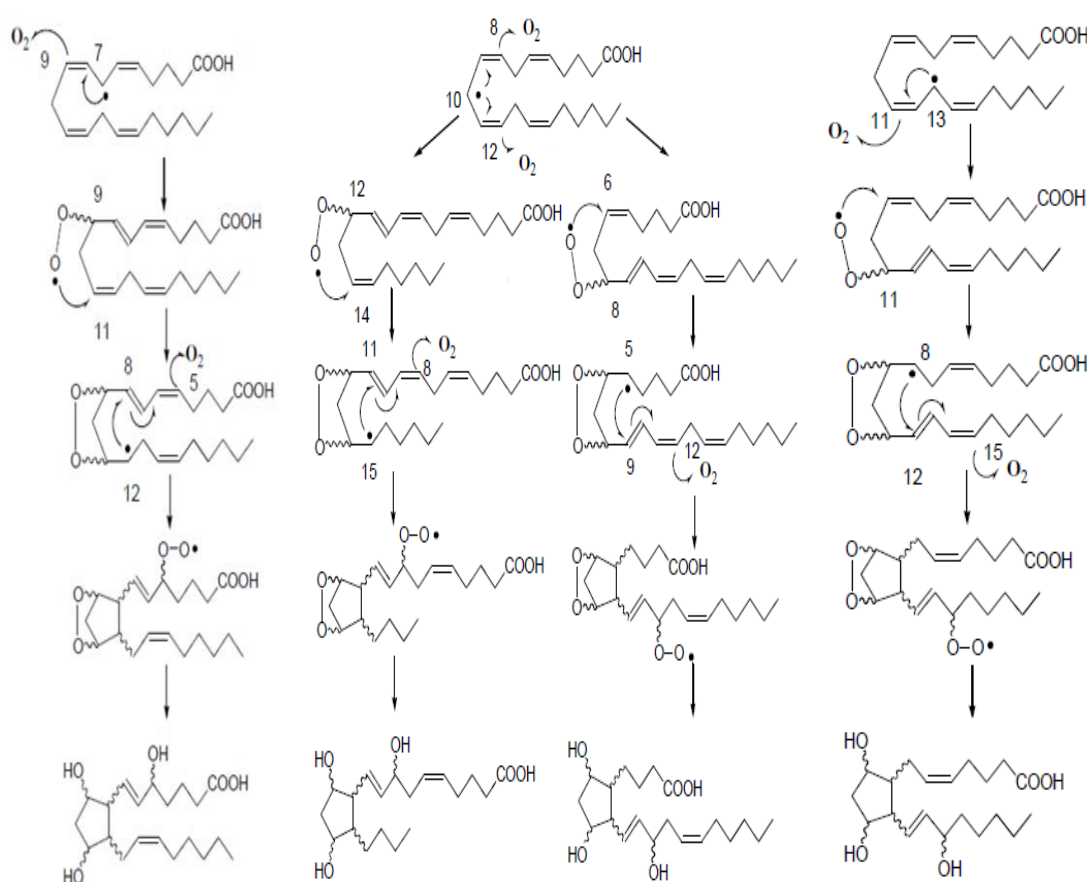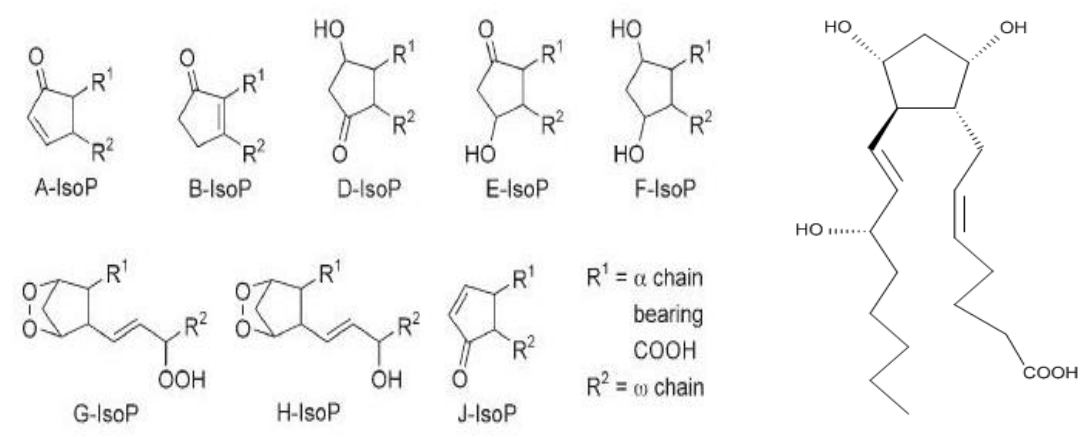
The top two images above the line shows the amount of different ring structures that can be formed under oxidation and the *cis*-dialkyl stereochemistry of an F-ring IsoP. The lower picture below the line, illustrates how F-ring IsoPs are formed by autoxidation. IsoPs form from initial hydrogen abstraction and oxygen attack, but instead of the hydroperoxide forming the radical attacks the PUFA backbone eventually resulting in the formation of the five member ring synonymous with prostanoic acid. Various methylene sites create multiple positional IsoPs. Illustrated is the mechanism of which the four regioisomers are formed, the 5-, 8-, 12- and 15-series IsoPs. Attack at methylene carbon 13 creates the 15-series; carbon 10 creates 8- and 12-series with carbon 7 resulting in 5-series IsoP.

Figure 11 – Phytoprostane formation from linolenic acid oxidation [154]

Type I - Phytoprostanes   **Type II - Phytoprostanes**
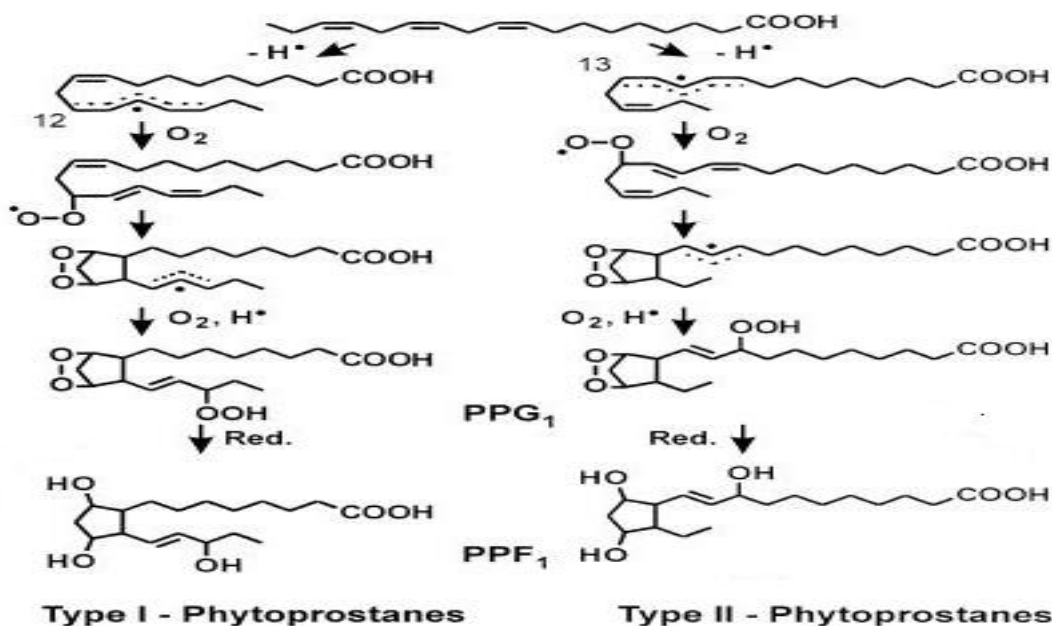
The mechanism of PP formation follows the same reaction principles seen in the IsoP scheme. With only two sites where the hydrogen can be abstracted from LNA, only two different sets of PPs are formed the 9- and 16-series from carbon 11 and carbon 14 respectively.

Figure 12 – Neuroprostane formation from docosahexaenoic acid oxidation [62]
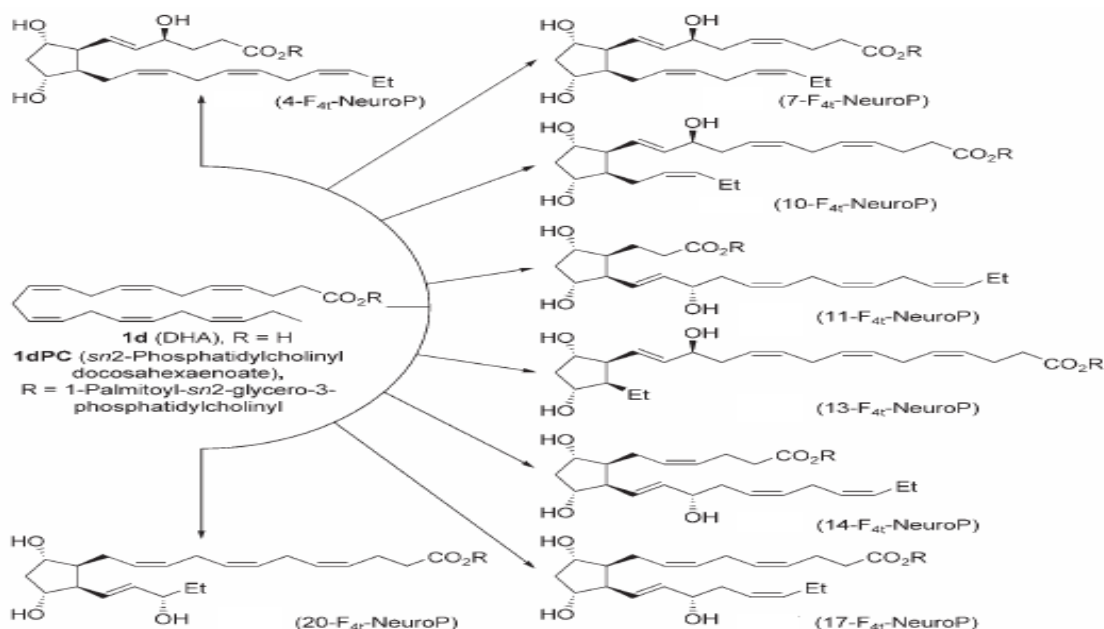
Due to the amount of positions where hydrogen abstraction can occur from within the DHA structure, the quantity of final oxidation products, the NPs, is vast. All these components are formed exactly in the same manner as the previous schemes. Abstraction at C6 gives 4-series, C9 – 7- and 11-series, C12 – 10- and 14-series, C15 – 13- and 17-series and finally C18 for 20-series NP.

1.5b – Biological properties of isoprostanes and neuroprostanes

Due to the role of IsoPs in the pathogenesis of disease, their cellular signalling pathways and biological effects have been explored [159-161]. In view of their structural similarity with enzymatically derived $PGF_{2\alpha}$, 15-series F_2-IsoPs interact with the enzymes that degrade PGs *in vivo* [47]. Such reactions include oxidation of the 15-alcohol group, saturation of the 13, 14-double bond, ω-oxidation, and β-oxidation. PG metabolites excreted in the urine are usually chain-shortened by two or four carbon atoms [152, 162, 163]. The configuration of the hydroxyl at C-15 must be *S* in the equivalent IsoP, as in the COX-derived PG, for 15-OH dehydrogenation to occur easily. The Δ13 double bond of PG cannot be reduced without prior 15-OH dehydrogenation. Another reaction that is markedly influenced by the steric structure is the reduction of the Δ5 double bond, which proceeds easily for $PGF_{2\alpha}$, but is more or less impeded for the various enantiomeric pairs of F_2-IsoPs.

Peroxisomal β-oxidation is crucial for several steps of lipid catabolism, such as shortening of very long chain fatty acids and degrading unusual fatty acids and oxygenated eicosanoids, including IsoPs. In humans, peroxisomal β-oxidation of IsoP mainly yields 2, 3 dinor 8-IsoP [119, 144, 163, 164]. DHA oxidation products can also undergo the same metabolism, for example 7-F_4t-NP is rapidly metabolised to 5-F_3t-IsoP by β-oxidation in rat liver homogenates [51]. F_2-IsoPs act as broncho- and vasoconstrictors, modulate platelet function, contract lymphatics, induce mitogenesis [165] and be carried by HDL in the lipoproteins of human plasma [166]. 15-F_2t-IsoP and its 2, 3-dinor, 5, 6-dihydro metabolite bind and activate the TxB receptor to potently induce vasoconstriction [128, 131, 134, 140, 144, 159].

1.6 - Defence against oxidation

Oxidative stress occurs *in vivo*, when antioxidant defences cannot inactivate the ROS generated either because of free radical overproduction or insufficient degradation of these radicals [167]. Primary defences are mainly preventative, whereas secondary defences have a "back-up" protective role, which might typically involve excision/repair of any lesions that do develop. Primary protection relies on the scavenging and inactivation of ROS or redox metal ions before lipid peroxidation takes place. Enzymes involved in primary protection include *i)* the copper/zinc-dependent and manganese dependent superoxide dismutases; *ii)* cytosolic and mitochondrial glutathione peroxidase,

which scavenges H_2O_2 at relatively low concentrations; and peroxisomal catalase, which scavenges H_2O_2 at high concentrations. Chain-breaking antioxidants such as α-tocopherol (vitamin e) and synthetic butylated hydroxytoluene (BHT) can afford primary as well as secondary stage protection. They compete with initial PUFAs for peroxyl radicals, generating LOOHs without creating another lipid radical, which stops propogation [104, 107]. A second line of enzymatic defence against peroxidation injury exists in most cells involving the removal of LOOHs at damage sites, followed by repair reactions [104].

<u>1.7 – Lipids and Alzheimer's disease</u>

Cell membrane lipid abnormalities described in AD brain contribute to Aβ deposition, neuronal dysfunction and are linked to the gene encoding the cholesterol transporter APOE [82]. As membranes comprise of phospholipids, their metabolism has been shown to be abnormal in AD brain cortex. Depletion of phospholipids correlates with accelerated membrane degeneration seen in AD brain [168]. Epidemiological and experimental studies have indicated that increased omega-3 fatty acid consumption decreases the risk of developing AD [71]. Supplementation of omega-3 fatty acids as a preventative measure or potential treatment of AD has been conducted in various studies, detailed in table 1.

In 2005, after reviewing 12 studies on the relationship between the intake of omega-3 fatty acids and cognitive function in the elderly, the US Department of Health and Human Services concluded that there was no definitive link between low omega-3 fatty acid intake and loss of cognitive function during aging [37]. Despite the findings by the US Department of Health and Human Services, literature still associates reduced DHA content in this disease, based upon the findings of <u>Söderberg et al</u> [169, 170]. This particular study represents the biggest *post-mortem* cohort sample size indicating lower DHA content in AD. *In vivo* studies in AD mouse models, reported that reduction of dietary intake of omega-3 PUFAs resulted in loss of post-synaptic proteins, while a DHA enriched diet could prevent these effects [20, 42]. Whether DHA is protective against AD or is a marker for disease pathogenesis is undefined, even with the plethora of literature studies (table 1). However, the disparity and lack of repetition between studies represents significant doubt.

Table 1 – Literature survey of association between AD and DHA

Author	Control Samples	Alzheimer Samples	Conclusion
Corrigan et al [88]	6	8	Reduced DHA levels
Reich et al [174]	11	9	No difference
Söderberg et al [170]	9	9	Reduced DHA levels
Skinner et al [175]	10	15	No difference
Lukiw et al [173]	6	6	Reduced DHA levels
Morris et al [41]	Progressive study of 815 patients over 4 years fish consumption		DHA reduced AD onset
Barberger-Gateau et al [172]	Progressive study of 1674 patients over 3 years		DHA content related to AD onset
Freund-Levi et al [176]	Progressive study of 204 patients over 12 months omega-3 supplementation		DHA reduced AD onset
Schaefer et al [171]	Progressive study of 899 patients over 10 years		DHA content related to AD onset

The first five rows of this table denote whether there was a reduction in DHA content with AD progression or there was no reduction in DHA in *post-mortem* brain samples. The four later rows depict whether DHA supplementation had an affect on AD onset, with all studies showing omega-3 intervention reduced AD progression. The two largest *post-mortem* studies highlighted that DHA was not lowered in AD, but the paper by Söderberg et al [170] is the most cited in linking lowering levels of DHA in AD pathogenesis.

AD brain is also under extensive oxidative stress, characterised by increasing levels of protein and lipid oxidation. Determining the level of oxidative damage in AD brain is central to understanding the pathogenesis of AD and will provide the basis for a rational development of experimental therapeutics [19, 177]. Alterations in brain phospholipids and increased oxidation, results in *sn-2* esterified PUFAs being suspect to free radical attack. The levels of these PUFAs are therefore predicted to decrease in AD brain, if lipid peroxidation were increased [21, 27].

F_2-IsoPs and NPs have been investigated in relation to AD and have been found to be increased in the frontal and temporal cortex of AD compared with control brains [13, 51, 71, 123, 139, 141, 177, 178] and in *post-mortem* ventricular cerebrospinal fluid (CSF) [13, 19, 21, 26, 51, 177], obtained from autopsy-verified AD cases, as well as in antemortem CSF from individuals diagnosed with AD. Results have been less consistent in regard to peripheral F_2-IsoPs, with several studies reporting increased plasma levels, and others reporting no significant difference in AD compared to controls. Similarly, urinary F_2-IsoPs have been reported to be increased [13] or unchanged [136]. The discrepancies concerning peripheral F_2-IsoPs may be due to differences in patient selection criteria between studies, as smoking and other conditions associated with oxidative stress, such as cardiovascular disease and diabetes, which can significantly alter IsoP levels [7]. Relationships between APOE Ɛ 4 genotype (positive [33], negative [19, 21, 26]), NFTs [19, 21, 26] and Aβ levels [21] have also been investigated.

1.8 – Research aims and objectives

The aim of this project was to distinguish whether there is a link between AD and higher lipid oxidation, as seen in the literature. This was assessed using various biomarkers in the oxidation mechanism, under a double-blind scenario to ensure the quality of the results. This project was carried out in collaborated with the Dementia Research Group, Institute of Clinical Neurosciences, University of Bristol, Frenchay Hospital who had access to South West Dementia Brain Bank, University of Bristol for brain tissue from both AD and age-matched controls with the project at Bristol being sponsored by the Alzheimer research trust (ART).

As discussed earlier a dietary intervention could halve AD onset, with omega-3 PUFAs and antioxidants being examples of proposed solutions. Various dietary supplementation

and *post-mortem* brain studies have not confirmed whether these can help prevent or treat AD, instead they have only created greater disparity. This is highlighted most in *post-mortem* studies, where low cohort sizes have commonly been used in conjunction with a couple of biomarker assays. All the assays have been explored in AD before as individual studies, but on different cohort sizes and brain regions which affect their conclusions. The advantage of this project is the use of over 70 patient samples including different brain regions, to elucidate any pathways or conclusions in differentiating between control and AD. By using this larger cohort size, multiple quantification assays and clinical data sets on the same tissue, we were able to investigate this, with greater statistical power.

The levels of DHA and AA were quantified, to evaluate the relationship between these PUFA and AD. Various oxidation markers were also investigated to reveal the role of lipid oxidation in the pathogenesis of AD. These markers included LOOHs and CDs, the cyclic IsoPs and NPs (highly specialised biomarkers formed from oxidation of AA and DHA respectively) and the levels of the aldehydes, which are the final endpoint products of fatty acid breakdown. The quantification of the cyclic compounds required the development of an appropriate method using GC-EI-MS. Total antioxidant levels were also determined to clarify the role of protection against oxidation in AD. All samples had a clinical breakdown of age, Braak staging, APOE genotype, sex and *post-mortem* delay accessible for multiple lines of enquiry. Each assay was investigated independently to find out its specific levels in AD to observe any differences, before statistical analysis was performed using the clinical data and creation of pathway oxidation models using correlation data.

2 Methodological approaches

FOREWORD

Methods conducted within Aston University are clearly marked, A=Aston, whilst the ones conducted by our collaborative group, (Dementia Research Group, Institute of Clinical Neurosciences, University of Bristol, Frenchay Hospital) are marked B=Bristol.

2.1 - Materials, methods and samples

2.1a - Chemicals

Solvents and reagents were obtained from Fisher Scientific or Sigma Aldrich and were HPLC grade or higher. Didocosahexaenoyl (diDHA) phospholipids were purchased from Avanti Lipids. The HPLC column was obtained from Wako Chemicals and the GC columns from Agilent. IsoP standards, deuterated and non-deuterated, were purchased from Cayman Chemicals. SPE cartridges were obtained from Waters and the vacuum manifold from Supleco. NP and PP standards were graciously provided by Dr Thierry Durand, Universitiés de Montepeiller I et II.

2.1b - Tissue

Tissue from 36 cases of neuropathologically confirmed AD (CERAD 'probable' or 'definite' AD, Braak tangle stage IV–VI) and 35 neuropathologically-normal controls without AD (Braak tangle stage 0-III) were obtained from the South West Dementia Brain Bank, University of Bristol. The age at death, gender, *post-mortem* delay and APOE genotype were known for all cases. Samples of mid-frontal cortex (Brodmann area 6) and superior temporal cortex (Brodmann area 22) carefully excluding underlying white matter were dissected from the frozen slices of left cerebral hemisphere. The study had approval from the Frenchay Research Ethics Committee. The AD cases, had a mean

age = 78 years and *post-mortem* delay mean = 45.0 hours and the controls, mean age = 81 years and *post-mortem* delay mean = 48.0 hours [179].

2.1c - (B) Preparation and extraction of lipids from tissue

50 mg tissue samples were added to 350 µl of methanol at 0°C and homogenised in a Bertin Technologies Precellys 24 mechanical homogeniser (Stretton Scientific, Stretton, UK) at 6,000 rpm for 29-30 s with 15–25 2.3 mm zirconia silica beads (Stratech Scientific, Newmarket, UK) in a screw cap 2 ml microcentrifuge tube (Fisher Scientific, Loughborough, UK) that had been washed by vortexing with 1 ml of chloroform three times. The sample was cooled on ice after homogenisation. Chloroform (350 µl) containing 100 mg/l butylated hydroxytoluene (BHT; 2,6-di-tert-butyl-4-methylphenol) was added and the resultant solution briefly vortexed. The homogenate was transferred to a centrifuge tube and the beads washed with a further 150 µl of chloroform/BHT:methanol 2:1 (v:v). The solutions were combined and centrifuged at 2,000g for 5 min and the supernatant was removed. The pellet was resuspended by vortexing in 200 µl of chloroform/BHT:methanol 1:1(v:v) and the solution was re-centrifuged. The supernatant was removed and combined with the first supernatant and, after the addition of a further 400 µl of chloroform/BHT and 325 µl of 0.9% w/v potassium chloride, the solution was vortexed and centrifuged. The purified lipid fraction was obtained in the lower chloroform phase [179].

2.1d - (B) Preparation of fatty acid methyl esters

The lower chloroform phase was removed under N_2, 250 µl of 1 M ethanolic KOH added and the solution left overnight at room temperature in a sealed tube. 1,250 µl of distilled water was added along with 400 µl of hexane: diethyl ether 1:1 (v:v) containing 100 mg/l BHT. The biphasic solution was vortexed, centrifuged usinf the conditions above and the upper phase discarded. The addition of hexane:diethyl ether/BHT, vortexing, centrifugation and removal of the upper phase were repeated three times. The pH of the lower phase was adjusted to pH 3 with concentrated hydrochloric acid. 400 µl of diethyl ether/BHT was added, vortexing, centrifugation and removal of the upper phase repeated twice, and the lower phase discarded. The two upper phases (containing the free fatty acids derived from the lipid extract) were combined and dried under N_2. Methyl esters of the free fatty acids were then prepared in a sealed tube with a solution of 2.5% concentrated H_2SO_4 in anhydrous methanol overnight at room temperature [179].

2.2 - (B) Analysis of total fatty acid methyl esters

FAMEs were analysed by GC and identified by co-chromatography with authenticated standards and by GC-MS. Heptadecanoic acid (200nmol) was used as the internal standard and quantified by flame ionisation detection (FID) (Bristol). All measurements were made in duplicate and the mean value calculated for each fatty acid as a molar percentage of the total fatty acid content [71, 179].

2.2a - (A) Thin layer chromatography and GC-EI-MS phospholipid composition analysis

Lipids were initially purified by TLC on Merck Kieselgel 60; separation of phospholipid classes and neutral lipid classes was achieved using a chloroform/methanol solvent system. PE and PC were major components together with PS and PI. Phospholipids were transmethylated using sulphuric acid-methanol and the corresponding fatty acid methyl esters were extracted into hexane and analysed by GC-MS (Agilent 6890 GC with 5973 MSD) on a 30m DB23 column using helium as a carrier gas. Lipids were quantified using heptadecanoic acid as an internal standard.

2.3 – (A + B) Conjugated diene and lipid hydroperoxide determination

PUFAs undergo free radical attack on their methylene interrupted carbon, resulting in the formation of the 1, 3 pentadiene motif. This motif is synonymous with lipid oxidation and is regarded as the first step in the oxidative breakdown of PUFAs. Formation of the conjugated system allows for UV detection at a separate wavelength to the methylene interrupted non-conjugated diene system. In conjunction with HPLC, lipid moieties can be separated out and then, UV spectroscopy can be used to show differences between conjugated and non-conjugated systems for individual phospholipids.

2.3a (A) - LC-UV-ESI-MS analysis

Separation of extracted lipids was achieved on an Agilent 1100 LC linked with a UV diode array detector and subsequent analysis carried out using an electrospray ion trap mass spectrometer using the method by Mawatari and Murakami [180]. Lipid samples were dissolved in *iso*hexane/*iso*propanol (3:1, v/v) and injected onto a Wakosil 5 NH$_2$ column (150mm x 4.6mm) held at 40°C at a flow rate of 1ml min^{-1}. Spectral information was obtained at wavelengths of 210 and 234nm for each of the eluting phospholipids.

The 234nm absorbance value is a measure of CD formation produced during the oxidation of PUFAs having the 1, 3 pentadiene motif and 210nm absorbance related to non-oxidised fatty acids.

2.3b (A) - UV spectrometer analysis

Lipids were dissolved in ethanol:water (9:1, v/v) and the ultraviolet absorption at 210 and 234 nm were measured (with a complementary blank), using a Jenway 6505 UV/vis spectrophotometer. The percentage difference between the maximum absorbance at 234nm of the peroxidised phospholipid and 210nm of the corresponding non-peroxidised phospholipid was also calculated. A molar extinction coefficient of 27,000 $M^{-1}cm^{-1}$ [181] was used for calculation of the CDs concentration [95, 182-185].

2.3c (B) - Lipid hydroperoxide assay (FOX)

The ferrous orange xylenoyl (FOX) assay kit was used to measure the amount of LOOHs. Briefly, brain homogenates (c. 50 mg frontal, temporal cortex) were prepared in 100% cold (5°C) HPLC grade methanol before being homogenised with 450 µl of buffer solution (1:9 w/v tissue). Into a 96-well plate the following was pipetted; 90 µl of 0.25 mM $FeSO_4$ (ferrous sulphate, prepared fresh for each assay), 35 µl of 25 mM H_2SO_4 (sulphuric acid), 35 µl of 0.1 mM xylenol orange and 160 µl of distilled water. Blanks were prepared by replacing the tissue extract with methanol. A volume of tissue extract was chosen that gave an absorbance at 580 nm of 0.7-0.8. Samples were incubated at room temperature until the reaction was complete, and absorbance at 580 nm was then measured. With tissue samples, a further addition was then made of 7 µL of 0.25 mM cumene hydroperoxide (CHP, 1.66 nmol), then incubation at room temperature for 30-60 minutes until the reaction had reached a stable end point, and the absorbance at 580 nm was re-measured. Levels of LOOHs are expressed as CHP equivalents [186].

2.4 - (B) Antioxidant assay

This spectrophotometric method involves the generation of the ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)] radical cation in a decolourisation technique in which the radical is generated directly in a stable form prior to reaction with putatative antioxidants. Trolox (6-hydroxy-2, 5, 7, 8-tetramethychroman-2-carboxylic acid) is used as a standard.

Figure 13 – Chromophore used for conjugated diene determination [187]
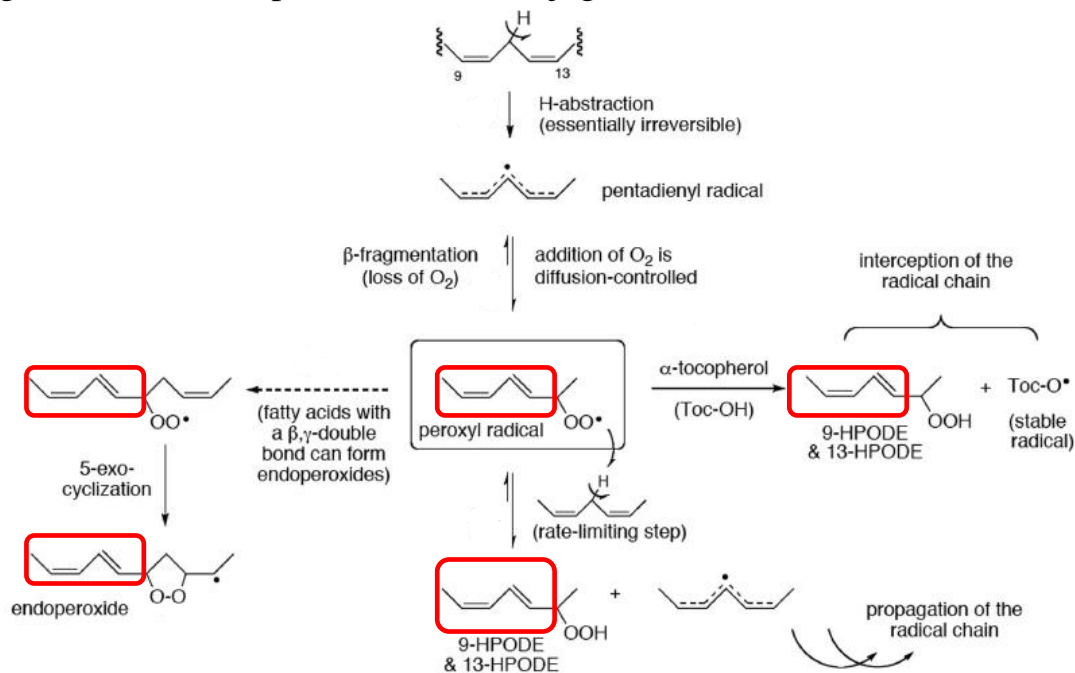
From initial hydrogen abstraction from methylene carbons under oxidation, the unconjugated diene bonds rearrange to form a CD system, denoted in the red boxes above. These bonds can be monitored by UV at 234nm. Non-conjugated bonds can be observed at 203-210nm wavelengths as discussed in the introduction.

Figure 14 – Chromophore used for lipid hydroperoxide determination
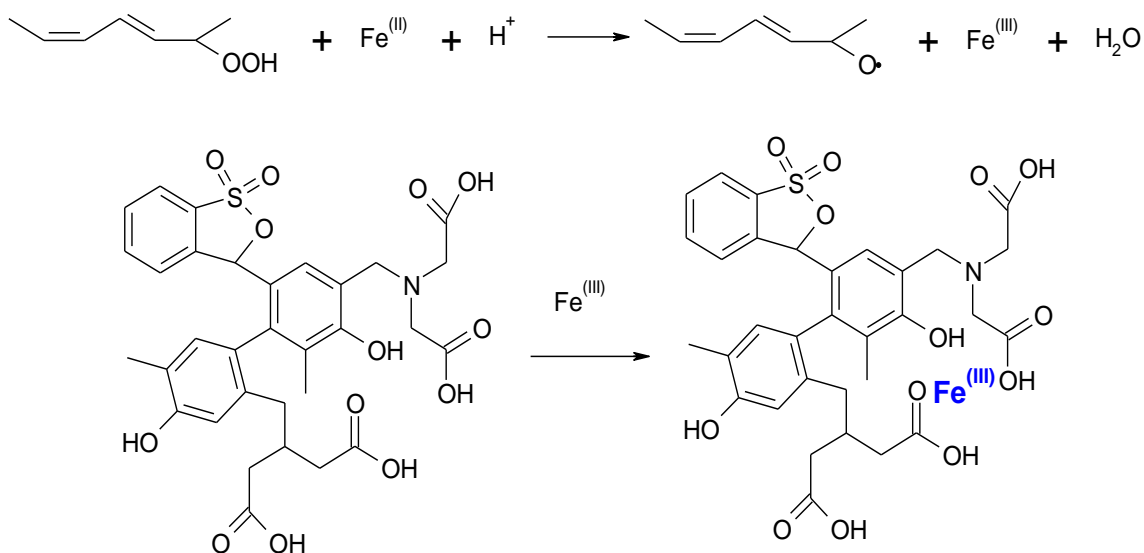
The FOX assay is a very specific determination of LOOH levels. The products from lipid peroxidation react upon addition of iron sulphate (Fe^{2+}) to produce a propagating lipid radical and oxidised iron (Fe^{3+}). Xylenoyl orange, depicted at the bottom, can trap metal ions and the xylenoyl-metal complex produces a blue solution which is observed spectrophotometrically.

The percentage inhibition of absorbance at 734 nm is calculated and plotted as a function of the concentration of antioxidants and of Trolox for the standard reference data. Briefly, the ABTS reagent (7mM in water) was reacted with potassium persulphate (2.45mM) and then diluted with phosphate buffered saline (ph7.4) to produce a solution with an absorbance of (±0.02) at 734 nm. The mixture was then allowed to equilibrate at 30 °C. Brain tissue samples (100mg) were homogenised in buffer solution before being applied, along with Trolox standards and blanks, into a 96-well plate. The diluted ABTS solution was added to each well (250μl) and the plate incubated at 30 °C for 6 minutes before reading the absorbance at 734 nm. The % inhibition of absorbance at 734 nm was calculated and plotted as a function of concentration of antioxidants and of Trolox for the standard reference data [188].

2.5 - (B) Aldehyde determination using TBARs assay

Measuring the end products of lipid peroxidation is one of the most widely accepted assays for oxidative damage. These aldehydic products of lipid peroxidation are generally accepted markers of oxidative stress. MDA forms a 1:2 adduct with thiobarbituric acid. The adduct formed from the reaction of MDA in samples with thiobarbituric acid (TBA) can be measured colorimetrically. TBARs levels are determined against a MDA standard calibration curve. A series of MDA standards were prepared by dilution from a starting concentration of 250μM down to 0μM for creation of a correlation curve. The TBA reagent was made by diluting the TBA acid with sodium hydroxide. Into a microcentrifuge tube the sample (50μl, in 1.15% KCl), MDA standard (50μl), SDS Lysis solution (50μl, mixed thoroughly and left for 5 minutes) and 125 μL of the TBA reagent were pipetted. Tubes were then incubated at 95°C for 45-60 minutes, cooled in an ice bath for 5 minutes before centrifuging at 3000rpm for 15 minutes. The supernatant (200μl) was taken and placed into a 96-well plate and the absorbance measured at 532nm [189]. The concentration of TBARs was assatained by using the MDA standard calibration curve.

2.6 (A) - Total (free and esterified) F_2-isoprostanes and F_4-neuroprostanes

Two extraction procedures for these compounds were utilised, however, the method of Morrow et al [190, 191], did not perform as well as the one detailed below by Nourooz-Zadeh et al [192]. To verify whether the extraction had worked, TLC was performed as described in Morrow et al [190, 191] and was unsuccessful.

53

Figure 15 – Loss of chromophore formed from antioxidant reaction [193]

ABTS·⁻ (λ_{max} = 734 nm)

+ antioxidant

– K_2SO_5

ABTS²⁻ (colorless)
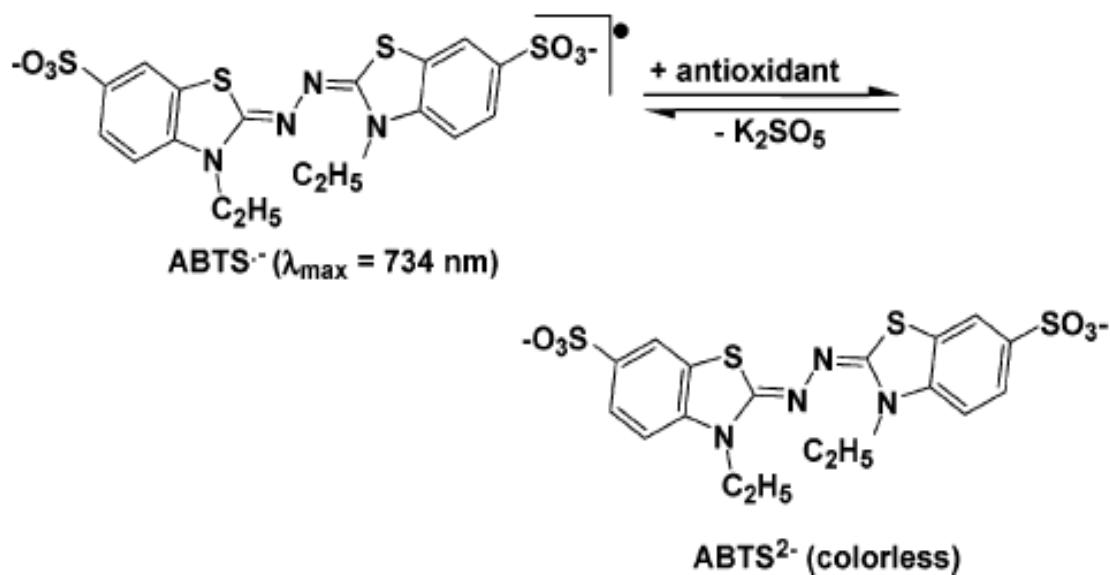
The antioxidant assay utilises the ABTS cation radical to quantify total levels. In this figure, the radical is formed by prior reaction with potassium persulphate in conjunction with Trolox, is used to form a calibration curve for later sample determination. Any antioxidants present react with the cation turning the coloured ABTS solution to a clear solution with no absorbance at 734nm wavelength.

Figure 16 – Chromogen formed for aldehydes quantification

Thiobarbituric acid

Malondialdehyde

H^+

Chromogen
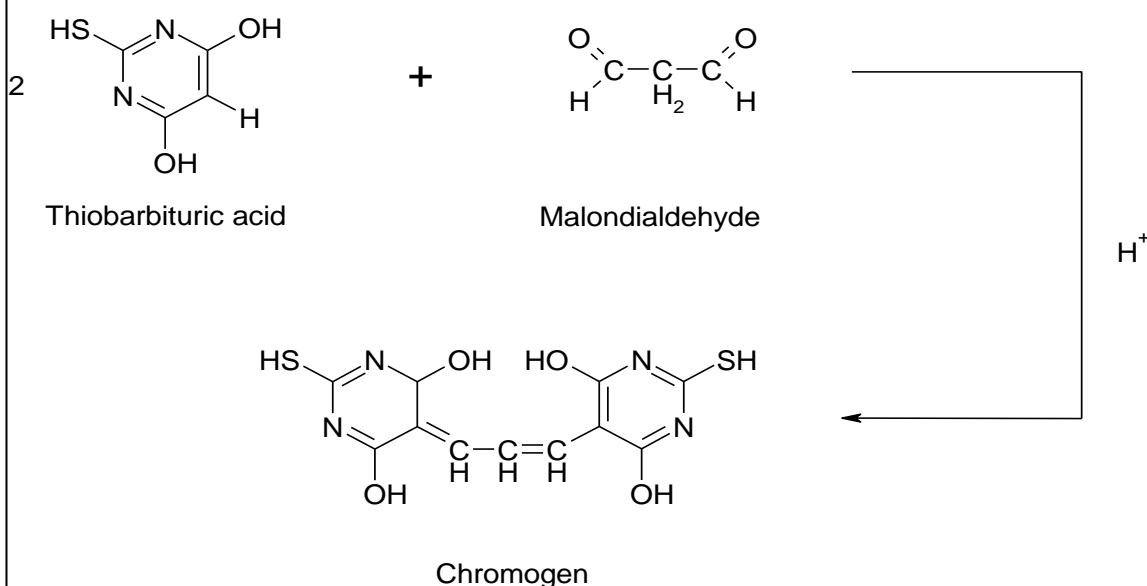
Quantification of aldehydes in this project was conducted using the classical TBA reaction with aldehydes which proceeds smoothly regardless of which aldehydes is present. Specifically in this scheme, MDA forms a 1:2 adduct with TBA. The MDA-TBA adduct formed from the reaction of MDA in samples with TBA can be measured colorimetrically or fluorometrically. The wavelength at 532nm was utilised for determination.

Hence the method by <u>Nourooz-Zadeh et al [192], which had also been checked by the same TLC method was chosen.</u> Samples were incubated with 1ml of an aqueous solution of potassium hydroxide (1.0M) at 45°C for 30 minutes, then acidified using 2ml of water adjusted to pH 2.0 with HCl and allowed to equilibrate at 4°C for 15 minutes. The sample was applied on a C18 cartridge (500mg) preconditioned with methanol and water pH 2.0. The cartridge was sequentially washed with 10ml of water (pH 2.0) and acetonitrile: water (15:85 v/v). Lipids were eluted by washing the cartridge with 5ml of hexane: ethyl acetate: *iso*-propanol (30:65:5 v/v). This eluate was then applied to an NH_2 cartridge (500mg) pre-conditioned with hexane (5ml). The NH_2 cartridge was sequentially washed with 10ml of hexane: ethyl acetate (30:70 v/v), acetonitrile: water (9:1v/v) and acetonitrile. Prostanoids were eluted from the NH_2 cartridge with 5ml of ethyl acetate: methanol: acetic acid (10:85:5 v/v). Addition of 30μl PP (3μg in ethanol) was used as internal standard. The sample was immediately transferred into a screw cap vial and the solvent was evaporated under nitrogen at room temperature [110, 121, 127, 140, 192, 194, 195].

The pentafluorobenzyl (PFB) ester was prepared by adding 40μl of PFB-Br (10% in acetonitrile) and 20μl of DIPEA (10% in acetonitrile) to the dried sample following solid-phase extraction. The vial was sealed with a PTFE-lined cap and kept at 45°C for forty five minutes. The solvent was removed under a stream of nitrogen. BSTFA containing 1% TMCS (30μl) was added; the vial was sealed and incubated at 70° for thirty minutes. The solvent was removed under nitrogen and the residue was reconstituted in *iso*-hexane (20μl). All the samples were stored at -20° until GC-MS analysis [110, 156, 190, 191].

The GC column was a fused-silica capillary column (HP-5MS, 5% phenyl methyl siloxane, 30m x 0.25mm ID 0.25μm film thickness). The initial oven temperature of 160ºC was increased at a rate of 20ºC/min to reach a maximum temperature of 310ºC, which was held for 2 mins. The injector system mode was splitless; carrier gas was helium at a constant flow-rate of 1ml/min. GC-MS temperatures were as follows: injector 300ºC, interface 250ºC, source 220ºC, and quadrupole 100ºC. The elution order in the GC chromatography went, PP first followed by IsoP then NP, this was confirmed using the commercially available IsoP and the synthesised PP and NP. The ion for quantification was the m/z 191 ion from the fragmentation of the F-ring of these prostanoids, along with the known mass of the internal standard.

3

Independent study analysis

FOREWORD

This chapter will discuss sets of results obtained by ourselves; separate from the larger cohort multiple assay study, detailed in the research aims and objectives. The larger study can be found in chapter 5, after the pathway models of lipid oxidation. These results are being utilised within this thesis to understand any relationships between AD and control in phospholipid compositions, *post-mortem* effect on CD levels and will also detail the discussion on the HPLC and prostanoid method development.

3.1 – Fatty acid analysis

SUMMARY

DHA is an important fatty acid within the CNS and has a plethora of roles as described in chapter 1, the depletion of which has been highlighted in AD. DHA is associated with phospholipids, so loss of this particular fatty acid may reduce phospholipid content as well. The loss of PUFA and phospholipid content will have effects on the properties of membranes, where they reside, which literature links to the effects of AD. Therefore, phospholipid fatty acid compositions were determined to find any differences, which may be specific for AD.

3.1a – Phospholipid composition

An aliquot of a brain lipid extract run on TLC allowed separation of phospholipids as stated in the methods section and enabled us to gain compositional data of phospholipids

In order from the bottom of the TLC plate, the retention of the phospholipids went PI, PS, PC, and PE with a non-polar band, comprising of triacylglycerols, cholesterols etc, running close to the solvent front. Use of an internal standard of known mass allowed for mass determination of each collected fraction *via* GC. Comparison between AD and control in the frontal lobe (table 2) showed a decrease in PC and PE with an increased level of PS. The decrease in PC and PE can be attributed to the theory of AD pathogenesis, either through metabolism deficits or through oxidation of specific membranes or membrane constituents. Fatty acid compositional analysis of the phospholipids clearly showed percentage differences in DHA between the three phospholipids postulated upon above. DHA appeared to be lower in PC, PE and PS; however this loss was greatest in PC. Justification for this loss, according to literature is down to AD pathogenesis and oxidation; however attention must be drawn to dietary intake and synthesis.

3.2 – Conjugated diene and phospholipid hydroperoxides

SUMMARY

LOOHs and CDs are the initial products of oxidation which can be readily quantified as described in the methods section for the specific products. The methylene carbon between the double bonds is susceptible to hydrogen abstraction by free radicals, before addition of molecular oxygen to form the peroxide radical which can stabilise to form the hydroperoxide. This mechanism also causes the double bond to rearrange and create the conjugated double bond system. Therefore increased oxidation will be correlated to higher primary oxidation products. AD and the role of lipid peroxidation were investigated to elucidate the role of primary products in differentiating between the disease and control samples.

3.2a – Separation and oxidation of single substrate phospholipids

Initial trials for the separation of phospholipids were investigated using the commercial samples of PC and PE, monitoring their elution at 210nm, as PUFAs absorb within the UV range 200-210nm. The phospholipids were well separated with relatively strong absorbance due to the high PUFA content in the samples. The LOOHs co-elute with the parent molecule for the phospholipid species. Since oxidation of mixed biological lipids is complex, we decided to investigate the oxidation of commercially obtained single substrates diDHA PC.

Table 2 – Mass and composition of each phospholipid class detected by thin layer chromatography in frontal control and AD samples

Control (n=4)			Alzheimer (n=4)		
Phospholipid	Mass (nmol)	% composition	Phospholipid	Mass (nmol)	% composition
PI	27.6 ± 1.2	3.8	PI	17.2 ± 4.3	2.3
PS	356.7 ± 23.6	49.4	PS	426.0 ± 36.7	57.1
PC	51.5 ± 8.7	7.1	PC	34.0 ± 12.1	4.6
PE	286.8 ± 35.1	39.7	PE	269.1 ± 41.8	36.0

Control samples phospholipid fatty acid composition (%)

	12:0	14:0	16:0	16:1	18:0	18:1	AA	EPA	DHA	U*
PI					80.0±23.9					20.0±10.9
PS		0.9±0.5	43.9±12.5		20.6±7.8	28.0±7.7			5.5±1.6	1.1±0.6
PC	4.9±1.4		23.9±15.6		23.0±11.2	16.9±5.6	15.9±7.9		9.8±4.2	5.6±2.7
PE			4.4±3.6		22.4±9.3	3.8±2.3	12.2±5.7	13.1±4.3	22.7±7.8	21.4±9.4

Alzheimer samples phospholipid fatty acid composition (%)

	12:0	14:0	16:0	16:1	18:0	18:1	AA	EPA	DHA	U*
PI					82.0±35.6					18.0±7.6
PS		0.8±0.3	39.2±7.8	1.0±0.3	21.3±11.7	32.5±13.6			3.5±2.1	1.6±1.1
PC	5.8±2.7		27.8±19.6		26.6±9.5	13.0±7.4	17.1±8.6			9.7±6.7
PE			5.8±3.7		18.8±7.4	7.9±4.6	14.2±9.9	9.9±5.4	19.7±9.3	23.7±11.2

From TLC, isolated phospholipid classes were analysed by GC-FID with heptadecanoic acid standard. The small sample size highlighted variation across the phospholipids and fatty acids, with AD samples having lower PC and PE content, but had increased PS content. Percentage composition of each phospholipid class indicated that the aminophospholipids were the highest in the brain, namely PS and PE. Additionally, across the fatty acids, DHA was seen to be lower in AD across these phospholipids and AA appeared to be higher in AD.

Rationalisation for this oxidation test was to justify whether DHA oxidises rapidly in phospholipids, with the view of evaluating the safest way of handling brain lipid extracts. Air oxidised samples from time course studies revealed that a 234nm absorbance increased with time. After 48h approximately 3.0% of the substrate had been oxidised. Exposure to air (up to 168h) resulted in increased CD formation which was detected at 234nm (data not shown). The four chromatograms in figure 17 showed the progressive formation of CD formation with time. It was observed that oxidation of this DHA phospholipid substrate, did not breakdown as quick as anticipated. Literature indicates that DHA oxidises quickly, but our results indicate a proportionatly substrate loss of 3% by LC-UV using 210nm and 234nm wavelengths. Attempts to increase oxidation of the phospholipids, by addition of hemin chloride and CHP or by the use of Fenton reagent (iron-ascorbic acid complex), were unsuccessful.

3.2b – HPLC phospholipid separation of biological samples

Lipid extracts from frontal AD brain samples and their age matched controls were dissolved in *iso*hexane/*iso*propanol (3:1, v/v) containing 0.01% BHT and separated by HPLC. The elution order of the phospholipids given in the method was PC, PE, PI and PS, which was verified using standards and MS fragmentation. Using both the MS and UV, phospholipid hydroperoxides in these samples were determined using the CD measurement at 234nm wavelength. Figure 18 shows an example of the chromatograms for both AD and control samples, with figure 19 detailing the corresponding levels of phospholipid hydroperoxides.

As stated previously, the peaks in the chromatogram relate to PUFA intensity not necessarily meaning they are of highest mass. PC and PE were observed to be the only two phospholipids which formed their respective hydroperoxides in the chromatograms. The highest 234nm absorbance found, was for PCOOH in the AD brain samples compared with the control samples. Also notably, 234nm absorbance for PE was visible in the AD cases and very little was observed in the control samples, indicating a difference in phospholipid oxidation between control and AD. This suggests that preferential oxidation appears to be happening to PC and PE in AD samples, which correlates with the earlier reduction in PC and PE DHA content observed in the phospholipid fatty acid compositional analysis.

Figure 17 – LC-UV chromatograms of a time course oxidation experiment on diDHA PC.

A (1h)

B (6h)

C (48h)

D (92h)
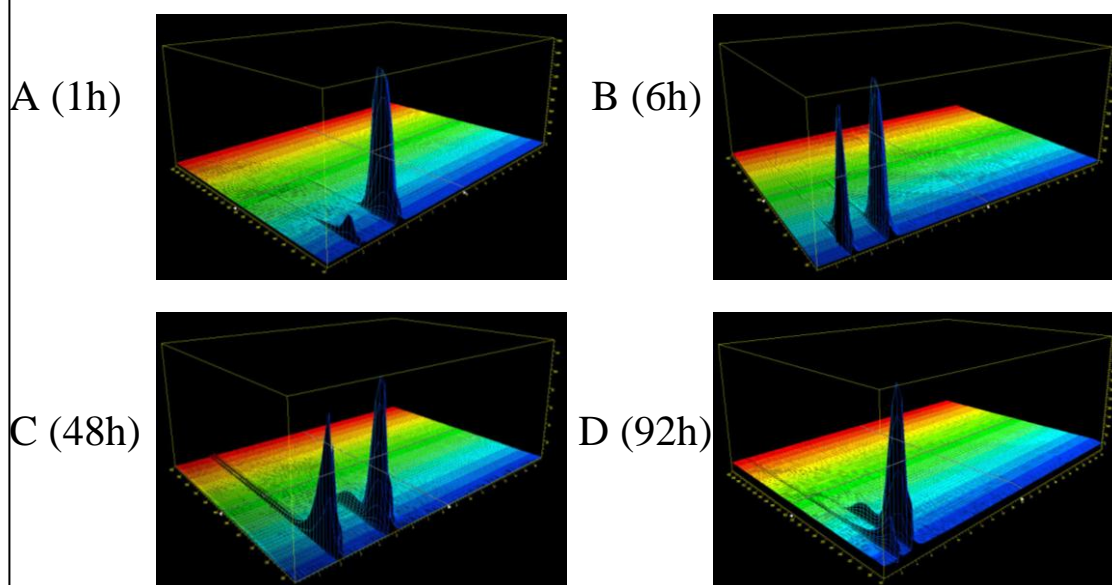
Table 3 - 210nm absorbance versus 234nm absorbance over a given time course at rtp (22ºC)

Time (hr)	210nm abs (mAU)	234nm abs (mAU)	% oxidation
0	1637.4 ± 142.6	16.9 ± 2.4	1.0 ± 0.2
0.5	1627.0 ± 175.8	17.0 ± 1.6	1.0 ± 0.1
1	1610.6 ± 156.4	18.3 ± 3.1	1.1 ± 0.2
2	1594.5 ± 236.3	19.5 ± 2.7	1.2 ± 0.1
4	1536.8 ± 163.2	20.2 ± 1.9	1.3 ± 0.4
6	1578.1 ± 189.2	21.5 ± 2.7	1.3 ± 0.2
24	1468.2 ± 135.1	25.7 ± 4.6	1.7 ± 0.5
48	1487.3 ± 126.9	47.6 ± 18.6	3.1 ± 0.8

The above figures and table are results obtained from oxidation of diDHA PC under RTP. Intensity of the unoxidised DHA PC appears to reduce proportionately over the given time frame reaching around 3.1% oxidation after 48 hours. The increase in 234nm absorbance can be clearly seen in figure 17. In the chromatogram, the elution order went injection solvent followed by the phospholipid. In image A there is only one peak for the phospholipid at 210nm and then as you follow the time course a secondary peak appears behind the 210nm wavelength. This can be observed in image D, where you can see another peak absorbing at 234nm indicating the presence of conjugated lipid hydroperoxides.

Figure 18 - LC-UV chromatograms of Alzheimer and control samples
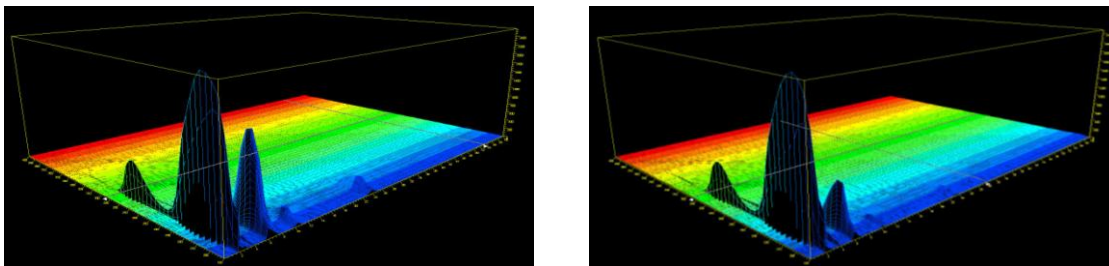
The elution order of samples in the chromatogram went injection solvent and antioxidant first, followed by SM second, PC third, PE fourth, PI fifth and then PS the last peak. The hydroperoxides elute at the same time but have a different wavelength to their non-peroxidised equivalents (210nm versus 234nm). This HPLC technique works differently to GC. In GC the largest abundance has the largest peak; here the largest peak refers to the strongest absorption. PS was higher in GC-MS determination, whereas PE is larger by this analysis due to its increased unsaturated content.

Figure 19 - Bar graphs showing levels of phospholipid hydroperoxides in AD and control
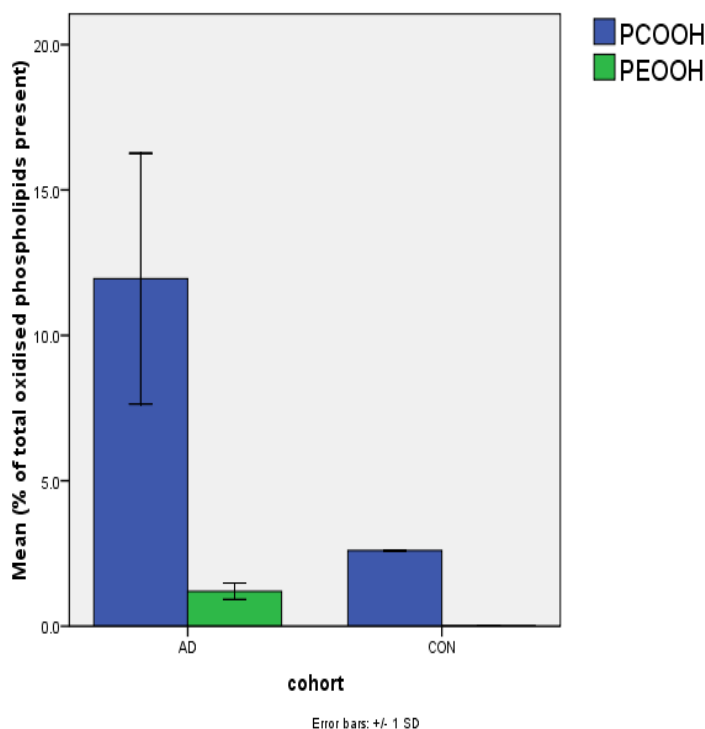
PC and PE hydroperoxides were observed to be lower in the AD samples compared with the control ones, PS and PI were detected but no oxidised derivative was found in the chromatograms hence their omission. This may indicate preferential oxidation in the AD state of PC over the other detectable phospholipids.

3.2c – *Post-mortem* delay and phospholipid hydroperoxide levels

Our collaborators provided us with the samples used in [71], where they confirmed that *post-mortem* tissue with time and temperature [RT (23ºC) and 4°C] as controllable variables, showed no detrimental reduction on the fatty acid levels. Additional to their study, phospholipid hydroperoxides were quantified to verify the levels of oxidation by HPLC-UV. On performing the analysis we were also able to confirm that there was no significant difference in CD levels, as observed in the figures overleaf. Each graph (figure 20) shows all the lipid classes in the chromatogram by percentage area of the designated peak. Across all the graphs there appear to be small changes in percentages of all detected lipids, whether nonperoxidised or peroxidised. This clearly supplements the conclusion that under these specific extraction and handling parameters, no sample deterioration is observed.

3.3 – Discussion of independent study

Phospholipid compositional analysis highlighted a percentage drop in PE, PC and PI with a corresponding reduction in PUFAs, which is in agreement with the general consensus of AD. DHA levels were lower in the AD samples compared with the controls across all the phospholipids. Of more importance is the increase in PS, with a reduced DHA content. PS, as previously stated, is the main phospholipid involved in apoptosis. Upon insult PS moves towards the cell surface, where the major PE and PC membranes exist, and acts as marker for damage. This damaged cell can then be removed by macrophages reducing potential damage. The change in composition of the affected membrane(s) would affect integrity, transmission and diffusion leading to cellular death. PI reduction indicates lowering synaptic transmission, affecting neural cells and their signalling pathways. PE and PC are core membrane based phospholipids and the reduction of these demonstrates further, the link between AD progression and the loss of membrane integrity.

The reduced level of DHA in the phospholipids of AD samples also agrees with the literature in table 1, whether this is due to breakdown or a dietary issue is undefined. Lower intake of DHA in AD may emphasise this loss or reduced metabolism of the DHA precursor, α-LNA. As we age the activity of desaturases, enzymes that introduce double bonds in the Sprecker pathway, begin to slow reducing production of key intermediates in fatty acid synthesis.

Figure 20 – HPLC-UV analysis of phospholipids and their respective hydroperoxides
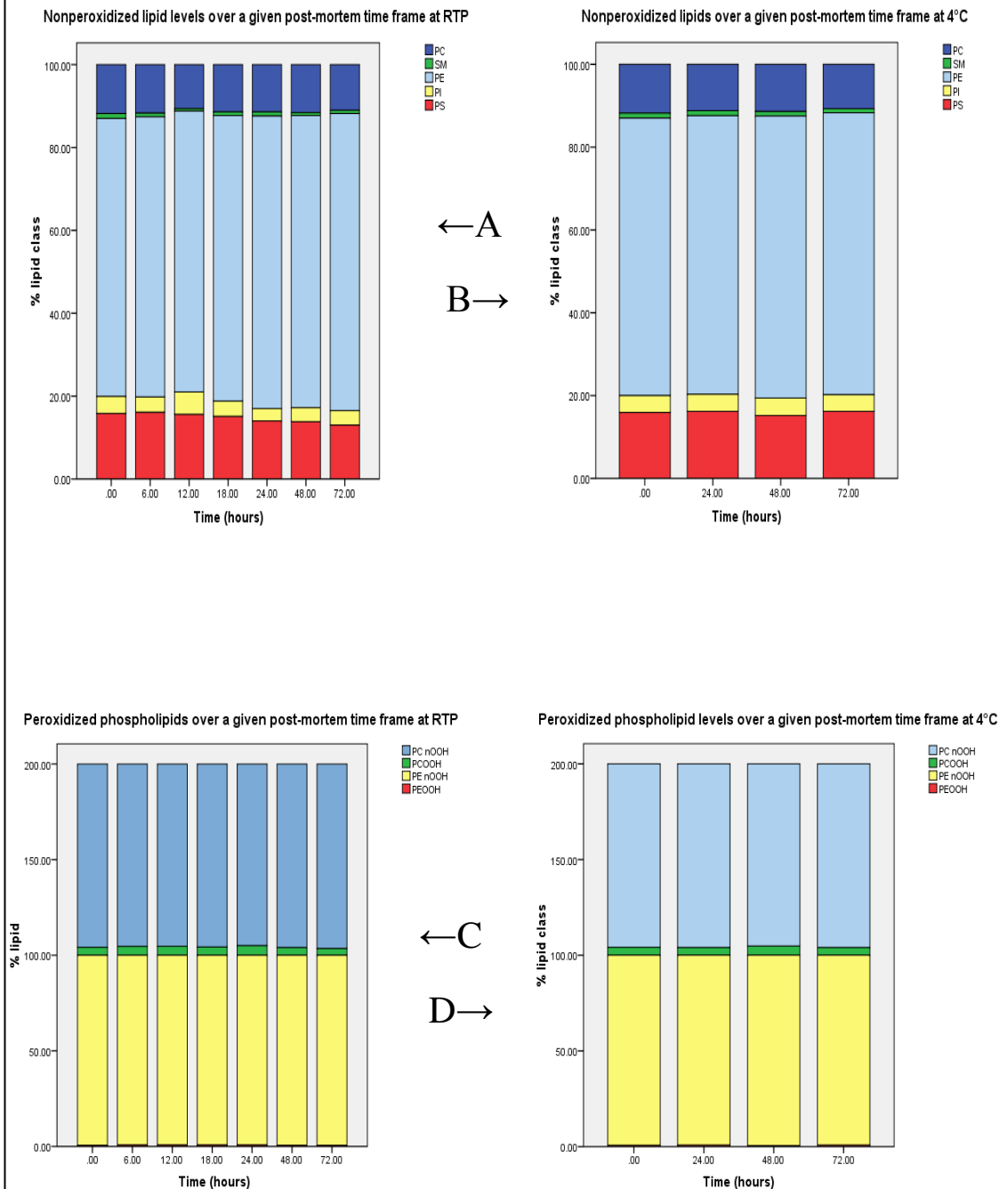
These four images show the affect of temperature and *post-mortem* delay on brain tissue samples (n=3) at six different times on phospholipid and phospholipid hydroperoxide levels. These charts represent the proportionality of each component in terms of their specific absorbance at the chosen wavelengths. At RTP over a 72 hour time frame (A), brain samples suffered no reduction in non-peroxidised components with the same conclusion at 4°C (B). The two lower graphs in the figure, show that the percentage levels of phospholipid hydroperoxides did not increase proportionately over the same time frame at RTP (C) and at 4°C (D).

DHA is critical to brain function, the brain can survive without it by replacing with DPA but this reduces the capabilities and will increase abnormalities [54]. Therefore supplementation of DHA in AD may be of therapeutic value.

The oxidation studies on diDHA phospholipids indicated no clear increase in conjugated lipid hydroperoxide over the time period. This clearly shows that DHA is not as susceptible to oxidation as is commonly believed [99, 196]. The large number of conformations and constant fluid motion of the PUFA chain makes hydrogen abstraction difficult. However once intiated the rate of oxidation would be elevated. The phospholipid, by which the PUFA is esterified onto, plays an extremely important role in defending the PUFA. The phospholipid headgroup can interact with oxidative aldehydes, through addition reactions, so reducing the potential oxidative damage [112]. Antioxidants present in the biological environment, would offer protection for the vulnerable PUFAs by either quenching lipid peroxidation or by providing an alternative substrate for hydrogen abstraction, therefore preventing further lipid membrane damage [104, 107].

Analysis of the samples used in [71], indicated that the *post-mortem* delay had no effect on phospholipid levels or their respectivehydroperoxides. Combining the outcomes of the diDHA time course and the *post-mortem* samples indicates that the breakdown of phospholipids and PUFAs *in vitro* is not detrimental. There was a specific increase in PCOOH in the AD state using a small cohort compared to the controls, which may again relate to exclusive oxidation of the membrane bound PC over the other major phospholipids. The reduction in DHA content within the PC pool as well as the increased PCOOH and PS levels, demonstrates that breakdown of PC and elevation of PS are unique to AD using this cohort.

3.4 – Discussion of method development

Analytically, we did not possess the GC-NICI-MS system and only had a GC-EI-MS system in which to employ a method for the quantification of the NPs. A number of EI-MS and CI-MS methods were thus evaluated to assess their performance and potential extension to NP quantification using the available instrumentation. Normally, a deuterated standard of a known mass would be used in IsoP and NP quantification, but

using EI-MS conditions they produce exactly the same retention times and fragments as the quantifiable material.

Using the same principal, seen in FAME quantification, a smaller chain prostanoid was implemented in union with a GC column which sorted fatty acids by chain length rather than degree of unsaturation. The smaller chain prostanoid in question was a PP. This nonenzymatic oxidation product of α-LNA, commonly found in plants, was generously given to us by Dr Thierry Durand along with the 4 series NP. In conjunction with the commercially available IsoP and acquired PP and NP, it allowed for simple chromatography to be obtained for method development, before quantification of these biomarkers in the biological samples was completed.

From thorough analysis of literature mass spectra, it was found that all the F-ring based prostanoids share two keys ions indicative of their structure. These ions were m/z 191 and 217 which are fragments of the cyclopentane ring where the two derivatised hydroxyls reside. The m/z 191 ion ($Me_3SiO+=CH-OSiMe_3$) is a rearrangement ion that is frequently a base ion of many F-ring compounds, and the m/z 217 ion ($Me_3SiO-CH^+-CH_2-CH^+-OSiMe_3$) [Figure 29] is usually a prominent ion in compounds with an F-ring [118, 157, 158, 163, 165]. From building the chromatographic library, it was discovered that the m/z 191 rearrangement fragment was increasingly more stable than the m/z 217 ion, therefore one SIM could be used for the quantification of all the F-ring IsoPs and NPs.

Using eleven carefully selected methods, only two methods were viable on the chosen system using the selected m/z 191 fragmentation ion. The nine methods which were not feasible included the following, Milatovic et al [132], Musiek et al [139], Imbusch et al [165], Wiswidel et al [194], Morrow et al [118, 148], Kyung Mee Kim et al [25, 155], Favreau et al [156], Praticó et al [157] and Waugh et al [158]. These methods either had issues with GC column thermal stability or quantification by EI-MS. This left only two methods with the potential to extend for NP quantification, Bessard et al [127] and Chiabrando et al [164]. Bessard et al [127] is a specific method for the quantification of IsoPs by EI-MS, in particular the 15 series IsoP. By monitoring the m/z 481 ion from 15-series IsoP fragmentation and quantification using a deuterated 15-series IsoP, it allows for definitive analysis on one series of IsoP.

Figure 21 – Prostanoid mass spectra and chromatography (A= IsoP [158], B= PP [154], C= NP [47] and D= Actual EI-MS spectra and chromatography)
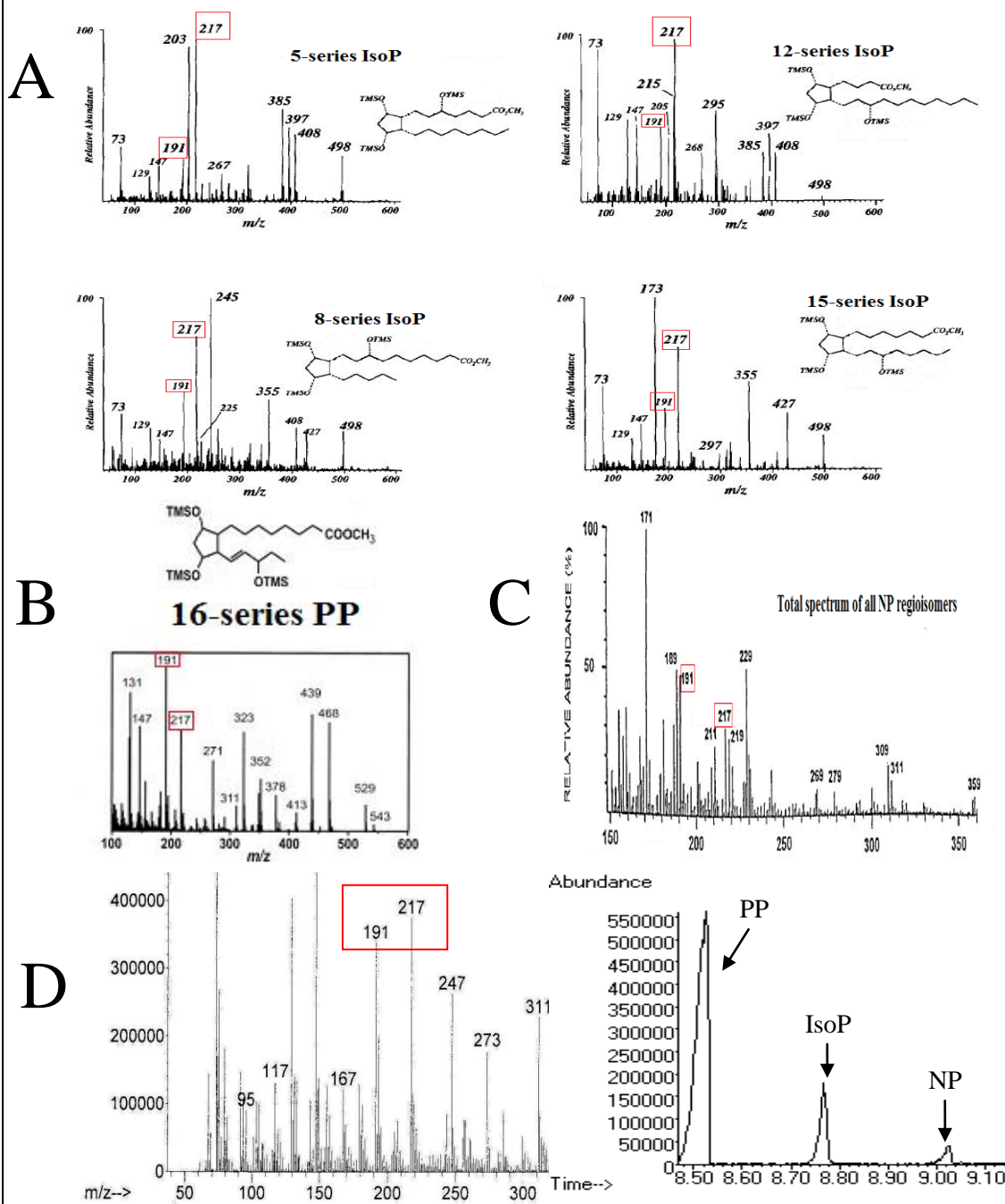
These four mass spectra are; (A) 5, 8, 12, 15 series IsoP, (B) 16-series PP, (C) all series of NPs and (D) actual NP spectra by EI-MS system and m/z 191 chromatograph of PP, IsoP and NP. The red boxes in the figures highlight the presence of the ions m/z 191 and 217 from several publications and (D) the actual fragments from PP using our system, along with SIM at m/z 191 showing PP, IsoP and NP. In the majority of spectra these two ions dominate within EI-MS chromatograms, which makes justification for there use in this new method important. Since PPs contain the same ions as the other classes, two ions can be monitored with carbon length changing the retention time, similar to FAMEs in GC.

However drawbacks for Bessard et al [127] included; specific quantification of one series rather than all F-rings, the chosen ions were not observed in the same intensity seen in paper when used and selective ion monitoring (SIM) chromatography signals were matched by noise. The other viable method was Chiabrando et al [164]. This utilised a short acquisition chromatographic runtime with a quick temperature ramp, best suited with the chosen column. Originally the method was used for IsoP quantification, but the chromatography allowed for the potential to extend towards NP quantification. Modification of the runtime, temperature ramp and using the target ions provided the means for NP quantification and more importantly a consistent platform for their analysis not performed using GC-EI-MS.

4

Pathway models of lipid oxidation

4.1 – Defining the pathway

A causal pathway model can be used to visualise the pattern of correlation between a set of variables to map the alternative pathway(s) of lipid oxidation in both AD and control states for both fatty acids, in terms of their specific endpoint markers. From either AA or DHA, initial CDs and LOOHs (denoted FOX in the pathway) can be formed in a combined conjugated LOOH component synonymous with classical oxidation mechanisms. The final endpoint biomarkers the aldehydes and IsoPs or NPs, can be created either from the peroxyl radical breaking the fatty acid in either a chain scission mechanism, or through endoperoxide formation which creates prostanoids.

The degree of unsaturation of both AA and DHA means that even the initial formation of the primary lipid oxidation product does not stop other methylene carbons from undergoing hydrogen abstraction, creating aldehydes or prostanoids from these sites. The putative links between all the markers used in this project are illustrated in the classical pathway model in figure 22. Not accounted for in this pathway are the roles of antioxidants, which can quench certain sections of the oxidation mechanism. These were independently correlated with each marker in the pathways to determine the roles antioxidants are having in defence or agonist in AD or control. Correlation data for each brain region was first acquired as a starting point for the pathway model to identify any initial relationships between all the assays utilised. Tables 4-7 show the correlation values (r) alongside the p values for each assay in the pathway. Following the pathway there appears to be a difference between the CD and LOOH in both brain regions, which was investigated prior to completing the pathway.

Figure 22 – Classical oxidation pathway linking several assays performed
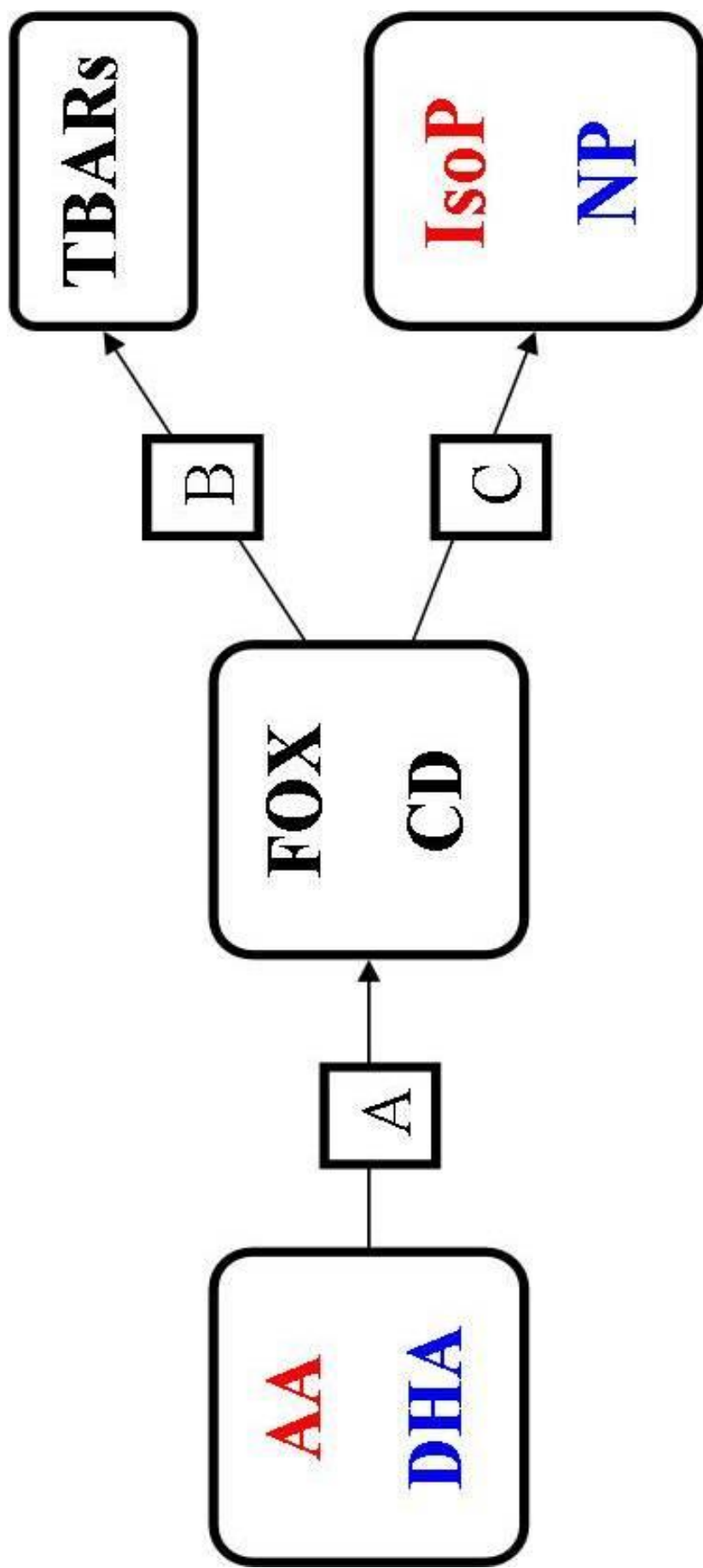
This pathway details how each assay is linked in the oxidation mechanism. Pathway A specifies the initial hydrogen abstraction produces a CD with hydroperoxide formation. From this combined primary product, two different final biomarkers can be produced namely the aldehydes, labelled pathway B, and IsoP and NP depending on whether AA or DHA is being oxidised as the alternative, pathway C. This model details the classical mechanism where both the initial CDs and LOOHs are formed at the same time.

In the frontal region, CDs were negatively correlated with LOOH (p = 0.034) and the temporal region showed no correlation between them (p = 0.822). There should be no difference between these two markers as they are formed *in situ* following the classical mechanism. However this disparity appearing in this analysis between these two products in the means of the two tests and the correlation results, supplements their removal from the pathway model.

These initial markers (CD and LOOH) of oxidation are transient intermediates in the mechanism and while the performing of the assays is standard, the results show a difference between these two. The chromophore for CD determination at 234nm can suffer from other components absorbing at this wavelength [197]. The FOX assay also has drawbacks, revolving around low linear range and low reproducibility [198]. Therefore FOX and CD results were combined to create a new variable known as FOXCD, rather than discard the pathway model and question the classical oxidation mechanism without any supplementary experiments. This new variable along with the initial fatty acids precursors was used to determine correlation values to see whether aldehydes or ring formation was favoured. Detailed analysis of each pathway for both fatty acids is discussed in the next two sections.

4.2 – Pathway model for DHA breakdown

When DHA is oxidised it can produce the primary oxidation product, the combined classical conjugated lipid hydroperoxide which can then follow independent pathways to final biomarkers (NPs and TBARs) as previously discussed. The tables below show the correlation data between each assay for each brain region and tissue state. In the control frontal lobe the FOXCD variable in the DHA mechanism appeared to show that aldehydes were being favoured in formation compared to the NPs. Specifically as the levels of FOXCD decreased the levels of aldehydes increased, however when correlating the initial fatty acid against the endpoint markers it was observed that correlation (positive) was greater with NPs than the aldehydes. The results obtained from the temporal region in the control cohort agreed with the previous frontal results, with the favoured endpoint being the aldehydes, but in this instance the initial fatty acid correlated greater with the aldehydes rather than the NPs.

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Table 4 – Correlation analysis of total data sets for DHA oxidation within the frontal lobe

Correlations

		FRdha	FRnp	FRcd	FRfox	FRtbars
FRdha	Pearson Correlation (*r*)		.172	.223	-.168	-.124
	Sig. (2-tailed) (p)		.192	.081	.196	.342
FRnp	Pearson Correlation (*r*)	.172		.067	.035	.206
	Sig. (2-tailed) (p)	.192		.595	.783	.102
FRcd	Pearson Correlation (*r*)	.223	.067		-.257*	-.061
	Sig. (2-tailed) (p)	.081	.595		.034	.620
FRfox	Pearson Correlation (*r*)	-.168	.035	-.257*		.219
	Sig. (2-tailed) (p)	.196	.783	.034		.072
FRtbars	Pearson Correlation (*r*)	-.124	.206	-.061	.219	
	Sig. (2-tailed) (p)	.342	.102	.620	.072	

*. Correlation is significant at the 0.05 level (2-tailed).

Table 5 – Correlation analysis of total data sets for AA oxidation within the frontal lobe

Correlations

		FRaa	FRip	FRcd	FRfox	FRtbars
FRaa	Pearson Correlation (*r*)		.347**	.128	-.181	-.386**
	Sig. (2-tailed) (p)		.006	.300	.145	.001
FRip	Pearson Correlation (*r*)	.347**		-.053	-.165	-.179
	Sig. (2-tailed) (p)	.006		.682	.205	.167
FRcd	Pearson Correlation (*r*)	.128	-.053		-.257*	-.061
	Sig. (2-tailed) (p)	.300	.682		.034	.620
FRfox	Pearson Correlation (*r*)	-.181	-.165	-.257*		.219
	Sig. (2-tailed) (p)	.145	.205	.034		.072
FRtbars	Pearson Correlation (*r*)	-.386**	-.179	-.061	.219	
	Sig. (2-tailed) (p)	.001	.167	.620	.072	

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

As observed in the classical oxidation pathway, both CDs and LOOHs are being regarded as the same component, addition of molecular oxygen to the pentadienyl radical causes the double bonds to flip into a conjugated system. In the frontal DHA correlation we see that DHA oxidises to form CDs (first yellow box), a positive association ($r = 0.223$, $p = 0.081$), over LOOHs (adjacent yellow box) which was a negative association ($r = -0.168$, $p = 0.196$). The opposite was observed with AA, a more significant negative correlation with LOOHs ($r = -0.181$, $p = 0.145$) over the CD ($r = 0.128$, $p = 0.300$). However in both tables, there is a clear dissociation between CD and FOX. Interpretation of this indicates that as CD decrease LOOHs increase or as LOOHs decrease the CD are increasing and is a significant negative correlation (green boxes; $r = -0.257$, $p = 0.034$).

Table 6 – Correlation analysis of total data sets for DHA oxidation within the temporal lobe

Correlations

		TPdha	TPnp	TPcd	TPfox	TPtbars
TPdha	Pearson Correlation (*r*)		.103	.099	.224	-.010
	Sig. (2-tailed) (p)		.420	.432	.075	.938
TPnp	Pearson Correlation (*r*)	.103		.044	-.075	-.048
	Sig. (2-tailed) (p)	.420		.723	.561	.711
TPcd	Pearson Correlation (*r*)	.099	.044		-.028	.269*
	Sig. (2-tailed) (p)	.432	.723		.822	.030
TPfox	Pearson Correlation (*r*)	.224	-.075	-.028		.171
	Sig. (2-tailed) (p)	.075	.561	.822		.164
TPtbars	Pearson Correlation (*r*)	-.010	-.048	.269*	.171	
	Sig. (2-tailed) (p)	.938	.711	.030	.164	

*. Correlation is significant at the 0.05 level (2-tailed).

Table 7 – Correlation analysis of total data sets for AA oxidation within the temporal lobe

Correlations

		TPaa	TPip	TPcd	TPfox	TPtbars
TPaa	Pearson Correlation (*r*)		-.099	-.039	.219	-.271*
	Sig. (2-tailed) (p)		.716	.758	.082	.030
TPip	Pearson Correlation (*r*)	-.099		-.249	-.152	.001
	Sig. (2-tailed) (p)	.716		.336	.574	.996
TPcd	Pearson Correlation (*r*)	-.039	-.249		-.028	.269*
	Sig. (2-tailed) (p)	.758	.336		.822	.030
TPfox	Pearson Correlation (*r*)	.219	-.152	-.028		.171
	Sig. (2-tailed) (p)	.082	.574	.822		.164
TPtbars	Pearson Correlation (*r*)	-.271*	.001	.269*	.171	
	Sig. (2-tailed) (p)	.030	.996	.030	.164	

*. Correlation is significant at the 0.05 level (2-tailed).

In the temporal DHA correlation we see that DHA oxidises to form LOOHs (first yellow box), a positive association ($r = 0.224$, p = 0.075), over CD (adjacent yellow box) which was also a positive association ($r = 0.099$, p = 0.432). The same was observed with AA, a more significant positive correlation with LOOHs ($r = 0.219$, p = 0.082) over the CD ($r = -0.039$, p = 0.758). However in both tables, there is a clear dissociation between CD and FOX. Interpretation of this indicates that CD and LOOHs have no significant correlation in either brain regions. In the classical pathway both CDs and LOOHs are formed in union, but this early correlation analysis appears to show that in the temporal region (green box) no correlation between the two ($r = -0.028$, p = 0.822), implying that one of these can be increasing or decreasing with no direct affect on the other.

Figure 23 – Pathway model for correlations between markers
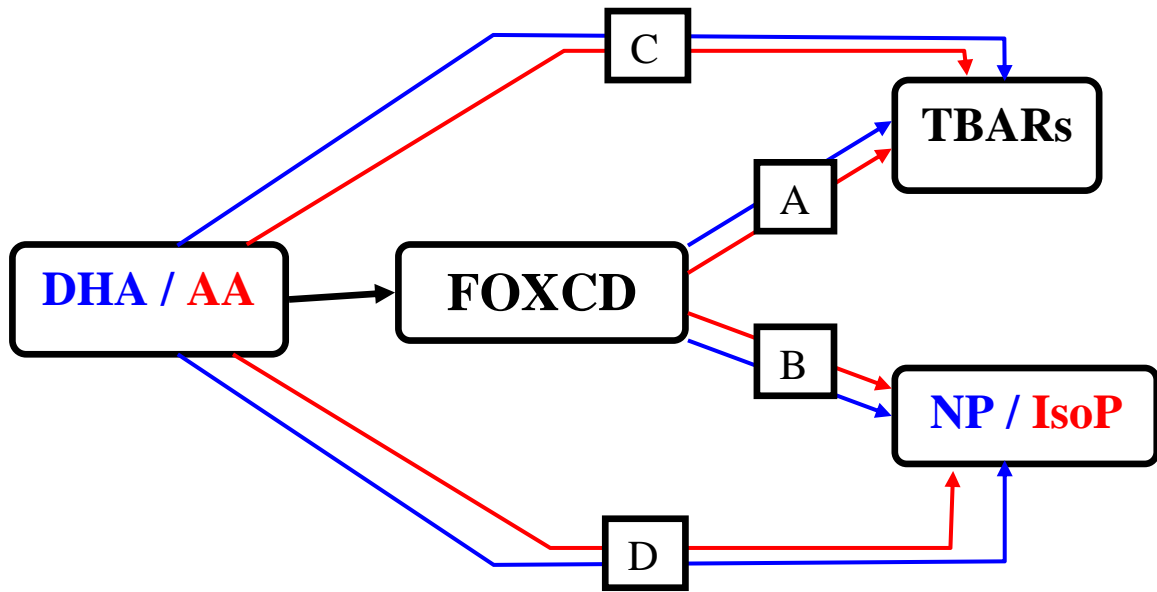
Table 8 – Correlation values for DHA oxidation (blue arrow)

Pathway	Control samples		AD samples	
	Frontal lobe	Temporal lobe	Frontal lobe	Temporal lobe
A	-0.370*	0.041	0.088	0.102
B	-0.050	0.015	0.122	0.159
C	-0.089	0.362*	-0.138	-0.205
D	0.336	-0.127	0.037	0.263
* = Correlation is significant at the 0.05 level (2-tailed)				
MODERATE relationship (± 0.6 - ± 0.4) = Blue, WEAK relationship (± 0.3 - ± 0.1) = Green				

Table 9 – Correlation values for AA oxidation (red arrow)

Pathway	Control samples		AD samples	
	Frontal lobe	Temporal lobe	Frontal lobe	Temporal lobe
A	-0.370*	0.041	0.088	0.102
B	0.145	-0.035	-0.013	0.621
C	-0.489**	-0.391*	-0.227	-0.244
D	0.470*	-0.172	-0.048	0.833
* = Correlation is significant at the 0.05 level (2-tailed)				
** = Correlation is significant at the 0.01 level (2-tailed)				
STRONG relationship (± 0.9 - ± 0.7) = Red, MODERATE relationship (± 0.6 - ± 0.4) = Blue, WEAK relationship (± 0.3 - ± 0.1) = Green				

With the AD results, the frontal region showed that the FOXCD variable had a stronger positive correlation with the NPs, once again DHA was observed to show a weak negative correlation to aldehydes rather then the NPs. The temporal lobe correlation results showed that both the FOXCD and DHA variables positively correlated with the NPs. Initial findings indicate that in the frontal and temporal regions DHA oxidised to yield aldehydes in the control samples, where as the AD favours the ring formation pathway creating NPs suggesting a specific oxidation pathway seen earlier in the elevated PCOOH assay.

4.3 – Pathway model for AA breakdown

The same pathway illustrated above can be performed on AA and its specific oxidation products for both regions and disease states. IsoPs are only created from AA oxidation, so their results are independent of the DHA derived NPs. In the frontal control lobe FOXCD was negatively correlated with the aldehydes over the IsoPs and this was also observed with the correlation between AA and TBARs. Of note in the frontal lobe was the positive correlation between AA and IsoPs which is in complete contrast to the aldehydes. The control temporal lobe samples appeared to show that FOXCD broke down to form aldehydes, with the AA variable negatively correlated to aldehydes. With the AD frontal samples, FOXCD and AA levels correlated better with the aldehydes than the IsoPs. In the AD temporal region, the strongest positive correlation values observed were between both FOXCD and AA with IsoPs. These outcomes differ from the previous DHA pathway model. In AD DHA oxidised to form the cyclic prostanoids in both brain regions, AA oxidation appears to depend upon the brain region. Frontal lobe results suggest that chain scission was occurring forming aldehydes, but in the temporal region ring formation is favoured.

4.4 – Antioxidant interactions in the pathway model

In the control cohort no significant findings were found between antioxidant assay and any of the markers, table 16. Of interest was the positive association with both IsoP and NP, as antioxidants increased these two appear to increase indicating the role of these protective compounds in the final reduction stage in the formation of prostanoids. This same characteristic seems to also apply to the aldehydes as they increase with elevating levels of antioxidants.

Figure 24 – Interaction between antioxidants and the assays used in the pathway models
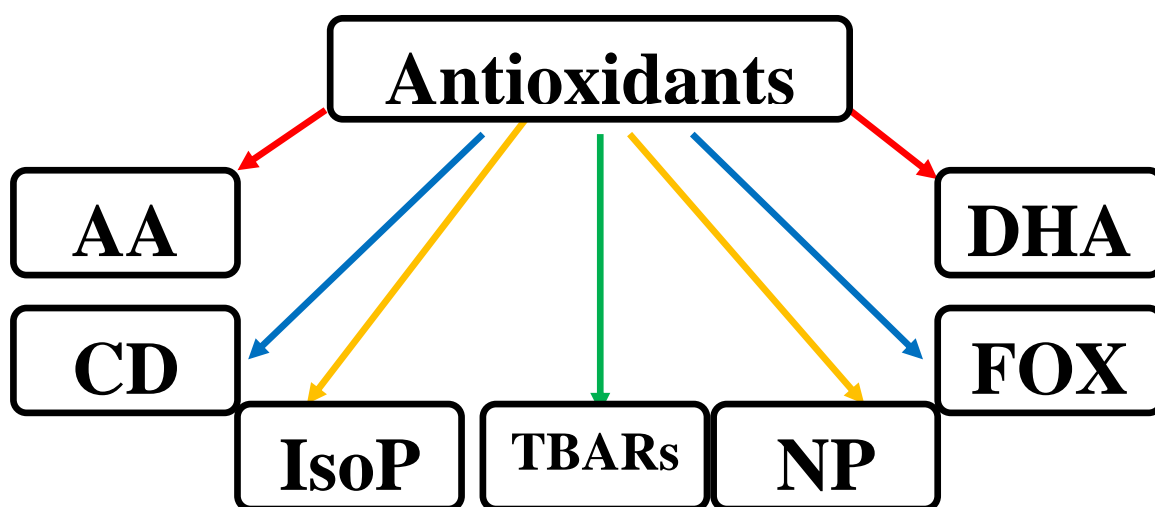
The coloured arrows represent the diversity of roles in which antioxidants can affect the respective marker. AA and DHA can be actively defended by antioxidants in reducing hydrogen abstraction by sacrificing themselves. CD and FOX can be quenched by antioxidants stopping propagation or by stopping the initial abstraction required for the formation of these two products. IsoP and NP are different, as antioxidants can perform two separate roles. They can stop the formation of these compounds initially, but they can also reduce the endoperoxide to form these components therefore actually increasing the amount of F-rings. Aldehydes can promote further oxidation which the antioxidants can suppress.

Table 10 – Correlation values between antioxidant levels and the assays used in the pathway model

Assay	Control samples		AD samples	
	Frontal lobe	**Temporal lobe**	**Frontal lobe**	**Temporal lobe**
AA	-0.065	-0.160	-0.065	-0.148
DHA	-0.005	-0.197	-0.212	-0.287
IsoP	0.283	-0.345	-0.094	-0.342
NP	0.188	0.245	0.301	-0.165
CD	-0.025	-0.062	-0.136	-0.137
FOX	0.035	-0.474**	0.264	0.396*
TBARs	0.222	-0.103	0.386*	0.282

* = Correlation is significant at the 0.05 level (2-tailed)

** = Correlation is significant at the 0.01 level (2-tailed)

MODERATE relationship (± 0.6 - ± 0.4) = Blue, WEAK relationship (± 0.3 - ± 0.1) = Green

The temporal region highlights a strong significant affect on the LOOH levels. As antioxidants are reduced in the control samples, this is causing the LOOHs to increase because of the lower defence mechanisms preventing the initial hydrogen abstraction and oxygen insertion. Within the frontal lobe of AD samples the aldehyde assay was the most significantly correlated, with the increasing antioxidants elevating the TBARs response. This contradicts the role antioxidants have in protecting vulnerable components from oxidation. Unless antioxidants are stabilising the lipid peroxyl radicals, yielding LOOHs which would then appear to be breaking down to aldehydes through chain scission.

Over the other assays it appears that the oxidation pathway is being ushered in favouring aldehyde formation. FOX and NP are also positively correlated with antioxidants, suggesting that the reduction processes are converting any unstable endoperoxides or peroxides to either F-ring prostanoids or LOOHs by supplying the hydrogen required for this process. In the temporal lobe the most significant correlated assay was the FOX, demonstrating that as the antioxidant levels increased the concentration of LOOHs raised with them.

4.5 – Significance of pathways for both DHA and AA breakdown

From the AA and DHA breakdown models the following pathways can be predicted to be significant biochemically. Damaged fatty acids appear to be breaking down into reactive aldehydes more than the cyclic prostanoids in both control brain regions. This suggests that the oxidised fatty acids are fragmenting into shorter chain products by chain scission. The frontal region highlighted negative correlations between FOXCD and TBARs, suggesting that conjugated lipid hydroperoxides are being broken down to aldehydes. In the temporal lobe a steady state of formation between FOXCD and TBARs variables appears to be happening. One difficulty faced with the TBARs assay is that the products of enzymatic and non-enzymatic formation of aldehydes cannot be differentiated so we cannot determine whether this is a true representation of non-enzymatic oxidation.

In AD samples, FOXCD followed NP formation instead of TBARs in a steady state formation cycle. From both lobes you can clearly see different processing in the DHA oxidation pathway with the control samples preferring aldehyde formation and the AD samples favouring NPs when analysing the correlational data. Following the same

principles governed in the DHA pathway, it is now possible to investigate if there were any differences in the AA oxidation process between lobes and disease state. For both brain regions in the control cohort it can clearly be observed that aldehydes are favoured in formation compared with the IsoPs. Frontal region analysis shows that both FOXCD and AA levels are reduced with increasing formation of aldehydes. The AD cohort in the frontal region highlights a different preferred endpoint biomarker to the temporal region. Within the frontal lobe greater correlation was observed with the aldehydes and in the temporal lobe the IsoPs. This suggests that damaged fatty acids undergo chain fragmentation in the frontal lobe and ring formation in the temporal of AD samples.

5

Large cohort statistical analysis

FOREWORD

The following chapters will discuss both sets of results obtained by us and from our collaborative group, (Dementia Research Group, Institute of Clinical Neurosciences, University of Bristol, Frenchay Hospital) highlighted at the beginning of the relevant sections similar to the system stated at the start of chapter 2. These results are being utilised within this thesis to understand relationships between multiple factors involving the disorder. The following assays were completed by our collaborators, DHA and AA composition, LOOHs, antioxidant status and aldehyde content while at Aston we completed CD and prostanoid quantification. Individual sample results can be found in appendix A and B.

5.1 – Cohort only statistical analysis

The advantages of this project are the copious variables available to investigate the involvement of lipid peroxidation and eventual breakdown in AD pathogenesis, through analytical assays and classification data. Independent variables (fatty acids, antioxidants, or other biomarkers) have been investigated in separate literature studies, often with fluctuating cohort sizes. An example of which is the PUFA data, Skinner et al [175] which used 25 *post-mortem* samples [largest quoted compiled of 10 control, 15 AD], we analysed 71 *post-mortem* samples [35 control, 36 AD] which is part of a larger 172 cohort [58 control, 114 AD [179]]. As discussed previously, Braak staging [section 1.1b], APOE gene [section 1.1d(ii)], along with *post-mortem* delay and age at death were introduced into the statistical analysis alongside the assays. These extra variables utilised

herein allow for a more in-depth examination into AD, with greater statistical power primarily from a larger cohort size and secondarily with the variables presented. In order to perform statistical analysis, the software SPSS, (SPSS Inc) was used to conduct analysis of variance and correlation coefficients.

Each assay was plotted against *post-mortem* delay and age at death to observe whether any detrimental effects occurred from these two time dependent factors (graphs can be found in the appendix C) either from breakdown under sample preparation or from normal ageing processes which would reduce the association with AD pathogenesis. The IsoP assay highlighted a slight dependence with *post-mortem* delay and age but this was attributed to outliers in the results. Agreeing with our collaborators [71] *post-mortem* delay was found to have no effect on any other assay for the entire cohort size, with no additional trends witnessed with age at death. On this basis both PM delay and age were removed from further statistical analyses.

The table overleaf denotes sample number, APOE genotype, Braak staging (0-VI), *post-mortem* delay (hours) and finally the age (years) at which the patient died. Statistical analysis will be performed on individual brain regions in the software; this is due to missing data from certain assays in specific regions which will reduce the inherent statistical power, this lowers the amount of samples viable for the determination. This is fundamental in analysis of the Braak staging and temporal lobe, where the IsoP assay had to be removed from the analysis.

alysis of the effects within each brain region was performed using a multivariate analysis of variance (MANOVA) with cohort as the lone independent variable. In this context, MANOVA provides an analysis technique to examine multiple dependent variables simultaneously without inflating the type one error rate as would results from multiple independent tests. The data for the frontal lobe were analysed with a single factor (control vs AD) MANOVA with AA, DHA, IsoP, NP, CD, ABTS, FOX and TBARs as dependent variables. The analysis revealed overall significant effect of cohort ($F_{(8, 45)} = 2.627$, $p = 0.019$; Wilks $\lambda = 0.682$). Wilks' lambda is the most commonly used F-value as advised by Tabachnick and Fidell (1997) [199].

Table 11 – Clinical data on Alzheimer and control samples

Control					Alzheimer				
Sample	1=no 2=yes	O-6 Braak stage	Post mortem delay (hours)	Age at death	Sample	1=no 2=yes	0-6 Braak stage	Post mortem delay (hours)	Age at death
16	1	0	46.00	95	190	1	5	71.00	89
36	1	2	24.00	78	196	2	6	77.00	78
57	1	3	35.00	82	203	1	2	88.00	79
79	1	2	103.00	81	205	2	5	9.00	78
98	1	2	62.00	88	211	1	1	44.00	83
103	1	2	106.00	76	213	2	6	42.00	81
106	1	0	72.00	88	219	2	4	43.00	77
110	1	2	18.00	93	224	1	4	53.00	96
112	1	0	92.00	80	228	2	5	72.00	87
120	1	2	28.00	88	241	2	5	67.00	87
122	1	2	30.00	82	242	1	3	70.00	79
127	1	3	48.00	84	243	1	6	79.00	88
130	1	2	48.00	75	284	2	6	24.00	82
147	1	1	33.00	73	287	2	4	58.00	85
150	1	2	66.00	69	288	2	5	43.00	83
206	1	1	59.00	73	289	1	3	43.00	91
254	1	3	40.00	90	290	1	5	82.00	89
269	1	2	24.00	83	302	2	6	25.00	70
295	1	2	3.00	82	318	2	2	42.00	71
321	1	.	24.00	79	326	2	5	48.00	69
333	1	1	17.00	84	330	2	5	50.00	74
336	2	2	37.00	82	341	1	3	48.00	95
352	1	0	24.00	72	508	2	5	5.00	80
355	1	1	48.00	78	538	1	6	43.00	63
390	1	2	56.00	82	558	2	6	9.00	64
402	1	2	23.00	76	561	2	5	24.00	74
407	2	2	60.00	91	568	2	6	21.00	78
412	2	3	96.00	82	573	2	5	39.00	89
461	2	3	10.00	77	580	1	6	90.00	65
467	2	3	6.00	75	592	1	6	22.00	65
563	2	4	79.00	48	601	1	6	61.00	68
678	2	2	216.00	84	683	1	5	48.00	83
714	2	3	35.00	73	685	1	5	48.00	74
721	2	2	5.50	90	687	.	6	5.00	60
733	2	3	37.75	93	713	2	6	24.50	62
					725	2	5	12.00	69

Additional data on the samples, this includes APOE genotype (1= 0 alleles, 2= 1 or more alleles denoted no and yes), Braak staging (0-6), *post-mortem* delay in hours and finally age at which the patient died. This data was released after the experiments were finished, under the double-blind study requirements to improve the validity of the work conducted.

Post-hoc analysis of the between group effects revealed that ABTS levels (antioxidant assay) were significantly different between groups. Evaluating the means of the ABTS results in the frontal lobe clearly showed an elevated level in the AD state, whether this is a host response to oxidation or overproduction is questionable and requires further investigation. The data acquired from the temporal lobe were also analysed with a single factor (control vs AD, table 12) MANOVA with AA, DHA, IsoP, NP, CD, ABTS, FOX and TBARs as dependent variables. The analysis revealed that there was no overall significant effect in the cohort ($F(8, 4) = 0.576$, p = 0.765; Wilks $\lambda = 0.465$). This outcome defers any *post-hoc* analysis in the temporal lobe because of the insignificance in this analysis. Contrary to the findings in the frontal analysis, no significant overall effect of cohort was found for any of the assays in the temporal lobe. The results suggest that pathogenesis of AD preferentially effects the frontal region under this cohort MANOVA analysis, with the frontal antioxidants being the most significant assay in differentiating between AD and control.

Table 13 shows the descriptive analysis of the cohort MANOVA, with the means and deviation for each assay. For the two fatty acids, across both brain regions, there was a reduction DHA and AA in the AD state from a mean-based analysis. This difference agrees with the common theory of lower fatty acids in AD compared to control samples however subjectively, these findings are not significant due to the variation in the standard deviation. DHA and AA levels appear to have no relevance in the determination or differentiation between AD and control samples which agress with Skinner et al [175].

CDs and LOOHs are closely correlated; formation of the hydroperoxide is accompanied by bonds migrating to form conjugated bonds. Initial analysis of the CD and LOOH levels shows that the control samples, regardless of brain region were higher than the AD samples. With the consensus on enhanced oxidation in AD, these primary products of lipid breakdown should be elevated in this disease. Nevertheless, CD and LOOH determination does not show how far the process of oxidation has progressed. If these compounds have broken down to further downstream biomarkers then a reduction in CD will occur unless you have a steady state of formation and conversion. Sample variation appears to also negate any conclusions, especially when the frontal samples have a larger deviation than the mean.

Table 12 – Cohort statistical analysis including variables significant in the MANOVA in both frontal and temporal regions

Frontal Multivariate Tests[b]

	Effect	Value	F	Hypothesis df	Error df	Sig.
cohort	Pillai's Trace	.318	2.627[a]	8.000	45.000	.019
	Wilks' Lambda	.682	2.627[a]	8.000	45.000	.019
	Hotelling's Trace	.467	2.627[a]	8.000	45.000	.019
	Roy's Largest Root	.467	2.627[a]	8.000	45.000	.019

Tests of Between-Subjects Effects in the Frontal region

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Cohort	FRdha	7.569	1	7.569	2.069	.156
	FRaa	.355	1	.355	.748	.391
	FRIsoP	259.283	1	259.283	1.294	.261
	FRnp	.263	1	.263	.008	.930
	FRcd	.568	1	.568	.205	.652
	FRabts	73.222	1	73.222	11.606	.001
	FRfox	1.202E8	1	1.202E8	2.838	.098
	FRtbars	.267	1	.267	.014	.906

Temporal Multivariate Tests[b]

	Effect	Value	F	Hypothesis df	Error df	Sig.
cohort	Pillai's Trace	.535	.576[a]	8.000	4.000	.765
	Wilks' Lambda	.465	.576[a]	8.000	4.000	.765
	Hotelling's Trace	1.151	.576[a]	8.000	4.000	.765
	Roy's Largest Root	1.151	.576[a]	8.000	4.000	.765

Tests of Between-Subjects Effects in the Temporal region

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Cohort	TPdha	4.617	1	4.617	1.971	.188
	TPaa	.124	1	.124	.338	.573
	TPIsoP	2.160	1	2.160	1.256	.286
	TPnp	32.611	1	32.611	.465	.509
	TPcd	.307	1	.307	.797	.391
	TPabts	5.005	1	5.005	.613	.450
	TPfox	4.561E7	1	4.561E7	1.573	.236
	TPtbars	12.939	1	12.939	1.084	.320

The data for the frontal lobe were analysed with a single factor (control vs AD) MANOVA with AA, DHA, IsoP, NP, CD, ABTS, FOX and TBARs as dependent variables. The analysis revealed overall significant effect of cohort ($F(8, 43) = 2.627$, $p = 0.019$; Wilks $\lambda = 0.682$). *Post-hoc* analysis of the between group effects revealed that ABTS levels (antioxidant assay) were significantly different between groups. In the temporal region there was no significant difference the variables between AD and control, making *post-hoc* analysis difficult unlike the frontal lobe.

Table 13 – Descriptive analysis of the cohort MANOVA analysis

1=Control 2=Alzheimer		FRdha (% of fatty acids)	FRaa (% of fatty acids)	FRip (µg/g tissue)	FRnp (µg/g tissue)	FRcd (mmol dm⁻³/g)	FRabts TEAC per g (µmol/g)	FRfox (CHPE/g wet weight)	FRtbars (nmol MDA/g tissue)
1	N	33	33	27	29	33	33	33	33
	Mean	**15.52**	**8.63**	**8.34**	**7.76**	**1.53**	**11.09**	**6859.68**	**12.01**
	Std. Deviation	1.36	0.80	23.83	7.07	1.85	2.13	6814.09	4.80
2	N	31	36	35	36	36	35	35	35
	Mean	**14.49**	**8.31**	**1.55**	**6.27**	**1.17**	**13.01**	**4210.37**	**12.63**
	Std. Deviation	2.32	0.63	3.91	5.70	1.59	2.91	5249.77	3.89

1=Control 2=Alzheimer		TPdha (% of fatty acids)	TPaa (% of fatty acids)	TPip (µg/g tissue)	TPnp (µg/g tissue)	TPcd (mmol dm⁻³/g)	TPabts TEAC per g (µmol/g)	TPfox (CHPE/g wet weight)	TPtbars (nmol MDA/g tissue)
1	N	34	34	12	31	33	30	33	33
	Mean	**15.01**	**8.96**	**5.76**	**4.15**	**1.08**	**10.64**	**5091.60**	**13.00**
	Std. Deviation	1.54	0.98	15.77	5.85	0.68	2.28	5316.74	2.77
2	N	33	33	5	35	35	30	35	35
	Mean	**14.62**	**8.45**	**0.10**	**6.52**	**0.84**	**12.98**	**1920.63**	**12.68**
	Std. Deviation	2.06	0.67	0.05	7.14	0.50	2.50	2980.72	3.99

Data from all the assays are summarised in the above two tables for both brain regions for control and AD. There were no differences between DHA and AA content, prostanoid quantification, CD levels and TBARs. LOOHs were lower in AD compared to the control, but there was also a large amount of variation making any conclusions difficult. *Post-hoc* analysis of the between group effects of the cohort MANOVA revealed that ABTS levels (antioxidant assay) were significantly different between groups. In the temporal region there was no significant difference the variables between AD and control. The data in the tables indicate that levels of antioxidants in the frontal region were higher in AD ($p = 0.001$), highlighted yellow in the first table.

With the newly developed method, the levels of the F-ring prostanoids were quantified in both frontal and temporal lobes in both disease tissue states. In the frontal and temporal lobes, control samples had higher IsoP averages compared to AD. NP levels were higher in the temporal region for AD samples, with little comparative difference observed in the frontal region. This demonstrates that in the temporal region, higher DHA oxidation is occurring, which may be of consequence of AD pathogenesis. However, interpretation of the results is difficult because of the standard deviations, which again nullify any conclusions.

Across the brain regions and individual sample groups, it was observed that there is no difference between AD and control in terms of the shorter chain aldehydes. The difference between the two is the lowest seen compared with the other assay averages. This can be concluded because the standard deviation is lower than the previous tests, which was so influencial in commenting on previous results. In previous oxidation tests (except CD) the mean and standard deviation varied by a large amount. FOX and prostanoid markers suffered from high variation in mean and the deviation was greater than the average, precluding the ability to draw conclusions. It would appear that the aldehydes are being regulated in terms of their formation in both circumstances as the difference between control and AD samples is minuscule.

In the frontal lobe, AD samples had slightly higher levels of antioxidants than the respective control cohort. Under the oxidation theory these protective compounds should be lower, because of the increased initiation and propagation stages of lipid peroxidation, but this is not the case. This same finding is also observed in the temporal region, where AD samples had higher levels of antioxidants than the control samples. Under progressive interpretation of all the results, the elevated levels of antioxidants support the notion that the AD cohort does not exhibit enhanced oxidation. The next statistical analysis that was performed involved the introduction of the genetic variable, to investigate the link between the clinical data and all the assays performed. Both regions were analysed even though the temporal region demonstrated no significant statistical findings thus far.

5.2 – MANOVA frontal and temporal analysis (Cohort and APOE)

Tissue was classified into two groups based on the number of APOE Ɛ 4 alleles present in the specimen's sample: the first group has no APOE Ɛ 4 alleles; the second group had

at least one allele of this risk gene. Once more the samples were processed in terms of the eight dependent variables but with addition of the APOE grouping (Control and AD with APOE1 vs APOE2 as the independent variables MANOVA with AA, DHA, IsoP, NP, CD, ABTS, FOX and TBARs as dependent variables). Evaluating the APOE genotype individually from cohort, appears to show no significant variation across the eight assays, demonstrating that the presence of more alleles does not correlate with any increased lipid breakdown or production of measurable metabolites.

The analysis revealed a strong interaction effect between cohort and risk ($F(8, 43) = 5.026$, $p < 0.001$; Wilks $\lambda = 0.517$), with *post-hoc* tests indicating that LOOHs (FRfox, $p = 0.001$) was the most robust assay with IsoPs ($p = 0.053$), NPs ($p = 0.075$), CD ($p = 0.072$) and antioxidant ($p = 0.056$) being over $p = 0.05$ but being referred to as being trend-level significant. Introduction of the genetic parameter appears to be a very significant outcome detailing the difference between AD and control. Table 15 depicts that in both genotype groups that IsoP levels were considerably higher in the control state compared to the AD, but this is not clearly seen in figure 25.

Next, the temporal region samples using the same conditions were examined for any correlations. From the tables overleaf it was discovered that no assays were significant in the temporal region under the same parameters which contrasts with the findings reported above for the frontal lobe. The temporal data was analysed with a two factor (control vs AD, APOE1 vs APOE2) MANOVA with AA, DHA, IsoP, NP, CD, ABTS, FOX and TBARs as dependent variables. As seen with the earlier MANOVA, the temporal region had no significant results with introduction of the APOE genotype. This then implies that the frontal lobe seems to be the most affected by the variables.

As discussed earlier the early discovery of higher levels of antioxidants in the AD state accounts for the reduction in oxidation and in particular this biomarker. Due to the emphasis attached with IsoPs in oxidation, there is lower AA oxidation in the frontal lobe in AD. Interestingly, when the control cohort have one or more alleles, the levels of IsoP increase dramatically which may authenticate the role in which the genetic parameters and oxidation share. NP levels were also of interest, being similar biomarkers to the IsoPs, within this analysis even with their respective p values. Discussing the two sample groups separately, it can be implied that the increased allele content correlates itself with higher levels of oxidation in terms of NPs. Therefore relating genetic variation to the precise non-enzymatic oxidation of DHA in the brain tissue.

Table 14 – Cohort and APOE statistical analysis, including *post-hoc* tests in both frontal and temporal lobes

Frontal Multivariate Tests[b]

Effect		Value	F	Hypothesis df	Error df	Sig.
Apoe	Wilks' Lambda	.833	1.080[a]	8.000	43.000	.395
cohort * apoe	Wilks' Lambda	.517	5.026[a]	8.000	43.000	.000

Tests of Between-Subjects Effects in the Frontal region

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
cohort * apoe	FRdha	.064	1	.064	.017	.897
	FRaa	.881	1	.881	1.852	.180
	FRIsoP	737.360	1	737.360	3.940	.053
	FRnp	103.144	1	103.144	3.316	.075
	FRcd	9.125	1	9.125	3.389	.072
	FRabts	22.406	1	22.406	3.838	.056
	FRfox	4.264E8	1	4.264E8	12.106	.001
	FRtbars	54.275	1	54.275	2.963	.091

Temporal Multivariate Tests[b]

Effect		Value	F	Hypothesis df	Error df	Sig.
Apoe	Wilks' Lambda	.086	1.335[a]	8.000	1.000	.588
cohort * apoe	Wilks' Lambda	.010	12.041[a]	8.000	1.000	.219

Tests of Between-Subjects Effects in the Temporal region

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
cohort * apoe	TPdha	.468	1	.468	.194	.671
	TPaa	.054	1	.054	.115	.743
	TPIsoP	.449	1	.449	.207	.661
	TPnp	56.280	1	56.280	.696	.428
	TPcd	.002	1	.002	.004	.951
	TPabts	4.689	1	4.689	1.218	.302
	TPfox	1.640E7	1	1.640E7	.525	.489
	TPtbars	26.721	1	26.721	2.382	.161

Once more the samples were processed in terms of the eight dependent variables but with addition of the APOE grouping. In both brain lobes there was no significant difference in the possession of increased alleles. APOE1 vs APOE2 MANOVA with AA, DHA, IsoP, NP, CD, ABTS, FOX and TBARs as dependent variables revealed a strong interaction effect between cohort and risk ($F(8, 43) = 5.026$, $p < 0.001$; Wilks $\lambda = 0.517$), with *post-hoc* tests indicating that IsoP, ABTS and FOX were significant ($p<0.05$), with trend significance seen in the NP and CD. Duplicate analysis in the temporal region with the same variables indicated no difference.

Table 15 – Descriptive analysis of the APOE and cohort MANOVA analysis

1=Control 2=Alzheimer	1= 0 alleles present 2= 1 or 2 alleles		FRIsoP (µg/g)	FRnp (µg/g)	FRcd (mmol dm^{-3}/g)	FRabts TEAC per g (µmol/g)	FRfox CHPE/g wet weight
1	1	Mean	5.72	7.65	1.79	11.26	8718.71
		Std. Deviation	16.51	7.03	2.04	2.40	6857.67
		N	21	22	25	25	25
	2	Mean	17.53	8.12	0.71	10.55	1050.20
		Std. Deviation	41.61	7.77	0.63	0.78	328.73
		N	6	7	8	8	8
2	1	Mean	1.81	4.23	0.86	11.71	2341.94
		Std. Deviation	5.61	3.19	1.05	2.11	2118.35
		N	15	15	15	14	14
	2	Mean	1.04	8.11	1.43	14.05	5694.23
		Std. Deviation	1.52	6.60	1.91	3.05	6380.12
		N	19	20	20	20	20

Once the MANOVA was conducted, descriptive analysis allowed for determination of the means and standard deviation of the significant assays in the *post-hoc* output. The genetic variable was grouped into two separate classes; group 1 consisted of samples, in both AD and control, which had no alleles in the APOE with group 2 having the samples which possessed 1 or 2 alleles.

Figure 25 – Frontal isoprostane levels between cohort and the APOE variable
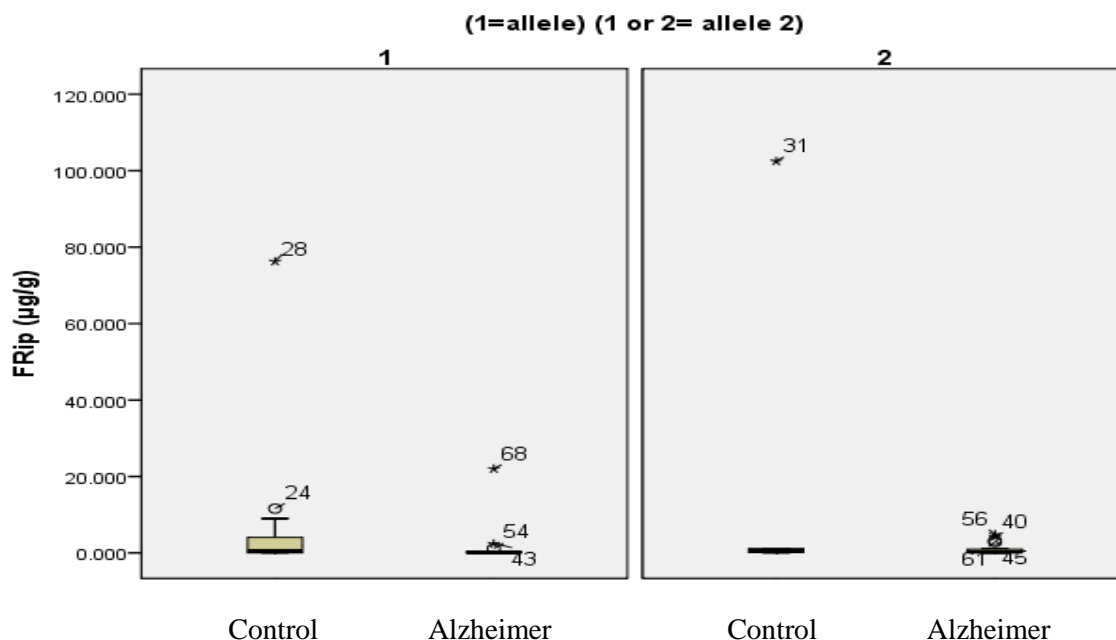
Frontal IsoPs were observed to be higher in the control cohort regardless of the amount of alleles present in the APOE gene. This observation was over $p = 0.05$, but can be considered as being trend-level significant.

The higher antioxidant status in AD tissue and the higher NP levels in this tissue when one or more alleles are present, demonstrates a link between the two. Any oxidised DHA in the prostanoid formation pathway will be reduced to form non-destructive components with antioxidant intervention, which will amplify stable biomarkers. The genotype itself may play a determining affect on the NP levels, increased alleles equates to higher risk of AD and with the consensus on oxidation in AD, this assay agrees with the notion of higher oxidation. Yet this differs from the previous IsoP determination. AD samples had lower levels of IsoP compared with controls and this then highlights the role of preferential DHA oxidation. Against this theory is the lack of statistical significance in both DHA and AA fatty acid levels for these samples, where no reduction was determined. Oxidation of DHA should lower that particular PUFA content regardless of disease state. However, a small percentage decrease in fatty acid levels (mg) will be magnified at the prostanoid level (μg) due to the mass variation.

CD levels were also statistically significant in the cohort and APOE genotypes MANOVA. Frontal CD levels were evaluated to be higher in the control state when no alleles are present on the APOE, but addition of one or more alleles shifts this result to being higher in the AD state. CDs are the primary products of PUFA oxidation and their reduction in the zero allele state can be attributed to the higher levels of antioxidants reduced hydrogen abstraction by free radicals therefore preventing PUFA oxidation. On the other hand, the introduction of the alleles appears to favour the negative role of antioxidants, where the biomarker levels such as CD and NPs are higher.

This supports the theory that oxidation appears to be of significance with increasing alleles in AD with two independent assays. Conversely when the control samples have one or more alleles the CD levels reduce dramatically along with IsoP and NPs indicating differences between them in oxidation. This implicates the link between genetic content and oxidation once again as previously stated in the link between DHA and Aβ pathway. LOOHs, shared with the CDs, are primary products of free radical hydrogen abstraction and oxidation and their findings should closely relate to the findings of the CD determination. Once more the same outcomes are observed with zero allele states having higher LOOH levels in the control group compared to the AD set. Introduction of the alleles shifts the results into favouring higher oxidation in AD, which is completely different to the means-based analysis that indicated higher antioxidant status in AD.

Figure 26 – Frontal neuroprostane levels between cohort and the APOE variable
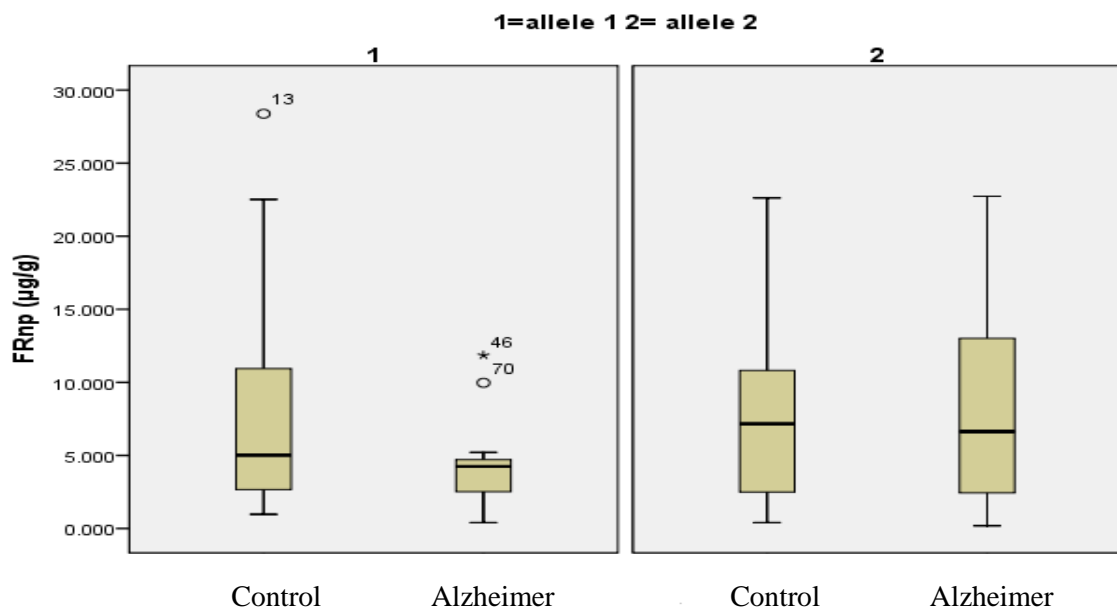
NPs were also trend-level significant (p = 0.075), but were higher in the samples which had 1 or 2 alleles present in the APOE gene, regardless of whether it was AD or control in comparison to having no alleles. This indicates that DHA oxidation, forming NPs, are affected by the genetic variable.

Figure 27 – Frontal conjugated diene levels controlling for APOE and cohort
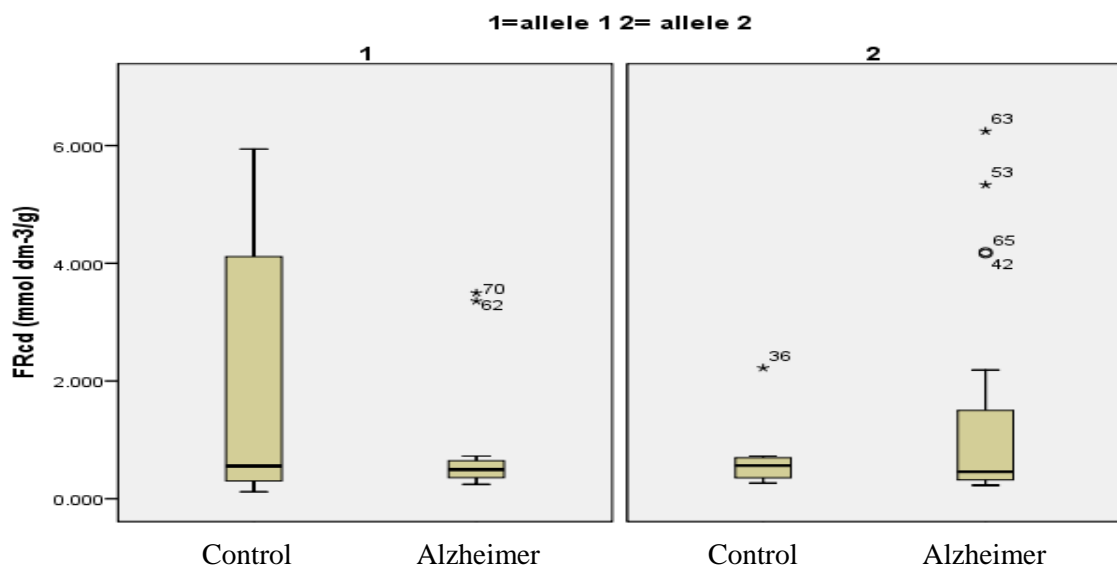
CDs, trend-level significant (p = 0.072), in the zero allele state had control samples with higher levels than the respective AD, interpretation of the second allele class indicates that AD had a higher amount of CD than the control samples. The second APOE allele group both the NPs and CDs have shown a higher level of oxidation, which is different to the previous means based analysis, standard deviation is also high.

Figure 28 – Frontal lipid hydroperoxide levels controlling for APOE and cohort
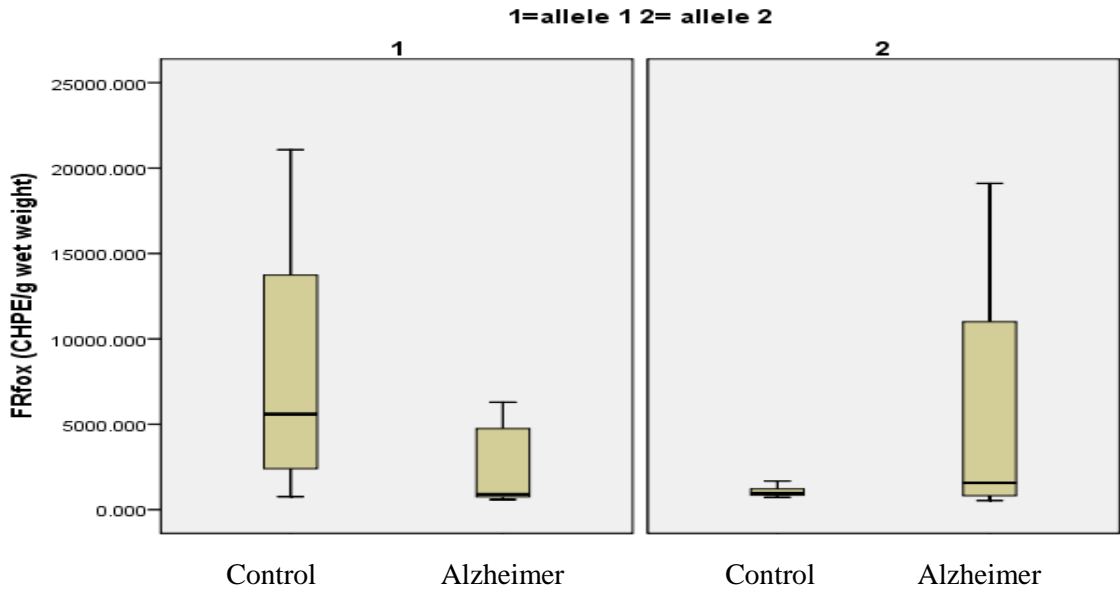
LOOHs (p = 0.001), were the most significant assay from the *post-hoc* analysis in the APOE and cohort MANOVA. When the sample has 1 or 2 alleles present, the levels of LOOHs are increased in AD, the opposite is observed in the 0 allele group with the control cohort having higher LOOH levels to the respective AD.

Figure 29 – Frontal antioxidant levels controlling for APOE and cohort
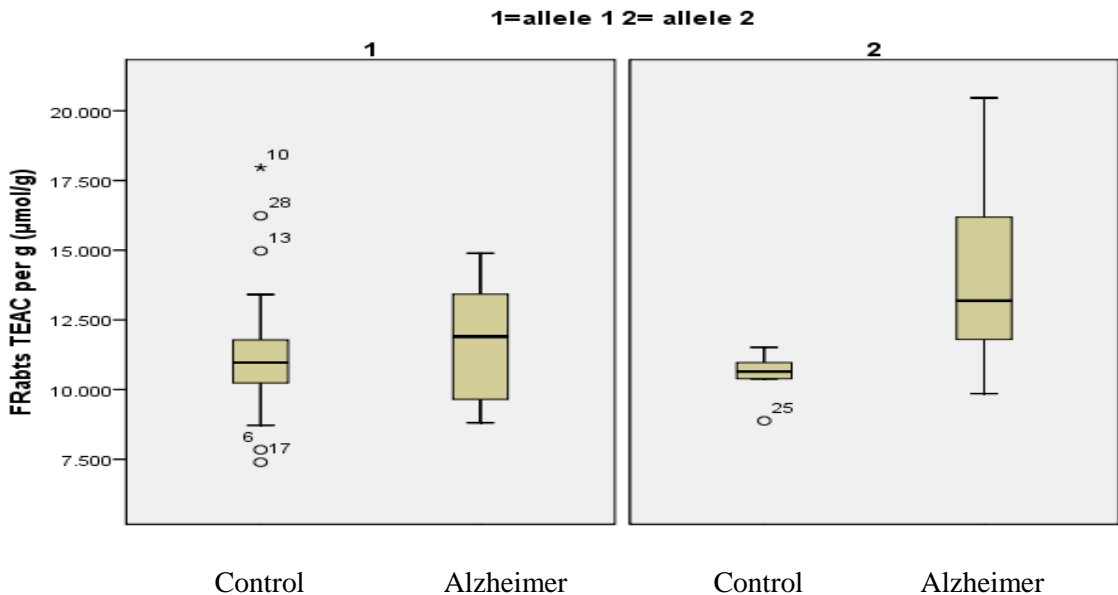
TEAC (p = 0.056), was not as significant as the earlier MANOVA without APOE in the frontal region. AD samples were observed to have higher levels of antioxidants than the control groups no matter how many alleles were present. Errors are greatly reduced unlike the previous assays from the *post-hoc* analysis.

5.3 – Refinement of MANOVA frontal analysis (Cohort and APOE)

In the previous section the MANOVA analysis was detailed, but in the majority of the graphs outliers were generated from the significant assays in the *post-hoc* breakdown. Therefore the samples denoted in each of the affected graphs, were removed to elucidate whether there is a significant difference under these variables, or to verify that the outliers in the boxplots were causing the variation in this analysis. APOE1 vs APOE2 MANOVA with IsoP, NP, CD, ABTS and FOX as dependent variables, these tests were the only ones significant in the previous cohort APOE *post-hoc* analysis hence the admission of the other tests.

The analysis revealed a strong interaction effect between cohort and risk ($F_{(5, 33)} = 3.367$, $p = 0.014$; Wilks $\lambda = 0.662$), with *post-hoc* tests indicating that only FOX was significant ($p = 0.010$), table 16. This modified MANOVA verifies that the significance of certain assays seen in the first APOΕ frontal analysis, in the *post-hoc*, was attributed to the influence of outliers. Essentially the conclusions discussed for the LOOH (FOX) in the first APOE MANOVA are applicable to the findings of this second MANOVA. When no alleles are present, the control samples have a higher level of LOOHs than the matched AD cohort. This reverses when 1 or more alleles are present in the sample. LOOHs were observed to be higher in the AD cohort with the increased allele presence, figure 30.

5.4 – MANOVA frontal and temporal analysis (Cohort and Braak staging)

From the genetic implications a clear observation was found. Specifically,possessing extra alleles promotes differences between IsoP, NP, CD, ABTS and LOOH assays. In section 1.1b, Braak staging was discussed in *post-mortem* tissue classification. Stages 0-VI has been regrouped into two definite groups because of the limitations with the MANOVA. Stages 0-III, defined as group one and stages IV-VI classed as group 2 as this evenly splits the data. Once again each region was dealt with separately. Frontal and temporal samples were independently processed in terms of the eight dependent variables but with addition of the Braak stages grouping. Frontal control and AD samples with Braak grouping, as independent variables, MANOVA with AA, DHA, IsoP, NP, CD, ABTS, FOX and TBARs as dependent variables.

Frontal Multivariate Tests[b]

Effect		Value	F	Hypothesis df	Error df	Sig.
Apoe	Wilks' Lambda	.841	1.248[a]	5.000	33.000	.310
cohort * apoe	Wilks' Lambda	.662	3.367[a]	5.000	33.000	.014

Tests of Between-Subjects Effects in the Frontal region

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
cohort * apoe	FRIsoP	4.053	1	4.053	1.074	.307
	FRnp	27.136	1	27.136	.868	.357
	FRcd	2.698	1	2.698	1.605	.213
	FRabts	8.966	1	8.966	2.050	.161
	FRfox	2.793E8	1	2.793E8	7.351	.010

Figure 30 – Lipid hydroperoxide levels controlling for APOE and cohort with removal of outliers
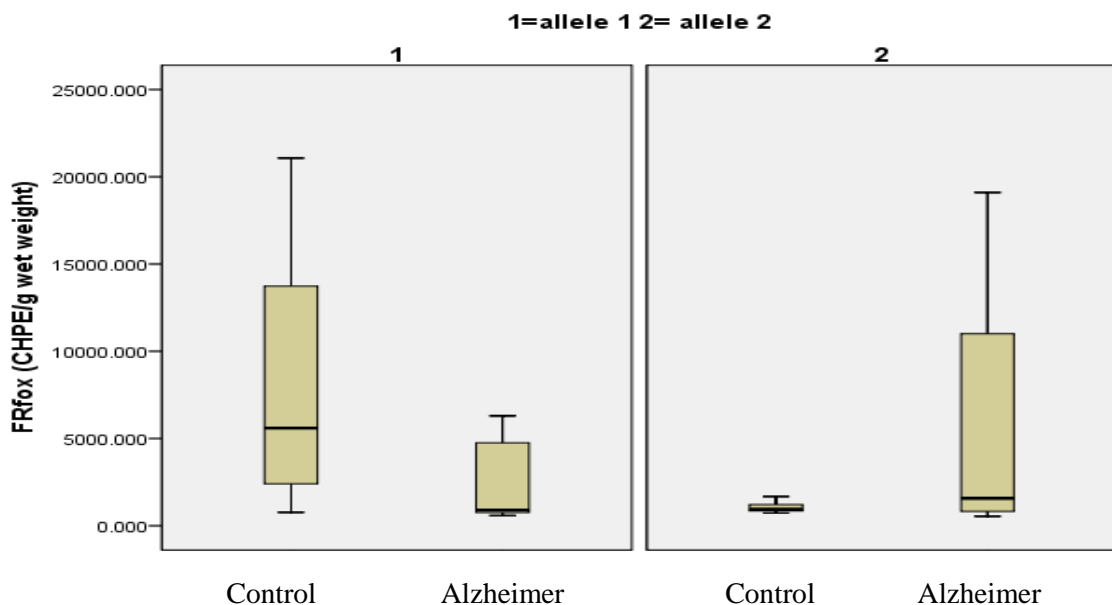
The removal of the outliers found in the previous MANOVA found that APOE1 vs APOE2 MANOVA with IsoP, NP, CD, ABTS and FOX as dependent variables. The analysis revealed a strong interaction effect between cohort and risk (F (5, 33) = 3.367, p = 0.014; Wilks λ = 0.662), with *post-hoc* tests indicating that only FOX was significant (p=0.010). This then implies that the findings in the first APOE MANOVA were down to the outliers affecting the mean of that sample size. In terms of FOX, control samples with no alleles had higher levels of LOOH compared to the AD equivalent. This was then reversed in the 1 or more allele state where AD samples had increased levels of FOX compared to the control.

<u>Table 17 – Cohort and Braak staging statistical analysis including variables significant in the MANOVA in both frontal and temporal regions</u>

Frontal Multivariate Tests[b]

Effect		Value	F	Hypothesis df	Error df	Sig.
cohort * Braak stages	Pillai's Trace	.174	1.106[a]	8.000	42.000	.379
	Wilks' Lambda	.826	1.106[a]	8.000	42.000	.379
	Hotelling's Trace	.211	1.106[a]	8.000	42.000	.379
	Roy's Largest Root	.211	1.106[a]	8.000	42.000	.379

Tests of Between-Subjects Effects in the Frontal region

Source	Dependent Variable	Type III Sum of Squares	Df	Mean Square	F	Sig.
cohort * Braak stages	FRdha	4.490	1	4.490	1.199	.279
	FRaa	1.308	1	1.308	2.785	.102
	FRIsoP	40.094	1	40.094	.189	.665
	FRnp	1.061	1	1.061	.030	.862
	FRcd	2.145	1	2.145	.875	.354
	FRabts	7.547E-6	1	7.547E-6	.000	.999
	FRfox	4.744E7	1	4.744E7	1.102	.299
	FRtbars	9.398	1	9.398	.473	.495

Temporal Multivariate Tests[b]

Effect		Value	F	Hypothesis df	Error df	Sig.
cohort * Braak stages	Pillai's Trace	.085	.557[a]	7.000	42.000	.786
	Wilks' Lambda	.915	.557[a]	7.000	42.000	.786
	Hotelling's Trace	.093	.557[a]	7.000	42.000	.786
	Roy's Largest Root	.093	.557[a]	7.000	42.000	.786

Tests of Between-Subjects Effects in the Temporal region

Source	Dependent Variable	Type III Sum of Squares	Df	Mean Square	F	Sig.
cohort * Braak stages	TPdha	.002	1	.002	.000	.984
	TPaa	.002	1	.002	.002	.962
	TPnp	12.197	1	12.197	.258	.614
	TPcd	.027	1	.027	.070	.793
	TPabts	8.059	1	8.059	1.468	.232
	TPfox	2.036E7	1	2.036E7	.981	.327
	TPtbars	12.768	1	12.768	.954	.334

Braak staging is divided into six different groups, each group having different sample sizes making MANOVA analysis difficult. To reduce such problems the Braak stages will be put into two groups of equal size to improve statistical analysis. Stages 0-III are defined as group 1 with the remaining IV-VI being in group 2. Addition of the Braak staging to the analysis had no relevance in differentiating between AD and control; IsoPs had to be removed from the temporal results due to missing data.

The analysis revealed no effect between cohort and Braak grading ($F_{(8, 42)} = 1.106$, $p = 0.379$; Wilks $\lambda = 0.826$), with *post-hoc* analysis being excluded because of the p value. Introduction of Braak staging appears to have no statistical affect on differentiating between the tissues. MANOVA processing of the temporal data controlling for cohort and Braak staging had to be modified due to lack of sample numbers in the IsoP assay. Thus only seven out of the eight dependent variables were used alongside the two independent factors. Control and AD with Braak staging in the temporal lobe was analysed as the two independent factors with the remaining AA, DHA, NP, CD, ABTS, FOX and TBARs as the dependent factors in the MANOVA. The analysis revealed that there was no interaction between cohort and Braak staging ($F_{(7, 42)} = 0.557$, $p = 0.786$; Wilks $\lambda = 0.915$). Both the frontal and temporal lobes, under cohort analysis, appear to show that Braak staging has no statistical and therefore no influence in the determination of AD for these assays.

5.5 – Discussion of statistical analysis

DHA and AA levels appear to have no relevance in the determination or differentiation between AD and control samples which agress with Skinner et al [175]. Our collaborators concluded from a larger cohort study that only the saturated stearic acid (18:0) and palmitic acid (16:0) had a statistical significance between the two states, with AD tissue having more of these two fatty acids the impact of which is more significant than both DHA and AA [179]. Palmitic and stearic acid were reported to induce hyperphosphorylation of tau [179] and paired with the associated increase in PS, where these fatty acids have been observed in large percentages, promote the externalisation of PS. Increasing saturated fatty acid phospholipid content, can be attributed to the high fat diets commonly observed within modern western diets [37, 58, 80]. Therefore these saturated fatty acids may be an influential component of AD pathogenesis, more than DHA and AA.

CD and LOOHs indicated no difference or at least no increase in the AD state, but of more interest was the standard deviation. In the FOX assay the variation was greater than the mean precluding any conclusion. Primary products, determined herein, only provide a snap shot of the state of oxidation, if the pathway has progressed past these compounds then these will appear reduced unless a steady state of formation and conversion is happening. Deviation between the samples may be attributed to the state of oxidation at that time point. Previous literature [71] confirmed that the extraction method used on

these same samples in collaboration with temperature and time delay suffered from no detrimental reduction in fatty acid and CD levels. This further augments the concept that the variation in LOOH is down to the progression of the oxidation pathway.

Analysis of the two independent end point markers confirmed that oxidation was not elevated in AD, except for temporal NP levels which were increased in the AD cohort. IsoPs and NPs had a large standard deviation similar to the LOOHs nullifying any potential conclusions. Levels of chain scission aldehydic products showed no difference between AD and control groups, but the results obtained had a reduced standard deviation than both the prostanoids and LOOHs. TEAC determination indicated that AD samples had higher antioxidant levels compared to the control. The suppression of all the oxidation markers in the AD samples is assumed to be linked to increased antioxidant protection. The means-based analysis implies that in the sample cohort, oxidation is not significant in AD using these biomarkers except for the levels of antioxidants. Following the general consensus in literature regarding increased oxidation in AD, it makes sense that up-regulated oxidation in turn promotes antioxidant levels to counteract the imbalance *in situ* as a host response. However, too many antioxidants can increase oxidation biomarkers as discussed previously. Formation of the prostanoids requires reduction which antioxidants can easily perform, therefore increasing levels of this particular biomarker. Either way the apparent variation in antioxidant levels can be argued in both theories with the results acquired.

Levels of TEAC were higher in AD regardless of the number of alleles present in the APOE genotype and more significantly the higher allele content in AD increased the level of antioxidants. Control samples had lower antioxidant levels when more than one allele was present on the APOE synonymous with the other statistically significant assays. This clear association between APOE and antioxidants in AD seems to enhance their role regardless of perception (positive or negative). Positively, these compounds reduce oxidation by competing with radicals and supplying themselves as hydrogen atom donors instead of the methylene carbon for example, hence protecting the vulnerable PUFAs. This would then lead to a marked reduction in oxidation markers. Negatively, when quenching the oxidation process any propagation reactions already occurring will be driven to their respective stable markers, therefore increasing the oxidation markers. Either way increased antioxidant levels are in contrast to the oxidation theory perceived in the literature regarding AD pathogenesis.

The increased levels of antioxidants were further investigated with correlations against Aβ, phospho-tau, post synaptic and dendritic spine proteins, neprilysin and three enzymes (insulin-degrading, angiotensin-converting and β-secretase) by our collaborators [200]. They found that antioxidant levels and insoluble Aβ were the only statistically significant variables, comparing APOE ε4 positive to APOE ε4 negative. The pathways illustrated in figure - 31 shows how both sAPPα and Aβ are made and their links to APOE and DHA.

The formation of sAPPα, through the non-amyloidogenic pathway, promotes cell survival and anti-inflammatory activities [17, 29, 49, 81]. Where as the formation of Aβ from the same starting compound, in the amyloidgenic pathway, causes more damage by elevating cell death and oxidative stress [201]. One of the determining factors on whether sAPPα or Aβ forms depends on which secretase enzyme cleaves the βAPP from the membrane. In the non-amyloidgenic pathway α-secretase splits βAPP to form a 83-residue protein known as sAPPα, however in the amyloidgenic pathway β-secretase breaks βAPP into a 99-residue protein which is then converted to Aβ by γ-secretase activity [202]. According to our collaborators there was no increase in β-secretase activity in AD, which starts the process of Aβ formation.

The formation of Aβ causes oxidative stress levels to increase, however our results differ from this. All the lipid oxidation biomarkers quantified in this study, show no elevation in AD where Aβ production is greater. The pathway shown in figure 31 shows that free DHA is converted to NPs under high Aβ synthesis and should increase in the amyloidgenic process. The results obtained herein show no increase in NPs or IsoPs in AD for both brain regions. This suggests that either Aβ is increasing oxidative stress and the host is responding by upregulated antioxidant levels, or the increase in oxidation is not breaking down the fatty acids. However, the reason behind the elevated Aβ and antioxidants in Tayler et al [200] and our study are unclear. Whether the elevated oxidation associated with Aβ is affecting other components like cholesterol and APOE, as seen in figure 31, requires further investigation.

Figure 31 – Nonamyloidgenic and amyloidgenic pathways [203]
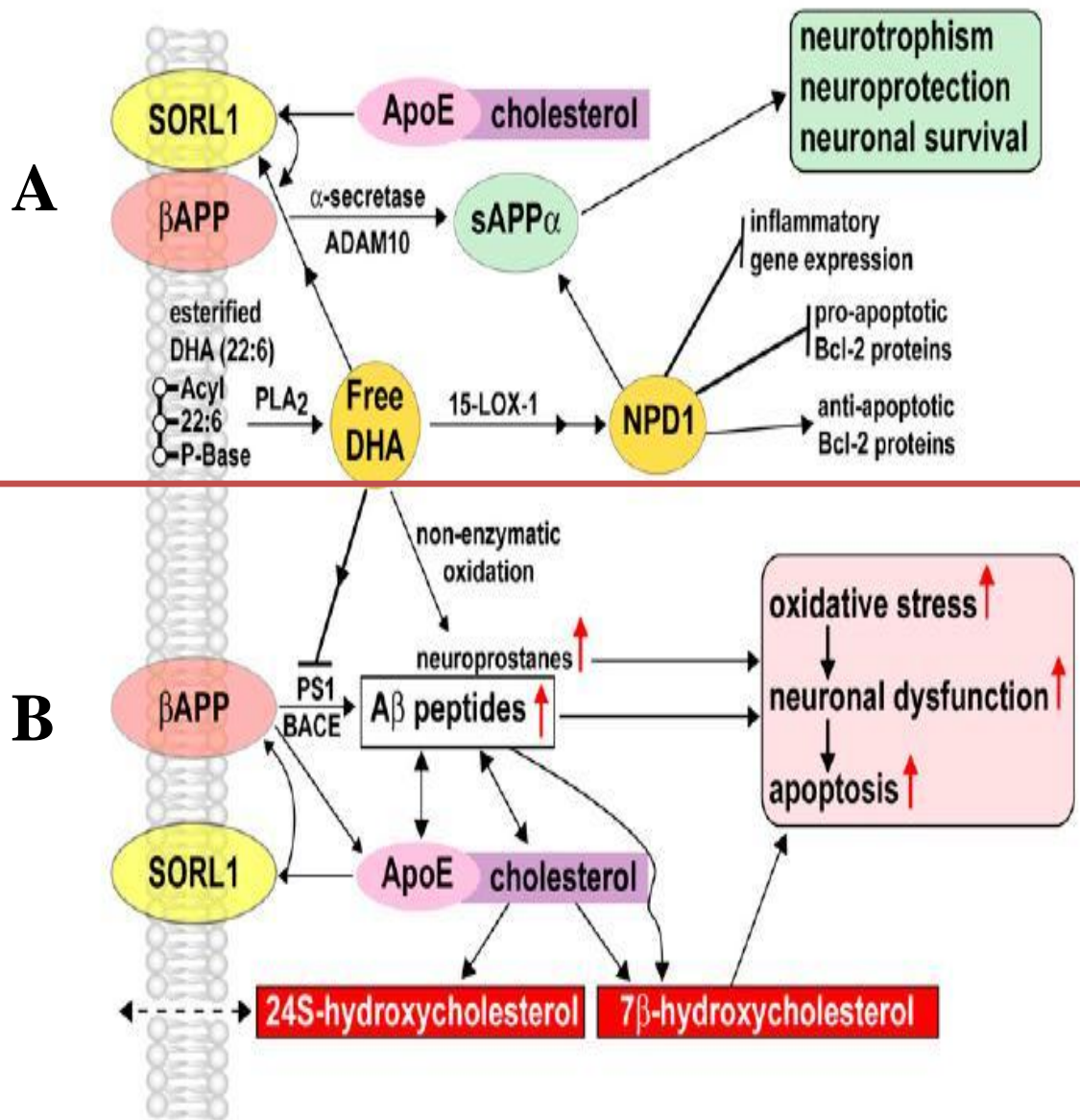
The diagram above shows two separate pathways denoted A and B. Pathway A describes the non-amyloidogenic pathway. SORL1, the βAPP membrane-integral sorting receptor sortilin-1, when near βAPP (in the presence of APOE) allows for processing of the precursor protein into secretory APPα under α-secretase. DHA actively supports this mechanism after enzymatic release by PLA$_2$. LOX derived neuroprotectin D1 supports sAPPα in general cell survival, reducing inflammatory processes and pro-apoptotic proteins. Pathway B denotes the amyloidogenic pathway where the formation of Aβ is formed instead of APP. When SORL-1 is not proximal to βAPP, the α-secretase pathway is replaced by β-secretase and presenlin-1 (PS1)/BACE activity that produce Aβ peptides. These peptides increase oxidation, leading to neuroprostane formation from DHA and neurotoxic sterols from cholesterol carrying APOE. Under these conditions oxidative stress, dysfunction and finally apoptosis are promoted.

The other variable which was linked to the elevated antioxidant levels was the APOE genotype, which is also linked to reducing fibril Aβ formation [204]. APOE is a carrier for lipids, cholesterol and antioxidants [205], but is also shown to have antioxidant activity [206] and may be responsible for the elevated levels of these defence compounds. The protein binding site of APOE that is associated with its antioxidant capacity lies between residues 141 and 155 of the protein [207]. However, there are many variants of APOE and each type has its own antioxidant activity and it has been shown that APOE2>APOE3>APOE4 in terms of oxidative defence [204, 208]. The reduced capacity of APOE4 as a antioxidant is down to the different proteins at 112 and 158 residues compared to both APOE2 and APOE3 [207]. Therefore more antioxidants would be recruited to sites where APOE4 was present. Such antioxidants as glutathione, glutathione peroxidase [209], superoxide dismutases [206] and catalase [205] have all been shown to be elevated in APOE4 samples.

5.6 – Future directions

The results and discussion in the previous sections have suggested that the role of lipid oxidation in AD pathogenesis is not justified. Various oxidation assays indicated no increase in AD, but questions remain over the variability in the results. Quantification of the transient intermediates involved in the initial stages of oxidation, the CD and LOOH assays, suggest that the results from these assays are independent of one another. This could be attributable to other compounds absorbing at the CD wavelength, reproducibility of both of these spectrophotometric methods or even the heterogeneity of the human brain tissue. The newly derived GC-EI-MS method requires further evaluation using other biological fluids (e.g. plasma and urine) to fully validate the method.

Antioxidant levels are higher in AD samples in both brain regions. Determination of water soluble antioxidant composition is required to find the reason for the increased levels in AD, preferably using LC. Candidate antioxidants likely to be involved in the brain include α-tocopherol, ascorbic acid or glutathione. The antioxidant capacity of APOE requires further investigation in AD. The ABTS assay works on radical scavengers such as gluthathione and vitamin e [210], where as APOE works by inhibiting metal induced oxidation [207]. Therefore the use of the Cu^{2+} reduction assay may demonstrate the antioxidant activity of APOE [211]. Genomic approaches such as DNA microarray could be used to evaluate whether genes encoding the biosynthesis pathways

of these molecules are the reason for elevated antioxidant levels in AD. Analysis of sterols being transported by APOE, in particular the neurotoxic sterols (24S- and 7β-hydroxycholesterol) formed upon interaction with the Aβ protein under the amyloidogenic pathway, may differentiate between AD and control as higher Aβ levels increase the presence of these modified cholesterol markers.

6 Conclusions

This project forms part of a large multiple variable analysis into the role of lipid oxidation in the pathogenesis of AD with clinical data sets. The causal pathway models for DHA oxidation indicate that control samples followed chain breakdown mechanisms to form aldehydes while the AD samples, went through ring formation to create the cyclic NPs. AA pathway models in the AD temporal region produced IsoPs with the frontal lobe forming aldehydes, similar to the control samples for both brain regions. This demonstrated that different lipid oxidation mechanisms in AD and control were occurring in both frontal and temporal lobes. Determination of the lipid oxidation marker levels showed that there was no significant difference between AD and control. The antioxidants, however, were higher in both brain regions of the AD samples which could indicate low lipid peroxidation and their lack of consumption in catabolism. Statistically, only frontal antioxidant levels were significant in differentiating between AD and control without any clinical variables.

Introduction of the APOE variable suggested that LOOHs were higher in the control when 1 or 2 alleles are present, which could be attributable to the lower antioxidant levels. Analysis of *post-mortem*, age at death and Braak staging did not show any differences between AD and control. Antioxidants were higher in AD and that may explain why the lipid oxidation markers were lower compared to the controls across the brain regions. Since elevated lipid peroxidation has been linked to AD pathogenesis (1.1d i Oxidation), the results obtained here appear to be counter intuitive with multiple oxidative biomarkers being reduced in AD.

Clinically, increased supplementation of omega-3 PUFAs and antioxidants has been a common suggestion as a dietary intervention for lowering the risk of AD in many large epidemiological studies, table 1. This study suggests that such an intervention would most likely be ineffective against AD *per se* although it may support improvement in other areas of general health. It can be argued that the elevated levels of antioxidants could be a negative consequence of AD and that the supplementation of such compounds could aggravate the condition further. In terms of lipid peroxidation, no definitive markers or target site for intervention have been revealed. Perhaps other non-lipid targets such as APOE hold the key to unravelling this condition.

7

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8 Appendices

Appendix A – Full results for control cohort, two pages

Appendix B – Full results Alzheimer cohort, two pages

Appendix C – Graphs demonstrating the reason why age and *post-mortem* were not included in statistical analysis

Appendix A – Results for the control cohort (continued onto the next page)

BB No	DHA F	DHA TP	AA F	AA TP	IP F	NP F	IP TP	NP TP	CD F	CD TP	ABTS F	ABTS TP	FOX F	FOX TP	TBARs F	TBARs TP
16	16.013	13.744	8.240	8.287	0.041	9.179		1.844	0.867	0.867	11.781		12778.768	777.803	21.550	13.669
36	15.506	16.267	7.825	8.859	7.707	15.178		0.271	0.978	0.963	12.151	.	13161.499	717.589	20.070	13.077
57	16.835	16.584	7.285	8.086	0.326	4.475		0.186	0.563	2.252	7.842	7.460	16891.815	14829.070	12.432	17.184
79	12.700	12.086	7.601	6.300	1.036	1.931		1.104	0.274	1.585	10.810	8.269	17404.480	1146.399	17.814	14.086
98	15.181	16.128	9.438	9.011	0.504	2.463		3.183	0.185	0.593	11.552	8.872	21071.108	11073.040	14.261	13.015
103	15.337	12.477	9.998	11.416				0.643	0.119	0.941	10.966	9.447	5596.456	984.998	5.498	9.728
106	14.498	16.580	8.552	10.203	0.056	5.724		0.768	0.333	1.474	17.962	1O.771	18399.150	8154.680	14.449	15.672
110	14.810	13.554	8.380	8.223	0.779	3.486		1.593	4.289	2.030	13.411	13.140	769.972	783.401	15.063	14.206
112	14.813	12.874	9.385	7.587	0.545	10.945			0.244		11.076		2554.697	839.382	10.406	13.760
120	18.729	18.098	7.997	8.589		28.379			2.933		14.972	11.667	9350.563	8561.043	17.750	14.525
122		15.215		8.882	0.017	2.596		2.399	0.556	0.585	10.634	8.042	5360.300	10513.481	12.223	12.805
127	17.132	17.011	8.871	9.057	0.053	4.995	55.565	2.093	0.148	2.156	8.719	11.357	13332.650	6598.448	7.056	15.523
130	16.162	15.264	8.191	8.448	0.018	11.857			0.333	0.311	9.187	8.366	4837.024	735.452	14.612	12.465
147		13.940		7.228		4.380	0.067	7.166	4.430	0.541	7.391	12.232	5075.434	13761.828	11.824	17.778
150	14.704	15.522	8.116	9.227	0.069	22.523	1.818	3.058	0.385	2.370	10.287	10.600	11136.456	1405.987	11.441	10.807
206	14.138	16.583	7.705	9.175	0.305	3.279	1.597	0.045	0.467	0.785	10.510	7.921	18968.654	7701.145	5.956	10.431

F – frontal, TP – temporal, BB No – Sample number, DHA and AA expressed as mean molar percentage, IP and NP (μg/ wet weight g), CD (nmol dm^{-3}/ wet weight g), ABTS (TEAC/g; μmol/ wet weight g), FOX (CHPE/ wet weightg), TBARs (nmol MDA/ wet weight g).

BB No	F DHA	TP DHA	F AA	TP AA	F IP	F NP	TP IP	TP NP	F CD	TP CD	F ABTS	TP ABTS	F FOX	TP FOX	F TBARs	TP TBARs
254	16.336	13.981	10.529	10.006		6.137	4.530		5.941	0.593	8.957	8.848	4866.873	3066.239	3.398	6.926
269	17.565	17.213	8.354	9.070	1.468	2.868	0.015	12.802	1.919	0.644	10.238	6.859	13730.655	16964.294	12.082	17.351
295	15.238	15.129	8.837	9.760	0.255	10.064		0.294	0.304	0.756	10.004	10.128	15270.619	10699.527	24.558	13.325
321	16.790	16.969	8.686	9.363	6.043	2.667	0.195	1.895	5.756	0.467	11.700	10.974	1388.984	1423.520	11.847	15.137
333	15.710		8.363		11.602	0.966		3.507	4.111	0.748	12.338	14.080	919.424	1223.002	6.409	14.432
336	13.511	15.704	7.919	8.417	0.542	1.924		10.606	0.667	3.333	8.877	8.747	1678.965	1035.367	12.064	16.636
352	15.023	14.464	8.568	10.960	4.081	5.031	0.638	0.376	4.326	0.770	11.135	9.616	2402.451	10213.159	10.758	12.504
355	15.239	13.002	8.898	9.271	0.029	11.631		2.118	0.356	0.615	10.736	12.234	999.755	746.954	6.951	12.350
390	15.361	16.221	8.322	8.190	76.218			9.634	0.244	1.111	16.233	15.715	764.332	1458.881	12.269	13.696
402	14.062	14.638	9.514	9.348	8.971	1.960	0.011	7.013	4.696	0.867	10.979	11.156	935.557	1179.061	8.048	13.194
407	12.799	15.821	7.845	9.341	0.105	3.065		4.196	0.407	0.719	10.399	9.505	952.755	17045.125	13.975	9.387
412	16.406	13.502	10.719	9.122	102.451	0.403		6.078	0.267	0.822	10.831	10.592	924.568	1503.851	5.960	12.732
461	14.322	16.121	8.733	9.586	1.138	13.358		1.328	0.622	0.963	11.099	11.432	1428.566	4187.995	13.456	14.745
467	16.635	15.664	8.557	8.798				3.822		0.644						
563	17.188	14.884	9.309	8.640	0.031	7.163	0.089	1.262	0.304	1.007	11.517	15.363	998.316	1136.337	9.998	10.037
678	14.243	14.129	7.756	8.640				0.510		0.889						
714	16.686	14.945	8.793	10.280		22.623		3.507	2.222	0.593	10.765	9.968	871.563	5399.822	6.935	8.658
721	16.421	13.098	8.372	8.597	0.907	8.279	4.623	30.333	0.504	0.689	10.526	13.413	822.668	1053.918	12.257	6.562
733	16.169	13.013	9.197	8.815			0.009	5.166	0.726	1.815	10.384	12.963	724.220	1101.875	13.121	12.568

F – frontal, TP – temporal, BB No – Sample number, DHA and AA expressed as mean molar percentage, IP and NP (μg/ wet weight g), CD (nmol dm^{-3}/ wet weight g), ABTS (TEAC/g; μmol/ wet weightg), FOX (CHPE/ wet weight g), TBARs (nmol MDA/ wet weight g).

Appendix B – Results for the Alzheimer cohort (continued onto the next page)

| BB No	DHA		AA		IP		NP		CD		ABTS		FOX		TBARs	
	F	TP	F	TP	F	TP	F	TP	F	TP	F	TP	F	TP	F	TP
190	9.639	16.565	7.835	8.506	0.227		5.215	10.509	0.429	0.822	14.704	13.836	4750.610	766.408	12.223	18.021
196	8.559	15.062	6.658	8.561	4.179		0.977	21.631	0.678	0.815	17.451	15.093	11965.399	6597.193	18.560	17.920
203	15.855	14.567	8.427	8.388	0.024	0.075	3.561	22.849	0.404	0.741	.	.	.	.		
205	11.209	15.666	7.109	8.477			1.555	20.031	4.193	2.081	10.619	10.411	5758.061	854.732	18.848	18.278
211	18.296	15.973	8.991	9.429	2.381		0.686	6.267	0.607	0.941	14.890	7.514	4864.031	1137.499	11.373	5.116
213	14.096	14.417	8.330	9.682	0.242		0.607	4.685	0.230	0.793	12.285	11.667	15241.046	782.654	9.914	12.089
219	14.070	14.917	7.599	8.561	3.257		0.704	16.593	0.689	0.556	9.853	13.534	12397.454	692.667	12.231	8.872
224	17.267	17.993	8.788	8.560	0.019		11.857	4.631	0.533	2.659	8.909	12.296	3349.141	1100.979	13.759	16.025
228	12.661	17.087	8.765	9.666	0.222		11.049	5.701	0.459	0.659	11.371	13.576	19103.798	14148.402	3.796	8.929
241	17.085	16.297	7.427	9.027	0.525		3.321	5.614	0.407	0.793	20.463	16.191	5340.951	1401.298	16.552	16.048
242	16.616	16.395	9.867	8.946	0.075		4.253	23.983	0.496	0.859	10.750	9.946	5447.945	1336.099	13.213	12.280
243	13.999	14.260	7.818	8.036	0.224		0.402	14.417	0.681	0.867	13.070	14.869	6294.800	1294.340	11.136	7.275
284	12.034	11.220	8.097	7.711	0.908		18.808	0.538	0.311	0.770	19.202	18.280	17826.473	10582.494	17.690	16.718
287	16.060	12.947	8.314	7.380	0.339		0.193	2.488	0.481	0.963	12.677	9.587	6045.734	879.162	15.735	9.042
288	17.509	12.667	8.918	7.938	0.005		12.652	1.232	5.333	0.941	15.290	12.377	1031.792	1481.610	8.256	19.260
289	14.197	17.243	7.661	8.362	1.182		4.120	0.930	0.726	0.837	8.808	10.295	588.089	1787.838	7.096	12.929
290	12.176	11.680	8.572	9.271	0.138	0.181	5.079	11.419	0.348	1.378	13.418	11.539	902.955	1493.283	10.121	11.751
302	15.664	9.451	8.425	6.905	4.786		4.358	0.719	0.459	1.044	11.779	11.954	934.015	955.792	16.597	19.369

F – frontal, TP – temporal, BB No – Sample number, DHA and AA expressed as mean molar percentage, IP and NP (μg/ wet weight g), CD (nmol dm^{-3}/ wet weight g), ABTS (TEAC/g; μmol/ wet weightg), FOX (CHPE/ wet weight g), TBARs (nmol MDA/ wet weight g).

| BB No	F DHA	TP DHA	F AA	TP AA	F IP	F NP	TP IP	TP NP	F CD	TP CD	F ABTS	TP ABTS	F FOX	TP FOX	F TBARs	TP TBARs
318	12.117	10.276	9.303	9.222	0.064	13.902	0.081	2.127	0.333	1.000	18.145	17.002	10035.030	6727.509	15.886	13.745
326	14.445	12.619	8.093	8.305	0.149	22.732		3.037	0.407	1.030	14.555	17.384	1100.646	965.123	17.306	11.932
330	15.011	17.739	8.359	9.642	0.043	3.869		13.257	0.304	0.889	10.517	11.000	2034.532	936.499	8.944	10.781
341	13.140	14.109	8.161	8.354	0.030	4.342		0.355	0.244	0.741	11.992	12.266	841.738	1021.559	11.136	17.085
508	16.378		9.011		2.863	15.260			0.815		14.010		784.868	682.443	11.727	14.628
538	17.171	14.384	8.891	7.722	0.010	1.708		2.478	3.496	0.785	10.122	15.253	727.171	898.551	8.540	11.882
558	17.366	16.522	9.076	8.277	1.036	12.750		6.628	6.244	0.793	12.490	12.930	917.882	965.628	20.210	10.110
561	15.106	15.982	8.028	8.708	0.118	7.946		0.414	2.185	0.393	11.814	13.342	666.460	698.902	9.193	17.594
568	14.050	13.613	8.106	7.656	0.055	13.264		2.185	4.163	0.481	13.702		853.078	740.041	16.063	13.290
573	14.497	15.206	8.558	8.671	0.078	6.926		0.765	0.370	1.607	17.081		690.282	492.210	11.657	5.155
580	14.784	13.489	8.651	8.200	0.021	4.262		9.392	0.370	0.800	12.393	12.451	1846.888	765.161	7.285	14.967
592	14.148	14.025	8.623	7.894	21.970	0.427		0.220	0.533	0.252	11.814		636.100	756.798	10.142	11.220
601	14.091		8.261		0.320	4.271		0.040	0.333	0.326	13.947	13.406	753.786	810.412	13.198	9.143
683			7.617		0.070	9.967		2.970	3.359	0.630	9.521	14.153	892.117	791.825	11.497	12.979
685	14.593		8.141	8.400	0.476	3.331	0.111	2.100	0.296	0.156	9.651	9.612	891.835	706.874	8.071	5.351
687	14.562		7.866	8.451	7.082	0.157	0.036	0.368	0.511	0.252	10.224	12.506	691.072	623.611	15.184	11.688
713	16.562		8.633	8.359	0.228	5.010		3.026	0.304	0.400	12.666	15.091	538.656	768.313	16.463	10.755
725	14.439		8.131	7.569	0.734	6.343		4.719	0.281	0.237	15.051		618.364	578.216	12.501	11.503

F – frontal, TP – temporal, BB No – Sample number, DHA and AA expressed as mean molar percentage, IP and NP (µg/ wet weight g), CD (nmol dm^{-3}/ wet weight g), ABTS (TEAC/g; µmol/ wet weightg), FOX (CHPE/ wet weight g), TBARs (nmol MDA/ wet weight g).

Appendix C – Age and *post-mortem* against assays conducted
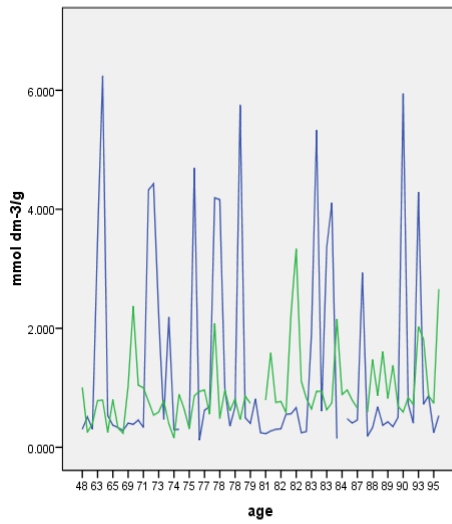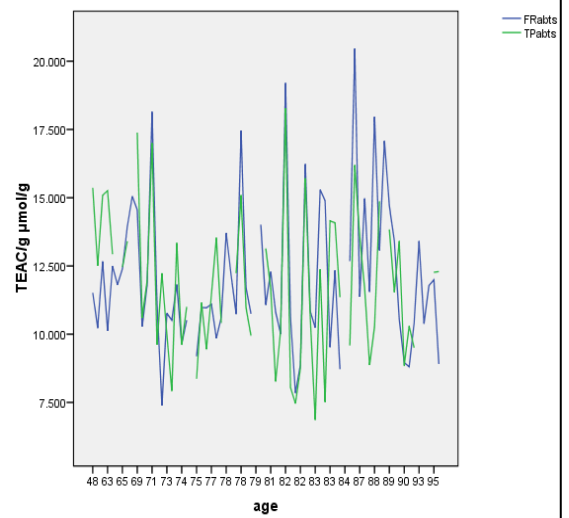

Fatty acid levels against age
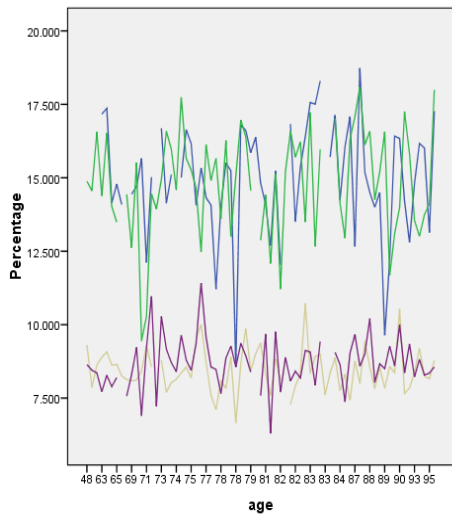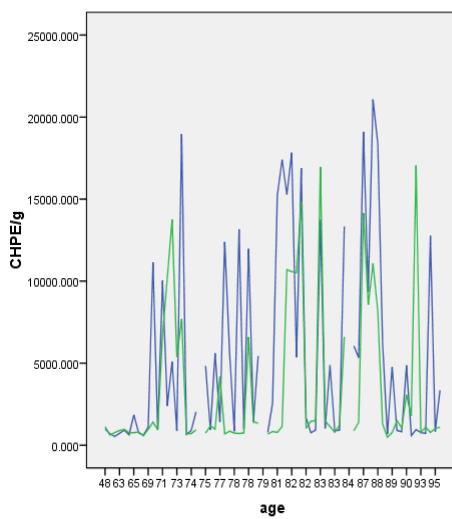

Isoprostane and neuroprostane levels against age
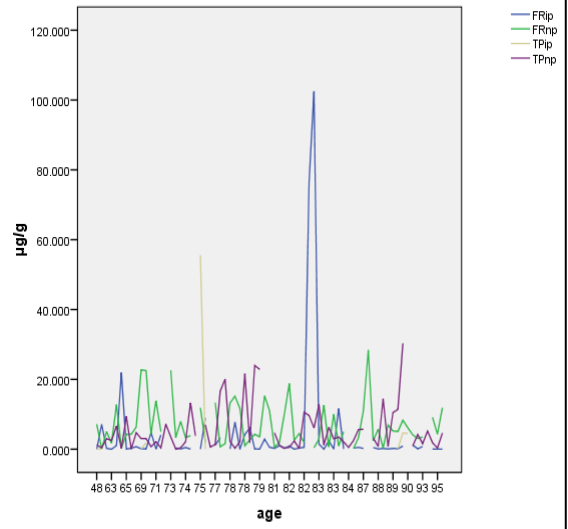
Conjugated diene levels against age

Antioxidant levels against age

Lipid hydroperoxide levels against age

Aldehyde levels against age

Fatty acid levels against post-mortem delay

Isoprostane and neuroprostane levels against post-mortem delay

Conjugated diene levels against post-mortem delay

Antioxidant levels against post-mortem delay

Lipid hydroperoxide levels against post-mortem delay

Aldehyde levels against post-mortem delay