DOCTOR OF PHILOSOPHY

Fatty acids, monocytes and ageing

Chathyan Pararasa

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Summary

Elevated free fatty acids (FFA) are a feature of ageing and a risk factor for metabolic disorders such as cardiovascular disease (CVD) and type-2 diabetes (T2D). Elevated FFA contribute to insulin resistance, production of inflammatory cytokines and expression of adhesion molecules on immune cells and endothelial cells, risk factors for CVD and T2D.

Molecular mechanisms of FFA effects on monocyte function and how FFA phenotype is affected by healthy ageing remain poorly understood. This thesis evaluated the effects of the two major FFA in plasma, oleate and palmitate on monocyte viability, cell surface antigen expression, and inflammatory activation in THP-1 monocytes.

Palmitate but not oleate increased cell surface expression of CD11b and CD36 after 24h, independent of mitochondrial superoxide, but dependent on de novo synthesis of ceramides.

LPS-mediated cytokine production in THP-1 monocytes was enhanced and decreased following incubation with palmitate and oleate respectively. In a model of monocyte-macrophage differentiation, palmitate induced a pro-inflammatory macrophage phenotype which required de novo ceramide synthesis, whilst oleate reduced cytokine secretion, producing a macrophage with enhanced clearance apoptotic cells.

Plasma fatty acid analysis in young and mid-life populations revealed age-related increases in both the SFA and MUFA classes, especially the medium and very long chain C14 and C24 fatty acids, which were accompanied by increases in the estimated activities of desaturase enzymes. Changes were independently correlated with increased PBMC CD11b, plasma TNF-α and insulin resistance.

In conclusion, the pro-atherogenic phenotype, enhanced LPS responses in monocytes, and pro-inflammatory macrophage in the presence of palmitate but not oleate is reliant upon de novo ceramide synthesis. Age-related increases in inflammation, cell surface integrin expression are related to increases in both the MUFA and SFA fatty acids, which in part may be explained by altered de novo fatty acid synthesis.

I would like to dedicate this thesis to my parents and my sister, for pushing towards better achievement and furthering myself

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ABBREVIATIONS

4-HHE	4-hydroxyhexenal
4-HNE	4-hydroxynonenal
AA	Arachidonic acid
ABCA1	ATP binding cassette transporter-1
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
Ac-DEVD-AMC	Acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin
ACC	Acetyl-CoA carboxylase
ADP	Adenosine disphosphate
AGPAT1	Acylglycerol-3-phosphate acyltransferase
Akt	Protein kinase B
AMC	7-amino-4-methylcoumarin
AMP	Adenosine monophosphate
ANP	Atrial natriuretic peptide
ANT	Adenine nucleotide translocator
Apaf-1	Apoptotic protease activating factor 1
ARCHA	Aston research centre for healthy ageing
ATF-6	Activating transcription factor 6
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BHT	Butylated hydroxytoluene
BMI	Body mass index

BP	Blood pressure
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CAT	Carnitine acylcarnitine translocase
C/EBPα	CCAAT enhancer binding protein α
C/EBP-β-LIP	CCAAT enhancer binding protein β
CERT	Ceramide transport protein
CETP	Cholesterol ester transport protein
CHD	coronary heart disease
CHOP	CCAAT enhancer binding protein homologous protein
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CPT1	Carnitine palmitoyl transferase 1
CPT2	Carnitine palmitoyl transferase 2
CRP1	C-reactive protein 1
CVD	Cardiovascular disease
D6D	Δ 6 desaturase
DAG	Diacylglycerol
DCF	2',7'-dichlorofluorescein
DCFDA	Dihydrodichlorofluorescein diacetate
DHE	Dihydroethidium
DMSO	Dimethyl sulphoxide
DTNB	5, 5'-Dithiobis(2-nitrobenzoic acid)

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
Elovl1	Elongase 1
Elovl3	Elongase 3
Elovl5	Elongase 5
Elovl6	Elongase 6
Elovl7	Elongase 7
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinases
FA	Fatty acid
FACS	Fatty acid Co-A synthetase
FAD	Fatty acid desaturase
FAME	Fatty acid methyl ester
FAS	Fatty acid synthetase
FAT	Fatty acid translocase/CD36
FATP	Fatty acid transporter protein
FB1	Fumonisin B1
FCCP	Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone
FFA	Free fatty acid
FITC	Fluorescein isothiocyanate
FOXO	Forkhead box protein
G3P	Glycerol-3-phosphate
GLUT4	Glucose transporter protein 4
GM-CSF	Granulocyte macrophage colony stimulating factor

GPAT	Glycerol-3-phosphate transferase
GSH	Glutathione
GSR	Glutathione-S-reductase
GSSG	Oxidised glutathione
HDL	High density lipoprotein
HETE	Hydroxyeicostrienoic acid
HMG-CoA reductase	Hydroxylmethylglutaryl CoA reductase
HOMA-B	Homeostatic model assessment-beta cell function
HOMA-IR	Homeostatic model assessment-insulin resistance
HPETE	Hydroxyperoxyeicosatrienoic acid
HRP	Horse radish peroxidase
HSL	Hormone sensitive lipase
HUVEC	Human umbilical vein endothelial cell
ICAM1	Intercellular adhesion molecule 1
IFNγ	Interferon gamma
IgG1	Immunoglobulin G1
IgG2a	Immunoglobulin G2a
IL-1β	Interleukin 1β
IL-1ra	Interleukin 1ra
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-10	Interleukin 10

IL-13	Interleukin 13
IMCL	Intramyocellular lipid
iNOS	inducible nitric oxide synthase
IP3	Inositol triphosphate
IP-10	Interferon gamma induced protein 10
IRE-1α	Inositol requiring enzyme 1 α
IRS-1	Insulin receptor substrate 1
JAK2	Janus Kinase 2
JC-1	5, 5, 6, 6,-Tetrachloro-1, 1, 3, 3,-tetraethyl-imidacarbocyanin iodide
JNK	C-Jun NH2 terminal kinase
LCFA-CoA	Long chain fatty acid CoA
LC-MS	Liquid chromatography mass spectrometry
LDL	Low density lipoproteins
LFA1	Lymphocyte function associated antigen 1
LOX	Lipooxygenase
LPA	Lysophosphatidic acid
LPS	Lipopolysaccharide
Ly6C	Lymphocyte antigen 6C
M-CSF	Macrophage colony stimulating factor
MAC1	Macrophage antigen 1
MAPK	Mitogen activated protein kinase
MAPP	D-erythro-2-(N-Myristoylamino)-1-phenyl-1-propanol)
MCP-1	Monocyte chemoattractant protein 1

MG	Monoaclyglycerol
MIP1α	Macrophage inflammatory protein
MMP	Mitochondrial membrane polarisation
MMP-9	Metallomatrix proteinase 9
MnTBap	Manganese (III) tetrakis (4-benzoic acid) porphyrin chloride
mRNA	messenger ribonucleic acid
MTT	(3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MUFA	Monounsaturated fatty acid
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NEFA	Non-esterified fatty acid
NO	Nitric oxide
Ob-Rb	Leptin subtype receptor B
P38 MAPK	p38 Mitogen activated protein kinase
PARP	Poly ADP ribose polymerase
PAP	Phosphatidic acid phosphohydrolase
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PDK4	Pyruvate dehydrogenase kinase 4
PG	Prostaglandin
PGE2	Prostaglandin E2
PI	Propidium iodide

PIP2	Phosphatidylinositol 4, 5-bisphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLA2	Phospholipase A2
PMA	Phorbol 12-myristate 13-acetate
PP2	Protein phosphatase 2
PPAR	Peroxisome proliferator activator receptor
PSGL1	P-selectin glycoprotein ligand 1
PTFE	Polytetrafluorethylene
PUFA	Polyunsaturated fatty acid
RPE	R-phycoerythrin
ROS	Reactive oxygen species
SAMP-6	Senescence accelerated mouse P6
ScAT	Subcutaneous adipose tissue
SCD-1	Stearoyl CoA desaturase 1
SFA	Saturated fatty acid
SLS	Sphingolipids
SMS1	Sphingomyelin synthase 1
SPT1	Serine palmitoyl transferase
SR-A	Scavenger receptor A
SSA	Sulfosalicylic acid
TAG	Triacylglycerol

TG	Triglycerides
TGF-β	Transforming growth factor β
TLR	Toll like receptor
TLR1	Toll like receptor 1
TLR2	Toll like receptor 2
TLR4	Toll like receptor 4
T2D	Type-2 diabetes
VAT	Visceral adipose tissue
VP	Vinylpyridine

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Chapter 1

Introduction

1 Chapter 1: Introduction

1.1 Lipids

Lipids consist of a large group of organic compounds which have been categorised into 8 different classes (Fahy, Subramaniam et al. 2005) and encompass fatty acids, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids , saccharolipids and polyketides. Glycerolipids are lipids which contain glycerol including the monoacylglycerols, diacylglycerols, and triacylglycerides, with and glycosylglycerols i.e. compounds which have 1 or more sugar residue. The fatty acid lipid class are incorporated into blood lipids, fat deposits, and within membranes. They are essential building blocks of more complex lipids such as triglycerides, sphingolipids (SLS) and phospholipids.The formation of three ester bonds between a carboxylic acid (on fatty acids) and an alcohol (on glycerol) yields a triacylglyceride or triglyceride (TG).. TGis the most prevalent lipid in tissues, consisting of a glycerol backbone attached to 3 fatty acyl groups (see figure 1.1).

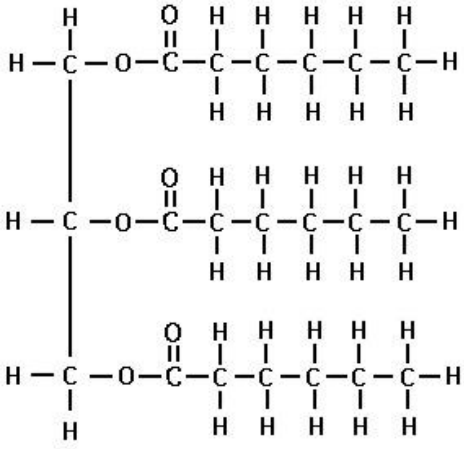


Figure 1.1: Triglyceride structure:

Glycerol backbone with three fatty acids attached by three ester bonds constitute a triglyceride molecule.

Glycerophospholipids are often referred to as phospholipids, being a major constituent of cell membranes e.g. phosphatidycholine, with a choline moiety attached to a phosphate providing a hydrophilic character suited for this purpose. Additionally these molecules serve as anchorage sites for both intracellular and exofacial proteins and may be metabolised to perform second messenger functions.

SLS share a common feature of a sphingoid base synthesised from serine and a long chain-fatty acyl-CoA. SLS can be converted into ceramides, phosphosphingolipids, and glycosphingolipids. The fatty

acid palmitate is the primary fatty acid used for sphingolipid synthesis conjugating to the sphingoid base, although other fatty acids of carbon length 14-26 can also be utilised.

Sterols are a group of lipids which share a common 4 ring structure with a hydrocarbon side chain and an alcohol group; the most common mammalian sterol is cholesterol which is an important cell membrane component. The sterols also include steroids such as estrogen and androsterone, glucocorticoids and mineralocorticoids.

Prenol lipids are synthesised from 5-carbon precursor's isopentenyl disphosphate and dimethlyallyl diphosphate in the mevalonic acid pathway. These compounds are known as simple isoprenoids, with successive addition of 5-carbon units generating polyterpenes. Simple isoprenoids include the carotenoids .e.g. vitamin A and tocopherols. Saccharolipids are fatty acids directly linked to a sugar backbone and they are highly localised in cell membrane bilayers, an example being the acylated glucosamine precursors of lipid A of lipopolysaccharides.

Polyketides are produced from the polymerization of acetyl and propionyl units by enzymes homologous to fatty acid synthases; they can further be modified by methylation, glycosylation, and oxidation performing functions such as virulence detection, with a diverse array of therapeutic applications (Hertweck 2009).

The physiological functions of fatty acids are primarily as a source of energy, as components of cell membranes and as second messengers. To act as a fuel source, fatty acids are first degraded with the successive removal of 2-carbon acetyl groups which bind the CoA derived from the active thiol on coenzyme A. The acetyl-CoA then enters the tricarboxylic acid cycle in the mitochondrial matrix undergoing oxidation to yield carbon dioxide and water, with the electrons extracted and passed onto co-factors (NAD^+/NADH or FAD/$FADH_2$). Electrons from NADH and $FADH_2$ enter the electron transport chain to generate energy in the form of adenine triphosphate. Several respiratory components, including flavoproteins, iron-sulphur clusters and ubisemiquinone, are capable of transferring one electron to oxygen and indeed most steps in the respiratory chain involve single-electron reactions, further favouring the monovalent reduction of oxygen to form superoxide anion radicals as a by-product.While this is relatively inert, superoxide is an important precursor for more reactive and potentially damaging reactive oxygen and nitrogen species (Turrens 2003). Normally, intracellular peroxides are scavenged by cellular glutathione, however, if oxidant production exceeds scavenging capacity, oxidative stress ensues (Sies and Cadenas 1985).

Phospholipids and glycolipids are major constituents of the cell membrane lipid bilayer.Different subclasses of fatty acids within the phospholipids and glycolipids impart differing effects on

membrane fluidity and subsequently membrane receptors.Higher saturation within the membrane reduces fluidity and reduces receptor numbers and the inverse is true of higher unsaturation in the membrane (Kalish, Fallon et al. 2012). Fatty acids can also serve as precursors to bioactive lipids such as eicosanoids and prostanoids which can have anti- and pro-inflammatory effects.

1.2 Fatty acid structure

Fatty acids (FA) are chains of methylene groups linked together by carbon-carbon bonds terminating with a carboxylic acid group; the nature of these bonds and chain length defines the lipid class and properties.

The extent of unsaturation (presence of carbon-carbon double bonds) is variable; FAs containing a single double bond are named as monounsaturated fatty acids (MUFA), those with more than one double bond classified as polyunsaturated fatty acids (PUFA); and those which do not possess double bonds called saturated fatty acids (SFA).

FAs are named by counting the number of carbons from the carboxylic acid group, and by the position of the double bonds with the denotation C: D (C represents the length of carbons and D represents the number of double bonds). Double bond position is denoted with the 'n minus' system followed by the location of the double bind from the methyl end of the fatty acid; this system is often used interchangeably with the omega system in place of 'n-', for example C18:3n6 or C18:3Ω6.

The saturated FA class is typically categorised further by length into short (4-6 carbons), medium (8-12 carbons), long (14-20 carbons) and very long chain (>20 carbons); whilst the monounsaturated fatty acids are divided into short (<19 carbons), long chain (20-24 carbons) and very long chains (>24 carbons).

The degree of saturation affects basic physical properties of FAs with the SFAs having higher melting points than their monounsaturated fatty acid counterparts. This is due to the double bond causing a 'kink' which prevents tighter packing compared to saturated fatty acids, therefore less energy is required to disrupt weak interactions between molecules allowing for a liquid state at room temperature.

Physiologically, the two main fatty acids are the SFA palmitate and the MUFA oleate, which constitute 60% of total fatty acids in tissues and blood. Whilst physical differences are noted between these two fatty acids, their metabolism and handling are considered to be broadly similar although regulatory control and enzyme affinity for substrates may differ (Bell and Coleman 1980).

1.3 Dietary lipid absorption, transport, metabolism and circulation to tissue

Digestion of lipids begins in the mouth with mastication and hydrolysis first by lingual lipase followed by gastric lipase in the stomach and the duodenum. In the duodenum, the presence of lipids stimulates both the gall bladder and pancreas to release bile and pancreatic juice, with the former emulsifying lipids for lipase action. The enzyme pancreatic lipase will act upon TG, specifically at the *sn-1* and *sn-3* positions to release free fatty acids (FFA), with the remaining *sn-2* undergoing non-enzymic conversion to mono-acylglycerol under the alkaline conditions of the small intestine(Brownlee, Forster et al. 2010).

In a stepwise fashion diacylglycerols, monoacylglycerol and FFA are generated, with the enzyme pancreatic lipase being more efficient at targeting TG with short or medium chains; the degree of unsaturation has a minimal impact on these processes. Long chain fatty acids emulsified with bile salts can enter the enterocyte by simple diffusion.

Within the enterocyte, the fatty acids are transported to the endoplasmic reticulum by fatty acid binding protein, and at this organelle are activated to a FA-acyl-CoA by acyl-CoA synthase. In order for FA to be transported around the body or stored they require conversion into more complex lipids. For FA this involves conversion into triglycerides by either the monoacylglycerol pathway (intestines) or the *sn* -glycero-3-phosphate pathway (liver and adipose tissue). The former involves the sequential esterification of FA with monoacylglycerol, to form diacylglycerol and finally TG, with the aid of acyl-CoA synthetase, monoacyglycerol acyltransferase, and diacylglyceroltransferase (DGAT1).

The TGs are bound to specific apoproteins to form chylomicrons processed in the Golgi apparatus; the newly synthesised TGs are transported into the bloodstream via the lymph vessels with the hydrophobic lipid core shielded by apoproteins which permit transport and recognition by specific receptors.

Circulating chylomicrons can be hydrolysed by cell surface lipoprotein lipase associated with the capillary endothelium. Released FFA and glycerol are freely able to cross endothelial cell membranes, with a portion of the FFA binding to albumin or are transported by protein facilitated mechanisms. Particle remnants from chylomicron catabolism consist of mainly ApoE, some cholesterol and TGs which return to the liver. At the liver, the ApoE is recycled to produce very low density lipoprotein (VLDL), the primary transporter of TGs.

TG hydrolysis within VLDL leads to the formation of intermediate and subsequently low density lipoproteins (LDL). LDL is primarily concerned with the transport of cholesterol, with high density lipoprotein (HDL) playing a crucial role in transporting a majority of cholesterol from the periphery to the liver for processing, and is responsible for transfer of cholesterol to VLDL via cholesterol ester transfer protein (CETP).

Released fatty acids are transported into the cell via fatty acid transport protein or fatty acid translocase (FATP or FAT/CD36), where they are either utilised as an immediate energy source and undergo oxidative phosphorylation, or as an energy store through triglyceride synthesis or for membrane biosynthesis through the synthesis of sphingolipids.

1.4 Metabolism of fatty acids in the mitochondria

Mitochondria are the energy generating organelles of the cell, able to oxidise substrates such as glucose and fatty acids to produce high energy phosphate molecules like ATP. Glucose metabolism is initiated with glycolysis, through phosphofructokinase 1 the substrate is metabolised to pyruvate which is then oxidised in the mitochondria. Entering mitochondria through the mitochondrial pyruvate carrier, pyruvate dehydrogenase converts the molecule to acetyl-CoA which then enters the tricarboxylic acid cycle (TCA).

Prior to fatty acid metabolism in the mitochondria, a CoA group is added to the fatty acid to produce fatty acyl-CoA by fatty acid CoA synthetase (FACS). The long chain acyl-CoA is converted to a long chain acylcarnitine by carnitine palmitoyl transferase 1 (CPT1), and carnitine acylcarnitine translocase (CAT) transports this molecule along the inner mitochondrial membrane, where CPT2 converts it back into an acyl-CoA. The long chain acyl-CoA undergoes β-oxidation.

β-oxidation consist of four enzyme reactions whereby the long-chain fatty acyl-CoA is shortened progressively releasing 2 carbon acetyl-CoA units at each of the four steps. A full cycle involves the dehydrogenation of an acyl-CoA ester yielding trans-2-enoyl-CoA, hydration of the double bond, the L-3-hydroxy-acyl-CoA is dehydrogenated to 3-keto-acy-CoA, with the final step involving thiolytic cleavage producing a two carbon shortened acyl-Coa and an acetyl-CoA group, with one nicotinamide adenine dinucleotide (NADH) and one flavin adenine dinucleotide ($FADH_2$) as electron carriers. The acyl-CoA re-enters β-oxidation, whilst the acetyl group enters the TCA, with the electron carriers entering the electron transport chain (Houten and Wanders 2010).

The electron transport chain is the principal site in mitochondria of oxidative phosphorylation. The system forms the basis of ATP production and consists of five complexes NADH dehydrogenase

(complex I), succinate dehydrogenase (complex II), cytochrome c reductase (complex III), cytochrome c oxidase (complex IV) and ATP synthase (complex V). Within the inner mitochondrial these complexes transport electrons from the reduced NADH and $FADH_2$ to molecular oxygen. Complexes I and II transfer electrons from the reduced carriers to ubiquinone. Complex III transfers electrons from ubiquinol to cytochrome c, and complex IV transfers electrons from this to molecular oxygen. These four complexes couple electron transport to a proton gradient which is utilised by complex V to catalyse the addition of a phosphate group to ADP to generate ATP (Boekema and Braun 2007).

1.5 De novo fatty acid synthesis

Fatty acids do not only originate from dietary sources but can originate endogenous through de novo synthesis. Fatty acids can be produced from excess carbohydrates involving their breakdown into 2 carbon acetate units. These units as acetyl-CoA are subsequently carboxylated with bicarbonate to generate malonyl-CoA under the enzymatic activity of acetyl-CoA carboxylase (ACC). The malonyl-CoA is progressively combined with further acetyl-CoA units by fatty acid synthase enzyme complex (FAS), and further units are added to produce C16 saturated fatty acid palmitate (Ratnayake and Galli 2009).

Once released from by FAS, palmitate is further metabolised by elongation and desaturation processes to generate fatty acids. There are various isoforms of both the desaturase and elongase enzymes and they work at various stages of the process of de novo synthesis. In the synthesis of saturated fatty acids, *Elovl6* elongates saturated fatty acids ranging with 12-16 carbons to a length of 18 carbons (Guillou, Zadravec et al. 2010). The process of elongation is achieved through the condensation of an acetyl-CoA and malonyl-CoA with a subsequent reduction step to generate β-hydroxyacyl-CoA. Dehydration results in an enoyl-CoA which is reduced to complete the elongation step. Stearate can be elongated further by *Elovl1, Elovl3* and *Elovl7* to produce arachidic acid (C20:0), with these enzymes capable of further elongation up toward vary long chain fatty acids i.e. >C20:0.

Desaturation occurs at the n-7 (Δ7) position of the carbon chain of palmitate, or at the n-9 (Δ9) position of palmitate and stearate generating palmitoleic and oleic acids respectively by the Δ9 desaturase, commonly referred to as stearoyl-CoA desaturase 1 (SCD-1). The n-7 fatty acids can be subject to elongation by *Elovl6* and *Elovl5*. The n-9 fatty acids are subject to elongation by *Elovl3* generating C20:1, C22:1, and C24:1, the final reaction being terminal in the production of monounsaturated fatty acids. In the absence of n-3 and n-6 fatty acids which cannot be synthesised are required in the diet, oleic acid can be subject to desaturation by Δ6 desaturase to produce the n-9 family of polyunsaturates (Ratnayake and Galli 2009, Guillou, Zadravec et al. 2010) (figure 1.2).

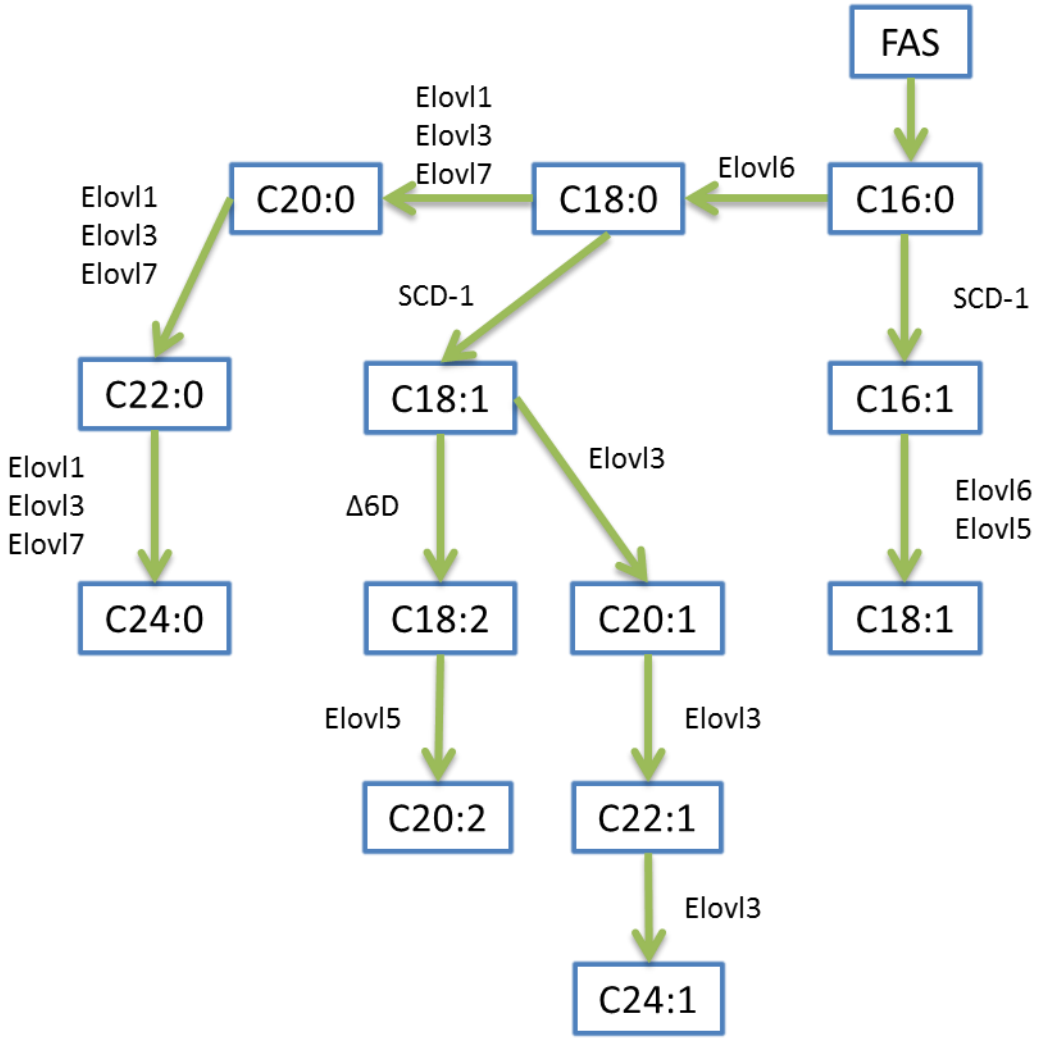


Figure 1.2: **De novo fatty acid synthesis:**

Fatty acid synthesis requires the elongase enzymes Elovl1, Elovl3, Elovl5, Elovl6 and Elovl7, in addition to the desaturation enzymes stearoyl-CoA desaturase-1 (SCD-1), Δ6 desaturase (Δ6D). Fatty acid synthase (FAS) generates the saturated fatty acid palmitate, and the activity of elongase and desaturase enzymes generates various fatty acids.

Essential fatty acids namely linoleic and α-linoleic acids cannot be synthesised as mammalian enzyme systems cannot introduce double bonds beyond the Δ10 carbon and the terminal methyl end, whilst in other animal and plants can introduce double bonds at Δ12 and Δ15 can be produced. Linoleic and α-linoleic acid are referred to as Ω-6 and Ω-3 polyunsaturated fatty acids respectively, and the conversion into longer and more desaturated fatty acids is required for eicosanoid synthesis (Ratnayake and Galli 2009, Guillou, Zadravec et al. 2010). The pathways of the Ω-6 and Ω-3 metabolism are independent without any convergence and are summarised below (figures 1.3).

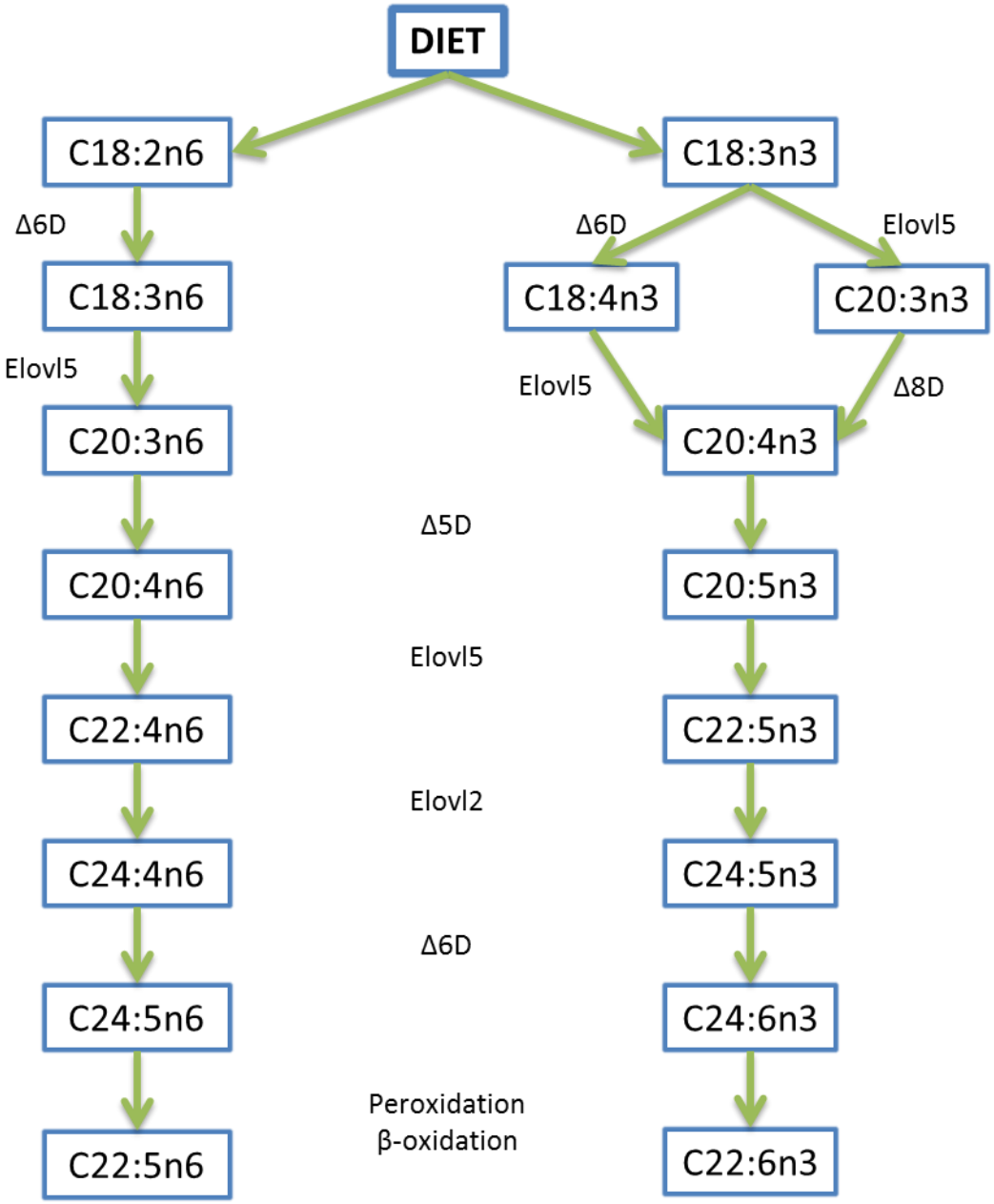


Figure 1.3: Synthesis of essential fatty acids from dietary sources

1.6 FFA are converted into TGs for energy storage

In the liver and adipose tissue the sn-glycero-3-phosphate pathway mediates TG synthesis which occurs in the rough endoplasmic reticulum. This process involves the direct addition of fatty acyl groups to sn-glycerol phosphate by glycerol-3-phosphate transferase (GPAT) to produce lysophosphatidic acid, which is acylated further to produce phosphatidic acid. A phosphate group is removed from the acid by phosphatidic acid phosphohydrolase (PAP) to yield a diacylglycerol, which is then acylated a final time to yield triacylglycerol or TG by diacylglyceroltransferase (DGAT).

Adipose tissue consists of mature adipocytes, connective tissue, non-adipocyte cells, vascular and neural tissue, with the non-adipocyte cell fraction consisting of macrophages, preadipocytes and fibroblasts. The mature adipocytes show variations in functional characteristics depending upon the site.

The visceral adipose depot is situated around the central organs withinthe abdomen and in the subcutaneous tissue above the central organs; in contrast main sites of subcutaneous adiposity are situated around the femoral-gluteal region and back, with up to 80% of body fat situated in these stores with the remainder from visceral stores (Wajchenberg 2000).

Adipocytes are considered lipid sinks storing free fatty acids as triglycerides, with smaller adipocytes capable of buffering against rapid increments in circulating FFAs. However, as adipocytes become larger they become dysfunctional. Subcutaneous adipose tissue (ScAT) contains small adipocytes which are generally more insulin sensitive having a greater FFA storage capacity, in contrast to visceral adipose tissue (VAT) which is primarily formed of large adipocytes which are insulin resistant, hyperlipolytic and resistant to the anti-lipolytic effect of insulin (Misra and Vikram 2003).

The degree of innervation and vascular supply to the adipose tissues appears to differ by site with the VAT having greater levels than that of ScAT (Ibrahim 2010). The expression of androgen and oestrogen receptors are increased in VAT and ScAT respectively (Mizutani, Nishikawa et al. 1994, Pedersen, Hansen et al. 1996, Bjorntorp 1997, Freedland 2004). Adrenergic receptor expression is higher in VAT compared to ScAT with elevated β_3 adrenergic receptors particularly prominent (Arner, Hellstrom et al. 1990, Hellmer, Marcus et al. 1992, Krief, Lonnqvist et al. 1993, Imbeault, Couillard et al. 2000).

A consequence of the increased innervation coupled with the greater expression of adrenergic receptor expression recorded in VAT compared ScAT there is an increased rate of basal lipolysis and metabolic rate (Lemieux and Despres 1994, Arner 1995), which in abdominally obese individuals has been observed as elevated FFA release (Freedland 2004). The increased FFA release by the visceral adipose depot leads to greater FFA exposure of the liver through venous blood derived from these sites draining into the portal vein, which is in contrast to the subcutaneous adipose depots where they principally drain into the systemic veins therefore diluting exposure of tissues to FFA (Marin, Andersson et al. 1992, Jensen 1997, Jensen 1997, Montagne and O'Rahilly 2000, Freedland 2004). A recent paper by Baglioni et al. described that the differences between the two adipose depots and the behaviour and characteristics of their respective adipocytes can be explained by differences in the respective adipocyte stem cells (Baglioni, Cantini et al. 2012), with the expression of genes

concerned with lipolysis and insulin signalling in stem cells reflecting that observed in a mature adipocyte.

The differences in the characteristics and functional nature of the visceral and subcutaneous adipocytes may aid in explaining the increase in metabolic disease and disorder risk associated with elevated visceral adiposity at these sites.

1.7 Membrane lipid synthesis and their metabolism to eicosanoids and ceramides

As described above, fatty acids are important precursors in the production of more complex lipids for cell membranes. The main site of glycerophospholipids, sphingolipids and sterols lipid synthesis is the endoplasmic reticulum.

Glycosphingolipids are the most abundant class of membrane lipids, and their synthesis begins in the ER with the acylation of glycerol-3-phosphate to form phosphatidic acid, and subsequently a polar head is added commonly serine, choline or ethanolamine. The addition of a choline group from CDP-choline to a diacylglycerol molecule produces phosphatidylcholine by CDP-choline:1,2-diacylglycerol cholinephosphotransferase, which is the most abundant (Blom, Somerharju et al. 2011), closely followed by the formation of phosphatidylethanolamine which is formed by the transfer of phosphoethanolamine from CDP-ethanolamine to diacylglycerol by CDP-ethanolamine:diacylglycerol ethanolaminephosphotransferase (Vance 2008). Phosphatidlyserine is formed through the transfer of the choline or ethanolamine polar head group in exchange for the serine by phosphatidylserine synthase.

Cholesterol is a major structural lipid of cell membranes, being particularly enriched at the plasma membrane. All cells are capable of synthesising cholesterol using acetate as a carbon source (derived from the breakdown of carbohydrates). The rate limiting step catalysed by hydroxymethylglutaryl CoA reductase (HMG CoA reductase) produces mevalonate and six further enzymes convert this compound into squalene. Committed cholesterol synthesis is undertaken by the enzyme squalene oxidase utilising molecular oxygen to generate lanosterol, a cyclic intermediate. The synthesis pathway requires a further 20 enzymes in either the Bloch or the Kandutsch-Russell pathway (Bloch 1992) to produce cholesterol.

Sphingolipids are typically found enriched at the cell membrane, and within this class the glycosphingolipids and sphingomyelin are particularly important structural lipids. These lipids participate in the formation of lipid rafts, membrane domains which can influence the function and distribution of membrane proteins (Coskun and Simons 2010). Precursors and less abundant

sphingolipids such as ceramides and sphingosine have important roles to play in intracellular signalling (Hannun and Obeid 2008, Hannun and Obeid 2011).

The first step in the synthesis of sphingolipids begins with the condensation of serine and palmitoyl-CoA under the action of serine palmitoyl transferase (SPT) producing 3-dehydrosphinganine which is then reduced to sphinganine under the action of 3-dehydrosphinganine reductase (Futerman and Riezman 2005, Sabourdy, Kedjouar et al. 2008). Sphinganine is then acylated under the activity of ceramides synthase to form dihydroceramide (Lahiri and Futerman 2007)with an acyl chain that will vary according the carbon chain length and unsaturation, which imparts variability on the sphingolipid generated. A desaturase will convert the dihydroceramide into a ceramide. This compound is the first to contain a sphingosine backbone, and is therefore considered central in the synthesis of other sphingolipids.

Further metabolism of ceramides can yield other sphingolipids through the addition of polar head groups or the exchange or removal of acyl groups. Newly synthesised ceramides are primarily utilised for synthesis of sphingomyelin and glycosphingolipids (Tafesse, Ternes et al. 2006). Sphingomyelin is the major sphingolipid of the plasma membrane with synthesis occurring at the endoplasmic reticulum and further processing in the Golgi apparatus. Ceramides are synthesised at the endoplasmic reticulum and are transported by ceramide transport protein (CERT) to the

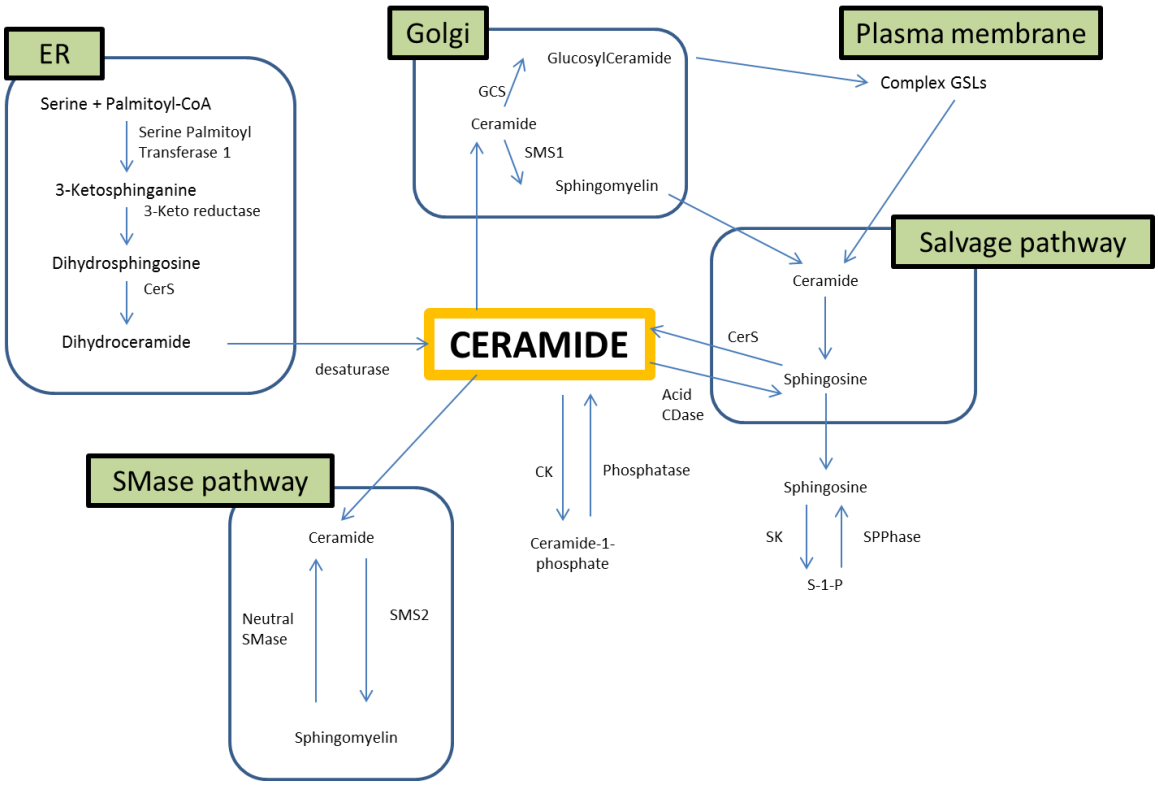


Figure 1.4: Overview of ceramide and complex sphingolipid synthesis and metabolism

cisternae of the Golgi where sphingomyelin synthase 1 (SMS1) converts ceramide to sphingomyelin (Hanada, Kumagai et al. 2009). Glycosphingolipid synthesis occurs also in the Golgi, beginning with the addition of a glucosyl moiety to the ceramide. The newly formed glucosylceramide is translocated to the luminar face of the Golgi for more complex glycosphingolipid synthesis (Blom, Somerharju et al. 2011). Furthermore the addition of a phosphate or phosphocholine group to ceramide can result in the formation of ceramide-1-phosphate.

Sphingolipids are readily recycled via salvage pathways. The breakdown of sphingolipids occurs in acidic organelles such as late endosomes and lysosomes (Kolter and Sandhoff 2005). Exohydrolases catalyse stepwise release of monosaccharides leading to the generation of ceramide, with sphingomyelin ultimately converted to ceramide also by acid sphingomyelinase (Riboni, Prinetti et al. 1994). Ceramides generated by these processes can ultimately be degraded further by acid ceramidase to form sphingosine and free fatty acids which are released by the acidic organelles into the cytoplasm (Riboni, Bassi et al. 1998). The long chain sphingolipids released can re-enter pathways of lipid synthesis, the latter generated via the activity of sphingosine kinases (Baumruker, Bornancin et al. 2005).

Beyond their roles as structural lipids, sphingolipids also perform important cellular signalling functions. For example, ceramides play roles in modulating whole body metabolism (Bikman and Summers 2011), with further roles in pathological conditions such as cardiovascular disease and non-alcoholic fatty liver disease have been extensively examined (Alewijnse and Peters 2008, Pagadala, Kasumov et al. 2012). Fatty acids can generate other lipids such as eicosanoids which play important roles in inflammation.

Eicosanoids, which encompass prostaglandins, leukotrienes and lipoxins, are intra- and inter-cellular signalling molecules which are generated primarily by the oxidation of arachidonic acid, a polyunsaturated fatty acid, but also from eicosapentaenoic acid and dihomo-γ-linoleic acid (Levin, Duffin et al. 2002, Wada, DeLong et al. 2007). These lipids exert control of many aspects of immunity such as cytokine production, antibody recognition, proliferation and migration, with a majority of eicosanoids produced by immune cells such as dendritic cells, neutrophils, and macrophages.

The synthesis of eicosanoids, like that of ceramides, is dependent upon the availability of a fatty acid, namely arachidonic acid. Cellular responses to hormones, cytokines or growth factors can trigger release of arachidonic acid (AA) by phospholipase A2 (PLA2). The metabolism of AA occurs via three pathways the P-450 epoxygenase, cyclooxygenase (COX), and lipoxygenase (LOX) pathways.

The P-450 epoxygenase pathway produces hydroxyeicosatetraenoic acids (HETE) and epoxides, whilst the LOX pathway generates hydroxyperoxyeicosatetranoic (HPETE) acid and HETE. The COX pathway generates PGG2 and PGH2. Theses metabolites are metabolised further to prostaglandins, prostacyclins and thromboxanes in the COX pathway, leukotrienes and lipoxins in the LOX pathway and HETEs and epoxides from the cytochrome P-450 pathway. Eicosanoid products have multiple immunomodulatory roles (Harizi, Corcuff et al. 2008). For example macrophages have been demonstrated to be an important source of eicosanoids (Back 2007) with production of PGs having an autocrine effect on TNF-α. Resolvins are a class of anti-inflammatory molecules produced from Ω-3 fatty acids e.g. docosahexaenoic acid and eicosapentaenoic acid, in the LOX pathway. These molecules resolvin D1 and resolvin E1 are involved in reducing both pain and inflammation in animal models and humans (Ji, Xu et al. 2011)

1.8 Hormonal control of lipid metabolism

Fatty acids are important in the production of structural lipids such as sphingolipids, the production of intercellular signalling molecules such as eicosanoids and intracellular signalling molecules such as ceramides. The production of these lipids is dependent upon the availability of fatty acids, and this is governed by a balance between rates of lipolysis and storage of free fatty acids in adipocytes and tissues such as skeletal muscle.

The storage of free fatty acids as triglycerides provides an energy reserve in times of fasting or exercise when energy is scarce through lipolysis, however a dysregulation in lipolysis may lead to metabolic abnormalities, with a reduction in lipolysis leading to the accumulation of TG and therefore obesity, conversely excessive lipolysis such as that observed in lipodystrophies are associated with a loss in adipose TG stores coupled with elevated circulating free fatty acids. Such abnormalities are associated with insulin resistance; highlighting the importance regulating lipolysis.

In adipocytes, TG is stored in the form of cytosolic lipid droplets. Lipolysis of these lipid droplets requires the enzymes adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL). Three consecutive steps convert TG into glycerol and free fatty acids, the first step involves the breakdown of TG by ATGL into diacylglycerol (DAG), followed by the breakdown of DAG by HSL to monoacylglycerol (MG) and subsequently monoacylglycerol lipase to hydrolyse MG (Chaves, Frasson et al. 2011)

In the fed state there is a necessity to store lipids and the most potent anti-lipolytic hormone is insulin. The effect of inhibiting lipolysis by insulin is achieved through insulin signalling through the

IRS-1 receptor activating phosphatidylinositol-3-kinase and phosphodiesterase 3B, which hydrolyses cAMP to AMP and loss of cAMP inhibits lipolysis. Alternatively, the activation of protein phosphatase-1 dephosphorylates HSL rendering it inactive and inhibits lipolysis (Zechner, Zimmermann et al. 2012).

Adiponectin is an adipokine which possesses insulin sensitising effects promoting energy storage. Studies in murine adipocytes demonstrate the ability of adiponectin to inhibit lipolysis through the inhibition of PKA mediated activation of HSL (Qiao, Kinney et al. 2011). Anti-lipolytic receptors identified on human fat cells include A1-adenosine receptors, EP3-prostaglandin E2 receptors and neuropeptide Y/peptide YY receptors suggest that other hormones are also involved in exerting anti-lipolytic activity (Chaves, Frasson et al. 2011, Lampidonis, Rogdakis et al. 2011).

Lipolysis is tightly regulated by the opposing action of catecholamines and insulin. Fat mobilisation is stimulated by catecholamines with the activation of β-adrenergic receptors coupled to Gαs proteins, the resulting increase in cyclic AMP, activation of protein kinase A (PKA) and phosphorylation of HSL. Activated HSL translocates from the cytoplasm to lipid droplets and catalyses lipolysis (Ahmadian, Wang et al. 2010).

Leptin is capable of promoting the lipolysis of TG, through its leptin subtype B receptor (OB-Rb) activating the protein JAK2, leading to tyrosine receptor autophosphorylation. This phosphorylated domain of the receptor provides a binding site for STAT proteins which translocate to the nucleus, induces an upregulation of ATGL mRNA and protein, and therefore lipolysis (Chaves, Frasson et al. 2011).

Other hormones stimulate lipolysis in a similar manner to catecholamines. Atrial-natriuretic peptide (ANP) and B-type natriuretic peptide stimulate lipolysis through a cyclic GMP and protein kinase G pathway act on type A natriuretic peptide receptor which leads to the phosphorylation of HSL and lipolysis (Lafontan 2008). TNF-α released locally from adipocytes has been demonstrated to induce lipolysis in an autocrine manner.

Whilst under tight regulatory control, elevated free fatty acid release from adipocytes can occur indisease states such as cardiovascular disease, diabetes(Bjorntorp 1990), and in ageing individuals(Pickart 1983), and increased circulating FFA concentrations are considered significant have pathological consequences.

1.9 Fatty acids, insulin resistance and cardiovascular disease

Free fatty acids themselves have been suggested to bind to toll like receptors and induce signalling pathways in a range of cells (Fessler, Rudel et al. 2009) including macrophages, monocytes, endothelial cells and skeletal muscle cells. Palmitate, being the most prevalent saturated fatty acid, has been demonstrated to induce insulin resistance in a wide range of cells. Elevated release of palmitate associated with obesity, leads to adipocyte dysfunction primarily through the induction of apoptotic pathways via oxidative stress, endoplasmic reticulum stress and generation of the complex lipid ceramide with protein kinase C activation (Ajuwon and Spurlock 2005, Guo, Wong et al. 2007, Takahashi, Yamaguchi et al. 2008). Insulin resistance is another feature of palmitate's effect on adipocytes and may be due to a reduction in the levels of adiponectin, an insulin sensitising adipokine(Kim, De Wall et al. 2007, Xi, Qian et al. 2007).

Different mechanisms have been postulated for palmitate mediated induction of insulin resistance with the formation of complex lipids e.g. ceramide and diacylglycerol (DAG) and modulation of PGC-1α possibly being involved. An overview of insulin signalling is shown in figure 1.4.

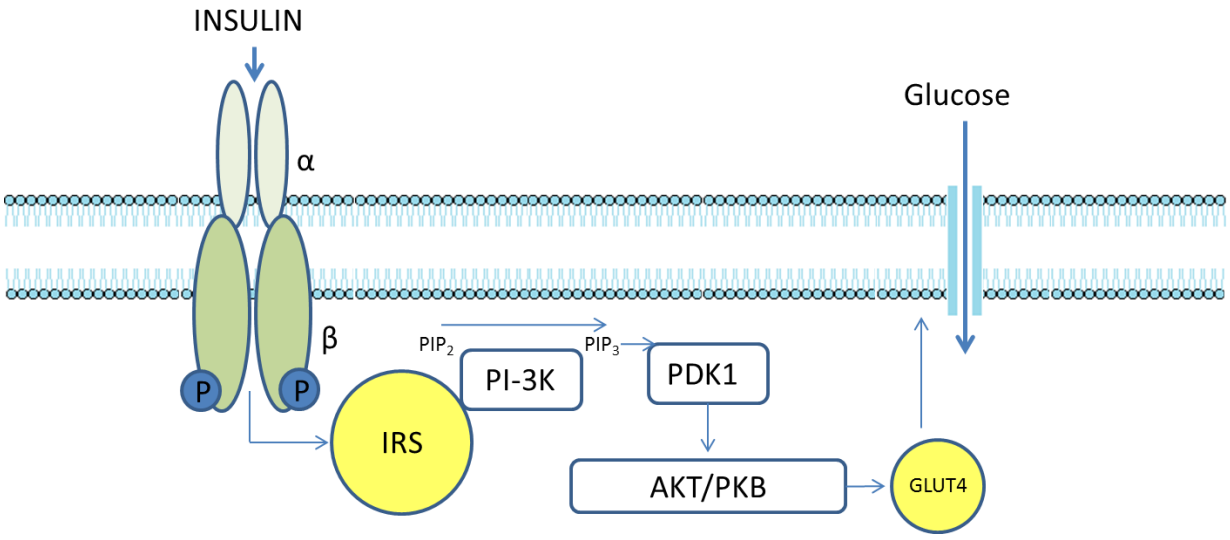


Figure 1.5: Insulin signalling pathway:

Insulin signalling is initiated with the binding of insulin to the α subunits of the insulin receptor, which induces the phosphorylating activity of the β-subunits, inducing their autophosphorylation at tyrosine residues and on other associated substrates including the insulin receptor substrate (IRS) family an Shc. These proteins interact with effector or adaptor proteins containing a Src homology 2 domain (SH2). The activated IRS proteins interact with the p85 subunit of PI-3K which generates phosphatidylinositol 3, 4, 5-triphosphate (PIP_3). The increase in PIP_3 activates PDK1 which in turn phosphorylates and activates AKT/PKB, which leads to GLUT4 translocation to the membrane and increased glucose transport.

Saturated fatty acids which are not metabolised are stored as triglycerides or utilised in the production of ceramides, the former pathway leads to DAG accumulation which is an intermediate in TG formation (section 1.3). In skeletal muscle of high fat diet fed rats(Yu, Chen et al. 2002), diabetic GK rats (Chibalin, Leng et al. 2008), and insulin resistant humans (Itani, Ruderman et al. 2002)significant increases in DAG are observed. In murine models fed a high fat diet rich in SFA there is evidence of increased macrophage tissue infiltration which is known to impair insulin sensitivity (Reynoso, Salgado et al. 2003). In mice, a high fat diet induced increases in adipose tissue mRNA and plasma levels of MCP-1, which lead to increased macrophage infiltration, and subsequently insulin resistance(Kanda, Tateya et al. 2006). Lumeng et al. characterised the behaviour of these infiltratingmacrophages demonstrating a unique profile expressing high levels of IL-6(Lumeng, DeYoung et al. 2007), a pro-inflammatory cytokine known to induce insulin resistance in adipocytes (Xu, Barnes et al. 2003).

The accumulation of DAG in the adipocytes leads to the activation of protein kinase C θ (PKC-θ) which desensitises adipocytes to insulin stimulation (Lobo and Bernlohr 2007, Su and Abumrad 2009). Activation of PKC-θ leads to the activation of the I-kappa-B kinase (IKK) and c-Jun N-terminal kinase pathways (JNK) pathways, resulting in the serine phosphorylation and degradation of the IRS-1 complex, with a secondary effect of pro-inflammatory cytokine induction (Gao, Zhang et al. 2004).

The elevation of intracellular DAG leads to the activation of the many isoforms of protein kinase C (PKC) such as PKCα, PKCδ, PKCθ, PKCε and PKCβ1 (Samuel, Petersen et al. 2010), although only PKCθ and PKCε are consistently linked to insulin resistance (Idris, Gray et al. 2001). DAG activated PKC isoforms down-regulate insulin signalling through PKCθ phosphorylation of IRS-1 serine residues Ser301 (Samuel, Petersen et al. 2010), Ser302 and Ser307 (Werner, Lee et al. 2004). Their activation is mediated through the upstream activation of stress kinases IκBα kinaseβ (IKKβ) and c-Jun NH2-terminal kinase (JNK), or can directly phosphorylate serine residues Ser1101 and Ser307 (Yu, Chen et al. 2002, Werner, Lee et al. 2004). PKCε mediates serine phosphorylation at Ser636/639 of IRS-1 (Mack, Ziv et al. 2008) in addition to direct association with insulin receptor which impairs its kinase activity (Ikeda, Olsen et al. 2001).

The production of ceramides and sphingolipids is initiated by the rate limiting step of condensing palmitate with serine catalysed by SPT1. In insulin-resistant animals (Turinsky, Osullivan et al. 1990, Powell, Turban et al. 2004), lipids-infused humans (Straczkowski, Kowalska et al. 2004), type 2 diabetics (Haus, Kashyap et al. 2009)and mouse models of SFA diets (Frangioudakis, Garrard et al.

2010) ceramides accumulate within skeletal muscle cells. Ceramides are now believed to modulate activity of protein kinase B/Akt. In a variety of cells such as adipocytes, smooth muscle cells, skeletal cells mammary cells and nerve cells, defects in protein kinase/Akt pathway have been induced by ceramides (Holland and Summers 2008). In fibroblasts it has been demonstrated that Akt/PKB translocation to the membrane is blocked (Stratford, DeWald et al. 2001), which in pericytes and smooth muscle cells is due to the activation of PKCζ (Bourbon, Yun et al. 2000), with similar findings in smooth muscle cells (Fox, Houck et al. 2007). PKCζ associates with and phosphorylates the PH domain of PKB/Akt on a Thr34/Ser34 residue preventing translocation to the cell membrane where it would normally be activated in response to insulin (Powell, Hajduch et al. 2003, Powell, Turban et al. 2004) in muscle cells, which has been demonstrated in rat aorta vascular smooth muscle cells (Fox, Houck et al. 2007). Dephosphorylation of PKB/Akt is increased by ceramides and is mediated through the ceramide dependent activation of protein phosphatase 2A in myoblasts (Chavez, Knotts et al. 2003), nerve cells (Salinas, Lopez-Valdaliso et al. 2000), brown adipocytes (Teruel, Hernandez et al. 2001) and in astrocyte cells (Zinda, Vlahos et al. 2001).

Ceramides promote retention of PKCζ and PKB/Akt in caveolin rich microdomains adjacent to the cell membrane. Such domains are typically enriched with ceramides, cholesterol and sphingolipids (Fox, Houck et al. 2007). Caveolae, and therefore microdomains, are particularly abundant in adipocytes, endothelial cells and muscle cells, thus it is plausible to suggest segregation of PKB/PKCζ in response to elevated ceramide can impair insulin signalling, demonstrated in both adipocytes and L6 myotubes (Hajduch, Turban et al. 2008).

An alternate mechanism for the induction of insulin resistance in skeletal muscle is through a reduction in the activity of PGC-1α (Coll, Jove et al. 2006), mediated through an increased p38 mitogen activate protein kinase (p38 MAPK) (Crunkhorn, Dearie et al. 2007). PGC-1α is responsible for induction of mitochondrial gene expression with a subsequent increase in oxidative phosphorylation and insulin stimulated glucose uptake (Handschin and Spiegelman 2006) in tissues such as skeletal muscle, liver, adipose and the heart. The metabolites of palmitate, DAG and ceramide appear to be the mediators of insulin resistance in skeletal muscle, with an increase in both associated with insulin resistance.

Associated with the increase in intracellular SFA in tissues is accelerated β-oxidation, the elevated flux of FFA through the mitochondria results in an increase in co-factors such as NADH/$FADH_2$ passing through the electron transport chain. This results in an increase in ROS production, which in itself is

described to induce insulin resistance in adipocytes (Lin, Berg et al. 2005). An insulin resistant state (Urakawa, Katsuki et al. 2003) associates with elevated oxidative stress, and the elevation in ROS is inversely associated with glycaemic control (NouroozZadeh, Rahimi et al. 1997), with elevated superoxide anion radical production observed in the skeletal muscle of insulin resistant animals (Shiuchi, Iwai et al. 2004).

In adipocytes and hepatoma cells, exposure to hydrogen peroxide reduced the IRS-1 protein content (Potashnik, Bloch-Damti et al. 2003), whilst exposure of vascular smooth muscle cells to angiotensin led to enhanced IRS-1 degradation, which could be ameliorated by catalase or anti-oxidants (Taniyama, Hitomi et al. 2005). In hepatoma cells, oxidative stress generated by hydrogen peroxide resulted in the serine phosphorylation at sites Ser307 and Ser632 on IRS-1, inhibiting receptor kinase activity (Bloch-Damti, Potashnik et al. 2006). Oxidants can affect the signal transduction following binding of insulin to IRS-1, with impairment in signal transduction from PI-3kinase to PKB, with disruption of spatial organisation, direct modification of insulin receptor proteins and alterations in their gene expression by oxidants also reported (Bashan, Kovsan et al. 2009).

Additionally, crosstalk between adipocytes and myotubes may contribute to skeletal muscle insulin resistance. An elegant study by Eckel et al.demonstrated that treatment of human skeletal muscle cells with adipocyte-conditioned medium decreased Akt activity, glycogen synthase phosphorylation and insulin-dependent glucose transporter 4 translocation, all of which could be rescued by adiponectin supplementation (Dietze-Schroeder, Sell et al. 2005). Sell et al.. demonstrated that monocyte chemoattractant protein 1 secreted from adipocytes were responsible for the diminished insulin signalling and glucose uptake by muscles (Sell, Dietze-Schroeder et al. 2006). In mice, a high fat diet reduced adiponectin levels (Zhou, Du et al. 2007) with the increase in FA downregulating insulin signalling and Akt signalling. Beyond adipocytes and myocytes, induction of insulin resistance by SFA has been observed in diverse array of cells including hepatocytes (Nakamura, Takamura et al. 2009, Achard and Laybutt 2012), endothelial cells (Lu, Qian et al. 2013, Zhang, Gao et al. 2013), myocardial cells (Nobuhara, Saotome et al. 2013), monocytes (Gao, Griffiths et al. 2009) and pancreatic cells (Watson, Macrae et al. 2011).

In contrast, the MUFA oleate does not impair insulin sensitivity (Hunnicutt, Hardy et al. 1994, Bastie, Hajri et al. 2004, Yuzefovych, Wilson et al. 2010) and may be beneficial for insulin sensitivity (Gao, Griffiths et al. 2009). Papackova et al.. adopted a high fat feeding study to demonstrate that a high MUFA diet enhances the alternative activation of resident hepatic tissue macrophages, Kupffer cells.

Kupffer cells mediate beneficial effects on insulin sensitivity (Papackova, Palenickova et al. 2012) by promoting the formation of anti-inflammatory, alternatively-activated macrophages which alleviated indices of insulin resistance. Hypertension, a CVD risk factor has been shown to be influenced by MUFA, with oleate decreasing total blood pressure (Ferrara, Raimondi et al. 2000) whilst replacing carbohydrates in the diet with MUFA provided similar benefits (Muzio, Mondazzi et al. 2007, Shah, Adams-Huet et al. 2007).

Saturated fatty acids have been consistently linked to elevated cardiovascular disease risk. From a dietary perspective, low SFA diets have been associated with reduced progression of coronary atherosclerosis (Sacks and Katan 2002, Griel and Kris-Etherton 2006). In a prospective cohort study SFA consumption was associated with elevated coronary heart disease (Jakobsen, O'Reilly et al. 2009) and epidemiological studies showed that of those with diets high in SFA intake provided associations with CHD (Mente, de Koning et al. 2009, Skeaff and Miller 2009, Siri-Tarino, Sun et al. 2010).

Circulating saturated fatty acids have been negatively associated with endothelial function index (Sarabi, Vessby et al. 2001, Lind, Sodergren et al. 2002), and pulse pressure (Schutte, Van Rooyen et al. 2003). In humans, dietary saturated fatty acids lead to a pro-atherogenic lipid profile, mainly via an increase in LDL cholesterol (Siri-Tarino, Sun et al. 2010) mediated by an inhibition of cholesterol receptor activity and an increase in apolipoprotein B containing lipoprotein production (Dietschy 1998).

Moreover dietary MUFA can reduce risk for cardiovascular disease by improving factors associated with the metabolic syndrome (Garg 1998, Kris-Etherton and Nutr 1999, Kris-Etherton and Nutrition 1999, Ros 2003); replacing SFA intake with that of MUFA improved the lipid profile, with an observed decrease in LDL cholesterol whilst maintaining the levels of HDL cholesterol (Hunter, Zhang et al. 2010).Others confirmed the reduction in LDL cholesterol in addition to reductions in non-HDL cholesterol and apolipoprotein B (Allman-Farinelli, Gomes et al. 2005, Appel, Sacks et al. 2005, Berglund, Lefevre et al. 2007) with increasing dietary MUFA.

Thus, beyond the physical differences between SFA and MUFA, the two fatty acid classes possess differential effects on insulin resistance and cardiovascular disease risk. Insulin resistance is mediated by SFA through generation of complex lipids DAG and ceramide which in turn lead to the activation of IKK and JNK pathways, with subsequent serine phosphorylation and degradation of IRS-1; in contrast the MUFA appear to have neutral or insulin sensitising effects. With respect to

cardiovascular disease risk SFA, in addition to the induction of insulin resistance, appear to generate a pro-atherogenic profile by increasing LDL cholesterol; whilst the reduction in cardiovascular disease risk maybe mediated by MUFA involves improvement in the lipid profile in addition to improvements in risk factors associated with the metabolic syndrome.

1.10 Demography and epidemiology of CVD and ageing

Age is the strongest predictor of CVD. As the population continues to grow due to the population living longer the incidence of CVD is likely to increase. The age-related deterioration in health has been attributed to a number of changes such as oxidative stress due to glutathione depletion, telomere shortening and senescence (Jiang, Ju et al. 2007, Liochev 2013). The evidence suggests that a big influence in determining age-related changes is the metabolic environment of the body.

1.11 Ageing metabolism

Ageing is associated with a host of physiological changes which results in deterioration of organism function over time. Of the phenotypic changes observed with ageing, one of the most visually apparent is the age-related change in body composition. The ageing phenotype is associated with a decrease in fat free mass i.e. sarcopenia (Sakuma and Yamaguchi 2010, Sakuma and Yamaguchi 2012, Sakuma and Yamaguchi 2012, Sakuma and Yamaguchi 2012), coupled with both an altered adipose tissue distribution(Kuk, Saunders et al. 2009) and increase in fat mass (Kuk, Lee et al. 2004, Kuk, Lee et al. 2005) and a redistribution of these tissues is observed with enhanced central and visceral adiposity. The observed age-related changes in body composition vary with ethnicity (Kuk, Saunders et al. 2009). Furthermore, the increases in fat mass occurs independent of total body weight change (Gallagher, Ruts et al. 2000, Zamboni, Zoico et al. 2003, Kuk, Saunders et al. 2009), which hides the true adiposity levels in older adults. Theage-related increase in central adiposity can be determined anthropometrically by measurement of waist circumference (Shimokata, Tobin et al. 1989, Carmelli, McElroy et al. 1991, Stevens, Knapp et al. 1991, Teh, Pan et al. 1996, Woo, Kwok et al. 1997, Zamboni, Zoico et al. 2003), and is not due to increased abdominal subcutaneous adipose deposition (Fuke, Okabe et al. 2005, Machann, Thamer et al. 2005).

The lower body and subcutaneous adipose stores have a reduced capacity to store fat during ageing, which has been demonstrated anthropometrically as reduced hip circumference and increasing waist to hip ratios (Enzi, Gasparo et al. 1986, Kotani, Tokunaga et al. 1994, Panotopoulos, Ruiz et al. 1996, Ishida, Kanehisa et al. 1997, Murakami, Arai et al. 1997, Hughes, Roubenoff et al. 2004, Van Pelt, Jankowski et al. 2011).

Ectopic adiposity is another feature of ageing. In skeletal muscle an age-related increase in both inter-muscular (Ryan and Nicklas 1999, Song, Ruts et al. 2004, Miljkovic, Cauley et al. 2009) and intra-muscular (Ryan and Nicklas 1999, Cree, Newcomer et al. 2004, Nakagawa, Hattori et al. 2007, Schwenzer, Martirosian et al. 2009) fat has been described.

Reports of age-related elevations in hepatic adiposity are somewhat mixed. Some studies observed an increase (Akahoshi, Amasaki et al. 2001, Cree, Newcomer et al. 2004, Fan, Zhu et al. 2005, Machann, Thamer et al. 2005, Frith, Day et al. 2009, Li, Wang et al. 2009) others do not find a change (Mahmood, Taketa et al. 1998, Seppala-Lindroos, Vehkavaara et al. 2002, Tiikkainen, Tamminen et al. 2002, Bedogni, Bellentani et al. 2006) and in some cases a negative correlation with age were found (Bedogni, Miglioli et al. 2005, Zhou, Li et al. 2007).

Studies of age-related fat deposition in cardiac tissues have shown increases in epicardial adipose tissue which resides between the myocardium and pericardium surrounding the heart (Silaghi, Piercecchi-Marti et al. 2008, Ueno, Anzai et al. 2009) and also increased fat deposition within myocardial muscle (van der Meer, Rijzewijk et al. 2008).

The age-related ectopic adiposity of ageing extends into the bone marrow (Yamada, Matsuzaka et al. 1995, Kugel, Jung et al. 1999, Jung, Kugel et al. 2000, Schellinger, Lin et al. 2000, Justesen, Stenderup et al. 2001, Brown and Rosen 2003, Yeung, Griffith et al. 2005, Rosen and Bouxsein 2006, Liney, Bernard et al. 2007) often associated with osteoporosis.

1.12 Impact of the ageing phenotype on muscle and effects on insulin sensitivity

The age-related ectopic fat redistribution has implications for the development of metabolic diseases. In lean tissues the enhanced intermuscular deposition of fat may negatively impact muscle strength and power, a common observation in diabetics and the obese (Hilton, Tuttle et al. 2008). In the elderly the enhanced adiposity of lean tissues has been observed to negatively affect mobility, muscle strength and consequently physical activity (Marcus, Addison et al. 2010). Gains in intramuscular adiposity with age are observed to reduce the improvements in muscle quality following exercise training (Marcus, Addison et al. 2013).

During obesity within intermuscular adipose tissue there is an enrichment of microRNAs linked with inflammation and immune responses, suggesting adipose deposition in muscle may be a metabolic risk factor in obesity (Ma, Yu et al. 2013). Skeletal muscle is a major site of glucose uptake and insulin action and as a consequence is the primary influence over global insulin sensitivity; intermolecular fat deposition increases insulin resistance (Lim, Son et al. 2009) in rodents and

humans (Kelley, Slasky et al. 1991, Phillips, Caddy et al. 1996, Pan, Lillioja et al. 1997, Russell, Shillabeer et al. 1998, Forouhi, Jenkinson et al. 1999, Jacob, Machann et al. 1999, Krssak, Petersen et al. 1999, Goodpaster, Theriault et al. 2000, Bachmann, Dahl et al. 2001, Greco, Mingrone et al. 2002, Sinha, Dufour et al. 2002, Gray, Tanner et al. 2003, Cree, Newcomer et al. 2004, Perseghin, Lattuada et al. 2008, Lim, Son et al. 2009). Only Stannard et al.(Stannard, Holdaway et al. 2007), suggested that elevated lipid storage as TG in muscle did not promotes insulin resistance in healthy Maori men.

Bonen et al. observed in diabetic and obese subjects, that TG accumulation is associated with changes in FAT/CD36 expression accompanied by altered FA transport which favours lipid storage in myocytes (Bonen, Parolin et al. 2004). Following a one week high fat diet study in human subjects Schrauwen-Hinderling et al. demonstrated an increase in lipid deposition with a reduction in β-oxidation (Schrauwen-Hinderling, Kooi et al. 2005), and attenuation of the insulin-mediated suppression of pyruvate dehydrogenase kinase (PDK4) (Tsintzas, Chokkalingam et al. 2007) is also predicted to increase lipid deposition.

Whilst associations between intramyocellular TG accumulation and insulin resistance are commonplace, further investigation indicated that a different lipid or effector is responsible. Prolonged insulin infusion can raise intramuscular triglycerides without affecting insulin sensitivity in type 2 diabetics (Anderwald, Bernroider et al. 2002), and in trained athletes elevated triglycerides in muscle is common despite high insulin sensitivity (Goodpaster, He et al. 2001, Thamer, Machann et al. 2003, van Loon and Goodpaster 2006, Dube, Amati et al. 2008).

Such findings suggest that other lipid mediators or effectors mediate insulin resistance e.g. long chain fatty acyl-CoAs and diacylglycerol (DAG), both precursors in the synthesis of triglycerides and the neutral lipid ceramide.

Long chain fatty acyl-CoA (LCFA-CoA) is a metabolically activated form of long chain fatty acids, which may act as a signalling molecule. LCFA-CoA activates AMP kinase and the transcription factor FadR which is involved in expression of fatty acid synthesis and metabolism genes in *E. coli*(Faergeman and Knudsen 1997) and a role for LCFA-CoA has been proposed in mammalian pancreatic β-cells (Prentki, Tornheim et al. 1997, Corkey, Deeney et al. 2000) . Elevations of LCFA-CoA in skeletal muscle of insulin resistant animals (Chen, Kaufman et al. 1992, Oakes, Bell et al. 1997, Laybutt, Schmitz-Peiffer et al. 1999, Kim, Fillmore et al. 2001) and humans (Ellis, Poynten et al. 2000) are associated with elevated myocellular lipid. Furthermore, obese individuals who have undergone weight loss improving insulin sensitivity have lower muscle LCFA-CoA content and improved insulin sensitivity (Houmard, Tanner et al. 2002).

LCFA-CoA mediated insulin resistance maybe through inhibition of hexokinase activity, which as the first step in glucose utilisation will reduce the effect of insulin (Thompson and Cooney 2000). Alternatively LCFA-CoA may activate muscle protein kinase C isoforms, a serine threonine kinase, which upon activation will phosphorylate serine Ser307 of the insulin receptor substrate 1 (IRS-1) preventing the tyrosine phosphorylation of the insulin receptor, and subsequent binding and activation of PI-3 kinase (Tanti, Gremeaux et al. 1994, Itani, Ruderman et al. 2002, Yu, Chen et al. 2002) necessary for normal insulin signalling (Orellana, Hidalgo et al. 1990, Nesher and Boneh 1994, Yaney, Korchak et al. 2000).

Disruption of insulin signalling and sensitivity may involve (DAG), and in insulin resistant states DAG levels are elevated in skeletal muscle and adipocytes (Heydrick, Ruderman et al. 1991, Cooper, Watson et al. 1993, Saha, Kurowski et al. 1994, Avignon, Standaert et al. 1995, SchmitzPeiffer, Browne et al. 1997, Qu, Seale et al. 1999). The mechanism of DAG mediated insulin resistance may be due to activation of PKC isoforms e.g. PKC-θ and –ε (Griffin, Marcucci et al. 1999, Laybutt, Schmitz-Peiffer et al. 1999, Qu, Seale et al. 1999, Yu, Chen et al. 2002) with effects as described previously. In obese diabetics PKC-θ activity has been associated with elevated DAG mass (Itani, Pories et al. 2001, Itani, Ruderman et al. 2002).

Ceramides are a neutral lipid which require LCFA-CoA, specifically palmitoyl-CoA, and serine as precursor molecules for their synthesis. In situations where elevated intramyocellular lipid content is expected such as obesity and diabetes, simultaneous increases in ceramide have been observed in muscle which is associated with insulin resistance (Adams, Pratipanawatr et al. 2004, Coen, Dube et al. 2010, Coen, Hames et al. 2013). Conversely exercise training in the obese or overweight improved insulin sensitivity with concomitant reductions in both ceramide and DAG content (Bruce, Thrush et al. 2006, Dube, Amati et al. 2008). Similarly, in sedentary subjects and endurance athletes, the obese subjects reported higher ceramide in the latter correlating with insulin resistance (Amati, Dube et al. 2011).

In contrast to these findings, Skovbro et al. demonstrated that in individuals with type-2 diabetes, endurance trained athletes, healthy controls and those with impaired glucose tolerance had similar levels of muscle ceramide content despite varied insulin sensitivity (Skovbro, Baranowski et al. 2008). The manner in which ceramides induce insulin resistance is believed to be reliant upon the activation of PKC-ζ through phosphorylation of the IRS-1 complex and protein phosphatase 2A which dephosphorylates the Akt/PKB complex at a separate site preventing downstream effector function (Chavez and Summers 2012).

The accumulation of intramyocellular lipids is detrimental for mitochondrial function in people with insulin resistant states (Kelley and Mandarino 2000, Petersen, Dufour et al. 2004) and the elderly (Petersen, Befroy et al. 2003), leading to a reduction in lipid oxidation and to elevated lipid deposition. Furthermore, the associated hyperglycaemia may increase malonyl-CoA, an endogenous inhibitor of carnitine palmitoyl transferase-1, subsequently increasing intramyocellular lipid deposition and insulin resistance (Krebs and Roden 2005). Indeed Yim et al. demonstrated that intermuscular adipose tissue was as strong an independent risk factor for cardiovascular disease as visceral adipose tissue fat (Yim, Heshka et al. 2007). These findings suggest that elevated muscle adiposity, as observed in ageing, are associated with negative metabolic effects such as insulin resistance and enhanced cardiovascular disease risk.

In the elderly population, intramuscular lipid content has been associated with the metabolic syndrome (Goodpaster, Krishnaswami et al. 2005), furthermore weight loss in elderly women led to reductions in low density lean tissue (adipose tissue in muscle) and was accompanied by improvements in insulin resistance (Mazzali, Di Francesco et al. 2006). Whilst data examining the link between muscle adiposity in the elderly and adverse metabolic changes is scarce, the effect of age per se on muscle metabolism has been examined in animal models in which and decreased Glut4 mRNA levels (Lin, Asano et al. 1991), decreased Glut4 protein (Oka, Asano et al. 1992, Houmard, Weidner et al. 1995) and reduced glucose oxidation (Gumbiner, Thorburn et al. 1992) was observed.

1.13 Impact of ageing on hepatic lipid deposition

There is a very strong correlation between metabolic syndrome and the subsequent development of non-alcoholic fatty liver disease, with a reciprocal relationship of hepatic steatosis being predictive for the development of metabolic syndrome with age (Fan, Zhu et al. 2005, Lazo and Clark 2008, Fan and Farrell 2009, Liu, Hung et al. 2010).Conversely, the age-related elevation in hepatic adiposity and insulin resistance can cause liver dysfunction. Fatty liver is associated with reduced insulin sensitivity and insulin resistance (Utzschneider and Kahn 2006, Takamura, Misu et al. 2012, Gaggini, Morelli et al. 2013).

Investigations into the causes or effects of fatty liver in the elderly are limited. A study by Flannery et al. indicated that skeletal muscle insulin resistance mediates the hepatic steatosis in the elderly through promotion of hepatic de novo lipogenesis and hyperlipidaemia (Flannery, Dufour et al. 2012). In a study of middle aged and elderly Chinese individuals, liver fat content was closely associated to carotid atherosclerosis (Li, Xia et al. 2012). Previously, Kagansky et al. described the

prevalence of non-alcoholic fatty liver disease in the elderly that was not associated with metabolic syndrome (Kagansky, Levy et al. 2004) suggesting that NAFLD is a common occurrence in the elderly.

1.14 Altered metabolic outcome associated with age-related redistribution of adipose depots

The age-related redistribution of adipose away from lower body and subcutaneous depots to central, visceral and ectopic sites in close proximity to the heart can increase diabetes, metabolic syndrome cardiovascular disease risk.

Age-related elevations in visceral adiposity were moderately linked to the decrease in insulin sensitivity, with unfavourable changes in lipid profile i.e. increased total cholesterol and LDL in non-obese women which may enhance cardiovascular disease risk (DeNino, Tchernof et al. 2001). Further associations have been described in elderly individuals between visceral adiposity and metabolic risk factors such as aortic stiffness (Sutton-Tyrrell, Newman et al. 2001), myocardial infarction (Nicklas, Penninx et al. 2004, Wannamethee, Shaper et al. 2005, Racette, Evans et al. 2006, Hansen, Ahlstrom et al. 2009), chronic heart failure (Nicklas, Cesari et al. 2006). However,elderly adults, central adiposity did not predict atherosclerotic plaque severity (Kim, Bergstrom et al. 2008).

Whilst the elevated risk of developing metabolic complications such as cardiovascular disease and diabetes is strongly associated with visceral adipose tissue, the inverse is true of lower body and subcutaneous adiposity. Lower body adiposity or gynoid adiposity is associated with reduced cardiovascular disease, or metabolic risk factors, in a range of populations including type 2 diabetics (Park, Cho et al. 2012), older individuals(Goodpaster, Krishnaswami et al. 2005, Snijder, Visser et al. 2005, Van Pelt, Jankowski et al. 2005, Aasen, Fagertun et al. 2008, Faloia, Tirabassi et al. 2009, Saunders, Davidson et al. 2009, Wu, Qi et al. 2010, Van Pelt, Jankowski et al. 2011, Fantin, Rossi et al. 2013), men (Kahn, Austin et al. 1996, Ferreira, Snijder et al. 2004, Snijder, Zimmet et al. 2004, Snijder, Zimmet et al. 2004, Boorsma, Snijder et al. 2008, Mason, Craig et al. 2008, Yim, Heshka et al. 2008, Heitmann and Frederiksen 2009, Hu, Bouchard et al. 2011, Lee, Choh et al. 2012), and women(Kahn, Austin et al. 1996, Ferreira, Snijder et al. 2004, Hara, Saikawa et al. 2004, Sakai, Ito et al. 2005, Mason, Craig et al. 2008, Aasen, Fagertun et al. 2009, Heitmann and Frederiksen 2009, Lee, Choh et al. 2012). Similarly, healthy, overweight, and obese individuals show a positive association between thigh fat and insulin sensitivity (McLaughlin, Lamendola et al. 2011), and on the other hand increased visceral adiposity correlated with reduced lower body and gynoidal adiposity (Lee, Shin et al. 2012), with diabetes have also been observed (Snijder, Dekker et al. 2003, Heshka, Ruggiero et al. 2008, Li, Ford et al. 2010, Shay, Secrest et al. 2010), (Snijder, Dekker et al. 2003, Snijder, Zimmet et al. 2004, Shay, Secrest et al. 2010).

1.15 Altered adipocyte function in ageing

Beyond these key differences, adipocyte function and the processes which govern their maturation from stem cells appear to be altered during of ageing. Adipogenesis is reported to be accelerated in a murine model of ageing using the senescence accelerated mice-P6 (SAMP6) (Kajkenova, LeckaCzernik et al. 1997), and a similar finding was observed also in human subjects biopsies (Justesen, Stenderup et al. 2001). Increased adipogenesis has been linked to age-relatedincrease in oxidative stress, low levels of vitamin D and loss of estrogen during menopause (Bethel, Chitteti et al. 2013). However a decline in adipose depot size which is due to a reduction in cell size (Kirkland, tchkonia 2002) and impaired differentiation from preadipocytes to a mature adipocyte.

There is also an age-related increase in lipotoxicity towards a range of cells due to the reduced capacity of adipose depots to store free fatty acids (Slawik and Vidal-Puig 2006). The underlying cause of this age associated elevation of FFA can be explained by altered maturation process of adipocytes. Replication and maturation into adipocytes depends upon the activity of CCAAT/enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated receptor-γ (PPAR-γ) (Wu, Bucher et al. 1996, Wu, Rosen et al. 1999, Zuo, Qiang et al. 2006) which are diminished in ageing (Kirkland, Hollenberg et al. 1990, Kirkland and Dobson 1997, Karagiannides, Tchkonia et al. 2001, Karagiannides, Thomou et al. 2006, Cartwright, Tchkonia et al. 2007). The process of differentiation and maturation of preadipocyte into an adipocyte depends upon the activity of CCAAT/enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated receptor-γ (PPAR-γ) (Wu, Bucher et al. 1996, Wu, Rosen et al. 1999, Zuo, Qiang et al. 2006). The process is initiated with the transient expression of C/EBP isoforms β and δ. Simultaneously there is an alleviation of adipogenic suppression by proteins such as C/EBP-β-LIP and CHOP. In ageing rat preadipocytes, C/EBP-α, C/EBP-δ and PPAR-γ expression is reduced compared to young rats with increases in the suppressors C/EBP-β-LIP and CHOP (Karagiannides, Tchkonia et al. 2001, Karagiannides, Thomou et al. 2006).

Moreover, age is negatively correlated to subcutaneous stromal preadipocyte cell differentiation (van Harmelen, Rohrig et al. 2004) possibly due to a loss in nucleotide excision repair enzymes Cockayne syndrome A and B, enhanced mutation rate and subsequent cell loss by apoptosis diminishing depot size (Berneburg 2010). The changes in maturation and differentiation of both the visceral and subcutaneous adipose depots, i.e. the reduced capacity to store lipids, led to an elevation in systemic FFA with age.

1.16 Metabolic health outcomes associated with elevated systemic FFA

In obesity, enhanced visceral adiposity is associated with greater lipolysis and consequently greater circulating FFA, whilst in the ageing phenotype diminished maturation of preadipocyte and increased levels of aberrant preadipocytes reduce the capacity to store FFA, therefore increased circulating FFA is observed (Cree, Newcomer et al. 2004).

Increased systemic FFA has negative metabolic impacts particularly on organs sensitive to the effects of insulin, for example in gluconeogenesis in hepatic tissues is promoted(Groop, Bonadonna et al. 1989), due to increased substrate availability (Boden, Chen et al. 1994, Lam, Carpentier et al. 2003); which suppresses glycogenolysis (Jenssen, Nurjhan et al. 1990). Whilst true of normal healthy subjects, in diabetic subjects there is a failure of this autoregulation with elevated gluconeogenesis and glycogenolysis (Gastaldelli, Miyazaki et al. 2004) which was related to abdominal adiposity and plasma FFA.

Increased FFA flux into hepatic tissues decreases insulin clearance (Hennes, Shrago et al. 1990) by inhibiting binding to its receptor and degradation of insulin,with subsequent induction of systemic hyperinsulinaemia (Svedberg, Bjorntorp et al. 1990). In association with this finding, loss of insulin suppression of hepatic glucose production (Ferrannini, Barrett et al. 1983, Bevilacqua, Bonadonna et al. 1987) ultimately leads to insulin resistance (Saloranta, Franssilakallunki et al. 1991, Rebrin, Steil et al. 1995).

Increased systemic FFA flux has an impact upon lipid synthesis and turnover within hepatic tissues. High FFA levels delivered to the hepatic tissues will increase their esterification and reduce the rate of apolipoprotein B degradation, with a subsequent increase in synthesis and secretion of small VLDL particles (Havel, Kane et al. 1970, Kissebah, Alfarsi et al. 1976, Kissebah, Alfarsi et al. 1976, Boden 1997); these particles have a reduced ratio of triglycerides to apolipoprotein B and consequently are smaller.

The lipolysis of these particles reduces the size and buoyancy of lipoproteins associated with VLDL particles generated by the liver. The lipoprotein lipase responsible for this process can become saturated under excess FFA conditions, with removal of lipids from HDL and LDL leading to smaller and denser LDL particles; the smaller and denser HDL particles are more rapidly cleared from the circulation lowering HDL-cholesterol levels (Hopkins and Barter 1986, Frenais, Nazih et al. 2001). The increase in TG within apolipoprotein B containing particles will lead to an increase in the transfer of TG for cholesterol esters via the cholesteryl ester transfer protein. The effect of these lipid synthesis

and metabolism changes is that the less dense LDL particles will be cleared through the VLDL pathway, therefore a higher proportion of cholesterol is cleared through the VLDL pathway, with such clearance occurring through atherogenic routes such as macrophages and smooth muscle cells which are a risk factor for cardiovascular disease (Eisenberg 1987). The pro-atherogenic lipid profile of elevated TG, low HDL-cholesterol, and smaller denser LDL particles, coupled with increased VLDL is encountered in insulin resistant subjects (Krauss 2004). The systemic effects of elevated FFA are not limited to hepatic tissues.

Skeletal muscle tissues are the primary site of glucose disposal and their metabolism is altered in the presence of FFA; reduced insulin stimulated peripheral glucose disposal is observed, which in healthy individuals is compensated by the FFA induced increase in glucose stimulated insulin secretion (Boden 1997). Furthermore, under conditions of elevated FFA increased intramyocellular lipid content been correlated to insulin sensitivity in non-obese and non-diabetic subjects (Krssak, Petersen et al. 1999). Further study in 20 healthy individuals revealed that those with increased intramyocellular lipid content had impairments in insulin signalling defined by reduced insulin receptor autophosphorylation and decreased signalling via IRS-1 and PI3-kinase, suggesting the insulin receptor tyrosine kinase signalling pathway is defective under conditions of excess FFA (Virkamaki, Korsheninnikova et al. 2001). These findings suggest elevated systemic FFA is associated with peripheral insulin resistance.

A key site of insulin action and glucose homeostasis is the pancreas. FFA impairs insulin secretion (Lee, Hirose et al. 1994, Zhou and Grill 1995) affecting pancreatic tissues.For example in Zuckef rat hyperglycaemia increased FFA levels and elevated cellular TG content precede aloss in glucose sensing by the β-cells (Mason, Goh et al. 1999).

Atherogenesis by FFA is also attributed to endothelial cell dysfunction which is likely to impede the secretion of vasodilatory factors such as nitric oxide, leading to stiffer major vessels and consequently hypertension. Dysfunctional endothelial cells will increase adhesion molecule expression e.g. ICAM-1 and VCAM-1 which promote the binding of immune cells including monocytes with their subsequent transmigration into the intima and, inflammatory cytokine release e.g. TNF-α and other signalling molecules e.g. monocyte chemo attractant protein 1; the sum effect will be deleterious arterial remodelling and atherosclerotic plaque formation (Jensen 2006).

Evidence for FFA mediated endothelial dysfunction has been determined in human volunteers (de Kreutzenberg, Crepaldi et al. 2000, de Jongh, Serne et al. 2004), where FFA have been shown to

reduce nitric oxide and prostacyclin synthesis (Endresen, Tosti et al. 1994, Kuroda, Hirata et al. 2001, Esenabhalu, Schaeffer et al. 2003), IL-8 synthesis (Suriyaphol, Fenske et al. 2002), promote endothelial cell apoptosis (Artwohl, Roden et al. 2004), reduce endothelial cell proliferation (Su, Tian et al. 2002), increase generation of ROS(Saraswathi, Wu et al. 2004), and accumulate intracellular TG (McCarty 2003).

1.17 Fatty acids and inflammation

In adipocytes, free fatty acid overload saturates the triglyceride synthesis pathway, consequently FFA accumulate in cells and leads to endoplasmic reticulum stress, oxidative stress and adipocyte dysfunction; the secretion of adipokines and cytokines by dysfunctional ageing adipocytes differs from those of healthy cells, with a reduction in adiponectin and increase in pro-inflammatory cytokines e.g. TNF-α, IL-6 and MCP-1 secretion (de Ferranti and Mozaffarian 2008).

The chronic low grade inflammation associated with elevated FFA increases neutrophil and macrophage recruitment to adipose tissue, and propagates the pro-inflammatory state (Elgazar-Carmon, Rudich et al. 2008). Dietary SFA can induce inflammatory cytokine production by adipose tissue (Kien, Bunn et al. 2005) via Toll-like receptor 4, CD14 and MD2, a cell surface glycolipid which interacts with the extracellular domain of TLR to provide greater specificity and responsiveness to lipopolysaccharide (Wright, Ramos et al. 1990, Shimazu, Akashi et al. 1999). Furthermore, the activation of MyD88 and TRIF pathways downstream of TLR4 leads to the activation of the NF-κB pathway (Wong, Kwon et al. 2009). Inflammatory cytokine secretion by macrophages (Laine, Schwartz et al. 2007) is also increased by palmitate.

The evidence for pro-inflammatory effects of SFA *in vitro* is supported by epidemiological data. Fung et al. examined dietary intake and plasma markers of cardiovascular disease, showing that C-reactive protein correlated with saturated fatty acid intake (Fung, Rimm et al. 2001). Furthermore Baer et al. demonstrated in an intervention study that stearic acid enhanced concentrations of acute phase protein reactant fibrinogen (Baer, Judd et al. 2004). A common feature of the metabolic syndrome is a pro-inflammatory state (Cleeman, Grundy et al. 2001), which is promoted by ectopic fat deposition (Montagne and O'Rahilly 2000).

In summary like obesity, ageing in humans and rodents is associated with an elevation in FFA (Bonadonna, Groop et al. 1994, Tessari 2000) that may mediate increased insulin resistance (Karakelides, Irving et al. 2010, Sakurai, Iimuro et al. 2010) and cardiovascular disease risk (Lakatta

2002, Lakatta 2003, Lakatta and Levy 2003, Lakatta and Levy 2003, North and Sinclair 2012). Ageing also associates with a pro-inflammatory state and an impaired immune system.

1.18 Inflammation in ageing

Ageing is associated with a progressive decline in the function of the immune system. In order to effectively provide host defence and immune surveillance, a co-ordinated response of both the innate and adaptive immune systems must be produced. The former comprises three effector mechanisms the phagocytes, the complement system and antimicrobial peptides, and via these mechanisms the immune system is able to repel invading microbial pathogens. Macrophages, eosinophil, monocytes and neutrophils encompass the phagocytic cells which can detect, ingest and kill bacteria through intracellular lipases, proteases, RNS and ROS generation (Auffray, Sieweke et al. 2009). In the resolution of inflammation, monocytes and macrophages are involved in the clearance of apoptotic cells coupled to the secretion of anti-inflammatory mediators such as TGF-β and prostaglandin E2 (Savill and Fadok 2000), with more recent data demonstrating that resolvins and protectins, anti-inflammatory class of eicosanoids, are also produced to aid in the resolution of inflammation (Schwab, Chiang et al. 2007, Lee and Surh 2013). In ageing, neutrophil numbers appear unaffected, but their ability to migrate directionally by chemotaxis appears reduced (Butcher, Chahel et al. 2000), phagocytosis is defective, ROS production is decreased (Gomez, Nomellini et al. 2008) and the rate of spontaneous apoptosis is increased. These changes may contribute to poor innate immunity, which is prevalent with ageing (Solana, Pawelec et al. 2006).

The functions of monocytes and macrophages may also be negatively impacted by age (Gomez, Nomellini et al. 2008). Chemotaxis by monocytes and macrophages was decreased in old mice due to lower macrophage inflammatory protein-1α, -1β and MIP-2 levels(Ortega, Garcia et al. 2000, Swift, Burns et al. 2001). The phagocytic activity of these cells was unaffected and phagocytic receptor expression was maintained (Liang, Domon et al. 2009). However, a reduction in endotoxin responses (Renshaw, Rockwell et al. 2002) and impaired pathogen dependent killing (Hajishengallis 2010) is observed with age despite TLR levels and cell surface expression being unaffected (Boehmer, Goral et al. 2004, Liang, Domon et al. 2009). This suggests that signalling pathways downstream of TLR receptors may be altered with age (Dunston and Griffiths 2010), including MyD88, NF-κB and MAPK.

Secretion of several cytokines including IL-6, TNF-α and IFN-γ is increased with age (Boehmer, Goral et al. 2004, Gomez, Nomellini et al. 2008) whilst production of prostaglandin E_2 (PGE_2) by aged human and mouse macrophages is elevated (Plowden, Renshaw-Hoelscher et al. 2004). Chronic low

grade systemic inflammation is common in ageing (Bartlett, Firth et al. 2012). Inflammageing (Franceschi, Bonafe et al. 2000), describes the systemic low grade increase in the levels of pro-inflammatory cytokines accompanied by a decrease in anti-inflammatory cytokines e.g. IL-10 (Lio, Scola et al. 2002).

1.19 Monocyte function

Monocytes are circulating white blood cells that are recruited to inflammatory sites and are capable of differentiating into macrophages or dendritic cells following stimulation with inflammatory cytokines and/or microbial components. The stages of monocyte recruitment from the blood encompass rolling, adhesion and transmigration.

The first stage of transmigration is the rolling of monocytes along the endothelial cells at the site of inflammation, a process which involves L-selectin expressed on monocytes with the expression of P- and E-selectin on inflamed endothelial cells (Kansas 1996) interacting with reciprocally expressed P-selectin glycoprotein ligand 1 (PSGL1) (McEver and Cummings 1997).

Integrins also function at this stage, between endothelial vascular cell-adhesion molecule 1 (VCAM1) and very late antigen 4 (VLA4) (Berlin, Bargatze et al. 1995), the latter are particularly prominent in monocytes and monocytic cell lines (Huo, Hafezi-Moghadam et al. 2000, Chan, Hyduk et al. 2001).

The β_2-integrin, CD18, is important during the rolling process, with lymphocyte function associated antigen 1 (LFA1; CD11a and CD18) transiently binding to intercellular adhesion molecule 1 (ICAM1) (Salas, Shimaoka et al. 2004, Chesnutt, Smith et al. 2006). Monocytes express CD11b which interacts with CD18 to form MAC1, which engages ICAM-1 on endothelial cells mediating slow rolling (Lo, Lee et al. 1991, Dunne, Ballantyne et al. 2002). Thus CD11b plays the role of mediating a firmer adhesion in comparison to L-selectin, with the importance of this molecule demonstrated by function blocking studies (Smith, Marlin et al. 1989, Issekutz and Issekutz 1992).

The subsequent step is the arrest of rolling and activation of the monocyte. The integrins which are important in slow rolling also function to mediate arrest, namely VLA4 and LFA1. The process is triggered by chemokines and chemoattractants acting upon their respective G-protein coupled receptors, with subsequent signalling triggering the firmer adhesion of β-integrins to endothelial cell ligands. For example platelets deposit CCL5 (RANTES), CXCL4 and CXCL5 onto the inflamed endothelium which trigger the arrest of rolling monocytes (von Hundelshausen, Weber et al. 2001, Huo, Schober et al. 2003). The arrest is mediated by conformational changes to integrins which serve

to increase affinity to adhesion molecules such as VCAM and ICAM (Chan, Hyduk et al. 2001, Chigaev, Zwartz et al. 2003, Kim, Carman et al. 2003).

Once the monocyte has stopped rolling and a firm adhesion has taken hold, the final step is transmigration. Prior to this step, the monocytes crawl along the endothelial cell reaching a preferable site of migration, achieved in a CD11b/ICAM-1 dependent manner (Schenkel, Mamdouh et al. 2004, Phillipson, Heit et al. 2006). The monocyte may migrate by paracellular or transcellular routes (Ley, Laudanna et al. 2007) and once through the barrier of the cells migrates through the endothelial basement membrane and pericyte layer.

Upon recruitment of cells and binding to pathogen, phagocytosis and release of ROS enables pathogens to be killed and in the process monocytes and diifferentiated macrophages will release cytokines e.g. TNF-α which promote inflammation and the further recruitment of immune cells. Monocytes and macrophages perform other functions such as pathogenic peptide presentation for the development of the adaptive immune response, in addition to the clearance of apoptotic cells through a CD14-dependent mechanism, removing debris and switching macrophage phenotype to reduce inflammation (Devitt, Pierce et al. 2003).

1.20 Monocyte and Macrophage heterogeneity

The population of monocytes is not uniform, with distinct populations of monocytes defined by differential expression of cell surface antigens coupled to distinct inflammatory cytokine production profiles. Currently three distinct monocyte populations have been identified based on the expression of CD14 and CD16 cell surface antigens. The major constituents are those monocytes which express high CD14 and low CD16 (CD14+++CD16-), and the remaining two subsets both express higher levels of CD16. The intermediate subset expresses higher CD14 with lower CD16 levels whilst the non-classical subset has lower CD14 with high CD16 levels. The contributions these three populations (major constituent classical CD14+++CD16-, intermediate CD14++CD16+, and non-classical CD14+CD16++) make with respect to inflammatory function is poorly defined (Wong, Yeap et al. 2012).

The differences in the monocytes subsets extend beyond cell surface antigen expression.Global gene expression analysis reveals the classical subset is distinct from the intermediate and non-classical populations, with the latter two displaying a gene expression profile(Wong, Tai et al. 2011, Zawada, Rogacev et al. 2011). These distinctions would suggest different behaviour and responses by these monocytes subsets.

Cytokine profiles recorded from the three populations of monocytes give a mixed picture. TNF-α production is high in either the intermediate (Frankenberger, Sternsdorf et al. 1996, Belge, Dayyani et al. 2002, Cros, Cagnard et al. 2010, Rossol, Kraus et al. 2012)or non-classical population of monocytes (Frankenberger, Sternsdorf et al. 1996, Belge, Dayyani et al. 2002, Wong, Tai et al. 2011) depending upon the study. Production of the anti-inflammatory IL-10 is high in intermediate monocytes (Skrzeczynska-Moncznik, Bzowska et al. 2008) and classical monocytes (Cros, Cagnard et al. 2010, Wong, Tai et al. 2011, Smedman, Ernemar et al. 2012) in response to LPS.

In mice, similar distinct profiles of monocytes have been observed, Geissman et al. performed profiling and fat mapping studies and determined the main monocyte population is Ly-6C^{high} CX3CR1^{low}CCR2^{high}CD62L+ which migrates into inflamed tissues, the second subset is smaller in size is Ly-6C^{low}CX3CR1^{high}CCR2$^{low/-}$CD62L^{-} is found within inflamed tissue and resting tissues. The Ly-6C^{high} monocytes were defined as inflammatory whilst the second population were resident (Geissmann, Jung et al. 2003). During infection the Ly-6C^{high} monocyte population undergoes a rapid increase within the circulation (Drevets, Dillon et al. 2004, Nahrendorf, Swirski et al. 2007), and under states of chronic inflammation e.g. atherosclerosis a progressive increase in this monocyte population has been observed (Swirski, Pittet et al. 2006, Swirski, Libby et al. 2007).

Monocyte to macrophage differentiation can generate distinct phenotypes, with different properties. In the presence of inflammatory triggers IFN-γ and LPS(Schwartz and Svistelnik 2012), bone marrow derived cells generate the classical pro-inflammatory M1 macrophage, whilst in the presence of cytokines associated with inflammatory resolution e.g. IL-10, IL-4 or IL-13, an alternatively-activated M2 macrophage is produced(Mackaness 1964, Vanfurth, Spector et al. 1972, Gordon 2003). The M1 macrophage typically expresses pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1 (Mosser and Edwards 2008). In contrast the M2 macrophages express IL-10, TGF-β, and are described as anti-inflammatory (Goerdt and Orfanos 1999, Mantovani, Sica et al. 2004).

In humans, macrophage heterogeneity was defined from the 1960s, which determined that molecules secreted from the activated T Helper 1 CD4^{+} (Th1) lymphocytes and natural killer cells, in particular IFNγ, TNF-α and LPS generated a classically-activated, whilst the cytokines typically released from T helper 2 CD4^{+} (Th2) IL-4 and IL-13 induce an alternatively-activated macrophage (Mackaness 1964, Vanfurth, Spector et al. 1972). Owing to the Th1/Th2 origins of cytokines and division of T helper cells the macrophages have been labelled M1 and M2 for classical and alternative activation.

Murine cells are classically-activated by IFNγ are identified by their production of nitric oxide (MacMicking, Xie et al. 1997, Hibbs 2002) which is absent in human macrophages(Weinberg, Misukonis et al. 1995). These macrophages also secrete elevated pro-inflammatory cytokines TNF-α, IL-1, IL-6 and IL-1, increased cell surface expression of MHC class II and CD86 with decreased expression of the mannose receptor (CD206), production of chemokines IP-10, MIP-1α and MCP-1(Mosser 2003) and improved phagocytosis(Wirth, Kierszenbaum et al. 1985, Higginbotham, Lin et al. 1992).

Murine M2 macrophages are defined by the expression of IL-10, TGF-β, and IL-RA, in addition to the induction of arginase(Rutschman, Lang et al. 2001), along with elevated expression of mannose receptor, scavenger receptors, CD23 and reduced cell surface expression CD14 and chemokine production of AMAC-1 (Mosser and Edwards 2008).

In human cells, markers for M1 and M2 macrophages are less clearly defined, owing to the lack of inducible NOS and arginase expression in human macrophages (Munder, Mollinedo et al. 2005). *In vitro* differentiation of human cells allows more precise delineation of M1 markers, with enhanced secretion of TNF-α, IL-1β, CXCL10 and cell surface CD80(Jaguin, Houlbert et al. 2013). Human M2 macrophages have been defined by the expression of galactose-type C-type lectins (Raes, Brys et al. 2005), elevated expression of CCL2, TGF-β, IL-10, cell surface SR-B1 and CD36 (Jaguin, Houlbert et al. 2013).

Gene expression analysis of isolated human monocytes differentiated to generate M1 and M2 macrophages revealed key differences (Martinez, Gordon et al. 2006); M1 and M2 polarised macrophages were treated with IFNγ and LPS (20 and 100ng/ml and 20ng/ml) and IL-4 respectively in the presence of M-CSF. Lipid metabolism was distinct between M1 and M2 macrophages with increased COX-2 in M1 and COX-1 in M2; enhanced sphingosine kinase and ceramide kinase in M1 and M2 macrophages, supporting the findings of others regarding cytokine expression.

Ly-6C^{high} differentiation into distinct M1 and M2 macrophages has been observed in kidney injury (Lin, Castano et al. 2009) and in response to *Toxoplasma gondii* infection, Ly-6C^{high} monocytes differentiated in to classical macrophages (Dunay, DaMatta et al. 2008). These finding would suggest the differentiation of monocytes to macrophages is not defined by a simple transition, and may in fact be dependent on the milieu of cytokines and signalling molecules present during or prior to differentiation.

1.21 Monocytes and FFA

Relatively little is known regarding the effects of elevations in free fatty acids on monocytes. A dietary study demonstrated elevated endothelial cell adhesion by monocytes in response to a high saturated fatty acid diet suggesting increased adhesion molecule expression (Mata, Alonso et al. 1996), with more recent data suggesting there is an increase in the expression of CD11b (a monocyte expressed cell surface integrin required for adhesion) on monocytes leads to enhanced binding to endothelial cells (Zhang, Schwartz et al. 2006).

Certain polyunsaturated fatty acids can induce the production of anti-inflammatory resolvins in monocytes (Jaudszus, Gruen et al. 2013), with the Ω-3 fatty acid eicosapentaenoic acid increasing the expression of IL-10 in monocytes (Satoh-Asahara, Shimatsu et al. 2012), whilst suppressing LPS stimulated TNF-α production (Endres, Ghorbani et al. 1989), inhibiting antigen presentation by monocytes (Hughes and Pinder 1997), inhibiting IL-8 (Mishra, Chaudhary et al. 2004), suppressing monocyte chemoattractant protein 1 gene expression (Baumann, Hessel et al. 1999) and increasing the bioavailability of the vasodilator nitric oxide (Shimokawa and Vanhoutte 1989). The mechanism through which these anti-inflammatory effects are mediated rely upon the activation of PPARα (Sethi, Ziouzenkova et al. 2002) and a decrease in nuclear translocation of NF-κB p65 subunit (Yamada, Yoshida et al. 2008).

The expression of urokinase type plasminogen activator receptor, a receptor that contributes to migration, is differentially affected by fatty acid treatment with the saturated fatty acids palmitate and stearate inducing greater expression than the monounsaturated or polyunsaturated fatty acids oleate and linoleate respectively (Assmann, Mohlig et al. 2008). In monocytes palmitate can induce insulin resistance (Gao, Bailey et al. 2009) and the expression of CD11b and CD36 (fatty acid translocase) (Gao, Pararasa et al. 2012), findings which suggest that saturated fatty acids induce changes in monocyte phenotype which may contribute to increased risk of atherosclerosis.

However the effects of saturated fatty acids are not limited to cell surfacephenotype changes, with the induction or promotion of inflammatory cytokine production a feature of the effects of saturated fatty acids. In murine monocytes SFA induces the expression of the pro-inflammatory COX-2 enzyme (Lee, Sohn et al. 2001), whilst in human monocytes palmitate can enhance LPS mediated pro-inflammatory cytokine expression (Schwartz, Zhang et al. 2010) and can activate the inflammatory NF-κB pathway (Huang, Rutkowsky et al. 2012). The production of pro-inflammatory cytokines has been demonstrated in THP-1 monocytes by palmitate but not oleate (Little, Madeira et al. 2012).

The induction of inflammation and promotion of pro-atherogenic cell surface antigen expression may increase risk of atherosclerosis.

1.22 Monocytes in disease

Cells of the innate immune system have an important role to play in metabolic and vascular disease. In atherosclerosis, monocytes, macrophages, and their derived inflammatory cytokines play a crucial role in the initiation and progression of atherosclerosis e.g. at sites of the vasculature which have disturbed laminar flow, typically sites of bifurcation (Moore and Tabas 2011). An early event is the accumulation of lipid, specifically LDL, within the sub-endothelial matrix underlying the endothelial cells, typically associated with elevated circulating lipids, hypertension or a pre-existing metabolic disorder such as diabetes.

Accumulated LDL is modified by ROS generating enzymes e.g. 12/15-lipoxygenase (Cyrus, Witztum et al. 1999), generating oxidised LDL which contributes the inflammatory signalling generated by the endothelial cells at the lesion site (Lusis 2000). The oxidised LDL (oxLDL) also impacts upon nitric oxide availability, reducing production (Mehta, Bryant et al. 1995, Chen, Mehta et al. 1996) and promoting hypertension, a promoter of atherosclerosis. The inflamed endothelial cells increase expression of adhesion molecules e.g. ICAM-1 which increase binding of monocytes as described above, with the expression of macrophage colony stimulating factor (M-CSF) stimulating proliferation and differentiation of monocytes into macrophages (Lusis 2000).

The oxLDL is further oxidised by enzymes such as myeloperoxidase and in this highly oxidised state binds to scavenger receptors, e.g. CD36 and SR-A (Suzuki, Kurihara et al. 1997, Febbraio, Abumrad et al. 1999), which take-up lipids in a non-saturable process to become foam cells (lipid-laden macrophages). The monocyte-derived macrophages contribute to the endothelial cell activation, promoting the recruitment of monocytes to the lesion.

The growing mass of the lesion consists of monocytes, macrophages and foam cells in a milieu of inflammatory cytokines and elevated lipids. In the next step of lesion progression, smooth muscle cells proliferate and the production of extracellular matrix components e.g. collagen, lead to the formation of a fibrous cap enclosing the region of the plaque exposed to blood flow.

The pathological impact of atherosclerosis depends on the stability of the plaque rather than the complete occlusion of the arterial vessel by the expanding plaque. Vulnerable plaques are those with relatively weak fibrous caps, are more likely to rupture leading to a distal thrombosis coupled to the release of necrotic components of the plaque.

1.23 Inflammation, ageing and atherosclerosis

Immune cells, specifically monocytes and macrophages, and inflammation are integrated in the process of atherosclerosis progression. The basic steps in atherosclerosis begin with an insult or stress of the endothelial monolayer which line the innermost arterial layer. Monocytes and macrophages are recruited with subsequent uptake of oxidised LDL and lipids leading to the propagation of inflammation and foam cell formation. Thus in the innermost layer of the artery, the tunica intima an atherosclerotic plaque begins to form (Libby, Ridker et al. 2011).

The macrophages resident within the atherosclerotic plaque exhibit pro-inflammatory functions characteristic of an M1 programme, defined by the expression of high levels of IL-1β and TNF-α (Bouhlel, Derudas et al. 2007). Progression of the atherosclerotic plaque requires the recruitment of resident smooth muscle cells from the tunica media (middle layer of the artery) into the tunica intima, and SMCs increase production of extracellular matrix proteins collagen and elastin generating a fibrous cap over the plaque.

The fibrous cap will deprive the plaque of substrate supply, the macrophages will undergo apoptosis, release their lipid contents which accumulate in the extracellular milieu of the plaque. The accumulation of apoptotic debris and poor clearance of these cells, termed efferoctyosis, leads to a lipid rich necrotic core (Tabas 2010).

Thus, monocytes/macrophages and inflammation are of critical importance during the atherosclerotic process which increases with age (de Tena 2005, Libby, Ridker et al. 2011). The pro-inflammatory cytokines, TNF-α (Paolisso, Rizzo et al. 1998, Bruunsgaard, Andersen-Ranberg et al. 1999) and IL-6 (Wei, Xu et al. 1992, Ershler, Sun et al. 1993, Hager, Machein et al. 1994, Cohen, Pieper et al. 1997, Ferrucci, Harris et al. 1999) are elevated in ageing (Bruunsgaard, Skinhoj et al. 2000, Song, Shen et al. 2012).

The importance of pro-inflammatory cytokines in atherosclerosis is highlighted by treatment strategies which serve to reduce the levels of TNF-α and IL-6 to reduce progression. Anti-TNF-α treatments, e.g. infliximab, used in the treatment of patients with chronic inflammatory conditions such as rheumatoid arthritis reduce cardiovascular events (Jacobsson, Turesson et al. 2005), in the absence of alterations in the lipid profile (Elhage, Maret et al. 1998, Pollono, Lopez-Olivo et al. 2010).

In murine models, using a monoclonal antibody against the IL-6 receptor elicited a similar decrease in atherosclerosis without altering the lipid profile (Schuett, Oestreich et al. 2012); however patients with rheumatoid arthritis receiving such therapy have an increase in lipids, that does not affect the

HDL-C:TC or atherogenic ratio (Maini, Taylor et al. 2006, Nishimoto, Ito et al. 2010, Kawashiri, Kawakami et al. 2011).

Furthermore, oxidised LDL induces pro-inflammatory cytokine expression via TLR4 activation and MyD88-null mice where TLR signalling is inhibited have reduced atherosclerosis (Bjorkbacka, Kunjathoor et al. 2004). Statins, a common treatment for metabolic disorders, mediate a downregulation in monocyte expressed TLR4, leading to reduced expression of downstream cytokines IL-6, IL-12, and TNF-α ex vivo (Methe, Kim et al. 2005).

1.24 Hypothesis

Currently, the risk of developing insulin resistance and cardiovascular disease increases with age is well documented. Furthermore, the ageing state is accompanied by an elevated low grade systemic inflammation possibly derived from adipocytes, monocytes and macrophages.

A causal factor in insulin resistance, inflammation and increased risk of developing cardiovascular disease is elevated circulating FFA, which is present in the ageing phenotype. The SFA sub-class of FFA are the most detrimental, and are established mediators of insulin resistance in cells crucial to euglycaemic uptake and storage of glucose such as adipocytes, hepatocytes and skeletal muscle cells. SFA mediated activation of inflammatory pathways e.g. TLR4 and NF-κB are demonstrated for a wide range of cells, with activation of TLR4 a possible pro-atherogenic mechanism (Poledne 2013).

Whilst there is evidence of increased FFA in ageing, such findings have tended not to be the focus of the study. Thus, the focus of this study will be test the hypothesis that ageing is associated with an elevation in FFA, specifically an increase in the SFA over the both the MUFA and PUFA sub-classes that contribute to the age related metabolic dysfunction, i.e. insulin resistance, and the low grade systemic inflammation.

Therefore a study using healthy volunteers will be required to determine the fatty acid profile in young and older adults, with the investigation expanding to indices of oxidative stress, insulin resistance and inflammation to determine whether there are any associations with the ageing fatty acid profile.

In the context of cardiovascular disease, as previously described, monocytes and macrophages play a critical role in the progression of atherosclerosis, serving to both enhance inflammation and supplying cells to the expanding lesion. Data characterising the effects of fatty acids on monocytes is relatively scarce, with the limited studies indicating effects are class dependent.

Thus, to address the lack of available data,a further objective of this study was to determine the effects of the MUFA oleate and SFA palmitate on THP-1 monocyte phenotype and cellular activation, to test the postulate that SFA generate pro-inflammatory and pro-atherogenic changes in monocytes. Furthermore, as oleate contributes a similar proportion to palmitate to total blood fatty acid content, and therefore the effect of this fatty acid on monocytes will also be investigated.

Following activation and recruitment, in response to stimuli such as M-CSF or GM-CSF, the monocyte will differentiate into a macrophage producing cytokines, increased ROS/RNS, and phagocytising bacteria. Whilst there are consistent in vitro models for the production of M1 classical and M2 alternatively activated macrophages, there is a paucity of data concerning the effects of endogenous compounds on the monocyte prior to or during the differentiation process, and whether any influence is exerted on the macrophage phenotype generated. Therefore, the role of both oleate and palmitate on modulating macrophage phenotype will be investigated, as macrophage phenotype appears to influence disease progression.

The mechanisms of SFA mediated effects of insulin resistance and inflammation are thought to rely upon the formation of neutral lipids such DAG and ceramide, production of ROS and fatty acid metabolism in the mitochondria, therefore an investigation into mechanisms of SFA mediated effects will also be undertaken.

Chapter 2

Materials and Methods

2 Materials and methods

General methods and volunteers

Phosphate buffered saline was made from137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 1.46 mM KH_2PO_4 in distilled H_2O.

2.1 Cell culture materials

THP-1 cell line was obtained from Health Protection Agency (HPA, UK). Cells utilised for experiments were within passages 6-55, and RPMI 1640 with stable glutamine was supplemented with 10% v/v foetal bovine serum and 1% v/v penicillin/streptomycin (50U/ml/50µg/ml, PAA, UK). Cell culture medium was stored at 4°C and used within a month.

2.1.1 Cell line background

THP-1 cells are human acute monocytic leukaemia cells derived from a 1 year old male. THP-1 cells have Fc and C3b receptors and lack surface and cytoplasmic immunoglobulins. These cells also stain positive for alpha-napthyl butyrate esterase, produce lysozyme and are phagocytic (both latex beads and sensitised erythrocytes).

2.1.2 Cell culture protocol

THP-1 cell line was cultured in RPMI 1640 with stable glutamine supplemented with 10% v/v fetal bovine serum and 1% v/v penicillin/streptomycin in $25cm^2$ and $75cm^2$ flasks grown to a density of 5 x 10^5 cells/ml. At each passage cells were seeded at a density of 2.5 x 10^5 cells/ml in either $25cm^2$ and $75cm^2$ flasks. Cells were counted using trypan blue under a haemocytometer when passaging or preparing experiments and analysing viability after treatments.

2.1.3 Volunteer recruitment and ethics

Volunteers were recruited from a pool of Aston staff and students to two age ranges 18-30 and 50 years and above for the young and mid-life cohorts respectively. Volunteers were free to withdraw themselves from participation at any time during the study with the removal of data. Blood was

obtained in the Aston Phlebotomy suite, with analysis of cholesterol, glucose and insulin measures obtained at this site immediately after obtaining blood. Submission of study outline and objectives were submitted and approval obtained from the Aston University Ethics Committee.

2.1.4 Blood collection

Whole blood (10ml) was obtained from the antecubital vein of fasted healthy male volunteers from consenting young (24-30 years old) and midlife (>50 years old) populations. Ethical approval for the study was granted by Aston University ethics committee. Blood was collected into potassium EDTA coated vacutainer. Samples were either analysed immediately or centrifuged for plasma and whole blood fractions and stored at -80°C.

2.1.5 Measurement of BMI, waist to hip ratio (WHR), blood pressure and heart rate

Prior to blood collection, the height and weight of volunteers was measured to calculate BMI; the waist and hip circumferences obtained to calculate waist to hip ratio; anautomated BP monitor (UA-767; A&D Co., Tokyo, Japan) device was used to obtain resting heart rate and blood pressure (mm/Hg).

2.1.6 Blood glucose, lipids, and liver enzymes

At point of blood collection, whole blood glucose, lipids, and liver enzymes were measured using Reflotron strips. Immediately after blood was obtained and placed into vacutainers, 30µl of blood was placed onto a reflotron strip and placed into Reflotron machine (Roche, UK) for analysis of blood glucose, HDL, total cholesterol or liver enzymes.

2.1.7 Analysis of plasma triglyceride content

Plasma samples were analysed for triglyceride content using a Randox kit, as per manufacturers' instructions.

2.1.8 Analysis of blood insulin

Insulin levels were determined from plasma sampled using a DRG Insulin ELISA kit, as per manufacturer's instructions.

2.1.9 Determination of insulin resistance and β-cell function

The Homeostatic Model assessment is a calculation derived from the work of Turner, Holman and colleagues (Turner, Holman et al. 1976)developing a mathematical equation describing glucose regulation by the β-cells of the pancreas as a feedback mechanism, with further development of the equation by Matthews et al.(Matthews, Hosker et al. 1985). The equations below allow for an approximate calculation of insulin resistance and %β-cell function using measured glucose and insulin values, with the results of these calculations providing comparable values to those derived by non-steady state means such as hyperinsulinaemic or hyperglycaemic clamps or intravenous glucose tolerance test.

Insulin Resistance: HOMA-IR (mmol/L) = (glucose x insulin)/22.5
B-cell function: HOMA-β (%) = (glucose x insulin)/(glucose-3.5%)

2.1.10 Analysis of Peripheral Blood Mononuclear Cell surface antigen expression and mitochondrial superoxides

Whole blood was collected from healthy male volunteers and placed into potassium EDTA coated vacutainers. 100µlaliquots of whole blood were placed into eppendorffs, and subsequently treated with either 2.5µM MitoSOX for 30min at 37°C, or mouse anti-human CD11b-RPE conjugated antibody, mouse anti-human CD36-FITC antibody, mouse anti-human CD16-RPE-Cy5 antibody or negative isotype IgG1 control (RPE, FITC or RPE-Cy5 conjugated) at a saturated concentration (10µl).for 30min on ice. Subsequently, whole blood aliquots were treated with 500µl of Optilyse C solution for 10min, followed by 500µl of PBS for 5min at room temperature, before analysis by flow cytometry.

2.2 Fatty acid conjugated bovine serum albumin (FA/BSA)

2.2.1 Reagents

Fatty acid free bovine serum albumin, sodium hydroxide, sodium oleate and sodium palmitate were obtained from Sigma (UK); DMEM (high glucose 4.5g/l without L-glutamine, serum-free) was obtained from PAA (UK) and ethanol was obtained from Thermo Fisher (UK).

2.2.2 Background

Lipid transport within the body requires carrier proteins owing to their hydrophobicity. Transport of fatty acids thus occurs in two forms; esterified in the form of triglycerides or as NEFA bound to albumin. The solubility of NEFA is very low, in the nanomolar range and thus the major fatty acid fraction in the blood will be protein bound with albumin. Therefore for *in vitro* cell culture experiments FA was conjugated to BSA as a carrier.

2.2.3 FA/BSA stock preparation

Stock solutions of sodium oleate and sodium palmitate were made by dissolving [200mM] of either fatty acid in 70% ethanol, 0.1M NaOH and 30% distilled water (278.4mg sodium palmitate and 304.44mg into 5ml of NaOH in 70% ethanol) at 60°C on a hot plate. Solutions were stored in -20°C until required. Working solutions of fatty acids were made by dissolving 5mM FA stock solutions into 5% BSA w/v in serum-free DMEM (fatty acid to BSA ratio of 6:1), placing on a hot plate at 37°C with stirring for 4 hours. Solutions were adjusted to pH7.4 with dilute sodium hydroxide and 0.2µM sterilised using a 0.2µM filter. All working solutions were stored in 4°C and used within 14 days(Gao, Griffiths et al. 2009).

2.3 Cell treatments

2.2.4 Cell treatment for the studying effects of FA on THP-1 monocytes

Working solutions of BSA-conjugated FA at 5mM were prepared as described above and stored at 4°C. To determine the effect of fatty acids on monocytes redox status, THP-1 cells were incubated with various concentrations of FA (50µM, 150µM and 300µM) or BSA equivalent for 6 and 24 hours in RPMI 1640 supplemented with 10% FBS and 100U/ml penicillin and 100µg/ml streptomycin at 37°C. THP-1 monocytes were incubated at 5 x 10^5 cells/ml into a 24 well plate (2ml/treatment) in 10% FBS and 1% P/S RPMI 1640 in the presence of FA/BSA for up to 24h.
The concentrations of FA used are based on plasma free fatty acid concentration, in which the two main physiological FA palmitate and oleate constitute approximately 30% of FFA human plasma. Control cells received BSA equivalent to the cells treated with the highest concentration of FA. Thus treatments below 300µM FA represent a healthy physiological level of FA, whilst 300µM represents

the upper limit of healthy and possibly similar to that seen in the insulin resistant state(Golay, Swislocki et al. 1987, Laws, Hoen et al. 1997, Salgin, Ong et al. 2012).

2.3 MTT assay

2.3.1 Reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium dodecyl sulphate and dimethyl formamide were from Sigma (UK). MTT (12mM) was prepared in PBS and subsequently stored in the dark at 4°C. Lysis buffer composed of 0.7M sodium dodecyl sulphate in 50% dimethyl formamide was adjusted to pH4.7 with glacial acetic acid.

2.3.2 MTT assay background

The reduction of MTT by mitochondrial succinate dehydrogenase within mitochondria of viable cells changes the colour from a yellow soluble tetrazole to an insoluble violet formazan crystal which once solubilised using a detergent solution can be quantified using a spectrophotometer. Whilst the assay does not allow for a differentiation between necrosis or apoptosis, it provides information regarding metabolic activity within the cell.

2.3.3 MTT assay protocol

Cultured cells treated with increasing fatty acid concentrations and BSA controls for up to 24 hours in 6-, 12-, or 24-well plates at a concentration of 5×10^5 cells per ml, cells were mixed and 100µl of cell suspension were removed and placed into a 96 well plates followed by 25µl of MTT solution. Following two hours of incubation at 37°C, the cells were lysed with MTT lysis buffer (100µl), and culture plates were left overnight at 37°C before being analysed spectrophotometrically at 570nm. Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) at a concentration of 50µM was incubated with cells for the same time period and was used as a positive control for inhibition of metabolic activity and cell viability. Values were expressed as absorbance @ 570nm(Grant, Barber et al. 2005).

2.4 Annexin-V/Propidium Iodide dual staining for apoptosis/necrosis

2.4.1 Reagents

Propidium iodide (PI) and FITC-conjugated annexin-V were obtained from Sigma (UK) and AbCam (UK) respectively. PI solution was made up at 100µg/ml PI in 10mM HEPES and 150mM NaCl; Annexin-V binding buffer consisted of 150mM NaCl, 10mM HEPES and 2.5mM $CaCl_2$ pH7.5.

2.4.2 Assay Background

Programmed cell death, apoptosis, is a key process in growth and development, immune responses and clearance of cancerous cells. Necrosis is a non-programmed cell death. PI is a non-cell-permeable DNA stain, and only under conditions in which the cell membrane becomes permeable or leaky i.e. cell stress and necrosis will the stain enter the cell, consequently PI can identify necrotic cells and an increase in PI fluorescence may be used as a measure of cellular necrosis.

2.4.3 Assay Protocol

Treated cells, samples or controls were washed in equal volume of PBS and subsequently centrifugedat 200g for 5min. PBS was removed and cells were resuspended in 500µl of Annexin-V binding buffer. Annexin-V (5µl) was added for 15 min in the dark and prior to analysis by flow cytometry 5µl of PI solution was added. Samples are analysed by flow cytometry until 10000 events (at 5 x 10^5 cells/ml, total population) were recorded (488nm argon laser using Quanta flow cytometry machine, using emission filters FL1 525nm and FL2 575nm) (Lam, Carmichael et al. 2012).

2.5 Cell Cycle analysis

2.5.1 Reagents

Propidium iodide (PI) solution was made up at 50µg/ml in 0.1% Triton-X and 0.1% sodium citrate in distilled water, and PBS was made up as previously described. All reagents were obtained from Sigma (UK).

2.5.2 Assay Background

Propidium iodide (PI) binds to DNA by intercalating between bases without preference, and because of its fluorescent nature, permits measurement by flow cytometry usually under the FL2 channel (excitation 518nm, emission 617nm). The ability of PI to bind to DNA is exploited to analyse the cell cycle in cells. The fluorescence intensity of cells treated with PI will reflect the amount of DNA within the cells and consequently the stage of the cell cycle they are in. Quiescent cells and those in G1 phase of the cell cycle will generally have a single copy of DNA. Cells which are in S phase will be replicating DNA and thus will have a mixed DNA content with between one and two copies of DNA, whilst cells in G2/M phase will have two copies of DNA; all these factors can be observed by flow cytometry with cells in G0/G1 having the lowest fluorescence intensity, with cells in G2/M phase having the highest and cells in S phase having a fluorescence intensity in between the two populations.

2.5.3 Assay protocol

Following experiments cells were washed in 1ml of PBS before resuspension in 1ml of PI solution and subsequently incubated for 24 hours at 4°C, before analysis by flow cytometry until 10,000 events are recorded (total cell population, excitation and emission at 518nm and 617nm, with data expressed as percentage of cells in G_0, G_1, S and G_2/M phase). (Woollard, Phillips et al. 2002).

2.6 Caspase-3 activity assay

2.6.1 Reagents

Tris-HCl, NaCl, NaH_2PO_4, NaF, Na_3VO_4, PMSF, DTT, glycerol, HEPES were all obtained from Sigma (UK), leupeptin was obtained from Enzo Life Sciences, and acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin (Ac-DEVD-AMC) and 7-amino-4-methylcoumarin were obtained from Calbiochem, Merck (UK). Cell lysis buffer consisted of 10mM Tris-HCl pH7.5, 130mM NaCl, 1% TritonX-100, 10mM NaH_2PO_4, 0.4mM PMSF, 0.2mM NaF, 0.2mM Na_3VO_4 and 0.3mg/ml leupeptin. Reaction buffer consisted of 20mM HEPES pH7.8, 10% glycerol and freshly added 2mM DTT.

2.6.2 Caspase-3 activity background

Programmed cell death, apoptosis, requires activation of proteins necessary for the degradation of cellular components and DNA. Caspases (cysteinyl aspartate specificproteases) are a family of proteases which which are instrumental in apoptotic processes mediating cell death. Caspase-3 processes caspase 2, 6, 7 and 9 proenzymes, and other key apoptotic proteins including poly ADP-ribose polymerase, the inhibitor of caspase-activated deoxyribonuclease (ICAD), gelsolin and fodrin. Therefore, during apoptosis caspase 3 is central in chromatin condensation, DNA fragmentation and cell blebbing(Porter and Janicke 1999).

The caspase-3 activity assay relies on enzymatic cleavage of an amino acid motif, to release a fluorophore. Using cellular lysates, the assay works on the basis that caspase-3 will hydrolyse the peptide substrate acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin (Ac-DEVD-AMC) to yield the fluorescent 7-amino-4-methylcoumarin (AMC) moiety which has excitation and emission wavelengths 360nm and 460nm respectively which can be measured fluorimetrically. Using cellular lysates the activity of caspase-3 can be determined by measuring the amount of AMC fluorescence generated, and comparing this to a standard curve generated from known AMC solutions relative to protein.

2.6.3 Assay protocol

Following treatment, THP-1 cells were collected and centrifuged at 250g for 5min then washed once with PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO4 and 1.46 mM KH_2 PO4 in distilled H_2O). 2million cells were subsequently treated with 100µl cell lysis buffer for 20 min on ice then spun at 14,000g for 30 seconds. The supernatants were then stored at -20°C prior to assay and BCA protein analysis. Into wells of a 96 well plate 175µl of reaction buffer and 25µl of supernatant were added, the caspase-3 assay was initiated by adding 50µl of Ac-DEVD-AMC (25µM) to the 96 well plate in addition to diluted AMC standard concentrations. The plate was left in the dark for 1hour at room temperature. The plate was read on a fluorimeter at excitation 360nm and emission 460nm, with results of caspase-3 activity calculated based on an AMC standard curve.

Protein content was determined from sample aliquots stored at -20°C. 400µl copper sulphate solution was added to 20ml of bicinchoninic acid (BCA), and into a 96 plate 10µl of sample and 200µl

of copper sulphate BCA solution was added, along with standards (0-1mg/ml), and protein content was determined by extrapolating from a standard curve.

2.7 MitoSOX red superoxide indicator

2.7.1 Reagents

MitoSOX Red reagent was obtained from Invitrogen, dimethyl sulphoxide (DMSO) was obtained from Sigma (UK) and PBS was made as previously described.

2.7.2 Background

Mitochondria are the principal site of energy generation, and a significant portion of cellular energy within this organelle is generated via the flow of electrons from reduced cofactors through the electron transport chain to oxygen. The process leaks electrons which interact with molecular oxygen yielding superoxide anions. MitoSOX Red is a fluorogenic dye which is highly selective for superoxide anions in mitochondria. Once in the mitochondria the reagent is readily oxidised by superoxide but not other ROS or reactive nitrogen species, generating fluorescence which can be measured by flow cytometry.

2.7.3 Assay protocol for mitochondrial ROS in THP1 cells

$1x10^6$ cells/ml were washed once and subsequently resuspended in PBS at 1×10^6 cells/ml, and incubated at 37°C for 5 min before addition of 2.5µM of MitoSOX (5µg resuspended in 52µl of DMSO in 1 vial) for exactly 30 min, cells were then analysed by flow cytometry.

2.7.4 Mitochondrial ROS production in PBMC population

Immediately after sample collection, 100µl of whole blood was aliquoted and treated with or without 2.5µM of MitoSOX for 30 min at 37°C in the dark. Samples were then treated with 500µl Optilyse C for 10 min in the dark, before 500µl of PBS for 5 min. Samples were analysed by cytometry until 10,000 events were recorded.

2.8 Reduced Glutathione assay

2.8.1 Reagents

DTNB, EDTA, glutathione (reduced, GSH), glutathione S reductase, NADPH, sodium phosphate and sulphosalicylic acid (SSA) were all obtained from Sigma (UK). 125mM sodium phosphate and 6.3mM disodium EDTA were dissolved in water for the stock buffer which can be stored at room temperature. 3µg NADPH was dissolved into 10mlstock buffer to make daily buffer which is made fresh prior to assay run. 6mM DTNB was prepared in 10ml stock buffer; 1g of SSA into 1ml dissolved in water. Glutathione stock solution was made up at 1mM aliquoted in dH_2O and stored at -20°C.

2.8.2 Background

The production of reactive oxygen species is inherent to energy generation in the mitochondria and innate immune function via the respiratory burst, and can damage cellular organelles namely the mitochondria and DNA preventing normal and viable cellular function. Cells possess various apparatus to deal with oxidative stress in the form of antioxidants and reducing enzymes. The primary cellular antioxidant is glutathione which is primarily found in its reduced, GSH, form which is oxidised in the presence of reactive oxygen species to yield GSSG.

2.8.3 Assay Protocol

After each experiment, 5 x 10^5 cells/ml were removed and washed in PBS (1ml), with 50µl retained for analysis of protein content. Cells were centrifuged (150g) for 5 min and the supernatant was removed 3.3µl SSA, and 96.6µl stock buffer were added. Standards in the range 0-80µM GSH were made up using equivalent concentrations/volumes of SSA and stock buffer. 25µl of standards and samples were added to a 96 well plate in triplicate, with 150µl daily buffer and 50µl DTNB solution and left for 5 min at 30°C before addition of 25µl of GSR. The plate was analysed at time points 0, 1, 2, 5 and 10 min post glutathione-S-reductase addition using plate reader at 410nm(Grant, Barber et al. 2005).

2.9 Oxidised Glutathione assay

2.9.1 Reagents

Reagents used are same as for glutathione assay above, with the addition of triethanolamine and 2-vinylpyridine (Sigma, UK). Oxidised glutathione stock solutions were made freshly, to a concentration of 5mM.

2.9.2 Background

The assay for oxidised glutathione works on the same principle as the glutathione assay, however 2-VP and TEA were added to each reaction condition in order to maintain a high pH, stabilise the GSH, GSSG and prevent oxidation of GSH to GSSG.

2.9.3 Assay protocol

The protocol follows that of the glutathione assay, except 2µl of 2-VP and 2µl of TEA were added to both standards and samples prior to the addition of GSR, then was measured at 410nm using a 96 well plate reader at 0, 1, 2, 5 and 10 min.

2.9.4 Calculations

The concentrations of GSSG were determined by calculating the maximal difference in sample and standard absorbance between two time points then interpolating this rate of GSSG conversion based on standards, the values of GSSG were subsequently normalised to protein content as determined by BCA assay. (Grant, Barber et al. 2005).

2.9.5 Analysis of plasma reduced and oxidised glutathione content

Plasma obtained after centrifugation was stored with 1% sulfosalicylic acid (SSA) to enable preservation of glutathione. These samples were then analysed for reduced (GSH) and oxidised (GSSG) glutathione content (Grant, Barber et al. 2005). GSH (0-80µM) and GSSG (0-1µM) standards were prepared, and 25µl of both standards and samples were aliquoted onto a 96 well plate in

triplicate as previously described, followed by 150µl daily buffer and 50µl 5-5'-dithiobis 2-nitrobenzoic acid (DTNB), and left for 5 min before the addition of 25µl of glutathione-S-reductase (GSR). The plates were analysed at time points 0, 1, 2, 5 and 10 min using a plate reader at 410nm.

2.10 Enzyme linked Immunosorbent Assay (ELISA)

2.10.1 Reagents

Human TNF-α, IL-6 and IL-10 ELISA development kits obtained from Peprotech; 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid ABTS, BSA, citrate phosphate, hydrogen peroxide, Tween-20 and NUNC MaxiSorp plates were obtained from Sigma (UK).

2.10.2 Background

Enzyme linked immunosorbent assay (ELISA) permits the detection of a specific antigenfrom a mixture, through the immobilization of specific proteins onto the wells of a microplate using capture antibodies. There are key steps in the ELISA protocol, coating, blocking, detection and measurement. The coating step involves addition of antibodies specific for a protein of interest to a plate (typically a 96 well plate). The blocking step adds a non-specific protein to areas of the well unbound by the antigen. The test sample and standards are added to separate wells of the plate. The detection step utilises another antibody to detect the specific protein, typically conjugated to enzyme or fluorophore, which is followed by signal measurement.

2.10.3 Assay protocol for cytokine secretion from THP1 cells

The ELISA kits supplied a capture and detection antibody, standards and avidin-horse radish peroxidase (avidin-HRP) conjugate and were prepared as per manufacturer's instructions. 10X PBS was made as previously described, and from this wash buffer (0.05% v/v Tween-20), diluent (0.05% v/v Tween-20 and 0.1% v/v BSA) and block buffer (1% v/v BSA) were produced. Capture antibody diluted to a 1µg/ml was added in triplicate to a 96 well plate (100µl/well) for each sample and standard to be analysed and subsequently was left overnight at room temperature. Before addition of standards and samples, the 96 well plate was rinsed 4x with wash buffer and blotted dry then block buffer was applied (300µl/well) for one hour. The plate was rinsed as previously described

then standards (2ng/ml-0ng/ml) and samples were applied (100µl/ml) and placed at room temperature for 2 hours. The plate was washed, and then detection antibody was added at 0.5µg/ml (100µl/well) and incubated for 2 hours at room temperature. The plate was washed, and then avidin-HRP conjugate was applied (100µl/well), for 30 min. ABTS liquid substrate solution was made up with 30ml citrate phosphate buffer, 200µl of ABTS (20mg/ml) and 10µl of hydrogen peroxide. After 30 min, the plate was washed and 100µl/well of ABTS liquid substrate was added, and placed in incubator at 37°C until colour development, the absorbance of the plate was read at 410nm, and levels of cytokines calculated using the internal standard curve.

2.10.4 Analysis of plasma cytokines

The cytokines IL-6, IL-10 and TNF-α were analysed in plasma using ELISA kit from Peprotech, as per manufacturer's instructions. Following validation, instead of using 100µl of sample per well, 75µl was used in order to generate results within the standard curve.

2.11 JC-1 assay

2.11.1 Reagents

JC-1 was obtained from Invitrogen (UK).

2.11.2 Background

The early stages of apoptosis are defined by changes in the potential of mitochondrial membranes, in part due to the formation and opening of a mitochondrial permeability transition pore, which permits the release of ions which causes the decoupling of the electron transport chain; this allows the release of cytochrome C. Mitochondrial dyes such as 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide CBIC2(3)JC-1 are cationic (positively charged) at normal membrane potentials permitting their accumulation in the negatively charged inner mitochondrial membrane. In this state, the JC-1 dye will form aggregates and fluoresce red (~590nm) and upon a membrane depolarisation the dye will be released. In the monomer form, the JC-1 dye will generate green fluorescence (~529nm). The membrane potential is thus measured as a ratio of red: green fluorescence. The dye can be excited by a standard argon 488nm laser found on flow cytometers which permits the analysis of both red and green fluorescence or by fluorescence microscopy

2.11.3 Protocol

Samples are washed in PBS twice and resuspended to concentration of 1 x 10^6 cells/ml, and are treated with JC-1 at a concentration of 10μg/ml and placed in the dark at 37°C for 10 min before analysis by flow cytometry.

2.12 Oil Red O lipid staining

2.12.1 Reagents

Oil Red O was obtained from Cayman (UK) and isopropanol from Fischer (UK).

2.12.2 Background

Oil Red O is a fat soluble dye used for the staining of neutral lipids such as triglycerides and lipoproteins e.g. LDL, which stains lipids red and can be visualised under microscopy or quantified using absorbance spectrometry.

2.12.3 Protocol

THP-1 monocytes treated for 24h with either oleate or palmitate (2x10^6 cells/ml) were subsequently washed in 1ml PBS and fixed in 1ml 4% paraformaldehyde solution before incubation with 500μl Oil Red O solution (Cayman, UK)for 15 min at room temperature. Cells were washed once in 1ml PBS before incubation with 1ml isopropanol for 5 min on rocker at a slow setting. Subsequently samples were centrifuged at 100g for 2min, with the supernatants transferred to a 96 well plate in triplicate before spectrophotometric analysis at 490nm.

2.13 Flow cytometry for monocyte surface antigen analysis

2.13.1 Reagents

Mouse anti-human CD14 RPE-Cy5 conjugated monoclonal antibody (clone 61D3, Abd Serotec); Mouse anti-human CD11b-RPE conjugated antibody (clone ICRF44, Abd Serotec); Mouse anti-human CD16-RPE Cy5 conjugated antibody (clone 3G8, Abd Serotec); Mouse-anti-human CD36-FITC conjugated antibody (clone SMO); Mouse IgG1 isotype control RPE, FITC and RPE-Cy5 antibodies and mouse IgG2a RPE-Cy5 isotype controls were all obtained from Abd Serotec (UK).

2.13.2 Background

Flow cytometry allows measurement of the characteristics of individual particles and cells. Cells are carried in a stream of fluid and passed through a laser. The properties analysed include particle size, granularity, and fluorescence intensity. As the cells are passed through the laser the light is scattered, and is collected by lenses which carry the light to appropriate detectors and generate proportional signals. Forward scatter light is proportional to cell size, and is a measure of diffracted light. Side scatter light is proportional to cellular granularity, and is a measure of light which is either refracted or reflected, and is usually collected at 90° to the laser beam.

The lasers present in a flow cytometer permit the measurement of specific fluorescence. Fluorochromes are fluorescent dyes which absorb light energy (emitted from a laser at 488nm) at a given wavelength; an electron within that compound is excited to a higher energy state. As the electron returns to its original/ground state the received energy is emitted at a longer wavelength as fluoresence. This is exploited in flow cytometry, as most flow cytometric equipment utilise an argon laser which excites more than one fluorochrome. Antibodies targeted against cell surface antigens can be labelled with fluorophores such as fluorescein isothiocyanate (FITC) and phycoerythrin (PE) permitting the quantification of cell surface expression of antigens. This can be used to measure changes in surface expression following treatment of cells; they can also be used for immunophenotyping to distinguish between healthy and diseased cell populations.

2.13.3 Assay protocol for THP1 cells

After completion of the experiment, cell samples are placed into eppendorff tube in ice with serum for 15 min to block receptors. Subsequently each sample is treated with a saturating concentration of antibody or negative control for 30 min on ice before analysis by flow cytometry (Beckman Coulter Quanta or Epics XCL).

2.13.4 Analysis of primary blood mononuclear cell (PBMC) cell surface antigen expression

Immediately after blood collection, 100µl of whole blood was aliquoted and subsequently labelled with 10µl of either mouse anti-human CD11b or mouse anti-human CD36 or mouse anti-human CD14, or respective mouse anti-human IgG negative control for 30 min on ice. Subsequently, 500µl of optilyse C was added and left in the dark at room temperature for 10 min, followed by 500µl of

PBS for 5 min. Samples were then analysed by flow cytometry using FL1 for CD36, FL2 for CD11b and FL4 for CD14 (table 2.1) until 10,000 events were recorded.

Antibody	Fluorophore	Filter (emission wavelength)
CD11b	RPE	FL2 (~575nm)
CD14 CD16	RPE-Cy5 RPE-Cy5	FL3 (~694nm)
CD36	FITC	FL1 (~525nm)

Table 2.1: Mouse anti-human antibodies with respective fluorophores and flow cytometry filters

2.14 Extraction of ceramides from monocytic cell lines and analysis under LC-MS

2.14.1 Reagents

PBS placed at 4°C prior to use; methanol, iso-propanol, water and ethyl acetate were acquired from Thermo-Fisher (UK) and C17 internal standard was obtained from Avanti Polar lipids (USA).

Ammonium formate and formic acid were obtained from Sigma (UK) and methanol was obtained from Thermo. Mobile phase A solution consisted of 1mM ammonium formate in methanol with 0.2% formic acid and mobile phase B solution consisted of 2mM ammonium formate in water with 0.2% formic acid.

2.14.2 Background

In order to fully understand how an increase in an initial substrate such as palmitate for de novo ceramide synthesis may impact on the cell, measurement of its metabolites such as ceramides can be undertaken. Efficient extraction and analysis of lipid content from samples for the sphingolipid class is required for such an analysis that permits both reproducibility and good yield and prevents oxidation or degradation of sample. Mass spectrometry provides is the best approach for the analysis of ceramide content.

2.14.3 Protocol

Monocytic cells (20ml 1x10^6 cells/ml) following 24 hour treatments were centrifuged at 200g for 5 min, and were resuspended in 10ml of ice cold PBS, and subsequently respun at 200g for 5 min before a second resuspension in 10ml ice cold PBS, 200µl was removed for protein analysis by BCA. After second washing, the cells were resuspended in 2ml of extraction solvent (iso-propanol: water: ethyl acetate (30:10:60; v:v:v)) containing C17:0 ceramide internal standards at 5µM in 15ml polypropylene Falcon tubes. The cells were subsequently vortexed 3 times for 30 seconds each before being spun at 500g for 10 min. Using a glass pipette, the extraction solvent was removed and placed into a glass vial. The remaining pellet was re-extracted with a further 2ml of extraction solvent, and lower phase placed back into the glass vial. The lipid containing phase is dried under a stream of nitrogen and placed into -80°C until analysis by LC-MS.

Lyophilised C17:0 ceramide spiked cellular lipids were redissolved in 80% Mobile phase A and 20% Mobile phase B. Lipids (10µL) were resolved by nano-liquid chromatography using a reverse phase C8 BioBasics 100mm x 0.075mm column (Integrafrit; Presearch, UK) and were eluted using a 90min gradient (A/B): 0-5 min 80/20; 5-40 min 80/20-99/1; 40-60 min 99/1; 60-65 min 99/1-80/20; 65-90 min 80/20. Eluted lipids were directly infused via nano-electrospray injection into a LXQ linear ion trap (ThermoFisher) with data dependent analysis to identify ceramide species. A standard curve was employed to determine absolute levels of C16 ceramide and the data was expressed relative to the C17 ceramide spiked into each sample.

2.15 Differentiation of THP-1 monocytes

2.15.1 Reagents

Phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharide (LPS) were obtained from Sigma (UK). Interleukin-4, Interleukin-13, and Interferon-γ were obtained from Peprotech (UK) and subsequently aliquoted in PBS containing 1% w/v BSA and stored at -20°C.

2.15.2 Background

In response to infection, circulating monocytes migrate from blood vessels to sites of infection, into the tissue under the influence of chemokines and other signalling molecules. In the tissues they

differentiate to macrophages, with the functions of the cell changing. The differentiation path and therefore type of function depends upon the milieu of cytokines and other signalling molecules present. Consequently the macrophage population will exhibit marked heterogeneity. The two principal phenotypes observed *in vitro* are the 'classically-activated' M1 macrophage, a pro-inflammatory and specialised antibacterial cell. The 'alternately activated' M2 macrophage functions to clear cell debris and mediate tissue repair.

In vitro, macrophages of differing phenotypes are modelled using differentiation programmes. The use of *in vitro* cell lines is complicated by the fact that various cell lines are at different stages of the differentiation process themselves. Typically differentiation agents such as dihydroxyvitamin D_3 or phorbol-12-myristate-13-acetate are utilised, combined with pro-(TNF-α) or anti-inflammatory (IL-10) cytokines or bacterial endotoxins (lipopolysaccharide or lipotechoic acid) to generate a desired macrophage phenotype. In such a manner other agents or molecules maybe used to determine their influence upon macrophage phenotype.

2.15.3 Protocol

THP-1 cells resuspended at 1 x 10^6 cells/ml were seeded into the wells of a 12 well plate at 3ml/well. The cells were treated with 20ng/ml IFN-γ and 100ng/ml LPS for 6 hours followed by 320nM PMA for a further 18-66 hours to generate a classical M1 phenotype. Non-classical M2 required 20ng/ml of both IL-4 and IL-13 for 6 hours followed by 320nM PMA for a further 18-66 hours. Monocyte controls were treated with cytokines for 6 hours with no PMA for a further 18-66 hours

2.16 Analysis fatty acid profile in plasma and red blood cells

2.16.1 Reagents

Chloroform, methanol, toluene, and hydrochloric acid were obtained from Thermo-Fisher. BHT and 37 fatty acid methyl ester mix were obtained from Sigma (UK).

2.16.2 Background

Analysis of fatty acids from biological material requires extraction and conversion to a volatile derivative followed by analysis. Fatty acids are not volatile enough to be analysed directly and

require conversion to fatty acid methyl esters (FAMEs), in this form they can be readily vapourised for analysis under gas chromatography (GC). At each stage of the extraction and conversion steps, care needs be taken to minimise oxidation and degradation of the lipid sample, this can be achieved through the use of low light and inert gas such as nitrogen. Antioxidants such as BHT are added to prevent oxidation of fatty acids during the extraction process. Analyses of FAMEs are executed by GC using a flame ionization detector with a hydrogen or helium carrier gas, with the use of a polar silica capillary column for optimal separation of fatty acids.

2.16.3 Protocol

The NEFA profile of fasted plasma samples was determined using a methodology adapted from Ichihara et al.(Ichihara and Fukubayashi 2010). Lipids were extracted from 500µl of plasma using chloroform methanol (2:1, 0.05% BHT), i.e. 1.5ml solvent mixture to 0.5ml of plasma in glass screw cap tube vortexed immediately and subsequently spun at 700g for 10 min. The chloroform layer was subsequently removed, and dried under nitrogen. The samples were subsequently methylated to generate fatty acid methyl esters using 200µl toluene, 1.5ml methanol and 0.3ml HCl in methanol, heated at 100°C for ~20min to methylate NEFA but not triglycerides or cholesterol esters in PTFE sealed glass vials. The FAMEs were subsequently extracted with 1ml of hexane and 1ml of water, removing the hexane layer. The extracted FAME sample was evaporated under nitrogen and resuspended in 50µl of hexane prior to analysis using a HP-5890 series II gas chromatography machine with a HP-Innowax polyethylene glycol polar column, with flame-ionisation detection.
Red blood cell membrane analysis followed a similar protocol; however, stored red blood cells were washed and diluted with PBS. Red blood cells stored in -80°C were defrosted, with 7ml of PBS added to 2ml of sample; this was subsequently returned to storage or immediately extracted. To 1ml of this sample, 2ml of chloroform and 1ml of methanol was added and vortexed for 30 seconds before centrifugation at 1000g for 10 min. The lower chloroform layer was removed, and the lipid layer was dried under nitrogen. The sample was methylated as described for plasma by heating to 100°C for 1 hour, before extraction of the FAMEs and analysis under GC conditions as described above.
Identification of fatty acids peaks was determined after running a Supelco 37 FAME mix standard, comparing run times and peak heights. The fatty acids in C20 range i.e. C20:0, C20:1, C20:2, C20:3n6, C20:3n3, C20:4n6 and C20:5n3, could not be identified individually although peaks within that range were present.

2.17 Statistical analysis

Data obtained from *in vitro* THP-1 monocyte cell work was tabulated and analysed using GraphPad Prism (v6.0). If paired data was analysed, data was subject to analysis by t-tests; otherwise one-way ANOVAs were performed. Data obtained from human samples was analysed byKolmogorov-Smirnov test for normality, and if normally distributed data was analysed by t-tests when comparing young and mid-life cohorts and if not normally distributed data was analysed using non parametric Mann-Whitney t-test.;whilst correlation analysis was carried out on the entire sample (including both young and mid-life) using GraphPad Prism. Data on NEFA and RBC fatty acid profiles was log transformed in order to accommodatevariability. Statistical significance is provided as *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$ and ****=$p<0.0001$, determined by the tests described.

Chapter 3

Effects of palmitate and oleate on monocyte oxidative stress and cell survival

3 Chapter 3: Effects of palmitate and oleate on monocyte oxidative stress and cell survival

3.1 Preface

This chapter describes an investigation of the effects of the MUFA and SFA oleate and palmitate respectively on monocyte redox status and apoptosis. The rationale for these experiments was to establish concentrations of fatty acids that were not toxic to monocytes. THP-1 monocytes were incubated with BSA-conjugated fatty acid (50μM, 150μM, 300μM) and BSA alone as control in order to determine fatty acid effects on cell growth, metabolic viability and redox status. Subsequent experiments determine if reactive oxygen species (ROS) generation and apoptosis were consistent effects of either saturated (myristate, palmitate and stearate) or monounsaturated (oleate) fatty acid treatment irrespective of chain length.

3.2 Introduction

Reactive oxygen species (ROS) encompass reactive oxygen intermediates including superoxide anion radical ($O_2^{\cdot-}$ or O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\cdot}OH$) in addition to both ozone (O_3) and singlet oxygen (Nathan and Ding 2010). ROS play important roles in normal physiological processes, for example in the immune system production of ROS is central in both the innate and adaptive responses (Nathan and Cunningham-Bussel 2013).

The production of ROS is part of the oxidative burst in activated phagocytes and represents the first line of defence against pathogens (Kohchi, Inagawa et al. 2009). ROS have also been demonstrated to contribute to polymorphonuclear leukocyte migration to a wound margin (Niethammer, Grabher et al. 2009) and subsequently promote their retention (Sakai, Kii et al. 2011).

ROS production and signalling is also important in adaptive immunity, for example activation of T cell receptors leads to ROS generation (Devadas, Zaritskaya et al. 2002), with the production of peroxides in these cells activating NF-κB which leads to IL-2 production (Los, Schenk et al. 1995) that is crucial for growth, proliferation and differentiation of T cells to become effector cells.

However oxidative stress arises when ROS production exceeds ROS scavenging (Cadenas and Sies 1985) and is a common observation in metabolic diseases such as cardiovascular disease, obesity, and type-2 diabetes. At a cellular level in the majority of non-phagocytic cells, ROS are principally derived from the electron transport chain of mitochondria (as superoxide anions) with minor contributions from NADPH oxidase, xanthine oxidase and uncoupled endothelial nitric oxide synthase (Nathan and Ding 2010).

In conditions such as obesity, diabetes mellitus, and cardiovascular disease the metabolic state can be described as an excess of energy substrates e.g. fatty acids and glucose. An excess of fatty acids can lead to peripheral insulin resistance via oxidative stress (see introduction), which is a prominent feature in metabolic disease.

The induction of insulin resistance by ROS has been demonstrated in both 3T3-L1 adipocytes (Rudich, Kozlovsky et al. 1997, Rudich, Tirosh et al. 1998, Tirosh, Potashnik et al. 1999)and in animal models of insulin resistance (Bonnard, Durand et al. 2008, Matsuzawa-Nagata, Takamura et al. 2008). Elevated ROS were causal in insulin resistance, and pharmacological intervention with scavengers of ROS improved insulin sensitivity (Anhe, Okamoto et al. 2012). Furthermore, a favoured hypothesis of diabetes aetiology suggests that superoxide production is the common pathological pathway inducing the impairments (insulin resistance, β-cell dysfunction, and impaired glucose tolerance) which precede type-2 diabetes (Ceriello and Motz 2004).

Elevations in intracellular and plasma ROS have been reported which correlate to obesity (Higdon and Frei 2003, Vincent, Innes et al. 2007, Hirao, Maruyama et al. 2010). In obese subjects and in animal models elevated markers of protein carbonyls and lipid peroxidation (markers of oxidative damage on proteins and lipids respectively) have been demonstrated (Vincent, Powers et al. 1999, Vincent, Powers et al. 2001, Uzun, Konukoglu et al. 2007).

The pathogenesis of cardiovascular disease is influenced by ROS through the disturbance of endothelial dysfunction, a key step in the early stages of atherosclerosis. One of the key functions of endothelial cells is the production of nitric oxide ($^{\bullet}$NO) to induce vasorelaxation by interaction with underlying SMCs in blood vessels; endothelial dysfunction is characterised by reduced $^{\bullet}$NO availability and increased ROS production. Atherosclerosis has many risk factors including type 2 diabetes, obesity, insulin resistance, hyperglycaemia and hyperlipidaemia, and an elevation in lipids.

In the process of fatty acid oxidation by mitochondria, ROS are generated in the electron transport chain. Mitochondrial ROS generation increases after addition of fatty acids in a diverse array of cells includingskeletal muscle cells (Lambertucci, Hirabara et al. 2008, Seifert, Estey et al. 2010), adipocytes (Subauste and Burant 2007, Gao, Zhu et al. 2010), pancreatic β-islet cells (Gehrmann, Elsner et al. 2010, Lin, Chen et al. 2012), macrophages (Kim, Seo et al. 2012)and hepatocytes (Zhang, Seitz et al. 2010). The literature suggests a difference in the cellular effects exerted between the SFA palmitate and the MUFA oleate in most cells except in β-islets.

Palmitate demonstrated far greater cytotoxicity than oleate at similar concentrations on a range of cells, both *in vitro* (Karaskov, Scott et al. 2006, Yuzefovych, Wilson et al. 2010) and *in vivo*. Palmitate

induces increases in mitochondrial ROS determined by MitoSOX (Koyama, Kume et al. 2011, Lin, Chen et al. 2012, Yuzefovych, Solodushko et al. 2012) or cytosolic ROS by dichlorodihydrofluorescein diacetate (DCFDA) (Morgan, Oliveira-Emilio et al. 2007, Han, Kargi et al. 2010, Lamers, Schlich et al. 2012); whilst such an effect is absent with oleate. The main site of palmitate mediated ROS production is the mitochondria, a site where the fatty acid has been shown to induce membrane depolarisation (Carlsson, Borg et al. 1999).

The cytotoxicity of palmitate extends to the induction of apoptosis and necrosis. Palmitate mediated cell death occurs via different mechanisms dependent upon the cell type, but the main mechanisms appear to be ROS-mediated DNA and mitochondrial damage leading to activation of caspases (Mishra and Simonson 2005)and poly ADP-ribose polymerase (Wei, Li et al. 2013), endoplasmic reticulum stress (Lu, Qian et al. 2013), activation of stress kinases (Lu, Wang et al. 2012) and production of ceramides (Listenberger, Ory et al. 2001).

The present study aims to investigate the effect that the two main physiological fatty acids (see introduction), palmitate and oleate, have on oxidative stress and ROS production in THP-1 monocytes, model cells for primary monocytes which are critical in atherosclerotic disease progression, in addition to determining if changes in such parameters will induce apoptosis or necrosis.

An exploration of oxidative stress was conducted with an examination of the main cellular antioxidant system glutathione and measurement of ROS production from mitochondria. Viability and toxicity measures were achieved through an analysis of mitochondrial membrane depolarisation with JC-1, cell cycle, caspase-3 activity, propidium iodide/annexin-V staining, MTT analysis and trypan blue cell counts. Consequently, analysis of these parameters will determine if subsequent studies of other measures of phenotype or function are a result of cytotoxicity or viable cells.

3.3 Materials and methods

3.3.1 Cell treatments

THP-1 monocytes were treated as described in Material and Methods section 2.3.1 and subsequently analysed for cell viability and redox status using trypan blue exclusion, MTT and glutathione assays, MitoSOX oxidation, JC-1 fluorescence, annexin-V/PI staining, caspase-3 activity, and PI cell cycle staining.

3.3.2 Statistical analysis

Data are expressed as mean±SEM of three independent experiments. Data collected under flow conditions are expressed as mean fluorescence relative to BSA control, whilst those data determined under an absorbance spectrometer data is expressed as mean absorbance. Statistical analyses were performed using one-way ANOVA with Dunnet's post variance analysis. Significance is reported where *p* values are less than 0.05.

3.4 Results

3.4.1 Effects of fatty acids on THP-1 monocyte viability

In order to determine if either palmitate or oleate treatment is toxic, THP-1 monocytes were treated for 6h or 24h before cell counting using trypan blue counts and MTT assays. Human THP-1 monocytic cell line was used to assess viability as these cells have been extensively characterised as a suitable *in vitro* model to study the function of human monocytes (Tsuchiya, Yamabe et al. 1980). Cells were treated for either 6 or 24 hours with FA/BSA then characterised for changes in viability and redox state.

3.4.2 Palmitate but not oleate reduces metabolic activity in THP-1 monocytes by MTT reduction but trypan blue exclusion is not affected

After 6h and 24h incubation with BSA with or without FA, no significant changes in indices of cell death were observed using the trypan blue exclusion assay when cells were viewed under a haemocytometer (figure 3.1A). However cell number was lower after 6h and 24h with palmitate treatment compared to BSA-treated controls. Cells were seeded at 500,000 cells/ml prior to treatment and BSA with or without oleate treated cells continued to proliferate reaching approximately 6-700,000cells/ml, whilst palmitate appears to reduce cell growth possibly eliciting growth arrest (figure 3.1B). In contrast oleate did not induce statisitically significant changes in MTT reduction.

The reduction of MTT to a purple insoluble formazan salt by mitochondrial reductases enables the determination of metabolic activity frequently used as a viability measure. In a dose-dependent manner, at both 6h and 24h, palmitate decreased MTT reducing capacity. These decreases were significant for both 150µM and 300µM palmitate (figure 3.1C, $p<0.01$ and $p<0.001$) at 6h, whilst at 24h the effect was only significant (figure 3.1D, $p<0.001$) with 300µM palmitate.

Metabolic activity was assessed in THP-1 monocytes following 24h treatment with the SFA myristate (C14:0) and stearate (C18:0) to determine the effect of chain length of saturated fatty acids altered effects on metabolic viability. In comparison to palmitate the shorter chain SFA myristate showed no significant reduction in metabolic activity which is contrasted by the longer chain SFA which demonstrates significant reductions in metabolic activity at both 150µM and 300µM (figure 3.1E, $p<0.01$ and $p<0.001$).

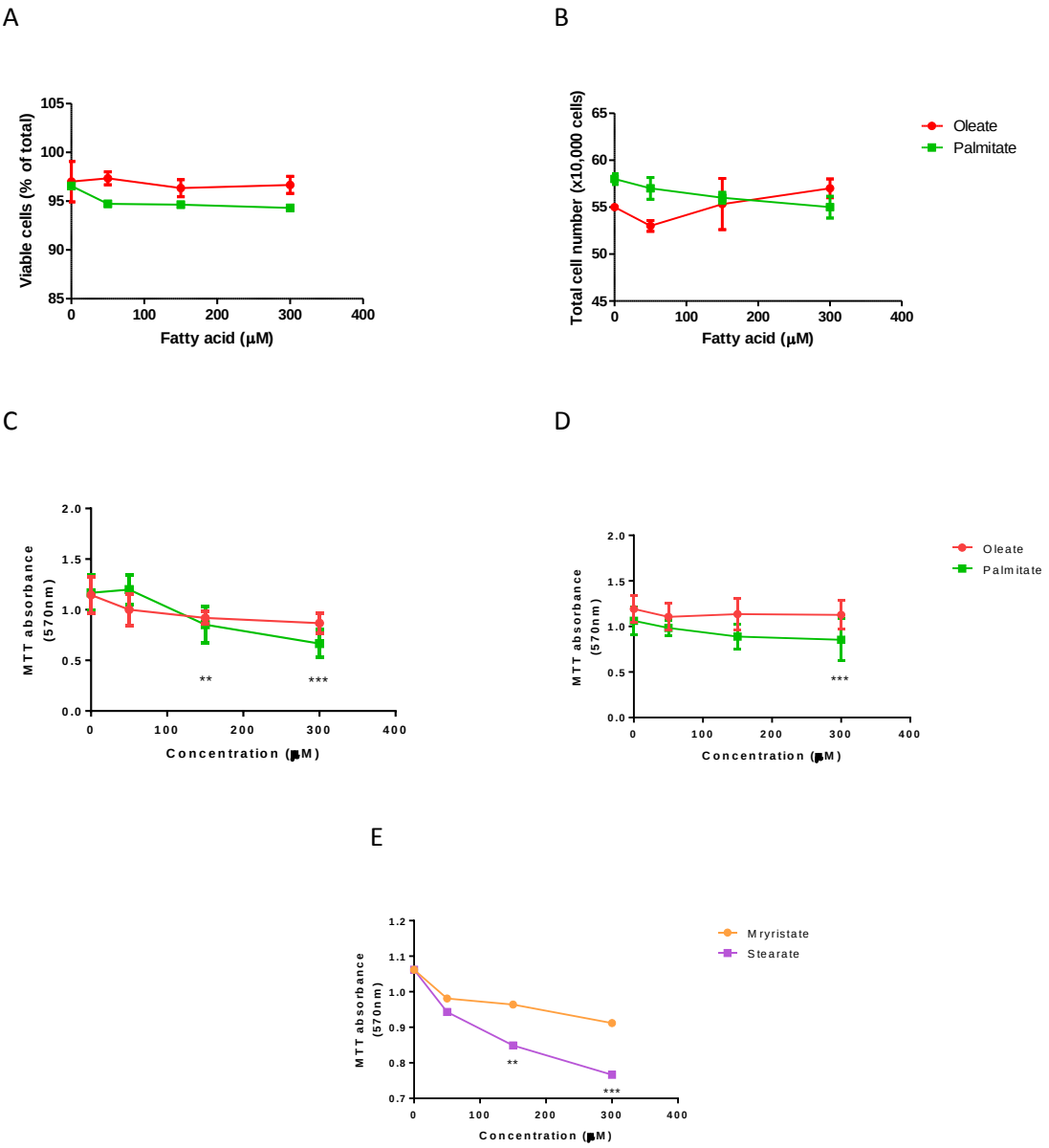


Figure 3.1: THP-1 monocytes treated for 24h with palmitate but not oleate have reduced metabolic activity:

THP-1 monocytes treated with FA/BSA or untreated for 6h and 24h were analysed for cell activity by MTT reduction and viability as trypan blue exclusion. Trypan blue exclusion assay was performed upon experimental completion, where cells were mixed 1:1 with trypan blue before counting under haemocytometer, data is displayed as percentage viable cells (A) and total cell numbers (B) following 24h FA/BSA treatment, where BSA is taken as 0µM FA. Metabolic activity was analysed 2h prior to the end of the experiment; 50,000 cells were transferred to a 96 well plate and incubated with MTT reagent until experimental completion, and lysed overnight with MTT lysis solution before analysis under absorbance spectrometry at 570nm. Effects 6h treatment with oleate and palmitate (C), 24h oleate and palmitate (D) and 24h myristate and stearate (E) are displayed as mean absorbance performed in triplicate and from at least three independent experiments, and statistical significance was determined through one-way ANOVA, where **=p<0.01 and ***=p<0.001.

3.4.3 Glutathione content is decreased in THP-1 monocytes treated with fatty acids

The metabolic inhibition shown as lower MTT reduction by palmitate warranted further investigation of the cellular metabolic state, specifically the primary intracellular antioxidant glutathione. Reduced glutathione was decreased significantly at both 6h (figure 3.2A, $p<0.01$) and 24h (figure 3.2B, $p<0.05$) with 50µM of either oleate or palmitate (~30% and 50% respectively at 6h and ~30% at 24h). Glutathione content at both 6h and 24h was not depleted with 300µM palmitate at 6h or 150µM or 300µM palmitate and 300µM oleate at 24h concentrations was not different to control levels (figure 3.2A and 3.2B).

Oxidised glutathione was not significantly changed with either fatty acid at any concentration studied, indicating that either FA treatment did not induce oxidation of glutathione or that oxidised glutathione was exported. Determination of the redox ratio, ratio of reduced and oxidised forms of glutathione, indicates that 50µM of either fatty acid induces a pronounced drop in redox status (figure 3.2C for 6h; and 3.2D for 24h, $p<0.001$).

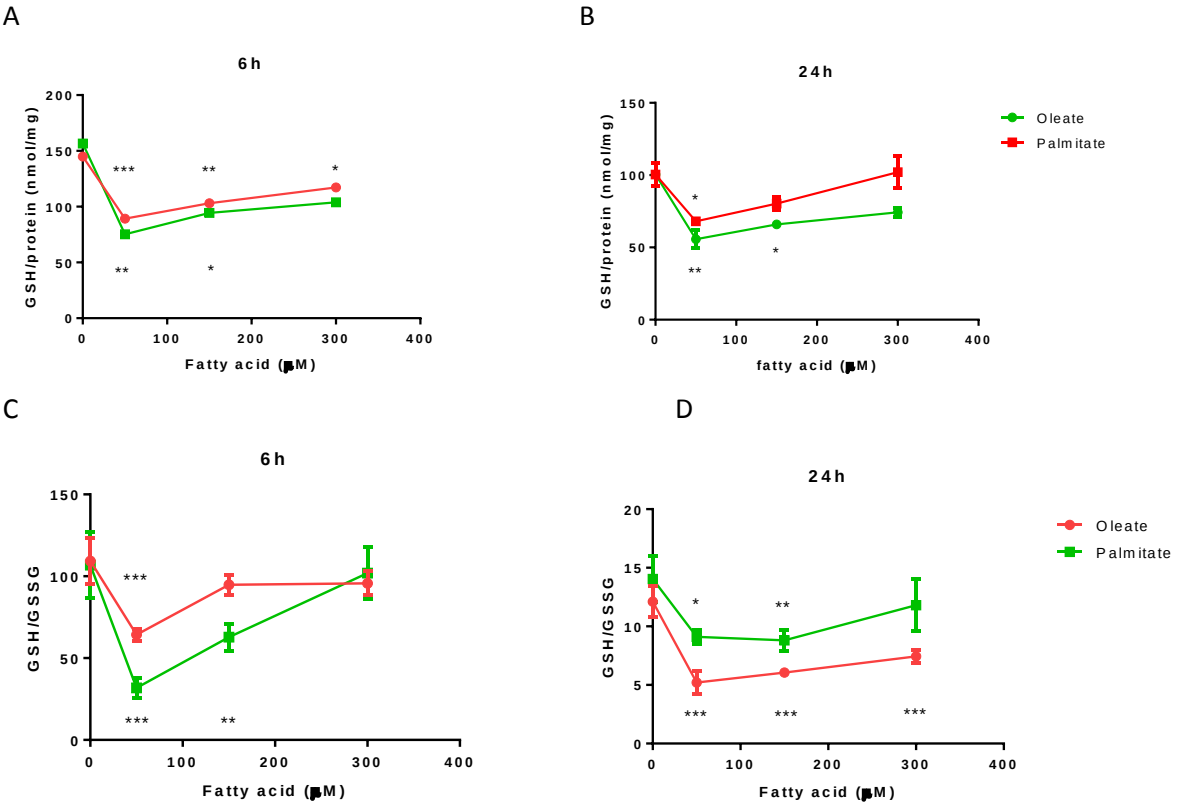


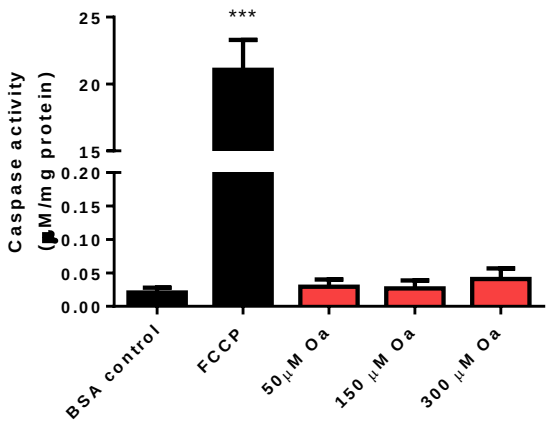
Figure 3.2: Fatty acid treatment alters monocyte glutathione after 6h and 24h treatment:

THP-1 monocytes were treated at $5x10^5$ cells/ml in the presence of 50-300μM FA or BSA control for either 6h or 24h and were analysed for glutathione (figure A and B respectively), and oxidised glutathione content for determination of GSH:GSSG ratio (figures C and D for 6h and 24h respectively). Data is displayed as glutathione content normalised for protein content, or as a ratio of GSH:GSSG content (both values corrected for protein content), presented as mean of at least 3 independent experiments. Statistical significance was determined by one-way ANOVA where *=p<0.05, **=p<0.01 and ***=p<0.001, compared to BSA control.

3.4.4 Palmitate but not oleate increases indices of early apoptosis

Caspase-3 activity was not significantly different from control after FA treatment (Figure 3.3). Apoptotic indices for monocytes with the highest concentration of palmitate were much lower than positive controls UV and FCCP. Further investigation of whether metabolic inhibition was associated with early apoptosis was undertaken after 24h treatment with FA or BSA by analysing annexin-V/PI co-staining and caspase-3 activity as determinants of apoptosis. Under annexin-V/PI co-staining, 300μM palmitate enhanced both early apoptosis and necrosis by ~5% (Figure 3.4C and 3.4D p<0.001). Oleate did not induce necrosis, or apoptosis (figure 3.4A).

A



B

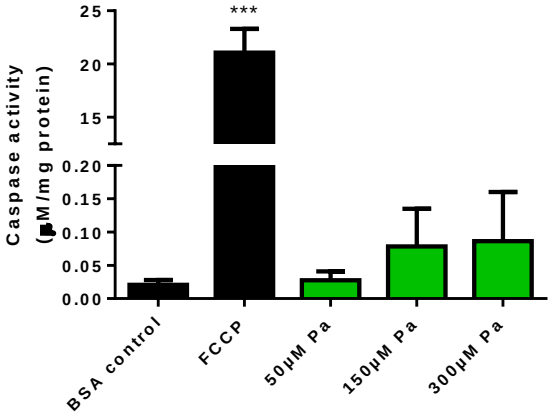
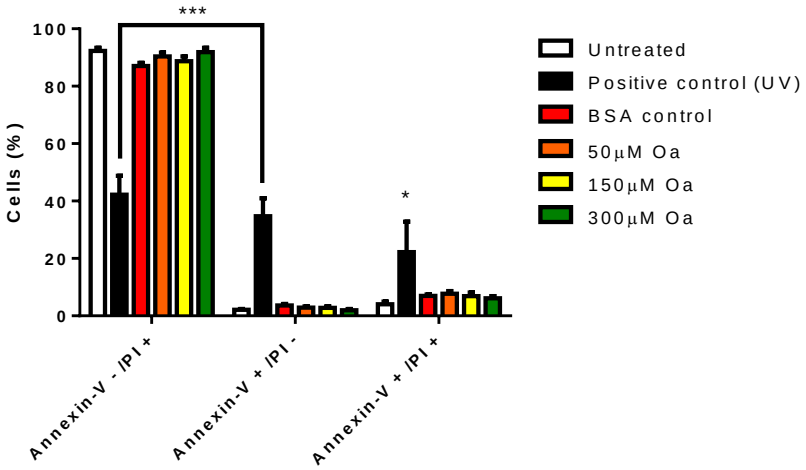


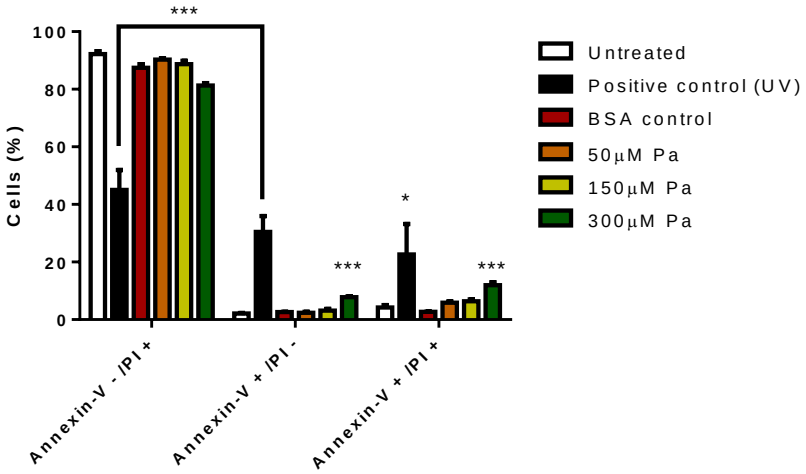
Figure 3.3: Caspase-3 activity in THP-1 is not affected by palmitate or oleate treatment:

THP-1 cells were treated with 50-300µM palmitate or oleate, or BSA control for 24h. Subsequently cells were washed in PBS then analysed for caspase-3 activity using Ac-DEVD-AMC as a substrate. Release of AMC was measured by fluorescence with excitation and emission wavelengths of 360nm and 460nm and was compared to standard curve of AMC concentrations. Oleate (A) and Palmitate (B) cells treated with FCCP for 1 hour at 50µM are used as a positive control. Data is presented as mean caspase activity in nmol/mg of protein performed in triplicate and from at least three independent experiments and statistical significance was determined through one-way ANOVA, where ***=$p<0.001$.

A


***
100
80
60
40
20
0
Cells (%)
*
Annexin-V - /PI +
Annexin-V + /PI -
Annexin-V + /PI +
Untreated
Positive control (UV)
BSA control
50µM Oa
150µM Oa
300µM Oa

B


***
100
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Cells (%)
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Annexin-V - /PI +
Annexin-V + /PI -
Annexin-V + /PI +
Untreated
Positive control (UV)
BSA control
50µM Pa
150µM Pa
300µM Pa

C D

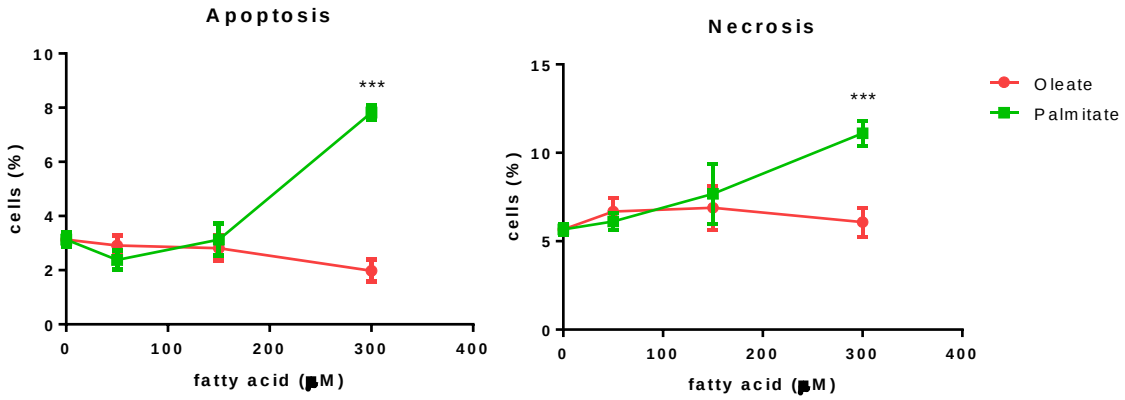


Figure 3.4: Palmitate but not oleate induces apoptosis at 24h:

THP-1 monocytes were incubated for 24h at a cell density of $5x10^5$ cells/ml with increasing concentrations of FA/BSA control and then stained with FITC labelled annexin-V/PI for 30 min prior to analysis under flow conditions. Annexin-V staining was analysed under FL1 (excitation 490nm and emission 525nm) and PI is analysed under FL2 (excitation 536nm and emission 617nm). Data is presented as percentage of cells in apoptosis or necrosis for oleate (A) and palmitate (B) treated cells, and percentage of apoptotic (C) and necrotic (D) cells. Data is presented mean from 3 independent experiments, where statistical significance is determined through one-way ANOVA comparing to BSA control, where ***=p<0.001.

3.4.5 Palmitate alters cell cycle dynamics of monocytes

On the basis of an apparent inhibition of cell growth with palmitate based on cell number counts and the low level induction of apoptosis observed by annexin-V/PI , cell cycle was investigated in FA/BSA treated cells for 24h. Oleate did not alter the proportion of nucleoids in any phase of the cell cycle, in contrast palmitate dose-dependently increased the proportion of apoptotic nucleoids by ~8points (figure 3.5A) in addition to enhancing nucleoids which were in G2/M phase (figure 3.5D); both changes were significant only at 300µM palmitate ($p<0.001$ and $p<0.05$). Dose-dependent decreases in G0/G1, (decreasing by 10% with 300µM, figure 3.5B, $p<0.05$) and S-phase (~3% decrease compared to control, figure 3.5C) were observed at all concentrations. The changes observed with palmitate but not oleate treatment suggest the saturated fatty may be inducing DNA damage causing cells to arrest at G2/M or maybe driving cells to differentiate.

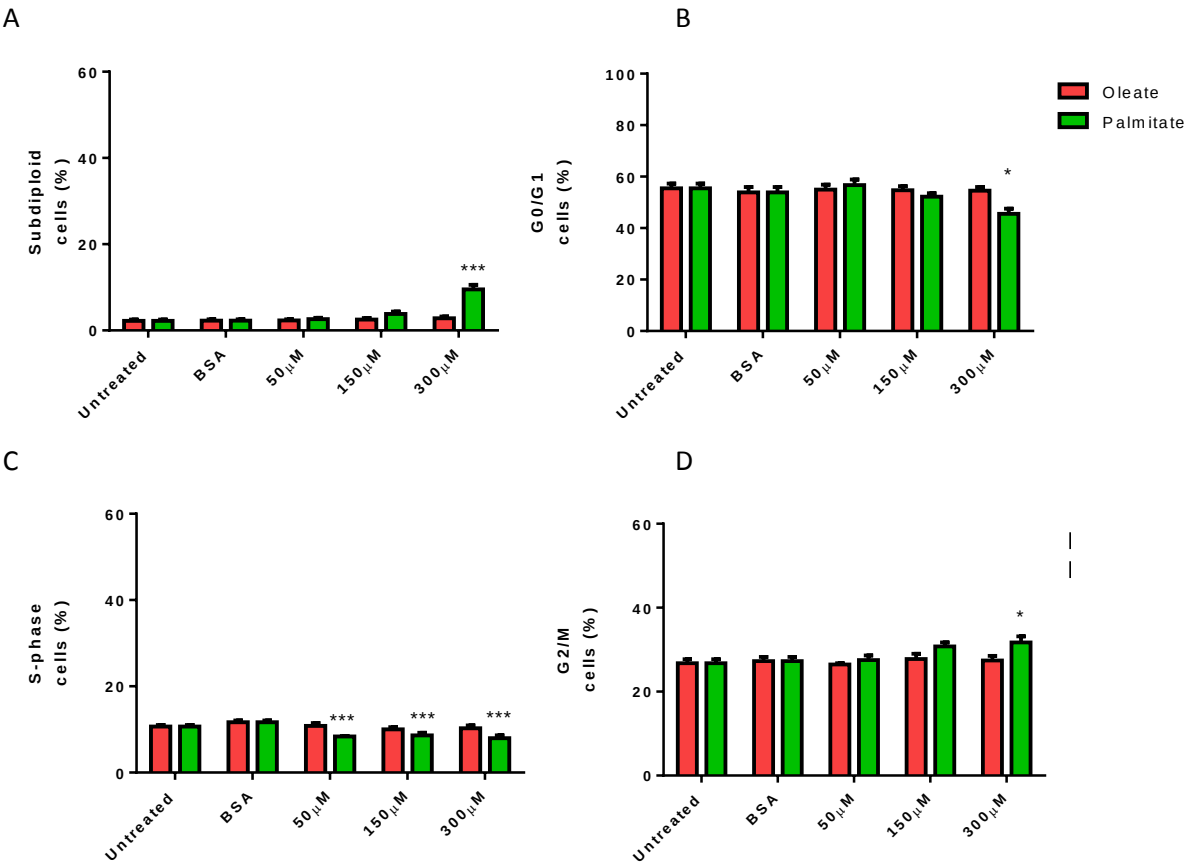


Figure 3.5: Palmitate induces cell cycle arrest at G2/M phase:

Treatment of THP-1 monocytes for 24h were analysed for cell cycle status using PI staining measured under flow conditions. Cells after 24h were treated with PI for 10 min before analysis under FL2 wavelength to determine populations of cells at sub-diploid/sub-G_0 (A), G1/G0 phase (B), S phase (C) and G2/M phase (D). Data is expressed as a percentage of cell population as the mean of n=5 independent experiments, and statistical significance is determined by one-way ANOVA in comparison to control where *=$p<0.05$, ***=$p<0.001$ and control are BSA treated cells.

3.4.6 Increased ROS production and altered membrane potential in mitochondria are induced by palmitate

In the presence of palmitate a dose-dependent increase in mitochondrial ROS production, as determined by MitoSOX fluorescence, was observed with palmitate which was significant at 300µM palmitate after both 6h and 24h (figure 3.6 and 3.7). A non-significant increase in mitochondrial ROS is observed with oleate.

Previously published work of palmitate's effect on mitochondria has suggested the SFA is capable of acting as an uncoupler within the organelle and inducing an alteration in mitochondrial membrane potential, thus JC-1 was used to investigate membrane potential change. The degree of red fluorescence reflects JC-1 dye concentrated within the mitochondria, whilst green fluorescence reflects leakage from mitochondria typically observed in apoptotic or necrotic cells. THP-1 cells treated with 300µM palmitate showed no significant change in membrane depolarisation over 24h (figure 3.8 and 3.9), whilst FCCP elicited a significant uncoupling.

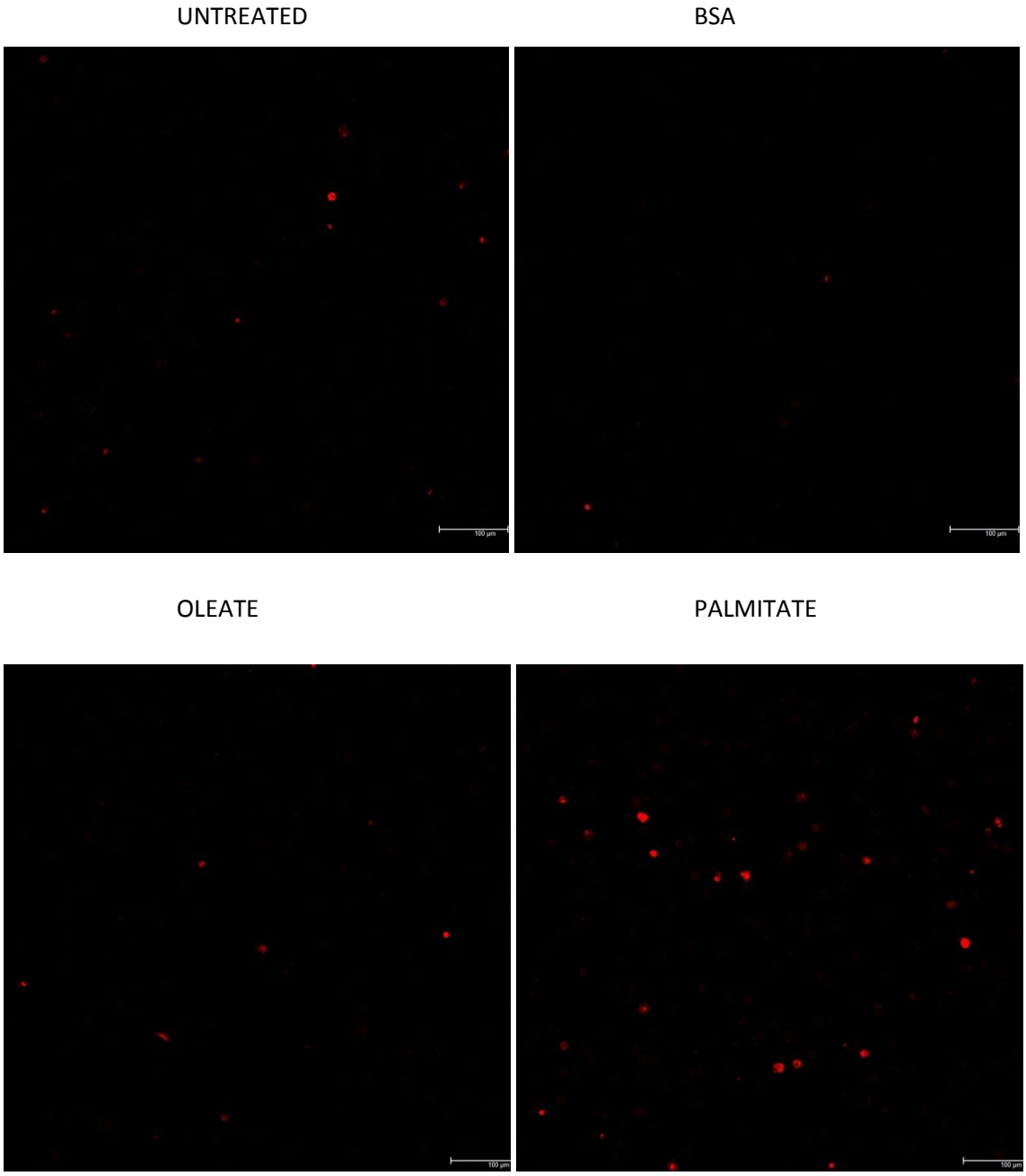


Figure 3.6: MitoSOX oxidation by THP-1 monocytes treated for 24h with FA/BSA:

THP-1 cells treated for 24h with FA/BSA (300µM) were washed with PBS prior to treatment with 2.5µM MitoSOX for 30 min in the dark at 37° before analysis under confocal microscopy. Untreated, BSA treated, 300µM oleate and 300µM palmitate images were obtained using Leica microscope under 20 x magnifications, with excitation at 488nm and emission at 580nm.

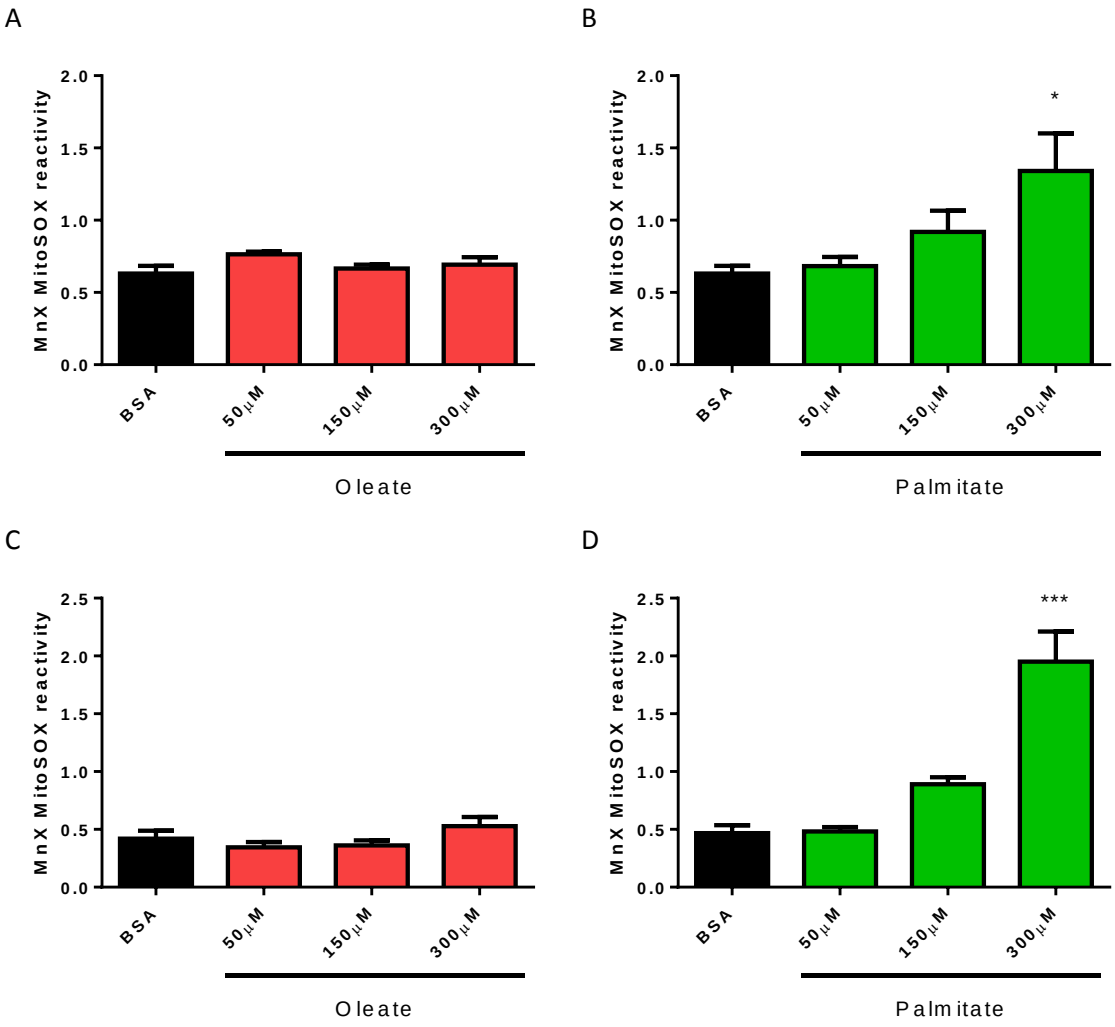


Figure 3.7: Palmitate but not oleate induces increases in mitochondrial superoxide formation:

THP-1 monocytes were treated with increasing concentrations of FA (50-300μM oleate or palmitate) or BSA control for 24h (A Oleate and B Palmitate) and 6h (C Oleate and D Palmitate) , before washing twice in PBS and treatment with 2.5μM MitoSOX in the dark at 37° for 30 min. Subsequently cells were analysed for MitoSOX fluorescence under flow conditions under the FL2 wavelength spectra (564-606nm). Data is presented as mean X value (MnX) of FL2, as an average of 3 independent experiments performed in triplicate; statistical significance is determined using one-way ANOVA with BSA as control, where ***=p<0.001 and * p<0.05.

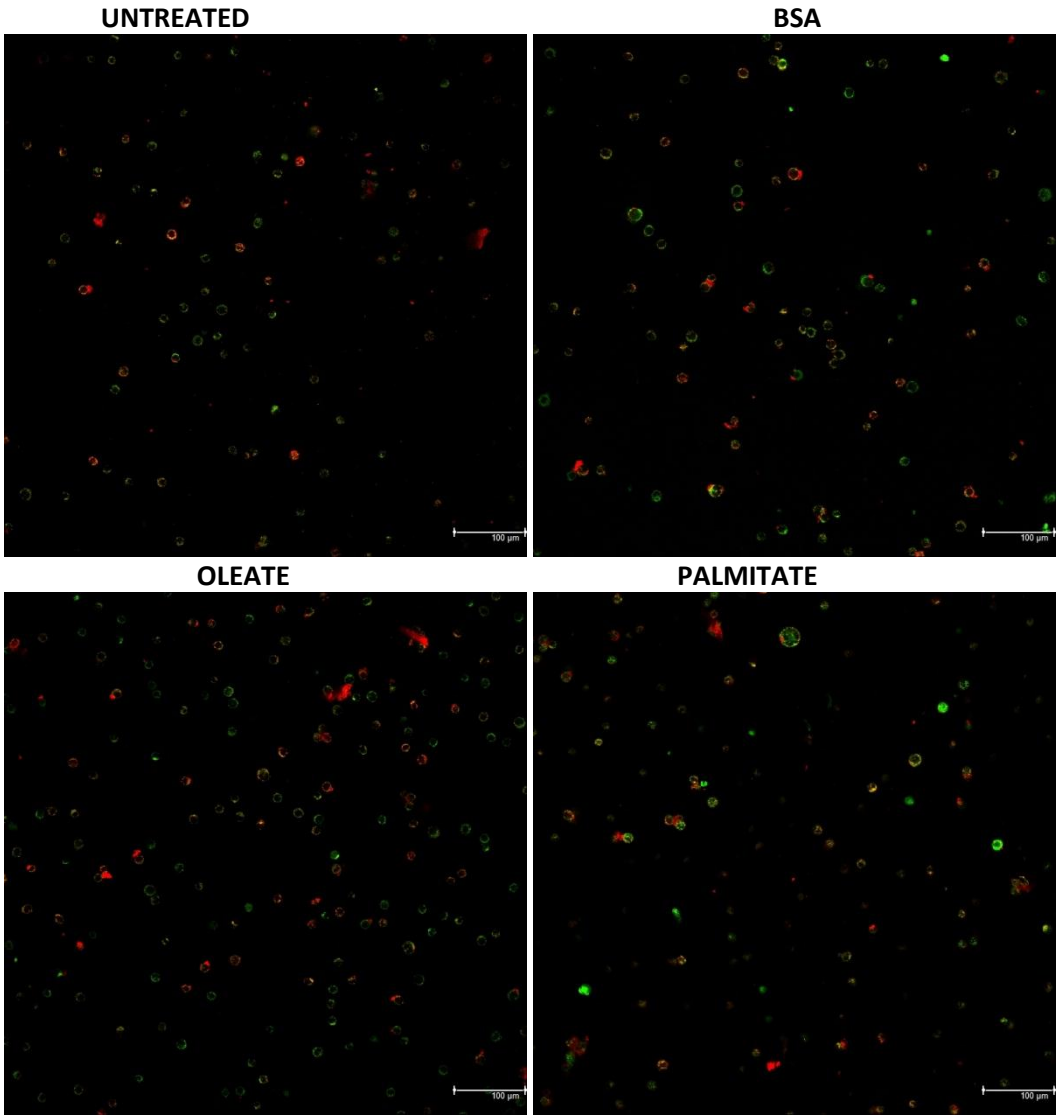


Figure 3.8: JC-1 fluorescence generated following 24h treatment of THP-1 monocytes with FA/BSA:

THP-1 cells treated for 24h with FA or BSA control were subsequently washed with PBS before treatment with 10µg/ml of JC-1 reagent at 37° in the dark before imaging using a Leica confocal microscope, (ARCHA imaging suite). Images of untreated (A), BSA control (B), oleate (C) and palmitate (D) treated cells were taken under 20x magnification.

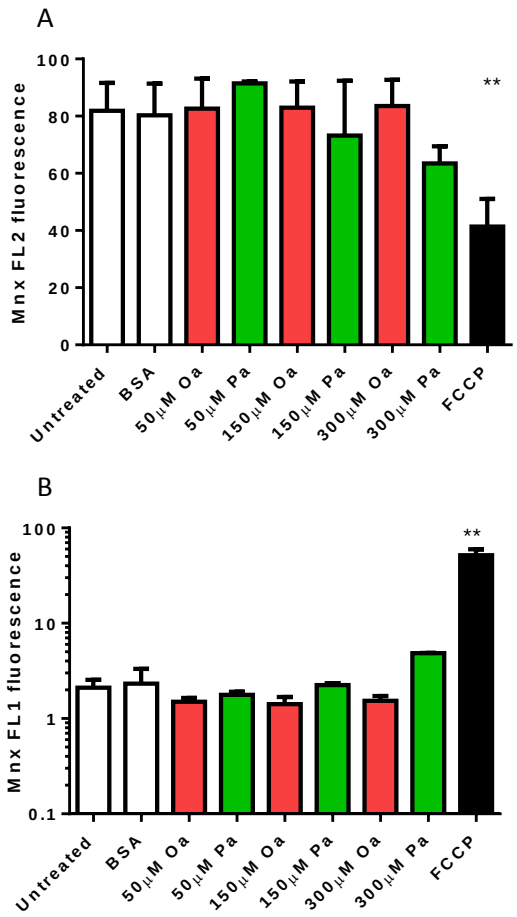


Figure 3.9: Membrane depolarisation of THP-1 monocyte mitochondria is not altered following palmitate or oleate treatment:

Cells were treated for 24h with FA or BSA control then analysed for JC-1 fluorescence, where red fluorescence is generated from aggregation in healthy mitochondria and green fluorescence is generated by monomers released by depolarised mitochondria, under flow conditions (excitation at 488nm emission for the green monomer form is 514-529nm and the emission for the red aggregate form is 585-590nm). Data is presented as FL2 fluorescence for aggregate form (red fluorescence) and FL1 fluorescence for monomer form (green fluorescence) displayed as mean of 3 independent experiments as mean X values (MnX). Statistical significance was determined from one-way ANOVA where **=$p<0.01$ compared to BSA control.

3.5 Discussion

The objectives of this chapter were to determine the effects of both the MUFA oleate and SFA palmitate on THP-1 monocytes with respect to cell and metabolic viability, any induction of apoptosis or necrosis, effect on redox status and mitochondrial ROS production. The results presented indicate differential effects of a SFA compared with a MUFA.

The MUFA oleate has no significant effect on viability, apoptosis/necrotic, mitochondrial ROS production or cell cycle dynamics, being no different to BSA or untreated background controls. The SFA palmitate adversely affects cell number and metabolism, mitochondrial ROS production and mitochondrial membrane potential in addition to increasing indices of early apoptosis and necrosis.

Novel effects of both fatty acids on redox status were observed; specifically the reduced but not oxidised forms of the cellular antioxidant glutathione were decreased significantly by both FA at 50μM. The similarity of effect observed with both fatty acids may relate to the handling of fatty acids at these concentrations. As discussed in the introduction, SFA and MUFA are metabolised differently, and possibly this differential handling does not yet occur at lower concentrations, therefore at 50μM both fatty acids are directed toward β-oxidation which leads to a decrease in reduced glutathione. An alternative possible explanation for the decrease in GSH with no increase in GSSG that would be associated with oxidative stress could be the conjugation of GSH with FA.

Another explanation could be the effect of fatty acids on the availability of $NADP^+$ synthesis. In its reduced form NADPH is required to restore GSH from GSSG, however the oxidised form of GSSG is not retained by the cell and is instead exported through the ABCA1 transporter. Thus as GSSG is exported, the GSH level will decline without any obvious effect on oxidised glutathione content.

Current data available on the interplay between fatty acids and glutathione focuses on the polyunsaturated fatty acids (PUFA). PUFA (more than one unsaturated bond) are grouped depending on where the carbon carbon double bonds reside, including ω-3 and ω-6 fatty acids and are required for human health (Spector 1999). The high degree of unsaturation of PUFA makes them vulnerable to lipid peroxidation leading to the generation of 4-hydroxyhexenal (4-HHE) and 4-hydroxy-2-nonenal (4-HNE) for ω-3 and ω-6 fatty acids respectively (Gueraud, Atalay et al. 2010). 4-HNE is highly reactive especially toward the nucleophillic sites present on DNA and proteins, in addition to high reactivity with phospholipids (Catala 2010).

The detoxification of 4-HNE is achieved through conjugation with glutathione (Srivastava, Chandra et al. 1998) or conversion to corresponding alcohol by aldehyde reductases (Reichard, Vasiliou et al.

2000, Alary, Gueraud et al. 2003, Siems and Grune 2003). At a cellular level the induction of stress via 4-HNE induces the antioxidant response element (*ARE*) via the activation of NF-E2-related factor (NRF2) (Iles and Liu 2005). The latter is under repressor control by KEAP-1, mitigating the degradation of NRF2, but under conditions of increased oxidative stress this interaction is disrupted allowing nuclear translocation of NRF2, dimerising with Maf proteins and binding to the *ARE*. Subsequently the cells have enhanced capacity to remove excess electrophiles and restore redox status with upregulation in glutamate cysteine ligase (Zhang, Court et al. 2007) the rate limiting enzyme in glutathione synthesis, therefore restoring cellular glutathione.

After ω-3 PUFA oxidation, the induction of NRF2 has been demonstrated, with a lipid peroxidation product of ω-3 PUFA, isoprostane which is analogous to the J-series cyclopentenone prostaglandins, inducing KEAP-1 dissociation from NRF2 (Levonen, Landar et al. 2004).

There is evidence that both MUFA and SFA can increase levels of glutathione. Dou et al. showed palmitate, in a dose-dependent manner, to increase GSH at 8h and 16h in hepatocytes (Dou, Wang et al. 2011). Such findings were replicated by Garcia et al. (2011) using HepG2/C3A hepatocytes and primary hepatocytes treated with either oleate or palmitate. By overloading primary cells with oleate and/or palmitate (1mM) an increase in GSH was induced, although the effect was not as pronounced with palmitate alone (Garcia, Amankwa-Sakyi et al. 2011). These concentrations are much higher than those investigated here and are non-physiological.

Whilst the activation of NRF2/*ARE* pathway may increase de novo glutathione synthesis via lipid peroxidation products of PUFA interacting with KEAP-1, whether this is the case for MUFA and SFA is unclear. The combination of both palmitate and oleate (2:1 ratio, 2mM for 72h) has been shown to increase lipid peroxidation in pancreatic islets (Piro, Rabuazzo et al. 2012) and hepatocytes (Anavi, Harmelin et al. 2012) which raises the possibility of NRF2 activation by SFA and MUFA or through their metabolites.

However the data concerning the production of MDA by either FA alone is mixed and may be cell type and concentration dependent. MDA levels are either elevated (Oh, Choi et al. 2012) or unaffected in response to palmitate treatment (Petrussa, Braidot et al. 1992, Song, Song et al. 2007, Dou, Wang et al. 2011); in T-lymphocytes MDA was elevated dose-dependently with palmitate (Stentz and Kitabchi 2006). Whilst palmitate can increase lipid peroxidation products oleate cannot suggesting that any mechanism of altering glutathione maybe independent of lipid oxidation. This is consistent with the lack of effect of either fatty acid on mitochondrial superoxide production at 50μM.

Thus, the picture regarding the effect of fatty acids on glutathione status remains unclear and warrants further investigation. Here, the SFA palmitate increased mitochondrial ROS at 6h and 24h but did not uncouple the mitochondria.

In many studies palmitate mediated increased ROS were measured using DCF, DCFDA or DHE fluorescence, whilst mitochondrial specific superoxide was not measured in other studies, although the data generated from all probes is still in agreement with data in this chapter. However, there are some cases where induction of ROS production by palmitate is not observed, for example Dou et al. did not find changes in DCF fluorescence following 16h incubation with 0.2mM palmitate in hepatocytes (Dou, Wang et al. 2011), although it can be argued that this concentration is not high enough to produce ROS formation or cellular antioxidant levels such as that of glutathione maybe higher in these cells.

The experimental time points maybe particularly important, as induction of ROS or superoxide production by fatty acids can be linked to uptake and metabolism within the cell. The findings of increased ROS production described here, specifically from the mitochondria, are the first example of monocytes generating mitochondrial ROS following palmitate but not oleate treatment and are in agreement with other cell lines demonstrating the ROS-generating nature of the SFA.

Few studies have considered the effects of oleate on ROS production. In human and rodent primary neutrophils, oleate dose-dependently increased ROS production measured by DCFDA fluorescence (Hatanaka, Levada-Pires et al. 2006, Carrillo, Cavia et al. 2011), and similar observations have been reported in INS beta cells (Maestre, Jordan et al. 2003, Koulajian, Desai et al. 2013) and fibroblasts (Hatanaka, Dermargos et al. 2013). In smooth muscle cells, oleic acid mediated vascular growth which relied upon the formation of ROS (Lu, Greene et al. 1998). It is likely that NOX activity is regulated by oleate in these studies. In human primary lymphocytes (Stentz and Kitabchi 2006), human hepatoblastoma C3A (Kohjima, Enjoji et al. 2009); and L6 myotubes (Yuzefovych, Wilson et al. 2010) no change in ROS production was observed with oleate.

However, oleate is capable of inducing endoplasmic reticulum stress in CaCo2 cells in a dose-dependent manner (Chen, Li et al. 2011), a cellular toxicity response and often observed prior to the induction of apoptosis. A recent paper focusing on the involvement of fatty acids in glucose stimulated insulin secretion proposed a possible mechanism for oleic acid-dependent ROS production. Santos et al. demonstrated that short term exposure INS-1 β-islet cells to oleic acid led to the activation of NADPH oxidase and subsequent ROS production, which were dependent upon the oxidation of oleic acid by the mitochondria (Santos, Rebelato et al. 2011).

Furthermore, antioxidant effects of oleate have been reported in human umbilical vein endothelial cells (HUVECs), with oleate counteracting the LPS induced depletion in glutathione and ROS scavengers (Massaro, Basta et al. 2002). Oh et al. demonstrated that LPS-mediated ROS production was quenched by oleate in stimulated microglial cells (Oh, Lee et al. 2009). Similarly, oleate counteracts antimycin A induced mitochondrial ROS production in epithelial cells (Duval, Auge et al. 2002); and in rat cardiomyocytes, oleate mitigates TNF-α induced oxidative stress (Al-Shudiefat, Sharma et al. 2013). Here, the MUFA oleate has no effect on THP-1 monocytes, with no significant changes on cell viability, apoptosis/necrosis or mitochondrial membrane potential or superoxide production.

The main site of ROS production in the non-phagocytic, respiring cell is the mitochondria, and as demonstrated here in THP-1 monocytes, palmitate dose-dependently increases mitochondrial ROS. These findings are supported by other data, with increased ROS production demonstrated in cortical , human umbilical vein endothelial (Zhang, Gao et al. 2013), human aortic endothelial (Stentz and Kitabchi 2006, Chinen, Shimabukuro et al. 2007, Maloney, Sweet et al. 2009), pancreatic beta(Lin, Chen et al. 2012, Maris, Robert et al. 2013), astroglial (Almaguel, Liu et al. 2009), muscle (Gao, Zhao et al. 2011, Tatebe and Morita 2011), adipocyte (Davis, Gabler et al. 2009, Han, Kargi et al. 2010), cardiomyoblast cell lines (Zhu, Yang et al. 2011, Wei, Li et al. 2013).

The elevated mitochondrial ROS observed following palmitate but not oleate treatment were not reflected by changes in some early apoptotic markers/necrosis. Analysis by annexin-V/PI co-staining and caspase-3 indicated no significant induction of apoptosis in the latter; this was substantiated by trypan blue exclusion analysis. However metabolic activity, as determined by MTT reduction, was significantly reduced by palmitate but not oleate. Analysis of annexin-V/PI co-staining in THP-1 monocytes treated with palmitate demonstrated an increase in apoptosis and necrosis, which is inconsistent with caspase-3 data. The elevated apoptosis and necrosis observed may be explained by the lack of compensation when dual staining, which can elevate the fluorescence observed.

The induction of apoptosis and reduction in cell viability is a common observation with palmitate treatment. In hepatocytes, treatment with 0.7mM palmitate increased annexin-V/PI co-staining, and caspase-3 activity (Zhang, Seitz et al. 2010)]. Palmitate induced apoptosis measured by caspase activity has been observed in human salivary gland epithelial cells (Shikama, Ishimaru et al. 2013), cardiomycocytes (Wei, Li et al. 2013), INS-1E pancreatic β-cells (Maris, Robert et al. 2013)and L6 myotubes (Yuzefovych, Solodushko et al. 2012).

The degree of apoptosis induced in previous studies is in response to higher concentrations of SFA, which could be supra-physiological and consequently may not accurately reflect the situation in vivo, as 0.2-0.4mM non-esterified fatty acids are typically observed (Frayn, Williams et al. 1996, Karpe, Dickmann et al. 2011). Therefore, the disparity in the lack of apoptosis observed here with THP-1 monocytes compared to other studies may reflect the use of higher FA concentrations and different cellular responses.

The lack of effect of oleate on cell viability and apoptosis/necrosis is in line with the majority of current literature, indeed Gao et al.. demonstrated the protective effect of oleate in preventing palmitate mediated toxicity in skeletal muscle cells (Gao, Griffiths et al. 2009). Nevertheless in INS-1 pancreatic β-cells 0.5mM oleate induced apoptosis (Karaskov, Scott et al. 2006)and in human primary blood mononuclear and Raji cells, oleic acid (0.1m-0.2mM) was sufficient to reduce cell viability, increase DNA fragmentation and annexin-V staining (Cury-Boaventura, Pompeia et al. 2005).

Thus a majority of studies support the findings that palmitate has a greater cytotoxic potential compared to oleate. In other studies, oleate appeared to mediate cytotoxicity when the fatty acids were prepared in ethanol and not conjugated to BSA prior to treatment of cells, i.e. as free fatty acid and the toxicity of NEFA is far greater if unconjugated. In this thesis, any effects of palmitate are unlikely to be attributed to induction of cell death, but are more likely a consequence of palmitate mediated changes signalling or metabolism.

The disparity between palmitate and oleate with respect to ROS production and cell growth effects led to an investigation of cell cycle dynamics using nucleoid staining with PI. The MUFA oleate did not elicit any changes on cell cycle dynamics, in contrast to palmitate. The SFA increased sub-diploid and G2/M nucleoid populations at 300µM palmitate at 24h, with a concomitant decrease in nucleoids in G0/G1. The altered cell populations reflect cells undergoing arrest at G2/M phase, normally due to increased DNA damage or inhibition of DNA repair. The DNA repair enzyme PARP is strongly influenced by cellular ATP and NAD.

In 3T3-L1 adipocytes and primary preadipocytes, 500µM palmitate mediates an ~8 fold increase in the sub-diploid/G_0 population measured by PI FACS (Guo, Wong et al. 2007). In human umbilical venous endothelial cells (HUVECs) 125µM palmitate increases the sub G_0 population (Choi, Kang et al. 2007), whilst in hepatocytes 0.7mM was sufficient to generate a small population of these cells (Zhang, Seitz et al. 2010). In human glioma cell lines, 150µM palmitate was sufficient to increase the sub G_0 population and induce G2/M phase arrest (Marcilla-Etxenike, Martin et al. 2012).

With regards to mitochondrial activity, both an increase in mitochondrial superoxide production and concordant reduction in MTT reducing capacity indicate possible mitochondrial dysfunction following treatment with the 300µM SFA rather than the MUFA. Fatty acids have previously been reported to induce uncoupling in mitochondria, diverting electrons directly to oxygen away from energy substrate production. Here, at 24h 300µM palmitate treatment had no significant effect on mitochondrial coupling. In HepG2 cells, 0.2mM palmitate over 6 hours induced a depolarisation of mitochondrial membrane potential by increased fluorescence generated by JC-1 (Denk, Kleiss et al. 2012). Palmitate induced mitochondrial membrane potential depolarisation has also been observed in primary mouse beta cell (Remizov, Jakubov et al. 2003), MIN6 β-islet cells (Joseph, Koshkin et al. 2004, Koshkin, Dai et al. 2008) and in isolated mitochondria (Mironova, Belosludtsev et al. 2007). A possible explanation of how palmitate induces altered membrane potential in other cells is via SFA induced cell death through cytochrome c release.

Mitochondria are closely linked to both metabolic activity and cell death. The mitochondria themselves consist of two membranes, the inner and outer membrane, with the former being impermeable to ions. This feature permits the respiratory complexes I-IV to form a proton gradient required to generate energy substrates during oxidative phosphorylation. It is this gradient that forms the inner mitochondrial membrane potential ($\Delta\psi$), and ultimately is used by ATP synthase to generate ATP. The outer membrane is freely permeable to small metabolites and solutes via the voltage dependent anion channel (VDAC). A common observation prior to apoptosis is mitochondrial membrane depolarisation (MMP) via the formation of a permeability transition pore (PTP), which involves a loss in $\Delta\psi$ (Basanez, Soane et al. 2012).

The PTP usually forms as a complex of proteins extending from the inner membrane to the outer membrane with VDAC and adenine nucleotide translocase (ANT) present in either membrane respectively. ANT is the most abundant of mitochondrial proteins, facilitating the exchange of ATP^{4-} with ADP^{3-} down the electrochemical gradient. The ANT can reside in either the 'm' or the 'c' conformation, with the former permitting translocation of adenine nucleotides, whilst the latter inhibits transport activity. Palmitate, as its fatty acyl CoA, interacts with ANT displacing ADP and inducing the 'c' conformation and consequently induces the formation of the inner component of the PTP (Skulachev 1998, Skulachev 1999, Kadenbach 2003). This effectively bypasses the electron transport chain and leads to uncoupling of mitochondria and enhanced ROS production, a finding which has been studied in great detail by Wojtczak et al.(Wojtczak and Schoenfeld 1993, Bernardi, Penzo et al. 2002, Schoenfeld and Wojtczak 2008).

In rat pancreatic β-cells, JC-1 oxidation was increased, a decrease in Δψ and increased ROS were recorded following 24h treatment with 0.2mM palmitate, which the authors attributed to mitochondrial uncoupling (Carlsson, Borg et al. 1999). In animal models of high dietary fat intake in which high proportions of energy intake come from palmitate, mitochondrial uncoupling has also been shown (Cole, Murray et al. 2011). Palmitate acts as a protonophore uncoupling mitochondria and enhancing mitochondrial ROS production. However in monocytes, mitochondrial ROS production was increased without significant uncoupling by palmitate but not oleate.

Palmitate once converted to palmitoyl-CoA enters the mitochondria through carnitine palmitoyl transferase-1 (CPT-1). During metabolism, palmitoyl-CoA may alter the conformation of ANT uncoupling respiratory metabolism and initialise the formation of the PTP, and the release of mitochondrial proteins including cytochrome c. In the cytosol, cytochrome c interacts with an adaptor protein Apaf-1, to process procaspase-9 to caspase-9. In turn caspase-9 cleaves and activates procaspases-3 and -7, and these effector caspases cleave proteins and permit apoptosis(Jeong and Seol 2008, Sinha, Das et al. 2013).

Here 300μM palmitate did not increase caspase-3 activity in THP-1 monocytes and the increase in apparent apoptotic/necrotic cells may be also in part due to growth failure. Furthermore observations of mitochondrial function suggest the palmitate primarily affects this organelle, and may not induce global toxicity, which is in agreement with trypan blue exclusion that demonstrated no effect under either palmitate or oleate. Certainly, data generated from cell cycle analysis suggest palmitate treated cells are leaving a proliferative state, and perhaps considering the fact that THP-1 cells are monocytes and have the potential to differentiate into macrophages, the possibility that cells are entering a differentiating state cannot be excluded.

In NIR-1 pancreatic cells, 0.5mM palmitate induced apoptosis over 12h, 24h and 48h through the induction of ROS largely derived from NOX, whilst only a small proportion was mitochondrial. Fatty acid treatment also activated stress kinases p38 and JNK, in addition to p53, all contributing to the mitochondrial activation of Bax, a pro-apoptotic mitochondrial protein (Yuan, Zhang et al. 2010). Similarly, in CHO cells, induction of apoptosis by palmitate was associated with ROS, and in the presence of antioxidants reduced caspase-3 activity and DNA laddering (Listenberger, Ory et al. 2001).

Another mechanism of SFA induced toxicity and/or stress may also relate to synthesis of ceramides. Ceramides are a lipid second messenger which in response to stressors such as TNF-α, heat shock

and ionising radiation are synthesised through sphingomyelin hydrolysis and activate ceramide activated protein kinases and protein phosphatases which may lead to apoptosis (Woodcock 2006).

In L6 myotubes, ceramides induce mitochondrial ROS production, caspase-3 activation, increase JNK activation, and induce apoptosis following 500μM palmitate which was via de novo ceramide synthesis (Yuzefovych, Wilson et al. 2010). Ceramide-dependent apoptosis following SFA treatment has also been demonstrated in retinal pericytes (Cacicedo, Benjachareowong et al. 2005), pancreatic β cells (Zhang, Ranta et al. 2009), leydig, Schwann cells (Suzuki, Akahane et al. 2011) and diabetic rats (Shimabukuro, Zhou et al. 1998). There is evidence for both oxidative stress and ceramide dependent pathway involvement in palmitate mediated toxicity and apoptosis. In addition palmitate can induce endoplasmic reticulum (ER) stress.

ER stress is sensed by inositol requiring enzyme (IRE) 1α, PKR ER kinase (PERK) and activating transcription factor 6α (ATF). These proteins activate signals to restore ER function, collectively known as the unfolded protein response, which down regulates protein translation, increases ER chaperone protein and protease synthesis which enhances protein refolding and degradation of misfolded proteins. Activated PERK will activate eIF2α reducing global protein translation, but also increasing translation of ATF4, which induces the transcription of pro survival genes. Under sustained stress ATF4 will activate CHOP to inhibit the expression of anti-apoptotic Bcl-2 and bind to the PUMA promoter upregulating pro apoptotic proteins (Jaeger, Bertrand et al. 2012).

In hepatocytes and hepatocarcinoma cell lines, palmitate increases ATF4 mRNA; findings replicated in INS-1 pancreatic β-cells. Observations of upregulated ER stress proteins in response to palmitate have been demonstrated in mesenchymal stem cells (Lu, Wang et al. 2012), adipocytes (Jeon, Leem et al. 2012), podocytes (Tao, Wen et al. 2012), and animal models (Mishra and Simonson 2005, Yuzefovych, Solodushko et al. 2012).

Induction of apoptosis reported by others following palmitate treatment often results from a concentration of SFA greater than the 300μM used in this study. Furthermore apoptosis induction at concentrations similar to or lower than that used in this study could reflect differences in preparation of fatty acids prior to treatment of cells. In this thesis, FA has been conjugated to BSA at a ratio of 6:1, which is commonly used by others (Mishra and Simonson 2005). This ratio was derived from the work of (Reed 1986) who showed bovine serum albumin binding capacity for FA of up to 6.9mol of palmitic acid per mol of BSA, where such ratio rarely induces toxicity. Other studies where palmitate toxicity has been shown rely on supplementing fatty acids directly into cell media (Listenberger, Han et al. 2001, Listenberger, Ory et al. 2001).

Many of the previously mentioned studies use skeletal muscle, adipocytes, hepatocytes or heptocarcinoma cell lines or pancreatic β-cells. A recent study by Choi et al. demonstrated that 200μM palmitate increased in JNK activity in THP-1 monocytes, which the authors related to ROS production and possibly ER stress but not apoptosis (Choi, Kim et al. 2011). The ROS generating effect of palmitate in monocytes has also been reported by Dasu et al.(Dasu and Jialal 2011). Ishiyama et al. demonstrated that palmitate induced upregulation of LOX-1 due to ER stress (0.2mM palmitate, FA:BSA ratio of 6:1), albeit these observations were seen in differentiated THP-1 macrophages (Ishiyama, Taguchi et al. 2011). Other studies conducted with THP-1 monocytes have focused on the inflammatory aspect of the immune cell in response to fatty acids (Bunn, Cockrell et al. 2010, Schwartz, Zhang et al. 2010). In these studies much of the focus has been on the phenotypic and functional changes effected by palmitate treatment using concentrations of fatty acid close to those used in this study (200-400μM), thus suggests that these are the concentrations in which palmitate induces signalling and effects which are unrelated to stress and cell death.

3.6 Conclusion

In summary, in this chapter the effects of the SFA and the MUFA palmitate and oleate respectively were investigated with respect to cell viability, apoptosis, oxidative stress and mitochondrial function. Palmitate but not oleate reduced metabolic activity and total cell number. Cell cycle analysis shows palmitate-treated cells entering cell cycle arrest. Furthermore mitochondrial ROS were increased but membrane potential was unchanged in response to palmitate but not oleate.

Whilst other studies do report cytotoxicity with palmitate, but not oleate, concentrations used are higher, (typically 400μM or above), than those used here. Furthermore previous studies using the THP-1 monocytes with similar concentrations of palmitate do not demonstrate overt toxicity, instead highlighting the phenotypic changes and activation of signalling pathways.

The observation of altered glutathione content which is mirrored in both palmitate and oleate treatment indicates a unique effect on antioxidant status irrespective of saturation or unsaturation which has not been reported by others, but requires further investigation due to the paucity of available data.

The results highlight the differential effects of saturated and SFAs with respect to mitochondrial ROS and metabolic activity. The signalling pathways and phenotypic changes induced by either fatty acid have not been thoroughly investigated in monocytes. Thus, the subsequent chapters will focus on

the phenotypic changes of fatty acid treatment on THP-1 monocytes with a focus on cell surface markers, and mechanisms for any changes observed.

Chapter 4

Effects of palmitate and oleate on monocyte cell surface expression of CD11b and CD36

4 Chapter 4: Effects of palmitate and oleate on monocyte cell surface expression of CD11b and CD36

4.1 Preface

Previously, the effects of fatty acids on THP-1 monocyte redox status and apoptosis were determined. Here, the phenotypic changes induced by fatty acid treatments are determined, with a particular focus upon cell surface antigen expression, and the mechanisms by which these changes are achieved. THP-1 monocytes were treated with varying concentrations of fatty acids over a 24 hour period to establish the effects on CD11b and CD36 expression. Further experimentation sought to establish whether observed changes were due to reactive oxygen species or complex lipids derived from fatty acids or from metabolites.

4.2 Introduction

Fatty acid handling, metabolism and fate are affected by the degree of saturation of the carbon chain (DeLany, Windhauser et al. 2000). Fatty acids, either endogenous or post prandial, are ultimately incorporated in triacylglycerol (TG) and packaged into chylomicrons (TG-CM). Whilst differential FA trafficking has been observed in animals, i.e. greater storage over oxidation with saturates vs. monounsaturates, in humans similar degrees of absorption, uptake and trafficking are found in adipocytes and skeletal muscle. Furthermore differences in whole body oxidative rates, determined through direct or direct calorimetry, between MUFA and SFA have been shown experimentally (Piers, Walker et al. 2002, Kien, Bunn et al. 2005).

In cases of metabolic excess, as observed in type-2 diabetes (T2D) and the obese, FA which cannot be metabolised via mitochondrial β-oxidation will be incorporated into complex lipids. FA-acyl-CoA can be converted to more complex lipids for purposes of storage, namely in the form of lipid droplets as triglycerides. Triglyceride synthesis mainly utilises the Kennedy or *sn*-glycerol-3-phosphate (G3P) pathway at the endoplasmic reticulum, an important site for lipid biosynthesis.

Rates of triglyceride synthesis are dependent on the saturation of fatty acid. Use of radiolabelled isotopes in cultured myotubes from control and diabetic subjects revealed greater radioactivity in DAG and NEFA with palmitate and oleate respectively (Gaster, Rustan et al. 2005). The preferential incorporation of SFA into DAG was also observed in a second radiolabelling study of muscle cells following 20 hour incubation (Montell, Turini et al. 2001), and is supported by other studies e.g. in adipocytes (Chavez and Summers 2003, Pickersgill, Litherland et al. 2007). In muscle cells accumulation of MAG (Bastie, Hajri et al. 2004), DAG and ceramides (Pickersgill, Litherland et al.

2007) are reported with SFA, whilst MUFA increases TG accumulation only (Pickersgill, Litherland et al. 2007).

The preferential synthesis of TG from MUFA may be explained by the apparent reduced affinity of DGAT2, the enzyme responsible in most mammalian cells to convert DAG to TG, for SFA compared to unsaturated FA (Cases, Stone et al. 2001, Lardizabal, Mai et al. 2001). Furthermore, at the endoplasmic reticulum DGAT2 and SCD-1 (an enzyme responsible for the desaturation of SFA to MUFA in de novo lipid synthesis) are co-localised suggesting channelling of MUFA substrate toward TG synthesis (Bergouignan, Momken et al. 2009).

The SFA pool of acyl-CoA during TG synthesis may accumulate as DAG, a complex lipid that is known to induce insulin resistance through the activation of PKC (Itani, Zhou et al. 2000, Li, Soos et al. 2004). Ceramides are elevated following treatment with SFA, such as palmitate. The rates of synthesis of ceramide are dependent upon the availability of long chain SFA and serine which are the substrates for the first and rate limiting step in *de novo* ceramide synthesis catalysed by serine palmitoyl transferase (Merrill 2002). Ceramides, like DAG, are thought to mediate many of the intracellular signalling and effects of SFA including insulin resistance through the activation of JNK or NF-κB (MohammadTaghvaei, Taheripak et al. 2012). Thus, the main mechanisms of SFA acid-induced insulin resistance rely upon the conversion of fatty acyl-CoA into complex lipids such as DAG and ceramide.

The effects of SFA are not limited to induction of insulin resistance and apoptosis. In endothelial cells, SFA are capable of increasing inflammatory cytokine production (Cheng, Handa et al. 2012), expression of adhesion molecules (Maloney, Sweet et al. 2009) and reducing nitric oxide production (Tian, Qiu et al. 2012). In immune cells, the activation of inflammatory signalling through TLR4-NF-κB signalling pathways are a common observation (Huang, Rutkowsky et al. 2012, Schilling, Machkovech et al. 2013).

In addition to influencing inflammatory processes, observations of pro-atherosclerotic changes mediated by palmitate have been demonstrated in endothelial cells and animal models. For example, mice fed a high fat diet to induce an animal model of atherosclerosis demonstrate an increase in matrix metalloproteinase enzymes, which breakdown components of the extracellular matrix facilitating atherosclerotic plaque progression(Jin, Jiang et al. 2012).

Integrins are components expressed on monocytes, macrophages and endothelial cells and play a crucial role in transmigration of immune cells to sites of inflammation. In the context of atherosclerosis, in response to inflammatory signals from lesions monocytes and macrophages are

recruited and adhesion molecules e.g. integrins are expressed to facilitate this process (Hyun, Lefort et al. 2009). Palmitate is known to induce expression of adhesion molecules such as vascular cell adhesion molecule VCAM-1 (Cacicedo, Yagihashi et al. 2004, Choi, Jang et al. 2010) and intercellular adhesion molecule ICAM-1 (Maloney, Sweet et al. 2009, Lin, Duan et al. 2010, Handa, Tateya et al. 2011) on endothelial cells. In such a manner, SFA can promote adhesion and recruitment of monocytes and macrophages to lesions and thus enhance atherosclerotic progression.

CD11b belongs to a group of integrin adhesion molecules which participate in leukocyte recruitment in response to inflammation. This cell surface antigen belongs to the β_2 integrin family sharing a common β chain (CD18) with α chains CD11a, CD11c and CD11d (Larson and Springer 1990, VanderVieren, LeTrong et al. 1995). CD11b is also described as macrophage antigen (Mac)-1, complement receptor 3 (CR3) (Gahmberg, Tolvanen et al. 1997), and is expressed on monocytes, T cells (McFarland, Nahill et al. 1992) and NK cells (Ault and Springer 1981). Firm attachment to the endothelium is mediated by members of the β_2 integrin family such as CD11a, CD11b and CD11c binding to their respective counterligands such ICAM-1 or extracellular matrix proteins e.g. fibrinogen (Plow and Zhang 1997).

In cardiovascular disease and atherosclerosis, CD11b is expression altered; for example in ischaemic heart disease increased CD11b/CD18 expression on peripheral leukocytes has been observed (Kassirer, Zeltser et al. 1999) and similarly in chronic heart failure, CD11b expression is increased (Dixon, Griggs et al. 2011). In acute coronary syndrome, CD11b expression is elevated in monocytes (Hillis, Dalsey et al. 2001); in unstable coronary artery disease and in unstable angina CD11b cell surface antigen expression is increased (Mazzone, Deservi et al. 1993, deServi, Mazzone et al. 1996).

CD36, or fatty acid translocase, is a membrane glycoprotein expressed in monocytes/macrophages, microvascular endothelial cells, adipocytes, skeletal muscle, dendritic cells and breast and retinal pigment epithelium (Febbraio, Hajjar et al. 2001). The glycoprotein belongs to the class B scavenger receptor family with ligands including long chain fatty acids, anionic phospholipids, apoptotic cells, collagens I and IV, in addition to both LDL and its modified counterparts e.g. oxidised LDL. As a scavenger receptor, CD36 performs a function within the innate immune system, recognising molecules on the surface of pathogens mediating their clearance through phagocytic cells, and has a role in the uptake of nonopsonised *Plasmodium falciparum* parasitized erythrocytes (McGilvray, Serghides et al. 2000). The scavenging role of CD36 is important in mediating the clearance CD8 + T-cells during the resolution of viral infections (Akbar, Savill et al. 1994), removal of photoreceptor outer segments by retinal epithelium (Ryeom, Sparrow et al. 1996), phagocytosis of eosinophils undergoing constitutive apoptosis (Stern, Savill et al. 1996), indeed the ability of macrophages to

phagocytose apoptotic cells is dependent upon CD36 (Moodley, Rigby et al. 2003, Truman, Ogden et al. 2004).

Previously LCFA transport across a membrane was believed to be reliant upon simple diffusion. The historic model involved a number of steps including dissociation of LCFA from albumin, movement to and into the outer phospholipid bilayer, 'flip-flop' across the membrane into the cytoplasm and finally binding to intracellular fatty acid binding proteins, thus maintaining a gradient for passive diffusion (Koonen, Glatz et al. 2005, Bonen, Chabowski et al. 2007). However more recent evidence indicates the process is protein facilitated (Abumrad, Coburn et al. 1999). Protein modification and inhibition indicated a majority of LFCA transport relies upon this process (Turcotte, Swenberger et al. 2000, Habets, Coumans et al. 2007, Kampf 2007); CD36 has been identified as a principal means of long chain fatty acid transport into cells (Abumrad, Elmaghrabi et al. 1993).

Further evidence for the involvement of CD36 in LCFA transport is provided by animal models. CD36 overexpression in skeletal muscle of mice induced enhanced fatty acid uptake and oxidation with a corresponding decrease in circulating NEFA, TG and fat deposition (Ibrahimi, Bonen et al. 1999). Conversely, null mutations in CD36 elevated plasma NEFA and reduced LCFA oxidation (Febbraio, Abumrad et al. 1999). Additionally the distribution of CD36 is consistent with a role in LCFA transport, with high expression observed in the intestine (Chen, Yang et al. 2001, Lobo, Huerta et al. 2001), cardiac (Abumrad, Elmaghrabi et al. 1993, Luiken, Schaap et al. 1999) and adipose tissue (Abumrad, Elmaghrabi et al. 1993). In skeletal muscle CD36 expression is highly expressed in fibers with a high oxidative capacity (Bonen, Dyck et al. 1999). In humans, CD36 deficiency is common in individuals of African (Love-Gregory, Sherva et al. 2008)or Asian (Yamashita, Hirano et al. 2007) descent, mutations which almost completely abolishing myocardial uptake (Tanaka, Nakata et al. 2001), which lead to hyperlipidaemia (as elevated TG), increased remnant lipoproteins, mildly elevated fasting glucose, and hypertension.

CD36 plays a role in metabolic adaptation, for example during skeletal muscle contraction in rodents sarcolemmal levels of the transporter were increased (Bonen, Luiken et al. 2000, Turcotte, Raney et al. 2005, Schwenk, Luiken et al. 2008). In response to insulin, GLUT4 is translocated to the surface of cells enhancing uptake of glucose into cells, and in a similar fashion insulin induces translocation of CD36 to the cell surface of skeletal (Luiken, Dyck et al. 2002, Han, Bonen et al. 2005) and heart tissue.

Many metabolic disease states are often associated with an increase in plasma NEFA and TG, especially insulin resistant states such as obesity and type-2 diabetes and (Roden, Price et al. 1996, SchmitzPeiffer, Browne et al. 1997, Shulman 2000). In obese and type 2 diabetic patients, an

increase in LCFA uptake and TG accumulation were strongly associated with increased skeletal muscle CD36 surface expression (Bonen, Parolin et al. 2004), findings replicated by other groups (Aguer, Foretz et al. 2011). Using obese Zucker rats as a model of insulin resistance, enhanced LCFA uptake is observed due to an increase in plasmalemmal CD36 content rather than increased expression (Luiken, Arumugam et al. 2001, Coort, Hasselbaink et al. 2004) in heart and skeletal muscle. Using the *db/db* mouse model of diabetes, elevated entry of LCFA into the heart is observed alongside relocation of CD36 and FABPm to the sarcolemma (Carley, Atkinson et al. 2007). Thus, CD36 is considered important in the aetiology of metabolic disease states.

This chapter aims to discern the phenotypic changes induced by fatty acids in THP-1 monocyte cell surface markers CD11b, a cell surface integrin typically expressed during transmigration of monocytes toward sites of inflammation and CD36, the principal fatty acid transporter.

In addition to determining cell surface marker changes, the mechanisms for any changes will also be explored through the use of pharmacological inhibitors. The superoxide dismutase mimetic MnTBap was used to determine the importance of palmitate mediated mitochondrial superoxide production inthese phenotype changes. Inhibitors of ceramide biosynthesis were also used based on previous observations characterising the importance of ceramide in mediating the effects of SFA following incubation with monocytes. Inhibition of ceramide synthesis via fumonisin B1 have been used to establish the importance of ceramide in any observed phenotype changes.

4.3 Materials and Methods

4.3.1 Inhibitor treatment for studying the effects of FA on THP-1 monocyte cell surface antigen expression and possible mechanisms

Prior to treatment of monocytes with fatty acids, as described in section 2.2.4, $1x10^6$ cells were treated with either MnTBap (200µM; superoxide scavenger), etomoxir (30µM; CPT1 inhibitor), FB1 (50µM; SPT inhibitor), 1S,2R-D-*erythro*-2-N-myristoylamino)-1-phenyl-1-propanol (MAPP;20µM; ceramidase inhibitor) or PPMP (0.05µM; glycosphingolipid synthesis inhibitor) for two min prior to co-incubation in the presence or absence of palmitate.

4.3.2 Statistical analysis

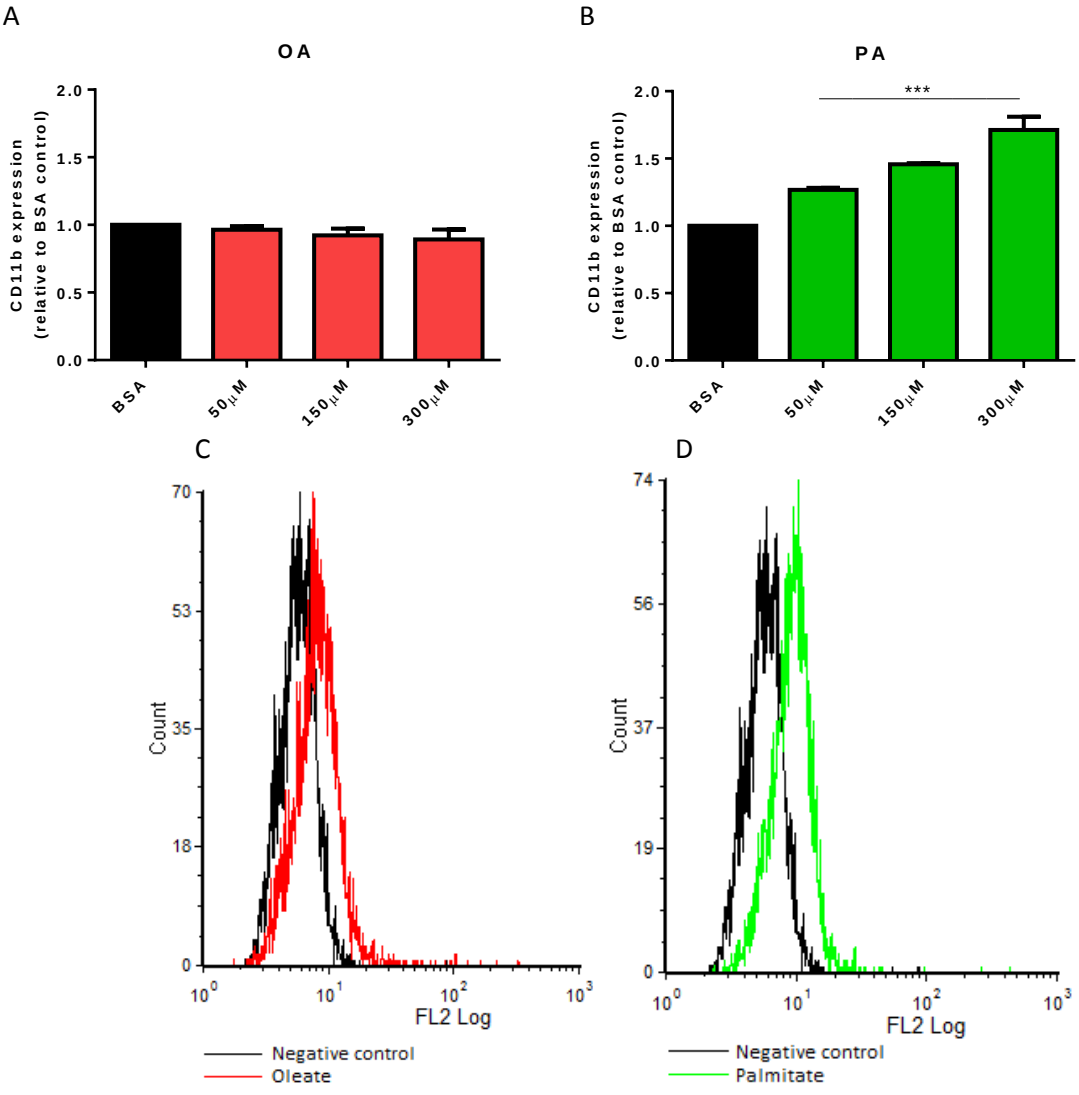
Data was expressed as means of at least 3 independent experiments performed in triplicate and one-way ANOVAs or t-tests were performed using GraphPad Prism.

4.4 Results

To determine the effect of either the MUFA oleate or SFA palmitate on cell surface expression of the β_2-integrin CD11b and fatty acid translocase CD36, THP-1 monocytes were treated with either fatty acid for 24h and surface expression subsequently analysed by flow cytometry.

4.4.1 CD11b expression is increased in the presence of palmitate but not oleate

The cell surface expression of integrin CD11b was assessed in THP-1 monocytes following 24h treatment with FA (50µM, 150µM and 300µM) or BSA control. Palmitate, but not oleate, demonstrated significant dose-dependent increases in CD11b expression compared to BSA control. The level of expression increases from 50µM to 300µM by 20% up to 50% above BSA control in the presence of palmitate (Figure 4.1B, $p<0.001$), whereas oleate did not affect CD11b expression.



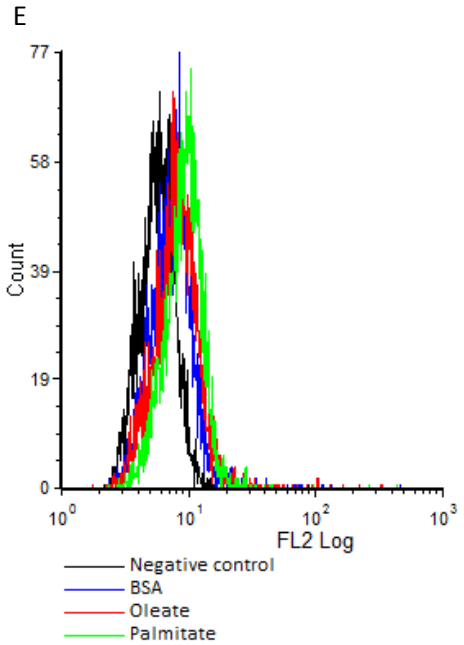
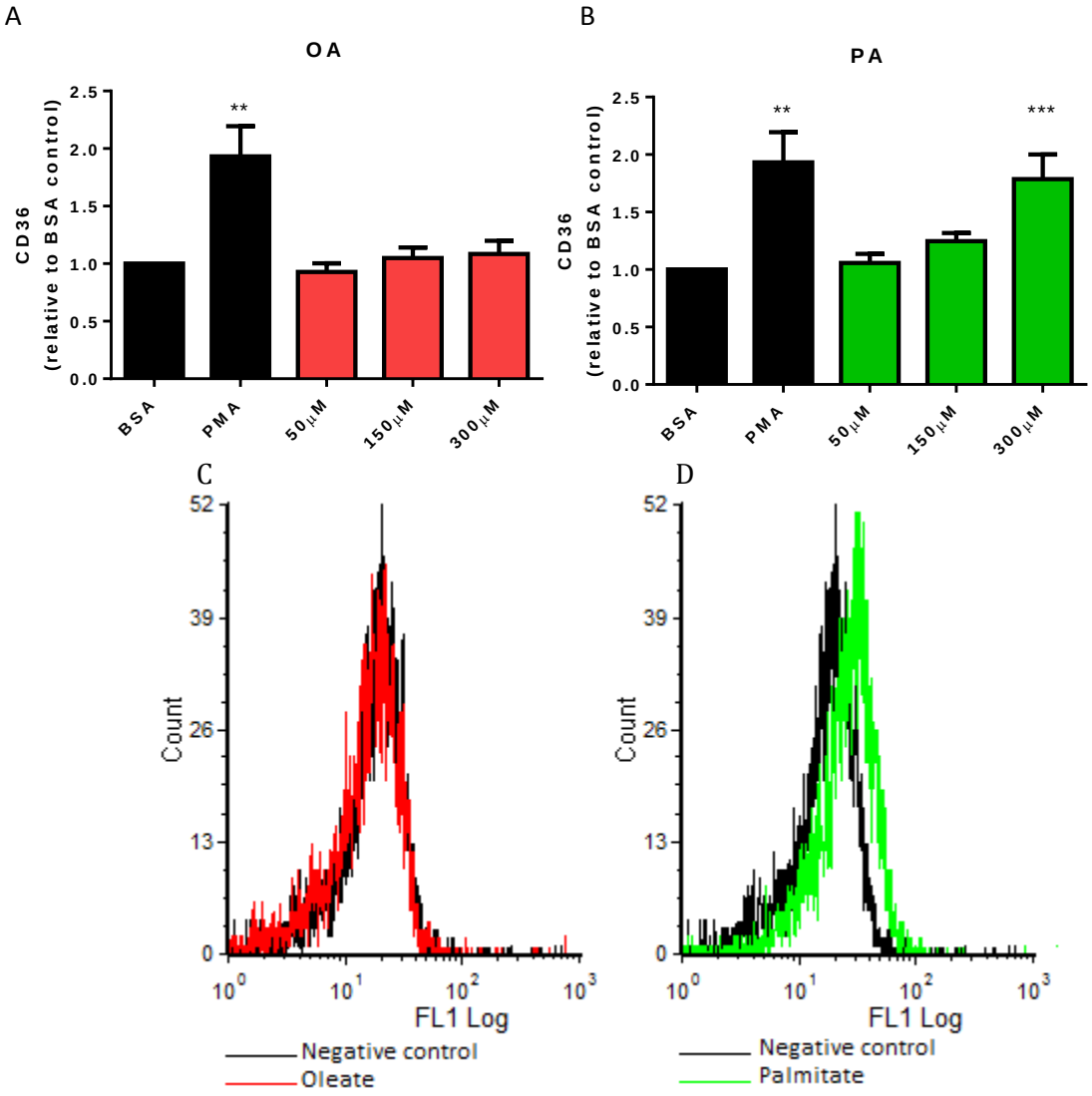


Figure 4.1: Palmitate but not oleate induces dose-dependent increases in THP-1 monocyte CD11b expression:

THP-1 monocytes treated with FA/BSA for 24h were analysed under flow conditions for cell surface CD11b expression. Upon experiment completion, cells were blocked with FCS for 15 min prior to treatment with saturating concentration of mouse anti-human CD11b RPE conjugated antibody, or IgG1 RPE conjugated negative control, for 30 min on ice. Oleate (A) and palmitate (B) treated monocytes were analysed under flow conditions using FL2 wavelength (564-606nm, on Beckman Coulter EpicXL flow cytometer), and data is displayed as mean expression relative to control obtained from 3 independent experiments with statistical significance determined through one-way ANOVA where ***=p<0.001. Histogram plots of CD11b expression (as FL2 log intensity) of negative isotype IgG1 controland oleate (C),negative IgG1 isotype control andpalmitate (D) and isotype IgG1 negative control, BSA, oleate and palmitate (E).

4.4.2 THP-1 monocyte cell surface fatty acid translocase (CD36) expression is enhanced in the presence of palmitate but not oleate

Cell surface expression of fatty acid translocase was assessed after 24h treatment with FA (50-300µM) or BSA control by flow cytometry. PMA was used as a positive control for CD36 induction. Cell surface expression of fatty acid translocase, CD36, is increased by palmitate in a dose-dependent fashion, but oleate has no effect. Expression is increased by 10% and 20% in the presence of 50µM and 150µM palmitate respectively when compared to BSA-control treated monocytes, with 300µM palmitate inducing a significant (figure 4.2B, p<0.001) 80% increase in CD36 expression.


A
OA
CD36 (relative to BSA control)
2.5
2.0
1.5
1.0
0.5
0.0
**
BSA
PMA
50μM
150μM
300μM
B
PA
CD36 (relative to BSA control)
**
***
BSA
PMA
50μM
150μM
300μM
C
52
39
26
13
0
Count
10^0
10^1
10^2
10^3
FL1 Log
Negative control
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0
Count
10^0
10^1
10^2
10^3
FL1 Log
Negative control
Palmitate

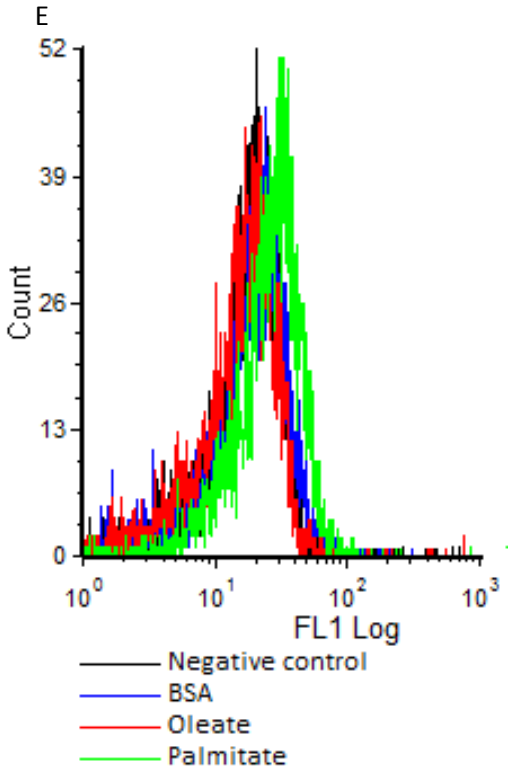


Figure 4.2: Cell surface expression of CD36/FAT in THP-1 monocytes is enhanced by palmitate but not oleate:

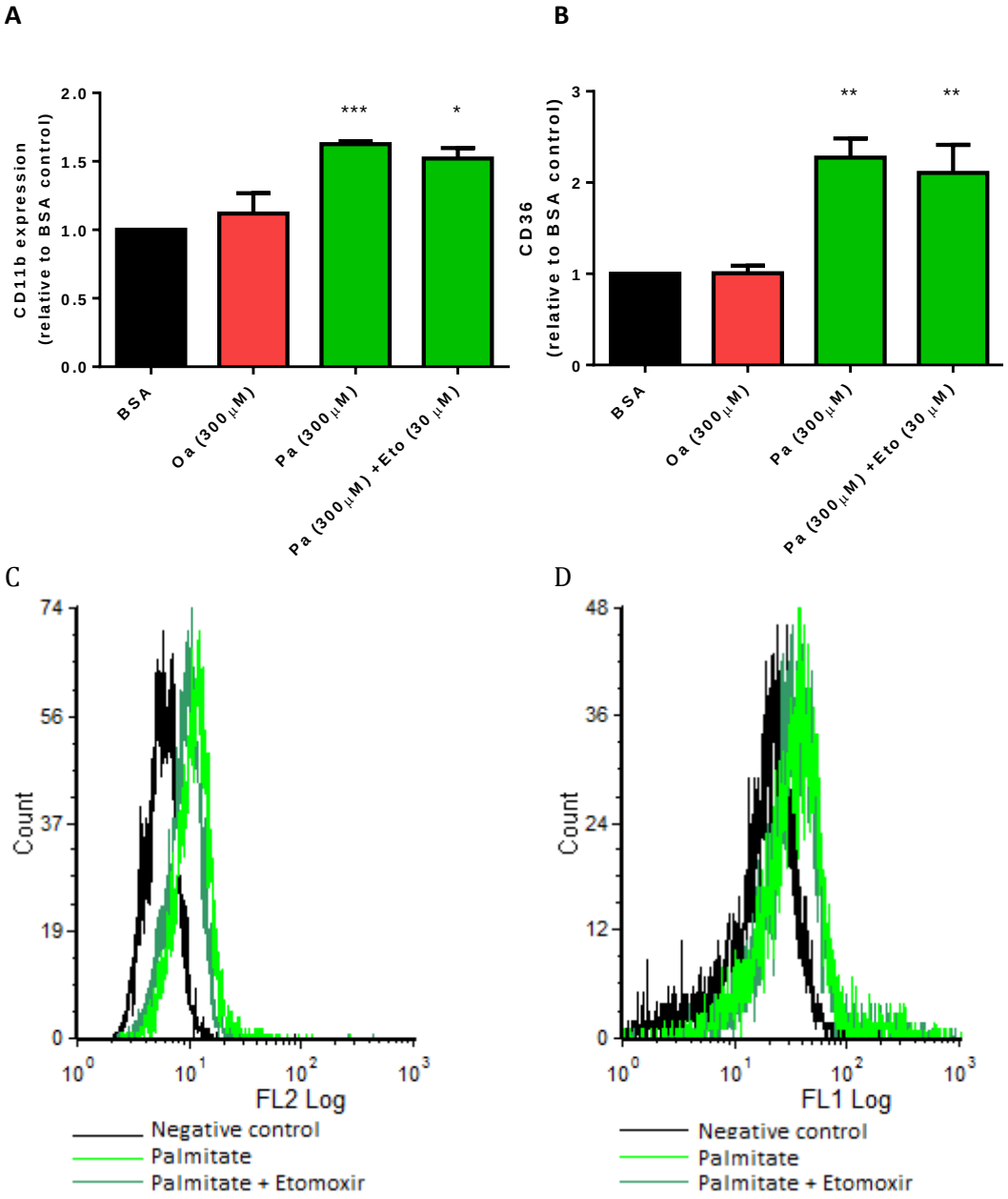
24h FA/BSA monocytes were analysed for CD36/FAT cell surface expression under flow conditions. At cessation of experiment, cells were resuspended in 5% FCS to block non-specific Fc receptors for 15 min on ice. Subsequently cells were treated with mouse anti-human FITC conjugated CD36 antibody or mouse anti-human FITC conjugated IgG1 for 30 min prior to analysis under flow conditions using FL1 wavelength (515-545nm). Oleate (A) and palmitate (B) treated cells are displayed as mean expression relative to control obtained from 3 independent experiments. Statistical significance was obtained using one-way ANOVA where **=p<0.01 and ***=p<0.001. Histogram plots of CD36 expression (as FL1 intensity) of negative isotype IgG1 control and oleate (C), negative IgG1 isotype control and palmitate (D) and isotype IgG1 negative control, BSA, oleate and palmitate (E).

4.4.3 CD11b and CD36 expression is not affected by inhibition of FA oxidation

To determine if oxidation of FA is important in the SFA mediated increase in cell surface expression of CD11b and CD36, etomoxir (an inhibitor of CPT-1 preventing entry of fatty acyl-CoA into the inner mitochondrial space and therefore preventing mitochondrial oxidation) was utilised. Monocytes were treated for 24h with BSA control, oleate, or palmitate in the presence or absence of etomoxir (50µM).

The increased expression of CD11b and CD36 by palmitate remained unaltered in the presence of etomoxir (figure 4.3A and 4.3B). However, MitoSOX oxidation by palmitate-treated cells in the presence of etomoxir showed a 50% reduction compared to palmitate alone and was not significantly

different to untreated cells (figure 4.3C), suggesting β-oxidation of palmitate results in elevated ROS production by the mitochondria but that it is not critical for changes in CD11b and CD36.


A
CD11b expression (relative to BSA control)
2.0
1.5
1.0
0.5
0.0
***
*
BSA
Oa (300 μM)
Pa (300 μM)
Pa (300 μM) +Eto (30 μM)
B
CD36 (relative to BSA control)
3
2
1
0
**
**
BSA
Oa (300 μM)
Pa (300 μM)
Pa (300 μM) +Eto (30 μM)
C
Count
74
56
37
19
0
10^0
10^1
10^2
10^3
FL2 Log
Negative control
Palmitate
Palmitate + Etomoxir
D
Count
48
36
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FL1 Log
Negative control
Palmitate
Palmitate + Etomoxir

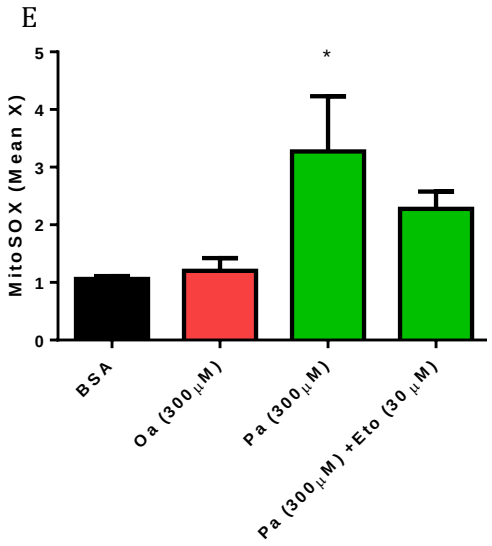


Figure 4.3: Etomoxir does not prevent palmitate mediated increases in CD11b and CD36 expression:

THP-1 cells treated with BSA or FA (300µM) oleate, palmitate ± etomoxir (30µM) for 24h were analysed for CD11b and CD36 expression and for MitoSOX reactivity independently. For antibody treatment, cells were resuspended in 5% FBS in media for 15 min on ice before treatment with saturating concentration of either FITC conjugated mouse anti-human CD36 or RPE conjugated mouse anti-human CD11b, or respective control. Cells were subsequently analysed under flow conditions as previously described. For MitoSOX analysis, cells were washed twice with PBS before addition of 2.5µM MitoSOX for 30 min at 37°C in the dark. Samples were analysed under flow conditions using FL2 wavelength. For all sampled analysed data was analysed until 10,000 events were recorded. CD11b (A) and CD36 (B) are displayed as mean of relative of control from 3 independent experiments, with statistical significance determined from one-way ANOVA analysis compared to BSA control, where *=p<0.05, **=p<0.01 and ***=p<0.001. Histogram plots of CD11b (C) and CD36 (D) expression showing isotype IgG1 negative control, palmitate and palmitate + etomoxir treated THP-1 cells. (E) MitoSOX reactivity is displayed as mean fluorescence from 3 experiments, with statistical significance determined by one way ANOVA where p=<0.05.

4.4.4 Palmitate mediated CD11b and CD36 increases are not affected by mitochondrial ROS quenching

The production of ROS and oxidative stress is a common effect of SFA supplementation, and has been reported to be involved in the activation of various signalling pathways e.g. JNK and p38 MAPK. Thus an investigation of whether increased ROS production is the primary factor involved in mediating the increased cell surface expression of CD11b and CD36 was warranted.

Palmitate increased CD11b and CD36 expression on monocytes (Figure 4.4A and B) whereas an equivalent concentration of oleate had no effect. However, in the presence of MnTBap, neither the palmitate-driven increase in CD11b nor CD36 expression was modulated, indicating that mitochondrial ROS is not a primary factor in determining the increased cell surface expression of

either the integrin or the fatty acid translocase in the presence of palmitate(figure 4.4). MnTBap the mitochondrial superoxide dismutase mimetic is known to be effective at quenching mitochondrial superoxide anion radicals. Palmitate-treated cells increase superoxide dependent fluorescence by ~50% compared to BSA treated controls, and in the presence of MnTBap this increase is abolished (Figure 4.4C).

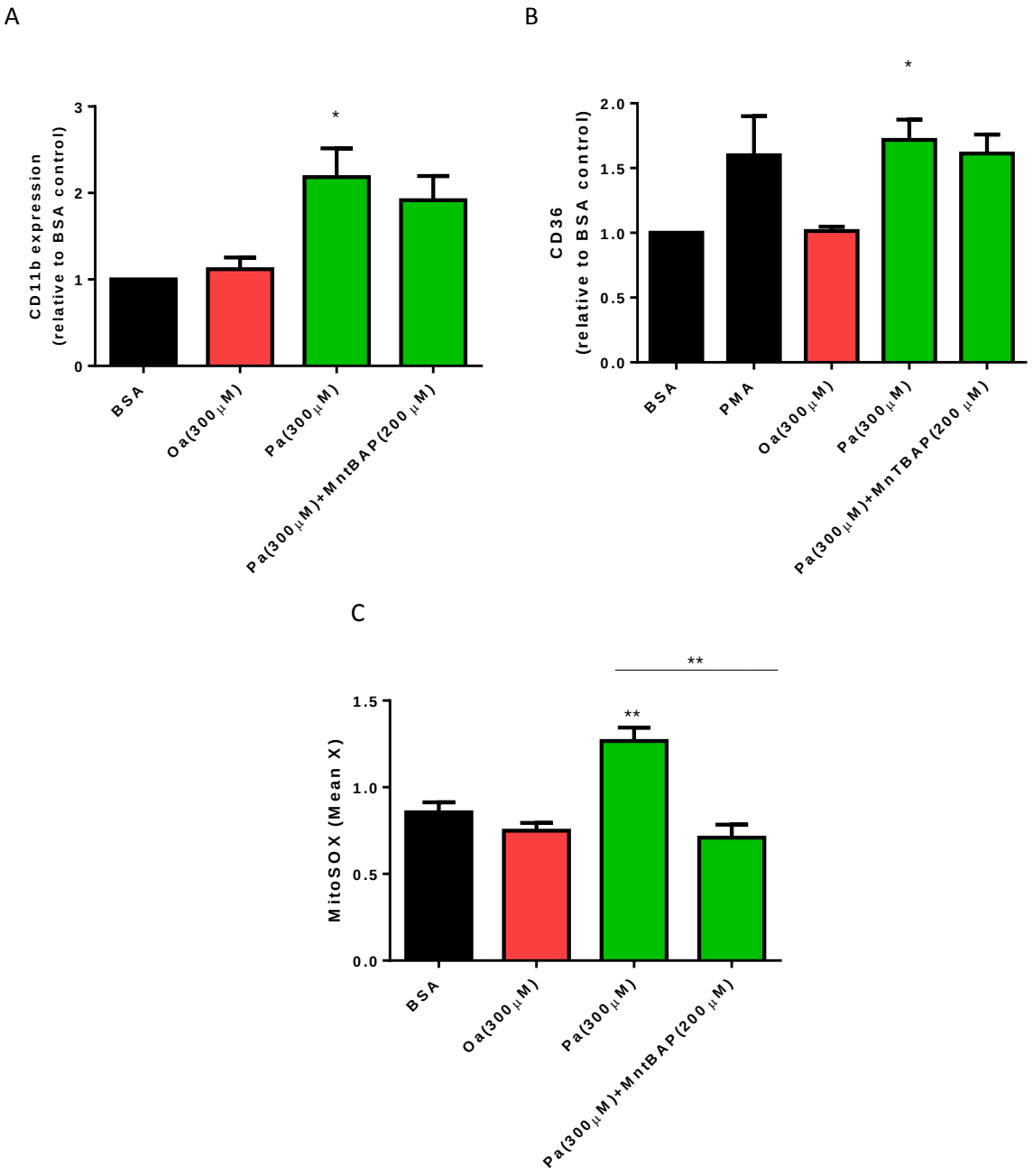


Figure 4.4: MnTBap does not alter palmitate induced cell surface expression of CD11b or CD36:

THP-1 monocytes were treated for 24h with BSA, FAs (300µM) oleate or palmitate ± MnTBap (200µM) and were analysed for cell surface CD11b or CD36 expression, and MitoSOX reactivity. Cells were treated and prepared as previously described. CD11b (A), CD36 (B) and MitoSOX (C) data was obtained from 3 independent experiments with statistical significance was determined for antibody data by one way ANOVA analysis where *=p<0.05, compared to BSA control, with t-test analysis to determine difference between palmitate alone and co-treatment with MnTBap, **=p<0.01 .

4.4.5 Enhanced expression of CD11b and CD36 are dependent on ceramide formation

Palmitate and serine are the two precursor molecules for the formation of ceramides in *de novo* synthesis. However, ceramides are not the final products in this pathway. Ceramides undergo further processing and can form sphingosine and glucosylceramide. Whilst ceramides are widely believed to be responsible for the effects of palmitate in insulin resistance, stress pathways and apoptosis, other sphingolipids are also believed to have key cellular functions (Lahiri and Futerman 2007).

In order to determine if ceramide synthesis is involved in the phenotypic effect of palmitate, the ceramide synthase inhibitor fumonisin B1 (FB1) was utilised in the presence of palmitate and effects of cell surface antigens were explored. In THP-1 monocytes, palmitate (300μM) induced a significant increase in CD11b and CD36 expression, and co-treatment with FB1 abrogates this increase; cell surface CD11b expression was lowered by ~25% and CD36 expression by ~20% respectively compared to palmitate alone (figure 4.5). This study confirms that in THP-1 monocytes the increased expression of cell surface markers CD11b and CD36 in response to the SFA palmitate but not MUFA oleate, is reliant upon ceramide formation.

To determine whether the true metabolite responsible is ceramide or a more complex ceramide metabolite, THP-1 monocytes were co-treated with palmitate and the inhibitors DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP), and 1S,2R)-D-erythro-2-(N-myristoylamino)-1-phenyl-1-propanol (MAPP), which inhibit glucosylceramidase and alkaline ceramidase respectively. In this manner, the conversion of ceramide to sphingosine-1-phosphate and glucosylceramides are prevented, and ceramide levels are artificially increased. In THP-1 monocytes, the palmitate-mediated increase in CD11b and CD36 expression were further increased or remained similar in the presence of palmitate and MAPP or PPMP (figure 4.5). This confirms the role of ceramide, and not a ceramide metabolite such as a glucosylceramide or sphingosine, in mediating the cell surface antigen expression induced by palmitate.

A B

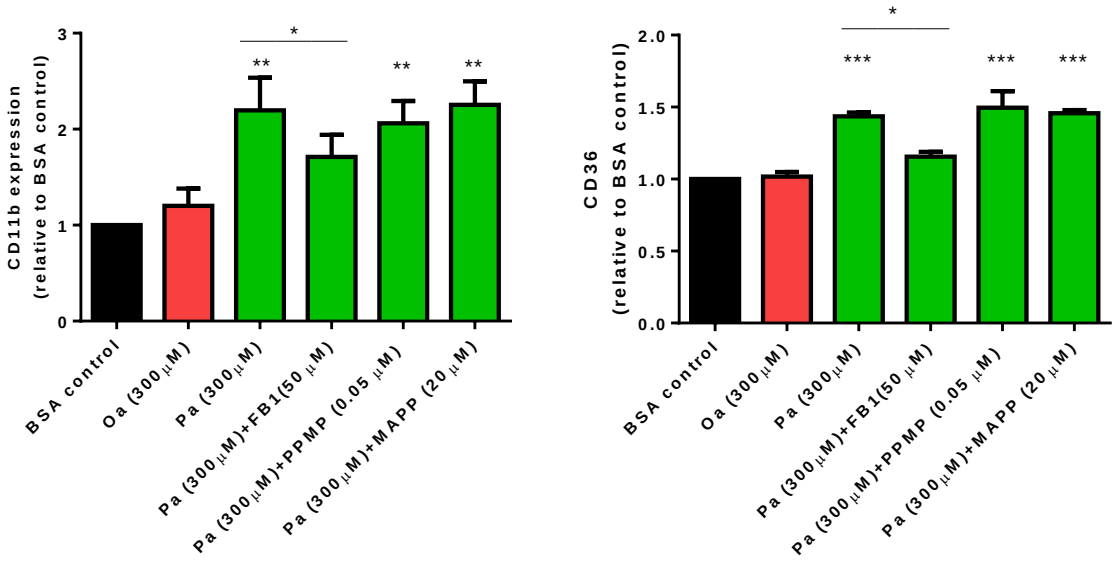
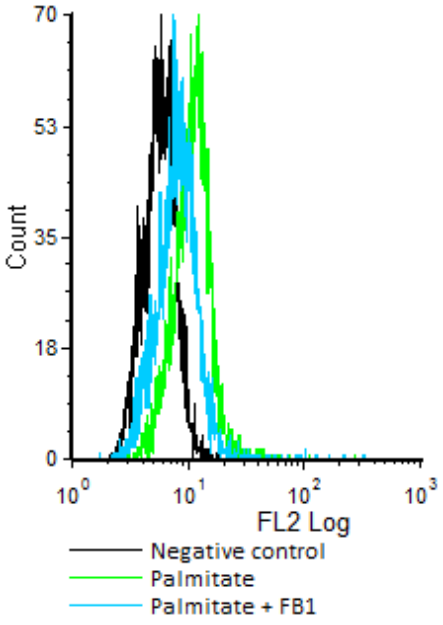


Figure 4.5: De novo synthesis of ceramide is required for the enhanced CD11b and CD36 expression in monocytes by palmitate treatment:

24h treatment of THP-1 cells with BSA, oleate (300µM), palmitate (300µM) ± FB1 (50µM), PPMP (0.05µM), or MAPP (20µM) were analysed under flow conditions for the expression of CD11b and CD36. Cells were treated with a saturating concentration of mouse anti-human CD11b or CD36 antibody for 30 min on ice before analysis under flow conditions, FL1 for CD36 and FL2 for CD11b with 10,000 events recorded. Data is presented as mean antigen expression relative to controls from 3 independent experiments. One way ANOVA determined statistical significance in comparison to BSA control, and t-tests were used to compare palmitate treatment alone to co-treatments with ceramide/sphingolipid synthesis inhibitors. *=p<0.05, **=p<0.01 and ***=p<0.001.

A B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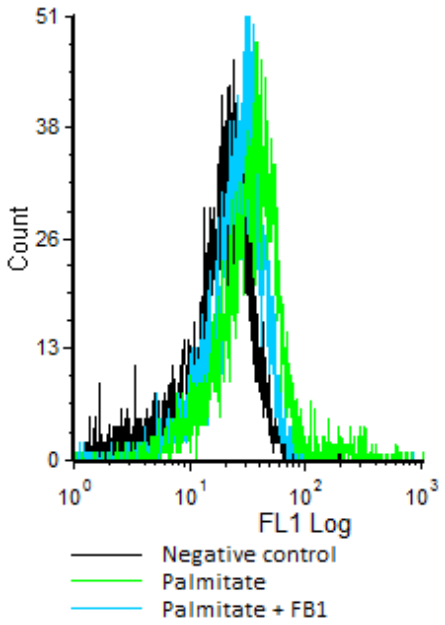
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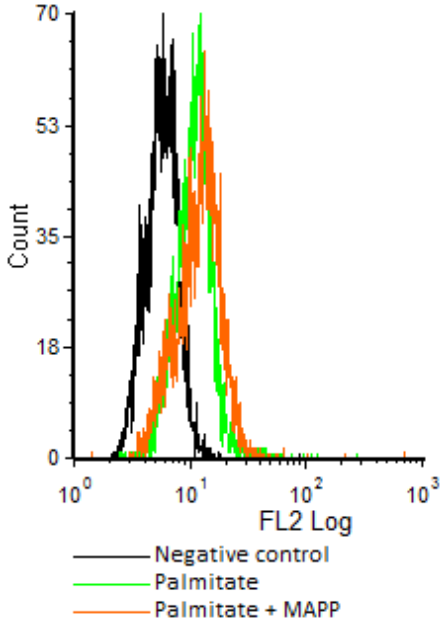
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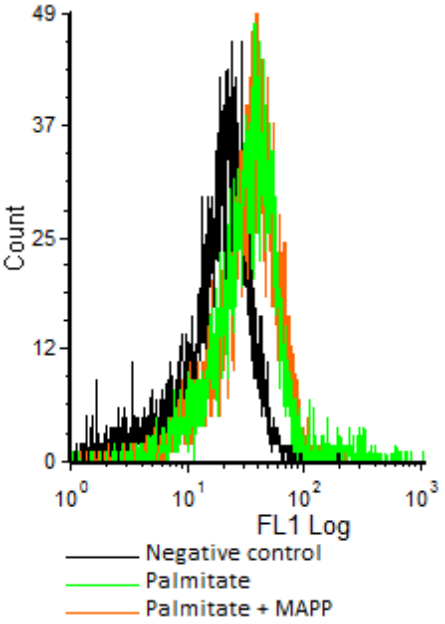
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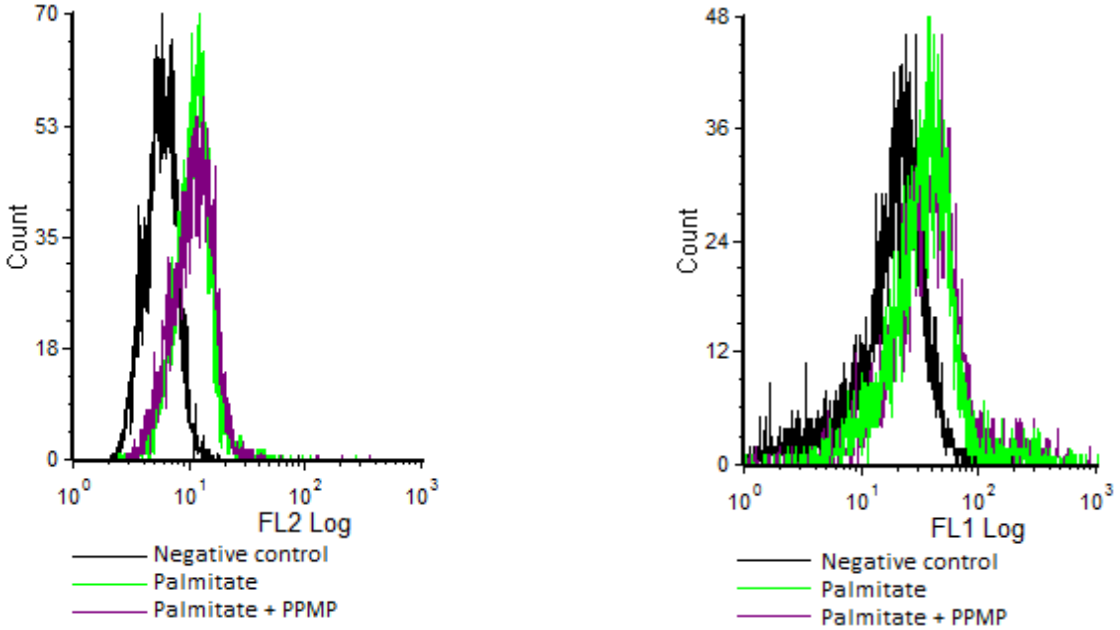


Figure 4.6: Histogram plots of CD11b and CD36 expression on THP-1 monocytes treated with palmitate in the presence or absence of ceramide and sphingolipid synthesis inhibitors:

CD11b (A – palmitate + FB1, C – palmitate + MAPP, and E – palmitate + PPMP) and CD36 (B – palmitate + FB1, D – palmitate + MAPP and F – palmitate + PPMP).

To confirm the palmitate-dependent formation of ceramide in monocytes, lipid extracts were obtained from THP-1 monocytes treated with BSA, oleate, palmitate, with or without FB1, MAPP or PPMP and analysed by mass spectrometry for ceramide content. In the presence of palmitate there was an increase in cellular ceramide content, which was reduced in the presence of FB1 (figure 4.7). This result confirms the ability of FB1 to inhibit SPT1 and therefore prevent formation of ceramide via de novo synthesis. Cells co-treated with palmitate and either MAPP or PPMP produced significant increases in ceramide content. Despite this increase in ceramide content with these inhibitors, no significant further increase in expression of CD11b or CD36 was observed (figure 4.5).

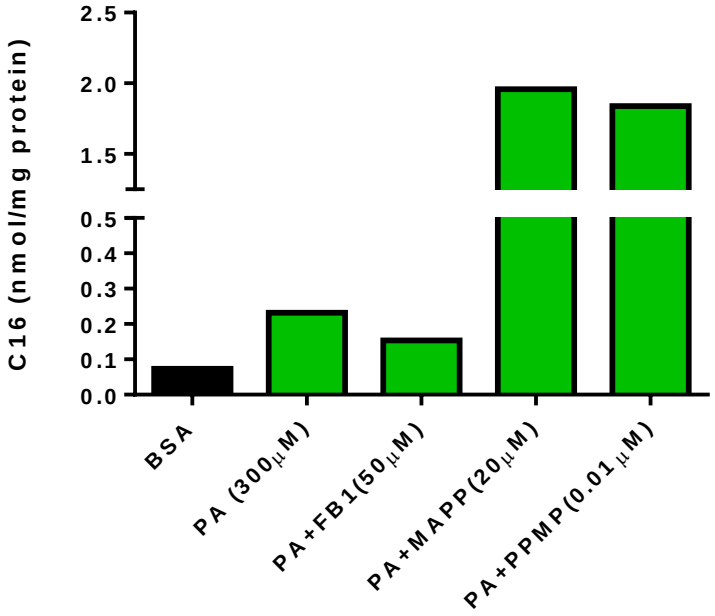


Figure 4.7: FB1 inhibits the formation of ceramides in THP-1 monocytes treated with palmitate:

Ceramide content of cells treated with palmitate in the presence or absence of ceramide and sphingolipid synthesis inhibitors were determined. Monocytes ($2x10^7$ cells/ml) were washed in PBS before ceramide and sphingolipids were extracted using iso-propanol: water: ethyl acetate (30:10:60) and dried under nitrogen. Samples were analysed by LC-MS using a reverse phase C18 column, with data dependent analysis to identify ceramides.

4.4.6 Triglyceride formation is unrelated to palmitate mediated phenotypic changes

Following cellular fatty acid uptake, conversion into fatty acyl-CoA is followed either by oxidation in the mitochondria or conversion into more complex lipids i.e. into triglycerides or in the case of SFA ceramides. Using Oil red O, a neutral lipid and triglyceride stain, the formation of complex lipids can be quantified in THP-1 monocytes following treatment with oleate or palmitate or BSA control.

Both fatty acids induced a dose-dependent increase in Oil red O staining. However, oleate elicited a greater and significant degree of neutral lipid formation in comparison in palmitate treated monocytes (figure 4.8).

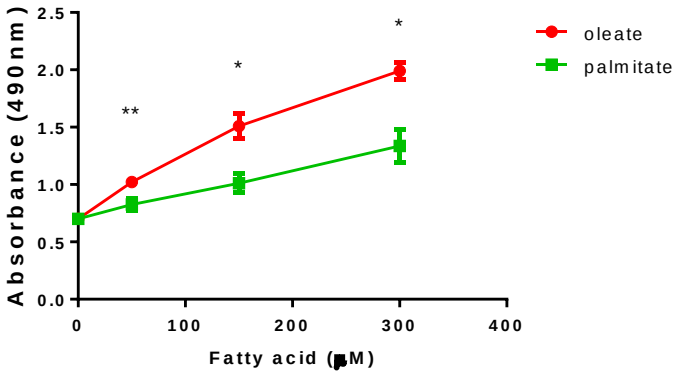


Figure 4.8: Triglyceride formation is greater in the presence of MUFA oleate than SFA palmitate:

THP-1 monocytes were incubated for 24h with oleate or palmitate and were subsequently analysed for triglyceride content using oil red O. Cells were washed in PBS prior to fixation with 1% paraformaldehyde at room temperature for 30 min, before resuspension in 60% isopropanol for 5 min. The cells were resuspended in oil red O for 5 min, washed in isopropanol before analysis under absorbance spectrometry at 490nm. Data is presented as mean of 3 independent experiments, where samples were analysed in duplicate in each experiment. Paired t-test analysis determined statistical significance between oleate and palmitate, where *=$p<0.05$ and **=$p<0.01$.

4.5 Discussion

Here, palmitate but not oleate has been shown to induce a dose-dependent increase in cell surface antigen expression of CD11b and CD36 over 24h. Furthermore, neither inhibition of fatty acid oxidation by etomoxir nor the quenching of mitochondrial ROS by superoxide dismutase mimetic MnTBap ameliorated the palmitate-induced increase in cell surface antigen expression. . In contrast, in the presence of FB1, serine palmitoyl transferase/ceramide synthase inhibitor, a decrease in expression of both antigens was observed. Use of MAPP and PPMP to inhibit the formation of downstream ceramide metabolites confirmed that *de novo* ceramide formation is required for the palmitate mediated increase in cell surface antigen expression. Evaluation of triglyceride and ceramide production showed that while both fatty acids generate triglycerides, ceramide formation is only observed with palmitate treatment and triglyceride production is greater with oleate.

CD11b and other adhesion molecules are measured as markers of increased immune cell-endothelial cell interaction, and therefore promotion of atherosclerotic plaque formation in animal models (Tropea, Huie et al. 1996, Aiello, Bourassa et al. 1999, Walker, Langheinrich et al. 1999, Strawn, Chappell et al. 2000, Reckless, Rubin et al. 2001, van Royen, Hoefer et al. 2003) and human studies (Mazzone, DeServi et al. 1997, Berliner, Rogowski et al. 2000, Hillis, Dalsey et al. 2001), and thus highlight the importance of CD11b and adhesion molecule expression in atherosclerosis. The data from this study demonstrates a clear dose-dependent increase in CD11b expression in response to SFA palmitate but not oleate in THP-1 monocytes, as previously reported by this lab (Gao, Griffiths et al. 2007). These observations are, to the best of our knowledge, the only observations of palmitate inducing CD11b expression in monocytes, although Zhang et al. demonstrated an increase in CD11b mRNA in THP-1 monocytes in response to a mixture of arachidonic, docosahexaenoic, eicosapentaenoic, oleic, palmitic and stearic non-esterified fatty acids at 0.2mM (Zhang, Schwartz et al. 2006), but the influence of other fatty acids is unclear.

Lipid mediated induction of CD11b expression in THP-1 monocytes is not exclusive to free fatty acids. Han et al. observed increased CD11b surface expression, protein and mRNA in response to 50µg/ml LDL (Han, Chen et al. 2003). In a similar fashion, C-reactive protein induces CD11b expression in THP-1 and primary monocytes (Woollard, Fisch et al. 2005). Elevations in CD11b expression can occur through increased translocation from intracellular vesicles (Woollard, Phillips et al. 2002), however in this chapter elevated cell surface expression of CD11b was observed after 24h suggesting the involvement of transcription factors or altered mRNA stability by ceramide.

Palmitate, but not oleate, induces an increase in CD36 expression in a similar fashion to CD11b. Perhaps the most prominent role of CD36 is in atherosclerosis. One of the ligands for CD36 is LDL and its oxidised counterpart oxidised LDL (oxLDL), the latter playing a crucial role in atherosclerotic progression. Within the artery LDL can be deposited and become oxidised to generate oxLDL, once internalised into the spaces of the arteries foam cells and cholesterol rich cells form (Weber and Noels 2011).

In light of the evidence of indicating a pro-atherosclerotic role for CD36, the finding here of a dose-dependent increase of CD36 expression on THP-1 monocytes suggests palmitate but not oleate induces phenotypic changes conducive to uptake of modified lipids such as oxLDL as well as further FFA. Additionally, the increase in CD11b and CD36 following palmitate treatment would favour binding of monocytes to vascular endothelium and the uptake of lipids leading to foam cell formation and consequently atherosclerotic plaque formation.

Febbraio et al. created a CD36 null mouse model, which was characterised by a significant reduction in oxLDL uptake in peritoneal macrophages (Febbraio, Abumrad et al. 1999). A CD36 and ApoE knockout mouse model fed a high fat diet had 70% fewer aortic lesions (Febbraio, Podrez et al. 2000). Bone marrow transplantation in the CD36/ApoE double knockout mouse model showed CD36 knockdown to impart atheroprotection (Febbraio, Guy et al. 2004). Such observations were reflected by a human study of individuals with a CD36 genetic polymorphism (Kashiwagi, Tomiyama et al. 1994). In humans, a role of CD36 in atherosclerosis was suggested by lipid laden macrophages derived from atherosclerotic plaques, whereby a high degree of immunoreactivity to CD36 is shown (Nakata, Nakagawa et al. 1999). Furthermore, in a long term Western diet feeding study, CD36 played a key role in the development of lesions through macrophage recruitment and foam cell formation (Guy, Kuchibhotla et al. 2007).

Whilst the significance of palmitate mediated increase in cell surface antigen expression is complex and contradictory as discussed above, the mechanisms underlying such changes have also been investigated. In this chapter the possibility that mitochondrial ROS production by palmitate but not oleate may drive CD11b and CD36 expression given ROS can play a role in signal transduction processes was explored (Janssen-Heininger, Mossman et al. 2008, Finkel 2011). During metabolic processes ROS, such as hydrogen peroxide, will oxidise protein thiols that exist in thiolate form under physiological conditions e.g. in an enzyme or transcription factor, which may alter their activity. Antioxidant systems such as thioredoxin with thioredoxin reductase or glutathione with glutathione reductase will reverse modifications. An example being the redox sensitive tyrosine phosphatases (Boivin, Yang et al. 2010).

CD11b upregulation by ROS has been reported by others. For example in microglia hydrogen peroxide rather than superoxide production was critical for CD11b expression in response to LPS (Roy, Jana et al. 2008), and in neutrophils differentiation programmes mediate increased CD11b expression which could be enhanced by ROS (Ogino, Ozaki et al. 2010). Furthermore Blouin et al. described a mechanism involving ROS and a tyrosine kinase in mediating CD11b expression on neutrophils in response to physiological agonists such TNF-α (Blouin, Halbwachs-Mecarelli et al. 1999), however in previous studies the source of ROS was not determined.

The involvement of ROS in cell surface antigen expression extends to CD36; in primary human monocytes the F(ab')2 fragment of adalimumab induced CD36 expression and mRNA expression via NADPH oxidase-generated ROS (Boyer, Balard et al. 2007). However, here MnTBap which inhibited MitoSOX oxidation presumably depleting mitochondrial ROS did not affect the expression of CD36, instead demonstrating ceramides are the principal mediators of both the CD11b and CD36 increase in expression in THP-1 monocytes in the presence of palmitate. Together these studies suggest that activation of NADPH oxidase might be an important effector of palmitate-mediated cell surface antigen expression in some cells but this has not been explored in monocytes.

To investigate the role of β-oxidation of fatty acids in the THP-1 monocyte cell surface changes, etomoxir an irreversible inhibitor of carnitine palmitoyl transferase-1 (CPT-1) was used to prevent entry of fatty acids into the mitochondria for β-oxidation. Co-treatment of THP-1 monocytes with palmitate and etomoxir indicates there is no requirement for oxidation of fatty acids for changes in either CD11b or CD36 expression.

Experimental observations by others co-treating cells with both palmitate and etomoxir provide mixed results. For example in β-islet cells, palmitate induction of the unfolded protein response and consequently endoplasmic reticulum stress was not reliant upon palmitate oxidation (Sargsyan, Sol et al. 2011) and in osteoblasts apoptotic induction was independent of palmitate oxidation (Kim, Ahn et al. 2008). In skeletal muscle and FAO hepatoma cells, induction of insulin resistance by DAG and PKC-θ could be abolished in the presence of etomoxir indicating the involvement of FA oxidation (Ruddock, Stein et al. 2008, Coll, Alvarez-Guardia et al. 2010). In skeletal muscle cells, palmitate oxidation is required to activate JNK via mitochondrial ROS (Nakamura, Takamura et al. 2009). In human microvascular mesangial cells, apoptotic induction by SFA including palmitate was potentiated by etomoxir co-treatment (Mishra and Simonson 2005).

An interesting report by Xu et al. highlighted the ability of etomoxir, at similar concentrations used here, to inhibit the activity of DGAT, the enzyme responsible for the final step in triglyceride

synthesis, by up to 81% (Xu, Taylor et al. 2003). In a study of skeletal muscle cells, Hommelberg et al. reported an increase in DAG with palmitate and etomoxir cotreatment which was responsible for induction of insulin resistance (Hommelberg, Plat et al. 2011). These reports suggest that a complex lipid could be responsible for the effects observed in THP-1 monocytes following palmitate treatment, and a likely candidate would be ceramides which require palmitate for their synthesis.

Ceramides are a member of the sphingolipid class of lipids which are important in cell membranes; furthermore they are bioactive species with key signalling functions in cell growth arrest, differentiation, senescence, apoptosis and inflammation (Gangoiti, Camacho et al. 2010, Bikman and Summers 2011, Boslem, Meikle et al. 2012). Ceramides are generated through three major mechanisms, the first being the *de novo* synthetic pathway (Gangoiti, Camacho et al. 2010, Bikman and Summers 2011, Boslem, Meikle et al. 2012, Hussain, Jin et al. 2012). The two precursor components for ceramides are palmitoyl-CoA and serine which are condensed under the enzymatic activity of serine palmitoyl transferase (SPT) to form 3-ketosphinganine(Hanada 2004). The latter is reduced to sphinganine (or dihydrosphingosine), which is subsequently acylated by ceramide synthase to dihydroceramide. The ceramide synthase has distinct isoforms for different fatty-acyl CoA isoforms hence the production of different ceramide species. A desaturase enzyme introduces a double bond to yield ceramide (figure 4.8).

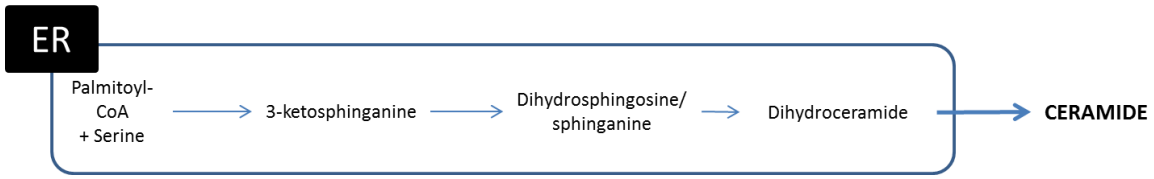


Figure 4.9: De novo synthesis of ceramides in the endoplasmic reticulum

Data in this chapter has demonstrated that inhibition of ceramide formation prevents the palmitate mediated increase in cell surface CD11b and CD36 expression. Experiments by Tettamanti determined in most differentiated cells much of the ceramides are generated by either *de novo* synthesis or the salvage pathway (Tettamanti 2004), thus it was important to determine whether other ceramide metabolites play a role in cell surface changes on THP-1 monocytes.

The glucosylceramide synthase inhibitor PPMP, and the alkaline ceramidase inhibitor MAPP were crucial in eliminating the involvement of glucosylceramides, sphingosine and sphingomyelin in surface antigen changes the use of these inhibitors are predicted to increase the levels of ceramides in the presence of palmitate. Under these conditions cell surface antigen expression was increased lending further support to the prominent role of ceramides.

The precise mechanisms which govern the expression of CD11b and CD36 havebeen determined. Our lab has previously shown that C-reactive protein upregulates cell surface CD11b expression in THP-1 monocytes, reliant upon tyrosine phosphorylation of Syk and subsequent calcium mobilisation, independently of intracellular ROS (Woollard, Fisch et al. 2005) but this interaction involved a Fcγ receptor interaction. Han et al. demonstrated the ability of LDL to increase CD11b expression in THP-1 monocytes, which required binding of LDL to its receptor, CD36, and calcium mobilisation (Han, Chen et al. 2003).

In neutrophils, the precise role of p38 MAPK upstream of CD11b expression during chemotaxis was defined using inhibitors (Heit, Colarusso et al. 2005, Xu, Hossain et al. 2013). In eosinophil migration, experimental observations indicate the involvement of protein kinase C and ERK-1/2 and p38 MAPK in both CD11b expression and cell motility (Langlois, Chouinard et al. 2009), and similar reports have been recorded in human neutrophils (Wyman, Dinarello et al. 2002, Evangelista, Pamuklar et al. 2007). Inhibition of neutral SMase altered polymorphonuclear neutrophil migration (Sitrin, Sassanella et al. 2011), suggesting a role of ceramide metabolites in this aspect of immune responses.

There are many reports of stress kinases JNK, ERK 1/2 and p38 being involved in ceramide signalling in a variety of cell lines including breast cancer (Malki and El Ashry 2012), ovarian cancer (Yang, Ji et al. 2012), pancreatic (Song, Wohltmann et al. 2012), erythrocytes (Gatidis, Zelenak et al. 2011), adipocytes (Mottillo, Shen et al. 2010), monocytes in addition to animal models (Lin, Lin et al. 2011, Liangpunsakul, Rahmini et al. 2012). In monocytes, one postulated mechanism of activation by SFA in inducing inflammation has been through generation of ceramides with subsequent activation of PKC-ζ which in turn activates MAP kinases including Erk, JNK and p38 (Schwartz, Zhang et al. 2010). Alternatively, the activation of protein phosphatase 2 enzymes are considered to link ceramides with the MAP kinases (Perry, Kitatani et al. 2012). Thus, it is plausible to suggest that SFA mediates the increase in THP-1 monocyte cell surface CD11b expression through the formation of ceramides with activation of either PKC or PP2 activation and subsequent activation of stress kinases, most likely p38. The reliance of palmitate on formation of ceramides may also explain the lack of effect of oleate on CD11b expression, on the basis that only the SFA are channelled toward ceramide production.

Control of CD36 expression is well characterised, with a critical regulator being PPARγ (Nagy, Tontonoz et al. 1998, Tontonoz, Nagy et al. 1998). Studies by Nagy and Totonoz et al. demonstrated the ability of oxLDL to increase the expression of CD36, effectively a feed forward loop to increase uptake. Once LDL and macrophages become trapped in the artery vessel wall, inflammatory stimuli

lead to the production of ROS and RNS. Oxidative stress induces modifications to LDL which are recognized by LDL. These ligands are internalised and generate PPARγ ligands with subsequent nuclear translocation and upregulation of target genes including *CD36* and *PPARγ*.

LCFA are also ligands for CD36, and as this study demonstrates induce a feed forward activation of CD36 expression in a similar manner to oxLDL. In adipocyte differentiation, increased CD36 expression was reliant upon LCFA presence (Sfeir, Ibrahimi et al. 1997). This raises the possibility that LCFA may mediate the increase in CD36 expression through the activation of PPARγ, with a role for both PKC and PPARγ in the induction of CD36 expression on RAW 2774 murine macrophages (Feng, Han et al. 2000), whilst Zhao et al. reported the involvement of p38 MAPK in PPARγ signalling and CD36 expression (Zhao, Liu et al. 2002). The raises the possibility of the involvement of ceramides in this process, and could explain phenotypic changes observed with palmitate but not oleate.

4.6 Conclusion

To conclude, the SFA palmitate but not the MUFA oleate increases cell surface expression of both CD11b and CD36. The physiological relevance of these changes have not been investigated here but are likely to be pro-atherogenic via CD11b binding to ICAM-1 expressed on vascular endothelial cells. Thus these cells are better able to adhere to the vasculature and thus promote atherogenesis in response to an insult or injury.

Similarly, elevated CD36 may promote atherosclerosis through the increased uptake of FA, LDL, and oxLDL promoting the formation of foam cells and release of inflammatory cytokines with further recruitment of monocytes.

There was no involvement of mitochondrial ROS in increasing the expression of both CD11b and CD36 in THP-1 monocytes. The mediators of SFA-induced effects in this model were ceramides, specifically generated by the *de novo* synthesis of ceramides from palmitate. Indeed oleate is preferentially shuttled toward triglyceride and palmitate to ceramides, thus explaining the differential impact of MUFA and SFA in THP-1 monocytes.

The data generated further highlights the differential effects of MUFA and SFA on phenotypic changes of THP-1 monocytes. The SFA induces a pro-atherogenic phenotype in monocytes which may aid pathological progression, whilst oleate has a neutral effect.

Chapter 5

Role of fatty acids in modulating monocyte cytokine production and differentiation

5 Chapter 5: Role of fatty acids in modulating monocyte cytokine production and differentiation

5.1 Preface

Here an investigation of the ability of MUFA and SFA to induce inflammatory phenotypes in monocytes is determined with respect to the generation of cytokines and cell surface marker expression in response to LPS following treatment with FA (50μM, 150μM and 300μM) or BSA. The influence of MUFA or SFA in influencing monocyte-macrophage differentiation and macrophage phenotype will be determined.

5.2 Introduction

The monocyte population in humans cannot be regarded as a purely homogeneous population, consisting of three identified monocyte phenotypes; classical, intermediate and non-classical which are defined by expression of CD14 and CD16. The differentiation of recruited monocytes to macrophages is widely acknowledged to be a critical for atherosclerotic plaque development. In response to environmental stimuli recruited monocytes will differentiate to macrophages with specific phenotypes. Macrophages also constitute a functionally heterogeneous population of cells. Depending upon the stimuli, the macrophage can be become polarised toward distinctive phenotypes, the two major classes of polarisation are defined as the classically-activated M1 or the alternatively-activated M2. The former demonstrate pro-inflammatory secretion of TNF-α and IL-6 whilst the latter secrete anti-inflammatory cytokines contributing to wound healing and regulation of inflammatory processes (Schwartz and Svistelnik 2012, Sica and Mantovani 2012, Mantovani, Biswas et al. 2013).

In type 1 diabetes, the prevailing metabolic state induces an increase in the expression of long chain acyl-CoA synthetase (ACSL1) in macrophages and promotes a pro-inflammatory phenotype (Kanter, Kramer et al. 2012). Monocytes from type-2 diabetes patients demonstrate a similar pro-inflammatory profile, although the reasons behind this are yet to be elucidated (Giulietti, van Etten et al. 2007)

In the context of atherosclerosis, macrophage polarisation has been implicated in altering the progression and stability of plaques. A pro-inflammatory environment typical of a plaque would favour M1 polarisation of macrophages however studies demonstrate the presence of M1 and M2 resident macrophages. In mouse models, the M2 macrophage containing plaques reside in stable regions of the plaque, typically away from the lipid core, reducing likelihood of plaque rupture and a possible coronary event (Khallou-Laschet, Varthaman et al. 2010, Ley, Miller et al. 2011). .

Pro-inflammatory cytokines e.g. TNF-α and IL-6 are capable of activating serine kinases including IKK and JNK (Gual, Le Marchand-Brustel et al. 2005) which promotes serine phosphorylation of IRS-1 and impairs insulin signalling therefore inducing insulin resistance (Schenk, Saberi et al. 2008). Alternatively-activated macrophages (M2) have been shown to counter these effects, through the activation of PPARγ. The induction of M2 phenotype requires IL-4/IL-13 activation of STAT6 pathway, and deficiency of the latter in animal models generates a phenotype prone to diabetes development.

In the context of metabolic disorders and ageing, increased circulating free fatty acids (FFA) are a common observation. However, the effect of specific FFA upon monocytes before and during differentiation has yet to be researched in detail. On the basis that SFA are capable of activating monocytes/macrophages through TLRs, it is possible that FFA can influence polarisation. In animal models, SFA and MUFA have been shown to induce classical and alternative polarisation in macrophages respectively (Lumeng, Bodzin et al. 2007, Samokhvalov, Bilan et al. 2009), but whether this translates to human cells has yet to be determined.

Here the inflammatory effects of SFA and MUFA on monocytes are examined with a focus on the production M1 pro-inflammatory TNF-α and IL-6, and the M2 anti-inflammatory cytokine IL-10.

To determine the effects of FA/BSA on monocyte to macrophage differentiation, monocytes were treated with FA/BSA or M1/M2 cytokines prior to induction of differentiation with PMA to determine if FA can influence polarisation toward a classical or alternatively-activated lineage, measured as cytokine secretion and cell surface marker profiles (CD11b, CD14, CD16 and CD36).

5.3 Results

In order to determine if either the MUFA oleate or the SFA palmitate were capable of inducing cytokine production, THP-1 monocytes were treated for 24h with either fatty acid, and the supernatants were analysed for cytokine content.

5.3.1 Cytokine production in THP-1 monocytes treated with palmitate or oleate is unaltered

The production of cytokines IL-6, IL-10 and TNF-α was assessed following 24h treatment with FA/BSA by an ELISA method. In the presence of fatty acids (figure 5.1) palmitate or oleate, no change in levels of cytokines released into the supernatant by THP-1 monocytes is observed above BSA control treated cells, nor was there a change in absolute levels of cytokines (0.1ng/ml for IL-6, IL-10 and TNF-α).

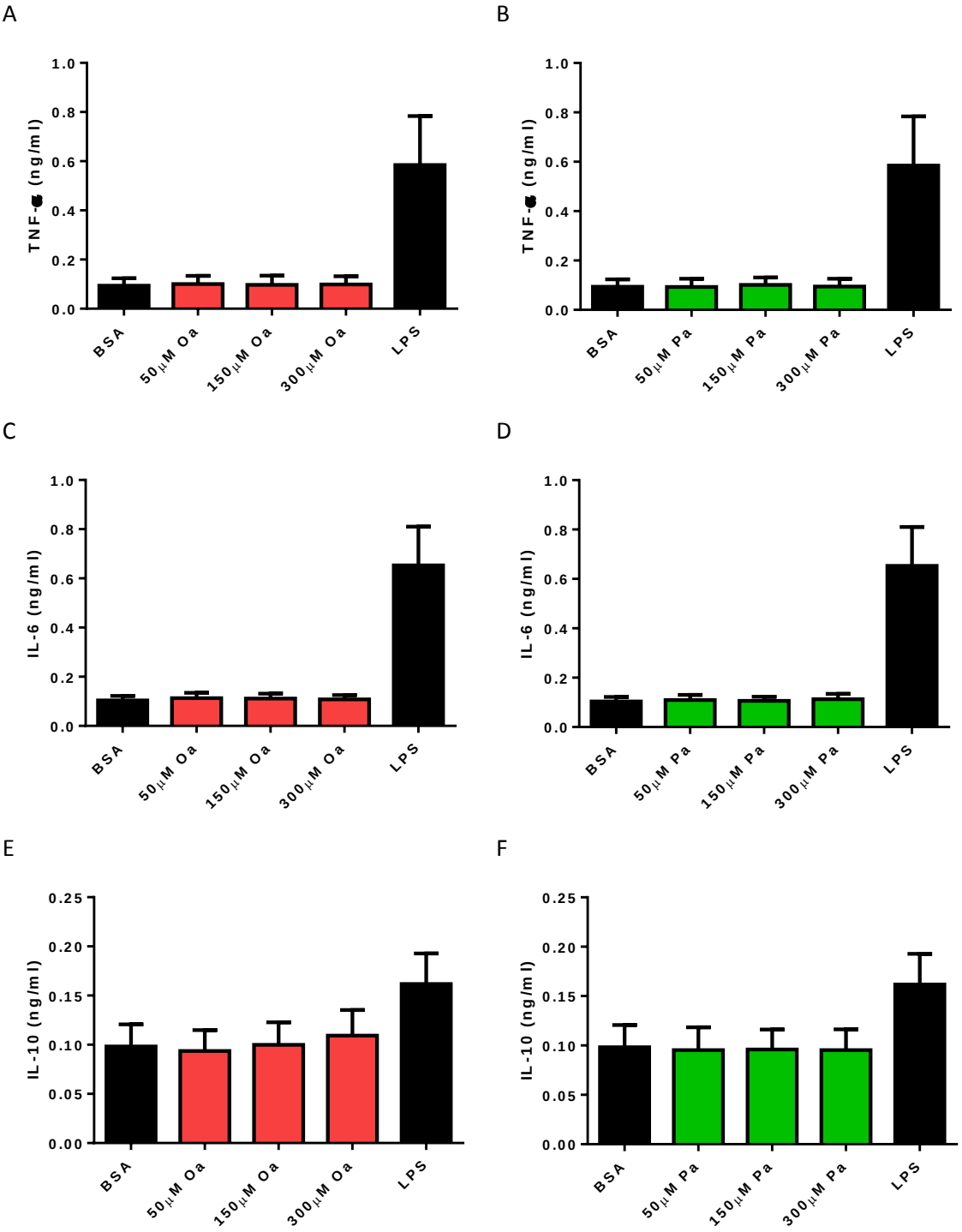


Figure 5.1: Fatty acids do not induce cytokine production in THP-1 monocytes:

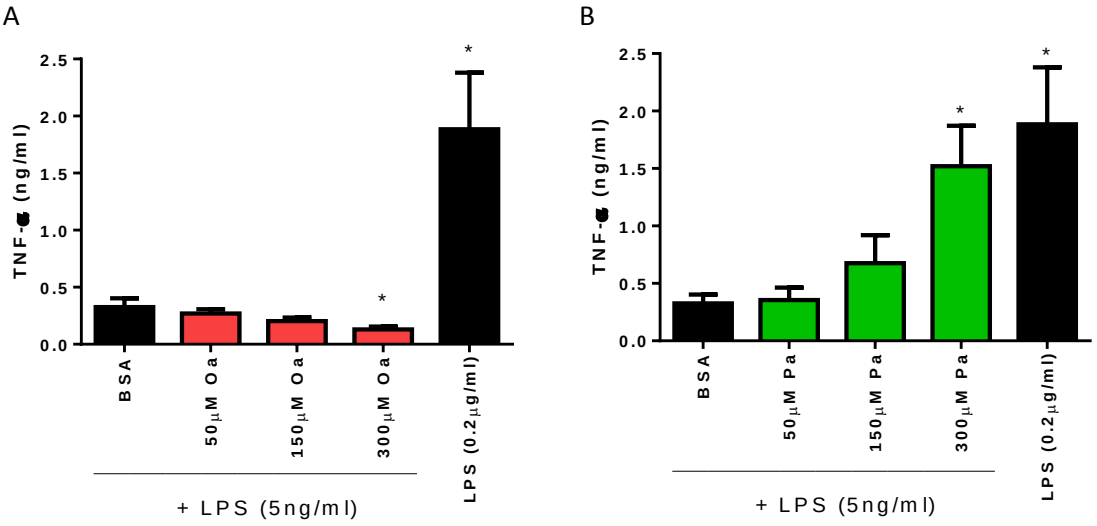
Upon completion of 24h treatment of (5 x 10^5/ml) THP-1 monocytes with BSA/FA (1:6 FA ratio, concentrations of 50µM, 150µM, 300µM), supernatants were either analysed for cytokines immediately or aliquoted and stored in -20°C, using TNF-α, IL-6 and IL-10 kits (Peprotech). The production of TNF-α by THP-1 monocytes in the presence of either (A) oleate (Oa) or (B) palmitate (Pa), IL-6 production from Oa (C) or Pa (D) treated monocytes, and IL-10 production from Oa (E) and Pa (F) is expressed as ng/ml of respective cytokine. Positive control utilised was LPS at 0.2µg/ml.

5.3.2 Priming of THP-1 monocyte proinflammatory response to LPS by palmitate but not oleate

There is evidence that immune responses can be modified by dietary components including glucose and fatty acids; additionally, the metabolic state can influence the differentiated phenotype of immune cells. Therefore, this study sought to determine if fatty acids could modulate the response of THP-1 monocytes to a sub-maximal LPS dose following pre-treatment for 6h with BSA, oleate or palmitate (50µM, 150µM and 300µM). Subsequently supernatants were separated from cells and analysed for the cytokines TNF-α, IL-6 and IL-10.

Pre-treatment of cells with 0.2µg/ml LPS for 6h appeared to influence the response to a stronger secondary stimulus of LPS in a FA-distinct manner (figure 5.2). In response to LPS, prior incubation with oleate reduced TNF-α secretion into the supernatant, conversely the SFA palmitate induced a dose-dependent increase in cytokine release (Figure 5.2A and B; $p<0.05$). The IL-6 response to LPS mimics the TNF-α response to oleate and palmitate (Figure 5.2C and D). These findings suggest that palmitate treatment primes the cells toward an enhanced response to inflammatory stimuli whereas oleate has a suppressive effect.

In contrast, 6h pre-treatment with FA prior to LPS on secretion of the anti-inflammatory cytokine IL-10 caused a dose-dependent trend to reduction in IL-10 production (Figure 5.2E and F). However, LPS at 5ng/ml or 0.2µg/ml does not induce an IL-10 response above BSA control.



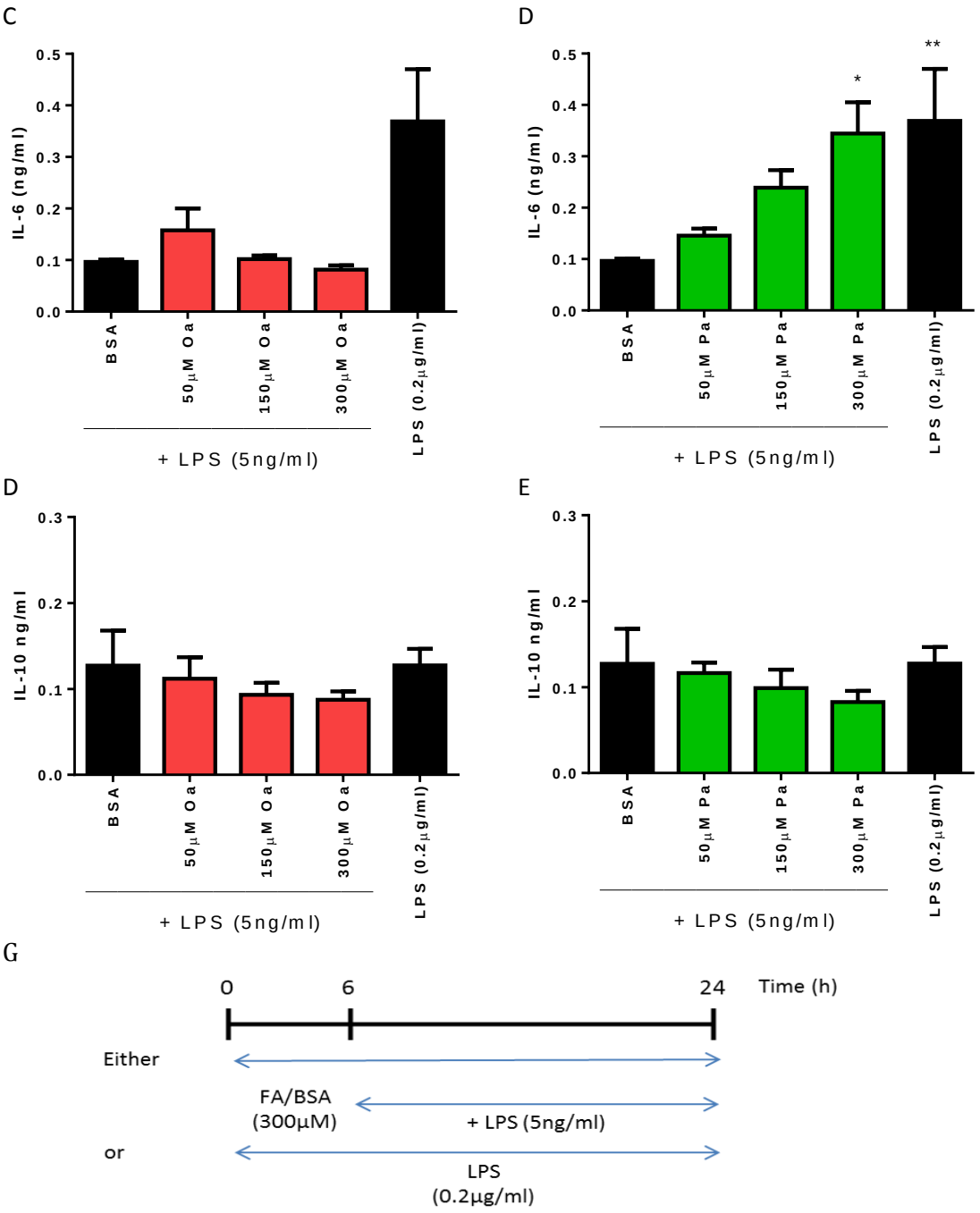


Figure 5.2: Pretreatment of THP-1 monocytes with palmitate enhances pro-inflammatory response to LPS:

THP-1 monocytes ($5x10^5$ cells/ml) were pretreated with BSA, oleate or palmitate for 6h prior to treatment with 0.2µg/ml of LPS for 18h in RPMI 1640 10% FBS and 1% P/S(G). Upon experimental completion cells were spun, the supernatant kept and the cell pellet discarded, with analysis of cytokine levels determined by ELISA method for TNF-α (A and B), IL-6 (C and D) and IL-10 (E and F), with 100µl of sample utilised in triplicate per assay, with data presented as mean from three independent experiments, with 0.2µg/ml LPS used as positive control for cytokine production.

5.3.3 Pre-treatment of THP-1 monocytes with FA does not alter responses to peptidoglycan

Peptidoglycan from *Staphylococcus aureus* as a secondary stimulus to fatty acids was investigated after LPS failed to induce significant increases in IL-10 production, as this endotoxin derived from Gram positive bacteria is a ligand for TLR2 which can induce IL-10 production. Initially, a dose-response was generated before confirming 125µg/ml PG as a co-activator, and with 250µg/ml PG as a positive control for IL-10 production (figure 5.3). In this model no priming of cytokine production was observed in the presence of either fatty acid, which would suggest that TLR2 mediated processes are not affected by palmitate or oleate priming (Figure 5.3B and 5.3C).

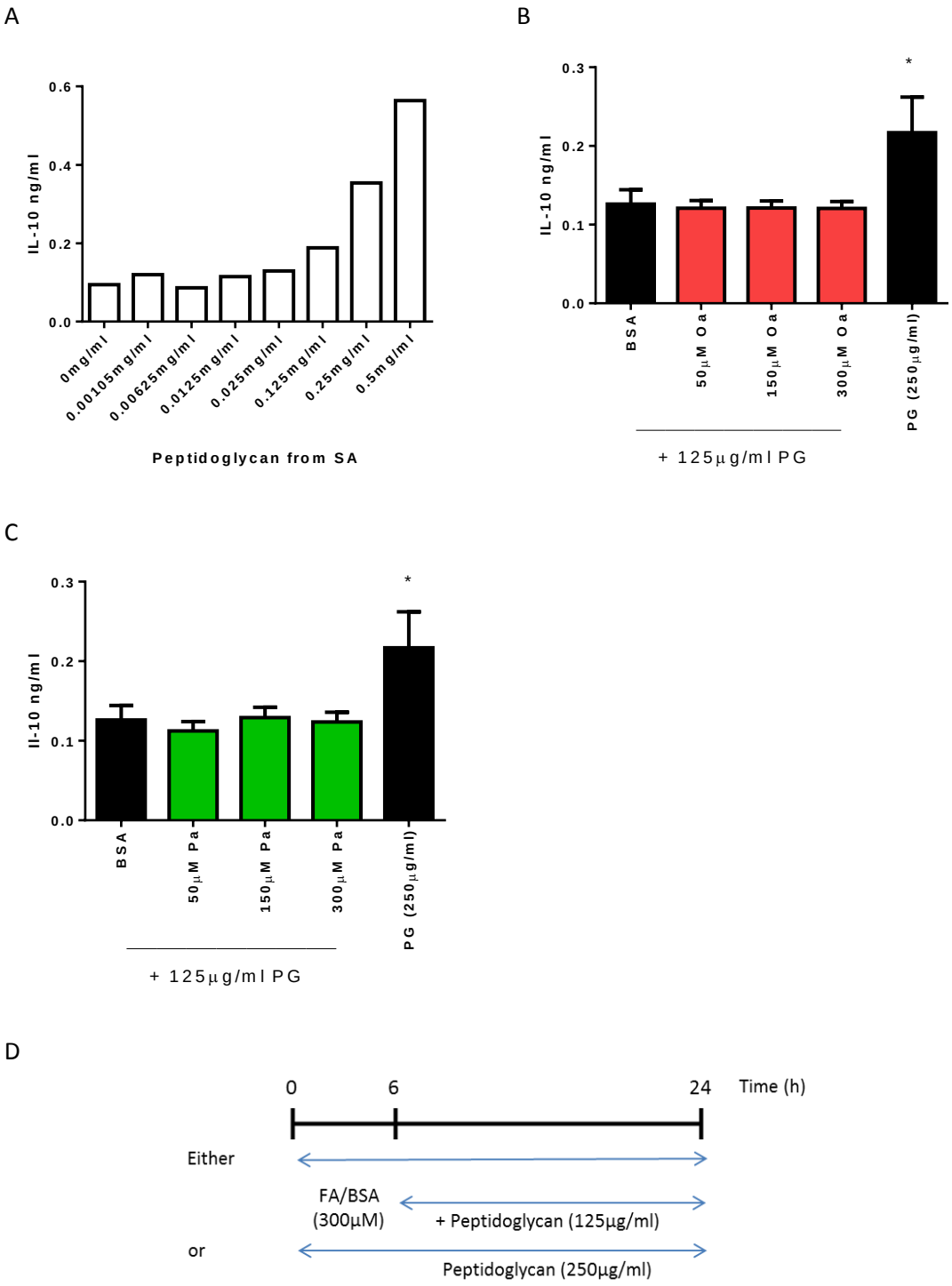


Figure 5.3: Fatty acid pre-treatment does not alter THP-1 monocyte responses to peptidoglycan from *Staphylococcus aureus*:

THP-1 ($5x10^5$ cells/ml) monocytes were treated with a titration of peptidoglycan in order to determine a suitable concentration to use in a dual stimulus model (FA + bacterial lipid), subsequently based on the dose response curve (A) data generated 125µg/ml PG-SA was utilised to induce IL-10 production when co-incubated with FA, with 250µg/ml was as a positive control. THP-1 monocytes were subsequently treated for 6h in the presence of FA or BSA, and then activated with 125µg/ml PG to determine the effect on IL-10 production (D). Effects of oleate (B) and palmitate (C) are displayed as the mean of 3 independent experiments, with one-way ANOVA performed to determine significance where *=$p<0.05$.

5.3.4 Differences in cytokine responses in oleate and palmitate pretreated cells cannot be explained by endotoxin contamination

The endotoxin content of BSA, oleate and palmitate working solutions were tested, with both the MUFA oleate and SFA palmitate demonstrating comparable levels of endotoxin, although both were elevated above BSA control (figure 5.4) in spite of solutions being sterile filtered prior to use. The finding is novel but does not explain the differential priming of monocytes exposed to either oleate or palmitate and secondly to LPS. The assay kit uses toxin derived from Gram-negative bacteria, but does not specify whether this related to or is LPS, thus one can only speculate that perhaps the assay recognises fatty acyl groups present on lipid endotoxins, hence the comparable levels of endotoxin observed in both oleate and palmitate working solutions.

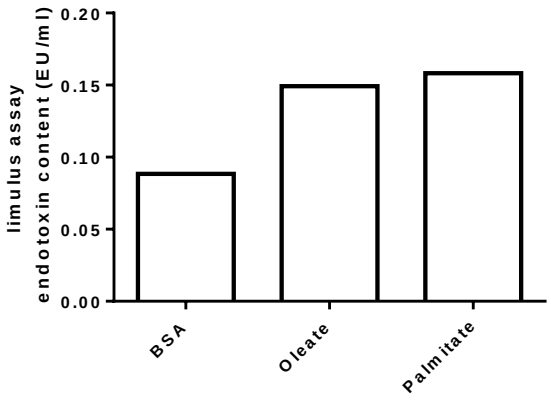


Figure 5.4: Oleate and palmitate (300μM) working solutions have comparable limulus assay positivity:

Using a Limulus assay, gram negative endotoxin contamination of working fatty acid solutions was assessed. Solutions were analysed according to kit instructions (Lonza, UK), with data displayed as mean of 3 wells on 96 well plates, n=1, where data is shown as endotoxin units (EU/ml).

5.3.5 Cell surface antigen expression of FA/BSA treated cells in a model of monocyte differentiation

In light of previous findings, and observations by others of the elevation of NEFA in a variety of metabolic conditions and ageing, the influence that either the MUFA oleate or the SFA palmitate has on monocyte differentiation was investigated for pro- and anti-inflammatory phenotype induction, or phenotypes which resemble M1 classically or M2 alternatively-activated monocytes

THP-1 monocytes were pre-treated with BSA, FA or LPS or were left untreated for 6h before treatment with PMA for a further 18h, 42h and 66h with total treatment times of 24h, 48h and 72h. Cells were analysed for cell surface marker expression of CD11b, CD14, CD16 and CD36 by flow cytometry.

In monocytes, over 72 hours, cell surface CD14 (figure 5.5C) expression did not change in BSA, FA or untreated monocytes, although LPS treated cells increased expression of CD14 from 24 hours which was maintained up to 72h ($p<0.0001$ at 24h, $p<0.001$ at 48h, and $p<0.01$ at 72h). With respect to CD11b (figure 5.5B), palmitate treatment mediated an increase in expression which is maintained for 72 hours. Whilst a % up-regulation in cell surface CD11b is induced by LPS, this is not maintained over 72 hours, peaking at 24h ($p<0.01$) and declining at 48h ($p<0.01$), becoming equivalent to untreated THP-1 cells at 72h. Cell surface expression of CD36 (figure 5.5D) increased ~2 fold in response to palmitate, above that of BSA, oleate and untreated cells, and continues to increase over a 72 hour period ($p<0.01$ at 72h). CD16 expression in monocytes (figure 5.5E) was not significantly different between treatments over the time course.

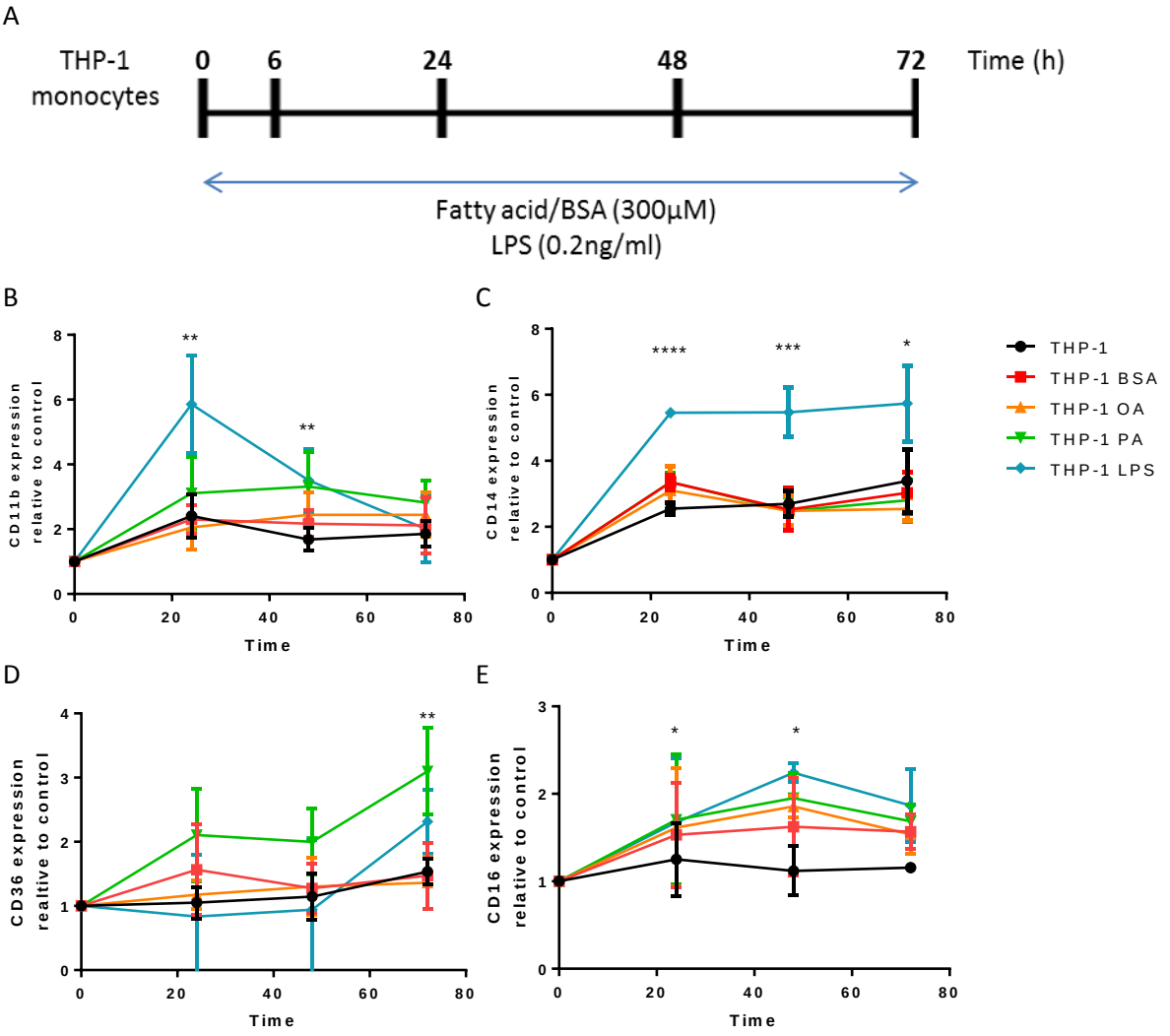


Figure 5.5: Changes in cell surface antigen expression of THP-1 monocytes:

Cells were treated 300µM of either oleate (OA) or palmitate (PA), BSA or LPS (0.2µg/ml) or untreated (as control) for 72h, with cell surface expression of CD11b (B), CD14 (C), CD16 (E) and CD36 (D) assessed at 24h, 48h and 72h under flow conditions (schematic figure 5A). Data is expressed as relative to untreated THP-1 monocytes t=0 with the mean of 3 independent experiments ± SEM, and data analysed by one-way ANOVA at 24h, 48h and 72h , where *=p<0.05, **=p<0.01, ***=p<0.001, and ****=p<0.0001 versus THP-1.

THP-1 monocytes treated with PMA after an initial 6h FA/BSA treatment, and phenotypic profile was measured over the course of 72h (figure 5.6A). The effect of PMA cell surface expression of CD14 was not different between BSA, LPS and untreated cells. In contrast, both oleate and palmitate demonstrated differential effects with the MUFA enhancing CD14 expression above PMA treatment alone over 72h, whilst SFA palmitate depressed cell surface CD14 expression to 72h when compared to cells treated with PMA alone (Figure 5.6B).

Comparing palmitate-treated cells with cells in the absence of PMA at zero hours, a similar level of CD14 expression was observed, suggesting the effect of palmitate treatment is to inhibit the effect of PMA. BSA, LPS and untreated cells demonstrate raised CD14 expression in the presence of PMA; oleate-treated cells amplified CD14 expression in the presence of PMA, suggesting oleate enhances PMA's effect (figure 5.6C).

CD11b expression is not affected by any treatment in the presence of PMA. Comparison of cells treated in the presence or absence of PMA reveal a mild increase in the level of CD11b expression; suggesting PMA mediated CD11b expression is not altered by FA, BSA or LPS (figure 5.6D).

Cell surface expression changes of CD36 induced by PMA over the 72h experimental period were not affected by FA, BSA or LPS treatment compared to cells treated with PMA alone. The presence of PMA caused an increase in CD36 (an expected increase given the common observations of increased CD36 observed during differentiation) expression suggesting there is no modulation of PMA's effect by any treatment (figure 5.6E).

Expression of cell surface marker CD16 does not differ with FA, BSA or LPS in the presence of PMA; a time dependent increase in CD16 over 72h was observed. Although elevated levels of CD16 were observed in cells treated in the presence of PMA, palmitate and LPS displayed slightly reduced levels compared to cells treated with oleate and PMA alone which were similar, but these differences were not significant (figure 5.6F).

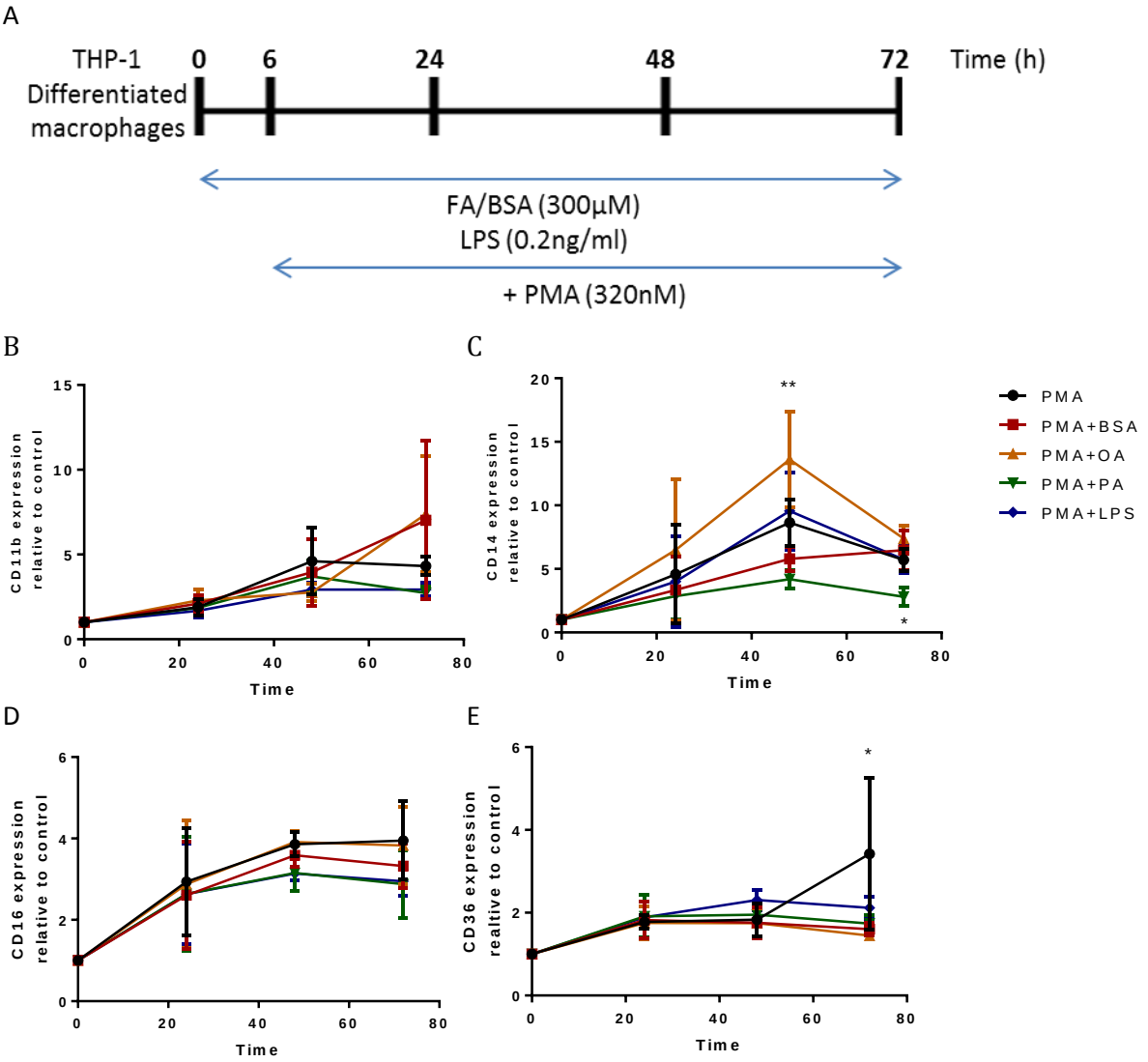


Figure 5.6: Expression of CD14 and CD11b are decreased in palmitate primed macrophages:

THP-1 monocytes were treated with FA, BSA, LPS or left untreated for 6h prior to stimulation with 320nM PMA to induce differentiation (A). Analysis of cell surface expression of CD11b (B), CD14 (C), CD16 (D) and CD36 (E) was determined by flow cytometry at 24h, 48h and 72h. Cells were then left on ice for 15min in RPMI 1640 10% FBS to block Fc receptors, subsequently treated with saturating concentration of antibody or respective negative IgG1 or IgG2α isotype control for 30 min on ice before flow cytometry. Data is displayed as mean of at least 3 independent experiments, expressed as relative to untreated THP-1 monocyte at t=0 ±SEM. Data was analysed by one way ANOVA whereversus PMA treated THP-1 cells at 24h, 48h and 72h, with *=p<0.05, and **=p<0.01.

5.3.6 Palmitate-primed macrophages generate a pro-inflammatory phenotype

Whilst the examination of cell surface markers is able to tell us about changes in receptors concerned with scavenging functions or cell migration, they do not provide information regarding any change in the inflammatory function due to FAs. In order to answer this question, levels of pro- and anti-inflammatory cytokines TNF-α, IL-6 and IL-10 respectively were quantified from supernatants after 24h treatment (6h FA/BSA + 18h PMA).

Earlier experiments demonstrated that no real elevation in cytokine production is induced in monocytes in response to FA/BSA alone; only LPS was capable of inducing increased expression of TNF-α and IL-6, whilst IL-10 induction was absent in the presence of LPS and FA/BSA treatments in monocytes (figure 5.2).

In the presence of PMA as a model to induce differentiation to macrophages, production and release of TNF-α was ~5-6 fold higher than control, BSA and oleate treated cells and when compared to monocytes; palmitate induces a ~10 fold increase and LPS induces a ~12 fold increase in TNF-α respectively ($p<0.01$) compared to monocytes treated with PMA. Comparison of palmitate- and oleate-treated macrophages to BSA control indicates that the MUFA marginally suppresses TNF-α and the SFA enhances its production (Figure 5.7A), with palmitate primed macrophages demonstrating significant difference when compared to all other macrophages except those treated with LPS. Production of IL-6 by PMA, BSA and oleate cotreated cells was unaltered. However, in the presence of palmitate a ~5fold increase in cytokine levels were recorded similar to LPS treated cells (Figure 5.7B).

The anti-inflammatory cytokine IL-10 was also measured to determine if alternate activation of macrophage during the differentiation process was induced by fatty acids. All treatments except palmitate induced a 2 fold increase in IL-10 above monocyte controls. Palmitate appeared to suppress in IL-10 production, with no significant difference between PMA treated macrophages and monocytes/controls (Figure 5.7C).

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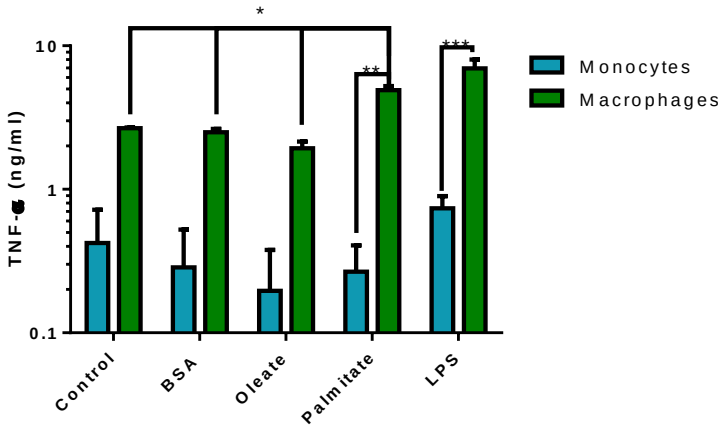

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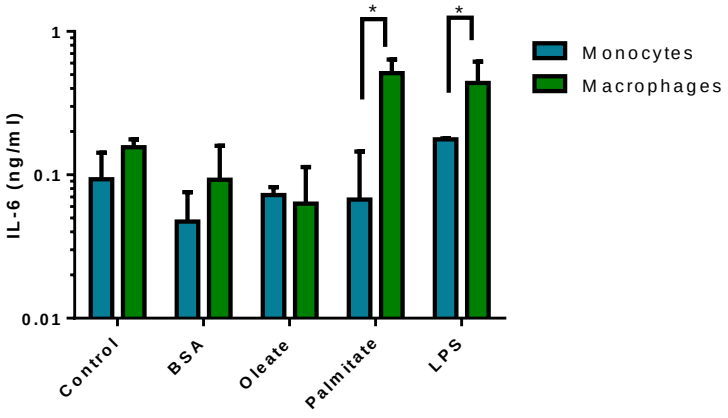

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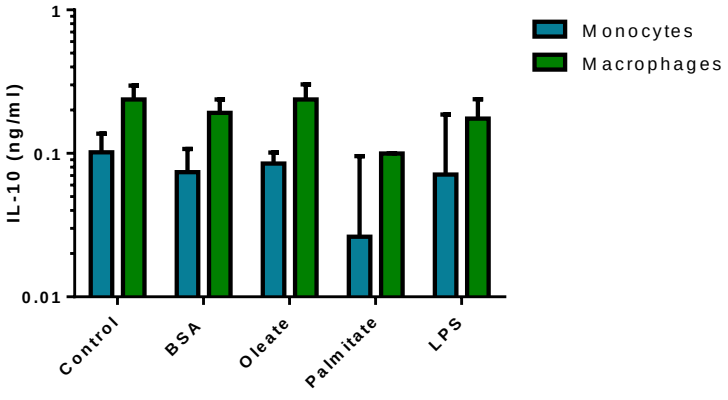


Figure 5.7: Distinct cytokine profiles are generated from FA-primed macrophages:

Monocytes and PMA-differentiated macrophages primed for 6h were analysed for supernatant TNF-α (A), IL-6 (B) and IL-10 (C) cytokine content using sandwich ELISA method. Briefly, following 24h cells were removed from cell conditions and spun gently to obtain supernatant. Data is presented as the mean of n=2 independent experiments, and significance was determined through tw0-way ANOVA comparing monocytes and PMA differentiated macrophages, with further analysis of individual macrophage treatments by One way ANOVA, where *=p<0.05, **=p<0.01 and ***=p<0.0001.

To determine if classical or alternative activation was induced by either FA, the apoptotic binding capacity of FA treated macrophages was examined through the ability of these differentiated cells to bind to apoptotic cells (figure 5.8). Whilst all macrophages have this capacity, M2 macrophages have a greater capacity to clear apoptotic cells, therefore measurement of apoptotic cell binding will determine if macrophages in the model used here are more M2 like. The apoptotic binding capacity of oleate-differentiated macrophages was ~20% greater than BSA or palmitate primed macrophages ($p<0.01$).

The cytokine data shows that palmitate enhances production of both the pro-inflammatory cytokines TNF-α and IL-6, with a simultaneous suppression of IL-10. Oleate appears to interfere with TNF-α production, but does not alter production of either IL-6 or IL-10. The differential effects observed would suggest that palmitate maybe inducing a pro-inflammatory phenotype.

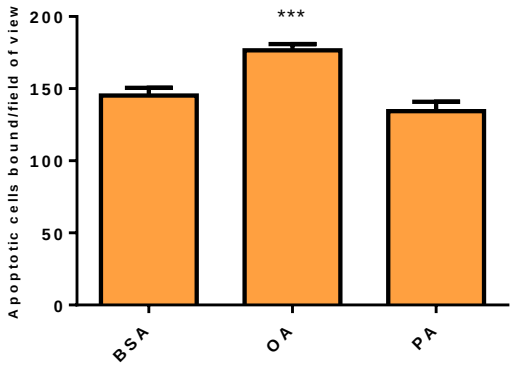


Figure 5.8: Increased apoptotic binding of oleate-primed macrophages:

THP-1 macrophages primed with BSA, oleate or palmitate were differentiated with PMA for 48hours on glass well slides to permit adhesion, subsequently unbound cells were washed off and adherent cells were incubated with Mu2 cells radiated with UV to generate apoptotic bodies for 1h at 37°C in 5% CO_2/95% air. Slides were subsequently washed to remove unbound cells, then stained and viewed under microscope to determine number of macrophages bound to apoptotic bodies in a field of view of up to 200. Data is displayed as mean of field of vision from 3 independent experiments, where ***=$p<0.001$ as determined by one way ANOVAversus BSA.

5.3.7 Distinct classical M1 and alternatively-activated M2 macrophage phenotypes produce unique cell surface and cytokine profiles

Certain differentiation programmes can be used to generate a classically (M1) or alternatively-activated (M2) THP-1 macrophages. To generate M1 or M2, IFN-γ (20ng/ml) and LPS (100ng/ml) for classic and IL-4 and IL-13 (20ng/ml) were used for alternatively-activated macrophage, treating monocytes with cytokines for 6h prior to PMA treatment. The M1 and M2 differentiation programme serve as a comparator to the macrophages that were differentiated in the presence of FA/BSA.

As with FA/BSA treatment programmes, cells treated in the absence PMA were utilised as monocyte controls (figure 5.9A) whilst cells treated with PMA were taken as macrophages (figure 5.10A). Similar levels of cell surface CD14 expression were observed between M1, M2 and untreated THP-1 monocytes but at 48h and 72h M2 monocytes produced higher cell surface CD14 expression (figure 5.9C). The cell surface expression of CD11b was elevated in M2 treated monocytes and were significantly elevated at 24h ($p<0.01$) remaining elevated for 72h, whilst M1 and untreated cells show no increases in CD11b (figure 5.9B). All treatments elicited a time dependent increase in the cell surface expression of CD36 (figure 5.9E), whilst monocyte CD16 expression was only significantly elevated for 72h by the M2 treatment ($p<0.01$).

Differentiation of THP-1 monocytes to M1 macrophages was associated with significantly reduced CD11b at 72h ($p<0.01$), with reduced expression compared to PMA and M2 treated cells; both M2 and PMA treated macrophages show elevated expression at 48h (figure 5.10B). The expression of CD14 was not significantly changed by PMA, M1 or M2, although elevated expression was noted in the M2 phenotype (figure 5.10C). Similarly cell surface expression of CD36 was unaffected by any treatment (figure 5.10E). M2 phenotype demonstrated a significant increase in CD16 expression at 24h, 48h and 72h ($p<0.001$ at 24h and 48h, and $p<0.01$ at 72h, figure 5.10D).

Comparison of the M1 and M2 phenotype in monocytes and macrophages indicate a more pronounced effect when cells were differentiated to macrophages in the presence of PMA on the expression of CD14, CD11b and CD16.

Oleate-differentiated macrophages demonstrate higher CD14 and CD11b expression, a finding which is similar to an extent to M2 macrophages, and which is in contrast to palmitate-differentiated macrophages. The impact of palmitate on CD14 and CD11b expression is similar to the profile of M1 macrophages. CD36 expression is common in both FA and BSA, and M1 and M2 differentiated

macrophages. The M2 phenotype was the only differentiation programme to mediate a significant increase in CD16 over 72h.

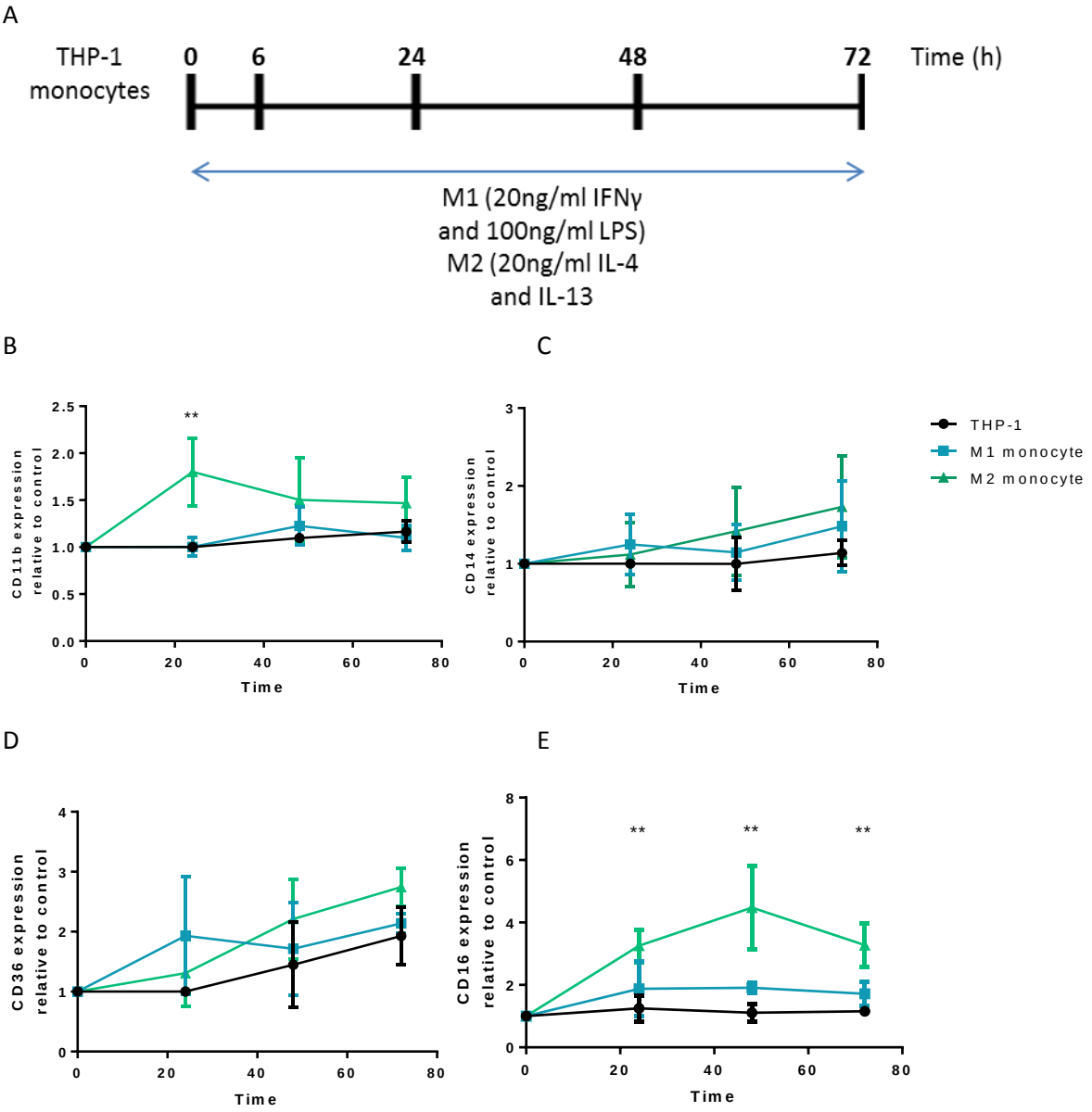


Figure 5.9: Enhanced CD11b, CD14 and CD16 in M2-cytokine treated THP-1 monocytes:

THP-1 cells were treated for 6h with 20ng/ml of IFN-γ and LPS for classically-activated M1 monocytes and 20ng/ml of IL4 and IL-13 for alternatively-activated M2 monocytes (A). At 24h, 48h and 72h cells were removed from experimental conditions, placed on ice for 15min before treatment with saturating concentration of mouse anti-human CD11b (B), CD14 (C), CD16 (D) or CD36 (E) antibody for 30min on ice before analysis under flow conditions. Data is expressed as the mean of 3 independent experiments relative to untreated THP-1 monocyte control at t=0, where *=p<0.05, **=p<0.01, ***=p<0.001 and ****=p<0.0001 as determined by one way ANOVA vs THP-1 at 24h, 48h and 72h

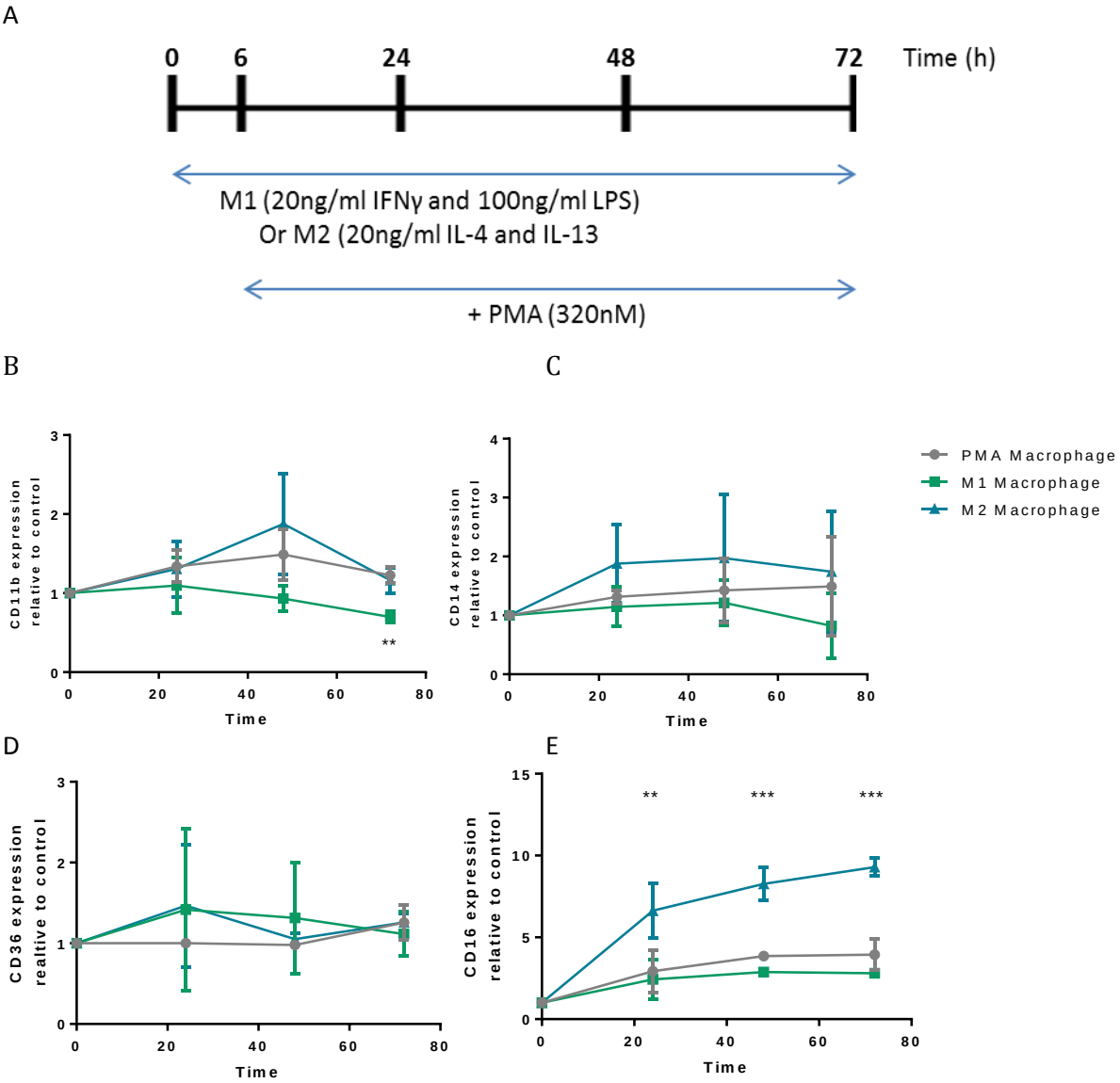
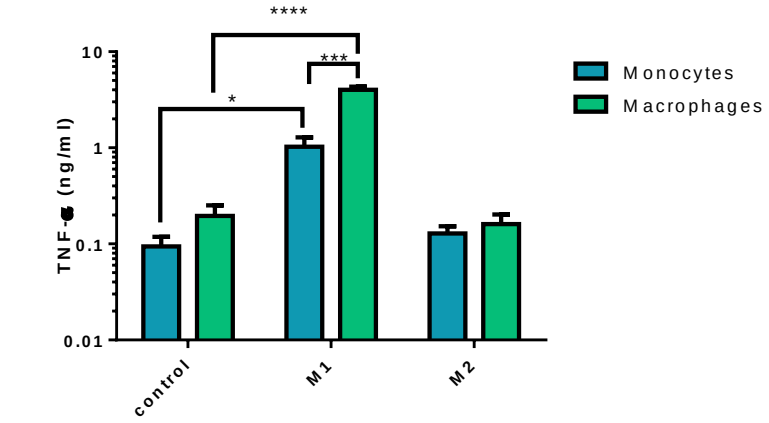


Figure 5.10: M2 macrophages display higher CD11b, CD14 and CD16 expression compared to M1 macrophages:

THP-1 monocytes were primed with M1 or M2 cytokines followed by PMA (A). At 24h, 48h and 72h cell surface antigen expression of CD11b (B), CD14 (C), CD36 (D) and CD16 (E) macrophage was determined under flow conditions, data being expressed as mean of 3 independent experiments, as relative tountreated THP-1 monocyte control at t=0, where ** =p.01 and ***=p<0.001=<0.05 determined by one way ANOVA vs PMA macrophages at 24h, 48h, and 72h. .

Differential expression of cytokines IL-6, IL-10 and TNF-αare observed with M1 and M2 macrophages. In monocytes and macrophage, treatment with M1 cytokines (IFN-γ and LPS, in the presence or absence of PMA) induced a significant increase in levels of both pro-inflammatory cytokines TNF-α and IL-6, whilst having no significant effect on the anti-inflammatory IL-10 (Figure 5.11). In contrast M2 macrophages failed to induce changes in TNF-α and IL-6 but induced a significant ~10 fold increase in IL-10.



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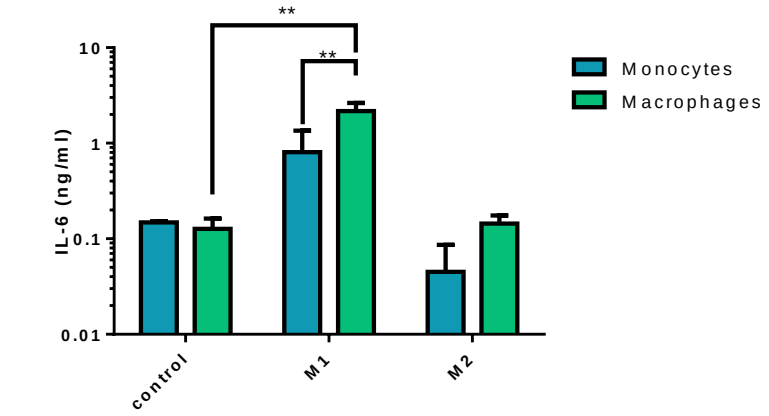

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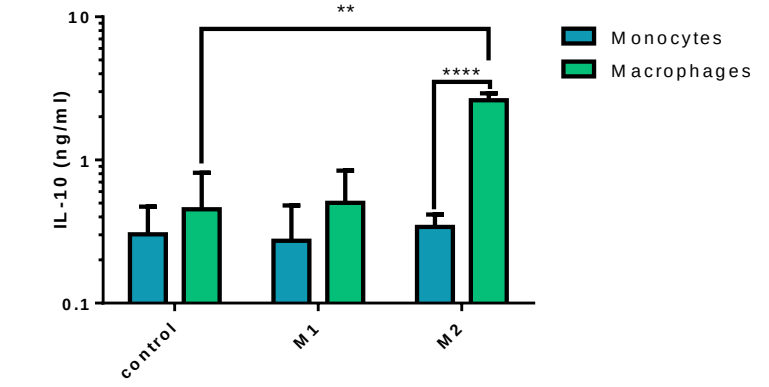

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Figure 5.11: M1 and M2 generate distinct pro- and anti-inflammatory macrophages:

Cytokine content of supernatants obtained after 24h incubation of M1, M2 or control primed macrophages was performed using sandwich ELISA. Cells were spun down gently at 150g for 5 min to obtain supernatants at which point cytokine analysis was performed immediately or store at -20°C prior to analysis. Cytokine levels of TNF-α (A), IL-6 (B) and IL-10 (C) were expressed as mean of n=3 experiments, where *=p<0.05, **=p<0.01, ***=p<0.001 and ****=p<0.0001 as determined by two way ANOVA when comparing monocytes to PMA differentiated macrophages, and then one way ANOVA to perform individual comparisons within the macrophage sub group.

5.3.8 Ceramide synthesis is required for the inflammatory phenotype of palmitate-primed macrophages

In the previous chapter, the importance of ceramides, generated through *de novo* synthesis, for mediating palmitate effects was demonstrated. To determine any role for ceramide generation induced by palmitate on macrophage phenotype, the effect of FB1 was investigated.

In the presence of BSA, palmitate or palmitate with FB1 co-treatment, THP-1 monocyte CD14 and CD16 expression was significantly altered (figure 5.12C and D). Cell surface expression of CD11b was significantly elevated by palmitate at 24h and 72h ($p<0.05$), and was significantly decreased at 48h by FB1 co-treatment ($p<0.05$) compared to palmitate alone (figure 5.12B). CD36 expression was significantly increased at 24h, 48h and 72h ($p<0.001$ at 24h and 48h, and $p<0.01$ at 72h) in palmitate-treated monocytes which was reducedin the presence of FB1 ($p<0.01$, figure 5.12E). The profile of expression for cell surface antigens except CD36 was an elevation at 24h followed a decline toward baseline expression levels at 72h.

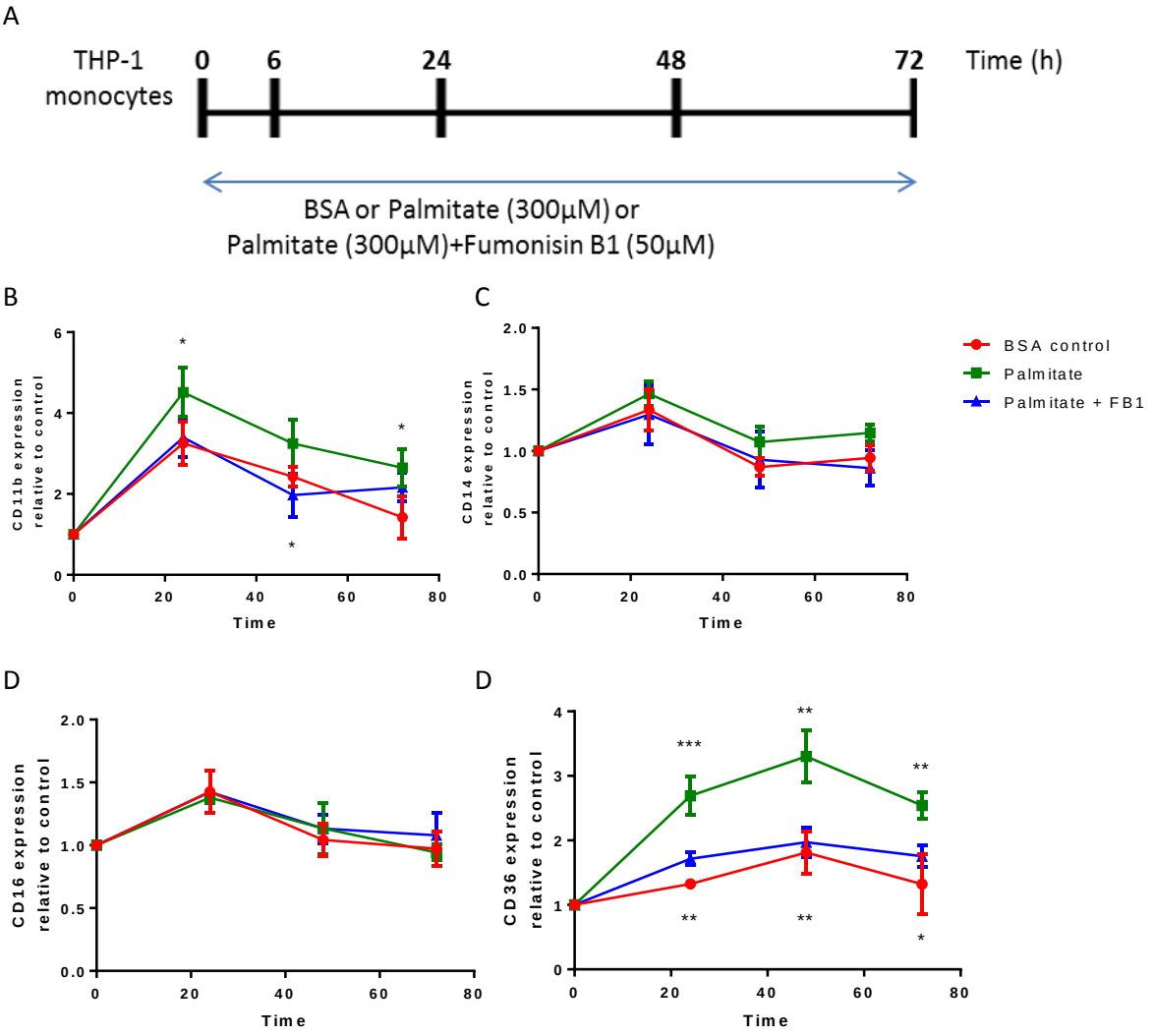

Figure 5.12: FB1 suppresses palmitate induced monocyte cell surface changes induced by palmitate treatment:

THP-1 monocytes treated with BSA, palmitate or palmitate and FB1 for 72h (A) were analysed for CD11b (B), CD14 (C), CD16 (D) and CD36 (E) cell surface expression. Upon cessation of experiment cells were placed on ice in 10% FBS RPMI 1640 for 15 min on ice, before treatment with mouse anti-human antibodies or respective IgG1 negative controls for 30min on ice. Subsequently cells were analysed under flow conditions. Data is calculated from 3 independent experiments of relative to untreated THP-1 monocyte control, with statistical analysis determined by one way ANOVA at each time point versus BSA, where *=p<0.05, **=p<0.01, ***=p<0.001 and ****=p<0.0001 represent significance comparing BSA to palmitate/palmitate + FB1 treated cells

The presence of PMA as a differentiating agent ameliorated any differences in the expression of CD11b, CD14, CD16 and CD36 (figure 5.13) due to palmitate and no further effect of FB1 was seen, with no significant differences between any treatment observed.

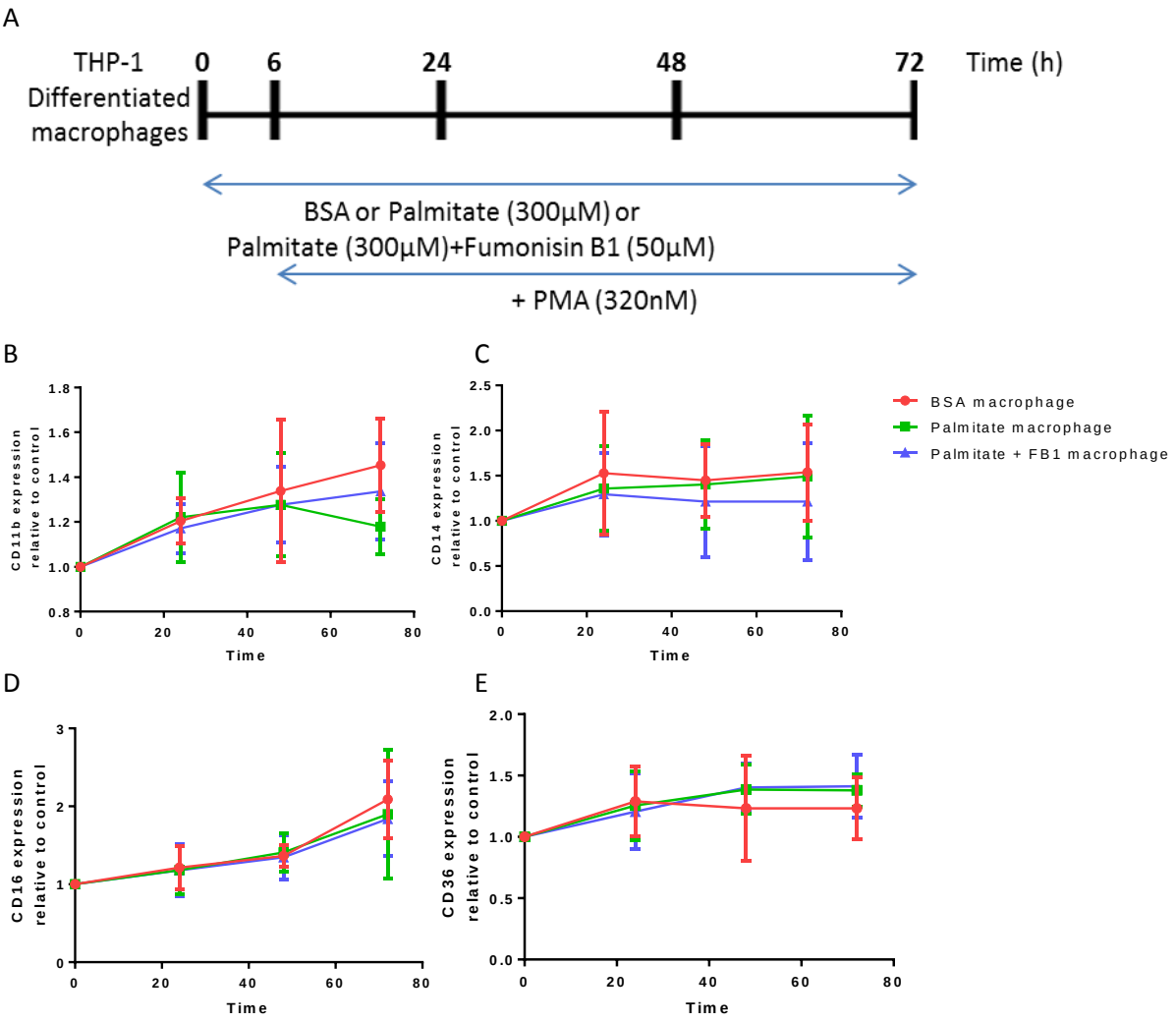

Figure 5.13: Cell surface antigen expression is not affected in palmitate primed THP-1 macrophages nor is it modulated by FB1:

THP-1 were primed with BSA, palmitate (300µM) or palmitate + FB1 (50µM) for 6h followed by PMA (320nM) for a further 66h (A). Cells were analysed at 24h, 48h and 72h for cell surface expression of CD11b (B), CD14 (C), CD16 (D) and CD36 (E), as previously described. Data is presented as mean relative to untreated THP-1 cell expression at 0h from n=3 experiment.

Palmitate-treated macrophages produce an increase in TNF-α and IL-6 secretion, whilst reducing IL-10 expression. In the presence of FB1, the palmitate-mediated increase in pro-inflammatory cytokines was significantly reduced. This effect decreased to control levels in the case of IL-6 (figure 5.14). These findings suggest palmitate mediates its pro-inflammatory effect through induction of ceramide synthesis.

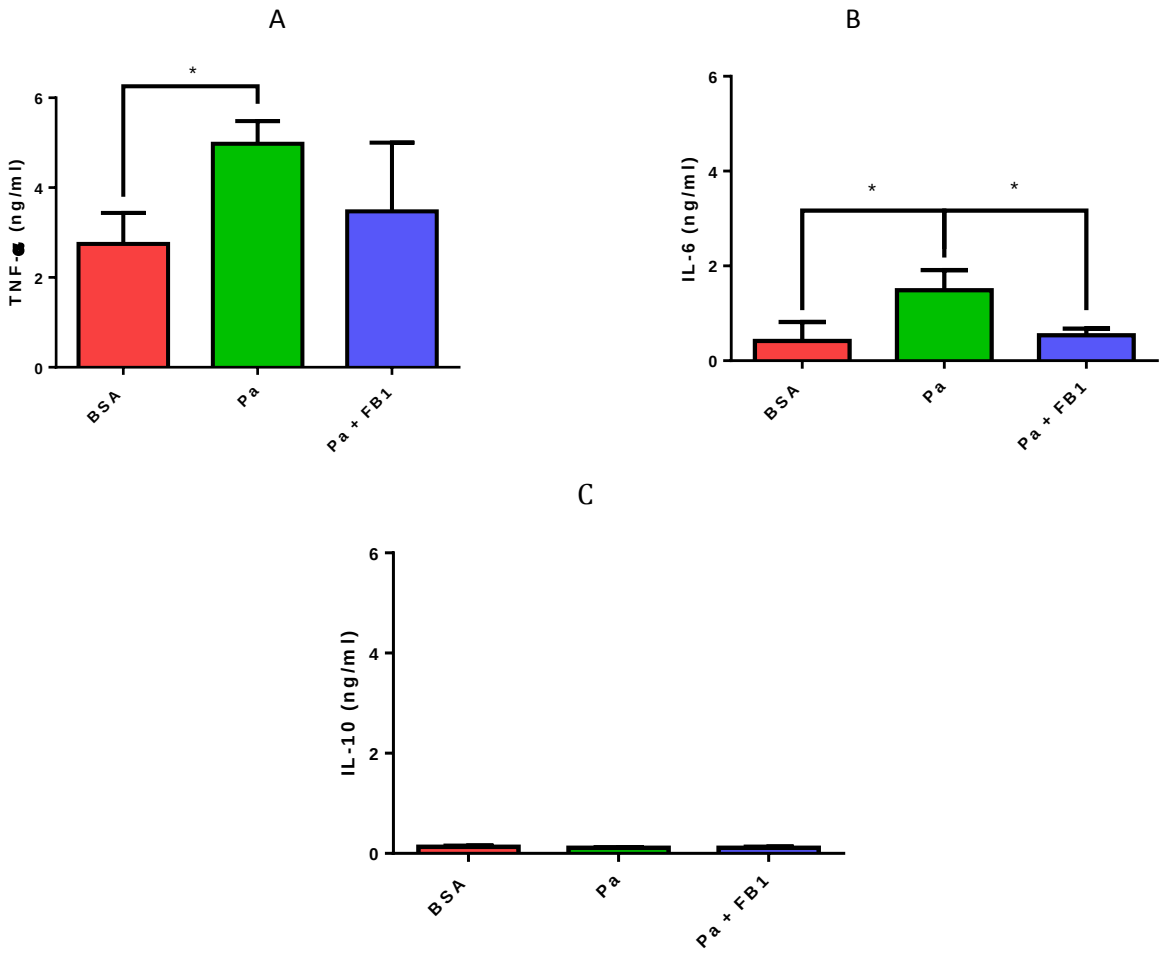

Figure 5.14: Ablation in inflammatory cytokine secretion of palmitate-primed macrophages by FB1:

THP-1 monocytes were primed with BSA, palmitate, palmitate + FB1 for 6h before PMA (320nM) treatment for a further 18h to induce differentiation into macrophages, before supernatants were obtained and analysed for cytokine content. TNF-α (A), IL-6 (B) and IL-10 (C) cytokine data was expressed as mean n=3, where data was analysed by t-tests comparing BSA, palmitate and palmitate + FB1 treated THP-1 cells, with **=p<0.01.

5.4 Discussion

In this chapter, fatty acids are shown to have no inflammatory effect on THP-1 monocytes, whilst in the presence of LPS only the SFA palmitate enhances the inflammatory response. This finding suggests that monocytes in a high SFA environment are primed for the production of amplified inflammatory response.

Modulation of inflammation by fatty acids is well described and is not limited to a single class of fatty acids, with alterations in immune response and cytokine production being common observations (Calder 2011, Calder 2013, Lesna, Suchanek et al. 2013). SFA have a proven effect to induce inflammation in adipocytes (Kennedy, Martinez et al. 2009, Chait and Kim 2010). Their effects on monocytes and the effects of MUFA are less clearly defined. In old rats fed a high oleate diet, oleate reduced both TNF-α and IL-1β levels in adipose tissue(Tardif, Salles et al. 2011). In a mouse model of non-alcoholic fatty liver disease mice fed an oleate rich diet increases in TNF-α typically associated with disease progression were ablated (Lee, Moon et al. 2011). Furthermore, analysis of plasma from overweight non-diabetic subjects revealed negative correlations between oleate in the NEFA pool and IL-8 concentrations (Staiger, Woll et al. 2009).

Jeffery et al. demonstrated the ability of olive oil and high oleic sunflower oil to decrease rat spleen lymphocyte proliferation (Jeffery, Yaqoob et al. 1996), likewise in human studies MUFA has been shown to reduce the expression of adhesion molecules in peripheral blood mononuclear cells (Yaqoob, Knapper et al. 1998).

SFA demonstrate a clear pro-inflammatory modulation of immune cells. In mice receiving diets with differing percentage of SFA (6%, 12% and 24%) Enos et al.. observed enhanced pro-inflammatory behaviour of macrophages with increased adipose tissue infiltration (Enos, Davis et al. 2013). In antigen presenting cells, palmitate inhibited the ability of APCs to activate naive T cells and reduced cell surface expression of MHC class I surface antigens (Shaikh, Mitchell et al. 2008).

Others have shown that oleate has a neutral effect on inflammation in a murine model of high fat feeding (Wen, Gris et al. 2011) microglial like THP-1 cells and SH-SY5Y neuroblastoma cells (Little, Madeira et al. 2012), THP-1 macrophages (Choi, Kim et al. 2011), THP-1 human monocytes (Dasu and Jialal 2011), and skeletal muscle cells (Coll, Eyre et al. 2008). SCD-1 deficient mice had reduced inflammation measured as lower TNF-α, MCP-1 and CCR2 mRNA in adipocytes, with adipocyte derived oleate in inducing inflammation (MCP-1 expression determined by PCR) in macrophages (Liu, Miyazaki et al. 2010). However, other groups failed to show inflammatory induction in adipocytes by

oleate (Han, Kargi et al. 2010). An *ex vivo* investigation of isolated human pancreatic islets treated with oleate revealed increased expression of inflammatory genes including C-reactive protein (Bikopoulos, Pimenta et al. 2008). In this thesis, the MUFA oleate did not activate monocyte cytokine production or antigen expression.

The induction of inflammatory cytokines requires the activity of NF-κB. Under non-stimulated conditions NF-κB is sequestered in the cytoplasm through interaction with inhibitor proteins of the IκB family encompassing IκBα, IκBβ, and IκBγ. Classical activation involving signalling by inflammatory cytokines (e.g. TNF-α), TLRs or viruses, leads to the activation of the β subunit of IκB kinase complex, which phosphorylates IκB proteins on two N-terminal serine residues. The phosphorylation of IκB proteins permits their recognition by ubiquitin ligases, polyubiquitination follows and then degradation. NF-κB is then free to translocate to the nucleus binding to promoter or enhancer regions of target genes (Hayden and Ghosh 2004) such as TNF-α and IL-6.

Measurement of NF-κB activity or protein levels is often utilised as a surrogate measure of inflammatory activation. Palmitate is known to activate NF-κB following TLR activation in both human and murine macrophages and human monocytes (Huang, Rutkowsky et al. 2012), as previously described through the classical pathway of NF-κB signalling.

Lamers et al. demonstrated that oleate in combination with conditioned media from human adipocytes increased IL-6 secretion, and alone palmitate induced a mild increase in NF-κB p65 subunit phosphorylation (Lamers, Schlich et al. 2012). In human endothelial cells, 48h treatment with oleate was sufficient to induce activation of NF-κB (Ciapaite, van Bezu et al. 2007).

The majority of studies indicate a pro-inflammatory effect of palmitate. In RAW264.7 cells palmitate induces gene expression and protein production of IL-1β, iNOS, MCP, IL-6 and TNF-α (Lu, Chen et al. 2013, Nasimian, Taheripak et al. 2013), with the production of IL-1β and TNF-α reported in human THP-1 macrophages (Nakakuki, Kawano et al. 2013) also. In human umbilical vein endothelial cells, palmitate induced TNF-α and IL-6 production and gene expression via ROS production (Zhang, Gao et al. 2013). In mouse primary Kupffer cells, palmitate upregulates MCP-1, TNF-α and IL-6 mRNA (Hoo, Lee et al. 2013). In skeletal muscle, palmitate treatment was sufficient to induce production of TNF-α and IL-6, furthermore the conditioned media from these cells induced activation of macrophages (Pillon, Arane et al. 2012). Inflammatory induction (measured as increased inflammatory cytokine mRNA or protein content) in response to palmitate treatment is observed in endothelial cells (Krogmann, Staiger et al. 2011, Liu, Zhao et al. 2012), fibroblasts (Paik, Cho et al. 2012), β-islets (Choi,

Hwang et al. 2012), hepatocytes (Choi, Choi et al. 2011), cardiac cells (Alvarez-Guardia, Palomer et al. 2011), and renal proximal cells (Soumura, Kume et al. 2010).

Thus a variety of cells demonstrate an inflammatory response to SFA, however, here production of either TNF-α, IL-6 or IL-10 in response to palmitate was not changed in THP-1 monocytes. A wide range of palmitate concentrations have been used in previous studies (from 200-500μM), therefore discrepancies cannot be explained by this variable. Alternatively, a number of studies measured mRNA levels, whereas this study evaluates the levels of cytokine secreted by THP-1 monocytes.

Bunn et al. stimulated THP-1 monocytes with 500μM palmitate, and demonstrated measurable increases in TNF-α and IL-6 protein and mRNA using a luminex system to measure protein levels of cytokines. Furthermore, cells were serum starved overnight prior to treatment with a mix of palmitate and stearate NEFA (Bunn, Cockrell et al. 2010). Dasu et al. utilised THP-1 monocytes to investigate the effects of free fatty acids (500μM) and high glucose on cytokines detected by an ELISA kit (Dasu and Jialal 2011).

Studies using THP-1 monocytes, apart from those previously mentioned, are rare with the majority using cells differentiated into macrophages (Haversen, Danielsson et al. 2009, Choi, Kim et al. 2011, Huang, Rutkowsky et al. 2012, Nakakuki, Kawano et al. 2013). Therefore, the discrepancies between this study and those of others can be explained by differences in methodology i.e. different cell protocol prior to treatment and higher SFA concentration.

In light of the immune-modulatory nature of fatty acids, and the lack of effect of MUFA and SFA oleate and palmitate respectively on THP-1 monocyte cytokine production shown here, THP-1 monocytes were subsequently pre-treated with oleate or palmitate and then stimulated with LPS to observe if modulation of immune response occurred. LPS induced TNF-α and IL-6 cytokine production in THP-1 monocytes was enhanced by palmitate, and an ablation in LPS response was observed with oleate pre-treatment.

It is worth noting that LPS induces ceramide synthesis. Barber et al. observed that stimulation of the ceramide synthetic pathway mimicked LPS stimulation of TNF-α cytokine production from murine peritoneal macrophages (Barber, Detore et al. 1996). Raetz et al. determined that lipid A, the moiety of LPS which possesses biological activity, are acylated with R-3-hydroxylaurate or myristate and the hydroxyl groups of these components being further acylated with either of the saturated fatty acids laurate, myristate or palmitate (Raetz 1990). The cleavage of these acyl groups dramatically reduces the toxicity of LPS (Munford and Hall 1986, Kitchens, Ulevitch et al. 1992), findings suggesting the importance of these saturated fatty acids in inducing the effects of LPS.

Whilst stimulation of the pro-inflammatory cytokines was induced by LPS, no significant production of IL-10 by fatty acid and LPS was detected. Consequently, peptidoglycan from *Staphylococcus aureus* was investigated as an inducer of IL-10, and to provide a secondary stimulus in the fatty acid co-treatment model. Neither palmitate nor oleate was able to mediate an increase in PG stimulated IL-10 cytokine levels. The expression of TLR2 on THP1 monocytes has not been investigated here.

Palmitate appears to prime monocytes for an enhancedinflammatory response, a finding which was demonstrated also by Schwartz et al.(Schwartz, Zhang et al. 2010). The group demonstrated the ability of palmitate to enhance IL-6 and IL-8 mRNA and protein after 4h and 48h respectively after LPS treatment that was dependent upon ceramide formation in THP-1 monocytes. Cells were treated with palmitate at 100µM for 16h before a wash step and subsequent treatment with 1ng/ml LPS in media; this contrasts to the present study in which cells were treated with 50-300µM palmitate for 6h and without a wash step were treated with 5ng/ml of LPS for a further 18h. The role of ceramides in mediating palmitate effects is further supported by data in this chapter in this chapter through the inclusion of FB1 which prevented the amplified cytokine production in response to palmitate, and supports findings of others (Schwartz, Zhang et al. 2010)

Experimental findings, albeit in THP-1-differentiated macrophages, by Haveson et al. demonstrated increased cytokine production to palmitate alone at 6h, with mRNA expression of TNF-α, IL-1β and IL-6 peaking at this time point. Further experimental study revealed the importance of ceramide, with maximal activation of p38 and JNK (postulated to be mediators of ceramide action) observed at 6h. Therefore such findings would support the notion that 6h is sufficient to generate ceramide in response to palmitate treatment, and subsequent signalling by ceramide is sufficient to enhance inflammatory signalling in response to TLR4 agonist LPS.

The experimental observations of this study are interesting in light of the ability of palmitate to activate the lipopolysaccharide receptor TLR4; saturated fatty acids to induce pro-inflammatory signalling through NF-κB and COX-2 expression which relied upon activation of TLR4 (Hwang 2001, Lee, Sohn et al. 2001) and other TLRs (Lee and Hwang 2006, Fessler, Rudel et al. 2009).

The interaction of fatty acids with TLR may also help to explain the suppressive effect of MUFA oleate in co-treated THP-1 monocytes. Oleate treated RAW 264.7 cells reduce signalling from TLR (Lee, Plakidas et al. 2003), and in subsequent work Lee et al. postulated that unsaturated fatty acids prevent SFA interaction with TLR4 (Lee, Ye et al. 2003). However, relatively little data is available on the effects of oleate on TLR interaction, and it can only be speculated that oleate interferes with TLR4 interaction with LPS in the context of the model used in this thesis.

The palmitate mediated amplificationof LPS responses in THP-1 monocytes and the ability to induce pro-inflammatory signalling in a wide variety of cells by TLR4 activation and ceramide generation may have consequences for metabolic disorders and ageing in which an elevation in NEFA is observed. Inflammation is a common feature of metabolic disorders. In type 2 diabetes, cross-sectional studies indicate elevations in IL-6 (Pickup, Mattock et al. 1997) and associations with systemic low grade inflammation (Herder, Illig et al. 2005) with risk of T2D increasing with elevations in IL-1β and IL-6 (Spranger, Kroke et al. 2003). Inflammation can play a mechanistic role in disease aetiology; hepatic and adipose production of IL-6 is thought to promote insulin resistance (Cai, Yuan et al. 2005, Mooney 2007).

Furthermore ablation of TLR4 signalling removed impaired insulin sensitivity in mouse models (Shi, Kokoeva et al. 2006) and myeloid specific deletion of TLR4 in an obese mouse model still allowed obesity but without insulin resistance (Saberi, Woods et al. 2009). Stearoyl-CoA desaturase-1 deficient mice in an atherosclerotic mouse model diet of elevated SFA, subsequently showed accelerated atherosclerosis. The peritoneal macrophages of these mice were engorged with SFA and produced greater responses to TLR ligands (Brown, Chung et al. 2008).

Therefore in the presence of LPS as a secondary stimulus, an amplified response is generated as MAPK is already activated with further activation by LPS of NF-κB which leads to the activation of gene expression of cytokines e.g. TNF-α and IL-6, hence the amplified response in palmitate-primed monocytes. Monocytes are an important cellular mediator of innate immunity, responding to inflammatory stimuli thereforeinfluencing behaviour can impact on function in both steady state and disease, thus it would be pertinent to highlight the distinctions of these cells.

In atherosclerosis and metabolic diseases, inflammation and elevated NEFA are common observations. In atherosclerosis, a requirement for disease progression is the recruitment of monocytes to plaques with subsequent differentiation into macrophages. This study sought to determine the influence of either the MUFA oleate or the SFA palmitate differentiation.

Models of differentiation using M1 and M2 treatments were established to confirm phenotypes in differentiated THP-1 cells and to serve as a comparison to FA or BSA programmes. This enabled a direct comparison between classically and alternatively-activated macrophages (table 5.1) to determine if either fatty acid could influence macrophage phenotype, which may be relevant for metabolic diseases.

Cell surface antigen	M1 monocytes	M2 monocytes	M1 macrophages	M2 macrophages
CD11b	+	++	-	++
CD14	+	++	-	++
CD16	-	++	-	++
CD36	NS	NS	NS	NS

Table 5.1: Summary of cell surface antigen expression in M1 and M2 cytokine-treated THP-1 monocytes and THP-1 differentiated macrophages in the presence of both cytokines and PMA.

M1 cytokines were 20ng/ml IFNγ and 100ng/ml LPS, whilst M2 cytokines were 20ng/ml of IL-4 and IL-13; macrophages were treated with these cytokines and with PMA (320nM). Replace + with ↑

In this chapter, the ability of oleate and palmitate to induce distinct macrophage phenotypes with differential expression of cell surface antigens (CD11b, CD14, CD16 and CD36) and inflammatory cytokines has been demonstrated. Palmitate and LPS co-incubation induces increased expression of CD11b and CD36. Pre-treatment with palmitate prior to PMA induces a macrophage phenotype with low cell surface expression of CD11b and CD14, and high production of cytokines TNF-α and IL-6. In contrast oleate treatment prior to PMA induces high levels of CD14 and CD11b, with suppression of the pro-inflammatory cytokines TNF-α and IL-6 in comparison to controls. Furthermore, oleate primed macrophages displayed greater apoptotic cell binding capacity which has been shown to be in part mediated by CD14, whilst palmitate primed cells did not. These findings suggest pro-inflammatory M1 phenotype is induced by palmitate, and the presence oleate generates an anti-inflammatory M2 phenotype. However, it is worth noting that differentiating monocytes with PMA in the presence of oleate failed to induce IL-10 whereas palmitate reduced basal IL-10 production or an increase in CD16 antigen expression although palmitate decreased CD16 expression.

The cytokine profile generated by palmitate-primed macrophages suggests the cell is pro-inflammatory; comparison with the classical M1 macrophage shows striking similarities, with enhanced production of TNF-α and IL-6, and suppression in IL-10. Whilst M2 macrophages do not change in TNF-α and IL-6 secretion, a dramatic IL-10 increase is observed; a finding which is absent in oleate primed macrophages, suggesting oleate produces a macrophage phenotype which less M2 like.

Fatty acid-driven alterations in differentiation pathways are a phenomenon that is not unheard of. In bone marrow derived mesenchymal and mouse embryonic stem cells, palmitate enhanced adipogenic differentiation (Polidori, Lomax et al. 2012, Park, Yeum et al. 2013). However, in the context of monocytes to macrophage differentiation relatively little is known regarding effects of fatty acid priming.

The classification of differentiated macrophages into distinct M1 classical and M2 alternatively-activated phenotypes is broad. Macrophages, like monocytes, display marked heterogeneity owing to the responsiveness to a wide range of endogenous and exogenous stimuli. Whilst M1 and M2 macrophages have been described, further distinctions within these groups can be made. The M1 group can be subdivided into classical M1a and innate M1b macrophages. M1a requires priming with IFN-γ with subsequent microbial stimuli e.g. TLR agonist LPS or a pro-inflammatory cytokine e.g. TNF-α (Gordon 2003, Martinez, Sica et al. 2008), such cells display enhanced phagocytic capacity and bactericidal machinery (Martinez, Sica et al. 2008). The M1b macrophages are a response to recognition by pathogen-associated molecular pattern receptors e.g. TLR or flagellin engaging pattern receptor, displaying similar characteristics to M1a but with reduced phagocytic activity (Janeway and Medzhitov 2002, Mukhopadhyay and Gordon 2004).

M2 macrophages display similar degrees of heterogeneity. The M1 macrophages are primarily concerned with enhancing inflammation and pathogen killing, whilst the M2 macrophages primarily deal with resolution of injury post-inflammation. The M2 class can be divided into M2a, M2b and M2c. The M2a macrophages are induced by IL-4 and IL-13, and are concerned with tissue repair expressing increased fibronectin and TGF-β (Gordon 2003, Mosser and Edwards 2008); such cells demonstrate enhanced expression of mannose receptor CD206, IL-1 receptor antagonist (which counters IL-1β), and have markedly reduced nitric oxide nitric oxide synthesis (Martinez, Sica et al. 2008).

Generation of M2b cells require priming by IgG immune complexes recognised by Fc receptors with subsequent stimulation with LPS, CD40 ligand or IL-1β (Anderson and Mosser 2002, Martinez, Helming et al. 2009), resulting in enhanced secretion of IL-10 in addition to the pro-inflammatory cytokines TNF-α, IL-6 and IL-1. M2c macrophages are generated in response to IL-10, TGF-β or glucocorticoids with the function of down regulating pro-inflammatory cytokines therefore limiting inflammation and they serve a debris scavenging and healing function (Martinez, Sica et al. 2008).

Apoptotic cell uptake has been described to induce a distinct phenotype of M2 macrophage (Savill, Dransfield et al. 2002, Erwig and Henson 2007), and IL-10 production is a hallmark of these cells.

Generation of these phenotypes*in vitro* may not translate to macrophage heterogeneity *in vivo*. The type of macrophage that develops will ultimately be dependent upon the levels of cytokines, intracellular signalling and nutrient status of immediate milieu. In this chapter, the pro-inflammatory nature of SFA and the anti-inflammatory nature of MUFA have been demonstrated. The priming of monocytes with SFA prior to PMA generates a pro-inflammatory phenotype akin to M1b, whilst priming with MUFA generates a macrophage with enhanced apoptotic debris scavenging capability, which makes the phenotype akin to that which aids in the resolution of apoptosis; however the reason for the lack of IL-10 production is puzzling.

Macrophage heterogeneity is not limited to normal physiology; in metabolic disorders distinct macrophage phenotypes have been observed which can be classified into either M1 or M2. In animal models of atherosclerosis macrophage heterogeneity is evident within the atherosclerotic plaque (Nahrendorf, Swirski et al. 2007, Tacke, Alvarez et al. 2007, Khallou-Laschet, Varthaman et al. 2010). However, no specific marker of either M1 or M2 human macrophages or their subgroups has been agreed upon. Despite this, distinct macrophage populations have been recorded, varying according to time of residence within the plaque, monocyte phenotype prior to differentiation and location (Poston and Hussain 1993, Waldo, Li et al. 2008).

Waldo et al. described the presence of CD14+ cells within plaques whilst infiltration of CD14- cells were located in disease-free regions (Waldo, Li et al. 2008). Macrophages in diffuse intimal lesions displayed high positivity for CD163 (associated with an anti-inflammatory phenotype), with the authors suggesting this would reduce the likelihood of plaque rupture (Komohara, Hirahara et al. 2006). Other authors have demonstrated the presence of both phenotypes e.g. the presence of MCP-1 and the mannose receptor within lesions, (Bouhlel, Derudas et al. 2007).

Beyond the M1/M2 paradigm, in both animals and humans there is evidence of macrophages that fit neither of these phenotypes. Oxidised LDL treated murine macrophages differentiated into a phenotype distinct from either M1 or M2, termed Mox (Kadl, Meher et al. 2010). Human monocytes induced to differentiate with either M-CSF or GM-CSF, CD14 and CD68 displayed gene expression associated with both M1 and M2 macrophages (Waldo, Li et al. 2008). *In vitro* differentiation of macrophages with M-CSF and CXCL4 produces a phenotype which is low in CD163 expression, termed the 'M4' macrophage, with such cells being found in vivo (Gleissner, Shaked et al. 2010).

In obesity and diabetes, similar macrophage heterogeneity has been observed. In obesity, both M1 and M2 macrophage populations have been observed in rodent and human adipose tissue, with the latter population being prevalent in lean subjects (Lumeng, Bodzin et al. 2007, Zeyda, Farmer et al.

2007). A study by Lumeng et al. determined that a high fat diet induced a phenotypic switch from M2 to the pro-inflammatory M1 in mice (Lumeng, Bodzin et al. 2007), furthermore Fujisaka et al. also showed insulin resistance is associated with elevated M1 cells and an increase in the M1:M2 ratio in mice (Fujisaka, Usui et al. 2010). Greater levels of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 and monocyte infiltration in the pancreas of diabetic patients in comparison to healthy controls (Bradshaw, Raddassi et al. 2009). In mice the involvement of M1 macrophages in type-2 diabetes was demonstrated through the analysis of adipose tissue, where 30% of expressed transcripts were for classical activation markers (Weisberg, McCann et al. 2003).

Such findings would suggest a pro-inflammatory M1, or similar, phenotype is pathogenic, whilst the M2 is associated with improvement in disease state, in atherosclerosis stabilisation of atherosclerotic plaque is associated with M2 macrophage inflitration. In the context of this study, the ability of palmitate to induce a pro-inflammatory phenotype suggests that elevations in FFA associated with metabolic disease states may promote atherosclerotic progression through phenotypic changes in U937 monocytes (Gao, Pararasa et al. 2012) and enhanced production of cytokines such as TNF-α and IL-6. In contrast, the phenotype generated by oleate-primed cells is one primarily concerned with clearance of apoptotic debris, with the lack of IL-10 production suggesting either a limitation in the ex vivo model or the poor detection.

In the previous chapter, de novo ceramide synthesis was demonstrated to be crucial in palmitate mediated increases in CD11b and CD36. Other groups have demonstrated the importance of TLR activation and ceramide signalling in mediating the inflammatory effects of palmitate. Here in the presence of FB1 and palmitate, cell surface marker expression resembles cells that have not received palmitate treatment, and the pro-inflammatory cytokine profile is markedly ablated. These findings strongly suggest the involvement of ceramides in the generation of a pro-inflammatory macrophage from palmitate primed monocytes.

5.5 Conclusion

In summary, this chapter demonstrates that neither the MUFA oleate nor the SFA palmitate induce changes in the production of cytokines TNF-α, IL-6 and IL-10 in THP-1 monocytes. In contrast, in the presence of FA and a secondary stimulus of LPS, and palmitate enhanced production of pro-inflammatory cytokines, with a trend of oleate to reduce pro-inflammatory cytokine expression. These findings indicated the monocytes were primed by FAs for either an enhanced or suppressed response. Such findings suggest that the type of FA can influence innate immune responses, with

considerable data available characterising the pro-inflammatory nature of SFA and neutral or anti-inflammatory nature of MUFA on immune cells.

Inflammation and differentiation of monocytes to macrophages are important steps in normal physiology and pathology of chronic, inflammatory and metabolic diseases, however both monocytes and macrophages are comprised of heterogeneous populations. The population of macrophages generated is somewhat dependent upon the milieu of cytokines, nutrients and other signalling molecules. Here, the production of a pro-inflammatory macrophage after palmitate priming, producing high amounts of TNF-α and IL-6 was demonstrated.

Use of FB1 in the palmitate primed-model indicated that the primary inflammatory inducer was ceramides, with inflammatory cytokines markedly reduced and cell surface antigen expression similar to BSA treated controls in the presence of palmitate and FB1. This finding highlights the importance of ceramide generation in both cell surface antigen changes and induction of inflammatory cytokine production by palmitate both at a monocytes and a differentiated macrophage stage.

In contrast, oleate-primed macrophages expressed higher levels of both CD14 and CD11b, but lacked the increase in IL-10 production. Furthermore, their enhanced apoptotic cell binding capability suggests these cells function to clear cell debris in the resolution of inflammation.

In disease pro-inflammatory macrophages are considered to favour pathological advancement of disease through generation of inflammatory cytokines, therefore the palmitate primed macrophage generated in this model would have negative consequences.

In the next chapter, an assessment of metabolic and inflammatory parameters during ageing will be undertaken, to address the hypothesis that age-associated changes to metabolism drive inflammageing. Insulin resistance and chronic low grade inflammation are observed in the context of an elevated NEFA level when compared to young adults. On this basis, the NEFA plasma profile in young and mid-life individuals has been investigated to examine whether a change in fatty acid profile is present that could contribute to the underlying inflammatory state in ageing.

Chapter 6

Age-related changes on fatty acid profile and relationship to inflammation, oxidative stress and insulin resistance

6 Chapter 6: Age-related changes on fatty acid profile and relationship to inflammation, oxidative stress and insulin resistance

6.1 Preface

In the present chapter, the effects of age on primary blood mononuclear cell phenotype, in relation to circulating plasma metabolic and inflammatory factors, and red blood cell membrane fatty acids are examined. Previous chapters have shown that cell surface antigens CD11b and CD36, inflammatory cytokines (TNF-α, IL-6 and IL-10) and mitochondrial ROS status are modulated by free fatty acids; these parameters are examined here relative to free fatty acids and age in blood from healthy male donors aged 20-30 or 50-75 years of age. Whole blood was also analysed for insulin, LDL, HDL, cholesterol, liver enzymes, glucose and plasma for glutathione content. Plasma and red blood cell membranes were analysed for non-esterified and membrane FA profiles. This investigation aims to determine whether ageing is associated with a change in the FA profile, specifically an increase in the levels of SFA, that may drive age-related inflammation or cell surface antigen expression.

6.2 Introduction

A common observation in ageing and many metabolic disorders is an increase in the plasma NEFA (Pilz, Scharnagl et al. 2006, Samuel, Petersen et al. 2010), which has been causally related to increased free fatty release from visceral adipose tissue (Bjorntorp 1990). Further analysis reveals NEFA contribute to cardiovascular mortality in the elderly (Smith and Wilson 2006). Acute increases in plasma NEFA decrease insulin stimulated glucose uptake and glycogen synthesis determined by indirect calorimetry (Boden, Chen et al. 1993), and in skeletal muscle inhibit PI3K and insulin action (Dresner, Laurent et al. 1999) therefore contributing to insulin resistance which is a metabolic disease risk factor known to increase with ageing (Fink, Kolterman et al. 1983, Ferrannini, Vichi et al. 1996).

Enhanced oxidative stress is another facet of the ageing phenotype in a wide variety of human tissue (Jackson and McArdle 2011, Massudi, Grant et al. 2012). Furthermore, mitochondria are the primary source of ROS within a non-phagocytic cell, and an age-related increase in oxidative stress and ROS generation has been shown in these organelles (Judge, Jang et al. 2005).

Ageing impacts negatively on cells of the immune system often referred to as immunosenescence. The effects of age on the adaptive B and T cells have been studied more extensively than cells of the innate immune system. Ageing is associated with a decline in the production of B-cells, coupled with

diminished responses to antigens by memory cells, (Cancro, Hao et al. 2009). In T cells, age-related changes are attributed to changes in CD4 or CD8 positivity, with the former showing reduced Th1 and Th2 effector differentiation with reduced cognate helper functions, whilst the latter population demonstrate altered clonal expansion (Haynes and Maue 2009).

Less is known regarding the innate immune system. Studies focusing on monocytes and macrophages indicate an age-related decrease in the levels of TLR1 (van Duin, Mohanty et al. 2007) coupled with a decrease in TLR induced TNF-α and IL-6 production in response to bacterial challenge. Analysis of monocyte function in the elderly revealed a reduction in phagocytosis of bacteria and apoptotic cells (Fietta, Merlini et al. 1994, Dunston and Griffiths 2010). Therefore the function of both the adaptive and innate immune systems is compromised in ageing.

Another notable aspect of the ageing body is the low grade systemic inflammation in ageing, which has been linked to the development of age-associated metabolic disorders. This inflammageing is characterised by an chronic sub-clinical elevation in inflammatory status with levels of pro-inflammatory cytokines IL-6 and TNF-α increased and anti-inflammatory cytokines e.g. IL-10 reduced (Bartlett, Firth et al. 2012).

Previous work in this thesis has shown that fatty acids can modulate monocyte phenotype, function and responses to agonists, however, it is not known how the specific free fatty acid pattern varies with age or whether this associates with inflammation and metabolic imbalance. Thus in this chapter, an examination of the ageing state will be undertaken, using healthy male volunteers from a young (24-30 years old) and midlife (>50 years old) population. In particular an examination of the NEFA plasma profile and red blood cell fatty acid profile will be undertaken to determine if an age-related shift in SFA, MUFA and PUFA is present which may positively or negatively affect risk of developing metabolic disorders and inflammation in ageing. The expression of cell surface markers CD11b and CD36 and cytokines (IL-6, IL-10 and TNF-α) will be considered as indices of inflammation, with mitochondrial ROS production, glutathione, cholesterol, glucose, and insulin and analysed as measures of metabolic health.

6.3 Results

6.3.1 Anthropometric changes associated with age

This study comprised of 14 healthy male volunteers in each of the young (24-30 years old) and midlife (>50 years old) cohorts, who were free from medication. Subjects with either existing metabolic disorders or on medication that could influence those parameters analysed e.g. anti-inflammatory, immunosuppressive and anti-diabetic drugs, were excluded from participation. The only anthropometric measurement that was significantly different was waist circumference, increasing by ~11% with age (table 6.1).

	Young (24-30 years old), n=14	Midlife (>50 years old), n=14
Age	27±2 (24-30)	58±7 (50-71)
Height (m)	1.79±0.07	1.81±0.08
Weight (Kg)	76.11±11.97	80.58±10.05
BMI (SI)	23.89±3.04	24.64±2.14
Waist circumference (cm)	88.92±6.82	98.54±7.07*
Hip circumference (cm)	99.16±6.94	104.3±5.23
Waist to Hip ratio (WHR)	0.896±0.06	0.945±0.07
Systolic pressure (mm/Hg)	123.1±16.82	134.9±14.31
Diastolic pressure mm/Hg)	76.22±11.21	89.42±10.33
Pulse	70±13	64±14

*=p<0.05

Table 6.1: Clinical characteristics of young and midlife cohorts:

Data was obtained following overnight fast from healthy male volunteers prior to blood collection for analysis of other parameters.

6.3.2 Age-related increase in plasma insulin and insulin resistance

Blood glucose did not change but plasma insulin increased significantly (~30%, $p<0.01$) with age as shown in figures 6.1A and 6.1B. The homeostatic model assessment (HOMA) is a model used to calculate insulin resistance and %β-cell function from blood glucose and insulin concentrations (Matthews, Hosker et al. 1985). Significant age-related increases in both calculated insulin resistance and a decrease in %β-cell function are present in the population studied here ($p<0.01$ and $p<0.05$ for HOMA-IR and HOMA-B respectively, figure 6.1C and 6.1D).

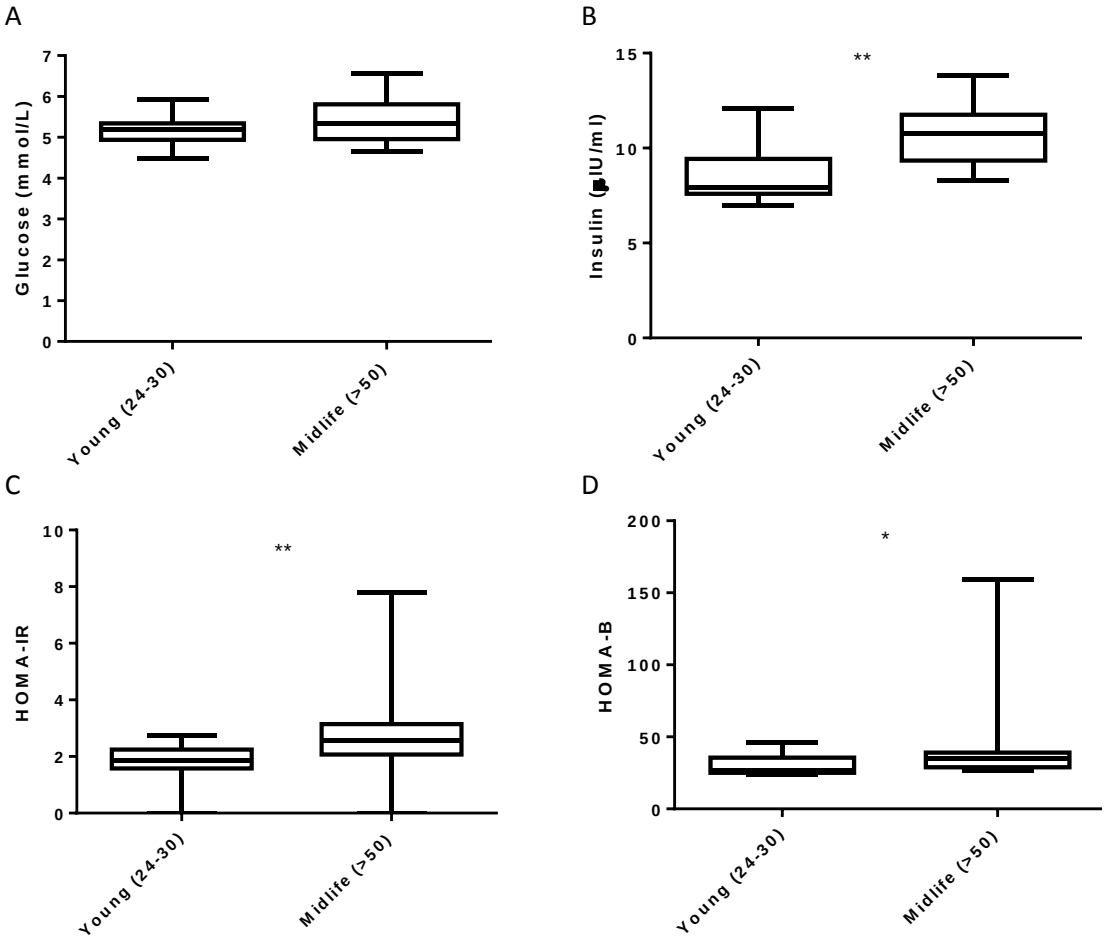

Figure 6.1: Age is associated with an increase in circulating insulin concentration and insulin resistance:

Whole blood obtained from young and midlife volunteers was immediately analysed for glucose at point of collection using reflotron strip measurement, whilst insulin determined in plasma by ELISA. Insulin and beta cell function was estimated by HOMA-IR and HOMA-B. Glucose (A), insulin (B), HOMA-IR (C) and HOMA-B (D) data is presented as box and whisker plots of n=14 per group displaying the minimum and maximum as the tails, with upper and lower quartiles and median representing the box with statistical analysis performed using t-tests where *=$p<0.05$ and **=$p<0.01$.

6.3.3 Cholesterol and triglyceride in the ageing cohort:

Plasma total cholesterol, LDL, HDL and triglycerides were not significantly elevated in healthy midlife individuals (figure 6.2).

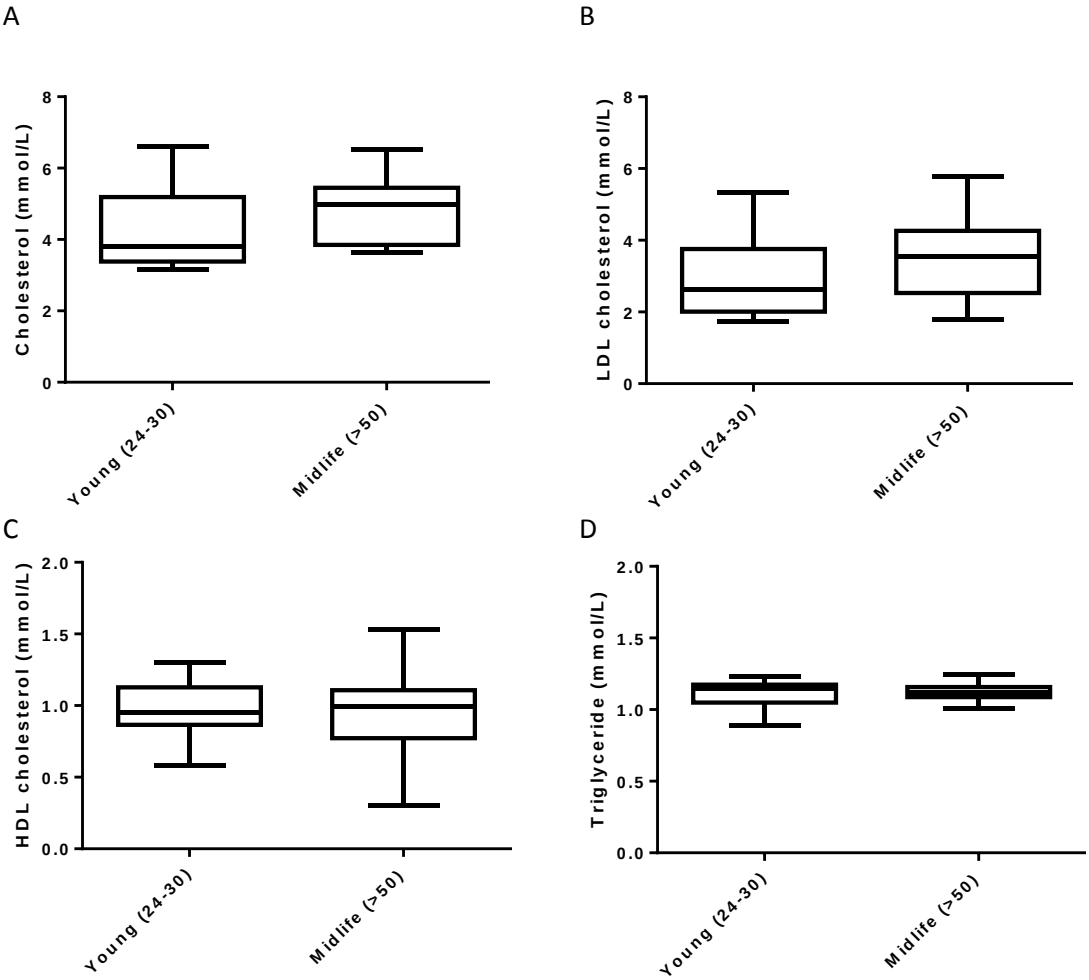

Figure 6.2: Lipid levels did not change with age in these cohorts:

Total cholesterol and HDL cholesterol were measured at point of obtaining blood from healthy young and midlife volunteers following an overnight fast using a reflotron , with LDL being subsequently calculated using the Friedwald formula. Triglycerides were evaluated following plasma extraction using a Randox triglyceride kit. Data is presented as box and whisker plots of n=14 per group displaying the minimum and maximum as the tails, with upper and lower quartiles and median representing the box.

6.3.4 Elevated oxidative stress in ageing cohort

To determine an index of extracellular redox state, glutathione content in plasma from young and midlife volunteers was quantified, as reduced and oxidised glutathione (GSH and GSSG respectively) using a DTNB recycling assay and the reduced/oxidised ratio was calculated. The ageing cohort demonstrated a significant decrease of 70% in GSH ($p<0.0001$, figure 6.3A), with a concomitant ~4 fold increase in GSSG ($p<0.0001$, figure 6.3B). The redox ratio determined from these outputs indicates a significant ~80% decline ($p<0.0001$ figure 6.3C), suggesting that in this ageing cohort there is a dramatic increase in oxidative stress. In peripheral blood mononuclear cells, mitochondrial ROS was significantly elevated ($p<0.0001$, figure 6.3D) with age measured as MitoSOX oxidation fluorescence. These findings support the notion that ageing is accompanied by elevated oxidative stress in cells and plasma.

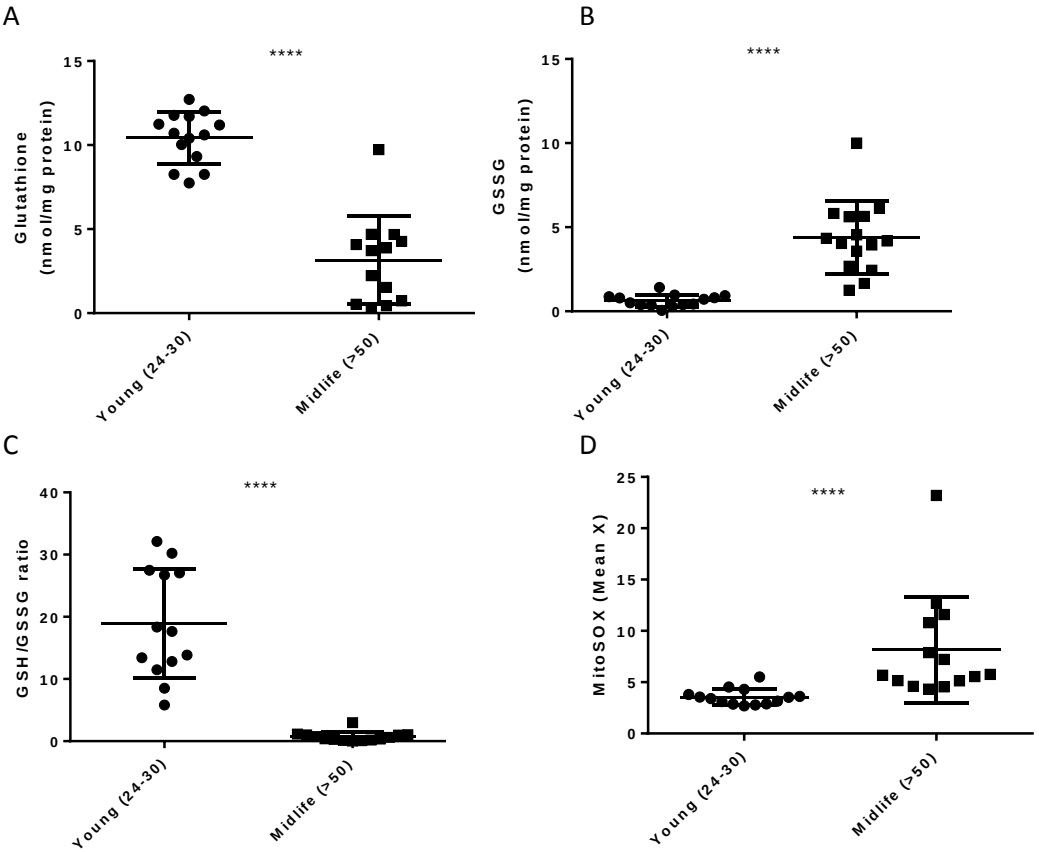

Figure 6.3: Age-related increase in oxidative stress in plasma and cells:

Plasma reduced and oxidised glutathione content, and mitochondrial superoxide levels in PBMCs was determined in whole blood from men of different ages. Glutathione parameters measured were determined using a DTNB recycling assay, whilst mitochondrial superoxide was measured in PBMCs. Following 30min treatment of whole blood at 37°C in the dark with 2.5µM MitoSOX, optilyse C and PBS was added to lyse red blood cells and fix PBMCs before analysis under flow conditions. Data is presented as scatter plots of n=14 per group SEM, statistical analysis performed using t-tests where ****=$p<0.0001$.

6.3.5 Increased cell surface marker expression on PBMCs with age

To investigate whether those cell surface markers that were increased by fatty acids *in vitro* are elevated during ageing, cell surface expression of CD14, CD11b and CD36 on PBMCs were determined by flow cytometry. CD11b and CD36 demonstrated an age-related increase (~10%, $p<0.05$, figure 6.4B and 6.4C respectively) and there was a trend for an increase in CD14 in older adults.

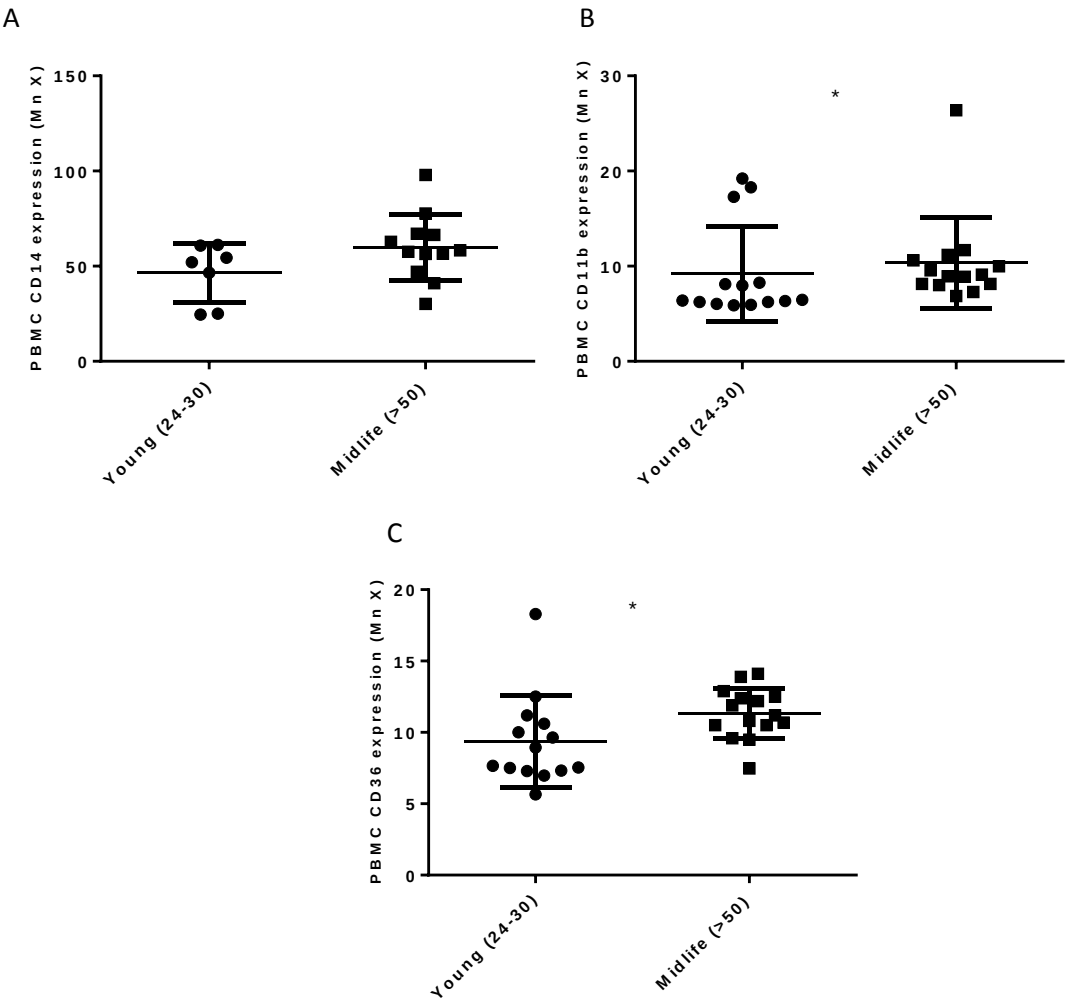

Figure 6.4: PBMC surface expression of CD11b and CD36 but not CD14 increase in the midlife cohort:

Whole blood samples obtained from young and midlife volunteers following an overnight fast were treated with a saturating concentration of mouse anti-human CD14-RPE Cy5, mouse anti-human CD11b-RPE or mouse anti-human CD36 FITC or respective isotype control for 30 min on ice, before treatment with optilyse and then analysis by flow cytometry. Data on CD11b and CD36 is presented as scatter plots of n=14 subjects, whilst CD14 data was obtained from n=7 young and n=10 midlife subjects; statistical significance is determined by t-test where *=$p<0.05$.

6.3.6 Ageing mediates an increase in plasma pro-inflammatory cytokine profile

To understand how the inflammatory environments in plasma change with age the pro-inflammatory cytokines TNF-α and IL-6, and the anti-inflammatory cytokine IL-10 in plasma were measured by ELISA. An increase in TNF-α was observed from the young to midlife cohorts of ~30%, whilst a similar significant increase in IL-6 was observed (figure 6.5A and 6.5B, $p<0.01$ and $p<0.001$ for TNF-α and IL-6 respectively). In contrast the anti-inflammatory cytokine IL-10 was decreased significantly (figure 6.5C, $p<0.05$) by ~25% in an age-related fashion. The alterations suggest ageing is associated with an elevation in the pro-inflammatory status.

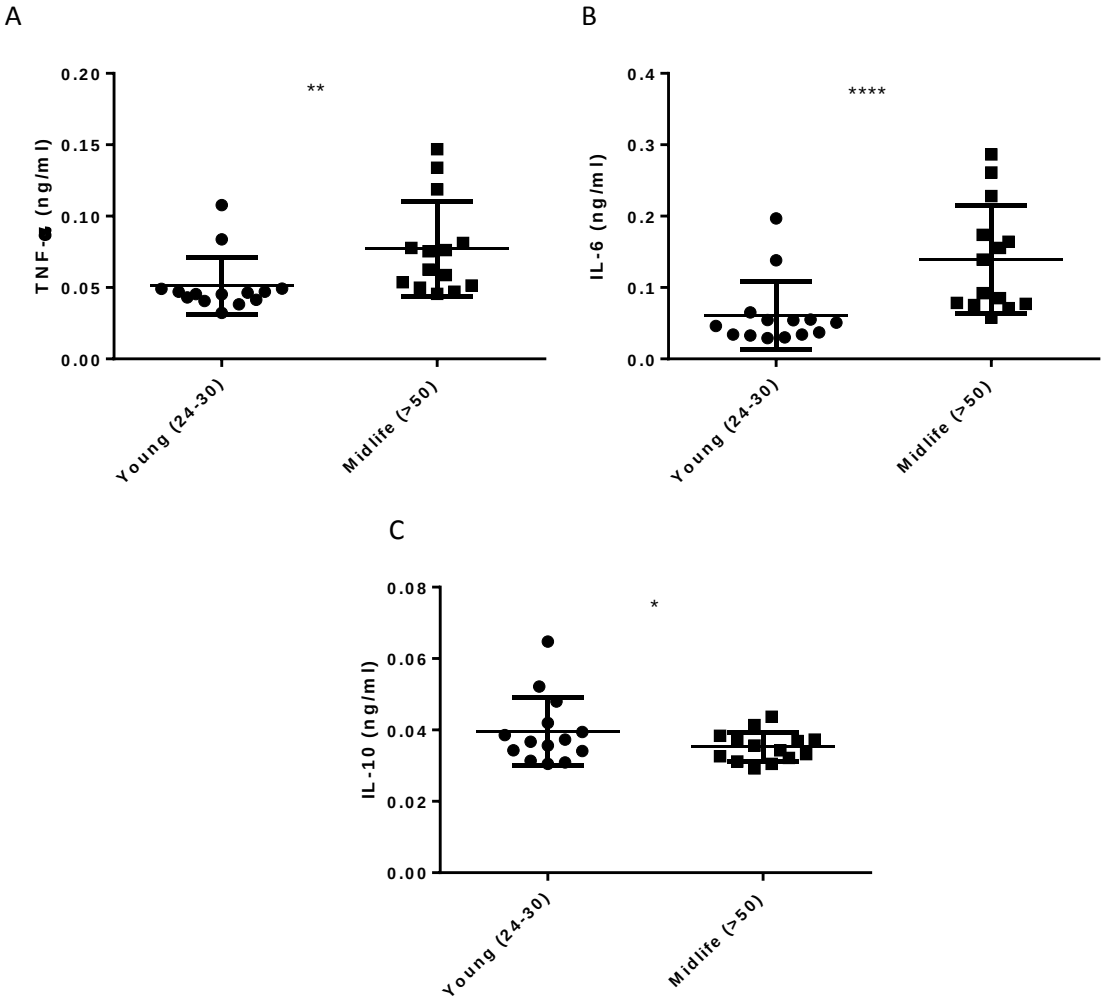

Figure 6.5: Elevation in the pro-inflammatory cytokine profile in ageing cohort:

Whole blood collected from healthy male volunteers were subsequently centrifuged at 650g to obtainplasma ,samples were stored at -80°C prior to analysis for levels of cytokines TNF-α, IL-6 and IL-10 by an ELISA kits from Peprotech, with75µl of plasma sample utilised in triplicate. Data is presented as scatter plots with means from n=14 individuals in both the young and midlife cohorts, where statistical significance was determined through t-tests, where *=$p<0.05$, **=$p<0.01$ and ****=$p<0.0001$.

6.3.7 Global increase in NEFA fatty acids in midlife cohort

Previous studies have described an increase in NEFA in older adults and subjects with insulin resistance but the profile of fatty acids has not been investigated in detail. The NEFA plasma profile was quantified by gas chromatography. Age-related increases in saturated fatty acids myristic, palmitate, stearate and lignoceric acids were observed in the NEFA profile, but there were not any significant changes with palmitate (figure 6.6A-D). Age-related increases in MUFA were also observed with statistically significant increases in oleate and myristoleate only (figure 6.7). No significant changes were observed regarding individual PUFA (figure 8), however an increase in total PUFA was seen ($p<0.01$, figure 6.9C).

Individual fatty acids demonstrate unique changes with age, by grouping the fatty acids into saturated, monounsaturated and polyunsaturated (Ω-3 and Ω-6) further highlights how the individual group profile changes. Globally the proportion of total fatty acids as saturated and monounsaturated fatty acids is maintained despite increasing concentrations in the midlife cohort, however within the polyunsaturated fatty acid group the Ω-3 fatty acids decrease whilst the Ω-6 remain unaltered, which may be important in the ageing phenotype, however the Ω-6/Ω-3 ratio was not significantly affected by age.

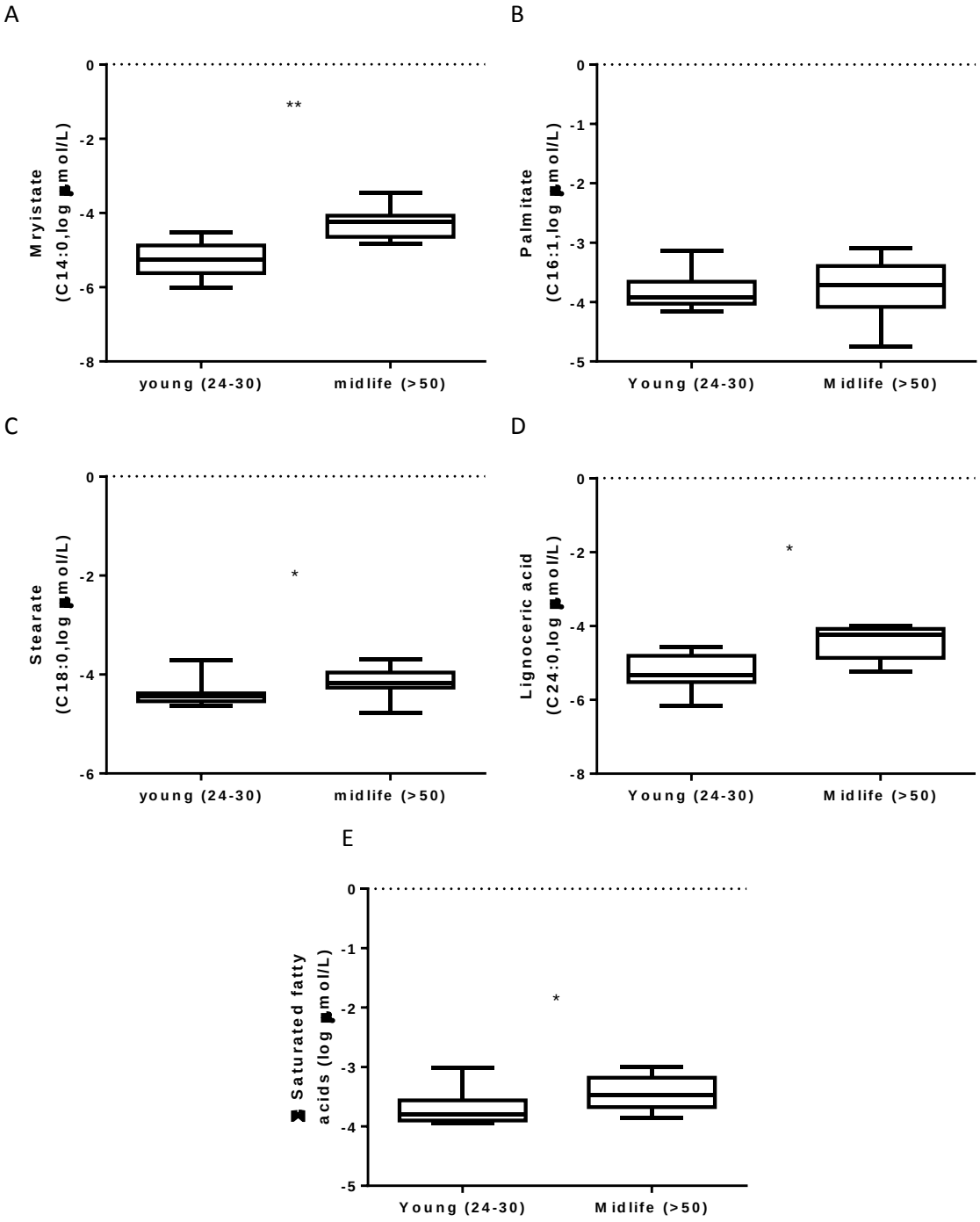

Figure 6.6: NEFA saturated fatty acids are elevated in the midlife cohort:

Fasting plasma samples obtained from subjects were first subject to lipid extraction using choloroform: methanol (2:1 v/v, 0.01% BHT), and concentration under nitrogen before conversion to methyl esters by hydrolysis before extraction with hexane and concentration under nitrogen. Samples were subsequently analysed by gas chromatography. Data is log transformed presented as box and whisker plots displaying minimum and maximum values as tails, with upper and lower quartiles and median representing the box plot n=14 samples per group, where statistical significance was determined by t-test analysis where *=$p<0.05$.

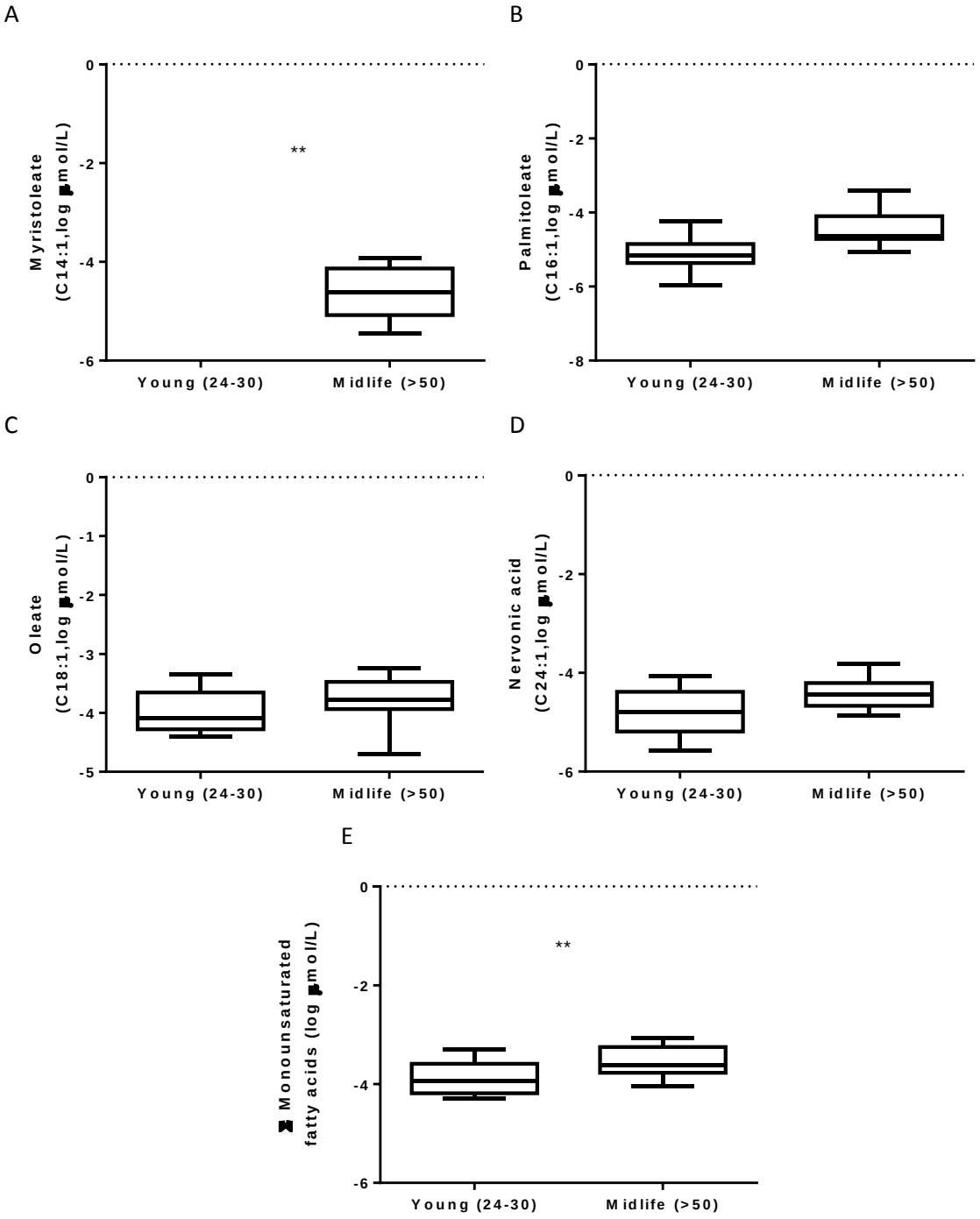

Figure 6.7: Plasma non-esterified monounsaturated fatty acids increase in an age dependent manner:

Plasma NEFA profiles were determined from fasted samples in young and midlife subjects. NEFA in the plasma were converted into methyl esters by acid hydrolysis subsequently extracted and concentrated before analysis by gas chromatography. Data is log transformed presented as box and whisker plots displaying minimum and maximum values as tails, with upper and lower quartiles and median representing the box plot n=14 samples per group.Data was determined to be non-parametric by Komogorov-Smirnov testing therefore statistical significance was determined by non-parametric Mann-Whitney test, **=p<0.01.

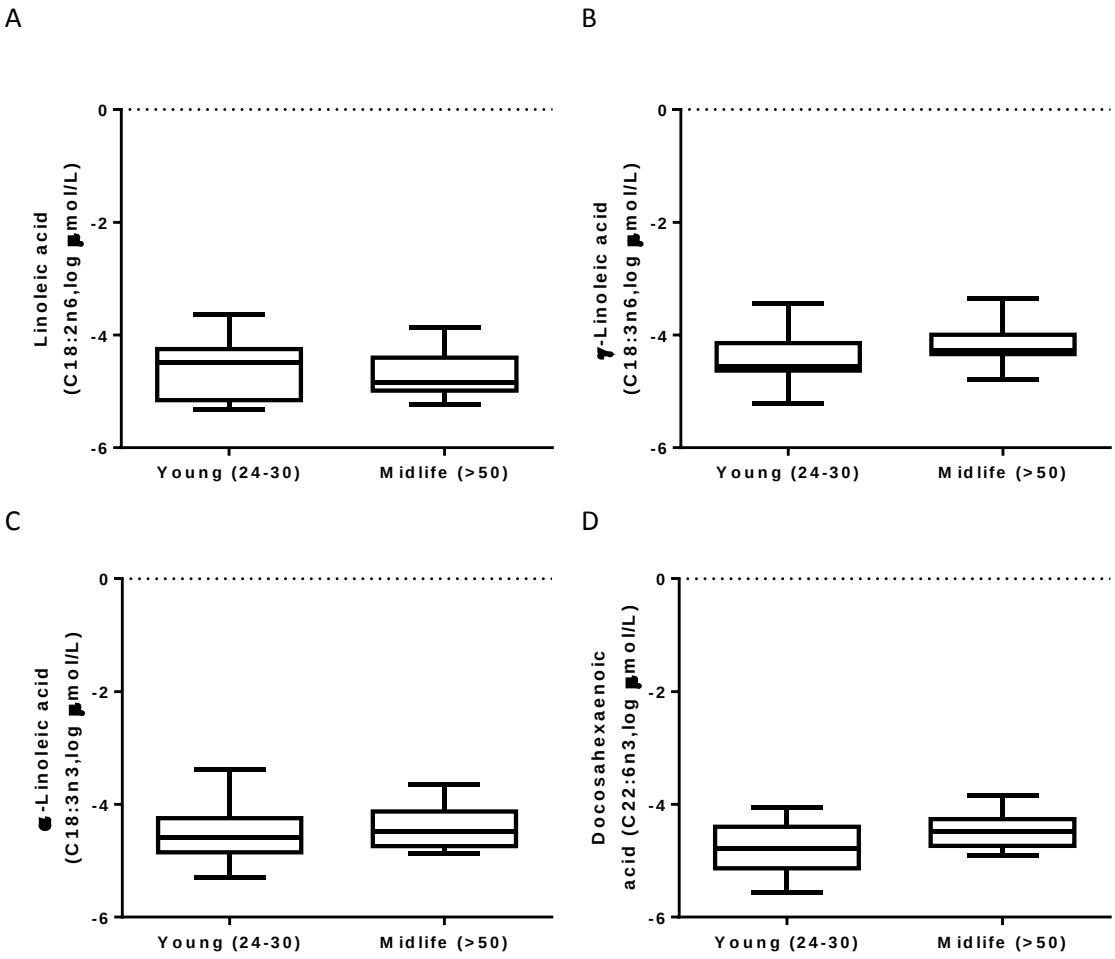

Figure 6.8: Plasma non-esterified PUFA profile is unchanged in ageing:

NEFA plasma profile of Ω-6 fatty acids analysed by gas chromatography following lipid extraction from plasma and subsequent concentration under nitrogen and conversion to fatty acid methyl esters. Data is presented as box and whisker plots with minimum and maximum values as tails, upper and lower quartiles and median values representing the box plotof n=14 individuals.

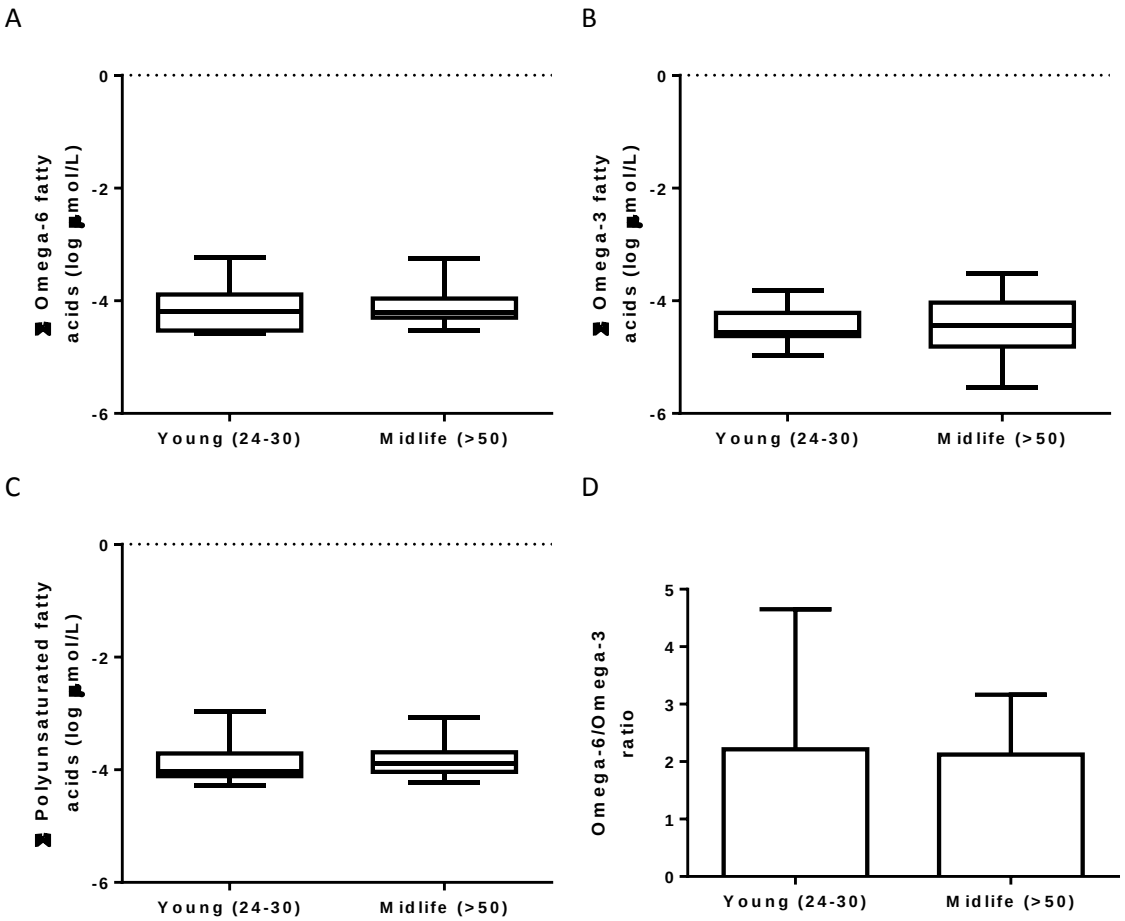

Figure 6.9: Ω-3 and Ω-6 PUFA levels in the plasma NEFA pool:

Plasma samples were analysed as previously described and displayed as box and whisker plots. The Ω-6/Ω3 ratio was calculated by the sum of fatty acids sum of all identified Ω-6 fatty acids over the sum of the Ω-3 fatty acids. Data are presented as box and whisker plots whilst Ω-6/Ω3 ratio were presented as mean ± SEM from n=14 samples per group.

6.3.8 Desaturase enzyme activity is elevated in ageing

The calculated enzyme activities of stearoyl CoA desaturase (SCD-1) and delta 6 desaturase (D6D) can be estimated through the product divided by the precursor. Stearoyl CoA desaturase converts the saturated fatty acids palmitate and stearate to their equivalent chain length monounsaturates palmitoleate and oleate; conversely the activity of D6D converts linoleic acid to dihomo-γ-linoleic acid. The ability of SCD-1 to act upon palmitate is elevated in the midlife cohort, whilst SCD-1 activity towards stearate remains unaltered (Figures 6.10A and B). The D6D activity is elevated in the ageing cohort ($p<0.05$), by ~2 fold (Figure 6.10C).

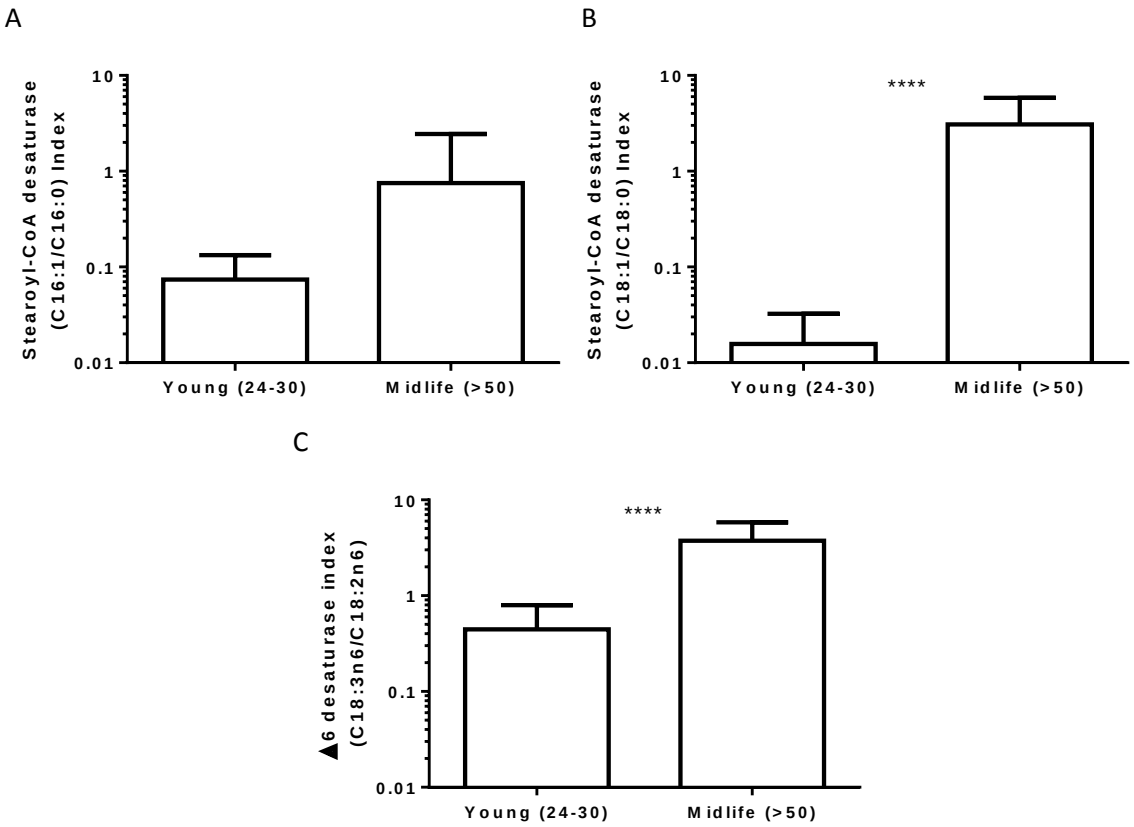

Figure 6.10: SCD-1 and D6D enzyme activity from NEFA fatty acids is elevated in midlife volunteers:

Enzyme activities of SCD-1 and D6D were calculated based on products/precursors (SCD-C16, C16:1/C16:0; SCD-C18, C18:1/C18:0; D6D, C18:2n6/C18:3n6) derived from the non-sterified fatty acid pool. Data is presented as mean±SEM of n=14 volunteers per group, with statistical significance determined through t-tests, where ****=p<0.0001.

6.3.9 Altered erythrocyte membrane fatty acid profile with age in erythrocyte membranes

Following analysis and quantification of the erythrocyte membrane fatty acid profile, the saturated fatty acids palmitate and lignoceric acid were increased, whilst stearate marginally decreased in the midlife cohort compared to the young; overall total saturated fatty acids in membranes were increased with age (figure 6.12). Palmitoleic, oleic, nervonic and total monounsaturated fatty acids are elevated in the ageing group, although palmitoleic acid is at the limit of detection (figure 6.13).

The Ω-6 fatty acids display a unique profile with age, with only linoleic acid increasing in the midlife cohort and γ-linoleic was marginally decreased; with the overall total Ω-6 fatty acids approximately the same. The membrane Ω-3 fatty acids in contrast increased with age (figure 6.14).

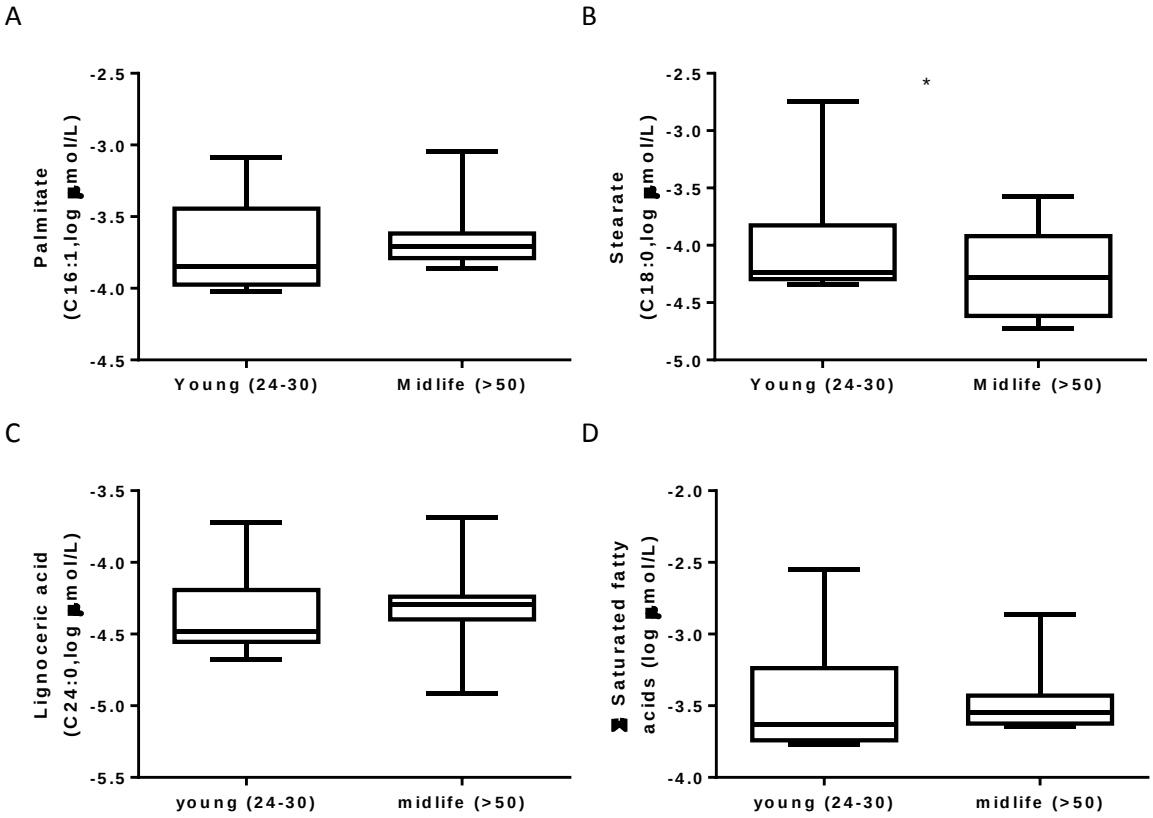

Figure 6.11: Red blood cell membrane saturated fatty acid profile in young and midlife volunteers:

Erythrocyte membrane lipids were obtained from red blood cells of healthy male volunteers by Folch extraction, and subsequently methylated by acid hydrolysis and analysed by gas chromatography. Data are presented as box and whisker plots where the minimum and maximum represent the tails, and the upper and lower quartiles and the median represent the box plot, from n=10 samples in both the young and midlife cohort. Data was analysed by t-tests where *=$p<0.05$.

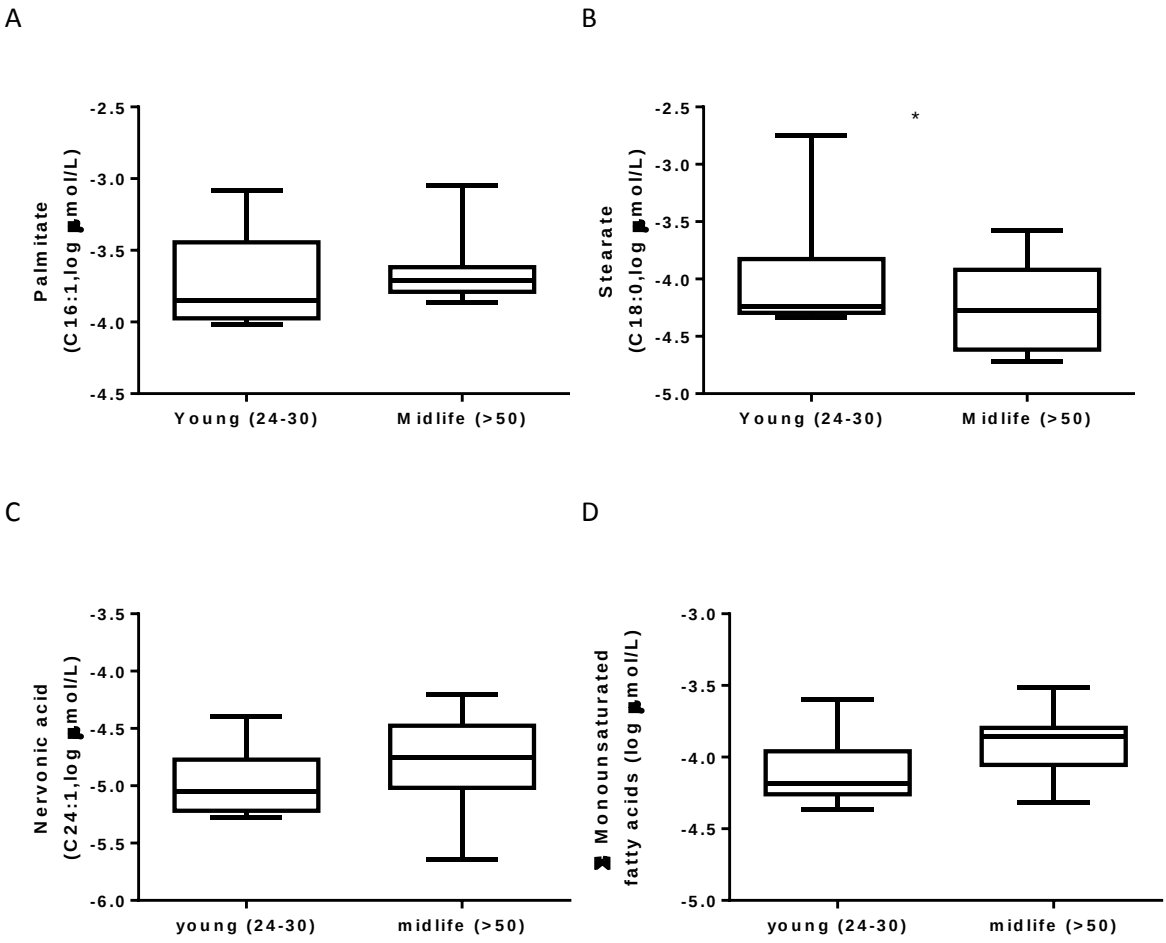

Figure 6.12: Erythrocyte membrane monounsaturated fatty acid increase with age:

Erythrocyte membrane fatty acid profiles were determined by gas chromatography, with data presented as box and whisker plots with minimum and maximum values represented by tails, the upper and lower quartiles and the median represented by the box plot, obtained from n=10 individuals. Statistical analysis was performed using non-parametric Mann-Whitneytests, where *=$p<0.05$.

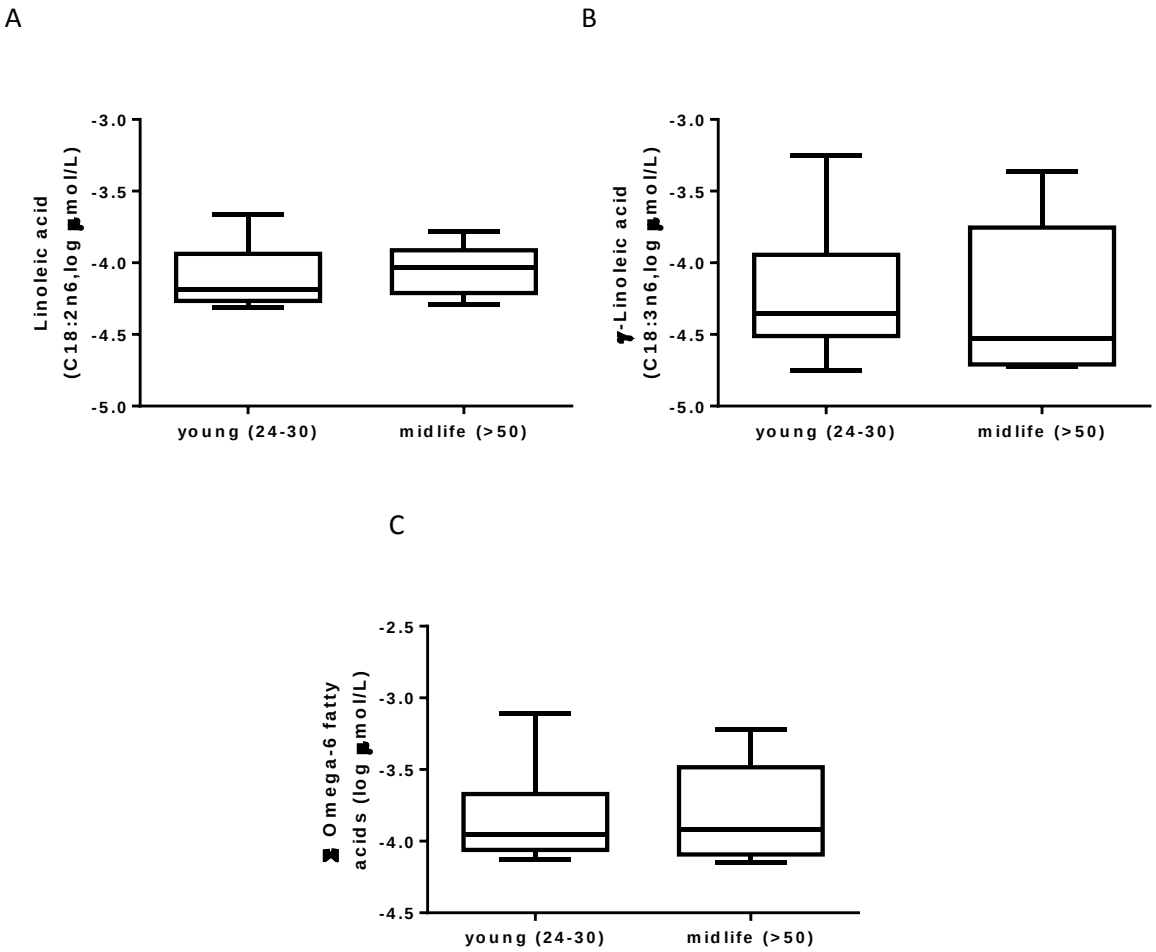

Figure 6.13: Membrane Ω-6 fatty acid profile is not different in ageing:

Erythrocyte membrane lipids were extractedusing Folch method, concentrated under nitrogen, and converted to fatty acid methyl esters beforeanalysisby gas chromatography. Data is presented as box and whisker plots where minimum and maximum is represented by the tails and the upper and lower quartiles and the median are shown as the box plot, with data derived from n=10 individuals.

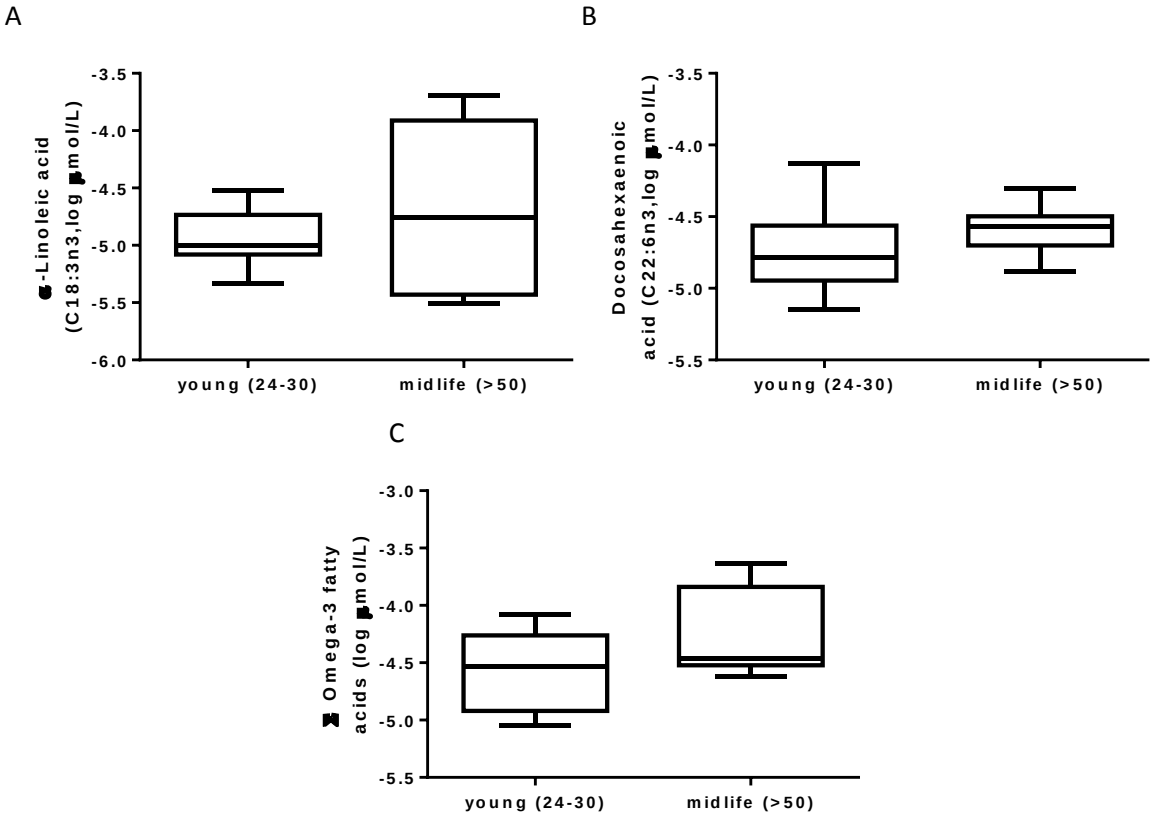

Figure 6.14: Membrane Ω-3 fatty acids according to age:

Erythrocyte fatty acids were extracted using Folch method, concentrated under nitrogen, and converted to fatty acid methyl esters before analysis by gas chromatography. Data is presented as box and whisker plots where minimum and maximum is represented by the tails and the upper and lower quartiles and the median are shown as the box plot, with data derived from n=10 individuals

6.3.10 Erythrocyte stearoyl-CoA desaturase but not D6 desaturase activity increased with age

Calculation of SCD-1 activity (for both C16 and C18 fatty acids) suggests an age associated increase in activity, however the conversion of linoleic to γ-linoleic acid decreases in the midlife cohort. With respect to SCD-1 activity, there appears to be a greater activity to act convert stearate to oleate than palmitate to palmitoleate (figure 6.15A and B). The Δ6 desaturase activity is not significantly affected by age (figure 6.15C).

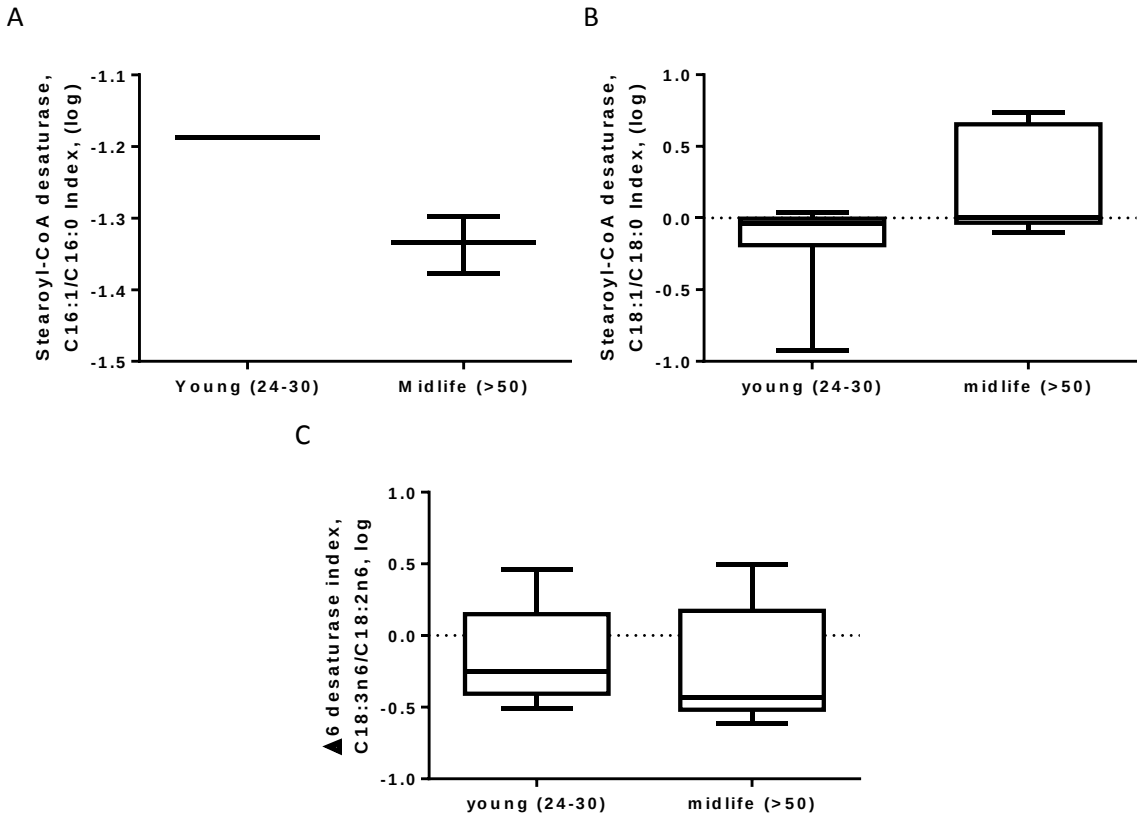

Figure 6.15: Altered erythrocyte SCD-1 and D6D activity in an ageing cohort:

SCD-1 and D6D activities are determined by the sum of the product over its precursor palmitoleate/palmitate (C16:1/C16), oleate/stearate (C18/C18:1) and γ-linoleic acid/linoleic acid (C18:3n6/C18:2n6) for SCD16, SCD18 and D6D respectively. Data is shown as box and whisker plots of upper and lower quartiles and the median representing the box plot with minimum and maximum whiskers.

6.3.11 Indices of Ω-3 and Ω-6 associated with midlife cohort are unaffected

As with the plasma NEFA profile, the erythrocyte membrane fatty acid shows no real change in n6.n3 ratio which may reflect no change in metabolic disease risk in this ageing population.

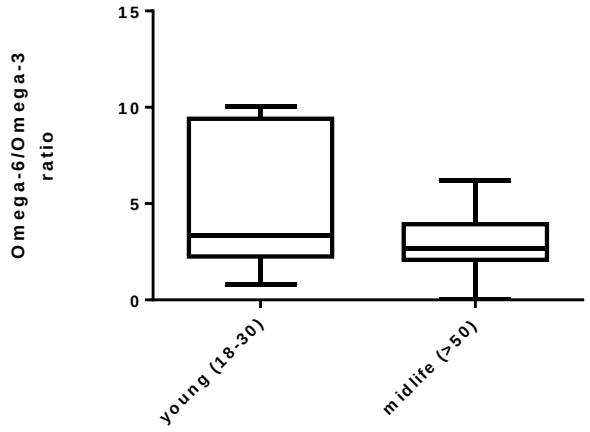

Figure 6.16: Erythrocyte Ω-6/Ω-3 ratio is lower in the ageing cohort:

Ω-6/Ω-3 ratio was determined by the sum of fatty acids sum of all erythrocyte Ω-6 fatty acids over the sum of the Ω-3 fatty acids, with data presented as box and whisker plots where the whiskers display the minimum and maximum values, and the box plot shows the upper quartile, median and the lower quartile of n=10 individuals

6.3.12 Regression analysis of metabolic, inflammatory and oxidative stress factors with and within age

Linear correlationanalysis was performed on all data combined into one single cohort to establish whether metabolic, inflammatory or oxidative factors were firstly significantly affected by age, and secondly to determine if within the context of age there are any relationships between these factors. Analysis of the relationships between age and blood lipids, glucose, insulin, PBMC cell surface antigens and mitochondrial superoxide levels, redox status, inflammatory cytokine levels and NEFA and RBC fatty acid profiles was determined (table 6.2). Age shares significant and positive linear relationships with insulin, HOMA-IR, MitoSOX, plasma GSSG, TNF-α, IL-6, NEFA SFA, NEFA, MUFA, NEFA PUFA, NEFA SCD(18) activity, and NEFA D6D activity. Negative and significant linear relationships were shown with GSH and GSH/GSSG.

NEFA SFA was demonstrated to have significant and positive linear relationships with PBMC CD14, PBMC CD11b, TNF-α, NEFA MUFA, NEFA PUFA, NEFA SCD(18), NEFA n6 FA, RBC MUFA, RBC D6D and

RBC n6 FA, and a negative correlation with plasma GSH. RBC SFA in contrast demonstrated a significant positive relationship with RBC MUFA.

Significant and positive linear relationships were observed between NEFA MUFA and insulin, HOMA-β, MitoSOX, plasma GSH, TNF-α, NEFA SFA, NEFA PUFA, and NEFA SCD (18). RBC SFA showed strong positive relationships with insulin, HOMA-IR, HOMA-β, RBC SFA and RBC n6 FA. NEFA PUFA demonstrated a significant positive relationship with glucose, NEFA SFA, NEFA MUFA, and RBC n3 FA. RBC PUFA demonstrated a strong significant and positive association with IL-10 and NEFA D6D.

Analysis of individual fatty acids revealed significant linear relationships not present when analysing total fatty acids for their respective class. Analysis of the NEFA SFA demonstrated positive relationships between myristate and D6D acitivity, RBC D6D activity and RBC n6 FA; palmitate was significantlycorrelated to TNF-α, CD11b, NEFA MUFA, NEFA PUFA, n6 FA, n3 FA and RBC D6D activity; stearate had positive linear relationships with MitoSOX, NEFA MUFA, NEFA PUFA and RBC D6D activity; lignoceric acid had positive linear relationships with TNF-α, IL-6, CD14, NEFA MUFA, NEFA PUFA, and D6D activity, in addition to negative linear relationship to plasma GSH.

Individual NEFA MUFA was demonstrated to have similar diverse relationships. Myristoleate demonstratedpositive linear relationships with insulin, HOMA-IR, HOMA-β, TNF-α, CD11b, NEFA SFA, NEFA n3 FA, NEFA D6D, RBC MUFA and RBC n6 FA, with a negative relationship to GSH. Palmitoleate demonstrated positive linearity to insulin, HOMA-IR, HOMA-β, and MitoSOX. Oleate showed significant positive linear relationships with TNF-α, NEFA SFA, NEFA PUFA, with a negative relationship with GSH. Nervonic acid showed positive linearity to GSSG, MitoSOX, and a negative relationship to GSH.

Linear correlation analysis was also performed on the individual RBC MUFA and SFA. Of the RBC SFA, both palmitate and lignoceric acid demonstrated significant positive linear relationships with insulin, HOMA-IR, HOMA-β and RBC MUFA, which is contrastedby RBC stearate which showed a significant relationship with RBC MUFA only.

Analysis of RBC MUFA revealed that indices of insulin resistance (insulin, HOMA-IR, HOMA-β) were strongly correlated to oleate and nervonic acid. Palmitoleate showed positive linearity to MitoSOX and RBC SCD16 activity. RBC oleate showed further associations with RBC SFA, whilst nervonic acid showed positive relationships to PUFA and RBC SFA, with a negative relationship with plasma GSH.

Further analysis of individual PUFA, inflammatory cytokines, blood lipids, oxidative stress and redox measures were also performed but are place in the appendix.

	AGE	
	r	p
Total cholesterol (mmol/L)	0.223294	0.2729
HDL cholesterol	-0.0053	0.9795
LDL cholesterol	0.192977	0.3449
TG (mmol/L)	0.152184	0.458
Glucose	0.283425	0.1606
Insulin (U/ml)	***0.392428***	***0.0353****
HOMA-IR	***0.434281***	***0.0186****
HOMA-ß	0.299449	0.1372
CD14	***0.455302***	***0.0501***
CD11b	0.15843	0.4207
CD36	0.357911	0.0566
MitoSOX	***0.43***	***0.0224****
Plasma GSH	***-0.90144***	***0.0001****
Plasma GSSG	***0.677643***	***0.0001****
GSH/GSSG	***-0.46054***	***0.0119****
TNF-α	***0.425441***	***0.0214****
IL-6	***0.456508***	***0.0128****
IL-10	-0.32404	0.0865
NEFA SFA	***0.368782***	***0.049****
NEFA MUFA	***0.413521***	***0.0258****
NEFA PUFA	***0.434856***	***0.0184****
NEFA SCD(16)	0.222014	0.247
NEFA SCD(18)	***0.667158***	***0.0001****
NEFA D6D	***0.753525***	***0.0001****
NEFA n6	0.115412	0.551
NEFA n3	0.190893	0.3212
NEFA n6/n3	-0.20746	0.3307
RBC SFA	-0.10045	0.6042
RBC MUFA	0.222733	0.2455
RBC PUFA	0.177961	0.3557
RBC SCD16	0.197484	0.4039
RBC SCD18	0.39064	0.0982
RBC D6D	-0.02514	0.9168
RBC n6	0.060581	0.7548
RBC n3	0.174671	0.3648
RBC n6/n3	-0.38601	0.0927

Table 6.2: Linear correlation analysis of age with FA profiles, metabolic, inflammatory and oxidative factors:

Linear regression analysis was performed with the above factors against age to determine any association. Significance is shown as *=p<0.05 and ****=p<0.0001determined from Spearman's rank correlation coefficient, with data compiled from n=28 individuals (n=14 young and n=14 midlife sample data).

	NEFA SFA		RBC SFA	
	r	p	r	p
Total cholesterol (mmol/L)	0.023195	0.9105	0.293138	0.1461
HDL cholesterol	0.031172	0.8798	0.246171	0.2254
LDL cholesterol	0.01318	0.9491	0.199875	0.3276
TG (mmol/L)	-0.04992	0.8086	0.295567	0.1427
Glucose	0.222621	0.2744	0.207171	0.3116
Insulin (U/ml)	0.252666	0.186	0.352987	0.0604
HOMA-IR	0.208399	0.278	0.335112	0.0756
HOMA-ß	0.190499	0.3512	0.370675	0.0623
CD14	***0.526498***	***0.0206****	0.058318	0.8226
CD11b	***0.472123***	***0.0112****	0.32665	0.0898
CD36	0.124097	0.5213	0.081945	0.6726
MitoSOX	0.16453	0.4028	0.204573	0.2964
Plasma GSH	***-0.38626***	***0.0423****	0.081541	0.68
Plasma GSSG	0.136748	0.4794	0.058626	0.7626
GSH/GSSG	0.18	0.3502	0.106958	0.5808
TNF-α	***0.405709***	***0.029****	0.075934	0.6954
IL-6	0.271662	0.154	0.173378	0.3684
IL-10	-0.07356	0.7045	0.052374	0.7873
NEFA SFA	-	-	0.007687	0.9684
NEFA MUFA	***0.711126***	***0.0001****	0.087812	0.6506
NEFA PUFA	***0.587282***	***0.0008****	0.039038	0.8406
NEFA SCD(16)	-0.07403	0.7027	0.08735	0.6523
NEFA SCD(18)	***0.450111***	***0.0143****	0.022419	0.9081
NEFA D6D	0.173983	0.3667	0.044721	0.8178
NEFA n6	***0.758156***	***0.0001****	0.042012	0.8287
NEFA n3	0.358608	0.0561	0.24368	0.2027
NEFA n6/n3	-0.11623	0.5887	0.044034	0.8381
RBC SFA	-0.00769	0.9684	0	
RBC MUFA	0.256223	0.1797	***0.768505***	***0.0001****
RBC PUFA	0.040988	0.8328	0.011623	0.9523
RBC SCD16	-0.18884	0.4252	0.170176	0.4732
RBC SCD18	-0.07034	0.7748	0.275536	0.2535
RBC D6D	***0.614003***	***0.004****	0.111086	0.6411
RBC n6	***0.396863***	***0.033****	0.336303	0.0745
RBC n3	-0.05371	0.782	0.030929	0.8735
RBC n6/n3	0.059093	0.8046	0.213378	0.3664

Table 6.3: Linear correlation of total NEFA and red blood cell (RBC) SFA against metabolic, inflammatory and oxidative factors.

Linear regression was performed on SFA derived from non-esterified and erythrocyte pools. Significance is shown as *=p<0.05 and ****=p<0.0001, determined from Spearman's rank correlation coefficient, with data compiled from n=28 individuals (n=14 young and n=14 midlife sample data).

	NEFA MUFA		RBC MUFA	
	r	p	r	p
Total cholesterol (mmol/L)	0.074431	0.1337	0.29722	0.1403
HDL cholesterol	0.166943	0.688	0.122434	0.5513
LDL cholesterol	0.106911	0.2774	0.233645	0.2507
TG (mmol/L)	0.121532	0.5542	0.310532	0.1226
Glucose	0.142724	0.4989	0.212321	0.2945
Insulin (U/ml)	***0.532823***	***0.0029****	***0.56356***	***0.0015****
HOMA-IR	0.330151	0.0802	***0.550727***	***0.002****
HOMA-ß	***0.53066***	***0.0053****	***0.584722***	***0.0017****
CD14	0.419047	0.0741	0.307116	0.2009
CD11b	0.234691	0.2267	0.233688	0.2314
CD36	0.140677	0.4667	0.056329	0.7717
MitoSOX	***0.481871***	***0.0094****	0.217692	0.2658
Plasma GSH	***0.489285***	***0.0082****	0.159248	0.4153
Plasma GSSG	0.3003	0.1135	0.188865	0.3265
GSH/GSSG	0.242858	0.2043	0.06229	0.7482
TNF-α	***0.461952***	***0.0117****	0.343802	0.0678
IL-6	0.219363	0.2529	0.060844	0.7539
IL-10	0.205913	0.2839	0.071218	0.7135
NEFA SFA	***0.711126***	***0.0001****	0.256223	0.1797
NEFA MUFA	-	-	0.2772	0.1455
NEFA PUFA	***0.383145***	***0.0402****	0.218472	0.2549
NEFA SCD(16)	0.021352	0.9125	0.164742	0.3931
NEFA SCD(18)	***0.56***	***0.0016****	0.243906	0.2023
NEFA D6D	0.113402	0.558	0.179944	0.3502
NEFA n6	***0.428486***	***0.0204****	0.095567	0.6219
NEFA n3	0.290017	0.127	0.311978	0.0984
NEFA n6/n3	0.167451	0.4341	0.024133	0.9109
RBC SFA	0.087812	0.6506	***0.768505***	***0.0001****
RBC MUFA	0.2772	0.1455	-	-
RBC PUFA	0.003776	0.9845	0.044091	0.8203
RBC SCD16	0.345688	0.1355	0.075578	0.7515
RBC SCD18	0.000154	0.9995	0.067045	0.7851
RBC D6D	0.126925	0.5938	0.108444	0.6491
RBC n6	0.140428	0.4675	0.4996	***0.0058****
RBC n3	0.004217	0.9827	0.069188	0.7214
RBC n6/n3	0.142688	0.5484	0.374433	0.1038

Table 6.4: Linear correlation of total NEFA and RBC MUFA against metabolic, inflammatory and oxidative stress factors.

Linear correlation analysis was performed on NEFA and erythrocyte MUFA pools. Significance is shown as *=p<0.05 and ****=p<0.0001determined from Spearman's rank correlation coefficient, with data compiled from n=28 individuals (n=14 young and n=14 midlife sample data).

	NEFA PUFA		RBC PUFA	
	r	p	r	p
Total cholesterol (mmol/L)	0.002847	0.989	0.077698	0.706
HDL cholesterol	0.200798	0.3253	0.046637	0.6922
LDL cholesterol	0.050646	0.8059	0.099955	0.821
TG (mmol/L)	0.00266	0.9897	0.014522	0.6271
Glucose	***0.394715***	***0.046****	0.061196	0.7665
Insulin (U/ml)	0.086879	0.6541	0.01555	0.9404
HOMA-IR	0.142969	0.4594	0.01555	0.9362
HOMA-ß	0.000589	0.9977	0.013442	0.948
CD14	0.25634	0.2895	0.116876	0.6337
CD11b	0.309079	0.1095	0.088199	0.6554
CD36	0.129885	0.5019	0.217394	0.2573
MitoSOX	0.132401	0.518	0.026023	0.8954
Plasma GSH	0.381838	0.0449	0.12025	0.5422
Plasma GSSG	0.090283	0.6414	0.108028	0.577
GSH/GSSG	0.176635	0.3593	0.067587	0.7276
TNF-a	0.127867	0.5086	0.369459	0.0586
IL-6	0.13914	0.4716	0.253772	0.1841
IL-10	0.064187	0.7408	***0.690145***	***0.0001****
NEFA SFA	***0.587282***	***0.0008****	0.040988	0.8328
NEFA MUFA	***0.383145***	***0.0402****	0.003776	0.9845
NEFA PUFA		-	0.074726	0.7001
NEFA SCD(16)	0.084172	0.6642	0.233431	0.223
NEFA SCD(18)	0.277687	0.1447	0.020337	0.9166
NEFA D6D	0.19264	0.3167	***0.435431***	***0.0182****
NEFA n6	***0.530377***	***0.0031****	0.062554	0.7472
NEFA n3	***0.428369***	***0.0204****	0.123612	0.523
NEFA n6/n3	0.104594	0.6266	0.062016	0.7734
RBC SFA	0.039038	0.8406	0.011623	0.9523
RBC MUFA	0.218472	0.2549	0.044091	0.8203
RBC PUFA	0.074726	0.7001		
RBC SCD16	0.237382	0.3136	0.120789	0.612
RBC SCD18	0.123814	0.6135	0.122923	0.6162
RBC D6D	0.339706	0.1429	0.118448	0.619
RBC n6	0.253712	0.1842	0.023403	0.9041
RBC n3	0.082395	0.6709	0.99945	***0.0001****
RBC n6/n3	0.047339	0.8429	0.329242	0.1564

Table 6.5: Linear correlation of total NEFA PUFA and RBC PUFA against metabolic, inflammatory and oxidative stress factors.

PUFA derived from NEFA and erythrocyte membranes were analysed for linear correlation. Significance is shown as *=p<0.05 and ****=p<0.0001, determined from Spearman's rank correlation coefficient, with data compiled from n=28 individuals (n=14 young and n=14 midlife sample data).

	Myristate (C14:0)		Palmitate (C16:0)		Stearate (C18:0)		Lignoceric acid C24:0)	
	r	p	r	p	r	p	r	p
Plasma GSH	-0.3001833	0.1207	-0.258631	0.1839	-0.2181055	0.2549	-0.5894913	***0.0005******
MitoSOX	-0.0549818	0.7811	0.08811356	0.6557	0.5781868	***0.0013****	0.01904993	0.9234
TNF-α	0.17467112	0.374	0.39698866	***0.0364****	0.01936492	0.9221	0.57792733	***0.0013****
IL-6	0.05305657	0.7886	0.20854256	0.2869	0.13475904	0.4941	0.59891569	***0.0008******
CD14	0.14768209	0.5462	0.45486262	0.0504	0.37309516	0.1157	0.52038447	***0.0224****
CD11b	0.00304729	0.9877	0.53469618	***0.0034****	0.16682326	0.3952	0.22891046	0.2413
MUFA	0.09401064	0.6276	0.6984268	***0.0001*****	0.54147945	***0.0024****	0.4241462	***0.045****
PUFA	0.11857487	0.5401	0.5230679	***0.0036****	0.50921508	***0.0048****	0.51254268	***0.0047****
n-6 PUFA	0.02610172	0.8931	0.84764379	***0.0001*****	0.33749074	0.0734	0.31244199	0.0989
n-3 PUFA	-0.0572014	0.7682	0.46151923	***0.0117****	-0.0433705	0.8232	0.21904337	0.2536
D6D	0.52459508	***0.0035****	-0.0744043	0.7013	0.14876155	0.4412	0.59791304	***0.0006******
RBC D6D	0.36742346	0.1111	0.5331979	***0.0155****	0.47127487	***0.0359****	0.23997917	0.3081
RBC Ω-6	0.37815341	***0.0431****	0.35014283	0.0626	0.00211518	0.9913	0.27429911	0.1577

Table 6.6: Linear correlation analysis of specific NEFA saturated fatty acids.

Individual NEFA derived SFA were subject to linear correlation analysis. Significance is shown as *=p<0.05 and ****=p<0.0001, determined from Spearman's rank correlation coefficient, with data compiled from n=28 individuals (n=14 young and n=14 midlife sample data).

	Myristoleate (C14:1)		Palmitoleate (C16:1)		Oleate (C18:1)		Nervonic acid (C24:1)	
	r	p	r	p	r	p	r	p
Insulin	0.714353	***0.0001****	0.80118662	***0.0001****	0.211518	0.277	0.04991	0.7971
HOMA-IR	0.639218	***0.0002****	0.435775171	***0.0181****	0.156812	0.4166	-0.07598	0.6953
HOMA-%β-cell	0.718331	***0.0001****	0.948156105	***0.0001****	0.141421	0.4908	-0.04904	0.8119
GSH	-0.42202	***0.0253****	-0.36138622	0.0588	-0.40559	***0.0322****	-0.20159	***0.03036****
GSSG	0.265612	0.1637	0.253594164	0.1844	0.16208	0.4009	0.39128	***0.0358****
MitoSOX	-0.03484	0.8603	0.777753174	***0.0001****	0.221495	0.2573	0.5781	***0.0013****
TNF-α	0.583952	***0.0011****	0.146184815	0.0644	0.452217	***0.0157****	-0.01867	0.9249
CD11b	0.431161	***0.022****	0.012429803	0.9499	0.210499	0.2823	0.246759	0.2056
NEFA SFA	0.452548	***0.0137****	0.01603122	0.0823	0.726842	***0.0001****	0.298597	0.1156
NEFA PUFA	0.184851	0.3371	0.022616366	0.7048	0.87761	***0.0001****	0.335708	***0.075***
NEFA PUFA n6	0.348712	0.0637	-0.064482556	0.7397	0.551453	***0.0019****	0.257468	0.1775
NEFA PUFA n3	0.573236	***0.0012****	0.134052229	0.4881	0.198394	0.3022	0.213448	0.2662
NEFA n6/n3	-0.07695	0.7208	-0.077967942	0.7173	0.390768	0.059	-0.17709	0.4078
NEFA D6D	0.488262	***0.0072****	0.064482556	0.7397	-0.01314	0.9461	-0.07178	0.7113
RBC SFA	0.283302	0.1364	0.212155603	0.2692	-0.02925	0.8803	-0.02476	0.8985
RBC MUFA	0.526783	***0.0033****	0.327719392	0.0827	0.162173	0.4006	-0.08717	0.653
RBC n6	0.435431	***0.0182****	0.047833043	0.8057	0.138708	0.473	-0.12276	0.5258

Table 6.7: Linear correlation analysis of specific monounsaturated fatty acids .

Individual NEFA pool derived MUFA were subject to linear correlation analysis. Significance is shown as *=p<0.05 and ****=p<0.0001, determined from Spearman's rank correlation coefficient, with data compiled from n=28 individuals (n=14 young and n=14 midlife sample data).

	RBC Palmitate (C16:0)		RBC Stearate (C18:0)		RBC Lignoceric acid (C24:0)	
	r	p	r	p	r	p
Insulin (U/ml)	0.658483	***0.0016******	0.084315	0.7238	0.650615	***0.0019******
HOMA-IR	0.626267	***0.0031******	0.091143	0.7023	0.610983	***0.0042******
HOMA-ß	0.66325	***0.0027******	0.083558	0.7417	0.662495	***0.0027******
RBC MUFA	0.861162	***0.0001********	0.547266	***0.0125****	0.900333	***0.0001********

Table 6.8: Linear regression analysis of erythrocyte specific saturated fatty acids.

Individual erythrocyte membrane derived SFA were analysed for linear correlation.Significance is shown as *=p<0.05 and ****=p<0.0001, determined from Spearman's rank correlation coefficient, with data compiled from n=28 individuals (n=14 young and n=14 midlife sample data).

	RBC Palmitoleate (C16:1)		RBC Oleate (C18:1)		RBC Nervonic acid (C24:1)	
	r	p	r	p	r	p
Insulin (U/ml)	0.016763	0.9441	0.675574	***0.0011****	0.622415	***0.0034****
HOMA-IR	0.10144	0.6705	0.662873	***0.0014****	0.647457	***0.002***
HOMA-ß	-0.03186	0.9001	0.680074	***0.0019****	0.590424	***0.0099****
mitosox	0.444972	***0.00563****	0.250938	0.3001	0.195551	0.4224
plasma GSH	-0.18512	0.4346	-0.36892	0.1094	-0.50468	***0.0232****
PUFA	0.318591	0.1711	0.019992	0.9333	0.432319	***0.057***
RBC SFA	-0.16814	0.4786	0.766159	***0.0001********	0.55109	***0.0118****
RBC SCD16	0.991716	***0.0001********	-0.04885	0.8386	0.261744	0.265

Table 6.9: Linear correlation analysis of erythrocyte specific monounsaturated fatty acids with factors that were significantly associated with total erythrocyte MUFA.

Individual erythrocyte membrane derived MUFA were analysed for linear correlation. Significance is shown as *=p<0.05 and ****=p<0.0001, determined from Spearman's rank correlation coefficient, with data compiled from n=28 individuals (n=14 young and n=14 midlife sample data).

	RBC Linoleic acid (C18:2n6)		RBC γ-linoleic acid (C18:3n6)		RBC α-linoleic acid (C18:3n3)		RBC Docosahexanoic acid (C22:6n3)	
	r	p	r	p	r	p	r	p
SCD(16)	-0.06104	0.7982	-0.10426	0.6618	0.893812	***0.0001****	-0.24118	0.2923
D6D	0.078064	0.7436	0.049325	0.8364	0.498096	***0.0254****	0.078122	0.7364
n3	0.53768	***0.0145****	0.401123	0.0796	-0.15569	0.5121	0.235499	0.3041
RBC SFA	0.54111	***0.0138****	0.031969	0.8936	-0.09535	0.6893	0.675648	***0.0008****
RBC MUFA	0.569912	***0.0087****	0.105736	0.6573	-0.22412	0.3421	0.613188	***0.0031****
RBC SCD18	-0.30315	0.2071	-0.09401	0.2164	-0.11432	***0.0001*****	0.045804	0.8523

Table 6.10: Linear correlation analysis of erythrocyte specific polyunsaturated fatty acids with factors that were significantly associated with total erythrocyte PUFA.

Erythrocyte membrane PUFAs were subject to linear correlation analysis. Significance is shown as *=p<0.05 and ****=p<0.0001, determined from Spearman's rank correlation coefficient, with data compiled from n=28 individuals (n=14 young and n=14 midlife sample data).

6.4 Discussion

In this chapter, age-related increases in PBMC cell surface expression of CD11b, CD14 and CD36 are observed, however only CD11b and CD36 show significance, which are accompanied by an age-related elevation in mitochondrial superoxide. The age-related increase in oxidative stress was not limited to an intracellular level, with increased oxidised glutathione and a decrease in reduced glutathione and redox ratio in the plasma. Pro-inflammatory cytokines TNF-α and IL-6 were elevated with age whilst the anti-inflammatory cytokine IL-10 was significantly reduced in older adults but did not correlate with age using regression analysis.

Analysis of fatty acid profiles, firstly of the NEFA profile in plasma and of the red blood cell membrane reveals age-related changes between young and midlife populations. The NEFA pool increased globally with age. In red blood cell membranes the levels of SFA and PUFA decline with age, whilst the level of MUFA remains the same, indicating a shift in the proportion of MUFA in the lipid membrane.

The paired observations of the increase in mitochondrial ROS and the decline in glutathione are a common observation in ageing. In animal models, glutathione levels in the brain undergo an age-related decline (Sasaki, Senda et al. 2001), whilst in the rat brain a down regulation of gamma glutamylcysteine synthetase regulatory subunit expression is observed (Liu 2002). In humans, glutathione status in blood has been extensively examined, with a decline in reduced glutathione in plasma and whole blood reported (Erden-Inal, Sunal et al. 2002). Similar decreases are observed in human lymphocytes (van Lieshout and Peters 1998), cultured human lenses (Rathbun and Murray 1991), and erythrocytes (Matsubara and Machado 1991, Rizvi and Maurya 2007). In certain other tissues the trend is somewhat different. In human skeletal muscle tissues, a majority of GSH is obtained from extracellular sources, and tends to remain the same throughout the ageing process, although reduction in GSH/GSSG ratio has been observed in adult males (Pansarasa, Castagna et al. 2000, Marzani, Felzani et al. 2005).

Thus, the ageing process causes a shift in the GSH/GSSG ratio that favours an oxidative state. A possible explanation for this would be an alteration in the redox capacity of the glutathione redox system. Rebrin and Sohal postulated that altered function of the rate limiting enzyme in glutathione synthesis, glutamate cysteine ligase, reduces the capacity for rapid biosynthesis of the antioxidant (Rebrin and Sohal 2008), therefore a decline in glutathione content presents.

Considering other enzymes of the glutathione antioxidant system, Ceballas-Picot et al. showed negative correlations between glutathione-S-transferase, GSSG-reductase and glutathione peroxidase and age in human erythrocytes (Ceballospicot, Trivier et al. 1992). Sekhar et al. demonstrated in healthy young and elderly individuals, there is an age-related reduction in the synthesis of glutathione; furthermore this reduction could be restored back to levels observed in their young population through supplementation with cysteine and glycine. Their data suggested an age-related reduction in both synthesis and substrate availability (Sekhar, Patel et al. 2011).

The reduction in glutathione content and the associated pro-oxidising shift is only one facet of the enhanced oxidative stress observed in ageing. Mitochondrial dysfunction is a common observation in ageing in animals. In this study, increased mitochondrial PBMC ROS production is demonstrated in humans with age. Components of the electron transport chain (ETC) are affected by age. Complex 1 efficiencies in both rodents and humans are decreased in liver (Lenaz, D'Aurelio et al. 2000), brain (Navarro, Lopez-Cepero et al. 2008), and heart (Choksi and Papaconstantinou 2008, Niemann, Chen et al. 2011). This age associated alteration will increase ROS leakage from the respiratory chain and cause oxidative damage. Damaged and defective mitochondria are removed by a process termed mitophagy (Kim, Rodriguez-Enriquez et al. 2007) which is triggered by enhanced ROS and a loss in mitochondrial membrane potential. In this manner a healthy population of mitochondria can be maintained. Ageing is associated with a reduction in autophagy (Klionsky and Emr 2000), consequently, the increase in ROS with age leads to a larger population of defective mitochondria which accumulates due to a poorer clean-up by the autophagic system.

A consequence of enhanced mitochondrial ROS, in addition to the enhanced oxidative stress and damage to proteins, is increased mitochondrial DNA damage e.g. point mutations and deletions (Hiona and Leeuwenburgh 2008). Defects in mitochondrial DNA may lead to a further decline in the wildtype genes encoding mitochondrial proteins, therefore reduced electron transport chain components and decreased oxidative phosphorylation (Short, Bigelow et al. 2005).

Other components in mitochondria concerning organelle function are affected with age. A protein involved in ROS signal transduction, $p66^{shc}$, which induces ROS production in response to stress is elevated in the elderly (Pandolfi, Bonafe et al. 2005). The protein enhances mitochondrial ROS production through regulation of NADH and oxygen consumption in the intermembrane space (Nemoto and Finkel 2002), furthermore in the mitochondrial space the protein becomes stabilised through ROS oxidation, in this manner acts as a ROS amplifier (Gertz, Fischer et al. 2008).

Age-related defects and deficiency in function of mitochondria play a significant role in pathologies of the ageing process (Verdin, Hirschey et al. 2010). Work by Petersen et al. in muscle indicated that the *in vivo* rates mitochondrial oxidation and phosphorylation were reduced by ~40% in elderly individuals compared to a young population; furthermore this was related to elevations in intramyocellular lipid content (IMCL) and reduced insulin sensitivity in this population. These findings suggest that the increased IMCL is due to defective mitochondrial function which in turn mediate insulin resistance (Petersen, Befroy et al. 2003). Others have reported an association between insulin resistance, impaired glucose tolerance and defective mitochondria in both elderly and type 2 diabetic populations (Kelley, He et al. 2002). Studies of skeletal muscle in individuals with insulin resistance and at risk of developing type 2 diabetes mitochondrial defects have been reported (Phielix, Szendroedi et al. 2011). Furthermore, in atherosclerotic disease progression has been postulated to involve cardiovascular disease mediated mitochondrial dysfunction (Puddu, Puddu et al. 2009). Therefore, the increased mitochondrial ROS production in PBMCs shown in this study may reflect greater mitochondrial dysfunction in these cells, which could promote insulin resistance and atherosclerotic disease progression.

Whilst reduced plasma glutathione content and enhanced mitochondrial ROS, are common observations in animal studies and human muscle, it is the first time it has been demonstrated in an ageing PBMC population. However, there is limited data available characterising the effects of age on cell surface antigen expression in immune cells. In this study, age is shown to associate with an increase the cell surface expression of CD14 and CD36 in PBMCs, with a trend to increase expression of CD11b. Whilst no significant elevation in CD11b is demonstrated, a confounding factor in the lack of significance may be the presence of three outliers in the young population analysed which have far greater CD11b expression compared to the rest of the population.

Studies of CD11b expression in young and older populations suggest an age dependent increase. In mouse brain, the microglial expression of CD11b was examined in populations of different ages, in which Hart et al. demonstrated a strong correlation with age(Hart, Wyttenbach et al. 2012), with similar findings in the rat (Blau, Cowley et al. 2012). In the frontal cortex of middle aged rats, an age dependent elevation on expression of CD11b mRNA was observed (Sarvari, Hrabovszky et al. 2012).

Hearps et al. isolated monocytes from young and old human populations and demonstrated an age-related increase in CD11b expression in monocytes (Hearps, Martin et al. 2012). Rea et al. determined the effect of age and in blood pressure on baseline and TNF-α stimulated CD11b expression on leucocytes. In an older population increased leucocyte CD11b expression was associated with elevated blood pressure and the authors infer that this is an age-related effect (Rea,

McNerlan et al. 2013). However, in polymorphonuclear leucocytes, Noble et al. did not demonstrate any age dependent increase in CD11b expression (Noble, Ford et al. 1999).

In these cases, the evidence suggests that ageing is associated with an increase in CD11b expression across a wide variety of cell types, and in those present within the vasculature, the elevated cell surface integrin expression increases the risk of monocyte and macrophage binding to endothelial cells, an event which is important in atherosclerosis (see introduction). Therefore ageing itself appears to promote risk of atherosclerotic development and progression.

In the PBMC population analysed here, an age-related and significant increase in CD36 expression is recorded, and to date this is the first time this has been demonstrated in human PBMCs. In animal models, CD36 expression is increased in cardiac and skeletal muscle (Tucker and Turcotte 2003, Koonen, Febbraio et al. 2007). The enhanced CD36 in cardiac tissues was linked to the age-related increase in cardiac dysfunction, whilst the increase in skeletal muscle CD36 was linked to elevated triglycerides in these tissues, and muscle triglyceride concentrations was negatively correlated to insulin sensitivity (Pagliassotti, Pan et al. 1995, Kelley, Goodpaster et al. 2002, Hoffman 2006).

The enhanced expression of CD36 in PBMCs may reflect a role for these cells in the development and progression of atherosclerosis or may serve an adaptive protective effect, indeed atherosclerosis is reduced in knockout of CD36 in the apoE -/- mice (Febbraio, Podrez et al. 2000). In a feed forward mechanism, enhanced expression of CD36 may increase the uptake of fatty acids and LDL, and in combination with pro-oxidising state of the cells by mitochondrial ROS lead to formation of oxidised lipids e.g. oxLDL. Consequently, monocytes may differentiate into macrophages promoting further recruitment of cells and plaque enrichment with both cells and lipids, depending on whether phenotype is skewed by M1 or M2 polarisation.

In examining the effect of age on CD14 expression, a significant increase was demonstrated between young and midlife PBMC populations. Monocytes within PBMCs are often characterised into individual populations based on cell surface CD14 and CD16 expression. Historically, monocytes were divided into two distinct populations based on this expression pattern, i.e. $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{+}$ subsets first classified by Passlick and colleagues (Passlick, Flieger et al. 1989). However, further analysis reveals further heterogeneity within the $CD16^{+}$ population, with a population expressing higher levels of CD14 but lower levels of CD16 termed intermediate and third population expressing low levels of CD14 and higher levels of CD16 termed the non-classical population (Ziegler-Heitbrock, Ancuta et al. 2010). Nyugen et al. evaluated the effect of age on these distinct populations, albeit in their study they looked at four populations of monocytes $CD14^{++\,(high)}$ $CD16^{-}$,

$CD14^{+(low)}$ $CD16^{-}$, $CD14^{++(high)}$ $CD16^{+}$, and $CD14^{+(low)}$ $CD16^{+}$and examined how these subpopulations varied with age comparing young (21-32 years old) and old (66-89 years old). They described observing increases in both CD16+ populations with a decrease in the $CD14^{+(low)}$ $CD16^{-}$ population (Nyugen, Agrawal et al. 2010). These findings were supported by Seidler et al. there was no real change in the classical $CD14^{++(high)}$ $CD16^{-}$ monocytes populations, but an increase in the non-classical $CD14^{+}CD16^{+}$ monocytes subpopulation (Seidler, Zimmermann et al. 2010).

Whilst this data would suggest the age dependent increase in CD14 observed in our study reflects an alteration in monocytes subpopulations, other studies do not necessarily agree. Heimbeck et al. analysed CD14 and CD16 from monocytes and demonstrated no effect of age, with no shift in individual subpopulations with age (Heimbeck, Hofer et al. 2010). However, our data shows a general increase in the CD14 expression levels in the ageing individual but in the absence of CD16 data it can only be speculated whether this is due to enhanced levels of $CD14^{++}/CD16^{-}$ or $CD14^{++}/CD16^{+}$ expressing monocytes.

Changes in monocyte subpopulations are observed in pathological conditions. For example, Rothe et al. observed high levels of atherogenic lipids (Rothe, Gabriel et al. 1996), and a unique scavenger receptor profile (Draude, Von Hundelshausen et al. 1999) in the $CD14^{+}CD16^{+}$ monocyte subset implicating these cells in the pathogenesis of atherosclerosis. $CD16^{+}$ subsets have been correlated to sub-clinical atherosclerosis in patients with renal disease (Heine, Ulrich et al. 2008, Ulrich, Heine et al. 2008, Rogacev, Seiler et al. 2011); atherosclerosis and inflammatory diseases (Merino, Buendia et al. 2011). The size of the $CD16^{+}$ population increases in familial hypercholesteremia (Mosig, Rennert et al. 2009), and correlates with atherosclerotic plaque vulnerability (Kashiwagi, Imanishi et al. 2010). These findings suggest changes in $CD14^{+}CD16^{+}$ population may play an important role in metabolic disorders, and the finding of elevated CD14 in midlife PBMCs merits further investigation for CD16 status.

In this study, an age-related elevation in the pro-inflammatory cytokines TNF-α and IL-6 with a minor reduction in the anti-inflammatory cytokine IL-10 were recorded. Increases in plasma levels of these cytokines are widely reported in the ageing individual (Paolisso, Rizzo et al. 1998, Bruunsgaard, Andersen-Ranberg et al. 1999, Bruunsgaard, Skinhoj et al. 2000, Kiecolt-Glaser, Preacher et al. 2003, Miles, Rees et al. 2008, Stowe, Peek et al. 2010, Coe, Love et al. 2011). However, there is conflicting data for example with IL-6, Beharka et al. observed no real change in serum IL-6 (Beharka, Meydani et al. 2001) as a consequence of age, which agrees with data by Peterson et al.(Peterson, Chao et al. 1994), and Fagiolo and colleagues (Fagiolo, Cossarizza et al. 1993) observed no change in TNF-α. Some studies demonstrated no change in IL-10 with age in humans(Peterson, Chao et al. 1994,

Stowe, Peek et al. 2010) and animals studies (Ye and Johnson 2001), whilst one indicated an decrease (Forsey, Thompson et al. 2003).

Reasons for these discrepancies can be related to variations in the methodologies applied to determine cytokine levels, lack of power or small sample size, the health or treatments of individual volunteers and to differences in their age. The volunteers used in this study are healthy male volunteers taken from 24-30 age groups (although a majority of individuals were above 24) with no underlying medication or disorders. In the midlife population, the age ranged from 50-, with a few individuals taking medication for underlying disorders however these were not expected to change inflammatory cytokine levels. A possible confounding factor is the evidence that inflammatory cytokines act as disease markers (Bruunsgaard, Andersen-Ranberg et al. 1999, Ferrucci, Harris et al. 1999, Bruunsgaard, Skinhoj et al. 2000, Krabbe, Pedersen et al. 2004), and may be higher in certain individuals with underlying and possibly undiagnosed conditions compared to very healthy midlife individuals.

Despite these discrepancies, this chapter highlights an age-related increase in pro-inflammatory cytokines in a healthy male population of young and midlife male volunteers. The source or underlying cause of the elevated inflammation in ageing remains unclear and has been postulated to be increased adipose tissue mass or an increase in macrophage infiltration. Cartier et al. examined the contribution of visceral adipose tissue to inflammation in the ageing process by examining male volunteers from ages 18-72 years old. CRP and IL-6 was strongly correlated with visceral adiposity in ageing (Cartier, Cote et al. 2009). Brinkley et al. used factor analysis in a population of 70-79 year old volunteers to determine whether individual inflammatory markers are associated with age-related parameters such as abdominal adiposity and demonstrated a positive association of IL-6, CRP and IL-1α with total and abdominal adiposity (Brinkley, Hsu et al. 2012). An anthropometric measure of abdominal adiposity is waist circumference, which was elevated in the midlife cohort sampled in this thesis, suggesting a possible contributory factor of adiposity to inflammation. However other anthropometric measurements, lipid, glucose, blood pressure and heart rates were evaluated in volunteers, and were not significantly different with age.

Furthermore evidence of age-related activation of redox sensitive NF-κB has been observed (Kim, Jung et al. 2002, Chung, Sung et al. 2006, Zou, Kim et al. 2009), and in rat aortas increased oxidative stress enhanced soluble adhesion molecular expression was observed, proteins under transcription control by NF-κB (Zou, Jung et al. 2003).

Analysis of plasma NEFA profiles from young and midlife individuals revealed a global increase in NEFA with age, with increases in individual MUFA, PUFA and SFA pools.

To date, only two studies have sought to determine the effect of age on the plasma NEFA profile, with one utilising HPLC methods (Shen, Qi et al. 2013)and the other using capillary gas chromatography (Fortier, Tremblay-Mercier et al. 2010). In both these studies global elevations in NEFA were observed, supporting the findings of this chapter, however the number of FA identified and therefore quantified are different to the other reported studies which may be related to differences in the gas chromatography column used. Another study by Sfar et al. examined age-related fatty acid profiles; however their data was expressed as overall composition with the contribution of each fatty acid given as a percentage (Sfar, Laporte et al. 2010) and absolute values were not available.

Here, the fatty acids myristate (C14:0), palmitate (C16:0), palmitoleate (C16:1), stearate (C18:0), oleate (C18:1), linoleic acid (C18:2), γ-linoleic acid (C18:3n6), linolenic acid (C18:3n3), docosahexanoic acid (C22:6), lignoceric acid (C24:0) and nervonic acid (C24:1) were identified and quantified by a gas chromatographic method in the NEFA pool of plasma. Shen and colleagues studied a greater sample size and demonstrated the age-related change in 9 FA (Shen, Qi et al. 2013) including lauric acid, whilst Fortier et al. were able to identify and quantify changes in 13 different FA (Fortier, Tremblay-Mercier et al. 2010). A similar number of fatty acids were identified by Fortier et al. using a gas chromatography methodology, with the exception of γ-linoleic acid, lignoceric acid and nervonic acid, however, in their study identified docosapentaenoic acid (C22:5) also.

Total NEFA measured in this study increase with ageing, but the polyunsaturated fatty acid profiles here are show no significant increase which differs to the general increase in all fatty acids observed by Shen et al. and Fortier et al. and were significantly increased in the midlife cohort.

Sample size is a key factor, with larger samples obtained by both Shen et al. and Fortier et al., at least n=24 and n=55 in each group for the latter and the former respectively, compared to n=14 in the young and midlife populations of this study. Whilst methodological differences have been described, the concentrations of fatty acids derived remain quite similar, and therefore this factor may not be as important in differences in fatty acids identified. The type of column used in the gas chromatography protocol used in this thesis may be more sensitive permitting identification of more fatty acids.

With respect to the monounsaturated fatty acid oleate and the saturated fatty acid palmitate, an age-related increase in both fatty acids was observed in the Aston cohort, suggesting that the hypothesis for an age-related alteration in NEFA profile favouring saturated fatty acids is not proven.

Analysis of fatty acid profiles of red blood cell membranes was also performed, in a subset and palmitate, stearate, oleate, linoleic acid, γ-linolenic acid, linolenic acid, docosahexanoic acid, nervonic and lignoceric acids by gas chromatography were identified. Measurement of fatty acids can provide information regarding the components involved in their processing and metabolism, and may provide information regarding disease risk. There was a trend for a higher percentage of MUFA, a lower percentage of SFA in older adult membranes compared with the younger cohort.

The desaturation index is a measure of the activity of stearoyl CoA-desaturase 1 (SCD-1) which is responsible for the production of monounsaturated fatty acids palmitoleate and oleate from palmitate and stearate respectively. An age-related elevation in SCD activity (calculated for NEFA and erythrocyte membrane fatty acids) was observed for both C16 and C18 fatty acids. In an elderly population and middle aged men, elevated plasma saturated fatty acids and high SCD-1 index were strongly correlated to plasma CRP and inflammation (Petersson, Lind et al. 2009), with the authors showing similar data in a population of middle aged men (Petersson, Basu et al. 2008). High SCD-1 activity has been correlated with development of the metabolic syndrome (a cluster of metabolic risk factors including abdominal obesity, hypertension, insulin resistance and dyslipidaemia) in men (Warensjo, Riserus et al. 2005). Mayneris-Perxachs et al. demonstrated a positive correlation between plasma SCD-1 activity and several risk factors of the metabolic syndrome (Mayneris-Perxachs, Guerendiain et al. 2013). In a Korean population, cardiometabolic disease risk has been associated with SCD-1 (Do, Chung et al. 2011). However SCD1 inhibition may not be a safe therapeutic target for the metabolic syndrome as it associates with increased membrane SFA and greater sensitivity to TLR4 activation (Brown, Chung et al. 2008)with accelerated atherosclerosis. This raises the intriguing possibility that SCD-1 activity increase is an adaptation to ageing which reduces inflammation and CVD.

SCD-1 activity has been correlated to insulin resistance (Warensjo, Rosell et al. 2009), in adipose tissue (Sjogren, Sierra-Johnson et al. 2008), and in the morbidly obese (Garcia-Serrano, Moreno-Santos et al. 2011). Analysis of erythrocytes revealed a link between elevated risk of diabetes development and SCD-1 activity in middle aged subjects (Kroeger, Zietemann et al. 2011), whilst Chow et al. demonstrated a positive association between incident diabetes and high SCD-1 activity estimated from plasma fatty acid profiles (Chow, Li et al. 2013).

The involvement and correlations between SCD-1 activity and pathology extends beyond metabolic diseases; brain samples from Alzheimer's disease patients have elevated SCD-1 mRNA, with a negative correlation to cognition (Astarita, Jung et al. 2011). In dialysis patients SCD-1 activity was

associated with age and IL-6, with high levels of activity was associated with increased mortality risk (Huang, Stenvinkel et al. 2013)

The enzymes Δ5 desaturase and Δ6 desaturase are also important in FA metabolism Δ5 desaturase (D5D) converts dihomo-γ-linoleic acid to arachidonic acid and Δ6 desaturase (D6D) converts linoleic acid to γ-linoleic acid (C18:2 to 18:3n-6). The data in this chapter demonstrated an increase in D6D derived from the NEFA pool but not the erythrocyte fatty pool. Age-related increases in the indices for SCD-1 and D6D observed in this study suggest elevated risk of developing metabolic complications. Krachler et al. demonstrated that the risk of developing type-2 diabetes increased with increments in D6D activity (Krachler, Norberg et al. 2008). High D6D and low D5D activity also predict development of the metabolic syndrome (Warensjo, Riserus et al. 2005, Warensjo, Rosell et al. 2009, Do, Chung et al. 2011, Kroeger, Zietemann et al. 2011, Kurotani, Sato et al. 2012, Mayneris-Perxachs, Guerendiain et al. 2013). Some studies did not find associations or correlations between Δ5 or Δ6 desaturation and metabolic risk factors; Ebbseson et al., correlated heart rate (a factor associated with arrhythmia and sudden death) with SCD but not Δ5 or Δ6 desaturase activity (Ebbesson, Lopez-Alvarenga et al. 2012).

Analysis of fatty acid profiles also provides information regarding polyunsaturated fatty acids. The ratio of the total ω-3 and ω-6 PUFAs have also been linked to disease risk, in particular cardiovascular disease, which is calculated based on total Ω-3 and total Ω-6. However the lack of change in Ω-6: Ω:3 ratio suggest no real change in metabolic disease risk, and may reflect the healthy state of the midlife volunteers used. Harris et al. pooled data from 13 case control and prospective studies to determine associations between the ω-6: ω-3 ratio and coronary heart disease risk. Their study concluded that whilst an association was present, the strongest association was between the two ω-3 fatty acids EPA and DHA (Harris, Assaad et al. 2006) showing an inverse relationship to disease risk. A review by Schmitz et al. focusing upon the effects of ω-3 and ω-6 fatty acids suggested a ratio favouring the latter could promote progression of coronary artery disease (Schmitz and Ecker 2008). Other studies like that of Leng et al. and Fujihara et al. focused on an ω-3 and ω-6 fatty acid measuring a ratio of EPA: AA, with both demonstrating a lower ratio in those with peripheral artery disease (Leng, Horrobin et al. 1994, Fujihara, Fukata et al. 2013).

In this study a significant age-related increase in myristic acid is observed in the plasma NEFA profile, suggesting elevated risk of metabolic pathology is a likely outcome. Individual fatty acids and classes have also been linked to adverse metabolic pathologies. Myristic (C14:0) and palmitoleic (C16:1) acids were elevated in non-alcoholic steatohepatitis (NASH), with the former being an independent predictor to differentiate between simple steatosis and NASH (Tomita, Teratani et al. 2011). Work by

other groups demonstrated an increase in myristic acid in individuals with impaired fasting glucose (Xu, Tavintharan et al. 2013), associations with heart failure(Yamagishi, Nettleton et al. 2008), myocardial infarction (Kabagambe, Baylin et al. 2003), and inversely related to endothelial vasodilatory function (Steer, Vessby et al. 2003).

The plasma NEFA profile demonstrates age-related elevations in both palmitate and stearate, whilst only the former is increased in erythrocyte membranes. Both these fatty acids are linked to poor metabolic health. Elevated saturated fatty acids in plasma have been associated with high blood pressure (Grimsgaard, Bonaa et al. 1999), the metabolic syndrome (Yu, Cai et al. 2012) and predict diabetes (Wang, Folsom et al. 2003). In erythrocyte membrane palmitate and total SFA have been linked to the metabolic syndrome (Kabagambe, Tsai et al. 2008).

The C24:0 SFA lignoceric acid exhibits a significant age-related increase in both plasma NEFA and erythrocytes. Data concerning interplay of this SFA with metabolic risk factors is rare. A study looking at serum fatty acid profiles in obese and lean subjects and the possible link to insulin resistance and inflammatory cytokines, demonstrated that the most insulin sensitive subjects had lower lignoceric acid content (Fernandez-Real, Broch et al. 2003). Matsumori et al. correlated elevated erythrocyte lignoceric acid to atherogenic lipoprotein profiles in individuals with metabolic syndrome (Matsumori, Miyazaki et al. 2013). Such findings suggest a link between elevated lignoceric levels and adverse metabolic outcomes.

Whilst SFA are typically associated with negative metabolic outcomes such as insulin resistance and atherosclerosis, correlations between both MUFA and PUFA groups which can have a role in metabolic disease risk factors were observed here.

Paradoxically MUFA in the diet associates with reduced cardiovascular risk, but relatively few studies examining the MUFA in plasma or blood at baseline. In serum phospholipids and in triglycerides increased MUFA have been correlated to risk factors associated with the metabolic syndrome such as insulin resistance and arterial stiffness (Kotronen, Velagapudi et al. 2009, Kim, Lim et al. 2013). An inverse association was present between MUFA and adverse heart dilation after analysis of serum fatty acid profiles (Rupp, Rupp et al. 2010). These studies suggest elevated serum MUFA is beneficial.

Individual MUFAs have been correlated to negative health outcomes; palmitoleic acid (C16:1) has been associated with adverse inflammatory profiles and metabolic syndrome in a middle aged and older Chinese population (Zong, Ye et al. 2012), whilst phospholipid palmitoleic acid was associated with cholesterol, LDL, HDL and insulin resistance but not incident diabetes in elderly subjects (Mozaffarian, Cao et al. 2010);and correlates with abdominal adiposity (Paillard, Catheline et al.

2008). Similar relationships have been observed for nervonic acid, C24:1, which has been linked to mortality in patients of chronic kidney disease (Shearer, Carrero et al. 2012) and to coronary risk factors (Oda, Hatada et al. 2005) indicating MUFA can be associated with negative health outcomes.

The possible effects of the age-related changes in the fatty acid profile of plasma NEFA and erythrocyte membranes between young and midlife volunteers has been postulated as above, however this does not take into account possible inter-relationships between fatty acids, markers of inflammation, metabolic risk factors, and oxidative stress as a function of age. Thus using linear regression, analysis was then used to further explore the relationships between age and risk factors grouped into fatty acid classes, metabolic factors (lipids, glucose and insulin measures), oxidative stress and inflammatory/immune factors (tables 6.3-6.16).

Positive associations between age and markers of insulin, insulin resistance, oxidative stress, pro-inflammatory cytokines TNF-α and IL-6, total NEFA MUFA, NEFA PUFA, NEFA SFA, NEFA SCD-18 and NEFA D6D activities were determined in this cohort. The strongest associations of monounsaturated fatty acids, both in the NEFA or erythrocyte membrane pools, were with insulin resistance. Furthermore, MUFA showed positive associations with mitochondrial superoxide production. Saturated fatty acids were associated with CD11b and CD14, in addition to insulin HOMA-IR and HOMA-B, and TNF-α, a relationship shared with MUFA. This suggests both the SFA and MUFA classes contribute to or change as an adaptation to insulin function, CD11b expression and inflammation.

Analysis of the saturated fatty acids revealed that myristic acid, showed relationships to D6D and erythrocyte Ω-6. All saturated fatty acids demonstrated strong positive inter-relationships with all other total MUFA and PUFA. Palmitate and lignoceric acid demonstrated a significant positive association with TNF-α, with the latter also showing a positive association with IL-6, possibly reflecting an association between saturated fatty acids and pro-inflammatory cytokines. The cell surface markers showed some positive relationships, with palmitate and lignoceric acid demonstrating positive linear relationships with CD11b and CD14 respectively. Stearate demonstrated a positive association with mitosox, and lignoceric acid showing a negative relationship with glutathione. These findings suggest that the SFA have a role to play in inflammation and oxidative stress in the ageing individual.

Relationships discovered when analysing the MUFA class were somewhat surprising. The shorter chain MUFAs myristoleic and palmitoleic acids demonstrated positive relationships to insulin, HOMA-IR and HOMA-B. NEFA MUFA demonstrated similarly strong relationships with markers of oxidative stress; all MUFA except palmitoleate showed significant negative correlations with glutathione,

whilst both palmitoleate and nervonic acid were positively associated with mitochondrial superoxide production. Surprisingly, myristoleate and oleate were positively associated with TNF-α, with also positively related to CD11b. MUFA appear to have a close association glutathione and insulin resistance.

Polyunsaturated fatty acids linoleic and γ-linoleic acid were positively associated with CD11b. Linoleic acid showed positive associations with NEFA SFA; γ-linoleic acid showed positive relationships with NEFA MUFA and SCD16, with α-linoleic acid also showed a positive relationship with NEFA derived SCD16. Docosahexanoic acid (C22:6n3) was positively associated with NEFA MUFA, SFA, SCD18 and RBC MUFA.

The erythrocyte membrane fatty acids were also evaluated for linear relationships with metabolic and inflammatory indices. Erythrocyte SFAs all showed positive associations with RBC MUFA, and unlike NEFA, both palmitate and lignoceric acid demonstrated positive relationships with insulin, HOMA-IR and HOMA-B. The RBC MUFAs shared similar relationships to the NEFA MUFA, with palmitoleate and nervonic acid having positive and negative relationships with glutathione and MitoSox respectively. Both erythrocyte membrane oleate and nervonic acid had positive relationships with insulin, HOMA-IR, HOMA-B and total erythrocyte SFA; a relationship which suggests the prominent contribution of MUFA in determining insulin levels and sensitivity.

Analysis of erythrocyte membrane PUFA revealed positive association of both linoleic acid and α-linoleic acid were positively associated erythrocyte total SFA and MUFA. α-linoleic acid was positively associated with NEFA SCD16 and D6D whilst a negative relationship was observed with erythrocyte SCD18 activity. DHA was negatively associated with HDL cholesterol.

Inter-relationships between measures of oxidative stress, lipids, cell surface markers and measures of glucose and insulin function were determined. PBMC cell surface markers CD11b and CD36 were positively associated with total cholesterol and LDL cholesterol. CD14 had a negative relationship with redox ratio but positive associations with TNF, IL-6 and NEFA SFA. CD11b was positively associated with CD36, TNF-α and IL-6 and NEFA SFA; whilst CD36 was negatively associated with reduced glutathione.

Linear correlation analysis of cytokines revealed positive relationships of both the pro-inflammatory cytokines TNF-α and IL-6 with CD14, CD36 whilst a negative association was present with glutathione for both cytokines. TNF-α showed positive relationships with insulin, HOMA-IR, HOMA-B, IL-6, NEFA MUFA, NEFA SFA, NEFA SCD18 and NEFA D6D. IL-6 showed positive relationship with oxidised

glutathione, TNF-α and NEFA SCD-1 activity. IL-10 showed a negative relationship with insulin resistance.

Analysis of lipids showed both total cholesterol and LDL were positively associated with both CD11b and CD36, whilst triglycerides (TG) were associated with mitochondrial superoxide production (MitoSOX) and NEFA MUFA. Glucose did not show relationships to other factors. Insulin, HOMA-IR and HOMA-B were associated with total TNF-α, NEFA MUFA, NEFA D6D activity and total RBC MUFA. Both insulin and HOMA-IR were negatively associated with glutathione; insulin positively with MitoSOX; HOMA-IR with triglycerides and negatively with IL-10.

Evaluation of the relationships of mitochondrial superoxide production and measures of glutathione by linear analysis revealed positive associations between mitochondrial superoxide with glucose, insulin, oxidised glutathione, NEFA MUFA and erythrocyte SCD-18 activity. Glutathione was negatively associated with insulin, HOMA-IR, CD36, mitochondrial superoxide production, TNF-α, IL-6, NEFA SFA, NEFA MUFA, NEFA PUFA, NEFA SCD18 and D6D activity. Oxidised glutathione was positively correlated with MitoSOX oxidation, IL-6, NEFA SCD18 and D6D activity; whilst redox ratio had a positive relationship with PBMC CD14 expression.

Data from previous chapters demonstrated an increase in both CD11b and CD36 in monocytes in response to palmitate, however, linear correlation analysis failed to demonstrate a relationship between SFA and CD36, instead total cholesterol and LDL cholesterol showed a positive and significant relationship suggesting cholesterol has a greater influence over cell surface expression of CD36. Analysis of the inter-relationships of inflammatory cytokines indicated both MUFA and SFA share a positive relationship with TNF-α.

6.5 Conclusions

In this population studied in this chapter, increases in oxidative stress, inflammation, immune function, lipid measures, insulin resistance were observed in an age-related manner when comparing a young (24-30 years old) and midlife (>50 years old) cohort.

The original hypothesis that ageing is associated with an increase of saturated fatty acids which would favour the development of insulin resistance and induction of inflammation observed in the ageing individual was not supported. However higher concentrations of total fatty acids were observed in the midlife compared to the young adult cohort present in the plasma NEFA fraction and these were associated with a metabolic and inflammatory shift.

Significant increases in the medium chain length C14 and very long chain length C24 fatty acids were observed with and coupled with elevated SCD-1 and D6D activity suggesting that the pathways and metabolic apparatus concerned with de novo synthesis of fatty acids may be altered in age.

Linear regression analysis revealed interrelationships between both SFA and MUFA with inflammatory cytokines and CD11b suggesting a possible influence of these fatty acids on inflammation and atherogenicity within the context of ageing, a relationship shared with LDL. The study also demonstrates an association between MUFA, oxidative stress and markers of insulin sensitivity, HOMA, which suggest involvement of oxidative stress age-related decline in glycaemic control and insulin resistance.

Thus, to conclude this study demonstrates age-related increases in oxidative stress, pro-inflammatory cytokines, insulin resistance and global fatty acids are observed which may reflect worsening metabolic state associated with age. Furthermore complex inter-relationships are revealed demonstrating the influence these factors may exert reciprocally in the context of ageing.

Chapter 7

Discussion, conclusion and further work

7 Chapter 7: Discussion, conclusion and further work

7.1 Discussion

The aims of the study presented here were to determine the effects of the monounsaturated fatty acid oleate and the saturated fatty acid palmitate on monocytes, specifically to determine their effects on THP-1 monocyte cellular and metabolic viability, antioxidant status, superoxide production, apoptosis and cell cycle status.

THP-1 monocytes treated with fatty acids demonstrate divergent effects, with palmitate inducing an increase in mitochondrial superoxides leading to a cell cycle arrest, which was absent in the presence of oleate. Furthermore minor increases in caspase-3 and annexin-V/PI staining coupled to a decrease in metabolic viability with palmitate but not oleate were observed.

These effects of palmitate are likely to be related to their effects in the mitochondria. Palmitate can uncouple the mitochondrial electron transport chain through the interaction of its acyl-CoA, palmitoyl-CoA with the mitochondrial protein ANT altering its conformation that leads to the formation of a permeability transition pore (PTP). PTP formation causes depolarisation of the mitochondrial membrane, (shown by altered JC1 fluorescence), that is a common feature observed prior to apoptosis.

This effect is likely to be responsible for the elevated mitochondrial superoxide production as the electron transport chain is uncoupled leading to the direct channelling of electrons to molecular oxygen. The formation of a PTP will release cytochrome c which will trigger apoptotic processes such as the caspase cascade which explains the increase in caspase-3 activity and annexin-V/PI expression associated with palmitate treatment; however none of the observed changes in apoptotic measures were significant. An alternative possibility is anincreased flux through the electron transport chain in the presence of palmitate.

Glutathione content in fatty acid treated THP-1 monocytes was similar, with reduced glutathione content observed at 50µM. Explanations as to how fatty acids mediate this reduction remain unclear.

Differential effects of oleate and palmitate were observed on THP-1 cell surface CD11b and CD36, with the SFA inducing dose-dependent increases in the expression of both cell surface antigens, which was absent in the presence of oleate. Mechanistic investigations were focused on whether the production of mitochondrial superoxides, β-oxidation and the formation of complex lipids were responsible for the increase in CD11b and CD36.

The mitochondrial superoxide dismutase mimetic MnTBap did not significantly affect the THP-1 expression of CD11b and CD36 in the presence of palmitate, even with amelioration of mitochondrial superoxides and improvements in metabolic viability, indicating that changes in cell surface antigen expression by palmitate are not related to a superoxide production or to a stress response. Etomoxir, an inhibitor of β-oxidation affected neither CD11b nor CD36 expression in THP-1 monocytes confirming that oxidation of fatty acids was not required to mediate the effects of palmitate.

Triglyceride content was examined in both oleate- and palmitate- treated THP-1 monocytes, with oleate treated cells demonstrating significantly greater storage/conversion of fatty acids into TG than palmitate. Complex lipids such as DAG and ceramide have consistently been linked to the effects of saturated fatty acids, and on the basis the palmitate is the primary precursor required for the synthesis of ceramides the effects of palmitate on THP-1 monocytes was examined in the presence of fumonisin B1, an inhibitor of serine palmitoyl transferase 1. Inhibition of de novo synthesis of ceramides prevented palmitate induced increases in both CD11b and CD36, indicating the involvement of ceramides; with increased expression of CD11b possibly mediated through the activation of stress kinases such as JNK, and p38 MAPK whilst the mechanism of increased CD36 expression by ceramides unclear.

Ceramides belong to the sphingolipid class of lipids, with a common 8 carbon backbone, sphingosine. Ceramide synthesis occurs in the endoplasmic reticulum, with the rate limiting step performed SPT1 condensing palmitate with serine producing 2-ketodihydrosphingosine, with three further steps converting this metabolite into ceramide. Ceramides are transported from the ER by CERT to the Golgi where either sphingomyelin or glycosphingolipids, which can then be utilised in the plasma membrane.

Ceramides can be degraded to yield sphingosine or converted to sphingomyelin under the activities of sphingosine kinase and sphingomyelin synthase respectively. The introduction of MAPP and PPMP to inhibit these enzymes enabled the determination of the true sphingolipid metabolite responsible for mediating the effects of palmitate. Neither PPMP nor MAPP could inhibit palmitate mediate CD11b or CD36 expression in THP-1 monocytes indicating ceramides are the primary mediator of the effects of palmitate.

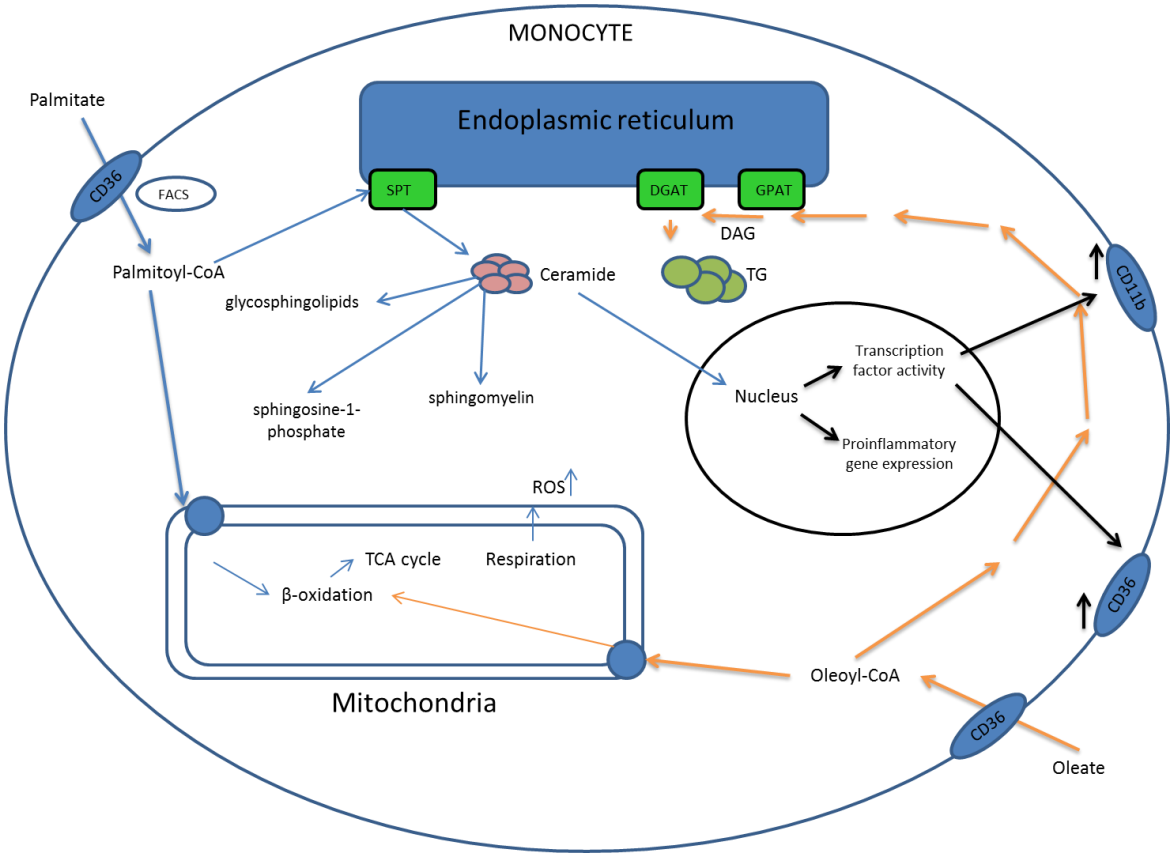

Figure 7.1: Schematic of the effects of palmitate and oleate on monocytes

The capacity to promote atherogenicity in palmitate but not oleate treated THP-1 monocytes by enhancing CD11b and CD36 expression has been shown. However effects of fatty acids in inflammatory cytokine production and their influence in monocyte-macrophage differentiation are poorly defined.

Monocytes did not display the capacity to induce inflammatory cytokine production in response to either fatty acid, however an amplified production of pro-inflammatory cytokines by palmitate pre-treated monocytes, whilst oleate treated cells appeared to suppress this LPS response. The finding suggests that in the presence of palmitate an inflammatory signalling pathway or mediators are induced. The involvement of ceramide in mediating the expression of CD11b and CD36 would suggest their involvement in this amplified response. Schwartz et al. demonstrated that ceramide formation in response to palmitate in THP-1 monocytes was responsible for an enhanced production of pro-inflammatory cytokines in response to LPS(Schwartz, Zhang et al. 2010).

The mechanism of this response may rely upon the activation of stress kinases such as JNK and p38 MAPK by ceramides. LPS induces an inflammatory response through activation of the TLR4 receptor, and in this signalling pathway activation of stress kinases is observed, which activate the NF-κB signalling pathway leading to the transcription of inflammatory cytokines. Therefore palmitate pre-

treatment will lead to the de novo synthesis of ceramides and consequently the activation of such stress kinases, which following an LPS stimulus will lead to further stress kinase activation and consequently an amplified production of pro-inflammatory cytokines.

Such findings, in combination with those in this study would suggest in the context of atherosclerosis and type-2 diabetes elevations in SFA are sufficient on their own, or in the presence of TLR ligands to greatly enhance inflammation and thus further disease progression. Furthermore, data generated here and from the previous chapter would suggest the ability of complex metabolites of SFA, namely ceramides, to promote insulin resistance and inflammation in the context of elevated NEFA typically observed in metabolic disorders.

From a signalling perspective, work by Medvedev et al.and Lakics et al.reveal that ceramide is capable of activating MAPK and AP-1, which is required in LPS signalling pathways(Lakics and Vogel 1998, Medvedev, Blanco et al. 1999). In human alveolar macrophages LPS exposure induced an increase in ceramide that mediated activation of PI3K/Akt pathway, but ceramide alone was insufficient to activate Nf-κB instead activating signalling pathways which can enhance inflammatory pathways(Monick, Mallampalli et al. 2001).

The ability of palmitate to enhance an LPS mediated increase in pro-inflammatory cytokine expression contrasts the anti-inflammatory effect of oleate on the monocyte LPS response. However in the presence of oleate and LPS, production of the anti-inflammatory cytokine was not induced, and neither was it induced on the presence of the IL-10 inducer peptidoglycan. This suggests that the effect of oleate is limited to suppressing the LPS response rather than the induction of anti-inflammatory cytokine production.

In addition to mechanisms described here, palmitate can modulate immune cell function directly through interaction with TLR4, thus the amplification of monocyte inflammatory cytokine production shown in this study or the direct activation of TLR4 leading to pro-inflammatory cytokine production can promote the attachment, recruitment and infiltration of these cells into the intima of arteries which contributes to atherosclerosis.

The capacity of fatty acids to modulate monocyte function and responses is not limited to SFA and MUFA. Human supplementation studies using the n-6 polyunsaturated arachidonic acid produced no change in immune responses such as natural killer cell activity (Kelley, Taylor et al. 1997), but an enhancement in both prostaglandin E2 and leukotriene B4 was described without affecting other immune parameters (Kelley, Taylor et al. 1998). In contrast to γ-linoleic acid decreases production of these mediators in human subjects (Johnson, Swan et al. 1997).

The n-3 polyunsaturates tend to generate anti-inflammatory effects. Reductions in lymphocyte proliferation (Meydani, Endres et al. 1991), IL-1 production (Endres, Ghorbani et al. 1989), IL-2 production (Soyland, Lea et al. 1994), TNF-α (Endres, Ghorbani et al. 1989)and NK cell activity (Yamashita, Maruyama et al. 1991, Kelley, Taylor et al. 1999)have been observed in human studies of n-3 intervention. For example supplementation of eicosapentaenoic was shown to decrease T cell proliferation and cytokine production (Molvig, Pociot et al. 1991, Gallai, Sarchielli et al. 1995), whilst docosahexaenoic acid reduced numbers of circulating neutrophils, NK-cell activity, and the production inflammatory cytokines IL-1β and TNF-α (Kelley, Taylor et al. 1999).

The modulatory capacity of oleate and palmitate on monocyte-macrophage differentiation has to date not been investigated. THP-1 monocytes pretreated with either oleate or palmitate was subsequently differentiated with PMA. In the co-treatment of fatty acids and LPS, palmitate induced a pro-inflammatory phenotype whilst oleate appeared to suppress pro-inflammatory cytokine production. As with the monocyte model, palmitate induced a pro-inflammatory macrophage with low CD14 and CD11b expression, which is contrasted by oleate treated cells which have high levels of both CD14 and CD11b, a low pro-inflammatory cytokine expression and a greater apoptotic binding capacity.

The pro-inflammatory nature of palmitate differentiated macrophages wasdemonstrated to be similar to classical M1 macrophages, however the oleate differentiated macrophages do not induce IL-10 or cell surface CD16 expression that is typical of M2 macrophages indicating that oleate primed macrophages are anti-inflammatory in the respect that they express high levels of CD14 and show a greater capacity to clear apoptotic cells. The pro-inflammatory nature of the palmitate differentiated macrophages was determined to be reliant upon the formation ceramides.

Thus, monocyte atherogenicity, promotion of LPS-mediated cytokine production, and the production of a pro-inflammatory macrophage in the presence of the SFA palmitate can be related to the formation of ceramides, with oleate preferentially converted to TG. Studies indicate that there are no significant differences in the digestion and assimilation of fatty acid classes, but differential handling occurs within the cell once lipoprotein lipase has broken down VLDL-TG particles and fatty acids have entered the cell.

Inside the cell, fatty acyl-CoA synthetase (FACS) converts fatty acids into fatty acyl-CoA which permits allows for either oxidation in the mitochondria for energy, storage as TG or conversion into complex lipids. In adipocytes, there is a preference for the storage of MUFA as TG, with higher proportions of MUFA in adipocytes than in the diet or prevailing metabolic profile (Hunter, Rimm et al. 1992,

Garland, Sacks et al. 1998), supported by findings in animal models (Raclot and Groscolas 1993, Connor, Lin et al. 1996) and human studies (Bergouignan, Momken et al. 2009).

β-oxidation or storage is the primary fate of fatty acyl-CoA in myocytes, although in these cells with a relatively high metabolic activity β-oxidation would feature more prominently. The oxidative fate of fatty acids is dependent upon the degree of saturation (DeLany, Windhauser et al. 2000), with indirect evidence indicating preference of carnitine palmitoyl transferase 1 (rate limiting step in β-oxidation, and controlling entry of fatty acyl-CoA into the mitochondria) favours palmitoyl-CoA over oleoyl-CoA (Gavino and Gavino 1991), which is supported by greater generation of palmitoylcarnitine than oleoylcarnitine from palmitate and oleate respectively in L6 myotubes (Koves, Ussher et al. 2008). This suggests greater oxidation of palmitoyl-CoA than oleoyl-CoA in myocytes.

In cultured muscle cells the incorporation of either MUFA or SFA acyl-CoAs differs. Incubation of radiolabelled palmitate and oleate with cultured muscle cells indicates that palmitate leads to accumulation of radioactivity in MAG(Bastie, Hajri et al. 2004), DAG(Montell, Turini et al. 2001, Chavez and Summers 2003, Bastie, Hajri et al. 2004) and ceramides (Pickersgill, Litherland et al. 2007)whilst oleate leads to elevated radioactivity in TG. These findings suggest that TG synthesis in the presence of saturated fatty acids e.g. palmitate is much lower than cells exposed to monounsaturated fatty acid oleate, which leads to the abnormal accumulation of DAG and/or ceramides.

The partitioning between SFA and MUFA can be explained by the affinity of enzymes involved in TG synthesis. TG synthesis occurs by either the glycerol phosphate pathway or the monoacylglycerol pathway, the former being the major pathway utilised by cells, whilst the monoacylglycerol pathway is utilised by the cells of the small intestines when handling absorbed dietary fatty acids (Johnston, Paultauf et al. 1970).

The first committed step in the synthesis of TG is the acylation of glycerol-3-phosphate by glycerol-3-phosphate acyltransferase (GPAT) in the endoplasmic reticulum (ER) producing lysophosphatidic acid (LPA), a further fatty acid is added to LPA by acylglycerol-3-phosphate acyltransferase (AGPAT) to generate phosphatidate. Phosphatidate is converted into DAG by phosphatidate phosphatase 1 (PAP1), with DAG converted into TAG into diacylglycerol transferase enzymes by adding a fatty acyl-CoA (Takeuchi and Reue 2009).

The final enzyme, DGAT has been shown to possess a preference for oleoyl-CoA over palmitoyl-CoA (Cases, Stone et al. 2001, Lardizabal, Mai et al. 2001) which would support the postulate that MUFA are preferentially stored as TG. The formation of ceramides is dependent upon the availability of

palmitate, however the addition of the second fatty acid can be either saturated or unsaturated (Merrill 2002). The lower rate of TG formation from DAG in the presence of palmitate will increase the available pool for the synthesis of ceramides, therefore palmitate treated monocytes will possess both greater DAG and ceramide formation compared to oleate treated cells which are preferentially converted into TG.

Elevations in fatty acids are a common feature of and risk factor for metabolic disorders such as diabetes and cardiovascular disease. Increased risk of developing such metabolic disorders is a hallmark of ageing, where increased circulating free fatty acids are observed. Other hallmarks of ageing are increased insulin resistance and low grade systemic inflammation, i.e. increased pro-inflammatory cytokines and decreased anti-inflammatory cytokines, both of which are can be induced by SFA. The data in this study demonstrates the detrimental effects of SFA in inducing pro-atherogenic and inflammatory changes in monocytes.

Plasma non-esterified fatty acid profile analysis revealed global increases in all fatty acid classes with age, with significant increases associated with the medium length myristate and myristoleate (C14:0 and C14:1 respectively), and the very long chain lignoceric and nervonic acids (C24:0 and C24:1 respectively), coupled to increased D6D and SCD-1 activity. These findings suggest that ageing is associated alterations in the pathways and metabolic apparatus associated with the de novo synthesis of fatty acids. Age was also associated with elevated oxidative stress, insulin resistance and pro-inflammatory cytokines, and PBMC cell surface expression of CD11b, CD14 and CD36 were also observed, and consequently linear regression was used to consolidate this data with changes in plasma fatty acid profile. Linear regression analysis revealed that both MUFA and SFA were positively associated with inflammatory cytokines, CD11b and insulin resistance, suggesting that in ageing both fatty acid classes can influence inflammation, atherogenicity and insulin resistance.

These changes reflect a declining metabolic state associated with age which cannot simply be explained simply by a change in fatty acid profile, with possible changes in de novo fatty acid synthesis. In the context of ageing, the altered activities of enzymes concerned with de novo fatty acid synthesis have been observed. In the aging human orbitofrontal cortex changes in fatty acid membrane profile were associated with increases in elongase and SCD-1 expression and activity (McNamara, Liu et al. 2008), however other data concerning enzyme activities with age are somewhat scarce.

7.2 Conclusion

In summary, *in vitro* experiments on THP-1 monocytes demonstrate the importance of ceramide formation in inducing cell surface antigen expression and inflammatory cytokine production in response to palmitate (summarised in figure 7.1). The association of age and SFA with insulin resistance and production of inflammatory cytokines led to an examination of blood plasma, PBMCs, oxidative stress and insulin resistance in a young and mid-life population. Ageing was associated with increases in both SFA and MUFA classes, coupled to increases in oxidative stress, pro-inflammatory cytokines and insulin resistance indicating that these effects cannot simply be explained by an increase in circulating SFA, pointing to derangements in de novo fatty acid synthesis.

7.3 Future work

The first area in which further investigation is perhaps warranted would be the capacity for fatty acids to induce endoplasmic reticulum stress (ER stress). In this thesis fatty acid-mediated effects on oxidative stress, metabolic activity and viability were investigated, with palmitate but not oleate inducing stress, possibly related to the production of ceramide and uncoupling in the mitochondria; however, their impact on endoplasmic reticulum were not investigated.

The endoplasmic reticulum is a key cellular organelle responsible for the synthesis of proteins and lipids. Associated with these organelles are various chaperone proteins and enzymes preventing aggregation and permitting regulated protein folding of newly synthesised proteins. During periods of stress the levels of protein entering the ER exceed the folding capacity, and induce the unfolded protein response or ER stress. Stressors can include changes in cellular pH, infection and changes in metabolic status. The ER stress response begins with an inhibition of translation, with an upregulation in genes associated with endoplasmic reticulum biogenesis, chaperone and folding proteins in addition to apparatus involved in protein degradation. This process is governed by three pathways involving the Inositol-requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6).

In mice, endoplasmic reticulum stress has a role to play in maintaining glucose homeostasis (Scheuner, Song et al. 2001), and has been purported to link obesity, insulin resistance and type-2 diabetes (Ozcan, Cao et al. 2004).There is evidence that ER stress is capable of augmenting inflammation and immune responses (Martinon 2012) with evidence these changes rely upon activation of stress kinases such as JNK with the activation of NF-κB inflammatory signalling pathway.

Saturated fatty acids have a well characterised ability to induce endoplasmic reticulum stress is a variety of cells (Cao, Dai et al. 2012, Tao, Wen et al. 2012, Cui, Ma et al. 2013) and in vivo (Nivala, Reese et al. 2012, Nivala, Reese et al. 2013) and in this thesis also induce changes in metabolic activity and augmentation of monocyte inflammatory responses to LPS, raising the possibility that there is involvement of ER stress in these processes.

During the investigation of the influence of fatty acids in the monocyte-macrophage differentiation process the anti- and pro-inflammatory nature of oleate and palmitate was demonstrated. Characterisation of anti- and pro-inflammatory phenotype was determined using the pro-inflammatory cytokines TNF-α and IL-6, and IL-10 and cell surface expression of CD16.

Other studies which have used THP-1 monocytes have tended to use a greater selection of markers, for example the cytokines IL-1β and TGF-β have been used to identify pro- and anti-inflammatory macrophages respectively (Tjiu, Chen et al. 2009). In other studies TNF-α and IL-6 are used to identify pro-inflammatory macrophages, but different cytokines are used to determine a M2 phenotype for example IL-1rα (Caras, Tucureanu et al. 2011). Therefore further characterisation of M2 macrophages by the production of other anti-inflammatory cytokines e.g. TGF-β may be required.

In this thesis the involvement of ceramides in palmitate mediated cell surface expression of both CD11b and CD36, enhancing LPS mediated cytokine production in monocytes, and generating a pro-inflammatory phenotype was demonstrated. However, fatty acids are capable of activating other signalling pathways. For example, peroxisome proliferator activated receptors (PPARs) and the forkhead box proteins (FOXO) have important roles to play in substrate utilisation, handling and metabolism (Gross, Wan et al. 2009, Poulsen, Siersbk et al. 2012), and they are both shown to be affected by the presence of specific fatty acids (Kamagate, Kim et al. 2010, Bindesboll, Berg et al. 2013). Such findings suggest that there are other pathways that are activated by fatty acids which may be dependent or independent of the formation of complex lipids such as ceramide and DAG, therefore an investigation of such pathways maybe warranted to determine what impact they have on monocyte function and phenotype.

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9 Appendices

9.1 Linear correlation analysis tables

	Linoleic acid (C18:2n6)		γ-linoleic acid (C18:3n6)		α-linoleic acid (C18:3n3)		Docosahexanoic acid (C22:6n3)	
	r	p	r	p	r	p	r	p
Total cholesterol	0.188785593	0.3557	0.227200352	0.1224	0.182756669	0.4679	0.040865633	0.8429
CD11b	0.596908703	***0.0008******	0.756571213	***0.0001********	0.072753007	0.7672	0.158018986	0.6847
NEFA MUFA	0.353694784	0.0598	0.769480344	***0.0001********	-0.030374331	0.8988	0.432087954	***0.0193****
NEFA SFA	0.643739078	***0.0002*****	0.439317653	0.171	-0.104307238	0.64	0.432897216	***0.019****
NEFA SCD16	-0.163920713	0.3955	-0.553895297	***0.0018*****	0.892692556	***0.0001********	-0.335261092	0.0754
NEFA SCD18	0.233302379	0.2232	0.088971906	0.6463	-0.048672374	0.8385	0.425323406	***0.0214****
RBC MUFA	-0.030426962	0.8755	0.151756384	0.432	0.053338541	0.7835	0.437492857	***0.0176****
RBC D6D	0.555427763	***0.011****	0.569912274	***0.0087****	0.65230361	***0.0018*****	0.374833296	0.1035
RBC n6	0.194602158	0.3117	0.399249296	***0.0319***	0.301330383	0.122	0.712881477	***0.0001********
RBCn6/n3	0.460325971	***0.0411****	0.154142791	0.5165	0.375366488	0.103	-0.22318154	0.3442

Table 9.1: Linear correlation analysis of specific polyunsaturated fatty acids that were significantly associated with total PUFA. Significance is shown as *=p<0.05 and ****=p<0.0001.

	CD14		CD11b		CD36	
	r	p	r	p	r	p
Total cholesterol (mmol/L)	-0.14826	0.5701	0.402119	***0.0463****	0.539166	***0.0045****
HDL cholesterol	0.119875	0.6467	-0.27601	0.1817	0.011593	0.9552
LDL cholesterol	-0.16214	0.5341	0.417253	***0.0379****	0.468508	***0.0158****
Glucose	0.012888	0.9608	0.236516	0.255	0.452769	***0.0202****
CD14	0		0.164165	0.5151	0.137368	0.5749
CD11b	0.164165	0.5151			0.397744	0.0361
CD36	0.137368	0.5749	0.397744	***0.0361****	0	
plasma GSH	-0.44553	0.0639	-0.12058	0.3079	-0.4046	***0.0327****
GSH/GSSG	-0.46551	***0.0446****	-0.12438	0.5283	-0.26542	0.1641
TNF-a	0.48156	***0.0368****	0.356651	0.0678	0.411339	***0.0297****
IL-6	0.486826	***0.0345****	0.361525	0.0639	0.422374	***0.0252****
SFA	0.526498	***0.0206****	0.472123	***0.0112****	0.124097	0.5213

Table9.2: Linear correlation analysis of PBMC cell surface markers: Significance is shown as *=p<0.05

	MitoSOX		GSH		GSSG		Redox ratio	
	r	p	r	p	r	p	r	p
Glucose	0.439318	***0.028****	-0.38406	0.058	-0.01906	0.9264	-0.10982	0.5933
Insulin (U/ml)	0.464112	***0.0129****	-0.40583	***0.0321****	0.35	0.0627	-0.21799	0.2559
HOMA-IR	-0.0908	0.6459	-0.4111	***0.0298****	0.328024	0.0823	-0.18966	0.3244
CD14	0.283337	0.2398	-0.44553	0.0639	0.231301	0.3407	-0.46551	***0.0446****
CD36	0.268533	0.1671	-0.4046	***0.0327****	0.357071	0.0573	-0.26542	0.1641
MitoSOX	0		-0.53385	***0.0041****	0.38833	***0.0411****	-0.88916	0.1472
plasma GSH	-0.53385	***0.0041****	0		-0.48405	***0.0091****	0.404846	***0.0326****
plasma GSSG	0.38833	***0.0411****	-0.48405	***0.0091****	0		-0.42166	***0.0227****
GSH/GSSG	-0.28118	0.1472	0.404846	***0.0326****	-0.42166	***0.0227****	0	
TNF-a	0.047864	0.8126	-0.4983	***0.0082****	0.331662	0.0847	-0.16706	0.3805
IL-6	0.255793	0.1978	-0.47645	***0.012****	0.456618	***0.0146****	-0.20135	0.3042
IL-10	-0.22795	0.2528	-0.28045	0.1565	-0.22057	0.2594	0.014741	0.9407
SFA	0.16453	0.4028	-0.38626	***0.0423****	0.136748	0.4794	-0.18	0.3502
MUFA	0.481871	***0.0094****	-0.48929	***0.0082****	0.3003	0.1135	-0.24286	0.2043
PUFA	0.132363	0.502	-0.3821	***0.0448****	0.090178	0.6418	-0.17666	0.3593
SCD(16)	0.033451	0.8658	-0.17683	0.368	0.243701	0.2045	-0.1362	0.4811
SCD(18)	0.116576	0.5547	-0.64684	***0.0002*****	0.49679	***0.0061*****	-0.29045	0.1264
D6D	0.116447	0.5551	-0.64382	***0.0002*****	0.485695	***0.0076*****	-0.33302	0.0776
RBC SCD(18)	0.6456	***0.0038*****	-0.39812	0.0913	-0.05863	0.7626	-0.23377	0.3354
RBCn6/n3	-0.30366	***0.02063****	0.337787	0.1452	-0.13799	0.5619	0.052402	0.8263

Table 9.3 Linear correlation analysis of oxidative stress markers

	TNF-α		IL-6		IL-10	
	r	p	r	p	r	p
Insulin (U/ml)	0.517204	***0.0048****	0.030744	0.8766	-0.25132	0.197
HOMA-IR	0.528488	***0.0038****	0.055902	0.7775	-0.51459	***0.0051****
HOMA-ß	0.469361	***0.0179****	-0.02678	0.8989	-0.21466	0.3028
CD14	0.48156	***0.0368****	0.486826	***0.0345****	-0.05354	0.8277
CD36	0.411339	***0.0297****	0.422374	***0.0252****	-0.37014	0.0526
plasma GSH	-0.4983	***0.0082****	-0.47645	***0.012****	0.280446	0.1565
plasma GSSG	0.331662	0.0847	0.456618	***0.0146****	-0.22054	0.2594
TNF-a	0		0.681836	***0.0001*****	-0.3618	0.0585
IL-6	0.681836	***0.0001*****	0		-0.18695	0.3408
IL-10	-0.3618	0.0585	-0.18695	0.3408	0	
SFA	0.416173	***0.0276****	0.266852	0.1698	-0.15218	0.4395
MUFA	0.497494	***0.0071****	0.225854	0.2478	-0.28448	0.1423
SCD(16)	0.164864	0.4018	0.489081	***0.0083****	0.079681	0.6869
SCD(18)	0.660454	***0.0001*****	0.436234	***0.0203****	-0.28277	0.1448
D6D	0.379605	***0.0463****	0.309855	0.1086	-0.22854	0.2421

Table 9.4: Linear correlation analysis of inflammatory cytokines

	Total cholesterol		HDL cholesterol		LDL cholesterol		TG	
	r	p	r	p	r	p	r	p
Total cholesterol (mmol/L)	0		-0.42297	***0.0313****	0.975705	***0.0001*****	-0.2836	0.1603
HDL cholesterol	-0.42297	***0.0313****	0		-0.61074	***0.0009*****	0.17047	0.4051
LDL cholesterol	0.975705	***0.0001*****	-0.61074	***0.0009*****	0		-0.30234	0.1333
CD11b	0.402119	***0.0463****	-0.27601	0.1817	0.417253	***0.0379****	-0.03603	0.8613
CD36	0.539166	***0.0045****	0.011593	0.9552	0.468508	***0.0158****	0.00311	0.9877
MitoSOX	0.31132	0.1298	-0.10526	0.6166	0.286705	0.1494	0.795424	***0.0001*****

Table9.5: Linear correlation analysis of lipids

	Glucose		Insulin		HOMA-IR		HOMA-B	
	r	p	r	p	r	p	r	p
TG (mmol/L)	0.004643	0.982	0.376165	0.0582	0.39064	***0.0485****	0.357771	0.0727
Glucose			-0.21185	0.2988	-0.00308	0.9881	-0.34957	0.0801
insulin (U/ml)	0.211849	0.2981	0		0.838033	***0.0001*****	0.98909	***0.0001*****
HOMA-IR	0.004643	0.9881	0.838033	***0.0001*****	0		0.935147	***0.0001*****
HOMA-ß	0.349571	0.0801	0.98909	***0.0001*****	0.935147	***0.0001*****	0	
MitoSOX	0.439318	0.028	0.464112	***0.0129****	-0.0908	0.6459	0.126531	0.5468
plasma GSH	0.384057	0.058	-0.40583	***0.0321****	-0.4111	***0.0298****	-0.30619	0.1366
TNF-a	0.089922	0.669	0.517204	***0.0048****	0.528488	***0.0038****	0.469361	***0.0179****
IL-10	0.238244	0.2514	-0.25132	0.197	-0.51459	***0.0051****	-0.21452	0.3028
MUFA	0.142724	0.4868	0.532823	***0.0029****	0.330151	0.0802	0.53066	***0.0053****
SCD(18)	0.458148	0.0186	0.28217	0.1681	0.379473	***0.0423****	0.184878	0.3659
D6D	0.003772	0.9854	0.422729	***0.0223****	0.518845	***0.0039****	0.397115	***0.0446****
RBC MUFA	0.213729	0.2945	0.56356	***0.0015****	0.550727	***0.002****	0.584722	***0.0017****

Table 9.6: Linear correlation analysis of glucose, insulin, HOMA-IR and HOMA-B

9.2 Published article: ‘Palmitate promotes atherogenicity via de novo ceramide synthesis’ Gao D., Pararasa C., Dunston C. R., Bailey C. J., and Griffiths H. R., Free Radic Biol Med 2012, 53(4): 796-806.