2015

Investigations of peroxidation of membrane phospholipids

Colin H. Cortie
University of Wollongong

UNIVERSITY OF WOLLONGONG

Investigations of peroxidation of membrane phospholipids

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

Doctor of Philosophy

from

The University of Wollongong

by

Colin H Cortie, BSc (Distinction)

School of Medicine, Illawarra Health and Medical Research Institute

2015

This work is dedicated to my beautiful wife and my wonderful family.

Thesis certification

I, Colin H Cortie, declare that this thesis, submitted in fulfilment of the requirements for the award of Doctor of Philosophy from the School of Medicine, University of Wollongong, is wholly my own work unless otherwise acknowledged. This document has not been submitted for qualifications at any other academic institution.

Mr. Colin H Cortie

Abstract

The deterioration of lipids by oxygen, termed peroxidation, is a constitutive process in living systems. Enzymatic peroxidation is required for some forms of cell signalling, but the non-enzymatic peroxidation of polyunsaturated fatty acids (PUFA) produces numerous toxic and mutagenic products associated with pathology and aging. Phospholipids containing PUFA are common in mammalian membranes, but an inverse relationship has been found between membrane susceptibility to peroxidation and maximal lifespan (MLS). Past studies of peroxidation have focused on the PUFA content of phospholipids, but other elements of phospholipid composition including class and saturated fatty acid (SFA) or monounsaturated fatty acid (MUFA) content may also influence peroxidation. This thesis presents three studies investigating the relationship between phospholipid composition and peroxidation.

In the first study, published data was analysed to examine the relationship between dietary fatty acids and the PUFA composition of cardiolipin and other classes of mitochondrial phospholipids. The composition of heart cardiolipin is commonly viewed as being strongly regulated due to its high levels of linoleic acid (18:2n-6), but the analysis showed that the percentage of 18:2n-6 in cardiolipin of heart conformed to dietary availability from 2–20% of total dietary fatty acids and was thereafter regulated. The percentage of 18:2n-6 in liver cardiolipin was lower than in heart, and was regulated across the dietary range examined. When both 18:2n-6 and docosahexaenoic acid (22:6n-3) were present in the diet, 22:6n-3 was incorporated into cardiolipin of heart and liver at the expense of 18:2n-6. The content of 22:6n-3 present in phosphatidylcholine and phosphatidylethanolamine also conformed to dietary levels, but not at the expense of 18:2n-6. This study suggests that dietary 22:6n-3 may increase the susceptibility of cardiolipin and other mitochondrial classes to peroxidation, and may therefore alter mitochondrial function.

In the second study, *in vitro* assays of iron-mediated peroxidation were used to investigate the effect of phospholipid composition on iron-mediated, non-enzymatic peroxidation. The influence of phospholipid class was examined for phospholipids of the cardiolipin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol and phosphatidic acid classes that were matched for 18:2n-6 content. Iron-initiated peroxidation of these phospholipids was tested in diffuse solution and in liposomes.

Cardiolipin and phosphatidic acid were found to peroxidise more readily than other classes in diffuse solution, but not in liposomes. Differences in peroxidation between classes were found to be related to head group charges in diffuse solution but head group sizes in liposomes. Following this work, *in vitro* assays of peroxidation were used to investigate whether phospholipids composed of SFA and MUFA influence the rates and phases of peroxidation. Phospholipids containing no PUFA, termed non-peroxidisable phospholipids (non-PPLs), were found to have an antioxidant-like effect that extended the duration of the lag phase of peroxidation several-fold through a weak inhibitory effect on rate of peroxidation during the lag phase. Non-PPLs are common in cellular membranes, suggesting that this antioxidant-like activity may be a widespread mechanism that augments traditional antioxidant defences to delay peroxidation.

In the third study, shotgun lipidomics was used to examine the phospholipid composition and peroxidation index of skeletal muscle, liver, and brain from mice (*Mus musculus*, MLS of 4 years), pigs (*Sus scrofa*, MLS of 27 years), and humans (*Homo sapiens*, MLS of 122 years). A comparison of mitochondrial membranes found a higher percentage of 22:6n-3 and a higher peroxidation index for each tissue in mice than humans. In comparison to mice, the mitochondrial membranes of humans contained a lower percentage of PUFA and a higher percentage of non-PPLs. The mitochondrial membranes of pigs shared characteristics of both mice and humans, supporting earlier findings that membrane composition is associated with lifespan. A second investigation of the importance of membrane composition to lifespan examined whether humans prevent peroxidation by excluding highly peroxidisable PUFA from their mitochondrial membranes. This exclusion has been suggested to be an evolutionary adaptation to extend longevity that is unique in humans. An intra-species comparison found few significant differences in the distributions of phospholipid classes, molecular phospholipids within classes, fatty acid composition, and the peroxidation index between the mitochondrial and microsomal membranes of humans, mice or pigs. This study is one of the most in-depth lipidomics analysis of human tissue to date, and suggests that human longevity may be related to a decrease in the susceptibility of all cellular membrane phospholipids to peroxidation.

Acknowledgements and thanks

I would like to acknowledge the following people for making the academic and technical aspects of this work possible:

- Prof. Paul Else, my primary supervisor, for encouraging me to undertake a Ph.D and guiding me with patience, humour, and a keen eye for detail.
- Assoc. Prof. Todd Mitchell, my co-supervisor, for his assistance with mass spectrometry techniques and his timely turnaround of whatever work I submitted to him.
- Prof. Tony Hulbert for his lessons on scientific writing and scientific thought, and for suggesting the comparative biology aspect of this thesis.
- Dr. Darryl McAndrew, for organizing and guiding the collection of human tissues.
- Ms. Sarah Norris, my Ph.D. sister, for her assistance with the mass spectrometry methods and general support during the trying times of Ph.D. life.
- Mr. Adam Zieba for his technical support in a wide range of areas.

In addition, I was lucky enough to have the support of many family and friends. My heartfelt thanks to:

- My parents for all their support, advice and kindness.
- My older brother, David, for his support and advice.
- My younger brother, Steve, for his unconditional sympathy while listening to my tedious complaints.
- My grandparents for all their quiet encouragement.
- Joel Mason, for always agreeing with me.
- My in-laws for all their support (and food).
- My scientific friends: Martin Engel, Lisa Franks, Franchesca Fernandez, Sarah Abbot, Megan Kelly, Peta Snikeris, Sai Ruthirakumar, Nicholas Martin, Adam Zieba and Sarah Norris for their friendship and support over the years.
- My friends at ‘cage’ soccer for their weekly dose of Sunday exercise.

And finally to my wife, Lill, for her endless love, support, and proof-reading during the good times and the bad.

Table of contents

List of figures

Chapter Six

Chapter Seven

Chapter Eight

List of tables

List of abbreviations

AAPH	2,2'–Azobis (2–amidinopropane) dihydrochloride
AGPAT	Acylglycerophosphate acyltransferase
A_{Lag}	Amount of peroxidation product produced by the end of the lag phase
ALCAT1	Lyso cardiolipin acyltransferease
ANOVA	Analysis of variance
BHCL	Cardiolipin isolated from bovine heart
BHT	Butylated hydroxytoluene
CL	Cardiolipin
Cu^{2+}	Copper
DBI	Double bond index
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ETC	Electron transport chain
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
FOX	Ferrous oxidation-xylenol orange assay
FOX-2	Ferrous oxidation-xylenol orange assay, version two
GC	Gas chromatography
HHE	4–Hydroxy–2(E)–hexenal
H_I	Hexagonal phase
H_{II}	Inverted hexagonal phase
HNE	4–Hydroxy–2(E)–nonenal
HPLC	High performance liquid chromatography
$L^{\bullet}$	Lipid radical
LH	Lipid methylene
$LOO^{\bullet}$	Peroxyl radical
LOOH	Lipid hydroperoxide
MBOAT	Membrane-bound O-acyltransferases
MDA	Malondialdehyde
MLS	Maximal lifespan
MS	Mass spectrometry
MUFA	Monounsaturated fatty acids
NMR	Nuclear magnetic resonance spectroscopy
Non-PPL	Non-peroxidisable phospholipids
NSW	New South Wales, Australia
ONE	4–Oxy–2(E)–nonenal
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PL	Phospholipids
PLA1	Phospholipase–1
PLA2	Phospholipase–2
PPL	Peroxidisable phospholipids

PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acids
R_{Lag}	Rate of peroxidation during the lag phase
R_{Max}	Maximum rate of peroxidation
ROS	Reactive oxygen species
SEM	Standard error of the mean
SFA	Saturated fatty acids
TBARS	Thiobarbituric acid reactive substances
T_{Lag}	Period of lag phase
TLC	Thin layer chromatography
T_{Max}	Total period of peroxidation reaction
UFA	Unsaturated fatty acid index
UI	Unsaturation index
UoW	The University of Wollongong

Publications and presentations arising from this thesis

Publications:

Cortie CH and Else PL, Dietary docosahexaenoic acid (22:6) incorporates into cardiolipin at the expense of linoleic acid (18:2): Analysis and potential implications. *International Journal of Molecular Sciences*, (2012). **13**(11): p. 15447–15463.

Cortie CH, and Else P.L., An antioxidant-like action for non-peroxidisable phospholipids using ferrous iron as a peroxidation initiator, *Biochimica et Biophysica Acta - Biomembranes*, 2015, **1846**(6): p. 1303-1307.

Cortie CH, Hulbert AJ, Norris SE, Mitchell TW, McAndrew D, and Else PL, Of mice, pigs and humans: a lipidomics analysis of mitochondrial phospholipids from mammals with very different maximal lifespans, *Experimental Gerontology*, 2015, **70**: 135-143.

Cortie CH, Hulbert AJ, Norris SE, Mitchell TW, McAndrew D, and Else PL, Is human longevity related to the unique ability to decrease membrane susceptibility to peroxidation? In preparation.

Presentations and posters:

Cortie CH, Hulbert AJ, Norris SE, McAndrew D, Mitchell TW, Else PL, Of Mice and men (and pigs): membrane composition and longevity. *Australian Lipid Meeting*, Wollongong,Australia 2014 (Presentation).

Cortie CH and Else PL, Comparison of lipid hydroperoxide production between phospholipid classes matched for linoleic acid: Cardiolipin is not an exceptional. *AussieMit,* Sydney,Australia 2010 (Presentation).

Cortie CH, Hulbert AJ, Norris SE, McAndrew D, Mitchell TW, Else PL, Investigating membrane composition of mitochondrial and microsomal fractions: Have humans reduced mitochondrial susceptibility to peroxidation to extend longevity? *Australian Society for Medical Research,* Sydney,Australia 2014 (Poster).

Cortie CH and Else PL, An antioxidant-like action for non-peroxidisable phospholipids using ferrous iron as a peroxidation initiator. *International Conference on the Bioscience of Lipids*, Bari, Italy, 2013, 2013, (Poster).

Cortie CH and Else PL, Dietary docosahexaenoic acid incorporates into cardiolipin at the expense of linoleic acid: analysis and potential implications. *Australian and New*

Zealand Society of Comparative Physiology and Biochemistry, Auckland, New Zealand, 2012 (Poster).

Chapter One

Preamble

1.1 Peroxidation and membrane composition

"We must answer the question, what is it that which, in natural objects, makes them easily destroyed?"

Aristotle, On the Length and Shortness of Life, paragraph 2 [1].

1.1.1 Peroxidation

Oxidative damage is a process that all aerobic organisms must accommodate in order to survive. Peroxidation is a form of oxidative modification that primarily affects the polyunsaturated fatty acids (PUFA) present in membrane and storage lipids. Many of the products of peroxidation are volatile, toxic and mutagenic [2-5] and while cells have adapted to make use of some of these products in enzymatically-controlled cell signalling pathways [6-9], the majority of products formed during peroxidation are due to non-enzymatic peroxidation [4]. Non-enzymatic peroxidation is commonly initiated by reactive oxygen species (ROS), reactive nitrogen species, and reactive chlorine species in a self-propagating reaction that produces multiple types of ROS [5, 10, 11]. This propagation may be problematic to cell function as it has the potential to greatly increase the oxidative stress placed on cells [11-14]. It is unsurprising, then, that peroxidation and its products are associated with numerous pathologies [3] and have been identified as a possible cause of aging [12]. The ROS produced by peroxidation may affect numerous components of cellular membranes including proteins, lipophilic antioxidants, and lipids. The major factor identified as determining membrane vulnerability to peroxidation is the PUFA content of membrane phospholipids, but phospholipid class and content of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) have also been suggested to influence peroxidation [15, 16]. The general aim of this thesis was to examine the relationships between membrane phospholipid composition and peroxidation.

1.1.2 Membrane phospholipid composition and peroxidation

Phospholipids are commonly composed of a class-defining head group, a glycerol backbone, and two fatty acids. Exceptions to this common structure include sphingomyelins with only one fatty acid, and cardiolipin (CL) with up to four fatty acids. Mammalian phospholipids are composed of SFA, MUFA and PUFA, with the distribution of these fatty acids differing between animal species, tissues, and classes of phospholipid [17-20]. The vulnerability of membrane phospholipids to peroxidation may be influenced by class [15] and proximity to sources of ROS production within a cell [12], but is primarily determined by fatty acid composition [5, 21-23]. Due to the abstraction energies required to initiate peroxidation, the vulnerability of membranes to peroxidation is principally determined by the percentage of PUFA present in membrane phospholipids, and the degree of unsaturation of PUFA [21]. Despite the risk posed by peroxidation, however, phospholipids containing PUFA are common in the majority of eukaryote membranes [24]. Between 40–80% of mammalian phospholipids contain a PUFA [18], and CL, a phospholipid class found primarily in mitochondria, appears to require a high PUFA content in order to interact with mitochondrial proteins of the electron transport chain (ETC) [25, 26]. It therefore appears that the incorporation of PUFA into membrane phospholipids is common, and is likely to be a trade-off between the associated risks and benefits.

Membrane phospholipid composition has been the subject of numerous studies including the influence of diet on membrane composition (e.g. [27, 28]), differences in membrane composition between animal species (reviewed in [14, 29]) and cellular organelles [17], and mechanistic studies showing how phospholipid properties may influence peroxidation and biological processes such as apoptosis [15, 30, 31]. A great deal still remains to be discovered regarding the biological importance of membrane composition and peroxidation. For example, although PUFA content may be the primary factor determining peroxidation, phospholipid class may also affect peroxidation [15, 30]. In addition, while non-peroxidisable phospholipids (non-PPLs) containing only saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) are not generally considered to influence peroxidation, non-PPLs may delay the onset of peroxidation [16]. Despite the importance of membrane composition and peroxidation to cellular health, relatively little is known regarding how PUFA and non-PPLs are distributed across phospholipid classes and whether this distribution differs between animal species or tissues. This distribution may be of particular importance as the

susceptibility of membrane phospholipids to peroxidation is inversely related to species longevity.

1.1.3 Peroxidation of membrane PUFA: A possible cause of aging?

The 'Free Radical Theory of Aging' [32, 33] proposes that ROS cause aging by irreversibly damaging cellular components, and peroxidation has been identified as a likely source of such ROS due to its self-propagating nature [12, 14]. Much of the work supporting the importance of membrane fatty acid composition and longevity comes from comparative animal studies that have established a strong and inverse correlation between membrane susceptibility to peroxidation and longevity. This relationship is best characterised for mammal muscle and liver [14, 34-36], but similar relationships have also been found for birds [12], molluscs [37], bees [38], and strains of *Caenorhabditis elegans* [39]. The relationship between membrane composition and aging caused by non-enzymatic lipid peroxidation was first proposed by Pamploma *et al.* under the name "Homeoviscous-Longevity" theory of aging [35] and formalised within the "Membrane Pacemaker Theory Of Aging" by Hulbert in 2005 [13]. This theory proposes that membrane composition and susceptibility to peroxidation is a key component of aging, but work in this area has been primarily based on fatty acid composition rather than phospholipid class or composition of molecular phospholipids. In recent years, advances in mass spectrometry have opened up new avenues of investigation into membrane phospholipid composition and longevity, but use of such technology has been limited to a small number of studies [40-42].

1.2 Thesis outline

Over 84 000 research articles have been published on the topic of peroxidation, with 50% of these published in the last decade (Web of Science). Many of these articles have focused on the chemistry of peroxidation and its complex product formation (reviewed in [4, 10, 43]), while others have focused on the presence of these products across a range of pathologies (reviewed in [3]). This thesis builds on this extensive body of work by examining the importance of phospholipid composition to peroxidation in three studies.

The first study was an analysis of all published *in vivo* dietary interventions that examined the influence of dietary PUFA on the fatty acid composition of cardiolipin

and other mitochondrial phospholipids. The results of these feeding trials are discussed in terms of recent reports of cardiolipin's role in ATP production and apoptosis in an attempt to understand the physiological relevance of changes in membrane composition. This work is presented in Chapter Three.

The second study examined *in vitro* iron-mediated peroxidation in liposomes of known membrane composition. This work investigated if phospholipid properties other than PUFA content influenced peroxidation. Chapter Four examined the influence of phospholipid head group on peroxidation independently of fatty acid content, and if the cardiolipin class of phospholipids is particularly susceptible to peroxidation compared to other phospholipid classes. This study used purified synthetic phospholipids to investigate the importance of phospholipid class to peroxidation independently of phospholipid associations with proteins, lipophilic antioxidants, and other membrane molecules that may influence peroxidation. In Chapter Five multiple measures of peroxidation were used to determine whether phospholipids containing only SFA and MUFA influence the onset of peroxidation by exerting an antioxidant-like effect. Such phospholipids are generally not thought to influence peroxidation, but are very common in membranes and have been suggested to delay the onset of peroxidation [16].

In the third study, a comparison of membrane composition of three mammal species with very different maximal lifespans (MLS) was conducted. The animal species examined were mice (*Mus musculus*, MLS of 4 years), pigs (*Sus scrofa*, MLs of 27 years) and humans (*Homo sapiens*, MLS of 122 years). Mice and pigs have typical lifespans for their body masses, but humans are exceptionally long-lived in comparison to other mammals with similar body masses (AnAge Database [44]). The reason for this longevity may be related to human membrane composition, but relatively little data for humans is available in the literature. Shotgun lipidomics was used to determine the molecular phospholipid composition of membranes from skeletal muscle, liver and brain. In Chapter Six, the differences in the membrane composition and susceptibility to peroxidation of mitochondria were compared between the three species. Chapter Seven continued this examination by testing the suggestion by Hulbert (2010) [45] that humans have a unique ability to decrease the susceptibility of their mitochondrial membranes peroxidation when compared to other cellular membranes. It was suggested that such an ability may prevent oxidative stress and therefore extend longevity. A major strength of these chapters was the use of human tissues, which is rarely included

in comparative studies. In addition, the use of shotgun lipidomics allowed a more detailed lipidomics analysis of membranes than previously possible.

The outline of this thesis is presented in Figure 1.1. Due to the different methods used in each study, separate introduction and method sections are provided for each chapter. A combined Bibliography is provided for all chapters.

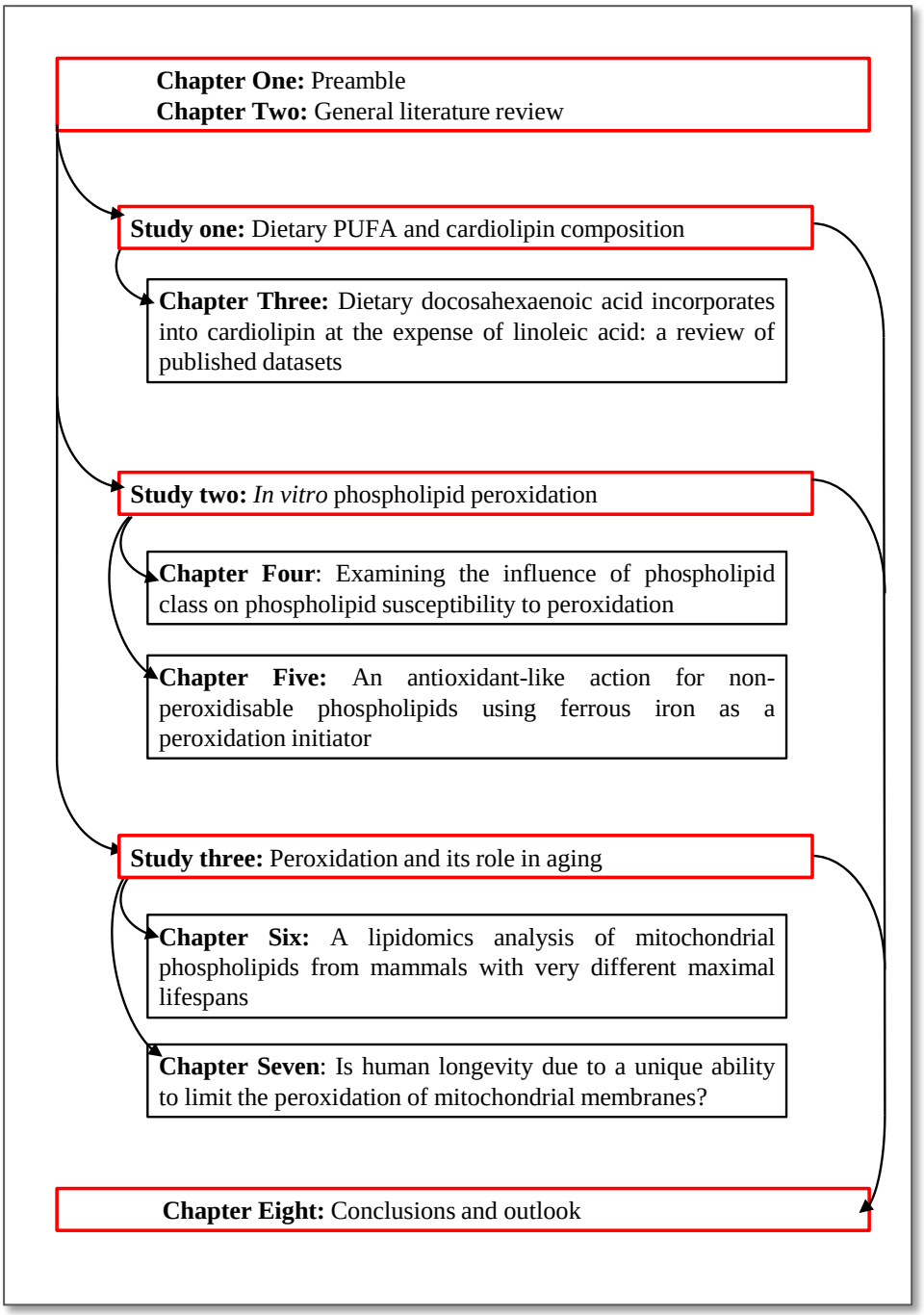

Figure 1.1. Thesis outline.

1.3 Aims and research questions

The overall aim of this thesis was to investigate the relationship between membrane composition and peroxidation with relevance to the aging process. The specific research questions addressed in this thesis are:

- How do dietary MUFA and PUFA influence the fatty acid composition of cardiolipin and other major classes of mitochondrial phospholipids in mammals?
- Do cardiolipin and other phospholipid classes affect peroxidation independently of PUFA composition?
- Do phospholipids composed of only SFA or MUFA influence the rates and lag phase of peroxidation of phospholipids containing PUFA through an antioxidant-like action?
- Are mitochondrial phospholipids less susceptible to peroxidation in humans than in mice or pigs?
- Are human mitochondria less susceptible to peroxidation than other human cellular membranes?

Chapter Two
General literature review

2.1 Overview

This chapter provides an overarching review of the literature relevant to this thesis. More specific literature reviews are provided within the introduction sections of each chapter. This chapter includes a discussion of cellular membranes and the role of phospholipids (2.2), phospholipid structure and regulation in membranes (2.3), the mechanisms and measurement of peroxidation of membrane lipids (2.4), the association between membrane composition and aging (2.5), and a brief conclusion (2.6).

2.2 Cellular membranes

Cellular membranes are ubiquitous structures in cells that are thin (6–8 nm), semi-permeable structures that define cell boundaries. Numerous interactions take place within and across cell membranes including the movement of molecules during signalling and transport, the formation of enzymes complexes to localise enzymatic reactions, and a great many anabolic and catabolic processes [46, 47]. In addition to the plasma membranes that define a cell's boundary, eukaryotes also have membrane-bound organelles that increase metabolic efficiency by compartmentalizing catabolic and anabolic functions [48]. Cellular membranes are complex structures composed of a diverse mixture of lipids, proteins, and carbohydrates, and this composition may differ between species, between tissues within a species, and even between organelles within a cell [17, 49-51]. The current model used to describe the interactions of the components in membranes is a modified form of the "Fluid-Mosaic Model" originally proposed by Singer and Nicolson in 1972 [49]. In this model, phospholipids form a thermodynamically stable, fluid and semi-permeable bilayer to which membrane proteins are associated by strong hydrophobic interactions (integral proteins), weaker hydrophilic bonds (peripheral proteins) [49], or through interactions that do not interfere with the intrinsic structure of cell membranes (membrane-associated proteins) [52] (Figure 2.1). The "Fluid-Mosaic Model" has been reviewed many times, and recent work in the area [46, 47, 53] supports the original "Fluid-Mosaic Model" with the

amendments that phospholipids are not organised randomly but rather as curved micro domains of varying fluidity including relatively viscous ‘rafts’ [54], that proteins are far more common within the bilayer than suggested in the original work [47, 53], and that these proteins may form aggregates with restricted lateral mobility [46].

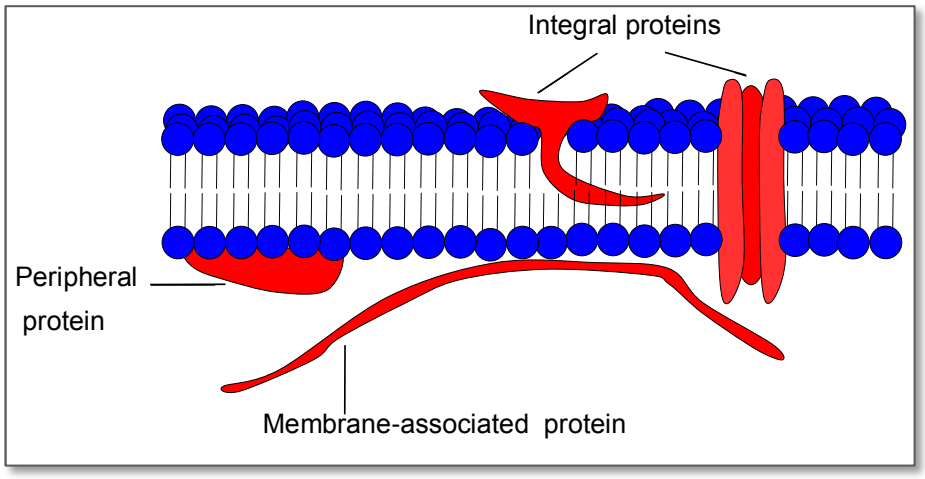

Figure 2.1. Diagram of protein interactions with the phospholipid bilayer. Proteins are in red; phospholipids in the bilayer are blue. Based on Goni *et al.* 2014 [47].

The phospholipid bilayer is a fundamental component of the cell membrane, and the phospholipids that comprise this bilayer account for 15–45% of the weight of biological membranes, with the remaining weight primarily proteins [17, 51]. As the average phospholipid molecule is much lighter than the average protein molecule, it has been estimated that cellular membranes contain 10–100 phospholipids per protein, making phospholipids the most numerous component of cellular membranes [50, 55]. In comparison to phospholipids, other lipid components of membranes such as sphingolipids, glycolipids and cholesterols are far less abundant [17]. In addition to forming the bilayer structure, phospholipids also have important roles in membranes as allosteric effectors of proteins [25, 56-58], substrates for enzymes during signalling cascades [8, 59-63], and in influencing key membrane properties including curvature [20], fluidity [64], and susceptibility to peroxidation [5, 22]. The peroxidation of phospholipids may disrupt the bilayer structure and permeability [65, 66] and damage membrane proteins [4, 5, 12], and the relative susceptibility of membrane phospholipids

to peroxidation is the focus of this thesis. This susceptibility is primarily determined by phospholipid fatty composition.

2.3 Phospholipids

2.3.1 Phospholipid structure

Phospholipids, which include all glycerophospholipids and some sphingosines, are composed of a polar head group joined by a phosphodiester linkage to a hydrophobic moiety. Due to this structure, phospholipids are amphipathic and self-orientate in aqueous media with head groups towards the aqueous plane and hydrophobic moieties away from the aqueous plane. This orientation forms a stable micelle or bilayer. Glycerophospholipids are composed of a head group joined by a phosphodiester link to the *sn*-3 position of a glycerol backbone and acyl chains at the *sn*-1 and *sn*-2 positions of the glycerol. An example of a common glycerol phospholipid is shown in Figure 2.2. In comparison, sphingomyelin phospholipids have a hydrophilic head group joined to a sphingosine backbone by a phosphodiester, and a single fatty acid joined to the backbone. As glycerophospholipids are the most common component of cell membranes and are the primary substrate of peroxidation, the subsequent use of 'phospholipids' in this thesis will refer to glycerolphospholipids only.

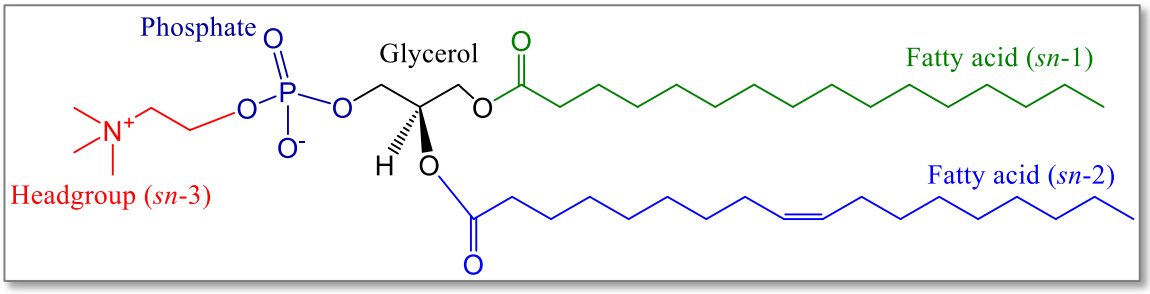

Figure 2.2. A typical mammalian phospholipid composed of a glycerol backbone with a phosphate-linked head group at the *sn*-3 position and hydrophobic acyl chains at the *sn*-1 and *sn*-2 positions.

2.3.2 Head groups

Phospholipids are classified according to the structure of the phosphate-ester head group present at the *sn*-3 position of the glycerol backbone. The structure of these phosphate-ester head groups determine the ionization constants, net charges, and relative shape of phospholipids [24]. The seven classes of glycerophospholipid that have been identified in mammals are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylinositol (PI), and di-phosphatidylglycerol which is more commonly known as cardiolipin (CL). The structures of these seven head groups are presented in Figure 2.3.

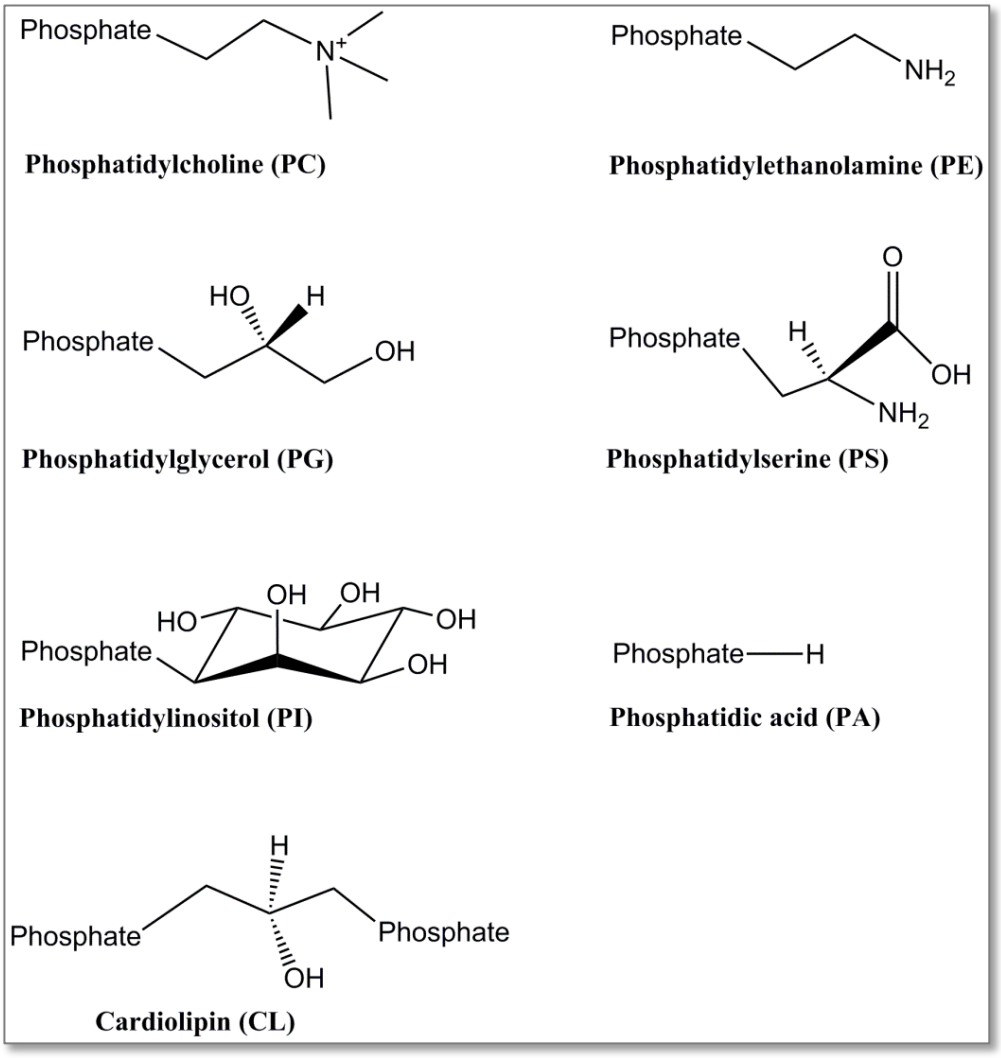

Figure 2.3. Structure of glycerophospholipid head groups.

2.3.3 Types of bonds between the glycerol and fatty acids

The *sn-2* position of a phospholipid glycerol is typically occupied by an ester-linked fatty acid, but the *sn*-1 position of the glycerol may be unoccupied, making a lyso-phospholipid, or occupied by an ester-linked acyl chain, a vinyl ether-linked acyl chain (commonly termed a plasmalogen), or an alkyl ether [67]. The structures of these three bonding motifs are shown in Figure 2.4. The purpose of these different types of fatty acid bonding motifs is unclear, but plasmalogen deficiencies have been reported in a number of developmental disorders [68], and it has been suggested that plasmalogens and alkyl ethers are important in membrane fusion events and as sacrificial molecules that prevent the propagation of peroxidation [67-69].

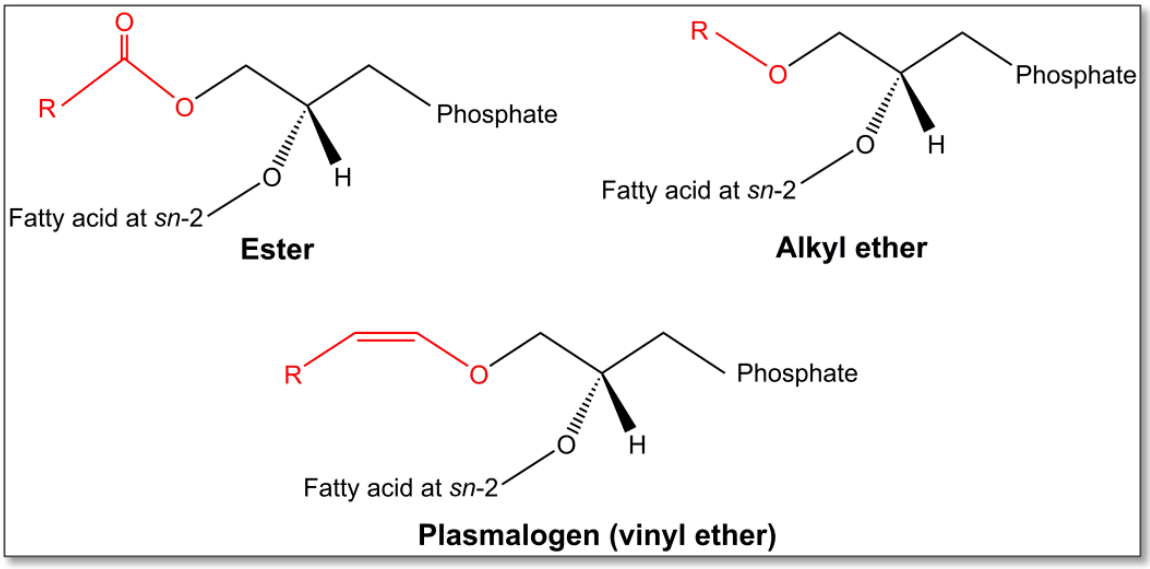

Figure 2.4. The structure of ester, alkyl ether and plasmalogen bonding motifs at the *sn*-1 position of a phospholipid's glycerol backbone. Differences in structures are highlighted in red.

2.3.4 Fatty acids

Fatty acids are long-chain carboxylic acids. Mammalian fatty acids are grouped into three structural categories based on the number of carbon-carbon bonds present [70]. The categories are: saturated fatty acids (SFA) that contain no double bonds, monounsaturated fatty acids (MUFA) that contain a single double bond, and polyunsaturated fatty acids (PUFA) that contain two or more double bonds. These double bonds are predominately in the *cis* (Z) configuration in mammalian tissues [71].

Isomers of MUFA and PUFA can be further classified by the position of the first double bond between carbons from the methyl end of the fatty acid chains [70]. This is termed the 'n' or omega position. Most MUFA are n-9 in structure, with the double bond beginning at the 9th carbon in from the methyl end of the acyl chain, while some are n-7 with the double bond beginning at the 7th carbon from the methyl end of the acyl chain. PUFA are most commonly n-6 or n-3, with the notable exception of mead acid which is an n-9 PUFA. Fatty acids can be abbreviated using the omega abbreviation system proposed by Holman (1964) [72] as the number of carbons: number of double bonds and the position of the 'n' double bond. The structures of 18-carbon long SFA, n-9 MUFA, n-6 PUFA and n-3 PUFA are presented in Figure 2.5. Examples of the names, structures, and abbreviations of common mammalian fatty acids are listed in Table 2.1.

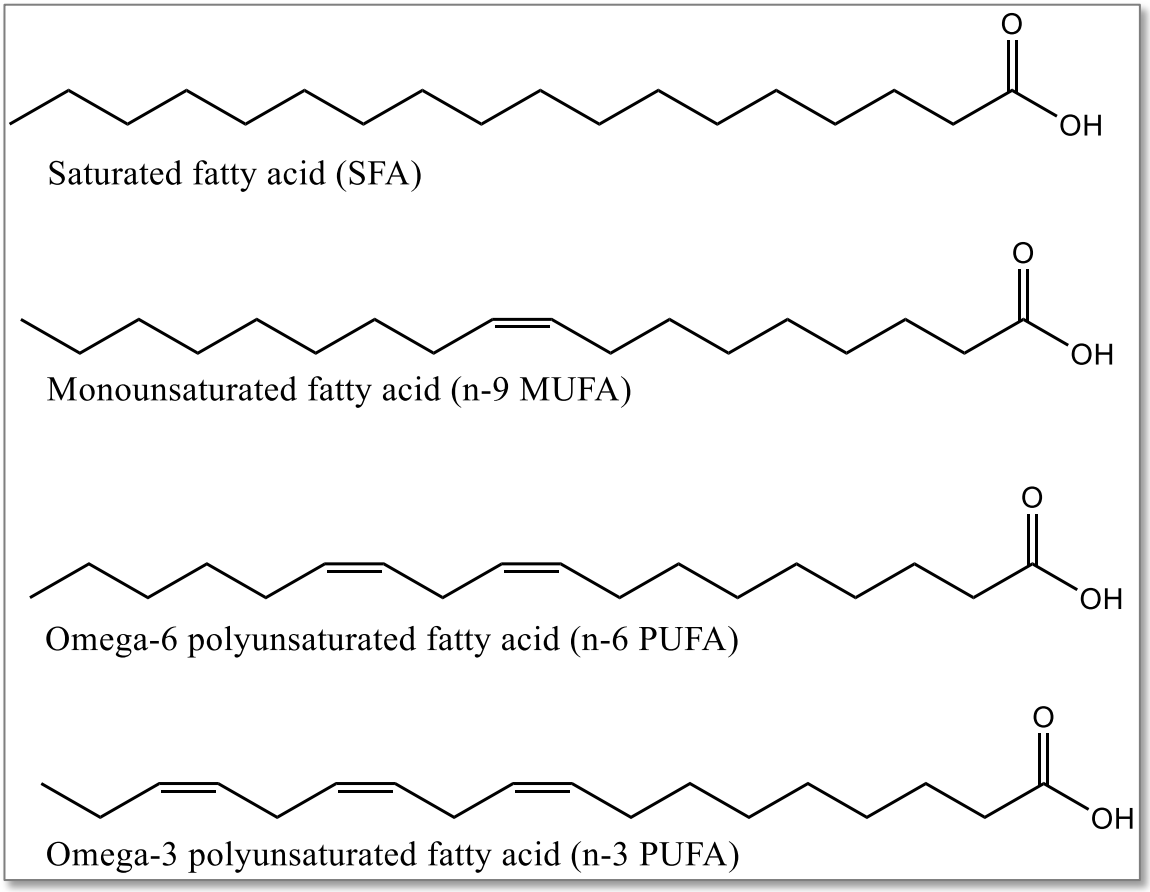

Figure 2.5. Structure of a saturated fatty acid (SFA), an omega-9 monounsaturated (n-9 MUFA), an omega-3 polyunsaturated fatty acid (n-3 PUFA) and an omega-6 polyunsaturated fatty acid (n-6 PUFA). All fatty acids shown are 18 carbons long.

Table 2.1. Common mammalian fatty acids

Trivial names	Systematic name	Carbons: Double bonds	Class
Saturated			
Myristic acid	Tetradecanoic acid	14:0	–
Palmitic acid	Hexadecanoic acid	16:0	–
Stearic acid	Octadecanoic acid	18:0	–
Monounsaturated			
Vaccenic acid	11–Octadecenoic acid	18:1	n-7
Oleic acid	9–Octadecenoic acid	18:1	n-9
Polyunsaturated			
Linoleic acid	9,12–Octadecadienoic acid	18:2	n-6
α–Linolenic acid	9,12,15–Octadecatrienoic acid	18:3	n-3
Arachidonic acid	5,8,11,14–Eicosatetraenoic acid	20:4	n-6
Clupanodonic acid	7,10,13,16,19–Docosapentaenoic acid	22:5	n-3
Cervonic acid	4,7,10,13,16,19–Docosahexaenoic acid	22:6	n-3

The introduction of a double bond into a fatty acid can affect a number of phospholipid properties including melting point [64]. Phospholipid melting points increase with fatty acid length but are decreased by the presence of double bonds, with double bonds having the greatest effect when present in the middle of the chain [24, 64]. For example, lyso-PC 18:0 has a melting point of 55 ºC, lyso-PC 18:1n-6 has a melting temperature of 10 ºC, and lyso-PC 18:1n-9 has a melting temperature of –20 ºC [64]. The presence of MUFA and PUFA in membranes may therefore greatly increases membrane fluidity.

The number and position of double bonds in the fatty acid chain is also the primary factor determining phospholipid susceptibility to hydrogen abstraction which may initiate peroxidation [5, 22, 73]. A SFA has no double bonds between carbons and can be considered to be a series of linked methylene groups with electrochemical reduction potentials of 1.90 V at the C–H bonds. The introduction of a single double bond produces a MUFA with two allylic methylene that have a C–H bond reduction potential of 0.96 V, and the introduction of multiple double bonds on alternative carbons produces a PUFA with bis-allylic methylene that have a reduction potential of only 0.60 V [73]. As a consequence of their lower reduction potential, the hydrogen in bis-allylic methylene groups are more likely to be abstracted than those in methylene or allylic methylene [11]. Due to their structure, SFA contain only methylene groups, MUFA contain two allylic methylene groups, and PUFA contain multiple allylic methylene groups and at least one bis-allylic methylene group depending on the degree

of polyunsaturation. For example, 18:2n-6 contains only one bis-allylic methylene while 18:3n-3 contains two, 20:4n-6 contains four, and 22:6n-3 contains five. Examples of a methylene group, allylic methylene group, and bis-allylic methylene group are illustrated in Figure 2.6.

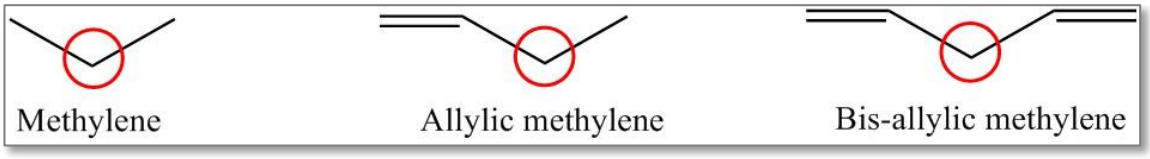

Figure 2.6. Structure of methylene, allylic methylene, and bis-allylic methylene groups. Red circles highlight the relevant methylene.

2.3.5 Nomenclature

Phospholipids have complex systematic names, and a number of ways to abbreviate these names have been proposed. This thesis follows the nomenclature for phospholipid abbreviation established by Liebish *et al* in 2013 [74]. Using this nomenclature, phospholipids are identified by abbreviation of phospholipid class (e.g. PC) and the number of carbon: number of double bonds of fatty acids present (e.g. 16:0, 22:6). Fatty acids are separated by a "/" when *sn*-position is known (*sn*-1/*sn*-2) or a "_" when *sn* position is not known (Lyso species can be indicated with either an "L" preceding the class or with a 0:0 indicating the empty *sn* position. Bond types other than esters are indicated with a "P" for plasmalogen or an "O" for alkyl ether in front of the fatty acid.

For example, lyso-phosphatidylcholine (LPC) composed of palmitic acid (16:0) has a systematic name of 1–hexadecanoyl–*sn*–glycero-3–phosphocholine and is abbreviated as LPC 16:0. Phosphatidylcholine (PC) containing a palmitic acid (16:0) and an oleic acid (18:1) has the systematic name 1–hexadecanoyl–2–(9-octadecenoyl) –*sn*–glycero–3–phosphocholine, and is abbreviated to PC 16:0_18:1 when *sn* position is unknown or PC 16:0/18:1 if *sn* position has been confirmed. Phosphatidylethanolamine (PE) with a plasmalogen-linked stearic acid (P–18:0) at the *sn*-1 position and a docosahexaenoic acid (22:6n-3) at the *sn*-2 position has the systematic name 1–(1–octadecenyl)–2–(4,7,10,13,16,19–docosahexaenoyl)–*sn*-glycero–3–phosphor-ethanolamine and is abbreviated as PE P–18:0/22:6.

2.3.6 Phospholipid composition influences membrane structure

The class and fatty acid composition of phospholipid properties may affect both their position within a membrane and the shape of the membrane itself. Phospholipids with head groups that are wider than their fatty acid moiety, such as lyso-phospholipids, have large positive curvatures and tend to form micelles in the hexagonal I (H_I) phase [75]. Phospholipids with head groups that are narrow relative to their fatty acid moiety, such as non-lyso molecular species of PE, PS and CL , tend to form membranes in the cubic phase (H_{II}) [75]. Phospholipids with head groups that are slightly wider than their fatty acid moiety, including non-lyso molecular species of PC, PG and PI, have small negative curvatures and tend to form in the lamellar phase which corresponds to the bilayer structure found in cell membranes. Figure 2.7 shows examples of phospholipids in the $H_{I,}$ H_{II} and lamellar phases. Within stable membranes, mixtures of phospholipids are likely to be asymmetrically distributed based on class, with the anionic classes such as PE and CL accumulating on the cytofacial leaflet while PC tends to accumulate in the exofacial leaflet [24]. This distribution is greatly aided by flippase, floppase, and scramblase activity [76]. Lipid-protein interactions are more likely to occur at the inner, negatively charged membrane leaflet, and proteins associated with this leaflet contain high levels of arginine and lysine [55, 77].

2.3.7 Composition and regulation of phospholipids in mammalian tissues

Although thousands of unique phospholipid structures are possible due to potential combinations of head groups, bonding motifs and fatty acids, mammalian membranes generally contain only a few hundred different molecular species of phospholipid. The composition of these molecular phospholipids may differ between species and tissues [17, 64], and even organelles within cells [17, 78]. The most common classes of phospholipids in mammal membranes are PC and PE, and together these two classes typically account for 60–90% of total phospholipids. CL is unusual amongst the phospholipid classes in that it accounts for approximately 15% of mitochondrial phospholipids but 1% or less phospholipids in other cell membranes [17]. The remaining phospholipid classes of PS, PG, PA and PI are less abundant, with their

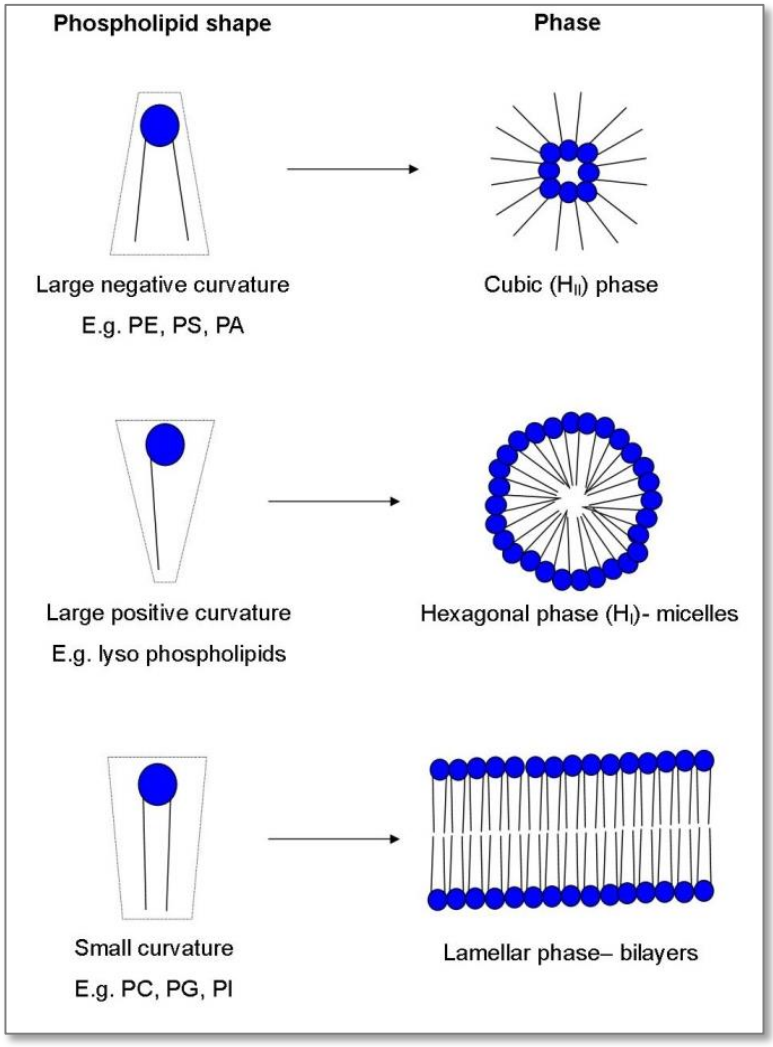

Figure 2.7. Influence of phospholipid structures on membrane structural phases.

distribution differing between membrane types. Plasmalogens and alkyl ethers are distributed evenly across phospholipid classes, being most abundant in PC and PE of brain, but are also found in a variety of other tissues [67]. PUFA are also unevenly distributed across phospholipid classes, with phospholipids of CL commonly containing multiple PUFA [79] while phospholipids containing more than one PUFA are rare in other classes [18, 24, 80]. Less is known about the composition of individual phospholipid species in mammal tissue, but PC 16:0_22:6, PE 18:0_22:6, and PS 18:0_22:6 have been found to be more common in smaller mammals than larger mammals [18], and more common in short-lived mammals compared to long-lived mammals [40]. In larger mammals, PC 16:0_18:1 and PE 18:0_18:2 are more common, possibly serving as replacements for phospholipid containing 22:6n-3.

The phospholipid composition of cellular membranes is regulated through synthesis [64], selective transfer between organelles, remodelling, and degradation [81]. The endoplasmic reticulum (ER) is the primary site of PC, PE, and PS synthesis within cells, mitochondria are the primary site of PG, PA, and CL synthesis and may also synthesize PE, and peroxisomes are the primary site of plasmalogen production [17, 82-85]. These phospholipids are subsequently transported between cellular membranes where they may undergo remodelling of their acyl content. For example, while mammalian cells normally produce only four acyl combinations (16:0/18:1, 16:0/18:2, 16:0/22:6 and 18:1/18:2) of PC and PE *de novo*, a variety of other molecular species of PC and PE are common suggesting that extensive acyl remodelling occurs [86]. This remodelling appears to be more common in PE, as PC 16:0_18:1 and PC 16:0_18:2 are dominant phospholipids in most mammal membranes and PC 16:0_22:6 is extremely common in small mammals like mice [18].

Phospholipid remodelling occurs by the Land cycle, a series of deacylation and reacylation reactions named after William E. Lands who conducted the seminal work in this area [87]. Phospholipid deacylation is initiated by the hydrolysing of a fatty acid at the *sn-1* position for the phospholipase 1 (PLA1) family or at the *sn*-2 position by member of the phospholipase 2 (PLA2) family [85]. Acyl transferases, such as those of the acylglycerophosphate acyltransferase (AGPAT) and membrane-bound O-acyltransferases (MBOAT) families, may then selectively reacylate the free *sn* position with a different fatty acid [85]. This selective reacylation can be influenced by availability of fatty acids substrates, with the types and amount of PUFA of particular importance in this respect. Unlike plants, mammals lack the desaturases required to produce n-3 and n-6 PUFA *de novo* and so must acquire 18:2n-6, 18:3n-3 and other PUFA from the diet [88]. In the absence of sufficient dietary PUFA, 18:1n-9 will be converted to mead acid (20:3n-9) in what may be an attempt by the organism to overcome this deficiency through alternate pathways [72]. This response is insufficient in the long term as animals that are not fed sufficient dietary PUFA die at an earlier age than controls [89]. In the presence of sufficient dietary n-3 and n-6 PUFA, the percentage of total dietary PUFA does not affect membrane composition but the ratio of n-3 PUFA to n-6 PUFA does as both are substrates for acyltransferases [28, 90, 91].

2.4 Peroxidation: The oxidation of lipids

2.4.1 Background

The term peroxidation refers to the oxidative modification of biological molecules to form peroxides, with lipids being the most common substrate for this reaction. Peroxidation was first observed in 1791 by the Swiss scientist Senebier who observed that the prolonged exposure of olive oil to air caused the oil to turn rancid [92]. This was followed by numerous investigations into the effect of peroxidation on food taste and stability (reviewed in Hammond (2011) [93]), including the finding in 1911 by the Swedish scientist Thunberg who reported that this process was catalysed by iron salts [94]. Despite this early work, the chemical mechanisms underlying peroxidation were finally elucidated by workers at the British Rubber Producers' Association in the 1940s [11, 93]. In more recent years, interest into the roles of non-enzymatic and enzymatic peroxidation in biology has greatly expanded.

2.4.2 Chemistry of peroxidation

Initiation, propagation, and termination

Peroxidation proceeds in a classical free radical chain reaction of initiation, propagation and termination that is most commonly initiated by reactive oxygen species (ROS) [4, 5, 10, 11]. Initiation of peroxidation can occur through the addition of ROS to a lipid methylene group (LH) (Equation 1) or the abstraction of a hydrogen from a LH by ROS (Equation 2) [11]. The abstraction of hydrogen is more common than the addition of ROS, but in either case a carbon radical ($L^{\bullet}$) is formed. This carbon radicals often stabilises by re-arranging to form a conjugated diene radical, and this diene may react with available oxygen to form a peroxyl radical ($LOO^{\bullet}$) (Equation 3). The $LOO^{\bullet}$ produced in this manner are sufficiently reactive to abstract hydrogen from another LH to produce a lipid hydroperoxide (LOOH) and a second $L^{\bullet}$, propagating the peroxidation reaction (Equations 4). The structures and progression of peroxidation are illustrated in Figure 2.8.

$LH + OH^{\bullet} \rightarrow LHOH^{\bullet}$ (alternatively written $L^{\bullet}$) Equation 1

$LH + R^{\bullet} \rightarrow L^{\bullet} + RH$ Equation 2

$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$ Equation 3

$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$ Equation 4

Termination of peroxidation may occur when two conjugated diene radicals cross-link (Equation 5), two lipid peroxide radicals react (Equation 6), or a conjugated diene radical or lipid peroxide radical reacts with an antioxidant (Equation 7).

$L^{\bullet} + L^{\bullet} \rightarrow L–L$ Equation 5

$LOO^{\bullet} + LOO^{\bullet} \rightarrow LOO–LOO$ Equation 6

$L^{\bullet}$ or $LOO^{\bullet}$ + antioxidant → non-radical products Equation 7

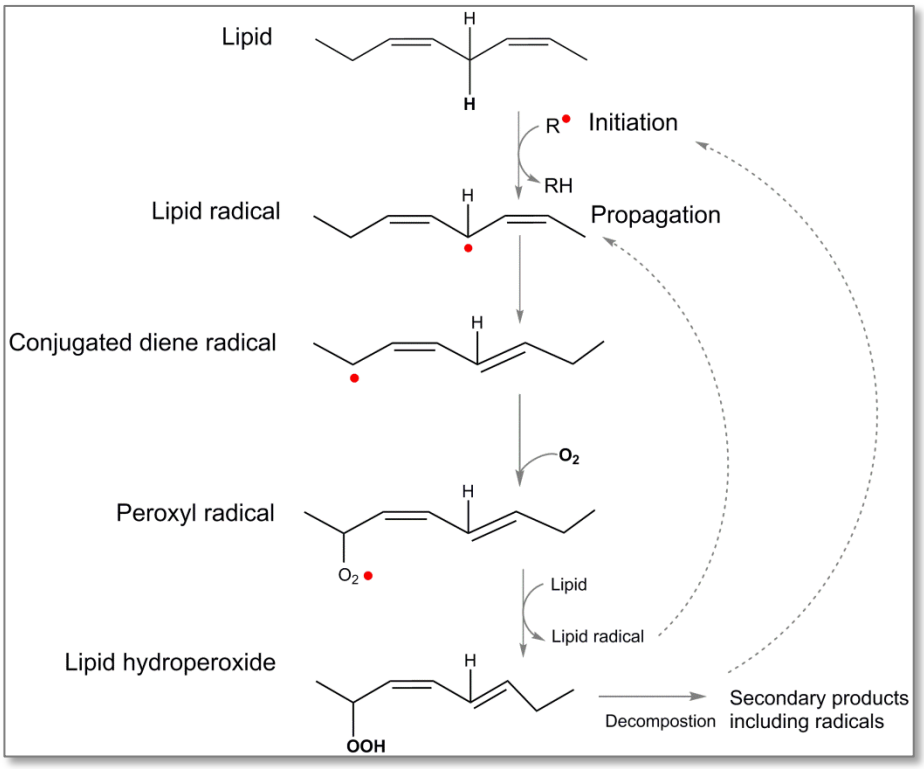

Figure 2.8. Initiation and propagation of peroxidation by hydrogen abstraction. Based on Yin *et al.* (2011) and Halliwell and Guetteridge (2007) [5, 11].

Initiators and targets of peroxidation

The energy required for hydrogen abstraction – and therefore the likelihood of abstraction occurring – is theoretically determined by difference in reduction potentials of substrates and oxidizing agents. Theoretically, a molecule can oxidise any compound with a lower standard reduction potential (V) [5, 11]. Table 2.2 shows standard reduction potentials of common targets and causes of oxidation in biological systems [73, 95]. As can be seen from the table, the bis-allylic methylene groups present in PUFA have a lower reduction potential than SFA, MUFA, proteins or DNA and are therefore more likely to undergo hydrogen abstraction. While numerous forms of ROS initiator may be present in cellular environments, *in vitro* studies of peroxidation generally use radical clocks such as 2,2'–azobis(2–amidinopropane)–dihydrochloride (AAPH) to generate ROS at a constant rate to allow the kinetics of peroxidation to be accurately determined [5, 96], or use iron and copper to initiate peroxidation through the generation of peroxyl and alkoxyl radicals [11, 96].

Table 2.2. Initiators and substrates of peroxidation

Type	Couple	Standard reduction potential (V)
Initiators		
Hydroxyl	$OH^{\bullet}, H^{+}/H_2O$	2.31
Alkoxyl	$RO^{\bullet}, H^{+}/ROH$	1.61
Hydroperoxyl	$HOO^{\bullet}, H^{+}/H_2O_2$	1.06
Peroxyl	$ROO^{\bullet}, H^{+}$ / ROOH	1.00
Targets		
SFA	$Methylene^{\bullet}, H^{+}$ /methylene–H	1.90
MUFA	$Allylic\ methylene^{\bullet}, H^{+}$/ allylic methylene–H	0.96
PUFA	$Bis–allylic\ methylene^{\bullet}, H^{+}$/bis–allylic methylene–H	0.60
Protein (Cysteine)	$ROS^{\bullet}, H^{+}$ / ROS–H	0.92
DNA (Guanine)	Various product species/guanine	0.96
Antioxidants		
α-Tocopherol (Vitamin E)	$TO^{\bullet}, H^{+}$/TOH	0.50
Ascorbate (Vitamin C)	$Ascorbate^{\bullet}, H^{+}$/ascorbate monoanion	0.28

Values are from [72].

Role of iron and other transition metals

Iron and copper greatly increase the rate of peroxidation, a process most extensively studied in ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron [11, 97-100]. High concentrations of Fe^{2+} may react with O_2 to form $OH^{\bullet}$ via H_2O_2 production and decomposition (Equation 4–7) or may react directly with H_2O_2 to produce $OH^{\bullet}$ through the Fenton reaction (Equation 6–7).

$$Fe^{2+} + O_2 \leftrightarrow Fe^{3+} + O_2^{\bullet -} \quad \text{Equation 4}$$

$$2O_2^{\bullet -} + 2H^{+} \rightarrow H_2O_2 \quad \text{Equation 5}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + OH^{\bullet} \quad \text{Equation 6}$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+}\ HO_2^{\bullet} + H^{+} \quad \text{Equation 7}$$

Although $OH^{\bullet}$ produced in this manner may initiate peroxidation, the presence of H_2O_2–scavenging proteins inhibits $OH^{\bullet}$ production but not peroxidation, suggesting that peroxidation is being initiation by ROS other than $OH^{\bullet}$. Such ROS may be produced when Fe^{2+} and Fe^{3+} decompose LOOH in a similar way as described for H_2O_2, with Fe^{2+} reacting with LOOH to produce $LOO^{\bullet}$ (Equation 8) and Fe^{3+} reacting with LOOH to produce alkoxyl radicals ($LO^{\bullet}$) (Equation 9) [99].

$$Fe^{3+} + LOOH \rightarrow Fe^{2+} + LOO^{\bullet} + H^{+} \quad \text{Equation 8}$$

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + OH^{-} + LO^{\bullet} \quad \text{Equation 9}$$

The $LOO^{\bullet}$ and $LO^{\bullet}$ radicals produced during this reaction are sufficiently reactive to abstract a hydrogen from a second bis-allylic methylene group, therefore initiating and propagating peroxidation (Equation 1). As Fe^{2+} and Fe^{3+} cannot abstract hydrogen directly, some LOOH must already be present in the lipid for mediation to occur through $LOO^{\bullet}$ and $LO^{\bullet}$ production. This is normally not a limitation for *in vitro* experiments as LOOH is commonly formed during purification, sonication and exposure to oxygen [101]. An added complexity of iron-mediated peroxidation is that Fe^{2+} and Fe^{3+} can also scavenge radicals under some conditions, terminating peroxidation (Equations 10–11).

$$Fe^{2+} + LO^{\bullet} + H^{+} \rightarrow Fe^{3+} + LOH \qquad \text{Equation 10}$$

$$Fe^{3+} + LOO^{\bullet} + H^{+} \rightarrow Fe^{2+} + LOOH \qquad \text{Equation 11}$$

A similar mechanism for redox cycling during peroxide decomposition has been reported for copper [11]. Although the chemistry of initiating peroxidation with iron and copper is complex [11, 96, 99, 101-103], poorly liganded iron is likely to be a major cause of peroxidation within cells [104, 105].

2.4.3 Products of peroxidation

It has been estimated that 120–150 different forms of lipid hydroperoxides may be produced in natural membranes during peroxidation [2], and the decomposition of these hydroperoxides produces a wide range of highly unstable secondary products including alkanes, aldehydes, ketones, alcohols and furanes [4]. This variety of products is due to the diversity off bis-allylic methylene groups position in PUFA, whether peroxidation occurs at one or multiple bis-allylic methylene groups, and whether cyclization or cross-liking occurs. In addition, the products of peroxidation may be produced in racemic mixtures [5]. Many of these products are highly volatile and can form adducts with proteins [106] and DNA [42, 107], with aldehydes being particularly reactive in this regard [4, 106]. Common examples of lipid peroxidation products include malondialdehyde (MDA) formed from PUFA with three or more double bonds, 4–oxy–2(E)–nonenal (ONE) produced from the decomposition of 18:2, 4–hydroxy–2(E)–nonenal (HNE) produced from the peroxidation of n-6 fatty acids, and 4–hydroxy–2(E)–hexenal (HHE) produced from the peroxidation of n-3 fatty acids [4]. The mixture of these products is therefore largely determined by the distribution of PUFA present in membrane phospholipids.

2.4.4 Phases of peroxidation

Peroxidation typically occurs in discrete phases of lag, propagation, and termination [96]. The phases and possible measures of peroxidation are presented in Figure 2.9. Following initiation, peroxidation enters a lag phase with a low rate of peroxidation (R_{Lag}). This phase is measured as a period of time (T_{Lag}) between the start of peroxidation and the beginning of the propagation phase. The amount of peroxidative product produced by the end of this phase is termed the (A_{Lag}). The T_{Lag} can be

lengthened and the the A_{Lag} increased by antioxidant defences that prevent the build-up of high levels of ROS. From a biological perspective, the T_{Lag} is important as it represents a window of opportunity for cells to prevent entry into the potentially more damaging propagation phase. The duration of T_{Lag} is therefore considered to be a measure of the antioxidant status of membranes [108].

The propagation phase begins when the lag phase ends. The peroxidation phase is when the maximal rate of peroxidation (R_{Max}) occurs. Propagation is likely to be the most damaging phase of peroxidation, and the R_{Max} of lipids has been studied in both solvents and liposomes [5, 21, 22, 109, 110]. The R_{Max} increases with the number of bis-allylic methylene present in PUFA, and this relationship has been reported being exponential in some studies [21, 109] but linear in others [5, 22, 110]. The termination phase of peroxidation occurs as the R_{Max} ebbs due to substrate exhaustion, radical crosslinking, or antioxidant quenching. The time at which this occurs is termed the $T_{Max,}$ and the maximal amount of peroxidation product that has been produced by this point is termed the A_{Max}.

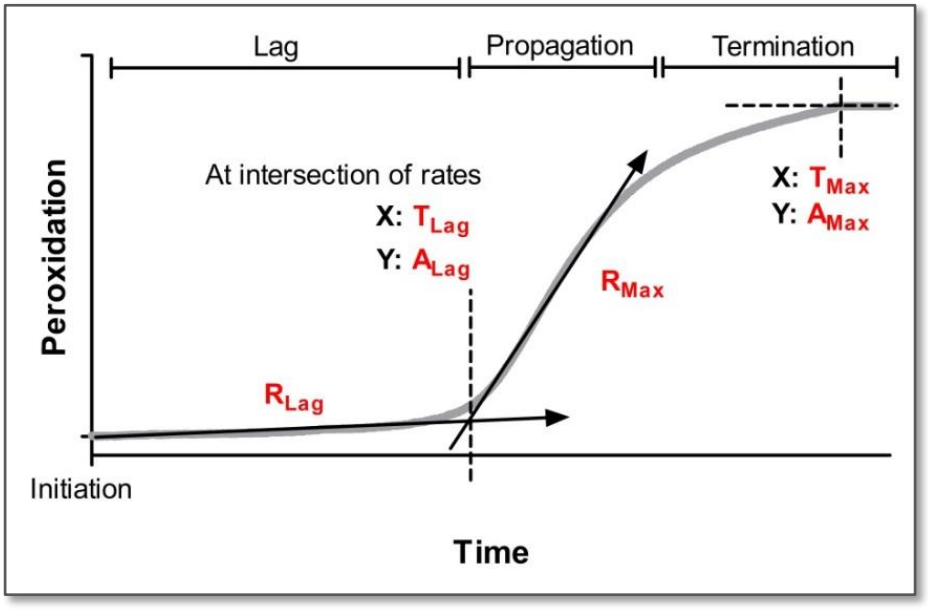

Figure 2.9. The progression of peroxidation during the lag, propagation and termination phases. Measures of peroxidation in each phase are highlighted in red. Based on Pinchuk and Lichtenberg, 2014 [96].

2.4.5 Measuring peroxidation

Lipid peroxidation produces conjugated dienes, consumes oxygen and PUFA, forms lipid hydroperoxides (LOOH), and produces numerous secondary products from LOOH decomposition. As a result, there are many ways of measuring peroxidation including substrate loss and product formation [11]. The earliest and simplest method used to measure peroxidation was to simply expose lipids to oxygen and measure the subsequent changes in lipid mass over time [94, 111]. Alternatively, the production of conjugated dienes, LOOH, and MDA can be used to measure peroxidation with various simple assays and absorbance spectrophotometry [111]. More complex techniques of measuring peroxidation include determining the loss of PUFA or production of secondary products measured with gas chromatography (GC), high performance liquid chromatography (HPLC), mass spectrometry, nuclear magnetic resonance spectroscopy (NMR), or a combination of these techniques [4, 10]. Much of the literature in this area has focused on the secondary products of peroxidation in *in vivo* samples, but *in vitro* studies also use more direct and continuous measures of peroxidation. In this thesis, peroxidation was measured using oxygen consumption and LOOH production.

Oxygen consumption was used as a measure of the reaction of conjugated diene radicals with oxygen to form a peroxyl radical. This reaction is an early and fundamental component of the peroxidation reaction. The consumption of oxygen during the formation of peroxyl radical can be measured using Clark-type oxygen electrodes [112]. Clark-type electrodes have been used in a large number of peroxidation studies including recent studies (e.g. [23, 30]). Modern Clark-type electrodes are extremely sensitive and can detect changes of as little as 500 nmol of oxygen per 0.5 seconds and produce results that are less variable than most measures of conjugated diene formation [111]. The continuous nature of oxygen consumption also has the added advantage of accurately measuring the different phases of peroxidation and measures within these phases.

The production of LOOH was also used to measure peroxidation in this thesis. This choice was based on LOOH being a primary product of peroxidation and a relatively stable molecule in comparison to other products of peroxidation [113]. LOOH production was measured using the ferrous oxidation-xylenol orange (FOX) method proposed by Wolff in 1994 [114] and recently updated to the FOX-2 assay by Bou *et al.* [113]. The FOX-2 method is considered an improvement on the earlier iodometric assay in terms of both sensitivity and reproducibility [111]. The FOX-2 method is based on

the oxidation of Fe^{2+} to Fe^{3+} by reaction with hydroperoxides formed during peroxidation under acid conditions (Equations 12–13). The subsequent reaction of Fe^{3+} with xylenol orange dye forms a chromophore complex that absorbs in the 540–600 nm range.

$$ROOH + Fe^{2+} \rightarrow Fe^{3+} + LO^{\bullet} + OH^{-} \qquad \text{Equation 12}$$

$$Fe^{3+} + XO \rightarrow Fe\text{–}XO \qquad \text{Equation 13}$$

The FOX-2 assay measures LOOH regardless of molecule. This is an advantage over the commonly used thiobarbituric acid reactive substances (TBARS) for MDA, as MDA does not form from the peroxidation of 18:2n-6. This limitation of the TBARS method is of particular relevance to the current thesis as 18:2n-6 was the primary PUFA examined in the peroxidation assays in this thesis.

2.4.6 Peroxidation in biological systems

The chemistry of peroxidation is well understood under controlled conditions, but conditions of *in vivo* peroxidation are far more complex and varied [11]. For example, although the initiation of peroxidation is theoretically determined by thermodynamic parameters, radicals must also be stable enough to reach a suitable target within the hydrophobic region of the cell membrane, a requirement that may rule out the most volatile or hydrophobic radicals [11]. Biological systems also have added complexity in terms of phospholipid compositions, pH conditions, concentrations of reactants, antioxidants, and the presence of poorly liganded iron present in haemoproteins such as complexes II, III and IV of the ETC as well as ferritin, the iron storage protein [105]. These conditions may even vary between organelles within a cell, with mitochondrial membranes particularly likely to undergo peroxidation as complexes I and III of the ETC are the main sources of cellular ROS production [14, 36]. To add to this complexity, organisms have evolved many strategies to use, repair or prevent peroxidation. Examples of this include the small number of products formed by enzymatic peroxidation involved in cell signalling activities including modulating inflammation and apoptosis [6-8], and the repair of peroxidation damage by turnover of 10% of membrane phospholipids over per hour [115]. Adaptations thought to prevent peroxidation include limiting the availability of iron and other catalysts within cells

[105], the formation of supercomplexes of potential enzymatic sources of ROS together in the ETC to limit electron leak, and production of antioxidants [11]. Substrate availability is also tightly controlled. For example, although the pO_2 of air is 21.1%, the pO_2 of human brain, liver and muscle is below 5% [116]. The percentage of PUFA in cell membranes can also be limited, with the membranes of longer-lived species generally having a lower degree of unsaturation than shorter-lived species [13]. Despite these adaptations, peroxidation is still considered a major cause of damage in cells and has been identified as a possible cause of aging.

2.5 The biological context of peroxidation: Focus on aging

2.5.1 Background

"The reasons for some animals being long-lived and others short-lived and, in a word, causes of the length and brevity of life call for investigation."

Aristotle, On Longevity and Shortness of Life, paragraph 1 [1].

How aging occurs – and whether aging can be prevented – has long been of interest to the scientific community and the general public alike. Due to its universal nature, aging was identified as an important aspect of biology by the very earliest scientists. The seminal mechanism of aging was proposed by Aristotle, who wrote that aging was caused by the damaging "fires of respiration" (and possibly also the soul). Aristotle based this theory on observations that larger mammals generally have longer lifespans than smaller mammals, but realised that this trend has many exceptions as "*a superior immunity from decay attaches neither to the largest animals (the horse has shorter life than man) nor to those that are small*" [1]. Modern science has a greater wealth of data to draw on than was available to Aristotle, and this data supports the early observations that lifespan shows an association with body mass, but with many exceptions including humans (Figure 2.10). More recent investigations of aging have replaced Aristotle's "fires of respiration" with multiple mechanistic and evolutionary theories of how aging progresses (reviewed in [117, 118]), yet the causes of aging still remain largely unresolved.

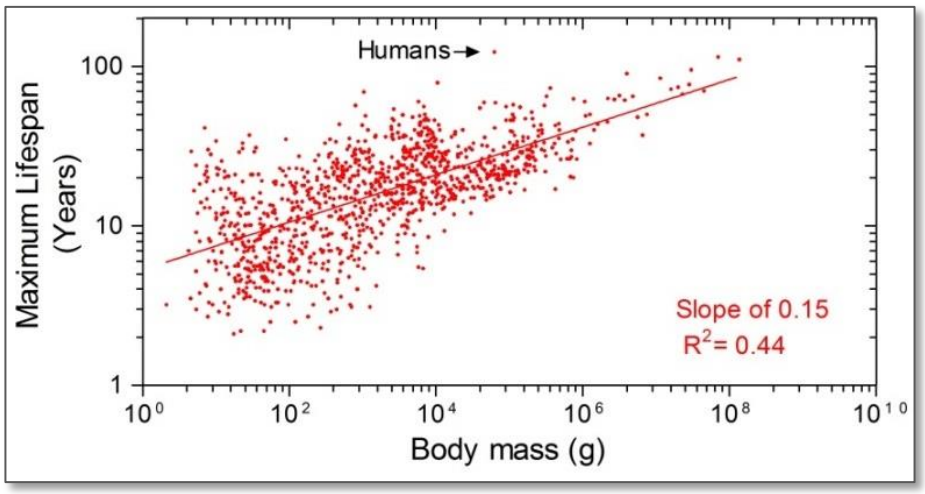

Figure 2.10. The association between mammalian longevity and body mass (*n*=1124). Data shown is from the AnAge Database.

Aging has been characterised as a universal and progressive accumulation of deleterious changes that lead to a loss of homeostasis and an increased likelihood of death [118-120]. One of the leading mechanistic theories of aging that fits this definition is the "Free Radical Theory of Aging", which was first proposed by Harman in 1956 [121]. This theory, which is also known as the "Oxidative Stress Theory of Aging", is based on findings that ROS produced during aerobic metabolism can be mutagenic and toxic. This theory has been updated in recent years by Harman [32, 33] and other researchers [9, 12, 14, 36, 122, 123], but is also debated by some scientists on the basis that oxidative damage is not always detrimental to lifespan and is only one possible form of damage to cells [124-126]. The arguments against the "Free Radical Theory" often based on comparisons of wild-type and mutant animals within a species [124-126] while much of the support for this theory has come from comparative animal studies that have examined aging-related properties across a range of mammals (reviewed in [12, 14, 127]). This approach, which was first used by Aristotle and his colleagues, is based on the key finding that longevity may differ more within a class than within a species. For example, the maximal lifespans (MLS) of mammals varies 100–fold between the short mammal lifespan of the shrew (*Myosorex varius*, MLS of 2.2) and the long life of the bowhead whale (*Balaena mysticetus*, estimated MLS of 211 years) (AnAge Database), but concerted efforts by the Methuselah Foundation (www.mfoundation.org) and other researchers have yet to extend the MLS of mice (*Mus musculus*) by more than 2–fold. Somewhat surprisingly, comparative studies of animal

sspecies have found only two cellular properties to be strongly related to aging: 1) the mitochondrial production of ROS and 2) the degree of membrane unsaturation [12, 14]. These two factors are related as ROS is both an initiator and product of the peroxidation of unsaturated fatty acids [5], supporting suggestions that the peroxidation of membrane phospholipids is a key component in the process of aging

2.5.2 The "Membrane Pacemaker Theory Of Aging."

In 1978, Gudbjarbason *et al.* [128] reported a positive correlation between the 22:6n-3 content of membranes and the heart rate of mammals ranging in size from mice to whales. This was one of the earliest findings linking membrane composition with body mass and metabolism, and suggested that membrane composition differs greatly between animals. Couture and Hulbert (1995) continued this work with a report that MUFA and PUFA contents of mammalian membranes vary with body mass [129]. The muscle, kidney, and liver of larger mammals were found to contain a higher percentage of 18:1n-9 and a lower percentage of 22:6n-3 than smaller animals. In contrast to these tissues, brain was found to contain high levels of 22:6n-3 in animals of all sizes which may reflect the importance is of 22:6n-3 to neural development and function [130].As larger mammals tend to live longer than shorter mammals, later work in this area found a similar relationship between membrane composition and longevity in liver mitochondria [12, 35, 131, 132]. Based on this work, it was proposed that the lower percent of PUFA and higher percent of MUFA seen in long-lived mammals than shorter-living mammals leads to a decrease in membrane susceptibility to peroxidation while maintaining membrane fluidity in what is termed a homeoviscous-longevity adaptation [35, 131]. The relationship between membrane composition and aging caused by lipid peroxidation was formalised by Hulbert as the "Membrane Pacemaker Theory Of Aging" in 2005 [13].

This theory includes the mechanistic aspects of the "Oxidative Stress Theory Of Aging" and is influenced by both the homeoviscous-longevity adaptation and the "Membrane Pacemaker Theory Of Metabolism" [12], and focusses on phospholipid composition and peroxidation as key determinants of oxidative damage. The key mechanistic aspects of this theory are based on the self-propagating nature of peroxidation (described in section 2.4.2), the damage caused by the highly volatile products of peroxidation (described in section 2.4.3) and the close relationship between

the membrane-bound enzymes that produce ROS and membrane phospholipids (described in sections 2.1 and 2.4.6). In order to establish a relationship between membrane composition and longevity, the "Membrane Pacemaker Theory Of Aging" and related work commonly distil the peroxidative capacity of complex membranes down to a simple theoretical measure of relative vulnerability to peroxidation.

2.5.3 Comparing membrane vulnerability to peroxidation

A number of ways to measure the relative susceptibility of membranes to peroxidation have been proposed by researchers in this area. The earliest measure used to calculate membrane susceptibility to peroxidation from membrane fatty acid composition was the number of unsaturated fatty acids (UFA) per 100 fatty acids. This calculation considers all MUFA and PUFA as UFA regardless of the number of double bonds present, despite MUFA being far less likely to undergo peroxidation than PUFA. As a result, the UFA is not a useful measure of membrane susceptibility to peroxidation. An alternative measure is the double bond index (DBI), also termed the unsaturation index (UI), which is calculated as the number of double bonds per 100 fatty acids [35]. Although popular with many researchers in this area, the DBI treats all double bonds as equally likely to undergo peroxidation regardless of whether they result in allylic or bis-allylic methylene groups [14, 45]. As a result, a membrane containing 100% 18:1n-9 with no bis-allylic methylene groups would be given the same DBI value as a membrane containing 50% 18:2n-6 with many bis-allylic methylene groups and therefore a higher susceptibility to peroxidation.

The "Membrane Pacemaker Theory Of Aging" combines membrane composition with R_{Max} values for fatty acids reported from experiments in chlorobenzene solution by Holman (1954) [21] to give a peroxidation index per 100 fatty acids. These rates of peroxidation increase exponentially with the number of bis-allylic methylene groups so that 18:2n-6 has a normalised value of 1, 18:3n-3 has a value of 2, 20:4n-6 has a value of 5 and 22:6n-3 has a value of 8. The peroxidation index of membranes is therefore calculated as: (% MUFA x 0.025) + (% PUFA with 1 bis-allylic methylene group x 1) + (% PUFA with 2 bis-allylic methylene groups x 2) + (% PUFA with 3 bis-allylic methylene groups x 4) + (% PUFA with 4 bis-allylic methylene groups x 6) + (% PUFA with 5 bis-allylic methylene groups x 8).

The peroxidation index is widely used in comparative physiology, but the exponential relationship between R_{Max} and bis-allylic methylene groups reported by Holman have been contested. Examinations of the peroxidation of fatty acids in chlorobenzene solution and liposomes have found a linear relationship between bis-allylic groups and R_{Max} [5, 22, 110], although there is support for an exponential rate from experiments in *in vitro* cell cultures [109]. Overall, the relationship between R_{Max} is widely considered to be linear in relation to bis-allylic methylene groups in contemporary chemistry. In order to maintain continuity with published physiology literature, however, the peroxidation index values determined by Holman (1954) [21] are used in this thesis.

2.5.4 Recent evidence for the "Membrane Pacemaker Theory of Aging"

A strong inverse relationship has been found between peroxidation index and longevity of mammals and other classes of animals [29, 127]. This relationship is best characterised for mammals, but relationships have also been found for birds [12], molluscs [37], bees [38], and strains of the nematode *Caenorhabditis elegans* [39]. The relationship between the peroxidation index of mammalian muscle and the maximal lifespan (MLS) of mammal species is shown in Figure 2.11 using data published in [29]. Although this is the most extensive dataset available, similar trends have been found for mammalian liver mitochondria [12]. Recent work in this area generally supports this relationship, with the membranes of skeletal muscle and liver mitochondria from naked mole rats (MLS of 28 years) found to have a lower peroxidation index than those from mice (MLS of 4 years) despite both animals having a similar body mass [133]. This difference was primarily due to phospholipids containing 22:6n-3 being less common in naked mole rat tissues than mouse tissues [40, 133]. A lower peroxidation index than predicted by body mass has also been reported for the echidna (*Tachyglossus aculeatu*), which lives 3–4 times longer than predicted for its mass, and the long-lived Ames mutant strain of mice (*Mus musculus* homozygosis for Prop1 phenotype) [44, 134, 135]. However, not all recent data support the "Membrane Pacemaker Theory Of Aging". The hoary bat (*Lasiurus cinereus*) has a predicted MLS of 4.5 years based on body mass and a reported MLS of 14 years, but contains a high percentage of 22:6n-3 in its muscle membranes and has a higher peroxidation index than predicted for a mammal of its lifespan [136]. Order Chiroptera

(bats) generally live longer than predicted for their body mass, but there is insufficient evidence available to determine if the entire order is an exception to the "Membrane Pacemaker" theory, or if this exception is limited to the hoary bat alone [29].

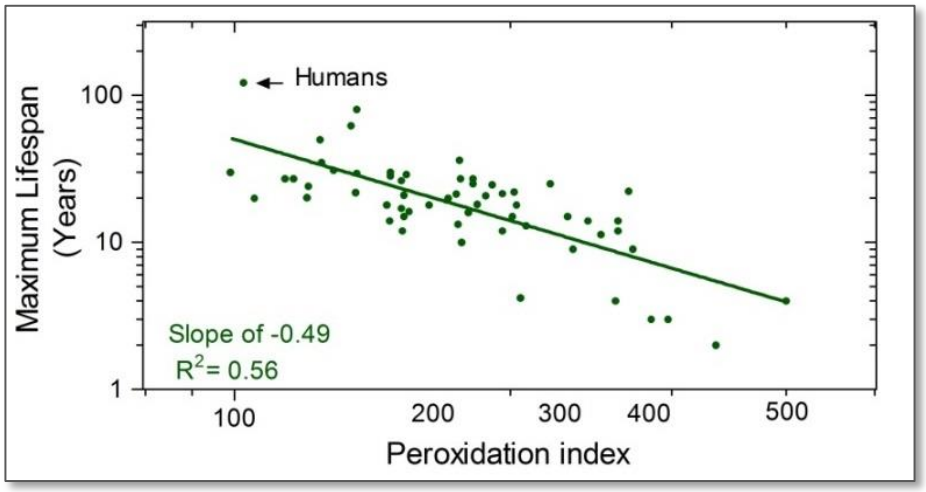

Figure 2.11. Correlation of the peroxidation index of mammal muscle and longevity (*n*=60). Data is from [29].

Relatively little is known regarding the peroxidation index of membrane phospholipids in humans (*Homo sapiens*). Humans have a predicted MLS of ~25 years based on their body mass (AnAge Database) but an actual MLS of 122 years [137]. This MLS is extremely long for a mammal, and is only exceeded by estimates for the bowhead whales. Although data on human membrane composition and peroxidation index is rare, there is some evidence to suggest human liver membranes contain a low percentage of PUFA [138, 139], and a recent comparison of mammalian plasma found humans had a lower DBI, a lower peroxidation index, and a lower level of peroxidation products than shorter lived mammals [42].

The link between membrane composition and peroxidation with longevity is best described in mammals, but similar relationships have also been found in other classes of animals. For birds, the membranes of long-lived petrels (order Procellariiformes) were less susceptible to peroxidation than those of the of short-lived fowl (order Galliformes) with comparable body masses [140]. Differences were not seen, however, in a comparison of long-lived parrots (order Psittaciformes) to fowl with similar body masses [141]. In invertebrates, membrane susceptibility to peroxidation was found to be lower in queen bees (MLS of 8 years) than worker bees from the same hive (MLS of 7

weeks) [38], lower in the Age–1 mutant strain of *C. elegans,* and lower in long-living molluscs than shorter-living molluscs with similar body masses and environments [37].

The majority of evidence for the "Membrane Pacemaker Theory Of Aging" comes from comparative animal studies, but some experimental interventions have been found to decrease the degree of membrane polyunsaturation and extend lifespan. Calorie restriction, which has been found to elongate lifespans in mice, rats and primates, has also been shown to decrease the peroxidation index of rats by increasing membrane MUFA content and decreasing membrane PUFA, particularly 22:6n-3 [29, 142-144]. In a recent study by Gómez *et al.* (2014) [145], the lifelong treatment of mice with atenolol, a β–blocker, was found to decrease the degree of polyunsaturation in cardiac and skeletal muscle primarily by decreasing 22:6n-3 and increasing 18:1n-9. This study, which was the first to examine the link between the peroxidation index and longevity experimentally in mammals, found that atenolol prevented oxidative modifications to proteins and decreased the loss of immune and behavioural functions related to aging, but did not increase lifespan. The authors of the study attributed the lack of an increase in lifespan to adverse side effects of atenolol itself that have been found to increase the likelihood of cardiovascular failure in humans. Despite not increasing of animal lifespan, this finding does provide support for the theory that a decrease in peroxidation slows the aging process.

2.5.5 Measuring and comparing membrane phospholipid composition

The majority of past investigations into membrane composition and longevity have used GC to compare relative percentages of fatty acids and peroxidation indexes between membranes (e.g. [80, 90, 129, 134]). Some studies have used additional purification steps with thin-layer chromatography (TLC) coupled with GC (e.g. [146-148]) or HPLC/GC (e.g. [80, 149, 150]) to better understand how fatty acids are distributed across classes, but these methods are relatively slow and labour intensive. More recent work has used mass spectrometry to examine the percentage of phospholipid class and molecular structure in membranes [18, 19, 40-42, 151], with some of the most detailed membrane studies using shotgun lipidomics techniques [18, 19, 151].

Shotgun lipidomics was first proposed by Han and Gross (1994) [152] as a method of direct infusion that *"exploits the unique chemical and physical properties of each lipid class to facilitate the high-throughput analysis of a cellular lipidome on a*

large scale directly from organic extracts of biological samples" [50]. This method uses electrospray ionization and other "soft" ionization methods in which each lipid gives rise to a single anion or cation depending on its molecular structure [153]. Using a triple quadrupole mass spectrometer, lipids may then be subjected to collision-induced dissociation to produce diagnostic fragmentation products, including for head group class and acyl chain content from which the molecular structure of the lipid can be deduced [50, 154]. Scan types used in such an analysis include product ion scans, precursor ion scans, and neutral loss ion scans [153]. Shotgun lipidomics has the advantage of requiring minimal sample preparation while having high sensitivity and high throughput in comparison to TLC/GC or HPLC/GC methods [50, 153, 154]. In addition, the data produced from shotgun lipidomics can be used to calculate total membrane fatty acid composition to give comparable results as those for GC [18]. The limitations of shotgun lipidomics include the potential for ion suppression and current lack of precursor scans to differentiate plasmalogens from alkenyl ethers. Despite these limitations, shotgun lipidomics is, an improvement on the use of GC and has the potential to give new insights into the association between membrane composition, peroxidation, and longevity.

2.6 Conclusion

The peroxidation of membrane phospholipids produces products that may cause cellular damage and aging. The susceptibility of membrane phospholipids to peroxidation is primarily determined by PUFA content, but other elements of phospholipid composition may also influence peroxidation. Many questions remain unanswered regarding the importance of phospholipid composition to peroxidation and aging. This dissertation addresses some of these questions through the use of three different methodologies. In Chapter Three, data is analysed to determine how dietary fatty acids influence the composition of mitochondrial CL, PC and PE, and the outcomes of this incorporation in terms of mitochondrial function and susceptibility to peroxidation. Chapter Four presents an *in vitro* examination of whether phospholipid class influences peroxidation, and Chapter Five investigates whether peroxidation is influenced by phospholipids that do not contain PUFA. This is followed by an examination of mice, pigs and human membranes using shotgun lipidomics that compares the mitochondrial membrane between species in Chapter Six, and intra-species differences in mitochondrial and

microsomal membranes from these animals in Chapter Seven. Chapter Eight provides an overview of the results of these studies.

Chapter Three

Dietary docosahexaenoic acid incorporates into cardiolipin at the expense of linoleic acid: An analysis of published datasets

3.1 Overview

Cardiolipin (CL), the signature phospholipid of mitochondria, is commonly viewed as having a highly regulated fatty acid content due to its high levels of linoleic acid (18:2n-6). In contrast to this view, numerous published studies have shown that dietary docosahexaenoic acid (22:6n-3) increases the percentage of 22:6n-3 present in CL suggesting that the fatty acid content of CL conforms to diet. This chapter reviews all previously published articles that examined the influence of dietary fatty acids on the fatty acid composition of CL, and examines the possible outcomes of changes in the fatty acid composition of CL in terms of mitochondrial function and susceptibility to peroxidation. The incorporation of 22:6n-3 into two other mitochondrial phospholipid classes, phosphatidylcholine and phosphatidylethanolamine, is also reviewed. This chapter is closely based on the published work 'Dietary docosahexaenoic acid (22:6) incorporates into cardiolipin at the expense of linoleic acid (18:2): Analysis and potential implications' by Cortie CH and Else PL, *International Journal of Molecular Sciences*, (2012). **13**(11): p. 15447–15463. The structure of the present chapter differs slightly from the published work to avoid repetition of material presented in Chapter Two. CHC proposed the study, collected and analysed the data, and produced the primary draft of the paper. PLE supervised all aspects of the work.

3.2 Introduction

Cardiolipin (CL) is the only prevalent di-phospholipid present in mammalian membranes [155-158] and is found almost exclusively in the inner leaflet of the inner mitochondrial membrane where it is synthesised [155]. CL has been called the signature phospholipid of mitochondria [156] as it has important roles in binding the mitochondrial proteins required for oxidative phosphorylation [25, 158] and initiating mitochondrial-induced apoptosis by reacting with the haem present in cytochrome *c* [156, 157, 159, 160]. These roles are related to CL's unusual di-phospholipid structure and fatty acid composition. CL possesses four fatty acids rather than the two fatty acids

present in other phospholipid classes, and while the combination of two PUFAs on the same phospholipid molecule is rare in other mammalian phospholipid classes [18, 161], CL containing four linoleic acid (18:2n-6) is highly common in mammalian muscle and other tissues [26]. Schlame *et al*. [26] reported that CL 18:2/18:2/18:2/18:2 comprises 80% of all CL in human heart, 70–77% in dog heart, 41–77% in rat heart and 50% in bovine heart, while Han *et al*. [162] reported that this same species of CL comprises 42% of CL in rat heart and 52% in rat liver. Other groups have reported the 18:2n-6 content of CL in liver and brain as lower than 60%, meaning that this form of CL may not be as common in some of these tissues [146, 147, 151]. CL 18:2/18:2/18:2/18:2 has been found to have a closer association with the mitochondrial ADP/ATP carrier than CL 16:0/16:0/16:0/16:0 [163], and to have a higher binding affinity for cytochrome *c* in comparison to CL molecules with high levels of oleic (18:1n-9), stearic (18:0) and palmitic acid (16:0) respectively [164].

Although a high 18:2n-6 content is potentially important in CL-protein interactions, the exposure of CL to free reactive oxygen species (ROS) produced in the mitochondria put the PUFA present in CL at high risk of oxidative damage [156]. The peroxidation of CL may decrease its affinity for protein binding and disrupt the structure of the inner mitochondrial membrane, and has been suggested to be an early event in the apoptotic cascade [164-168] or even a trigger of apoptosis [159, 169]. Changes in the degree of unsaturation in CL's PUFA content could therefore be expected to affect both its interaction with proteins and susceptibility to peroxidation, modifying its function in mitochondria. Such changes are thought to be unusual, as one of the widely held beliefs about mammalian CL fatty acid composition is that it is highly regulated, primarily due to its high levels of 18:2n-6 and the prevalence of a CL 18:2/18:2/18:2/18:2 in cardiac mitochondria, but also due to an apparent resistance to dietary manipulation [26]. This review examines the evidence that, contrary to popular belief, CL's PUFA composition does conform to dietary fatty acid intake in some circumstances, particularly when diets are rich in 22:6n-3

3.3 Changes in the fatty acid composition of cardiolipin due to diet

3.3.1 Dietary trials investigating cardiolipin in animal models

It has been known for some time that dietary fats affect phospholipid composition [148], and CL seems to be no exception. The fatty acid composition of CL has been

reported in numerous studies examining the effects of various dietary fatty acids on the phospholipid composition of heart and liver mitochondria. Most (~75%) of these studies have used rats [56, 146-149, 170-182] or mice [150, 183-186], and these studies supply the raw data used in the present study. The primary focus of many of these studies has been on n-3 and n-6 PUFA that, unlike SFA and MUFA, mammals cannot form *de novo* from basic carbon sources [89]. The sources of dietary SFA, MUFA and PUFA used in these studies were generally from sources commonly eaten by humans including fish, linseed, corn, olive, rapeseed and soybean, although one study used crocodile oil [185] (Table 3.1). The majority of the studies examined predate the use of mass spectrometry, instead using combinations of thin layer chromatography (TLC) or high-performance liquid chromatography (HPLC) together with gas chromatography (GC). One limitation of these techniques (versus a lipidomics approach) is that they cannot readily determine the abundance of specific phospholipid molecular species within each phospholipid class. However, these studies did provide the total fatty acid composition of the CL present in membranes,

The incorporation of dietary fatty acids was found to be a selective process in these studies, and so this analysis did not include all fatty acids. Although some researchers reported SFA in very low levels in CL, presumably due to the presence of immature CL (discussed in section 3.4), SFA content was not examined in this review as not all work reported SFAs shorter than 18:0. Linolenic acid (18:3n-3) was also not examined as, although it is consumed at moderate levels in the diet [187], little if any appears in most phospholipids [18] including CL [26], presumably due to its conversion through to the longer chained omega-3 fats. Arachidonic acid (20:4n-6) was not included in the present analysis due to limited data in this area but has been reported in CL at levels ranging from 0–12% [148, 170, 184]. As eicosapentaenoic acid (EPA, 20:5n-3) can be converted to 22:6n-3, diets containing greater than 10% EPA or diets in which 20:5n-3 levels exceeded those of 22:6n-3 were excluded to prevent confounding effects. This review therefore focussed on the influence of dietary 18:1n-9, 18:2n-6 and 22:6n-3 on CL composition.

Table 3.1 Details of species, sources of fat, measurement method used, and feeding period of studies examined.

Reference	Source of fat	Method	Dietary fat (w/w)	Feeding period (weeks)
Rats				
Yamaoka *et al*., 1988 [147]	Corn oil, sardine oil	TLC, GC	20%	4
McGee *et al*., 1996 [146]	Not stated	TLC, GC	20%	4
Charnock *et al*., 1986 [148]	Sunflower oil, tuna, vegetable oil	TLC, GC	4% or 16%	60
Astorg *et al*., 1991 [149]	Sunflower seed oil, linseed oil	HPLC/GC	10%	20
Ikeda *et al*., 1996 [170]	Safflower oil, perilla oil, palm oil, methyl 20:4	TLC/GC	10%	3
Innis and Clandinin, 1981 [171]	Soya-bean, rapeseed	TLC, GC	20%	5
Jahouvey *et al*., 1990 [172]	Palm oil, olive oil, sunflower oil, linseed oil, menhaden oil	HPLC, GC	15%	4
Kramer, 1980 [173]	Corn oil, zephyr oil	TLC, GC	20%	16
Novak *et al*., 2006 [174]	Standard chow diet	TLC, GC	3.5%	9
Power *et al*., 1994 [175]	Coconut oil, olive oil, safflower oil, menhaden oil	TLC, GC	2% or 20%	10
Taniguchi *et al*., 1993 [177]	20:5 and 22:6 methylesters	TLC, GC	15%	2
Yamaoka *et al*., 1990 [178]	Corn oil, sardine oil	TLC, HPLC, GC	20%	2
Hoy and Holmer, 1990 [179]	Marine oil, olive oil, sunflower seed oil	TLC, GC	20%	10
Charnock *et al*., 1984 [180]	Sunflower seed oil, sheep kidney fat	TLC, GC	4%, 12%	20
Charnock *et al*., 1991 [181]	Sunflower, fish oil	TLC, GC	16%	44
Robblee and Clandinin, 1984 [182]	Beef tallow, soybean oil	TLC, GC	7–23%	2
Lee *et al*., 2006 [188]	Soybean oil and meal, fish meal, alfalfa meal, corn meal	TLC, GC	4%	16
Mice				
Croset and Kinsella, 1989 [183]	Crocodile oil, soybean oil	TLC, GC	7%	13
Berger and German, 1990 [184]	Coconut oil, safflower oil, flaxseed oil	HPLC, MS	10%	17
Watkins *et al*., 2001 [185]	Crocodile oil, soybean oil	TLC, GC	7%	13
Hussein *et al*., 2009 [150]	Coconut oil, safflower oil, flaxseed oil	HPLC, MS	10%	17

TLC: thin layer chromatography; GC: gas chromatography; HPLC: high performance liquid chromatography; MS: mass spectrometry.

A small number of exclusion criteria were applied for data selection. As 18:2n-6 is the most readily consumed PUFA in the human diet (~42% of PUFA consumed [187]) and an essential fatty acid in mammals, the analysis excluded any diets containing less than 2% of 18:2n-6 as a proportion of total dietary fatty acid. Based on the work of Owen *et al*. [189], two weeks of dietary intervention is the minimum period required in order to allow adequate time for phospholipid remodelling to occur, so any dietary interventions shorter than two weeks were excluded. Where different feeding periods were used in the same study, the period closest to four months was chosen as it represented an average common feeding period across studies. In the entire analysis, the only study excluded as a gross outlier was that of Berger *et al.* from 1992 [186]. This study reported that the percentage of 22:6n-3 in mouse heart CL was increased from 12 to 48% of total fatty acids after 18 days on a diet containing 22:6n-3 at 11% of total fatty acids [186]. This percentage was approximately double that reported by any other study, and has not been replicated since its original publication.

3.3.2 Conformer-regulator model

The conformer-regulator model was used to examine the relationship between dietary fatty acid composition and phospholipid fatty acid composition. In this model, the percent of a fatty acid in the diet (e.g. 18:1n-9, 18:2n-6 or 22:6n-3) is plotted against its level or that of another fatty acid present in CL. A slope of 1 represents a perfect conformation between dietary fatty acid composition and phospholipid fatty acid composition, and a slope of zero suggests that the fatty acid composition of phospholipids is independent to that of diet and is therefore perfectly regulated [90]. Slopes not significantly different from zero indicate regulation, slopes significantly greater than zero but below 1 indicate weak conformity, and slopes of 1 or more represent very strong, active dietary conformity. Previous work using this model has shown that total membrane SFA, MUFA and PUFA content are likely to show a regulator pattern with diet, but n-3 and n-6 PUFA show varying levels of dietary conformity, particularly at low dietary levels where presumably their essential dietary nature is displayed [28, 190].

Line fit and slopes in all figures presented in this study were determined using segmental linear regression with an unconstrained line intercept available in GraphPad Prism 5.04 (GraphPad Software, CA, USA). All statistical analyses were performed

using the same software package and slope values are reported as slope ±95% confidence interval.

3.3.3 Modelling fatty acid composition of diet and cardiac cardiolipin

The three fatty acids most commonly reported in CL were 18:1n-9, 18:2n-6 and 22:6n-3 [146, 147, 149, 150, 170-174, 176, 177, 183-185, 188]. Figure 3.1 shows the percentage of 18:1n-9 and 18:2n-6 in CL against their percentage in the diet in heart and liver. No significant trend was apparent for 18:1n-9, with a slope of 0.036±0.094 (P= 0.886) across a wide dietary range (0–78%), indicating a regulated pattern with 18:1n-9 comprising around ~10% of cardiac CL fatty acid content. The incorporation of 18:2n-6 into cardiac CL showed a distinctive biphasic pattern with a rapid rise in CL 18:2n-6 levels from 2–20% of dietary fat indicating a conformer pattern as indicated by a slope of 1.17±1.10 (P= 0.038). At these lower levels of dietary 18:2n-6 intake, the incorporation of 18:2n-6 into CL appeared to be an active and selective process as would be predicted from the high 18:2n-6 content generally reported for CL. At dietary levels where 18:2n-6 was above 20% (range 21–68%) of dietary fat, the incorporation of this fatty acid was reduced to a regulated pattern with a slope of 0.036±0.524, levelling off at ~70% of CL fatty acid being 18:2n-6 (or ~65% when rat and mouse values are combined), supporting reports that 18:2n-6 levels in CL is largely a regulated phenomenon

Analysis of 22:6n-3 levels in heart CL showed that a small percentage (2–8%) of 22:6n-3 was present in CL even when 22:6n-3 was absent from the diet (Figure 3.2). This was presumably due to the elongation and desaturation of shorter chained PUFA such as 18:3n-3 to 22:6n-3 by the liver [191] and the subsequent incorporation of 22:6n-3 into heart CL. When 22:6n-3 was present in the diet at levels of up to 20% of total fatty acid, it was readily incorporated into CL in a strong active conformer pattern with a slope of 1.43±0.58 (P< 0.0001). Although 22:6n-3 was examined over a more extended range (0–37%) than 18:2n-6, dietary levels of 22:6n-3 above 20% of total dietary fatty acid indicated some degree of regulation with the plateauing of 22:6n-3 levels at ~20% of fatty acid CL content. Therefore 18:2n-6 and 22:6n-3 seemed to be readily incorporated into heart CL when their levels in the diet were up to 20% of total dietary fatty acid, indicating a strong active conformer pattern, but at higher dietary levels a regulated pattern was suggested.

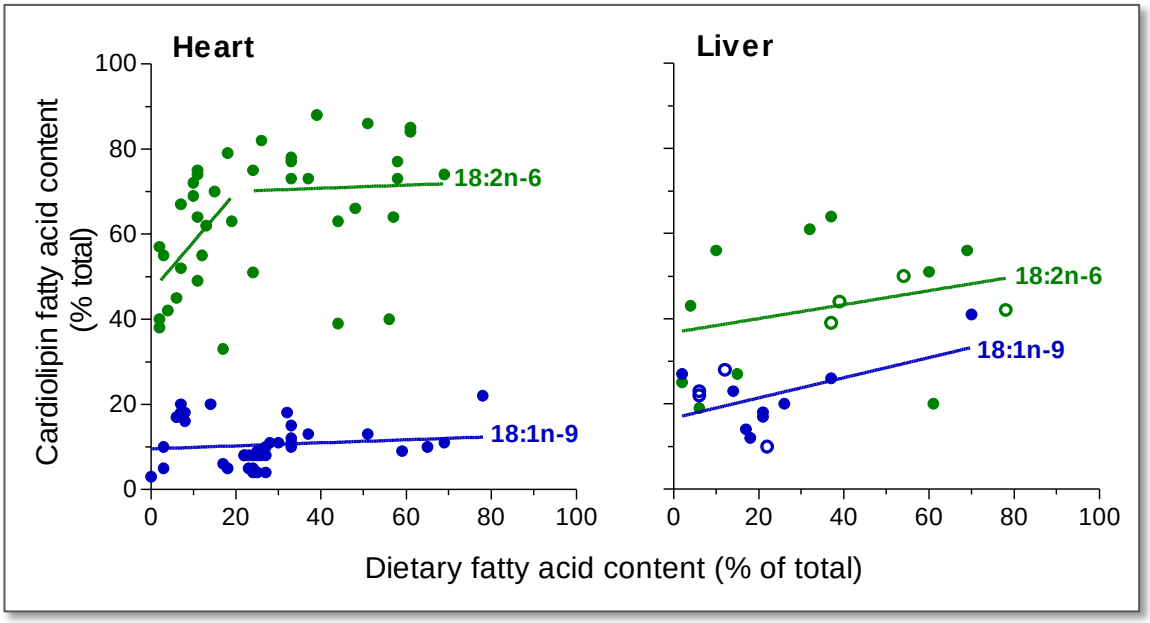

Figure 3.1. The relative content of 18:1n-9 and 18:2n-6 as percentages of the fatty acid content of cardiolipin and total dietary fat. Values for heart are for rat (●) and those for liver are for rat and mice (○). Data for heart is from [146-149, 170-174, 177-182, 188] and liver data is from [147, 149, 175, 177, 179, 184, 185].

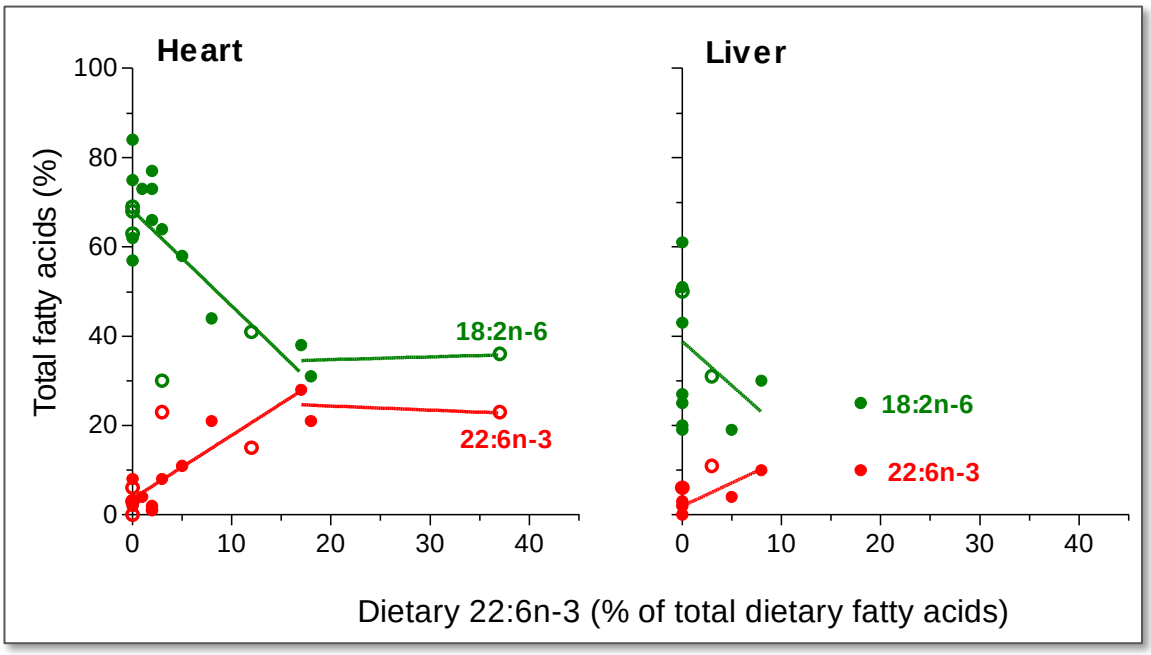

Figure 3.2. The relationship between the percentage of 18:2n-6 and 22:6n-3 in heart and liver cardiolipin and the percentage of 22:6n-3 present in dietary fat. Data is from studies using both rats (●) and mice (○) and included the following experimental groups: control diet [180], sunflower oil and tuna oil diets [148], diet 1 and 4 [183], 4 month time point [188], high 18:3n-3 diet, long chain n-3 diet [150], control, high 22:6 [177], soy oil and crocodile oil [185], sardine oil, corn oil [147], 18:2 and long chain n-3 [172].

3.3.4 Modelling fatty acid composition of diet and liver cardiolipin

Fewer studies have examined CL acyl remodelling in liver compared to heart, but what was available [149, 175, 177, 184, 185] is presented in Figure 3.1 for 18:1n-9 and 18:2n-6, and in Figure 3.2 for 22:6n-3. Both figures use data for mice and rats. In general, the percentage of 18:1n-9 was higher (at ~20%) in liver than in heart and the percentages of 18:2n-6 and 22:6n-3 were lower. Presumably 18:1n-9 was acting as an alternative for some of the 18:2n-6 and 22:6n-3 fatty acids in liver, with some studies reporting 18:1n-9 levels of 25% or higher in liver CL [175, 179, 184]. The incorporation of 18:1n-9 into liver CL was regulated at a low level with a very mild and non-significant accrual (slope of 0.237±0.266; P= 0.076) throughout the dietary range examined (2–70%). Similarly, 18:2n-6 showed little indication of any strong trend to incorporate into liver CL at either low or high dietary 18:2n-6 intakes (Figure 3.1) with a slope of 0.164±0.352; (P= 0.33) across a broad dietary range, again indicating that the incorporation of 18:2n-6 into CL is highly regulated. Although there was little data available for the influence of dietary 22:6n-3 on the composition of liver CL (Figure 3.2), what was available suggested a conformer pattern when 22:6n-3 levels were below 10% of total dietary fatty acid with a slope of 1.04±0.85 (P= 0.02).

3.3.5 The effect of dietary 22:6n-3 on the incorporation of 18:2n-6 into heart and liver cardiolipin

All studies that incorporated both 22:6n-3 and 18:2n-6 into the same diet and measured the fatty acid composition of CL were analysed (Figure 3.2) for heart [147, 148, 150, 172, 177, 180, 181, 183, 185, 188] and liver [147, 175, 177, 185, 186]. Owing to the more limited number of studies available that included both 22:6n-3 and 18:2n-6 in the same diet, the analysis includes data for both rat and mouse tissues. No statistical relationship was found between dietary levels of 22:6n-3 and 18:2n-6. For example, when 22:6n-3 was at its highest level in the diet (37%) the level of 18:2n-6 was also one of the highest of any of the diets (56%) indicating that changes in 18:2n-6 levels in heart CL were not simply due to dietary 22:6n-3 displacing dietary 18:2n-6 [183]. Figure 3.2 clearly shows the deposing influence of 22:6n-3 on 18:2n-6 levels in cardiac CL at 22:6n-3 levels of up to 20% of total dietary fatty acid. At dietary levels where 22:6n-3 was less than 20% of total fatty acids, 22:6n-3 was incorporated into cardiac CL (slope of 1.43±0.58; P= 0.0001) while percentages of 18:2n-6 in CL decreased (slope of

–2.14±1.24; *P*= 0.002). At percentages of dietary 22:6n-3 above 20% of fatty acids, the percentage of 18:2n-6 appeared to plateau at just under 40% of total fatty acids in heart CL which was below its "normal level" of ~70% (Figure 3.1), whereas 22:6n-3 plateaued at just above ~20% of total fatty acid making up most of the difference (Figure 3.2).

Liver CL also appeared to accrue 22:6n-3 at the expense of 18:2n-6 (slope of 1.041±0.846; *P*= 0.022) although less data was available for liver than heart (Figure 3.2). A study that examined the effects of very high levels of dietary 22:6n-3 on CL composition reported liver CL did not incorporate 22:6n-3 even when dietary 22:6n-3 exceeded 10% of total fatty acid in the diet [177]. At this point 18:2n-6 plateaued at slightly over ~20% of CL fatty acid content, down from the normal ~30–60% with 22:6n-3 at ~13% of CL fatty acid making up a large proportion of the difference. Therefore CL in heart and liver maintained 18:2n-6 levels at ~70% and 50% respectively, and in the case of heart readily accrued 18:2n-6 from the diet when this fatty acid is at lower levels (<20%). However, when 22:6n-3 was available in the diet it was incorporated into CL at the expense of 18:2n-6 until 22:6-3 made up ~20% of CL fatty acid content in heart and ~13% in liver, after which both 18:2-6 and 22:6n-3 levels seemed to be constant and therefore regulated.

3.3.6 Comparison of 22:6n-3 incorporation into other mitochondrial phospholipids

Although functionally important, CL is not the major phospholipid class in mitochondria, comprising 5–20% of mitochondrial phospholipids [17, 51]. The two major mammalian mitochondrial phospholipids classes are phosphatidylcholine (PC) and phosphatidylethanolamine (PE), which make up ~45% and ~35% of total mitochondrial phospholipid respectively [192]. Some of the studies examined here [28, 56, 164, 167, 168, 170, 172, 173, 175, 176, 178-185] included data for PC and PE in addition to CL, and a brief analysis of 18:1n-9, 18:2n-6 and 22:6n-3 incorporation into PC and PE of heart and liver mitochondria was conducted (Figure 3.3). There were no significant trends in the incorporation of 18:1n-9 and 18:2n-6 into PC and 18:1n-9 into PE of cardiac mitochondria across a broad range of dietary supplementation (2–78% of total fatty acids), indicating a regulated pattern for these fatty acids in heart. In comparison, the incorporation of 18:2n-6 into heart PE showed a weak but significant conformer trend with increasing levels across the same dietary range with a slope of

0.056±0.051 (*P*= 0.034). The incorporation of 22:6n-3 in both PC and PE in heart mitochondria displayed a strong conformer trend with slopes of 0.93±0.45 (*P*= 0.0003) for PC and 0.89±0.58 (*P*= 0.005) for PE when 22:6n-3 composed up to 38% of dietary fatty acids. In liver mitochondria the incorporation of 18:1n-9, 18:2n-6 into PC and 18:1n-9 into PE also showed no significant trends in across a broad dietary range. Liver mitochondrial PE showed a weak conformer uptake for 18:2n-6 (slope 0.045±0.038; *P*= 0.024) but both liver PC and PE showed a strong dietary conformer pattern for 22:6n-3 uptake with slopes of 0.95±0.32 (*P*< 0.0001) for PC and 1.33±0.79 (*P*= 0.0025) for PE across a dietary range of 22:6n-3 of up to 20%. Unlike in CL, the incorporation of 22:6n-3 into PC and PE was not found to be at the expense of 18:2n-6.

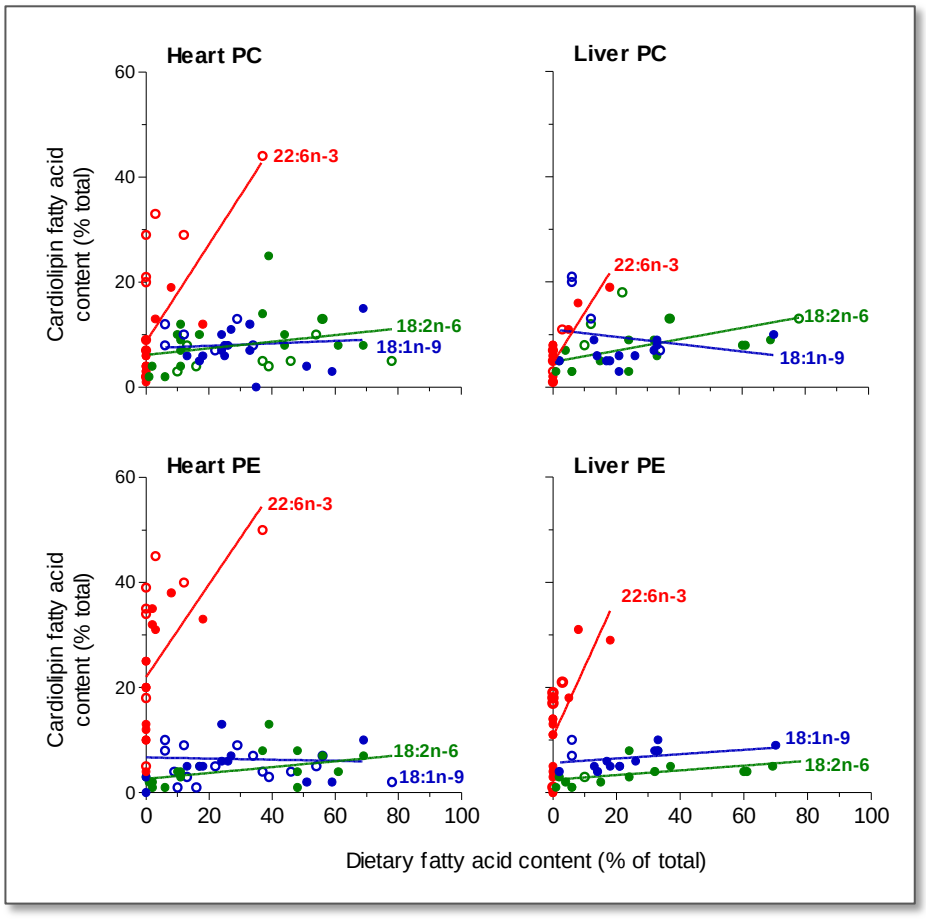

Figure 3.3. Levels of 18:1n-9, 18:2n-6 and 22:6n-3 as a percentage of total fatty acids in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) against total dietary fatty acids. Each figure includes both rat (●) and mouse (○) data. Data used for heart PC was from [147-150, 171, 172, 174, 177, 178, 180-185], for heart PE was from [56, 149, 183, 184], for liver PC and PE were from [147, 149, 170, 175, 177, 184, 185]. Data excluded from the analysis included diets that contained higher 20:5n-3 than 22:6n-3.

3.4 Cardiolipin remodelling

This analysis used a comprehensive compilation of studies to show that dietary 18:2n-6 is selectively incorporated into heart and liver CL, but that the incorporation of dietary 22:6n-3 has an even greater selectivity. The analysis indicated a strong conformer pattern when dietary levels of 18:2n-6 were between 2–20% of total dietary fatty acid, with higher dietary availability of 18:2n-6 showing regulated levels. In liver, no trend was evident for 18:2n-6 incorporation into CL across a broad range of dietary 18:2n-6 intakes, indicating a regulated level of 18:2n-6 into CL of liver mitochondria. When 22:6n-3 and 18:2n-6 were present in the same diet and 22:6n-3 was between 0–20% of total dietary fatty acids, CL preferentially incorporated 22:6n-3 into its structure at the expense of 18:2n-6. This preference for the incorporation of 22:6n-3 over 18:2n-6 seems to be present in both heart and liver. The replacement of 18:2n-6 by 22:6n-3 has previously been noted in phospholipids of heart. For example, in a dose-response study of dietary 22:6n-3 (0–32%) examining myocardial phospholipids, Owen *et al*. [189] found that 22:6n-3 replaced 18:2n-6, and to a lesser extent 18:1n-9. Other studies comparing diets low and high in 22:6n-3 have noted similar trends in cardiac phospholipids [183, 185] and CL specifically [147, 149, 178, 185]. The present analysis found that 22:6n-3 will replace 18:2n-6 in CL even when dietary levels of 18:2n-6 are high, but this replacement will occur at levels of dietary 22:6n-3 of up to 20% in heart and ~10% in liver.

The selectivity of 22:6n-3 over 18:2n-6 in CL and the preferential incorporation of these two fatty acids over others is likely due to the complex nature of CL synthesis and remodelling. CL is synthesised from the condensation of a PG molecule with cytidine diphosphate diacylglycerol resulting in what is commonly referred to as immature CL with a mixed acyl content that is then remodelled to produce mature CL with high 18:2n-6 content [156]. The selected incorporation of fatty acids during remodelling was recently investigated by Kiebish *et al.* [193], who reported that only 1–5% of the fatty acids incorporated into CL during the remodelling process come from PC and PE, with this process having a low acyl selectivity. The majority of fatty acids are drawn from the pool of acyl-CoA fatty acids and this incorporation is highly selective [193]. Interestingly, heart acyltransferases were reported to have 1.3 times the specificity for 22:6n-3 in comparison to 18:2n-6, which in turn had specificity many times higher than that of any other fatty acid. In contrast, liver acyltransferases had a far higher specificity

for 18:2n-6 over 22:6n-3 or any other fatty acid [193]. The specificities of these acyltransferases and tissue differences are likely to explain the underlying mechanism by which 22:6n-3 replaces 18:2n-6 in heart CL but does not explain the response in liver.

3.5 Consequences of the incorporation of 22:6n-3 into cardiolipin

The accrual of 22:6n-3 into phospholipids in a conformer fashion probably reflects the essential dietary nature of omega-3 fats generally and the readiness of tissues to use preformed fatty acid if available. Based on the roles CL performs in mitochondria, the functional consequences of accruing 22:6n-3 into the structure of CL are likely to be significant. The incorporation of 22:6n-3 in CL might increase the fluidity and permeability of inner mitochondrial membranes and may adversely affect CL's interactions with proteins [163]. These changes would be expected to decrease the efficiency of oxidative phosphorylation, and the three dietary studies in the analysis that investigated the effect of 22:6n-3 incorporation into CL on mitochondrial oxidative phosphorylation found no change [183] or a decrease in activity [147, 182]. More recent work in this area has shown a similar trend of dietary 22:6n-3 either decreasing or not affecting mitochondrial respiration [194, 195].

As 18:2n-6 contains only one bis-allylic methylene group but 22:6n-3 contains five, the incorporation of 22:6n-3 over 18:2n-6 into CL, PC and PE will increase membrane susceptibility to peroxidation, which is an important mechanism in cellular health and disease [3-5]. Furthermore, the products formed during peroxidation of n-3 PUFA are proposed to be more reactive than those produced from n-6 PUFA [196]. CL has been shown to be more susceptible to peroxidation than other phospholipid classes both *in vitro* [30] and *in vivo* [166] due to its high level of 18:2n-6, proximity to ROS production, and close association with the haem-containing cytochrome *c* [25, 197, 198]. An increase in CL's susceptibility to peroxidation due to the incorporation of 22:6n-3 may also increase cell sensitivity to apoptosis as the peroxidation of CL is thought to be an early event in mitochondrial-induced apoptosis related to cytochrome *c* release and disruption of the inner and outer mitochondrial membranes [164-166, 199]. Overall, the replacement of 18:2n-6 with 22:6n-3 has the potential to adversely affect cell function, but the finding that CL selectively accrues 22:6n-3 in place of 18:2-6

suggests that the incorporation of 22:6n-3 into CL is not a disadvantageous under normal dietary circumstances.

3.5.1 Limitations

This chapter shows both the limitations and importance of analysing data from published works. The incorporation of 22:6n-3 into the CL of mammalian heart and liver is likely to increase the peroxidation index of these tissues, but the exact changes in the peroxidation index could not be determined from the available data. In addition, physiological changes due to increased 22:6n-3 in CL composition were not examined, and so it could not be determined if the incorporation of 22:6n-3 into the CL of healthy organisms has an overall positive or negative influence on longevity and health. The advantage of the present study, however, is that it combines similar findings from multiple sources to provide the best evidence available that the fatty acid composition of CL can be influenced by diet, particularly dietary 22:6n-3.

3.6 Conclusions

The high level of 18:2n-6 reported in CL has led to the view that the fatty acid composition of CL is regulated interdependently of diet [26]. This analysis has, however, shown that the 18:2n-6 and 22:6n-3 content of CL is influenced by the percentage of these fatty acids in the diet. The accrual of 18:2n-6 into cardiac CL increased when 18:2n-6 was present at 2–20% of dietary fatty acids, and was regulated at ~70% of CL when 18:2n-6 was present at higher percentages in the diet. In comparison, liver showed no indication of accrual at lower 18:2n-6 dietary levels and overall levels of 18:2n-6 in liver CL are lower. This analysis also clearly shows that the incorporation of 22:6n-3 into heart CL is favoured over the incorporation of 18:2-n6 when 22:6n-3 is present at up to 20% of dietary fatty acids, but not at higher percentages of dietary 22:6n-3. There is less evidence that this is occurring in liver, suggesting that selectivity may differ between tissues. The replacement of the single bis-allylic methylene group present in 18:2n-6 with the five bis-allylic methylene groups in 22:6n-3 may result in changes in cellular respiration, membrane peroxidation index, and in the initiation of mitochondrial-mediated apoptosis.

Chapter Four

Comparison of lipid hydroperoxide production between phospholipid classes matched for linoleic acid: Cardiolipin is not an exception

4.1 Overview

Phospholipid peroxidation is primarily a function of polyunsaturated fatty acid (PUFA) content, but a number of studies have also found phospholipid class to influence susceptibility to peroxidation. This chapter presents an *in vitro* comparison of iron-mediated peroxidation of cardiolipin and other phospholipid classes in diffuse solutions and in liposomes. Unlike many past works, the phospholipids compared in this chapter all contained linoleic acid at equivalent amounts in order to remove fatty acid composition as a variable in comparisons.

4.2 Introduction

The oxidative damage of phospholipids by reactive oxygen species (ROS), a process termed peroxidation, is intrinsic to aerobic organisms [3-5, 200, 201]. Although the head groups that define phospholipid class do not normally peroxidises, differences in head group structures have been suggested to influence phospholipid peroxidation [15, 22, 30, 202-204]. Work in this area is limited and has resulted in contradictory findings due to differences in initiators, phospholipid mixtures, buffers and measurement technique used [15]. For example, negatively charged phosphatidylethanolamine (PE) has been reported to increase iron-initiated peroxidation in some conditions but inhibit it in others [202, 203]. Phosphatidic acid (PA) and phosphatidylglycerol (PG), both small head groups, have been reported to inhibit peroxidation [204] but the di-phospholipid cardiolipin (CL), also a small head group in respect to molecule size, has greatly increased peroxidation in comparison to phosphatidylcholine (PC) or PE [30]. The peroxidation of CL is of particular interest due to CLs close association with the early stages of apoptosis involving the release of cytochrome *c* into the cytosol [30, 197, 205, 206].

CL is the only prevalent di-phospholipid class present in mammalian membranes and is found almost exclusively in mitochondria where it facilitates oxidative phosphorylation by binding cytochrome *c* and other mitochondrial proteins [197, 205,

206]. Mammalian CL often contains multiple linoleic acids (18:2n-6) and occasionally also 22:6n-3, as described in Chapter Three, but other phospholipid classes in mammalian tissue rarely contain more than one polyunsaturated fatty acid [18]. The presence of multiple PUFA in a single phospholipid, particularly the presence of 22:6n-3, is likely to make CL highly susceptible to peroxidation. The peroxidation of CL is an early event in apoptosis, and some researchers have suggested that the peroxidation of CL by cytochrome *c* is a trigger of apoptosis [156, 157, 159, 160]. This idea has resonated recently in a number of studies that have reported preferential peroxidation of CL compared to other phospholipid classes during oxidative stress [30] and hyperoxia [169], with reports of CL having twice the peroxidisability of comparable amounts of 18:2n-6 in methyl ester form [207]. However, measurement of CL peroxidation via its production of lipid hydroperoxides has never been compared to other phospholipid classes with similar fatty acid compositions.

This chapter examines the influence of the phospholipid head groups by comparing the auto and iron-mediated peroxidation of CL, of PC, PE, PG and PA matched for amounts of 18:2n-6. Phospholipids were examined as either diffuse in solution or as liposomes in mixtures with a carrier phospholipid, PC 16:0/16:0. All phospholipids contained comparable concentrations of 18:2n-6 to ensure comparable peroxidation indexes between experiments. Peroxidation was initiated using ferrous iron (Fe^{2+}) and measured as LOOH levels due to their high level of production and relative stability products [113, 203, 208-212].

4.3 Methods

4.3.1 Materials

The bovine heart cardiolipin (BHCL) had a purity of >97% and fatty acid composition of 90% 18:2, 5% 18:1 and 5% unknown. The synthetic phospholipids had a purity of >99% and included: PC 16:0/16:0, PC 16:0/18:2, PC 18:2/18:2, PE 16:0/18:2, PE 18:2/18:2, PG 16:0/18:2, PA 16:0/18:2. All phospholipids were purchased from Avanti Polar Lipids Inc. (Alabama, USA) without any added butylated hydroxytoluene (BHT) and kept as 5 mg/ml stock solutions maintained under nitrogen in sealed containers at –20℃. Ammonium ferrous sulfate $(NH_4)_2Fe(SO_4)_2.6H_2O$, xylenol orange and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich (USA). Methanol and chloroform (HPLC grade) were purchased from Crown Scientific (Australia). Sulfuric

acid (98%) was purchased from Ajax Chemicals (Australia). All chemicals used were of analytical grade.

4.3.2 Matching the concentration of bis-allylic methylene groups between phospholipids

Phospholipids were made-up from stock solutions to a final concentration of bis-allylic methylene of group concentration of 194 mM. This concentration of bis-allylic methylene groups reflected original work performed using soy PC to ensure a comparable peroxidative index between phospholipid classes. Based on this concentration, 54 mM of BHCL (containing 90% 18:2n-6) had an equivalent peroxidation index of 194 mM of phospholipids composed of 16:0/18:2 or 97 mM of phospholipids composed of 18:2/18:2.

4.3.3 Sample preparation

Initial methanol experiments were carried out in 250 µM ammonium ferrous sulfate in a solution of 90% methanol, 10% 2.5 mM sulfuric acid (v/v) [113]. As CL, PE, and PA do not readily form liposomes in aqueous media [24] it was necessary to stabilise them in liposomes of PC 16:0/16:0, a non-peroxidisable phospholipid commonly used to produce liposomes. During initial experiments, a ratio of 1:3 BHCL:PC 16:0/16:0 (w/w) was found to have low levels of auto peroxidation and high levels of iron initiated peroxidation. Based on these results, all phospholipids tested were mixed with 0.3 mg/ml of PC 16:0/16:0 and enough peroxidisable phospholipid to yield 194 mM of bis-allylic methylene groups. To form liposomes, lipid mixtures were dried under nitrogen for 60 min at 37 ºC and then hydrated with 140 mM NaCl solution (pH 3.4) for 10 min at 45 ºC. The solution was maintained at pH 3.4 to ensure that the iron used to initiate peroxidation remained in the ferrous form. Following hydration, mixtures were vortexed for 15 min and sonicated on ice using a Sanyo Soniprep 150 with an exponential probe. The probe sonicator output frequency was 23 kHz with an amplitude of 63 microns. Sonication occurred in 3 bouts of 45 seconds separated by 5 second rest periods. Liposomes were maintained at 45 ºC and used immediately after preparation. Mixtures of the peroxidisable phospholipids were also tested in solution of 90% methanol, 10% water without PC 16:0/16:0. Solutions were assessed for particle formation (e.g. liposomes or micelles) using a Zetasizer APS (Malvern Instruments Ltd,

UK) but no discernible populations of particles were detected. The pH of methanol and aqueous solutions was kept at between pH 3–4 [213].

4.3.4 Peroxidation

Peroxidation was initiated by the addition of ferrous iron in the form of ammonium ferrous sulfate. Peroxidation involved two components: that associated with ferrous iron and that independent of ferrous iron i.e. auto peroxidation, both occurring in the same incubations. To separate these contributions, liposomes were incubated both with and without ferrous iron. Auto peroxidation (without ferrous iron) was then taken away from total peroxidation (i.e. that with ferrous iron) in order to distinguish peroxidation initiated by the presence of ferrous iron only.

Lipid hydroperoxide (LOOH) concentration was used as a measure of peroxidation using the xylenol orange (FOX-2) assay as previously described [203] and as reviewed by Bou *et al.* [113]. All results were reported as lipid hydroperoxides produced per 18:2n-6 (mmol LOOH per mol 18:2n-6). No LOOH was detected in the concentrated stock solutions (maintained under nitrogen). In order to determine conditions for maximum LOOH production, liposomes composed of BHCL and PC 16:0/16:0 at a ratio of 1:3 BHCL:PC 16:0/16/0 (w/w) were tested against increasing concentrations of ferrous iron using an incubation time of ten min. A second series of incubations was conducted using incubation times of between 5–120 min and 100 μM ferrous iron to ensure the maximum level of product was formed. Based on these results subsequent experiments using liposomes were carried out over 10 min with 100 μM ferrous iron used to initiate peroxidation. Data was analysed by one-way ANOVA with Tukey post hoc tests using GraphPad Prism 5.04 (GraphPad Software, San Diego, USA) and IBM SPSS Statistics 19 (IBM, New York, USA).

4.4 Results

4.4.1 LOOH formation in diffuse solution

In diffuse solution (90% methanol, 10% water), the total lipid hydroperoxide formation (mmol LOOH per mol 18:2n-6) was higher for BHCL (305.3±13.6) and PA (356.6±9.2) than PC, PE, or PG phospholipids (all below 200) (Figure 4.1A). PC 18:2/18:2, PE 16:0/18:2 and PE 18:2/18:2 produced significantly higher total LOOH than PC

16:0/18:2 and PG 16:0/18:2, and neither PC 16:0/18:2 nor PG 16:0/18:2 produced more total LOOH than PC 16:0/16:0, the negative control. While auto peroxidation (without added ferrous iron) was below 50 mmol LOOH per mol 18:2n-6 for most phospholipids, levels of 61.78±17.9 and 100.3±17.7 were reached for PC 18:2/18:2 and PE 18:2/18:2 respectively, with both values significantly higher than PC 16:0/16:0 (Figure 4.1B). This auto peroxidation accounted for the major component of total peroxidation of these two phospholipids. Iron-mediated LOOH production (i.e. total peroxidation minus auto peroxidation) was highest in PA 16:0/18:2 (355.6±8.4), followed by BHCL (296.4±12.0) and PE 18:2/18:2 (71.6±14.0) (Figure 4.1C).

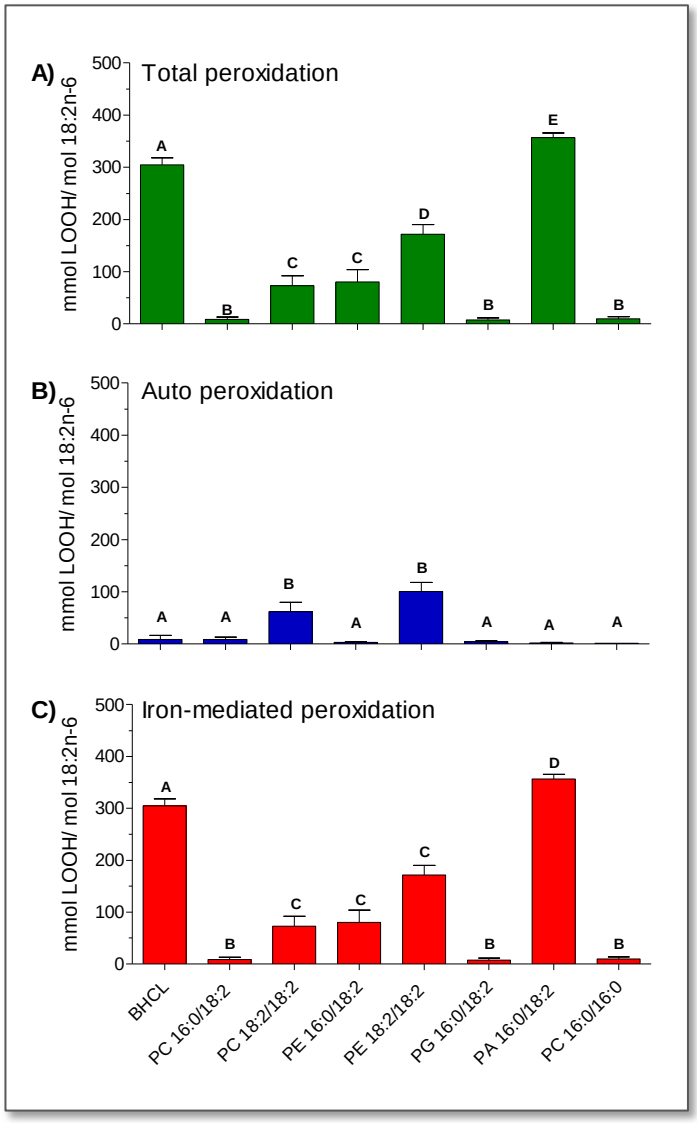

Figure 4.1. Lipid hydroperoxide production (mmol LOOH per mol 18:2n-6) of phospholipids matched for linoleic acid (18:2n-6) content in a solution of 1:9 water to methanol (v/v). Peroxidation was initiated by the addition of 100 µM ferrous iron in the form of ammonium ferrous sulfate for total peroxidation (A) or without iron to determine auto peroxidation (B). Peroxidation due to initiation with ferrous iron alone (C) was calculated as the difference between total peroxidation minus auto peroxidation. LOOH levels were tested after 60 min of reaction. Data are presented as mean ±SEM, n=6. Non-identical letters indicate significant differences within each graph for P<0.05

4.4.2 Maximizing LOOH formation in liposomes

Initial experiments found that BHCL alone did not readily peroxidise in aqueous solution, but LOOH production was greatly increased when BHCL was incorporated into liposomes with PC 16:0/16:0 (Figure 4.2). Auto peroxidation was maximal when only BHCL was present and reduced with increasing levels of PC 16:0/16:0. The maximal level of ferrous initiated BHCL peroxidation occurred at a ratio of 1:3 (w:w) BHCL to PC 16:0/16:0. This ratio produced LOOH levels significant greater 1:1 BHCL to PC 16:0/16:0. Based on these results, all subsequent comparisons between head groups and their ability to peroxidise were carried out at a ratio of 1:3 of peroxidisable phospholipids to PC 16:0/16:0. This ratio was also used to optimise concentration of ferrous iron and time of incubation necessary to produce maximal LOOH in liposomes (Figure 4.3). Results from BHCL in diffuse solution are shown for comparison. The concentration of ferrous iron found to maximise LOOH production of peroxidisable phospholipids was 100 µM (Figure 4.3A). The maximum rate of LOOH production was 35.3±1.1 (mmol LOOH per mol 18:2n-6 per min), which occurred within the first 5 min of incubation. The rate then dropped to 3.2±1.2 (mmol LOOH per mol 18:2n-6 per min) between 5 to 10 min (Figure 4.3B). LOOH amounts were stable after ten min at a ratio of 1:3 (BCHL:PC 16:0/16:0).

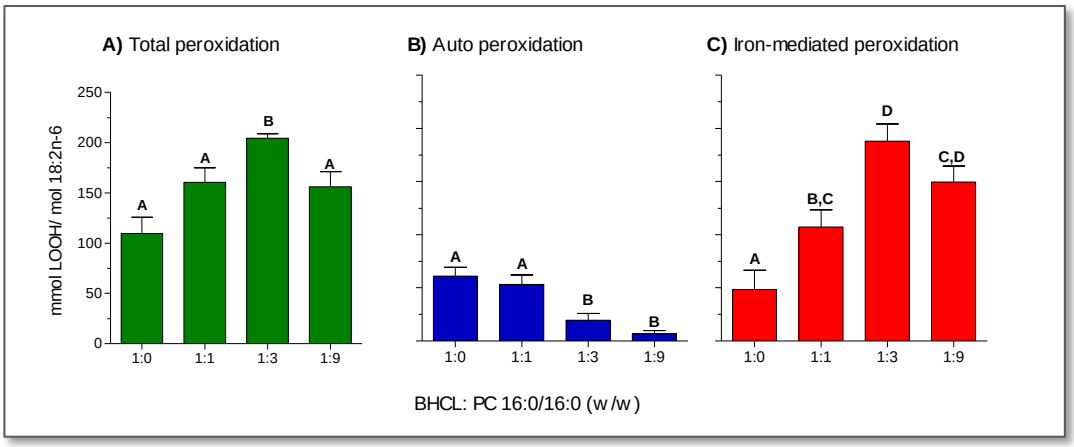

Figure 4.2 Production of lipid hydroperoxides (mmol LOOH per mol 18:2n-6) after sixty min of reaction of bovine heart cardiolipin (BHCL) in liposomal mixtures of BHCL and PC 16:0/16:0. Total peroxidation (A) was initiated by the addition of 100 µM ferrous iron in the form of ammonium ferrous sulfate while auto peroxidation (B) was not initiated by iron. Peroxidation due to initiation with ferrous iron alone (C) calculated as the difference between total peroxidation minus auto peroxidation. Data are presented as mean ±SEM, *n*=6. Non-identical letters indicate significant differences within each graph for $P<0.05$.

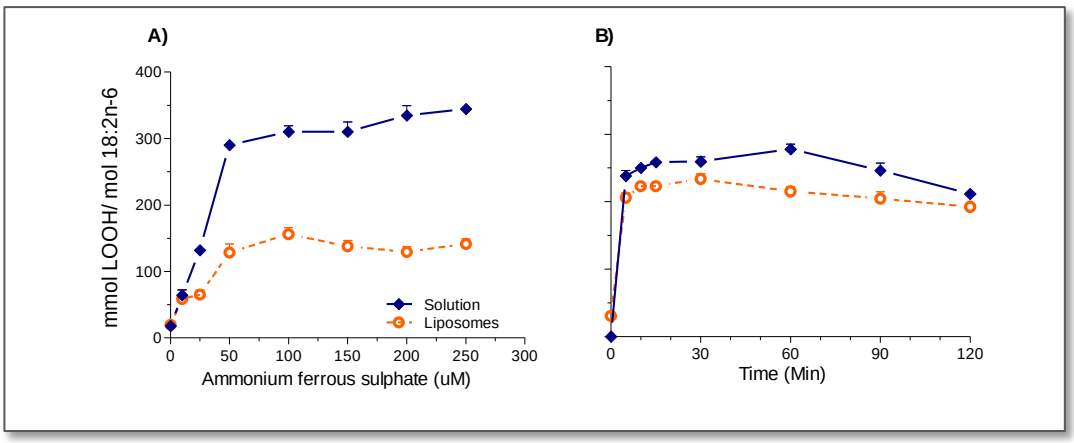

Figure 4.3. Production of lipid hydroperoxides (mmol LOOH per mol 18:2n-6) from bovine heart cardiolipin (BHCL) in liposomes containing PC 16:0/16:0 at a ratio of 1:3 (w/w) or BHCL diffuse in a solution of 1:9 water to methanol (v/v). All experiments contained 194 mM of 18:2n-6. A) values varying concentrations of ammonium ferrous sulfate after 60 min of incubation and B) values with increased incubation time and with 100 µM ammonium ferrous sulfate. Values are means ±SEM, *n*=4.

4.4.3 Comparison of LOOH production of phospholipid classes in liposomes

All phospholipids incorporated into liposomes in aqueous media peroxidised readily and had significantly higher levels of total LOOH production than the negative control (Figure 4.4A). There was a more uniform level of auto peroxidation in the liposomes in comparison to diffuse solutions and PE 16:0/18:2, PE 18:2/18:2, and PG 16:0/18:2 had higher levels than the negative control, PC 16:0/16:0 (Figure 4.4B). Overall, iron-initiated peroxidation was the dominant contribution to total peroxidation in all phospholipids tested (Figure 4.4C). Iron-initiated production of LOOH, the primary outcome of these experiments, was less homogenous in outcome than auto peroxidation. The phospholipid with the highest level of iron-initiated peroxidation (mmol LOOH per mol of 18:2n-6) was PC 18:2/18:2, followed by PE 16:0/18:2, PE 18:2/18:2 and PC 16:0/18:2, BHCL, PG 16:0/18:2, and PA 16:/18:2. The amount of iron-initiated LOOH production ranged from 103.3 to 296.1 (mmol LOOH per mol of 18:2n-6). Although BHCL had a lower level of iron-initiated production of LOOH (202.5±5.3) than other phospholipids when normalised for 18:2n-6 content, it was only statistically lower than PC 18:2/18:2.

4.5 Discussion

4.5.1 Differences in diffuse solution and liposomes

This chapter compared the iron-initiated peroxidation of cardiolipin (CL) and species of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidic acid (PA) matched for linoleic acid (18:2n-6) content when diffuse in solution or in liposomes in aqueous medium. Interest in this comparison came from experiments conducted by Paul Else examining phospholipid peroxidation using the updated ferrous oxidation-xylenol orange (FOX-2) assay for lipid hydroperoxides (LOOH). In these experiments very high levels of peroxidation were observed for bovine heart CL (BHCL) but not for PC or PE phospholipids containing 18:2n-6. Initial work showed that this peroxidation was taking place in the FOX-2 solution rather than in prior assays, suggesting that the CL head group influences peroxidation in ways that PC and PE do not. When phospholipid classes were tested in diffuse solution, the ranking of iron-mediated lipid hydroperoxide production was PA> BHCL>> PE> PC, PG. The cause of the retarded peroxidation of PG, PC and PE and much higher peroxidation for the phospholipids PA and BHCL is not obvious, but may be related to the potential of the head groups of these classes to hold double

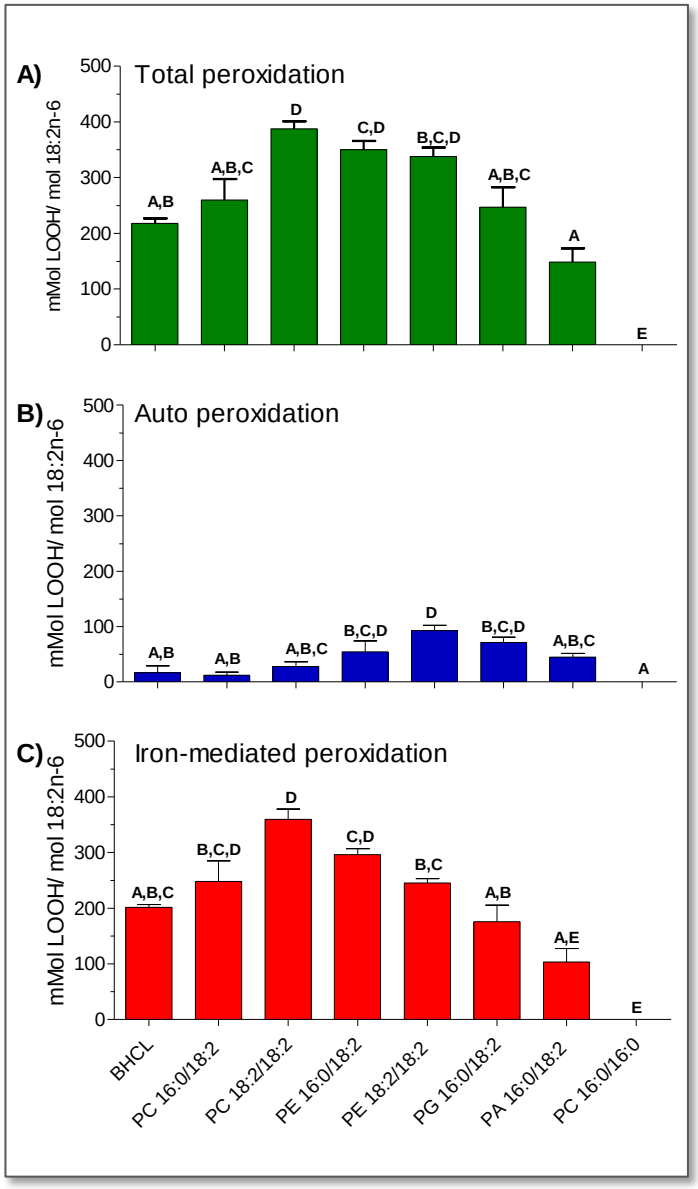

Figure 4.4 Production of lipid hydroperoxides (mmol LOOH per mol 18:2n-6) by phospholipid classes matched for 18:2n-6 in liposomes containing PC 16:0/16:0. Peroxidation was initiated by the addition of 100 µM ferrous iron in the form of ammonium ferrous sulfate for total peroxidation (A) or without iron to determine auto peroxidation (B). Peroxidation due to initiation with ferrous iron alone (C) was calculated as the difference between total peroxidation minus auto peroxidation. LOOH levels were tested after ten min of reaction. Data are presented as mean±SEM, n=6. Non-identical letter indicate significant differences within each graph for P<0.05, as determined by Tukey post-hoc tests.

negative charges [24]. Auto peroxidation in methanol did not follow the same trends as iron-mediated peroxidation, with PC 18:2/18:2 and PE 18:2/18:2 the only phospholipids to show significantly higher LOOH production.

In contrast, all phospholipid classes showed similar levels of iron-mediated peroxidation when tested in liposomes. The overall ranking of iron-initiated lipid

hydroperoxide production in liposomes was PC> PE> CL> PG> PA. PC and PE are the largest head groups relative to phospholipid size and were uncharged under the experimental conditions used while the head groups of PG, CL and PA are relatively smaller and negatively charged. Previous studies have suggested that negatively charged phospholipid head groups can increase peroxidation by attracting ferrous iron [202], but this was not strongly supported by the present study. The differences between the results found in liposomes compared to those produced in methanol highlights the importance of the models used to test peroxidation.

4.5.2 Cardiolipin and peroxidation

The present results are in contrast to previous reports that CL peroxidises more readily than other head groups [30, 169, 207]. One reason for this difference is in the use of free iron rather than cytochrome *c*, as cytochrome *c* forms a tight complex with CL but not with other phospholipids [159, 169]. A second reason is the choice of stabilizing agents for liposome preparation. In order to maximise iron-initiated peroxidation and ensure the availability of CL, BHCL and the other phospholipids were incorporated into liposomes containing a non-peroxidisable 'carrier' phospholipid (PC 16:0/16:0). A recent study by Roginsky [207] reported CL peroxidises at twice the rate of the methyl ester of 18:2 per bis-allylic methylene group when used with Triton X-100 (a detergent) to form CL micelles. CL and 18:2 methyl esters were incubated at different concentrations (0.6–2 mM to 5–20 mM respectively) in the same concentration of Triton X-100. However, Triton X-100 contains endogenous peroxide (0.22%: see Sigma MSDS sheet) that can accelerate rates of peroxidation and potentially total product formation. This factor may have influenced the results. In addition, methyl esters are far more resistant peroxidation than those in phospholipids containing the same peroxidisable fatty acids (Else and Kraffe, in preparation).

Another explanation as to why CL may appear to peroxidise more readily and produce more product is that peroxidation can be measured as oxidation per bis-allylic methylene group, as in the present study and [207], or per phospholipid. As a di-phospholipid rich in 18:2n-6, mammalian CL is far more likely to peroxidise on a per phospholipid basis than other classes that typically contain only up to one PUFA [79, 214]. Wiswedel *et al*. [30] compared the peroxidation of CL, PC and PE from rat brain during oxidative stress and reported that CL content of the mitochondria dropped away

more rapidly than both PE and PC. Using values for the acyl composition of mouse brain mitochondria for CL, PE and PC [215], the peroxidative index of these phospholipids can be calculated to be 106, 170 and 76 respectively. This might indicate that the early peroxidation of CL is disproportionate to its peroxidative potential. However, peroxidation was measured by the 'disappearance' of phospholipids from a mass spectrum, a process that measures peroxidation per phospholipid rather than per bis-allylic methylene group. Due to its structure, CL is approximately twice as likely to 'disappear' as PE or PC with comparable peroxidative indexes. This re-evaluated peroxidative potential of the molecule is in-line with the relative disappearance of the different phospholipids found by Wiswedel *et al.* [30]. Similarly, the relative early disappearance of CL from other studies on rat brain mitochondria during oxidative stress [216] and in lungs during acute hyperoxic lung injury [169] can be similarly explained without requiring CL to show exceptional peroxidative capacity.

4.5.3 Limitations

The limitation of the FOX-2 assay to measure *in vitro* peroxidation are due to its slow sampling speed which cannot accurately be used to measure the phases of peroxidation. The use of the FOX-2 assay is an accurate measure of the maximum product of peroxidation but does not record data faster enough to identify distinct lag and propagation phases of peroxidation (Figure 4.3, [202]). The maximum rate of peroxidation was measured for BHCL in diffuse solutions and liposomes, but the length of the lag phase and rate of peroxidation during this phase could not be measured. This limitation is shared by similar assays including the thiobarbituric acid reactive substances (TBARS) assay. These limitations are addressed in Chapter Five, which describes results obtained following the purchase of a six-channel oxygen meter (Strathkelvin Instruments) that provided a more sensitive and continuous measurement of peroxidation. A further limitation was the use of iron as an initiator of peroxidation. The 100 µM ferrous iron used in these experiments is likely to be far in excess of iron concentrations found in under physiological conditions, and iron is only one of many possible initiators that may be used to study peroxidation. Future work in this area could consider physiologically relevant concentrations of iron alongside other initiators such as radical clocks to ensure heterogeneity of results.

4.6 Conclusions

The experiments presented in this Chapter show that the CL and PA classes increased peroxidation products in diffuse solution when compared to other phospholipid classes independently of PUFA composition. In comparison, peroxidation in liposomes was similar between classes, with the small differences in peroxidation related to head group size. In contrast to past studies, CL did not have higher peroxidation (as measured by product formation) compared to other phospholipid classes in liposomes, suggesting that the CL head group does not have exceptional peroxidative capacity in terms of product formation.

Chapter Five

An antioxidant-like action for non-peroxidisable phospholipids using ferrous iron as a peroxidation initiator

5.1 Overview

Phospholipids with saturated (e.g. PC 16:0/16:0) and monounsaturated fatty acids (e.g. PC 16:0/18:1) are some of the most common phospholipids found in membranes, and under normal conditions are generally non-peroxidisable. This chapter presents an *in vitro* investigation into an antioxidant-like effect of PC 16:0/16:0 and PC 16:0/18:1 during iron-mediated peroxidation in liposomes. This study used more sophisticated techniques than Chapter Four to measure multiple aspects of peroxidation including the period of the lag phase and the associated rates of peroxidation. This chapter is closely based the article 'An antioxidant-like action for non-peroxidisable phospholipids using ferrous iron as a peroxidation initiator' by Cortie CH and Else PL, *BBA Biomembranes*, 2015, **1846**(6): p. 1303-1307. The structure of the chapter differs from the accepted article to avoid repetition with earlier chapters. CHC developed the method used in this study and collected all data except that presented in Figure 5.5. CHC analysed all data and drafted the work. PLE proposed the study, collected the data for Figure 5.5, and supervised all aspects of the work.

5.2 Introduction

Peroxidation is a constitutive process in living systems and is part of healthy functions but also pathology and aging [217, 218]. Peroxidation is a free radical reaction that typically progresses through three well-recognised phases; i) the lag phase, a duration of time (T_{Lag}) with a slow rate of reaction (R_{Lag}) and product formation, ii) the propagation phase, with a fast rate of reaction (R_{Max}) and product formation and, iii) termination due to substrate exhausting or antioxidant quenching (Figure 2.9, [96]). From a biological perspective, the lag phase is important as it offers the opportunity to prevent peroxidation entering into the more damaging propagation phase. It is within the lag phase that membrane antioxidants and enzymes can stop the peroxidation reaction, and the T_{Lag} is commonly considered a measure of the antioxidant status of a membrane [108].

The chemistry of peroxidation is complex [5, 219], but the peroxidation of cellular membranes can be mainly attributed to the polyunsaturated fatty acids (PUFA) present in membrane phospholipids. As a result of the differences in the bond energies of hydrogen to the carbons of fatty acids, PUFA are far more likely to undergo peroxidation than saturated fatty acids (SFA) or monounsaturated fatty acids (MUFA) ([73], Table 2.2). As a consequence, phospholipids that contain one or more PUFA are peroxidisable phospholipids (PPLs) while phospholipids containing only SFA and/or MUFA are non-peroxidisable phospholipids (non-PPLs). Despite the challenge PUFA present to living organisms in terms of peroxidation, PPLs are prevalent in membranes. In mammals, for example, membrane phospholipids typically possess between 30 to 60% of their total fatty acids as PUFA [18, 220], making PPLs a major portion of membrane phospholipids.

The current research examines if non-PPLs can protect PPLs from peroxidising. This work examines the separate peroxidation of two PPLs (a natural phospholipid, soy phosphatidylcholine, and a synthetic phospholipid phosphatidylcholine, PC 16:0/18:2) in the presence of one of two different non-PPLs (PC 16:0/16:0 and PC 16:0/18:1) to determine if any protection occurs. Peroxidation was induced by low levels of ferrous iron rather than the more common high concentrations of azo-initiator such as AAPH in order to produce a more natural peroxidative stimulus. Although the mechanism by which iron initiates lipid peroxidation is still under debate [100], poorly-ligated iron is a problem common to a number of pathologies [105]. The results of this study suggest that the presence of non-PPLs decreases the rates of peroxidation resulting in the extension of the duration of the lag phase producing an antioxidant-like action.

5.3 Methods

5.3.1 Materials

The phosphatidylcholines PC 16:0/16:0, PC 16:0/18:1, PC 16:0/18:2 and Soy PC (fatty acid composition of 14.9% 16:0, 3.7% 18:0, 11.4% 18:1n-9, 63% 18:2-6, 5.7% 18:3n-3 and 1.2% unknown) were purchased from Avanti Polar Lipids (Alabaster, USA) without added butylated hydroxytoluene (BHT) as antioxidant. Ammonium ferrous sulfate ($(NH_4)2Fe(SO_4)2.6H_2O$), sodium sulfate and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich (St Louis, USA). Methanol (HPLC grade) and

sulfuric acid (98%) were purchased from Crown Scientific (Rowville, Australia). All chemicals used were of analytical grade.

5.3.2 Liposome preparation

Stock solutions of phospholipids (125 mM) of both peroxidisable (PC 16:0/18:2 and Soy PC) and non-peroxidisable (PC 16:0/16:0 or PC 16:0/18:1) phosphatidylcholines were made-up in methanol and stored at –20 ^{o}C under nitrogen. Phospholipids from each stock solution were combined to produce the required phosphatidylcholine mixtures. Each phospholipid mixture was dried down under a stream of nitrogen for a minimum of 90 min (or until completely dry) at 42 ^{o}C, made-up to 10 mM using deionised water (42 ^{o}C, pH 3), stirred continuously for 45 min (42 ^{o}C) then passed nine times across a 0.1 μm pore polycarbonate membrane (Avanti Polar Lipids) using a Mini-Extruder (Avanti Polar Lipids) at (42 ^{o}C). Following extrusion, samples were diluted to 2.5 mM. Samples of liposomes were taken from each preparation, before and after peroxidation, in order to measure liposome size. BHT was added to prevent further peroxidation. In addition, the lipid hydroperoxide (LOOH) level of each liposomal preparation was measured prior to initiation of peroxidation and only those preparations with no measureable levels of LOOH were used.

5.3.3 Measurement of lipid peroxidation and liposome size

Peroxidation was measured using oxygen consumption as a measure of peroxyl formation. Oxygen consumption was measured using Clarke-type microelectrodes (Strathkelvin Instruments) with up to six electrodes used simultaneously. Oxygen consumption measurements were made over 2 hr periods at 37 ^{o}C using a RC-650 six-electrode respirometer. Oxygen consumption data was acquired each second from each microelectrode using a Six-Channel Oxygen Meter (Strathkelvin Instruments). Oxygen electrodes were prepared fresh daily using high sensitivity, fast response membranes (YSI Life Sciences, Morningside, Australia). Care was taken to remove any trace of lipid hydroperoxides adhering to the respiration wells between experiments. Prior to any measurement, microelectrodes were allowed to stabilise and thermally equilibrate to 37^{o}C for a minimum period of 30 min. Incubations were stirred continuously during experimentation. Microelectrode calibration was performed as per manufacturer's instruction using air saturated, filtered deionised water with sodium sulfate used to

determine oxygen range. At 37 ^{o}C the oxygen concentration used was 6.73 mg or 210.3 µmol of oxygen/L of water at prevailing atmospheric pressure.

Peroxidation was initiated using 10 µM ferrous iron derived from ammonium ferrous sulfate (pH 3–4). Liposomes were made up to a final concentration of 2.5 mM, with PPLs present at between 2.5 and 0.5 mM and non-PPLs added from 0.5 to 2.0 mM (20–80%). Graphs show the decrease in PPL and increase in non-PPL from left to right. The inset in each figure show the same experiment carried for liposomes composed of the same amount of PPL only (2.5–0.5 mM). Except for the period of the lag phase (T_{Lag}), all measurements are normalised for the concentration of PPL. Measurements made during peroxidation included: rate of peroxidation during the lag phase (R_{Lag}), maximum rate of peroxidation during the propagation phase (R_{Max}), duration of the lag phase (T_{Lag}) and total amount of oxygen consumed during the lag phase (A_{Lag}), as shown in Figure 2.9. R_{Lag} and R_{Max} were determined using segmental linear regression analysis GraphPad Prism 5.04 (GraphPad Software, San Diego, USA) where the model divided the data into R_{Lag} and R_{Max} segments for each replicate and using an iterative process proceeded to determine the best fit for each segment. The duration of the T_{Lag} was taken as the period between the point of ferrous iron addition to the point of intersection between the derived R_{Lag} and R_{Max} segments [96].

Liposome size was determined using a Malvern Zetasizer (Malvern, UK) at a refractive index of 1.46 at 37 ^{o}C. Measurements were taken before and after each experiment with liposomes found to vary in size between 140 and 155nm. No significant change in liposome size was detected as a result of time of incubation (120 min), phospholipid composition or peroxidation. All results were analysed using 2-way ANOVAs with Bonferroni post-hoc tests using GraphPad Prism 5.04.

5.4 Results

5.4.1 Extension of the lag phase

The duration of the lag phases (T_{Lag}) of liposomes composed of the peroxidisable phospholipids (PPLs) Soy PC (Figure 5.1A) and PC 16:0/18:2 (Figure 5.1B) were extended by the presence of the non-PPLs PC 16:0/16:0 or PC 16:0/18:1. The insets in each figure show T_{Lag} in the absence of non-PPLs at the same concentration of PPL as in the main graphs (2.5 mM down to 0.5 mM). In the absence of non-PPLs, concentrations of 2.5 to 0.5 mM of Soy PC and PC 16:0/18:2 showed no change in the

duration of their lag phases (Figure 5.1 insets). The presence of either non-PPL with Soy PC or PC 16:0/18:2 increased the T_{Lag} significantly when present at 60% and 80% of liposomal phospholipids. The presence of PC 16:0/16:0 increased the T_{Lag} of Soy PC by up to 4.7–fold and that of PC 16:0/18:2 by up to 2.6–fold. In comparison, the presence of PC 16:0/18:1 increased the T_{Lag} of Soy PC by up to 3.1–fold and that of PC 16:0/18:2 by up to 5.3–fold. One major difference between the two PPLs was the duration of T_{Lag} in the absence of non-PPLs which was approximately 9–fold longer for Soy PC compared to PC 16:0/18:2 (3.9–7.0 min for Soy PC compared to 0.43–0.79 min for PC 16:0/18:2).

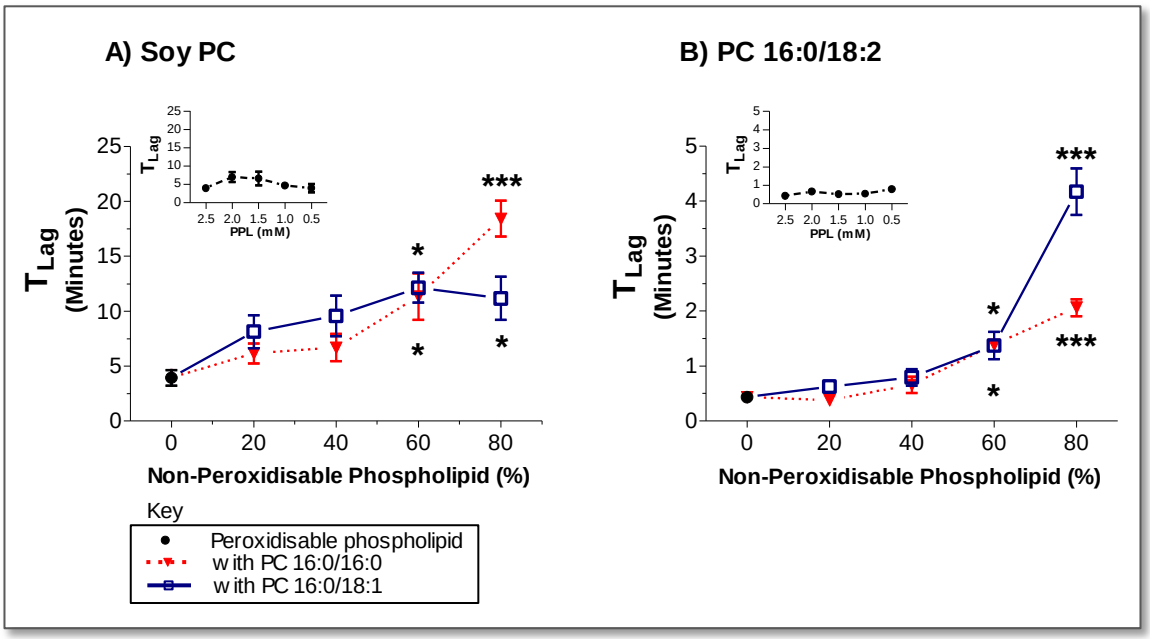

Figure 5.1. The period of the lag phase (T_{Lag}) during iron-mediated peroxidation of the peroxidisable phospholipids (PPLs) Soy PC (A) and PC 16:0/18:2 (B) in the presence of the non-peroxidisable phospholipids (non-PPLs) PC 16:0/16:0 and PC 16:0/18:1. PPLs were measured at concentrations between 2.5 and 0.5 mM, with non-PPLs added to make all final concentrations of phospholipid 2.5 mM. The inset in each figure shows the response of each PPL in the absence of any non-PPL across the same concentration range (2.5–0.5 mM). Peroxidation was initiated with 10 µM ammonium ferrous sulphate. Values are averages ±SEM, *n*=6. Significant differences between PPLs in the presence (main graph) and absence (inset) of non-PPLs are indicated with * for $P<0.05$ and *** for $P<0.001$.

5.4.2 Changes in rate

The ability of non-PPLs to increase the duration of the lag phase of PPLs appears to be due to their capacity to reduce rates of peroxidation (Note: with the exception of T_{Lag} all other parameters were normalised to account for the differences in PPL concentration). This is shown in the rates of peroxidation during the lag (R_{Lag}, Figure 5.2A and B) and propagation (R_{Max}; Figure 5.3A and B) phases of Soy PC and PC 16:0/18:2 respectively. The R_{Lag} and R_{Max} values remain constant across an increasing percentage of both non-PPLs. The R_{Lag} and R_{Max} were lower for Soy PC than PC 16:0/18:2, and both non-PPLs had similar effects on the rates. In the absence of non-PPLs, the R_{Lag} of Soy PC increased 2.1–fold (from 1.4 to 3.0 mmol of O_2 per mole of Soy PC per minute, Figure 5.3A inset) and that of PC 16:0/18:2 increased 3.2–fold (from 3.6 to 11.7 mmol of O_2 per mole of PC 16:0/18:2 per minute, Figure 5.3B inset). Similarly, in the absence of non-PPLs, the R_{Max} of Soy PC increased 2.1–fold (Figure 5.3A inset) and that of PC 16:0/18:2 by 1.3–fold (Figure 5.3B inset).

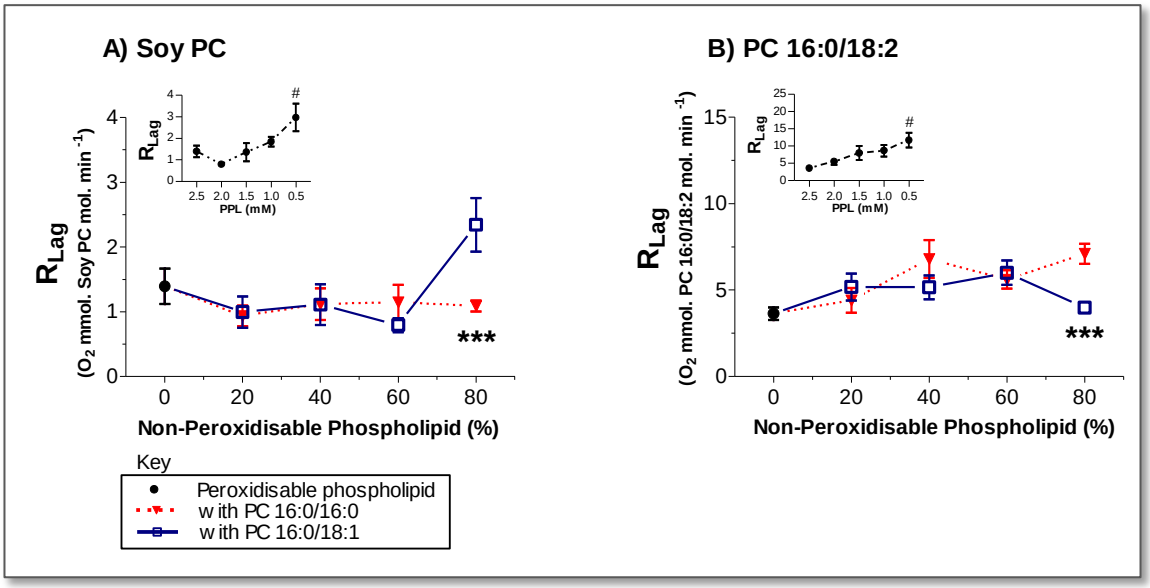

Figure 5.2. Rates of iron-initiated peroxidation during the lag phase (R_{Lag}) of the peroxidisable phospholipids (PPLs) A) Soy PC and B) PC 16:0/18:2 in the presence of the non-peroxidisable phospholipids (non-PPLs) PC 16:0/16:0 and PC 16:0/18:1. PPLs were measured at concentrations between 2.5 and 0.5 mM, with non-PPLs added to make all final concentrations of phospholipid 2.5 mM. The inset in each figure shows the response of each PPL in the absence of any non-PPL across the same concentration range (2.5–0.5 mM). Rates were normalised for differences in the concentration of PPL. Peroxidation was initiated with 10 µM ammonium ferrous sulphate. Values are averages ±SEM, n=6. Significant differences between PPLs in the presence and absence of non-PPLs are indicated with * for P<0.05 and *** for P<0.001. In the insets, # indicates significant differences at P<0.05 for values in comparison to the original PPL concentration of 2.5 mM.

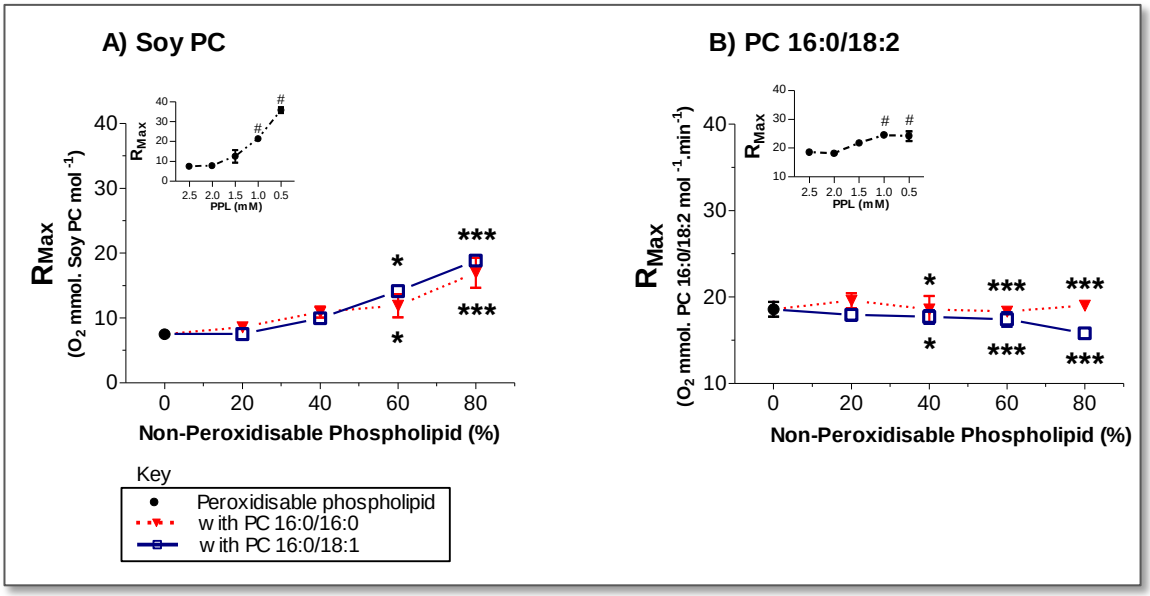

Figure 5.3. Rates of iron-initiated peroxidation during the propagation phase (R_{Max}) of the peroxidisable phospholipids (PPLs) A) Soy PC and B) PC 16:0/18:2 in the presence of the non-peroxidisable phospholipids (non-PPLs) PC 16:0/16:0 and PC 16:0/18:1. PPLs were measured at concentrations between 2.5 and 0.5 mM, with non-PPLs added to make all final concentrations of phospholipid 2.5 mM. The inset in each figure shows the response of each PPL in the absence of any non-PPL across the same concentration range (2.5–0.5 mM). Rates were normalised for differences in the concentration of PPL. Peroxidation was initiated with 10 µM ammonium ferrous sulphate. Values are averages ±SEM, *n*=6. Significant differences between PPLs in the presence and absence of non-PPLs are indicated with * for $P<0.05$ and *** for $P<0.001$. In the insets, # indicates significant differences at $P<0.05$ for values in comparison to the original PPL concentration of 2.5 mM.

The ability of non-PPLs to inhibit the R_{Lag} was supported by measurements of the total amount of oxygen consumed during the lag phase (A_{Lag}) by Soy PC and synthetic PC 16:0/18:2. The presence of non-PPLs increased the total amount of oxygen consumed during the lag phase of both Soy PC (Figure 5.4A) and PC 16:0/18:2 (Figure 5.4B), being statistically significant when present at 80% of liposomal phospholipid. This inhibition is likely due to the ability of each PPL to reach a critical level of peroxidation product (presumably peroxyl radicals) needed to transition into the propagation phase.

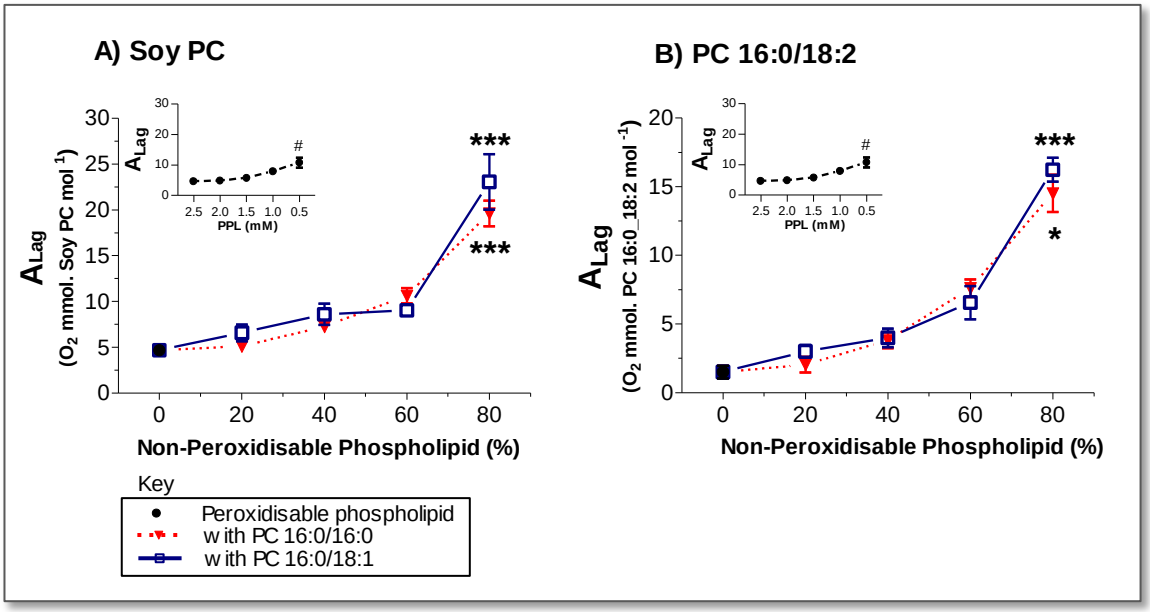

Figure 5.4. Amount of O_2 consumed by the end of the lag phase (A_{Lag}) of iron-mediated peroxidation of the peroxidisable phospholipids (PPLs) A) Soy PC and B) PC 16:0/18:2 in the presence of the non-peroxidisable phospholipids (non-PPLs) PC 16:0/16:0 and PC 16:0/18:1. PPLs were measured at concentrations between 2.5 and 0.5 mM, with non-PPLs added to make all final concentrations of phospholipid 2.5 mM. The inset in each figure shows the response of each PPL in the absence of any non-PPL across the same concentration range (2.5–0.5 mM). All parameters measured were normalised for differences in the concentration of PPL. Values are averages ±SEM, *n*=6. Significant differences between PPLs in the presence and absence of non-PPLs are indicated with * for $P<0.05$ and *** for $P<0.001$. In the insets, # indicates significant differences at $P<0.05$ for values in comparison to the original PPL concentration of 2.5 mM.

5.4.3 The importance of the ratio of Fe^{2+} to PPL

The increases in normalized rates of peroxidation of PPLs seen with decreasing PPL concentration can be explained by changes in the ratio of Fe^{2+} to PPL. This presumably occurs because 10 µM ferrous iron did not produce a maximal peroxidation response, and as the concentration of each PPL decreased (in the absence of non-PPLs) the ratio of Fe^{2+} to PPL increased from 1:250 to 1:50. To test the importance of relative concentrations of Fe^{2+} to PPLs, the R_{Max} of Soy PC was measured at both variable (1:250 to 1:50) and constant (1:250) Fe^{2+}:PPL ratios. The result shows that increases in the R_{Max} were removed once the ratio of Fe^{2+} to PPL ratio was held constant (Figure 5.5).

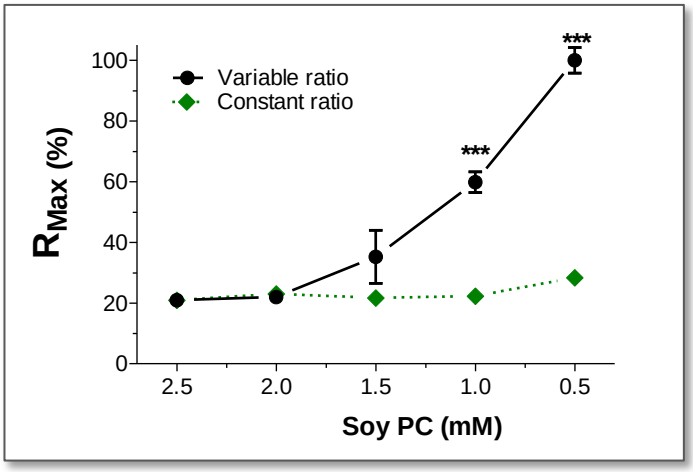

Figure 5.5. The relative maximal rates of peroxidation (R_{max}) of Soy PC at either constant (1:250) or increasing (1:250 to 1:50) ratios of Fe^{2+} to Soy PC. Statistical differences show comparisons between measurements at the same Soy PC concentration but different Fe^{2+}:Soy PC ratios. Values are averages ±SEM, *n*=6, *** indicates a significant difference at *P*<0.001.

5.5 Discussion

5.5.1 Non-peroxidisable phospholipids have an antioxidant-like action

Antioxidant molecules and enzymes are considered key defences in controlling the radical-generating reactions of peroxidation. These defences act by extending the lag phase of peroxidation, thus preventing the transition to the more damaging propagation phase [96, 108], and so the duration of the lag phase is commonly considered to reflect the antioxidant status of membranes [108]. Many factors influence peroxidation including both physical and chemical aspects of the lipid and their packing within membranes, with the polyunsaturated fatty acid (PUFA) content being particularly important [15, 64]. The role of non-peroxidisable phospholipids (non-PPLs) containing saturated fatty acids (SFA) or monounsaturated fatty acids (MUFA) in influencing peroxidation has been only examined in a limited number of studies [16, 221]. The present work suggests that these non-PPLs can produce an antioxidant-like action that protects peroxidisable phospholipids (PPLs), therefore preventing the propagation of peroxidation in membranes. This action appears to be accomplished by decreasing the overall rate of peroxidation during the lag phase and impeding the build-up of peroxidation product necessary to enter into propagation phase. As a result, the presence of non-PPLs extends the duration of the lag phase which is likely to be the most important antioxidant-like action produced by these phospholipids in membranes. Non-PPLS commonly account for about half of the phospholipid molecules found in

membranes [18], and it is at this level that the significant antioxidant-like activity of non-PPLs extends the lag phase by 2.5–fold to 5.3–fold.

The antioxidant-like action of non-PPLs was found to have a greater influence on the extracted Soy PC than the synthetic PC 16:0/18:2 even though these two phospholipids have a similar acyl composition. Soy lecithin, which is primarily soy PC, has a high level of antioxidants [222] and so the extended lag phase of Soy PC is likely due to the presence of antioxidants co-extracted during purification. If this is the case, the greater influence of non-PPLs on Soy PC than PC 16:0/18:2 suggests a synergistic action of the antioxidants present in Soy PC with the antioxidant-like action of the non-PPLs. Such an action could reduce the levels of antioxidant needed in membranes to control peroxidation reactions by providing more time for antioxidants to be recycled or for damaged phospholipids to be removed.

Indications of some antioxidant-like actions for non-peroxidisable phospholipids have been previously reported by Lee *et al.* (1998) [16] who examined liposomes containing phosphatidylcholines with a constant proportion of 18:2n-6 (37.5%) and varying amounts of 18:1n-9 and 16:0. This study found that "*when 16:0 was replaced by 18:1 there was a marked increase in the lag time*". The study of Lee *et al*. did not vary the concentration of non-PPLs and emphasised the role of 18:1n-9. In the present work, the concentration of non-PPLs varied and both PC 16:0/16:0 and 16:0/18:1 were found to be capable of extending the lag phase of Soy PC and PC 16:0/18:2. An antioxidant-like action for PC 16:0/16:0 has also been shown in the study of Soto-Arriaza *et al*. (2008) [221] examining the peroxidation of liposomes made-up of egg PC and varying amounts of PC 16:0/16:0 (0–60 mol%). This study found that PC 16:0/16:0 inhibited peroxidation "*beyond that expected from the unsaturated lipid dilution*". The antioxidant action was interpreted as being due to a rigidification of the bilayer and 'capture' of initiating radicals. This explanation is slightly contrary to the result of the present study where PC 16:0/16:0 and PC 16:0/18:1 were both found to display antioxidant-like activity even though their transition temperatures are quite different, (41.5 ^{o}C for PC 16:0/16:0 and –2 ^{o}C for PC 16:0/18:1 [223]). It is therefore possible that the antioxidant-like action of these non-PPL works by steric hindrance without requiring rigidification. The peroxidation reaction requires lipid radicals to come in contact with each other in the plane of the monolayer in order to continue peroxidation and this requires diffusion as peroxidation appears not to cross monolayers within the bilayer [23]. The presence of non-PPL may 'cage' lipid radicals within the

monolayer and thus slow rates of peroxidation. The ability of different non-PPLs to perform this task is likely to depend upon their individual structure and acyl composition. This mechanism appears not to be a simple linear dilution effect as shown for PC 16:0/18:2 where a 20% increase in PC 16:0/18:1 (60–80%) increased lag duration 3–fold.

5.5.2 Methodological considerations

One reason the antioxidant-like action of non-PPL has only been recognised in a small number of studies is that most peroxidation studies use high levels of initiator (e.g. AAPH, Cu^{2+}, Fe^{2+}, or radiation) to maximize the peroxidation response. The levels of ferrous iron needed to produce a maximal peroxidation response (100–1000 µM) would have overwhelmed any protective effect of the non-PPL. In the present study, the use of a submaximal ferrous iron concentration (10 µM) added extra complexity due to variable ratios of Fe^{2+} to PPLs, but did allow non-PPLs to exhibit their antioxidant capacity. As even a 10 µM ferrous iron peroxidation stimulus is likely to be extremely high *in vivo*, the strength of the antioxidant-like action of non-PPLs in the membrane of normal working cells is likely to be biological relevant.

5.5.3 Limitations

The use of oxygen consumption to measure peroxidation addresses the limitations of the methods used in Chapter Four. A general limitation of the *in vitro* studies presented in this thesis was the use of a single initiator, iron, in peroxidation assays. Iron-initiated peroxidation is biologically relevant but chemically complex. Radical clocks such as AAPH produce ROS at a known rate and could have been used to better determine the kinetics of the peroxidation reactions [5, 96], but these kinetics are still complex [224] and initiating peroxidation in this manner may not accurately model physiological conditions. Future studies of peroxidation would benefit from the use of multiple initiators including AAPH, iron, copper and haem to ensure that results are not limited to a single initiator. In addition, future work could also be extended to examine the importance of other major components of membranes such as proteins to this antioxidant-like activity.

5.6 Conclusion

Phospholipids composed of SFA and MUFA are present in cellular membranes at sufficient concentrations to extend the lag phase of peroxidation. This study shows these non-PPLs may play an important role in protecting membranes from peroxidation by extending the lag phase in a newly-described antioxidant-like action. It is suggested that this antioxidant-like action may assist cells at controlling peroxidation in membranes by working synergistically with other antioxidant defences. Future would in this area could test the homogeneity of these results with peroxidation caused by AAPH, and would also benefit from examining this effect in other phospholipid classes.

Chapter Six

Of mice, pigs and humans: A lipidomics analysis of mitochondrial phospholipids from mammals with very different maximal lifespans

6.1 Overview

Maximal lifespan (MLS) has been found to be inversely related to the degree of membrane unsaturation, particularly of polyunsaturated fatty acids (PUFA). Previous comparative work in this area has examined membrane composition at the level of fatty acids, but little is known regarding the influence of phospholipid classes and individual phospholipid molecules. In addition, data for humans is extremely rare in this area. This chapter presents a shotgun lipidomics analysis of mitochondrial membranes and the peroxidation index of skeletal muscle, liver, and brain in three mammals that span the range of mammalian longevity. This work is closely based on 'Of mice, pigs and humans: A lipidomics analysis of mitochondrial phospholipids from mammals with very different maximal lifespans' by Cortie CH, Hulbert AJ, Norris SE, Mitchell TW, McAndrew D, and Else PL, published in *Experimental Gerontology*, 2015, **70**: 135-143. This chapter differs from the submitted work to avoid repetition with earlier chapters. CHC collected and analysed the samples, wrote the Excel algorithms used to identify molecular phospholipids and determine fatty acid composition, and drafted the article. SEN and TWM assisted in developing the mass spectrometry methods used. PLE and AJH designed the study, supervised the work, and assisted with drafting the article.

6.2 Introduction

Animal longevity has been inversely correlated with mitochondrial production of reactive oxygen species (ROS) and the degree of fatty acid unsaturation in membranes [12, 14]. These factors are closely related as ROS are both a cause and a product of the peroxidation of polyunsaturated fatty acids (PUFA) [5]. Propagation of peroxidation can adversely affect cell function by forming adducts with DNA and proteins, disrupting the structure of cell membranes, and propagating damage to lipids [4, 65]. The peroxidation of mitochondrial membranes is particularly important as mitochondria are the major source of ROS within cells [14] and mitochondrial failure is an indicator of aging [118]. Peroxidation primarily occurs at bis-allylic methylene groups as the reduction potential

of these groups is lower than that of other methylene groups [73]. Bis-allylic methylene groups are present in PUFA, but saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) do not contain these groups and are resistant to peroxidation. The number of bis-allylic methylene groups present in PUFA, and therefore the relative susceptibility of membranes to peroxidation, increases with the degree of polyunsaturation [21, 22]. Common mammalian PUFA include linoleic acid (18:2n-6) with one bis-allylic methylene group, arachidonic acid (20:4n-6) with three groups, and docosahexaenoic acid (22:6n-3) with five groups.

The relative susceptibility of cellular membranes to peroxidation, termed a peroxidation index, can be determined from membrane composition and experimentally derived rates of peroxidation [12, 35]. A strong inverse correlation has been established between the peroxidation index and maximal lifespan (MLS) of skeletal muscle and liver mitochondria for a range of mammals [34, 35]. This relationship is best characterised for mammals, but similar relationships have also been found for birds [12], molluscs [37], bees [38], and strains of *Caenorhabditis elegans* [39]. Despite the breadth of prior work in this area it is not known 1) whether results for muscle mitochondrial membranes correspond to whole muscle, 2) if brain mitochondria exhibit a similar relationship between the peroxidation index and MLS as found in skeletal muscle and liver and 3) how different phospholipid classes and phospholipid molecules contribute to the peroxidation index.

To address these questions, an in-depth lipidomics analysis was conducted using the common mouse (*Mus musculus*, MLS of 4 years), the domestic pig (*Sus scofa*, MLS of 27 years), and humans (*Homo sapiens*, MLS of 122 years). Mice and humans were chosen as examples of the extremes of mammal longevity (AnAge Database), and as data for human mitochondria is extremely rare. Pigs were chosen as they have a similar body mass to humans but a much shorter maximal lifespan. Shotgun lipidomics was used to determine phospholipid composition of mitochondrial-enriched fractions of skeletal muscle, liver and brain of each animal, and calculated the fatty acid composition and peroxidation index of mitochondrial membranes from phospholipid values.

6.3 Methods

6.3.1 Materials

Methanol and chloroform (HPLC grade or higher) were supplied by VWR International (Queensland, Australia). Analytical grade ammonium acetate was obtained from Crown Scientific (New South Wales, Australia). Sucrose, Tris, and ethylenediaminetetraacetic acid (EDTA) were supplied by Astral Scientific (New South Wales, Australia). Pierce BCA Protein Assay Kits were obtained from Thermo Fisher Scientific (Victoria, Australia). Butylated hydroxytoluene (BHT) was supplied by Sigma-Aldrich, (New South Wales, Australia). Phospholipid internal standards were purchased from Avanti Polar Lipids through Auspep (Victoria, Australia) and consisted of 20 µM of PC 19:0_19:0, PE 17:0_17:0, and PS 17:0_17:0 and 10 µM of lyso-PC 17:0 and lyso-PE 14:0. Internal standards were stored in 2:1 chloroform to methanol at –20 °C.

6.3.2 Animals and tissues

Skeletal muscle, liver and brain tissue samples were harvested from six mice (*Mus musculus* C57BL6; female*)* 12 months of age, six domestic pigs (*Sus scrofa domesticus*; mixed gender) approximately 6 months of age and six humans (*Homo sapiens*; mixed gender*)* ranging in age from 64 to 104 years. Mouse tissue was harvested immediately following euthanasia by CO_2 inhalation. Pig tissue was harvested immediately following the death of each animal at the Wollondilly Abattoir (Picton, New South Wales, Australia). Human tissue was provided by the University of Wollongong body donation program (ethics number HE12/373). The post-mortem interval for human tissue was between three to seven days with the bodies maintained at 2–4 ^{o}C. Skeletal muscle was harvested from the intercostal muscle of all three mammals. Human liver was harvest by percutaneous liver biopsy. Similar regions of mouse and pig liver were taken following removal of the whole liver. Brain was harvested from humans through a burr hole approximately 20 mm superior to the external acoustic meatus by means of a surgical drill and a cutting biopsy needle. A similar region of the brain was harvested from mice and pigs following decapitation.

6.3.3 Subcellular fractionation and lipid extraction

Approximately 100 mg of tissue was extracted in 10% homogenising solution containing 20 mM Tris buffer, 250 mM sucrose and 2 mM EDTA, pH 7.4. The tissue was homogenated in glass-glass homogenizers and the homogenate was centrifuged (10 min, 1,000 x *g*) to give a pellet containing nuclei and large cellular debris. Supernatant was collected and centrifuged (35 min, 10,000 x *g)* to produce a mitochondria-enriched pellet. All centrifugation steps were performed at 4°C. Characterization of the mitochondrial fraction was performed in preliminary work using lamb brain using cytochrome *c* as a mitochondrial marker and Na^+/K ATPase as a marker for microsomal membrane. This assessment found a high level of cytochrome *c* but low levels of Na^+/K ATPase in the mitochondrial fraction but low levels of cytochrome *c* in the microsomal fraction. Mitochondrial pellets were resuspended in milliQ H_2O and total protein content was determined. Protein concentrations were determined using a BCA assay. Aliquots containing 75 µg of total protein and 50 µL of an internal standard mixture were added to 2 mL chloroform: methanol (2:1 v/v with 0.01% BHT). Phospholipid internal standards consisted of 20 µM each of PC 19:0/19:0, PE 17:0/17:0, and PS 17:0/17:0: and 10 µM lyso-PC 17:0 and lyso-PE 14:0 in chloroform: methanol were purchased from Avanti Polar Lipids through Auspep (VIC, Australia). Lipids were extracted using a modified Folch method as previously described in Deeley *et al* (2008) [225]. Lipid extracts were reconstituted in 1 mL chloroform:methanol (1:2 v/v) and stored at -20°C until analysed.

6.3.4 Mass spectrometry and bioinformatics analysis

Nano-electrospray ionization mass spectrometry of lipid extracts was performed using a hybrid triple quadrupole linear ion trap mass spectrometer (QTRAP® 5500 AB Sciex, MA, USA) equipped with an automated chip-based nano-electrospray source (TriVersa Nanomate™, Advion Biosciences, NY, USA). Samples were diluted to approximately 10 µM for total phospholipid, and 50 µl of diluted sample was spiked with 50 µl of ammonium acetate in chloroform and methanol (1:2 v/v) for a final concentration of 5 mM of ammonium acetate. Samples were loaded onto 96–well plates. Plates were centrifuged (10 min, 2200 x *g*) prior to direct infusion. Spray parameters were set at a gas pressure of 0.4 psi and a voltage of 1.2 kV and 1.1 kV for positive and negative ion mode respectively for all acquisitions.

Phospholipid data was acquired by targeted ion scans using multi-channel acquisition. All scans used are listed in Appendix A. As a result of the ammonium acetate, phospholipids of all classes were protonated $(M+H)^+$ in the positive ion mode, phospholipids of the PC class formed acetate ions $(M+OAc)^-$ in the negative ion mode, and phospholipids of PE, PS, PG and PA formed anions $(M-H)^-$ in the negative ion mode. Target lists for these charged phospholipids were made using the Glycerophospholipid MS/MS Prediction tool (www.lipidmaps.org) and converted to targeted ion lists for use in Lipidview™. Phospholipids of the PC, PE and PS classes were quantified from internal standards using positive ion scans for head groups while phospholipids in the PG and PA classes were quantified from internal standards using negative precursor ion scans for fatty acids. Phospholipid molecular species were determined from positive and negative scans was using custom-written spreadsheets in Microsoft Excel 2010 (Microsoft Corporation, Redmond, USA). Phospholipids were first quantified at the sum composition level, e.g. PC 34:1 from head group scans before the molecular level composition was determined, e.g. PC 16:0_18:1. Negative precursor ion scans were also used to quantify phospholipid isobaric species within classes. Isobaric phospholipid species containing either alkyl ethers or vinyl ethers (plasmalogens) could not be differentiated using this method and therefore the sum of alkyl ethers and plasmalogens were reported. Processing settings in LipidView™ software were set at a mass tolerance of 0.5 Da, with a minimum intensity of 0.1% and a minimum signal-to-noise ratio of 10. Phospholipid species that comprised less than 0.5% of each phospholipid class across the tissue were present in less than 66% of the tissue samples were removed from analysis. Values are normalized relative to total concentration of phospholipid in each phospholipid class. Phospholipid structure was reported using the nomenclature proposed by Liebisch *et al.* [74].

6.3.5 Fatty acid composition

Membrane fatty acid composition was calculated from quantified molecular phospholipid species using custom Excel calculators. The fatty acid composition of the combined phospholipid classes (PC, PE, PS, PG, and PA) were calculated, and the fatty acid composition of PC, PE and PS were calculated for each class. The sum of saturated fatty acids (SFA) included ether-linked species. As double bond position with fatty

acids could not be determined using the method of analysis, all fatty acids are reported without omega-designations.

6.3.6 The peroxidation index

The peroxidation index was calculated from membrane composition as (% MUFA x 0.025) + (% PUFA with 1 bis-allylic methylene group x 1) + (% PUFA with 2 bis-allylic methylene groups x 2) + (% PUFA with 3 bis-allylic methylene groups x 4) + (% PUFA with 4 bis-allylic methylene groups x 6) + (% PUFA with 5 bis-allylic methylene groups x 8) based on experimental values from [21]. This gives a peroxidation index per 100 fatty acids. The relative contribution of each phospholipid class to the peroxidation index was calculated as the peroxidation index of each class multiplied by the relative abundance of each class. This calculation gave the relative contribution of each class as a percentage of the total peroxidation index. Similarly, the contributions of molecular phospholipids to the peroxidation index were calculated from he peroxidation index and relative abundance of molecular phospholipids

6.3.7 Statistical analysis

Two-way ANOVAs (animal x measure) with Bonferroni post-hoc tests were used for all comparisons. All statistical analyses were completed using GraphPad Prism 5.04 (GraphPad Software, San Diego, USA).

6.4 Results

6.4.1 Phospholipid composition of skeletal muscle mitochondria

Greater than 85% of phospholipids found in the mitochondria of skeletal muscle were phosphatidylcholines (PC) and phosphatidylethanolamines (PE), 10% were phosphatidylserines (PS) and less than 5% were phosphatidylglycerols (PG) and phosphatidic acids (PA) (Figure 6.1A). Phosphatidylinositol (PI) was not examined due to a PI standard not being available for use. Mice and pigs possessed a lower percentage of PC and a higher percentage of PE than humans. No significant difference was found in the relative distribution of PS between species. PA was present in mouse muscle mitochondria but was absent in human and pig muscle. The muscle mitochondria of mice possessed a much higher percentage of phospholipids containing multiple bis-

allylic methylene groups than humans or pigs (Figure 6.2A). This occurred throughout the phospholipid classes and included PC 16:0_20:4 with three bis-allylic methylene groups, and PC 16:0_22:6, PE 18:0_22:6 and PS 18:0_22:6 which each have five bis-allylic methylene groups. In contrast, human and pig mitochondria had much lower levels of these highly peroxidative phospholipids but had a higher abundance of phospholipids containing 18:2, the PUFA containing only one bis-allylic methylene group. These phospholipids included PC 16:0_18:2 and PE 18:0_18:2. Mouse and pig muscle shared similar levels of phospholipid resistant to peroxidation such as PC 16:0_18:1 with humans having more of these peroxidation resistant phospholipids, including PC 16:0_18:1 as well as PC 18:1_18:1.

6.4.2 Phospholipid composition of liver mitochondria

The relative distribution of phospholipid classes in liver mitochondria was similar to that found for muscle mitochondria, with PC, PE and PS accounting for the majority (>90%) of phospholipids (Figure 6.1B). The percentages of PC, PE and PS did not differ between mice and humans whereas pigs possessed a lower percentages of PC and a higher percentage of PE than mice or humans. PS was also significantly higher in mice than in the pigs. Mice and humans shared a similar high percentage of PE 18:0_20:4, whereas mice and pigs shared a high level of PS 18:0_20:4. Mice tended to possess phospholipids with more bis-allylic methylene groups than humans (Figure 6.2B). Mice and humans shared similarly high levels of peroxidation-resistant PC 16:0_18:1 compared to pigs. Overall, differences between species were smaller in the mitochondria of liver than found in skeletal muscle.

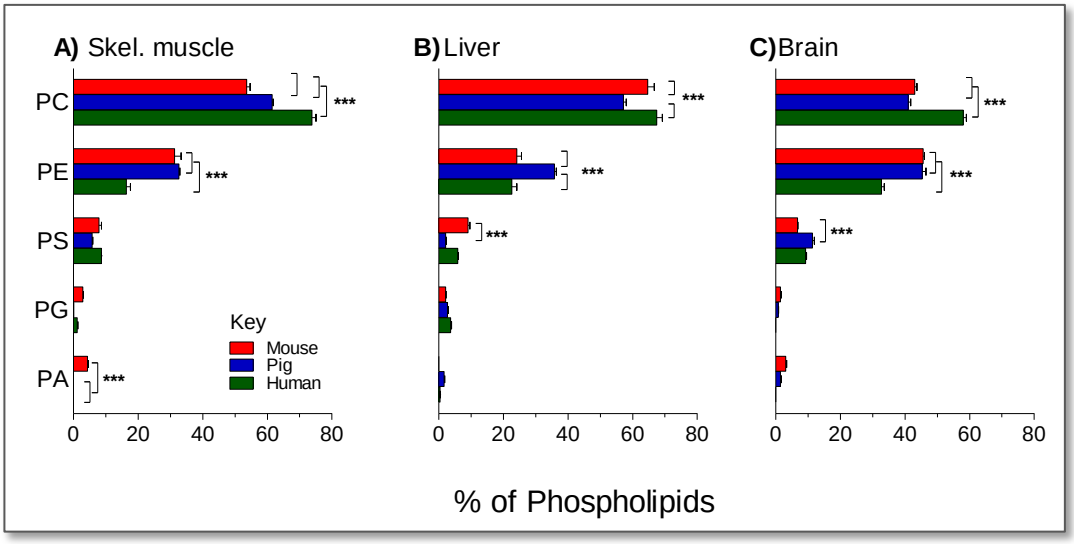

Figure 6.1. Distribution of phospholipid classes in A) skeletal muscle mitochondria, B) liver mitochondria and C) brain mitochondria of mice (*M. musculus*), pigs (*S. scrofa*) and humans (*H. sapiens*). Values are presented as mean mol% of total phospholipids ±SEM, *n*=6. Significant differences are indicated with *** for *P*<0.001. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol PA, phosphatidic acid.

6.4.3 Phospholipid composition of brain mitochondria

The distribution of PC and PE phospholipids in brain mitochondria was very similar in mice and pigs, whereas humans had significantly more PC and less PE (Figure 6.1C). The only other major difference was that PS was statistically lower in the mitochondria of mice than pigs. The phospholipids with the most bis-allylic methylene groups in brain mitochondria were species of PE and PS (Figure 6.2C). Of these, mice brain contained a higher percentage of PE 16:0_22:6 but similar levels of PE 18:0_22:6 to humans. Pigs had less PE 16:0_22:6 than mice and less PE 18:0_22:6 than mice and humans. Interestingly, humans had more PS 18:0_22:6 than the other two species. Two of the most common phospholipids in brain mitochondria were the peroxidation resistant PC 16:0_16:0 and PC 16:0_18:1, with mice having more PC 16:0_16:0 and humans more PC 16:0_18:1.

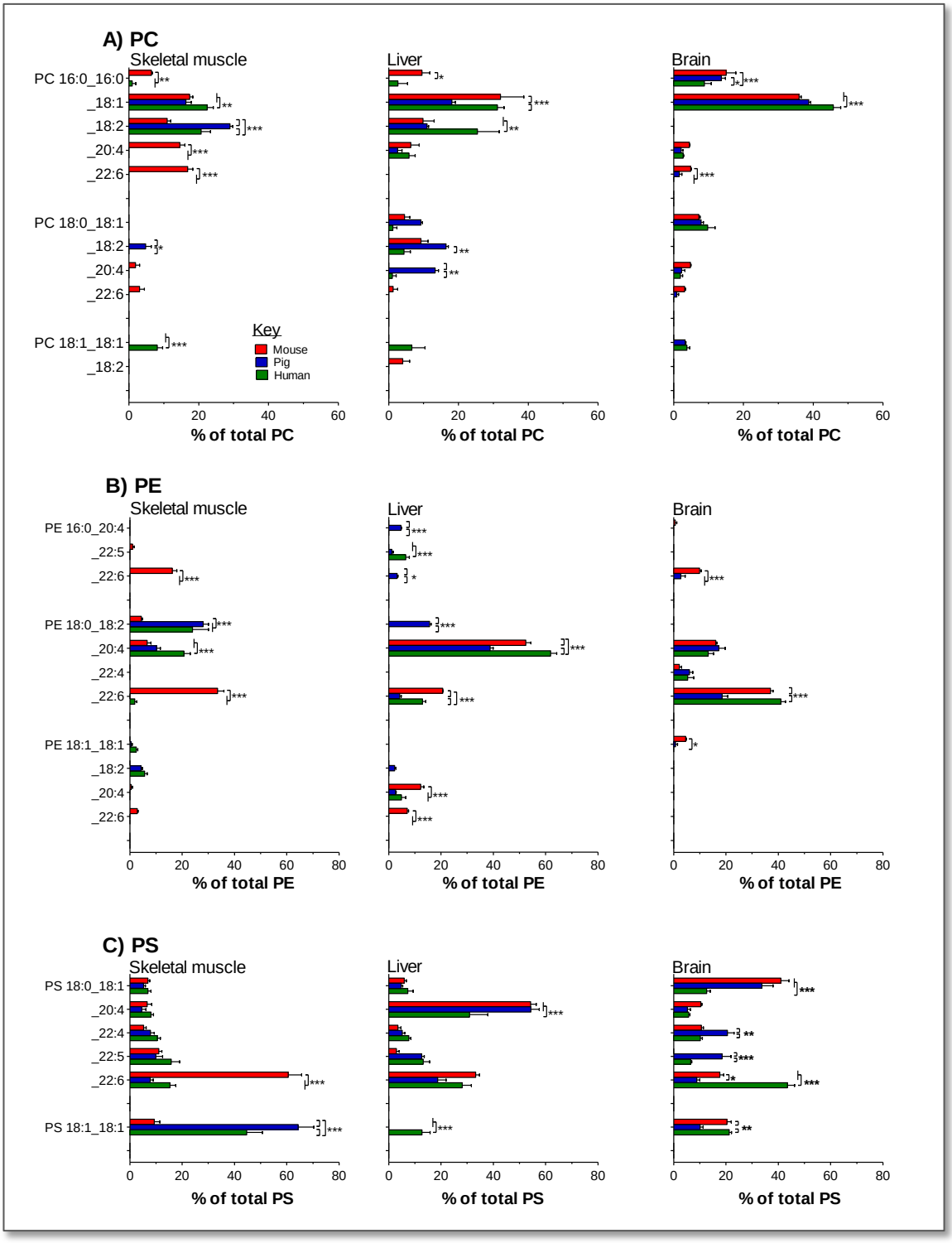

Figure 6.2. Common phospholipid compositions for A) phosphatidylcholine (PC), B) phosphatidylethanolamine (PE), and C) phosphatidylserine (PS) of mitochondria isolated from the tissues of mice (*M. musculus*), pigs (*S. scrofa*) and humans (*H. sapiens*). Values are presented as mean mol% of total phospholipids in each class ±SEM, *n*=6. Significant differences are indicated with * for $P<0.05$, ** for $P<0.01$ and *** for $P<0.001$.

6.4.4 Fatty acid composition by class

The fatty acid composition of total phospholipids (PC, PE, PS, PG, and PA) for muscle, liver and brain mitochondria of mice, pigs, and humans are shown in Table 6.1. The most common types of fatty acids in the combined phospholipid fraction were saturated fatty acids (SFA) including alkenyl ethers and vinyl ethaers (plasmalogens), which comprised 39–46% of fatty acids in muscle mitochondria, 44–45% in liver mitochondria, and 50–54% in brain mitochondria. The mitochondria of mice and pigs contained around 13–20% monounsaturated fatty acids (MUFA), but the mitochondria of humans had a higher percentage of MUFA. Mouse and pig mitochondria contained higher percentages of polyunsaturated fatty acids (PUFA) in muscle, liver, and brain compared to humans. Mouse mitochondria invariably contain higher percentages of 22:6, the fatty acid with the most bis-allylic methylene groups, than either pigs or humans. For all tissues, mouse mitochondria contained a higher percentage of 22:6. Pig mitochondria contained more 18:2 in muscle, 22:5 in liver and brain, and 22:4 in brain. All species possessed high levels of 20:4 in all tissues.

Table 6.2 shows the PUFA composition of the major phospholipid classes (PC, PE, and PS) in each tissue for each species. In most cases, PE contained the highest percentage of PUFA and the highest levels of 22:6, with the only exception being PS in liver. Muscle mitochondria in mice were unique as it was the only tissue in which 22:6 was present at high percentages in PC, PE and PS. Human mitochondria contained a lower percentage of PUFA than mice or pigs in PC, PE, and PS. Of the tissues examined, liver mitochondria showed the smallest difference in total PUFA and 22:6 content between the three animals. In most tissues, values for pig mitochondria were intermediate between values for mouse and human mitochondria. Interestingly, PUFA was lower in the PC (3.4%) and higher in the PE (54%) of human brain mitochondria than in any other tissue mitochondria examined.

As alkyl ethers and plasmalogens could not be differentiated and were reported together. These ethers were most commonly found in the PE phospholipids of mitochondria, and were lower in the mitochondria of muscle and brain of mice than in humans and pigs (Table 6.3).). Alkyl ethers and plasmalogens were largely absent in the PC and PS phospholipids for all tissue (<3%) with the exception of pig muscle.

Table 6.1. Acyl composition (mol%) of the combined phospholipids of mitochondria from mouse, pig, and human tissues

	Skeletal Muscle			Liver			Brain		
	Mouse	Pig	Human	Mouse	Pig	Human	Mouse	Pig	Human
Fatty acid									
16:0	26.0±0.7^{P}	19.2±0.4^{M,H}	25.9±2.5^{P}	23.5±1.9^{P}	14.3±0.3^{M,H}	27.8±2.5^{P}	23.9±1.6^{P}	19.6±1.2^{M,H}	23.9 ±1.8^{P}
18:0	19.4±0.9^{P,H}	12.6±0.8^{M}	10.5±1.2^{M}	20.5±1.0^{P}	28.8±0.6^{M,H}	16.6±1.5^{P}	26.3±1^{H}	24.7±0.6	22.5 ±1.4^{M}
18:1	10.9±0.7^{P,H}	15.2±0.9^{M,H}	25.5±1.3^{M,P}	18.0±0.9	15.0±0.7^{H}	21.0±2^{P}	18.5±0.4^{H}	19.1±0.9^{H}	24.5±1.0^{M,P}
18:2	7.8±0.8^{P,H}	30.5±1^{M,H}	20.4±1.2^{M,P}	12.7±1.8	13.2±0.5	12.3±1.6	0.3±0.1	0.6±0.2	0.3±0.1
20:4	8.3±0.8	7.2±0.6	6.5±1.3	16.9±2.5	16.9±0.8	13.2±1.9	8.2±0.1^{H}	7.0±0.7	4.3±0.2^{M}
22:4	0.9±0.4	1.4±0.2	0.8±0.2	0.7±0.2	0.9±0.1	0.5±0.1	1.9±0.1^{P}	6.3±0.6^{M}	5.1±0.5
22:5	3.8±0.6	1.3±0.3	1.6±0.5	0.1±0.0	2.7±0.2	1.8±0.4	1.6±0.3^{P}	6.2±0.6^{M}	2.5±0.4
22:6	19.6±1.4^{P,H}	0.6±0.1^{M}	1.4±0.2^{M}	5.3±0.5	3.0±0.3	2.9±0.4	14.3±0.3^{P,H}	8.2±0.4^{M}	9.9±0.9^{M}
ΣSFA	46.2±0.8^{P,H}	39.7±0.4^{M}	39.2±1.4^{M}	44.5±1.7	44.0±0.4	45.6±1.3	54.4±0.4^{P}	50.1±0.9^{M}	51.3±0.8
ΣMUFA	13.0±0.5^{H}	15.5±0.8^{H}	28.4±1.3^{M,P}	18.5±1.0	16.5±0.8^{H}	23.2±1.9^{P}	18.6±0.4^{H}	20.0±0.9^{H}	26±1.2^{M,P}
ΣPUFA	40.4±0.7^{H}	41.0±0.9^{H}	30.7±2.0^{M,P}	35.6±1.8	36.7±0.7^{H}	30.7±2.9^{P}	26.2±0.4^{H}	28.4±0.7^{H}	22.1±1.2^{M,P}

Values are mean mol% of the combined phospholipid fraction ±SEM, *n*=6. Superscript indicates significant differences of mean within the corresponding tissue at $P<0.05$: ^{M}, mouse (*M. musculus)*; ^{P}, pig (*S. scrofa)* and; ^{H}, human (*H. sapiens*). Combined acyl composition was calculated from quantified molecular species of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, and phosphatidic acid of mitochondria. Acyls less than 3 mol% of all tissues are not listed but have been included in all calculations. ∑SFA, total saturated fatty acids including acyl ethers; ∑MUFA, total monounsaturated fatty acids; ∑PUFA, total polyunsaturated fatty acids.

Table 6.2. Polyunsaturated fatty acid composition (mol% of total fatty acids) in mitochondrial phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine.

	Skeletal muscle			Liver			Brain		
	Mouse	Pig	Human	Mouse	Pig	Human	Mouse	Pig	Human
Phosphatidylcholine									
18:2	10.9±1.0^{P,H}	32.1±1.0^{M,H}	21.8±1.2^{M,P}	18.7±2.2	16.3±0.4	16.9±2.7	0.6±0.1	0.5±0.2	0.5±0.1
20:4	10.8±0.9^{P,H}	4.1±0.2^{M}	4.1±0.9^{M}	8.2±1.0	9.8±0.6^{H}	4.5±1.3^{P}	5.4±0.2^{H}	2.8±0.8	2.8±0.2^{M}
22:4	0.5±0.2	0.5±0.2	0.4±0.2	0.0±0.0	0.2±0.0	0.0±0.0	0.0±0.0^{P}	1.6±0.6^{M,H}	0.0±0.0^{P}
22:5	0.9±0.2	0.4±0.1	0.9±0.3	0.0±0.0	1.9±0.2	0.8±0.2	0.0±0.0	0.0±0.0	0.0±0.0
22:6	11.2±1.1^{P,H}	0.2±0.1^{M}	0.1±0.0^{M}	2.0±0.5	1.6±0.2	1.0±0.3	4.1±0.2^{P,H}	2.3±0.3^{M,H}	0.0±0.0^{M,P}
ΣSFA	49.5±0.5^{P,H}	42.5±0.4^{M}	39.8±1.5^{M}	45.2±2.8	46.0±0.3	46.7±2.0	64.6±0.4^{P,H}	61.5±0.7^{M,H}	57.4±1.3^{M,P}
ΣMUFA	15.9±0.5^{H}	18.0±0.9^{H}	31.4±1.2^{M,P}	24.0±2.9	21.0±0.9	29.6±2.9	24.1±0.4^{P,H}	30.8±0.7^{M,H}	38.4±1.0^{M,P}
ΣPUFA	34.2±0.6	37.3±1.0^{H}	27.4±2.1^{P}	28.9±2.1	29.7±0.7	23.1±2.7	10.0±0.0^{H}	7.1±0.5^{H}	3.4±0.3^{M,P}
Phosphatidylethanolamine									
18:2	6.7±2.1^{P}	28.3±1.5^{M}	19.3±3.0	0.0±0.0	10.1±0.5	0.0±0.0	0.0±0.0	1.0±0.5	0.0±0.0
20:4	9.6±4.7	13.6±1.4	16.5±1.7	38.2±3.7	25.8±0.6	38.6±3.1	12.4±0.3	12.8±1.3	7.6±0.9
22:4	1.7±0.7	3.5±0.5	3.3±0.8	2.6±0.8	2.0±0.2	1.4±0.6	3.4±0.3	10.2±0.7	14.8±1.6
22:5	7.6±1.7	3.3±0.6	4.0±1.2	0.0±0.0	3.6±0.2	4.3±1.2	3.8±0.7	9.7±0.9	6.8±1.1
22:6	23.4±5^{P,H}	0.4±0.1^{M}	1.1±0.4^{M}	9.3±2.9	4.4±0.5	5.2±1.4	26.8±0.5	15.6±1.0	24.8±1.3
ΣSFA	43.0±1.6^{P,H}	33.3±1.6^{M}	36.4±1.2^{M}	41.7±1.8^{H}	43.6±0.4	47.0±0.8^{M}	43.6±0.5	39.8±1.5^{H}	43.8±0.7^{P}
ΣMUFA	7.6±1.7^{H}	10.0±0.7	15.5±2.5^{M}	8.4±1.8^{H}	7.7±0.5^{H}	2.5±0.8^{P,H}	9.6±0.6^{H}	7.8±1.5^{H}	2.1±0.6^{M,P}
ΣPUFA	49.0±0.6	49.2±0.8	44.2±2.2	50.0±0.0^{P}	45.9±0.2^{M}	49.5±0.2	46.2±0.9^{H}	49.3±0.9^{H}	53.9±1.0^{M,P}
Phosphatidylserine									
18:2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
20:4	2.7±0.9	20.0±0.7	3.4±0.8	27.1±1.1^{H}	27.2±1.6^{H}	15.5±3.4^{P,M}	5.2±0.2	2.6±0.5	2.9±0.2
22:4	2.7±0.5	4.8±1.1	5.3±0.6	1.8±0.5	2.6±0.5	3.8±0.4	5.3±0.4^{P}	10.3±1.3^{M,P}	5.1±0.4^{P}
22:5	5.5±0.6	4.2±1.3	7.9±1.6	1.5±0.5	6.3±0.5	6.6±1.2	0.0±0^{P,H}	9.3±1.7^{M,H}	3.3±0.2^{M,P}
22:6	30.3±2.6^{P,H}	3.3±0.8^{M}	7.7±1.1^{M}	16.6±0.7^{P}	10.1±1.6^{M}	14.1±1.7	8.9±0.7^{P,H}	4.5±0.5^{M,H}	21.8±1.3^{M,P}
ΣSFA	45.3±1.1^{P,H}	17.0±2.6^{M}	27.7±3.0^{M}	50.0±0^{H}	49.3±0.1	43.6±1.5^{M}	39.8±0.8	4.9±0.5^{H}	39.4±0.4^{P}
ΣMUFA	13.4±2.5^{P,H}	68.9±5.1^{M,H}	48.1±6.1^{M,P}	3.0±0.4^{H}	4.6±0.4^{H}	16.4±3.7^{M,P}	40.9±0.7^{P,H}	28.4±2.6^{M}	27.6±1.2^{M}
ΣPUFA	41.2±1.5^{P,H}	14.2±2.5^{M}	24.2±3.1^{M}	47.0±0.4	46.1±0.3	40.0±2.2^{P}	19.3±0.9^{P,H}	26.6±2.4^{M,H}	33.1±0.9^{M,P}

Values are mean mol% of the phospholipid classes, ±SEM, *n*=6. Superscript indicates significant differences of mean within the corresponding tissue at *P*<0.05: ^{M}, mouse (*M. musculus)*; ^{P}, pig (*S. scrofa)*, and; ^{H}, human (*H. sapiens*). ∑SFA, total saturated fatty acids including acyl ethers; ∑MUFA, total monounsaturated fatty acids; ∑ PUFA, total polyunsaturated fatty acids.

Table 6.3. Alkenyl ether and vinyl ether composition (mol% of total acyls, alkenyl ethers, and vinyl ehters) present in the combined phospholipids, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine fractions of mitochondria.

	Skeletal muscle mitochondria			Liver mitochondria			Brain mitochondria		
	Mouse	Pig	Human	Mouse	Pig	Human	Mouse	Pig	Human
Combined PL	0.4±0.1^{P,H}	7.8±0.6^{M,H}	2.1±0.3^{M,P}	0.5±0.2	0.8±0.1	1.1±0.3	0.4±0.2^{P,H}	3.2±0.4^{M}	3.2±0.6^{M}
Phosphatidylcholine	0.0±0.0^{P,H}	10.0±0.7^{M,H}	2.2±0.3^{M,P}	0.0±.0.0	0.4±0.0	0.9±0.3	0.0±0.0^{H}	0.0±0.0^{H}	2.6±0.7^{M,P}
Phosphatidylethanolamine	1.0±0.3^{P,H}	3.6±0.4^{M}	2.8±0.5^{M}	2.6±0.8	1.3±0.1	2.3±0.7	0.9±0.4^{P,H}	6.9±0.7^{M}	5.3±1.0^{M}
Phosphatidylserine	0.7±0.3	0.0±0.0	0.0±0.0	0.0±0.0	1.5±0.2	0.0±0.0	0.0±0.0	1.4±0.4	0.0±0.0

Values are mean mol% of acyls of the phospholipid fraction, ±SEM, *n*=6. Superscript indicates significant differences of mean within the corresponding tissue at $P<0.05$: ^{M}, mouse (*M. musculus)*; ^{P}, pig (*S. scrofa)*, and; ^{H}, human (*H. sapiens*). Acyl ethers represents the sum of acyls identified as plasmalogens and alkyl ethers, but may also include odd-chain fatty acids. Combined phospholipid composition (combined PL) was calculated from quantified molecular species of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, and phosphatidic acid fractions of mitochondria.

6.4.5 The contributions of phospholipid class to peroxidation index

The peroxidation index of muscle, liver and brain mitochondria was higher in mice than in humans, with this difference greater in muscle than liver and brain (Figure 6.3A). The muscle mitochondria of mice had a peroxidation index 3 times that of humans, and the peroxidation index of liver and brain mitochondria was higher in mice than humans. The peroxidation index for pigs were more similar to those of humans for muscle, but more similar to mice for liver and brain. Tissue-dependent differences in the peroxidation index were not consistent between mice, humans and pigs. The peroxidation index of mouse muscle mitochondria was nearly two times that of mouse liver and brain mitochondria. In contrast, the peroxidation index of humans muscle was lower than liver or brain. Overall, the peroxidation index of pigs was more similar to humans than mice.

The relative contributions of PC, PE and PS to the peroxidation index were more similar between animals than between tissues (Figure 6.3B). The relative contributions of PC and PE phospholipids to the total peroxidation index were comparable in muscle mitochondria, but PE was a larger contributor in liver mitochondria and the dominant contributor in brain mitochondria where it accounts for over 80% of the total peroxidation index. Interestingly, the relative contribution of PC to the total peroxidation index of brain mitochondria decreased with MLS while the relative contribution of PS increased.

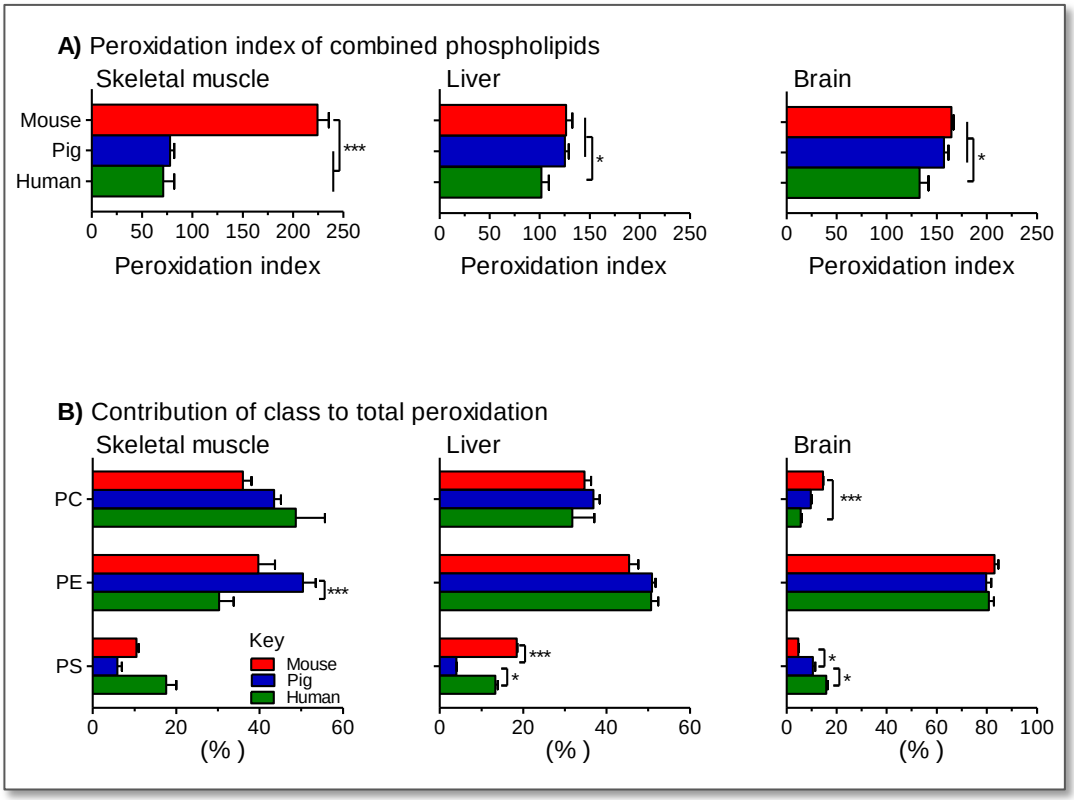

Figure 6.3. The peroxidation index of A) the total phospholipid fractions and B) the contribution of phospholipid class as a percentage of the peroxidation index in muscle, liver and brain mitochondria of mice (*M. musculus*), pigs (*S. scrofa*) and humans (*H. sapiens*). The contributions of each phospholipid class to peroxidation index was calculated as the peroxidation index of each class multiplied by the percentage of each class (Figure 6.1). Values are means ±SEM, n=6. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine. Significant differences are indicated with * for P<0.05, ** for P<0.01 and *** for P<0.001.

6.5 Discussion

6.5.1 Peroxidation, membrane composition, and maximum life span

An inverse relationship has been found between maximal lifespan (MLS) and membrane peroxidation index for mammals and other animal classes [12, 37-39]. In this study, shotgun lipidomics was used to examine this relationship at the level of molecular phospholipids and fatty acid composition in the mitochondria of muscle, liver, and brain. This chapter is one of the most in-depth studies of the phospholipid composition of mammalian mitochondrial membranes published to date. The peroxidation index of muscle mitochondria of mice and humans was found to fit the trends for whole skeletal muscle [29], and the peroxidation indexes for liver mitochondria were similar to published trends [45]. The peroxidation index of brain

mitochondria from mice and humans has not been previously described, but would seem to fit with the differences found in other tissues of mice and humans with high values for mice and lower values for humans. The peroxidation values of pig mitochondria were similar to mice for liver and brain mitochondria but more similar to humans for skeletal muscle mitochondria. Overall, the peroxidation index of pigs was lower than expected for muscle and liver [29, 34], but the membrane composition of pig mitochondria is consistent with past studies of membrane composition in pig muscle that used gas chromatography to determine membrane composition [226].

The higher peroxidation values of mice mitochondria were due to both a higher percentage of PUFA and a higher number of bis-allylic methylene groups per PUFA than for humans. Muscle mitochondria of mice contained a high percentage of 22:6, the PUFA with the most bis-allylic methylene groups and therefore the PUFA most vulnerable to peroxidation. In comparison, human muscle mitochondria contained a high percentage of 18:2, the PUFA with the least number of bis-allylic methylene groups and therefore less susceptible to peroxidation. A similar difference was found in brain mitochondria, but not in liver. No difference in PUFA profile was seen between liver mitochondria of mice and humans, and differences in the peroxidation index were due to the higher percentage of total PUFA in mice than humans. In contrast, pig mitochondria had a higher percentage of total PUFA but a similar PUFA profile to human mitochondria. These results support past findings that the percentage of 22:6 is lower in the muscle and liver of longer-lived mammals [40, 220].

Differences in the peroxidation indexes of each tissue varied by species. Mice had a higher peroxidation index in the muscle mitochondria than liver or brain mitochondria while humans and pigs had a lower peroxidation index in muscle and liver mitochondria than brain mitochondria. The primary PUFA in muscle mitochondria was 22:6 in mice but 18:2 in humans and pigs, which partly explained the large differences seen in the peroxidation indexes of muscle mitochondria. The primary PUFA in liver was 20:4, but 22:6 was also found at low levels in all animals. This contrasts to the only previous investigation of human liver mitochondria which reported no 22:6 [138], but supports the finding that membrane composition varies less between species in liver than muscle [220]. This may be explained by the ability of liver to regenerate liver, and it is possible that the peroxidation of membrane lipids has less long-term ramifications to cell health in liver compared to post-mitotic tissues such as skeletal muscle and brain.

The primary PUFA in brain mitochondria was 22:6, which has previously been found to be at similar levels in a range of mammalian species [220] suggesting that peroxidation index of brain is high and comparable in mammals. In this study, however, the peroxidation index of mitochondria in the mouse brain was greater than that of humans. As peroxidation is associated with numerous pathologies, including neuronal diseases [227], the high 22:6 content found in brain has the potential to be detrimental to long-term survival. An alternative view proposed by Yavin *et al.* (2002) suggests that 22:6 may not be as susceptible to peroxidation might be expect from its structure [228]. It is also possible that 22:6 may protect the brain from inflammation by serving as a precursor for resolvins [229]. In the current study, a negative association was found between the 22:6 content of brain mitochondria and lifespan for the combined phospholipid, PC, and PE. Brain PS was found to contain more 22:6 in humans than mice or pigs, suggesting a possible positive correlation in PS, but overall these results support the view that the 22:6 content of brain is inversely correlated with MLS. The enzymatic peroxidation of PUFA, however, is involved in a broad range of cell signalling pathways and is required for healthy brain function [8]. For example, the loss of 22:6 in brain is associated with severe brain anomalies including Zellweger syndrome [230] and Alzheimer's disease [231]. As a speculative hypothesis for the difference in the peroxidation indexes of muscle and brain mitochondria, I propose that that muscle membranes are exposed to more ROS than brain membranes, and that oxidative damage to muscle is more detrimental to organism survival than oxidative damage to the central nervous system.

6.5.2 Contributions of phospholipids to the peroxidation index

For all three mammals, PC and PE had relatively equal contributions to the peroxidation index of muscle mitochondria, but the relative contribution of PE was the dominant contributor in liver and brain mitochondria. PUFA are generally less common in the PC classes than the PE or PS classes [20], and the high ratio of PC to PE present in the mitochondria of human muscle and brain may represent an adaptation to limiting membrane peroxidation independently of fatty acid composition. The significance of the distribution of PUFA across phospholipid to peroxidation remains to be established as data in this area is uncommon and often contradictory due to differences in methods used [15, 202].

The peroxidation index was largely determined by a surprisingly low number of molecular phospholipids (Figure 6.4). The greatest difference between animal species was the contributions of PC 16:0_22:6 and PE 18:0_22:6 in mice muscle mitochondria and the contributions of PC 16:0_18:2 and PC 18:0_18:2 in pig and humans mitochondria. The higher percentages of PC 16:0_22:6 and PE 18:0_22:6 found in the muscle of mice in comparison to pigs or humans supports is similar to comparisons between mice and naked mole rats (*Heterocephalus glaber*, MLS of 28 years) [40, 44]. As PC 16:0_22:6 is synthesized *de novo* by mammals but PE 18:0_22:6 is not [86], mammals with longer lifespans may have adapted to decrease the peroxidation index of muscle membranes by decreasing the *de novo* synthesis of PC 16:0_22:6, increase remodelling of PC 16:0_22:6 to other molecular species of PC, and limiting the synthesis of PE 18:0_22:6 during remodelling.

This replacement of PUFA susceptible to peroxidation with the less susceptible 18:2 seen in muscle was not found in liver mitochondria where the phospholipids with the largest contribution to the peroxidation index was PE 18:0_20:4 in all species, or in brain mitochondria where the greatest contributor was PE 18:0_22:6 for all three species. The high contribution of PE to the peroxidation index in brain has been previously identified as being due to high levels of 22:6, but had not previously been identified as being due to the presence of a single molecular species [80]. Despite having a potentially high risk of undergoing peroxidation, the percentage of PE 18:0_22:6 in the mitochondria of healthy adult human brain remains relatively constant during normal adult aging [232] and 22:6 has been even been identified as having a possible role in protecting brain from ROS damage by some researches [228].

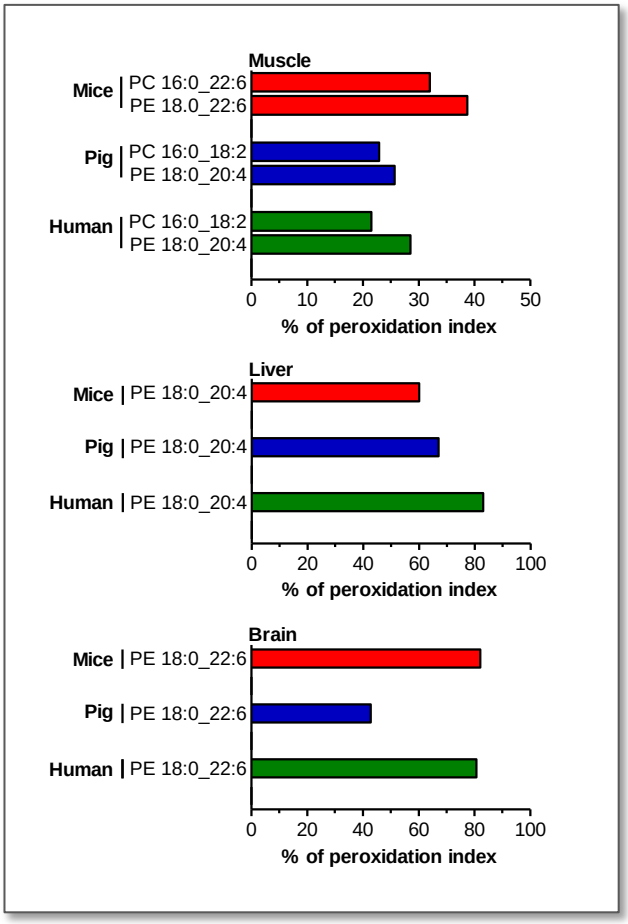

Figure 6.4. Molecular phospholipids with the largest contribution to the peroxidation index of mitochondria from mouse (*M. musculus*), pig (*S. scrofa*) and human (*H. sapiens*) tissues. The percentage of peroxidation index is determined from the peroxidation index and relative abundance of molecular phospholipids.

6.5.3 Importance of non-peroxidising phospholipids

The most abundant peroxidation-resistant phospholipid found in mitochondria was PC 16:0_18:1 in all tissues, suggesting that this phospholipid may be important to membrane function. As PC 16:0_18:1 is one of the few molecular phospholipids that mammalian cells can assemble *de novo*, high levels may indicate that comparatively limited phospholipid remodelling of this phospholipid is taking place [86]. The advantages of PC 16:0_18:1 include a lower melting point than phospholipids containing SFA [24] and an increased resistance to peroxidation in comparison to phospholipids containing PUFA [21, 73]. These benefits are similar to those of non-methylene interrupted PUFA associated with long-lived molluscs [37]. Another interesting feature of PC 16:0_18:1 is its apparent capacity to delay the onset of peroxidation by extending the lag phase of peroxidation as described in Chapter Five. It

is likely that PC 16:0_18:1 serves as a substitute for phospholipids containing PUFA and therefore may be a useful biomarker of membrane resistance to oxidative damage.

Phospholipids with vinyl-ethers at the *sn*-1 position (commonly termed plasmalogens) may also increase membrane resistance by acting as a sacrificial target for ROS [233, 234]. Plasmalogens have been positively associated with longevity in mammals [40] but not in consistently in molluscs [37], suggesting that the link between longevity and the presence of plasmalogens in membranes remains unclear. In the current study, isobaric alkyl ethers and plasmalogens could not be differentiated as the method used cannot identify the position of bonds and therefore the vinyl ether of plasmalogens cannot be differentiated from an alkyl ether that contains a double bond elsewhere along it's chain. This is an important distinction as only the vinyl ether inhibits the loss of the phosphoethanolamine head group, and therefore the method underestimated PE-plasmalogens when using a neutral loss of 141 to identify PE phospholipids [235]. The present study used the conservative approach of reporting the sum of alkyl ethers and plasmalogens together without using a correction factor for plasmalogens [40, 236]. The sum of alkyl ethers and plasmalogens comprised <3% of the PE fraction of mitochondria and were largely absent from the PC and PS fractions. An exception to this finding was the high percentage of alkyl ethers in the PC fraction of pig muscle mitochondria. This is possibly a case of incorrect identification because odd-chain fatty acids cannot be distinguished from alkyl ethers or plasmalogens with the present mass spectrometry method with complete certainty. However, odd-chain fatty acids were not reported in a review of studies of pig tissues [226]. Overall, the mitochondria of muscle and brain contained a lower percentage of alkyl ethers and plasmalogens in mice than in pigs or humans, suggesting that alkyl ethers and plasmalogens may be positively associated with MLS in mammals.

6.5.4 Limitations

Although the use of human tissues is very valuable, working with human tissues presents several difficulties including the precious nature of the samples and the inevitable post-mortem interval prior to lipid extraction. The average post-mortem interval of the present study was four days. Lipid composition has been reported to show only minor changes with post-mortem intervals of three days [237] and five days [238]. We are not aware of any studies examining lipid degradation over longer-post-

mortem intervals, although such a study would be useful. The relative stability of membrane lipids during the post-mortem interval suggests that the post-mortem interval of the human tissue used in this study was unlikely to greatly affect the overall conclusions. The inclusion of human tissue in this study also limited the amount of sample available to approximately 100 mg per sample, resulting in mitochondrial yield that was sufficient for the analysis but did not allow for extensive purification steps.

A further limitation of the present study is that cardiolipin (CL), a phospholipid class that comprises 10-15% of mitochondrial phospholipids [17], was not able to be measured with the methods used. CL is a di-phospholipid with a highly conserved fatty acid composition of CL 18:2/18:2/18:2/18:2 in mammalian tissues [79] that is considered to be important in lipid-proteins interactions with proteins of the eletron transport (ETC) chain and early apoptotic evens [165, 166, 169, 239]. CL as been reporeted to have a similar contribution to the peroxidation index across a range of mammal species [80, 138], and although the composition of CL can be influenced by dietary fatty acids and may therefore differ between species (Chapter Three) this is not expected to be the case for mice or pigs and is unlikely in humans. The absence of CL from the analysis underestimates the peroxidation index of each animal, and the values presented may be lower than reported values generated with alternative methods for mammalian muscle and liver [12, 29]. This underestimation is unlikely to greatly affect the large differences in peroxidation index between species.

An additional consideration of this study (and many aging studies) is the comparison of animals at very different stages of their MLS. The mice examined in the present study had reached 25% of the MLS, the pigs examined had reached only around 2% of their MLS, and humans varied from 50-80% of their MLS. The phospholipid composition of mammalian membranes does appear to change gradually over the adult lifespan. In human brain, small increases in phospholipids containing 22:6 and small decreases in phospholipids containing 20:4 were found with increasing age, but the percentage of mitochondrial PE 18:0_22:6 was found to be consistent across a wide range of ages [232]. An increase in the 22:6 content and peroxidation index with age has also been reported for mouse muscle membranes [145], but overall differences in the membrane PUFA composition and peroxidation index across a species lifespan seem minor in comparison to differences between species with very different lifespans.

6.6 Conclusion

This work has shown that the mitochondria of muscle, liver and brain of mice are highly susceptible to peroxidation while the tissues of pigs and humans are less susceptible to peroxidation. Human membranes differed from mouse and pig membranes by containing a lower percentage of PUFA, suggesting that this may be a protective form of lipid remodelling. Somewhat surprisingly, a relatively small number of molecular phospholipids that contributed the majority of the peroxidation index in each tissue. The differences in these molecular species varied across tissues and suggests that differences in lipid remodelling in muscle may be particularly important for lifespan. Overall, this study shows that the link between membrane susceptibility and peroxidation is more complex than PUFA content and the peroxidation index. Other elements of membrane composition found to be associated with lifespan were the percentages of alkenyl ethers and plasmalogens, the ratio of PC to PE, and the percentage of peroxidation resistant-phospholipids such as PC 16:0_18:1. These findings supported the 'membrane-pacemaker' theory of aging and the importance of peroxidation as a mechanism of aging. Future work in this area would benefit from examining these measures across a wider range of mammals and other animal classes.

Chapter Seven

Is human longevity due to a unique ability to limit the peroxidation of mitochondrial membranes?

7.1 Overview

The damage to mitochondria caused by peroxidation is associated with aging, and it has been suggested that humans have the unusual ability to exclude highly peroxidisable phospholipids from their mitochondrial membranes. Past comparisons of the peroxidation index of mitochondrial and microsomal membranes of human tissues has shown high variability, and no single study has examined both fractions using the same methods. This chapter presents a shotgun lipidomics analysis of mitochondrial and microsomal membranes from skeletal muscle, liver, and brain from humans. Mice and pig tissues were also examined to determine if differences in mitochondrial and microsomal fractions were unique to humans.

7.2 Introduction

The maximal lifespan (MLS) of humans (*Homo sapiens*) is 122 years, which is 3–4 fold longer than other mammal species with similar body masses (AnAge database, Figure 2.10). No single explanation for human longevity has yet been universally accepted, but one possible explanation is that human membranes are resistant to the damage of cellular components caused by peroxidation [4, 65]. Peroxidation is initiated by reactive oxygen species (ROS) and primarily affects the bis-allylic methylene groups found in polyunsaturated fatty acids (PUFA) present in membranes [14, 36, 240]. The relative vulnerability of membranes to peroxidation, termed a peroxidation index, can be calculated from the percentage of membrane PUFA, the number of bis-allylic methylene groups present in this PUFA, and experimentally derived rates of peroxidation [35]. Chapter Six of this thesis found that the mitochondrial membranes of skeletal muscle, liver and brain contained a lower percentage of PUFA in humans than in mice (*Mus musculus*, MLS of 4 years) or pigs (*Sus scrofa*, MLS of 27 years). As a result, the mitochondria of these organs had a lower peroxidation index in humans than in mice or pigs, suggesting that human mitochondria are less susceptible to peroxidation than mouse or pig mitochondria. An inverse relationship between MLS and the peroxidation

index has also been reported in a variety of other mammals [29], birds [12], molluscs [37], bees [38], and strains of *Caenorhabditis elegans* [39] suggesting a strong link between longevity and membrane composition.

The composition of mitochondrial phospholipid is of particular importance to age-related peroxidation as mitochondria are a major source of ROS within cells [14, 36] and mitochondrial failure is an indicator of aging [118]. Based on differences in the membrane fatty acid composition reported for whole liver [241-243] and liver mitochondria [138], Hulbert (2010) suggested that humans exclude highly peroxidisable fatty acids from their mitochondrial membranes to prevent peroxidation damage and extend longevity [45, 240]. Unfortunately, human data in this area is rare and highly variable due to the differences in methods used by different laboratory groups. In the few studies available, the peroxidation indexes calculated for whole cell membranes were 110 [241], 170 [242] and 280 [243], while the peroxidation index calculated for mitochondria was 50 [138]. If these findings are correct, mitochondria are far less susceptible to peroxidation than other cellular membranes in humans, but not in other exceptionally long-lived mammals or in mammals with MLS close to those predicted for their mass, [45, 134, 244, 245]. On this basis, it was hypothesized that 1) mitochondrial membranes have a lower peroxidation index than other cellular membranes in human tissues and 2) there is no difference in the peroxidation index between mitochondrial membranes and other cellular membranes in other mammals.

To test these hypotheses, this chapter used shotgun-lipidomics to compare the membrane composition and peroxidation index of mitochondrial and microsomal fractions from humans, mice (*Mus musculus*, MLS of 4 years) and pigs (*Sus scrofa*, MLS of 27 years). Microsomal fractions, which contain the phospholipids from the plasma membrane, endoplasmic reticulum (ER), Golgi apparatus, secretory vesicles and endosomes [246], represent an average cell membrane in the absence of mitochondria. Mice and pigs were chosen as examples of mammals that have lifespans similar to other mammals of their mass (AnAge Database) and therefore serve as control species for exceptionally long-lived humans.

7.3 Methods

The samples and methods used in this chapter are described in Chapter Six (6.3. Methods). In addition to these methods, a microsomal pellet and supernatant were

isolated from the supernatant by centrifuging at 100,000 x *g* for 40 min at 4 ^{o}C [246]. In the present study, two-way ANOVAs (tissue x fraction) with Bonferroni post-hoc tests were used to compare mitochondrial and microsomal fractions within each species for each tissue GraphPad Prism 5.04 (GraphPad Software, San Diego, USA).

7.4 Results

7.4.1 Phospholipid composition

The distribution of phospholipid classes in the mitochondrial and microsomal membranes of skeletal muscle, liver and brain are shown for mice, pigs and humans in Table 7.1. In skeletal muscle, the percentage of phosphatidylcholine (PC) was higher in the mitochondrial fraction than the microsomal fraction for humans and mice but lower in the mitochondrial fractions than the microsomal fraction for pigs. The percentage of phosphatidylethanolamine (PE) did not differ significantly between the mitochondrial and microsomal fractions for humans and mice, but was higher in the mitochondrial fraction than the microsomal fraction for pigs. No significant differences were found between the mitochondrial and microsomal fractions for phosphatidylserine (PS), phosphatidylglycerol (PG) or phosphatidic acid (PA). Phospholipid composition did not differ greatly between the mitochondrial and microsomal fractions of mouse or pig skeletal muscle, but some differences were seen for human muscle (Table 7.2). The percentage of PC 16:0_16:0 was higher in the mitochondrial fraction than the microsomal fraction in mice and the percentage of PE 18:0_18:2 was higher in the mitochondrial fraction than the microsomal fraction of pigs, but no other significant differences were observed. In humans, the percentages of PC 16:0_18:1, PC 18:0_18:2 and PE 18:0_18:2 were lower in the mitochondrial fraction than the microsomal fraction. The full list of phospholipids identified in the mitochondrial and microsomal fraction of skeletal muscle is presented in Appendix Table B1.

In liver, the only difference in the percentages of phospholipid classes was a statistically higher level of PE in pig mitochondrial membranes (Table 7.1). Within classes, however, liver contained more differences in the relative amounts of molecular phospholipids between fractions than seen for muscle (Table 7.2). For mice, higher percentages of PC 16:0_16:0, PC 16:0_18:1, PE 18:0_20:4 and PS 18:1_18:1 were seen in mitochondria compared to microsomes, but a far lower percentage of PE 16:0_22:6 was seen in mitochondria. Similarly, pigs were found to have a higher percentage of PE

16:0_20:4 but a lower percentage of PE 18:0_18:2 in their mitochondria than their microsomes, and human mitochondria contained a higher percentage of PE 18:0_20:4 than human microsomes. The full list of phospholipids identified in the mitochondrial and microsomal fraction of liver is presented in Appendix Table B12.

For the brain mitochondrial and microsomal fractions, PE was higher in the mitochondrial fraction of both mice and pigs, PC was higher in the mitochondrial fraction of humans, and PA was lower in the mitochondrial fraction for all three animals (Table 7.1). No significant differences were seen for the other phospholipid classes. Multiple differences were seen in the relative abundance of molecular phospholipids of brain (Table 7.2). A higher percentage of PC 16:0_16:0 was found in the mitochondrial fraction than the microsomal fraction of mouse brain while the percentages of both PE 18:0_22:5 and PE 18:0_22:6 were higher in the mitochondrial fraction than the microsomal fraction of human brain. The percentage of PS 18:0_18:1 was higher in the mitochondrial fraction than the microsomal fraction of mice and pigs, and the percentage of PS 18:1_18:1 was higher in the mitochondrial fraction than the microsomal fraction of pigs and humans. Differences in the distribution of peroxidisable species of PS were mixed, with the percentage of PS 18:0_20:4 higher in the mitochondrial fraction of mouse, the percentage of PS 18:0_22:5 higher in the mitochondrial fraction of pigs but the percentage of PS 18:0_22:6 lower in the mitochondrial fraction of pigs. The full list of phospholipids identified in the mitochondrial and microsomal fraction of brain is presented in Appendix Table B13.

Table 7.1. Distribution of phospholipid classes in the mitochondrial and microsomal fractions of mouse, pig, and human tissues.

		Mouse		Pig		Human	
		Mitochondrial	Microsomal	Mitochondrial	Microsomal	Mitochondrial	Microsomal
Muscle	PC	62.1±0.4	53.6±2.7*	61.5±0.7	68.6±2.8**	73.8±3.0	65.4±2.7**
	PE	27.7±0.5	31.3±5.2	32.6±0.6	18.6±2.1***	16.3±3.1	21.0±2.1
	PS	6.1±0.5	8.0±1.8	5.8±0.6	7.4±2.4	8.7±0.2	12.5±0.7
	PG	1.2±0.2	2.9±0.5	0.2±0.0	0.4±0.1	1.2±0.2	0.8±0.3
	PA	3.0±0.2	4.3±0.8	0.0±0.0	5.1±2.1	0.0±0.0	0.3±0.2
Liver	PC	64.6±5.0	65.3±1.7	57.1±2.2	60.4±2.0	67.5±3.8	66.5±2.4
	PE	24.2±3.6	27.8±1.9	35.8±1.6	30.3±1.4*	22.7±3.4	23.9±1.2
	PS	9.1±1.6	4.1±0.3	2.1±0.6	2.9±0.2	5.9±0.4	5.3±0.7
	PG	2.2±0.2	0.9±0.1	2.7±0.7	4.5±1.3	3.6±0.6	4.0±0.8
	PA	0.0±0.0	2.0±0.2	1.7±0.4	2.0±0.2	0.4±0.1	0.3±0.1
Brain	PC	43.0±1.6	43.9±2.6	41.0±1.8	39.1±1.0	58.0±2.3	37.4±2.8***
	PE	45.6±0.9	34.0±1.4***	45.4±2.3	29.3±2.1***	32.7±2.2	36.2±1.5
	PS	6.7±0.3	4.8±0.5	11.4±1.2	15.3±1.0	9.3±0.7	14.8±1.5
	PG	1.5±0.4	5.6±0.9	0.8±0.3	4.4±0.8	0.0±0.0	0.0±0.0
	PA	3.2±0.6	11.7±2.7***	1.5±0.7	1.9±3.4***	0.0±0.0	1.6±1.3***

Species examined were mice (*M. musculus)*, pigs (*S. scrofa)* and humans (*H. sapiens*). Values are presented as mean mol% of total phospholipids ±SEM, n=6. Significant differences between mitochondrial and microsomal fractions are indicated with * for P<0.05, ** for P<0.01, and *** for P<0.001. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol PA, phosphatidic acid.

Table 7.2. Significant differences in the acyl composition of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) between the mitochondrial and microsomal membranes of mouse, pig, and human tissue.

				Mol%		
Tissue	Animal	Class	Phospholipid	Mitochondrial	Microsomal	P
Muscle	Mouse	PC	PC 16:0_16:0	6.4±0. 3	0.0±0.0	*P*< 0.0001
		PE	None	–	–	–
		PS	None	–	–	–
	Pig	PC	None	–	–	–
		PE	PE 18:0_18:2	28.0±2.1	16.2±3.5	*P*< 0.05
		PS	None	–	–	–
	Human	PC	PC 16:0_18:1	22.4±1.8	29.4±1.9	*P*< 0.05
			PC 18:0_18:2	20.7±2.7	26.9±2.7	*P*< 0.05
		PE	PE 18:0_18:2	0.0±0.0	7.1±0.6	*P*< 0.05
		PS	None	–	–	–
Liver	Mouse	PC	PC 16:0_16:0	9.5±2.3	0.0±0.0	*P*< 0.05
			PC 16:0_18:1	32.0±6.7	14.1±0.8	*P*< 0.0001
		PE	PE 16:0_22:6	0.0±0.0	31.5±1.3	*P*< 0.0001
			PE 18:0_20:4	64.4±8.3	20.8±0.6	*P*< 0.0001
		PS	PS 18:0_18:1	6.0±0.8	0.1±0.1	–
	Pig	PC	None	–	–	–
		PE	PE 16:0_20:4	4.7±0.2	0.0±0.0	*P*< 0.001
			PE 18:0_18:2	15.6±0.6	19.5±1.9	*P*< 0.01
		PS	None	–	–	–
	Human	PC	None	–	–	–
		PE	PE 18:0_20:4	69.5±7.8	50.6±5.7	*P*< 0.05
		PS	None	–	–	–
Brain	Mouse	PC	PC 16:0_16:0	15±2.8	4.9±2.1	*P*< 0.0001
		PE	None	–	–	–
		PS	PS 18:0_18:1	41.0±3.1	30.8±3.0	*P*< 0.01
			PS 18:0_20:4	10.4±0.4	0.0±0.0	*P*< 0.01
			PS 18:1_18:1	20.4±1.6	12.1±3.0	*P*< 0.05
	Pig	PC	None	–	–	–
		PE	None	–	–	–
		PS	PS 18:0_18:1	33.7±4.2	4.7±0.6	*P*< 0.0001
			PS 18:0_22:6	8.9±1.1	47.1±3.9	*P*< 0.0001
			PS 18:0_22:5	18.5±3.4	9.1±0.9	*P*< 0.05
	Human	PC	None	–	–	
		PE	PE 18:0_20:4	13.9±1.8	6.9±0.9	*P*< 0.05
			PE 18:0_22:6	38.5±2.8	25.3±1.3	*P*< 0.001
		PS	PS 18:1_18:1	12.7±1.3	8.4±1.1	*P*< 0.05

Species examined were mice (*M. musculus)*, pigs (*S. scrofa)* and humans (*H. sapiens*). Values are presented as mean mol% of total phospholipids in each class ±SEM, *n*=6. Significant differences between fractions are indicated with * for *P*<0.05, ** for *P*<0.01 and *** for *P*<0.001.

7.4.2 Fatty acid composition

Although phospholipid composition varied between tissues and between species, the fatty acid compositions of the mitochondrial and microsomal fractions within tissues were generally similar (Table 7.3). In skeletal muscle, small but significant differences were seen in the 18:0 content of mice and the 16:0 content of pigs, but no differences were seen for humans. In liver, the mitochondrial fraction of mice contained a higher percentage of 18:1, a lower percentage of 22:6, but a higher percentage of PUFA overall. Similar patterns were not seen in the mitochondrial fraction of pigs or humans, although pig mitochondria had a slightly higher percentage of 20:4. In brain, 16:0 was higher in the mitochondrial fraction of all three animals, 18:0 was higher in the mitochondrial fraction of pigs, and 18:1 was higher in the mitochondrial fraction of mice. Overall, MUFA was lower in the mitochondrial fraction of pig brain, but higher in the mitochondrial fraction of human brain. Acyl ethers were also not found to differ significantly between mitochondrial and microsomal membranes in any tissue.

Table 7.3. Acyl composition (mol%) of the combined phospholipids of mitochondrial and microsomal fractions of mouse, pig, and human tissue.

		Mouse		Pig		Human	
	Fatty acid	Mitochondrial	Microsomal	Mitochondrial	Microsomal	Mitochondrial	Microsomal
Muscle	16:0	26.0±0.7	27.7±0.5	19.2±0.4	23.0±2.1*	25.9±2.5	26.3±1.2
	18:0	19.4±0.9	16.3±0.4**	12.6±0.8	11.6±0.6	10.5±1.2	9.7±0.6
	18:1	10.9±0.7	12.2±0.2	15.2±0.9	12.7±1.4	25.5±1.3	26.3±2.0
	18:2	7.8±0.8	9.0±0.5	30.5±1.0	32.6±0.9	20.4±1.2	23.5±1.4
	20:4	8.3±0.8	9.1±0.4	7.2±0.6	6.4±0.3	6.5±1.3	5.4±0.6
	22:6	19.6±1.4	18.5±0.8	0.6±0.1	0.9±0.4	1.4±0.2	1.5±0.2
	Acyl ethers	1.0±.0.2	0.4±.1	7.3±0.7	7.8±0.6	0.5±0.1	2.1±0.3
	∑SFA	46.2±0.8	45.5±0.4	39.7±0.4	41.8±1.1	39.2±1.4	36.8±1.0
	∑MUFA	13.0±0.5	13.3±0.2	15.5±0.8	13.0±1.5	28.4±1.3	29.7±2.1
	∑PUFA	40.4±0.7	40.1±0.5	41.0±0.9	42.1±0.8	30.7±2.5	32.5±1.8
Liver	16:0	23.5±1.9	26.4±1.3	14.3±0.3	13.9±0.5	27.8±2.5	29.5±2.4
	18:0	20.5±1.0	16.3±0.9	28.8±0.6	29.4±0.7	16.6±1.5	15.6±1.5
	18:1	18.0±0.9	13.1±0.8*	15.0±0.7	17.2±0.7	21.0±2.0	18.6±2.4
	18:2	12.7±1.8	11.3±0.8	13.2±0.5	13.6±0.7	12.3±1.6	12.6±1.4
	20:4	16.9±2.5	13.9±0.6	16.9±0.8	14.2±1.0*	13.2±1.9	14.8±2.2
	22:5	0.1±0.0	1.1±0.2	2.7±0.2	2.5±0.2	1.8±0.4	1.5±0.4
	22:6	5.3±0.5	12.4±0.6**	3.0±0.3	4.7±0.5	2.9±0.4	3.4±0.8
	Acyl ethers	0.8±0.1	0.5±0.2	0.8±0.1	0.7±0.1	0.0±0.0	1.1±0.3
	∑SFA	44.5±1.7	43.5±0.8	44.0±0.4	44.2±0.6	45.6±1.3	45.1±1.2
	∑MUFA	18.5±1.0	13.8±0.9*	16.5±0.8	17.9±0.7	23.2±1.9	20.8±2.2
	∑PUFA	35.6±1.8	40.5±0.5**	36.7±0.7	35.8±0.8	30.7±2.9	34.1±3.0
Brain	16:0	23.9±1.6	18.1±1.4**	19.6±1.2	15.2±1.7*	23.9±1.8	15.0±1.3**
	18:0	26.3±1.0	29.9±0.8*	24.7±0.6	25.1±0.9*	22.5±1.4	25.7±1.3
	18:1	18.5±0.4	14.2±1.1**	19.1±0.9	21.6±1.1	24.5±1.0	22.2±1.2
	20:4	8.2±0.1	7.8±1.4	7.0±0.7	7.8±0.3	4.3±0.2	4.0±0.3
	22:5	1.6±0.3	3.6±0.7	6.2±0.6	5.5±1.3	2.5±0.4	6.0±1.7
	22:6	14.3±0.3	14.3±0.9	8.2±0.4	9.9±1.2	9.9±0.9	7.9±0.9
	Acyl ethers	1.5±0.4	0.4±.02	1.5±0.3	3.2±0.4	4.5±0.8	3.2±0.6
	∑SFA	54.4±0.4	55.1±2.0	50.1±0.9	43.7±1.5	51.3±0.8	48.9±0.6
	∑MUFA	18.6±0.4	14.7±1.2	20.0±0.9	26.7±1.5*	26.0±1.2	23.0±1.1**
	∑PUFA	26.2±0.4	27.3±1.2	28.4±0.7	28.4±0.5	22.1±1.2	23.8±1.9

Species examined were mice (*M. musculus*), pigs (*S. scrofa*) and humans (*H. sapiens*). s double bond positon could not be ascertained with the method used, fatty acids are reported as carbon: double bonds without omega position Values are mean mol% of the combined phospholipid fraction ±SEM, n=6. Significant differences between fractions are indicated with * for P<0.05, ** for P<0.01 and *** for P<0.001. Combined acyl composition was calculated from quantified molecular species of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, and phosphatidic acid of mitochondria. Acyls less than 3 mol% of all tissues are not listed but have been included in all calculations. ∑SFA, total saturated fatty acids including acyl ethers; ∑MUFA, total monounsaturated fatty acids; ∑PUFA, total polyunsaturated fatty acids.

7.4.3 Susceptibility to peroxidation

The peroxidation index of combined phospholipids did not differ greatly between mitochondrial and microsomal fractions of skeletal muscle, liver and brain for humans mice, or pigs (Figure 7.1). The only exception was mouse liver in which the peroxidation index was significantly lower in the mitochondrial fraction than the microsomal fraction. The contributions of phospholipid classes to the peroxidation index of the mitochondrial and microsomal fractions showed numerous but inconsistent differences (Figure 7.2). A higher contribution of PE was found in pig mitochondria for muscle, liver and brain, but this was the only difference that was consistent across tissues. For muscle, human had a higher contribution for PC but a lower contribution for PS in the mitochondrial the microsomal fraction and no differences were seen for mice (Figure 7.2A). In liver, no difference was seen for humans but a lower contribution of PC and a higher contribution of PS was seen in the mitochondrial that the microsomal fraction of mice (Figure 7.2B). Differences between the contributions to the peroxidation index of mitochondrial and microsomal fractions were most common in brain (Figure 7.2C). The relative contribution of PC to the peroxidation index was higher in the mitochondrial fraction than the microsomal fraction of brain for humans and pigs, but lower in the mitochondrial fraction of mice. The relative contribution of PE was higher in the mitochondrial fraction than the microsomal fraction of pig brain, and the contribution of PS was higher in the microsomal fraction of pig and human brain.

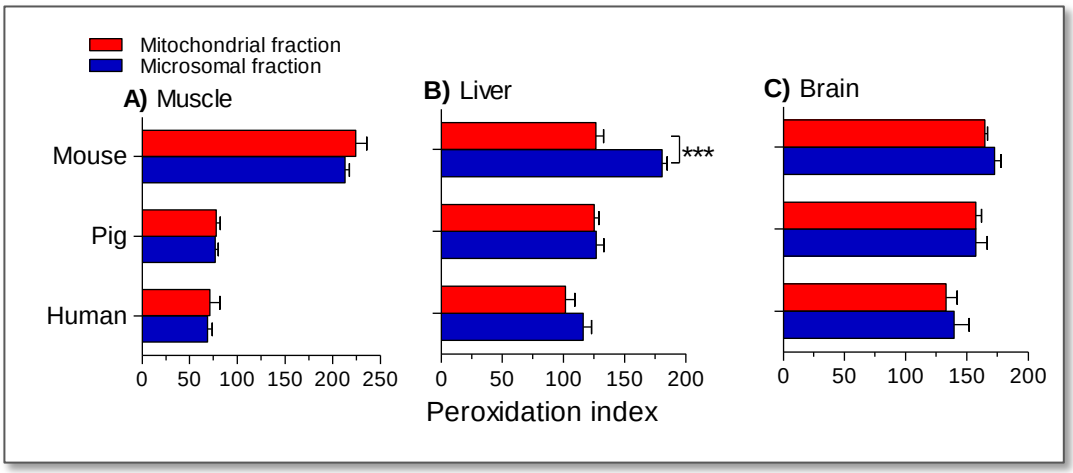

Figure 7.1. The peroxidation index of the in microsomal fraction and mitochondrial-enriched fraction of the A) skeletal muscle, B) liver, and C) brain of mouse (*M. musculus*), pigs (*S. scrofa*), and humans (*H. sapiens*). The peroxidation index is calculated using values from Holman [21]. Values are means ±SEM, n=6. Significant differences of P<0.001 are indicated with ***.

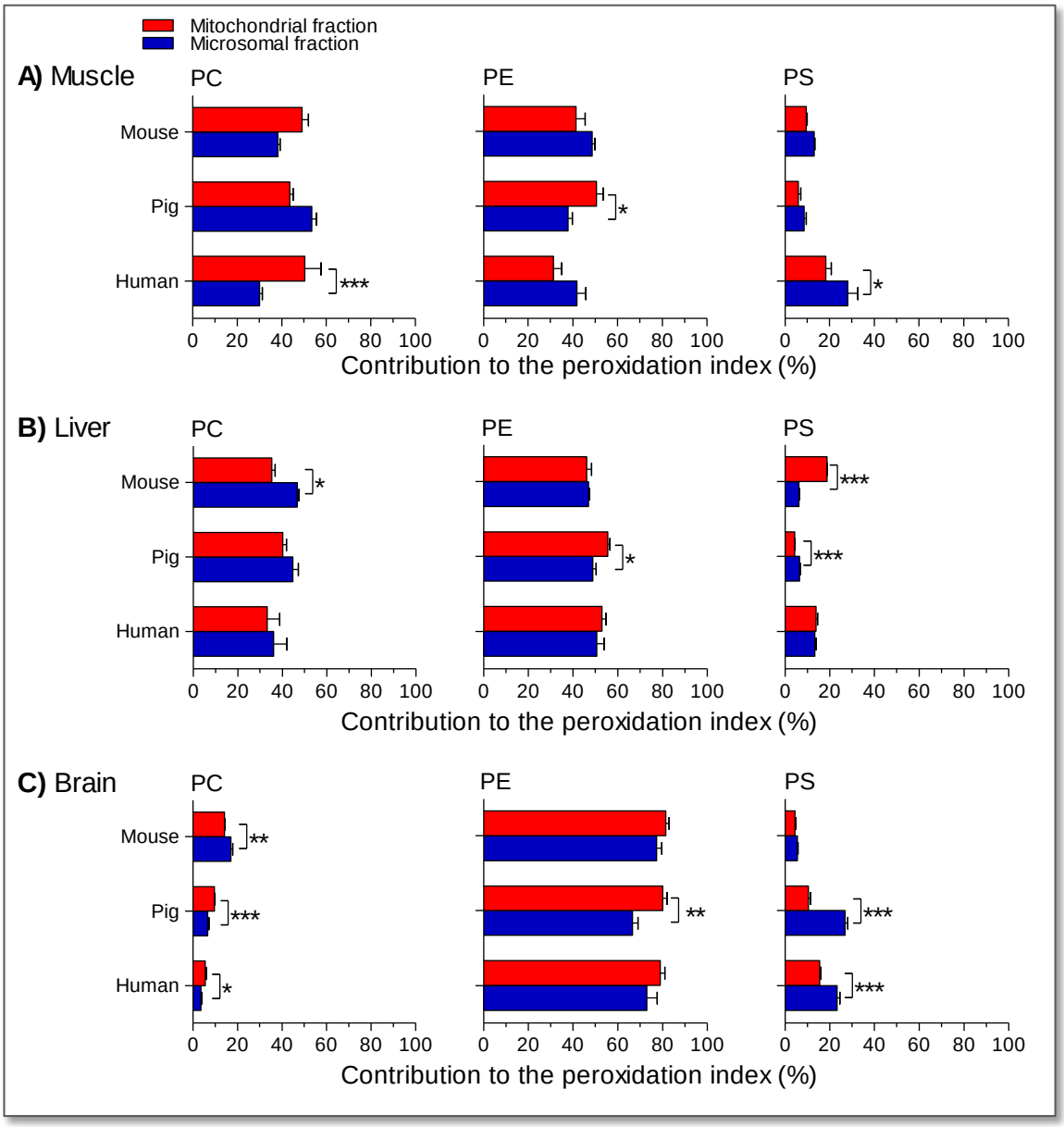

Figure 7.2. Relative contributions of the major phospholipid classes to the peroxidation index of the microsomal fraction and mitochondrial fraction of A) skeletal muscle, B) liver, and C) brain of mouse (*M. Musculus*), pigs (*S. scrofa*), and humans (*H. sapiens*). Values are means ±SEM, n=6. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine. Significant differences are indicated with * for P<0.05, ** for P<0.01 and *** for P<0.001.

7.5 Discussion

7.5.1 Composition of mitochondrial and microsomal membranes

Humans (*Homo sapiens*) have a maximal lifespans (MLS) of 122 years, which is far higher than the MLS of mammals with similar body masses (AnAge Database). The peroxidation of mitochondrial membranes is considered a major cause of aging [4, 65, 118], and one possible explanation for human longevity is that human mitochondria are less susceptible to peroxidation damage than expected for a mammal of their mass (Chapter Six). It has also been suggested that human mitochondria are less susceptible to peroxidation-related aging than other cellular membranes in humans [45, 240] and that this may be an evolutionary mechanism to extend longevity that is unique to humans [45, 134, 244, 245]. Unfortunately human data is rare in this area and only a limited number of studies from different laboratory groups have reported the fatty acid composition of whole liver [241-243] and liver mitochondria [138]. Unlike comparisons of this past work that found differences between the fatty acid composition and peroxidation index of liver mitochondria to whole liver cells [45, 240], the present study found few differences between the mitochondrial and microsomal fractions of muscle, liver and brain in humans. The peroxidation indexes of the mitochondrial and microsomal fractions did not differ significantly, with the peroxidation index of human liver mitochondria lower than previously reported [138] but the peroxidation index of human liver microsomes higher than previous reports for whole liver [241-243] (Figure 7.3.). Unlike humans, mice (*Mus musculus*, MLS of 4 years) and pigs (*Sus scrofa*, MLS of 27 years) are examples of mammals with MLS similar to those of other mammals of their body mass. Comparisons of the mitochondrial and microsomal membranes of, found similar peroxidation indexes between mitochondrial and microsomal fractions of skeletal muscle, liver, and brain for each animal. Overall, mammalian mitochondria and microsomal membranes were found to be similar in their susceptibility to peroxidation for humans, mice and pigs.

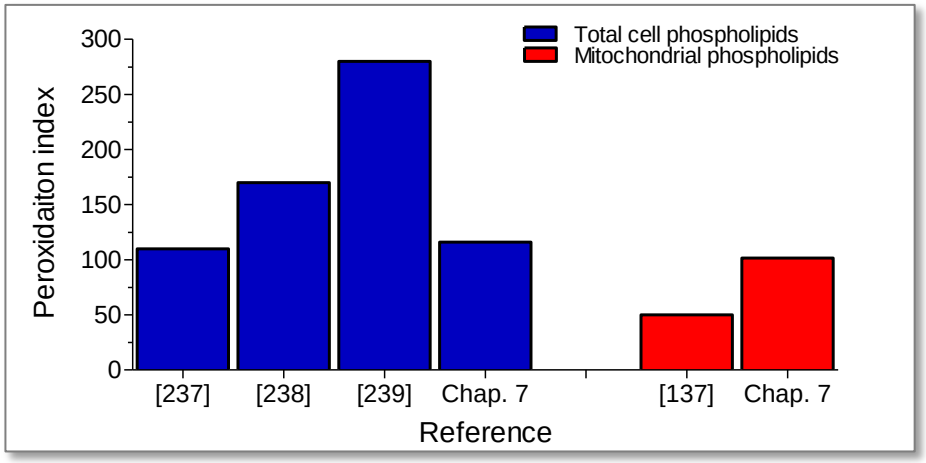

Figure 7.3. Comparison of the peroxidation index of human (*H. sapiens*) liver from this chapter with published values for humans.

A notable exception was mouse liver, where a higher peroxidation index was found for the microsomal fraction than the mitochondrial fraction. This difference was primarily due to the higher percentage of 22:6, a highly peroxidisable PUFA, in the microsomal fraction and may be related to 22:6n-3 synthesis. Standard mouse diets are generally low in long chain omega-3 fatty acids including 22:6n-3, and the primary source of 22:6 present in mouse tissues is 22:6n-3 synthesized in liver [247] by the fatty acid elongase 'elongation of very long-chain fatty acids type 2' (ELOVL–2) [248]. ELOVL–2 is highly expressed in mouse liver [248] where it is located in the endoplasmic reticulum (ER) [83], [88]. The ER is a major component of the microsomal fraction, and so high levels of 22:6n-3 synthesis in liver are likely to be reflected in a higher percentage of 22:6 and a higher peroxidation index found in the microsomal fraction than the mitochondrial fraction. A high level of 22:6n-3 synthesis in the microsomal fraction may also account for the higher percentage of PE 16:0_22:6 in mouse liver mitochondria as PE 16:0_22:6 is one of only four fatty acid compositions of PE that are synthesized *de novo* in mammals [86]. In comparison to mice, no difference was seen in the peroxidation index or percentage of 22:6 in liver mitochondrial and microsomal fractions for pigs or humans, suggesting that 22:6 synthesis is more common in mice liver than pig or human liver. This difference in 22:6 synthesis is likely to be related to the requirements of higher percentages of 22:6 in the tissues of mice than in the tissues of pigs or humans (Chapter Six).

The relative abundance of phospholipid classes did not differ significantly between the mitochondrial and microsomal fractions. The similarities found for

mitochondrial and microsomal PC and PS may reflect the common origin of these classes in the ER [82, 83]. The PC and PS produced in the ER are transported to mitochondrial and other cellular membranes either through vesicular pathways involving endosomal compartments and the Golgi apparatus or non-vesicular pathways using lipid transporter proteins [68]. Differences in the levels of PC found in muscle suggest some variation in how PC is imported into cellular membranes. Unlike PC and PS that must be imported into mitochondria, PE may be either imported from the ER or synthesised in mitochondria by the decarboxylation of PS [83]. The ability of mitochondria to synthesise PE independently of the ER may explain the high PE found in the mitochondrial fraction of all pig tissues and in mouse brain. This increase could also be associated with a higher importation of PE into mitochondria as no significant decrease in mitochondrial PS was observed. No difference in the levels of mitochondrial and microsomal PE was observed in other mouse or human tissues, suggesting pigs might be unusual in their regulation of PE levels. Mitochondria also synthesise phosphatidic acid (PA) and phosphatidylglycerol (PG) [83], but the only consistent finding in all three animals was a lower percentage of PA in the mitochondrial fraction of brain, which contrasts with a past report that phospholipid class was similar in the mitochondrial and microsomal fractions of rat brain [249]. The low amount of PA in mitochondria may be due a high degree of export to the microsomal membranes, or may be related to the role of PA as a substrate in the mitochondrial synthesis of PG and cardiolipin (CL) [83].

The mitochondrial and microsomal membranes of muscle, liver and brain generally contained similar molecular species of phospholipid. This is perhaps surprising considering that mitochondria possess different isoforms of enzymes for both fatty acid synthesis and phospholipid remodelling than the cytosol [17, 84]. The similarities between the phospholipid composition of the mitochondria suggest that fraction-specific enzyme isoforms do not greatly differ in their selectivity or function. Interestingly, in the few instances where molecular species of PC did differ between fractions, there was a trend for PC 16:0_16:0 and PC 16:0_18:1 to be higher in the mitochondrial fraction. As these phospholipids are synthesized *de novo*, this finding suggests that slightly less remodelling of PC is occurring in the mitochondria than in the microsomes [86]. No significant differences in percentages of PS as a class were found for any tissue, but the percentage of molecular species of PS did differ significantly in the mitochondrial and microsomal fractions of brain. The result of this asymmetrical

distribution was an increase in the percentage of two non-peroxidisable phospholipids, PS 18:0_18:1 in mice and pigs and PS 18:1_18:1 in humans. The increase in the percentages of PS 18:0_20:4 in mice and humans and PS 18:0_22:5 in pigs may be related to the role of PS oxidation in plasma membranes during pro-apoptotic signalling [250].

7.5.2 Phospholipid contributions to the peroxidation index

With the exception of mouse liver, the peroxidation indexes of mitochondrial and microsomal membranes did not differ greatly in mice, pig or human tissues. Greater differences between fractions were seen in the relative contribution of phospholipid classes to the peroxidation index, but these differences were not generally consistent within species or tissues. The relative contribution of phospholipid classes to the peroxidation index was determined by both the relative abundance of phospholipid class and distribution of molecular phospholipids within that class (Table 7.4). The largest differences in phospholipid distribution were higher levels of non-peroxidisable phospholipids such as PC 16:0_16:0 , PC 16:0_18:1, PS 18:0_18:1 and PS 18:1_18:1 in mitochondrial membranes compared to microsomal membranes. In addition to replacing phospholipids containing PUFA, two non-peroxidisable phospholipids, PC 16:0_16:0 and PC 6:0_18:1, have been found to exhibit an antioxidant-like activity (Chapter Five). It has yet to be determined if a similar antioxidant-like effect is caused by non-peroxidisable phospholipids such as PS 18:0_18:1 or PS 18:1_18:1.

Table 7.4. Contributions of phospholipid classes and molecular phospholipids to the peroxidation index in the mitochondrial fraction relative to the microsomal fraction of mouse, pig, and human tissues.

Tissue	Animal	Class	Contribution relative to microsomal fraction	Reason	
				Class	Phospholipid species
Muscle	Mouse	PC	–		
		PE	–		
		PS	–		
	Pig	PC	–		
		PE	Increased	Higher PE	Higher PE 18:0_18:2
		PS	–		
	Human	PC	Increased	Higher PC	None
		PE	Decreased	Lower PE*	None
		PS	Decreased	Lower PS*	None
Liver	Mouse	PC	Decreased		Higher PC 16:0_16:0, Higher PC 16:0_18:1
		PE	–		
		PS	Increased	Higher PS*	None
	Pig	PC	–		
		PE	Increased	Higher PE	Higher PE 16:0_20:4
		PS	Decreased		
	Human	PC	–		
		PE	–		
		PS	–		
Brain	Mouse	PC	–		
		PE	Increased	Higher PE	None
		PS	–		
	Pig	PC	Increased	Lower PE,PS*	
		PE	Decreased	Lower PE,	None
		PS	Decreased	Lower PS*	Higher PS 18:0_18:1
	Human	PC	Increased	Higher PC	None
		PE	–		
		PS	Decreased	Lower PS*	Higher PS 18:1_18:1

Species examined were mice (*M. musculus*), pigs (*S. scrofa*) and humans (*H. sapiens*).
Notes: *Not significant differences.

A difference in the percentages of phospholipid with alkenyl ethers and vinyl ethers (plasmalogens)between membranes may also indicate an adaptation to decrease peroxidation. Plasmalogens are phospholipids with vinyl-ether bonds at the *sn*-1 position, and this vinyl bond has been suggested to have an antioxidant-like action by acting as a sacrificial target for ROS [233]. Unlike other phospholipids, plasmalogens are synthesised in peroxisomes [83], and PE plasmalogens are transported into cellular membranes via a non-vesicular, ATP dependent pathway. The majority of alkenyl ethers and plasmalogens are present in the PE class. Plasmalogens in this class inhibit the loss of the phosphoethanolamine head group, and therefore the method used underestimated PE-plasmalogens when using a neutral loss of 141 to identify PE phospholipids [235]. A correction factor of 3.45 can be used to correct for this, but only if the plasmalogens can be differentiated from alkenyl ethers. This differentiation is not possible in the present study due to the methodology used, and so the present study took the conservative approach of reporting the sum of alkyl ethers and plasmalogens together without using a correction factor for plasmalogens [40, 236]. If a correction factor was used, the percentages of plasmalogens in human brain would be comparable to past findings [251]. In the present study, the percentage of alkenyl ethers and plasmalogens did not differ between mitochondrial and microsomal fractions of any tissue, and the high levels reported in the membranes of pig muscle mitochondria (Chapter Six) were also found in the membranes of pig muscle microsomes. These results suggest that acyl ethers do not confer additional protection to mammal mitochondria. This work contrasts to a previous finding of higher percentages of plasmalogens in mitochondrial membranes compared to other cell membranes of molluscs [37].

7.5.3 Limitations

This chapter shares the limitations addressed in Chapter Six (Section 6.5.4) with regards to the post-mortem interval of the human tissue used, the absence of cardiolipin (CL) from the present analysis, and the comparison of species from different stages of their MLS. As a result of CL not being included in the analysis, the peroxidation indexes of mitochondrial membranes presented in this study are an underestimation. This underestimation does not, however, affect the conclusion of this study as the data did not support the hypothesis that the peroxidation index of mitochondrial membranes is

lower than that of microsomal membranes in humans. In addition, the analysis shows the high degree of similarity in mitochondrial and microsomal membranes (with the exception of CL) and suggests that the inclusion of CL into mitochondria does not influence the composition of molecular phospholipids present in other classes within the membrane. This work is also limited to a small number of species, which prevents the use of linear slopes to compare trends in cellular fractions. Future work could address this limitation by extending the number of species examined. A number of caveats should also be applied to the isolation techniques used to separate the mitochondrial and microsomal fractions of tissues. The techniques used were not tissue specific, and it is possible that synapsosomes may have co-sedimented out with brain mitochondria. These membranes would not have been as abundant as mitochondrial membranes and are unlikely to have greatly affected mitochondrial purity but should still be considered in future studies.

7.6 Conclusion

The present study used shotgun lipidomics to compare the membrane composition and peroxidation index of mitochondrial and microsomal membranes of skeletal muscle, liver and brain from humans, mice and pigs. The membrane composition of both fractions were similar for all three species, which does not support the hypothesis that humans prevent mitochondrial damage by excluding highly peroxidisable phospholipids form mitochondria. This one of the first lipidomics studies of the membrane composition and peroxidation index of cellular fractions, and suggests that membrane composition differs more between tissues that cellular fractions in mammals. Future work in this area could examine other cellular fractions within mammal tissues or in long-lived animals of other classes.

Chapter Eight

Conclusion and outlook

8.1 Membrane composition, peroxidation and longevity

"It remains for us to discuss youth and age, life and death. To come to a definite understanding of these things would complete our study on animals."

Aristotle [1], 'On Longevity and Shortness of Life', Part 6.

Aristotle may have been optimistic in his belief that a "definite understanding" of life could ever be reached, and continuing work in this area has shown biological systems to be some of the most complex systems yet encountered. This thesis has focussed on one fundamental element of cellular structure, membrane glycerophospholipids, in an attempt to better understand the importance of peroxidation of membrane phospholipids to oxidative stress, cellular damage, and aging. This thesis contains three studies that used different methodologies. The first study examined the relationship between dietary fats and mitochondrial membrane composition in Chapter Three, the second study investigated the importance of phospholipid composition to peroxidation in Chapter Four and Chapter Five, and third study examined the relationship between the susceptibility of membrane phospholipids to peroxidation and species longevity in Chapter Six and Chapter Seven. This chapter summarises the outcomes and significance of these findings and suggests future directions for this work.

8.1.1 Cardiolipin composition is influenced by dietary fats

Cardiolipin (CL) is widely considered to be primarily composed of linoleic acid (18:2n-6). This is probably due the persistent citation of early reports that found a high content of 18:2n-6 in CL (reviewed in [20, 51]), and a high percentage of CL 18:2/18:2/18:2/18:2 in mammalian muscle [79]. Numerous published studies have reported that docosahexaenoic acid (22:6n-3) is selectively incorporated into CL, but these studies are frequently overlooked. The analysis of dietary data presented in Chapter Three reviews all available studies that have examined the influence of dietary fatty acids on CL composition in mice and rats. Interestingly, this review showed that when both 18:2n-6 and 22:6n-3 were present in the same diet, 22:6n-3 was incorporated

into heart CL at the expense of 18:2n-6. This occurred in heart when 22:6n-3 was up to ~20% of dietary fatty acids and in liver when 22:6n-3 is up to 10% of total dietary fatty acids, suggesting that the regulation of CL composition may differ between tissues. This finding contrasts to the general view that CL has a highly regulated 18:2n-6 content that is not influenced by diet, and suggests that molecular species of CL containing 22:6n-3 may be more common than previously considered. The incorporation of 22:6n-3 into CL and mitochondrial phosphatidylcholine (PC) and phosphatidylethanolamine (PE) reported in this analysis would greatly increase the peroxidation index of mitochondrial membranes, and may make mitochondria more susceptible to apoptosis induced by peroxidation [199].

8.1.2 The importance of phospholipid composition to peroxidation

Most *in vivo* studies of peroxidation have focused on the role of the PUFA content of phospholipids, but other elements of phospholipid composition may also influence peroxidation. Chapter Four presents a comparison of the amount of peroxidation product produced by phospholipids of the CL, PC, PE, phosphatidic acid (PA) and phosphatidylglycerol (PG) classes that were matched for PUFA content. This comparison arose from observations made in the Else laboratory that bovine heart CL readily undergoes peroxidation in methanol, but that phospholipid of PC and PE do not despite have a similar peroxidation index. Upon further investigation, the ranking of iron-mediated lipid hydroperoxide production was found to be greater in CL and PA when tested in diffuse solutions (1:9 water to methanol v/v), but this was not found for CL and PA tested in liposomes in aqueous solutions. The order of lipid hydroperoxide production from highest to lowest for the phospholipid classes tested was PA> CL>> PE> PC, PG in methanol but PC> PE> CL> PG> PA in liposomes. These differences in class suggest that head group charge may influence peroxidation in methanol, but relative head group size appears to have a greater influence on peroxidation in liposomal bilayers. It was concluded that whatever properties make phospholipids of the CL and PA classes readily susceptible to peroxidation in methanol did not influence peroxidation in liposomes, and therefore may not be physiologically relevant. Past comparisons of phospholipid classes have had similar difficulties in establishing a relationship between phospholipid class and peroxidation, with some studies reporting

an influence of class due to charge, others due to phospholipid shape, and a few due to protein interactions [15].

A more sophisticated study of peroxidation was carried out in Chapter Five using oxygen consumption as a measure of peroxidation. This chapter examined the ability of non-peroxidisable phospholipids (non-PPL) to extend the lag phase through an antioxidant-like action. While most i*n vitro* studies of peroxidation focus on either the maximal rate of peroxidation or the products of peroxidation, Chapter Five contained multiple measures of peroxidation as proposed by Pinchuk and Lichtenberg (2014) [96] including rates of peroxidation during the lag phase, the length of the lag phase, and rates of peroxidation during the propagation phase. The length of the lag phase is a particularly important measure of peroxidation as the lag phase is a period of limited oxidative stress where cells can nullify oxidative damage in order to maintain a healthy physiological state [108]. It is possible that healthy cells exist in this phase indefinitely by limiting reactive oxygen species (ROS) production with lipid-soluble antioxidants such as α–tocopherol. The antioxidant-like ability of non-PPLs to delay peroxidation through steric hindrance or 'caging' of ROS may be particularly efficient in maintaining the lag phase as non-PPLs are unlikely to be damaged or consumed in the same manner as other antioxidants. This effect may also be widespread as non-PPLs are major component of most cells. In addition, the antioxidant-like action of non-PPL is predicted to work synergistically with other antioxidant systems to greatly enhance their effectiveness as shown in Figure 8.1.

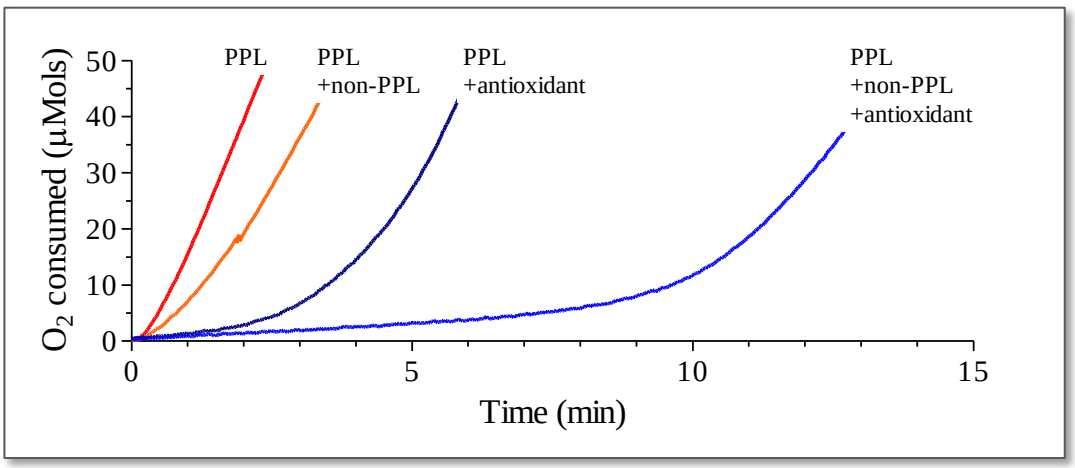

Figure 8.1. Oxygen consumption as a measure of the iron-mediated peroxidation of peroxidisable phospholipids (PPL) alone, with non-PPLs, with antioxidants, and with both non-PPL and antioxidants. Lines are typical examples of oxygen consumption curves measured experimentally.

8.1.3 Membrane composition and lifespan

Chapter Six and Chapter Seven of this thesis investigated the relationship between membrane susceptibility to peroxidation and the maximal lifespan (MLS) of three mammals. Skeletal muscle, liver and brain from mice (*Mus musculus*, MLS of 4 years), pigs (*Sus scrofa*, MLS of 27 years) and humans (*Homo sapiens*, MLS of 122 years) were examined using shotgun lipidomics. In Chapter Six, an interspecies comparison found a link between mitochondrial membrane composition and longevity, with mouse mitochondria containing high percentages of highly unsaturated PUFA in all phospholipid classes, human mitochondria containing low percentages of PUFA content with a low degree of unsaturation, and pig mitochondria sharing characteristics of both mice and human. The results for the peroxidation indexes calculated for skeletal muscle mitochondria are compared with published values for mammal species in Figure 8.2. As can be seen from the figure, the values of Chapter Six, particularly for pigs, are slightly lower than the published trend for mammals. This low value may be a result of using shotgun lipidomics as this method has been found to report a lower percentage of membrane PUFA than the gas chromatography (GC) methods used in the majority of work in this area [18]. This difference may be due to the lipidomics analysis not including phosphatidylinositol and CL, which are both classes of phospholipid with high PUFA content [20]. Despite these small differences to past work, however, the comparison of membrane composition between mice, pigs and humans supports the link between membrane composition and longevity proposed in the "Membrane Pacemaker Theory Of Aging" and similar theories.

This thesis also presents one of the first investigations into how phospholipid classes and individual species of molecular phospholipids contribute to the peroxidation index. Somewhat surprisingly, the peroxidation index of membranes was primarily determined by a limited number of molecular phospholipids species (Figure 6.4). Molecular species of both PC and PE were large contributors to the peroxidation index in skeletal muscle, PE 18:0_20:4 was the major contributor in liver, and PE 18:0_22:6 was the major contributor in brain. Another molecular phospholipid species of particular interest was PC 16:0_18:1, a non-PPL found to increase the lag phase of peroxidation in Chapter Five that was far more common in the tissues of human than of mice or pigs. This finding suggests that human membranes are more likely to be protected by the antioxidant-like action of this phospholipid in comparison to the membranes of mice or pigs.

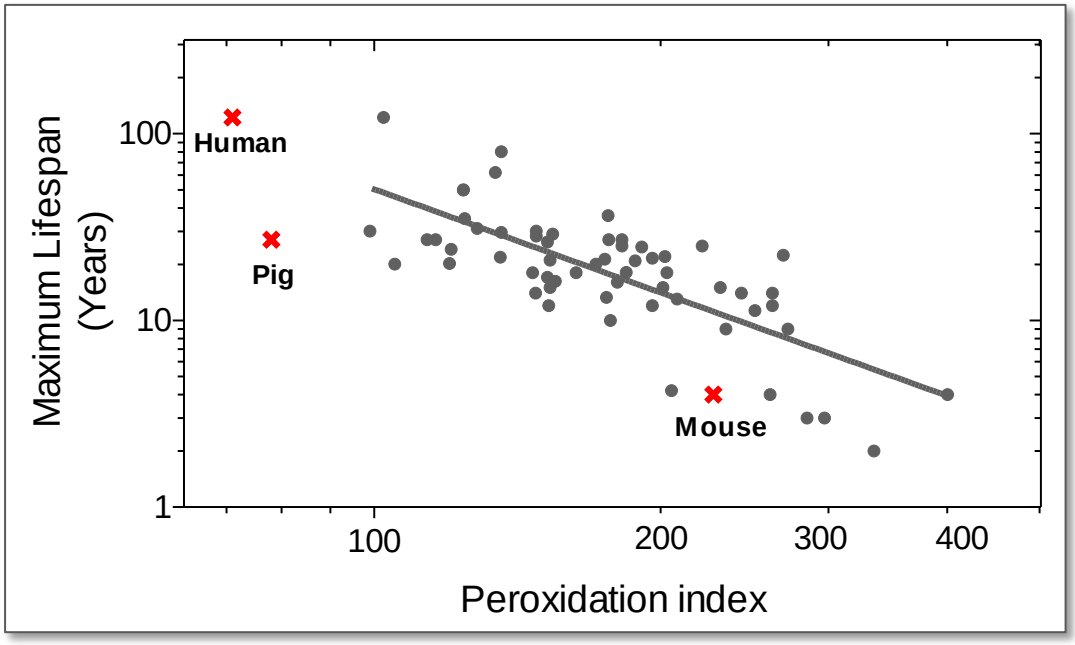

Figure 8.2. Comparison of the peroxidation index of mitochondria from mice, pig and human skeletal muscle values from Chapter Six, (red crosses) to published values for mammal muscle from [29] (grey circles).

Chapter Seven used the same methods and samples as Chapter Six to investigate whether humans are capable of excluding highly peroxidisable fatty acids from their mitochondrial membranes. If this were the case, it would represent an ability to decrease the rate of aging caused by mitochondrial damage and may explain why humans have exceptionally long lifespans in comparison to others mammals. This ability was thought to be unique to humans, and was not be expected to be present in mammals such as mice or pigs that have lifespans close to those predicted for their body mass. An intra-species comparison of the mitochondrial and microsomal membranes in humans, mice, and pigs found a high degree of similarity between organelle membrane composition within the tissues of each species, although some small differences were seen for individual phospholipid classes. Mouse liver had a higher peroxidation index in the microsomal fraction, possibly due to to 22:6n-3 synthesis. With the exception of this tissue, however, no differences were found in the peroxidation indexes of mitochondrial and microsomal fractions of tissues of human, mice or pigs. This finding suggests that the exceptionally longevity of humans is not due to a unique ability to decrease the susceptibility of their mitochondria membranes to peroxidation, and that humans are typical mammals in this regard.

A significant outcome of this thesis was the lipidomics profiling of human tissues to a level of detail not previously described. To the best of my knowledge, the work presented in this thesis is one of the most comprehensive examinations of the membrane composition and peroxidation index of humans and other mammals to date. Working with human tissues has many challenges, but results for human tissues are of particular relevance both to the understanding of human physiology and studies of mammalian longevity and aging. Overall, the finding that the membranes of human tissues contained a low percentage of PUFA and a high percentage of non-PPLs suggests that human longevity may be due to an ability to prevent the propagation of peroxidation in all cellular membranes, and thereby limit oxidative stress and damage.

8.2 Outlook and future directions

The work presented in this thesis supports the association between membrane susceptibility to peroxidation and lifespan, but emphasise the need for caveats on the traditional measures of the peroxidation index. Traditional calculations of the peroxidation index are based on GC measures of fatty acid composition and do not take into account the potential other elements of phospholipid composition such as class, non-PPLs, and plasmalogens to influence peroxidation. This thesis has shown the importance of measuring the contribution of such elements to peroxidation using *in vitro* peroxidation studies in addition to the distribution of these elements in animal tissues using a lipidomics analysis. It would also be valuable to identify how components of membranes such sterols, proteins, and lipophilic antioxidants may influence peroxidation. Future work in this area could incorporate the importance of these elements into the traditional measure of the peroxidation index to reach a better understanding of the interaction between membrane composition and oxidative stress in natural systems. My suggestions for such future work include:

1) Examining how the incorporation of dietary 22:6n-3 into CL affects membrane susceptibility to peroxidation and animal longevity.
2) Investigations of whether CL and other phospholipid classes influence the rates and lag phases of peroxidation in liposomal systems.
3) Replicating the antioxidant-like effect of non-PPLs using different combinations of phospholipid classes, fatty acid compositions, and initiators of peroxidation to clarify the underlying mechanism.

4) Proving experimentally that the antioxidant-like effect of non-PPLs affect works synergistically with lipophilic antioxidants present in cellular membranes.
5) Comparative studies of tissues from humans and mammals of similar sizes but shorter lifespans to determine membrane composition using shotgun lipidomics and membrane susceptibility to peroxidation using *in vitro* assays to test the lag phase of membrane peroxidation.

Reference list

1. Aristotle, *The complete works of aristotle*. Bollingen series, ed. J Barnes. Vol. LXXI.2. 1984, Princeton: Princeton University Press.
2. Esterbauer H. Cytotoxicity and genotoxicity of lipid-oxidation products. *American Journal of Clinical Nutrition,* (1993). **57**(5): p. 779-786.
3. Negre-Salvayre A, Auge N, Ayala V, Basaga H, Boada J, Brenke R, Chapple S, Cohen G, Feher J, Grune T, Lengyel G, Mann GE, Pamplona R, Poli G, Portero-Otin M, Riahi Y, Salvayre R, Sasson S, Serrano J, Shamni O, Siems W, Siow RCM, Wiswedel I, Zarkovic K, and Zarkovic N. Pathological aspects of lipid peroxidation. *Free Radical Research,* (2010). **44**(10): p. 1125-1171.
4. Gueraud F, Atalay M, Bresgen N, Cipak A, Eckl PM, Huc L, Jouanin I, Siems W, and Uchida K. Chemistry and biochemistry of lipid peroxidation products. *Free Radical Research,* (2010). **44**(10): p. 1098-1124.
5. Yin H, Xu L, and Porter NA. Free radical lipid peroxidation: Mechanisms and analysis. *Chemical Reviews,* (2011). **111**(10): p. 5944-5972.
6. Komprda T. Eicosapentaenoic and docosahexaenoic acids as inflammation-modulating and lipid homeostasis influencing nutraceuticals: A review. *Journal of Functional Foods,* (2012). **4**(1): p. 25-38.
7. Higdon A, Diers AR, Oh JY, Landar A, and Darley-Usmar VM. Cell signalling by reactive lipid species: New concepts and molecular mechanisms. *Biochemical Journal,* (2012). **442**: p. 453-464.
8. Davies SS and Guo LL. Lipid peroxidation generates biologically active phospholipids including oxidatively n-modified phospholipids. *Chemistry and Physics of Lipids,* (2014). **181**: p. 1-33.
9. Liochev SI. Reactive oxygen species and the free radical theory of aging. *Free Radical Biology and Medicine,* (2013). **60**(0): p. 1-4.
10. Reis A and Spickett CM. Chemistry of phospholipid oxidation. *Biochimica Et Biophysica Acta-Biomembranes,* (2012). **1818**(10): p. 2374-2387.
11. Halliwell B and Gutteridge JMC, eds. *Free radicals in biology and medicine*. 4th ed. 2007, Oxford University Press: Oxford, UK.
12. Hulbert AJ, Pamplona R, Buffenstein R, and Buttemer WA. Life and death: Metabolic rate, membrane composition, and life span of animals. *Physiological Reviews,* (2007). **87**(4): p. 1175-1213.
13. Hulbert AJ. On the importance of fatty acid composition of membranes for aging. *Journal of Theoretical Biology,* (2005). **234**(2): p. 277-288.
14. Barja G. Updating the mitochondrial free radical theory of aging: An integrated view, key aspects, and confounding concepts. *Antioxidants & Redox Signaling,* (2013). **19**(12): p. 1420-1445.
15. Schnitzer E, Pinchuk I, and Lichtenberg D. Peroxidation of liposomal lipids. *European Biophysics Journal with Biophysics Letters,* (2007). **36**(4-5): p. 499-515.
16. Lee C, Barnett J, and Reaven PD. Liposomes enriched in oleic acid are less susceptible to oxidation and have less proinflammatory activity when exposed to oxidizing conditions. *Journal of Lipid Research,* (1998). **39**(6): p. 1239-1247.
17. Horvath SE and Daum G. Lipids of mitochondria. *Progress in Lipid Research,* (2013). **52**(4): p. 590-614.
18. Nealon JR, Blanksby SJ, Mitchell TW, and Else PL. Systematic differences in membrane acyl composition associated with varying body mass in mammals

occur in all phospholipid classes: An analysis of kidney and brain. *Journal of Experimental Biology,* (2008). **211**(19): p. 3195-3204.
19. Mitchell TW, Ekroos K, Blanksby SJ, Hulbert AJ, and Else PL. Differences in membrane acyl phospholipid composition between an endothermic mammal and an ectothermic reptile are not limited to any phospholipid class. *Journal of Experimental Biology,* (2007). **210**(19): p. 3440-3450.
20. Marsh D, *Crc handbook of lipid bilayers*. 1st ed. 1990, Boca Raton, Fla: CRC Press. 387.
21. Holman. RT, ed. *Autoxidation of fats and related substances*. Progress in chemistry of fats and other lipids, ed. L WO and M T. Vol. 2. 1954, Pergamon Press: London. pp 51-98.
22. Cosgrove J, Church D, and Pryor W. The kinetics of the autoxidation of polyunsaturated fatty acids. *Lipids,* (1987). **22**(5): p. 299-304.
23. Else PL and Kraffe E. Docosahexaenoic and arachidonic acid peroxidation: It's a within molecule cascade. *Biochimica et Biophysica Acta (BBA) - Biomembranes,* (2015). **1848**(2): p. 417-421.
24. Marsh D. *Phospholipids,* in *Handbook of lipid bilayers, second edition*. 2013, CRC Press: Boca Raton. p. 43-860.
25. Claypool SM. Cardiolipin, a critical determinant of mitochondrial carrier protein assembly and function. *Biochimica Et Biophysica Acta-Biomembranes,* (2009). **1788**(10): p. 2059-2068.
26. Schlame M, Ren MD, Xu Y, Greenberg ML, and Haller I. Molecular symmetry in mitochondrial cardiolipins. *Chemistry and Physics of Lipids,* (2005). **138**(1-2): p. 38-49.
27. Song JH, Fujimoto K, and Miyazawa T. Polyunsaturated (n-3) fatty acids susceptible to peroxidation are increased in plasma and tissue lipids of rats fed docosahexaenoic acid-containing oils. *Journal of Nutrition,* (2000). **130**(12): p. 3028-3033.
28. Abbott SK, Else PL, Atkins TA, and Hulbert AJ. Fatty acid composition of membrane bilayers: Importance of diet polyunsaturated fat balance. *BBA-Biomemembr.,* (2012). **1818**(5): p. 1309-1317.
29. Hulbert AJ, Kelly M, and Abbott S. Polyunsaturated fats, membrane lipids and animal longevity. *Journal of Comparative Physiology B,* (2014). **184**(2): p. 149-166.
30. Wiswedel I, Gardemann A, Storch A, Peter D, and Schild L. Degradation of phospholipids by oxidative stress-exceptional significance of cardiolipin. *Free Radical Research,* (2010). **44**(2): p. 135-145.
31. Samhan-Arias AK, Ji J, Demidova OM, Sparvero LJ, Feng W, Tyurin V, Tyurina YY, Epperly MW, Shvedova AA, Greenberger JS, Bayır H, Kagan VE, and Amoscato AA. Oxidized phospholipids as biomarkers of tissue and cell damage with a focus on cardiolipin. *Biochimica et Biophysica Acta (BBA) - Biomembranes,* (2012). **1818**(10): p. 2413-2423.
32. Harman D. The free radical theory of aging. *Antioxidants & Redox Signaling,* (2003). **5**(5): p. 557-561.
33. Harman D. The biologic clock: The mitochondria? *Journal of the American Geriatrics Society,* (1972). **20**(4): p. 145-147.
34. Hulbert AJ. Explaining longevity of different animals: Is membrane fatty acid composition the missing link? *Age,* (2008). **30**(2-3): p. 89-97.

35. Pamplona R, Portero-Otin M, Riba D, Ruiz C, Prat J, Bellmunt MJ, and Barja G. Mitochondrial membrane peroxidizability index is inversely related to maximum life span in mammals. *Journal of Lipid Research,* (1998). **39**(10): p. 1989-1994.
36. Barja G. *The mitochondrial free radical theory of aging*, in *Mitochondrion in aging and disease*, HD Osiewacz, Editor. 2014, Elsevier Academic Press Inc: San Diego. p. 1-27.
37. Munro D and Blier PU. The extreme longevity of arctica islandica is associated with increased peroxidation resistance in mitochondrial membranes. *Aging Cell,* (2012). **11**(5): p. 845-855.
38. Haddad LS, Kelbert L, and Hulbert AJ. Extended longevity of queen honey bees compared to workers is associated with peroxidation-resistant membranes. *Experimental Gerontology,* (2007). **42**(7): p. 601-609.
39. Reis RJS, Xu LL, Lee H, Chae M, Thaden JJ, Bharill P, Tazearslan C, Siegel E, Alla R, Zimniak P, and Ayyadevara S. Modulation of lipid biosynthesis contributes to stress resistance and longevity of c. Elegans mutants. *Aging-Us,* (2011). **3**(2): p. 125-147.
40. Mitchell TW, Buffenstein R, and Hulbert AJ. Membrane phospholipid composition may contribute to exceptional longevity of the naked mole-rat (heterocephalus glaber): A comparative study using shotgun lipidomics. *Experimental Gerontology,* (2007). **42**(11): p. 1053-1062.
41. Gonzalo H, Brieva L, Tatzber F, Jove M, Cacabelos D, Cassanye A, Lanau-Angulo L, Boada J, Serrano JCE, Gonzalez C, Hernandez L, Peralta S, Pamplona R, and Portero-Otin M. Lipidome analysis in multiple sclerosis reveals protein lipoxidative damage as a potential pathogenic mechanism. *Journal of Neurochemistry,* (2012). **123**(4): p. 622-634.
42. Jove M, Naudi A, Aledo JC, Cabre R, Ayala V, Portero-Otin M, Barja G, and Pamplona R. Plasma long-chain free fatty acids predict mammalian longevity. *Scientific Reports,* (2013). **3**.
43. Niki E, Yoshida Y, Saito Y, and Noguchi N. Lipid peroxidation: Mechanisms, inhibition, and biological effects. *Biochemical and Biophysical Research Communications,* (2005). **338**(1): p. 668-676.
44. *Anage: The animal aging and longevity database*. [cited 2014; Available from: http://genomics.senescence.info/species/.
45. Hulbert AJ. Metabolism and longevity: Is there a role for membrane fatty acids? *Integrative and Comparative Biology,* (2010). **50**(5): p. 808-817.
46. Nicolson GL. The fluid-mosaic model of membrane structure: Still relevant to understanding the structure, function and dynamics of biological membranes after more than 40 years. *Biochimica Et Biophysica Acta-Biomembranes,* (2014). **1838**(6): p. 1451-1466.
47. Goni FM. The basic structure and dynamics of cell membranes: An update of the singer-nicolson model. *Biochimica Et Biophysica Acta-Biomembranes,* (2014). **1838**(6): p. 1467-1476.
48. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, and Walter P, eds. *Molecular biology of the cell*. Fourth edition ed. 2002, Garland Science: New York, New York.
49. Singer SJ and Nicolson GL. Fluid mosaic model of structure of cell-membranes. *Science,* (1972). **175**(4023): p. 720-&.
50. Han XL and Gross RW. Shotgun lipidomics: Electrospray ionization mass spectrometric analysis and quantitation of cellular lipidomes directly from crude

extracts of biological samples. *Mass Spectrometry Reviews,* (2005). **24**(3): p. 367-412.
51. Daum G. Lipids of mitochondria. *Biochimica Et Biophysica Acta,* (1985). **822**(1): p. 1-42.
52. Nicolson GL. Transmembrane controls of receptors on normal and tumor cells 1. Cytoplasmic influence over cell-surface components. *Biochimica Et Biophysica Acta,* (1976). **457**(1): p. 57-108.
53. Engelman DM. Membranes are more mosaic than fluid. *Nature,* (2005). **438**(7068): p. 578-580.
54. Brown DA and London E. Functions of lipid rafts in biological membranes. *Annual Review of Cell and Developmental Biology,* (1998). **14**: p. 111-136.
55. Contreras FX, Ernst AM, Wieland F, and Brugger B. Specificity of intramembrane protein-lipid interactions. *Cold Spring Harbor Perspectives in Biology,* (2011). **3**(6).
56. Lee AG. Lipid-protein interactions in biological membranes: A structural perspective. *Biochimica Et Biophysica Acta-Biomembranes,* (2003). **1612**(1): p. 1-40.
57. Lee AG. How lipids affect the activities of integral membrane proteins. *Biochimica Et Biophysica Acta-Biomembranes,* (2004). **1666**(1-2): p. 62-87.
58. Acehan D, Malhotra A, Xu Y, Ren MD, Stokes DL, and Schlame M. Cardiolipin affects the supramolecular organization of atp synthase in mitochondria. *Biophysical Journal,* (2011). **100**(9): p. 2184-2192.
59. Dobrian AD, Lieb DC, Cole BK, Taylor-Fishwick DA, Chakrabarti SK, and Nadler JL. Functional and pathological roles of the 12-and 15-lipoxygenases. *Progress in Lipid Research,* (2011). **50**(1): p. 115-131.
60. Hersberger M. Potential role of the lipoxygenase derived lipid mediators in atherosclerosis: Leukotrienes, lipoxins and resolvins. *Clinical Chemistry and Laboratory Medicine,* (2010). **48**(8): p. 1063-1073.
61. Schwab JM, Chiang N, Arita M, and Serhan CN. Resolvin e1 and protectin d1 activate inflammation-resolution programmes. *Nature,* (2007). **447**(7146): p. 869-874.
62. Serhan CN. Novel chemical mediators in the resolution of inflammation: Resolvins and protectins. *Anesthesiology clinics,* (2006). **24**(2): p. 341-64.
63. Serhan CN, Chiang N, and Van Dyke TE. Resolving inflammation: Dual anti-inflammatory and pro-resolution lipid mediators. *Nature Reviews Immunology,* (2008). **8**(5): p. 349-361.
64. Marsh D. Structural and thermodynamic determinants of chain-melting transition temperatures for phospholipid and glycolipids membranes. *Biochimica et Biophysica Acta (BBA) - Biomembranes,* (2010). **1798**(1): p. 40-51.
65. Greenberg ME, Li XM, Gugiu BG, Gu XD, Qin J, Salomon RG, and Hazen SL. The lipid whisker model of the structure of oxidized cell membranes. *Journal of Biological Chemistry,* (2008). **283**(4): p. 2385-2396.
66. Lis M, Wizert A, Przybylo M, Langner M, Swiatek J, Jungwirth P, and Cwiklik L. The effect of lipid oxidation on the water permeability of phospholipids bilayers. *Physical Chemistry Chemical Physics,* (2011). **13**(39): p. 17555-17563.
67. Nagan N and Zoeller RA. Plasmalogens: Biosynthesis and functions. *Progress in Lipid Research,* (2001). **40**(3): p. 199-229.
68. Braverman NE and Moser AB. Functions of plasmalogen lipids in health and disease. *Biochimica Et Biophysica Acta-Molecular Basis of Disease,* (2012). **1822**(9): p. 1442-1452.

69. Lessig J and Fuchs B. Plasmalogens in biological systems: Their role in oxidative processes in biological membranes, their contribution to pathological processes and aging and plasmalogen analysis. *Current Medicinal Chemistry,* (2009). **16**(16): p. 2021-2041.
70. IUPAC-IUB, The nomenclature of lipids (recommendations 1976). Iupac-iub commission on biochemical nomenclature, in *IUPAC-IUB Commission on Biochemical Nomenclature*. 1978: Journal of Lipid Research. p. 114-28.
71. Gunstone FD, *Fatty acid and lipid chemistry*. 1996: Springer US.
72. Holman RT. The ratio of trienoic: Tetraenoic acids in tissue lipids as a measure of essential fatty acid requirement. *The Journal of nutrition,* (1960). **70**: p. 405-10.
73. Buettner GR. The pecking order of free-radicals and antioxidants- lipid peroxidation, alpha-tocopherol and ascorbate. *Archives of Biochemistry and Biophysics,* (1993). **300**(2): p. 535-543.
74. Liebisch G, Vizcaino JA, Kofeler H, Trotzmuller M, Griffiths WJ, Schmitz G, Spener F, and Wakelam MJO. Shorthand notation for lipid structures derived from mass spectrometry. *Journal of Lipid Research,* (2013). **54**(6): p. 1523-1530.
75. Jouhet J. Importance of the hexagonal lipid phase in biological membrane organization. *Frontiers in Plant Science,* (2013). **4**: p. 494.
76. Daleke DL. Regulation of transbilayer plasma membrane phospholipid asymmetry. *Journal of Lipid Research,* (2003). **44**(2): p. 233-242.
77. Peng T, Yuan XQ, and Hang HC. Turning the spotlight on protein-lipid interactions in cells. *Current Opinion in Chemical Biology,* (2014). **21**: p. 144-153.
78. Andreyev AY, Fahy E, Guan ZQ, Kelly S, Li XA, McDonald JG, Milne S, Myers D, Park H, Ryan A, Thompson BM, Wang E, Zhao YH, Brown HA, Merrill AH, Raetz CRH, Russell DW, Subramaniam S, and Dennis EA. Subcellular organelle lipidomics in tlr-4-activated macrophages. *Journal of Lipid Research,* (2010). **51**(9): p. 2785-2797.
79. Schlame M, Brody S, and Hostetler KY. Mitochondrial cardiolipin in diverse eukaryotes - comparisons of biosyntgetic reactions and molecular acyl species. *European Journal of Biochemistry,* (1993). **212**(3): p. 727-735.
80. Portero-Otin M, Bellmunt MJ, Ruiz MC, Barja G, and Pamplona R. Correlation of fatty acid unsaturation of the major liver mitochondrial phospholipid classes in mammals to their maximum life span potential. *Lipids,* (2001). **36**(5): p. 491-498.
81. Hermansson M, Hokynar K, and Somerharju P. Mechanisms of glycerophospholipid homeostasis in mammalian cells. *Progress in Lipid Research,* (2011). **50**(3): p. 240-257.
82. Schenkel LC and Bakovic M. Formation and regulation of mitochondrial membranes. *International Journal of Cell Biology,* (2014). **2014**: p. 13.
83. Fagone P and Jackowski S. Membrane phospholipid synthesis and endoplasmic reticulum function. *Journal of Lipid Research,* (2009). **50**: p. S311-S316.
84. Hiltunen JK, Schonauer MS, Autio KJ, Mittelmeier TM, Kastaniotis AJ, and Dieckmann CL. Mitochondrial fatty acid synthesis type ii: More than just fatty acids. *Journal of Biological Chemistry,* (2009). **284**(14): p. 9011-9015.
85. Yamashita A, Hayashi Y, Nemoto-Sasaki Y, Ito M, Oka S, Tanikawa T, Waku K, and Sugiura T. Acyltransferases and transacylases that determine the fatty acid composition of glycerolipids and the metabolism of bioactive lipid

mediators in mammalian cells and model organisms. *Progress in Lipid Research,* (2014). **53**: p. 18-81.
86. Schmid PC, Deli E, and Schmid HHO. Generation and remodeling of phospholipid molecular species in rat hepatocytes. *Archives of Biochemistry and Biophysics,* (1995). **319**(1): p. 168-176.
87. Lands WEM. Metabolism of glycerolipids: A comparison of lecithin and triglyceride synthesis. *Journal of Biological Chemistry,* (1958). **231**(2): p. 883-888.
88. Sprecher H and Chen Q. Polyunsaturated fatty acid biosynthesis: A microsomal-peroxisomal process. *Prostaglandins Leukotrienes and Essential Fatty Acids,* (1999). **60**(5-6): p. 317-321.
89. Burr G and Burr M. On the nature and role of the fatty acids essential in nutrition. *Journal of Biological Chemistry,* (1930). **86**: p. 587-621.
90. Abbott SK, Else PL, and Hulbert AJ. Membrane fatty acid composition of rat skeletal muscle is most responsive to the balance of dietary n-3 and n-6 pufa. *British Journal of Nutrition,* (2010). **103**(4): p. 522-529.
91. Lands WE, Inoue M, Sugiura Y, and Okuyama H. Selective incorporation of polyunsaturated fatty acids into phosphatidylcholine by rat liver microsomes. *Journal of Biological Chemistry,* (1982). **257**(24): p. 14968-72.
92. Senebier J, *Encyclopédie méthodique*. Vol. 11. 1791, Paris, France: Diderot and D'Alembert.
93. Hammond E and White P. A brief history of lipid oxidation. *Journal of the American Oil Chemists' Society,* (2011). **88**(7): p. 891-897.
94. Thunberg T. Untersuchungen über autoxydable substanzen und autoxydable systeme von physiologischem interesse (the special position of the iron under the heavy metals with respect to the catalytic acceleration of the oxygen absorption the lecithins). *Skandinavisches Archiv Für Physiologie,* (1911). **24**(1): p. 94-96.
95. Psciuk BT and Schlegel HB. Computational prediction of one-electron reduction potentials and acid dissociation constants for guanine oxidation intermediates and products. *Journal of Physical Chemistry B,* (2013). **117**(32): p. 9518-9531.
96. Pinchuk I and Lichtenberg D. Analysis of the kinetics of lipid peroxidation in terms of characteristic time-points. *Chemistry and Physics of Lipids,* (2014). **178**: p. 63-76.
97. Morrill GA, Kostellow A, Resnick LM, and Gupta RK. Interaction between ferric ions, phospholipid hydroperoxides, and the lipid phosphate moiety at physiological ph. *Lipids,* (2004). **39**(9): p. 881-889.
98. Repetto MG, Ferrarotti NF, and Boveris A. The involvement of transition metal ions on iron-dependent lipid peroxidation. *Archives of Toxicology*. **84**(4): p. 255-262.
99. Mozuraityte R, Rustad T, and Storro I. The role of iron in peroxidation of polyunsaturated fatty acids in liposomes. *Journal of Agricultural and Food Chemistry,* (2008). **56**(2): p. 537-543.
100. Cheng ZY and Li YZ. What is responsible for the initiating chemistry of iron-mediated lipid peroxidation: An update (vol 107, pg 748, 2007). *Chemical Reviews,* (2007). **107**(5): p. 2165-2165.
101. Welch KD, Davis TZ, and Aust SD. Iron autoxidation and free radical generation: Effects of buffers, ligands, and chelators. *Archives of Biochemistry and Biophysics,* (2002). **397**(2): p. 360-369.

102. Tadolini B and Hakim G. The mechanism of iron (iii) stimulation of lipid peroxidation. *Free Radical Research,* (1996). **25**(3): p. 221-227.
103. Min B and Ahn DU. Mechanism of lipid peroxidation in meat and meat products - a review. *Food Science and Biotechnology,* (2005). **14**(1): p. 152-163.
104. Carlsen CU, Moller JKS, and Skibsted LH. Heme-iron in lipid oxidation. *Coordination Chemistry Reviews,* (2005). **249**(3-4): p. 485-498.
105. Kell DB. Iron behaving badly: Inappropriate iron chelation as a major contributor to the aetiology of vascular and other progressive inflammatory and degenerative diseases. *Bmc Medical Genomics,* (2009). **2**.
106. Esterbauer H, Schaur RJ, and Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radical Biology and Medicine,* (1991). **11**(1): p. 81-128.
107. Porter NA. A perspective on free radical autoxidation: The physical organic chemistry of polyunsaturated fatty acid and sterol peroxidation. *Journal of Organic Chemistry,* (2013). **78**(8): p. 3511-3524.
108. Cadenas E and Sies H. The lag phase. *Free Radical Research,* (1998). **28**(6): p. 601-609.
109. Wagner BA, Buettner GR, and Burns CP. Free radical-mediated lipid peroxidation in cells: Oxidizability is a function of cell lipid bis-allylic hydrogen content. *Biochemistry,* (1994). **33**(15): p. 4449-4453.
110. Gardner HW. Oxygen radical chemistry of poly-unsaturated fatty acids. *Free Radical Biology and Medicine,* (1989). **7**(1): p. 65-86.
111. Moore K and Roberts LJ. Measurement of lipid peroxidation. *Free Radical Research,* (1998). **28**(6): p. 659-671.
112. Clark LC, Jr., Wolf R, Granger D, and Taylor Z. Continuous recording of blood oxygen tensions by polarography. *Journal of Applied Physiology,* (1953). **6**(3): p. 189-93.
113. Bou R, Codony R, Tres A, Decker EA, and Guardicila F. Determination of hydroperoxides in foods and biological samples by the ferrous oxidation-xylenol orange method: A review of the factors that influence the method's performance. *Analytical Biochemistry,* (2008). **377**(1): p. 1-15.
114. Wolff SP. *Ferrous ion oxidation in presence of ferroc ion indicator xylenol orange for measurements of hydroperoxides*, in *Oxygen radicals in biological systems, pt c,* L Packer, Editor. 1994, Elsevier Academic Press Inc: San Diego. p. 182-189.
115. GironCalle J, Schmid PC, and Schmid HHO. Effects of oxidative stress on glycerolipid acyl turnover in rat hepatocytes. *Lipids,* (1997). **32**(9): p. 917-923.
116. Carreau A, El Hafny-Rahbi B, Matejuk A, Grillon C, and Kieda C. Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. *Journal of Cellular and Molecular Medicine,* (2011). **15**(6): p. 1239-1253.
117. Jin KL. Modern biological theories of aging. *Aging and Disease,* (2010). **1**(2): p. 72-74.
118. López-Otín C, Blasco MA, Partridge L, Serrano M, and Kroemer G. The hallmarks of aging. *Cell,* (2013). **153**(6): p. 1194-1217.
119. Strehler BL, *Time, cells and aging*. 1962, New York: Academic Press.
120. Harman D. *Aging: Overview*, in *Healthy aging for functional longevity: Molecular and cellular interactions in senescence,* SC Park, ES Hwang, HS Kim, and WY Park, Editors. 2001. p. 1-21.

121. Harman D. Aging: A theory based on free radical and radiation chemistry. *Journal of Gerontology,* (1956). **11**(3): p. 298-300.
122. Muller FL, Lustgarten MS, Jang Y, Richardson A, and Van Remmen H. Trends in oxidative aging theories. *Free Radical Biology and Medicine,* (2007). **43**(4): p. 477-503.
123. Edrey YH and Salmon AB. Revisiting an age-old question regarding oxidative stress. *Free Radical Biology and Medicine,* (2014). **71**: p. 368-378.
124. Gladyshev VN. The free radical theory of aging is dead. Long live the damage theory! *Antioxidants & Redox Signaling,* (2014). **20**(4): p. 727-731.
125. Gems D and Doonan R. Antioxidant defense and aging in c. Elegans: Is the oxidative damage theory of aging wrong? *Cell Cycle,* (2009). **8**(11): p. 1681-1687.
126. Lapointe J and Hekimi S. When a theory of aging ages badly. *Cellular and Molecular Life Sciences,* (2010). **67**(1): p. 1-8.
127. Naudí A, Jové M, Ayala V, Portero-Otín M, Barja G, and Pamplona R. Regulation of membrane unsaturation as antioxidant adaptive mechanism in long-lived animal species. *Free Radicals and Antioxidants,* (2011). **1**(3): p. 3-12.
128. Gudbjarnason S, Doell B, and Oskarsdottir G. Docosahezaenoic acid in cardiac metablism and function. *Acta Biologica et Medica Germanica,* (1978). **37**(5-6): p. 777-784.
129. Couture P and Hulbert AJ. Membrane fatty-acid composition of tissues is related to body-mass of mammals. *Journal of Membrane Biology,* (1995). **148**(1): p. 27-39.
130. Innis SM. Dietary omega 3 fatty acids and the developing brain. *Brain Research,* (2008). **1237**: p. 35-43.
131. Pamplona R, Barja G, and Portero-Otin M. *Membrane fatty acid unsaturation, protection against oxidative stress, and maximum life span - a homeoviscous-longevity adaptation?*, in *Increasing healthy life span: Conventional measures and slowing the innate aging process*, D Harman, Editor. 2002, New York Acad Sciences: New York. p. 475-490.
132. Pamplona R. Membrane phospholipids, lipoxidative damage and molecular integrity: A causal role in aging and longevity. *Biochimica Et Biophysica Acta-Bioenergetics,* (2008). **1777**(10): p. 1249-1262.
133. Hulbert AJ, Faulks SC, and Buffenstein R. Oxidation-resistant membrane phospholipids can explain longevity differences among the longest-living rodents and similarly-sized mice. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences,* (2006). **61**(10): p. 1009-1018.
134. Hulbert AJ, Beard LA, and Grigg GC. The exceptional longevity of an egg-laying mammal, the short-beaked echidna (tachyglossus aculeatus) is associated with peroxidation-resistant membrane composition. *Experimental Gerontology,* (2008). **43**(8): p. 729-733.
135. Valencak TG and Ruf T. Phospholipid composition and longevity: Lessons from ames dwarf mice. *Age,* (2013). **35**(6): p. 2303-2313.
136. McGuire LP, Fenton MB, and Guglielmo CG. Phenotypic flexibility in migrating bats: Seasonal variation in body composition, organ sizes and fatty acid profiles. *Journal of Experimental Biology,* (2013). **216**(5): p. 800-808.
137. Coles LS. Demography of human supercentenarians. *Journals of Gerontology Series a-Biological Sciences and Medical Sciences,* (2004). **59**(6): p. 579-586.
138. Benga G, Hodarnau A, Bohm B, Borza V, Tilinca R, Dancea S, Petrescu I, and Ferdinand W. Human liver mitochondria- relation of a particular lipid

composition to mobility of spin-labelled lipids. *European Journal of Biochemistry,* (1978). **84**(2): p. 625-633.
139. Andersson A, Nalsen C, Tengblad S, and Vessby B. Fatty acid composition of skeletal muscle reflects dietary fat composition in humans. *American Journal of Clinical Nutrition,* (2002). **76**(6): p. 1222-1229.
140. Buttemer WA, Battam H, and Hulbert AJ. Fowl play and the price of petrel: Long-living procellariiformes have peroxidation-resistant membrane composition compared with short-living galliformes. *Biology Letters,* (2008). **4**(4): p. 351-354.
141. Montgomery MK, Hulbert AJ, and Buttemer WA. Metabolic rate and membrane fatty acid composition in birds: A comparison between long-living parrots and short-living fowl. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology,* (2012). **182**(1): p. 127-137.
142. Faulks SC, Turner N, Else PL, and Hulbert AJ. Calorie restriction in mice: Effects on body composition, daily activity, metabolic rate, mitochondrial reactive oxygen species production, and membrane fatty acid composition. *Journals of Gerontology Series a-Biological Sciences and Medical Sciences,* (2006). **61**(8): p. 781-794.
143. Cefalu WT, Wang ZQ, Bell-Farrow AD, Terry JG, Sonntag W, Waite M, and Parks J. Chronic caloric restriction alters muscle membrane fatty acid content. *Experimental Gerontology,* (2000). **35**(3): p. 331-341.
144. Chen YN, Hagopian K, McDonald RB, Bibus D, Lopez-Lluch G, Villalba JM, Navas P, and Ramsey JJ. The influence of dietary lipid composition on skeletal muscle mitochondria from mice following 1 month of calorie restriction. *Journals of Gerontology Series a-Biological Sciences and Medical Sciences,* (2012). **67**(11): p. 1121-1131.
145. Gómez A, Sánchez-Roman I, Gomez J, Cruces J, Mate I, Lopez-Torres M, Naudi A, Portero-Otin M, Pamplona R, De la Fuente M, and Barja G. Lifelong treatment with atenolol decreases membrane fatty acid unsaturation and oxidative stress in heart and skeletal muscle mitochondria and improves immunity and behavior, without changing mice longevity. *Aging Cell,* (2014). **13**(3): p. 551-560.
146. McGee CD, Lieberman P, and Greenwood CE. Dietary fatty acid composition induces comparable changes in cardiolipin fatty acid profile of heart and brain mitochondria. *Lipids,* (1996). **31**(6): p. 611-616.
147. Yamaoka S, Urade R, and Kito M. Mitochondrial-function in rats is affected by modification of membrane phospholipids with dietary sardine oil. *Journal of Nutrition,* (1988). **118**(3): p. 290-296.
148. Charnock JS, Abeywardena MY, and McLennan PL. Comparative changes in the fatty-acid composition of rat cardiac phospholipids after long-term feeding of sunflower seed oil-supplemented or tuna fish oil-supplemented diets. *Annals of Nutrition and Metabolism,* (1986). **30**(6): p. 393-406.
149. Astorg PO and Chevalier J. Phospholipid fatty acid composition and respiratory properties of heart and liver mitochondria from rats fed with or deprived of linolenic acid. *Nutrition Research,* (1991). **11**(1): p. 71-77.
150. Hussein N, Fedorova I, Moriguchi T, Hamazaki K, Kim H-Y, Hoshiba J, and Salem N. Artificial rearing of infant mice leads to n-3 fatty acid deficiency in cardiac, neural and peripheral tissues. *Lipids,* (2009). **44**(8): p. 685-702.
151. Cheng H, Mancuso DJ, Jiang XT, Guan SP, Yang JY, Yang K, Sun G, Gross RW, and Han XL. Shotgun lipidomics reveals the temporally dependent, highly

diversified cardiolipin profile in the mammalian brain: Temporally coordinated postnatal diversification of cardiolipin molecular species with neuronal remodeling. *Biochemistry,* (2008). **47**(21): p. 5869-5880.

152. Han XL and Gross RW. Electrospary-ionisation mass spectroscopic analysis of human erythrocyte plasma-membrane phospholipids. *Proceedings of the National Academy of Sciences of the United States of America,* (1994). **91**(22): p. 10635-10639.
153. Blanksby SJ and Mitchell TW. Advances in mass spectrometry for lipidomics. *Annual Review of Analytical Chemistry, Vol 3,* (2010). **3**: p. 433-465.
154. Han XL, Yang K, and Gross RW. Multi-dimensional mass spectrometry-based shotgun lipidomics and novel strategies for lipidomic analyses. *Mass Spectrometry Reviews,* (2012). **31**(1): p. 134-178.
155. Schlame M and Haldar D. Cardiolipin is synthesized on the matrix side of the inner membrane in rat-liver mitochondria. *Journal of Biological Chemistry,* (1993). **268**(1): p. 74-79.
156. Claypool SM and Koehler CM. The complexity of cardiolipin in health and disease. *Trends in Biochemical Sciences,* (2012). **37**(1): p. 32-41.
157. Houtkooper RH and Vaz FM. Cardiolipin, the heart of mitochondrial metabolism. *Cellular and Molecular Life Sciences,* (2008). **65**(16): p. 2493-2506.
158. Haines TH. A new look at cardiolipin. *Biochimica Et Biophysica Acta-Biomembranes,* (2009). **1788**(10): p. 1997-2002.
159. Kagan VE, Bayir HA, Belikova NA, Kapralov O, Tyurina YY, Tyurin VA, Jiang JF, Stoyanovsky DA, Wipf P, Kochanek PM, Greenberger JS, Pitt B, Shvedova AA, and Borisenko G. Cytochrome c/cardiolipin relations in mitochondria: A kiss of death. *Free Radical Biology and Medicine,* (2009). **46**(11): p. 1439-1453.
160. Koshkin V and Greenberg ML. Oxidative phosphorylation in cardiolipin-lacking yeast mitochondria. *Biochemical Journal,* (2000). **347**: p. 687-691.
161. Norris S, Membrane phospholipid peroxidation: Effect of fatty acid pairing and head group, in *School of Health Sciences*. 2010, University of Wollongong: Wollongong.
162. Han XL, Yang K, Yang JY, Cheng H, and Gross RW. Shotgun lipidomics of cardiolipin molecular species in lipid extracts of biological samples. *Journal of Lipid Research,* (2006). **47**(4): p. 864-879.
163. Schlame M, Beyer K, Hayerhartl M, and Klingenberg M. Molecular-species of cardiolipin in relation to other mitochondrial phospholipids - is there an acyl specificity of the interaction between cardiolipin and the adp/atp carrier? *European Journal of Biochemistry,* (1991). **199**(2): p. 459-466.
164. Belikova NA, Vladimirov YA, Osipov AN, Kapralov AA, Tyurin VA, Potapovich MV, Basova LV, Peterson J, Kurnikov IV, and Kagan VE. Peroxidase activity and structural transitions of cytochrome c bound to cardiolipin-containing membranes. *Biochemistry,* (2006). **45**(15): p. 4998-5009.
165. Tyurin VA, Tyurina YY, Kochanek PM, Hamilton R, DeKosky ST, Greenberger JS, Bayir H, and Kagan VE. *Oxidative lipidomics of programmed cell death*, in *Programmed cell death, general principles for studying cell death, pt a*, R KhosraviFar, Z Zakeri, RA Lockshin, and M Piacentini, Editors. 2008. p. 375-393.
166. Tyurina YY, Tyurin VA, Kapralova VI, Wasserloos K, Mosher M, Epperly MW, Greenberger JS, Pitt BR, and Kagan VE. Oxidative lipidomics of gamma-

radiation-induced lung injury: Mass spectrometric characterization of cardiolipin and phosphatidylserine peroxidation. *Radiation Research,* (2011). **175**(5): p. 610-621.

167. Liu W, Porter NA, Schneider C, Brash AR, and Yin H. Formation of 4-hydroxynonenal from cardiolipin oxidation: Intramolecular peroxyl radical addition and decomposition. *Free Radical Biology and Medicine,* (2011). **50**(1): p. 166-178.
168. Das UN. Essential fatty acids enhance free radical generation and lipid peroxidation to induce apoptosis of tumor cells. *Clinical Lipidology,* (2011). **6**(4): p. 463-489.
169. Tyurina YY, Kini V, Tyurin VA, Vlasova, II, Jiang JF, Kapralov AA, Belikova NA, Yalowich JC, Kurnikov IV, and Kagan VE. Mechanisms of cardiolipin oxidation by cytochrome c: Relevance to pro- and antiapoptotic functions of etoposide. *Molecular Pharmacology,* (2006). **70**(2): p. 706-717.
170. Ikeda I, Mitsui K, and Imaizumi K. Effect of dietary linoleic, alpha-linolenic and arachidonic acids on lipid metabolism, tissue fatty acid composition and eicosanoid production in rats. *Journal of Nutritional Science and Vitaminology,* (1996). **42**(6): p. 541-551.
171. Innis SM and Clandinin MT. Dynamic modulation of mitochondrial inner-membrane lipids in rat-heart by dietary-fat. *Biochemical Journal,* (1981). **193**(1): p. 155-167.
172. Javouhey A, Rocquelin G, Rochette L, and Juaneda P. Comparative effects of equivalent intakes of 18-3 (n-3) and of marine (n-3) fatty-acids on rat cardiac phospholipid contents and fatty-acid compositions. *Nutrition Research,* (1990). **10**(3): p. 291-301.
173. Kramer JKG. Comparative studies on composition of cardiac phospholipids in rats fed different vegetable-oils. *Lipids,* (1980). **15**(9): p. 651-660.
174. Novak F, Tvrzicka E, Hamplova B, Kolar F, and Novakova O. Postnatal development of phospholipids and their fatty acid profile in rat heart. *Molecular and Cellular Biochemistry,* (2006). **293**(1-2): p. 23-33.
175. Power GW, Yaqoob P, Harvey DJ, Newsholme EA, and Calder PC. The effect of dietary-lipid manipulation on hepatic mitochondrial phospholipid fatty-acid composition and carnitine palmitoyltransferase-i activity. *Biochemistry and Molecular Biology International,* (1994). **34**(4): p. 671-684.
176. Swanson JE and Kinsella JE. Dietary normal-3 polyunsaturated fatty-acids - modification of rat cardiac lipids and fatty-acid composition. *Journal of Nutrition,* (1986). **116**(4): p. 514-523.
177. Taniguchi H, Suzuki K, Takita T, Chung SY, Hayakawa T, Nakamura K, and Innami S. Comparative effects of eicosapentaenoic acid and docosahexaenoic acid on n-6 and n-3 fatty-acid profiles of phospholipid classes in several tissues of rats fed a hypertriglyceridemic diet. *Journal of Clinical Biochemistry and Nutrition,* (1993). **14**(3): p. 151-162.
178. Yamaoka S, Urade R, and Kito M. Cardiolipin molecular-species in rat-heart mitochondria are sensitive to essential fatty-acid deficient dietary lipids. *Journal of Nutrition,* (1990). **120**(5): p. 415-421.
179. Hoy CE and Holmer G. Influence of dietary linoleic-acid and trans-fatty acids on the fatty acid profile of cardiolipin in rats. *Lipids,* (1990). **25**(8): p. 455-459.
180. Charnock JS, Abeywardena MY, McMurchie EJ, and Russell GR. The composition of cardiac phospholipids in rats fed different lipid supplements. *Lipids,* (1984). **19**(3): p. 206-213.

181. Charnock JS, Abeywardena MY, Tan D, and McLennan PL. Omega-3 and omega-6 pufas have different effects on the phospholipid fatty acid composition of rate myocardial muscle when added to a saturated fatty acid dietary supplement. *Nutrition Research,* (1991). **11**(9): p. 1013-1024.
182. Robblee NM and Clandinin MT. Effect of dietary-fat level and poly-unsaturated fatty-acid content on the phospholipid-composition of rat cardiac mitochondrial-membranes and mitochondrial atpase activity. *Journal of Nutrition,* (1984). **114**(2): p. 263-269.
183. Croset M and Kinsella JE. Changes in phospholipid fatty-acid composition of mouse cardiac organelles after feeding graded amounts of docosahexaenoate in presence of high-levels of linoleate - effect on cardiac atpase activities. *Annals of Nutrition and Metabolism,* (1989). **33**(3): p. 125-142.
184. Berger A and German JB. Phospholipid fatty-acid composition of various mouse-tissues after feeding alpha-linolenate (18-3n-3) or eicosatrienoate (20-3n-3). *Lipids,* (1990). **25**(8): p. 473-480.
185. Watkins SM, Lin TY, Davis RM, Ching JR, DePeters EJ, Halpern GM, Walzem RL, and German JB. Unique phospholipid metabolism in mouse heart in response to dietary docosahexaenoic or alpha-linolenic acids. *Lipids,* (2001). **36**(3): p. 247-254.
186. Berger A, Gershwin ME, and German JB. Effects of various dietary fats on cardiolipin acyl composition during ontogent of mice. *Lipids,* (1992). **27**(8): p. 605-612.
187. Ailhaud G, Massiera F, Weill P, Legrand P, Alessandri JM, and Guesnet P. Temporal changes in dietary fats: Role of n-6 polyunsaturated fatty acids in excessive adipose tissue development and relationship to obesity. *Progress in Lipid Research,* (2006). **45**(3): p. 203-236.
188. Lee HJ, Mayette J, Rapoport SI, and Bazinet RP. Selective remodeling of cardiolipin fatty acids in the aged rat heart. *Lipids in Health and Disease,* (2006). **5**.
189. Owen AJ, Peter-Przyborowska BA, Hoy AJ, and McLennan PL. Dietary fish oil dose- and time-response effects on cardiac phospholipid fatty acid composition. *Lipids,* (2004). **39**(10): p. 955-961.
190. Hulbert AJ, Turner N, Storlien LH, and Else PL. Dietary fats and membrane function: Implications for metabolism and disease. *Biological Reviews,* (2005). **80**(1): p. 155-169.
191. Gao F, Kiesewetter D, Chang L, Ma K, Bell JM, Rapoport SI, and Igarashi M. Whole-body synthesis-secretion rates of long-chain n-3 pufas from circulating unesterified alpha-linolenic acid in unanesthetized rats. *Journal of Lipid Research,* (2009). **50**(4): p. 749-758.
192. Daum G and Vance JE. Import of lipids into mitochondria. *Progress in Lipid Research,* (1997). **36**(2-3): p. 103-130.
193. Kiebish MA, Bell R, Yang K, Phan T, Zhao ZD, Ames W, Seyfried TN, Gross RW, Chuang JH, and Han XL. Dynamic simulation of cardiolipin remodeling: Greasing the wheels for an interpretative approach to lipidomics. *Journal of Lipid Research,* (2010). **51**(8): p. 2153-2170.
194. Khairallah RJ, Kim J, O'Shea KM, O'Connell KA, Brown BH, Galvao T, Daneault C, Des Rosiers C, Polster BM, Hoppel CL, and Stanley WC. Improved mitochondrial function with diet-induced increase in either docosahexaenoic acid or arachidonic acid in membrane phospholipids. *PLoS ONE,* (2012). **7**(3).

195. Aoun M, Feillet-Coudray C, Fouret G, Chabi B, Crouzier D, Ferreri C, Chatgilialoglu C, Wrutniak-Cabello C, Cristol JP, Carbonneau MA, and Coudray C. Rat liver mitochondrial membrane characteristics and mitochondrial functions are more profoundly altered by dietary lipid quantity than by dietary lipid quality: Effect of different nutritional lipid patterns. *British Journal of Nutrition,* (2012). **107**(5): p. 647-659.
196. Long EK and Picklo MJ. Trans-4-hydroxy-2-hexenal, a product of n-3 fatty acid peroxidation: Make some room hne. *Free Radical Biology and Medicine,* (2010). **49**(1): p. 1-8.
197. Kagan VE, Tyurin VA, Jiang JF, Tyurina YY, Ritov VB, Amoscato AA, Osipov AN, Belikova NA, Kapralov AA, Kini V, Vlasova, II, Zhao Q, Zou MM, Di P, Svistunenko DA, Kurnikov IV, and Borisenko GG. Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors. *Nature Chemical Biology,* (2005). **1**(4): p. 223-232.
198. Balakrishnan G, Hu Y, Oyerinde OF, Su J, Groves JT, and Spiro TG. A conformational switch to beta-sheet structure in cytochrome c leads to heme exposure. Implications for cardiolipin peroxidation and apoptosis. *Journal of the American Chemical Society,* (2007). **129**(3): p. 504-505.
199. Watkins SM, Carter LC, and German JB. Docosahexaenoic acid accumulates in cardiolipin and enhances ht-29 cell oxidant production. *Journal of Lipid Research,* (1998). **39**(8): p. 1583-1588.
200. McCord JM. The evolution of free radicals and oxidative stress. *American Journal of Medicine,* (2000). **108**(8): p. 652-659.
201. Girotti AW. Lipid hydroperoxide generation, turnover, and effector action in biological systems. *Journal of Lipid Research,* (1998). **39**(8): p. 1529-1542.
202. Montfoort A, Bezstarosti K, Groh MMJ, and Metsaketela TJA. The influence of the lipid composition on the degree of lipid peroxidation of liposomes. *Biochemistry International,* (1987). **15**(3): p. 525-543.
203. Norris SE, Mitchell TW, and Else PL. Phospholipid peroxidation: Lack of effect of fatty acid pairing. *Lipids,* (2012). **47**(5): p. 451-460.
204. Szebeni J and Toth K. Lipid peroxidation in hemoglobin-containing liposomes-effects of membrane phospholipid composition and cholesterol content. *Biochimica Et Biophysica Acta,* (1986). **857**(2): p. 139-145.
205. Sinibaldi F, Fiorucci L, Patriarca A, Lauceri R, Ferri T, Coletta M, and Santucci R. Insights into cytochrome c-cardiolipin interaction. Role played by ionic strength. *Biochemistry,* (2008). **47**(26): p. 6928-6935.
206. Ivanov I, Heydeck D, Hofheinz K, Roffeis J, O'Donnell VB, Kuhn H, and Walther M. Molecular enzymology of lipoxygenases. *Archives of Biochemistry and Biophysics,* (2010). **503**(2): p. 161-174.
207. Roginsky V. Oxidizability of cardiac cardiolipin in triton x-100 micelles as determined by using a clark electrode. *Chemistry and Physics of Lipids,* (2010). **163**(2): p. 127-130.
208. Devasagayam TPA, Boloor KK, and Ramasarma T. Methods for estimating lipid peroxidation: An analysis of merits and demerits. *Indian Journal of Biochemistry and Biophysics,* (2003). **40**(5): p. 300-308.
209. Timabud T, Sanitchon J, and Pongdontri P. A modified ferrous oxidation-xylenol orange assay for lipoxygenase activity in rice grains. *Food Chemistry,* (2013). **141**(3): p. 2405-2411.

210. Spickett CM, Wiswedel I, Siems W, Zarkovic K, and Zarkovic N. Advances in methods for the determination of biologically relevant lipid peroxidation products. *Free Radical Research,* (2010). **44**(10): p. 1172-1202.
211. Villaverde JJ, Santos SAO, Simões MMQ, Neto CP, Domingues MRM, and Silvestre AJD. Analysis of linoleic acid hydroperoxides generated by biomimetic and enzymatic systems through an integrated methodology. *Industrial Crops and Products,* (2011). **34**(3): p. 1474-1481.
212. Grintzalis K, Zisimopoulos D, Grune T, Weber D, and Georgiou CD. Method for the simultaneous determination of free/protein malondialdehyde and lipid/protein hydroperoxides. *Free Radical Biology and Medicine,* (2013). **59**: p. 27-35.
213. Abrash HI. The relative acidities of water and methanol. *Journal of Chemical Education,* (2001). **78**(11): p. 1496-1498.
214. Cortie CH and Else PL. Dietary docosahexaenoic acid (22:6) incorporates into cardiolipin at the expense of linoleic acid (18:2): Analysis and potential implications. *International Journal of Molecular Sciences,* (2012). **13**(11): p. 15447-15463.
215. Kiebish MA, Han XL, Cheng H, Chuang JH, and Seyfried TN. Cardiolipin and electron transport chain abnormalities in mouse brain tumor mitochondria: Lipidomic evidence supporting the warburg theory of cancer. *Journal of Lipid Research,* (2008). **49**(12): p. 2545-2556.
216. Sen T, Sen N, Tripathi G, Chatterjee U, and Chakrabarti S. Lipid peroxidation associated cardiolipin loss and membrane depolarization in rat brain mitochondria. *Neurochemistry International,* (2006). **49**(1): p. 20-27.
217. Sevanian A and Hochstein P. Mechanisms and consequences of lipid-peroxidation in biological systems. *Annual Review of Nutrition,* (1985). **5**: p. 365-390.
218. Volinsky R and Kinnunen PKJ. Oxidized phosphatidylcholines in membrane-level cellular signaling: From biophysics to physiology and molecular pathology. *Febs Journal,* (2013). **280**(12): p. 2806-2816.
219. Porter NA. A perspective on free radical autoxidation: The physical organic chemistry of polyunsaturated fatty acid and sterol peroxidation. *Journal of Organic Chemistry,* (2013). **78**(8): p. 3511-3524.
220. Hulbert AJ, Rana T, and Couture P. The acyl composition of mammalian phospholipids: An allometric analysis. *Comp. Biochem. Physiol. B-Biochem. Mol. Biol.*, (2002). **132**(3): p. 515-527.
221. Soto-Arriaza MA, Sotomayor CP, and Lissi EA. Relation between lipid peroxidation and rigidity in l-α -phosphatidylcholine-dppc vesicles. *Journal of Colloid and Interface Science,* (2008). **323**: p. 7-74.
222. Judde A, Villeneuve P, Rossignol-Castera A, and Le Guillou A. Antioxidant effect of soy lecithins on vegetable oil stability and their synergism with tocopherols. *Journal of the American Oil Chemists Society,* (2003). **80**(12): p. 1209-1215.
223. Silvius JR, *Thermotropic phase transitions of pure lipids in model membranes and their modifications by membrane proteins*. 1982, New York: John Wiley & Sons, Inc.
224. McPherson PAC, Bole A, Cruz KA, Young IS, and McEneny J. A curvilinear approach to the kinetic analysis of linoleate peroxidation in aqueous liposomes by 2,2 ' azobis(2-amidoinopropane) dihydrochloride. *Chemistry and Physics of Lipids,* (2012). **165**(6): p. 682-688.

225. Deeley JM, Mitchell TW, Wei XJ, Korth J, Nealon JR, Blanksby SJ, and Truscott RJW. Human lens lipids differ markedly from those of commonly used experimental animals. *BBA-MOL. Cell. Biol. L.*, (2008). **1781**(6-7): p. 288-298.
226. Wood JD, Enser M, Fisher AV, Nute GR, Sheard PR, Richardson RI, Hughes SI, and Whittington FM. Fat deposition, fatty acid composition and meat quality: A review. *Meat Science*, (2008). **78**(4): p. 343-358.
227. Adibhatla RM and Hatcher JF. Lipid oxidation and peroxidation in cns health and disease: From molecular mechanisms to therapeutic opportunities. *Antioxidants & Redox Signaling*, (2010). **12**(1): p. 125-169.
228. Yavin E, Brand A, and Green P. Docosahexaenoic acid abundance in the brain: A biodevice to combat oxidative stress. *Nutritional Neuroscience*, (2002). **5**(3): p. 149-157.
229. Weylandt KH, Chiu CY, Gomolka B, Waechter SF, and Wiedenmann B. Omega-3 fatty acids and their lipid mediators: Towards an understanding of resolvin and protectin formation omega-3 fatty acids and their resolvin/protectin mediators. *Prostaglandins & Other Lipid Mediators*, (2012). **97**(3-4): p. 73-82.
230. Martinez M. Abnormal profiles of polyunsaturated fatty-acids in the brain, liver, kidney and retina of patients with peroxisomal disorders. *Brain Research*, (1992). **583**(1-2): p. 171-182.
231. Martín V, Fabelo N, Santpere G, Puig B, Marin R, Ferrer I, and Diaz M. Lipid alterations in lipid rafts from alzheimer's disease human brain cortex. *Journal of Alzheimers Disease*, (2010). **19**(2): p. 489-502.
232. Norris SE, Friedrich MG, Mitchell TW, Truscott RJW, and Else PL. Human prefrontal cortex phospholipids containing docosahexaenoic acid increase during normal adult aging, whereas those containing arachidonic acid decrease. *Neurobiology of Aging*, (2015)(0).
233. Broniec A, Klosinski R, Pawlak A, Wrona-Krol M, Thompson D, and Sarna T. Interactions of plasmalogens and their diacyl analogs with singlet oxygen in selected model systems. *Free Radical Biology and Medicine*, (2011). **50**(7): p. 892-898.
234. Engelmann B. Plasmalogens: Targets for oxidants and major lipophilic antioxidants. *Biochemical Society Transactions*, (2004). **32**: p. 147-150.
235. Berry KAZ and Murphy RC. Electrospray ionization tandem mass spectrometry of glycerophosphoethanolamine plasmalogen phospholipids. *Journal of the American Society for Mass Spectrometry*, (2004). **15**(10): p. 1499-1508.
236. Abbott SK, Jenner AM, Mitchell TW, Brown SHJ, Halliday GM, and Garner B. An improved high-throughput lipid extraction method for the analysis of human brain lipids. *Lipids*, (2013). **48**(3): p. 307-318.
237. Fraser T, Tayler H, and Love S. Low-temperature improved-throughput method for analysis of brain fatty acids and assessment of their post-mortem stability. *Journal of Neuroscience Methods*, (2008). **169**(1): p. 135-140.
238. Ylaherttuala S and Nikkari T. Effect of post-mortem time on the biochemical composition of coronary arteries. *Atherosclerosis*, (1985). **56**(1): p. 1-10.
239. Korytowski W, Basova LV, Pilat A, Kernstock RM, and Girotti AW. Permeabilization of the mitochondrial outer membrane by bax/truncated bid (tbid) proteins as sensitized by cardiolipin hydroperoxide translocation: Mechanistic implications for the intrinsic pathway of oxidative apoptosis. *J Biol Chem*, (2011). **286**(30): p. 26334-43.
240. Pamploma R and Barja G, Longevity, mitochondria and oxygen free radicals, SG Pandalai, Editor. 2010, Research Signpost: Trivandrum, India.

241. Burke PA, Ling PR, Forse RA, Lewis DW, Jenkins R, and Bistrian BR. Sites of conditional essential fatty acid deficiency in end stage liver disease. *Journal of Parenteral and Enteral Nutrition,* (2001). **25**(4): p. 188-193.
242. Sekine K. Hepatic role in the storage and utilization of fish-oil fatty-acids in humans- studies on liver surgery patients. *Internal Medicine,* (1995). **34**(3): p. 139-143.
243. Elizondo A, Araya J, Rodrigo R, Poniachik J, Csendes A, Maluenda F, Diaz JC, Signorini C, Sgherri C, Comporti M, and Videla LA. Polyunsaturated fatty acid pattern in liver and erythrocyte phospholipids from obese patients. *Obesity,* (2007). **15**(1): p. 24-31.
244. Gutierrez AM, Reboredo GR, Arcemis CJ, and Catala A. Non-enzymatic lipid peroxidation of microsomes and mitochondria isolated from liver and heart of pigeon and rat. *International Journal of Biochemistry & Cell Biology,* (2000). **32**(1): p. 73-79.
245. Onibi GE, Scaife JR, Murray I, and Fowler VR. Supplementary alpha-tocopherol acetate in full-fat rapeseed-based diets for pigs: Effects on the alpha-tocopherol content and fatty acid composition of pig muscle microsomal and mitochondrial membranes and membrane lipid peroxidation. *Journal of the Science of Food and Agriculture,* (2000). **80**(11): p. 1633-1639.
246. Graham J. Preparation of crude subcellular fractions by differential centrifugation. *TheScientificWorldJournal,* (2002). **2**.
247. Gao F, Kim HW, Igarashi M, Kiesewetter D, Chang L, Ma KZ, and Rapoport SI. Liver conversion of docosahexaenoic and arachidonic acids from their 18-carbon precursors in rats on a dha-free but alpha-lna-containing n-3 pufa adequate diet. *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids,* (2011). **1811**(7-8): p. 484-489.
248. Pauter AM, Olsson P, Asadi A, Herslof B, Csikasz RI, Zadravec D, and Jacobsson A. Elovl2 ablation demonstrates that systemic dha is endogenously produced and is essential for lipid homeostasis in mice. *Journal of Lipid Research,* (2014). **55**(4): p. 718-728.
249. Modi HR and Katyare SS. Cadmium exposure-induced alterations in the lipid/phospholipids composition of rat brain microsomes and mitochondria. *Neuroscience Letters,* (2009). **464**(2): p. 108-112.
250. Tyurina YY, Shvedova AA, Kawai K, Tyurin VA, Kommineni C, Quinn PJ, Schor NF, Fabisiak JP, and Kagan VE. Phospholipid signaling in apoptosis: Peroxidation and externalization of phosphatidylserine. *Toxicology,* (2000). **148**(2-3): p. 93-101.
251. Farooqui AA, Horrocks LA, and Farooqui T. Glycerophospholipids in brain: Their metabolism, incorporation into membranes, functions, and involvement in neurological disorders. *Chemistry and Physics of Lipids,* (2000). **106**(1): p. 1-29.

Appendix A – Scans used in shotgun lipidomics analysis.

Appendix Table A. Targeted ion scans used to acquired phospholipid data

	Ion Mode	Scan	DP	EP	CE	CXP	Mass Range	Da/ second
Fatty acid chain scans								
PC	Positive	PI 184.1	100	10	47	8	640–1000	200
Lyso–PC	Positive	PI 184.1	100	10	47	8	450–600	200
PE	Positive	NL 141	100	10	30	8	685–950	200
Lyso–PE	Positive	NL 141	100	10	30	8	400–600	200
PS	Positive	NL 185	100	10	30	8	755–965	200
Fatty acid chain scans								
14:0	Negative	PI 227.2	-100	-10	-55	-11	580–900	1000
16:1	Negative	PI 253.2	-100	-10	-55	-11	600–900	1000
16:0	Negative	PI 255.2	-100	-10	-55	-11	600–900	1000
17:0	Negative	PI 269.3	-100	-10	-55	-11	560–900	1000
18:3	Negative	PI 277.2	-100	-10	-40	-11	600–900	1000
18:2	Negative	PI 279.2	-100	-10	-40	-11	600–900	1000
18:1	Negative	PI 281.3	-100	-10	-55	-11	600–900	1000
18:0	Negative	PI 283.3	-100	-10	-55	-11	600–900	1000
19:0	Negative	PI 297.3	-100	-10	-55	-11	600–900	1000
20:5	Negative	PI 301.2	-100	-10	-40	-11	500–1000	1000
20:4	Negative	PI 303.2	-100	-10	-40	-11	600–1000	1000
20:3	Negative	PI 305.2	-100	-10	-40	-11	600–1000	1000
20:2	Negative	PI 307.2	-100	-10	-40	-11	600–1000	1000
20:1	Negative	PI 309.2	-100	-10	-55	-11	600–1000	1000
20:0	Negative	PI 311.2	-100	-10	-55	-11	600–1000	1000
22:6	Negative	PI 327.2	-100	-10	-40	-11	700–1000	1000
22:5	Negative	PI 329.2	-100	-10	-40	-11	700–1000	1000
22:4	Negative	PI 331.2	-100	-10	-40	-11	700–1000	1000
22:3	Negative	PI 333.3	-100	-10	-40	-11	600–1000	1000
22:2	Negative	PI 335.2	-100	-10	-40	-11	700–1000	1000
22:1	Negative	PI 337.3	-100	-10	-55	-11	700–1000	1000
22:0	Negative	PI 339.3	-100	-10	-55	-11	600–1000	1000
24:1	Negative	PI 365.3	-100	-10	-55	-11	700–1000	1000

Mass shifting was prevented in negative ion mode by increasing number of summed scans. PI precursor ion, NL neutral loss, DP declustering potential, EP entrance potential, CE collision energy, CXP collision cell exit potential, PC phosphatidylcholine; PE phosphatidylethanolamine; PS phosphatidylserine.

Appendix B – Distribution of phospholipids in mitochondrial and microsomal fractions of mouse, pig and human tissues.

Appendix Table B1. Distribution of phospholipid classes in the mitochondrial and microsomal fractions of mouse, pig, and human skeletal muscle

	Human		Mouse		Pig	
	Mitochondria	Microsomal	Mitochondria	Microsomal	Mitochondria	Microsomal
Phosphatidylcholine						
PC 16:0_16:0	0.0±0.0	0.0±0.0	6.4±0.3	0.0±0.0	0.0±0.0	0.0±0.0
PC 16:0_18:1	22.4±1.8	29.4±1.9	17.4±0.9	16.8±0.6	16.3±1.5	14.8±1.5
PC 16:0_18:2	20.7±2.7	26.9±2.7	11.0±1.0	11.9±0.5	29.0±0.8	31.8±2.2
PC 16:0_20:4	0.0±0.0	0.0±0.0	14.6±1.4	13.8±0.6	0.0±0.0	0.0±0.0
PC 16:0_22:6	0.0±0.0	0.0±0.0	16.9±1.5	15.3±1.0	0.0±0.0	0.0±0.0
PC 18:0_18:2	0.0±0.0	7.1±0.6	0.0±0.0	0.0±0.0	4.8±1.6	0.0±0.0
PC 18:1_18:1	8.1±1.5	9.1±1.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
PC 8:1_18:2	0.0±0.0	3.4±1.6	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Phosphatidylethanolamine						
PE 16:0_20:4	0.0±0.0	0.0±0.0	2.3±1.9	1.6±0.5	0.0±0.0	0.0±0.0
PE 16:0_22:6	0.0±0.0	0.0±0.0	13.5±3.1	17.3±1.5	0.0±0.0	0.0±0.0
PE 18:0_18:2	23.9±6.1	14.8±1.9	9.5±5.0	4.4±0.4	20.8±2.1	16.2±3.5
PE 18:0_20:4	20.7±2.3	11.9±2.1	14.5±8.0	7.3±0.9	10.2±1.5	18.8±11.7
PE 18:0_22:4	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
PE 18:0_22:6	1.9±0.8	0.0±0.0	28.0±5.9	29.6±2.3	0.0±0.0	0.0±0.0
PE 18:1_18:1	2.5±0.6	1.9±0.9	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
PE 18:1_18:2	5.7±0.9	4.9±0.5	0.0±0.0	0.0±0.0	4.3±0.5	3.2±0.8
PE 18:1_22:6	0.0±0.0	0.0±0.0	2.4±0.5	2.7±0.6	0.0±0.0	0.0±0.0
Phosphatidylserine						
PS 18:0_18:1	6.9±1.2	6.8±1.3	7.0±0.6	3.1±0.3	5.5±0.6	5.1±0.5
PS 18:0_20:4	8.1±0.9	8.5±0.5	6.6±1.8	5.6±1.1	4.5±1.6	5.4±0.6
PS 18:0_22:6	15.4±2.2	18.4±5	60.6±5.1	65±3.6	7.8±1.2	6.4±0.8
PS 18:0_22:5	15.8±3.2	16.6±2.2	11.1±1.1	14.7±2.0	9.9±2.6	9.5±2.2
PS 18:0:_22:4	10.5±1.2	15.1±3.5	5.3±1.0	8.1±0.8	9.6±2.1	8.7±1.1
PS 18:1_18:1	44.7±6.0	43.2±7.1	9.3±2.2	5.2±1.0	66.1±5.2	65.1±3.7

Values are means ±SEM, *n*=6.

Appendix Table B2. Distribution of phospholipid classes in the mitochondrial and microsomal fractions of mouse, pig, and human liver

	Human		Mouse		Pig	
	Mitochondria	Microsomal	Mitochondria	Microsomal	Mitochondria	Microsomal
Phosphatidylcholine						
PC 16:0_16:0	0.0±0.0	0.0±0.0	9.5±2.3	0.0±0.0	0.0±0.0	0.0±0.0
PC 16:0_18:1	31.2±1.8	33.9±3.3	32±6.7	14.1±0.8	18.1±1.0	19.1±0.9
PC 16:0_18:2	25.5±6.2	23.7±3.5	9.8±3.2	16.3±1	11.0±0.5	11.2±0.6
PC 16:0_20:4	5.8±1.7	10.5±1.3	6.4±2.4	11.1±0.8	0.0±0.0	0.0±0.0
PC 16:0_22:6	0.0±0.0	0.0±0.0	0.0±0.0	9±0.1	0.0±0.0	0.0±0.0
PC 18:0_18:1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	9.2±0.4	9.9±0.5
PC 18:0_18:2	4.4±1.8	5.3±1.8	9.3±2	10.5±1.2	16.4±0.7	17.1±0.4
PC 18:0_20:4	0.0±0.0	0.0±0.0	0.0±0.0	7.9±1.6	13.3±0.9	12.9±1.5
Phosphatidylethanolamine						
PE 16:0_20:4	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	4.7±0.2	0.0±0.0
PE 16:0_22:5	5.2±1.7	9.0±2.6	0.0±0.0	2.8±0.7	0.0±0.0	2.8±0.6
PE 16:0_22:6	0.0±0.0	0.0±0.0	0.0±0.0	31.5±1.3	3.2±0.3	3.8±0.2
PE 18:0_18:2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	15.6±0.6	19.5±1.9
PE 18:0_20:4	69.5±7.8	50.6±5.7	64.4±8.3	20.8±0.6	38.8±1.1	40.9±1.8
PE 18:0_22:4	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
PE 18:0_22:6	10.4±2.8	0.0±0.0	13.7±4.3	8.9±0.2	4.2±0.6	5.1±0.5
PE 18:1_18:2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	2.3±0.5	2.0±0.4
PE 18:1_20:4	0.0±0.0	0.0±0.0	12.0±3.1	10.1±0.8	2.7±0.2	3.3±0.2
PE 18:1_22:6	0.0±0.0	0.0±0.0	4.7±1.5	5.1±0.1	0.0±0.0	0.0±0.0
Phosphatidylserine						
PS 18:0_18:1	7.3±2.0	5.6±0.9	6.0±0.8	0.1±0.1	4.8±0.5	5.9±1.0
PS 18:0_20:4	31.0±6.9	25.2±8	54.3±2.1	55.7±1.5	54.4±3.2	54.3±2.4
PS 18:0_22:6	28.1±3.4	32.0±5.5	33.3±1.4	34.4±2.4	18.7±3.2	18.5±3.0
PS 18:0_22:5	13.2±2.4	9.6±0.7	3±1.1	3.1±1.2	12.6±1	12.7±1.1
PS 18:0:_22:4	7.7±0.7	7.8±0.9	3.6±1.1	4.8±0.4	5.1±1.0	5.1±1.0
PS 18:1_18:1	12.7±3.1	7.8±0.9	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Values are means ±SEM, *n*=6.

Appendix Table B3. Distribution of phospholipid classes in the mitochondrial and microsomal fractions of mouse, pig, and human brain

	Human		Mouse		Pig	
	Mitochondria	Microsomal	Mitochondria	Microsomal	Mitochondria	Microsomal
Phosphatidylcholine						
PC 16:0_16:0	8.8±2.0	5.5±1.8	15.0±2.8	4.9±2.1	13.7±1.2	0.0±0.0
PC 16:0_18:1	45.7±2.2	41.3±2.1	35.9±0.6	34.8±1.5	38.8±0.6	42.1±13.9
PC 16:0_18:2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	12.2±7.3
PC 16:0_20:4	2.7±0.2	2.8±0.2	4.5±0.1	5.9±0.5	2.1±0.6	3.4±1.4
PC 16:0_22:6	0.0±0.0	0.0±0.0	4.9±0.2	6.7±0.6	0.0±0.0	0.0±0.0
PC 16:1_18:1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.5±0.5	12.4±6.3
PC 18:0_18:2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
PC 18:0_20:4	1.9±0.6	2.3±0.6	4.8±0.2	7.4±0.5	2.2±1.0	4.6±1.5
PC 18:0_22:6	0.0±0.0	0.0±0.0	3.2±0.2	0.0±0.0	0.0±0.0	0.0±0.0
PC 18:1_18:1	3.8±0.8	3.4±0.9	0.0±0.0	0.0±0.0	3.3±0.2	11.0±3.1
PC 18:1_18:2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Phosphatidylethanolamine						
PE 18:1_18:1	0.0±0.0	0.0±0.0	4.4±0.3	0.0±0.0	0.0±0.0	0.0±0.0
PE 18:0_22:6	38.5±2.8	25.3±1.3	36.6±0.9	38.3±3.2	19.4±1.9	20.5±2.0
PE 18:0_22:4	4.5±2.1	5.3±0.3	2.4±0.8	2.4±1.6	5.7±1.0	7.5±0.8
PE 18:0_20:4	13.9±1.8	6.9±0.9	16.2±0.3	13.1±2.3	13.8±3.9	14.8±3.7
PE 16:0_22:6	0.0±0.0	0.0±0.0	10.2±0.5	10.1±2.7	0.0±0.0	0.0±0.0
Phosphatidylserine						
PS 18:0_18:1	12.7±1.3	8.4±1.1	41.0±3.1	30.8±3.0	33.7±4.2	4.7±0.6
PS 18:0_20:4	5.8±0.5	7.9±1.1	10.4±0.4	0.0±0.0	5.3±1.1	5.5±0.6
PS 18:0_22:6	43.5±2.7	44.0±3.5	17.7±1.4	21.2±2.3	8.9±1.1	47.1±3.9
PS 18:0_22:5	6.6±0.4	8.0±1.0	0.0±0.0	0.0±0.0	18.5±3.4	9.1±0.9
PS 18:1_18:1	21.2±0.9	14.6±1.3	20.4±1.6	12.1±3.0	10.2±1.1	12.5±1.1

Values are means ±SEM, *n*=6.