UNIVERSITY OF SOUTHAMPTON

Modifications to LDL and endothelial cells by oxidation and n-3 fatty acids.

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Abstract

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Modifications to endothelial cell function and LDL by n-3 fatty acids.

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Initial endothelial dysfunction is critical in the pathogenesis of atherosclerosis leading to an accumulation of lipids and inflammatory cells in the sub-endothelial space, resulting in the formation of an atherosclerotic plaque. There is growing interest in dietary intervention into the role of n-3 fatty acids, found at high levels in oily fish as potential anti-atherogenic agents. Mechanisms of action may possibly include the modification of membrane or plasma phospholipid compositions, resulting in changes to the cell signalling or LDL oxidation. The products of LDL oxidation are potential substrates Lp-PLA_2, which is increasingly considered to be an independent marker of cardiovascular disease. Consequently, ESI-MS was used together with stable isotope labelling to determine the temporal responses of cell phosphatidylcholine (PC) composition, synthesis de novo and acyl remodelling by human umbilical vein endothelial cells (HUVEC) cultured with exogenous fatty acids, the resultant changes in cytokine production were measured. The modifications to PC composition of plasma were also measured by ESI-MS following *in vivo* supplementation with either DHA or EPA fatty acids for four weeks. LDL was isolated from the modified plasma and subjected to copper oxidation in the presence and absence of an Lp-PLA_2 inhibitor and the oxidised PC species produced were compared to those formed in control LDL.

These results demonstrated that HUVEC PC composition was resistant to change in response to the added fatty acids, small changes in composition were achieved primarily by acyl remodelling of existing PC species rather than by changing the specificity of synthesis de novo; changes to cytokine production in the cells was also largely unaffected. *In vivo* fatty acid supplementation resulted in large changes to specific PC species in plasma. However, following LDL isolation and oxidation, the same oxidised species were produced for the fatty acid modified LDLs and the control LDL. Oxidation increased three groups of PC species: lysoPC and low and high mass oxidised PC. A higher proportion of low mass oxidised species were produced in the fatty acid modified LDLs but there was no accumulation of the high mass species, which demonstrated the largest increase in the control LDL. The inclusion of the Lp-PLA_2 inhibitor in the oxidation reaction identified lysoPC and low mass oxidised PC species as the products and substrates respectively of the inhibitor. High resolution mass spectrometry allowed the exact masses and elemental formulae to be assigned for each of the oxidised species produced.

Declaration of Authorship

The research reported in this thesis is my own original work, except where otherwise stated, and has not been submitted for any other degree.

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List of Abbreviations

AA	Arachadonic acid
BSA	Bovine serum albumin
CID	Collision induced dissociation
EDTA	Ethylenediaminetetraacetic acid
DAG	Diacylglycerol
DHA	Docosahexaenoic acid
ELISA	Enzyme linked immunoassay
EPA	Eicosapentaenoic acid
ESI-MS	Electrospray ionisation mass spectrometry
FACS	Flow cytometry Fluorescence Activated Cell Sorting
HBSS	Hanks Balanced Salt Solution
HDL	High density lipoprotein
HPLC/MS	High pressure liquid chromatography/ mass spectrometry
HUVECS	Human umbilical vein endothelial cells
ICAM-1	Intracellular adhesion molecule 1
IL-1β	Interleukin 1β

IL-6	Interleukin-6
IL-8	Interleukin-8
LDL	Low Density Lipoprotein
$LpPLA_2$	Lipoprotein-associated phospholipase A_2
lysoPC	Lysophosphatidylcholine
mmLDL	Minimally modified LDL
m/z	Mass to charge ratio
NaCl	Sodium chloride
NFκB	Nuclear factor kappa B
oxLDL	Oxidised LDL
oxPC	Oxidised phosphatidylcholine
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PECAM-1	Platelet/endothelial cell adhesion molecule-1
PG	Phosphatidyglycerol
PI	Phosphotidylinositol
PS	Phosophatidylserine

PUFAs	Polyunsaturated fatty acids
QToF	Quadrupole Time-of-Flight
TBARS	Thiobarbituric acid-reactive substance
TCA	Tricholoacetic acid
TLC	Thin layer chromatography
TNFα	Tumour necrosis factor α
VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very Low Density Lipoprotein
8-isoPGF2a	8-iso-prostaglandin $F_{2\alpha}$

Chapter One

Introduction

1.1 Overview

The endothelial cell is situated at the interface between the circulating blood and the vessel wall. It serves as a sensor and transductor of signals within the circulatory system and is integral to maintaining the homeostatic balance of the vessel through the production of factors that regulate vessel tone, coagulation state, lipid transport, cellular responses and leucocyte trafficking. It is widely recognised that injury to the endothelium is pivotal in the development of atherosclerosis and its complications. Atherosclerosis is an inflammatory disease, in which lipids and cellular waste products are deposited in the sub-endothelial space of large and medium arteries following endothelial damage. The accumulation of waste products, or plaque, can continue to develop over a person's lifetime, leading to significantly reduced blood flow through the artery. Ultimately, this leads to ischemia of the heart, brain or extremities, resulting in infarction. Atherosclerosis and its complications continue to be the leading cause of mortality and morbidity throughout the developed world (Glass et al. 2001). The lipid products that build up in the artery are generally thought to be low density lipoprotein (LDL) which has been modified, usually by oxidation, thereby changing its properties and function. There is very little known about the structure and composition of modified LDL *in vivo* or if LDL oxidised *in vitro* has a similar structure. Elevated serum cholesterol particularly the apolipoprotein B containing form, is an important aetiological factor in the pathogenesis of atherosclerosis. However there is wide variation in the level of cholesterol that infers coronary heart disease, so there are, therefore, other factors involved.

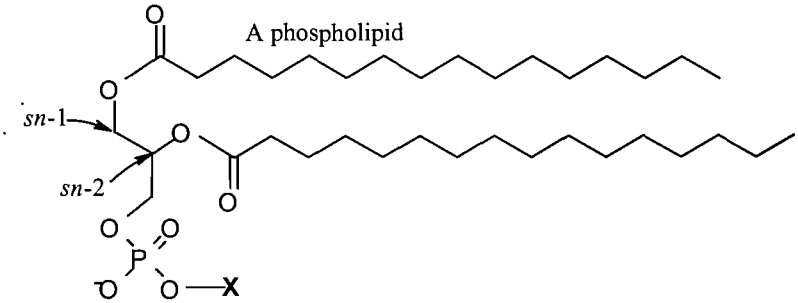
Epidemiology evidence suggests that dietary intervention can result in protection against the development of cardiovascular diseases. The consumption of n-3 fatty acids, found naturally in high amounts in oily fish, has been suggested to be beneficial for decreasing the risk of cardiovascular disease. However, this theory is largely based upon epidemiology evidence and there is very little evidence to suggest how these fatty acids exert their protective affects. There are two major areas on which this thesis will concentrate. The first area will focus on the oxidation of control and DHA or EPA modified LDL in the presence and absence of an Lp-PLA_2 inhibitor. The second area will concentrate on the phospholipid changes endothelial cells *in vitro* after supplementation with n-3 fatty acids, and the resultant changes in cell signalling.

1.2 Phospholipids

1.2.1 Phospholipid overview

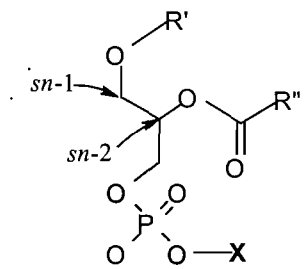
Phospholipids are lipid molecules in which phosphate ester groups feature as the common component. There are two main classes of phospholipid; glycerophospholipids which contain glycerol, a three-carbon alcohol and sphingophospholipids which contain sphingosine, a complex alcohol. This thesis will focus upon glycerophospholipids.

The general structure of a glycerophospholipid is that of a three carbon sugar molecule with a fatty acyl or alkyl group at the sn-1 position and fatty acyl group at the sn-2 position, figure 1.1. The polar headgroup attached to the phosphate defines the class of glycerophospholipid, of which there are six main classes. These are: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosophatidylserine (PS), phosphotidylinositol (PI) phosphatidyglycerol (PG) and phosphatidic acid (PA), figure 1.1. The molecular species composition within each phospholipid class is determined by the combination of fatty acids attached to the glycerophosphate backbone. The fatty acids usually contain an even number of carbon atoms, and may be saturated or unsaturated. Table 1.1 lists the most common fatty acids and their structures. Phospholipids are particularly important as constituents of cell membranes, where the different classes of phospholipids are present in different amounts depending on the function of the cell, although PC is generally the major species present.

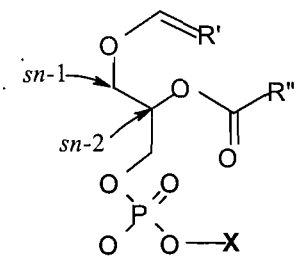


The class of a phospholipid is defined by the nature of the nitrogenous base or polyol esterified to the phosphate group (**X**). The species distribution within any phospholipid class is determined by the fatty acyl substitutes at the *sn*-1 and *sn*-2 positions of the glycerol backbone. The dipalmitoyl shown here would be designated PC16:0/16:0 if X was choline. If arachidonic acid was esterified at *sn*-2, the molecule would be designated PC16:0/20:4.

In the diacyl species shown above, fatty acids are attached by ester linkages. For *sn*-1-alkyl-*sn*-2-acyl species, the *sn*-1 fatty acid is attached by an ether bond. For *sn*-1alkenyl-*sn*-2-acyl species, the*sn*-1 fatty acid is attached by a vinyl ether linkage.



sn-1-alkyl-*sn*-2acyl species



sn-1alkenyl-*sn*-2-acyl species

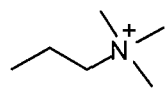
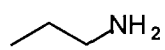
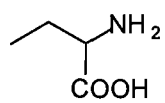
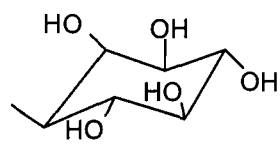
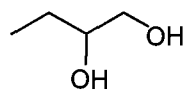
Phospholipid	**Headgroup(X)**
Phosphatidylcholine (PC)	
Phosphatidylethanolamine(PE)	
Phosphatidylserine (PS)	
Phosphatidylinositol (PI)	
Phosphatidylglycerol (PG)	
Phosphatidic acid (PA)	

Figure 1.1: Molecular structures of phospholipids, illustrating the different classes of phospholipids.

No. carbons: double bonds	Name	Structure
Saturated		
14:0	Myristic	$CH_3(CH_2)_{12}COOH$
16:0	Palmitic	$CH_3(CH_2)_{14}COOH$
18:0	Stearic	$CH_3(CH_2)_{16}COOH$
Unsaturated		
16:1 (ω-7)	Palmitoleic	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$
18:1 (ω-9)	Oleic	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$
18:2 (ω-6)	Linoleic	$CH_3(CH_2)_4CH=CHCH_2CH=CH-(CH_2)_7COOH$
20:4 (ω-6)	Arachidonic	$CH_3(CH_2)_4(CH=CHCH_2)_4CH_2$-CH2COOH
20:5 (ω-3)	Eicosapentaenoic	$CH_3CH_2(CH=CHCH_2)_5(CH_2)_2COOH$
22:6 (ω-3)	Docosahexaenoic	$CH_3CH_2(CH=CHCH_2)_6CH_2COOH$

Table 1.1: Fatty acid names and structures.

1.3 Mass spectrometry

A mass spectrometer is an analytical tool that determines the molecular weight of chemical compounds by separating molecular ions according to their mass to charge ratio (m/z) (Biemann 1963). In order to analyse a sample by mass spectrometry, a sample at atmospheric pressure must be introduced into the instrument such that the vacuum within remains largely unchanged.

In order for a molecule to be detectable by mass spectrometry it must carry a charge. Molecular ions are generated by inducing either the loss or gain of a proton in the ionisation source. Charged molecules are then separated according to their m/z by the mass filter/ analyser and subsequently detected by the ion detector. The result of ionisation, ion separation and detection is a mass spectrum that can provide molecular weight and structural information. The fundamental components of a mass spectrometer are shown in figure 1.2.

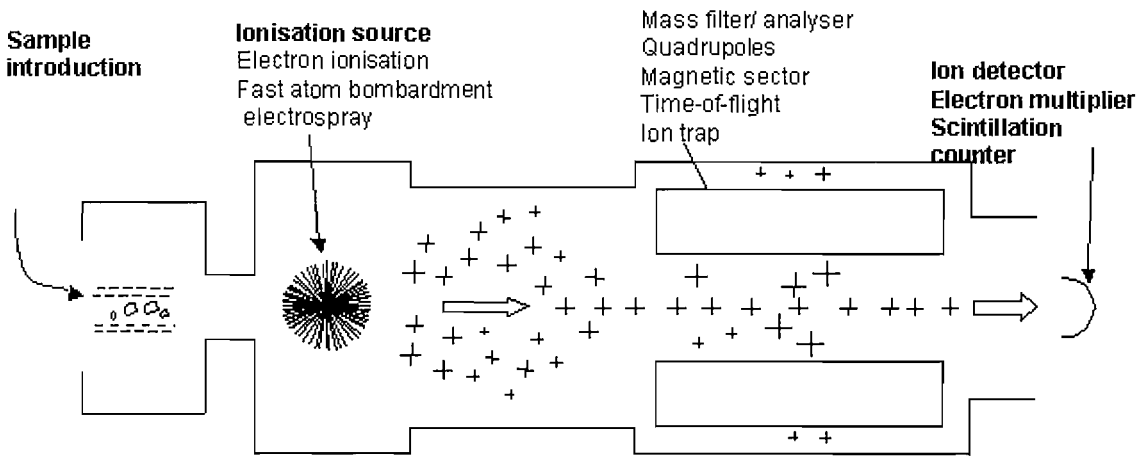


Figure 1.2: Fundamental components of a mass spectrometer. The sample enters the mass spectrometer where it becomes ionised in the ionisation source, is analysed in the quadrupoles and is then detected.

1.3.1 Electrospray ionisation (ESI-MS)

Electrospray ionisation is a technique used to generate gaseous ionised molecules from a liquid. The sample of interest is sprayed out of a fine capillary, across which a high voltage is applied (typically 1.5 – 4 kVolts). This produces charged droplets which are

electrostatically attracted to the mass spectrometer inlet. Dry heated gas is applied to the droplets before they enter the vacuum of the mass spectrometer causing the solvent to evaporate from the droplet surface. As the droplets decrease in size, the electric field density on its surface increases. The repulsion between like charges on the droplet surface becomes so great that they eventually exceed the force of surface tension and ions begin to leave the droplet through a Taylor cone, figure 1.3. This soft ionisation technique is capable of producing non-fragmented molecular ions.

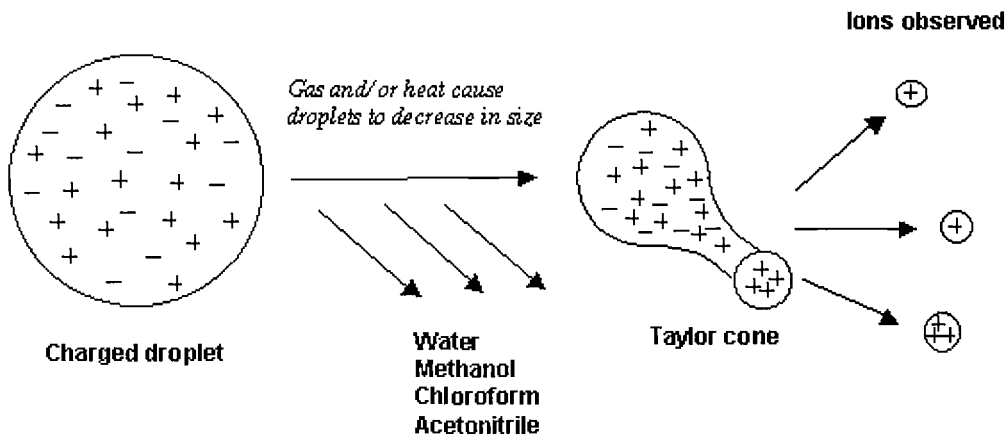


Figure 1.3: Ion formation from an electrospray ion source. The charged droplet decreases in size, causing the repulsion between like charges to increase and resulting in the formation of the Taylor cone.

1.3.2 Phospholipid analysis by ESI-MS

Conventional methods for the characterisation of phospholipids involve laborious multi-step procedures. Mass spectrometry offers fast universal analysis of complex mixtures with unequalled levels of detection and sensitivity. Most phospholipids gain a single charge (except phospholipids with two or more phosphate groups, such as cardiolipins or polyphosphoinositides; therefore, m/z is analogous to molecular weight, providing relatively simple analysis.

1.3.3 Tandem electrospray ionisation mass spectrometry (ESI-MS/MS)

Tandem MS/MS is a facility only available on mass spectrometers which possess two mass analysers which are linked in series and separated by a collision cell, the tandem mass spectrometer used throughout this project is an electrospray mass spectrometer (although there are other types of tandem mass spectrometers). Molecular ions which

enter the first mass analyser are then fragmented in the collision cell by bombarding into an inert gas (typically argon) and the fragments separated in the second mass analyser prior to detection. Tandem MS/MS aids in identifying the structural conformation of compounds as well as providing a way to confirm the identity of molecular ions.

1.3.4 Product ion scanning

Product ion analysis can be used both for the structural elucidation of various mass ions as well as in the determination of fragments specific to certain classes of molecules such as the phospholipids. In this analysis mode, a specific ion of interest is selected in MS1; all other molecular ions are ignored. The selected molecular ion is then fragmented in the collision cell through collision induced dissociation (CID). The fragment or product ions originating from the selected precursor molecule are then separated in the second mass analyser and detected, figure 1.4.

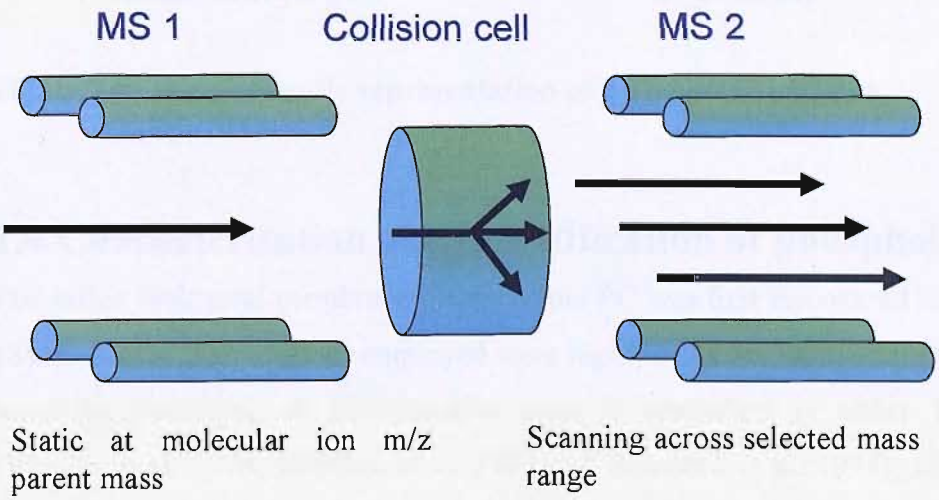


Figure 1.4: Diagrammatic representation of a product ion scan.

1.3.5 Precursor ion scanning

In this acquisition mode MS1 is set to scan across a selected mass range, allowing any molecular ion within that selected range to pass through the first mass analyser. These molecular ions all undergo CID in the collision cell. The resulting fragments then pass through into MS2. However, MS2 is set to monitor only one specific fragment ion of interest; therefore only fragments of the selected m/z will pass through the second mass

analyser to the detector. Because the two mass analysers are connected the mass spectrometer is able to determine which molecular ions were generated from the selected product ion. Precursor ion analyses can be used to identify and confirm known groups of compounds that produce a common product ion upon fragmentation, figure 1.5.

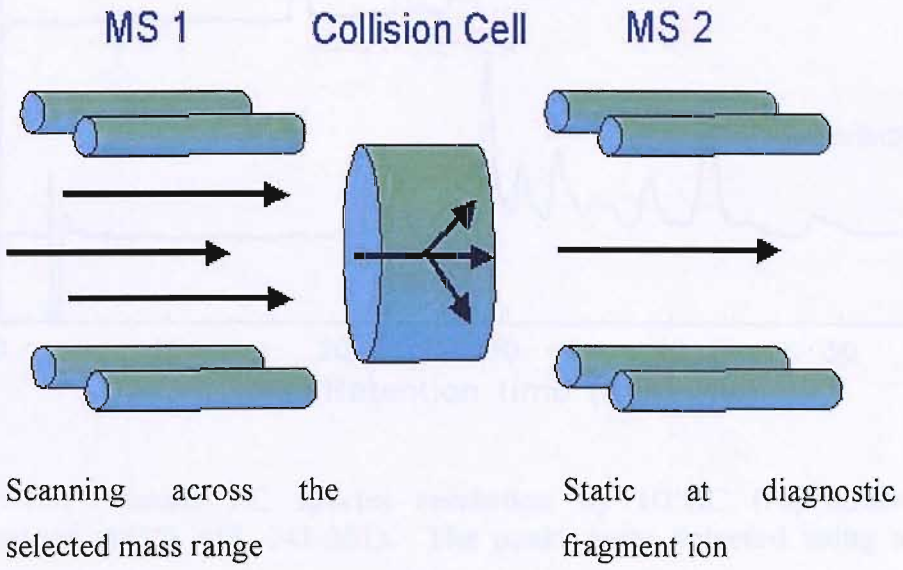


Figure 1.5: Diagrammatic representation of a precursor ion scan.

1.4 Characterisation and quantification of phospholipids

The major biological membrane phospholipid PC was first discovered in 1847 (Gobley 1847). Methods of analysis employed were highly complex multi-step procedures often involving hydrolysis or derivatisation prior to resolution by either TLC or HPLC (Vitiello et al. 1978, Gilfillan et al. 1983 and Bernhard et al. 1994), which were time consuming and frequently required large amounts of sample that were not always available. Methods were subsequently developed to allow analysis of PC without hydrolysis or derivatisation prior to measurement. Figure 1.6 is an example of HPLC analysis of intact PC eluted peaks which were detected by a sensitive post-column fluorescence system using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene. This detection system permitted direct quantification of PC species and eliminated the need for elaborate pre-column derivatisation.

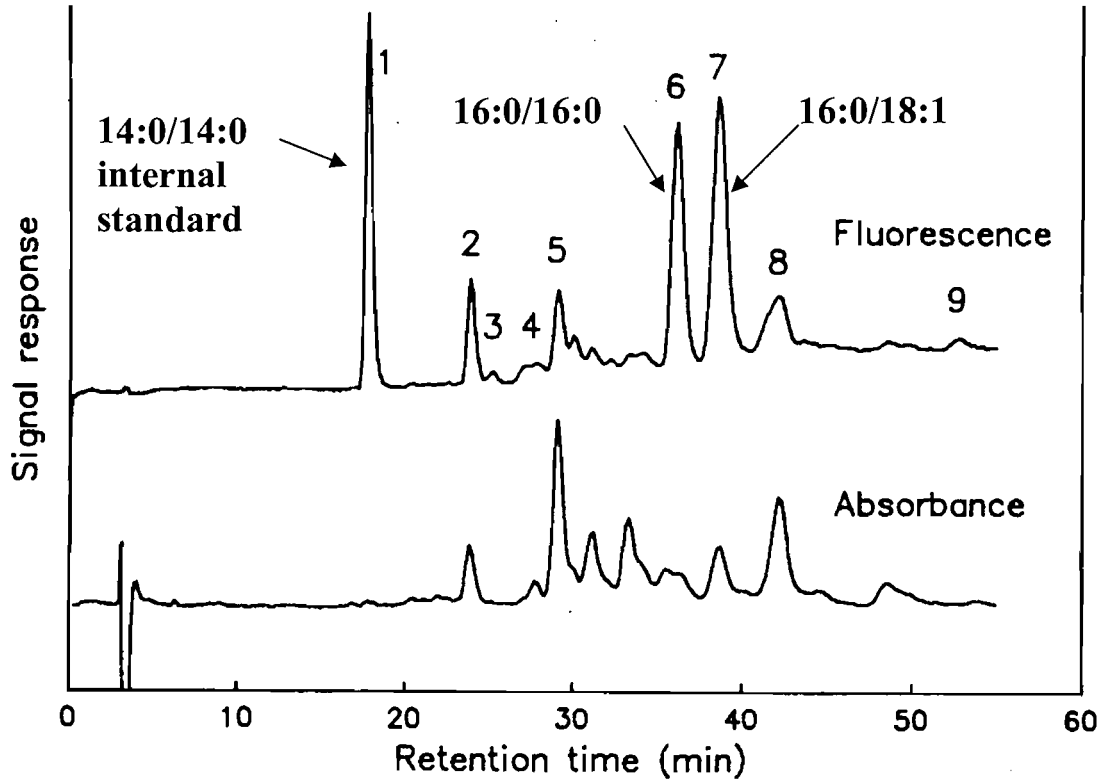


Figure 1.6: Intact PC species resolution by HPLC (reproduced from J Chromatogr (1987) 415, 241-251). The peaks were detected using a florescent probe.

Electrospray ionisation mass spectrometry (ESI-MS) represented a major change in phospholipid analysis; method development involved the use of phospholipid standards (Kayganich et al. 1991). The first study in which electrospray ionisation was validated for structural identification and quantitative analysis of synthetic and naturally derived phospholipids was conducted by Gross et al. (1994) and paved the way for the development in the analysis of phospholipids. Much of this type of analysis has been conducted by making static measurements of lipid molecular species composition at specific time points. However, this does not generate information regarding underlying mechanisms affecting the kinetics of synthesis and turnover in specific metabolic pathways, nor how this may be affected in different disease states.

The analysis of phospholipid synthesis was performed with radioactively labelled precursors, such as [^{3}H] and [^{14}C] labelled choline headgroups through PC molecular species (Burdge et al. 1993 and 1994). The use of radioactive labels was not ideal due to the expense, limited detection due to small incorporations, poor resolution of molecular species by HPLC and low efficiency of counting. The combination of ESI-MS/MS and stable isotope labelling using deuterium has provided a powerful

alternative tool which is more sensitive, more specific and cheaper than radio labelling. The availability of stable isotope labelled choline headgroups, most notably choline-d_9, choline-d_{13} and [^{13}C]-labelled alternatives, combined with precursor ion scanning mass spectrometry have enabled analysis of PC metabolism in subcellular organelles (Hunt et al. 2002). The methodology was developed using choline-d_9 substrate to characterise PC synthesis in whole cells by Delong et al. (2002).

Labelling PC molecules with deuterium makes them readily distinguishable from endogenous PC by mass spectrometry. Substitution of the nine protons on the phosphocholine headgroup with nine deuterons increases the molecular mass of the molecule by nine mass units. Breakdown of these labelled lipid molecules in the collision cell of the tandem mass spectrometer generate a diagnostic labelled fragment nine mass units higher the endogenous fragment, figure1.7. Besides the minor increase in mass, these labelled molecules are to all intensive purposes physiologically identical to their non-labelled counterparts. ESI-MS/MS of PC species labelled with specific deuteriated precursors makes it possible to distinguish between newly synthesised and endogenous PC.

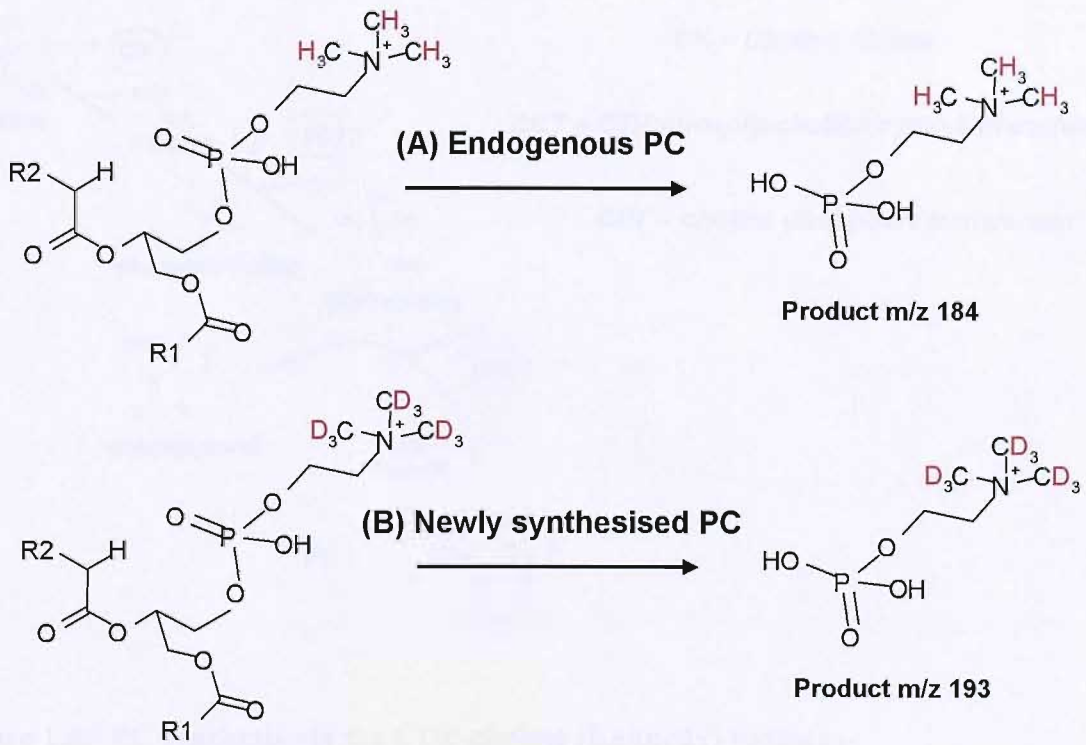


Figure 1.7: Fragmentation of endogenous (A) and newly synthesised (B) PC by tandem MS/MS producing fragments with different masses to allow the two to be readily distinguishable.

1.5 Lipid synthesis

1.5.1 Phosphatidylcholine synthesis

This project will focus on phosphatidylcholine (PC) which is a major phospholipid present in all mammalian cells. The molecular diversity of PC and other phospholipids is dictated by the combination of different chain lengths, number of double bonds and type of linkage; acyl, alkyl or alkenyl. Mammalian cells contain many phospholipids estimated to be in the range of at least one thousand molecular species. There are two main pathways by which PC can be produced in mammalian cells and an additional pathway unique to hepacytes.

1.5.1.1 CDP-Choline pathway

In most mammalian cells, PC is synthesised via the CDP-choline pathway. This pathway uses choline as an initial substrate and is catalysed by three enzymes: choline kinase (CK), CTP:phosphocholine cytidylyltransferase (CCT) and cholinephosphate transferase (CPT) with CPT as the rate limiting enzyme, figure 1.8. The CDP-choline pathway uses exogenous choline as the initial substrate and generates PC molecular species (Kennedy et al. 1956).

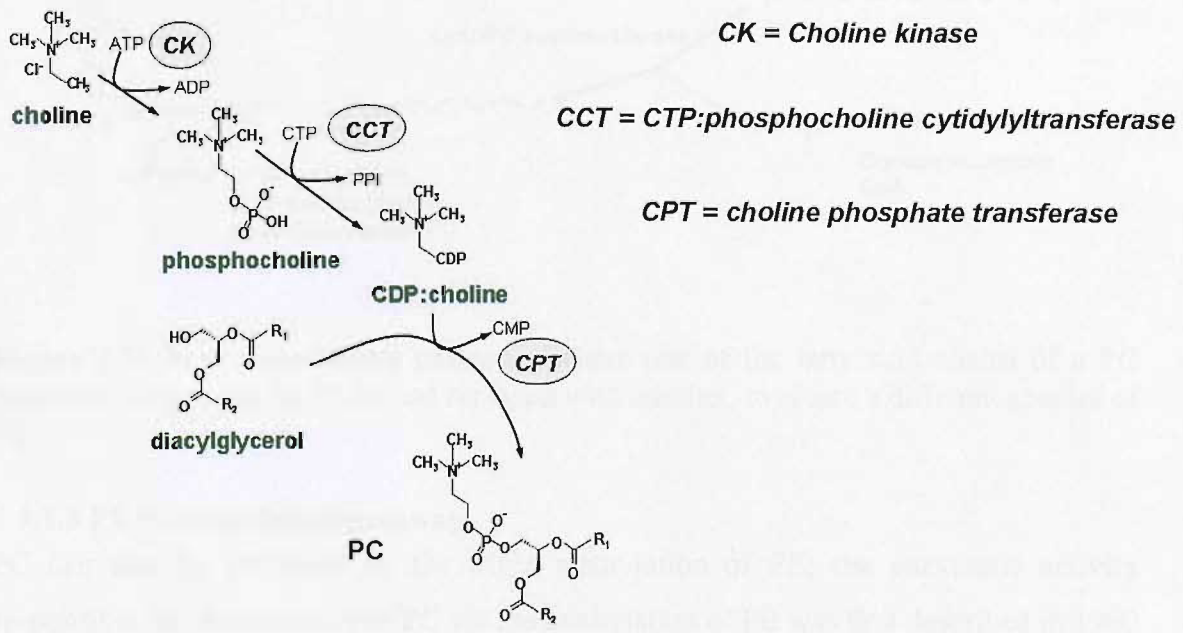


Figure 1.8: PC synthesis via the CDP-choline (Kennedy) pathway.

In the liver however, the CDP-choline pathway accounts for approximately 80% of PC synthesis (Vance et al. 1988). In this case the CDP-choline pathway predominately

produces species with short chains (16 and 18 carbons in length), while the longer chain species are produced by the PE *N*-methylation pathway (section 1.5.13).

1.5.1.2 Acyl remodelling

Newly synthesised PC undergoes a series of modifications which fine tune the fatty acid species present in the cell. This involves the deacylation and reacylation of the phospholipids, which is known as the acyl remodelling pathway. Phospholipase A_2 cleaves the fatty acid in the sn-2 position from the molecule to form a lyso-PC molecule and a free fatty acid which is reacylated by acyl-CoA (Lands et al. 1976). Lysophopholipid acyltransferase then catalyses the addition of a different fatty acid onto the lyso-PC, figure 1.9.

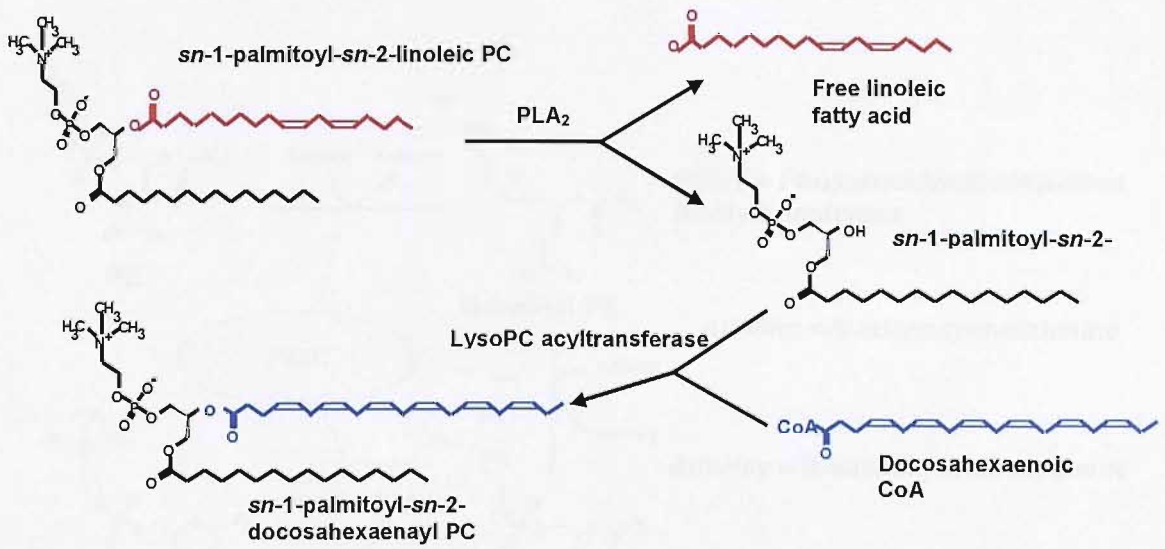


Figure 1.9: Acyl remodelling pathway, where one of the fatty acid chains of a PC molecule is removed by PLA_2 and replaced with another, to create a different species of PC.

1.5.1.3 PE *N*-methylation pathway

PC can also be produced by the triple methylation of PE; the enzymatic activity responsible for the synthesis of PC via the methylation of PE was first described in 1960 by Bremer and Greenberg. Phosphatidylethanolamine methyltransferase (PEMT) catalyses the conversion of PE to PC (Vance et al. 1988) using the general enzyme donor adenosylmethionine (AdoMet) as a substrate (Walkey et al. 1998). PEMT catalyses the stepwise transfer of three methyl groups from AdoMet to the amino headgroup of PE, figure 1.10. Methylation of PE in the liver is highly responsive to the

dietary content of components that may have metabolic consequences on methylation reactions. *In vitro* investigation of primary hepatocytes (DeLong et al. 1999) and rat hepatocarcinoma cell lines (DeLong et al. 2002) by ESI-MS/MS and deuterium labelling allowed for accurate and sensitive evaluation of PC molecular species synthesised by the two pathways. PC produced by the CDP-choline pathway is comprised mainly of medium-chain, saturated and mono-unsaturated species (16:0/18:0, 16:0/18:1), whereas PC species produced via PEMT activity were comprised of longer chain polyunsaturated fatty acid species (e.g. 16:0/20:4, 18:0/22:6). As yet there has been little or no investigation of these differences in synthesis in humans as the appropriate safe technologies have only just been developed. Therefore the PC species produced via these different pathways may have important consequences for the handling of n-3 fatty acid supplementation *in vivo* and *in vitro* as *in vitro* the cells will not be able to ultilise the PE methylation pathway.

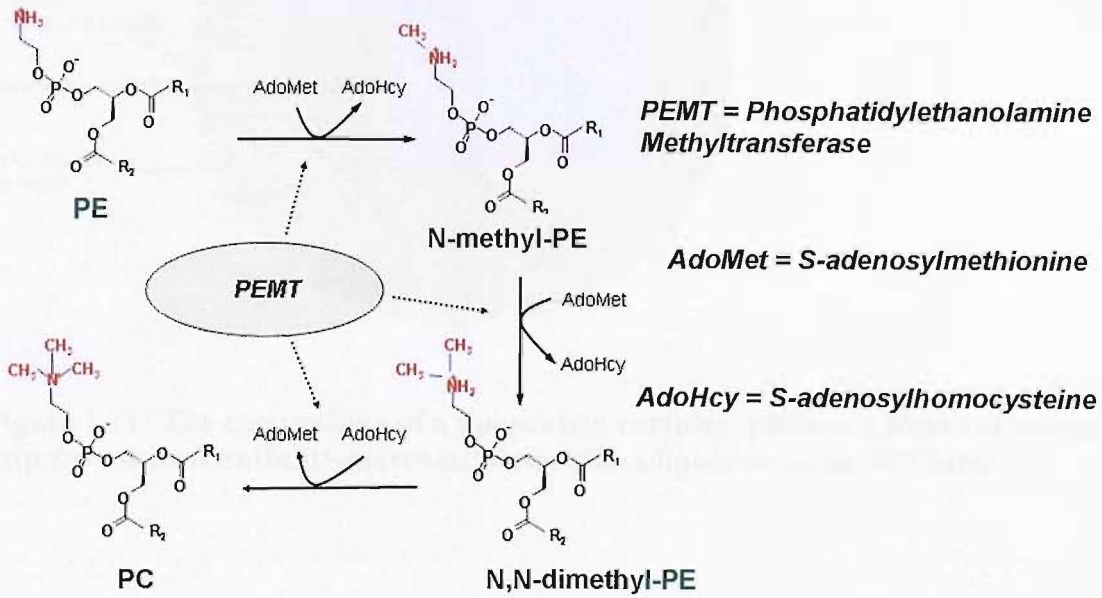


Figure 1.10: PC synthesis by the PE methylation pathway.

The lipid metabolism of endothelial cells in culture has been poorly investigated (Whatley et al. 1990). The majority of work on endothelial cells has focused on the production of signalling molecules after omega-3 fatty acid supplementation. However, in this project the phospholipid composition of endothelial cells will be investigated before and after fatty acid supplementation with the use of stable isotope choline d-9.

1.5.2 Lipoprotein synthesis

The lipids present in plasma do not circulate in their free form. Free fatty acids are generally bound to albumin, whereas cholesterol, triglycerides and phospholipids are transported in lipoprotein complexes. These complexes increase the solubility of the lipids, in general consisting of a hydrophobic core of triglycerides and cholesterol esters surrounded by phospholipids and protein (Ganong 1999), figure 1.11. The protein constituents of lipoproteins are called apoproteins, the major types are APO E, APO C and APO B. The lipoproteins are graded in size and lipid content, the density of these lipoproteins is inversely proportionate to their lipid content, table 1.2.

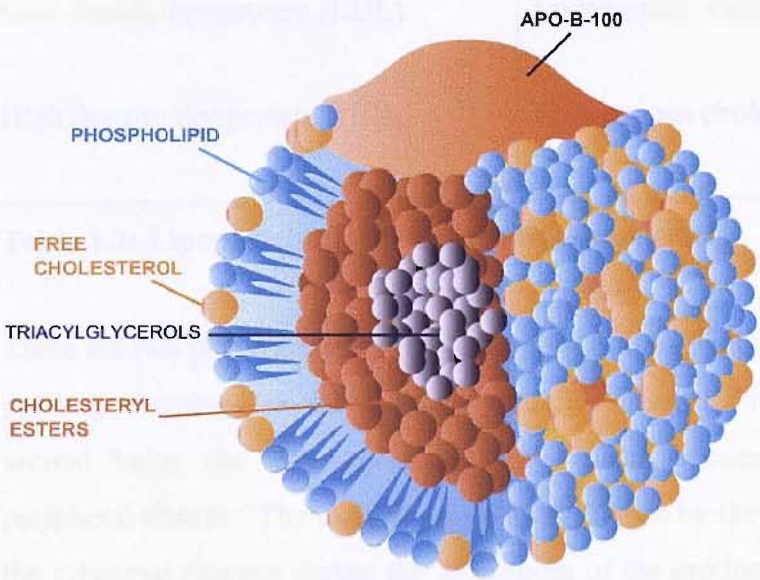


Figure 1.11: The composition of a lipoprotein complex, picture is from the website http://www.med.unibs.it/~marchesi/biotec/slides/lipoproteine/sld002.htm.

Lipoproteins	Major core lipids	Apoproteins
Chylomicron	Dietary triacylglycerols	B48, C, E
Chylomicron remnant	Dietary cholesterol esters	B48, E
Very low density lipoprotein (VLDL)	Endogenous triacylglycerols	B100, C, E
Intermediate density lipoprotein (IDL)	Endogenous cholesterol esters	B100, E
Low density lipoprotein (LDL)	Endogenous cholesterol esters	B100
High density lipoprotein (HDL)	Endogenous cholesterol esters	A

Table 1.2: Lipoprotein particles in increasing density

There are two pathways involved in the transport of lipids, the first being the exogenous pathway, involved in the transport of lipids from the intestine to the liver, and the second being the endogenous pathway, which transports lipids to and from the peripheral tissues. The exogenous pathway begins by the formation of chylomicrons in the intestinal mucosa during the absorption of the products of fat digestion. These are very large lipoprotein complexes that enter the circulation via the lymphatic ducts and are present in large quantities in the blood after meals. The chylomicrons are cleared from circulation by lipoprotein lipase, located on the endothelium of capillaries, resulting in the breakdown of the triglyceride to free fatty acids and glycerol, which either enter adipose cells or remain in the circulation bound to albumin. The remains of the chylomicrons are known as chylomicron remnants, and are taken up by the liver and degraded in lysosomes.

The second pathway is the endogenous pathway which transports lipids to and from the tissues, which consist of VLDL, IDL, LDL and HDL. VLDL is formed in the liver and transports the triglycerides formed from fatty acids and carbohydrates in the liver to extrahepatic tissues. The triglyceride is removed by the action of lipoprotein lipase to become IDL. The IDL is taken up by the liver and processed or converted to LDL by the removal of more triacylglycerols. LDL is the major carrier of cholesterol in the blood to the peripheral tissues; it has a density of between 1.019-1.063 gml^{-1}, it is 20 nm

in size, composed of 20% apoprotein B-100, 21% phospholipids and 46% cholesterol (Ganong 1999). LDL is taken up by receptor mediated endocytosis which is inhibited when the amount of LDL in the cells becomes too high.

There is also a scavenger system present in some cells, such as macrophages, which are able to take up LDL at an uninhibited rate; such receptors appear to show a higher affinity for modified LDL (Goldstein et al. 1979). Cholesterol leaving the cells is absorbed by HDL, transported to the liver and excreted in the bile, thereby lowering plasma cholesterol levels.

1.6 Overview of atherosclerosis disease progression

1.6.1 Artery structure

Atherosclerosis is a complex disease characterised by the accumulation of lipids in macrophages in the large and medium arteries. The normal artery wall consists of three layers; each has a distinctive composition of cells and an extracellular matrix. The layer immediately next to the lumen is the endothelium, which consists of a single continuous layer of endothelial cells and lines the luminal surface of arteries. The cells sit on a basement membrane of extracellualar matrix and proteoglycans and are boarded by elastin. The middle layer is the intima consisting primarily of smooth muscle cells arranged in layers with an extracellular matrix primarily consisting of elastic fibres and collagen. The outermost layer comprises the adventitia, which consists of a loose matrix of elastin, smooth muscle cells, fibroblasts and collagen. The three layers of the artery wall are separated by layers of elastin, see figure 1.12.

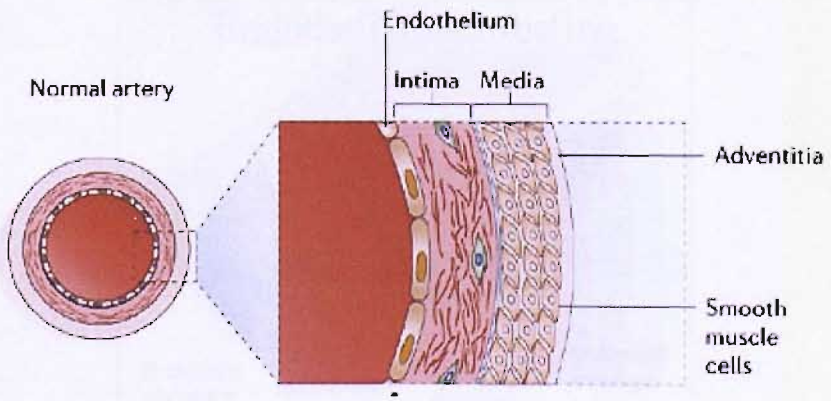


Figure 1.12: Diagram of the structure of a normal artery. (http://www.nature.com/nrg/journal/v7/n3/fig_tab/nrg1805_ft.html).

The functional integrity of the endothelium is vital in dividing blood from tissue. It is characterised by tight junctions between each cell which restrict the movement of macromolecules and cells into the sub-endothelial space. The main functions of the endothelium are to maintain blood circulation and fluidity, regulate vascular tone, and modulate leukocyte and platelet adhesion and leukocyte transmigration. The endothelium is essential to the haemostatic processes of cell adhesion and migration, thrombosis and fibrinolysis. Endothelial cells express adhesion molecules such as P-selectin, E-selectin, intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on the cell surface that are involved in leukocyte recruitment and platelet adhesion during thrombosis and inflammation. When the vascular endothelium encounters inflammatory stimuli it undergoes several changes, including the up-regulation of surface and soluble cell adhesion molecules and the release of cytokines, known as endothelial activation.

The commencement and progression of atherosclerosis involves damage to the endothelium (Henriksen et al. 1981), which has been attributed to several possible causes. These include: elevated and modified LDL, free radicals caused by cigarette smoking, hypertension and diabetes, genetic alterations, infectious micro-organisms such as herpesviruses or *Chlamydia pneumoniae* and combinations of these factors (Ross 1999). The consequential endothelial dysfunction leads to the altering of the homeostatic properties of the endothelium. Endothelial cells in atherosclerosis express increased surface bound selectins and adhesion molecules, resulting in increased migration of inflammatory cells into the sub-endothelial space, figure 1.13.

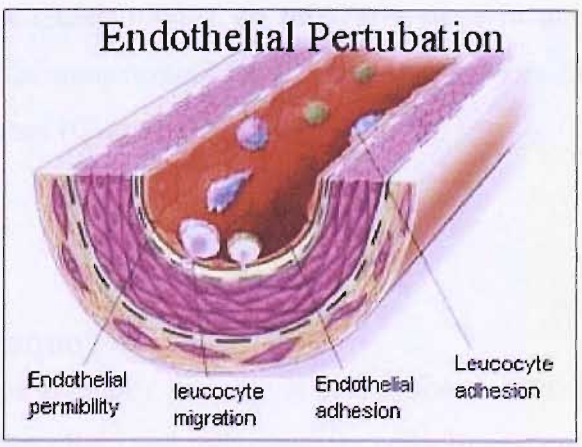


Figure 1.13: An artery with increased adhesion of blood cells to the endothelium and their subsequent migration into the intima, from Ross 1999.

1.6.2 Fatty streak

The continued inflammatory response is responsible for chemo attraction, migration and subsequent activation of monocytes within the sub-endothelial space. Once present in the intima, monocytes differentiate into macrophages which ingest the trapped oxidised lipids via a scavenger receptor, leading to the development of foam cells (Bolgar et al. 1996), figure 1.14. This is the early plaque, known as the fatty streak, which can be present in young children and is clinically silent.

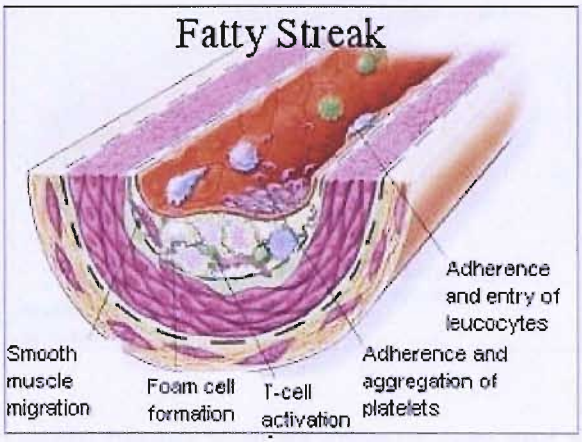


Figure 1.14: The formation of the early plaque, from Ross 1999.

In early atherosclerosis the macrophage has a predominantly beneficial role in neutralising potentially harmful lipid components in the vessel wall. Activated inflammatory cells also express a variety of pro-inflammatory cytokines and growth factors that contribute both beneficially and detrimentally to the evolution of the plaque. Activated macrophages have a high rate of apoptosis, and release their lipid content into the core of the plaque (Ross 1999). At this early stage in plaque development the thickening artery wall is compensated for by dilation, therefore the lumen of the blood vessel remains unaffected (Glaqov et al. 1987).

1.6.3 Advanced plaque

Atherosclerotic plaques gradually increase in size to form an advanced plaque with the addition of more oxidised lipid and inflammatory cells, in particular monocytes. These inflammatory cells produce cytokines, chemokines, hydrolytic enzymes and growth factors, instigating further damage to the endothelium (Libby et al. 1996 and Raines et al. 1996). As the monocytes and foam cells die they become incorporated into an

enlarging necrotic lipid laden plaque core. Fibrosis is essential if the fatty streak is to progress to a mature lesion, which is comprised of a fibrous cap beneath the arterial endothelium. The collagen of the fibrous cap is laid down by fibroblasts which are derived from the proliferation and transformation of smooth muscle cells in the arterial wall (Berliner et al. 1995); this is particularly aided by IL-6 which is produced by the foam cells and other arterial wall cells (Ross 1999), figure 1.15.

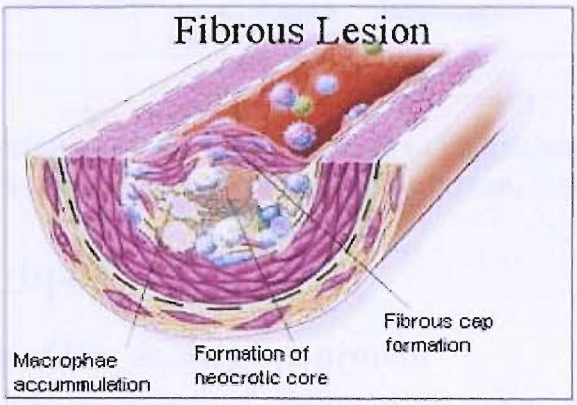


Figure 1.15: The advanced plaque with its necrotic core and a fibrous cap over the plaque, from Ross 1999.

Foam cells can secrete metalloproteinases which may digest the collagen and thus contribute to the fissuring of the cap (Galis et al. 1994). The strength of the fibrous cap will depend on the amount of collagen produced from the smooth muscle cell derived fibroblasts. At this stage the lesion is no longer compensated for and will begin to protrude into the lumen of the blood vessel (Ross 1999).

1.6.4 Plaque rupture

Atherosclerotic plaques can become life threatening if they initiate clot formation in the arterial lumen. Following fibrous cap rupture, there is exposure of the highly thrombogenic extracellular matrix of the cap and the tissue rich lipid core to circulating platelets, figure 1.16. This may result in the triggering of the clotting cascade, thrombus formation and if extensive complete vessel occlusion.

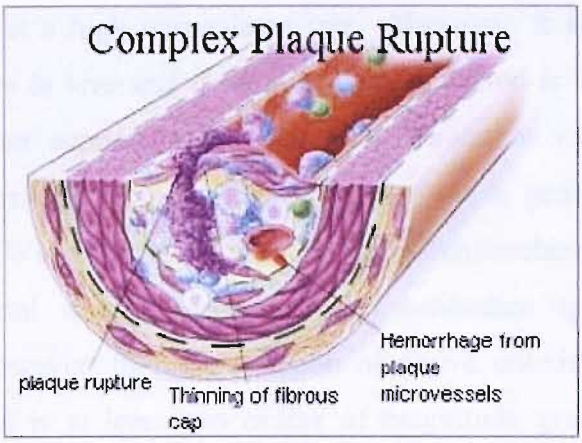


Figure 1.16: Degradation of the fibrous cap leads to plaque rupture and the release of the thrombogenic contents of the plaque into the lumen, from Ross 1999.

1.7 Low density lipoprotein

1.7.1 Modification of low density lipoprotein

The oxidative modification hypothesis of atherosclerosis suggests that LDL modified by oxidation is an early event in the progression of atherosclerosis (Henriksen et al. 1981). The site of this oxidation is thought to be in the intima as opposed to within the circulatory system, where there are high levels of anti-oxidants (Frei et al. 1988). Oxidation can occur in both the lipid (which includes sterols, fatty acids in phospholipids, cholesterol esters and triglycerides), and the protein moieties. There are many remaining uncertainties about the mechanism of LDL oxidation in vivo; it is not known what the cellular sources of free radicals are or how LDL is oxidised. *In vitro* oxidation of LDL is largely performed by the transition metal ions such as copper or iron. These conditions are strongly oxidising resulting in the production of oxidised LDL capable of generating foam cells *in vitro* (Lougheed et al. 1996), although *in vivo* there is little evidence to suggest that such strong oxidising conditions exist. Products formed early in the oxidation process *in vitro* are described as minimally modified LDL (mmLDL), which do not produce foam cells when incubated with macrophages (Jessop et al. 2004). mmLDL has usually undergone extensive oxidation of the arachidonic acid containing phospholipids but very little of the linoleic acid containing phospholipids, and with little or no protein modification (Berliner et al. 1990). Products formed later in the process are identified as being oxidised to a greater extent and upon incubation with macrophages produce foam cells by cholesterol accumulation (Berliner et al. 1995, Gillotte et al. 2000). Oxidised LDL is taken up by scavenger receptors on the cell surface, such as CD36, which are unable to recognise native LDL but are able to uptake

uptake oxidised LDL at a high unregulated rate. However, it is not known to what extent oxidation occurs *in vivo* and if the extent of oxidation is important. There are many observations that support a role for LDL oxidation in atherosclerosis, for example: the LDL extracted from atherosclerotic lesions is generally at least in part oxidativley modified (Watson et al. 1997), and immunohistochemistry has shown that lesions contain material which is reactive with antibodies against oxidised LDL (Steinberg 1997). However, the concentration of native unoxidised phospholipid in atherosclerotic plaques is at least two orders of magnitude greater than that of the oxidised phospholipid species present and studies into antioxidant treatment have resulted in varying results. Two major trials, the Alpha-Tocopherol, Beta-Carotene Cancer prevention (The Alpha-Tocopherol, Beta-Carotene Cancer prevention Study Group 1994) and The Collaborative Primary Prevention Project (PPP) (PPP 2001), both showed no effect of vitamin E supplementation on myocardial infarction, stroke or cardiovascular disease after 3-6 years. In contrast, the Cambridge Heart Antioxidant Study (CHAOS) reported reduction in the incidence of nonfatal acute myocardial infarction with vitamin E supplements, although this was associated with a non-significant increase in fatal myocardial infarction (Stephens et al. 1996), and Secondary Prevention with Antioxidants of Cardiovascular disease in End-stage renal disease (SPACE) resulted in a significant decrease in acute myocardial infarction in haemodialysis patients (Boaz et al. 2000). These discrepancies raise doubts about the extensive role of oxLDL in atherosclerosis in humans, as it would be presumed that if oxidation was the whole picture then antioxidants would do more to slow the progression of the disease. In summary; trials in atherosclerosis related cardiovascular disease indicate that supplements with vitamin E alone do not consistently convey cardiovascular benefit. It must also be remembered that the susceptibility of LDL *in vivo* will also be different to that *in vitro*, as *in vivo* the LDLs microenvironment, including local antioxidant concentration, transition metal availability, pH and the presence of specific enzymes, among other factors, will greatly influence oxidation and will therefore influence the results.

There is increasing evidence indicating a role for oxidised phospholipids in atherosclerosis disease progression in general and the mediation of inflammatory processes within the plaque (Berliner 2002). Disease development is associated with the accumulation of foam cells and other oxidised phospholipid products within the plaque (Furnkranz 2004). However, there has been little investigation into the exact

molecular changes in phospholipid composition that occur after oxidation. There has been some structural analysis of specific oxidised phospholipids but this has mainly been HPLC based, which is a time consuming process and there has been little investigation into the development of a rapid way of analysing the products formed LDL oxidation. There has also been little characterisation of the structure of the oxidised lipids formed during LDL oxidation and the compositional changes that occur to LDL during oxidation. Mass spectrometry will be used to characterise the changes phospholipid composition of LDL after oxidation *in vitro*.

1.7.2 Lipoprotein associated phospholipase A_2

As discussed above atherosclerotic disease development is invariably linked to the formation and accumulation of a variety of lipids within the plaque, which include oxidation products of LDL. It has become clear that many phospholipid oxidation products are bioactive species with pro-inflammatory actions that contribute to plaque progression and destabilisation (Berliner 2002). LDL is linked with lipoprotein associated phospholipase A_2 (Lp-PLA_2) which is a 50- kDalton enzyme belonging to the A_2 phospholipase superfamily (Tew et al. 1996). Lp-PLA_2 is alternatively known as platelet activating factor acetylhydrolase (PAF-AH). Lp-PLA_2 is produced by macrophages and lymphocytes and 80% circulates bound to LDL with the remainder distributed across HDL and VLDL (Caslake et al. 2000). There is increasing evidence from both animal (Hakkinen et al. 1999) and human studies to suggest its action is pro-atherogenic (Packard et al. 2000). Packard et al. (2000) was the first study to show Lp-PLA_2 to be an independent marker of coronary heart disease (West of Scotland Coronary Prevention Study (WOSCOPS). This study showed that in the highest Lp-PLA_2 group there was a 60% increase in the risk of artery complications. Lp-PLA_2 remains latent until the LDL undergoes oxidation, when it immediately acts, independent of calcium, to cleave PC into two biologically active products: lyso-PC and oxidised free fatty acids. The biological actions of the free oxidised fatty acid are not fully defined as their structures have not yet been fully characterised. The biological actions of lyso-PC have been well described to include impairment of the vascular endothelium, which is crucial to the progression of atherosclerosis (Zalewski and Macphee 2005). There is evidence to suggest a role for type II secretory phospholipase A_2 (sPLA_2) (Leitinger et al. 1999 and Kugiyama et al. 1999) in atherosclerotic development. Non-pancreatic sPLA_2 is distinct from Lp-PLA_2 because it is calcium dependent and hydrolyses phospholipids at cell membranes to produce lyso-PC and free

oxidised fatty acids whereas Lp-PLA_2 acts in solution. The production of $sPLA_2$ is upregulated by inflammatory cytokines including TNFα, IL-6 and IL-1β (Hurt-Camejo et al. 2000). The involvement of $sPLA_2$ in atherosclerosis development is further supported by Kugiyama et al. (1999), who observed significantly higher levels of plasma $sPLA_2$ in coronary artery disease patients compared to controls.

Novel and potent inhibitors of Lp-PLA_2 have been used to confirm the role of Lp-PLA_2 during the *in vitro* oxidation of LDL (Tew et al. 1998); inhibition in the Watanabe heritable hyperlipidaemic rabbit led to significant reduction in plaque development (Benson et al. 2000). Patients undergoing carotid endarterectomy showed that the use of the compound SB-480848 demonstrated a dose-dependent inhibition of Lp-PLA_2 activity in plasma and atherosclerotic plaque, with the maximal dose resulting in an 80% inhibition of the enzyme activity after only 14 days (Johnson et al. 2004). Despite the interest in these drugs there has been little investigation into the action of such drugs at the molecular level. With the use of mass spectrometry the changes within oxLDL *in vitro* after treatment with such an inhibitor shall be systematically explored.

1.8 Omega-3 fatty acids

1.8.1 Background to n-3 fatty acids

A high dietary intake of n-3 fatty acids, found naturally in oily fish, has been found to decrease ischemic heart disease risk and mortality. This theory is largely based upon epidemiological data which demonstrated that cardiovascular disease was much rarer in populations with a very high intake of fish, such as the Alaskan Native Americans (Newman et al. 1993), Greenland Eskimos (Kromann et al. 1980 and Bjerregaard et al. 1988) and Japanese fishing villages (Yano et al. 1988). The n-3 fatty acids thought to be protective against cardiovascular disease are docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), although it is not known if they are equally important. Large cohort secondary prevention studies using fish oil demonstrated a marked decrease in cardiovascular mortality in post myocardial infraction patients. Diet and Reinfarction Trial (DART, 1989) and the GISSI-Prevenzione Trial (1999) have studied the effect of omega-3 fatty acids on fatality in patients after coronary heart disease. DART advised men who had previously suffered a heart attack to increase their oily fish intake, resulting in a 29% reduction in total mortality after two years. The GISSI-Prevenzione trial randomly assigned four different daily supplementation treatments; 1. DHA and EPA; 2. Vitamin E; 3. Vitamin E, DHA and EPA; 4. No supplement. Daily

treatment with DHA and EPA reduced the death rate by 10-15% and significantly reduced non-fatal myocardial infarction and stroke over 3.5 years. Combined treatment with vitamin E and n-3 fatty acids resulted in similar reductions while no effect was seen for vitamin E alone. Both studies confirmed a beneficial role for n-3 fatty acids in coronary heart disease for both fatal and non-fatal cardiovascular events. However, the mechanisms by which these protective effects are exerted are not understood, though one possible theory suggested has been to stabilise the plaque (Rapp et al. 1991) although the study was not placebo controlled. Plaque incorporation of n-3 fatty acids was further investigated by Theis et al. 2003, in which patients under going carotid endarterectomy were randomly assigned either 6g of placebo, sunflower oil or fish oil every day until surgery (varying from 7- 189 days); it was found that the fish oil fatty acids (DHA and EPA) had been incorporated into blood plasma, LDL and plaque tissue, and there was a decrease in the number of macrophages in the plaque. These results demonstrated that plaques are dynamic and responsive to dietary modification, and the lowering of macrophages within the plaque could contribute to increased stability of the plaque. The reduced number of macrophages in the plaque would either be achieved by a decrease in the expression of adhesion molecules, thereby ceasing the movement of these inflammatory cells, or a decrease in the production of chemoattractants and inflammatory cytokines thereby decreasing the inflammatory response. In this project, the changes in the composition of plasma from volunteers receiving either DHA or EPA supplementation will be measured. The LDL will then be isolated from the plasma and subjected to oxidation treatment to compare the oxidised phospholipids formed in these LDLs to control volunteer oxidised LDL.

Investigation into the mechanisms for n-3 fatty acid action *in vitro* is largely based upon fatty acid supplementation of human umbilical vein endothelial cells (HUVECS), followed by cell stimulation and the measuring of the resultant changes in adhesion molecules and inflammatory cytokines (De Caterina et al. 1994). In resting endothelial cells there is no change in adhesion molecule or cytokine production, but following endothelial stimulation with IL-1β, TNFα or LPS the expression of ICAM-1, VCAM-1 and cytokines decreased compared to control cells. *In vivo* these changes would be expected to result in a decreased adherence and transmigration of cells into the vascular intima, and therefore decrease plaque development and possibly stabilise the plaque by reducing the number of macrophages. There are several other possible mechanisms upon which omega-3 fatty acids may exert their effects. These include alterations to the

cell membrane and phospholipid structure of the endothelial cells and blood cells, which in turn may alter cell signalling, gene expression, and eicosanoid formations. The phospholipid composition of blood cells and endothelial cells after fatty acid supplementation has been poorly investigated for molecular specificity of changes caused by fatty acid supplementation. By supplementing HUVECS with omega-3 fatty acids, followed by treatment with choline-d9, mass spectrometry will allow the changes in phospholipid composition and synthesis of HUVECS *in vitro* to be characterised.

1.8.2 Possible mechanism for omega-3 fatty acid action in endothelial cells

The mechanism by which dietary supplementation exerts its effect in endothelial cells is poorly understood; one possible mechanism is via NFκB activation. NFκB and its dependent genes have been associated with several pathological conditions including atherosclerosis, cancer and arthritis (Barnes et al. 1997). NFκB is associated with the inhibitory protein IκB in the cytoplasm to maintain NFκB in its inactive state. In response to inflammatory signals such as TNFα, IL-1 and LPS, IκB (of which there are several forms, including IκBα and IκBβ) is rapidly degraded to release NFκB from its inhibitor. This degradation is brought about by phosphorylation of IκB at two serine residues by the IκB kinases. The phosphorylated IκB undergoes ubiquitination by degradation of IκB in the proteasome. Hence this process disrupts the inhibitors associated with NFκB and results in its activation and migration to the nucleus where it stimulates the transcription of the target genes (Baeurle et al. 1994), figure 1.17. In response to NFκB activation there is an increase in the transcription of the inhibitor IκBα which is able to enter the nucleus to aid dissociation of NFκB from the nucleus, although the continuation of certain inducing agents, such as LPS, causes NFκB to be maintained in the nucleus despite the presence of IκBα.

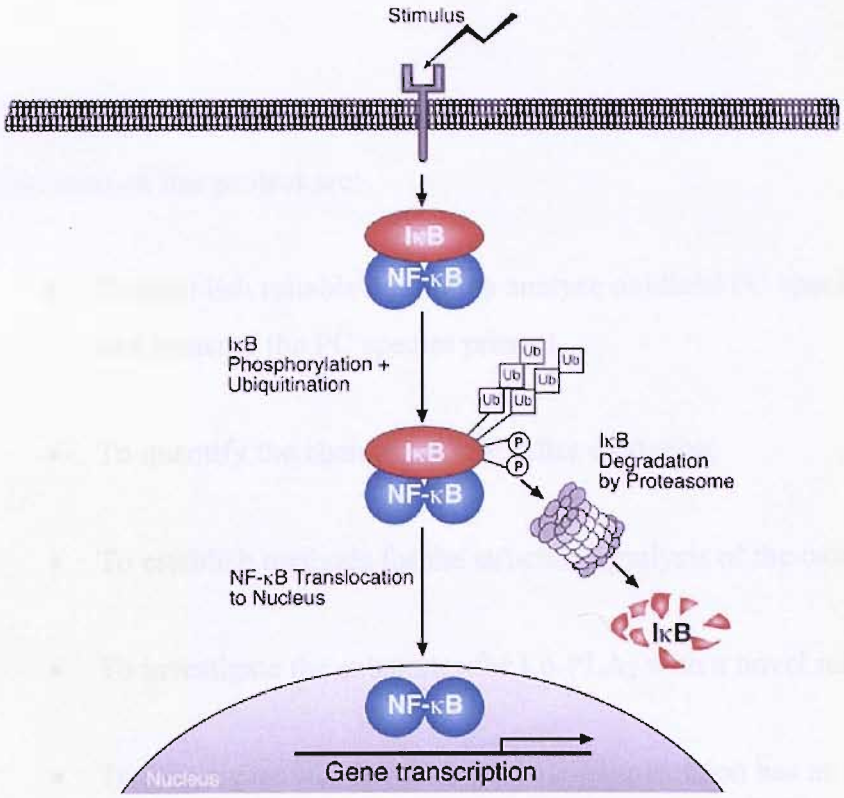


Figure 1.17: Diagram of NFκB activation pathway, demonstrating activation by an external stimulus which allows release of NFκB from its inhibitory protein, resulting in translocation to the nucleus and transcription of inflammatory genes. (http://www.biochemj.org/bj/382/0393/bj3820393f02.gif)

NFκB is a dimer of members of the Rel family of DNA binding proteins, which are characterised by the presence of the 'Rel homology domain' (RHD), which is a 300 amino acid sequence at the N-terminus. It is this sequence that is responsible for DNA binding and interaction with the inhibitory cytoplasmic IκB proteins as well as containing a nuclear localisation sequence which promotes NFκB translocation to the nucleus. NFκB dimers can consist of various combinations of the Rel family proteins (Ghosh et al. 1998), but the most abundant dimer is composed of p50 and p65 subunits.

As most adhesion molecules are not expressed in basal conditions, cytokine activation requires the initiation of transcription. One possible mechanism of action for omega-3 fatty acids is via membrane phospholipids of endothelial cells. When an inflammatory signal, such as TNFα, binds to the membrane, there is a rapid release of diacylglycerol (DAG) via the hydrolysis of PC. The increase in DAG acts to release NFκB from its inhibitorary protein allowing inflammatory cytokines to be released. However, by altering the composition of endothelial cells the DAG species released from the membrane may modify inflammatory response within the cells.

1.9 Aims

The aims of this project are:

- To establish reliable method to analyse oxidised PC species in LDL and plaques, and measure the PC species present.
- To quantify the changes in LDL after oxidation.
- To establish methods for the structural analysis of the oxidised phospholipids.
- To investigate the substrates for Lp-PLA_2 with a novel inhibitor of the enzyme.
- To investigate whether fatty acid supplementation has an effect on cytokine and NFκB activation in HUVECs.

Chapter 2

Materials and Methods

Materials

Jurkat cells a were gift from Chris Pynn, Southampton General Hospital. HL60 cells were a gift from Dr Alan Hunt, Southampton General Hospital.

All reagents were from Sigma, Poole, UK unless otherwise stated. All solvents were HPLC grade from Fisher, Loughbourgh, UK except chloroform which was HPLC grade from Baker.

2.1 Mass spectrometry of phospholipids

2.1.1 Lipid extraction

For total lipid extractions of 0.8ml LDL (isolated in section 2.21) or plaque samples (section 4.2.1) or pelleted cells (section 2.3.4) made to 0.8ml with 0.9% NaCl solution were used and total lipid was extracted with chloroform and methanol according to the method of Bligh and Dyer (1959). Samples were made up to 800µl with 0.9% NaCl solution, followed by 1ml chloroform and 2ml methanol, with thorough shaking, to form a single phase solution, containing both hydrophobic and hydrophilic components. A further 1ml chloroform was added followed by 1ml distilled water, with shaking between additions, to generate a biphasic system with the chloroform-rich lower layer containing the lipid and an aqueous upper phase. Sharp resolution of the two phases was achieved by centrifugation at 1000xg for 10 minutes. The chloroform layer was carefully decanted to into smaller glass tubes and dried at 40°C, under stream of nitrogen, to remove any water or methanol contamination.

2.1.2 Solid Phase extraction

Phospholipids were separated by the method of Kaluzny et al (1985) using disposable bond elut NH_2 columns. Dried lipid extracts were resuspended in 1ml chloroform and applied to the bond elut column, which had been pre-conditioned with 1ml chloroform. The column was washed with two 1ml additions of chloroform under vacuum. The PC fraction was eluted with 1ml chloroform:methanol (60:40) under vacuum and PE fraction with 1ml methanol under vacuum. The acidic phospholipids (including PA, PS and PG) were eluted with 3 x 1ml washes of methanol/water/phosphoric acid (96:4:1) which contained 40mM choline chloride, this fraction was dried under nitrogen, then back extracted with the addition of 100µl water, 200µl methanol and 200µl chloroform,

the fraction was mixed, followed by the addition of a further 100µl water. The chloroform layer was removed and all fractions were dried under nitrogen and stored at -20°C prior to mass spectrometry analysis.

2.1.3 Phospholipid analysis by Mass Spectrometry

2.1.3.1 Mass spectrometry using the Micromass Ultima mass spectrometer

Mass spectrometry performed using a triple quadrupole electrospray mass spectrometer. (Quattro Ultima, Micromass UK) equipped with an electrospray ionisation interface. All samples were analysed by direct infusion (5µl min^{-1}) using methanol/chloroform/water/ ammonia (in the ratio of 70:20:7:3) as the solvent. Spectra were acquired in both positive and negative ionisation mode in m/z range 450 - 900 with a scan time of 12 seconds and a resolution setting of 0.1 mass units. The mass spectrometer tune parameters were optimised for PC analysis and the typical tune page settings for ESI-MS/MS are given in table 2.1.

Settings	Positive ionisation mode (ES+ve)	Negative ionisation mode (ES-ve)
Capillary (kV)	2.5	2.5
Cone (V)	90	90
Hexapole 1 (V)	1	0.2
Apperture 1 (V)	0.9	0.2
Hexapole 2 (V)	0	0.3
Source temperature (°C)	80	80
Collision energy (eV)	35	35

Table 2.1: Typical settings for the ESI-MS/MS used for analysing samples in both positive and negative ionisation mode for Quattro Ultima mass spectrometer.

2.1.3.2 Analysis of plasma and LDL using the Quattro Ultima mass spectrometer

All samples were injected in the mass spectrometer and were analysed by precursor scans of 184 for endogenous PC (Hunt et al 2001).

2.1.3.3 Analysis of cell PC synthesis using the Quattro Ultima mass spectrometer

Precursor scans of samples were injected into a Micromass Quattro Ultima, the samples were analysed in positive ionisation for precursor scans of 184, endogenous PC, (Hunt et al 2001) and 193, newly synthesised PC, (DeLong et al 1999).

2.1.3.4 Mass spectrometry using the Micromass QToF mass spectrometer

Samples were dissolved into 3ml of QToF solvent, methanol/chloroform/water/ammonia and 0.01% acetic acid in the ratio of 70:20:8:2 (acetic acid was used to form acid adducts of PC) all samples were analysed by direct infusion (8µl min^{-1}), each sample was run 6 times before combining. The QToF was used in negative ionisation mode and the settings of the instrument are shown in table 2.2:

Settings	Function one: TOF MS	Function two: TOF daughter
Cycle time (secs)	0.990	2.00
Scan duration (secs)	0.89	1.90
Interscan delay (secs)	0.10	0.10
Run time (mins)	51	51
Mass range	50-1500	50-901
Source temperature (°C)	120	120
Collision energy (V)	30	30
Cone voltage (V)	35	35

Table 2.2: Settings used for the QToF for analysing samples in negative ionisation mode.

2.1.3.5 Mass spectrometry using the LTQ Orbitrap™ mass spectrometer

Exact mass measurement was performed on a hybrid linear ion trap, LTQ Orbitrap™ mass spectrometer (Thermo Electron, Bremen) used in positive ionisation mode. Samples were dissolved in 1ml of chloroform and infused into the Orbitrap mass spectrometer at a flow rate of 5µl/min using an integrated syringe pump, and ionised in a standard electrospray source with a sheath gas (nitrogen) flow 10 units, capillary temperature of 275°C and a spray voltage of 4.5kV. The capillary and tube lens voltages for positive ionisation analysis were set to 50V and 120V respectively. The linear ion trap was operated with a target value $3e^4$; the corresponding target value for the Orbitrap mass analyser was $2e^4$. The instrument was calibrated externally according to the manufacturer instructions. The spectra were obtained with resolving power settings of 100,000 at m/z 400 (Full Width at Half-Maximum, FWHM) corresponding to the acquisition time period of 1.5 s/scan.

2.1.4 Processing Ultima mass spectrum data

Data generated by the mass spectrometer was visualised as a spectrum with the mass/charge ratio (m/z) plotted against intensity. Individual molecular species were identified according to their m/z ratio or molecular weight, as phospholipids are singularly charged (z=1). Spectral data were typically averaged for 10 scans, background noise was subtracted and the data was smoothed using MassLynx NT software. The mass spectrometer collects 10 intensity values per Dalton. In order to analyse the data a numerical value must be assigned to each mass ion. The mass spectrum was centred to obtain one single numerical value for each mass. The height of this single peak is proportional to the sum of the individual intensities for that particular mass. These numerical values were then transferred to excel spreadsheets. An excel macro programmed in visual basic had been developed within the laboratory which enables the extraction and isolation of ion intensities corresponding to specific phospholipid molecular species of interest. The corresponding selected intensities were combined in a results spreadsheet where they were corrected for ^{13}C isotope effects and reduced response with increasing m/z values (Hunt et al 2001).

2.2 LDL preparation

2.2.1 Isolation of LDL from whole blood

LDL was isolated from human plasma by ultracentrifugation in successive KBr gradients (Schmaker et al 1986). Each plasma preparation was from a single donor. Thawed plasma (8 x 4.8ml in 8 Beckman 355646 10ml polycarbonate tubes) was adjusted to 1.019g/ml by addition of 0.2ml per tube of 1.34g/ml KBr solution (containing 1mM EDTA) and centrifuged at 75000 rpm (Beckman OptimaMax ultracentrifuge, MLA.80 rotor) at 4°C for 14 hours. The top 1ml from each tube (containing VLDL) was removed and discarded; the remainders from each tube were pooled, mixed thoroughly, and distributed into 8 x 3.25ml in fresh polycarbonate tubes. Density was adjusted to 1.063g/ml by addition of 0.595 ml of 1.34g/ml KBr (containing 1mM EDTA) and volume was adjusted to 5ml by addition of 1.063g/ml KBr (containing 1mM EDTA). Tubes were centrifuged again (as before), and the top 1ml from each (containing LDL) was pooled and dialysed overnight in a Pierce Slide-a-lyser cassette (3-12ml, 3500 MWCO) against 2 changes of PBS (4L each) containing 1mM EDTA. Dialysate was sterilised by filtration through a 0.2 micron unit (Minisart) and

stored at 4°C for a maximum of 4 weeks before use. The plasma was obtained from volunteers at Addenbrookes Hospital, with informed consent, and stored at -70°C.

2.2.2 Protein assay

The protein concentrations of the LDL samples were determined using the Sigma BCA kit for protein determination (Smith et al, 1985). Solution A (BCA) was mixed with solution B (10% $CuSO_4$) at 1:50 ratio. Bovine serum albumin (BSA) of 1mg/ml was used to construct a standard curve, with a range of 0-100μg protein. 20μl of standards and samples were added to a 96 well plate followed by the addition of 200μl BCA reagent mix before incubation at 20°C for 20 minutes to allow for colour formation, subsequently measured at an absorbance of 540nm.

2.2.3 Oxidation of LDL

Before oxidation LDL samples were dialysed into PBS without EDTA, LDL was incubated with 40μM copper sulphate for 20 hours at 37°C. For non- oxidised samples EDTA (1mM) and BHT (50μg/ml) were added prior to addition of 40μM copper sulphate. After incubation EDTA and BHT were then added to the oxidised samples. Treated LDL samples were stored at -20°C before lipid extraction.

2.2.4 Lp-PLA_2 inhibition during oxidation

Lp-PLA2 inhibitor (SB222657, dissolved in DMSO to 0.3μM), or vehicle (DMSO, 0.1%), was added to LDL and tubes were then vortexed before incubation at room temperature for 20 minutes. For non-oxidised LDL EDTA (1mM) and BHT (50μg/ml) were added prior to addition of 40μM copper sulphate; for oxidised samples EDTA and BHT were omitted. All tubes were incubated for 20 hours at 37°C; EDTA and BHT were then added to the oxidised samples. Treated LDL samples were stored at -20°C before lipid extraction.

2.3 Human carotid endarterectomy samples

2.3.1 Prepartion of plaque samples

Patients under going carotid endarterectomy were randomly assigned placebo, 40mg or 80mg Lp-PLA_2 inhibitor (SB480848) a day in tablet form for 14 days prior to surgery, the samples were stored at -20°C. Whilst still frozen the samples were powdered under liquid nitrogen using a biopulveriser, then suspended in PBS containing BHT (50µg/ml) by vigorous vortexing before being subjected to immediate lipid extraction as described in chapter 2 section 2.1.1.

2.4 Cell culture

2.4.1 HL60 cell and Jurkat cell culture

HL60 cells and Jurkat cells were grown in medium containing: RPMI 1640, 10% fetal calf serum (FCS), 1% penicillin and streptomycin and 1% L-glutamine. Cells were incubated at 37°C under 5% CO_2 humid atmosphere. At a density of approximately 1 x 10^6 cells/ml, the cells were sub-cultured with warmed cell medium to produce a density of 5 x 10^5 cells/ml.

2.4.2 Cell count

Cells were diluted (1:1) with trypan blue stain and left to stand for 5 minutes. The stained cell suspension was visualised with an Improved Neubauer manual haemocytometer, dead cells were identified by the blue stain. The concentration of live cells was determined by multiplying the number of cells by 2 (to account for the dilution with trypan blue), and then multiplying by 10^4.

2.4.3 Isolation of HUVECs

Umbilical cords were collected from normal placentas at the Princess Anne Hospital, Southampton, after informed consent was obtained from the mother prior to birth; Ethics number 021/00, Southampton and SW Hampshire Research Ethics Committee. Intact cords were placed in sterile PBS by the midwives and stored at 4°C until collection. Isolation of HUVECs (Jaffe et al 1973) was carried out in a class II tissue laminar airflow culture hood, using sterile technique. At the time of processing all areas of the cord with clamp marks were removed. The umbilical vein was cannulated with sterile kwills (a plastic tube, approximately 5mm in diameter, with a lip at the end inserted into the vein), and clamped in place. The vein was perfused with Hanks balanced salt solution (HBSS) to wash the blood out. All HBSS was evacuated before

5ml 0.1% w/v collagenase (Lorne Diagnostics) was injected. The kwills were closed with a bung and incubated at 37°C for 20 minutes. After incubation the cord was massaged to aid cell detachment. The cell suspension was collected by syringe and centrifuged for 5 minutes at 4°C at 1500rpm, the cells were washed then resuspended in 5ml medium which consisted of: M199 medium supplemented with 20% heat inactivated fetal calf serum (FCS), L-Glutamine (2mM) Penicillin (100μg/ml) Streptomycin (100μg/ml), endothelial cell growth factor (3mg/ml) and heparin (13mg/100ml). The cells were cultured in $25cm^3$ flasks at 37°C under 5% CO_2 humid atmosphere. The culture medium was replaced at 24 hours to remove non-adherent cells and blood contamination. Thereafter, cell medium was changed every 48 hours until confluence was reached, approximately 10-14 days after isolation.

2.4.4 HUVEC passage

At confluence the cell medium was removed and 5ml 1x trypsin EDTA was added to the flasks for 5 minutes. Cell detachment was facilitated by repeat pipette aspirations. After cell detachment, trypsin activity was stopped with the addition of 5ml culture medium. The cell suspension was centrifuged for 5 minutes at 1500rpm at 4°C. The supernatant was discarded and the cells were washed with HBSS and resuspended in culture medium before transferral into new tissue culture flasks or plates.

2.5 Fatty acid modifications

2.5.1 DHA and EPA supplementation of volunteers

All volunteers were recruited by the University of Reading and were asked to complete a health and lifestyle questionnaire before giving a fasting blood sample to ascertain their suitability for the study. Volunteers were excluded if there was any evidence of heart disease, stroke, diabetes, liver function disease or any other kind of endocrine disorder or were taking any medications that would affect lipoprotein metabolism, were taking any supplements (antioxidant vitamins or fish oil), had restrictions on their diet or were competitive athletes. The inclusion criteria for participating in the study were as follows: only males, between 18 and 70 years old, body mass index (BMI) less than 32 kg/m^2, plasma triglycerides between 1-4mmol/L, plasma cholesterol less than 8mmol/L, glucose concentrations of less than 7mmol/L, haemoglobin higher than 11g/dL. In total 43 volunteers were recruited to participate in the study.

The study was a placebo controlled, double blind cross over design. Subjects consumed placebo, EPA or DHA capsules for 4 weeks followed by a 10 week wash out period before crossing over to another treatment group. The placebo (a fatty acid mixture comparable to a typical UK diet), EPA and DHA enriched oils were produced by Ocean Nutrition (Nova Scotia, Canada). The fatty acid composition of the treatment capsules are detailed in Table 2.3. Subjects consumed 6g oil capsules per day enriched by either 3.3g EPA or 3.7g DHA, or 6g of placebo for the treatment periods. Two capsules were taken with each of the three main meals. Volunteers attended the Hugh Sinclair Unit at the University of Reading having been fasted for 10-12 hours. Fasting blood samples (37ml) were then taken at the beginning and end of each treatment. The plasma samples were stored at -80°C and a selection of plasma samples from the DHA and EPA groups was kindly donated for mass spectrometry analysis.

Fatty acid	Nomenclature	EPA	DHA	Placebo
Myristic	14:0	1.1	0.7	1.4
Palmitic	16:0	0.4	0.4	29.0
Stearic	18:0	6.6	0.3	4.3
Oleic	18:1n-9	9.4	1.1	41.1
Linoleic	18:2n-6	1.3	0.3	20.8
Alpha linolenic	18:3n-3	0.6	0.4	0.4
Arachidonic	20:4n-6	3.8	0.7	0.0
EPA	20:5n-3	54.4	10.3	0.0
DPAn-6	22:5n-6	0.0	1.9	0.0
DPAn-3	22:5n-3	1.0	11.2	0.0
DHA	22:6n-3	6.6	62.0	0.0

Table 2.3: Percent fatty acid composition of treatment and placebo capsules

DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid.

2.5.2 Binding of fatty acids to albumin for supplementation to cells

100mg DHA, EPA or AA were dissolved in 10ml HBSS, this solution was mixed with BSA in HBSS to give 3mM stock solution of fatty acids at a ratio of 2mole:1mole of fatty acids:albumin (due to the presence of two binding sites on albumin for fatty acids). All the fatty acid-albumin complexes were filter sterilised and stored at -20°C.

2.5.3 Incubation of Jurkat, HL-60 cells and HUVECs with fatty acids

1ml cell suspension of approx 1 x 10^5 cells were added to 24 well plates. Cell medium was changed every 48 hours for 5 days with medium containing 30μM or 100μM of fatty acids bound to albumin.

2.5.4 Time course analysis of PC synthesis using D9 choline

HUVECs, Jurkats and HL60 cells were supplemented with 100μM DHA, EPA and AA to their normal medium. Following fatty acid supplementation, 0.2mg/ml d_9 choline (choline-trimethyl-d_9 chloride, Aldrich Chem Co.) was added to the cells at 0, 1.5, 3 hours and 5 days, for three hours with incubation at 37°C, followed by phospholipid extraction as in section 2.1.1.

2.6 Cytokine and adhesion molecule measurement

2.6.1 Flow cytometry analysis of PECAM-1

Endothelial cells were recovered as for passage and the cells were washed with HBSS twice. Cells were incubated with 10 FCS% in FACS buffer (1%BSA in PBS) at 4°C for 30 minutes. Cells were washed twice with PBS followed by incubation with 100μl of either 1:100 FITC labelled anti-human PECAM-1 (CD31) anti-human PECAM-1 antibody (BD Biosciences, Oxford, UK), or 100μl of 1:100 mouse IgG1 FITC (Chemicon, Hampshire UK), isotype control, for 30 minutes at 4°C. Endothelial cells were washed twice with FACS buffer. 200μl of a 1:200 dilution of Alexa 488 goat anti-mouse (Molecular probes) was added to the tubes for 30 minutes at 4°C in the dark. The cells were washed twice with FACS buffer and fixed in 4% PFA.

2.6.2 Determining the amount of TNFα needed for HUVEC stimulation

Confluent Passage 1 HUVECs were stimulated with TNF α (Serotec Ltd, Oxford, UK) at concentrations 1000, 500, 200, 100, 50, 10 and 0 U/ml for 1, 2, 4, 6, and 8 hours. Supernatants were collected and IL-6 and IL-8 ELISAs (sections 2.5.3 and 2.5.4) were

carried out. Cells were removed with trypsin (section 2.3.4) and the cells analysed by FACS for ICAM-1 changes (section 2.5.5).

2.6.3 Enzyme-linked immunosorbent assay (ELISA) for interleukin-6 (IL-6) concentration

The concentration of total IL-6 was measured in 100µl of HUVEC supernatant using a commercially available ELISA kit (PeliKine Compact) according to the manufacturer's instructions. All reagents were supplied in pre-determined concentrations, and used at the recommended dilutions. 100µl monoclonal anti-human IL-6 was coated onto flat bottomed microtitre plates during an overnight incubation at room temperature in a 0.1M carbonate/bicarbonate buffer pH 9.6. The plates were washed five times with 0.01M PBS containing 0.05% tween (PBS-T). In order to prevent non-specific binding of the antibody the wells were incubated with 200µl with the supplied blocking reagent at room temperature for one hour. The plates were washed five times in PBS-T. Human IL-6 standards in the range of 0-450pg/ml and HUVEC supernatants were diluted in the supplied buffer and added to the plate before incubation for a further hour at room temperature. After another five washes the plates were incubated with 100µl biotinylated IL-6 antibody for one hour at room temperature. After another five washes in PBS-T the plates were incubated with 100µl streptavidin-horse radish peroxidase conjugate for 30 minutes. After a final five washes the plates were incubated in 100µl per well with hydrogen peroxide and 3,5,3',5' tetramethylbenzidine (TMB) (BD Biosciences, Oxford, UK), for 30 minutes in the dark. The reaction was stopped with 100µl per well of 1.8M sulphuric acid. The coloured product was quantified on a plate reader at an absorbance of 450nm, and the concentration of IL-6 in the samples was determined from the standard curve.

2.6.4 Enzyme-linked immunosorbent assay (ELISA) for interleukin-8 (IL-8) concentration

The concentration of total IL-8 was measured in 100µl of HUVEC supernatant using a commercially available ELISA kit (PeliKine Compact), the method was performed as described for IL-6 in section 2.5.3. Human IL-8 standards in the ELISA were in the range 0-240pg/ml.

2.6.5 Flow cytometry analysis of intracellular cell adhesion molecule-1

Trypsin was used to remove HUVECs as in section 2.3.4, and cells were centrifuged at 1500rpm for 5 minutes at 4°C, followed by washing twice with 200µl FACS buffer. Cells were incubated with 10 FCS% in FACS buffer at 4°C for 30 minutes. Cells were washed twice with PBS followed by incubation with 100µl (1:100 dilution) Mouse Anti-Human CD54 (ICAM-1) FITC conjugate (Chemicon, Hampshire UK) to the fatty acid supplemented cells, or 100µl of 1:100 mouse IgG1 FITC for control cells (Chemicon, Hampshire UK). These were then incubated in the dark at 4°C for 30 minutes. Cells were centrifuged at 1500rpm for 5 minutes at 4°C, and then washed twice with FACS buffer as before. 250µl FACS buffer containing 4% PFA was added to fix cells.

2.7 NFκB measurments

2.7.1 Preparation of cells for NFκB measurements

HUVECs were plated onto 10cm dishes then rested for 3 days prior the addition of fatty acid supplemented medium at 100µM or oxidised LDL at a protein concentration of 50mg/ml for 5 days followed by stimulation with TNFα at 200U/ml for 0, 0.5,1 and 2 hours.

2.7.2 Preparation of nuclear extracts

Nuclear extracts were prepared by a modified method of Digman et al (1983) as published by Elsharkawy et al (1999). Cells were harvested, washed twice in PBS, and lysed in 10mmol/L HEPES (pH 7.9) containing: 1.5mmol/L $MgCl_2$, 10mmol/L 4(2-aminoethyl) benzenesulfonyl fluoride KCl, 0.5mmol/L dithiothreitol, 0.2% NP40, and 0.5 mmol/L 4(2-amino-ethyl) benzenesulfonyl fluoride (AEBSF). Cell lystaes were centrifuged for 10 seconds at 13,000rpm to collect crude nuclear pellets. Cell lysates were centrifuged for a further 5 minutes at 13,000 rpm and supernatants collected for use as crude cytoplasmic extracts. Nuclear pellets were washed twice in lysis buffer and resuspended in 20mmol/L HEPES (pH7.9) containing: 25% glycerol, 0.42 mol/L NaCl, 1.5 mmol $MgCl_2$, 0.5 mmol/L dithiothreitol, 0.2 mmol/L EDTA, and 0.5mmol/L AEBSF. Pellets were incubated on ice for 10 minutes, followed by centrifugation for

30 seconds at 13,000 rpm; the supernatant contains the nuclear extracts. Cytoplasmic and nuclear extracts were stored at -80°C.

2.7.3 Electrophoretic Mobility Shift Assays (EMSA)

NFκB DNA binding activities were detected using a ^{32}P labelled double stranded oligonucleotides containing a consensus NFκB binding site (Promega, Southampton, UK). Protein concentration of the nuclear extracts was determined as in section 2.2.2. EMSA reactions (20µl volumes) consisted of 1µg poly dI:dC and 3µg nuclear protein extract, incubated at 4°C for 25minutes, followed by the addition of 0.2ng double stranded radio labelled oligonucleotide and a further incubation of 20 minutes at 4°C. Reactions were fractionated through a native polyacrylamide gel containing 0.25x Tris-Borate-EDTA (TBE) at 10mA constant current in a 0.5x TBE running buffer. Gels were transferred to 3mm Whatman paper, dried, and autoradiographed.

2.7.4 Sodium Dodecyl Sulfate-Polyacrylamide Gel electrophoresis and Immunoblotting

Equal quantities of nuclear and cytoplasmic extracts were fractionated through a 12.5% sodium dodecyl sulphate (SDS)- polyacrylamide gel. Gels were run at 20mA constant current for 1.5 hours before transfer for 1 hour at 0.8mA/cm^2 onto nitrocellulose using a Trans-blot semidry protein blot apparatus (Biorad, Hertz, UK). Nitrocellulose membranes were blocked for non-specific protein binding by incubation for 1 hour in PBS containing 5% Marvel. Blots were incubated for two hours with primary antibodies diluted in PBS/tween 20 (0.05%) containing 5% Marvel. Affinity-purified sheep IgG was obtained from animals either immunised with recombient p50, p65 or IκBα. Antibodies were used at the following concentrations anti-p50 at 1:500 and anti p65 and anti-IκBα at 1:3,000. Blots were washed twice in PBS/tween 20 before incubation for 1 hour with donkey anti-sheep horseradish peroxide conjugated antibody at 1:2,000. Blots were extensively washed with PBS/tween 20 before detection using the ECL system (Amersham, Bucks, UK).

2.8 Statistical analysis

Statistical analysis was performed in Excel using T-tests to signify if results were significant (<0.05).

Chapter 3

Development of Mass Spectrometry Methods for Oxidised Phospholipids

3.1 Introduction

Mass spectrometry revolutionised the way in which phospholipids were analysed. As explained in the introduction, it allowed analysis to develop from a time consuming process involving many steps prior to measurement by HPLC or TLC to a rapid procedure. This chapter will examine the mass spectrometry methods that are utilised throughout this thesis to obtain detailed phospholipid compositions. All of the mass spectrometers used contain an electrospray source. The vast majority of mass spectrometry analysis was performed on the Quattro Ultima (Micromass, Wythenshaw, UK), which is used to analyse changes to the compositions of LDL and cell phospholipids; the basic principles of this instrument were described in chapter one. For more detailed information on the changes in the LDL after oxidation a Quadrupole time of flight mass spectrometry (QToF) (Micromass, Wythenshaw, UK) was used; this mass spectrometer is composed of a quadrupole coupled to a time of flight analyser and has a resolution capacity of 10,000 fwhm. Ions enter the mass spectrometer from an electrospray source, the instrument then accelerates all the ions to a detector using a set amount of energy and the m/z ratio of the ions determines when they arrive at the detector. The ions with a lower m/z ratio reach the detector first because of their greater velocity whereas the heavier ions are slower; therefore the m/z is determined by the length of time it takes the ions to reach the detector. This project used the QToF to provide a two-dimensional analysis of phospholipid components by multiple precursor scans. In this mode each sequential precursor ion is selected in the quadrupole and fragmented prior to entering the time of flight region where the ions are accelerated and measured. The equation that relates the m/z obtained to the time-of-flight is:

$$m/z = 2Es(t/d)^2$$

where E is the extraction pulse potential of the mass spectrometer, *s* is the length fo the flight tube over which E is applied, t is the time of flight of the ion and D is the length of field free drift zone.

The final mass spectrometer used is the LTQ Orbitrap™ (Thermo Electron, Bremen); this is a hybrid mass spectrometer which enables sensitive detection and identification of compounds at a resolution of 100,000. The ions are collected in the linear ion trap, and then axially injected into a static electric/magnetic ion trap (C-Trap), from where the ions are squeezed into a small cloud and are electrostatically trapped, while rotating around the central electrode and performing axial oscillation in the Orbitrap. Detectors

measure the electrical signal of ions over time producing cyclical signal, see figure 3.1. The oscillating frequency of these ions is then used in the equation:

$$\omega = \sqrt{\frac{k}{m/z}}$$

In which ω= oscillation frequency, κ= instrument constant and m/z is the mass-to-charge ratio. The frequency of the oscillation is directly related to the mass-to-charge ratio, allowing the frequency to be readily converted into a mass spectrum. The ions in the Orbitrap generate a complex signal whose frequencies are determined using a Fourier Transformation.

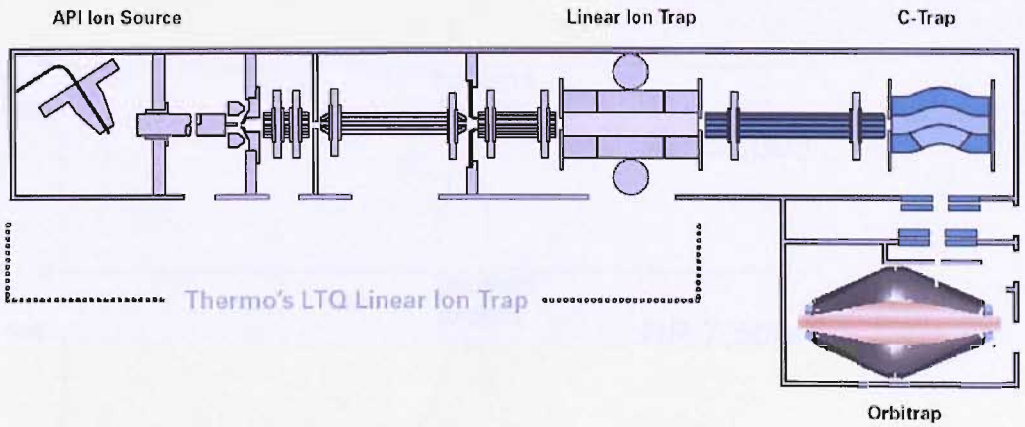


Figure 3.1: A diagrammatic representation of the Orbitrap™ mass spectrometer, which is composed of the linear ion trap, coupled to the C-Trap, which is linked to the Orbitrap™ (Diagram from Theremo Electron).

This project requires the use of mass spectrometers with high resolution as this allows the determination of a high density of mass peaks within a single mass unit. The resolving power of a mass spectrometer is measured as the ratio of the mass of the peak divided by its width. Usually, the peak width is taken as the full width at half maximum intensity (fwhm). Figure 3.2 demonstrates that increasing resolution creates a smaller width peak with standard PC18:18:1 on the Orbitap™, at resolutions of 7500, 15000, 30,000, 60,000 and 100,000 (all resolutions quoted for m/z 400), there is also a slight shift in the mass. Therefore, in a complex sample a high resolution mass spectrometer would be required to distinguish between many peaks in a single mass unit.

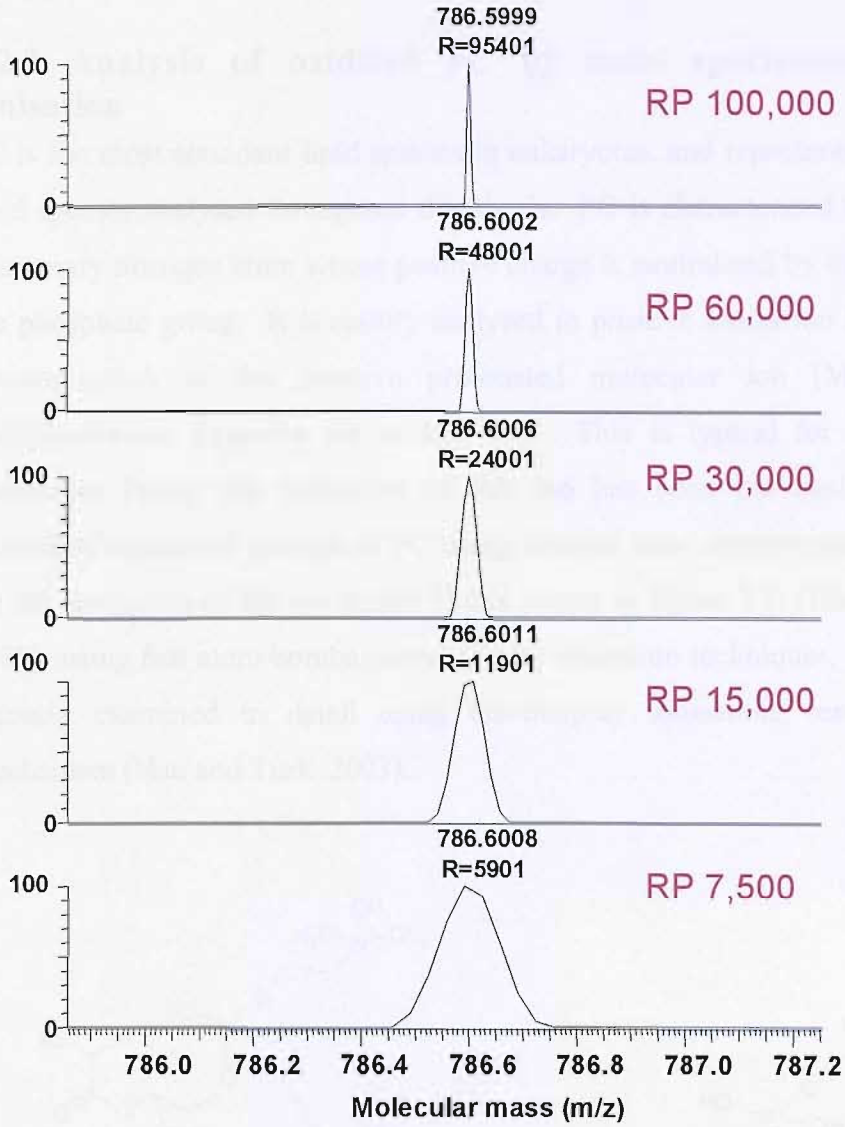


Figure 3.2: The effect of increasing resolution on the precursor ion of standard PC18:1/18:1, using the LTQ Oritrap™ mass spectrometer injected at a flow rate of 5µl/min using an integrated syringe pump.

Aims: To establish reliable mass spectrometry methods to allow the identification of oxidised PC species in LDL and plaque samples.

3.2 Results

3.2.1 Analysis of oxidised PC by mass spectrometry in positive ionisation

PC is the most abundant lipid species in eukaryotes, and represents the majority of the lipid species analysed throughout this thesis. PC is characterised by the presence of a quaternary nitrogen atom whose positive charge is neutralised by the negative charge of the phosphate group. It is readily analysed in positive ionisation by collision induced decomposition of the positive protonated molecular ion $[M+1]^+$, yielding the phosphocholine fragment ion at m/z 184. This is typical for all phosphocholine-containing lipids; the formation of this ion has been the basis for characterising individual molecular species of PC using tandem mass spectrometry. The mechanism for the formation of the ion at m/z 184 is shown in figure 3.3, (Haroldsen and Gaskell, 1989), using fast atom bombardment (FAB) ionisation techniques. This has been more recently examined in detail using electrospray ionisation, resulting in the same mechanism (Hsu and Turk, 2003).

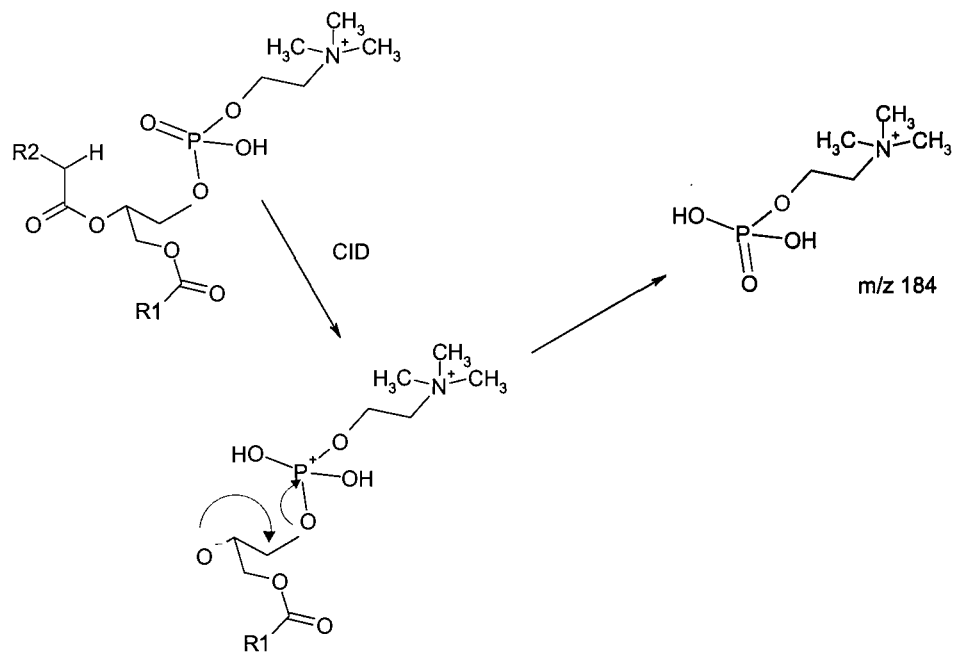
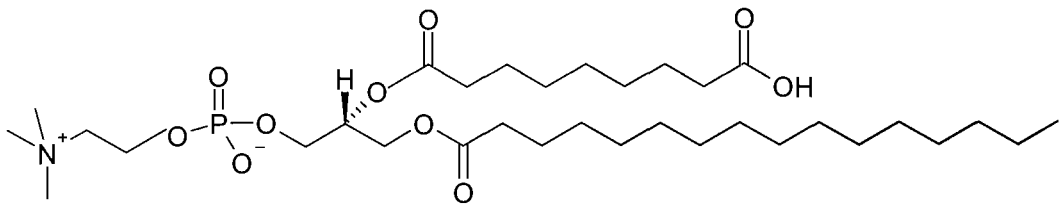


Figure 3.3: Mechanism for the collision induced formation of the phosphocholine ion at m/z 184. This product ion is used as a diagnostic tool specific for phosphatidylcholine.

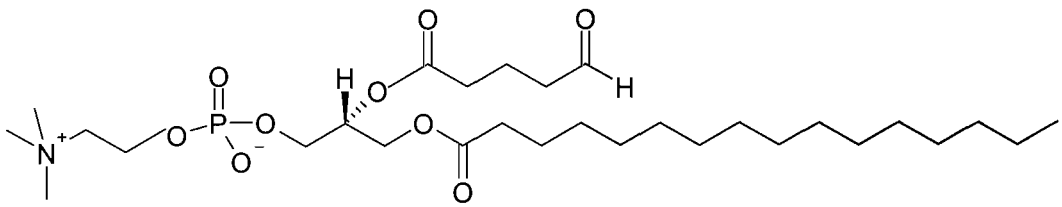
The formation of the phosphocholine ion at m/z 184 was initially confirmed for oxidised PC using standards purchased from Avanti Polar Lipids (Instruchemie, The Netherlands). The standards were analysed by a product scan of the mass of the

standard. The standards used were 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (16:0-09:0(COOH)PC) mass 665.85, 1-palmitoyl-2-(5'-oxo-valeroyl)sn-glycero-3-phosphocholine (16:0-05:0(ALDO)PC mass 593.74, 1-palmitoyl-2-glutaroyl-sn-glutaroyl-sn-glycero-3-phosphocholine (16:0-05:0(COOH)PC) mass 609.74 and 1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine (16:0-09:0(ALDO)PC mass 649.85. The structures of the standards are shown in figure 3.4.

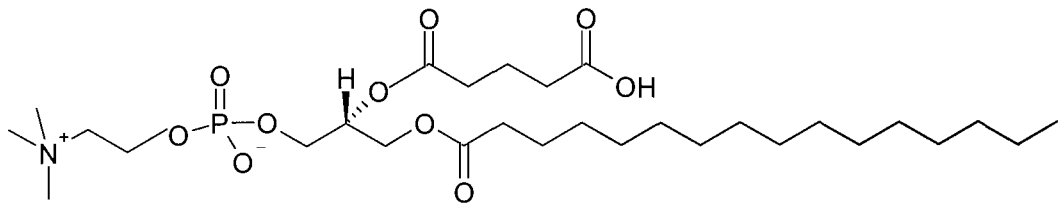
16:0-09:0(COOH)PC mass 665.85



16:0-05:0(ALDO)PC mass 593.75



16:0-05:0(COOH)PC mass 609.74



16:0-09:0(COOH)PC mass 665.85

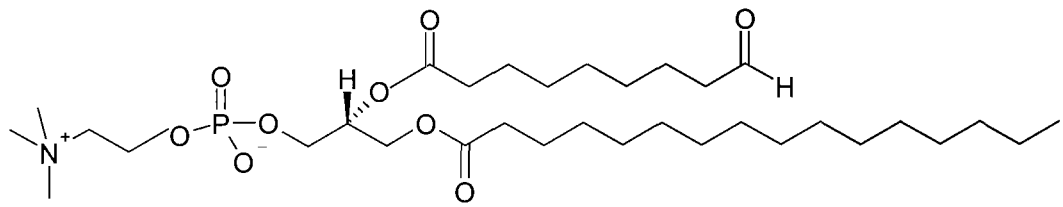


Figure 3.4: Structures of the oxidised PC standards.

The standards clearly demonstrated the formation of this ion at m/z 184, figure 3.5. There were very few other ion peaks formed, a small amount of the parent ion and a small amount of choline at m/z 86, demonstrating the specificity of this scan for PC.

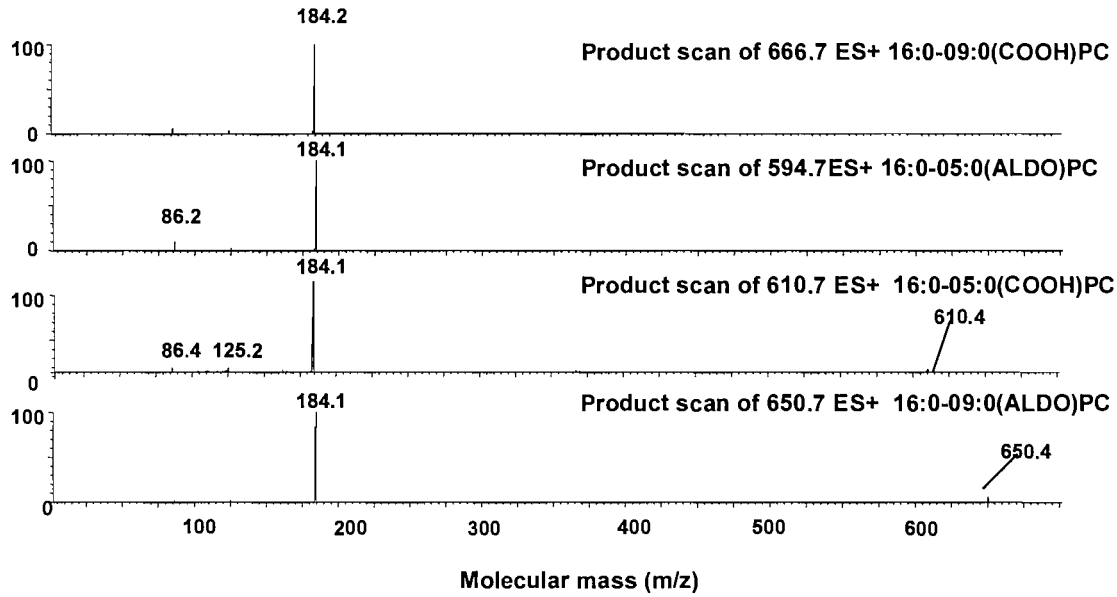


Figure 3.5: Product scans of oxidised PC standards; demonstrating the formation of the PC headgroup in positive ionisation mode.

Therefore, a scan of the product ion at m/z 184 in positive mode can be used for the analysis of oxidised PC, as well as for endogenous PC and lysoPC species. This scan resulted in a peak at m/z corresponding to the mass of the standard, which is demonstrated in figure 3.6 for the standard 1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine (16:0-09:0(ALDO)PC).

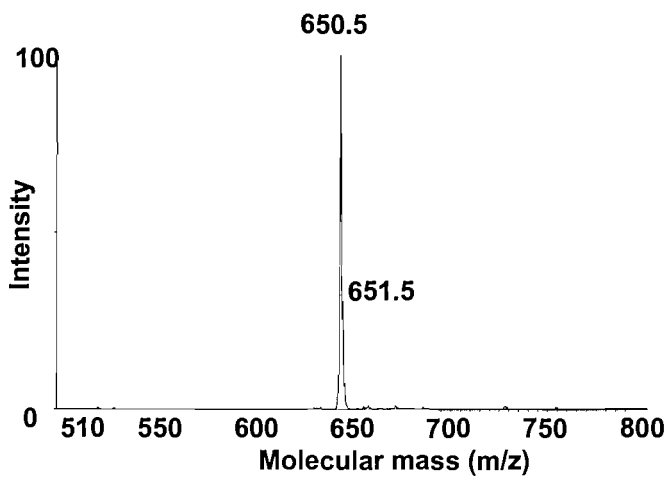


Figure 3.6: Precursor scan of m/z 184; for oxidised standard mass 650, 1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine (16:0-9:0(ALDO)PC).

Lyso-, oxidised and endogenous PC standards were combined and the response of each standard at different dilutions was measured by precursor scans of 184 using the Quattro Ultima. The concentrations of the lysoPC and oxidised PC standards were 10 times lower than the endogenous PC standards. The lysoPC standards used were: 1-myristoyl-2-hydroxy-*sn*-glycero-3-phosphocholine mass 468, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine mass 496 and 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine mass 522. The oxidised PC standards used were: 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (16:0-09:0(COOH)PC) mass 665.85, 1-palmitoyl-2-(5'-oxo-valeroyl)sn-glycero-3-phosphocholine (16:0-05:0(ALDO)PC mass 593.74 and 1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine (16:0-09:0(ALDO)PC mass 649.85. The endogenous PC standards used were: myristoylpalmitoyl PC (PC14:0/16:0) mass 706.5, dipalmitoyl PC (PC16:0/16:0) mass 734.5, palmitoylinoleoyl PC (PC16:0/18:2) mass 758.5, palmitoyloleoyl PC (PC16:0/18:1) mass 760.5, palmitoylarachidonoyl PC (PC16:0/20:4) mass 782.5 and stearoyllinoleoyl PC (PC18:0/18:2) mass 786.5. These standards were analysed on the Quattro Ultima mass spectrometer using precursor scans of 184. The standard mixture was diluted to 0.03 ,0.1, 0.3, 1 and 3nM. The endogenous standards gave linear responses and the oxidised PC standards gave a near linear response at a ten times lower intensity, figure 3.7. Therefore in the samples the oxidised PC species were calibrated by the PC standard and not the lysoPC standard.

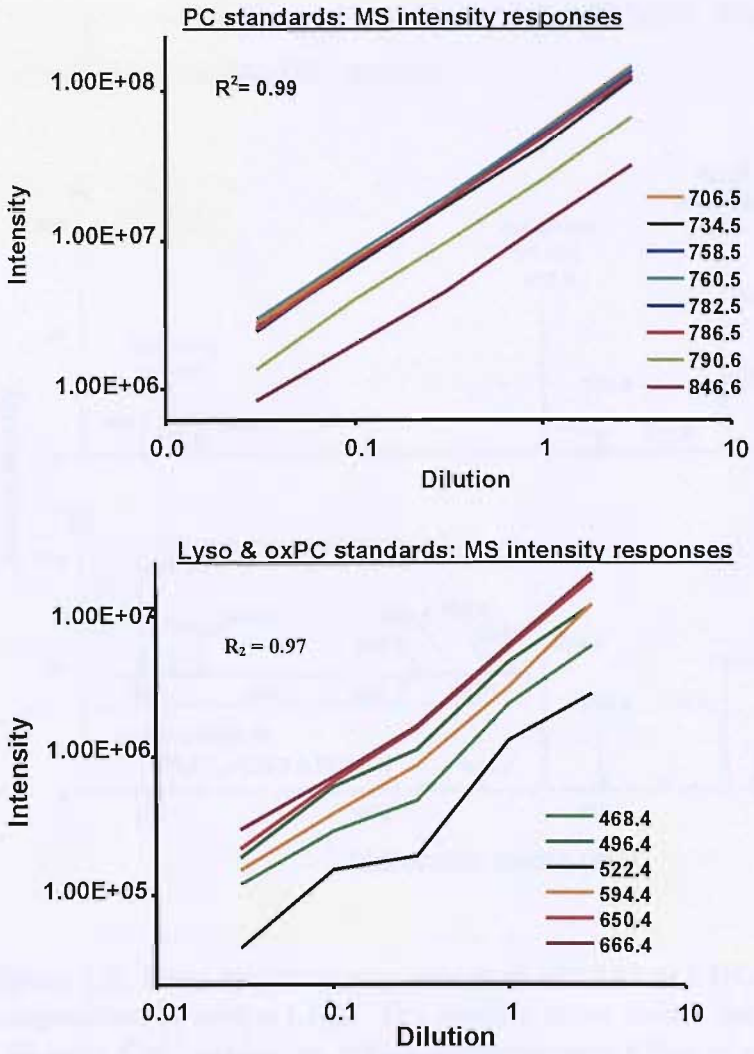


Figure 3.7: Analysis of oxidised and endogenous PC standards in positive ionisation on the Quattro Ultima mass spectrometer at different dilutions, demonstrating that oxidised PC acts in the same way as endogenous PC so the endogenous PC standard can be used for calibration.

The majority of oxidised PC analysis was performed in positive ionisation mode, supplemented with additional fragmentation information from negative ionisation mode. The compositional analysis of LDL and oxLDL took place on the Quattro Ultima mass spectrometer. Figure 3.8 is a typical mass spectra in the m/z range 490-830 for both native LDL (top panel), and LDL after 20 hour oxidation with 40μM $CuSO_4$ (bottom panel). Native LDL had low concentrations of both lysoPC and of PC species in m/z range 594-666, and was enriched with unsaturated PC species, including m/z 758 (PC 16:0/18:2), 786 (PC18:0/18:2), 806 (PC 16:0/22:6) and 810 (PC18:0/20:4). By contrast, oxidised LDL demonstrated decreased ion intensities in LDL PC species at m/z 758, 782, 786, 806 and 810. Oxidation also resulted in increased ion intensities for lysoPC

species and ions in the m/z range 594-666 and 772-830, thought to represent short chain and long chain oxidised PC species.

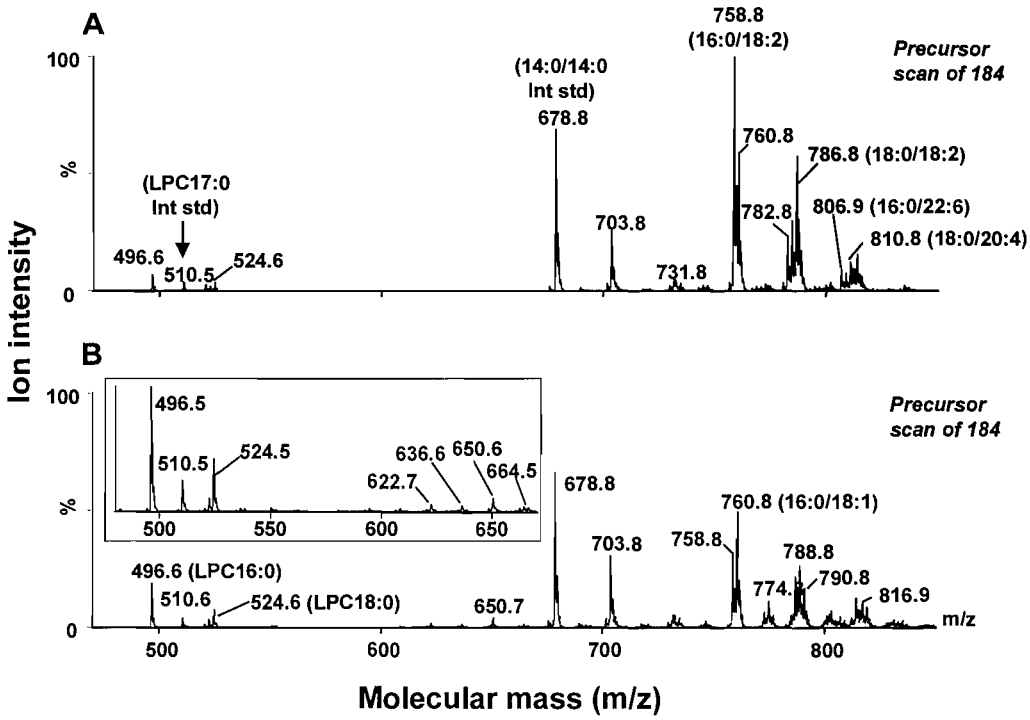


Figure 3.8: Mass spectrometry traces of m/z 184 of LDL. The top trace shows the composition of native LDL. The bottom trace shows the composition of LDL after a 20 hour Cu^{2+} oxidation, which demonstrates a loss of polyunsaturated species, but increases of lyso PC species and species in the m/z range 594-666 and m/z 772-830.

3.2.2 Analysis of oxidised PC by mass spectrometry in negative ionisation

3.2.2.1 Analysis of low mass oxidised PC species

In negative ionisation, PC is normally detected as either $[M-15]^-$ ion (loss of methyl group) (Zirrolli et al 1991), or $[M+35]^-$ (addition of chloride ion). Figure 3.9 demonstrates the analysis of standard PC14:0/14:0 in both positive (bottom panel) and negative ionisation (top panel) mode. Positive ionisation showed a predominant ion peak at m/z 678 with a small contribution from the sodium adduct at m/z 700; negative ionisation resulted in major ion peaks at m/z 662, $[M-15]^-$ and 712, $[M+35]^-$. Although a small ion peak at m/z 676 possibly corresponded to the $[M-1]^-$ ion, clearly the $[M-15]^-$ and $[M+35]^-$ ion peaks predominated. The ion peak at m/z 665 in the upper panel related to a PG standard in this sample.

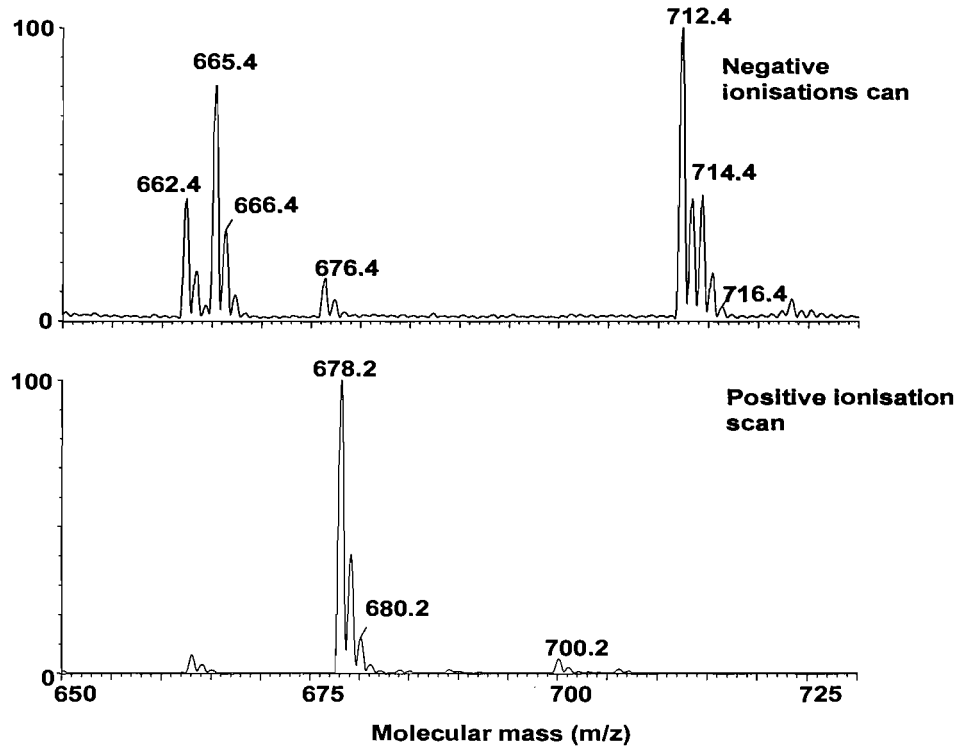


Figure 3.9: Analysis of standard PC14:0/14:0 in negative ionisation (top panel) and positive ionisation (bottom panel), demonstrating the measurement of PC in negative as either 15 mass units lower or 35 mass units higher than in positive ionisation mode.

However, this was not the case with the oxidised PC standards which were detected as either $[M-1]^-$ or $[M-15]^-$. Two examples of the standards used were; 1-palmitoyl-2-(9'-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine, mass 649.85 and 1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine, mass 665.85 (structures of the standards are shown in figure 3.4). Analysis of these standards in negative mode resulted in predominant ions at m/z 648 for 1-palmitoyl-2-(9'-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine and at m/z 664 1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine, these corresponded to $[M-1]^-$ ions, figure 3.10, with a little of the $[M-15]^-$ species detectable for 1-palmitoyl-2-(9'-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine at 634, figure 3.10 (top panel), but none detectable for 1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine at 650 in the lower panel.

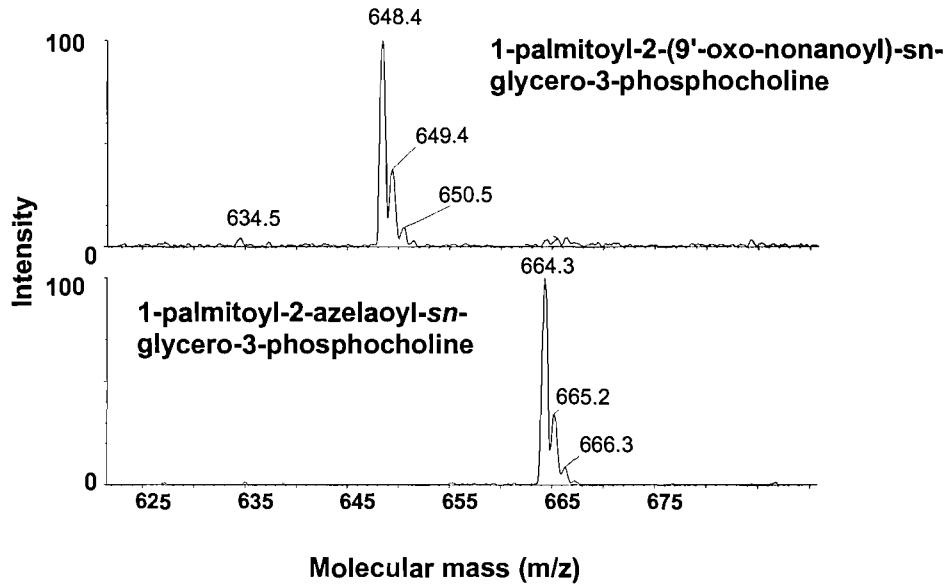


Figure 3.10: Negative ionisation spectra of oxidised standards 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine and 1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine, demonstrating the formation of the [M-1]- ion.

This was unexpected, as Kayganich-Harrison and Murphy (1994) reported the $[M-15]^-$ pattern of ionisation for ω–carboxyl sn-2 oxidised PCs in negative ionisation (not for aldehydes), using FAB ionisation, but it is clear from figure 3.10 that the $[M-1]^-$ ion was preferentially formed. Therefore, this discrepancy with 1-palmitoyl-2-(9'-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine standard was investigated. Further mass spectrometry analysis showed the ion fragmentation was largely dependent on the energy of ionisation (figure 3.11), as the ratio of ion intensities at m/z 648 and 634 varied with cone voltage. There was a greater proportion of the ion at m/z 648 produced at lower energies, reaching a maximum at 55 volts, which fell sharply with increasing voltage. The ion at m/z 634 was much lower in intensity at low cone voltage and reached a maximum at 65 volts.

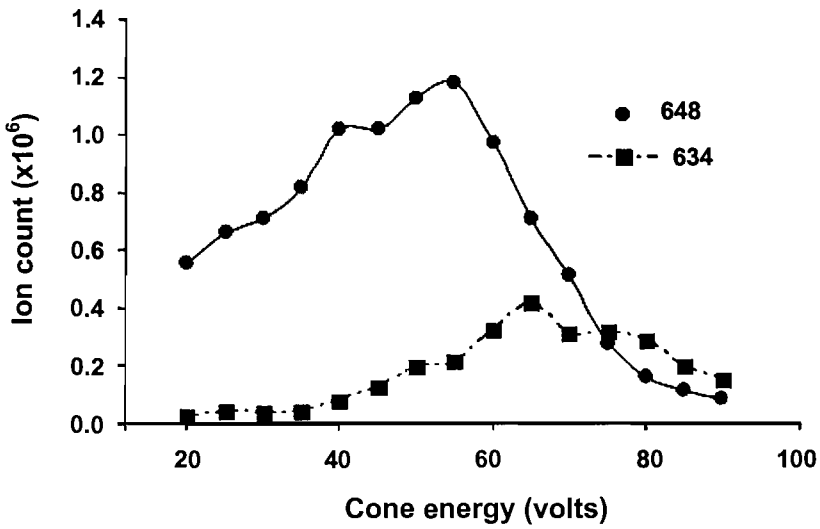


Figure 3.11: Energy dependent generation of the $[M-1]^-$ (648)and $[M-15]^-$ (634) from oxidised standard 1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine.

Product scans of m/z at 634, 648 and 664 were performed in negative ionisation mode. The product scan of m/z 664 from 1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine fragmented to predominantly m/z 255 (palmitate), 201 and 125, figure 3.12 (upper panel). For 1-palmitoyl-2-(9'-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine, the 648 peak fragmented to ions with m/z 255 (palmitate) and 185 with a small peak at m/z 392 which corresponded to neutral loss of palmitic acid (256), figure 3.12 (centre panel). The 634 species generated the expected products, with m/z values corresponding to the masses of palmitate (255) and 9-oxo-nonanate (171), figure 3.12 (lower panel). The structures for these products are shown in figure 3.13. The results clearly showed that when authentic oxidised PC standards were analysed under the low cone voltage conditions described, $[M-1]^-$ species were the predominant negative ions for both the carboxyl and aldehyde molecules, suggesting a common mechanism of ionisation. Thus it appears likely that an intramolecular rearrangement, involving transfer of an *N*-methyl group to the sn-2 ω–carboxyl or aldehyde gives an abundant negative ion with a neutral tertiary nitrogen in place of the choline group. Figure 3.13 shows the formulae and probable mechanism for: 1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine (top panel), with 1-palmitoyl-2-(9'-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine $[M-1]^-$ (middle panel) and $[M-15]^-$ (bottom panel).

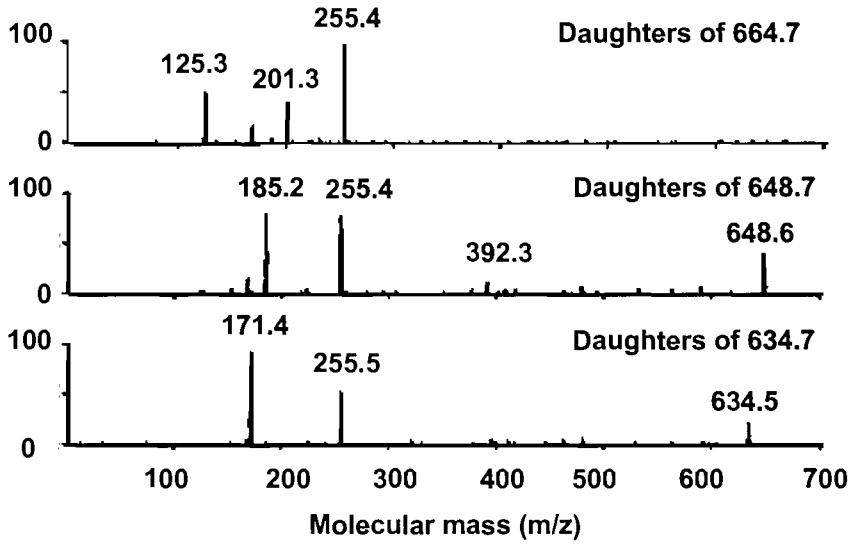
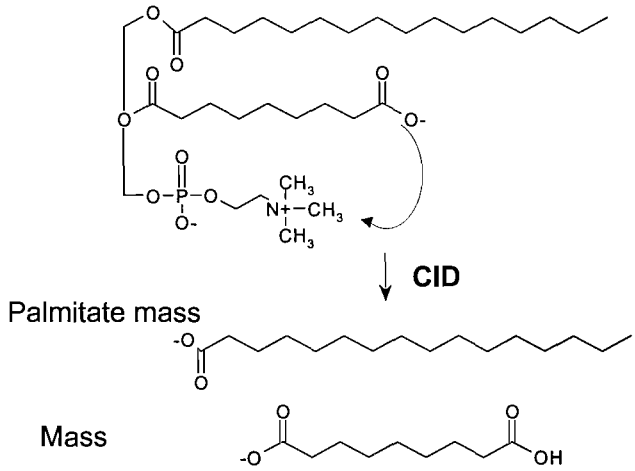
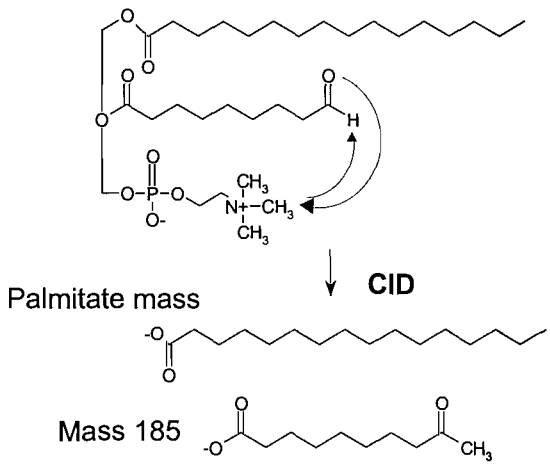


Figure 3.12: ESI MS/MS fragmentation in negative ionisation mode of m/z 664.7 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine and m/z 648.7 and 634.7 for 1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine.

1-palmitoyl-2-Azelaoyl-sn-glycero-3-phosphocholine, mass 664 [M-1]-



1-palmitoyl-2(9-oxo-Nonanoyl)-sn-glycero-3-phosphocholine, mass 648 [M-1]-



1-palmitoyl-2(9-oxo-Nonanoyl)-sn-glycero-3-phosphocholine, mass 648 [M-15]-

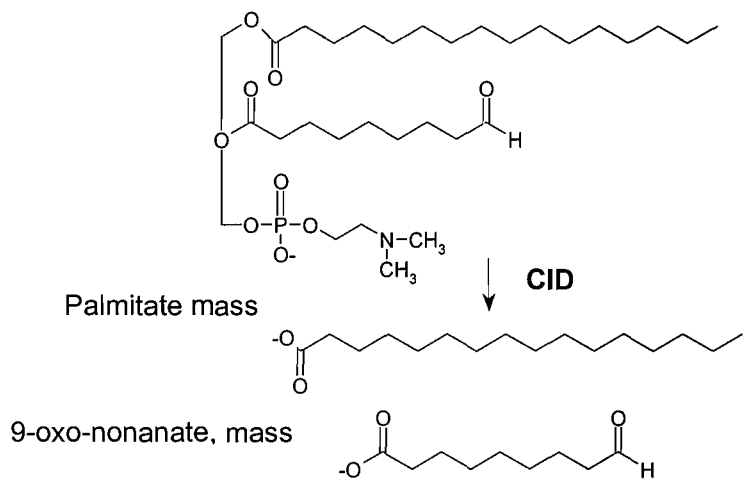


Figure 3.13: Mechanisms for the breakdowns of palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine as the [M-1]- and 1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine as both the [M-1]- and [M-15]- ions.

3.2.2.2 Analysis of high mass oxidised PC species

Figure 3.14 shows spectra from fragmentation analysis of the $[M-15]^-$ peaks in negative ion mode which corresponded to ion peaks in positive ionisation at m/z 788 for both native LDL and oxLDL and 790 for oxLDL only, as there was very little detectable 790 in the native LDL. In native LDL the daughters of 772 spectrum showed the expected pattern for PC18:0/18:1, that is predominant peaks at m/z 283 (stearate) and 281 (oleate) (the peak at m/z 279 is a carry over from 18:0/18:2 (m/z 786)). These peaks were also present in the 772 daughter ion spectrum from oxLDL, but there were also prominent peaks at m/z 255 and 309. This provides good evidence that an oxidised product was formed during LDL oxidation from an original palmitoyl-containing PC; the fragmentation product at m/z 309 is likely to represent an (unknown) product of oxygenation of a polyunsaturated substituent at the sn-2 position of this precursor. Similarly the oxLDL PC product at 790 showed predominant fragmentation products (daughters of 774 in negative) at 255 and 311, corresponding to palmitate and another unknown oxidised fatty acid.

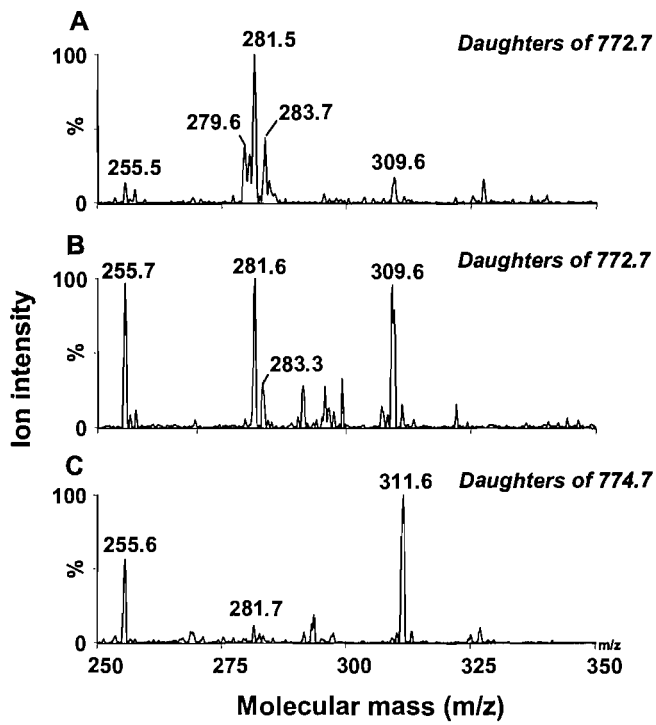


Figure 3.14: Negative ionisation fragmentation of A: m/z 772.7 from native LDL, B: m/z 772.7 and C: m/z774.7 from copper oxidised LDL.

3.2.3 Further characterisation of oxidised PC

3.2.3.1 Comparison of peak resolution on the Quattro Ultima and QToF

To allow further characterisation of the oxidised species within oxLDL and plaque samples, good resolution of the peaks within a single mass unit was required. The Quattro Ultima mass spectrometer, used above, was not able to distinguish between the high density of peaks in a single mass unit. Therefore, a higher resolution mass spectrometer was required such as a Micromass QToF. However, the spectra produced were not of a high enough quality to distinguish between the peaks that were present in the oxidised LDL, partly due to the low sample amounts. Figure 3.15 clearly demonstrates the limitations of these two mass spectrometers under the conditions used; which would make it difficult to characterise these lipids, as there is probably more than one species present in a peak.

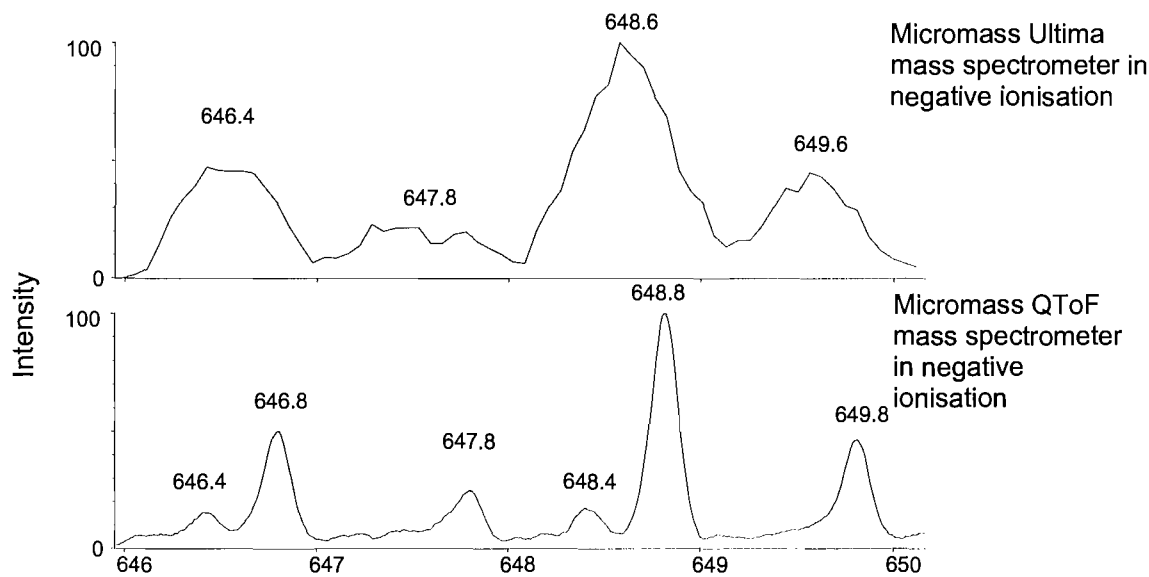


Figure 3.15: Comparison of mass spectrometry traces from the Quattro Ultima and QTOF. The QTOF has better resolution of the species in a mass range, allowing two species within a mass unit to be identified, but there is not good enough separation to allow accurate characterisation.

3.2.3.2 Separation of phospholipids classes

To allow clearer characterisation of the oxidised phospholipids by mass spectrometry, samples were separated into three fractions: phospholipid classes PC, PE and acidic phospholipids. Plaque samples from patients undergoing carotid endarterectomy were used for this analysis, as they contained the most complex mix of phospholipids. To isolate the lipid fractions, dried lipid extract was redissolved in 1ml chloroform and applied to a Bond Elut NH_2 disposable solid phase extraction column, the phospholipids were separated into the classes, by sequential washing of the Bond Elut column with solvent, as detailed in chapter 2 section 2.2.2. Precursor scans of m/z 184 were performed on each of the eluted fractions; the oxidised species were eluted from the column with the increasing polarity of the solvents, with the acidic phospholipids, figure 3.16.

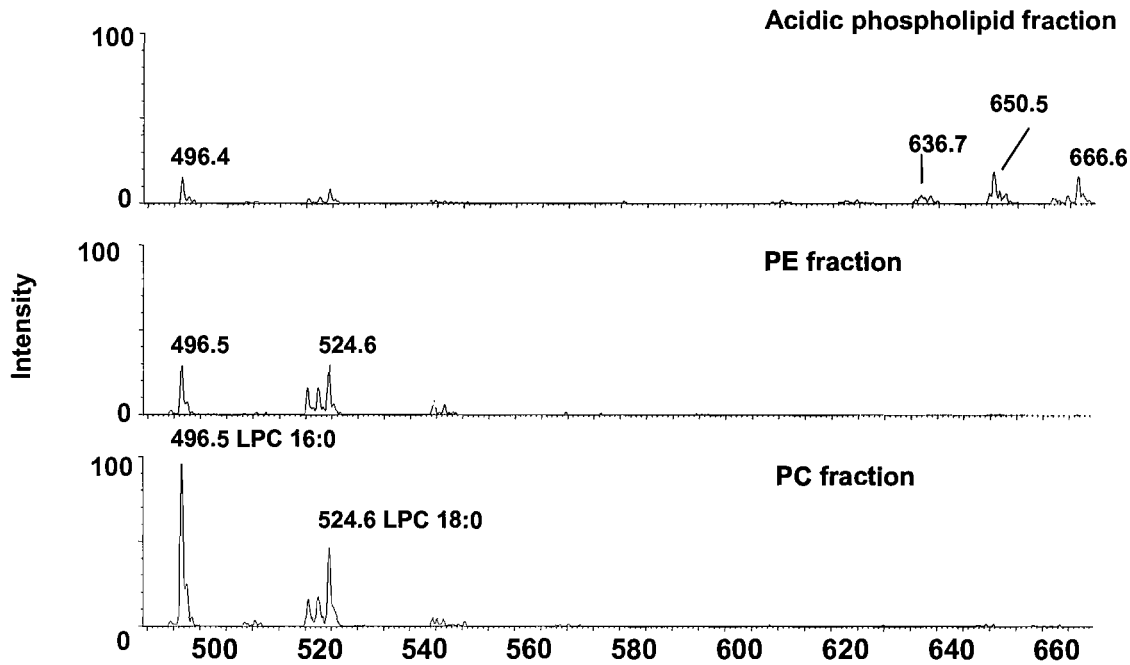


Figure 3.16: Precursor scans of m/z 184 on each of the Bond Elut fractions from a plaque sample, demonstrating that oxidised phospholipids were predominately eluted in the acidic phospholipid fraction.

This method was an easy way to separate the lipids in the plaque samples, thus permitting straight forward analysis of the oxidised lipids which were present in a low concentration compared to the other PC species and other lipid classes. The separated plaque samples were analysed by product ion scans corresponding to the masses of

oxidised standards. These scans resulted in preliminary identification of one of the species present in the plaque sample which corresponded to the oxidised standard 1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine, figure 3.17.

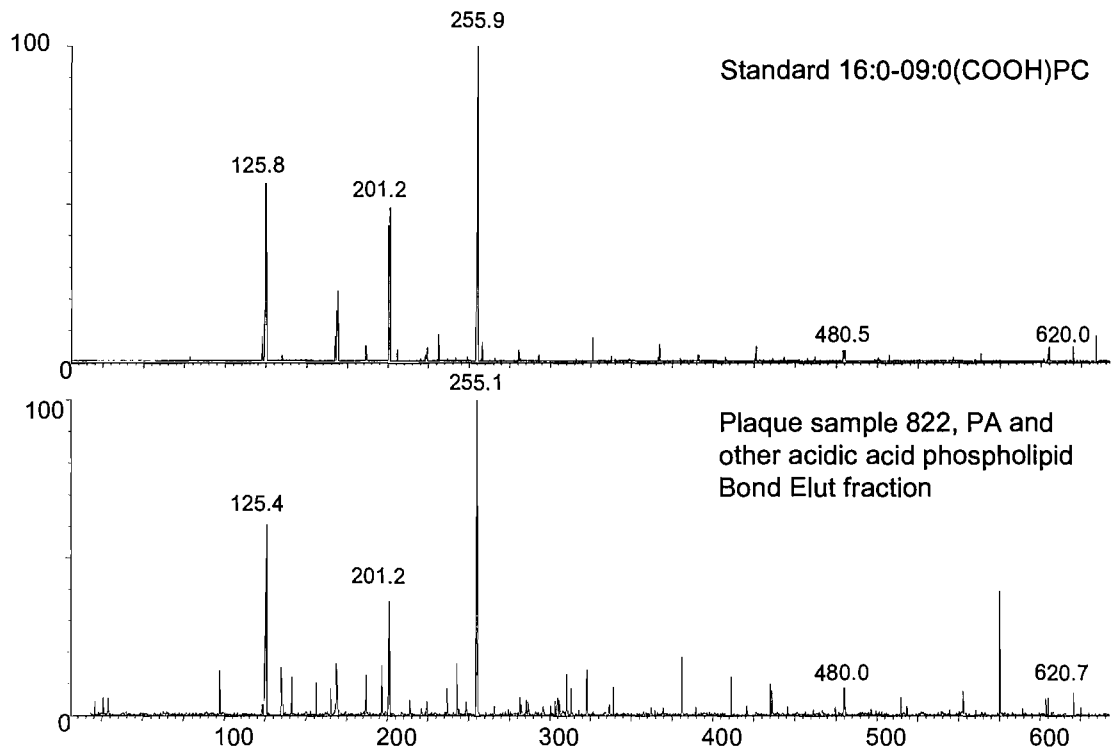


Figure 3.17: Product ion scans of m/z 664 in negative ionisation mode, the top panel is the oxidised standard 1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine and the bottom panel is for a plaque sample.

The other standards did not correspond to the oxidised species present in the samples. For example, the predominate ion peak from the m/z 648 in the oxidised LDL had an ion peak at m/z 392, which was consistent with the neutral loss of palmitic acid (mass 256), with only trace amounts at m/z 255 and 185, the predominant peaks of the standard 1-palmitoyl-2-(9'-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine, figure 3.18. Therefore, it was concluded that either the major species measured at m/z 650 in oxidised LDL and plaques in precursor scans of 184 was not 1-palmitoyl-2-(9'-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine, or the species with an ion peak at m/z 648 in negative did not correspond to the oxidised species at m/z 650 in positive.

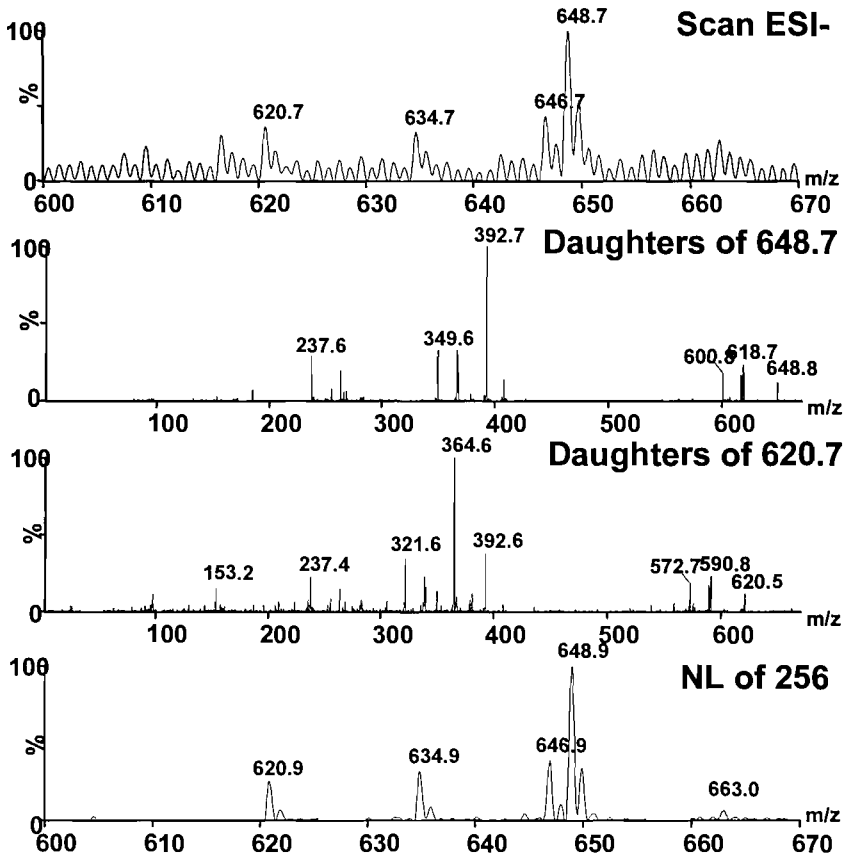


Figure 3.18: Tandem MS/MS analysis of copper oxidised LDL in the presence of Lp-PLA2 inhibitor SB222657. The upper panel is a typical negative spectrum of the low mass oxidised species. The middle two spectra are product scans of two of the major oxidised species, both demonstrating a major peak at m/z 392, which is 256 mass units lower than the oxidised species. The bottom panel demonstrates the presence of these oxidised species after a scan for the loss of 256.

However, when this method was attempted with other plaque samples and oxidised LDL, the oxidised phospholipids did not always elute with the acidic phospholipids as completely. The addition of phosphoric acid for the elution of acidic phospholipids also occasionally affected the mass spectrometry by resulting in the addition of extra ion peaks. Therefore, this method could not be relied upon alone under the conditions used, so other ways for achieving separation were investigated. One option was to establish an LC/MS/MS method, but this would have been time consuming to establish correct eluent conditions and a lack of sufficient free instrument time prevented this being satisfactorily completed. The other option was to use a higher resolution mass spectrometer capable of good separation of phospholipids, such as the recently available LTQ Orbitrap™.

3.2.3.3 Detailed analysis of oxidised PC by mass spectrometry in positive ionisation of the low mass oxidised species

For the characterisation of the oxidised phospholipids the LTQ Orbitrap™ mass spectrometer was used as it was capable of producing accurate masses for each of the oxidised PC species present in the oxLDL and allowed ions of a particular mass range to be measured as individual species. Upon the identification of the individual species in a mass unit it was possible to use software to aid the generation of formulae for each of the species. Figure 3.19 demonstrates the different species present in the ion peak at m/z 650 and the formulas related to each species. The top panel is the original trace obtained, showing that there are several species for the m/z 650, these peaks are then analysed individually and elemental formulae assigned.

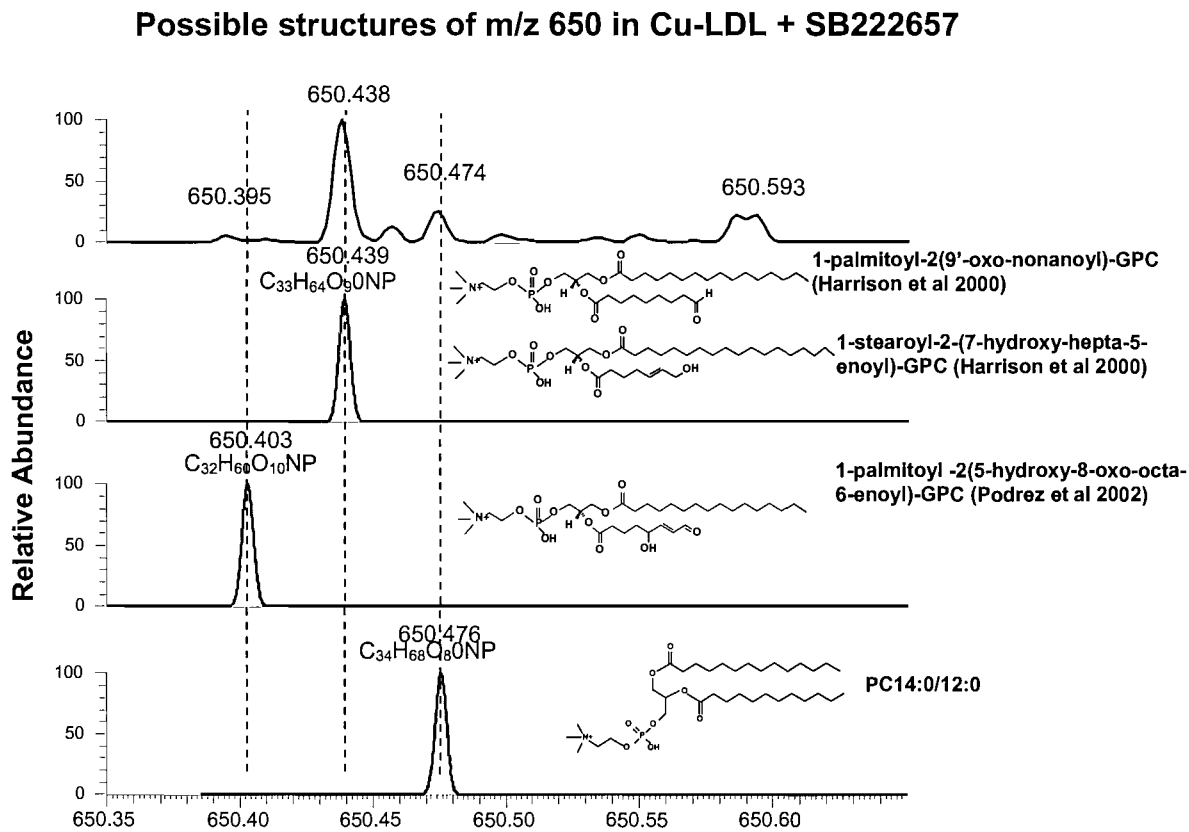


Figure 3.19: LTQ Orbitrap™ analysis of m/z 650 in oxidised LDL. The top panel shows the original data obtained for m/z 650. The lower panels are simulations of each of the species obtained and the elemental formulae allocated to each species.

The most abundant peak in this range had a mass of 650.4387; the other components of this peak are minor constituents, the mass 650.4387 has a molecular formulae of $C_{33}H_{65}O_9NP$, which is the same formula for the commercially available oxidised standard 1-palmitoyl-2-(9'-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine. Therefore

confirming the ion peak at m/z in negative ionisation mode did not correspond to the ion peak at m/z 650 in positive ionisation. These results from the LTQ Orbitrap™ confirm that if there are several species within a single mass unit which are not separated correctly the results obtained can lead to misleading conclusions.

3.2.3.4 Detailed analysis of oxidised PC by mass spectrometry in positive ionisation of the high mass oxidised species

There were also a group of oxidised PC species present in the m/z range 788-854, as shown in figure 3.8; these species are the probable products of oxygen addition at a double bond in the polyunsaturated fatty acid chain. In this section the m/z range focused upon is 788-790 to allow the method development to be presented. The full results are presented in chapter 4. Several of the ion peaks that were present in the higher mass range were also present in the non-oxidised LDL, but the mass related to a completely different species, figure 3.20. In the oxidised LDL the major species for m/z 788 had a mass 788.5432, the molecular formula calculated for this species was $C_{42}H_{79}O_{10}NP$. The ion peak at m/z 790 was calculated to have a molecular formulae of $C_{42}H_{81}O_{10}NP$, this species is likely to correspond to the addition of two oxygens to PC16:0/18:2 (m/z 758). There was also a large quantity of a species with the mass 788.6160, which corresponded to a non-oxidised species, PC18:0/18:1 ($C_{44}H_{87}O_8NP$). In the native LDL it was clear that the non-oxidised species is the major peak at 788 and there was very little m/z 790 present.

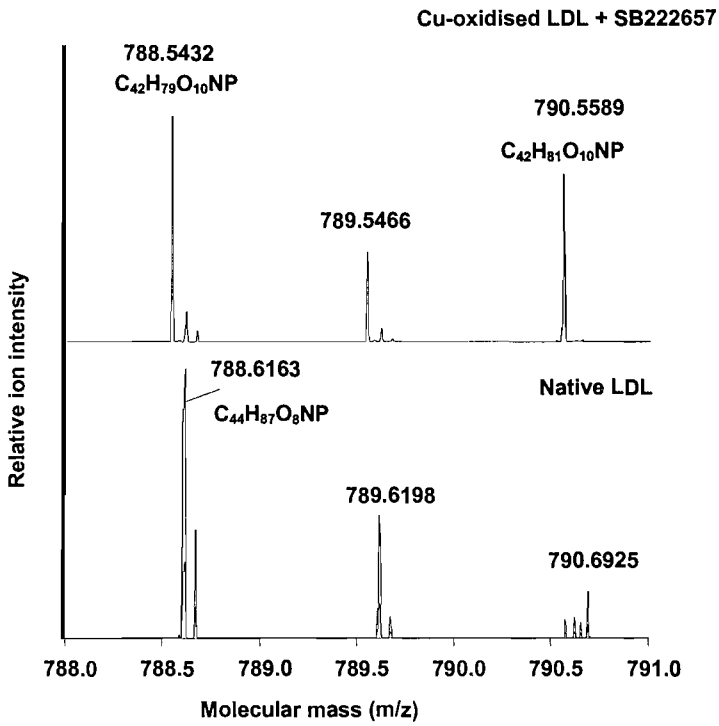


Figure 3.20: Accurate mass analysis in positive ionisation of native and oxidised LDL in m/z 788- 790 region. Panel A is from native LDL and the top panel B is from oxidised LDL.

3.3 Discussion

This chapter demonstrated the methods that were developed to allow detailed analysis of oxidised PC species in both oxidised LDL and plaque samples. Structural analysis was initially performed on the Quattro Ultima mass spectrometer with commercially available oxidised PC standards; the standards were first confirmed to be PC species, demonstrated by the formation of the ion peak at m/z 184, followed by precursor ion scans of m/z 184, which resulted in the formation of the ion peak at the m/z corresponding to the mass of the standard. Further characterisation of these lipids was obtained in negative ionisation mode, which demonstrated some crucial differences between oxidised PC and non-oxidised PC. The oxidised standard 1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine (16:0-09:0(ALDO)PC mass 649.85, resulted in the formation of the $[M-1]^-$ peak. However, at a higher ionisation energy the peak produced was the $[M-15]^-$, but the predominant peak formed was the $[M-1]^-$, although the standard 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (16:0-09:0(COOH)PC) mass 665.85 resulted in the formation of the $[M-1]^-$ peak only, figure 3.13. In contrast, non-oxidised PC has predominate peaks at $[M-15]^-$ and $[M+35]^-$ in negative ionisation mode. The results clearly demonstrated that the products produced by oxidised PC standards were largely dependent upon the ionisation energy; under the initial conditions used both standards ionised as predominant negative ions at $[M-1]^-$. This is in contrast to Kayganich-Harrison and Murphy (1994) who reported this pattern using FAB ionisation for ω–carboxyl, but not for aldehydes.

MS/MS analysis of the 184 precursor of m/z 788 illustrated that the material detected in this peak was both oxidised and non-oxidised. In negative ionisation this peak was analysed as m/z 772, $[M-15]^-$, unlike the low mass oxidised species, demonstrating similar behaviour to non-oxidised PC. In native LDL the products of 772 spectrum showed that this peak was composed almost entirely of PC18:0/18:1, whilst in oxLDL there was also a significant amount of palmitoyl PC, whose other substituent (presumably a product of oxidation of the original, polyunsaturated, sn-2 fatty acid) had a mass of 309. Similarly, the ion peak detected at m/z 790, figure 3.8, in oxLDL was a palmitoyl PC whose other substituent had a mass of 311, figure 3.14.. Interestingly, the oxidised fatty acids of masses 309 and 311 have been previously identified in precursor species of masses 788 and 790 from soybean PC extracts with the use of nano-electrospray Fourier transform ion cyclotron resonance MS (Ishida et al 2004). This data suggested these PC species each had a palmitoyl substituent, m/z 255.2325 and an

oxidised substituent containing two additional oxygen atoms, m/z 309.2073, which corresponded to a fatty acid chain 18:3 with two additional oxygen atoms and 311.2221, consistent to 18:2 with two extra oxygens (Ishida et al 2004). The Orbitrap™ data obtained for the species with ion peaks at m/z 788 and 790, in positive ionisation, confirmed the presence of an oxidised species at m/z 788 which was not present in native LDL. Therefore, it seems likely that the species identified in the oxidised LDL are comparable to those present in the soybean.

To allow further characterisation of these oxidised phospholipids present in real samples, such as oxidised LDL and plaques, good separation of the species present in an individual mass must be obtained. This was initially performed on the QToF mass spectrometer, which resulted in better resolution for the oxidised standards. However, once used with oxidised LDL and plaque samples the resolution on this machine was not capable of distinguishing between the many additional peaks in the samples compared to pure standards, particularly as in plaques the oxidised species represent a very small percentage of the total phospholipid. Therefore, a means of separating oxidised lipids from other lipids was required. HPLC (Watson et al 1997) would have been a suitable option, but the instrument time needed to optimise a method was not available. Consequently, solid phase extraction was attempted with Bond Elut columns (Leitinger et al 1999), initially with promising results, but these results were not reproducible under the conditions used in the laboratory. As a result the LTQ Orbitrap™ was used at Thermo for structural and exact mass analysis. The samples were mainly analysed in positive ionisation due to problems experienced when analysing m/z 648 in negative ionisation mode. The next chapter will use these methods to investigate the compositional changes in the PC composition of LDL following *in vitro* copper oxidation, in the presence and absence of an irreversible Lp-PLA_2 inhibitor with Quattro Ultima mass spectrometer. This will allow the identification of oxidised species in the LDL, which will then be analysed with the LTQ Orbitrap™ and exact masses and elemental formulae will be produced.

Chapter 4
Oxidation of LDL

4.1 Introduction

There is considerable evidence to suggest that oxidative modifications of LDL are key events in the pathogenesis of atherosclerosis at every stage from foam cell formation through to the destabilisation and rupture of the plaque (Hansson 2005). In addition, lipid products of LDL oxidation are hypothesised to act as pro-inflammatory mediators within the plaque and there is increasing evidence indicating a role for oxidised lipids in the development of atherosclerosis in general (Berliner 2002 and Furnkranz 2004). Plaque development is linked to the formation and accumulation of a variety of lipids, which include products of LDL oxidation. These species are potentially bioactive and pro-inflammatory, and their actions may contribute to the advancement and destabilisation of the lesion (Witztum 1998, Berliner 2001, 2002). Both pro- and anti-inflammatory properties have been attributed to phospholipids that have under gone a variety of oxidation modifications to the polyunsaturated *sn*-2 fatty acyl constituents (Watson et al 1997, 1999, Subbanagounder et all 2000, 2002), and some of these species have been identified in both oxidised LDL (Watson et all 1997, Harrison at al 2000 and Podrez et al 2002) and atherosclerotic plaques (Podrez et al 2002 and Hoff et al 2003). Electrospray ionisation mass spectrometry will be used to identify the changes in PC composition of LDL after copper oxidation and compare the species that are formed to those that are present in plaque samples.

Epidemiological evidence suggests that consumption of n-3 polyunsaturated fatty acids, found in oily fish and fish oils, protects against fatal myocardial infarction (Daviglus et al 1997). Secondary prevention studies, which provided n-3 fatty acids to myocardial infarction patients, demonstrated significant benefit (Burr et al 1989, GISSI-Prevenzione Trial 1999). Long chain n-3 fatty acids, such as DHA and EPA, are usually consumed in small quantities in the diet and are therefore found in relatively low proportions in plasma and tissue lipids, but are abundant in the brain. However, an increased consumption of these fatty acids is marked by an increase in their proportion in various blood and tissue lipid pools, including LDL cholesterol esters and phospholipids. Paradoxically, enrichment of LDL with n-3 fatty acids is thought to enhance its susceptibility to oxidation (Suzukawa et al 1995, Hau et al 1996, Oostenbrug et al 1997). Electrospray mass spectrometry will be used to determine the changes in the PC composition of LDL before and after oxidation in DHA and EPA enriched LDL.

Lipoprotein associated phospholipase A_2 (Lp-PLA_2) predominately circulates as a constituent of LDL particles (Stafforini et al 1987 and 1999) and hence primary products of LDL oxidation may be subject to Lp-PLA_2 mediated hydrolysis. Identification of a high circulating concentration of Lp-PLA_2, as an independent risk factor in cardiovascular disease (Packard et al 1999 and Caslake et al 1999), together with increasing evidence for the presence of the enzyme in atherosclerotic lesions (Hakkinen et al 1999, Papaspyridonos et al 2006 and Kolodgie et al 2006), has provided rationale to support the development of Lp-PLA_2 inhibitors as possible anti-atherogenic drugs (Schumaker et al 1986, Suckling et al 2002 and Macphee et al 2006). Despite this interest, the consequences of Lp-PLA_2 inhibition at the molecular level have not been systematically explored, either *in vitro* or *in vivo*. Therefore, electrospray ionisation mass spectrometry will be used to quantify the changes to PC of purified human LDL caused by *in vitro* oxidation in the presence and absence of an irreversible Lp-PLA_2 inhibitor SB222657, the LTQ Orbitrap™ mass spectrometer will be used to determine the exact mass and molecular formulae of the oxidised species. Plaque samples from patients randomly assigned Lp-PLA_2 inhibitor SB480484 prior to carotid endarterectomy will also be analysed for changes in PC composition. The identification of PC species within human atherosclerotic lesions and LDL, could lead to potential *in vivo* markers of atherosclerotic disease progression and response to Lp-PLA_2 inhibitor therapy.

Aims: To measure changes in PC composition by mass spectrometry in LDL before and after oxidation in the presence and absence of an Lp-PLA_2 inhibitor and to use exact mass mass spectrometry to obtain exact masses for oxidised species. The oxidised species will be measured in control LDL and LDL from volunteers receiving DHA or EPA supplements.

4.2 Results

4.2.1 Oxidised LDL

Incubation of control LDL (n=6) with 40μM $CuSO_4$ for 20 hours caused substantial and reproducible alterations to the PC composition of LDL. It was assumed that oxidation under these conditions had proceeded to completion on the basis that no further enhancements of thiobarbituric acid reactive substances (TBARS) or lipid hydroperoxides were detected for oxidation reactions extended beyond this time (data not shown). Copper oxidised LDL was analysed by mass spectrometry by precursor scans of m/z 184, in positive ionisation mode using a Quattro Ultima mass spectrometer. An example of mass spectra representative of the changes observed in LDL PC composition incubated with and without $CuSO_4$ was shown in chapter 3, figure 3.7. The traces demonstrate that oxidation decreased ion intensities of PC species at m/z 758, 782, 786, 806 and 810 and increased the intensity of the ions at m/z 772, 774, 788, 790, 800, 802, 816 and 818. Oxidation also increased the abundance of lysoPC species at m/z 496 and 524 and a group of ions in the m/z 594 to 666 range. The altered concentrations of these species after Cu-induced oxidation are shown in figure 4.1 as a mean of six LDL preparations; values for each mass peak have been calculated as a percentage of the total PC measured in the native LDL for each preparation before averaging. The extent of the oxidation for diacyl PC species correlated with the degree of fatty acyl unsaturation. Thus there was a loss of most PC species containing arachidonate (20:4) or docosahexaenoate (22:6), significant loss of di- and tri-unsaturated fatty acid-containing species and negligible change in observed concentration of the major mono-unsaturated species, PC16:0/18:1, figure 4.1.

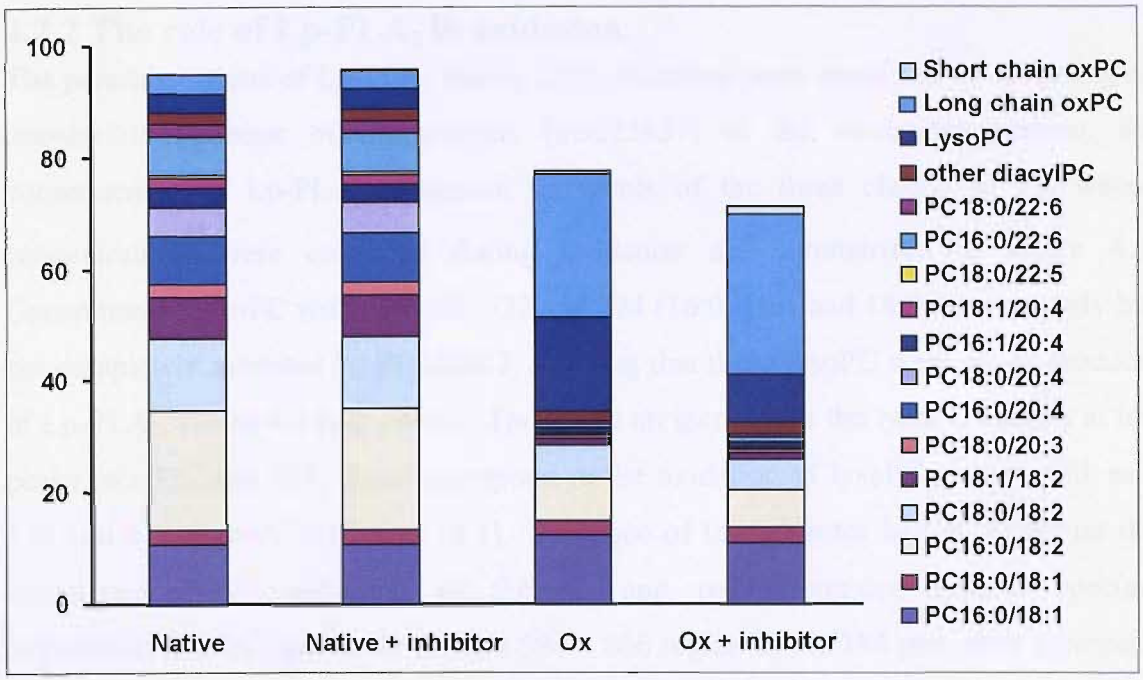


Figure 4.1: PC composition of native and copper-oxidised LDL. Bars represent mean values from native and oxidised LDL isolated from 6 separate preparations; values for each mass peak were calculated as a percentage of the total PC measured in the native LDL for each donor before averaging.

The concentration of measurable PC species in the oxidised LDL was considerably less than that in the native. This would suggest that this material had either degraded, during the oxidation reaction, to a point where it was no longer extractable in the chloroform phase, or had formed species (possibly protein adducts) that were either no longer extractable or were not detectable in the range analysed.

Three categories of PC molecules were increased by oxidation. Firstly, the most significant increases were to the species detected over m/z range 772-830. It is presumed that these corresponded to products with the addition of oxygen at double bonds in the fatty acids. Secondly, oxidation significantly increased the concentrations of saturated and monounsaturated lysoPC species, 16:0, 18:0 and 18:1 and decreased all the polyunsaturated species, 18:2, 20:4 and 22:6. Finally, oxidation increased the concentrations of species detected between the m/z range 594-666, particularly that of m/z 650. These are likely to represent oxidatively truncated products of polyunsaturated fatty acid containing PC.

4.2.2 The role of Lp-PLA_2 in oxidation

The possible actions of Lp-PLA_2 during LDL oxidation were examined by including an irreversible inhibitor of the enzyme (SB222657) in the oxidation reaction; the consequences of Lp-PLA_2 inhibition on levels of the three classes of PC whose concentrations were enhanced during oxidation are summarised in figure 4.2. Generation of lysoPC with m/z 496, 522 and 524 (16:0, 18:1 and 18:0) was strongly but not completely inhibited by SB222657, showing that these lysoPC were major products of Lp-PLA_2, figure 4.2 (top panel). There was an increase in the lysoPC species at ion peaks m/z 536 and 538; these correspond to the oxidation of lysoPC species with m/z 520 and 522 (lysoPC 18:2 and 18:1). Presence of the inhibitor had no effect on the oxidation-mediated reduction of the di- and polyunsaturated lysoPC species. Importantly, the PC species in the m/z 594 – 666 region of the 184 precursor spectrum, whose levels were increased by oxidation, were all further enhanced by at least two-fold in the presence of inhibitor, figure 4.2 (middle panel). This result strongly suggests that these ion peaks represent molecules that are substrates of Lp-PLA_2. The inhibitor further enhanced concentrations of the higher mass PC species, although the proportional increase in these species was considerably less than that observed for the lower m/z products, suggesting that these PC species were less efficiently hydrolysed by Lp-PLA_2 during LDL oxidation, figure 4.2 (bottom panel).

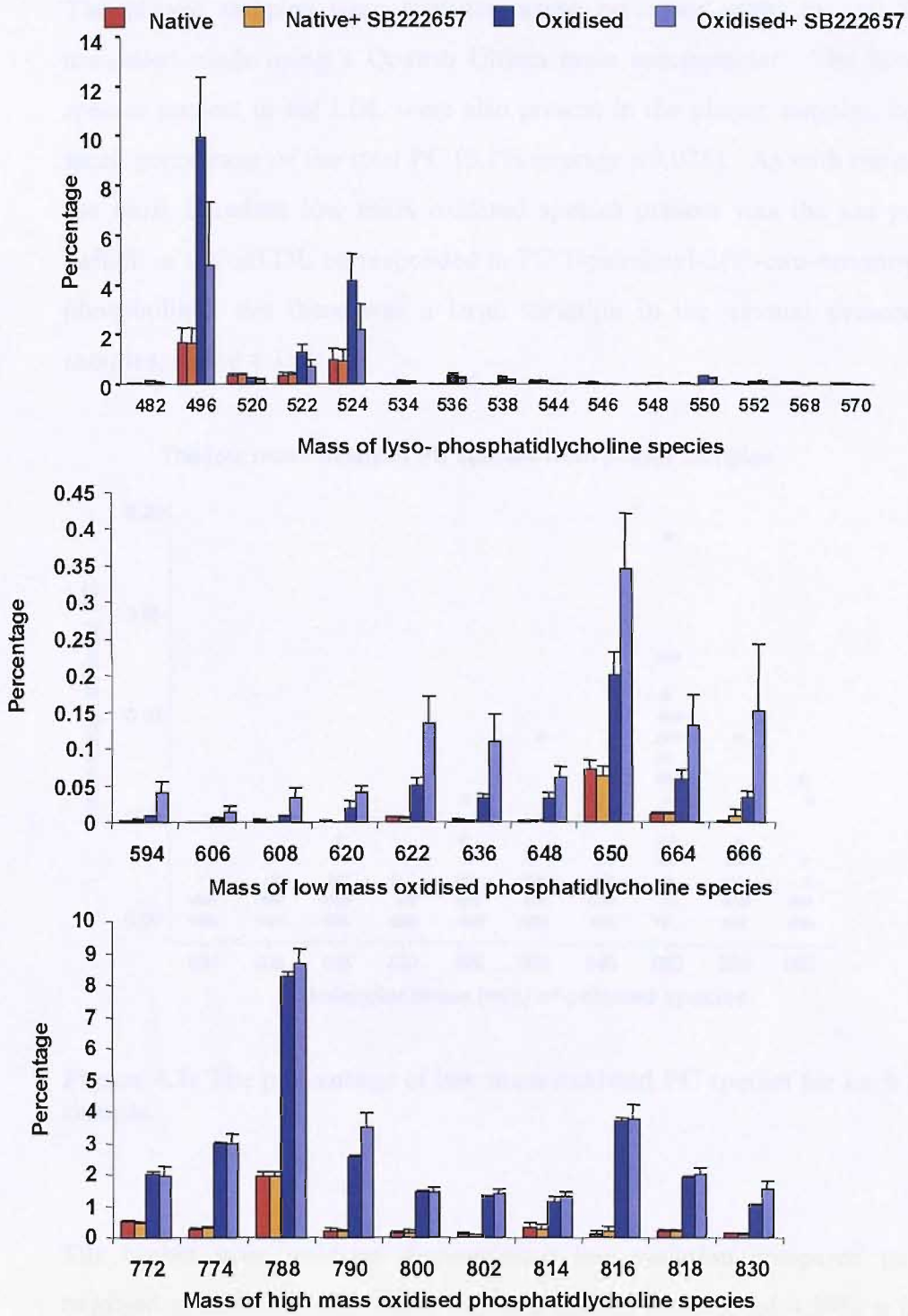


Figure 4.2: Effects of Lp-PLA2 inhibition during LDL oxidation on levels of lysoPC (top: lysoPC m/z of peaks indicated), truncated oxidised PC (middle: m/z of peaks indicated), and other PC whose levels were enhanced by oxidation (bottom: m/z of peaks indicated). Peak 788 in native LDL is principally PC18:0/18:1 but in oxidised LDL corresponds to an oxidised PC species.

4.2.3 Plaques

Patients undergoing carotid endarterectomy (n= 91) received either placebo or Lp-PLA_2 inhibitor (SB480848) tablets at either 40 or 80mg a day for 14 days prior to surgery. The plaque samples were analysed using precursor scans of m/z 184 in positive ionisation mode using a Quattro Ultima mass spectrometer. The low mass oxidised species present in the LDL were also present in the plaque samples, but contributed a small percentage of the total PC (0.1% average ±0.078). As with the oxLDL samples, the most abundant low mass oxidised species present was the ion peak at m/z 650 (which in the oxLDL corresponded to PC 1-palmitoyl-2(9'-oxo-nonanoyl)sn-glycero-3-phospholine), but there was a large variation in the amount present in the plaque samples, figure 4.3.

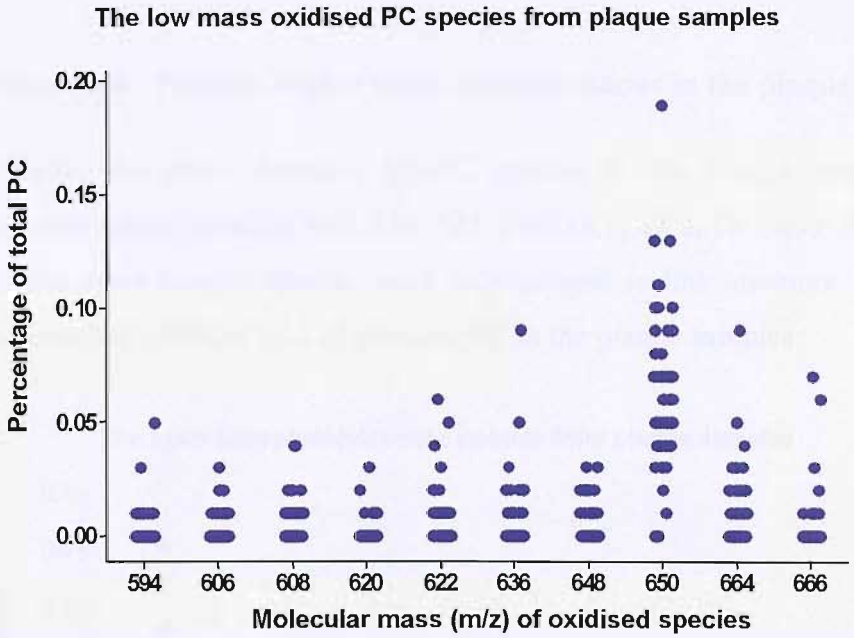


Figure 4.3: The percentage of low mass oxidised PC species for each plaque sample.

The higher mass oxidised demonstrated less variation compared to the low mass oxidised species, the high mass oxidised species contributed 4.20% ± 0.92 to the total PC in the plaque samples. The species with ion peaks at m/z 788 was the most abundant, but it will contain some of non-oxidised PC18:0/18:1, figure 4.4.

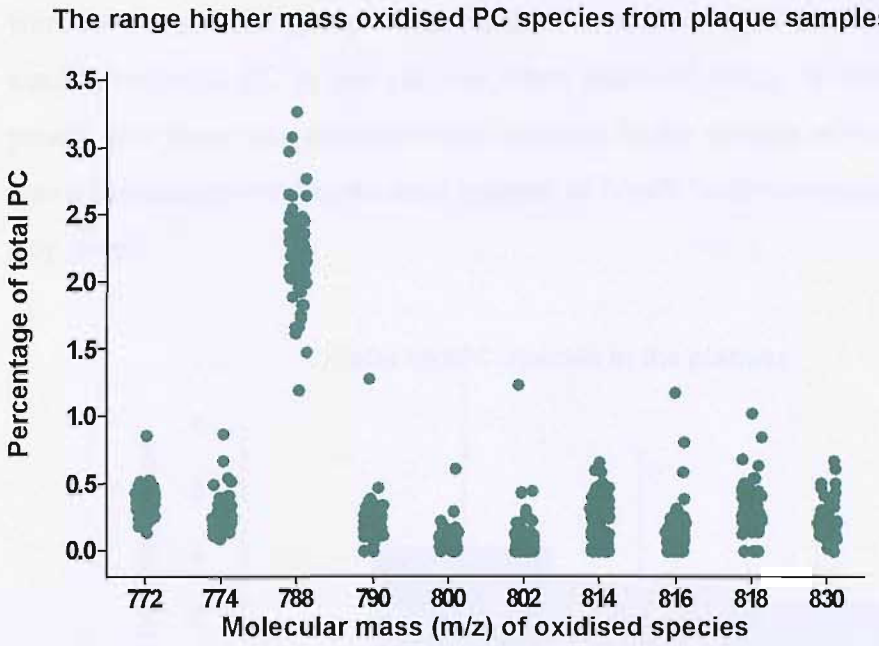


Figure 4.4: Possible higher mass oxidised species in the plaque samples.

Finally, the most abundant lysoPC species in the plaque samples had ion peaks corresponding to m/z at 496, 520, 522, 524 (16:0, 18:2, 18:1 and 18:0), figure 4.5. Most of the other lysoPC species were only present in low amounts. The lysoPC species constituted 1.08% ± 0.32 of the total PC in the plaque samples.

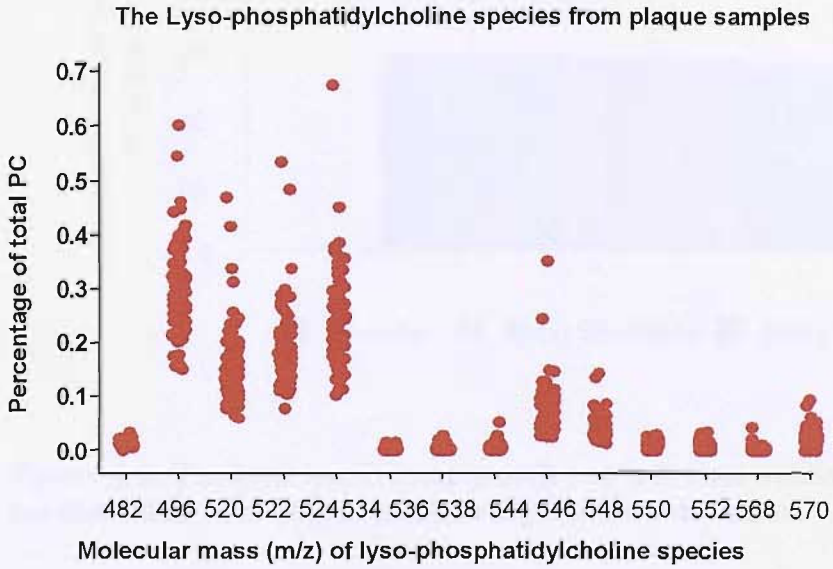


Figure 4.5: The percentage of lysoPC species in each of the individual plaques.

Patient allocation within the trial was roughly equal for the three groups: 30 subjects received 40mg, 32 individuals received 80mg of the inhibitor SB480484 and 29 subjects

were in the placebo group. The results showed no significant difference in the total amount oxidised PC in the plaques when analysed using T-test, figure 4.6 (bottom panel), and there was no significant decrease in the amount of lysoPC, although there was a decreasing trend in the total amount of lysoPC with increased inhibitor, figure 4.6 (top panel).

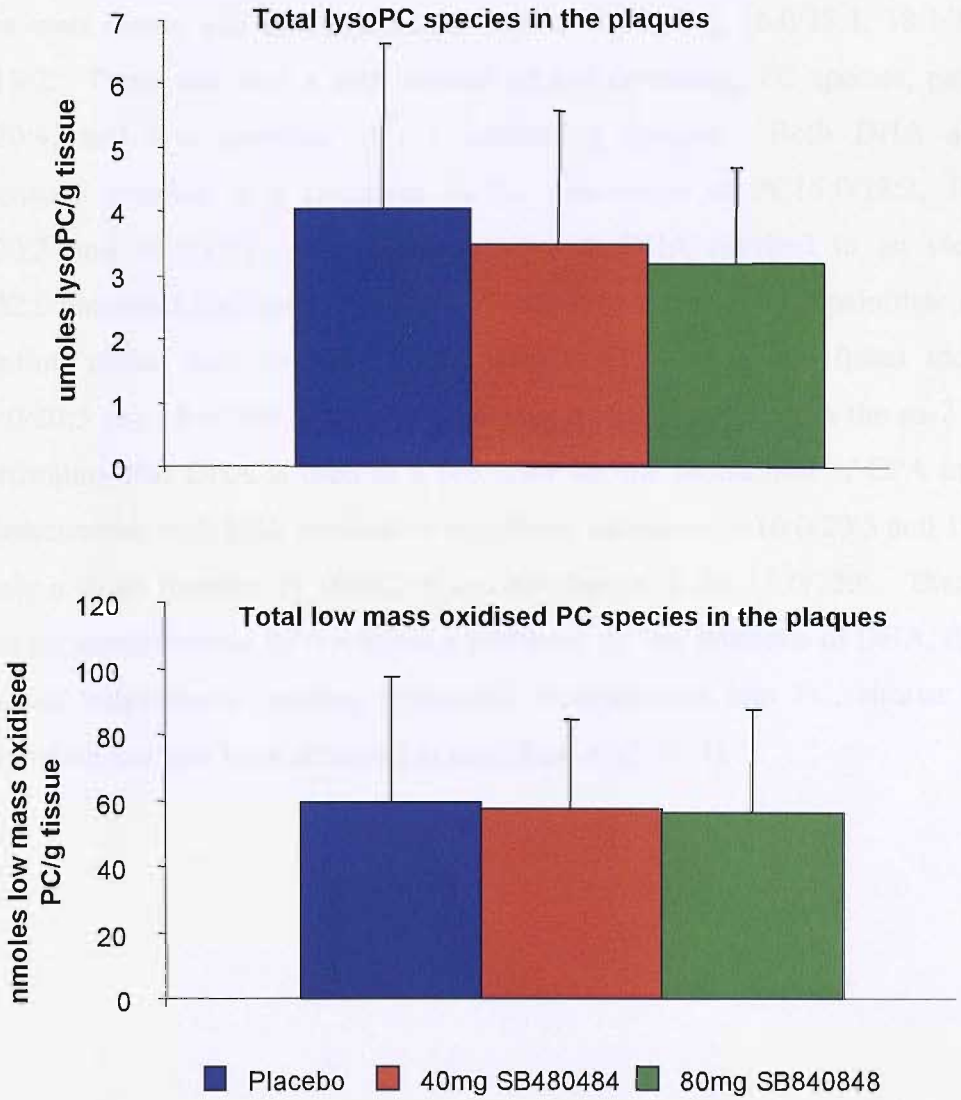


Figure 4.6: The total lysoPC (top panel) and low mass oxidised PC (bottom panel) for the different trial groups, showing standard deviation.

4.2.4 PC changes in plasma from volunteers taking n-3 supplements

Healthy volunteers (criteria for inclusion in study were detailed in chapter 2) were given n-3 supplements of either DHA (n=47) or EPA (n=47) for 4 weeks, after which a blood sample was taken (plasma samples were kindly donated by the University of Reading).

The changes in plasma PC were analysed to gain a strong indication of the changes to the composition of LDL. The control group in figure 4.7 shows the typical lipid composition of human plasma. These control plasma samples were from another study, but were also EDTA plasma so were a suitable control (n=28). The species chosen to be included in the graph were the major PC species of plasma, and the species that were expected to show a change after the supplements. The major PC species in the control plasma were mono- and di- unsaturated, such as 16:0/18:2, 16:0/18:1, 18:1/18:2, and 18:0/18:2. There was also a high amount of n-6 containing PC species, particularly 16:0/20:4, and low amounts of n-3 containing species. Both DHA and EPA supplements resulted in a reduction in the percentage of PC16:0/18:2, 16:0/20:4, 18:0/20:3 and 18:1/18:2. Supplementation with DHA resulted in an increase in 16:0/22:6 but very little change in 18:0/22:6, showing specificity for palmitate in the sn-1 position rather than stearate. DHA also resulted in a significant increase in PC16:0/20:5 and 18:0/20:5, which both contain an EPA fatty acid in the sn-2 position, demonstrating that DHA is used as a precursor for the production of EPA in plasma. Supplementation with EPA resulted in significant increases in 16:0/20:5 and 18:0/20:5, but only a small increase in 16:0/22:6 and no change in the 18:0/22:6. Therefore, in plasma the supplemented EPA was not a precursor for the synthesis of DHA, figure 4.7. Fatty acid supplements resulted in specific incorporation into PC, similar selective incorporation has also been observed in rats (Kew et al 2003).

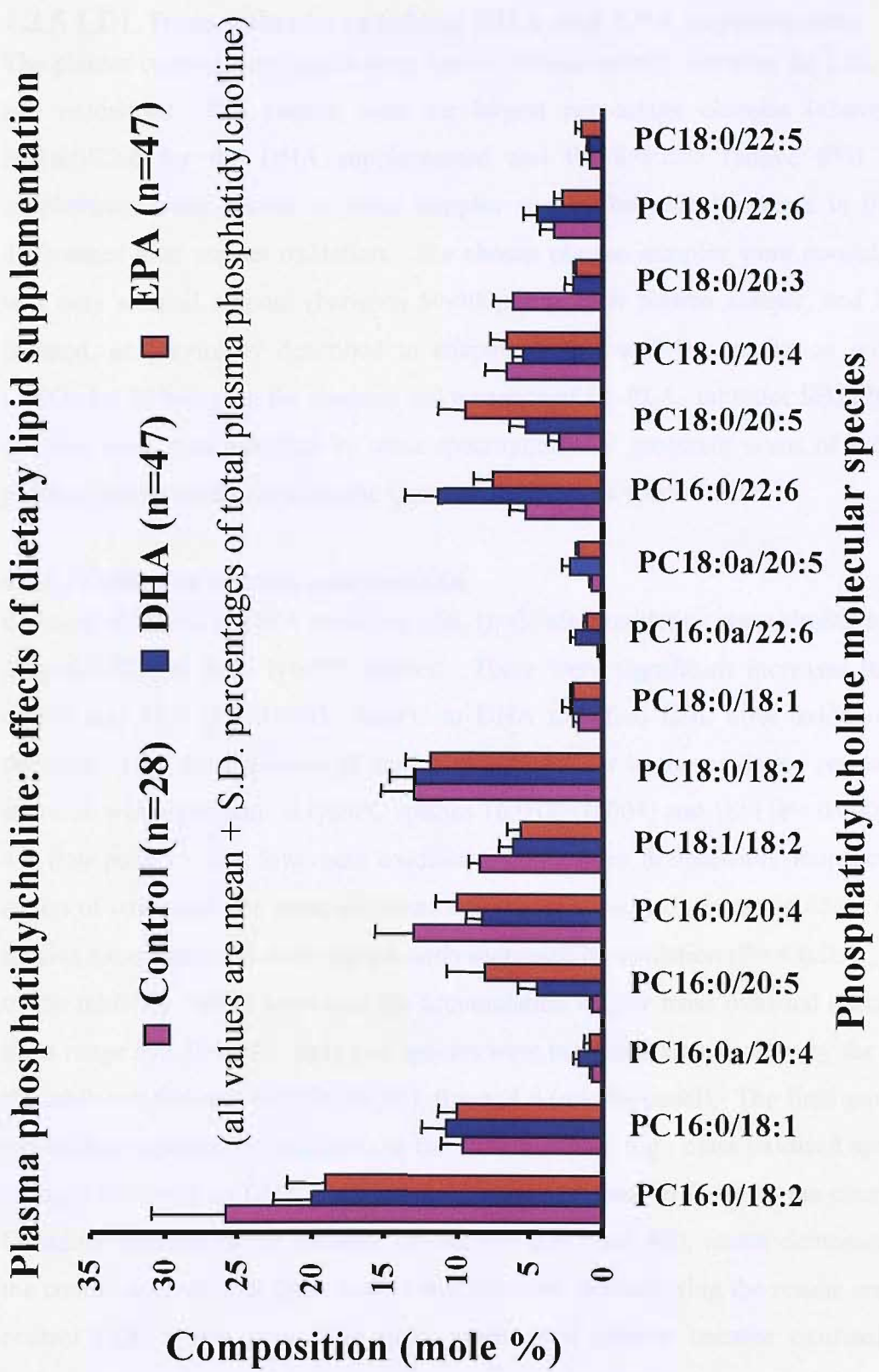


Figure 4.7: Plasma PC composition for control plasma and DHA and EPA plasma after supplementation.

4.2.5 LDL from volunteers taking DHA and EPA supplements

The plasma composition results were used to choose specific samples for LDL isolation and oxidation. The plasma with the largest percentage changes (above 9%) in PC16:0/22:6 for the DHA supplemented and PC18:0/20:5 (above 8%) for EPA supplements were chosen as these samples should theoretically result in the largest differences after copper oxidation. The chosen plasma samples were pooled, as there was only a small amount (between 50-800μl) of each plasma sample, and LDL was isolated, as previously described in chapter 2, followed by incubation with 40μM $CuSO_4$ for 20 hours, in the absence and presence of Lp-PLA_2 inhibitor SB222657. The samples were then analysed by mass spectrometry by precursor scans of m/z 184, in positive ionisation mode using the Quattro Ultima mass spectrometer.

4.2.5.1 Oxidation of DHA modified LDL

Changes observed in DHA modified LDL (n=3) after oxidation were similar to those in control LDL for both lysoPC species. There were significant increases to 16:0 (P =0.08) and 18:0 (P =0.0003) lysoPC in DHA modified LDL after oxidation, which decreased after the inclusion of the Lp-PLA_2 inhibitor in the oxidation reaction. This decrease was significant in lysoPC species 16:0 (P= 0.004) and 18:0 (P= 0.0005), figure 4.8 (top panel). The low mass oxidised species were dramatically increased by the action of oxidation; the most abundant oxidised species had an m/z of 650. All of the species except m/z 664 were significantly increased by oxidation (P= < 0.05). Inclusion of the inhibitor further increased the accumulation of low mass oxidised species in the mass range m/z 594-666, only two species were increased significantly by the action of the inhibitor: 636 and 648 (P= <0.02), figure 4.8 (middle panel). The final group of PC molecules increased by oxidation in the LDL were the high mass oxidised species; the changes observed in DHA modified LDL were different to those in the control LDL. Oxidation resulted in the decrease of 788 (P= 0.02) and 800, which demonstrated that the control species with these masses was oxidised, contradicting the results seen for the control LDL where very little mono-unsaturated species became oxidised. After inclusion of the inhibitor in the oxidation reaction these species increased, but not to the extent observed in the control LDL. Two of the species were significantly increased by the action of oxidation 790 (P= 0.02) and 818 (P= 0.01), figure 4.8 (bottom panel).

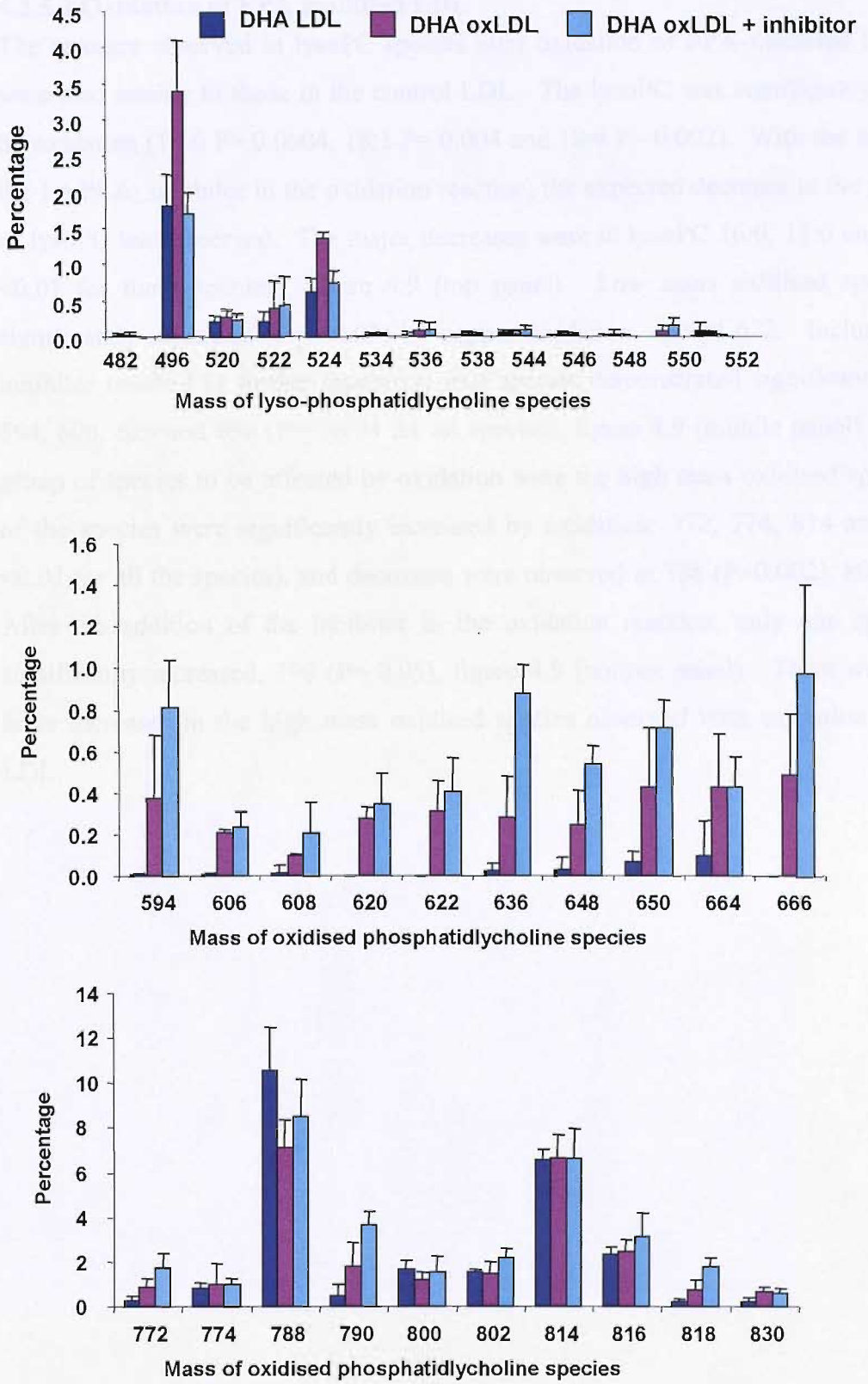


Figure 4.8: The effects of 20 hours copper oxidation and Lp-PLA2 inhibition on levels of lysoPC (top panel), low mass oxidised PC (middle panel) and higher mass PC species (bottom panel) in DHA LDL.

4.2.5.2 Oxidation of EPA modified LDL

The changes observed in lysoPC species after oxidation of EPA modified LDL (n=3) were also similar to those in the control LDL. The lysoPC was significantly increased by oxidation (16:0 P= 0.0004, 18:1 P= 0.004 and 18:0 P= 0.002). With the inclusion of the Lp-PLA_2 inhibitor in the oxidation reaction, the expected decrease in the percentage of lysoPC was observed. The major decreases were in lysoPC 16:0, 18:0 and 18:1 (P= <0.01 for these species), figure 4.9 (top panel). Low mass oxidised species were significantly increased (P= <0.03) by copper oxidation, except 622. Inclusion of the inhibitor resulted in further increases, four species demonstrated significant increases, 594, 606, 620 and 650 (P= <0.04 for all species), figure 4.9 (middle panel). The final group of species to be affected by oxidation were the high mass oxidised species, four of the species were significantly increased by oxidation: 772, 774, 814 and 830 (P= <0.02 for all the species), and decreases were observed in 788 (P=0.002), 800 and 802. After the addition of the inhibitor in the oxidation reaction, only one species was significantly increased, 790 (P= 0.05), figure 4.9 (bottom panel). There were not the large increases in the high mass oxidised species observed with oxidation of control LDL.

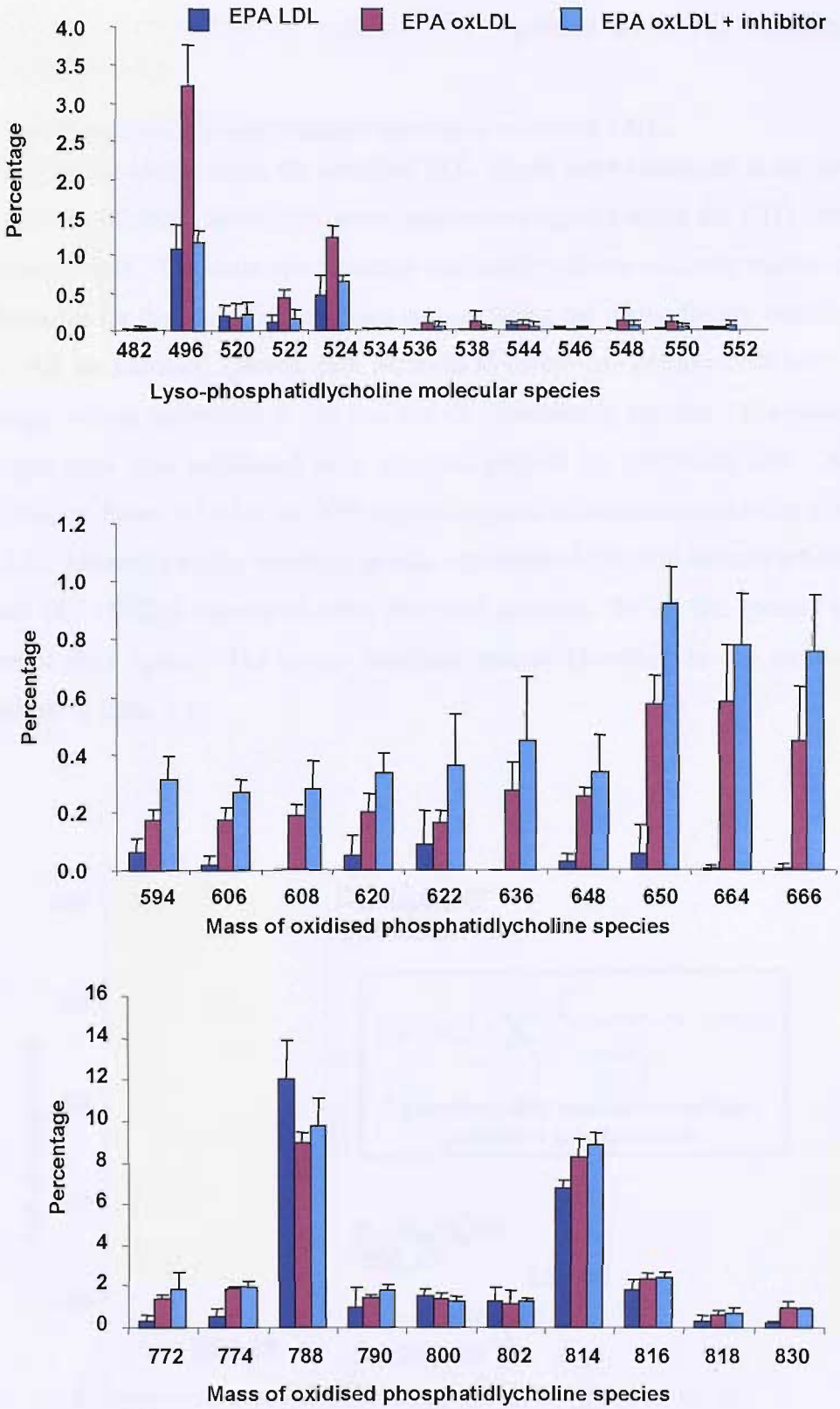


Figure 4.9: The effects of 20 hours copper oxidation and Lp-PLA2 inhibition on levels of lysoPC (top panel), low mass oxidised PC (middle panel) and high mass oxidised PC species (bottom panel) in EPA LDL.

4.2.6 Identification of oxidised PC species by LTQ Orbitrap™ mass spectrometry

4.2.6.1 Identification of oxidised species in oxidised LDL

The masses identified in the oxidised LDL which were enhanced in the presence of the Lp-PLA_2 inhibitor SB222657 were further investigated using the LTQ Orbitrap™ mass spectrometer. The mass spectrometer was used to define accurate masses and elemental formulae for the identified oxidised species using the methodology described in chapter 3. All the oxidised species were resolved to create one predominate peak in their mass range, which correlated to O_9, O_{10} and O_{11} containing species. The percentage of the major peak was calculated from the total present for that mass unit. An example is shown in figure 4.10 for m/z 650 region in positive ionisation mode for control oxidised LDL; where the major oxidised species represents 43% total species present in m/z 650 and PC14:0/12:0 represents 16%, the mass accuracy for all the species identified was better than 3ppm. The major oxidised species identified in the oxidised LDLs are shown in table 4.1.

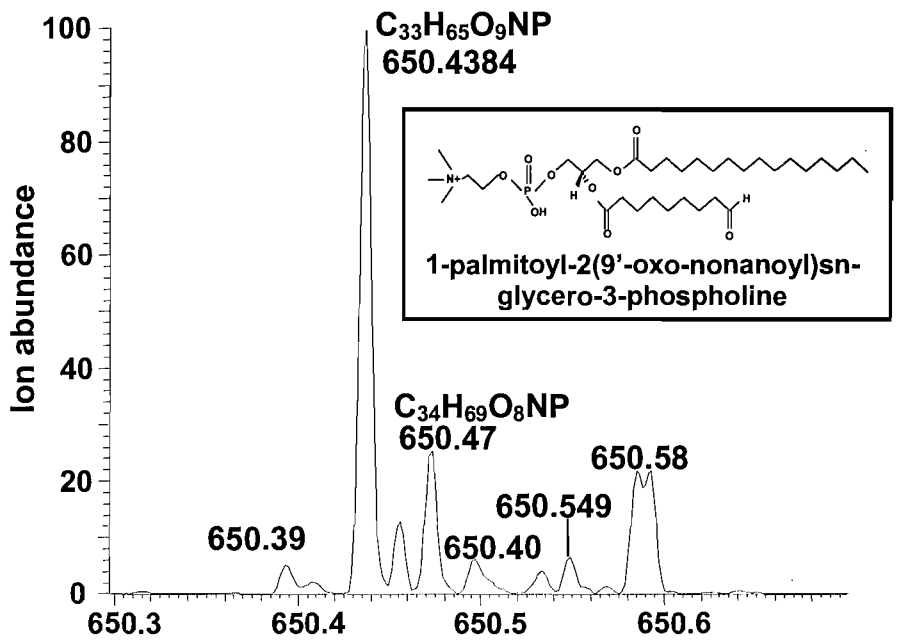


Figure 4.10: Accurate mass analysis of oxidised control LDL in m/z 650 in positive ionisation mode using LTQ Orbitrap™ mass spectrometer.

Mass	Elemental formula	Percentage of mass unit		
		Control	DHA	EPA
594.3763	$C_{29}H_{57}O_9N_1P_1$	13	96	36
606.3691	$C_{30}H_{57}O_9N_1P_1$	7	51	50
608.3924	$C_{30}H_{59}O_9N_1P_1$	26	38	37
622.4076	$C_{31}H_{63}O_9N_1P_1$	58	79	70
636.4231	$C_{32}H_{61}O_9N_1P_1$	45	70	69
648.4225	$C_{33}H_{63}O_9N_1P_1$	24	40	35
650.4389	$C_{33}H_{65}O_9N_1P_1$	43	64	86
664.4182	$C_{33}H_{63}O_{10}N_1P_1$	47	3	41
666.47	$C_{34}H_{69}O_9N_1P_1$	26	3	3
772.5484	$C_{42}H_{79}O_9\ N_1P_1$	5	78	80
774.564	$C_{42}H_{81}O_9\ N_1P_1$	5	74	83
788.5432	$C_{42}H_{79}O_{10}\ N_1P_1$	85	30	40
790.5589	$C_{42}H_{81}O_{10}\ N_1P_1$	96	78	91
800.5796	$C_{44}H_{83}O_9\ N_1P_1$	71	73	75
802.5954	$C_{44}H_{85}O_9\ N_1P_1$	60	39	50
814.5588	$C_{44}H_{81}O_{10}\ N_1P_1$	58	33	30
816.5745	$C_{44}H_{83}O_{10}\ N_1P_1$	85	51	45
818.59	$C_{44}H_{85}O_{10}\ N_1P_1$	96	61	93
830.554	$C_{44}H_{81}O_{11}\ N_1P_1$	95	54	85
832.5684	$C_{44}H_{83}O_{11}\ N_1P_1$	9	45	37
834.5854	$C_{44}H_{85}O_{11}\ N_1P_1$	7	12	41
856.569	$C_{46}H_{83}O_{11}\ N_1P_1$	53	63	81
858.585	$C_{46}H_{85}O_{11}\ N_1P_1$	83	73	98
860.6012	$C_{46}H_{87}O_{11}\ N_1P_1$	30	25	86

Table 4.1: Masses and elemental formulae of oxidised PC species identified in oxidised control DHA and EPA LDL by LTQ Orbitrap™ mass spectrometry and the percentage of the oxidised species present in the mass unit. This was performed on one control, DHA modified and EPA modified oxidised LDL.

In the control oxidised LDL the low mass oxidised species were less predominant than in the fatty acid modified LDLs. For example; the oxidised species at m/z 650 in control LDL contributed 43% to the total mass unit while in DHA LDL it contributed 64% and in EPA modified LDL constituted 86% to the total species in the mass unit. In comparison, the fatty acid modified LDLs generally had lower proportions of the high mass oxidised LDL than the control. For example; the oxidised PC species at m/z 788 constituted 85% of the mass unit in control oxidised LDL but only 30% and 40% respectively for DHA and EPA modified LDLs, with the remainder comprised of non-oxidised PC species or non-PC components. The fatty acid modified LDLs were less oxidised than the control samples, in many cases the PC species remaining unoxidised

corresponded to PC species increased by the n-3 supplements. Figure 4.11 demonstrates the presence of oxidised and non-oxidised species in EPA oxidised LDL within the same mass unit. The non-oxidised PC is the predominate species, demonstrating that n-3 fatty acid containing PC were not as readily oxidised as within control LDL, in which oxidation resulted in almost complete loss of the polyunsaturated species.

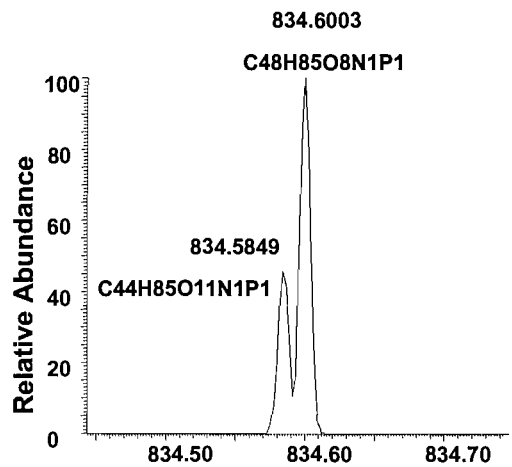


Figure 4.11: The oxidised and non-oxidised species with the same masses in oxidised EPA LDL.

4.2.6.2 Identification of isoprostane containing oxidised PC species in LDL

The higher signal to noise ratio of the LTQ Orbitrap™ permitted detailed examination of the PC species within the m/z range 830-860. Isoprostanes are prostaglandin like molecules formed from arachidonic acid via a free radical catalysed mechanism (Gopaul et al 1994), which is independent of cyclooxygenase (Patrono et al 1997); and are a known marker of lipid peroxidation. Isoprostanes can be formed at the membrane and are released by phospholipases to circulate in their free forms (Cracowski 2004). Masses consistent with isoprostanes bound in the sn-2 position of PC were identified in oxidised LDL. Possible PC bound isoprostanes had m/z which corresponded to either a palmitate species in the sn-1 position, figure 4.12 (top panel) or those that had a stearate in the sn-1 position, figure 4.12 (bottom panel). These species would have been formed from native PC species with arachidonate in the sn-2 position.

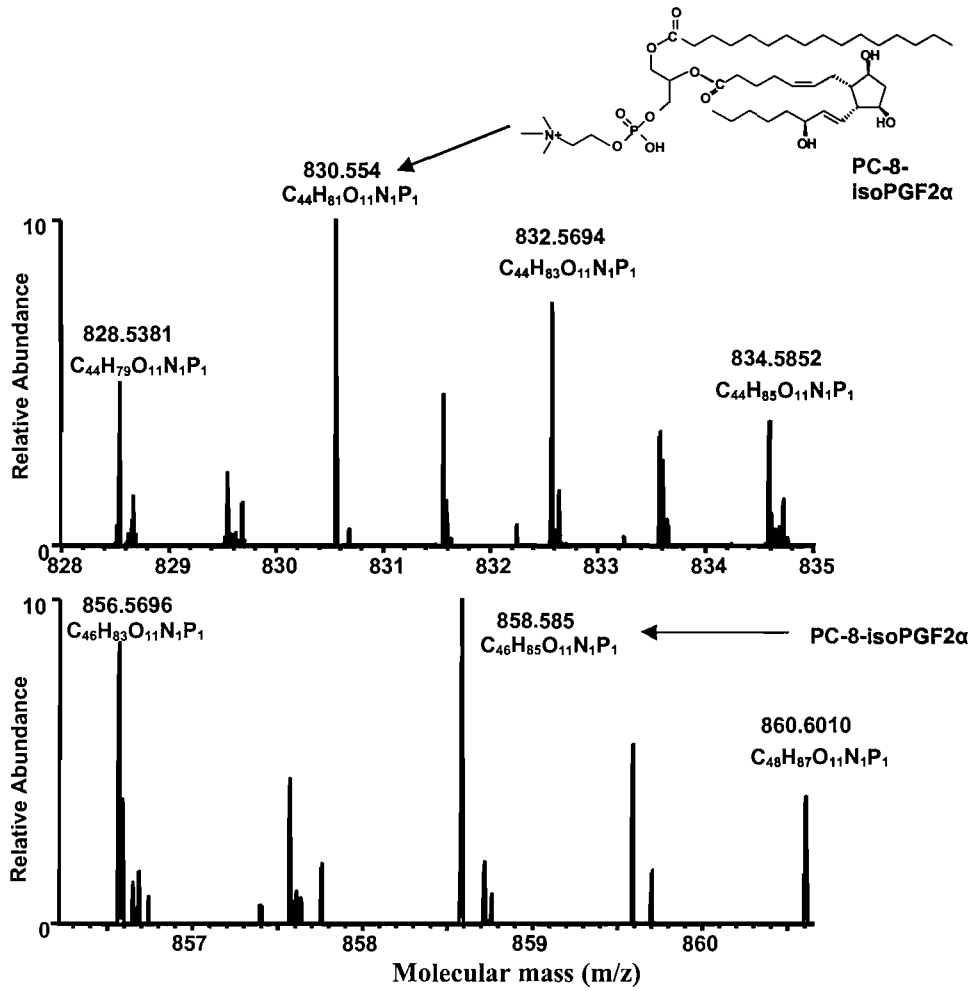


Figure 4.12: Copper oxidised LDL demonstrating the presence of PC bound isoprostanes.

There was no MS/MS analysis performed, so it is not possible to confirm whether these PC species contained isoprostanes; therefore the species in the m/z range 854-858 also have masses consistent with neuroprostanes bound in the sn-2 position with a palmitate fatty acid in sn-1.

4.2.6.3 Identification of oxidised species in plaque samples

Plaque samples (n=2) were analysed by LTQ Orbitrap™ mass spectrometry. The major component of the ion peak at m/z 650 was not PC and there were no detectable oxidised PC species. Figure 4.13 demonstrates the isolation of the peaks in the m/z range 650-651, the major species at m/z 650.5944 did not breakdown to give a peak at m/z 184, therefore it is not PC. This was the case with all of the ion peaks that related to oxidised PC species, there were many ion peaks that corresponded to non-PC components and it

was difficult to detect oxidised PC species, as they would have been present in low amounts in comparison to other phospholipids.

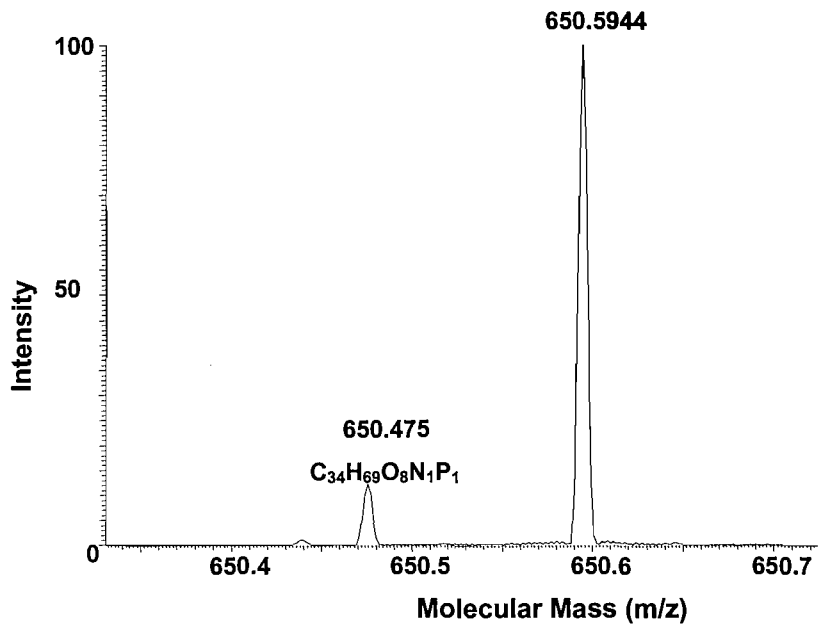


Figure 4.13: Isolation of the ion peak at m/z 650 in a plaque sample by LTQ Orbitrap mass spectrometry. The major component was not PC and there was no detectable oxidised PC species.

4.3 Discussion

Detailed analysis of plasma composition in humans after n-3 fatty acid supplementation was performed by mass spectrometry. Previous studies have focused on the changes to the fatty acid composition by gas chromatography (Bronsgeest-Schoute et al 1981) or by HPLC (Subbaiah et al 1993). The results obtained suggest that measurement of fatty acids alone does not provide enough information on the specificity of fatty acid incorporation into PC, as DHA supplements resulted in specific incorporation into PC species with a 16:0 fatty acid in the sn-1 position, as well as conversion to EPA resulting in increases to PC16:0/20:5 and PC18:0/20:5. By contrast, EPA supplements resulted in incorporation into both PC16:0/20:5 and PC18:0/20:5 with very little effect on DHA containing PC species. A previous study using HPLC to measure the plasma PC changes after combined DHA and EPA supplements for 28 days reported an increase in PC species 16:0/20:5, 16:0/22:6 and 18:0/22:5, and reported no change in PC16:0/18:1, 16:0/18:2 and 16:0/20:4 (Subbaiah et al 1993). These results generally support the observations of this project, confirming the specific PC incorporation of n-3 fatty acid supplements into plasma PC.

Initially, precursor scans of m/z 184 were used to identify the changes in PC content of LDL following copper oxidation *in vitro*. Oxidation resulted in almost complete loss of the peaks corresponding to diacyl PCs with polyunsaturated fatty acyl substituents and a substantial loss of those corresponding to PCs with di-unsaturated substituents (figure 4.1). However, the levels of PCs containing only mono-unsaturated and saturated substituents remained fairly constant in the control oxidised LDL. It is clear from the quantitative analysis of the 184 precursor spectrum that most of the PC oxidised during the reaction were not modified into species that were subsequently detectable by this method. This disappearance was likely to be caused by the formation of covalent adducts between the oxidised molecules and the proteins present in the LDL particle (Trostchansky et al 2001). LDL was also oxidised in the presence of an Lp-PLA_2 inhibitor, the levels of 16:0, 18:1 and 18:0 lysoPC (and of other less abundant monounsaturated and saturated lysoPCs) were significantly enhanced in oxidised LDL, compared to native LDL. In the presence of SB222657 the generation of lysoPC was largely, although incompletely, reversed; suggesting that the majority of lysoPC was indeed formed by Lp-PLA_2 action during oxidation. A more complete inhibition of lysoPC formation has been reported in LDL subjected to only 4 hours copper oxidation with SB222657 present at 0.1μM (Macphee et al 1999). This difference shows that

there was a low rate generation of lysoPC associated LDL oxidation independent of Lp-PLA_2, likely to be other PLA_2 that co-purified with the LDL under these conditions. There were also significant increases in the levels of PC species in m/z range 594-666 after oxidation, which were further enhanced by the presence of SB222657. The changes in lysoPC and short chain oxidised PC were the main changes observed between LDL oxidised in the presence and absence of inhibitor. This would suggest that the ion peaks in the m/z 594-666 range were substrates for Lp-PLA_2 and were likely to be oxygen-containing PC products of oxidative fragmentation of n-6 fatty acids at the sn-2 position of diacyl PC molecules. The results thus identified a class of molecules present in human LDL that represent the major source of Lp-PLA_2-generated lysoPC during LDL oxidation. The long chain oxidised PCs represented quantitatively the most abundant PC products of control LDL oxidation. However, it was clear from the use of SB222657 that these PC species were less efficient substrates for Lp-PLA_2 than the short chain oxidised PCs, as the increase in these species in the presence of the inhibitor was not as considerable when compared to oxidation alone.

The oxidised peaks detected in the LDL samples were compared to those measurable in plaque samples; the same species were present in the plaque samples as in the oxidised LDL but in smaller quantities. This may have been due to the difference in oxidation *in vitro* and *in vivo*, as copper oxidation is not comparable to the oxidation processes occurring *in vivo*. The plaque data showed variation in the lysoPC and oxidised PC species between the samples, the variation in the oxidised PC species was not related to the treatment group; therefore must be related to disease severity or individual specificity. There was no significant difference in the lysoPC between the Lp-PLA_2 groups, although there was a decreasing trend in lysoPC with increasing Lp-PLA_2 dose.

Oxidation of DHA and EPA fatty acid modified LDL resulted in a significant increase in lysoPC species, from 3.55% to 5.83%, falling to 3.47% in the presence of the inhibitor in DHA modified LDL. In EPA modified LDL the changes were 1.96% to 5.58% and 2.49%. The presence of the inhibitor completely reversed the formation of lysoPC species in the fatty acids modified LDLs, unlike the control LDL. The total low mass oxidised PC in fatty acid modified LDL was higher than in control LDL. These species are likely to be products from the oxidation of the n-3 containing PC species, confirmed by the greater percentage present in the fatty acid modified LDLs compared to control. The fatty acid modified LDLs had smaller changes to high mass oxidised

species, particularly in the ion peak at m/z 788, which decreased, a phenomenon not seen in the control oxidised LDL. The PC species produced by oxidation were the same for fatty acid modified LDLs and control LDL, which was surprising. Some studies have suggested that increased n-3 fatty acid content in LDL results in increased lag time prior to the onset of oxidation in the presence of oxidants (Suzukawa et al 1995). Although there are discrepancies in this data, it has also been suggested that increased polyunsaturated fatty acids cause LDL to become more susceptible to oxidation (Hau et al 1996, Oostenberg et al 1997 and Leigh-Firbank et al 2002). The results would suggest that increased n-3 fatty acids did not result in an increased susceptibility to oxidation, as fatty acid modified LDLs were less completely oxidised than control LDL, confirmed by native n-3 containing PC species remaining in the oxidised samples at m/z 806, 808 and 834 (PC 16:0/22:6, 18:1/22:5 and 18:0/22:6).

Detailed analysis of *in vitro* copper oxidised LDL with the LTQ Orbitrap™ mass spectrometer revealed that 1-palmitoyl-2-(9'-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine was a large constituent of the 650 peak in all oxidised LDLs. There have been previous studies characterising components of the m/z 650 peak in oxidised LDL (Harrison et al 2000 and Podrez et al 2002), and material from atherosclerotic lesions (Podrez et al 2002, Hoff et al 2003). Harrison et al (2000) separated phospholipids isolated from oxidised LDL by reverse phase HPLC, and identified components of m/z 650 peak as 1-stearoyl-2-(7-hydroxyhepta-5-enoyl)-*sn*-glycero-3-phosphocholine and 1-palmitoyl-2-(9-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine. The molecular formula of both these was $C_{33}H_{65}O_9NP$; therefore, either or both could have been detected by the LTQ Orbitrap™ mass spectrometer. By contrast, Hoff et al (2003) used positive ion monitoring MS/MS to track 650 to 184 transitions in an HPLC separation of phospholipids extracted from either oxidised LDL or human atherosclerotic lesions, concluding that the major component of the peak was 1-palmitoyl-2-(5-hydroxy-8-oxo-6-octenoyl)-*sn*-glycero-3-phosphocholine, molecular formulae $C_{32}H_{61}O_{10}NP$. However, the data obtained on the LTQ Orbitrap™ would suggest that there were only small amounts of compounds with this molecular formulae present in the oxidised LDL. The discrepancies between these sets of data may relate to the difference between the LDL preparations, oxidation conditions and the difference in individual plaque samples, which can vary greatly in composition. The analysis of plaque samples by LTQ Orbitrap™ mass spectrometer could not detect any of the major oxidised species identified in the oxidised LDL. For example, in the plaque samples the

major component of the ion peak at m/z 650 was not PC, concluding that the oxidised PC components in plaques could not be detected due to the low quantities compared to the other lipid products.

The results have highlighted the possible presence of PC bound isoprostanes and/or neuroprostanes. The possible presence of neuroprostanes is interesting as the formation of neuroprostanes from DHA is particularly important in neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington disease (Simonian et al 1996, Knight 1997 and Markesbery 1997), due to the enrichment of DHA in the neurons of the brain. Therefore, increased intake of such fatty acids in the diet may increase the formation of these molecules in LDL which may, in turn, result in detrimental inflammatory responses, although confirmation of the structure of these species is required by MS/MS analysis. It has been previously shown that Lp-PLA_2 has a high affinity for the release of isoprostanes from PC, but at a slower rate than other substrates of the enzyme (Stafforini et al 2006). These findings support the results observed in this project that the high mass oxidised PC species are not as efficient substrates for Lp-PLA_2 hydrolysis.

This was the first time mass spectrometry has been used to quantify the changes to PC composition of control LDL and fatty acid modified LDL before and after *in vitro* oxidation and the PC composition of plaque samples. Oxidation was also performed in the presence of an Lp-PLA_2 inhibitor which demonstrated that the low mass oxidised species were the major substrates of the enzyme, while saturated and mono-unsaturated lysoPC were the major products. The LTQ Orbitrap™ was used to aid the identification of oxidised species in LDL and provided exact masses and elemental formulae for the species. However, in plaque samples this was not possible due to interference from other lipids in the sample; therefore greater purification would be required to obtain the exact mass and elemental formulae

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Chapter 5

Fatty Acid Modification of Cell PC Composition and Alterations to Cytokine Production

5.1 Introduction

Consumption of n-3 fatty acids, found naturally in oily fish and fish oil, has been implicated in a decreased ischemic heart disease risk and mortality as a result of several large studies on different populations (Bjerregaard et al 1988 and Yano et al 1988). In addition, secondary prevention studies using fish oil have demonstrated a marked decrease in cardiovascular mortality in post myocardial infarction patients (Diet and Reinfarction Trial (DART) 1989 and GISSI-Prevenzione Trial 1999). Endothelial cell activation is critical for the development of the atherosclerotic plaque (Ross 1999), and is consequently a prime potential target for the beneficial effects of the n-3 fatty acids. However, the mechanism(s) by which fish oil exerts these effects is not understood, although many suggested mechanisms of action involve modulation of cell membrane phospholipid composition (Endres et al 1995). Proposed downstream effectors of such compositional changes include: modifying the specificity and magnitude of signalling responses transduced by phospholipase C and D, altering the balance between generation of pro- and anti-inflammatory eicosanoids by inflammatory cells (Simopoulos 1994), influencing membrane receptor conformation and altering the binding of, for instance, cytokines (De Caterina et al 1994 and Collie-Duguid et al 1996), and altering the structure and composition of LDL leading to altered generation of pro-inflammatory oxidation products. Perhaps surprisingly, given the extensive nutritional research conducted on the biological and clinical effects of n-3 fats, there is very little information about how n-3 fatty acids alter cell membrane phospholipid composition and synthesis at the molecular level. Analysis in terms of phospholipid molecular species rather than phospholipid fatty acids is essential to evaluate these proposed mechanisms of n-3 fatty acid effects.

There are two main components of fish oil considered important in anti-atherogenic roles; DHA and EPA. These will be added to the medium of HUVECs to observe the changes seen *in vitro* and will be coupled with the use of d-9 choline, to measure the changes in PC synthesis. In addition to the n-3 fatty acids, the cells will also be supplemented with an n-6 polyunsaturated fatty acid (arachidonic acid (AA)) for comparison, as n-6 derived eicosanoids are known to be inflammatory and represent the most common polyunsaturated fatty acid in western diets. The compositional changes measured in the HUVECs will then be analysed in relation to downstream signalling events in the cell by measuring changes in ICAM-1 (Abe et al 1998), and inflammatory cytokines IL-6 (De Caterina et al 2000) and IL-8 (Yeh et al 2001). The resultant

changes to the NFκB signalling pathway will then be measured, as it is strongly implicated in the regulation of these inflammatorily mediators in endothelial cells (Hamaguchi et al 2003). The oxidised species formed by the oxidation of LDL may effect the production of cytokines in endothelial cells; therefore oxidised LDL will be incubated with HUVECs following supplementation with fatty acids. Previous work using standard 1-palmitoyl-2-oxovaleroyl-sn-glycero-3-phosphorylcholine (POVPC) resulted in HUVECs binding to peripheral blood monocytes (Berliner et al 1997); POVPC has also been reported to have been found in atherosclerotic lesions of animals and humans and in the membranes of apoptotic cells (Watson et al 1997, Subbanagounder et al 2000, Huber et al 2002 and Chang et al 2004).

Aims: To measure the changes in PC composition and PC synthesis of endothelial cells after the addition of DHA and EPA to the culture medium. The cells will then be stimulated to measure any effect of the change in phospholipid composition on downstream signalling within the cells.

5.2 Methods

5.2.1 Cell culture and supplementation

Endothelial cells were isolated from umbilical veins as described in section 2.3.3 and grown until confluence when the cells were passaged onto 24 well plates. For comparison with the HUVECs, transformed cells in long term culture (approximately 1 x 10^5), HL60 cells or Jurkat cells were added to 24 well plates for fatty acid supplementation experiments. Fatty acids were bound to fat free albumin in a 2:1 molar ratio and added to cell medium at a final concentration of either 30μM or 100μM. as described in section 2.4.3. For analysis of PC synthesis, *(methyl-d_9)*-choline chloride was added as described in section 2.4.4. The HL60 and Jurkat cells were removed from the plate by pipetting and the HUVECs were removed by cell scraping prior to lipid extraction as described in section 2.1.1, followed by mass spectrometry of the PC composition of the cells.

5.2.2 Stimulation of fatty acid supplemented HUVECs with oxLDL

HUVECs were supplemented with fatty acids as in section 2.4.3, after which the cells were stimulated with 20 hour copper oxidised LDL for 4 hours. The supernatant was collected and IL-6 and IL-8 measured as in sections 2.5.3 and 2.5.4.

5.3 Results

5.3.1 Measuring the purity of HUVECs

Cells isolated from umbilical cords were analysed to ensure the majority were of endothelial origin, as the isolation procedure can result in contamination from other cells, such as fibroblasts. The adhesion molecule platelet/endothelial cell adhesion molecule-1 (PECAM-1) was used as a marker of endothelial cells as it was constitutively expressed by endothelial cells but not by other cells that may have been isolated during the procedure (such as fibroblasts). The FACS histograms are shown in figure 5.1; non-specific binding of the PECAM-1 antibody (left hand side), specific binding of the PECAM-1 antibody (right hand side), demonstrated on the histogram by the M1 bar starting where the non-specific binding ended. The histograms demonstrate the frequency distributions within the 10,000 cells counted that express the antibody. This was repeated four times, and with the average number of cells expressing PECAM-1 at 90.9% ±0.74, confirming the majority of cells surviving in culture were of endothelial origin.

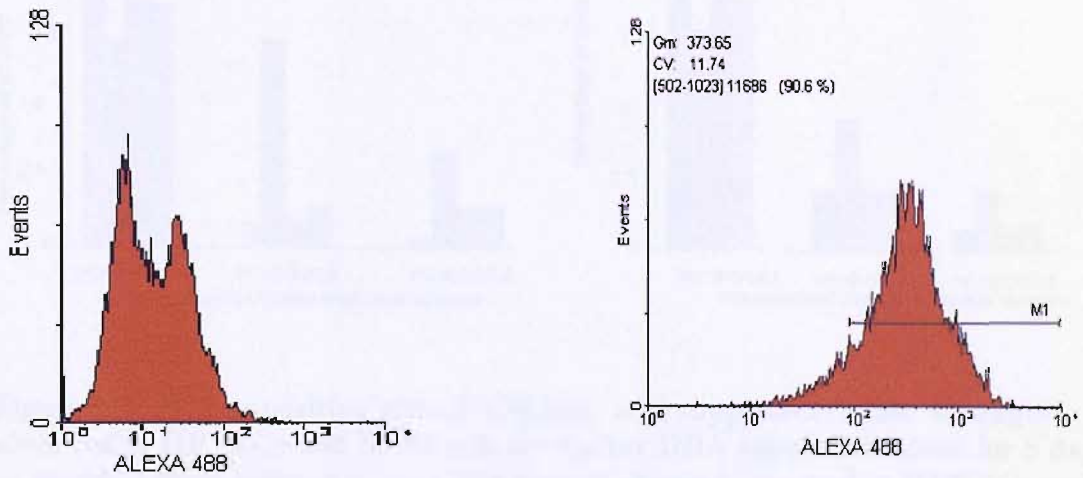

Figure 5.1: FACS histograms demonstrating specificity of PECAM-1 binding. Non-specific binding of the PECAM-1 antibody (left hand side); specific binding of the PECAM-1 antibody (right hand side).

5.3.2 Fatty supplementation of HUVECs and HL60 cells at 30μM

Fatty acid supplementation of HL60 cells previously showed 30μM supplementation achieved substantial changes in phospholipid composition (Hunt et al 2004). Therefore, cells used previously (HL60), and HUVECs, were supplemented with either DHA or EPA, followed by mass spectrometry analysis of the PC composition using a precursor scan of 184 using a Quattro Ultima mass spectrometer. Figure 5.2 demonstrates the changes observed in three selected PC species from these cells (n=1), PC16:0/18:1 (the major PC species), and two of the species most increased after supplementation, PC16:0/22:6 and PC18:0/22:6 for DHA and PC16:020:5 and PC18:0/20:5 for EPA. The changes in the HL60 cells were larger than those in the HUVECs after 30μM supplementation. These results suggested that the HUVECs were resistant to change by fatty acid supplementation compared to the cell line; therefore the concentration of fatty acid in the medium was increased in an attempt to increase the changes observed in the HUVECs.

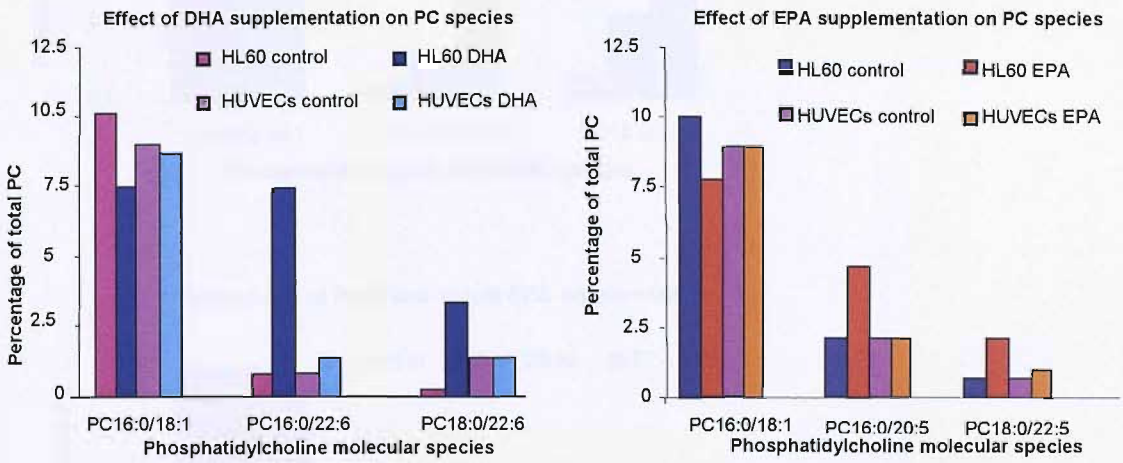

Figure 5.2: PC composition after 30μM fatty acid supplementation. Changes observed in HUVECs and HL60 cells (n=1)after DHA supplementation for 5 days at 30μM in three selected species (left panel); changes observed in HUVECs and HL60 cells after EPA supplementation for 5 days at 30μM in three selected PC species (right panel).

5.3.3 Comparing fatty acid supplementation at 30μM and 100μM in HUVECs

HUVECs were supplemented with 100μM fatty acid for 5 days, followed by lipid extraction and analysis of PC composition by mass spectrometry. The control cells from both concentrations had similar phospholipid compositions, as would be expected, so have been combined (n=1). The same three species shown previously in section 5.3.2 are illustrated in figure 5.3, to demonstrate the major changes observed with each fatty acid. Supplementation with 100μM fatty acid resulted in larger changes for both the DHA and EPA containing species compared to the 30μM.

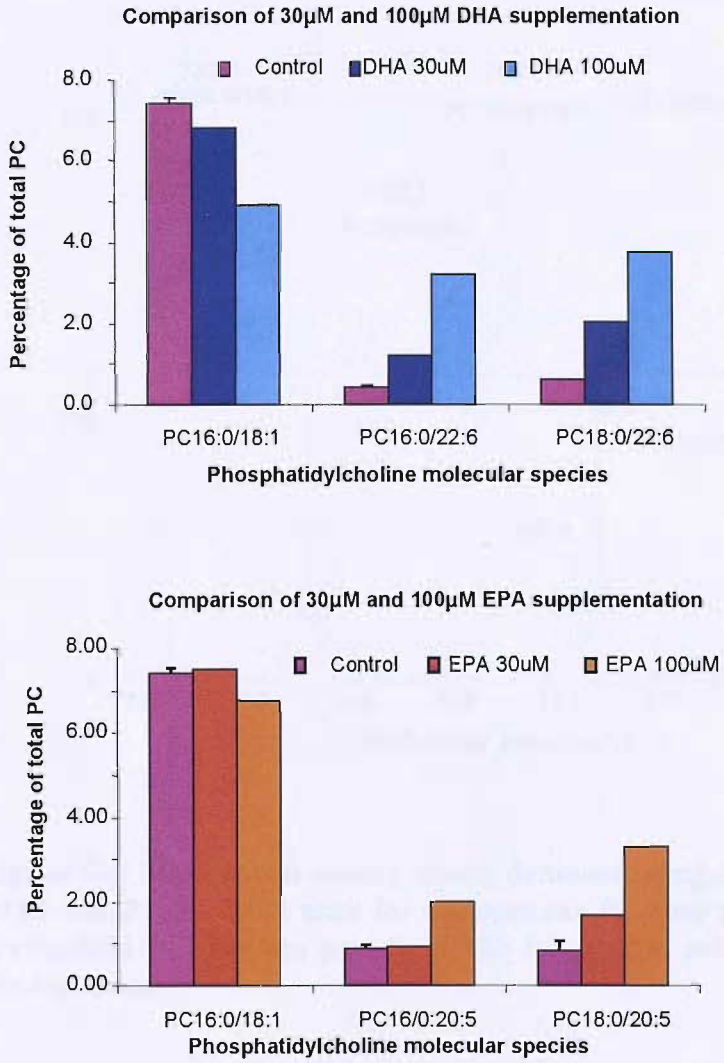

Figure 5.3: The changes observed in HUVECS after 5 days supplementation at 30μM and 100μM in three selected species. Changes observed after DHA supplementation (top panel); changes after EPA supplementation (bottom panel) (n=).

5.3.4 Fatty acid supplementation of three cell types at 100μM

Methyl d9 choline was added to the cells for three hours in a 20 fold excess at 0, 1.5, 3 hours and 5 days after the initial fatty acid supplement was added to the cells. Therefore, all newly synthesised PC would contain the labelled choline which will be nine mass units higher than the endogenous PC species when analysed by mass spectrometry, and so will be detected with a precursor scan of 193 rather than 184. Figure 5.4 is an example of a mass spectrometry trace, a small mass range is shown that demonstrates that newly synthesised PC (bottom panel) is nine mass units higher than endogenous PC (top panel), for example the ion peak at m/z 760 (PC16:0.18:1) corresponds to the ion peak at m/z 769 in the lower trace.

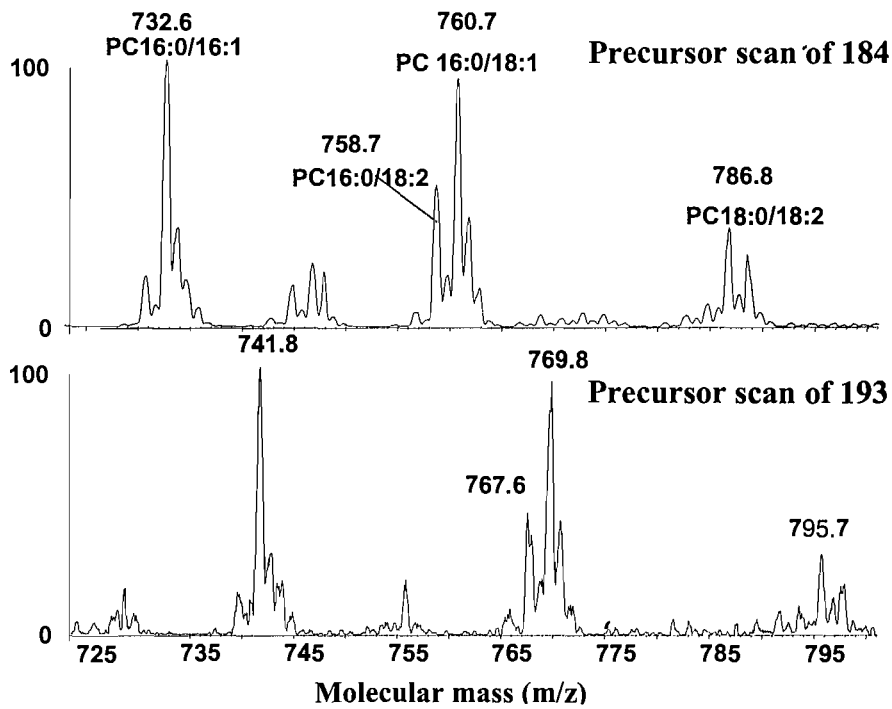

Figure 5.4: Mass spectrometry traces demonstrating differences observed between P184 and P193. P184 scan for endogenous PC (top panel); P193 trace for newly synthesised PC (bottom panel), all the PC species are 9 mass units higher than in the top trace.

5.3.4.1 Control cell endogenous PC composition

The control data (cells grown in normal medium) for all the cells was fairly consistent across all time points, as expected. In all cells types the major species present was PC16:0/18:1; the composition of the cells was primarily of mono-, di- and tri-unsaturated PC species. The HUVECs had higher levels of the polyunsaturated species than the cell lines, although these species represent the smallest group of PCs in all the control cells. Only the polyunsaturated species have been chosen for the figures. The changes observed in the endogenous PC composition of the cells after fatty acid supplementation were similar for all of the cell types.

5.3.4.2 Endogenous PC composition after supplementation with DHA

The percentage changes in all the cell types were similar in the endogenous PC, but with different species preferentially increased in the HUVECs compared to the HL60 and jurkat cells. In the cell lines PC16:0/22:6 ($P = <0.01$ over all time points) demonstrated the largest change; it rose and fell rapidly, but remained higher than the control cells at five days. The changes in HUVECs PC composition were more gradual and sustained, with the largest increase in PC18:0/20:5 ($P = <0.01$ at all time points). Interestingly, this species contained an EPA fatty acid in the sn-2 position rather than a DHA, demonstrating that the cells were converting DHA into EPA. By contrast, the cell lines did not show large changes in EPA containing PC species. For DHA containing species the changes in the HUVECs were mainly in the species with a saturated chain in the sn-1 position (16:0 and 18:0), whereas the cell lines did not show specificity for PC containing a saturated fatty acid in the sn-1 position. The composition of HL60 cells are in the top panel, the middle panel for jurkat cells and HUVECs are shown in the bottom panel of figure 5.5.

Endogenous PC composition of HL60, jurkats, HUVECs after DHA supplementation

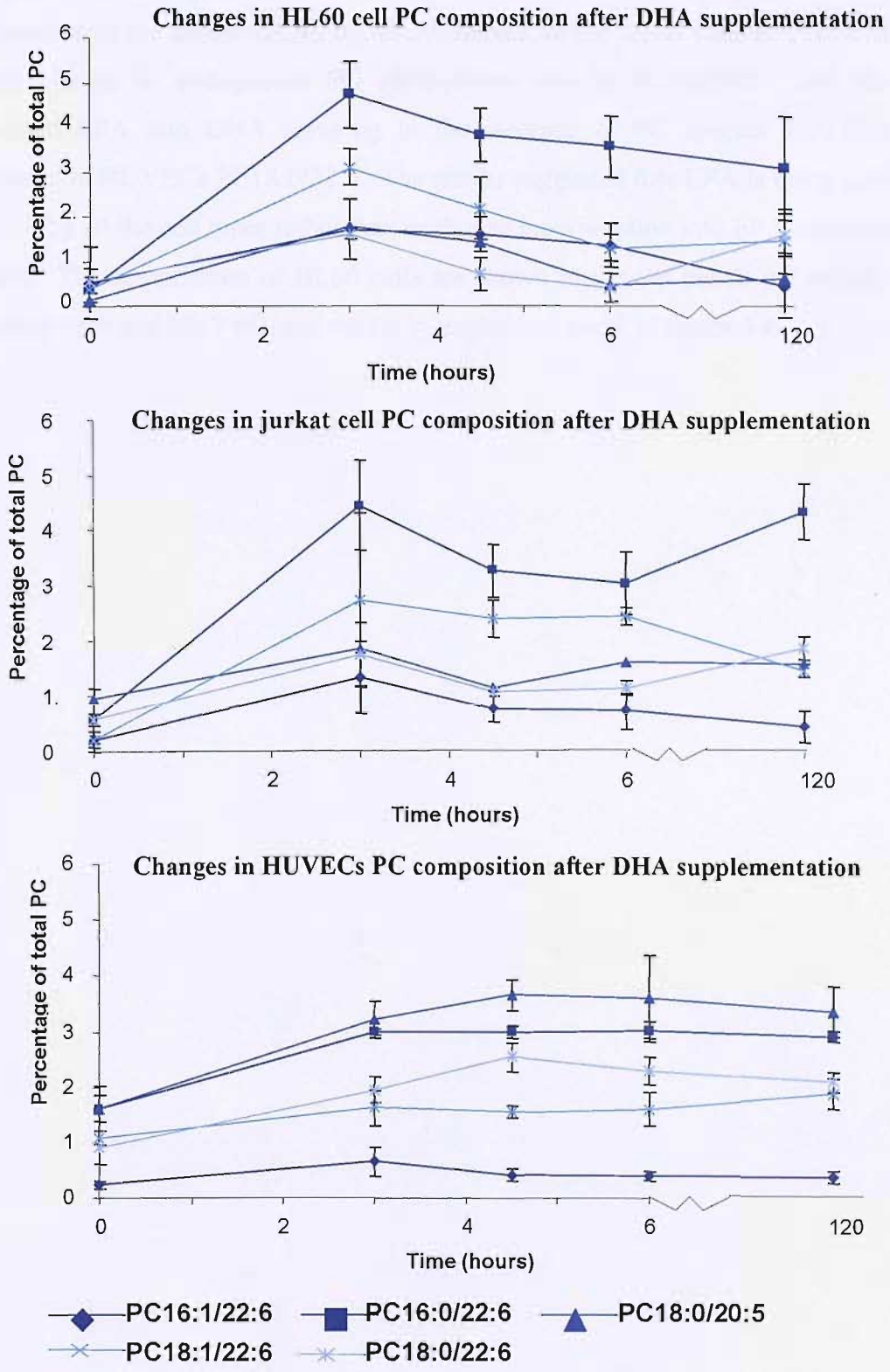

Figure 5.5: Changes to the endogenous PC composition after 100μM DHA supplementation. The top panel shows HL60 cells, the middle jurkat cells and the bottom HUVECs.

5.3.4.3 Endogenous PC composition after supplementation with EPA

Supplementation with EPA resulted in selective incorporation into both EPA and DHA PC species. EPA supplementation of HL60 cells resulted in the largest changes to PC composition to the species PC16:0/20:5. Whereas, in the jurkat cells and HUVECs the largest change to endogenous PC composition was in PC18:/20:5. All the cells converted EPA into DHA resulting in the increase of PC species 16:0/22:6, and additional in HUVECs PC18:0/22:6. The results suggested that EPA is being converted to DHA by all the cell types rather than exclusive incorporation into EPA containing PC species. The composition of HL60 cells are shown in the top panel, the middle panel for jurkat cells and HUVECs are shown in the bottom panel of figure 5.6.

Endogenous PC composition for HL60, jurkat cells and HUVECs after EPA supplementation

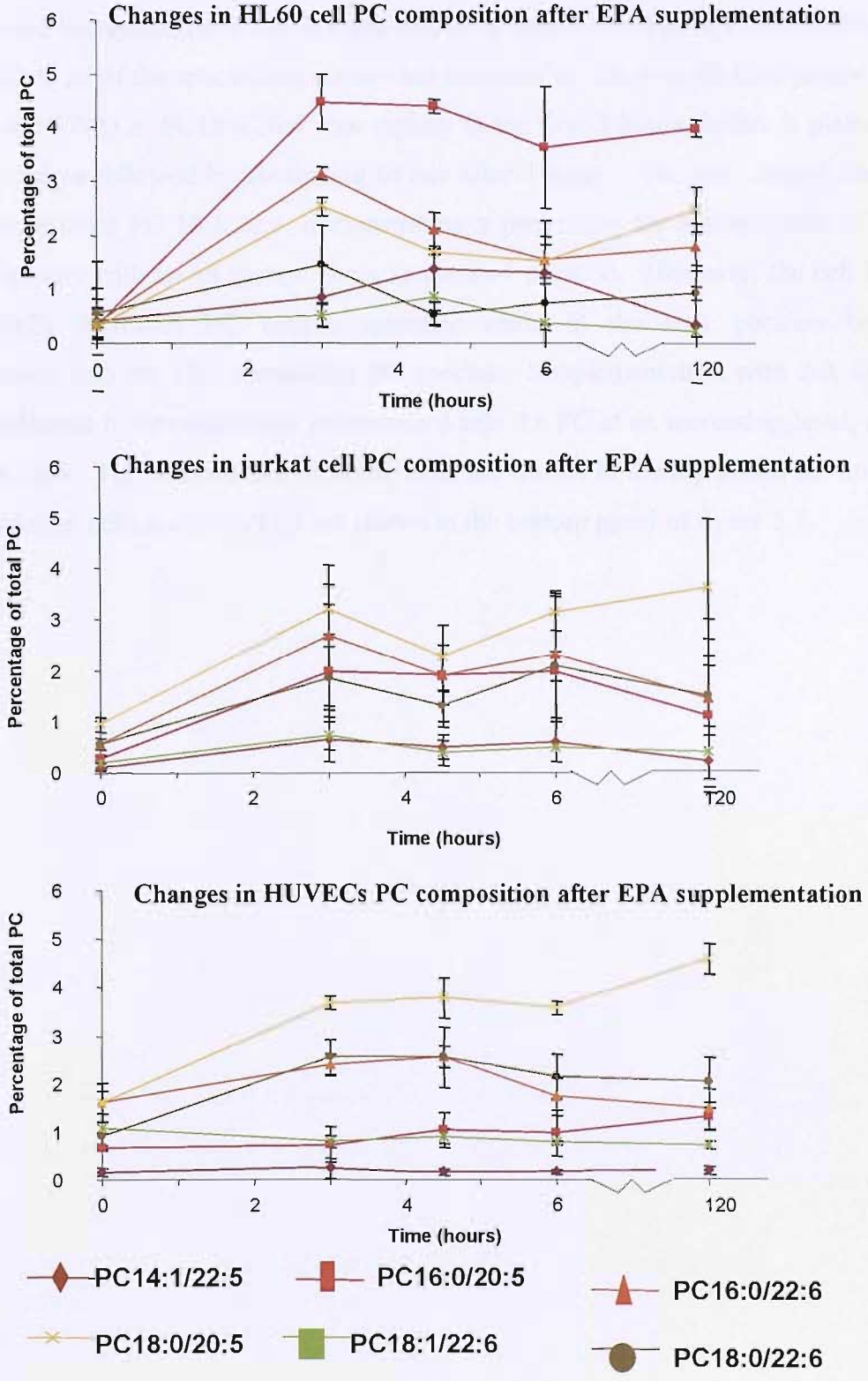

Figure 5.6: Changes to the endogenous PC composition after EPA supplementation. The top panel shows HL60 cells, the middle jurkat cells and the bottom HUVECs.

5.3.4.4 Endogenous PC composition after supplementation with AA

In all the cell types the increases to the AA containing PC species were still rising after 5 days, which was unlike the changes observed after DHA and EPA supplements, which had begun to decline after by five days. The endogenous PC composition of the cell lines showed incorporation of the AA was primarily into PC16:0/20:4, PC18:0/20:4 and PC18:1/20:4; all of the species rise slowly but continue to rise over all time points. By contrast in HUVECs, PC18:0/20:4 rose rapidly in the first 3 hours, before it plateaued for a short time followed by continuing to rise after 6 hours. The next largest change was in the species PC 18:1/20:4, demonstrating a preference for incorporation of AA into PC species with an 18 carbon chain in the sn-1 position. However, the cell lines preferentially increased PC with a saturated chain in the sn-1 position before incorporation into the 18:1 containing PC species. Supplementation with AA is the only supplement to be continually incorporated into the PC at an increasing level, even after five days. The composition of HL60 cells are shown in the top panel, the middle panel for jurkat cells and HUVECs are shown in the bottom panel of figure 5.7.

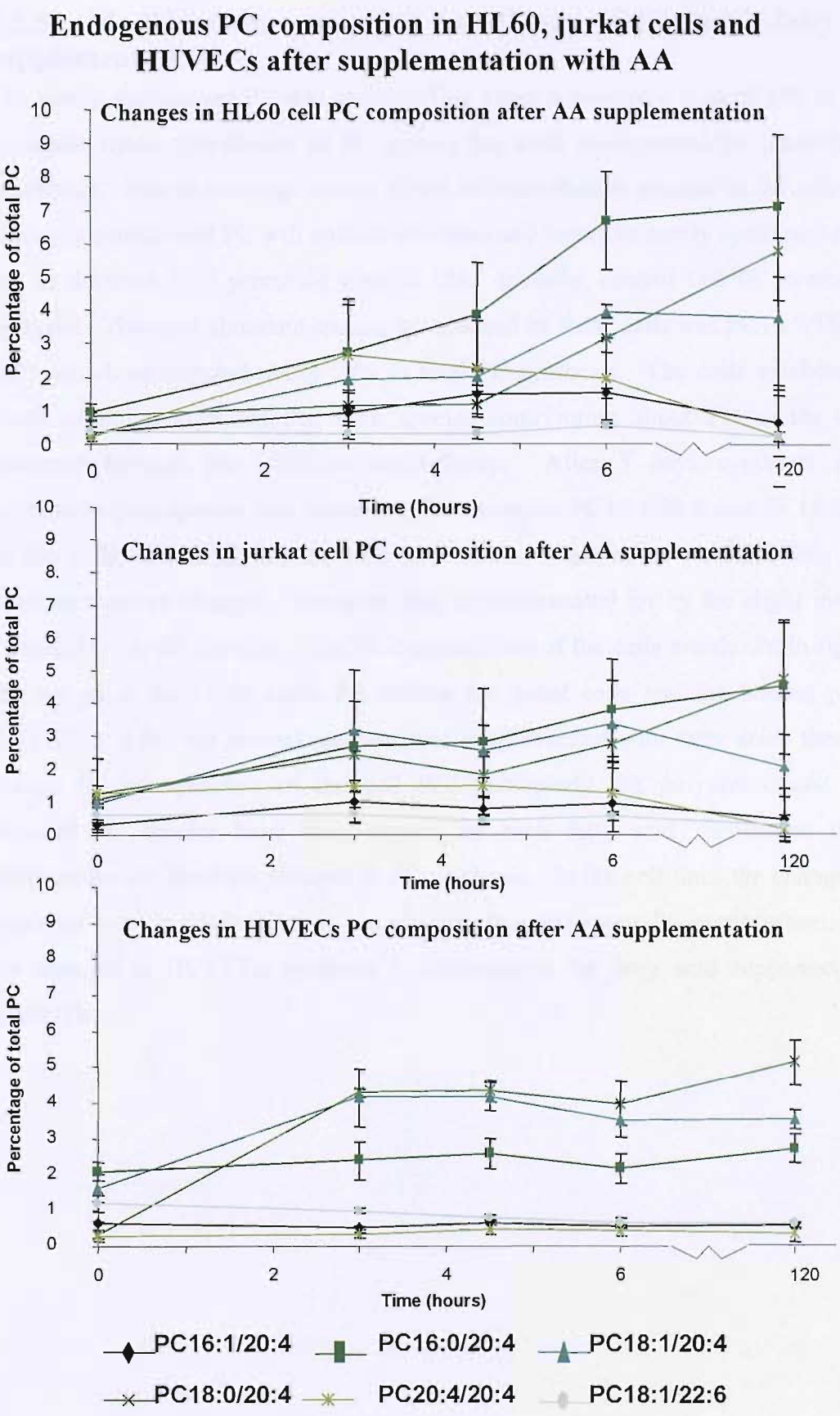

Figure 5.7: Changes to the endogenous PC composition after AA supplementation. The top panel shows HL60 cells, the middle jurkat cells and the bottom HUVECs.

5.3.5 Modifications to PC synthesis following fatty acid supplementation

The newly synthesised PC was measured by using a precursor scan of 193 in positive ionisation mode; this detects all PC species that have incorporated the labelled choline headgroup. Due to the large excess of the labelled choline present in the cell medium all newly synthesised PC will contain this label and therefore newly synthesised PC will not be detected by a precursor scan of 184. Initially, control cell PC synthesis was analysed. The most abundant species synthesised by these cells was PC16:0/18:1 (mass 760), which contributed about 10% to total PC synthesis. The cells synthesised low levels of polyunsaturated PC, each species contributing about 2% to the total PC produced through the CDP-choline pathway. After 5 days synthesis of some polyunsaturated species had decreased; for example, PC18:1/22:6 and PC18:0/22:6 in all the cells, indicating that the cells had become deprived of essential fatty acids in between medium changes. However, this is compensated for by the slight increase in PC16:0/18:1 in all the cells. The PC compositions of the cells are shown in figure 5.8; the top panel for HL60 cells, the middle for jurkat cells and the bottom panel for HUVECs. After the normal medium was supplemented with fatty acids there was a change in the synthesis of the cell PC, particularly the polyunsaturated species. Selected PC species have been chosen for each fatty acid supplement that best demonstrate the resultant changes in PC synthesis. In the cell lines the changes in PC synthesis were much larger than the changes to endogenous PC composition, whereas the changes in HUVECs synthesis in response to the fatty acid supplements were negligible.

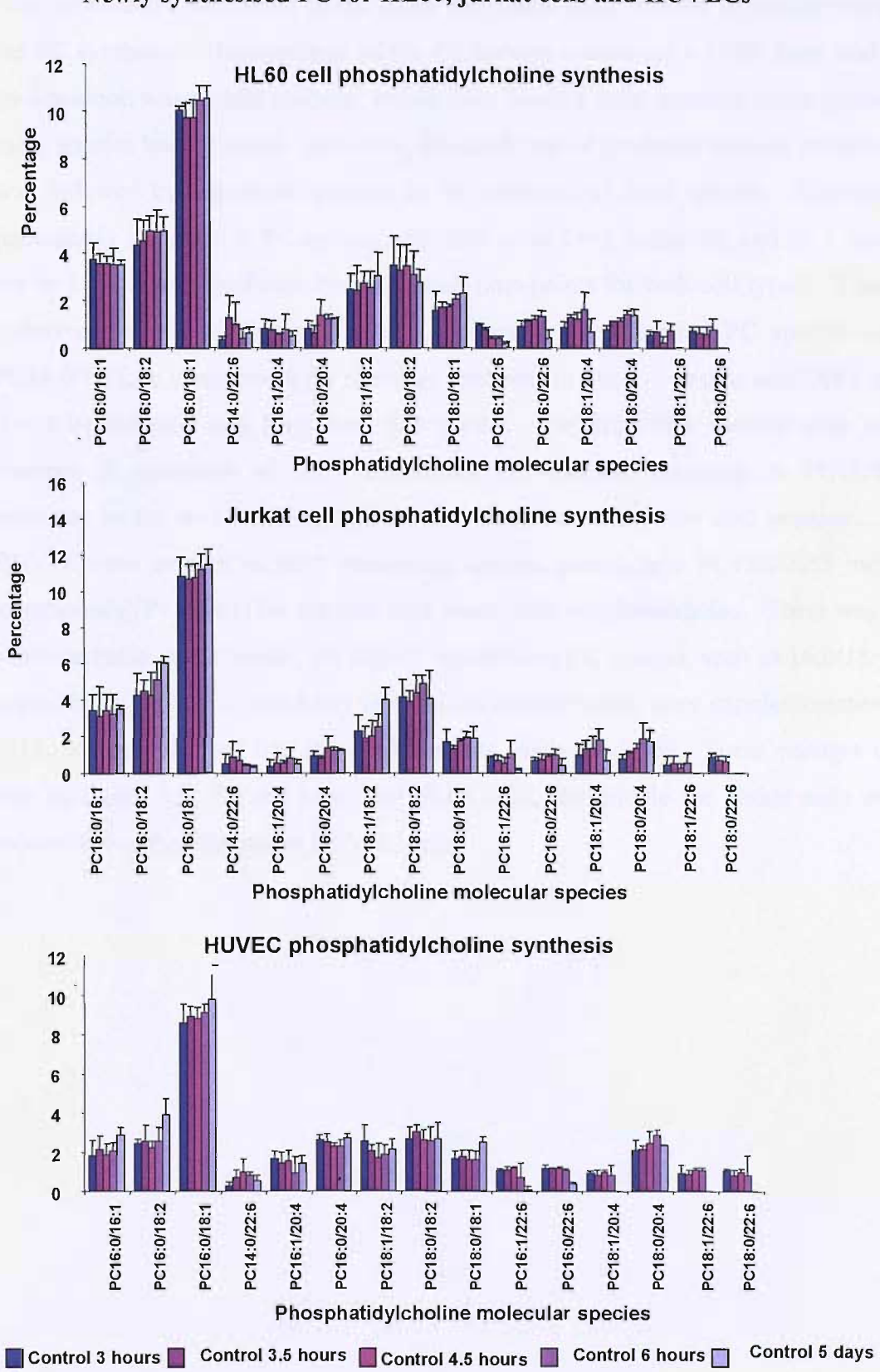

Figure 5.8: PC synthesis in control cells. PC synthesis for HL60 cells in the top panel, jurkat cells in the middle and HUVECs in the bottom panel, error bars indicate standard deviation.

5.3.5.1 Newly synthesised PC after supplementation with DHA

Supplementation with DHA to the HL60 and jurkat cells resulted in similar changes to the PC synthesis. The synthesis of the PC species containing a DHA fatty acid in the sn-2 position was rapidly adapted, within three hours a large increase in the synthesis of these species had occurred. However, this high rate of synthesis was not sustained and was followed by a gradual decrease in the synthesis of these species. Synthesis was particularly increased in PC species with fatty acids 16:0, palmitate, and 18:1, oleate, in the sn-1 position (significant P=<0.02 at all time points for both cell types). There was a decrease in the synthesis of mono-, di- and tri- unsaturated PC species such as PC16:0/18:1, to compensate for the large increases in the polyunsaturated DHA species (P=<0.01 for both cell lines time 6-9 hours). The HUVECs showed only a small increase in synthesis of DHA containing PC species, primarily to PC16:0/22:6, palmitate in the sn-1 position, and PC18:0/22:6, stearate in the sn-2 position. In the HUVECs the amount of EPA containing species, particularly PC18:0/22:5 increased significantly (P= <0.01) for the first nine hours after supplementation. There was also a small decrease in the mono-, di- and tri- unsaturated PC species, such as 16:0/18:1. PC synthesis in HUVECs was fairly resistant to modifications after supplementation with DHA, whereas the cell line PC synthesis was easily modified. These changes can be seen in figure 5.9; the top panel for HL60 cells, the middle for Jurkat cells and the bottom shows the changes to HUVEC cells.

Newly synthesised material for HL60, jurkat cells and HUVECs after DHA supplementation

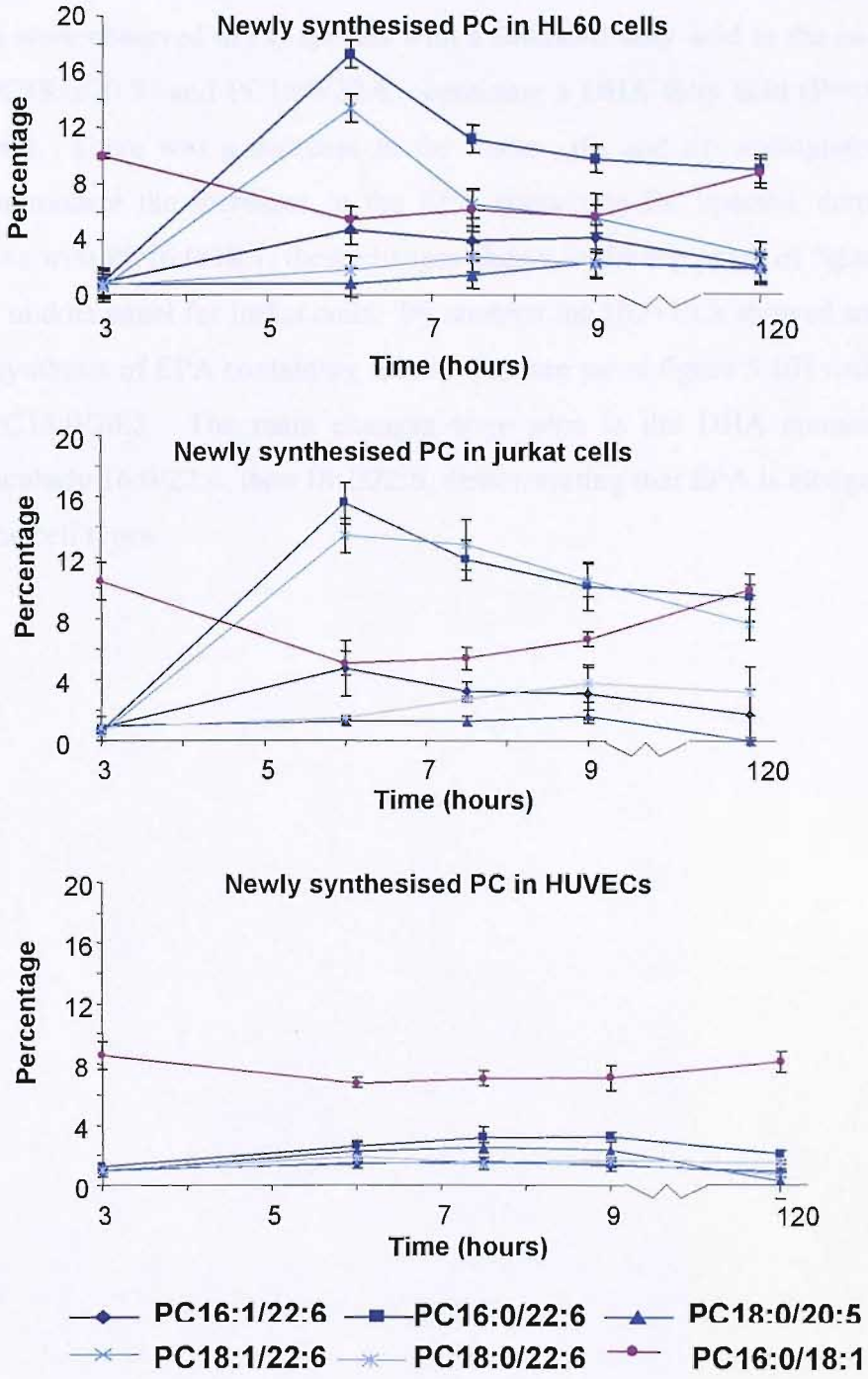

Figure 5.9: Changes to the newly synthesised PC composition after DHA supplementation. The top panel shows HL60 cells, the middle jurkat cells and the bottom HUVECs.

5.3.5.2 Newly synthesised PC after supplementation with EPA

As anticipated, EPA supplementation resulted in a large increase in the synthesis of PC species containing an EPA fatty acid in the sn-2 position. The largest changes in cell lines were observed in PC species with a saturated fatty acid in the sn-1 position; such as PC18:0/20:5 and PC16:0/22:6, containing a DHA fatty acid ($P=<0.02$ for all time points). There was a decrease in the mono-, di- and tri- unsaturated PC species to accommodate the increases in the EPA containing PC species, demonstrated in the graphs with PC16:0/18:1, these changes shown in the top panel of figure 5.10 for HL60 cell, middle panel for jurkat cells. By contrast the HUVECs showed smaller changes to the synthesis of EPA containing species (bottom panel figure 5.10) with small increases to PC18:0/20:5. The main changes were seen in the DHA containing PC species particularly 16:0/22:6, then 18:1/22:6, demonstrating that EPA is elongated into DHA in all the cell types.

Newly synthesised PC for HL60, jurkat cells and HUVECs after supplementation with EPA.

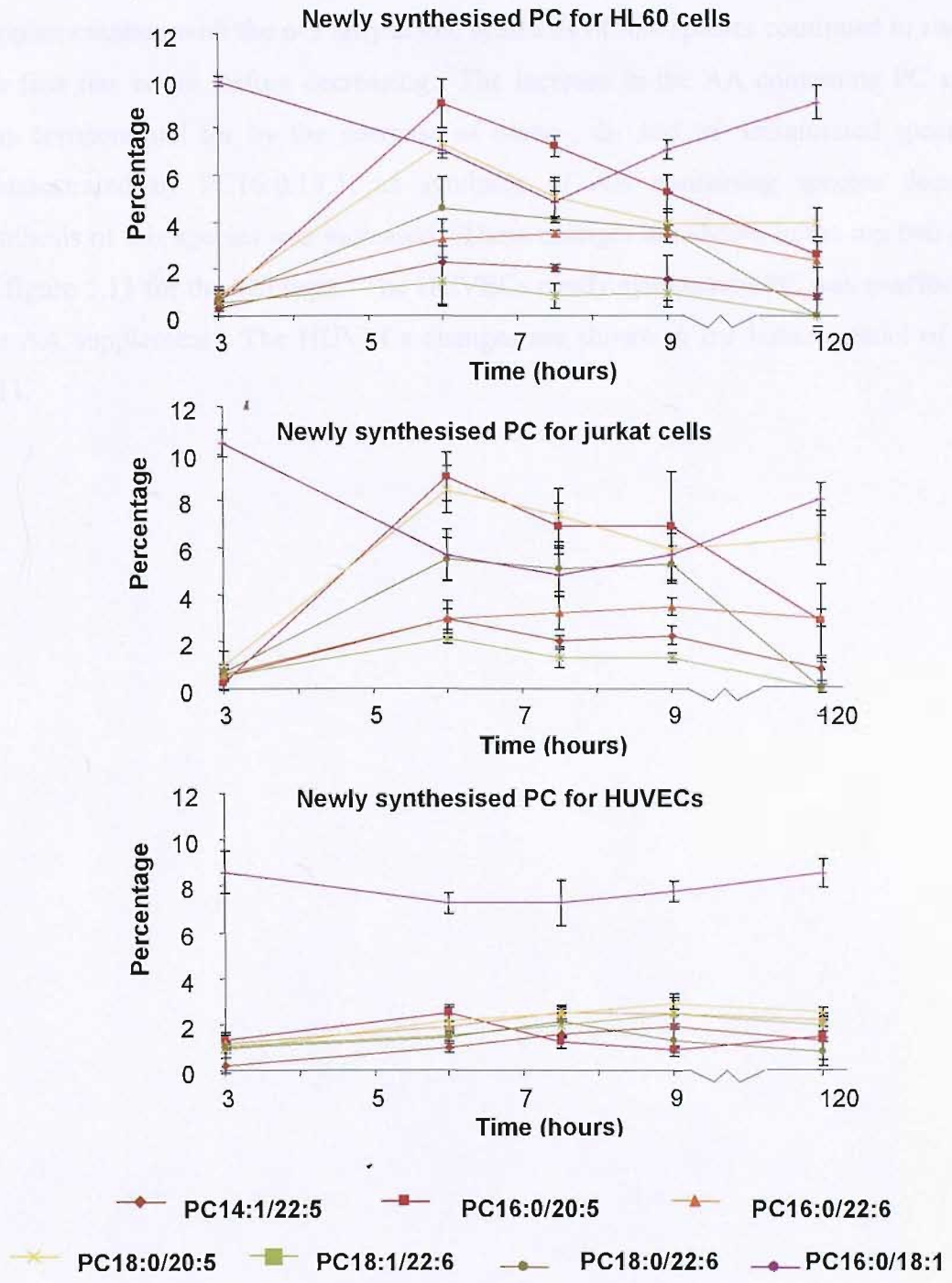

Figure 5.10: Changes to the newly synthesised PC composition after EPA supplementation. The top panel shows HL60 cells, the middle jurkat cells and the bottom HUVECs.

5.3.5.3 Newly synthesised PC after supplementation with AA

Arachidonic acid was swiftly incorporated into the newly synthesised PC of the cell lines. The most dramatic increase was in the species PC20:4/20:4, although unlike supplementation with the n-3 fatty acids, synthesis of this species continued to rise over the first few hours, before decreasing. The increase in the AA containing PC species was compensated for by the decrease of mono-, di- and tri- unsaturated species; as demonstrated by PC16:0.18:1; as synthesis of AA containing species decreased, synthesis of this species was increased. These changes are shown in the top two panels of figure 5.11 for the cell lines. The HUVECs newly synthesised PC was unaffected by the AA supplement. The HUVECs changes are shown in the bottom panel of figure 5.11.

Newly synthesised PC after supplementation with AA in HL60, jurkat cells and HUVECs

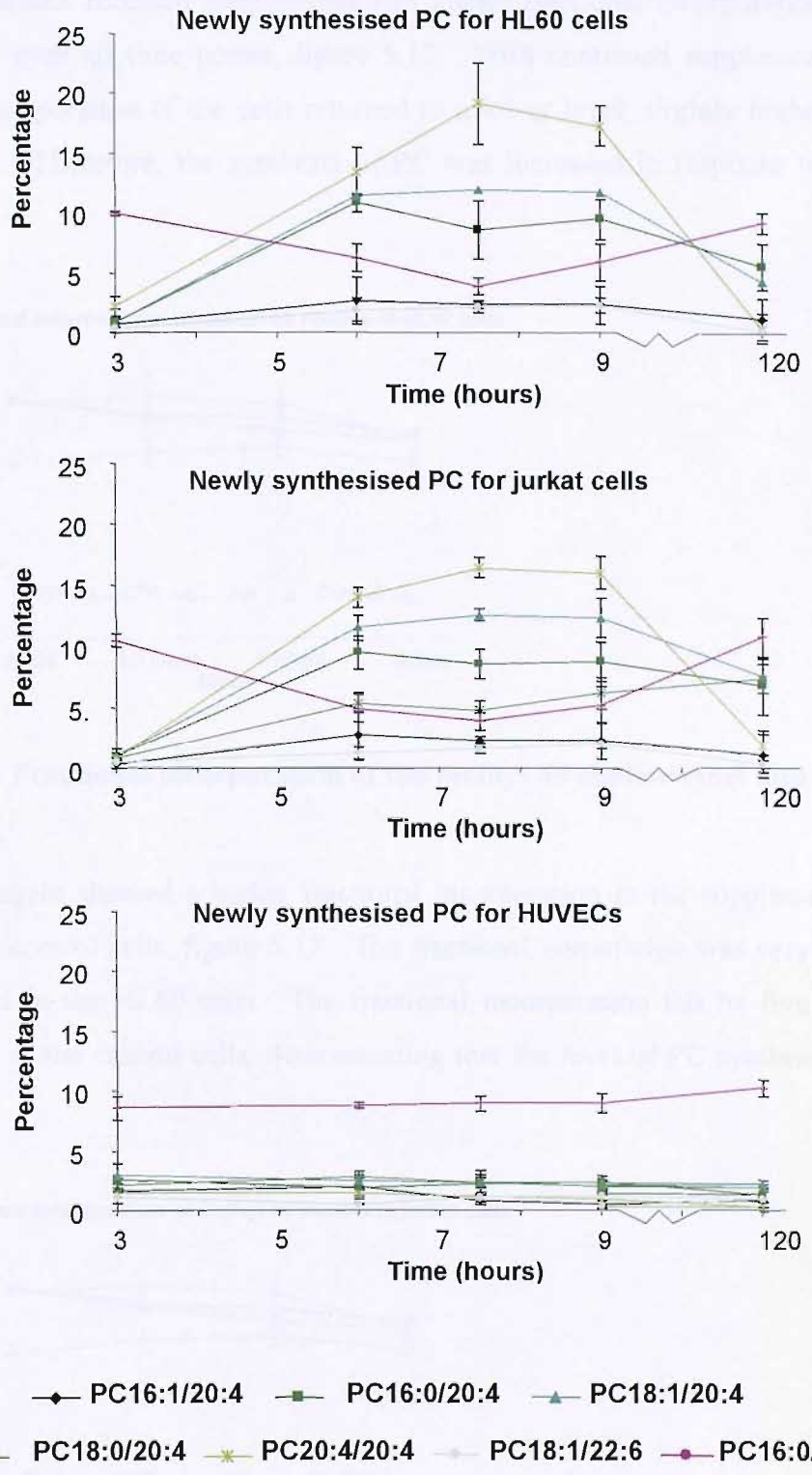

Figure 5.11: Changes to the newly synthesised PC composition after AA supplementation. The top panel shows HL60 cells, the middle jurkat cells and the bottom HUVECs.

5.3.6 Incorporation of deuterium label into three cell types

Incorporation of *methyl*-D_9 label into cell PC through the CDP-choline pathway was detectable within the first three hours after fatty acid supplementation in all cell types. HL60 cells which received supplements had higher fractional incorporation than the control cells over all time points, figure 5.12. With continued supplementation the fractional incorporation of the cells returned to a lower level, slightly higher than the control cells. Therefore, the synthesis of PC was increased in response to the fatty acids.

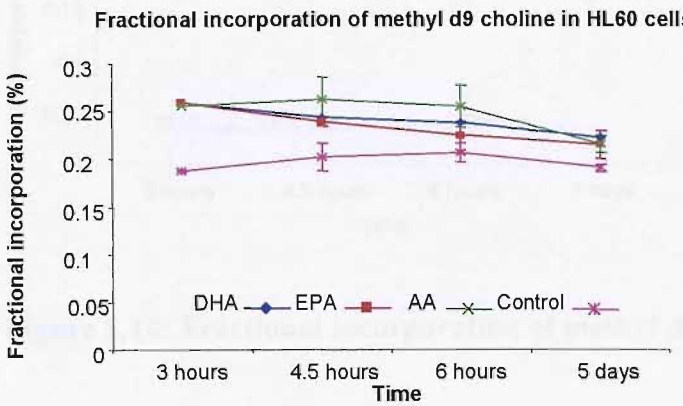

Figure 5.12: Fractional incorporation of the methyl d9 choline label into the HL60 cells.

Jurkat cells again showed a higher fractional incorporation in the supplemented cells compared to control cells, figure 5.13. The fractional corporation was very similar to that observed in the HL60 cells. The fractional incorporation fell by five days to a similar level as the control cells, demonstrating that the level of PC synthesis has also decreased.

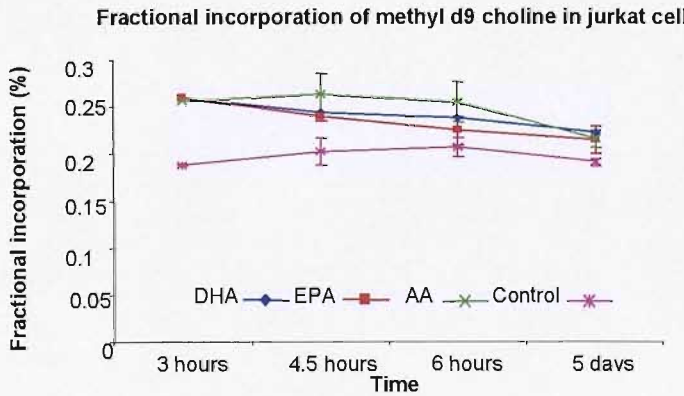

Figure 5.13: Fractional incorporation of the methyl d9 choline into the jurkat cells.

HUVECs had a slightly lower incorporation of the label than the cell lines. Synthesis in the control cells was slightly higher than the supplemented cells, indicating that the fatty acids supplements resulted in decrease in the synthesis of PC. With continued supplementation PC synthesis in the supplemented cells increased to a similar level as the control cells, figure 5.14.

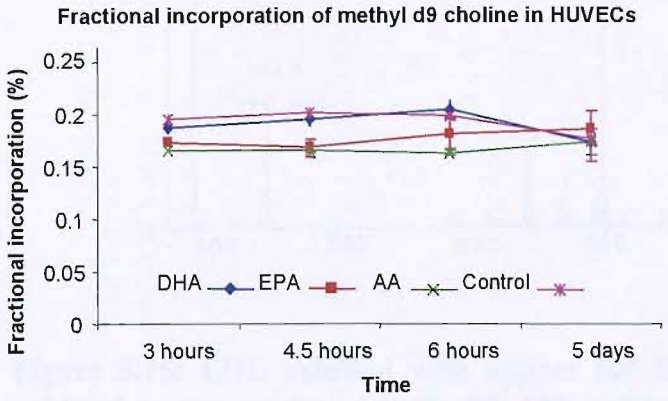

Figure 5.14: Fractional incorporation of methyl d9 choline in HUVECs.

5.3.7 Cytokine production in HUVECs after stimulation with oxLDL

After the changes in PC composition had been established, supplemented HUVECs were stimulated with 20 hour copper oxidised LDL (from a single donor) to determine the effect the supplements had on the production of inflammatory cytokines. The PC composition of the oxidised LDL, figure 5.15, which was used for stimulation of the cells was analysed by mass spectrometry. This showed that the oxidised PC was high in lysoPC species, which have chemo attractant properties for inflammatory cells (Macphee et al 1999), and was high in oxidised phospholipids.

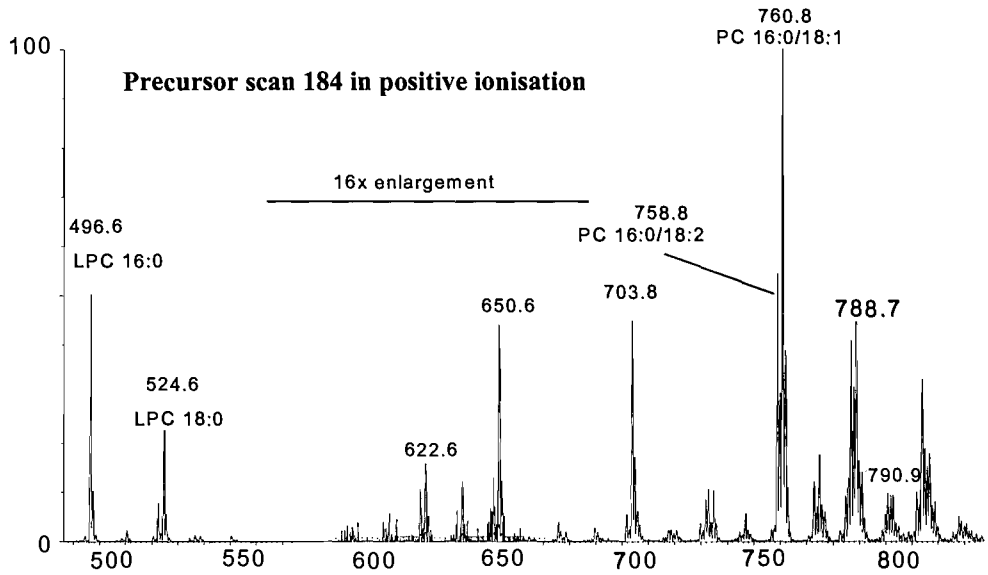

Figure 5.15: LDL oxidised with copper for 20 hours, shows a large amount of oxidised species produced in the 590-650 region and a large amount of lyso-PC.

HUVEC cell medium was supplemented with DHA, EPA or AA at 100μM for five days, as before, followed by stimulation with copper oxidised LDL. The resultant changes in IL-6 and IL-8 were measured by ELISAs; there was a small decrease in these cytokines in the DHA and EPA supplemented cells compared to the control, which was not significant. The AA supplemented cells did not produce more cytokines after stimulation with oxidised LDL, figure 5.16. This would suggest that the oxidised LDL used in these experiments alone was not enough to cause any significant changes to the cytokines measured and that there must be other factors involved in the process.

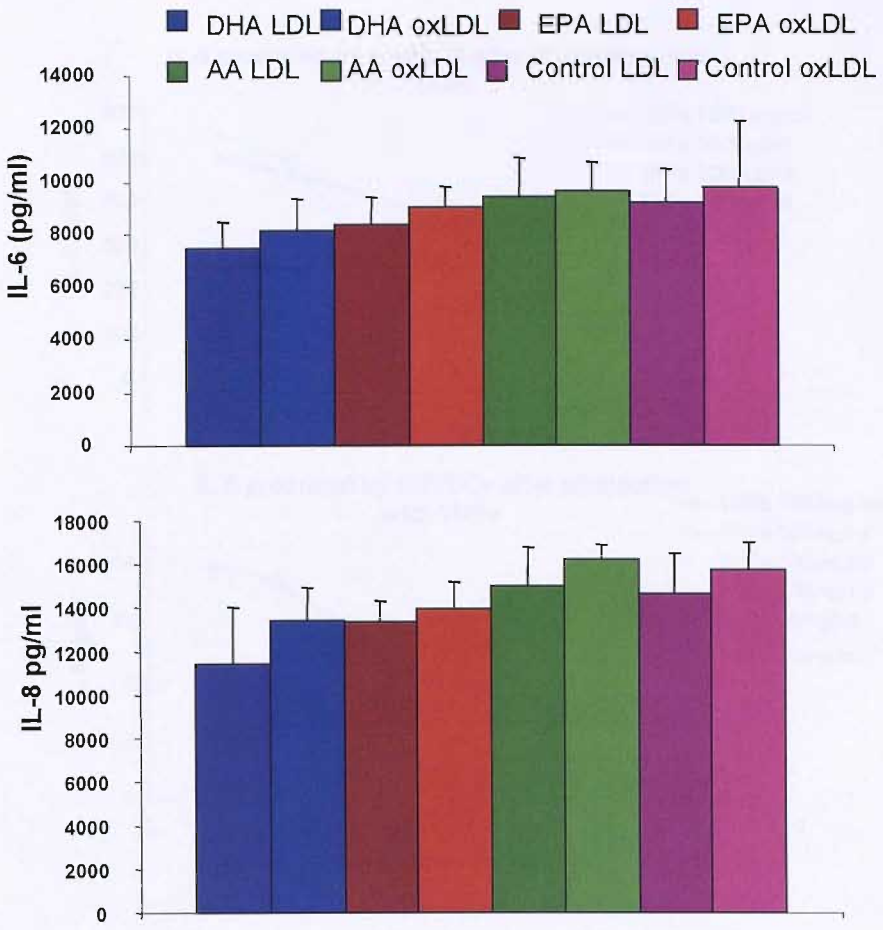

Figure 5.16: Cytokine production in HUVECs after the addition of oxidised LDL. The top panel shows the production of IL-6 in HUVECs after fatty acid supplementation for five days followed by stimulation with either LDL or oxLDL for 2hours. The bottom panel shows the production of IL-8 in HUVECs.

5.3.8 Cytokine production from HUVECs fatty acid supplementation

Stimulation of the cells was changed to TNFα, a known inflammatory cytokine which should result in the up-regulation of cytokines. The optimum stimulation of HUVECs with TNFα was established in dose response measurements for both IL-6 and IL-8 (these experiments were carried out with Rebecca Wiltshire, an intercalated BSc student 2005-2006). Figure 5.17 demonstrates that at the levels of TNFα used the HUVECs produced a maximal response with 200U/ml TNFα for two hours for both IL-6 (top panel) and IL-8 (bottom panel).

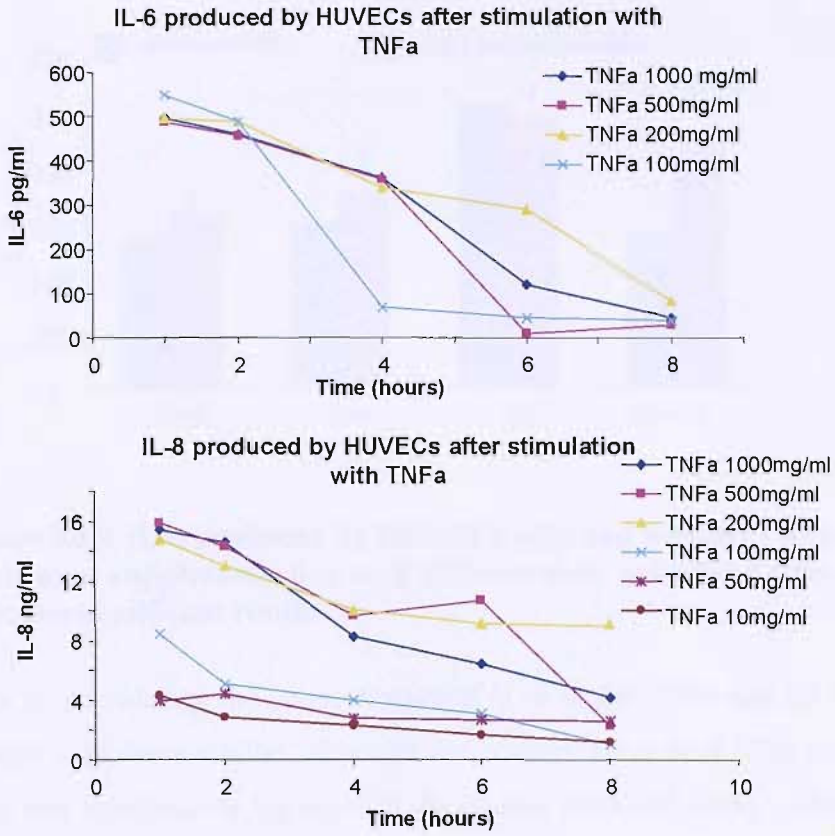

Figure 5.17: Production of IL-6 and IL-8 in response to TNFα stimulation at different concentrations. These panels show the amount of IL-6 (top panel) and IL-8 (bottom panel) produced by HUVECs.

HUVECs were supplemented with fatty acids as before, after five days the cells were stimulated with TNFα for two hours before the supernatants were collected (n=3). The non-stimulated cells showed no difference between the amounts of IL-6 produced in the DHA, EPA and control cells, whereas in the AA supplemented cells the amount of IL-6 was significantly higher than that of the control cells . After TNFα stimulation the concentration of IL-6 present in the supernatants increased. The DHA and EPA supplemented cells demonstrated a lower increase in the expression IL-6 than control cells, whereas AA supplemented cells produced more IL-6 than that of the control cells, though none of the changes were significant, figure 5.18.

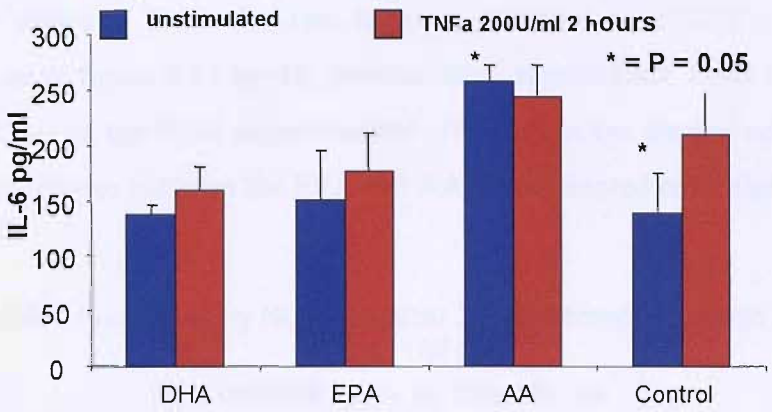

Figure 5.18: IL-6 produced by HUVECs with and without TNFα stimulation for 2 hours after supplementation with different fatty acids for 5 days at 100μM, * indicates significant results.

Prior to stimulation the concentration of IL-8 in the DHA and EPA supplemented and control cells were similar, although the concentration of IL-8 in the AA supplemented cells was significantly higher than the control cells (P= 0.05). After TNFα stimulation IL-8 production increased, although the increase was not significant difference between the control, DHA and EPA supplemented cells. There was a significant increase in the AA supplemented cells compared to the unstimulated cells (n=3), figure 5.19.

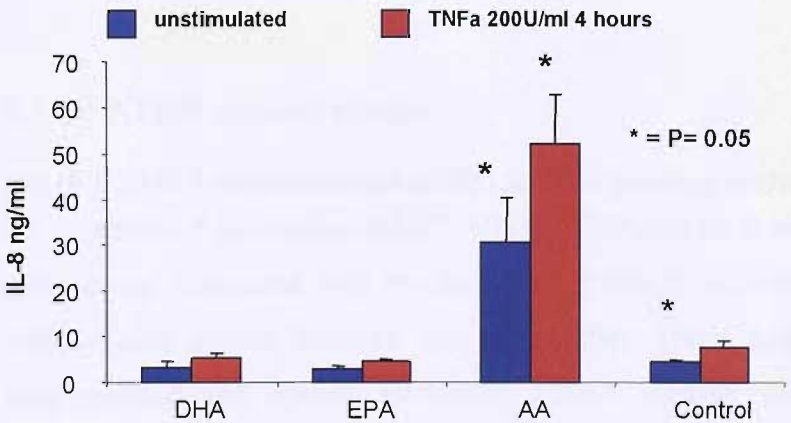

Figure 5.19: IL-8 produced by HUVECs with and without TNFα stimulation for 2 hours after supplementation with different fatty acids for 5 days at 100μM, * indicates significant results.

5.3.9 ICAM-1 levels expressed by HUVECs

The effect of TNFα for two hours at 200U/ml on ICAM-1 expression HUVECs is shown in figure 5.21 (n=3), demonstrating significantly lower (P= 0.026) expression of ICAM-1 in the DHA supplemented cells than in the control cells. However, there was no difference between the EPA and AA supplemented cells, figure 5.20.

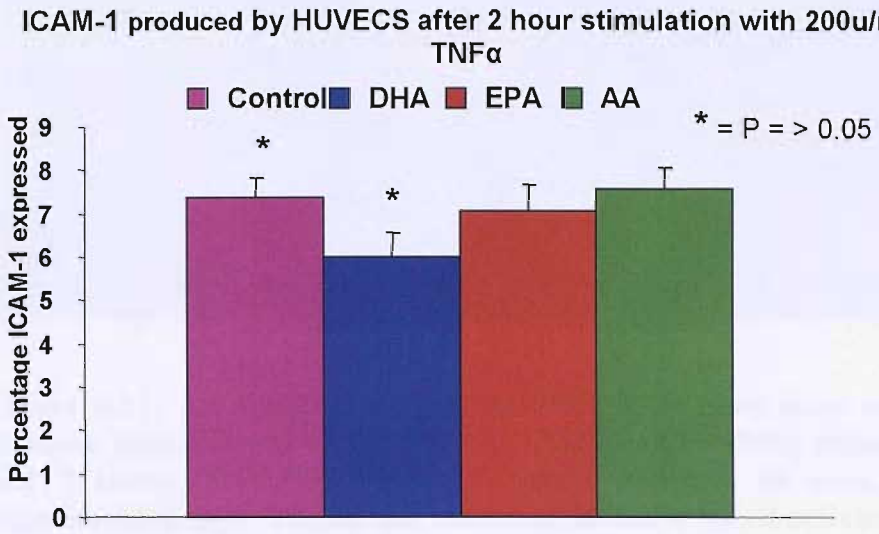

Figure 5.20: ICAM-1 produced by HUVECs with and without TNFα stimulation for 2 hours after supplementation with different fatty acids for 5 days at 100μM, * indicates significant changes.

5.3.10 NFκB measurement

5.3.10.1 EMSA measurement of NFκB DNA binding activities

To determine if activation of HUVECs by TNFα (even at the low levels shown by the results) was associated with the induction of NFκB, nuclear extracts were prepared at various time points between 0-2 hours after TNFα addition for both fatty acid supplemented and control HUVECs. DNA binding was determined by EMSA. Although the blot is not of a high quality it is the clearer of the two performed and it does show NFκB activation in these cells, figure 5.21. The bands show time points of 0, 0.5, 1 and 2 hours after TNFα addition. The results for control, DHA, EPA and AA were all very similar, showing NFκB activation, but it would be difficult to obtain much more information from the plot. The final four bands show oxLDL supplemented cells. All time points show slightly higher activation than the control cells, activation is particularly high at 0.5 hours after TNFα addition. Running of the EMSA gels was

carried out by Dr Ahmed Elsharkawy, The Liver Group, School of Medicine, University of Southampton.

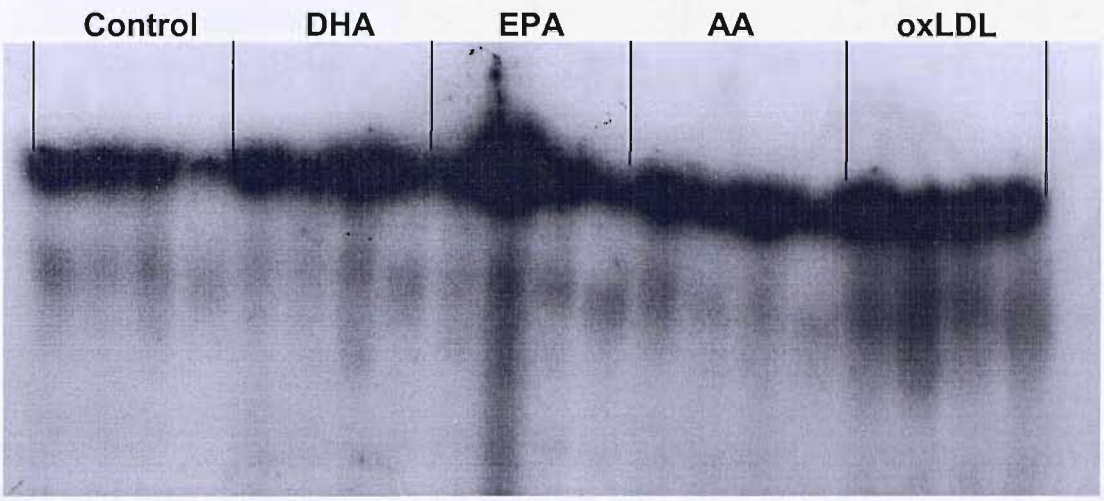

Figure 5.21: An EMSA plot for HUVECs that have been supplemented with different fatty acids or LDL for 5 days. Followed by TNFα stimulation for 0, 0.5, 1 and 2 hours, these are the four bands that can be seen for each of the supplemented cells. The darker the band the more NFκB activation in the cells.

5.3.10.2 Regulation of p50, p65 and IκBα proteins after HUVEC activation with TNFα

A change in NFκB DNA binding activities requires the degradation of IκB, typically IκBα, followed by transport of active NFκB to the nucleus. To investigate this, the levels of p50, p65 and IκBα in the nucleus were measured by immunoblotting. Figure 5.22 shows p50 in the top panel and p65 in the bottom panel. The levels of the p65 subunit in the nucleus are much lower than the levels of p50. Again the blots are not of a high quality but suggest small changes in p65 levels in the different supplemented cells. In general it would appear that there was less p65 and p50 present in the DHA, EPA and AA supplemented cells than the control cells. For oxidised LDL there is probably more p65 and p50 present.

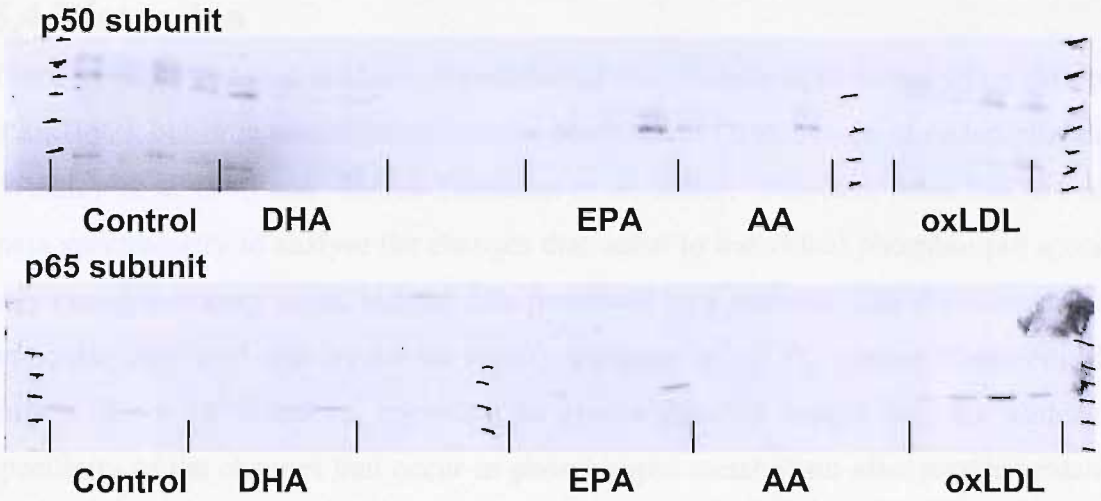

Figure 5.22: An immunoblot of the p50 and p65 subunits of NFκB for HUVECs that have been supplemented with different fatty acids or oxidised LDL for 5 days. Followed by TNFα stimulation for 0, 0.5, 1 and 2 hours, these are the four bands that can be seen for each of the supplemented cells.

The levels of IκBα in the nucleus are shown in figure 5.23. This was the best blot obtained, demonstrating that although IκBα is initially raised in the nucleus in the DHA cells it has disappeared after 2 hours. However, the EPA cells show the opposite occurring, with very little IκBα in the nucleus at time 0, although this increases with time. The AA and oxidised LDL cells had less IκBα in the nucleus at all time points than the control cells. All immunobloting in this section was carried out by Dr Ahmed Elsharkawy, The Liver Group, School of Medicine, University of Southampton.

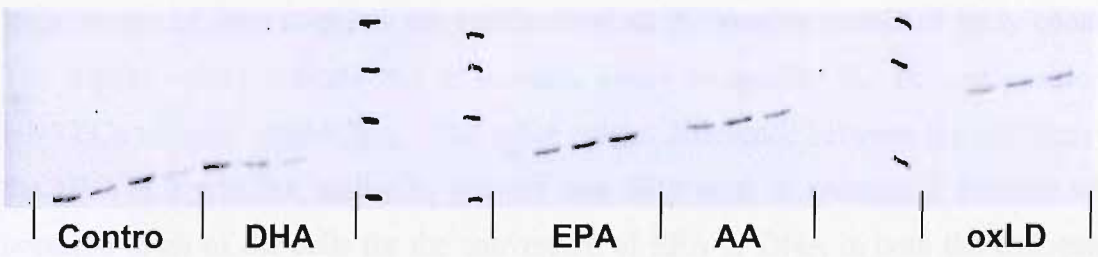

Figure 5.23: IκBα immunoblot for HUVECs that have been supplemented with different fatty acids or LDL for 5 days. Followed by TNFα stimulation for 0, 0.5, 1 and 2 hours, these are represented by the four bands that can be seen for each of the cells.

5.4 Discussion

There has been detailed analysis of endothelial cell phospholipid composition (Murphy et al, 1992), but little investigation into the phospholipid metabolism of endothelial cells (Whatley et al 1990 and Helies-Toussaint et al, 2006). Previous work has not used mass spectrometry to analyse the changes that occur to individual phospholipid species, only changes in fatty acids, but the data presented here demonstrates that changes in a particular fatty acid species do not signify a change in all PC species containing that fatty acid. It is, therefore, important to give a detailed insight into the molecular specificity of the changes that occur in phospholipid metabolism after supplementation with different fatty acids, which can be performed easily and quickly with mass spectrometry.

The changes in total PC composition were measured in HUVECs, HL60 and Jurkat cells at 3, 4.5, 6 hours and 5 days after the initial fatty acid supplementation for both endogenous PC composition (acyl remodelling pathway), and newly synthesised PC (CDP-choline pathway). The fatty acid supplements resulted in similar levels of change to the endogenous PC composition of the cells. However, the CDP-choline pathway results for the cell lines were very different to those of the HUVECs. The PC synthesis of the cell lines was rapidly altered to produce large increases in polyunsaturated PC, within three hours of the fatty acid supplement being added, although this rate of synthesis was not sustained and quickly fell to a more maintainable level. The HUVECs showed a very low ability to modify this pathway even in the presence of a large excess of fatty acids; as the synthesis of all PC species remained fairly constant. The results would indicate that it is much easier to modify the PC composition of HUVECs via acyl remodelling. The other crucial difference between the cell lines and the HUVECs was the ability to convert one fatty acid to another, a process which occurred in all of the cells for the conversion of EPA to DHA in both the endogenous and newly synthesised PC. However, the HUVECs also showed a high level of retro conversion of the DHA to EPA, previously observed in cultured retinoblastoma cells by Yorek et al (1984). Following EPA supplements there was also an increase in the fatty acid 22:5 in the sn-2 position; this fatty acid is an intermediate for the conversion of EPA to DHA and has been raised in previous studies measuring changes in fatty acids after DHA and EPA supplements (Philbrich et al 1987). The results for the HUVECs were similar to Helies-Toussaint (2006), which used HPLC to separate phospholipids of endothelial cells after fatty acid supplements; demonstrating that acyl remodelling

occurred at a higher rate than synthesis through the CDP-choline pathway. The difference in synthesis between the cell lines and the HUVECs cannot be due to the umbilical origin of the cells. Laura Swain, an intercalated BSc student 2005-2006, used umbilical cord blood to demonstrate a rapid change in endogenous composition and synthesis of PC species after supplementation with linoleic acid, figure 5.24. The results show larger changes in cord blood compared to adult blood, particularly in the newly synthesised species 16:0/18:2 and 16:0/20:4 with a decrease in 16:0/16:1 after six hours.

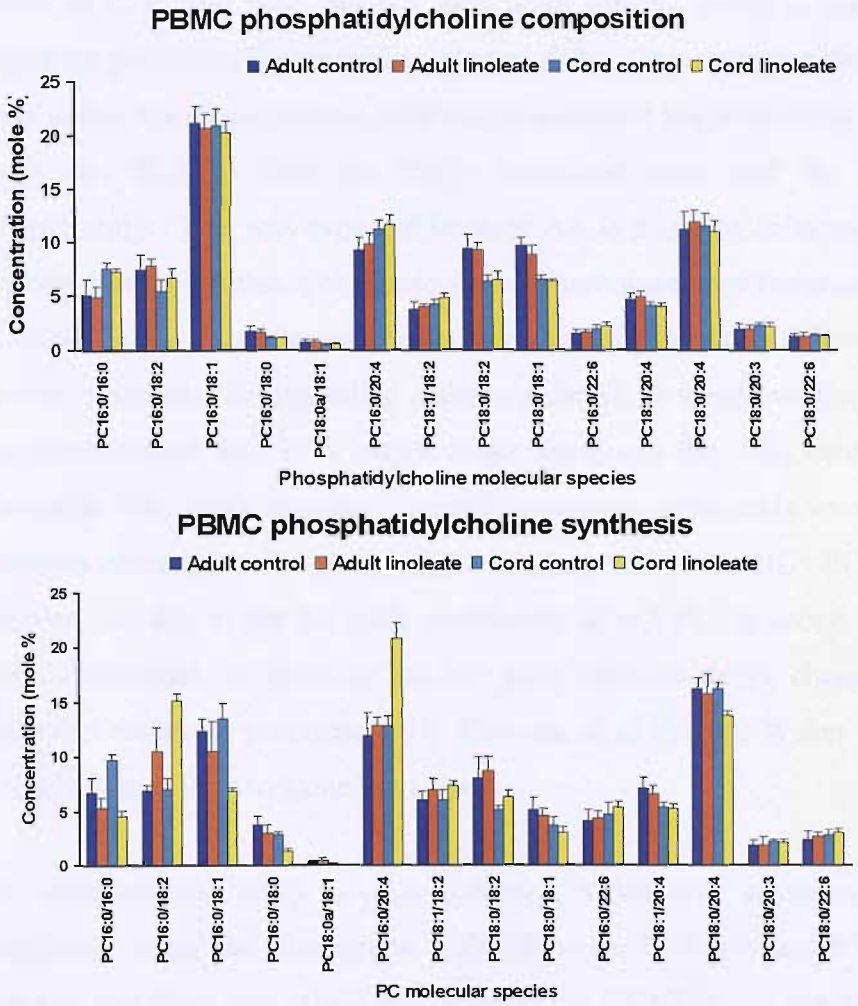

Figure 5.24: The changes in PC composition in both adult and cord blood PBMCs with and without a linoleic acid supplement at 30μM for 6 hours. These results demonstrated cord blood PC synthesis was rapidly altered by the addition of the supplement, unlike the HUVECs.

The unresponsive nature of the HUVECs was more likely to be caused by the vessel origin, as previous studies have reported that responses to oxidised LDL in arterial derived endothelial cells may be more sensitive to extracellular oxidative signals than

venous derived endothelial cells such as HUVECs (Khan et al 1995). The HUVECs were supplemented with a large excess of fatty acids, which would not be reproducible by diet. Therefore, as the changes to PC composition and synthesis were small in the endothelial cells *in vitro* any changes observed in the endothelium *in vivo* through dietary supplementation would be even smaller and it is questionable whether this is a plausible mechanism of n-3 polyunsaturated fatty acid action.

The modified HUVECs were stimulated with TNFα and oxidised LDL and the resultant changes in IL-6, IL-8 and ICAM-1 were measured. The cells have a higher background level of IL-8 than IL-6, but n-3 fatty acids did not result in significant reduction to cytokine production compared to control cells. The only significantly different result was in the AA supplemented cells which increased the production of IL-6 in the resting cells and IL-8 in both the TNFα stimulated cells and the non-stimulated cells significantly. This was expected because AA is a known inflammatory agent which is involved in the synthesis of eicosonoids. There were significant changes in the level of ICAM-1 in the cells after supplementation with DHA but no difference with EPA. This demonstrates that the signalling pathways altered by these two fatty acids must vary, as supplementation with EPA caused large changes to the DHA containing PC species in the cells. The small changes observed in cytokine production were related to the small changes observed in the phospholipid composition of the HUVECs; this would further suggest that this is not the main mechanism of n-3 PUFA action. These were not the results expected as previous studies have reported large changes in cytokine and adhesion molecule production; De Caterina et al (1994), Weber et al (1995), Collie-Duguid et al (1996) to name but a few.

To complete the study of this pathway NFκB was measured in the cells after supplementation and stimulation with TNFα for 0, 0.5, 1 and 2 hours. EMSA blots showed that there was NFκB activation in the HUVECs but the blot was not of a high enough quality to provide firm information. Therefore, to examine the role of NFκB further immunblotting was performed to analyse the changes in the p50 and p65 subunits, and the inhibitory IκBα. The results showed less p65 was present than p50 in the cells, therefore there was likely to be more p50:p50 dimers induced in response to TNFα rather than p65:p50 in HUVECs. Once more these blots were not of a high quality, though it appeared there were less of these subunits present in the nucleus in the DHA and EPA supplemented cells than in the control cells, indicating NFκB activity

was down regulated by the n-3 fatty acids. The oxidised LDL supplemented cells showed more NFκB subunits present in the nucleus demonstrating increased NFκB activation. This was supported by the presence of more inhibitory IκBα in the nucleus of the DHA and EPA supplemented cells than in the control, AA and oxidised LDL supplemented cells. There needs to be further investigation into the pathway that leads to the change in activation of NFκB in these cells following n-3 fatty acid supplementation. These experiments would need to be performed again to confirm the results because of the poor standard of the blots. These were not repeated as I did not have the required radioactivity training to conduct the experiments myself, and were further inhibited by the liver group moving to another university. Previous work has demonstrated oxidised LDL triggering a rapid dose dependent activation of NFκB in endothelial cells (Maziere et al 1996) therefore would have expected a higher level of NFκB activation in the cells with oxidised LDL added.

This chapter has examined the species molecular changes in PC composition and synthesises in HUVECs, HL60 and Jurkat cells, the results have demonstrated that the PC synthesis pathway in HUVECs was extremely hard to modify even in the presence of a large excess of fatty acids in the medium. By comparison the endogenous composition of these cells was readily modified. These small changes in the membrane composition of PC will have affected the cytokines produced by the cells in response to stimulation and have lead to the conclusion that under the conditions used this pathway is not a viable mechanism for the action of n-3 fatty acids.

Chapter Six
General Discussion

During the past few years, there has been an increase in both scientific and public interest in the role of n-3 fatty acids found in fish and fish oils in the prevention and management of cardiovascular disease. Controlled-intervention trials *in vivo* have indicated a favourable modifying effect of dietary fish oils on various risk factors for cardiovascular disease independent of their lowering of blood cholesterol (DART, 1989) and the GISSI-Prevenzione Trial (1999). The mechanism(s) through which the n-3 fatty acids exert their protective effects may include: changes to cell membranes and their functioning, modifications to cell signalling, changes in gene expression and biosynthetic processes, alterations in the oxidation of LDL and alterations to eicosanoid formations. This thesis has investigated changes to the PC composition of endothelial cells in culture, followed by the resultant modifications to cytokine production and NFκB activations. Changes to the PC composition of plasma and LDL were analysed after n-3 supplementation. Comparisons were made between the oxidation of the fatty acid modified LDLs and control LDL in the presence and absence of an Lp-PLA_2 inhibitor (SB222657). The oxidised species produced were compared to the oxidised species formed *in vivo* within atherosclerotic plaques.

Initially this project undertook the changes in PC composition and synthesis of HUVECs and two cell lines; HL60 and jurkat cells. Fatty acid incorporation into the endogenous PC was similar for all cells, but the incorporation of the fatty acid was more selective in HUVECs than the cell lines. The fatty acid supplements had virtually no effect on the PC synthesis in HUVECs whereas the PC synthesis of the cell lines was dramatically altered, supported by previous data for other cells in culture (Hunt et al 2004). The results raise questions over whether HUVECs are a suitable phospholipid model for the adult endothelium; the age of the cells was carefully controlled to ensure similarity to their *in vivo* composition, therefore resistance to change was not related to the transformation of the cells in culture. Helies-Toussaint (2006) used HUVECs at passage three and also reported little change in PC synthesis in response to fatty acid supplements. The small changes in PC synthesis may relate to HUVECs previous exposure to high levels of polyunsaturated phospholipid species as *in vivo* maternal blood is enriched with PC species containing a 22:6 fatty acid (Postle et al 1995), and polyunsaturated fatty acids pass from the mother to the foetus (Dutta-Roy 2000). This exposure to high levels of polyunsaturated fatty acids *in vivo* may cause the cells to become immune to changes *in vitro*. This is also supported by larger responses to oxidised LDL in arterial derived endothelial cells than venous derived endothelial cells

such as HUVECs (Khan et al 1995). Possible mechanisms underlying the small changes to PC synthesis may be related to modifications of DAG, of which there are different pools for synthesis of phospholipids and signalling responses within the cell (Burdge et al 1994), if there is little change to these species then PC synthesis will not be altered. In HUVECs there appears to be specific fatty acid incorporation into either acyl CoA (Holub et al 1980) or lysoPC (Vianen et al 1978) used in acyl remodelling, therefore resulting in the changes observed for endogenous PC composition. This project only measured changes in PC composition in HUVECs, it would be important to measure the changes to other phospholipids which also have important functions in cells, which would include: PI is the main source of DAG for signalling and PS is an essential co-factor of PKC, therefore changes to these phospholipid compositions may alter NFκB activation. PA is a second messenger within cells; therefore changes in its composition could alter inflammatory responses. Supplementation with the fatty acids should result in changes to DAG composition, a precursor of phospholipid synthesis; therefore changes to the fatty acids in DAG would influence synthesis. DAG is also a signalling molecule; therefore resulting in different signalling responses within the cells. The use of stable isotope labelled fatty acids would permit the investigation into the handling of n-3 fatty acids in HUVECs. The modification to cytokine production in HUVECs following n-3 fatty acid supplements did not fit with the literature, this was unexpected.

Modifications to PC composition of plasma *in vivo* with dietary n-3 fatty acid supplements were much more pronounced than the *in vitro* changes produced in HUVECs; previous studies have focused upon the changes to fatty acid composition, not changes to PC as a whole molecule. The results obtained would suggest that analysis of fatty acid composition alone does not provide enough information on the incorporation of n-3 fatty acids as both DHA and EPA supplements resulted in specific molecular changes to PC composition; DHA was incorporated primarily into PC16:0/22:6 whereas EPA was incorporated into PC16:0/20:5 and PC18:0/20:5. This suggests that the synthesis and turnover of PC16:0/22:6 is more rapid than PC18:0/22:6 in plasma; this is supported by previous unpublished work in Child Health, Southampton General Hospital which demonstrated higher rates of synthesis PC16:0/22:6 rather than PC18:0/22:6 in the plasma of mice. However, the results would suggest that the synthesis and turnover of both PC16:0/20:5 and PC18:0/20:5 are at a similar level. There was a decrease in the arachidonyl containing PC though this,

again, was particularly in PC16:0/20:4 rather than PC18:0/20:4 and the major decrease in mono-unsaturated species was PC16:0/18:2. These results show that the turnover of the PC containing palmitic acid, 16:0, in the sn-1 position is more rapidly altered in response to dietary fatty acids, suggesting the 16:0 containing species may process greater biological significance. There was little elongation of EPA into DHA in the plasma from this study. However, plasma PC composition indicates the conversion of DHA to EPA with particular incorporation into PC16:0/20:5; evidence for the retro conversion of DHA into EPA has previously been described *in vivo* after DHA supplements (Von Schacky et al 1985), in cultured retinoblastoma cells grown in DHA enriched medium (Yorek et al 1984), and in the liver of rats after DHA supplements (Schenk et al 1969). From the data collected in this project, it is not known which PC synthesis pathway was used in plasma to incorporate the fatty acids. To investigate this, a combination of ESI/MS/MS and *in vivo* stable isotope labels, such as choline-d9 (Bernhard et al 2004), would permit changes via the different pathways to be analysed; phosphocholine headgroup fragments with m\z 184 and 193 could be used for endogenous and newly synthesised PC respectively. Analysis of the intermediates at m/z 187 and 190 (i.e. with one or two labeled methyl groups) could show indirect incorporation of deuterium into chlorine headgroups via the *N*-methylation pathway.

Oxidation of the fatty acid modified LDLs was difficult to achieve, this may have been due to: methodological problems or the LDLs were more resistant to oxidation than expected. Previous studies have suggested that increasing the number of double bonds can result in a greater susceptibility to oxidation (Suzukawa et al 1995, Hau et al 1996, Oostenbrug et al 1997), but this was not supported by these results, as after 20 hours copper oxidation there were native n-3 containing PC species remaining in the LDL. The literature suggests that n-3 fatty acids increase the onset of oxidation; therefore it would be of interest to monitor the oxidation products formed in fatty acid modified LDLs over a time course extended beyond 20 hours. In addition, the fatty acid modified LDLs were less completely oxidised than the control LDL, indicating resistance to oxidation. This is the first detailed analysis of the oxidised species formed in the presence and absence of an Lp-PLA_2 inhibitor, which allowed the identification of the substrates and products of this enzyme. Under the oxidation conditions used lysoPC was produced while in the presence of the Lp-PLA_2 inhibitor formation of lysoPC was completely reversed. However, in the oxidised control LDL, there remained a low level of lysoPC formed independent of Lp-PLA_2. The DHA and EPA LDLs showed

accumulation of the low mass oxidised LDLs in the presence of the Lp-PLA_2 inhibitor at a higher rate than the control LDL but there was not a build up of the high mass oxidised PC species. Therefore the absence of these species may indicate n-3 fatty acids stop the formation of these species, which may possess more inflammatory biological actions than the short chain oxidised species. However, in response to oxidation there is a build up of the high mass oxidised species in the control LDL, although in all of the LDLs the high mass oxidised species are not as efficient substrates for Lp-PLA_2 action as the low mass oxidised species. By contrast plaque samples from patients receiving Lp-PLA_2 inhibitor prior to surgery did not show significant reduction to lysoPC, although there was a decreasing trend with increasing inhibitor.

The analysis of oxidised LDL and plaque samples required the development of methods to allow the identification of the exact masses and likely structure of the oxidised species. This was performed using a LTQ Orbitrap™ mass spectrometer, which enabled the identification of the oxidised species in the oxidised LDL samples. However, as plaques contain many other phospholipid classes there was increased interference, which prevented the identification of the oxidised species. To isolate the oxidised species in the plaque, an LC method would need to be established, to separate the phospholipids prior to MS/MS analysis using LTQ Orbitrap™.

In the view of the increasing evidence of a contributory role for Lp-PLA_2 in the development and progression of atherosclerotic lesions it would be of great interest to measure these molecules in plaques, as well as in the circulation of vulnerable individuals. The *in vitro* oxidation of n-3 modified LDLs in the presence of the Lp-PLA_2 inhibitor completely stopped lysoPC formation and there was no accumulation of the high mass oxidised species. Therefore, a placebo controlled trial that assigned both n-3 fatty acid supplements and Lp-PLA_2 inhibitor prior to carotid endarterectomy, would allow the effects of n-3 fatty acid incorporation into plaque composition (Rapp et al 1991, Theis et al 2003), and plasma composition to be investigated in combination with an Lp-PLA_2 inhibitor.

Analysis of *in vivo* oxidised LDL will allow comparisons to be made between oxidised species that may have the potential to provide clinical information of atherosclerotic disease progression, as well as response to potential Lp-PLA_2 inhibitor therapy. A method involving LC separation and multiple reaction monitoring (MRM), in which the

first quadrupole of the mass spectrometer is set at specific precursor m/z and the second quadrupole is set at a specific product m/z would allow the specific determination of product ions in relation to the precursor ion, therefore confirming the structure of the PC species.

This thesis has analysed the specific molecular changes to the PC composition of HUVECs, plasma and LDL after n-3 fatty acid supplementation, demonstrating the specific handling of the fatty acids both *in vivo* and *in vitro* and raising the question of biological activity of the different PC effected. Due to the low modifications obtained for PC synthesis in HUVECs, it also raises questions over the suitability of these cells for a model of phospholipid synthesis of the endothelium *in vivo*. The development of exact mass spectrometry methods using the LTQ Orbitrap™ mass spectrometer resulted in the identification of oxidised PC species formed during LDL oxidation *in vitro*. Oxidation resulted in variations in the oxidised lipids formed demonstrating that oxidation is a complex process. This was also confirmed by the plaque data, which showed large variations in the amount of lysoPC and oxidised species, in the presence and absence of the inhibitor. The identified oxidised PC species formed in LDL *in vitro* in both control and fatty acid oxidised LDL have identified species for further investigation of clinical markers as well as identified the changes in PC composition of plasma and LDL after n-3 fatty acids.

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