ANGLIA RUSKIN UNIVERSITY

FACULTY OF SCIENCE AND TECHNOLOGY

PATHOPHYSIOLOGICAL EFFECTS OF FATTY ACIDS

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ABSTRACT

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Elevated plasma free fatty acid levels have been associated with the development of type II diabetes and cardiovascular diseases, and are thought to be present in the obese state. Studies suggest that cellular exposure to free fatty acids can result in intracellular lipid accumulation and lipotoxicity, leading to cellular dysfunction and cell death. In turn, this can promote a range of systemic consequences.

This project employed a multifactorial approach to better understand the pathophysiological effects of fatty acids. The effects of the two most common circulating fatty acids, palmitic acid and oleic acid, were investigated on hepatocyte, skeletal myocyte and washed platelet models *in vitro*. In addition, a systematic review and meta-analysis was conducted to calculate average circulating concentrations of plasma lipid species. This was to determine whether plasma free fatty acids and other lipid species are in fact elevated in obesity.
In vitro studies determined that palmitic acid induced cell death in both the hepatocyte and skeletal myocyte models, whilst oleic acid caused intracellular lipid accumulation. However, there was no correlation between lipid accumulation and cell death. In platelets, palmitic acid had no effect. In contrast, oleic acid evoked platelet aggregation that was not characteristic of platelet activation. The meta-analysis determined that circulating plasma free fatty acids are higher in obese subjects than in non-obese subjects and higher yet in type II diabetics.

In conclusion, fatty acids exert deleterious effects on a range of cells *in vitro,* and are raised in the obese state. This lends weight to the proposed association between elevated plasma free fatty acids and comorbidities of obesity, further highlighting the importance of future work to understand the mechanisms responsible.

Keywords: free fatty acids, palmitic acid, oleic acid, lipotoxicity

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Abbreviations

AA	arachidonic acid
ACBP	acyl-CoA-binding protein
ADP	adenosine diphosphate
ATCC	American Type Culture Collection
ATGL	adipose triglyceride lipase
ATP	adenosine triphosphate
BAPTA	(1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid)
BMI	body mass index
BSA	bovine serum albumin
CCK-8	cell counting kit 8
CE	cholesterol ester
CHD	coronary heart disease
CMC	critical micellar concentration
CoA	coenzyme A
CPT1	carnitine palmitoyltransferase 1
CPT2	carnitine palmitoyltransferase 2
CRP-XL	collagen-related peptide
CVD	cardiovascular disease
DAG	diacylglycerol
DMEM	Dulbecco's Modified Eagle Medium
DNL	*de novo* lipogenesis
EMCL	extramyocellular lipid
ER	endoplasmic reticulum
FA	fatty acids
FABP	fatty acid-binding protein
FATP	fatty acid transport protein
FFA	free fatty acids
FITC	fluorescein isothiocyanate
GLUT4	glucose transporter 4
Gly-3-P	glycerol-3-phosphate
HCC	hepatocellular carcinoma
HDL	high density lipoprotein
HSL	hormone-sensitive lipase
IDL	intermediate density lipoprotein
IDL	intermediate density lipoprotein
IMCL	intramyocellular lipid
IRS	insulin receptor substrate
JNK	c-jun N-terminal kinase
LD	lipid droplet
LDH	lactate dehydrogenase
LDL	low density lipoprotein
LPA	lysophosphatidic acid
LPL	lipoprotein lipase
LTA	Light transmission aggregometry

MAG	monoacylglycerol
MG	monoacylglycerol
mRNA	messenger ribonucleic acid
MUFA	monounsaturated fatty acid
NAFLD	non-alcoholic fatty liver disease
NEFA	non-esterified fatty acid
OA	oleic acid
PA	palmitic acid
PGE1	prostaglandin E1
PI	propidium iodide
PL	phospholipid
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
SFA	saturated fatty acid
T2DM	type II diabetes mellitus
TAG	triacylglycerol/ triglyceride
TG	triglyceride/triacylglycerol
TPEN	N,N,N′,N′-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine
UPR	unfolded protein response
VLDL	very low density lipoprotein

1 Introduction

The purpose of this project was to investigate the pathophysiological effects of fatty acids as elevated plasma free fatty acid levels, which have been reported in the obese state, have been associated with the development of type II diabetes and cardiovascular diseases. *In vitro* studies were designed to establish the effects of palmitic acid and oleic acid in cell death and lipid accumulation in two cell models: hepatocytes and skeletal myocytes, and activation and aggregation in washed human platelets. A meta-analysis was planned to calculate normal levels of different circulating plasma lipids in the general population and subpopulations for the creation of normal reference ranges. The final analysis aimed to identify associations between plasma lipid species and different subpopulations, especially diseased states and obesity.

Research in this area is vital as obesity is a growing epidemic associated with a plethora of co-morbidities. Currently more than one billion people worldwide are overweight or obese (Aguilera*, et al.*, 2013) with obesity worldwide tripling within the last 30 years. As of 2016, 39% of the world's adults were overweight and 13% were obese (World Health Organization, 2018). This is putting strain on healthcare services across the globe, but especially on state run services such as the NHS.

1.1 The pathology of obesity

Obesity is the accumulation of excess body fat, which may impact health negatively. Obesity is characterised by increased visceral and subcutaneous adiposity, and accumulation of lipids in non-adipose tissue, in a phenomenon called ectopic lipid accumulation (van Herpen & Schrauwen-Hinderling, 2008).

The universally accepted measurement of adiposity is body mass index (BMI), assessing weight relative to height regardless of age or sex. BMI is divided into categories (Table 1.1). As BMI increases above 25 kg/m^2 , the associated health risks increase exponentially, with

risk of developing type II diabetes increasing over 40-fold with a BMI of >35 kg/m^2 (Day & Bailey, 2011).

$$BMI = \frac{Weight\ (kg)}{Height^2\ (m)}$$

Studies on the comorbidities of obesity indicate that abdominal adiposity is a greater risk factor than the overall extent of obesity, due to increased visceral fat (Chang*, et al.*, 2012; Day*, et al.*, 2011; Faria*, et al.*, 2015). Visceral fat stored in the abdominal cavity, is more harmful than subcutaneous fat, stored beneath the skin, in the development of comorbidities of obesity including hypertension, atherosclerosis, hyperlipidaemia and type II diabetes mellitus (Farb & Gokce, 2015). Furthermore, obesity affects not only physical but also psychological wellbeing (Table 1.2) (Ogunbode*, et al.*, 2009), and as such access to clinical psychologists for people struggling with obesity is a fundamental part of Tier 3 weight management services, recommended by NICE and the British Psychological Society. However, this service is tremendously limited within the current NHS provisions (Atter*, et al.*, 2011; NICE, 2014)

Table 1.1 Clinically accepted categories of BMI

	BMI (kg/m^2)
Underweight	<18.5
Healthy	18.5 – 24.9
Overweight	25.0 – 29.9
Obese	30.0 – 39.9
Morbidly obese	≥40

Table 1.2 Risks associated with obesity

Categories of problems	Health risks of obesity
Medical	• Hypertension (Landsberg*, et al.*, 2013) • Cerebrovascular accident, otherwise known as stroke (Kwon*, et al.*, 2016) • Hypertriglyceridaemia and hyperlipidaemia (Taskinen*, et al.*, 2011) • Coronary heart disease (Logue*, et al.*, 2011) • Non-alcoholic fatty liver disease (NAFLD) (Chang*, et al.*, 2016) • Obesity hypoventilation syndrome (Piper & Grunstein, 2011) • Gastro-oesophageal reflux disease (Friedenberg*, et al.*, 2008) • Type II diabetes mellitus (Eckel*, et al.,* 2011; Sharma & Lau, 2013) • Osteoarthritis (Thijssen*, et al.*, 2015) • Gout (Juraschek*, et al.*, 2013) • Cholethiasis, otherwise known as gall stones (Erlinger, 2000) • Obstructive sleep apnoea (Romero-Corral*, et al.*, 2010) • Cancer (De Pergola & Silvestris, 2013) • Cutaneous infections including: cellulitis, folliculitis, candidiasis, impertigo, furunculosis, erythrasma and tinea cruris (Scheinfeld, 2004) • Dermatological issues including, eczema, psoriasis, dermatitis, skin ulcers, acanthosis nigricans,

	hyperandrogenism and hirsutism (Silverberg & Simpson, 2014; Yosipovitch*, et al.*, 2007) • Fluid retention (Sartorio*, et al.*, 2005) • Stretch marks (Cho*, et al.*, 2006) • Low back pain (Shiri*, et al.*, 2010)
Surgical	• Difficult intravenous access (Guss & Bhattacharyya, 2006; Nafiu*, et al.*, 2010) • Altered wound healing (Mitchell & Fantasia, 2016; Pierpont*, et al.*, 2014) • Longer operation time (Silber*, et al.*, 2013) • Increased risk of wound infection (Guss*, et al.*, 2006; Thelwall*, et al.*, 2015) • Greater intraoperative blood loss (Triantafyllopoulos*, et al.*, 2015) • Wound dehiscence (Pierpont*, et al.*, 2014) • Increased risk of deep vein thrombosis (Guss*, et al.*, 2006; Klovaite*, et al.*, 2015)
Reproductive	• Menstrual irregularities (Zhang*, et al.*, 2012) • Reduced fertility in males and females (Katib, 2015) • Polycystic ovary syndrome (Álvarez-Blasco*, et al.*, 2006) • Gestational diabetes mellitus (Chu*, et al.*, 2007) • Gestational hypertension (Gaillard*, et al.*, 2011) • Pre-eclampsia (Roberts*, et al.*, 2011) • Difficult labour with increased risk of maternal and foetal complications (Gupta & Faber, 2011) • Stress incontinence (Mishra*, et al.*, 2008) • Erectile dysfunction (Skrypnik*, et al.*, 2014)

Social and psychological	• Poor self-esteem (Griffiths*, et al.*, 2010; O'Dea, 2006) • Depression and anxiety (Fox*, et al.*, 2016; Gariepy*, et al.*, 2010) • Social disadvantage and discrimination (Cawley, 2004) • Decreased libido (Kolotkin*, et al.*, 2012; Nackers*, et al.*, 2015; Østbye*, et al.*, 2011) • Reduced cognitive function (van den Berg*, et al.*, 2009)

Table adapted from Kelly*, et al.,* (2013) listing possible medical, surgical, reproductive, social and psychological consequences of obesity.

1.2 Mechanisms of obesity

Obesity occurs gradually when a person's total energy intake regularly exceeds their total energy expenditure causing an energy imbalance. The chronic state of surplus energy in obesity is thought to saturate the normal triacylglycerol storage capacity of adipose tissue by increasing fatty acid uptake (Kusminski*, et al.*, 2009). This is initially contained by hypertrophy and hyperplasia of adipocytes within the adipose tissue (Kusminski*, et al.*, 2009). However, when adipose tissue can no longer accommodate excess fatty acids and lipid products, the adipose tissue goes into a state of dysfunction and surplus is deposited in non-adipose tissue, such as the liver and skeletal muscle, and named ectopic fat (Blüher, 2009; Blüher, 2013; Kusminski*, et al.*, 2009). Ectopic fat is defined as occurring when more than 5% of an organ's volume is made of fat or the presence of lipid droplets exceeds 5% of the organ's cells (Snel*, et al.*, 2012). The accumulation of ectopic fat and the dysfunction of the adipose tissue are thought to cause pathological responses associated with a number of sequelae (Carobbio*, et al.*, 2011)

As shown in Table 1.2, obesity has been linked to a number of potentially fatal conditions such as type II diabetes mellitus (T2DM) (Hu*, et al.*, 2001), cardiovascular diseases (CVD) (Ortega*, et al.*, 2016) and hypertension (Jiang*, et al.*, 2016; Re, 2009). Despite the consensus about the positive correlation between obesity and these conditions, the

association between them has yet to be fully understood. Studies have identified links between obesity and these comorbidities involving altered fatty acid metabolism (Abel, 2010; Blaak, 2003; Fukushima & Lopaschuk, 2016), insulin resistance (Kahn & Flier, 2000; Ye, 2013), proinflammatory cytokines (interleukin-6 and tumor necrosis factor) (Redinger, 2007; Sulistyoningrum*, et al.*, 2017; Yudkin*, et al.*, 2000), endoplasmic reticulum stress (Cnop*, et al.*, 2012) and mitochondrial dysfunction (Højlund*, et al.*, 2008; Parish & Petersen, 2005). However, these interactions are complex and their importance vaguely defined (Eckel*, et al.*, 2011).

1.2.1 Obesity and type II diabetes

Type II diabetes mellitus (T2DM) is a chronic metabolic disorder characterised by hyperglycaemia (Olokoba*, et al.*, 2012). T2DM develops as a result of insulin resistance in peripheral tissues including muscle, liver, and adipose, followed by impaired insulin secretion by the pancreatic β-cells (Pratley, 2013). Insulin resistance and decreased circulating insulin leads to decreased glucose uptake into cells and, thus, elevated blood glucose levels. This hyperglycaemia can cause serious chronic sequelae including cardiovascular disease, diabetic retinopathy, neuropathy and diabetic nephropathy, in addition to acute sequelae that can lead to a diabetic coma and death (Zimmet*, et al.*, 2001). The aetiology of T2DM is complex. Several risk factors that play a role in the pathophysiology of T2DM have been recognised, including gender, ethnicity, age, history of smoking, generalised obesity and central obesity, low fibre and high saturated fat diets, family history, low physical activity, dyslipidaemia, elevated blood pressure and use of certain drug treatments including diuretics (Lyssenko & Laakso, 2013). Whilst evidence suggests genetics have a strong influence over the development of type II diabetes with studies of monozygotic twins exhibiting ~70% concordance for T2DM as opposed to ~25% for dizygotic twins (Barnett*, et al.*, 1981; Kaprio*, et al.*, 1992; Newman*, et al.*, 1987). In addition to a 3.5-fold greater risk of developing type II diabetes than the general population, if one parent has the disorder (Groop*, et al.*, 1996; Köbberling, 1982; Scott*, et al.*, 2007)

with inheritance more commonly from the mother (Cox, 1994; Groop*, et al.*, 1996). Much evidence supports a causal relationship between obesity and the development of T2DM, including molecular (Kadowaki*, et al.*, 2003), epidemiological (Chan*, et al.*, 1994), genetical (McCarthy, 2010; Morris*, et al.*, 2012) and clinical studies (Dixon*, et al.*, 2008; Lim*, et al.*, 2011). Furthermore, Type II diabetes is potentially reversible by implementing a substantial negative energy balance by either dietary intervention or bariatric surgery (Taylor, 2013), with one study finding that bariatric surgery reversed type II diabetes in 73% of patients (Ferrannini & Mingrone, 2009). Following bariatric surgery, daily calorie intake is dramatically reduced thereby diminishing pressure on lipid metabolism pathways, which in turn profoundly alters intracellular concentrations of lipid metabolites, including those found to be toxic.

Three distinct mechanisms have been offered to connect obesity to insulin resistance and predisposition to T2DM. *1*) mitochondrial dysfunction, due to decreased mitochondrial biogenesis and/or reduced functional capacity (Bournat & Brown, 2010; Højlund*, et al.*, 2008). 2) increased production of adipokines/cytokines, including retinol-binding protein 4 tumor necrosis factor-α and interleukin-6, that contribute to insulin resistance (Deng & Scherer, 2010). 3) ectopic fat accumulation, particularly in the liver and skeletal muscle, leading to dysmetabolic sequelae (Cusi, 2010; Eckel*, et al.*, 2011; Levelt*, et al.*, 2016)

Decreases in mitochondrial oxidative activity and mitochondrial adenosine triphosphate (ATP) synthesis were found in subjects with severely insulin resistant muscle. This finding supports the theory that insulin resistance can arise from defects in mitochondrial fatty acid oxidation. These defects likely increase intracellular fatty acid metabolites such as fatty acyl CoA and diacylglyerol, thereby disrupting insulin signalling (Lowell, 2005).

Other papers have published the presence of low-grade chronic inflammation in obesity (Koca, 2017) with the adipose tissue secreting higher levels of proinflammatory cytokines such as interleukin 6 and tumor necrosis factor-alpha (Greenberg & Obin, 2006; Hotamisligil*, et al.*, 1995; Monteiro & Azevedo, 2010). These cytokines are potential

mediators that link obesity-derived low-grade chronic inflammation with insulin resistance (Kim*, et al.*, 2009).

1.2.2 Obesity and cardiovascular diseases

Obesity can cause physical dysfunction of the heart and vasculature *via* lipid accumulation. For example, obesity can lead to an almost 2-fold increase in the fat pads around the base of ventricles (Montani*, et al.*, 2004), which causes diastolic and systolic dysfunction (Christoffersen*, et al.*, 2003; Szczepaniak*, et al.*, 2003), and deposition of fat along major blood vessels, which can lead to alterations in blood pressure (Dwyer*, et al.*, 1995). However, the main links between obesity and CVD are thought to encompass many of the same mechanisms as with obesity and T2DM, sharing similar chronic inflammatory processes and oxidative injury (Vincent*, et al.*, 2001; Witztum & Lichtman, 2014), however, these associations are less clear. In fact, the question of whether obesity is an individual risk factor for coronary heart disease (CHD) is currently under debate. This does not suggest that obesity is not a causative risk factor for CHD. It is agreed that in the least obesity is a contributing cause of directly atherogenic risk factors, so must be in the chain of causality somewhere. Moreover, the induction of several major risk factors by obesity may actually make it a more significant in the development of atherosclerotic disease than if it were an individual risk factor (Grundy, 2002).

Research indicates that the relationship between obesity and CVD is mediated through major risk factors including total cholesterol (Xu*, et al.*, 2007), HDL (Mahdy Ali*, et al.*, 2012; Wilkins*, et al.*, 2014), hypertension (Artham*, et al.*, 2009; Montani*, et al.*, 2004), and T2DM (Hanley*, et al.*, 2002; Wilson*, et al.*, 1998; Xun*, et al.*, 2012), all of which are affected by obesity (Grundy, 2002; Wilson*, et al.*, 1998). In spite of this, obesity is thought to play a more active role in the development of atherosclerosis, which encompasses complex interactions between serum lipids, platelets inflammatory cells and the vascular endothelium (Williams*, et al.*, 2002). Atherosclerosis is initiated by endothelial cell dysfunction, which is induced by a reduction in the bioavailability of nitric oxide (Ross,

1999). Obesity has been found to reduce the biosynthesis of nitric oxide in endothelial cells causing dysfunction (Joost & Tschöp, 2007; Toda & Okamura, 2013), vasoconstriction and reduced inhibition of thrombocyte aggregation (Joost*, et al.*, 2007; Leite*, et al.*, 2016; Vignini*, et al.*, 2008). Furthermore, impaired sensitivity of platelets to inhibition by nitric oxide has been found in obesity, all of which combine to increase the risk of atherosclerosis (Russo*, et al.*, 2010).

1.3 The physiological role of platelets

Platelets are the smallest cells of the body and yet vital for the maintenance of the cardiovascular system, especially following vascular injury. In normal conditions, platelet function is regulated by three types of signal; inhibitory signals which encourage platelet quiescence in healthy vasculature, activatory signals that trigger adhesion and aggregation at sites of vascular injury, and negative regulatory signals that provide feedback in order to control thrombus formation following platelet activation (Bye*, et al.*, 2016).

Platelets, otherwise known as thrombocytes, are activated when they come into contact with a damaged blood vessel wall. The process of activation involves the reorganisation of the cytoskeleton to facilitate rapid shape change, granule release to promote activation through soluble agonists, and integrin activation to enable platelet-platelet and platelet-endothelium interactions. However, this is a process that requires strict regulation in order to prevent dysfunction resulting in mortalities from haemorrhagic or thrombotic pathologies. Therefore, understanding the causes and mechanisms of platelet activation is instrumental in the fight against cardiovascular disease (CVD). Platelet signalling and their role in haemostasis will be discussed in more detail in this chapter.

1.3.1 Platelet formation

Platelets are formed from megakaryocytes (MKs), derived from haematopoietic stem cells of the myeloid lineage. As megakaryocytes differentiate they lose their ability to proliferate, whereby the normal mitotic cycle is arrested at anaphase B and re-enters in G1 phase in a process called endomitosis (Geddis, 2010; Patel*, et al.*, 2005). Multiple cycles of endomitosis occur causing the replication of chromosomes and the beginnings of cleavage furrow formation without telophase and cytokinesis (Geddis, 2010; Patel*, et al.*, 2005), resulting in the production of megakaryocytes each with a multilobulated, polyploid nucleus containing up to 512N chromosomes (Malherbe*, et al.*, 2016). Differentiated megakaryocytes then undergo maturation involving the development on an extensive membrane system known as the demarcation membrane system (DMS) (Eckly*, et al.*, 2014; Kautz & Demarsh, 1954). The DMS divides the cytoplasm of the megakaryocyte into membrane-delineated platelet territories that ultimately forms the cell membrane of the platelets produced (Eckly*, et al.*, 2014; Kautz*, et al.*, 1954).

Two hypotheses exist to explain platelet formation from mature megakaryocytes; the fragment model and the proplatelet model (Geddis, 2010). The fragment model suggests that mature megakaryocytes leave the bone marrow and travel to the lungs to be fragmented into platelets in the microvasculature, which has been found in infants (Sharnoff & Scardino, 1960). The proplatelet model asserts that within the bone marrow, megakaryocytes form long branching processes called proplatelets that extend into sinusoids of the bone marrow and shed platelets into the vasculature (Machlus & Italiano, 2013). The proplatelet model has more support including live cell microscopy showing elaborate proplatelet networks extending from megakaryocytes in culture (Italiano*, et al.*, 1999) and in vivo imaging of fluorescently-labelled megakaryocytes releasing particles, presumed to be platelets, into sinusoids of the marrow vasculature (Junt*, et al.*, 2007).

The platelet formation process takes approximately 10 days and leads to the release of approximately 4000 platelets per megakaryocyte, and is an important step in understanding the structure and function of platelets (Hoffbrand & Moss, 2015).

1.3.2 Platelet structure

Platelets are anuclear, biconvex discoid cells with an average diameter of 2.5 µm. They contain two types of granules α-granules and dense granules that store clotting factors and mediators to platelet activation, respectively. These granules are released *via* the canicular system upon platelet activation. This system also serves as a pathway of transport into the platelet (Figure 1.10). Inactivated platelets circulate in a biconvex discoid shape, but upon activation undergo rapid morphological changes by extending pseudopodia which increase surface area and aid in the formation of a stable platelet plug (Figure 1.11).

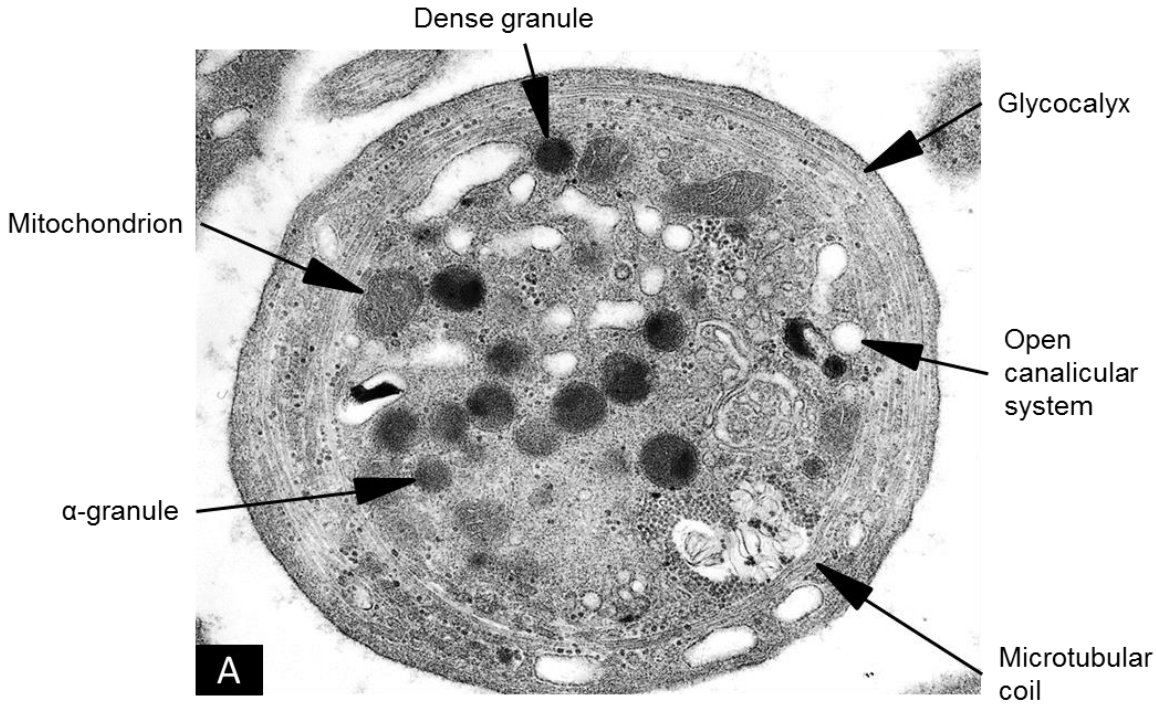


Figure 1.1 Scanning electron microscopy image of a platelet

The image demonstrates the structure and organelles of a platelet. The platelet's phospholipid bilayer is encased in an outer membrane called the glycocalyx. The structure of the platelet is maintained by the cytoskeleton consisting of microtubular coils and actin filaments. The cytoplasm contains numerous components required for their role in thrombosis such as α-and dense granules. Adapted from (White, 2007).

Table 1.3 Components of platelet granules

α-Granules	Dense Granules	Lysosomes
Albumin	Serotonin	Cathepsin D
Fibrinogen	ATP	Cathepsin E
Fibronectin	ADP	Carboxypeptidase A
Vitronectin	Calcium	Carboxypeptidase B
Osteonectrin	Pyrophosphate	Proline Carboxypeptidase
Von Willebrand Factor		β-N-acetyl-D-hexosaminidase
Von Willebrand antigen II		β-D-Glucuronidase
Thrombospondin		β-D-Galactosidase
Platelet Factor 4		α-D-Mannosidase
IgG, IgA, IgM		α-L-Arabinofuranosidase
C1 Inhibitor		α-D-Galactosidase
Plasminogen		α-L-Fucosidase
Plasminogen Activator Inhibitor-1		β-D-Fucosidase
Platelet-Derived Collagenase Inhibitor		β-D-Glucosidase
High Molecular Weight Kininogen		α-D-Glucosidase
Protein S		Acid Phosphatase
α2-antitrypsin		Arylsulphatase
α2-macroglobulin		
α2-antiplasmin		
Multimerin		
Platelet Basic Protein		
β-thromboglobulin		
Histidine-Rich Glycoprotein		
Connective Tissue-Activation Protein III		
Neutrophil-Activation Protein II		
Platelet-Derived Growth Factor		
Transforming Growth Factor β		
Vascular Endothelial Cell Growth Factor		
Coagulation Factor V		
Coagulation Factor VIII		

A table listing the contents of α-granules, dense granules and lysosomes of platelets. Taken from (McNicol & Israels, 1999).

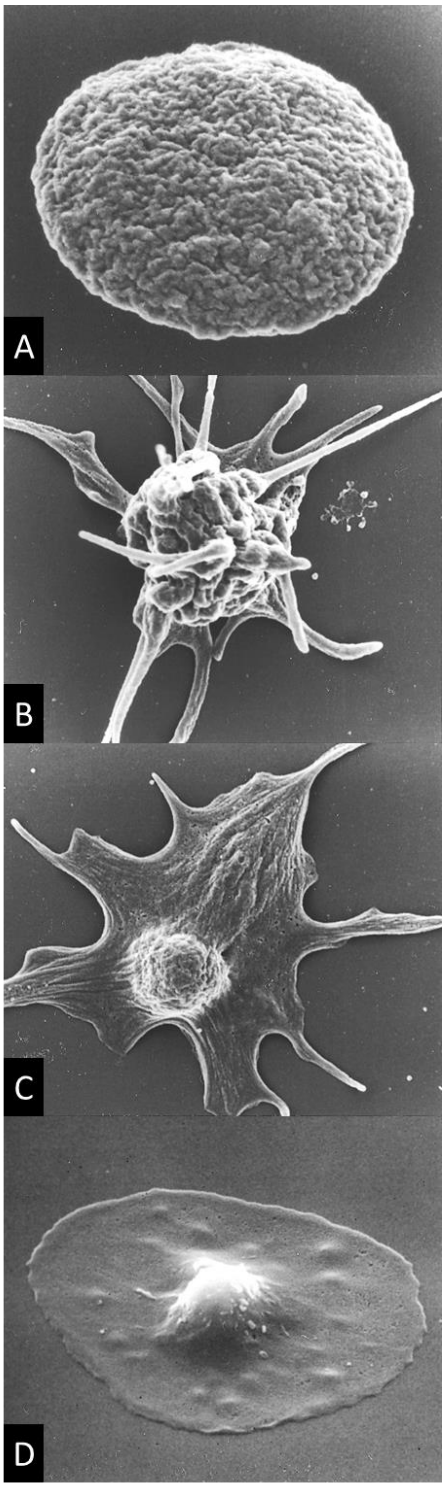


Figure 1.2 Platelet shape change

Scanning electron microscope images of platelets. (A) An unstimulated platelet with the normal round discoid morphology with the surface having a convoluted appearance. (B) Protrusions called “pseudopodia” burst from the surface of the platelet during early shape change triggered by activation. (C) Lamellipodia spread between the pseudopodia after adherence to a surface. (D) The platelet achieves a fully spread morphology approximately 20 minutes after adhering to a surface, thereby increasing surface area (Bearer*, et al.*, 2002). Adapted from (White, 2007).

1.3.3 Formation of the platelet plug

Exposure of vWF in the sub-endothelium transiently tethers platelets through interactions with the platelet membrane receptor glycoprotein (GP) complex GPIb-V-IX (Gibbins, 2004) However, vWF-dependant interactions are insufficiently stable to support thrombus formation and are superseded by the binding of collagen *via* integrin $\alpha_2\beta_1$ and GPVI receptors on the platelet surface (Clemetson, 2012). Ca^{2+} release, degranulation and shape change are triggered downstream of GPVI, which in turn amplifies platelet activation (Nieswandt*, et al.*, 2001). Platelets contain two types of granule with distinct cargos and function (Table 1.4). Alpha (α)-granules contain a plethora of proteins that comprise the majority of the platelet secretome, whilst dense granules mainly contain small molecules (Whiteheart, 2011). α-granules contain coagulation factors, angiogenic factors, anti-angiogenic factors, proteases, growth factors, necrotic factors, other cytokines, fibrinogen and adhesion molecules (Blair & Flaumenhaft, 2009; Whiteheart, 2011). Dense granules contain serotonin, ATP, ADP and Ca^{2+} (King & Reed, 2002; Whiteheart, 2011). ADP-induced platelet activation stimulates the synthesis and release of the secondary agonist TxA_2 (Paul*, et al.*, 1999). ATP and ADP are also secondary mediators of aggregation that perpetuate platelet activation and, thus, aggregation (Fung*, et al.*, 2007). Shape change is caused by the reorganisation of the actin cytoskeleton following stimulation by an agonist. The platelets lose their discoid shape, increasing surface area and facilitate the recruitment of passing platelets to the forming thrombus by becoming rounded and extending pseudopodia out (Paul*, et al.*, 1999). With interactions between GPIb-V-IX and vWF being too weak to mediate platelet adhesion, additional, more robust interactions between collagen and $\alpha_5\beta_1$, $\alpha_2\beta_1$ and $\alpha_6\beta_1$ receptors and collagen, laminin and fibronectin are formed (Gibbins, 2004; Grüner*, et al.*, 2003; Inoue*, et al.*, 2006; Maurer*, et al.*, 2016). *In vivo* interactions between platelets and multiple endothelial matrix proteins were shown to be necessary to form a stable foundation for subsequent thrombus formation (Kuijpers*, et al.*, 2007; Schaff*, et al.*, 2013). Intraplatelet adhesion is then facilitated by the binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$ (Bennett, 1996). Furthermore, phosphatidylserine (PS) is exposed on the surface of a subset of platelets, which provides the membrane surface for

thrombin formation (Munnix*, et al.*, 2007). The thrombin generated activates other platelets and coagulation factors, and generates an insoluble mesh by cleaving fibrinogen to fibrin, thereby further stabilising the thrombus (Binnie & Lord, 1993).

1.3.4 Thrombosis and Haemostasis

Damage to the sub-endothelium of a blood vessel exposes matrix proteins which upon contact with platelets cause them to activate and undergo dynamic shape change (Figure 1.11), adherence and aggregation to prevent blood loss, in a process called haemostasis. However, thrombosis, the dysregulation of this process can restrict blood flow to vital organs such as the heart and brain, and can be fatal. A reduction in blood flow causes decreased oxygenation of these vital organs causing ischaemia. Severe reduction to the heart and brain cause anoxia and ultimately myocardial infarction and cerebrovascular accident (stroke), respectively. Diseases associated with thrombosis are the leading cause of cardiovascular-related mortality globally (ISTH Steering Committee for World Thrombosis Day, 2014).

Major haemorrhage resultant from traumatic injury or a medical condition is fatal in the absence of an adequate haemostatic response. However, dysregulated haemostasis through excessive stimulation or insufficient inhibition can be equally life threatening. In a balanced haemostatic state, the endothelial cells of vasculature express heparin proteoglycans (de Agostini*, et al.*, 1990) and thrombomodulin (Esmon & Esmon, 2011), and release nitric oxide and prostacyclin (PGI_2) to maintain platelet quiescence thereby preventing activation of the coagulation cascade (Gryglewski*, et al.*, 2001). Haemostasis is instigated *via* exposure to sub-endothelial matrix proteins such as collagen (Roberts*, et al.*, 2004), exogenous agonists such as ADP and ATP (Born & Cross, 1964) or by mechanical deformation from shear stress (Goncalves*, et al.*, 2005), all of which promote procoagulant activity and thrombus formation. However, a fine balance between activation and inhibition exists as excessive stimulation or insufficient inhibition of these processes can lead to occlusion and may end in myocardial infarction, cerebrovascular and pulmonary embolism.

Injury to the vessel endothelium exposes von Willebrand factor (vWF) and matrix proteins such as collagen, laminin and fibronectin which bind to platelets and initiate activation pathways (Gibbins, 2004; Grüner*, et al.*, 2003; Schaff*, et al.*, 2013). Platelet activation is further induced in response to thrombin, ADP and thromboxane A_2 (TxA_2) that are produced or secreted by platelets. These inside-out signalling pathways lead to activation of integrin $\alpha_{IIb}\beta_3$ receptors, which bind to fibrinogen forming bridges to cross-link platelets, developing a stable platelet plug (Ye*, et al.*, 2011).

In cases of dysfunction, such as in the rupture of lipid-rich atherosclerotic plaques, the formation of thrombi may lead to myocardial infarction and/ or ischemic stroke.

1.3.5 Atherosclerotic plaques

Atherosclerosis is a potentially serious condition in which arteries are narrowed due to the formation of lipid-rich plaques (Bentzon, et al., 2014). These plaques can be caused by an increased concentration of low-density lipoproteins (LDL) in the blood, or they may develop at lower concentrations of LDL in combination with other risk factors such as hypertension and smoking (Bentzon, et al.,2014; Riccioni, et al., 2003).

The LDL accumulates in the intima where it is oxidised. Monocytes-macrophages are recruited to the area where macrophage scavenger receptors uptake the oxidised LDL and transform into foam cells. A fibrous cap containing smooth muscle cells is then formed over the plaque to stabilise it. Each stage of the process is also mediated by inflammatory cytokines (Tegui & Mallat, 1999).

Atherosclerosis and obesity share common pathophysiological features with lipids contributing critically to both conditions. Both oxidized LDL associated with atherosclerotic disease and free fatty acids associated with obesity can trigger inflammation and initiate disease (Rocha & Libby, 2009).

1.4 Lipids

1.4.1 Triacylglycerol digestion and transport

The most common form of lipid in the human diet is triacylglycerol (TAG), a 3C alcohol with three fatty acids conjugated to glycerol (Figure 1.5), which is insoluble in the aqueous environment of the gastrointestinal tract and must be solubilised to be absorbed.

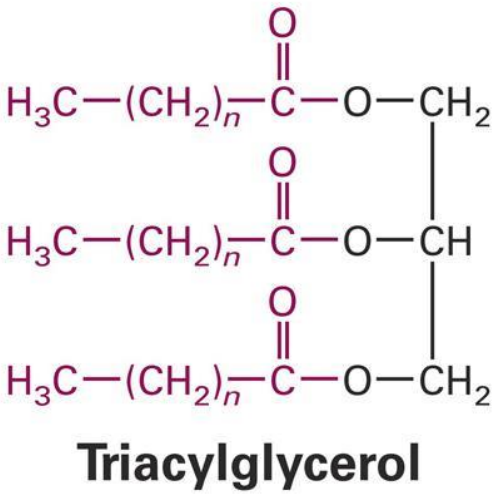


Figure 1.3 Generic triacylglycerol structure
Glycerol backbone depicted in black with three generic fatty acids in mauve attached to hydroxyl groups. These fatty acids vary, but all three are usually different and contain 16, 18 or 20 carbons.

The absorption process starts in the stomach, where mechanical digestion exposes lipids to hydrolysis by gastric lipase which breaks down up to 20% of the fatty acids (Lowe, 2002). Expulsion of chyme from the stomach into the duodenum follows, where bile acids from the gall bladder, are released to emulsify the lipid components (Favé*, et al.*, 2004). Emulsion is achieved through hydrophobic domains of bile acids intercalating into the lipids, which forms a complex oil interface with the aqueous environment that achieves maximal lipase

activity (Lowe, 2002). This oil interface is fluid in its composition and physical properties, and continuously changes as lipolysis proceeds, increasing the surface area by breaking down large aggregates into smaller droplets. The increase in surface area allows greater accessibility of pancreatic lipase and pancreatic phospholipase A2 (PLA2) to degrade dietary triacylglycerols into their acylglycerol and free fatty acid components (Mun*, et al.*, 2007). The products then enter enterocytes lining the intestine *via* simple diffusion or facilitated by fatty acid binding protein (FABP) (Favé*, et al.*, 2004). Within the endoplasmic reticulum (ER) of enterocytes triacylglycerols are reformed and passed through the Golgi apparatus where they are packaged with fatty acids, cholesterol and lipoproteins to form chylomicrons (Lambert*, et al.*, 2001). Chylomicrons are exuded from the Golgi and exocytosed in vesicles into the lymphatic system, from which they flow into the systemic circulation (Figure 1.5). Nascent chylomicrons consist of 85% TAG and some cholesterol, with apolipoptein B-48 as the main apolipoprotein (Table 1.3). As chylomicrons circulate, they exchange components with high-density lipoproteins (HDL). As the chylomicrons circulate, components are delivered to various parts of the body, and the remnant is then returned to the liver for endocytosis and breakdown.

VLDL are synthesised in the liver, and their function is to deliver TAG to the body. As they circulate, VLDL particles are stripped of TAG and become less dense, thus, transforming into LDL (Table 1.3). The function of LDL (so called ‘bad’ cholesterol) is to deliver cholesterol to the cells, which is required for plasma membrane function or synthesis into steroid hormones. Cells take up cholesterol *via* receptor-mediated endocytosis. HDL (so called ‘good’ cholesterol) is involved in reverse cholesterol transport, and the function of the liver is to remove LDL from the circulation. Familial hypercholesterolaemia, a dominant gene disorder resulting in early heart attack and stroke, results from a defect in the LDL receptor.

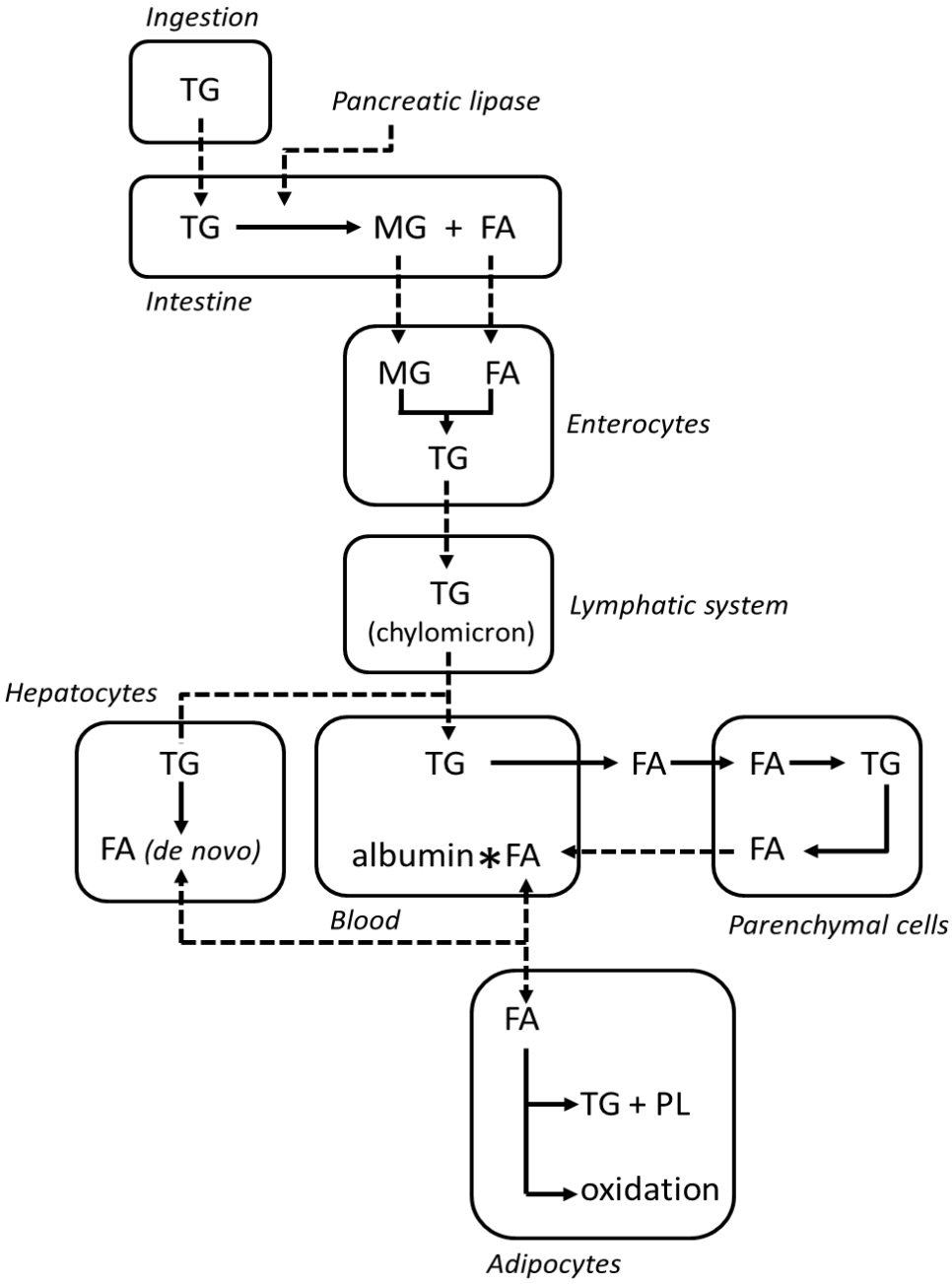


Figure 1.4 Uptake, transport and distribution of lipids in the body

In the intestine TG is broken down by lipase to produce MG and FA, which are absorbed by enterocytes and re-esterified to TG. This reformed TG is packaged into chylomicrons and released into the lymph system then the circulation. The TG is taken up by hepatocytes and metabolised into FA, which is released into the circulation and bound to albumin. Simultaneously, lipoprotein lipases within blood vessels metabolise TG to FA. This FA is delivered to parenchymal cells for storage and use, and to adipocytes for storage.

(abbreviations: TG – triacylglyceride, MG – monoacylglyceride, FA – fatty acids, PL - phospholipid)

Table 1.4 Lipoproteins: their composition and associated apolipoproteins

	Chylomicron	VLDL	IDL	LDL	HDL
Density (g/mL)	<0.950	0.950-1.006	1.006-1.019	1.019-1.063	1.063-1.090
Diameter (nm)	80-1000	30-80	25-30	20-22	9-15
Apolipoproteins	B48, A1, A2, C, E	B100, C, E	B100, C, E	B100	A1, A2, C, E
Protein (%)	~1	~10	~18	~20	~50
Triglyceride (%)	~90	~65	~34	~10	~20
Cholesterol (%)	~5	~13	~22	~45	~18
Phospholipids (%)	~4	~13	~22	~23	~30

Adults consume approximately 70g of fat per day, most of this in the form of triacylglycerol (TAG). TAG is digested in the gastrointestinal tract to release free fatty acids (FFA), diacylglycerol (DAG) and monoacylglycerol (MAG), which are then absorbed in the small intestine. FFAs are re-esterified to triacylglycerols in the intestinal epithelial cells, which are transported in chylomicrons *via* the lymph system and into the circulation (Figure 1.5). Due to their low solubility, FFAs are bound to albumin in order to be transported in the circulation, from where they are taken up by cells *via* protein transporters in the cell membrane. Once inside the cell, FFAs are transported *via* fatty acid-binding proteins (FABP) to the endoplasmic reticulum (ER) and the outer mitochondrial membrane for activation with CoA to form fatty acyl-CoA. Afterwards they are transported to the mitochondria, peroxisomes, and ER *via* acyl-CoA-binding protein (ACBP). In the mitochondria and peroxisomes they are broken down *via* β-oxidation to produce ATP, whilst in the ER they are esterified to different lipid species.

1.5 Fatty Acids

Fatty acids (FAs) are energy-rich long hydrocarbon chain molecules that exist both in a free state and as part of complex lipids, usually forming either triglycerides (three fatty acids + glycerol) or phospholipids (two fatty acids plus glycerol plus phosphate and a changed head group). They play vital roles in metabolism, are essential components of cell membranes, and are important in signal transduction and gene regulation (Rustan & Drevon, 2005; van der Vusse, 2009).

Fatty acids are defined as carboxylic acids with a long unbranched aliphatic chain and share a common structure with diversity based on chain length and degree of saturation (Duplus*, et al.*, 2000). The general formula for fatty acids is $CH_3(CH_2)_nCOOH$, where n is the chain length (Figure 1.1), although the chain length of biological fatty acids is usually an even number between 4 and 28. Fatty acids are synthesised from 2C acetyl CoA, thus, the even number of carbons. Fatty acids have trivial names, as well as using systematic nomenclature. FAs over 16 carbons in length can be either saturated, monounsaturated (MUFA) or polyunsaturated (PUFA) depending upon the presence of none, one or more double bonds respectively, in the carbon chain (Duplus*, et al.*, 2000).

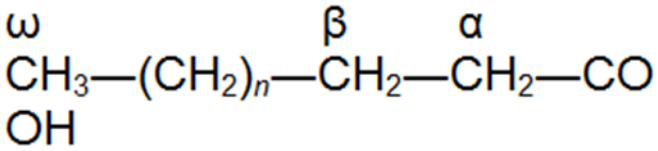


Figure 1.5 Nomenclature for fatty acids

Fatty acids are commonly named by systematic or colloquial nomenclature. The systematic approach is to describe fatty acids in relation to the methyl (ω) end. It is from this end that the position of double bonds are described the position of double bonds from the end of the fatty acid. The letter *n* may also be used to denote the position of the double bond.

Fatty acids have a low solubility in water in their undissociated (acidic) form. Their solubility is dramatically influenced by temperature and pH, and they have a strong tendency to associate in lamellar structures and micelles thereby reducing their bioavailability. The formation of micelles occurs over a limited range of concentrations and conditions with the point of association known as the critical micellar concentration (CMC). The CMC is not a fixed value, but rather represents a concentration range at which the conditions of a solution strongly affect the lipids and cause them to exhibit a tendency to associate rather than remain as single molecules (Rustan*, et al.*, 2005) (Figure 1.2). Therefore, in the circulation FFAs are bound to the transporter protein albumin to increase solubility.

The unbound FFA at pH 7.4 in the final concentrations used in this study may form lamellar fatty acid/soap or crystalline 1:1 acid-soap aggregates, but not micelles, as fatty acids do not form micelles in solutions below pH 9 (Figure 1.2) (Cistola*, et al.*, 1988). Furthermore, the melting point for palmitic acid is 62.20°c and oleic acid is 12.82°c, which further support this assumption (Figure 1.2) (Knothe & Dunn, 2009). A ratio of FFA:BSA greater than a 7:1 also increases the chances of unbound fatty acid crystallising (Cistola*, et al.*, 1987). All stock solutions contained 1% w/v BSA, which equates to approximately 151 μM, therefore, the upper limit of FFA concentration before crystallisation was 1057 μM. Thus, concentrations used in this study were below this threshold.

The structure of a fatty acid plays a significant role in the manifestation of its physical properties. For example, fatty acids with odd numbers of carbon atoms have higher melting points, whilst monounsaturated fatty acids with a cis double bond have lower melting points due to their inability to pack closely. The complete saturation of saturated fatty acids makes them very stable, whereas unsaturated fatty acids are more susceptible to oxidation due to instability caused by their double bonds. The more double bonds, the greater their instability (Cistola*, et al.*, 1988).

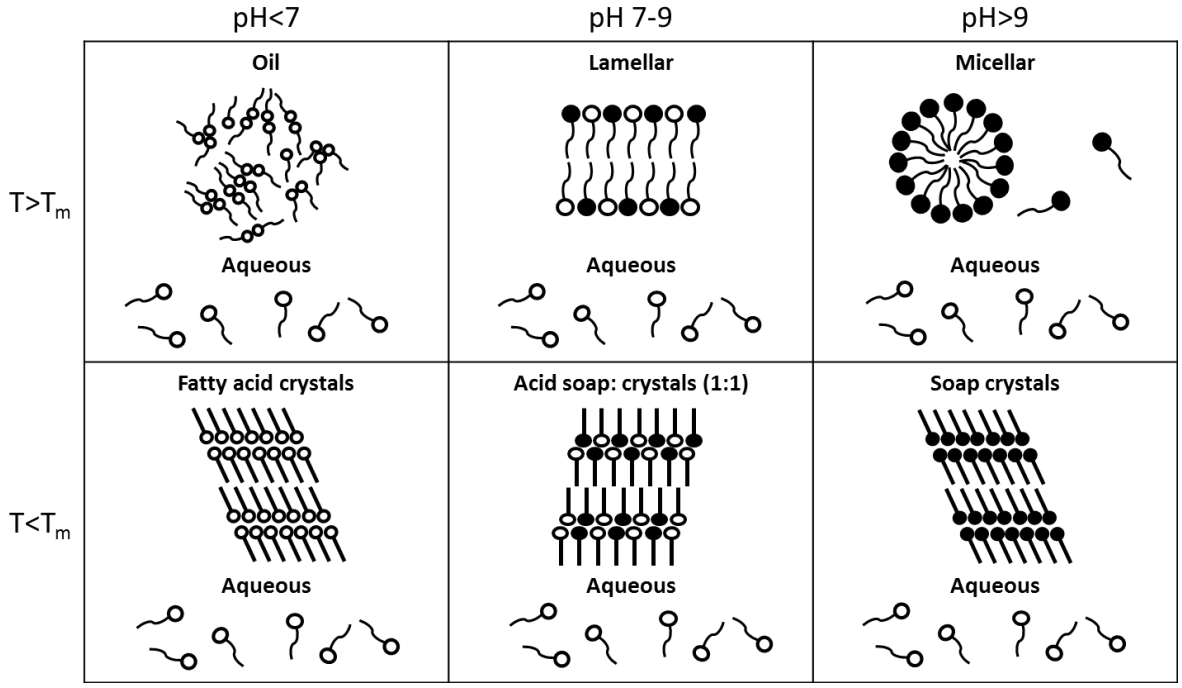


Figure 1.6 Formation of fatty acids in aqueous solutions

The aqueous phase is a saturated solution of fatty acid, acid-soap, or soap, and the concentration of these molecules varies with ionization state and hydrocarbon chain length. The closed circles represent ionized (anionic) carboxylate groups and the open circles protonated carboxyl groups. The straight lines represent ordered hydrocarbon chains and the curved lines disordered (liquid) hydrocarbon chains.

1.5.1 Saturated fatty acids

Saturated fatty acids (SFAs) derive their name from the fact that all of their carbon atoms are fully saturated with hydrogen. Most SFA are linear hydrocarbon chains with an even number of carbons, with the most abundant containing between 12-22 carbon atoms (Rustan*, et al.*, 2005).

1.5.2 Unsaturated fatty acids

Unsaturated fatty acids are not fully saturated with carbon atoms due to the presence of one or more double bonds. Monounsaturated fatty acids (MUFAs) have one carbon-carbon double bond, which can occur at different carbon positions and produce different configurations, namely cis and trans isomers. In cis isomers the carbon atoms are present

on the same side of the double bond, whilst in trans isomers the carbon atoms are on opposite sides of the double bond (Figure 1.2). The presence of a double bond not only alters the structure and stability of fatty acids, but also has important physiological implications. The double bond in cis isomers causes a kink in the molecular shape and reduces the thermodynamic stability, this kink reduces the ability of cis fatty acids to pack tightly thereby causing lower melting points than their saturated or trans unsaturated counterparts (Rustan*, et al.*, 2005). The most common monounsaturated fatty acids are between 16-22 carbons in length and exist in the cis configuration. If the double bond is present between the seventh and eighth carbon atoms, these are called ω-7 fatty acids, and if the double bond is between the ninth and tenth carbon atoms, these are called ω-9 fatty acid. Trans fatty acids are less common as they are produced *via* the industrial process of hydrogenation or by biohydrogenation in the gastrointestinal tract of ruminants (Iqbal, 2014). Furthermore, trans monounsaturated fatty acids have similar effects in organisms as saturated fatty acids due to their similarity in shape (Doyle, 1997).

Polyunsaturated fatty acids (PUFAs) have two or more double bonds and are categorised based on the position of the first double bond. If the first double bond is present between the third and fourth carbons, these are called ω-3 fatty acids, and if the first double bond exists between the sixth and seventh carbon atoms, these are called ω-6 fatty acids. PUFAs are produced by plants and are metabolised in the human body by the addition of carbons, and the extraction of hydrogen in a process called desaturation. Fatty acids are also broken down by β-oxidation in the mitochondria.

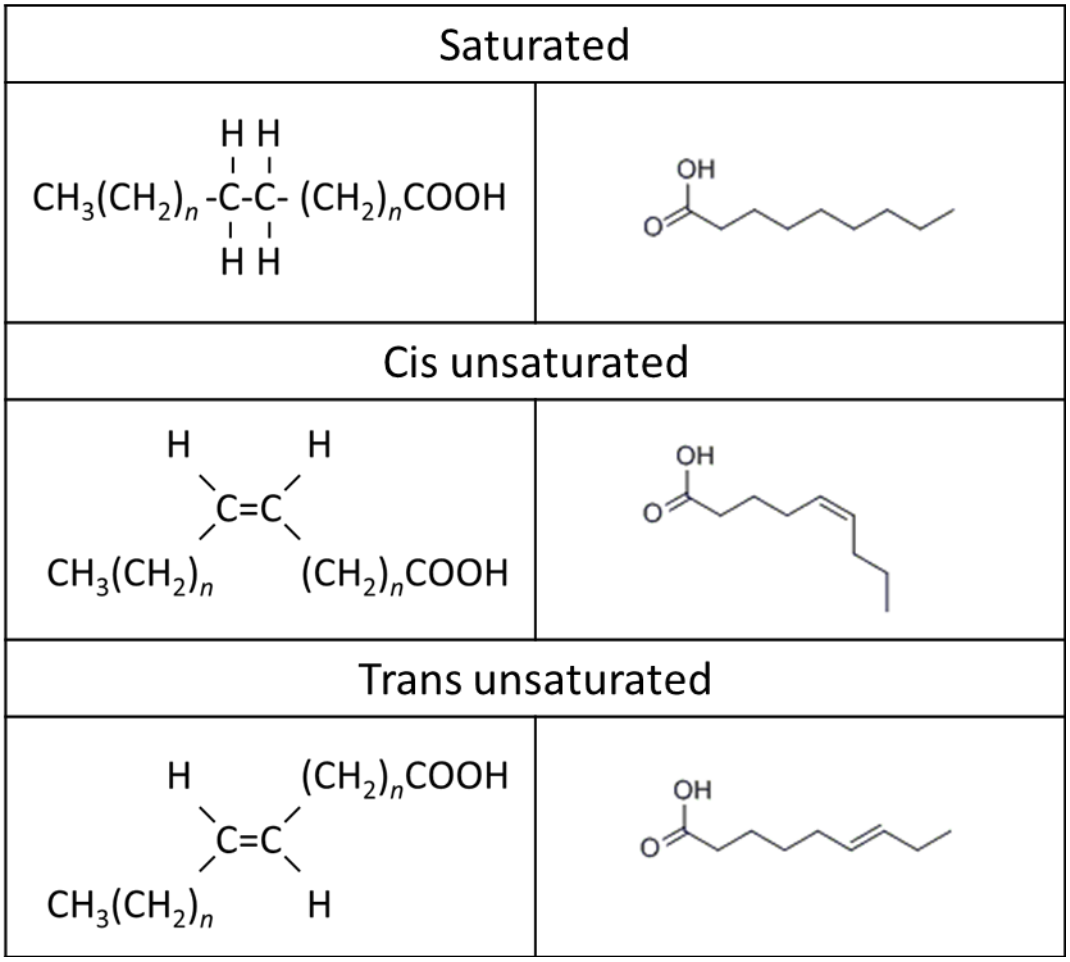


Figure 1.7 Fatty acid formulae and example molecular shapes

Double bonds are represented by two parallel lines. Cis isomers are bent as the carbon atoms are present on the same side of the double bond causing repulsion between electrons.

1.5.3 Palmitic acid and oleic acid

Two of the most populous circulating fatty acids are palmitic acid (PA) and oleic acid (OA), and, thus, their common use in research. Palmitic acid has a lipid number of 16:0, and is classified as a saturated fatty acid due to all of its 16 carbon atoms being fully saturated with hydrogen atoms (Figure 1.3). Oleic acid is a fatty acid classified as a monounsaturated omega-9 fatty acid with the lipid number 18:1 cis-9, meaning it is 18 carbon atoms in length with a double bond at the 9th carbon, with the chain continuing on the same side of the double bond (Figure 1.3).

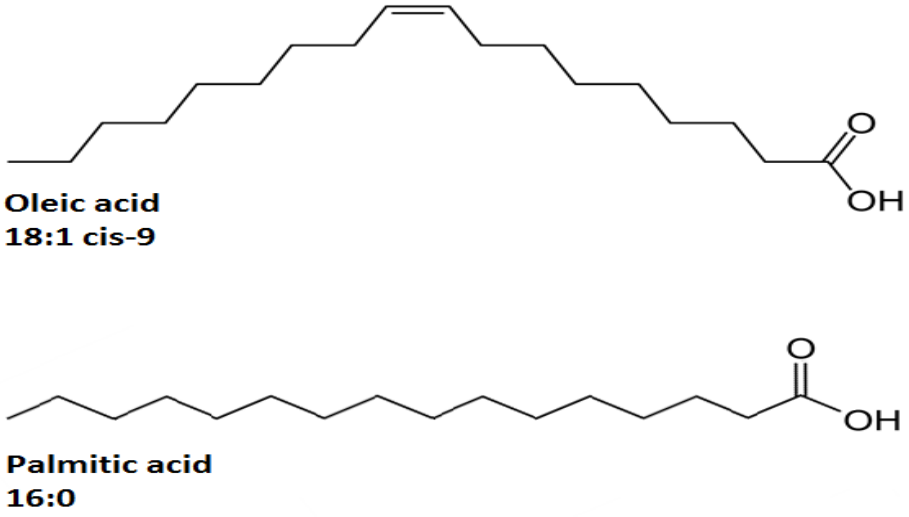


Figure 1.8 The structure of palmitic acid and oleic acid

1.5.4 Lipid uptake, storage and utilisation

Fatty acids are transported around the body in the vasculature and lymphatic system, in which they are transported as triacylglycerols in lipoproteins (Guo*, et al.*, 2009) and chylomicrons (see below), or as non-esterified fatty acids (NEFA) (van der Vusse, 2009)(van der Vusse, 2009). NEFA is another term for free fatty acids, meaning the fatty acids have not been incorporated into other lipid species.

Upon reaching cells, fatty acids are released from the triacylglycerol component of lipoproteins by lipoprotein lipase and from albumin. Free fatty acid uptake into cells occurs *via* two routes -passive diffusion, and *via* specific membrane-bound transport proteins, namely fatty acid transport protein (FATP) and CD36 (Bonen*, et al.*, 2004; Ehehalt*, et al.*, 2006; Glatz*, et al.*, 2002). Following uptake, fatty acids are hydrolysed to produce acyl groups, which bind to hydroxyl groups in glycerol esterification, forming acylglycerols. Monoacylglycerol (MAG), diacylglycerol (DAG) and triacylglycerol (TAG) (also known at triglyceride TG) are produced following the addition of one, two and three fatty acids respectively to the three hydroxyl groups of glycerol (Figure 1.6) (Gunstone & Herslöf, 2000).

The TAG pool is in constant flux, termed the glycerolipid/free fatty acid cycle (Karpe*, et al.*, 2011). At normal levels of free fatty acid uptake, cells convert surplus, unoxidised fatty acids into neutral triacylglycerol. The production of TAG occurs *via* the glycerol-3-phosphate pathway and/or the monoaclyglycerol pathway, dependant on the tissue type (Figure 1.8). When there is a short-term increase in demand for ATP due to low-intensity exercise or short-term fasting, TAG turnover increases without an effect on the overall TAG content of the pool, indicating a matched rate of synthesis to lipolysis (Figure 1.7) (Karpe*, et al.*, 2011).

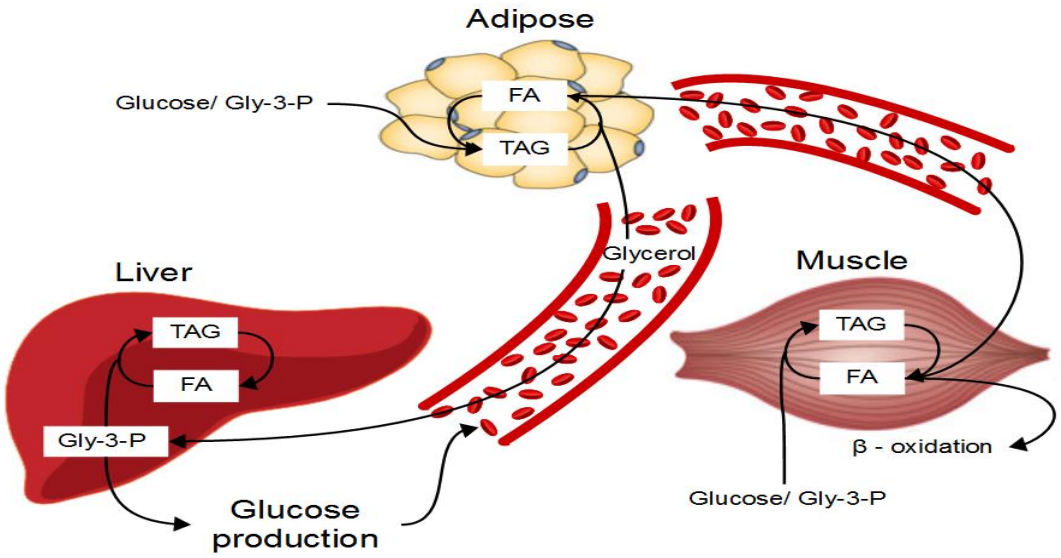


Figure 1.9 The glycerolipid/free fatty acid cycle

The intracellular lipid pool is in constant flux. At normal levels of fatty acid uptake into cells, TAG is formed. Once storage has reached capacity the pathways are reversed, leading to the formation of toxic lipid species. However, high intensity exercise also causes the breakdown of TAG in muscle cells, fatty acid products from which are neutralised by oxidation (Karpe*, et al.*, 2011). (abbreviations: Gly-3-p – glycerol-3-phosphate, otherwise known as Gro3P)

Once formed these acylglycerols along with sterol esters are stored in lipid droplets (LD) as an energy reservoir with high ATP-generating capacity as part of normal lipid accumulation (Thiam*, et al.*, 2013). The lipid droplets are localised to the endoplasmic reticulum and mitochondria, bound in a phospholipid monolayer expressing a variety of proteins involved in lipolysis and esterification (Thiam*, et al.*, 2013). The main repository of fat is the adipose tissue, with skeletal muscle and liver playing a key role. It is said that skeletal muscle contains ~300g of tryglyceride in adults with a normal BMI, whilst the liver contains ~100g (Frayn*, et al.*, 2006; Frayn & Blaak, 2007).

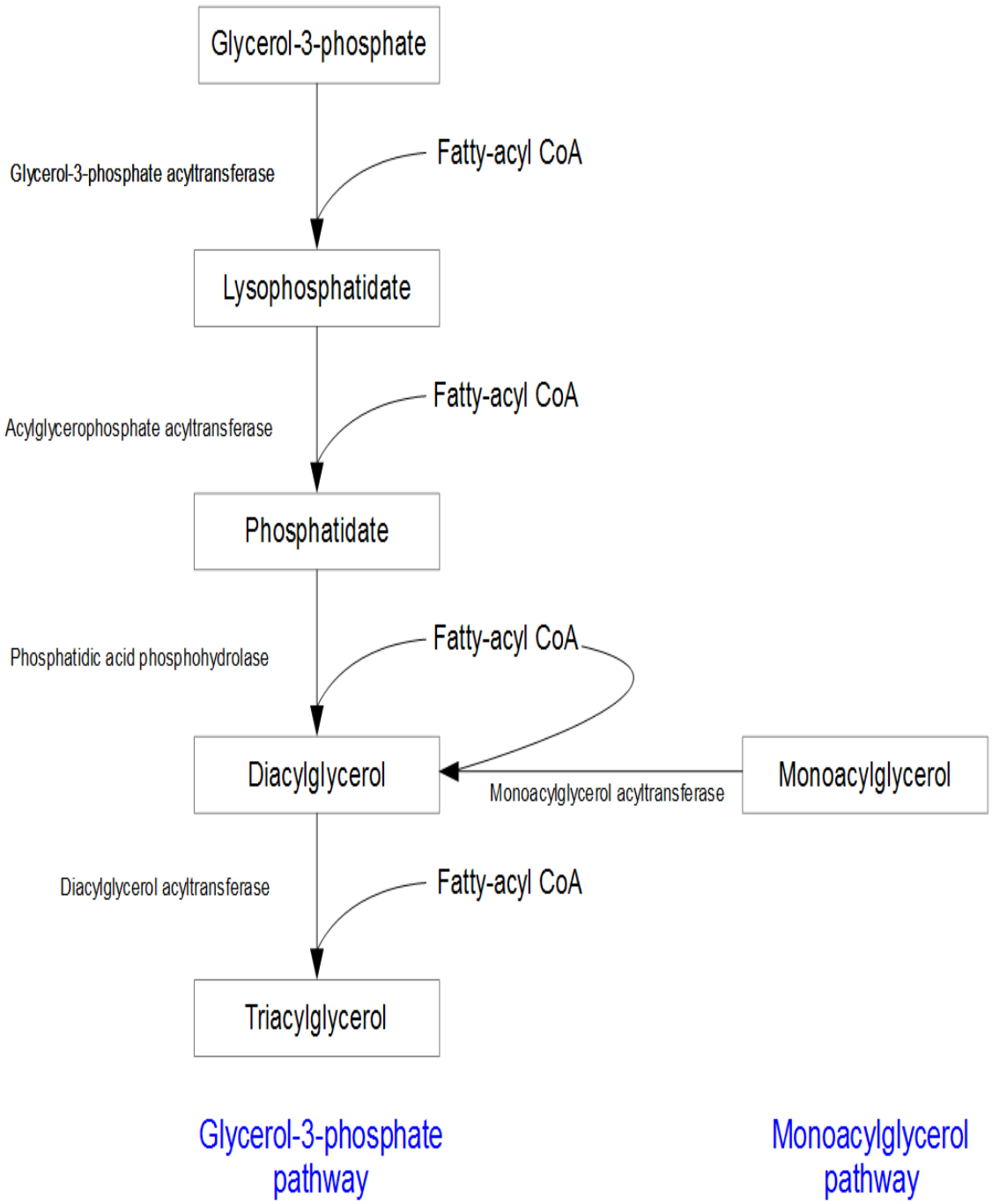


Figure 1.10 Normal pathway of lipid accumulation

Normal levels of free fatty acid uptake lead to the production of TAG *via* the glycerol-3-phosphate pathway and/or the monoacylglycerol pathway, neutralising unoxidised fatty acids (Karpe*, et al.*, 2011). TAG turnover increases when demand for ATP increases without affecting the TAG pool, indicating a matched rate of synthesis to lipolysis. However, intracellular TAG content is reduced by high-intensity exercise by favouring fatty acid oxidation over fatty acid esterification and production of TAG (Karpe*, et al.*, 2011).

1.5.5 Lipid droplets

Neutral lipids, such as TG, found in lipid droplets (LDs) are synthesised in the endoplasmic reticulum, however, the mechanism of formation is largely unknown. One hypothesis, the ER-budding model, postulates that a layer of oil forms in the ER membrane, from which sections of lipid bilayer 'bud'. This model has significant backing, but no direct evidence supporting it (Walther*, et al.*, 2009; Zhang & Zhang, 2012). A variation on this, the ER-domain model, theorises that lipid droplets are essentially protrusions of specialised ER domain containing lipids, however, little research supports it (Raghunathan*, et al.*, 2017).

Other theories for the formation of LDs have been offered. The bicelle model (Ploegh, 2007) suggests that accumulation of neutral lipids occurs between the leaflets of the ER bilayer and that upon excision from the membrane they remove phospholipids from the luminal and cytosolic leaflets (Walther*, et al.*, 2009). Another model, vesicular-budding, posits that small bilayer vesicles form in the ER membrane and remain tethered, whilst neutral lipids fill the intermembrane space until the internal bilayer is overwhelmed (Farese*, et al.*, 2009; Walther*, et al.*, 2009). More research is required to shed light on LD formation.

1.5.6 Normal fat metabolism in adipose tissue

Adipose tissue is a connective tissue mainly composed of adipocytes, specialised to synthesise and store TG. The storage of TG varies greatly between individuals with ~ 30% of the 'standard' adult female body weight accounted for by fat. This number is lower in males at ~20% (Gallagher*, et al.*, 2000). Adipocytes are highly specialised for the storage of fat, but plays an important role in the secretion of hormones such as adiponectin and leptin, and a cytokines termed 'adipokines'. The composition of fatty acids stored in the adipose tissue directly reflects dietary intake with most arising from the TG fraction of plasma lipids, whilst a minority are from the direct uptake of circulating FFAs (Coppack*, et al.*, 1999). However, the control of uptake by adipocytes is unclear.

Adipocytes synthesise lipoprotein lipase (LPL) and export it to the surface of endothelial cells in the lumen of capillaries. However, a pool remains within the adipose tissue to degrade TG in the fasting state, with only a percentage becoming active endothelial-bound LPL (Gonzales & Orlando, 2007). Upon feeding, LPL is diverted away from the degradative pathway within the adipose tissue and the secretory pathway is up-regulated (Bergö*, et al.*, 1996). Chylomicrons are the main substrate for secreted LPL as chylomicron-TG is hydrolysed more rapidly than VLDL-TG. The FAs liberated by LPL in the lumen migrate across the capillary endothelium and into adipocytes beneath by a proposed combination of diffusion- and protein-mediated uptake, likely involving CD36 (Thompson*, et al.*, 2010; Wiczer*, et al.*, 2006). Inside the adipocyte, FAs are esterified to CoA in an ATP-dependent process.

Adipose tissue LPL produce an excess of FAs, a proportion of which are taken up by adipocytes, subject to their nutritional state. However, there is always a surplus or 'overspill' of fatty acids that are release into the plasma. The uptake of LPL-derived FAs is regulated by insulin, which increases the percentage taken up. Nonetheless, even in the postprandial state ~50% are released as NEFA, making them available for uptake by parenchymal cells of other tissues including the liver and skeletal muscle (Koutsari*, et al.*, 2008). It is thought that insulin stimulation controls FA uptake *via* recruitment of FA transporters FATP1, ACSL1 and CD36 to the cell surface (Lobo & Bernlohr, 2007; Wiczer*, et al.*, 2006). Insulin also stimulates the esterification of FAs to produce TG within adipocytes. Despite consensus that insulin stimulates intracellular TG synthesis, the regulation of enzymes involved in this process is not fully understood (Czech*, et al.*, 2013; Holm, 2003). Furthermore, insulin is possibly the stimulant for glucose uptake and glycolysis, supplying the Gro3P required for FA esterification as the direct uptake of acylglycerols is very limited in adipocytes (Frayn*, et al.*, 2006).

Within adipocytes TG hydrolysis is regulated by two enzymes, hormone-sensitive lipase (HSL), which is active against two fatty acid chains on a TG molecule, and monoglycerol lipase to catalyse the final stage of TG hydrolysis (Fredrikson*, et al.*, 1986). Long-term

regulation of HSL is by transcriptional control with starvation causing an increase in HSL mRNA abundance, whilst obesity causes a reduction. But on a daily basis, HSL is regulated *via* reversible phosphorylation by protein kinase A at its three phosphorylation sites (Holm, 2003). Phosphorylation rapidly increases the activity of HSL *via* signalling that increases cAMP concentrations, thereby increasing adipocyte fat mobilisation. On the other hand, insulin suppresses HSL activity *via* signalling that reduces cAMP concentration. Concurrent to HSL phosphorylation, a protein that coats the intracellular lipid droplet, perilipin, is also phosphorylated. This enables the translocation of HSL from the cytosol to the lipid droplet surface in order to achieve lipolysis (Miyoshi*, et al.*, 2006), without which HSL would be excluded.

HSL as the main enzyme for lipolysis in adipocytes was challenged by results in HSL-deficient mice finding relatively normal adipose tissue mass with functional lipolysis (Haemmerle*, et al.*, 2002; Kraemer & Shen, 2006). This led to the discovery of adipose triglyceride lipase (ATGL) which has a specificity for triacylglycerols and not di- or mono-acylglycerols (Kershaw*, et al.*, 2006). ATGL is now considered to be responsible for basal adipose lipolysis with HSL responsible for catecholamine- stimulated lipolysis (Rydén*, et al.*, 2007).

1.5.7 Normal fat metabolism in the liver

The normal TG content of the liver is up to 10% by weight, but can enlarge significantly in non-alcoholic fatty liver disease. The liver obtains FAs from the uptake of lipoprotein remnants and the hydrolysis of circulating TG with uptake of FAs through diffusion- and protein-mediated process involving FAT/CD36, FATP2 and FATP5 (Wilson*, et al.*, 2016). LPL is not normally expressed in adult liver, instead hepatic lipase (HL), a related enzyme is employed. HL acts preferentially on smaller lipoprotein particles than LPL, removing TG from VLDL particles, HDL particles and from particles forming LDL (Carr*, et al.*, 2002). The liver is the main site for uptake of remnant lipoprotein particles formed by the LPL removal of TG from TG-rich chylomicrons and VLDL. These remnants typically contain about one-

third of the original TG content, which is considerable as chylomicron particles comprise ~106 molecules of TG (Frayn*, et al.*, 2006). These processes deliver 20–30 g/day of dietary TG to the liver.

Additionally, the liver has the enzymatic ability for *de novo lipogenesis* (DNL), the synthesis of FAs from glucose and other carbohydrate precursors. This process makes a small contribution in people consuming high fat diets, but is activated when intake of carbohydrate exceeds energy requirements (Hellerstein*, et al.*, 1996). Isotopic data indicates that ~10% of adipose tissue FAs arise from DNL (Strawford*, et al.*, 2004). DNL involves the formation acetyl-CoA in mitochondria followed by conversion to malonyl-CoA by acetyl-CoA carboxylase, and finally elongation by the sequential addition of two carbon groups by fatty acid synthase (Sanders & Griffin, 2016).

FAs taken up into hepatocytes are esterified to CoA. Acyl-CoA may then be oxidised in the mitochondria or act as a substrate for TG and phospholipid synthesis, in a process called glycerolipid synthesis. This is the vital regulatory point, regulated by entry into the mitochondria, *via* carnitine palmitoyltransferase 1 (CPT1), which is allosterically controlled by the concentration of cytosolic malonyl-CoA due to inhibitory properties. The concentration of malonyl-CoA is linked to insulin levels and is increased when glucose is readily available, essentially during conditions when FA synthesis is required over FA oxidation. FA oxidation is favoured when insulin is low in the fasting state (Lee*, et al.*, 2016). The cytosolic TG stores within hepatocytes are precursors for VLDL-TG. The generation of FAs *via* lipolysis of the cytosolic pool is required for esterification into new TG, occurring in the ER where VLDL is assembled. However, the regulation and lipase involved remain unclear. Insulin acutely inhibits the secretion of VLDL-TG, causing the storage of hepatic FAs as TG when insulin levels are high. Thus, intracellular TG stores fluctuate, accumulating following meals and depleting during fasting due to the secretion of VLDL. It is approximated that 10% of fat consumed is stored in the liver within a few hours of consumption (Ravikumar*, et al.*, 2005).

1.6 Normal fat metabolism in skeletal muscle

The total TG content throughout skeletal muscle is ~300 g across two main sites (Frayn & Blaak, 2007). In muscle TG is stored interleaved between muscle fibres in adipocytes and within skeletal myocytes themselves (intramyocellular TG). Intramyocellular TG form lipid droplets in close proximity with mitochondria. Skeletal myocytes contain many of the same enzymatic processes for TG and FA metabolism as adipocytes. FA uptake is from the hydrolysis of circulating TG by LPL, and from albumin-bound plasma FFA with uptake *via* facilitated diffusion utilising FATP1, FABPpm and CD36 expressed in skeletal muscle (Harasim*, et al.*, 2008). The TG present in skeletal muscle arises exclusively from plasma FAs (NEFA or TG). The regulation of FA oxidation is *via* malonyl-CoA, in the same manner as described above for liver, however, skeletal muscle is unable to synthesise FAs (Foster, 2012; Saha*, et al.*, 1997).

Intramyocellular TG is hydrolysed to make FAs for oxidation with hormone-sensitive lipase (HSL) the likely enzyme controlling the process. No direct evidence exists to support this other than the expression of HSL in skeletal muscle and its activation upon muscle contraction (Donsmark*, et al.*, 2005), which is reinforced by the formation of large intramyocellular lipid droplets in HSL-deficient mice (Hansson*, et al.*, 2005). FAs released from TG lipolysis are substrates for oxidation after activation into CoA and transit into the mitochondria. This lipolysis is not as sensitive to hormone regulation as lipolysis in adipocytes. Insulin is ineffective at stimulating muscle lipolysis, whilst catecholamines are effective (Donsmark*, et al.*, 2005; Moberg*, et al.*, 2002; Qvisth*, et al.*, 2006).

It remains unclear whether, skeletal muscle releases FAs into the circulation as adipocytes do, however, it is agreed that the muscle never gives a net contribution, always extracting from the circulating FFA pool overall. Adipose tissue is the only tissue that is a net contributor to circulating plasma FFAs.

1.6.1 Fatty acid oxidation

1.6.1.1 Carnitine transport

Within the cytosol, fatty acids are converted into fatty acyl-CoA by acyl-CoA synthetases, which enable the formation of a thioester bond between the carboxyl group of the fatty acid and the thiol group of coenzyme A (CoA) in an ATP-dependent reaction. Fatty acyl-CoA then attaches to the hydroxyl group of carnitine in the presence of carnitine palmitoyltransferase I (CPT1), forming fatty acyl-carnitine. Fatty acyl-carnitine is transported across the outer mitochondrial membrane in exchange for carnitine *via* carnitine-acylcarnitine translocase facilitated diffusion. Inside the mitochondrial matrix, carnitine acyltransferase II (CPT2) on the inner mitochondrial membrane catalyses the transfer of the acyl group from fatty acyl-carnitine to a different CoA, thus, forming fatty acyl-CoA and free carnitine. Fatty acyl-CoA is then ready for β-oxidation (Longo*, et al.*, 2016).

1.6.1.2 Beta oxidation of fats

Within the mitochondrial matrix, fatty acyl-CoA is dehydrogenated in the presenceof acyl-CoA dehydrogenase to form trans-Δ^2-enoyl-CoA with a trans double bond between the two carbons closest to the carboxyl end. Electrons from this reaction are transferred to FAD reducing it to $FADH_2$, which is converted to ATP *via* the electron transport system. Trans-Δ^2-enoyl-CoA is then hydrated *via* enoyl-CoA hydrase to form β-hydroxyacyl-CoA, which is dehydrogenated in the presence of β-hydroxyacyl-CoA dehydrogenase to form β-ketoacyl-CoA. This results in the transfer of electrons to NAD^+ to form NADH + H^+ which enters the electron transport system to produce ATP. The final step is the thiolysis of β-ketoacyl-CoA; in the presence of acyl-CoA acetyltransferase and CoA, acetyl-CoA and a fatty acid CoA with n-2 carbons. This process is repeated for the new fatty acyl-CoAuntil the entire molecule has been converted to acetyl-CoA. Acetyl-CoA molecules enter the Kreb's cycle (Longo*, et al.*, 2016).

1.6.2 Ectopic fat accumulation and lipotoxicity

In 2003, Roger Unger proposed the 'lipid overflow' to explain the accumulation of ectopic fat accumulation (Unger, 2003). This hypothesis suggests that obesity causes adipose tissue to expand to capacity, and upon continuation of excess energy intake surplus lipids spillover into non-adipose tissues for storage, whereby 'lipotoxicity' is induced. This, in combination with leptin resistance causes the metabolic dysfunction associated with obesity (Farooqi & O'Rahilly, 2009). Lipotoxicity is the accumulation of intracellular lipids in non-adipose tissue, causing cellular dysfunction and/or cell death.

However, the flaw in this hypothesis is that there is no evidence for a limit to subcutaneous adipose tissue expansion. Furthermore, this theory is completely contradicted by findings that endurance trained athletes have markedly high insulin sensitivity, despite elevated intramyocellular lipid content that often exceeds that of obese, diabetic subjects (Goodpaster*, et al.*, 2001; van Loon*, et al.*, 2004).

Perhaps, a more fitting hypothesis is the theory of adiposopathy, or sick fat. In 2004, Harold Bays suggested that metabolic disease may be caused by dysfunctional adipose tissue and its interaction with the body, in a condition termed 'adiposopathy' (Bays*, et al.*, 2004). This concept has been further discussed and enhanced over the past decade (Bays, 2014; Bays*, et al.*, 2005; Bays, 2011; Dalamaga & Christodoulatos, 2015; McCrindle, 2014). According to this hypothesis, increase in adiposity leads to metabolic disease when the adipose tissue expands *via* hypertrophy rather than hyperplasia due to adipocyte dysfunction, which impairs adipogenesis (Klöting*, et al.*, 2010). This dysfunction leads to excess free fatty acid release, and ultimately systemic inflammation and insulin resistance *via* apoptosis, ER stress, hypoxia, macrophage infiltration and inflammation, and peripheral insulin resistance (Mittendorfer, 2011). Moreover, the dysfunctional adipose tissue not only releases excess free fatty acids, but also down-regulates uptake (McQuaid*, et al.*, 2011), through decrease fatty acid transporter expression (Fabbrini*, et al.*, 2009). Both of which lead to increased levels of plasma free fatty acid and, therefore, increased availability to non-adipose tissues (McQuaid*, et al.*, 2011).

Healthy humans store surplus energy in adipose tissue in the form of triglycerides. However, in an obese state, the storage capacity of adipose tissue is exceeded leading to the formation of ectopic fat in non-adipose tissues. Triglycerides in themselves are considered neutral and, therefore, not toxic, although they act as a reservoir for metabolites of non-oxidative pathways that cause toxic effects (Samuel*, et al.*, 2010). Furthermore, excess triacylglycerol production eventually surpasses storage capacity of the cell and drives hydrolysis of neutral TAG back into fatty-acyl CoAs and glycerol (Figure 1.9).

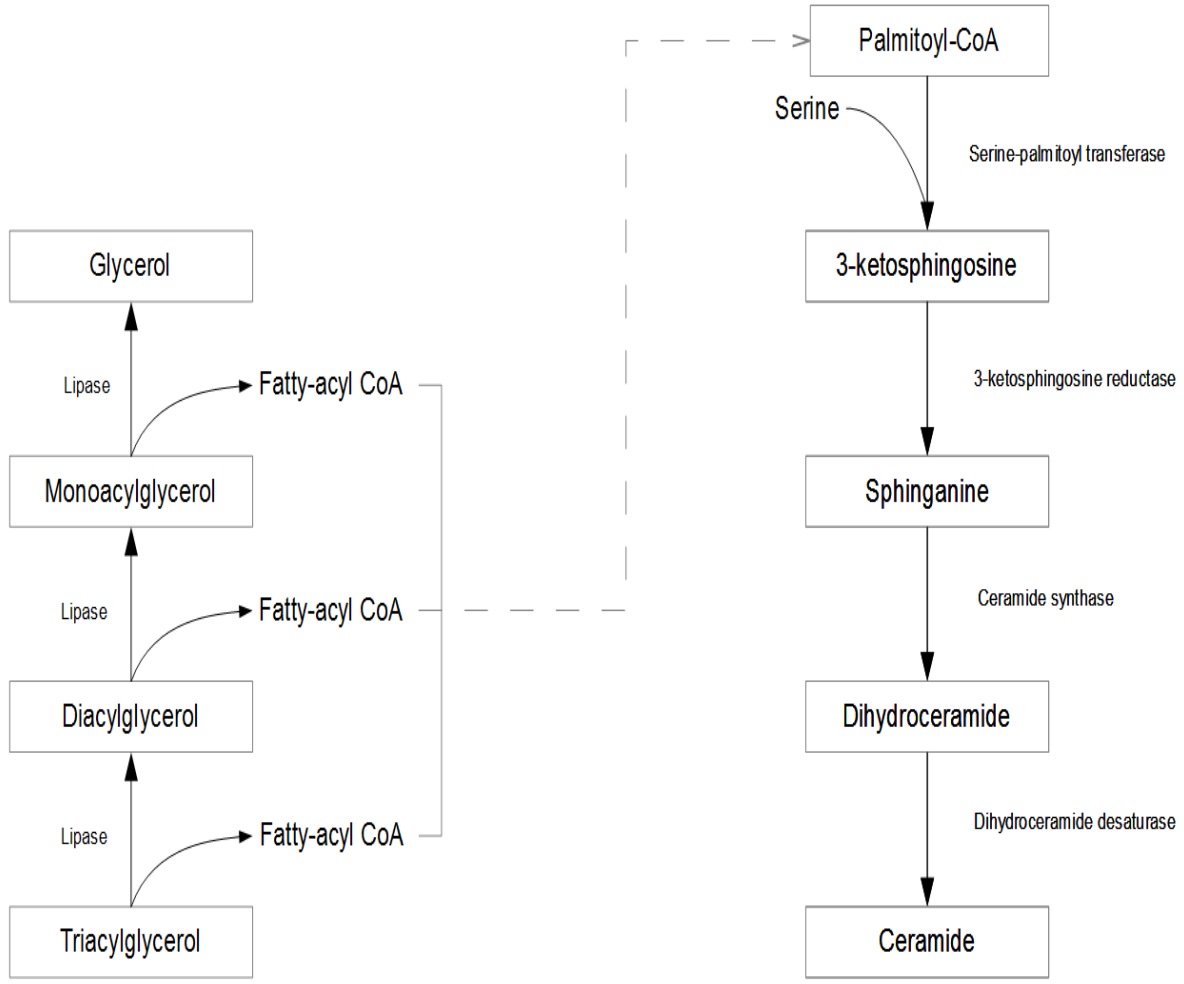


Figure 1.11 Triacylglycerol breakdown and accumulation of fatty acid species

When capacity of TAG storage is reached, hydrolysis back into fatty-acyl coA is favoured. This in turn strains the oxidative capacity of the cell and drives reactions towards non-oxidative pathways of fatty acid metabolism (Unger & Zhou, 2001). These pathways lead to the production of toxic lipid species such as ceramide.

The increase in fatty-acyl CoA strains the cell's oxidative capacity, causing spillover into non-oxidative pathways of fatty acid metabolism that produce toxic lipid intermediates

(Unger*, et al.*, 2001). It is these lipid intermediates from lipolysis and esterification in the glycerolipid/fatty acid cycle that are hypothesised to initiate signalling that alters insulin sensitivity (Samuel*, et al.*, 2010) (Figure 1.9). The *de novo* ceramide pathway is one such pathway feeding into the pool of reactive lipid species with deleterious effects (Summers, 2010).

Lipotoxicity describes cellular dysfunction caused by chronically elevated intracellular lipid accumulation in non-adipose tissue. Lipotoxic effects are reported to occur *via* several mechanisms, including the production of reactive oxygen species (ROS), disruption to intracellular signalling pathways, damage to organelles, release of proinflammatory factors and lipoapoptosis, and lipid-induced apoptosis. Lipotoxicity has been found to cause cell death and insulin resistance and is a possible link between obesity, insulin resistance and type II diabetes (Day*, et al.*, 2011). It is proposed that reactive lipid metabolites rather than FA themselves are deleterious in terms of insulin signalling and cell death. Such species include fatty acyl-CoAs (especially long-chain), diacylglycerol (DAG) (Shmueli*, et al.*, 1993), phosphatidic acids (Zhang*, et al.*, 2013) and ceramides (Górski, 2012; Reali*, et al.*, 2017). Ceramide accumulation is thought to hinder insulin action by activating Akt inhibition, and inducing mitochondrial dysfunction and ER stress (Stratford*, et al.*, 2004).

These metabolites induce chronic activation of a serine/threonine kinase cascade, leading to altered insulin signalling through serine phosphorylation of IRS (Morino*, et al.*, 2006). The serine-phosphorylated IRS1 is then unable to activate downstream signalling, leading to decreased GLUT4 translocation and hence decreased glucose uptake. Excess intracellular fatty acids and toxic lipid species such as fatty acyl-coA, ceramide and DAG have been shown to promote insulin resistance and exert deleterious effects on a range of tissues (Samuel*, et al.*, 2010). DAG activates both novel and conventional protein kinases C (PKC), which are serine/threonine kinases shown to inhibit IRS *via* serine phosphorylation leading to insulin resistance (Erion & Shulman, 2010; Huang, 1989; Wang, 2006).

Lipotoxicity also results in the induction of apoptosis with many mechanisms such as; synthesis of nitric oxide and reactive oxygen species (Gehrmann*, et al.*, 2010), ceramide

production (Lupi*, et al.*, 2002), endoplasmic reticulum stress (Cui*, et al.*, 2013), being implicated. Furthermore, a one study indicates that lipotoxicity may cause cell death by necrosis *via* the mitochondrial necrotic pathway (Rockenfeller*, et al.*, 2010). Many studies suggest that ectopic fat accumulation in insulin-sensitive tissues is linked to insulin resistance, however, understanding of the mechanisms by which the reactive lipid species alter insulin signalling and induce apoptosis, in liver and skeletal muscle is limited (Yki-Järvinen, 2002).

1.7 Adipose stress in obesity

When adipocytes become too large a number of stress pathways are triggered casuing adipose dysfunction. This includes stress of the endothelial reticulum (ER), which increases the number of misfolded proteins and leads to an unfolded protein response (UPR) (Gregor & Hotamisligil, 2007). Under normal conditions, the ER is an important site of protein synthesis and is involved in the transport and release of proteins. Under acute ER stress the UPR is activated to increases protein folding, degradation, protein ubiquitination and various post-translational protein modifications in order to combat the ER stress (Ron & Walter, 2007). However under chronic ER stress conditions, UPR induces apoptosis. The ER demonstrates a state of chronic stress in obese and insulin resistant patients. The involvement of the UPR is thought to trigger the activation of inflammatory pathways, causing the release of chemokines and cytokines able to interfere with insulin signalling (Snel*, et al.*, 2012). However, an increase in circulating insulin has been shown to upregulate the UPR (Boden*, et al.*, 2014). Furthermore, UPR activation has been implicated in the development of insulin resistance *via* an array of mechanisms, including activation of c-jun N-terminal kinase (JNK), oxidative stress and inflammation (Cnop*, et al.*, 2012).

The dysfunction of adipose tissue results in adipocyte hypertrophy and apoptosis, both of which have the potential to induce the secretion of pro-inflammatory adipokines leading to the recruitment of pro-inflammatory immune cells to the site of dysfunction causing chronic, low-grade inflammation. In turn, this is associated with the activation of stress-signalling

pathways, insulin resistance and increases in autophagy and apoptosis resulting in a vicious cycle (Cancello*, et al.*, 2005; Cinti*, et al.*, 2005; Harman-Boehm*, et al.*, 2007).

1.7.1 Ectopic fat accumulation in liver

Obesity, especially central obesity associated with increased visceral fat plays a primary role in ectopic fat accumulation and the pathogenesis of non-alcoholic fatty liver disease (NAFLD) (Marchesini*, et al.*, 2008). Intrahepatocellular fat accumulation varies with the degree of obesity; small changes to weight are evident in changes in steatosis (Hamaguchi*, et al.*, 2005). Compared with individuals of a normal weight, obese people have a 4.6-fold increased risk of developing NAFLD (Bellentani*, et al.*, 2000). NAFLD is associated with metabolic abnormalities linked with excess adiposity and is a risk factor for type II diabetes mellitus, cardiovascular disease, and subsequent mortality (Anstee*, et al.*, 2013). More than 90% of obese type II diabetic patients also have non-alcoholic fatty liver disease (NAFLD) (Perry*, et al.*, 2014), but weight loss is capable of normalising metabolic abnormalities responsible for the pathogenesis of NAFLD (Klein*, et al.*, 2006).

Accumulation of fat in the liver is mostly dependent on levels of free fatty acids (FFAs) recirculating from the adipose tissue with an estimated 59% of liver triglycerides from this source (Donnelly*, et al.*, 2005). It is these FFAs rather than circulating triglycerides that promote hepatocellular injury (Yamaguchi*, et al.*, 2007). Furthermore, the lipolytic potential of visceral adipose tissue is greater than that of the more abundant subcutaneous adipose tissue, and it deposits FFAs directly into the portal circulation adding more significantly to hepatic injury and steatosis (Björntorp, 1990; Jensen, 2008). Studies have shown that a 1% increase in subcutaneous fat increases intrahepatocellular lipids by 20%, whereas an increase of 1% in visceral fat increases intrahepatocellular lipids by 50% (Thomas*, et al.*, 2005).

NAFLD leads to localised hepatic insulin resistance, caused by decreased tyrosine phosphorylation of IRS-2 and, thus, the inability to suppress hepatic glucose production and

active glycogen synthesis (Kubota*, et al.*, 2016). Increased levels of hepatic DAG is thought to be the vehicle by which insulin signalling is hindered with the link being attributed to the activation of novel PKC-ε, which binds directly to and inhibits insulin receptor kinase activity (Erion*, et al.*, 2010; Perry*, et al.*, 2014; Schmitz-Peiffer & Biden, 2008). In turn, this causes a reduction in downstream phosphorylation and consequently Akt activation; thereby impairing insulin stimulation of glycogen synthesis and insulin inhibition of gluconeogenesis (Perry*, et al.*, 2014). Furthermore, Kumashiro*, et al.,* (2011) showed that increased hepatic DAG levels in obese, non-diabetic subjects was associated with insulin resistance *via* PKC-ε activation.

Additionally, hepatocellular carcinoma (HCC) has been associated with obesity (Ioannou*, et al.*, 2003) with obesity-related metabolic abnormalities considered to initiate hepatic neoplasm (Yang*, et al.*, 2001). In comparison with people of a normal weight, people with a BMI greater than 35 kg/m^2 have a 4.52 relative risk of dying from HCC (Calle*, et al.*, 2003).

Exposure of the liver to high levels of FFAs can also lead to acute lipotoxicity, causing oxidative stress, ER stress (Pardo*, et al.*, 2015), disturbances to the cell membrane, apoptosis and necrosis and can ultimately result in chronic liver injury and end-stage liver disease (Akazawa & Nakao, 2016; Marra*, et al.*, 2008; Trauner*, et al.*, 2010; Zámbó*, et al.*, 2013). However, mechanisms of lipotoxicity are not fully understood.

In vitro studies on hepatocytic cell lines suggest that FFAs induce cell death mediated by ER stress, and mitochondrial dysfunction (Egnatchik*, et al.*, 2014; Han & Kaufman, 2016; Yao*, et al.*, 2011). Many studies suggest that cell death is *via* apoptosis (Akazawa*, et al.*, 2016; Malhi*, et al.*, 2006; Zámbó*, et al.*, 2013), however, further research is required to understand the mechanisms and consequences involved.

1.7.2 Ectopic lipid accumulation in skeletal muscle

In skeletal muscle, lipids are present as extracellular lipids (EMCL) and intramyocellular lipids (IMCL). EMCL are lipids located in adipocytes between myofibers and IMCL are lipids located in droplets within muscle cells (Boesch*, et al.*, 2006). In obese individuals, excess lipids are capable of accumulating as intramyocellular lipids (Unger*, et al.*, 2010). This lipid overload affects not only insulin sensitivity, but also muscle repair and regeneration (Akhmedov & Berdeaux, 2013).

Skeletal muscle is an important insulin-sensitive tissue heavily involved in glucose metabolism and, therefore, plays a crucial role in the pathogenesis of insulin resistance. Exposure to FFAs and intramyocellular lipid accumulation is able to cause lipotoxicity associated with localised insulin resistance, an indicator of systemic insulin resistance in non-obese offspring of type II diabetic patients (Guebre-Egziabher*, et al.*, 2013). The relationship between the accumulation of intramyocellular triglycerides and insulin sensitivity was well established by Kelley & Goodpaster (2001). Intramyocellular accumulation of DAG was found to activate novel PKC-θ and in turn inhibit insulin signalling (Schmitz-Peiffer*, et al.*, 2008; Szendroedi*, et al.*, 2014).

Another mechanism of lipotoxicity is the reduction in mitochondrial oxidative capacity caused either by a defect in the respiratory pathway or by a decrease in mitochondrial content. Much research suggests impairment in mitochondrial fatty acid oxidation causes an accumulation of toxic lipid intermediates. However, contradictory research indicates that insulin resistance is unlikely to be a consequence of diminished mitochondrial oxidative capacity caused by lipid accumulation (Summers, 2010; Turner*, et al.*, 2007; Unger*, et al.*, 2001). A less common idea is that increased passage of fatty acids through mitochondria promotes incomplete oxidation and/or oxidative stress, resulting in a compensatory impairment in glucose utilisation (Guebre-Egziabher*, et al.*, 2013). However, evidence indicates that mitochondrial dysfunction is not a primary defect, but rather a consequence of insulin resistance (Sleigh*, et al.*, 2012). Therefore, it is interesting that although endurance-trained athletes have high intramyocellular lipid content they also exhibit high insulin

sensitivity (Goodpaster, *et al.*, 2001). Thus, it is thought that the cell's oxidative capacity determines whether the accumulated lipids play a pathological or physiological role (van Loon & Goodpaster, 2006).

In vitro studies on skeletal muscle cell lines indicate that exposure to FFAs causes irreversible impairments to cellular functions by inducing ER stress, mitochondrial dysfunction and ultimately apoptosis, and/or necrosis (Brøns & Vaag, 2009; Han, *et al.*, 2016; Martins, *et al.*, 2012; Turpin, *et al.*, 2009).

1.7.3 Mechanisms of cell death

Apoptosis is defined as programmed cell death and is a normal physiological process that removes damaged cells and aids in the homeostasis of tissue. However, apoptosis is also involved in the pathological progression of a number of diseases (Favaloro, *et al.*, 2012) including type II diabetes mellitus (Butler, *et al.*, 2003). The process of apoptosis involves characteristic morphological changes (Saraste & Pulkki, 2000), including shrinkage of the cytoplasm (Bortner & Cidlowski, 2002), cleavage of chromatin into internucleosomal fragments (Hughes & Cidlowski, 1997; Iglesias-Guimarais, *et al.*, 2012), and damage to the cell membrane such as blebbing and asymmetry (Coleman, *et al.*, 2001). Asymmetry of the membrane is an early feature of apoptosis and is accompanied by the translocation of phospholipid phosphatidylserine (PS) from the internal surface of the phospholipid bilayer to the external surface (Fadok, *et al.*, 2001; Rysavy, *et al.*, 2014).

On the other hand necrosis was once considered as a passive, unregulated form of cell death usually resulting from insult to the cell. However, research over the past decade has found links between apoptosis and necrosis, indicating the involvement of signalling pathways (Hitomi, *et al.*, 2008), yet much is left to be understood. The characteristics of necrosis are undefined, but can be distinguished from apoptosis by the rapid loss of membrane integrity early in the death process resulting in leakage of cellular content (Ziegler & Groscurth, 2004).

1.7.4 Fats as signalling molecules

The capacity of lipids to act as signalling molecules was first acknowledged in the 1930s upon the discovery of cysteinyl leukotrienes and prostaglandins as muscle stimulants (Feldberg*, et al.*, 1938; von Euler, 1936). The major breakthrough in lipid signalling was the identification of arachidonic acid as the source of prostaglandins and leukotrienes, which has resulted in our present understanding of eicosanoid signalling. This proved that a single lipid signalling molecule could induce a range of cell type- and pathway-dependent responses.

Eicosanoids are a family of lipid signalling molecules including leukotrienes, prostaglandins and lipoxins, most of which are derived from arachidonic acid (AA) oxidation. These molecules modulate a vast array of physiological processes such as cell differentiation, migration, proliferation, antibody production, antigen presentation and cytokine formation (Harizi*, et al.*, 2008). Cells of innate immunity are significant manufacturers of eicosanoids, which play a vital role in innate immunity by eliciting their effects locally in a paracrine and autocrine manner. Furthermore, they interact with cytokines and chemokines in the regulation of both inflammatory and homeostatic disorders. AA and its bioactive metabolites, thromboxane A2 and/or prostaglandin H2 are also potent activators of platelet aggregation (Hornberger & Patscheke, 1989; Parise*, et al.*, 1984; Rao & White, 1985; Sangkuhl*, et al.*, 2011).

Research into lipid bilayers uncovered that phospholipase C could hydrolyse phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) to DAG and inositol-1,4,5-trisphosphate (Ins(1,4,5)P3). Both of which were determined to be secondary messengers, triggering PKC activation and Ca^{2+} release from the internal pool, respectively. Phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3) was also discovered as a signalling molecule regulating cell proliferation, growth, migration and survival (Leslie*, et al.*, 2007; Wymann*, et al.*, 2003).

The anti-proliferative and pro-apoptotic properties of ceramides and sphingosines have been well published (Futerman & Hannun, 2004; Pettus*, et al.*, 2002; Woodcock, 2006).

Yet, sphingosine-1-phosphate (S1P) is involved in the promotion of cell proliferation, growth, survival and mobility *via* G protein-coupled receptors (GPCRs) and an unknown intracellular mechanism (An*, et al.*, 2000; Goetzl*, et al.*, 2004).

Lysophosphatidic acid (LPA) is yet another signalling lipid that regulates gene expression through lipid-sensing transcription activators (Oyesanya*, et al.*, 2008; Semple*, et al.*, 2006). Furthermore, LPA induces platelet aggregation *via* an unclear mechanism that is thought to rely on the release of intracellular Ca^{2+} (Gerrard*, et al.*, 1979).

The contribution of lipid signalling dysregulation to the progression of many diseases has been established including allergic reactions, cancer, and cardiovascular, degenerative, and metabolic diseases to name a few (Wymann*, et al.*, 2003).

1.8 Physiological fatty acid concentrations

Circulating fatty acids have been associated with a number of diseases including cardiovascular disease (Imamura*, et al.*, 2012) and type II diabetes (Eckel*, et al.*, 2005). Moreover, different groups within the subpopulation are said to have different levels of circulating FAs with studies indicating that circulating plasma FFAs are increased in obesity (Arner & Rydén, 2015; Ni*, et al.*, 2015). As levels of circulating FAs may predispose subsets of the population to specific pathologies, it is important to understand natural variation in circulating lipid species and to find associations with disease. Especially as established references ranges for the concentration of each fatty acid for healthy individuals have yet to be standardised (Abdelmagid*, et al.*, 2015).

A review of the literature uncovered a handful of studies reporting average PA and OA in plasma phospholipids (De Vriese*, et al.*, 2003; Song*, et al.*, 2016). Many of the studies reviewed published relative percentages of fatty acid fractions rather than absolute concentrations (Bermúdez-Cardona & Velásquez-Rodríguez, 2016; Glaser*, et al.*, 2010; Imamura*, et al.*, 2017; Phillips & Dodges, 1967; Saadatian-Elahi*, et al.*, 2008). Song*, et al.,* (2016) measured the concentration of fatty acid fractions in phospholipids and their relative

percentages in 279 participants. Average PA in phospholipids was reported as 36.49 mg/dL, and OA as 1.67 mg/dL. Most absolute values correlated with relative values, however, correlation between the concentration of PA in phospholipids and the relative weight percentage of PA in phospholipids was low (Song*, et al.*, 2016). Another paper found 36.8 ± 8.3 mg/dL of PA and 10.5 ± 3.4 mg/dL (Yeh*, et al.*, 1994).

Most papers reporting average PA and OA in cholesterol esters also published relative values rather than absolute concentrations (Lewis-Barned*, et al.*, 2000; Lindeberg*, et al.*, 1996; Salomaa*, et al.*, 1990). Additionally, studies commonly omit whether the relative value is based on weight or concentration, which could lead to confusion (Lindeberg*, et al.*, 1996). Papers reporting absolute concentrations were exceedingly difficult to find. Yeh*, et al.,* (1994) measured fatty acids fractions in plasma cholesterol esters in 104 postmenopausal women and found 11.2 ± 2.6 mg/dL of PA and 14.8 ± 3.9 mg/dL of OA.

Average PA and OA in plasma TG was more commonly published as a percentage relative to weight or concentration than absolute concentration (Koletzko & Decsi, 1994; Peterson*, et al.*, 1994), with papers containing concentrations difficult to find. However, Yeh*, et al.,* (1994) measured 17.9 ± 13.5 mg/dL of PA in plasma TG and 20.0 ± 13.5 mg/dl of OA in plasma TG.

Once again, average circulating free palmitic acid and oleic acid were more frequently measured as a molar percentage (Laaksonen*, et al.*, 2002; Raatz*, et al.*, 2001; Yli-Jama*, et al.*, 2002). A moderate number of papers publishing concentrations were also found, however, the range of reported values was large. Itakura *et al*., (2011) established circulating free fatty acid concentrations in 8076 participants, uncovering an average PA concentration of 74.8 ± 31.3 mg/dL and average OA concentration of 69.1 ± 37.0 mg/dL. Han*, et al.,* (2011) found an average free PA of 25.6 ± 4.6 mg/dL and free OA of 47.9 ± 9.0 mg/dl in 30 subjects. However, previous research has found that levels of plasma FFA increase during extended periods of fasting (Gjesdal*, et al.*, 1976) and in states of increased stress (Taggart & Carruthers, 1971).

Relative measures and absolute concentrations of PA and OA in total plasma lipids were published equally in the literature (Abdelmagid*, et al.*, 2015; Risé*, et al.*, 2007; Yeh*, et al.*, 1994). However, the usage of the units mmol/L were more common than mg/dL. Abdelmagid*, et al.,* (2015) studied concentrations in 826 subjects. Average total PA and OA were 1.63 ± 0.46 mmol/L and 1.29 ± 0.42 mmol/L, respectively. Concentrations ranged as high as 4.1 mmol/L for PA and 3.2 mmol/L for OA. From this paper 'normal ranges' (2.5th - 97.5th percentile) of PA were found to be 0.71 -2.5 mM, whilst for OA this was slightly lower, 0.45 -2.1 mM.

1.8.1 Dietary effects of saturated versus unsaturated fatty acids

The issue of dietary fat intake is particularly relevant considering the extensive intake of saturated fats in the western 'McDonald's' diet. The recommendation (based on an average-sized woman doing an average amount of exercise) for total fat intake is less than 70g, with saturates less than 20g (NHS, 2017). While the average total fat intake for the UK population is close to recommended values, that for saturated fats remains about 1.7% over the recommended limit (British Nutrition Foundation, 2018). Morever, there is a proportion of the population with a significantly higher intake of fat, with 1 in 4 adults and 1 in 4 children aged 10-11 classified as obese.

The fat composition of dietary intake is important as the diet is able to alter circulating fatty acid compositions (Ahrens*, et al.*, 1957; Liu*, et al.*, 2015; Raatz*, et al.*, 2001). Furthermore, the composition of TG in the adipose tissue is directly related to composition of ingested fats (Coppack*, et al.*, 1999).

For the past few decades, controversy regarding 'good' fats and 'bad' fats has led to sensational stories in the media, and an increase in the number of meta-analysis and cohort studies being published. It is claimed that the consumption of dietary SFA is bad and leads to the development of T2DM, CVD and CHD. Whereas both unsaturated fatty acids,

MUFA and PUFA, are considered ‘good’ with their consumption decreasing these risk factors.

The majority of large-scale studies concluded that the consumption of SFA was not associated with the development of CHD (de Souza*, et al.*, 2015; Li*, et al.*, 2015; Nettleton*, et al.*, 2017; Siri-Tarino*, et al.*, 2010), CVD (de Souza*, et al.*, 2015; Siri-Tarino*, et al.*, 2010), stroke (de Souza*, et al.*, 2015; Li*, et al.*, 2015; Nettleton*, et al.*, 2017), T2DM (de Souza*, et al.*, 2015; Guasch-Ferré*, et al.*, 2017; Liu*, et al.*, 2018) or all—cause mortality (de Souza*, et al.*, 2015). However, Zong*, et al.,* (2016) found that high dietary intake of SFA was associated with CHD, whilst Paquet*, et al.*, (2014) discovered a link between dietary SFA and T2DM. Some studies concluded that the total intake of SFA is not as important as the source of those SFA (Praagman*, et al.*, 2016), for example Guasch-Ferré*, et al.,* (2017) found no association between baseline dietary SFA intake and T2DM, but did find a significant association between the consumption of cheese and butter and its development. In contrast, the consumption of whole yoghurt was found to reduce the risk of developing T2DM. Additionally, a link between the consumption of SFA from processed foods and the development of CVD was uncovered (Guasch-Ferré*, et al.*, 2015).

Conversely to SFA, the dietary intake of PUFA and MUFA was inversely associated with all-cause mortality (Guasch-Ferré, *et al.*, 2015) and omega-3 fatty acids were negatively associated with the development of T2DM (Paquet, *et al.*, 2014). Furthermore, it was established that the replacement of dietary SFA with PUFA decreased the risk of developing CHD (Jakobsen, *et al.*, 2009; Li, *et al.*, 2015; Nettleton, *et al.*, 2017; Siri-Tarino, *et al.*, 2010). Additionally, the replacement of SFA with PUFA or MUFA decreased the risk of CVD (Guasch-Ferré, *et al.*, 2017).

The consumption of trans fats was also linked with CHD and all-cause mortality (de Souza, *et al.*, 2015). The replacement of which with MUFA was found to decrease the risk of CVD (Guasch-Ferré, *et al.*, 2015).

1.9 Models

1.9.1 Solubility of fatty acids in solutions

The fact that long-chain fatty acids have low solubility in aqueous solutions presents as the main limitation for *in vitro* studies. However, this may be bridged by conjugating FFAs to albumin, which in turn enhances the physiological relevance of the FFA solutions. Plasma albumin has 7 binding sites for fatty acids ranging from high to moderate affinity, and also acts to facilitate fatty acid uptake at cells (van der Vusse, 2009). Albumin makes up about 60% of the total protein in blood. The reference range for albumin in serum is approximately 35-50 g/L, with a serum half-life of approximately 20 days. Ratios greater than 7 exceed solubility, potentially inducing artefacts. Therefore, the FFA/albumin molar ratio is an important factor in determining cellular responses.

Unbound FFAs are considered accessible for cellular uptake, the concentration of which is reliant on the ratio of albumin to total FFAs (Spector, 1975). This suggests that biological effects can be augmented to mimic pathological conditions by reducing the concentration of bovine serum albumin (BSA) present, thereby increasing bioavailable FFA. Despite albumin having 7 FA-binding sites (van der Vusse, 2009), only 2 FA molecules are conjugated to each albumin in the circulation (Oliveira*, et al.*, 2015). It is said that the FFA:albumin ratio can rise to 6 in certain disease states (Kleinfeld*, et al.*, 1996; Nehra*, et al.*, 2001), however, many studies use higher ratios to study mechanisms of lipotoxicity.

Experimentally, FFA to BSA molar ratios between 8:1 and 2:1 were used in lipotoxicity studies. 8:1 (Hatanaka*, et al.*, 2014), 7:1 (Mei*, et al.*, 2011; Song*, et al.*, 2007), 6.6:1 (Hall*, et al.*, 2014; Lai*, et al.*, 2008; Listenberger*, et al.*, 2003a; Mantzaris*, et al.*, 2011), 6:1 (Chu*, et al.*, 2010; Martinez*, et al.*, 2008; Nagaoka*, et al.*, 2015; Schulz*, et al.*, 2013), 5:1 (Briaud*, et al.*, 2001; Laurens*, et al.*, 2016), 3.5:1 (Lou*, et al.*, 2014) and 2:1 (Cacicedo*, et al.*, 2011; He*, et al.*, 2016; Hetherington*, et al.*, 2016; Stoianov*, et al.*, 2015). The FFA to BSA used in this study was guided by the literature and did not exceed 7:1 (Table 2.2).

1.9.2 HepG2

HepG2 are a widely used hepatocellular carcinoma cell line that are highly differentiated, displaying many genotypical traits of normal liver cells (Gerets*, et al.*, 2012; Sassa*, et al.*, 1987). Yet, they have a basal gene expression profile distinct from primary human hepatocytes (Jennen*, et al.*, 2010). This may be due to oncogenic transformation from their origin as hepatocellular carcinoma cells, but may also be a result of extensive passaging leading to genetic mutations (Marx, 2014).

The metabolic capacity of HepG2 is also low in comparison to primary hepatocytes (Xu*, et al.*, 2004), meaning they are more suited to testing the toxicity of molecules than their metabolites. Additionally, HepG2 cells are partially deficient in cytochromes whilst levels of phase II enzymes, excluding UDP-glucuronosyl transferases, are expressed in normal levels (Westerink & Schoonen, 2007).

Regardless of these drawbacks, the sensitivity of HepG2 in the detection of hepatotoxic treatments was established to be 90% (O'Brien*, et al.*, 2006).

1.9.3 L6

Most established immortalised skeletal muscle cell lines exhibit little insulin-dependent glucose uptake and GLUT4 translocation, except for rat L6 and mouse C2C12 myotubes (Nedachi & Kanzaki, 2006). However, L6 myotubes exhibit greater glucose uptake following insulin stimulation than C2C12 cells (Sarabia*, et al.*, 1990), thereby highlighting L6 myotubes as the most promising *in vitro* skeletal muscle cell model for this project. Furthermore, L6 maintain many of the same characteristics as mature skeletal muscle cells and grow in a monolayer, thus, allowing even accessibility to substrates (Klip*, et al.*, 1986).

1.9.4 3T3-L1

3T3-L1 is an immortalised cell line developed from murine Swiss 3T3 cells (Mehra*, et al.*, 2007). The cells are morphologically indistinguishable from fibroblasts yet are already committed to the adipocyte lineage (Mehra*, et al.*, 2007). Differentiation is achieved through treatment with pro-differentiation agents for approximately 5 days followed by a period of maintenance, which results in the development of mature adipocytes characterised by the formation of intracellular lipid droplets (Zebisch*, et al.*, 2012).

3T3-L1 are used extensively as a model for insulin signalling studies as they utilise two isoforms of glucose transporters; a constitutive glucose transporter, GLUT1 and an insulin-sensitive transporter, GLUT4 (Thomson*, et al.*, 1997). The fibroblast phenotype expresses only GLUT1, whilst upon differentiation the adipocyte phenotype additionally expresses GLUT4 (Thomson*, et al.*, 1997). 3T3-L1 are thought to represent a valid model for insulin signalling studies as evidence indicates their metabolism mimics that of adipocytes isolated from adipose tissue (Macdougald*, et al.*, 1995; Mehra*, et al.*, 2007).

1.9.5 Washed human platelets

Three platelet models exist for in vitro studies: whole blood, platelet rich plasma (PRP) and washed platelets. Despite being more physiologically relevant, whole blood and PRP still contain many components of the blood that could potentially interfere and/or mask the effects being studied. The plasma in whole blood and PRP contain fluctuating FFA concentrations which would make the study of the effects of individual fatty acids impossible. Therefore, a washed platelet model was selected.

2 Materials and methods

2.1 Chemicals and reagents

Palmitic acid ≥99% (catalogue no. P0500) and oleic acid ≥99% (catalogue no. O1008) were purchased from Sigma-Aldrich (Dorset, UK).

Human hepatocellular carcinoma cells (HepG2 -ATCC® HB-8065™) were purchased from American Type Culture Collection (ATCC) (Virginia, USA), cultured in Roswell Park Memorial Institute (RPMI, catalogue no. 21875-034) medium from Thermo Fisher (Massachusetts, USA) and used between passage 6-15. Rat skeletal muscle cells (L6 - ATCC® CRL-1458™) were purchased from ATCC (Virginia, USA), cultured in Dulbecco's Modified Eagle Medium (DMEM, catalogue no. 41965-039) from Thermo Fisher (Massachusetts, USA) and used between passage 4-15. Mouse fibroblasts (3T3-L1 - ATCC® CL-173™) were purchased from ATCC (Virginia, USA), cultured in Dulbecco Modified Eagle Medium (DMEM, catalogue no. 41965-039) from Thermo Fisher (Massachusetts, USA) and used between passage 4-6. All medium contained 10% foetal bovine serum (catalogue no. F2442) and 1% penicillin/streptomycin (catalogue no. P4333) from Sigma-Aldrich (Dorset, UK). FITC Annexin V Apoptosis Detection Kit (catalogue no. 556419) was purchased from BD Pharmingen (Wokingham, UK) and EnzChek® Caspase-3 Assay Kit #1 (catalogue no. E13183) was obtained from Thermo Fisher (Massachusetts, USA). All other reagents were purchased from Sigma Aldrich (Dorset, UK).

Human platelets were obtained from volunteers. Thrombin (catalogue no. T9326) was obtained from Sigma-Aldrich (Dorset, UK) and CRP-XL was donated by the Department of Pharmacology at Cambridge University, UK. Antibodies against markers of activation, anti-CD62P (catalogue no. MCA2420PE) and anti-CD63 (catalogue no. MCA4754) were obtained from Bio-Rad (California, USA). Anti-PAC1 (catalogue no. MABT893) was purchased from Merck Millipore (Massachusetts, USA). Pierce™ LDH Cytotoxicity Assay Kit (catalogue no. 88953) was acquired from Thermo Fisher (Massachusetts, USA). All other reagents were purchased from Sigma Aldrich (Dorset, UK).

2.1.1 Fatty acid solutions

Fatty acid stock solutions were created by dissolving palmitic acid (PA) or oleic acid (OA) in 100% methanol (catalogue no. 34860, Sigma Aldrich, Dorset UK) to a concentration of 100 mM. These stock solutions were diluted in cell culture medium containing 1% w/v BSA (catalogue no. A7030, Sigma Aldrich, Dorset UK) to produce the final working concentrations prior to treatment of the cells. Treatment medium was dispensed onto the cells for 24 hours. Final treatment concentrations are shown in Tables 2.1 and 2.3. Concentrations of free fatty acids in the medium prior to the addition of PA and/or OA could not be ascertained due to the use of a proprietary formula of Thermo Fisher Scientific, however, they were said to be low.

1% w/v BSA was added to each treatment solution (151 µM). BSA: FFA molar ratios are presented in Table 2.2.

Table 2.1 Final concentrations of fatty acids used in cell culture experiments in medium containing 1% BSA

	Concentration (mM)							
	PA	**OA**	**PA**	**OA**	**PA**	**OA**	**PA**	**OA**
Palmitic acid	0.17	-	0.33	-	0.67	-	1.0	-
Oleic acid	-	0.17	-	0.33	-	0.67	-	1.0
2 : 1 combination OA : PA	0.17		0.33		0.67		1.0	
	0.06	0.11	0.11	0.22	0.22	0.45	0.33	0.67

Table 2.2 BSA : fatty acid molar ratio in final concentrations

Concentration (mM)	FFA to BSA molar ratio
0.06	0.38:1
0.11	0.73:1
0.17	1.13:1
0.22	1.46:1
0.33	2.19:1
0.45	2.98:1
0.67	4.44:1
1.00	6.62:1

Fatty acids in platelet experiments were added to calcium free Tyrode's solution (Table 2.4) without BSA as its addition altered the turbidity of washed platelets thereby preventing analysis by light transmission aggregometry. Initial concentrations are shown below in Table 2.3. A wide range of concentrations were used as lower levels of unbound FFA are present in the plasma than bound to albumin. Eventually, only the effects of 0.1 mM oleic acid were investigated.

Table 2.3 Final concentrations of fatty acids used in platelet experiments using a logarithmic scale

	Concentration (mM)						
Palmitic acid	0.001	0.003	0.01	0.03	0.1	0.3	1
Oleic acid	0.001	0.003	0.01	0.03	0.1	0.3	1

2.1.2 Calcium Free Tyrode's (CFT) buffer

CFT buffer is the most commonly used buffer in platelet research and was selected as the presence of above normal levels of calcium can inhibit platelet aggregation, thus, invalidating results (Herrmann*, et al.*, 1970). The formulation can be found in Table 2.4. NaCl (catalogue no. S7653), KCl (catalogue no. P9333), HEPES (catalogue no. H3375), NaH_2PO_4 (catalogue no. S8282), $NaHCO_3$ (catalogue no. S5761), $MgCl_2$ (catalogue no. M8266) and glucose (catalogue no. D9434) were all ordered from Sigma Aldrich, Dorset UK. The pH of the solution was adjusted to pH 7.4 and checked every morning before use.

Table 2.4 Calcium-free Tyrode's (CFT) buffer formulation (500 ml of 1 mM stock)

CFT buffer	**Mass (g)**
NaCl	68.5
KCl	1.35
HEPES	5.0
NaH_2PO_4	0.2
$NaHCO_3$	5.95
$MgCl_2$	0.55
Glucose	2.8

2.1.3 Oil Red O solution

350 mg of Oil Red O was dissolved in 100 ml of 100 % isopropanol and filtered with 5-8 µm pore filter paper. The stock solution was stored at room temperature. Oil Red O working solution was freshly prepared by mixing the stock solution in a 3:2 ratio with double distilled water (ddH_20), and vortexed prior to use.

2.2 Cell culture methods

2.2.1 Cell culture

HepG2 (ATCC® HB-8065™) cells were purchased from ATCC. Upon arrival the vial was thawed by gentle agitation in a 37°C water bath, transferred to a centrifuge tube containing 9 ml of Roswell Park Memorial Institute medium (RPMI) and spun at 125 x g for 5 minutes. The pellet was resuspended in 20 ml of pre-incubated, supplemented RPMI and transferred into a 75 cm^2 flask (T-75).

L6 (ATCC® CRL-1458™) cells were purchased from ATCC. Upon arrival the vial was thawed by gentle agitation in a 37°C water bath and transferred into a T-75 containing 20 ml of pre-incubated, supplemented Dulbecco's Modified Eagle's Medium supplemented (DMEM). The myocytes were differentiated to form multinucleated myotubes and striated fibres by allowing them to mature at a high confluence over 5 days (Figure 2.1). Cell fusion gradually declined with passage, so cells with a passage higher than 15 were not used in this study.

3T3-L1 (ATCC® CL-173™) cells were purchased from ATCC. Upon receipt the vial was thawed by gentle agitation in a 37°C water bath, transferred to a centrifuge tube containing 9 ml of DMEM and spun at 125 x g for 5 minutes. The pellet was resuspended in 10 ml of pre-incubated, supplemented DMEM and transferred into a 25 cm^2 flask (T-25).

All cells were incubated in a humidified incubator with an atmosphere of 5% CO_2 at 37°C.

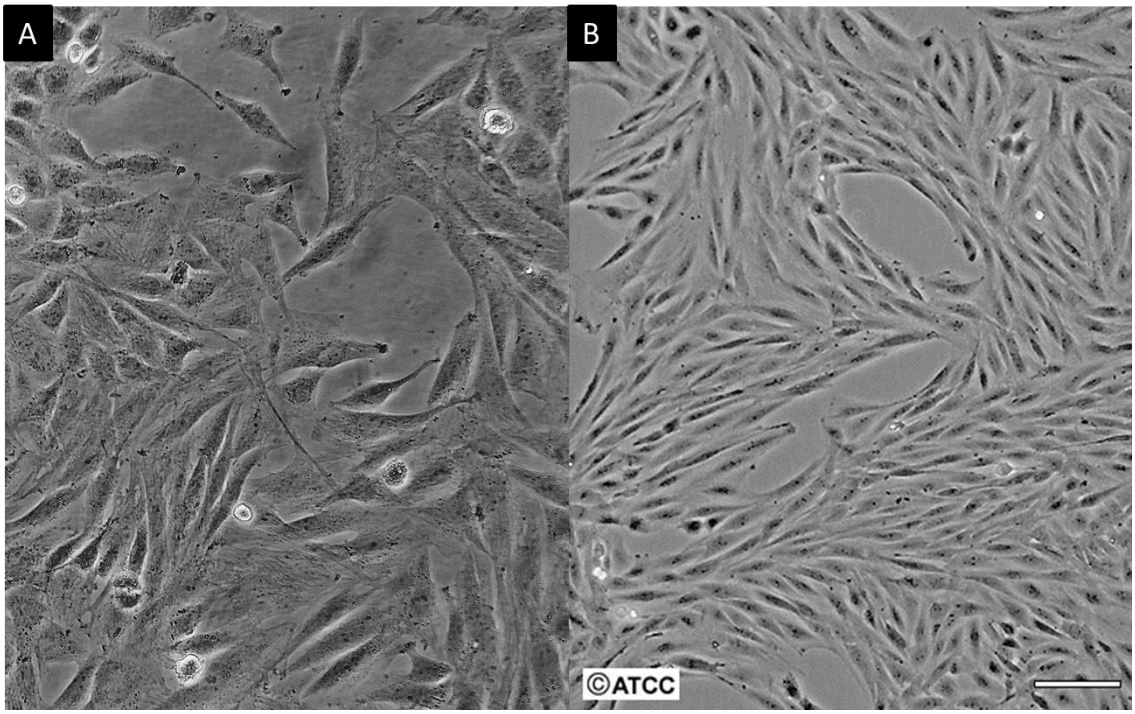


Figure 2.1 Image of differentiated L6 myotubes

A) Inverted microscopy image of L6 myotubes following differentiation at 1000x magnification. B) Image of differentiated L6 myotubes supplied in ATCC literature (ATCC, 2017).

2.2.1.1 Subculturing Procedure

Cells were subcultured before reaching 70% confluence. The culture medium was removed and discarded, and cells were washed gently with phosphate-buffered saline (PBS). 1 ml of trypsin-EDTA solution was added per 25 cm^2 of flask and observed under an inverted microscope until the cell layer dispersed. Dispersal was often facilitated by placing flasks in an incubator at 37°C. Upon detachment, medium was added to neutralise the trypsin-EDTA and cells were aspirated *via* pipette. Appropriate aliquots of the cell suspension were transferred to new culture vessels. A subcultivation ratio of between 1:4 and 1:6 was used for HepG2 (ATCC, 2018b), whilst a ratio of between 1:20 and 1:40 was used for L6 (ATCC, 2017). For 3T3-L1, new flasks were inoculated with 2 X 10^3 cells/cm^2 (ATCC, 2018a).

2.2.2 Assessment of cell viability

2.2.2.1 Cell Counting Kit 8

For experimental conditions, a 96 well-plate was plated with 100 µl/well of 16,000 cells/ml cell suspension. The plate was incubated in a humidified incubator at 37°C with 5% CO_2 for 24 hours. Cells were treated with control and fatty acid solutions and returned to the incubator for a further 24 hours. 10 µl CCK-8 reagent was added to each well, and the plate was returned to the incubator for 2 hours. Following this incubation period, the absorbance was measured at 450 nm (Tecan Sunrise absorbance microplate reader).

Initially, a standard curve (Figure 2.2A) was created to assess the linearity of absorbance, by diluting a cell suspension of 100,000 cells/well in increments of 2000 cells/well in a 96-well plate. The above method was used and and the results used to select a seeding density for the following experimentation.

The confidence interval for the line of best-fit was defined by the confidence bands on either side. The curved nature of the dashed confidence bands indicate the boundaries of all possible lines of best-fit, rather than indicating that the line of best-fit may be a curve. Given the assumptions of linear regression, the confidence bands encapsulated the true best-fit line of non-linear regression with a 95% certainty (Figure 2.2A). They indicated a consistent linear relationship between number of cells and absorbance below 20000 cells, therefore, allowing use of the line of best-fit to calculate percentage viability.

The slight inverted u-shaped distribution on the residuals plot indicated that a non-linear regression was preferable over linear regression. However, the spread appeared to be random and, thus, did not allow the prediction of error, indicating that only random error remained and the non-linear regression model was a good fit (Figure 2.2B).

A non-linear relationship was found between the number of cells and absorbance above 22,000 cells per well, where the curve plateaued and standard error increased due to saturation of the solution in the wells. Therefore, a final cell density of ~16,000 cells per well were utilised to maximise effect size without invalidating results by reaching saturation.

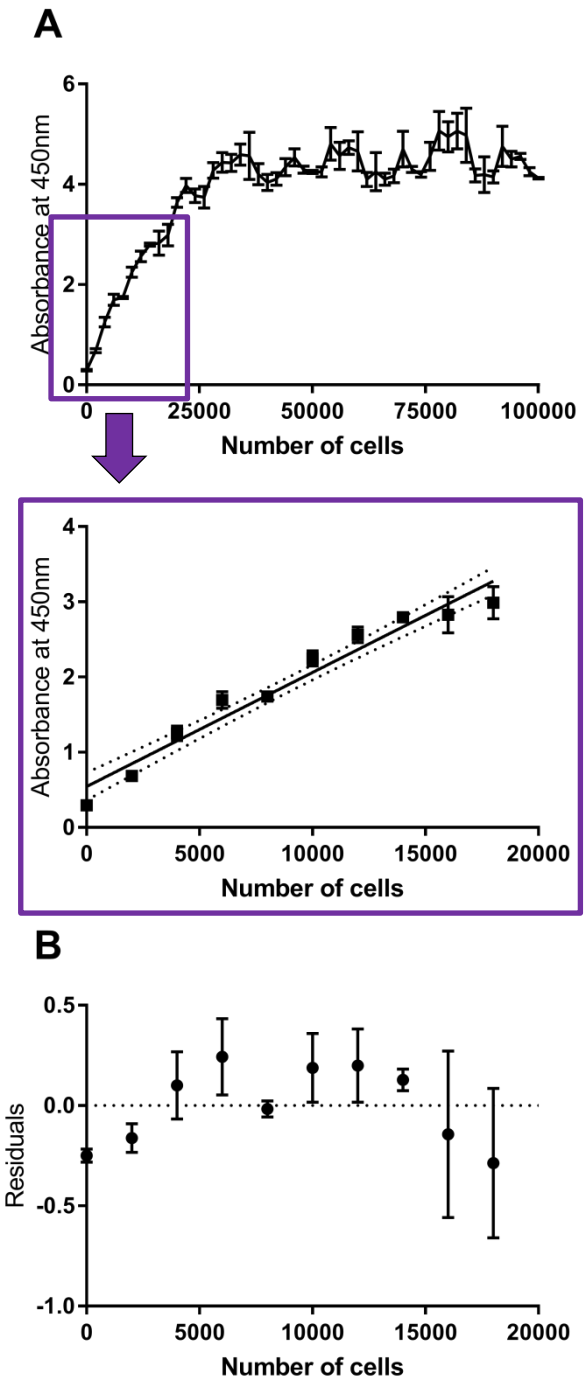


Figure 2.2 CCK-8 produced a linear relationship between the number of cells and absorbance

A) Up to 20000 cells per well, the absorbance of CCK-8 was directly proportional to the number of cells present. B) The random distribution of points on the residuals plot indicated that the model used for the line of best-fit was correct.

2.2.3 Stages of cell death determined by flow cytometric analysis

2.2.3.1 Annexin V/propidum iodide assay

Flow cytometric apoptosis assays exploit exposed phosphatidylserine (PS) *via* the use of fluorophore conjugated annexin V, a phospholipid-binding protein with high affinity for PS. The translocation of PS precedes the loss of plasma membrane integrity, which is evident in the subsequent stages of apoptotic and necrotic progression. Therefore, the use of a nuclear counter stain such as propidium iodide (PI) with annexin V is vital for flow cytometric analysis of cell states ranging from viable to apoptotic, necrotic and dead. The intact, but damaged membranes of early apoptotic cells are targeted by the annexin V, but are impermeable to PI, however, as apoptosis and/or necrosis progress the membrane is further damaged, becoming permeable to PI allowing for the identification of the later stages of cell death.

Flow cytometric analysis of annexin V/PI stained cells is divided into 4 quadrants (Figure 2.4). Annexin V-and PI-negative cells are considered viable, as PS is not located extracellularly. Annexin V-positive and PI-negative cells are classified as early apoptotic due to the translocation of PS to the external surface of the cell membrane. Annexin V-negative and PI-positive cells are late apoptotic/necrotic, portraying external cellular damage as PS has not translocated to the extracellular surface of the cell membrane, yet nuclear content is leaking. Annexin V-and PI-positive cells are considered to be dead as both PS and nuclear material can be accessed by the antibodies.

Cells were grown in T-25 flasks and treated with fatty acids for 24 hours once 70% confluent. After treatment the cells were trypsinised, neutralised with medium and transferred to 15 ml centrifuge tubes.

A FITC Annexin V Apoptosis Detection Kit was used to assess cell death. Binding buffer supplied in the kit was diluted 1:9 with ddH_2O. The cells were washed with 5 ml of chilled cell wash then centrifuged, twice. The pellets were resuspended in 300 µl of binding buffer solution and 150 µl of each sample was transferred to a 5 ml Falcon tube. 5 µl of annexin V

and 5 µl of PI was added to each Falcon tube. The cells were gently vortexed and incubated in the dark, at room temperature for 15 minutes. 300 µl of binding buffer solution was then added to each tube and agitated prior to analysis.

Samples were analysed on a BD Accuri™ C6 (BD Biosciences), with 10,000 events per sample acquired. Control flow cytometry plots were used to gate the cell population and exclude debris from further analysis. A forward scatter vs. side scatter plot included in a template supplied by BD Biosciences for the FITC Annexin V Apoptosis Detection Kit was used to gate the visible cell population (Figure 2.3).The template was also used with the gated flow cytometry results to separate plots into four quadrants; live, dead, early apoptotic and late apoptotic/necrotic (Figure 2.4). Raw data and the percentage of events in each quadrant was collected and analysed in conjunction with visual analysis of the plots. Forward scattered light and side scattered light were measured and converted to percentage change from the control.

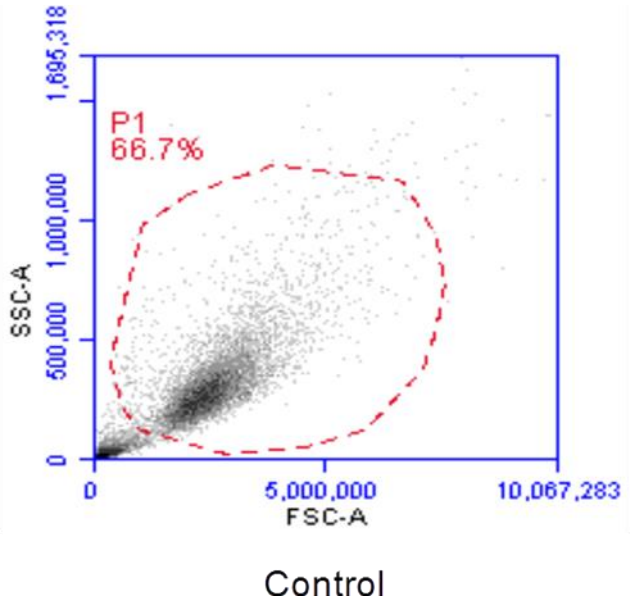


Figure 2.3 Exclusion of debris using the BD Bioscience kit template

A gate drawn around the visible cell population in the control samples excluded low FSC and SSC events, which were likely to be debris.

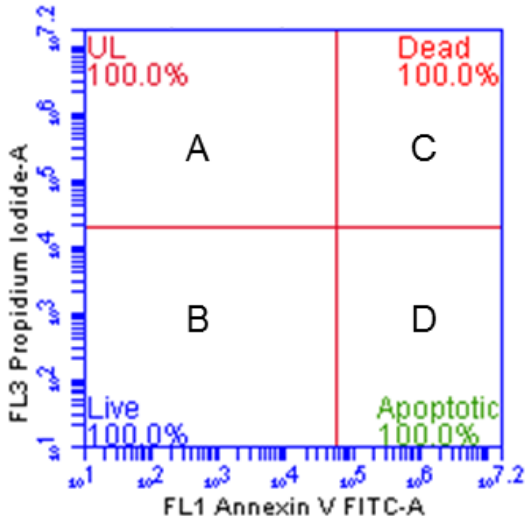


Figure 2.4 BD Biosciences template used to gate different stages of cell death

A) Annexin V-negative/PI-positive quadrant where late apoptotic/necrotic events were recorded. B) Annexin V-and PI-negative quadrant where live cells were logged. C) Annexin V-and PI-positive quadrant where dead cells were measured. D) Annexin V-positive/PI negativequadrant where apoptotic events were counted.

2.2.4 Determination of apoptosis by caspase 3/7 assay

Apoptosis, a form of programmed cell death, plays a vital role in many disease states and disorders (Favaloro*, et al.*, 2012). Apoptosis is distinct from necrosis, both biochemically and morphologically and, thus, can be distinguished from one another. Apoptosis causes condensation of the nuclear chromatin and shrinkage of the cytoplasm whilst fragmenting the genome and degrading a number of cellular proteins including cytokeratins, alpha-fodrin, poly (ADP-ribose) polymerase (PARP), and nuclear mitotic apparatus protein (NuMA) (Slee*, et al.*, 2001). The caspase CED-3/ICE family of proteases are thought to be crucial mediators of these detrimental biochemical processes. Caspase-3 cleaves a range of proteins, a number of which are important in the initiation of apoptosis through its specificity for the amino acid sequence Asp-Glu-Val-Asp (DEVD) (Asakura*, et al.*, 1999; Shiah*, et al.*, 1999; Wolf*, et al.*, 1999).

2.2.4.1 EnzChek® Caspase-3 Assay Kit #1

The EnzChek® Caspase-3 Assay Kit #1 enables the detection of apoptosis by measuring increases in caspase-3 activity and the activity of other DEVD-specific proteases such as caspase-7. The assay capitalises on the use of Z-DEVD–AMC substrate which is mildly fluorescent in its native state (excitation/emission ~330/390 nm), but which produces a strong blue fluorescent product (excitation/emission ~342/441 nm) upon proteolytic cleavage (Thermo Fisher, 2003). Fluorescence measured *via* a fluorescence microplate reader is directly proportional to caspase-like activity. Furthermore, this activity can be quantified with the use of a reference standard and the result verified with the use of an inhibitor (Blaak*, et al.*, 2007).

The EnzChek® Caspase-3 Assay Kit #1 (Thermo Fisher) was utilised to assess apoptosis in L6 cells. The assay was not used for HepG2 cells as these cells were found to clump in culture, thereby risking high levels of background cell death.

Cells were cultured in T-25 flasks following the aforementioned procedure until 60% confluent, and differentiated in to myotubes prior to treatment. Cells were incubated for 24 hours with the fatty acid solutions at 37^{o}C with 5% CO_2. Negative controls were also prepared by treating cells with medium containing 1% BSA, but no fatty acids.

Following treatment, cells were harvested by trypsinisation *via* the aforementioned method and washed in ice cold phosphate-buffered saline (PBS) three times prior to use of the EnzChek® Caspase-3 Assay Kit #1. Cell pellets were resuspended in 50 µL of cell lysis buffer, frozen in a dry ice-ethanol bath for 5 minutes and then thawed. The lysed cells were centrifuged at 5000 rpm for 5 minutes in a micro-centrifuge to pellet cell debris, and 50 µL of each supernatant aliquoted into individual wells of a 96-well plate. 50 µL of cell lysis buffer was used to determine background fluorescence as a no-enzyme control. 1 µL of 1 mM Ac-DEVD-CHO inhibitor was added to specific samples. These samples were covered with foil and incubated at room temperature for 10 minutes. Meanwhile, 1 µL of DMSO was added to another set of samples without the inhibitor and incubated under the same conditions, whilst the remaining samples were stored on ice for 10 minutes. 50 µL of substrate working solution, containing Z-DEVD–AMC substrate and reaction buffer was added to each sample and control. The microplate was covered with foil and incubated at room temperature for 30 minutes. Meanwhile, a 7-amino-4-methylcoumarin (AMC) standard curve was created. 10 mM AMC stock solution was diluted in reaction buffer to yield a concentration range between 0-100 µM. 100 µL of each concentration was dispensed into empty microplate wells. Fluorescence was measured using a PerkinElmer fluorescence plate reader at an excitation of 342 nm and emission of 441 nm every 10 minutes for 5 hours.

The reference standard, AMC was used to enable the quantitation of AMC released, and, thus, caspase activity (Figure 2.6B). A reversible aldehyde inhibitor, Ac-DEVD-CHO was employed to confirm that caspase-3-like proteases were responsible for the observed fluorescence signal in control and treated cell populations.

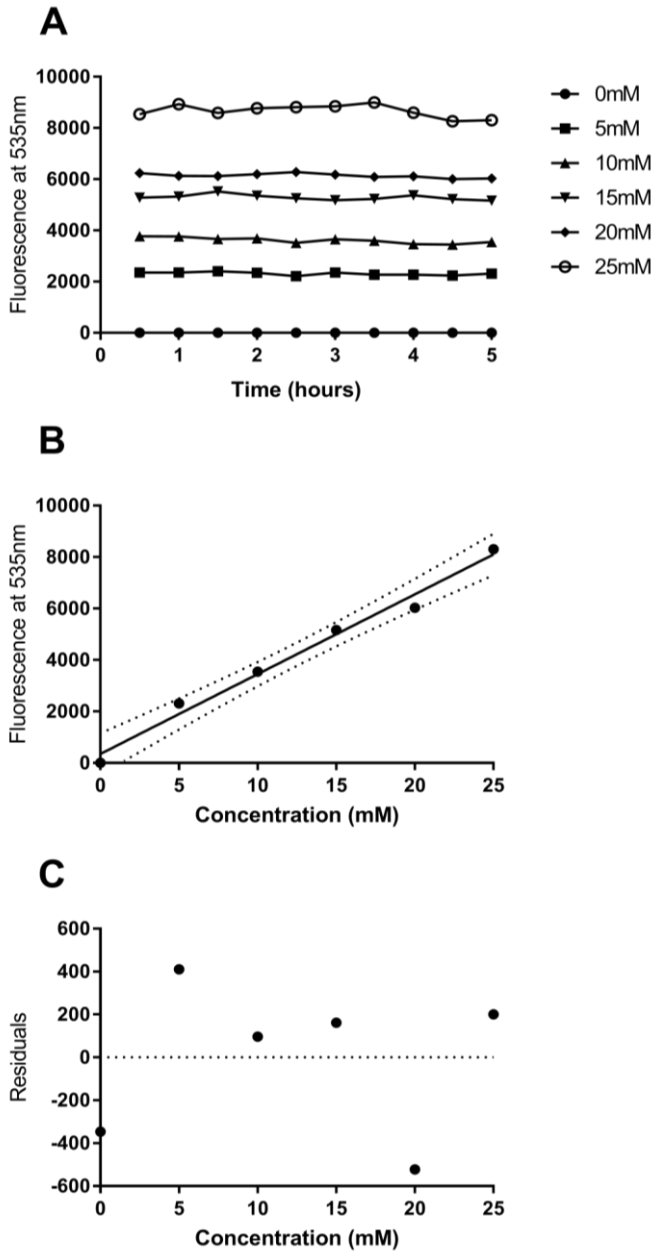


Figure 2.5 Fluorescence of the reference standard, AMC, over time at increasing concentrations

The reference samples did not increase in fluorescence over time and produced a linear line of best-fit. A) Plotted over time, the fluorescence produced by all concentrations of the reference did not increase over time. B) At 5 hours, there was a linear relationship between concentration of the reference standard and fluorescence. C) A residuals plot showed random distribution indicating the correct model for the line of best-fit was selected.

Readings were taken every 10 minutes for 5 hours to find the optimum time point at which the samples were adequately fluorescent for statistical analysis of the data (Figure 2.6). The AMC reference standard was also read every 10 minutes for 5 hours to ensure evaporation was not occurring (Figure 2.5A). The 5 hour time point was selected.

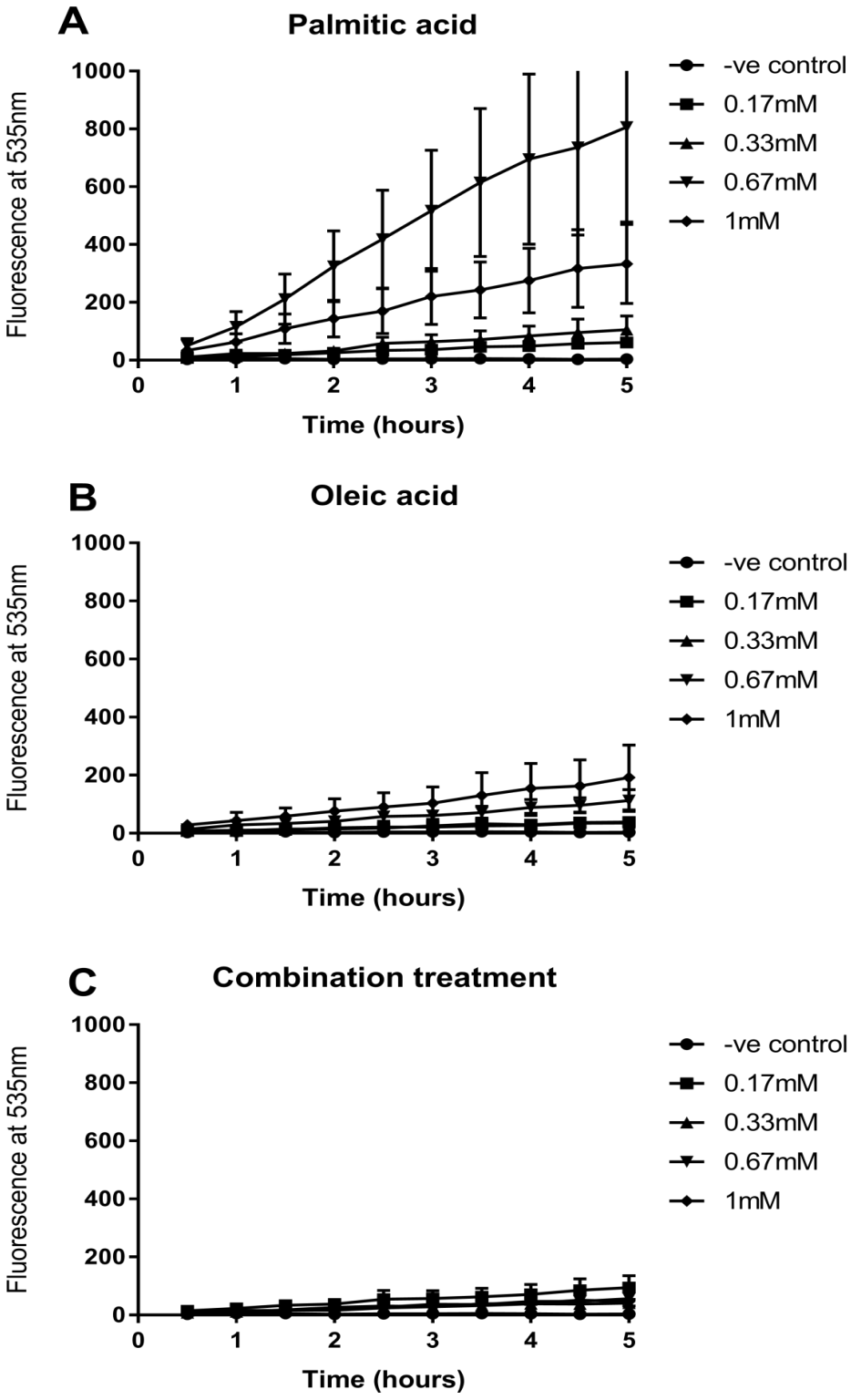


Figure 2.6 Fluorescence produced by all treatments increased linearly over time

Fluorescence produced by palmitic acid treated cells (A), oleic acid treated cells (B) and combination treated cells (C) increased linearly over time and produced maximal fluorescence at the 5 hour time point.

2.2.5 Intracellular lipid accumulation

2.2.5.1 Oil Red O assay

Cells were seeded at 8 x 10^4/ml onto coverslips in 6 well plates. Cells were treated at 60% confluence for 24 hours. After 24 hours, the medium was removed and the cells were washed with PBS. 2 ml of 10% formalin was dispensed into each well and incubated at room temperature for 10 minutes. The formalin was discarded and replaced with 2 ml of fresh 10% formalin and incubated at room temperature for a further 2 hours. The formalin was then removed and the cells were washed twice with ddH_20. 2 ml of 60% isopropanol was dispensed into the wells and the cells were incubated at room temperature for 5 minutes. The isopropanol was removed and the cells were left to dry. 1 ml of Oil Red O working solution was added to the cells and incubated at room temperature for 10 minutes. Following incubation the Oil Red O was removed and the cells were immediately washed 4 times with ddH_20.

Intracellular Oil Red O was eluted out of the cells by 1 ml of 100% isopropanol and incubated on an agitator for 10 minutes. The solution was mixed *via* pipette and transferred to glass cuvettes for spectrophotometric analysis. The absorbance was measured at 500 nm with 100% isopropanol as a blank (Tecan Sunrise absorbance microplate reader).

2.2.5.2 ImageJ analysis of intracellular lipid accumulation

Cells were grown to 60% confluence and treated in T-75 flasks as described above. Medium was discarded and cells studied *via* inverted microscopy at 1000x magnification. A camera was attached to the microscope and photos were taken of random fields for ImageJ analysis.

A review of the literature identified two methods for the identification of lipid droplets *via* open-source ImageJ software (National Institutes of Health), using Macros MorphoLibJ morphological segmentation (INRA-IJPB Modeling and Digital Imaging lab) and MRI Lipid Droplets Tool (MRI). The ability of these two tools to count and/or measure lipid droplets in

unstained L6 cell images were assessed. However, neither method was capable of distinguishing lipid droplets to a high enough degree of accuracy (Figure 2.7 and 2.8, respectively). Therefore, a new method was developed to count lipid droplets in unstained images.

2.2.5.3 Method development for ImageJ analysis of unstained intracellular lipid droplets

Images from inverted microscopy were assigned unique identifiers and analysed blind. Their identity was revealed after all data collection had taken place. The image to be analysed was randomly selected from the pool of images, without knowing to which treatment or concentration it belonged. The photograph was opened, viewed at 100% size, converted to an 8-bit greyscale image, and the contrast increased using the sharpen tool. The area of interest was randomly selected by dragging the image across the field aperture, and then cropped (Figure 2.9A).The image was thresholded by eye to include the maximum number of lipid droplets without introducing artefacts (Figure 2.9B). This was performed on the acquired images to segment background from lipid droplets, which was possible as the edge of lipid droplets were more highly saturated than other structures within the cell. The images were converted to binary images and processed to fill holes and view outlines. Lipid droplets that had not been adequately captured by thresholding were corrected using the ImageJ pencil tool. The watershed feature was also used to separate adjoining outlines (Figure 2.9C). The images were manually compared against the original image to ensure consistency and correct binary conversion prior to measuring the number of lipid droplets present per field. Finally, the image was analysed using the 'analyse particles' feature with limits set at 10 pixels to infinity, to include all levels of sphericity, and to produce an image of outlines including holes along with the number of lipid droplets present (Figure 2.9D). Data was loaded into GraphPad Prism 7 for statistical analysis.

Note that the limits for the final analysis of particles could have been lowered from 10, however, this would likely have included artefacts in the results.

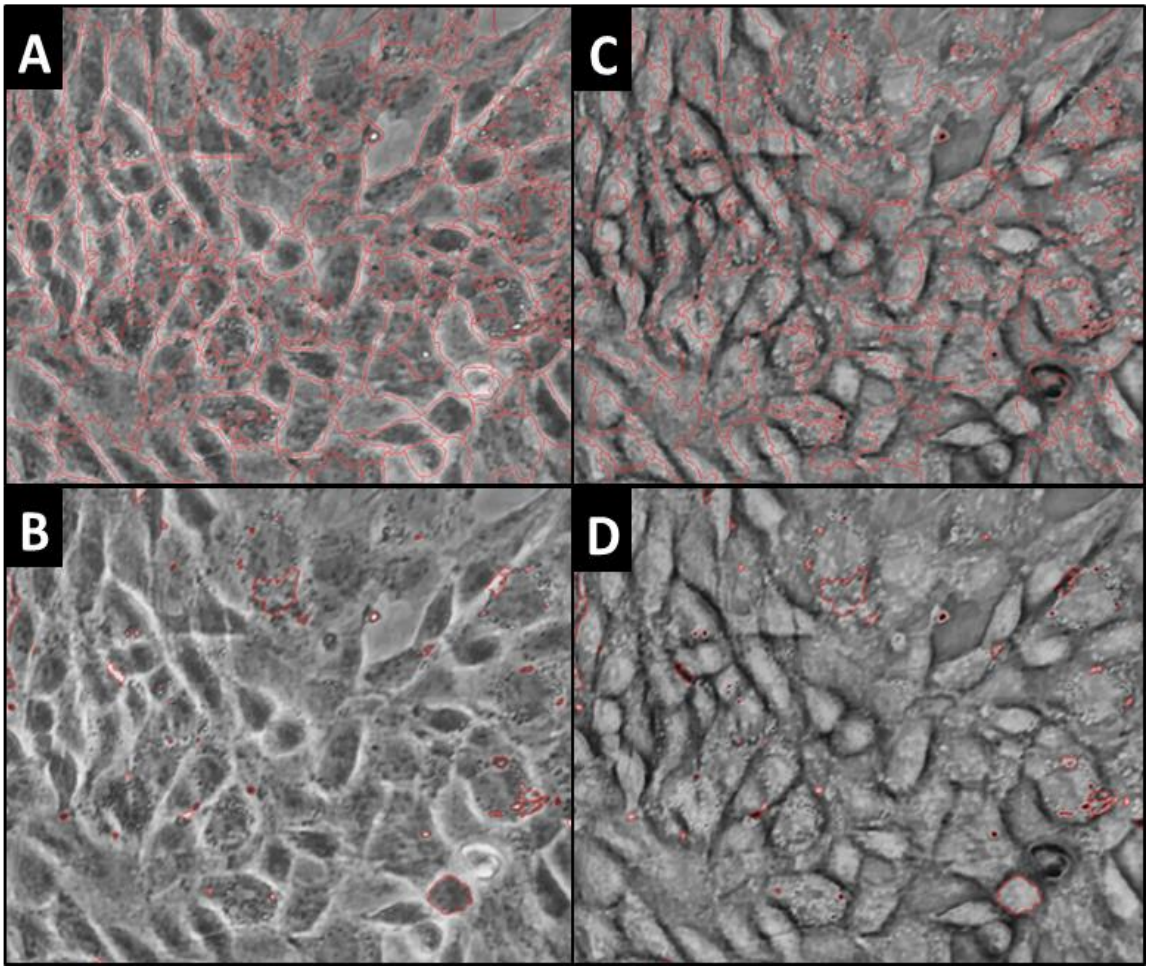


Figure 2.7 MorphoLibJ morphological segmentation analysis of lipid accumulation did not produce acceptable results

A) Normal 8-bit image analysed as a 'Border Image'. Watershedding identified whole cells not lipid droplets. B) Normal 8-bit image analysed as an 'Object Image'. Watershedding outlined miscellaneous areas of the image, not lipid droplets. C) Inverted colour 8-bit image analysed as a 'Border Image'. Watershedding was unable to identify any structures. D) Inverted colour 8-bit image analysed as an 'Object Image'. Watershedding outlined miscellaneous areas of the image, not lipid droplets.

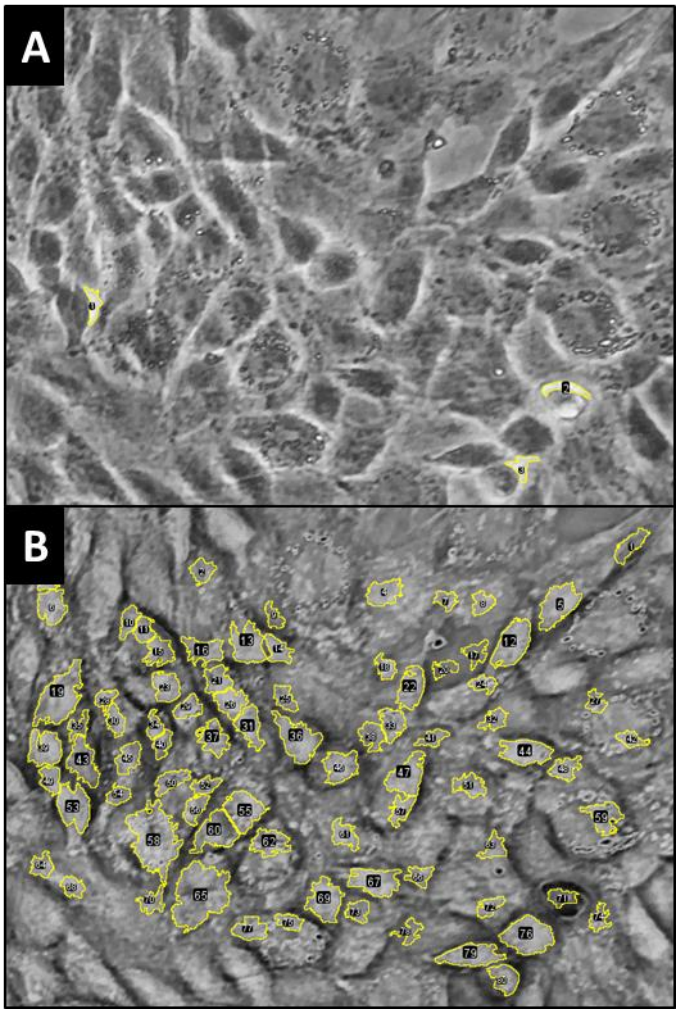


Figure 2.8 MRI Lipid Droplets Tool did not identify lipid droplets

A) Normal 8-bit image following analysis. Watershedding identified few structures, mainly interfaces between cells and not lipid droplets. B) Inversed colour 8-bit image following analysis. Watershedding outlined cells not intracellular lipid droplets.

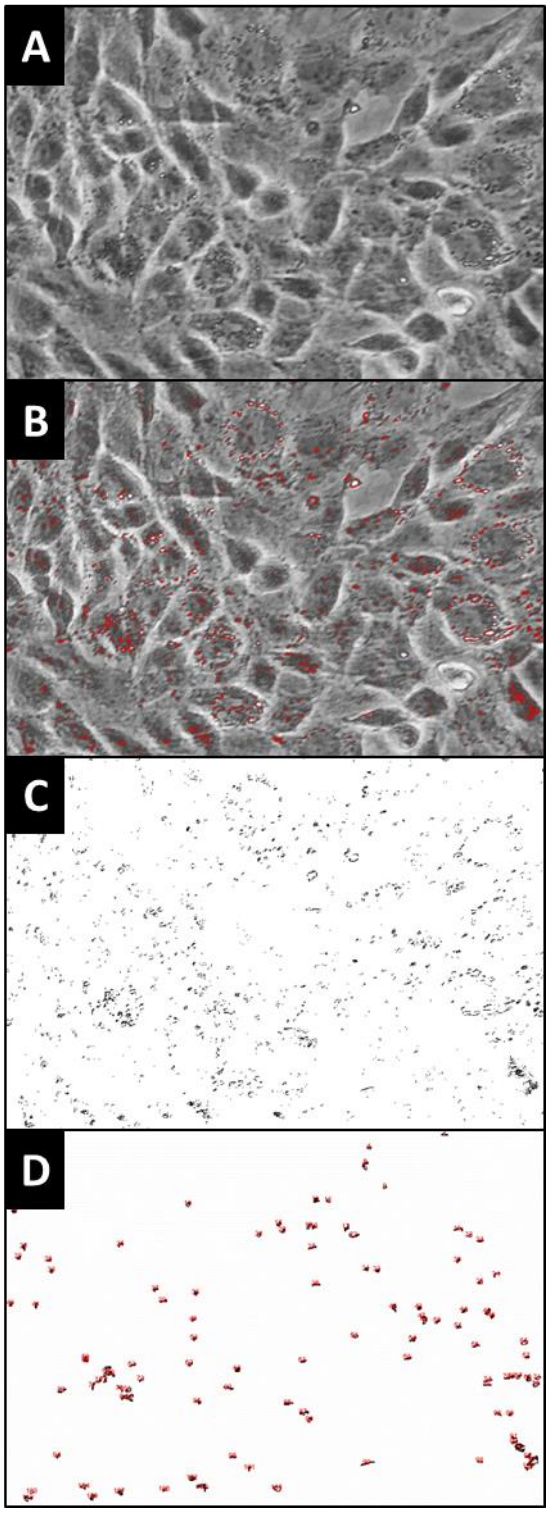


Figure 2.9 Visual representation of novel method to count unstained intracellular lipid droplets using ImageJ

A) 8-bit sharpened and cropped image. B) Thresholded lipid droplets. C) Binarised image containing only lipid droplets and background. D) The counted lipid droplets.

2.3 Platelet methods

2.3.1 Blood collection

The use of human blood in this project was granted ethical approval by the Research Ethics Panel of the Faculty of Science and Technology, Anglia Ruskin University, UK. Donors were selected at random from a pool of healthy adult volunteers, with no exclusion criteria for smokers and/or consumers of alcohol. Before donation, the volunteers provided informed consent and confirmed that medication, such as aspirin, that may affect platelet function, had not been taken within 14 days prior to blood donation. Blood was drawn by venepuncture of the median cubital vein in the cubital fossa using a 21G butterfly needle. Blood was collected into 50 ml syringes containing trisodium citrate (11 mM) in a 1:5 (v/v) concentration.

2.3.1.1 Isolation of platelets from donor blood

Following collection, blood was transferred into 15 ml Falcon tubes and centrifuged at 125 x g at room temperature for 15 minutes. Centrifugation separated the blood into three layers, the bottom-most red layer of erythrocytes, the cloudy middle layer of leukocytes (buffy coat) and the upper layer of platelet-rich plasma (PRP). The PRP was gently aspirated off and dispensed into clean 15 ml Falcon tubes, with care taken to leave the buffy coat undisturbed. Prostaglandin E1 (PGE1), a reversible inhibitor of platelet activation, was added at a concentration of 1 µM and gently inverted to mix before centrifugation at 200 x g for 15 minutes. The supernatant, otherwise known as platelet-poor plasma (PPP), was aspirated off and discarded. The platelet pellet was gently resuspended in CFT (Table 2.4) and incubated in a water bath at 37 °C for 1 hour before being counted and adjusted to achieve the required platelet count.

2.3.2 Methodology for the investigation of platelet aggregatory responses

2.3.2.1 Light transmission aggregometry

Platelet aggregation was measured in a Helena Biosciences AggRam, light transmission aggregometer (Helena Biosciences Europe). Washed platelet preparations (250 µl) were aliquoted from stock stored in a water bath at 37°C into glass cuvettes, and incubated in the AggRam for 2 minutes without stirring to allow the platelets to equilibrate prior to stimulation. The program was then initiated, and the appropriate agonists were introduced at the 30-second time point, under stirred conditions (1000 rpm). Aggregatory responses were measured for 15 minutes.

In order to study pathways of platelet activation in isolation, a number of inhibiting agents were utilised. In experiments to investigate secondary-mediated signalling, washed platelets were pre-treated with aspirin (1 mM), Prostaglandin E1 (PGE1) (2 µM) and N,N,N',N'-tetrakis(2-pyridylmethyl)ethane-1,2-diamine (TPEN) (50 µM), and incubated in a water bath at 37°C for 15 minutes prior to light transmission aggregometry. Aspirin was utilised to prevent the generation of thromboxane A_2 (TxA_2); PGE1 was employed to inhibit adenosine diphosphate (ADP) signalling; and TPEN, a Zn^{2+} chelating agent, was used to as a general inhibitor of secondary aggregation. To investigate protein kinase C (PKC)-mediated signalling, platelet suspensions were pre-incubated with GF109203X (10 µM) for 15 minutes prior to aggregometry, to inhibit PKC. To study integrin $\alpha_{IIb}\beta_3$-dependent signalling, washed platelets were pre-incubated with GR144053 (10 µM) and 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) (10 µM) for 15 minutes preceding aggregation analysis. GR144053 was used to competitively inhibit integrin $\alpha_{IIb}\beta_3$, whilst BAPTA, a Ca^{2+} chelator, was employed to inhibit the activation of $\alpha_{IIb}\beta_3$.

2.3.2.2 Fixation of platelets

To explore the reliance of platelet aggregation on intracellular signalling, washed platelets were fixed prior to LTA.

Washed platelets were fixed with the addition of paraformaldehyde in order to determine whether oleic acid-induced aggregation was dependent on intracellular signalling. A final concentration of 4% (v/v) paraformaldehyde was added to washed platelets and incubated in a water bath at 37^{o}C for thirty minutes. After incubation aggregometry studies were performed as mentioned above.

2.3.3 Flow cytometric analysis of platelet activation markers

The exposure of markers of platelet activation following stimulation was investigated using PE anti-CD62P, Alexa Fluor 647® anti-CD63, FITC anti-PAC1. Anti-CD62P is an antibody against P-selectin, and anti-CD63 is directed against CD63, both of which are expressed on the outer platelet membrane following α-granule release and dense granule release, respectively. Anti-PAC1 is directed against activated integrin $\alpha_{IIb}\beta_3$, the vital product of platelet activation. PE, FITC and Alexa Fluor 647® conjugated antibodies were chosen to enable simultaneous analysis *via* different FL channels. The excitation/emission wavelengths of FITC are 495/519 nm, and, thus, anti-PAC1 was analysed *via* the FL1 channel using a laser at 488 nm and a filter at 533 nm with a band pass of 30 nm. The excitation/emission wavelengths of PE are 496/576 nm. Therefore, anti-CD62P was measured using the FL2 channel with a 488 nm laser and a filter at 585 nm with a band pass of 40 nm. Alexa Fluor 647® is excited at 650 nm and emits at 665 nm, consequently anti-CD63 was analysed *via* the FL4 channel using a laser at 640 nm and a filter at 675 nm with a band pass of 25 nm (Abcam, 2012; BD Biosciences, 2011).

Tubes containing CFT (50 µl) and 1.5µl of each antibody were prepared once the washed platelets had been rested. The washed platelets (50µl) were added to each tube and mixed gently by flicking. Samples were incubated for 20 minutes at room temperature before being analysed continuously over 5 minutes. After 30 seconds, 1.5 µl of agonist or

treatment was added and mixed *via* pipette, to assess platelet activation in real-time for 5 minutes. Samples were analysed on a BD Accuri™ C6.

FITC annexin V was used separately to study the exposure of phosphatidylserine (PS) on the outer platelet membrane, which occurs following platelet activation. It was measured using the FL1 channel due to the conjugated FITC, using the method above.

Prior to experimentation, unstained platelets, isotype controls, untreated platelets with primary antibody only, untreated platelets with secondary antibody only, and untreated platelets with both primary and secondary antibodies were used to control background fluorescence.

2.3.4 Methodology for the investigation of platelet death

2.3.4.1 LDH assay

Platelet cell death was quantified using a Pierce™ LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific), following a modified version of the manufacturer's protocol. Platelets were stimulated in the aggregometer for 15 then centrifuged at 125 x g to remove the CFT containing released LDH. Aliquots of the supernatant (50 µl) were dispensed into a 96 well microplate, 50 µl reaction mixture was added and the plate was incubated at room temperature for 30 minutes, protected from light. Following incubation 50 µl of Stop Solution was added to each sample and mixed by gentle tapping. The absorbance was measured at 490 nm and 680 nm. The 680 nm value was subtracted from the 490 nm value to remove background signal from the instrument.

A standard curve was created to ascertain the optimum number of platelets required to produce a level of absorbance measurable with acceptable levels of error and without saturating the absorbance (Figure 2.10). The above method was used to measure LDH release in up to 2×10^7 platelets that had been lysed with lysis buffer. The optimum number of platelets for the LDH assay was assessed to be 2×10^6, which was used for the samples treated with fatty acids.

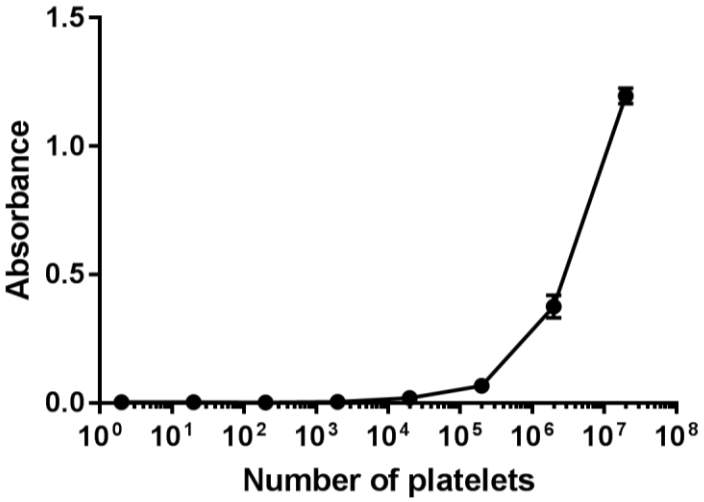


Figure 2.10 LDH standard curve demonstrated the absorbance produced by lysed platelets

The standard curve demonstrated the absorbance and hence total LDH released from platelets destroyed using lysis buffer. This was used to assess the optimum number of platelets required for the LDH assay to detect a significant increase in absorbance upon platelet death. This was determined to be 2×10^6 platelets.

2.3.4.2 Calcein determination of viability

Platelet viability was established using calcein AM, a cell-permeant, non-fluorescent dye that is hydrolysed by intracellular esterases in viable cells, thereby becoming fluorescent.

Calcein was added to freshly washed platelets to a final concentration of 2 µg/mL and incubated for 1 hour in a water bath at 30°C. After incubation, 1 µM PGE1 was added to the platelets and centrifuged at 1,000 × g for 10 minutes. The supernatant was discarded and the platelets washed with PBS supplemented with 1% BSA (w/v) and 1 µM PGE1. The platelets were centrifuged again at 1,000 g for 10 minutes, the supernatant discarded and resuspended in CFT. Calcein AM fluorescence has excitation and emission wavelengths of 495/515 nm respectively, so was measured using the FL1 channel on the BD Accuri™ C6 flow cytometer due to the appropriate laser and filter setup. Before experimentation, unstained platelets and stained untreated platelets were used as controls to determine background fluorescence.

2.3.5 Confocal microscopy

Confocal microscopy was performed to ensure platelets were in fact aggregating and not producing artefactual results *via* light transmission aggregometry.

Washed platelets were prepared and treated as aforementioned. The cuvettes were placed in the aggregometer and testing initiated. Cuvettes were removed at time points of 1, 2, 5, 10 and 15 minutes and 1 µL as immediately pipetted on to a cover slip. Samples were studied on a confocal microscope (Zeiss Axiovert 200M) at 600X magnification. Images were taken of random fields.

2.3.6 Analysis of competing hypotheses

Analysis of competing hypothesis software (ACH) was used to determine which hypothesis was mostly likely following experimentation and statistical analysis.

Analysis of competing hypotheses (ACH) is an analytical process that aims to provide an unbiased methodology for evaluating competing hypotheses through systematic evaluation of whether data is consistent or inconsistent with each hypothesis. This enables the rejection of hypotheses that are too inconsistent with the data. The concept works on the principle that data consistent with a hypothesis does not prove said hypothesis, as it may be consistent with multiple hypotheses and further research could reveal inconsistencies in the future. On the other hand, even a single piece of inconsistent data may be enough to disprove said hypothesis, and allow for its rejection (Heuer Jr., 1999).

ACH was performed with the help of ACH software (Palo Alto Research Center). Competing hypotheses present in this study were entered into the software, along with primary evidence collected in this project, and secondary evidence in the form of key pieces of literature. Each piece of evidence was individually assessed for its relevance to this study and its credibility as a source of evidence. Each piece of evidence was then judged against each hypothesis for inconsistences and marked as CC for very consistent, C for consistent, N for neutral, I for inconsistent and II for very inconsistent. Upon completion, the software computed weighted inconsistency scores based upon the relevance and credibility ratings.

2.4 Systematic review and meta-analysis

2.4.1 Search Strategy

Primary research articles and meta-analysis reviews were identified through electronic searches of the following databases: PubMed, Web of Knowledge, MEDLINE, SCOPUS and Google Scholar. Search terms included: free fatty acids, non-esterified fatty acids, palmitic acid, oleic acid, plasma, serum, phospholipids, cholesterol esters, triglycerides. The full list of search terms can be found in Appendix F. Articles identified through databases were supplemented by reviewing reference lists of review articles to identify additional eligible articles. All databases were searched by one reviewer, initially in April 2016, and again in January/February 2018.

2.4.2 Inclusion Criteria

Any study of adult plasma regardless of the date of publication, methodology or number of participants was included. The data must also have been presented in mol%, mass%, mg/L, mg/dL, mmol/L or µmol/L. Multiple articles from a single dataset were included when minimal overlap between factors existed.

No restrictions were imposed on publication date or methodology used for lipid measurement, although studies in adolescents, and those studying specific medical conditions were excluded.

2.4.3 Exclusion criteria

For the analysis of average concentrations of plasma lipid species and the comparison of lipid species between subpopulations, studies were excluded if they were missing information such as BMI, and SD or SEM. Also, groups of subjects with any reported medical condition, smokers, BMI below 20 kg/m^2 or following a specific diet were also excluded.

2.4.4 Data extraction

Articles were reviewed by one reviewer on two separate occasions. Those not meeting the eligibility criteria were discarded as were duplicates.

Data meeting the eligibility criteria was collated using the standard BMI measures of obesity (20-24.9 for normal, 25-29.9 for overweight and over 30 for obese). However, authors often broadened the standard BMI categories, using a BMI of up to 25 for lean participants. Many authors also used morbidly obese participants with a BMI of over 40. The categories for this meta-analysis were adjusted accordingly with non-obese including BMIs between 20-27.9, and overweight/obese using BMIs over 28. In studies that arranged the results by health condition or smoking status, only the control groups were used for analysis of average concentrations of plasma lipid species.

Data was extracted into a Microsoft Excel 2010 spreadsheet and separated by lipid species and unit of measurement, BMI, and subpopulation i.e. sex, medical condition, smoking status, etc

For analysis of average plasma triglycerides all data was converted to mmol/L, whilst all data of average plasma FFAs was convered to µmol/L using the average molecular weight calculated in Table 5.3.

All data included in this study can be found in Appendix F.

2.5 Data analysis

The software used for statistical analysis throughout this project was GraphPad Prism 7. For all chapters, $P<0.05$ was accepted as statistically significant with a single asterisk on graphs to represent significance. The minimum number of times an experiment was repeated was three, with the exact n number provided in the figure legend or text. All data in chapters 3 and 4 were presented as mean ± SEM. All data presented in chapter 5 is mean ± SD, as this was more suitable for a meta-analysis.

2.5.1 Chapter 3 specific data analysis

One-way ANOVAs with Dunnett's multiple comparisons test were used to analysis data with a single variable. Two-way ANOVAs were performed with Dunnett's multiple comparisons test to analyse two factors concurrently. Correlation coefficients were used to assess the relationship between lipid accumulation and alterations in cell viability. The Oil Red O data was plotted on the X-axis against data from one of the cell viability experiments on the Y-axis, to establish whether any associations existed between lipid accumulation and cell death, apoptosis or late apoptosis.

Pearson correlation coefficient was calculated to analyse the relationship between intracellular lipid accumulation and HepG2 viability measured by CCK-8 assay. Pearson

correlation coefficient, a parametric test, was judged as suitable for this analysis as n=16. However, non-parametric testing was used to study the remaining associations because normality was uncertain due to small sample sizes which would have affected the validity of parametric testing had the data not had a Gaussian distribution. In particular, Spearman's rho (also known as Spearman's rank correlation coefficient) was selected to examine the correlation between variables. Spearman's rho is a measure of rank correlation, which determines the direction of the monotonic relationship between two variables.

Both correlation coefficients result in an r value between 1 and -1. A positive number indicates positive correlation and a negative number indicates a negative correlation. The closer the r value is to 1 or -1, the stronger the correlation. P values were also calculated as a measure of the statistical strength of the correlation.

Datasets were normalised relative to the control and presented as either fold change or percentage change. The formulas below were used for these calculations.

Fold change was calculated with the following formula:

$$Fold\ change = (\frac{test\ result - control}{control})$$

Percentage change was calculated using the following formula:

$$Percentage\ change = (\frac{test\ result - control}{control}) \times 100$$

2.5.2 Chapter 4 specific data analysis

One-way ANOVAs with Dunnett's multiple comparisons test were performed for the analysis of all data. Helena AggRAM (Helena Biosciences Europe, UK) software was used to graph aggregation traces. ACH software was used to calculate inconsistency scores.

2.5.3 Chapter 5 specific data analysis

Averages were calculated of Microsoft Office Excel 2010. An average for each set of data was calculated without weighting the average of each study, and, thus, the number of participants per study had no influence. In addition, a weighted average was calculated for each set of data by weighting the average of each study based on the number of participants included. Weighted standard deviations were calculated using the following formula:

$$\sqrt{\frac{\sum_{i=1}^{N} w_i (x_i - \bar{x}^*)^2}{\frac{(M-1)}{M} \sum_{i=1}^{N} w_i}},$$

where

N is the number of observations.

M is the number of nonzero weights.

w_i are the weights

x_i are the observations.

$\bar{x}^*$ is the weighted mean.

Simple T-tests were performed on GraphPad Prism 7 to compare the average and weighted means of the data. GraphPad Prism 7 was also used to analyse Gaussian distribution by fitting Gaussian distribution non-linear regressions to % frequency histograms of the data. The frequency histograms plotted the percentage of data points falling within a certain interval in order to show the distribution of data.

Residual plots of the non-linear regression lines were also produced and assessed to determine the ability to create reference ranges from the data. A residual is the difference between the observed value of the dependent variable and the value predicted by the

regression model. A residual plot graphs these differences on the Y-axis against the independent variable on the X-axis in order to assess the fit of the regression model.

Results from the non-linear regression were presented as an adjusted R^2 value. A value of 1 indicates a model that perfectly predicts values in within that subpopulation and a value that is less than or equal to 0 indicates a model that has no predictive value. In the real world, adjusted R^2 lies between these values.

Reference range between the 2.5th and 97.5th percentile were calculated with the formula:

$$Reference\ range = \ Mean\ \pm 2\ standard\ deviations$$

RevMan 5 was used to statistically compare the levels of lipid species between different subpopulations. Tau^2, Chi^2 and I^2 were automatically calculated by the RevMan software and were used in combination to assess heterogeneity.

The unit molar percentage (mol%) is the percentage of moles of a particular component within the total moles in a mixture. Whereas the unit mass percentage (mass%) is the percentage of mass of a particular component within the total mass in a mixture.

3 The effects of palmitic acid and oleic acid on cell viability

3.1 Background

Obesity worldwide has tripled within the last 30 years with 39% of the world's adults overweight and 13% obese as of 2016 (World Health Organization, 2018). The increasing incidence of obesity, and more specifically its associated health burden, continues to place strain on healthcare systems across the world. It is hypothesised that a key factor in obesity-associated sequelae is the elevation of circulating plasma free fatty acid levels (Guenther Boden, 2008; Karpe*, et al.*, 2011). Therefore, it is essential that we understand how metabolic changes cause cellular changes, and play a role in the pathologies associated with this condition.

The central importance of fatty acids in nutrition and disease is widely agreed, and yet, surprisingly few comprehensive studies have been conducted to assess how fatty acids directly affect cell viability, intracellular lipid accumulation and the link between the two. Alterations in fatty acid metabolism have been widely linked with development of insulin resistance (Karpe*, et al.*, 2011), amongst other diseases such as cancer (Saavedra-García*, et al.*, 2018). Thus, an understanding of how fatty acids affect different insulin-sensitive cells is crucial.

Two cell lines, HepG2 (human hepatocarcinoma) and L6 myotubes (rat myoblasts), were selected to expand current knowledge regarding the differential effects of palmitic acid and oleic acid on insulin-sensitive cells. Treatments with palmitic acid and oleic acid were selected as they are the most abundant saturated fatty acid and monounsaturated fatty acid in the plasma, respectively (Abdelmagid*, et al.*, 2015). The cells were treated with these fatty acids alone and combined, in a 2:1 molar ratio of oleic acid:palmitic acid, as suggested by previous studies.

Yao*, et al.,* (2011) showed that treatment of HepG2 cells with a 2:1 ratio of oleic acid to palmitic acid at concentrations between 1-3 mM caused intracellular lipid accumulation and cell death. Lipid accumulation and cell death occurred in a dose-dependent manner and led to apoptosis and necrosis (Yao*, et al.*, 2011). However, the research demonstrated the effects of these fatty acids in combination, not in isolation, and only studied their effects in HepG2 cells, which provides a limited view of the differential effects of palmitic acid and oleic acid on insulin-sensitive cells. Furthermore, the 3 mM concentration of fatty acids is above physiological concentrations as demonstrated by the systematic review performed in this study (Chapter 5). Thus, this project investigated these results and studied the effects of palmitic acid up to 1 mM, oleic acid up to 2 mM and the combined treatment up to 3 mM (see appendix). The treatments were refined to more physiologically relevant concentrations up to 1 mM as the systematic review found that normal plasma free fatty acid levels are between 0.2 – 0.9 mM (Figure 5.5A). At these concentrations, a combination of oleic acid and palmitic acid (2:1 ratio, respectively) caused intracellular lipid accumulation and apoptosis in HuH7 (human hepatocarcinoma cells) (Chavez-Tapia*, et al.*, 2012), however, the study's range of concentrations was limited and only hepatocarcinoma cells were studied.

Moravcová, *et al.,* (2015) also found that PA exhibited a dose-dependent cytotoxic effect in primary rat hepatocytes, whilst OA caused cytoplasmic membrane damage at concentrations above 1 mM. Yet, in combination OA:PA induced lower cytotoxicity than palmitic acid alone. Furthermore, both oleic acid and palmitic acid were found to increase intracellular triacylglycerol content in hepatocytes, however the purposes of the study was to identify the best model for steatosis, and as such did not study the link between lipid accumulation and cytotoxicity.

Many studies investigating the effects of fatty acids across a range of cell lines report a decrease in cell viability following treatment with palmitic acid (Cheon & Cho, 2014; El-Assaad*, et al.*, 2003; Listenberger*, et al.*, 2003). For example, 0.5 mM of PA caused apoptosis in Chinese Hamster Ovary (CHO) cells (Listenberger*, et al.*, 2003b), and 0.3 mM

of PA induced endoplasmic reticulum stress in cardiomyocytes leading to cell death (Haffar*, et al.*, 2015). In β-pancreatic cells, 0.4 mM PA was found to synergise with high levels of glucose to cause cell death (El-Assaad*, et al.*, 2003) and the same concentration induced membrane phase separation in HeLa cells (Shen*, et al.*, 2017). However, all such studies would not be directly comparable to hepatocytes and skeletal myocytes due to different functional outputs and signalling pathways.

More relevant to this study, palmitic acid induced cell death in C2C12 skeletal myocytes in a dose dependent manner up to 1.5 mM with death occurring *via* apoptosis as a result of mitochondrial membrane potential loss (Cheon & Cho, 2014). Ricchi*, et al.,* (2009) studied the differential effects of PA on three hepatocytic cell lines (HepG2, HuH7, WRL68), and demonstrated that palmitic acid induced apoptosis in all three at concentrations of 0.66 mM.

Research indicates that toxicity may result from intracellular accumulation (Chavez-Tapir, *et al.*, 2012). It is widely agreed that obesity leads to elevated levels of circulating free fatty acids, which cause adipose dysfunction and lead to the accumulation of lipids outside of the adipose tissue, in and around other tissues. Previous papers indicate that palmitic acid is poorly incorporated into neutral lipids and is, thus, free to exert its lipotoxic effects (Listenberger*, et al.*, 2003b). Others report that PA accumulates intracellularly as diacylglycerol (DAG) and triacylglycerol (TAG) (Gaster*, et al.*, 2005); the accumulation of DAG is considered toxic in many cells (Akoumi*, et al.*, 2017; Erion*, et al.*, 2010). Whilst Lee, *et al.*, (2017) found that treatment with palmitic acid increases exosome production in LX-2 cells, and alters the exosomal miRNA profile which could act as a mediator of toxicity.

On the other hand, research across a range of different cell lines reported no decrease in cell viability following treatment with oleic acid (El-Assaad*, et al.*, 2003; Listenberger*, et al.*, 2003b; Ricchi*, et al.*, 2009), yet this finding was not consistently observed with one study noting oleic acid-induced cell death. This study demonstrated that 0.2 mM OA caused modest levels of apoptosis in HepG2 and primary mouse hepatocytes MRH 7777 (Malhi*, et al.*, 2006). However, at concentrations of 1.32 mM OA was not found to induce cell death in three hepatocytic cell lines (HepG2, HuH7, WRL68) (Ricchi*, et al.*, 2009). It is thought that

the lack of toxicity exerted by OA is due to its intracellular accumulation as neutral TAG which is well tolerated (Listenberger*, et al.*, 2003b), however, Gaster*, et al.,* (2005) reported that oleic acid accumulates as intracellular free fatty acids (FFA).

Interestingly, researchers claim that the addition of oleic acid is able to reduce palmitic acid-induced cell death (Cheon & Cho, 2014; Haffar*, et al.*, 2015; Listenberger*, et al.*, 2003). For example, the addition of 0.2 mM OA to 0.5 mM PA protected CHO cells from cell death induced by 0.5 mM PA alone (Listenberger*, et al.*, 2003b). In cardiomyocytes it was found that the addition of just 0.05 mM of OA rescued ER stress induced by 0.3mM PA (Haffar*, et al.*, 2015), whilst 0.0625 mM of oleic acid reduced palmitic acid-induced cell death in HepG2 cells (Cheon & Cho, 2014). Conversely, one study found that treatment with 2 mM of oleic acid and palmitic acid in a molar ratio of 2:1 caused cell death in three hepatocytic cell lines (HepG2, HuH7, WRL68) (Ricchi*, et al.*, 2009). Furthermore, combination of oleic acid and palmitic acid was found to induce greater intracellular lipid accumulation than either fatty acid alone.

The lack of clarity is most likely due to differences in how different cells respond to fatty acids under normal conditions, and how these responses are exacerbated under pathological changes. There is much debate regarding whether decreased cell viability occurs *via* apoptotic or necrotic processes. The majority of reports support apoptosis, although this by no means rules out necrosis (Chavez-Tapia*, et al.*, 2012; Cheon*, et al.*, 2014; Malhi*, et al.*, 2006; Ricchi*, et al.*, 2009). For example, Yao*, et al.*, (2011) found no signs of early apoptosis following treatment of HepG2 cells with free fatty acids. Therefore, it was important to perform experiments in this study to tease out the mechanism of cell death and to identify the defining factors responsible for differences in toxicity between treatments. It was thought the aforementioned differences in toxicity between palmitic acid and oleic acid may be due to differing rates of transport across the plasma membrane or differing rates of metabolism within the cell. Perhaps, explained by differences in structure with palmitic acid being a 16C saturated fatty acid and oleic acid, an 18:1 cis-9 unsaturated fatty acid (see Figure 1.4).

Therefore, it was important to investigate whether elevated levels of palmitic acid and/or oleic acid could induce intracellular lipid accumulation in HepG2 and L6 cells, and moreover, evaluate whether an association exists between intracellular lipid accumulation and cell viability. Finally, it was considered vital to compare the differential effects of PA and OA between HepG2 and L6 cells, to understand whichtissue is potentially more vunerable to elevated levels of FFAs, This was important due to potential implications in the progression of obesity-associated disorders and may highlight links between obesity and its sequelae.

3.1.1 Aims

- To ascertain whether palmitic acid and/or oleic acid cause cell death and to identify whether death is *via* apoptotic or necrotic mechanism.
- To establish whether palmitic acid and/or oleic acid cause intracellular lipid accumulation.
- To understand the correlation, or lack of, between fatty acid-induced intracellular lipid accumulation and cell death.
- To compare the effect of palmitic acid and oleic acid on cell death, lipid accumulation and potential association of the two between HepG2 cells and L6 cells.

3.2 Methods

Refer to Chapter 2 Materials and methods. The minimum number of times an experiment was repeated was three, with the exact n number provided in the figure legend.All data is expressed as mean ± SEM. *P<0.05

N.B. The caspase assay was not performed on HepG2 cells, as these cells had a tendency to clump in culture which led to marked decreases in viability of the controls in the annexin V/PI assay (image in Appendix B). This clumping would, therefore, risk the production of artefactual results, especially without access to the sophisticated software used for flow cytometric analysis. ImageJ analysis was also not used for HepG2 cells as the aforementioned clumping prevented the formation of a monolayer which made ImageJ analysis difficult and unreliable.

3.2.1 Concentration of fatty acids

Initial experiments used concentrations of palmitic acid up to 1 mM, oleic acid up to 2 mM and combined up to 3 mM, similar to those found throughout the literature (Appendix Dl). However, these concentrations were found to be too high to be physiologically relevant both in the literature and *via* meta-analysis conducted in this project (Chapter 5; Abdelmagid*, et al.*, 2015). Concentrations up to 1 mM were selected for subsequent experimentation with the range chosen to enable direct comparison between treatments of palmitic acid and oleic acid alone and in combination (Table 2.1).

3.3 Results

3.3.1 HepG2 Viability in response to fatty acids

3.3.1.1 Toxicity of the FFA vehicle in HepG2 cells

Initial experiments were carried out to ensure that methanol, the solvent for FFA, did not affect HepG2 cell viability. Methanol at concentrations equivalent to those used in the fatty acid solutions did not cause cell death, as assessed by CCK-8 assays (P=0.1343, Figure 3.1A) and flow cytometric analysis of annexin V/PI staining (P=0.9736, Figure 3.1B).

As methanol did not affect HepG2 cells adversely, experimentation using methanol as the solvent was continued.

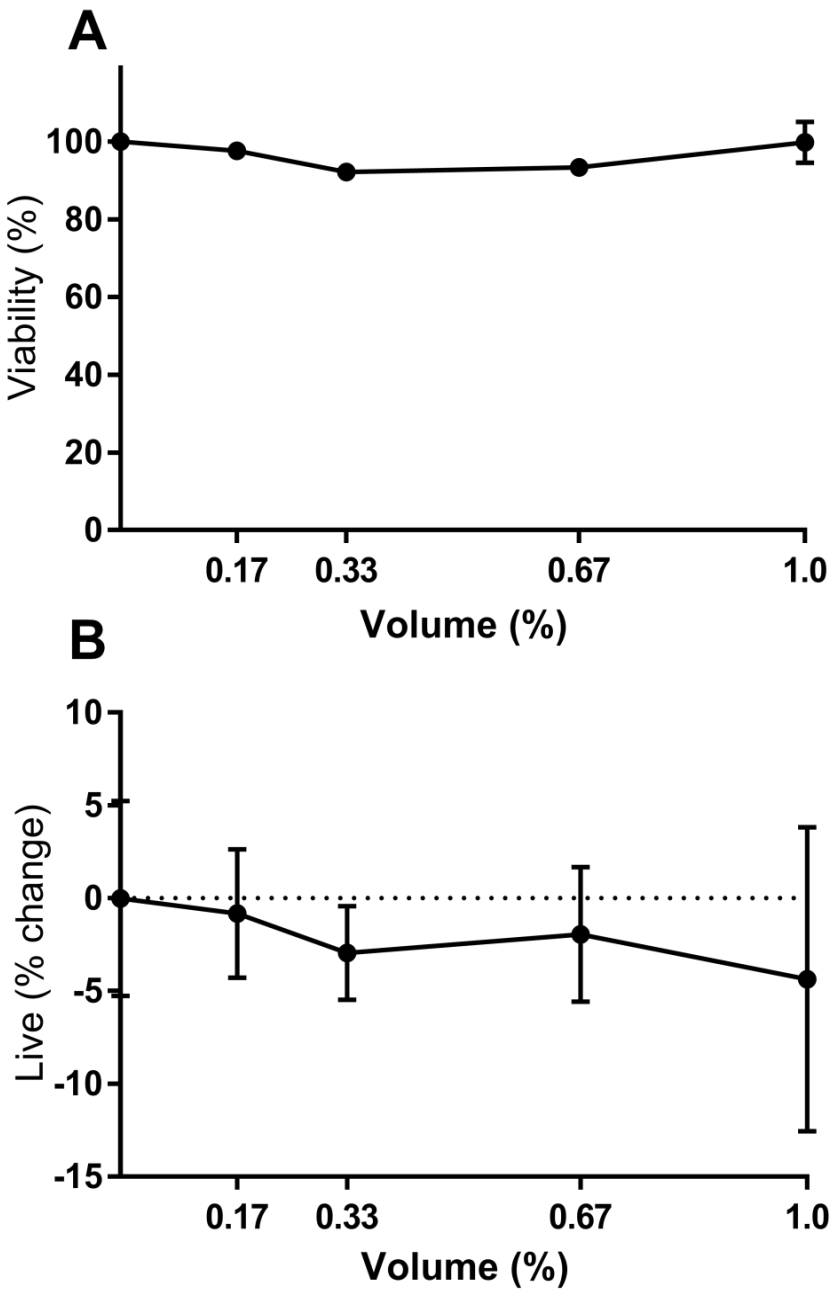


Figure 3.1 Methanol did not cause cell death in HepG2 cells

A) Methanol did not affect the viability of HepG2, as assessed by CCK-8 assays (P=0.1343) (n=8). B) Flow cytometric analysis of annexin V/PI staining showed that methanol did not affect the original percentage of live HepG2 cells (P=0.9736) (n=3). Due to the use of 100 mM stock solutions, the percentage of methanol equalled that of the fatty acid concentrations. The use of % change is described in 3.2.2.

3.3.1.2 Cell death in response to palmitic acid and oleic acid

Section 3.3.1.1. demonstrated that the FFA vehicle, methanol, had no effect on HepG2 cell death, the next experiment sought to measure cell death in these cells in response to FAs. The CCK-8 assay used to determine the effects of palmitic acid and oleic acid, alone and in combination, demonstrated that low concentrations of palmitic acid (0.17-0.33 mM) did not affect the viability of HepG2 cells (P=0.9976 and 0.9991, respectively). In contrast, 0.67 mM palmitic acid caused significant cell death (P=0.0001), with the highest concentration of 1 mM palmitic acid causing the greatest decrease in HepG2 viability, resulting in an average viability of 31.6 ± 1.86 % following treatment (P=0.0001) (Figure 3.2A). In contrast, treatment with oleic acid did not produce a significant decrease in HepG2 viability, however, 1 mM oleic acid resulted in an average viability of only 89.38 ± 2.53% following treatment (P=0.0786). The results for individual treatments of PA and OA were similar to those produced by Moravcová, *et al.,* (2015), however the combination of the two was found to be far more cytotoxic in this project. At the lowest concentration, 0.17 mM of the combined treatment conferred little effect on the viability of HepG2 (P=0.1617). However, treatment with higher concentrations (0.33–1 mM) led to significant decreases in cell viability (P=0.0467, 0.0145 and 0.0052, respectively). Yet it is worth noting that 1 mM of the combination treatment only decreased the viability of HepG2 cells by an average of 15.1 ± 4.90%, unlike 1 mM of palmitic acid which produced an average reduction of 68.4 ± 1.86%.

Before assessing whether palmitic acid caused a loss in cell viability *via* apoptosis or necrosis, it was important to validate the CCK-8 results, by studying the percentage of live HepG2 cells remaining following treatment. This was achieved *via* flow cytometric analysis of annexin V/PI staining, and was vital as CCK-8 assays measure all cells with functioning dehydrogenase activity, and, thus, may include cells that are undergoing cell death processes. However, flow cytometric techniques are able to quantify cells that are live and not undergoing any cell death processes *via* the combination of one probe of apoptosis (annexin V) and one probe of necrosis (PI), which allows for the gating of apoptosis- and necrosis-negative cells (live cells).

Analysis of annexin V- and propidium iodide-negative cells following 24 hour treatment largely corroborated the CCK-8 results (Figure 3.2B). The three lowest concentrations of palmitic acid, 0.17 mM, 0.33 mM and 0.67 mM, had little effect on the percentage of live HepG2 cells following treatment as compared to the control (P=0.6499, 0.9084 and 0.2071, respectively). However, 1 mM palmitic acid caused an average reduction of 62.84 ± 0.70% in live HepG2 cells (P=0.0001). In contrast to the CCK-8 results, the lowest concentrations of oleic acid, 0.17 mM, 0.33 mM and 0.67 mM, produced significant reductions in the percentage of live cells by up to 35.8 ± 5.42% (P=0.0078, 0.0007 and 0.0001, respectively). Whereas, 1 mM oleic acid did not have a significant effect on the percentage of live cells in comparison to the control (P=0.1478). Conversely, 0.17-0.33 mM of the combined treatment did not cause a decrease in the live cell percentage (P=0.9999 and 0.9983, respectively), whilst 0.67 mM resulted in an average reduction of 22.65 ± 4.06% (P=0.0077). Interestingly, the highest concentration of 1 mM caused an increase of 14.36 ± 3.23% in the percentage of live HepG2, however, this was not statistically significant (P=0.1293) and not evident in the CCK-8 results.

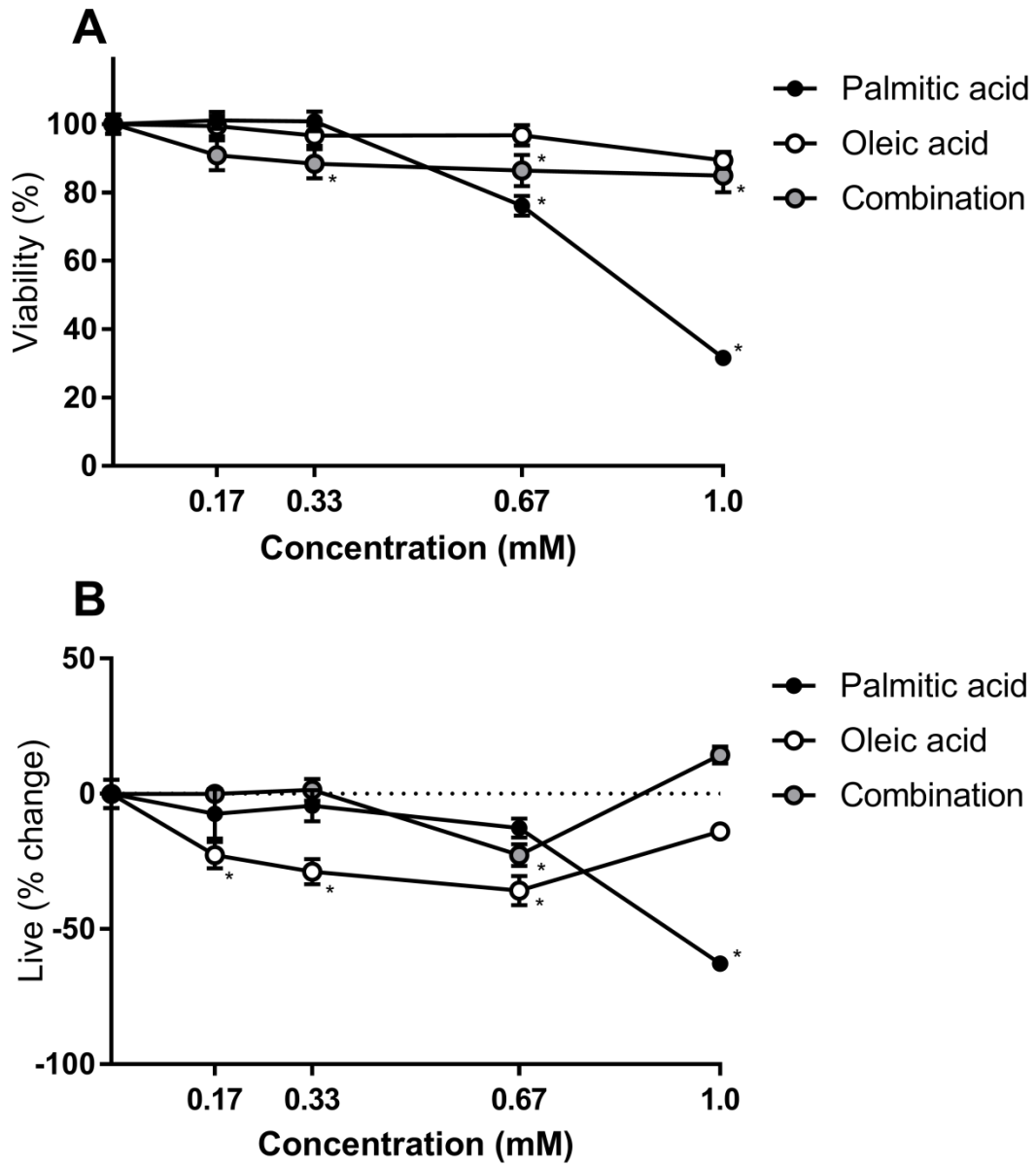


Figure 3.2 Palmitic acid caused cell death in HepG2 cells

■ = palmitic acid □ = oleic acid ■ = combination treatment

A) Changes in cell viability following treatment with palmitic acid and oleic acid, alone and in combination were assessed by CCK-8 assay (n=16). Palmitic acid caused significant decreases in HepG2 viability (P=0.0001), whilst oleic acid did not (P=0.0786). In combination, viability was significantly reduced, but not to the extent caused by palmitic acid alone (P=0.0052). B) Flow cytometric analysis of annexin V/PI staining determined the percentage change in live cells following treatment (n=3). Palmitic acid produced significant reductions in the percentage of live HepG2 cells (P=0.0001). Low concentrations of oleic acid had a similar effect (P=0.0001), however, the highest concentration did not cause significant reductions in live cells (P=0.1478). The combination treatment (0.67 mM) caused a decrease in live cell percentage (P=0.0077), whilst 1 mM produced a non-significant increase (P=0.1293).

3.3.1.3 Stages of cell death in HepG2 cells

Results from the cell viability studies found that all treatments reduced the percentage of live HepG2 cells, and, thus, further investigations into cell death processes were required. Flow cytometric analysis of annexin V/PI staining was performed to ascertain the mechanisms involved (Figure 3.3). Annexin V was used as a probe for apoptosis and propidium iodide as a probe for necrosis with results broken down into four gates. Annexin V- and PI-negative cells were considered to be live, annexin V-positive and PI-negative cells were said to be apoptotic, annexin V-negative and PI-positive were judged to be late apoptotic/necrotic and annexin V- and PI-positive cells were deemed to be dead.

At the lowest concentration of 0.17 mM, palmitic acid, oleic acid nor the combination treatment caused an increase in percentage of dead cells in comparison to the control (P=0.3555, 0.9814 and 0.0575, respectively) (Figure 3.3A). At a concentration of 0.17 mM, none of the treatments induced changes in the percentage of apoptotic HepG2 cells (P=0.8380, 0.1804, and 0.4674, respectively), nor the percentage of late apoptotic HepG2 cells (P=0.5476, 0.0736 and 0.7818, respectively). The only treatment to have an effect was 0.17 mM, which produced a significant decrease in the percentage of live cells (P=0.0078).

Palmitic acid and oleic acid, alone and in combination at a concentration of 0.33 mM did not alter the percentage of apoptotic HepG2 cells (P=0.9169, 0.6660 and 0.9463, respectively) (Figure 3.3B). However, at this concentration oleic acid induced an increase of 66.83 ± 14.30% in late apoptotic cells (P=0.0073), whilst palmitic acid and the combination treatment did not significantly alter the percentage of apoptotic HepG2 (P=0.9567 and 0.4718, respectively). In contrast, 0.33 mM of the combined treatment significantly reduced the percentage of dead cells by 47.8 ± 0.61% (P=0.0002), whilst individually palmitic acid and oleic acid had little effect (P=0.9999 and 0.5088, respectively).

Higher concentrations of the treatments (0.67 mM) had greater effects on HepG2 cells (Figure 3.3C). 0.67 mM of the combined treatment significantly reduced apoptotic HepG2 cells by 36.26 ± 3.55% and dead cells by 44.12 ± 1.05% (P=0.0483 and 0.0006,

respectively), whilst it increased the percentage of late apoptotic cells by 76.74 ± 7.82% (P=0.0019). The same concentration of palmitic acid did not alter the percentages of apoptotic, late apoptotic or dead cells significantly (P=0.9999, 0.5887 and 0.9814, respectively). On the other hand, 0.67 mM of oleic acid did not cause significant percentage change of apoptotic or dead HepG2 (P=0.5647 and 0.9936, respectively), but did induce an increase of 70.18 ± 20.94% in late apoptotic cells (P=0.0045).

Palmitic acid at the highest concentration of 1 mM, increased the percentage of apoptotic HepG2 cells by 98.69 ± 10.69%, and the percentage of late apoptotic/necrotic cells by 114.1 ± 6.16% (P=0.0001 and 0.0001, respectively) (Figure 3.3D). However, the percentage of dead cells was not altered by this treatment (P=0.5499). In contrast, 1 mM of the combination treatment did not cause changes to the percentages of apoptotic or late apoptotic/necrotic HepG2 cells (P=0.8147 and 0.9943, respectively), and yet reduced the percentage of dead HepG2 cells by 57.14 ± 0.26% (P=0.0001). Individually, 1 mM oleic acid did not significantly affect the percentage of apoptotic cells (P=0.6106), but did increase late apoptosis/necrosis by 59.02 ± 6.85% (P=0.0187). Furthermore, 1 mM oleic acid significantly decreased the percentage of dead HepG2 cells by 46.61 ± 7.51% (P=0.0003).

It is worth noting that for all concentrations, the combination of oleic acid and palmitic acid in a 2:1 ratio caused significantly less cell death than palmitic acid or oleic acid alone (Figure 3.3).

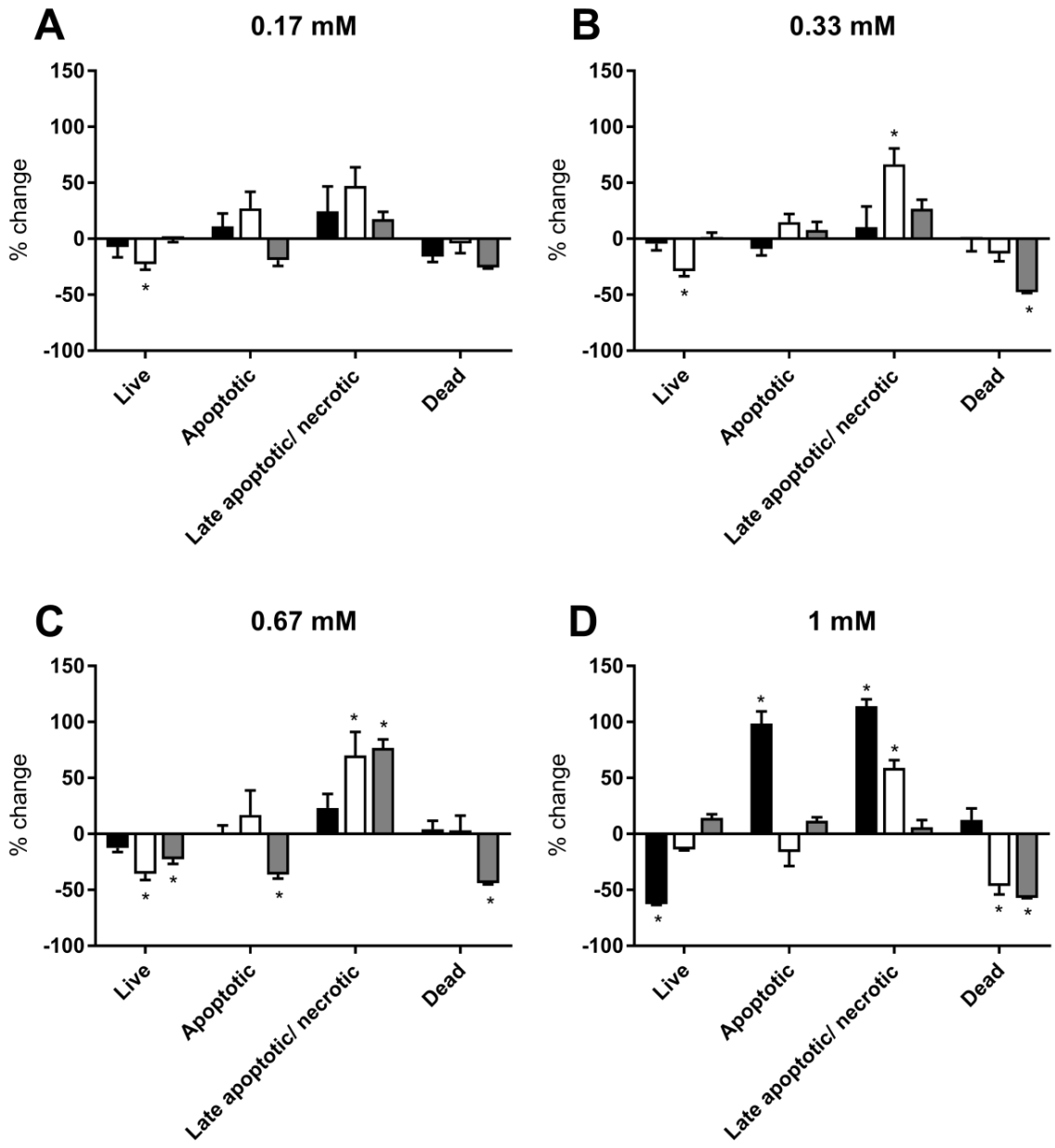


Figure 3.3 Palmitic acid increased early apoptosis, whilst oleic acid increased late apoptosis/necrosis, and the combination treatment decreased the percentage of dead HepG2 cells

■ = palmitic acid □ = oleic acid ■ = combination treatment

A, B, C, D) Flow cytometric analysis of annexin V/PI staining identified changes in the percentage of live, apoptotic, late apoptotic/necrotic and dead cells. HepG2 cells were treated for 24 hours with oleic acid, palmitic acid or a 2:1 combination in the following concentrations: 0.17 mM (A), 0.33 mM (B), 0.67 mM (C) and 1 mM (D) (n=3).

Forward scatter and side scatter of the cells were analysed to aid in the determination of the mechanism of fatty acid-induced cell death. The results produced by this analysis were by no means prescriptive, but rather enabled the formation of a semi-qualitative overview.

The lowest concentration of palmitic acid and the combination treatment, 0.17 mM, did not cause significant changes to forward scatter (P=0.1245 and 0.9927, respectively) (Figure 3.4A). However, 0.17 mM oleic acid did induce a significant reduction in forward scatter (P=0.0059). At concentrations of 0.33 mM, 0.67 mM and 1 mM, all treatments caused statistically significant decreases in forward scatter with 1 mM oleic acid producing the largest reduction of 86.61 ± 0.80% (P=0.0001).

Side scatter produced by HepG2 cells was significantly increased by 30.5 ± 10.74% after treatment with 0.17 mM oleic acid and by 31.94 ± 10.05 after treatment with 0.17 mM of the combined treatment (Figure 3.4B). Whereas, 0.67 mM oleic acid reduced side scattered light by 44.14 ± 6.23% (P=0.0001). Side scatter was further decreased by 1 mM palmitic acid which caused a 48.71 ± 2.57% reduction and 1 mM oleic acid which induced a 67.04 ± 2.56% reduction (P=0.0001 and 0.0001, respectively).

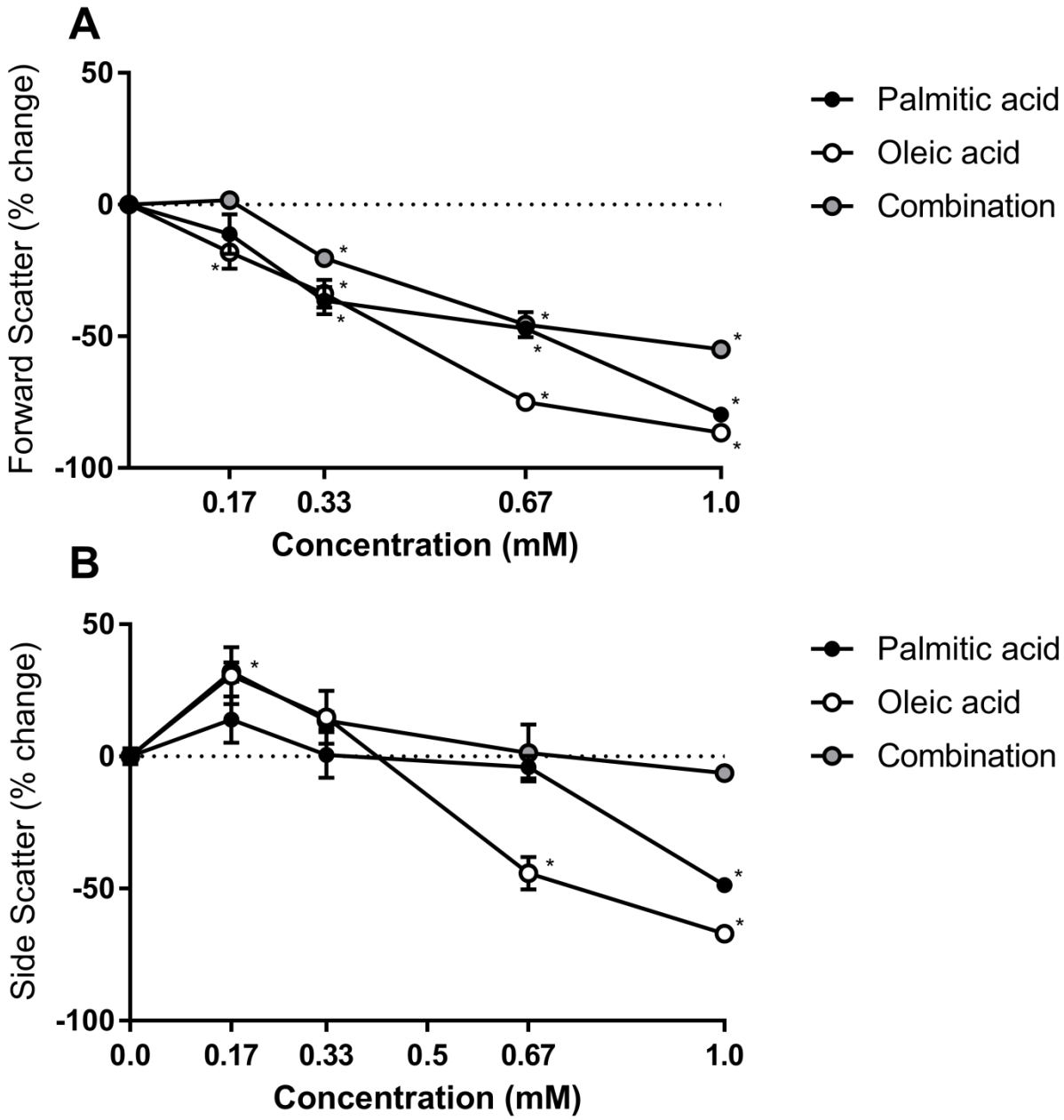


Figure 3.4 All treatments caused significant reductions in forward scatter whilst palmitic acid and oleic acid caused decreases in side scatter

■ = palmitic acid □ = oleic acid ■ = combination treatment

A) Flow cytometric analysis identified changes in forward scatter (n=3). All treatments caused a significant decrease in forward scatter (P=0.0001). B) Flow cytometric analysis identified changes in side scatter (n=3). Side scattered light was reduced by 1 mM palmitic acid and 1 mM oleic acid (P=0.0001 and 0.0001).

3.3.2 Lipid accumulation in HepG2

Oil red O staining was used to quantify the extent of lipid accumulation in HepG2 cells.

At even the highest concentration of palmitic acid (1 mM) did not induce significant intracellular lipid accumulation with a 37.84 ± 21.79% increase from the control (P=0.1562) (Figure 3.5), which contradicts results from Moravcová, *et al.,* (2015). Conversely, the two highest concentrations of the combination treatment, 0.67 mM and 1 mM, produced significant dose-dependent lipid accumulation up to 105.1 ± 18.13% (P=0.0024 and 0.0001, respectively). Oleic acid caused the most intracellular lipid accumulation with 0.33 mM, 0.67 mM and 1mM producing significant results in a dose-dependent manner (P=0.0072, 0.0001 and 0.0001, respectively). The largest percentage increase in intracellular lipid accumulation, 164.9 ± 8.01% was produced by 1 mM oleic acid.

Of note is that the combination treatment induced 63.7%, approximately two thirds, of the lipid accumulation produced by oleic acid (105.1 ± 18.13% and 164.9 ± 8.01%, respectively). This was consistent with the composition of the combined treatment, which was composed of $\frac{2}{3}$ oleic acid and $\frac{1}{3}$ palmitic acid (Figure 3.5).

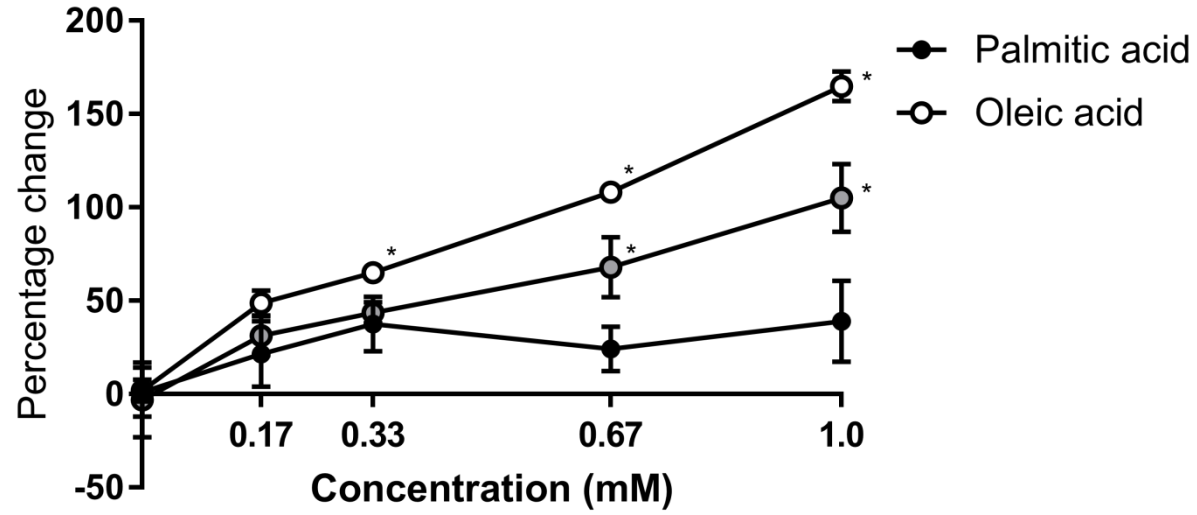


Figure 3.5 Oleic acid and the combination treatment caused intracellular lipid accumulation in HepG2 cells

■ = palmitic acid □ = oleic acid ■ = combination treatment

Oil Red O staining quantified intracellular lipid accumulation following treatment (n=3). Oleic acid and the combination treatment induced lipid accumulation in a dose-dependent manner (P=0.0001 and 0.0001), whilst even the highest concentration of palmitic acid did not (P=0.1562).

3.3.3 Correlation between lipid accumulation and viability

The correlation between intracellular lipid accumulation tested by Oil Red O staining and cell viability of HepG2 assessed by CCK-8 (Figure 3.6A, 3.6B, 3.6C) was determined. The correlation between intracellular lipid accumulation from ORO experimentation and live cells (Figure 3.6D, 3.6E, 3.6F), apoptotic cells (Figure 3.7A, 3.7B, 3.7C), late apoptotic/necrotic cells (Figure 3.7D, 3.7E, 3.7F) and dead cells (Figure 3.8) measured by flow cytometric analysis of annexin V/PI staining was studied. The data was presented in percentage change in viability, live cells, apoptotic cells, late apoptotic cells and dead cell in order to standardise the results and enable better visual comprehension.

The correlation between lipid accumulation and cell viability was assessed by plotting Oil Red O results on the X-axis with CCK-8 results on the Y-axis (Figure 3.6A, 3.6B, 3.6C). Palmitic acid induced-intracellular lipid accumulation was linked with a decrease in cell viability, however, this result was statistically non-significant ($r=-0.512$, $P=0.3778$). Oleic acid-induced lipid accumulation was significantly correlated with a decrease in the viability of HepG2 cells ($r=-0.925$, $P=0.0244$), as was lipid accumulation induced by the combined treatment ($r=-1$, $P=0.0301$).

Oil Red O results on the X-axis were plotted against annexin V-and PI-negative results on the Y-axis, in order to study the correlation between lipid accumulation and the percentage of live cells following treatment (Figure 3.6D, 3.6E, 3.6F). Intracellular lipid accumulation resulting from treatment with palmitic acid was negatively correlated with live cell percentage, yet not significantly ($r=-0.7$, $P=0.2333$). A mild, non-significant correlation existed between oleic acid-induced lipid accumulation and a decrease in live HepG2 cells ($r=-0.4$, $P=0.5167$). Conversely, lipid accumulation induced by the combined treatment was mildly associated with an increase in live cell percentage, although not significantly ($r=0.3$, $P=0.6833$).

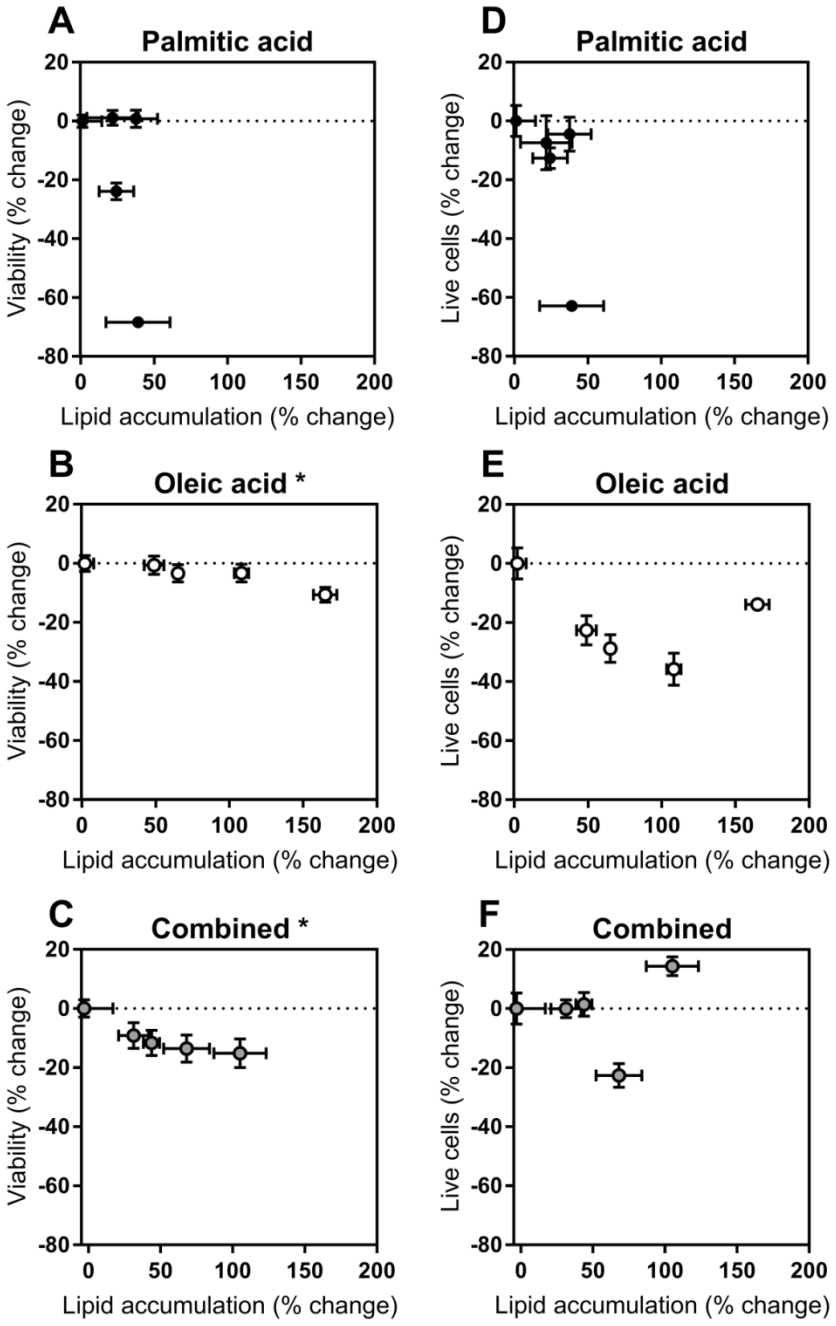


Figure 3.6 Lipid accumulation induced by oleic acid and the combination treatment is associated with decreases in cell viability

■ = palmitic acid □ = oleic acid ■ = combination treatment

A, B, C) Analysis of the correlation between intracellular lipid accumulation and the viability of HepG2 as determined by CCK-8 assay (ORO n=3, CCK-8 n=16). Combination-and oleic acid-induced lipid accumulation was correlated with decreased cell viability (P=0.0301 and 0.0244, respectively), whilst palmitic acid-induced lipid accumulation was not (P=0.3778). D, E, F) Analysis of the correlation between intracellular lipid accumulation and percentage of apoptotic HepG2 cells found no significant links following 24h of exposure to any of the treatments (ORO n=3, flow cytometry n=3).

On the X-axis, Oil Red O results were plotted against annexin V-positive/PI-negative results on the Y-axis, which allowed the correlation between lipid accumulation and the percentage of apoptotic cells to be studied (Figure 3.7A, 3.7B, 3.7C). Intracellular lipid accumulation as a result of treatment with palmitic acid was linked with a slight increase in apoptotic HepG2 cells, which was not significant (r=0.3, P=0.6833). Lipid accumulation induced by oleic acid was mildly correlated with a decrease in the percentage of apoptotic cells, but not significantly (r=-0.3, P=0.6833). Whilst, combination treatment-induced lipid accumulation was also slightly associated with a non-significant decrease in apoptotic HepG2 cells (r=-0.3, P=0.6833).

Oil Red O results were plotted on the X-axis with annexin V-negative/PI-positive results on the Y-axis to analyse the correlation between lipid accumulation and the percentage of late apoptotic/necrotic following treatment (Figure 3.7D, 3.7E, 3.7F). Intracellular lipid accumulation induced by all three treatments was associated with an elevation in late apoptotic/necrotic HepG2 cells, however, none were statistically significant. Oleic acid-induced lipid accumulation was the most strongly correlated (r=0.7, P=0.2333) followed by palmitic acid were (r=0.6, P=0.3500), and the combined treatment (r=0.4, P=0.5167), however, these too did not reach significance.

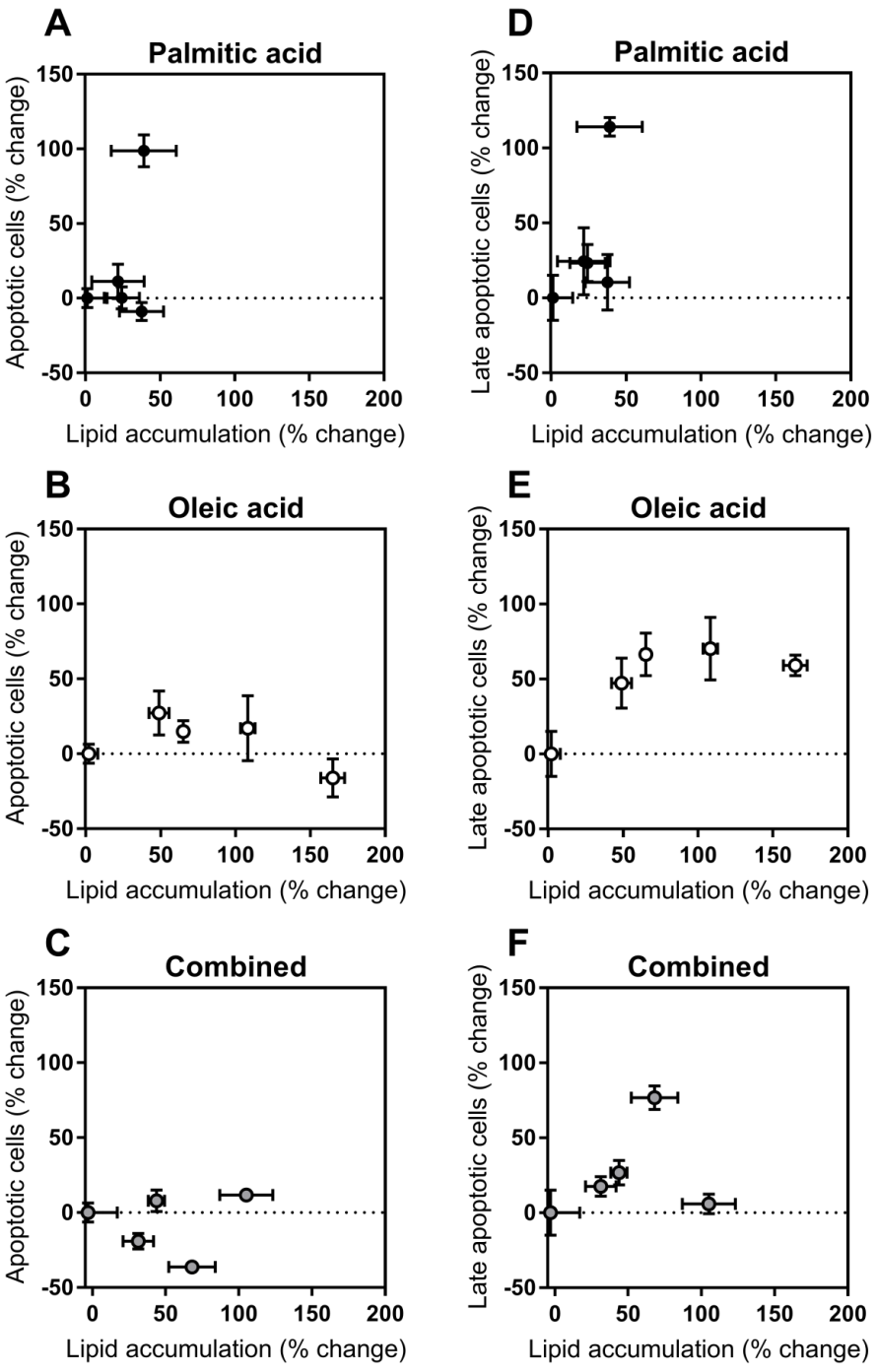


Figure 3.7 There was no significant correlation between lipid accumulation and apoptosis or late apoptosis/necrosis in HepG2

■ = palmitic acid □ = oleic acid ■ = combination treatment

A, B, C) Analysis of the correlation between intracellular lipid accumulation and percentage of apoptotic HepG2 found no significant associations following 24h of exposure to any of the treatments (ORO n=3, flow cytometry n=3). D, E, F) Study of the correlation between intracellular lipid accumulation and percentage of late apoptotic/necrotic HepG2 cells determined no significant links for any of the treatments (ORO n=3, flow cytometry n=3).

Oil Red O results on the X-axis were plotted against annexin V-and PI-positive results on the Y-axis, to analyse the correlation between lipid accumulation and the percentage of dead HepG2 cells (Figure 3.8A, 3.8B, 3.8C). Intracellular lipid accumulation induced by palmitic acid was associated with a mild increase in the percentage of dead HepG2 cells, yet this associations was non-significant (r=0.5, P=0.4500). On the other hand, oleic acid-induced lipid accumulation was mildly linked with a non-significant decrease in the percentage of dead cells (r=-0.4, P=0.5167). Furthermore, lipid accumulation produced by the addition of the combination treatment was strongly correlated with a decrease in the dead cell percentage, however, this result was not statistically significant (r=-0.9, P=0.0833).

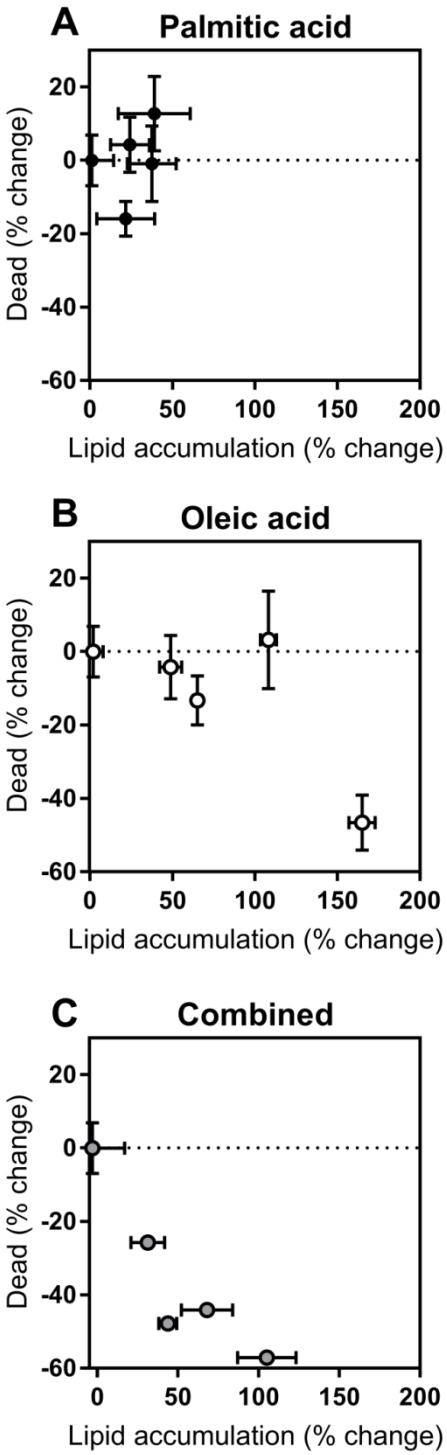


Figure 3.8 There was no significant correlation between lipid accumulation and percentage of dead HepG2 cells

■ = palmitic acid □ = oleic acid ■ = combination treatment

A, B, C) Statistical analysis uncovered no significant associations between intracellular lipid accumulation and percentage of dead HepG2 cells following treatment with palmitic acid and oleic acid, alone and in combination (ORO n=3, flow cytometry n=3).

3.3.4 L6 Viability in response to fatty acids

Following investigations into the differential effects of palmitic acid and oleic acid, alone and in combination, on HepG2 hepatocytes, the next study aimed to characterise the effects on skeletal myotubes. L6 cells were cultured and allowed to differentiate before the above experimentation was replicated in this model (Figure 2.1).

3.3.4.1 Toxicity of the FFA vehicle in L6 cells

Experiments were performed to confirm that methanol did not adversely affect L6 cells (Figure 3.9). Even at the highest volume percentage, methanol did not cause significant changes in the viability of L6 cells in CCK-8 assays (P=0.6833). Also, flow cytometric analysis of annexin V/PI staining demonstrated that the addition of methanol did not alter the percentage of live L6 cells (P=0.9677).

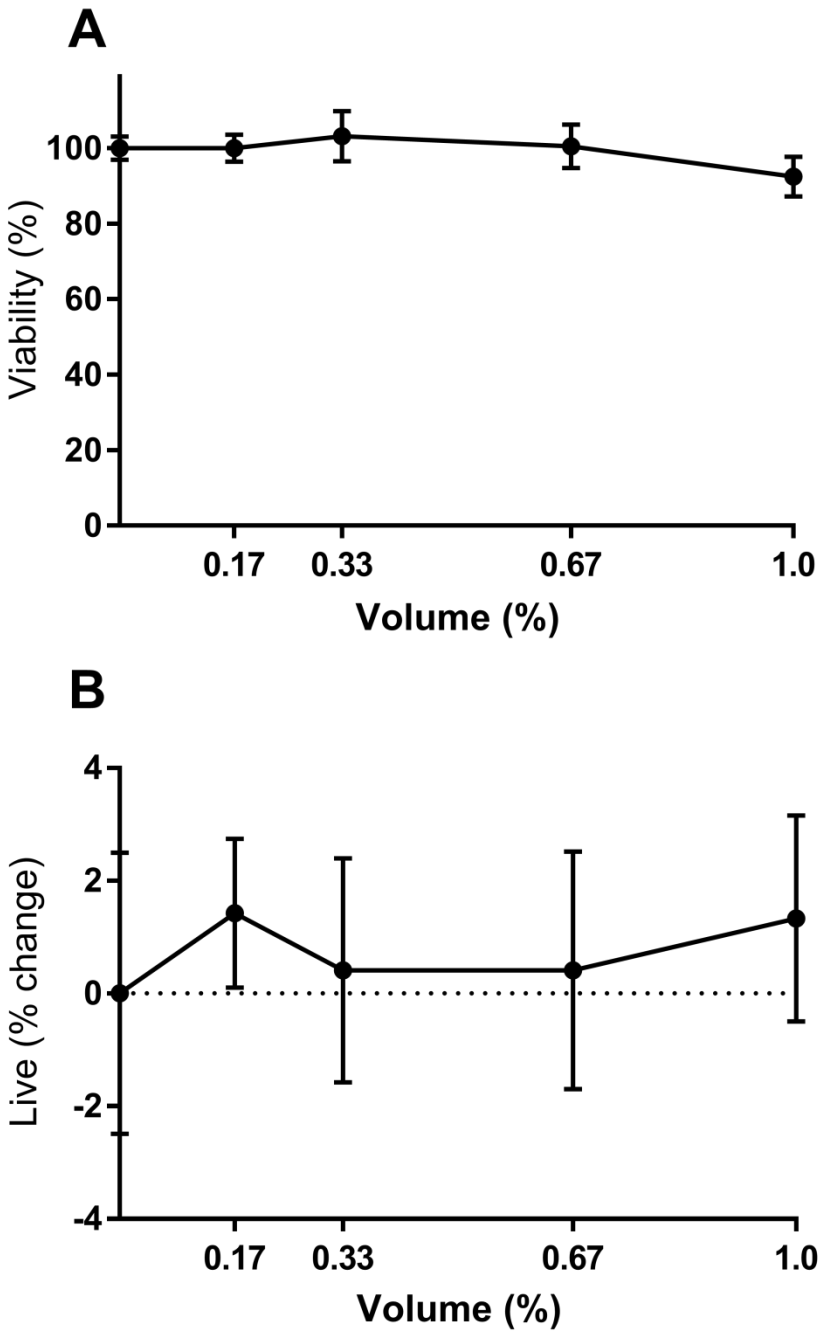


Figure 3.9 Methanol did not cause cell death in L6 cells

A) CCK-8 assays determined that methanol did not alter the viability of L6 cells (P=0.6833) (n=15). B) Flow cytometric analysis of annexin V/PI staining found that methanol had no effect on the percentage of live L6 cells following treatment (P=0.9677) (n=3). Due to the use of 100 mM stock solutions, the percentage of methanol equalled that of the fatty acid concentrations. The use of % change is described in 3.2.2.

3.3.4.2 Cell death in response to palmitic acid and oleic acid

The CCK-8 assay used to examine the differential effects of palmitic acid and oleic acid on L6 cells demonstrated that a combination of the two fatty acids did not alter cell viability, even at the highest concentration of 1 mM (P=0.9311) (Figure 3.10A). At low concentrations (0.17-0.33 mM), palmitic acid had little effect on the viability of L6 cells (P=0.9990 and 0.9911, respectively). Whilst, 0.67 mM palmitic acid caused a significant reduction of 29.01 ± 6.04% in cell viability (P=0.0004), and 1 mM palmitic acid decreased cell viability by 87.87 ± 0.37% (P=0.0001). Similarly, the treatment of L6 cells with 0.17 mM oleic acid had no significant effect on cell viability (P=0.1426). However, oleic acid at concentrations of 0.33 mM, 0.67 mM and 1 mM produced significant decreases in cell viability (P=0.0086, 0.0005 and 0.0001, respectively).

It is worth noting that although palmitic acid and oleic acid both induced significant decreases in the viability of L6, reduction caused by palmitic acid was far greater. For example, percentage viability following treatment with 1 mM oleic acid was 65.4 ± 2.66%, as opposed to 12.13 ± 0.37% following treatment with 1 mM palmitic acid.

Live cell percentage results from flow cytometric analysis of annexin V/PI staining were studied to verify the CCK-8 results as CCK-8 classifies all cells with functioning dehydrogenase activity as viable, which may result in the inclusion cells undergoing death processes. On the other hand, flow cytometric techniques are able to quantify cells that are live without cell death processes initiated.

Live cell percentage results were similar to the above cell viability results (Figure 3.10B). Alone, the lowest concentration of palmitic acid and oleic acid, 0.17 mM, had little effect on the percentage of live L6 cells following treatment (P=0.0764 and 0.3700, respectively). However, 0.17 mM of the combined treatment caused a small yet significant decrease in live cell percentage of 2.38 ± 0.24% (P=0.0159). At a concentration of 0.33 mM, palmitic acid, oleic acid and the combined treatment all produced small, but significant reductions in the percentage of live L6 (P=0.0001, 0.0001 and 0.0449, respectively). However, 0.67 mM of the combination treatment did not alter the live cell percentage (P=0.3548), whilst 0.67

mM oleic acid reduced live L6 cells by 8.33 ± 0.39% and 0.67 mM palmitic acid decreased the live cell percentage by 84.66 ± 0.48% (P=0.0001 and 0.0001, respectively). All treatments at the highest concentration of 1 mM diminished the percentage of live L6 cells. However, the percentage change varied wildly from a reduction of 5.22 ± 0.35% produced by the combination treatment, to a decrease of 25.95 ± 1.06% by oleic acid, to a 97.64 ± 0.13% by palmitic acid (P=0.0001, 0.0001 and 0.0001, respectively).

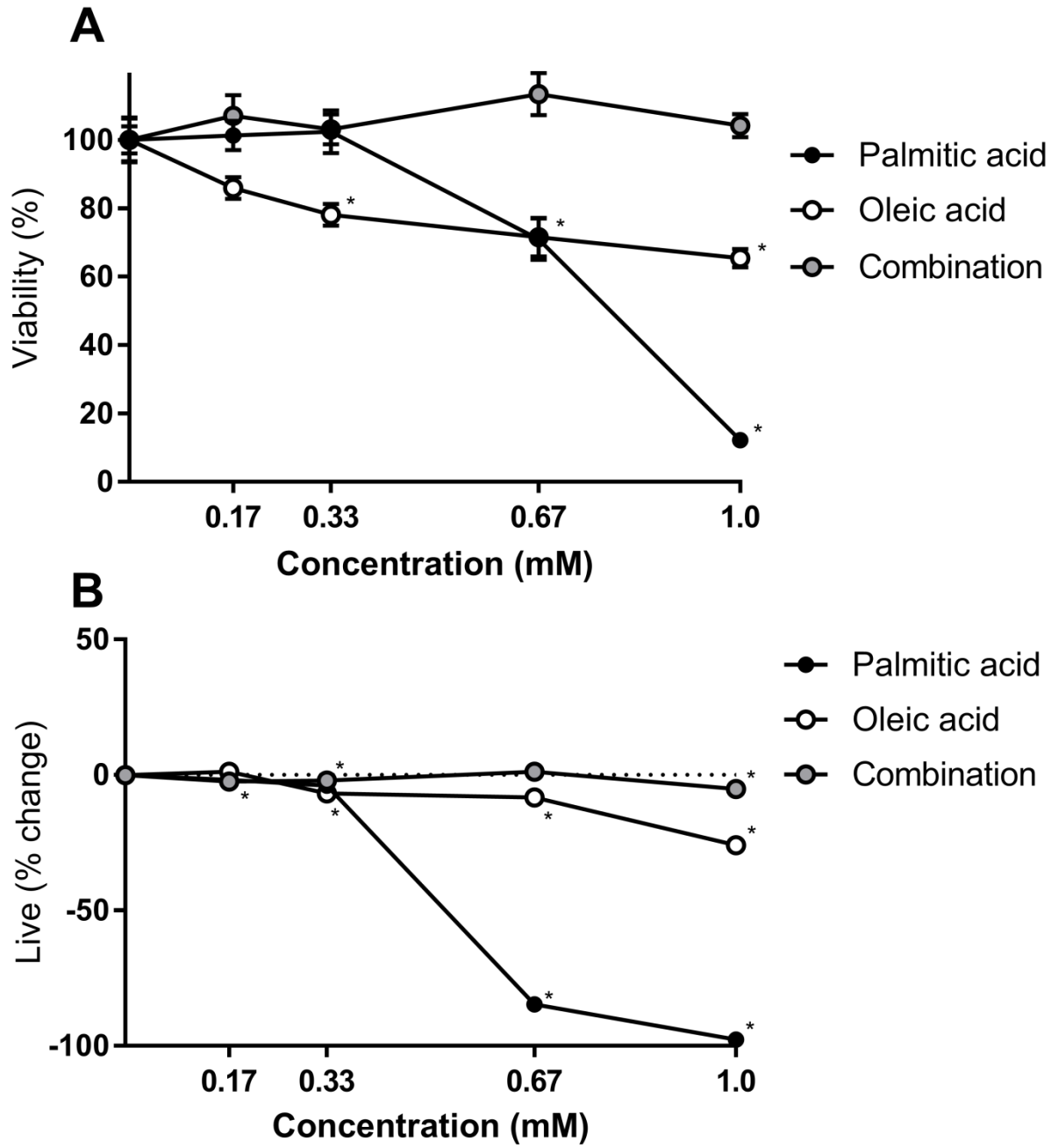


Figure 3.10 All treatments caused a reduction in the percentage of live L6 cells

= palmitic acid = oleic acid = combination treatment

A) CCK-8 assays illustrated that palmitic acid and oleic acid caused significant decreases in cell viability (P=0.0001 and 0.0001, respectively) (n=5). B) Flow cytometric analysis of annexin V/PI staining determined that all treatments caused significant decreases in live L6 cells (P=0.0001), with palmitic acid causing the greatest reduction (n=3).

3.3.4.3 Stages of cell death in L6 cells

The above studies determined that all treatments decreased the percentage of live L6 cells, and, therefore, further experimentation was required to understand the stage of cell death affected by FAs. As for HepG2, flow cytometric analysis of annexin V/PI staining was performed to examine the mechanisms responsible (Figure 3.11).

At the lowest concentration of 0.17 mM, all treatments caused a significant decrease in the percentage of apoptotic L6 in comparison to the control (Figure 3.11A). Palmitic acid reduced apoptosis by 54.09 ± 7.70%, oleic acid by 68.44 ± 1.37% and the combination treatment by 49.93 ± 3.53% (P=0.0001, 0.0001 and 0.0001, respectively). Conversely, all treatments caused non-significant increases in late apoptosis/necrosis (P=0.0990, 0.8768 and 0.1519, respectively). Furthermore, palmitic acid and the combined treatment both increased the percentage of dead L6 cells by 79.01 ± 27.2% and 107.8 ± 5.54%, respectively (P=0.0002 and 0.0001, respectively).

At a concentration of 0.33 mM, palmitic acid, oleic acid and the combination treatment all significantly reduced the percentage of apoptotic cells, however, to a lesser extent than at 0.17 mM (P=0.0001, 0.0001 and 0.0003, respectively) (Figure 3.11B). On the other hand, all treatments increased late apoptosis/necrosis to a greater and statistically significant extent (P=0.0001, 0.0001 and 0.0292, respectively). Palmitic acid continued to cause a greater percentage of dead L6 cells than the control (P=0.0043), and oleic acid also caused a significant increase of 104.23 ± 5.21% in dead cell percentage (P=0.0001), whilst the combined treatment did not (P=0.0646).

As the concentration of the treatments increased to 0.67 mM, palmitic acid and the combined treatment continued to produce decreases in the percentage of apoptotic L6 cells by 50.79 ± 2.66% and 53.8 ± 4.1%, respectively (P=0.0001 and 0.0001, respectively), whilst reduction caused by oleic acid decreased to 16.36 ± 7.82% (P=0.0642) (Figure 3.11C). Oleic acid and palmitic acid also continued to increase the percentage of late apoptosis/necrosis by 285.27 ± 22.56% and 3234.56 ± 17.85%, respectively (P=0.0001 and 0.0001, respectively), however, the combination treatment did not induce a significant

increase (P=0.5984). Furthermore, oleic acid and palmitic acid continued to cause greater percentages of dead L6 cells than the control, whilst the combination treatment did not (P=0.0001, 0.0001 and 0.1343, respectively).

At 1 mM, oleic acid caused small non-significant decreases in apoptosis of 1.15 ± 8.14%, whilst the combination treatment caused a small non-significant increase in apoptosis (P=0.9991 and 0.1258, respectively) (Figure 3.11D). Only 1 mM palmitic acid caused greater percentages in apoptotic cells than the control (P=0.0001). Very high levels of late apoptosis/necrosis were induced by 1 mM of all treatments. The combined treatment increased the percentage of late apoptotic/necrotic L6 cells by 166.67 ± 15.17%, oleic acid by 827.96 ± 40.38% and palmitic acid by 3026.73 ± 8.00% (P=0.0001, 0.0001 and 0.0001, respectively). At this concentration, all treatments also caused larger percentages of dead L6 cells than the control. The combined treatment increased the dead cell percentage by 49.74 ± 3.23%, oleic acid by 336.16 ± 8.03% and palmitic acid by 1457.14 ± 14.83% (P=0.0173, 0.0001 and 0.0001, respectively).

It is of note that, in spite of all 1 mM treatments causing significant late apoptosis/necrosis and cell death, palmitic acid caused significantly greater levels than oleic acid, and oleic acid caused significantly greater levels than the combined treatment.

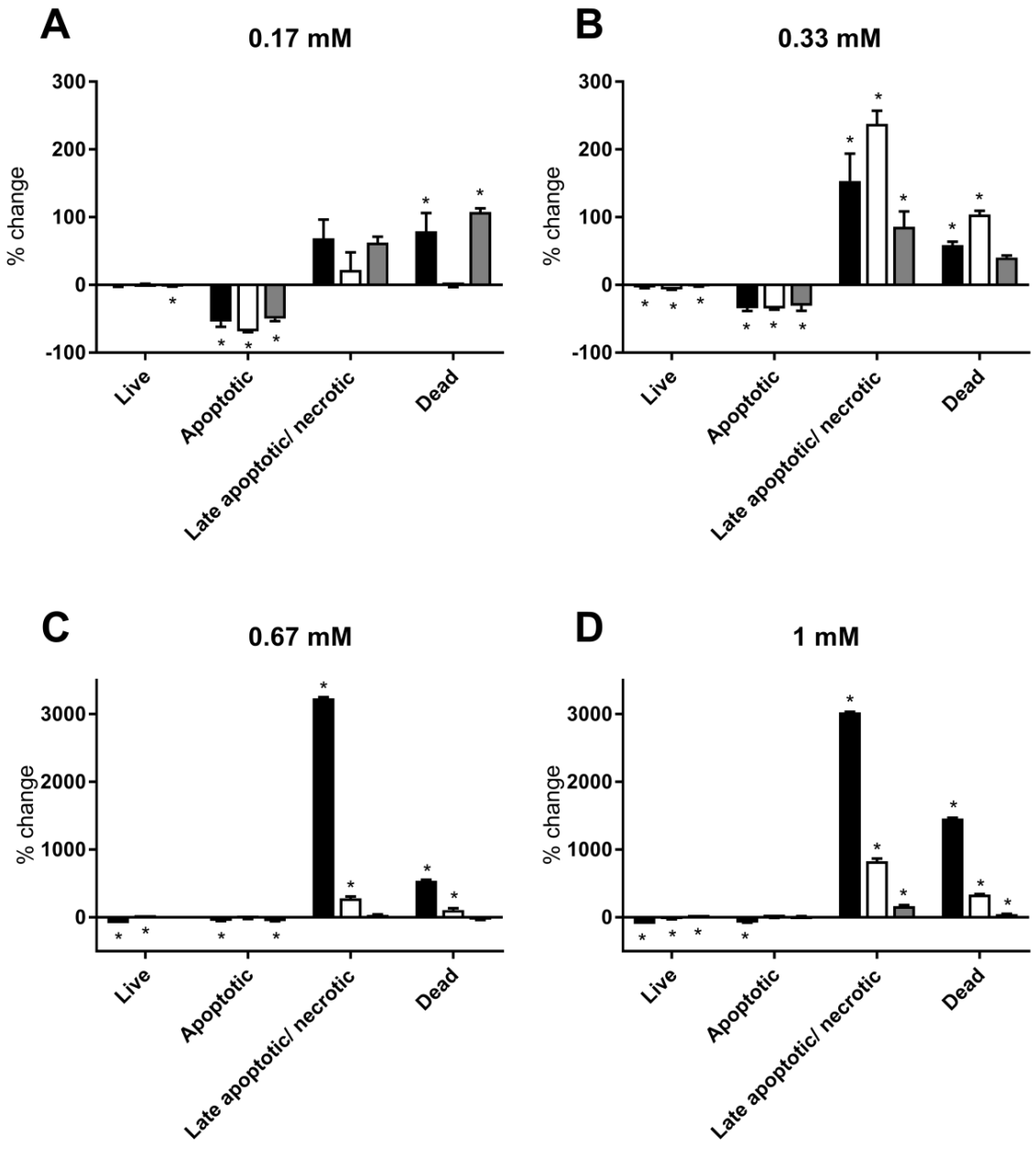


Figure 3.11 Palmitic acid and oleic acid increased late apoptosis/necrosis and cell death in L6

■ = palmitic acid □ = oleic acid ■ = combination treatment

A, B, C, D) Flow cytometric analysis of annexin V/PI staining examined changes in the percentage of live, apoptotic, late apoptotic/necrotic and dead L6 cells. Experiments were performed following 24 hours of treatment with palmitic acid, oleic acid, alone or combined, in the following concentrations: 0.17 mM (A), 0.33 mM (B), 0.67 mM (C) and 1 mM (D) (n=3).

Similar to the HepG2 study, forward scatter and side scatter of L6 were examined as an additional tool in understanding the stage of cell death induced by fatty acids. The results formed by this experiment were judged holistically to give a qualitative summary, which carried little weight in the discussion.

Neither palmitic acid, oleic acid nor the combination treatment at 0.17 mM induced significant alterations to the forward scatter produced by L6 (P=0.3828, 0.0653 and 0.1040, respectively) (Figure 3.12A). At 0.33 mM, oleic acid had no significant effect on forward scattered light (P=0.9505), however, palmitic acid induced a 13.55 ± 2.5% decrease whilst the combined treatment produced a 12.53 ± 1.2% increase (P=0.0179 and 0.0309, respectively). Palmitic acid continued to cause reductions to forward scatter at 0.67 mM with a decrease of 72.84 ± 0.25%, yet oleic acid and the combination treatment did not induce significant changes (P=0.0001, 0.1999, and 0.6876, respectively). Interestingly, 1 mM of the combined treatment did not produce significant alterations to forward scattered light (P=0.8885), but oleic acid caused a 15.62 ± 0.98% decrease and palmitic acid a 60.22 ± 0.16% reduction (P=0.0056 and 0.0001, respectively).

Side scatter produced by L6 cells remained unchanged by the majority of treatments, but 0.67 mM oleic acid which caused an increase of 95.49 ± 66.37% (P=0.0023) (Figure 3.12B).

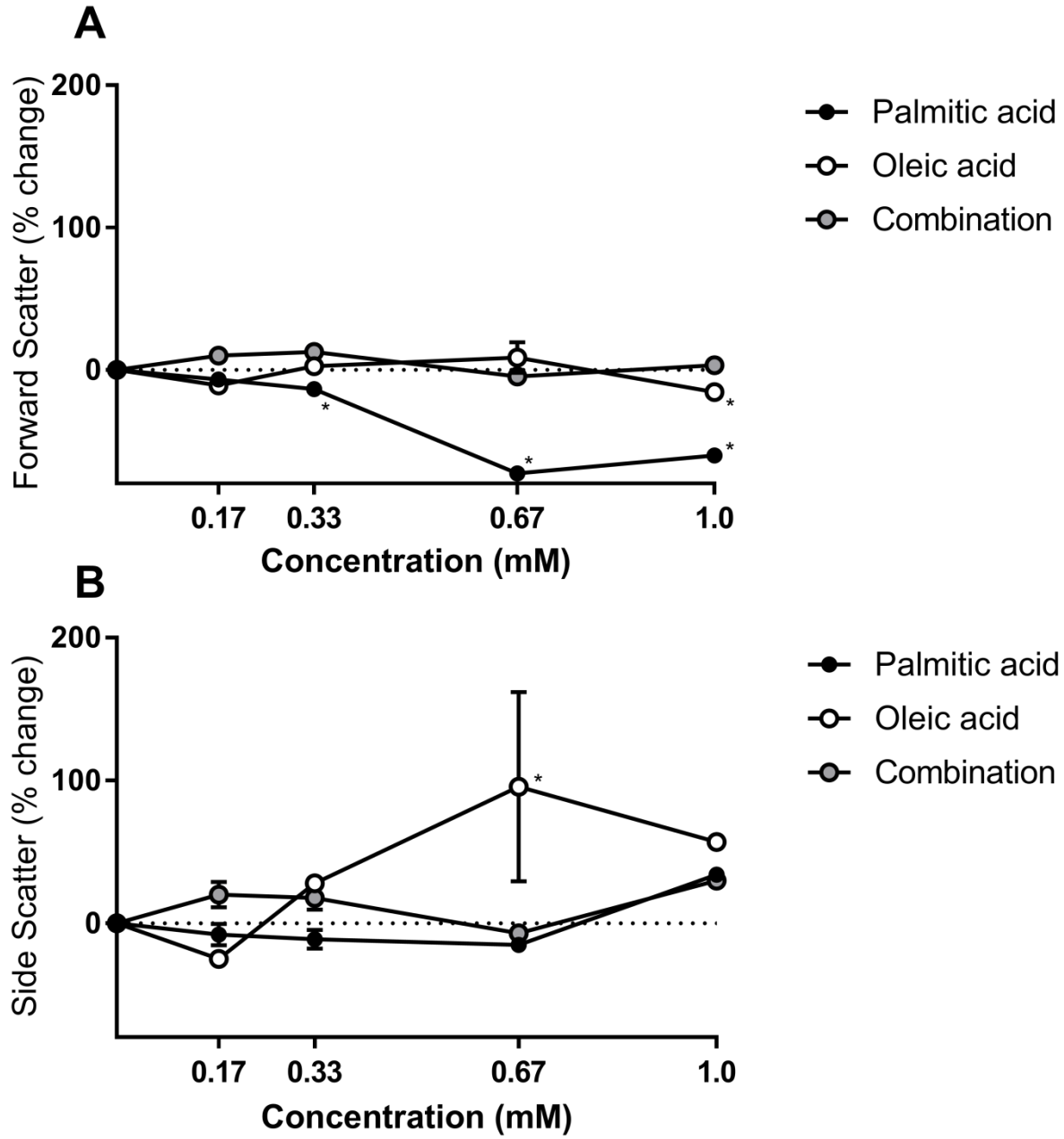


Figure 3.12 Palmitic acid and oleic acid reduced forward scatter

= palmitic acid = oleic acid = combination treatment

A) Flow cytometric analysis of annexin V/PI staining showed that palmitic acid caused a significant decrease in forward scatter (P=0.0001) (n=3). B) None of the treatments had a significant effect on side scattered light (n=3).

3.3.4.4 Apoptosis in L6 cells

The results from flow cytometric analysis of annexin V/PI staining were equivocal as the cells appeared to bypass early apoptosis with large increases in late apoptosis/necrosis. Therefore, additional experiments were performed to determine whether the fatty acid treatments induced cell death *via* apoptosis or necrosis. To investigate this, L6 caspase 3/7 activity was measured *via* an assay kit following treatment.

Results from the caspase assay in L6 corroborated those from the late apoptosis/necrosis gate of the annexin V/PI analysis (Figure 3.13). Low concentrations of palmitic acid, 0.17 and 0.33 mM had little effect on caspase 3/7 activity (P=0.9830 and 0.8817, respectively). Whereas, the addition of 0.67 mM palmitic triggered a significant 17811.11 ± 7288.50% increase in activity (P=0.0001). At the higher concentration of 1 mM, palmitic acid induced caspase activity of only 7288.89 ± 3038.05% (P=0.0753). In contrast, even the highest concentration of oleic acid and the combination treatment, 1 mM, did not cause significant percentage change in caspase 3/7 activity. Oleic acid induced an increase of 4166.67 ± 2483.79% and the combined treatment produced an increase of 944.44 ± 309.25% (P=0.4753 and 0.9943, respectively).

It is worth noting that caspase 3/7 activity following treatment with 1 mM palmitic acid was lower than following treatment with 0.67 mM palmitic acid. This was likely due to the very large increases in late apoptosis/necrosis shown in Figure 3.11C.

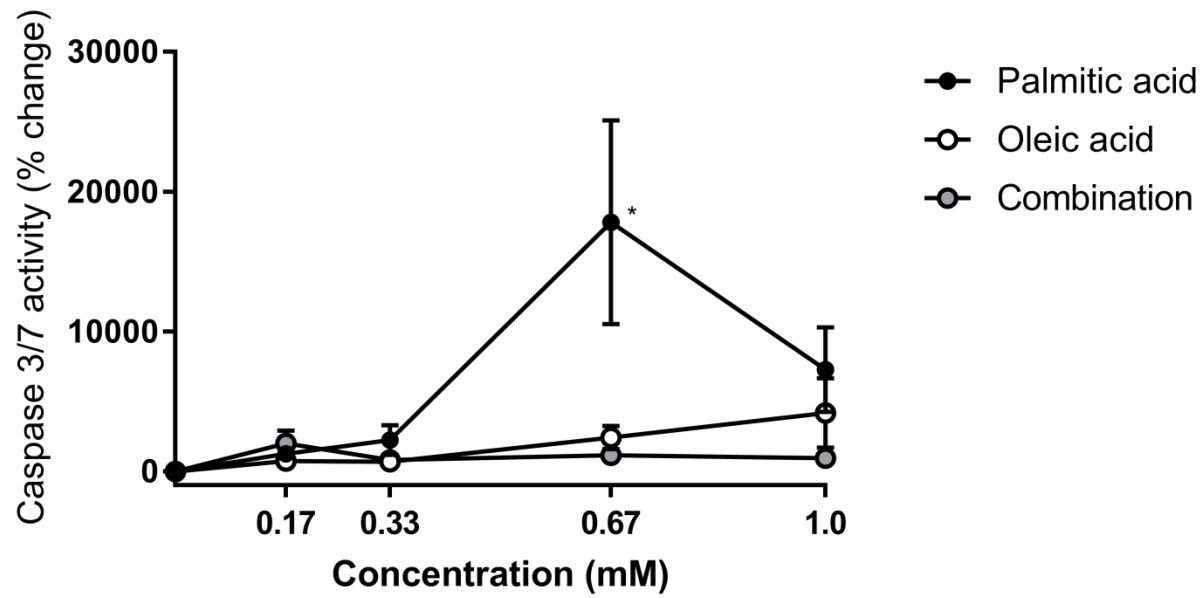


Figure 3.13 Palmitic acid triggered caspase 3/7 activity

= palmitic acid = oleic acid = combination treatment

A caspase 3/7 assay determined that palmitic acid induced caspase 3/7 activity (P=0.0753), whilst oleic acid and the combination treatment did not (n=3).

3.3.5 Lipid accumulation in L6

Oil red O staining was utilised to measure intracellular lipid accumulation in L6 cells and ImageJ analysis was used to quantify the number of lipid droplets present.

Results determined that palmitic acid did not trigger significant intracellular lipid accumulation in L6 cells (Figure 3.14A). The highest concentration of palmitic acid, 1 mM produced a 41.71 ± 19.33% increase in intracellular lipids (P=0.1113). On the other hand, oleic acid and the combination treatment induced lipid accumulation in a dose-dependent manner. The lowest concentration, 0.17 mM of the combination treatment did not cause significant lipid accumulation (P=0.0878), however, 0.33 mM, 0.67 mM and 1 mM did, with a maximal increase of 152.80 ± 9.74% (P=0.0168, 0.0001 and 0.0001, respectively). Oleic acid produced the highest levels of intracellular lipid accumulation in L6 cells with all concentrations, 0.17 mM, 0.33 mM, 0.67 mM and 1 mM producing statistically significant results (P=0.0083, 0.0002, 0.0001 and 0.0001, respectively). Maximal lipid accumulation of 185.61 ± 22.77% was produced by 1 mM oleic acid.

Interestingly, the combined treatment induced greater than two thirds of the lipid accumulation produced by oleic acid. This was odd as the combination treatment was composed of $\frac{2}{3}$ oleic acid and $\frac{1}{3}$ palmitic acid.

Image J analysis examined the number of lipid droplets per field of confluent L6 cells and deduced that neither palmitic acid nor the combination treatment caused a significant increase in lipid droplet formation (Figure 3.14B). At the highest concentration of 1 mM, oleic acid did not induce the formation of any lipid droplets, whilst the combination treatment produced 2.33 ± 0.67 lipid droplets per field (P=0.9969). However, 1 mM of oleic acid resulted in a marked increase in the number of lipid droplets per field to 106 ± 24.58 (P=0.0001).

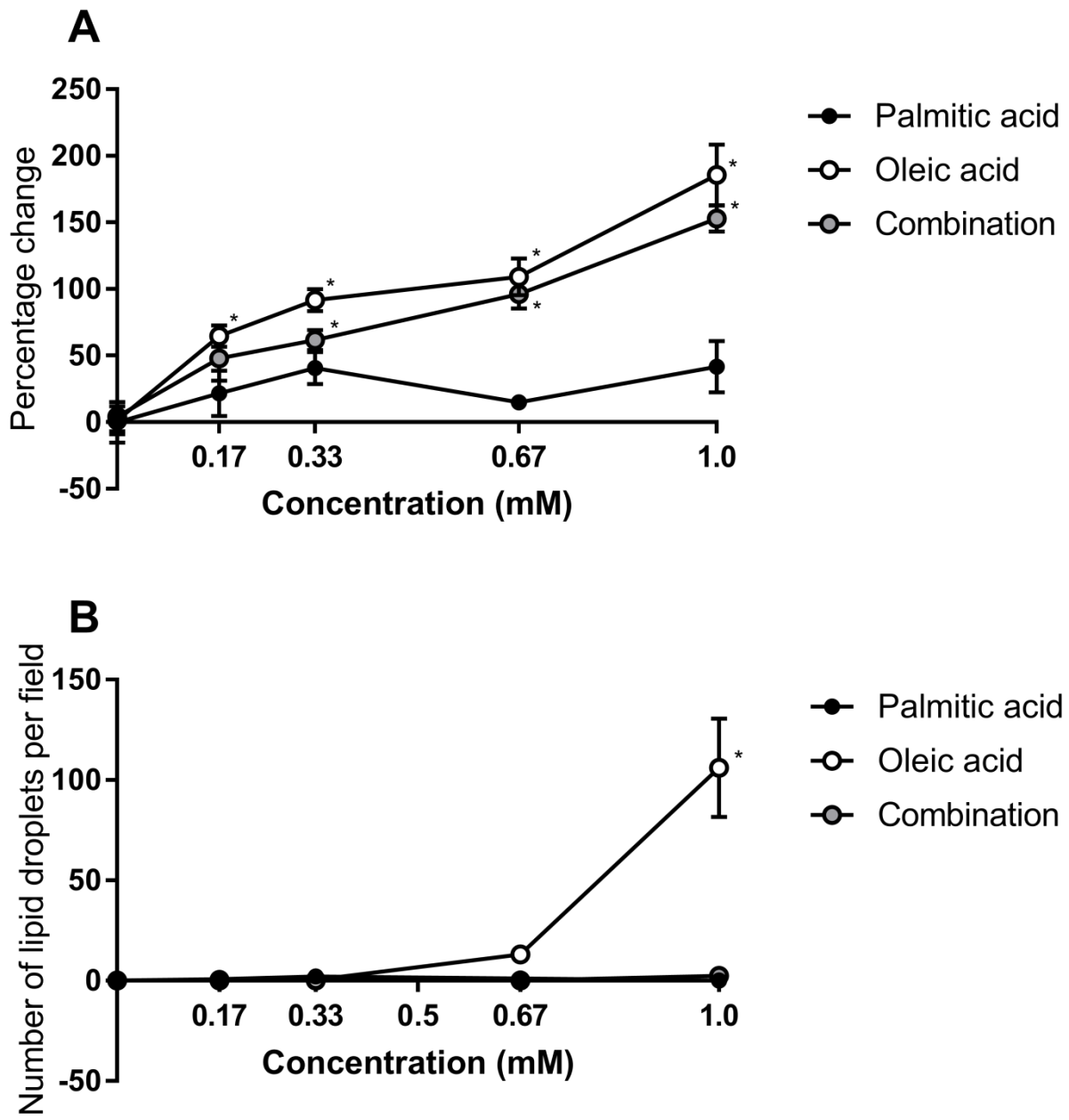


Figure 3.14 Oleic acid and the combined treatment induced significant lipid accumulation, whilst oleic acid caused the formation of lipid droplets in L6 cells

■ = palmitic acid □ = oleic acid ■ = combination treatment

A) Oil Red O staining showed that oleic acid produced significant intracellular lipid accumulation (P=0.0001), as did the combination treatment (P=0.0001) (n=3). B) Analysis of lipid droplets *via* ImageJ showed that oleic acid caused the formation of intracellular lipid droplets in L6 (P=0.0001).

3.3.6 Correlation between lipid accumulation and viability

As with the HepG2 study, the correlation between intracellular lipid accumulation and varying L6 cell death parameters were analysed. The correlation between intracellular lipid accumulation, tested by Oil Red O staining and cell viability of L6 determined by CCK-8 (Figure 3.15A, 3.15B, 3.15C) was examined. The correlation between intracellular lipid accumulation and live cells (Figure 3.15D, 3.15E, 3.15F), apoptotic cells (Figure 3.16A, 3.16B, 3.16C), late apoptotic/necrotic cells (Figure 3.16D, 3.16E, 3.16F) and dead cells (Figure 3.17A, 3.17B, 3.17C) quantified by flow cytometric analysis of annexin V/PI staining was studied. The results were presented in percentage change to standardise the data and permit better visual understanding.

On the X-axis, Oil Red O results were plotted against CCK-8 results on the Y-axis, to determine the correlation between lipid accumulation and cell viability (Figure 3.15A, 3.15B, 3.15C). Intracellular lipid accumulation resulting from treatment with palmitic acid was not associated with changes in cell viability (r=-0.1, P=0.9500). Oleic acid-induced lipid accumulation was moderately, but non-significantly correlated with a reduction in the viability of L6 cells (r=-0.7, P=0.2333). On the other hand, lipid accumulation induced by the combination treatment was correlated with a moderate increase in cell viability, however, this result was not significant (r=0.5, P=0.4500).

Oil Red O results were plotted on the X-axis against annexin V-and PI-negative results on the Y-axis, to examine the association between lipid accumulation and the percentage of live cells following treatment (Figure 3.15D, 3.15E, 3.15F). Palmitic acid-induced intracellular lipid accumulation was negatively, yet not significantly linked with live cell percentage (r=-0.7, P=0.2333). Lipid accumulation produced by treatment with oleic acid was strongly associated with a decrease in the percentage of live L6 cells, however, the result was not significant (r=-0.9, P=0.0833). Lipid accumulation induced by the combination treatment was mildly associated with a non-significant decrease in live cell percentage (r=-0.3, P=0.6833).

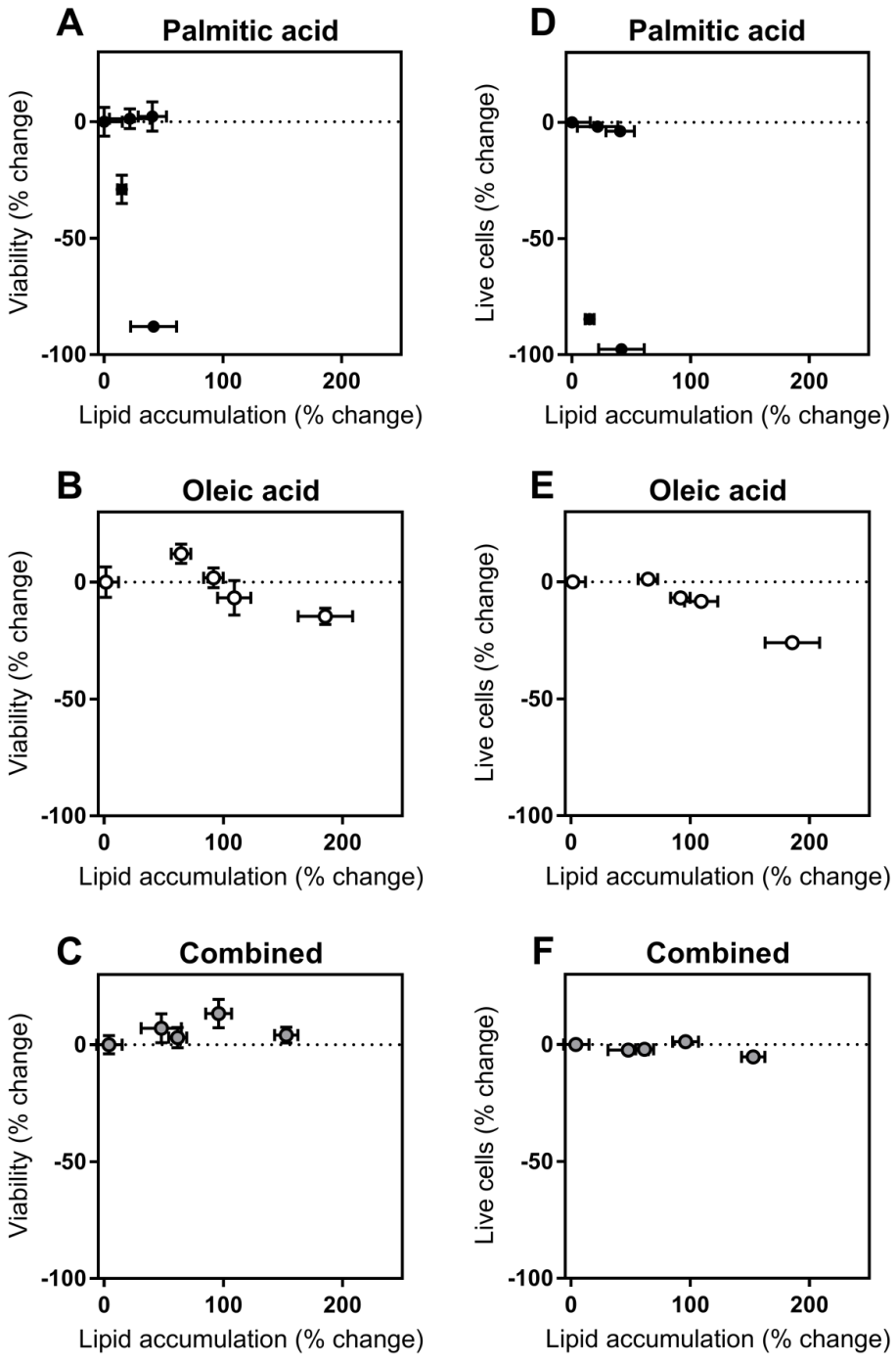


Figure 3.15 Intracellular lipid accumulation was not significantly associated with cell viability or the percentage of live L6 cells

■ = palmitic acid □ = oleic acid ▒ = combination treatment

A, B, C) Analysis of the correlation between lipid accumulation and cell viability of L6 was determined by comparison of ORO and CCK-8 results. There was no significant correlation following exposure to any of the treatments (ORO n=3, CCK-8 n=5). D, E, F) Analysis of the association between intracellular lipid accumulation and percentage of apoptotic L6 cells found no significant links for any of the treatments (ORO n=3, flow cytometry n=3).

Correlation between lipid accumulation and the percentage of apoptotic L6 cells was determined by plotting ORO results on the X-axis against annexin V-positive and PI-negative results on the Y-axis (Figure 3.16A, 3.16B, 3.16C). Palmitic acid-induced lipid accumulation was moderately linked with a reduction in the percentage of early apoptotic L6 cells, yet non-significantly ($r=-0.7$, $P=0.2333$). There was no correlation between oleic acid-induced lipid accumulation and the percentage of apoptotic L6 cells ($r=0$, $P>0.9999$). Furthermore, there was no correlation between intracellular lipid accumulation induced by the combination treatment and apoptosis in L6 cells ($r=0.1$, $P=0.9500$).

On the X-axis, Oil Red O results were plotted against annexin V-negative and PI-positive results on the Y-axis, in order to study the correlation between lipid accumulation and the percentage of late apoptotic/necrotic L6 cells (Figure 3.16D, 3.16E, 3.16F). Intracellular lipid accumulation produced by palmitic acid was linked with a non-significant increase in late apoptotic/necrotic cell percentage ($r=0.4$, $P=0.9500$). Oleic acid-induced lipid accumulation was significantly linked to an increase in the percentage of late apoptotic/necrotic L6 cells ($r=1$, $P=0.0167$). Lipid accumulation caused by the combination treatment was also moderately associated with an elevation in late apoptotic/necrotic cells, however, this result was not significant ($r=0.7$, $P=0.2333$).

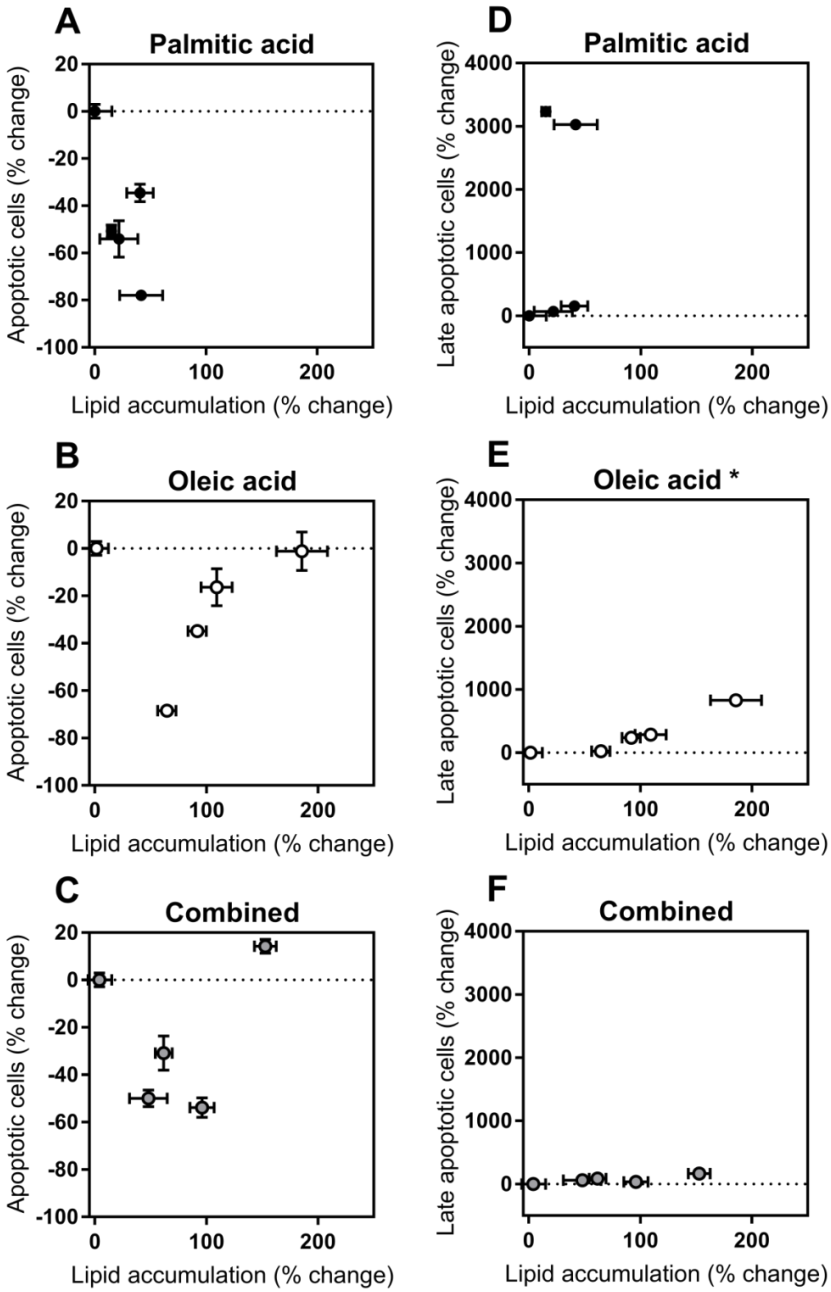


Figure 3.16 Oleic acid-induced intracellular lipid accumulation was associated with an increase in late apoptosis/necrosis.

■ = palmitic acid □ = oleic acid ■ = combination treatment

A, B, C) Study of the correlation between intracellular lipid accumulation and percentage of early apoptotic L6 found no significant links following exposure to any of the treatments (ORO n=3, flow cytometry n=3). D, E, F) Examination of the association between intracellular lipid accumulation and late apoptotic/necrotic L6 cells found that oleic acid-induced lipid accumulation was significantly correlated with an increase in late apoptosis/necrosis (P=0.0167), whilst palmitic acid and the combined treatment were not (P=0.9500 and 0.2333, respectively) (ORO n=3, flow cytometry n=3).

The association between lipid accumulation and the percentage of dead L6 cells was examined by plotting ORO results on the X-axis against annexin V- and PI-negative results on the Y-axis (Figure 3.17A, 3.17B, 3.17C). Intracellular lipid accumulation as a result of palmitic acid treatment was moderately correlated with elevated cell death, yet not significantly ($r=0.6$, $P=0.3500$). A strong association existed between oleic acid-induced lipid accumulation and the percentage of dead L6 cells, however, this correlation was not statistically significant ($r=0.8$, $P=0.1333$). There was zero correlation between intracellular lipid accumulation induced by the combination treatment and the percentage of dead L6 cells ($r=0$, $P>0.9999$).

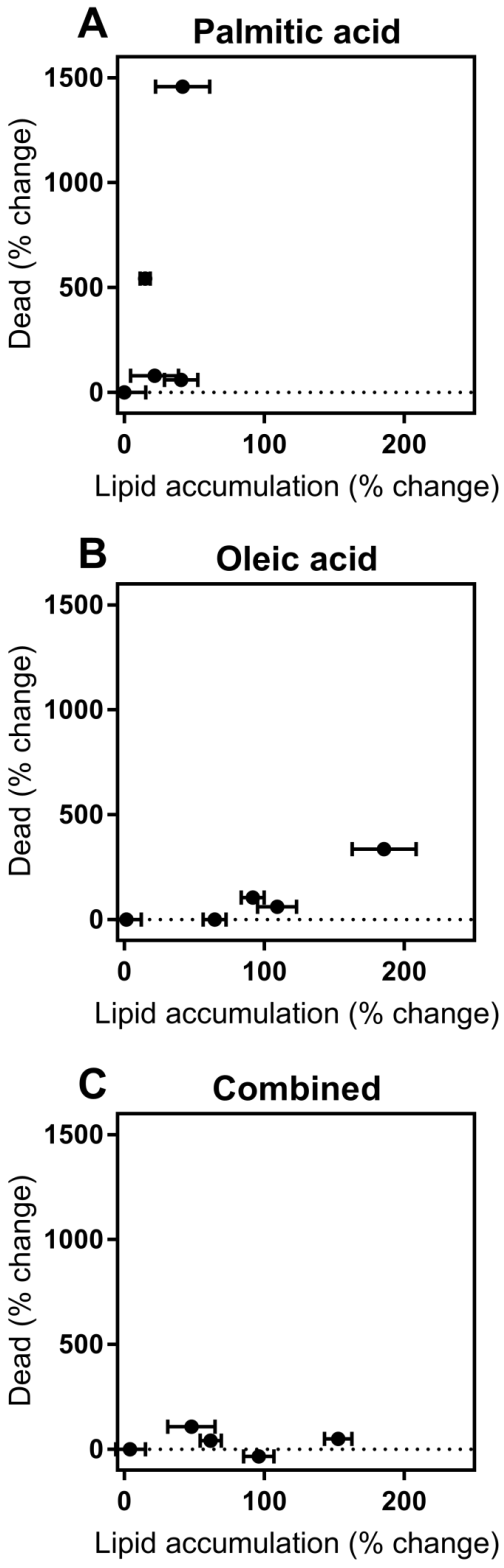


Figure 3.17 No significant associations between intracellular lipid accumulation and L6 cell death existed for any of the treatments

■ = palmitic acid □ = oleic acid ▒ = combination treatment

A, B, C) Upon comparison of intracellular lipid accumulation and dead cell percentage, no significant associations were found (ORO n=3, flow cytometry n=3).

On the X-axis, Oil Red O results were plotted against caspase assay results on the Y-axis, to determine the link between lipid accumulation and caspase 3/7 activity (Figure 3.18A, 3.18B, 3.18C). Intracellular lipid accumulation induced by palmitic acid was mildly, but non-significantly associated with elevated caspase 3/7 activity in L6 cells (r=0.4, P=0.5167). Oleic acid caused significant lipid accumulation, which was strongly correlated with an increase in caspase 3/7 activity, however, the result was not statistically (r=0.9, P=0.0833). Lipid accumulation induced by the combination treatment was mildly and non-significantly linked with increased caspase 3/7 activity (r=0.3, P=0.6833).

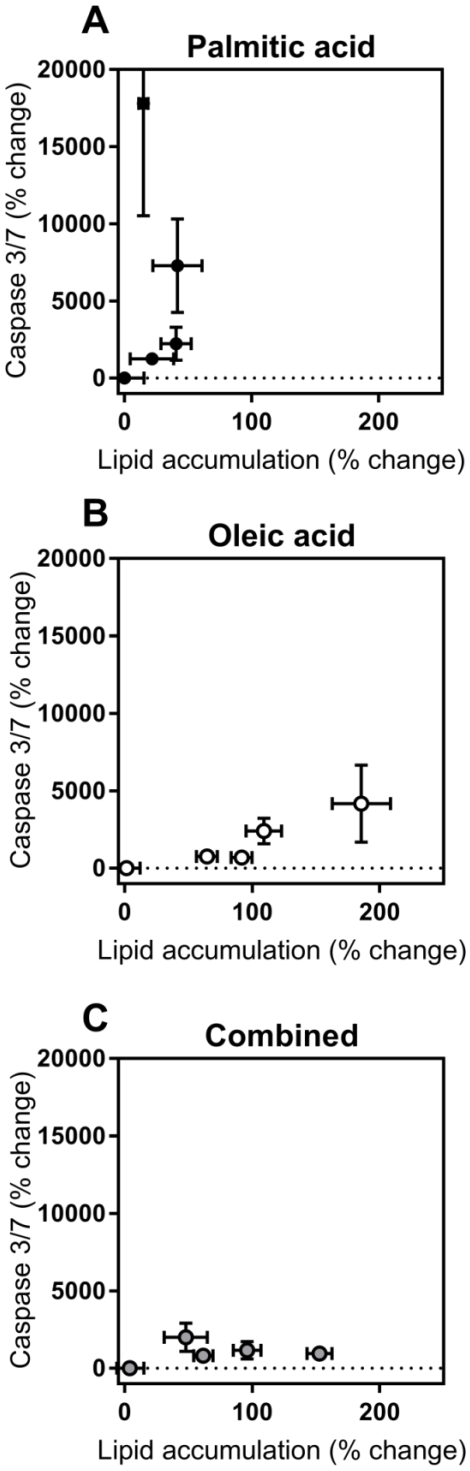


Figure 3.18 Intracellular lipid accumulation was not linked with caspase 3/7 activity in L6 cells.

■ = palmitic acid □ = oleic acid ■ = combination treatment

A, B, C) Examination of the correlation between intracellular lipid accumulation and caspase 3/7 activity in L6 cells found no significant links following 24 hours of treatment with palmitic acid and oleic acid, individually and in combination (ORO n=3, caspase assay n=3).

3.3.7 The differential effect of palmitic acid and oleic acid in HepG2 versus L6

Given the different findings from the two cell types, and the lack of understanding on the effect of FA in these two tissues, the final study in this chapter sought to compare the effect of PA and OA in HepG2 versus L6 cells.

Comparison of cell viability data between HepG2 and L6 revealed consistent trends across the range of concentrations (Figure 3.19). Palmitic acid caused greater loss in cell viability in L6 cells. At the highest concentration, 1 mM palmitic acid reduced the viability of HepG2 cells to 31.6 ± 1.86%, and L6 cells to the significantly lower 12.13 ± 0.37% (P=0.0086). Furthermore, all but the lowest concentration of oleic acid caused significantly greater loss in viability in L6 cells. Oleic acid at 1 mM decreased the viability of HepG2 to 89.38 ± 2.53%, but reduced the viability of L6 to 65.40 ± 2.66% (P=0.0010). In contrast, the combination treatment caused a greater loss to the viability of HepG2 cells than L6 cells, yet at higher concentrations caused reductions in the viability of HepG2 cells and increases in viability in L6 cells. The 1 mM combined treatment decreased the viability of HepG2 cells to 82.91 ± 4.90% and yet increased the viability of L6 to 104.20 ± 3.43% (P=0.0095).

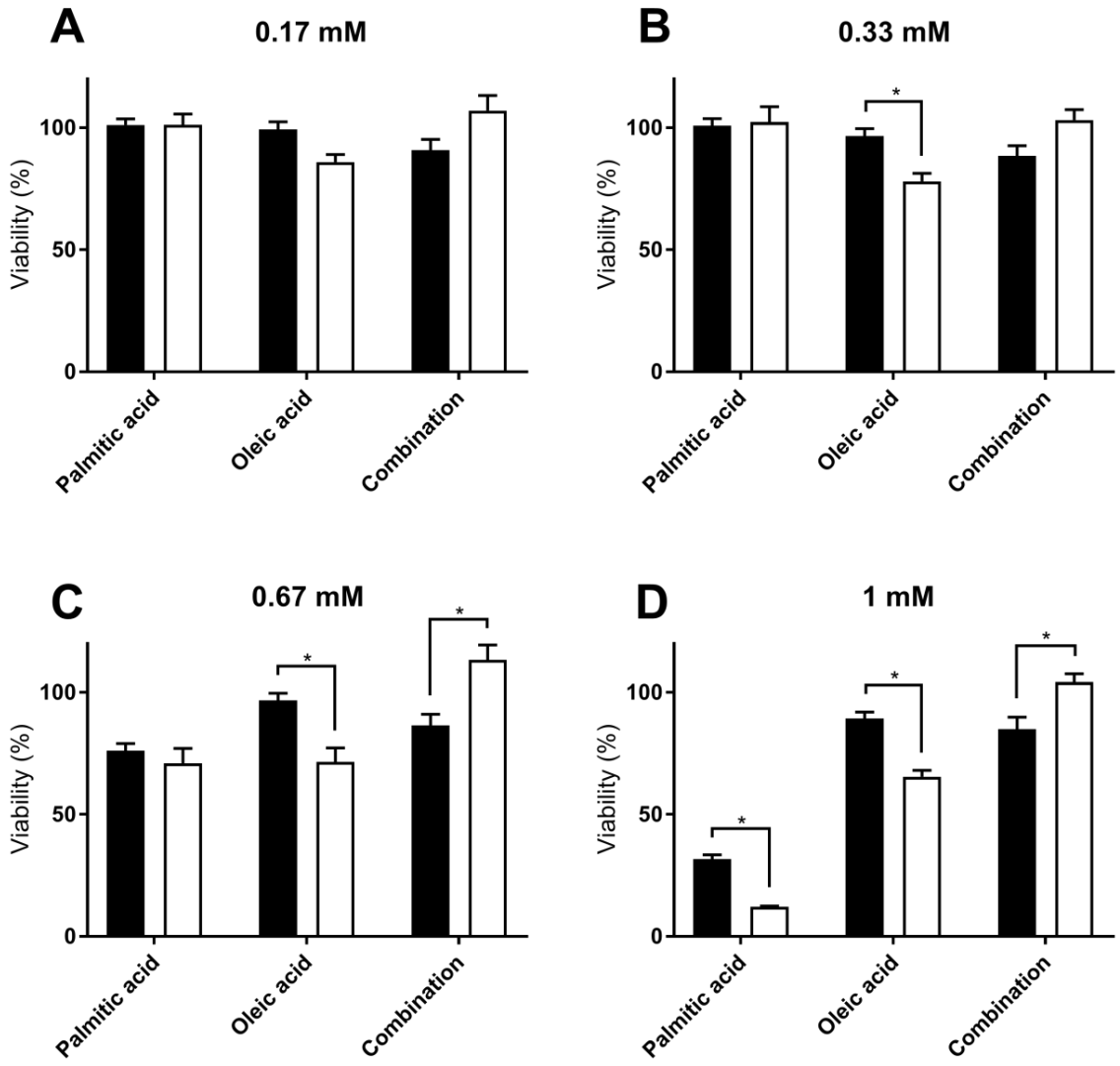


Figure 3.19 Palmitic acid and oleic acid caused greater viability loss in L6 cells, whilst the combined treatment produced the opposite effect

■ = HepG2 □ = L6

Comparision of HepG2 and L6 viability following FA treatment with results from the CCK-8 assay. A) 0.17 mM. B) 0.33 mM. C) 0.67 mM. D) 1 mM.

Live cell percentage data was compared between HepG2 and L6 cells to identify trends. At low concentrations, 0.17 mM and 0.33 mM palmitic acid produced larger, but non-significant reductions in live HepG2 cells as opposed to L6 (P=0.7834 and 0.9992, respectively) (Figure 3.20). However, at higher concentrations of 0.67 mM and 1 mM this was reversed and palmitic acid caused significantly greater reductions in the percentage of live L6 in comparison to HepG2. At 1mM, oleic acid decreased live HepG2 percentage by 62.84 ± 0.70% yet reduced live L6 percentage by 97.64 ± 0.13% (P<0.0001). Oleic acid produced a similar trend in that low concentrations caused significantly greater reductions in live HepG2 cells than L6. For example, 0.67 mM oleic acid decreased the percentage of live HepG2 by 35.80 ± 5.42% and L6 by only 8.32 ± 0.39% (P=0.0001). However, 1 mM of oleic acid caused a reduction in live HepG2 cells by 13.89 ± 0.71% and yet decreased live L6 by 25.95 ± 1.06%, and, thus, demonstrated the opposite trend (P=0.0002). On the other hand, the combination treatment produced little difference between HepG2 and L6 at 0.17 mM and 0.33 mM (P=0.9769 and 0.8660, respectively), and yet produced contradictory results for 0.67 mM and 1 mM. At 0.67 mM, the combined treatment caused a reduction in live HepG2 cells by 22.65 ± 4.06% and a slight increase in live L6 cells of 1.21 ± 0.33% (P=0.0005). But conversely at 1 mM, elevated the percentage of live HepG2 cells by 14.36 ± 3.23% and decreased live L6 cells by 5.22 ± 0.35% (P<0.0001).

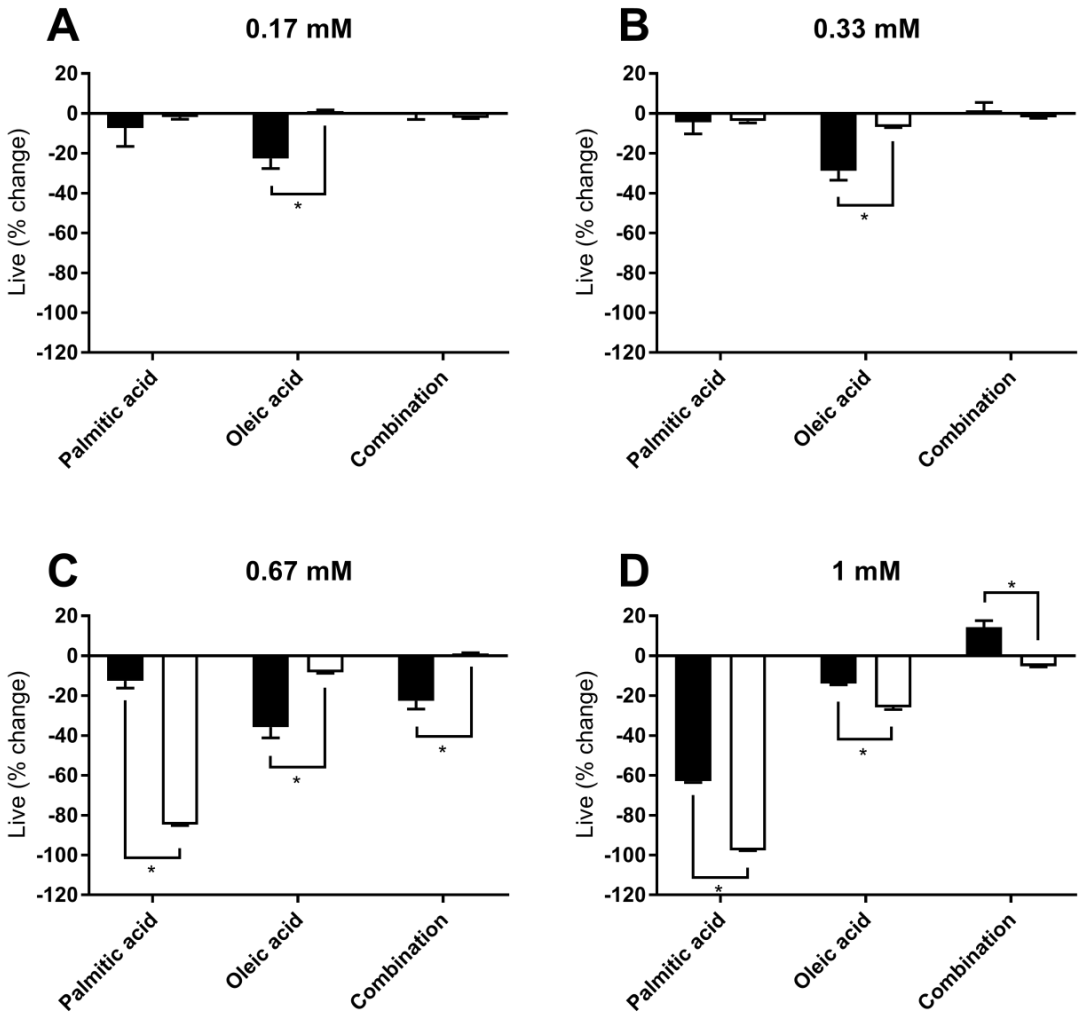


Figure 3.20 Palmitic acid caused greater reductions in the percentage of live L6 cells in comparison to HepG2

■ = HepG2 □ = L6

Comparison of live cell percentage change in HepG2 and L6 following treatment with PA and/or OA. Results from flow cytometric analysis of annexin V/PI staining. A) 0.17 mM. B) 0.33 mM. C) 0.67 mM. D) 1 mM.

The comparison of apoptosis data demonstrated that palmitic acid induced greater levels in HepG2 than L6 cells (Figure 3.21). Furthermore, palmitic acid consistently decreased apoptosis in L6 cells, but had a tendency to increase it in HepG2. At 1 mM, palmitic acid induced a 98.69 ± 10.69% increase in the percentage of apoptotic HepG2 cells, yet reduced apoptosis by 77.91 ± 1.65% in L6 cells ($P<0.0001$). Oleic acid produced a similar trend at concentrations of 0.17 mM and 0.33 mM ($P<0.0001$ and $P=0.0002$, respectively). However, there was no significant difference between the effects of higher concentrations of oleic acid on HepG2 cells and L6 cells. The combination treatment did not produce a trend between HepG2 and L6, but did produce a significant result at 0.33 mM ($P=0.0017$).

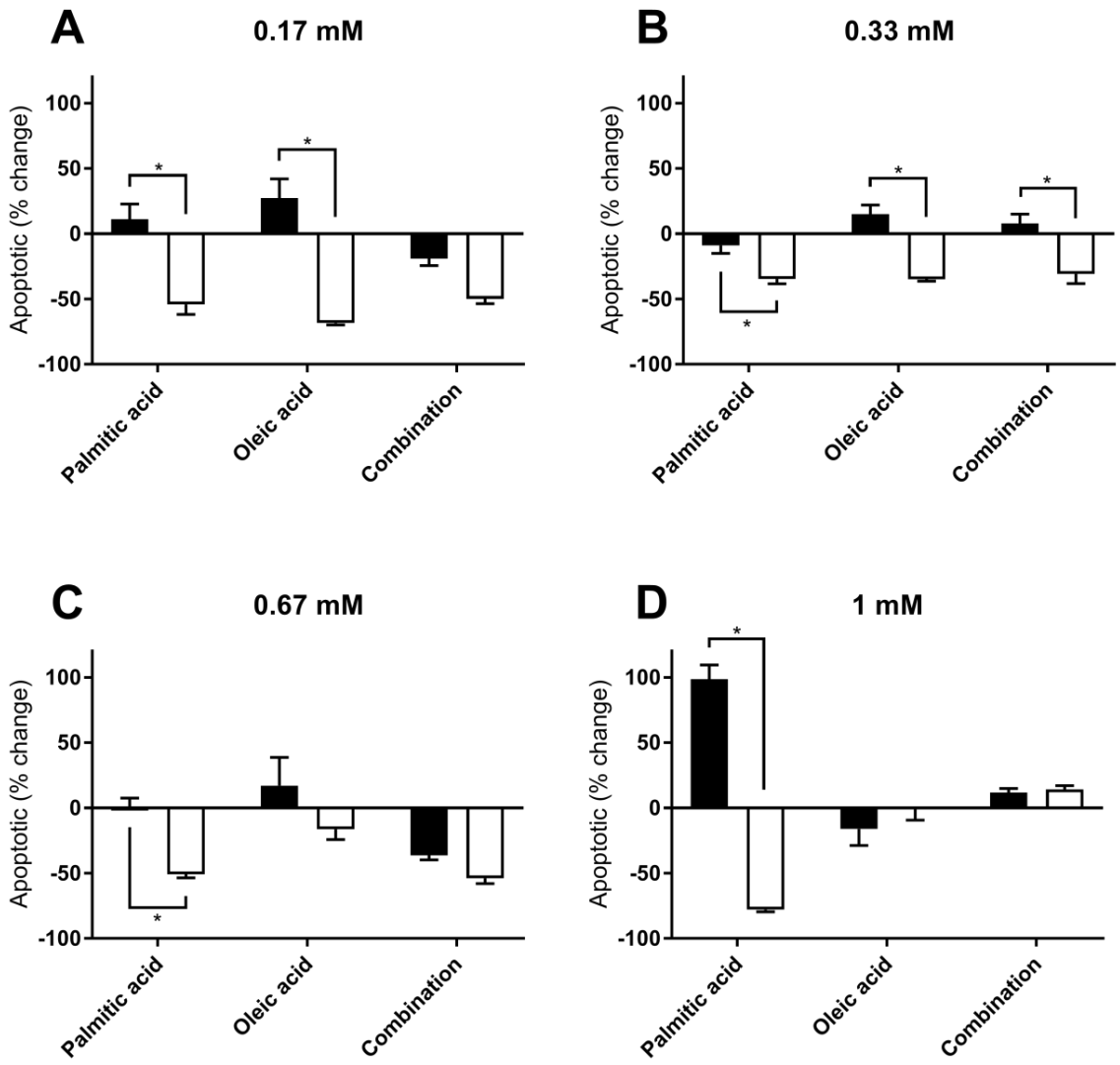


Figure 3.21 Palmitic acid induced greater levels of early apoptosis in HepG2 than L6 cells

■ = HepG2 □ = L6

Comparison of apoptotic cell percentage change in HepG2 and L6 following FA treatment. Results from flow cytometric analysis of annexin V/PI staining. A) 0.17 mM. B) 0.33 mM. C) 0.67 mM. D) 1 mM.

The percentage of late apoptotic/necrotic cells was compared between HepG2 and L6. Palmitic acid at concentrations of 0.33mM, 0.67 mM and 1 mM induced significantly higher percentages of late apoptosis/necrosis in L6 cells compared to HepG2 cells ($P=0.0025$, $P<0.0001$ and $P<0.0001$) (Figure 3.22). At 1 mM, palmitic acid increased late apoptotic/necrotic L6 cells by 3026.73 ± 8.00%, whilst the percentage of late/necrotic HepG2 cells only increased by 114.11 ± 6.16%. Oleic acid demonstrated the same trend and caused significantly greater late apoptosis/necrosis in L6 cells following treatment with 0.33 mM, 0.67 mM and 1 mM oleic acid ($P=0.0006$, $P<0.0001$ and $P<0.0001$, respectively). At 1 mM, oleic acid elevated the percentage of late apoptotic/necrotic HepG2 cells by 59.02 ± 6.85% yet increased late apoptosis/necrosis by 827.96 ± 40.38% in L6 cells. The combination treatment also followed the same pattern, however, only1 mM produced a significant difference between HepG2 and L6. At 1 mM, the combination treatment increased late apoptotic/necrotic HepG2 cells by only 5.95 ± 6.53% yet elevated late apoptosis/necrosis by 166.67 ± 15.17% in L6 cells ($P=0.0001$).

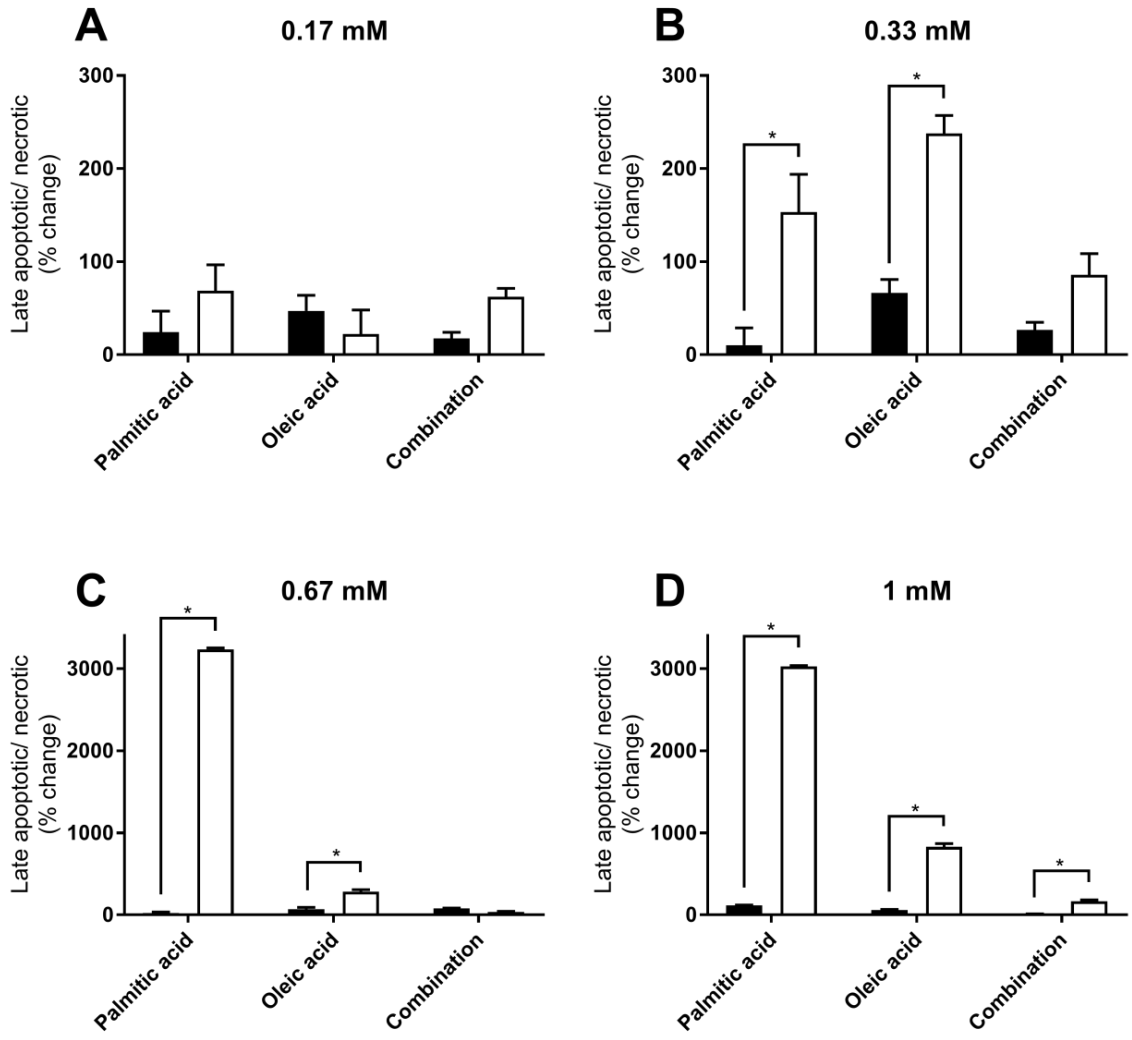


Figure 3.22 Palmitic acid and oleic acid induced vastly greater levels of late apoptosis/necrosis in L6 cells than HepG2 cells

■ = HepG2 □ = L6

Comparison of late apoptotic/ necrotic cell percentage change in HepG2 and L6 following FA treatment. Results from flow cytometric analysis of annexin V/PI staining. A) 0.17 mM. B) 0.33 mM. C) 0.67 mM. D) 1 mM.

Comparison of dead cell percentages between HepG2 and L6 demonstrated that all treatments induced greater levels of cell death in L6 cells (Figure 3.23). All concentrations of palmitic acid produced a significant difference, however, at 0.17 mM and 0.33 mM palmitic acid reduced cell death in HepG2 cells, yet increased cell death in L6 ($P=0.0004$ and $P<0.0001$, respectively). At higher concentrations, palmitic acid induced minimal levels of cell death in HepG2 cells, but high levels in L6. At 1 mM, cell death was increased by 12.70 ± 10.17% in HepG2 and 1457.14 ± 14.83% in L6 ($P<0.0001$). Oleic acid produced the same trend except at 0.17 mM, which caused little cell death in either cell line ($P=0.9938$). At 0.33 mM, 0.67 mM and 1mM oleic acid induced greater percentages of cell death in L6 than HepG2 ($P<0.0001$, $P=0.0004$ and $P<0.0001$, respectively). The pattern was also similar for the combination treatment, however, all concentrations caused a reduction in cell death in HepG2. At 1 mM, the combined treatment decreased the dead HepG2 percentage by 57.14 ± 0.26% and elevated cell death in L6 by 49.74 ± 2.23% ($P<0.0001$).

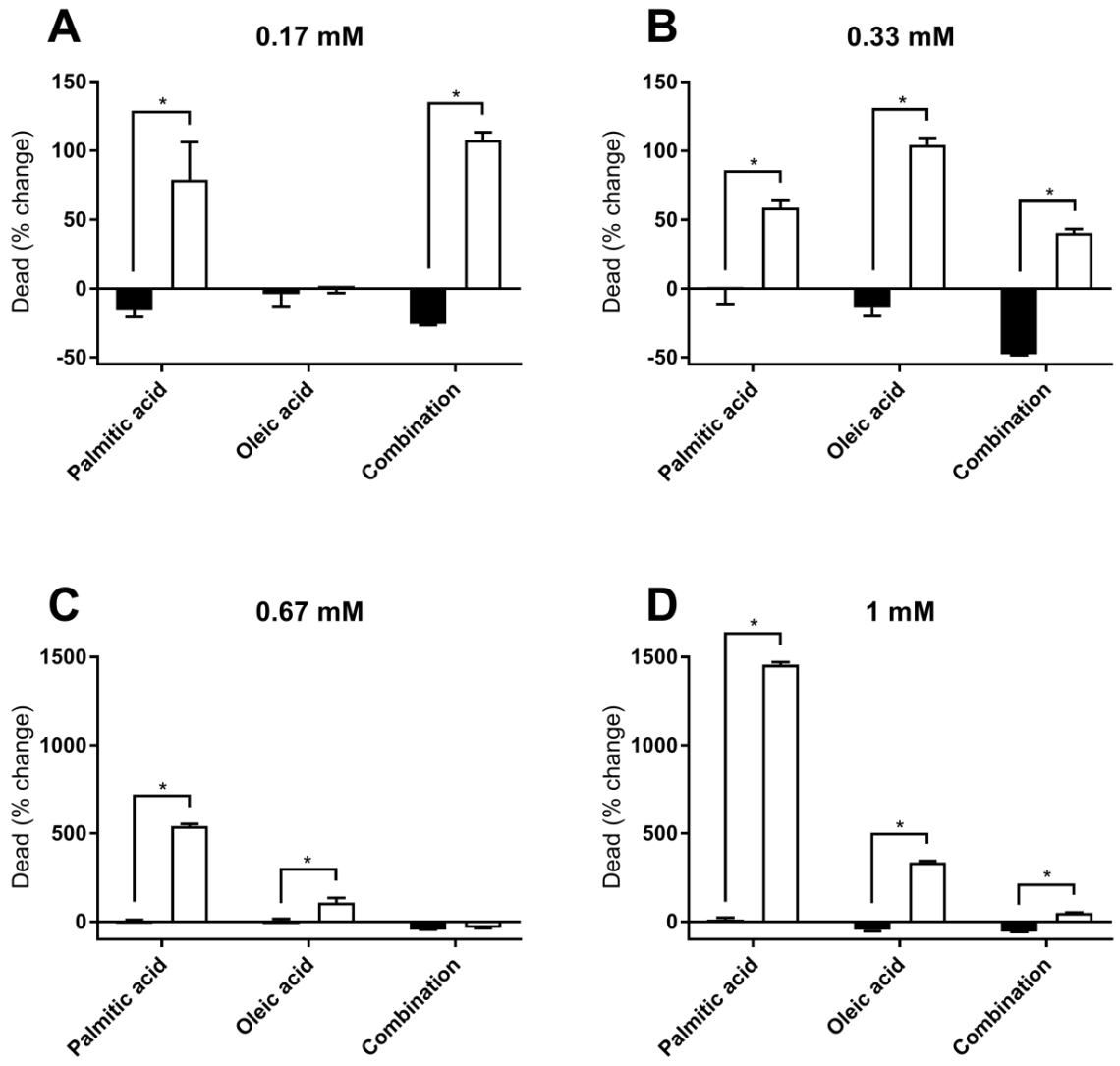


Figure 3.23 All treatments caused greater cell death in L6 cells as opposed to HepG2 cells

■ = HepG2 □ = L6

Comparison of late apoptotic/ necrotic cell percentage change in HepG2 and L6 following treatment with PA and/or OA. Results from flow cytometric analysis of annexin V/PI staining. A) 0.17 mM. B) 0.33 mM. C) 0.67 mM. D) 1 mM.

Intracellular lipid accumulation was compared between HepG2 and L6 cells. This section of the study was vital to understand whether disparities in lipid accumulation could be a factor in the differing effects of palmitic acid and oleic acid between the two cell types.

Comparisons found that levels of palmitic acid-induced lipid accumulation was equal between HepG2 and L6 cells, even at 1 mM (P=0.9994) (Figure 3.24). On the other hand, both oleic acid and the combined treatment caused greater lipid accumulation in L6 than HepG2, however, this observation was not statistically significant even at the highest concentration (P=0.8064 and 0.2190, respectively).

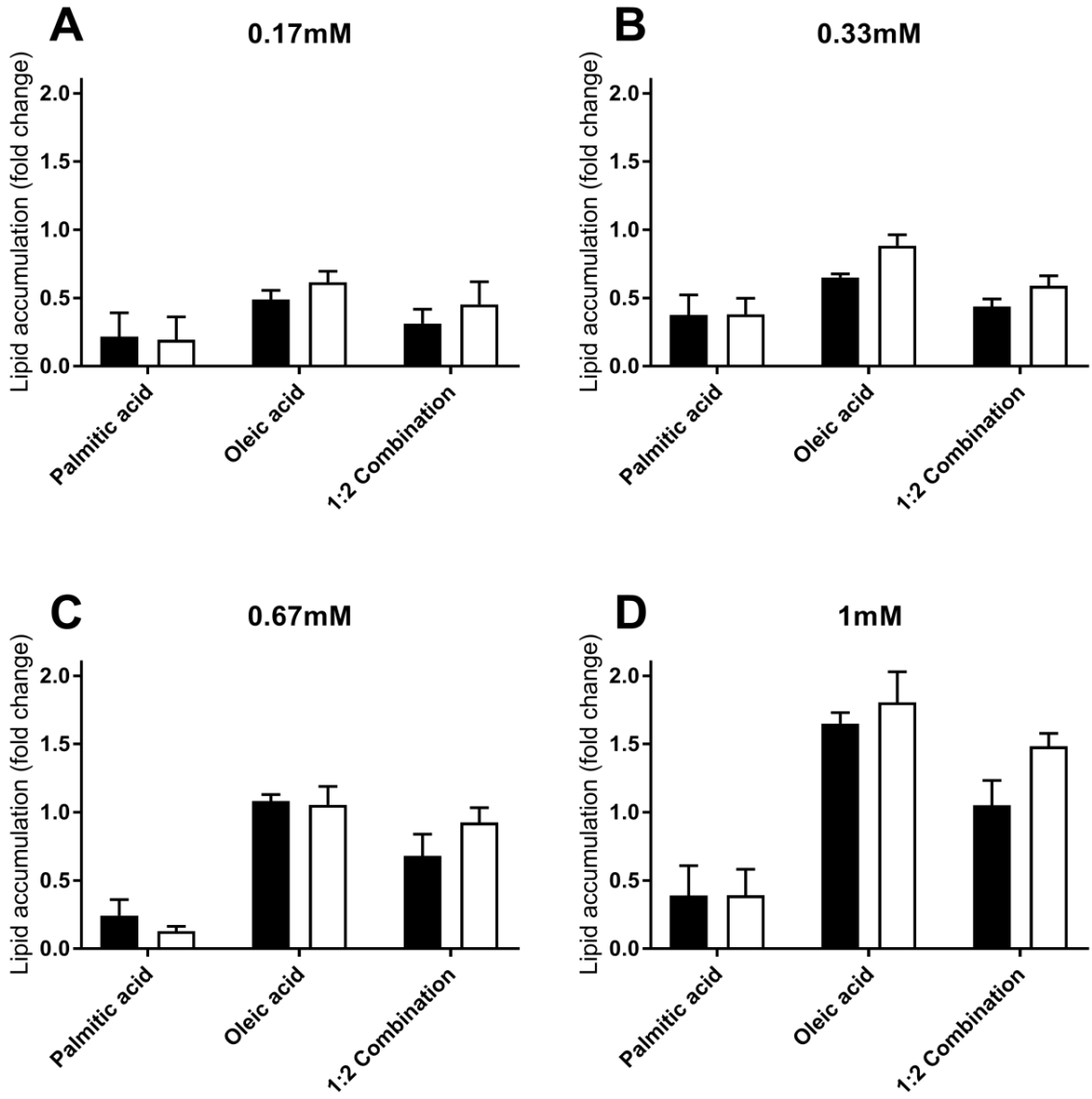


Figure 3.24 Lipid accumulation induced by any of the treatments did not differ between HepG2 and L6 cells

■ = HepG2 □ = L6

Comparison of intracellular lipid accumulation fold change in HepG2 and L6 following FA treatment. Results from analysis of ORO staining. A) 0.17 mM. B) 0.33 mM. C) 0.67 mM. D) 1 mM.

3.4 Discussion

The aim of experiments in this chapter was to examine the effects of palmitic acid and oleic acid, alone and in combination, on cell death and intracellular lipid accumulation in HepG2 and L6 cells. This extended to the determination of associations between lipid accumulation and phase of cell death, and finally to the comparison of all results between the two cell lines to clarify contradictory literature in the field and gain a better understanding of the differential effects caused by these fatty acids. Results highlighted that palmitic acid instigated loss of viability in both HepG2 and L6 cells, whilst oleic acid only reduced cell viability in L6 cells. The results from the combination treatment were more interesting when compared to results from its constituent parts. The 1 mM combination treatment was composed of 0.67 mM oleic acid and 0.33 mM palmitic acid and produced differing effects in the two cell lines. In L6, the combination of the two fatty acids abolished the deleterious effects of oleic acid, whilst in HepG2 the combination was more toxic than the individual treatments alone. These results highlighted the differential effects between the two cell lines and indicated potential differences in fatty acid metabolism between HepG2 and L6 cells.

Further experimentation investigated the stages of viability loss and demonstrated that all treatments reduced the percentage of live cells relative to the control in both cell lines. In L6 cells, low concentrations of palmitic acid and oleic acid, alone and in combination reduced early apoptosis. Conversely, at the highest concentration they all elevated late apoptotic/necrotic and dead cells. In HepG2 cells, trends were less clear. The highest concentration of palmitic acid increased late apoptosis/necrosis, but unlike L6 also increased early apoptosis. Oleic acid on the other hand, caused an increase in late apoptosis/necrosis, but decreased dead HepG2 cells. In contrast to L6, the combination treatment reduced the percentage of dead cells in HepG2. The results indicated that palmitic acid was inducing cell death *via* apoptosis as increases in early and late apoptosis were found. However, the mechanism of oleic acid-induced cell death was less certain, and, thus, further analysis was required.

Forward scatter and side scatter were examined to gain a better insight into the possible modes of cell death. In HepG2, palmitic acid, oleic acid and the combination treatment decreased forward scattered light. Only palmitic acid and oleic acid alone reduced FSC in L6 cells. Palmitic acid and oleic acid alone, also reduced side scatter in HepG2, but no treatment altered this parameter in L6 cells. In spite of these results being tentative, they added further weight to the theory that palmitic acid was causing cell death *via* apoptosis. However, results for the other treatments were equivocal. Therefore, a caspase assay was used to study apoptosis in L6 cells, but not in HepG2 cells due to aforementioned issues with clumping in culture. Palmitic acid greatly increased caspase 3/7 activity in L6 cells, whilst oleic acid and the combination treatment did not. This confirmed that palmitic acid was eliciting its effects *via* apoptosis, and suggested that oleic acid and the combination treatment were causing cell death by another mechanism.

Intracellular lipid levels were measured to examine the link between intracellular lipid accumulation and cell death. In both cell lines, oleic acid and the combination treatment induced lipid accumulation. In HepG2, the combined treatment caused an increase in lipid accumulation, whilst oleic acid caused maximal accumulation from the control. Slightly higher increases were found in L6 with both the combination treatment and oleic acid. Further analysis studying the number of lipid droplets was performed in L6. This experiment was not performed on HepG2 as in culture the cells clumped rather than grow in a monolayer, which made analysis unreliable. This test was important to understand how the different treatments altered lipid storage. Only oleic acid caused a significant increase in the number of lipid droplets in L6. These results suggested that palmitic acid did not exert its deleterious effects through intracellular lipid accumulation, and that oleic acid caused intracellular lipid accumulation in the form of lipid droplets. Furthermore, they suggested that combination treatment-induced lipid accumulation differed from oleic acid-induced lipid accumulation. However, further statistical analysis was performed to fully understand the relationships between intracellular lipid accumulation and cell death processes.

The correlation between intracellular lipid accumulation and cell viability, percentage of live cells, early apoptosis, late apoptosis/necrosis and percentage of dead cells was examined, and only three associations found. In HepG2, lipid accumulation induced by oleic acid and the combination treatment were both associated with a loss of cell viability. In addition, oleic acid-induced lipid accumulation was correlated with late apoptosis/necrosis in L6 cells. These results confirmed that palmitic acid was not eliciting its toxicity *via* intracellular lipid accumulation. However, oleic acid-induced lipid accumulation may physically damage cells, thus, supporting necrosis as the proposed mechanism of cell death.

3.4.1 Palmitic acid induces cell death in HepG2 and L6 cells

Many studies have investigated the effects of FFA on cells in vitro (Chavez-Tapia*, et al.*, 2012; Cheon*, et al.*, 2014; El-Assaad*, et al.*, 2003; Gaster*, et al.*, 2005; Haffar*, et al.*, 2015; Listenberger*, et al.*, 2003b; Malhi*, et al.*, 2006; Ricchi*, et al.*, 2009; Shen*, et al.*, 2017; Yao*, et al.*, 2011), however, there are a number of contradictions in the literature regarding which fatty acids cause cell death, and whether the loss in viability is as a result of intracellular lipid accumulation. Furthermore, most papers suggest that unsaturated fatty acids are able to prevent the detrimental effects produced by saturated fatty acids (Cheon*, et al.*, 2014; El-Assaad*, et al.*, 2003; Haffar*, et al.*, 2015; Listenberger*, et al.*, 2003b; Shen*, et al.*, 2017), whilst a small number have found these 'protective' unsaturated fatty acids to cause cell death in their own right (Malhi*, et al.*, 2006).

Research on pancreatic β-cells is the most abundant possibly due to links between obesity and type II diabetes (Halban*, et al.*, 2014; Linnemann*, et al.*, 2014; Tomita, 2016). Studies focusing on hepatocytes are less frequent, although due to implications for non-alcoholic fatty liver disease are still relatively common (Al Fadel*, et al.*, 2016; Kochan*, et al.*, 2017). However, investigations into the effects of FFA on skeletal muscle cells are somewhat lacking (Cheon*, et al.*, 2014; Peng*, et al.*, 2011).

The literature in this area reports that fatty acids have a common effect across cell types. Most papers report that palmitic acid causes cell death, whilst oleic acid seems to have little

effect, however, this was not always the case. Azevedo-Martins*, et al.,* (2006) found that both palmitic acid and oleic acid cause DNA fragmentation leading to apoptosis in RINm5F pancreatic β-cells, while Maestre*, et al.*, (2003) found that apoptosis is accelerated in β(INS-1)-cells). One study found that oleic acid causes limited cell death in pancreatic β-cells, whilst exerting higher toxicity than palmitic acid in non–β islet cells (Cnop*, et al.*, 2001). Oleic acid has also been shown to induce cell death in AS-30D rat hepatoma cells (Dymkowska*, et al.*, 2006), Jurkat human T lymphocytes and Raji B lymphocytes (Cury-Boaventura*, et al.*, 2006), J774 macrophages (Martins de Lima*, et al.*, 2006) and human neuroblastoma SH-SY5Y cells (Zhu*, et al.*, 2005). Other studies report that palmitic acid induces apoptosis in hepatocytes, whilst oleic acid induces autophagy (Mei*, et al.*, 2011).

This discord in the literature highlights the importance of investigating the effects of palmitic acid and oleic acid on the viability of hepatocytes and skeletal myocytes.

The results from this study determined that palmitic acid and oleic acid elicited different effects on different cell types. Palmitic acid was highly toxic to HepG2 and L6 cells, causing 68.4 ± 1.86% and 87.9 ± 0.37% cell death, respectively. Flow cytometric analysis of annexin V/PI staining found that palmitic acid caused a significant increase in late apoptotic/necrotic cells in both HepG2 and L6 cells (P=0.0001 and 0.0001, respectively. Furthermore, 0.67 mM palmitic acid significantly increased caspase 3/7 activity in L6 cells (P=0.0001). These results were consistent with previous research in HepG2 that found palmitic acid causes mitochondrial dysfunction, by inducing oxidative stress *via* the activation of NADPHox (García-Ruiz*, et al.*, 2015). A study in L6 myocytes determined that palmitic acid induces cell death through the loss of mitochondrial membrane potential (Cheon & Cho, 2014). However, many mechanisms of action have been proposed; for example a study using L6 and C2C12 myocytes found that palmitic acid induces ER stress (Peng*, et al.*, 2011).

It was found that oleic acid, at concentrations of 1 mM caused significant cell death in L6 cells, (34.6 ± 2.66% of cells), whilst triggering cell death in only 10.6 ±2.53% in HepG2 cells (P=0.0010). This finding was confirmed by flow cytometric analysis of annexin V/PI, which

highlighted a significant increases in late apoptotic/necrotic cells in L6 (827.96 ± 40.38%), and smaller, but still significant elevations in late apoptotic/necrotic HepG2 of 59.02 ± 6.85% following treatment with oleic acid (P=0.0001 and 0.0187, respectively). This is in contrast to research carried out in L6 and C2C12 myocytes, which found that oleic acid does not cause ER stress (Peng*, et al.*, 2011), and, thus, does not trigger cell death.

The combination of palmitic acid and oleic acid appeared to exert protective effects over low level toxicity in L6 cells. In HepG2 cells, the combination treatment induced greater losses to cell viability than its constituent parts. These results are similar to findings in L6 and C2C12 myocytes which indicate that a combination the addition of oleic acid relieved palmitic acid-induced ER stress (Peng*, et al.*, 2011). Others have found that the effects of palmitic acid are completely abolished with the equimolar addition of unsaturated fatty acids in mouse L-M fibroblasts (Doi*, et al.*, 1978). However, the combination of palmitic acid and oleic acid increasing cell viability in any cell type has not been evidenced in the literature. However, this was a small effect, and, thus, within the experimental range.

There are a number of mechanisms through which oleic acid may protect cells against the deleterious effects of palmitic acid. For example, oleic acid may block the uptake of palmitic acid into the cells by forming aggregates or *via* competitive inhibition. However this was disproven in C2C12 cells as it was shown that oleic acid recovers Akt phosphorylation by relieving palmitic acid-induced ER stress (Peng*, et al.*, 2011).

A significant number of studies suggest that oleic acid forms neutral lipid droplets within the cell which sequesters the palmitic acid, thereby preventing it from eliciting lipotoxic effects (Cheon & Cho, 2014; El-Assaad*, et al.*, 2003; Haffar*, et al.*, 2015; Listenberger*, et al.*, 2003; Shen*, et al.*, 2017). Results from this study suggested that oleic acid did form lipid droplets, however, these were not necessarily neutral as oleic acid caused toxicity in L6 cells. Furthermore, these lipid droplets did not appear to sequester palmitic acid.

There is also some confusion as to whether fatty acids cause cell death *via* apoptotic or necrotic pathways. The majority of papers suggest that fatty acids trigger apoptosis, yet a number of papers challenge the dogma, suggesting that lipotoxicity is executed *via*

mitochondrion-dependent necrosis (Rockenfeller*, et al.*, 2010), or *via* a combination of the two (Hoffmann*, et al.*, 2014). Mitochondrion-dependent necrosis is initiated by the formation of the mitochondrial permeability transition pore following stress resulting in membrane depolarisation and uncoupling of oxidative phosphorylation, which leads to ATP depletion and necrotic cell death (Halestrap, 2009; Lemasters*, et al.*, 1998). The results from the current study concur with those from Yao*, et al.,* (2011), who found that HepG2 treated with a combination of palmitic and oleic acid showed increases in the late apoptotic/necrotic quadrant as demonstrated by flow cytometric analysis of annexin V/PI staining. Yao*, et al.,* (2011) suggested that these results indicate a combination of apoptosis and necrosis is taking place. However, results from this project indicated that palmitic acid was undergoing apoptosis. This theory appears to be highly likely as the increase in caspase 3/7 following treatment with palmitic acid was a strong indicator of apoptosis. This finding is supported by the literature, as palmitic acid causes LDH release in cultured human endothelial cells (Zhang*, et al.*, 1992), DNA laddering in neonatal rat ventricular myocytes (de Vries*, et al.*, 1997), and DNA fragmentation in pancreatic β-cells (Maedler*, et al.*, 2001). It was hypothesised that the lapse of time between the initiation of apoptosis and the performance of the assay resulted in some cells falling into the late apoptotic/necrotic category. However, oleic acid appears to initiate necrotic processes as signs of early apoptosis were not evident.

Despite differences in the mechanism, the evidence strongly supports the concept that mitochondria and the ER play a role in fatty acid-induced lipotoxicity (Egnatchik*, et al.*, 2014; Hauck & Bernlohr, 2016). A number of studies have shown that fatty acids cause structural and functional changes to mitochondria (Chan & Higgins, 1978; Penzo*, et al.*, 2002). One of the most recognised deleterious effects of fatty acids is the uncoupling of oxidative phosphorylation through a number of metabolite transporters (Hermesh*, et al.*, 1998; Samartsev*, et al.*, 2011; Samartsev*, et al.*, 2013). The interaction between fatty acids and mitochondrial carrier proteins leads to membrane depolarisation, and/or the transformation of the mitochondrial carrier protein into a pore (Penzo*, et al.*, 2002). The opening of this permeability transition pore initiates apoptosis (Rial*, et al.*, 2010). Some

authors believe that *de novo* ceramide synthesis following treatment with palmitic acid also leads to activation of the apoptotic mitochondrial pathway (Maedler*, et al.*, 2001). Results from research investigating effects on the ER indicate that palmitic acid induces the production of phospholipids, which cause imbalances in the ER membrane leading to ER expansion, disrupting the normal functioning of the ER (Peng*, et al.*, 2011). However, other papers suggest that palmitic acid causes the formation of solid lipid deposits in the ER membrane (Shen*, et al.*, 2017), which produce cleft-like dilations displacing the normal granular contents of the ER (Gordon, 1977). This project did not find significant lipid accumulation following treatment with palmitic acid which could favour the theory of incorporation into the ER membrane, leading to ER stress and ultimately cell death. However, further investigations would be required to confirm this hypothesis.

3.4.2 Oleic acid induces lipid accumulation

Yao*, et al.,* (2011) showed that as the concentration of fatty acid solution increases, so does intracellular lipid accumulation ($P<0.01$), which is implicated in triggering cell death and alterations to intracellular signalling. There is much debate regarding intracellular lipid accumulation and the mechanisms by which it damages cell viability, if at all. Some studies have linked the accumulation of intracellular lipids with cell death (Lee*, et al.*, 1994; Unger, 1997). However, other studies have found an inverse relationship between TAG accumulation and lipotoxicity in pancreatic β-cells (Cnop*, et al.*, 2001).

Results from study determined that palmitic acid did not induce intracellular lipid accumulation in either cell line. Furthermore, palmitic acid-induced intracellular lipid accumulation was not correlated with cell death or any of its processes in either cell line, yet palmitic acid was the most toxic treatment tested. Therefore, this project found that lipid accumulation was not inherently responsible for cell death. Many authors agree that palmitic acid treatment results in minimal lipid accumulation, however, some studies have found it to accumulate in primary neonatal cardiomyocytes (Haffar*, et al.*, 2015), jejunal

epithelial cells (McKay*, et al.*, 1967) and C2C12 myocytes (Chavez*, et al.*, 2003; Peng*, et al.*, 2011).

The presence of increasing concentrations of oleic acid resulted in significant intracellular lipid accumulation in both HepG2 and L6 cells. This lipid accumulation was correlated with the loss of HepG2 viability and associated with late apoptosis/necrosis in L6 cells. There is a strong body of evidence that suggests that oleic acid causes intracellular lipid accumulation in lipid droplets (Cheon & Cho, 2014; Cnop*, et al.*, 2001; Gordon, 1977; Haffar*, et al.*, 2015; Hawley & Gordon, 1976; Listenberger*, et al.*, 2003; Peng*, et al.*, 2011; Rosenthal, 1981). However, most studies did not find a link between intracellular lipid accumulation and cell death. The common consensus is that palmitic acid and oleic acid cause the intracellular accumulation of different lipid species in different structures, and, thus, trigger very different consequences. The results from treatment with palmitic acid and oleic acid individually, highlighted that cell death occurred regardless of lipid accumulation. Therefore, it was hypothesised that intracellular lipid metabolites were formed following treatment, and it was those lipid species that resulted in differential toxic effects.

The combination treatment of 2:1, oleic acid to palmitic acid also resulted in significant intracellular lipid accumulation, but to a lesser extent than oleic acid alone. In HepG2 cells, lipid accumulation induced by the combination treatment appeared to be relative to the ⅔ oleic acid composition, and was correlated with a loss of viability. Whereas, lipid accumulation induced in L6 by the combination treatment was greater than could be accounted for by the ⅔ oleic acid composition. However, there were no associations between lipid accumulation and any cell death process. It was theorised that in HepG2 the lipid droplets formed by oleic acid did not sequester palmitic acid, thus, leading to lower lipid accumulation and higher toxicity than in L6 cells. On the other hand, the larger percentage of lipid accumulation cause by 1 mM of the combination treatment than 0.67 mM oleic acid alone indicated that palmitic acid was partially sequestered.

It is thought that the disparity in toxicity between palmitic acid and oleic acid is because oleic acid is stored in neutral TAG lipid droplets within the cell, whilst palmitic acid leads to

the production of toxic lipid species such as ceramide and DAG (Itami*, et al.*, 2018; Listenberger*, et al.*, 2003; Lu*, et al.*, 2016). One study (Gordon, 1977), measured intracellular triglycerides in L mouse fibroblasts using the colorimetric methods of Soloni (1971) and found that unsaturated FFAs increased intracellular TAG concentrations over 2000%, whilst maintaining normal cell growth (Gordon, 1977). On the other hand, saturated FFAs increased intracellular TAG by 800%, markedly less than caused by unsaturated FFAs (Gordon, 1977). Treatment with palmitic acid causes an increase in intracellular DAG in human skin fibroblasts (Rosenthal, 1981), HeLa cervical epithelial cells (Shen*, et al.*, 2017), and C2C12 myocytes (Chavez*, et al.*, 2003; Peng*, et al.*, 2011). In addition, increases in intracellular ceramide levels have been shown in H4IIE liver cells (Wei*, et al.*, 2006), pancreatic β-cells (Maedler*, et al.*, 2001; Shimabukuro*, et al.*, 1998; Unger, 1997), HeLa cervical epithelial cells (Shen*, et al.*, 2017), haematopoietic cell lines (Paumen*, et al.*, 1997), and C2C12 myocytes (Chavez*, et al.*, 2003). However, palmitic acid-induced ER stress is not prevented by inhibition of *de novo* ceramide synthesis, demonstrating that palmitic acid disrupts cells and induces apoptosis independently of ceramide accumulation (Wei*, et al.*, 2006).

Many studies suggest that whilst oleic acid accumulates in neutral lipid droplets in the cytoplasm (Cheon & Cho, 2014; Cnop*, et al.*, 2001; Gordon, 1977; Haffar*, et al.*, 2015; Hawley & Gordon, 1976; Leamy*, et al.*, 2014; Listenberger*, et al.*, 2003; Peng*, et al.*, 2011; Rosenthal, 1981), palmitic acid accumulates in other forms. A study by Cnop*, et al.,* (2001) showed that oleic acid caused the formation of large spherical lipid droplets, whilst palmitic acid forms small crescent-like particles. Haffar*, et al.,* (2015) also found that the pattern of lipid accumulation differs between treatments. Oleic acid resulted in the formation of spherical lipid droplets, whilst palmitic acid triggers the formation of irregular shaped deposits. The generation of larger spherical droplets by oleic acid in contrast to those produced by palmitic acid may suggest differences in converting fatty acids into neutral lipid species (Peng*, et al.*, 2011). Interestingly, co-administration of oleic acid with palmitic acid causes the formation of smaller spherical lipid droplets than oleic acid on its own (Haffar*, et al.*, 2015). One study found that arachidonic acid generates considerable neutral TAG lipid

droplets, which are able to limit the lipotoxic effects of palmitic acid by sequestering it. Oleic acid may act through the same mechanism, but to a lesser extent (Cheon & Cho, 2014; Leamy*, et al.*, 2014).

A number of papers take this hypothesis further by claiming that these palmitic acid deposits cause alterations to the ER membrane. Scarpelli*, et al.,* (1974) found that feeding trout a diet of cyclopropenic fatty acids led to damage of ER membranes in their hepatocytes. Palmitic acid specifically has been shown to cause alterations to ER morphology, namely ER expansion in C2C12 myocytes (Peng*, et al.*, 2011). This ER expansion occurred after 3 hours of PA treatment, whereas ER stress does not become significant until 6 hours after PA treatment, indicating the morphological changes may be responsible for inducing ER stress (Peng*, et al.*, 2011). It is thought that an imbalance of phospholipids in the ER membrane may disrupt the normal functioning of the ER as approximately 22% of palmitic acid applied is incorporated into phospholipids in primary hepatocytes (Leamy*, et al.*, 2014) and C2C12 myocytes (Peng*, et al.*, 2011). However, many studies suggest that it isn't the incorporation of phospholipids in the ER membrane that induces ER stress, but the formation of seemingly solid lipid deposits that physically damages the membrane. Hawley & Gordon (1976) report that human neutrophils incubated *in vitro* with oleic acid develop neutral lipid droplets within the cytoplasm, whilst those exposed to palmitic acid form elongated cleft-like dilations in the ER. In L mouse fibroblasts, Gordon (1977) also demonstrated that saturated FFAs cause deposits of lipid species in elongated needle-like dilations to form in the ER without the production of intracellular lipid droplets. These dilations displace the normal granular contents of the ER and appear as angular, electron-translucent spaces (McKay*, et al.*, 1967). An explanation of these structural alterations is that crystallisation of the lipids may be occurring due to the high melting point of palmitic acid (Gordon, 1977). This hypothesis is confirmed by Shen*, et al.,* (2017) who report that palmitic acid causes accumulation of TAG, DAG, phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylcholine (PC) and ceramide, in structures other than lipid droplets (Borradaile*, et al.*, 2006). These lipid structures are not readily stained by Nile Red and are more widely distributed in size than lipid droplets. However, they exhibit

weaker, more uniform intensity, suggesting a lamellar organisation indicative of new membrane (Shen*, et al.*, 2017). This may explain why lipid droplets were not found *via* ImageJ analysis in this study. Further investigations found palmitic acid was being incorporated into membrane lipids, and in fact developing new ER membrane in the form of lamellar cisternae, distinct from native ER membrane (Borradaile*, et al.*, 2006; Deguil*, et al.*, 2011; Shen*, et al.*, 2017). However, the lipids synthesised and incorporated into the new membrane as a result of palmitic acid treatment are high melting temperature lipids that drive phase separation from the 'normal' fluid phase membrane to a solid phase membrane. The result is a highly ordered acyl chain packing and ultraslow lateral motion. The addition of oleic acid is able to channel palmitic acid into lipid droplets and reduce solid domains (Shen*, et al.*, 2017).

3.4.3 Comparison of findings from HepG2 and L6 cells

Both palmitic acid and oleic acid were more toxic to L6 than HepG2 cells. However, in combination the opposite was true, perhaps indicating different processing or metabolic pathways in the two cell types. It is also worth noting that not only was the combination treatment more damaging to HepG2 cells, but in contrast, it increased the viability of L6 cells, although not significantly. Higher levels of lipid accumulation were found in L6 cells as opposed to HepG2 cells, with oleic acid causing the highest degree of intracellular lipid accumulation in both cell lines. Lipid accumulation in HepG2 was negatively correlated with viability for all treatments, however, individually palmitic acid and oleic acid were positively correlated with viability in L6.

Hepatocytes and skeletal myocytes have vastly different roles within the body, which may cause them to respond differently to FFAs. The major functions of hepatocytes are bile production, toxin removal, lipid synthesis, carbohydrate metabolism and storage, and protein synthesis and storage, whilst skeletal myocytes are mainly involved in muscle contraction. However, both liver and skeletal muscle are important in fat storage and fatty acid metabolism (Frayn*, et al.*, 2006).

Skeletal muscle is the second main site for triacylglycerol storage after adipose tissue, containing ~300g. However, there are two sites of storage in muscle, in adipocytes between muscle fibres and within the muscle cells themselves (intramyocellular). Liver is the third main tissue in triacylglycerol storage, containing ~100g (Frayn*, et al.*, 2006). Liver and muscle both hydrolyse the stored triaclyglycerol to release fatty acids (Akie & Cooper, 2015; Jensen, 2002). Skeletal muscle uses fatty acids as a substrate for oxidation whilst the liver uses them as a substrate for re-esterification to make triacylglycerol for the secretion of very-low-density lipoproteins (VLDL).

The normal triacylglycerol content of the liver is up to 10% by weight, however, this can expand significantly in non-alcoholic fatty liver disease (NAFLD). The liver receives fatty acids from the plasma, from the uptake of remnant particles of lipoproteins and from the hydrolysis of plasma triglyceride. The liver can also synthesise fatty acids from glucose and and other precursors in a process called *de novo lipogenesis* (Akie*, et al.*, 2015; Clark*, et al.*, 1974; Goodridge, 1973).

Skeletal muscle receives fatty acids from the plasma and from the hydrolysis of plasma triglycerides. Muscle lipolysis is unaffected by insulin, but is affected by catecholamines, whilst FFA uptake and oxidation is increased by vigourous exercise (Jensen, 2002).

The ability of the liver to store excess fat and its advanced role in lipid metabolism in comparison to skeletal muscle, could explain why HepG2 had a greater tolerance to FFAs than L6 cells. This may also explain why the earliest detectable abnormality of T2DM is insulin resistance in skeletal muscle (Petersen*, et al.*, 2012), and why exercise has a positive effect on type II diabetes independent of its weight loss effects (Ansari, 2009; Zou*, et al.*, 2016).

4 The effects of palmitic acid and oleic acid on platelet function

4.1 Introduction

Platelets are crucial in haemostasis, but have also been shown to play a key role in atherosclerosis and its sequelae by adhering and aggregating on exposed vascular surfaces leading to activation of the clotting cascade in a process called atherothrombosis (Huo*, et al.*, 2003; Karshovska*, et al.*, 2015; Massberg*, et al.*, 2002; Murphy*, et al.*, 2013). Platelets perpetuate inflammation in atherosclerosis by recruiting inflammatory cells towards sites of plaque formation, and release a variety of inflammatory mediators (Lievens & Hundelshausen, 2011). Furthermore, they have been shown to transport regulatory molecules which drive the inflammatory response and mediate atherosclerotic progression (Badimon*, et al.*, 2012). Platelets are in constant contact with fatty acids within the plasma and at sites of atherosclerotic plaque formation. Therefore, it is important to understand the effects fatty acids may have on platelets in populations with elevated circulating FFA levels.

Atherosclerosis is a progressive, systemic inflammatory disease characterised by the accumulation of lipids, macrophages and lymphocytes within the intima of large arteries, in formations otherwise known as plaques (Badimon*, et al.*, 2012). The formation of plaques often compromises the integrity of the arterial walls leading to ischaemic events distal to the arterial stenosis, and is thereby the underlying cause of most cases of coronary artery disease, peripheral arterial disease and many cases of stroke (Badimon*, et al.*, 2012).

The formation of atherosclerotic plaques is multifaceted and combines interactions between cells of the arterial intima and leukocytes, with disturbances in local blood flow and lipids. Once formed, plaques display a fibrous cap surrounding a central core of extracellular lipids and debris, otherwise known as the atheroma. The fibrous tissue

provides structural integrity to the plaque as the atheroma is soft and highly thrombogenic (van der Wal & Becker, 1999). Atherosclerotic plaques are high in extracellular lipids and are bordered by macrophages. These macrophages phagocytose vast quantities of oxidised LDL resulting in the formation of foam cells, the death of which perpetuates the formation and growth of the atheroma, in combination with extracellular binding of lipids to collagen fibres and proteoglycans (van der Wal*, et al.*, 1999).

In addition to LDL, atherosclerotic plaques accumulate cholesterol esters which are hydrolysed in the later stages of plaque development to release cholesterol and free fatty acids (Lundberg, 1985; Smith*, et al.*, 1967). As plaques advance they become unstable and susceptible to erosion and rupture. This damage not only initiates platelet activation exacerbating atherosclerotic plaque formation, but also exposes platelets to additional levels of free fatty acids. The hydrolysis of cholesterol esters specifically causes an increase in palmitic acid (Smith*, et al.*, 1967) with palmitic acid accounting for 20.64% of total fatty acids within atherosclerotic plaques, whilst oleic acid accounts for 29.85% (Stachowska*, et al.*, 2004). Free fatty acids are also released into the plasma following carotid artery stenting, where they could contribute to the higher rate of periprocedural stroke (Abe*, et al.*, 2012). Furthermore, saturated fatty acids are taken up more readily than other fatty acids by macrophages in cell culture and could lead to greater lipid accumulation within arteries (De Pascale*, et al.*, 2006). However, it is unknown whether free fatty acids play a role in platelet aggregation at sites of plaque damage, and moreover, the specific effects of palmitic acid and oleic acid on platelets in general has yet to be fully elucidated.

Research into the influence of free fatty acids on the behaviour of platelets is surprisingly limited. Interactions between platelets and FFAs may occur either in the plasma (where levels of FFA are raised in populations with obesity and/ or diabetes), or as a consequence of plaque rupture, or both. Exposure to elevated levels of plasma FFAs may contribute to sensitisation of circulating platelets, resulting in heightened

reactivity at sites of plaque rupture. In fact, a study by Connor*, et al.,* (1963) found that infusion of FFAs into canines results in mortality from extensive thrombosis, thus, indicating a pro-thrombotic effect *in vivo*. Light transmission aggregometry revealed increases in light transmission of washed human platelet suspensions following treatment with 0.35 mM stearic acid or oleic acid (Hoak*, et al.*, 1967). In platelet-rich plasma (PRP), stearic acid increases light transmission whilst oleic acid does not (Srivastava & Awasthi, 1983). This possibly reflects a buffering effect by plasma proteins such as albumin on plasma FFAs. Furthermore, elevated plasma FFA levels induced by heparin injected intravenously results in increased platelet sensitivity to ADP and collagen (Burstein*, et al.*, 1978). Other studies suggest an inhibitory role for oleic acid during platelet activation by ADP and collagen in PRP (Srivastava*, et al.*, 1983).

Early work found that most free fatty acids have the ability to initiate platelet aggregation, with reports suggesting that saturated fatty acids are more potent than unsaturated fatty acids (Hoak*, et al.*, 1967; Mahadevan*, et al.,* 1966). The majority of studies found that oleic acid evoked a robust aggregatory response in washed platelets (Miles *et al*.,. 1988; Hashimoto*, et al.*, 1985; Zentner*, et al.*, 1981; Connor*, et al.*, 1969; Hoak*, et al.*, 1967). Similarly, it was reported that palmitic acid caused platelet aggregation (Zentner*, et al.*, 1981, Connor*, et al.*, 1969), yet this was disputed by the minority of studies (Hashimoto*, et al.*, 1985). Additionally, oleic acid was found to activate protein kinase C (PKC), whilst palmitic acid did not (Yoshida*, et al.*, 1992). The activation of PKC is a critical event in platelet aggregation as it modulates granule secretion, synthesis of thromboxane A2 and integrin $\alpha_{IIb}\beta_3$ activation (Bye*, et al.*, 2016; Konopatskaya*, et al.*, 2011; Yacoub*, et al.*, 2006).

The role that these interactions may play in atherosclerosis and the obese population, and the lack of consensus regarding the effects of palmitic acid and oleic acid on platelets, highlights the importance of investigating this further.

Owing to the contradictory reports highlighted above, experiments were designed to establish whether exposure to oleic acid or palmitic acid results in platelet activation and/or aggregation. This study is one of the few to investigate this effect in detail; it was, therefore, important to characterise these effects further by comparing the effects of FFAs to responses triggered by physiological agonists to investigate the mechanisms involved. In particular, it was important to dissect platelet activation into its component signalling pathways and study the influence of these pathways in the aggregatory response to fatty acids.

Initial light transmission aggregometry experiments were performed to establish the potential of oleic acid and palmitic acid to induce aggregation in washed platelet suspensions. These experiments provided the foundation for future studies, to determine 1) whether aggregation was triggered *via* known pathways, 2) whether aggregation was mediated by secondary signalling, and 3) whether aggregation exhibited conventional characteristics of platelet activation. In order to fully understand the platelet-aggregating properties of fatty acids, responses to two conventional platelet agonists (thrombin and CRP-XL) were studied. Analyses included the quantification of maximum aggregation, minimum aggregation, derived from aggregometry traces. This approach ensured that the washed platelet suspensions were functional, and acted as points of comparison for typical platelet activation. Thrombin and collagen-related peptide (CRP-XL), are widely used in platelet researchand function through different signalling pathways. Thrombin utilises G protein-mediated signalling, whilst CRP-XL triggers tyrosine kinase-based signalling *via* engagement of the Ig superfamily member, GpVI. The two signalling pathways converge on PLC isoforms, resulting in PKC activation. PKC regulates many downstream activatory responses, including integrin activation, granule release and PS exposure.

Maximum aggregation was measured as it is probably the most important analysis in LTA, as it measures the maximum aggregation achieved during the period of analysis regardless of time point. In typical agonist-induced aggregation the measure of

maximum aggregation would be very similar to final aggregation. However, in cases of platelet dissociation, maximum aggregation would produce a meaningful result whilst final aggregation would not. Minimum aggregation was analysed as an important measure of platelet shape change, considered to be the first measurable physiological response to agonists (Kim and Kunapuli, 2011), detectable in the first seconds of platelet activation (Redondo*, et al.*, 2006). Shape change is the transition that platelets undergo from flat, concave discs to spherical forms with pseudopodial and lamellipodial protrusions, which occur as a result of actin reorganisation (Hartwig 2006; Bearer*, et al.*, 2002). Upon addition of an agonist, the initial shape change results in an increase in optical density, and hence decrease in light transmittance during aggregometry (Aslan, 2017). The extent of the decrease is quantified as the minimum aggregation.

PS exposure on the outer surface of the platelet was analysed as platelet activation causes its translocation from the inner leaflet of the platelet membrane to the outer surface (Bonomini*, et al.*, 2004). The reason for this remains unclear, however, it is thought to be a major regulatory event in coagulation as it binds to discrete regulatory sites on factors V(a) and X(a), thereby allosterically changing their proteolytic activities (Lentz, 2003). Furthermore, PS exposure is correlated with an increase in thrombin generation (Lentz, 2003; Monroe*, et al.*, 2002).

4.1.1 Aims

- To determine whether palmitic acid and oleic acid cause platelet aggregation
    - If so, to understand the mechanism by which they elicit a response
    - To compare fatty acid- induced responses with those of known agonists
- To establish whether exposure to palmitic acid and oleic acid initiates apoptosis

4.2 Methods

Refer to Chapter 2 Materials and methods. The minimum number of times an experiment was repeated was three, with the exact n number provided in the figure legend. All data is expressed as mean ± SEM. *P<0.05

4.3 Results

4.3.1 Interpretation of platelet aggregation in washed platelets

4.3.1.1 Platelet aggregation in response to thrombin and collagen-related peptide

Initial experiments were performed to ensure that platelet activity following preparation of washed suspensions from PRP was retained for subsequent investigation. Washed platelets suspensions at a concentration of $2x10^6$/mL were stimulated with collagen-related peptide (CRP-XL) and thrombin. Both agonists were used to verify the platelet aggregation response *via* light transmission aggregometry to ensure washed platelets were functional following processing and as a control. Only thrombin was used in subsequent experiments to confirm platelet activation.

Once responses of the platelet suspensions to agonist stimulation were established, effects of palmitic acid and oleic acid on platelet aggregation were investigated

The addition of CRP-XL (0.03-1 µg/ml) to washed platelet suspensions evoked a concentration-dependent increase in light transmission, consistent with platelet aggregation (Figure 4.1). While 0.03-0.1 µg/ml induced only a partial aggregatory response, 0.3 µg/ml caused 77.2 ± 13.7% aggregation and 1 µg/ml induced maximal aggregation of 85.7 ± 7.9%. Thrombin evoked a similar concentration-dependent increase in platelet aggregation with 0.03-0.1 U/ml producing little aggregation, whilst 0.3 U/ml induced 70.8 ± 32.5% and 1 U/ml caused maximal aggregation of 88.7 ± 2.2% (Figure 4.1A).

Thrombin (1U/ml) caused minimum aggregation of -7.7 ± 3.9%, whilst CRP-XL (1µg/ml) caused minimum aggregation of -8.7 ± 5.0%, both demonstrating shape change typical of platelet activation (Figure 4.1D).

The time-to-half maximum aggregation and the gradient at 25% aggregation were analysed and used in combination with the aggregation traces to assess the overall progression of the aggregatory response. Thrombin (1 U/ml) produced a very short lag phase, the time immediately following stimulation until shape change has changed to the start of platelet aggregation. CRP-XL (1 µg/ml) produced a longer lag phase initially, however, there was no significant difference between the time-to-half maximum aggregation for thrombin (1 U/ml) and CRP-XL (1 µg/ml), of 44.9 ± 23s and 46.6 ± 10.2s, respectively (P=8468) (Figure 4.1E). Similarly, the aggregation traces and gradient at 25% of completion show that both agonists achieved the majority of their aggregatory response rapidly, within the first couple of minutes following addition of the agonists (Figure 4.1F). These results are typical of agonists causing full platelet activation.

These results established the functionality of the platelet preparations. Thrombin (1U/ml) was selected as a control for subsequent experiments as it demonstrated maximum aggregation (Figure 4.1C) and typical aggregations traces (Figure 4.1B) produced by a full aggregatory response.

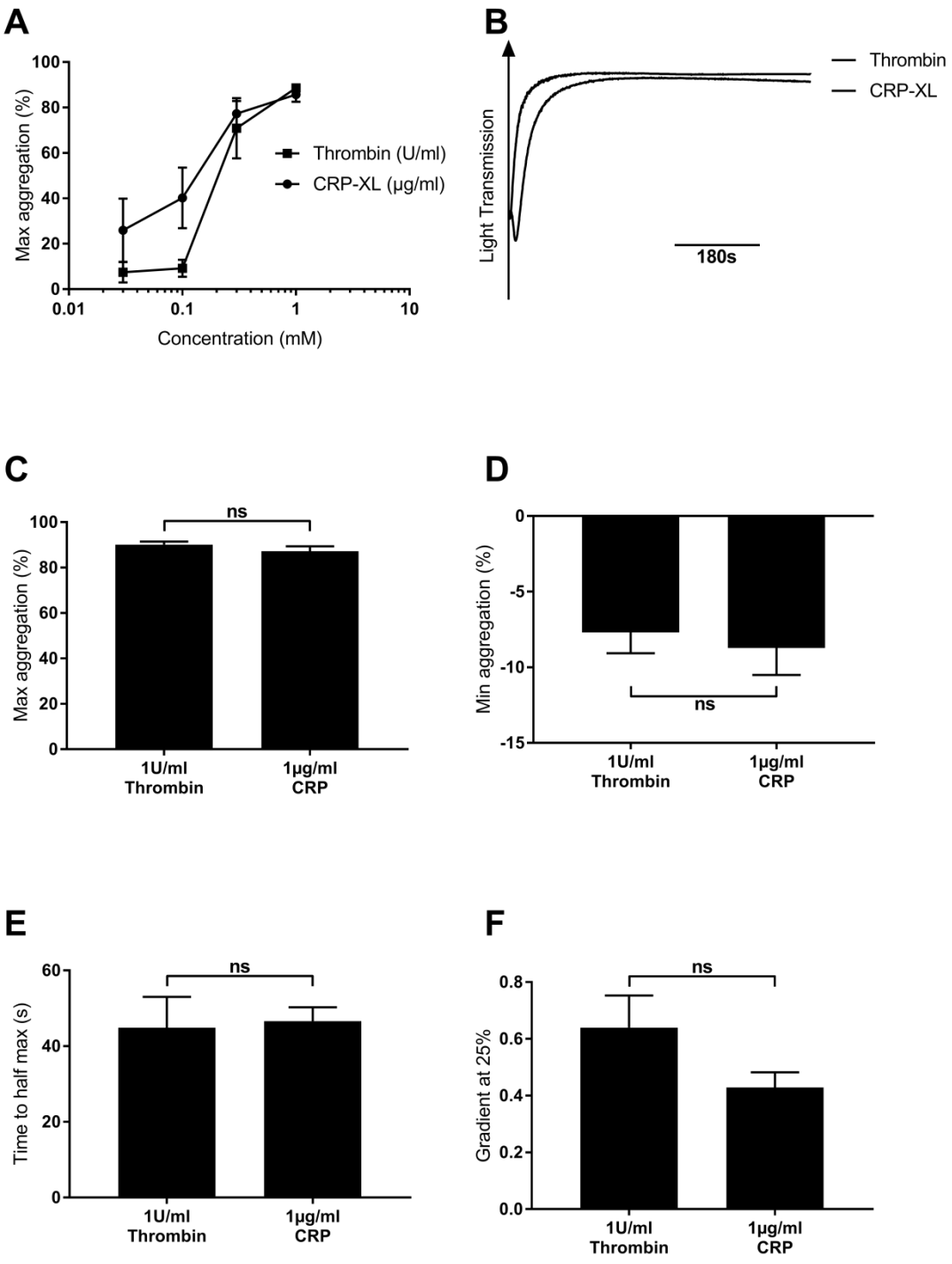


Figure 4.1 Platelet preparations were responsive to physiological agonists

The functionality of the washed platelet preparations were confirmed *via* LTA (n=6). A) CRP-XL and thrombin induced platelet aggregation in a dose-dependent manner. B) Representative traces of aggregation induced by CRP-XL and thrombin at concentrations of 1 mM. C, D, E, F) At 1 mM, the agonists produced similar maximum aggregation (C), minimum aggregation (D), time to half maximum (E) and gradient at 25% of completion (F). All of which were indicative of a full aggregatory response.

4.3.1.2 Maximum aggregation in response to oleic acid and palmitic acid

The aggregatory response of platelets to a range of concentrations of PA and OA was exmined in order to select a concentration for subsequent experimentation. Maximum aggregation and traces were analysed and compared with responses to CRP-XL and thrombin.

The addition of palmitic acid (0.001-1 mM) caused aggregation in a concentration-independent manner. While 0.001-0.3 mM palmitic acid had little effect on platelet aggregation, 1 mM caused maximal aggregation of 32.2 ± 6.5% (P=0.0028) (Figure 4.2A). Similarly, the addition of oleic acid (0.001-1 mM) induced aggregation in a concentration-independent manner. Low concentrations (0.001-0.003 mM) caused little aggregation, whilst 0.01 mM OA evoked partial aggregation of 28.8 ± 8.1%, and 0.1 mM induced maximal aggregation of 73.0 ± 2.0%.

The 1 mM concentration of both palmitic acid and oleic acid caused significant platelet aggregation, 32.1 ± 6.5% and 29.8 ± 7.2%, respectively (P=0.0028 and 0.0052, respectively) (Figure 4.2). Maximal aggregation produced by palmitic acid (32.2 ± 6.5%) did not resemble aggregation induced by thrombin or CRP, in that the period of shape change was elongated, thereby delaying the log phase of aggregation (Figure 4.2B). This profile was not consistent with full platelet activation as the trace indicated that palmitic acid did not initiate secondary aggregation as shown by the trace gradually evened out before full aggregation.

Aggregation caused by 1 mM oleic acid showed a swift, but small log phase, possibly caused by primary activation, then dissociation where the platelets separated from their aggregates. This was followed by a gradual increase to maximal aggregation of 29.8 ± 7.2% (Figure 4.2C). In a similar manner to PA, the profile was also not consistent with full platelet activation. The gradual increase to maximum aggregation could have been caused by a delay in secondary aggregation, however, similar traces have not been found in the literature.

A concentration of 1 mM was selected for further use in this study, because at this concentration both PA and OA induced significant aggregation.

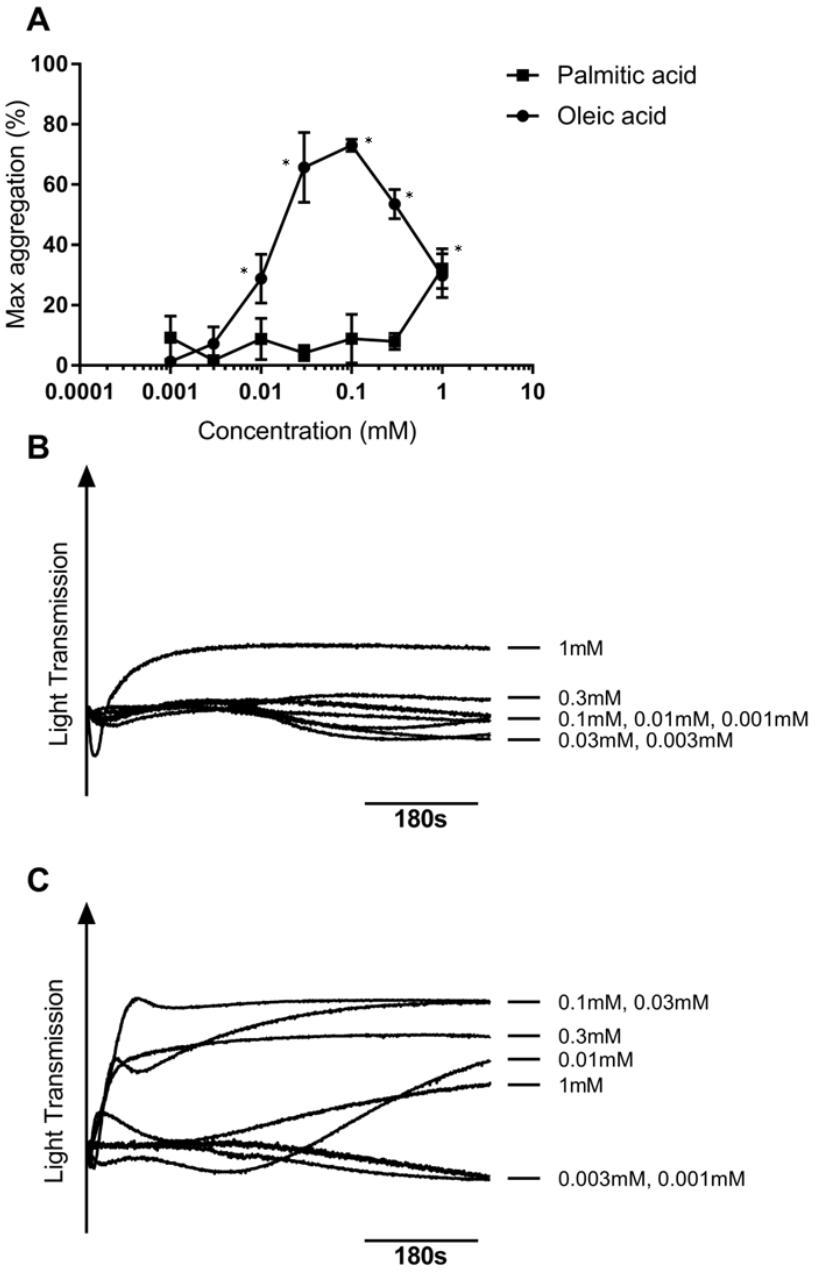


Figure 4.2 Platelet aggregation in response to varying concentrations of fatty acids.

The aggregatory responses of washed platelets to a range of concentrations of PA and OA were examined *via* LTA (n=6). Aggregation was measured every 0.5 seconds to produce representative aggregation traces. A) Comparision of maximum aggregation caused by PA and OA found that oleic acid produced greater responses. B) Representative traces of PA-induced aggregation. C) Representative traces of OA-induced aggregation.

4.3.1.3 Platelet aggregation in response to 1 mM of palmitic acid or oleic acid

As mentioned above, 1 mM palmitic acid caused maximal aggregation of 32.2 ± 6.5% and 1 mM oleic acid 29.8 ± 7.2% (Figure 4.3B). These levels of aggregation were significantly different from the vehicle control (1.2 ± 0.2%, P=0.0028 and 0.0052, respectively); and were also significantly lower than maximal aggregation induced by thrombin (90.1 ± 1.3%, P<0.0001).

Oleic acid (1 mM) caused minimum aggregation of -3.2 ± 2.5%. This is consistent with minimal aggregation induced by thrombin (1 U/ml, values) and CRP-XL (1 µg/ml, values, Figure 4.3C). Thereby, indicating that OA induced a similar shape change response to that seen following activation with conventional agonists. Conversely, palmitic acid (1mM) caused minimum aggregation of -17.4 ± 8.7%, which was significantly different from that produced by thrombin (1 U/ml) and CRP-XL (1 µg/ml) (P=0.0084 and 0.0216, respectively). The shape change results produced by palmitic acid were not representative of platelet activation and may have indicated structural changes to the platelet membrane, perhaps expansion from necrotic process. Alternatively, palmitic acid-induced aggregation may be elicited *via* mechanisms other than platelet activation.

The representative aggregation traces evoked by palmitic acid and oleic acid (1mM) showed partial aggregation in comparison to that caused by thrombin (Figure 4.3A). The palmitic acid trace appeared to show activation of the primary wave of aggregation without secondary mediated signalling to produce the second wave, seen as the trace gradually evens out before full aggregation. Additionally, the time-to-half maximum (138.2 ± 104.6s) was significantly greater than that of thrombin or CRP-XL (P=0.0128 and 0.0151, respectively), which further suggested the lack of secondary signalling to perpetuate activation (Figure 4.3D). The oleic acid aggregation trace also showed a lack of full platelet activation, and instead indicated activation of the primary wave of aggregation, followed by disaggregation before initiation of the secondary wave of aggregation. Gradient at 25% of completion was negative and, thus, showed the

disaggregation (-3.7 ± 4.4) (Figure 4.3E). However, the reliability of gradient at 25% of completion was questioned as the result produced by 1 U/ml thrombin was not significantly different from the untreated control.

It is also worth noting the high variability of maximal aggregation between platelet donors. A range of 9 - 81% maximum aggregation was observed across 6 individual platelet donors for the 0.03 mM dose of oleic acid.

However, whilst performing experiments, it was observed that the fatty acids may not have been fully dissolving in the platelet suspension at concentrations of 1mM, as shown in Figure 4.4A. This was of consequence, as if correct, it may have caused artefactual aggregation traces and enhanced maximum aggregation readings. This observations was examined in the test section with testing performed to ascertain whether this observation was correct.

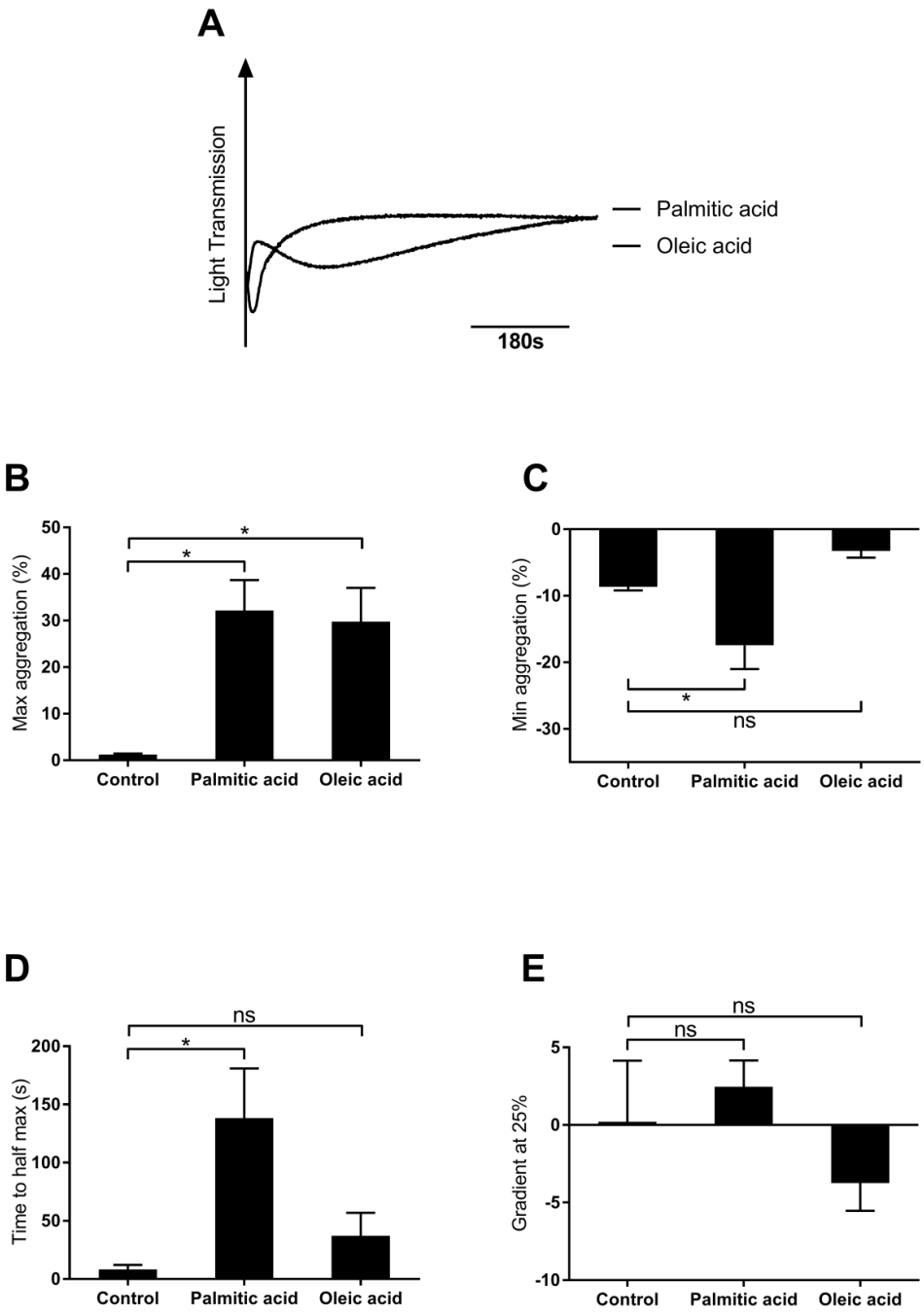


Figure 4.3 Platelet aggregation in response to 1 mM of PA or OA

The aggregatory responses of washed platelets to 1 mM of PA or OA were established *via* LTA (n=6). A) Aggregation was measured every 0.5 seconds to produce representative aggregation traces. B) At 1 mM, PA and OA induced significant maximum aggregation, but only PA produced significant minimum aggregation (C). (D) Time for PA to produce half maximal results was significantly different to unstimulated platelets, but neither PA nor OA induced a significant change in gradient at 25% of completion (E).

4.3.1.4 Palmitic acid induced platelet aggregation is an artefact of insolubility in platelet preparations

To ascertain whether the fatty acids were coming out of solution following their addition to washed platelet preparations, as mentioned before, the samples were removed from the aggregometer immediately prior to analysis and placed into a spectrophotometer, and their absorbance read at 600 nm. An unstirred, untreated platelet preparation was used to blank the spectrophotometer, and an untreated platelet preparation processed in the same way as the treated samples was used as a control. At concentrations of 1 mM, both oleic acid and palmitic acid samples displayed significant turbidity (P=0.0075 and P=0.0001, respectively), whilst at 0.1 mM, neither were significantly different from the control (P=0.9999 and P=0.9997, respectively) (Figure 4.4B). Thus, the anecdotal observation was judged to be correct.

These results invalidated the aggregometry data for both 1mM oleic acid and 1mM palmitic acid, as the accuracy of the data produced could not be reliably proven. Therefore, the aggregation results for 1 mM of PA and OA were disregarded and this concentration was not used for subsequent experiments. Lower concentrations of palmitic acid were unable to evoke statistically significant platelet aggregation, however, analysis was initially continued with 0.1 mM in order to study the potential of PA inducing platelet shape change. On the other hand, 0.1 mM of oleic acid caused significant maximal aggregation without affecting turbidity, therefore, this treatment was selected for the remainder of the study.

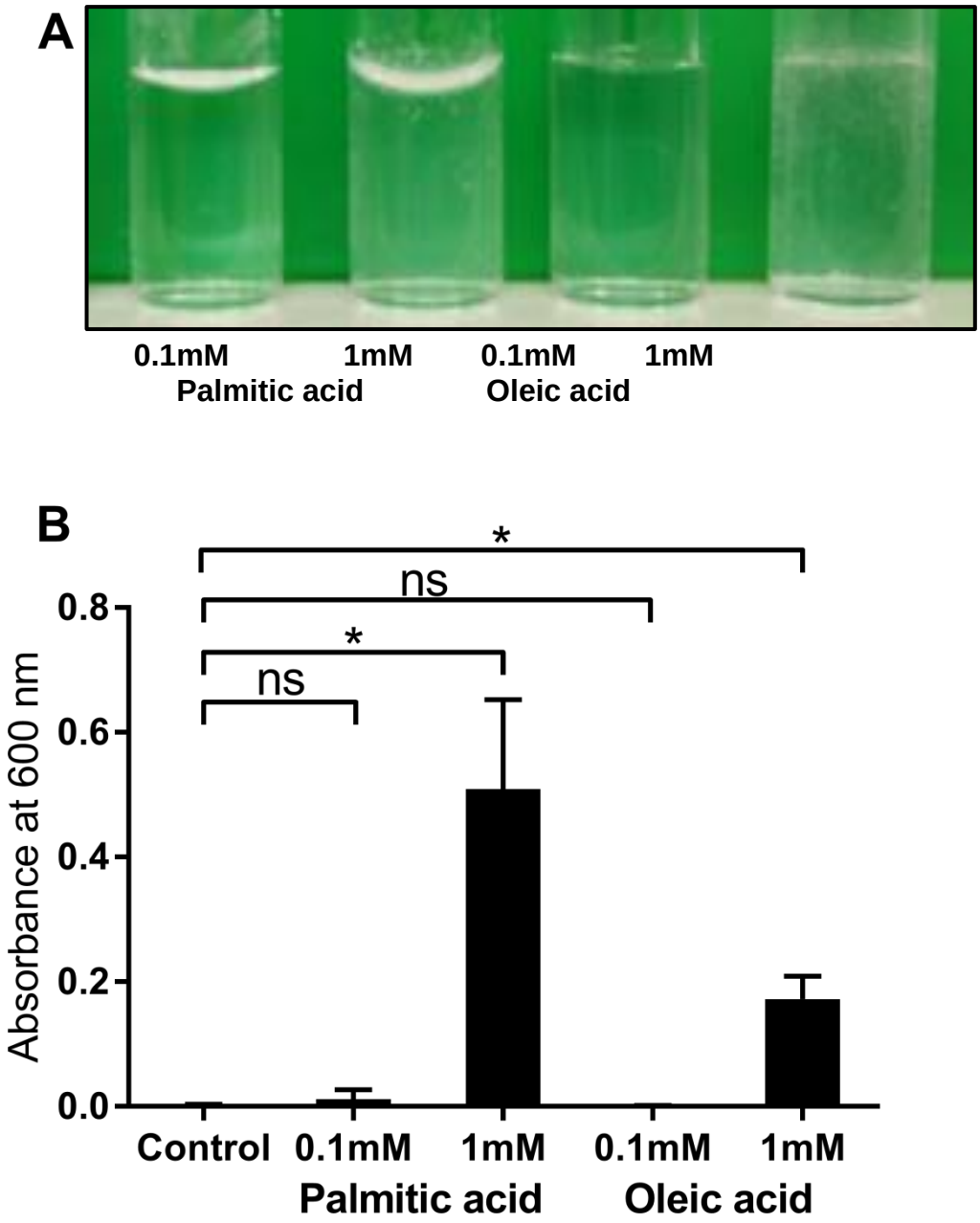


Figure 4.4 1 mM fatty acid treatments were insoluble in CFT buffer

A) A photograph of the perceived lack of solubilisation shows cloudiness and the presence of grains stuck to the cuvettes in both 1 mM fatty acid solutions. B) Spectrophotometric analysis determined that 1 mM solutions of both PA and OA in CFT were significantly more turbid that CFT alone.

4.3.1.5 Changes in light transmission of platelet suspensions by OA represents conventional platelet aggregation

As with 1 mM oleic acid, 0.1 mM oleic acid produced a biphasic aggregation trace suggestive of activation of the primary wave of aggregation, followed by disaggregation before the initiation of secondary messenger-mediated signalling to perpetuate the aggregatory response (Figure 4.5A). Biphasic traces are frequently indicative of weak agonists. Furthermore, minimum aggregation (-12.0 ± 5.9%) was consistent with that produced by thrombin (P=0.1213), which suggested a similar shape change (Figure 4.5C). Time-to-half maximum (57.1 ± 9.6s) produced by 0.1mM oleic acid was significantly different from that of the control (P<0.0001), but was not statistically different from that caused by thrombin (P=0.2478) (Figure 4.5D). This indicated that oleic acid may have been caused primary activation including shape change in a less rapid manner than thrombin. There were no significant differences between the gradient at 25% of control compared to thrombin or oleic acid (Figure 4.5E).

As 1 mM of PA did not induce changes in maximum aggregation (P=0.3704), minimum aggregation (P=0.7363), time to half maximum aggregation (P=0.2292) or gradient at 25% of completion (P=0.1664), its use was discontinued.

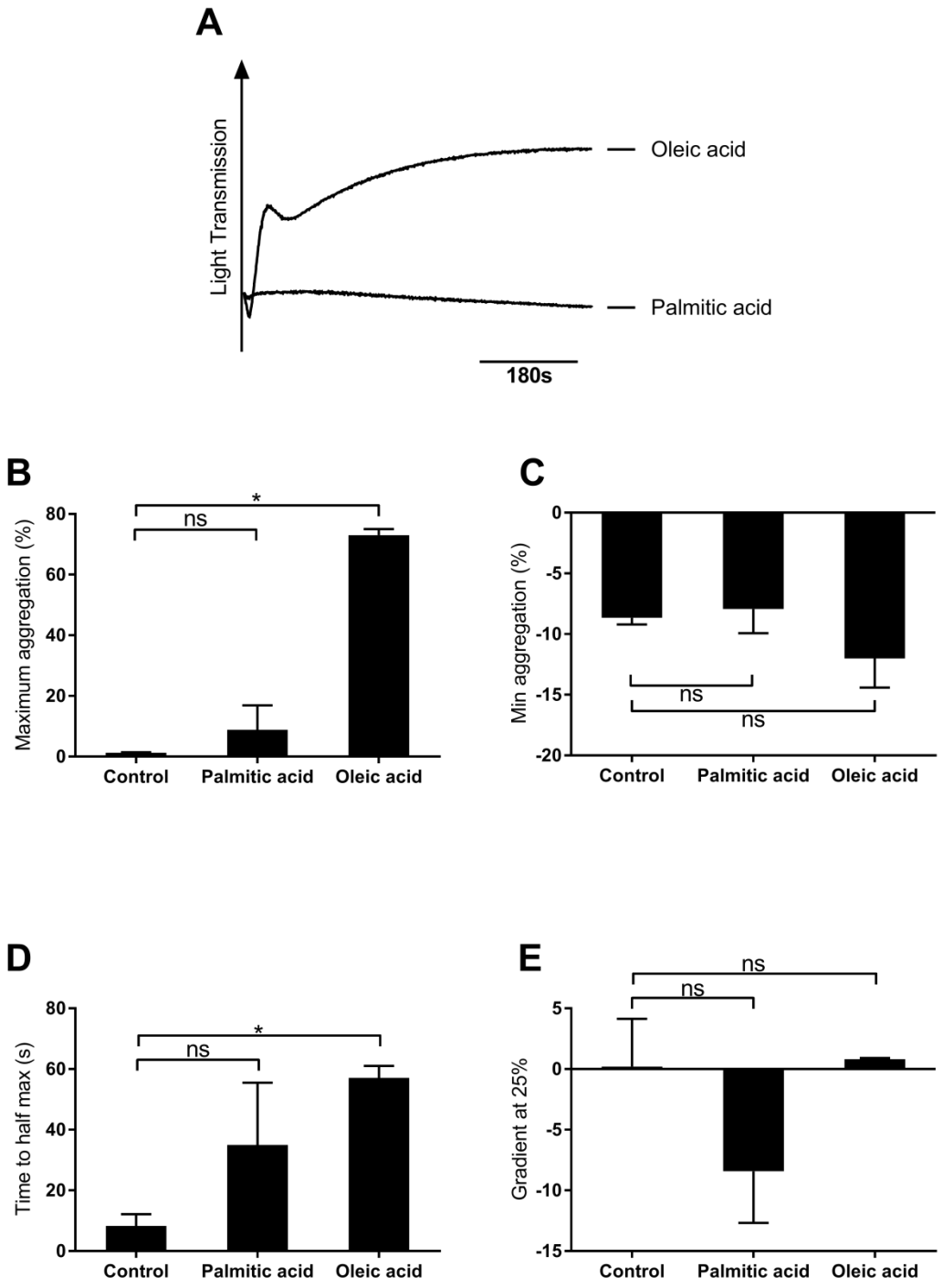


Figure 4.5 Oleic acid induced aggregation comparable to physiological agonists

The aggregatory responses of washed platelets to 0.1 mM of PA or OA were examined *via* LTA (n=6). A) Aggregation was measured every 0.5 seconds to produce representative aggregation traces. B) 0.1 mM OA induced significant maximum aggregation, and time to half maximum (D), but did not cause significant mimum aggregation (C) or gradient at 25% of completion (E) in comparision to unstimulated platelets.

4.3.2 Characterisation of platelet activation in washed platelets

4.3.2.1 Oleic acid induced aggregation was not reliant on secondary signalling *via* granule secretion and thromboxane release

Following platelet activation, second messengers enhance the platelet aggregation response through the release of ADP from dense granules, the generation of thromboxane A_2 (TxA_2) and the release of zinc, sometimes causing a biphasic aggregation trace in the process. Therefore, it was important to establish whether oleic acid-induced aggregation was dependent on second messenger-mediated signalling and to understand the biphasic aggregation trace produced by 0.1mM oleic. To study this, the platelets were treated with 1 mM aspirin, 2 µM prostaglandin E1 (PGE1) or 50 µM N,N,N',N'-tetrakis(2-pyridylmethyl)ethane-1,2-diamine (TPEN) prior to stimulation with oleic acid. Aspirin is an irreversible inhibitor of the enzyme cyclooxygenase, preventing the generation of TxA_2, whilst PGE1 increases cyclic AMP (cAMP) levels, which in turn inhibits ADP signalling. TPEN is a chelating agent with high affinity for Zn^{2+}, without which secondary aggregation is reduced.

The ability for these reagents to evoke platelet aggregation on their own without an agonist was examined to ensure results seen were produced by oleic acid alone (Figure 4.6B). None of the reagents was found to cause platelet aggregation in the absence of agonists.

To establish the effectiveness of these reagents in the presence of agonists, aggregation was observed in response to thrombin following incubation (Figure 4.6A). Aspirin, PGE1 and TPEN all caused a significant reduction in thrombin-stimulated platelet aggregation, reducing maximum aggregation to 8.00 ± 3.86%, 5.43 ± 4.05%, and 1.74 ± 0.32%, respectively (P=0.0001, 0.0001 and 0.0001, respectively). Therefore, these reagents were confirmed to inhibit second messenger-mediated signalling and were used to examine the mechanism of oleic acid-induced aggregation.

Following inhibition of second messenger signalling with aspirin, PGE1 or TPEN, platelets were treated with 0.1 mM oleic acid. OA alone induced aggregation of 71.4 ± 4.5%. Aspirin decreased aggregation to 60.54 ± 9.14%, PGE1 reduced aggregation to 63.76 ± 12.99%, and TPEN decreased aggregation to 68.55 ± 6.9%. However, neither aspirin nor PGE1 nor TPEN significantly inhibited oleic acid-induced aggregation (P=0.6822, 0.8250 and 0.9564, respectively) (Figure 4.6C). However, these molecules did have different effects on the shape of the aggregation traces produced (Figure 4.6D). Aspirin caused very little shape change and went straight into a gradual log phase. PGE1 significantly increased shape change and doubled the period of shape change. It also caused a biphasic response. TPEN significantly increased the period of shape change and resulted in a very gradual log phase. These results strongly suggest that although oleic acid-induced aggregation is not reliant on second messengers, they may play a small role in mediating signalling responses.

4.3.2.2 Oleic acid induced aggregation is not mediated by PKC

In order to understand the pathway through which oleic acid elicits platelet aggregation, a further experiment was performed. Here, platelets were incubated with 10 µM GF109203X prior to stimulation with oleic acid. GF109203X is an inhibitor of protein kinase C (PKC). PKC is an important enzyme, with a number of platelet activation pathways converging on this target downstream of integrin $\alpha_{IIb}\beta_3$. Inhibition of PKC by GF109203X prevents collagen- and thrombin-induced aggregation, in addition to collagen-triggered ATP secretion, without affecting ADP-induced, reversible aggregation. Initially, aggregation in response to thrombin was studied to establish the effectiveness of the reagent.

The effectiveness of GF109203X to inhibit aggregation was examined in response to thrombin (Figure 4.6A). It reduced aggregation to 7.16 ± 3.80% (P=0.0001), and, thus, was confirmed to inhibit PKC-mediated aggregation.

The ability of GF109203X to induce platelet aggregation without the addition of an agonist was also tested to ensure the presence of no background aggregation (Figure 4.6B). GF109203X alone did not cause platelet aggregation.

OA alone induced aggregation of 71.4 ± 4.5%. The addition of oleic acid to GF109203X pre-treated platelets produced 67.39 ± 12.29% aggregation, and thereby did not significantly inhibit oleic acid-induced aggregation, P=0.7671 (Figure 4.6C). However, the trace showed that GF109203X slightly increased the period of shape change and significantly altered the gradient of the log phase, making it far less steep than that produced by oleic acid alone. These results indicate that oleic acid-induced aggregation is not reliant on PKC signalling, although PKC is likely to play a role in the process.

4.3.2.3 Oleic acid induced aggregation is not integrin dependent

To verify that aggregatory responses induced by oleic acid were indeed a result of platelet activation and integrin-dependent aggregation, as opposed to platelet agglutination, additional experiments were performed. Platelets were stimulated in the presence of 10 µM GR144053, and 20 µM of the Ca^{2+} chelator, BAPTA. GR144053 is a peptidomimetic antagonist of integrin $\alpha_{IIb}\beta_3$, containing the amino acid sequence, arginine, glycine and aspartic acid, mimicking the sequence on the fibrinogen Aα chain, which binds to integrin $\alpha_{IIb}\beta_{3,}$ the point at which all platelet activatory pathways converge. It thereby blocks interactions between fibrinogen and the receptor, preventing all known pathways of aggregation through competitive inhibition. BAPTA is a chelating agent with high affinity for Ca^{2+}; increases in intracellular calcium are essential for the activation of integrin $\alpha_{IIb}\beta_3$, without which aggregation would be inhibited.

Aggregation following the addition of GR144053 or BAPTA was measured to confirm that these reagents did not cause aggregation on their own (Figure 4.6B). Neither GR144053 nor BAPTA induced platelet aggregation.

Firstly, the effectiveness of the reagents was confirmed with the use of the known agonist, thrombin (Figure 4.6A). GR144053 reduced maximum aggregation to 2.36 ± 1.08%, whilst BAPTA decreased it to 4.75 ± 2.70% (P=0.0001 and 0.0001, respectively). Therefore, the effectiveness of these reagents to inhibit the activation of integrin $\alpha_{IIb}\beta_3$ was established.

OA alone induced aggregation of 71.4 ± 4.5%. The addition of 0.1 mM oleic acid to platelets incubated with GR144053 or BAPTA produced aggregation of 60.50 ± 5.73% and 56.36 ± 11.41%, respectively (P=0.1739 and 0.3509, respectively). Therefore, inhibition of the activation of integrin $\alpha_{IIb}\beta_3$ did not significantly affect oleic acid-induced aggregation (Figure 4.6C). The traces revealed that GR144053 greatly increased oleic acid-induced shape change and severely elongated the period of shape change. Furthermore, the integrin $\alpha_{IIb}\beta_3$ antagonist altered the usual log phase from a steep curve to a gradual increase in aggregation. BAPTA had no effect on the magnitude of shape change, but did increase the period of shape change, and reduced the gradient of the log phase. These results suggested that although oleic acid-induced platelet aggregation was not dependent on the activation of integrin $\alpha_{IIb}\beta_{3,}$ this signalling step may be involved in the aggregatory process. However, all known pathways of platelet activation resulting in platelet aggregation culminate in the activation and expression of integrin $\alpha_{IIb}\beta_3$, therefore, these results indicate that oleic acid is not eliciting a response *via* platelet activation.

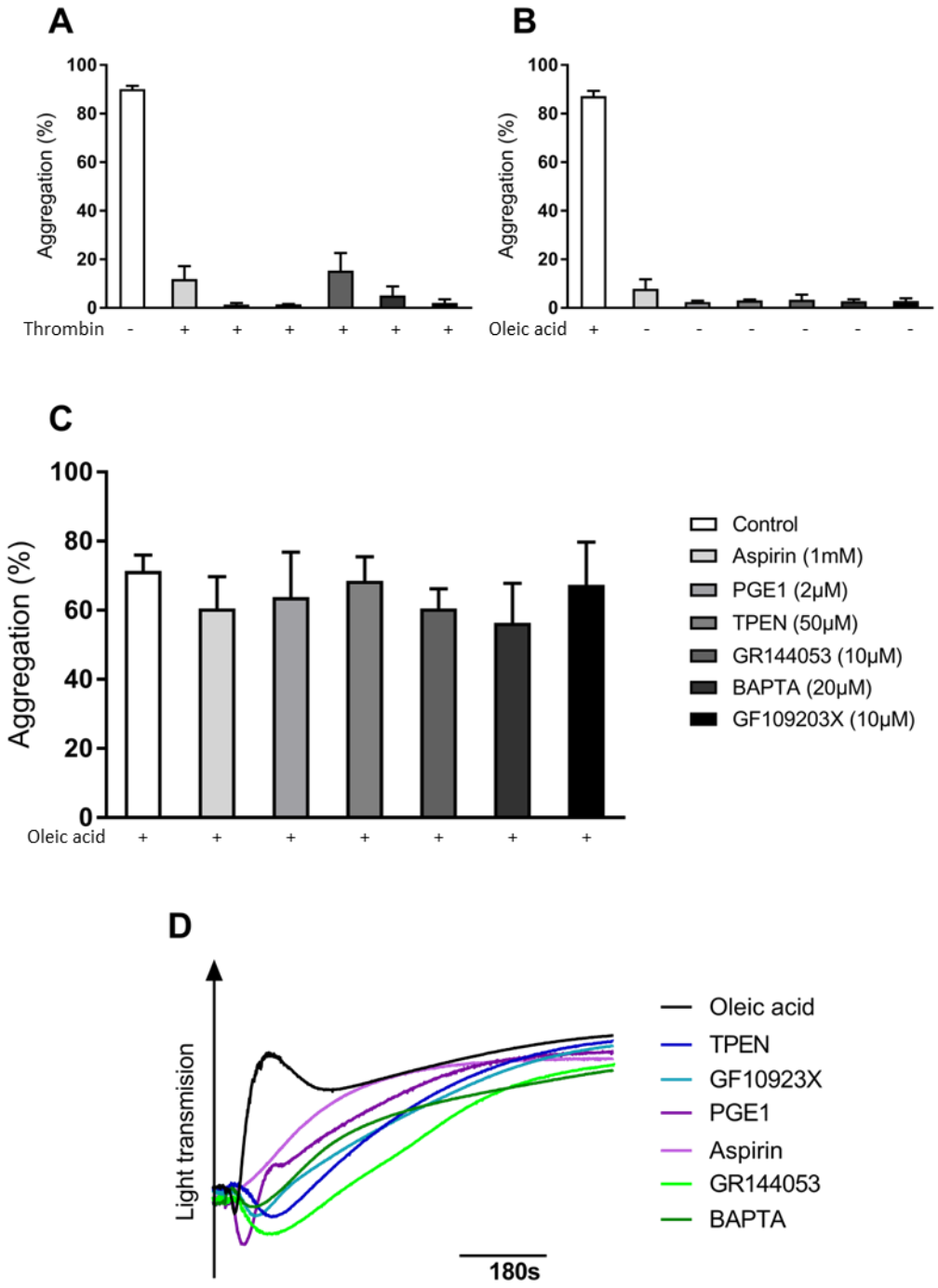


Figure 4.6 Inhibitors of vital components of platelet activation were unable to prevent oleic acid-induced aggregation

Platelets pre-treated with inhibitors for a range of vital activatory signalling events were stimulated and their responses measured *via* LTA (n=5). A) All inhibitors were effective against thrombin stimulation. B) None of the inhibitors displayed an ability to activate platelets themselves. C) None of the inhibitors could significantly reduced OA-induced platelet aggregation, however, they did alter the traces produced.

4.3.2.4 Oleic acid-induced aggregation is reliant on intracellular signalling

Further experiments were required to understand the results produced *via* aggregometry. It was important to distinguish whether oleic acid-induced platelet activation led to typical platelet aggregation, or whether oleic acid resulted in superficial clumping and platelet agglutination. To investigate this, formalin was used to fix platelets prior to stimulation in order to establish whether the effects of oleic acid were dependent on intracellular signalling.

Aggregation through integrin $\alpha_{IIb}\beta_3$/ fibrinogen interactions, as a consequence of platelet activation, is only one way in which platelets are able to aggregate. Agglutination is a process by which platelets can aggregate through interactions between surface receptors and molecules, independent of intracellular signalling. Agglutination is typically used to refer to aggregate formation caused by interactions between antibodies and surface antigens. To ascertain whether oleic acid-induced platelet aggregation is reliant on intracellular signalling, or is alternatively a result of agglutination, washed platelets were fixed with formalin prior to stimulation. Formalin contains formaldehyde, a reactive electrophilic species, which reacts readily with various functional groups of macromolecules in order to form cross-links (Thavarajah*, et al.*, 2012). This cross-linking prevents molecules performing their function, whilst preserving their structure and spatial relationship to the cell, thereby preventing intracellular signalling (Thavarajah*, et al.*, 2012). To confirm the effectiveness of fixation, aggregation was observed in response to thrombin and CRP-XL.

Fixation significantly reduced the aggregatory responses produced by thrombin and CRP-XL, producing maximal aggregation of 6.6 ± 6.5% and 9.0 ± 4.6%, respectively ($P < 0.0001$) (Figure 4.7A). The aggregation traces following fixation showed no shape change or aggregation, and, thus, established that neither agonist elicited a response (Figure 4.7C). This confirmed that fixation with formalin blocked the intracellular signalling required for activation-dependent platelet aggregation, and could be used to understand the mechanism of oleic acid-induced aggregation.

Formalin fixation significantly reduced oleic acid-induced aggregation, producing maximal aggregation of 3.3 ± 1.4% ($P<0.0001$) (Figure 4.7A). Furthermore, the aggregation trace was visually the same as those produced by thrombin and CRP-XL following fixation without shape change or aggregation (Figure 4.7C). This strongly suggested that oleic acid-induced aggregation was dependent on intracellular signalling and was, therefore, not a result of agglutination. However, previous experiments were unable to identify the pathway through which oleic acid evoked its response, possibly suggesting a novel pathway of platelet activation or a novel mechanism of aggregation, independent of platelet activation.

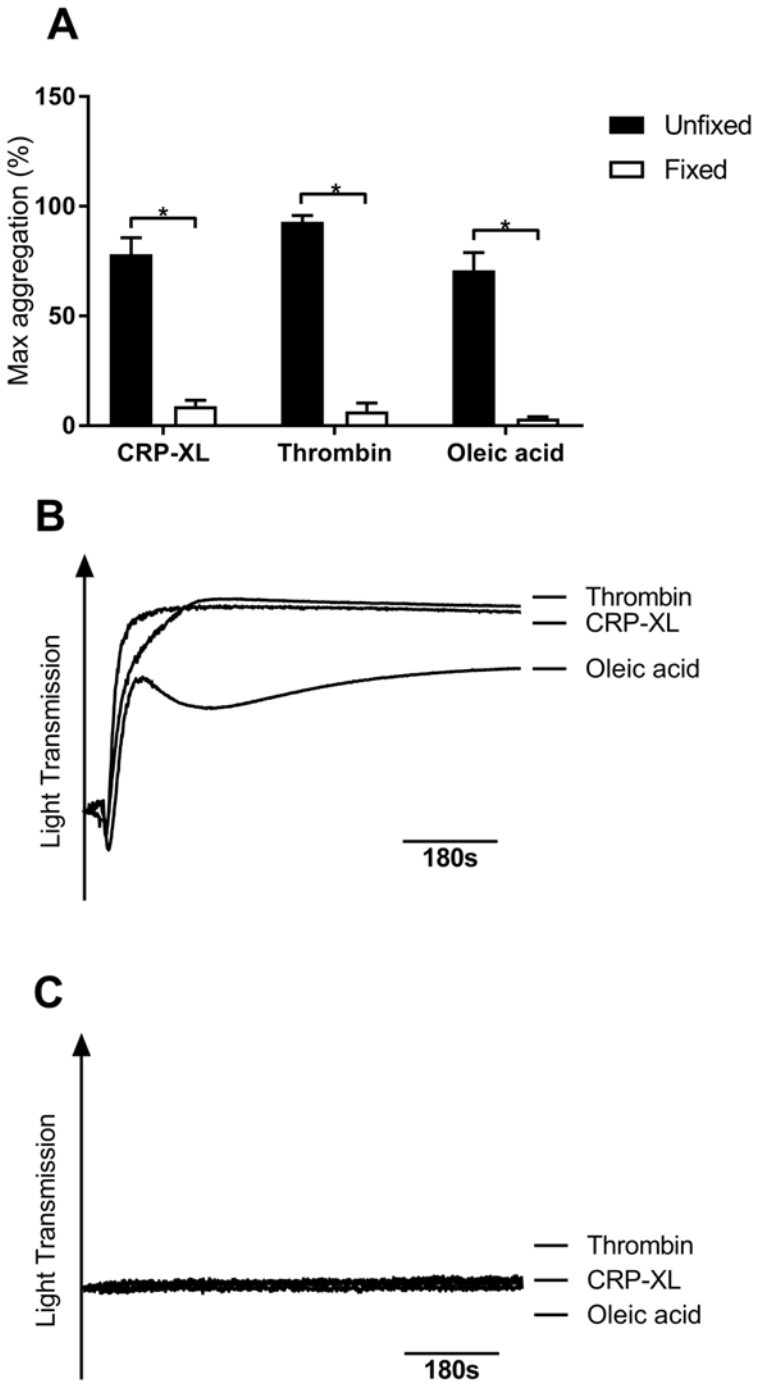


Figure 4.7 Fixation of platelets with formalin prevents OA-induced aggregation

A) Formalin fixation of washed platelets prevented physiological agonist-induced platelet aggregation and OA-induced aggregation. B) Representative traces prove the function of unfixed cells to agonist stimulation. C) Representative traces show a complete lack of response in fixed platelets.

4.3.2.5 Oleic acid does not cause activation marker expression

In order to establish whether oleic acid was eliciting a response *via* a pathway of platelet activation or not, antibodies against markers of platelet activation were employed. Platelets were incubated with fluorescently labelled anti-PAC1, anti-CD62P and anti-CD63 prior to stimulation and the fluorescence was measured on a flow cytometer. PAC1 antibody is directed against activated integrin $\alpha_{IIb}\beta_3$, the product of all known pathways of aggregation as a result of platelet activation. Anti-CD62P is an antibody against P-selectin, which is expressed on the membrane of α-granules in resting platelets, but, translocates to the surface of the platelet following activation (Berman*, et al.*, 1986; Stenberg*, et al.*, 1985). Anti-CD63 is directed against CD63, a protein present in the membrane of dense granules and lysosomes (Nishibori*, et al.*, 1993), which translocates to the outer platelet membrane upon release of dense granules, associating with the integrin $\alpha_{IIb}\beta_3$-CD9 complex, and with the actin cytoskeleton in an $\alpha_{IIb}\beta_3$-dependent manner, following platelet activation (Hamamoto*, et al.*, 1994). To confirm functionality of the antibodies and to understand levels of activation markers present on activated platelets, the response following stimulation with a known agonist, thrombin, was observed.

Thrombin evoked a significant increase in the expression of all three markers of activation, activated integrin $\alpha_{IIb}\beta_3$, CD62P and CD63 ($P=0.0021$, 0.0001, and 0.0012, respectively) (Figure 4.8). These results confirmed the functionality of the antibodies, and established that all three markers were significantly increased by platelet activation *via* known pathways.

Oleic acid did not cause a significant increase in the expression any of the markers of activation (Figure 4.8). Therefore, it was determined that oleic acid did not cause the release of α- or dense granules, both of which are vital to all known pathways of platelet activation. Moreover, oleic acid did not cause platelets to aggregate *via* binding of activated integrin $\alpha_{IIb}\beta_3$ to fibrinogen, currently the only known mechanism through which activated platelets aggregate. These results strongly suggested that oleic acid

did not cause platelet activation, but rather acted through another mechanism of aggregation, independent of platelet activation.

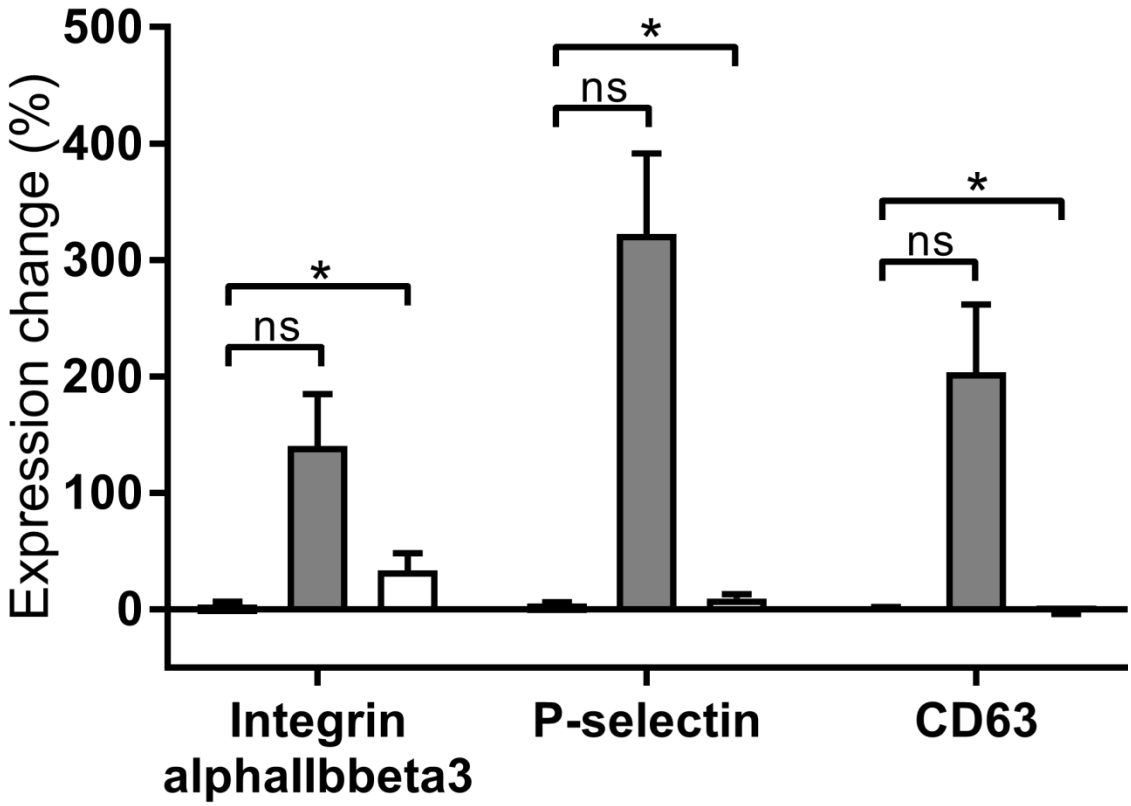


Figure 4.8 Oleic acid does not increase the expression of markers of platelet activation

Platelet activation markers were probed with fluorescence conjugated antibodies and analysed in real-time for 5 minutes with unstimulated platelets as a control (n=4). Expression change was measured at the 5 minute time point. Thrombin vastly increased the expression of all three markers of activation; integrin $\alpha_{IIb}\beta_3$, P-seletin and CD6, whilst oleic acid did not.

4.3.2.6 Oleic acid induced phosphatidylserine exposure

Exposure of phosphatidlyserine (PS) on the outer platelet membrane is another marker of platelet activation. However, unlike the previously investigated markers, PS exposure can also be regulated by signalling pathways independent of platelet activation (Schoenwaelder*, et al.*, 2009). At rest, phosphatidylserine (PS) is distributed asymmetrically across the plasma membrane of platelets, with the majority on the cytoplasmic face (Chap*, et al.*, 1977; Schick*, et al.*, 1976; Verkleij*, et al.*, 1973). This asymmetry is maintained through ATP-dependent aminophospholipid translocase activity (Devaux, 1991). However, upon activation or cellular damage, PS is translocated the outer surface of the platelet membrane through a currently unknown mechanism. To study the notion that oleic acid may be inducing platelet aggregation *via* signalling independent of the activatory pathways, annexin V-FITC, a fluorescent phosphatidylserine-binding protein was employed to measure PS exposure. In order to establish functionality of the probe, and to understand levels of PS exposure present on activated platelets, the response following stimulation with thrombin was assessed.

Thrombin caused an increase in external PS exposure of 71.1 ± 31.8% in comparison to the control, although, this increase was not significant, and, thus, indicated that platelet activation led to small increases in PS exposure (P=0.4968) (Figure 4.9).

On the other hand, oleic acid, induced significant PS exposure of 291.2 ± 203.8%, (P=0.0014) (Figure 4.9). These results indicated that oleic acid mediated platelet aggregation *via* intracellular signalling linked to PS exposure. PS exposure is also associated with cell death processes, which in turn have been linked with platelet aggregation, and, thus, it was vital to investigate whether oleic acid induced platelet death.

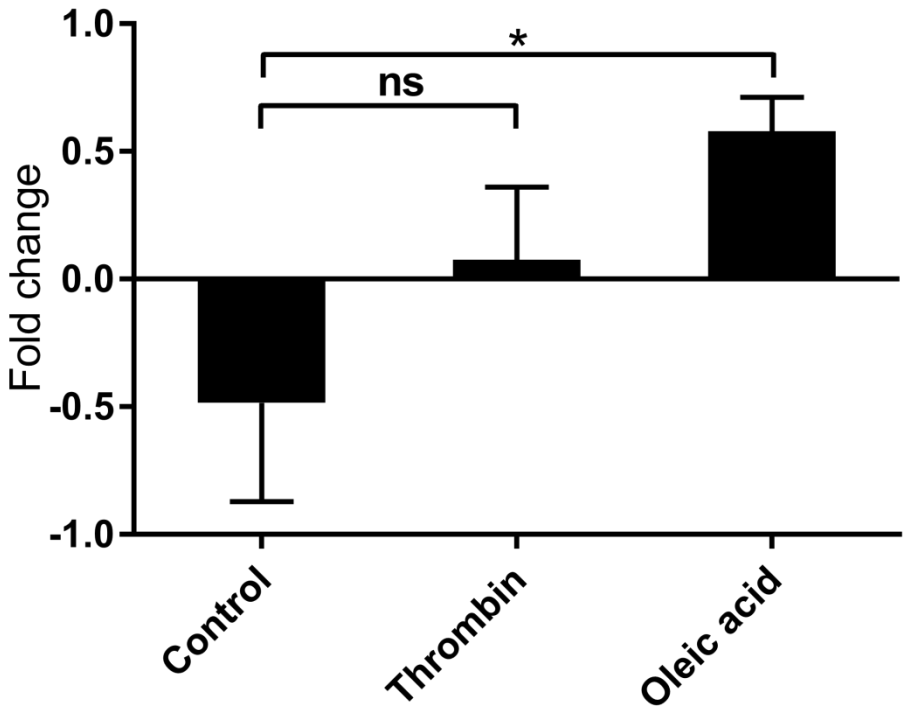


Figure 4.9 Oleic acid induced high levels of PS exposure

Flow cytometric analysis of annexin V stained platelets determined PS exposure following stimulation with unstimulated platelets as a control (n=6).Thrombin induced small, non-significant PS translocation, whilst oleic acid induced significant PS exposure.

4.3.3 Investigation of the effect of oleic acid on other platelet functions

4.3.3.1 Oleic acid caused significant cell death

To ascertain whether cell death signalling was involved in oleic acid-induced PS exposure, a lactate dehydrogenase (LDH) assay and a calcein AM experiment were performed following platelet stimulation. LDH absorbance is inversely proportional to cell viability, and, thus, were converted from absorbance to percentage viability, theory can be found in Appendix C .Calcein AM is a cell-permeant, non-fluorescent dye that is converted to green-fluorescent calcein by esterases in live cells, and leaks from dead cells or cells with damaged membranes, thereby acting as a measure of platelet

viability. In order to understand differences between platelet activation and platelet death, the known agonist thrombin was used as a model of typical aggregation.

Thrombin did not cause significant alterations to platelet viability as measured by the LDH assay (P=0.5177) (Figure 4.10A). The calcein AM experiment also found that thrombin had no effect on the viability of washed platelets (P=0.7039) (Figure 4.10B). These results determined that typical platelet activation and aggregation did not cause a decrease in platelet viability.

The addition of 0.1 mM oleic acid to washed platelets caused a significant increase in LDH release, which represented a final viability of 59.62 ± 6.27% (P=0.0011) (Figure 4.10A). Flow cytometric analysis of calcein AM leakage found that oleic acid significantly decreased platelet viability to 49.79 ± 4.76% (P<0.0001) (Figure 4.10B). These results determined that oleic acid evoked platelet death, and, thus, suggested that intracellular signalling in oleic acid-induced aggregation was important in cell death processes, not platelet activation. However, these results enabled further understanding of platelet signalling and PS exposure following treatment with oleic acid, but did not explain how the platelets were aggregating, or in fact whether they were aggregating at all. Aggregometry relies on a rudimentary measure of optical density to measure aggregation, which is liable to a wide range of artefactual results from anything that alters the turbidity of the solution. For example, platelet lysis and crystallisation of a solution can both lead to convincing aggregation results and traces.

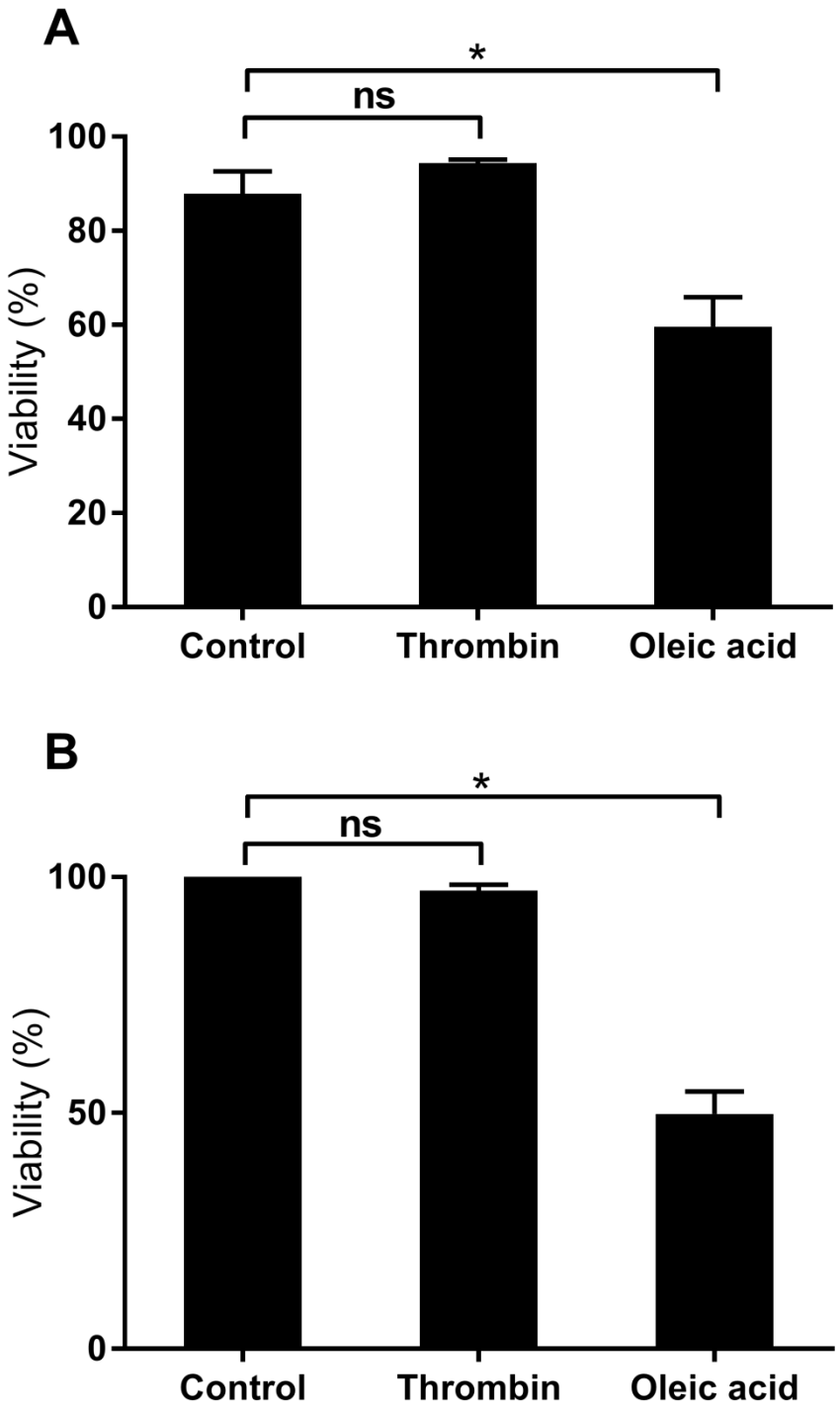


Figure 4.10 Oleic acid decreased platelet viability

Two experiments to assess platelet viability following stimulation were employed, LDH assay and calcein-AM staining with unstimulated platelets as a control (n=6 and 5, respectively. A) Oleic acid-induced loss in platelet viability measured by LDH assay. B) Oleic acid-induced reduction in platelet viability measure by calcein-AM staining.

4.3.4 The formation of oleic acid-induced platelet aggregates was confirmed

It was important to establish whether platelets were indeed aggregating following stimulation with oleic acid. Therefore, confocal microscopy was used to view the washed platelets at a range of time points throughout the aggregometry experiment.

As shown in Figure 4.11, oleic acid did induce the formation of platelet aggregates, and thereby indicated that the results produced during the aggregometry experiments were not artefactual. Close examination of the images indicated that oleic acid appeared to cause platelet shrinkage, however, this could not be measured using the confocal software as the magnification was too low. This observation contradicted platelet activation, as this would cause platelets to increase surface area through the extension of pseudopodia. It also contradicted a loss in platelet viability *via* necrosis, as this would have caused expansion and blebbing. Therefore, it was important to measure the size of platelets following treatment to gain a better understanding of aggregation and platelet death processes.

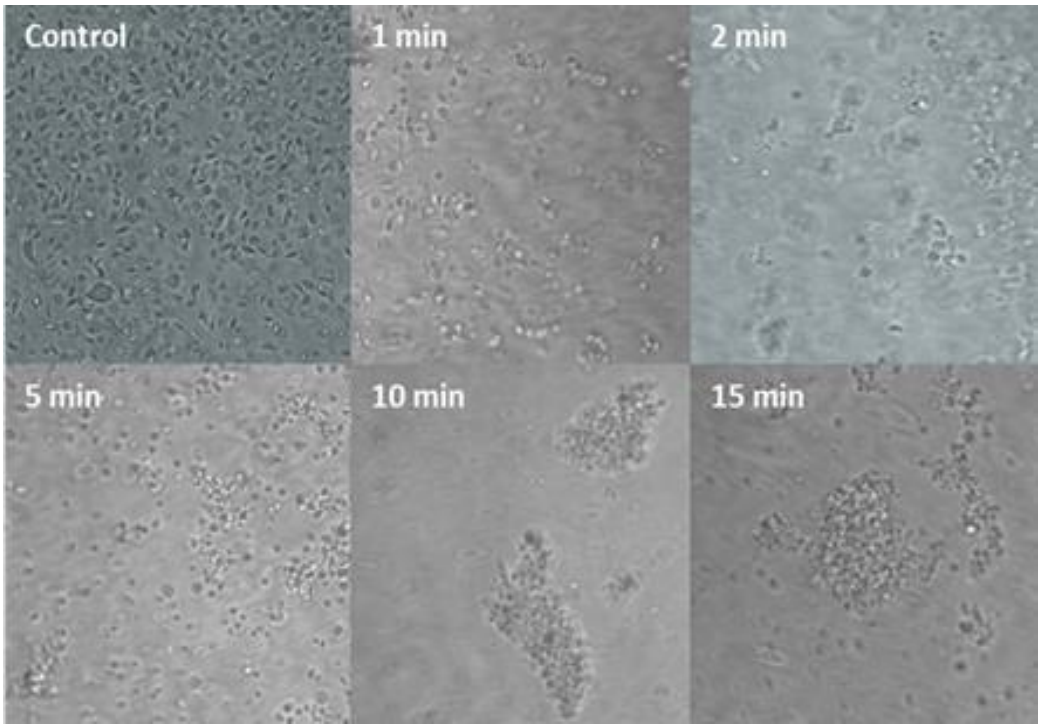


Figure 4.11 Oleic acid-induced aggregates were visualised *via* confocal microscopy

Platelets were removed from the aggregometer at time points of 0, 1, 2, 5, 10 and 15 minutes and images taken at 600x magnification. These confirmed that physical aggregation of platelets was occurring following OA stimulation rather than an artefact.

4.3.4.1 Oleic acid caused platelet shrinkage

The relative size of platelets following treatment in comparison to their size before treatment can be indicative of a number of different processes. The confocal microscopy images were qualitatively analysed and it was observed that platelets were shrinking (Figure 9). However, a more robust technique was required for quantitative analysis. Flow cytometric analysis was employed to measure relative cell size following platelet stimulation. To understand alterations in platelet size caused by activation, forward scattered light was analysed following stimulation with thrombin to act as a comparison for oleic acid treated samples.

Thrombin caused significant platelet enlargement of 47.69 ± 17.06%, P=0.0127 (Figure 4.12). These results were consistent with the literature and are explained by the cytoskeletal reorganisation induced by platelet activation that facilitates platelet-platelet interactions *via* increased surface area.

Oleic acid, on the other hand, induced significant platelet shrinkage of 48.81 ± 7.52%, P=0.0032 (Figure 4.12). These results further indicated that oleic acid was not inducing platelet activation. In combination with the platelet viability results, the significant platelet shrinkage was a potential indicator of apoptotic processes at play. This was also consistent with the significant PS exposure found.

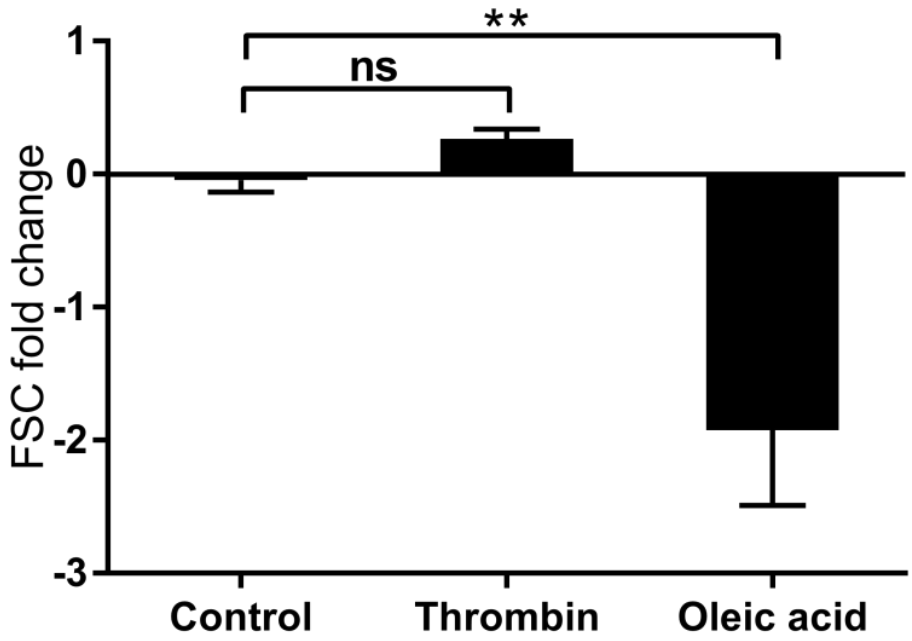


Figure 4.12 Oleic acid caused a decrease in platelet size

Forward scatter, indicative of platelet size, was measured flow cytometry (n=6). Thrombin induced small, non-significant increases in FSC as shown in the literature. Oleic acid caused significant decreases in FSC.

4.3.5 Analysis of competing hypotheses

The interpretation of results from this chapter was complex due to their novelty and conflicting nature. In order to view the results objectively and make unbiased conclusions, a methodology called analysis of competing hypotheses (ACH) was utilised. ACH was developed for the Central Intelligence Agency to overcome cognitive bias.

Primary data from this study was combined with a couple of key pieces of literature in the ACH matrix (Table 4.1). Each piece of evidence was listed on the left hand side and its credibility and relevance assessed. The credibility of each piece of evidence was rated on the accuracy and precision of the methodology, and the relevance of the

evidence was rated on how important that result was in reaching a conclusion. The evidence was then rated for each hypothesis on a five-point-scale of inconsistency.

CC = very consistent, C = consistent, N = neutral, I = inconsistent and II = very inconsistent.

The ACH software was used to form weighted inconsistency scores, taking the credibility and relevance of evidence into consideration (Figure 4.13). These scores were used to objectively assess each hypothesis and draw conclusions about this study. The theory that inconsistency outweighs consistency was used in this analysis.

The hypothesis that oleic acid was causing platelet activation produced the highest weighted inconsistency score of 30. This strongly indicated that this hypothesis should be rejected due to a number of large flaws in the argument. The theory of platelet agglutination also received a high weighted inconsistency score of 19, which also suggested the rejection of this hypothesis based on a lack of support in the data. On the other hand, hypotheses of oleic acid-induced platelet death produced low weighted inconsistency scores. Necrosis received a score of 3, whilst apoptosis was favoured with a score of 0. Only the determination that oleic acid caused platelet shrinkage was contradictory to the necrosis theory, whilst none of the results produced in this study could discount the possibility that apoptosis was the mechanism of action of oleic acid. These results, therefore, strongly indicate that oleic acid does not cause platelet activation or agglutination, but does in fact induce platelet death *via* the probable mechanism of apoptosis.

Table 4.1 Analysis of competing hypotheses matrix

	Type	Credibility	Relevance	Activation	Agglutination	Necrosis	Apoptosis
Aggregation trace	1ary	Low	Medium	CC	CC	N	N
Oleic acid produces maximum platelet aggregation of 61.4 ± 22.5%	1ary	Low	Medium	C	C	N	N
Oleic acid-induced aggregation is not reliant on secondary-	1ary	High	Low	N	C	C	C
Oleic acid-induced aggregation is not mediated by PKC	1ary	High	High	II	C	C	C
Oleic acid-induced aggregation is not integrin dependent	1ary	High	High	II	C	C	C
Oleic acid-induced aggregation is reliant on intracellular signalling	1ary	High	High	CC	II	N	CC
Oleic acid does not activate integrin $\alpha_{IIb}\beta_3$	1ary	High	High	II	CC	CC	CC
Oleic acid does not cause α-granule release	1ary	High	High	II	CC	CC	CC
Oleic acid does not cause dense granule release	1ary	High	High	II	CC	CC	CC
Oleic acid induces PS exposure	1ary	Medium	High	C	II	CC	CC
Oleic acid evokes platelet death	1ary	High	High	I	II	CC	CC
Oleic acid causes platelet shrinkage	1ary	Medium	High	II	II	II	CC
Inhibitors of platelet activation and chelators of calcium were unable to inhibit PS exposure in platelets undergoing apoptosis	2ary	High	High	II	II	C	CC
Amphipathic molecules can induce aggregation of PS along with leakage of cellular content	2ary	High	High	II	II	CC	CC

Objective analysis of evidence

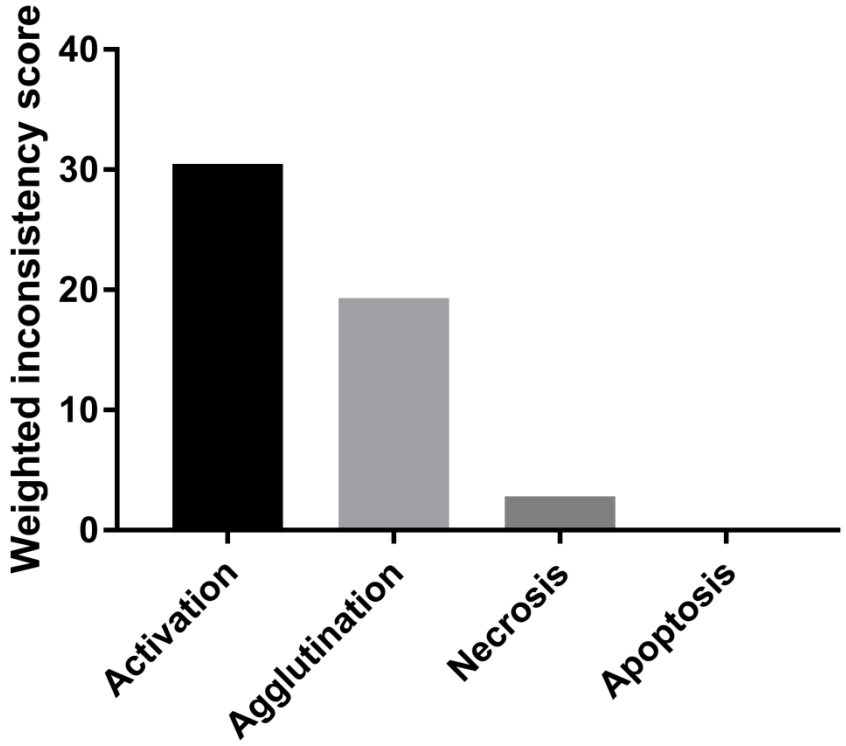


Figure 4.13 Analysis of competing hypotheses rejects platelet activation

Weighted inconsistency scores were produced by ACH software to indicate the strength of the proposed hypotheses. Platelet activation was the theory least supported by the data in this chapter, whilst apoptosis was the most supported.

4.4 Discussion

The objective of this study was to characterise the effect of palmitic acid and oleic acid on platelet aggregation, and specifically platelet activation. This extended from aggregometry to the analysis of activation markers, and finally the analysis of LDH release to clarify contradictions in the literature regarding fatty acid-induced aggregation, and to gain a better understanding of the mechanisms through which fatty acids may elicit their response. Results confirmed that oleic acid induced platelet aggregation, in contrast to palmitic acid. Furthermore, oleic acid-induced aggregation was found to be reliant on intracellular signalling, but independent of traditional activation pathways. This potentially indicated the action of a novel mechanism that may or may not be related to oleic acid-induced platelet death, established in this study.

4.4.1 Oleic acid induces platelet aggregation *via* an unknown mechanism

In this study, oleic acid was shown to evoke a robust aggregatory response in washed platelets, which is supported by the majority of literature (Miles *et al*.,. 1988; Hashimoto*, et al.*, 1985; Zentner*, et al.*, 1981; Connor*, et al.*, 1969; Hoak*, et al.*, 1967), and contradicted by a small minority (Nunez*, et al.*, 1990). In contrast, palmitic acid did not induce significant aggregation, which is both supported (Hashimoto*, et al.*, 1985) and contradicted by the literature (Zentner*, et al.*, 1981, Connor*, et al.*, 1969).

Oleic acid-induced aggregation appeared to be biphasic due to the presence of two waves, as seen on the aggregation trace, suggesting a lag between primary and secondary signalling, and, thus, indicating that 0.1mM oleic was not as potent as the physiological agonists used in this study (Huang, 1989). Additionally, it suggested an important role for secondary mediators of signalling such as ADP and TxA_2, in oleic acid- induced aggregation. However, inhibitors of secondary signalling pathways did not significantly reduce the aggregation response to oleic acid, indicating that it is not perpetuated by ADP or the generation of TxA_2. These results suggest another mechanism may be responsible for the secondary wave of aggregation, seen on the biphasic aggregation traces.

The observations that the inhibition of PKC also had little effect on oleic acid-induced aggregation, suggested that oleic acid does not act through the $P2Y_1$, thromboxane-prostanoid (TP), PAR1/ 4, GPVI or α2β1 receptors. This left $P2Y_{12}$ as a possible candidate for the signal transduction receptor, as $P2Y_{12}$ has been found to activate integrin $\alpha_{IIb}\beta_3$ concomitantly with $P2T_{AC}$, and is vital for ADP-induced aggregation (Kauffenstein*, et al.*, 2001; Jin and Kunapuli, 1998). The inability of integrin $\alpha_{IIb}\beta_3$ inhibition to reduce platelet aggregation as a result of oleic acid caused swift dismissal of this possibility, and strongly suggested that oleic acid caused aggregation independently of platelet activation. However, the possibility of a novel mechanism of aggregation, or a novel pathway of activation could not be ruled out until the lack of CD62P, CD63, and most importantly activated integrin $\alpha_{IIb}\beta_3$ on the outer platelet membrane following stimulation with oleic acid, contradicted the latter.

The result that oleic acid-induced responses were resistant to a broad spectrum of inhibitors was surprising yet not inconsistent with some forms of agglutination. For example, some snake venom proteins such as echicetin, ristocetin and botrocetin induce agglutination and intracellular signalling *via* GpIb (Kini & Evans, 1990; Navdaev*, et al.*, 2001). Conventional agglutination requires activation of von Willebrand's factor (vWF), either *via* biochemical interactions or by exposure to elevated shear rates. Activated vWF subsequently binds to GpIb/V/IX, which crosslinks platelets and form aggregates. However, the changes invoked in light transmission aggregometry in response to oleic acid were apparent in washed platelets, from which plasma vWF had been removed. This strongly indicated that vWF-dependent agglutination was also unlikely to be the mechanism through which oleic acid induced responses.

4.4.2 Oleic acid as a signalling molecule

A number of studies have suggested that oleic acid may have signalling properties in a range of nucleated cell types (Siafaka-Kapadai*, et al.*, 1997). Oleic acid activates soluble protein kinase C (Khan*, et al.*, 1992). In rat β islet cells, OA evokes Ca^{2+} signalling *via*

interaction with GPCRs of the FFAR family (Alvarez-Curto & Milligan, 2016; Fujiwara*, et al.*, 2005). Additionally, OA interacts with CD36 (GpIV) to induce foam cell formation and enhance atherosclerotic lesion development in smooth muscle cells (Ma*, et al.*, 2011). Interestingly, CD36 is also expressed on platelet membranes, where it contributes to platelet activation (Nergiz-Unal*, et al.*, 2011). Furthermore, oleic acid regulates the activity of α- and β-adrenergic receptors during blood pressure regulation (Terés*, et al.*, 2008).

Oleic acid is an omega-9 monounsaturated C18 fatty acid. The oleoyl- group is common amongst numerous signalling molecules in both nucleated cells and platelets. For example, 1-oleoyl lysophosphatidic acid (LPA) is a phospho-oleoyl ester which evokes platelet aggregation *via* interactions with LPA receptors (Haserück*, et al.*, 2004; Lin*, et al.*, 2010). Additionally, 1-oleoyl-2-acetyl-sn-glycerol (OAG) is regularly employed in platelet research as a diacylglycerol (DAG) analogue to induce platelet aggregation (Albarran*, et al.*, 2014; Ilkan*, et al.*, 2017; Kroll*, et al.*, 1988; Marumo & Wakabayashi, 2017). N-oleoyl-dopamine (OLDA) is a vanilloid receptor agonist which inhibits platelet aggregation in response to ADP and collagen (Almaghrabi*, et al.*, 2016; Almaghrabi*, et al.*, 2014). In nucleated cells, oleoyl-ethanolamide (OEA) activates the nuclear receptor PPAR-α (Chen*, et al.*, 2015; Fu*, et al.*, 2005; Guzmán*, et al.*, 2004). Furthermore, OEA activates GPR119, a Gαs-coupled GPCR leading to enhanced insulin secretion in pancreatic β cells (Moran*, et al.*, 2014) and increased glucagon-like peptide-1 (GLP-1) secretion from intestinal L-cells (Lauffer*, et al.*, 2009). Other lipids containing the oleoyl- group also activate the GPR119 receptor, including oleoyl-lysophosphatidylcholine (LPC), 2-oleoyl-glycerol (2OG) and N-oleoyl-dopamine (ODA) (Hansen*, et al.*, 2012; Ning*, et al.*, 2009). N-oleoyl-glycine (OLGly) is an endocannabinoid receptor ligand associated with activation of Akt signalling in 3T3-L1 adipocytes (Wang*, et al.*, 2015). Other compounds containing the oleoyl group with signalling roles in nucleated cells include oleoyl-CoA, oleamide and oleoyl-glycerol (Bell*, et al.*, 2007; Hsu & Powell, 1975; Liu*, et al.*, 2001).

These studies provide substantial evidence of roles for FFAs, and more specifically oleoyl-group containing compounds as signalling molecules in a variety of cells *via* heterogeneous

mechanisms, and, thus, it is plausible that oleic acid activates signalling pathways in platelets other than those of activation. Additionally, a mounting body of evidence suggests that many characteristics of platelet activation may be mimicked by the initiation of cell death signalling within platelets.

4.4.3 Oleic acid causes platelet death

A number of studies have found a link between procoagulant platelet activity and apoptosis (Zhu*, et al.*, 2016; Cohen*, et al.*, 2007; Cohen*, et al.*, 2004). Procoagulant platelets exhibit key characteristics of apoptotic cells, including phosphatidylserine (PS) exposure, membrane blebbing and microvesiculation (Schoenwaelder*, et al.*, 2009). One study demonstrated that there is often overlap between activation and cell death when platelets are stimulated with known agonists (Schoenwaelder*, et al.*, 2009). It was determined that PS exposure on the surface of agonist-stimulated platelets is associated with the proteolytic cleavage of a number of cellular substrates present in cell death processes. Furthermore, inhibitors of platelet activation and chelators of calcium were unable to inhibit the cleavage of these proteins in platelets undergoing apoptosis, in contrast to agonist-stimulated platelets (Schoenwaelder*, et al.*, 2009). Consistent with this, studies have also demonstrated that caspase inhibitors do not prevent the procoagulant function of activated platelets (Kulkarni & Jackson, 2004; Schoenwaelder*, et al.*, 2009; Zauli*, et al.*, 1997).

Results produced by this study are consistent with the literature in that a vast range of inhibitors could not reduce oleic-acid induced aggregation, and two assays measuring cell death found that oleic acid caused significant loss in platelet viability. However, this does not explain the physical aggregation of oleic acid stimulated platelets, but may indicate that signalling other than platelet activation is responsible for this process or at least partially involved.

4.4.4 Oleic acid may cause aggregation *via* changes to the platelet membrane and*/* or *via* platelet fusion

The mechanism responsible for OA-induced changes in platelet behaviour is not known. Conventional receptor-ligand mediated responses might provide the best explanation. However, a number of other possible mechanisms may also play a role.

The incubation of nucleated cells with oleoyl-group compounds has previously been shown to alter cell signalling. For example, fusion of cells with dioleoyl-phosphatidylcholine (DOPC) liposomes resulted in a loss of β-adrenergic receptor sites, in comparison to cells treated with dipalmitoyl-phosphatidylecholine (DPPC) liposomes (Bakardjieva*, et al.*, 1979). This loss in function was associated with a decrease in adenylate cyclase activity and a reduction in cAMP. In platelets, if a similar effect were to occur, the reduction in cAMP would potentiate platelet activation.

A second potential mechanism of oleic acid-induced platelet aggregation is through modulation of membrane fluidity and anisotropy of membrane lipids (Feijge*, et al.*, 1990; Mateo*, et al.*, 1991; Vélez*, et al.*, 1995). Oleic acid has been demonstrated to incorporate into the plasma membrane (Rao*, et al.*, 1994) and Miles*, et al.,* (1988) found that high concentrations of oleic acid produced changes in membrane fluidity that caused cellular damage. Additionally, membrane fluidity is often cited as a contributing factor in a range of disorders such as leukaemia (Popov*, et al.*, 2014), Alzheimer's disease (Yang*, et al.*, 2011) and obesity (Cazzola*, et al.*, 2004).

Another potential explanation for the effects of oleic acid on platelets is fusogenicity, in which the phospholipid bilayers of adjacent cells merge *via* interactions with membrane lipids and proteins. Cell fusion is a familiar phenomenon in the differentiation of some nucleated cells, although a role in platelet function has not been reported. Ahkong*, et al.,* (1973b) found that oleic acid and oleoyl-glycerol induced fusion in erythrocytes and erythrocyte ghosts in physiological time periods of 5-10 minutes at 37^{o}C. Interestingly, palmitic acid did not produce the same response and was shown to possess no fusogenic properties (Ahkong*, et al.*, 1973b). The mechanism by which lipids with fusogenic properties

induce cell fusion remains unclear. It has been proposed that oleoyl-induced erythrocyte fusion occurs due to increased membrane fluidity, which is likely caused by an elevated percentage of hydrocarbons with low melting points. Lipids with melting points below body temperature exist in a relatively liquid state and, thus, their incorporation into the membrane would alter fluidity (Ahkong*, et al.*, 1973a; Ahkong*, et al.*, 1973b). Alternatively, the incorporation of exogenous lipids into the plasma membrane may create protein–free regions that can interact with similar regions of opposing cells. Contact of these regions causes fusion *via* the formation of a multi-lamellar lipid membrane (Tsivion & Sharon, 1981). Throughout the literature search, only one paper confirmed platelet-platelet fusion. The study incidentally observed platelet fusion in response to the cholinergic agent, carbachol in vitro (White, 1999). However, this was independent of time and concentration, and, thus, the pathophysiological significance of this observation is unclear.

Thus, oleoyl-induced changes in membrane fluidity or fusion of platelets may be a contributing factor to occlusive thrombi formation at sites of plaque rupture. If so, such effects may explain correlations between plasma FFA levels and pathogenicity of stroke, cardiovascular disease and diabetes and may be a significant contributing factor for platelet thrombus formation during plaque rupture.

5 Meta-analysis of plasma lipid composition: implications for pathologies

5.1 Background

Lipid transport, storage and metabolism is complex, with the involvement of lipoproteins, formation of large plasma lipid and protein complexes, and involvement of the digestion system, liver and target tissues of muscle and adipose tissue. Most lipids (about 90%) are carried in the form of fatty acid esters in the form of triacylglycerol (TAG), cholesterol esters and phospholipids, contained in circulating lipoprotein particles. A smaller proportion of lipids (up to10%) are transported as non-esterified (NEFA) fatty acids (also called free fatty acids -FFA) that circulate in the plasma, the functions of which were largely elucidated by Vincent Dole (1956) and Robert Gordon (1956; 1957), at the Rockefeller Institute and the National Institutes of Health respectively. FFAs are an important source of energy *via* fatty acid β-oxidation (Janssen & Stoffel, 2002). They also play a role in signalling, interacting with nuclear peroxisome proliferator-activated receptors (PPARs) to maintain lipid homeostasis (Varga*, et al.*, 2011). Furthermore, they are vital in the production of plasma membranes (Abbott*, et al.*, 2012).

As they diffuse easily across cell membranes, circulating FFA are the primary fuel source in the body, as well as having a number of other important functions, with significant involvement in oxidative stress, cell signalling and membrane stability. Being hydrophobic, free fatty acids bind to plasma albumin for transport in the circulatory system, and are the vehicle by which triacylglycerol is transported from storage in the adipose tissue to sites of utilisation. Turnover of FFA is rapid, with a plasma half-life of 1.8-3.9 minutes (Eaton*, et al.*, 1969).

In the normal overnight fasting state, FFA are almost entirely produced by the hydrolysis of triacylglycerol within adipocytes of abdominal subcutaneous fat, with small amounts arising from leg adipose tissue and intra-abdominal adipose tissue (Nielsen*, et al.*, 2004), thereby

maintaining a steady-state reported to be approximately 300-600 µmol/L, with levels higher in woman than in men (Abdelmagid*, et al.*, 2015; Frayn*, et al.*, 1996). Levels of circulating FFA can also be altered by physiological states; for example, extended fasting of 72 hours increases levels of plasma FFA up to ~1800 µmol/L (Gjesdal*, et al.*, 1976). States of increased stress as found in racing drivers before a race are associated with elevated circulating FFA, up to 1 720 µmol/L (Taggart*, et al.*, 1971).

In addition, varied dietary habits generate a wide variation in intake of amounts of fat, and the proportions of different types of fatty acids. Different countries and ethnicities have significantly varying diets, for instance palm oil producing island nations such as Samoa and Kiribati consume high levels of saturated fat (27.5% and 27% of total energy intake, respectively). Other island nations consume high levels of omega-3 such as the Maldives, whilst unsurprisingly sub-Saharan Africa have a very low intake (Micha*, et al.*, 2014). In addition, the daily nutritional intake recommendations vary between men and women, which may lead to differences in circulating FFA.

It is also hypothesised that disparities in circulating plasma FFA exist between different BMI groups. As plasma FFA mainly arises from adipose tissue in the fasting state, and adipose tissue mass is increased in obesity, it has been hypothesised that circulating concentrations of FFA are elevated in these individuals (Björntorp*, et al.*, 2009; Karpe*, et al.*, 2011; Lafontan & Langin, 2009). Increased circulating FFAs may trigger peripheral insulin resistance, as well as inhibiting production and release of insulin from pancreatic beta cells (Boden, 1999; Sears & Perry, 2015). Furthermore, increases in circulating FFAs has also been linked with the devlopment and progression of cardiovascular disease (Breitling*, et al.*, 2011; Pilz*, et al.*, 2006; Roy*, et al.*, 2013).

Elevated plasma FFA in obese individuals was found in early research (Opie & Walfish, 1963), however, more recent studies have shown that the difference in fasting circulating FFA between obese and non-obese patients is highly variable and equivocal (Arner*, et al.*, 2015; Frias*, et al.*, 2000; Horton*, et al.*, 1995; Jocken*, et al.*, 2008). One meta-analysis of 43

articles showed that FFA levels in obese patients were elevated only by an average of 70 μmol/L, with no direct association with BMI. The range of increase across the studies, however, was highly variable, ranging from a slight decrease, to an increase of over 500 μmol/L. Another study following this meta-analysis conducted on obese vs non-obese subjects involved in a genetic trial found a level in FFA in obese subjects 30% higher than non-obese (Arner*, et al.*, 2015).

As elevated plasma FFA concentrations are possibly associated with a number of diseases, it is essential to understand whether we can distinguish groups within the population with increased total circulating FFA, which may predispose to specific pathologies. Moreover, it is important to understand whether changes in plasma FFA profiles are evident in the population, and more importantly whether these are linked to unfavourable health outcomes. Small-scale studies lack enough statistical power to overcome within-person variation. It is, therefore, important to aggregate studies in a meta-analysis, in order to compute the relationship between such complex variables more reliably.

An essential aspect in developing our understanding is to know what a 'normal' lipid and FFA profile looks like, and be able to compare this to an 'abnormal' one. However, robust normal reference ranges for plasma lipids and FFA do not currently exist, thereby hindering this line of clinical investigation. In this study, we aimed to aggregate data in order to produce a robust set of reference ranges. Studies often rely upon the literature as a foundation for continuing research, using previously reported values as fact in new investigations. However, reported values are commonly produced from experimentation with small sample sizes and differing methodology, leading to an unstable foundation for future work. Therefore, it is important to draw together results and information from systematic reviews to create more accurate values for future research.

Furthermore, with focus shifting towards lipids, FFAs, and their roles in health and disease, it is vital to create reference ranges of certain key lipid species to be used in healthcare settings.

Plasma triglycerides are one of the most commonly measured lipid species within plasma, and are measured within the National Health Service (NHS) as an indicator of different disease states such as type II diabetes, nephrosis and liver obstruction. Average plasma triglyceride concentrations and NHS reference ranges are ‘common knowledge’ (Table 5.1). This study aimed to determine whether variability in results exists between multiple sources, even for the most common of lipid species.

Like plasma triglycerides, plasma free fatty acids are also tested within the NHS and have established reference ranges. However, their testing is not as common, but may possibly more be important as triglycerides are deemed to be inert lipids (Sørensen, 2011), while free fatty acids are biologically active and potentially toxic (Carpentier*, et al.*, 2000; Masi*, et al.*, 2011; Plötz*, et al.*, 2017).

Table 5.1 Plasma triglyceride reference ranges across NHS Trusts

NHS Trust	Reference range (mmol/L)
South Tees Hospitals NHS Foundation Trust	<40 years old: 0.3 – 1.8 >40 years old: 0.3 – 2.1
Leeds Teaching Hospitals NHS Trust	<1.7
Southend University Hospital NHS Trust	0.6 – 2.3
North Bristol NHS Trust	0.5 – 1.7
Gloucestershire Hospitals NHS Foundation Trust	0.6 – 1.9
Southern Health NHS Foundation Trust	<1.7
The Newcastle upon Tyne Hospitals NHS Trust	0.8 – 1.9
University Hospitals of North Midlands NHS Trust	0.8 – 1.8
Epsom and St. Helier University Hospitals NHS Trust	0.5 – 2.0
East Sussex Healthcare NHS Trust	0.0 – 1.7
Homerton University Hospital NHS Trust	<1.8
Warrington and Halton Hospitals NHS Foundation Trust	0.2 – 2.0
University Hospitals Birmingham NHS Foundation Trust, 2018	<1.7
Barking Havering and Redbridge University Hospitals NHS Trust	0.5 – 2.3
Tameside and Glossop Integrated Care NHS Foundation Trust	<1.8
Bedford Hospital NHS Trust	0.4 – 1.8
Kingston Hospital NHS Foundation Trust	0.2 – 1.7
Sheffield Children's NHS Foundation Trust	Neonate: <1.8 Infant: 0.3 – 1.7 Child: 0.4 – 2.1 Adult: <2.5
The Ipswich Hospital NHS Trust	0.3 – 2.3
University Hospital Southampton NHS Foundation Trust	<2.3

Table 5.1 continued

St. George's University Hospitals NHS Foundation Trust	0.8 – 2.0
University College London Hospitals NHS Foundation Trust	<2.3
Coventry and Warwickshire Partnership NHS Trust	0.4 -1.9
London North West Healthcare NHS Trust	<2.3
University Hospitals of Leicester NHS Trust	0.0 – 2.0
North Cumbria University Hospitals NHS Trust	<2.3
Nottingham University Hospitals NHS Trust	Female: 0.4 – 1.53 Male: 0.45 – 1.81
Lewisham and Greenwich NHS Trust	0.5 – 1.5
Derby Teaching Hospitals NHS Foundation Trust	0.5 – 2.0
Hampshire Hospitals NHS Foundation Trust	0.28 – 2.20
Dartford and Gravesham NHS Trust	<1.7
Portsmouth Hospitals NHS Trust	<2.3
Mid Cheshire Hospitals NHS Foundation Trust	0.4 – 1.7
Wirral University Teaching Hospital NHS Foundation Trust	0.8 – 1.8
Salford Royal NHS Foundation Trust & Wigan and Leigh NHS Foundation Trust	<1.7
Whittington Health NHS Trust	0.0 – 2.2
NHS Borders	<1.7
NHS Greater Glasgow and Clyde	<2.3
York Teaching Hospital NHS Foundation Trust	<1.7
Royal Liverpool and Broadgreen University Hospitals NHS Trust	<2.3
Worcestershire Acute Hospitals NHS Trust	0.0 – 1.8
Surrey and Sussex Healthcare NHS Trust	<2.3
Royal Berkshire NHS Foundation Trust	Female: 0.4 -1.54 Male : 0.45 -1.82

Table 5.1 continued

County Durham and Darlington NHS Foundation Trust	<2.0
Countess of Chester Hospital NHS Foundation Trust	0.6 – 1.7
NHS Fife	0.85 – 2.0
Frimley Health NHS Foundation Trust	<1.8
Royal Cornwall Hospitals NHS Trust	0.5 – 1.86
Manchester University NHS Foundation Trust	<1.7
Central Manchester University Hospitals NHS Foundation Trust	<1.7
St. Helens and Knowsley Teaching Hospitals NHS Trust	<2.0

This table illustrates the disparity between plasma triglyceride references ranges across NHS Trusts

5.1.1 Aims

- To establish the normal levels and reference ranges for different circulating lipid species in different subpopulations
- To establish the relationship between different lipid species in different populations
- To establish the relationship between different lipid species and diseased states

5.2 Methods

Refer to Chapter 2 Materials and methods. The number of studies and participants per analysis is provided in the figure legend or text. The number of participants per study is also indicated on each graph by the size of the circle symbol used (Table 4.2).

Table 5.2 Key to indicate the number of participants in each study

Plasma triglycerides	Symbol	Fatty acids
0 < n ≤ 25		0 < n ≤ 5
25 < n ≤ 50		5 < n ≤ 10
50 < n ≤ 75		10 < n ≤ 25
75 < n ≤ 100		25 < n ≤ 50
100 < n ≤ 250		50 < n ≤ 75
250 < n ≤ 500		75 < n ≤ 100
500 < n ≤ 750		100 < n ≤ 250
750 < n ≤ 1000		250 < n ≤ 500
1000 < n ≤ 2500		500 < n ≤ 750
2500 < n ≤ 5000		750 < n ≤ 1000
n > 5000		n > 1000

All data is expressed as mean ± SD.

5.3 Results

5.3.1 Average molecular weight of plasma free fatty acids

To measure the concentration of fatty acids within plasma lipid species, both mg/dL and mmol/L are equally acceptable. However, one source states mg/L as the SI unit for free fatty acids (Young, 1988), whilst another states μmol/L for plasma free fatty acids and mmol/L for total serum fatty acids (Lehmann, 1976) and another g/L (American Diabetes Association, 1991).

An average molecular weight (MW) for free fatty acids in plasma was required for conversion between these units to collate data from studies and to provide a consistent reference range to healthcare providers. A search of literature revealed that most studies do not convert units, whilst online calculators and kit inserts use the molecular weight of oleic acid as the average molecular weight for total free fatty acids (DIALAB, 2008; DiaSys Diagnostic Systems GmbH, 2017).

The literature was searched for reports with the most comprehensive profiling of plasma lipids and with a large number of participants. One study was found, and the data extracted to create a weighted average molecular weight for plasma free fatty acids (Abdelmagid*, et al.*, 2015). Results show that oleic acid is not the most abundant FFA in circulation (Figure 5.1), but rather that polyunsaturated fatty acids, 18 carbons in length with 2 double bonds (18:2) such as linoleic acid are, making up 33.07% of total plasma FFAs. The most frequent monounsaturated fatty acids are 18:1, which includes oleic acid and accounts for 21.83% of total plasma FFAs, whilst palmitic acid (16:0) is the most abundant saturated fatty acid at 23.38%. Therefore, the use of the molecular weight of oleic acid (282.47g/mol) as the average molecular weight of plasma FFA is inaccurate.

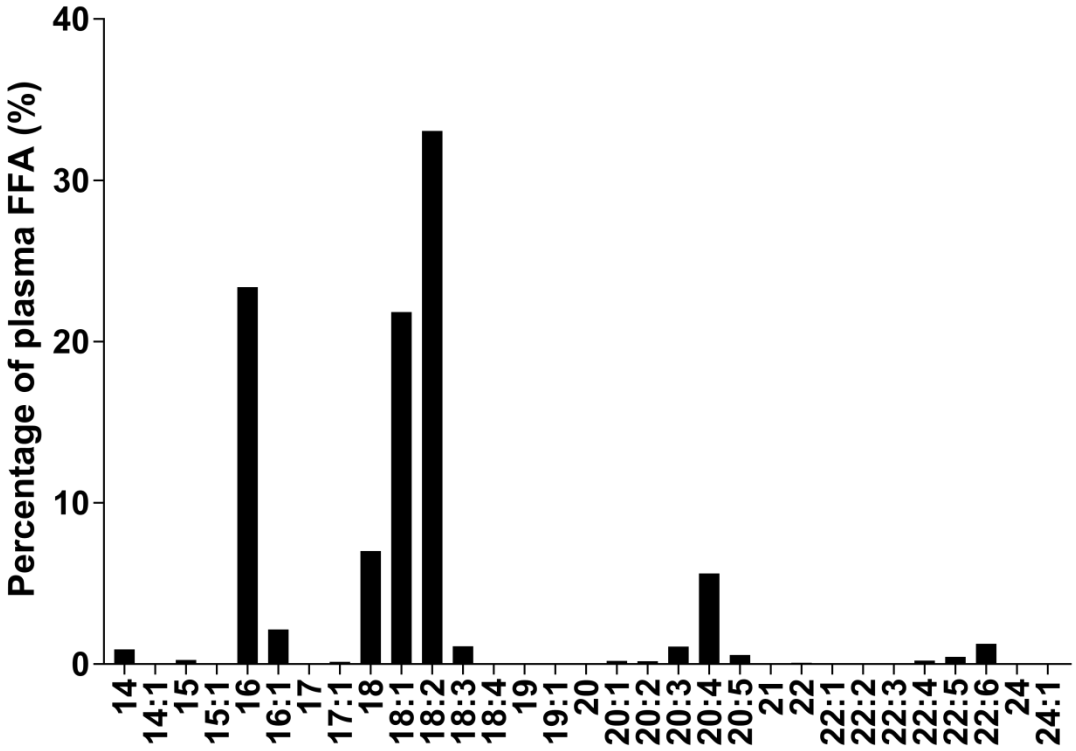


Figure 5.1 Relative percentage of each free fatty acid in plasma

Data for this graph was calculated from comprehensive plasma FFA profiling published by Abdelmagid*, et al.,* (2015). n=826

An average molecular weight for total plasma FFA was calculated by weighting the molecular weight of each individual fatty acid based on their abundance within the plasma (Table 5.3). The sum of the weighted averages was divided by 100 to compute the result.

$$\text{Average MW} = \frac{\sum \%_{14} \times MW_{14} + \%_{14:1} \times MW_{14:1} + \%_{15} \times MW_{15} + \%_{15:1} \times MW_{15:1} \ldots}{100}$$

Table 5.3 Weighted compositions (abundance x MW) of plasma FFA used in the calculation of an average FFA MW

Fatty acid (C:D)	Abundance (%)	MW (g/mol)	Weighted composition
14	0.911	228.378	2.082
14:1	0.039	226.378	0.088
15	0.255	242.405	0.618
15:1	0.001	240.405	0.003
16	23.376	256.432	59.942
16:1	2.150	254.432	5.469
17:1	0.150	268.459	0.404
18	7.015	284.486	19.957
18:1	21.828	282.486	61.660
18:2	33.072	280.486	92.762
18:3	1.116	278.486	3.109
18:4	0.003	276.486	0.008
19	0.062	298.513	0.184
19:1	0.007	296.513	0.021
20	0.070	312.540	0.219
20:1	0.205	310.540	0.636
20:2	0.188	308.540	0.579
20:3	1.088	306.540	3.334
20:4	5.632	304.540	17.152
20:5	0.578	302.540	1.747

Table 5.3 continued

21	0.021	326.567	0.070
22	0.096	340.594	0.327
22:1	0.056	338.594	0.189
22:2	0.044	336.594	0.150
22:3	0.009	334.594	0.029
22:4	0.221	332.594	0.734
22:5	0.457	330.594	1.511
22:6	1.273	328.594	4.182
24	0.020	368.648	0.074
24:1	0.057	366.648	0.210
Average MW	**277.454 g/mol**		

Carbon:double bond – ratio of number of carbons in the hydrocarbon chain to the number of double (unsaturated) bonds. Data for this table was calculated from a plasma FFA profile published by Abdelmagid*, et al.,* (2015). n=826

The average molecular weight of plasma FFA was found to be 277.454 g/mol. This new value was 1.79% different from the molecular weight of oleic acid, which in turn can create disparities of up to ~20 µmol/L in the conversion of FFA from mg/dL to µmol/L.

5.3.2 Average plasma triglyceride levels

This study collated 139 sets of data from 87 studies with n=52,128 (Appendix F). All data was converted to mmol/L. All BMIs were included, but diseased states were excluded. Although not perfect with the risk of large variance, this was assessed to be the best way to capture data representative of the general population.

The results (Figure 5.2) showed a vast range of average plasma triglyceride concentrations; the lowest reported value was 0.7 mmol/L (Jocken*, et al.*, 2008) whilst the highest was 5-fold greater at 3.5 mmol/L (Stojiljkovic*, et al.*, 2002). The average plasma triglyceride concentration was 1.424 ± 0.476 mmol/L, which was significantly different to the weighted average based on the number of participants per study (1.578 ± 0.409 mmol/L) (p=0.0002).

The Gaussian qualities of the data were examined to assess whether it could be representative of the general population for the calculation of reference ranges. Analysis was achieved by fitting a non-linear regression line of best fit with confidence intervals onto a histogram of the data (Figure 5.3A). The adjusted R^2=0.8305 indicated a relatively good fit for the bell curve. However, visual inspection of the line of best fit found that it could not predict low concentrations of plasma triglycerides. Furthermore, two intervals of the histogram were outliers, 0.6 mmol/L and 1.8 mmol/L. Analysis of the residuals initially showed a random spread, which indicated a good fit of the non-linear regression model (Figure 5.3B). However, as values of plasma triglyceride concentrations increased, the residuals became more regular and formed a sigmoidal curve, indicating flaws in the fit of the bell curve. In all, the data was perfectly normally distributed, which prevented the modelling of a high quality bell curve, but was adequate for the calculation of references ranges.

A standard 95% reference range was calculated using the equation in section 5.2.8. The reference range included values between the 2.5 percentile and the 97.5 percentile, leaving 5% of ‘normal’ results outside of the range. The reference range for plasma triglycerides was 0.47 - 2.38 mmol/L.

This systematic review uncovered a wide range of reported average plasma triglyceride concentrations, which may be explained by natural variation, differences in methodology or due to influencing factors such as BMI. The correlation between plasma triglycerides and BMI was analysed and a moderate, but statistically significant positive correlation was found ($r=0.5608$, $P<0.0001$) (Figure 5.28). Differences in plasma triglyceride concentrations between different subpopulations such as non-obese vs. obese vs. type II diabetic and men vs. women were studied later in this chapter (Section 5.3.15 and 5.3.16, respectively). Furthermore, a reference range was calculated that could be used to better inform NHS trusts, as many use ranges that are significantly different (Table 5.3).

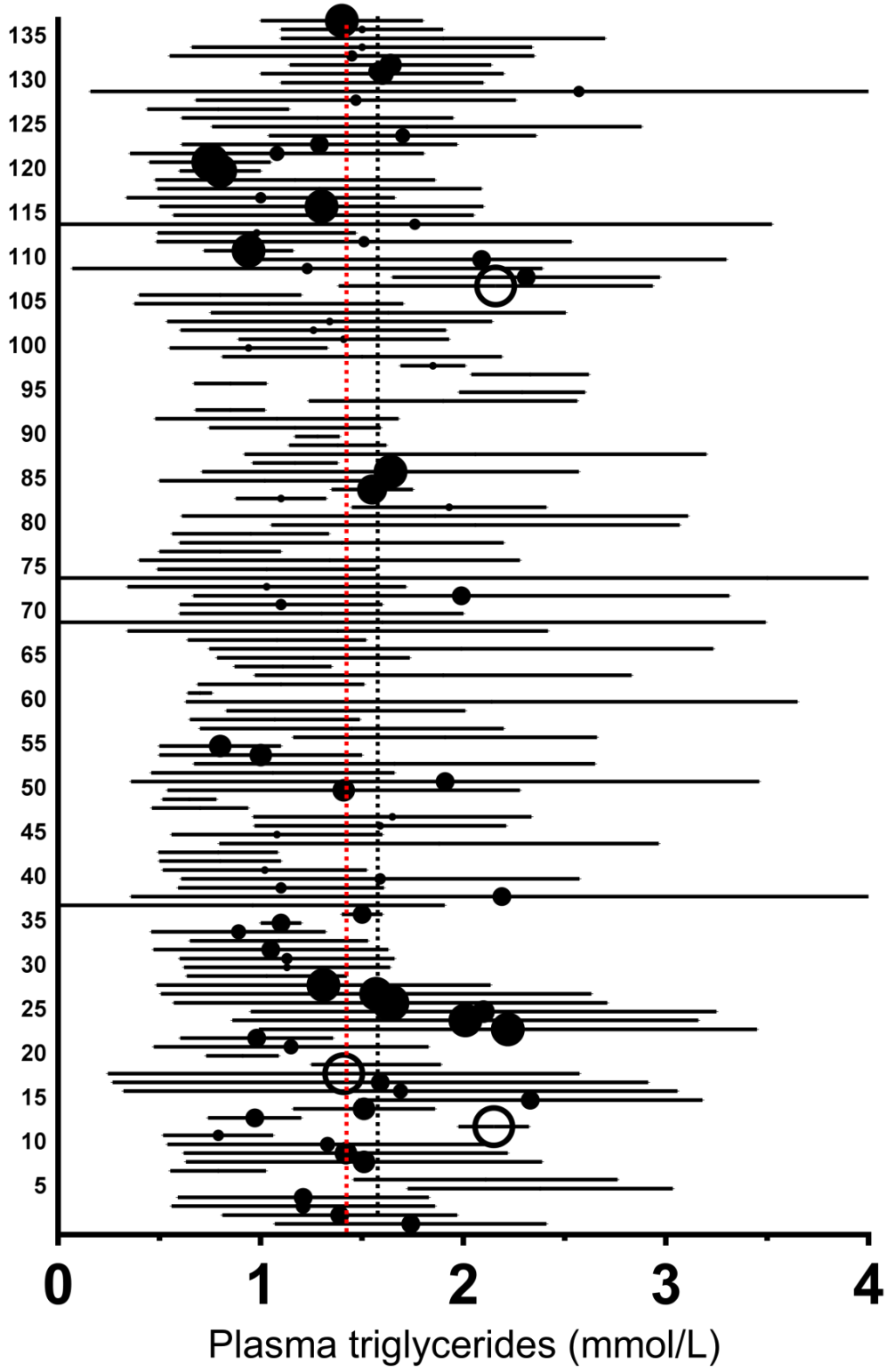


Figure 5.2 Average plasma triglyceride levels from systematic review

Black circles indicate mean plasma triglycerides published by the study. The size of the circle is representative of the number of participants in the study (Table 5.2). Black dots mark the average of the datasets. Red dots mark the weighted average of the dataset. A table of the data can be found in Appendix F. n=52,128

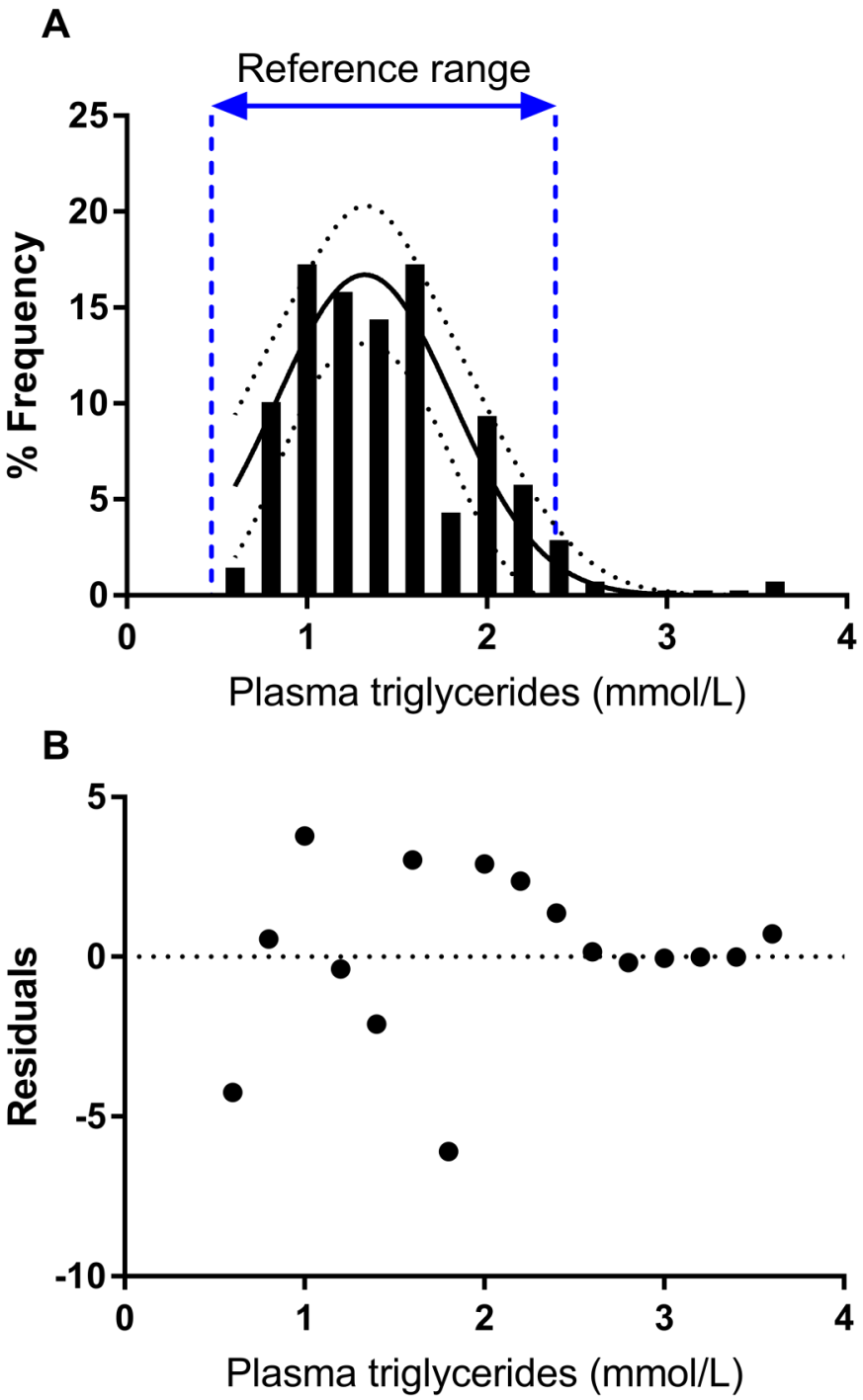


Figure 5.3 Plasma triglyceride frequency histogram with Gaussian curve shows passable normal distribution

A) % frequency histogram showing the distribution of data amongst brackets of 0.2 mmol/L. The Gaussian curve was fitted to test normality. Confidence intervals show where the Gaussian curve falls with a 95% certainty. The blue dashed lines indicate the calculated reference range. B) Residuals plot was used to assess the fit of the Gaussian distribution curve. n=52,128

5.3.3 Average plasma free fatty acid levels

This study analysed 125 sets of data across 71 studies with n=8847 (Appendix F). All BMIs were included, but diseased states were excluded. The results, illustrated in Figure 5.4, show a wide range of average plasma FFA concentrations, with the highest value at 990 µmol/L (Xiao*, et al.*, 2006), over 4-fold more than the lowest average value of 223 µmol/L (Sundell*, et al.*, 2003). The unweighted average plasma FFA concentration was found to be 562.1 ± 172.881 µmol/L, whilst the calculated weighted average was significantly lower, at 397.9 ± 134.7µmol/L ($p<0.0001$).

The adjusted R^2 value was examined to assess the fit of the non-linear regression model and found a relatively good fit (adjusted $R^2=0.8234$), with no outliers (Figure 5.5A). However, visual inspection showed that the model was not adequate at predicting low concentrations of plasma free fatty acids. Analysis of residuals found a random spread, which further indicated a normal distribution (Figure 5.5B). Therefore, it was concluded that this dataset could be used to calculate reference ranges, although there was room for improvement.

A 95% reference range was computed using the equation in section 5.2.8. The reference range included values between the 2.5 and the 97.5 percentile, leaving 2.5% of 'normal' results above this range and 2.5% below this range. The reference range for plasma free fatty acids was 216.3 - 907.9 µmol/L.

This systematic review unearthed a range of average plasma free fatty acid concentrations, which could be accounted for by differences in methodology used in each study, natural variation and/or by influencing factors such as BMI. Unfortunately, the correlation between FFA and BMI could not be computed in this project as BMI was a controlled variable in this study. However, further analyses of plasma FFA concentrations between different subpopulations were studied later in this chapter (Section 5.3.15 and 5.3.16, respectively). Furthermore, a relatively robust reference range was calculated for plasma FFAs that could

be used in a clinical setting to identify those at risk of obesity-related sequelae. Additionally, it could be used as a foundation upon which to base research in this field.

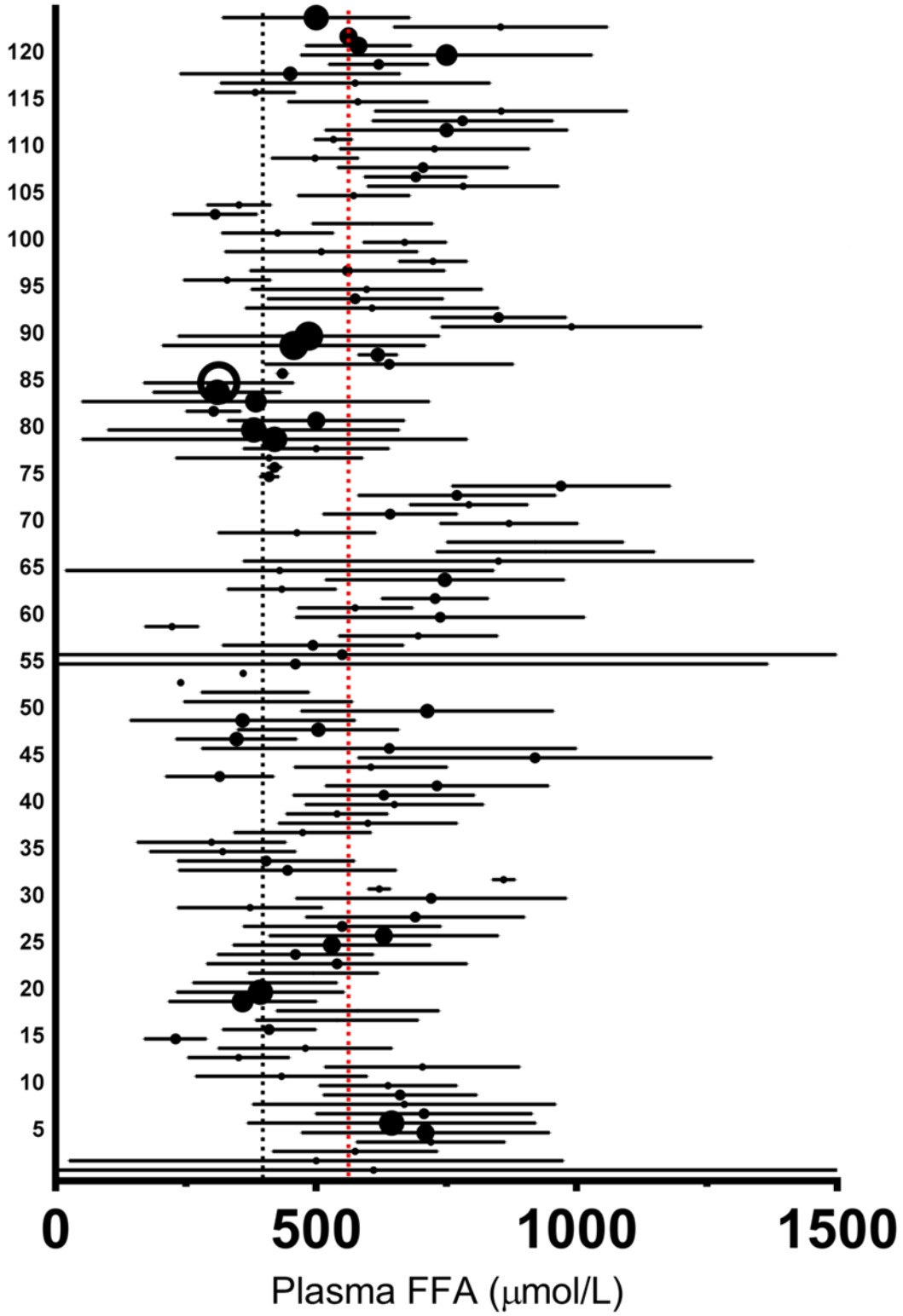


Figure 5.4 Average plasma FFA from systematic literature review

Black circles indicate mean plasma triglycerides published by the study. The size of the circle is representative of the number of participants in the study (Table 5.2). Black dots mark the average of the datasets. Red dots mark the weighted average of the dataset. A table of the data can be found in Appendix F. n=8847

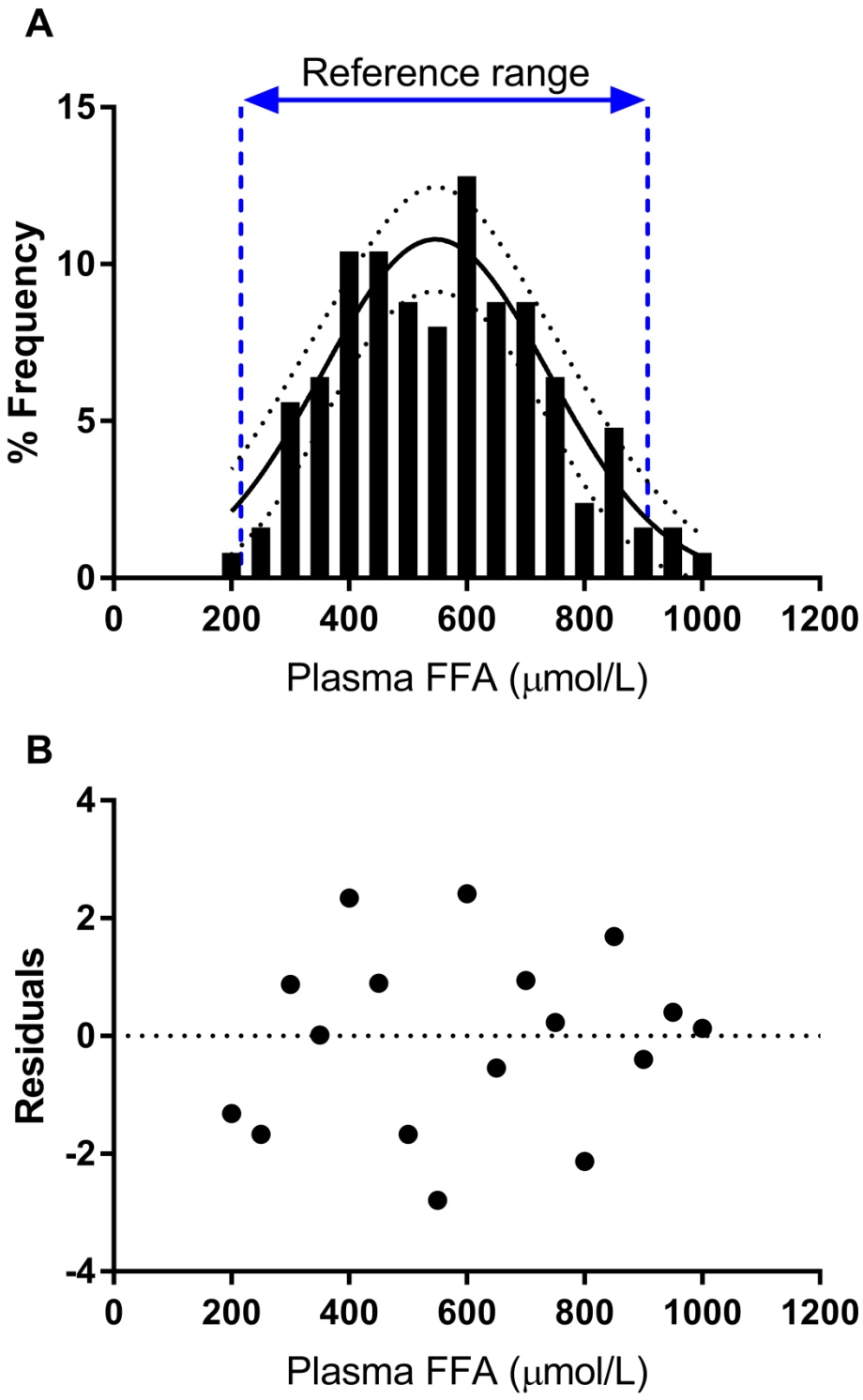


Figure 5.5 Plasma FFA frequency histogram with Gaussian curve reveals normal distribution

A) % frequency histogram showing the distribution of data amongst brackets of 50 µmol/L. The Gaussian curve was fitted to test normality. Confidence intervals show where the Gaussian curve falls with a 95% certainty. The blue lines indicate the computed reference range. B) Residuals plot was used to assess the fit of the Gaussian distribution curve. n=8847

5.3.4 Plasma phospholipid composition – molar percentage of palmitic and oleic acids

The fatty acid fractions of plasma phospholipids are the most commonly analysed lipid species in the literature. Therefore, one would assume a consensus amongst studies as to average concentrations found in the circulation. Changes to plasma phospholipid composition, especially increases in palmitic acid incorporation, have been associated with a number of diseases including prostate cancer (Crowe*, et al.*, 2008) and coronary heart disease (Simon*, et al.*, 1996).

Molar percentage is the most common measurement of fatty acid composition of phospholipids (mol % -percentage of total moles of a particular component), which is more susceptible to error than absolute concentration measurements as it is related to the other fractions (Schwertner & Mosser, 1993). Therefore, it is important to collate multiple sources of data to calculate averages in the general population.

This study examined 74 datasets over 31 studies with n=41,288 for palmitic acid and 73 datasets across 30 studies with n=26,124 for oleic acid (Appendix F).

Results in Figure 5.6A showed the spread of data for palmitic acid with a range of 15 mol%. However, the average and weighted average of palmitic acid in plasma phospholipids were remarkably similar at 27.24 ± 3.51 mol% and 27.62 ± 2.62 mol%, respectively (p=0.3603). The fit of the Gaussian distribution curve to the palmitic acid in phospholipids (mol%) data was not ideal (adjusted R^2 =0.7603) (Figure 5.7A). Furthermore, examination of the residuals showed that predictions calculated by the model were consistently too low (Figure 5.7B). Therefore, distribution of the data was not deemed adequately normal for the computation of reference ranges.

The range of oleic acid data (Figure 5.6B) was just less than 11 mol%. However, the average and weighted average were significantly different, measuring 10.56 ± 1.94 mol% and 11.20± 2.03 mol%, respectively (p<0.0001). The fit of the Gaussian distribution curve to

the oleic acid in phospholipids (mol%) data was good, even with an outlier in the 10 mol% bracket (adjusted R^2 =0.8715) (Figure 5.7C). Furthermore, analysis of the residuals plot showed a random spread, which suggested a good fit of the bell curve (Figure 5.7D). In all, this dataset was adequately Gaussian to enable the calculation of reference ranges, however, due to small underestimations of the model more of the population fell outside the upper limit of the reference range than the lower limit.

An equation in section 5.2.8 was used to calculate the 95% reference range for oleic acid in phospholipids (mol%). The reference range included values between the 2.5 and the 97.5 percentile, leaving 5% of 'normal' results outside this range. The reference range was 6.68 – 14.44 mol%.

This systematic review highlighted that molar percentage is the most common measure of palmitic acid and oleic acid in plasma phospholipids. A reference range was calculated for oleic acid in phospholipids (mol%), but not palmitic acid. The use of such reference ranges could be important in the measurement of risk factors as mol% of fatty acids in phospholipids have been linked to prostate cancer risk with a high mol% of palmitic acid in phospholipids being positively associated and oleic acid being inversely associated (Bassett*, et al.*, 2013).

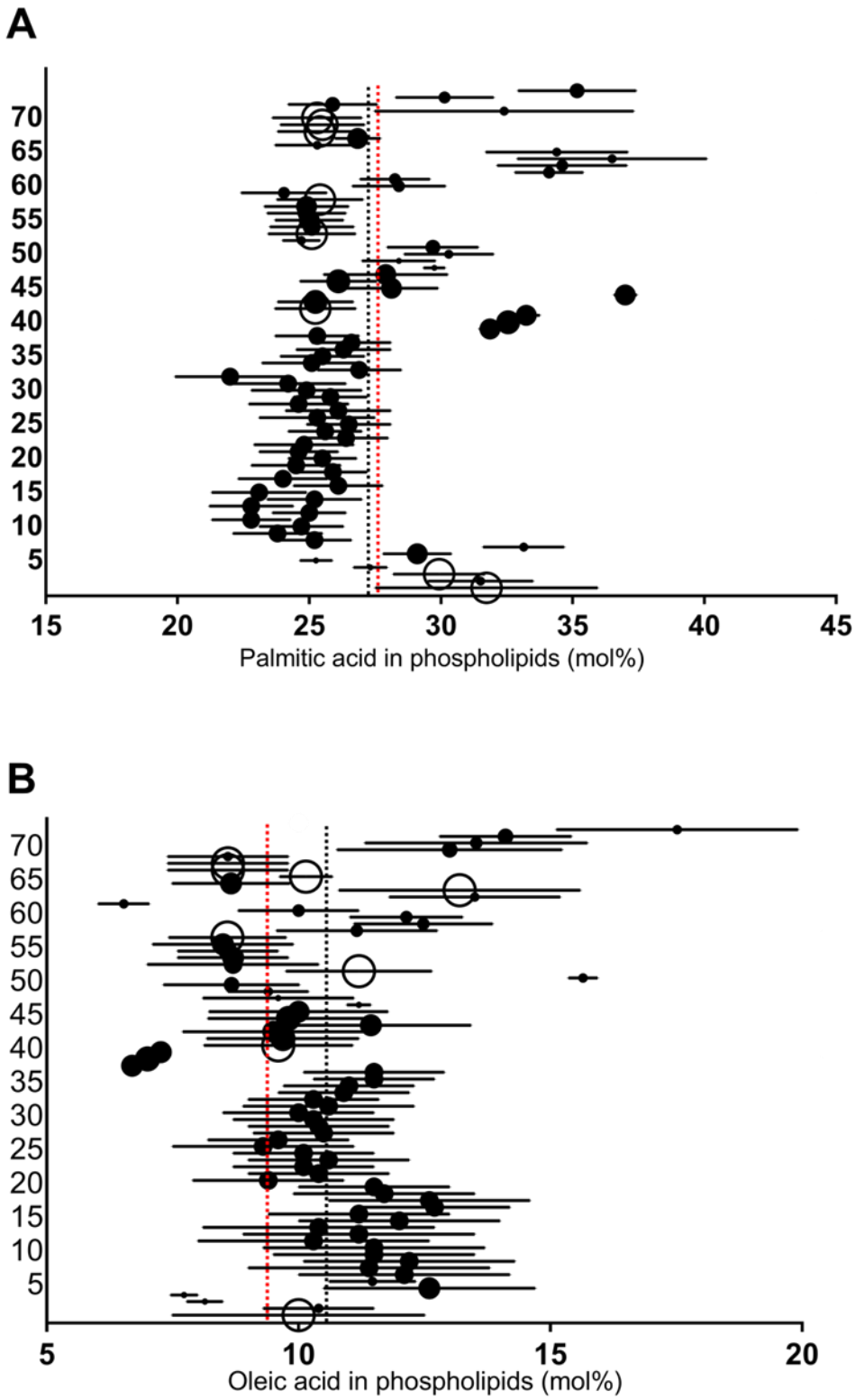


Figure 5.6 Average molar percentage of palmitic and oleic acid in phospholipids

Black circles indicate mean plasma triglycerides published by the study. The size of the circle is representative of the number of participants in the study (Table 5.2). Black dots mark the average of the datasets. Red dots mark the weighted average of the dataset. A table of the data can be found in Appendix F. A) n=41,288 B) n=26,124

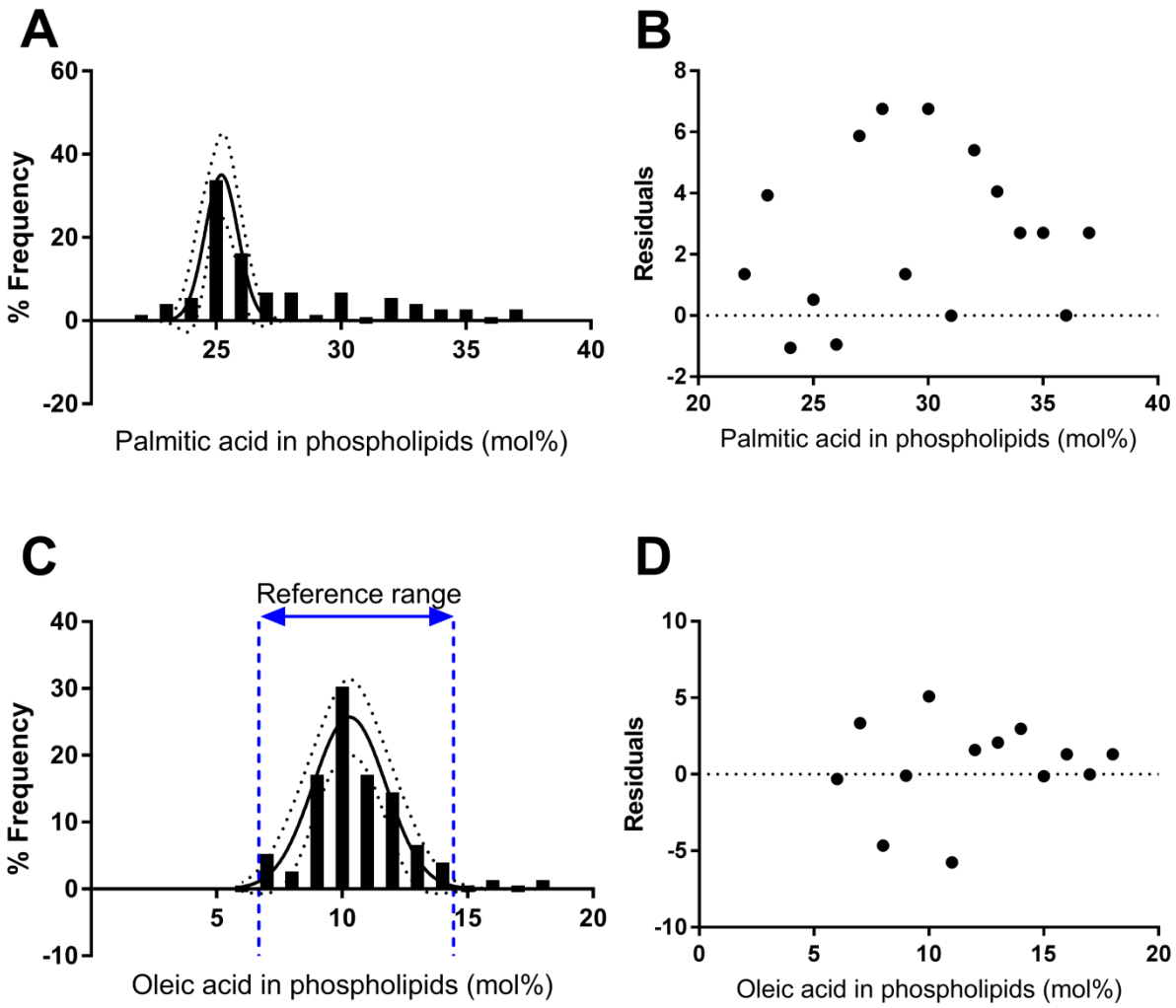


Figure 5.7 Frequency histogram with Gaussian curve established normality of oleic acid in phospholipids, but not palmitic acid in phospholipids (mol%)

A, C) % frequency histogram showing the distribution of data amongst brackets of 1 mol%. The Gaussian curve was fitted to test normality. Confidence intervals show where the Gaussian curve falls with a 95% certainty. The blue dashed lines indicate the calculated reference range. B, D) Residuals plot was used to assess the fit of the Gaussian distribution curve. A, B) n=41,288 C, D) n=26,124

5.3.5 Palmitic acid in phospholipids

This study examined 5 datasets over 4 studies with n=316 for mass%, 9 sets of data across 8 studies with n=901 for mg/dL and 3 datasets in 3 studies with n=8,480 for mmol/L (Appendix F).

The mass% results found a small range of 4.59 % across data points with similar average and weighted average of 27.94 ± 2.20 mass% and 26.49 ± 1.90 mass%, respectively (p=0.2147) (Figure 5.8A). A Gaussian distribution curve could not be fitted to the palmitic acid in phospholipids (mass%) data, and hence reference ranges could not be calculated (Figure 5.9A and 5.9B). The data was not convergent, probably due to the small sample size.

The spread of data was very large for mg/dL of palmitic acid in phospholipids with a range of 126.21 mg/dL. This caused a significant disparity between the average and weight average, 47.08 ± 39.46 mg/dL and 36.40 ± 14.46 mg/dL, respectively (p<0.0001) (Figure 5.8B). The Gaussian distribution curve for the palmitic acid in phospholipid (mg/dL) data produced a high adjusted R^2 value (0.9169) (Figure 5.9C). However, two of the histogram intervals were outliers, 80 mg/dL and 140 mg/dL, and visual inspection of the curve showed that it did not adequately fit the datat. Examination of the residuals revealed a lack of random scatter around zero, which further indicated the inadequacy of the model, and hence the inability to use this dataset for the computation of reference ranges (Figure 5.9D).

The spread of data was also large for mmol/L with a range of 0.83 mmol/L (Figure 5.8C). The difference between the average and weighted average was 50%, 0.88 ± 0.42 mmol/L and 1.22 ± 0.13 mmol/L, respectively. However, this difference was not significant (p=0.3010). The Gaussian curve did not fit the palmitic acid in phospholipids (mmol/L) data whatsoever (adjusted R^2 =-0.1111) (Figure 5.9E). The inadequacy of the model was further shown by the lack of random scatter of residuals, and hence the data could not be used to calculate reference ranges (Figure 5.9F).

In summary, few studies analysed the mass%, mg/dL or mmol/L of palmitic acid in plasma phospholipids, even though links have been found with diseased states such as prostate cancer (Bassett*, et al.*, 2013; Crowe*, et al.*, 2008) and metabolic dysfunction (Lemaitre*, et al.*, 2015). This small volume of data prevented in-depth analysis and computation of reference ranges, but did highlight gaps in the literature. The mg/dL and mmol/L data was not interconverted and presented together as this approach had the potential to introduce error into some of the values. However, the average and weighted average of the mg/dL data was converted to mmol/L in order run statistical analysis. The converted average of 1.84 ± 1.54 was significantly higher than the average of the mmol/L data at 0.38 ± 0.42 (P=0.0144). On the other hand, the converted weighted average of 1.42 ± 0.61 was not significantly different to the unconverted weighted average of 1.22 ± 0.13 (P=0.3504).

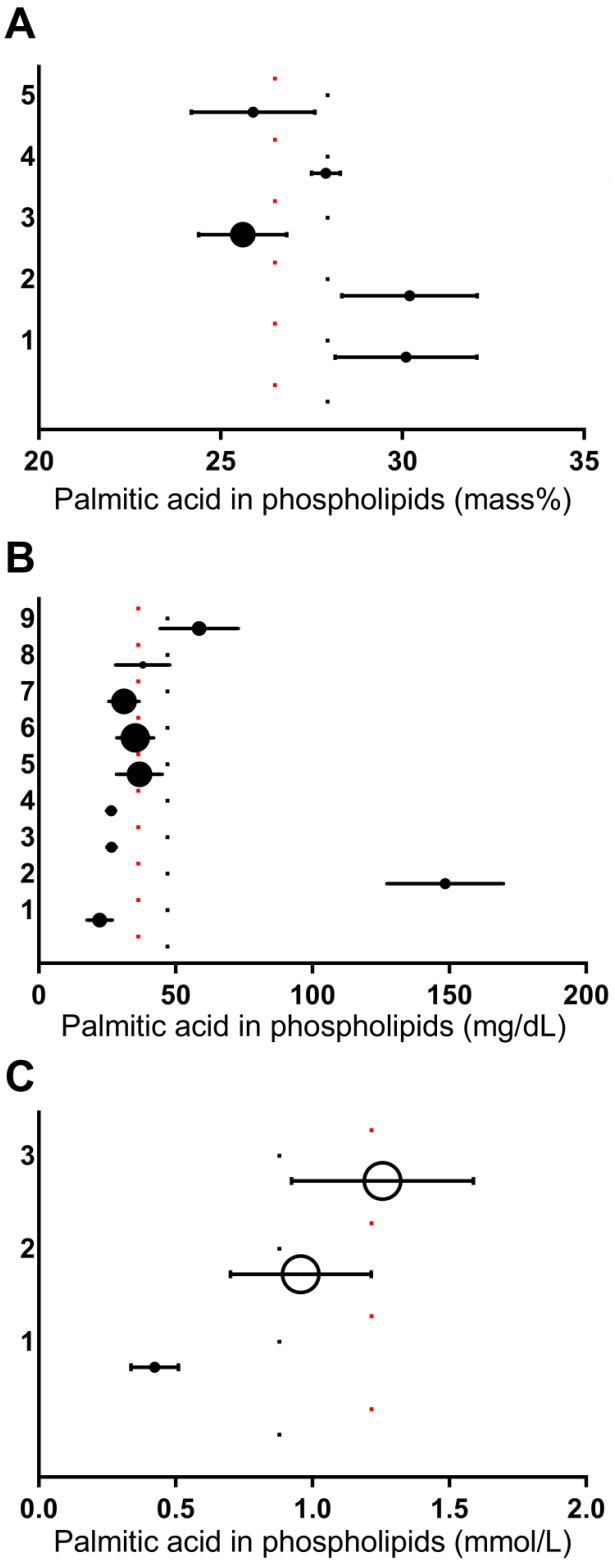


Figure 5.8 Palmitic acid in phospholipids in literature

Black circles indicate mean plasma triglycerides published by the study. The size of the circle is representative of the number of participants in the study (Table 5.2). Black dots mark the average of the datasets. Red dots mark the weighted average of the dataset. A table of the data can be found in Appendix F. A) n=316 B) n=901 C) n=8,480

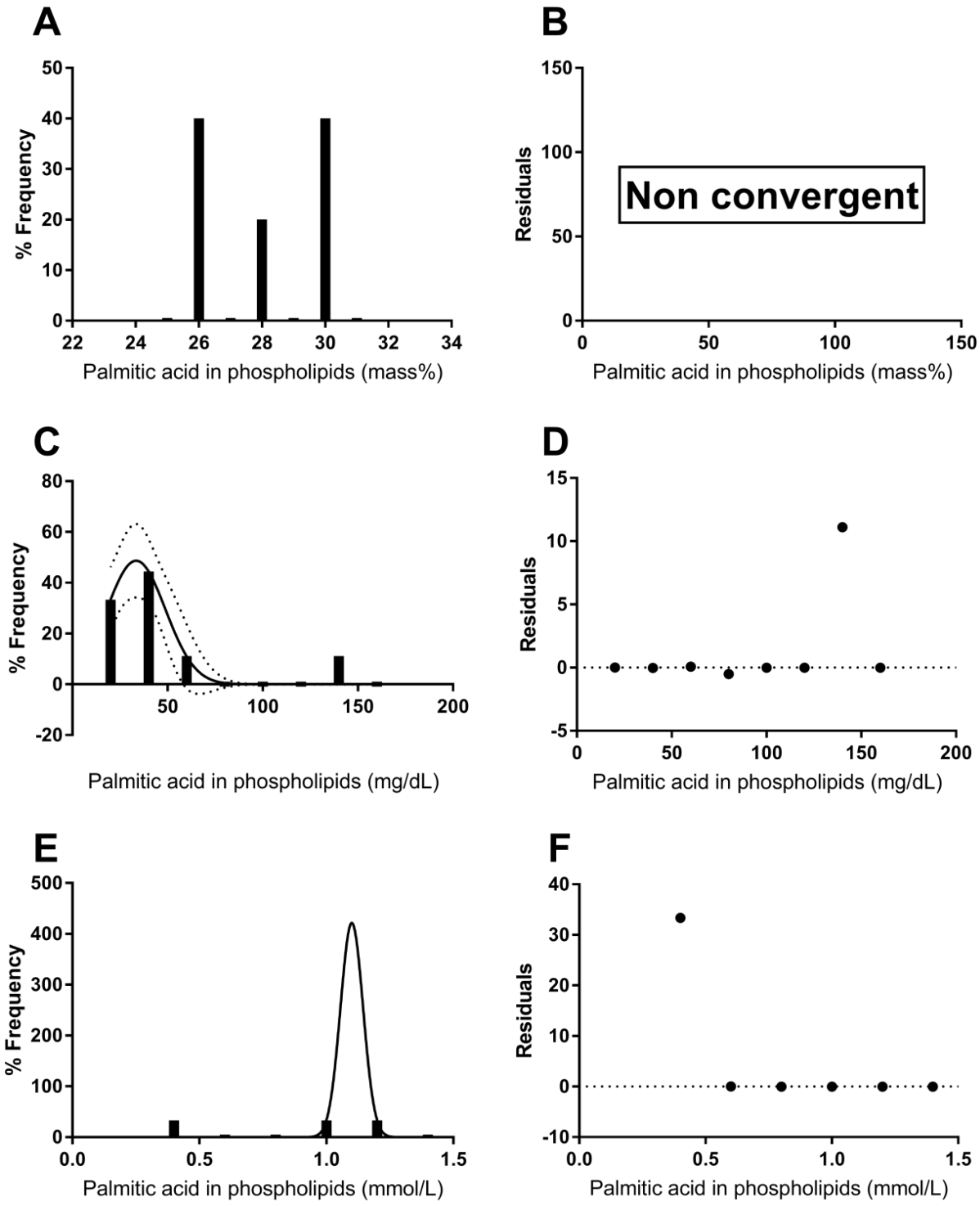


Figure 5.9 Frequency histograms with Gaussian curves demonstrated the lack of normality of palmitic acid in phospholipids (mass%, mg/dL, mmol/L)

A, C, E) % frequency histogram showing the distribution of data amongst brackets of 1 mass%, 20 mg/dL and 0.2 mmol/L. The Gaussian curve was fitted to test normality. Confidence intervals show where the Gaussian curve falls with a 95% certainty. B, D, F) Residuals plot was used to assess the fit of the Gaussian distribution curve.
A, B) n=316 C, D) n=901 E, F) n=8,480

5.3.6 Oleic acids in phospholipids

This study analysed 5 sets of data across 4 studies with n=316 for mass%, 9 datasets in 8 studies with n=901 for mg/dL and 3 datasets across 3 studies with n=8,480 for mmol/L (Appendix F).

The results showed a range of 4.1 % for mass% data with a non-significant difference between the average and weighted average, 11.06 ± 1.80 mass% and 9.76 ± 1.19 mass%, respectively (p=0.1816) (Figure 5.10A). The Gaussian curve fitted to the oleic acid in phospholipids (mass%) data did not fit at all (adjusted R^2=-0.7241), which was further illustrated by the lack of random scatter of the residuals (Figure 5.11A and 5.11B). Therefore, this dataset could not be used to compute reference ranges.

The spread of data for mg/dL was small except for one data point that was over 5.5-fold greater than the weighted average (Schwertner*, et al.*, 1993); including this point the range was 66.09 mg/dL and excluding this point the range was 17.39 mg/dL (Figure 5.10B). This data point in question was produced by TLC, an outdated technique, for the measurement of the lipid species, which may explain the anomalous result. The average and weighted average were 20.50 ± 21.53 mg/dL and 13.79 ± 8.59 mg/dL respectively, which were not significantly different (p=0.3769). The normal distribution curve fitted to the oleic acid in phospholipids data (mg/dL) had a high adjusted R^2 value (0.9315), which indicated a good fit of the model (Figure 5.11C). However, visual examination of the curve and residuals plot found that the curve did not fit the data and two outliers were present at 30 mg/dL and 80 mg/dL intervals (Figure 5.11D). Therefore, references ranges could not be calculated from this dataset.

The range of data for mmol/L of oleic acid in plasma phospholipids was 0.21 mmol/L (Figure 5.10C). The average and weighted average were 0.39 ± 0.11 mmol/L and 0.47 ±0.02 mmol/L, respectively, and were not significantly different (p=0.3495). The adjusted R^2 value for the bell curve fitted to the oleic acid in phospholipids (mmol/L) data was low, which suggested a poor fit of the model (0.5238) (Figure 5.11E). Examination of the histogram

and lack of random spread of residuals further indicated the inadequacy of the model, and hence, this dataset could not be used to calculate reference ranges (Figure 5.11F).

In summary, the study of mass%, mg/dL or mmol/L of oleic acid in plasma phospholipids was not particularly common, in spite of research inversely associating it with the risk of developing prostate cancer (Bassett*, et al.*, 2013; Crowe*, et al.*, 2008). The lack of data did not enable the calculation of reference ranges or detailed statistical analysis, but did illustrate the need for further research in this field. The conversion of data points from mg/dL to mmol/L was not performed as this would have introduced undue error. However, the average and weighted average of the mg/dL data was converted to mmol/L to compare them statistically. The converted average of 0.73 ± 0.76 was not statistically different to the unconverted average of 0.39 ± 0.11 (P=0.2027). Additionally, the converted weighted average of 0.49 ± 0.30 was similar to the unconverted weighted average of 0.47 ± 0.02 (P=0.8443).

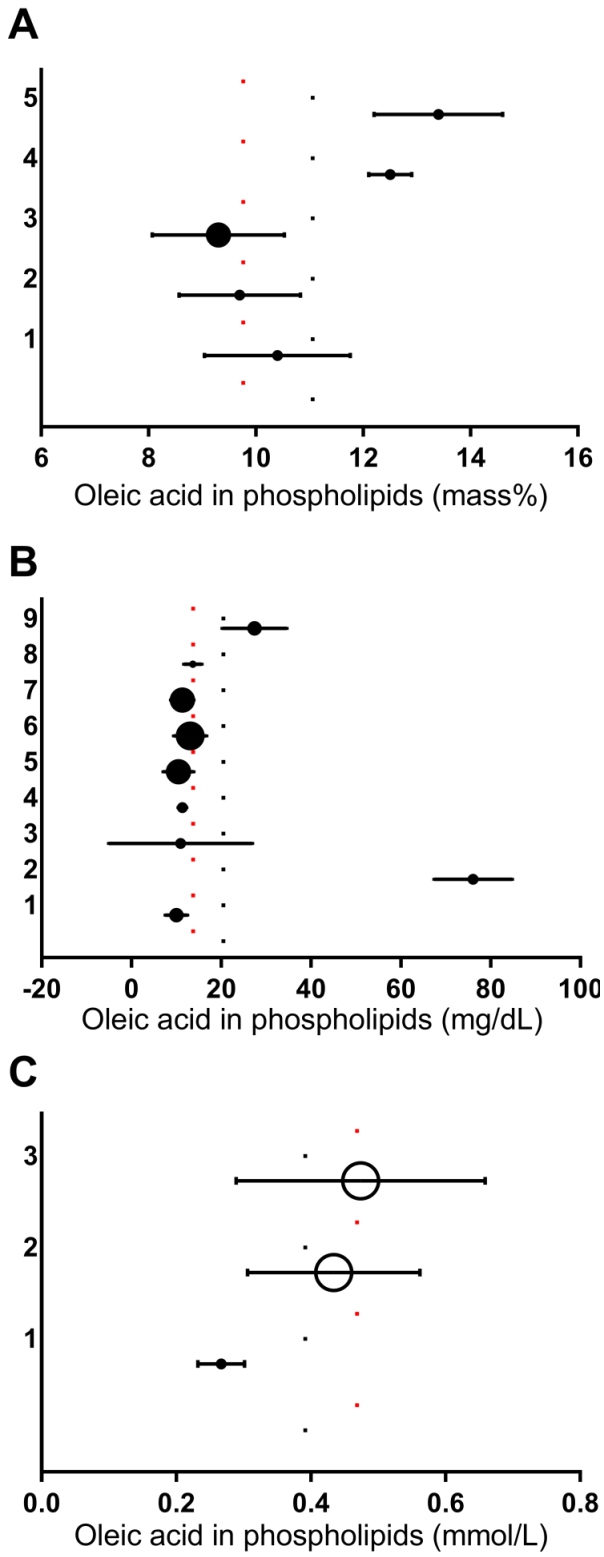


Figure 5.10 Oleic acid in phospholipids from systematic review

Black circles indicate mean plasma triglycerides published by the study. The size of the circle is representative of the number of participants in the study (Table 5.2). Black dots mark the average of the datasets. Red dots mark the weighted average of the dataset. A table of the data can be found in Appendix F.A) n=316 B) n=901 C) n=8,480

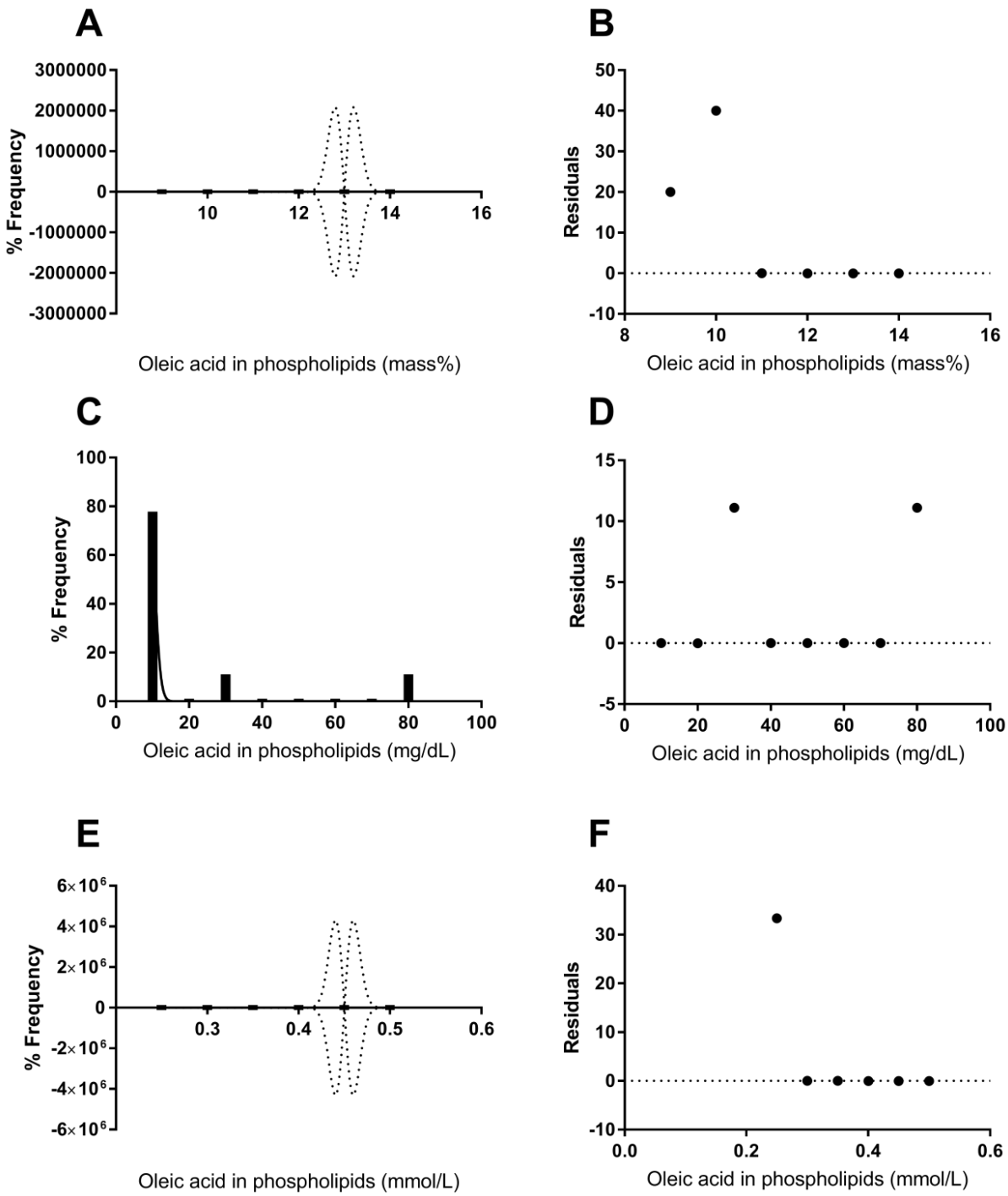


Figure 5.11 Frequency histograms with Gaussian curves demonstrated the lack of normality of oleic acid in phospholipids (mass%, mg/dL, mmol/L)

A, C, E) % frequency histogram showing the distribution of data amongst brackets of 1 mass%, 10 mg/dL and 0.05 mmol/L. The Gaussian curve was fitted to test normality. Confidence intervals show where the Gaussian curve falls with a 95% certainty. B, D, F) Residuals plot was used to assess the fit of the Gaussian distribution curve. A, B) n=316 C, D) n=901 E, F) n=8,480

5.3.7 Palmitic acid in cholesterol esters

Cholesterol esters and their fractions are the second most frequently investigated lipid species in the literature. Long chain cholesterol esters have been linked to Alzheimer's disease (Proitsi*, et al.*, 2015), whilst increased esterification rates predict coronary heart disease (Tanaka*, et al.*, 2013). Furthermore, the fatty acid composition of cholesterol esters has been associated with visceral and subcutaneous fat deposition, which in turn is a predictor of cardiometabolic disease (Rosqvist*, et al.*, 2017).

This study collated 29 datasets from 20 studies with n=20,294 for molar percentage, 5 datasets across 4 studies with n=185 for mg/dL and 5 sets of data from 3 studies with n=146 for mmol/L (Appendix F).

The mol% data produced a range of 5.73 % with an average and weighted average of 11.63 ± 1.30 mol% and 10.92 ± 0.97 mol%, respectively. This small difference between averages was statistically significant (p=0.0068) (Figure 5.12A). The fit of the Gaussian distribution curve to the palmitic acid in cholesterol esters (mol%) data was assessed to be poor due to a low adjusted R^2 value (0.4401) (Figure 5.13A). Further examination found one outlier at the 10 mol% interval, but a good random spread of residuals (Figure 5.13B). Once the results were amalgamated, the model was considered inadequate for the calculation of reference ranges.

The range for mg/dL was 3.37 mg/dL when excluding a data point that was over 4.4-fold greater than the weighted average (Schwertner*, et al.*, 1993) (Figure 5.12B). This outlier was likely due to the TLC methodology utilised in the study. Including this point the range was 52.44 mg/dL. The average and weighted average were not significantly different, measuring 20.28 ± 22.42 mg/dL and 13.82 ± 13.75 mg/dL, respectively (p=0.5547) (Figure 7B). The adjusted R^2 value (0.8991) for the bell curve fitted to the palmitic acid in cholesterol esters (mg/dL) data was high and no outliers were found, which indicated a good fit of the model (Figure 5.13C). However, visual examination found that the curve did

not fit the wide spread of data, and the residuals were not randomly spread (Figure 5.13D). Thus, the dataset was deemed inadequate for the creation of reference ranges.

The range of data for mmol/L was large, spanning 11.46 mmol/L (Figure 5.12C). Unfortunately, the two very small values were from the same paper, whilst the two large values were also from the same paper. The average and weighted average were 4.42 ± 6.00 mmol/L and 2.57 ± 5.20 mmol/L, respectively. The difference between the two averages was not statistically significant (p=0.5290). The normal distribution curve to the palmitic acid in cholesterol esters (mmol/L) data produced a moderately low adjusted R^2 value (0.5962) (Figure 5.13E). Visual analysis of the histogram revealed that the model did not fit the data and the residuals plot showed a lack of random spread (Figure 5.13F). Therefore, the dataset was determined to be unsuitable for the computation of reference ranges.

In summary, mol% was the most common measure of palmitic acid in plasma cholesterol esters with mg/dL and mmol/L included in only a few studies. A good volume of data existed for mol%, however, no reference ranges could be calculated as none of the datasets were adequately normally distributed. Furthermore, the mg/dL individual data points were not converted to mmol/L as this would have included error. However, the average and weighted average of the mg/dL data was converted to mmol/L to enable statistical analysis. The converted average of 0.79 ± 0.87 was not statistically smaller than the average of the mmol/L dataset, 4.42 ± 6.00 (P=0.2174). Additionally, the converted weighted average of 0.54 ± 0.54 was not significantly smaller than the unconverted weighted average of 2.57 ± 5.20 (P=0.4105). These statistical observations were due to the large standard deviations.

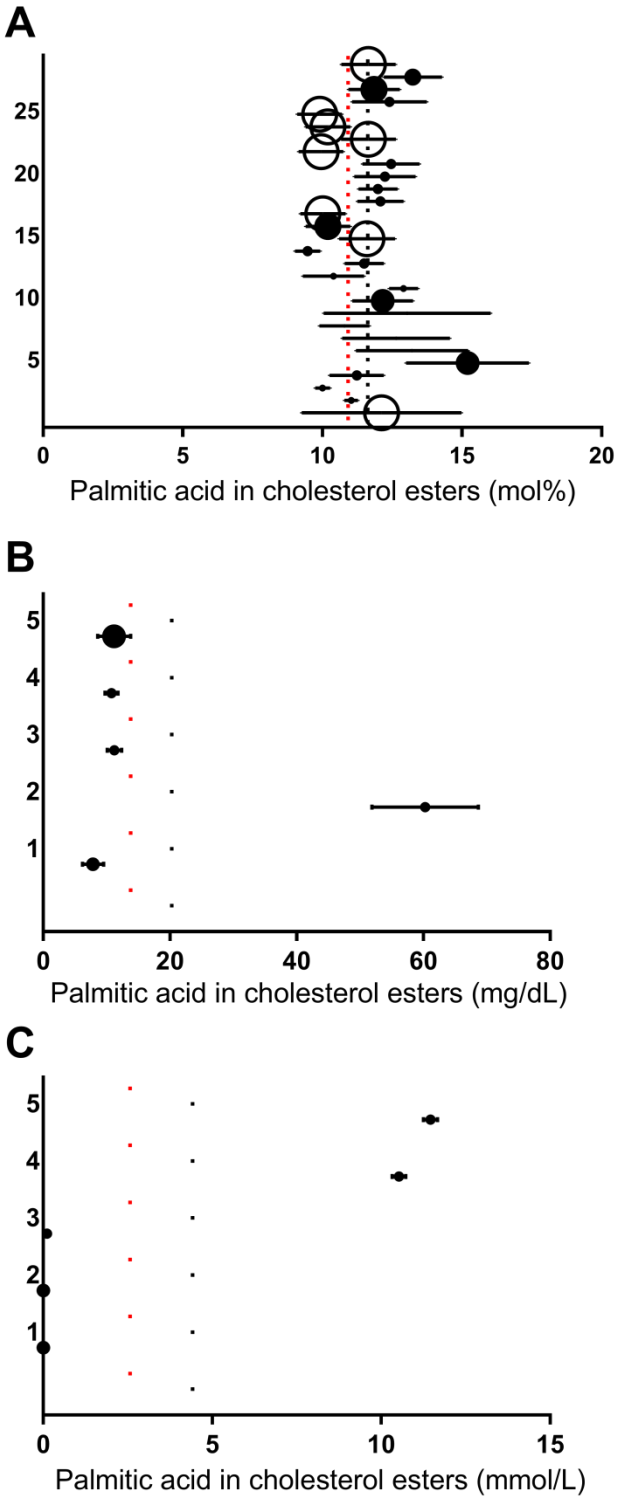


Figure 5.12 Palmitic acid in cholesterol esters in literature

Black circles indicate mean plasma triglycerides published by the study. The size of the circle is representative of the number of participants in the study (Table 5.2). Black dots mark the average of the datasets. Red dots mark the weighted average of the dataset. A table of the data can be found in Appendix F. A) n=20,294 B) n=185 C) n=146

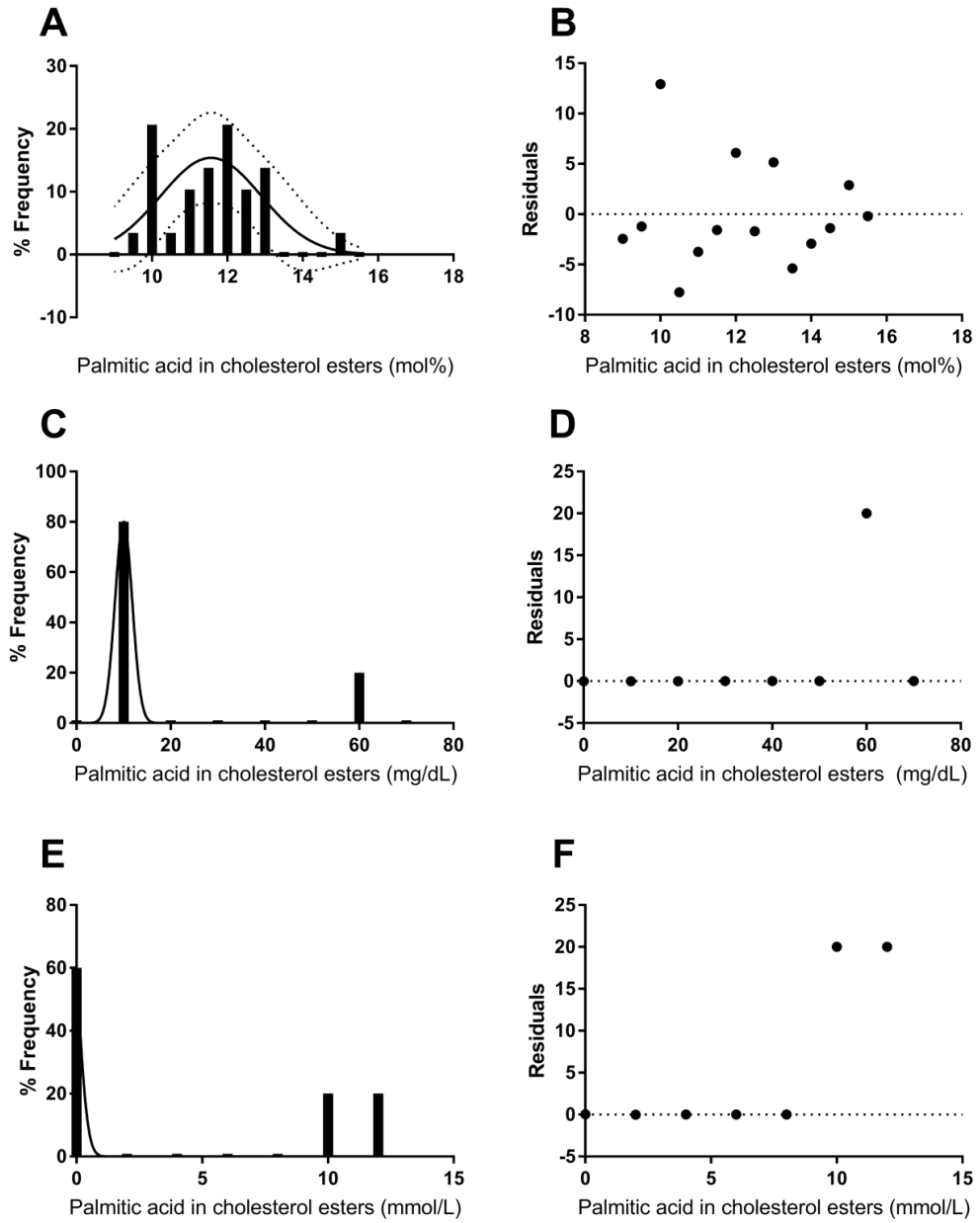


Figure 5.13 Frequency histograms with Gaussian curves demonstrated the lack of normality of palmitic acid in cholesterol esters (mol%, mg/dL, mmol/L)

A, C, E) % frequency histogram showing the distribution of data amongst bands of (0.5 mol%, 10 mg/dL and 2 mmol/L. The Gaussian curve was fitted to test normality. Confidence intervals show where the Gaussian curve falls with a 95% certainty. B, D, F) Residuals plot was used to assess the fit of the Gaussian distribution curve. A, B) n=20,294 C, D) n=185 E, F) n=146

5.3.8 Oleic acid in cholesterol esters

This investigation analysed 29 datasets from 20 studies with n=20,294 for molar percentage, 5 datasets from 4 studies with n=185 for mg/dL and 5 sets of data across 3 studies with n=146 for mmol/L (Appendix F).

The range of mol% data was large, measuring 10.77 %, and the average and weighted average were significantly different, at 18.67 ± 2.40 mol% and 17.26 ± 1.88 mol%, respectively (p=0.0037) (Figure 5.14A). The normal distribution curve fitted to the oleic acid in cholesterol esters (mol%) data was assessed to be poor due to a low adjusted R^2 value (0.4707) (Figure 5.15A). Further analysis found one outlier at the 16 mol% interval, and an inadequate spread of residuals around zero (Figure 5.15B). All of which indicated that the dataset was not suitable for the creation of reference ranges.

The mg/dL range of data was 3.2 mg/dL when excluding a data point that was 4.65-fold greater than the weighted average (Schwertner*, et al.*, 1993) (Figure 5.14B). This outlier may be explained by the use of TLC, an outdated technique, for the measurement of the lipid species. Including this point the range was 81.35 mg/dL. With inclusion of this anomalous data point, the difference between the average and weighted average was not statistically significant, measuring 32.19 ± 35.76 mg/dL and 20.68 ± 22.25 mg/dL, respectively (p=0.5116). The adjusted R^2 value (0.8701) for the Gaussian distribution curve fitted to the OA in cholesterol esters (mg/dL) data was moderately high with no outliers, which suggested an adequate fit of the model (Figure 5.15C). However, visual analysis of the histogram found that the bell curve was vastly inadequate. The residuals were also not spread randomly, and hence the dataset was considered inadequate for the calculation of reference ranges (Figure 5.15D).

The data for mmol/L was very widely spread with a range of 28.15mmol/L (Figure 5.14C). Even though the range of data was large the difference between the two averages was non-significant (p=0.5353). The average and weighted average were 9.89 ± 13.67 mmol/L

and 5.75 ± 11.77 mmol/L, respectively. The bell curve for the oleic acid in cholesterol esters (mmol/L) data was deemed to be inadequate due to its low adjusted R^2 value (0.5962) (Figure 5.15E). Examination of the histogram identified that the model did not fit the data and the lack of random spread of residuals further confirmed flaws in the model (Figure 5.15F). Thus, it was concluded that the dataset was unsuitable for the creation of reference ranges.

In summary, mol% was the most common measure of oleic acid in plasma cholesterol esters, whilst few studies used mg/dL and mmol/L. Despite the good volume of mol% data, no reference range could be calculated as it wasn't adequately Gaussian. Data for the other units was also inadequately normally distributed due to the smll number of studies, and, thus, no reference ranges could be calculated. Furthermore, the mg/dL data was not converted to mmol/L and presented together as this would have introduced undue error. However, the average and weighted average of the mg/dL data was converted to mmol/L to enable an overall comparison. The difference between the converted average of 1.14 ± 1.26 and unconverted average of 9.89 ± 13.67 was not statistically significant (P=0.1919). The difference between the converted weighted average of 0.73 ± 0.79 and the unconverted weighted average of 5.75 ± 11.77 (P=0.3692) was also not significant. These statistical observations were most probably due to the large standard deviations of the unconverted mmol/L data.

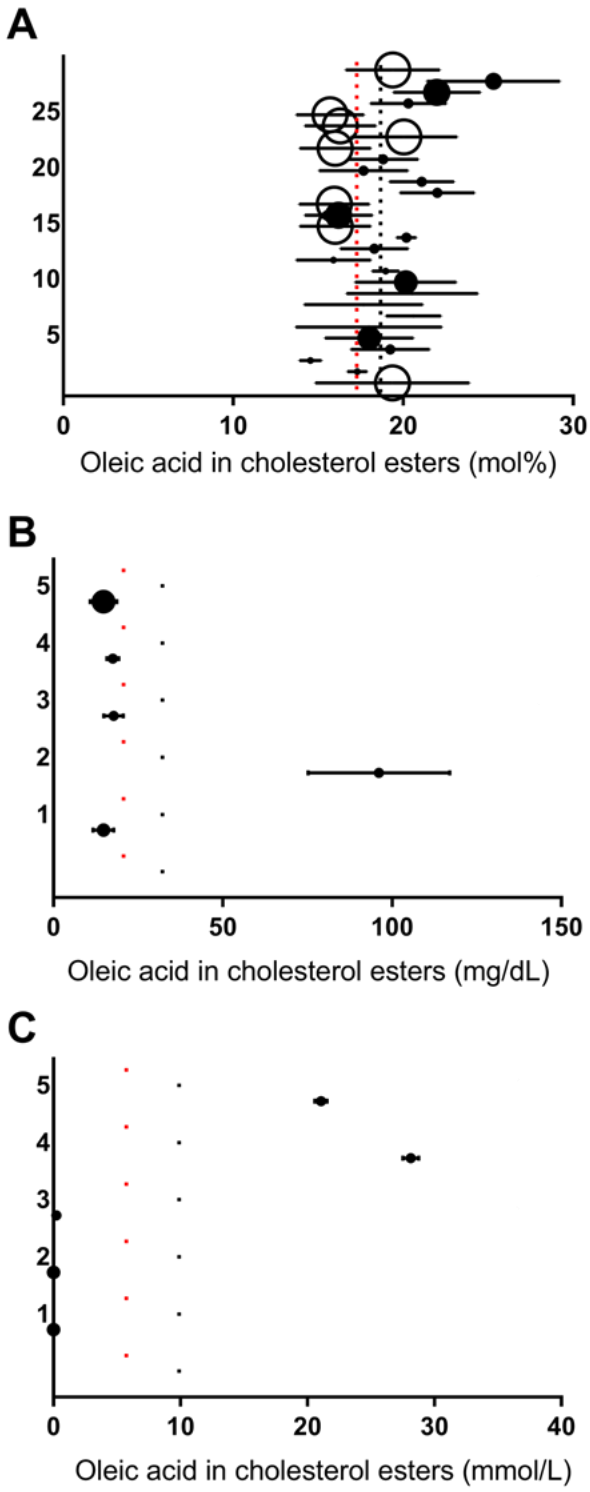


Figure 5.14 Oleic acid in cholesterol esters from systematic review

Black circles indicate mean plasma triglycerides published by the study. The size of the circle is representative of the number of participants in the study (Table 5.2). Black dots mark the average of the datasets. Red dots mark the weighted average of the dataset. A table of the data can be found in Appendix F. A) n=20,294 B) n=185 C) n=146

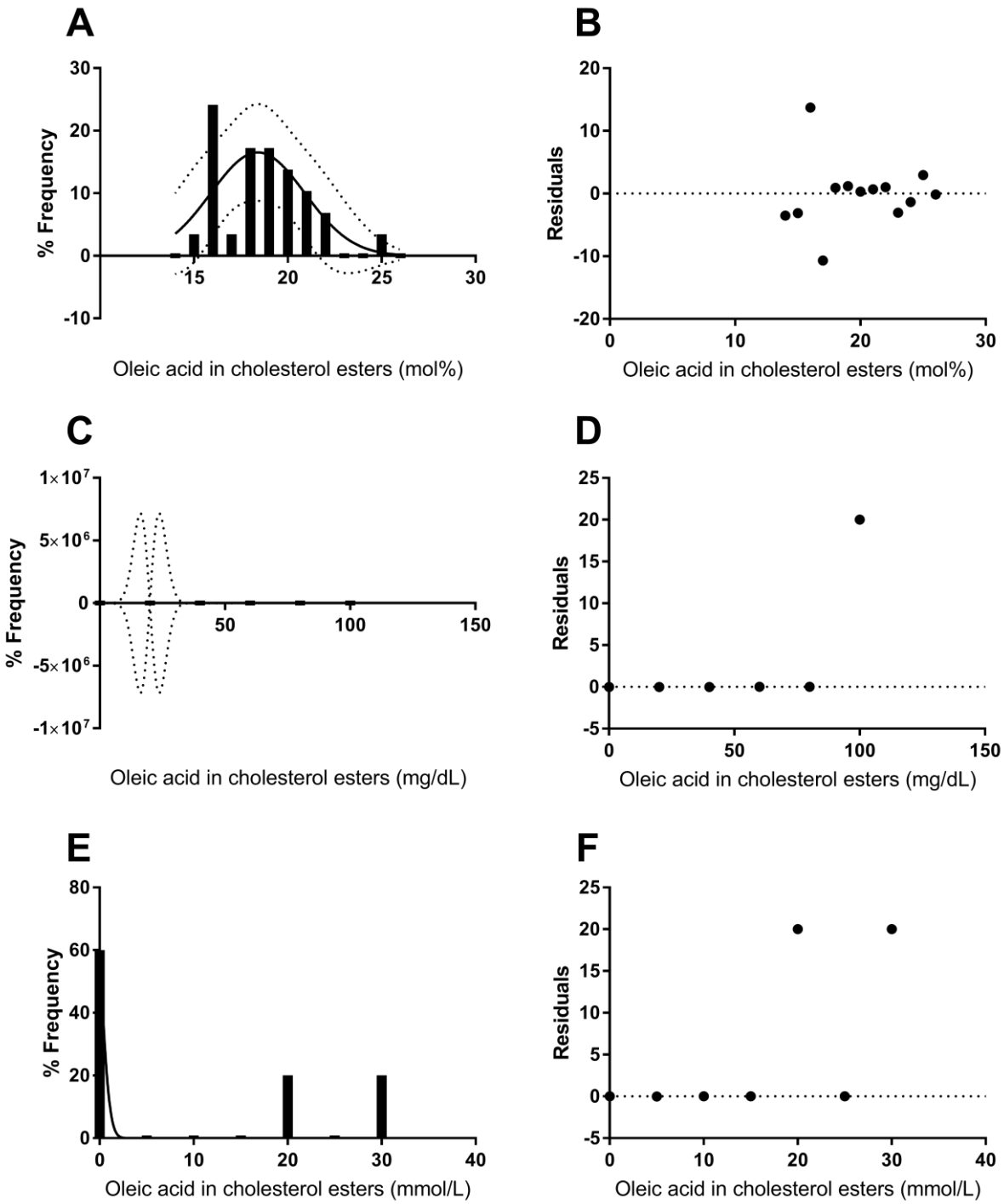


Figure 5.15 Frequency histograms with Gaussian curves established the absence of normality of oleic acid in cholesterol esters (mol%, mg/dL, mmol/L)

A, C, E) % frequency histogram showing the distribution of data amongst bands of 1 mol%, 20 mg/dL and 5 mmol/L. The Gaussian curve was fitted to test normality. Confidence intervals show where the Gaussian curve falls with a 95% certainty. B, D, F) Residuals plot was used to assess the fit of the Gaussian distribution curve. A, B) n=20,294 C, D) n=185 E, F) n=146

5.3.9 Palmitic acid in triglycerides

Although the analysis of total plasma triglycerides is common, the study of the fatty acid composition of plasma triglycerides is not as frequently published. Research in this area is important as a reduction in some long chain plasma triglyceride species has been found to preceed cardiovascular disease (Fernández-Real*, et al.*, 2003).

This analysis studied 18 sets of data from 12 studies with n=2797 for molar percentage, 3 datasets across 2 studies with n=144 for mg/dL and 3 datasets over 2 studies with n=43 for mmol/L (Appendix F).

The spread of mol% data was large with a range of 18.02%. The average and weighted average were significantly different, measuring 26.12 ± 4.21 mol% and 29.11 ± 1.52 mol%, respectively (p=0.0077) (Figure 5.16A). The adjusted R^2 value for the bell curve fitted to the palmitic acid in triglycerides mol% data was moderately good (0.7518) with no outliers found (Figure 5.17A). Furthermore, the spread of residuals was deemed adequate (Figure 5.17B). However, visual examination of the curve found that the fit at the upper and lower concentration limits were not good. In all, these results suggested the data could be used for rough references ranges.

A 95% reference range for palmitic acid in plasma triglycerides (mol%) was calculated using the equation in section 5.2.8. The reference range included values between the 2.5 and the 97.5 percentile, leaving 2.5% of 'normal' results above the range and 2.5% below the range. The reference range was 17.71 – 34.53 mol%.

The range of data for mg/dL was 9.17 mg/dL, whilst the average and weighted average were 23.28 ± 4.79 mg/dL and 20.14 ± 4.48 mg/dL, respectively, and were not significantly different (p=0.3740) (Figure 5.16B). A bell curve could not be fitted to the palmitic acid in triglycerides (mg/dL) data as it was not convergent, and hence, reference ranges could not be calculated (Figure 5.17C and 5.17D). Non-convergence was due to separation of the data, likely a result of the small sample size.

The mmol/L data points were vastly spread with the range measuring 25.53 mmol/L (Figure 5.16C). However, the difference between the average and weighted average was not statistically significant, at 15.96 ± 13.86 mmol/L and 18.92 ± 12.01 mmol/L, respectively (p=0.7474). The fit of the Gaussian distribution curve to the palmitic acid in triglycerides (mmol/L) data was assessed as poor due to a low adjusted R^2 value (0.125 (Figure 5.17E). Furthermore, one outlier in the 0 mmol/L interval was found, and the residuals did not vary from zero (Figure 5.17F). Therefore, the dataset was considered unsuitable for the calculation of reference ranges.

In summary, mol% was the most common measure of palmitic acid in plasma triglycerides with a good number of studies found. On the other hand, limited studies were found to report mg/dL and mmol/L. The mol% data was confirmed to be adequately Gaussian, which enabled the calculation of a reference range. However, reference ranges could not be calculated for the other datasets. Moreover, individual mg/dL and mmol/L data points were not interconverted as this would have included error. Yet, the average and weighted average of the mg/dL data was converted to mmol/L to identify differences through statistical analysis. Although much smaller, the difference between the converted average of 0.91 ± 0.19 and the unconverted average of 15.96 ± 13.86 was not statistically different (P=0.1332). Also, the converted weighted average of 0.79 ± 0.17 was not significantly smaller than the unconverted weighted average of 18.92 ± 12.01 (P=0.0591). These results were likely due to that large standard deviations of the unconverted averages.

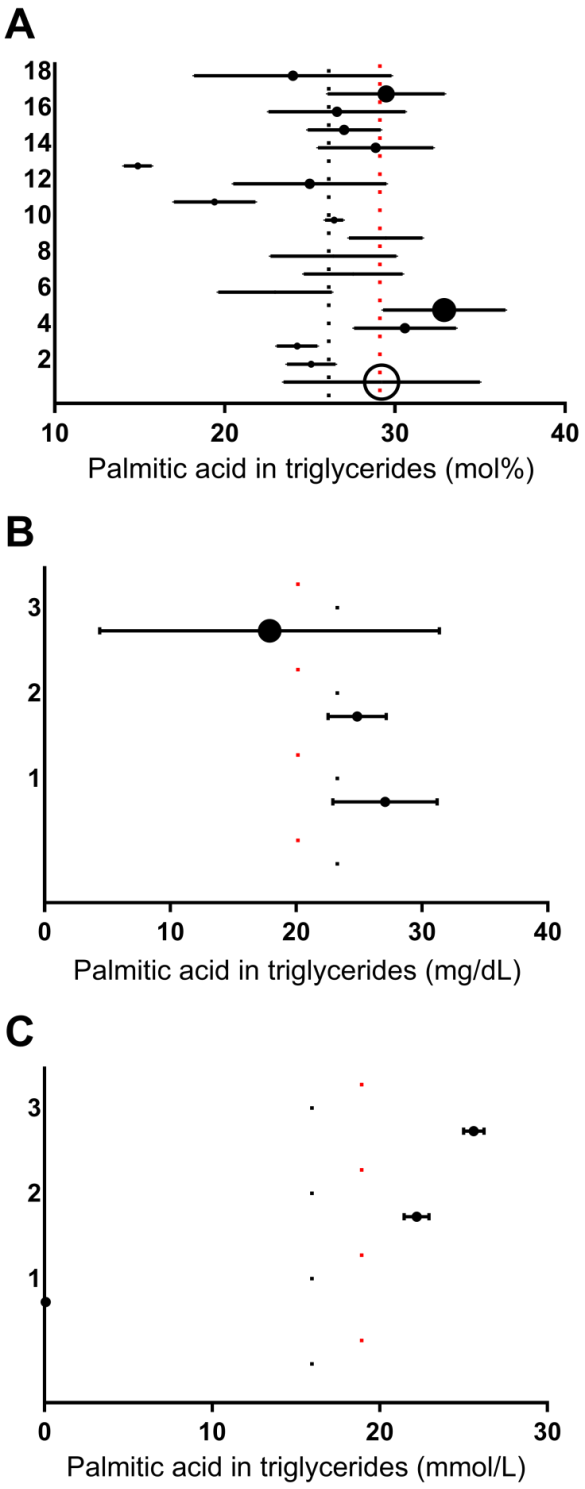


Figure 5.16 Palmitic acid in triglycerides from literature review

Black circles indicate mean plasma triglycerides published by the study. The size of the circle is representative of the number of participants in the study (Table 5.2). Black dots mark the average of the datasets. Red dots mark the weighted average of the dataset. A table of the data can be found in Appendix F. A) n=2797 B) n=144 C) n=43

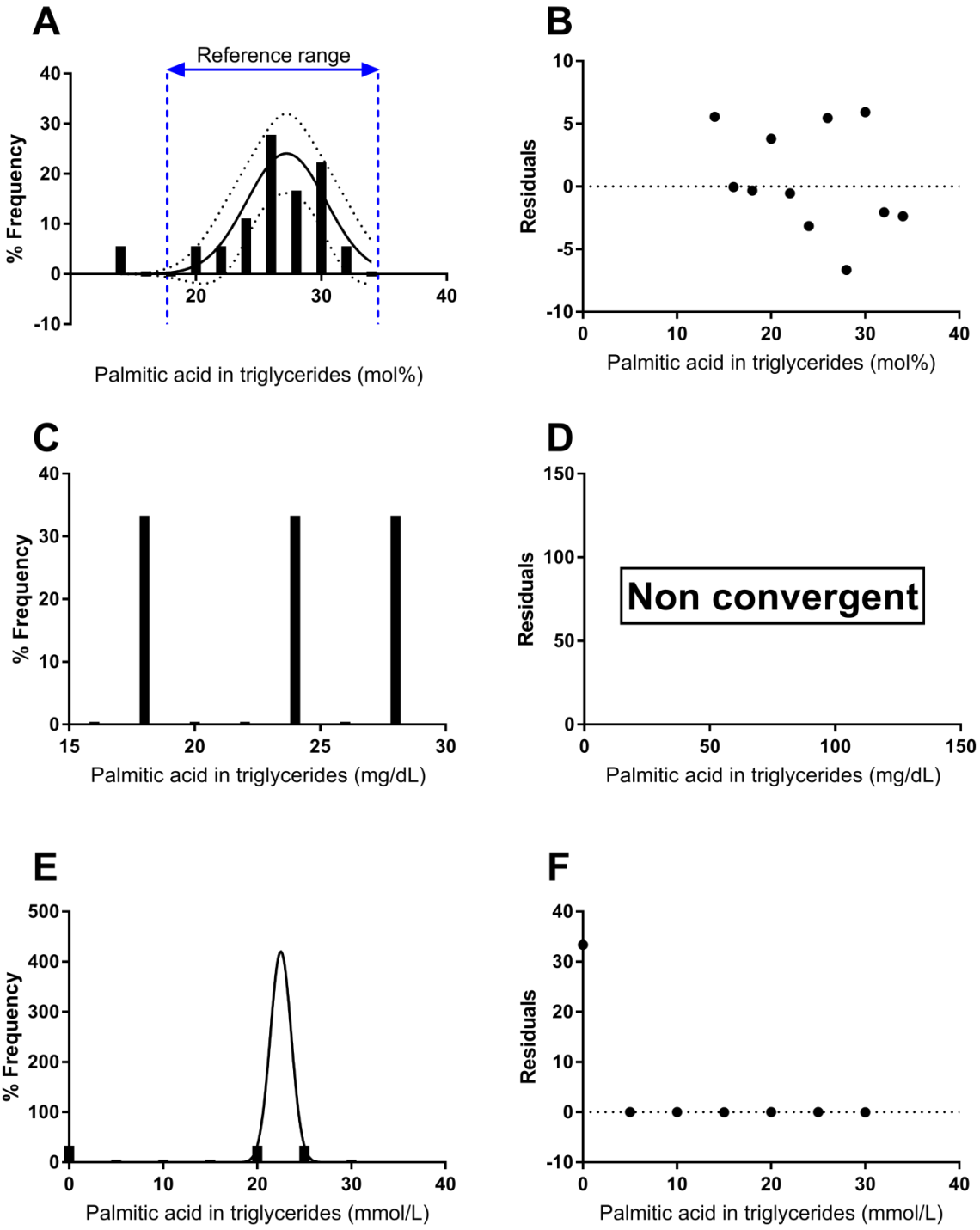


Figure 5.17 Frequency histograms with Gaussian curves revealed the normal distribution of palmitic acid in triglycerides (mol%)

A, C, E) % frequency histogram showing the distribution of data amongst bands of 2 mol%, 2 mg/dL and 5 mmol/L. The Gaussian curve was fitted to test normality. Confidence intervals show where the Gaussian curve falls with a 95% certainty. The blue lines indicate the computed reference range. B, D, F) Residuals plot was used to assess the fit of the Gaussian distribution curve. A, B) n=2797 C, D) n=144 E, F) n=43

5.3.10 Oleic acid in triglycerides

This comparison examined 18 datasets across 12 studies with n=2798 for molar percentage, 3 datasets across 2 studies with n=144 for mg/dL and 3 sets of data from 2 studies with n=43 for mmol/L (Appendix F).

The range of mol% data was 9.00%, whilst the difference between the two averages was statistically significant (p=0.0003). The average and weighted average were 38.02 ± 2.95 mol% and 34.90 ± 1.90%, respectively (Figure 5.18A). The Gaussian distribution curve for the oleic acid in triglycerides (mol%) data was not an adequate fit due to an adjusted R^2 value below zero (-0.5198) (5.19A). Furthermore, the residuals were skewed which caused an under prediction of many data points (Figure 5.19B). Therefore, the dataset was not suitable for the creation of reference ranges.

The spread of data points for mg/dL was large with a range of 18.98 mg/dL (Figure 5.18B). The average and weighted average were 31.74 ± 10.25 mg/dL and 24.89 ± 9.70 mg/dL, respectively. The difference between the two averages was not statistically significant (p=0.3667). The low adjusted R^2 value for the curve fitted to the oleic acid in triglycerides (mg/dL) data indicated a poor fit (Figure 5.19C). Furthermore, three outliers were identified at the intervals 20 mg/dL, 34 mg/dL and 40 mg/dL and the residuals lacked a random spread about zero (Figure 5.19D). Thus, the dataset was inadequate for the calculation of reference ranges.

The mmol/L range of data was vast, measuring 47.56 mmol/L (Figure 5.18C). But, this large spread of data did not confer a statistically significant difference between the two averages (p=0.7846). The average and weighted average was 28.61 ± 25.10 mmol/L and 33.13 ± 22.51 mmol/L, respectively. The normal distribution curve on the oleic acid in triglycerides (mmol/L) data was a poor fit by an adjusted R^2 value below zero (-0.1111) (Figure 5.19E). Furthermore, there was one outlier in the 0 mmol/L interval, and the residuals were not randomly distributed about zero (Figure 5.19F). Thus, the dataset was inadequate for the computation of reference ranges.

In summary, a good number of studies measuring the mol% of oleic acid in plasma triglycerides were uncovered, but papers using mg/dL and mmol/L were limited. Despite a good volume of data for mol%, no reference range could be calculated as it was not normally distributed. Reference ranges could not be computed for mg/dL or mmol/L either as the number of dtata points was too small to produce a Gaussian distribution. Also, individual data points for mg/dL were not converted to mmol/L in order to limit errors. But, the average and weighted average of the mg/dL data was converted to mmol/L and their difference analysed. The converted average of 1.12 ± 0.36 was much smaller than the average of the mmol/L dataset, 28.61 ± 25.10 (P=0.1307), however, the difference was not statistically significant due to a large standard deviation for the original mmol/L data. This was also true for the difference between the converted weighted average of 0.88 ± 0.34 and the unconverted weighted average of 33.13 ± 22.51 (P=0.0681).

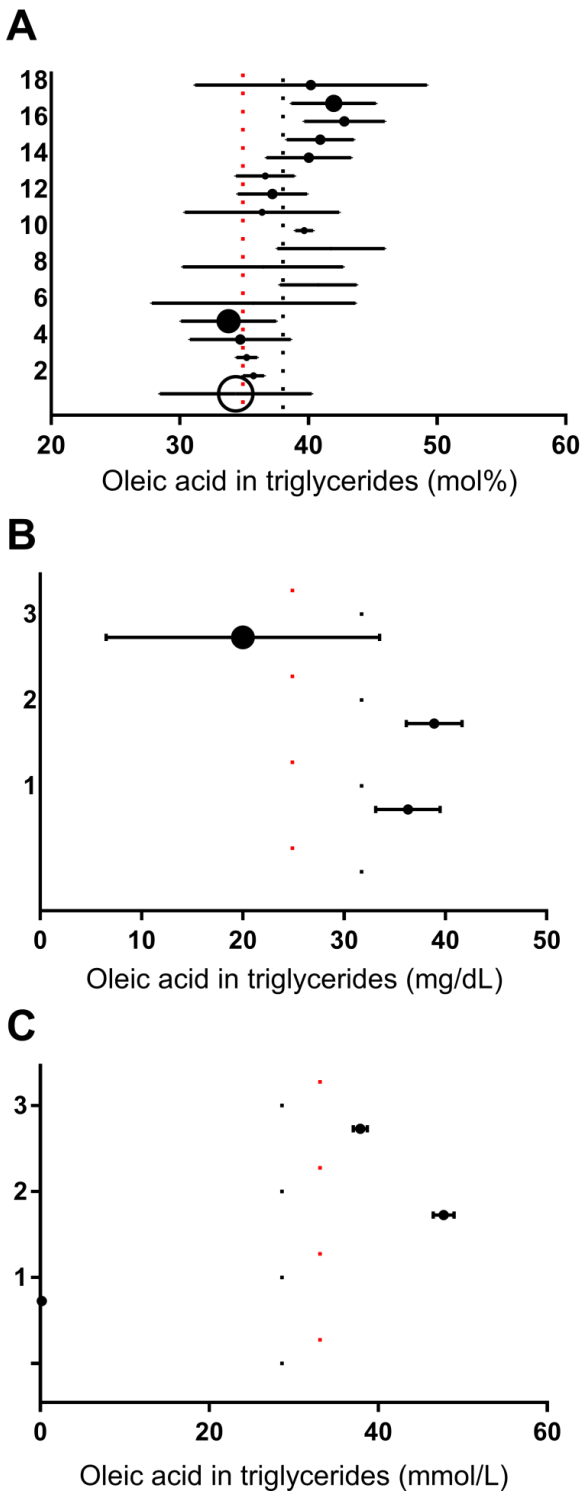


Figure 5.18 Oleic acid in triglycerides in literature

Black circles indicate mean plasma triglycerides published by the study. The size of the circle is representative of the number of participants in the study (Table 5.2). Black dots mark the average of the datasets. Red dots mark the weighted average of the dataset. A table of the data can be found in Appendix F. A) n=2798 B) n=144 C) n=43

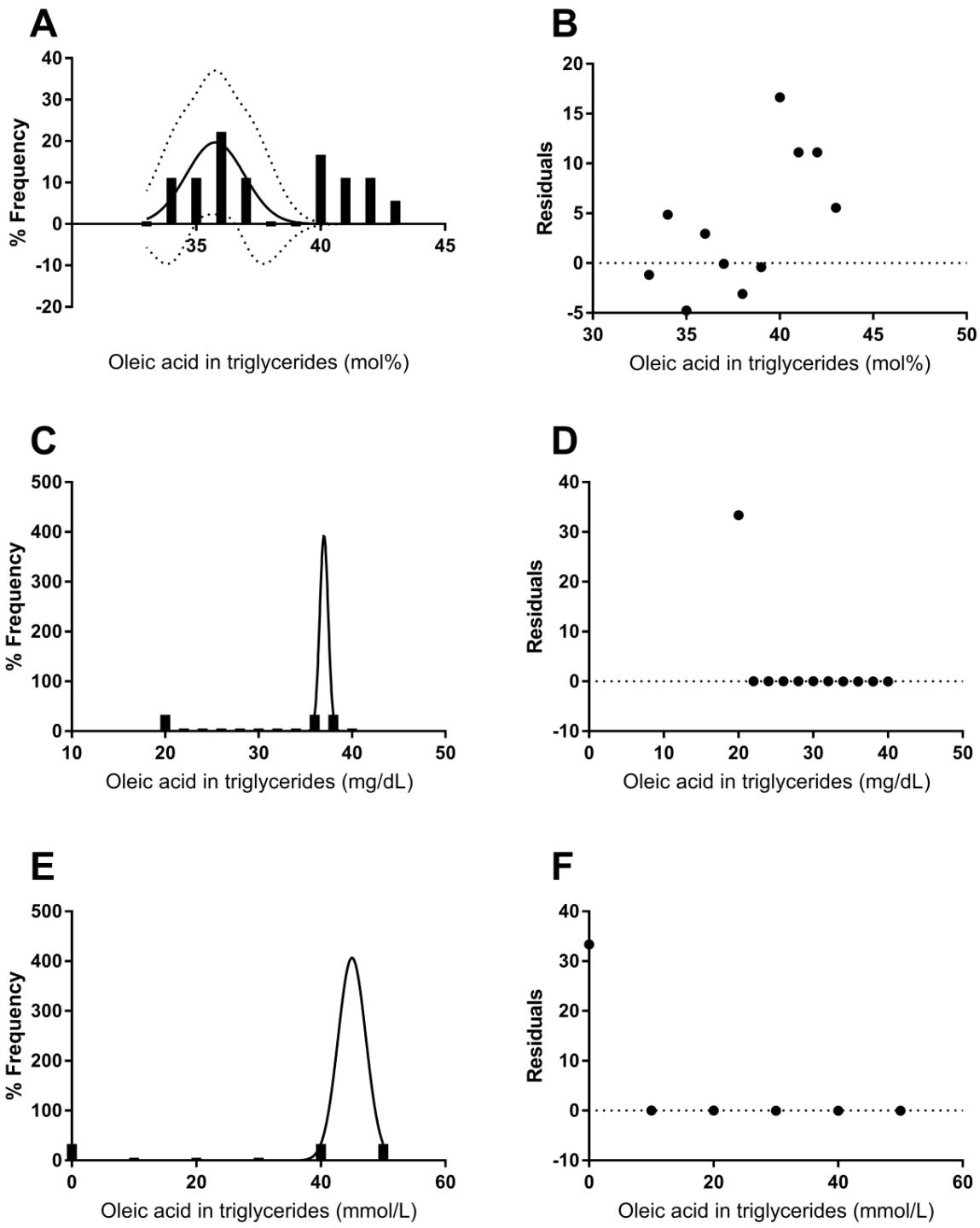


Figure 5.19 Frequency histograms with Gaussian curves demonstrated the lack of normality of oleic acid in triglycerides (mol%, mg/dL, mmol/L)

A, C, E) % frequency histogram showing the distribution of data amongst bands of 1 mol%, 2 mg/dL and 10 mmol/L. The Gaussian curve was fitted to test normality. Confidence intervals show where the Gaussian curve falls with a 95% certainty. B, D, F) Residuals plot was used to assess the fit of the Gaussian distribution curve. A, B) n=2798 C, D) n=144 E, F) n=43

5.3.11 Free palmitic acid levels

Whilst numerous studies have been published regarding high levels of plasma FFAs on health and disease, far fewer have studied the effects of the composition of plasma FFAs. Studies that have found palmitic acid to be directly correlated with coronary heart disease (Simon*, et al.*, 1995) and increases in systolic blood pressure (Simon*, et al.*, 1996). Whereas low plasma oleic acid levels have been found in colorectal cancer patients (Butler*, et al.*, 2017).

Data for three comparisons was collated from 14 sets of data across 8 studies with n=1039 for molar percentage, 7 datasets from 6 studies with n=8256 for mg/dL and 6 datasets over 6 studies with n=169 for mmol/L (Appendix F).

The mol% data had a range of 12.07 %, whilst the difference between the two averages was non-significant (p=0.0528). The average and weighted average were 24.53 ± 3.30 mol% and 26.41 ± 1.45 mol%, respectively (Figure 5.20A). The fit of the Gaussian distribution curve for the free palmitic acid (mol%) data was considered poor due to a moderate adjusted R^2 value (0.5948) (Figure 5.21A). Furthermore, visual examination of the histogram revealed that the model did not account for low molar percentages of palmitic acid. The spread of residuals was also skewed, which showed a consistent underestimation of the data (Figure 5.21B). In combination these results indicated that the dataset was not suitable for the calculations of references ranges.

The mg/dL data spread was substantial with a range of 73.71 mg/dL (Figure 5.20B), however, this was not due to a single anomalous data point. The average and weighted average were significantly different, measuring 33.54 ± 23.36 mg/dL and 73.73 ± 8.32 mg/dL, respectively (p=0.0039), meaning the larger studies found higher concentrations of palmitic acid. The bell curve fitted to the free palmitic acid (mg/dL) data was a poor fit with a low adjusted R^2 value (0.1345) (Figure 5.21C). Visually the curve did not fit the lower and upper limits, even though the residuals were randomly distributed (Figure 5.21D). Therefore, reference ranges could not be reliably calculated from this dataset.

The range of data for mmol/L was small at 0.124 mmol/L, and the two averages were not significantly different (p=0.7712) (Figure 5.20C). The average and weighted average was 0.124 ± 0.047 mmol/L and 0.118 ± 0.041 mmol/L, respectively. The adjusted R^2 value for the fit of the Gaussian distribution curve to the free palmitic acid (mmol/L) data was moderate (0.6267) (Figure 5.21E). However, the dataset contained two outliers at the intervals of 0.16 mmol/L and 0.20 mmol/L. Furthermore, visual examination found that the curve did not adequately fit the data and the residuals were not random about zero (Figure 5.21F). Thus, the dataset was inadequate for the creation of reference ranges.

In summary, a moderate number of studies used mol%, mg/dL and mmol/L to measure free palmitic acid in the plasma, but too few used mass% for analysis. No reference ranges could be computed as the datasets were not normally distributed. Furthermore, individual mg/dL data points were not converted to mmol/L or vice versa as this would have introduced undue error. However, the average and weighted average of the mg/dL data was converted to mmol/L to allow for statistical analysis. The converted average was significantly larger than unconverted average (1.308 ± 0.91 and 0.124 ± 0.047, respectively) (P=0.0049). Additionally, the difference between the converted weighted average and the weighted average of the mmol/L dataset was also statistically significant (2.876 ± 0.32 and 0.118 ± 0.041, respectively) (P<0.0001).

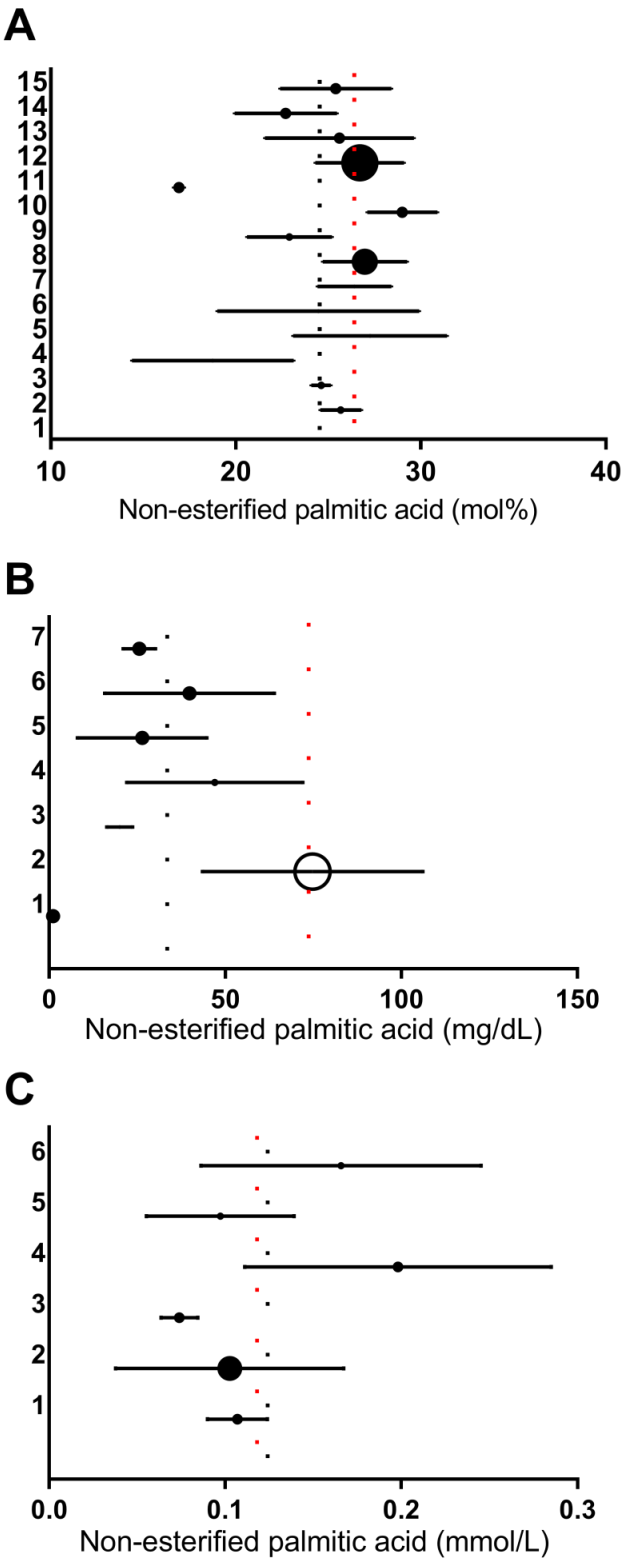


Figure 5.20 Free palmitic acid concentrations from the literature

Black circles indicate mean plasma triglycerides published by the study. The size of the circle is representative of the number of participants in the study (Table 5.2). Black dots mark the average of the datasets. Red dots mark the weighted average of the dataset. A table of the data can be found in Appendix F. A) n=1039 B) n=8256 C) n=169

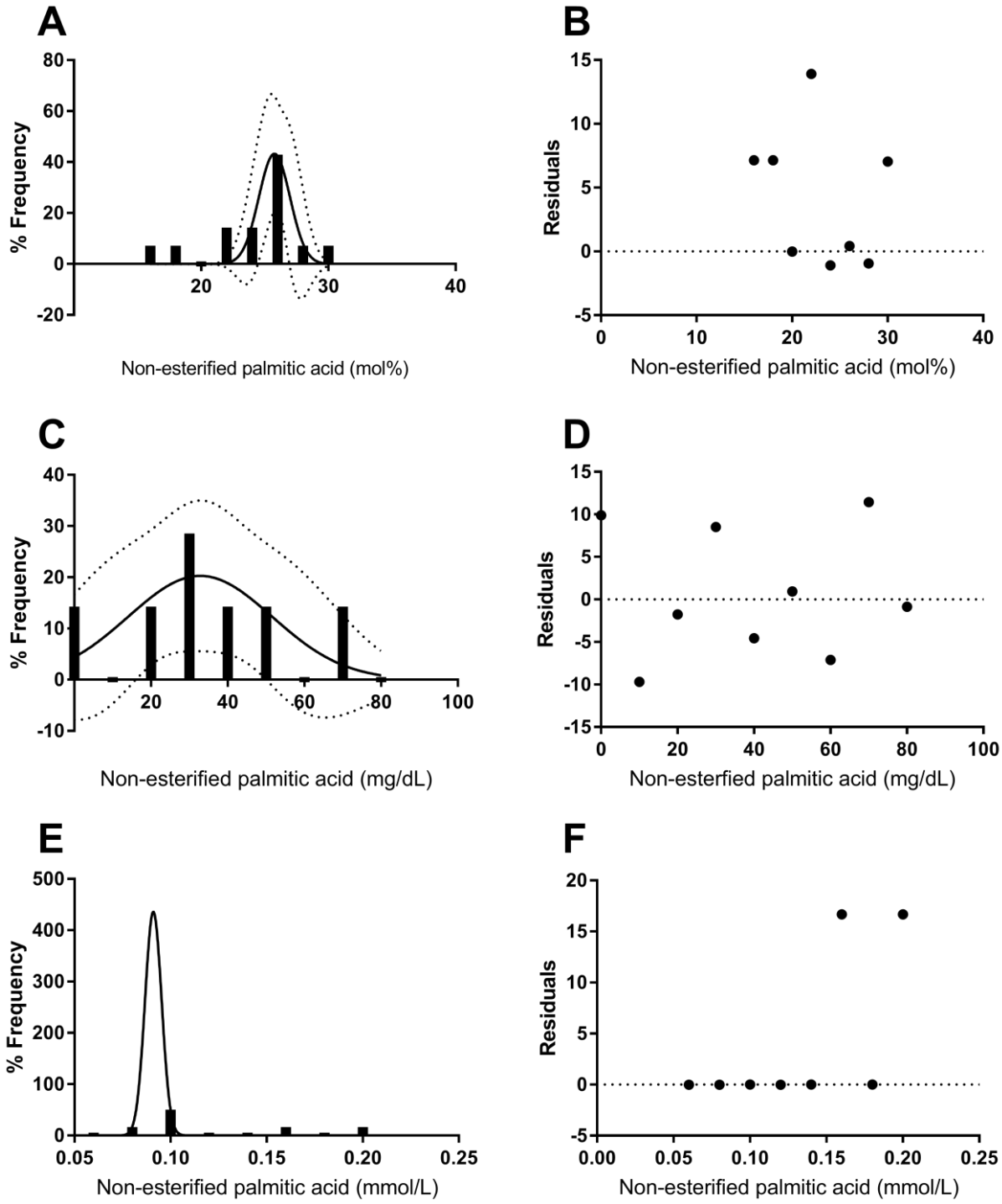


Figure 5.21 Frequency histograms with Gaussian curve established the absence of normality of free oleic acid (mol%, mg/dL, mmol/L)

A, C, E) % frequency histogram showing the distribution of data amongst bands of 2 mol%, 10 mg/dL and 0.02 mmol/L. The Gaussian curve was fitted to test normality. Confidence intervals show where the Gaussian curve falls with a 95% certainty. B, D, F) Residuals plot was used to assess the fit of the Gaussian distribution curve. A, B) n=1039 C, D) n=8256 E, F) n=169

5.3.12 Free oleic acid levels

Three groups of data looking at free oleic acid levels were obtained: 13 sets of data from 7 studies with n=233 for molar percentage, 7 datasets over 6 studies with n=8256 for mg/dL and 5 sets of data across 5 studies with n=163 for mmol/L (Appendix F).

The range of mol% data was 14.06 %. The average and weighted average measured 33.44 ± 4.27 mol% and 33.90 ± 3.85 mol%, respectively, and were not significantly different (p=0.7091) (Figure 5.22A) 12A). The fit of the bell curve for the free oleic acid (mol%) data was assessed as poor by a low adjusted R^2 value (0.1406) (Figure 5.23A). Visual analysis of the curve further confirmed the poor fit of the model, even though residuals were randomly spread about zero (Figure 5.23B). It was concluded that this dataset was inadequate for the creation of references ranges.

The spread of data for mg/dL was substantial with a range of 68.63 mg/d, however, this was not due to an outlying data point (Figure 5.22B). The difference between the average and weighted average was statistically significant (p=0.0066), with the two measuring 32.86 ± 22.93 mg/dL and 68.06 ± 7.96 mg/dL, respectively. A Gaussian distribution curve could not be fitted to the free oleic acid (mg/dL) data as it was not converged, and hence could not be used to calculate reference ranges (Figure 5.23C and 5.23D). This was likely due to separation of the data, resulting from the small sample size.

The mmol/L data had a range of 0.28 mmol/L. The average and weighted average were 0.192 ± 0.112 mmol/L and 0.151 ± 0.073 mmol/L (Figure 5.22C), respectively. The difference between the two was not statistically significant (p=0.7712). The adjusted R^2 value for the fit of the normal distribution curve to the free oleic acid (mmol/L) data was low (0.2844), which indicated a poor fit (Figure 5.23E). Additionally, visual analysis of the curve revealed a poor fit that did not account for high concentrations of oleic acid, whilst the residuals showed a skew in under predicting the data (Figure 5.23F). Hence, reference ranges were not computed from the data.

In summary, a moderate number of studies measured free oleic acid in all three units, mol%, mg/dL or mmol/L, however, too few were found using mass% for analysis. This made analysis and the calculation of reference ranges difficult. Reference ranges could not be produced as the datasets were not normally distributed. Additionally, mg/dL and mmol/L were not presented on the same graph as conversion would have introduced error into some values. Yet, the average and weighted average of the mg/dL data was converted to mmol/L to enable statistical analysis. The converted average of 1.63 ± 0.81 was significantly larger than the unconverted average, 0.192 ± 0.112 ($P=0.0085$). The converted weighted average of 2.409 ± 0.28 was also significantly higher than the average of the mmol/L dataset, 0.157 ± 0.073 ($P<0.0001$).

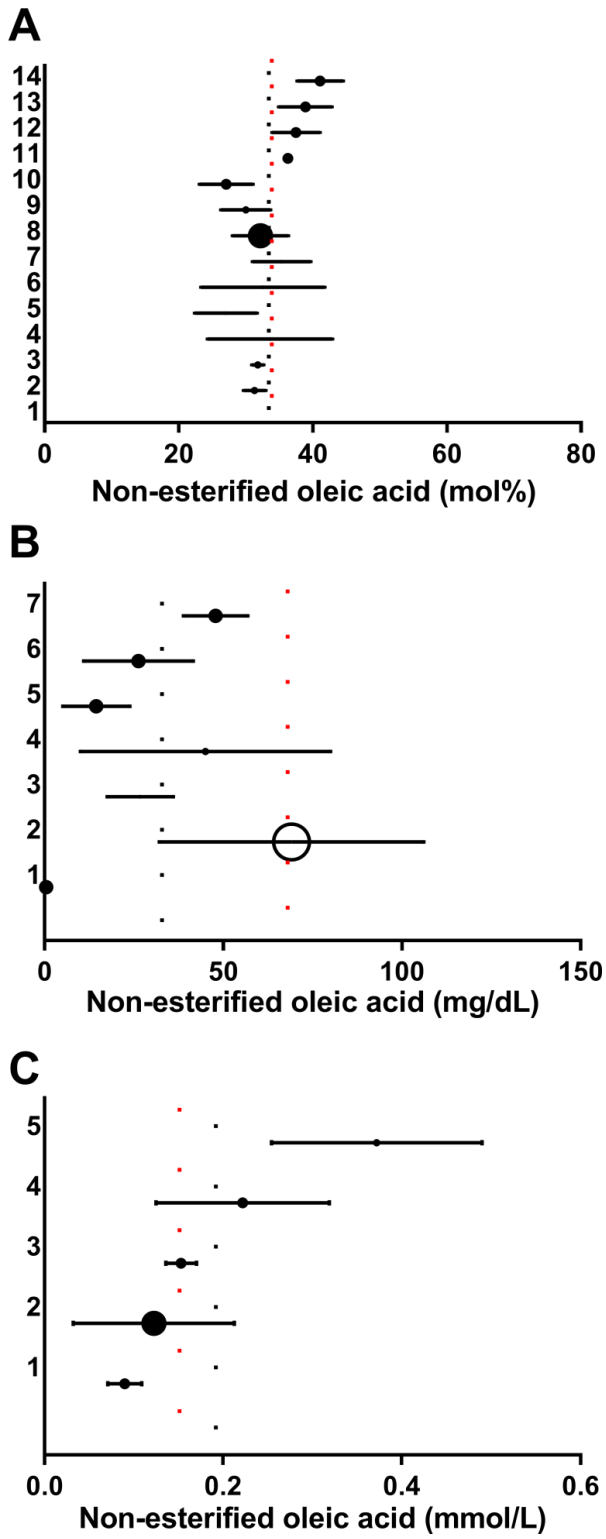


Figure 5.22 Free oleic acid concentrations from the literature

Black circles indicate mean plasma triglycerides published by the study. The size of the circle is representative of the number of participants in the study (Table 5.2). Black dots mark the average of the datasets. Red dots mark the weighted average of the dataset. A table of the data can be found in Appendix F. A) n=233 B) n=8256 C) n=163

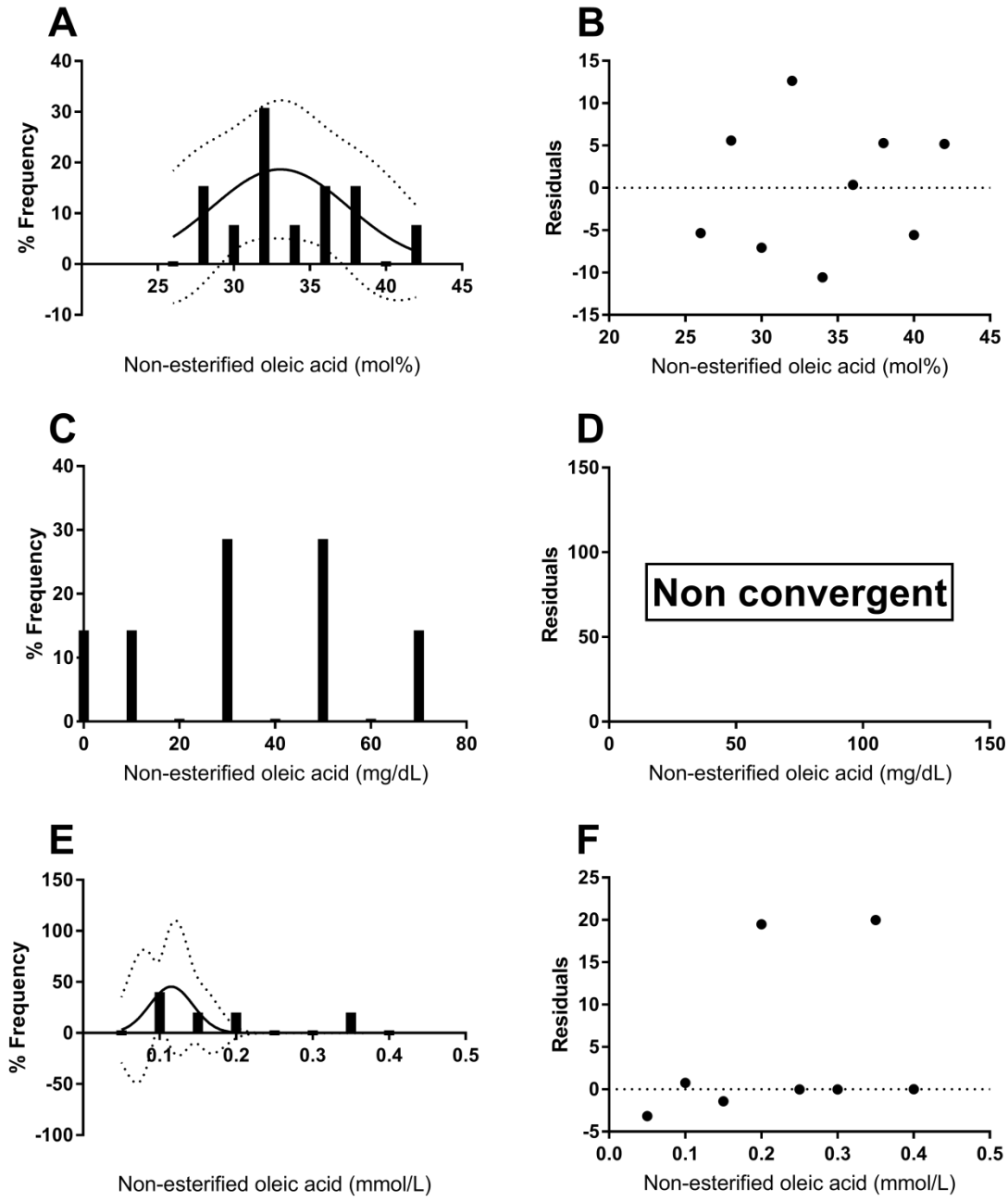


Figure 5.23 Frequency histograms with Gaussian curve demonstrated the lack of normality of free oleic acid (mol%, mg/dL, mmol/L)

A, C, E) % frequency histogram showing the distribution of data amongst bands of 2 mol%, 10 mg/dL and 0.05 mmol/L). The Gaussian curve was fitted to test normality. Confidence intervals show where the Gaussian curve falls with a 95% certainty. B, D, F) Residuals plot was used to assess the fit of the Gaussian distribution curve. A, B) n=233 C, D) n=8256 E, F) n=163

5.3.13 Total palmitic acid in plasma lipids

Total palmitic acid in plasma lipids is the sum of palmitic acid across all plasma lipid species including phospholipids, cholesterol esters, triglycerides and free fatty acids. Despite a moderate number of studies and datasets uncovered, there is very little research available of the effect of total plasma lipid composition on disease. Most studies included in this research were fatty acid profiles.

This research analysed 26 datasets across 13 studies with n=1687 for molar percentage, 15 datasets from 4 studies with n=392 for mass%, 3 sets of data from 2 studies with n=160 for mg/dL and 9 sets of data across 4 studies with n=1162 for mmol/L (Appendix F).

The mol% data had a range of 8.9% and the average and weighted average were not statistically different, measuring 21.63 ± 2.53 mol% and 21.18 ± 2.65 mol%, respectively (p=0.3803) (Figure 5.24A). The fit of the Gaussian distribution curve to the total palmitic acid (mol%) data was poor with a low adjusted R^2 value (0.3449) (Figure 5.25A). Furthermore, visual examination found that the curve did not account for high molar percentages of total palmitic acid and the positive residuals were quite large (Figure 5.25B). Hence this dataset was deemed unsuitable for the calculation of references ranges.

The range for mass% data was 7.9% with no significant difference between the average and weighted average, 21.67 ± 2.84 mass% and 22.29 ± 2.67 mass%, respectively (P=0.5373) (Figure 5.24B). The bell curve fitted to total palmitic acid (mass%) data as deemed to be inadequate due to a moderate adjusted R^2 value (0.6044) (Figure 5.25C). Further examination found that the curve did not visually fit the data and the residuals were not random about zero (Figure 5.25D). Therefore, it was concluded that the dataset was not adequate for the formation of reference ranges.

The data spread for mg/dL was large with a range of 68.54 mg/dL (Figure 5.24C). However, this did not confer statistical significance to the difference between the averages (p=0.4723). The average and weighted average measure 46.35 ± 34.81 mg/dL and 28.70 ±

34.68 mg/dL, respectively. A normal distribution curve could not be fitted to the total palmitic acid (mg/dL) data as it was not converged, and hence could not be used to calculate reference ranges (Figure 5.25E and 5.25F). This was due to separation of the data, a result of the small sample size.

The range of mmol/L data was 1.27 mmol/L. The difference between the average and weighted average was statistically significant, measuring 2.41 ± 0.52 mmol/L and 1.92 ± 0.50 mmol/L, respectively (p=0.0222) (Figure 5.24D). The adjusted R^2 value for the fit of the Gaussian distribution curve to the total palmitic acid (mmol/L) data was low (0.3476), which suggested a poor fit (Figure 5.25G). This was supported by visual analysis, which revealed a poor fitting curve, in addition to large residuals over double figures (Figure 5.25H). Thus, reference ranges could not be calculated from the data.

In summary, mol% was the most common unit for the measurement of total palmitic acid in plasma lipids, whilst mass%, mg/dL and mmol/L were only used in a few studies. A good number of studies existed for mol%, however, no reference ranges could be calculated as none of the datasets were Gaussian in their distribution. Moreover, individual mg/dL data points were not converted to mmol/L as this would have incorporated error. But, the average and weighted average of the mg/dL data was converted to mmol/L to allow statistical comparison. The converted average of 1.81 ± 1.36 was similar to the unconverted average of 2.41 ± 0.52 (P=0.5148). Additionally, the converted weighted average of 0.89 ± 1.35 was not significantly smaller than the unconverted weighted average of 1.92 ± 0.50 (P=0.2830), probably due to the large standard deviation of the converted average.

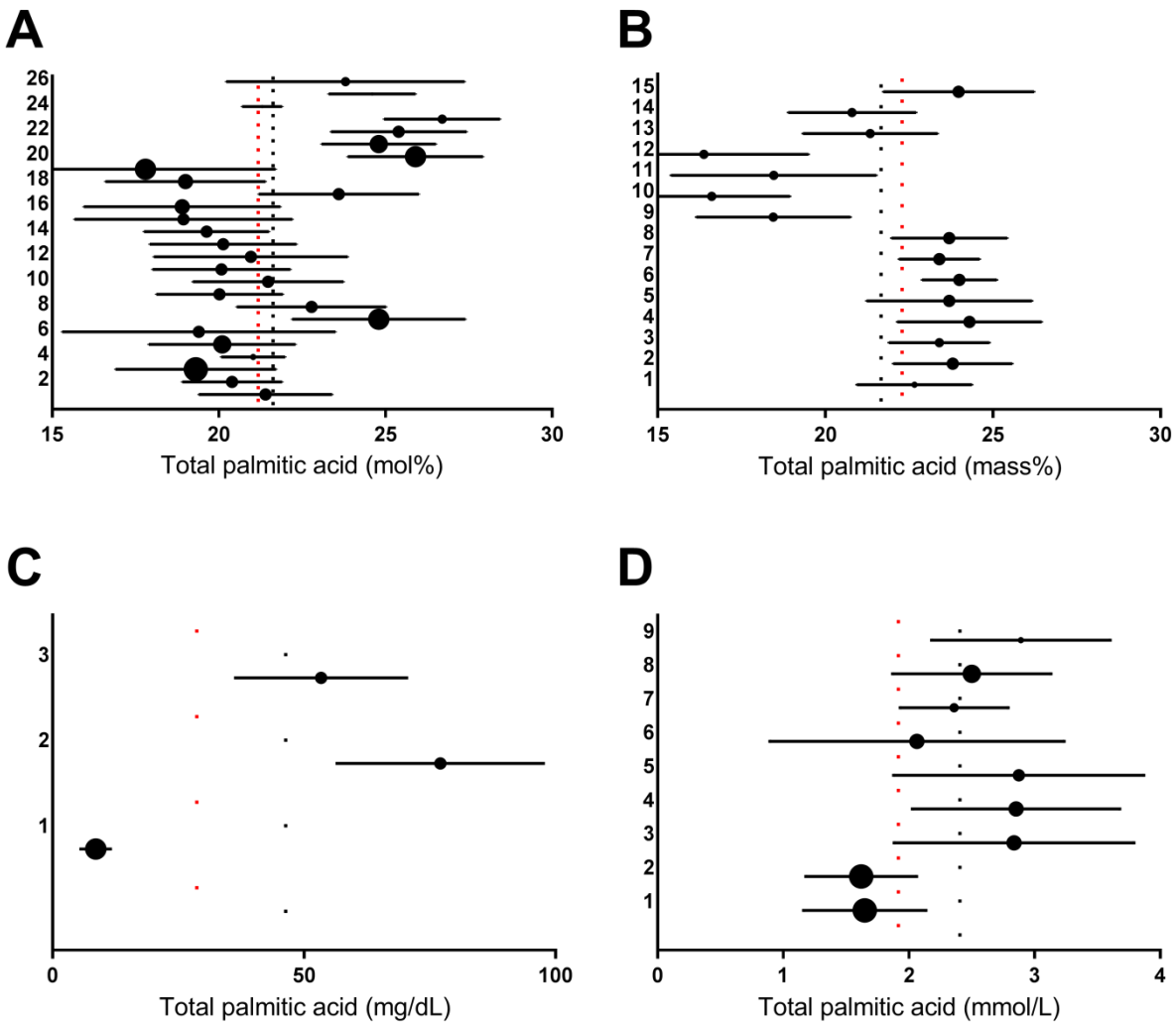


Figure 5.24 Total plasma palmitic acid from systematic review

Black circles indicate mean plasma triglycerides published by the study. The size of the circle is representative of the number of participants in the study (Table 5.2). Black dots mark the average of the datasets. Red dots mark the weighted average of the dataset. A table of the data can be found in Appendix F. A) n=1687 B) n=392 C) n=160 D) n=1162

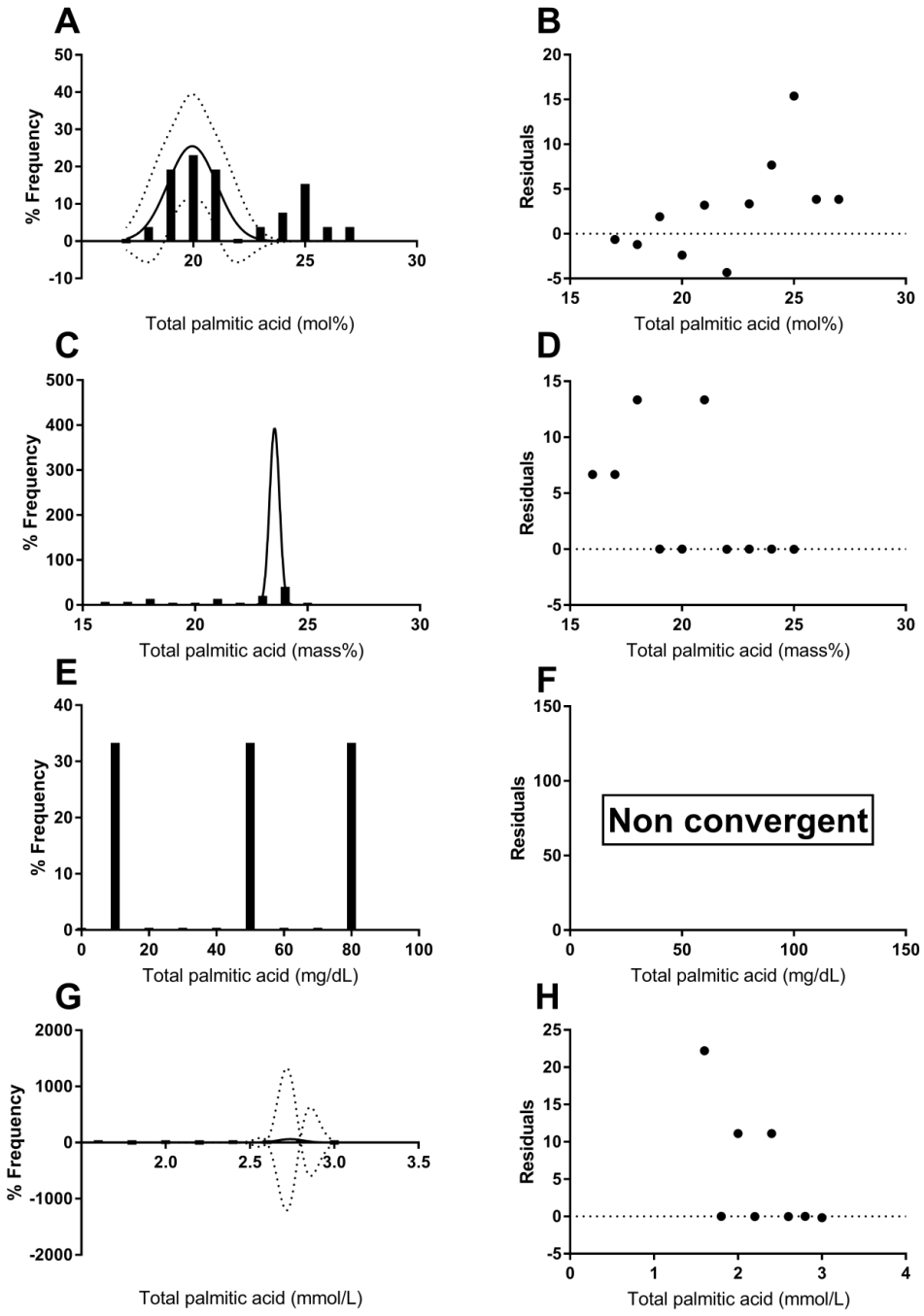


Figure 5.25 Frequency histograms with Gaussian curve established the absence of normality of palmitic acid in total plasma lipids (mol%, mass%, mg/dL, mmol/L)

A, C, E, G) % frequency histogram showing the distribution of data amongst bands of 1 mol%, 1 mass%, 10 mg/dL and 0.2 mmol/L. The Gaussian curve was fitted to test normality. Confidence intervals show where the Gaussian curve falls with a 95% certainty. B, D, F, H) Residuals plot was used to assess the fit of the Gaussian distribution curve. A, B) n=1687 C, D) n=392 E, F) n=160 G, H) n=1162

5.3.14 Total oleic acid in plasma lipids

This research examined 26 datasets from 13 studies with n=1687 for molar percentage, 15 datasets from 4 studies with n=392 for mass%, 3 datasets across 2 studies with n=160 for mg/dL and 8 datasets over 3 studies with n=1156 for mmol/L (Appendix F).

The range of mol% data was 10.64 %. The difference between the two averages was statistically significant (p=0.0152). The average and weighted average were 22.51 ± 2.79 mol% and 21.08 ± 2.29 mol%, respectively (Figure 5.26A). The adjusted R^2 value for the fit of the bell curve to the total oleic acid (mol%) data was moderately low (0.4334), which suggested a poor fit (Figure 5.27A). Further analysis revealed that the curve did not account for high molar percentages even though the residuals were well distributed about zero (Figure 5.27B). Therefore, reference ranges could not be calculated for this dataset.

The range for mass% data was 5.0% with similar average and weighted average, 19.26 ± 1.57 mass% and 19.16 ± 1.65 mass%, respectively (P=0.8662) (Figure 5.26B).The normal distribution curve fitted to total oleic acid (mass%) data was inadequate as the adjusted R^2 value was below zero (-0.02229) (Figure 5.27C). This was corroborated by visual examination of the histogram, which found that the curve did not account for high mass percentages of total oleic acid. Furthermore, analysis of the residuals found a pattern that was not completely random with one large residual (Figure 5.27D). It was concluded that the dataset was unsuitable for the calculation of reference ranges.

The mg/dL data had a range of 39.51 mg/dL with an average and weighted average of 13.46 ± 20.62 mg/dL and 19.57 ± 2.79 mg/dL, respectively (Figure 5.26C). The difference between these two averages was non-significant (p=0.4570). The adjusted R^2 value for the Gaussian distribution curve fitted to the total oleic acid (mg/dL) data was below zero, which deemed the curve unsuitable (-0.241) (Figure 5.27E). Visual analysis found that it did not account for low concentrations of total oleic acid, and the residuals were not initially random, both conferring the poor fit of the curve (Figure 5.27F). Thus, references ranges could not be formulated for this dataset.

The range of mmol/L data was 2.15 mmol/L. The average and weighted average measured 2.35 ± 0.77 mmol/L and 1.71 ± 0.75 mmol/L, respectively, the difference between which was non-significant (p=0.0516) (Figure 5.26D). The normal distribution curve fitted to the total oleic acid (mmol/L) data was a poor fit with a very low adjusted R^2 (0.008014) (Figure 5.27G). Visual examination confirmed this, as did the residuals, which were not random below zero, and hence the dataset was inadequate for the calculation of reference ranges (Figure 5.27H).

In summary, mol% was the most common measure of total oleic acid in plasma lipids with mass%, mg/dL and mmol/L used in only a few studies. Unfortunately, no reference ranges could be computed as the datasets were not normally distributed, in spite of the good volume of data for mol%. The mg/dL data was not converted to mmol/L and presented on the same graph as this would have introduced error. However, the average and weighted average of the mg/dL data was converted to mmol/L for statistical analysis. The converted average was significantly smaller than the average of the mmol/L dataset (0.48 ± 0.73 and 2.35 ± 0.77, respectively) (P=0.0379). Conversely, the difference between the converted weighted average and the unconverted weighted average was not significant (0.69 ± 0.74 and 1.71 ± 0.75) (P=0.1689).

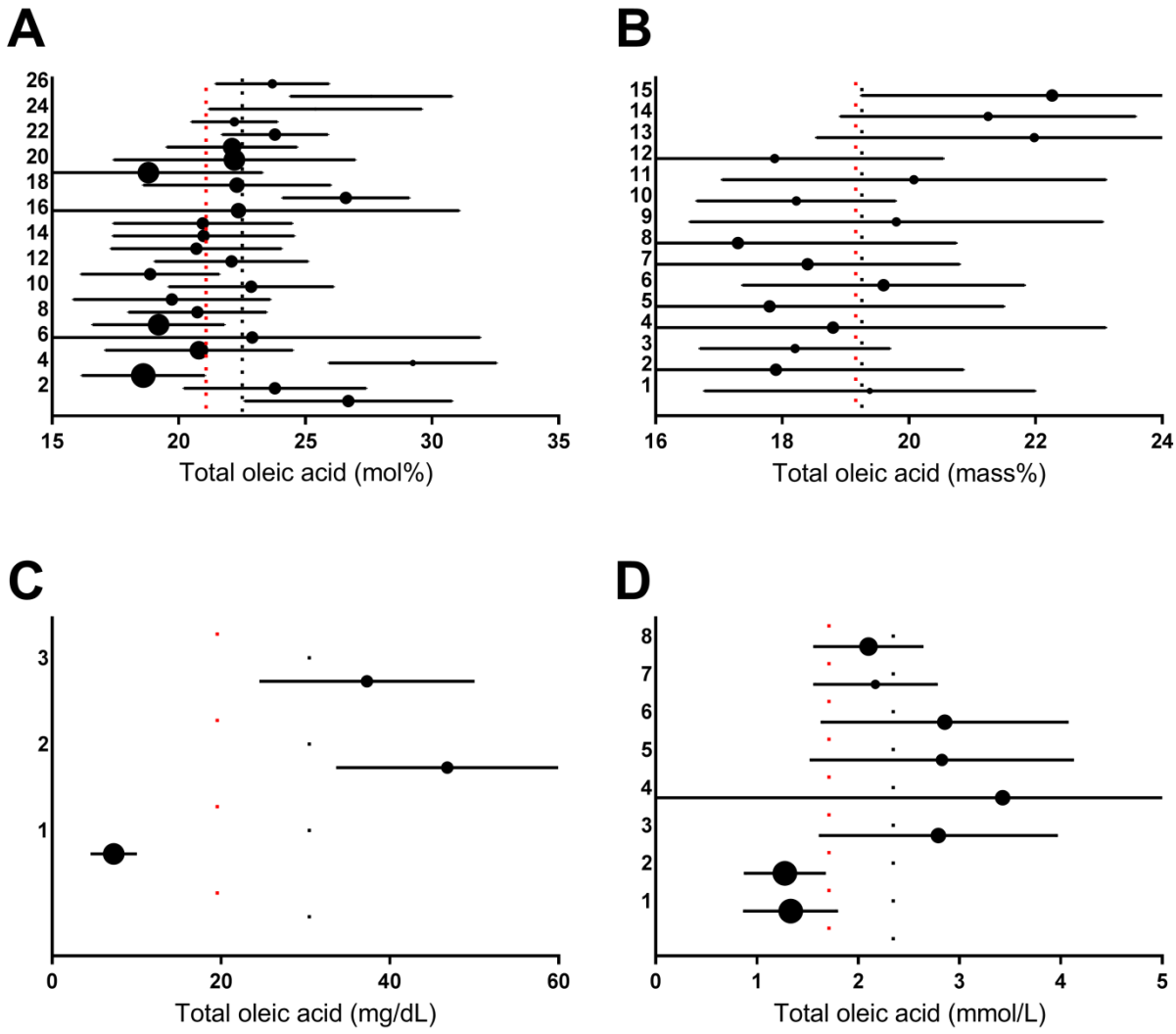


Figure 5.26 Total plasma oleic acid in literature

Black circles indicate mean plasma triglycerides published by the study. The size of the circle is representative of the number of participants in the study (Table 5.2). Black dots mark the average of the datasets. Red dots mark the weighted average of the dataset. A table of the data can be found in Appendix F. A) n=1687 B) n=392 C) n=160 D) n=1156

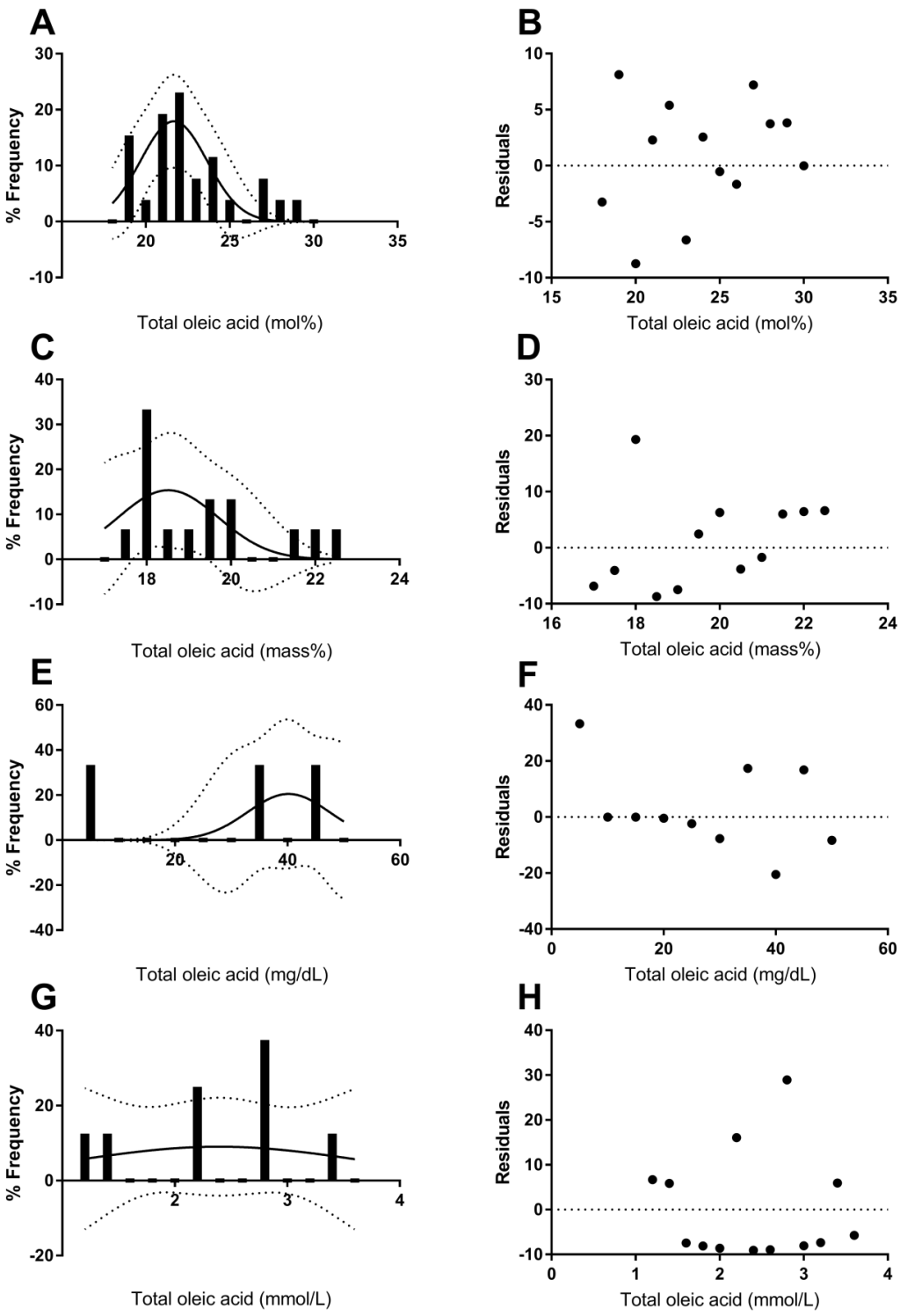


Figure 5.27 Frequency histograms with Gaussian curve revealed the lack of normality of oleic acid in total plasma lipids (mol%, mass%, mg/dL, mmol/L)

A, C, E, G) % frequency histogram showing the distribution of data amongst bands of 1 mol%, 0.5 mass%, 5 mg/dL and 0.2 mmol/L). The Gaussian curve was fitted to test normality. Confidence intervals show where the Gaussian curve falls with a 95% certainty. B, D, F, H) Residuals plot was used to assess the fit of the Gaussian distribution curve. A, B) n=1687 C, D) n=392 E, F) n=160 G, H) n=1156

Table 5.4 Summary of average concentrations

		Mol%		Mass%		mg/dL		mmol/L		mg/dL converted to mmol/L	
		Average	Weighted average	Average	Weighted average	Average	Weighted average	Average	Weighted average	Average	Weighted average
Phospholipid	PA	27.24 ± 3.51	27.62 ± 2.62	27.94 ± 2.20	26.49 ± 1.90	47.08 ± 39.46	36.40 ± 15.65	0.38 ± 0.42	1.22 ± 0.13	1.84 ± 1.54*	1.42 ± 0.61
	OA	10.56 ± 1.94	11.20 ± 2.03	11.06 ± 1.80	9.76 ± 1.19	20.50 ± 21.53	13.79 ± 8.59	0.39 ± 0.11	0.47 ± 0.02	0.73 ± 0.76	0.49 ± 0.30
Cholesterol esters	PA	11.63 ± 1.30	10.92 ± 0.97			20.28 ± 22.42	13.82 ± 13.75	4.42 ± 6.00	2.57 ± 5.20	0.79 ± 0.87	0.54 ± 0.54
	OA	18.67 ± 2.40	17.26 ± 1.88			32.19 ± 35.57	20.68 ± 22.25	9.89 ± 13.67	5.75 ± 11.77	1.14 ± 1.26	0.73 ± 0.79
Triglycerides	PA	26.12± 4.21	29.11 ± 1.52			23.28 ± 4.79	20.14 ± 4.48	15.96 ± 13.86	18.92 ± 12.01	0.91 ± 0.19	0.79 ± 0.17
	OA	38.02 ± 2.95	34.90 ± 1.90			31.74 ± 10.25	24.89 ± 9.70	28.61 ± 25.10	33.13 ± 22.51	1.12 ± 0.36	0.88 ± 0.34
Free	PA	25.53 ± 3.31	26.41 ± 1.45			33.54 ± 23.36	73.73 ± 8.32	0.124 ± 0.047	0.118 ± 0.041	1.308 ± 0.91*	2.876 ± 0.32*
	OA	33.44 ± 4.27	33.90 ± 3.85			32.86 ± 22.93	68.06 ± 7.96	0.192 ± 0.112	0.157 ± 0.073	1.163 ± 0.81*	2.409 ± 0.28*
Total	PA	21.63 ± 2.53	21.18 ± 2.65	21.67 ± 2.84	22.29 ± 2.67	46.35 ± 34.81	22.70 ± 34.68	2.41 ± 0. 52	1.92 ± 0.50	1.81 ± 1.36	0.89 ± 1.35
	OA	22.51 ± 2.79	21.08 ± 2.29	19.26 ± 1.57	19.16 ± 1.65	13.46 ± 20.62	19.57 ± 20.79	2.35 ± 0.77	1.71 ± 0.75	0.48 ± 0.73	0.69 ± 0.74

5.3.15 Ratio of palmitic acid: oleic acid in lipid species

This research examined 90 datasets from 45 studies with n=35821 for PA:OA in phospholipids, 41 datasets from 29 studies with n=20852 for PA:OA in cholesterol esters, 24 datasets from 16 studies with n=2985 for PA:OA in triacylglycerol, 27 datasets from 22 studies with n=8675 for PA:OA in free fatty acids, and 52 datasets from 22 studies with n=3395 for PA:OA in total plasma lipids.

The range of ratios of PA:OA in phospholipids was 3.27. The difference between the two averages was not statistically significant (p=0.0806). The average and weighted average were 2.62 and 2.52, respectively (Figure 5.28A). The adjusted R^2 value for the fit of the Gaussian distribution curve to the data was high (0.9640), which suggested a very good fit suitable for the creation of a reference range (Figure 5.28B). Analysis of the residuals confirmed that this dataset could be used for the calculation of a reference range as points were randomly distributed around zero (Figure 5.28C).

A 95% reference range was calculated using the equation in section 5.2.8, leaving 2.5% of results above this range and 2.5% below this range. The reference range for the ratio of PA:OA in phospholipids was 1.51 – 3.73.

The range for ratios of PA:OA in cholesterol esters was 0.47 with similar average and weighted average, 0.62 and 0.63, respectively (P=0.1300) (Figure 5.29A).The bell curve fitted to the data was more than adequate as the adjusted R^2 value was high (0.9053) (Figure 5.29B), and the residuals were close to zero and randomly distributed (Figure 5.29C). It was concluded that the dataset was suitable for the calculation of reference ranges.

Using the equation in section 5.2.8, a 95% reference range was computed. The reference range including ratios of PA:OA in cholesterol esters between the 2.5 and the 97.5 percentile was 0.45 – 0.78.

The ratios of PA:OA in TAG data had a range of 0.57 with an average and weighted average of 0.68 and 0.83, respectively (Figure 5.30A), and the difference between the two averages statistically significant ($p<0.0001$). The adjusted R^2 value for the Gaussian distribution curve was moderate, but not adequate for the creation of reference ranges (0.7620) (Figure 5.30B). Analysis of the residuals found a persistent underestimation of the data (Figure 5.30C). Thus, references ranges could not be calculated for this dataset.

The range of ratios of PA:OA in free fatty acids was 1.88. The average and weighted average measured 0.91 and 1.1, respectively, the difference between which was statistically significant ($p=0.0473$) (Figure 5.31A). The normal distribution curve was a relatively good fit with an R^2 (0.8989) (Figure 5.31B). Visual examination found a tail of data that had been excluded from the curve, and residuals showed a lack of randomness about zero. The non-liner regression systematically underestimated then overestimated the dataset (Figure 5.31C). Therefore, this dataset was inadequate for the creation of reference ranges.

The ratios of PA:OA in total plasma lipids had a range of 0.93 with an average and weighted average of 1.1 and 1.1, respectively (Figure 5.32A). The difference between these two averages was non-significant ($p=0.1061$). The normal distribution curve had moderate fit but did not account for data on the lower end of the scale. The adjusted R^2 value was 0.7548 (Figure 5.32B). The residuals were randomly distributed around zero, however, the fit of the curve was too poor to be used in the calculation of reference ranges (Figure 5.32C).

In summary, calculating the ratio of palmitic acid: oleic acid values published in the collated studies was an effective way of combining data regardless of measurement unit. This increased the number of participants per analysis and in turn increased the quality of the results, which led to the calculation of two reference ranges.

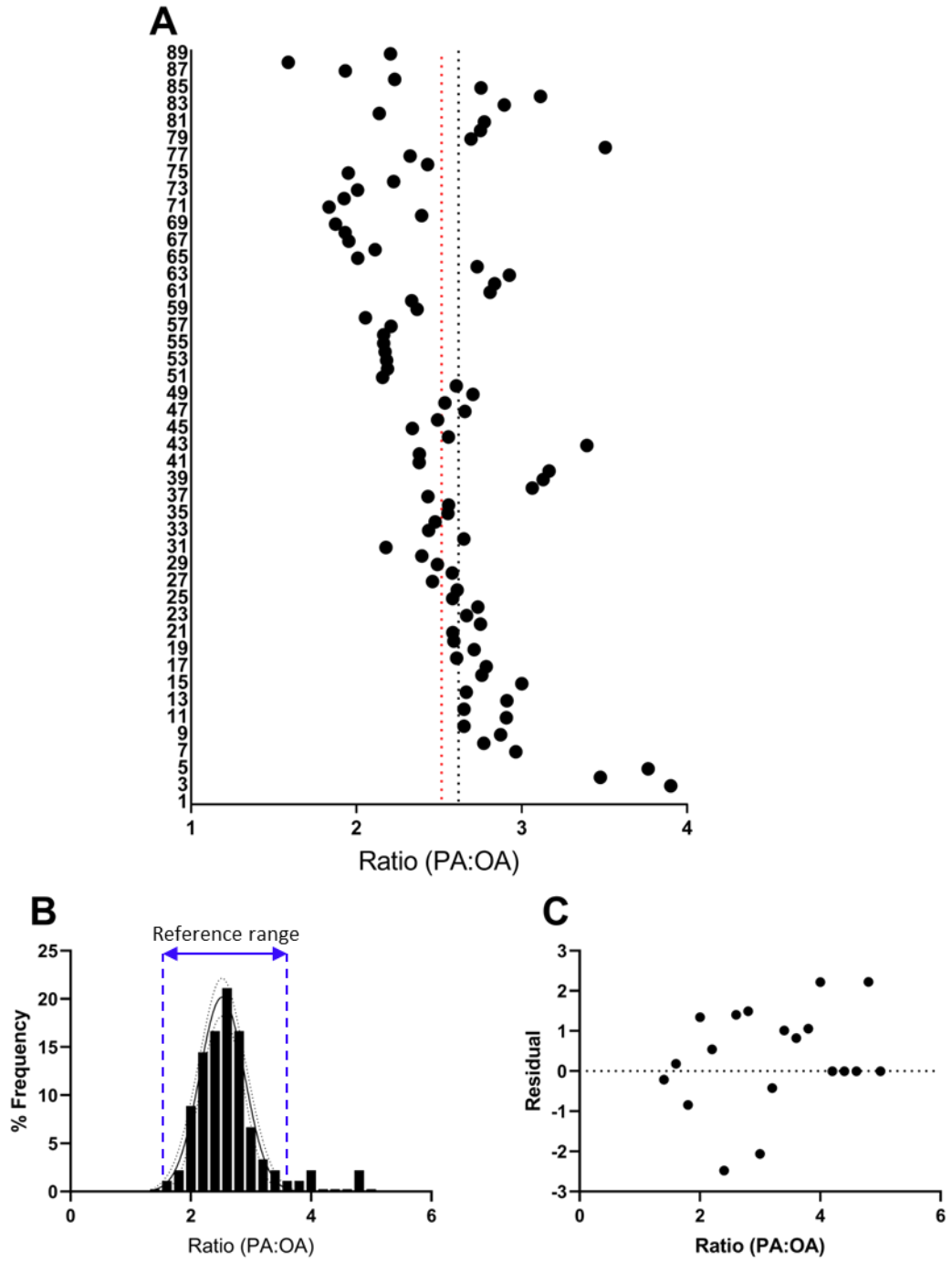


Figure 5.28 Ratio of PA:OA in phospholipids

A) Black circles indicate the ratio of PA:OA published by the study. Black dots mark the average of the datasets. Red dots mark the weighted average of the dataset. B) % frequency histogram showing the distribution of data amongst bands of 0.2. The Gaussian curve was fitted to test normality. Confidence intervals show where the Gaussian curve falls with a 95% certainty. C) Residuals plot was used to assess the fit of the Gaussian distribution curve. n=35821

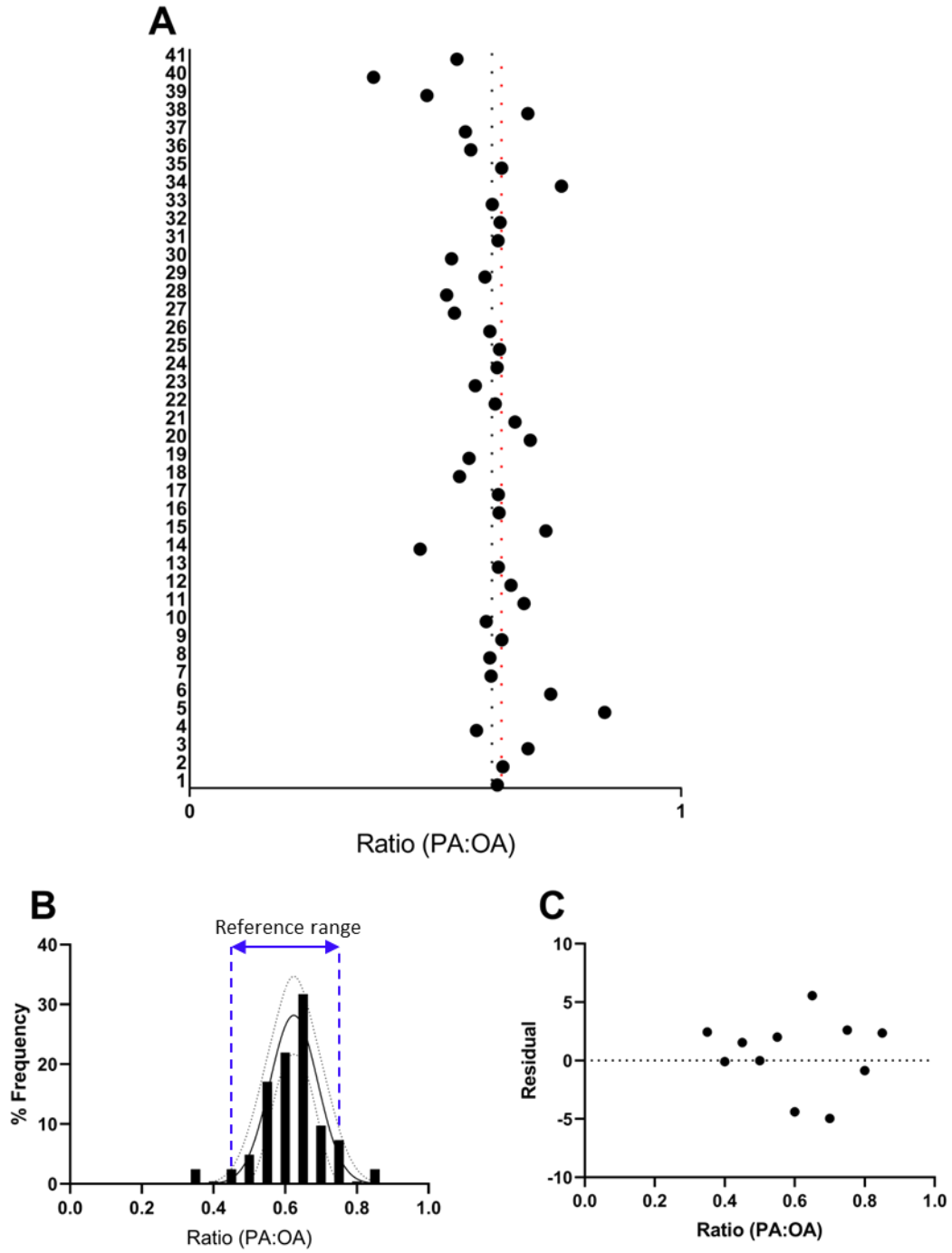


Figure 5.29 Ratio of PA:OA in cholesterol esters

A) Black circles indicate the ratio of PA:OA published by the study. Black dots mark the average of the datasets. Red dots mark the weighted average of the dataset. B) % frequency histogram showing the distribution of data amongst bands of 0.05. The Gaussian curve was fitted to test normality. Confidence intervals show where the Gaussian curve falls with a 95% certainty. C) Residuals plot was used to assess the fit of the Gaussian distribution curve. n=20852

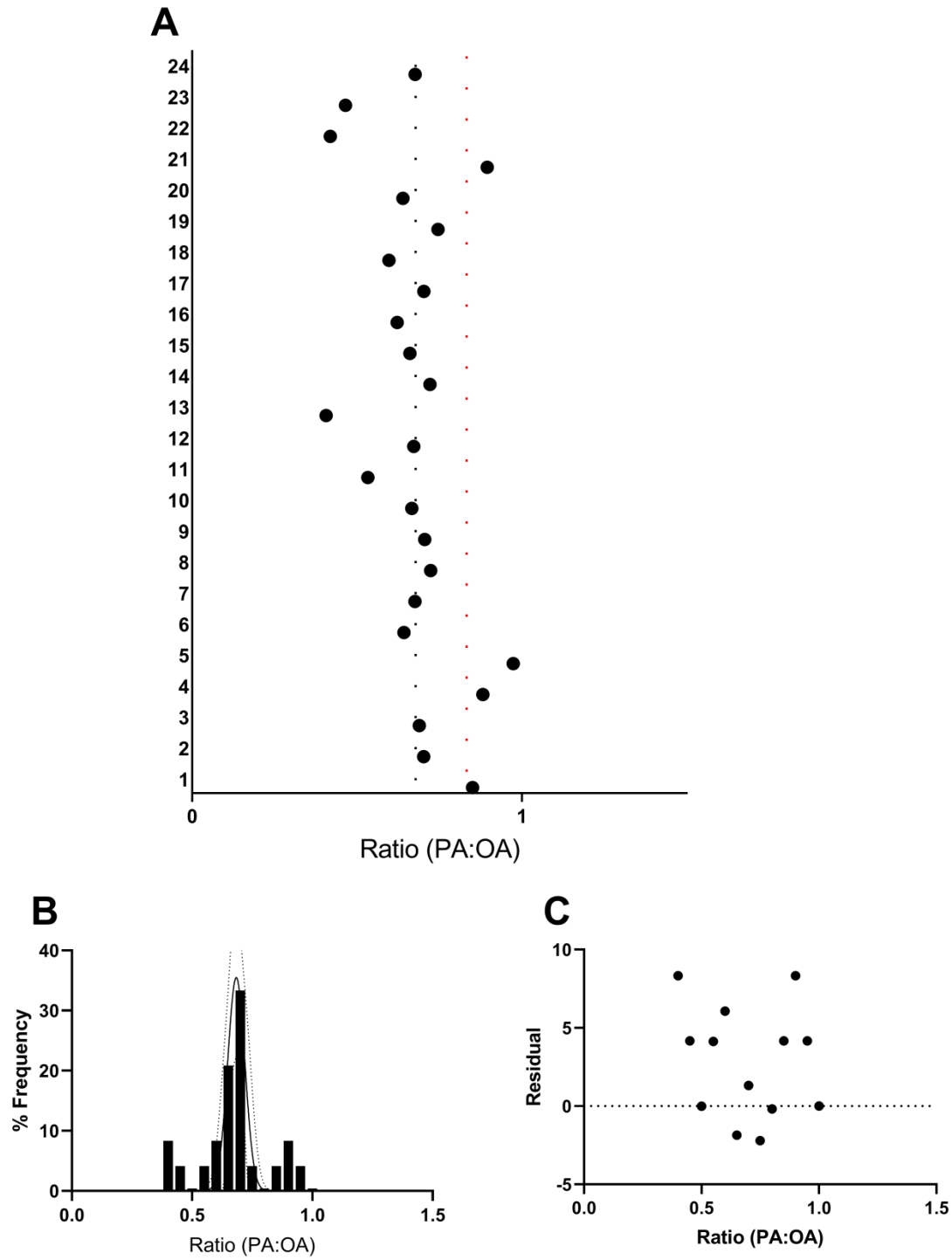


Figure 5.30 Ratio of PA:OA in triacylglycerol

A) Black circles indicate the ratio of PA:OA published by the study. Black dots mark the average of the datasets. Red dots mark the weighted average of the dataset. B) % frequency histogram showing the distribution of data amongst bands of 0.05. The Gaussian curve was fitted to test normality. Confidence intervals show where the Gaussian curve falls with a 95% certainty. C) Residuals plot was used to assess the fit of the Gaussian distribution curve. n=2985

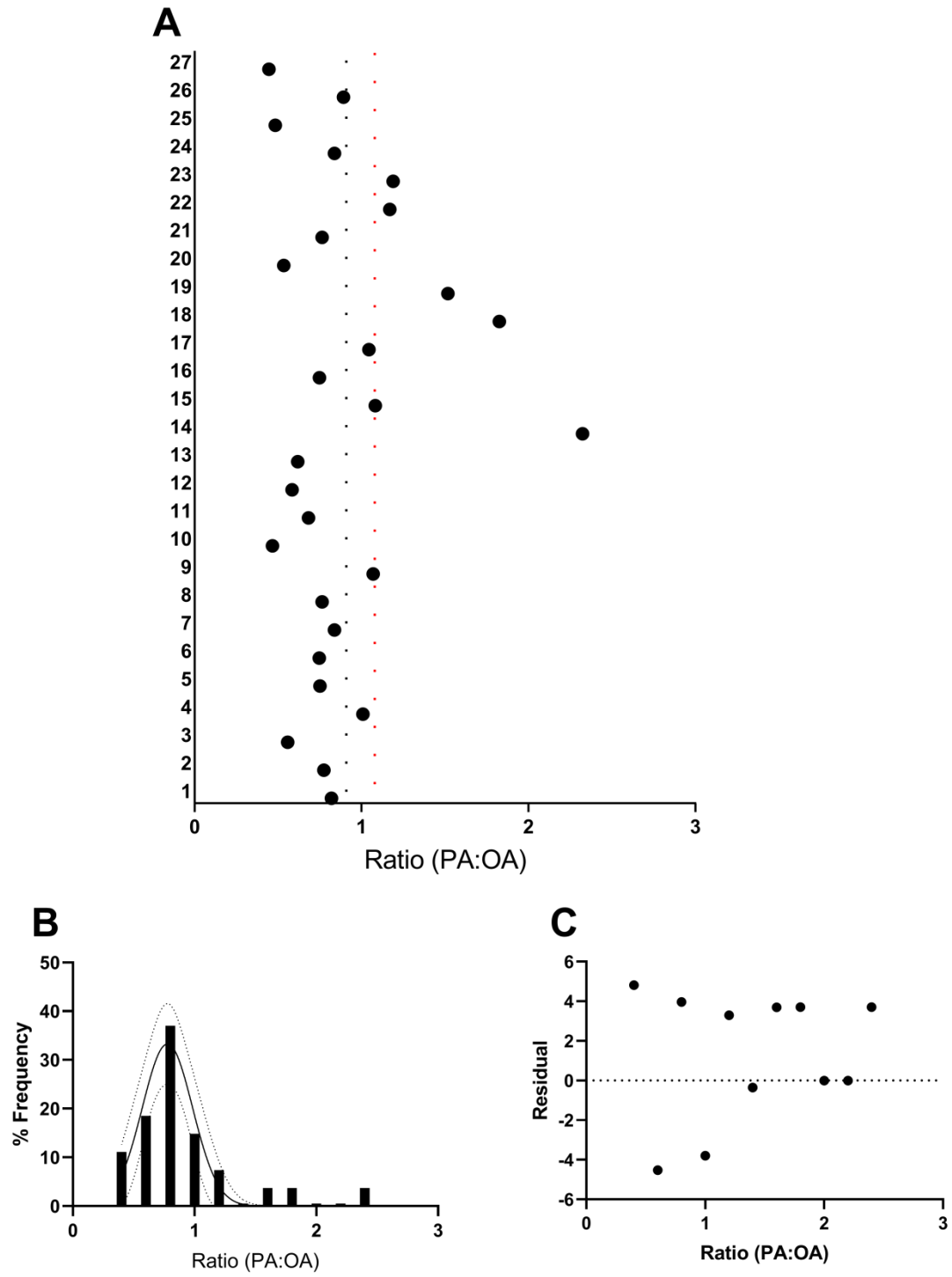


Figure 5.31 Ratio of PA:OA in fatty acids

A) Black circles indicate the ratio of PA:OA published by the study. Black dots mark the average of the datasets. Red dots mark the weighted average of the dataset. B) % frequency histogram showing the distribution of data amongst bands of 0.2. The Gaussian curve was fitted to test normality. Confidence intervals show where the Gaussian curve falls with a 95% certainty. C) Residuals plot was used to assess the fit of the Gaussian distribution curve. n=8675

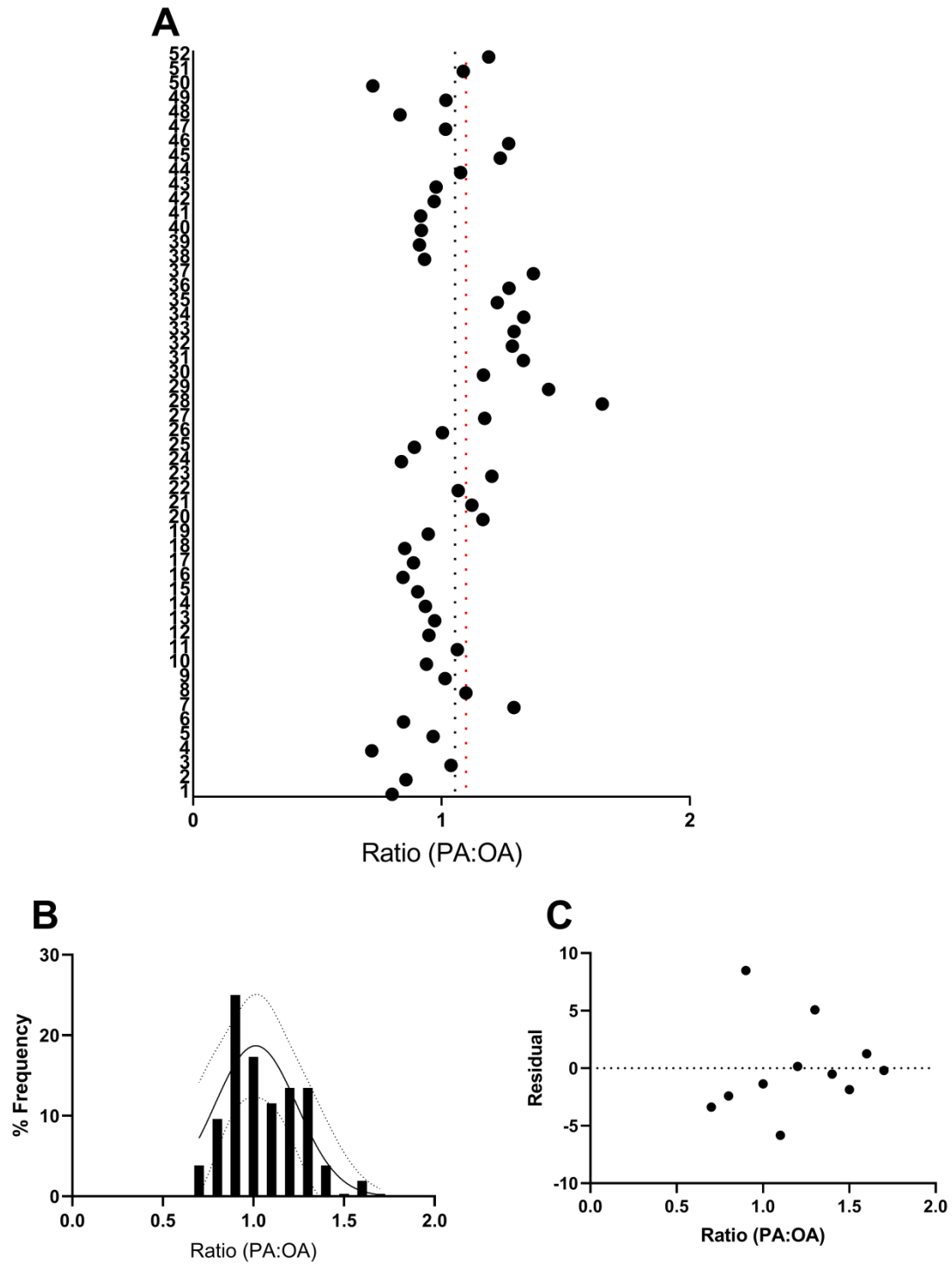


Figure 5.32 Ratio of PA:OA in total plasma lipids

A) Black circles indicate the ratio of PA:OA published by the study. Black dots mark the average of the datasets. Red dots mark the weighted average of the dataset. B) % frequency histogram showing the distribution of data amongst bands of 0.1. The Gaussian curve was fitted to test normality. Confidence intervals show where the Gaussian curve falls with a 95% certainty. C) Residuals plot was used to assess the fit of the Gaussian distribution curve. n=3395

5.3.16 Comparison of lipid profiles in men vs. women

As concentrations of plasma lipids have been linked with the prediction and development of a range of diseases it is important to understand which subpopulations may be at risk. For example, research suggests that plasma triglycerides are a risk factor for the development of coronary heart disease (Iso*, et al.*, 2001), and that there is a higher and earlier incidence of coronary heart disease in the male population (Sanchis-Gomar*, et al.*, 2016). Therefore, its important to establish whether the male population has higher plasma triglyceride levels than the female population, however, research to date has been rather contradictory (Ebbesson*, et al.*, 2015; Glew*, et al.*, 2010; Svanborg*, et al.*, 2009; Yamagishi*, et al.*, 2008).

For this study all BMIs were included, whilst diseased states were excluded. This approach aimed to capture data representative of the general population.

5.3.17 Plasma triglyceride levels in males vs females

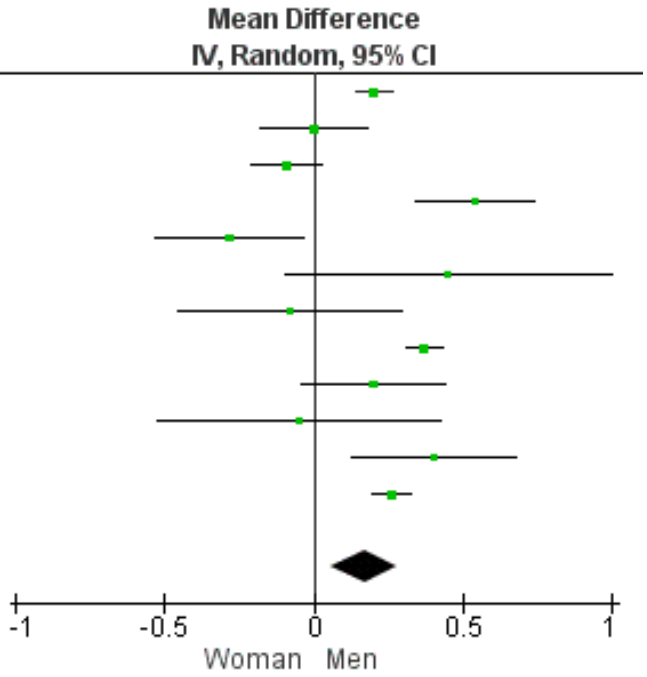
The average plasma triglyceride concentrations from 12 studies were compared between male and female subjects, n=5051 and n=5943, respectively.

Meta-analysis of the data (Table 5.5) demonstrated that men had significantly higher concentrations of plasma triglycerides than women ($p=0.003$) with the average triglyceride concentration in men being almost 3 SD greater than in women ($Z=2.95$).

Table 5.5 Plasma triglycerides - men vs. women

Study or Subgroup	Men Mean	Men SD	Men Total	Woman Mean	Woman SD	Woman Total	Weight	Mean Difference IV, Random, 95% CI
Abdelmagid et al., 2015	1	0.5	327	0.8	0.3	499	12.0%	0.20 [0.14, 0.26]
Baylin et al., 2005	1.21	0.65	99	1.21	0.62	101	9.5%	0.00 [-0.18, 0.18]
Ebbesson et al., 2015	1.42	0.8	337	1.51	0.87914	419	10.9%	-0.09 [-0.21, 0.03]
Fernández-Real et al., 2005	1.33	0.791	76	0.79	0.2712	40	9.0%	0.54 [0.34, 0.74]
Glew et al., 2010	1.03	0.3955	22	1.31	0.5085	29	7.7%	-0.28 [-0.53, -0.03]
Hotta et al., 2000	1.76	1.7636	54	1.31	0.7408	28	3.1%	0.45 [-0.09, 0.99]
Judd et al., 1994	1.26	0.655	29	1.34	0.802	29	5.2%	-0.08 [-0.46, 0.30]
Khaw et al., 2012	2.01	1.15	2246	1.64	1.07	2684	12.0%	0.37 [0.31, 0.43]
Kuriki et al., 2003	1.09	0.44	15	0.89	0.43	79	7.9%	0.20 [-0.04, 0.44]
Svanborg et al., 2009	0.94	0.39	30	0.99	0.4	3	3.8%	-0.05 [-0.52, 0.42]
Vognild et al., 1998	1.5	1.2207	149	1.1	1.1136	124	7.0%	0.40 [0.12, 0.68]
Yamagishi et al., 2008	1.57	1.0622	1667	1.31	0.8249	1908	12.0%	0.26 [0.20, 0.32]
Total (95% CI)			**5051**			**5943**	**100.0%**	**0.17 [0.06, 0.28]**

Heterogeneity: Tau² = 0.03; Chi² = 86.80, df = 11 (P < 0.00001); I² = 87%
Test for overall effect: Z = 2.95 (P = 0.003)



Chi^2 =86.80 was much greater than the degrees of freedom (11), which indicated heterogeneity. However, the number of studies included was too small to produce a reliable result. I^2 =87% estimated high levels of variation amongst study results. Tau^2 =0.03 found small levels of between-study variance. In combination, these results point to a level of heterogeneity greater suited to the use of a random-effects model.

5.3.18 Total palmitic acid and oleic acid levels in men vs. women

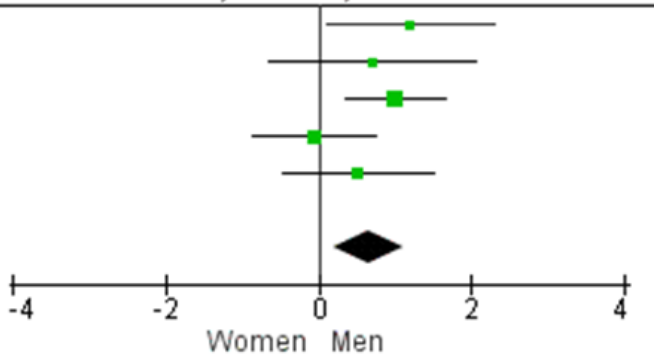
The average molar percentage of palmitic acid and oleic acid across plasma lipids from 3 studies between male and female participants (n=277 and n=266, respectively).

Results (Table 5.6 and 5.7) showed that men had significantly higher molar percentages of total plasma palmitic acid than women (p=0.006), however, there was no significant difference between men and women for molar percentages of total plasma oleic acid (p=0.31). Average mol% of total plasma palmitic acid was over 2 SD higher in men than women (Z=2.73).

Table 5.6 Total palmitic acid (mol%) - men vs. women

	Men			Women				Mean Difference	Mean Difference
Study or Subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, Random, 95% CI	IV, Random, 95% CI
Fernandez-Real et al., 2003	20.13	2.2	64	18.94	3.27	45	15.0%	1.19 [0.09, 2.29]	
Fernández-Real et al., 2003	20.1	2.2	76	19.4	4.1	40	10.3%	0.70 [-0.66, 2.06]	
Mayneris-Perxachs et al., 2014	21.4	2	62	20.4	1.5	49	32.4%	1.00 [0.35, 1.65]	
Melchert et al., 1987	20.02	1.9	38	20.08	2.07	62	24.8%	-0.06 [-0.85, 0.73]	
Melchert et al., 1987	21.48	2.26	37	20.96	2.91	70	17.5%	0.52 [-0.48, 1.52]	
Total (95% CI)			**277**			**266**	**100.0%**	**0.65 [0.18, 1.12]**	

Heterogeneity: Tau² = 0.07; Chi² = 5.18, df = 4 (P = 0.27); I² = 23%
Test for overall effect: Z = 2.73 (P = 0.006)

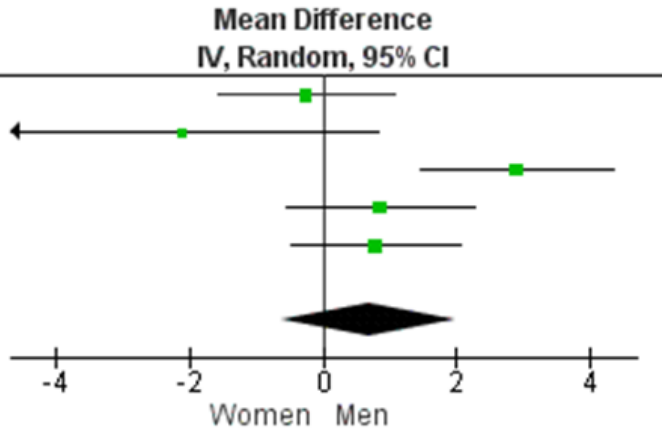


Chi^2 =5.18 was slightly greater than the degrees of freedom (4), which confers acceptable levels of heterogeneity. However, in this instance Chi^2 was not reliable due to the small number of samples. I^2 =23% estimated small amounts of inconsistency amongst study results, which was acceptable. Tau^2 =0.07 also indicated small levels of heterogeneity. However, these results were not significant (p=0.27), and, therefore, not reliable. A random-effects model was used as a precaution to diminish the impact potential heterogeneity, especially as all studies included had a similar number of subjects, thereby reducing small sample bias.

Table 5.7 Total oleic acid (mol%) – men vs. women

Study or Subgroup	Men Mean	Men SD	Men Total	Women Mean	Women SD	Women Total	Weight	Mean Difference IV, Random, 95% CI
Fernandez-Real et al., 2003	20.69	3.37	64	20.95	3.53	45	22.3%	-0.26 [-1.58, 1.06]
Fernández-Real et al., 2003	20.8	3.7	76	22.9	9	40	11.9%	-2.10 [-5.01, 0.81]
Mayneris-Perxachs et al., 2014	26.7	4.1	62	23.8	3.6	49	21.4%	2.90 [1.47, 4.33]
Melchert et al., 1987	19.73	3.89	38	18.87	2.73	62	21.6%	0.86 [-0.55, 2.27]
Melchert et al., 1987	22.86	3.25	37	22.08	3.03	70	22.7%	0.78 [-0.49, 2.05]
Total (95% CI)			**277**			**266**	**100.0%**	**0.68 [-0.63, 1.99]**

Heterogeneity: Tau² = 1.54; Chi² = 14.52, df = 4 (P = 0.006); I² = 72%
Test for overall effect: Z = 1.01 (P = 0.31)



For the comparison of total plasma oleic acid in men vs. women (Figure 20B), Chi^2 =14.52 was greater than the degrees of freedom (4), and thereby indicated heterogeneity. However, the sample size in this study was too small to produce reliable results. I^2 =72% estimated moderate variability amongst study results. Whilst, Tau^2 =1.54 was greater than 1, which confirmed definite heterogeneity. Therefore, a random-effects model was used.

5.3.19 Molar percentage of palmitic acid in phospholipids in men vs. women

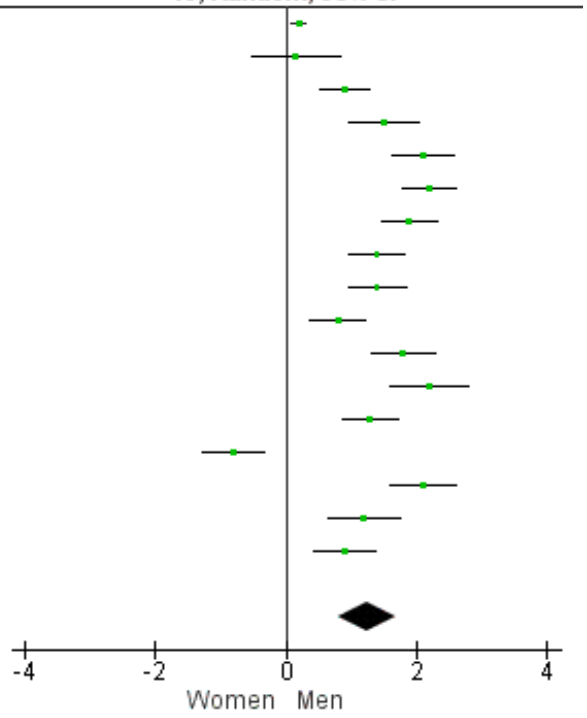
The average molar percentage of palmitic acid in plasma phospholipids between male and female subjects was compared, n=3173 and n=3383, respectively. 17 datasets across 3 studies were analysed.

The results, shown in Table 5.8, demonstrated that men had significantly higher molar percentages of palmitic acid in plasma phospholipids than women ($p<0.00001$). The average mol% of palmitic acid in plasma phospholipids was over 5 SD greater in male participants than female participants ($Z=5.56$).

Table 5.8 Palmitic acid in plasma phospholipids (mol%) - men vs. women

Study or Subgroup	Men Mean	Men SD	Men Total	Women Mean	Women SD	Women Total	Weight	Mean Difference IV, Random, 95% CI
Ma et al., 1995	25.5	1.6	1712	25.3	1.6	1858	6.3%	0.20 [0.09, 0.31]
Nikkari et al., 1995	28.4	1.77	41	28.25	1.33	41	5.5%	0.15 [-0.53, 0.83]
Saadatian-Elahi et al., 2009	25.5	1.3	96	24.6	1.5	100	6.0%	0.90 [0.51, 1.29]
Saadatian-Elahi et al., 2009	26.1	2	95	24.6	1.9	100	5.8%	1.50 [0.95, 2.05]
Saadatian-Elahi et al., 2009	26.1	1.7	90	24	1.7	99	5.9%	2.10 [1.61, 2.59]
Saadatian-Elahi et al., 2009	25	1.4	96	22.8	1.5	100	6.0%	2.20 [1.79, 2.61]
Saadatian-Elahi et al., 2009	24.7	1.6	93	22.8	1.5	100	5.9%	1.90 [1.46, 2.34]
Saadatian-Elahi et al., 2009	25.9	1.3	94	24.5	1.7	99	6.0%	1.40 [0.97, 1.83]
Saadatian-Elahi et al., 2009	25.2	1.4	91	23.8	1.7	100	5.9%	1.40 [0.96, 1.84]
Saadatian-Elahi et al., 2009	26.4	1.6	95	25.6	1.4	96	6.0%	0.80 [0.37, 1.23]
Saadatian-Elahi et al., 2009	26.9	1.6	96	25.1	1.9	100	5.9%	1.80 [1.31, 2.29]
Saadatian-Elahi et al., 2009	24.2	2.2	95	22	2.1	100	5.6%	2.20 [1.60, 2.80]
Saadatian-Elahi et al., 2009	26.6	1.5	94	25.3	1.6	99	5.9%	1.30 [0.86, 1.74]
Saadatian-Elahi et al., 2009	25.5	1.6	100	26.3	1.8	95	5.9%	-0.80 [-1.28, -0.32]
Saadatian-Elahi et al., 2009	25.2	1.8	94	23.1	1.8	100	5.8%	2.10 [1.59, 2.61]
Saadatian-Elahi et al., 2009	26.5	1.6	96	25.3	2.2	96	5.8%	1.20 [0.66, 1.74]
Saadatian-Elahi et al., 2009	25.8	1.4	95	24.9	1.9	100	5.9%	0.90 [0.43, 1.37]
Total (95% CI)			**3173**			**3383**	**100.0%**	**1.25 [0.81, 1.69]**

Heterogeneity: Tau² = 0.80; Chi² = 353.35, df = 16 (P < 0.00001); I² = 95%
Test for overall effect: Z = 5.56 (P < 0.00001)



Chi^2 =353.35 was much greater than the degrees of freedom (16) and, thus, suggested heterogeneity. However, the number of studies included was too small to produce a reliable result. I^2 =95% which strongly suggested variation amongst study results. Tau^2 =0.8 was close to 1, which also strongly indicated heterogeneity. Therefore, a random-effects model was utilise to diminish the impact of between-study variance. Multiple values from Saadatian-Elahi*, et al.,* (2008) represent datasets from different countries.

5.3.20 Molar percentage of oleic acid in phospholipids in men vs. women

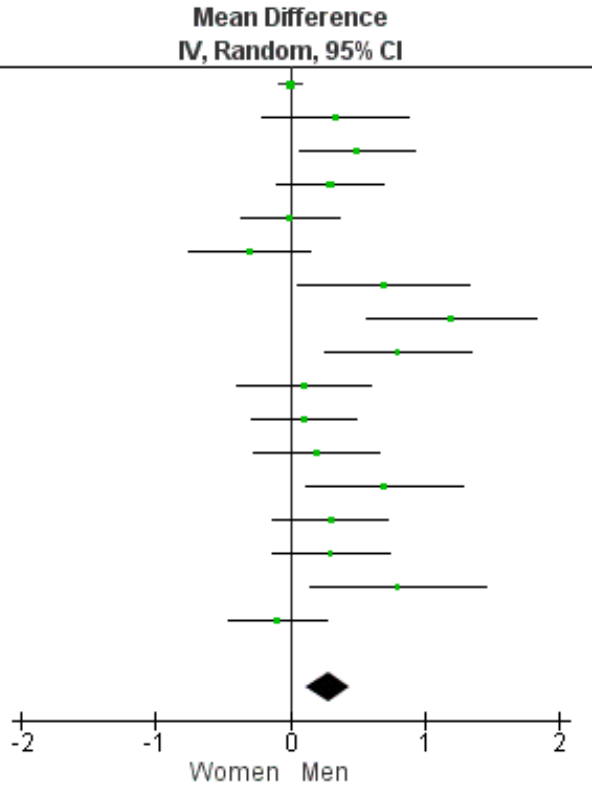
The next comparison looked at the average molar percentage of oleic acid in plasma phospholipids between male and female participants, n=3173 and n=3382, respectively. 17 datasets were analysed from 3 studies.

The meta-analysis (Table 5.9) found that men had significantly higher molar percentages of oleic acid in plasma phospholipids than women (p=0.0007) with the average mol% of the male subjects over 3 SD higher than the average mol% of the female subjects (Z=3.41).

Table 5.9 Oleic acid in plasma phospholipids (mol%) - men vs. women

Study or Subgroup	Men Mean	Men SD	Men Total	Women Mean	Women SD	Women Total	Weight	Mean Difference IV, Random, 95% CI
Ma et al., 1995	8.6	1.2	1712	8.6	1.2	1858	10.4%	0.00 [-0.08, 0.08]
Nikkari et al., 1995	12.48	1.38	41	12.14	1.12	41	4.9%	0.34 [-0.20, 0.88]
Saadatian-Elahi et al., 2009	10.6	1.6	96	10.1	1.4	99	6.3%	0.50 [0.08, 0.92]
Saadatian-Elahi et al., 2009	10.4	1.4	95	10.1	1.4	96	6.6%	0.30 [-0.10, 0.70]
Saadatian-Elahi et al., 2009	11.5	1.2	94	11.5	1.4	99	7.0%	0.00 [-0.37, 0.37]
Saadatian-Elahi et al., 2009	9.3	1.8	95	9.6	1.4	100	5.9%	-0.30 [-0.75, 0.15]
Saadatian-Elahi et al., 2009	12.1	2.1	91	11.4	2.4	100	4.1%	0.70 [0.06, 1.34]
Saadatian-Elahi et al., 2009	11.5	2.2	96	10.3	2.3	100	4.2%	1.20 [0.57, 1.83]
Saadatian-Elahi et al., 2009	12	2	90	11.2	1.8	99	4.9%	0.80 [0.26, 1.34]
Saadatian-Elahi et al., 2009	12.7	1.5	94	12.6	2	100	5.4%	0.10 [-0.40, 0.60]
Saadatian-Elahi et al., 2009	10.5	1.4	95	10.4	1.4	100	6.6%	0.10 [-0.29, 0.49]
Saadatian-Elahi et al., 2009	11.7	1.8	96	11.5	1.5	96	5.7%	0.20 [-0.27, 0.67]
Saadatian-Elahi et al., 2009	12.2	2.1	93	11.5	2	100	4.6%	0.70 [0.12, 1.28]
Saadatian-Elahi et al., 2009	10.6	1.7	96	10.3	1.3	100	6.2%	0.30 [-0.12, 0.72]
Saadatian-Elahi et al., 2009	10.3	1.6	95	10	1.5	100	6.1%	0.30 [-0.14, 0.74]
Saadatian-Elahi et al., 2009	11.2	2.3	94	10.4	2.3	99	4.0%	0.80 [0.15, 1.45]
Saadatian-Elahi et al., 2009	10.9	1.3	100	11	1.3	95	7.0%	-0.10 [-0.47, 0.27]
Total (95% CI)			**3173**			**3382**	**100.0%**	**0.29 [0.12, 0.45]**

Heterogeneity: Tau² = 0.07; Chi² = 47.47, df = 16 (P < 0.0001); I² = 66%
Test for overall effect: Z = 3.41 (P = 0.0007)



Chi^2 =47.47 was much larger than the degrees of freedom (16) and consequently indicated heterogeneity. However, the result was unreliable due to the small number of studies included. I^2 =66% which indicated moderate variability amongst study results. Tau^2 =0.07 suggested small levels of heterogeneity. In combination these results suggested levels of heterogeneity which were better suited to a random-effects model in order to combat the impact of between-study variance. Multiple values from Saadatian-Elahi*, et al.,* (2008) represent datasets from different countries.

5.3.21 Lipid profiles in non-obese vs overweight/obese vs type II diabetic subjects

5.3.22 Plasma triglyceride levels in non-obese vs. obese subjects

Having established concentration ranges of triglycerides and fatty acids in their various plasma constituents, the next step was to compare average total plasma triglyceride concentrations in non-obese versus overweight/obese subjects without medical conditions. Data was extracted from 21 studies with non-obese and overweight/obese participants, n=692 and n=658, respectively.

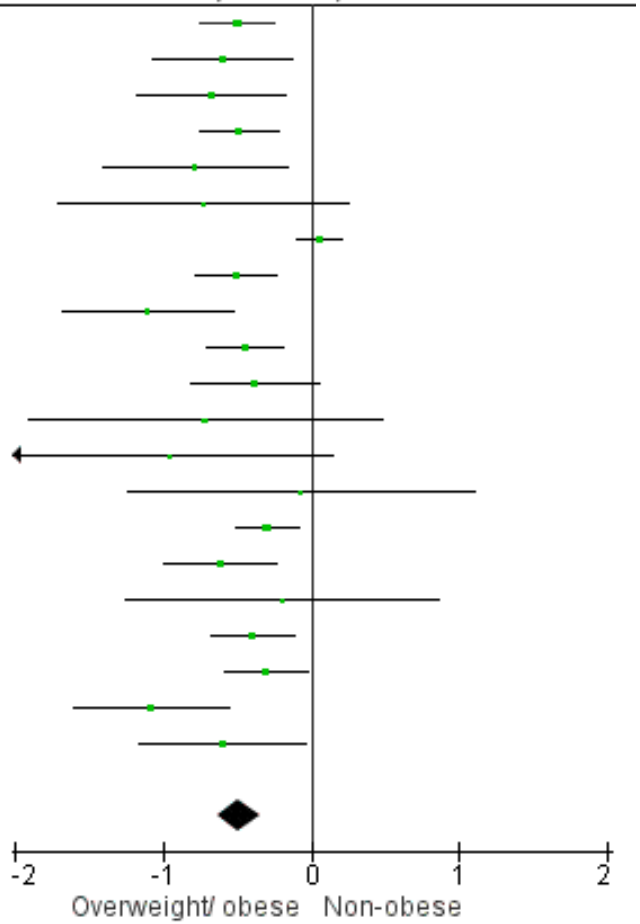
Meta-analysis of the data demonstrated that overweight/obese subjects had significantly higher concentrations of plasma triglyceride than non-obese subjects ($p<0.00001$) (Table 5.10). The average plasma triglyceride concentration of overweight/obese participants was over 6 SD greater than the average plasma triglyceride concentration of non-obese participants ($Z=6.78$).

Table 5.10 Plasma triglyceride levels – non-obese vs. overweight/obese

	Non-obese			Overweight/ obese				Mean Difference
Study or Subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, Random, 95% CI
Buemann et al., 2006	1.41	0.87	269	1.91	1.55	174	7.3%	-0.50 [-0.75, -0.25]
de Jongh et al., 2004	0.8	0.3	16	1.4	0.8	12	4.6%	-0.60 [-1.08, -0.12]
Després et al., 1990	0.79	0.35	25	1.47	0.79	10	4.3%	-0.68 [-1.19, -0.17]
Fernández-Real et al., 2003	1.1	0.5085	64	1.59	0.9831	70	7.1%	-0.49 [-0.75, -0.23]
Frias et al., 2000	1.11	0.24	9	1.9	0.93	9	3.3%	-0.79 [-1.42, -0.16]
Goodpaster et al., 2002	1.26	0.4762	7	1.99	1.2435	7	1.7%	-0.73 [-1.72, 0.26]
Jocken et al., 2008	0.701	0.238	13	0.648	0.1328	10	8.5%	0.05 [-0.10, 0.21]
Kawashima et al., 2009	1.08	0.5198	27	1.59	0.6215	43	7.0%	-0.51 [-0.78, -0.24]
Kruszynska et al., 2003	0.95	0.3873	15	2.06	1.07	15	3.7%	-1.11 [-1.69, -0.53]
Manco et al., 2004	1.1	0.2236	5	1.55	0.2012	5	7.1%	-0.45 [-0.71, -0.19]
Manning et al., 2008	1.07	0.42	14	1.45	0.75	15	5.0%	-0.38 [-0.82, 0.06]
Pollare et al., 1991	1.42	0.59	7	2.14	1.51	7	1.2%	-0.72 [-1.92, 0.48]
Ranganath et al., 1999	1.03	0.6879	7	1.99	1.3229	7	1.4%	-0.96 [-2.06, 0.14]
Riedel et al., 1995	1.86	1.25	5	1.93	0.48	5	1.3%	-0.07 [-1.24, 1.10]
Stefan et al., 2008	1.08	0.4409	54	1.38	1.0379	133	7.8%	-0.30 [-0.51, -0.09]
Straczkowski et al., 2006	1.02	0.52	33	1.64	0.93	30	5.7%	-0.62 [-1.00, -0.24]
Sundell et al., 2003	1.1	0.5	10	1.3	1.64	10	1.5%	-0.20 [-1.26, 0.86]
van der Merwe et al., 2001	0.7	0.1897	10	1.1	0.4111	10	6.9%	-0.40 [-0.68, -0.12]
Vanttinen et al., 2005	1.03	0.54	72	1.34	0.94	52	6.8%	-0.31 [-0.59, -0.03]
Woods et al., 2009	0.79	0.2949	13	1.88	1.0848	18	4.1%	-1.09 [-1.62, -0.56]
Wuesten et al., 2005	1.06	0.6	17	1.66	0.99	16	3.8%	-0.60 [-1.16, -0.04]
Total (95% CI)			**692**			**658**	**100.0%**	**-0.49 [-0.63, -0.35]**

Heterogeneity: $Tau^2 = 0.06$; $Chi^2 = 55.22$, df = 20 ($P < 0.0001$); $I^2 = 64\%$

Test for overall effect: $Z = 6.78$ ($P < 0.00001$)



The Chi^2 was calculated at 55.22, which was much larger than the degrees of freedom (20), indicating heterogeneity. However, the number of studies included was not large enough to produce a reliable Chi^2. I^2 was equal to 64%, indicating moderate inconsistency in study results. Tau^2 is the most reliable measure of heterogeneity, and a value of 0.06 was derived, which suggested that small amounts of between-study variance existed. Together, these measures indicated that samples of the population used in each study varied between studies, producing heterogeneity, therefore, a random-effects model was used.

The forest plot in Table 5.10 demonstrated that obese subjects had higher plasma triglyceride concentrations than non-obese subjects, however, it was important to identify whether a direct correlation between BMI and plasma triglyceride levels existed.

Results found a moderate, statistically significant positive correlation between BMI and plasma triglyceride concentrations (r=0.5608, P<0.0001).

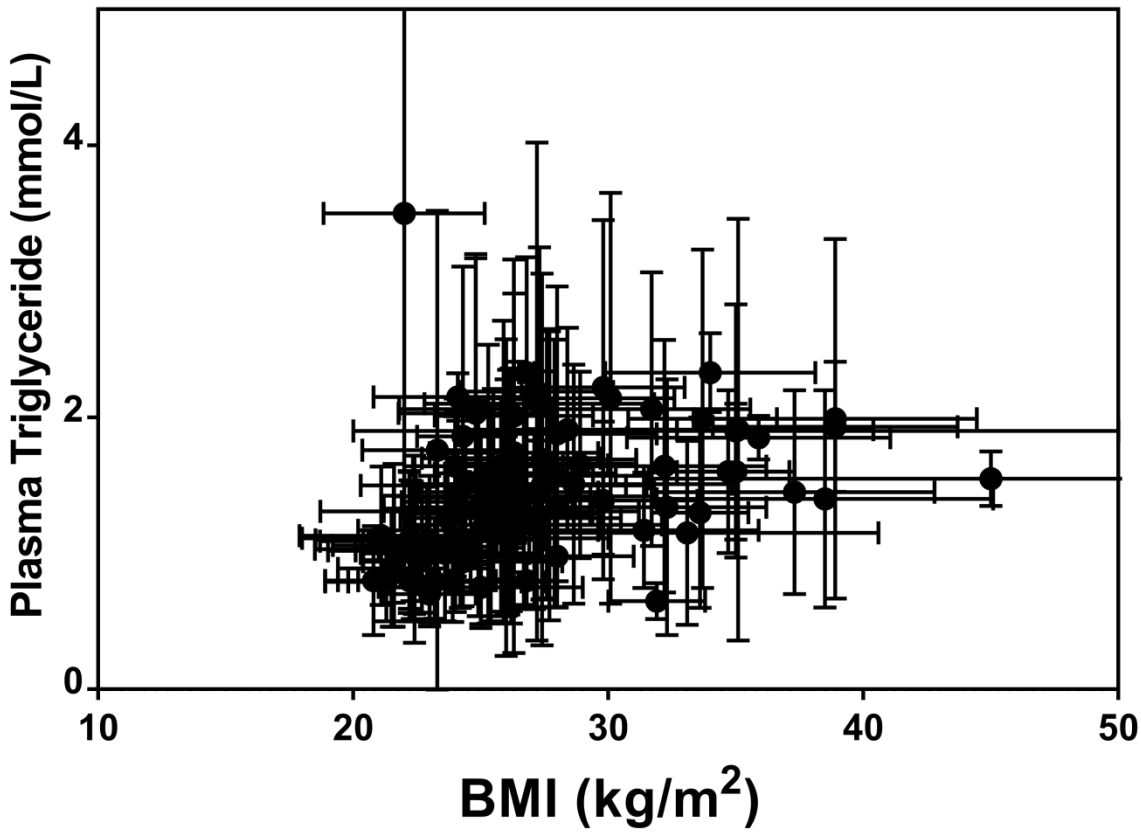


Figure 5.33 Correlation between BMI and plasma triglycerides

Analysis of the correlation between BMI and plasma triglycerides found a positive correlation between the two (r=0.5608, P<0.0001) (n=100).

5.3.23 Plasma free fatty acid levels in non-obese vs. obese subjects

This study compared average plasma free fatty acid concentrations from 44 studies between non-obese and overweight/obese participants, n=864 and n=854, respectively.

Results of the meta-analysis are shown in Table 5.11, and showed that overweight/obese subjects had significantly higher concentrations of plasma free fatty acids than non-obese subjects ($p<0.00001$). The average plasma FFA concentration of overweight/obese participants was over 7 SD higher than the average plasma FFA concentration of non-obese participants ($Z=7.06$).

Table 5.11 Plasma free fatty acids – non-obese vs. overweight/obese

	Non-obese			Overweight/ obese				Mean Difference	Mean Difference
Study or Subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, Random, 95% CI	IV, Random, 95% CI
Adams et al., 2004	630	221.3594362	10	880	221.3594362	10	2.0%	-250.00 [-444.03, -55.97]	
Allick et al., 2004	540	97.9796	6	650	171.4643	6	2.3%	-110.00 [-268.02, 48.02]	
Belfiore et al., 2001	359	216.1533867	34	713	242.6083566	27	2.8%	-354.00 [-470.85, -237.15]	
Buemann et al., 2006	359	142	269	393	161	174	3.5%	-34.00 [-63.33, -4.67]	
Chen et al., 1987	572	108	6	782	184	6	2.2%	-210.00 [-380.72, -39.28]	
de Jongh et al., 2004	550	190	16	690	210	12	2.4%	-140.00 [-290.95, 10.95]	
Felber et al., 1987	383	78	9	580	134.7219	6	2.7%	-197.00 [-316.24, -77.76]	
Frias et al., 2000	240	150	9	360	240	9	2.0%	-120.00 [-304.90, 64.90]	
Gautier et al., 2000	640	360	11	920	340	11	1.2%	-280.00 [-572.63, 12.63]	
Gautier et al., 2001	990	250	10	850	130	10	2.1%	140.00 [-34.65, 314.65]	
Golay et al., 2002	630	174.2843	15	732	214.7744	12	2.4%	-102.00 [-252.15, 48.15]	
Goodpaster et al., 2002	474	132.2876	7	599	171.9738	7	2.3%	-125.00 [-285.73, 35.73]	
Groop et al., 1992	642	129.3483	11	793	113.7673	7	2.8%	-151.00 [-264.78, -37.22]	
Horton et al., 1995	320.8	140.55	9	299.1	143.0293	7	2.5%	21.70 [-118.51, 161.91]	
Ianello et al., 1998	373	139.1402	10	721	260	25	2.6%	-348.00 [-481.51, -214.49]	
Jansson et al., 1998	494	162.6653	15	696	207	9	2.3%	-202.00 [-360.32, -43.68]	
Jocken et al., 2008	661	147.8276	13	638	132.8156	10	2.8%	23.00 [-92.04, 138.04]	
Kruszynska et al., 2003	445	209.1411	15	404	170.4112	15	2.5%	41.00 [-95.52, 177.52]	
Macor et al., 1997	434	105	9	747	229.4559	26	2.8%	-313.00 [-424.74, -201.26]	
Manco et al., 2004	540	156.525	5	580	156.525	5	2.0%	-40.00 [-234.03, 154.03]	
Manning et al., 2008	410	190	14	420	140	15	2.7%	-10.00 [-132.17, 112.17]	
Mari et al., 2006	230	60	22	410	90	12	3.3%	-180.00 [-236.76, -123.24]	
Morin-Papunen et al., 2000	460	907.0832	17	550	948.3142	17	0.4%	-90.00 [-713.81, 533.81]	
Owen et al., 1992	402	138.6362	5	495	125.2198	5	2.3%	-93.00 [-256.75, 70.75]	
Paquot et al., 2002	430	411.0961	10	850	489.8979	6	0.6%	-420.00 [-887.52, 47.52]	

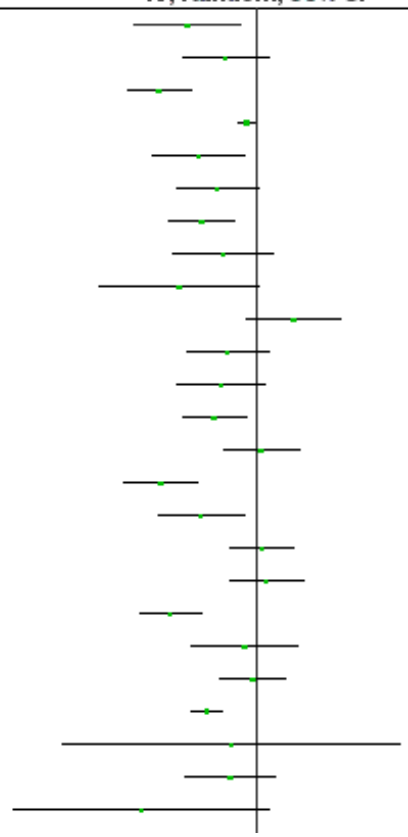
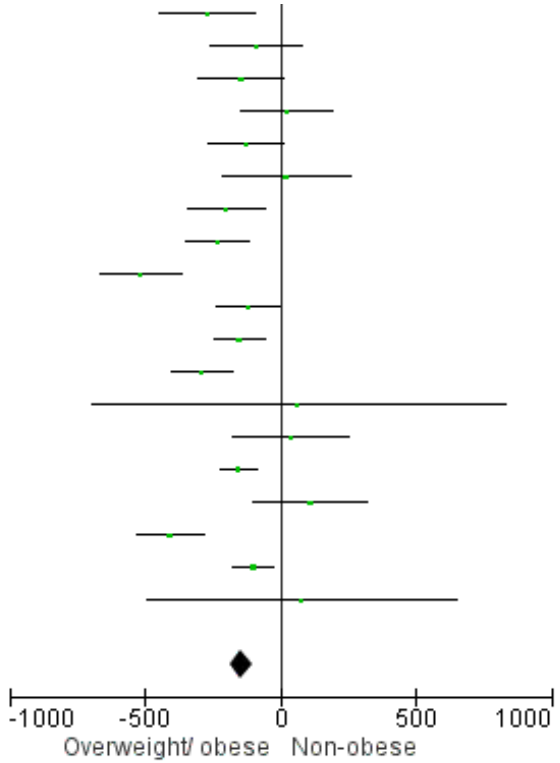


Table 5.11 continued

Pincelli et al., 2001	434	144.9609	6	704	212.8529	10	2.1%	-270.00 [-445.66, -94.34]	
Pollare et al., 1991	410	180	7	500	140	7	2.2%	-90.00 [-258.93, 78.93]	
Ranganath et al., 1999	575	158.7451	7	720	142.87	7	2.3%	-145.00 [-303.21, 13.21]	
Reeds et al., 2006	408	162.1149	5	383	103.7536	5	2.2%	25.00 [-143.71, 193.71]	
Reyna et al., 2008	351	97.8928	7	479	166.8773	8	2.5%	-128.00 [-264.50, 8.50]	
Riedel et al., 1995	940	210	5	920	170	5	1.6%	20.00 [-216.82, 256.82]	
Robertson et al., 1992	770	190	14	970	210	16	2.5%	-200.00 [-343.16, -56.84]	
Santomauro et al., 1999	329	84	9	560	187.4887	13	2.8%	-231.00 [-346.75, -115.25]	
Sbraccia et al., 2002	223	51.8614	10	738	277.631	14	2.4%	-515.00 [-663.94, -366.06]	
Serlie et al., 2007	430	117.5	8	550	105	6	2.8%	-120.00 [-237.00, -3.00]	
Solini et al., 1997	575	111	9	728	102.8154	11	3.0%	-153.00 [-247.61, -58.39]	
Soriguer et al., 2008	315	103.923	12	605	147	9	2.8%	-290.00 [-402.61, -177.39]	
Stefan et al., 2008	710	2,383.6824	54	645	2,769.377	164	0.3%	65.00 [-699.10, 829.10]	
Stojiljkovic et al., 2002	707	207.8461	12	669	290.9295	10	1.8%	38.00 [-177.28, 253.28]	
Straczkowski et al., 2006	347	116	33	504	155	30	3.2%	-157.00 [-225.14, -88.86]	
Sundell et al., 2003	610	300	10	500	150	10	1.8%	110.00 [-97.89, 317.89]	
van der Merwe et al., 2001	463	151.7893	10	870	132.8157	10	2.7%	-407.00 [-532.01, -281.99]	
Vanttinen et al., 2005	530	190	72	630	220	52	3.2%	-100.00 [-174.17, -25.83]	
Wuesten et al., 2005	540	1,030.776	17	460	600	16	0.4%	80.00 [-491.42, 651.42]	
Total (95% CI)			**864**			**852**	**100.0%**	**-145.42 [-185.80, -105.04]**	

Heterogeneity: Tau² = 11869.91; Chi² = 188.87, df = 43 (P < 0.00001); I² = 77%
Test for overall effect: Z = 7.06 (P < 0.00001)



Chi^2 =188.87 was much larger than the degrees of freedom (43) and, thus, indicated heterogeneity. I^2 =77% indicated moderate inconsistency in study results. Tau^2 =11869.91 was far greater than 1, thereby confirming definite heterogeneity between the included studies, therefore, a random-effects model was selected.

The forest plot in Table 5.11 found that obese subjects had higher plasma free fatty acid concentrations than non-obese subjects. But, it was imperative to study the relationship BMI and plasma free fatty acid concentrations.

The study revealed a small, yet statistically significant positive correlation between BMI and plasma free fatty acid concentrations (r=0.3734, P=0.0001).

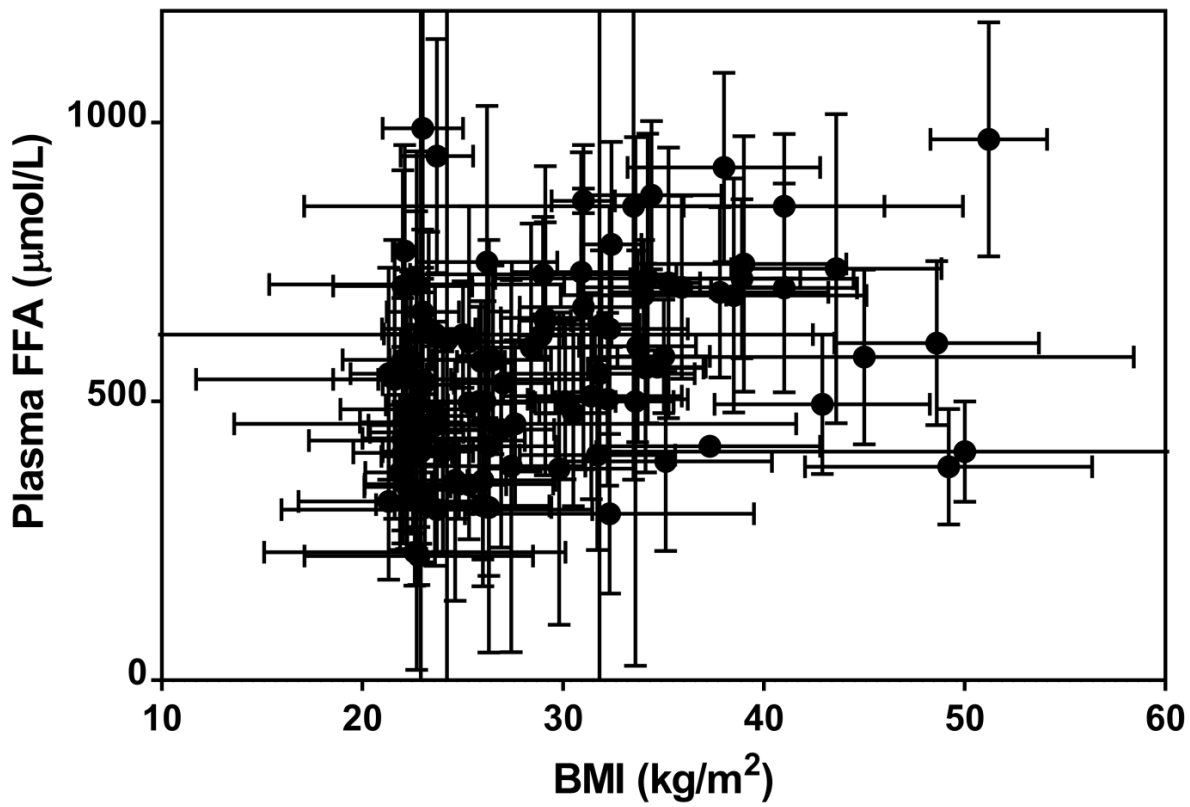


Figure 5.34 Correlation between BMI and plasma free fatty acids

Analysis of the correlation between BMI and plasma free fatty acids found a positive correlation between the two (r=0.3734, P=0.0001) (n=103).

5.3.24 Plasma triglyceride levels in non-obese and obese subjects vs. type II diabetics

Following on from the comparison of triglyceride levels in non-obese vs. obese subjects (Table 5.10), this study compared average plasma triglyceride concentrations from 11 studies between non-obese participants without medical conditions and participants with type II diabetes regardless of BMI, n=8688 and n=1093, respectively. Average plasma triglyceride concentrations were also compared across 11 studies between overweight/obese participants without medical conditions and participants with type II diabetes regardless of BMI, n=2646 and n=2069, respectively.

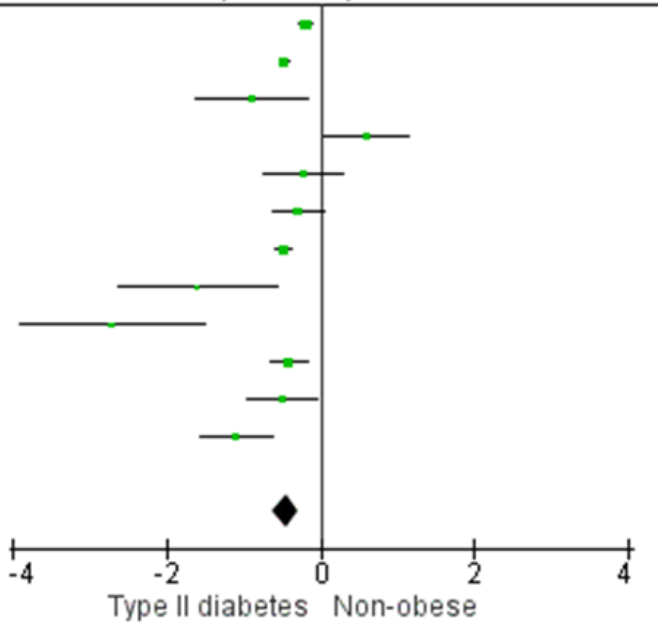
The meta-analysis (Table 5.12) found that subjects with type II diabetes had significantly higher concentrations of plasma triglyceride than both non-obese and overweight/obese controls without type II diabetes ($p<0.00001$). The average concentrations of plasma triglyceride in participants with type II diabetes was over 5 SD greater than both control groups ($Z=5.27$ non-obese, $Z=5.97$ overweight/obese).

Table 5.12 Plasma triglycerides – non-obese vs. type II diabetes

	Non-obese			Type II diabetes				Mean Difference
Study or Subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, Random, 95% CI
Brancatti et al., 2000	1.08	0.7255	1316	1.27	0.6847	293	14.9%	-0.19 [-0.28, -0.10]
Brancatti et al., 2000	1.29	0.6798	4621	1.77	0.6134	418	15.2%	-0.48 [-0.54, -0.42]
Frias et al., 2000	1.11	0.24	9	2.01	1.11	9	3.9%	-0.90 [-1.64, -0.16]
Herrero et al., 2006	1.5	0.69	11	0.9	0.6	11	6.0%	0.60 [0.06, 1.14]
Hotta et al., 2000	1.76	1.7636	54	1.99	1.0866	82	6.2%	-0.23 [-0.76, 0.30]
Hotta et al., 2000	1.31	0.7408	28	1.6	0.6691	37	9.3%	-0.29 [-0.64, 0.06]
Laaksonen et al., 2002	1.17	0.21	806	1.65	0.315	34	14.6%	-0.48 [-0.59, -0.37]
Pollare et al., 1991	1.42	0.59	7	3.02	1.28	7	2.2%	-1.60 [-2.64, -0.56]
Prior et al., 2005	0.75	0.3	19	3.47	2.78	21	1.8%	-2.72 [-3.92, -1.52]
Stefan et al., 2008	1.08	0.4409	54	1.49	1.0778	96	11.7%	-0.41 [-0.66, -0.16]
van der Merwe et al., 2001	0.7	0.1897	10	1.2	0.7273	10	7.1%	-0.50 [-0.97, -0.03]
Vessby et al., 1994	2.06	1.14	1753	3.16	2.09	75	7.0%	-1.10 [-1.58, -0.62]
Total (95% CI)			**8688**			**1093**	**100.0%**	**-0.46 [-0.62, -0.29]**

Heterogeneity: $Tau^2 = 0.05$; $Chi^2 = 74.23$, df = 11 (P < 0.00001); $I^2 = 85\%$

Test for overall effect: Z = 5.29 (P < 0.00001)



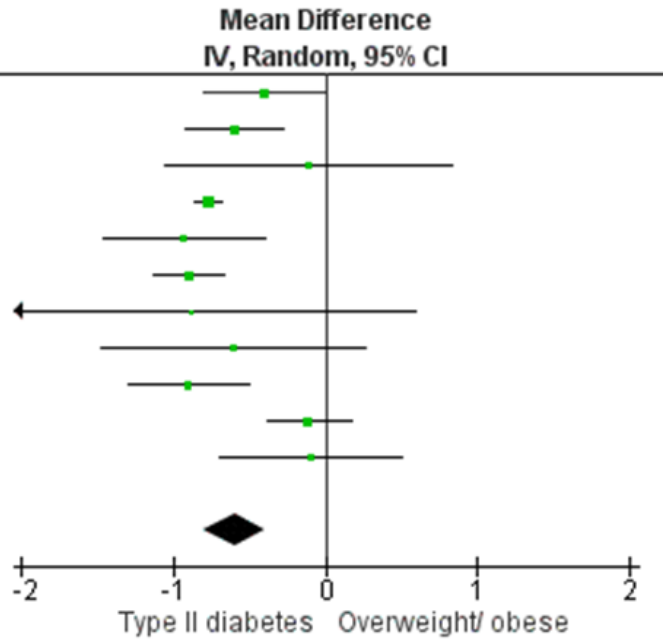
Chi^2 =74.23 was much greater than the degrees of freedom (11), which indicated heterogeneity. However, the number of studies included was too small to produce a reliable result. I^2 =85% indicated high levels of inconsistency amongst study results. Tau^2 =0.05 suggested a small level of heterogeneity, however, in combination these results indicated the presence of between-study variance and hence a random-effects model was chosen.

Table 5.13 Plasma triglycerides – overweight/obese vs. type II diabetes

Study or Subgroup	Overweight/ obese Mean	SD	Total	Type II diabetes Mean	SD	Total	Weight	Mean Difference IV, Random, 95% CI
Carpentier et al., 2000	1.17	0.4233	7	1.57	0.3439	7	10.1%	-0.40 [-0.80, 0.00]
Chen et al., 2006	1.3	0.8	59	1.9	1	61	11.9%	-0.60 [-0.92, -0.28]
Frias et al., 2000	1.9	0.93	9	2.01	1.11	9	3.5%	-0.11 [-1.06, 0.84]
Isomaa et al., 2001	1.29	0.8	1988	2.06	1.6	1697	17.1%	-0.77 [-0.85, -0.69]
Liu et al., 2013	1.17	0.69	60	2.1	2.49	96	7.8%	-0.93 [-1.46, -0.40]
Nappo et al., 2002	0.8	0.2	20	1.7	0.5	20	14.1%	-0.90 [-1.14, -0.66]
Pollare et al., 1991	2.14	1.51	7	3.02	1.28	7	1.6%	-0.88 [-2.35, 0.59]
Rijzewijk et al., 2008	1	0.6614	28	1.6	2.6199	38	4.0%	-0.60 [-1.47, 0.27]
Salomaa et al., 1990	1.5	0.84	325	2.4	1.11	31	10.2%	-0.90 [-1.30, -0.50]
Stefan et al., 2008	1.38	1.0379	133	1.49	1.0778	96	13.0%	-0.11 [-0.39, 0.17]
van der Merwe et al., 2001	1.1	0.4111	10	1.2	0.7273	7	6.7%	-0.10 [-0.70, 0.50]
Total (95% CI)			**2646**			**2069**	**100.0%**	**-0.60 [-0.79, -0.40]**

Heterogeneity: Tau² = 0.06; Chi² = 32.18, df = 10 (P = 0.0004); I² = 69%

Test for overall effect: Z = 5.97 (P < 0.00001)



Chi^2 =32.18 was greater than the degrees of freedom (10), which suggested heterogeneity. However these results were unreliable in a sample of this size. I^2 =69% which suggested moderate variability amongst study results. Tau^2 =0.06 indicated small amounts of between-study variance, however, when amalgamated these results indicated a level of heterogeneity that was greater suited to the use of a random-effects model.

5.3.25 Plasma free fatty acid levels in non-obese and obese subjects vs. type II diabetics

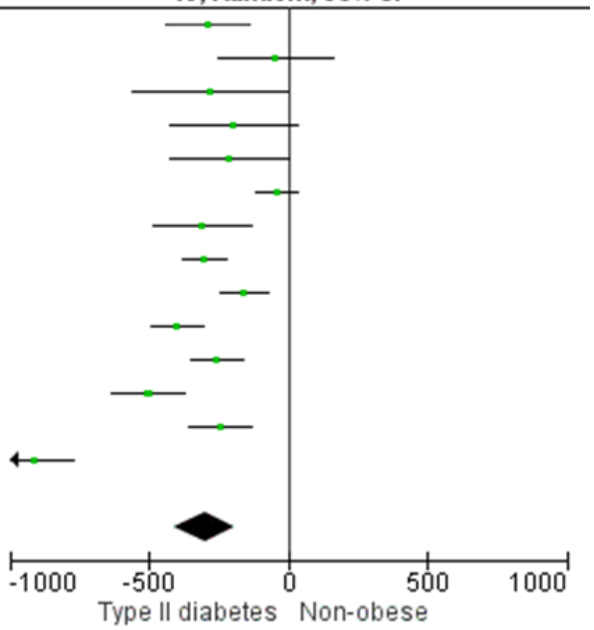
Following on from the comparison of FFA levels in non-obese vs obese subjects, this study compared average free fatty acid concentrations from 14 studies between non-obese participants and participants with type II diabetes, n=178 and n=152, respectively. Average plasma FFA concentrations were also compared across 13 studies between overweight/obese subjects and participants with type II diabetes, n=153 and n=151, respectively.

The results (Table 5.14 and 5.15) showed that participants with type II diabetes had significantly higher concentrations of plasma FFA than non-obese participants without type II diabetes ($p<0.00001$). Furthermore, subjects with type II diabetes also had significantly higher concentrations of plasma FFA than overweight/obese participants without type II diabetes ($p=0.003$).The average concentration of plasma FFA in participants with type II diabetes was almost 3 SD more than the average plasma FFA concentration for the overweight/obese controls ($Z=2.99$), and over 5 SD greater than the average of the non-obese control group ($Z=5.37$).

Table 5.14 Plasma free fatty acids – non-obese vs. type II diabetes

	Non-obese			Type II diabetes				Mean Difference	Mean Difference
Study or Subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, Random, 95% CI	IV, Random, 95% CI
Belfiore et al., 2001	359	216.1533867	34	648.64	216.3103	11	7.2%	-289.64 [-436.67, -142.61]	
Boden et al., 1998	597	222.2431	7	643	166.6823	7	6.4%	-46.00 [-251.80, 159.80]	
Carlsten et al., 2009	854	205.5	9	1,133.4	432.6663	12	5.4%	-279.40 [-558.60, -0.20]	
Chen et al., 1987	572	108	6	767	269	6	6.0%	-195.00 [-426.94, 36.94]	
Felber et al., 1987	575	259.2836	7	785	122.4745	6	6.3%	-210.00 [-425.63, 5.63]	
Fraze et al., 1985	306	81.3327	15	346	127.8085	15	8.0%	-40.00 [-116.66, 36.66]	
Golay et al., 2002	630	174.2843	15	940	246.6577	10	6.8%	-310.00 [-486.49, -133.51]	
Mari et al., 2006	230	60	22	530	130	11	8.0%	-300.00 [-380.81, -219.19]	
Prior et al., 2005	620	95.8958	19	780	174.1379	21	7.9%	-160.00 [-246.06, -73.94]	
Reyna et al., 2008	351	97.8928	7	750	108.5081	14	7.9%	-399.00 [-491.14, -306.86]	
Santomauro et al., 1999	329	84	9	584	129.3484	11	7.8%	-255.00 [-349.10, -160.90]	
Soriguer et al., 2008	315	103.923	12	817	211.3102	12	7.4%	-502.00 [-635.23, -368.77]	
Swislocki et al., 1987	533	36.7423	6	775	134.7219	6	7.7%	-242.00 [-353.74, -130.26]	
van der Merwe et al., 2001	463	151.7893	10	1,373	173.9253	10	7.3%	-910.00 [-1053.08, -766.92]	
Total (95% CI)			**178**			**152**	**100.0%**	**-297.84 [-406.49, -189.18]**	

Heterogeneity: Tau² = 36923.47; Chi² = 143.28, df = 13 (P < 0.00001); I² = 91%
Test for overall effect: Z = 5.37 (P < 0.00001)

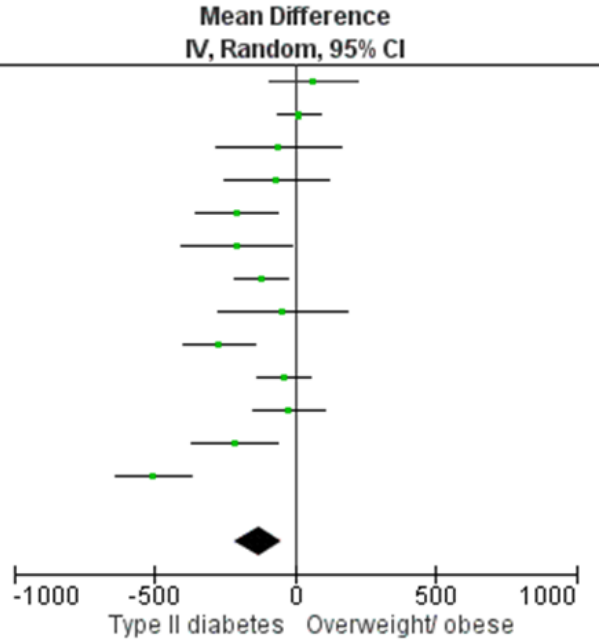


Chi^2 =143.28 was much larger than the degrees of freedom (13), which strongly suggested heterogeneity. However, the number of studies included was not large enough to produce a dependable result. I^2 =91% indicated very high levels of inconsistency amongst study results. Tau^2 =36924.47 was vastly greater than 1, which confirmed definite heterogeneity.

Table 5.15 Plasma free fatty acids – overweight/obese vs. type II diabetes

Study or Subgroup	Overweight/ obese Mean	SD	Total	Type II diabetes Mean	SD	Total	Weight	Mean Difference IV, Random, 95% CI
Belfiore et al., 2001	713	242.6083566	27	648.64	216.3103	11	7.5%	64.36 [-92.85, 221.57]
Blaak et al., 2000	724	66	8	710	87	8	9.4%	14.00 [-61.67, 89.67]
Carpentier et al., 2000	510	185.2026	7	570	238.1176	7	6.0%	-60.00 [-283.47, 163.47]
Chen et al., 1987	782	184	6	848	149	6	6.7%	-66.00 [-255.45, 123.45]
Felber et al., 1987	580	134.7219	6	785	122.4745	6	7.8%	-205.00 [-350.68, -59.32]
Golay et al., 2002	732	214.7744	12	940	246.6577	10	6.6%	-208.00 [-403.29, -12.71]
Mari et al., 2006	410	90	12	530	130	11	9.1%	-120.00 [-212.17, -27.83]
Mensink et al., 2001	727	182.25568	7	770	251.3464	7	5.8%	-43.00 [-273.00, 187.00]
Reyna et al., 2008	479	166.8773	8	750	108.5081	14	8.2%	-271.00 [-399.85, -142.15]
Rijzewijk et al., 2008	450	211.6601	28	490	184.9324	38	8.9%	-40.00 [-138.00, 58.00]
Santomauro et al., 1999	560	187.4887	13	584	129.3483	11	8.3%	-24.00 [-151.40, 103.40]
Soriguer et al., 2008	605	147	9	817	211.3102	12	7.6%	-212.00 [-365.35, -58.65]
van der Merwe et al., 2001	870	132.8157	10	1,373	173.9253	10	8.1%	-503.00 [-638.63, -367.37]
Total (95% CI)			**153**			**151**	**100.0%**	**-129.52 [-214.47, -44.56]**

Heterogeneity: $Tau^2 = 18525.69$; $Chi^2 = 62.06$, df = 12 ($P < 0.00001$); $I^2 = 81\%$
Test for overall effect: $Z = 2.99$ ($P = 0.003$)



Chi^2 =62.06 was greater than the degrees of freedom (12), which suggested heterogeneity. However, these results were unreliable in a sample of this size. I^2 =81% showed moderate variability amongst study results. Tau^2 =18525.69 was significantly larger than 1, which identified definite heterogeneity. Therefore, a random-effects model was selected in both instances.

5.3.26 Molar percentage of palmitic acid and oleic acid in cholesterol esters in control and type II diabetic subjects

A further comparison was made comparing the average molar percentage of palmitic acid and oleic acid in cholesterol esters between control subjects regardless of BMI and participants with type II diabetes across 3 studies, n=4735 and n=358, respectively.

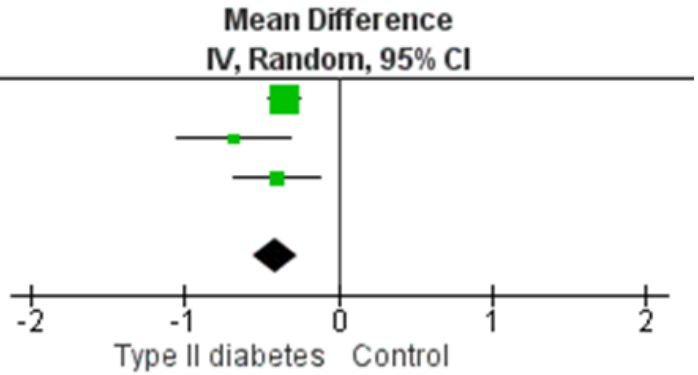
Results found that participants with type II diabetes had significantly higher molar percentages of palmitic acid in plasma cholesterol esters than the control group ($p<0.00001$) (Table 5.16 and 5.17). Furthermore, subjects with type II diabetes also had significantly higher molar percentages of oleic acid in plasma cholesterol esters than the control subjects ($p=0.02$). The average mol% of palmitic acid in plasma cholesterol esters was over 5 SD higher in type II diabetics than in the control group ($Z=5.32$), whilst the average mol% of oleic acid in cholesterol esters was over 2 SD greater in type II diabetics than in the control participants ($Z=2.38$).

Table 5.16 Palmitic acid in cholesterol esters (mol%) – control vs. Type II diabetes

	Control			Type II diabetes				Mean Difference
Study or Subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, Random, 95% CI
Patel et al., 2010	9.95	0.8	2657	10.3	0.8	252	63.6%	-0.35 [-0.45, -0.25]
Salomaa et al., 1990	11.85	0.92	325	12.53	1.01	31	14.0%	-0.68 [-1.05, -0.31]
Wang et al., 2003	11.65	0.98	1753	12.05	1.2	75	22.4%	-0.40 [-0.68, -0.12]
Total (95% CI)			**4735**			**358**	**100.0%**	**-0.41 [-0.56, -0.26]**

Heterogeneity: $Tau^2 = 0.01$; $Chi^2 = 2.87$, df = 2 (P = 0.24); $I^2 = 30\%$

Test for overall effect: Z = 5.32 (P < 0.00001)

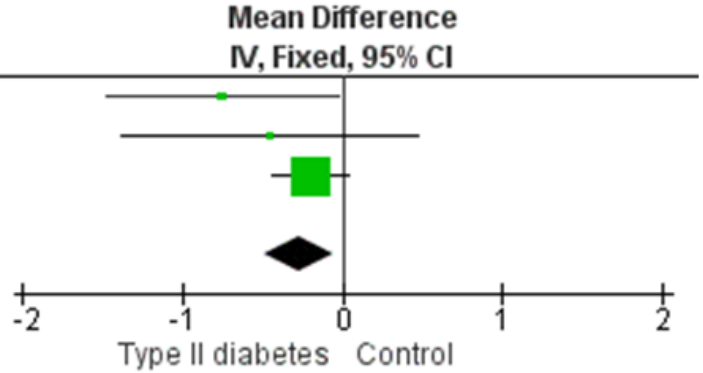


Chi^2 =2.87 was slightly greater than the degrees of freedom (2), which provided marginally acceptable levels of heterogeneity. However, in this study Chi^2 was unreliable due to the small number of samples. I^2 =30% estimated small to moderate levels of inconsistency amongst study results, which was just outside the realms of acceptable. Tau^2 =0.01 was small, which indicated low levels of between-study variance, however, when combined these results indicated an unacceptable level of heterogeneity for the assumptions in a fixed-effects model. Therefore, a random-effects model was selected.

Table 5.17 Oleic acid in cholesterol esters (mol%) – control vs. Type II diabetes

	Control			Type II diabetes				Mean Difference
Study or Subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, Fixed, 95% CI
Patel et al., 2010	19.29	2.68	1753	20.04	3.13	75	9.2%	-0.75 [-1.47, -0.03]
Salomaa et al., 1990	21.98	2.57	325	22.43	2.51	31	5.5%	-0.45 [-1.38, 0.48]
Wang et al., 2003	16	2.1	2657	16.2	1.8	252	85.3%	-0.20 [-0.44, 0.04]
Total (95% CI)			**4735**			**358**	**100.0%**	**-0.26 [-0.48, -0.05]**

Heterogeneity: Chi² = 2.19, df = 2 (P = 0.33); I² = 9%
Test for overall effect: Z = 2.38 (P = 0.02)



For the comparison of oleic acid plasma cholesterol esters in men vs. women (Figure 23B), Chi^2 =2.19 was just larger than the degrees of freedom (2), and thereby suggested a low and acceptable level of between-study variance. I^2 =9% estimated a very small amount of inconsistency amongst study results. Tau^2 =0.01 was small and, thus, indicated acceptably low levels of heterogeneity, therefore, a fixed-effects model was selected.

5.3.27 Molar percentage of palmitic acid in plasma phospholipids between control and type II diabetic subjects

This research examined the average molar percentage of palmitic acid in plasma phospholipids between control subjects and subjects with type II diabetes across 4 studies, n=21396 and n=12929, respectively.

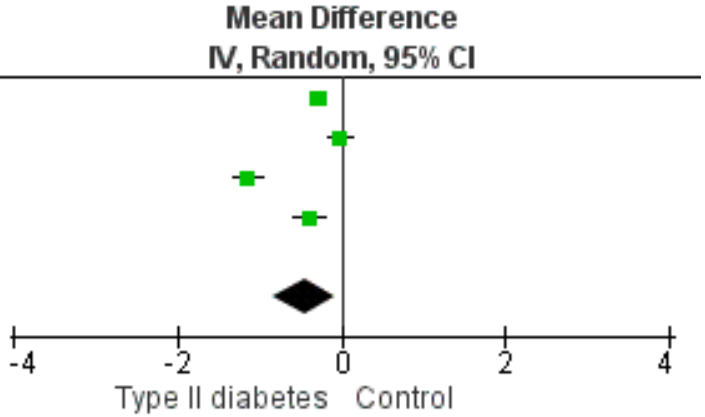
Results (Table 5.18) showed that participants with type II diabetes had significantly higher molar percentages of palmitic acid in plasma phospholipids than the control subjects (p=0.002). The average mol% of palmitic acid in plasma phospholipids was over 2 SD higher in the type II diabetes group than in the control group (Z=2.40).

Table 5.18 Palmitic acid in phospholipids (mol%) - control vs. type II diabetes

	Control			Type II diabetes				Mean Difference
Study or Subgroup	**Mean**	**SD**	**Total**	**Mean**	**SD**	**Total**	**Weight**	**IV, Random, 95% CI**
Forouhi et al., 2014	29.93	1.74	15164	30.21	1.67	12132	26.1%	-0.28 [-0.32, -0.24]
Hodge et al., 2007	25.23	1.54	3391	25.24	1.44	346	25.0%	-0.01 [-0.17, 0.15]
Patel et al., 2010	26.84	0.87	184	27.99	1	199	24.5%	-1.15 [-1.34, -0.96]
Wang et al., 2003	25.3	1.6	2657	25.7	1.5	252	24.4%	-0.40 [-0.59, -0.21]
Total (95% CI)			**21396**			**12929**	**100.0%**	**-0.46 [-0.83, -0.08]**

Heterogeneity: Tau² = 0.14; Chi² = 93.50, df = 3 (P < 0.00001); I² = 97%

Test for overall effect: Z = 2.40 (P = 0.02)



Chi^2 =93.50 was much greater than the degrees of freedom (3), which strongly suggested between-study variance. However, the small number of studies produced an unreliable result. I^2 =97% estimated very high levels of variability amongst study results. Tau^2 =0.14 suggested moderate levels of heterogeneity. When studied in combination, these results demonstrated levels of heterogeneity unsuitable for a fixed-effects model, hence the selection of a random-effects model.

5.3.28 Lipid profile in patients with cardiovascular disease

5.3.29 Plasma triglyceride concentrations between control participants and subjects with cardiovascular disease

Because of the expected involvement of lipids in the development of cardiovascular disease, this research examined the average plasma triglyceride concentrations between control participants and subjects with cardiovascular disease. This analysis collated 7 studies including 12151 control subjects and 3259 subjects with cardiovascular disease.

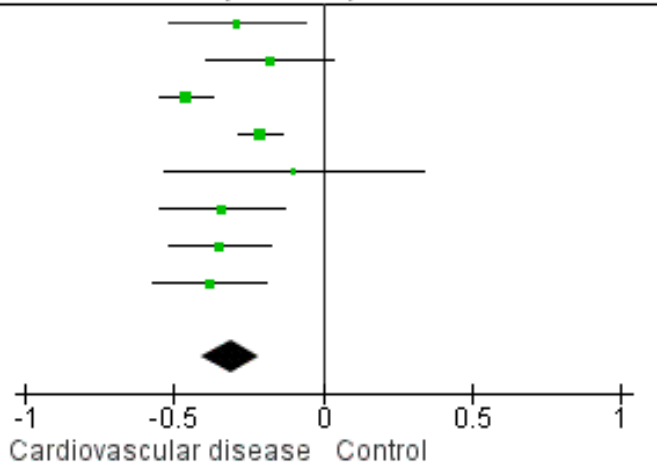
Results (Table 5.19) found that subjects with cardiovascular disease had significantly higher concentrations of plasma triglyceride than participants in the control group ($p<0.00001$). The average plasma triglyceride concentration of the cardiovascular disease group was over 6 SD higher than the average for the control subjects ($Z=6.25$).

Table 5.19 Plasma triglycerides - control vs. cardiovascular disease

	Control			Cardiovascular disease				Mean Difference	Mean Difference
Study or Subgroup	**Mean**	**SD**	**Total**	**Mean**	**SD**	**Total**	**Weight**	**IV, Random, 95% CI**	**IV, Random, 95% CI**
Drexel et al., 1994	1.51	1.0247	105	1.8	1.1047	339	10.1%	-0.29 [-0.52, -0.06]	
Jouven et al., 2001	1.41	1.1639	5014	1.59	1.3221	145	10.6%	-0.18 [-0.40, 0.04]	
Khaw et al., 2012	1.64	1.07	2684	2.1	1.15	776	18.9%	-0.46 [-0.55, -0.37]	
Khaw et al., 2012	2.01	1.15	2246	2.22	1.23	1595	19.8%	-0.21 [-0.29, -0.13]	
Miettinen et al., 1982	1.9	0.8	64	2	1.1489	33	4.1%	-0.10 [-0.54, 0.34]	
Pirro et al., 2002	1.7	0.66	98	2.04	0.78	88	11.1%	-0.34 [-0.55, -0.13]	
Yli-Jama et al., 2002	1.39	0.58	104	1.74	0.67	103	13.3%	-0.35 [-0.52, -0.18]	
Öhrvall et al., 1996	2.03	1.14	1836	2.41	1.26	180	12.1%	-0.38 [-0.57, -0.19]	
Total (95% CI)			**12151**			**3259**	**100.0%**	**-0.31 [-0.41, -0.21]**	

Heterogeneity: $Tau^2 = 0.01$; $Chi^2 = 20.14$, df = 7 (P = 0.005); $I^2 = 65\%$

Test for overall effect: Z = 6.25 (P < 0.00001)



Chi^2 =20.14 was greater than the degrees of freedom (7), and, thus, indicated heterogeneity. However, this result was unreliable due to the small number of studies included. I^2 =65% predicted moderate levels of inconsistency amongst study results. Tau^2 =0.01 was small and suggested small amounts of between-study variance. However, once combined these results indicated moderate heterogeneity, and, thus, a random-effects model was performed.

5.3.30 Plasma free fatty acid concentrations between control subjects and participants with cardiovascular disease

This study compared average plasma free fatty acid concentrations between control subjects and participants with cardiovascular disease across 3 studies, n=5216 and n=336, respectively.

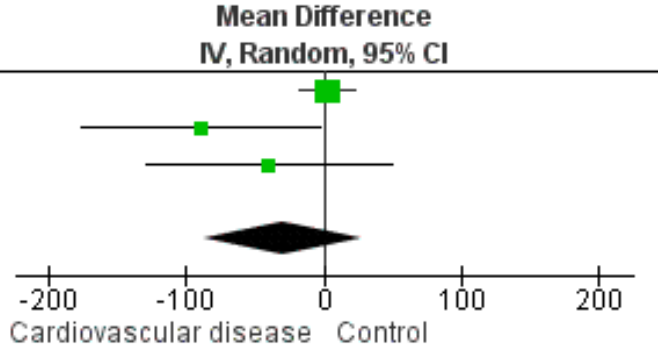
The meta-analysis (Table 5.20) showed that there was no significant difference between average plasma FFA in subjects with cardiovascular disease and participants in the control group (p=0.31).

Table 5.20 Plasma free fatty acids - control vs. cardiovascular disease

Study or Subgroup	Control Mean	Control SD	Control Total	Cardiovascular disease Mean	Cardiovascular disease SD	Cardiovascular disease Total	Weight	Mean Difference IV, Random, 95% CI
Jouven et al., 2001	313	144	5014	310	123	145	51.9%	3.00 [-17.41, 23.41]
Pirro et al., 2002	750	280	98	840	320	88	24.5%	-90.00 [-176.85, -3.15]
Yli-Jama et al., 2002	380	280	104	420	370	103	23.7%	-40.00 [-129.45, 49.45]
Total (95% CI)			**5216**			**336**	**100.0%**	**-29.93 [-87.37, 27.52]**

Heterogeneity: Tau² = 1547.42; Chi² = 4.84, df = 2 (P = 0.09); I² = 59%

Test for overall effect: Z = 1.02 (P = 0.31)



Chi^2 =4.84 was greater than the degrees of freedom (2) and suggested heterogeneity. However, the Chi^2 was unreliable due to the small number of studies included. I^2 =59% which suggested moderate inconsistencies amongst study results. Tau^2 =1547.42 was vastly greater than 1, and, thus, confirmed heterogeneity. Therefore, a random-effects model was selected to take between-study variance into account.

5.3.31 Molar percentage of palmitic acid and oleic acid in cholesterol esters between control and cardiovascular disease subjects

The next step was to compare the average molar percentage of palmitic acid and oleic acid in cholesterol esters between control subjects and participants with cardiovascular diseases across 3 studies, n=5209 and n=495, respectively.

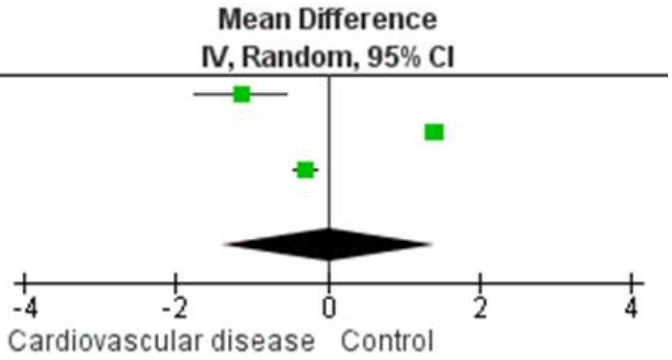
The meta-analysis (Table 5.21 and 5.22) demonstrated that there was no difference between the molar percentage of palmitic acid in cholesterol esters in participants with cardiovascular disease compared to the control group (p=0.99). However, subjects with cardiovascular disease did have a significantly higher molar percentage of oleic acid in plasma cholesterol esters than the control participants (p=0.02). The average mol% of oleic acid in plasma cholesterol esters was over 2 SD higher in the cardiovascular disease group than in the control group (Z=2.37), whilst the average mol% of palmitic acid in cholesterol esters was marginally greater in the cardiovascular group versus the control group (Z=0.02).

Table 5.21 Palmitic acid in cholesterol esters (mol%) - control vs. cardiovascular disease

	Control			Cardiovascular diease				Mean Difference
Study or Subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, Random, 95% CI
Miettinen et al., 1982	13.24	1.04	64	14.38	1.6085	33	32.1%	-1.14 [-1.75, -0.53]
Wang et al., 2003	11.6	1	3309	10.2	0.8	282	34.0%	1.40 [1.30, 1.50]
Öhrvall et al., 1996	11.65	0.97	1836	11.94	1.06	180	33.9%	-0.29 [-0.45, -0.13]
Total (95% CI)			**5209**			**495**	**100.0%**	**0.01 [-1.39, 1.42]**

Heterogeneity: $Tau^2 = 1.51$; $Chi^2 = 350.51$, df = 2 (P < 0.00001); $I^2 = 99\%$

Test for overall effect: Z = 0.02 (P = 0.99)

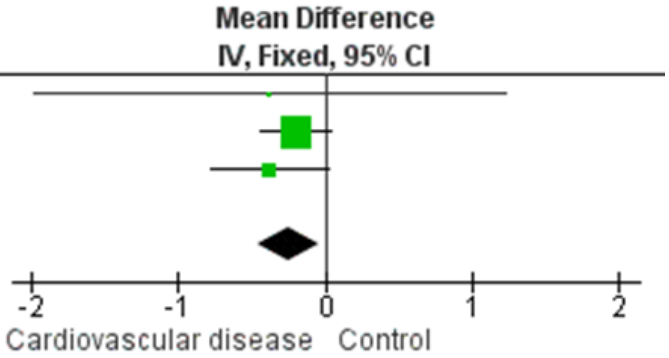


Chi^2 =350.51 was significantly greater than the degrees of freedom (2), which demonstrated unacceptable levels of heterogeneity. I^2 =99% estimated large, unacceptable levels of inconsistency amongst study results. Tau^2 =1.51 was large, and, thus, suggested high levels of between-study variance. Therefore, a random-effects model was selected.

Table 5.22 Oleic acid in cholesterol esters (mol%) - control vs. cardiovascular disease

	Control			Cardiovascular disease				Mean Difference
Study or Subgroup	**Mean**	**SD**	**Total**	**Mean**	**SD**	**Total**	**Weight**	**IV, Fixed, 95% CI**
Miettinen et al., 1982	25.31	3.92	64	25.69	3.7914	33	1.7%	-0.38 [-1.99, 1.23]
Wang et al., 2003	16	2.1	3309	16.2	2	282	72.0%	-0.20 [-0.44, 0.04]
Öhrvall et al., 1996	19.38	2.77	1836	19.76	2.62	180	26.4%	-0.38 [-0.78, 0.02]
Total (95% CI)			**5209**			**495**	**100.0%**	**-0.25 [-0.46, -0.04]**

Heterogeneity: Chi² = 0.59, df = 2 (P = 0.75); I² = 0%
Test for overall effect: Z = 2.37 (P = 0.02)



Chi^2 =0.59 was smaller than the degrees of freedom (2), which conferred an acceptable level of heterogeneity. I^2 =0% estimated no variability amongst study results. Tau^2 =0.00 also suggested no between-study variance, and, thus, a fixed-effects model was performed. N.B. the heterogeneity results produced a large non-significant p value (p=0.75), however, the results were deemed strong enough to use a fixed-effects model regardless.

5.4 Discussion

A comprehensive systematic review of the literature revealed that many papers stated average plasma lipid concentrations obtained from small samples or from niche populations. Further primary studies have then used these averages as a baseline for subsequent work, thereby creating an unstable and inaccurate foundation for research in this field.

The rationale behind this study was that with the amalgamation of results collected through systematic review, robust averages and reference ranges of total lipids, free fatty acids, and levels of palmitic acid and oleic acid in different plasma lipid species could be derived, thereby informing future studies in this field. Furthermore, this study aimed to assess the levels of different lipid species in subpopulations and diseased states, in order to understand the potential of a clinical relationship between palmitic and oleic acid levels and disease. The majority of this was achieved with the exception of reference ranges for all lipid species; only four could be calculated reliably.

5.4.1 Units of measurement and average molecular weight of plasma free fatty acids

A systematic review of the literature found that two units of measurement are commonly used for plasma free fatty acids. The majority of papers used the SI unit mmol/L, although many used mg/dL. However, not one paper converted between the two units. This result was surprising, as instead of pooling research for the advancement of knowledge in the field, researchers are continuing down one of the two separate paths.

It was important for this systematic review to pool data in order to give a better overview, and calculate more robust averages. Therefore, the literature was interrogated for a conversion factor between the two units, however, one could not be found. A number of online calculators exist that use the molecular weight of oleic acid as the average molecular

weight for plasma free fatty acids (DIALAB, 2008; DiaSys Diagnostic Systems GmbH, 2017). None of the websites included references evidencing the validity of this conversion factor, and, therefore, it was important to calculate the average molecular weight of plasma free fatty acids for subsequent use in this study.

Systematic review identified one comprehensive lipid profiling study with a large sample size (n=826) (Abdelmagid*, et al.*, 2015). From this study data was extracted and an average molecular weight calculated based on the fatty acid percentage composition of plasma in the study. This study calculated the average molecular weight of plasma free fatty acids to be 277.454 g/mol as opposed to 282.47 g/mol, the MW of oleic acid. This difference can create disparities of ~2% in the conversion from mg/dL to mmol/L. We recommend the use of the lower value in future conversions.

5.4.2 Average concentrations of plasma lipids

Systematic review identified and collated data on total plasma triglycerides and total free fatty acids levels. In addition, palmitic acid and oleic acid content in phospholipids, cholesterol esters, and triglycerides were calculated, as well as free palmitic acid and oleic acid levels. The range of available data varied hugely from plasma triglycerides with 139 datasets included to palmitic acid and oleic acid in phospholipids with only 3 datasets.

Plasma triglycerides are the most frequently measured plasma lipid, and are measured by the NHS to evaluate risk factors of coronary heart disease and pancreatitis (Southend University Hospital NHS Trust, 2018). As such, average plasma triglyceride concentrations are widely published and reference ranges have been calculated (Table 5.1). This study considered it important to check for any disparities between published averages for the most common lipids in order to illustrate the vast range in data that existed, and to highlight the need for greater standardisation. Results from plasma triglyceride data found that the average concentration across the 139 datasets was significantly different compared to the average when weighted for the number of participants per study. A reference range was

calculated to be 0.47 - 2.38 mmol/L, which was quite different to many being used by the NHS (Table 5.1). This new reference range could be used nationwide, across the NHS to standardise results, as currently reference ranges are used on a trust by trust basis (Table 5.1).

Plasma free fatty acid levels are also commonly measured, however, few reference ranges exist. The difference between the average plasma FFA concentration across datasets and the weighted average was very large. However, the data was assessed to be normally distributed and a reference range of 216.3 - 907.9 µmol/L was calculated. This could also be used by the NHS to standardise patient results.

The data available for palmitic acid and oleic acid in different lipid fractions was scarce and not helped by the existence of four different seemingly acceptable units of measurement that were found in the literature. The majority of analyses contained too few datasets to compute reliable statistics and most were not normally distributed, and hence could not be used to form reference ranges. However, reference ranges could be calculated for oleic acid in phospholipids (mol%) and palmitic acid in triglycerides, 6.68 – 14.44 mol% and 17.71 – 34.53 mol%, respectively.

Many studies chose to report palmitic acid and oleic acid in different lipid fractions as a percentage of the total moles or total weight, instead of absolute concentrations. However, inaccuracy in the measurement of one fraction *via* this method can have substantial knock-on effects on the percentages of the other fatty acids measured.

Most importantly, for the analysis of palmitic acid and oleic acid in different plasma lipid fractions, none of the units of individual data points were converted, as conversion would have introduced error. However, the average and weighted average were converted to mmol/L to enable statistical analysis, which highlighted vast disparities. For example, the converted weighted average of free palmitic acid was significantly larger than the unconverted weighted average ($P<0.0001$) (Table 5.4). However, ratios of palmitic acid to oleic acid were calculated for all lipid species in order to amalgamate data from all sources

regardless of unit. This effectively increased the amount of usable data per analysis, thereby, increasing the quality of the output. Furthermore, this analysis was performed as the previous two chapters indicated the importance of measuring the ratio of saturated to unsaturated fatty acids.

This study strongly highlighted the need for larger scale studies to act as a foundation for future research, and standardisation of units and measurements across the field.

5.4.3 Lipid species amongst the population

There is much contradiction in the literature regarding lipid species in different populations. For example, most studies report that men have higher plasma triglyceride concentrations than women (Abdelmagid*, et al.*, 2015; Fernandez-Real*, et al.*, 2005; Hotta*, et al.*, 2000; Khaw*, et al.*, 2012; Kuriki*, et al.*, 2003; Vognild*, et al.*, 1998; Yamagishi*, et al.*, 2008) , but many authors have published results contradicting this (Baylin*, et al.*, 2005; Ebbesson*, et al.*, 2015; Glew*, et al.*, 2010; Svanborg*, et al.*, 2009). Therefore, taken individually studies could be misleading with the possibility of negatively influencing future research. We felt it important to conduct a systematic review and meta-analysis to view the data as a whole and draw more informed conclusions.

Results found that most lipid species are higher in men. Men have ~3 SD higher levels of plasma triglycerides, ~2 SD higher total palmitic acid (mol%), ~5 SD higher palmitic acid in phospholipids (mol%) and ~ 3 SD higher oleic acid in phospholipids (mol%) than women. For total oleic acid (mol%) the values from men and women were the same.

Overweight/obese, non-diabetic subjects have significantly higher levels of plasma triglycerides and plasma free fatty acids than non-obese subjects, ~6 SD and ~7SD, respectively. Furthermore, there was a positive correlation between BMI and plasma triglyceride concentration, and BMI and plasma FFA. Previous studies have reported similar results, but in smaller, niche populations. For example, one study found a correlation between BMI and blood triglycerides in 208 South Korean subjects (Kim*, et al.*, 2012),

whilst another in 155 Iraqi diabetic premenopausal women during their pre ovulatory period (Ali & Al-Zaidi, 2011).

5.4.4 Lipid species in disease

In the past couple of decades an increasing number of studies have examined the relationship between different lipid species and a range of diseases including cancer (Butler*, et al.*, 2017; Crowe*, et al.*, 2008), cardiovascular diseases (Fernández-Real*, et al.*, 2003), cardiometabolic diseases (Rosqvist*, et al.*, 2017), and even Alzheimer's disease (Proitsi*, et al.*, 2015). However, studies are often small and don't utilise meta-analyses which can enhance statistical power. Therefore, this study considered it to be important to study the relationship between lipids and prominent diseased states by collating data.

Participants with type II diabetes had significantly higher concentrations of plasma triglycerides and plasma FFAs than non-obese and overweight/obese participants without the disease. Furthermore, participants with type II diabetes also had higher molar percentages of palmitic acid and oleic acid in cholesterol esters, and palmitic acid in phospholipids than control subjects.

Participants with cardiovascular disease had significantly higher concentrations of plasma triglycerides and a higher molar percentage of oleic acid in cholesterol esters than control subjects. On the other hand, subjects with cardiovascular diseases did not have significantly higher concentrations of plasma free fatty acids or higher molar percentage of palmitic acid in cholesterol esters.

There was, however, no significant difference between the molar percentages of palmitic acid or oleic acid in phospholipids in control subjects and participants with cancer, which was contradictory to research by Crowe*, et al.,* (2008) who found a positive association between palmitic acid in phospholipids and prostate cancer.

This study worked with the available resources to generate the results above. The systematic review process highlighted the lack of data available in this field and established the need for more large scale studies to act as a foundation for future research.

6 General discussion

This project took a multifactorial approach with an aim to better understanding the pathophysiological effects of elevated plasma FFA throughout the body and in different populations in order to clarify contradictions in the literature. Originally the aim of this project was to study the effects of FFA on insulin sensitive cells and platelets in depth as many scientists believe that plasma FFAs are elevated in the obese community. If true, research into their effects are vital in the process of understanding how to prevent the deleterious effects of the obesity, which are pandemic across the whole and cause significant strain on global healthcare services. However, throughout the course of research large disparities and contradictions started to appear in the foundations of the literature, which caused questions as to the importance of FFAs in obesity. For example, many papers have been published regarding the measurement of lipid species in different subpopulations without coming to a common consensus on average plasma levels of lipid species in the normal population. Even the NHS uses vastly different reference ranges for the normal population across the country. Furthermore, researchers also disagree about whether certain lipids are elevated in certain subpopulations or not, and use different measurement units which cannot be directly converted. Additionally, many disagree about lipid risk factors for CVD, CHD, mortality etc. Therefore, it was deemed important to try to debunk this area of research before continuing with the cellular research.

The final project and thesis was achieved *via in vitro* studies on hepatocyte, skeletal myocyte and washed platelet models to determine whether fatty acids accumulate intracellularly and induce lipotoxicty. In addition to a systematic review and meta-analysis to determine whether certain plasma lipid fractions are in fact associated with obesity and diseased states.

Initially this project investigated the effects of physiological levels of free palmitic acid and oleic acid on hepatocyte, skeletal myocyte and platelet models in order to understand the potential effects elevated plasma FFAs may have at a cellular level. The selection of fatty

acid concentrations for cell and platelet studies was difficult as there was a limited consensus regarding normal physiological levels and levels in obesity, yet after consulting the literature, concentrations up to 1 mM were selected. In the hepatocyte and skeletal muscle cell models, palmitic acid was found to cause cell death, probably *via* apoptotic mechanisms, whilst oleic acid induced intracellular lipid accumulation. These results were in agreement with the most common theory that lipotoxicity occurs when fatty acids cannot be stored intracellularly as neutral lipid species. This is highly dependent on fatty acid structure, and the cell's storage capacity. It is thought that due to their higher melting temperatures, saturated fatty acids are less capable of forming neutral lipids intracellularly so are more lipotoxic individually, however, unsaturated fatty acids are able to sequester them into neutral lipid storage thereby ameliorating their deleterious effects in combination. This leads to thinking that the physiological combination of plasma fatty acids within the circulation would not cause cellular damage unless potentially highly elevated, or the ratio of saturated to unsaturated fatty acids was abnormal.

Conversely, palmitic acid had no effect on washed platelets whilst oleic acid induced platelet aggregation. Attempts to reduce oleic acid-induced aggregation *via* the inhibition of vital signalling events in platelet activation failed. Additionally, no markers of platelet activation were found following stimulation, except the exposure of PS which is also a hallmark of apoptosis. Two separate experiments were performed to examine cell death; oleic acid caused significant loss of platelet viability, giving credence to the hypothesis that oleic acid evokes platelet aggregation *via* apoptotic mechanisms. Due to the rapid response of platelets upon stimulation with oleic acid, it is likely that apoptosis is occurring due to alterations to the platelet membrane. The mechanisms are likely to be different to those initiated by palmitic acid in HepG2 and L6 cells. However, the three cells studied in this project do share common signalling pathways, such as caspase activation in apoptosis (Mutlu*, et al.*, 2012). These results are produced in isolation, and would not be present if treated with the physiological combination of fatty acids found within the blood. However,

once again it is possible that these results would be seen *in vivo* in the presence of an abnormal ratio of saturated to unsaturated fatty acids.

Systematic review and meta-analysis was firstly used to analyse circulating lipids in healthy subjects. The aim was to find average values and reference ranges, and produce average PA:OA ratios for different lipid species. Results confirmed that 1 mM of fatty acids was appropriate for the laboratory experiments, and suggested averages and reference ranges for future use by the wider community including researchers and the NHS. Systematic review uncovered the usage of four different measurement units across the literature that could not be accurately converted, which caused confusion when trying to ascertain a congruent answer for average circulating lipids. Analysis found that 25% of the converted values were statistically different to the values originally measured in mmol/L. This finding was worrying and unexplained, hence the recommendation that the research community favours one unit and does not convert between the two. Furthermore, there is not always a good correlation between the relative (mol% and mass%) and absolute (mg/dL and mmol/L) metrics (Song*, et al.*, 2016). This may not affect the interpretation of all results in studies of association between a lipid species and disease outcomes, especially if a covariate of total lipids is provided. However, for lipid species with low correlation between the two measures, choosing which to draw inferences from may significantly affect the outcome (Song*, et al.*, 2016).

Fortunately, following analysis from the cellular and platelet work in this project it was identified that the ratio of PA:OA was potentially more important that the absolute values, and all data regardless of units could be used for these calculations. The results produced had a smaller variance and effectively amalgamated all of the data in addition to producing reliable average ratios and reference ranges.

The main point highlighted by the research was the vastly differing results for all lipid species studied. Even without the additional issue of units, the range of data in the literature

varied massively and as such affected results. Often there was a statistical difference between the average and weighted average, suggesting outliers or study bias, where the weight of small studies is overestimated or the weight of large studies is underestimated. This, and a lack of available data for some lipid fractions led to data that was not Gaussian with no improvement upon log transformation, therefore, many reference ranges could not be calculated. The wide range of data published throughout the literature was believed to be due to a number of factors. Firstly, there is no gold-standard for the measurement of lipids in the blood or plasma, so different approaches were used across published works. Secondly, the inclusion and exclusion criteria for 'healthy subjects' were not only widely different across research articles, but failed to identify the effects characteristics such as age, obesity and menopause may have upon the validity of the results. For example, most researchers did not consider asking female participants about whether they had started the menopause even though they included women of pre- and post- menopausal age. Furthermore, none included in this study asked whether post-menopausal women were taking hormone replacement therapy. This could have significant effects on the results as hormone levels have been shown to alter the lipid profile (Bade, *et al.,* 2014).

Regardless of the issue with the data, reliable reference ranges for the general population were calculated for plasma FFA, plasma triglyceride, oleic acid in phospholipids (mol%) and palmitic acid in triglycerides (mol%). The reference range for plasma triglycerides could be adopted across the NHS to standardise testing, as currently reference ranges vary greatly between trusts. In spite of being a niche test in the NHS, the plasma FFA reference range could also be utilised. Unfortunately, the other two mean little as stand-alone reference ranges.

The levels of lipid species in different subpopulations were then studied to identify groups with elevated lipids such as obese and type II diabetic patients. Many studies linked elevated levels of plasma FFA with the development of a range of cardiovascular and metabolic diseases. In the 1960s, individuals classified as obese with a BMI over 30 kg/m^2 were found to have greater levels of circulating FFA (Opie*, et al.*, 1963). However, as more

studies have been published and the analysis widened to include more and more subsets of the population the results have become variable. More recently, a number of studies have been published that show no difference in plasma FFA levels between obese and non-obese subjects, which contradicted the results of this study (Arner*, et al.*, 2015; Frias*, et al.*, 2000; Horton*, et al.*, 1995; Jocken*, et al.*, 2008; Johns*, et al.*, 2014). And yet, research continues to be published based on the assumption that FFAs are raised in obesity, without further large scale systematic review and meta-analysis.

The meta-analysis confirmed that circulating levels of plasma TG and FFAs are indeed raised in the obese population, but furthermore, that they are even more elevated in type II diabetics. This could be due to systemic insulin resistance leading to hyperinsulinaemia which in turn leads to reduced TG synthesis and storage, and increased lipolysis in the adipose tissue, thereby, causing increased circulating levels (Czech, *et al.*, 2013; Shah*, et al.*, 2003). However, it could also be due to a causal effect. In order to qualify as a physiological link between obesity and insulin resistance, the substance should be elevated in the blood of obese people; increasing plasma levels (within physiologic limits) should enhance insulin resistance, and reducing plasma levels should ameliorate insulin resistance. FFAs have been found to meet all three criteria (Boden, 2008; Boden, *et al.,* 1994; Reaven, *et al.,* 1988; Santomauro, *et al.,* 1999).

In contradiction to this literature suggests that plasma TG is rarely elevated in well controlled diabetics (Fisher, 1991), which may indicate that type II diabetics, in general, have poor glycaemic control. Glycated haemoglobin (HbA1c), the gold-standard test for glycaemic control should be lower than 7% in diabetics. However, numerous systematic reviews found that over half of type II diabetics had HbA1c readings higher than 7% across a range of interventions (Li, *et al.,* 2018; Srinivasan, *et al.,* 2008). Additionally, type II diabetics have higher molar percentages of palmitic acid in phospholipids and cholesterol esters than the non-diabetic population. This is directly contradicted by Chuang*, et al.,* (2012) who found that palmitic acid in phospholipids was decreased in the type II diabetic population, yet, supported by Salomaa*, et al.,* (1990) who discovered that palmitic acid in

cholesterol esters increased with increasing glucose intolerance. Subjects with cardiovascular disease had greater levels of plasma triglyceride, which is not surprising as high circulating TG levels are a risk factor in the development of cardiovascular diseases (Han*, et al.*, 2016; Harchaoui*, et al.*, 2009; Yuan*, et al.*, 2007). The molar percentage of oleic acid in cholesterol esters is also increased in subjects with cardiovascular disease, whilst plasma FFA is not. However, many studies have found that elevated FFA is a risk factor for cardiovascular diseases (Djoussé*, et al.*, 2013; Pirro*, et al.*, 2002), and is linked to the progression of CVD (Jin*, et al.*, 2017).

In summary, this project provides insights into the pathophysiological effects of fatty acid on hepatocytes, skeletal myocytes and platelets. In addition to providing average plasma triglyceride and free fatty acid levels, and palmitic acid and oleic acid content of plasma lipid species, in the general population and subpopulations. It was established that palmitic acid caused cell death in hepatocytes and skeletal muscle cells, whilst oleic acid caused intracellular lipid accumulation. Oleic acid also caused platelet death, likely *via* apoptosis. These results highlighted the physiological importance of the saturated:unsaturated fatty acid ratio in the blood.

Results from the meta-analysis showed that men have greater concentrations of plasma FFA. Furthermore, they confirmed that circulating levels of FFAs are indeed raised in the obese population, and even more so in type II diabetics. This has large implications as high levels of FFA are associated with a variety of acute and chronic conditions in cells and tissues across the body.

In addition to understanding basic processes involved in lipotoxicity. This study highlights gaps in the foundations of research in this field and suggests that more work goes into understanding differing levels of lipid species amongst the population in order to better understand subpopulations at risk of developing cardiovascular and/or metabolic disease. All of this should be with the aim of reducing obesity and its comorbidities in order to lessen the burden on healthcare services across the world.

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8 Appendices

8.1 Appendix A

8.1.1 Establishing an adipocyte model

8.1.2 Chemically-induced Differentiation of 3T3-L1

3T3-L1 cells were seeded at approximately 3 x 10^3 cells per cm^2 of the culture flask. The culture was subcultured before reaching 70% confluence using trypsin. Prior to differentiation pre-adipocyte expansion medium (PEM) was used to maintain the cells. PEM consists of DMEM supplemented with 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin.

For differentiation the cells were subcultured and seeded into the appropriate vessels at the following densities:

Table 8.1 Cell density used in different cell culture vessel

Culture Vessel	Cell Density
6 well plate	8 x 10^4 cells per well
96 well plate	2 x 10^3 cells per well

Subcultures were maintained with PEM until reaching 100% confluence. The fully confluent cells were incubated for 48 hours, then the PEM was replaced with differentiation medium (DM) and incubated for a further 48 hours. DM consists of DMEM supplemented with 10% FBS, 1.0 µM dexamethasone, 0.5 mM methylisobutylxanthine, 1.0 µg/ml of bovine insulin and 1% penicillin-streptomycin. After 48 hours DM was replaced with adipocyte maintenance medium (AMM) and maintained as per the usual protocol. AMM consists of DMEM supplemented with 10% FBS, 1.0 µg/ml of bovine insulin and 1% penicillin-streptomycin. The cells were incubated between 7 and 20 days until fully differentiated, as evidenced by the formation of lipid droplets.

8.1.3 Measurement of GLUT4 translocation

The 3T3-L1 cells were serum starved overnight. They were trypsinised and seeded in to a 24 well-plate at 1 x 10^5 cells/well in serum-free medium. The cells were placed into the incubator for 2 hours to recover from trypsinisation.

5 µl of primary anti-GLUT4 was mixed with 1 µl of secondary goat anti-mouse IgG (conjugated to Alexa Fluor 488) for every well used. The antibodies were incubated in the dark, at room temperature for 10 minutes. A 2X working stock of insulin was prepared by diluting it in DMEM.

6 µl of the antibody mix and 500 µl of insulin solution were added to each well. The well-plates were then incubated in an incubator with 5% CO_2 at 37°C for 30 minutes. Halfway through incubation 15 nM insulin solution was added to the positive samples.

The cells were fixed by the addition of 500 µl of 1% paraformaldehyde/PBS solution to each well and incubated in the dark, at room temperature for 20 minutes.

The cell suspension was transferred to 1.5 ml Eppendorf tubes and centrifuged at 1000 rpm for 5 minutes. The pellet was washed twice and recentrifuged at 1000 rpm for a further 5 minutes each. After washing, the pellet was resuspended in 400 µl of 1% paraformaldehyde/PBS solution. The samples were run through the BD Accuri™ C6 flow cytometer and data was acquired from 10,000 events per tube.

8.1.4 Results from adipocytes

This study was conducted to evaluate the differentiation of 3T3-L1 from fibroblasts into adipocytes following a differentiation procedure. GLUT4 translocation was tested to assess the feasibility of the differentiated cells as an adipocyte model for following research.

Differentiation of 3T3-L1 from fibroblasts into adipocytes was attempted three times following the recommended ATCC chemically-induced differentiation procedure (2011). Twice the adherent cell layer detached due to the fragility of the cell line and a pH imbalance. The process reached completion on one attempt. Throughout the procedure differentiation was assessed *via* the formation of intracellular lipid droplets visualised by inverted microscopy. Following 20 days of differentiation, 5 more than the stated requirement, lipid droplets were only visibly apparent in ~50% of 3T3-L1 cells as evidenced by photographs (Figure 8.1). The effectiveness of differentiation was not in keeping with the reference material from ATCC (Figure 1d). This indicates incomplete differentiation from fibroblasts to adipocytes, as upwards of 85% of cells were expected to undergo the transformation.

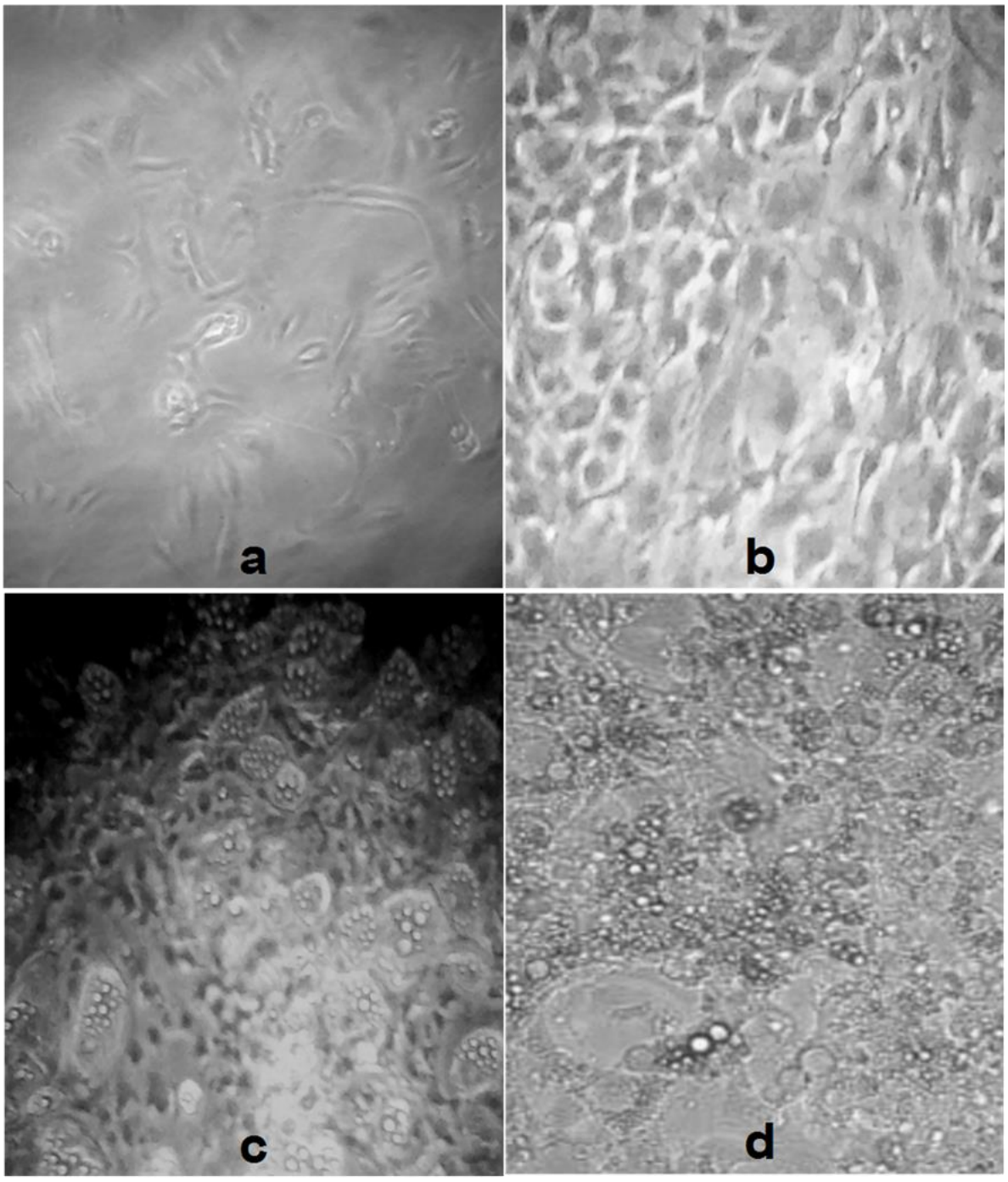


Figure 8.1 Progression of 3T3-L1 differentiation following treatment with differentiation and maintenance medium.

a) Cells were subcultured into 6 well plates at approximately 30% confluence. b) After 3 days cell reached 100% confluence and the differentiation process began. c) Intracellular lipid droplets were visible after 15 days of differentiation. d) An example of fully differentiated 3T3-L1 cells from ATCC (2011).

The success of differentiation was measured by GLUT4 translocation studies. The rationale behind this was that only cells sensitive to insulin, thereby causing GLUT4 translocation upon its presence, were fully differentiated and hence a feasible adipocyte model in this research. Results from these studies show GLUT4 translocation occurred in a maximum of 6% of cells with no significant difference between basal and insulin-induced levels of GLUT4 translocation (P=0.8422) (Figure 8.2). Therefore, the differentiation process was ineffective, with the vast majority of cells not acquiring the insulin sensitive mechanisms of adipocytes, rendering the 3T3-L1 cells an inadequate adipocyte model for further investigations in this research.

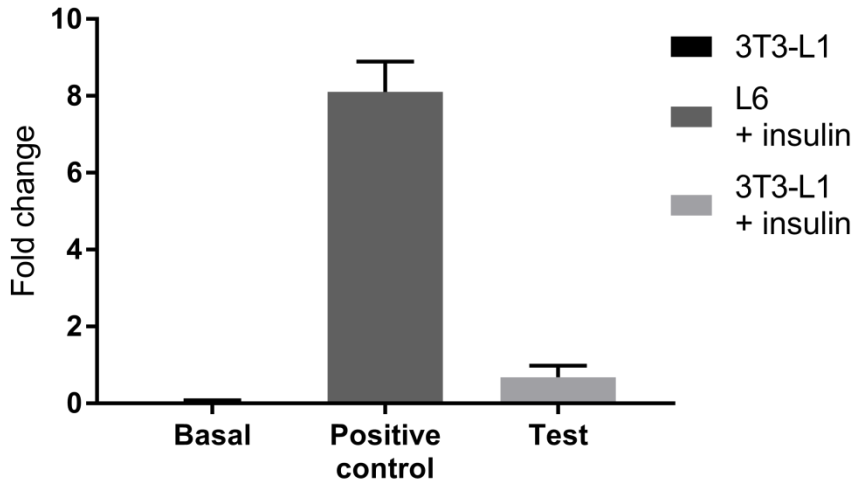


Figure 8.2 Insulin stimulation of differentiated 3T3-L1 cells caused no significant GLUT4 translocation.

A maximum of 6% of cells exhibited GLUT4 translocation following insulin activation, with no significant difference between the negative and positive control (P=0.8422, n=3).

8.2 Appendix B

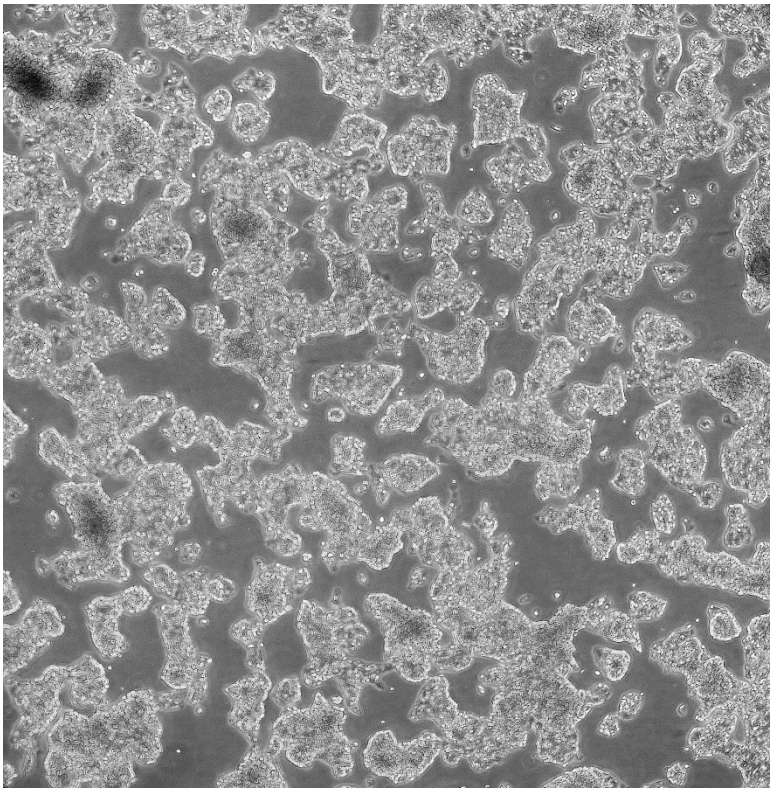


Figure 8.3 Inverted microscope image of HepG2 clumping at 400x magnification

8.3 Appendix C

8.3.1 Background of methods

8.3.1.1 Cell Counting Kit 8

A number of assays can be employed to assess the viability of cells including the Cell Counting Kit-8 (CCK-8), which measures the proportion of viable cells in a sample. CCK-8 utilises a colourless, water-soluble tetrazolium salt-8 (WST-8), which is oxidised by intracellular NADH, NADPH and dehydrogenases to form an orange water-soluble WST-8 formazan dye (Dojindo, 2009). Enzyme action only occurs in cells with their dehydrogenase activity intact, hence the correlation between absorbance at 450 nm and viable cells. A standard curve was obtained to check for the assay's limits of detection.

8.3.1.2 Flow cytometry theory

Flow cytometry utilises fluidics, lasers, filters and detectors to measure a range of characteristics of individual cells. Flow cytometers employ hydrodynamic focusing to create a single file stream of cells by injecting them into the core of sheath fluid being pumped through a laminar flow chamber. This flow of single file cells is transported to the interrogation point, where the laser intersects the sample, causing light scatter and/or the excitation of fluorescence tagged antibodies, which is measured by different detectors (Figure 8.4).

The two main characteristics measured by the detection of scattered light upon interrogation are cell size, measured by forward scatter (FSC), and cell granularity, measured by side scatter (SSC). However, these measurements are only reliable for regular shaped cells. In order to measure other characteristics, fluorescent dyes and fluorescently labelled antibodies against a target of interest can be incubated with cells prior to analysis to investigate the presence of specific antigens within the cell or on the cell surface. Lasers appropriate for the excitation wavelengths of the fluorophores being used are directed at cells passing through the interrogation point. Upon contact, the fluorophore

is excited and emits a different wavelength of light, which are measured separately to FSC and SSC by a range of detectors.

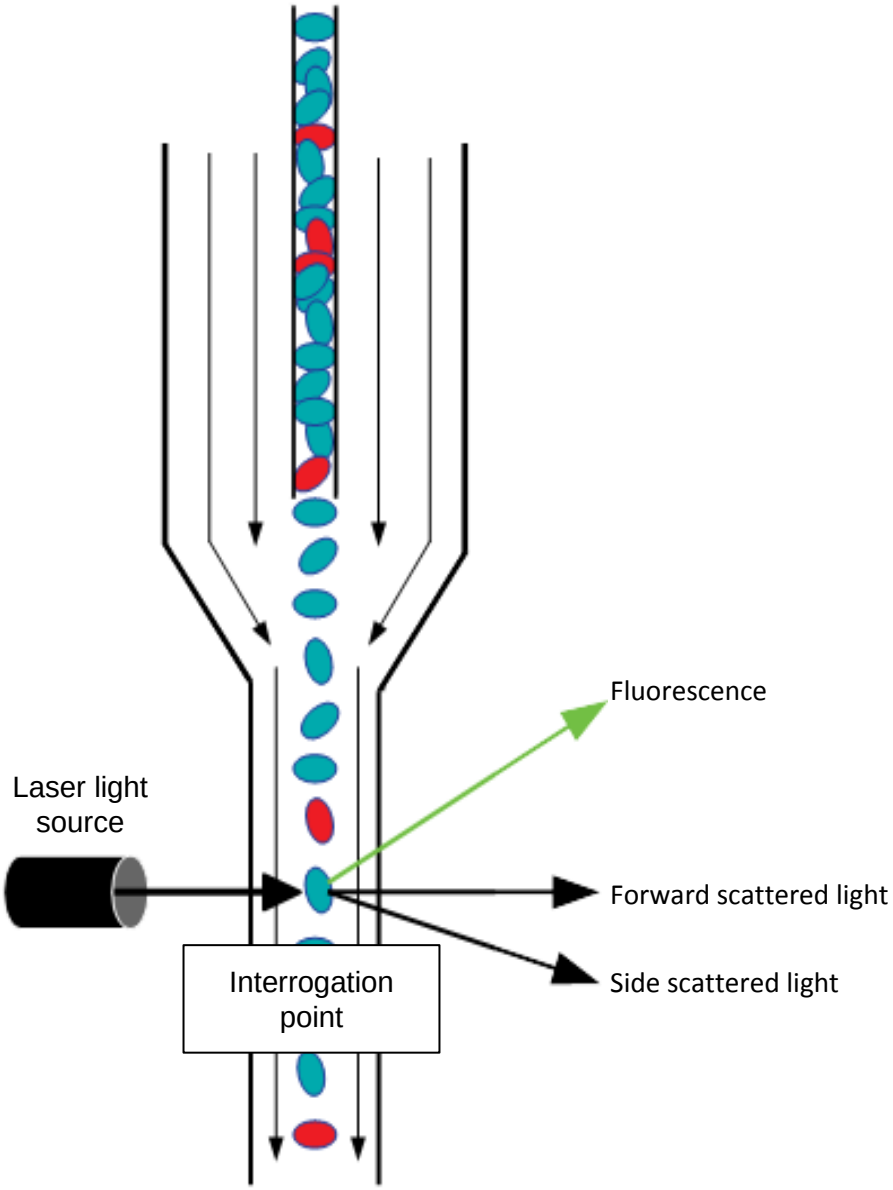


Figure 8.4 Representation of the internal workings of a flow cytometer

Cells are passed single file through a laser beam at the interrogation point. Upon contact with the cell, light scatters based on the cell's properties. Lasers are also able to excite fluorophores conjugated to bound antibodies, which emit different wavelengths of fluorescent light.

8.3.1.3 Light transmission aggregometry theory

First described by Born (1962), light transmission aggregometry (LTA) is now the 'gold standard' technique for *in vitro* platelet function testing. LTA is a turbidimetric assay that measures the decrease in intensity of transmitted light due to the scattering effect of particles suspended in the solution. The technique relies on platelets being distributed evenly throughout the suspension, forming an optically dense medium that significantly refracts the passage of light (Figure 8.5A). This enables a suspension of resting platelets to be set as a representative of 0% aggregation and platelet-free CFT to represent 100% aggregation (ie. full light transmission as all platelets are assumed to be aggregated).

Washed platelet preparation is dispensed into a glass cuvette and placed into the aggregometer, where it rests between a light source and a photometer, under stirring conditions at 37°C. Upon the addition of an agonist, platelet activation is initiated and they undergo rapid cytoskeletal reorganisation causing the extension of filopodia and lamellipodia, resulting in a dramatic increase in surface area, otherwise referred to as platelet shape change. This shape change causes an increase in turbidity due to the increased surface area of the platelets, which results in a decrease in light transmission from the resting platelet baseline (Figure 8.5B). After shape change, activatory signalling continues *via* a number of different pathways until integrin $\alpha_{IIb}\beta_3$ receptors on the platelet surface are activated to a high-affinity state. Active integrin $\alpha_{IIb}\beta_3$ binds fibrinogen released from α-granules of other activated platelets. As fibrinogen can bind to two activated integrin receptors simultaneously, this leads to a cascade of platelet binding that initially forms microaggregates, and later forms macroaggregates. As aggregates start to form, the turbidity of the platelet suspension decreases, allowing increased light transmission through the sample. Therefore, following an initial dip in light transmission, it increases past 0% towards 100%, with the rate of aggregation and maximal aggregation being influenced by the potency of the agonist. The photometer measures the amount of light transmitted through the platelet suspension periodically, and the computer software plots this data on a graph, otherwise known as an aggregation trace (Figure 2.11B).

The formation of platelet aggregates upon stimulation transforms the solution from one containing a vast number of small particles to one containing a smaller number of larger particles. This increases the space between particles, thus, light transmission increases (Cardinal & Flower, 1980; Cattaneo*, et al.*, 2012). As the platelets form larger and larger aggregates, the space between them continues to increase, thereby, allowing greater and greater transmission of light through the solution. When agonist stimulated sample is compared to the platelet-free CFT sample, there is a correlation between the amount of light transmission and the extent of platelets aggregation.

The potency of the agonist dictates which of three types of aggregation the platelets undergo. Weak agonists induce primary aggregation, which is reversible, allowing platelets to return back to a resting state. Stronger agonists can evoke either biphasic or secondary wave of aggregation, both of which are irreversible and result from the release of mediators of secondary signalling, however, there is a lag between primary and secondary signalling in biphasic aggregation. Biphasic aggregation may be caused by agonists with medium potency. The representative trace in Figure 2.11B is biphasic.

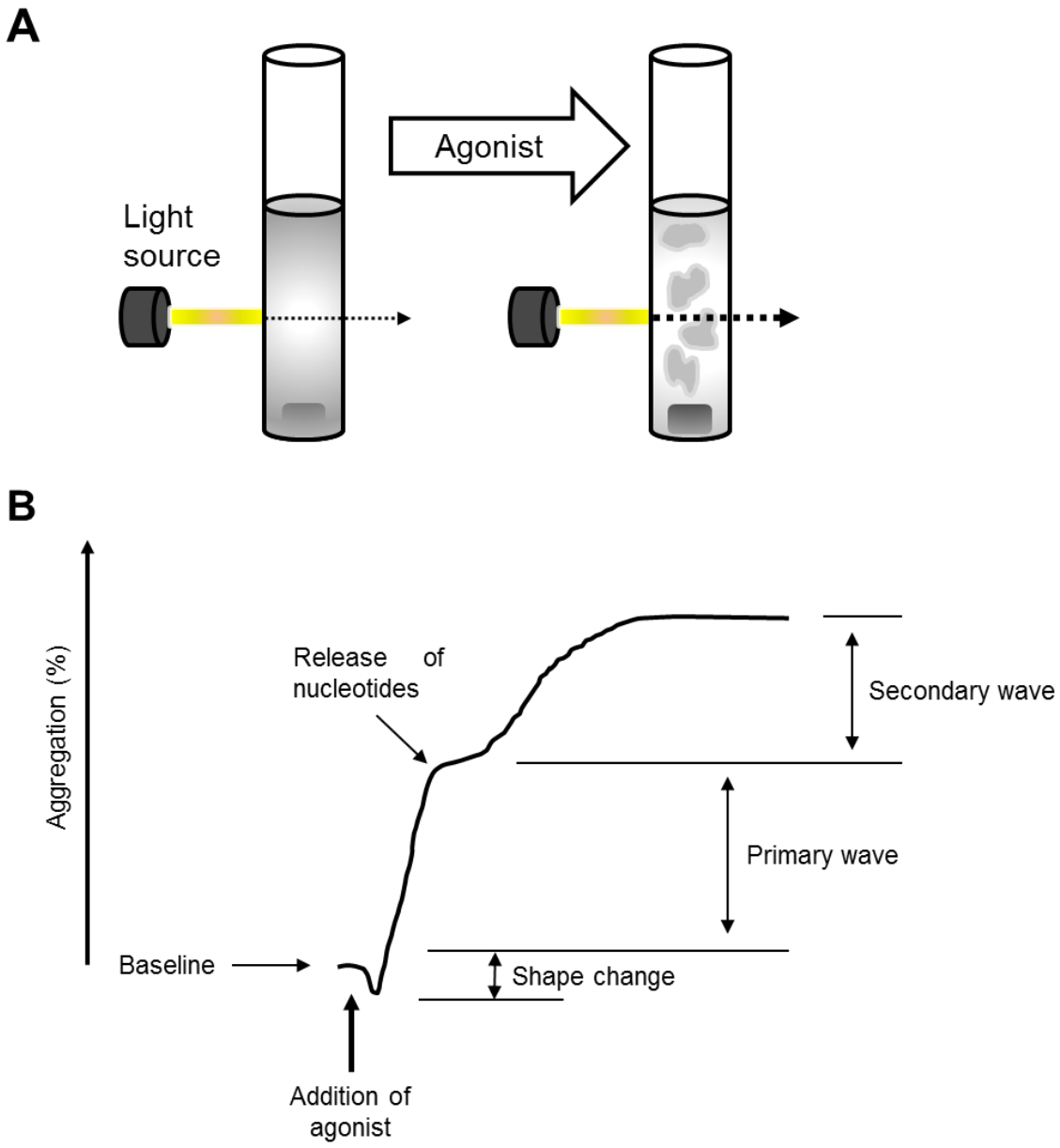


Figure 8.5 Light transmission aggregometry of washed platelets

A) An illustration of aggregation of washed platelets in an aggregometer. B) Aggregation trace of biphasic aggregation with an explanation of trace analysis. Upon addition of an agonist, platelets undergo shape change that increases turbidity, before a rapid decrease in turbidity as a result of platelet activation.

8.3.1.4 Lactate dehydrogenase assay theory

Lactate dehydrogenase (LDH) is a stable oxidoreductase enzyme present in nearly all living cells that is released upon damage to the plasma membranes. The LDH assay determines LDH activity *via* a two-step enzymatic reaction (Figure 8.6). Firstly, LDH catalyses the

oxidation of lactate to pyruvate while reducing NAD^+ to NADH and H^+. Then, diaphorase uses NADH and H^+ to catalyse the reduction of the tetrazolium salt to a formazan dye, which changes colour from pale yellow to red. The magnitude of this reaction depends on the quantity of LDH released, which is proportional to the quantity of cells with damaged plasma membranes. The formazan salt is water-soluble with a broad absorbance spectrum and maxima at 500 nm, whereas the tetrazolium salt produces minimal absorbance at these wavelengths, making it ideal for experimental use (abcam, 2016; Cayman chemical, 2012; Thermo Scientific, 2016).

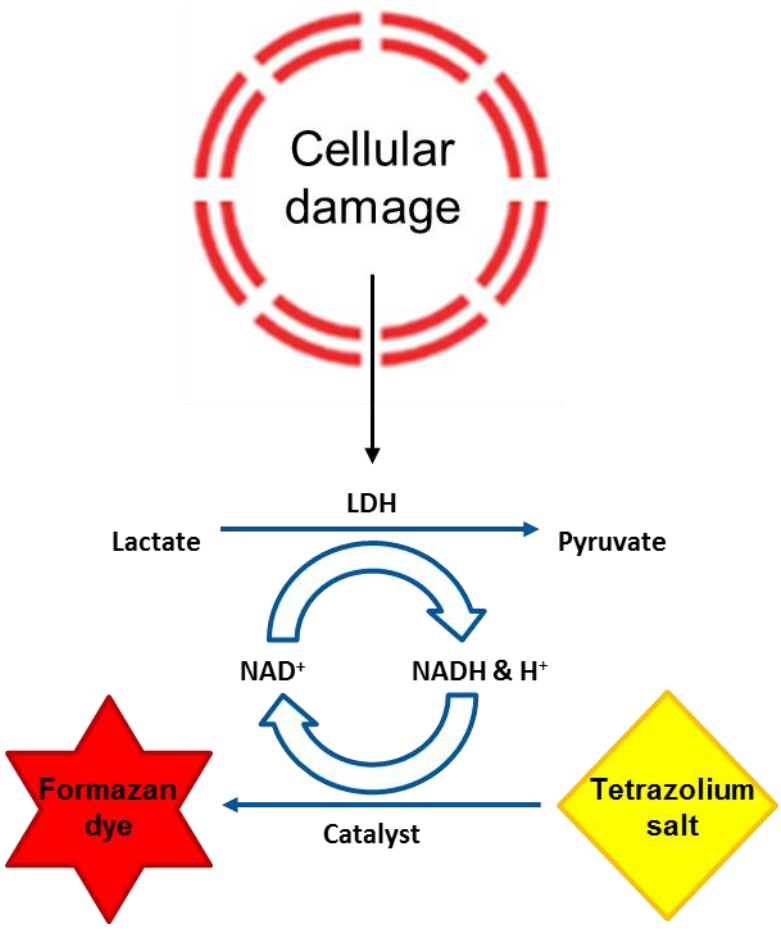


Figure 8.6 Diagram of reactions involved in the LDH assay

Cellular damage releases LDH which catalyses the production of NADH and H^+, which are used in the production of a formazan dye. The absorbance of this dye is proportional to LDH release.

8.4 Appendix D

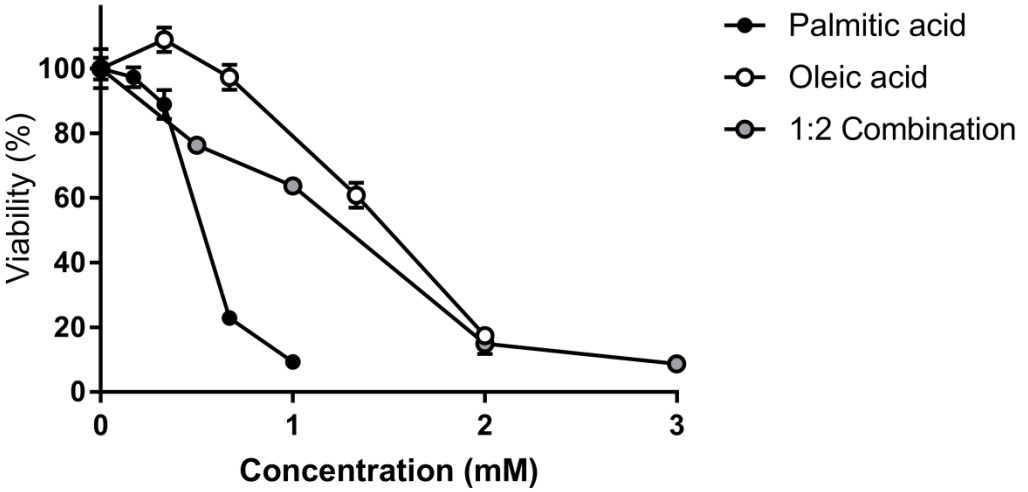


Figure 8.7 Loss of HepG2 viability following treatment with fatty acids upto 3 mM

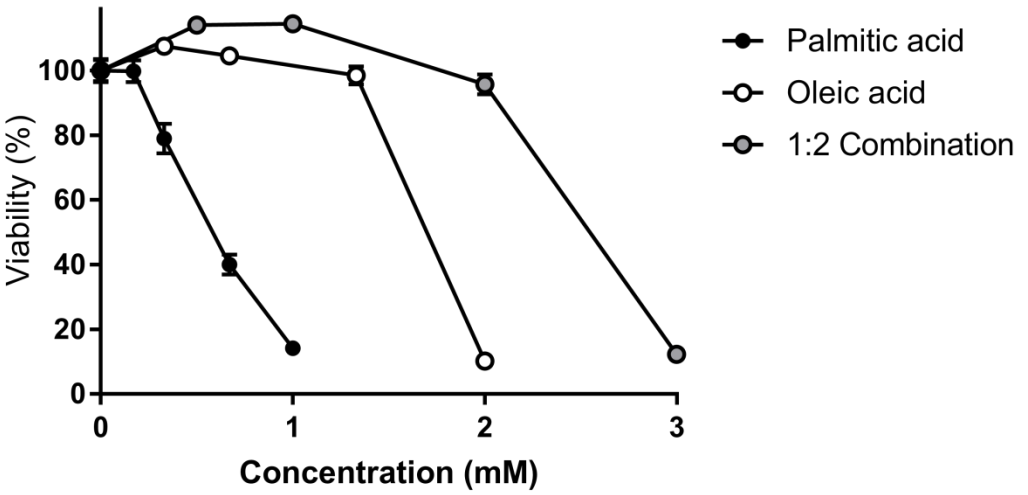


Figure 8.8 Loss of L6 viability following treatment with fatty acids upto 3 mM

8.5 Appendix E

8.5.1 Molar percentage of palmitic acid and oleic acid in phospholipids between control participants and subjects with cancer

This research studied the average molar percentage of palmitic acid and oleic acid in phospholipids between control participants and subjects with cancer across 4 studies, n=540 and n=379, respectively.

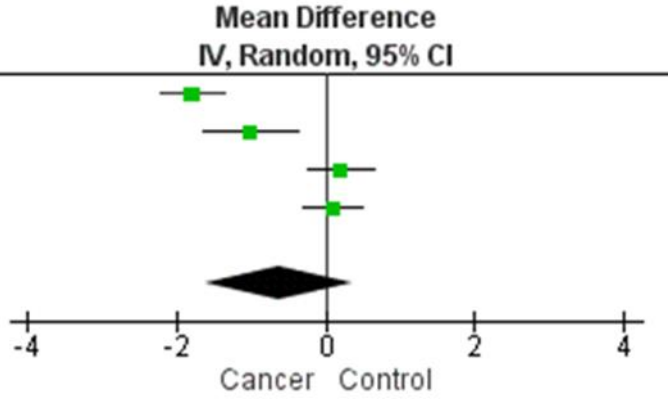
The meta-analysis (Table 8.2 and 8.3) demonstrated that there were no significant differences in the molar percentages of palmitic acid and oleic acid in plasma phospholipids in cancer patients versus the control group (p=0.22 and 0.94, respectively).

Table 8.2 Palmitic acid in phospholipids (mol%) - control vs. cancer

Study or Subgroup	Control Mean	Control SD	Control Total	Cancer Mean	Cancer SD	Cancer Total	Weight	Mean Difference IV, Random, 95% CI
Harvei et al., 1997	26.1	1.46	282	27.9	2.36	141	25.3%	-1.80 [-2.23, -1.37]
Okuno et al., 2013	29.69	1.73	61	30.7	1.54	41	24.0%	-1.01 [-1.65, -0.37]
Saadatian-Elahi et al., 2002	25.1	1.6	91	24.9	1.5	91	25.2%	0.20 [-0.25, 0.65]
Saadatian-Elahi et al., 2002	25	1.3	106	24.9	1.6	106	25.5%	0.10 [-0.29, 0.49]
Total (95% CI)			**540**			**379**	**100.0%**	**-0.62 [-1.63, 0.38]**

Heterogeneity: $Tau^2 = 0.99$; $Chi^2 = 56.36$, df = 3 ($P < 0.00001$); $I^2 = 95\%$

Test for overall effect: $Z = 1.22$ ($P = 0.22$)



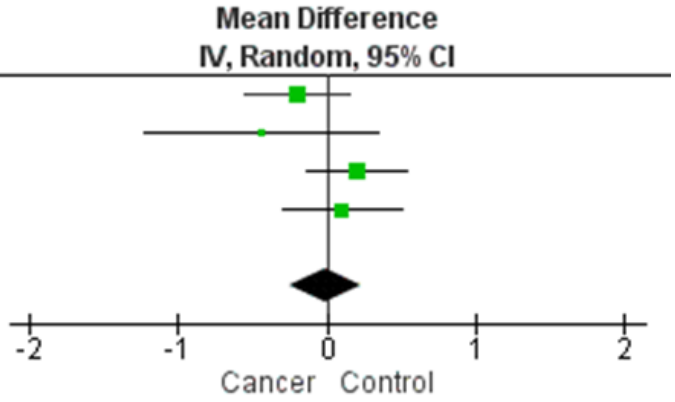
Chi^2 =56.36 was far larger than the degrees of freedom (3), which suggested significant heterogeneity. However, Chi^2 was unreliable due to the small number of studies included. I^2 =95% estimated very high levels of variability amongst study results. Tau^2 =0.99 was very close to 1, which confers definite heterogeneity. Therefore, between-study variance was present and a random-effects model was chosen.

Table 8.3 Oleic acid in phospholipids (mol%) - control vs. cancer

Study or Subgroup	Control Mean	Control SD	Control Total	Cancer Mean	Cancer SD	Cancer Total	Weight	Mean Difference IV, Random, 95% CI
Harvei et al., 1997	9.8	1.6	282	10	1.78	141	32.1%	-0.20 [-0.55, 0.15]
Okuno et al., 2013	8.67	1.35	61	9.11	2.31	41	8.7%	-0.44 [-1.22, 0.34]
Saadatian-Elahi et al., 2002	8.7	1.1	106	8.5	1.4	106	33.3%	0.20 [-0.14, 0.54]
Saadatian-Elahi et al., 2002	8.7	1.7	91	8.6	1	91	26.0%	0.10 [-0.31, 0.51]
Total (95% CI)			**540**			**379**	**100.0%**	**-0.01 [-0.25, 0.23]**

Heterogeneity: $Tau^2 = 0.02$; $Chi^2 = 4.05$, df = 3 (P = 0.26); $I^2 = 26\%$

Test for overall effect: Z = 0.08 (P = 0.94)



Chi^2 =4.05 was far greater than the degrees of freedom (3), and, thus, suggested between-study variance. However, the result was unreliable as number of studies included was too small to produce reliable results. I^2 =26% predicted moderate variability amongst study results. However, Tau^2 =0.02 was fairly small. However, when analysed in combination, the results showed the heterogeneity within the study that required the use of a random-effects model.

8.6 Appendix F

Table 8.4 Full list of search terms

Search terms
fatty acids, FA, free fatty acids, FFA, non-esterified fatty acids, NEFA, palmitic acid, oleic acid, plasma, serum, phospholipids, PL, cholesterol esters, CE, triglycerides,TG, triacylglycerol, TAG, circulating, composition, fractions, levels, concentrations, percentages, proportions

A combination of theses search terms were used to find studies for this project

Table 8.5 Plasma triglyceride data

	Study	n	TAG (mmol/L)	SD
1	Yli-Jama*, et al.*, 2002	103	1.74	0.67
2	Yli-Jama*, et al.*, 2002	104	1.39	0.58
3	Baylin*, et al.*, 2005	99	1.21	0.65
4	Baylin*, et al.*, 2005	101	1.21	0.62
5	Forsythe*, et al.*, 2008	20	2.38	0.65427
6	Forsythe*, et al.*, 2008	20	2.11	0.65088
7	Rise*, et al.*, 2007	10	0.79	0.23617
8	Ebbesson*, et al.*, 2015	419	1.51	0.87914
9	Ebbesson*, et al.*, 2015	337	1.42	0.80004
10	Fernandez-Real*, et al.*, 2005	76	1.33	0.791
11	Fernandez-Real*, et al.*, 2005	40	0.79	0.2712
12	Itakura*, et al.*, 2010	8076	2.15	0.17402
13	Kim *et al*., 2013	159	0.97	0.23
14	Kim *et al*., 2013	331	1.51	0.35
15	Kim *et al*., 2013	103	2.33	0.85
16	Jouven*, et al.*, 2001	91	1.69	1.3673
17	Jouven*, et al.*, 2001	145	1.59	1.3221
18	Jouven*, et al.*, 2001	5014	1.41	1.1639
19	Raatz*, et al.*, 2001	8	1.57	0.32
20	Raatz*, et al.*, 2001	8	0.91	0.18
21	Talreja*, et al.*, 2014	76	1.15	0.678
22	Mayneris-Perxachs *et al*., 2014	112	0.98	0.37742
23	Khaw*, et al.*, 2012	1595	2.22	1.23
24	Khaw*, et al.*, 2012	2246	2.01	1.15
25	Khaw*, et al.*, 2012	776	2.1	1.15
26	Khaw*, et al.*, 2012	2684	1.64	1.07

27	Yamagishi*, et al.*, 2008	1667	1.57	1.0622
28	Yamagishi*, et al.*, 2008	1908	1.31	0.8249
29	Glew*, et al.*, 2010	22	1.03	0.3955
30	Glew*, et al.*, 2010	29	1.13	0.5085
31	Lee*, et al.*, 2000	60	1.13	0.53
32	Lee*, et al.*, 2000	133	1.05	0.58
33	Kuriki*, et al.*, 2003	15	1.09	0.44
34	Kuriki*, et al.*, 2003	79	0.89	0.43
35	Vognild*, et al.*, 1998	124	1.1	0.1
36	Vognild*, et al.*, 1998	149	1.5	0.1
37	Vicario*, et al.*, 1998	10	0.96	0.9486832981
38	Simon*, et al.*, 1996	156	2.19	1.83
39	Ferdandz-Real*, et al.*, 2003	64	1.1	0.5085
40	Ferdandz-Real*, et al.*, 2003	70	1.59	0.9831
41	Yerlikaya*, et al.*, 2011	45	1.02	0.50285
42	Tholstrup*, et al.*, 2004	17	0.8	0.3
43	Woods*, et al.*, 2009	13	0.79	0.29493
44	Woods*, et al.*, 2009	18	1.88	1.0848
45	Kawashima*, et al.*, 2009	27	1.08	0.5198
46	Kawashima*, et al.*, 2009	43	1.59	0.6215
47	Kim*, et al.*, 2010	30	1.65	0.6870084039
48	Jocken*, et al.*, 2008	13	0.701	0.2379663842
49	Jocken*, et al.*, 2008	10	0.648	0.1328156617
50	Buemann*, et al.*, 2006	269	1.41	0.87
51	Buemann*, et al.*, 2006	174	1.91	1.55
52	Wuesten*, et al.*, 2005	17	1.06	0.6
53	Wuesten*, et al.*, 2005	16	1.66	0.99
54	Abdelmagid*, et al.*, 2015	327	1	0.5
55	Abdelmagid*, et al.*, 2015	499	0.8	0.3
56	Chrisholm*, et al.*, 1998	21	1.91	0.75
57	Manning*, et al.*, 2008	15	1.45	0.75
58	Manning*, et al.*, 2008	14	1.07	0.42
59	Pollare*, et al.*, 1991	7	1.42	0.59
60	Pollare*, et al.*, 1991	7	2.14	1.51
61	van der Merwe*, et al.*, 2001	10	0.7	0.06
62	van der Merwe*, et al.*, 2001	10	1.1	0.4110960958
63	Frias*, et al.*, 2000	9	1.9	0.93
64	Frias*, et al.*, 2000	9	1.11	0.24
65	Goodpaster*, et al.*, 2002	7	1.26	0.476235236
66	Goodpaster*, et al.*, 2002	7	1.99	1.243503116
67	Stefan*, et al.*, 2008	54	1.08	0.4409081537
68	Stefan*, et al.*, 2008	133	1.38	1.037930634
69	Stefan*, et al.*, 2008	31	1.6	1.893039883
70	Sundell*, et al.*, 2003	10	1.3	0.7
71	Sundell*, et al.*, 2003	10	1.1	0.5
72	Ranganath*, et al.*, 1999	7	1.99	1.322875656

73	Ranganath*, et al.*, 1999	7	1.03	0.6878953409
74	Stojiljkovic*, et al.*, 2002	10	3.5	3.794733192
75	Vanttinen*, et al.*, 2005	72	1.03	0.54
76	Vanttinen*, et al.*, 2005	52	1.34	0.94
77	de Jongh*, et al.*, 2004	16	0.8	0.3
78	de Jongh*, et al.*, 2004	12	1.4	0.8
79	Kruszynska*, et al.*, 2003	15	0.95	0.3872983346
80	Kruszynska*, et al.*, 2003	15	2.06	1.00697567
81	Riedel*, et al.*, 1995	5	1.86	1.25
82	Riedel*, et al.*, 1995	5	1.93	0.48
83	Manco*, et al.*, 2004	5	1.1	0.2236067977
84	Manco*, et al.*, 2004	5	1.55	0.201246118
85	Straczkowski*, et al.*, 2006	33	1.02	0.52
86	Straczkowski*, et al.*, 2006	30	1.64	0.93
87	Laaksonen*, et al.*, 2002	806	1.17	0.21
88	Vessby*, et al.*, 1994	1753	2.06	1.14
89	Kashyap*, et al.*, 2003	8	1.38	0.24
90	Kashyap*, et al.*, 2003	13	1.28	0.11
91	Carpentier*, et al.*, 2000	7	1.17	0.4233202098
92	Ferrannini*, et al.*, 1993	6	1.08	0.6
93	Diraison*, et al.*, 2003	6	0.85	0.171464282
94	Grundy*, et al.*, 1986	11	1.9	0.6633249581
95	Friday*, et al.*, 1989	8	2.29	0.3111269837
96	Tripathy*, et al.*, 2003	10	0.85	0.18
97	Kelley*, et al.*, 2004	17	2.33	0.29
98	Kelley*, et al.*, 2004	22	1.85	0.16
99	Herrero*, et al.*, 2006	11	1.5	0.69
100	Svanborg*, et al.*, 2009	30	0.94	0.39
101	Abbey*, et al.*, 1994	16	1.41	0.52
102	Judd*, et al.*, 1994	29	1.26	0.655
103	Judd*, et al.*, 1994	29	1.34	0.802
104	Ramirez-Tortosa*, et al.*, 2010	30	1.63	0.876356092
105	Ramirez-Tortosa*, et al.*, 2010	26	1.04	0.6628725368
106	De Almeida*, et al.*, 2002	6	0.8	0.4
107	Jacobsen*, et al.*, 1983	22	2.16	0.775
108	Jacobsen*, et al.*, 1983	20	2.31	0.66
109	Ducimetiere*, et al.*, 1980	7246	1.23	1.16
110	DeFronzo and Goodman, 1995	146	2.09	1.208304597
111	Couillard*, et al.*, 2001	62	0.94	0.22
112	Drexel*, et al.*, 1994	105	1.51	1.024695077
113	Schaefer*, et al.*, 1978	1088	0.98	0.49
114	Hotta*, et al.*, 2000	54	1.76	1.763632615
115	Hotta*, et al.*, 2000	28	1.31	0.7408103671
116	Chen*, et al.*, 2006	59	1.3	0.8
117	Rijzewijk*, et al.*, 2008	28	1	0.6614378278
118	Isomaa*, et al.*, 2001	1988	1.29	0.8

119	Liu*, et al.*, 2013	60	1.17	0.69
120	Nappo*, et al.*, 2002	20	0.8	0.2
121	Prior*, et al.*, 2005	19	0.75	0.3
122	Brancati*, et al.*, 2000	1316	1.08	0.7255342859
123	Brancati*, et al.*, 2000	4621	1.29	0.679779376
124	Pirro*, et al.*, 2002	98	1.7	0.66
125	Logan*, et al.*, 1978	107	1.82	1.06
126	Logan*, et al.*, 1978	82	1.28	0.67
127	Després*, et al.*, 1990	25	0.79	0.35
128	Després*, et al.*, 1990	10	1.47	0.79
129	Després*, et al.*, 1990	10	2.57	2.41
130	Esposito*, et al.*, 2003	60	1.6	0.5
131	Esposito*, et al.*, 2003	60	1.6	0.6
132	Bonanome and Grundy, 1988	11	1.64	0.4974937186
133	Vessby*, et al.*, 1994b	215	1.45	0.9
134	Salomaa*, et al.*, 1990	325	1.5	0.84
135	Miettinen*, et al.*, 1982	64	1.9	0.8
136	Harper*, et al.*, 2006	27	1.5	0.4
137	Harper*, et al.*, 2006	22	1.4	0.4
138	Baer*, et al.*, 2004	50	1.149	0.5727564928
139	Öhrvall*, et al.*, 1996	1836	2.03	1.14
		n= 52128	Average = 1.424158273	
			Weighted average = 1.577840757	

Table 8.6 Plasma FFA data

	Study	n	FFA (µmol/L)	SD
1	Sundell*, et al.*, 2003	10	610	948.6832981
2	Sundell*, et al.*, 2003	10	500	474.341649
3	Ranganath*, et al.*, 1999	7	575	158.7450787
4	Ranganath*, et al.*, 1999	7	720	142.8705708
5	Stefan*, et al.*, 2008	54	710	238.3843418
6	Stefan*, et al.*, 2008	164	645	276.9991545
7	Stojiljkovic*, et al.*, 2002	12	707	207.8460969
8	Stojiljkovic*, et al.*, 2002	10	669	290.9295447
9	Jocken*, et al.*, 2008	13	661	147.8276023
10	Jocken*, et al.*, 2008	10	638	132.8156617
11	Pincelli*, et al.*, 2001	6	433.6	164.8751546
12	Pincelli*, et al.*, 2001	10	703.7	187.1435919
13	Reyna*, et al.*, 2008	7	351	97.89279851

14	Reyna*, et al.*, 2008	8	479	166.8772004
15	Mari*, et al.*, 2006	22	230	60
16	Mari*, et al.*, 2006	12	410	90
17	Manco*, et al.*, 2004	5	540	156.5247584
18	Manco*, et al.*, 2004	5	580	156.5247584
19	Buemann*, et al.*, 2006	269	359	142
20	Buemann*, et al.*, 2006	174	393	161
21	Owen*, et al.*, 1992	5	402	138.6362146
22	Owen*, et al.*, 1992	5	495	125.2198067
23	Wuesten*, et al.*, 2005	17	540	250
24	Wuesten*, et al.*, 2005	16	460	150
25	Vanttinen*, et al.*, 2005	72	530	190
26	Vanttinen*, et al.*, 2005	52	630	220
27	de Jongh*, et al.*, 2004	16	550	190
28	de Jongh*, et al.*, 2004	12	690	210
29	Iannello*, et al.*, 1998	10	373	139.140217
30	Iannello*, et al.*, 1998	25	721	260
31	Adams*, et al.*, 2004	10	621	22.13594362
32	Adams*, et al.*, 2004	10	860	22.13594362
33	Kruszynska*, et al.*, 2003	15	445	209.1411007
34	Kruszynska*, et al.*, 2003	15	404	170.4112672
35	Horton*, et al.*, 1995	9	320.8	140.55
36	Horton*, et al.*, 1995	7	299.1	143.0293159
37	Goodpaster*, et al.*, 2002	7	474	132.2875656
38	Goodpaster*, et al.*, 2002	7	599	171.9738352
39	Allick*, et al.*, 2004	6	540	97.97958971
40	Allick*, et al.*, 2004	6	650	171.464282
41	Golay*, et al.*, 2002	15	630	174.2842506
42	Golay*, et al.*, 2002	12	732	214.7743001
43	Soriguer*, et al.*, 2008	12	315	103.9230485
44	Soriguer*, et al.*, 2008	9	605	147
45	Gautier*, et al.*, 2000	11	920	340
46	Gautier*, et al.*, 2000	11	640	360
47	Straczkowski*, et al.*, 2006	33	347	116
48	Straczkowski*, et al.*, 2006	30	504	155
49	Belfiore*, et al.*, 2001	34	359	216.1533867
50	Belfiore*, et al.*, 2001	27	713	242.6083566
51	Reeds*, et al.*, 2006	5	408	162.1149284
52	Reeds*, et al.*, 2006	5	383	103.7535542
53	Frias*, et al.*, 2000	9	240	0.15
54	Frias*, et al.*, 2000	9	360	0.24
55	Morin-Papunen*, et al.*,2000	17	460	907.0832376
56	Morin-Papunen*, et al.*,2000	17	550	948.3142939
57	Jansson*, et al.*, 1998	15	494	174.2842506
58	Jansson*, et al.*, 1998	9	696	153
59	Sbraccia*, et al.*, 2002	10	223	51.86135363

60	Sbraccia*, et al.*, 2002	14	738	277.6309781
61	Solini*, et al.*, 1997	9	575	111
62	Solini*, et al.*, 1997	11	728	102.8153685
63	Macor*, et al.*, 1997	9	434	105
64	Macor*, et al.*, 1997	26	747	229.4558781
65	Paquot*, et al.*, 2002	10	430	411.0960958
66	Paquot*, et al.*, 2002	6	850	489.8979486
67	Riedel*, et al.*, 1995	5	940	210
68	Riedel*, et al.*, 1995	5	920	170
69	van der Merwe*, et al.*, 2001	10	463	151.7893277
70	van der Merwe*, et al.*, 2001	10	870	132.8156617
71	Groop*, et al.*, 1992	11	642	129.3483668
72	Groop*, et al.*, 1992	7	793	113.7673064
73	Robertson*, et al.*, 1992	14	770	190
74	Robertson*, et al.*, 1992	16	970	210
75	Manning*, et al.*, 2008	14	410	19
76	Manning*, et al.*, 2008	15	420	14
77	Pollare*, et al.*, 1991	7	410	180
78	Pollare*, et al.*, 1991	7	500	140
79	Yli-Jama*, et al.*, 2002	103	420	370
80	Yli-Jama*, et al.*, 2002	104	380	280
81	Smedman*, et al.*, 1999	62	500	170
82	Mougios*, et al.*, 2003	13	303	53
83	Jouven*, et al.*, 2001	91	384	334
84	Jouven*, et al.*, 2001	145	310	123
85	Jouven*, et al.*, 2001	5014	313	144
86	Lopes*, et al.*, 1991	12	435	11
87	Hagenfeldt, 1971	25	640	239
88	Kim*, et al.*, 2010	30	618.2	38.3
89	Abdelmagid*, et al.*, 2012	327	457.5	252.5
90	Abdelmagid*, et al.*, 2012	499	485.9	250.8
91	Gautier*, et al.*, 2001	10	990	250
92	Gautier*, et al.*, 2001	12	850	130
93	Kashyap*, et al.*, 2003	8	607	243.2447327
94	Kashyap*, et al.*, 2003	13	575	169.4609099
95	Boden*, et al.*, 1998	7	597	222.2431101
96	Santomauro*, et al.*, 1999	9	329	84
97	Santomauro*, et al.*, 1999	13	560	187.4886663
98	Blaak*, et al.*, 2000	8	724	66
99	Carpentier*, et al.*, 2000	7	510	185.2025918
100	Ferrannini*, et al.*, 1993	6	670	80
101	Diraison*, et al.*, 2003	6	426	107.7775487
102	Diraison*, et al.*, 2003	5	608	116.2755348
103	Fraze*, et al.*, 1985	15	306	81.33265027
104	Tripathy*, et al.*, 2003	10	352	62
105	Chen*, et al.*, 1987	6	572	108

106	Chen*, et al.*, 1987	6	782	184
107	Kelley*, et al.*, 2004	17	691	98.95453501
108	Kelley*, et al.*, 2004	22	705	164.1645516
109	Cusi*, et al.*, 2007	9	498	84
110	Mensink*, et al.*, 2001	7	727	182.5568405
111	Swislocki*, et al.*, 1987	6	533	36.74234614
112	Svanborg*, et al.*, 2009	47	750	233
113	Svanborg*, et al.*, 2009	15	781	174
114	Rich*, et al.*, 1959	7	855	243
115	Felber*, et al.*, 1987	6	580	134.7219359
116	Felber*, et al.*, 1987	9	383	78
117	Felber*, et al.*, 1987	7	575	259.2836285
118	Rijzewijk*, et al.*, 2008	28	450	211.6601049
119	Prior*, et al.*, 2005	19	620	95.89577676
120	Pirro*, et al.*, 2002	98	750	280
121	Esposito*, et al.*, 2003	60	581	102
122	Esposito*, et al.*, 2003	60	562	0.6
123	Carlsten*, et al.*, 2009	9	854	205.5
124	Vessby*, et al.*, 1994b	215	500	180
125	Xiao*, et al.*, 2006	7	460	211.6601049
		n= 8847	Average = 562.1344	
			Weighted average = 397.8530688	

Table 8.7 Palmitic acid in phospholipids (mol%) data

	Study	n	Palmitic acid in phospholipids (mol%)	SD
1	Bradbury*, et al.*, 2011	2416	31.73	4.22
2	Clemmesen*, et al.*, 2000	11	31.5	2
3	Forouhi*, et al.*, 2014	15164	29.93	1.74
4	Raatz*, et al.*, 2001	10	27.32	0.64
5	Raatz*, et al.*, 2001	10	25.26	0.62
6	Clarke*, et al.*, 2009	239	29.1	1.3
7	Skeaff*, et al.*, 2006	19	33.14	1.54
8	Saadatian-Elahi*, et al.*, 2009	91	25.2	1.4
9	Saadatian-Elahi*, et al.*, 2009	100	23.8	1.7
10	Saadatian-Elahi*, et al.*, 2009	93	24.7	1.6
11	Saadatian-Elahi*, et al.*, 2009	100	22.8	1.5
12	Saadatian-Elahi*, et al.*, 2009	96	25	1.4
13	Saadatian-Elahi*, et al.*, 2009	100	22.8	1.6
14	Saadatian-Elahi*, et al.*, 2009	94	25.2	1.8

15	Saadatian-Elahi*, et al.*, 2009	100	23.1	1.8
16	Saadatian-Elahi*, et al.*, 2009	90	26.1	1.7
17	Saadatian-Elahi*, et al.*, 2009	99	24	1.7
18	Saadatian-Elahi*, et al.*, 2009	94	25.9	1.3
19	Saadatian-Elahi*, et al.*, 2009	99	24.5	1.7
20	Saadatian-Elahi*, et al.*, 2009	96	25.5	1.3
21	Saadatian-Elahi*, et al.*, 2009	100	24.6	1.5
22	Saadatian-Elahi*, et al.*, 2009	96	24.8	1.9
23	Saadatian-Elahi*, et al.*, 2009	95	26.4	1.6
24	Saadatian-Elahi*, et al.*, 2009	96	25.6	1.4
25	Saadatian-Elahi*, et al.*, 2009	96	26.5	1.6
26	Saadatian-Elahi*, et al.*, 2009	99	25.3	2.2
27	Saadatian-Elahi*, et al.*, 2009	95	26.1	2
28	Saadatian-Elahi*, et al.*, 2009	100	24.6	1.9
29	Saadatian-Elahi*, et al.*, 2009	95	25.8	1.4
30	Saadatian-Elahi*, et al.*, 2009	100	24.9	2.1
31	Saadatian-Elahi*, et al.*, 2009	95	24.2	2.2
32	Saadatian-Elahi*, et al.*, 2009	100	22	2.1
33	Saadatian-Elahi*, et al.*, 2009	96	26.9	1.6
34	Saadatian-Elahi*, et al.*, 2009	100	25.1	1.9
35	Saadatian-Elahi*, et al.*, 2009	100	25.5	1.6
36	Saadatian-Elahi*, et al.*, 2009	95	26.3	1.8
37	Saadatian-Elahi*, et al.*, 2009	94	26.6	1.5
38	Saadatian-Elahi*, et al.*, 2009	99	25.3	1.6
39	Kim*, et al.*, 2013	159	31.858	0.415
40	Kim*, et al.*, 2013	331	32.544	0.29
41	Kim*, et al.*, 2013	103	33.235	0.519
42	Hodge*, et al.*, 2007	3391	25.23	1.54
43	Hodge*, et al.*, 2007	346	25.24	1.44
44	Yeh *et al*., 1994	104	37	0.44
45	Simon*, et al.*, 1996	156	28.12	1.77
46	Harvei*, et al.*, 1997	282	26.1	1.46
47	Harvei*, et al.*, 1997	141	27.9	2.36
48	Vicario*, et al.*, 1998	10	29.75	0.41
49	Agren*, et al.*, 1995	8	28.4	1.4
50	Agren*, et al.*, 1995	11	30.3	1.7
51	Okuno*, et al.*, 2013	61	29.69	1.73
52	Lopes*, et al.*, 1991	12	24.7	0.72
53	Thiebaut*, et al.*, 2009	1114	25.1	1.66
54	Saadatian-Elahi*, et al.*, 2002	91	25.1	1.6
55	Saadatian-Elahi*, et al.*, 2002	106	25	1.3
56	Saadatian-Elahi*, et al.*, 2002	91	24.9	1.5
57	Saadatian-Elahi*, et al.*, 2002	106	24.9	1.6
58	Yamagishi*, et al.*, 2008	3575	25.41	1.64
59	Zeleniuch-Jacquotte*, et al.*, 2000	46	24.04	1.63
60	Nikkari*, et al.*, 1995	41	28.4	1.77

61	Nikkari, *et al.*, 1995	41	28.25	1.33
62	Sabel, *et al.*, 2009	30	34.1	1.3
63	Kim, *et al.*, 2010	30	34.6	2.464751509
64	Woods, *et al.*, 2009	13	36.5	3.6
65	Woods, *et al.*, 2009	18	34.4	2.7
66	Wang, *et al.*, 2003	2657	25.3	1.6
67	Patel, *et al.*, 2010	184	26.84	0.87
68	Folsom, *et al.*, 1996	3403	25.4	1.6
69	Ma, *et al.*, 1995	1712	25.5	1.6
70	Ma, *et al.*, 1995	1858	25.3	1.7
71	Caren and Corbo, 1966	20	32.4	4.9193495 5
72	Manku, *et al.*, 1984	60	25.89	1.69
73	Manku, *et al.*, 1984	41	30.14	1.86
74	Miettinen, *et al.*, 1982	64	35.17	2.24
		n= 41288	Average = 27.24036486	
			Weighted average = 27.61610761	

Table 8.8 Oleic acid in phospholipids (mol%) data

	Study	n	Oleic acid in phospholipids (mol%)	SD
1	Bradbury, *et al.*, 2011	2416	6.53	2.51
2	Clemmesen, *et al.*, 2000	11	6.699	1.1
3	Raatz, *et al.*, 2001	10	7.003	0.36
4	Raatz, *et al.*, 2001	10	7.269	0.27
5	Clarke, *et al.*, 2009	239	7.73	2.1
6	Skeaff, *et al.*, 2006	19	8.14	0.86
7	Saadatian-Elahi, *et al.*, 2009	91	8.5	2.1
8	Saadatian-Elahi, *et al.*, 2009	100	8.59	2.4
9	Saadatian-Elahi, *et al.*, 2009	93	8.6	2.1
10	Saadatian-Elahi, *et al.*, 2009	100	8.6	2
11	Saadatian-Elahi, *et al.*, 2009	96	8.6	2.2
12	Saadatian-Elahi, *et al.*, 2009	100	8.6	2.3
13	Saadatian-Elahi, *et al.*, 2009	94	8.66	2.3
14	Saadatian-Elahi, *et al.*, 2009	100	8.67	2.3
15	Saadatian-Elahi, *et al.*, 2009	90	8.7	2
16	Saadatian-Elahi, *et al.*, 2009	99	8.7	1.8
17	Saadatian-Elahi, *et al.*, 2009	94	9.3	1.5
18	Saadatian-Elahi, *et al.*, 2009	99	9.4	2

19	Saadatian-Elahi*, et al.*, 2009	96	9.4	1.8
20	Saadatian-Elahi*, et al.*, 2009	100	9.5	1.5
21	Saadatian-Elahi*, et al.*, 2009	96	9.6	1.5
22	Saadatian-Elahi*, et al.*, 2009	95	9.6	1.4
23	Saadatian-Elahi*, et al.*, 2009	96	9.6	1.4
24	Saadatian-Elahi*, et al.*, 2009	96	9.69	1.6
25	Saadatian-Elahi*, et al.*, 2009	99	9.8	1.4
26	Saadatian-Elahi*, et al.*, 2009	95	10	1.8
27	Saadatian-Elahi*, et al.*, 2009	100	10	1.4
28	Saadatian-Elahi*, et al.*, 2009	95	10	1.4
29	Saadatian-Elahi*, et al.*, 2009	100	10	1.4
30	Saadatian-Elahi*, et al.*, 2009	95	10.1	1.6
31	Saadatian-Elahi*, et al.*, 2009	100	10.1	1.5
32	Saadatian-Elahi*, et al.*, 2009	96	10.15	1.7
33	Saadatian-Elahi*, et al.*, 2009	100	10.3	1.3
34	Saadatian-Elahi*, et al.*, 2009	100	10.3	1.3
35	Saadatian-Elahi*, et al.*, 2009	95	10.3	1.3
36	Saadatian-Elahi*, et al.*, 2009	94	10.4	1.2
37	Saadatian-Elahi*, et al.*, 2009	99	10.4	1.4
38	Kim*, et al.*, 2013	159	10.4	0.147
39	Kim*, et al.*, 2013	331	10.4	0.095
40	Kim*, et al.*, 2013	103	10.5	0.159
41	Hodge*, et al.*, 2007	3391	10.6	1.48
42	Hodge*, et al.*, 2007	346	10.6	1.51
43	Yeh *et al*., 1994	104	10.9	1.8
44	Simon*, et al.*, 1996	156	11	1.99
45	Harvei*, et al.*, 1997	282	11.16	1.6
46	Harvei*, et al.*, 1997	141	11.2	1.78
47	Vicario*, et al.*, 1998	10	11.2	0.24
48	Agren*, et al.*, 1995	8	11.2	1.5
49	Agren*, et al.*, 1995	11	11.2	0.8
50	Okuno*, et al.*, 2013	61	11.4	1.35
51	Lopes*, et al.*, 1991	12	11.44	0.29
52	Thiebaut*, et al.*, 2009	1114	11.47	1.45
53	Saadatian-Elahi*, et al.*, 2002	91	11.5	1.7
54	Saadatian-Elahi*, et al.*, 2002	106	11.5	1.1
55	Saadatian-Elahi*, et al.*, 2002	91	11.5	1
56	Saadatian-Elahi*, et al.*, 2002	106	11.5	1.4
57	Yamagishi*, et al.*, 2008	3575	11.5	1.17
58	Zeleniuch-Jacquotte*, et al.*, 2000	46	11.7	1.6
59	Nikkari*, et al.*, 1995	41	12	1.38
60	Nikkari*, et al.*, 1995	41	12.1	1.12
61	Sabel*, et al.*, 2009	30	12.14	1.2
62	Kim*, et al.*, 2010	30	12.2	0.5110772936
63	Woods*, et al.*, 2009	13	12.48	1.7

64	Woods*, et al.*, 2009	18	12.6	2.4
65	Wang*, et al.*, 2003	2657	12.6	1.17
66	Patel*, et al.*, 2010	184	12.7	0.5275
67	Folsom*, et al.*, 1996	3403	13	1.2
68	Ma*, et al.*, 1995	1712	13.2	1.2
69	Ma*, et al.*, 1995	1858	13.5	1.2
70	Caren and Corbo, 1966	20	13.53	2.23606 7977
71	Manku*, et al.*, 1984	60	14.11	2.21
72	Manku*, et al.*, 1984	41	15.65	1.31
73	Miettinen*, et al.*, 1982	64	17.52	2.4
		n= 26124	Average = 10.5579589	
			Weighted average = 11.20448434	

Table 8.9 Palmitic acid in phospholipids (mass%) data

	Study	n	Palmitic acid in phospholipids (mass%)	SD
1	Glew*, et al.*, 2010	22	30.1	1.95
2	Glew*, et al.*, 2010	29	30.2	1.86
3	Hjartaker*, et al.*, 1997	234	25.61	1.21
4	Conquer*, et al.*, 2000	19	27.9	0.4
5	Dyerberg, 1968	12	25.9	1.7
		n= 316	Average = 27.942	
			Weighted average = 26.49253165	

Table 8.10 Palmitic acid in phospholipids (mg/L) data

	Study	n	Palmitic acid in phospholipids (mg/L)	SD
1	Durieu*, et al.*, 2007	29	222.86	45.29
2	Schwertner and mosser, 1993	12	1485	212
3	Forsythe*, et al.*, 2008	20	266.2	16.4
4	Forsythe*, et al.*, 2008	20	264.6	15.9
5	Yeh*, et al.*, 1994	104	368	83
6	Harvei*, et al.*, 1997	423	353.1	66.8

7	Hjartaker, *et al.*, 1997	234	311.76	54.37
8	Cunnane, *et al.*, 2012	10	380	98
9	Begin, *et al.*, 1989	49	586	142
		n= 901	Average = 470.8355556	
			Weighted average = 364.0378246	

Table 8.11 Palmitic acid in phospholipids (mmol/L) data

	Study	n	Palmitic acid in phospholipids (mmol/L)	SD
1	Lopes, *et al.*, 1991	12	0.424	0.08660254038
2	Thiebaut, *et al.*, 2009	1114	0.9577	0.2575
3	Khaw, *et al.*, 2012	7354	1.256	0.3323
		n= 8480	Average = 0.879233333	
			Weighted average = 1.21563559	

Table 8.12 Oleic acid in phospholipids (mass%) data

	Study	n	Oleic acid in phospholipids (mass%)	SD
1	Glew, *et al.*, 2010	22	10.4	1.36
2	Glew, *et al.*, 2010	29	9.7	1.13
3	Hjartaker, *et al.*, 1997	234	9.3	1.23
4	Conquer, *et al.*, 2000	19	12.5	0.4
5	Dyerberg, 1968	12	13.4	1.2
		n= 316	Average = 11.06	
			Weighted average = 9.761392405	

Table 8.13 Oleic acid in phospholipids (mg/L) data

	Study	n	Oleic acid in phospholipids (mg/L)	SD
1	Durieu*, et al.*, 2007	29	100.14	24.7
2	Schwertner and mosser, 1993	12	761	87
3	Forsythe*, et al.*, 2008	20	109.5	16
4	Forsythe*, et al.*, 2008	20	113.8	9.4
5	Yeh*, et al.*, 1994	104	105	34
6	Harvei*, et al.*, 1997	423	131.1	36.5
7	Hjartaker*, et al.*, 1997	234	113.36	25.68
8	Cunnane*, et al.*, 2012	10	137	20
9	Begin*, et al.*, 1989	49	274	72
		n= 901	Average = 204.9888889	
			Weighted average = 137.8463929	

Table 8.14 Oleic acid in phospholipids (mmol/L) data

	Study	n	Oleic acid in phospholipids (mmol/L)	SD
1	Lopes*, et al.*, 1991	12	0.267	0.03464101615
				2
3	Khaw*, et al.*, 2012	7354	0.474	0.1849
		n= 8480	Average = 0.3916666667	
			Weighted average = 0.4684523585	

Table 8.15 Palmitic acid in cholesterol esters (mol%) data

	Study	n	Palmitic acid in cholesterol esters (mol%)	SD
1	Bradbury*, et al.*, 2011	2393	12.12	2.86
2	Raatz*, et al.*, 2001	9	11.03	0.24
3	Raatz*, et al.*, 2001	9	10.01	0.27
4	Skeaff*, et al.*, 2006	19	11.23	0.97
5	Yeh *et al*., 1994	104	15.2	2.2

6	Melchert, *et al.*, 1987	5	13.21	2.01
7	Melchert, *et al.*, 1987	5	12.64	1.93
8	Melchert, *et al.*, 1987	5	10.79	0.9
9	Melchert, *et al.*, 1987	5	13.04	2.99
10	Simon, *et al.*, 1996	156	12.16	1.09
11	Vicario, *et al.*, 1998	10	12.91	0.52
12	Agren, *et al.*, 1995	8	10.4	1.1
13	Agren, *et al.*, 1995	11	11.5	0.7
14	Lopes, *et al.*, 1991	12	9.47	0.45
15	Wang, *et al.*, 2003	3309	11.6	1
16	Wang, *et al.*, 2003	282	10.2	0.8
17	Yamagishi, *et al.*, 2008	3575	10.02	0.81
18	Nikkari, *et al.*, 1995	41	12.08	0.83
19	Nikkari, *et al.*, 1995	41	11.99	0.7
20	Abel, 2005	27	12.24	1.1
21	Abel, 2005	43	12.46	1.02
22	Wang, *et al.*, 2003	2657	9.95	0.8
23	Vessby, *et al.*, 1994	1753	11.65	0.98
24	Ma, *et al.*, 1995	1712	10.2	0.8
25	Ma, *et al.*, 1995	1858	9.9	0.8
26	Caren and Corbo, 1966	20	12.4	1.34164 0786
27	Salomaa, *et al.*, 1990	325	11.85	0.92
28	Miettinen, *et al.*, 1982	64	13.24	1.04
29	Öhrvall, *et al.*, 1996	1836	11.65	0.97
		n= 20294	Average = 11.62551724	
			Weighted average = 10.91811028	

Table 8.16 Palmitic acid in cholesterol esters (mg/L) data

	Study	n	Palmitic acid in cholesterol esters (mg/L)	SD
1	Durieu, *et al.*, 2007	29	78.62	16.57
2	Schwertner and mosser, 1993	12	603	84
3	Forsythe, *et al.*, 2008	20	112.3	11.3
4	Forsythe, *et al.*, 2008	20	107.9	10.3
5	Yeh, *et al.*, 1994	104	112	26
		n= 185	Average = 202.764	

			Weighted average = 138.2052973	

Table 8.17 Palmitic acid in cholesterol esters (mmol/L) data

	Study	n	Palmitic acid in cholesterol esters (mmol/L)	SD
1	Sansone*, et al.*, 2016	50	0.0018	0.0003111269837
2	Sansone*, et al.*, 2016	50	0.001453	0.000261629509
3	Lopes*, et al.*, 1991	12	0.114	0.04156921938
4	Tholstrop*, et al.*, 2004	17	10.53	0.2
5	Tholstrop*, et al.*, 2004	17	11.46	0.21
		n= 146	Average = 4.4214506	
			Weighted average = 2.570963356	

Table 8.18 Oleic acid in cholesterol esters (mol%) data

	Study	n	Oleic acid in cholesterol esters (mol%)	SD
1	Bradbury*, et al.*, 2011	2393	19.36	4.54
2	Raatz*, et al.*, 2001	9	17.3	0.58
3	Raatz*, et al.*, 2001	9	14.54	0.65
4	Skeaff*, et al.*, 2006	19	19.24	2.32
5	Yeh *et al*., 1994	104	18	2.6
6	Melchert*, et al.*, 1987	5	17.98	4.3
7	Melchert*, et al.*, 1987	5	20.6	1.62
8	Melchert*, et al.*, 1987	5	17.66	3.49
9	Melchert*, et al.*, 1987	5	20.53	3.86
10	Simon*, et al.*, 1996	156	20.15	2.98
11	Vicario*, et al.*, 1998	10	18.97	0.8
12	Agren*, et al.*, 1995	8	15.9	2.2
13	Agren*, et al.*, 1995	11	18.3	2
14	Lopes*, et al.*, 1991	12	20.19	0.58
15	Wang*, et al.*, 2003	3309	16	2.1
16	Wang*, et al.*, 2003	282	16.2	2
17	Yamagishi*, et al.*, 2008	3575	15.95	2.07
18	Nikkari*, et al.*, 1995	41	22	2.19

19	Nikkari*, et al.*, 1995	41	21.09	1.9
20	Abel, 2005	27	17.66	2.62
21	Abel, 2005	43	18.82	2.06
22	Wang*, et al.*, 2003	2657	16	2.1
23	Vessby*, et al.*, 1994	1753	20.04	3.13
24	Ma*, et al.*, 1995	1712	16.3	2.1
25	Ma*, et al.*, 1995	1858	15.7	2
26	Caren and Corbo, 1966	20	20.3	2.236067977
27	Salomaa*, et al.*, 1990	325	21.98	2.57
28	Miettinen*, et al.*, 1982	64	25.31	3.92
29	Öhrvall*, et al.*, 1996	1836	19.38	2.77
		n= 20294	Average = 18.67068966	
			Weighted average = 17.25613925	

Table 8.19 Oleic acid in cholesterol esters (mg/L) data

	Study	n	Oleic acid in cholesterol esters (mg/L)	SD
1	Durieu*, et al.*, 2007	29	147.52	30.43
2	Schwertner and mosser, 1993	12	961	209
3	Forsythe*, et al.*, 2008	20	177.7	28.9
4	Forsythe*, et al.*, 2008	20	175.2	17.7
5	Yeh*, et al.*, 1994	104	148	39
		n= 185	Average = 321.884	
			Weighted average = 206.8112432	

Table 8.20 Oleic acid in cholesterol esters (mmol/L) data

	Study	n	Oleic acid in cholesterol esters (mmol/L)	SD
1	Sansone*, et al.*, 2016	50	0.003207	0.000763675323 7
2	Sansone*, et al.*, 2016	50	0.002111	0.000502045814 6
3	Lopes*, et al.*, 1991	12	0.236	0.05888

				972746
4	Tholstrop*, et al.*, 2004	17	28.15	0.62
5	Tholstrop*, et al.*, 2004	17	21.07	0.48
		n= 146	Average = 9.8922636	
			Weighted average = 5.752314384	

Table 8.21 Palmitic acid in triglycerides (mol%) data

	Study	n	Palmitic acid in triglycerides (mol%)	SD
1	Bradbury*, et al.*, 2011	2402	29.22	5.77
2	Raatz*, et al.*, 2001	8	25.08	1.43
3	Raatz*, et al.*, 2001	8	24.25	1.19
4	Skeaff*, et al.*, 2006	19	30.59	3
5	Yeh *et al*., 1994	104	32.9	3.6
6	Melchert*, et al.*, 1987	5	22.95	3.34
7	Melchert*, et al.*, 1987	5	27.55	2.91
8	Melchert*, et al.*, 1987	5	26.38	3.7
9	Melchert*, et al.*, 1987	5	29.46	2.17
10	Vicario*, et al.*, 1998	10	26.43	0.54
11	Agren*, et al.*, 1995	8	19.4	2.4
12	Agren*, et al.*, 1995	11	25	4.5
13	Lopes*, et al.*, 1991	9	14.88	0.82
14	Nikkari*, et al.*, 1995	41	28.87	3.39
15	Nikkari*, et al.*, 1995	41	27.02	2.14
16	Caren and Corbo, 1966	20	26.6	4.024922359
17	Miettinen*, et al.*, 1982	64	29.49	3.44
18	Simpson*, et al.*, 1982	32	24	5.8
		n= 2797	Average = 26.115	
			Weighted average = 29.11078298	

Table 8.22 Palmitic acid in triglycerides (mg/L) data

	Study	n	Palmitic acid in triglycerides (mg/L)	SD
1	Forsythe, *et al.*, 2008	20	270.7	41.4
2	Forsythe, *et al.*, 2008	20	248.6	23.1
3	Yeh, *et al.*, 1994	104	179	135
		n= 144	Average = 232.7666667	
			Weighted average = 201.4027778	

Table 8.23 Palmitic acid in triglycerides (mmol/L) data

	Study	n	Palmitic acid in triglycerides (mmol/L)	SD
1	Lopes, *et al.*, 1991	9	0.08	0.042
2	Tholstrop, *et al.*, 2004	17	22.2	0.74
3	Tholstrop, *et al.*, 2004	17	25.61	0.61
		n= 43	Average = 15.96333333	
			Weighted average = 18.91837209	

Table 8.24 Oleic acid in triglycerides (mol%) data

	Study	n	Oleic acid in triglycerides (mol%)	SD
1	Bradbury, *et al.*, 2011	2402	34.36	5.9
2	Raatz, *et al.*, 2001	8	35.73	0.82
3	Raatz, *et al.*, 2001	8	35.21	0.81
4	Skeaff, *et al.*, 2006	19	34.71	3.92
5	Yeh *et al*., 1994	104	33.8	3.7
6	Melchert, *et al.*, 1987	5	35.74	7.92
7	Melchert, *et al.*, 1987	5	40.77	2.99
8	Melchert, *et al.*, 1987	5	36.47	6.23
9	Melchert, *et al.*, 1987	5	41.76	4.18
10	Vicario, *et al.*, 1998	10	39.68	0.69
11	Agren, *et al.*, 1995	8	36.4	6
12	Agren, *et al.*, 1995	11	37.2	2.7

13	Lopes*, et al.*, 1991	10	36.64	2.27
14	Nikkari*, et al.*, 1995	41	40.03	3.3
15	Nikkari*, et al.*, 1995	41	40.92	2.6
16	Caren and Corbo, 1966	20	42.8	3.130495168
17	Miettinen*, et al.*, 1982	64	41.96	3.28
18	Simpson*, et al.*, 1982	32	40.2	9
		n= 2798	Average = 38.02111111	
			Weighted average = 34.90315225	

Table 8.25 Oleic acid in triglycerides (mg/L) data

	Study	n	Oleic acid in triglycerides (mg/L)	SD
1	Forsythe*, et al.*, 2008	20	363.1	31.8
2	Forsythe*, et al.*, 2008	20	389.1	27.5
3	Yeh*, et al.*, 1994	104	200	135
		n = 144	Average = 317.4	
			Weighted average = 248.9166667	

Table 8.26 Oleic acid in triglycerides (mmol/L) data

	Study	n	Oleic acid in triglycerides (mmol/L)	SD
1	Lopes*, et al.*, 1991	10	0.191	0.07589466384
2	Tholstrop*, et al.*, 2004	17	47.75	1.23
3	Tholstrop*, et al.*, 2004	17	37.88	0.82
		n= 44	Average = 28.607	
			Weighted average = 33.12772727	

Table 8.27 Free palmitic acid (mol%) data

	Study	n	Free palmitic acid (mol%)	SD
1	Raatz*, et al.*, 2001	8	25.67	1.11
2	Raatz*, et al.*, 2001	8	24.61	0.53
3	Melchert*, et al.*, 1987	5	18.75	4.37
4	Melchert*, et al.*, 1987	5	27.25	4.16
5	Melchert*, et al.*, 1987	5	24.46	5.46
6	Melchert*, et al.*, 1987	5	26.41	2
7	Yli-Jama*, et al.*, 2002	104	26.98	2.28
8	Agren*, et al.*, 1995	8	22.9	2.3
9	Agren*, et al.*, 1995	11	29	1.9
10	Lopes*, et al.*, 1991	12	16.93	0.29
11	Laaksonen*, et al.*, 2002	806	26.7	2.4
12	Caren and Corbo, 1966	20	25.6	4.024922359
13	Jacobsen*, et al.*, 1983	22	22.7	2.77
14	Jacobsen*, et al.*, 1983	20	25.4	3.01
		n= 1039	Average = 24.52571429	
			Weighted average = 26.40757459	

Table 8.28 Free palmitic acid (mg/L) data

	Study	n	Free palmitic acid (mg/L)	SD
1	Yerlikaya*, et al.*, 2011	45	10.9	5.7
2	Itakura*, et al.*, 2010	8076	748	313
3	Schwertner and mosser, 1993	5	200.2	36.6
4	Cunnane*, et al.*, 2012	10	470	250
5	Teubert*, et al.*, 2013	45	264.29	183.52
6	Teubert*, et al.*, 2013	45	398.62	241.02
7	Han*, et al.*, 2011	30	256	46
		n= 8256	Average = 335.43	
			Weighted average = 736.9852774	

Table 8.29 Free palmitic acid (mmol/L) data

	Study	n	Free palmitic acid (mmol/L)	SD
1	Hadj Ahmed*, et al.*, 2017	13	0.107	0.017
2	Yli-Jama*, et al.*, 2002	104	0.102524	0.064752
3	Lopes*, et al.*, 1991	12	0.074	0.01039230485
4	Lankinen*, et al.*, 2015	25	0.198	0.087
5	De Almeida*, et al.*, 2002	6	0.0972	0.0418862746
6	Carlsten*, et al.*, 2009	9	0.1657	0.0795
		n= 169	Average = 0.1240706667	
			Weighted average = 0.1181419882	

Table 8.30 Free oleic acid (mol%) data

	Study	n	Free oleic acid (mol%)	SD
1	Raatz*, et al.*, 2001	8	31.33	1.68
2	Raatz*, et al.*, 2001	8	31.79	0.88
3	Melchert*, et al.*, 1987	5	33.62	9.35
4	Melchert*, et al.*, 1987	5	27.04	4.65
5	Melchert*, et al.*, 1987	5	32.56	9.24
6	Melchert*, et al.*, 1987	5	35.35	4.35
7	Yli-Jama*, et al.*, 2002	104	32.2	4.16
8	Agren*, et al.*, 1995	8	30	3.7
9	Agren*, et al.*, 1995	11	27.1	4
10	Lopes*, et al.*, 1991	12	36.27	0.34
11	Caren and Corbo, 1966	20	37.5	3.577708764
12	Jacobsen*, et al.*, 1983	22	38.9	3.97
13	Jacobsen*, et al.*, 1983	20	41.1	3.45
		n= 233	Average = 33.44307692	
			Weighted average = 33.89592275	

Table 8.31 Free oleic acid (mg/L) data

	Study	n	Free oleic acid (mg/L)	SD
1	Yerlikaya, *et al.*, 2011	45	4.69	2.6
2	Itakura, *et al.*, 2010	8076	691	370
3	Schwertner and Mosser, 1993	5	267.7	92.5
4	Cunnane, *et al.*, 2012	10	450	350
5	Teubert, *et al.*, 2013	45	144.81	93.97
6	Teubert, *et al.*, 2013	45	262.76	153.53
7	Han, *et al.*, 2011	30	479	90
		n= 8256	Average = 328.5657143	
			Weighted average = 680.6293847	

Table 8.32 Free oleic acid (mmol/L) data

	Study	n	Free oleic acid (mmol/L)	SD
1	Hadj Ahmed, *et al.*, 2017	13	0.09	0.019
2	Yli-Jama, *et al.*, 2002	104	0.12236	0.09016
3	Lopes, *et al.*, 1991	12	0.153	0.01732050808
4	Lankinen, *et al.*, 2015	25	0.222	0.097
5	Carlsten, *et al.*, 2009	9	0.372	0.1179
		n= 163	Average = 0.191872	
			Weighted average = 0.1511008589	

Table 8.33 Palmitic acid in total plasma lipids (mol%) data

	Study	n	Palmitic acid in total plasma lipids (mol%)	SD
1	Mayneris-Perxachs, *et al.*, 2014	62	21.4	2
2	Mayneris-Perxachs, *et al.*, 2014	49	20.4	1.5
3	Sun, *et al.*, 2007	306	19.31	2.41
4	Marangoni, *et al.*, 2004	6	21.03	0.96
5	Fernandez-Real, *et al.*, 2005	76	20.1	2.2
6	Fernandez-Real, *et al.*, 2005	40	19.4	4.1

7	Yeh *et al*., 1994	104	24.8	2.6
8	Baylin*, et al.*, 2005	63	22.78	2.24
9	Melchert*, et al.*, 1987	38	20.02	1.9
10	Melchert*, et al.*, 1987	37	21.48	2.26
11	Melchert*, et al.*, 1987	62	20.08	2.07
12	Melchert*, et al.*, 1987	70	20.96	2.91
13	Kuksis, 1978	64	20.13	2.2
14	Kuksis, 1978	70	19.63	1.88
15	Kuksis, 1978	45	18.94	3.27
16	Kuksis, 1978	57	18.9	2.95
17	Solakivi*, et al.*, 2010	32	23.6	2.4
18	Lee*, et al.*, 2000	60	19	2.4
19	Lee*, et al.*, 2000	133	17.8	3.9
20	Wheeler*, et al.*, 2011	142	25.9	2.025783799
21	Wheeler*, et al.*, 2011	91	24.8	1.717090563
22	Wheeler*, et al.*, 2011	32	25.4	2.036467 53
23	Wheeler*, et al.*, 2011	18	26.7	1.739482682
24	Faas*, et al.*, 1988	5	21.3	0.6
25	Faas*, et al.*, 1988	5	24.6	1.3
26	Caren and Corbo, 1966	20	23.8	3.577708764
		n= 1687	Average = 21.62538462	
			Weighted average = 21.18267931	

Table 8.34 Palmitic acid in total plasma lipids (mass%) data

	Study	n	Palmitic acid in total plasma lipids (mass%)	SD
1	Rise*, et al.*, 2007	10	22.66	1.72
2	Vognild*, et al.*, 1998	35	23.8	1.774823935
3	Vognild*, et al.*, 1998	25	23.4	1.5
4	Vognild*, et al.*, 1998	29	24.3	2.154065923
5	Vognild*, et al.*, 1998	38	23.7	2.465765601
6	Vognild*, et al.*, 1998	31	24	1.113552873
7	Vognild*, et al.*, 1998	36	23.4	1.2
8	Vognild*, et al.*, 1998	33	23.7	1.72336

				8794
9	Parkinson*, et al.*, 1994	20	18.45	2.3
10	Parkinson*, et al.*, 1994	20	16.61	2.34
11	Parkinson*, et al.*, 1994	20	18.46	3.06
12	Parkinson*, et al.*, 1994	20	16.38	3.12
13	Parkinson*, et al.*, 1994	13	21.34	2.01
14	Parkinson*, et al.*, 1994	13	20.8	1.91
15	Begin*, et al.*, 1989	49	23.98	2.25
		n= 392	Average = 21.66533333	
			Weighted average = 22.29397959	

Table 8.35 Palmitic acid in total plasma lipids (mmol/L) data

	Study	n	Palmitic acid in total plasma lipids (mmol/L)	SD
1	Abdelmagid*, et al.*, 2015	327	1.6486	0.4879
2	Abdelmagid*, et al.*, 2015	499	1.62	0.4405
3	Kuksis, 1978	64	2.837	0.954
4	Kuksis, 1978	70	2.853	0.825
5	Kuksis, 1978	45	2.875	0.996
6	Kuksis, 1978	57	2.064	1.171
7	Kuriki*, et al.*, 2003	15	2.36	0.43
8	Kuriki*, et al.*, 2003	79	2.5	0.63
9	De Almeida*, et al.*, 2002	6	2.8907	0.71133 18213
		n= 1162	Average = 2.405366667	
			Weighted average = 1.915677625	

Table 8.36 Oleic acid in total plasma lipids (mol%) data

	Study	n	Oleic acid in total plasma lipids (mol%)	SD
1	Mayneris-Perxachs*, et al.*, 2014	62	26.7	4.1
2	Mayneris-Perxachs*, et al.*, 2014	49	23.8	3.6
3	Sun*, et al.*, 2007	306	18.6	2.43
4	Marangoni*, et al.*, 2004	6	29.24	3.31

5	Fernandez-Real	76	20.8	3.7
6	Fernandez-Real	40	22.9	9
7	Yeh *et al*., 1994	104	19.2	2.6
8	Baylin*, et al.*, 2005	63	20.74	2.72
9	Melchert*, et al.*, 1987	38	19.73	3.89
10	Melchert*, et al.*, 1987	37	22.86	3.25
11	Melchert*, et al.*, 1987	62	18.87	2.73
12	Melchert*, et al.*, 1987	70	22.08	3.03
13	Kuksis, 1978	64	20.69	3.37
14	Kuksis, 1978	70	20.98	3.58
15	Kuksis, 1978	45	20.95	3.53
16	Kuksis, 1978	57	22.36	8.72
17	Solakivi*, et al.*, 2010	32	26.6	2.5
18	Lee*, et al.*, 2000	60	22.3	3.7
19	Lee*, et al.*, 2000	133	18.8	4.5
20	Wheeler*, et al.*, 2011	142	22.2	4.766550115
21	Wheeler*, et al.*, 2011	91	22.1	2.575635844
22	Wheeler*, et al.*, 2011	32	23.8	2.093036072
23	Wheeler*, et al.*, 2011	18	22.2	1.697056275
24	Faas*, et al.*, 1988	5	25.4	4.2
25	Faas*, et al.*, 1988	5	27.6	3.2
26	Caren and Corbo, 1966	20	23.7	2.236067977
		n= 1687	Average = 22.50769231	
			Weighted average = 21.08078838	

Table 8.37 Oleic acid in total plasma lipids (mass%) data

	Study	n	Oleic acid in total plasma lipids (mass%)	SD
1	Rise*, et al.*, 2007	10	19.38	2.61
2	Vognild*, et al.*, 1998	35	17.9	2.958039892
3	Vognild*, et al.*, 1998	25	18.2	1.5
4	Vognild*, et al.*, 1998	29	18.8	4.308131846
5	Vognild*, et al.*, 1998	38	17.8	3.698648402
6	Vognild*, et al.*, 1998	31	19.6	2.227105745
7	Vognild*, et al.*, 1998	36	18.4	2.4
8	Vognild*, et al.*, 1998	33	17.3	3.446737588

9	Parkinson*, et al.*, 1994	20	19.8	3.26
10	Parkinson*, et al.*, 1994	20	18.22	1.57
11	Parkinson*, et al.*, 1994	20	20.08	3.03
12	Parkinson*, et al.*, 1994	20	17.88	2.66
13	Parkinson*, et al.*, 1994	13	21.98	3.44
14	Parkinson*, et al.*, 1994	13	21.25	2.33
15	Begin*, et al.*, 1989	49	22.26	3.01
		n= 392	Average = 19.25666667	
			Weighted average = 19.1584949	

Table 8.38 Oleic acid in total plasma lipids (mmol/L) data

	Study	n	Oleic acid in total plasma lipids (mmol/L)	SD
1	Abdelmagid*, et al.*, 2015	327	1.3327	0.4545
2	Abdelmagid*, et al.*, 2015	499	1.275	0.3908
3	Kuksis, 1978	64	2.791	1.165
4	Kuksis, 1978	70	3.424	5.047
5	Kuksis, 1978	45	2.825	1.29
6	Kuksis, 1978	57	2.853	1.209
7	Kuriki*, et al.*, 2003	15	2.17	0.6
8	Kuriki*, et al.*, 2003	79	2.1	0.53
		n= 1156	Average = 2.3463375	
			Weighted average = 1.711520675	