Anti-atherogenic actions of dihomo-gamma-linolenic acid and its key metabolites

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Table of Contents

Table of Figure

Table of tables

Abstract

Atherosclerosis, an inflammatory disease of medium and large arteries, is the underlying cause of cardiovascular diseases. Current pharmaceutical therapies against atherosclerosis are not fully effective. Nutraceuticals, such as polyunsaturated fatty acids (PUFA), represent promising alternatives but require detailed understanding of their mechanisms of action.

Dihomo-gamma-linolenic acid (DGLA), an omega-6 PUFA, attenuates atherosclerosis in the apolipoprotein-E-deficient mouse model of the disease. Previous research in the laboratory showed that DGLA inhibited several key macrophage processes associated with atherosclerosis *in vitro* and increased the levels of prostaglandin E_1 (PGE_1) and 15-S-hydroxyeicosatriaenoic acid (15-S-HETrE) in human macrophages, thereby suggesting that these metabolites are potentially responsible for the anti-atherogenic actions of DGLA. Unfortunately, the effects of PGE_1 and 15-S-HETrE on key atherosclerosis-associated processes are poorly understood and formed the focus of studies in this project.

Similar to DGLA, PGE_1 and 15-S-HETrE attenuated chemokine-driven monocytic migration together with macropinocytosis, oxidised low-density lipoprotein (oxLDL) uptake and the expression of key scavenger receptors in human macrophages *in vitro*. The use of Atherosclerosis RT^2 Profiler PCR Arrays showed that DGLA, PGE_1 and 15-S-HETrE inhibited the expression of 16, 12 and 21 genes respectively in human macrophages *in vitro* with 3 common regulated genes. These genes included those that play key roles in stress response and apoptosis, lipid transport and metabolism, and regulation of inflammation. The use of pharmacological inhibitors against cyclooxygenases and lipoxygenases together with bone marrow-derived macrophages from wildtype and 12/15-lipooxygenase deficient mice showed the key roles of these enzymes in the actions of DGLA *in vitro*. In addition, DGLA supplementation produced changes in signalling lymphocyte activation molecule and cell lineage populations within the bone marrow of LDL receptor deficient mice fed a high fat diet. Overall, these studies provide novel insights into the anti-atherogenic actions of DGLA and its metabolites and inform on its therapeutic potential.

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Abbreviations

Abbreviations	Full name
AA	Arachidonic acid
ABC	ATP binding cassette
ABCA1	ABC transporter A1
ABCG1	ABC transporter G1
ACAT	Acetyl-CoA cholesterol acetyltransferase
acLDL	Acetylated LDL
ALA	α-linoleic acid
ANOVA	One-way analysis of variance
AP	Alkaline phosphatase
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
BMDMs	Bone marrow derived macrophages
BSA	Bovine serum albumin
BSH	Bile salt hydrolase
CANTOS	Canakinumab Anti-inflammatory Thrombosis Outcomes Study
CCL2	Chemokine (C-C motif) ligand 2
CCR2	C-C chemokine receptor 2
CD36	Cluster differentiation 36
ChIP	Chromatin immunoprecipitation
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
COX	Cyclo-oxygenase
CPT	Carnitine palmitoyl transferase
CRP	C-reactive protein
CV	Crystal violet
CVD	Cardiovascular disease
CXCL1	Chemokine (C-X-C motif) ligand 1
DAMPS	Danger associated molecular patterns
DC	Dendritic cells
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid

DPA	Docosapentaenoic acid
ECM	Extracellular matrix
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid
ERK	Extracellular signal-regulated kinase
FFA	Free fatty acids
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
GDC	Genomic DNA control
GLA	γ-linolenic acid
GMP	Granulocyte-macrophage progenitor
GPCR	G protein-coupled receptor
GSB	Gel sample buffer
HASMCs	Human aortic smooth muscle cells
HBEGF	Heparin-binding EGF-like growth factor
HDL	High density lipoprotein
HETE	Hydroxy-eicosatetraenoic acids
HETrE	Hydroxy-eicosatriaenoic acids
HFD	High fat diet
HI-FCS	Heat-inactivated foetal calf serum
HL	Hepatic lipase
HMDM	Human monocyte derived macrophages
HMG-CoAR	3-hydroxy-3-methyl-glutaryl-CoA reductase
HPC II	Haematopoietic progenitor cells II
HPF	High power fields
HSC	Haematopoietic stem cell
HSPCs	Haematopoietic stem/progenitor cells
HTA	Heptanoic acid
ICAM1	Intercellular adhesion molecule 1
IDL	Intermediate density lipoprotein
IFN	Interferon
IFN-γR	Interferon γ receptor
IL	Interleukin

IL-1ra	IL-1 receptor antagonist
iNOS	Inducible nitric oxide synthase
INT	Tetrazolium salt
JAK	Janus kinase
LA	Linoleic acid
LAL	Lysosomal acid lipase
LCAT	Lecithin cholesterol acyl transferase
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LDLr	Low density lipoprotein receptor
LK	Lin^{-} c-Kit^{+}
LIPE	Hormone sensitive lipase
LOX	Lipo-oxygenase
LPL	Lipoprotein lipase
LPS	Lipopolysaccaride
LT	Leukotriene
LXR	Liver X receptor
LY	Lucifer yellow
MA	Myristic acid
Mac-1	Macrophage-1-antigen
MAPK	Mitogen-activated protein kinases
MCP-1	Monocyte chemotactic protein 1
M-CSF	Macrophage colony stimulating factor
MEP	Megakaryocyte-erythroid progenitor
Mig	Monokine induced by IFN-γ
mmLDL	Minimally modified LDL
MMLV	Molony leukemia virus
MMPs	Matrix metalloproteinase
MPP	Multipotent progenitor cells
MUFA	Mono-unsaturated fatty acids
NF-κB	Nuclear factor κB
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NPC1L1	Niemann-Pick C1-like protein

NLR	NOD-like receptor
NO	Nitric oxide
NOD	Nucleotide oligomerization domain
NPC	Niemann-Pick type C
OA	Oleic acid
oxLDL	Oxidised LDL
PA	Palmitic acid
PAD	Peripheral artery disease
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PFA	Paraformaldehyde
PG	Prostaglandin
PI3K	Phosphoinositide-3-kinase
PLA2	Phospholipase A2
PMA	Phorbol 12-myristate 13-acetate
PPAR	Peroxisome proliferator activated receptors
PPRE	PPAR response elements
PRRs	Pathogen recognition receptors
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PUFA	Poly-unsaturated fatty acids
RCT	Reverse cholesterol transport
ROS	Reactive oxygen species
RT-qPCR	Real time quantitative PCR
RTC	Reverse transcription control
SELE	E-selectin
SLAM	Signalling lymphocytic activation molecule
SDS	Sodium dodecyl sulphate
SFA	Saturated fatty acids
SMC	Smooth muscle cells

SRA	Scavenger receptor A
SRB1	Scavenger receptor B1
SREBP	Sterol regulatory element binding protein
STA	Stearic acid
STAT	Signal transducer and activator of transcription
TAG	Triacylglycerol
TBE	Tris/borate/EDTA
TBHP	Tert-butyl hydroperoxide
TFA	Total fatty acids
TGs	Triglycerides
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TX	Thromboxane
VCAM-1	Vascular cellular adhesion molecule 1
VEGF	Vascular endothelial growth factor
VLDL	Very low-density lipoprotein
WHO	World Health Organisation
WT	Wild type

Chapter 1 Introduction

1.1 Cardiovascular disease

1.1.1 Overview of cardiovascular disease

Cardiovascular Disease (CVD) is the primary cause of death in the West and in developing countries (World Health Organization 2019), being responsible for about one in three reported deaths. CVD includes heart disease, stroke and peripheral artery disease (PAD). Total deaths caused by CVD are estimated to be around 26% in the UK (BHF, 2018) and around 28% in developing countries (Gaziano et al. 2010). According to the World Health Organization (2018), CVD was responsible for about 31% of all global deaths. Although CVD is common in older people, young people have recently experienced an unprecedented rise in the disease (World Health Organization 2019). CVD is a major economic burden and costs the UK economy about £19 billion per year (BHF 2018) . Due to the expected rise in mortality rates from CVD in the future and associated healthcare burden, it is important to investigate the causes of CVD and to identify new targets and treatment avenues.

1.1.2 Atherosclerosis

The most common cause of CVD is atherosclerosis, which is an inflammatory disease characterised by the accumulation of lipids in the walls of medium and large arteries (Michael et al. 2012). Over time, debris from immune cells and other cells accumulates in atherosclerotic plaques, which decreases the elasticity of the arterial wall and reduces the size of the lumen. Consequently, there is reduced blood flow to the affected artery-supported tissues, which eventually causes oxygen deprivation (Hansson and Hermansson 2011).

1.1.3 Risk factors

A knowledge of risk factors for CVD can provide significant insights into prevention and highlight the causes of this substantial public health problem. While not all risks can be changed, many can prevent CVD and reduce its burden. The more risk factors a person has, the higher the chance is of that individual developing CVD, as some risk factors act in a synergistic manner (World Health Organization 2019). The risk of atherosclerosis development is determined largely by common modifiable cardiovascular risk factors, such as dyslipidaemia, tobacco smoking (Lubin et al. 2017), hypertension (Petruski-Ivleva et al. 2016), diets rich in saturated fats, diabetes and obesity (Kim et al. 2014; Groh et al. 2018). As well as the lifestyle factors, which may be modified, an individual's risk for developing atherosclerosis depends on factors that cannot be modified, including age above 50, male sex (Nanayakkara et al. 2018), ethnicity and family predisposition (Gijsberts et al. 2015). Some of the risk factors are described further in Table 1.1. Because of the importance of lipids and inflammation in atherosclerosis, these factors are discussed later in more detail.

Table 1. 1 Modifiable and non-modifiable risk factors for atherosclerosis and CVD.

Category	Factors	Contributions to CVD	References
Modifiable risk factors	Hypertension	The incidence of CVD is reduced by the control of blood pressure, and hypertension also harms the kidneys.	(McLaren *et al.* 2011a; Petruski-Ivleva *et al.* 2016); McLaren et al. 2011a)
	Diabetes	The risk of atherosclerosis and CVD is significantly increased in those with diabetes.	(Lusis 2000; Lee *et al.* 2017; Groh *et al.* 2018)
	Smoking	The possibility of CVD and heart failure rises with smoking. Within one year of stopping smoking, the risk of heart failure and coronary heart disease decreases to around half that of a smoker.	(Lubin et al. 2017)
	Obesity	Reduced plasma levels of high-density lipoprotein (HDL) and increased levels of Triacylglycerol (TAGs), both of which increase the chances of developing CVD, are associated with obesity and weight gain.	(Lusis 2000)
Non-modifiable risk factors	Age	The occurrence of CVD correlates with age, with the incidence in individuals below 50 reported to be less than 1%. However, this figure is 25% between the ages of 70 and 79 and 40% above the age of 80.	(Hinton et al. 2018)
	Hereditary	These include hypercholesterolemia due to mutations in the low-density lipoprotein receptor (LDLr) gene, which increases plasma concentrations of low-density lipoprotein (LDL) and mutations in the ApoA1 gene that lowers HDL levels.	(Lusis et al. 2004; Gijsberts et al. 2015)

	Gender	Men are likely to have more CVD compared with age-matched females before menopause.	(Kouvari et al. 2018)

1.2 Lipids in atherosclerosis

Atherosclerosis is characterised by the accumulation of lipid-rich plaques in the walls of medium and large arteries due to dysfunctional lipid metabolism (Lusis et al. 2004). The important role of lipids in atherosclerosis can be gauged by the decrease in CVD mortality obtained by statins that reduce cholesterol biosynthesis (Kreisberg and Oberman 2002). Statins are probably the most effective drugs used to prevent atherosclerosis and other CVD (Libby and Ridker 2004). Statins act by inhibiting 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoAR), a rate limiting enzyme in *de novo* cholesterol synthesis, thus reducing circulating LDL levels (Michael et al. 2012). Statins have pleiotropic actions related to their anti-inflammatory properties and have also been found to increase circulating levels of HDL, control the expression of macrophage scavenger receptors, and decrease the levels of both oxidative stress and oxidised LDL (oxLDL) (Jain and Ridker 2005).

1.2.1 Fatty acids

Fatty acids play important roles in the body by facilitating proper cell function and metabolism, and are also involved in the processing of dietary lipids or in their *de novo* synthesis (see Figure 1.1 for general presentation of fatty acid metabolism) (Fernandez et al. 2019). They are a key source of energy for metabolic processes, act as gene regulators and are key elements of the cell membrane in the form of phospholipids (Orsavova et al. 2015). In the diet, fatty acids generally exist in the form of Triglycerides (TG) (Orsavova et al. 2015), which consists of glycerol esterified with two or more fatty acids (Hunter et al. 2010). Fatty acids can be categorised as saturated fatty acids (SFA), which have no double bonds between carbon atoms, or unsaturated fatty acids, which contain a minimum of one double bond between carbon atoms (Hunter et al. 2010). For example, stearic acid (SA, 18:0) is a non-essential SFA present mainly in meat, fish, eggs and milk products together with cereals, fruit and vegetables (Hunter et al. 2010). The average SA intake is 5-8 g/day, making it one of the most prevalent SFAs consumed beside palmitic acid (Kris-Etherton et al. 2003; Hunter et al. 2010). Polyunsaturated fatty acids (PUFAs) are defined

as fatty acids with more than one double bond (Rustan and Drevon 2005). Special interest has focused on omega-3 (ω-3) and omega-6 (ω-6) PUFAs, which include the first double bond between the third and fourth or sixth and seventh carbon atoms from the methyl group, respectively (Rustan and Drevon 2005). Figure 1.2 illustrates the classification and primary dietary sources of fatty acids.

PUFAs are generated mainly by plants and phytoplankton, but are important for all mammals (Rustan and Drevon 2005). Linoleic acid (LA, 18:2n-6) and alpha-linolenic acid (ALA, 18:3n-3) are precursors of ω-6 and ω-3 fatty acids respectively that are required by humans (Mangold 2012).

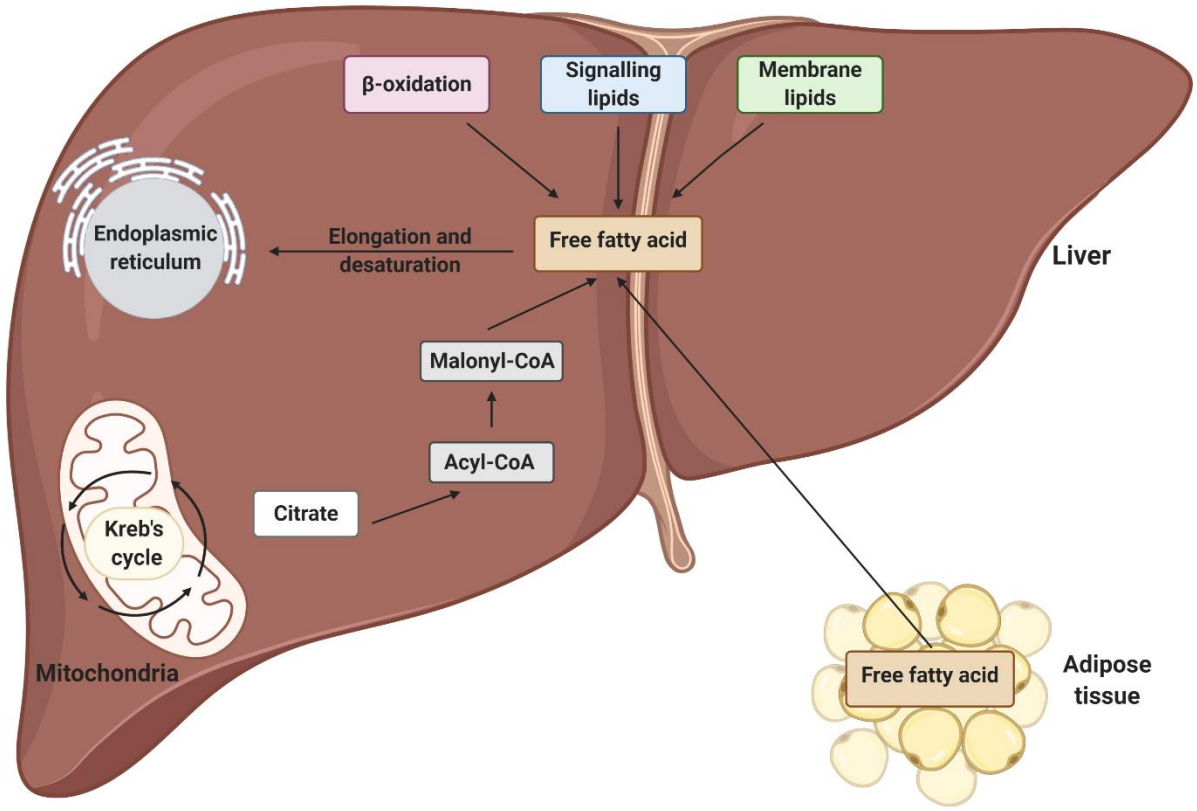


Figure 1. 1 Fatty acid metabolism.

Via sequential addition of 2-carbon groups of malonyl-CoA up to a length of 22-24 carbons, the microsomal system extends saturated or unsaturated fatty acyl-CoA. The malonyl-CoA donor and NADPH are necessary for elongation and are affected by the intake of dietary fatty acids. *De novo* synthesis products consist primarily of 16 carbon fatty acids such as palmitic acid and, in addition to shorter chain fatty acids, small amounts of the 18-carbon stearic acid are also produced. Adapted from (Mato *et al.* 2019).

Enzymes in animals and humans metabolise these fatty acids by addition of new double bonds and elongation of the carbon chain (Mangold 2012). Interconversion of ω-6 PUFAs and ω-3 PUFAs cannot be achieved, which underlines the relevance of a healthy diet that contains the essential fatty acids (EFA) (Tvrzicka et al. 2011). LA and ALA are commonly found in plant oils, including sunflower, canola and corn together with fish and red meat (Mourente et al. 2005). DGLA, an essential component of the phospholipid membrane and for eicosanoid synthesis, additionally may be metabolised into arachidonic acid (AA) (Sergeant et al. 2016). Fatty acids can be stored in the adipose tissue as TAG, used in muscle cells to produce energy, or metabolised into bioactive signalling molecules by multiple enzymes (German and Dillard 2006). Table 1.2 provides a list of the most common fatty acids.

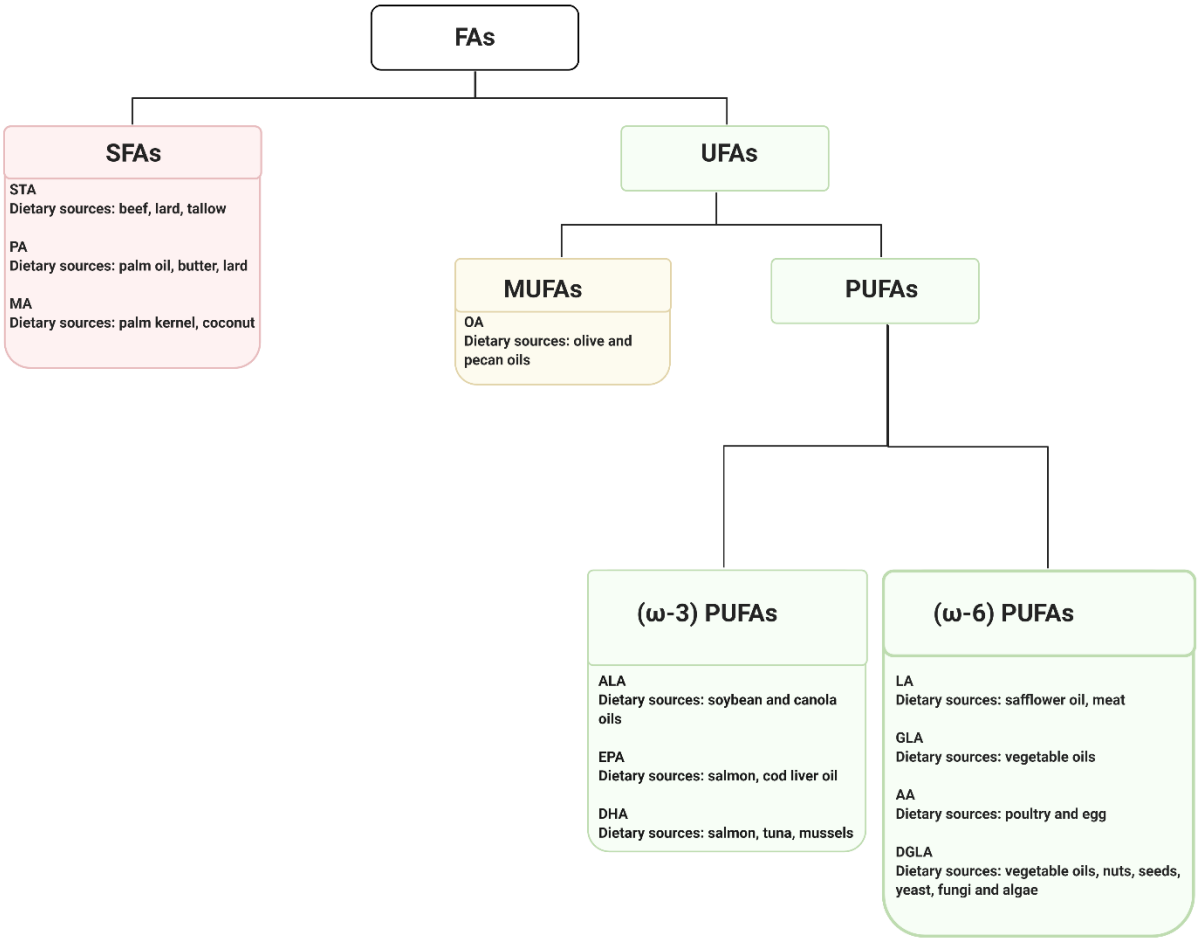


Figure 1. 2 Classification and primary dietary sources of fatty acids (FAs).
Adapted from (Radzikowska et al. 2019). Abbreviations: ALA, α-Linolenic acid; AA, arachidonic acid; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAs, fatty acids; GLA, γ-linolenic acid; LA, linoleic acid; MA, myristic acid; MUFAs, mono-unsaturated fatty acids; PA, palmitic acid; PUFAs, poly-unsaturated fatty acids; OA, oleic acid; SFAs, saturated fatty acids; STA, stearic acid; UFAs, unsaturated fatty acids. Created with BioRender.com.

Table 1. 2 Common natural fatty acids.

Chemical name	Shorthand	Common name	Abbreviation
Tetradecanoic	14:0	Myristic acid	MA
Hexadecanoic	16:0	Palmitic acid	PA
Cis-9-hexadecenoic	16:1n7	Palmitoleic acid	-
Cis-7-hexadecenoic	16:1n9	-	-
Octadecanoic	18:0	Stearic acid	STA
Cis-11-octadecenoic	18:1n7	Cis-Vaccenic acid	-
Cis-9-octadecenoic	18:1n9	Oleic acid	OA
Cis-9,12-octadecadienoic	18:2n6	Linoleic acid	LA
Cis-9,12,15-octadecatrienoic	18:3n3	α-Linolenic acid	ALA
Cis-6,9,12-octadecatrienoic	18:3n6	γ-Linolenic acid	GLA
Cis-8,11,14-eicosatrienoic	20:3n6	Dihomo-γ-linolenic acid	DGLA
Cis-5,8,11,14-eicosatetraenoic	20:4n6	Arachidonic acid	AA/ARA
Cis-5,8,11,14,17eicosapentaenoic	20:5n3	Eicosapentaenoic acid	EPA
Cis-7,10,13,16,19-docosapentaenoic	22:5n3	Docosapentaenoic acid	DPA
Cis-4,7,10,13,16,19-docosahexaenoic	22:6n3	Docosahexaenoic acid	DHA

1.2.2 Membrane lipids; Phospholipids

Phospholipids consist of fatty acids esterified to the glycerol base at position sn-1 and sn-2 and an additional phosphate polar head group at the sn3-position (Brown and Marnett 2011) (Figure 1.3). The key components of eukaryotic membranes are phospholipids, together with proteins, other lipids such as cholesterol, and carbohydrate components on the outside (van Meer et al. 2008). Various head groups are present in different classes of phospholipids; the most common in eukaryotic membranes are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) (Van Meer et al. 2008). Phospholipid

cleavage enables production of fatty acids and their metabolites that are involved in signal transduction networks as lipid messengers (Van Meer et al. 2008).

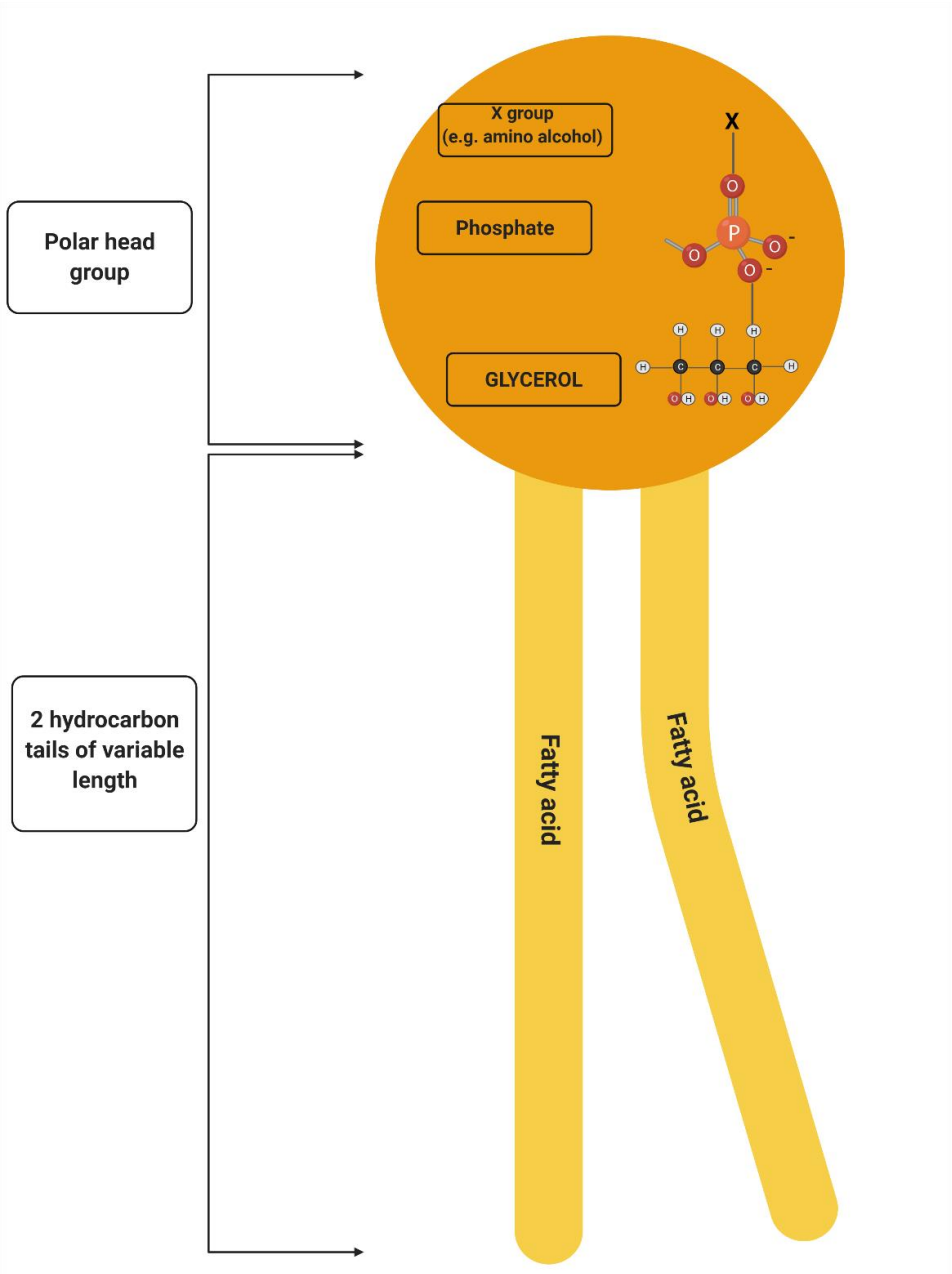


Figure 1. 3 General structure of phospholipids.
A wide variety of phospholipids can be produced by various combinations of fatty acid groups linked to a glycerol backbone. The amino alcohol group can include, for example, choline, ethanolamine, serine, or inositol, which form PC, PE, PS and PI, respectively. Created with BioRender.com.

1.2.3 Lipid storage; triacylglycerol

Fatty acids can be used for energy storage because of their condensed state. TAG as lipid droplets is used for energy storage (Van Meer et al. 2008).TAG consists of three fatty acids bonded to the backbone of glycerol. Due to the supply of carbon in a reduced form, these structures are useful energy stores that will generate optimum energy when they undergo oxidation.

1.2.4 Cholesterol

Cholesterol is a sterol with major functions in the formation of membranes and regulation of membrane fluidity and lipid raft formation (Figure 1.4) (McLaren *et al.* 2011a). High cholesterol levels in the body are a significant risk factor for atherosclerosis (McLaren *et al.* 2011a). Cholesterol can be synthesised in the liver from dietary lipids (McLaren *et al.* 2011a) via several enzymatic reactions from acetate (Ikonen 2006). The HMG-CoAR enzyme that produces mevalonate from acetate is an essential regulatory step in the synthesis of cholesterol (Ikonen 2006).

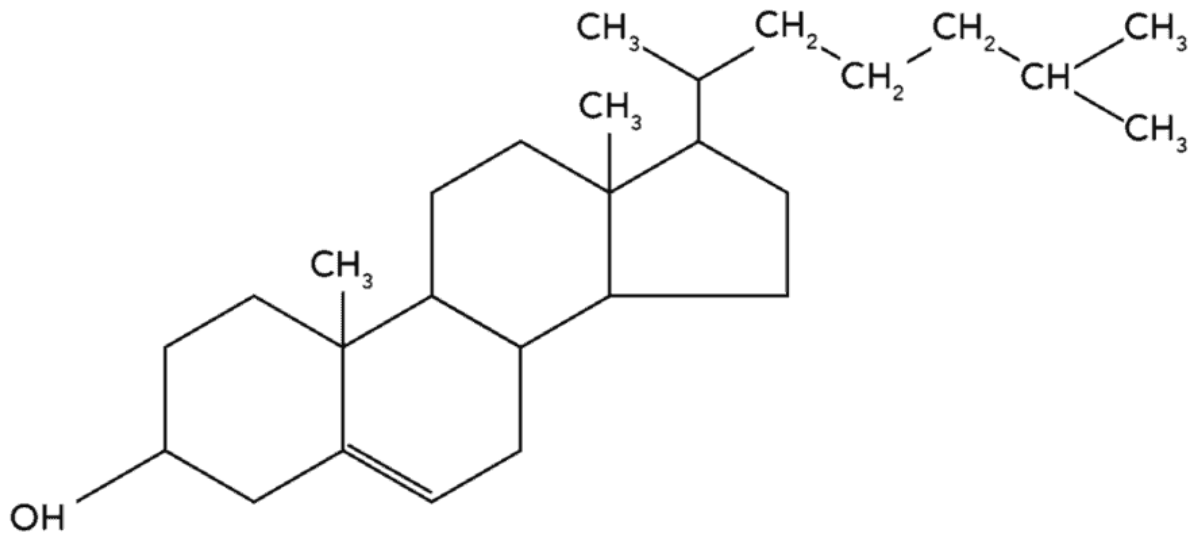


Figure 1. 4 Structure of cholesterol.
Cholesterol consists of a bonded tetracyclic ring. It is an essential element of lipid rafts and cell membranes (particularly plasma membranes). Created with BioRender.com.

1.2.5 Metabolism of lipids and transport

Lipid transport in the body is a dynamic process involving multiple enzymes and lipoproteins (Shah et al. 2013). It is important to understand these processes, as they may reveal possible imbalances which can lead to atherosclerosis. In the pathology of atherosclerosis, the synthesis and transport across the body of cholesterol and fatty acids play an important role (Shah et al. 2013).

1.2.6 Roles of lipoproteins

Lipids are transported as lipoprotein particles in the circulation (Lusis et al. 2004). Lipoproteins consist of a monolayer of hydrophilic lipids (i.e., phospholipids and free cholesterol) surrounding a core of hydrophobic lipids (TAG and cholesterol esters) (Figure 1.5) (Lusis et al. 2004). Moreover, they contain apolipoproteins that act in the processing and release of lipids, and also as ligands for cell surface receptors (Buckley and Ramji 2015). Chylomicrons, VLDL, intermediate density lipoprotein (IDL), LDL and HDL are forms of lipoproteins (Buckley and Ramji 2015). A list of the most common type of lipoproteins and their functions is presented in Table 1.3. The form of lipoprotein in which a lipid is packaged is based on the source (Buckley and Ramji 2015). Chylomicrons carry dietary TAG, whereas those synthesised in the liver are packaged into VLDL (McLaren *et al.* 2011a). IDL and LDL are formed via the metabolism and processing of VLDL by the action of lipoprotein lipase (LPL) and hepatic lipase (HL) catalysed reactions that hydrolyse the TAG element (McLaren *et al.* 2011a). LPL-mediated hydrolysis of chylomicrons can also form IDL and LDL. LDL is used to transport cholesterol to peripheral tissues, where it can be utilised in membranes or participate in the production of steroid hormones (Van der Velde 2010). HDL plays a role in the transfer of excess cholesterol from peripheral tissues to the liver for biliary secretion (Van der Velde 2010). Atherosclerosis is associated with changes in the levels of circulating lipoproteins (McLaren *et al.* 2011a). Both elevated blood LDL levels and lower circulating HDL have been associated with an increased risk of atherosclerosis (McLaren *et al.* 2011a). Figure 1.6 illustrates the cholesterol transport process.

Cholesterol, TAG and dietary fatty acids are processed by the intestine (Lusis et al. 2004). Dietary TAG is hydrolysed to fatty acids and monoglycerides by the digestive process and can also form

micelles in the intestinal lumen with bile acids (Georgiadi and Kersten 2012). The fatty acids are re-esterified to TAG together with cholesterol by the intestinal epithelial cells and packaged into chylomicrons containing Apo-B, -A, -C and -E (McLaren *et al.* 2011a). Then, TG in circulating chylomicrons are hydrolysed by LPL, which is found on the capillary endothelium surface, to generate non-esterified fatty acids and 2-monoacyl glycerol for uptake by different tissues (Georgiadi and Kersten 2012). TAG-derived fatty acids or free fatty acids are the majority of fatty acids taken up by the cells (Georgiadi and Kersten 2012).

Cholesterol esters in LDL are hydrolysed following uptake into cells to free cholesterol via lysosomal acid lipase (Buckley and Ramji 2015). Free cholesterol that is in excess can be re-esterified through the enzyme acyl-coenzyme A acyltransferase (ACAT) (Buckley and Ramji 2015). Cholesterol esters decrease solubility and facilitate storage in lipid droplets located in the cytoplasm (Buckley and Ramji 2015). Excess accumulation of cholesterol ester and TAG plays a major role in atherosclerosis development (McLaren *et al.* 2011a). However, under normal conditions, cellular homeostatic mechanisms effectively respond to excess cholesterol (McLaren *et al.* 2011a).

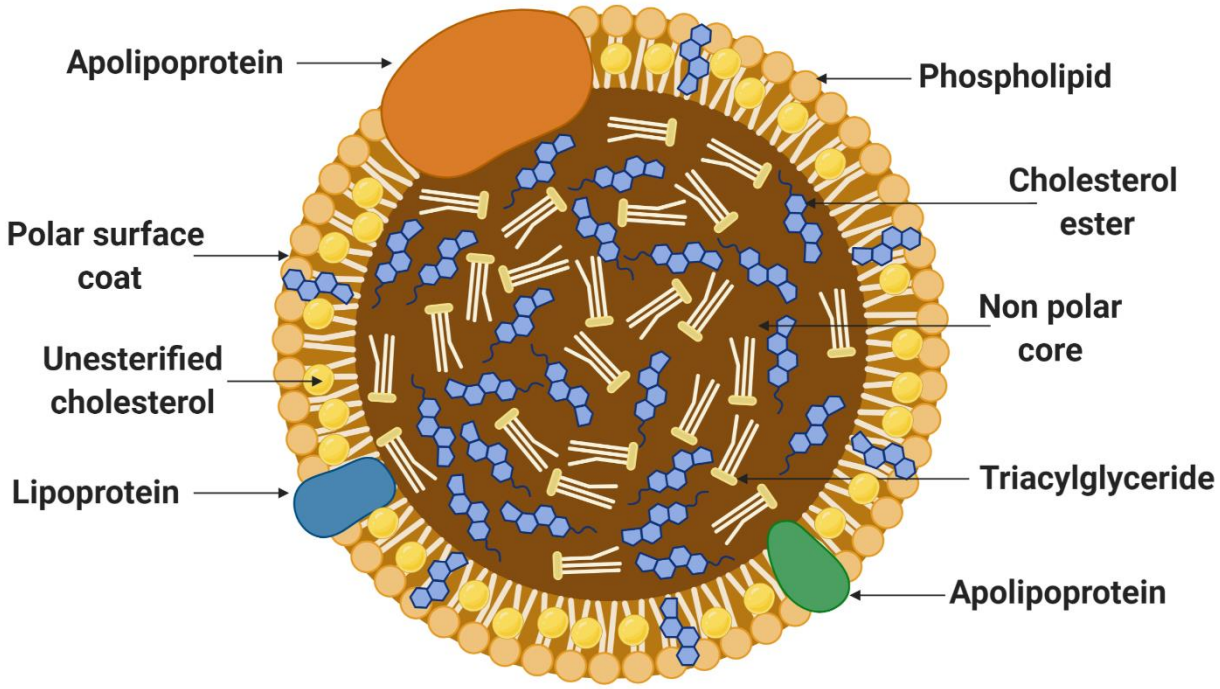


Figure 1. 5 Lipoprotein composition.
A core of TAG and cholesterol ester is surrounded by a phospholipid monolayer. Lipoproteins can be differentiated on the basis of their density and the apolipoproteins they contain, which also influence the particle's structural and functional properties (Wasan et al. 2008). Created with BioRender.com.

Table 1. 3 The most common types of lipoproteins and their functions.

Lipoproteins	Major lipids	Apoprotein	Major functions	References
Chylomicrons	TAG	Apo B-48, Apo C, Apo E, Apo A-I	Transports ingested fat and fat-soluble vitamins	(McLaren *et al.* 2011a)
HDL	Cholesterol Phospholipids	Apo A-I, Apo C, Apo E	Reverse cholesterol transport	(Buckley and Ramji 2015)
IDL	TAG Cholesterol	Apo B-100, Apo E, Apo C	Transports cholesterol to peripheral tissues or the liver	(Buckley and Ramji 2015)
LDL	Cholesterol	Apo B-100	Delivers cholesterol to cells	(Lusis et al. 2004; Buckley and Ramji 2015)
VLDL	TAG	Apo B-100, Apo E, Apo C	Transports TAG synthesised in the liver	(Buckley and Ramji 2015)

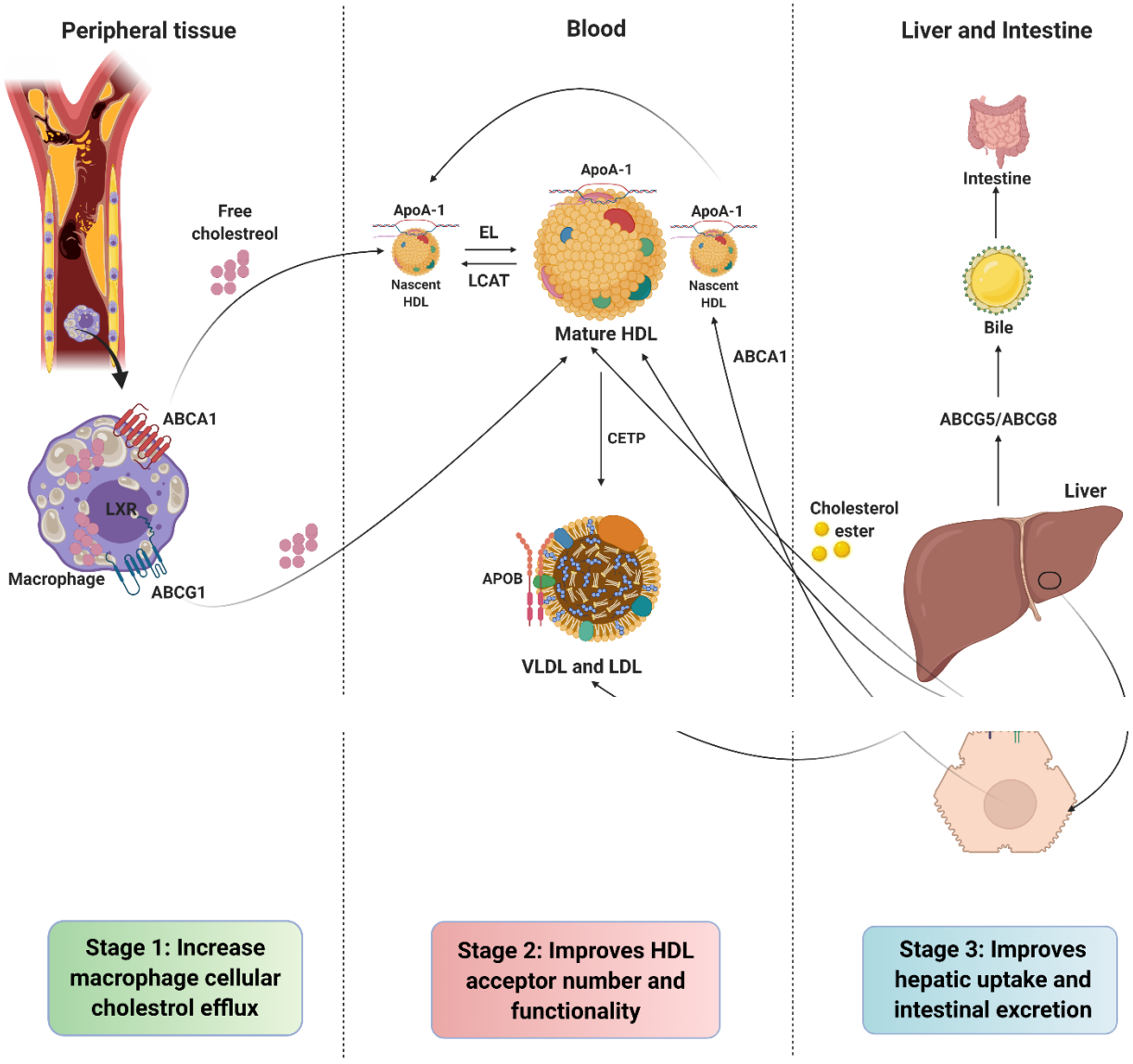


Figure 1. 6 Mechanism for the reverse transport of cholesterol.

Apolipoprotein A-I (ApoA-I), which is secreted in a lipid-poor form, is synthesised by both the liver and the intestine via the hepatocyte ATP-binding cassette A1 (ABCA1) transporter. These particles are lapidated with both phospholipids and free cholesterol to form nascent high-density lipoprotein (HDL). In peripheral tissues, through the macrophage ABCA1 transporter, these HDL particles obtain extra free cholesterol. Lecithin cholesterol acyltransferase (LCAT) causes maturation of HDL by esterifying free cholesterol into cholesterol esters. Through the macrophage ATP-binding cassette G1 (ABCG1) pathway, these larger HDL particles act as additional cholesterol efflux acceptors. The Liver X receptor (LXR) controls the expression of both ABCA1 and ABCG1 in macrophages (Khera and Rader 2010). Abbreviations: CETP, cholesterol ester transfer protein; EL, endothelial lipase; LDL, low density lipoproteins; LDLr, low density lipoproteins receptor; SR-BI, scavenger receptor class B type 1; VLDL, very low-density lipoproteins. Created with BioRender.com.

1.3 Inflammation in atherosclerosis

The acute inflammation response of the body is critical for protection against invasion by pathogens and other cellular insults. The immune response is a complex system that requires the input and regulation of different cell types and signals (Calder 2012). Although negative feedback mechanisms typically minimise excessive damage, chronic inflammatory diseases such as atherosclerosis occur, if inflammation persists (Calder 2002). Atherosclerosis is a focal inflammatory disease that occurs in the walls of large and medium arteries as a response to various risk factors, particularly the accumulation of cholesterol and other lipids. Atherosclerosis contributes to several adverse events such as coronary artery disease, myocardial infarction, stroke and disease of the peripheral arteries (Taleb 2016). ApoB, which is present in several lipoproteins, such as chylomicron remnants, LDL, VLDL and IDL, is one of the most significant causative agents of atherosclerosis (Shapiro and Fazio 2017). The disease is thought to be initiated by the retention of ApoB-containing lipoproteins, especially LDL, in arteries, particularly in areas where blood flow is disturbed by folding or branching (Shapiro and Fazio 2017). The resulting inflammation plays a crucial role in each phase of the disease. Initial inflammatory reactions involve several cell types, particularly monocytes and T-lymphocytes, leading to plaque development in atherosclerosis (Moore et al. 2013). As part of the inflammatory response, the arterial endothelium becomes activated or dysfunctional, leading to the secretion of several chemokines and the expression of many adhesion molecules on the cell surface. This causes the invasion of monocytes/lymphocytes in the sub-endothelium (Tedgui and Mallat 2006) and leads to plaque development. Figure 1.7 provides a summary of different stages during atherosclerosis development.

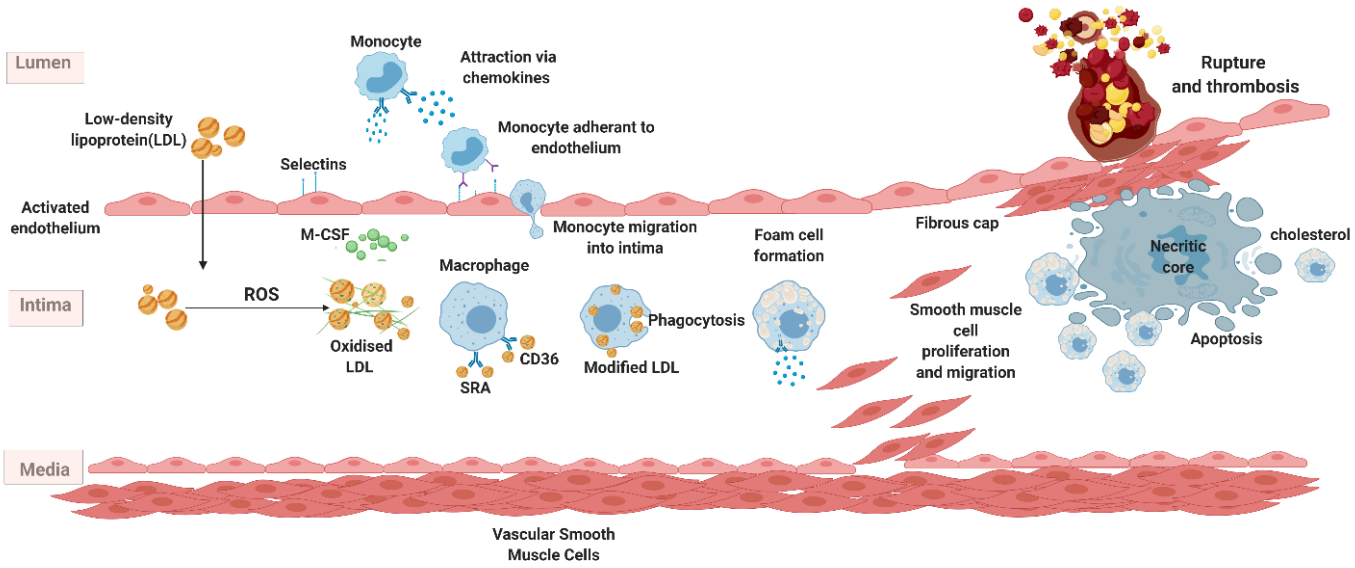


Figure 1. 7 The development of an atherosclerotic plaque.

LDL accumulates in the intima via passive diffusion or SR-BI-mediated transcytosis, where it undergoes modification, particularly oxidation to oxidised LDL (oxLDL), in an environment associated with oxidative stress. In the adjacent endothelial cells, oxLDL causes an inflammatory response, causing them to secrete a range of chemokines to the surroundings and to express adhesion molecules on their surface. Such adhesion molecules permit the rolling of monocytes and their adherence to the artery wall at the affected site. Then, the monocytes move to the subendothelial space, where they differentiate into macrophages and start taking up modified LDL via scavenger receptors (e.g., SRA and CD36) to transform into lipid-laden foam cells. These foam cells undergo apoptosis and necrosis over time because increased intracellular cholesterol accumulation is toxic to the cells. The deposited lipids lead to the formation of a necrotic core that further increases the inflammatory response, in part via the activation of the inflammasome. This lipid-rich necrotic core causes plaque destabilisation. As a protective mechanism, the normally quiescent smooth muscle cells migrate from the media to the intima, where they produce extracellular matrix (ECM) proteins that form the plaque-stabilising fibrous cap. Matrix metalloproteinases (MMPs) and other proteases can destabilise the plaque by digestion of the ECM proteins, which can cause plaque rupture and subsequent thrombotic response. The resulting blood clot could obstruct the artery and can either induce a myocardial infarction or a stroke depending on the position of the plaque. Abbreviations: CCR2, C-C chemokine receptor 2; CCR5, C-C chemokine receptor 5; CD36, cluster differentiation 36; ICAM-1, intercellular adhesion molecule 1; LDL, low density lipoprotein; MCP-1, monocyte chemotactic protein 1; M-CSF, macrophage colony stimulating factor; oxLDL, oxidised LDL; ROS, reactive oxygen species; SMC, smooth muscle cell; SRA, scavenger receptor A; SRB1, Scavenger receptor B1; VCAM-1, vascular cellular adhesion molecule 1; VSMC, vascular smooth muscle cells. Created with BioRender.com

1.3.1 Initiation of atherosclerosis

Activation of the arterial endothelial cells (ECs) and endothelial cell dysfunction resulting from various risk factors, particularly the increased blood circulation of ApoB-containing lipoproteins such as LDL, are key initiating factors in atherosclerosis development (Tabas et al. 2007; Insull Jr 2009; Sedding et al. 2018). The LDL passes into the subendothelial layer via either passive diffusion or SRBI-mediated transcytosis (Ng 2004). The LDL undergoes modification, particularly oxidation under conditions of oxidative stress to form oxLDL. The oxidation process involves many enzymes, including myeloperoxidases and lipoxygenases (LOX), as well as reactive oxygen species, peroxynitrite and nitric oxide (NO) produced by them or other effectors (Wen and Leake 2007). As a result of endothelial cell activation and dysfunction, the cells express various adhesion molecules [e.g., vascular cell adhesion molecule-1 (VCAM-1)], P- and E selectins and intercellular adhesion molecule-1 (ICAM-1)], and secrete several cytokines and chemokines, including monocyte chemotactic protein-1 (MCP-1, also known as CCL2). The chemokines attract immune cells, particularly monocytes, and the adhesion proteins regulate the mobilisation and transmigration of immune cells into the subendothelial space. In the subendothelial space, monocytes undergo differentiation into different polarised macrophages. Several macrophage phenotypes have been identified, with two major groups: M1 and M2 macrophages, polarised by cytokines of T-helper 1 and T-helper 2 cells, respectively (Wang et al. 2014; Jinnouchi et al. 2020). Recent studies have demonstrated additional macrophage phenotypes, such as M (Hb), Mox and M4 (Jinnouchi et al. 2020). M1 macrophages are usually activated by bacterial products such as lipopolysaccharide (LPS), releasing inflammatory cytokines, whereas M2 macrophages are anti-inflammatory (Wang et al., 2014). Additionally, M1 macrophages tend to predominate in atherosclerotic plaques (Moore et al. 2013). In atherosclerosis, the function of M2 macrophages has been related to plaque regression, together with decrease in plaque size, macrophage content, cholesterol content and inflammatory condition (Bartlett et al. 2019). In mouse atherosclerosis, Mox macrophages are observed where they are stimulated by oxLDL and have pro-atherogenic properties (Chistiakov et al. 2015a; Jinnouchi et al. 2020). M4 macrophages are produced by platelet CXCL4 chemokine and are charecterised by deficiency of haemoglobin-haptoglobin scavenger receptor (SR) CD163 and expression of a combination of CD68, S100A8 and MMP7 (Chistiakov et al. 2015a; Jinnouchi et al. 2020). Developed plaques may also undergo intraplaque haemorrhage (IPH) due to rupture of red blood cells into the plaque and its subsequent uptake via haptoglobin binding through the CD163 receptor, which triggers

differentiation into M(Hb) or Mhem macrophages (Jinnouchi et al. 2020). Figure 1.8 provides a summary of subtypes of key macrophages found in atherosclerotic lesions.

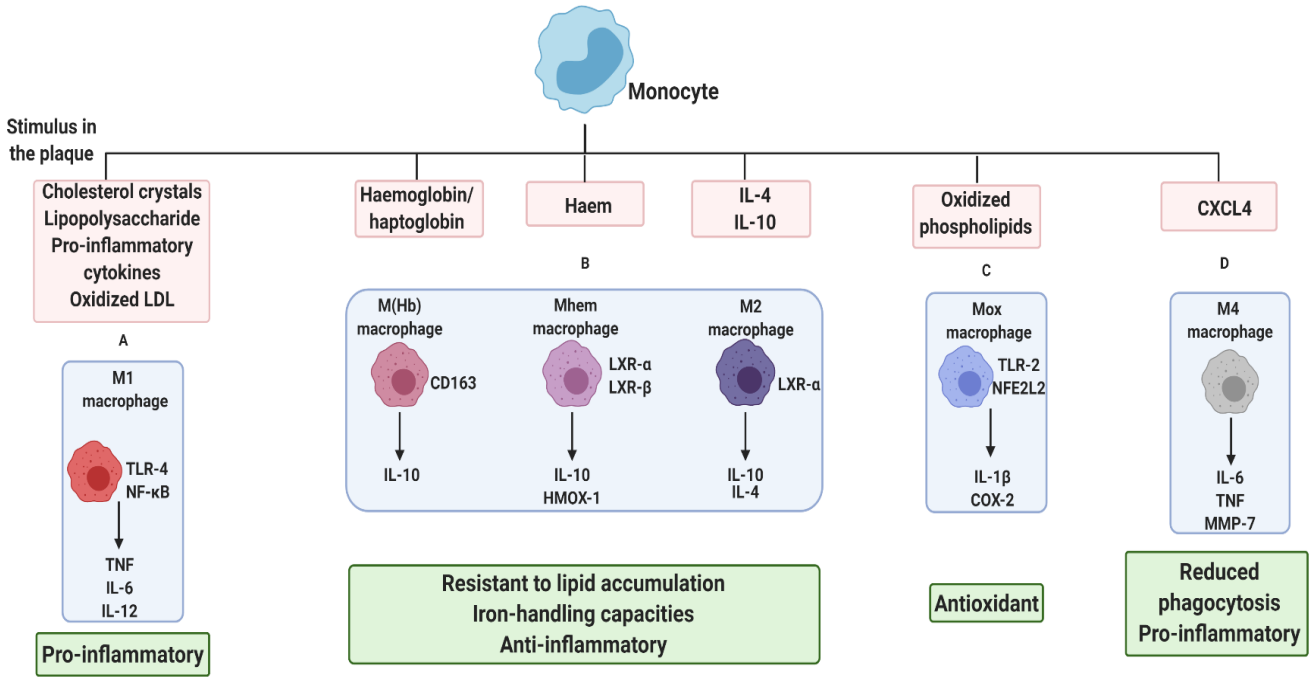


Figure 1. 8 Subtypes of key macrophages in atherosclerotic lesions.
The differentiation of monocytes into various macrophage phenotypes is directed by stimuli present in atherosclerotic lesions. (A) M1 macrophages express proinflammatory cytokines. (B) The macrophages M(Hb), Mhem and M2 are resistant to lipid accumulation, have iron-handling ability and also have anti-inflammatory properties. (C) An antioxidant gene expression profile is demonstrated by Mox macrophages. (D) M4 macrophages, like M1 macrophages, are proinflammatory but have reduced phagocytosis ability. Abbreviations: COX-2, cyclooxygenase; CXCL4, C-X-C motif chemokine 4; HMOX-1, haem oxygenase (decycling) 1; LDL, low-density lipoprotein; LXR, liver X receptor; MMP-7, matrix metalloproteinase-7; NFE2L2, nuclear factor (erythroid-derived 2)-like 2; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TLR, toll-like receptor; TNF, tumour necrosis factor (Jinnouchi et al. 2020). Created with BioRender.com.

1.3.2 Macrophage foam cell formation

At all phases of atherosclerotic lesion development, from initial lesions to advanced plaques, foam cells perform a key function (Chistiakov et al. 2015b). A small proportion of foam cells derive from ECs, with a substantial proportion from macrophages and vascular smooth muscle cells (VSMCs) (Sun et al. 2016). The process of macrophage foam cell formation is summarised in Figure 1.9. Foam cell formation is a consequence of disturbance in the normal homeostatic mechanisms that control macrophage metabolism, and associated with increased intake of modified lipoproteins and oxLDL, as well as decreased cholesterol efflux resulting in the accumulation of intracellular cholesterol esters (Chistiakov et al. 2017). Foam cells contribute through several mechanisms to the development of atherosclerotic plaque, including the release of high levels of proinflammatory cytokines and chemokines, secretion of MMPs that lead to plaque destabilisation, and release of their contents by apoptosis and necrosis, which drives the progression of the disease and the formation of a necrotic core (Groh et al. 2018). LDL undergoes modifications such as glycation, acylation, aggregation or oxidation when trapped in the subendothelial layer (Orsó et al. 2011). The most studied atherogenic modification of LDL is oxidation to produce oxLDL, a highly pro-inflammatory and pro-atherogenic particle (Chistiakov et al. 2016). The differentiation of monocytes into macrophages is associated with increased expression on the cell surface of a number of pattern-recognition receptors (PRR), including the scavenger receptors and the toll-like receptors (TLRs), that are an integral part of the innate immune response, as they recognise molecular patterns in pathogens (Chou et al. 2008). Several SRs, including SR-A, SR-BI and CD36 (Zani et al. 2015), are capable of recognising molecular patterns on modified LDL, particularly oxLDL, and mediate its uptake by receptor-mediated endocytosis (McLaren *et al.* 2011a). The uptake of LDL by the LDLr is under negative feedback regulation by increased intracellular levels of cholesterol, so cannot contribute to excessive accumulation of this sterol (McLaren *et al.* 2011a). However, uptake by SRs is not under such negative feedback regulation, so can contribute to uncontrolled uptake of cholesterol. Other non-receptor-mediated processes also contribute to the uptake of LDL/modified LDL, including phagocytosis, a characteristic feature of leukocytes, and macropinocytosis. The latter is a type of fluid-phase endocytosis that has been implicated as a significant contributor to the development of foam cells (Kruth 2011). Receptor-mediated uptake and degradation of modified LDL by macrophages are mediated primarily via CD36 and SR-A (Jiang et al. 2012). In addition, the main type of modified LDL internalised through scavenger receptors is oxLDL (Plüddemann et al. 2007).

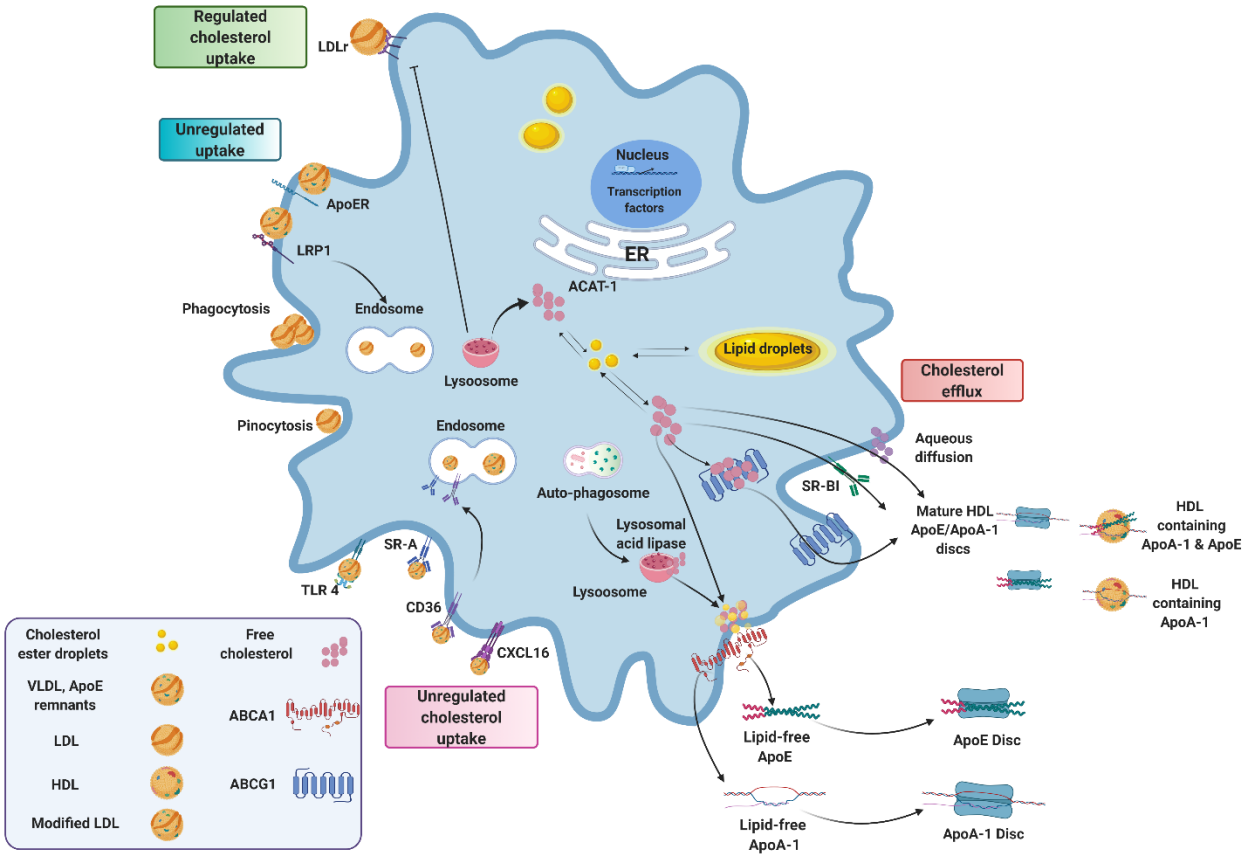


Figure 1. 9 Lipid homeostasis during the development of macrophage-derived foam cells. The LDL receptor (LDLr) recognises native LDL. The LDL is endocytosed and transferred to the lysosomes, where the lysosomal acid lipase hydrolyses the cholesterol ester (CE) to free cholesterol (FC). The FC is transported to the endoplasmic reticulum (ER) for esterification by acyl CoA: cholesterol acyltransferase (ACAT) and storage of cholesterol esters as lipid droplets. Increased FC activates a signalling cascade in the ER that results in down-regulation of the expression of the LDL receptor. In the setting of hypercholesterolemia, cholesterol regulation of the LDLr prevents the development of foam cells through this receptor. Through the interaction of ApoE with ApoE receptors, including LRP1 and VLDL receptors, that are not controlled by cellular cholesterol, ApoA-1 containing lipoproteins which also contain ApoE (e.g., ApoE remnants, VLDL) can cause cholesterol accumulation. Native LDL uptake through fluid phase pinocytosis can also lead to the formation of foam cells. Lipoprotein-containing modifications of ApoA-1 causes substantial accumulation of cholesterol through a variety of mechanisms. Oxidation and/or glycation promotes internalisation through a range of receptors, including CD36, scavenger receptor A (SRA), lectin-like (LOX) and toll-like receptors (e.g., TLR4). Cytoplasmic CE can be cleared by two main pathways. In one pathway, the removal of FC from the plasma membrane stimulates the transport of FCs produced by neutral cholesterol esterase away from ACAT to the plasma membrane. Cytoplasmic CE is alternatively packaged via autophagosomes that are transferred to fuse with lysosomes, where lysosomal acid lipase hydrolyses the CE and then the resulting FC is transported to the plasma membrane. Figure adapted from (Yuan et al. 2012). Abbreviations: ACAT, Acyl CoA cholesterol acyltransferase; Apo, Apolipoprotein; CD36, Cluster differentiation 36; CE, cholesterol ester; ER, endoplasmic reticulum, FC, free cholesterol; LDL, Low density lipoprotein; LDLr, Low density lipoprotein receptor; LOX, Lectin-like; SRA, Scavenger receptor A, TLR, Toll-like receptors, VLDL, Very low-density lipoprotein. Created with BioRender.com.

In addition to the abnormal uptake of modified lipoproteins, the processes that regulate cholesterol efflux are also affected in atherosclerosis, leading to decreased efflux and consequently the accumulation of excess intracellular cholesterol (Y Litvinov et al. 2016). The efflux machinery acts under normal conditions for the transfer of intracellular cholesterol from the cell, either by passive diffusion or via specific transporter proteins such as ATP-binding cassette transporter (ABC)-A1/G1 to extracellular lipid acceptors such as HDL for hepatic removal by reverse cholesterol transport (RCT) (Jeong et al. 2017). RCT is a necessary cholesterol excretion process of peripheral tissues where the sterol can be removed via the bile system into the faeces (McLaren *et al.* 2011a; O'Morain and Ramji 2020). Figure 1.6 presents an outline of RCT; free cholesterol is transferred from the cell to lipid-free ApoA1 in the initial phases, producing nascent HDL. On the surface of HDL particles, free cholesterol is esterified by lecithin cholesterol acyltransferase (LCAT) and mature HDL particles are formed by the additional acquiring of cholesterol (Vaziri 2009). After that, HDL cholesterol can be transferred directly to the liver via SR-B1 (Ohashi et al. 2005). Conversely, through CETP-mediated transfer, HDL cholesterol can be supplied to the liver indirectly (Trajkovska and Topuzovska 2017). In exchange for TGs, CEs from HDL are transferred to ApoB-containing LDL/VLDL particles and are consequently delivered through LDL receptors to the liver (Trajkovska and Topuzovska 2017).

ABCA1 and ABCG1 are the main transporters involved in cholesterol efflux, and their expression is upregulated specifically by liver X receptors (LXRs), which are activated in response to intracellular cholesterol accumulation (Bobryshev et al. 2016). ABCA1 and ABCG1 are intrinsic plasma membrane proteins that facilitate the transfer of intracellular cholesterol to the plasma membrane (Favari et al. 2015). Although other ABC cholesterol-responsive transporters have been identified, their precise roles in atherogenic formation of foam cells and RCT are not fully understood (Fu et al. 2013; Favari et al. 2015). ApoE is a lipoprotein component synthesised in response to stimuli like cytokines and lipid enrichment by several cell types, such as hepatocytes and macrophages (Favari et al. 2015). In the absence of cholesterol acceptors, ApoE secreted from cholesterol-rich macrophages is known to induce cholesterol efflux (Huang et al. 2001). In addition, genetic ApoE deficiency in mice contributes to the progression of atherosclerosis, with protein re-expression decreasing the severity of the disease (Greenow et al. 2005). Indeed, ApoE-deficient mice and LDLr-deficient mice are two of the widely used mouse models of atherosclerosis (Zadelaar et al. 2007). The class B scavenger receptor, SR-B1, is also involved

in the efflux of cholesterol; furthermore, it internalises modified LDL and hence plays a complex role in atherosclerosis (Yancey et al. 2003; Huang et al. 2019a).

1.3.3 Disease progression

The development of foam cells causes the secretion of several inflammatory cytokines from the cells, including tumour necrosis factor α (TNF-α), interleukin 1(IL-1) and interleukin 6 (IL-6), which modulate other cells within the plaque microenvironment and enhance the inflammatory response. The high intracellular levels of free cholesterol and cholesterol ester impact intracellular membranes and cause endoplasmic reticulum (ER) stress that promotes apoptosis of foam cells (Kavurma et al. 2017; Bäck et al. 2019). The death of foam cells by apoptosis and necrosis causes the formation of a lipid-rich necrotic core (Lusis et al. 2004). Efferocytosis (apoptotic cell clearance) is essential for the elimination of cell debris and apoptotic cells in early lesions (Tajbakhsh et al. 2018). However, apoptosis of foam cells becomes more excessive than efferocytosis as the plaque progresses (Tabas 2009). Increased apoptotic cell death combined with impaired efferocytosis leads primarily to dead or dying macrophages, which are characteristic of advanced atherosclerotic plaques (Bobryshev et al. 2016).

Another critical process in the development of the disease includes the migration of VSMCs from the media to the arterial intima, where they secrete several ECM proteins (Fernández-Hernando et al. 2009). ECM proteins contribute to plaque stability via formation of a protective fibrous cap (Vassiliadis et al. 2013). VSMCs exist largely in a quiescent state in the medial layer of healthy arteries (Rudijanto 2007). In atherosclerotic conditions, they become proliferative and migrate to the intima, where they act to remodel the ECM (Buckley and Ramji 2015). This remodelling results in the deposition of a dense protective cap of collagenous tissue throughout the endothelium and the lipid-rich necrotic core, which provides a protective barrier between thrombogenic plaque and the bloodstream (Rohwedder et al. 2012). A thick fibrous cap is maintained by a balance between the production and degradation of ECM components, which is an essential factor in the prognosis of long-term disease (Rohwedder et al. 2012). When degradation exceeds ECM protein generation, the fibrous cap is reduced and the plaque remains unstable and vulnerable to rupture, causing thrombus formation and associated clinical complications (Newby et al. 2009).

The specific processes of thinning of the fibrous cap remain unknown, but a variety of factors have been implicated, including increased production and activity of MMP, which play a critical role in ECM protein degradation (Tousoulis et al. 2016). Macrophage foam cells are a source of abundant serine and MMP that actively degrade ECM, which include collagen, elastin and other ECM proteins (Newby et al. 2009). The development of the necrotic core is also associated with increased VSMC death and reduced ECM protein production (Tabas 2009). Due to the plaque disruption hypothesis, only a minority of plaques become unstable and rupture, and this mechanism is more closely correlated with the size of the necrotic core than with the size of the plaque itself (Tabas 2009). As inflammation is central to all the different stages of atherosclerosis development, it is important to consider the role and the signalling mechanisms of key cytokines that regulate the various cellular processes associated with the disease (Tousoulis et al. 2016).

1.4 Cytokines in atherosclerosis

Cytokines are a class of secreted glycoproteins that typically play a major role in the control of inflammation (McLaren *et al.* 2011a). They are divided into several classes, including interleukins (ILs), tumour necrosis factors (TNFs), interferons (IFNs), transforming growth factors (TGFs), colony-stimulating factors (CSFs) and various chemokines. Depending on their actions in the immune response, they are classified as pro- or anti-inflammatory (McLaren *et al.* 2011a). Cytokines are involved in all stages of atherosclerosis, from initiation to plaque rupture (Lusis 2000; Sprague and Khalil 2009). During the inflammatory response, foam cells develop because of the actions of proinflammatory cytokines such as IL-1, IL-6, IL-12 and TNF-α (Hansson 2001; Bobryshev 2006; Wang and Butany 2017). It has been found that cytokines also play a major role in the migration of immune cells (e.g., monocytes, neutrophils, lymphocytes) during endothelium dysfunction by upregulation of the expression of adhesion proteins and the synthesis of chemokines (Lusis 2000). In addition, pro-inflammatory cytokines promote the migration of SMCs from the media to the intima (Lusis 2000). Table1.4 presents the function of key selected cytokines in the pathogenesis of atherosclerosis. The *in vivo* studies have mostly been carried out in two common mouse models of the disease: ApoE$^{-/-}$ mice and LDLr$^{-/-}$ mice that are fed a high-fat diet (HFD) (Jawień et al. 2004; Wouters et al. 2005). Cytokine therapy represents a potential avenue for combating atherosclerosis, as demonstrated by the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) trial (Ridker et al. 2011).

Table 1. 4 The role of cytokines in atherogenesis.

Cytokines	Role in atherosclerosis	References
IL-1β	In ApoE$^{-/-}$ mice, lack of IL-1β decreased the expression of inflammation markers MCP-1 and ICAM-1 and reduced atherosclerosis. Similar findings have been demonstrated in ApoE$^{-/-}$/IL1 receptor knockout mice and anti-IL-1β antibody-targeted mice.	(Kirii et al. 2003; Chamberlain et al. 2009; Bhaskar et al. 2011)
IL-6	Injection of IL-6 into ApoE$^{-/-}$ mice increased the levels of pro-inflammatory cytokines and lesion size. However, inhibition of *trans*-signalling by IL-6, involving soluble glycoprotein 130, decreased atherosclerosis, endothelial cell activation, infiltration of mesenchymal stem cells (MSCs) and monocyte recruitment.	(Zhang et al. 2012; Hartman and Frishman 2014)
IL-17	The role of IL-17 in atherosclerosis tends to be context dependent. Inhibition of IL-17 in ApoE-deficient mice via a neutralising antibody prevented the progression of atherosclerotic lesions by reducing the inflammatory burden and cellular infiltration, and enhanced lesion stability. In a rat carotid artery model system, IL-17 exacerbated arterial thrombosis caused by ferric chloride.	(Van Es et al. 2009; Chen et al. 2010; Taleb et al. 2010)
IL-10	Double knockout ApoE$^{-/-}$/IL-10$^{-/-}$ mice demonstrated increased size of atherosclerotic lesions along with pro-coagulant plaque activity, suggesting that this cytokine plays a role in plaque stability. Furthermore, treating LDLr$^{-/-}$ mice with IL-10-encoding viral vector caused a reduction in atherosclerotic lesions. Additionally, in LDLr$^{-/-}$ mice, IL-10-expressing macrophages inhibited atherosclerosis. There was also a decrease in cholesterol ester accumulation and an increase in cholesterol uptake and efflux by macrophages.	(Liu et al. 2006; Han et al. 2010; Bhaskar et al. 2011)
IL-35	IL-35 is a novel anti-inflammatory cytokine that has been found to play an important role in the maintenance of the regulatory T cells (Treg)-mediated immune homeostasis. A previous study found that IL-35 decreased atherosclerosis in ApoE-deficient mice.	(Tao et al. 2016; Huang et al. 2019b)

IFN-γ	Deficiency of IFN-γ receptors was correlated with decreased atherosclerotic lesion size in ApoE-deficient mice, and HFD-induced atherosclerosis was considerably decreased in LDLr$^{-/-}$ lacking IFN-γ. In addition, intraperitoneal injection of IFN-γ promoted atherosclerosis in ApoE-deficient mice.	(Gupta et al. 1997; Whitman et al. 2000)
TNF-α	A pro-atherogenic cytokine produced by cells such as activated monocytes and macrophages. Atherosclerosis development was reduced in ApoE-deficient mice lacking TNF-α and was associated with reduced levels of pro-inflammatory cytokines, chemokines and adhesion molecules. There was also a reduction in oxLDL uptake and SRA expression in macrophages in another study using the same knockout model.	(Ohta et al. 2005; Xiao et al. 2009)

1.5 Prevention of atherosclerosis and current and emerging therapies

1.5.1 Lifestyle changes

Many lifestyle modifications have been recommended by the WHO as a cost-effective measure to prevent atherosclerosis and CVD, including increased fruit and vegetable intake, reduced saturated fat and salt consumption, increased physical activity, cessation of smoking, reduction in body mass index and moderate exercise of 30-minute duration five times in a week (World Health Organization 2019). However, pharmaceutical intervention is required once the disease has been established.

1.5.2 Therapy with medications

Statins have been the most common and widely used treatment for reducing plasma LDL cholesterol levels in recent years. Statins act by inhibiting cholesterol biosynthesis in the liver by acting as competitive inhibitors of a rate-limiting step catalysed by 3-hydroxymethylglutaryl HMG CoA reductase (Singh et al. 2014; Ramkumar et al. 2016). Statins also have pleiotropic effects beyond reducing plasma LDL cholesterol levels, including inhibiting endothelial cell dysfunction and acting in an anti-inflammatory manner (Oesterle et al. 2017). Statins have been effective in

reducing the burden of CVD, as demonstrated by several large clinical trials and meta-analysis (Miao et al. 2019b). However, statin therapy is associated with marked residual risk for CVD, with suggestions that statins have only contributed to about 30% to the recent reduction in CVD morbidity and mortality seen in the West (McLaren *et al.* 2011a). Their over-prescription and the possible adverse effects of statin therapy have also been a growing concern. Some patients stop using statins because of concerns about the potential long-term effects, such as acute rhinitis, diarrhoea, muscle aches and a significant risk of diabetes and liver dysfunction (Logue et al. 2015; Ramkumar et al. 2016). In addition, some patients are unable to tolerate statins, whereas others are not able to achieve target LDL cholesterol levels even with the maximal dose of the drug. Therefore, other lipid-lowering therapies have to be considered.

Ezetimibe functions at the small intestine and prevents the absorption of dietary and biliary cholesterol by inhibiting the transporter Niemann-Pick C1-Like 1 (NPC1L1) (Kurobe et al. 2011). Ezetimibe treatment may be used alone or in combination with statins for patients who cannot achieve target LDL plasma levels even with the highest dose of statins (Hayashi and Kawamura 2013). The use of ezetimibe with statin therapy produced a significant reduction of cardiovascular disease in the IMPROVE-IT trial (Improved Reduction of Outcomes: Vytorin Efficacy International Trial) of patients with acute coronary syndrome (Cannon et al. 2015). In comparison to statin monotherapy, the co-therapy of statin and ezetimibe significantly reduced deaths from CVD (Miao et al. 2019a).

The LDLr is normally recycled back to the cell surface after delivery of the LDL particles to the lysosomes. Proprotein convertase subtilisin/kexin type 9 (PCSK9) is an LDLr-bound serine protease that causes intracellular degradation of the receptor, and therefore reduces the clearance of LDL cholesterol, as there are fewer LDLrs present on the cell surface. Alirocumab and evolocumab, monoclonal antibodies against PCSK9, have been successful in decreasing plasma LDL cholesterol (Maxwell et al. 2005). These monoclonal antibodies are now widely licenced for use in hypercholesterolaemia patients who otherwise do not respond to statin therapies (Preiss and Baigent 2017a; Saborowski et al. 2018; Sun et al. 2018).

Anti-inflammatory agents represent a potential treatment approach for the decrease of cardiovascular risk due to the inflammatory aspect of atherosclerosis disease; nevertheless, several promising candidates have failed in clinical trials (Libby et al. 2009; Charo and Taub 2011). Treatment with canakinumab, a monoclonal antibody that prevents inflammation by blocking IL-1β, resulted in a substantially reduced occurrence of atherosclerotic events in patients with elevated levels of the inflammatory marker C-reactive protein (CRP) compared with placebo in the CANTOS trial (Dolgin 2017). However, patients were more vulnerable to infection, and so medication may be limited to high-risk patients. Experiments with alternative anti-inflammatory agents have demonstrated fewer positive results, despite the success with IL-1β blockers to date. Methotrexate, for instance, is one possible anti-inflammatory that is commonly used in the prevention of rheumatoid arthritis, which is associated with high levels of atherosclerosis, but a low dose of Methotrexate failed in the CIRT trial (Roubille *et al.* 2015; O'Morain and Ramji 2020). Furthermore, the highly anticipated lipoprotein-associated phospholipase A2 (Lp-PLA2) inhibitor darapladib, developed by the pharmaceutical giant GlaxoSmithKline, failed to decrease cardiovascular risk in two separate clinical trials: STABILITY (Stabilisation of Atherosclerotic Plaque by Initiation of Darapladib Therapy) and SOLID-TIMI 522 (The Stabilisation Of pLaques usIng Darapladib-Thrombolysis In Myocardial Infarction 52) (Mullard 2014). As the search continues for successful anti-inflammatory therapies, the ability of anti-inflammatory agents to reduce the occurrence of CVD remains the topic of intense research. Modulating cytokine signalling is another possible therapeutic avenue.

Considering the above-mentioned issues with statins and the high costs associated with monoclonal antibody therapies, the latter will have to be restricted to people at high risks (Baylis et al. 2017); alternative therapies are required. Since CVD treatments are of long-term duration (typically 30-60 years), it is difficult to gauge side effects unless there is genetic evidence such as loss of function mutations, as is the case for PCSK9 (Nilsson and Hansson 2015; Kramer et al. 2018). Because natural products often have an excellent safety profile, current research on their use for the prevention of atherosclerosis and as add-ons with current pharmaceutical agents has increased (Moss and Ramji 2016b).

1.5.3 Nutraceuticals

Nutraceuticals are characterised as foods with health benefits beyond their nutritional values. Several studies have demonstrated cardiovascular health benefits of diets that are rich in berries, herbs, seafood, oily fish, cereals and olive oil (Sosnowska et al. 2017; Moss et al. 2018). Omega-3 PUFAs, hydroxytyrosol, flavanols, phytosterols, butyrate and vitamins C and E are among the most researched in human trials (Moss and Ramji 2016b). They have demonstrated anti-inflammatory actions and are being researched as potential anti-atherogenic treatments (Moss and Ramji 2016b). Hydroxytyrosol, a polyphenol compound found in olive oil that is identified to have anti-inflammatory properties, is a nutraceutical that has demonstrated special promise (Moss and Ramji 2016b). Anti-atherogenic benefits of hydroxytyrosol, including decreased serum oxLDL levels, increased HDL cholesterol (HDL-C) levels, decreased expression of inflammatory markers and improved endothelial function, have been reported in a large number of clinical trials (Gimeno et al. 2007; Valls et al. 2015; Al-Ahmadi 2019). Likewise, flavanols, plant metabolites usually found in fruits and vegetables, have been found to have antiatherogenic effects, with a range of human research indicating decreased LDL cholesterol (LDL-C), decreased pro-inflammatory marker expression and improved endothelial function (Matsuyama et al. 2008; Sansone et al. 2015). In the last two decades, omega-3 PUFAs have also been highly researched for their therapeutic effects, as their dietary supplementation has been found to minimise the occurrence of cardiovascular events in both preclinical and human studies (Moss and Ramji 2016b). It should, however, be emphasised that nutraceutical research has lagged behind that of pharmaceuticals in two key aspects: general lack of large clinical trials and lack of deeper insight into the mechanisms underlying their actions (Moss and Ramji 2016b). Figure 1.10 provides an overview of the actions of some nutraceuticals in atherosclerosis. The Reduction of Cardiovascular Events with Icosapent Ethyl-Intervention Trial (REDUCE-IT), a clinical trial aimed to resolve residual cardiovascular risk in raised TAG levels in statin-treated patients, has recently shown promise of omega-3 PUFAs in decreasing cardiovascular risk (Bhatt 2019). A correlation between atherosclerosis-associated CVD and fatty acids has therefore been highlighted in recent studies as potential treatment for atherosclerosis intervention (e.g., the REDUCE-IT trial mentioned above) (Nomura et al. 2019). The role of fatty acids in atherosclerosis is discussed in the next section.

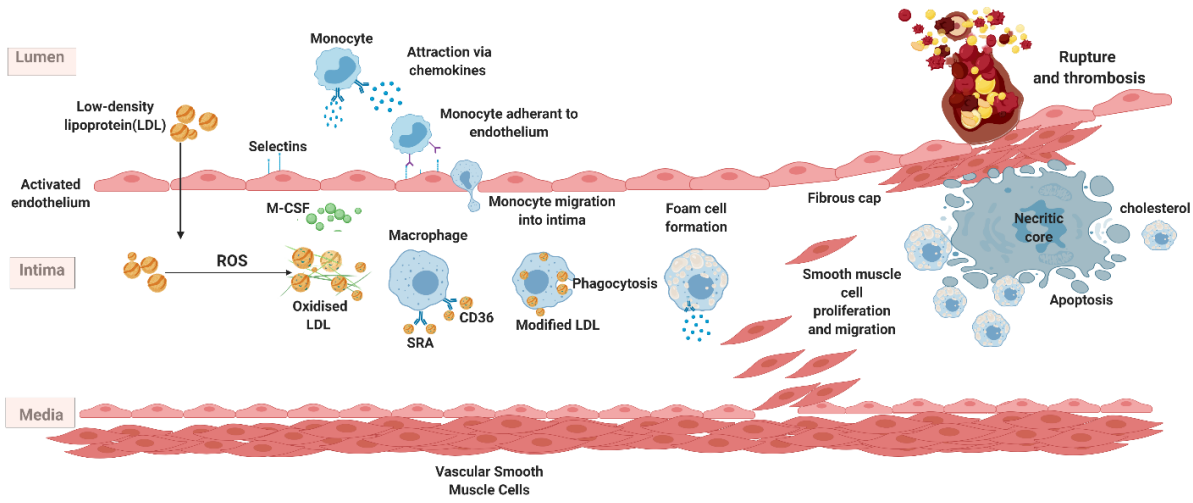
	Endothelial dysfunction	Monocyte recruitment/ Proinflammatory gene expression	foam cell formation	Plaque stability
Key steps through atherosclerosis development				
Nutraceuticals	Hydroxytyrosol Flavanols Vitamin C	Omega-3 PUFAs Omega-6 PUFAs Hydroxytyrosol Allicin Dietary fibre	Omega-3 PUFAs Allicin Phytosterols	Omega-6 PUFAs Dietary fibre

Figure 1. 10 The actions of some common nutraceuticals on atherosclerosis.

1.6 Fatty acids and atherosclerosis

Fatty acids can be characterised as saturated and unsaturated, as previously explained. Fatty acids have also been found to have an impact on several atherosclerosis-associated pathways such as lipid peroxidation, inflammation and the haemostatic system. Table 1.5 describes some roles of fatty acids in atherosclerosis. PUFA hydrocarbon chains that have more than one double bond are essential molecules for signalling. SFA are commonly known as "bad fat" as their dietary intake often relates to increased levels of plasma cholesterol and inflammatory processes, which increases the risk of CVD (Xu et al. 2006). On the other hand, PUFA-rich diets demonstrate considerable athero-protective function (Dyerberg 1989), which has been attributed in part to

regulation of inflammation (Suresh and Das 2003a, b; Wang et al. 2012; Dawczynski et al. 2013). The next section will discuss two key classes of PUFAs: omega-3 and omega-6 fatty acids.

Table 1. 5 Fatty acids and their roles in the development of atherosclerosis.

Class	Name	Possible role in atherosclerosis	References
SFA	Palmitic acid	Palmitic acid may lead to atherosclerosis by enhancing oxLDL uptake through macrophage upregulation of LOX-1 expression.	(Ishiyama et al. 2010)
SFA	Myristic acid	Intake of myristic acid in a Mediterranean population is linked to low plasma HDL cholesterol and increased HDL catabolism.	(Noto et al. 2016)
MUFA	Oleic acid	Oleic acid demonstrated antioxidant properties against LDL oxidation in a mouse model. Significantly inhibited uptake of oxLDL in THP-1 macrophages.	(Cho et al. 2010)
MUFA	Vaccenic acid	In $LDLr^{-/-}$ mice, vaccenic acid decreased cholesterol-induced hyperlipidaemia and atherosclerosis.	(Bassett et al. 2010)
n-6 PUFA	Linoleic acid	Decreased atherosclerosis by reducing total cholesterol levels in $LDLr^{-/-}$ mice fed an omega-6 linoleic acid-rich diet.	(Machado et al. 2012)
n-6 PUFA	GLA	In rats fed a high-fat diet, it reduced total cholesterol levels and oxLDL.	(Shi et al. 2008)
n-6 PUFA	DGLA	Reduced DGLA was linked with an elevated death rate in patients with heart failure. Atherosclerosis was attenuated by DGLA supplementation in $ApoE^{-/-}$ mice and associated with decreased expression of inflammatory markers.	(Ouchi et al. 2017)
n-6 PUFA	AA	The pro-inflammatory role and pro-thrombotic actions of metabolites of AA (for example PGE_2) has been documented in atherosclerosis.	(Levin et al. 2002) (Gomolka et al. 2011; Wang and

			Bennett 2012)
n-3 PUFA	α-linolenic acid	α-linolenic acid was successful in decreasing plasma lipid concentrations in ApoE$^{-/-}$ mice. The expression of inflammatory cytokines in ApoE$^{-/-}$ mice was decreased by α-linolenic acid.	(Xiao et al. 2016)
n-3 PUFA	EPA	EPA dietary supplementation in ApoE$^{-/-}$ mice reduced atherosclerotic plaques and decreased the content of immune cells.	(Harris et al. 2008; Nakajima et al. 2011)
n-3 PUFA	DPA	DPA reduced total cholesterol in plasma and cholesterol associated with non-HDL in hamsters.	(Harris et al. 2008; Chen et al. 2012)
n-3 PUFA	DHA	Increased consumption has been found to be related to a decrease in CVD in humans.	(Harris et al. 2008)

1.6.1 PUFAs: ω-6s v/s ω-3s

Linoleic acid and α-linolenic acid are the EFAs of the Omega-3 and -6 PUFA class (Das 2007). EFAs are an essential component of cell membranes (Das 2007). Production of eicosanoids can be modulated by changes in the dietary intake of EFAs (McDaniel et al. 2011). Eicosanoids are a group of 20 carbon PUFAs formed by the cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (cytP450) pathways (Calder 2020). The eicosanoid family includes prostaglandins (PG), leukotrienes (LT), thromboxanes (TX) and multiple hydroxy fatty acids (McDaniel et al. 2011). These mediators act rapidly and locally and are active until they are degraded (Arita 2012). Eicosanoids carry out a variety of functions that are pro-inflammatory or anti-inflammatory. Figure 1.11 provides a summary of omega-3 and -6 pathways.

Two major categories of PUFAs, namely ω-6s and ω-3s, are essential components of the cellular systems and have diverse bioactivities in the human body (Kaur et al. 2014). The two types of PUFAs are classified by the position of the first C=C double bond in the backbone (Hammad et al. 2016). Those with the first double bond at the sixth carbon atom (ω-6 carbon), for instance,

are called ω-6 fatty acids and include LA, GLA, DGLA and AA. On the other hand, those with the first C=C double bond at the third carbon atom (ω-3 carbon) from the methyl are ω-3 PUFAs. Examples include ALA, EPA and DHA (Xu 2017). As there is no enzyme present to add double bonds to fatty acids between carbons 9 and 10 on the carboxylic side, the human body cannot synthesise them *de novo* (Walsh and Metz 2013). These therefore need to be obtained via diet, and hence many ω-6s and ω-3s are considered as EFA (Huerta-Yépez et al. 2016). Evening primrose oil, borage oil, currant oil and hemp oil are rich in GLA (Huerta-Yépez et al. 2016), whereas ALA is present in canola, perilla, flaxseed and walnut oil (Simopoulos 2016). The majority of EPA and DHA is found in deep, cold water fish oil (Schuchardt et al. 2011).

Dietary GLA is converted by elongase into DGLA, introducing two extra carbon atoms. Delta-5 desaturase (also classified as fatty acid desaturase 1 or FADS 1) metabolises DGLA into AA, creating more C=C double bonds (Kim et al. 2012). Similarly, upon consumption, ALA, the precursor of ω-3 PUFAs, can be metabolised via elongase, delta-6-desaturase and delta-5-desaturase to produce various downstream PUFAs, including EPA and DHA (Simopoulos 2016). Studies on the impact of increasing ω-6 levels in the diet have variously demonstrated either an increased risk of CVD, a decreased risk or no correlation (Harris et al. 2009). For example, the alpha-tocopherol beta-carotene cancer prevention study found that there was no correlation between the LA content of the diet and CVD (Pietinen et al. 1997). LA dietary content was inversely correlated with CVD risk, whereas AA was not, in a meta-analysis of 25 case control studies (Harris et al. 2007). There was also evidence that high LA intake is not associated with acute myocardial infarction risk (Kark et al. 2003). Overall, it has been proposed that reducing the consumption of ω-6 fatty acids in the diet could reduce the risk of CVD (Harris et al. 2009). However, this proposition is not applicable to all ω-6 PUFAs, as dietary interventions with GLA and DGLA ω-6 fatty acids revealed significant anti-inflammatory actions in several inflammatory diseases (Kawashima *et al.* 2008; Takai *et al.* 2009a; Wang and Bennett 2012) .

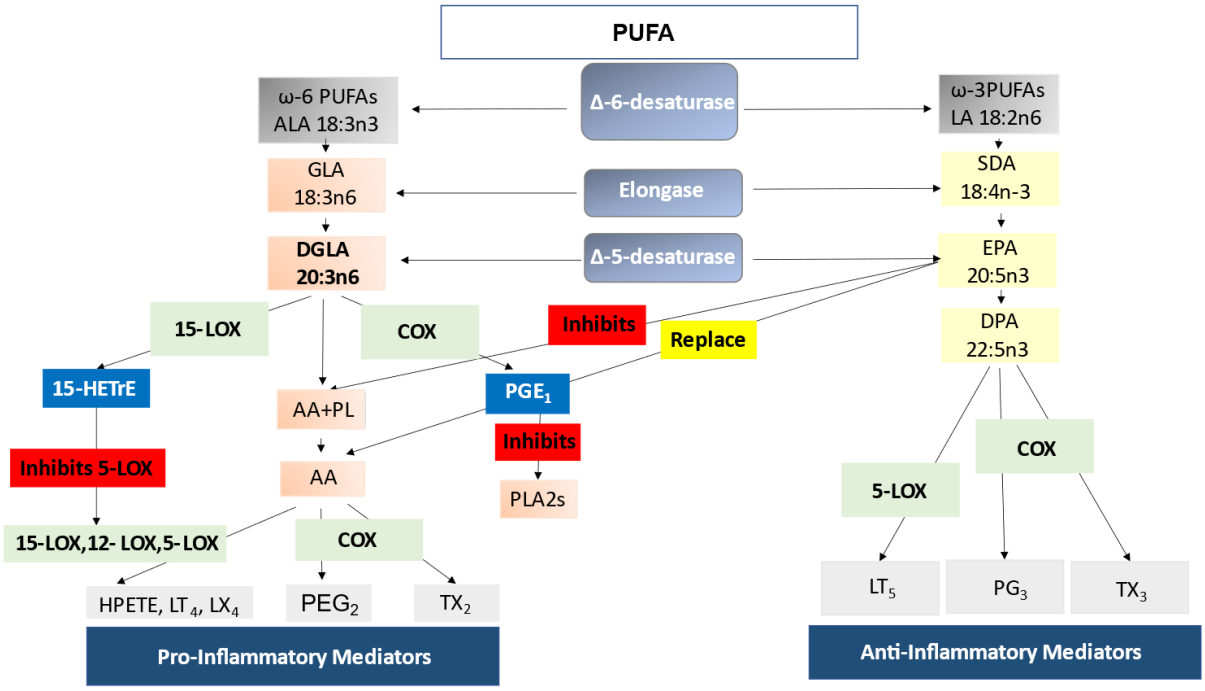


Figure 1. 11 Summary of omega-3 and -6 pathways.
ω-3 and ω-6 PUFAs, metabolites, such as eicosanoids, and their potential inflammatory consequences are indicated. The PUFAs are metabolised by several pathways after intake to produce active mediators that either promote inflammation or facilitate resolution of inflammation. Abbreviations: AA, Arachidonic acid; ALA, α-Linolenic acid; COX, cyclooxygenase; DGLA, Dihomo-γ-linolenic acid; DPA, docosapentaenoic acid; EPA, Eicosapentaenoic acid; HETrE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxy eicosatetraenoic acid; GLA, γ-Linolenic acid; LOX, lipoxygenase; LT, leukotriene; PG, prostaglandin; PGE2, prostaglandin E2; PL, phospholipid; PUFAs, polyunsaturated fatty acids; SDA, stearidonic acid; TX, thromboxane (Pantzaris et al. 2013).

1.7 DGLA

1.7.1 Role of DGLA in disease

Due to the activities of metabolites of AA, ω-6 PUFAs are generally referred to as pro-inflammatory (Saini and Keum 2018). Nevertheless, it has been found that metabolic precursors of AA have key anti-inflammatory functions. In several inflammatory diseases, dietary supplementation with GLA has demonstrated effectiveness (Fan et al. 2001). GLA intake, for instance, dramatically decreased symptoms in rheumatoid arthritis patients and those with atopic eczema (Umeda-Sawada et al., 2006). Atherosclerosis and smooth muscle cell proliferation have also been found to be inhibited by GLA in $ApoE^{-/-}$ mice (Fan et al. 2001). GLA also attenuated the release of IL-1β in dose-dependent manner in human monocytes (Furse et al. 2001). GLA is elongated into DGLA, which is then cleaved or metabolised into cell membranes to generate anti-inflammatory mediators (Teraoka et al. 2009). To increase the fatty acid pool of DGLA indirectly, several studies have used GLA (Teraoka et al. 2009). However, this method may not cause sufficient increase in the levels of this fatty acid (Teraoka et al. 2009). Because of the lack of availability, many previous studies have not used direct DGLA supplementation but instead have relied on production via GLA. More recently, a limited number of studies have investigated the anti-inflammatory functions of DGLA, some relating to diseases, including atherosclerosis, leading to the exploration of new sources of this fatty acid.

DGLA has several positive effects on CVD as well as on other inflammatory conditions (Takai *et al.* 2009a). ApoE-deficient mice given a normal diet with DGLA supplementation demonstrated a significant decrease in atherosclerosis development (Takai *et al.* 2009a). In addition, DGLA treatment decreased the expression of ICAM-1 and VCAM-1 inflammation markers, along with a reduction in the p22phox and gp91phox NADPH oxidase subunits, suggesting a possible increase in vascular relaxation (Takai *et al.* 2009a).

In a mouse model of atopic dermatitis, supplementation with DGLA oil in the diet also significantly decreased the risk of skin scores and scratching actions (Kawashima et al. 2008). In mice given DGLA orally, there was also a substantial decrease in ear inflammation after induction using an inflammatory stimulus. After 7 days of therapy, DGLA prevented swelling and cell infiltration (Watanabe et al. 2014). Table 1.6 summarises the functions of DGLA in diseases.

Table 1. 6 Summary of roles of DGLA in diseases.

Role	Reference
In ApoE-deficient mice fed a normal diet supplemented with DGLA, there was a significant reduction in the development of atherosclerosis.	(Takai *et al.* 2009b)
In a free radical-dependent mechanism, DGLA has shown cytotoxic activity in drug-sensitive and resistant cancer cells.	(Das 2011)
In NC/Tnd mice, a model of atopic dermatitis, DGLA supplementation demonstrated a significant accumulation of DGLA in the skin that reduced the severity of atopic dermatitis.	(Amagai *et al.* 2015)
Reduced circulating DGLA levels are related to increased death rate in patients with chronic heart disease.	(Ouchi et al. 2017)

1.7.2 Synthesis and metabolism

DGLA (20:3, n-6) is an ω-6 PUFA carbon metabolised from linolenic acid. (Khan et al. 2019). Linolenic acid is an important fatty acid essential for humans, and to avoid deficiency, about 1-2% of total dietary energy should consist of this fatty acid (Wang and Bennett 2012). DGLA synthesis via the ω-6 pathway is described in detail in Figure 1.12. The ω-6 pathway interacts with the ω-3 pathway because both have a similar set of desaturase enzymes (Calder 2012).

The ω-6 pathway consists of two desaturation steps which are catalysed by desaturase enzymes (Kapoor and Huang 2006). Desaturation step is very slow, which means that the transformation of LA to GLA is limited and may lead to deficiencies of GLA, especially under inflammatory conditions such as arthritis (Kapoor and Huang 2006). Reduced GLA-processing capacity has been related to obesity, hypertension, atopic dermatitis, rheumatoid arthritis, leukaemia and CVD (Fan and Chapkin 1998; Kapoor and Huang 2006; Wang and Bennett 2012). DGLA is also slowly unsaturated to AA, and only a few DGLA molecules are converted into AA (Fan and Chapkin 1998). The direct ingestion of DGLA would therefore bypass the first stage of desaturation and would increase the availability of DGLA, which can be incorporated into the cell membranes and metabolised without significant increases in AA accumulation (Fan and Chapkin 1998).

AA-derived eicosanoids have been reported to be involved in inflammation initiation and chronic inflammation (Bannenberg and Serhan 2010). The 5-LOX metabolite LTB4 and the COX-2 metabolite PGE_2 are efficient leukocyte chemoattractants (Bannenberg and Serhan 2010). However, DGLA also produces two essential protective metabolites: PGE_1 and 15-hydroxy-platelet aggregation, facilitates cell cholesterol efflux and attenuates the biosynthesis of cholesterol together with numerous other anti-inflammatory actions (Horrobin 1991). 5-LOX and 12-LOX are inhibited by 15-HETrE, which thus inhibits the production of pro-inflammatory eicosanoids derived from the activities of these enzymes on AA (Horrobin 1991). Figure 1.12 shows the synthesis of DGLA.

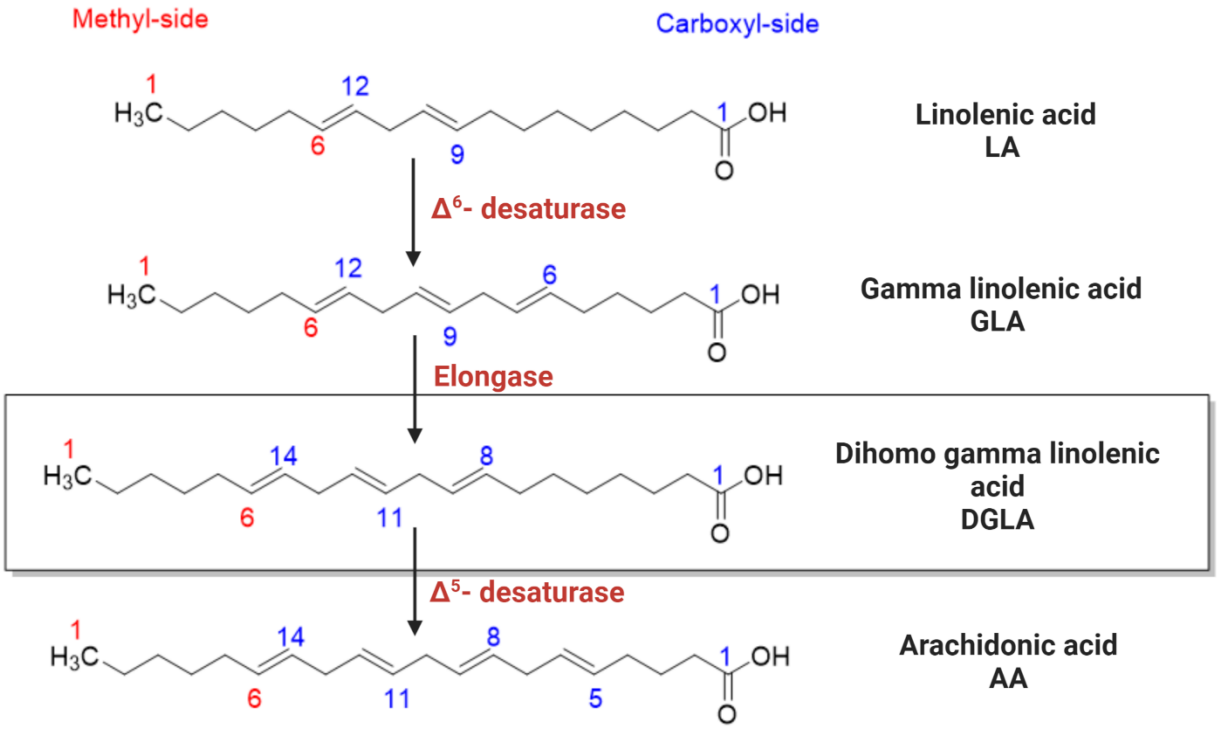


Figure 1. 12 DGLA synthesis. Metabolism of linolenic acid to other ω-6 fatty acids.

1.7.3 Sources of DGLA

The primary sources of omega-3 PUFAs are fish oils. Fish resources are constantly decreasing due to both increased fish consumption and marine pollution (Genot et al. 2016). It is therefore important to study other PUFAs such as DGLA. Previous studies have demonstrated that DGLA is found in vegetable oils, nuts and seeds, while new sources of DGLA have been obtained by

manipulating the fatty acid synthesis pathways in yeast, fungi and algae. These could represent alternative sources for this PUFA in the future.

To produce DGLA, the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) has been genetically manipulated. As a product of the desaturase enzyme Δ9 desaturase, *S. cerevisiae* generates oleic acid. This permits the yeast to transform oleic acid to LA and then DGLA through genetically introducing Δ5 and Δ6 desaturase enzymes (Watanabe et al. 2014). *Parietochloris incisa* (*P. incisa*), a mutant of phototrophic green microalgae, has recently been used in the production of DGLA (Iskandarov et al. 2011). *P. incisa* generates large quantities of AA, but chemical mutagenesis of the Δ5 desaturase gene produced a nonsense mutation (Iskandarov et al. 2011). As a result, the percentage of DGLA in total fatty acids (TFA) increased substantially. In contrast to mutants, wild-type algae produce approximately 1% DGLA and 58% AA as TFA. However, in the mutant strain, the percentage of AA became insignificant, and the percentage of DGLA increased to 32% (Iskandarov et al. 2011). By lipid extraction, DGLA can be purified and consequently used for experimentation. Table 1.7 illustrates the main sources of DGLA from generally modified organisms.

Table 1. 7 Main sources of DGLA.

Microbial sources	Modification	% of DGLA	References
S. cerevisiae	Δ5 and Δ6 desaturase	2.74%	Watanabe et al., 2014
P. incisa	Δ 5 desaturase	~ 30%	Iskandarov et al., 2011

1.8 Project aims

Atherosclerosis is the primary cause of CVD and a leading cause of death in Western societies. As healthcare systems are under increasing financial strain and the disease is economically costly, there is a pressing need to find effective interventions to prevent or treat the disease, especially as the prediction is that CVD events will rise. Currently, statins are the main pharmacological intervention for atherosclerosis, but the maximum improvement achievable by statins is 30%, and not all patients respond to the therapy (Mishra and Routray 2003). Nutraceuticals such as DGLA represent promising alternatives in the prevention and treatment of atherosclerosis but require an in-depth understanding of the mechanisms underlying their actions together with large clinical trials in the future. Atherosclerosis is a chronic condition in which lipids and immune cells accumulate in the wall of the artery. Macrophages are central to the progression of atherosclerosis because they form foam cells that are the hallmark of the initial and latter stages of the disease. This identifies macrophages as a candidate target for intervention. The studies in this project, therefore, focused mostly on macrophages.

The studies in this project also focused on DGLA because of previous promising anti-atherogenic actions in macrophages and mouse model systems (Takai *et al.* 2009a; Gallagher 2016) (Figure 1.13-1.14). Previous studies in the laboratory indicated that the levels of PGE_1 and 15-S-HETrE were increased following incubation of human macrophages with DGLA. In addition, PGE_1 attenuated IFN-γ-induced pro-inflammatory gene expression and monocyte migration, and thus could represent one mechanism by which DGLA produces anti-inflammatory responses. *In vitro* and *in vivo* experiments have also demonstrated that DGLA was incorporated into plasma membranes of macrophages following addition to the cells and was also found in the plasma, liver and kidney lipids following ingestion in supplemented diet in mice (Gallagher 2016). Therefore, intracellular metabolites, particularly PGE_1 and 15-S-HETrE, are potentially involved in mediating DGLA actions. This has been supported by limited previous studies on PGE_1 where, like DGLA, it was found to attenuate monocytic migration and pro-inflammatory gene expression. However, no work was performed on 15-S-HETrE, and therefore this forms the focus of the current project together with a more in-depth study on the actions of PGE_1. Moreover, as an independent project, Dr. Jessica Williams in the laboratory was investigating plaque burden and cellular content along with the plasma lipid profile in $LDLr^{-/-}$ mice fed HFD alone or HFD supplemented with DGLA for 12 weeks (progression study) and following switching to chow diet for 4 weeks or to chow diet supplemented with DGLA for 4 weeks (regression study). The availability of these mice provided

opportunities to expand research from *in vitro* to an *in vivo* context by investigating the impact of DGLA on haematopoietic stem and progenitor cell profile in the bone marrow (Figure 1.15).

Overall, the project had four aims:

1. To analyse the effects of DGLA, 15-S-HETrE and PGE_1 on several processes associated with atherosclerosis *in vitro*. This objective expands on previous work on DGLA in areas such as monocytic migration, foam cell formation and cytokine-induced pro-inflammatory gene expression *in vitro*;

2. To determine DGLA-mediated changes in gene expression in human macrophages and to compare them to those produced by 15-S-HETrE and PGE_1. This involves the use of reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)-based atherosclerosis arrays;

3. To study the mechanisms of DGLA and metabolites using bone marrow-derived macrophages from wild-type mice and 12/15-Lox knockout mice together with use of pharmacological inhibitors. This objective expands on the role of the cyclooxygenase and lipoxygenase pathways in the actions of DGLA in areas such as foam cell formation and cytokine-induced pro-inflammatory gene expression *in vitro*;

4. To investigate the action of DGLA on the haematopoietic stem and progenitor cell profile in the bone marrow of $LDLr^{-/-}$ from both progression and regression studies.

An illustrated overview of the experimental approach is presented in Figures 1.16, 1.17 and 1.18.

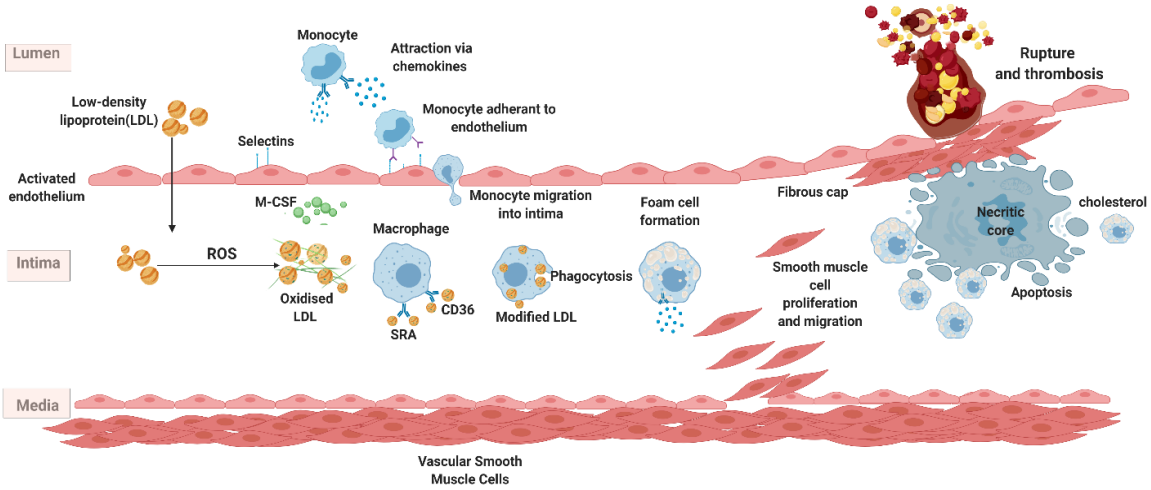
	Endothelial dysfunction	Monocyte recruitment/ foam cell formation	SMC invasion	Plaque stability
				
DGLA	↓Pro-inflammatory cytokine (IFN-γ, IL-1β, TNF-α) induced expression of MCP-1 and ICAM-1 in macrophages *in vitro*. ↓STAT-1 SER727 phosphorylation, involved in activation of IFN-γ signalling in human macrophages *in vitro.*	↓ Monocyte migration. ↓ Macropinocytosis in human macrophages *in vitro.* ↓ OxLDL uptake in human macrophages *in vitro.* ↓ Expression of scavenger receptors SRA1 and CD36 in human macrophages *in vitro.* ↓AcLDL induced cholesteryl ester accumulation. ↑ Cholesterol efflux in human macrophages *in vitro.*		
PGE_1	↓IFN-γ induced MCP-1 and ICAM-1 expression in human macrophages *in vitro.*	↓ Monocyte migration.		

Figure 1. 13 Summary of previous *in vitro* experiments.
Abbreviations: acLDL, Acetylated LDL; CCR2, C-C chemokine receptor 2; CCR5, C-C chemokine receptor 5; CD36, Cluster differentiation 36; IFN-γ, Interferon γ; ICAM-1, Intercellular adhesion molecule 1; IL-1β, Interleukin-1; LDL, Low density lipoprotein; MCP-1, Monocyte chemotactic protein 1; M-CSF, Macrophage colony stimulating factor; oxLDL, Oxidised LDL; ROS, Reactive oxygen species; SMC, Smooth muscle cell; SRA, Scavenger receptor A; SRB1, Scavenger receptor B1; STAT-1, Signal transducer and activator of

transcription-1 TNF-α,Tumour necrosis factor; VCAM-1, Vascular cellular adhesion molecule 1; VSMC, Vascular smooth muscle cells. Created with BioRender.com.

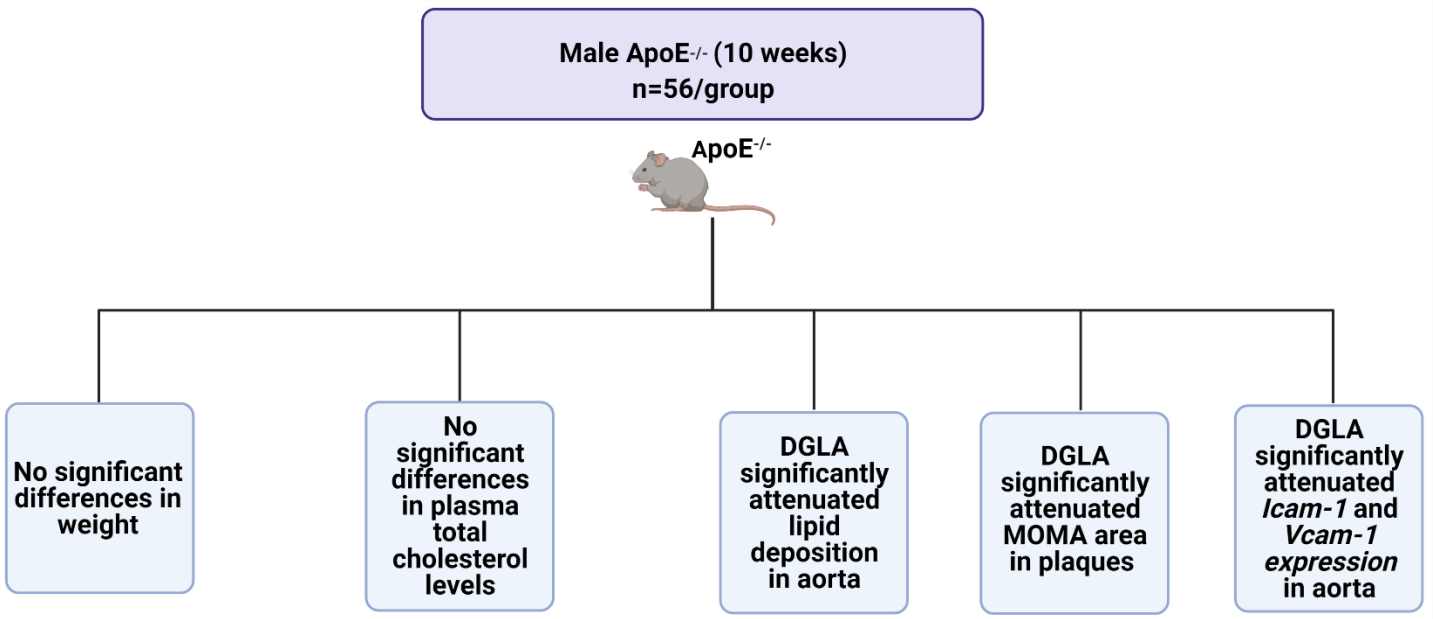


Figure 1. 14 Summary of previous *in vivo* progression studies.
(Takai *et al.* 2009b). Created with BioRender.com.

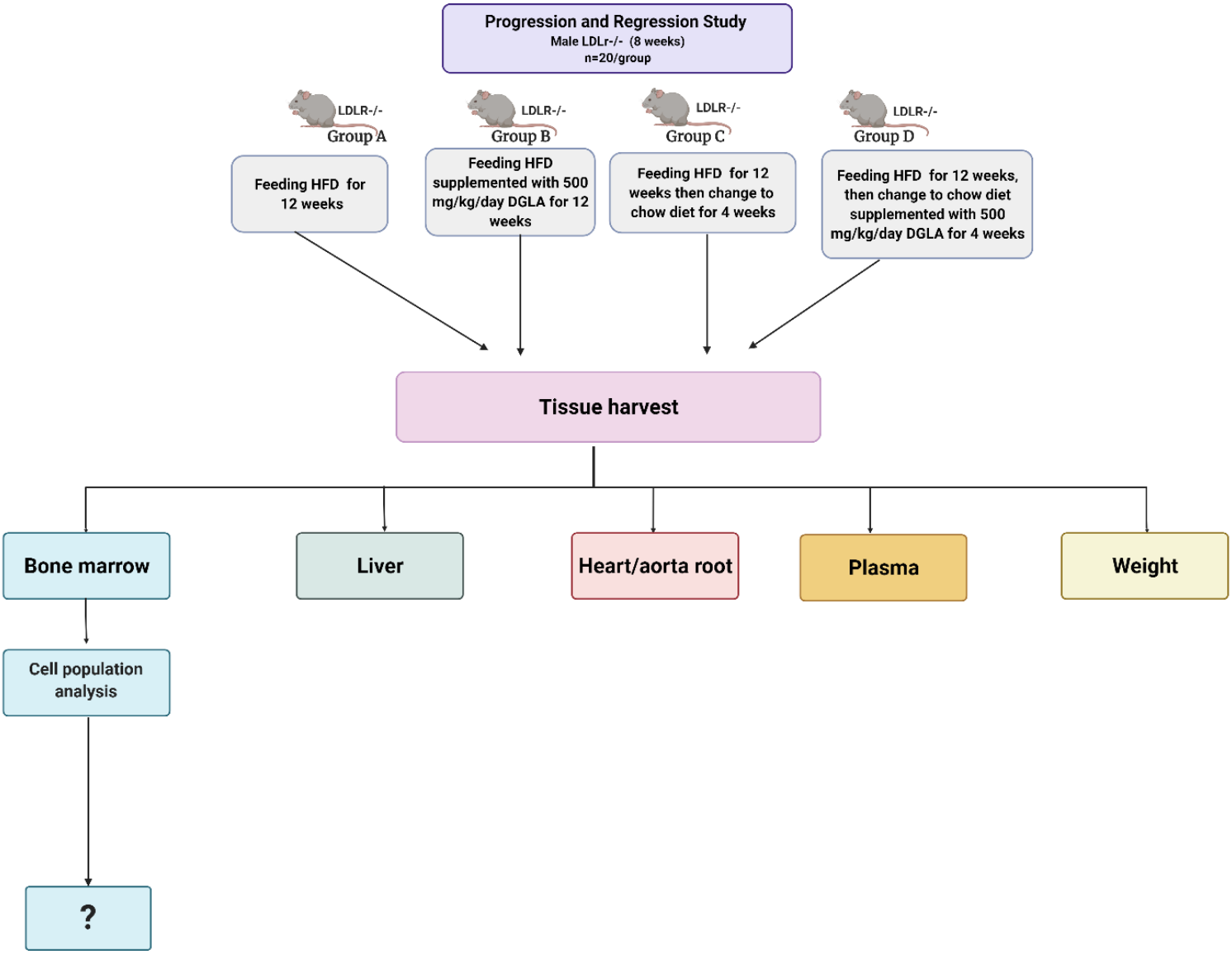


Figure 1. 15 Summary of *in vivo* study.
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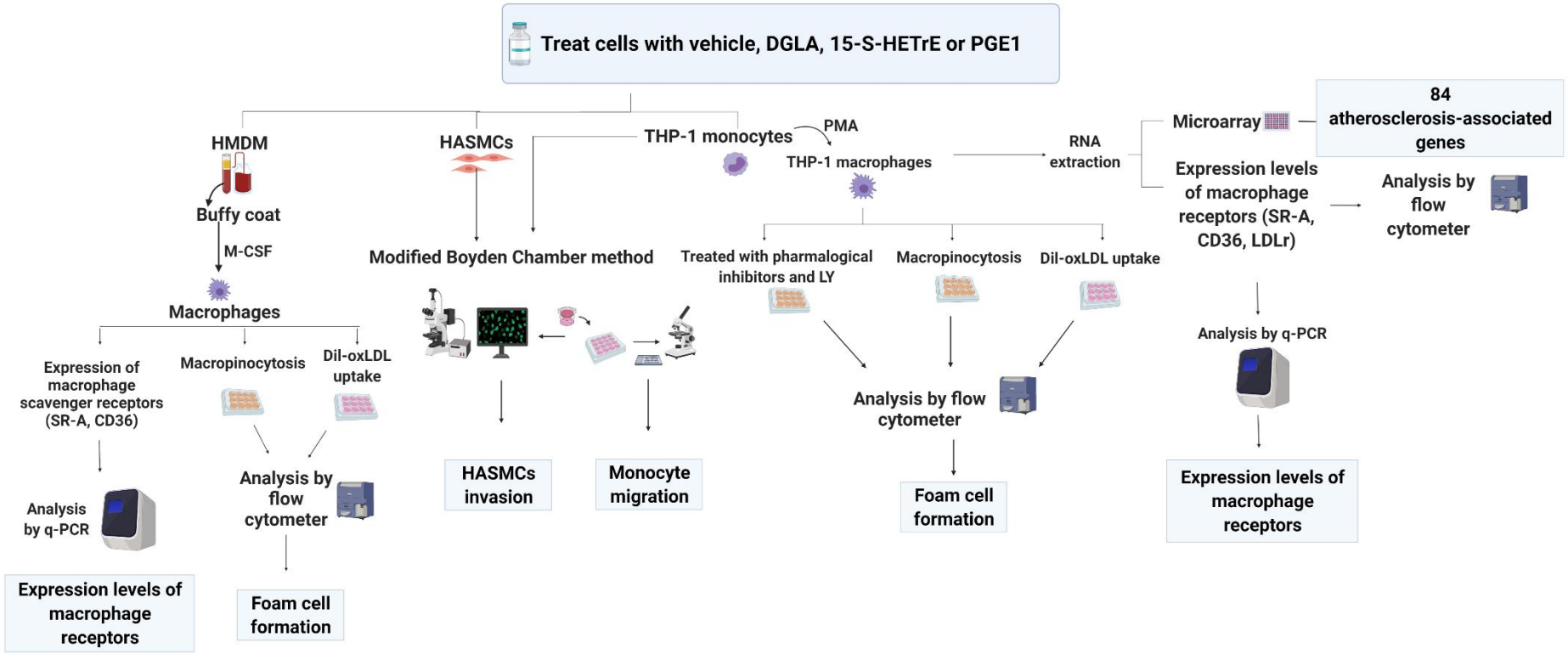

Figure 1. 16 Experimental strategy used to assess the mechanisms of DGLA and metabolites in macrophages and smooth muscle cells *in vitro*.

Abbreviations: BMDM, bone marrow derived macrophage; CD36, cluster differentiation 36; LY, Lucifer yellow; oxLDL, oxidised LDL; RT-qPCR, real time quantitative PCR; SRA, scavenger receptor A. Created with BioRender.com.

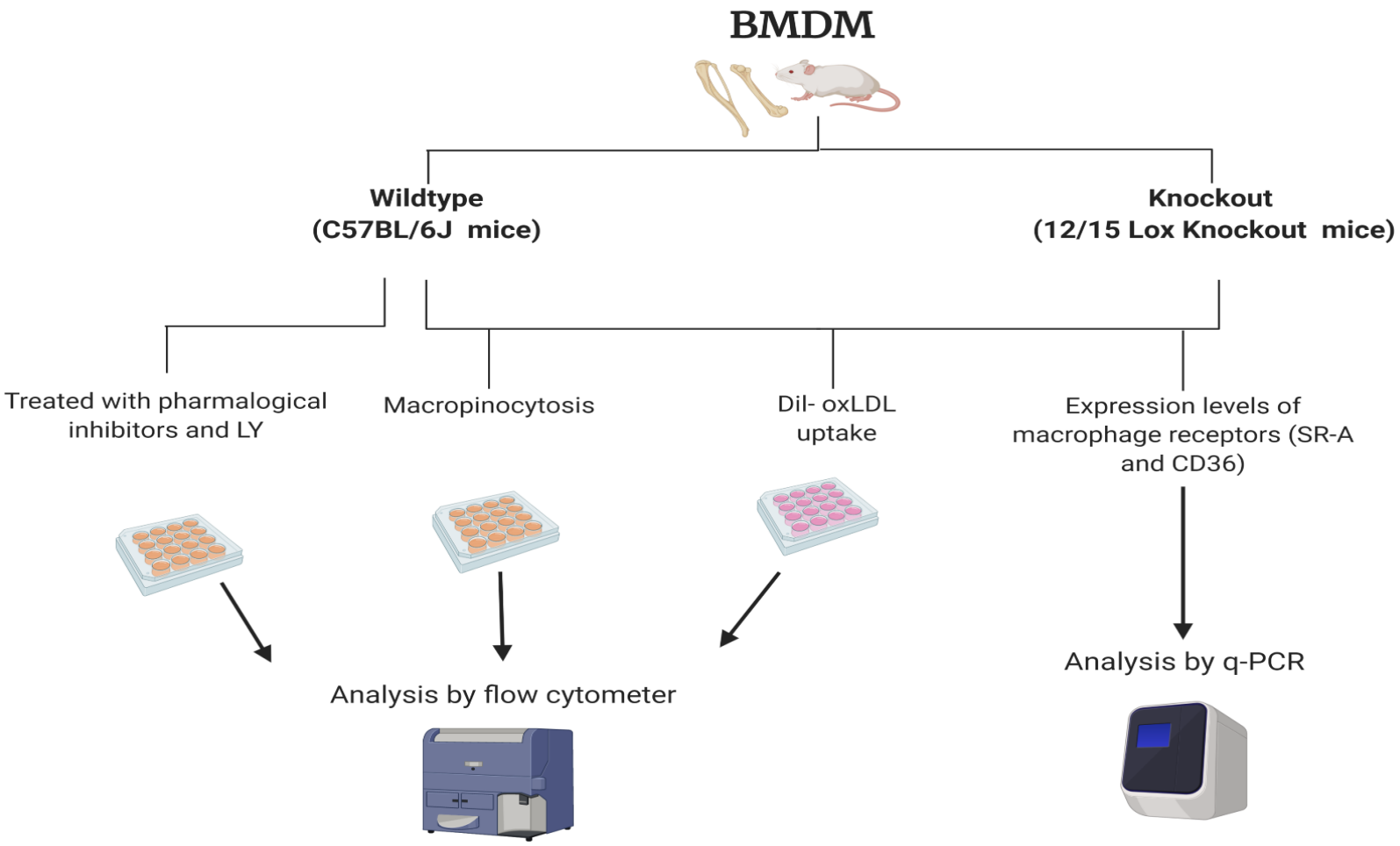

Figure 1. 17 Experimental approach to analyse the impact of DGLA and its metabolites on key atherosclerosis-associated cellular processes using BMDM from C57BL/6J and 12/15 Lox knockout mice.

Abbreviations: CD36, cluster differentiation 36; DGLA, dihomo-ɣ-linolenic acid; LY, Lucifer yellow; oxLDL, oxidised LDL; RT-qPCR, real time quantitative PCR; SRA, scavenger receptor A. Created with BioRender.com.

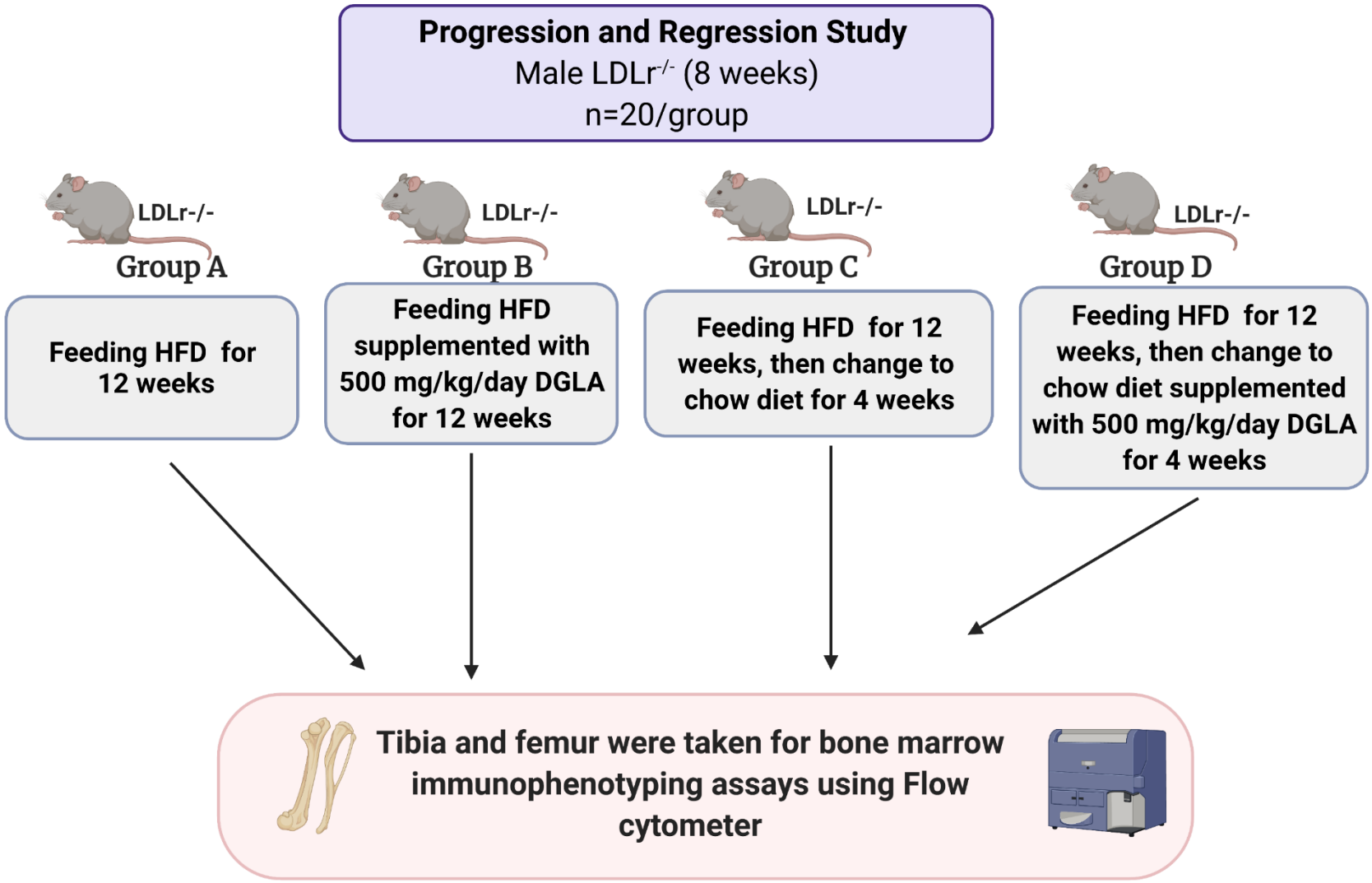

Figure 1. 18 Experimental strategy for the profiling of haematopoietic stem and progenitor cell populations in the bone marrow. Created with BioRender.com.

Chapter 2 Materials and Methods

2.1 Materials

Table 2. 1 List of materials and reagents used.

Company	Reagent
Abcam, UK	Anti-rabbit secondary antibody IgG- Alkaline phosphatase (AP) Anti-goat secondary antibody IgG-AP
BD Bioscience, UK	40/70 µm sterile cell strainer FITC-conjugated anti-mouse CD34
Biolegend, USA	Allophycocyanin (APC)-conjugated anti-mouse c-Kit APC/Cy7-conjugated anti-mouse Sca-1 Brilliant Violet (BV)650-conjugated anti-mouse CD127 FITC-conjugated anti-mouse CD48 Phycoerythrin (PE) -conjugated anti-mouse Sca-1 PE/ Cyanine7(Cy7)-conjugated anti-mouse CD150 PE/Cy7-conjugated anti-mouse
Biotrend, Germany	Dil-oxLDL
Cayman Chemicals, USA	15-(S)-HETrE PGE_1
Fisher Scientific, UK	Agarose Chloroform 4', 6-diamidino-2-phenylindole (DAPI) nuclear stain Dulbecco's Modified Eagle's Medium (DMEM) Methanol Nuclease-free water Scalpel
Helena Biosciences, UK	Cell scraper
Life Technologies, UK	Foetal calf serum (FCS) qPCR plate seals Tris-borate EDTA (TBE) Penicillin Streptomycin
Lonza, UK	RPMI 1640 with L-Glutamine medium
Marienfeld, UK	Haemocytometer

NU-CHEK, USA	DGLA
Nycomed Pharmaceuticals, Switzerland	Lymphoprep™
Peprotech, UK	IFN-γ MCP-1 M-CSF PDGF-BB
Promega, UK	dNTPs MMLV reverse transcriptase MMLV RT 5x buffer Random primers RNasin ribonuclease inhibitor
Qiagen, UK	Atherosclerosis RT^2 Profiler PCR Arrays (human)
Roche, UK	Roche light cycler
Sigma-Aldrich, UK	10/25 mL pipettes 12/24/96-well plates 15/50 mL Falcon tubes Apolipoprotein A-I Bovine serum albumin (BSA) Cell culture plates Crystal violet Dimethyl sulfoxide (DMSO) Ethanol Ethidium bromide Ethylenediaminetetraacetic acid (EDTA) Indomethacin Isopropanol Lucifer yellow CH dipotassium salt (LY) Pharmacological inhibitor ML350 PCR primers Phosphate buffer saline (PBS) tablets Red blood cells lysis buffer RNA Zap Sodium hydroxide Smooth muscle cells SYBR Green Jumpstart Taq polymerase

	THP-1 cells Tissue culture flasks (25/75 cm^2) Tris-borate EDTA (TBE) Trypsin EDTA
Special Diets Services, Jackson Laboratory UK	High-fat diet [21% (w/w) pork lard and 0.15% (w/w) cholesterol]
Animal Facility at School of Medicine, Cardiff University	WT C57BL/6J 12/15 Lox knockout mice
Starlabs, UK	qPCR 96-well plates
Helena Biosciences, UK	Haemocytometer with a 5 x 5 grid
Fisher Scientific, UK	2x RNA loading dye. Gel sample buffer (GSB) Nanodrop™ ND2000 Paraformaldehyde (PFA) PBS Tween Pierce lactate dehydrogenase (LDH) cytotoxicity assay kit RiboRuler high range RNA ladder RiboZol™ RIPA buffer Streptavidin
VWR Jencons, UK	Falcon® 12-well companion plates Falcon® cell culture inserts (8 µm pore size) Glass slides

Abbreviations: AP, alkaline phosphatase; APC, allophycocyanin; BSA, bovine serum albumin; Cy7, cyanine7; DAPI, 4', 6-diamidino-2-phenylindole; DGLA, dihomo-γ-linolenic acid; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide; dNTPs, deoxynucleoside triphosphate; EDTA, ethylenediaminetetraacetic acid; FCS, foetal calf serum; FITC, fluorescein isothiocyanate; GSB, gel sample buffer; HETrE, hydroxyeicosatriaenoic acids; IFN, interferon; IL, interleukin; LDLr, LDL receptor; MCP-1, monocyte chemotactic protein-1; LY, Lucifer yellow; M-CSF, macrophage colony-stimulating factor; MMLV, Moloney murine leukemia virus; PDGF, platelet-derived growth factor; PE, phycoerythrin; PFA, paraformaldehyde; PG, prostaglandin; qPCR, quantitative PCR; LDH, lactate dehydrogenase; RNA, ribonucleic acid; RIPA buffer, radioimmunoprecipitation assay buffer; TBE, Tris-borate EDTA.

2.2 Methods

2.2.1 Cell culture

2.2.1.1 The human acute monocytic leukaemia cell line THP-1

THP-1 is widely utilised in the investigation of atherosclerosis and associated cellular pathways involving monocytes/macrophages (Qin 2012). THP-1 monocytes were plated in 75cm^2 tissue culture flasks with RPMI medium supplemented with 10% (v/v) heat-inactivated foetal calf serum (HI-FCS) (56ºC for 30 minutes) along with penicillin (100 U/mL) and streptomycin (100 µg/mL) (pen/strep) (complete medium). The cells were split at a ratio of approximately 1:30, and cells between passage 1 and 10 were used at a concentration of 1 x 10^6 cells per mL unless otherwise stated. Incubation of the cells was performed at 37ºC in a humidified 5% CO_2 (v/v) incubator.

Differentiation of THP-1 monocytes into macrophages was carried out by incubation of the cells for 24 hours using 160 nM phorbol 12-myristate 13-acetate (PMA). Differentiated THP-1 macrophages were incubated with agents such as DGLA and vehicle control as required.

2.2.1.2 Human aortic smooth muscle cells (HASMCs)

HASMCs play a key role in atherosclerosis, particularly migration from the media to the intima, where they produce plaque-stabilising ECM (Jiang et al. 2009; Boehme et al. 2018), and are hence used extensively in research. HASMCs were cultured in 15 mL Smooth Muscle Cell Growth Medium (Sigma-Aldrich) in 75cm^2 flasks at 37°C, 5% (v/v) CO_2. When the cells reached approximately 80% confluency, they were washed with Hanks Balanced Salt Solution (HBSS). Then, the cells were incubated briefly in 8 mL of 0.05% (w/v) trypsin-EDTA in the flask to dissociate the cells. The dissociated cells were placed in 8 mL of fresh DMEM. The cells were pelleted by centrifugation at 250 x g for 5 minutes at room temperature (RT) and the pellet was resuspended in 1 mL of fresh culture medium for cell counting using a haemocytometer.

2.2.1.3 Primary human monocyte-derived macrophages (HMDM)

THP-1 monocyte-derived macrophages demonstrate the characteristics of primary human macrophages, with experimental findings generally conserved between THP-1 macrophages and HMDM (McLaren et al. 2010). However, to confirm that the results were not due to the modified nature of cell lines, key findings were confirmed in HMDMs. Human blood buffy coats

were collected from the National Blood Service Wales. Ethical approval was granted by the National Blood Service Wales for use of such buffy coats in research. White blood cells were prepared by adding 25 mL of buffy coat to 15 mL of Lymphoprep™ on the top of 50 mL Accuspin™ separation tubes. The tube was then centrifuged for 1 minute at 800 x g to move Lymphoprep to the bottom. Centrifugation of the tubes was then carried out for 30 minutes at 250 x g at RT to remove the plasma layer and collect the peripheral blood mononuclear cell (PBMC) layer. The layer was transferred to a new 50 mL Falcon tube, and an equal volume of cold 0.4% (w/v) tri-sodium citrate in PBS was added, followed by centrifugation for 7 minutes at 350 x g at 4°C to lyse any red blood cell contamination. Then, the cells were resuspended in 10 mL of ice-cold PBS and centrifuged again for 7 minutes at 250 xg at 4°C. This step was repeated 4-6 times to remove any contaminating platelets. The cells were then resuspended in 30 mL of complete RPMI-1640 medium containing 10% (v/v) HI-FCS and pen/strep and counted. To differentiate the monocytes to macrophages, the cells were incubated with HM-CSF (20 ng/mL) at 37°C for 10 days. Before starting the experiments, the cells were washed extensively 3 times with complete RPMI medium to remove all unattached cells and debris.

2.2.1.4 Isolation of bone marrow-derived macrophages (BMDM)

The bone marrow of the leg bones of mice was collected from mice using the tibiae and femurs. Muscles and cartilage were carefully removed from the bones after the isolation of the mouse tibiae and femur before sterilising them with 70% (v/v) ethanol. The ends of the bone were cut, and the bone marrow flushed 2–3 times with complete RPMI medium [containing 2 mM L-glutamine supplemented with 10% (v/v) HI-FCS and 100 U/mL penicillin/100 µg/mL streptomycin (pen/Sep)] using a syringe with 25G needle. The mixture was centrifuged at 250 xg for 5 minutes and the pellet resuspended in 1 mL of red blood cell lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4) for less than 1 minute. The solution was then topped up to 10 mL with RPMI complete medium. The clumped cell debris was removed by passing the cell suspension through a 40 µm cell strainer before centrifuging once again as described above. The pellet was resuspended in 20 mL of differentiation medium [DMEM containing 10% (v/v) HI-FCS and 100 U/mL penicillin, 100 µg/mL streptomycin, and 20 ng/mL M-CSF] following three times washing with RPMI only after centrifugation steps as above. The cells were plated in 5 mL of differentiation medium in four 100 mm^2 plates. After three days incubation at 37°C and 5% (v/v) CO_2, 10 mL of differentiation medium was added to each plate. On day six of incubation, the cells were washed three times with PBS to remove any non-adherent cells. The cells were harvested from the plate by scraping the monolayer of the cells with a cell scraper, which were then centrifuged at 250 xg for 5 minutes at RT.

Macrophages were counted as outlined further in Section 2.2.2.8 and plated in complete DMEM medium containing 10% (v/v) HI-FCS, 100 U/mL penicillin and 100 µg/mL streptomycin without M-CSF as needed for experiments.

2.2.2 Sub-culturing of cells

2.2.2.5 THP-1

THP-1 cells were cultured in 75cm^2 tissue culture flasks until they reached 60% confluency (about 6 x 10^5 cells per mL). The cell suspension was then transferred into a polypropylene tube (Falcon) and centrifuged at room temperature at 110 xg for 5 minutes. The pellet was resuspended in 5 mL of fresh complete medium containing 10% (v/v) HI-FCS and pen/strep. The cells were split at a ratio of around 1:30. Cells between passage 1 and 10 were typically used for experiments.

2.2.2.6 Freezing down cell lines

The cells were centrifuged at 110x g for 5 minutes and resuspended in HI-FCS with 10% (v/v) DMSO. Cells were then transferred into 1 mL cryotubes (about 5 x 10^6 cells/tube) and placed in a Nalgene™ Cryo 1ºC freezing tube at 1˚C overnight at -80˚C. The cell stocks were then transferred to liquid nitrogen for long-term storage. The freezing of cells was only carried out when they were passage 3 or less.

2.2.2.7 Growing up frozen cell lines

The cell stock from liquid nitrogen was defrosted in a 37˚C water bath. At RT, the cells were then transferred to 10 mL of HI-FCS and centrifuged at 110 x g for 5 minutes. Cell pellet was resuspended in a pre-heated complete culture medium.

2.2.2.8 Counting cells

A haemocytometer with a 5 x 5 grid (Helena Biosciences, UK) was used to count cells. The pelleted cells were resuspended in 5 mL of fresh complete medium containing 10% (v/v) HI-FCS and pen/strep. After that, coverslip was placed on the slide and 7.5 μl of cell suspension was introduced by capillary action into the counting space. An average number of cells within the four outer 5 x 5 grids of the haemocytometer were counted and the total numbers of cells calculated by multiplying the average by 10^4.

2.2.3 Cellular assays

2.2.3.1 Lactate dehydrogenase (LDH) assay

LDH is a cytosolic enzyme that can be released into the medium following damage to the cells. The LDL in the medium can be assayed using the LDL assay kit, in which LDH catalyses a reaction that reduces NAD^+ to NADH. Diaphorase in the assay uses the latter to reduce tetrazolium salt (INT) to a red product that can be measured by monitoring the absorbance at 490 nm. The concentration of LDH is directly proportional to the amount of red product formed. The cells were seeded in 96 well plates at a density of 140,000 cells in complete RPMI medium. Then the cells were treated with vehicle (0.1% DMSO) or different concentrations of agents (DGLA, 15-(S)-HETrE and PGE_1) for 24 hours. A positive control was included, which involved the addition of a cell lysis buffer (provided in the kit) to achieve 100% cellular lysis following incubation at 37ºC for 45 minutes. The negative control contained medium alone for background reading. At the end of the experimental period, the supernatant (50 µL) was transferred into a separate well of a 96-well plate and mixed with an equal volume of LDH assay buffer (supplied with the kit) followed by incubation for 30 minutes at 37ºC. The absorbance was then read at 490 nm on a Dynex Technology MRX Model 680 Microplate reader from BioRad. Negative control values were subtracted from the other readings and changes in viability expressed as fold-change in comparison to the control.

2.2.3.2 Crystal violet

Crystal violet stain is an effective technique for the quantification of cell numbers that therefore provides indication of cell proliferation. In this process, crystal violet dye binds to proteins and DNA in adhered cells. The stain is released in solution following cell lysis, where the level of

purple stain correlates with DNA quantity and consequently to the cell number. The cells were cultured according to the instructions in Section 2.2.3.1. The cells were washed with 300 µL of PBS after the supernatant was removed from the original 96-well plate for the LDH assay. The cells were stained with crystal violet solution (0.2% (w/v) crystal violet in 10% (v/v) ethanol) for 5 minutes at RT. The cells were washed three times with 1x PBS followed by the addition of 250 µL of solubilisation buffer (0.1 M NaH_2PO_4 in 50% (w/v) ethanol). The plate was left for 5 minutes at room temperature on a rocking platform before determining the absorbance at 590 nm using a Bio-Rad microplate reader. The results were calculated as a percentage of the control conditions (i.e., vehicle).

2.2.3.3 Monocyte migration

In this method, the migration of THP-1 monocytic cells in response to the chemoattractant MCP-1 was compared in the presence of the vehicle control or DGLA,15-(S)-HETrE or PGE_1. This experimental setup contained a cell insert with 8 µm pore size (to mimic the endothelial cell layer of arteries) placed within a 12-well companion plate (modified Boyden chamber) (Figure 2.1). Undifferentiated THP-1 monocytes (5 x 10^5 cells) in complete RMPI medium supplemented with 10% (v/v) FCS and pen/strep together with vehicle or appropriate concentration of agent (DGLA ,15-(S)-HETrE or PGE_1) were placed in the upper chamber. The bottom chamber contained 0.5 mL of complete RMPI medium supplemented with 10% (v/v) FCS, pen/strep and the chemoattractant MCP-1 (20 ng/mL) (refer to Figure 2.1 for experimental setup). After 3 hours incubation at 37°C and 5% (v/v) CO_2, the medium in the upper chamber was removed and discarded. The underside of the membrane was then washed with PBS to ensure that all the migrated cells were washed into the medium underneath. This was then centrifuged at 110 x g for 5 minutes at room temperature to pellet the cells, which were then resuspended in 1 mL of PBS. The numbers of migrated monocytes were counted using a haemocytometer. Monocyte migration was expressed as a fold-change compared to the total input cells.

2.2.3.4 HASMC Invasion

The impact of DGLA and its metabolites on the invasion of SMCs was evaluated using HASMCs in a modified Boyden chamber like that required for monocyte migration. This assay was carried out using a cell insert with 8 µm pore size coated with Matrigel (Sigma-Aldrich) for

30 minutes at 37°C to allow the gel to set. SMC in complete DMEM supplemented with 10% (v/v) FCS and pen/strep (1 x 10^5 cells) were added to the upper layer (i.e., above the membrane) and returned to the incubator to allow the cells to adhere. After incubation for 2 hours at 37°C to allow the cells to settle down, the medium was changed to 500 µL of serum-free DMEM and the incubation carried out for 48 hours at 37°C. The serum-free DMEM was then replaced with 500 µL of vehicle control (negative control) or with PDGF (20 ng/mL: vehicle control and other wells) or DGLA, 15-(S)-HETrE or PGE_1 in serum-free DMEM. The lower chamber contained DMEM supplemented with 10% (v/v) FCS and pen/strep together with the chemoattractant 20 ng/mL PDGF-BB. Following incubation for 4 hours at 37°C, the inserts were removed from the wells and the cells on the upper side of the membrane were removed using cotton swabs. The membranes were cut and stained with DAPI. The number of cells in 5 different fields of view were counted to get an average sum of cells that had migrated through the membrane, and migration was calculated as a percentage of cells added to the cell culture insert.

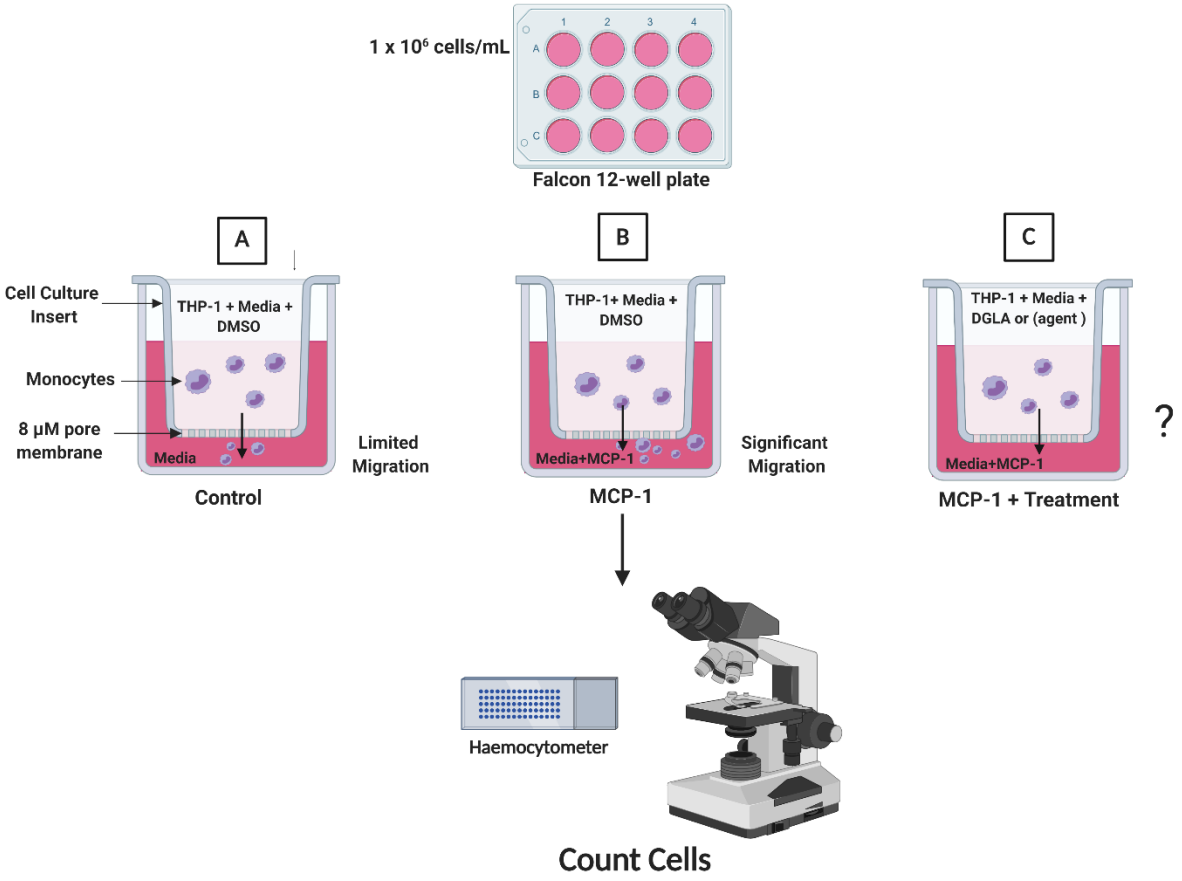

Figure 2. 1 Experimental setup for migration assays.
THP-1 monocytes were suspended in either vehicle control or agent and added to the cell culture inserts (upper chamber). Cells can migrate through the 8 µM pore membrane to the bottom layer (lower chamber) in the presence of MCP-1. Migrated cells in the lower chamber were counted and fold-change in migration calculated. (A) Negative control representing physiological migration in the absence of chemoattractant stimulus, with cells plus vehicle in the upper chamber and medium only in the lower chamber. (B) Vehicle control with cells plus vehicle in the upper chamber and medium plus MCP-1 in the lower chamber. (C) Treatment wells with cells plus DGLA, PGE_1 and15-(S)-HETrE in the upper chamber and medium plus MCP-1 in the lower chamber. Created with BioRender.com.

2.2.4 RNA/DNA techniques

2.2.4.1 Cytokine stimulation

The cells were incubated with vehicle or appropriate concentration of agent for 24 hours in complete RPMI medium. IFN-γ (250 U/mL) was added to some cells for the desired length of time to induce the expression of the target genes (Gallagher 2016).

2.2.4.2 RNA isolation

Since mRNA is produced from activated genes, the quantity of target mRNA can be calculated to evaluate the levels of gene expression. The experimental setup is illustrated in Figure 2.2. RNA was prepared using RiboZol™ (1 mL), a single-phase phenol, with homogenisation of the samples by passing the lysate through a pipette several times according to the manufacturer's instructions (Sigma-Aldrich). Samples were left for 10-15 minutes at room temperature and then 200 µL of chloroform was added per 1 mL of RiboZol™ and mixed vigorously. Then, the samples were incubated for 2-3 minutes at room temperature and centrifuged at 12,000 xg for 20 minutes at 4°C. The colourless upper aqueous phase, containing the RNA, was then carefully transferred into a new tube. RNA was precipitated by adding 0.5 mL of isopropanol per 1 mL of RiboZol™. Samples were incubated overnight at -80°C and then centrifuged at 12,000 xg for 20 minutes at 4°C to pellet the RNA. The isopropanol was decanted carefully from the tube without disturbing the RNA pellet. The pellet was washed three times with 75% (v/v) ethanol. After the final wash, the pellet was left to air-dry to evaporate any excess ethanol. Dried RNA pellets were dissolved in 30-50 µL of nuclease-free water and incubated for 10 minutes at 56°C to completely re-dissolve the RNA.

The concentration of RNA was determined using the spectrophotometer (Nanodrop™ ND2000) and a small aliquot was also analysed by agarose gel electrophoresis. The absorbance ratios A260/A280 and A230/A260 were used to assess RNA purity, where values of 1.8 – 2.0 were considered acceptable.

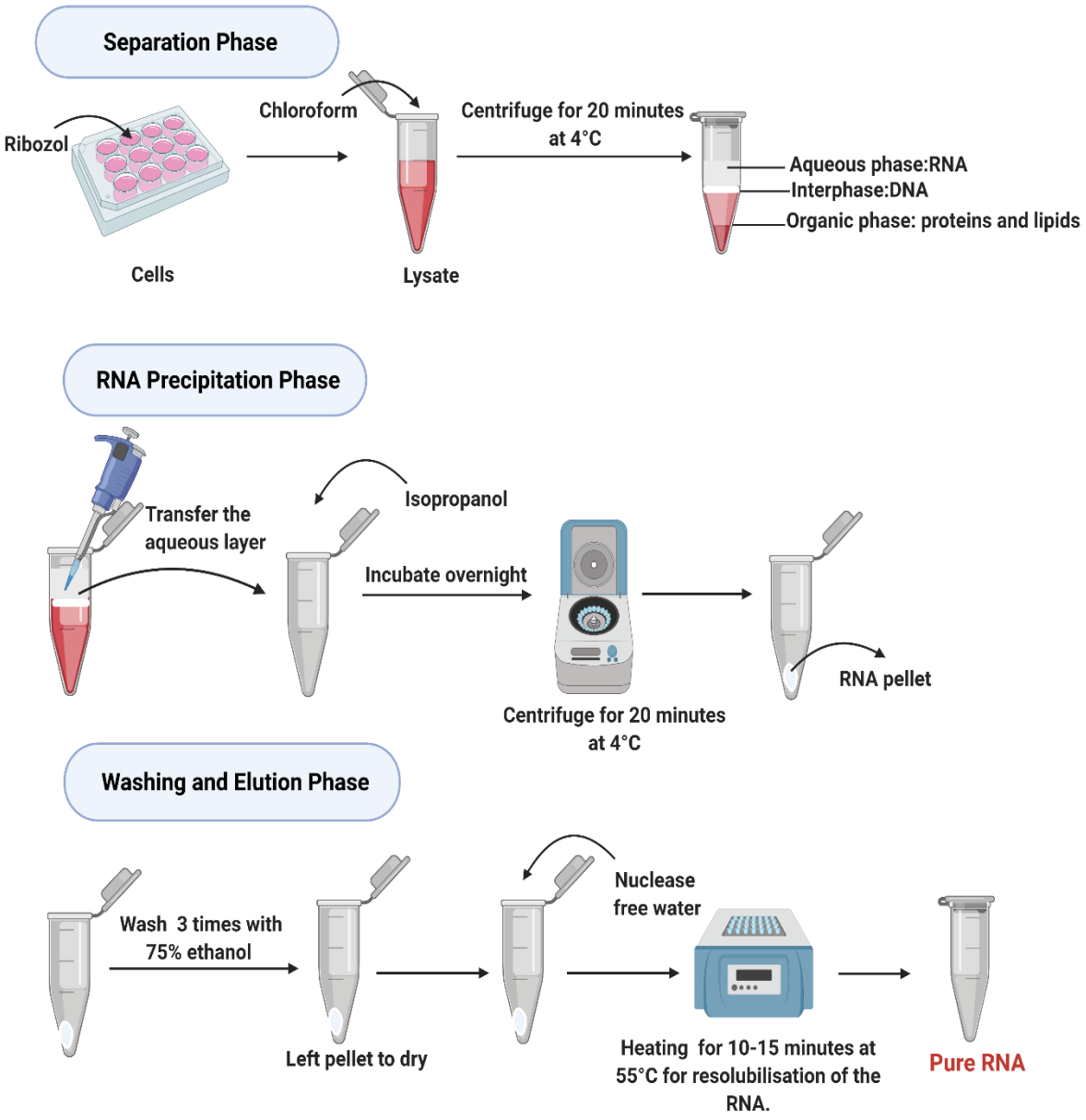

Figure 2. 2 RNA extraction technique.
Description of the steps involved in RNA extraction using RiboZolTM. Created with BioRender.com.

2.2.4.3 Agarose Gel Electrophoresis

A 1.5% (w/v) agarose gel was used to examine the purity of the extracted RNA. Firstly, 1.5 g of agarose was added to 1x Tris/borate/EDTA buffer (TBE; 89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, pH 8.3). This mixture was heated in a microwave oven until the agarose had dissolved, and then 0.5 µg/mL of ethidium bromide was added for visualisation of RNA. Then, 4 µL of RNA samples were mixed with 4 µL of 2x RNA Loading Dye (Thermo Fisher Scientific), loaded onto the wells of the agarose gel and subjected to electrophoresis at 100 V in 1x TBE buffer for 45 minutes. The quality of RNA was examined by the appearance of two distinct bands corresponding to 28S rRNA and 18S rRNA with the 28S rRNA being twice as intense as that for 18S rRNA (i.e., ratio of 2:1) using a Syngene Gel Documentation system with UV light.

2.2.4.4 Preparation of cDNA

For RT-qPCR evaluation, the extracted RNA had to be reverse transcribed into cDNA to examine gene expression. The cDNA synthesis was performed by mixing 1 µg of total RNA (0.5 µg if the total RNA yield was low; the same starting quantity of RNA was used in specific experiments), random hexamer primers (200 pmol), 10 mM of dNTP mix (dATP, dGTP, dTTP, dCTP) and nuclease-free water to make a total volume of 14 µL. Then the mixture was centrifuged at 5,000 x g for 30 seconds to collect the contents at the bottom of the tube. These were then placed on a thermal block at 70˚C for 5 minutes. The tubes were then placed on ice before adding 6 µL of master-mix composed of 200 U of Moloney murine leukaemia virus (M-MLV) reverse transcriptase, 40 U/mL of recombinant RNase inhibitor, and 5X reverse transcription buffer (provided in the cDNA synthesis kit from Promega). Then the tubes were put back on the thermal block and incubated at 37°C for 1 hour. Following quick centrifugation at 5,000 x g for 30 seconds, the reaction was terminated by incubation at 92°C for 2 minutes. A –RT sample (minus reverse transcriptase) was also included as a negative control. Samples were used immediately for RT-qPCR or stored at - 20°C.

2.2.4.5 RT –qPCR

qPCR is a method which enables gene expression quantification by simultaneous amplification and quantification of appropriate cDNA. The quantity of the PCR product produced during the reaction corresponds to starting cDNA concentration within the

exponential PCR amplification cycle. RT-qPCR reactions were performed using SYBR Green Taq Ready mix, which is a jumpstart Taq ready mix that incorporates the Taq Polymerase. Therefore, the reactions require only specific primers (Table 2.2) and a cDNA template.

2.2.4.6 Microarray

Microarrays permit the quantification of several genes in a single experiment. Therefore, more data can be obtained in a shorter timeframe (Efron et al. 2001). The arrays were used because of their high sensitivity, good reproducibility and wide dynamic quantification range. Atherosclerosis RT² ProfilerTM PCR Array (Qiagen) was used to determine the effects of DGLA, PGE_1 and 15-HETrE on the expression levels of a large number of genes related to atherosclerosis. The plate has 84 primers for atherosclerosis-associated genes and 5 housekeeping genes as well as 3 negative, 3 positive and 1 genomic DNA contamination controls (Figure 2.3).

Table 2. 2 Primer sequences used in RT-qPCR.

	Species	Forward sequence (5'-3')	Reverse sequence (5'-3')
BCL2	Human	TCTCCCCAGTCCTAGGTAGC	ACAAGCTAGACACAAAAGGACA
CD36	Human	AGCCATTTTAAAGATAGCTTTCC	AAGCTCTGGTTCTTATTCACA
GAPDH	Human	CTTTTGCGTCGCCAGCCGAG	GCCCAATACGACCAAATCCGTTGACT
ICAM1	Human	GACCAGAGGTTGAACCCCAC	GCGCCGGAAAGCTGTAGAT
LPL	Human	GAGATTTCTCTGTATGGCACC	CTGCAAATGAGACACTTTCTC
SR-A	Human	GTCCAATAGGTCCTCCGGGT	CCCACCGACCAGTCGAAC
VCAM1	Human	GTTGAGATCTCCCCTGGACC	GGATTCACAGCCATGACAC

Abbreviations: CD36, cluster differentiation 36; BCL2, B cell leukaemia/lymphoma; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MCP-1, monocyte chemotactic protein-1; ICAM-1, intercellular adhesion molecule-1; SR-A, scavenger receptor-A; LPL, lipoprotein lipase; ACAT1, acyl-CoA acetyltransferase 1.

Samples (25 µL) were prepared in a 96-well plate reaction mix as detailed in Table 2.3.

Table 2. 3 Reaction of 1X master mix for RT-qPCR and microarray reactions using SYBR® Green.

Reagent	1x Master Mix for regular qPCR (µL)	1x Master Mix for Microarray (µL)
SYBR® Green	12.5	12.5
Forward primer (10 ng/µL)	1	-
Reverse primer (10 ng/µL)	1	-
cDNA	2	2
Nuclease-free water	10.5	12.24

Standard RT-qPCR was performed on a Roche light cycler. A typical “run” consisted of 2 minutes pre-incubation at 94°C followed by 40 cycles of three-step amplification (denaturation at 95°C for 30 seconds, annealing at a varying temperature depending on the primers for 60 seconds and elongation at 72°C for 60 seconds). However, for qPCR microarrays, the amplification process involved only two steps, melting and annealing and extension, over 45 cycles. This was followed by melting curve analysis (95°C for 10 seconds, 65°C for 60 seconds and 97°C for 1 second). The three-step amplification is detailed in Table 2.4. The fold changes in expression were determined using $2^{-(\Delta Ct1-\Delta Ct2)}$, where ΔCt represents the difference between the threshold cycles (Ct). The primers used in this study were specifically designed to span an intron using the Primer Blast web site (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers have been optimised previously for other studies in the laboratory. GAPDH was chosen as a suitable control gene for normalisation of the data as its expression has been found to be independent of the experimental conditions in human macrophages in previous studies.

Table 2. 4 RT-qPCR and microarray reactions conditions using SYBR® Green.

	RT-qPCR			qPCR microarray		
Stage	Temperature (°C)	Duration (seconds)		Temperature (°C)	Duration (seconds)	
Preincubation	94	120		95	600	
Denaturation	95	30	X40	95	15	X45
Annealing	60	60		60	60	
Primer extension	72	60				

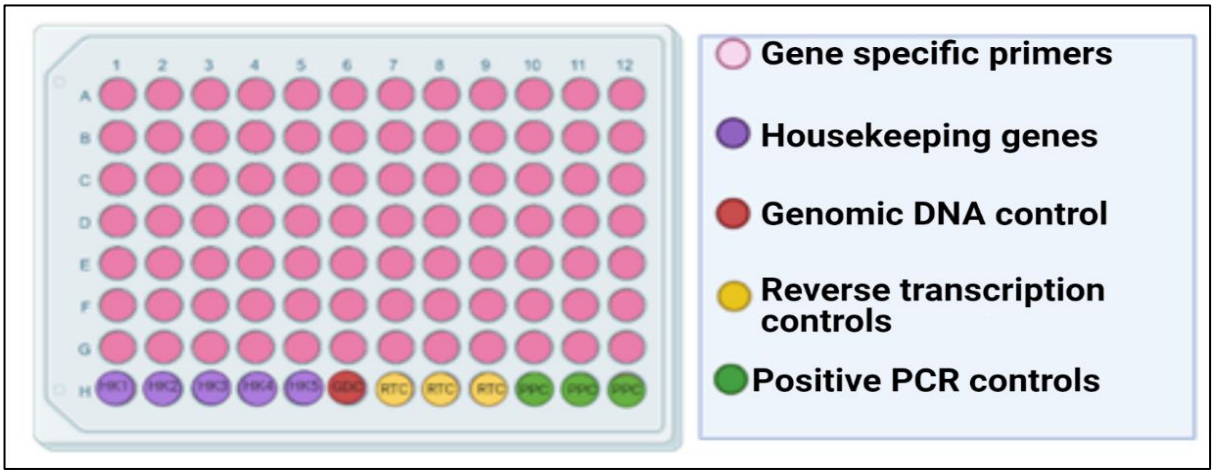

Figure 2. 3 Atherosclerosis-associated gene array plate layout.
Created with BioRender.com.

2.2.5 Lipid methods

2.2.5.1 Dil-oxLDL uptake

An important stage in the transformation of macrophages into foam cells is the uptake of modified lipoproteins, including oxLDL (Xia et al., 2013). Dil-labeled oxLDL (Dil-oxLDL), a fluorescent label, has been used to quantify the uptake of oxLDL by macrophages (Xu et al., 2010). THP-1 monocytes were differentiated to macrophages and then incubated for 24 hours with vehicle (DMSO) or the required concentration of agent (DGLA, 15-(S)-HETrE or PGE_1) in complete RPMI medium containing 10% (v/v) HI-FCS and pen/strep. After incubation, the

medium was changed to RPMI medium supplemented with 0.2% (w/v) fatty acid-free BSA and the cells were treated with 5 µg/mL Dil-oxLDL for 24 hours. Then the medium was removed, and the cells were detached from the plastic plate using 0.05% (w/v) Trypsin-EDTA for 30 minutes. The detached cells were transferred into Eppendorf tubes and subjected to centrifugation at 9,000 xg for 5 minutes at room temperature to pellet the cells. Dil-oxLDL uptake was analysed by flow cytometry on a BD FACS Canto flow cytometer after the pellet was resuspended in 2% (w/v) Paraformaldehyde (PFA) with at least 10,000 events counted for each sample.

2.2.5.2 Macropinocytosis

Lucifer yellow CH dilithium salt (LY) is a fluorescent dye which can enter the cells through the macropinocytosis pathway (McLaren et al., 2010, Lim and Gleeson, 2011). The cellular uptake of LY is therefore often used as an indicator of macropinocytosis (Lim and Gleeson, 2011). THP-1 macrophages were incubated with vehicle or appropriate concentration of agent for 24 hours in complete RPMI medium containing 10% (v/v) HI-FCS and pen/strep. The medium was then removed, and the cells incubated for 24 hours with 100 µg/mL LY in RPMI medium supplemented with 0.2% (v/v) fatty acid-free BSA. Then, the medium was discarded, and the cells detached from the plastic surface using 0.05% (w/v) Trypsin-EDTA. Cells were transferred into Eppendorf tubes and subjected to centrifugation at 9,000 xg for 5 minutes at room temperature to pellet the cells. The supernatant was removed, and the pellet was resuspended in 2% (w/v) paraformaldehyde (PFA). LY uptake was analysed by flow cytometry on a BD FACS Canto flow cytometer with at least 10,000 events counted for each sample.

2.2.5.3 Effect of pharmacological inhibitors

In the case of pharmacological inhibitors, THP-1 macrophages were pre-treated with DGLA or DMSO vehicle with 1 µM indomethacin or 10 µM ML351 for 24 hours, followed by 100 µg/mL LY for a further 24 hours. The analysis is described in Section 2.2.5.2.

2.2.5.4 Analysis of cell surface expression of proteins by flow cytometry

THP-1 monocytes were differentiated into macrophages and treated with vehicle or DGLA as described in Section 2.2.2.1. The overlying medium was then discarded, and the cells were detached using 0.5 mL of Trypsin-EDTA (0.05%, w/v), which was added to each well and incubated for 30 minutes. The resulting mixture was then pipetted up and down several times to ensure that the cells were no longer adherent and subjected to centrifugation at 9,000 xg for 5 minutes to pellet the cells. The pellets were re-suspended in 2% (w/v) PFA, and the cells were stained at 4°C for 30 minutes with a marker antibody for CD36, SR-A and LDLr or appropriate isotype control. To analyse the receptor-expressing populations, the following antibodies were used: PE Mouse Anti-Human MSR1; Anti-Human LDLr APC Conjugated Mouse IgG1; and FITC Mouse Anti-Human CD36 clone (CLB-IVC7) (BD Biosciences, USA). After initial staining, the cells were washed with 2% (v/v) PBS. The cells were ready to be analysed by FACS Canto flow cytometer with at least 10,000 events counted for each sample.

2.2.6 *In vivo* methods

2.2.6.1 Animals and feeding

The effect of DGLA supplementation on plaque and other parameters *in vivo* was assessed using homozygous $LDLr^{-/-}$ mice with the LDLrtm1Her mutation and backcrossed to the C57BL/6J strain (Jackson Laboratory). The breeding and experimental treatment of these mice was carried out by Dr. Jessica Williams. The Cardiff University Institutional Ethics Review Committee and the United Kingdom Home Office authorized all experiments and procedures, and studies were carried out in accordance with the Guide to the Care and Use of Laboratory Animals (NIH Publication No. 85-23, updated 1996; experimental licence 30/3365).

Male-8-week-old $LDLr^{-/-}$ mice were housed in a light and temperature-controlled facility (12-hour light/dark cycle, 22°C) with free access to water and food. The mice (80) were randomly assigned between four groups (20 per group based on previous studies in the laboratory) and fed high-fat diet (HFD) [21% (w/w) pork lard and 0.15% (w/w) cholesterol]; or HFD supplemented with 500 mg/kg/day DGLA for a period of 12 weeks (Progression study); or HFD for 12 weeks followed by chow diet for 4 weeks; or HFD for 12 weeks followed by chow diet supplemented with 500mg/kg/day DGLA for 4 weeks (Regression study). All mice were sacrificed at the end of the experiment via increasing CO_2 levels, and death was confirmed through the absence of a pulse.

Previous studies have suggested that a HFD diet could affect the percentage of haematopoietic signalling lymphocyte activation molecule (SLAM) and progenitor cells in the bone marrow (Chan et al., 2012; Wu et al., 2013; Adler et al., 2014; van den Berg et al., 2016). The effect of DGLA feeding, either HFD or with 500 mg/kg/day DGLA in progression studies together with those in regression study, on bone marrow cell population in LDLR$^{-/-}$ mice was therefore analysed (Figure1.19). Table 2.5 presents the antibodies used for profiling.

Table 2. 5 Composition of antibody cocktails used in immunophenotyping of bone marrow cell populations.

Class	Antibody	Fluorochrome	Volume (µL)	(2%, v / v) HIFCS-PBS (µL)
SLAM	Ly-6A/E (Sca-1)	PE	4	32
	CD48	FITC	2	
	CD150	PE/Cy7	1	
	CD117 (c-Kit)	APC	1	
	Lineage cocktail		10	
Progenitor	Ly-6A/E (Sca-1)	APC/Cy7	4	76
	CD34	FITC	4	
	CD16/32	PE/Cy7	4	
	CD127	PE	1	
	CD117 (c-Kit)	APC	1	
	Lineage cocktail		10	
Lineage	Ly-6G/Ly-6C (Gr-1)	PE/Cy7	0.1	100
	CD11b (Mac-1)	PE	0.1	
	CD45R/B220	APC	0.1	
	CD3	FITC	0.1	
	TER-119	APC/Cy7	0.1	
Lineage cocktail	Biotin CD3		25	537.5
	Biotin CD4		12.5	
	Biotin CD8a		25	
	Biotin Ly-6G/Ly-6C (Gr-1)		50	
	Biotin CD11b		100	
	Biotin CD45R/B220		50	
	Biotin TER-119		200	

2.2.6.2 Harvesting Bone Marrow Cells

The tibia and femur were ground with a pestle and mortar until there were no noticeable bones. The extracted marrow was then homogenised in a mortar with 10 mL of PBS and 2% HI-FCS (v/v) (Figure 2.4). Then, the homogenised marrow was passed through a sterile 70 µm filter

and collected in a 50 mL Falcon tube. This process was repeated twice to obtain the final 30 mL bone marrow cell suspension. The cell suspension was then transferred to another tube and pelleted for 5 minutes at 500 x g at 4°C. The pelleted cells were resuspended in 2% (v/v) HI-FCS for cell counting at room temperature. The total cell count was then determined, and the SLAM and progenitor cell populations respectively were separated in distinct Falcon tubes for staining (10 $x10^6$) cells/tube). In addition, cell suspension for lineage positive cell populations analysis was also separated for staining (2 to 5 x 10^5 cells/ tube).

2.2.6.3 Immunophenotyping /Antibody Staining

The bone marrow cells were stained with a biotinylated mix of lineage positive markers to separate population of SLAM and progenitor cells at 4°C for 30 minutes in the dark. The following antibodies were used to analyse the SLAM cell populations: phycoerythrin (PE)/ cyanine7 (Cy7)-conjugated anti-mouse CD150; FITC-conjugated anti-mouse CD48; allophycocyanin (APC)-conjugated anti-mouse c-Kit; and PE-conjugated anti-mouse stem cell antigen (Sca)-1. The progenitor cell population was analysed using the following antibodies: BV650-conjugated anti-mouse CD127; PE/Cy7-conjugated anti-mouse CD16/32; FITC-conjugated anti-mouse CD34; APC-conjugated anti-mouse c-Kit; and APC/Cy7-conjugated anti-mouse Sca-1 (10 $x10^6$) cells/tube). Then 3 mL of 2% (v/v) PBS-FCS was added and centrifuged at 500 x g at 4 °C for 5 minutes to remove the unbound antibodies. The pellet was resuspended in pacific blue conjugated streptavidin and incubated for 30 minutes at 4°C in the dark. Subsequently, SLAM and progenitor tubes were washed in 2% (v/v) PBS-FCS by centrifugation at 500 x g at 4°C for 5 minutes and resuspended in 600 uL of 2% (v/v) HI-FCS for cell sorting by FACS.

Analysis of the lineage positive cell population was also performed concurrently. For this, cells (2 to 5 x 10^5 cells/ tube) were incubated for 30 minutes at 4°C with APC-conjugated anti-mouse B220, FITC-conjugated anti-mouse CD3, PE/Cy7-conjugated anti-mouse Gr1, PE-conjugated anti-mouse Mac1 and APC/Cy7-conjugated anti-mouse Ter119. After incubation, the cells were washed and resuspended in 2% (v/v) HI-FCS for FACS analysis. Table 2. 6 illustrated markers used in the immunophenotyping of bone marrow cell populations.

Table 2. 6 Markers used in the immunophenotyping of bone marrow cell populations.

Class	Cell type	Lineage
SLAM	Lineage -	
	LSK	Lin- Sca-1+ c-Kit+
	HSC	CD150+ CD48-
	MPP	CD150- CD48-
	HPC I	CD150- CD48+
	HPC II	CD150+ CD48+
Progenitor	Lineage -	
	LK	Lin- Sca-1- c-Kit+
	CMP	CD34+ CD16/32-
	MEP	CD34- CD16/32-
	GMP	CD34+ CD16/32+
	CLP	CD127+
Lineage	Lineage +	
	Granulocyte	GR1+ Mac1-
	MDSC	GR1+ Mac1+
	Macrophages	GR1- Mac1-
	B-Cell	B220+
	T-Cell	CD3+

Abbreviations: HSC, haematopoietic stem cell; MPP, multipotent progenitors; HPC, haematopoietic progenitor cell; CMP, common myeloid progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-macrophage progenitor; MDSC, myeloid-derived suppressor cells; CLP, common lymphoid progenitor; SLAM, signalling lymphocytic activation molecule.

2.2.6.4 FACS Acquisition

When all preparations were completed for SLAM, progenitor, lineage and fluorochrome single cells, the samples were then filtered through sterile 40 µm pore filters into FACS tubes used for the FACS machine. The samples were analysed on BD LSRFortessa™. Viability dye DAPI (20 ng/mL, v/v) was added (6 µL per tube; 1 µL/100 µL of the sample) to identify dead cells within SLAM, progenitors and lineage populations (dead cells should be <10% of total nucleated cells). For valid statistical calculation, $3X10^6$ events in SLAM (LSK Gate) and $1.5x10^6$ events for progenitor (LK Gate) had been collected. Lineage samples and single-cell stains were analysed for collect $2x10^4$ events.

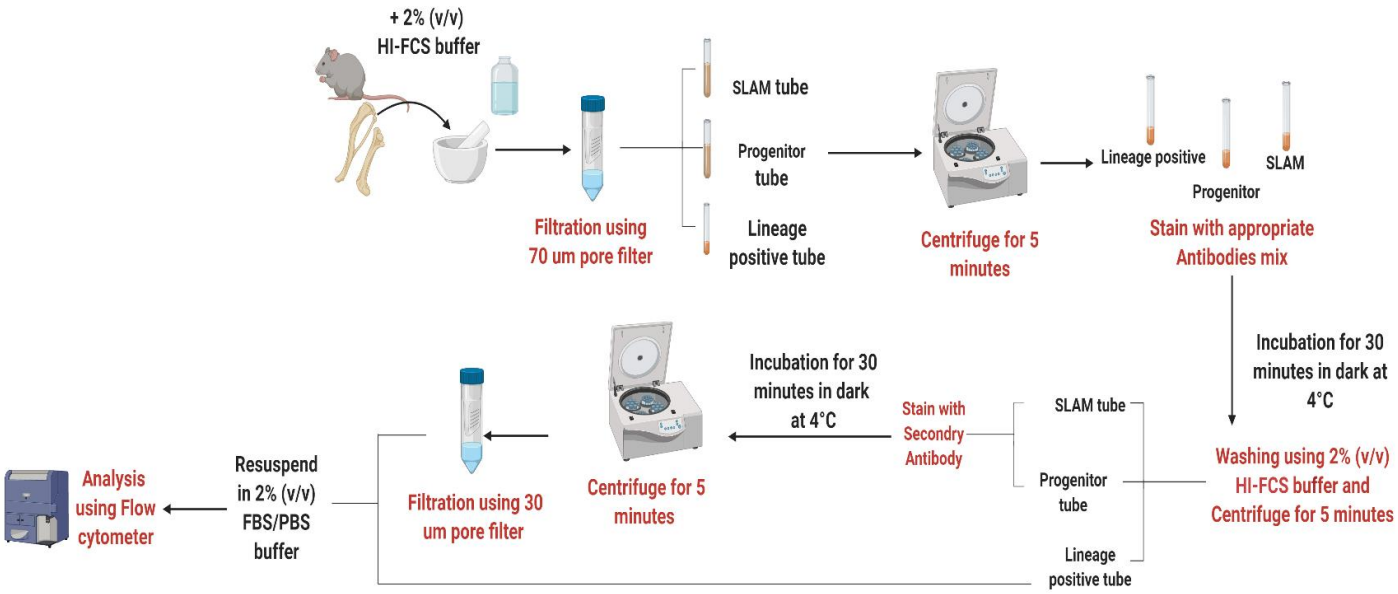

Figure 2. 4 Schematic representation of the procedures used to analyse bone marrow cell populations.
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2.2.7 Statistical analysis

Data were tested for normality using the Shapiro-Wilks test, histograms and Q-Q plots, and any data transformations have been stated. For multiple comparisons between various groups with equal variances, a One-way analysis of variance (ANOVA) was used with Tukey's post hoc test. To compare between multiple groups with unequal variances, Welch's test for equality was carried out using GraphPad Prism followed by Games Howell/Dunnett's T3 post hoc test. For comparisons between two groups, either a Student's t-test (where data displays normal distribution) or Mann Whitney U test (where normality was not attained) was performed. All statistical analysis was performed using GraphPad Prism 8 software. Statistical significance was defined when P-value was ≤0.05.

Chapter 3 The effects of DGLA and its metabolites on key atherosclerosis-associated cellular processes in vitro

3.1 Introduction

DGLA has protective actions in many diseases, including inhibition of platelet aggregation in human studies (Kernoff et al. 1977) and a significant reduction in inflammation of the ears after an inflammatory stimulus in mice (Watanabe et al. 2014). The effects of DGLA on key cellular processes in such diseases have been suggested to be mediated via its metabolites (Wang and Bennett 2012; Xu et al. 2014).

Previous studies in the laboratory demonstrated several anti-atherogenic actions of DGLA *in vitro*, such as decrease in the macrophage uptake of modified LDL by receptor-mediated endocytosis and macropinocytosis, in part via reduction in the expression of the two main SRs, SR-A and CD36, and the induction in cholesterol efflux from foam cells. Together, these changes contribute to DGLA-mediated inhibition of the development of foam cells (Gallagher et al. 2019). In addition, previous studies in the laboratory using mass spectrometry demonstrated that the levels of PGE_1 and 15-(S)-HETrE increased dramatically following treatment of human macrophages with DGLA (Gallagher 2016). This suggested that the actions of DGLA were potentially mediated by these two metabolites. Indeed, PGE_1 was found to attenuate IFN-γ-induced expression of MCP-1 and ICAM-1 in human macrophages and chemokine-driven migration of human monocytes (Gallagher et al. 2019). In addition, small interfering RNA-mediated knockdown of Cox-1/2, which are responsible for the production of PGE_1, attenuated the inhibition by DGLA of IFN-γ-induced MCP-1 expression in human macrophages (Gallagher 2016). The effect of 15-(S)- HETrE, however, was not investigated. The aim of studies presented in this chapter was to address this and to compare with the actions of DGLA and PGE_1. The optimal concentration of DGLA and PGE_1 was determined previously in the laboratory. However, dose response experiments were required in the case of 15-(S)-HETrE.

This study therefore focused on the effects of DGLA and its metabolites on the viability and proliferation of cells, monocyte migration, VSMC invasion, lipoprotein uptake via macropinocytosis and receptor-mediated endocytosis (e.g., Dil-oxLDL uptake) together with the expression of key scavenger receptors and specific pro-atherogenic genes whose expression was induced by cytokines.

3.1.1 Monocyte recruitment and migration

Monocytes play a key role in the innate immune response and the maintenance of tissue homeostasis (Geissmann et al. 2003; Moore et al. 2013). Different signals, such as cytokines and chemokines produced by tissue cells, are used to recruit circulatory monocytes at sites of damage or pathogen invasion (Chistiakov et al. 2016). MCP1 (also known as CCL2) is a major pro-inflammatory chemokine necessary for the recruitment of monocytes and primary lesion development (Braunersreuther et al. 2007; Ramji and Davies 2015; Moss and Ramji 2016b). A previous study in atherosclerotic mouse models presented smaller lesions in animals with deficiency of this chemokine (Magnusson et al. 2015; Moss and Ramji 2016a). Previous research also found that DGLA can significantly inhibit MCP-1-driven monocyte migration *in vitro* (Gallagher et al. 2019). The current study analysed the impact of DGLA and its metabolites on monocyte migration. A modified Boyden chamber was used to assess the MCP-1-driven recruitment of monocytes via a 8 µm pore membrane that mimicked the arterial endothelial layer.

3.1.2 VSMC invasion

VSMC, through stabilisation of lipid-rich necrotic cores of atherosclerotic plaques by the formation of fibrous caps, plays an important role in reducing the clinical consequences of the disease (Alexander et al. 2012). In atherosclerosis, the proliferation of VSMCs is mainly believed to be reparative and thought not to be a key factor in plaque development (Bennett et al. 2016). The precise functions of VSMC migration in atherosclerosis are still unclear and under intense research (Bennett et al. 2016). During atherogenesis, VSMCs migrate from the media to the intima in response to many cytokines, chemokines and growth factors (Welser et al. 2007). One of the most effective mitogens and chemoattractants for VSMC is platelet-derived growth factor-BB (PDGF-BB) (Pan et al. 2019). A modified Boyden chamber method, as described in Section 2.2.3.4, was used in this study to investigate the effect of DGLA and its metabolites on VSMC invasion. The ability of HASMCs treated with either a vehicle control, DGLA or its metabolites to migrate towards a PDGF-BB stimulus was investigated.

3.1.3 Lipid uptake

3.1.3.1 Macropinocytosis

A major mechanism for atherosclerotic plaque development is macrophage foam cell formation (Kruth et al. 2005). Interestingly, it was previously assumed that receptor-mediated uptake of only modified forms of LDL, including oxidised and aggregated LDL, was primarily involved in the development of macrophage foam cells (Kruth et al. 2002). However, fluid-phase endocytosis, termed macropinocytosis, also plays an important role, and this is facilitated by the stimulation of human macrophages with, for example, protein kinase C activator PMA (Kruth et al. 2005; Lim et al. 2012). Macropinocytosis also mediates several physiological processes by non-selective uptake by macrophages and dendritic cells (DC) of nutrients and antigens (Figure 3.1) (Lim et al. 2012; Michael et al. 2013). Previous studies in the laboratory have demonstrated that cytokines also regulate macropinocytosis in human macrophages (Michael et al. 2013). Recently, the effect of DGLA on macropinocytosis was investigated, and it was found that this fatty acid inhibited this process *in vitro* (Gallagher et al. 2019).

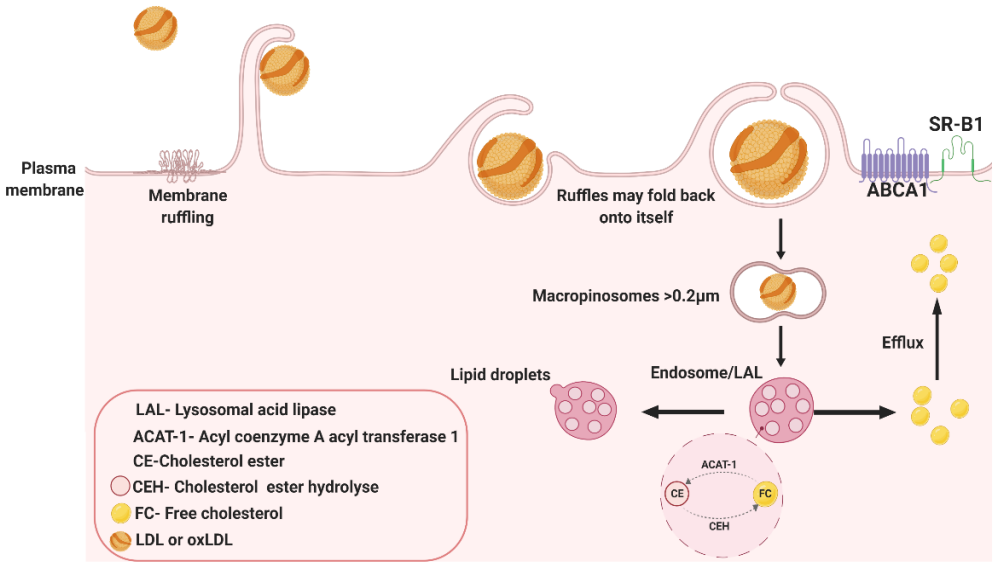

Figure 3. 1 Macropinocytosis.
This involves the rearrangement of the actin cytoskeleton at the plasma membrane that causes circular ruffles during macropinosome formation. In macropinosomes, ruffles may self-assemble independently and attach at the plasma membrane base, trapping compounds. At late endosome/lysosome, the LDL or oxLDL component of the macropinosomes is then metabolised to CE and FC. Efflux can occur via ABCA1 or SR-BI to ApoA1; this is probably because FC is recycled back to the plasma membrane. In the cytoplasm, CE can form lipid droplets resulting in the development of foam cells (Lim et al. 2012). Created with BioRender.com.

3.1.3.2 Dil-oxLDL uptake

OxLDL is one of the most important pro-atherogenic particles involved in foam cell formation (Choi et al. 2010; Liu et al. 2017). OxLDL is taken up via a range of receptors, such as the class A SR SR-A and the class B SR CD36 of macrophages, which also promotes an inflammatory response (Libby 2012; Liu et al. 2017). Moreover, oxLDL is also involved in other cellular processes in atherosclerosis, including mediating dysfunction of EC and stimulating proliferation of VSMC (Erbel et al. 2011; Di Pietro et al. 2016). In cholesterol uptake and efflux studies *in vitro*, both acetylated LDL (acLDL) and oxLDL are commonly used and both present comparable effects on macrophage foam cell formation; however, acLDL is generally taken up more avidly than oxLDL (McLaren *et al.* 2010; McLaren *et al.* 2011a). A recent study by our laboratory demonstrated that DGLA inhibits the uptake of Dil-oxLDL by THP-1

macrophages (Gallagher 2016). This finding led to the study here of the effect of DGLA metabolites on scavenger receptor-mediated uptake of oxLDL.

3.1.3.3 Scavenger receptors

SRs are membrane-bound glycoproteins that bind to modified LDL and specific patterns on pathogens (Goldstein et al. 1979; Hooper et al. 2006). The expression and activities of SRs are regulated in vascular tissues by intracellular signals, lipid accumulation and autophagy associated with atherosclerosis (Stephen et al. 2010). There is a 'superfamily' of SRs that have been categorised into ten classes (Classes A-J) based on their tertiary structure and biological function (Peiser and Gordon 2001; Zani et al. 2015). In atherosclerosis, SRs, in particular CD36 and SRA, play a key role in foam cell formation, which leads to the accumulation of cholesteryl esters in cells, a hallmark of foam cells, because of their ability to recognise and internalise modified LDL (Kzhyshkowska et al. 2012).

Class A SRs include SR-A1, also called MSR1 or CD204, which is expressed on the surface of macrophages and other cells (de Winther et al. 2000; Zani et al. 2015). In 1979, SR-A1 was first identified in peritoneal macrophages of mice by Brown and Goldstein (Brown et al. 1980). In atherosclerotic lesions, SR-A1 presented significantly increased expression in human macrophages (Matsumoto et al. 1990; Mäkinen et al. 2010). Additionally, previous studies that used SR-A1$^{-/-}$/ ApoE$^{-/-}$ mice demonstrated that atherosclerotic plaque burden was reduced in comparison to ApoE$^{-/-}$ mice despite high cholesterol levels in the plasma.

CD36 is a cell-surface glycoprotein that is a member of the SR class B family, which interacts with a wide range of ligands, such as thrombospondin-1, oxidised phospholipids/lipoproteins, long-chain fatty acids, modified lipid particles, apoptotic cells and pathogens (Febbraio et al. 2004; Zani et al. 2015). CD36 in macrophages is involved in the uptake of oxLDL and the development of foam cells together with promotion of apoptosis, angiogenesis and inflammation (Febbraio et al. 2004; Kennedy et al. 2011; Zani et al. 2015). A previous study by Kennedy et al. (2011) *in vivo* in CD36/ApoE null mice demonstrated that CD36 in the vascular system can play a significant role in promoting the formation of foam cells, plaque development and thrombus formation (Kennedy et al. 2011). Additionally, another study indicated that in macrophages, SR-A and CD36 accounted for a large proportion (75–90%) of

modified LDL uptake (Kunjathoor et al. 2002). Previous studies in the laboratory have found that in human macrophages, DGLA causes a dramatic reduction in the expression of SR-A and CD36 (Gallagher et al. 2019). The studies presented in this chapter extended to the effects of DGLA metabolites on the expression of SR-A and CD36.

3.1.4 IFN-γ signalling.

IFN-γ is a pro-inflammatory cytokine which plays a crucial role in the regulation of immune functions, is expressed at high levels in atherosclerotic lesions and is involved in the initiation of the inflammatory process (Harvey and Ramji 2005; McLaren and Ramji 2009). Previous studies have addressed the main effects of IFN-γ on many atherosclerosis-associated processes, including the recruitment of monocytes, plaque development and the expression of pro-inflammatory genes (Harvey and Ramji 2005; Ramji and Davies 2015). The remarkable characteristic of IFN-γ is that it regulates the expression of a large number of pro-inflammatory genes implicated in atherosclerosis, including MCP-1 and ICAM-1, and 30% of the macrophage transcriptome (Watanabe and Fan 1998; Inagaki et al. 2002). MCP-1 and ICAM1 play a dominant role in the migration of monocytes (Watanabe and Fan 1998; Inagaki et al. 2002; Melgarejo et al. 2009). Studies in $ApoE^{-/-}$ mice have indicated that atherosclerosis is attenuated by the inhibition of IFN-γ actions (Zhou and Liao 2009). In *in vitro* studies, IFN-γ activated the apoptosis of macrophages and promoted the production of MCP-1, thereby contributing to the development and progression of atherosclerosis (Inagaki et al. 2002). Recent research in our laboratory revealed that in human and mouse macrophages, DGLA and its main metabolite PGE_1 inhibited the mRNA expression of MCP-1 and ICAM-1 induced by IFN-γ (Gallagher et al. 2019). However, these studies involved pre-treatment of the cells with DGLA, so its actions in established inflammation (i.e., following stimulation of IFN-γ) were not clear. Therefore, the studies presented in this chapter aimed to determine the effects of DGLA, PGE_1 and 15-S-HETrE treatment in human macrophages on IFN-γ-induced MCP-1 and ICAM1 expression in different conditions (e.g., pre-treatment with DGLA or its metabolites followed by IFN-γ, addition of DGLA/metabolites and IFN-γ together or addition of DGLA/metabolites after IFN-γ-induced inflammation).

3.2 Experimental design

The key focus of studies presented in this chapter was to investigate the effect of DGLA and its metabolites on main processes linked to the progression of atherosclerosis as presented in Figures 3.2 – 3.3. Chapter 2 describes the different methods used in the studies.

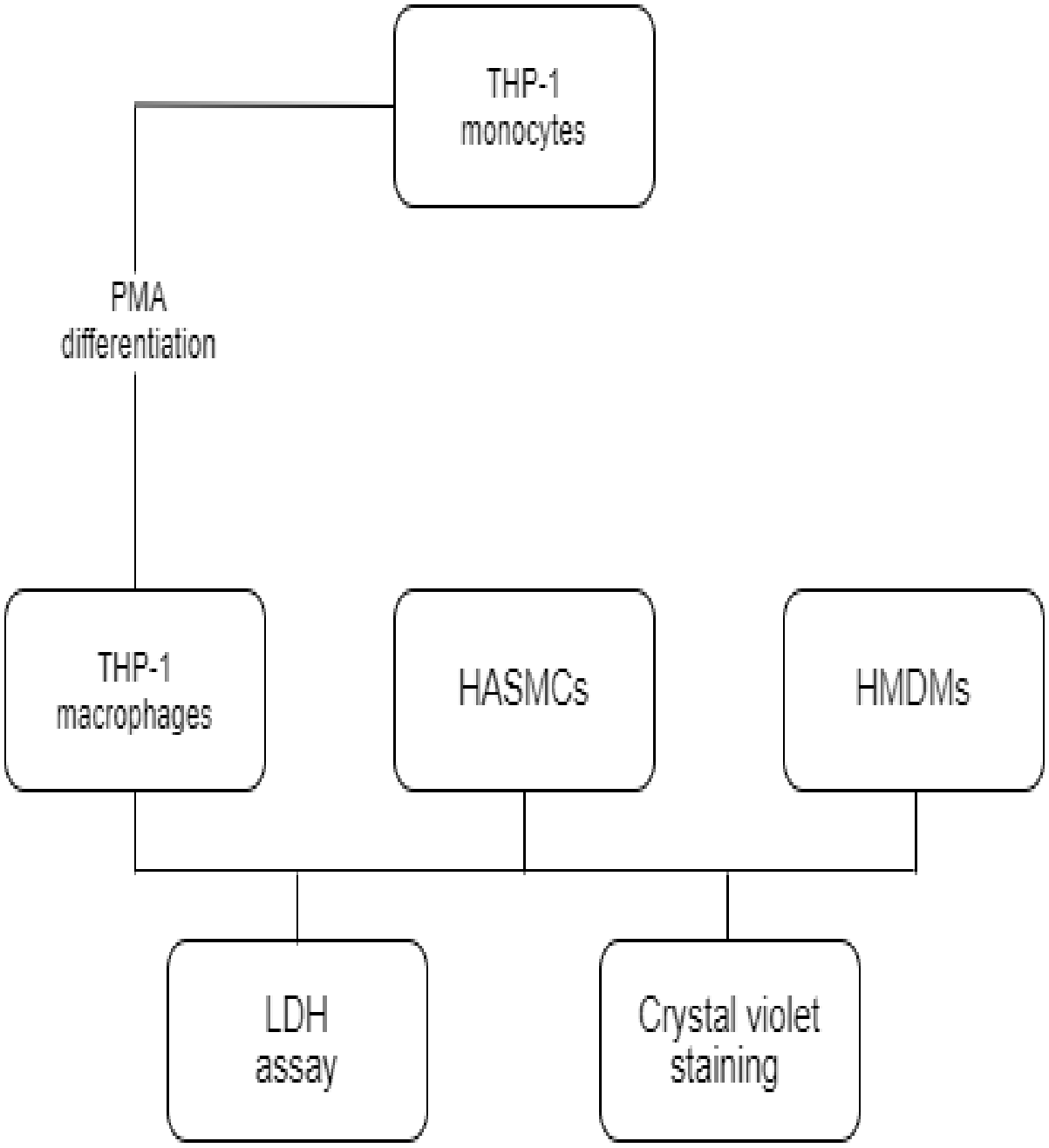

Figure 3. 4 Experimental strategy for cell viability and proliferation.
Abbreviations: HASMCs, human aortic smooth muscle cells; HMDMs, human monocyte-derived macrophages; LDH, lactate dehydrogenase.

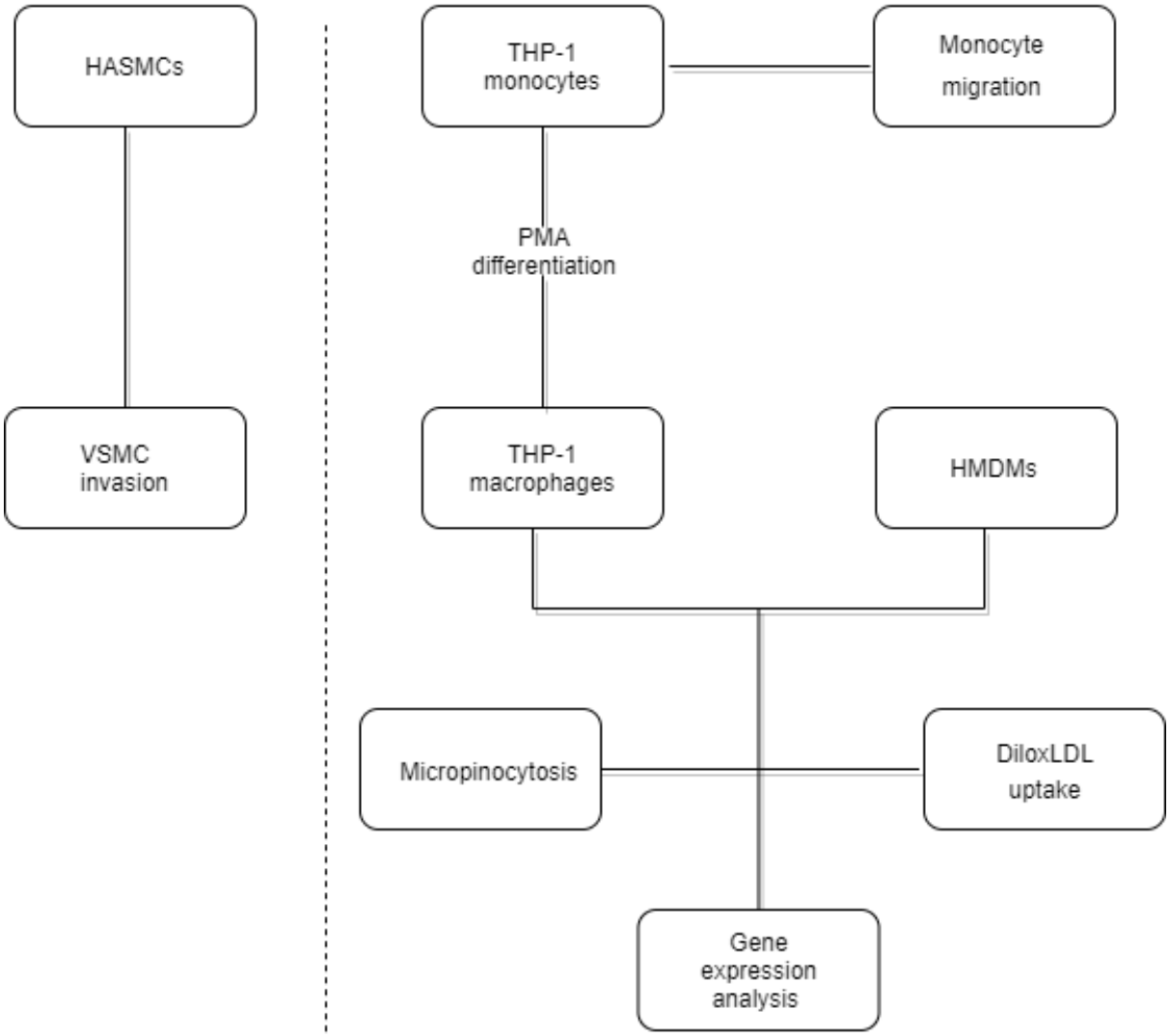

Figure 3. 5 Experimental strategy to evaluate the effects of DGLA and its metabolites on key atherosclerosis-associated cellular processes.

Abbreviations: HASMCs, human aortic smooth muscle cells; HMDMs, human monocyte-derived macrophages; VSMC, vascular smooth muscle cells.

3.3 Results

3.3.1 DGLA and its metabolites do not affect cell viability of key cell types used for *in vitro* studies.

LDH, a cytosolic enzyme, exists in all cell types, and its extracellular release is an indicator of cellular membrane damage. Therefore, the activity of LDH in the medium of cultured THP-1 macrophages or HASMCs treated with vehicle, DGLA or its metabolites was compared as an indicator of compromised cell viability. As some of the assays with HASMCs are of longer duration, viability was determined at two time points (24 hours and 8 days). The previously found optimum concentrations of DGLA (50 μM) and PGE_1 (10 μM), together with varying concentrations of 15-(S)-HETrE (1 μM, 2 μM, 5 μM and 10 μM), were utilised in these assays. Figures 3.4. and 3.5 demonstrate that there were no significant changes in LDH activity in the medium following treatment of the cells with DGLA (50 μM), 15-(S)-HETrE (1μM, 2μM, 5μM and 10μM) and PGE_1 (10 μM) when compared to the 0.1% DMSO vehicle control in both human macrophages and HASMCs. These reagents were therefore utilised for subsequent investigations with confidence that the outcomes were due to their direct actions rather than any indirect effect on the cell viability of THP-1 macrophages or HASMCs.

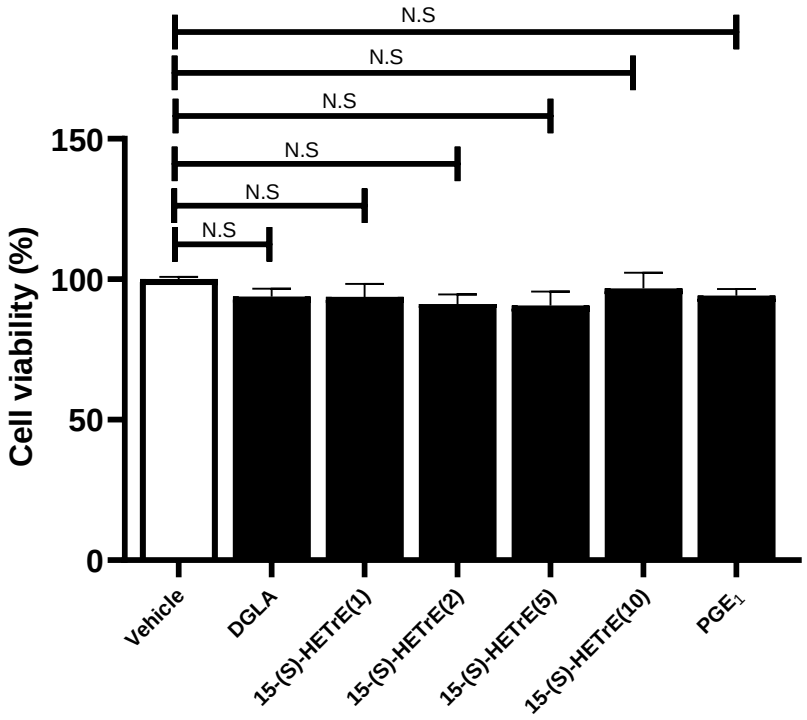

Figure 3. 6 DGLA, 15-(S)-HETrE or PGE_1 have no effect on the cell viability of macrophages.

THP-1 macrophages were incubated with 50 μM DGLA, varying concentrations of 15-(S)-HETrE (presented as numbers in μM in brackets) and 10 μM PGE_1 or the vehicle control for 24 hours. The results are presented as percentage (mean +/- SEM) to the vehicle control (arbitrarily assigned as 100%) from four independent experiments. Statistical analysis was carried out by One-way ANOVA with Dunnett post-hoc analysis, comparing each individual treatment to the control, and no significant changes were observed (N.S, not significant).

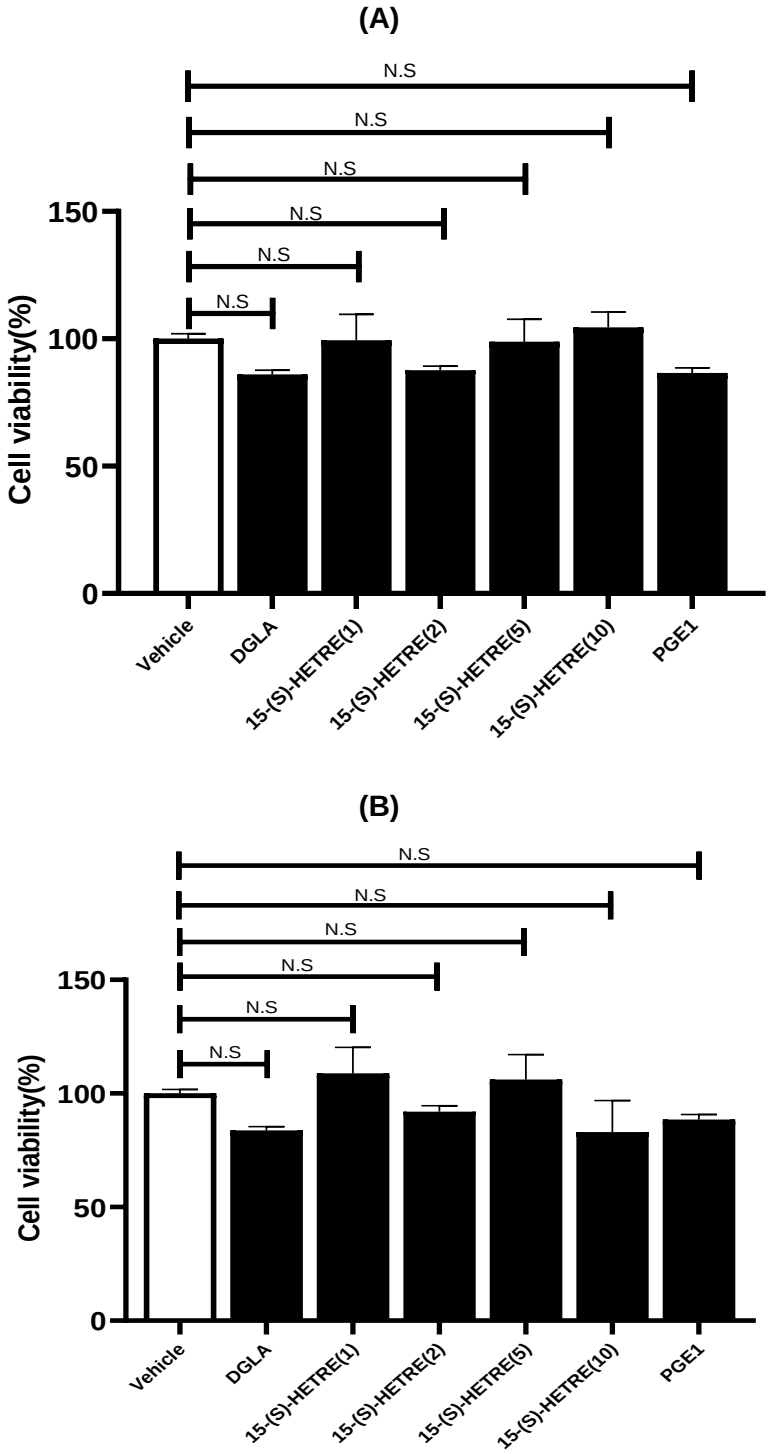

Figure 3. 7 DGLA, 15-(S)-HETrE or PGE_1 have no significant effect on the viability of HASMCs at two time points over an 8-day period.

HASMCs were incubated with 50 μM DGLA, varying concentrations of 15-S-HETrE (presented as numbers in μM in brackets), 10 μM PGE_1 or the vehicle control for 24 hours (A) or for 8 days (B). LDH in the medium was determined using the LDH Assay Kit. The results are presented as a percentage relative to the vehicle control, which was set to 100% (mean +/- SEM) from three independent experiments. Statistical analysis was carried out by One-way ANOVA with Dunnett post-hoc analysis, comparing each individual treatment to the control, and no significant changes were observed (N.S, not significant).

3.3.2 DGLA and metabolites have no detrimental effects on the proliferation of cells used for *in vitro* studies.

Crystal violet staining was also undertaken for adherent cells. This binds to the DNA in the cell nucleus and therefore indicate cell numbers and, hence, proliferation. Figure 3.6 demonstrates that there was no significant change in cell proliferation between THP-1 macrophages treated with the vehicle control, DGLA, 15-(S)-HETrE or PGE_1 under all experimental conditions. For HASMCs, whilst cell proliferation following treatment of the cells with DGLA (50 μM), varying concentrations of 15-(S)-HETrE (1 μM, 2 μM and 5 μM) or PGE_1 (10 μM) was generally lower compared to the vehicle control, none of the changes were significant (Figure 3.7). Whereas 15-(S)-HETrE (10 μM) treatment resulted in a trend of reduction ($p=0.057$) (Figure 3.7E). Overall, these results suggest that cell proliferation was not significantly affected by these agents.

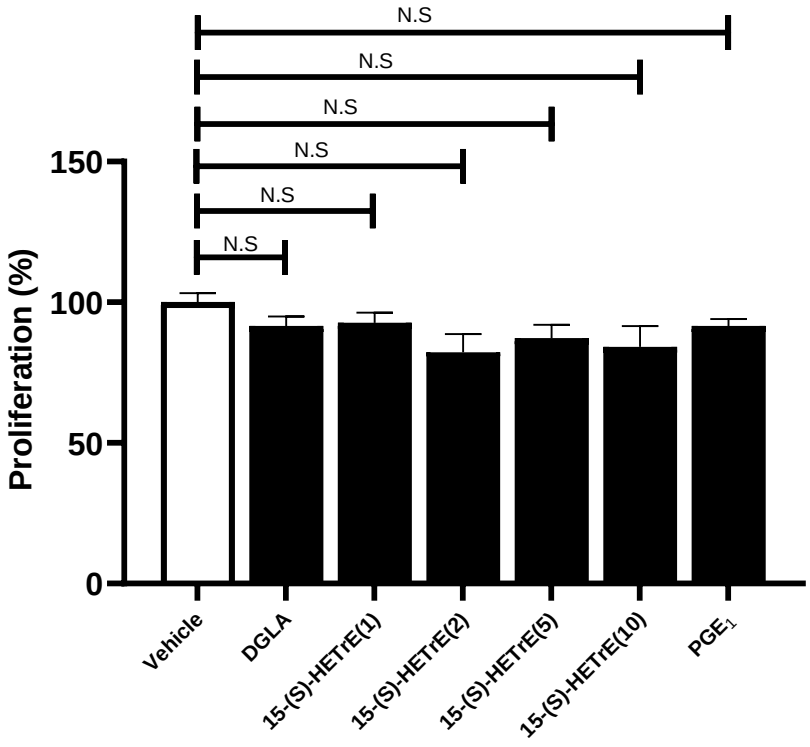

Figure 3. 8 DGLA, 15-(S)-HETrE or PGE_1 did not affect macrophage proliferation.
THP-1 macrophages were incubated with 50 μM DGLA, varying concentrations of 15-S-HETrE (shown as numbers in μM in brackets), 10 μM PGE_1 or the vehicle control for 24 hours. The results are shown as percentage (mean +/- SEM) to the vehicle control (arbitrarily assigned as 1) from four independent experiments. Statistical analysis was carried out by One-way ANOVA with Dunnett post-hoc analysis, comparing each individual treatment to the control, and no significant changes were observed (N.S, not significant).

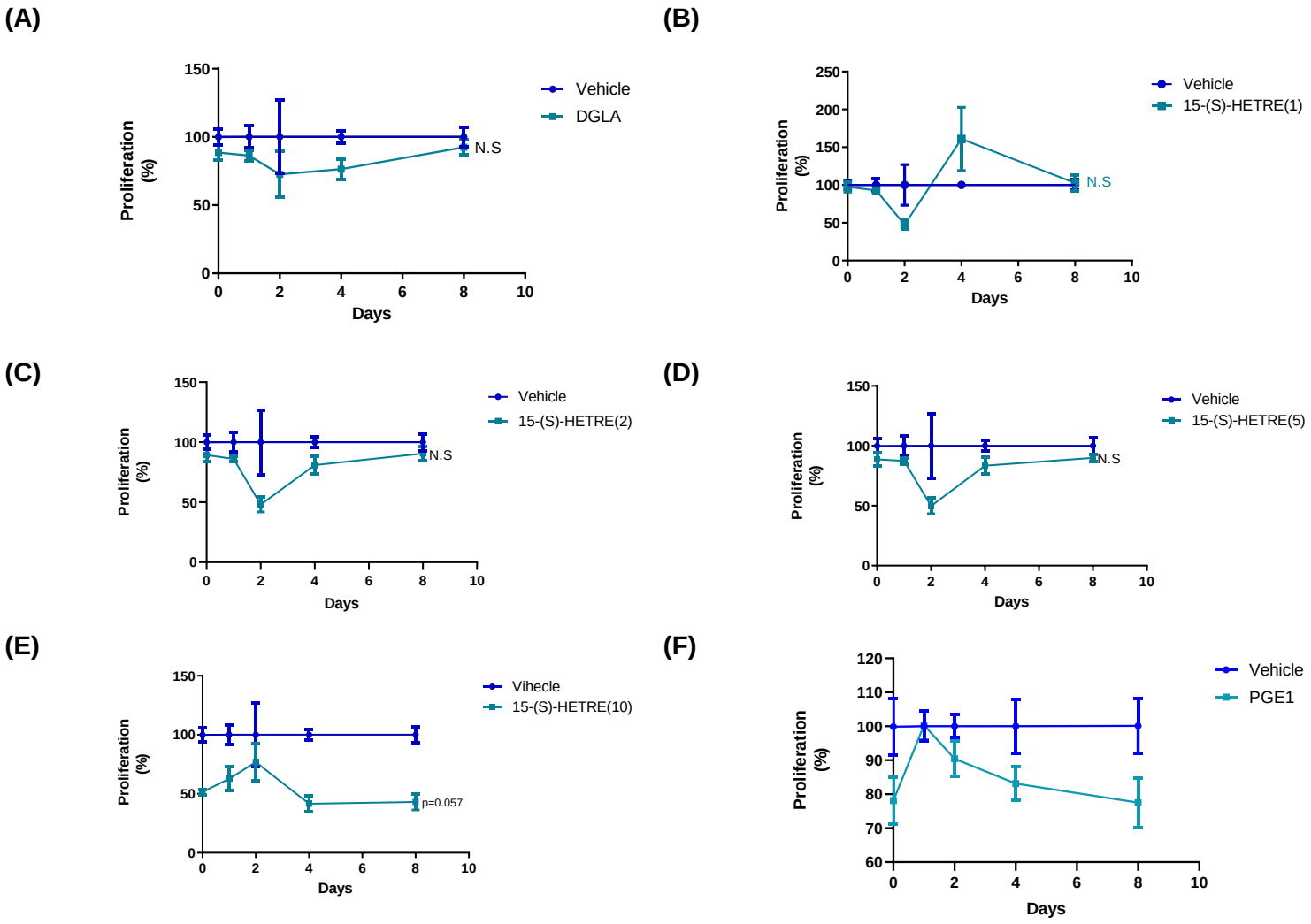

Figure 3. 9 The effect of DGLA, 15-(S)-HETrE or PGE_1 on HASMCs proliferation over a 8-day period.

HASMCs were incubated with 50 μM DGLA, varying concentrations of 15-S-HETrE (shown as numbers in μM in brackets), 10 μM PGE_1 or the vehicle control for 8 days. The results are shown as percentage (mean +/- SEM) to the vehicle control from four independent experiments. Statistical analysis was carried out by linear regression, comparing each individual treatment to the control, and no significant changes were observed (N.S, not significant).

3.3.3 DGLA, 15-(S)-HETrE and PGE_1 significantly inhibit the MCP-1-induced migration of THP-1 monocytes.

Previous studies had indicated that DGLA and its metabolite PGE_1 reduced monocytic migration (Gallagher et al. 2019). The effect of 15-(S)-HETrE on monocytic migration has not been determined and so was investigated in comparison with DGLA and PGE_1. Cell inserts with 8 μm pores were used to represent the arterial endothelial layer. Figure 3.8 demonstrates that the migration of THP-1 monocytes through the cell insert was significantly increased in the presence of MCP-1. However, the inclusion of DGLA resulted in a significant average decrease of 69.9% ($p=0.045$) of this MCP-1-driven monocytic migration. PGE_1 (10 μM) produced a trend towards reduction of 68.12% ($p=0.076$), whilst 2 μM, 5 μM and 10 μM of 15-(S)-HETrE produced significant reductions in migration of 65.77% ($p=0.035$), 47.30% ($p=0.030$) and 91.17% ($p\leq0.001$) respectively. There was also a reduction of 42% ($p>0.999$) in migration seen with 1 μM of 15-(S)-HETrE, but this failed to reach significance.

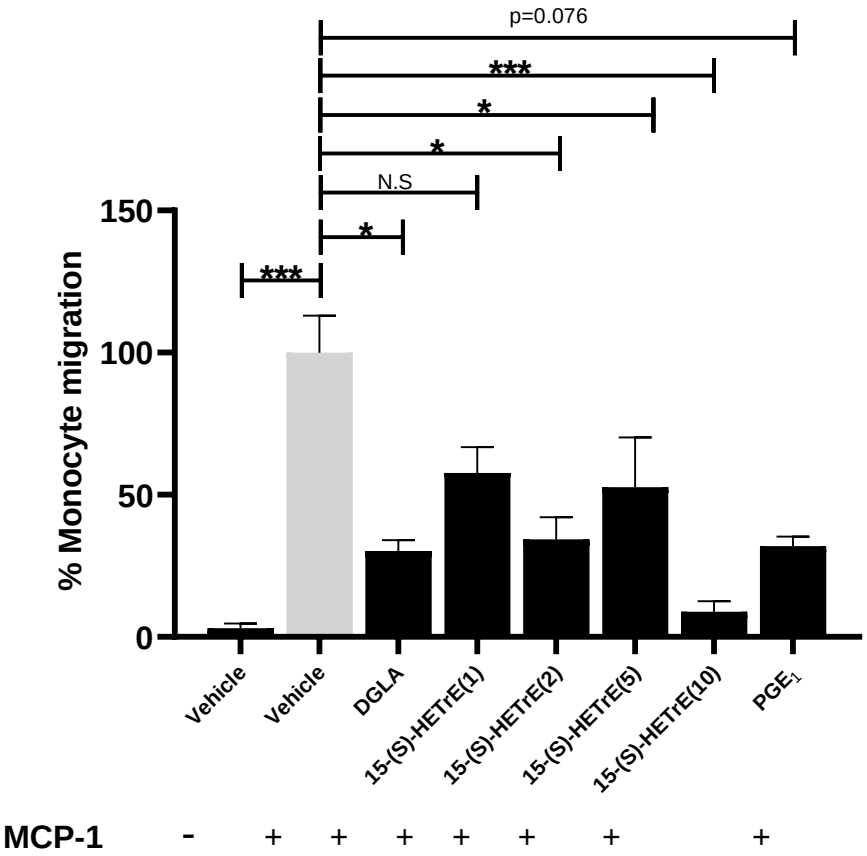

Figure 3. 10 - DGLA, 15-(S)-HETrE and PGE_1 significantly inhibit the MCP-1 induced migration of THP-1 monocytes.

THP-1 monocytes were incubated for 3 hours with DMSO vehicle control in the absence or presence of MCP-1 (20 ng/mL), or with MCP-1 in the presence of 50 μM DGLA, varying concentrations of 15-(S)-HETrE (1μM, 2μM, 5μM or 10μM; shown in brackets) or 10 μM PGE_1. Migration of monocytes (expressed as a percentage of total input cells) was determined by counting the number of cells that successfully migrated over a cell insert. The value from the DMSO-treated cells in the presence of MCP-1 has been arbitrarily assigned as 100% and compared with the others. The results are mean +/- SEM from four independent experiments. Statistical analysis was carried out using One-way ANOVA followed by Tukey's post hoc test (* P≤0.05, ** P≤0.01, *** P≤0.001, P=0.076 and N.S, not significant).

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3.3.4 DGLA and metabolites reduce macropinocytosis in macrophages.

Previous studies have identified macropinocytosis as a mechanism for fluid-phase uptake of LDL and modified LDL with involvement of both cell signalling and cytoskeletal components (Kruth et al. 2005; Michael et al. 2013). Uptake of LY by FACS was used to determine the effect of DGLA, PGE_1 and 15(S)-HETrE on macropinocytosis. LY is a fluorescent pigment frequently reported as a suitable marker to determine uptake via macropinocytosis (Swanson and Watts, 1995, Jones and Willingham, 1999, Michael et al., 2013). THP-1 macrophages were pre-treated for 24 hours with the DMSO vehicle, 50 μM DGLA, various concentrations of 15-(S)-HETrE (1 μM, 2 μM, 5 μM and 10 μM) or 10 μM PGE_1, followed by 100 μg/mL LY for 24 hours. LY uptake from the control, vehicle-treated cells was arbitrarily assigned as 100%. The results demonstrate a significant reduction of LY uptake seen in vehicle-treated cells by 50 μM DGLA (18.7%; p=0.008), 2 μM 15-(S)-HETrE (17.5%; p=0.023) and 10 μM PGE_1, (27.0%; p=0.024) only (Figure 3.9).

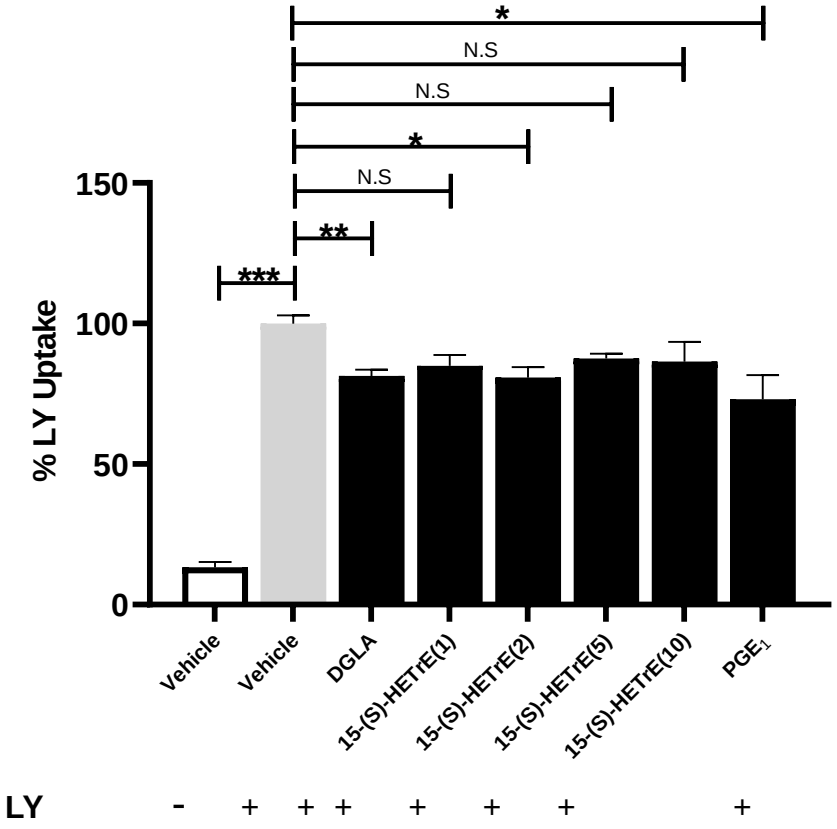


Figure 3. 11 - Effect of DGLA, PGE_1 and 15-(S)-HETrE on LY uptake by macropinocytosis in THP-1 macrophages.

THP-1 macrophages were incubated with DMSO vehicle control, 50 μM DGLA, 2 μM 15-(S)-HETrE, 5 μM 15-(S)-HETrE or 10 μM PGE_1 for 24 hours followed by 100 μg/mL LY (+). Cells treated with DGLA in the absence of LY were also included for comparison. Flow cytometry (FACS Canto) was employed to determine LY uptake. LY uptake is reported as a percentage value (mean +/- SEM from four independent experiments) with the vehicle control (LY and DMSO) arbitrarily assigned at 100%. Statistical analyses were performed using a One-way ANOVA and Dunnett 2-sided post-hoc test (* P≤0.05, ** P≤0.01, *** P≤0.001 and N.S, not significant).

3.3.5 DGLA and its metabolites inhibit oxLDL uptake in macrophages.

Evaluation of the potential mechanisms contributing to DGLA, PGE_1 and 15-(S)-HETrE effects on cholesteryl ester accumulation in macrophages was undertaken by the determination of modified LDL uptake (Shashkin et al. 2005; Lusis 2012). FACS analysis was performed to measure the uptake of oxLDL labelled with a fluorescent marker (Dil). Dil-oxLDL uptake from the control-vehicle-treated cells was arbitrarily assigned as 100%. The results indicate significant attenuation of Dil-oxLDL uptake seen in vehicle-treated THP-1 macrophages by 50 μM DGLA (15.9%; p=0.015), 2 μM 15-(S)-HETrE (17.9%; p= 0.016), 10 μM 15-(S)-HETrE (21.7%; p= 0.036) and 10 μM PGE_1 (14.0%; p=0.048) (Figure 3.10).

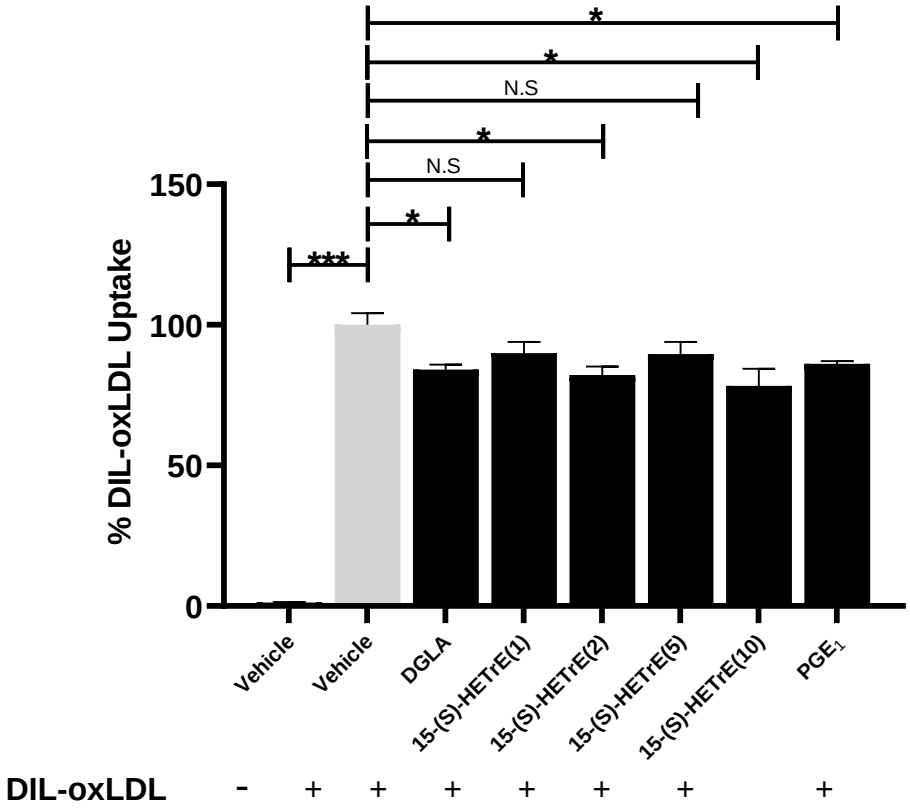


Figure 3. 12 Significant attenuation of Dil-oxLDL uptake in THP-1 macrophages by DGLA.

THP-1 macrophages were incubated with DMSO vehicle control, 50 μM DGLA, varying concentrations of 15-(S)-HETrE (1 μM, 2 μM, 5 μM and 10 μM; shown in brackets) or 10 μM PGE_1 for 24 hours prior to the addition of 5 μg/mL Dil-oxLDL for a further 24 hours. Macrophages were resuspended in 2% PFA, and FACS analysis was carried out to determine uptake, counting 10,000 events. Results are mean +/- SEM from three independent experiments. Statistical analysis was carried out by one-way ANOVA followed by Tukey's post hoc test (* P≤0.05, *** P≤0.001 and N.S, not significant).

3.3.6 Expression of macrophage SRs was inhibited by DGLA, PGE_1 and 15-(S)-HETrE treatments

SRs present on the macrophage cell surface recognise and cause extensive, uncontrolled internalisation of large quantities of modified LDL, resulting in their transformation to foam cells (de Winther et al. 2000; Shashkin et al. 2005; Park 2014). CD36 and SRA are the main SRs expressed by macrophages during atherosclerotic lesion formation, and mouse models of atherosclerosis with deficiency of these genes have been reported to exhibit significant atherosclerotic protection (Endemann et al. 1993; Sakaguchi et al. 1998; Kunjathoor et al. 2002; Febbraio et al. 2004). Previous studies had indicated that DGLA inhibited the expression of SRA and CD36 mRNA and protein expression in THP-1 macrophages (Gallagher et al. 2019). One of the aims of this project was to determine the effect of DGLA, PGE_1 and 15-(S)-HETrE on the expression of 84 genes in atherosclerosis arrays (Chapter 4) and to delineate whether common genes are regulated by the three agents. On the basis of previous experiments, 2 μM 15-(S)-HETrE was used for subsequent experiments as the lowest concentration that affected monocyte migration, macropinocytosis and Dil-oxLDL uptake. Thus, THP1 macrophages were incubated with 50 μM DGLA, 2 μM 15-(S)-HETrE, 10 μM PGE_1 or vehicle for 24 hours before RNA extraction. The expression of SRA and CD36 was determined to examine whether the previously noted response with DGLA could be replicated, which would also act as a positive control for the use of the RNA for subsequent array analysis together with the actions of metabolites. As shown in Figures 3.11, the expression of SR-A was reduced in the presence of DGLA, 15-(S)-HETrE and PGE_1 by 58.6% (p=0.019), 6.6% (p=0.057) and 58.9% (p=0.029) respectively. Gene expression of CD36 was significantly reduced in the presence of DGLA and PGE_1 by 35.5% (p=0.029) and 49.8% (p=0.029) respectively but was non-significantly attenuated in the presence of 15-(S)-HETrE by 30.0% (p=0.497).

The cell-surface expression of these two scavenger receptors was also investigated to confirm that there was a correlation between mRNA and protein expression. LDLr was also included in the comparison, as this is involved in the uptake of native LDL. Flow cytometry was used to determine the surface expression of SR-A, CD36 and LDLr. THP-1 macrophages were incubated with 50 μM DGLA, 2 μM 15-(S)-HETrE, 10 μM PGE_1 or DMSO vehicle control for 24 hours. As shown in Figure 3.12, the expression of SR-A was significantly reduced in the presence of DGLA by 37.4% (p=0.026) but was not significantly affected by 15-(S)-HETrE and PGE_1. The expression of CD36 was also significantly reduced in the presence of DGLA and PGE_1 by 36.0% (p=0.029) and 41.1% (p=0.029) respectively

but was non-significantly decreased in the presence of 15-(S)-HETrE by 26.4%. Moreover, the expression of LDLr was significantly reduced in the presence of DGLA, 15-(S)-HETrE and PGE_1 by 22.9% (p=0.005), 37.5% (p<0.001) and 26.0% (p=0.023) respectively.

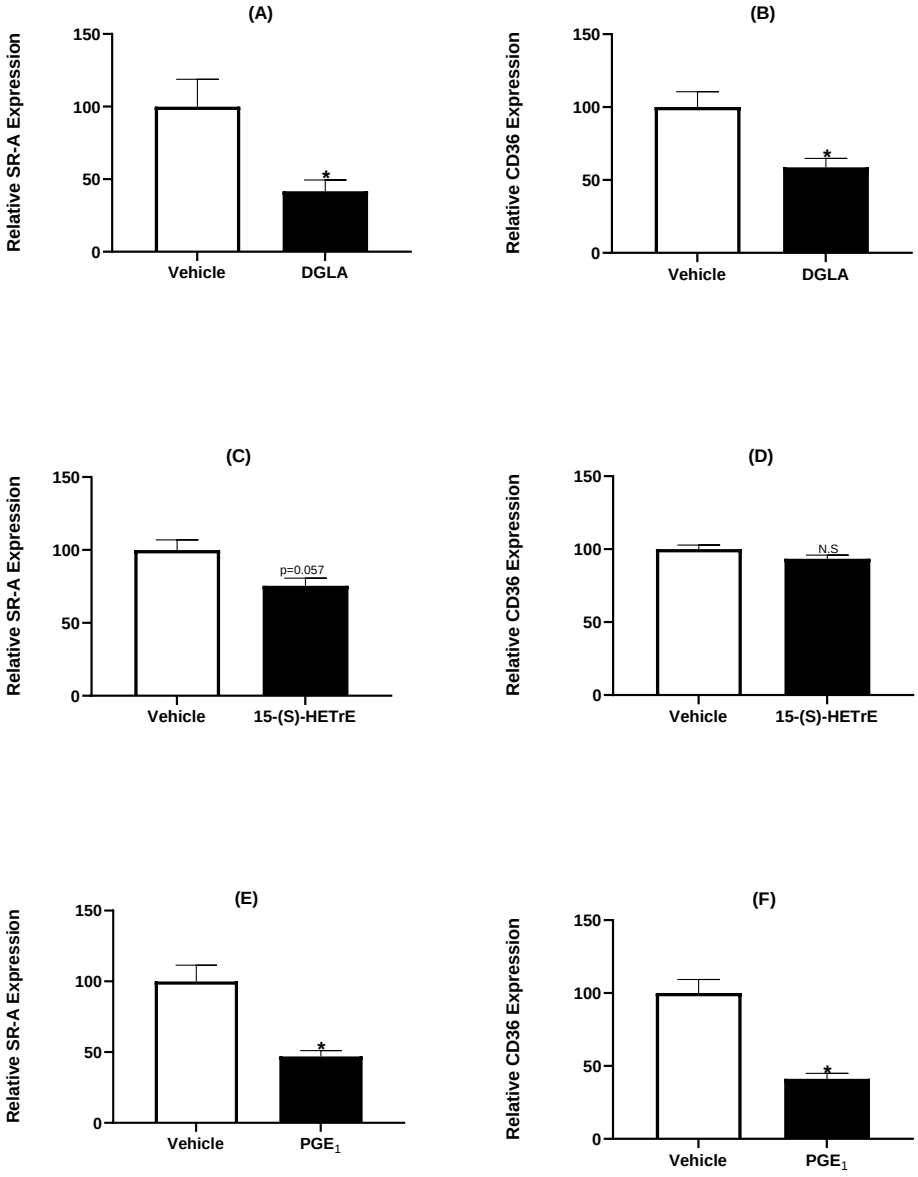


Figure 3. 13 DGLA and its metabolites modulate the expression of CD36 and SR-A in THP-1 macrophages.

THP-1 macrophages were incubated with 50 μM DGLA (A, B), 2 μM 15-(S)-HETrE (C, D), 10 μM PGE_1 (E, F) or control DMSO vehicle for 24 hours. Total RNA was subjected to RT-qPCR with primers specific for SRA, CD36 or GAPDH control. The results show average gene expression (mean +/- SEM) (control arbitrarily assigned as 1) from four independent experiments. Statistical analysis was carried out using an unpaired Student's two-tailed t-test (* $P \leq 0.05$, N.S, not significant).

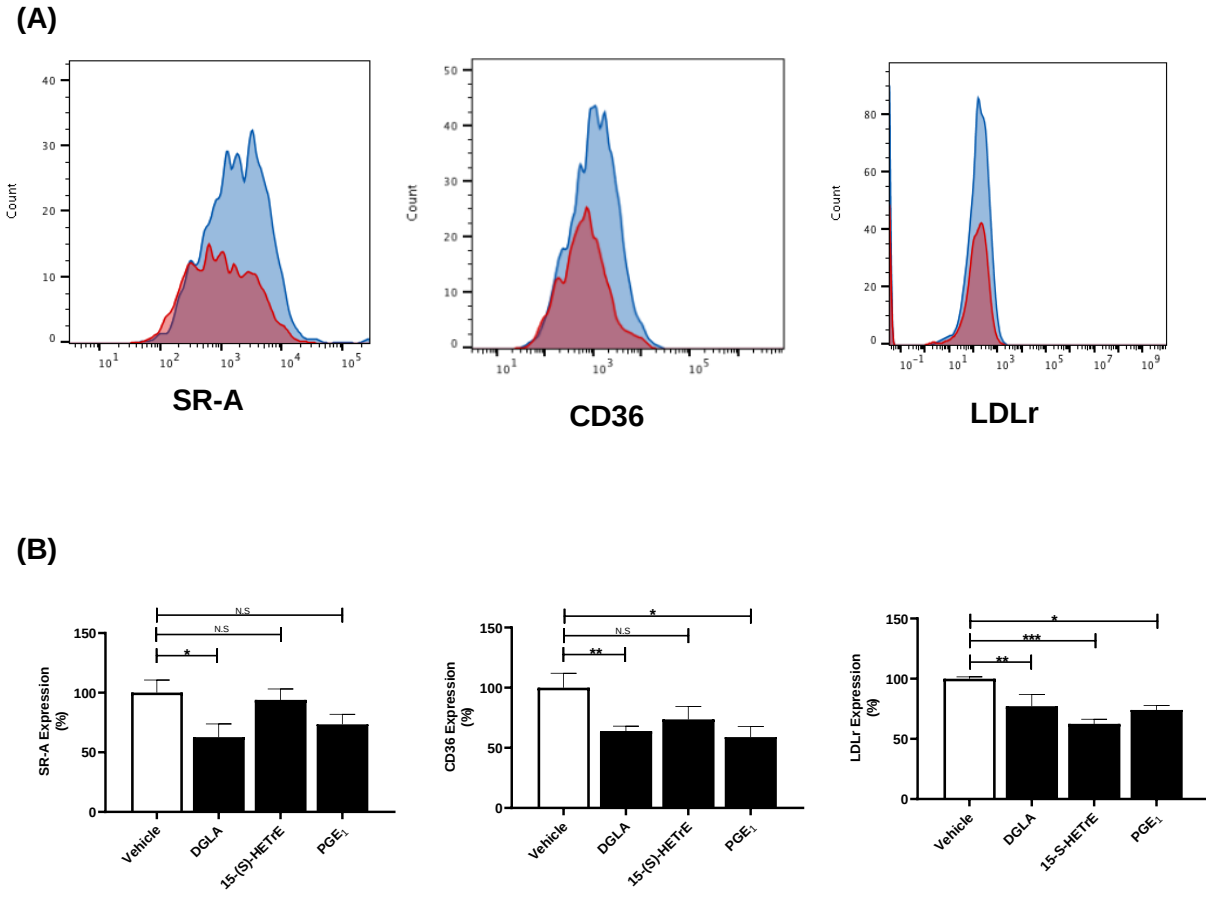


Figure 3. 14 The effect of DGLA and its metabolites on the cell surface expression of SR-A, CD36 and LDLr.

THP-1 macrophages were incubated for 24 hours with control vehicle or 50 μM DGLA, 2 μM 15-(S)-HETrE and 10 μM PGE_1. The cell surface expression of SR-A, CD36 and LDLr was determined by flow cytometry. (A), representative data from flow cytometry; (B), graphs showing mean expression +/- SEM from four independent experiments (levels in cells treated with the vehicle have been arbitrarily assigned as 1). Blue histogram represents control vehicle and red histogram represents 50 μM DGLA, 2 μM 15-(S) HETrE or 10 μM PGE_1. Statistical analysis was performed using an unpaired Student's two-tailed t-test (*, P ≤0.05; **, P ≤ 0.01, *** P≤0.001 and N.S, not significant).

3.3.7 The effect of DGLA and its metabolites on the expression of MCP-1 and ICAM-1 induced by IFN-γ in THP-1 macrophages under various conditions.

Previous research has shown that IFN-γ is a pro-inflammatory cytokine that promotes atherosclerosis (McLaren and Ramji 2009). It is often defined as one of the key atherosclerosis regulators because of its multiple functions in the initiation and development of the disease (McLaren and Ramji 2009; Li et al. 2010). Therefore, the effect of DGLA and its metabolites on IFN-γ induced pro-inflammatory gene expression was analysed using MCP-1 and ICAM-1 as model genes. Additionally, the treatment of stimulation with IFN-γ (250 U/mL) was varied to delineate the effects of DGLA and its metabolites under three different conditions: pre-treatment with DGLA or metabolites before IFN-γ stimulation (i.e., prevention of inflammation); DGLA and metabolites added with IFN-γ (i.e., effect at start of inflammatory stimulus); and DGLA and metabolites added after IFN-γ stimulation (i.e., ability to inhibit inflammation once this has taken place). The effect of DGLA and its metabolites on IFN-γ-induced expression of ICAM-1 and MCP-1 mRNA expression was evaluated by qPCR.

As shown in Figures 3.13, when THP-1 macrophages were first treated with IFN-γ (250 U/ mL) and then followed with treatment with DGLA or its metabolites, 50 μM DGLA had no significant effect on IFN-γ induced expression of either MCP-1 or ICAM-1 genes (panels A and B). However, the IFN- γ induced expression of ICAM-1 was significantly reduced by 1.45-fold ($p=0.005$) by 2 μM 15-(S)-HETrE (Figure 3.13D), whereas 10 μM PGE_1 produced a significant increase of the IFN-γ induced expression of MCP-1 by 0.59-fold ($p=0.001$) (Figure 3.13E). No significant changes were observed with 15-(S)-HETrE for IFN-γ induced MCP-1 expression (Figure 3.13C) or for PGE_1 for IFN-γ induced ICAM-1 expression (Figure 3.13F).

Incubation of the cells with IFN-γ (250 U/mL) together with DGLA and its metabolites was not found to significantly attenuate the IFN-γ induced expression of either MCP-1 or ICAM-1 genes except for 10 μM PGE_1, where an inhibition of MCP-1 expression by 1.74-fold ($p=0.039$) was observed (Figure 3.14).

In contrast to the findings above, pre-treatment of the cells with DGLA or its metabolites attenuated the subsequent IFN-γ induced MCP-1 and ICAM-1 expression. Thus, the IFN-γ induced MCP-1 and ICAM-1 expression was attenuated by 1.76-fold ($p=0.007$) and 1.58-fold ($p=0.013$) respectively by 50 μM DGLA (Figure 3.15 A-B). Treatment with 2 μM 15-(S)-HETrE

produced a significant reduction of the IFN-γ-induced expression of MCP-1 by 1.65-fold (p=0.027) and ICAM-1 by 1.38-fold (p=0.037) (Figures 3.15 C-D). In addition, PGE_1 inhibited the IFN-γ induced MCP-1 and ICAM-1 expression by 1.59-fold (p=0.076) and 1.53-fold (p=0.003) respectively (Figure 3.15E-F). In all cases, the basal expression of the genes seen in vehicle treated cells was not significantly affected in the presence of DGLA of its metabolites.

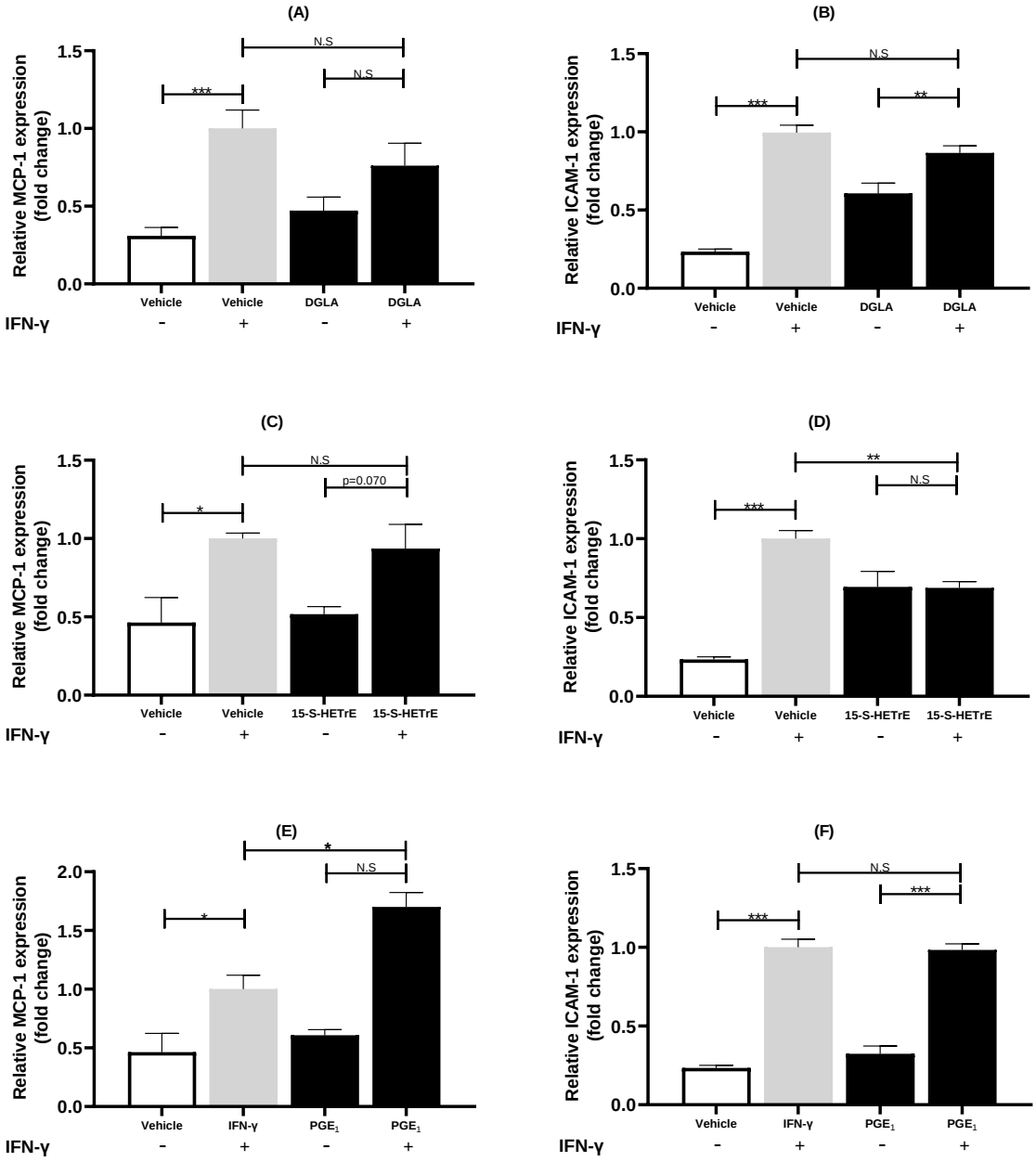


Figure 3. 15 The *in vitro* effects of DGLA, 15-S-HETrE and PGE_1 on IFN-γ induced MCP-1 and ICAM-1 expression in human macrophages following prior stimulation with the cytokine.

THP-1 macrophages were pre-treated with 250 U/ml IFN-γ (+) for 3 hours followed by incubation with 50 μM DGLA, 2 μM 15-S-HETrE and 10 μM PGE_1 or DMSO vehicle control (−) for 24 hours. RT-qPCR was used to measure the expression of MCP-1 (A, C and E), ICAM-1(B, D and F) or the housekeeping gene GAPDH. The level of the mRNA expression was determined using a comparative ΔΔCT method and normalised to the housekeeping gene with values from cells treated with the DMSO vehicle and IFN-γ arbitrarily assigned as 1. The results show average gene expression (mean +/- SEM) from four independent experiments. Statistical analysis was performed using a One-way ANOVA with Tukey's post hoc analysis (* P≤0.05, ** P≤0.01, *** P≤0.001 and N.S, not significant).

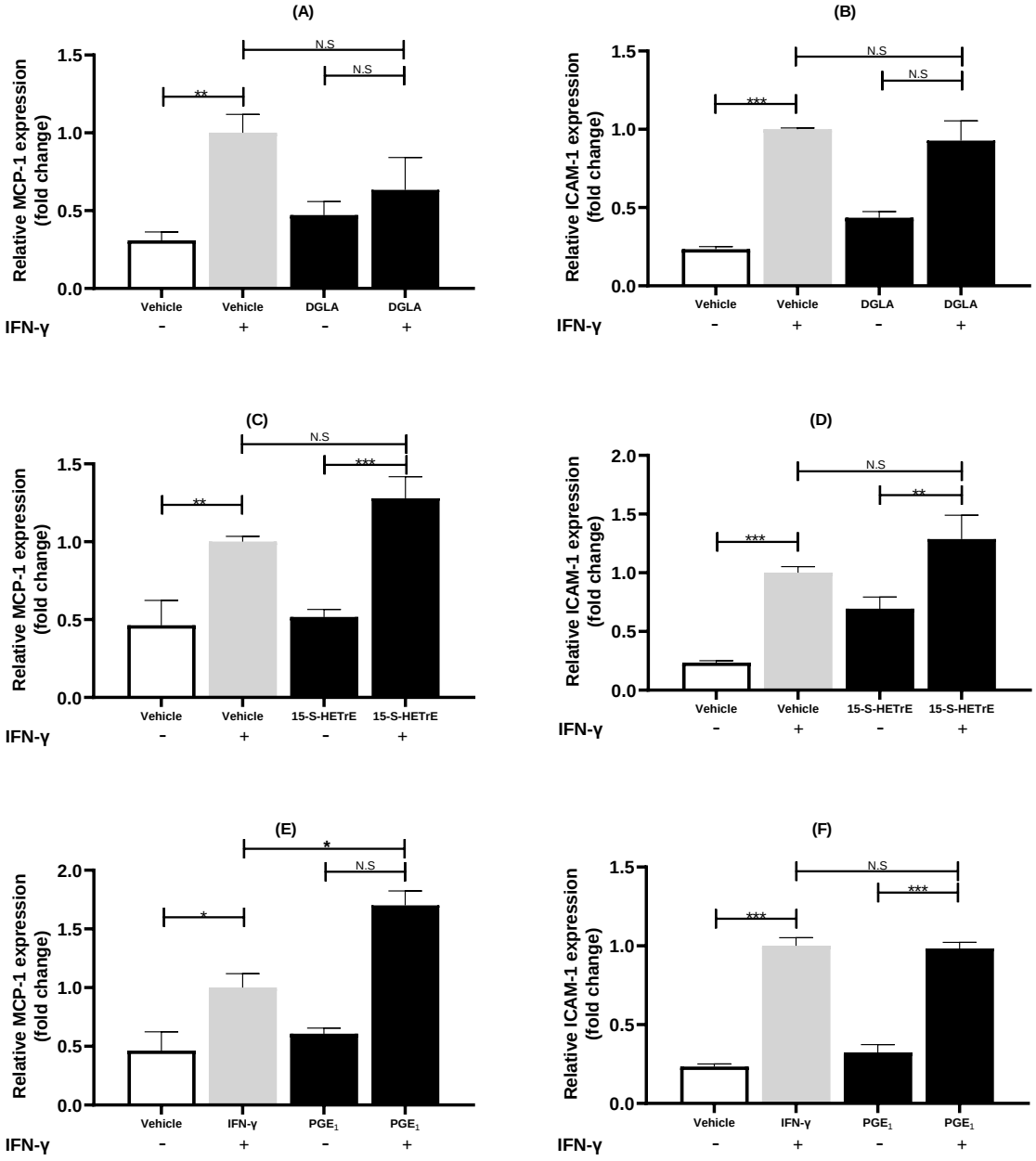


Figure 3. 16 The *in vitro* effects of DGLA, 15-S-HETrE and PGE_1 on IFN-γ induced MCP-1 and ICAM-1 expression in THP-1 macrophages.

The cells were incubated with DMSO vehicle control, 50 μM DGLA, 2μM 15-S-HETrE or 10μM PGE_1 together with 250 U/ml IFN-γ (+) for 24 hours. RT-qPCR was used to measure the expression of MCP-1 (A, D and E), ICAM-1(B, D and F) or the housekeeping GAPDH. The mRNA levels were determined using the comparative ΔΔCT method and normalised to the housekeeping gene with values from cells in the DMSO vehicle control and IFN-γ arbitrarily assigned as 1. The results show average gene expression (mean +/- SEM) from four independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey's post hoc analysis (* P≤0.05, ** P≤0.01, *** P≤0.001, N.S, not significant).

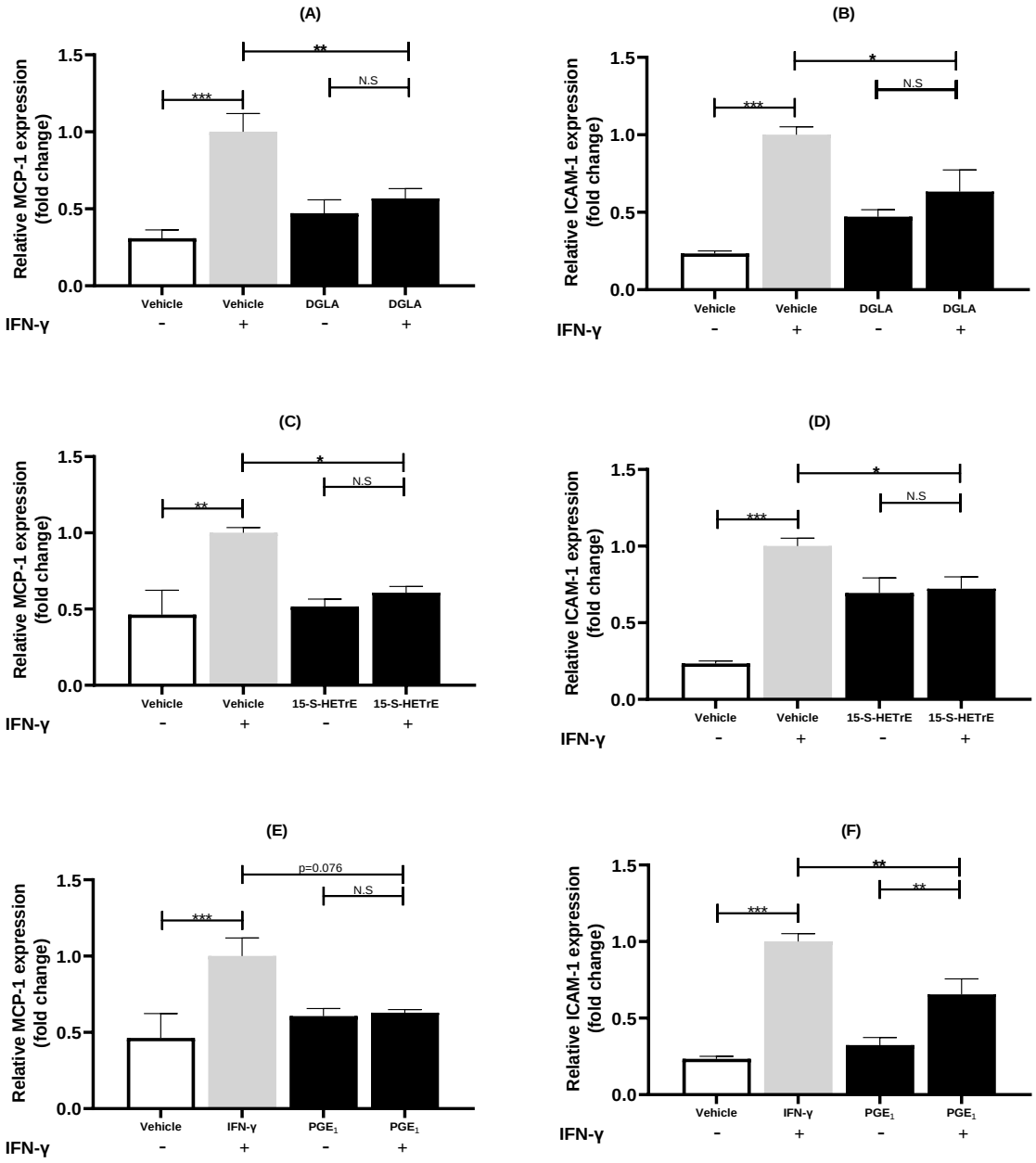


Figure 3. 17 The *in vitro* effects of pre-treatment of THP-1 macrophages with DGLA, 15-S-HETrE and PGE_1 on subsequent IFN-γ induced MCP-1 and ICAM-1 expression.

The cells were incubated with DMSO vehicle control, 50 μM DGLA, 2 μM 15-S-HETrE or 10 μM PGE_1 for 24 hours followed by 250 U/ml IFN-γ (+) for 3 hours. RT-qPCR was used to measure the expression of MCP-1 (A, D and E), ICAM-1(B, D and F) or the housekeeping gene GAPDH. The levels of mRNA were determined using the comparative ΔΔCT method and normalised to the housekeeping gene with values from cells in the DMSO vehicle control with IFN-γ arbitrarily assigned as 1. The results show average gene expression (mean +/- SEM) from four independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey's post hoc analysis (* P≤0.05, ** P≤0.01, *** P≤0.001, N.S, not significant).

3.3.8 DGLA and its metabolites do not affect cell viability and proliferation of HMDM used for *in vitro* studies.

To confirm that the results obtained with THP-1 macrophages were not due to the transformed nature of the cell line, key findings were replicated in primary cultures of HMDMs. After a range of concentrations of 15-(S)-HETrE (1 µM, 2 µM, 5 µM and 10 µM) with THP-1 macrophages were evaluated to establish an optimal concentration, as detailed above, 2 µM of 15-(S)-HETrE was used for these studies.

The viability and proliferation of HMDM under different conditions were first investigated using LDH and crystal violet assays as respectively described above for THP-1 macrophages. As shown in Figure 3.16, no significant effects on cell viability and proliferation were observed following treatment of the cells with 50 µM DGLA, 2 µM 15-(S)-HETrE or 10 µM PGE_1 for 24 hours when compared to the vehicle control.

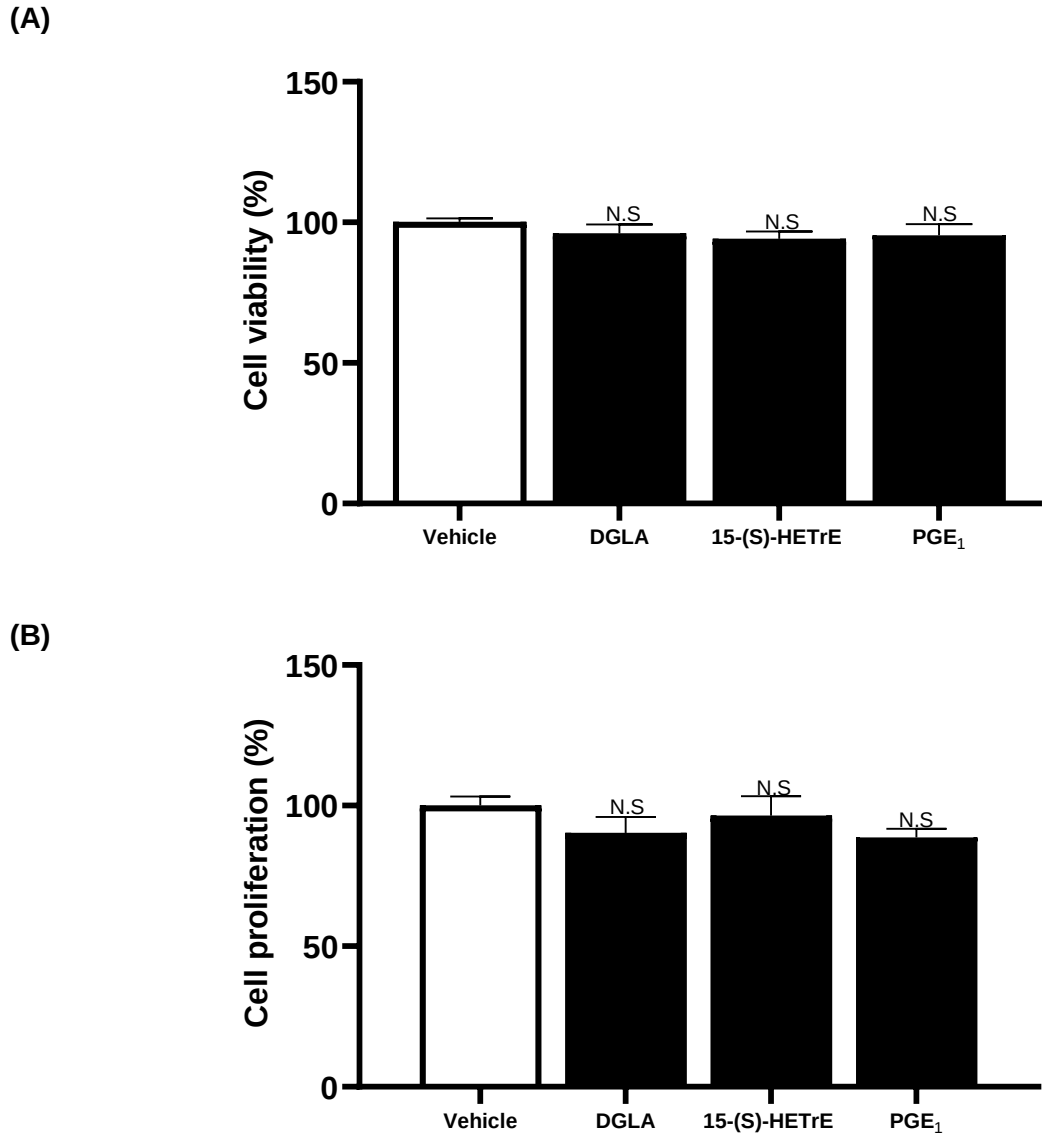


Figure 3. 19 DGLA, 15-(S)-HETrE or PGE_1 have no effect on HMDM viability and proliferation.

HMDM were incubated with 50 μM DGLA, 2 μM 15-S-HETrE, 10 μM PGE_1 or the DMSO vehicle for 24 hours. The results are shown as percentage (mean +/- SEM) to the DMSO control (arbitrarily assigned as 1) from four independent experiments. Statistical analysis was carried out by One-way ANOVA, comparing each individual treatment to control, and no significant changes were observed. N.S, not significant.

3.3.9 Macropinocytosis

The previously observed inhibition of macropinocytosis by DGLA and its metabolites in THP-1 macrophages (Figure 3.9) was next analysed in HMDM. As shown in Figure 3.17, 50 μM DGLA, 2 μM 15-(S)-HETrE or 10 μM PGE_1 reduced macropinocytosis seen in vehicle-treated cells by 31.7% (p= 0.088; trend towards significance), 34.1% (p= 0.030) and 37.1% (p= 0.012, respectively.

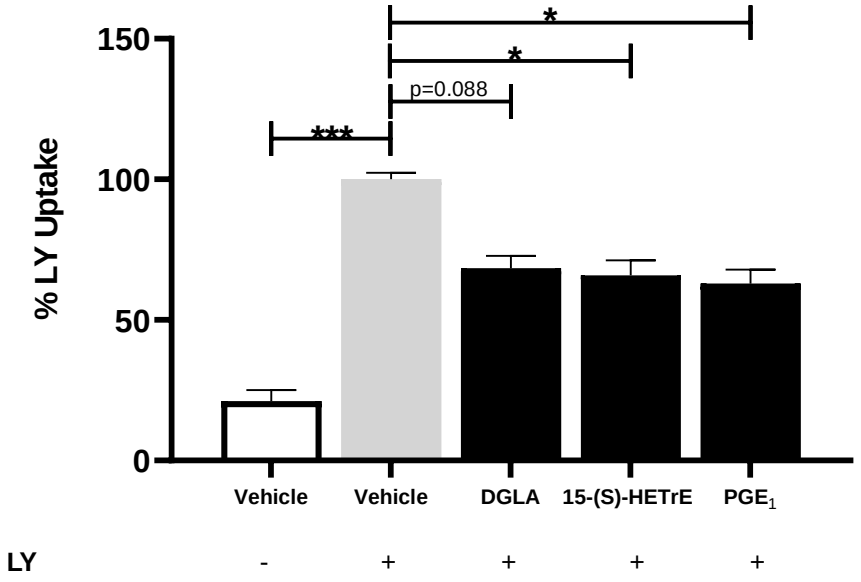


Figure 3. 20 The effect of DGLA, PGE_1 or 15-(S)-HETrE on LY uptake by macropinocytosis in HMDMs.

The cells were incubated with DMSO vehicle control, 50 μM DGLA, 2 μM 15-(S)-HETrE or 10 μM PGE_1 for 24 hours prior to addition of 100 μg/ml of LY for a further 24 hours. Cells treated with DGLA in the absence of LY were also included for comparison. Macrophages were resuspended in 2% PFA and FACS analysis was performed to determine uptake, counting 10,000 events. Results are mean +/- SEM from four independent experiments. Statistical analysis was carried out by One-way ANOVA followed by Dunnett 2-sided post-hoc test (* P≤0.05 *** P≤0.001).

3.3.10 Dil-oxLDL uptake

The uptake of Dil-oxLDL uptake in HMDMs was next analysed, as this is another critical event in macrophage foam cell formation. As shown in Figure 3.18, Dil-oxLDL uptake in control vehicle treated cells was reduced by 50 μM DGLA (11.4%; p=0.013), 2 μM 15-(S)-HETrE (18.4%; p<0.001) or 10 μM PGE_1 15.1% (P<0.001).

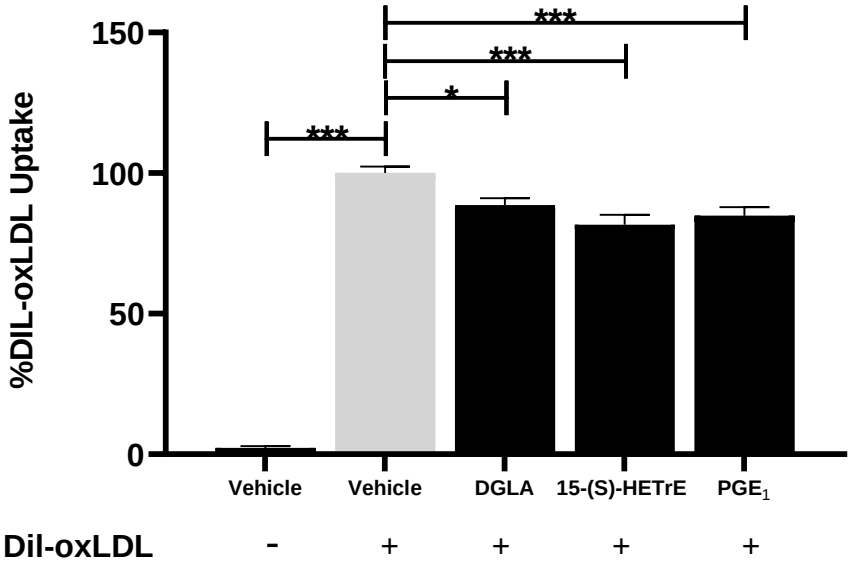


Figure 3. 21 The effect of DGLA, PGE_1 or 15-(S)-HETrE on oxLDL uptake by HMDMs.
The cells were incubated with DMSO vehicle control, 50 μM DGLA, 2μM 15-(S)-HETrE or 10 μM PGE_1 for 24 hours prior to the addition of 5 μg/ml Dil-oxLDL for a further 24 hours. Macrophages were resuspended in 2% PFA, and FACS analysis was employed to determine uptake, counting 10,000 events. Results are mean +/- SEM from three independent experiments. Statistical analysis was carried out by One-way ANOVA followed by Tukey's post hoc test (* P≤0.05 and *** P≤0.001).

3.3.11 Expression of macrophage SRs SR-A and CD36 in HMDM

The impact of DGLA and its metabolites on the main genes involved in modified LDL uptake was also analysed at the mRNA level by RT-qPCR as for THP-1 macrophages detailed above. As shown in Figure 3.19, the expression of SR-A was significantly reduced in the presence of DGLA and PGE_1 by 35.5% (p=0.006) and 49.8% (p=0.029) respectively but was non-significantly reduced in the presence of 15-(S)-HETrE by 30.0%. Gene expression of CD36 was also significantly reduced in the presence of DGLA by (70.9%, p= 0.029) but was non-significantly reduced in the presence of 15-(S)-HETrE or PGE_1.

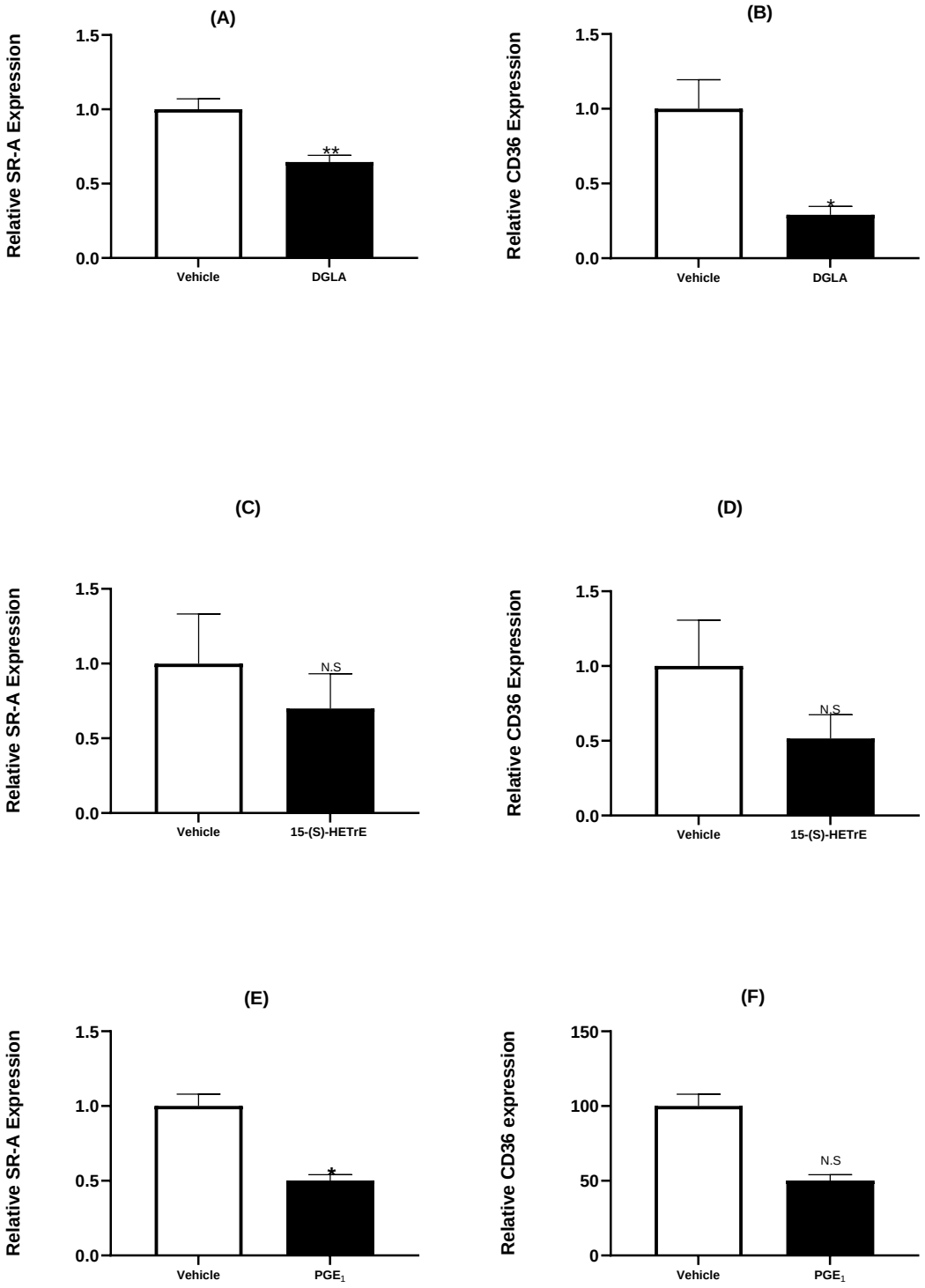


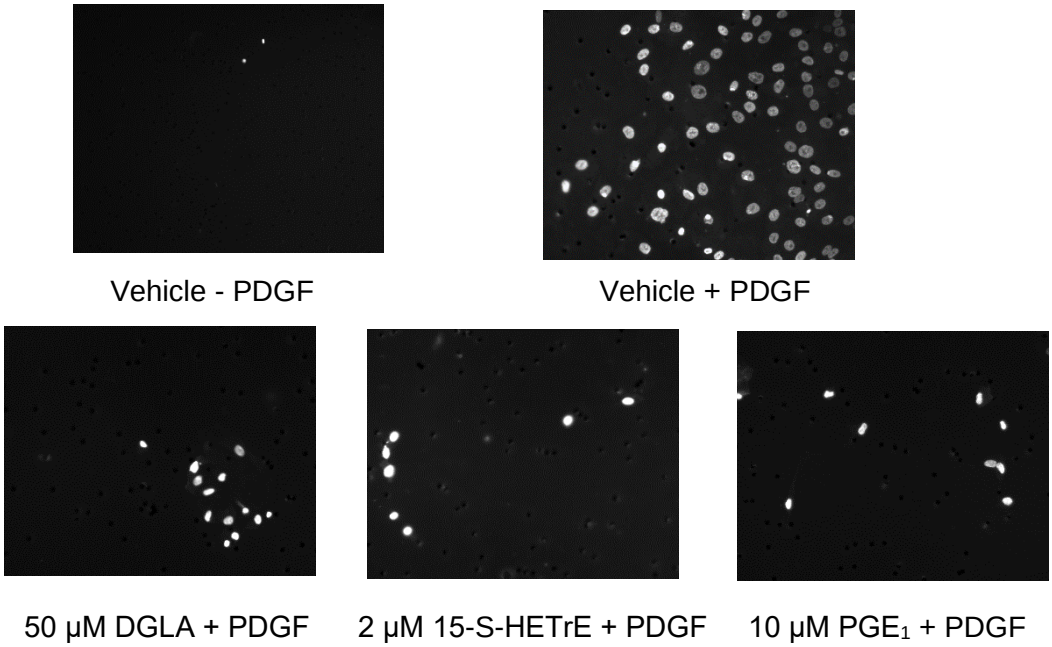
Figure 3. 22 DGLA and its metabolites inhibit the expression of SR-A and CD36 in primary human macrophages.

HMDMs were incubated with 50 μM DGLA (A, D), 2 μM 15-(S)-HETrE (B, D) or 10 μM PGE_1 (E, F) or control DMSO vehicle for 24 hours. Total RNA was subjected to RT-qPCR with primers specific for SRA, CD36 or GAPDH control. The results show average gene expression (mean +/- SEM) (control arbitrarily assigned as 1) from four independent experiments. Statistical analysis was carried out using a two-tailed, unpaired Student's t-test (* $P \leq 0.05$, ** $P \leq 0.01$ and N.S, not significant).

3.3.12 VSMC invasion

VSMC migration plays a key role in atherogenesis, such as in the formation of a fibrous cap, and is also a characteristic of advanced plaques (Bennett et al. 2016). The effect of DGLA and its metabolites on PDGF-BB-mediated migration of HASMCs was therefore determined. Figure 3.20 shows that the PDGF-induced migration of HASMCs was significantly reduced in the presence of 50 μM DGLA, 2 μM 15-(S)-HETrE or 10 μM PGE_1 by 80.3% (p=0.049), 79.7% (p=0.040) and 82.2% (p=0.035) respectively.

(A)



(B)

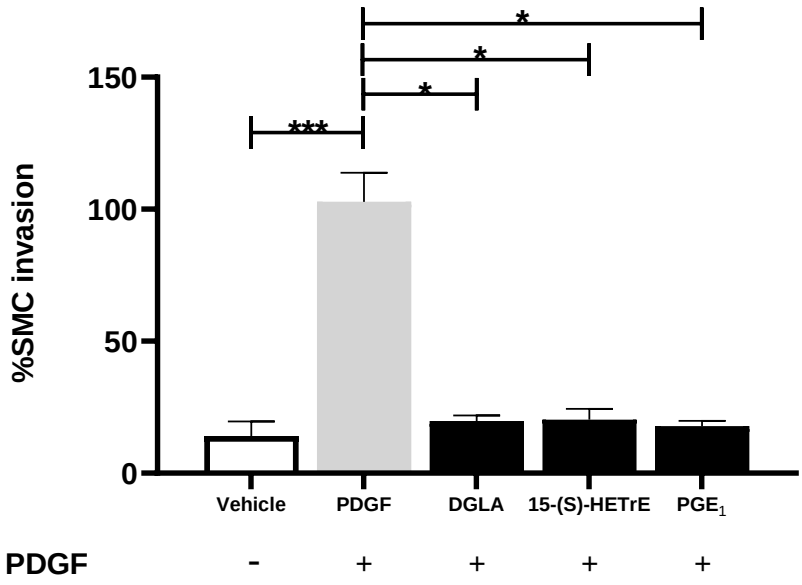


Figure 3. 23 DGLA, 15-(S)-HETrE and PGE_1 significantly inhibits the migration of HASMCs.

HASMCs were incubated with the DMSO vehicle control in the absence or the presence of PDGF or with PDGF in the presence of 50 µM DGLA, 2µM 15-(S)-HETrE or 10 µM PGE_1. SMC invasion was determined by counting the number of cells that successfully migrated over a cell insert. The number of migrated cells were counted and averaged per five high power fields (HPF), and the value from the PDGF vehicle control has been arbitrarily assigned as 100%. The results are mean +/- SEM from four independent experiments. Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc test (* P≤0.05, ** P≤0.01, *** P≤0.001 and N.S, not significant).

3.4 Discussion

The results presented in this chapter show that DGLA, 15-(S)-HETrE and PGE_1 inhibit monocytic migration, macropinocytosis and oxLDL uptake in human macrophages. In addition, they provided information about the effect of DGLA and its metabolites on the expression of a group of genes which are involved in regulating several key cellular processes and functions during atherosclerosis. These results suggest a potentially important role for PGE_1 and 15-(S)-HETrE in mediating the anti-atherogenic actions of DGLA, which requires further investigation *in vitro* and *in vivo*. Tables 3.1, 3.2 and 3.3 outline all the processes that were analysed.

Table 3. 1 Summary of the effect of DGLA and its metabolites on various cellular processes in THP-1 macrophages.

Cellular processes	DGLA		15-(S)-HETrE		PGE_1	
	Effect	**P-value**	**Effect**	**P-value**	**Effect**	**P-value**
Viability	NC	NS	NC	NS	NC	NS
Proliferation	NC	NS	NC	NS	NC	NS
Monocyte migration	↓	0.045(*)	↓	0.030(*)	↓	0.077(T)
Macropinocytosis	↓	0.008(**)	↓	0.023(*)	↓	0.024(*)
Dil-oxLDL uptake	↓	0.015(*)	↓	0.016(*)	↓	0.048(*)
SR CD36 expression (qPCR)	↓	0.027(*)	-	0.200(NS)	↓	0.029(*)
SR SRA1 expression (qPCR)	↓	0.027(*)	↓	0.057(T)	↓	0.029(*)
SR CD36 levels (FACS)	↓	0.009(**)	-	0.232(NS)	↓	0.080(T)
SR SRA1 levels (FACS)	↓	0.036(*)	-	>0.100(NS)	-	0.607(NS)

LDLR levels (FACS)	↓	0.005(**)	↓	<0.001(***)	↓	0.023(*)
Effect on prior IFN-γ treatment on MCP-1 expression	-	0.414(NS)	-	0.980(NS)	↑	0.002(**)
Effect of treatments with IFN-γ together on MCP-1 expression	-	0.225(NS)	-	0.301(NS)	↓	0.040(*)
Effect of pre-treatment on subsequent IFN-γ induced MCP-1 expression	↓	0.007(**)	↓	0.027(*)	↓	0.076(T)
Effect on prior treatments with IFN-γ on ICAM-1 expression	-	0.300(NS)	↓	0.005(**)	-	0.993(NS)
Effect of treatments with IFN-γ together on ICAM-1	-	0.171(NS)	-	0.275(NS)	-	0.712(NS)
Effect of pre-treatment on subsequent IFN-γ induced ICAM-1 expression	↓	0.013(*)	↓	0.037(*)	↓	0.003(**)

Abbreviations: NC-No changes in relation to control; NS- no significant effect; T-trend; ↓downregulation; ↑upregulation

Table 3. 2 Summary of the effect of DGLA and metabolites on various cellular processes in HMDMs.

Cellular processes	DGLA		15-(S)-HETrE		PGE_1	
	Effect	P-value	Effect	P-value	Effect	P-value
Viability	NC	NS	NC	NS	NC	NS
Proliferation	NC	NS	NC	NS	NC	NS
Macropinocytosis	↓	0.028(*)	↓	0.041(*)	↓	0.003
Dil-oxLDL uptake	↓	0.029(*)	-	0.234(NS)	↓	0.080(T)
SR CD36 expression (qPCR)	↓	0.029(*)	-	0.486(NS)	-	0.343(NS)
SR SRA1 expression (qPCR)	↓	0.006(**)	-	0.343(NS)	↓	0.029(*)

Abbreviations: NC-No changes in relation to control; NS- no significant effect; T-trend; ↓downregulation; ↑upregulation.

Table 3. 3 Summary of the effect of DGLA and metabolites on various cellular processes in HASMCs.

Cellular processes	DGLA		15-(S)-HETrE		PGE1	
	Effect	P-value	Effect	P-value	Effect	P-value
Viability	NC	NS	NC	NS	NC	NS
Proliferation	NC	NS	NC	NS	NC	NS
Invasion	↓	0.013(*)	↓	0.011(*)	↓	0.001(**)

Abbreviations: NC-No changes in relation to control; NS- no significant effect; T-trend; ↓downregulation; ↑upregulation.

3.4.1 Monocyte migration

Previous research into MCP-1 expression in atherosclerosis has explained how this chemokine transfers blood-borne monocytes to the arterial intima through the endothelium (Bobryshev, 2006). It is also clear from previous research that DGLA can inhibit MCP-1 expression caused by pro-inflammatory cytokines (Gallagher et al. 2019). MCP-1-induced monocyte migration through a barrier mimicking an arterial endothelial wall should therefore be inhibited by DGLA, 15-(S)-HETrE and PGE_1. Indeed, the MCP-1-induced monocytic migration was inhibited by all these agents (Figure 3.8). *In vivo* suppression of monocyte migration has been demonstrated to be atheroprotective in the $ApoE^{-/-}$ mouse model of atherosclerosis. For instance, gremlin-1 suppressed the *in vivo* migration of monocytes, resulting in a reduced monocyte and macrophage content in plaques and increased atheroprogression (Mueller et al. 2013). Moreover, the fully oxidized phospholipid small molecule named VB-201 selectively inhibited monocyte chemotaxis by 90% *in vitro*, and administration of the compound prevented the progression of atherosclerosis *in vivo* (Feige *et al.* 2013). The role of DGLA in inhibiting monocyte migration is also consistent with decreased macrophage accumulation observed in a previous *in vivo* study (Takai *et al.* 2009a).

3.4.2 HASMCs invasion

SMC migration and proliferation play important roles in atherosclerosis pathology (e.g., the formation of a fibrous cap), and the process is tightly regulated by various factors released by resident macrophages, endothelial cells and T-cells (McLaren *et al.* 2011a; Michael *et al.* 2012). Studies have demonstrated that PDGF-BB and the PDGF-β receptor (PDGFRβ) are expressed in atherosclerotic plaques within VSMCs, and their deficiencies reduce the migration and proliferation of SMCs (He *et al.* 2015; Ricci and Ferri 2015; Wang and Butany 2017) . The inhibitor of tyrosine kinase, Imatinib, blocks PDGFR activity, and in diabetic $ApoE^{-/-}$ mice treated with imatinib, the overall size of the plaque was decreased, together with PDGF-BB expression and PDGFR-β phosphorylation (Lassila *et al.* 2004; Ricci and Ferri 2015). The results of the migration analysis showed that 50 μM DGLA, 2 μM 15-(S)-HETrE or 10 μM PGE_1 reduced SMC migration by around 80% compared with the DMSO vehicle control (Figure 3.20). This provides a better understanding of the wider role of DGLA and its metabolites on plaque development. In addition, the reduction of VSMC invasion is consistent with reduced levels of these cells in plaques *in vivo* produced by dietary DGLA supplementation (Takai *et al.* 2009a).

3.4.3 Macropinocytosis

Macropinocytosis of solute macromolecules is a type of non-selective endocytosis (Swanson and Watts 1995) that is involved in LDL and to a certain extent in modified LDL uptake by macrophages and the development of foam cells. McLaren et al. (2011) previously investigated the impact of omega-3 PUFAs on macropinocytosis; the THP-1 macrophage uptake of acLDL via macropinocytosis was found to be inhibited by eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Ulven et al. 2011). The effect of DGLA on macropinocytosis was also assessed in PMA-differentiated macrophages. PMA, a phorbol ester, has been widely used in published studies to induce macropinocytosis in differentiated macrophages (Swanson 1989). For example, PMA improved macropinocytosis in BMDM macrophages (Swanson 1989) and LDL and modified LDL uptake by HMDM (Kruth et al. 2002; Kruth et al. 2005) and THP-1 macrophages (McLaren et al. 2011b; Michael et al. 2013). Cellular macropinocytosis can be assessed by monitoring LY uptake by FACS, and this technique has been used in several previous studies (Swanson and Watts 1995; Jones and Willingham 1999; McLaren et al. 2011b; Michael et al. 2013). Following on from monocyte migration, we initially assessed the effect of DGLA and its metabolites on macropinocytosis by macrophages and found a significant attenuation of LY uptake with 50 μM DGLA, 2 μM of 15-(S)-HETrE or 10 μM PGE_1 (Figure 3.9). Inhibition of macropinocytosis involving the uptake of LDL and modified forms of LDL (including acLDL) by DGLA, PGE_1 and 15-(S)-HETrE could be responsible for the inhibition of cholesteryl ester accumulation and thus foam cell formation. However, further research is required into such an impact of DGLA and its metabolites on the uptake of native LDL *in vivo*.

3.4.4 Modified LDL uptake in macrophages

During atherosclerosis, macrophage receptor-mediated endocytosis may lead to the unregulated uptake of large amounts of modified LDL particles. A characteristic process in the development of foam cells is the storage of excess cholesterol as lipid droplets, which accumulate in the cytoplasm as cholesteryl esters (Shashkin et al. 2005; Lusis 2012). Consequently, suppressing the uptake of modified forms of LDL prevents the accumulation of excess cholesterol and the transformation of macrophages into foam cells. A fluorescently labelled modified form of LDL, DiI-oxLDL, was used to evaluate uptake. DiI-oxLDL uptake in both THP-1 macrophages and HMDMs was significantly attenuated by 10-20% following treatment with DGLA, 15-S-HETrE, and PGE_1 (Figures 3.10 and 3.18). Comparable findings using omega-3 fatty acid DHA were reported by McLaren et al. (2011b), who found that DHA

inhibited Dil-acLDL and Dil-oxLDL uptake by THP-1 macrophages. This pathway was reported to rely on both scavenger receptors and macropinocytosis (McLaren et al., 2011b). When THP-1 macrophage gene expression was evaluated and analysed, treatment with DGLA and its metabolites was found to significantly decrease the expression of scavenger receptors, SR-A and CD36 (Figure 3.11). DGLA and its metabolites were also found to attenuate SR-A and CD36 expression in HMDMs (Figure 3.19).

Overall, the experiments addressed in this chapter showed that DGLA and its metabolites significantly reduced macropinocytosis in human macrophages (Figure 3.9), and that macrophages also attenuated uptake of oxLDL, which was, at least in part, due to inhibition inhibiting macrophage scavenger receptors at the level of gene expression.

3.4.5 Scavenger receptors

Macrophage binding and internalisation of multiple molecules, such as pathogens and modified LDL, are achieved through receptor-mediated endocytosis. Scavenger receptors are expressed on the surface of macrophages, and the uptake of modified LDL is dynamically regulated (Kunjathoor et al. 2002). SRA and CD36 have been shown to uptake significant amounts of oxLDL and acLDL and to contribute to foam cell formation and atherosclerosis (Febbraio et al. 2000; Kunjathoor et al. 2002; Kuchibhotla et al. 2007). The expression levels of SRA and CD36 decreased following the treatment of THP-1 macrophages and HMDMs with DGLA and its metabolites. Song et al. (2013) have suggested that such changes are often related to a substantial reduction in the cholesterol content of foam cells, and comparable results have been observed in previous studies analysing the impacts of SRA and CD36 deficiency. In SRA and CD36 knockout mice, cholesterol ester accumulation in macrophages was impaired (Kunjathoor et al. 2002). $SRA^{-/-}$ macrophages had 70% reduced acLDL uptake compared with those from wildtype mice, and in particular, the binding of acLDL was decreased by 44% (Kunjathoor et al. 2002). CD36 appears to have a minor role in acLDL binding and degradation, as this was 28% less bound and 13% less degraded in $CD36^{-/-}$ macrophages (Kunjathoor et al. 2002). OxLDL uptake by CD36 has been shown to carry out other pro-inflammatory functions in addition to its role in foam cell formation. CD36 uptake of oxLDL reduced macrophage efflux from atherosclerotic plaques and facilitated macrophage trapping and increased plaque development (Park 2014). Additionally, oxLDL-binding to CD36 has been demonstrated to stimulate platelet aggregation and to assist with pro-thrombotic events (Chen et al. 2008). Eventually, in either the presence or absence of oxLDL, the $CD36^{-}$

$^{/-}$ macrophages expressed reduced amounts of IFN-α, MCP-1 and TNF-α (Kennedy et al. 2011).

The results obtained in this study confirm previous findings on the anti-atherogenic action of DGLA on macrophages (Gallagher 2016). The results show broadly that DGLA, 15-(S)-HETrE and PGE_1 attenuate monocyte migration, macropinocytosis and modified LDL uptake. Therefore, it seems likely that the actions of DGLA are mediated by PGE_1 and 15-(S)-HETrE, and this warrants further in-depth investigations.

3.4.6 IFN-γ

This study also aimed to determine the roles of DGLA in pro-inflammatory cytokine signalling and cytokine-induced expression of inflammatory markers, MCP-1 and ICAM1, in relation to atherosclerosis. IFN-γ has been identified as a master regulator of atherosclerosis because of its pro-inflammatory actions throughout the various phases of disease development, from the formation of foam cells to lesion rupture (McLaren and Ramji, 2009). The expression of several pro-inflammatory genes, including MCP-1 and ICAM-1, is regulated by IFN-γ and is often essential for monocyte recruitment and adhesion (Rimbach et al., 2000, Li et al., 2010, Grandaliano et al., 1994, Lusis, 2000, Chang et al., 2002). As shown in Figures 3.13, 3.14, and 3.15, pre-treatment with DGLA, 15-(S)-HETrE, and PGE_1 respectively produced a significant reduction in the IFN-γ-induced expression of MCP-1 and ICAM-1 in THP-1 macrophages. These studies also showed that DGLA was effective following pre-treatment of the cells before IFN- γ (i.e., prevention) rather when added together or following induction of inflammation with IFN- γ. This inhibition of IFN-γ-mediated pro-inflammatory signals by DGLA is important because a deficiency in IFN-γ has been shown to substantially decrease atherosclerotic lesions in mouse models (Gupta et al., 1997), and IFN-γ functions have been extensively investigated for possible atherosclerosis treatment approaches. These findings are consistent with the study by Gallagher et al. (2019) who showed DGLA and PGE_1 effectively decreased the expression of MCP-1 and ICAM-1 in macrophages induced by IFN-γ. DGLA or its metabolites had no significant effect on the basal expression of MCP-1 and ICAM-1 genes seen in vehicle-treated cells.

3.5 Summary and future work

In this study, we intended to provide a comprehensive overview of the roles of DGLA and its metabolites in the inhibition of several major atherosclerosis processes, such as monocyte migration and foam cell formation (Figure 3.21). This chapter reports on the inhibition of monocyte migration, macrophage micropinocytosis and VSMC invasion by DGLA and its metabolites. In addition, we found that treatment of human macrophages *in vitro* with DGLA and its metabolites attenuated the receptor-mediated uptake of modified LDL and substantially attenuated the expression of scavenger receptors genes, SR-A and CD36. Treatment with DGLA and its metabolites, therefore, tended to have a range of favourable effects on macrophage activity. Based on these findings, we decided to expand the investigations to the effects of DGLA and its metabolites on human atherosclerosis-associated genes using qPCR microarrays. These studies are presented in the next chapter.

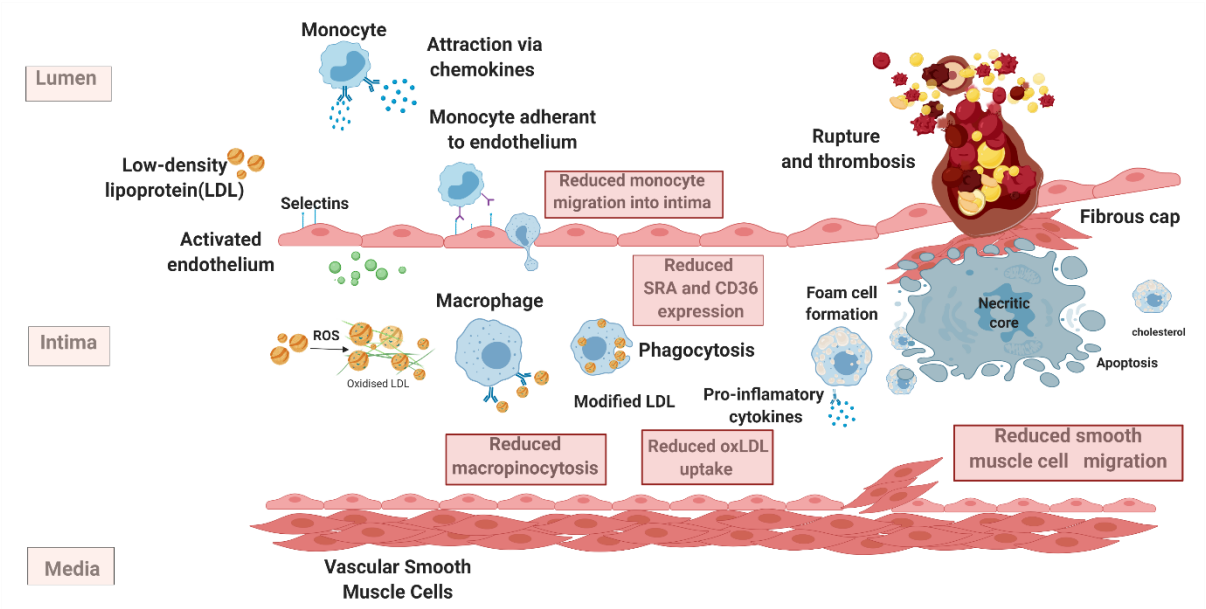


Figure 3. 24 Summary of the anti-atherogenic properties of DGLA and its metabolites. The steps reduced in atherosclerosis disease progression following DGLA and its metabolites treatment are highlighted in red. Created with BioRender.com.

Chapter 4 Regulation of gene expression in human macrophages by DGLA and its metabolites

4.1 Introduction

As presented in Chapter 3, DGLA and its metabolites have demonstrated a range of anti-atherogenic activities in *in vitro* experiments, including inhibition of chemokine-driven monocyte migration, reduced modified LDL uptake and foam cell formation, and inhibition of pro-inflammatory cytokine-induced gene expression. These promising findings on several atherosclerosis-associated processes suggest the need for further in-depth studies on the effects of DGLA and its metabolites on the expression of many genes using a qPCR microarray. Such analysis could potentially guide new fields of *in vitro* and *in vivo* investigations that have not been previously addressed. The genes in the qPCR microarray used (RT^2 Atherosclerosis arrays) are categorised according to their key functions in this disease. They include apoptosis, blood coagulation, cell adhesion, cell proliferation, metabolism of lipids, response to stress and transcriptional regulation. The critical roles of some of these genes and their encoding proteins in the development of atherosclerosis are summarised in Figure 4.1 and Table 4.1. Due to their essential functions in contributing to the development of atherosclerosis, it would be expected that DGLA and its metabolites might have a therapeutic value by inhibiting the expression of such pro-inflammatory genes and thereby secretion of some pro-inflammatory factors such as cytokines into the blood.

In the initial stages of atherosclerosis progression, cytokines have been found to play a crucial role from foam cell formation through to plaque progression and its rupture (Moss and Ramji 2016a). Studies on macrophages have considerably enhanced our understanding of the function of cytokines and their signalling pathways on these cells through disease development, together with macrophage processes in atherosclerosis. An analysis of gene expression in atherosclerosis indicated that macrophages are one of the most common sources of changes in this disease (Li et al. 2005). In the early stages of atherosclerotic lesion formation, endothelial cells undergo activation or dysfunction by oxLDL or cytokines such as IFN-γ or TNF-α (Ramji and Davies 2015), leading to monocyte recruitment via adhesion molecules such as VCAM-1 and ICAM-1 and their subsequent differentiation into macrophages (Stoneman and Bennett 2004). When macrophages transform into foam cells, they secrete several pro-inflammatory cytokines (Stoneman and Bennett 2004; Moss and Ramji 2016a). They eventually undergo apoptosis, which leads to the release of their intracellularly stored lipids into the intima of the artery, and also to the release of pro-inflammatory molecules like TNF-α and CCL2 (Stoneman and Bennett 2004; Moss and Ramji

2016a). The generation of oxidised lipids via ROS production by macrophages is another critical event in the additional recruitment of monocytes, which leads to a continuous pro-inflammatory response (Pamukcu et al. 2010). Apoptotic cell death continues to increase during plaque development, thereby encouraging the lipid-rich necrotic core to grow via the actions of pro-inflammatory cytokines such as TNF-α, IFN-γ and IL-1β (Stoneman and Bennett 2004; Bobryshev et al. 2016). As VSMC apoptosis increases via the action of cytokines such as IFN-γ produced by macrophages and foam cells, the fibrous cap becomes unstable and ruptures. This process is related more to the necrotic core size than to the size of the plaque itself (Stoneman and Bennett 2004; Tabas 2009).

4.2 Aims

Macrophages play a key role in all stages of atherosclerosis, from foam cells to plaque rupture (Moore and Tabas 2011).The formation of macrophage-derived foam cells is an important contributor to atherosclerosis development (Bobryshev 2006). The anti-atherogenic effect of DGLA has been shown in previous research, and its actions on macrophage gene expression are of substantial importance given its potential in the prevention and treatment of atherosclerosis. Previous studies have also shown that DGLA produces two key metabolites in human macrophages: PGE_1 and 15-(S)-HETrE. Studies presented in Chapter 3 showed that DGLA, PGE_1 and 15-(S)-HETrE regulated several atherosclerosis-associated cellular processes in monocytes/macrophages and VSMC such as inhibition of monocyte migration, macrophage micropinocytosis and VSMC invasion. Additionally, DGLA and its metabolites *in vitro* attenuated the receptor-mediated uptake of modified LDL and substantially attenuated the expression of scavenger receptor genes SR-A and CD36. It would therefore be of substantial interest to delineate the effects of DGLA, 15-(S)-HETrE and PGE_1 on atherosclerosis-associated gene expression in human macrophages to identify common and mediator-specific genes. This aspect formed the focus of studies in this chapter using Atherosclerosis Arrays containing 84 genes.

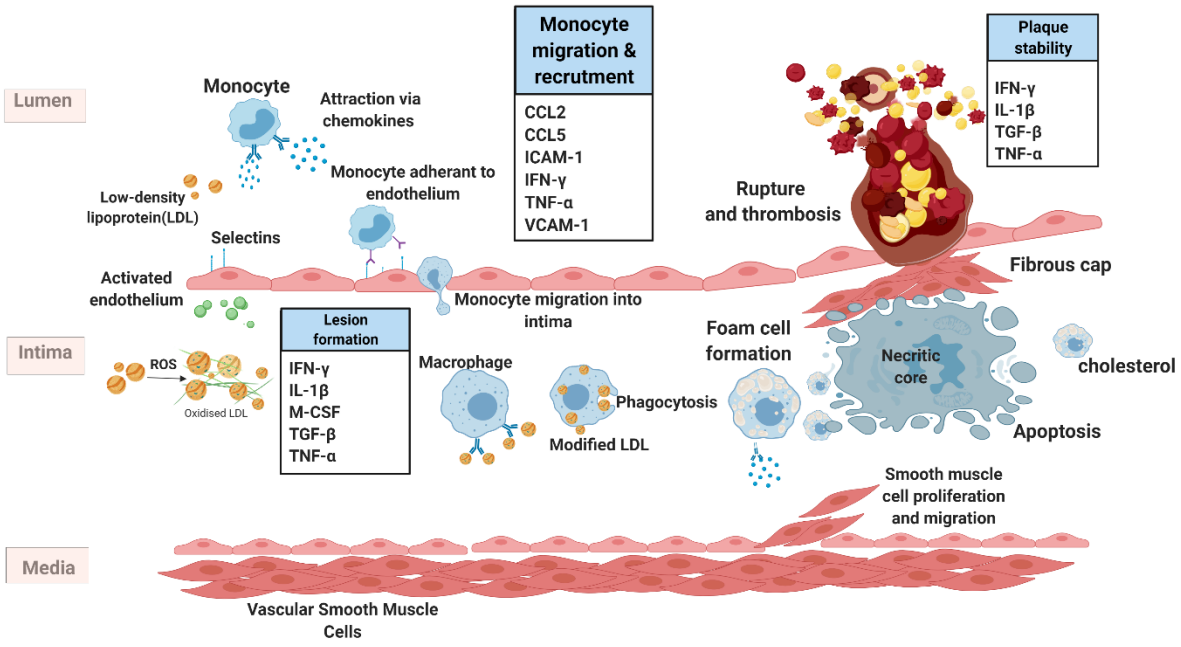


Figure 4. 1 Atherosclerosis development and the role of macrophages in key pro-inflammatory genes and cytokines.

Modified LDL accumulation in the intima of arteries causes an inflammatory response in the surrounding endothelial cells. Such cells secrete cytokines and chemokines, which are pro-inflammatory and recruit circulating monocytes. The monocytes differentiate to macrophages, which then take up modified LDL to transform into foam cells. Because accumulation of cholesterol is toxic, foam cells undergo apoptosis or necrosis, which then causes the development of a necrotic core. Smooth muscle cells migrate from the media to the intima, creating a plaque-stabilising fibrous cap over the necrotic core because of production of extracellular matrix proteins from these cells. Chronic inflammatory response in atherosclerosis ultimately results in an unstable atherosclerotic plaque that can rupture, causing a thrombotic reaction which might lead to myocardial infarction or stroke, depending on where the plaque occurs. List of abbreviations: CCL, chemokine (C-C motif) ligand; IFN, interferon; IL, interleukin; LDL, low-density lipoprotein; M-CSF, macrophage colony stimulating factor; TGF, transforming growth factor; TNF, tumour necrosis factor (Moss and Ramji, 2016).

Table 4. 1 The function of key pro- and anti-inflammatory genes and macrophages in atherosclerosis.

Protein	Role in atherosclerosis
ApoE	ApoE is produced by the liver and macrophages and constitutes a component of lipoprotein particles, as a ligand for lipoprotein receptors. It is an essential contributor to the anti-atherogenic lipoprotein metabolism and transport in general and also acts in an anti-inflammatory manner. A previous study showed that ApoE$^{-/-}$ mice develop complicated atherosclerotic lesions as a consequence of impaired cholesterol-enriched particle clearance, which leads to their accumulation in their plasma. (Curtiss 2000; Greenow et al. 2005)
CCL2	Plays an active role in the recruitment of monocytes to the arterial wall during atherosclerosis. In ApoE$^{-/-}$ mice, silencing of the gene using adenoviral vectors causes the stabilisation of the plaques. (Aiello et al. 1999; Winter et al. 2018)
CXCL1	Produced by macrophages and found at increased levels in CVD patients. It also plays a key role in neutrophil chemoattraction and leukocyte recruitment. (Zernecke and Weber 2010; Wan and Murphy 2013)
ICAM1	Involved in atherosclerosis by regulating monocyte recruitment into areas susceptible to atherosclerosis. In ApoE$^{-/-}$ mice, deficiency of Icam1 is associated with reduced size of vascular lesions. Potential therapeutic target as it is involved in the early stages of atherosclerosis by causing the recruitment of monocytes. (Nageh et al. 1997; Bourdillon et al. 2000; Galkina and Ley 2007)
IFN-γ	Actively involved in the pathogenesis of atherosclerosis. It also stimulates the development of foam cells, the formation of plaques and the adaptive immune response of Th1 cells.(McLaren and Ramji 2009)
MSR1 and CD36	Scavenger receptors such as MSR1 (SRA) and CD36 are primarily involved in foam cell formation in atherosclerosis. A previous study that used CD36–/–/ApoE–/– and Msr1–/–/ApoE–/– mice fed with an atherogenic diet confirmed the pro-atherogenic roles of these receptors via promotion of modified LDL uptake.(Moore et al. 2005; Kzhyshkowska et al. 2012)
LPL	Determines the metabolism of lipoproteins and their transport. Responsible for catalysing the hydrolysis of TG present in chylomicrons and VLDL. LPL expressed by macrophages is considered pro-atherogenic as it aids in the uptake of lipoproteins/modified lipoproteins via a “bridging action”. However, LPL expressed elsewhere is considered anti-atherogenic because it aids in the clearance of circulating lipoprotein particles.(Mead et al. 2002)

VCAM1	VCAM1 expression is stimulated in arterial endothelial cells in response to cholesterol accumulation. Involved in the recruitment of monocytes in the early stages of atherosclerosis.(Galkina and Ley 2007)
TNF-α	It helps leukocyte adhesion and its expression also stimulates apoptosis, thrombin formation and macrophage cholesterol uptake. ApoE$^{-/-}$/ TNF-α$^{-/-}$ mice demonstrate a reduction in lipid accumulation compared to control mice and a decrease in IL-1β, IFN-γ, ICAM1, VCAM1, MCP-1 and GM-CSF expression along with NF-κB activation. Additionally, compared to ApoE$^{-/-}$ control mice, ApoE$^{-/-}$/ TNF-α$^{-/-}$ mice show a decrease in the size of atherosclerotic plaques present in the aortic luminal surface and the aortic sinus. (Ohta et al. 2005; Bradley 2008; Xiao et al. 2009; Ramji and Davies 2015)

4.3 Experimental design

The studies presented in this chapter aimed to assess the effect of DGLA and its metabolites on atherosclerosis-associated gene expression in human macrophages. Figure 4.2 presents an outline of the experimental approach.

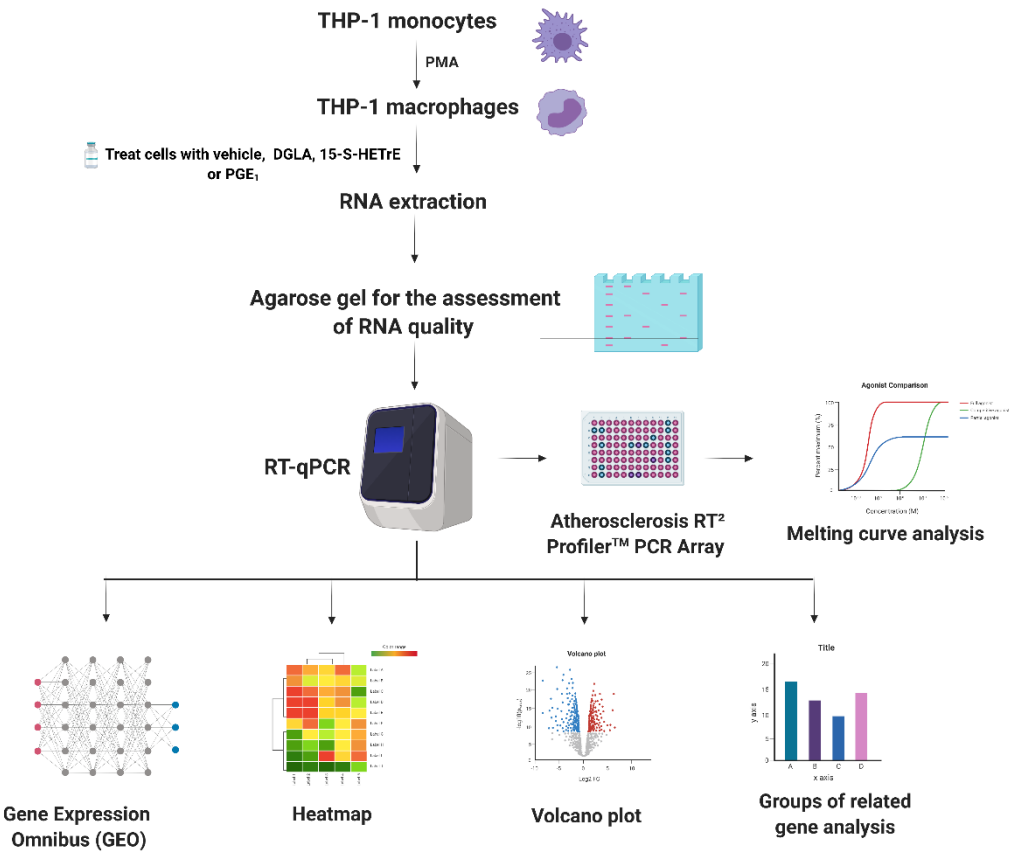


Figure 4. 2 The experimental strategy for the evaluation of gene expression. Created with BioRender.com.

4.4 Results

4.4.1 The effect of DGLA and its metabolites on the expression of key atherosclerosis-associated genes in human macrophages

Studies in chapter 3 showed that DGLA and Its metabolites significantly attenuate gene expression, particularly that of scavenger receptors SRA and CD36 (Figure 3.22). This is likely to be associated with decreased uptake of modified LDL observed *in vitro* (Figure 3.21). More in-depth analysis of the expression of a range of atherosclerosis-associated genes should therefore allow identification of other potential contributory mechanisms for the anti-atherogenic actions of DGLA and its metabolites. Atherosclerosis qPCR arrays were therefore used to investigate the expression of a large number of genes implicated in this disease.

Microarrays allow the expression of various genes to be quantified in a single experiment. Consequently, more data can be generated in shorter periods (Efron et al. 2001). A human atherosclerosis array was used to determine the overall effects of 50 μM DGLA, 2 μM 15-(S)-HETrE or 10 μM PGE_1 along with the vehicle control on the expression of 84 genes implicated in this disease. RNA preparations were evaluated for quality by agarose gel electrophoresis before performing qPCR reactions. Standard undegraded RNA generates two distinct 28S and 18S rRNA bands in approximately a 2:1 ratio. Figure 4.3 shows the RNA quality that was typically obtained from the studies described in this chapter (i.e., undegraded RNA of good quality was obtained). Additional parameters of good RNA quality and concentration were, measured with a NanoDrop™ ND2000 spectrophotometer. High quality RNA was suggested from ratios between 1.8-2.1 for the A260/A280 and the A230/A260 ratios.

The effects of DGLA, 15-S-HETrE and PGE_1 on the expression of 84 genes in the arrays grouped according to their functions, as defined by the manufacturer (Qiagen), are shown in Tables 4.2, 4.3 and 4.4, respectively. Such functions included stress reactions, cholesterol homeostasis, cell growth and proliferation, blood coagulation and control of inflammation. In addition, the expression of the housekeeping genes: β-actin (Actb); β-2-microglobulin (B2m); Gapdh; β-glucuronidase (Gusb); and heat shock protein HSP 90-β (Hsp90ab1); were found to be stable during the assay and were therefore used to calculate gene expression fold changes induced by DGLA and its metabolites. The data presented are the averages from different experiments from control and DGLA and its metabolites, with normalised values from control cells arbitrarily assigned as 1. A list of full gene names is included in Table 4.5. The findings showed that DGLA, 15-(S)-HETrE and PGE_1 significantly attenuate the expression of

15, 19 and 12 genes respectively and a further 2 (DGLA) and 6 (15-(S)-HETrE) indicate a trend in change in expression (p between 0.050 and 0.100; none in this category for PGE_1.

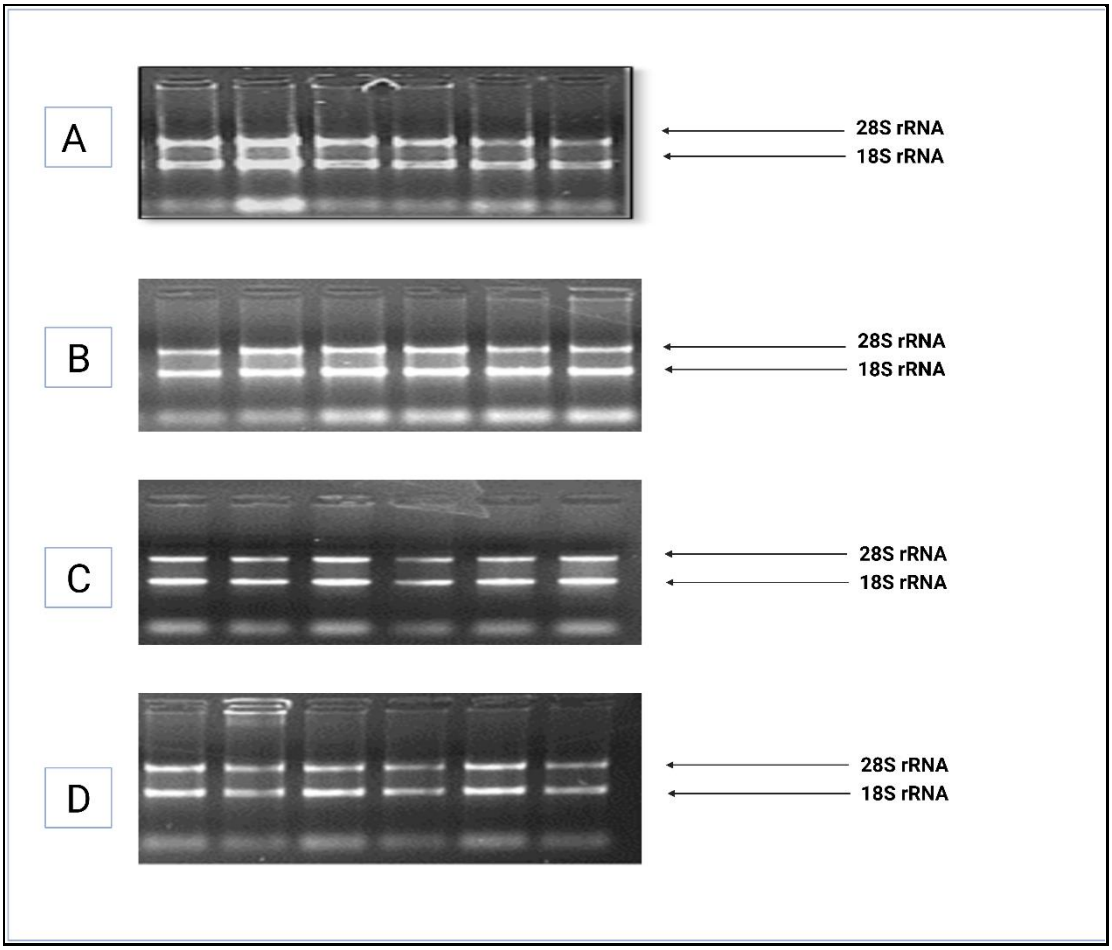


Figure 4. 2 Agarose gel photographs of RNA quality.
These data are indicative of the quality of RNA in this project. RNA was analysed by agarose gel electrophoresis, and the 28S rRNA and 18S rRNA bands are shown: (A) Vehicle (B) 50 μM DGLA (C) 2 μM 15-S-HETrE and (D) 10 μM PGE_1.

Table 4. 2 The effect of DGLA on the expression of atherosclerosis-associated genes in THP-1 macrophages.

		Control		DGLA			
Gene	**GenBank ID**	**N**	**Mean ± SEM**	**N**	**Mean ± SEM**	**Fold-change**	***P* value**	**%Change**
Stress Responses								
Ccl2	NM_002982	7	1±0.45	5	0.27±0.14	0.27	0.009**	↓72.58
Ccl5	NM_002985	7	1±0.49	6	1.51±0.46	1.5	0.361	↑50.70
Ccr1	NM_001295	6	1±0.51	5	1.75±0.55	1.74	0.287	↑74.80
Ccr2	NM_001123396	7	1±0.44	6	0.34±0.12	0.34	0.003**	↓72.58
Fn1	NM_002026	7	1±0.71	6	0.97±0.42	0.97	0.952	↓2.880
Il1r1	NM_000877	7	1±0.53	6	0.94±0.29	0.94	0.848	↓6.40
Il1r2	NM_004633	6	1±0.64	5	4.08±1.46	4.08	0.132	↑308.4
Itgb2	NM_000211	7	1±0.68	6	1.79±0.88	1.79	0.45	↑79.02
Pparg	NM_015869	7	1±0.54	6	0.69±0.22	0.695	0.267	↓30.52
Nfkb1	NM_003998	7	1±0.71	5	0.87±0.33	0.87	0.738	↓13.04
Nos3	NM_000603	6	1±0.69	6	0.93±0.70	0.93	0.931	↓6.91
Sele	NM_000450	5	1±0.52	4	0.43±0.28	0.43	0.178	↓56.76
Sod1	NM_000454	7	1±0.52	5	0.55±0.23	0.55	0.162	↓44.52
Spp1	NM_000582	7	1±1.42	6	0.86±0.28	0.86	0.658	↓14.19
Tnf	NM_000594	7	1±0.56	6	0.76±0.32	0.76	0.513	↓24.48
Apoptosis								
Bax	NM_004324	6	1±0.31	5	0.40±0.12	0.4	0.011*	↓60.36
Bcl2	NM_000633	7	1±0.38	6	0.36±0.19	0.36	0.029*	↓64.19
Bcl2a1	NM_004049	7	1±0.28	4	1.49±0.22	1.49	0.154	↑48.74
Bid	NM_001196	6	1±0.44	4	1.76±0.98	1.76	0.548	↑76.02
Birc3	NM_001165	7	1±0.50	6	1±0.46	0.99	0.979	↓1.435
Cflar	NM_003879	7	1±0.27	6	1.25±0.41	1.25	0.605	↑24.68
Fas	NM_000043	4	1±0.57	3	0.91±0.03	0.91	0.109	↓37.77
Il1a	NM_000575	6	1±0.50	5	4.71±1.64	4.71	0.113	↑371.2
Il5	NM_000879	4	1±0.39	3	0.78±0.32	0.78	0.628	↓22.19
Nfkb1	NM_003998	7	1±0.71	5	0.87±0.33	0.87	0.738	↓13.04
Tnfaip3	NM_006290	6	1±0.51	5	1.04±0.70	1.04	0.965	↑3.62
Blood Coagulation & Circulation								
Apob	NM_000384	5	1±0.67	3	1.07±0.81	1.07	0.951	↑6.887
Eln	NM_000501	6	1±0.38	5	0.88±0.50	0.88	0.842	↓11.83

Eng	NM_000118	5	1±0.71	4	0.63±0.19	0.63	0.195	↓37.01
Fga	NM_000508		1±0.31	2	1.22±0.85	1.22	0.886	↑21.76
Itga2	NM_002203	7	1±0.67	6	1.08±0.54	1.08	0.904	↑79.02
Npy	NM_000905	7	1±0.67	6	0.47±0.18	0.47	0.044*	↓52.83
Pdgfa	NM_002607	7	1±0.39	6	1.12±0.30	1.12	0.721	↓9.704
Pdgfrb	NM_002609	7	1±0.62	4	0.14±0.07	0.14	0.002**	↓85.86
Adhesion Cell Molecules								
Cd44	NM_000610	7	1±0.29	5	0.84±0.17	0.84	0.463	↓15.67
Cdh5	NM_001795	7	1±0.60	4	0.27±0.13	0.27	0.017*	↓73.27
Ctgf	NM_001901	6	1±0.42	4	1.12±0.08	1.12	0.286	↑12.13
Icam1	NM_000201	7	1±0.62	5	0.37±0.15	0.37	0.021*	↓63.20
Sell	NM_000655	5	1±0.59	4	0.97±0.41	0.97	0.961	↓56.76
Selplg	NM_003006	6	1±0.35	5	0.63±0.29	0.63	0.31	↓37.31
Thbs4	NM_003248	6	1±0.03	5	0.84±0.49	0.84	0.788	↓15.72
Tnc	NM_002160	6	1±0.74	5	1.33±0.81	1.33	0.736	↑32.72
Vcam1	NM_001078	6	1±0.00	5	0.34±0.15	0.34	0.016*	↓66.31
Vwf	NM_000552	6	1±0.00	5	0.78±0.26	0.78	0.493	↓21.63
Lipid Transport & Metabolism								
Abca1	NM_005502	6	1±0.37	5	0.62±0.17	0.62	0.106	↓38.40
Apoa1	NM_000039	7	1±0.35	6	0.51±0.21	0.51	0.085(T)	↓48.76
Fabp3	NM_004102	6	1±0.39	4	0.62±0.30	0.62	0.359	↓37.77
Ldlr	NM_000527	7	1±0.77	6	0.39±0.16	0.39	0.029*	↓60.87
Lpa	NM_005577	3	1±0.62	3	0.40±0.24	0.4	0.176	↓59.67
Lpl	NM_000237	7	1±0.49	6	0.54±0.15	0.54	0.036*	↓46.37
Msr1	NM_002445	7	1±0.61	6	0.85±0.20	0.85	0.514	↓15.19
Ptgs1	NM_000962	7	1±0.54	5	0.48±0.17	0.48	0.053(T)	↓52.12
Cell Growth & Proliferation								
Csf1	NM_000757	7	1±0.68	6	1.56±0.54	1.56	0.387	↑56.42
Csf2	NM_000758	6	1±0.44	5	0.88±0.24	0.88	0.677	↓12.25
Fgf2	NM_002006	7	1±0.33	6	0.85±0.32	0.85	0.685	↓15.31
Hbegf	NM_001945	7	1±0.56	6	1.76±0.89	1.76	0.470	↑76.23
Il3	NM_000588	5	1±0.54	5	1.46±0.70	1.46	0.593	↑45.67
Il5	NM_000879	4	1±0.39	3	0.78±0.32	0.78	0.628	↓22.19
Kdr	NM_002253	6	1±0.43	5	2.38±1.51	2.38	0.460	↑138.3
Lif	NM_002309	7	1±0.84	5	2.29±0.96	2.29	0.296	↑129.0

Tgfb1	NM_000660	7	1±0.68	6	1.50±0.51	1.5	0.412	↑50.18

The table shows the gene, GenBank reference, number of independent experiments (N), fold-change in expression, % change in gene expression and *P* value (*P ≤ 0.05, **P ≤ 0.01). ↓ = Down-regulation of gene expression and ↑ = Up-regulation of gene expression. Abbreviations, a list of full gene names is included in Table 4.5. Red highlight genes whose expression was significantly increased or decreased (*P ≤ 0.05, **P ≤ 0.01). Green highlight genes where there was trend of increased or decreased expression (P between 0.5 and 0.1).

Table 4. 3 The effect of 15-(S)-HETrE on the expression of atherosclerosis-associated genes in THP-1 macrophages.

		Control		15-(S)-HETrE				
Gene	**GenBank ID**	**N**	**Mean ± SEM**	**N**	**Mean ± SEM**	**Fold-change**	**P value**	**%Change**
Stress Responses								
Ccl2	NM_002982	6	1±0.56	4	0.2	0.20±0.06	0.001**	↓79.62
Ccl5	NM_002985		1±0.85	5	4.87	4.87±3.82	0.444	↑387.3
Ccr1	NM_001295	6	1±0.83	4	3.54	3.54±1.80	0.308	↑254.1
Ccr2	NM_001123396	5	1±0.65	6	2.94	2.94±1.42	0.289	↑193.7
Fn1	NM_002026	6	1±0.67	5	0.230	0.23±0.10	0.002**	↓77.00
Il1r1	NM_000877	6	1±0.53	5	0.800	0.80±0.51	0.749	↓19.61
Il1r2	NM_004633	6	1±0.36	4	0.550	0.55±0.14	0.072(T)	↓44.89
Itgb2	NM_000211	5	1±0.62	4	0.480	0.48±0.11	0.025*	↓52.34
Pparg	NM_015869	5	1±0.67	5	0.536	0.54±0.19	0.089	↓46.38
Nfkb1	NM_003998	6	1±0.70	6	4.74	4.47±3.90	0.439	↑374.1
Nos3	NM_000603	6	1±0.67	6	0.700	0.70±0.29	0.408	↓30.16
Sod1	NM_000454	4	1±0.71	5	0.650	0.65±0.18	0.156	↓34.66
Spp1	NM_000582	6	1±0.61	5	0.74	0.74±0.21	0.346	↓25.52
Tnf	NM_000594	6	1±0.62	6	0.39	0.39±0.20	0.052(T)	↓60.73
Apoptosis								
Bax	NM_004324	5	1±0.65	4	0.87	0.87±0.11	0.376	↓13.19
Bcl2	NM_000633	6	1±0.66	6	0.36	0.31±0.11	0.029*	↓64.19
Bcl2a1	NM_004049	6	1±0.35	4	6.89	6.89±1.29	0.065(T)	↑588.9
Bid	NM_001196	5	1±0.86	4	3.72	3.72±2.47	0.409	↑272.5
Birc3	NM_001165	6	1±0.59	4	1.26	1.26±0.77	0.809	↑26.00
Cflar	NM_003879	6	1±0.45	5	0.34	0.34±0.07	0.004**	↓65.60
Il1a	NM_000575	3	1±0.84	3	1.03	1.03±0.38	0.952	↑3.163
Il5	NM_000879	5	1±0.67	2	0.23	0.23±0.15	0.166	↓77.35

Nfkb1	NM_003998	3	1±0.70	6	4.74	4.47±3.90	0.439	↑374.1
Tnfaip3	NM_006290	6	1±0.55	3	1.59	1.59±0.95	0.666	↑58.57

Blood Coagulation & Circulation								
Eln	NM_000501	6	1±0.59	4	0.58	0.58±0.18	0.132	↓41.57
Eng	NM_000118	6	1±0.81	3	1.21	1.21±0.58	0.795	↑21.06
Itga2	NM_002203	6	1±0.89	4	0.48	0.28±0.15	0.025*	↓72.30
Npy	NM_000905	3	1±0.72	6	0.89	0.89±0.28	0.745	↓10.71
Pdgfa	NM_002607	5	1±0.43	5	0.57	0.57±0.16	0.101	↓42.71
Adhesion Cell Molecules								
Cd44	NM_000610	6	1±0.71	5	0.68	0.68±0.07	0.034*	↓32.04
Cdh5	NM_001795	5	1±0.33	4	0.86	0.86±0.26	0.675	↓14.01
Ctgf	NM_001901	5	1±0.43	4	1.89	1.89±0.56	0.321	↑89.43
Icam1	NM_000201	6	1±0.56	5	0.89	0.89±0.38	0.814	↓10.62
Selplg	NM_003006	5	1±0.57	3	0.47	0.47±0.29	0.272	↓53.25
Thbs4	NM_003248	5	1±0.85	6	1.77	1.77±0.94	0.505	↑76.63
Tnc	NM_002160	5	1±0.72	6	0.45	0.45±0.24	0.11	↓55.14
Vcam1	NM_001078	5	1±0.36	3	0.91	0.91±0.68	0.928	↓8.506
Vwf	NM_000552	6	1±0.80	6	0.24	0.24±0.12	0.005**	↓76.15
Lipid Transport & Metabolism								
Abca1	NM_005502	5	1±0.64	4	0.41	0.41±0.14	0.036*	↓59.41
Apoa1	NM_000039	6	1±0.41	4	2.99	3.00±0.93	0.223	↑198.9
Fabp3	NM_004102	6	1±0.66	5	5.02	5.02±2.66	0.282	↑401.7
Lldr	NM_000527	6	1±0.60	4	0.35	0.35±0.09	0.008**	↓64.88
Lpl	NM_000237	6	1±0.79	6	0.35	0.35±0.03	0.0001***	↓65.24
Msr1	NM_002445	6	1±0.85	4	0.43	0.43±0.12	0.025*	↓56.66
Ptgs1	NM_000962	6	1±0.61	6	0.45	0.45±0.16	0.04*	↓54.89
Cell Growth & Proliferation								
Csf1	NM_000757	6	1±0.52	5	0.24	0.24±0.10	0.008**	↓76.47
Csf2	NM_000758	5	1±0.51	4	0.91	0.91±0.46	0.878	↓8.929
Fgf2	NM_002006	6	1±0.55	4	0.385	0.38±0.10	0.08(T)	↓61.54
Hbegf	NM_001945	6	1±0.70	5	0.749	0.75±0.41	0.617	↓25.08
Il3	NM_000588	4	1±0.47	3	1.24	1.24±0.66	0.798	↑23.66
Kdr	NM_002253	5	1±0.45	4	1.46	1.46±0.52	0.503	↑45.90
Lif	NM_002309	6	1±0.85	6	1.19	1.19±0.53	0.761	↑19.37

Tgfb1	NM_000660	6	1±0.52	5	0.4	0.40±0.14	0.036*	↓60.11
Tgfb2	NM_003238	5	1±0.51	6	0.92	0.92±0.33	0.83	↓8.350
Vegfa	NM_003376	6	1±0.55	6	1.51	1.51±0.87	0.627	↑51.22

The table shows the gene, GenBank reference, number of independent experiments (N), fold-change in expression, % change in gene expression and P value (*P ≤ 0.05, **P ≤ 0.01). ↓ = down-regulation of gene expression and ↑ = up-regulation of gene expression. Abbreviations: a list of full gene names is included in Table 4.5. Red highlights genes whose expression was significantly increased or decreased (*P ≤ 0.05, **P ≤ 0.01). Green highlights genes where there was a trend of increased or decreased expression (P between 0.5 and 0.1).

Table 4. 4 The effect of PGE_1 on the expression of atherosclerosis-associated genes in THP-1 macrophages.

		Control		PGE_1				
Gene	**GenBank ID**	**N**	**Mean ± SEM**	**N**	**Mean ± SEM**	**Fold-change**	***P* value**	**% Change**
Stress Responses								
Ccl2	NM_002982	6	1±0.56	6	0.27±0.20	0.27	0.049*	↓73.24
Ccl5	NM_002985	6	1±0.85	4	0.11±0.05	0.11	0.001**	↓88.92
Ccr1	NM_001295	5	1±0.83	3	0.73±0.58	0.73	0.739	↓27.02
Ccr2	NM_001123396	6	1±0.65	6	2.48±1.61	2.48	0.439	↑147.8
Fn1	NM_002026	6	1±0.67	5	1.10±0.73	1.099	0.910	↑9.894
Il1r1	NM_000877	6	1±0.53	5	0.72±0.54	0.72	0.663	↓28.44
Il1r2	NM_004633	5	1±0.36	4	0.86±0.31	0.86	0.726	↓13.92
Itggb2	NM_000211	5	1±0.62	4	1.85±1.58	1.85	0.672	↑85.35
Pparg	NM_015869	6	1±0.67	6	2.50±1.69	2.500	0.454	↑150.0
Nfkb1	NM_003998	6	1±0.70	4	1.27±1.07	1.27	0.844	↑26.53
Nos3	NM_000603	5	1±0.67	4	0.52±0.40	0.52	0.367	↓48.40
Sele	NM_000450	4	1±0.63	3	1.29±0.81	1.29	0.799	↑28.68
Sod1	NM_000454	6	1±0.71	5	3.88±2.75	3.88	0.403	↑287.6
Spp1	NM_000582	6	1±0.61	5	4.47±2.73	4.47	0.319	↑347.3
Tnf	NM_000594	6	1±0.62	6	1.37±0.85	1.37	0.706	↑37.17
Apoptosis								
Bax	NM_004324	5	1±0.65	4	1.55±1.29	1.55	0.735	↑55.44
Bcl2	NM_000633	6	1±0.66	4	0.06±0.03	0.06	0.0001***	↓93.90
Bcl2a1	NM_004049	6	1±0.35	3	0.02±0.01	0.02	0.0001***	↓97.97
Bid	NM_001196	5	1±0.86	5	1.22±0.96	1.22	0.849	↓21.73
Birc3	NM_001165	6	1±0.59	5	5.19±4.63	5.19	0.463	↑418.8
Cflar	NM_003879	6	1±0.45	6	4.14±1.86	4.14	0.184	↑314.1
Il1a	NM_000575	3	1±0.84	4	2.51±2.11	2.51	0.58	↑150.6
Il5	NM_000879	5	1±0.67	3	0.54±0.36	0.54	0.402	↓46.43
Nfkb1	NM_003998	3	1±0.70	4	1.27±1.07	1.27	0.844	↑26.53
Tnfaip3	NM_006290	6	1±0.55	5	0.29±0.16	0.29	0.017*	↓70.85

Blood Coagulation & Circulation								
Eln	NM_000501	5	1±0.43	4	1.62±0.70	1.62	0.495	↑62.19
Itga2	NM_002203	6	1±0.59	5	0.57±0.33	0.57	0.305	↓43.22
Npy	NM_000905	6	1±0.81	4	0.18±0.09	0.18	0.004**	↓81.97
Pdgfa	NM_002607	6	1±0.89	5	1.32±0.70	1.32	0.704	↑32.06
Pdgfrb	NM_002609	3	1±0.72	3	1.18±0.85	1.18	0.879	↑17.85
Adhesion Cell Molecules								
Cd44	NM_000610	6	1±0.71	4	3.17±2.67	3.170	0.532	↑217.0
Cdh5	NM_001795	5	1±0.33	4	3.48±1.16	3.478	0.162	↑247.8
Ctgf	NM_001901	5	1±0.43	5	2.34±1.01	2.343	0.300	↑134.3
Icam1	NM_000201	6	1±0.56	5	3.52±1.96	3.52	0.316	↑251.6
Sell	NM_000655	4	1±0.43	4	1.00±0.43	1	0.998	↓0.106
Selplg	NM_003006	5	1±0.57	4	0.11±0.06	0.11	0.001***	↓88.82
Thbs4	NM_003248	5	1±0.85	6	2.86±2.43	2.86	0.517	↑185.5
Tnc	NM_002160	5	1±0.72	6	1.24±0.90	1.37	0.706	↑24.09
Vcam1	NM_001078	5	1±0.36	4	0.21±0.08	0.21	0.003**	↓79.13
Vwf	NM_000552	6	1±0.80	4	0.09±0.06	0.09	0.001***	↓91.01
Lipid Transport & Metabolism								
Abca1	NM_005502	5	1±0.64	4	2.79±2.36	2.79	0.559	↑178.7
Apoa1	NM_000039	6	1±0.41	5	2.99±1.52	2.99	0.307	↑198.7
Fabp3	NM_004102	6	1±0.66	4	1.70±1.22	1.7	0.656	↑69.67
Ldlr	NM_000527	6	1±0.60	4	1.17±0.97	1.17	0.891	↑16.63
Lpl	NM_000237	6	1±0.79	4	0.04±0.01	0.04	0.0001***	↓95.61
Msr1	NM_002445	6	1±0.85	4	0.15±0.07	0.15	0.002**	↓84.85
Ptgs1	NM_000962	6	1±0.61	6	2.79±1.72	2.79	0.384	↑179.4
Cell Growth & Proliferation								
Csf1	NM_000757	6	1±0.52	5	0.95±0.58	0.95	0.941	↓5.09
Csf2	NM_000758	5	1±0.51	4	0.83±0.42	0.83	0.75	↓17.04
Fgf2	NM_002006	6	1±0.55	5	3.35±1.84	3.35	0.315	↑235.4
Hbegf	NM_001945	6	1±0.70	6	3.15±2.21	3.15	0.416	↑214.8
Il3	NM_000588	4	1±0.47	4	2.94±1.38	2.94	0.311	↑194.2
Il5	NM_000879	3	1±0.67	3	0.54±0.36	0.54	0.402	↓46.43
Kdr	NM_002253	5	1±0.45	4	2.83±1.27	2.83	0.299	↑183.2
Lif	NM_002309	6	1±0.85	4	0.53±0.26	0.53	0.22	↓47.18

The table shows the gene, GenBank reference, number of independent experiments (N), fold-change in expression, % change in gene expression and P value (*P ≤ 0.05, **P ≤ 0.01). ↓ = down-regulation of gene expression and ↑ = up-regulation of gene expression. Abbreviations: a list of full gene names is included in Table

4.5. Red highlights genes whose expression was significantly increased or decreased (*P ≤ 0.05, **P ≤ 0.01). Green highlights genes which were trend increased or decreased (P values between 0.5 and 0.1).

Table 4. 5 Full list of genes included in the RT² Atherosclerosis Array.

Symbol	Description
Abca1	ATP-binding cassette, sub-family A (ABC1), member 1
Ace	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1
Apoa1	Apolipoprotein A-I
Apob	Apolipoprotein B
Apoe	Apolipoprotein E
Bax	Bcl2-associated X protein
Bcl2	B-cell leukaemia/lymphoma 2
Bcl2a1a	B-cell leukaemia/lymphoma 2 related protein A1a
Bcl2l1	Bcl2-like 1
Bid	BH3 interacting domain death agonist
Birc3	Baculoviral IAP repeat-containing 3
Ccl2	Chemokine (C-C motif) ligand 2
Ccl5	Chemokine (C-C motif) ligand 5
Ccr1	Chemokine (C-C motif) receptor 1
Ccr2	Chemokine (C-C motif) receptor 2
Cd44	CD44 antigen
Cdh5	Cadherin 5
Cflar	CASP8 and FADD-like apoptosis regulator
Col3a1	Collagen, type III, alpha 1
Csf2	Colony stimulating factor 2 (granulocyte-macrophage)
Ctgf	Connective tissue growth factor
Cxcl1	Chemokine (C-X-C motif) ligand 1
Eln	Elastin
Eng	Endoglin
Fabp3	Fatty acid binding protein 3, muscle and heart
Fas	Fas (TNF receptor superfamily member 6)
Fga	Fibrinogen alpha chain
Fgb	Fibrinogen beta chain
Fgf2	Fibroblast growth factor 2

Fn1	Fibronectin 1
Hbegf	Heparin-binding EGF-like growth factor
Icam1	Intercellular adhesion molecule 1
Ifng	Interferon gamma
Il1a	Interleukin 1 alpha
Il1b	Interleukin 1 beta
Il1r1	Interleukin 1 receptor, type I
Il1r2	Interleukin 1 receptor, type II
Il2	Interleukin 2
Il3	Interleukin 3
Il4	Interleukin 4
Il5	Interleukin 5
Itga2	Integrin alpha 2
Itga5	Integrin alpha 5 (fibronectin receptor alpha)
Itgax	Integrin alpha X
Itgb2	Integrin beta 2
Kdr	Kinase insert domain protein receptor
Klf2	Kruppel-like factor 2 (lung)
Lama1	Laminin, alpha 1
Ldlr	Low density lipoprotein receptor
Lif	Leukemia inhibitory factor
Lpl	Lipoprotein lipase
Lypla1	Lysophospholipase 1
Mmp1a	Matrix metallopeptidase 1a (interstitial collagenase)
Mmp3	Matrix metallopeptidase 3
Msr1	Macrophage scavenger receptor 1
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in Bcells 1, p105
Npy	Neuropeptide Y
Nr1h3	Nuclear receptor subfamily 1, group H, member 3
Pdgfa	Platelet derived growth factor, alpha
Pdgfb	Platelet derived growth factor, B polypeptide
Pdgfrb	Platelet derived growth factor receptor, beta polypeptide
Plin2	Perilipin 2
Ppara	Peroxisome proliferator activated receptor alpha
Ppard	Peroxisome proliferator activator receptor delta

Pparg	Peroxisome proliferator activated receptor gamma
Ptgs1	Prostaglandin-endoperoxide synthase 1
Rxra	Retinoid X receptor alpha
Sele	Selectin, endothelial cell
Sell	Selectin, lymphocyte
Selp	Selectin, platelet
Selplg	Selectin, platelet (p-selectin) ligand
Serpinb2	Serine (or cysteine) peptidase inhibitor, clade B, member 2
Serpine1	Serine (or cysteine) peptidase inhibitor, clade E, member 1
Sod1	Superoxide dismutase 1, soluble
Spp1	Secreted phosphoprotein 1
Tgfb1	Transforming growth factor, beta 1
Tgfb2	Transforming growth factor, beta 2
Thbs4	Thrombospondin 4
Tnc	Tenascin C
Tnf	Tumour necrosis factor
Tnfaip3	Tumour necrosis factor, alpha-induced protein 3
Vcam1	Vascular cell adhesion molecule 1
Vegfa	Vascular endothelial growth factor A
Vwf	Von Willebrand factor homologue

4.4.2 Summary of unique and common regulated genes for DGLA and its metabolites

A Venn diagram for gene expression that is shared between, or is specific to, DGLA, 15-(S)-HETrE and PGE_1 is shown in Figure 4.4. Overall, the expression of three common genes (*Ccl2, Bcl2 and Lpl*) was regulated by DGLA, 15-(S)-HETrE and PGE_1. DGLA specifically regulated the expression of ten genes (*Apoa1, Bax, Bcl2l, Ccr2, Cdh5, Icam1, Pdgfrb, Ptgs1, Col3a1 and Tgfb2*) with an additional four shared with 15-(S)-HETrE (Ccl2, Bcl2, Lpl and Ptgs1) and five shared with PGE_1 (*Ccl2, Bcl2, Lpl, Npy and Vcam1*). 15-(S)-HETrE specifically regulated the expression of thirteen genes (*Abca1, Fn1, Ilr2, Itga2, Itgb2, Itgax, Pparg, Rxra, Serpine1 and Tnf, Tgfb1, Itga5 and Ppard*) with an additional four shared with DGLA (Ccl2, Bcl2, Lpl and Ptgs1) and four shared with PGE_1 (*Bcl2a1, Mmp3, Msr1 and Vwf*). PGE_1 specifically regulated the expression of three genes (*Ccl5, Selplg and Tnfaip3*) with an additional five shared with DGLA (Ccl2, Bcl2, Lpl, Npy and Vcam1) and seven shared with 15-(S)-HETrE (*Bcl2, Bcl2a1, Ccl2, Lpl, Mmp3, Msr1 and Vwf, Bcl2a1*).

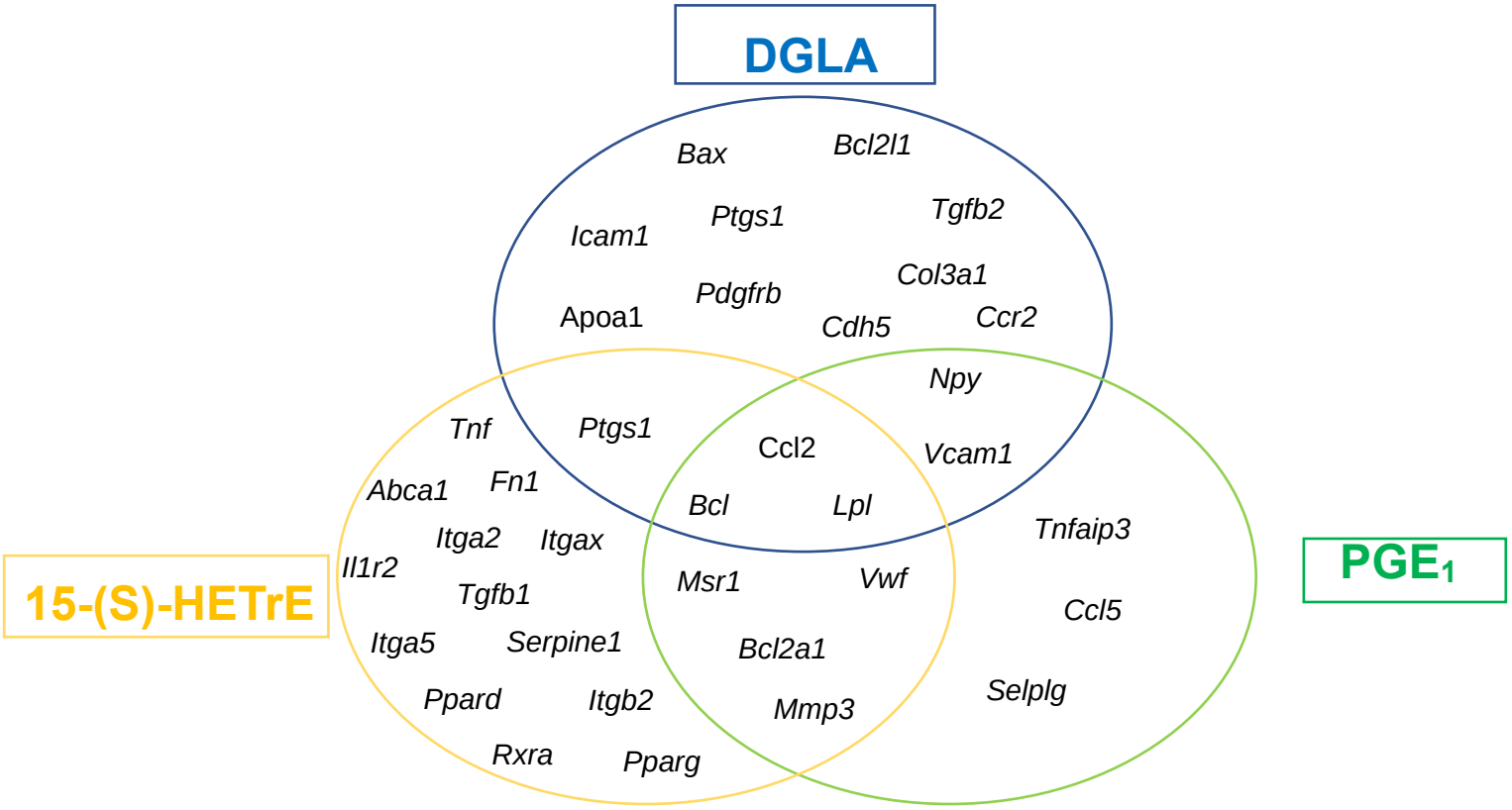


Figure 4. 4 A Venn diagram showing genes whose expression is exclusive to, or shared between, DGLA, 15-(S)-HETrE and PGE_1.

The blue circle indicates DGLA-regulated genes, the yellow circle indicates 15-(S)-HETrE-regulated genes, and the green circle represents PGE_1-regulated genes. The Venn diagram demonstrates that three of these genes, *Ccl2, Bcl2* and *Lpl*, are common to all three treatments. Abbreviations: A list of full gene names is included in Table 4.5.

4.4.3 Volcano plot analysis of regulation of gene expression in human THP-1 macrophages by DGLA and its metabolites *in vitro*.

Volcano plots that illustrate the general significant and non-significant gene expression changes were also prepared using the R software (Figures 4.5-4.7).

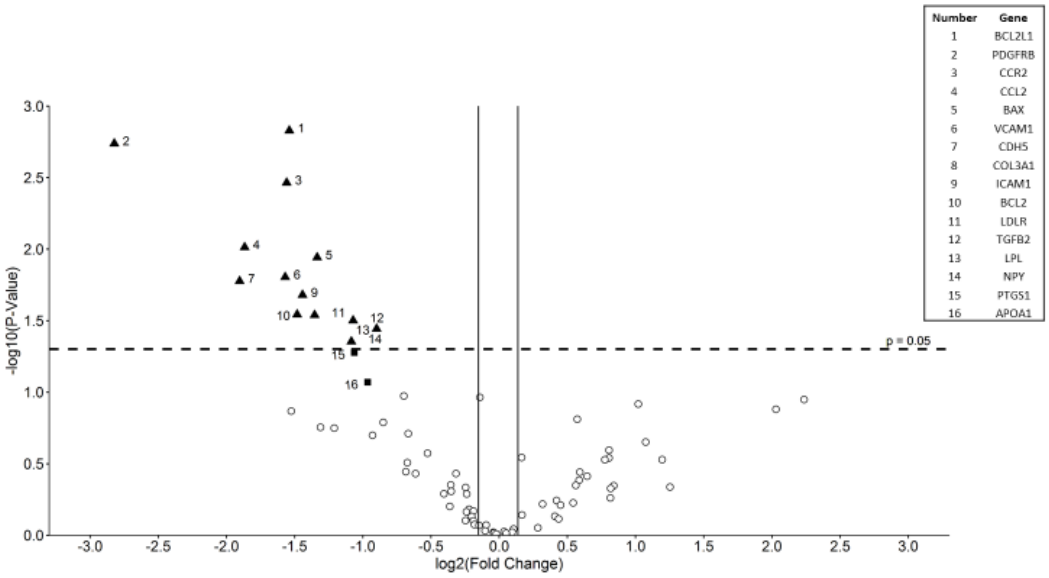


Figure 4. 5 Volcano plot indicating gene expression changes in THP-1 macrophages by DGLA compared to the control.

The effect of DGLA on the expression of atherosclerosis-associated genes was evaluated in human THP-1 macrophages, which were treated with either the vehicle control or 50 μM DGLA for 24 hours. Eighty-four genes were plotted in the Qiagen RT^2 PCR Array profiler. DGLA data were compared with vehicle control and described as mean fold change in gene expression. The log2 fold change in gene expression is presented on the x-axis. The y-axis indicates the p value -log10. A p value of 0.05 (dashed line) and a fold change of ±10% (solid lines) are also indicated. A black triangle indicates genes that have significant expression changes, and a black square indicates genes that show a trend towards significance (p values between 0.050 and 0.100). Abbreviations: A list of full gene names is included in Table 4.5.

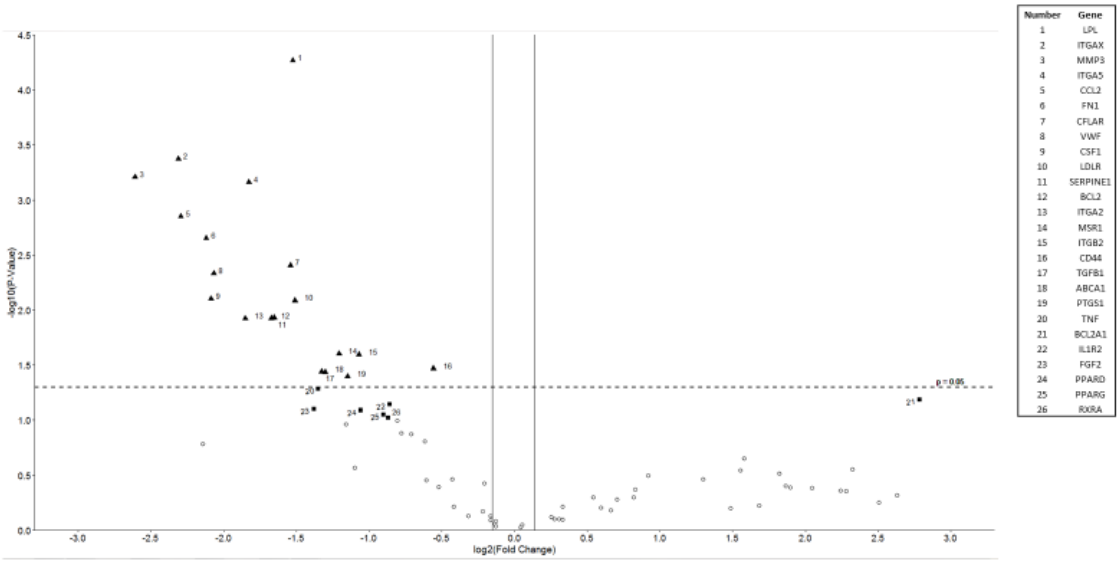


Figure 4. 6 Volcano plot indicating gene expression changes produced by 15-(S)-HETrE treatment of THP-1 macrophages.

The effect of 15-(S)-HETrE on the expression of atherosclerosis-associated genes was evaluated in human THP-1 macrophages, which were treated with either the vehicle control or 2 μM 15-(S)-HETrE for 24 hours. Eighty-four genes were plotted in the Qiagen RT^2 PCR Array profiler. 15-(S)-HETrE data are compared with the vehicle control and shown as a mean fold change in gene expression. The log2 fold changes in gene expression is presented on the x-axis. The y-axis indicates the p value -log10. A p value of 0.05 (dashed line) and a fold change of ±10% (solid lines) are also indicated. A black triangle indicates genes that have significant changes in gene expression and a black square represents gene that have a trend towards significance (p between 0.050 and 0.100). Abbreviations: A list of full gene names is included in Table 4.5.

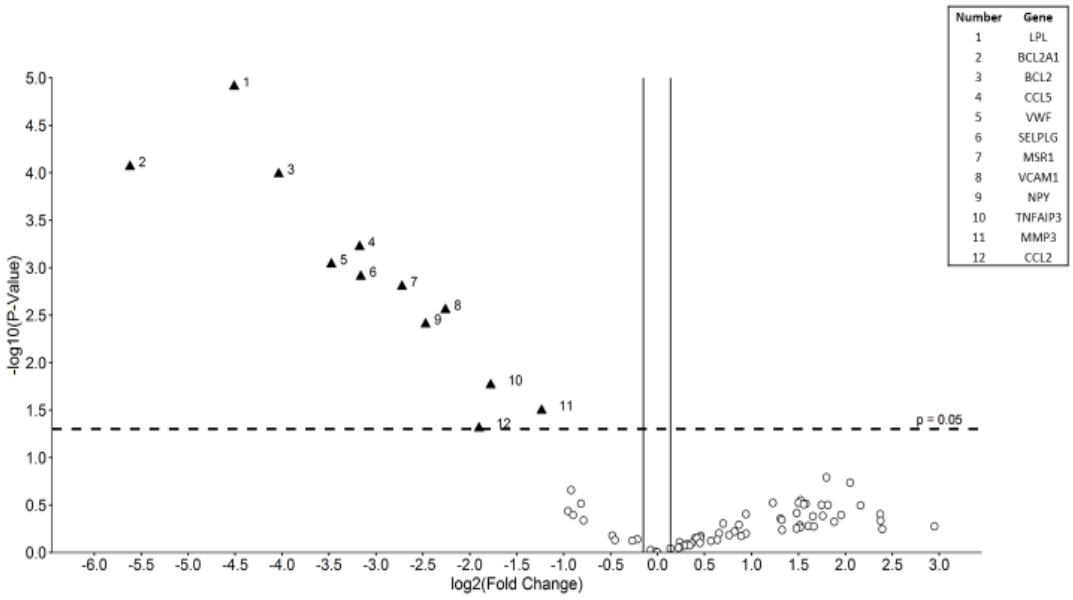


Figure 4. 7 Volcano plot indicating gene expression changes by PGE_1 treatment compared to control in THP-1 macrophages.

The effect of PGE_1 on the expression of atherosclerosis-associated genes was evaluated in human THP-1 macrophages, which were treated with either the vehicle control or 10 μM PGE_1 for 24 hours. Eighty-four genes were plotted in the Qiagen RT^2 PCR Array. PGE_1 data are compared with the vehicle control and are described as a mean fold change in gene expression. The log2 fold change in gene expression is presented on the x-axis. The y-axis indicates the p value -log10. A p value of 0.05 (dashed line) and a fold change of ±10% (solid lines) are also indicated. A black triangle indicates genes that have significant changes in expression and a black square identifies genes that show a trend towards significance (p between 0.05 and 0.100). Abbreviations: A list of full gene names is included in Table 4.5.

4.4.4 Genes whose expression was significantly regulated by DGLA in THP-1 macrophages or showed a trend towards significance.

Figure 4.8 shows graphical representation of significant DGLA regulated genes or those that showed a trend towards significance according to their functions suggested by the manufacturer (Qiagen). Some genes have more pleiotropic functions and hence shown in more than one category. From the genes implicated in stress responses, the expression of three genes was significantly reduced by DGLA compared to the control: *Ccl2* (p=0.010); *Ccr2* (p=0.003); and *Tgfβ2* (p=0.032) (Figure 4.8A). The expression of three genes involved apoptosis was also decreased [*Bax* (p=0.011), *Bcl2* (p=0.078) and *Bcl2l1* (p=0.001)] (Figure 4.8B). There was also significantly reduced expression of two genes involved in blood coagulation and circulation (*Npy* p=0.044) and *Pdgfrb* (p=0.002) (Figure 4.8C). The expression of *Cdh5* (p= 0.017), *Icam1* (p=0.021), and *Vcam1* (p=0.016), which are all involved in cell adhesion, was also significantly reduced (Figure 4.8D). Finally, the expression of three genes implicated in lipid transport and metabolism [*Apoa1* (p=0.085), *Ldlr* (p=0.029) and *Lpl* (p=0.036)] and the *Tgfβ2* (p=0.032) gene implicated in the control of cell growth and proliferation was also decreased (Figures 4.8E-F).

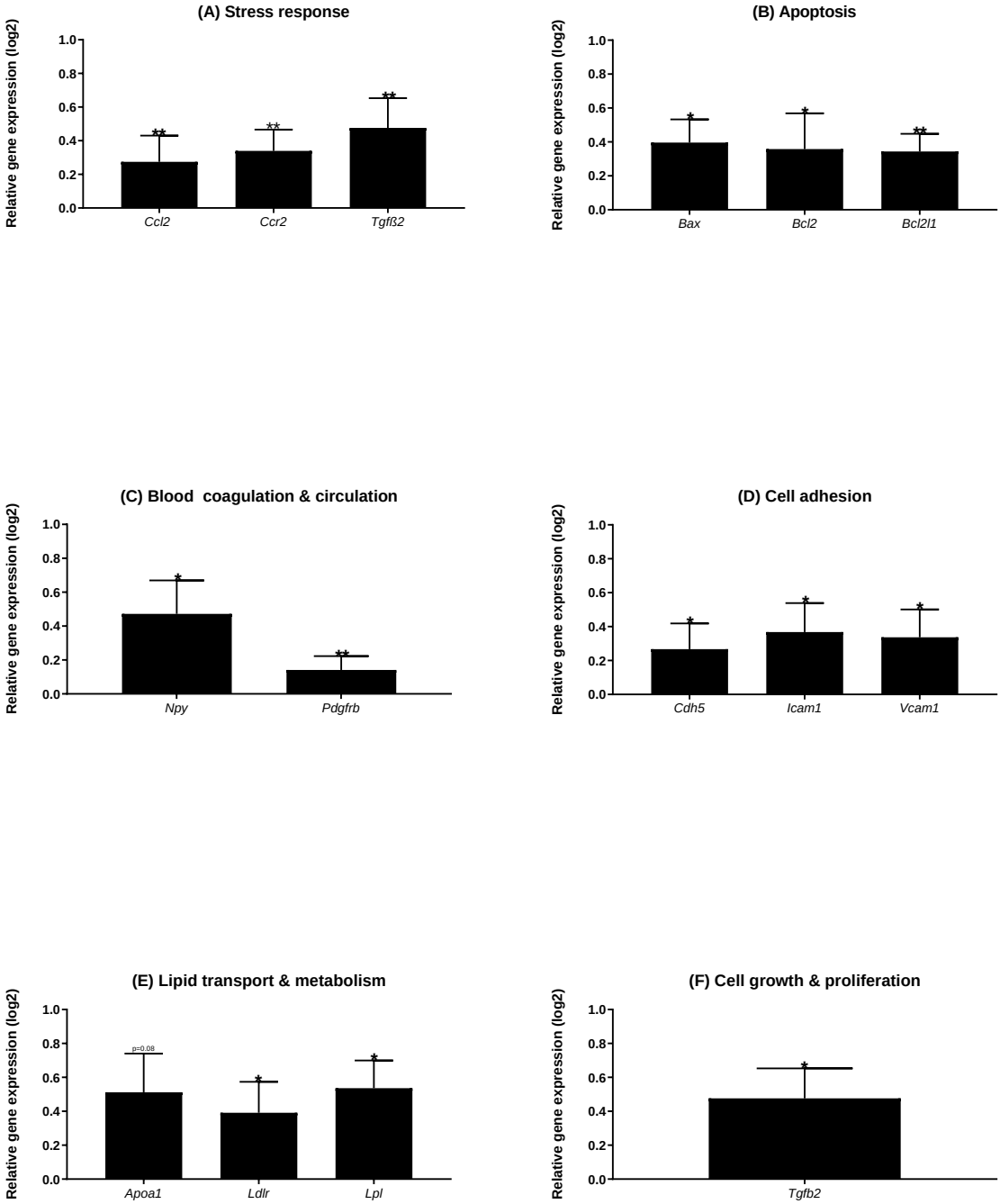


Figure 4. 8 DGLA decreased the expression of several genes implicated in specific atherosclerosis-associated processes.

The effect of DGLA on the expression of atherosclerosis-associated genes was evaluated in THP-1 macrophages. Genes displaying significant expression changes or trends towards significance were grouped into related classes associated with: (A) stress response, (B) lipid transport and metabolism, (C) cell adhesion, (D) cell growth and proliferation, (E) transcriptional regulation, (F) apoptosis and (G) blood coagulation and circulation. Statistical analysis was performed using an unpaired Student's t-test where * P ≤0.05; **, P ≤ 0.01 and *** P≤0.001. Abbreviations: A list of full gene names is included in Table 4.5.

4.4.5 The expression of genes involved in atherosclerosis that were significantly affected or showed a trend towards significance by 15-(S)-HETrE.

Figure 4.9 is a graphical representation of changes in the expression of atherosclerosis-associated genes regulated by 15-(S)-HETrE according to their functions. From the genes implicated in the regulation of stress responses, there were reduction in the expression of *Ccl2* (p=0.001), *Fn1* (p=0.002), *Il1r2* (p=0.072), *Itgb2* (p=0.025), *Pparg* (p=0.089) and *Tnf* (p=0.052) by 15-(S)-HETrE compared to the control (Figure 4.9A). For genes involved in apoptosis, there was significant decrease in the expression of *Bcl2* (p=0.012) and *Cflar* (p=0.004) (Figure 4.9B). For blood coagulation and circulation, there was significantly reduced expression of *Itga2* (p= 0.012) and a trend towards decreased expression of *Pdgf* (p=0.101) (Figure 4.9C). The expression of *Cd44* (p=0.034) and *Vwf* (p=0.005), both of which are involved in cell adhesion, was also significantly reduced (Figure 4.9D). Other genes whose expression was decreased by 15-(S)-HETrE included: *Abca1* (p=0.036), *Ldlr* (p=0.008), *Lpl* (p=0.001), *Msr1* (p=0.025) and *Ptgs1* (p=0.040), implicated in lipid transport and metabolism; *Csf1* (p=0.008), *Fgf2* (p=0.08) and *Tgfβ1* (p= 0.036), involved in cell growth and proliferation; transcriptional regulators *Ppard* (p=0.081), *Pparg* (p=0.089) and *Rxra* (p=0.096); and those coding for components in the regulation of ECM production [*Mmp3* (p=0.001) and Serpine1 (p=0.012)] (Figures 4.9E-H).

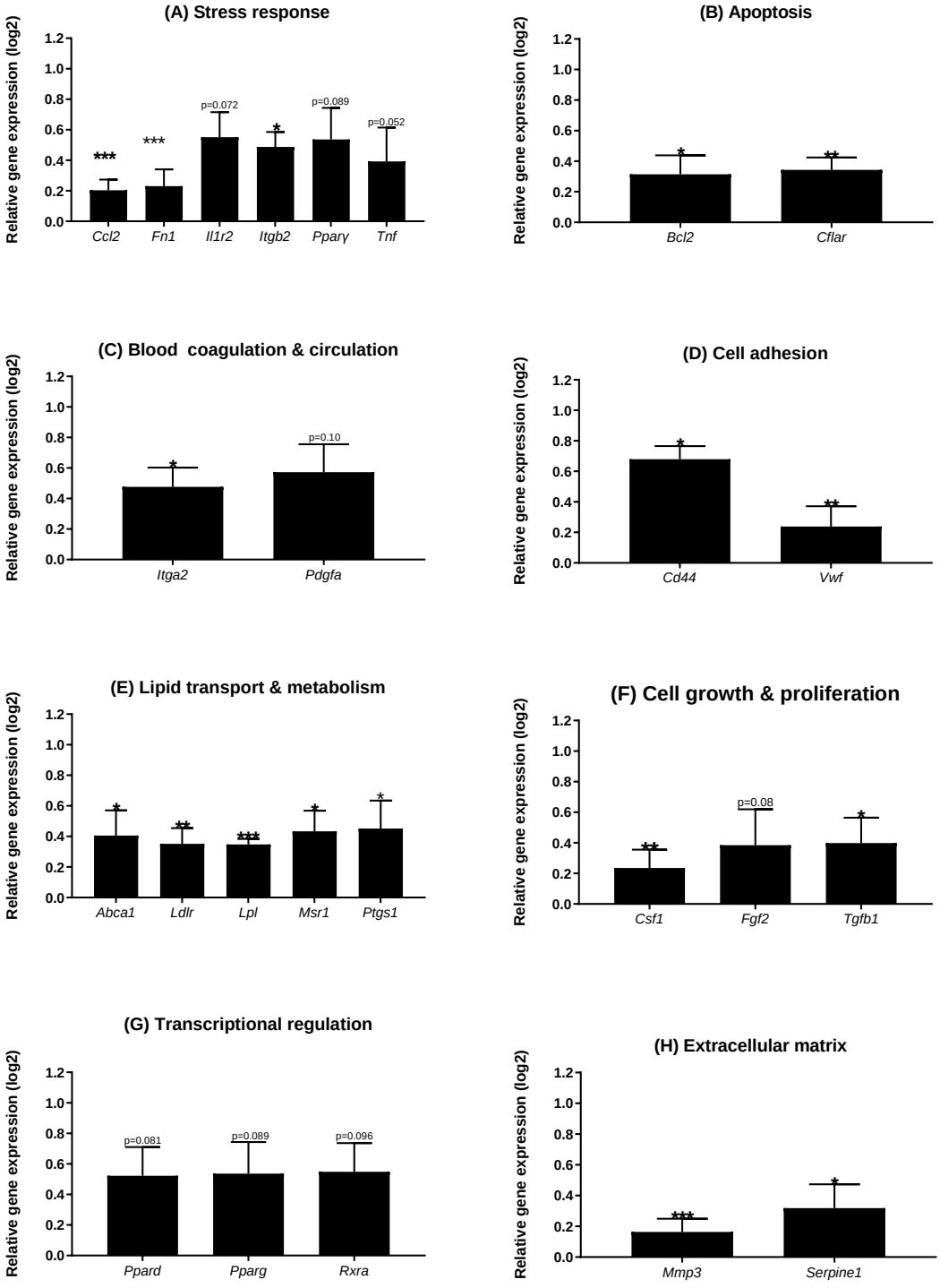


Figure 4. 9 15-(S)-HETrE decreased the expression of genes associated with specific atherosclerosis-associated processes.

The effect of 15-(S)-HETrE on the expression of atherosclerosis-associated genes was evaluated in THP-1 macrophages. Genes displaying significant changes in expression or a trend towards changes were grouped into related classes associated with: (A) stress response, (B) apoptosis, (C) blood coagulation and circulation, (D) cell adhesion molecules, (E) lipid transport and metabolism, (F) cell growth and proliferation, (G) lipid transport and metabolism, and (H) extracellular matrix. Statistical analysis was performed using an unpaired Student's t-test where * P ≤0.05; **, P ≤ 0.01 and *** P≤0.001. Abbreviations: A list of full gene names is included in Table 4.5.

4.4.6 The expression of genes involved in atherosclerosis affected by PGE_1.

Figure 4.10 shows graphical representation of genes whose expression was significantly decreased by PGE_1 according to function. PGE_1 produced significant reduction of the following genes in each category: *Ccl2* (p=0.001) and *Ccl5* (p=0.001) in stress responses; *Bcl2* (p=0.001), *Bcl2a1* (p=0.001) and *Tnfa1p3* (p=0.017) in the control of apoptosis; *Npy* (p=0.004) implicated in blood coagulation and circulation; *Selplg* (p=0.001), *Vcam1* (p=0.003) and *Vwf* (p=0.001) involved in cell adhesion; *Lpl* (p=0.001) and *Msr1*(p=0.002) involved in lipid transport and metabolism; and *Mmp3* (p=0.032) implicated in the control of ECM.

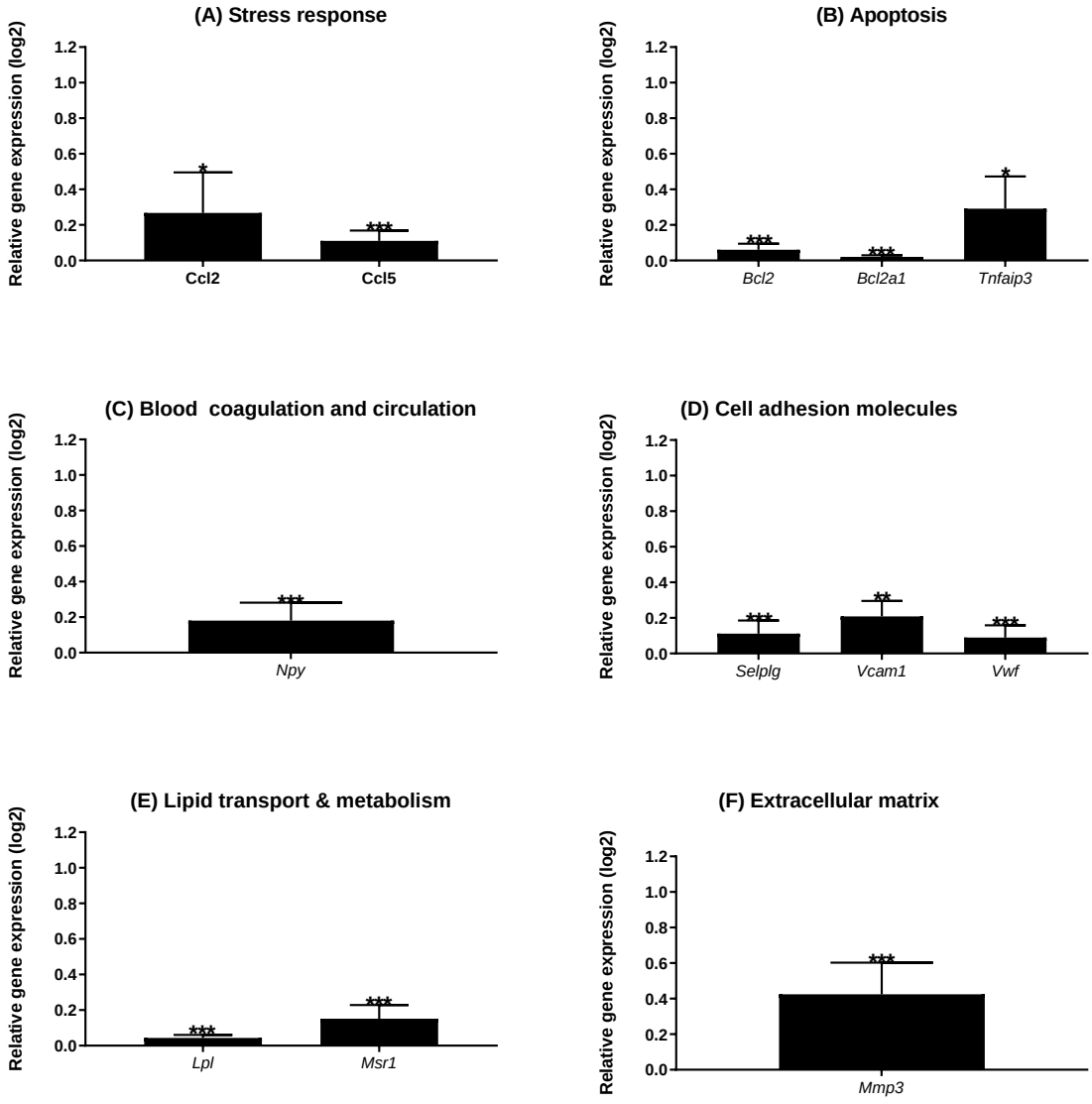


Figure 4. 10 PGE_1 decreases the expression of genes associated with specific atherosclerosis-associated processes.

The effect of PGE_1 on the expression of atherosclerosis-associated genes was evaluated in THP-1 macrophages. Genes displaying significant expression changes were grouped into related classes associated with: (A) stress response, (B) apoptosis, (C) blood coagulation and circulation, (D) cell adhesion molecules, (E) lipid transport and metabolism and (F) extracellular matrix. Statistical analysis was performed using an unpaired Student's t-test where * $P \leq 0.05$; **, $P \leq 0.01$ and *** $P \leq 0.001$. Abbreviations: A list of full gene names is included in Table 4.5

4.4.7 Heatmap of gene expression changes associated with atherosclerosis produced by DGLA, 15-(S)-HETrE and PGE_1

In the heat maps, data are presented as log^2 and displayed in a grid where each row represents a gene and each column represents a sample (i.e., DGLA, 15-(S)-HETrE or PGE_1). A cluster analysis was carried out for each group of related genes and a heatmap was generated using the online tool Morpheus, where colour and intensity visually reflect changes in the gene expression of all genes. Clustering on the heat map is based on the similarity in the pattern of expression between the specific genes in each related group. Red represents up-regulated genes and blue represents down-regulated genes. White represents unchanged expression. The expressions of some genes were undetectable and are shown by grey boxes (Figure 4.11).

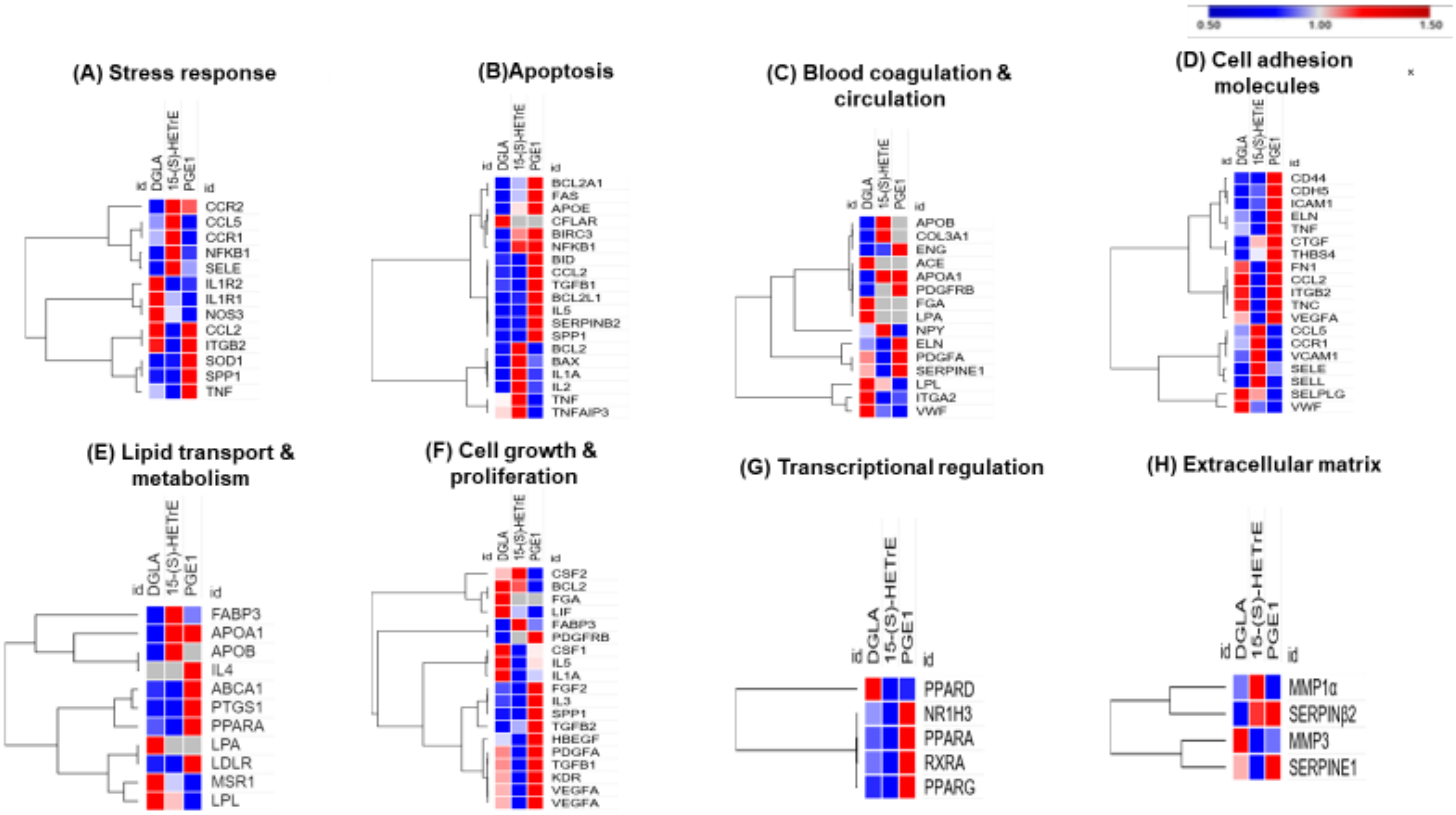


Figure 4. 11 Combined heatmap of gene expression in THP-1 macrophages regulated by DGLA, 15-(S)-HETrE and PGE_1 illustrating a visual image of fold-changes in expression.

Hierarchical clustering heat maps of the 84 genes at the transcript level were evaluated in THP-1 macrophages treated for 24 hours with 50 μM DGLA, 2 μM 15-(S)-HETrE and 10 μM PGE_1 using DMSO as a vehicle control. The colour and intensity of the boxes describe the fold-changes in gene expression according to the scale. The ΔΔCT method was used to detect fold-changes in gene expression. Cluster analysis was conducted and heatmaps were created using Morpheus software. Abbreviations: A list of full gene names is included in Table 4.5.

4.4.8 Construction of Hub Gene Network

To classify the molecular networks and pathways, gene ontology (GO) research tools, STRING software platforms and Cytoscape online analyses tools were used. The STRING website was used to obtain information on potential protein–protein interactions (PPI) (associations between candidate genes/proteins). In addition, a network term that evaluates the cohesiveness of the neighbourhood of a node is the clustering coefficient. A complex network of chosen differentially expressed genes (DEGs) was constructed as shown in Figure 4.12. The networks included cytokine ligand–receptor interaction pathway, containing 16, 25 and 12 hub nodes (genes) and 38, 111 and 25 edges (interactions) to this node respectively for significant gene expression changes for DGLA, 15-(S)-HETrE or $PGE_{1,}$ respectively. The results showed that the genes in this module were mainly involved in proliferation, the recruitment of monocytes, cholesterol efflux, the aggregation of platelets, migration of vascular smooth muscle cells, apoptosis, and programmed cell death (Figure 4.12)

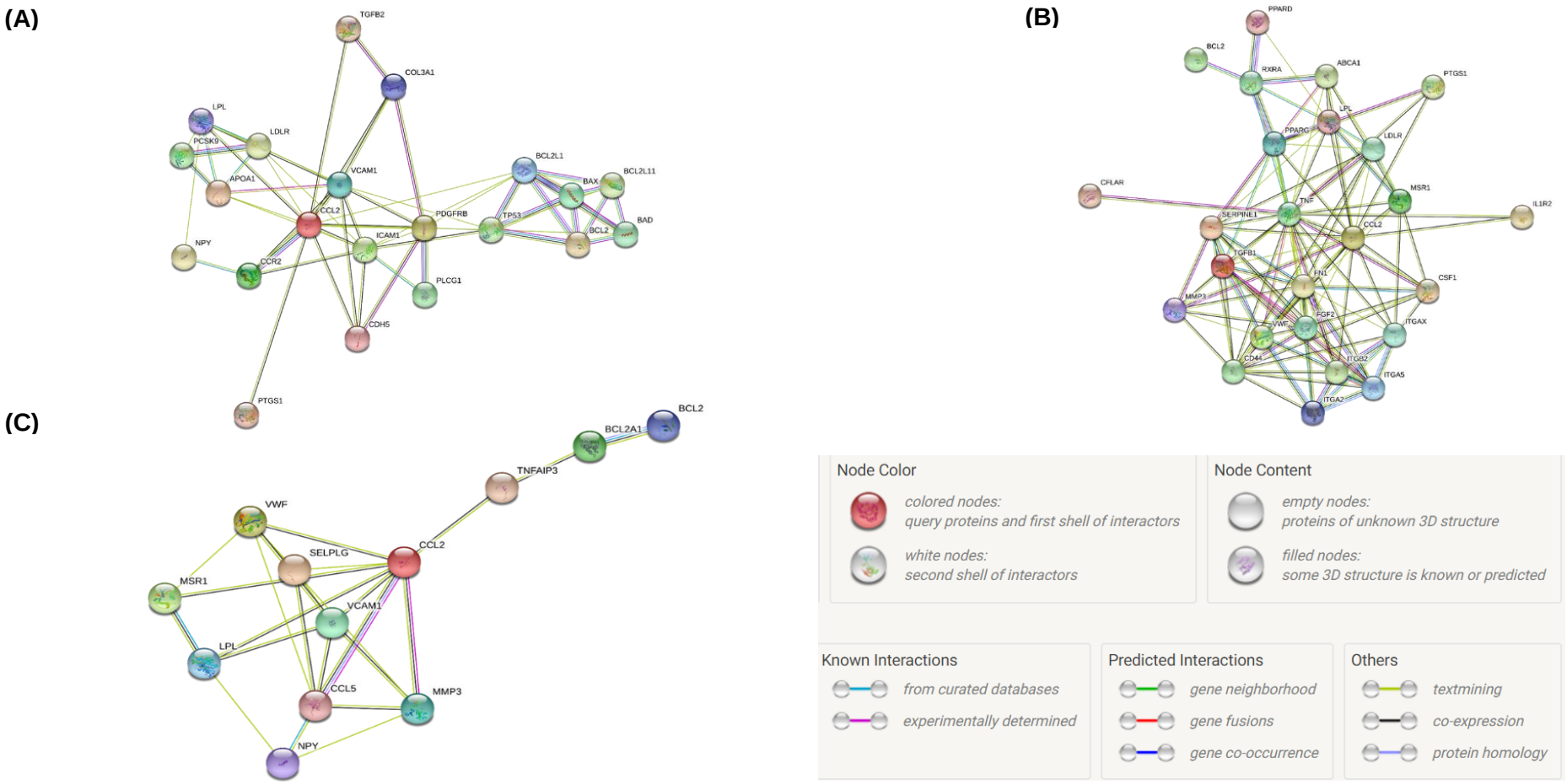


Figure 4. 13 Gene network construction and identification of key genes for DGLA, 15-(S)-HETrE or PGE_1 actions.

The effect of DGLA and its metabolites on the expression of key atherosclerosis-associated genes was investigated in THP-1 macrophages treated for 24 hours either with a vehicle control or with (A) 50 μM DGLA, (B) 2 μM of 15-(S)-HETrE or (C) 10 μM PGE1. Data were evaluated using STRING to determine the networks of interactions involved in atherosclerosis. There are 16, 25 and 12 nodes and 38, 111 and 25 edges respectively in the network. The nodes display genes, and the lines reflect the corresponding interactions. Abbreviations: A list of full gene names is included in Table 4.5.

4.5 Discussion

The results obtained from this study show that DGLA and its metabolites regulate many atherosclerosis-associated genes that modulate key cellular processes associated with this disease. The RT-qPCR array simultaneously evaluated the expression of 84 genes that play a crucial role in atherosclerosis (Table 4.1). The expression of three common genes (*Ccl2, Bcl2* and *Lpl*) was regulated by DGLA, 15-(S)-HETrE and PGE_1. Tables 4.5, 4.6 and 4.7 list genes and key atherosclerosis-associated functions of genes whose expression was significantly changed by DGLA and its metabolites *in vitro*.

Table 4. 6 Genes whose expression was significantly altered by DGLA *in vitro*.

Gene	Role in atherosclerosis	Change	References
Apoa1	Anti-atherogenic: Part of HDL that mediates reverse cholesterol transport from peripheral tissues to the liver by stimulating cholesterol efflux from the cells.	Decrease	(Smith 2010)
Bax	Anti-atherogenic: Modulates programmed cell death.	Decrease	(Aiello et al. 1999)
Bcl2	Anti-atherogenic: A key regulator of apoptosis.	Decrease	(Ismail et al. 2013)
Bcl2l1	Anti-atherogenic: A potent inhibitor of cell death.	Decrease	(Gustafsson and Gottlieb 2007)
Ccl2	Pro-atherogenic: Stimulates recruitment of monocytes to activated arterial endothelial cells.	Decrease	(Winter et al. 2018)
Ccr2	Pro-atherogenic: Plays a crucial role in the recruitment of monocytes/macrophages to inflammatory sites.	Decrease	(Boring *et al.* 1998)
Cdh5	Pro-atherogenic: Promotes pro-atherogenic and inflammatory leukocyte rolling and adherence, endothelial dysfunction, and inflammation in the vasculature.	Decrease	(Nus et al. 2016)

Icam1	Pro-atherogenic: Controls the attachment of leukocytes to the endothelium and subsequent peripheral transmigration.	Decrease	(Frank and Lisanti 2008)
Ldlr	Anti-atherogenic: Controls the clearance of circulating LDL by uptake via peripheral cells/tissues.	Decrease	(Linton et al. 1999)
Lpl	Pro- or anti-atherogenic depending on expression site. Regulates lipid metabolism and transport. Enzyme expressed in vascular endothelial cells, muscles and adipose tissue promotes hydrolysis of TG in VLDL and chylomicrons and is anti-atherogenic. The LDL expressed by macrophages is pro-atherogenic as it promotes lipoprotein uptake.	Decrease	(Mead et al. 2002)
Npy	Anti-atherogenic: Affects vascular endothelial cell dysfunction, the development of foam cells, vascular smooth muscle cell proliferation, local inflammatory plaque responses and platelet activation and aggregation.	Decrease	(Zhu et al. 2016)
Pdgfrb	Anti-atherogenic: Modulates VSMC proliferation and migration.	Decrease	(Zhou et al. 1999)
Ptgs1	Pro-atherogenic: Plays a crucial role in atherosclerotic development through regulation of prostaglandin production.	Decrease	(Helmersson *et al.* 2009)
Tgfb2	Anti-atherogenic: Plays a vital role in VSMC proliferation and migration.	Decrease	(Xie et al. 2015)
Vcam1	Pro-atherogenic: Plays a key role in atherosclerosis development. Activates cell adhesion and signal transduction associated with leukocyte-endothelial cell interaction.	Decrease	(Ley and Huo 2001)

Vwf	Pro-atherogenic: Controls adhesion of platelets and modulates coagulation of the blood.	Decrease	(Van Galen et al. 2012)

Table 4. 7 Genes whose expression was significantly altered by 15-(S)-HETrE *in vitro*.

Gene	Role in atherosclerosis	Change	References
Abca1	Anti-atherogenic: Stimulates cholesterol efflux and RCT.	Decrease	(Bielicki et al. 2010)
Bcl2	See Table 4.5.	Decrease	
Ccl2	See Table 4.5.	Decrease	
Cd44	Pro-atherogenic: Facilitates the recruitment of monocytes/macrophages to atherosclerotic lesions.	Decrease	(Cuff et al. 2001)
Cflar	Anti-atherogenic: Suppresses cardiovascular risk correlated to metabolic disorders. Moreover, plays a key role in inhibiting cell apoptosis by converting pro-apoptotic signals to anti-apoptotic signals by stimulation of caspase 8.	Decrease	(Wang and Butany 2017)
Csf1	Pro-atherogenic: plays a key role in the regulation of monocyte differentiation, proliferation, and migration.	Decrease	(Shaposhnik et al. 2010)
Fgf2	Anti-atherogenic: Controls cell proliferation, differentiation, growth, adherence, and migration. Mediates processes such as vasculogenesis, angiogenesis and remodelling of blood vessels.	Decrease	(Oulion et al. 2012; Liu et al. 2013)
Fn1	Anti-atherogenic: Plays a critical role in regulating endothelial cell inflammation.	Decrease	(Kusumi et al. 2015)
Il1r2	Pro-atherogenic: Modulates inflammatory responses.	Decrease	(Peters et al. 2013)
Itga2	Pro-atherogenic: This is an adhesion molecule, which encourages the aggregation	Decrease	(Rivera et al. 2009)

	of platelets and leads to blood clot development.		
Ldlr	See Table 4.5.	Decrease	
Lpl	See Table 4.5.	Decrease	
Mmp3	Pro-atherogenic: Promotes breakdown of ECM.	Decrease	(Vacek et al. 2015)
Msr1	Pro-atherogenic: Mediates uncontrolled uptake of modified LDL.	Decrease	(Manning-Tobin et al. 2009)
Pparδ	Anti-atherogenic: Modulates development of lesions and suppresses chemoattractant signalling through down-regulation of chemokine expression.	Decrease	(Barish et al. 2008; Ehrenborg and Skogsberg 2013)
Pparγ	Anti-atherogenic: Plays a substantial role in the formation of atherosclerotic plaques, such as increasing the expression of the scavenger receptor CD36 to improve the binding of oxidised LDL to macrophages, and also reduces inflammation.	Decrease	(Lopez-Liuchi and Meier 1998)
Ptgs1	See Table 4.	Decrease	
Rxr1	Pro-atherogenic: Promotes the biosynthesis of cholesterol and regulators of inflammatory gene expression such as those coding for TNF-α and IL-1β.	Decrease	(Rőszer et al. 2013)
Serpine1	Pro-atherogenic: Regulates the migration of vascular smooth muscle cells.	Decrease	(Mo et al. 2015)
Tgfβ1	Anti-atherogenic: A key growth factor in the accumulation of vascular matrix by SMC; also acts in an anti-inflammatory manner and inhibits foam cell formation.	Decrease	(Pham et al. 2010)
Tnf	Pro-atherogenic: Enhances the adhesion of leukocytes and endothelial cell migration and promotes thrombosis.	Decrease	(Bradley 2008)

Table 4. 8 Genes whose expression was significantly altered by PGE_1 *in vitro*.

Cytokines	Role in atherosclerosis	Change	References
Bcl2	See Table 4.5.	Decrease	
Bcl2a1	Anti-atherogenic: Inhibits apoptosis.	Decrease	(Erbel et al. 2011)
Ccl2	See Table 4.5.	Decrease	
Ccl5	Pro-atherogenic: Plays a critical role in leukocyte recruitment to the arterial wall.	Decrease	(Boring et al. 1998)
Lpl	See Table 4.5.	Decrease	
Mmp3	See Table 4.6.	Decrease	
Msr1	See Table 4.6.	Decrease	
Npy	See Table 4.5.	Decrease	
Selplg	Pro-atherogenic: Recruits leukocytes to the endothelium.	Decrease	(Tregouet et al. 2003)
Tnfaip3	Anti-atherogenic: Suppresses apoptosis in the progression of atherosclerosis at different stages.	Decrease	(Vozzi et al. 2018)
Vcam1	See Table 4.5.	Decrease	
Vwf	See Table 4.5.	Decrease	

The expression of several genes that are involved in cell adhesion was reduced considerably by DGLA and its metabolites compared to the control (Figure 4.8). The genes coding for *Icam1, Vcam1, Mcp1* and *E-selectin* are expressed at high levels on the surface of activated endothelial cells as a response to pro-inflammatory cytokines, such as TNF-α, and this is associated with stimulation of monocyte migration and recruitment (Hoffman et al., 2018). Previous *in vivo* studies have shown decreased size of atherosclerotic lesions in mice deficient in cell adhesion molecule *Icam1* (Collins *et al.* 2000; Galkina and Ley 2007). In addition, in male C57BL/6J mice, the expression of numerous adhesion molecule genes, including *Icam1*, was attenuated by anti-atherogenic PUFAs present in diets rich in fish oil (Miles *et al.* 2000). DGLA reduced *Mcp1* and *Icam1* expression produced by three cytokines – IFN-γ, IL-1β and TNF-α – in human macrophages *in vitro* and this reflects a possible mechanism for decreased expression of *Icam1* and *Vcam1* in ApoE deficient mice *in vivo* (Takai et al. 2009). Additionally, $ApoE^{-/-}$ mice that were treated orally with DGLA showed a reduction in circulating levels of MCP-11 (Watanabe et al., 2014). This result is consistent with the significant decrease observed in pro-inflammatory gene expression in studies presented in this chapter and the reduction in cell migration previously shown in Figure 3.17. In the current study, the expression of *Ccl2* (*Mcp1*), *Icam-1* and *Vcam-1* was also decreased by DGLA and its metabolites

compared to the control (Figures 4.8, 4.9 and 4.10). This is likely to be important for the function of DGLA as previous studies have shown that *in vivo,* reducing the expression of *Mcp1* and *Icam1* significantly prevents the progression of atherosclerosis (Gosling *et al.* 1999; Kitagawa *et al.* 2002). The findings in this chapter, combined with previously published work, show that DGLA is a critical factor in the inhibition of expression of pro-atherogenic chemokines and adhesion molecules.

LPL, expressed in the atherosclerotic plaque by macrophages leads to the initiation and development of foam cells, which are crucial for the onset of the disease. LPL has a crucial function in lipid metabolism and transport (Evans 2005). In the current study, *Lpl* expression was decreased considerably by DGLA and its metabolites compared to the control, which is therefore consistent with its anti-foam cell action seen in previous studies (Argmann et al.,2001, Akiyama et al. 2002) (Table 4.5). LPL can enhance the ability of uptake of lipoproteins by associating with them (Serri et al. 2004). LPL also plays a key role in increasing the production of proinflammatory cytokine TNF-α, monocyte adhesion to endothelial cells and VSMC proliferation (Serri et al. 2004). These findings support the promising impact of treatment of the cells with DGLA and its metabolites by reducing the expression of *Lpl*.

LDLr controls the uptake of LDL in most peripheral tissues. *Ldlr* expression is inhibited by negative feedback as cholesterol concentration in cells increases (Ghosh et al. 2010). A previous *in vivo* study, using C57BL/6 mice, which had bone marrow transplantation, to define the role of LDLr in the uptake of LDL and the endocytosis of β-VLDL, found that LDLr is capable of mediating lipid accumulation during the formation of foam cells (Linton *et al.* 1999). As shown in Figures 4.7 and 4.8. While PGE_1 upregulated the expression of *Ldlr* (Figure 4.9). the expression of *Ldlr* was significantly downregulated by DGLA and 15-(S)-HETrE, correlating with results in Chapter 3 where the cell surface expression of LDLr protein was significantly reduced in the presence DGLA and its metabolites (Figure 3.12). Decreased gene levels could probably reduce the formation of foam cells and suppress disease progression, although this might also encourage atherogenesis by lowering plasma LDL clearance.

Receptor-mediated endocytosis is an effective mechanism for the attachment and internalisation by macrophages of a variety of molecules, such as pathogens and LDL (Swanson and Watts 1995). On the surface of macrophages, scavenger receptors contribute to unregulated uptake of modified forms of LDL (Goldstein *et al.* 1979; Peiser and Gordon

2001; Park 2014). CD36 and SRA have been demonstrated to cause the accumulation of significant quantities of oxLDL leading to the development of foam cells and atherosclerosis (Febbraio *et al.* 2000; Kunjathoor *et al.* 2002; Kuchibhotla *et al.* 2007). The atherosclerotic lesions were shown to have a lower expression of inflammatory genes and a decrease in plaque necrosis in aortic root lesions in a previous study using the ApoE$^{-/-}$/CD36$^{-/-}$/MSR1$^{-/-}$ animal model that also established the functions of MSR1 and CD36 for atherogenesis and lesion macrophage apoptosis (Manning-Tobin *et al.* 2009). There was a 30% decrease in macrophage apoptosis in these mice due to increased expression of *Bcl2* and *Bcl2l1* anti-apoptotic genes. The expression levels of *Msr1* were decreased by 15-(S)-HETrE and PGE_1 in this study (Figures 4.9 and 4.10) and DGLA suppressed the development of foam cells by reducing the expression of the two main SRs: SR-A and CD36. Because DGLA and its metabolites inhibited oxLDL uptake and the development of foam cells *in vitro* (Figure 3.12), this decreased expression of *Msr1* is likely to be functionally significant.

Vwf plays an important role in regulating the adherence of platelets to vascular sites and induces coagulation of the blood (van Galen et al., 2012). In a clinical trial, the extent of atherosclerosis in both aortic and carotid arteries and the levels of VWF (Sonneveld et al. 2013). Animal studies also indicate that the deficiency VWF correlated with decreased atherosclerosis and atherothrombosis effects, including acute myocardial and ischemic stroke incidents (Shim et al. 2015; Doddapattar et al. 2018). 15-(S)-HETrE and PGE_1 significantly downregulated the expression of *Vwf* (Figures 4.9 and 4.10). Reduction in the expression of this might potentially implicate reduced platelet adhesion to vascular sites and prevention of disease progression, although this might also encourage atherogenesis by reducing blood coagulation.

Atherosclerotic lesions have classified macrophage-derived foam cells as a major source of MMPs, including MMP-3 (Beaudeux et al. 2003). The most noticeable key function of MMPs is the degradation of the fibrous cap leading to plaque instability and rupture (Newby *et al.* 2009). In a previous *in vivo* study that investigated the role of Mmp-3 in atherosclerotic aortas, macrophages in the atherosclerotic vessel wall and SMCs, plaques were significantly reduced in size around the whole thoracic aorta in ApoE$^{-/-}$/MMP-3$^{+/+}$ mice than in ApoE$^{-/-}$/MMP-3$^{-/-}$ (Silence et al. 2001). In the current study, the expression of *Mmp3* was significantly reduced by 15-(S)-HETrE and PGE_1 treatment compared to the control, suggesting a potential avenue for improving plaque stability (Figures 4.9 and 4.10).

Only three common genes were regulated by DGLA and its metabolites. It would be expected that this number would be higher on the basis of the hypothesis that the metabolites are mediating DGLA actions. The exact reason for the low number is unclear but it should be noted that DGLA and metabolites were added externally to the cells and uptake and metabolism of metabolites may have impacted gene expression. Future studies should compare the effects of DGLA on gene expression in normal cells with those where the metabolites are absent or produced at low levels (e.g. knockout or knockdown of enzymes that produce the metabolites).

A summary of key findings is shown in Figure 4.13. The gene expression changes produced by DGLA and its metabolites could be essential for preventing and treating atherosclerosis. DGLA and its metabolites can potentially be used as agents to treat inflammatory diseases and to reduce damage due to chronic inflammation. A substantial reduction in the expression of genes participating in cell adhesion (Figures 4.8, 4.9 and 4.10) was seen, which correlated with a decrease in monocyte migration demonstrated during *in vitro* studies (Figure 3.8). Furthermore, downregulation of expression of genes implicated in foam cell formation (Figures 4.8, 4.9 and 4.10) may help to suppress serum LDL-C and raise HDL-C levels. More research that thoroughly evaluate the efficacy of DGLA, 15-(S)-HETrE and PGE_1 will improve our understanding of the activities of DGLA and its metabolites and could help in the development of alternative treatment and prevention approaches to eliminate the global prevalence of CVD. Based on the findings outlined in this chapter, research has continued to focus on explaining the mechanisms underlying these anti-atherosclerotic effects. In the studies presented in the next chapter, pharmacological inhibitors were utilised to explore the roles of the COX and LOX pathways in THP-1 macrophages together with BMDM from C57BL/6J and 12/15 Lox deficient mice identify the effect of DGLA and its metabolites on key processes in atherosclerosis.

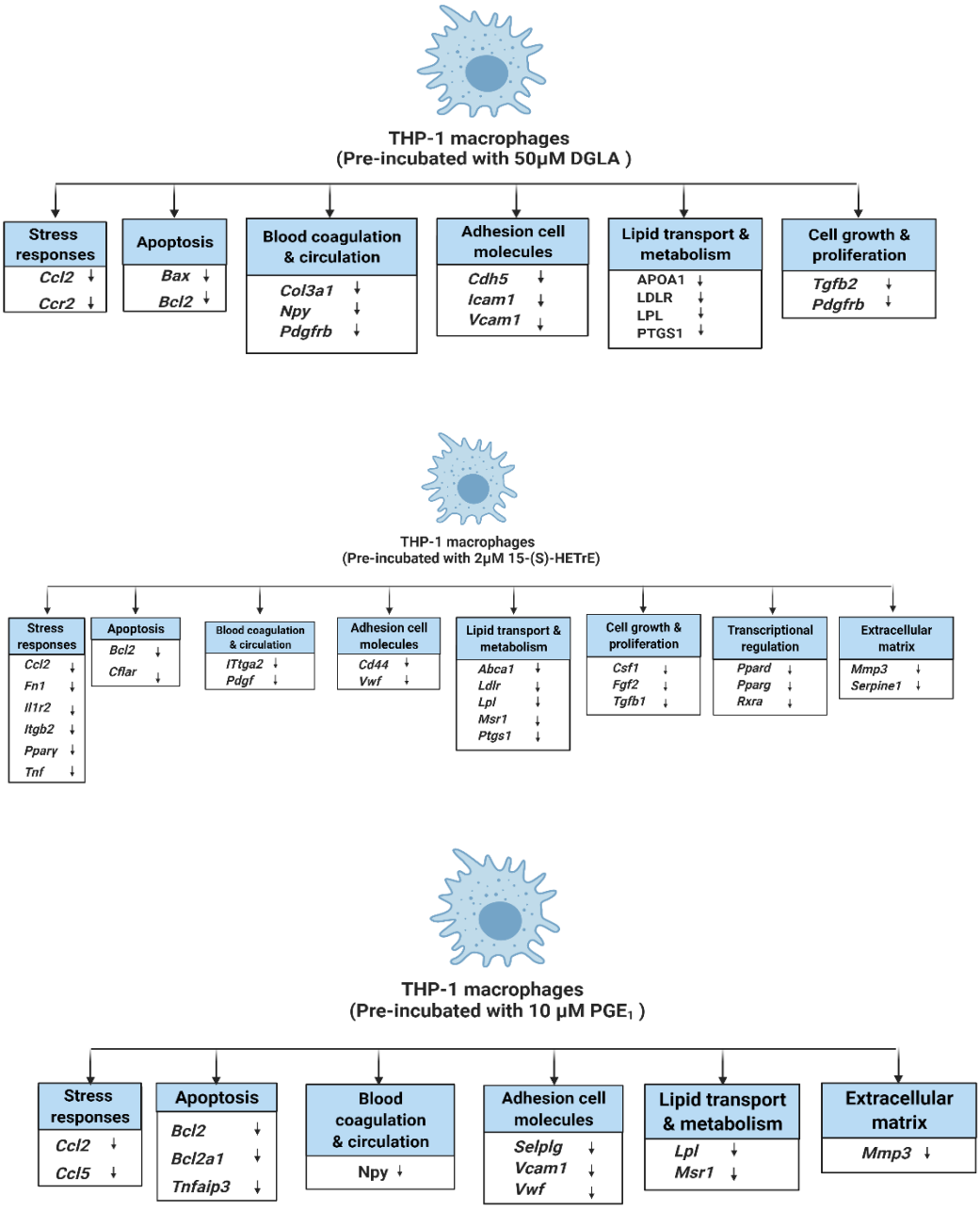


Figure 4. 14 Summary of the potential role of DGLA and its metabolites in decreasing the expression of key genes implicated in atherosclerosis.
Created with BioRender.com.

Chapter 5 The potential mechanisms underlying the anti-atherogenic actions of DGLA and its metabolites.

5.1 Introduction

The studies described in Chapter 3 showed that DGLA and its metabolites have several protective actions *in vitro*, including decreased monocyte migration and suppressed macrophage foam-cell formation. The data generated in this work, therefore, were quite positive regarding the potential of DGLA and its metabolites as anti-atherogenic agents. Although these *in vitro* experiments were highly instructive and facilitate a better understanding of the potential of DGLA and its metabolites as preventative/therapeutic agents, more deeper insights into the underlying molecular mechanisms are required. It was therefore decided to extend our analysis of the beneficial actions of DGLA and its metabolites further to potential mechanisms using pharmacological inhibitors and BMDM from wild type mice and those that are deficient in a key gene potentially involved in cellular DGLA metabolism.

5.1.1 Production and function of eicosanoids in diseases

Changes in the dietary intake of fatty acids, such as increased levels of PUFAs, results in improved eicosanoid production (Calder and Grimble 2002). The two main pathways involved in the production of eicosanoids are known as the COX pathways and the LOX pathways (Seo and Oh 2017). Eicosanoids play key roles in addition to their direct intracellular effects; for example, they can activate signalling pathways by interacting with cell surface receptors such as on the platelet cell surface that can subsequently influence platelet activation and aggregation (JIN et al. 2005). The production of eicosanoids requires the availability of lipid or phospholipid cleaved fatty acid substrates (Wang et al. 2012). DGLA can be catalysed into a wide range of products, including prostaglandins, lipoxins, leukotriene, thromboxane and hydroxy eicosanoids by the COX and LOX enzymes (Wang et al. 2012). The production of eicosanoids from DGLA include several anti-inflammatory mediators (e.g., PGE_1, PGD1, 5-HETE, 12-HETE and 15-HETrE) (Iversen et al. 1992). Because AA, an eicosanoid that promotes inflammation, can also be produced from DGLA in the omega-6 pathway by delta-5 desaturase, it is also important to determine the roles of LOX and COX in DGLA actions in THP-1 macrophages to determine how DGLA treatment affects eicosanoid production. Specific metabolites of DGLA are detailed in Figure 5.1 and Table 5.1.

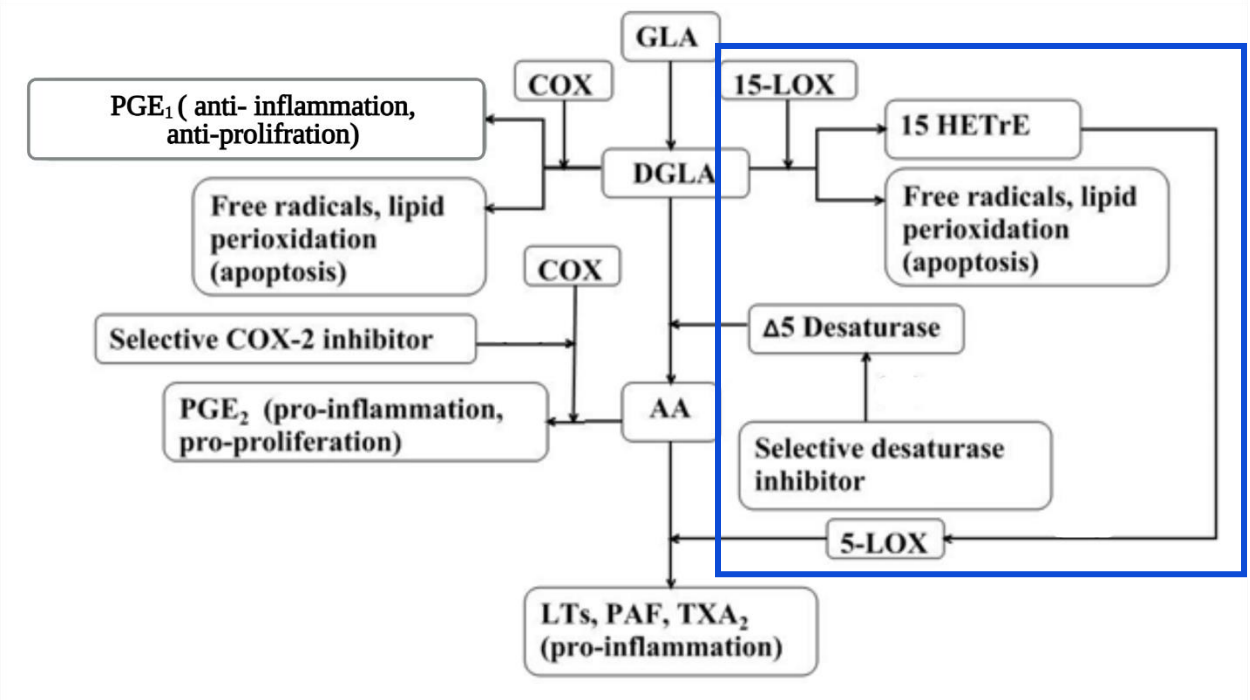


Figure 5. 3 DGLA metabolism.

GLA is transformed to AA by an alternating sequence of Δ6 desaturation, chain elongation and Δ5 desaturation. GLA reaches the desaturation stage and is elongated to DGLA, with only a very limited amount being desaturated to AA by Δ5 desaturase. DGLA can be converted to PGE_1 via the cyclooxygenase pathway and/or converted to 15-HETrE via the 15-lipoxygenase pathway. Abbreviation: AA, Arachidonic acid; COX, Cyclo-oxygenase; DGLA, Dihomo-γ-linolenic acid; GLA, γ-linolenic acid; HETrE, Hydroxy-eicosatriaenoic acids; LOX, Lipo-oxygenase (Wang et al. 2012).

Table 5. 1 Functions of specific metabolites of COX and LOX from DGLA or AA.

Name	Abbreviation	LOX/COX and substrate	Function	References
12-hydroxyeicosatetraenoic acid	12-HETE	12-LOX from AA	Promotes ICAM-1 expression and atherogenesis in association with insulin resistance and obesity.	(Funk 2006; Kühn and O'Donnell 2006)
Prostaglandin E_2	PGE_2	COX from AA	The lack of PGE_2 in LDLR knockout mice showed a substantial decrease in plaque. In NC/Tnd mice, increased levels of PGE_2 lead to atopic dermatitis.	(Suzuki *et al.* 2011; Amagai *et al.* 2015)
Leukotriene B_4	LTB4	5-LOX from AA	Effective monocyte chemoattractant, increasing MCP-1 expression. Promotes macrophage transformation to foam cells through increasing the expression of CD36.	(Subbarao et al. 2004; Neels 2013)
Lipoxin A_4	LXA4	5,15-LOX from AA	Attenuates TNF-α simulated adherence of neutrophils to endothelial cell monolayers together with the release of IL-8 and MCP-1.	(Goh et al. 2001; Cho et al. 2010)
Prostaglandin E_1	PGE_1	COX from DGLA	Decreases blood pressure, prevents platelet aggregation, facilitates cell cholesterol efflux, and inhibits the biosynthesis of cholesterol. In cancer cell lines, it plays a key role in anti-proliferation.	(Zurier *et al.* 1977; Horrobin 1991)

Prostaglandin D_1	PGD_1	COX from DGLA	in AC/Tnd mice, inhibits atopic dermatitis progression.	(Amagai et al. 2015)
15-hydroxyeicosatrienoic acid	15-HETrE	15-LOX from DGLA	Prevents 5-LOX and 12-LOX levels. Effective blocker of LBT4 actions.	(Iversen et al. 1992)

5.2 Aims

The study described in this chapter aimed to determine the mechanisms through which DGLA and its metabolites attenuate cellular processes associated with atherosclerosis, particularly the roles of the COX and LOX pathways using pharmacological inhibitors and, for the first time, cells from gene knockout mice. Figures 5.2-5.3 provides a schematic representation of the approaches used. This extended our investigation of DGLA to more in-depth understanding of the underlying molecular mechanisms.

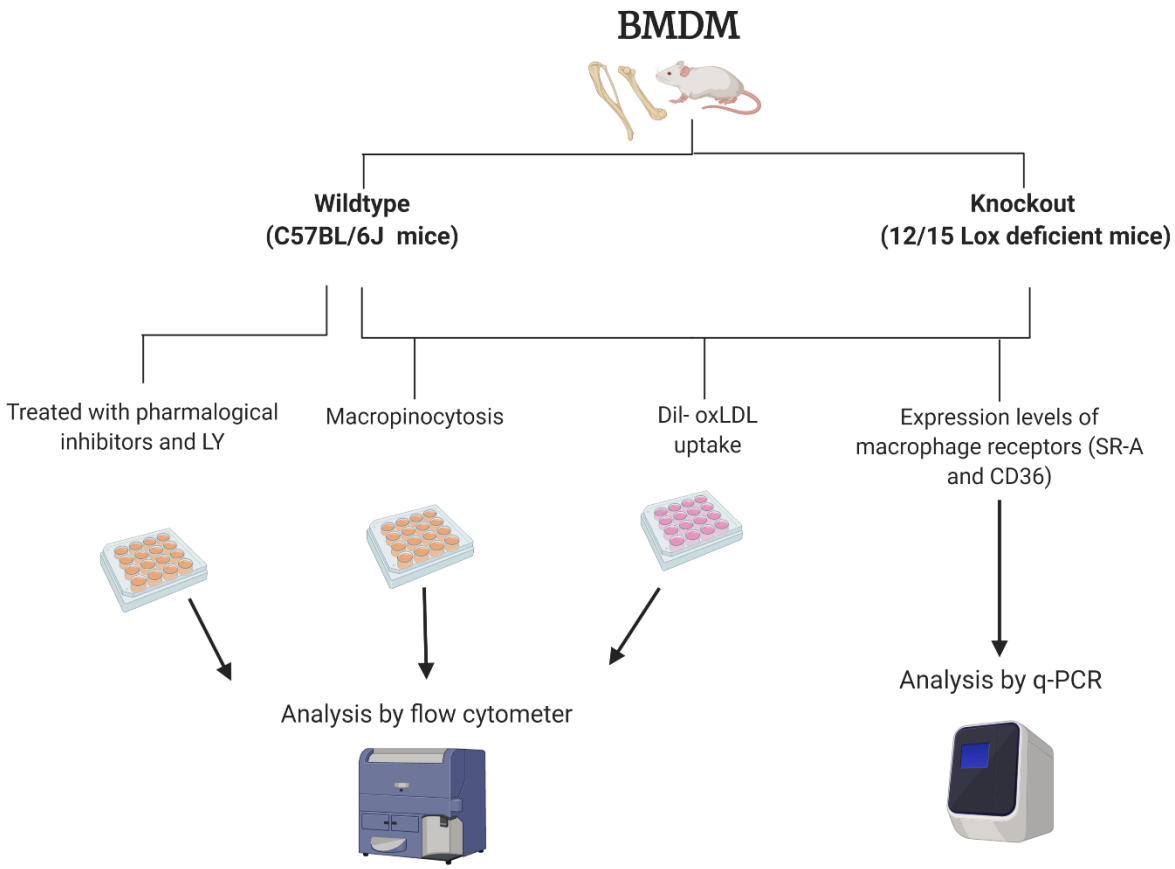


Figure 5. 4 Experimental workflow for assessing the effects of DGLA using pharmacological inhibitors and BMDM from C57BL/6J and 12/15 Lox knockout mice on micropinocytosis.

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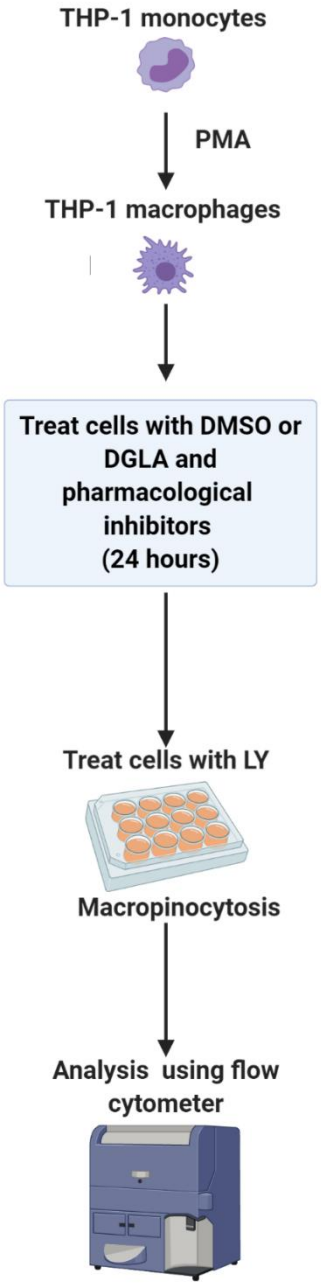

Figure 5. 5 Experimental workflow for the analysis of the effects of pharmacological inhibitors.
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5.3 Results

5.3.1 The effects of pharmacological inhibitors on DGLA actions *in vitro*.

To investigate the roles of COX and LOX pathways in DGLA-mediated inhibition of macropinocytosis, the effects of the COX inhibitor indomethacin and the 15-LOX-1 inhibitor ML351 was investigated. Previous studies had used COX inhibitor indomethacin at 1 μM (Abrial et al. 2015) and 15-LOX inhibitor ML351 at 10 μM (Abrial et al. 2015). THP-1 macrophages were incubated with the DMSO vehicle or 50 μM DGLA in the presence of 1 μM indomethacin or 10 μM ML351 followed by 100 μg/ml LY for 24 hours as described in Section 2.2.5.2. LY was used to monitor macropinocytosis as previous studies (Swanson and Watts 1995; Jones and Willingham 1999; Michael et al. 2013). The amount of LY uptake in the control, vehicle-treated cells was arbitrarily assigned as 100%. As shown in Figure 5.4A-B, both indomethacin and ML351 had no significant effect on LY uptake observed in cells treated with vehicle alone (i.e., there was no effect on constitutive macropinocytosis). DGLA produced a significant reduction in LY uptake compared to that observed in cells treated with vehicle alone ($p=0.029$ for panel A and $p=0.007$ for panel B). Such a DGLA-mediated decrease in LY uptake was attenuated following the inclusion of indomethacin, thereby indicating a role for the COX pathway in the action of the fatty acid. The inclusion of ML351 also produced some attenuation as there was a trend towards reduction of LY uptake by DGLA (14.3% decrease, $p=0.056$) compared to significant reduction produced in the absence of the inhibitor. This suggests that the LOX pathway is also involved in the actions of DGLA though further experiments are required for confirmation.

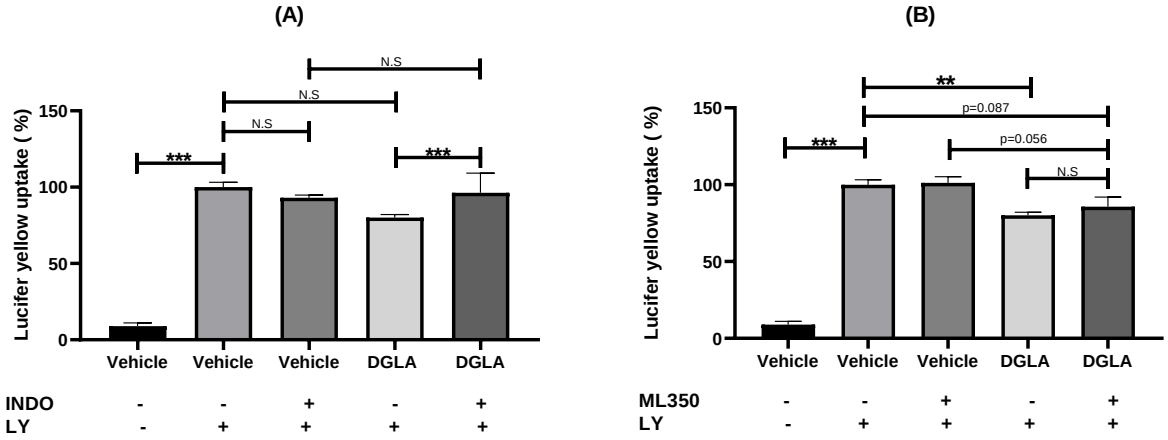

Figure 5. 6 The effect of pharmacological inhibitors on DGLA-mediated LY uptake by macropinocytosis in THP-1 macrophages.

THP-1 macrophages were incubated with DMSO vehicle control or 50 μM DGLA for 24 hours with 1 μM indomethacin or 10 μM ML351 or their corresponding vehicle followed by 100 μg/ml LY (+) for a further 24 hours. Cells treated with vehicle in the absence of LY were also included for comparison. Flow cytometry (FACS Canto) was employed to determine LY uptake. LY uptake is reported as a percentage value (mean +/- SEM from four independent experiments) with the vehicle control (LY and DMSO with no pharmacological agent) arbitrarily assigned at 100%. Statistical analyses were performed using a One-way ANOVA and Dunnett two-sided post-hoc test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$; N.S, not significant).

5.3.2 DGLA, 15-(S)-HETrE and PGE_1 have no effect on BMDM cell viability.

At the time of these studies, 12/15-Lox-deficient animals became available that allowed us to probe the requirement of 15-(S)-HETrE, which are produced by 12/15-Lox in mice, in DGLA-mediated actions in more detail using BMDM from these animals. As none of our previous studies on the effects of DGLA were carried out on BMDM, it was first necessary to confirm that key responses were indeed conserved in these cells before moving to knockout animals. The viability of these cells to treatment with 50 μM DGLA, 2 μM 15-(S)-HETrE or 10 μM PGE_1 was first determined by following the release of the LDH enzyme. As shown in Figure 5.5, DGLA and its metabolites had no effect on the viability of these cells.

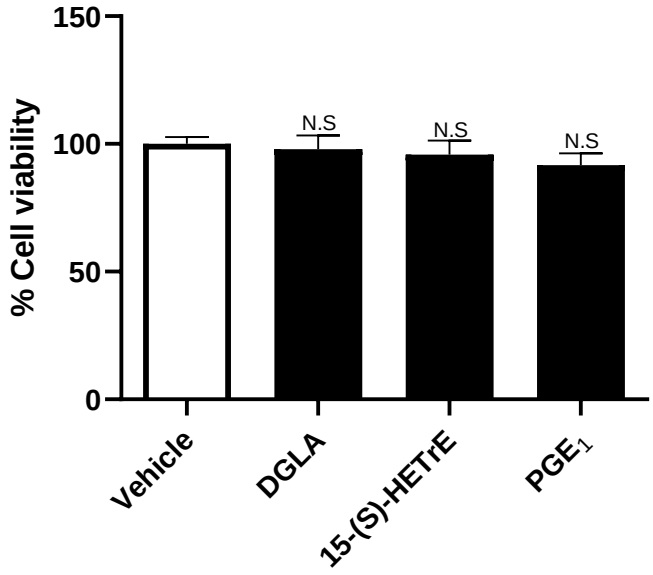

Figure 5. 7 The effects of DGLA, 15-(S)-HETrE or PGE_1 on BMDM cell viability.
BMDM from C57BL/6J mice were incubated with 50 μM DGLA, 2 μM 15-(S)-HETrE, 10 μM PGE_1 or the vehicle control for 24 hours. The results are shown as percent change (mean +/- SEM) compared to the vehicle control (arbitrarily assigned as 100%) from four independent experiments. Statistical analysis was carried out by One-way ANOVA with Dunnett post-hoc analysis comparing each individual treatment to the control. No significant changes were observed (N.S, not significant).

5.3.3 DGLA and its metabolites had no effects on the proliferation of BMDM.

A CV assay was used to determine the effects of DGLA and its metabolites on proliferation of BMDM from C57BL/6J mice. As shown in Figure 5.6, there were no significant changes in cell proliferation in cells treated with 50 μM DGLA, 2 μM 15-(S)-HETrE or 10 μM PGE_1 compared to the vehicle control.

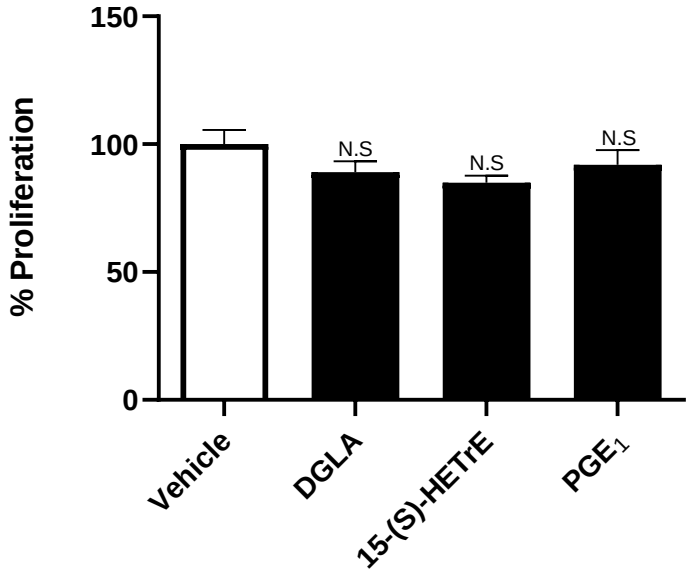

Figure 5. 8 DGLA, 15-(S)-HETrE and PGE_1 did not affect macrophage proliferation.
BMDM from C57BL/6J mice were incubated with 50 μM DGLA, 2 μM 15-(S)-HETrE, 10 μM PGE_1 or the vehicle control for 24 hours. The results are shown as percent change (mean +/- SEM) compared with the vehicle control (arbitrarily assigned as 100%) from four independent experiments. Statistical analysis was carried out by One-way ANOVA with Dunnett post-hoc analysis comparing each individual treatment to the control. No significant changes were observed (N.S, not significant).

5.3.4 DGLA and its metabolites inhibit oxLDL uptake in BMDM from C57BL/6J but not 12/15 Lox deficient mice.

The effect of DGLA and its metabolites on the uptake of Dil-oxLDL was first investigated using BMDM from C57BL/6J mice. As shown in Figure 5.7, Dil-oxLDL uptake seen in the vehicle-treated control cells was reduced by treatment with 50 μM DGLA (20.3%; p =0.017), 2 μM 15-(S)-HETrE (19.9%; p=0.030) and 10 μM PGE_1 (20.0%; p=0.023).

The effect of 12/15 Lox deficiency on this DGLA-mediated inhibition of Dil-oxLDL was next investigated. In BMDM from C57BL/6J mice, there was a significant attenuation of Dil-oxLDL uptake in the cells treated with 50 μM DGLA (15.9%; p=0.001) compared to the vehicle control. However, such a DGLA-mediated reduction in Dil-oxLDL uptake was not observed in BMDM from 12/15 Lox knockout mice. Instead, there was a significant increase in Dil-oxLDL uptake of 12.2% (p= 0.032) (Figure 5.8B).

As BMDM from 12/15 Lox deficient mice should lack metabolites such as15-(S)-HETrE, further experiments were carried out to determine whether the loss of DGLA-mediated inhibition of Dil-oxLDL uptake in BMDM from 12/15 Lox deficient mice could be overcome by the addition of the metabolites 15-(S)-HETrE and PGE_1. Similar experiments were also carried out with BMDM from C57BL/6J mice for comparison. Figure 5.9A shows that there was significant attenuation of Dil-oxLDL uptake in the BMDM from C57BL/6J by 50 μM DGLA alone (13.4%; p= 0.004) or 50 μM DGLA together with 2 μM 15-(S)-HETrE (10.5%; p = 0.030) or 10 μM PGE_1 (14.2%; p= 0.023). No further reduction of DGLA-mediated inhibition of Dil-oxLDL uptake was observed by the inclusion of 15-(S)-HETrE or PGE_1. In contrast to these findings, DGLA produced a non-significant increase in Dil-oxLDL uptake compared to the vehicle control in BMDM from 12/15 Lox deficient mice (Figure 5.9B). However, a reduction of 9.1% was observed when 15-(S)-HETrE (p= 0.074) was added with DGLA and a decrease of 12.3% was observed when PGE_1 was added with DGLA (p= 0.020) (Figure 5.9B).It was therefore of interest to determine the precise effect.

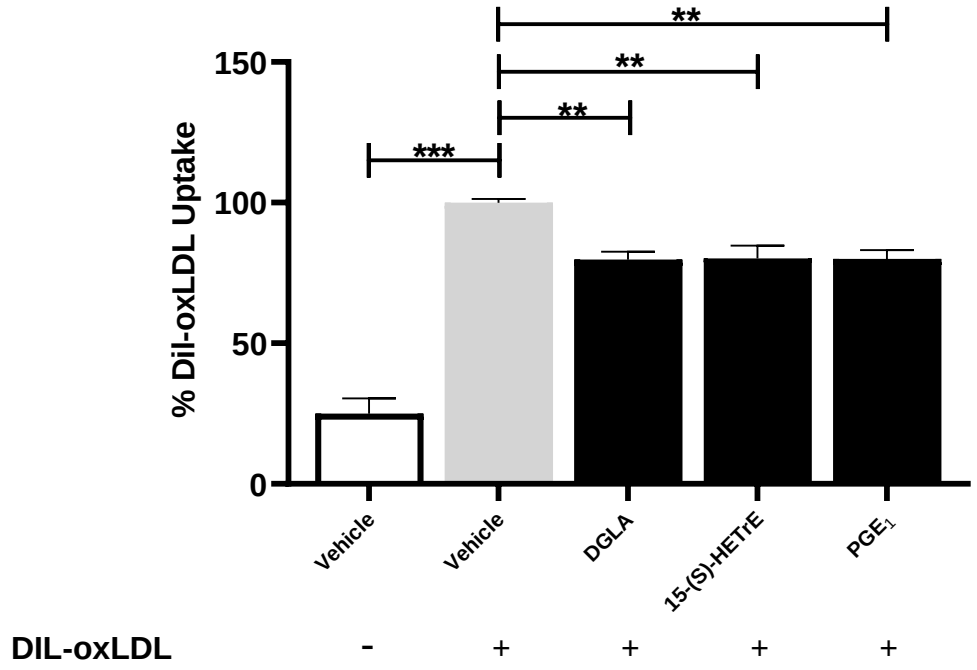

Figure 5. 9 Significant attenuation of Dil-oxLDL uptake by BMDM from C57BL/6J mice treated with DGLA, 15-(S)-HETrE or PGE_1

BMDM were incubated with DMSO vehicle control, 50 μM DGLA, 2 μM 15-(S)-HETrE or 10 μM PGE1 for 24 hours prior to the addition of 5 μg/ml Dil-oxLDL for a further 24 hours. Cells incubated with vehicle in the absence of Dil-oxLDL were also included for comparison. Macrophages were resuspended in 2% PFA and FACS analysis was carried out to determine uptake by counting 10,000 events. Results are mean +/- SEM from three independent experiments (Dil-oxLDL uptake in cells treated with vehicle has been arbitrarily assigned as 100). Statistical analysis was carried out by One-way ANOVA followed by Dunnett post hoc test (** $P \leq 0.01$ and *** $P \leq 0.001$).

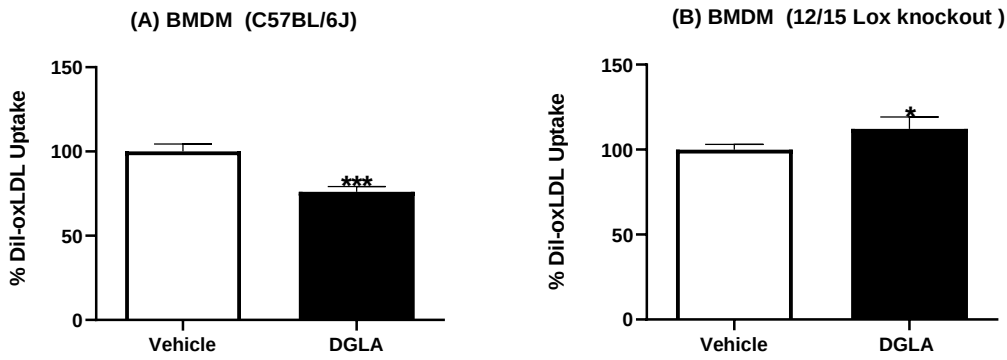

Figure 5. 10 The effect of DGLA on oxLDL uptake by BMDM from C57BL/6J and 12/15 Lox deficient mice.

BMDM cultures from (A) C57BL/6J and (B) 12/15 Lox knockout mice were incubated with DMSO vehicle control or 50 μM DGLA for 24 hours followed by 5 μg/ml Dil-oxLDL. Flow cytometry (FACS Canto) was employed to determine Dil-oxLDL uptake. Dil-oxLDL uptake is reported as a percentage value (mean +/- SEM from five independent experiments) with the vehicle control (Dil-oxLDL and DMSO) arbitrarily assigned at 100%. Statistical analysis was carried out using an unpaired Student's two-tailed t-test (* $P \leq 0.05$ and *** $P \leq 0.001$).

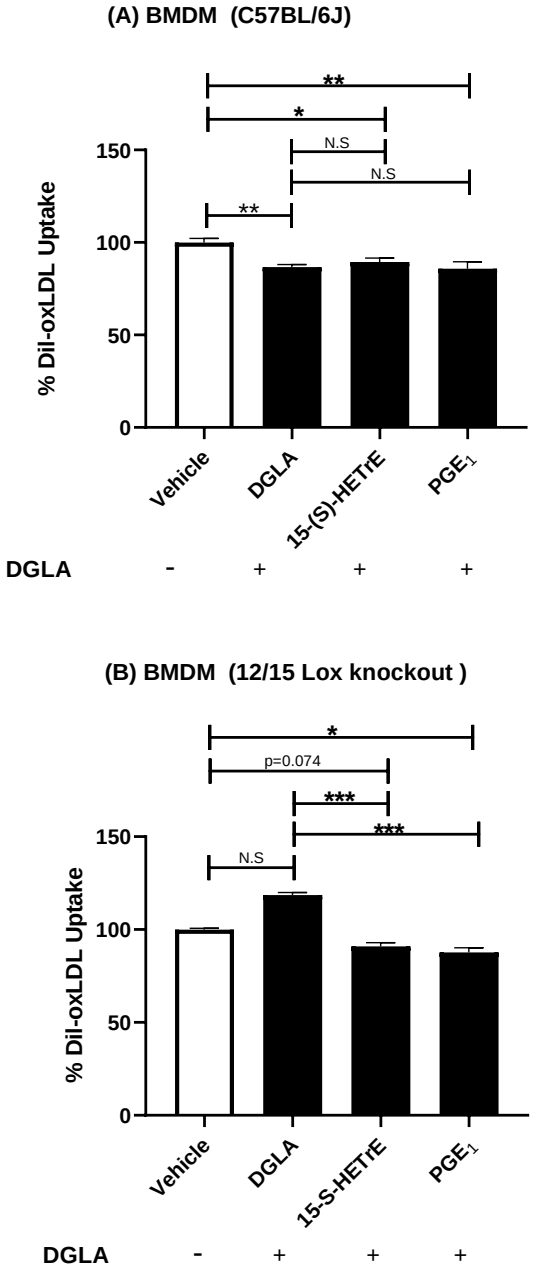

Figure 5. 11 The effect of DGLA alone or with 15-(S)-HETrE or 10 μM PGE_1 on oxLDL uptake by BMDM from C57BL/6J and 12/15-Lox deficient mice.

BMDM cultures from (A) C57BL/6J and (B) 12/15 Lox knockout mice were incubated with DMSO vehicle control or 50 μM DGLA alone or with 2 μM 15-(S)-HETrE or 10 μM PGE_1 for 24 hours followed by 5 μg/ml Dil-oxLDL. Flow cytometry (FACS Canto) was employed to determine Dil-oxLDL uptake. Dil-oxLDL uptake is reported as a percentage value (mean +/- SEM from five independent experiments) with the vehicle control (Dil-oxLDL and DMSO) arbitrarily assigned at 100%. Statistical analysis was carried out using an unpaired Student's two-tailed t-test (* $P \leq 0.05$, *** $P \leq 0.001$, $P = 0.074$; N.S, not significant).

5.3.5 DGLA and metabolites reduce macropinocytosis in BMDM from C57BL/6J mice.

The effect of DGLA and its metabolites on macropinocytosis was evaluated using the LY fluorescent marker as outlined in section 2.2.6.2. BMDM cultures from C57BL/6J mice were pre-incubated with the DMSO vehicle or 50 μM DGLA, 2 μM 15-(S)-HETrE or 10 μM PGE_1 for 24 hours followed by 100 μg/ml LY for 24 hours. Cells incubated with vehicle in the absence of LY treatment were also included for comparative purposes. The cellular uptake of LY was inhibited by DGLA and its metabolites though this failed to reach significance with 15-(S)-HETrE and a trend towards reduction was observed for DGLA (24.8%; p=0.098). A significant decrease in LY uptake was observed for PGE_1 (27.9%; p=0.034) (Figures 5.10).

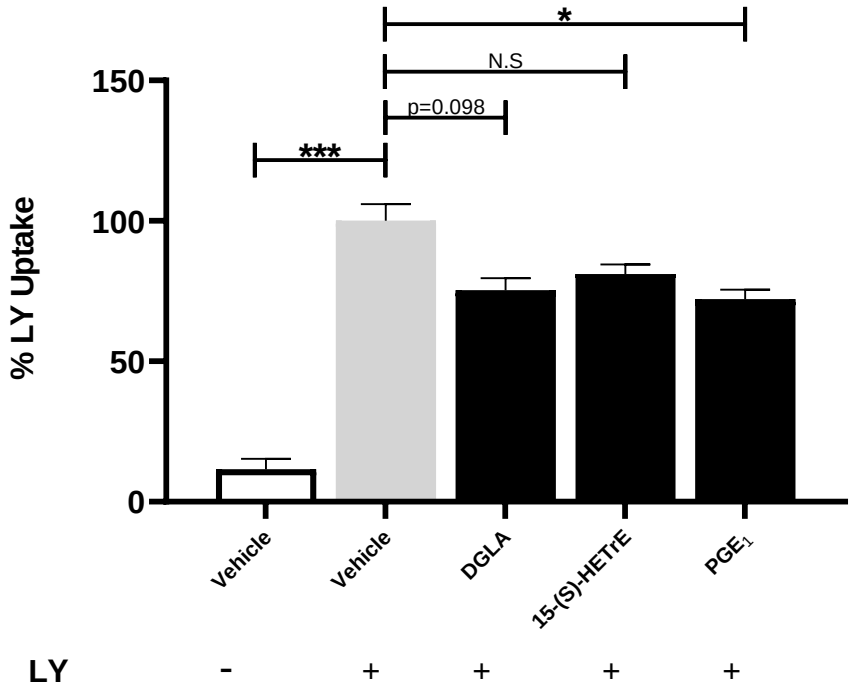

Figure 5. 12 Attenuation of LY uptake by BMDM from C57BL/6J mice by DGLA, 15-(S)-HETrE and PGE_1.

BMDM were incubated with DMSO vehicle control, 50 μM DGLA, 2 μM 15-(S)-HETrE or 10 μM PGE_1 for 24 hours prior to the addition of 100 μg/ml LY (+) for a further 24 hours. Cells treated with vehicle without LY were also included for comparison. The macrophages were resuspended in 2% PFA and FACS analysis was carried out to determine uptake by counting 10,000 events. Results are mean+/- SEM from three independent experiments. Statistical analysis was carried out by One-way ANOVA followed by Dunnett post-hoc test (* $P \leq 0.05$, *** $P \leq 0.001$, $P = 0.098$; N.S, not significant).

5.3.6 The DGLA-mediated inhibition of LY uptake in BMDM from C57BL/6J mice is attenuated in cells from12/15 Lox knockout mice.

We next investigated whether 12/15 Lox deficiency impacted DGLA-mediated changes in the uptake of LY by micropinocytosis. In this series of experiments, DGLA produced a significant decrease in LY uptake (5.6%; $p \leq 0.05$) (Figure 5.11A). However, such an inhibition of LY uptake was not observed with BMDM from 12/15 Lox deficient mice where instead a trend towards increase in LY uptake was seen (24.5%; p=0.099) (Figure 5.11B).

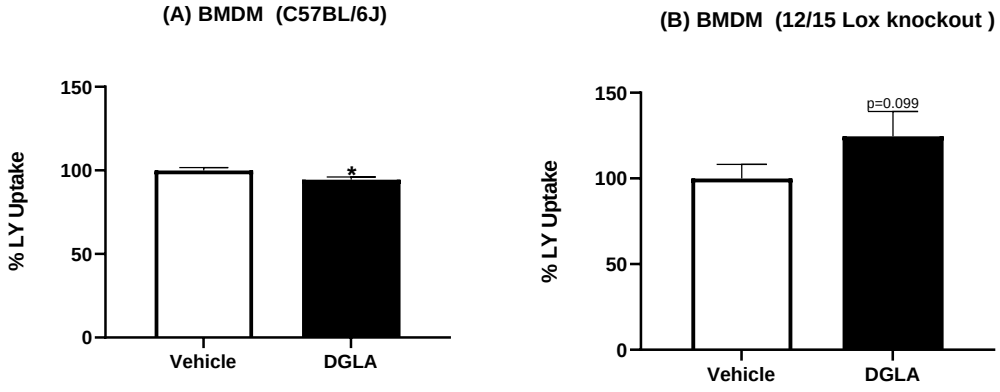

Figure 5. 13 The effect of DGLA on LY uptake by macropinocytosis in BMDM from C57BL/6J and 12/15 Lox deficient mice.

BMDM cultures from (A) C57BL/6J and (B) 12/15 Lox knockout mice were incubated with DMSO vehicle control or 50 μM DGLA for 24 hours followed by 100 μg/ml LY (+). Flow cytometry (FACS Canto) was employed to determine LY uptake. LY uptake is reported as a percentage value (mean +/- SEM from five independent experiments) with the vehicle control (LY and DMSO) arbitrarily assigned at 100%. Statistical analysis was carried out using an unpaired Student's two-tailed t-test (* $P \leq 0.05$ and $P = 0.099$).

5.3.7 The expression of SR-A was inhibited by DGLA, PGE_1 and 15-(S)-HETrE treatment of BMDM from C57BL/6J mice.

To investigate the effect of DGLA and its metabolites on key SRs involved in the uptake of modified LDL, BMDM from C57BL/6J mice were treated with either vehicle control, DGLA, 15-(S)-HETrE or PGE_1 for 24 hours. RNA was extracted and reverse transcribed into cDNA, and gene expression was determined by real-time qPCR as described in Section 2.2.5. As shown in Figure 5.12 A-F, gene expression of SR-A was reduced in the presence of 50 μM DGLA, 2 μM 15-(S)-HETrE and 10 μM PGE_1 by 27.7% (p=0.029), 34.3% (p=0.057), and 56.5% (p=0.029), respectively. Gene expression of CD36 showed a trend towards reduction in the presence of 50 μM DGLA by 33.8% (p= 0.057) whereas this was reduced non-significantly in the presence of 2 μM 15-(S)-HETrE and 10 μM PGE_1 by 18.6% (p = 0.686) and 44.9% (p=0.200) respectively (Figure 5.13 D-F). Because of the relatively low yields of RNA from BMDM from 12/15 Lox knockout mice, the effects of its deficiency on the expression of these SRs could not be determined.

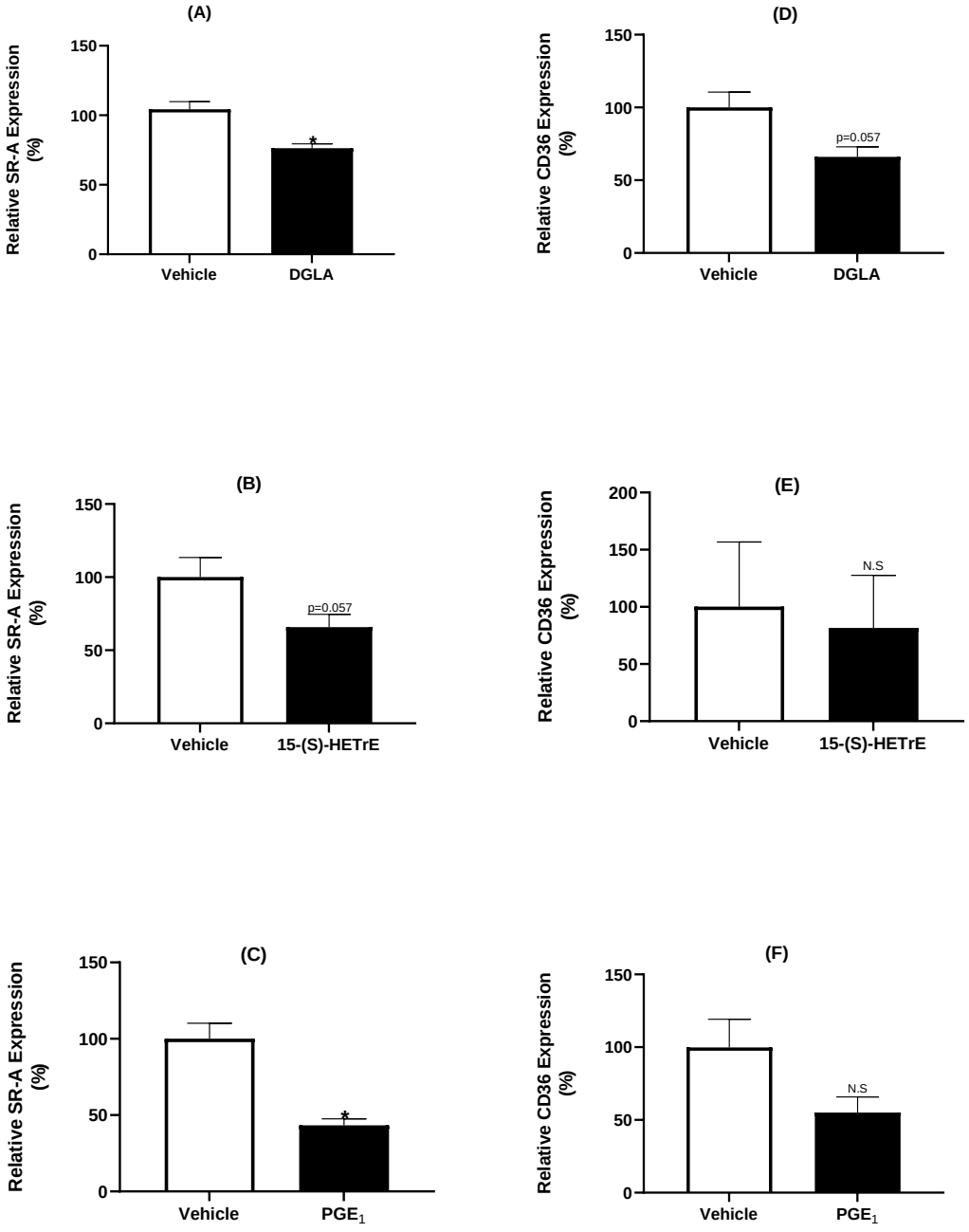

Figure 5. 14 Inhibition of expression of CD36 and SR-A in BMDM by DGLA and its metabolites.

BMDM were incubated with 50 μM DGLA, 2 μM 15-(S)-HETrE, 10 μM PGE_1 or DMSO vehicle control for 24 hours. Total RNA was subjected to RT-qPCR with primers specific for SR-A, CD36 or GAPDH control. The results show the average levels of gene expression (mean +/- SEM) (control arbitrarily assigned as 1) from four independent experiments. Statistical analysis was carried out using an unpaired Student's two-tailed t-test (* $P \leq 0.05$ and $P = 0.057$).

5.4 Discussion

In this chapter, the role of COX and LOX pathways was investigated using inhibitors in THP-1 macrophages and with BMDM from C57BL/6J mice and 12/15 Lox deficient mice. Tables 3.1-3.3 provide a summary of the results obtained in this study. The results show that the COX and LOX inhibitors attenuate DGLA-mediated inhibition of macropinocytosis (Figure 5.4). In addition, they provided information about the effect of DGLA and its metabolites on macropinocytosis, oxLDL uptake and expression of SRs in BMDM from C57BL/6J mice (Figures 5.12). The use of BMDM from 12/15-Lox deficient mice showed their role in DGLA-mediated changes in oxLDL uptake (Figure 5.8) and macropinocytosis (Figure 5.11).

Table 5. 2 Summary of the effects of pharmacological inhibitors on DGLA actions *in vitro*.

	Indomethacin		ML350	
	Effect	**P-value**	**Effect**	**P-value**
LY- vs. DGLA-	↓ (19.9%)	0.030(*)	↓ (19.9%)	0.007(**)
LY- vs. DGLA+	- (34.7%)	0.467(NS)	↓ (14.3%)	0.087 (T)
LY+ vs. DGLA-	- (14.4%)	0.276(NS)	↓ (21.1%)	0.004 (**)
LY +vs. DGLA+	- (29.2%)	>0.999(NS)	↓ (15.5%)	0.054(T)

Abbreviations: NC-No changes in relation to control; NS- no significant effect; ↓Downregulation; ↑Upregulation; +, with inhibitors; -, without inhibitors.

Table 5. 3 Summary of the effect of DGLA and its metabolites on various cellular processes in BMDM from C57BL/6J mice.

Cellular processes	DGLA		15-S-HETrE		PGE_1	
	Effect	P-value	Effect	P-value	Effect	P-value
Viability	NC	NS	NC	NS	NC	NS
Proliferation	NC	NS	NC	NS	NC	NS
Macropinocytosis	↓	0.0976(T)	-	0.5814(NS)	↓	0.0339(*)
Dil-oxLDL uptake	↓ (20.3%)	0.0016(**)	↓ (19.9%)	0.002(**)	↓ (20.0%)	0.0019(**)
SR (SR-A)	↓ (27.7%)	0.0286(*)	↓ (34.3%)	0.0571(T)	↓ (56.5%)	0.0286(*)
SR (CD36)	↓ (33.8%)	0.0571(T)	- (18.6%)	0.6857(NS)	↓ (44.9%)	0.2000(NS)

Abbreviations: NC, No changes in relation to control; NS- no significant effect; T, Trend; ↓, Downregulation; ↑, Upregulation.

Table 5. 4 Summary of the effect of DGLA on macropinocytosis and oxLDL uptake in 12/15 Lox deficient mice.

Cellular processes	DGLA		15-(S)-HETrE+ DGLA		PGE_1+ DGLA	
	Effect	P-value	Effect	P-value	Effect	P-value
Macropinocytosis	↑ (24.5%)	0.0987(T)	NT	NT	NT	NT
Dil-oxLDL uptake	↑ (12.2%)	0.0320 (*)	NT	NT	NT	NT
Reversing by adding PGE_1 and 15-(S)-HETrE	- (13.4%)	0.3022 (NS)	↓ (10.5%)	0.0744 (T)	↓ (14.2%)	0.0196 (*)

Abbreviations: NS, no significant effect; T-trend; ↓, Downregulation; ↑, Upregulation; NT, Not tested.

5.4.1 Eicosanoid production from THP-1 macrophages

5.4.1.1 COX metabolites

DGLA is a substrate for COX enzymes and causes metabolism into prostaglandin series 1. Thus, via the cyclooxygenase pathway, DGLA can be metabolised to PGE_1 and increased levels of this eicosanoid have been reported after treatment of mouse peritoneal macrophages with GLA, an upstream precursor of DGLA (Chapkin and Coble 1991), and in human mononuclear leukocytes with DGLA (Iversen et al. 1992). PGE_1 was the key prostaglandin (PG) species observed in THP-1 macrophages following treatment with DGLA. PGE_1 has been demonstrated to have many beneficial anti-inflammatory actions in a number of diseases (Sinzinger et al. 1991; Palumbo et al. 2000; Wang et al. 2012). Also, PGE_1 is noted for its anti-thrombosis properties (Zhao et al. 2017). It has been hypothesised that the metabolism of PUFA to prostaglandin may be the process by which DGLA exerts its anti-inflammatory and anti-atherogenic actions (Gallagher et al., 2016). This seems to be likely considering the ability of PGE_1 to mimic main responses of DGLA. For example, as shown in Chapter 3, PGE_1 produced the inhibition of several major atherosclerosis-associated processes, such as monocyte migration and foam cell formation. Additionally, studies in Chapter 4 showed that PGE_1 plays a key role in the regulation of expression of many atherosclerosis-associated genes. More importantly, in the studies presented in this chapter, the inhibition of COX in THP-1 macrophages using indomethacin was associated with attenuation of DGLA-mediated macropinocytosis (Figure 5.4A). Previously, oral administration of DGLA was found to significantly attenuate atherosclerosis in ApoE-deficient mice (Takai *et al.* 2009b). This clearly suggests that PGE_1 is a key mediator for the anti-atherosclerotic effect of DGLA.

5.4.1.2 LOX metabolites

The LOX pathway relates to the activities of 5/12/15-LO to generate leukotrienes, lipoxins and some HETEs in leukocytes (Kikut et al., 2020); hydroxy eicosanoids derived from AA have a variety of pro-inflammatory functions (Kang and Weylandt 2008). They can also act as substrates to yield several other eicosanoids, with both pro- and anti-inflammatory roles (Samuelsson et al. 1987; Neels 2013). For instance, the action of 5-LOX on AA produces 5-HPETE, that is a precursor for the production of proinflammatory LT (Samuelsson et al. 1987; Neels 2013). In comparison, 15-HETE metabolism from AA serves as a precursor for the production of lipoxin,

which has been linked to resolution of inflammation (Goh et al. 2001; Cho et al. 2010). 15-HETrE is well known for suppressing pro-inflammatory LTB4 production (Johnson et al. 1997) and the production of LBT4 in rat macrophages has been found to be decreased significantly by DGLA (Nakamura et al. 1993).

As shown in the previous chapters, several key atherosclerosis-associated processes are inhibited by 15-(S)-HETrE, including foam cell formation in macrophages, monocyte migration, pro-inflammatory cytokine induced gene expression, modified LDL uptake, scavenger receptor expression and macropinocytosis. In the presence of ML351, the extent of DGLA mediated reduction of LY uptake was also attenuated (Figure 5.4B) thereby indicating an important of the LOX pathway in DGLA action. ML351 has also been shown to inhibit 15-LOX-1 associated with significant inhibition of the LPS-induced production of CCL2 and CCL3 (Abrial et al. 2015).

5.4.2 Modified LDL uptake by BMDM macrophages

The development of foam cells leading to excessive oxLDL uptake and imbalance in lipid efflux is important in the formation of atherosclerotic lesions (McLaren *et al.* 2011a). Treatment of BMDM from male C57BL/6J mice with 1-palmitoyl-2-arachidonoyl phosphatidylcholine (PAPC) PUFAs have been shown previously to have a lipid-lowering activity and inhibit foam cell formation (Lu et al. 2017). Also, 12/15 Lox is expressed in a variety of cells, including vascular cells and macrophages (Huo et al. 2004). Furthermore, increased 12/15-LOX expression has been demonstrated to increase the degradation of cholesterol ester, resulting in decreased development of foam cells (Belkner et al. 2005). In this study, the effect of DGLA and its metabolites on the uptake of Dil-oxLDL was investigated for the first time in BMDM from C57BL/6J mice and 12/15 Lox deficient mice. Uptake of Dil-oxLDL was significantly attenuated in BMDM from C57BL/6J mice treated with DGLA, 15-(S)-HETrE and PGE_1 (Figure 5.9 (A)). Whilst there was a significant attenuation of Dil-oxLDL uptake in BMDM from C57BL/6J mice, there was a significant increase in Dil-oxLDL uptake in BMDM from 12/15 Lox knockout mice (Figure 5.9(B)). Additional experiments were performed to establish if the loss of DGLA-mediated inhibition of Dil-oxLDL uptake in BMDM from 12/15 Lox deficient mice could be overcome by the addition of 15-(S)-HETrE or PGE_1 metabolites (note that it would be expected that 15-(S)-HETrE would not be produced in these cells because of deficiency of 12/15 Lox). DGLA or DGLA plus 15-(S)-HETrE

or PGE_1 inhibited oxLDL uptake in BMDM from C57BL/6J mice. In contrast to these findings, in BMDM from 12/15 Lox deficient mice, DGLA increased Dil-oxLDL uptake while pre incubation with DGLA plus 15-(S)-HETrE or PGE_1 inhibited Dil-oxLDL uptake by approximately 20%. These results indicate that in macrophages, DGLA supplementation changes eicosanoid production, stimulating the anti-inflammatory mediators PGE_1 and 15-(S)-HETrE. Also, DGLA effects are likely to be mediated via the metabolites PGE_1 and 15-(S)-HETrE.

This study is the first to investigate the effect of DGLA and its metabolites treatment on oxLDL uptake in BMDM from C57BL/6J mice and 12/15 Lox deficient mice with no similar studies appearing in the literature to date. However, a previous study investigating the effects of 12/15 Lox deficiency showed decreased lesions in ApoE-deficient mice associated with reduced antibodies directed against oxLDL epitopes (Cyrus et al. 1999), and this indicates pro-atherogenic actions of Lox metabolites.

5.4.3 Macropinocytosis

Macropinocytosis is the process via which macrophages take up cholesterol crystals, LDL and modified LDL (Bobryshev 2006). Results show significant attenuation of LY uptake with 50 μM DGLA, 2 μM 15-(S)-HETrE or 10 μM PGE_1 in BMDM from C57BL/6J mice (Figure 5.10) However, such DGLA-mediated inhibition of LY uptake was not observed in BMDM from 12/15 Lox deficient mice, instead there was an increase LY uptake (Figure 5.11). This suggests a requirement of metabolites produced by the action of 12/15 Lox in DGLA-mediated inhibition of micropinocytosis. As the effect of DGLA treatment on macropinocytosis in BMDM from 12/15 Lox deficient mice has previously not been studied, these results represent a possible mechanism leading to an attenuation of foam cell formation during atherosclerosis development.

5.4.4 Scavenger receptors

$ApoE^{-/-}$ mice lacking SR-A and CD36 have shown that these two SRs are responsible for most of the modified LDL uptake by macrophages (Kunjathoor et al. 2002). Also, SR-A and CD36 deficient

macrophages failed to accumulate cholesterol esters, thus indicating that other scavenger receptors fail to compensate for the absence of SR-A and CD36 (Kunjathoor et al. 2002). A further recent research showed a significant decrease in the aortic lesion region in either CD36 (61-74% decrease) or SR-A (32% decrease) deficiency in ApoE$^{-/-}$ mice but no additional decrease was seen with the combined absence of CD36 and SR-A (Kuchibhotla et al. 2007). Gene expression analysis in BMDM from C57BL/6J mice showed that the expression of scavenger receptors SR-A and CD36 was attenuated by DGLA and its metabolites (Figure 5.12). Thus, the DGLA-mediated attenuation of oxLDL uptake by BMDM is at least in part by inhibition of macrophage scavenger receptors at the gene expression level. The results of this chapter support previous results on the anti-atherogenic action of DGLA and its metabolites in both THP-1 macrophages and HMDMs presented in chapter 3 and also in a previous study in our laboratory (Gallagher 2016).

5.5 Summary and future work

In the laboratory, previous research has shown that DGLA has a direct effect or acts by producing PGE_1 (Gallagher 2016). The studies presented in this chapter provided insight into the roles of 15-(S)-HETrE and PGE_1 produced from DGLA in the regulation of the inflammatory response in atherosclerosis, particularly several key atherosclerosis-associated processes. The summary of the potential anti-atherogenic properties of DGLA and its metabolites in BMDM from C57BL/6J mice are summarised in Figure 5.13. The ability of DGLA and its metabolites to reduce the development of foam cells is an important result in terms of anti-atherosclerotic therapy, considering the important impact of foam cell accumulation on atherosclerosis. In combination with the beneficial impacts identified in Chapter 3, it can hypothesise that DGLA and its metabolites are a potential candidate for anti-atherosclerotic treatment. Studies discussed in the next chapter will use bone marrow to investigate the impact of DGLA on haematopoietic stem and progenitor cell populations *in vivo*.

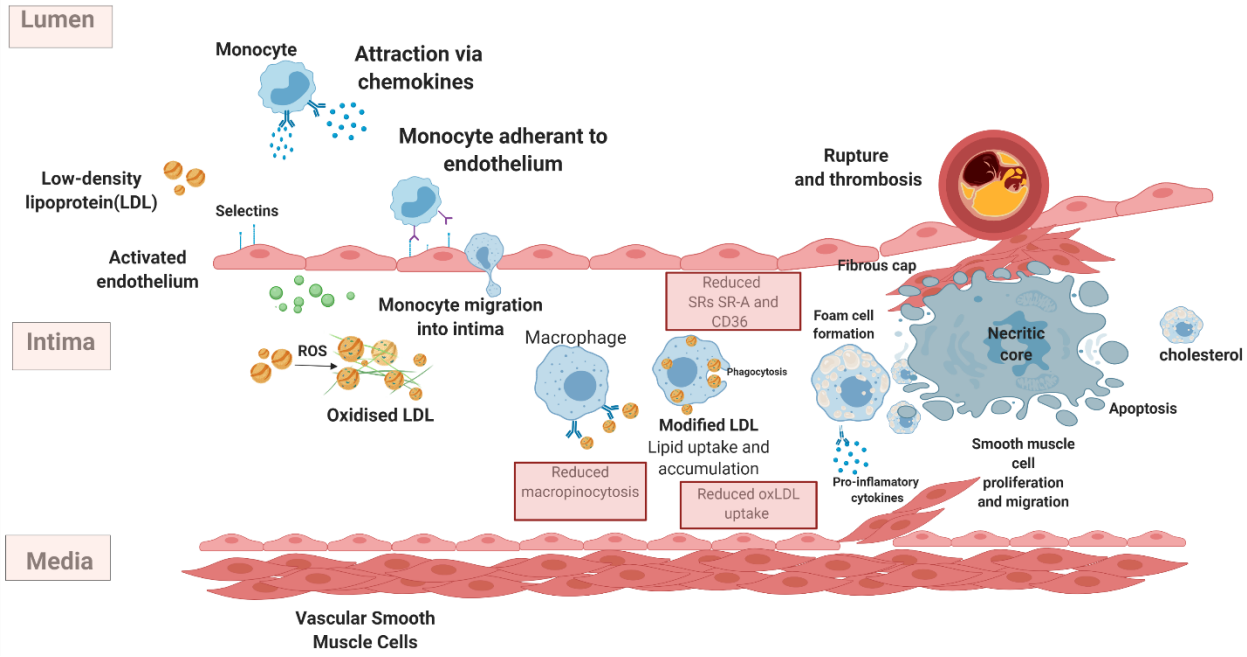

Figure 5. 15 Summary of the potential anti-atherogenic properties of DGLA and its metabolites in BMDM from C57BL/6J mice.

The steps potentially involved in the reduction of atherosclerosis disease progression following DGLA and its metabolites treatment are highlighted in red. Possible mechanisms for these changes are also included.

Chapter 6 The effects of DGLA on haematopoietic cells in the bone marrow of LDLr$^{-/-}$ mice fed a HFD.

6.1 Introduction

Research presented in Chapter 5 investigated the effects of DGLA and its metabolites in BMDM macrophages from wild type and 12/15-Lox deficient mice. Other studies in the laboratory carried out by a Postdoctoral Research Associate, Dr. Jessica Williams, were investigating the effect of DGLA on plaque development and regression in LDLr$^{-/-}$ mice *in vivo*. This involved feeding of these mice *ad libitum*: (i) HFD alone for 12 weeks; (ii) HFD supplemented with DGLA (500 mg/kg/day in diet) for 12 weeks; (iii) HFD for 12 weeks followed by chow diet for 4 weeks; and (v) HFD for 12 weeks followed by chow diet supplemented with 500 mg/kg/day DGLA for 4 weeks. (i) and (ii) allowed monitoring of effect of DGLA on plaque progression, and (i) also acted as baseline for regression studies. (iii) and (iv) allowed monitoring of the plaque regression parameters (i.e., whether switching to chow diet causes regression of plaques seen following feeding of mice HFD for 12 weeks and whether any such regression was affected by the inclusion of DGLA in the chow diet. Dr. Jessica Williams was monitoring plaque burden and cellular content together with plasma lipid profile (Figure 1.15). The availability of these mice provided an excellent opportunity to investigate effects on haematopoietic stem and progenitor cell profiles.

Haematopoiesis is a dynamic, ongoing mechanism for the production of human and animal blood cells that occurs principally in the bone marrow (Ramalho-Santos and Willenbring 2007). Studies that have analysed mature bone marrow components have previously revealed the considerable range of cells with various differentiation stages (Till and McCulloch 1980). They suggested the possibility that these cells were derived from a standard precursor cell, the haematopoietic stem cell (HSC) (Wilson and Trumpp 2006). Ernst Haeckel invented the word "stem cell" in the middle of the nineteenth century (Ramalho-Santos and Willenbring 2007). He reported that in the event of failure of the bone marrow due to exposure to ionising radiation, its function could be restored, at least in part, through injections of spleen or other bone marrow cells (Ramalho-Santos and Willenbring 2007). In recent years, advanced techniques such as flow cytometry have been used to investigate the mechanisms behind both murine and human haematopoiesis (Orkin and Zon 2008; Anjos-Afonso *et al.* 2013).

Stem and progenitor cells can undergo self-renewal and produce all the other types of cells in the body. Earlier research showed that there were changes in the stem and progenitor cell populations in the bone marrow of mice fed a HFD (Chan 2012; Adler *et al.* 2014; van den Berg *et al.* 2016). Such modified populations of bone marrow cells have been found to perform crucial functions in hypercholesterolemia and in the progression of atherosclerosis (Lang and Cimato 2014; Ma and Feng 2016). Certain cells, including myeloid-derived suppressor cells (MDSC), are directly linked to chronic inflammatory diseases such as atherosclerosis and are frequently present in higher numbers during the development of the disease (Foks *et al.* 2016). Bone marrow stem cells, progenitor cells and differentiated lineage cells were therefore studied using procedures commonly used in the field (Moss et al. 2018). Figure 6.1. shows a simplified representation of the bone marrow cell populations.

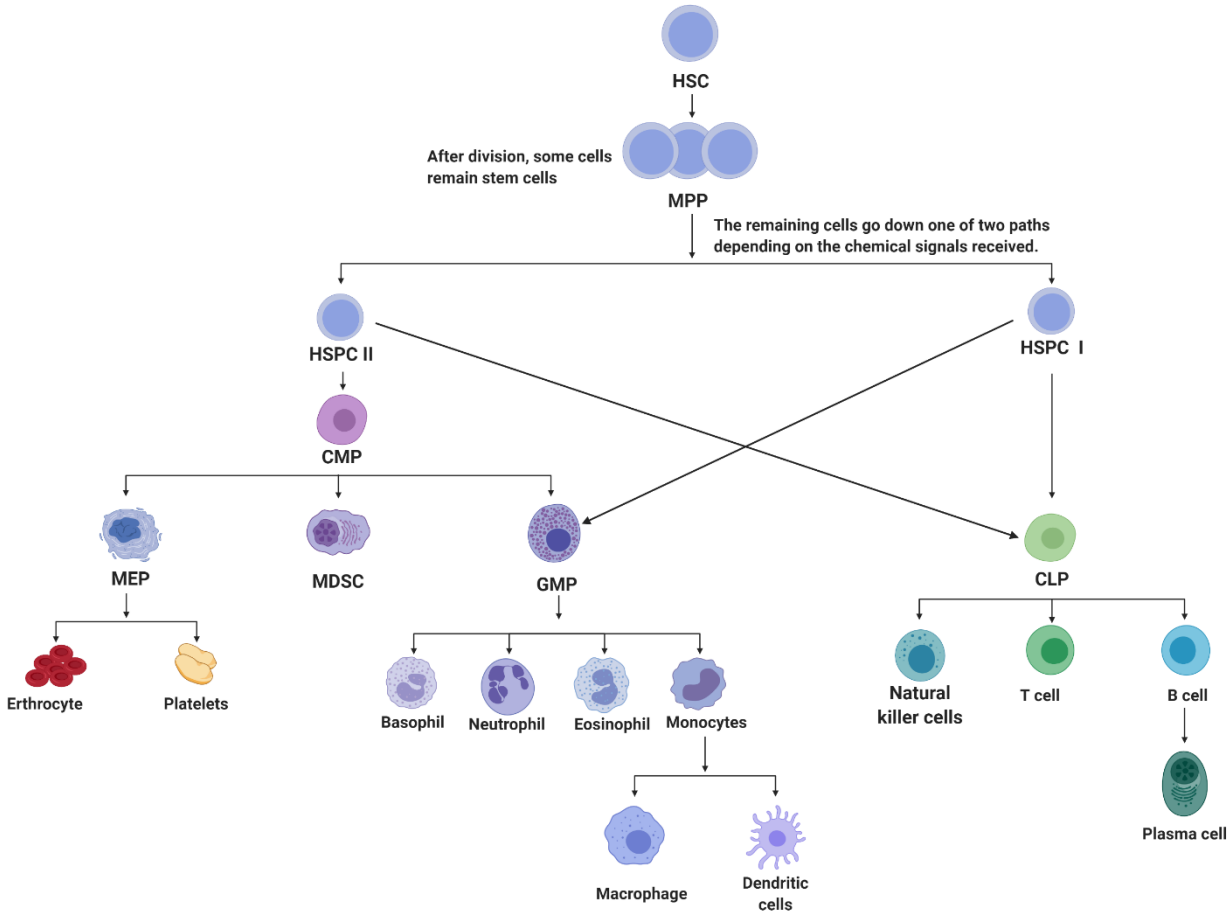

Figure 6. 1 Classification of the populations of bone marrow cells.
Abbreviations: HSC, haematopoietic stem cell; MPP, multipotent progenitor; HPC, haematopoietic progenitor cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-macrophage progenitor; MDSC, myeloid-derived suppressor cell. Created with BioRender.com.

6.2 Experimental Aims

The purpose of this chapter was to evaluate the effect of DGLA treatment on bone marrow cell populations in LDLr$^{-/-}$ mice following 12 weeks of HFD or HFD supplementation with DGLA (i.e., atherosclerosis progression), and following switching to chow diet for 4 weeks or to chow diet supplemented with DGLA for 4 weeks (i.e., during potential regression). Section 2.2.6 provides detailed methods for this chapter and a brief experimental plan for Chapter 6 is presented in Figure 6.2. The population of stem cells included HSCs, MPP cells and HPC-I and -II cells, which were identified via CD150 and CD48 antibodies directed to the cell surface glycoproteins. CD150 is also known as the signalling lymphocyte activation molecule (SLAM), and these cell populations were identified as SLAM cells. CMP, MEP and GMP, and CD34 and CD16/32 were used as identifiers for the progenitor cell populations. To identify the positive lineage differentiated cells, the marker B220 was used for B cells; CD3 was used for T cells; and GR1 and Mac1 were used for macrophages, granulocytes and MDSCs. Table 2.5 outlines the composition of antibody combinations used to identify SLAM, progenitor and lineage-positive cell populations.

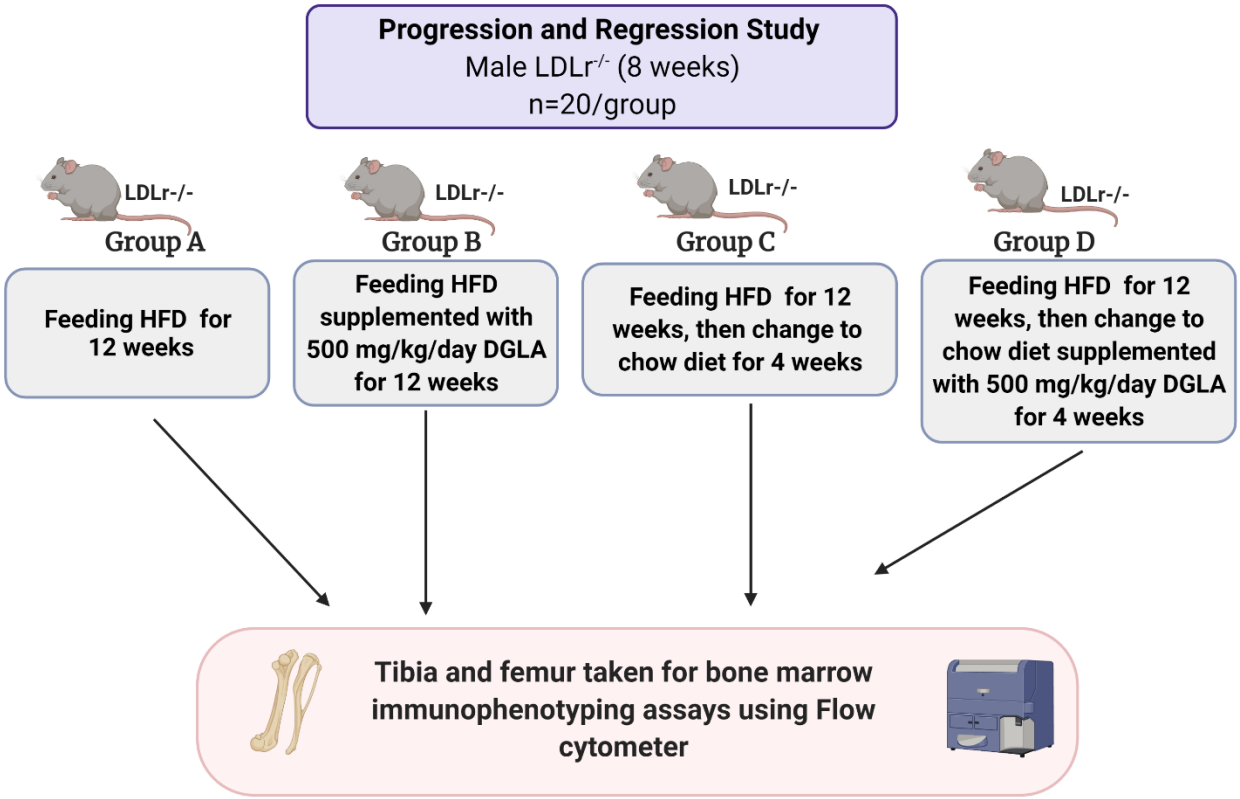

Figure 6. 2 Experimental strategy for the analysis of bone marrow cell populations in LDLr$^{-/-}$ mice.
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6.3 Results

6.3.1 The effects of DGLA supplementation on key bone marrow cell populations in LDLr$^{-/-}$ mice

Bone marrow was extracted, and the cell populations were analysed by immunophenotyping after feeding the mice for 12 weeks with a HFD (control) or an HFD supplemented with 500mg/kg/day DGLA or feeding with an HFD for 12 weeks and then changing to either chow diet or chow diet supplemented with 500mg/kg/day DGLA for 4 weeks, as described in Section 2.2.6. Initially, total white blood cells (WBC) count in the bone marrow was evaluated, and a significant increase in WBC count was found in the DGLA/HFD group compared with the control HFD-only group (Figure 6.4; $p<0.001$). The WBC counts in mice switched to chow diet showed a trend towards increase compared to feeding of HFD alone for 12 weeks ($p=0.096$) whereas the cell numbers in animals feed chow diet supplemented with DGLA was significantly higher than those fed HFD alone for 12 weeks ($p<0.001$). Because of such changes, cell numbers rather than frequency were used for investigation of other cell populations in the bone marrow.

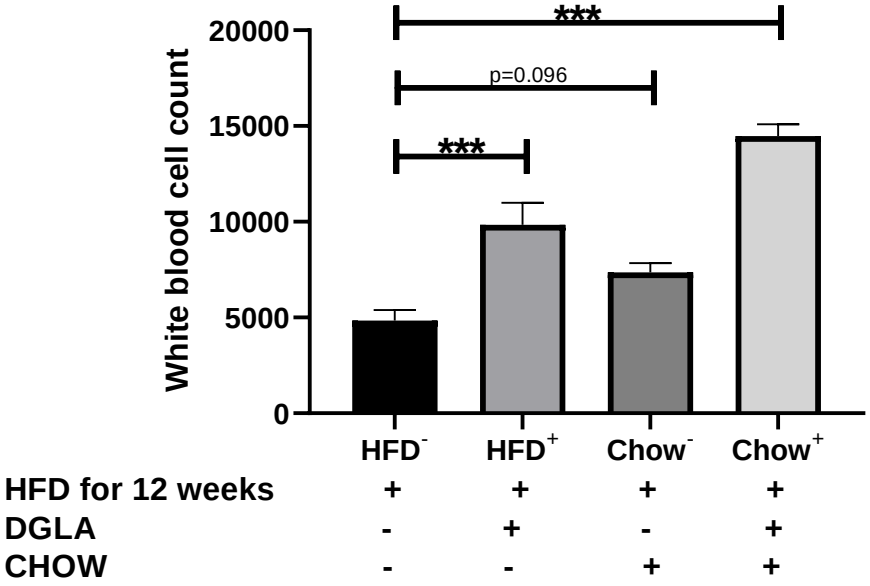


Figure 6. 3 . DGLA treatment significantly increased white blood cell counts within the bone marrow in LDLr$^{-/-}$ mice.

Immunophenotyping of LDLr$^{-/-}$ mouse bone marrow cells was carried out after 12 weeks of feeding mice with either HFD (control) for 12 weeks of HFD supplemented with DGLA for 12 weeks, or 12 weeks of feeding with HFD and then changing to either chow diet alone or chow diet supplemented with DGLA for 4 weeks. The graph displays the total number of white blood cells in various groups. Results are mean ± SEM from 19 HFD-, 19 HFD+, 18 Chow- and 19 Chow+ mice (- and + indicates absence or presence respectively of DGLA in the diet). Statistical analysis was performed using a One-way ANOVA with Tukey's post-hoc analysis where *** $p < 0.001$.

6.3.2 The effects of DGLA administration on populations of SLAM cells within the bone marrow in LDLr$^{-/-}$ mice

SLAM cells, including LSK, HSC, MPP, and HSPC-I and HSPC-II, were detected using an antibody mixture against CD150 and CD48 cell surface glycoproteins following the measurement of WBCs within the bone marrow. Figure 6.4 shows the gating strategy used for evaluating the different cell populations with the quantitative data shown in Figure 6.5. There was a significant increase in the numbers of LSK ($p=0.040$), HSC ($p=0.005$), HSPC II ($p=0.008$) and MPP ($p=0.045$) cells in the bone marrow of mice fed a HFD for 12 weeks supplemented with DGLA compared to those fed HFD alone (Fig. 6.5). However, there was a trend towards decrease in the number of HSPC I in mice fed HFD supplemented with DGLA compared to those fed HFD alone ($p=0.078$) (Fig. 6.5C). In addition, the numbers of HSPC I cells in mice fed high fat diet supplemented with DGLA switched to chow diet for 4 weeks was significantly lower than the baseline group of mice fed HFD alone ($p=0.023$) (Fig. 6.5C). For HSPC II cells, there was a significant increase in numbers in mice switched to chow diet for 4 weeks supplemented with DGLA compared to the baseline group of mice fed HFD alone ($p=0.004$) (Fig. 6.5D). Inclusion of DGLA to the chow diet also produced a trend towards increase compared to chow diet alone ($p=0.055$) (Fig. 6.5D).

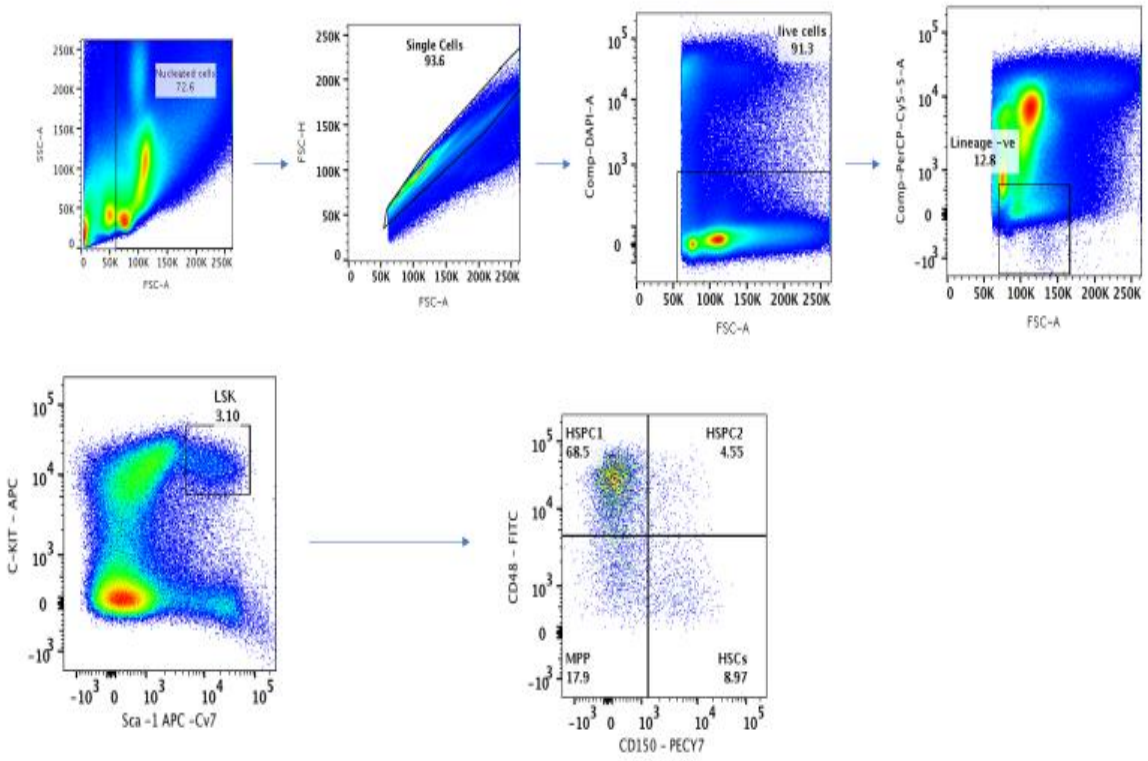


Figure 6. 4 Gating strategy used for investigation of SLAM cell populations within the bone marrow in LDLr$^{-/-}$ mice.

The graphs are flow plots illustrating the gating strategy used by FlowJo v.10 software to analyse the SLAM cell populations. The forward scatter (FSC-A) allows the size of the cells to be determined by calculating the amount of light passing through them. Side scattering (SSC-A) is proportional to cell granularity by evaluating the amount of light inside the cells that is reflected by particles. Specific filters and gating were applied to classify various populations. The first plot omits debris and nucleated red blood cells (RBCs). Then, gating for single WBCs was carried out that excludes doublets. DAPI-negative populations were defined as live cells. The LSK population gated from live cells. LIN-, lineage-negative; HPC, haematopoietic progenitor cell; MPP, multipotent progenitor; HSCs, haematopoietic stem cell.

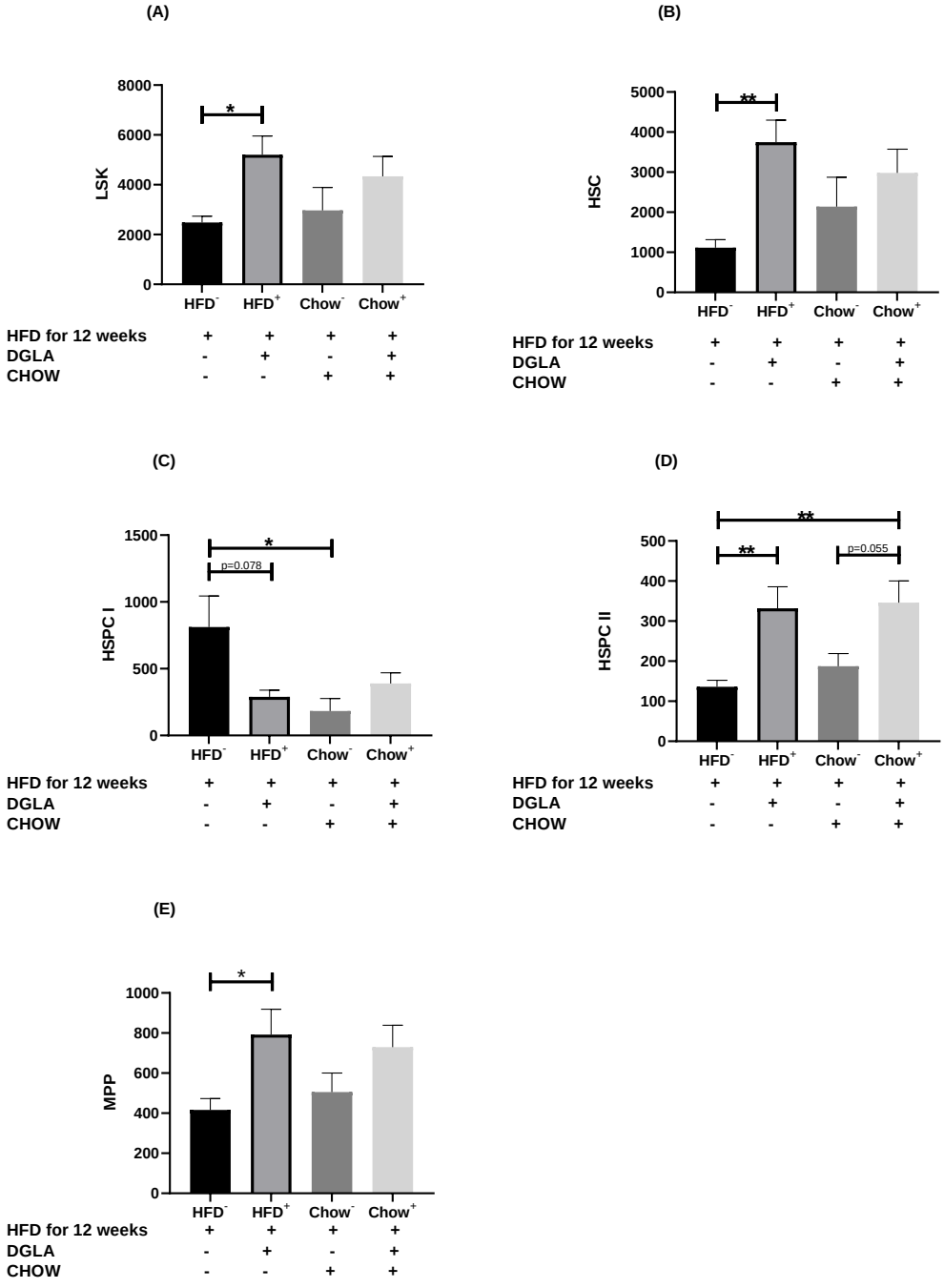


Figure 6. 5 The effect of DGLA administration on bone marrow stem cell populations in LDLr$^{-/-}$ mice.

Populations of cells were evaluated in bone marrow of mice fed a HFD (control) or HFD supplemented with DGLA for 12 weeks or following feeding of HFD for 12 weeks and then either changing to chow diet or chow diet supplemented with DGLA for 4 weeks. The bar charts demonstrate the numbers of (A) LSK, (B) HSC, (C) HSCP I, (D) HSCP II and (E) MMP in the total number of bone marrow cells. The data are shown as the mean ± SEM of 19 HFD-, 19 HFD+, 18 Chow- and 19 Chow+ mice (- and + indicates absence or

presence respectively of DGLA in the mice). Statistical analysis was performed using a One-way ANOVA with Tukey's post-hoc analysis where * $p < 0.05$ and ** $p < 0.01$.

6.3.3 The effect of DGLA treatment on the progenitor cell populations in the bone marrow of LDLr$^{-/-}$ mice

All myeloid cells are produced from a population of LK cells; the cell populations CMP, GMP, MEP and CLP are classes of LK cells. The gating strategy for evaluating these different cell populations is shown in Figure 6.6 with the quantitative data on numbers of the different cell populations in the bone marrow shown in Figure 6.7. No significant changes were seen in the different cell populations within the bone marrow when the mice were fed a HFD alone or that supplemented with DGLA for 12 weeks (Fig. 6.7). Similarly, no significant changes were seen when mice that were fed a HFD for 12 weeks were switched for 4 weeks to a chow diet alone or that supplemented with DGLA (Fig. 6.7).

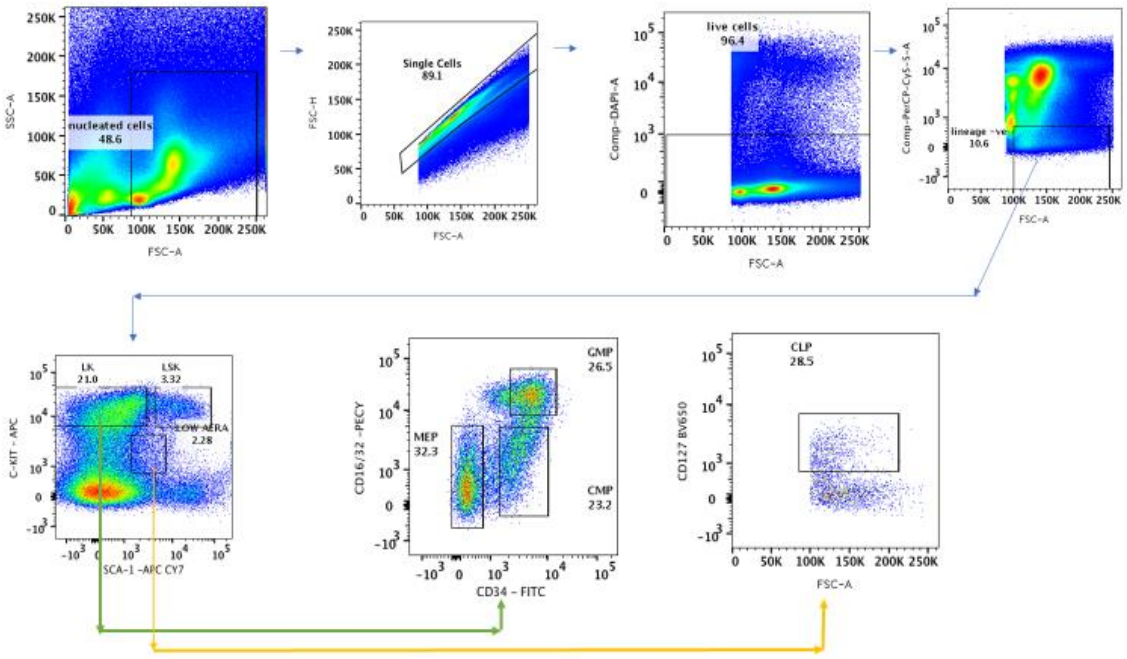


Figure 6. 6 Sequential gating plots for evaluation of LK cell population in the bone marrow in LDLr$^{-/-}$ mice.

The arrows illustrate how the previous gating strategy was used to classify each cell population. The axis represents the various stains used to differentiate cell populations. The LK cell population (Lin– Sca-1- c-kit+) was used to identify their three downstream cells GMP, CMP and MEP.

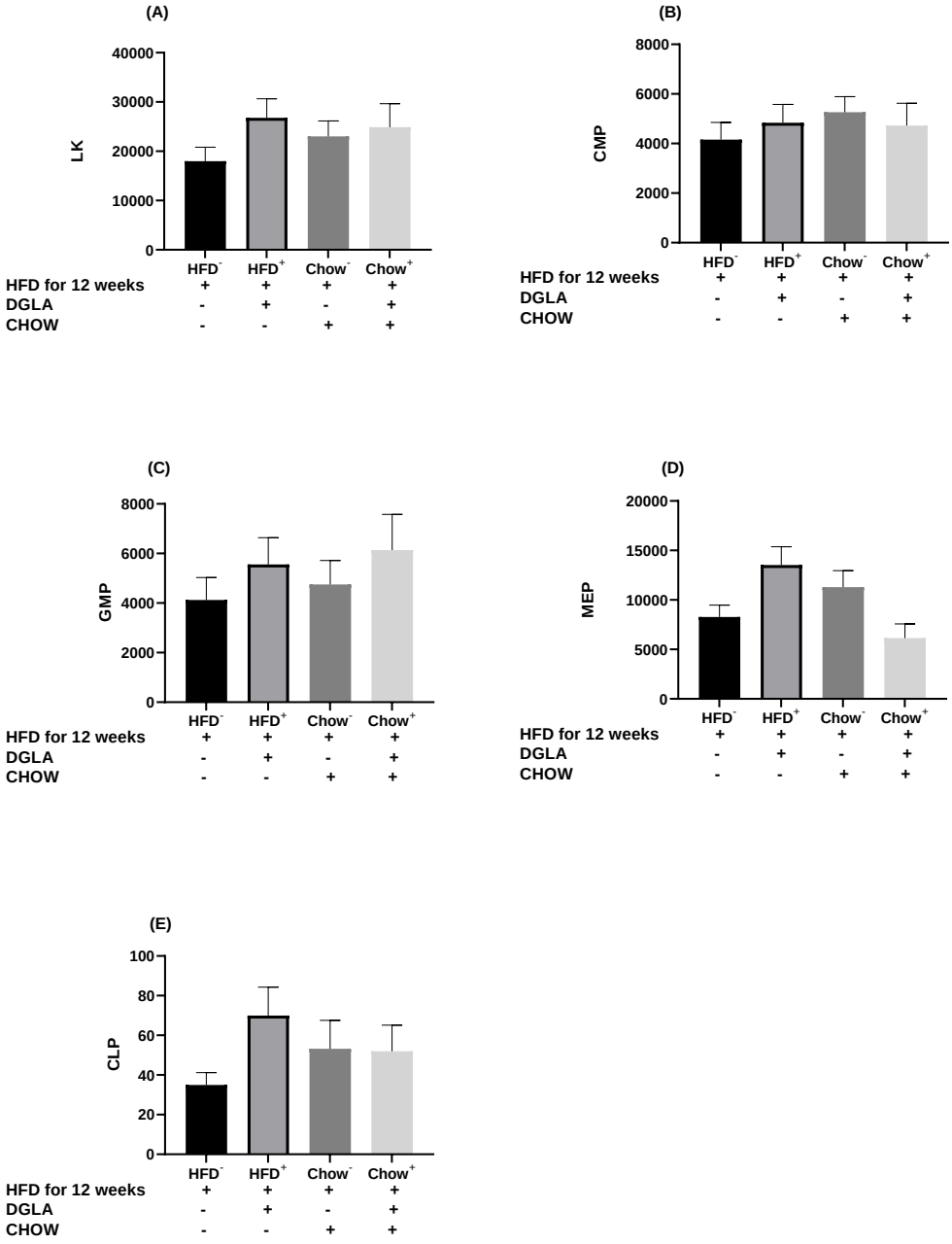


Figure 6. 7 The effect of DGLA administration on LK cell populations in the bone marrow in LDLr$^{-/-}$ mice.

Changes in the populations of cells in the bone marrow were evaluated after feeding mice a HFD alone or HFD supplemented with DGLA for 12 weeks or feeding with HFD for 12 weeks and then changing to either a chow diet or a chow diet supplemented with DGLA for 4 weeks. The bar charts show the numbers of (A) LK, (B) CMP, (C) GMP, (D) MEP and (E) CLP in the bone marrow. The data are provided as the mean ± SEM of 19 HFD-, 19 HFD+, 18 Chow- and 19 Chow+ mice (- and + indicates absence or presence respectively of DGLA in the diet). Statistical analysis was performed using a One-way ANOVA with Tukey's post-hoc analysis.

6.3.4 The effect of DGLA treatment on lineage cell populations within the bone marrow of LDLr$^{-/-}$ mice

MDSC (Mac1+/Gr1+), Macrophage (Mac1+), B-cell (B220+), T-cell (CD3+) and RBC (Ter119+) populations in the bone marrow of LDLr-/- mice were also examined. Figure 6.8 shows the gating strategy used for investigating the various cell populations with quantitative data shown in Figure 6.9. For lineage-positive differentiated cells, no significant changes were seen in the different cell populations within the bone marrow when the mice were fed a HFD alone or that supplemented with DGLA for 12 weeks (Figure 6.9). When mice fed a HFD for 12 weeks were switched to a chow diet alone for 4 weeks. Panel C shows a significant reduction in T cells ($p=0.014$) for Chow without DGLA, a significant increase in B cells ($p<0.001$; Figure 6.9D) and erythroid cells ($p<0.001$; Figure 6.9 E). For mice fed chow diet supplemented with DGLA, there was a significant increase in MDSC ($p=0.036$), B cells ($p <0.001$) and erythroid cells ($p <0.001$) compared to baseline group of mice fed HFD alone for 12 weeks. In addition, the presence of DGLA in the chow diet caused a significant reduction in T cells ($p=0.014$) and a significant increase in B cells ($p <0.001$) and erythroid cells ($p <0.001$) compared to chow diet alone.

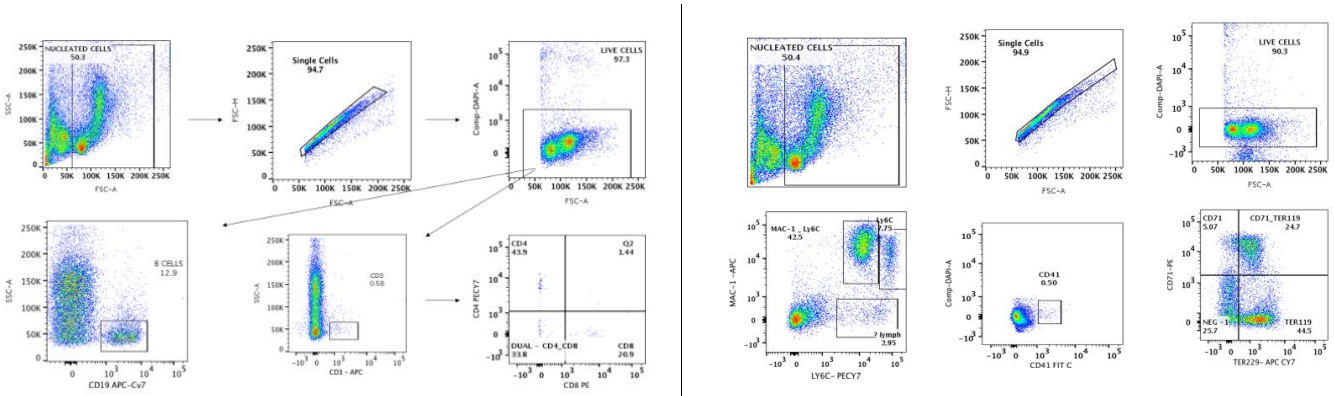


Figure 6. 8 Gating strategy for monitoring the effect of DGLA on lineage positive cells in the bone marrow in LDLr$^{-/-}$ mice.

The arrows illustrate how the previous gating strategy would classify each cell population. The axis represents the various markers used to differentiate cell populations. MAC-1 GR-1 represents myeloid-derived suppressor cells (MDSC); MAC-1 represents the macrophage population and Ter-119 represents the erythroid cells within the bone marrow.

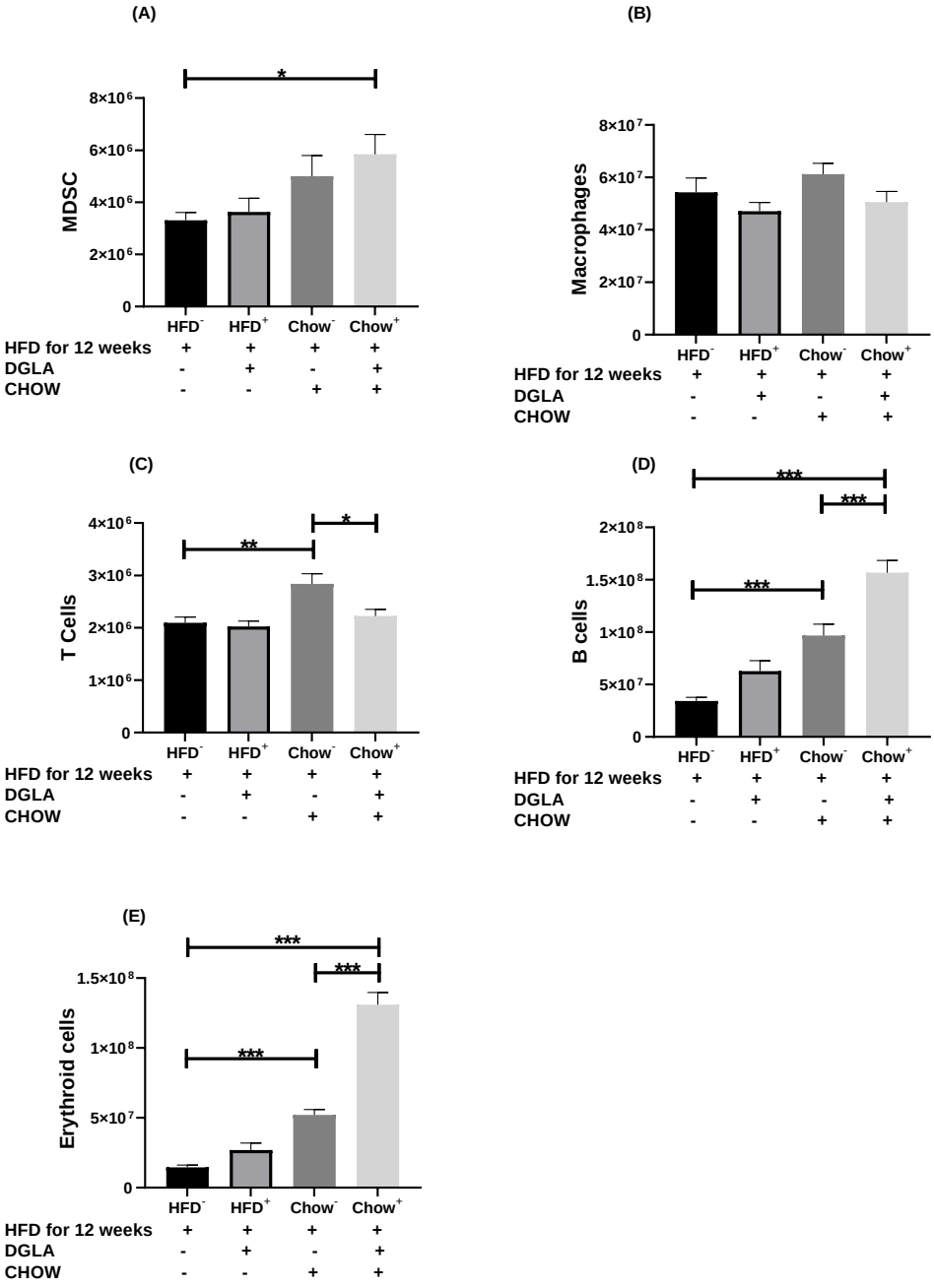


Figure 6. 9 The effect of DGLA supplementation on bone marrow lineage cell populations in LDLr$^{-/-}$ mice.

Changes in the populations of cells in the bone marrow were evaluated following feeding of the mice with either a HFD alone or HFD supplemented DGLA for 12 weeks or in mice fed a HFD for 12 weeks before being changed to either a chow diet or chow diet supplemented with DGLA for 4 weeks. The bar charts demonstrate the numbers of (A) MDSC (GR-1+CD11b+), (B) macrophages (MAC; GR-1-CD11b+), (C) T Cells (CD3+B220-), (D) B Cells (CD3-B220+) and (E) Erythroid cells (CD71-Ter119). Data are presented as mean ± SEM from 19 HFD-, 19 HFD+, 18 Chow- and 19 Chow+ mice (- and + indicates absence or presence respectively of DGLA in the diet). Statistical analysis was performed using a One-way ANOVA with Tukey's post-hoc analysis where *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

6.4 Discussion

Many studies support the idea that obesity-induced inflammation and the pro-inflammatory activation of leukocytes, along with adjustments to HSCs and early-bone marrow myeloid progenitors, which generate proinflammatory macrophages, significantly contribute to diseases such as CVD in mice and humans (Phillips and Perry 2013; Singer et al. 2014). Furthermore, changes in the cell profiles in the bone marrow were reported for atherosclerosis pathogenesis, and these changes may serve as independent risk indicators (Dotsenko 2010). Nutraceutical supplementation such as omega-3 PUFAs have also been shown to change bone marrow cell populations (Takano et al. 2004; Betiati et al. 2013; Chang and Deckelbaum 2013). The effects of DGLA treatment on bone marrow cell populations were therefore investigated using an immunophenotyping approach. Bone marrow extract was incubated with antibodies against specific cell surface markers and flow cytometry was performed to detect the cell populations of interest. Tables 6.1 and 6.2 outline a summary of findings from investigation of haematopoietic cell population of the effects of DGLA in progression and regression study in LDLr-/- mice fed HFD.

Table 6. 1 Summary of key findings on the effects of DGLA on haematopoietic cells in progression study.

Bone marrow cell populations	DGLA⁺ HFD v/s HFD alone	
	Effect	P-value
WBCs	↑	<0.001 (***)
LSK	↑	0.041 (*)
HSC	↑	0.005 (**)
HSPCs I	↓	0.078 (T)
HSPCs II	↑	0.008 (**)
MPP	-	0.045(*)
LK	-	0.484 (NS)
CMP	-	0.998 (NS)
GMP	-	0.816 (NS)
MEP	-	0.101 (NS)
CLP	-	0.209 (NS)
MDSC	-	0.987 (NS)
Macrophages	-	0.660 (NS)
T cells	-	0.984 (NS)
B cells	-	0.218 (NS)
Erythroid cells	-	0.430 (NS)

Table 6. 2 Summary of key findings on the effects of DGLA on haematopoietic cells in regression study.

Bone marrow cell populations	$DGLA^{+}$ $chow^{+}$ v/s $chow^{-}$	
	Effect	P-value
WBCs	↑	<0.001 (***)
LSK	-	0.545 (NS)
HSC	-	0.706 (NS)
HSPCs I	-	0.811 (NS)
HSPCs II	↑	0.055 (T)
MPP	-	0.413 (NS)
LK	-	0.984 (NS)
CMP	-	0.957 (NS)
GMP	-	0.831 (NS)
MEP	-	0.111(NS)
CLP	-	1.00 (NS)
MDSC	-	0.781 (NS)
Macrophages	-	0.279 (NS)
T cells	↑	0.014 (*)
B cells	↑	<0.001 (***)
Erythroid cells	↑	<0.001 (***)

Cell types were identified and the number of each type of cell within the WBC numbers was estimated. WBC levels have been used as indicators of the inflammatory condition. Previous research reported a link between elevated cholesterol and HSPC counts, which resulted in an increase in WBCs (Ma and Feng 2016). In addition, previous studies found that WBC numbers usually increase during the development of atherosclerosis (Ates et al. 2011; Mozos et al. 2017). DGLA treatment resulted in an increased WBC count in the bone marrow possibly due to high number of B cells, which suggests that DGLA has a pro-inflammatory effect and can lead to the development of atherosclerosis. However, this contradicts the anti-atherogenic roles of DGLA identified in published studies (Gallagher et al. 2019) and those presented in previous chapters in this thesis. It is therefore important to investigate whether these changes correlate with WBCs in the plasma and atherosclerotic plaques of the animals.

The LSK populations were HSC, HSCP-I, HSCP-II and MPP. The overall results from the investigation of bone marrow samples showed a significant increase in LSK HSC, the most important cell in bone marrow following supplementation of HFD with DGLA (Figure 6.5A). In addition, the populations of HSC and HPCS-II cells were significantly increased in bone marrow

following supplementation of HFD with DGLA (Figure 6.5B and D). Based on previous studies, HFD have an inverse relationship to atherosclerosis development, more HFD, more atherosclerosis through the ability of HFD to change the frequency of the population of haematopoietic stem/ progenitor cells in the bone marrow of mice (Adler et al. 2014; Lang and Cimato 2014; Ma and Feng 2016). The rise in the proliferation of HSCs in bone marrow is related to an increased development of leukocytes (Hanna and Hedrick 2014). Moreover, an increase in HSPC is associated with hypercholesterolaemia (Feng et al. 2012; Lang and Cimato 2014). Hypercholesterolaemia contributes to enhanced proliferation of HSCs, bloodstream mobilisation and differentiation into inflammatory monocytes, which are then involved in an inflammatory response (Lang and Cimato 2014; Ma and Feng 2016).

In the bone marrow, the LK cell population produces myeloid progenitors and, consequently, all myeloid cells. The LK cell population usually expands during atherosclerosis development and relocates to the spleen where monocytes can differentiate, which can target the atherosclerotic lesion (Robbins et al. 2013). The CMPs are responsible for the production of both MEPs and GMPs. In the current study, CMP, GMP, MEP and CLP populations were non-significantly increased following DGLA supplementation. Proangiogenic cells (PACs), originating from bone marrow, such as CMPs and GMPs, have been shown to enhance neovascularisation and tissue repair (Wara et al. 2011); however, the function of angiogenesis in atherosclerosis is unclear. Angiogenesis is necessary for tissue repair in CVD disorders, especially myocardial ischemia, and peripheral artery disease (Wara et al. 2011), although angiogenesis may encourage the development and dysfunction of atherosclerotic plaques (Camaré et al. 2017). However, clinical studies using anti-angiogenic drugs have not been successful in reducing the progression of atherosclerosis (Tian et al. 2013; Katsi et al. 2014; Camaré et al. 2017).

Previous research found that MDSCs significantly inhibits the proliferation of T cells in bone marrow of $LDLr^{-/-}$ mice under atherosclerotic conditions, especially pro-inflammatory Th1 and Th17 cells (Foks et al. 2016). DGLA supplementation in the present study led to an increase in MDSCs (Figure 6.10). Moreover, in the current study, DGLA supplementation had no effect on the CLP cell population (Figure 6.8E) and resulted in a significant decrease in T cell population (Figure 6.10C) and significant increase in B cell (Figure 6.10D) population in regression study (DGLA+ $chow^{+}$ v/s $chow^{-)}$. The development and maturation of T cells is complicated and their

function in atherosclerosis depends on several variables, including the particular sub-set of phenotypes (Sykes 1990). The number of T cells can be used as an indirect inflammatory status marker, thus the decrease in bone marrow T cells demonstrates a potential anti-inflammatory and anti-atherogenic impact.

Finally, the modifications in the population of bone marrow cells following DGLA treatment indicated that DGLA acts on progenitor cells and potentially causes a minor pro-atherogenic shift. Additional experiments are critically important for evaluating the effects of these changes on the immune cell populations resulting from DGLA treatment. The cell populations inside the atherosclerotic plaque and blood will need to be investigated. This would address the weaknesses of this study, which was limited to only testing the bone marrow cell populations. When identifying changes in cell populations within atherosclerotic plaques, we cannot directly assume that the changes in the bone marrow will be accompanied by corresponding changes in the plaque. The summary of the changes within the bone marrow following 12 weeks of HFD, HFD supplementation with DGLA, change to chow diet for 4 weeks or change to chow diet supplementation with DGLA for 4 weeks are shown in Figure 6.11 and 6.12. The aim of future work will be to further identify and evaluate the proportion of these cell populations in the blood, atherosclerotic lesion, thymus, and spleen using a similar method of immunophenotyping.

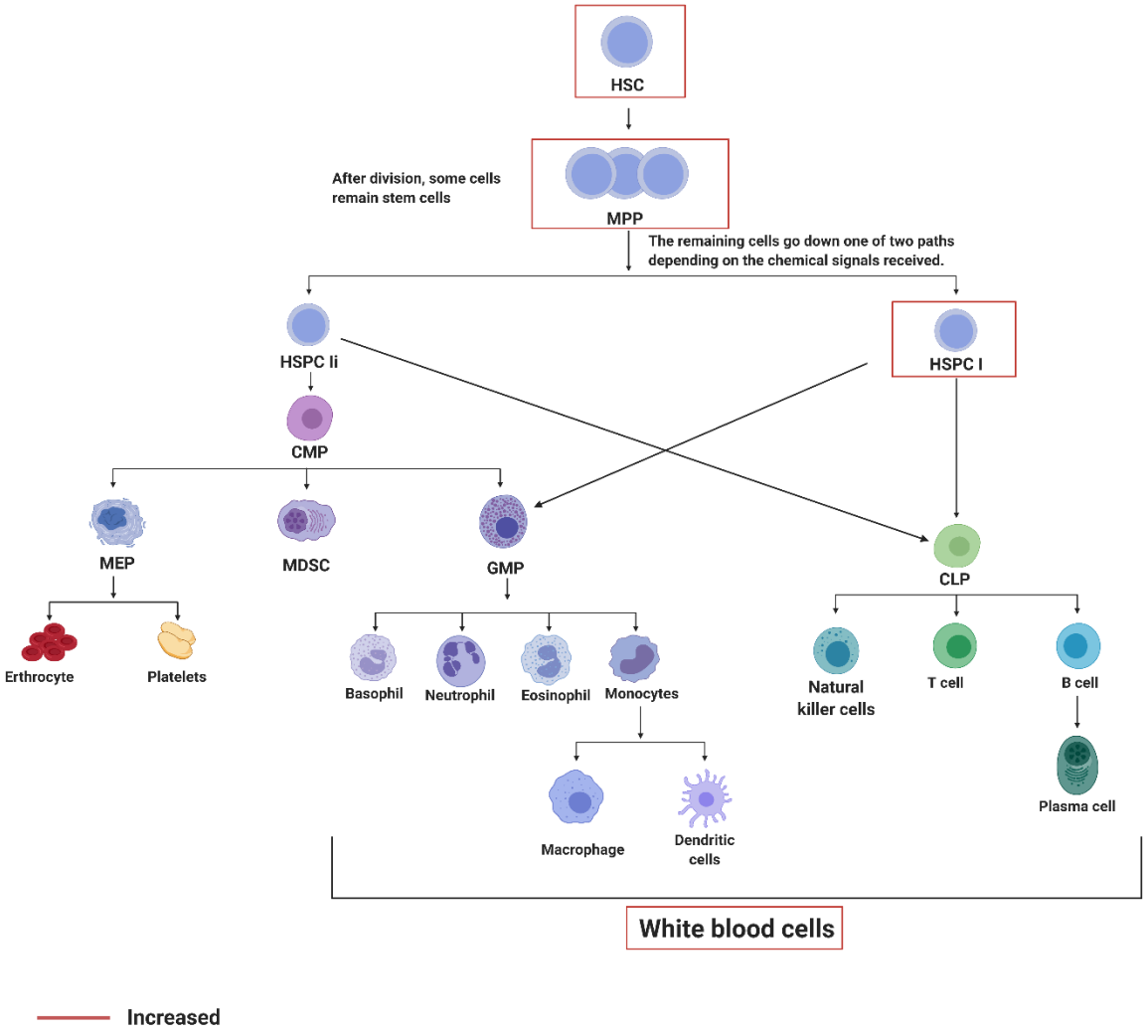


Figure 6. 10 Bone marrow cell populations showing changes due to DGLA treatment in progression of atherosclerosis.

Simplified description of bone marrow cell populations. Red squares highlight cell populations which were significantly increased in the bone marrow of DGLA supplemented mice compared to the control (DGLA+ HFD+ v/s HFD+ alone). HSC, haematopoietic stem cell; MPP, multipotent progenitor; HPC, haematopoietic progenitor cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-macrophage progenitor; MDSC, myeloid-derived suppressor cell. Created with BioRender.com.

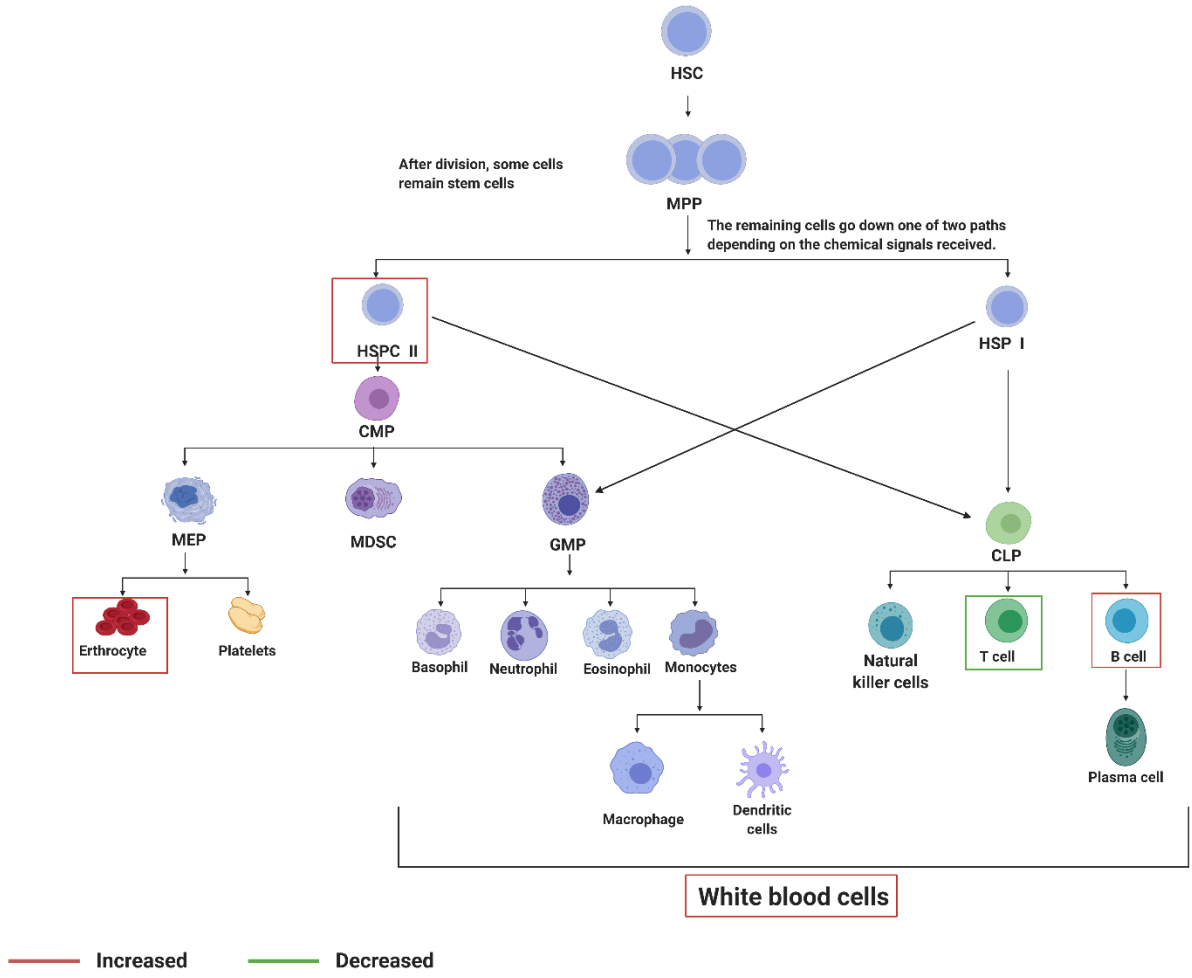


Figure 6. 11 Bone marrow cell populations showing changes due to DGLA treatment in regression of atherosclerosis.

Simplified description of bone marrow cell populations. Red squares highlight cell populations which were significantly increased, or green squares highlight cell populations which were significantly decreased in the bone marrow of mice fed HFD for 12 weeks and then change to chow diet supplementation with DGLA for 4 weeks compared to the control ($DGLA^{+}$ $chow^{+}$ v/s $chow^{-}$). HSC, haematopoietic stem cell; MPP, multipotent progenitor; HPC, haematopoietic progenitor cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-macrophage progenitor; MDSC, myeloid-derived suppressor cell. Created with BioRender.com.

Chapter 7 General Discussion

7.1 Introduction

CVD is currently the world's largest cause of death, with an estimated 1 in 3 deaths due to the disease. The incidence of CVD is a major financial burden, costing the UK economy nearly £19 billion every year. Atherosclerosis, a chronic inflammatory disorder of medium and large arteries, is the primary cause of CVD-related disease and mortality. Atherosclerosis develops during the lifetime of an individual and has a number of risk factors such as cigarette smoke, hypertension, hyperglycaemia, dyslipidaemia and genetic susceptibility. First-line pharmaceutical agents that are mainly administered for treatment are statins, a class of lipid-lowering drugs that inhibit the activity of the HMG CoAR enzyme. Statins have demonstrated some effectiveness as lipid-lowering drugs; nevertheless, statin-therapy has some limitations with a substantial cardiovascular risk maintained even in combination with life-style modifications (Cannon et al. 2015). In addition, some patients have intolerable side effects with statin therapy and some fail to achieve their target LDL-C levels even with the highest dose (Whayne Jr 2013; Stroes et al. 2015). In the light of such limitations with statin therapy, research continues for further alternative agents and has revealed the promise of PCSK9 inhibitors, ezetimibe and monoclonal antibodies against IL-1β (Preiss and Baigent 2017b; Moss 2018).

Nutraceuticals, as preventive or limiting agents, are potentially promising drug alternatives. However, it is important to identify the molecular mechanisms that underlie the beneficial actions of nutraceuticals *in vitro* and *in vivo*. DGLA is an omega-6 PUFA that has protective actions in a number of diseases like atopic eczema and atherosclerosis, where it plays anti-inflammatory roles (Kawashima *et al.* 2008; Takai *et al.* 2009a). This PUFA has also been shown to decrease hypertension in rats in response to saturated fatty acids (Hassall and Kirtland 1984), reduce inflammatory responses induced with croton oil in mice and encourage antithrombotic effects in humans (Kernoff et al. 1977)and rats (Watanabe et al. 2014). *In vivo* studies have revealed an excellent safety profile of DGLA in mice, rats, and humans (Umeda-Sawada *et al.* 2006; Takai *et al.* 2009a; Teraoka *et al.* 2009; Tanaka *et al.* 2012). Previous studies in the laboratory have shown that PGE_1 and 15-(S)-HETrE levels are increased after incubation of macrophages with DGLA. This suggests that these metabolites are potentially involved in mediating the beneficial actions of DGLA. This was confirmed by limited previous studies on PGE_1, which found monocytic

migration and pro-inflammatory gene expression to be attenuated in similar manner to DGLA (Gallagher 2016; Gallagher et al. 2019) .

Therefore, the general objective of this project was to evaluate the antiatherogenic effects of DGLA,15-(S)-HETrE and PGE_1 on key processes associated with atherosclerosis *in vitro*. Research was carried out using human monocytes, macrophages and VSMCs *in vitro*. Key findings from human THP-1 macrophages were replicated in primary human macrophages. In addition, the effect of DGLA supplementation on haematopoietic stem and progenitor cell profile was determined in $LDLr^{-/-}$ mice *in vivo*.

7.2 Summary of key findings.

The overall results from this project have highlighted the beneficial effects of DGLA,15-(S)-HETrE and PGE_1 on several atherogenic processes. The main anti-atherosclerotic actions of DGLA and its metabolites are outlined in Figure 7.1. This section also summarises the results of each experimental chapter and presents key findings from *in vitro* and *in vivo* studies in Tables 7.1, 7.2, 7.3, 7.4 and 7.5.

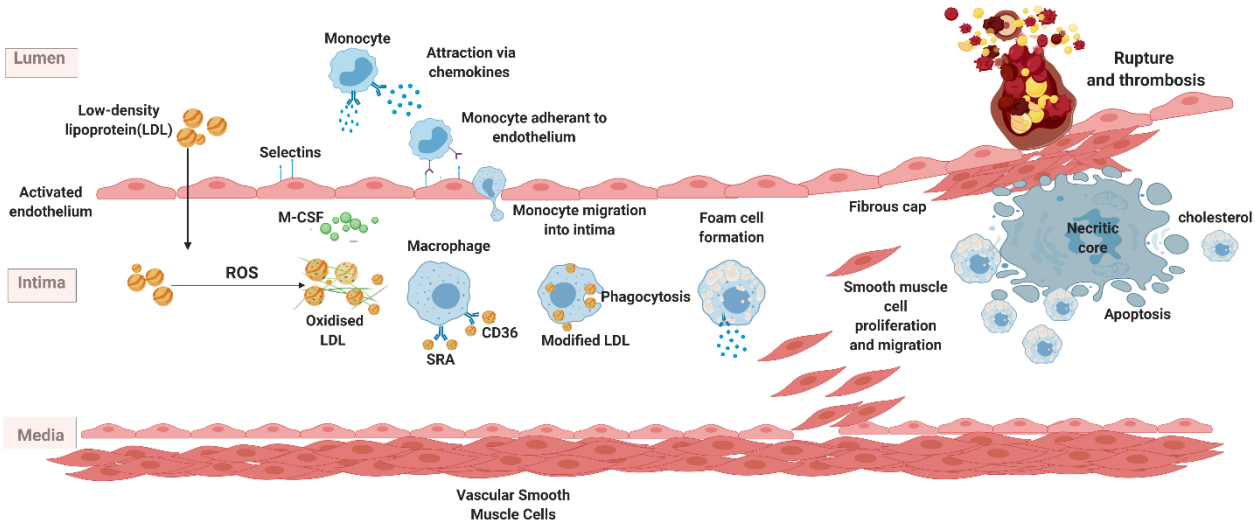
	Endothelial dysfunction	Monocyte recruitment/ foam cell formation	SMC invasion	Plaque stability
				
DGLA	↓ Expression of key genes encoding cell adhesion molecules (e.g., *Cdh5*, *Vcam1* and *Icam1*).	↓ Monocyte migration. ↓ Expression of *Ccl2* and *Ccr2*. ↓ Macropinocytosis. ↓ oxLDL uptake. ↓ Expression of scavenger receptors SRA1 and CD36. ↓ Expression of lipid transport and metabolism genes (e.g., *Apoa1*, *Ldlr* and *Lpl*).	↓SMC invasion.	↓ Expression of apoptosis-associated genes (e.g., *Bax*, *Bcl2* and *Bcl2l1*). ↓ T cells.
15-(S)-HETrE	↓ Expression of key genes implicated in cell adhesion (e.g., *Cd44* and *Vwf*).	↓ Monocyte migration. ↓ Expression of *Ccl2* and *Tnf-α*. ↓ Macropinocytosis. ↓ oxLDL uptake. ↓ Expression of SR SRA1 and CD36. ↓ Expression of lipid transport and metabolism genes (e.g., *Abca1*, *Ldlr*, *Lpl* and *Ptgs1*)	↓SMC invasion.	↓ Expression of apoptosis genes (e.g., *Bcl2* and *Cflar*). ↓ expression of genes involved in the control of ECM (e.g., *Mmp3* and SERPINE1).
PGE_1	↓ Expression of key genes involved in cell adhesion (e.g., *Selplg*, *Vcam1* and *Vwf*).	↓ Monocyte migration. ↓ Expression of *Ccl2* and *Ccl5*. ↓ Macropinocytosis. ↓ oxLDL uptake. ↓ Expression of scavenger receptors SRA1 and CD36. ↓ Expression of lipid transport and metabolism genes (e.g., *Ldlr* and *Lpl*).	↓SMC invasion.	↓ Expression of apoptosis-associated genes (e.g., *Bcl2*, *Bcl2a1* and *Tnfaip3*). ↓ Expression of genes involved in the regulation of ECM (e.g., *Mmp3*).

Figure 7. 1 Key anti-atherosclerotic effects of DGLA, 15-(S)-HETrE and PGE_1.

Illustrates the key outcomes from the *in vitro* studies together with the mechanisms that underlie the benefits of DGLA, 15-(S)-HETrE and PGE_1 at different stages of atherosclerosis development, including endothelial dysfunction, monocyte recruitment, foam cell formation, SMC invasion and plaque stability. ↓ = Down-regulation of gene expression. Abbreviations: *Apoa1*, Apolipoprotein A-I; *Bax*, Bcl2-associated X protein; *Bcl2a1*, B-cell leukaemia/lymphoma 2 related protein A1a; *Bcl2*, B-cell leukaemia/lymphoma 2; *Bcl2l1*, Bcl2-like 1; *Ccl2*, Chemokine (C-C motif) ligand 2; *Ccr2*, C-C chemokine receptor 2; *Ccr5*, C-C chemokine receptor 5; *Cd36*, Cluster differentiation 36; *Cd44*, CD44 antigen; *Cdh5*, Cadherin 5; *Cflar*, CASP8 and FADD-like apoptosis regulator; ECM, Extracellular matrix; *Icam1*, Intercellular adhesion molecule 1; *Ldl*, Low density lipoprotein; *Ldlr*, Low density lipoprotein receptor; *Lpl*, Lipoprotein lipase; MCP-1, Monocyte chemotactic protein 1; M-CSF, Macrophage colony stimulating factor; *Mmp3*, Matrix metallopeptidase 3; oxLDL, Oxidised LDL; *Ptgs1*, Prostaglandin-endoperoxide synthase 1, ROS, Reactive oxygen species; *Selplg*, Selectin, platelet (p-selectin) ligand; SMC, Smooth muscle cell; *Serpine1*, Serine (or cysteine) peptidase inhibitor, clade E, member 1; SRA, Scavenger receptor A; SRB1, Scavenger receptor B1; *Tnfaip3*, Tumour necrosis factor, alpha-induced protein 3; *Vcam1*, Vascular cellular adhesion molecule 1; VSMC, Vascular smooth muscle cells; *Vwf*, Von Willebrand factor homolog. Created with BioRender.com.

Chapter 3. Initially, the effect of DGLA and its metabolites on several key processes in atherosclerosis were determined *in vitro* using THP-1 monocytes, PMA-differentiated THP-1 macrophages, and primary cultures of HMDMs and VSMC. Main findings from this initial work indicate that DGLA, 15-(S)-HETrE and PGE_1 can all attenuate monocyte migration (Figure 3.8), inhibit macropinocytosis by macrophages (Figure 3.10), attenuate the receptor-mediated uptake of modified LDL (Figure 3.10), substantially inhibit the expression of scavenger receptor genes SR-A and CD36 (Figure 3.10) and decrease VSMC invasion (Figures 3.20). Following an investigation of the effect of DGLA and its metabolites on inflammatory gene expression, the IFN-γ-induced expression of MCP-1 and ICAM1 genes was found to be attenuated by DGLA,15-(S)-HETrE and PGE_1 (Figure 3.27). Overall, therefore, the findings from this chapter indicated the anti-atherosclerotic ability of DGLA and its metabolites in key processes in the development of atherosclerosis.

Chapter 4. To investigate the potential mechanisms underlying the anti-atherosclerotic effects demonstrated in Chapter 3, macrophage gene expression was analysed. Gene expression was determined by qPCR using the Atherosclerosis RT^2 Profiler PCR Array and the fold-change in gene expression was calculated using the ΔΔCT method. Of the 84 genes tested, DGLA, 15-(S)-HETrE and PGE_1 significantly attenuated the expression of 16, 21 and 12 genes, respectively (Table 4.2, 4.3 and 4.4). From these genes, many were found to encode several key proteins involved in atherosclerotic processes, including inflammatory responses, cell

adhesion, lipid transport and regulation of the ECM. Overall, results from this chapter correlate well with the anti-atherogenic effects observed in the previous chapter and provide insight into the mechanisms by which DGLA and its metabolites may regulate many atherosclerosis-associated genes that modulate key cellular processes associated with this disease.

Chapter 5. Following on from the positive findings of *in vitro* experiments, studies in this chapter further evaluated the anti-atherosclerosis effects of DGLA and its metabolites using BMDM from C57BL/6J and 12/15 Lox deficient mice together with pharmacological inhibitors against COX-1/2 and 12/15 LOX. Studies presented in this chapter showed that the DGLA-mediated inhibition of macropinocytosis in human macrophages was attenuated by pharmacological inhibitors against COX-1/2 and 12/15 LOX pathways (Figure 5.4). Furthermore, we found that treatment of BMDM from C57BL/6J mice with DGLA and its metabolites attenuated the receptor-mediated uptake of modified LDL together with macropinocytosis and inhibited the expression of scavenger receptors genes, SR-A and CD36 (Figure 5.12). In addition, the DGLA-mediated inhibition of receptor-mediated uptake of oxLDL and micropinocytosis seen in BMDM from C57BL/6J mice was attenuated in such cells from 12/15-Lox deficient mice (Figure 5.11). Overall, these studies demonstrated an important role of COX-1/2 and 12/15 LOX in DGLA actions and substantiate the proposition that this fatty acid mediates its actions via PGE_1 and 15-(S)-HETrE. The ability of DGLA and its metabolites to reduce the development of foam cells is an important result in terms of anti-atherosclerotic therapy considering the important impact of foam cell accumulation on atherosclerosis. In combination with the beneficial actions identified in Chapters 4, it can hypothesise that DGLA and its metabolites are a potential candidate for anti-atherosclerotic treatment and warrant further investigation *in vivo* and in clinical trials.

Chapter 6. To extend the *in vitro* findings presented in Chapters 3-5 to an *in vivo* context, bone marrow cell populations were analysed in $LDLr^{-/-}$ mice fed for 12 weeks with HFD, HFD supplemented with DGLA (progression study), change to chow diet for 4 weeks after 12 weeks on HFD or change to chow diet supplemented with DGLA for 4 weeks (regression study). Immunophenotyping of bone marrow cell populations was carried out by flow cytometry and changes in the levels of key cell types were identified. WBCs were found to be increased in mice for both studies ($DGLA^+$ HFD v/s HFD alone and $DGLA^+$ $chow^+$ v/s $chow^-$) (Figure 6.3). The numbers of SLAM cells, including LSK, HSC, HSPCs II and MPP, were significantly increased in

the DGLA group compared to the control in progression study. In addition, myeloid progenitor cell populations were increased in progression study ($DGLA^+$ HFD v/s HFD alone) (Figure 6.7). For the lineage cell populations in regression study, T cell populations were significantly reduced (Figure 6.9) and populations of B cells and erythroid cells were significantly increased in the DGLA group compared to the control (Figure 6.9D-E).

Table 7. 1 Summary of key findings from *in vitro* investigations of the effects of DGLA, 15-(S)-HETrE and PGE_1 on key processes in atherosclerosis.

Cellular processes	DGLA	15-(S)-HETrE	PGE_1
Monocyte migration	↓	↓	↓
Macropinocytosis	↓	↓	↓
Receptor-mediated uptake of oxLDL	↓	↓	↓
SR expression	↓	↓	↓
VSMC invasion	↓	↓	↓
Gene expression by microarray	Downregulation of 16 genes	Downregulation of 21 genes	Downregulation of 12 genes
Effect of treatments with IFN-γ on *Mcp1* and *Icam1* expression	↓	↓	↓

Table 7. 2 Summary of key findings from studies on BMDM on the effects of DGLA, 15-(S)-HETrE and PGE_1 on key processes in atherosclerosis.

Cellular processes	DGLA	15-(S)-HETrE	PGE_1
Macropinocytosis	↓	↓	↓
Receptor-mediated uptake of oxLDL	↓	↓	↓
SR SRA1	↓	↓	↓
SR CD36	↓	↓	↓

Table 7. 3 Summary of the effect of DGLA on macropinocytosis and oxLDL uptake in 12/15 Lox deficient mice.

Cellular processes	DGLA		15-(S)-HETrE+ DGLA		PGE_1+ DGLA	
	Effect	P-value	Effect	P-value	Effect	P-value
Macropinocytosis	↑	0.0987(T)	-	-	-	-
Dil-oxLDL uptake	↑	0.0320 (*)	-	-	-	-
Reversing by adding PGE_1 and 15-(S)-HETrE	↑	0.3022 (NS)	↓	0.0744 (T)	↓	0.0196 (*)

Table 7. 4 Summary of key findings on the effects of DGLA on haematopoietic cells in progression study.

Bone marrow cell populations	$DGLA^+$ HFD v/s HFD alone
WBCs	↑
LSK	↑
HSC	↑
HSPCs I	↓
HSPCs II	↑
MEP	↑

Abbreviations: HSC, haematopoietic stem cell; MEP, multipotent progenitor; LSK, Lineage - Sca-1+ C-kit+; HSPC, haematopoietic stem progenitor cell; MEP, megakaryocyte-erythroid progenitor; WBCs, white blood cells.

Table 7. 5 Summary of key findings on the effects of DGLA on haematopoietic cells in in regression study.

Bone marrow cell populations	$DGLA^{+}$ $chow^{+}$ v/s $chow^{-}$
WBCs	↑
HSPCs II	↑
T cells	↑
B cells	↑
Erythroid cells	↑

Abbreviations: HSPC, haematopoietic stem progenitor cell; WBCs, white blood cells.

7.3 Eicosanoids

Eicosanoids perform an important role in the function of platelets as can both stimulate and inhibit platelet aggregation (Crescente *et al.* 2019). DGLA has several beneficial effects on platelets because it effectively inhibits the risk of atherosclerotic and thrombus development (Yeung *et al.* 2016). These effects of DGLA have frequently been attributed to its metabolism to PGE_1 via COX-1/2 and 15-(S)-HETrE via 12/15-LOX in mice. PGE_1 and 15-(S)-HETrE have been shown to play several anti-inflammatory functions (Zurier *et al.* 1977; Horrobin 1991). Furthermore, *in vivo* studies have shown that DGLA uptake increases the production of several lipid mediators, including PGE_1 and 15-HETrE, that may therefore be directly or indirectly involved in the regulation of atopic dermatitis symptoms and provide the benefit of DGLA supplementation (Amagai *et al.* 2015).

In the present study, to confirm the contributions of COX-1/2 and 15-LOX metabolites of DGLA to the anti-atherosclerotic effect, the effect of COX and LOX inhibitor, indomethacin and ML351 respectively, was examined in DGLA treated THP-1 macrophages. It was found that DGLA-mediated macropinocytosis was attenuated inhibition of COX via indomethacin and LOX via ML350 in THP-1 macrophages by around 29.2% and 15.5% respectively (Figure 5.4). These inhibitors were chosen based on previous studies such as on lung cancer cells (Amagai et al. 2015). For example, in *in vitro* activation of human lung tissues, 10 μM ML351 decreased the release of LPS- and Th2 cytokine-mediated chemokines secretion, and indomethacin also revealed the inhibitory effect on the release of TNF-α (Abrial *et al.* 2015).

Building upon the data from the use of pharmacological inhibitors, a series of experiments were carried out using BMDM from C57BL/6J and 12/15 Lox deficient mice. A key role of 12/15 LOX, which produce a range of pro- and anti-inflammatory mediators, in atherogensis was provided in 1999 when Cyrus et al. (1999) found that the disease in ApoE deficient mice decreased in the absence of 12/15 LOX (Cyrus *et al.* 1999). Another research showed that the deficiency of 12/15-LOX gene resulted in decreased development of atherosclerotic lesions caused by HFD in $LDLr^{-/-}$ mice consistent with the results in ApoE-knockout mice (George *et al.* 2001). DGLA and its metabolites significantly attenuated Dil-oxLDL uptake in BMDM from C57BL/6J mice by around 20% (Figure 5.7). In addition, it was found that on treatment with Dil-oxLDL, BMDM from 12/15 Lox deficient mice showed a significant increase in Dil-oxLDL uptake of 12.2% (Figure 5.8), while this was significantly decreased when a mixture of DGLA and 15-(S)-HETrE or PGE_1 was added to the cells (Figure 5.9B). These findings demonstrate that DGLA treatment, which alters eicosanoid production, potentially promotes the production of anti-inflammatory mediators PGE_1 and 15-(S)-HETrE.

7.4 Monocyte migration

In monocyte recruitment, several cytokines and cell adhesion molecules (e.g., MCP-1, TNF-α, M-CSF, E- and P-selectins, integrins, VCAM1 and ICAM1) are involved in adhesion to endothelial cells and eventually their transmigration into the arterial intima (Gerhardt and Ley, 2015). MCP-1 performs an essential function in monocyte recruitment, as mentioned above (Section 4.1.2.2.1) (Gosling et al. 1999). The migration of monocytes across a cell insert that mimics endothelial cells was significantly induced by MCP-1 (Figure 3.8) and this was significantly reduced after co-incubation with DGLA and its metabolites. DGLA's impact on monocyte migration induced by MCP-1 was an important outcome. This is one possible mechanism by which the fatty acid has anti-inflammatory and anti-atherosclerotic properties. Previous studies have shown that suppressing the migration of monocytes can substantially protect against atherosclerosis *in vivo* (Feige et al. 2013; Mueller et al. 2013). In *in vitro* gene expression analysis, THP-1 macrophages treated with DGLA and its metabolites showed significant changes in the expression of several genes that code for proteins involved in the recruitment and adhesion of monocytes (*Cdh5, Cd44, Icam1, Selplg, Vcam1* and *Vwf*) (Figure 4.8, 4.9 and 4.10). For example, treatment of macrophages with DGLA led to a significant decrease in the expression of *Icam1* and *Vcam1*, two key cell adhesion molecules (Figure 4.8).

7.5 Foam cell formation

The main step in atherosclerosis after monocyte migration into the arterial intima is their differentiation into macrophages. Macrophages can uptake modified forms of LDL, particularly oxLDL, in an unregulated manner via SR, resulting in the accumulation of cholesterol that is stored as cholesteryl esters lipid droplets in the cytoplasm (Ghosh et al. 2010). This leads to their foamy appearance (Brown *et al.* 1980). A crucial event in the development of atherosclerosis is the apoptosis and necrosis of foam cells, and the identification of novel agents capable of interrupting this mechanism could be highly effective in the prevention or even regression of atherosclerosis (Chistiakov et al., 2017). On the surface of macrophages, scavenger receptors are expressed and uptake unregulated amounts of modified LDL. As previously mentioned, SRA and CD36 were selected for analysis because of their main functions in oxLDL uptake (Febbraio et al. 2000; Kunjathoor et al. 2002; Kuchibhotla et al. 2008). Although previous research in our laboratory has shown that DGLA influences the development of macrophage foam cells (Gallagher ,2016), no studies have examined the impact of PGE_1 and 15-(S)-HETrE on the development of macrophage foam cells.

In Chapter 3, *in vitro* studies showed that both DGLA and its metabolites were able to significantly reduce receptor-mediated uptake of oxLDL at the cellular level by at least 10-20%, with a significant reduction in SR-A and CD36 SRs at the level of gene expression and cell surface expression (Figure 3.11 and 3.12). In addition, in Chapter 5, a significant decrease also of around 20% in receptor-mediated uptake of modified LDL with DGLA and its metabolites treatment was seen in BMDM from C57BL/6J mice (Figure 5.7). The mRNA expression of CD36 and SRA1 was also reduced by DGLA and its metabolites in BMDM from C57BL/6J mice (Figure 5.12). The association between the expression of SR-A/CD36 and oxLDL uptake is well identified (Crucet et al., 2013; Sun et al., 2007). Thus, a key anti-atherogenic mechanism for DGLA and its metabolites is to reduce oxLDL uptake through attenuation of SR expression.

FACS analysis of the marker, LY, evaluated receptor-independent uptake by macropinocytosis. Macropinocytosis is required in order to take up native and modified forms of LDL and to significantly contribute to foam cell formation in macrophages (McLaren et al. 2011b; Michael et al. 2013). The uptake of LY by macropinocytosis was significantly reduced when treating cells with DGLA and its metabolites (Figure 3.9).

7.6 Additional mechanisms of action

Atherosclerosis is a chronic inflammatory disease and as such proinflammatory cytokines are associated in each phase of the formation and development of this disease. Consequently, inflammation is a common target for intervention in atherosclerosis and research into effective anti-inflammatory products has increased recently (Fava and Montagnana, 2018). DGLA has been recently recognised as having immunomodulatory impact on the production of inflammatory cytokines such as IFN-γ and TNF-α (Gallagher 2016). In this study, IFN-γ was the primary cytokine used as it has previously been extensively studied in the laboratory and identified as a key regulator of atherosclerosis (McLaren and Ramji 2009). Moreover, in recent study, DGLA and PGE_1 reduced the expression of *Mcp-1* and *Icam-1* in macrophages stimulated with IFN-γ (Gallagher et al., 2019). These results are also consistent with current investigation showing that DGLA, PGE_1 and 15-(S)-HETrE produced a significant decrease in *Mcp-1* expression induced by IFN-γ in THP-1 macrophages (Figures 3.15).

Attenuating SMC migration and plaque progression is another process by which DGLA and its metabolites can have anti-atherogenic impact. A key event in advanced atherosclerosis is the presence of a thick fibrous protective cap which is regulated by the production and degradation of ECM molecules (Lusis 2000). The fibrous cap is compromised if degradation is increased via enzymes such as MMPs that are released from apoptotic SMCs and resident macrophages and T cells, which breakdown the ECM network of the plaque (Magdalena et al., 2006). The link between the activity of MMP and atherosclerosis is well known; increased expression of some MMPs is correlated with increased atherosclerotic lesion formation and enhanced migration of SMC (Magdalena et al., 2006; Prescott et al., 1999). In the current study, *in vitro* gene expression analysis revealed a significant reduction of *Mmp-3* expression *in vitro* by 15-(S)-HETrE and PGE_1 compared to the control (Table 4.3 and 4.4). In a previous *in vivo* study exploring the effect of deficiency of MMP3 in atherosclerotic aorta in $ApoE^{-/-}$/MMP-$3^{+/+}$ mice (Silence et al., 2001), atherosclerotic plaques around the entire thoracic aorta were significantly reduced in size, also macrophages, and SMCs were decreased. Additionally, DGLA and its metabolites produced a significant reduction in SMC migration (Figure 3.20). Overall, these findings imply that DGLA has immunomodulatory effects; nevertheless, inflammatory atherosclerosis pathways are extremely complex and more specific studies are required on the actions of DGLA and its metabolites on specific inflammation-associated pathways such as Mitogen activated protein kinase (MAPK)

pathways and Janus kinase-Signal transducer and activator of transcription (JAK-STAT) pathways.

7.7 From bench side to clinical trials

This research has shown that DGLA and its metabolites are valuable natural anti-inflammatory agents that have the potential to exert many protective anti-atherosclerotic effects in our *in vitro* and *in vivo* atherosclerotic models (Chapters 4-6). It could therefore be expected that the treatment of DGLA and its metabolites can decrease plaque size in humans (Moss and Ramji, 2016).

As most nutraceutical therapies are likely to be used for prevention of atherosclerosis or in combination with other therapies (e.g., statins), the effects of DGLA and its metabolites requires to be evaluated in combination with selected current treatment (e.g., statins). This will inform on whether there are any potential beneficial cardiovascular actions, or any adverse effects associated with the intake of traditional drugs and nutraceutical treatments for atherosclerosis (D'Addato *et al.* 2017). Only once the safe and efficient dietary supplementation of DGLA and its metabolites have been observed in these studies, should it proceed into introductory human clinical trials for examination as adds-on treatment. It is important to prove if DGLA and metabolites are more efficient in achieving greater cardiovascular protective effects when used as a preventive or when used in conjunction with other medications.

7.8 Future perspectives

Although the studies in this thesis have provided key insights into the anti-atherogenic actions of DGLA and its metabolites, there are several limitations such as most of the experiments being performed *in vitro*. In addition, although previous studies have shown that DGLA is taken up by cells and accumulates in membrane lipids and also in plasma, liver and adipose tissues of animals fed with diet containing this fatty acid (Gallagher 2016), concentrations of DGLA and metabolites should be determined in each experimental series in cells or plaques using advanced mass spectrometry approaches to allow more direct comparisons of concentration dependent effects. The exact physiological concentration of DGLA and its metabolites in humans is not known so

dose response experiments should have been carried out in all cases, particularly in LDLr$^{-/-}$ mice, as these would allow more informed comparisons. In addition, multiple time points should have been used for *in vivo* studies (e.g., early and late lesions). Some other *in vitro* and *in vivo* studies for future work are described below.

7.8.1 *In vitro* assays

Atherosclerosis is a chronic disorder that includes different types of cells and involves interactions between several cellular processes and many molecular mechanisms are therefore involved (Lusis 2000; Tabas et al., 2015). Results obtained in the thesis provided a significant overview into the function of DGLA and its metabolites in several aspects that correlate with *in vitro* formation of macrophage foam cells. DGLA and its metabolites also showed the ability to reduce migration of monocytes and pro-inflammatory gene expression. A popular method to studying the effect of a single gene product that performs a key role in atherosclerosis includes alteration of expression via genetic engineering (e.g., knock out or knock in). This is accompanied by an *in vitro* investigation of their targets using cells as in this study or *in vivo* to test the progression of the disease and to identify new therapeutic targets (Von Scheidt et al. 2017). In addition, such approaches (e.g., knockdown of key regulators) can be used to delineate their roles in the actions of DGLA and its metabolites on processes such as monocytic migration, macropinocytosis and foam cell formation. Some studies such as cholesterol efflux and efferocytosis were not conducted due to time constraints and could be carried out in the future to build on the results found so far.

Future research will aim to assess the effect of 15-(S)-HETrE and PGE_1 on DGLA-regulated changes such as stimulation of cholesterol efflux and the effect on inflammasome activation. Also, DGLA and its metabolites were found to inhibit the IFN-γ mediated expression of main pro-inflammatory genes in THP-1 macrophages in studies decreased in Chapter 3. Given the data presented in previous studies (Gallagher 2016) detailing that this may be a result of the inhibition of Signal transducer and activator of transcription-1 (STAT-1) and SER^{727} phosphorylation, involved in activation of IFN-γ signalling in THP-1 macrophages, this aspect needs to be investigated in relation to metabolites produced by DGLA. Although STAT1 translocation into the nucleus and transcription activation can occur independently of SER^{727} phosphorylation, inhibiting phosphorylation at this site has previously been found to attenuate pro-inflammatory gene

expression induced by IFN-γ, including *Mcp-1* and *Icam-1* (Varinou et al. 2003; Li et al. 2010). Signalling via STAT1 also involves controlling other proinflammatory pathways, such as IL-1 and TLR4 (Sikorski et al. 2011). A range of inflammasome complexes have also been classified, but the inflammasome NLRP3 has been found as an important regulator for the production of IL-1β and has been reported to be involved in atherosclerosis (Moore et al. 2013). The function of inflammasome NLRP3 in the production of IL-1β was also reported in THP-1 macrophages (Rajamäki et al. 2010). Previous work has shown that the IL-1β- and TNF-α-induced expression of *Mcp-1* and *Icam-1* was significantly decreased by DGLA and returned to levels comparable to control levels (Gallagher 2016). Following on from this, the effect of DGLA and its metabolites on other aspects of cytokine signalling may subsequently be studied such as TNF-α, NLRP3 and IL-1 *in vitro* and *in vivo*. Moreover, a variety of techniques may be used to assess the subsequent effect of STAT1 phosphorylation inhibition by DGLA. For example, the translocation of STAT1 from the cytoplasm into the nucleus can be determined using immunofluorescence analysis. Using chromatin immunoprecipitation (ChIP) assays, STAT1 binding to DNA can also be analysed. The information provided by such studies might assist to identify the precise mechanisms underlying DGLAs effects in inflammatory mechanisms.

In addition to macrophages, several other cell types play an important role in atherosclerosis pathology such as endothelial cells and HASMCs. Endothelial cell dysfunction is a key early event in the initiation of atherosclerosis (McLaren et al. 2011a; Michael et al. 2012). Future work could include the effect of DGLA and its metabolites on endothelial dysfunction by techniques such as measuring cell viability, apoptosis, ROS and Nitric oxide (NO) production, expression of pro-inflammatory markers and altered gene expression profile of cell adhesion molecules that could potentially result in improved endothelial function. Moreover, for HASMCs, the effect of DGLA and its metabolites could be examined on ROS and NO production, expression of pro-inflammatory markers, altered gene expression profile and determination of plaque lipid content (Oil Red O). An overview of the future work for *in vitro* research is shown in Figure 7.2.

7.8.2 *In vivo* experiments

Additional *in vivo* experiments with PGE_1 and 15-(S)-HETrE may also be undertaken to extend initial findings on their actions in atherosclerosis. This will involve the use of a mouse models of atherosclerosis. For this, $LDLr^{-/-}$ mice could be fed a HFD containing vehicle or 15-(S)-HETrE or

PGE_1 for 12 weeks. The consequences of PGE_1 or 15-(S)-HETrE on plaque morphology and stabilisation of plaques could be examined by various plaque morphometry and immunohistochemical techniques, such as determination of plaque lipid content (Oil Red O) or cell content by immunohistochemical staining for markers of macrophages, SMCs and T-cells, foam cell quantification and collagen staining (Miller et al., 2008, McLaren et al., 2010).Furthermore, the actions of PGE_1 or 15-(S)-HETrE on various other parameters could also be investigated such as immune cell profiles in the bone marrow by FACS. Plasma concentrations of cytokines and chemokines in response to DGLA, 15-(S)-HETrE or PGE_1 feeding could be assessed *in vivo* together with global changes in gene expression by RNA-seq of RNA from the aorta. An overview of the future work for *in vivo* research for PGE_1 or 15-(S)-HETrE is shown in Figure 7.3.

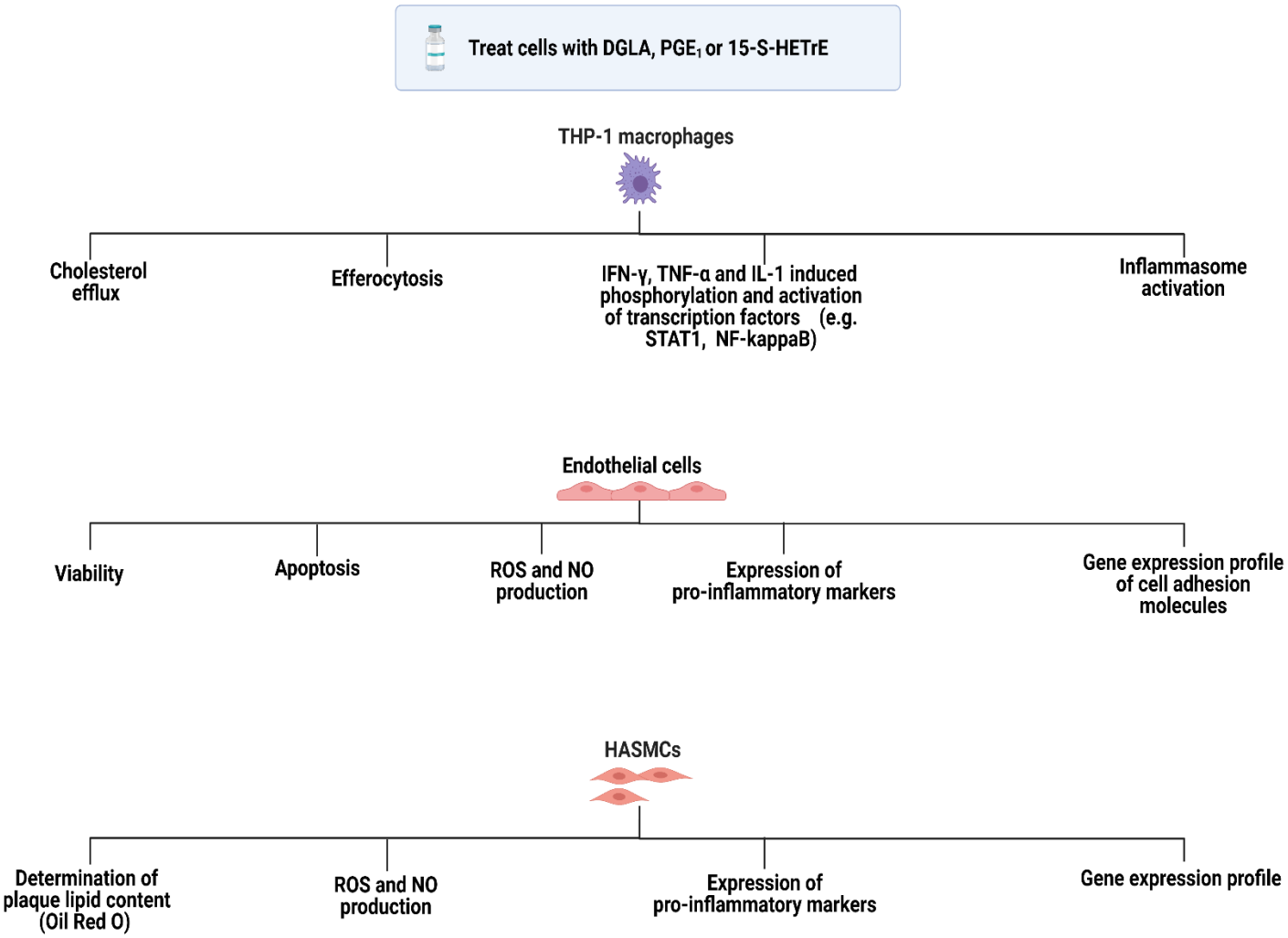


Figure 7. 2 Overview of potential avenues for *in vitro* investigations.
Created with BioRender.com.

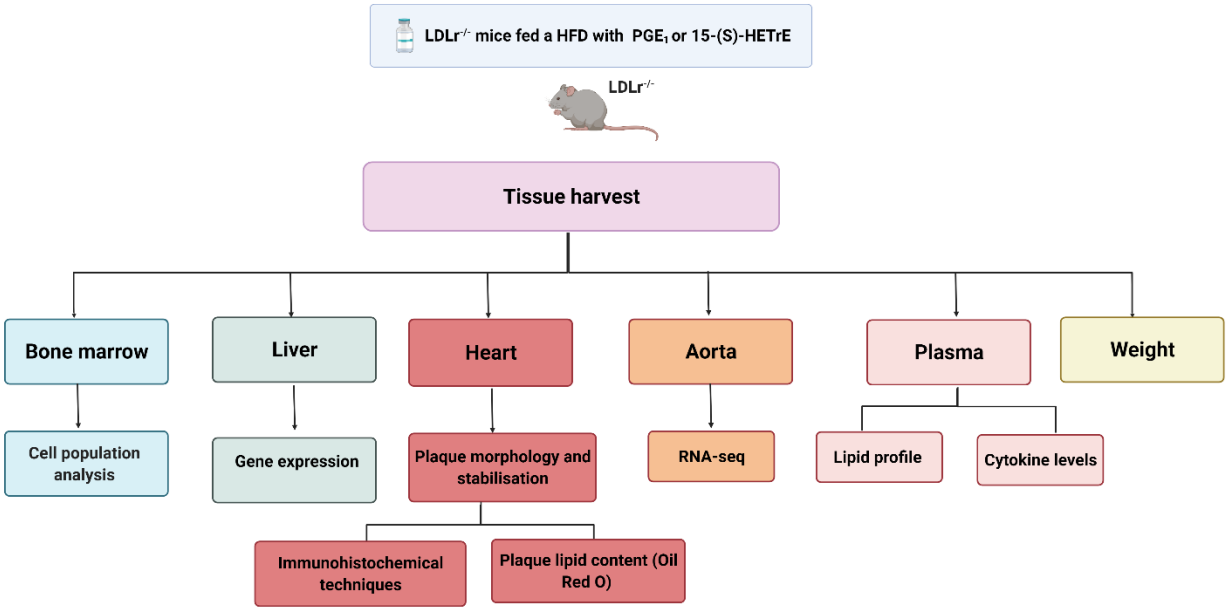


Figure 7. 3 Overview of investigations *in vivo* for PGE_1 or 15-(S)-HETrE planned.
Created with BioRender.com.

7.9 Conclusions

An anti-inflammatory and anti-atherogenic function for DGLA and its metabolites has been identified by *in vitro* research. Treatment with DGLA and its metabolites regulates multiple important pro-atherosclerotic mechanisms. These include attenuation of monocyte migration, actions of pro-inflammatory cytokines, pro-inflammatory gene expression and modified LDL uptake via pathways that are receptor-dependent and independent. Furthermore, as carried out in the current research, immunophenotyping of bone marrow cell populations provided more understanding on the effects of DGLA on bone marrow populations such as specific T cells and B cells *in vivo*. The data obtained in the thesis complements the previous limited *in vitro* studies performed with DGLA and its metabolites. Overall, studies have shown that DGLA and its metabolites can be an efficient and safe anti-inflammatory treatment with cardiovascular protection effects and are thus a promising potential alternative atherosclerosis therapy.

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