Anti-inflammatory actions of nutraceuticals:
Novel emerging therapies for atherosclerosis?

Joe Moss BSc (Hons) MRes

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Cardiff School of Biosciences
Sir Martin Evans Building
Cardiff University
Museum Avenue
Cardiff
CF10 3AX

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Abstract

Background

Cardiovascular disease (CVD)-related events such as myocardial infarction and stroke remain the world's leading cause of death. The incidence of CVD-related events is expected to rise in the future due to the increase in the global prevalence of obesity and diabetes, in addition to less economically developed countries adopting a western style diet. Atherosclerosis is a chronic inflammatory disease which is the underlying cause of CVD and characterised by the build-up of fatty deposits within the walls of medium and large arteries. Macrophages play critical roles during the pathogenesis of atherosclerosis, including the uptake of modified low-density lipoproteins to form foam cells. CardioWise is a dietary supplement developed by Cultech Limited which contains the anti-inflammatory compounds ω-3 PUFAs, flavanols and phytosterols. The aim of this project was to assess the cardiovascular protective effects of CardioWise and its individual components in isolation using *in vitro* and *in vivo* model systems.

Results

Foam cell formation was attenuated in human THP-1 macrophages treated with CardioWise. In addition, CardioWise reduced pro-inflammatory gene expression, monocyte recruitment and M1 macrophage phenotype polarisation. CardioWise was also found to increase HDL cholesterol levels and attenuate circulating levels of pro-inflammatory cytokines in wild type mice. Further investigation identified (+)-catechin within CardioWise as a key beneficial molecule to explore in greater detail. *In vitro* experiments demonstrated that catechin reduced monocyte migration and reactive oxygen species generation. Wild type mice treated with catechin were also found to receive anti-atherogenic benefits such as increased HDL cholesterol levels and reduced pro-inflammatory cytokine levels.

Conclusion

The findings of this study show that CardioWise and catechin are capable of exerting strong anti-inflammatory effects on several stages of atherosclerosis disease development *in vitro*. Furthermore initial *in vivo* studies using wild type mice revealed that both treatments are also capable of exerting several cardiovascular protective effects. Reasons for these beneficial effects have been proposed in this thesis and future studies outlined.

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Abbreviations

Abbreviation	Full Term
ABC	ATP-binding cassette
ACAT1	Acyl-CoA acyltransferase 1
acLDL	Acetylated LDL
ANOVA	One-way analysis of variance
Apo	Apolipoprotein
APT1	Acyl-protein thioesterase
Arg2	Arginase 2
BCL2A1A	BCL2 related protein A1
BMDMs	Bone marrow derived macrophages
BSA	Bovine serum albumin
C2TA	Class 2 transcription activator of the major histocompatibility complex
CAD	Coronary artery disease
CCR2	C-C chemokine receptor type 2
CE	Cholesterol esters
CEC	Cholesterol efflux capacity
CETP	Cholesterol ester transfer protein
CFLAR	CASP8 And FADD like apoptosis regulator
CHD	Coronary heart disease
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
COX	Cyclooxygenase
CSF2	Colony stimulating factor 2
CVD	Cardiovascular disease
CXCL	Chemokine (C-X-C motif) ligand
DAMPs	Danger-associated molecular patterns
DCFDA	2’7’-dichlorofluorescin diacetate
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
ECM	Extracellular matrix
ECs	Endothelial cells
ELISA	Enzyme-linked immunosorbent assay
ENG	Endoglin
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
FACS	Flow assisted cell sorting
FAD	Fatty acid desaturase
FC	Free cholesterol
FFA	Free fatty acid
FG	Fibrinogen
FGF2	Basic fibroblast growth factor
FMD	Flow-mediated dilation
FRET	Fluorescent resonance energy peptide
G-CSF	Granulocyte colony-stimulating factor

GLM	Generalised linear model
GLMM	Generalised linear mixed model
GMP	Granulocyte-macrophage progenitor
GPR	G-protein coupled receptor
HBEGF	Heparin-binding EGF-like growth factor
HDL	High density lipoproteins
HET-E	Heterokaryon incompatibility
HI-FCS	Heat-inactivated foetal calf serum
HL	Hepatic lipase
HMDM	Human monocyte-derived macrophages
HMG CoA reductase	3-hydroxy-3-methylglutaryl-CoA reductase
HPC II	Haematopoietic progenitor cells II
HRP	Horseradish-peroxidase
HSC	Haematopoietic stem cell
HSPCs	Haematopoietic stem/progenitor cells
HUVEC	Human umbilical vein endothelial cells
ICAM	Intercellular adhesion molecule
IDL	Intermediate density lipoproteins
IFN	Interferon
IL	Interleukin
iNOS	Inducible NOS
ITG	Integrin subunit
JNK	Janus kinase
KDR	Kinase insert domain receptor
LAMA1	Laminin subunit alpha 1
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LDLr	LDL receptor
LIF	Leukaemia inhibitory factor
LK	Lin- cKIT+
LOX	Lipoxygenase
LPL	Lipoprotein lipase
LMPP	Lymphomyeloid progenitor
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LXR	Liver X receptor
LY	Lucifer yellow
MCP	Monocyte chemotactic protein
M-CSF	Macrophage colony-stimulating factor
MDA	Malondialdehyde
MDSC	Myeloid derived suppressor cells
MEP	Megakaryocyte-erythroid progenitor
MI	Myocardial infarction
MMP	Matrix metalloproteinase
MPP	Multipotent progenitor cells
MSR-1	Macrophage scavenger receptor 1
NACHT	NAIP, C2TA, HET-E and TP1
NAIP	Neuronal apoptosis inhibitor protein

NAP	Neutrophil-activating peptide
NF-κB	Nuclear factor-kappa B
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NO	Nitric oxide
NOS	Nitric oxide synthase
NPLC1L1	Niemann-Pick C1-like protein
NR1H3	Nuclear receptor subfamily 1 group H member 3
oxLDL	Oxidised LDL
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCSK9	Proprotein convertase subtilisin/kexin type 9
PDGFB	Platelet-derived growth factor subunit B
PLA2	Phospholipase A2
PMA	Phorbol-12-myristate-13-acetate
PPAR	Peroxisome proliferator-activated receptor
PUFAs	Polyunsaturated fatty acids
PVAT	Perivascular adipose tissue
PWV	Pulse wave velocity
PYD	Pyrin domain
qPCR	Quantitative polymerase chain reaction
RCT	Reverse cholesterol transport
ROS	Reactive oxygen species
SCAP	SREBP cleavage-activating protein
SELE	E-selectin
SELL	L-selectin
SELPLG	Selectin P ligand
SLAM	Signalling lymphocytic activation molecule
SOD	Superoxide dismutase
SR	Scavenger receptor
SREBP2	Sterol regulatory element-binding protein 2
TBE	Tris/borate/EDTA
TBHP	Tert-butyl hydrogen peroxide
TG	Triacylglycerol
TGF	Transforming growth factor
THBS4	Thrombospondin 4
TIMPs	Tissue inhibitors of metalloproteinases
TLC	Thin layer chromatography
TNC	Tenascin C
TNF	Tumour necrosis factor
TP1	Telomerase-associated protein 1
TPL	Total polar lipid
TRAF3IP2	TNF receptor associated factor 3 interacting protein 2
UCP1	Uncoupling protein 1
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoproteins
VSMC	Vascular smooth muscle cells
WHO	World Health Organisation

Chapter 1

Introduction

1.1 Prevalence of atherosclerosis

The World Health Organisation (WHO) has estimated that one in three global deaths are due to the total number of cardiovascular disease (CVD)-related events, such as myocardial infarction (MI) and stroke (WHO 2017). The global number of CVD-related deaths in 2015 was estimated to be 17.7 million (WHO 2017). Furthermore during 2013 in England and Wales, there were 142,000 registered CVD-related deaths, equating to 28% of the total number of deaths that year (ONS 2014). The levels of CVD-related events have remained consistent in England and Wales since 2011 (ONS 2012; ONS 2013; ONS 2014) whereas the global incidence of CVD is predicted to increase significantly due to rises in the rates of obesity and diabetes in combination with the uptake of a westernised diet in developing countries. Obesity and diabetes are two major risk factors for CVD and therefore increased prevalence of these two conditions will result in a rise in the number of reported CVD-related events (Lavie *et al.* 2016; Dongerkery *et al.* 2017). Atherosclerosis is a chronic inflammatory disease which is the underlying cause of CVD and therefore therapeutic strategies that can attenuate disease development must be pursued further in order to reduce its prevalence. Atherosclerosis is triggered by the dysfunctional transportation of lipids and the resulting build-up of cholesterol within the walls of medium and large arteries.

1.2 Lipid dysfunctions in atherosclerosis

Cholesterol can be obtained from two sources, *de novo* synthesis and dietary intake, with both sources requiring transport around the body to the liver or peripheral tissues (McLaren *et al.* 2011a). Due to the hydrophobic nature of lipids, there are several hydrophilic lipoproteins which each have a different function and allow lipids to be transported in the blood (Fig. 1.1; Table 1.1). Newly synthesised triacylglycerols (TGs) in the liver can be transported to peripheral tissues by very low density lipoproteins (VLDL), whereas TGs synthesised in the intestine from the digestion products of dietary fats are transported to the liver or peripheral tissues by chylomicrons (McLaren *et al.* 2011a). Low density lipoproteins (LDL) are the most abundant lipoprotein found in the bloodstream and transports cholesterol from the liver to the peripheral tissues, whereas intermediate density lipoproteins (IDL) are found at very low levels and forms an intermediate step between VLDL and LDL. VLDL can receive apolipoprotein (Apo)-E, -A and -C from high density lipoproteins (HDL) and transfers phospholipids in return. The TGs in VLDL can then be hydrolysed by lipoprotein lipase (LPL) and hepatic lipase (HL)

altering the VLDL into an IDL particle. LDL can be formed from IDL following further HL-mediated TG hydrolysis and the transfer of apolipoproteins out of the IDL (McLaren *et al.* 2011a). LDL particles enter cells primarily by receptor-mediated endocytosis using the LDL receptor (LDLr). The key role LDL plays during atherosclerosis development can be demonstrated in studies focusing on patients with mutations in their LDLr gene, a condition known as familial hypercholesterolemia (Buckley and Ramji 2015; Kovacic and Bakran 2012). It is thought that approximately one in a million people are heterozygous sufferers of the condition and are at a greater risk of MIs at an earlier age than non-sufferers due to their plasma LDL levels being six to ten times greater than non-sufferers (Kovacic and Bakran 2012).

Table 1.1. The major lipoproteins/apolipoproteins and their functions.

Molecule	Major function	Reference
ApoA1	The major protein present in HDL. Induces cholesterol efflux within cells via ABCA1 and allows nascent HDL to collect free cholesterol.	McLaren et al. 2011a
ApoB	The primary apolipoprotein found in CM, VLDL, IDL and LDL particles.	Piepoli *et al.* 2016
ApoC-III	Prevents the removal of ApoB rich lipoproteins from the blood stream.	Sacks 2015
ApoE	Combines with cholesterol within HDL molecules to reduce its mobility and aid in transportation of cholesterol back to the liver for excretion.	van der Velde 2010
CM	Transports triacylglycerols synthesised in the intestine from digested food to the liver or peripheral tissues.	McLaren *et al.* 2011a
CMR	Peripheral tissues hydrolyse the triacylglycerols within CM via LPL into Fas and 2-monoacylglycerol which are then transported to the liver by CMR.	McLaren *et al.* 2011a
HDL	Transports surplus cholesterol from the peripheral tissues to the liver for removal in a process known as RCT.	McLaren *et al.* 2011a
IDL	Forms upon the degradation of VLDL and can be further degraded into LDL.	McLaren *et al.* 2011a
LDL	Transports cholesterol from the liver to the peripheral tissues. It is the most abundant lipoprotein within the blood stream.	McLaren *et al.* 2011a
VLDL	Transports triacylglycerols synthesised in the liver to peripheral tissues.	McLaren *et al.* 2011a

Apo, apolipoprotein; CM, chylomicrons; CMR, CM remnants; FAs, fatty acids; HDL, high density lipoprotein; IDL, intermediate lipoprotein; LDL, low density lipoprotein; LPL, lipoprotein lipase; RCT, reverse cholesterol transport; VLDL, very low density lipoprotein.

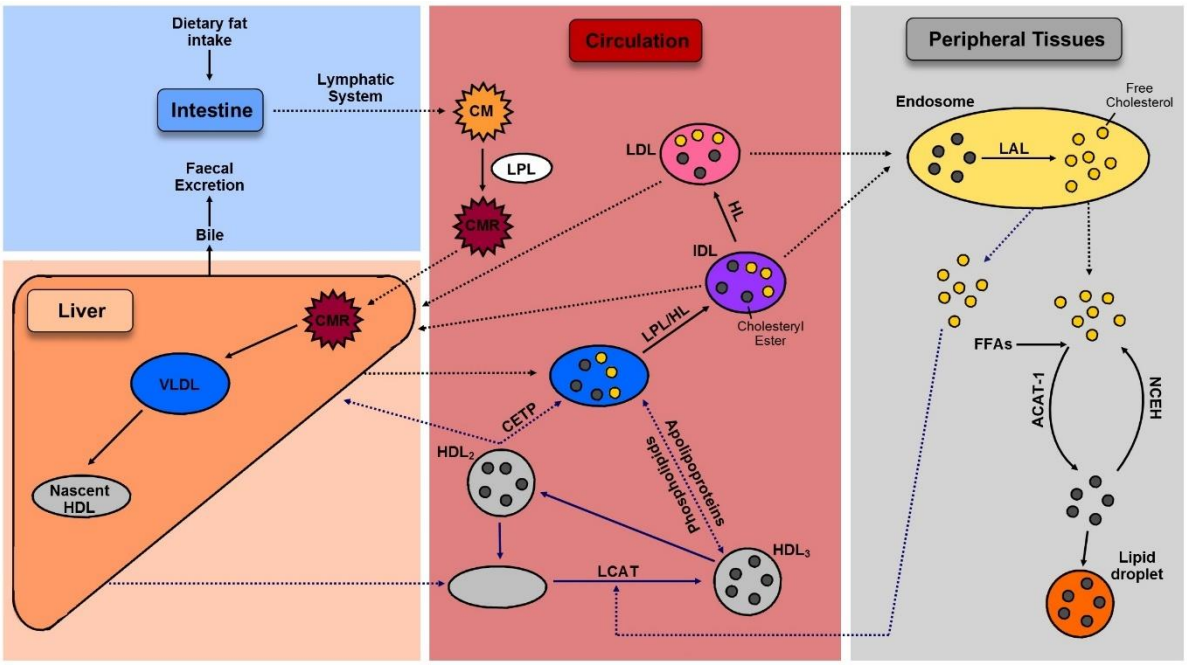

Fig. 1.1. Cholesterol metabolism. Several lipoprotein particles are involved in the transport of cholesterol and other lipids around the body through the bloodstream. Triacylglycerols absorbed in the intestine from dietary intake are transported to the liver and peripheral tissues by chylomicrons, whereas triacylglycerols synthesised *de novo* are transported from the liver to the peripheral tissues by VLDL. IDL bridges the gap between the VLDL and LDL pathways. LDL particles can be formed when the triacylglycerols in VLDL particles are hydrolysed by LPL and HL. Cholesterol is transported from the liver to the peripheral tissues via LDL. The CEs are then hydrolysed into free cholesterol and FFAs by LAL. The excess cholesterol in the peripheral tissues can either be transported out of the cells or stored. The free cholesterol can be re-esterified to CEs by ACAT-1 and stored within the cells as lipid droplets. The remaining cholesterol is transported back to the liver by HDL for excretion in bile through the reverse cholesterol transport pathway (indicated by the blue arrows). However some of the cholesterol esters can be transferred to VLDL and other lipoprotein particles by CETP-mediated processes. The stored CEs can be hydrolysed back into free cholesterol by NCEH. LCAT is an enzyme that is able to re-esterify free cholesterol into CEs and sequester it in a nascent HDL particle, causing a one way reaction, to form a newly synthesised HDL particle. ACAT-1, acyl-coenzyme A acyltransferase 1; CETP, cholesterol ester transfer protein; CEs, cholesteryl esters; FFAs, free fatty acids; HDL, high density lipoproteins; HL, hepatic lipase; IDL, intermediate density lipoproteins; LAL, lysosomal acid lipases; LCAT, lecithin-cholesterol acyltransferase; LDL, low density lipoproteins; LPL, lipoprotein lipase; NCEH, neutral cholesterol ester hydrolase; VLDL, very low-density lipoproteins. Figure adapted from *McLaren et al.* (2011a).

In order to maintain a balance in cholesterol metabolism, HDL transports excess cholesterol from the peripheral tissues back to the liver for excretion by a process known as reverse cholesterol transport (RCT; McLaren *et al.* 2011a). Initially HDL and ApoA1 stimulate cholesterol efflux in cells within the peripheral by stimulating the transporter ATP-binding cassette A1 (ABCA1; McLaren *et al.* 2011a). Therefore increased levels of ApoA1 is seen as a marker of increased cholesterol efflux. ApoA1 also allows nascent HDL particles to collect the released cholesterol for transportation. To reduce the mobility of cholesterol being transported by HDL, it is esterified and combined with ApoE to decrease its net electrical polarity (van der Velde 2010). Some of the cholesteryl esters (CEs) are transferred to VLDL

particles via cholesterol ester transfer protein (CETP)-mediated processes, while the rest are delivered to the liver (McLaren *et al.* 2011a). Once the HDL particles reach the liver, CEs detach in a process involving HDL particles briefly binding to the cell membrane of hepatocytes via scavenger receptor (SR)-B1 and releasing the esterified cholesterol into the cytosol of these cells (van der Velde 2010; Bhatt and Rohatgi 2016). The esterified cholesterol is processed into bile salts which are then excreted into the small intestine (van der Velde 2010). However only 5% of the biliary cholesterol is excreted in faeces as the rest is reabsorbed in the intestine as they are used to emulsify lipid droplets formed within the intestine following dietary intake (van der Velde 2010). This rotation of bile salts is known as the enterohepatic circulation. Sufferers of a genetic disease called Tangier disease have mutations in their *ABCA1* gene, which plays a key role in cholesterol efflux, and as a result have low plasma levels of HDL and develop atherosclerosis much earlier compared to the general population (Van Eck 2014). The association of high HDL plasma levels and cardiovascular protective effects has been demonstrated in both human and animal studies (Van Eck 2014; Rader and Hovingh 2014; Kingwell *et al.* 2014; Calabresi *et al.* 2015). Recent human and murine studies have suggested that rather than the concentration of circulating levels of HDL cholesterol, it is its ability to induce cholesterol efflux which results its cardiovascular protective effects (Bhatt and Rohatgi 2016). This has led to the cholesterol efflux capacity (CEC) of the RCT system being investigated as a potential therapeutic target.

During normal healthy conditions, the metabolism and transport of cholesterol is highly regulated and the influx and efflux of cholesterol in cells is well balanced. The development of atherosclerosis can begin when these transport systems become unbalanced in favour of increased cholesterol influx or decreased efflux (discussed in greater detail in Section 1.3). High LDL and low HDL serum levels have been associated with reduced endothelium function such as induction of a pro-inflammatory state, increased pro-thrombotic effects and attenuated vasodilation (Vogel 1997; Rajendran *et al.* 2013). Therefore increased LDL levels are thought of as being pro-atherogenic whereas elevated HDL levels are considered to be anti-atherogenic (Vogel 1997). It is important that therapies for atherosclerosis aim to lower plasma LDL levels as well as increase the efflux capacity of the RCT system through HDL.

Naturally mice do not develop atherosclerosis but *LDLr* and *ApoE* deficient mouse models have been developed which are prone to atherosclerosis lesion formation while on their natural diet, however this can be accelerated by feeding them a high fat or high cholesterol diet. Mice lacking the LDLr develop atherosclerosis due an increase in the circulating levels of VLDL and LDL as they cannot be cleared by the liver (Kapourchali *et al.* 2014). ApoE deficient mice develop atherosclerotic lesions as the HDL molecules cannot be removed from the blood stream and they are eventually metabolised into VLDL and LDL particles, resulting in atherosclerosis disease development (Kapourchali *et al.* 2014). Mouse model systems have

significantly improved our understanding of the pathology of atherosclerosis disease progression.

1.3 Pathophysiology

Atherosclerosis is often characterised by the build-up of fatty deposits and the formation of plaque structures in the walls of large and medium arteries, as well as a strong immunological response to the fatty deposit accumulation (Fig. 1.2). The initial fatty deposit build up in the intima of arteries, often referred to as a fatty streak, is composed of ApoB containing lipoproteins, in particular LDL, and other lipoprotein remnants (McLaren *et al.* 2011a). Vascular cells produce reactive oxygen species (ROS) which are thought to act as secondary messengers in vascular homeostasis (Vara and Pula 2014). During aerobic metabolism, ROS are generated as a product of the electron transport chain as well as being produced by a variety of enzymes including cyclooxygenase (COX) and nitric oxide synthase (NOS; Vara and Pula 2014). However ROS can also modify the LDL by oxidation to oxidised LDL (oxLDL; McLaren *et al.* 2011a). LDL can also undergo under several other modifications to form a variety of modified LDL molecules including aggregated LDL and glycated LDL (gLDL; Alique *et al.* 2015). The later modification occurs in diabetics who are at an increased risk of forming gLDL, which increases their risk of developing atherosclerosis (Rashid *et al.* 2006).

An inflammatory response is then generated by the presence of oxLDL in the neighbouring endothelial cells (ECs) which start to produce pro-inflammatory cytokines and chemokines (McLaren *et al.* 2011a; Lusis 2000; Ramji and Davies 2015). The role of different chemokines and cytokines has been extensively reviewed and can be generally classified as either pro- or anti-inflammatory (Buckley and Ramji 2015; Ramji and Davies 2015; Moss and Ramji 2016a). Pro-inflammatory cytokines include interferon (IFN)-γ, tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-18, whereas anti-inflammatory cytokines include IL-10, IL-33 and transforming growth factor (TGF)-β (Ramji and Davies 2015; Moss and Ramji 2016a; Moss and Ramji 2015). However this distinction is not always possible with some cytokines and chemokines capable of exerting both pro- and anti-inflammatory effects; for example macrophage colony-stimulating factor (M-CSF) and granulocyte colony-stimulating factor (G-CSF; Ramji and Davies 2015).

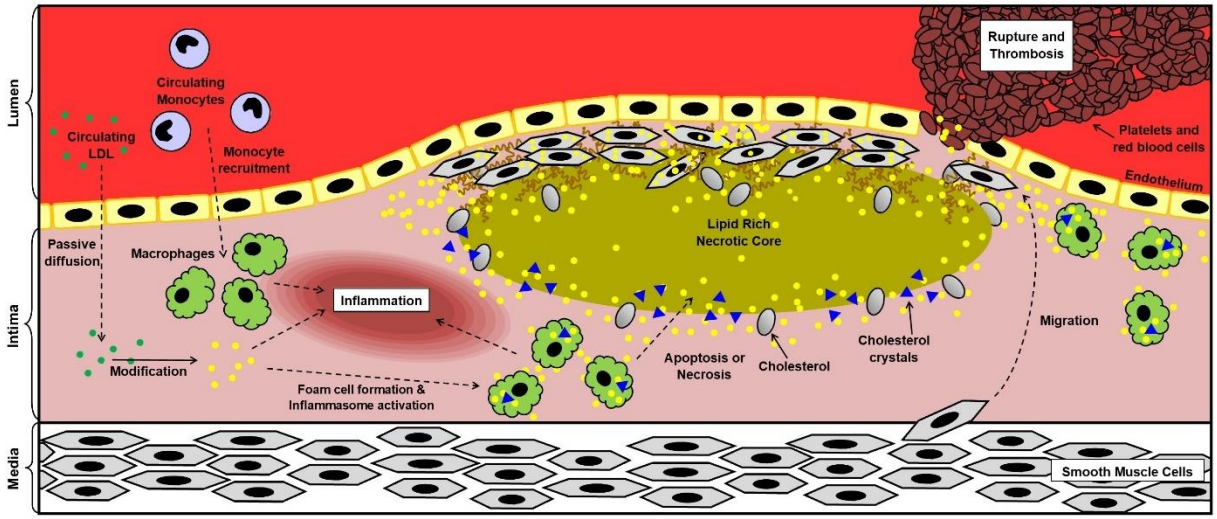

Fig. 1.2. The progress of an atherosclerotic plaque formation. Initially circulating LDL within the lumen of the artery passively diffuses into the intima where it becomes modified, usually by oxidation into oxidised LDL. The presence of modified LDL triggers an inflammatory response in the neighbouring endothelial cells causing them to express cell adhesion molecules on their surface. These cell adhesion molecules allow monocytes to roll and adhere to the wall of the artery at the affected site. The monocytes then proceed to migrate into the intima of the artery, where they differentiate into macrophages and begin to take up the modified LDL causing their transformation into lipid laden foam cells. Over time these foam cells undergo apoptosis or necrosis as accumulation of intracellular cholesterol is toxic. Foam cells then release their lipid contents into the wall of the artery which accumulate and form a lipid-rich necrotic core. The necrotic core continues to add to the initial inflammatory response by triggering inflammasome activation due to lipid and cholesterol accumulation. Inflammasome activation results in the continuous recruitment and formation of foam cells. As the lipid-rich necrotic core grows in size it can become unstable. Smooth muscle cells migrate from the media of the artery to the top of the necrotic core and deposit collagen and other proteins to form a fibrous cap to provide the plaque with extra stability. Eventually the fibrous cap deteriorates and ruptures due to a build-up of matrix metalloproteinases and other proteases capable of degrading the ECM. Once ruptured the internal components of the plaque become exposed to the lumen. This triggers a thrombotic reaction and the formation of a blood clot due to the aggregation of platelets, which can block the artery and depending on the location of the plaque can result in either a myocardial infarction or stroke.

One of the major pro-atherogenic chemokines released by the activated ECs is monocyte chemotactic protein (MCP)-1, which guides the circulating monocytes and T-lymphocytes towards the oxLDL build-up. Atherosclerotic mouse models lacking MCP-1 or its receptor (CCR2) have been shown to develop smaller lesions (Gu *et al.* 1998; Boring *et al.* 1998; Öhman *et al.* 2010; Liu *et al.* 2012b; Olzinski *et al.* 2010; Majmudar *et al.* 2013), highlighting the importance of MCP-1 in the development of this disease. The presence of oxLDL further enhances the inflammatory response by inhibiting the production of nitric oxide (NO), possibly by reducing the availability of its precursor L-arginine (Knowles *et al.* 2000; Wang *et al.* 2011b). Due to the role of NO in vasodilation, inhibiting its production leads to an increase in blood pressure and enhances atherosclerosis in mouse models (Knowles *et al.* 2000). Furthermore, oxLDL has been shown to directly induce endothelial dysfunction and reduce vasorelaxation by stimulation of endothelial cell death as well as enhancing the expression of the cytoplasmic adapter protein TNF receptor associated factor 3 interacting protein 2 (TRAF3IP2; sometimes

referred to as CIKS or Act1; Valente *et al.* 2014). TRAF3IP2 is known to regulate the expression of nuclear factor-kappa B (NF-κB) and Janus kinase (JNK; Leonardi *et al.* 2000; Li *et al.* 2000), both of which are capable of modulating the immune response and contributing towards atherosclerosis progression. The activated ECs also express cell adhesion markers on their cell surface, such as P- and E-selectins, vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 (McLaren *et al.* 2011a). These cell surface molecules bind to the corresponding receptors and ligands expressed on the surface of circulating monocytes, allowing them to roll and adhere to the site of activation in the endothelium, aiding their migration to the oxLDL accumulation in the arterial walls (Dong *et al.* 1998; Collins *et al.* 2000; Shih *et al.* 1999). Pro-inflammatory cytokines and chemokines, including IFN-γ, IL-1β and TNF-α, have been shown to aid cellular recruitment to the atherosclerotic lesion by inducing the expression of *VCAM-1*, *ICAM-1* and *MCP-1* in activated ECs (Charo and Taubman 2004; Blankenberg *et al.* 2003; Chung *et al.* 2002; Kirii *et al.* 2003; Xiao *et al.* 2009). Mouse models deficient in IFN-γ, IL-1β and TNF-α have reduced lesion sizes due to a decrease in the number of macrophages (Kirii *et al.* 2003; Gupta *et al.* 1997; Whitman *et al.* 2000; Whitman *et al.* 2002; Koga *et al.* 2007b; Koga *et al.* 2007a; Chamberlain *et al.* 2009; Branen *et al.* 2004; Ohta *et al.* 2005; Xiao *et al.* 2009). Mice deficient in a variety of chemokines and cytokines have been shown to have reduced or enhanced atherosclerotic plaque formation depending on whether they are pro- or anti-inflammatory. These are extensively reviewed by *Ramji and Davies* (2015).

Once the monocytes have migrated into the intima of the arteries, they become exposed to M-CSF and differentiate into macrophages, a process that is associated with increased expression of pattern recognition receptors on their cell surface (McLaren *et al.* 2011a; Lusis 2000). These receptors allow macrophages to identify foreign bodies during an innate immune response, however they are cable of recognising domains found within modified LDL. Research continues to emerge which suggests that macrophages are capable of polarising into broad classes, either the pro-inflammatory M1 phenotype or the anti-inflammatory M2 phenotype (Geeraerts *et al.* 2017; Rojas *et al.* 2015; Komohara *et al.* 2016). The factors which affect macrophage phenotype polarisation are discussed in Chapter 3.

Macrophages are able to uptake oxLDL via SR-mediated endocytosis, macropinocytosis or phagocytosis (Li and Glass 2002; Bobryshev 2006). Although there are several SRs, SR-A and CD36 are thought to be of particular importance and play a significant role in oxLDL uptake as shown by mouse models deficient in either receptor developing smaller atherosclerotic lesions (Febbraio *et al.* 2000; Kuchibhotla *et al.* 2008; Mäkinen *et al.* 2010). Both SR-A and CD36 are thought to be responsible for 90% of macrophage oxLDL uptake (Chistiakov *et al.* 2016). The uptake of LDL via the LDLr is under a negative feedback loop which is tightly regulated by intracellular cholesterol levels (Zhang *et al.* 2016). The proteins sterol regulatory

element-binding protein 2 (SREBP2) and SREBP cleavage-activating protein (SCAP) are essential for the expression of the LDLr. These two proteins form a dimer which is transferred from the endoplasmic reticulum (ER) to the golgi apparatus for maturation. Once in the golgi, SREBP2 is cleaved by proteases and separated from SCAP to form the active nuclear SREBP (nSREBP), which is then able to bind to the promotor region of the LDLr gene and induce its transcription (Zhang *et al.* 2016). Increased intracellular cholesterol levels induce SCAP to undergo a conformation change which results in the SREBP2/SCAP dimer unable to transfer from the ER to the golgi leading to the attenuation of LDLr gene expression as well as cholesterol uptake (Zhang *et al.* 2016). On the other hand, oxLDL uptake via SRs is unregulated and not controlled by a negative feedback loop and therefore results in the continuous uptake of oxLDL (Stephen *et al.* 2010). Constant uptake of oxLDL leads to the formation of CE lipid droplets in the cytoplasm of macrophages, giving them a foamy appearance and induce the development of foam cells (Yu *et al.* 2013).

Pro-inflammatory cytokines are capable of inducing foam cell formation by altering cholesterol homeostasis in macrophages to lower cholesterol efflux and increase the uptake and retention of oxLDL, and are thought to mediate these effects by regulating the expression of key cholesterol metabolism and transport genes including *ApoE*, *ABCA1*, acetyl-CoA acyltransferase 1 (*ACAT1*) and *SR-A* (McLaren *et al.* 2011a; Ramji and Davies 2015; Harvey and Ramji 2005; Panousis and Zuckerman 2000; Wuttge *et al.* 2004; Ohta *et al.* 2005). The formation of foam cells is summarised in Fig. 1.3. Foam cells then begin to accumulate and form an initial lesion that can be seen as a fatty streak in arteries which then matures into an atherosclerotic plaque.

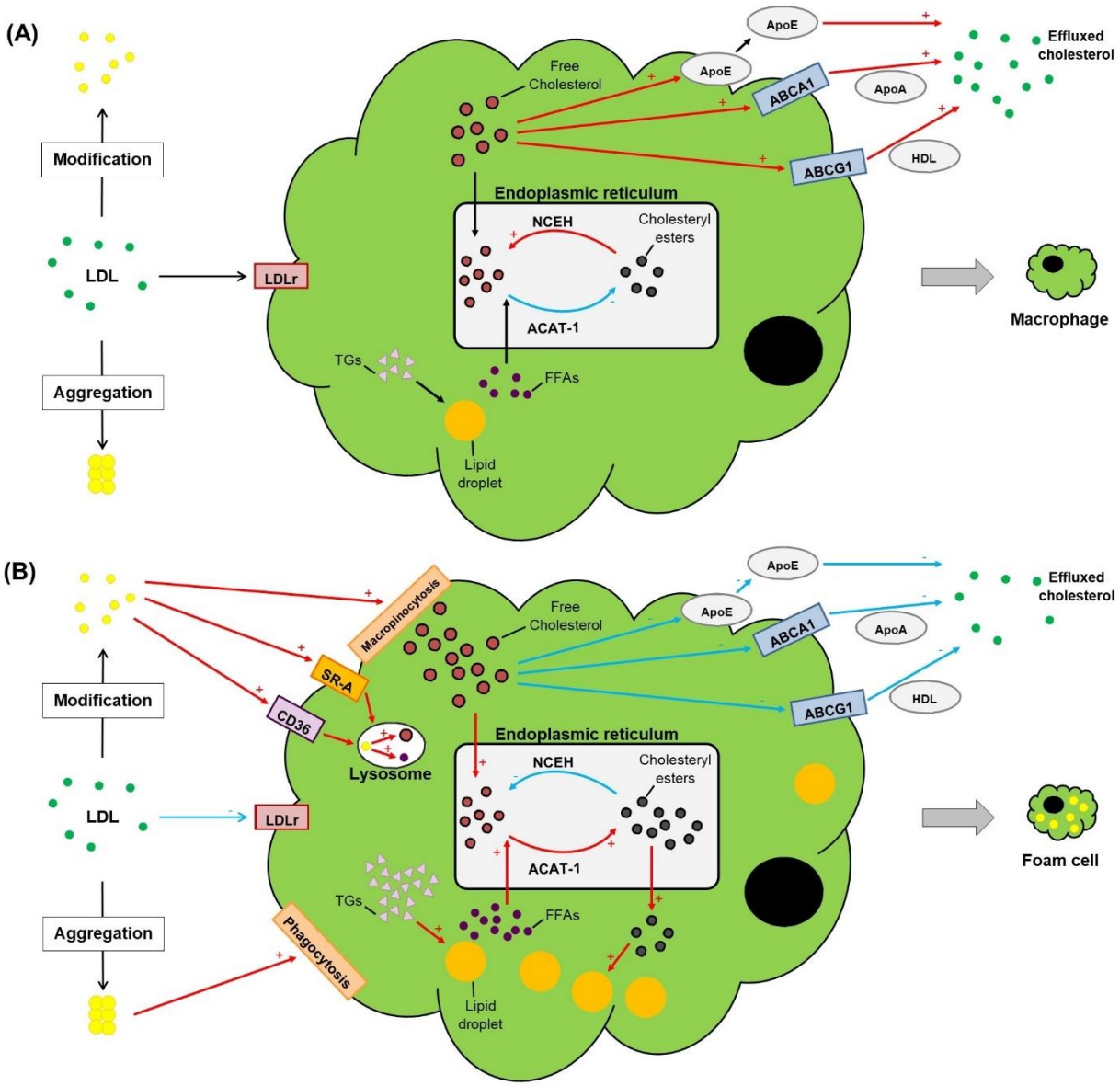

Fig. 1.3. Foam cell formation. The mechanism of cholesterol homeostasis and the cholesterol transport processes used to prevent foam cell formation by balancing cholesterol uptake and efflux (A). The uptake of LDL via the LDL receptor (LDLr) is precisely controlled by a negative feedback loop. The intracellular cholesterol is stored as free cholesterol and effluxed via ApoE and the cholesterol efflux transporters ATP-binding cassette (ABC)A1 and ABCG1. When macrophage homeostasis becomes imbalanced to favour cholesterol uptake and reduced cholesterol efflux, foam cells begin to form (B). Modified LDL is taken up via scavenger receptors (SRs), SR-A and CD36, as well as macropinocytosis and phagocytosis. SR-mediated uptake of modified LDL is not under a negative feedback loop and therefore results in the continuous uptake of modified LDL. The efflux of cholesterol is also attenuated which results in an increase of intracellular cholesterol levels. The free cholesterol is esterified into cholesteryl esters, leading to an increase in lipid droplet formation and macrophages developing into foam cells. Red arrows indicate processes which are increased, blue arrows indicate processes which are attenuated. ACAT-1, acyl-coenzyme A acyltransferase 1; Apo, apolipoprotein; FFAs, free fatty acids; HDL, high density lipoprotein; NCEH, neutral cholesterol ester hydrolase; TGs, triacylglycerols. Figure adapted from *McLaren et al.* (2011a).

During atherosclerosis lesion maturation, the accumulated foam cells begin to undergo apoptosis and necrosis as increased cholesterol accumulation is toxic and results in ER stress (Sozen and Ozer 2017). Foam cell death causes them to release their fatty contents, including

components from the metabolism of oxLDL such as cholesterol, into the intima of the arteries. The apoptotic cells and the fatty contents accumulate to form a lipid-rich necrotic core. The accumulated lipids within the core, including oxLDL and cholesterol crystals, are capable of exacerbating the inflammatory response by triggering inflammasome activation (Hoseini *et al.* 2017). The inflammasome is a multi-protein complex capable of enhancing the innate immune response and can be triggered by a variety of stimuli including pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs; Hoseini *et al.* 2017). The activated inflammasome is involved in the maturation and activation of the pro-inflammatory cytokines IL-1β and IL-18. This has led to inflammasome activation being associated with atherosclerosis disease progression (Guo *et al.* 2015). Patients considered to be at high risk of suffering a CVD-related event (hypercholesterolaemic, diabetic, hypertensive and smokers) often have an increased level of inflammasomes in their aortas (Zheng *et al.* 2013). Furthermore silencing the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome leads to improved plaque stability in *ApoE* deficient mice (Zheng *et al.* 2014). Additionally all mechanisms which activate inflammasomes are also involved in atherosclerosis disease progression (Hoseini *et al.* 2017). Therefore the inflammasome represents a possible link between aberrant lipid metabolism and inflammation in atherosclerosis (Hoseini *et al.* 2017).

The necrotic core can be covered by a fibrous cap formed by the extracellular matrix (ECM) produced by the vascular smooth muscle cells (VSMCs; McLaren *et al.* 2011a; Katsuda and Kaji 2003). During the latter stages of plaque progression, macrophages, endothelial cells and T-cells stimulate VSMC proliferation and migration towards the oxLDL accumulation and form a fibrous cap over the lipid core (McLaren *et al.* 2011a; Chistiakov *et al.* 2015). This process occurs when the phenotype of the VSMCs is altered from an inactive quiescent state to an active contractile one, triggered by pro-inflammatory factors which induce the expression of integrins on the surface of VSMCs that are then able to bind to fibronectin in ECM and allow VSMC migration (Rudijanto 2007; Schonbeck *et al.* 2000; Barillari *et al.* 2001). The CD40 receptor is expressed on antigen presenting cells, including macrophages and T-cells, and its activation has been linked with the release of chemokines and pro-inflammatory cytokines (Legein *et al.* 2013). Furthermore, CD40 activation has been shown to stimulate ECM degradation and remodelling, allowing the VSMCs to migrate more easily from the media to the intima to form the fibrous cap (McLaren *et al.* 2011a). Atherosclerotic mouse models deficient in CD40 have smaller and less inflammatory atherosclerotic lesions (Lusis 2000; Lutgens *et al.* 2010), stressing its important role in plaque progression.

The fibrous cap provides the lesion with stability, therefore the balance of ECM deposition and degradation is a key element in the clinical complications of atherosclerosis. ECM degrading enzymes are released from macrophages during an inflammatory response as well as when

they undergoing apoptosis, shifting the balance towards ECM degradation and increasing the risk of a plaque rupture (Newby 2006; McLaren *et al.* 2011a). Matrix metalloproteinases (MMPs), a class of proteases capable of degrading ECM proteins, have been shown to be produced by macrophages and VSMCs during inflammatory conditions (Schonbeck *et al.* 1997). Murine studies have also demonstrated that the levels and activity of MMP-2 and MMP-9 are increased within advanced atherosclerotic plaques, highlighting their key role in plaque stability (Wågsäter *et al.* 2011). No external symptoms of plaque development are observable until the plaque ruptures. Once ruptured a thrombotic reaction is generated and platelet aggregation occurs, which can quickly impede or obstruct blood flow though the artery (Lusis 2000). Due to the global prevalence of CVD and its economic burden on healthcare services around the world, many therapeutic strategies have been/are being developed to retard atherosclerosis progression. The benefits and limitations of these therapeutics will be discussed below in greater detail.

1.4 Aetiology

An individual's risk of developing atherosclerosis can be affected by both their life style and genetic predisposition (Lusis 2000). The life style of the patient can often be modified to reduce their risk of a CVD-related event, however a family history of CVD and other unmodifiable factors can indicate whether an individual is genetically susceptible to atherosclerosis (McLaren *et al.* 2011a).

One of the major modifiable life style risk factors is smoking. The susceptibility of LDL to oxidation, vasoconstriction and platelet aggregation, all of which are involved in the development of a CVD-related event, are all promoted by nicotine (Vogel 1997). Indeed in those who quit smoking there was an improvement in endothelial function within 1 year (Johnson *et al.* 2010). Furthermore serum levels of LDL and HDL cholesterol can be influenced both directly and indirectly by a high fat diet and obesity (McGill *et al.* 2002), therefore obesity is another key risk factor in determining whether an individual will suffer a CVD-related event. Additionally, a lack of exercise is associated with an increased risk of developing atherosclerosis as improved endothelial function has been observed in patients following regular exercise (Hambrecht *et al.* 2003; Beck *et al.* 2014).

There are many unmodifiable genetic factors which can lead to a predisposition to CVD including family history, age and gender. Those with a family history of CVD-related events such as MI, stroke, aortic disease and peripheral arterial disease are more likely to develop atherosclerosis (Vogel 1997). Age is strongly correlated with increased vasoconstriction and build-up of cholesterol within atherosclerotic plaques and therefore it is also correlated with an increased risk of suffering a CVD-related event (Vogel 1997; Niccoli and Partridge 2012).

Furthermore men are more likely to suffer a CVD-related event than aged matched pre-menopausal women, leading to what is known as the 'gender gap' (Frink 2009). This 'gender gap' narrows in post-menopausal women. As previously discussed, the genetic conditions familial hypercholesterolemia and Tangier disease cause patients to have mutations in their *LDLr* and *ABCA1* genes respectively (Kovacic and Bakran 2012; Van Eck 2014; Buckley and Ramji 2015). As a result these patients have increased circulating LDL cholesterol levels or reduced plasma levels of HDL cholesterol compared to non-sufferers, giving them a higher risk of developing atherosclerosis (Kovacic and Bakran 2012; Van Eck 2014).

There are many modifiable and unmodifiable risk factors that can influence an individual's risk of suffering a CVD-related event, therefore it is key that doctors can accurately identify high risk patients (McLaren *et al.* 2011a). It is essential that educational drives to help individuals recognise their risk factors start much earlier to allow early intervention rather than wait until advanced atherosclerosis disease development has occurred. Once high risk patients have been identified, their risk can be reduced by developing strategies that either modify their lifestyle or involve the use of pharmaceuticals such as statins.

1.5 Current and emerging therapeutics: Benefits and limitations

The most commonly and successfully used cholesterol lowering class of pharmaceutical therapies are statins, which inhibit the enzyme involved in the rate limiting step during cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl-CoA (HMG CoA) reductase, resulting in a reduction in circulating LDL cholesterol (Schonbeck 2004; Haslinger-Loffler 2008). However there is still a discernible residual risk of a CVD-related event in patients taking statins, in addition to a significant minority of individuals who are unable to achieve target plasma cholesterol levels despite receiving the maximum recommended dosage (McLaren *et al.* 2011a; Leitersdorf 2001). The maximum reduction in CVD mortality that can be attributed to statins is approximately 30%, stressing the need to develop alternative therapies to target the remaining 70% (McLaren *et al.* 2011a). Approximately for every 6% reduction in plasma LDL levels past the baseline achieved with the initial statin treatment levels, the dosage needs to be doubled (Knopp 1999). However high-dose statin therapy is known to exert side effects such as nose bleeds, non-allergic rhinitis, headaches, muscle pain and an increased risk of developing diabetes (Parker *et al.* 2013; Ridker *et al.* 2008; NHS 2016). This highlights the significant need to develop new therapeutics that can either be taken alone or in combination with statins (McLaren *et al.* 2011a; Leitersdorf 2001).

Ezetimibe therapy in combination with statins is providing some hope for lowering LDL concentrations in those individuals that are unable to achieve target levels whist receiving the maximal dose of statins. It is designed to inhibit the Niemann-Pick C1-like protein (NPLC1L1)

in the intestine and reduce cholesterol uptake. Ezetimibe in combination with statins reduces LDL levels by approximately 23% compared to statin therapy alone (Ballantyne *et al.* 2004; Morrone *et al.* 2012). Two recent trials known as Improved Reduction of Outcomes: Vytorin Efficacy International Trial (IMPROVE-IT) and Plaque Regression With Cholesterol Absorption Inhibitor or Synthesis Inhibitor Evaluated by Intravascular Ultrasound (PRECISE-IVUS) which involved 18,144 and 229 participants respectively, have shown that ezetimibe plus statin therapy lowered patient LDL cholesterol levels and improved cardiovascular outcomes (Cannon *et al.* 2015; Tsujita *et al.* 2015). However a previous study involving 3,827 individuals found no evidence that ezetimibe therapy in combination with statins significantly reduced mortality compared to the statin only group (Patel *et al.* 2013), thereby implicating the need for more larger clinical trials to evaluate efficacy.

Another emerging strategy involves targeting proprotein convertase subtilisin/kexin type 9 (PCSK9) with human monoclonal antibodies at present and possibly specific inhibitors in the future. PCSK9 is able to bind to the LDLr and direct it towards the lysosome for degradation during LDLr metabolism and results in increased plasma LDL cholesterol levels (Giugliano and Sabatine 2015). Loss-of-function in PCSK9 leads to protection against cardiovascular events regardless of other risk factors by increasing LDLr expression. This results in a reduction in the circulating LDL cholesterol levels, making it an exciting emerging therapeutic target (Giugliano and Sabatine 2015). Phase I and II clinical trials have shown that inhibition of PCSK9 by human monoclonal antibodies reduced circulating LDL levels without exerting any severe side effects (Giugliano and Sabatine 2015; Zhang *et al.* 2015). Phase III clinical trials are currently underway or completed recently using three antibodies, evolocumab, alirocumab and bococizumab, in trials known as FOURIER (Further Cardiovascular Outcomes Research with PCSK9 Inhibition in Subjects with Elevated Risk), ODYSSEY Outcomes (Study to Evaluate the Effect of Alirocumab on the Occurrence of Cardiovascular Events in Patients Who Have Experienced an Acute Coronary Syndrome), SPIRE (Studies of PCSK9 Inhibition and the Reduction of Vascular Events)-1 and SPIRE-2 involving 28,000, 18,000, 17,000 and 10,000 participants respectively (U.S. National Institutes of Health 2017b; Ridker *et al.* 2017b; Sabatine *et al.* 2017). The ODYSSEY trial is expected to be completed in 2018. The FOURIER trial found evolocumab was able to reduce patients LDL cholesterol levels by 59% when compared to those receiving the placebo without exerting any adverse effects (Sabatine *et al.* 2017). However evolocumab failed to lower an individual's risk of suffering a CVD-related event. The efficacy of bococizumab was assessed in both low risk patients (SPIRE-1) and high risk individuals (SPIRE-2; Ridker *et al.* 2017b). These studies were prematurely stopped as other trials involving bococizumab showed it was capable of inducing the development of high-titer antidrug antibodies (Ridker *et al.* 2017b). Despite this, pooled data from both SPIRE studies demonstrated that bococizumab resulted in a decrease in circulating LDL levels by 56% after 14 weeks. This reduction in LDL levels was maintained throughout the study period.

High risk patients treated with bococizumab were found to have a reduced CVD-related event risk, however no reduction was observed in low risk patients (Ridker *et al.* 2017b). These trials show that PCSK9 inhibitors are capable of further reducing LDL levels in patients already receiving statins, however due to the high cost of this treatment and potential adverse effects the use of these therapies should be limited to high risk patients. Indeed alirocumab has already been approved for high risk patients in both USA and Europe (FDA 2015; EMA 2015b), whereas as the use of evolocumab has only been granted in Europe (EMA 2015a).

A number of other targets have been explored for therapeutic intervention of atherosclerosis though most have proved disappointing at the clinical level. For example, despite the extensive evidence associating increased plasma HDL levels with reduced risk of CVD, clinical trials using orally active, HDL raising agents have largely failed (Rader and Hovingh 2014; Kingwell *et al.* 2014; Cimmino *et al.* 2015; Kaur *et al.* 2014). Other trails with agents that are known to augment HDL levels in addition to other beneficial effects at the pre-clinical level, such as niacin and fibrates, have also been disappointing (Sando and Knight 2015). This probably reflects the complex roles of HDL, its heterogeneity and its interactions with numerous proteins (Rader and Hovingh 2014; Kingwell *et al.* 2014; Cimmino *et al.* 2015). Future research is focusing on attaining a deeper understanding of HDL biology and enhancing its capacity to stimulate cellular cholesterol efflux and RCT. CETP is involved in the bidirectional transfer of cholesteryl esters in HDL for TGs in ApoB containing lipoproteins such as LDL and VLDL (Sando and Knight 2015; Ladeiras-Lopes *et al.* 2015). Inhibition of CETP should therefore be beneficial, as this would increase the cholesteryl ester content of HDL for RCT and reduce that in pro-atherogenic LDL particles. However, initial large-scale clinical trials with two CETP inhibitors, torcetrapip and delcetrapib, were disappointing due to off target effects (Sando and Knight 2015; Ladeiras-Lopes *et al.* 2015). Ongoing clinical trials are evaluating the efficacy of two more potent CETP inhibitors, anacetrapib and evacetrapib (Sando and Knight 2015; Ladeiras-Lopes *et al.* 2015). Other approaches such as inducing the levels of ApoA1, the major apolipoprotein in HDL, HDL mimetics and microRNAs that modulate plasma HDL levels together with agents that augment the levels/activities of proteins involved in cellular cholesterol efflux are being evaluated (Sando and Knight 2015; Ladeiras-Lopes *et al.* 2015).

As atherosclerosis is an inflammatory disorder, approaches that dampen inflammation are also being pursued. Members of the phospholipase A2 (PLA2) are involved in producing atherogenic lipids via their actions on lipoprotein remodelling (Ladeiras-Lopes *et al.* 2015). Inhibitors of these enzymes therefore represent a promising avenue for limiting atherosclerosis. However, clinical trials with an inhibitor of a lipoprotein-associated PLA2 (Derapladib) and secretory PLA2 (Verespladib) have been disappointing (Ladeiras-Lopes *et al.* 2015). Based on the beneficial effects of low dose methotrexate in other inflammatory disorders such as arthritis, CIRT (Cardiovascular Inflammation Reduction Trial) is evaluating

its potential in limiting cardiovascular events in patients with prior MI and either type II diabetes or metabolic syndrome (Ridker 2013). The outcomes of the CIRT trial will clearly open up further avenues for limiting inflammation and the actions of other cytokines. Another approach is focussed on IL-1β given that it is a major cytokine involved in the disease and produced by the inflammasome that is activated by modified LDL and cholesterol crystals (Ramji and Davies 2015). The CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcomes Study) trial evaluated the potential of an IL-1β neutralising antibody in reducing cardiovascular events in patients with prior MI (Ridker 2016). Despite no changes in lipid levels or the rate of all-cause mortality, treatment with 150 mg of canakinumab every 3 months was found to reduce an individual's risk of a recurrent CVD-related event when compared to those receiving the placebo (Ridker *et al.* 2017a). It should however be noted that because of the risks associated with manipulating systemic inflammation, such as predisposition to infections, these approaches will have to be restricted to high risk patients until effective technologies to target them to the atherosclerotic lesions are developed. The use of nanoparticles and other drug delivery systems have been explored as possible options to directly target cytokines and their receptors within atherosclerotic plaques (Chacko *et al.* 2011; Jayagopal *et al.* 2010; Lewis *et al.* 2011; Chung 2016). Although these drug delivery systems have been used to deliver antibodies to atherosclerotic lesions to enhance imaging techniques, their use in therapeutic drug delivery remains largely unexplored (Chacko *et al.* 2011; Jayagopal *et al.* 2010; Lewis *et al.* 2011). A recent study has shown that nanoparticle-directed drug was capable of improving plaque stability in LDLr deficient mice in comparison to the control mice (Fredman *et al.* 2015). However until anti-cytokine and anti-chemokine antibodies and molecules can be targeted within atherosclerotic plaques, it is important that further research is carried out on alternative approaches that limit inflammation and other pro-atherogenic changes in the disease.

One potential avenue being explored for novel therapeutics for the prevention of atherosclerosis are natural products, known as nutraceuticals, which are thought to have anti-inflammatory properties. Unless there is genetic evidence, it is often difficult to predict the safety profiles of pharmaceuticals as patients receive them for several years, whereas nutraceuticals are natural products which are generally considered safe. Therefore nutraceuticals may represent effective treatments to provide to patients before traditional pharmaceuticals. Nutraceuticals can be broken down into two broad classes, functional foods or dietary supplements. There are several nutraceuticals being explored as potential anti-atherogenic therapies which are comprehensively reviewed in *Moss and Ramji* (2016b), however a summary of the preclinical and human studies are presented in Tables 1.2 and 1.3. Diets which are rich in fruit, vegetables, fish, cereal grains or olive oil have all been associated with cardiovascular health benefits (Slavin and Lloyd 2012; Wall *et al.* 2010; Granados-Principal *et al.* 2010; Moss and Ramji 2016b). The aim of this study is to assess the key nutraceutical components ω-3 PUFAs, flavanols and phytosterols in order to determine their

potential uses for the prevention of atherosclerosis development. Therefore these nutraceuticals are discussed in greater detail in Sections 1.6 – 1.8.

Table 1.2 The potential anti-atherogenic effects of key nutraceuticals from pre-clinical studies.

Nutraceutical	Cardiovascular health benefits	References
Allicin	- Murine macrophages stimulated with LPS have reduced expression of pro-inflammatory cytokines (*IL-1β*, *IL-6* and *TNF-α*).	Lee *et al.* 2012
	- Reduced leukocyte adherence and inflammatory response in murine models.	Zanardo 2006
	- Reduced foam cell formation due to attenuated expression of *SR-A*, *ACAT-1* and *CD36* in human monocyte-derived macrophages.	Zhao *et al.* 2011
	- Dietary supplementation retards atherosclerosis development in *ApoE* deficient mice by reducing lesion size.	Zhang *et al.* 2012
Berberine	- Reduces *MCP-1*, *iNOS*, *IL-1β* and *IL-6* gene expression in mouse macrophages.	Jeong *et al.* 2009
	- Attenuates monocyte recruitment.	Cheng *et al.* 2015
	- Attenuates intracellular accumulation of oxLDL in human macrophages by increasing *ABCA1* gene expression.	Lee *et al.* 2010a
	- Lowers total cholesterol serum levels and the number of atherosclerotic lesions in *ApoE* deficient mice.	Hu and Davies 2010; Wang *et al.* 2011a
Butyrate	- Reduces pro-inflammatory cytokine and nitric oxide production in murine macrophages.	Liu *et al.* 2012a
	- Decreases monocyte and macrophage migration in *ApoE* deficient mice resulting in reduced plaque size.	Aguilar *et al.* 2014
Carnosine	- Protects against foam cell formation *in vitro.*	Rasid *et al.* 2007
	- Improves atherosclerotic plaque stability in murine diabetes-associated atherosclerosis models.	Brown *et al.* 2014
Coenzyme Q10	- Induces macrophage reverse cholesterol transport and slow the development of atherosclerosis possibly via miR-378 in *ApoE* deficient mice.	Wang *et al.* 2014a
Curcumin	- Reduces pro-inflammatory cytokine (MCP-1, IL-1β and TNF-α) production in primary human monocytes.	Abe *et al.* 1999
	- Induces an anti-inflammatory M2 macrophage phenotype *in vitro.*	Gao *et al.* 2015
	- Decreases oxidative stresses and LDL oxidation in addition to reducing aortic fatty streak development in rabbits.	Ramírez-Tortosa *et al.* 1999; Quiles *et al.* 2002
	- *ApoE* and *LDLr* double knockout mice had reduced atherosclerotic plaque development.	Olszanecki *et al.* 2005
Flavanols	- Decreases atherosclerotic plaque size in ApoE*3-Leiden mice.	Morrison *et al.* 2014
	- Reduces endothelial exocytosis (the process of releasing pro-inflammatory cytokines and chemokines into the extracellular space) in HUVECs	Yamakuchi *et al.* 2008

Hydroxytyrosol	- The expression of the pro-inflammatory adhesion proteins *VCAM-1* and *ICAM-1* is attenuated in HUVECs.	Dell'Agli *et al.* 2006
	- Increased plasma HDL levels and lower LDL plasma levels in Wistar rats compared to control rats.	Mangas-Cruz *et al.* 2001; Gorinstein *et al.* 2002
	- Attenuates atherosclerotic plaque formation in hyperlipemic mice.	González-Santiago *et al.* 2006
ω-3 PUFAs	- The expression of several key atherosclerotic markers in both human and murine macrophages is reduced.	Hughes *et al.* 1996; Miles *et al.* 2000
	- Induces the expression of "cholesterol efflux" genes as well as decreasing the expression of "LDL uptake" genes.	Song *et al.* 2013
	- Raises plasma HDL levels and attenuates atherosclerotic lesion formation in LDLr deficient mice.	Brown *et al.* 2012; Nakajima *et al.* 2011
ω-6 PUFAs	- PGE1, a metabolite of DGLA, is capable of improving plaque stability in atherosclerosis in rabbits by increasing the thickness of the fibrous cap.	Bai *et al.* 2012
	- Decreased blood pressure in hypertensive rats.	Engler 1993
	- Attenuates atherosclerotic plaque formation in mice.	Takai *et al.* 2009
Phytosterols	- Enhanced cholesterol efflux in human THP-1 macrophages.	Sabeva *et al.* 2011
	- Reduced atherosclerotic plaque formation and plasma LDL levels in *ApoE* deficient mice.	Moghadasian 2006; Nashed *et al.* 2005
Resveratrol	- Attenuates oxidation of LDL *in vitro.*	Frankel *et al.* 1993
	- Enhanced cholesterol efflux in human THP-1 macrophages resulting in reduced foam cell formation.	Voloshyna *et al.* 2013
	- Reduced atherosclerotic lesion formation by approximately 50% in ApoE*3-Lieiden.CEPT mice.	Berbée *et al.* 2013
Vitamin C	- Improved eNOS activity and restored endothelium function in *ApoE* deficient mice.	d'Uscio 2002; Matsumoto *et al.* 2003

ACAT-1, acyl-CoA acetyltransferase-1; ApoE, apolipoprotein E; CHD, coronary heart disease; CVD, cardiovascular disease; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; eNOS, endothelial nitric oxide synthase; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; H_2S, hydrogen sulphide; HDL, high density lipoprotein; HUVECs, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; LDL, low density lipoprotein; LDLr, LDL receptor; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; miR, micro RNA; MMP, matrix metalloproteinases; NO, nitric oxide; oxLDL, oxidised LDL; PGE1, prostaglandin E1; PUFAs, polyunsaturated fatty acids; SR-A, scavenger receptor-A; TNF-α, tumour necrosis factor α; VCAM-1, vascular cellular adhesion molecule-1.

Table 1.3. Summary of the major findings on nutraceuticals from human studies.

Nutraceutical	Number of participants	Study type	Major Findings	References
Fibre	116	CL	No additional benefit when combined with other lipid lowering therapies.	Ramos *et al.* 2011
	46,032	EP	Increased fibre intake associated with reduced peripheral arterial disease risk.	Merchant *et al.* 2003
	78,779	EP	Increased fibre intake reduced the risk of stroke in women.	Oh *et al.* 2005a
	39,876	EP	Increased fibre consumption was correlated with reduced CVD risk.	Liu *et al.* 2002
Flavanols	90	CL	Reduced blood pressure and lipid peroxidation.	Mastroiacovo *et al.* 2015
	100	CL	Increased FMD and HDL plasma levels. Decreased blood pressure, arterial stiffness, total and LDL cholesterol levels.	Sansone *et al.* 2015
	7,172	EP	Increased flavanol intake correlated with reduced CVD risk.	Tresserra-Rimbau *et al.* 2014a
	57	CL	Improved FMD and reduced blood pressure.	Rassaf *et al.* 2016
ω-3 PUFAs	11,324	CL	15% reduction in total number of deaths and non-fatal CVD-related events. 45% reduction in sudden cardiac deaths.	Anon. 1999
	18,645	CL	19% reduction in major CVD-related events. Most effective as a secondary preventative.	Yokoyama *et al.* 2007
	600	CL	Lowered TG, VLDL and oxLDL levels. No additional benefit to those already taking lipid lowering therapies.	Franzese *et al.* 2015
	3,851	CL	No cardiovascular protective effects observed after 1 year follow-up.	Rauch *et al.* 2010
	12,536	CL	No cardiovascular protective effects observed after 6 years follow-up.	The ORIGIN Trial Investigators 2012
	229	CL	Lowered TG levels.	Bays *et al.* 2011
	702	CL	Lowered TG, non-HDL, LDL and total cholesterol levels in statin receiving patients.	Ballantyne *et al.* 2012

ω-6 PUFAs	258	CL	Optimisation of Omega 6:3 ratio to 3:1 reduces triglyceride and LDL levels.	Griffin *et al.* 2006
	401	CL	No cardiovascular protective effects observed.	Sluijs *et al.* 2010
	340,000	EP	Replacement of saturated fats with PUFA reduces CVD risk.	Katan 2009
	78,778	EP	Inverse relationship between PUFA and CVD in women with BMI over 25.	Oh *et al.* 2005b
	2,682	EP	Linoleic acid is cardio protective and reduces overall mortality.	Laaksonen 2005
	2,206	EP	Serum CRP levels correlate with increased omega-6 serum levels present.	Tomiyama *et al.* 2011
Polyphenols	200	CL	Correlation between polyphenol intake and HDL plasma levels Reduced TG and oxidative stress levels.	Covas *et al.* 2006
	134	CL	Reduced blood pressure.	Tjelle *et al.* 2015
	101	CL	Reduced blood pressure and improved lipid profile in haemodialysis patients.	Shema-Didi *et al.* 2014
	117	CL	Improved inflammatory and oxidative condition of metabolic syndrome patients.	Panahi *et al.* 2015
	70	CL	No change in blood pressure.	Ras *et al.* 2013
	78	CL	No change in blood pressure.	Bondia-Pons *et al.* 2014
	7,447	CH	Increased polyphenol intake resulted in a reduced mortality risk.	Tresserra-Rimbau *et al.* 2014b
	200	CH	Polyphenol intake reduced blood pressure and increased NO plasma levels.	Medina-Remón *et al.* 2015
	573	CH	Reduced blood pressure in elderly individuals. Decreased TG levels but no changes in total, HDL or LDL levels.	Guo *et al.* 2016
	1,139	CH	Reduced plasma levels of several inflammatory biomarkers.	Medina-Remón *et al.* 2017
Vitamins	14,641	CL	No positive effects of Vitamins C or E on any major CVD outcomes. Vitamin E increased risk of stroke.	Sesso 2008
	1,302	CL	Vitamin C and E increased number of plaques and decreased pulse wave velocity.	Zureik 2004
	353	CL	No cardiovascular protective effects observed following vitamin E supplementation.	Hodis 2002

520	CL	Vitamin C and E reduced carotid artery atherosclerosis in smoking men.	Salonen *et al.* 2000
39,876	CL	24% reduction in CVD mortality in women receiving vitamin E.	Lee *et al.* 2005
840	EP	High serum carotenes is protective in early atherosclerosis.	Karppi *et al.* 2011

CH, cohort study; CL, clinical trial; EP, epidemiological study.

1.6 Omega-3 polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFAs) are fatty acids which contain at least two carbon-carbon double bonds. PUFAs can be classified by the position of the nearest carbon-carbon double bond to the methyl terminus of the fatty acid (Wall *et al.* 2010). During normal physiological conditions, PUFAs play important roles in influencing both blood clotting and blood pressure (Wall *et al.* 2010). More importantly PUFAs are also capable of manipulating the production of key regulators of inflammation, known as eicosanoids and resolvins, which play key roles in enhancing or attenuating the inflammatory response respectively (Fig. 1.4; Wall *et al.* 2010; Fredman and Tabas 2017). It is essential that diets are rich in both ω-3 and ω-6 PUFAs as they cannot be synthesised *in vivo* and therefore must be acquired from exogenous sources. Nuts, flax seeds and especially oily fish are key sources of ω-3 PUFAs, whereas animal fats and vegetable oils are able to provide ω-6 PUFAs (Wall *et al.* 2010).

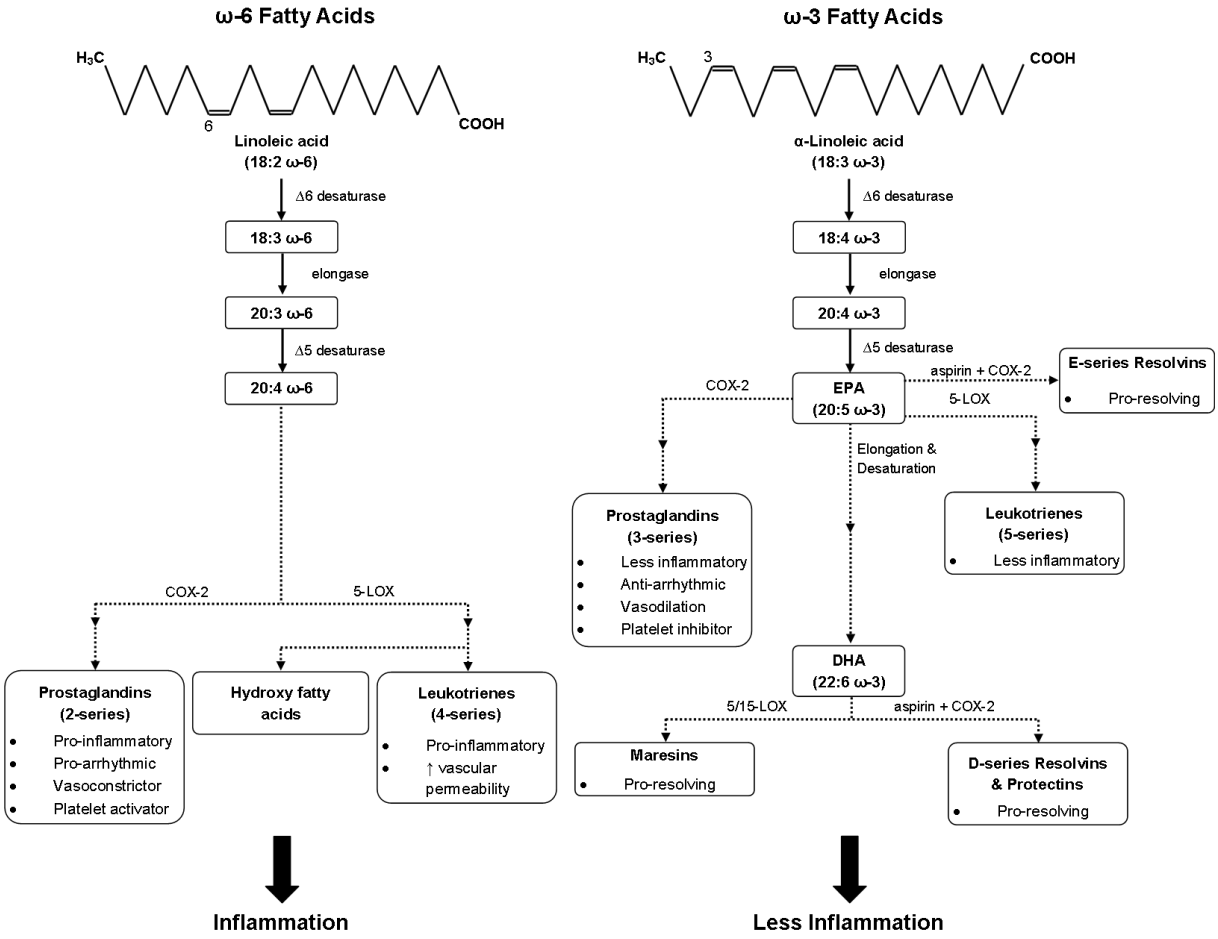

Fig. 1.4. The structure and metabolism of ω-6 and ω-3 PUFAs. The metabolism of ω-6 PUFAs leads to the formation of a variety of compounds which can result in an increased inflammatory state, whereas the metabolism of ω-3 PUFAs results in compounds which can improve inflammation resolution. See text for more details. COX, cyclooxygenase; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LOX, lipoxygenase. Figure adapted from *Adkins and Kelley* (2010).

The cardiovascular protective effects of ω-3 PUFAs have been known for the last 60 years. The Inuit population of Greenland were found to have a lower incidence rate of CVD and other CVD-related events despite having a diet consisting mainly of fish and whale meat and lacking fruit and vegetables, meaning their diets were rich in saturated fats (Bang and Dyerberg 1980). Since this initial epidemiological observation, the findings that ω-3 PUFAs may exert cardiovascular health benefits has been repeated in numerous epidemiological and clinical trials (Table 1.3; Lee *et al.* 2008; Lavie *et al.* 2009; Moss and Ramji 2016b). Both eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are considered the two most potent ω-3 PUFAs and exert the greatest cardiovascular protective effects (Moss and Ramji 2016b).

Although both ω-3 and ω-6 PUFAs are essential within the diet, they must be consumed in the correct proportion. A ratio of 1:4 for ω-3:ω-6 is considered to be ideal, however since the introduction of vegetable oils (which are rich in ω-6 PUFAs) in Western countries for the use

in cooking, it is thought the current ratio is closer to approximately 1:15 (Simopoulos 2003). Over the last few decades in Europe the incidence of inflammatory based diseases has increased significantly, correlating with the increased dietary intake of ω-6 PUFAs which has risen by approximately 50% (Sanders 2000). Diets which are low in ω-3 but rich in ω-6 PUFAs have been associated with increased expression of pro-inflammatory eicosanoids (James *et al.* 2000). Furthermore an increased sensitivity of LDL to oxidation into oxLDL *ex vivo* has been linked to diets with high levels of ω-6 PUFA intake (Tsimikas *et al.* 1999). Despite the association of ω-6 PUFAs with enhancing the pro-inflammatory nature of atherosclerosis disease progression, some ω-6 PUFAs, such as dihomo-γ-linolenic acid (DGLA), have been shown to exert anti-inflammatory effects and provide cardiovascular protective effects. However the anti-inflammatory effects of DGLA do not fall within the scope of this project and further discussion of its potential health benefits can be found in *Moss and Ramji* (2016b).

The ability of ω-3 PUFAs to attenuate atherosclerosis disease development has been demonstrated in several *in vitro* and *in vivo* studies performed in both murine and human participants. The expression of pro-atherogenic markers, including *ICAM-1* and *SR-A*, in both murine and human macrophages can be significantly reduced following treatment with fish oil (Hughes *et al.* 1996; Miles *et al.* 2000). The use of human THP-1 macrophages *in vitro* has shown that ω-3 PUFA treatment is capable of attenuating cholesterol uptake while simultaneously enhancing cholesterol efflux, resulting in reduced intracellular cholesterol accumulation (McLaren *et al.* 2011b; Lada *et al.* 2003). These changes in cholesterol accumulation can be explained by the upregulation in the expression of genes involved in cholesterol efflux and a reduction in the expression of “LDL uptake” genes (Song *et al.* 2013). The recruitment of monocytes to the activated ECs and their subsequent migration into the intima of the artery to the site of modified LDL build up is one the key processes in atherosclerosis disease progression. Studies performed *in vitro* and *in vivo* have shown that treatment with ω-3 PUFA results in reduced monocyte migration (Brown *et al.* 2012), which highlights the possible use of ω-3 PUFAs to attenuate one of the key initial stages of plaque formation.

The use of mouse model systems have greatly enhanced our knowledge of the effects of ω-3 PUFA dietary supplementation on atherosclerosis disease progression. The use of fish oil dietary supplementation in *LDLr* deficient mice while they received a high fat diet for 16 weeks resulted in a decrease of approximately 50% in monocyte migration to the atherosclerotic lesion (Brown *et al.* 2012). In contrast to the control mice, those receiving ω-3 PUFAs developed smaller atherosclerotic plaques and had significantly reduced plasma cholesterol levels (Brown *et al.* 2012). Conversely when the study was repeated using *ApoE* deficient mice no significant differences in monocyte migration or lesion size was observed between the control group and those receiving fish oil dietary supplementation (Brown *et al.* 2012). However

it should be noted that *ApoE* deficient mice are a more aggressive model of atherosclerosis disease progression and therefore a shorter time point was possibly required in order to study any possible differences (Getz and Reardon 2016). Encouraging results have also been seen in a plaque regression study. After 8 weeks of feeding *LDLr* deficient mice a high fat diet they were switched to a standard chow diet in the presence or absence of 5% EPA for 4 weeks (Nakajima *et al.* 2011). Compared to the control mice, those that received the EPA supplementation had increased serum HDL levels and their plaques were approximately 20.9% smaller (Nakajima *et al.* 2011). The study also found that the EPA receiving mice had attenuated expression of pro-inflammatory genes such as *ICAM-1*, *IL-10*, *IFN-γ* and *TNF-α* within the atherosclerotic plaques (Nakajima *et al.* 2011), indicating that EPA dietary supplementation may exert strong anti-atherogenic effects.

The mechanisms behind the anti-inflammatory effects of ω-3 PUFA rich diets are slowly being uncovered (Calder 2015). Both EPA and DHA can be metabolised via COX, lipoxygenase (LOX) and CYTP450 pathways to form anti-inflammatory molecules capable of attenuating inflammation known as resolvins (Calder 2015; Fredman and Tabas 2017). Both human and murine studies have shown levels of resolvins in the blood to increase following diets rich in ω-3 PUFAs (Hong *et al.* 2003; Mas *et al.* 2012). Another anti-inflammatory effect of ω-3 PUFA dietary supplementation is the reduced production of pro-inflammatory cytokines and adhesion molecules (Nakajima *et al.* 2011; Calder 2015). ω-3 PUFAs are thought to achieve these effects by attenuating the expression of the pro-inflammatory transcription factor *NF-κB* and upregulating the action of the anti-inflammatory transcription factor peroxisome proliferator-activated receptor (*PPAR*)-*γ* (Calder 2013; Calder 2015). These transcription factors influence the expression of several genes involved in resolvin formation as well as cytokine and cell adhesion molecules (Calder 2015). Upon further investigation it was found that the G-protein coupled receptor (GPR)120 is also capable of binding to PUFAs in addition to attenuating the expression of *NF-κB* (Oh *et al.* 2010; Calder 2015). Indeed GPR120 agonists have been shown to induce similar anti-inflammatory effects that are normally associated with ω-3 PUFA dietary supplementation (Oh *et al.* 2014). Therefore GPR120 appears to be a key receptor involved in the anti-inflammatory properties of ω-3 PUFA dietary supplementation.

As previously mentioned achieving a better ω-3:ω-6 ratio is considered key for reducing the speed of atherosclerosis development. ApoE mice which also expressed the *Fat-1* gene from *Caenorhabditis elegans* have been used to demonstrate the importance of the ω-3:ω-6 ratio (Wan *et al.* 2010). In theory these mice should achieve a tissue ω-3:ω-6 ratio of approximately 1:1 due to the ability of Fat-1 to metabolise ω-6 into ω-3 PUFAs by an ω-3 fatty acid desaturase (FAD; Wan *et al.* 2010). The *ApoE* deficient/*Fat-1* mice developed smaller atherosclerotic plaques in addition to attenuated expression of *IFN-γ* and *MCP-1* in comparison to *ApoE* deficient only mice following 14 weeks of high fat diet feeding (Wan *et al.* 2010). Despite these

changes neither serum LDL, HDL or total cholesterol levels were altered. This study reinforces the idea of using EPA and DHA dietary supplements in order to achieve a ω-3:ω-6 ratio which is closer to the ideal 1:4 to attenuate atherosclerosis disease progression and reduce an individual's risk of suffering a CVD-related event.

Relevant clinical data in humans

During the last 30 years, there have been three large robust trials which used either EPA, DHA or both dietary supplementation in high risk patients. These trials are known as the DART (Diet and Reinfarction Trial), GISSI (Gruppo Italiano per lo Studio della Sopravvivenza nell' Infarto Miocardico)-Prevenzione and the JELIS (Japan EPA Lipid Intervention Study) trials (Burr *et al.* 1989; Anon. 1999; Yokoyama *et al.* 2007). Approximately 2,000 men who had recently suffered a MI were involved in the DART trial (Burr *et al.* 1989). The participants were then randomised to receive three different diets for 2 years and compared to a control group. The diets being assessed were either increased cereal fibre intake; improved ratio of polyunsaturated to saturated fat by reducing the amount of dietary fat; or using oily fish or fish capsules to increase ω-3 PUFAs intake (Burr *et al.* 1989). The participants receiving the enhanced ω-3 PUFAs intake diet were found to have a 29% reduction in all-cause mortality, almost completely due to a substantial decrease in the number of CVD-related events (Burr *et al.* 1989). However no significant reduction in the mortality rates was observed in the participants receiving the other diets (Burr *et al.* 1989). This study highlights the possible benefits of using ω-3 PUFAs as secondary preventatives to reduce an individual's risk of suffering a subsequent CVD-related event following their initial MI.

During the GISSI-Prevenzione trial, 11,323 patients were recruited and all participants had recently suffered a MI (Anon. 1999). The participants randomly received either ω-3 PUFA dietary supplementation or continued to only receive traditional care for CVD (Anon. 1999). After a 6 month follow up, there were no significant changes in total, LDL or HDL cholesterol levels in the patient's serum (Anon. 1999). Despite these disappointing results at the 6 month time point, at the 1 year follow up participants on the ω-3 PUFA supplemented diet showed a 45% reduction in sudden cardiac death when compared to the control group (Anon. 1999). This reduction in cardiac death contributed to the observed 15% decrease in all-cause mortality and non-fatal CVD-related events in the ω-3 PUFA receiving group (Anon. 1999).

The JELIS trial involved 18,645 hypercholesterolaemic individuals who were randomly assigned to receive either statin only therapy or a combination of statin and EPA supplementation (Yokoyama *et al.* 2007). During the average follow up time point of 4.6 years, no changes in serum HDL or LDL levels were observed in those receiving the additional EPA supplementation compared to those receiving statins only. However the extra dietary intake of EPA was associated with a 19% relative reduction in major CVD-related events (Yokoyama *et*

al. 2007). Further evidence of the cardiovascular protective effects of ω-3 PUFA dietary supplementation has been generated in a recent study involving 95 patients who had been treated with a high dose of statins for at least 6 months before also being given an EPA supplement for 6 months (Niki *et al.* 2016). A reduction in the levels of pro-inflammatory cytokines, such as MCP-1, was found in the participants receiving EPA compared to those on the statin only therapy (Niki *et al.* 2016). Furthermore EPA supplementation appeared to improve plaque stability by reducing the amount of lipid build up within the lesion and enhancing collagen deposition in the fibrous cap, indicating a reduction in the risk of a plaque rupturing and therefore reducing the chance of a CVD-related event (Niki *et al.* 2016). The evidence observed in these trials indicates that increasing an individual's dietary intake of ω-3 PUFAs, in particular EPA and DHA, either alone or as a combination with statins can provide cardiovascular protective effects which may reduce an individual's risk of a major CVD-related event.

A reduction in the markers of atherothrombotic risk was observed in 600 Caucasian men with CVD following dietary fish oil supplementation (Franzese *et al.* 2015). In a study involving 29 patients who received daily ω-3 PUFA supplementation for 12 weeks, there was a significant improvement in endothelial function and arterial stiffness as assessed by flow-mediated dilation (FMD) and pulse wave velocity (PWV; Tousoulis *et al.* 2014). This observation was confirmed by a later epidemiological study involving 160 Japanese patients which found a correlation between low serum levels of DHA and attenuated endothelium function when measured by FMD (Yagi *et al.* 2015).

Even though many studies provide evidence for the cardiovascular protective effects of increased ω-3 PUFA dietary intake, their effectiveness is still debated in the literature with several meta-analysis studies failing to find an association between ω-3 PUFA intake and a reduced risk of CVD-related events. One meta-analysis involving a total of 36,913 individuals across 48 randomised studies failed to find any significant reduction in CVD-related mortality following 6 months of ω-3 PUFA supplementation (Hooper *et al.* 2006). Another study investigating 14 randomised double blind trials and 20,485 total participants with a history of CVD, found no correlation between ω-3 PUFA supplementation and any cardiovascular protective effects (Kwak *et al.* 2012). These findings have also been shown in another study involving 68,680 patients in 20 randomised clinical trials, in which ω-3 PUFA supplementation did not alter patient's risk of either all-cause or CVD-related mortality (Rizos *et al.* 2012). ω-3 PUFA supplementation also failed to reduce the number of CVD-related events in individuals with peripheral arterial disease (Enns *et al.* 2014). Furthermore a recent clinical trial involving 74 patients with atherosclerotic vascular disease and type 2 diabetes failed to find any changes in the inflammatory state of participants following daily treatment of EPA and DHA for 3 months (Poreba *et al.* 2017).

Recent trials did not observe any additional cardiovascular protective effects from taking ω-3 PUFAs in combination with traditional pharmaceuticals. The MAGMA (Magnetic Resonance Imaging Evaluation of Mineralocorticoid Receptor Antagonism in Diabetic Atherosclerosis) trial failed to find any further reductions in the levels of TG, VLDL and oxLDL following fish oil supplementation when patients were already receiving traditional therapy (Franzese *et al.* 2015). However fish oil supplementation was shown to be effective at lowering these factors within the general population. A total of 3,851 and 12,536 high risk patients were recruited for the OMEGA and ORIGIN (Outcome Reduction with an Initial Glargine Intervention) trials respectfully (Rauch *et al.* 2010; The ORIGIN Trial Investigators 2012). Neither of these studies found any benefits from ω-3 PUFA dietary supplementation, however these trials used relatively low doses of ω-3 PUFAs and conducted the trial in patients with typical TG serum levels (a risk factor for CVD; Bhatt *et al.* 2017; Nordestgaard 2016).

To assess the effect of ω-3 PUFA dietary supplementation in patients with raised TG levels, two studies known as the MARINE (Multi-centre, Placebo-controlled, Randomized, Double-blind, 12-week study with an open-label Extension) and ANCHOR trials were conducted (Bays *et al.* 2011; Ballantyne *et al.* 2012). These preliminary studies observed a reduction in serum TG levels as well as a decrease in a variety of inflammatory markers without increasing LDL levels following EPA ethyl ester treatment (Bays *et al.* 2013). These preliminary trials formed the basis of the large international trial known as REDUCE-IT (Reduction of Cardiovascular Events with EPA – Intervention Trial; Bhatt *et al.* 2017; U.S. National Institutes of Health 2017a). The REDUCE-IT trial is expected to involve 8,000 participants and investigate the effect of Vascepa® (icosapent ethyl) in combination with statins on the incidence rate of CVD-related events (U.S. National Institutes of Health 2017a). Vascepa® is a purified ethyl ester of EPA and has been designed to treat hyperglyceridaemia. Another large international study known as the STRENGTH (Statin Residual Risk Reduction With EpaNova in HIGh CV Risk PatienTs With Hypertriglyceridemia) trial involves 13,000 individuals and is establishing whether a combined treatment of statins and Epanova® (ω-3 carboxylic acids) can reduce the number of CVD-related events compared to those receiving statins only (U.S. National Institutes of Health 2017c). It is hoped these studies will provide greater evidence on whether additional ω-3 PUFA supplementation taken in combination with statins is capable of exerting enhanced cardiovascular protective effects.

When drawing conclusions from the results of clinical trials, caution must be taken due to the heterogeneity in the experimental design of the studies (Enns *et al.* 2014). Trials often differ in how the ω-3 PUFA supplementation is provided and whether it is given alone or in combination with statins and therefore the combined therapy may exert synergistic effects. Furthermore trials can differ in their dose and length of ω-3 PUFA dietary intervention. The biggest difference within the trials are the populations which are selected. For example, dietary intake

of ω-3 PUFAs is estimated to be roughly 15 times higher in the Japanese population when compared to their Western counterparts (Iso 2006; Kobayashi *et al.* 2003). Therefore this may alter the outcomes of these trials as the Western population is more likely to see health benefits of extra ω-3 PUFA dietary supplementation in contrast to the Japanese population. These key differences in the designs of the studies may result in the different outcomes observed within the clinical trials and meta-analysis.

1.7 Flavanols

The cardiovascular protective effects of diets with high intakes of fruit and vegetables has long been known (Slavin and Lloyd 2012; Moss and Ramji 2016b). Flavonoids are a class of plant secondary metabolites which are commonly found in fruit and vegetables. Flavanols, a subclass of flavonoids, have the possibility to be used as a nutraceutical therapy for atherosclerosis and therefore their potential must be explored in greater detail (Falcone Ferreyra *et al.* 2012). Catechin (Fig. 1.5) is a common flavanol found within green tea and cocoa and has been shown to reduce endothelial exocytosis in human umbilical vein endothelial cells (HUVECs; Yamakuchi *et al.* 2008). Activated ECs release stored pro-inflammatory cytokines and chemokines from intracellular endothelial granules into the extracellular space via a process called endothelial exocytosis. Therefore catechin and its isomers may be capable of attenuating vascular inflammation during the initial atherosclerotic lesion formation, highlighting the need to develop a greater insight into the effects and potential of this compound.

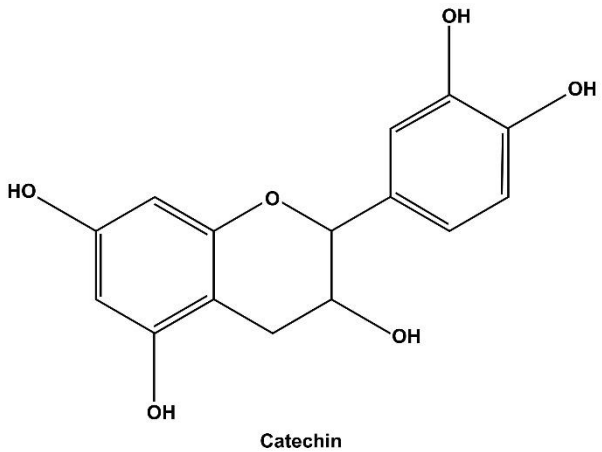

Fig. 1.5. The chemical structure of catechin. Catechin is a major flavanol found within green tea and cocoa and shows promising cardiovascular protective effects.

The size of severe lesions in ApoE*3-Leiden mice were found to be significantly smaller following 20 weeks of a high fat diet supplemented with 0.1% epicatechin (Morrison *et al.* 2014). The mice receiving the epicatechin supplement were found to have altered expression of 173 genes, including 77 that had their expression reversed when compared to the mice

receiving the high fat diet only. Furthermore a large number of the 173 genes played a role in cell recruitment (Morrison *et al.* 2014), indicating that epicatechin may reduce lesion size by attenuating one of the key steps during early atherosclerosis lesion formation.

Relevant clinical data in humans

NO production was increased in 27 healthy individuals who received a flavanols-rich cocoa (epicatechin and catechin) diet for 5 days (Fisher *et al.* 2003). The increased NO production was also accompanied by an increase in vasodilation, highlighting the possibility of using catechin to reduce an individual's blood pressure which is a risk factor for CVD-related events (Fisher *et al.* 2003). Subsequent studies have also found an improvement in vasodilation following 5 weeks of green tea extract dietary supplementation as well as a reduction in serum oxLDL levels (Babu *et al.* 2008; Tinahones *et al.* 2008). Increased dietary intake of catechin in obese and near-obese children resulted in a significant reduction in the levels of circulating LDL after 24 weeks of daily supplementation (Matsuyama *et al.* 2008). FMD was improved in 57 patients with end stage renal disease, a population at high risk of developing CVD, following 30 days of cocoa flavanol-rich dietary supplementation (Rassaf *et al.* 2016). The vascular function of 100 healthy individuals without any previously reported cases of CVD was also improved after 30 days of cocoa flavanol consumption (Sansone *et al.* 2015). The use of cocoa flavanol dietary supplementation for 14 days in 22 young and 20 elderly patients resulted in decreased arterial stiffness and increased FMD in both participant groups (Heiss *et al.* 2015). This study highlights the use of flavanols in order to reduce blood pressure in the elderly, a high risk population for suffering a CVD-related event. These trials indicate that the use of flavanols may not only be used as a preventative in healthy individuals but it may also provide cardiovascular protective effects in high risk patients.

The levels of several pro-inflammatory mediators including soluble TNF-receptor 2, IL-6 receptor, IL-8, Fas ligand and neutrophil-activating peptide (NAP)-2 were reduced following catechin treatment, highlighting its anti-inflammatory properties (Hsu *et al.* 2007). A small trial involving 17 healthy men receiving a daily catechin treatment for 3 weeks observed a significant reduction in the total:HDL cholesterol ratio compared to the control group, however other cardiovascular risk factors, including blood pressure were unaffected (Frank *et al.* 2009). A later study also failed to find a significant improvement in blood pressure or FMD after 4 weeks of daily cocoa flavanol supplementation in 30 overweight adults, however there was a significant reduction in arterial stiffness within the women subgroup (West *et al.* 2014). The lack of reduction in blood pressure in these studies contradicts the decreases found in the previously mentioned studies (Fisher *et al.* 2003; Babu *et al.* 2008; Tinahones *et al.* 2008). These differences in outcomes may be partly due to the limited number of trial participants and therefore larger and more robust clinical trials are required to provide stronger conclusions about the benefits of catechin supplementation (Moss and Ramji 2016b).

The three largest studies investigating the potential cardiovascular health benefits of flavanol dietary supplementation are known as CoCoA (Cocoa, Cognition, and Aging) study, the Flaviola Health study and the PREDIMED (Prevención con Dieta Mediterránea) trial (Mastroiacovo *et al.* 2015; Sansone *et al.* 2015; Tresserra-Rimbau *et al.* 2014a). The CoCoA study observed a significant reduction in the amount of lipid peroxidation, in addition to an improvement in blood pressure and insulin resistance in 90 elderly participants who had received either a high or intermediate dose of daily flavanols for 8 weeks compared to those that only received a low dose (Mastroiacovo *et al.* 2015). These results were also found in the subsequent Flaviola Health study which involved 100 healthy individuals with no prior history of CVD receiving cocoa flavanol dietary supplementation for 1 month (Sansone *et al.* 2015). Those receiving the flavanols showed improvements in FMD and HDL levels as well as a reduction in blood pressure, serum LDL levels and arterial stiffness compared to the control group (Sansone *et al.* 2015). The PREDIMED trial is a large dietary intervention study which ran for 4 years and recruited 7,172 participants (Tresserra-Rimbau *et al.* 2014b). After controlling for other risk factors and nutrients together with lipid lowering pharmaceutical usage, the PREDIMED study found a significant correlation between flavanol intake and an individual's risk of suffering a CVD-related event (Tresserra-Rimbau *et al.* 2014a). Despite these larger studies showing associations between flavanol intake and cardiovascular health benefits such as lowering an individual's blood pressure and circulating LDL levels, two CVD risk factors, the efficacy of flavanol treatment needs to be assessed further in both *in vitro* and *in vivo* studies.

1.8 Phytosterols

Phytosterols are almost identical in structure to cholesterol but are derived from plant sources instead of animal sources (Fig. 1.6). Due to their similar structure, phytosterols are able to compete with cholesterol within the intestine during dietary and biliary cholesterol uptake and therefore reduce an individual's cholesterol level and provide cardiovascular health benefits (Andersson *et al.* 2004). Indeed low serum LDL levels have been associated with diets containing high levels of phytosterols (Katan *et al.* 2003; Andersson *et al.* 2004). An *in vitro* study observed an increase in the expression of the "cholesterol efflux" gene *ABCA1* as well as a decrease in *LDLr* expression in murine macrophages (Sabeva *et al.* 2011). These changes in gene expression correlated with increased ApoA1- and HDL-mediated cholesterol efflux from human THP-1 macrophages following phytosterols treatment (Sabeva *et al.* 2011).

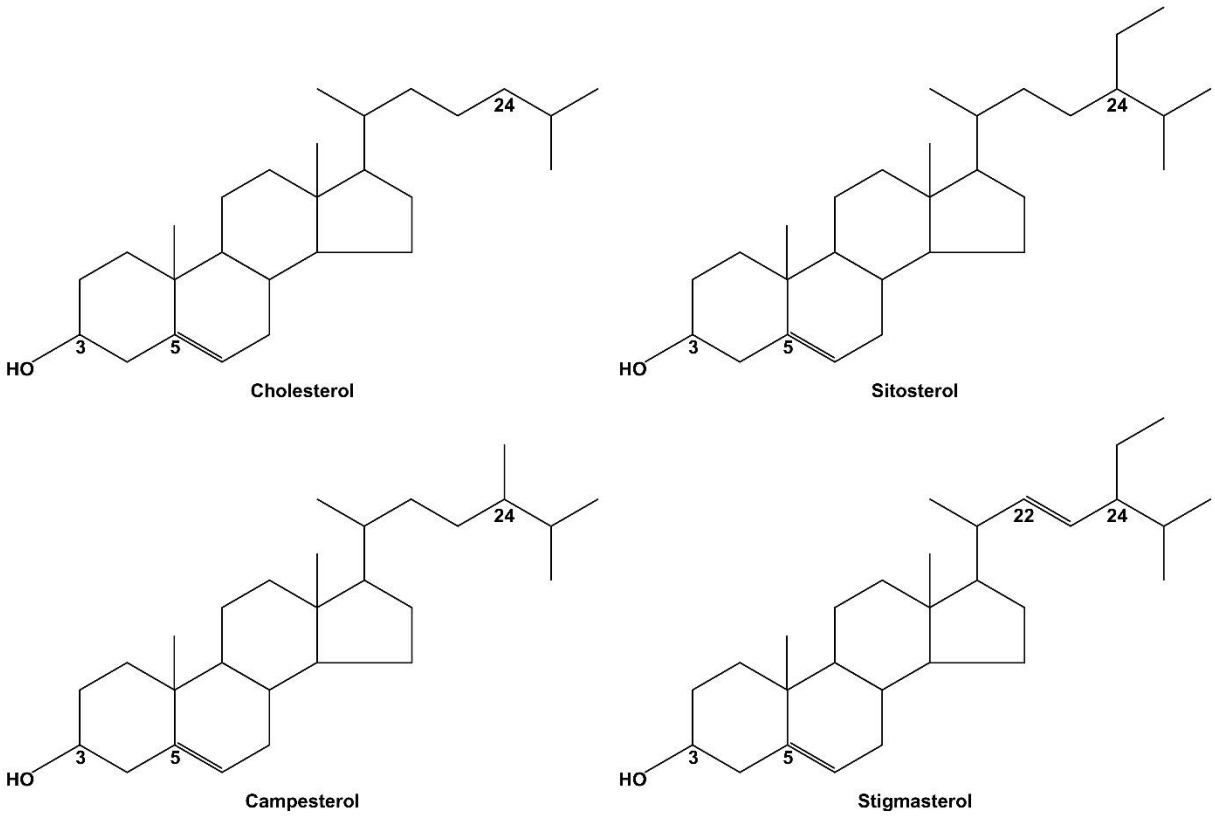

Fig. 1.6. The chemical structure of cholesterol and phytosterols. The structure of cholesterol is almost identical to the phytosterols sitosterol, campesterol and stigmasterol. Due to their similar structure it is hoped that phytosterols can be used to lower an individual's cholesterol level by out competing it in the gut and preventing cholesterol uptake.

The anti-inflammatory potential of phytosterols has also been demonstrated in *in vivo* studies. After 20 weeks of a high fat diet supplemented with a 2% phytosterols mixture, ApoE mice developed atherosclerotic plaques which were approximately 50% smaller than the mice which received a high fat only diet (Moghadasian *et al.* 1999). The same study also observed a decrease in serum LDL levels in addition to a moderate increase in HDL levels in the mice which had received the phytosterols supplement (Moghadasian *et al.* 1999). Later studies also observed smaller lesion formation and reduced serum LDL levels in *ApoE* deficient mice following 12 and 14 weeks of a 2% phytosterol supplemented diet when compared to the control mice (Yeganeh *et al.* 2005; Moghadasian 2006). The levels of pro-inflammatory cytokines, IL-6 and TNF-α, were reduced whereas there was an increase in the anti-inflammatory cytokine IL-10 within the spleen of ApoE mice fed a 2% phytosterol supplemented high fat diet for 2 weeks (Nashed *et al.* 2005). These mice also developed lesions which were approximately 60% smaller in the mice receiving the phytosterol supplemented diet (Nashed *et al.* 2005). A study involving *ApoE* deficient mice found that 132 genes were differentially expressed, including several genes implicated in the control of sterol metabolism such as *FAD1*, *FAD2*, PPAR-γ coactivator 1β (*Ppargc1B*) and hydroxysteroid (17-beta) dehydrogenase 7 (*Hsd17b7*), after receiving a 2% phytosterol supplemented diet when compared to the control group (Xu *et al.* 2008).

Relevant clinical data in humans

A recent clinical trial involving 233 patients receiving phytosterol dietary supplementation for 12 weeks found a decrease in their serum LDL levels compared to the control group, however there were no changes in FMD or PWV (Ras *et al.* 2015). Furthermore low serum LDL levels were associated with higher dietary intake of phytosterols in an epidemiological study involving 22,256 men and women (Andersson *et al.* 2004). Despite the potential cardiovascular protective effects of phytosterols discussed so far, there are still several studies which indicate that high dietary intake of phytosterols may exert adverse effects and further impact on atherosclerosis development (Chan *et al.* 2006). One trial found that an increased serum phytosterol:cholesterol ratio was associated with a greater risk of developing coronary heart disease (CHD) when assessed in 48 postmenopausal women with existing disease (Rajaratnam *et al.* 2000). However several of studies which have concluded that phytosterols actually exert pro-atherogenic effects, and increase an individual's risk of suffering a CVD-related event, often lack appropriate controls or fail to match risk factors between those receiving the phytosterols and the control group. Therefore caution should be taken when interpreting the findings (extensively reviewed by *Chan et al.* (2006)). In one study, serum phytosterol levels were significantly higher in 159 patients who had suffered a MI or sudden cardiac death in contrast to 318 control individuals (Assmann *et al.* 2006). However a major flaw in this study was the failure to account for differences in total cholesterol, TG and LDL levels, as well as blood pressure, all of which are considered risk factors for CVD-related events, between the two sets of participants (Chan *et al.* 2006). All of these risk factors were found to be higher in the CVD-related event group. Additionally the serum phytosterol:cholesterol ratio between the two groups was not significant, consequently this study does not provide any substantial evidence which directly links increased phytosterol levels with increased risk of CVD-related events (Chan *et al.* 2006).

1.9 Project aims

Due to the adoption of a westernised diet in many developing countries around the world and the expected rise in diabetes and obesity worldwide, CVD-related events are predicted to continue their increase in global prevalence. As atherosclerosis, the primary cause of CVD, is a chronic inflammatory disease, novel therapeutics that are able to reduce many of the inflammatory stages involved in disease progression are required. These key inflammatory stages include; the recruitment of immune cells to the affected area, the expression of key pro-inflammatory genes and the formation of foam cells. Therefore novel therapies that are able to attenuate these steps may be helpful in reducing the global incidence of CVD-related events by reducing atherosclerosis development.

The main hypothesis of this study was that CardioWise, as well as its constituent components, are capable of attenuating key steps in atherosclerosis development *in vitro* and *in vivo* by exerting anti-inflammatory effects. Initial experiments were performed using monocytes and macrophages *in vitro* as they are involved in all of the major steps in atherosclerosis progression and therefore any anti-atherogenic effects observed would denote an encouraging therapeutic avenue to pursue. Once a potential anti-atherogenic nutraceutical was identified, it was then used in an initial *in vivo* study to determine its effects in wild type C57/BL6 mice. The major project objectives are outlined below and in Fig. 1.7:

- Research started with CardioWise as it is a unique combination of nutraceuticals produced by Cultech Ltd with no previous studies on atherosclerosis. Its effect on several macrophage processes associated with atherosclerosis development such as *MCP-1* and *ICAM-1* gene expression, cholesterol efflux and M1 macrophage phenotype formation was therefore investigated (Chapter 3).
- Chapter 4 describes the effects of CardioWise dietary supplementation in wild type C57/BL6 mice for 3 weeks while being fed a high fat diet. The potential anti-inflammatory effects of CardioWise were determined by analysing circulating cholesterol and cytokine levels, gene expression studies and investigating the proportion of haematopoietic stem/progenitor cells within the bone marrow.
- Investigation of the efficacy of various ingredients present in CardioWise on a key pro-atherogenic process, monocyte migration, identified a major role of the flavanol (+)-catechin (Chapter 5).
- Chapter 6 describes studies on the anti-inflammatory effects of catechin on several pro-atherogenic processes including gene expression changes, ROS generation and MMP activity.
- The anti-atherogenic effects of catechin dietary supplementation was assessed in wild type C57/BL6 mice fed a high fat diet for 3 weeks (Chapter 7). The cardiovascular protective effects of catechin were determined by monitoring circulating cholesterol and cytokine levels, gene expression analysis and the proportion of haematopoietic stem/progenitor cells within the bone marrow.

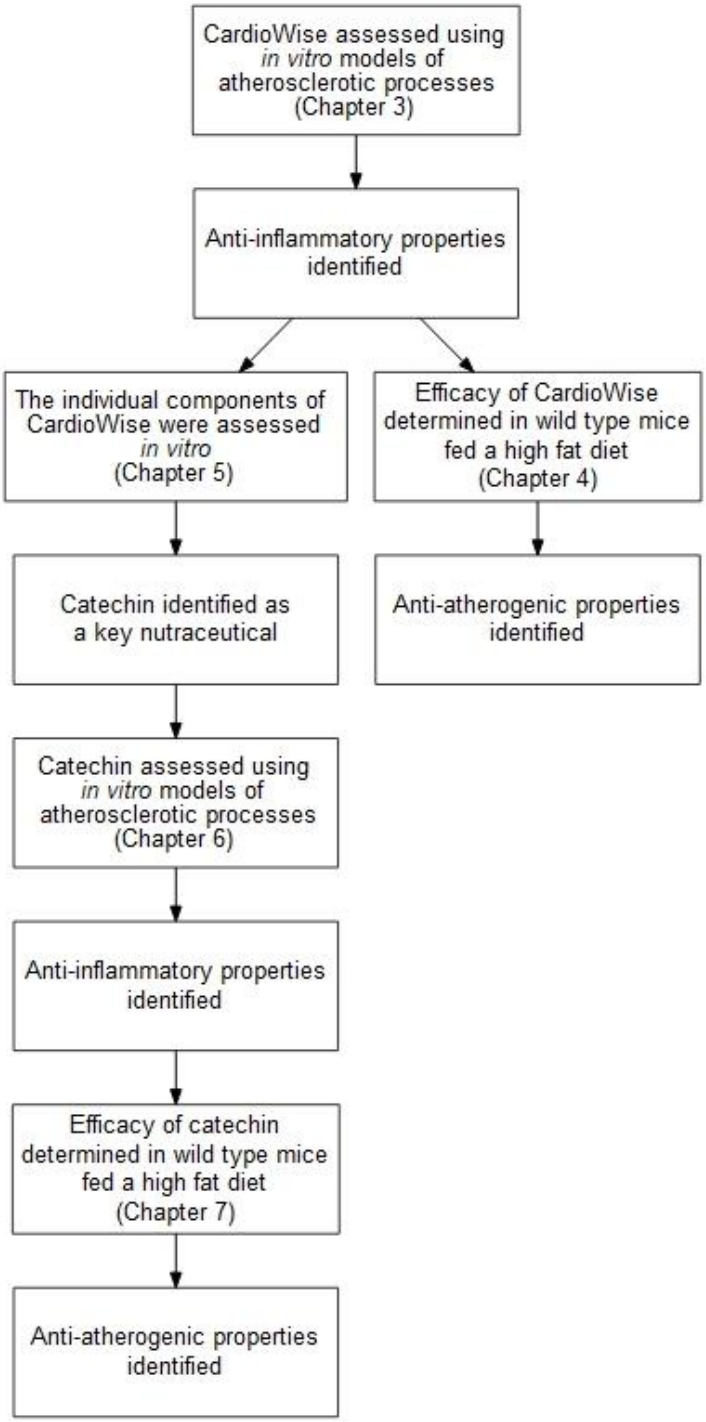

Fig. 1.7. An overall summary of the aims addressed within this project. Further details on the specific methods used to assess the anti-inflammatory properties of CardioWise and catechin, as well as the rationale for each experiment, can be found in Chapter 2 – 7.

Chapter 2

Materials and Methods

2.1 Materials

A list of all the suppliers and materials used in this project have been outlined below in Table 2.1.

Table 2.1. The materials used to carry out the project and their suppliers.

Supplier	Material
Abcam, UK	Annexin V-FITC apoptosis detection kit; DCFDA Cellular ROS Detection Assay Kit; MMP Activity Assay Kit (Fluorometric - Green); HDL and LDL/VLDL cholesterol assay kit; Triacylglycerol quantification assay kit
Amersham, UK	[4-^{14}C]cholesterol
BD Bioscience, UK	FITC-conjugated anti-mouse CD34
Biolegend, USA	APC-conjugated anti-mouse c-Kit; PE-conjugated anti-mouse Sca-1; FITC-conjugated anti-mouse CD48; PE/Cy7-conjugated anti-mouse CD150; APC/Cy7-conjugated anti-mouse Sca-1; PE/Cy7-conjugated anti-mouse CD16/32; BV650-conjugated anti-mouse CD127
Biotrend, Germany	Acetylated LDL; Dil-oxLDL
Cayman Chemical, USA	EPA; DHA; β-sitosterol; Campesterol
Cell Biolabs, USA	OxiSelect *in vitro* ROS/RNS Assay kit (green fluorescence); OxiSelect TBARS Assay Kit (MDA Quantification)
Cultech Limited, UK	CardioWise; CardioWise Plus
Dutscher, UK	40/70 µm sterile cell strainer
Fisher Scientific, UK	Agarose
Helena Biosciences, UK	Cell scrappers
Jackson Laboratory, USA	Male C57 black 6 wild type mice
Life Technologies, UK	TBE; Penicillin; Streptomycin; Foetal calf serum; miRNA probes and Taqman master mix; Amplex Red kit; qPCR plate seals
Lonza, UK	RPMI 1640 with L-Glutamine media
Nycomed, Switzerland	Lymphoprep
Peprotech, UK	IFN-γ, MCP-1; M-CSF; TNF-α
Promega, UK	dNTPs; MMLV reverse transcriptase; RNasin ribonuclease inhibitor; random primers; MMLV RT 5x buffer

Qiagen, UK	Atherosclerosis RT^2 Profiler PCR Arrays (human and mouse)
R&D Systems, UK	Human CCL2 DuoSet ELISA; Human IL-1β DuoSet ELISA; DuoSet ancillary reagent kit 2
Sigma-Aldrich, UK	Phorbol-12-myristate-13-acetate (PMA): SYBR Green; Dimethyl sulfoxide (DMSO); RAW264.7 cells; Ethylenediaminetetraacetic acid (EDTA); Ethanol; PCR primers; Isopropanol; Ipegal; Bovine serum albumin (BSA); Hexane; Tissue culture flasks; 12/24/96-well plates; 10/25ml strippets; 15/50ml Falcon tubes; Cell culture plates; Crystal violet; THP-1 cells; Apolipoprotein A-I; 5-Bromo-2′-deoxy-uridine Labeling and Detection Kit III; Lucifer yellow CH dipotassium salt (LY); Stigmasterol; (+)-Catechin; Catechin hydrate; Accuspin tubes
Special Diets Services, UK	High fat diet [21% (w/w) pork lard and 0.15% (w/w) cholesterol]
Starlabs, UK	qPCR 96-well plates
Thermo Scientific, UK	Micro BCA™ Protein Assay kit; 2x RNA loading dye; RiboRuler high Range RNA ladder; Pierce lactate dehydrogenase (LDH) cytotoxicity assay kit; Streptavidin; Cryomolds; OCT
VWR Jencons, UK	Falcon® cell culture inserts (8 µm pore size); Falcon® 12-well companion plates

APC, allophycocyanin; Cy7, cyanine7; DCFDA, 2'7'-dichlorofluorescin diacetate; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FITC, fluorescein isothiocyanate; HDL, high density lipoprotein; IFN, interferon; LDL, low density lipoprotein; MCP-1, monocyte chemotactic protein-1; M-CSF, macrophage colony-stimulating factor; MDA, malondialdehyde; MMLV, moloney murine leukaemia virus; oxLDL, oxidised LDL; PCR, polymerase chain reaction; PE, phycoerythrin; RNS, reactive nitrogen species; ROS, reactive oxygen species; Sca-1, stem cell antigen-1; TBARS, thiobarbituric acid reactive substances; TBE, tris/borate/EDTA buffer; TNF-α, tumour necrosis factor α; VLDL, very low density lipoprotein.

2.2 CardioWise Composition

CardioWise is a unique combinational product supplied by Cultech Limited containing a variety of nutraceuticals thought to exert anti-inflammatory effects. In order to create the different treatment dosages of CardioWise used in this study (Table 2.2), stock CardioWise was diluted (1:10 v/v) in culture media. Culture media consists of RPMI 1640 with L-Glutamine media, 10% (v/v) heat-inactivated (56ºC for 30 minutes) foetal calf serum (HI-FCS), as well as penicillin and streptomycin (both 100 µg/ml). To preserve the sterility of the media, the HI-FCS, penicillin and streptomycin were passed through a sterile filter (0.2 µm) before being added to the media. The doses initially used in the study ranged from 0.25x to 4x physiological dose, with 1x physiological dose based on the reported levels of each component found in the blood stream following consumption (Garaiova *et al.* 2007; Batta *et al.* 2006; Manach *et al.* 2005).

Table 2.2. Dosages of CardioWise used in the study.

Compound	0.25x PD	0.5x PD	1x PD	2x PD	4x PD
EPA (µg/ml)	7.5	15.0	30.0	60.0	120.0
DHA (µg/ml)	5.0	9.9	19.7	39.4	78.8
Total Phytosterol Esters (µg/ml)	14	28	56*	112	224
Flavanols (µmol/ml)	1.25	2.50	5.00	10.00	20.00
FA free BSA (µg/ml)**	25	50	100	200	400

FA, fatty acid; PD, physiological dose. * Stigmasterol: 10 µg/ml, Campesterol: 13.9 µg/ml, β-sitosterol: 27.2 µg/ml, Brassicasterol: 1.672 µg/ml. ** used as an emulsifier and protein carrier unless stated otherwise.

2.3 Cell Culture Techniques

2.3.1 Cell Lines Utilised

The RAW264.7 and THP-1 cell lines were predominately used throughout the study. The RAW264.7 cell line is a mouse macrophage-like cell line which was originally from a tumour developing in a BALB/c IgH strain of BAB/14 mouse which had been injected with Abelson murine leukaemia virus (Hartley *et al.* 2008). Since their first discovery 35 years ago, the RAW264.7 cell line has become one of the most common mouse macrophage-like cell lines used in atherosclerosis research for gene expression and macrophage function studies (Hartley *et al.* 2008). These cells can also be efficiently transfected with exogenous DNA and therefore extensively used in promoter analysis studies (Hartley *et al.* 2008).

In order to make the results of this study more applicable to humans, experiments were also performed using the THP-1 human monocytic leukaemia cell line. Unlike the RAW264.7 cells which are adherent to tissue culture dishes, THP-1 monocytes grow in suspension and require treatment with phorbol-12-myristate-13-acetate (PMA) for at least 24 hours in order to differentiate into macrophages. PMA differentiated THP-1 macrophages make ideal cell models for atherosclerosis gene expression, macrophage function and cell signalling studies as they exhibit similar traits to human monocyte-derived macrophages (HMDMs) both *in vitro* and *in vivo* (Auwerx 1991; Qin 2012; Chanput *et al.* 2014). For this reason the THP-1 cell line has become well established and makes for an excellent *in vitro* model of HMDMs.

Some assays were also performed using primary HMDMs from buffy coats in order to rule out the possibility that the results achieved were due to the use of a cell line. HMDMs were obtained from buffy coats using the following protocol. The National Blood Service Wales provided the human blood buffy coats and the white blood cells were separated using Lymphoprep. In order to place the Lymphoprep in the bottom of a 50 ml Accuspin tube, 15 ml

of Lymphoprep was centrifuged at 1000 *x g* for 60 seconds at room temperature. Subsequently 25 ml of blood was added to the tube and centrifuged at room temperature at 800 *x g* for 10 minutes. No brake was used to slow the centrifuge at this step. The mononuclear layer of cells was aspirated and placed in a fresh Falcon tube where an equal volume of ice cold 0.4% (w/v) tri-sodium citrate phosphate buffered saline (PBS) was added. The mixture was centrifuged at 1000 *x g* for 5 minutes at room temperature. Red blood cells were lysed by re-suspending the cell pellet in 10 ml of 0.2% (w/v) saline and placed on ice for 30 seconds before addition of an extra 10 ml of 1.6% (w/v) saline to the mixture. It was then centrifuged at 1000 *x g* for 5 minutes at room temperature. In order to remove platelets, the resulting pellet was re-suspended in ice cold 0.4% (w/v) tri-sodium citrate PBS and centrifuged at 800 *x g* for 5 minutes at room temperature 6 – 10 times. The final cell pellet could be re-suspended in culture media and seeded into 12 well plates and incubated at 37ºC in 5% (v/v) CO_2. The monocytes required 10 days to differentiate into HMDMs before they could be used experimentally. The HMDMs were washed before use in order to remove non-adhering leukocytes. The primary human macrophages were utilised in the same way as the THP-1 macrophages, therefore the following methods will only refer to THP-1 monocytes/macrophages.

2.3.2 Thawing Frozen Cells

For long term storage of the cell lines they were either stored in liquid nitrogen or at -80ºC, therefore careful thawing of the frozen stock was required when fresh cells were needed. The cells were warmed to 37ºC using a water bath and once thawed, the cells were immediately added to 10 ml of pre-incubated culture media and centrifuged at 250 *x g* for 5 minutes at room temperature. Once centrifuged the supernatant was aspirated and a further 5 ml of pre-incubated culture media was used to re-suspend the cells, which were then maintained and sub-cultured according to the instructions outlined further in Section 2.3.3.

2.3.3 Cell Line Maintenance and Sub-culturing of Cells

Cell lines were maintained and incubated in culture media at 37ºC in 5% (v/v) CO_2 in either tissue culture dishes (RAW264.7 due to their adherent nature) or tissue culture flasks (THP-1 cells as they grow in suspension). When the RAW264.7 or THP-1 cells became confluent (80% or 60% respectively) they were sub-cultured. A cell scraper was used to remove the adherent RAW264.7 cells from the surface of the tissue culture dishes to create a cell suspension. Both of the RAW264.7 and THP-1 cell suspensions were then transferred to a Falcon tube and centrifuged at room temperature at 250 *x g* for 5 minutes. The supernatant was then removed and the cell pellet re-suspended in 5 ml of fresh pre-warmed culture media and split in a ratio of approximately 1:30. The freshly split cells were then maintained at 37ºC in 5% (v/v) CO_2 until the process needed to be repeated. Cells between passage 3 and 12 were used in experiments.

2.3.4 Counting Cells

In order to accurately seed the correct number of cells for each experiment a haemocytometer was used. The surface of the haemocytometer is covered in a 5 x 5 grid which can be used to count the number of cells by adding 7.5 µl of either the RAW264.7 or THP-1 cell suspension and using a glass cover slip over the surface of haemocytometer to evenly spread the suspension. The average number of cells in the four corners and centre of the grid was multiplied by 10^4 in order to calculate the number of cells/ml.

2.3.5 Cytokine stimulation

RAW264.7 and THP-1 cells were seeded at 5.13 x 10^4 cells/cm^2 and 1.28 x 10^5 cells/cm^2 respectively in total volumes of 2 ml and 1 ml of culture media unless stated otherwise. Due to their adherent nature, the RAW264.7 cells could be left for 24 hours to allow them to "stick" to the surface of the experimental plate at 37ºC and 5% (v/v) CO_2. However THP-1 monocytes required the culture media to be supplemented with 0.16 µM PMA for 24 hours at the same incubation conditions as the RAW264.7 cells to induce macrophage differentiation. Following their 24 hour PMA differentiation, the THP-1 cells were washed using 0.5 ml of PBS (x1, pH 7.4). The required cytokine could be then added to the cells for the desired length of time to induce the expression of the target genes. The concentrations of the cytokines used are listed below in Table 2.3 as previous optimisation studies in the laboratory had shown these concentrations to be effective (Gallagher 2016).

Table 2.3. The concentrations of cytokines used in the study (unless stated otherwise).

Cytokine	Concentration
IFN-γ	250 U/ml
IL-4	20 ng/µl
LPS	100 ng/µl
MCP-1	20 ng/ml
TNF-α	100 ng/ml

IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MCP, monocyte chemotactic protein-1; TNF-α, tumour necrosis factor α.

2.4 Cell Based Assays

2.4.1 Cell Viability

The effect of nutraceuticals on cell viability was assessed in THP-1 cells for 24 hours using the Pierce lactate dehydrogenase (LDH) cytotoxicity and crystal violet assays simultaneously. This time frame was chosen as it corresponds to the longest assay time used in other cell based assays in this study. Initially, THP-1 monocytes (4.11 x 10^5 cells/cm^2) were plated per well of

a 96-well plate and differentiated into macrophages using 0.16 µM PMA and incubation at 37°C and 5% (v/v) CO_2 for 24 hours. The culture media was aspirated and substituted with 200 µl of culture media encompassing a nutraceutical along with both a negative (vehicle) and positive control (complete lysis) and returned to the incubator for a further 24 hours.

After 24 hours, 20 µl of 10x lysis buffer (provided in kit) was added to the positive control wells to create a maximum LDH release control. In order to compensate for the increased volume in the positive control wells, 20 µl of dH_2O was added to all of the other wells. The plate was then incubated for 45 minutes using the conditions listed above. Subsequently, 50 µl of supernatant from each well was transferred to a new 96-well plate to which 50 µl of reaction mixture (provided in kit and made according to manufacturer's instructions) was added and incubated for 30 minutes. LDH is an intracellular enzyme whose function is to catalyse the conversion of lactate and NAD+ to pyruvate and NADH. Upon cell membrane rupture (cell death or damage), LDH is released into the surrounding media and is then able to freely react with the lactate and NAD+ in the reaction mixture. The reaction mixture also contains the water soluble salt 2-(4-iodophenyl)-3-94-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT), which is reduced to an insoluble red formazan salt by NADH. Therefore the darker the red colour the more LDH leakage has occurred and indicating a reduction in cell viability (Korzeniewski and Callewaert 1983; Decker and Lohmann-Matthes 1988). After the final incubation, 50 µl of stop solution (provided in kit) was added to each well.

Once the supernatant was removed from the original 96-well plate, the remaining media was removed and the cells washed with 50 µl PBS (1x, pH 7.4). A 0.2% (w/v) crystal violet solution (which also contained 10% (v/v) ethanol) was prepared and 100 µl added to each well for 5 minutes at room temperature to stain the cells. The crystal violet solution was removed and the cells washed three times with 50 µl PBS (1x, pH 7.4). Then 100 µl solubilisation buffer (0.1 M NaH_2PO_4 in 50% ethanol) was added to every sample. Crystal violet is a dye capable of binding to DNA within a cell (Feoktistova *et al.* 2016). Therefore the darker the purple colour the more viable cells are present as unviable cells will have become detached and removed during the assay. As crystal violet binds to DNA it can also be used to assess the level proliferation occurring within the cells. The absorbance of both the LDH and crystal violet plates were read in a microplate reader at 570 nm. The negative vehicle control was arbitrarily assigned as 100% cell viability and all other samples standardised to it.

2.4.2 Cell Proliferation

Cell proliferation was assessed using three different methods including crystal violet assay (see Section 2.4.1 for further details), counting the number of cells and a bromouridine enzyme-linked immunosorbent assay (ELISA). The simplest way to measure cell proliferation is count the changes in cell numbers over a prolonged period of time. To determine the effect of (+)-catechin on cell proliferation by cell counting, 200,000 undifferentiated THP-1 monocytes

were grown at 37ºC in 5% (v/v) CO_2 for 7 days. The cells were cultured in complete cell media with the vehicle control or in culture media also containing the desired nutraceutical. The media was replaced every 24 hours for the first 4 days. The cell number was determined using a haemocytometer (see Section 2.3.4 for further details) on days 0, 1, 2, 3, 4 and 7.

As bromouridine is simply a uridine with one of its carbon substituted with a bromine, it is incorporated into RNA just as regular uridine would be. Higher levels of RNA would indicate an increase in the number of cells and therefore increased cell proliferation. Bromouridine can be detected using fluorescently labelled antibodies, consequently an increase in the fluorescent signal would suggest an increase in cell proliferation. To assess cell proliferation by bromouridine ELISA, PMA differentiated THP-1 macrophages (4.11×10^5 cells/cm^2) were incubated at 37ºC at 5% (v/v) CO_2 for 24 hours in either complete media with the vehicle control or complete media containing the nutraceutical. Following initial incubation, 10 µM bromouridine labelling solution (provided by the manufacturer) was added and the cells returned to the incubator for a further 3 hours. The media was then carefully aspirated and the cells washed twice with complete media. Pre-cooled fixative (0.5 M HCl in 70% (v/v) ethanol) was added to the cells which were then incubated at -20ºC for 30 minutes, before being washed a further three times with complete media. After the final wash when all media had been removed, the cells were kept at 4ºC overnight. The following morning the cells were washed once with complete media and then incubated with nuclease solution (provided by the manufacturer) at 37ºC at 5% (v/v) CO_2 for 30 minutes. The cells were then washed three times with complete media and returned to the incubator for 30 minutes in anti-BrdU-POD, Fab fragments solution (provided in kit and made according to manufacturer's instructions). A washing buffer (provided by the manufacturer) was used to wash the cells three times before they were incubated in peroxidase substrate solution (provided by the manufacturer) at room temperature in the dark for 30 minutes. Fluorescence was measured in a fluorescence microplate reader at 405 nm with a secondary reference wave-length of 490 nm. The vehicle control was then arbitrarily assigned as 1.0 and all other data were presented relative to this.

2.4.3 Cell Apoptosis

PMA differentiated THP-1 macrophages (4.11×10^5 cells/cm^2) were used to assess the effect of nutraceuticals on apoptosis. To induce apoptosis cells were subjected to a combination of serum starvation (by using culture media lacking HI-FCS) and TNF-α stimulation for 3 hours. Cells were incubated during that time at 37ºC in 5% (v/v) CO_2 in the presence of either the vehicle control or nutraceutical of choice. Following the induction of apoptosis, the 96-well plate was centrifuged at 250 *x g* for 5 minutes and the media aspirated. The cells were then gently washed with PBS (x1, pH 7.4) and 100 µl of annexin V-FITC solution (made according to manufacturer's instructions from the reagents provided) was added and the cells incubated at room temperature in the dark for 5 minutes. Annexin V is protein capable of binding to markers

of apoptosis and therefore the levels of apoptosis can be assessed by using a fluorescently labelled annexin V protein. After incubation, fluorescence was measured in a fluorescence microplate reader, with excitation at 485 nm and emission detected at 535 nm. The vehicle control was then arbitrarily assigned as 1.0 and all other data were presented relative to this.

2.4.4 Monocyte Migration

2.4.4.1 Setting Up the Boyden Chamber

Monocyte migration was determined using a modified Boyden chamber (Fig. 2.1), which involved using a porous membrane to act like the endothelium layer in the artery. Cells were placed on one side of the membrane and the levels of migration determined by the percentage of cells which moves across the membrane in response to a chemoattractant. To assess monocyte migration, Falcon® cell culture inserts and Falcon® 12-well companion plates were used. The cell culture inserts contained a membrane with pore sizes of 8 µm (mimicking the arterial wall) and were used to split the wells of the companion plate into separate halves but still allowing monocytes to migrate. To induce migration, the chemokine MCP-1 was added in a total of 1 ml of culture media to the bottom half of all the wells except for the control (No MCP-1). Undifferentiated THP-1 monocytes (1.28 x 10^5 cells/cm^2) were added to the top half of the well. The top halves of the wells were then made up to a total volume of 0.5 ml using culture media either containing the vehicle control or nutraceutical. The modified Boyden chambers were then incubated at 37°C in 5% (v/v) CO_2 for 3 hours.

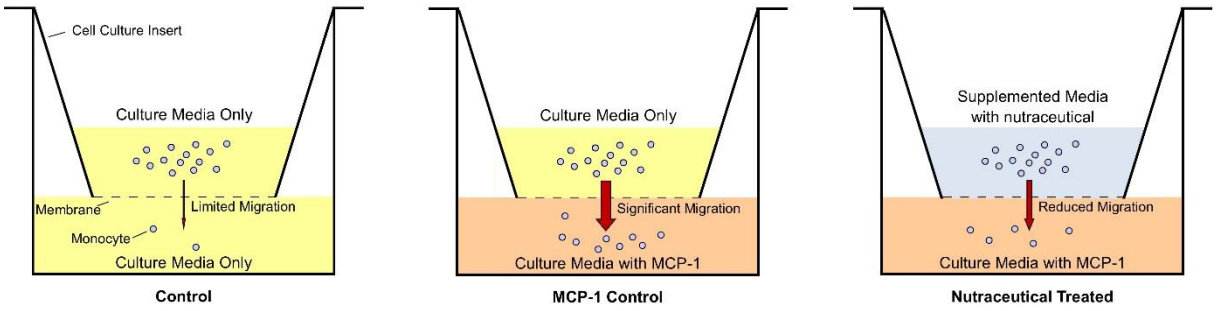

Fig. 2.1. The experimental set up of the modified Boyden chambers used to assess monocyte migration. Monocytes within the control group are not exposed to the chemoattractant MCP-1 in the bottom half of the chamber and therefore basal or limited migration should occur. Migration was induced within the MCP-1 control group by using culture media supplemented with MCP-1 in the bottom half of the chamber. As the THP-1 monocytes in the top half of the chamber are treated with vehicle control a significant level of migration across the membrane should occur. The final group were also exposed to MCP-1 within the bottom half of the chamber, however the cells are suspended in culture media containing the nutraceutical of choice. A successful nutraceutical treatment would result in a reduction in the percentage of monocytes migrating from the top to the bottom half of the chamber.

2.4.4.2 Assessing Migration

After 3 hours of migration, the media in the top half of the wells was removed and the underside of the membrane washed with 0.5 ml of PBS (x1, pH 7.4) into the bottom well to remove any adherent cells. The media in the bottom of the wells was then transferred to a 10 ml Falcon

tube and centrifuged at 250 *x g* for 5 minutes at room temperature. The supernatant was aspirated and the cells resuspended in 2 ml of culture media and counted using the haemocytometer (Section 2.3.4). The number of cells that had migrated from the top to the bottom half of the well was calculated and expressed as a percentage of the number of cells originally added.

2.4.5 MMP Activity

To assess MMP activity, PMA differentiated THP-1 macrophages (4.11 x 10^5 cells/cm^2) were cultured for 3 or 24 hours with either culture media containing vehicle control or the nutraceutical of choice at 37ºC in 5% (v/v) CO_2. These time points were chosen to assess both short term and long term effects of nutraceuticals on MMP activity. Following the desired incubation time period, 50 µl of the media was transferred to a new plate for analysis. An equal volume of MMP green substrate solution (provided by the manufacturer) was added to each well and the plate returned to the incubator for a further 30 minutes. The substrate solution contains a fluorescent resonance energy transfer (FRET) peptide consisting of two subunits, an emitter and a quencher. When the peptide is intact, the quencher prevents the emitter from releasing a fluorescent signal upon excitation. However the FRET peptide has been designed to be broken down by MMPs, causing the separation of the emitter and quencher. Once separated from the quencher, the fluorescence of the emitter is recovered. Therefore a reduction in MMP activity would result in more FRET peptides remaining intact and a reduced fluorescent signal detected. Following incubation, fluorescence was measured in a fluorescence microplate reader, with excitation at 485 nm and emission detected at 535 nm. The vehicle control was then arbitrarily assigned as 1.0 and all other data were presented relative to this.

2.4.6 ROS Production

To assess whether nutraceuticals possessed any anti-oxidant properties a 2'7'-dichlorofluorescin diacetate (DCFDA) Cellular ROS Detection Assay Kit was used. DCFDA is a non-fluorogenic compound capable of passively diffusing into cells where it is deacetylated by cellular esterases. This new molecule is oxidised by ROS into a highly fluorescent compound. Therefore the higher the fluorescent signal, the more ROS generation which has occurred within the cell. Undifferentiated THP-1 monocytes and PMA differentiated THP-1 macrophages (4.11 x 10^5 cells/cm^2) were added per well of a 96-well plate in 50 µl. Culture media containing 2x concentration of the desired nutraceutical were also added to the cells in a volume of 50 µl, resulting in a 1x concentration in a total volume of 100 µl. Tert-butyl hydrogen peroxide (TBHP) was used as a positive control as it is capable of stimulating ROS production in cells. The nutraceuticals were used in combination with TBHP to assess whether they were capable of reducing ROS production. All reagents were supplied as part of the DCFDA Cellular ROS Detection Assay Kit unless stated otherwise.

Before cell treatment could begin, the cells were washed using 1x buffer before being stained with 20 µM DCFDA in 1x buffer for 30 or 45 minutes (for monocytes or macrophages respectfully) in the dark at 37°C in 5% (v/v) CO_2. A longer incubation period is required for macrophages as it takes more time for the DCFDA compound to diffuse into adherent cells. Once the cells had been stained, they were washed once with 1x buffer before being transferred to a 96-well plate in 50 µl of culture media. The different treatment groups were then added to the wells of the plate which was incubated at 37°C in 5% (v/v) CO_2 for 3 hours. After incubation, fluorescence was measured in a fluorescence microplate reader, with excitation at 485 nm and emission detected at 535 nm. The vehicle control was then arbitrarily assigned as 1.0 and all other data were presented relative to this.

2.4.7 ELISAs for Pro-inflammatory Cytokines/Chemokines

PMA differentiated THP-1 macrophages (1.28×10^5 cells/cm^2) were stimulated with IFN-γ in the presence or absence of a variety of nutraceuticals in 0.2% (w/v) bovine serum albumin (BSA) cell culture media for 24 hours. Cell culture media containing BSA was used as it is present in the blocking buffer and used as a carrier for other substrates during this assay. As a result BSA media was used in order to avoid any possible interference from HI-FCS containing media. A DuoSet ELISA kit was used to determine the levels of MCP-1 and IL-1β secreted from the cells into the surrounding media. Initially a capture antibody, which is fixed to the surface of microplate, binds to the target cytokine/chemokine present in the aspirated cell media. A detection antibody, which contains biotin, is then added and also binds to the target antigen. Streptavidin is protein which has a high affinity to biotin. By attaching the enzyme horseradish-peroxidase (HRP) to streptavidin, it means the more target cytokine/chemokine captured from the aspirated cell media the more HRP will be present at the end of the assay. A substrate solution is then added which undergoes a colour change in the presence of HRP. Therefore the more colour change which occurs, the more target cytokine/chemokine was secreted by the cells into the surrounding media.

A microplate was incubated with the desired capture antibody (provided in kit) overnight at room temperature to allow adequate time for adhesion. The following morning, the microplate was washed three times with wash buffer (provided in kit) which was then aspirated and blotted on a paper towel to ensure it was completely dry. All subsequently described washing steps used the same procedure. Once dry, a blocking buffer (provided in kit) was added for 1 hour at room temperature, followed by further washing and drying. The samples and standard curve (100 µl) were then incubated with the adhered capture antibody for 2 hours at room temperature, before being washed and subsequently incubated with the detection antibody (provided in kit) for a further 2 hours. The standard curve consisted of known concentrations of the target cytokine/chemokine and made according the manufacturer's instructions from reagents provided in the kit. The MCP-1 ELISA has an assay range of 15.6 pg/ml to 1000

pg/ml, whereas the IL-1β ELISA has an assay range of 3.91 pg/ml to 250 pg/ml. Samples being assessed were diluted if they exceeded this maximum limit of detection. After being washed again, the following steps were considered light sensitive and therefore all further incubation periods were carried out in the dark. A conjugate of streptavidin and HRP (provided in kit) was added to the microplate and left at room temperature for 20 minutes. After another wash, the microplate was then incubated with substrate solution (provided in kit) for an additional 20 minutes before the stop solution (provided in kit) was added. The absorbance of the microplate was read in a microplate reader using wavelength correction with the first reading measured at 450 nm and a second reading at 590 nm. Wavelength correction is a process by which more accurate absorbance readings are achieved. A second wavelength is used to determine the level of background interference and remove it from the desired primary wavelength readings.

2.5 Nucleic Acid Based Techniques

2.5.1 RNA Extraction

As mRNA is produced by activated genes, it can extracted and the amount of target mRNA can be determined in order to assess gene expression levels. Prior to RNA extraction, the culture media was aspirated and the cells washed with 0.5 ml of PBS (x1, pH 7.4). RiboZol™ (1 ml) was added to the cells which were then lysed by passing them several times through the tip of a pipette. The mixture was then left to stand for 10 minutes at room temperature to guarantee the complete dissociation of nucleoprotein complexes. To isolate the RNA in the mixture, 200 µl of chloroform was added to the samples and shaken for 15 seconds and then centrifuged at 12,000 *x g* for 15 minutes at 4ºC. This process separates the mixture into three phases: a lower red, phenol-chloroform phase; a white interphase; and a colourless upper phase where the RNA is present. In order to avoid contaminating the RNA phase, approximately only 80% of the RNA phase was transferred to a clean RNase-free tube. The RNA was then precipitated using isopropanol (500 µl) and incubated at -80ºC for at least one hour and subsequently centrifuged at 12,000 *x g* at 4°C for 10 minutes. The supernatant was removed and the RNA pellet washed three times using 1 ml 75% (v/v) ethanol and vortexing the samples before centrifugation at 7,500 *x g* for 5 minutes at 4ºC. Following the final RNA pellet wash, the pellet was left to air dry for 10 minutes at room temperature before re-suspending the pellet in 15 µl of RNase-free water and incubating the samples at 55-60ºC for 10 minutes. A NanoDrop 2000 spectrophotometer was used to determine the quality and concentration of the extracted RNA. A result between 1.8 – 2.1 for the A_{260}/A_{280} and the A_{230}/A_{260} ratios indicated high quality RNA. Gel electrophoresis was also used to assess the quality of a small quantity of the extracted RNA (Section 2.5.4).

2.5.2 Reverse Transcription

In order to be able to assess gene expression, the extracted RNA had to be reverse transcribed into cDNA for quantitative polymerase chain reaction (qPCR; Section 2.5.3). The initial step involved adding 0.5 µl of random hexamer primers (200 pmol) to 500 or 1000 ng of extracted RNA (depending on the yield) and ddH_2O in a total volume of 13.5 µl. The samples were then heated to 70ºC for 5 minutes and immediately afterwards placed on ice to prevent RNase activity. The reagents listed in Table 2.4 were then added and the reaction mixture incubated at 37ºC for 1 hour with a final enzyme inactivation phase of 90ºC for 2 minutes. Following reverse transcription, ddH_2O was added until a final concentration of 10 ng/µl of cDNA was achieved. The cDNA could then be kept at 4ºC for short-term storage or -20ºC if long-term storage was required.

Table 2.4. Composition of the master mix used in the reverse transcription reaction.

Reagent	1x Master Mix (µL)
100mM dNTPs mixture	1.0
5x MMLV buffer	4.0
RNase inhibitor (40 U/µl)	0.5
MMLV reverse transcriptase (200 U/µl)	0.5

MMLV, moloney murine leukaemia virus.

2.5.3 qPCR

qPCR is a technique that allows for the quantification of gene expression by simultaneously amplifying and quantifying target cDNA. This technique achieves this because the amount of PCR product produced in the reaction is proportional to the starting amount of cDNA during the exponential phase of PCR amplification. In this study SYBR Green JumpStart™ Taq Readymix™ was used as it is a fluorescent dye that is only detectable when bound to double stranded DNA, therefore the strength of the fluorescent emission is proportional to the amount of cDNA template. The method determines when the strength of the fluorescent emission reaches a pre-set threshold value (C_T value), and compares this value for the target gene of interest to a housekeeping gene (whose expression should not alter under the experimental conditions) using the comparative C_T ($\Delta\Delta C_T$) method (Schmittgen and Livak 2008). The housekeeping genes used in this study were *β-actin* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) for RAW264.7 and THP-1 cells respectively as their expression has been shown to be stable under different experimental conditions (Mehta *et al.* 2010), in addition to their extensive use previously in the laboratory (McLaren *et al.* 2010a; Li *et al.* 2010). During the project two forms of qPCR were utilised, the first will be referred to as ‘regular qPCR’ and was performed using the method outlined above and sequence of the primers employed can be found in Table 2.5. The second form of qPCR will be referred to as ‘qPCR array’. Unless

stated otherwise, all primers used for regular qPCR were designed by members of the laboratory to be intron spanning to avoid genomic DNA amplification.

qPCR arrays use specialist plates (Atherosclerosis RT^2 Profiler PCR Arrays) which contain the primers for 84 atherosclerotic and 5 housekeeping genes as well as 3 negative, 3 positive and 1 genomic DNA contamination controls. The advantage of the qPCR arrays is that they are able to provide a large volume of data compared to regular qPCRs in the same time frame. The cycling conditions for the qPCRs can be found in Tables 2.6 and 2.7. A reverse transcriptase negative control was included in all regular qPCR experiments to confirm that there was no genomic DNA contamination. Melting curve analysis was used for both regular qPCRs and qPCR arrays to ensure the primers produced a single product.

Table 2.5. Sequences of qPCR primers and their targets.

Species	Target Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
Mouse	*β-actin**	ACACCCGCCACCAGTTCGCCAT	CACACCCTGGTGCCTAGGGCGGCCCACGATG
	*MCP-1**	GCTCAGCCAGATGCAGTTAACG	GCTTGGTGACAAAAACTACAGCTTC
	*ICAM-1**	ACGTGCTGTATGGTCCTCGG	GTCCAGTTATTTTGAGAGTGGTACAGTA
	Arg2[#]	ATATGGTCCAGCTGCCATTCGAGA	CCACTTCAGCCAGTTCCTGGT
	iNOS[##]	CAGTTCTGCGCCTTTGCTCAT	GGTGGTGCGGCTGGACTTT
	IL-6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA
	IL-1β	GAGGACATGAGCACCTTCTTT	GCCTGTAGTGCAGTTGTCTAA
Human	*GAPDH*	CTTTTGCGTCGCCAGCCGAG	GCCCAATACGACCAAATCCGTTGACT
	MCP-1	CGCTCAGCCAGATGCAATCAATG	ATGGTCTTGAAGATCACAGCTTCTTTGG
	ICAM-1	GACCAGAGGTTGAACCCCAC	GCGCCGGAAAGCTGTAGAT
	ABCA1	AGTGGAAACAGTTAATGACCAG	GCAGCTGACATGTTTGTCTTC
	ABCG1	GGTGGACGAAGAAAGGATACAAGACC	ATGCCCGTCTCCCTGTATCCA

* Previous work in my MRes has shown these primers to produce a single PCR product of the target gene by sequence analysis (Moss 2014). [#] primers from *Jin et al.* (2015). [##] primers from *Chang et al.* (2010). All other primers were designed by members of the laboratory.

Table 2.6. Composition of the qPCR master mix for gene expression.

Reagent	1x Master Mix for regular qPCR (µL)	1x Master Mix for qPCR array (µL)
SYBR Green JumpStart™ Taq Readymix™ for quantitative PCR	13.75	12.5
Forward Primer (100 µM)	0.55	-
Reverse Primer (100 µM)	0.55	-
cDNA (10 ng/µl)	1.10	0.26
ddH_2O	11.55	12.24

The master mix was prepared in excess before 25 µl was transferred to the qPCR plate.

Table 2.7. The qPCR conditions used for gene expression.

	Regular qPCR		qPCR array	
PCR step	Temperature (°C)	Time (s)	Temperature (°C)	Time (s)
Preincubation	94	120	95	600
Melting	95	30	95	15
Annealing	60	60	60	60
Extension	72	60		
Melt curve	95	10	95	10
	60	60	60	60
	97*	2	97*	2

The regular qPCR is a three step amplification process and cycles 40 times, whereas the qPCR array involves a two-step amplification (as the annealing and extension steps occur simultaneously) and cycles 45 times. * Increased from 55°C by 0.5°C per cycle over 74 cycles.

2.5.4 Agarose Gel Electrophoresis

A 2% (w/v) agarose gel was used to determine the integrity of the extracted RNA. Agarose was dissolved in 1x Tris/borate/EDTA (TBE; 89 mM Tris-borate, 89 mM Boric acid, 2 mM EDTA, pH 8.3) buffer by heating it in a microwave. Initially, 10 µl of the RNA samples were added to 10 µl 2x RNA Loading Dye and heated at 70°C for 10 minutes and then immediately transferred to ice for 3 minutes. Electrophoresis was carried out using a horizontal gel unit at 100 V in 1x TBE for 45 minutes. The size of RNA products were determined using a RiboRuler High Range RNA Ladder. UV light was used to picture the gel using a Sygene Gel Documentation system.

2.6 Cholesterol Homeostasis

2.6.1 Cholesterol Uptake

Reduced cholesterol uptake can also be a sign of decreased foam cell formation, therefore nutraceuticals which are capable of attenuating cholesterol uptake would represent potential anti-atherogenic therapies. Using fluorescently labelled lipoprotein, the amount of uptake into cells can be assessed by flow assisted cell sorting (FACS) analysis as the stronger the fluorescent signal, the more cholesterol uptake has occurred. PMA differentiated THP-1 macrophages (1.28×10^5 cells/cm^2) were incubated with a fluorescently labelled oxLDL known as Dil-oxLDL (5 µg/ml) for 24 hours in the presence of vehicle or nutraceuticals in media containing 0.2% (v/v) fatty acid free BSA. HI-FCS containing media was not used during this assay due to its ability to induce cholesterol efflux. Following incubation, the media was aspirated and the cells washed with PBS (x1, pH 7.4) and the cells "lifted" from the plate using Trypsin EDTA (0.05% w/v) for 10 minutes at 37 °C. Once the cells were in suspension, they were centrifuged at 9000 *x g* for 5 minutes at room temperature. The supernatant was removed and the pellet re-suspended in 2% (w/v) paraformaldehyde (PFA) in order to fix the cells for FACS analysis. A BD FACS Canto flow cytometer was then used to assess Dil-oxLDL uptake. At least 10,000 counts were captured for each sample.

2.6.2 Macropinocytosis

Macropinocytosis is another mechanism by which cholesterol may be taken up into macrophages during foam cell formation. Lucifer yellow (LY) is a fluorescent dye which enters cells via macropinocytosis. Therefore measuring the fluorescent signal within macrophages treated with LY by FACS analysis can be used as a maker for the level of macropinocytosis which has occurred within the cells. Cholesterol crystals are primarily taken up by macrophages via micropinocytosis (Moore *et al.* 2013). Therefore a macropinocytosis assay was carried out to determine the effect of nutraceuticals on PMA differentiated THP-1 cells (1.28×10^5 cells/cm^2). Once differentiated, the THP-1 cells were incubated at 37°C with 5% CO_2 (v/v) for 1 hour with either 1 ml of media only (for both the negative and positive control) or media containing the nutraceutical. Due to the effect of HI-FCS on cholesterol efflux, media containing 0.2% (v/v) fatty acid free BSA was used throughout this assay. Following incubation for 1 hour, LY (100 µg/ml) was added to both the positive control and nutraceutical treated cells and incubated for 24 hours.

After incubation with the LY, the media was removed and the cells washed with PBS (1x, pH 7.4). Subsequently, 0.5 ml Trypsin EDTA (0.05% w/v) was then added to each well and incubated for 10 minutes at 37°C. Culture medium (0.5 ml) was added to each well to inactivate the Trypsin EDTA to prevent cell lysis. The resulting mixture was then pipetted up and down several times to ensure the cells were no longer adherent to the surface of the well. The cell suspension was then centrifuged at 12,000 *x g* for 5 minutes. The resulting pellet was washed

with PBS and centrifuged again and the subsequent pellet resuspended in 500 µl 2% (w/v) PFA. LY uptake was determined using at least 10,000 detection events for each sample with a FACS flow cytometer.

2.6.3 Cholesterol Efflux

Cholesterol efflux can be measured using radioactively labelled cholesterol. Cells are allowed to take up the radioactive cholesterol during foam cell formation for 24 hours before its efflux is stimulated by ApoA1 into the surrounding media. The more radioactive cholesterol found within the media compared to the amount found within the cell indicates increased cholesterol efflux and therefore a possible reduction in foam cell formation. The following cholesterol efflux assay was adapted from a previously reported protocol (McLaren *et al.* 2010a). Initially, THP-1 macrophages (1.28 x 10^5 cells/cm^2) were incubated with acetylated LDL (acLDL; 25 µg/ml) and [4-^{14}C]cholesterol (0.5 mCi/ml) in media containing 0.2% (v/v) fatty acid free BSA for 24 hours before subsequent treatment with ApoA1 (10 mg/ml) with vehicle or the desired nutraceutical in 0.2% (v/v) BSA media. No HI-FCS was used in the culture media once the cells had differentiated due to the ability of FCS to stimulate cholesterol efflux from the cells and therefore affecting the accuracy of the assay. After 24 hours, the media was collected and 0.2 M NaOH (100 µl) was used to solubilise the cells. A liquid scintillation counter was used to measure the radioactivity within the supernatant and cellular fractions. Radioactivity was determined by the number of disintegrations per minute (dpm) within the samples. Cholesterol efflux was calculated as the percentage radioactivity in the supernatant versus total radioactivity (cells and supernatant).

2.6.4 Lipid Analysis

2.6.4.1 Lipid extraction

As well as determining the effect of nutraceuticals on cholesterol homeostasis, their effects on intracellular lipids was also assessed. Once intracellular lipids have been extracted, the major classes can be separated by thin layer chromatography (TLC) and quantified (Section 2.6.4.2). Previous work within the laboratory has shown RAW264.7 to produce a high quantity of intracellular lipids compared to THP-1 macrophages (which only contain very low levels of cholesteryl esters), improving the sensitivity of the data (Gallagher 2016). For this reason RAW264.7 macrophages were used for TLC analysis. Initially, RAW264.7 macrophages (2.08 x 10^5 cells/cm^2) were cultured for 24 hours in culture media at 37ºC in 5% (v/v) CO_2 to allow the cells to adhere. Once adhered, the macrophages were treated for 24 hours with either the vehicle control or nutraceutical of choice in the presence or absence of acLDL (25 µg/ml) and [^{14}C]acetate (1 µCi/ml). Following treatment, the media was aspirated and the cells washed once with PBS (x1, pH 7.4). The macrophages were collected by scrapping the wells using PBS (x1, pH 7.4) and transferred into Eppendorf tubes. Cells were pelleted by centrifugation

at 9000 *x g* for 5 minutes and resuspended in 1 ml of dH_2O and transferred into a glass tube to prevent the adherence of lipids to a plastic surface. Once in the glass tube, 2 ml of chloroform:methanol (2:1, v/v) solution was added and the mixture vortexed and incubated at room temperature for 15 minutes. Following incubation, 1 ml of chloroform and 2 ml of Garbus solution (2M KCl in 0.5 M potassium phosphate buffer, pH 7.6; Garbus *et al.* 1963) was added and the resulting mixture vortexed and centrifuged at 220 *x g* for 5 minutes in order to separate the chloroform and aqueous layers. A glass pipette was used to transfer the chloroform aqueous layer into a clean glass tube and subsequently evaporated under nitrogen and resuspended in 300 µl of chloroform.

2.6.4.2 TLC

A 10 x 10 cm silica gel G Merck plate was used to separate the different lipid classes during TLC. In order to separate total polar lipid (TPL), free cholesterol (FC), free fatty acid (FFA), TG and CE lipid classes from one another, a solution of hexane: diethyl ether: acetic acid (80:20:1, v/v/v) was used. A solvent mixture of chloroform: methanol: ammonium hydroxide (65:35:4, v/v/v) was used for one dimensional separation on the TLC silica gel plate. Following separation, the plates were left to dry before being sprayed with 8-anilino-4-naphthosulphonic acid (ANSA; 0.05% v/v in methanol). ANSA solution allows the lipids to become detectable under UV light. The location of the bands was then marked on the plates which were subsequently sprayed with water for scrapping into liquid scintillation vials. The radioactivity of each band was measured by a liquid scintillation counter.

2.7 *In Vivo* Techniques

2.7.1 Feeding and weighing

Once CardioWise and catechin were found to exert anti-inflammatory effects in the *in vitro* models of atherosclerosis disease development, an initial *in vivo* study involving high fat fed wild type mice was established. This study was designed to determine whether the benefits associated with CardioWise and catechin treatment *in vitro* were also observed *in vivo*.

To assess the effects of CardioWise and catechin *in vivo*, 8 week old male C57 black 6 (C57BL/6) wild type mice were used. The mice were housed in a light and temperature controlled facility (lights on 7 am to 7 pm, 22°C). Based upon previously published studies by Cultech Limited (Michael *et al.* 2017) and others in the literature (Schreyer *et al.* 1998; Shimizu *et al.* 2014; Zanotti *et al.* 2013), it was found that group sizes of 6 to 8 are required to calculate significant differences between our various parameters at the $p<0.05$ levels with a power of 0.8 (standard deviation ~30% and differences between groups ~40%). However, the number of mice used during the studies were based on the number of wild type mice available at the time of experiments. As a result during the CardioWise study, 6 mice were designated to

receive the vehicle control and 7 mice to receive CardioWise. A total of 16 mice were used to assess catechin with 8 in both the vehicle and catechin treatment groups.

The number of mice used during the studies were based on the number of wild type mice available at the time of experiments. All mice in the study received a high fat diet [21% (w/w) pork lard and 0.15% (w/w) cholesterol] for 3 weeks based on previous unpublished experiments performed in laboratory and previous studies within the field (Michael *et al.* 2017). During the feeding period, the mice were gavaged daily with their respective treatments. A 1x human equivalent dose of CardioWise was calculated based on the surface area of the mice and consisted of 410 mg/kg of EPA/DHA, 410 mg/kg of phytosterols as well as 41 mg/kg of catechin (Nair and Jacob 2016). To prevent the fish oil separating from the rest of the treatment, xanthan gum was used as an emulsifying agent, which was therefore also used as the vehicle control. Based on a similar study which gavaged rats every day for 3 weeks, a concentration of 200 mg/kg of catechin hydrate was chosen (Potenza *et al.* 2007). Catechin hydrate was used in this study due to the extremely high cost of using pure (+)-catechin. *In vitro* studies were used to confirm that (+)-catechin and catechin hydrate exert similar effects in experiments (Fig. 7.3). The catechin hydrate was dissolved in PBS which was also used as the vehicle control.

Body weight was measured at the start of the study and then approximately every 2 days. At the end of the study, all mice were sacrificed under anaesthesia by cardiac puncture and death confirmed by an absence of a pulse. All studies and protocols were approved by the Cardiff University Institutional Ethics Review Committee and the United Kingdom Home Office, and experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996; Experimental licence 30/3365).

2.7.2 Blood and tissue collection

Blood from cardiac puncture was collected into Eppendorfs containing heparin and the plasma obtained by centrifugation (12,000 *x g*) for 5 minutes. Rear legs were removed and placed into Falcon tubes containing PBS supplemented with 10% (v/v) HI-FCS for cell population analysis (Section 2.7.7). The fat pads and organs were placed in weighing-boats and weighed on an electronic scale. Samples from the fat pads, spleen, perivascular aortic tissue and liver were placed in cryomolds, covered in OCT and placed on dry ice. The remainder of the fat pads and liver, along with the brain, intestine and heart were snap frozen in liquid nitrogen. All samples were then stored at -80°C.

2.7.3 Serum cholesterol levels

As high levels of LDL cholesterol and low levels of HDL cholesterol are considered to be pro-atherogenic, it was essential to determine the effect of CardioWise and catechin treatment exerted on cholesterol levels in wild type mice receiving a high fat diet. Cholesterol levels were

determined with a HDL and LDL/VLDL cholesterol assay kit. To assess total cholesterol levels no additional preparation of the collected serum was required, however additional steps were needed to separate the HDL and LDL/VLDL fractions. The serum was mixed with precipitation buffer (provided by the manufacturer) in order to separate the LDL/VLDL fraction from the rest of the serum. The mixture was then incubated at room temperature for 10 minutes. The sample was then centrifuged at 2,000 *x g* for 10 minutes. The HDL fraction is located in the supernatant, which was removed and placed in to a clean Eppendorf tube. The remaining sample was re-centrifuged under the same conditions and any remaining supernatant aspirated. The pellet was resuspended in PBS (1x, pH 7.4), this is the LDL/VLDL fraction. The levels of free and total cholesterol was measured in all three fractions and compared to a standard curve which was prepared according to manufacturer's instructions. The absorbance was measured in a microplate reader at 570 nm. The assay is designed to detect up to 1 µg of cholesterol. As a result, samples being assessed were diluted if they exceeded this maximum limit of detection.

2.7.4 Serum TG levels

TG levels were determined with a triacylglycerol quantification assay kit. During the assay, all TGs within the serum were broken down into FFAs and glycerol. The glycerol is then oxidised to produce a product which is capable of interacting with target probe and induce a colour change. Therefore the greater the colour change, the more TGs are present in the serum. Initially the serum samples were mixed with lipase for 20 minutes at room temperature in order to convert the TGs into glycerol and fatty acids. The samples were then combined with an assay buffer, probe and enzyme mix according to manufacturer's instructions and incubated at room temperature for 1 hour while protected from light. The colour change was measured in all samples and compared to a standard curve which was prepared according to manufacturer's instructions. The absorbance was measured in a microplate reader at 570 nm. The assay is designed to detect between 2 pmol and 10 nmol of TG. As a result, samples being assessed were diluted if they exceeded this maximum limit of detection.

2.7.5 Oxidative Stress State

2.7.5.1 ROS Levels

To assess the levels of ROS production in the mice following treatments, an OxiSelect *in vitro* ROS/reactive nitrogen species (RNS) Assay kit (green fluorescence) was used. The principle of the assay is similar to that described in Section 2.4.6. A dichlorodihydrofluorescin (DCFH) probe is oxidised by ROS into a highly fluorescent probe and therefore the stronger the fluorescent signal detected the more ROS were present in the serum. No additional steps were required in the preparation of the samples and 50 µl of the sample and H_2O_2 standard curve (made according to manufacturer's instructions) were transferred to a 96-well plate. An equal volume of catalyst solution (provided in kit) was added to each well and the plate incubated for

an additional 5 minutes at room temperature while protected from light. The samples were then mixed with a DCFH solution (provided in kit), a probe which is broken down in the presence of H_2O_2 into a fluorescent form, for 30 minutes in the dark. After incubation, fluorescence was measured in a fluorescence microplate reader, with excitation at 485 nm and emission detected at 535 nm, and compared to the H_2O_2 standard curve. The vehicle control was then arbitrarily assigned as 1.0 and all other data were presented relative to this. The assay has a sensitivity limits of 10 pM.

2.7.5.2 MDA Levels

Lipid peroxidation is a useful indicator of the level of oxidative stress which is occurring. One product of lipid peroxidation is malondialdehyde (MDA) and therefore measuring its levels using an OxiSelect thiobarbituric acid reactive substances (TBARS) Assay Kit can reveal useful insights into the oxidative stress state of the mice. Thiobarbituric acid reacts with MDA to produce a fluorescent molecule, as a result the strength of the fluorescent signal produced by this reaction can be an indicator for the level of lipid peroxidation which has occurred. Initially to prevent further oxidation, 0.05% (v/v) butylated hydroxytoluene solution was added to the samples. An equal volume of SDS lysis solution was added to the samples and incubated at room temperature for 5 minutes. Thiobarbituric acid (TBA) was added to the mixture and the samples were subsequently incubated at 95°C for 30 minutes before being cooled to room temperature on ice. Samples were then centrifuged at 500 *x g* for 15 minutes. The supernatant was transferred to a 96-well plate and fluorescence was measured in a fluorescence microplate reader, with excitation at 535 nm and emission detected at 590 nm. The levels of MDA measured in all samples were compared to a standard curve (prepared according to manufacturer's instructions). The assay is designed to detect between 0 µmol and 125 µmol of MDA. As a result, samples being assessed were diluted if they exceeded this maximum limit of detection.

2.7.6 Plasma cytokine levels

Plasma cytokine levels were determined by the Central Biotechnology Services at the school of Medicine using a V-PLEX Plus Pro-inflammatory Panel1 Mouse Kit and 25 µl of serum from each mouse.

2.7.7 Liver gene expression

Approximately 50 mg of liver was placed in a mortar along with 1 ml of RiboZol™ and the tissue samples were homogenised with a pestle. RNA was then extracted following the method outlined in Section 2.5.1. The RNA was then used in a reverse transcription reaction (Section 2.5.2) before gene expression was assessed by a qPCR array (Section 2.5.3).

2.7.8 Cell populations within the bone marrow analysis

Previous studies have shown that a high fat diet is capable of altering the proportion of haematopoietic stem/progenitor cells within the bone marrow (Chan *et al.* 2012; Wu *et al.* 2013; Adler *et al.* 2014; van den Berg *et al.* 2016). The bone marrow of wild type mice was collected following treatment with either CardioWise or catechin hydrate or appropriate control (see Section 2.7.2) to determine whether either nutraceutical was capable of preventing high fat diet induced changes in haematopoietic cell populations.

The day after collection, the tibia and fibia were placed in a mortar along with 10 ml of PBS (1x, pH 7.4) which was supplemented with HI-FCS (2% v/v). The bones were then crushed until no visible bone marrow remained in the bones and the extracted marrow had been homogenised. The mixture was placed on a sterile filter (pore size of 70 μm) and allowed to pass through into a large Falcon tube. Further 2% (v/v) PBS was added to the filter until a final volume of 30 ml had been collected in the Falcon tube. The total number of cells was then calculated and either 10 or 8 million cells were placed in separate Falcon tubes to analyse the signalling lymphocytic activation molecule (SLAM) and progenitor cell populations respectively. As the SLAM and progenitor cells form a small proportion of the total number of cells extracted, these large cell numbers were required to ensure a sufficient number of target cells were detected during FACS analysis. The cells were then centrifuged at 5,000 *x g* for 5 minutes at 4°C (these setting were used for centrifugation unless stated otherwise).

Cells from the bone marrow were stained at 4°C for 30 minutes with a biotinylated mix of lineage marker antibodies present within the SLAM and progenitor cell populations. To analyse the SLAM cell population the following antibodies were used: 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. After initial staining, the cells were washed with 2% (v/v) PBS and resuspended in PerCP-Cy5.5-conjugated streptavidin and incubated for a further 15 minutes at 4°C. The SLAM and progenitor cell populations were now ready to be analysed by FACS.

While the SLAM and progenitor cell populations are being primed for FACS analysis, the preparation of lineage cell population was carried out simultaneously. Cells were incubated at 4°C for 20 minutes 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. All markers of the haematopoietic cell populations analysed in this study are presented in Table 2.8. Following incubation, the cells were washed and resuspended in 2% (v/v) PBS.

Table 2.8. Markers used to identify haematopoietic cell populations within the bone marrow.

Class	Cell type	Identifiable marker
SLAM	LSK	Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$
	HSC	$CD150^+$ $CD48^-$ LSK
	MPP	$CD150^-$ $CD48^-$ LSK
	HPC I	$CD150^-$ $CD48^+$ LSK
	HPC II	$CD150^+$ $CD48^+$ LSK
Progenitor	LK	Lin^- $Sca\text{-}1^-$ $c\text{-}Kit^+$
	CMP	$CD34^+$ $CD16/32^-$ LK
	MEP	$CD34^-$ $CD16/32^-$ LK
	GMP	$CD34^+$ $CD16/32^+$ LK
	CLP	$CD127^+$
Lineage	Granulocytes	$GR1^+$ $Mac1^-$
	Macrophages	$GR1^-$ $Mac1^+$
	MDSCs	$GR1^+$ $Mac1^+$
	B-cells	$B220^+$
	T-cells	$CD3^+$
	RBCs	$Ter119^+$

HSC, haematopoietic stem cell; MPP, multipotent progenitors; HPC, hematopoietic 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.

Before the samples could be analysed on the FACS machine, they were vortexed and filtered (pore size 40 µm) to prevent the cells from clumping together. Due to the high concentration of cells within the SLAM and progenitor samples, extra 2% (v/v) PBS was added. DAPI stain was added to all of the SLAM, progenitor and lineage samples in order to identify viable cells. A BD FACS Canto flow cytometer was then used to assess the composition of the cell populations and all possible cell counts were collected from each sample. The gating strategy used in this assay is summarised in Fig. 2.2 and the overall assay is summarised in Fig. 2.3. The process of backgating was performed to ensure the accuracy of the gating strategy. This is achieved by overlaying the final gated population over the preceding parent populations in order to determine whether all of the target cells had been isolated correctly.

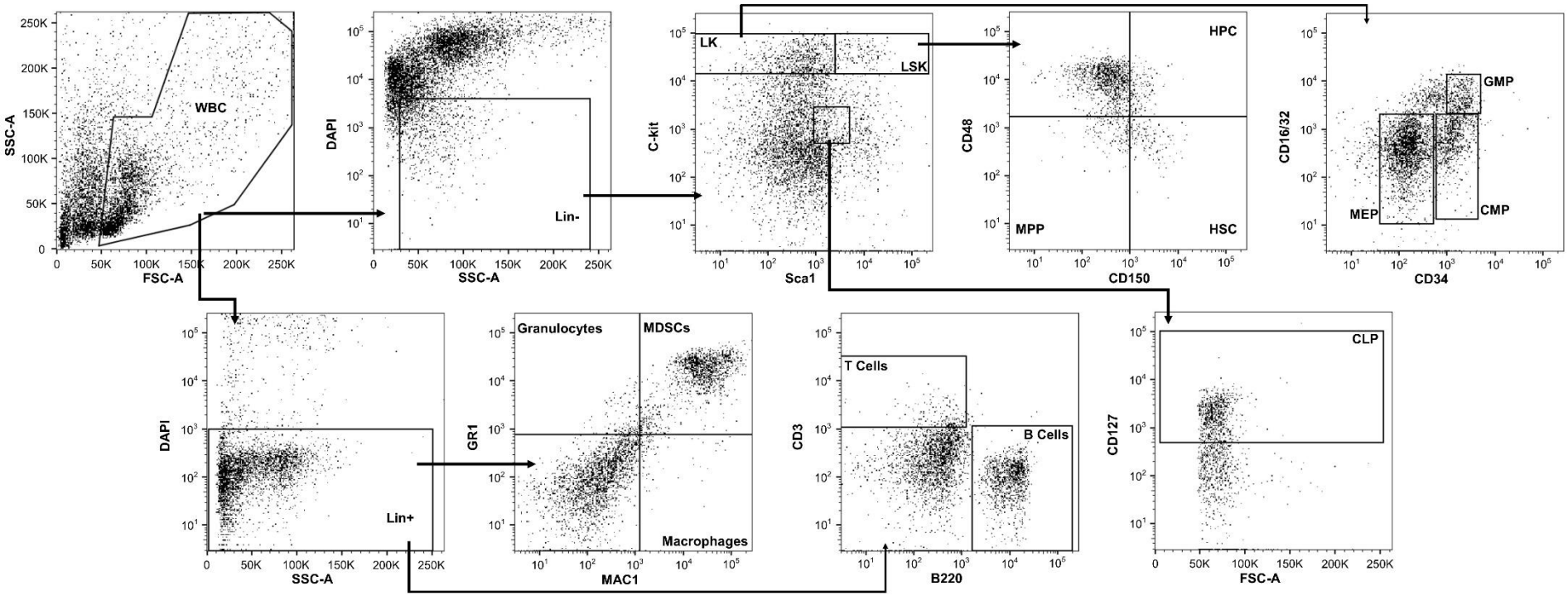

Fig. 2.2. An example of the gating strategy used during this study to identify and quantify the different haematopoietic cell populations within the bone marrow of wild type mice. The arrows indicate how each haematopoietic stem/progenitor cell population can be identified within the preceding gating strategy. The axis represent the various stains used to separate the cell populations (see Table 2.8 for further details). The forward scatter (FSC-A) is a measurement of cell size by determining the amount of light which passes around it. The side scatter (SSC-A) is a measurement of the amount of light which is reflected by particles within the cells and therefore can be used to determine the granularity of cells. HSC, haematopoietic stem cell; MPP, multipotent progenitors; HPC, hematopoietic progenitor cell; CMP, common myeloid progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-macrophage progenitor; MDSC, myeloid-derived suppressor cells; CLP, common lymphoid progenitor; LIN, lineage; WBC, white blood cell.

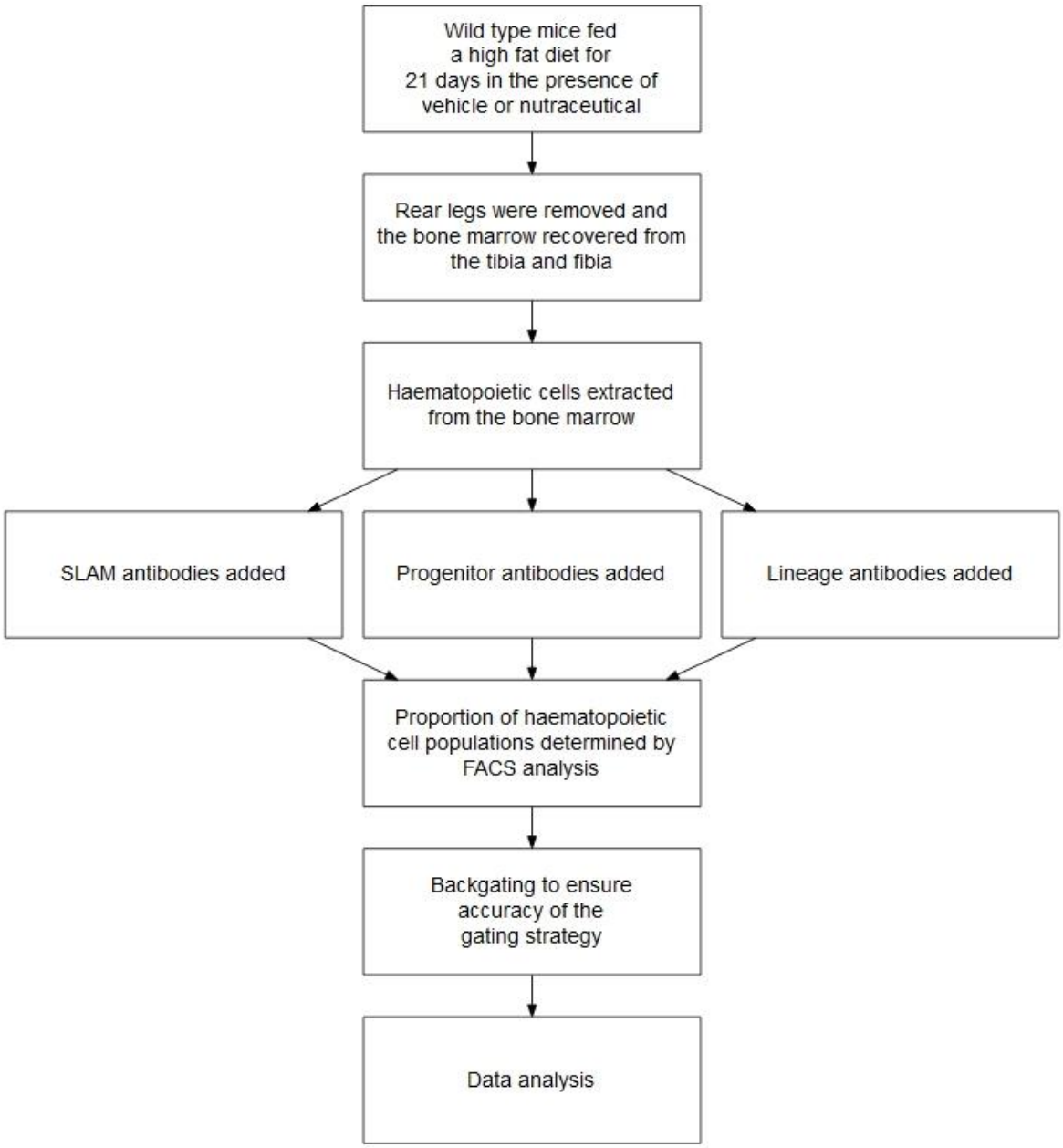

Fig. 2.3. Overall summary of the assay used to determine haematopoietic cell populations within the bone marrow of wild type mice. Further details such as antibodies used, incubation periods and cell extraction can be found within the main text and Table 2.8. FACS, flow assisted cell sorting; SLAM, signalling lymphocytic activation molecule.

2.8 Data Analysis

Normality of the data sets was tested using Shapiro-Wilk test, histograms and Q-Q plots and any data transformations have been stated. Data values outside two standard deviations of the mean were classed as outliers and removed before statistical analysis. To compare the means of two groups either a t-test (normal data distribution) or a Mann-Whitney test (non-normal distribution) were used. To compare the means of multiple groups a one-way analysis of variance (ANOVA) was used with a variety of post-hoc tests which were determined by the variation within the data. If there was equal variation between the different test groups either a Tukeys or Dunnett post-hoc analysis was performed, whereas Dunnett T3 or Games-Howell post-hoc tests were utilised in cases of unequal variation. In cases where a combination of continuous and categorical variables were assessed simultaneously a generalised linear model (GLM) was used. Where necessary a generalised linear mixed model (GLMM) was used to avoid pseudoreplication. All data analysis was performed in the R statistical software package (R Core Team 2015). Each independent experiment contained three technical replicates for all *in vitro* assays (except for qPCR array which were only performed once). Data are presented as the average of all experiments. Significance was defined as $p \leq 0.05$.

Chapter 3

CardioWise can attenuate key processes in atherosclerosis development *in vitro*

3.1 Introduction

CardioWise is a unique emulsion of nutritionally active compounds which are thought to exert strong anti-inflammatory effects (Moss and Ramji 2016b), therefore it represents a promising nutraceutical combinational therapy for the prevention and treatment of atherosclerosis and must be investigated further. It contains the two major ω-3 PUFAs EPA and DHA, derived from fish oil, as well as cocoa flavanols in the primary form of catechin. As previously discussed the health benefits of ω-3 PUFAs have long been known while flavanols are rapidly emerging as potential anti-oxidants for use in atherosclerosis (Moss and Ramji 2016b). Initially CardioWise was designed to either contain or not contain phytosterols. Although there is some promising cardiovascular health benefits associated with phytosterols, there is still a concern about their possible adverse effects (Moss and Ramji 2016b; Köhler *et al.* 2017). Therefore two forms of CardioWise were utilised to discern any detrimental effects *in vitro* following the inclusion of phytosterols.

CardioWise has been designed as a convenient way for an individual to boost their ω-3 PUFA intake and achieve current guidelines. The current advice given by the UK government and WHO is that individuals should be consuming two portions of fish every week with at least one being considered rich in ω-3 PUFAs (SACN 2004; WHO 2007). A fish containing 200-500 mg of EPA and DHA are considered to be one portion of fish (WHO 2007). Increasing an individual's EPA and DHA dietary intake through supplementation represents the easiest way to achieve target levels (Garaiova *et al.* 2007). It is essential easy solutions are found to increase ω-3 PUFA intake as the majority of the UK public consume less than one portion of fish every week (SACN 2004). Nutraceutical combinational therapies have previously been shown to improve lipid parameters in patients, therefore the combination of anti-inflammatory nutraceuticals within CardioWise make it a promising therapy (Pirro *et al.* 2016; Spigoni *et al.* 2017). However any potential health benefits must be assessed using *in vitro* atherosclerotic model systems before moving on to *in vivo* analysis.

A variety of doses and model systems were used to analyse the efficacy and safety of CardioWise in the presence or absence of phytosterols. Initially a range of physiological doses of CardioWise were used to assess the effect of CardioWise on cell viability (Table 2.2; Manach *et al.* 2005; Batta *et al.* 2006; Garaiova *et al.* 2007). As CardioWise is a new product and a novel combination of nutraceuticals, it is important to determine whether it exerts any adverse

effects. Cell viability was determined using two independent assays, the first was a crystal violet stain which binds to protein and DNA (Feoktistova *et al.* 2016). As the assay was conducted in adherent cells, those that underwent apoptosis or necrosis would become detached and removed during the assay procedure. Consequently the amount of crystal violet stain present is proportional to amount of DNA present and therefore cell viability and cell proliferation. To confirm these results, cell viability was also determined using a LDH assay. LDH is normally localised within the cytoplasm of the cell, however when cells undergo apoptosis or necrosis their membranes can become leaky or lyse causing the secretion of LDH into the surrounding media (Chan *et al.* 2013). Therefore the amount of LDH present in the media surrounding the cells correlates to the amount of cell death which has occurred.

After determining that CardioWise did not exert any adverse effects on cell viability, its effects on IFN-γ induced pro-inflammatory gene expression was determined. Due to their role in immune cell recruitment to the site of oxLDL accumulation during early atherosclerosis lesion formation, *MCP-1* and *ICAM-1* have become two robust markers of atherosclerosis. Their expression is also tightly regulated by IFN-γ. The expression of nearly 25% of genes can be induced in macrophages by IFN-γ (McLaren and Ramji 2009). IFN-γ is often considered a master regulator of atherosclerosis disease progression due to its ability to influence all three major stages of plaque development including immune cell recruitment, foam cell formation and plaque stability (see *Moss and Ramji* (2015) for more detail). A 60% reduction in lipid accumulation within atherosclerotic lesions in addition to a general attenuation in disease development was observed in *ApoE* deficient mice when the IFN-γ receptor was knocked out, highlighting the important role of IFN-γ in atherosclerosis (Gupta *et al.* 1997). Therefore being able to attenuate IFN-γ induced gene expression with CardioWise would signal strong anti-inflammatory effects. In this assay both a 1x and 2x physiological dose was used to determine whether any adverse effects would occur if someone accidentally took double the recommended dosage, once it was determined safe only a 1x dose was used for the remaining assays.

Another key step in atherosclerosis disease development is the migration of monocytes from the artery lumen into the wall of artery at the site of oxLDL accumulation. Monocyte recruitment was assessed using a modified Boyden chamber, which involves monocytes crossing a porous membrane. The membrane mimics the endothelial layer in the artery by having pores which monocytes can pass through towards a chemoattractant. MCP-1 was used as the chemoattractant due to its major role in the initial stages of lesion formation (Moss and Ramji 2016a; Ramji and Davies 2015). The importance of MCP-1 has been demonstrated in atherosclerotic mouse models where smaller atherosclerotic lesions formed in animals that were either deficient in the cytokine or its receptor (Öhman *et al.* 2010; Yadav *et al.* 2010).

Decreasing monocyte recruitment, the initial step in lesion formation, with CardioWise would represent a promising preventative therapy for atherosclerosis.

The formation of foam cells occurs when cholesterol homeostasis becomes disrupted in favour of reduced cholesterol efflux and enhanced cholesterol uptake, resulting in higher levels of intracellular cholesterol (Lusis 2000; McLaren *et al.* 2011a; Buckley and Ramji 2015). The effect of CardioWise on the cholesterol efflux potential of human macrophages was determined using radioactively labelled cholesterol. Stimulating the efflux of radioactive cholesterol from preloaded macrophages with ApoA1 into the surrounding media allows determination of the cholesterol efflux capability. Increasing the radioactivity in the surrounding media with CardioWise would indicate an increase in cholesterol efflux and therefore a reduction in foam cell formation. Furthermore increased levels of ROS are associated with an increase in oxLDL levels (Lusis 2000; McLaren *et al.* 2011a; Buckley and Ramji 2015). In the presence of ROS the non-fluorescent probe DCFDA is broken down into a highly fluorescent molecule which can be measured on a microplate reader. Therefore a reduction in the fluorescent signal following CardioWise treatment would mean it is capable of attenuating ROS generation. Increasing cholesterol efflux and reducing ROS production as a result of CardioWise treatment would mean it exerted strong anti-atherogenic effects.

More recently greater emphasis has been placed on the differing roles of macrophage phenotypes during atherosclerosis plaque formation. As research continues to emerge in this area it appears the situation is relatively complex with a large variety of subclass whose roles are not always well defined or easily disguisable. However there is broad agreement that two of the major classes of macrophages which exist can be defined as those which are classically activated (M1 phenotype) or those that are alternatively activated (M2 phenotype; Geeraerts *et al.* 2017; Rojas *et al.* 2015; Komohara *et al.* 2016). M1 macrophages play a key role in enhancing an inflammatory reaction in the innate immune system in response to bacterial infection, whereas the M2 macrophages are able to influence inflammatory resolution and repair (Leitinger and Schulman 2013). This has led to the consensus that M1 macrophages are pro-inflammatory whereas M2 macrophages are anti-inflammatory. The formation of M1 macrophages can be induced by a variety of pro-inflammatory cytokines including IFN-γ and IL-1β, and they are able to exert their harmful effects by secreting pro-inflammatory cytokines such as TNF-α, IL-6 and IL-12 (Moss and Ramji 2016a). The anti-inflammatory cytokines IL-4 and IL-13 are capable of inducing M2 macrophage phenotype formation, leading to the release of anti-inflammatory cytokines TGF-β and IL-10 (Moss and Ramji 2016a). Other macrophage phenotypes including M2b, M2c, M2d and M4 have been identified but their function remains inadequately defined (Moss and Ramji 2016a). Due to the critical role macrophages play during atherosclerosis disease progression, targeting their phenotype formation may represent an ideal therapeutic target. If the balance can be influenced to reduce M1 phenotype while

simultaneously enhancing M2 formation, it may result in a retardation in plaque formation. As the M1 and M2 phenotypes have unique cell surface markers it is possible to assess the effect of CardioWise on M1 macrophage phenotype formation by assessing the gene expression levels of identifiable markers inducible NOS (*iNOS*) and arginase 2 (*Arg2*).

3.2 Experimental Aims

Details of specific methodologies for each experimental aim are outlined in Chapter 2 and the experimental strategies for Chapter 3 are presented in Figs. 3.1 – 3.6.

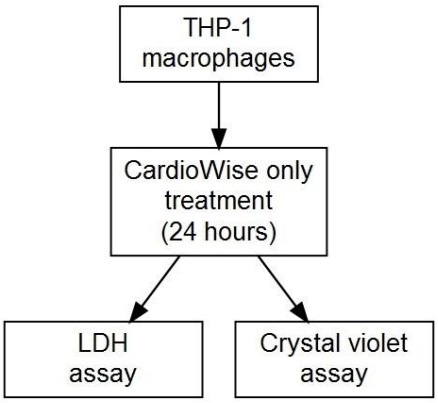

Fig. 3.1. Cell viability experimental strategies. LDH, lactate dehydrogenase. A 24 hour time point was chosen for this assay to ensure sufficient time for determination of changes in the level of cell proliferation.

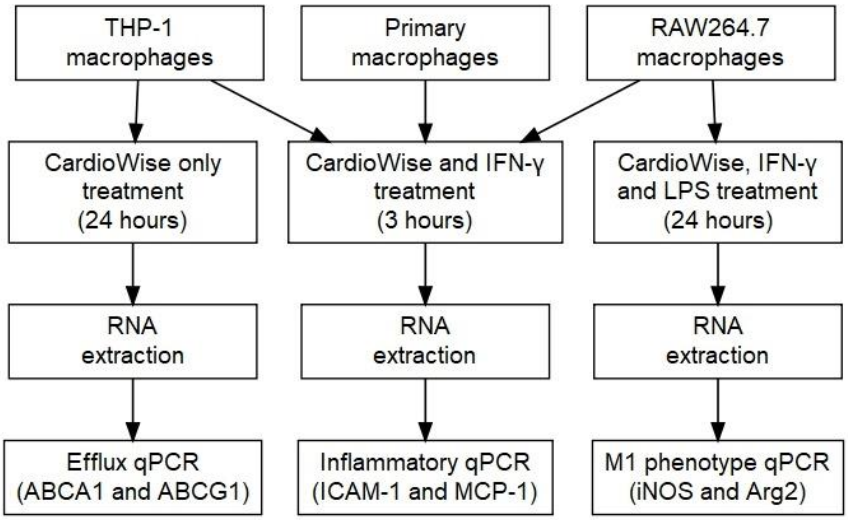

Fig. 3.2. Strategy for assessing gene expression. Previous studies have suggested that differentiating THP-1 monocytes into macrophages with PMA can result in an established M1 phenotype (Park *et al.* 2007). For this reason the murine RAW264.7 macrophages were used to assess the effect of catechin on M1 macrophage phenotype formation in this study. A 24 hour time point was chosen to ensure sufficient time for determination of changes in the level of cholesterol efflux and M1 phenotype gene expression. A 3 hour time point has previously been shown to be sufficient to study pro-inflammatory gene expression levels (Moss 2014). ABC, ATP-binding cassette; Arg2, arginase 2; ICAM, intercellular adhesion molecule; IFN, interferon; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MCP, monocyte chemotactic protein.

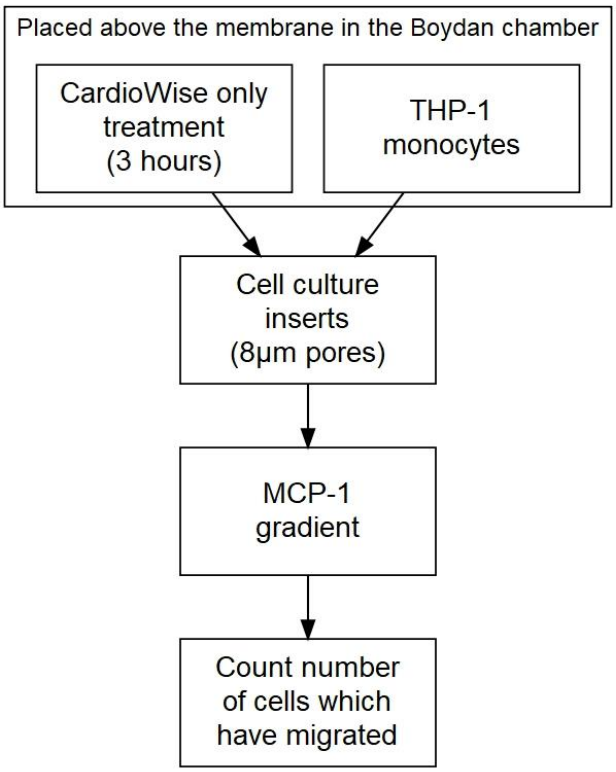

Fig. 3.3. Monocyte migration experimental strategy. A 3 hour time point has previously been shown to be sufficient to study monocyte migration levels (Moss 2014). MCP, monocyte chemotactic protein.

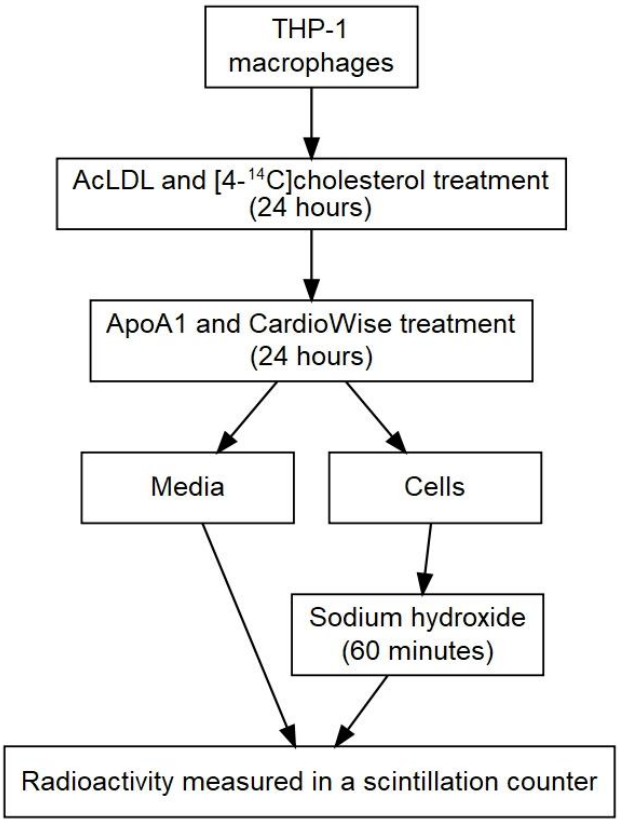

Fig. 3.4. Experimental strategy for determining cholesterol efflux. A 24 hour time point was chosen to ensure sufficient time for cholesterol efflux to occur. AcLDL, acetylated LDL; ApoA1, apolipoprotein A1.

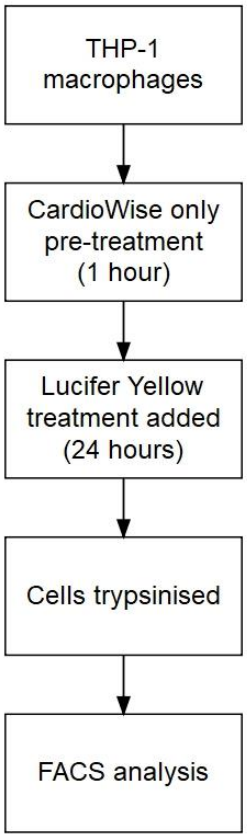

Fig. 3.5. Strategy for assessing macropinocytosis. A 24 hour time point was chosen to ensure sufficient time for Lucifer Yellow to be taken up by the cells. FACS, flow assisted cell sorting.

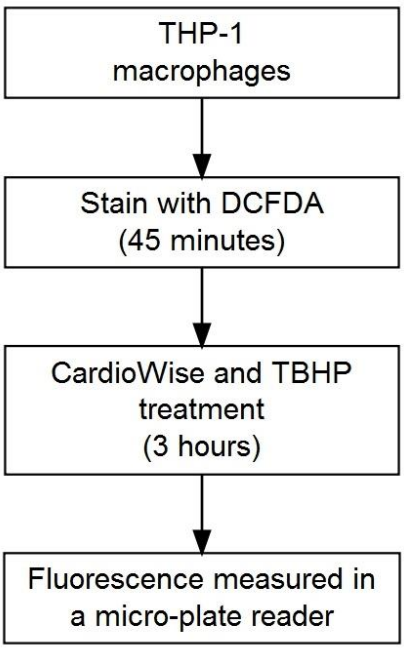

Fig. 3.6. ROS detection experimental strategy. A 3 hour time point has previously been shown to be sufficient to study ROS generation in macrophages (Moss 2014). DCFDA, 2'7'-dichlorofluorescin diacetate; TBHP, Tert-butyl hydrogen peroxide.

3.3 Results

3.3.1 The viability of human macrophages is unaffected by CardioWise *in vitro*

The viability of human THP-1 monocyte-derived macrophages was determined by either a crystal violet assay or a LDH assay. Furthermore due to the possible adverse effects of phytosterols, CardioWise was assessed in the presence or absence of phytosterols. As shown in Fig. 3.7 there were no significant differences in cell proliferation (Fig. 3.7A and 3.7C; crystal violet assay) or cell viability (Fig. 3.7B and 3.7D; LDH assay) following treatment of the cells with either the complete CardioWise or CardioWise without phytosterols for 24 hours in comparison to the vehicle control.

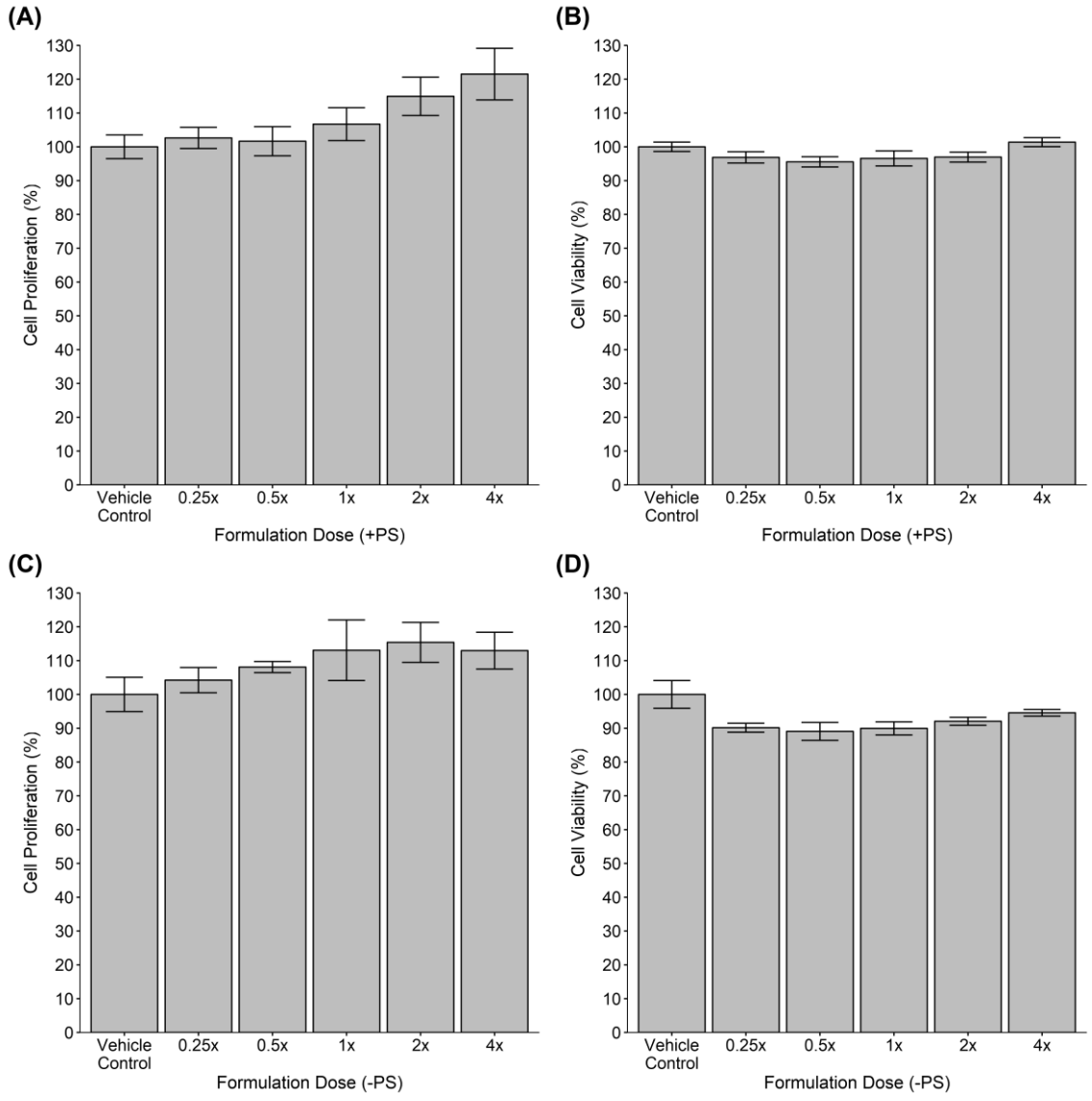

Fig. 3.7. No detrimental effects were observed in human macrophages following treatment with physiologically relevant doses of CardioWise. Crystal violet (A and C) or lactate dehydrogenase (B and D) assays were used to asses cell viability in PMA differentiated THP-1 macrophages that were treated with vehicle (vehicle control) or various doses of the complete CardioWise formulation (+PS) or various doses of CardioWise lacking phytosterols (-PS) for 24 hours. Data were normalised to the vehicle control that has been arbitrarily assigned as 100%. The data are presented as the mean ± SEM from three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnett T3 post-hoc analysis.

3.3.2 CardioWise attenuates the expression of pro-inflammatory genes following IFN-γ stimulation in human macrophages

IFN-γ is capable of inducing the expression of two key markers of inflammation, *MCP-1* and *ICAM-1*, which also play major roles in the development of atherosclerosis development (Moss and Ramji 2016a; Li *et al.* 2010). The expression of *MCP-1* in human PMA differentiated THP-1 macrophages was significantly increased by 7.83 and 10.13 fold ($p<0.001$ and $p<0.001$) following IFN-γ stimulation (250 U/ml) for 3 hours (Fig. 3.8A and 3.8C). Furthermore the transcript levels of *ICAM-1* were increased by 2.55 and 1.99 fold ($p=0.014$ and $p=0.001$) when compared to the vehicle control (Fig. 3.8B and 3.8D). When treated with either a 1x or 2x physiological dose of the complete CardioWise formulation, the IFN-γ stimulated *MCP-1* expression was attenuated by 71.3% ($p=0.004$) and 73.6% ($p=0.001$) respectively (Fig. 3.8A). A non-significant trend of decrease (51.1%; $p=0.108$) in IFN-γ induced expression of *ICAM-1* was observed when cells were incubated with 1x dose of complete CardioWise, whereas the 2x dose reduced *ICAM-1* expression by 57.5% ($p=0.010$; Fig. 3.8B). Similar observations were seen in human THP-1 monocyte-derived macrophages which were treated with CardioWise in the absence of phytosterols (Figs. 3.8C and D). The expression of IFN-γ induced *MCP-1* expression was reduced by 79.1% ($p=0.001$) and 78.2% ($p=0.002$) when treated with the 1x or 2x physiological dose respectively (Fig. 3.8C). In comparison to the IFN-γ stimulated cells, the expression of ICAM-1 was attenuated in the presence of CardioWise lacking phytosterols at the 1x and 2x dose by 52.8% ($p<0.001$) and 64.7% ($p<0.001$) respectively (Fig. 3.8D). These results were confirmed in primary HMDM (see Section 3.3.3). Furthermore similar observations were seen in murine RAW264.7 macrophages (Supplementary Fig. S.1).

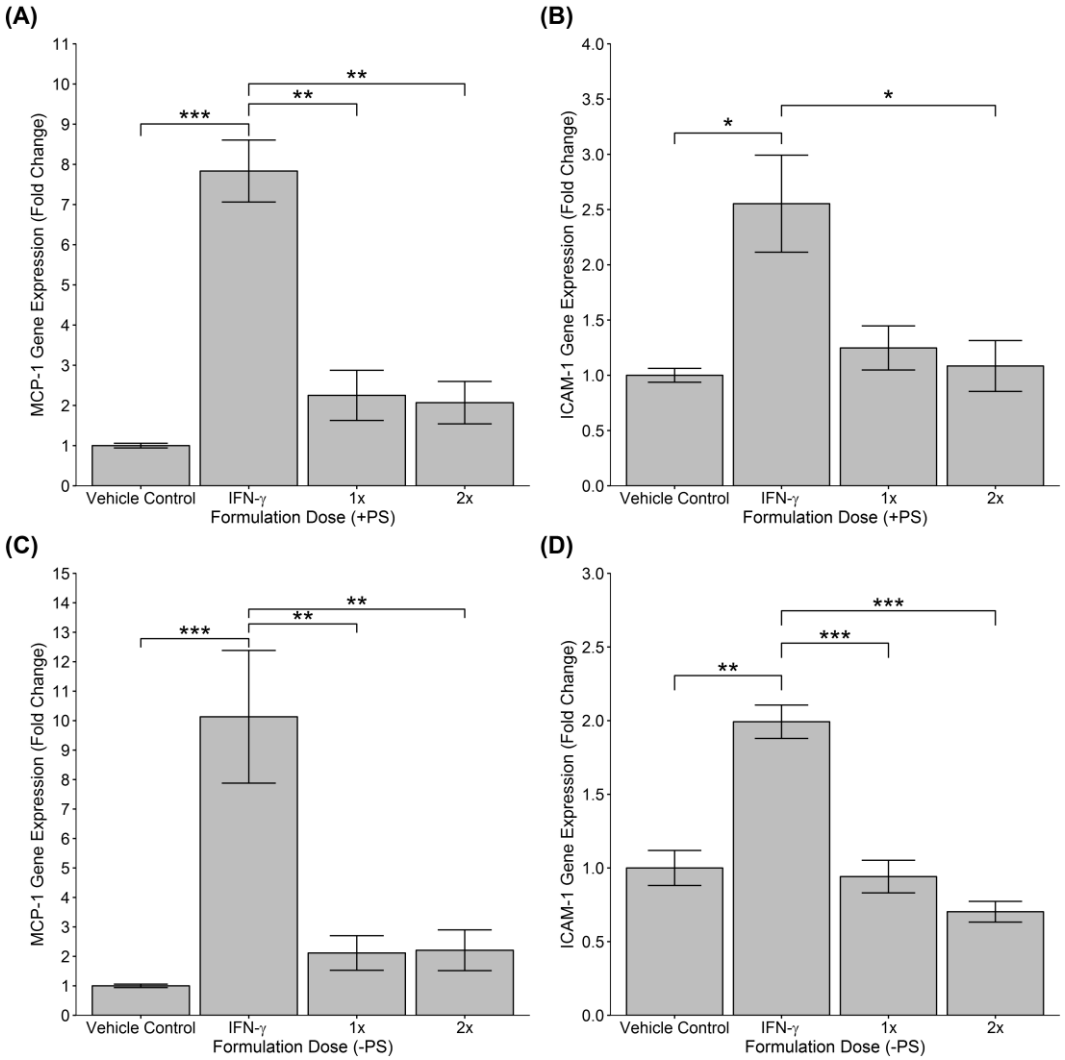

Fig. 3.8. CardioWise can inhibit the IFN-γ induced expression of *MCP-1* and *ICAM-1* at physiologically relevant doses in human macrophages. The expression of *MCP-1* (A and C) and *ICAM-1* (B and D) were assessed in PMA differentiated THP-1 macrophages that were either treated with vehicle (vehicle control) or with IFN-γ (250 U/ml) or IFN-γ (250 U/ml) in the presence of the complete CardioWise formulation (+PS; A and B) or with IFN-γ (250 U/ml) in the presence of CardioWise lacking phytosterols (-PS; C and D) for 3 hours. Gene expression levels were assessed using qPCR and calculated using the comparative Ct method and normalised to *GAPDH* levels with values from vehicle treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from three independent experiments. Statistical analysis was performed using a one-way ANOVA with Dunnett T3 post-hoc analysis on log-transformed data where * p <0.05, ** p <0.01 and *** p <0.001.

3.3.3 CardioWise can attenuate pro-inflammatory gene expression in primary human macrophages

To rule out the possibility that the results observed in THP-1 macrophages were due to the cell line used, gene expression analysis was repeated in primary cultures of HMDMs. IFN-γ stimulation (250 U/ml for 3 hours) significantly increased the transcript levels of *MCP-1* (Fig. 3.9A; 3.13-fold increase (p<0.001)) and *ICAM-1* (Fig. 3.9B; 1.75-fold increase (p=0.016)) in primary HMDMs when compared to the vehicle control. However in the presence of 1x dose of CardioWise with phytosterols, significant 30.7% (p=0.011) and 62.9% (p=0.002) reductions, in IFN-γ induced *MCP-1* and *ICAM-1* transcript levels respectively were observed when

compared to cells treated with IFN-γ alone. Likewise, 28.2% (p=0.025) and 50.6% (p=0.013) reductions respectively were seen in IFN-γ induced *MCP-1* and *ICAM-1* expression in response to treatment with the formulation lacking phytosterols.

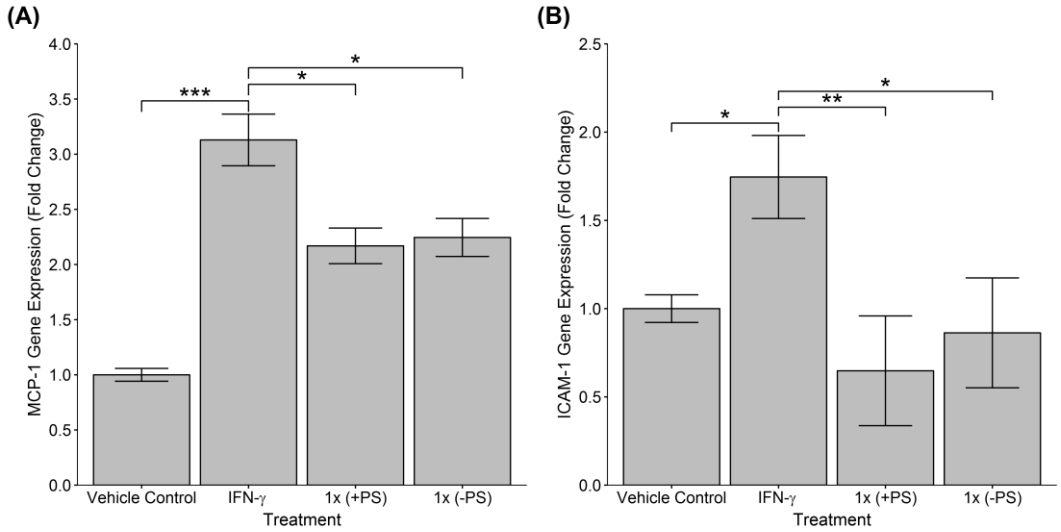

Fig. 3.9. A physiologically relevant dose of CardioWise inhibits pro-inflammatory gene expression in primary human macrophages. Gene transcript levels of *MCP-1* (A) and *ICAM-1* (B) were assessed in HMDMs that were either treated with vehicle (vehicle control) or IFN-γ (250 U/ml) or IFN-γ (250 U/ml) in the presence of the complete CardioWise formulation (+PS) or IFN-γ (250 U/ml) in the presence of the formulation lacking phytosterols (-PS) for 3 hours. Gene transcript levels were calculated using the comparative Ct method and normalised to *GAPDH* levels with values from vehicle treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from three (A) or four (B) independent experiments. Statistical analysis was performed on the log-transformed data using a one-way ANOVA with either a Tukeys (A) or Dunnett T3 (B) post-hoc analysis where * p <0.05, ** p <0.01 and *** p <0.001.

3.3.4 The migration of human monocytes is prevented by CardioWise

During the progression of atherosclerosis disease development, the recruitment of monocytes to the affected area is a key mechanism in lesion formation. The chemokine MCP-1 (20 ng/ml for 3 hours) is capable of recruiting monocytes and this was confirmed in our *in vitro* model as a significant 6.84 fold increase (p<0.001) in monocyte migration was observed in comparison to the vehicle control (Fig. 3.10). A 1x physiological dose of the complete CardioWise formulation reduced MCP-1 driven monocyte recruitment by 37% (p=0.019) in comparison to MCP-1 only. A similar decrease in monocyte recruitment (38%; p=0.001) was observed in cell treated with CardioWise in the absence of phytosterols. There was no significant difference in monocyte recruitment between the cells receiving CardioWise with or without phytosterols.

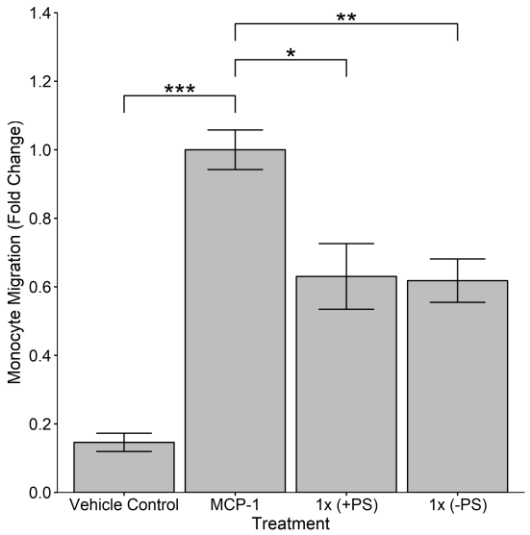

Fig. 3.10. The migration of human monocytes towards MCP-1 is inhibited by physiologically relevant doses of CardioWise. THP-1 monocytes were treated with vehicle (vehicle control) or treated with MCP-1 (20 ng/ml) alone or with MCP-1 (20 ng/ml) in the presence of CardioWise (+PS) or with MCP-1 (20ng/ml) in the presence the CardioWise lacking phytosterols (-PS) for 3 hours. Monocyte migration was then determined as described in Section 2.4.4. Data were normalised to the percentage of cells that migrated from the apical compartment of the modified Boyden chamber into the basolateral compartment in response to MCP-1 only treatment (arbitrarily assigned as 1). The data are presented as the mean ± SEM from four independent experiments. Statistical analysis was performed using a one-way ANOVA with a Games-Howell post-hoc analysis where * p <0.05, ** p<0.01 and *** p <0.001.

3.3.5 The efflux of cholesterol from human macrophages is increased following CardioWise treatment

Human PMA differentiated THP-1 macrophage foam cells, pre-loaded with cholesterol, were treated with ApoA1 (10 µg/ml) for 24 hours to stimulate the efflux of their intracellular radiolabelled cholesterol. Following ApoA1 only stimulation, the THP-1 macrophages were able to release approximately 20% of their cholesterol into the surrounding media, and this was arbitrarily assigned as 1 and the remaining data normalised to this (Fig. 3.11). Treatment with either a 1x physiological dose of CardioWise in the presence or absence of phytosterols significantly increased cholesterol efflux by 1.61 (p=0.002) and 1.53 (p=0.001) fold respectively. The effects on cholesterol efflux from the two forms of the CardioWise formulation were not significantly different from one another.

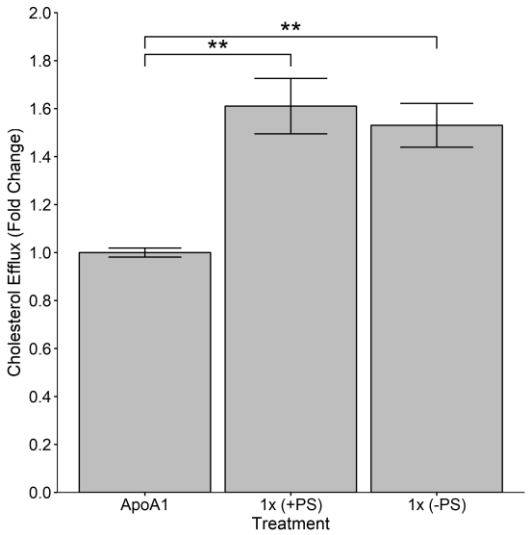

Fig. 3.11. CardioWise can induce cholesterol efflux from human macrophage foam cells at physiologically relevant doses. PMA-differentiated THP-1 cells were loaded with [4-^{14}C]cholesterol and acLDL for 24 hours prior to a further 24 hour treatment with ApoA1 (10 µg/ml) in the presence of vehicle or ApoA1 in the presence of CardioWise (+PS) or ApoA1 in the presence of CardioWise lacking phytosterols (-PS). The vehicle control (ApoA1) was arbitrarily assigned as 1 and remaining data normalised to this. The data are presented as the mean ± SEM from three independent experiments. Statistical analysis was performed using a one-way ANOVA with Dunnett T3 post-hoc analysis where ** p <0.01.

3.3.6 CardioWise can attenuate macropinocytosis in human macrophages

The uptake of cholesterol via macropinocytosis is an important early step in the development of atherosclerosis. Upon the inclusion of CardioWise in the absence of phytosterols (1x), macropinocytosis was significantly reduced (22.8%, p<0.001) when compared to cells treated with vehicle control (Fig. 3.12). However, no significant difference in macropinocytosis was observed between the complete CardioWise formulation and vehicle control.

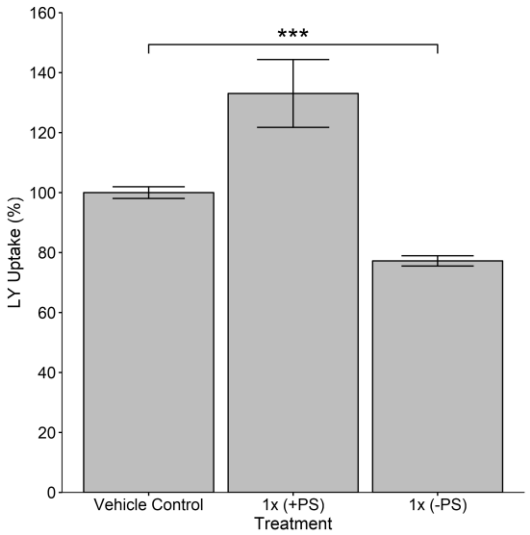

Fig. 3.12. A physiologically relevant dose of CardioWise can attenuate macropinocytosis in human macrophages. Macropinocytosis was assessed in PMA differentiated THP-1 macrophages that were either treated with vehicle (vehicle control) or with complete CardioWise (+PS) or with CardioWise lacking phytosterols (-PS) for 1 hour. Following pre-treatment, Lucifer yellow (LY; 100 µg/ml) was also added to all cells for a further 24 hours. LY uptake was measured using a BD FACS Canto flow cytometer and at least 5,000 counts were measured for each sample. LY uptake in the vehicle control was given an arbitrary value of 100%. The data are presented as the mean ± SEM from three independent experiments. Statistical analysis was performed using a one-way ANOVA with Dunnett T3 post-hoc analysis where *** p <0.001.

3.3.7 The expression of two cholesterol efflux genes is attenuated by the formulation

ABCA1 and *ABCG1* are both cholesterol efflux genes and therefore changes in their expression may correlate to possible changes in foam cell formation. As the effect of CardioWise on cholesterol efflux was assessed after 24 hours (Fig. 3.11), the expression of *ABCA1* and *ABCG1* was also determined after 24 hours. In the presence of 1x doses of CardioWise, there was a trend of decreased *ABCA1* (Fig. 3.13A) and *ABCG1* (Fig. 3.13B) expression of 44.4% and 54.7% respectively when compared to cells treated with vehicle control. Likewise, 43.4% and 57.8% reductions were observed in *ABCA1* and *ABCG1* expression in response to treatment with the formulation lacking phytosterols. However these results are based on PMA differentiated THP-1 macrophages, rather than THP-1 macrophages loaded with acLDL in order to form foam cells. Furthermore the results are from one independent experiment and therefore must be repeated using human macrophage foam cells before any firm conclusions can be drawn.

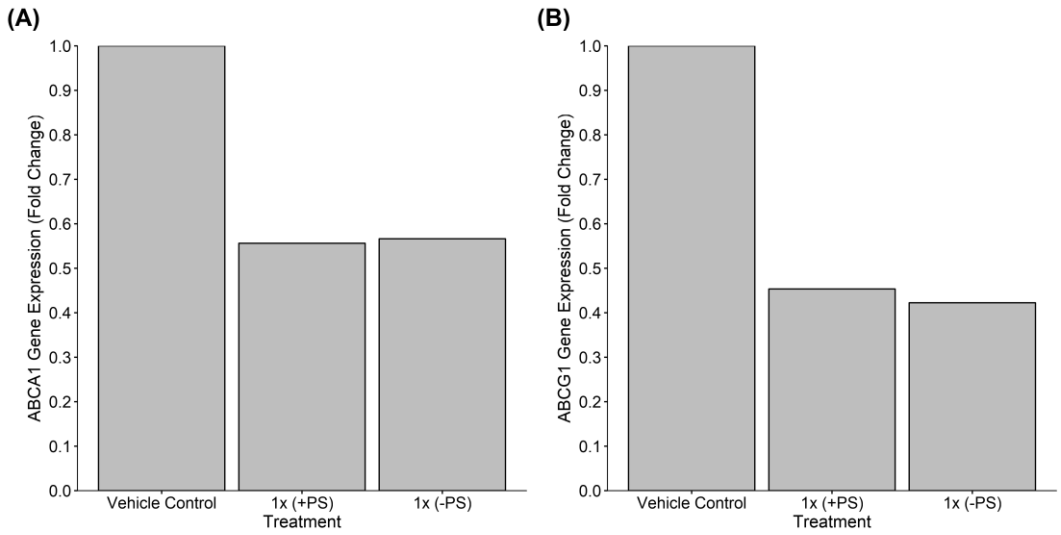

Fig. 3.13. A physiologically relevant dose of CardioWise appears to reduce the expression of two cholesterol efflux genes in human macrophages. Gene transcript levels of *ABCA1* (A) and *ABCG1* (B) were assessed in PMA differentiated THP-1 macrophages that were either treated with vehicle (vehicle control) or with CardioWise (+PS) or with the formulation lacking phytosterols (-PS) for 24 hours. Gene transcript levels were calculated using the comparative Ct method and normalised to *GAPDH* levels with values from vehicle treated cells given an arbitrary value of 1. The data are presented as the mean from one independent experiment performed in triplicate.

3.3.8 CardioWise can induce ROS production in human macrophages

The formation of ROS is a key step in the generation of oxLDL and therefore foam cell formation. The production of ROS can be induced in macrophages by treating them with tert-Butyl hydroperoxide (TBHP; 50 µM for 3 hours) and this was confirmed in our system as a significant 1.56-fold induction (p<0.001) in the formation of ROS in cells treated with TBHP alone was observed when compared to the vehicle control (Fig. 3.14). Upon the inclusion of the complete CardioWise formulation (1x), ROS production in response to TBHP was significantly increased (21.3%, p=0.026) when compared to cells treated with TBHP alone. Similarly, incubation with CardioWise lacking phytosterols resulted in a 77.7% (p<0.001) increase in ROS formation when compared to TBHP treated cells. However ROS production in the cells treated with the complete formulation was 31.7% lower (p<0.001) compared to those which has received the formulation lacking phytosterols.

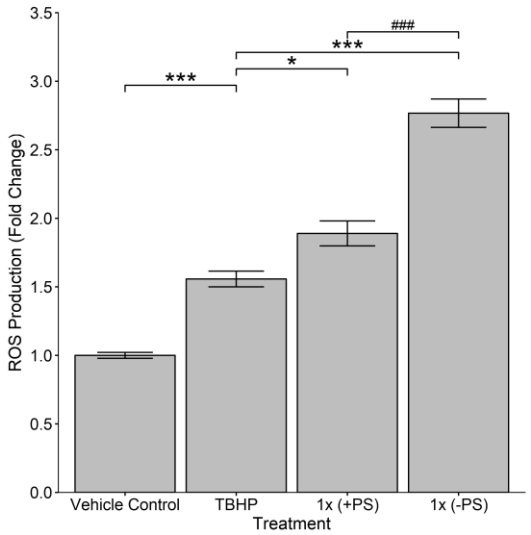

Fig. 3.14. A physiologically relevant dose of CardioWise can induce ROS production in human macrophages. ROS production was assessed in PMA differentiated THP-1 macrophages that were either treated with vehicle (vehicle control) or tert-Butyl hydroperoxide (TBHP; 50 μM) or TBHP (50 μM) in the presence of CardioWise (+PS) or TBHP (50 μM) in the presence of CardioWise lacking phytosterols (-PS) for 3 hours. ROS production was measured in a fluorescence microplate reader, with excitation at 485 nm and emission detected at 535 nm. Vehicle control was given an arbitrary value of 1. The data are presented as the mean ± SEM from three independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukeys post-hoc analysis where * p <0.05 and *** p <0.001 when compared to TBHP. $^{###}$ p <0.001 when comparing +PS to –PS.

3.3.9 CardioWise attenuates the expression of M1 phenotype markers in murine macrophages

Due to the possibility of PMA differentiating THP-1 monocytes resulting in an established M1 macrophage phenotype, murine RAW264.7 macrophages were used during this assay (Park *et al.* 2007). Previous studies have demonstrated that a combined treatment of IFN-γ and lipopolysaccharide (LPS) is capable of inducing M1 macrophage polarisation and both *iNOS* and *Arg2* are robust markers of the M1 phenotype (Khallou-Laschet *et al.* 2010; Dugo *et al.* 2017). For this reason the expression of *iNOS* and *Arg2* was determined following co-stimulation with IFN-γ and LPS (250 U/ml and 100 ng/ml respectively for 24 hours) to determine the effect of CardioWise on macrophage polarisation. The expression of *iNOS* (Fig. 3.15A; p<0.001) and *Arg2* (Fig. 3.15B; p<0.001) was significantly increased following co-stimulation treatment. Incubation with the complete CardioWise formulation resulted in a decrease in the expression of both *iNOS* and *Arg2* by 48% (p=0.004) and 42% (p=0.015) respectively. Similar results were observed in cells treated with CardioWise lacking phytosterols, where a significant decrease in *iNOS* expression (45%; p=0.002) and a trend of decrease in *Arg2* expression (37%; p=0.056) was seen when compared to the IFN-γ and LPS co-stimulation only group. The presence or absence of phytosterols had no significant effect on either *iNOS* or *Arg2* expression.

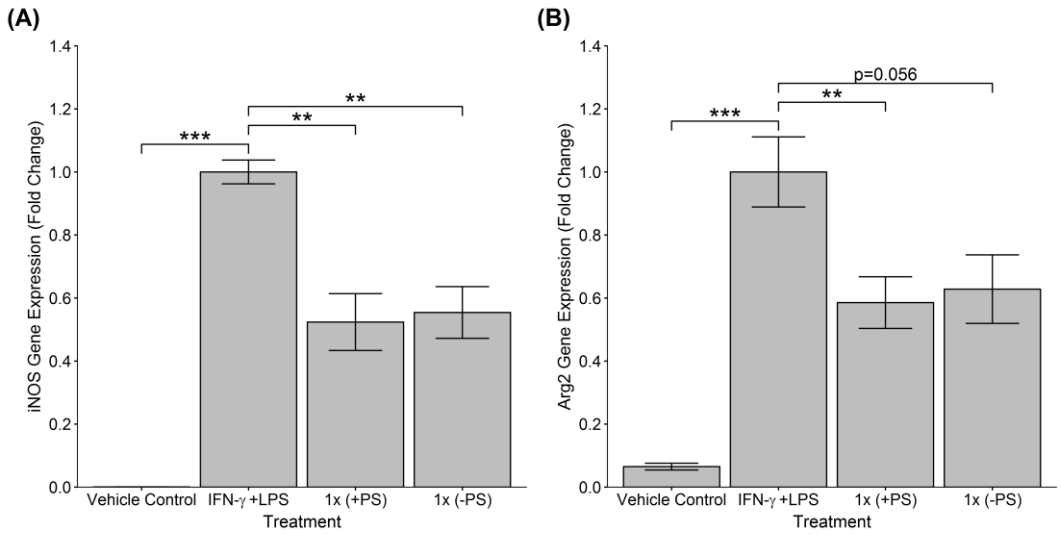

Fig. 3.15. M1 polarisation in murine macrophages is inhibited by a physiologically relevant dose of CardioWise. The expression of *iNOS* (A) and *Arg2* (B) were assessed in murine RAW264.7 macrophages that were either treated with vehicle (vehicle control); with IFN-γ (250 U/ml) and LPS (100 ng/ml); with IFN-γ (250 U/ml) and LPS (100 ng/ml) in the presence of the complete CardioWise formulation (+PS) or with IFN-γ (250 U/ml) and LPS (100 ng/ml) in the presence of CardioWise lacking PS (-PS) for 24 hours. Gene expression levels were assessed using qPCR and calculated using the comparative Ct method and normalised to *β-actin* levels with values from IFN-γ and LPS treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from five independent experiments. Statistical analysis was performed using a one-way ANOVA with Dunnett T3 post-hoc analysis on square root-transformed data where * p <0.05, ** p <0.01 and *** p <0.001.

3.4 Discussion

The various atherosclerosis-associated assays used for the different experiments in this chapter demonstrate the ability of the unique nutraceutical combination, known as CardioWise, to attenuate the expression of IFN-γ induced pro-atherogenic genes, reduce monocyte recruitment, enhance cholesterol efflux while simultaneous decreasing macropinocytosis and attenuate the polarisation of macrophages into their pro-inflammatory M1 phenotype. Although CardioWise did increase ROS generation, overall it appears to exert strong anti-atherogenic effects. This unique combination of ω-3 PUFAs, flavanols and phytosterols has been shown to work on a variety of pro-inflammatory processes associated with atherosclerosis *in vitro*, highlighting its possible use as a preventative during early atherosclerotic lesion formation.

Previous studies have not investigated the effects of a nutraceutical combination consisting of fish oils, cocoa extracts and phytosterols either *in vitro* or *in vivo*. Several studies focusing on the individual nutraceuticals have observed potential anti-inflammatory effects. However a variety of studies have also found fish oils, cocoa extracts and especially phytosterols to be capable of either exerting adverse effects and contributing to atherosclerosis lesion formation or having no observable anti-atherogenic properties (reviewed in Moss and Ramji 2016b). There are many reasons why these negative observations may have been made including: a

single agent being tested which does not take into consideration the possible synergistic effects between nutraceuticals that may occur during dietary intake; pure forms of the compounds being utilised rather than the naturally and most abundant forms which ignores possible beneficial metabolic by-products produced by side chains not present on the pure molecule; nutraceuticals being used at dosages that are unrealistic and unachievable from dietary absorption; differences in the genetic makeup of clinical trial participants; presence or absence of other risk factors such as high blood pressure, increased cholesterol levels and age; and patients also receiving high levels of pharmaceutical therapies such as statins. In order to try and account for some of these limitations, this study used the nutraceuticals in their most widely occurring natural forms rather than the pure compound, as previous studies have shown them to be capable of influencing macrophage function (Oh *et al.* 2010; Sabeva *et al.* 2011; Byun *et al.* 2014). The study also used the nutraceuticals at doses that have been previously observed following dietary intake (Manach *et al.* 2005; Batta *et al.* 2006; Garaiova *et al.* 2007). This makes the observations seen in this study more translatable and more representative of the effects which may occur following CardioWise consumption. THP-1 monocytes and macrophages were used in this study due to their significant role in all stages of atherosclerosis disease progression and also because the responses observed in THP-1 cells are highly conserved in HMDM and *in vivo* studies (Michael *et al.* 2012b; McLaren *et al.* 2010a; McLaren *et al.* 2010b). Indeed the key finding of attenuated IFN-γ-induced expression of *MCP-1* and *ICAM-1* (Fig. 3.8) was directly confirmed in primary HMDM cultures (Fig. 3.9).

As CardioWise is a novel formulation it was essential to initially confirm it did not exert any detrimental effects on either cell proliferation or viability of human macrophages *in vitro* (Fig. 3.7). These results also confirm the alterations in gene expression and cell functions observed in other assays were as a result of CardioWise rather than as a consequence of altered cell viability. The primary function of phytosterols is to reduce dietary cholesterol uptake from the lumen of the intestine, however they are also known to cross the gut-blood barrier at extremely low levels (Chan *et al.* 2006; Gylling *et al.* 2014). Furthermore individual phytosterols have been found to cause adverse effects on macrophages *in vitro,* including the phytosterols sitosterol and stigmasterol, which are present in CardioWise (Sabeva *et al.* 2011). Therefore during this study, CardioWise in the presence or absence of phytosterols was used in all of the assays to ensure no detrimental effects were observed following exposure of the cells to the total dose of phytosterols. The inclusion of phytosterols did not alter the effect of CardioWise on cell viability (Fig. 3.7), pro-inflammatory gene expression (Figs. 3.8 and 3.9), monocyte migration (Fig. 3.10), cholesterol efflux (Fig. 3.11), “cholesterol efflux” gene expression (Fig. 3.13) and M1 macrophage polarisation (Fig. 3.15), providing no evidence to support its removal from the formulation. However the inclusion of phytosterols did affect macropinocytosis and ROS production (Figs. 3.12 and 3.14 respectively; reasons for this are discussed in greater detail later).

IFN-γ is a major pro-inflammatory cytokine which is considered to be one of the key orchestrators of atherosclerosis disease progression (Moss and Ramji 2016a; Moss and Ramji 2015; Ramji and Davies 2015). Several immune cells found within atherosclerotic plaques, such as macrophages, natural killer T-cells and T-lymphocytes, produce and release IFN-γ at prominent levels (van Boxel-Dezaire and Strak 2007; Kleemann *et al.* 2008). Furthermore due to its role in all stages of atherosclerotic plaque formation, IFN-γ is thought of as a potential therapeutic target (Moss and Ramji 2015; Moss and Ramji 2016a). Previous work in the laboratory has shown that IFN-γ is capable of stimulating the expression of the pro-inflammatory genes *MCP-1* and *ICAM-1* (Li *et al.* 2010). Products of these genes are capable of significantly driving monocyte recruitment during early atherosclerosis lesion formation at the site of oxLDL accumulation. In this study we show that a physiologically relevant dose of CardioWise in the presence or absence of phytosterols is capable of reducing IFN-γ-mediated expression of *MCP-1* and *ICAM-1* in PMA differentiated THP-1 macrophages (Fig. 3.8), primary HMDMs (Fig. 3.9) and murine macrophages (Supplementary Fig. S.1). These results highlight the possibility of using CardioWise in order to attenuate monocyte migration to the activated endothelium during the early stages of atherosclerosis disease progression. The pro-inflammatory stimuli induced expression of *ICAM-1* and *MCP-1* has also been reduced in a variety of cell types following individual treatment with ω-3 PUFAs, flavanols and phytosterols in other studies (Hughes *et al.* 1996; Miles *et al.* 2000; Nakanishi *et al.* 2010; Wang *et al.* 2014b; Bustos *et al.* 2008).

In addition to attenuating *MCP-1* gene expression, a 1x physiological dose of CardioWise was also capable of decreasing the recruitment of monocytes towards a MCP-1 stimulus (Fig. 3.10). In theory these results suggest that a physiological dose of CardioWise would be doubly effective at reducing the size of atherosclerotic lesions. Firstly it is capable of reducing the levels of MCP-1 produced by activated cells and secondly it then decreases the responsive of monocytes to move towards the MCP-1 stimuli. This makes CardioWise an extremely promising anti-atherogenic therapy. Previous studies using ω-3 PUFAs, flavanols and phytosterols individually have also observed reductions in monocyte recruitment (Brown *et al.* 2012; Melgarejo *et al.* 2009; Bustos *et al.* 2008). The alterations observed in *MCP-1* gene expression (Figs. 3.8 and 3.9) may mechanistically explain the changes seen in monocyte migration (Fig. 3.10). These results imply that CardioWise may reduce the number of macrophages which accumulate at the site of oxLDL build-up in atherosclerotic lesions and therefore result in a smaller lesion. However this hypothesis still requires extensive *in vivo* investigation in mouse models of atherosclerosis which was unfortunately not performed during this study due to time limitations.

The unbalancing of cholesterol homeostasis towards increased cholesterol uptake and reduced efflux within macrophages leads to the formation of foam cells, which form the initial

fatty streak in atherosclerosis disease progression. Following treatment with CardioWise in the presence or absence of phytosterols, there was a significant increase in ApoA1-mediated cholesterol efflux from cholesterol laden human macrophage foam cells (Fig. 3.11), indicating a possible attenuation in foam cell formation. These results correlate with previous *in vitro* studies which assessed the effects of individual nutraceuticals on cholesterol efflux (Lada *et al.* 2003; Bursill and Roach 2006; Sabeva *et al.* 2011). Macropinocytosis is the mechanism by which cholesterol crystals are taken up by macrophages (Bobryshev 2006). Cholesterol crystals are able to activate inflammasomes, an intracellular multi-protein complex in the innate immune response which is capable of converting the pro-inflammatory cytokines IL-1β and IL-18 into their mature forms (Hoseini *et al.* 2017). In the absence of phytosterols CardioWise was able to decrease macropinocytosis uptake (Fig. 3.12), showing it is capable of increasing cholesterol efflux while simultaneously reducing cholesterol uptake. However the effect of CardioWise on receptor-mediated cholesterol uptake remains to be assessed. In contrast, CardioWise with phytosterols showed a non-significant trend of increased macropinocytosis. This is the first result in which the addition of phytosterols altered the effect of CardioWise, this may be due to the fact that phytosterols and cholesterol have almost identical structures. The PMA differentiated THP-1 macrophages may have recognised the presence of phytosterols in the same way it recognises cholesterol, which in turn triggered a slight increase in macropinocytosis. As the vast majority of phytosterols will not be taken up from the intestine and therefore unlikely to come into contact with macrophages in atherosclerotic lesions, the trend of increase in macropinocytosis seen is this *in vitro* study is not of concern but should be kept in mind when analysing future *in vivo* studies.

The expression of two “cholesterol efflux” genes, *ABCA1* and *ABCG1*, was reduced following CardioWise treatment (Fig. 3.13). This result is unusual because it shows the opposite result of the cholesterol efflux assay (Fig. 3.11), however it is worth noting that the gene expression assay is only based on one independent experiment and therefore requires repeating in order to draw firm conclusions. Furthermore mRNA expression levels do not always match protein abundance levels (Vogel and Marcotte 2012), so it is possible to have reduced mRNA expression levels but increased ABCA1 and ABCG1 protein levels, this would have to determined using a western blot. Furthermore there are a variety of other cholesterol efflux genes including liver X receptors (*LXRs*) and *ApoE* which may have their expression increased following CardioWise treatment and therefore their expression levels should be assessed. Previous *in vitro* studies have observed increases in “cholesterol efflux” gene expression following treatment with ω-3 PUFAs and phytosterols (Song *et al.* 2013; Sabeva *et al.* 2011).

The normal physiological function of vascular cells, including VSMC contraction and endothelial homeostasis, are reliant on ROS for cell signalling (Chen *et al.* 2017c). Failure to maintain a balance in ROS formation can have serious detrimental effects and increased ROS

production has been associated with increased cell damage, lipid peroxidation, immune cell recruitment and MMP activation (Chen *et al.* 2017c). Therefore reducing ROS formation represents a potential therapeutic avenue for attenuating atherosclerosis disease progression. In this study both forms of CardioWise significantly increased hydrogen peroxide-induced ROS formation (Fig. 3.14), however ROS levels were significantly higher following treatment with CardioWise in the absence of phytosterols when compared to the formulation containing phytosterols. These results imply that the addition of phytosterols provides a level of anti-oxidant protection that is not present in the base formulation, indeed a previous *in vitro* study has demonstrated that β-sitosterol (a phytosterol present in CardioWise) is capable of increasing the activity of superoxide dismuatase (SOD; Vivancos and Moreno 2005). Superoxide dismutase is an anti-oxidant enzyme which converts oxygen free radicals into oxygen molecules or hydrogen peroxide. However as previously mentioned, only a small amount of the phytosterols will be absorbed in the intestine and therefore CardioWise without phytosterols is a more accurate indication of the effect it will exert on macrophages within atherosclerosis lesions. This is the first assay which indicates that CardioWise may exert detrimental pro-inflammatory effects, this results needs to be confirmed *in vivo*.

Due to the pro-atherogenic role of M1 macrophages, it is important to target macrophage polarisation in order to reduce the levels of M1 macrophages within lesions. Furthermore it is emerging that M1 macrophages also contribute to plaque instability and rupture after it was found they tend to be localised within the rupture prone shoulder regions of atherosclerotic plaques (Stöger *et al.* 2012). Following the treatment of murine macrophages with CardioWise in the presence or absence of phytosterols, the expression of two robust markers of the M1 phenotype, *iNOS* and *Arg2*, was significantly attenuated (Fig. 3.15). These results indicate that CardioWise may be able to stabilise mature atherosclerotic plaques and reduce the risk of them rupturing, however this hypothesis needs to be scrutinised *in vivo*. The findings in this study are similar to previous *in vitro* studies which have found that ω-3 PUFAs and flavanols are capable of reducing M1 polarisation and activation (De Boer *et al.* 2014; Chang and Deckelbaum 2013; Dugo *et al.* 2017).

Overall this is the first *in vitro* study which has demonstrated that a unique combination of ω-3 PUFAs, flavanols and phytosterols exerts strong anti-atherogenic effects at several key atherosclerosis disease progression steps. CardioWise was found to significantly attenuate pro-inflammatory gene expression, monocyte recruitment, M1 macrophage polarisation as well as enhancing cholesterol efflux (summarised in Fig. 3.16). As CardioWise is a combination of nutraceuticals it is important to also assess them individually to identify the most effective compounds (Chapter 5). The results of this *in vitro* study provides support for the use of CardioWise in an initial *in vivo* feeding study. Indeed Chapter 4 discusses the anti-atherogenic effects of CardioWise treatment in wild type mice fed a high fat diet for 3 weeks.

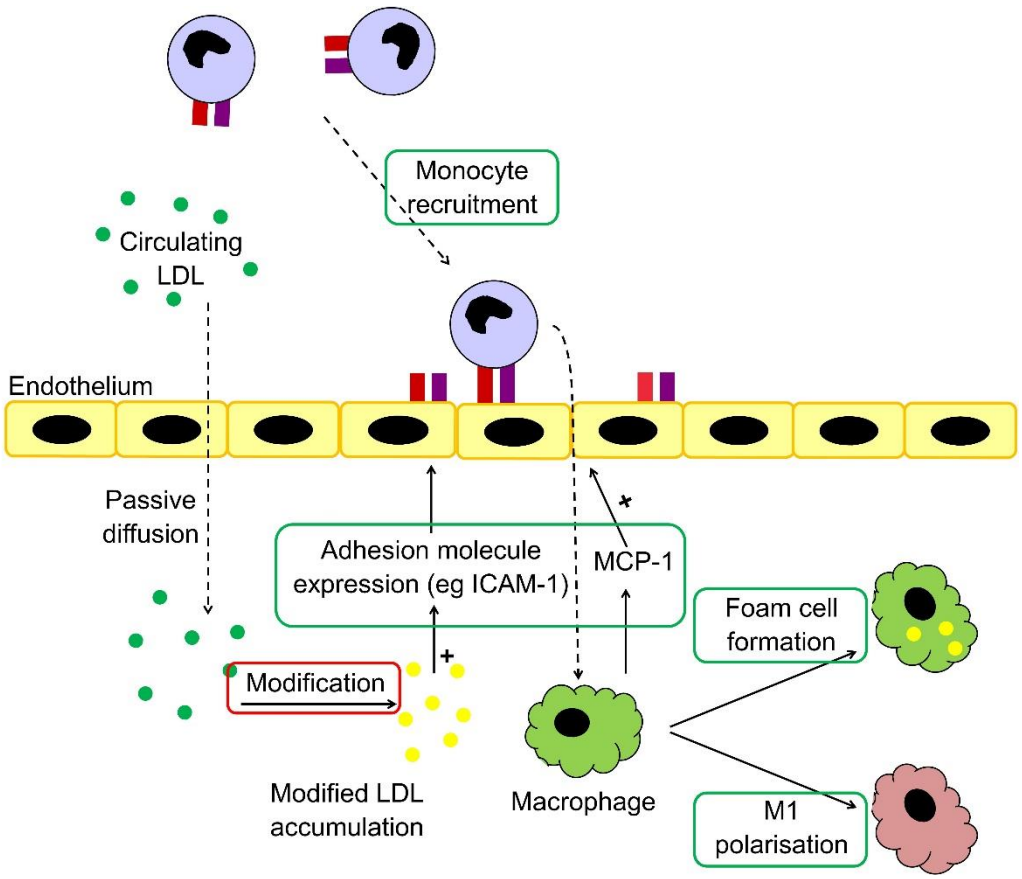

Fig. 3.16. Summary of the anti-atherogenic properties of CardioWise. The steps reduced in atherosclerosis disease progression following CardioWise treatment are highlighted in green whereas those increased by CardioWise are highlighted in red.

Chapter 4

The effects of CardioWise in wild type mice fed a high fat diet

4.1 Introduction

The results produced by the full CardioWise formulation in the *in vitro* assays covered in Chapter 3 justified its use in an initial mouse study to further explore its potential as an anti-atherogenic nutraceutical. The use of *in vivo* models allows for a greater understanding of the effect of novel treatments that cannot currently be replicated *in vitro*, due to the potential unknown effects which a whole body system may exert. To ensure the results of the preclinical study were more translatable to human subjects, the mice were treated with a 1x human equivalent physiological dose based on a body surface area calculation (Nair and Jacob 2016). The exact concentrations of the components of CardioWise can be found in Section 2.7.1. Wild type mice fed a high fat diet for 21 days were used as previous work in the laboratory has shown these conditions to induce markers of atherosclerosis development (Wijdan Al-Ahmadi personal communication). These markers include weight gain, changes in plasma cholesterol and TG levels, levels of pro-inflammatory cytokines within the serum, as well as changes in gene expression in the liver. Furthermore a study using wild type mice found that a high fat diet also raised the levels of these markers after just 14 days (Michael *et al.* 2017), indicating our study period is sufficient to observe potential anti-atherogenic effects of nutraceuticals. Therefore this *in vivo* model was utilised to assess the potential cardiovascular protective effects of CardioWise.

Initially, basic physical and physiological parameters were measured to ensure no detrimental effects occurred as a result of CardioWise treatment. Weight gain was assessed using a GLMM in order to determine the effects of both time, treatment and their interaction simultaneously. Furthermore as the study involved repeatedly measuring the same mice over the 21 day trial period, it suffered from a phenomenon known as pseudoreplication. In this study the weight of the 13 mice was measured 10 times. However traditional statistical methods, such as an ANOVA, would inappropriately interpret the data as 130 independent measurements rather than 13, making the p values associated with the ANOVA meaningless. Therefore to avoid pseudoreplication each mouse was given a unique ID tag and this tag was included within the random model portion of the GLMM.

The weight of the different fat pads within the mice was also measured to determine whether CardioWise affected fat accumulation or the location where the fat was stored. This is because recent studies have found white fat, brown fat and perivascular adipose tissue (PVAT) to

potentially have different roles in the progression of atherosclerosis (Gómez-Hernández *et al.* 2016; Hoeke *et al.* 2016; van Dam *et al.* 2017). White fat, such as subcutaneous, inguinal, gonadal and renal fat are used by the body to store excess dietary fat for organs to use as energy when required (van Dam *et al.* 2017). On the other hand, brown fat has been shown to be used by the body for maintaining body temperature by combusting fatty acids for heat generation (van Dam *et al.* 2017). PVAT is unique due to its location surrounding systemic blood vessels (van Dam *et al.* 2017). Unlike white and brown fat, PVAT maintains the normal function of blood vessels by providing structural support. During obesity and inflammatory disorders, white fat releases fatty acids into the plasma making it pro-atherogenic, whereas brown fat begins to store circulating TG in lipid droplets indicating it is anti-atherogenic (van Dam *et al.* 2017). The role of PVAT during atherosclerosis remains unclear as it has been shown to be both pro- and anti-atherogenic (van Dam *et al.* 2017). Therefore if CardioWise affects the proportion of white fat, brown fat and PVAT, it may imply that it is capable of exerting cardiovascular protective effects.

Previous studies have shown that ω-3 PUFAs, catechin and phytosterol consumption is capable of altering an individual's lipid profile (Franzese *et al.* 2015; Matsuyama *et al.* 2008; Ras *et al.* 2015). We therefore assessed the effect of CardioWise treatment on the plasma levels of total, LDL and HDL cholesterol. It has long been established that elevated LDL levels are associated with an increased risk of CVD whereas low HDL levels are considered pro-atherogenic (Buckley and Ramji 2015). CardioWise would be considered a strong anti-atherogenic nutraceutical combination if it was capable of lowering LDL levels as well as increasing HDL levels. However plasma concentrations of LDL and HDL in isolation are not always ideal predictors of an individual's risk of CVD and often the ratio of total/HDL cholesterol and LDL/HDL cholesterol are more useful as clinical risk indicators (Millán *et al.* 2009). The effect of CardioWise on the total/HDL, total/LDL and LDL/HDL cholesterol ratios was also determined. TG levels within the blood are another lipid parameter which can be used as an indicator of an individual's risk of suffering a CVD-related event (Talayero and Sacks 2011). Furthermore the ratio of TG/HDL cholesterol has been previously shown to be strongly associated with CVD (da Luz *et al.* 2008). Therefore it was expected that CardioWise would lower TG levels and TG/HDL cholesterol ratio in the plasma of wild type mice fed a high fat diet for 21 days.

During our *in vitro* studies CardioWise was found to potentially increase ROS generation (Fig. 3.14), therefore it was vital to assess the effect of CardioWise on ROS production *in vivo*. Furthermore the levels of lipid peroxidation were also measured to determine the full effect of CardioWise on ROS generation and its consequences as increased oxidative stress is associated with increased lipid peroxidation (Ayala *et al.* 2014; Chen *et al.* 2017c). Previous studies have shown that unregulated lipid peroxidation results in reducing the strength of the

arterial wall and increasing the risk of formation of an aneurysm by triggering the apoptosis of aortic cells (Kinnunen *et al.* 2012; Ayala *et al.* 2014). MDA is a major by-product of lipid peroxidation found within the blood, consequently measuring the effect of CardioWise on MDA levels can be used as an indicator for its effects on ROS damage and lipid peroxidation (Ayala *et al.* 2014).

The circulating concentrations of pro- and anti-inflammatory cytokines were also assessed in the plasma from wild type mice fed a high fat diet for 21 days following CardioWise treatment. The roles of the cytokines in atherosclerosis disease development are outlined in Table 4.1 and extensively reviewed by *Moss and Ramji* (2016) and *Ramji and Davies* (2015). These pro-inflammatory cytokines are key contributors to atherosclerosis progression and therefore it is hoped that CardioWise treatment is able to attenuate their secretion into the blood.

Table 4.1. The role of pro- and anti-inflammatory cytokines analysed during this study.

Cytokine	Role in atherosclerosis	Reference
CXCL1	Produced by macrophages and has been found to be increased in patients with CVD. *LDLr* deficient mice also lacking *CXCL1* were found to develop smaller atherosclerotic lesions.	Boisvert *et al.* 2006; Breland *et al.* 2008
IFN-γ	Genetic deletion in *ApoE* deficient mice results in reduced atherosclerotic lesion size and lipid accumulation.	Whitman *et al.* 2002; Koga *et al.* 2007a
IL-1β	*ApoE* mice which are also *IL-1β* deficient have reduced expression of pro-inflammatory genes and attenuated atherosclerosis development.	Kirii *et al.* 2003; Bhaskar *et al.* 2011
IL-2	Blocking IL-2 signalling in *ApoE* deficient mice retards atherosclerosis, whereas IL-2 injections enhances disease progression.	Dinh *et al.* 2012; Upadhya *et al.* 2004
IL-4	Induces a pro-inflammatory state in vascular endothelium. Deficiency in *ApoE* mice resulted in smaller plaque formation.	Davenport and Tipping 2003; Lee *et al.* 2010c
IL-5	Transplanting *IL-5* deficient bone marrow into *LDLr* deficient mice resulted in accelerated atherosclerosis.	Binder *et al.* 2004
IL-6	IL-6 injections in *ApoE* deficient mice resulted in increased pro-inflammatory cytokine levels and plaque size. However other studies have shown that both *ApoE* and *LDLr* mice which also lack *IL-6* suffer from enhanced atherosclerosis.	Schuett *et al.* 2012; Madan *et al.* 2008
IL-10	Genetic deletion in *ApoE* mice enhanced atherosclerosis disease development.	Potteaux *et al.* 2004; Eefting *et al.* 2007
TNF-α	Deficiency in *ApoE* mice reduced atherosclerosis development by decreasing foam cell formation and the expression of multiple pro-inflammatory genes.	Xiao *et al.* 2009; Xanthoulea *et al.* 2009

In order to determine whether the changes in physical and physiological parameters were as a result of altered gene expression, the expression of genes in the liver of wild type mice fed a high fat diet for 21 days was assessed by qPCR array. Furthermore, gene expression changes were measured within the liver due to the critical role it plays during metabolic disorders (Pietrangelo 2009). One of the major advantages of using qPCR arrays over regular qPCRs is that they produce more data in a shorter space of time, this is due to the plate being able to determine mRNA expression levels of 84 genes simultaneously. As different mice were used for the vehicle control group and the CardioWise treatment group, an average base line of gene expression had to be established for all the vehicle control treated mice rather than an independent base line for each mouse. Any p value of approximately 0.1 or lower was considered to show a trend of change and was included and discussed in the results section. It should be noted that due to the low expression levels of some genes, obtaining a Ct value was not achievable for every repeat. Mice with undetectable Ct readings were removed before statistical analysis and need to be investigated in future experiments by possibly increasing the starting amount of cDNA.

Stem and progenitors cells are capable of self-renewal and give rise to all the other cell types within the body. The haematopoietic stem cells (HSCs) give rise to all types of blood cells. Previous studies have observed changes in the stem and progenitor cell populations within the bone marrow of mice following a high fat diet (Chan *et al.* 2012; Wu *et al.* 2013; Adler *et al.* 2014; van den Berg *et al.* 2016). Alteration in bone marrow cell populations are also associated with atherosclerosis disease progression (Lang and Cimato 2014; Murphy *et al.* 2014; Ma and Feng 2016). For example an increase in HSC proliferation and differentiation has been observed in hypercholesterolaemic conditions (Lang and Cimato 2014; Ma and Feng 2016). Other cells such as the myeloid derived suppressor cell (MDSC) population is associated with chronic inflammatory diseases such as atherosclerosis and is often found in increased numbers during disease development (Foks *et al.* 2016). However the current role of MDSCs during atherosclerosis remains unclear. The formation of all other cell types from HSCs is outlined in Fig. 4.1. Using CardioWise treatment may represent a novel way of restoring the cell populations to their natural state during atherosclerosis.

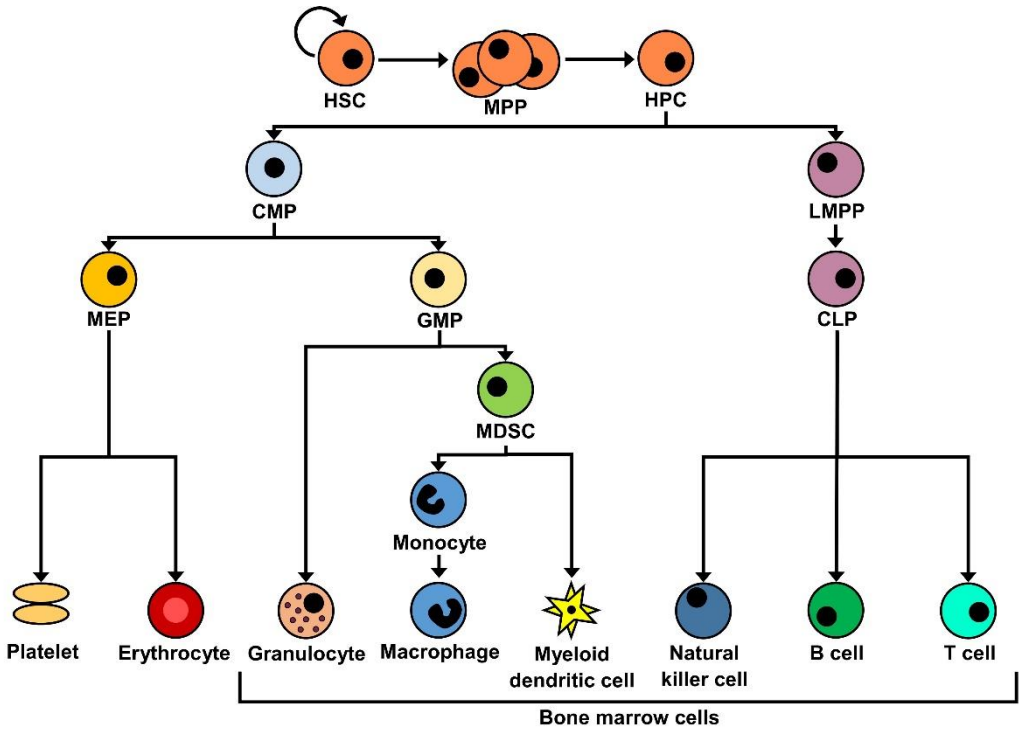

Fig. 4.1. The formation of haematopoietic cells from a haematopoietic stem cell. HSC, haematopoietic stem cell; MPP, multipotent progenitors; HPC, haematopoietic progenitor cell; CMP, common myeloid progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-macrophage progenitor; MDSC, myeloid-derived suppressor cells; LMPP, lymphomyeloid progenitor; CLP, common lymphoid progenitor.

4.2 Experimental Aims

Details of the specific *in vivo* methodologies are outlined in Chapter 2 and a brief experimental strategy for Chapter 4 is presented in Fig. 4.2.

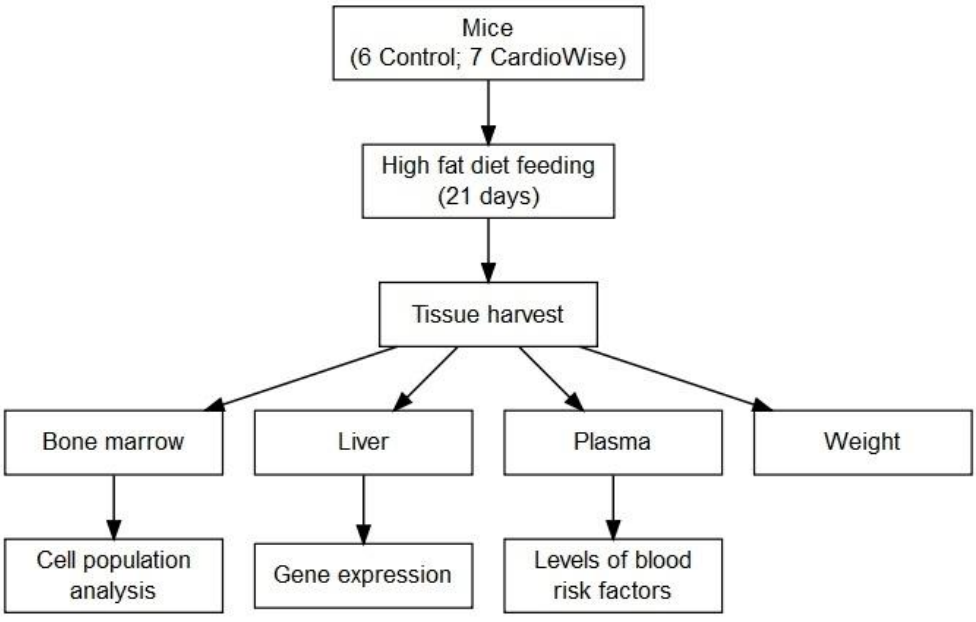

Fig. 4.2. Experimental strategy used to assess the effect of CardioWise *in vivo*.

4.3 Results

4.3.1 CardioWise had no effect on weight gain in wild type mice receiving a high fat diet

Mouse weight gain was positively correlated with time (Fig. 4.3), with mice gaining approximately 0.09 g per day resulting in a total average increase in weight of 1.89 g (p<0.001; adj-R^2=0.86). However there was no significant effect observed for CardioWise treatment or interaction between time and treatment type.

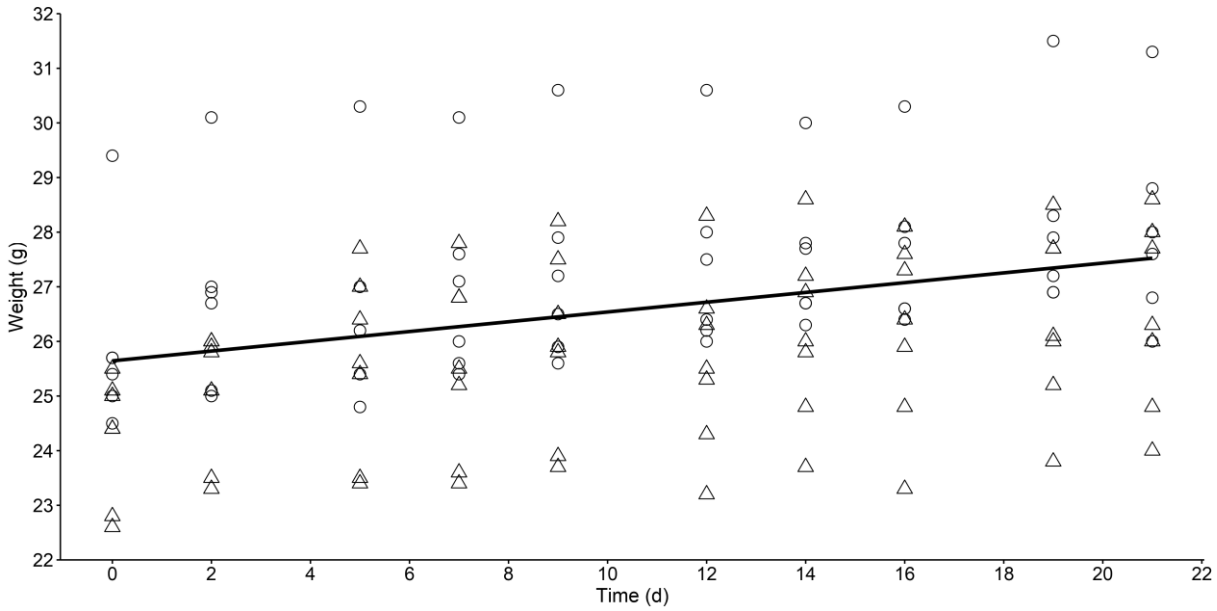

Fig. 4.3. The rate of weight gain in wild type mice fed a high fat diet was not altered by CardioWise. The weight of mice was measured every 2 or 3 days for the entire duration of the study (21 days). Mice were fed a high fat diet which was either supplemented with vehicle control (circle) or a 1x human equivalent physiological dose of CardioWise (triangle). The prediction of the average rate of weight gain, as calculated by a generalised linear mixed model with a Gamma error distribution and identity link function, is displayed from 13 mice (control 6; CardioWise 7). To avoid pseudoreplication the identity of each mouse was included within the model as a random term. The raw data from each mouse is also presented to provide a visual representation of the spread within the data.

4.3.2 The weight of fat pads in wild type mice fed a high fat diet was not significantly affected by CardioWise

As shown in Fig. 4.4, there were no significant differences in the weight of subcutaneous (Fig. 4.4A), inguinal (Fig. 4.4B), gonadal (Fig. 4.4C) or total (Fig. 4.4G) fat pads observed following CardioWise treatment in wild type mice fed a high fat diet in comparison to the vehicle control. The weight of renal fat and PVAT showed a non-significant trend of increase of 20.8% (p=0.139) and 79.9% (p=0.105) respectively following CardioWise treatment (Fig. 4.4D and F), whereas the brown fat pad weight was non-significantly reduced by 38.3% (p=0.217) when compared to the vehicle control (Fig. 4.4E).

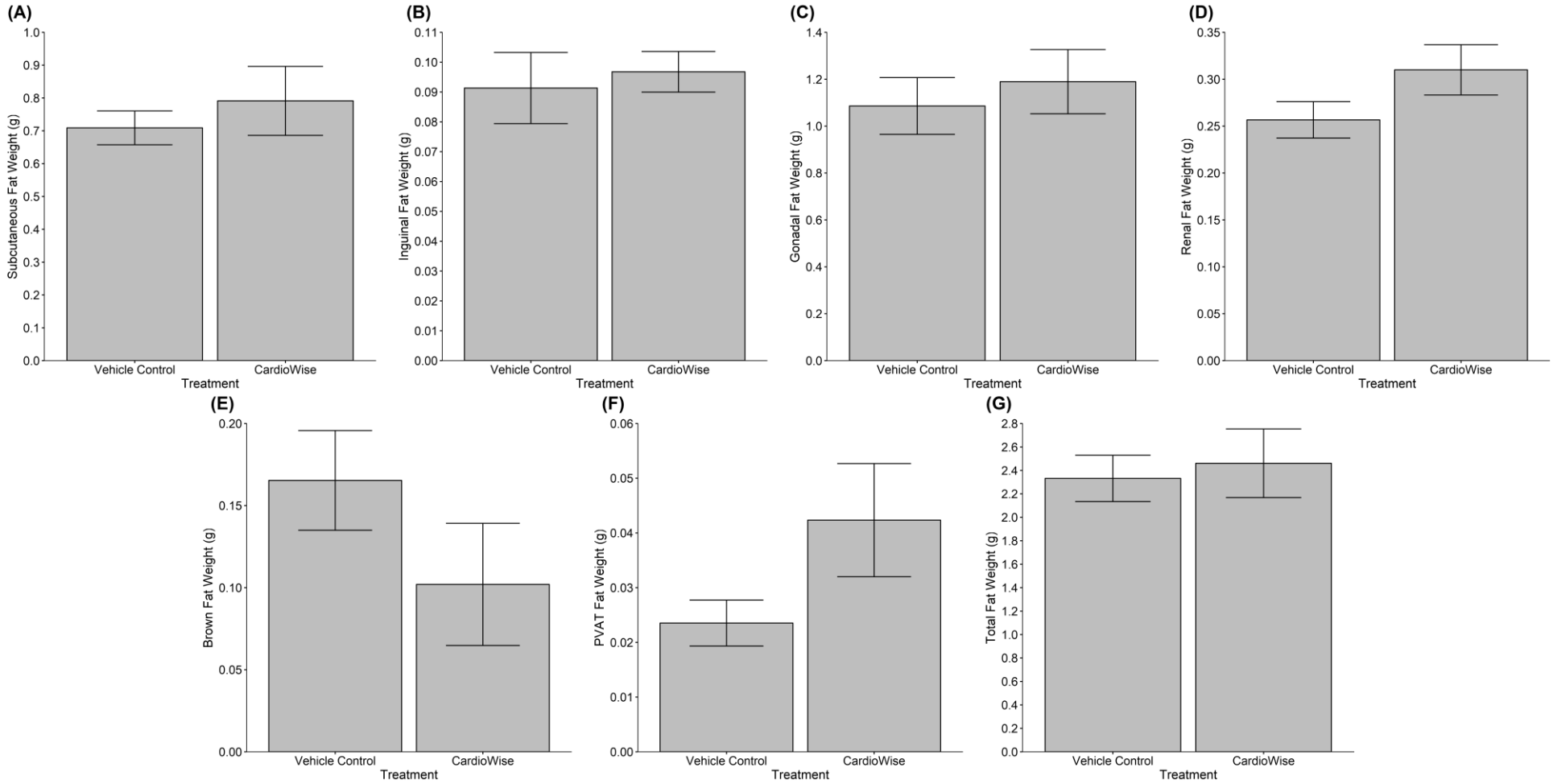

Fig. 4.4. No changes in fat pad weight were observed in wild type mice following feeding of a high fat diet and treatment with CardioWise. Subcutaneous (A), inguinal (B), gonadal (C), renal (D), brown (E), PVAT (F) and total (G) fats from wild type mice were weighed after 21 days of a high fat diet and treatment with either a vehicle control or a 1x human equivalent physiological dose of CardioWise. The data are presented as the mean ± SEM from 13 mice (control 6; CardioWise 7). Statistical analysis was performed using a t-test (equal variance) on either untransformed data (C, D, E and F) or log-transformed data (A, B and G).

4.3.3 CardioWise increases HDL cholesterol levels in wild type mice fed a high fat diet

The levels of HDL within the plasma of the mice were increased by 1.60 fold (p=0.054) following 21 days of CardioWise treatment (Fig. 4.5B). The levels of total cholesterol were also non-significantly increased (Fig. 4.5A), whereas no changes in LDL levels were observed in contrast to the vehicle control treated mice (Fig. 4.5C). The ratios of total cholesterol/HDL, total cholesterol/LDL and LDL/HDL were also assessed. CardioWise treatment did not affect the total cholesterol/HDL and total cholesterol/LDL ratio (Fig. 4.5D and E). However a non-significant trend of decrease of 38% (p=0.091) was observed in the ratio of LDL/HDL cholesterol in the mice receiving CardioWise (Fig. 4.5F). This result means that CardioWise resulted in more HDL per LDL cholesterol molecule in comparison to the vehicle control mice.

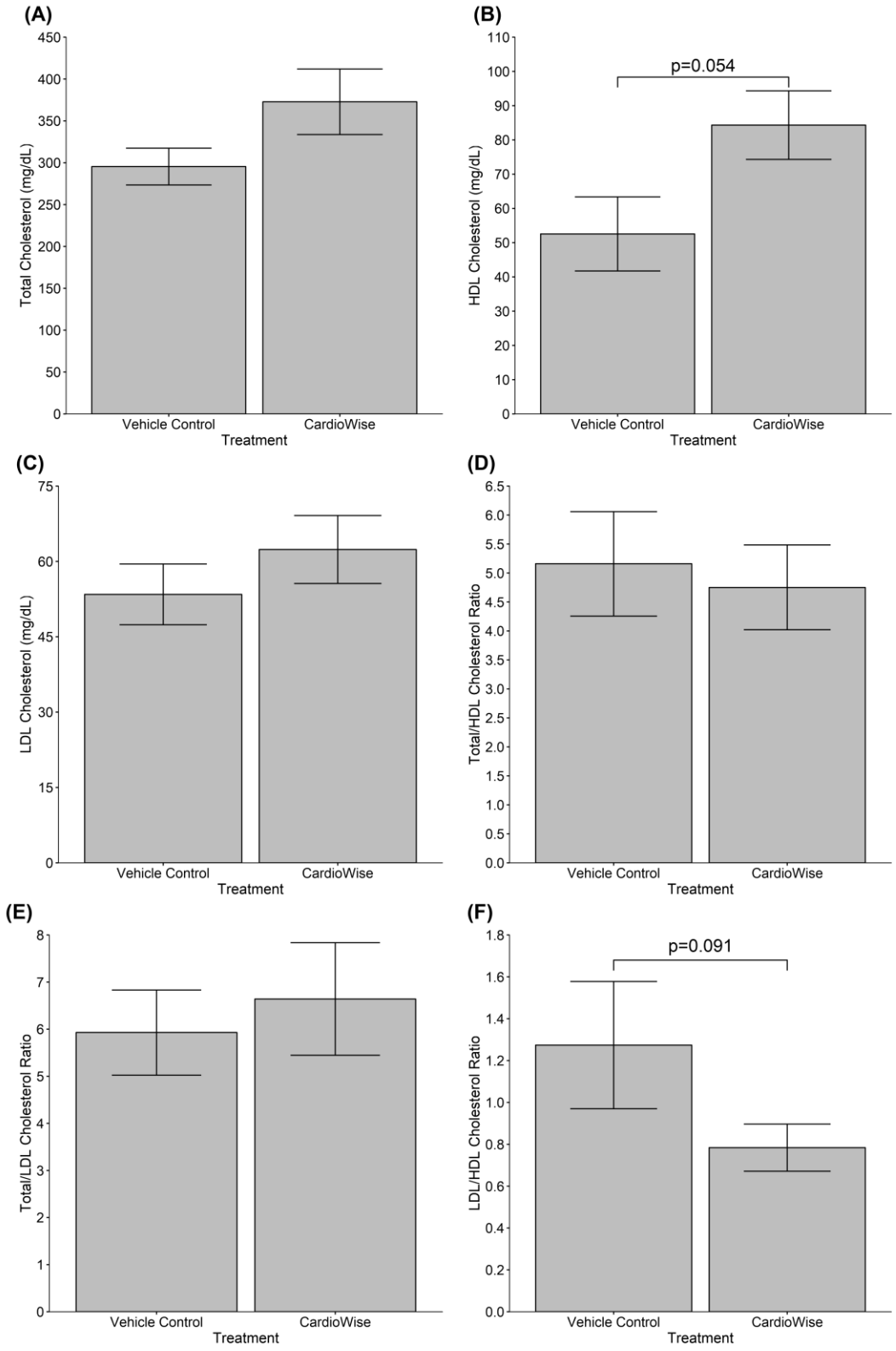

Fig. 4.5. Wild type mice on a high fat diet and treated with CardioWise had increased HDL cholesterol levels. Total cholesterol (A), HDL (B) and LDL (C) from wild type mice were measured after 21 days of a high fat diet and treatment with either a vehicle control or a 1x human equivalent physiological dose of CardioWise. The ratios of total cholesterol/HDL (D), total cholesterol/LDL (E) and LDL/HDL (F) were also assessed. The data are presented as the mean ± SEM from 13 mice (control 6; CardioWise 7). Statistical analysis was performed using either a t-test (equal variance; A, B, C, E and F) or a Mann-Whitney test (D) on untransformed data (B, D and E), reciprocal-transformed data (A), square rooted-transformed data (C) or log-transformed data (F).

4.3.4 CardioWise increases TG levels in wild type mice fed a high fat diet

Increased serum TG levels can be seen as a risk factor for developing atherosclerosis and CVD-related events. CardioWise treatment significantly increased TG levels by 96% ($p<0.001$) in wild type mice fed a high fat diet for 21 days (Fig. 4.6A). The ratios of TG/total cholesterol, TG/HDL and TG/LDL were also assessed. The level of TGs to total or LDL cholesterol levels showed significant increases of 43% and 71% ($p=0.046$ and $p=0.032$) respectively following CardioWise treatment when compared to the vehicle control (Fig. 4.6B and D). A non-significant trend of increase of 39% ($p=0.193$) was observed for the TG/HDL cholesterol ratio in the mice which had received the CardioWise treatment (Fig. 4.6C). These results show that CardioWise treatment increases the levels of TG within the plasma in relation to the levels of the various types of cholesterol.

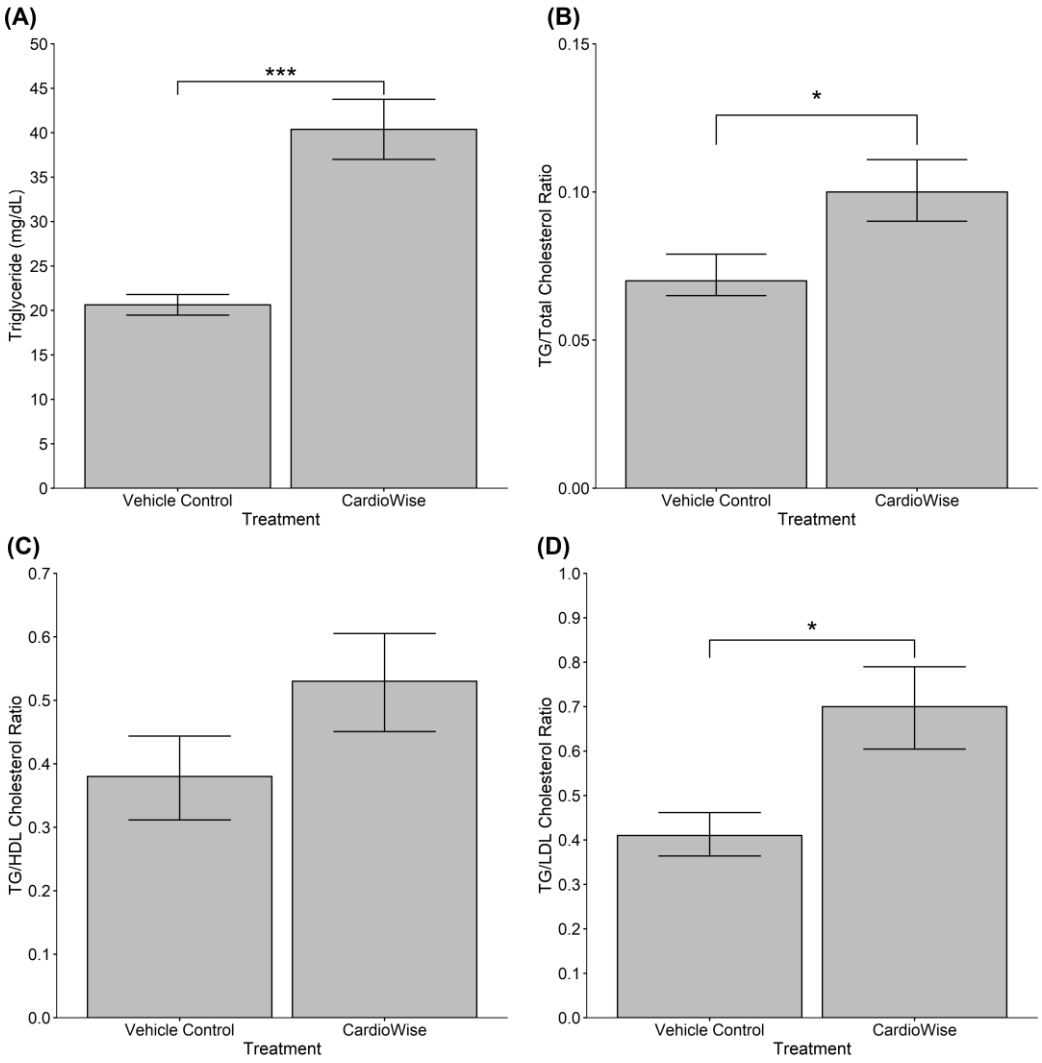

Fig. 4.6. CardioWise in wild type mice increased TG levels while receiving a high fat diet. TG levels (A) from wild type mice were measured after 21 days of a high fat diet and treatment with either a vehicle control or a 1x human equivalent physiological dose of CardioWise. The ratios of TG/total cholesterol (B), TG/HDL cholesterol (C) and TG/LDL cholesterol (D) were also assessed. The data are presented as the mean ± SEM from 13 mice (control 6; CardioWise 7). Statistical analysis was performed using a t-test (equal variance) on either untransformed data (A, B and C) or log-transformed data (D) where * $p \leq 0.05$ and *** $p \leq 0.001$.

4.3.5 CardioWise increases lipid peroxidation in wild type mice fed a high fat diet

Upon treatment with a human equivalent physiologically relevant dose of CardioWise the levels of ROS generation did not significantly differ from the mice receiving the vehicle control (Fig. 4.7A). However the levels of MDA, a by-product of lipid peroxidation, were increased by 2.07 fold (p=0.053) in mice receiving CardioWise treatment in contrast to the mice receiving the vehicle control (Fig. 4.7B).

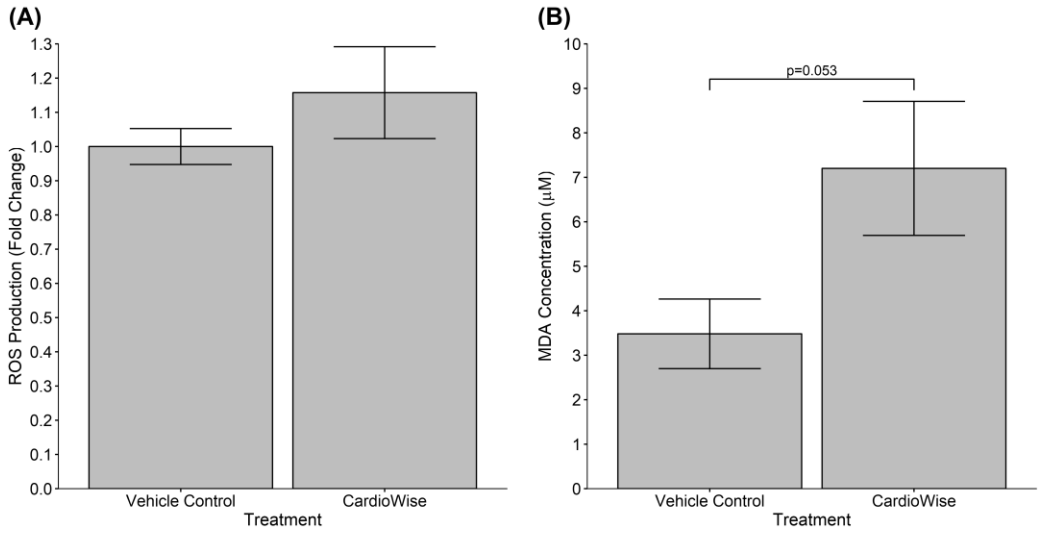

Fig. 4.7. CardioWise shows a trend of induction for lipid peroxidation in wild type mice fed a high fat diet. ROS production (A) and MDA levels (B) were assessed in wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or a 1x human equivalent physiological dose of CardioWise. ROS production was measured in a fluorescence microplate reader, with excitation at 485 nm and emission detected at 535 nm. Vehicle control was given an arbitrary value of 1. MDA levels were measured in a fluorescence microplate reader, with excitation at 535 nm and emission detected at 590 nm. The data are presented as the mean ± SEM from six control mice and either six (B) or seven (A) CardioWise treated mice. Mice with undetectable assay readings were removed before statistical analysis. Statistical analysis was performed using a t-test (equal variance) on either untransformed data (B) or log-transformed data (A).

4.3.6 CardioWise reduces the levels of several pro- and anti-inflammatory cytokines in the plasma of wild type mice fed a high fat diet

The protein levels of pro- and anti-inflammatory cytokines IFN-γ, IL-1β, IL-2, IL-5, IL-6, IL-10, TNF-α and chemokine (C-X-C motif) ligand (CXCL)1 (see Table 4.1 for further details) were assessed in the plasma of wild type mice fed a high fat diet that was supplemented with either vehicle control or a human equivalent physiological dose of CardioWise for 21 days (Fig. 4.8). Following CardioWise treatment, a non-significant trend of decrease was observed for CXCL1 of 18.1% (p=0.106). Although the levels of IL-2, IL-5, IL-6, IL-10 and TNF-α were found to be reduced these changes were found not to be significant, 61.9%, 42.9% (p=0.426), 46.5% (p=0.275), 21.1% (p=0.210) and 15.4% (p=0.414) respectively. No changes in IFN-γ or IL-1β levels were seen in mice receiving CardioWise in contrast to the vehicle control.

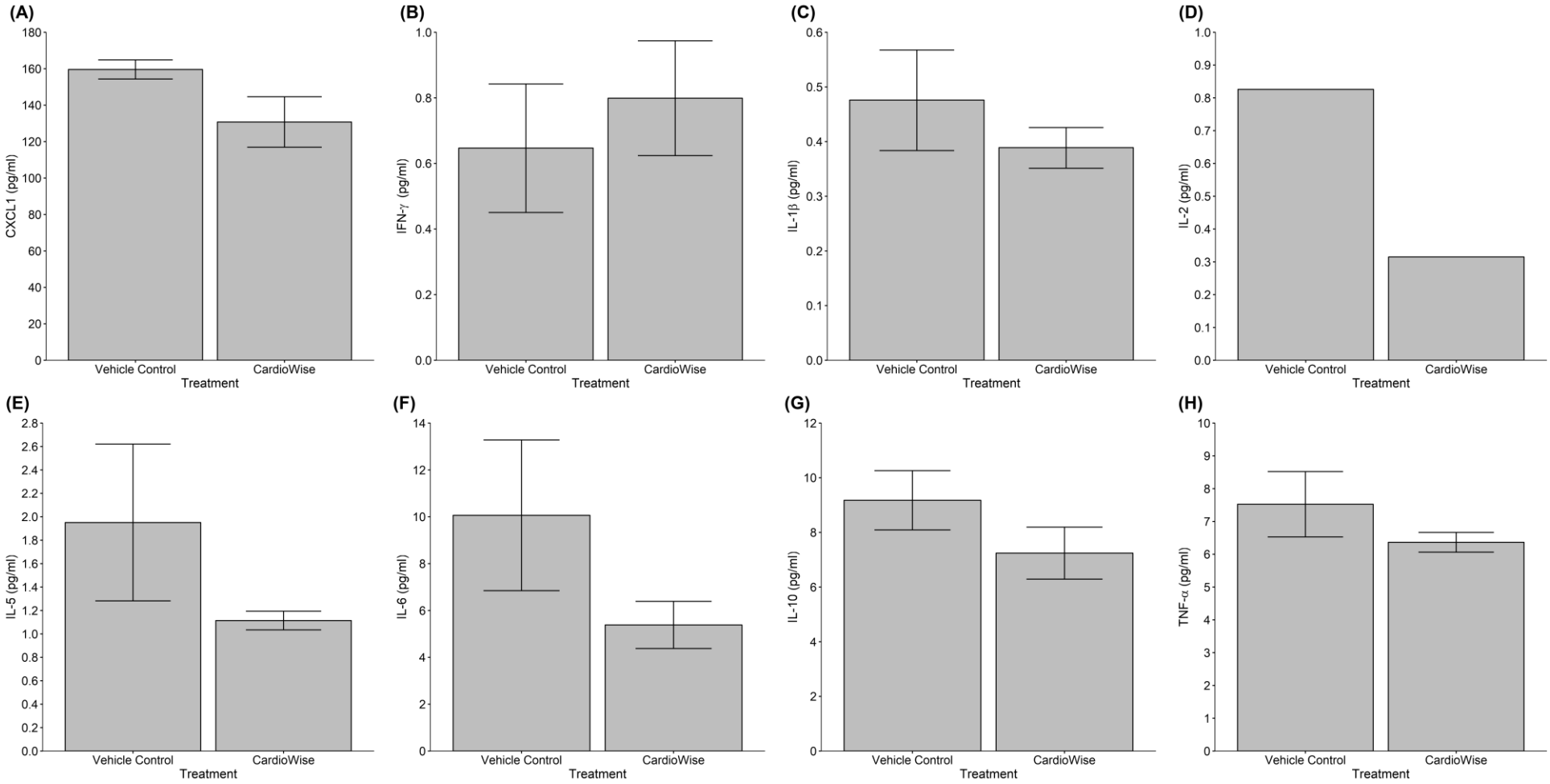

Fig. 4.8. Wild type mice on a high fat diet and treated with CardioWise had a trend of reduced levels of several cytokines. CXCL1 (A), IFN-γ (B), IL-1β (C), IL-2 (D), IL-5 (E), IL-6 (F), IL-10 (G) and TNF-α (H) levels within the plasma of wild type mice were measured after 21 days of a high fat diet and treatment with either a vehicle control or a 1x human equivalent physiological dose of CardioWise. The data are presented as the mean ± SEM from four (D), five (A and C) or six (B, E, F, G and H) control mice and either one (D), five (B) or six (A, C, E, F, G and H) CardioWise treated mice. Mice with undetectable assay readings were removed before statistical analysis. Statistical analysis was performed using a t-test (equal variance) either on untransformed data (A, C and G) or log-transformed data (B, E, F and H).

4.3.7 The expression of all genes present on the qPCR array following CardioWise treatment in wild type mice fed a high fat diet

Fig. 4.9 shows the global significant and non-significant gene expression changes in the livers of wild type mice fed a high fat diet for 21 days following treatment with a human equivalent physiological dose of CardioWise compared to vehicle control treated mice. The housekeeping genes: *β-actin*; 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 CardioWise. A total of 49 genes were found to have their expression altered by at least 10% and 8 of those genes were found to be significantly altered. The genes have been classed by their function (according to the literature provided by Qiagen which accompanied the Atherosclerosis RT^2 Profiler PCR Arrays) and will be explored in greater detail in the subsequent sections.

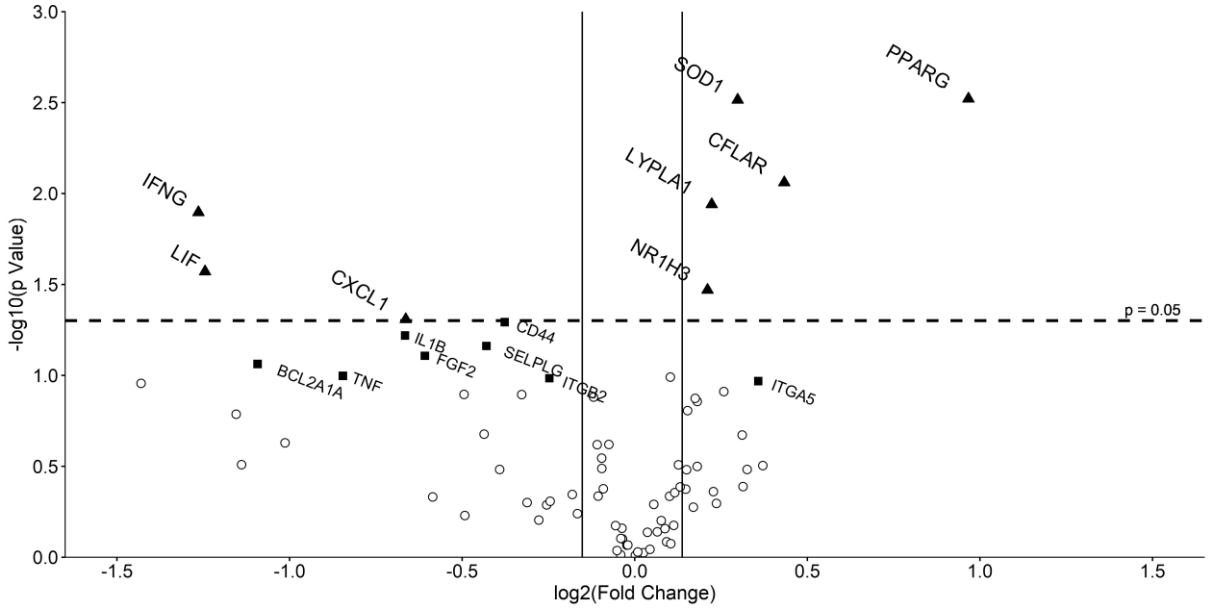

Fig. 4.9. Volcano plot showing global gene expression changes in the liver of wild type mice treated with CardioWise vs vehicle control. Gene transcript levels of 84 genes were assessed in the livers of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or a 1x human equivalent physiological dose of CardioWise. The data are presented as the mean CardioWise induced fold change when compared to the vehicle control treated mice. Results are based on at least three control and three catechin treated mice. All genes present on the Qiagen RT2 profiler PCR Array were plotted. The log fold change in the treated cells versus the vehicle control cells is represented on the x-axis. The y-axis shows the -log10 of the p value. A p value of 0.05 (dashed line) and a fold changes of ±10% (solid lines) are indicated. Significantly altered gene expressions are indicated by a black triangle and non-significant trends of change (approximately $p<0.1$) are indicated by a black square.

4.3.8 CardioWise affects the expression of genes involved in the regulation of apoptosis in wild type mice fed a high fat diet

Both BCL2 related protein A1 (*BCL2A1A*) and CASP8 and FADD-like apoptosis regulator (*CFLAR*) are two genes involved in the regulation of cell apoptosis. Following treatment with

CardioWise there was a trend of decrease in the expression of *BCL2A1A* in the liver of wild type mice of 53.1% when compared to the vehicle control treated mice (p=0.086; Fig. 4.10A). On the other hand, the expression of *CFLAR* was found to be significantly increased by 35.0% (p=0.009) within the livers of CardioWise treated mice (Fig. 4.10B).

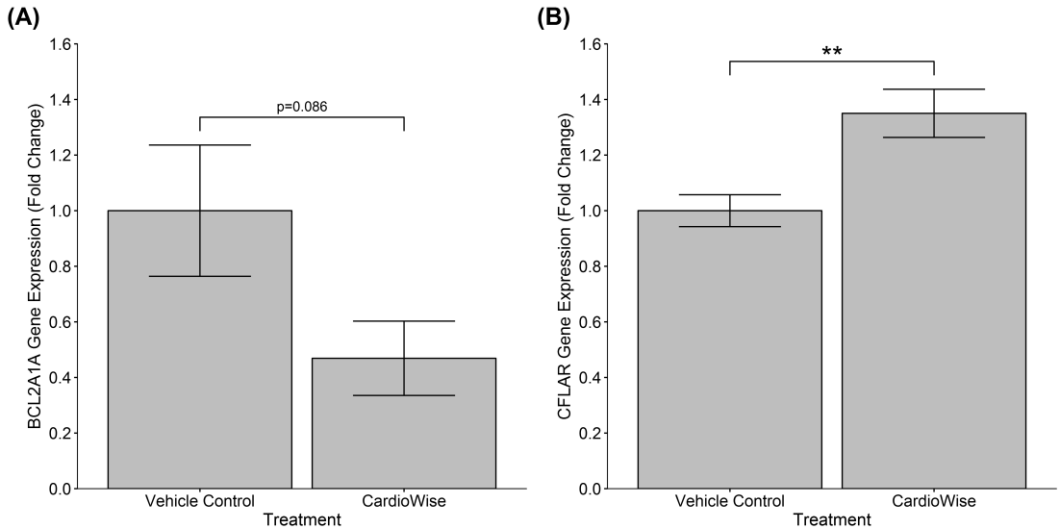

Fig. 4.10. CardioWise alters the expression of genes involved in apoptosis regulation. Gene transcript levels of *BCL21A* (A) and *CFLAR* (B) were assessed in the livers of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or a 1x human equivalent physiological dose of CardioWise. Gene transcript levels were calculated using the comparative Ct method and normalised to five housekeeping genes (*β-actin*, *B2M*, *GAPDH*, *GUSB* and *HSP90AB1*) with values from the vehicle treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from six control and six CardioWise treated mice. Mice with undetectable Ct readings were removed before statistical analysis. Statistical analysis was performed using a t-test (unequal variances) where ** $p \leq 0.01$.

4.3.9 CardioWise attenuates the expression of cell adhesion molecules in wild type mice fed a high fat diet

The recruitment and migration of immune cells can be influenced by a variety of genes including *CD44*, *CXCL1*, integrin subunit (*ITG*)*A5*, *ITGB2* and selectin P ligand (*SELPLG*), therefore attenuating their expression may lead to reduced atherosclerosis plaque formation. The expression of *CD44*, *ITGB2* and *SELPLG* showed trends of decrease following CardioWise treatment of 23.0% (p=0.051), 15.8% (p=0.108) and 25.8% (p=0.069) respectively when compared to the vehicle control treated cells (Fig. 4.11A, D and E). The expression of *ITGA5* showed a trend of increase of 28.1% in the presence of CardioWise (p=0.108; Fig. 4.11C). CardioWise treatment significantly attenuated the expression of *CXCL1* in the liver of wild type mice fed a high fat diet for 21 days by 36.9% (p=0.049) in comparison to the vehicle control receiving mice (Fig. 4.11B).

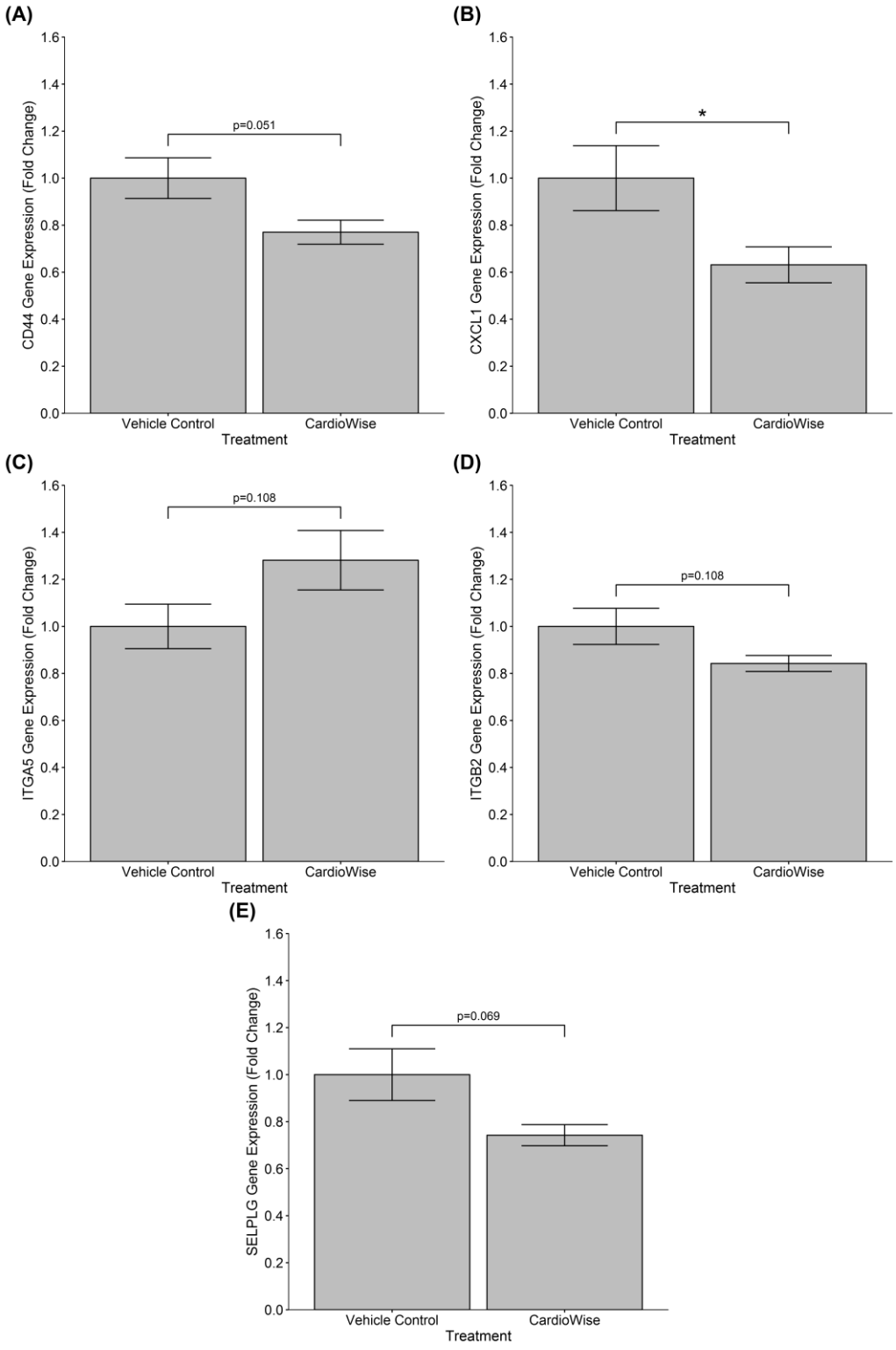

Fig. 4.11. CardioWise decreases the expression of cell adhesion molecules *in vivo*. Gene transcript levels of *CD44* (A), *CXCL1* (B), *ITGA5* (C), *ITGB2* (D) and *SELPLG* (E) were assessed in the livers of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or a 1x human equivalent physiological dose of CardioWise. Gene transcript levels were calculated using the comparative Ct method and normalised to five housekeeping genes (*β-actin*, *B2M*, *GAPDH*, *GUSB* and *HSP90AB1*) with values from the vehicle treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from six control mice and either five (B) or six (A, C, D and E) CardioWise treated mice. Mice with undetectable Ct readings were removed before statistical analysis. Statistical analysis was performed using a t-test (unequal variances) where * $p \leq 0.05$.

4.3.10 CardioWise reduces the expression of cell growth and proliferation regulators in wild type mice fed a high fat diet

Colony stimulating factor (*CSF*)*2*, basic fibroblast growth factor (*FGF2*) and *IL-3* are three pro-inflammatory genes capable of influencing cell growth and proliferation, whereas leukaemia inhibitory factor (*LIF*) is an anti-inflammatory gene which plays a key role in the terminal differentiation of myeloid leukemic cells. CardioWise treatment resulted in a trend of decreased expression of *CSF2*, *FGF2* and *IL-3* in the livers of wild type mice by 23.3%, 34.4% (p=0.078) and 62.3% respectively (Fig. 4.12A, B and C). The expression of *LIF* was significantly attenuated by CardioWise treatment by 57.8% (p=0.027) compared to the vehicle control mice (Fig. 4.12D).

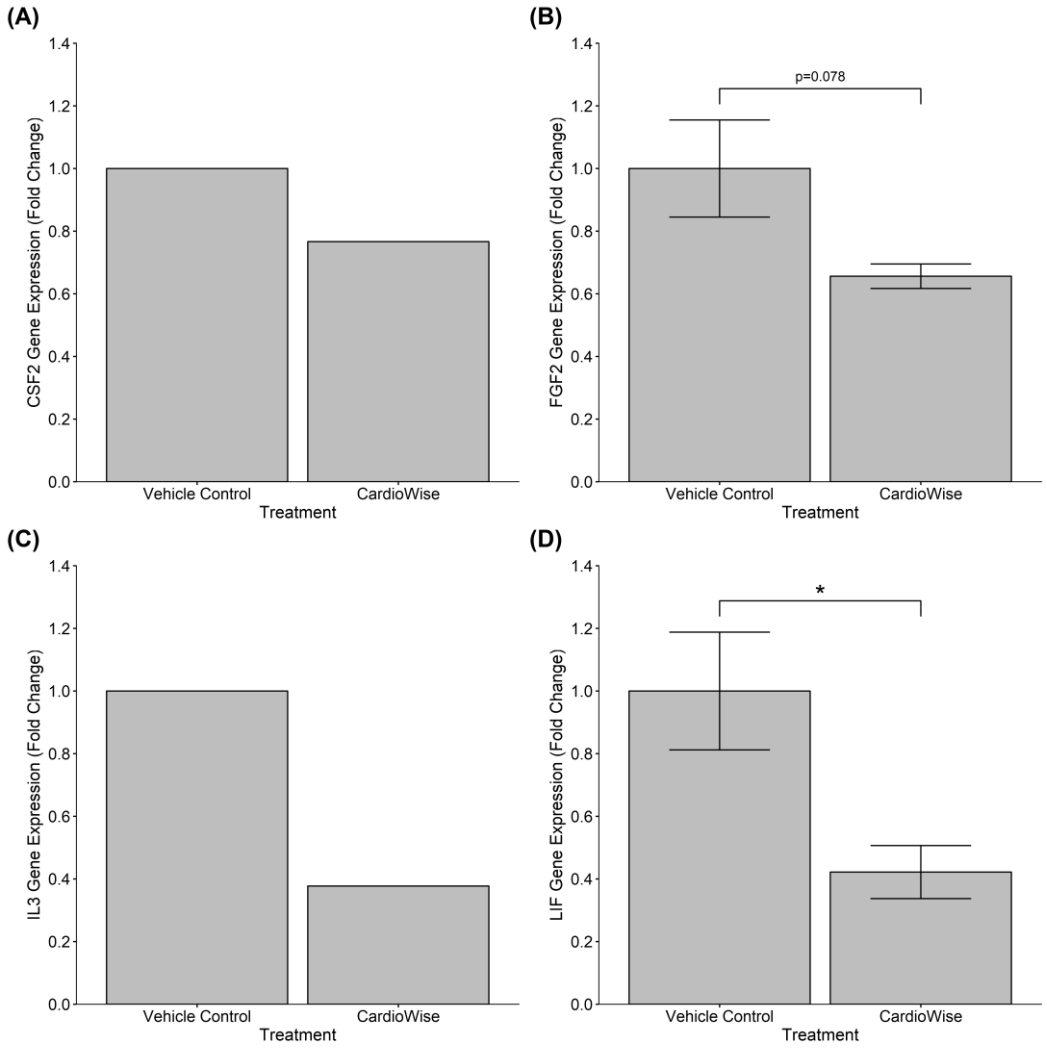

Fig. 4.12. CardioWise decreases the expression of cell growth and proliferation regulators. Gene transcript levels of *CSF2* (A), *FGF2* (B), *IL3* (C) and *LIF* (D) were assessed in the livers of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or a 1x human equivalent physiological dose of CardioWise. Gene transcript levels were calculated using the comparative Ct method and normalised to five housekeeping genes (*β-actin*, *B2M*, *GAPDH*, *GUSB* and *HSP90AB1*) with values from the vehicle treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from four (A and C) or six (B and D) control mice, and either two (A and C) or five (B and D) CardioWise treated mice. Mice with undetectable Ct readings were removed before statistical analysis. Statistical analysis was performed using a t-test (unequal variances) where * $p \leq 0.05$.

4.3.11 CardioWise treatment increases the expression of a lipid transport and metabolism gene in wild type mice fed a high fat diet

The *LYPLA1* gene controls the expression of acyl-protein thioesterase 1 (APT1), a lipase involved in lipid metabolism. The expression of *LYPLA1* within the livers of wild type mice was significantly increased by 16.7% (p=0.011) following CardioWise treatment when compared to the vehicle control mice (Fig. 4.13).

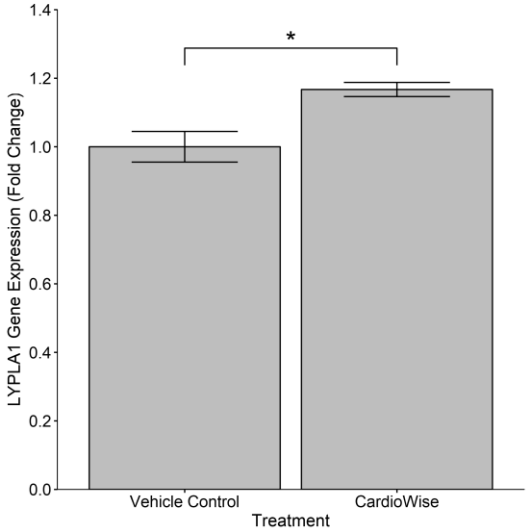

Fig. 4.13. CardioWise increases the expression of a gene involved in the regulation of lipid transport and metabolism. Gene transcript levels of *LYPAL1* were assessed in the livers of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or a 1x human equivalent physiological dose of CardioWise. Gene transcript levels were calculated using the comparative Ct method and normalised to five housekeeping genes (*β-actin*, *B2M*, *GAPDH*, *GUSB* and *HSP90AB1*) with values from the vehicle treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from six control and six CardioWise treated mice. Mice with undetectable Ct readings were removed before statistical analysis. Statistical analysis was performed using a t-test (unequal variances) where * $p \leq 0.05$.

4.3.12 CardioWise alters the expression of genes involved in stress responses in wild type mice fed a high fat diet

IFN-γ, IL-1β and TNF-α are all major pro-inflammatory cytokines capable of influencing the progression of atherosclerosis disease progression and the development of atherosclerotic plaques. SOD1 is an enzyme capable of metabolising ROS, therefore increasing its expression may represent a level of protection against ROS formation. The expression of *IFN-γ* was significantly reduced following treatment with CardioWise by 58.4% (p=0.013) when compared to the vehicle control mice (Fig. 4.14A). A non-significant trend of decreased expression of *IL-1β* and *TNF-α* (36.9% and 44.3%; p=0.060 and p=0.101 respectively) was also observed in the livers of mice which had received CardioWise (Fig. 4.14B and D). CardioWise treatment also resulted in a significant increase in the expression of *SOD1* by 23.0% (p=0.003; Fig. 4.14C).

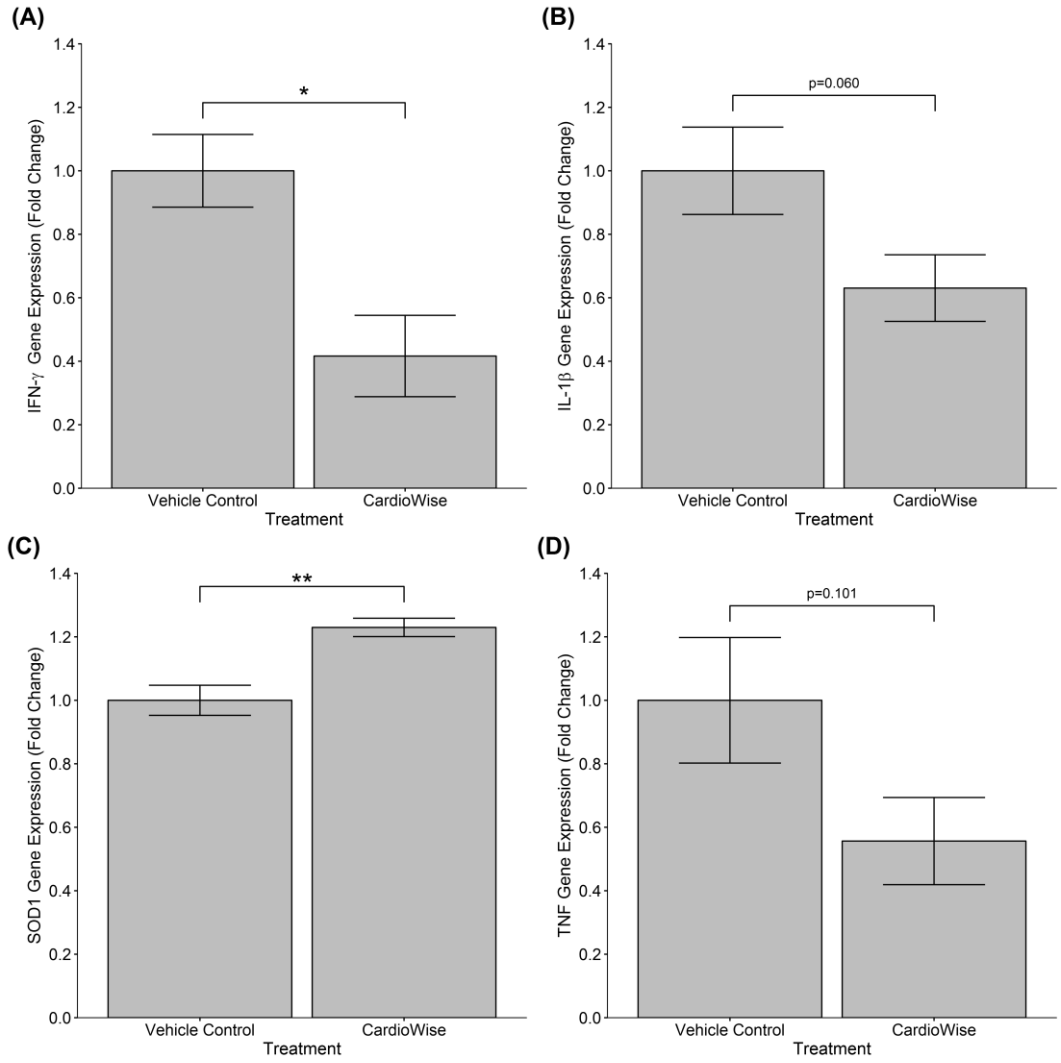

Fig. 4.14. CardioWise exerts anti-inflammatory effects on the expression of genes involved in stress responses. Gene transcript levels of *IFN-γ* (A), *IL-1β* (B), *SOD1* (C) and *TNF-α* (D) were assessed in the livers of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or a 1x human equivalent physiological dose of CardioWise. Gene transcript levels were calculated using the comparative Ct method and normalised to five housekeeping genes (*β-actin*, *B2M*, *GAPDH*, *GUSB* and *HSP90AB1*) with values from the vehicle treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from five (A) or six (B, C and D) control mice, and either four (A), five (D) or six (B and C) CardioWise treated mice. Mice with undetectable Ct readings were removed before statistical analysis. Statistical analysis was performed using a t-test (unequal variances) where * $p \leq 0.05$ and ** $p \leq 0.01$.

4.3.13 CardioWise increases the expression of transcriptional regulators in wild type mice fed a high fat diet

Nuclear receptor subfamily 1 group H member 3 (NR1H3) and PPAR-γ are two anti-inflammatory regulators of transcription, therefore increasing their expression with CardioWise treatment would represent a strong anti-inflammatory effect. CardioWise treatment caused a significant increase in the expression of *NR1H3* and *PPAR-γ* within the livers of wild type mice fed a high fat diet for 21 days by 15.7% and 95.4% respectively (p=0.034 and p=0.003) when compared to the vehicle control treated mice (Fig. 4.15).

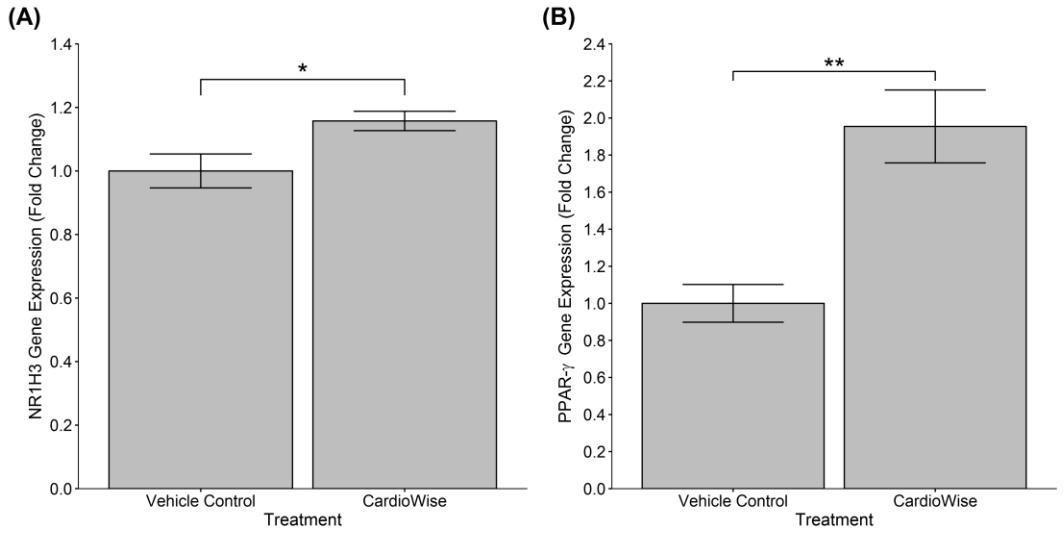

Fig. 4.15. CardioWise increases the expression of genes involved in transcriptional regulation in wild type mice. Gene transcript levels of *NR1H3* (A) and *PPAR-γ* (B) were assessed in the livers of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or a 1x human equivalent physiological dose of CardioWise. Gene transcript levels were calculated using the comparative Ct method and normalised to five housekeeping genes (*β-actin*, *B2M*, *GAPDH*, *GUSB* and *HSP90AB1*) with values from the vehicle treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from six control and six CardioWise mice. Mice with undetectable Ct readings were removed before statistical analysis. Statistical analysis was performed using a t-test (unequal variances) where * $p \leq 0.05$ and ** $p \leq 0.01$.

4.3.14 CardioWise treatment reduces the number of total bone marrow cells in wild type mice fed a high fat diet

The number of white blood cells within the bone marrow can be an indication of the inflammatory status of an individual. Wild type mice treated with CardioWise were found to have the proportion of white blood cells within their bone marrow reduced by 8% (p=0.039) in contrast to the mice treated with the vehicle control (Fig. 4.16).

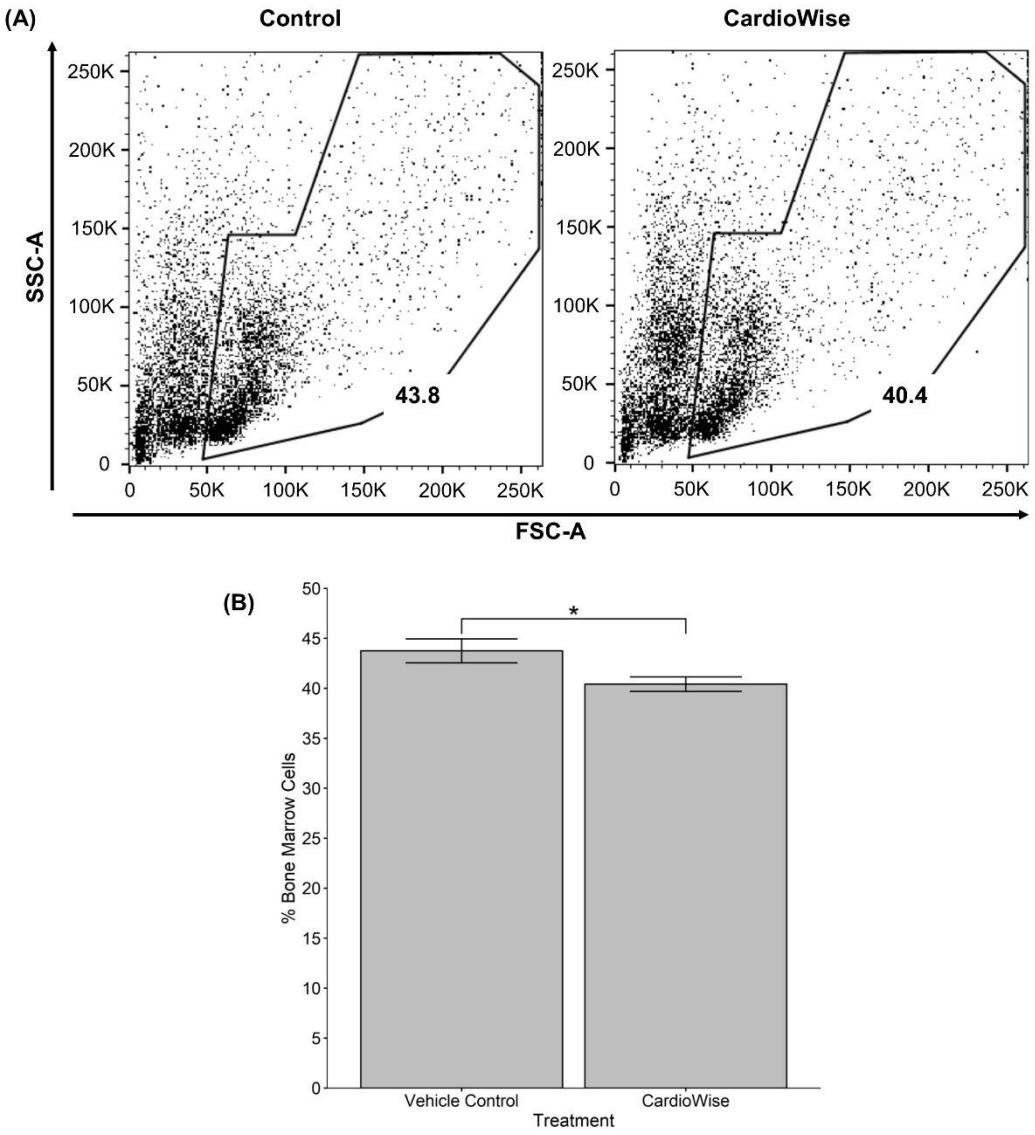

Fig. 4.16. CardioWise decreases the proportion of total bone marrow cells. Cell populations were assessed in the bone marrow of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or a 1x human equivalent physiological dose of CardioWise. Representative flow plots of the total cell population in the bone marrow (A). Numbers on the flow plots indicate the proportion of the cell population as a percentage of total bone marrow cell count. The forward scatter (FSC-A) is a measurement of cell size by determining the amount of light which passes around it. The side scatter (SSC-A) is a measurement of the amount of light which is reflected by particles within the cells and therefore can be used to determine the granularity of cells. Cumulative bar graphs showing the frequency of total bone marrow cells as a percentage of total cell count (B). The data are presented as the mean ± SEM from six control and six CardioWise treated mice. Statistical analysis was performed using a t-test (equal variances) where * $p \leq 0.05$.

4.3.15 The number of SLAM and progenitor cells within the bone marrow of wild type mice fed a high fat diet following CardioWise treatment

The flow plots for the number of SLAM and progenitor cells present in the bone marrow of wild type mice fed either a vehicle control or CardioWise are displayed in Fig. 4.17 and the results are explored in greater detail in the subsequent sections.

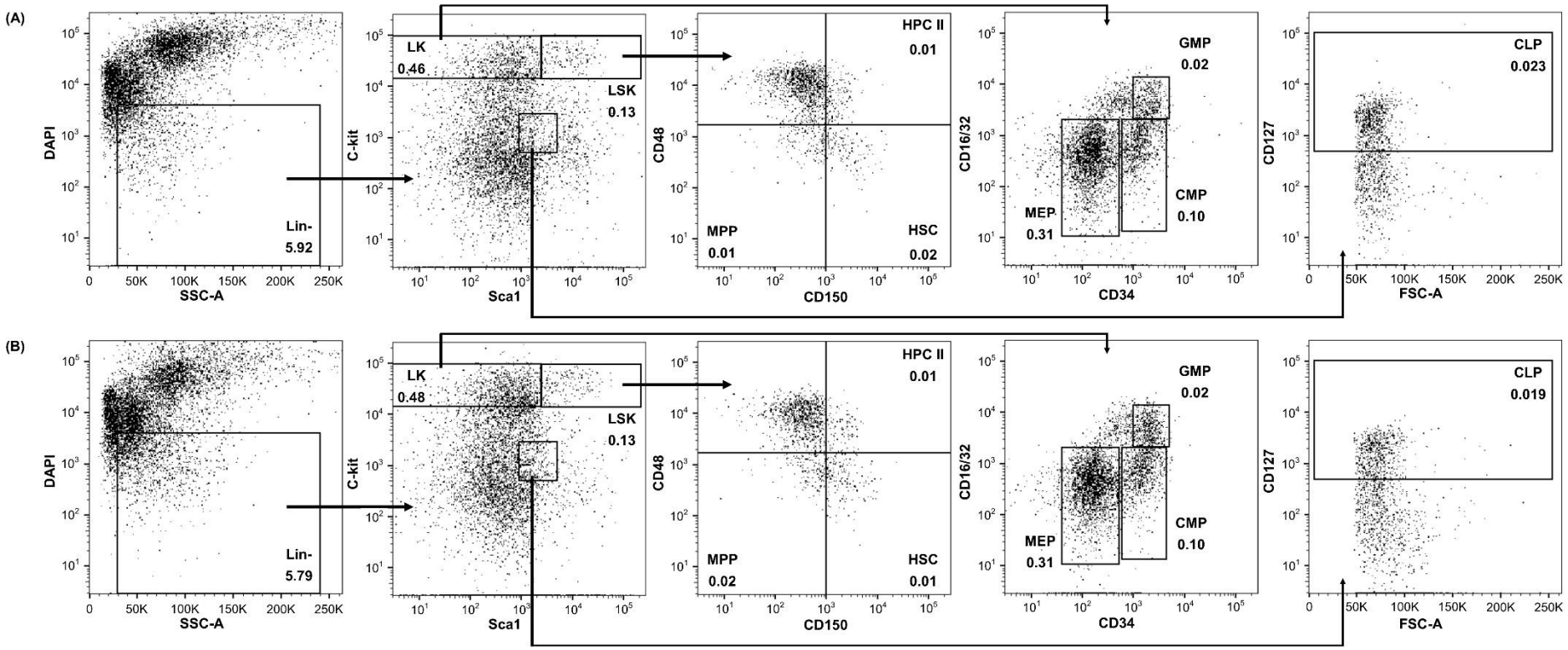

Fig. 4.17. The effect of CardioWise on the proportion of SLAM and progenitor cells present in the bone marrow. Cell populations were assessed in the bone marrow of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control (A) or a 1x human equivalent physiological dose of CardioWise (B). Representative flow plots of the SLAM and progenitor populations in the bone marrow are presented. Numbers on the flow plots indicate the proportion of the cell population as a percentage of total bone marrow cell count. Cumulative bar graphs showing the cell populations as a percentage of total bone marrow cell count are shown in Figs. 4.18 - 4.20. The arrows indicate how each haematopoietic stem/progenitor cell population can be identified within the preceding gating strategy. The axis represent the various stains used to separate the cell populations (see Table 2.8 for further details). The forward scatter (FSC-A) is a measurement of cell size by determining the amount of light which passes around it. The side scatter (SSC-A) is a measurement of the amount of light which is reflected by particles within the cells and therefore can be used to determine the granularity of cells. HSC, haematopoietic stem cell; MPP, multipotent progenitors; HPC, hematopoietic progenitor cell; CMP, common myeloid progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-macrophage progenitor; MDSC, myeloid-derived suppressor cells; CLP, common lymphoid progenitor; LIN, lineage.

4.3.16 CardioWise treatment attenuates the number of HPC cells within the bone marrow of wild type mice fed a high fat diet

All mature haematopoietic cells such as macrophages, T-cells and B-cells express lineage markers and are considered to be lineage-positive (Lin+). All of the remaining haematopoietic cells are lineage-negative (Lin-), this population contains all of the stem and progenitor cells. Examining the Lin- population further can reveal the effects on the multipotent blood cells within the bone marrow as these are found exclusively within the LSK population. The proportion of Lin- and LSK cells within the bone marrow of wild type mice treated with CardioWise was found to be unaffected when compared to the vehicle control treated mice (Fig. 4.18A and B). HSC, multipotent progenitor cells (MPP) and haematopoietic progenitor cells II (HPC II) give rise to all myeloid and lymphocyte cells within the bone marrow. The HPC II population in mice treated with CardioWise was reduced by 38% (p=0.222) when compared to the vehicle control mice, however this change was found not to be significant (Fig. 4.18C). CardioWise treatment had no effect on the proportion of MPP and HSC populations found within the bone marrow (Fig. 4.18D and E).

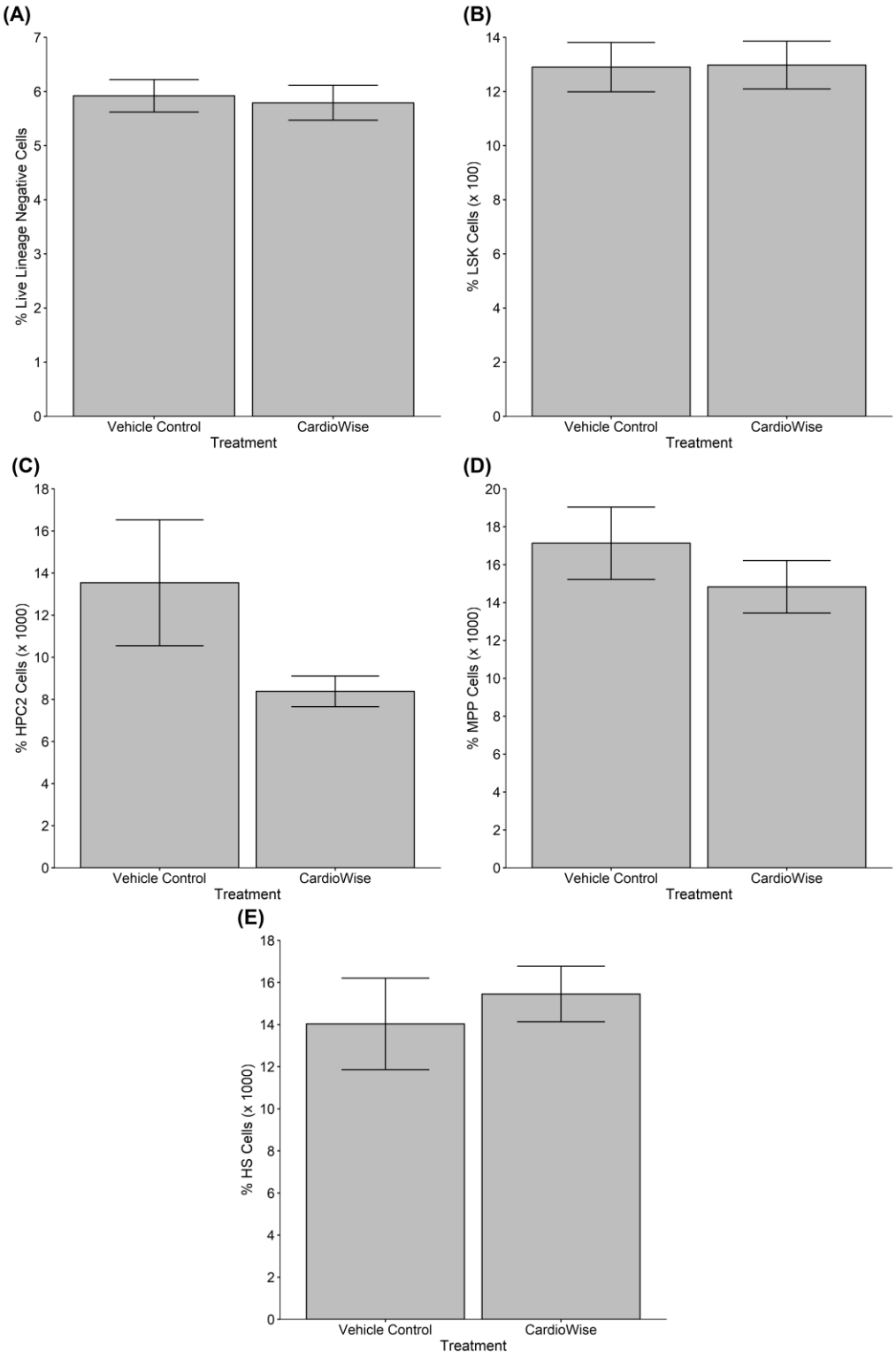

Fig. 4.18. CardioWise decreases the proportion of HPC cells present in the bone marrow. Cell populations were assessed in the bone marrow of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or a 1x human equivalent physiological dose of CardioWise. Cumulative bar graphs showing the frequency of live lineage negative (A), LSK (B), HPC II (C), MPP (D) and HSC (E) populations in the bone marrow as a percentage of total bone marrow cell count. Where appropriate, the data has been multiplied by powers of 10 for ease of presenting the result clearly. The data are presented as the mean ± SEM from six control mice and either six (A and B) or seven (C, D and E) CardioWise treated mice. Statistical analysis was performed using either a t-test (equal variances; A, B, D and E) or a t-test (unequal variances; C) on either untransformed (A, B, D and E) or log-transformed (C) data.

4.3.17 CardioWise treatment does not alter the number of LK, GMP, MEP or CMP cells within the bone marrow of wild type mice fed a high fat diet

The Lin- cKIT+ (LK) cell population within the bone marrow gives rise to all of the myeloid cells. Wild type mice treated with CardioWise had no change in the proportion of LK cells within their bone marrow in contrast to the mice treated with the vehicle control (Fig. 4.19A). The common myeloid progenitor (CMP) cells gives rise to both the granulocyte-macrophage progenitor (GMP) and megakaryocyte-erythroid progenitor (MEP) populations. Mature cells including monocytes and granulocytes are formed from GMP cells, whereas megakaryocytes and red blood cells are derived from the MEP population. The proportion of CMP, GMP and MEP cells within the bone marrow of wild type mice treated with CardioWise was found to be unaffected when compared to the vehicle control treated mice (Fig. 4.19B - D).

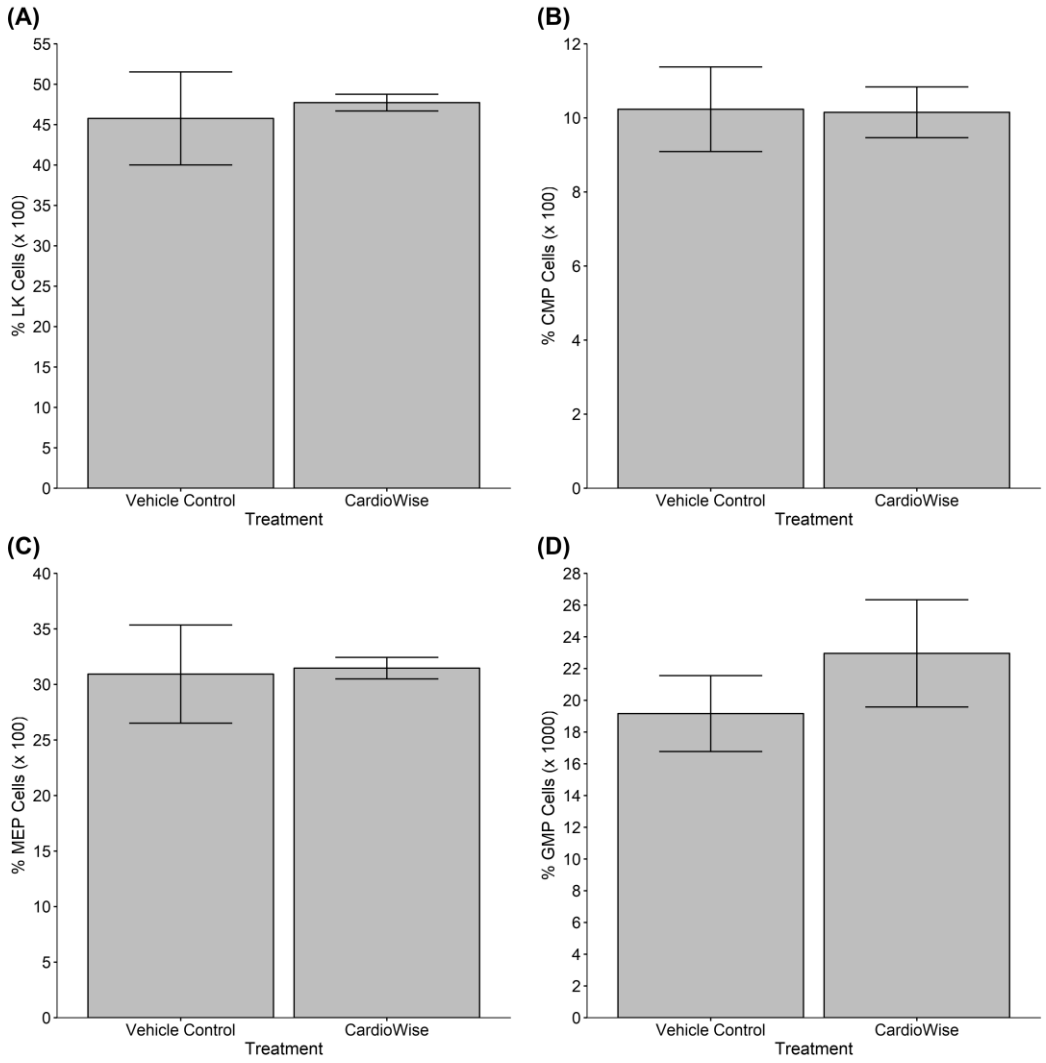

Fig. 4.19. CardioWise does not alter the proportion of LK, GMP, MEP or CMP cells present in the bone marrow. Cell populations were assessed in the bone marrow of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or a 1x human equivalent physiological dose of CardioWise. Cumulative bar graphs showing the frequency of LK (A), CMP (B), MEP (C) and GMP (D) cell populations in the bone marrow as a percentage of total bone marrow cell count. Where appropriate, the data has been multiplied by powers of 10 for ease of presenting the result clearly. The data are presented as the mean ± SEM from six control and seven CardioWise treated mice. Statistical analysis was performed using either a t-test (equal variances; B and D) or a t-test (unequal variances; A and C) on either untransformed (A), log-transformed (B and D) or squared-transformed (C) data.

4.3.18 CardioWise treatment reduces the number of CLP cells within the bone marrow of wild type mice fed a high fat diet

The common lymphoid progenitor (CLP) cell population gives rise to B and T-cells. A non-significant decrease in the CLP cell population of 17% (p=0.241) was observed in mice treated with CardioWise when compared to the vehicle control mice (Fig. 4.20).

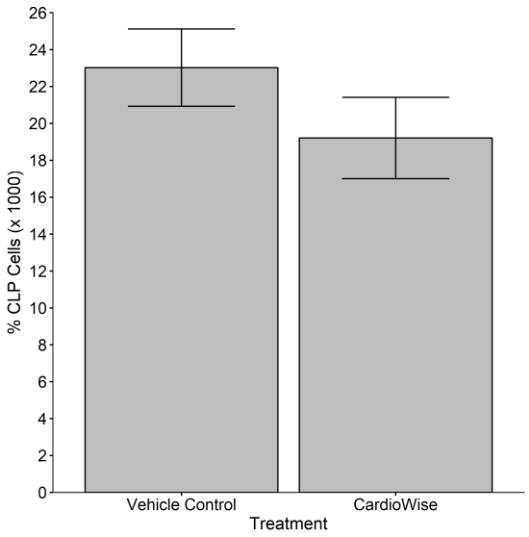

Fig. 4.20. CardioWise non-significantly reduces the proportion of CLP cells present in the bone marrow. Cell populations were assessed in the bone marrow of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or a 1x human equivalent physiological dose of CardioWise. Cumulative bar graphs showing the frequency of the CLP populations in the bone marrow as a percentage of total bone marrow cell count. The data has been multiplied by 1000 for ease of presenting the result clearly. The data are presented as the mean ± SEM from six control and seven CardioWise treated mice. Statistical analysis was performed using a t-test (equal variances).

4.3.19 CardioWise treatment reduces the number of macrophages and MDSCs within the bone marrow of wild type mice fed a high fat diet

Monocyte and macrophage cell populations are MAC1+ and GR1- and can be distinguished from the MDSC population which are MAC1+ and GR1+. Wild type mice treated with CardioWise showed a significant decrease in the proportion of monocytes and macrophages within their bone marrow by 37% (p=0.011) in contrast to the mice treated with the vehicle control (Fig. 4.21B). Additionally, CardioWise treatment significantly reduced the proportion of MDSCs by 24% (p=0.009) when compared to the vehicle control treated mice (Fig. 4.21C).

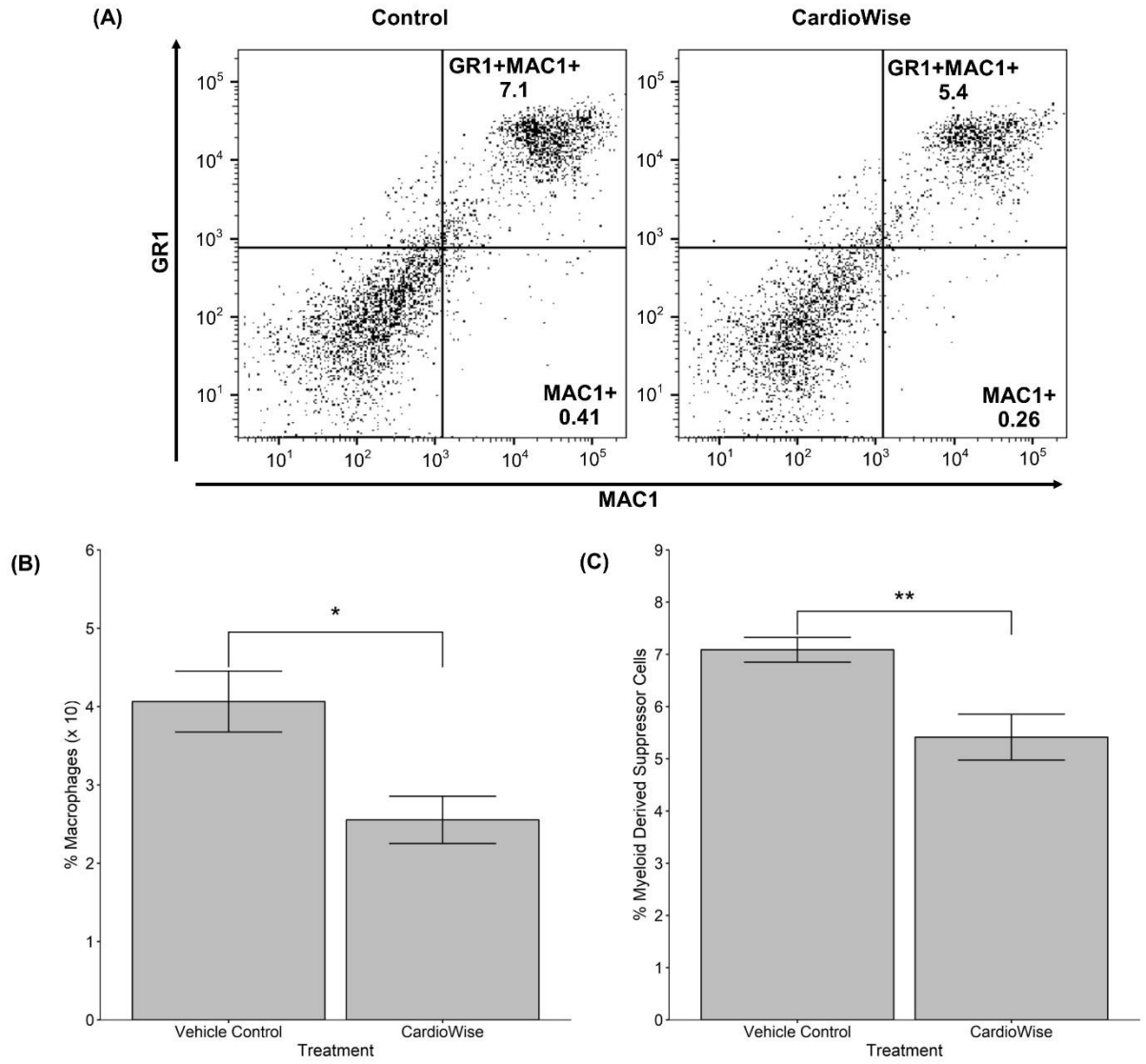

Fig. 4.21. CardioWise reduces the proportion of macrophages and MDSCs present in the bone marrow. Cell populations were assessed in the bone marrow of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or a 1x human equivalent physiological dose of CardioWise. Representative flow plots of the macrophage (MAC1+) and MDSCs (GR1+MAC1+) populations in the bone marrow (A). Numbers on the flow plots indicate the proportion of the cell population as a percentage of total bone marrow cell count. Cumulative bar graphs showing the frequency of monocytes and macrophages (B) and MDSCs (C) in the bone marrow as a percentage of total bone marrow cell count. Where appropriate, the data has been multiplied by powers of 10 for ease of presenting the result clearly. The data are presented as the mean ± SEM from six control and seven CardioWise treated mice. Statistical analysis was performed using either a t-test (equal variances) where * $p \leq 0.05$ and ** $p \leq 0.01$.

4.3.20 CardioWise treatment reduces the number of B- and T-cells within the bone marrow of wild type mice fed a high fat diet

The levels of B- and T-cells can be used as an indirect marker of an individual's inflammatory status. Wild type mice treated with CardioWise showed a significant decrease in the proportion of T-cells within their bone marrow by 23% (p=0.033) in contrast to the mice treated with the vehicle control (Fig. 4.22B). In addition, CardioWise treatment showed a non-significant trend

of reduction in the proportion of B-cells by 25% (p=0.082) when compared to the vehicle control treated mice (Fig. 4.22C).

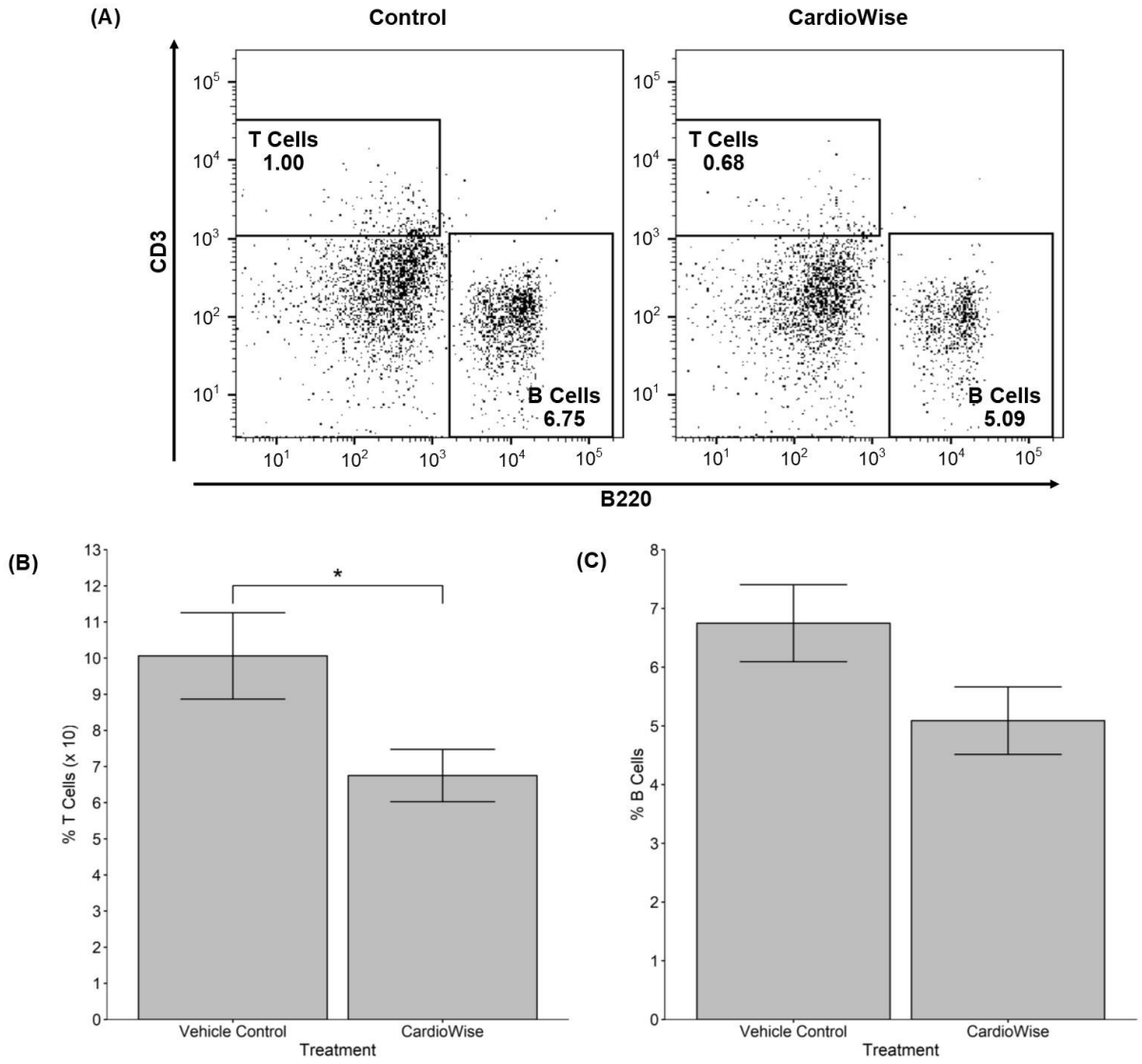

Fig. 4.22. CardioWise reduces the proportion of T and B-cells present in the bone marrow. Cell populations were assessed in the bone marrow of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or a 1x human equivalent physiological dose of CardioWise. Representative flow plots of the T and B-cell populations in the bone marrow (A). Numbers on the flow plots indicate the proportion of the cell population as a percentage of total bone marrow cell count. Cumulative bar graphs showing the frequency of T-cells (B) and B-cells (C) in the bone marrow as a percentage of total bone marrow cell count. Where appropriate, the data has been multiplied by powers of 10 for ease of presenting the result clearly. The data are presented as the mean ± SEM from six control and seven CardioWise treated mice. Statistical analysis was performed using a t-test (equal variances) where * $p \leq 0.05$.

4.4 Discussion

4.4.1 The effect of CardioWise on physical parameters in wild type mice

The results of this *in vivo* study show the great potential CardioWise has as a novel nutraceutical combinational therapy for retarding the development of atherosclerosis and reducing an individual's risk of suffering a CVD-related event. In wild type mice fed a high fat diet for 21 days, CardioWise was found to increase both HDL cholesterol levels and improve the LDL/HDL ratio, while simultaneously attenuating pro-inflammatory gene expression in the liver and reducing pro-inflammatory cytokine levels in the blood. Furthermore CardioWise may exert anti-atherogenic effects on the cell populations within the bone marrow. Despite observing some pro-atherogenic actions of CardioWise, such as increased lipid peroxidation, overall it appears to exert strong anti-inflammatory effects and this study provides further evidence for the cardiovascular protective effects of CardioWise that were seen during the *in vitro* studies (Chapter 3).

The effect of CardioWise on weight change was assessed as sudden changes in weight may imply CardioWise was exerting a detrimental effect in the mice. After 21 days of a high fat diet, both the vehicle control and CardioWise treated mice put on weight at the same rate, indicating that no detrimental effects occurred as a result of CardioWise (Fig. 4.3). However as this was a short term feeding study, beneficial changes in weight gain may occur gradually over time and should therefore continue to be explored in future long term feeding studies. Indeed a recent study failed to show any difference in the weight of wild type mice treated with EPA or DHA after 3 weeks but a decrease in body weight was observed following 10 weeks of fish oil supplementation (Kim *et al.* 2016). Another study involving wild type mice fed a high fat diet did not observe any changes in body weight of mice following 4 weeks of fish oil supplementation, however after 12 weeks the mice had gained less weight when compared to the vehicle control treated mice (de Sá *et al.* 2016). The weight gained in mice on a high fat diet was also unaffected by green tea extracts during short term feeding studies (Murase *et al.* 2002; Sugiura *et al.* 2012). However a high fat diet supplemented with either 0.2% or 0.5% catechins resulted in reduced weight gain in mice after 42 weeks (Murase *et al.* 2002). Furthermore mice fed a high fat diet supplemented with 2% phytosterols for 32 weeks gained the same amount of weight as mice receiving the high fat diet only (Calpe-Berdiel *et al.* 2008). The results from this study for CardioWise shows that the combined product of ω-3 PUFAs, flavanols and phytosterols acts similarly to other short term studies which have investigated the effect of the individual components.

The accumulation of adipose tissue in obesity is a clinical risk factor of developing CVD and suffering a CVD-related event. Two risk factors of atherosclerosis, systemic inflammation and hyperlipidaemia, have been associated with obesity (Hotamisligil 2006; Nordestgaard 2016). However how a build-up of fat is directly linked to atherosclerosis remains poorly understood

(van Dam *et al.* 2017). The fat deposits around the body all contribute to the hydrolysis of TG-rich lipoproteins via LPL into FFAs and glycerol (Cinti 2009; van Dam *et al.* 2017). How the different fat deposits subsequently process the remaining lipids differs and each have unique functions, as a result they can be classified as white, brown and PVAT (Cinti 2009; van Dam *et al.* 2017). White fat is the most common fat type throughout the body and predominately maintains energy regulation by storing excess dietary fatty acids as TGs, and subsequently breaking them down by lipolysis in order to release FFAs for the energy needs of organs (van Dam *et al.* 2017). The regulation of lipid and glucose homeostasis has shown to be influenced by white fat (Kershaw and Flier 2004). During obesity, as white fat deposits continue to increase, they become dysfunctional due to reduced TG removal from the adipose cells and increased TG storage (Arner *et al.* 2011). If the turnover of lipids starts to decrease, it can result in lipids being shunted to the liver and increase plasma TG levels (Arner *et al.* 2011). Furthermore there was an indication that CardioWise treatment results in lower lipid turnover as an increase in circulating TG levels were also observed (Fig. 4.6). Previous studies have found that both ω-3 PUFAs and catechin supplementation resulted in reduced white fat accumulation *in vivo* (de Sá *et al.* 2016; Janovská *et al.* 2013; Tokimitsu 2004; Nagao *et al.* 2007). However other studies have found both catechin and phytosterols had no effect on the size of the white fat deposits (Sugiura *et al.* 2012; Calpe-Berdiel *et al.* 2008).

Brown adipose tissue breaks down fatty acids to generate heat in a process known as non-shivering thermogenesis in order to maintain body temperature (van Dam *et al.* 2017). The lipolysis of TGs generates the fatty acids for brown fat to utilise (Cannon and Nedergaard 2004). Once the lipid stores have been used, brown fat begins to take up glucose and TG-derived fatty acids from the plasma (van Dam *et al.* 2017). This makes brown fat an appealing anti-atherogenic therapeutic target because its activation results in a reduction in circulating TG levels (Boon *et al.* 2014; Sun *et al.* 2014; van Dam *et al.* 2015; Berbée *et al.* 2015). However recently it has been shown that short term activation of brown fat is capable of increasing serum TG levels (Hoeke *et al.* 2017). In this study, CardioWise treatment in wild type mice fed a high fat diet for 21 days resulted in a smaller weight of brown fat (Fig. 4.4E). However perhaps this reduction in brown fat weight is due to its activation causing a depletion of its stored lipids and resulting in a smaller brown fat deposit. Indeed previous studies have shown ω-3 PUFAs and other GPR120 agonists (the receptor ω-3 PUFAs are thought to exert their cardiovascular protective effects) are capable of activating brown fat (Pahlavani *et al.* 2017; Quesada-López *et al.* 2016). An increase in the expression of *NR1H3* and *PPAR-γ* are also associated with brown fat activation, both of which were found at increased levels in the liver following CardioWise treatment in this study (Fig. 4.15; Steffensen *et al.* 2003; Nedergaard *et al.* 2005; Laurencikiene and Rydén 2012). As previously mentioned brown fat activation is associated with reduced plasma TG levels. However when brown fat replenishes its lipid stores, it does so by taking up circulating fatty acids following lipolysis of TGs in the blood stream (Khedoe *et*

al. 2015). This means the circulating levels of glycerol would potentially increase. Every sample had its glycerol background level tested which were found to be increased by 22% following CardioWise treatment (data not shown). Furthermore the increase in TG levels observed in this study may be due to the short term activation of brown fat (Hoeke *et al.* 2017). Additionally brown fat activation has been shown to result in increased HDL cholesterol levels (Chen *et al.* 2017b; Hoeke *et al.* 2017), which has also been seen in this study (Fig. 4.5). These results provide some evidence for the activation of brown fat following CardioWise treatment in wild type mice, highlighting a possible mechanism by which CardioWise exerts its cardiovascular protective effects. The effect of CardioWise treatment on brown fat activation requires further studies *in vivo* as well as determining the expression of brown fat related genes within the fat pads.

PVAT is a unique fat deposit within the body due to its specific location surrounding systemic blood vessels and its ability to provide mechanical support and regulate tension within the blood vessels (van Dam *et al.* 2017). It is thought to achieve these effects by releasing hormones which have a paracrine effect on the vascular system (Löhn *et al.* 2002). However the phenotype of PVAT can differ depending on its location, with thoracic PVAT considered to be similar to brown fat whereas abdominal PVAT is thought to be like white fat (Fitzgibbons *et al.* 2011; Padilla *et al.* 2013). During this study, only the thoracic PVAT was harvested and therefore the discussion will focus on what role that PVAT within the thorax plays during atherosclerosis disease progression. Following 21 days of a high fat diet and CardioWise treatment, wild type mice were found to have increased thoracic PVAT (Fig. 4.4F). Evidence is emerging which suggests PVAT can influence atherosclerosis due its paracrine properties and its location surrounding the adventitia of blood vessels (van Dam *et al.* 2017). During obesity PVAT deposits grow in size and become dysfunctional, characterised by the recruitment of immune cells and the release of pro-inflammatory chemokines and cytokines (Greenstein *et al.* 2009; Police *et al.* 2009). Interestingly CardioWise treatment was found to reduce the levels of circulatory pro-inflammatory cytokines (Fig. 4.8). An increase in PVAT inflammation has been shown to be triggered by a high fat diet and due to its location surrounding blood vessels, it is also thought to have the ability to initiate an inflammatory response within the wall of arteries (Chatterjee *et al.* 2009; Lee *et al.* 2014; Wang *et al.* 2009; Zou *et al.* 2016). In a similar way to brown fat, PVAT is thought to be able to contribute to fatty acid clearance via thermogenesis (van Dam *et al.* 2017). Previous studies have shown that reduced thermogenesis caused by *PPAR-γ* deficiency can lead to a loss of PVAT and increased atherosclerosis (Chang *et al.* 2012). In this study, CardioWise was found to increase the expression of *PPAR-γ* in the liver of wild type mice (Fig. 4.15B). This increase in *PPAR-γ* expression may explain the increase in the size of the thoracic PVAT deposit if it is required for its proper function. These results indicate that CardioWise is able to maintain and enhance thoracic PVAT despite exposure to a high fat diet as shown by increased thoracic PVAT weight,

increased *PPAR-γ* expression and a reduction in circulatory pro-inflammatory cytokine levels. Overall it appears CardioWise exerts anti-inflammatory effects on fat deposits around the body by potentially increase in brown and thoracic PVAT activation, however this hypothesis needs to be investigated further in future *in vivo* studies.

4.4.2 The effect of CardioWise on blood parameters in wild type mice

The levels of cholesterol and TG within the blood can be used as clinical markers of CVD risk. HDL cholesterol is involved in the RCT process and aids in cholesterol excretion by transporting excess cholesterol to the liver (McLaren *et al.* 2011a). For this reason, increased levels of HDL are considered to be anti-atherogenic. This study showed CardioWise is capable of increasing circulating HDL levels in wild type mice fed a high fat diet for 21 days (Fig. 4.5B). This result implies CardioWise is capable of providing anti-atherogenic effects and protecting against exposure to a high fat diet. Previous studies involving *ApoE* deficient mice have also shown that EPA dietary supplementation for 28 days is capable of increasing serum HDL levels (Nakajima *et al.* 2011). Furthermore an increase in HDL levels has also been observed in rats and mice treated with green tea extract or phytosterols (Chisaka *et al.* 1988; Babu *et al.* 2006; Moghadasian *et al.* 1999). The increase in HDL levels following CardioWise treatment therefore correlates with the results of the individual nutraceutical components and the unique combination did not inhibit their cardiovascular protective effects. High circulating levels of LDL have been associated with atherosclerosis disease development, therefore low serum levels of LDL are considered less pro-atherogenic (McLaren *et al.* 2011a). Following treatment with a human equivalent physiological dose of CardioWise, there were no observable changes in LDL cholesterol levels (Fig. 4.5C). This results contradicts previous studies which have found that ω-3 PUFAs, catechins and phytosterols are capable of reducing serum LDL levels (Kamat and Roy 2016; Matsuyama *et al.* 2008; Moghadasian *et al.* 1999). Despite the lack of reduction in circulating LDL levels, CardioWise was able to reduce the LDL/HDL cholesterol ratio in wild type mice. This means CardioWise was able to reduce the amount of pro-atherogenic LDL cholesterol in relation to the increase in anti-atherogenic HDL cholesterol. Indeed there is growing evidence that LDL or HDL cholesterol levels in isolation are not always associated with cardiovascular health and the LDL/HDL ratio is a better clinical risk marker of atherosclerosis and sudden cardiac death (Millán *et al.* 2009; Kunutsor *et al.* 2017). The evidence from this study suggest that CardioWise is able to alter the lipid profile of mice to a potentially anti-atherogenic one.

Although not presently thought to play a direct role in atherosclerosis disease development, TGs are a useful biomarker of TG-rich lipoproteins such as VLDL and chylomicron remnants which are able to enhance atherosclerosis independently of LDL (Talayero and Sacks 2011). CardioWise treatment caused an increase in serum TG levels in wild type mice fed a high fat diet (Fig. 4.6A). However this result is surprising as previous studies have shown ω-3 PUFAs

and phytosterols to be capable of reducing circulating TG levels (Kamat and Roy 2016; Schonewille *et al.* 2014; Perez-Ternero *et al.* 2017). The use of flavanols has failed to significantly alter TG levels in both mice and humans (Matsuyama *et al.* 2008; Friedrich *et al.* 2012). This suggests the novel combination of CardioWise may be having interacting effects which cause an increase in TG levels which is not seen when the individual components are tested alone. However as previously discussed above, the short term activation of brown fat has been shown to directly increase plasma TG levels (Hoeke *et al.* 2017). The use of circulating TG levels as risk indictor for atherosclerosis and CVD has remained controversial since it was first proposed in 1980 (Hulley *et al.* 1980; Miller *et al.* 2011). This is because an increase in plasma TG levels is also correlated with a reduction in HDL levels and therefore it is hard to separate the two to determine the best indicator of CVD risk (Miller *et al.* 2011; Dron and Hegele 2017). Some meta-analysis studies have found that circulating TG levels is associated with CVD risk after controlling for changes in HDL levels (Harchaoui *et al.* 2009). However whether alterations in TG levels can be used as an independent risk marker of CVD or whether it is just a consequence of altered HDL levels remains debated. To overcome the controversy around TG levels as a CVD risk marker, the use of the TG/HDL cholesterol ratio has been found to be a better indicator of atherosclerosis disease progression (da Luz *et al.* 2008). Although not significant, CardioWise treatment resulted in a strong trend of increased TG/HDL cholesterol ratio in wild type mice following a high fat diet for 21 days. Despite this result, as the rise in TG levels is not associated with a reduction in HDL or an increase in LDL levels, CardioWise appears to provide cardiovascular protective effects by increasing circulating levels of HDL after exposure to a high fat diet.

Our *in vitro* studies showed that CardioWise treatment had the potential to increase ROS production (Fig. 3.14), therefore it was essential to assess the effect of CardioWise treatment on ROS generation in wild type mice following a high fat diet for 21 days. No significant changes in ROS production were observed after CardioWise treatment when compared to the vehicle control treated mice (Fig. 4.7A). Several recent *in vivo* studies have demonstrated that ω-3 PUFAs, flavanol and phytosterol dietary supplementation is capable of attenuating oxidative stress (Han *et al.* 2015; Ali *et al.* 2015; Tak *et al.* 2016; Firat *et al.* 2017; Nakamura *et al.* 2017; Wiest *et al.* 2017; Yu *et al.* 2017; Oyama *et al.* 2017). The increased gene expression of *SOD1*, an enzyme responsible for neutralising ROS within the body, which also occurred following CardioWise treatment (Fig. 4.14C) may explain why ROS species generation was not significantly increased *in vivo*. Despite no changes in ROS generation, CardioWise treatment did significantly increase lipid peroxidation, a consequence of increased ROS levels, as measured by plasma MDA levels (Fig. 4.7B). This result contradicts other studies in the literature which have shown that the individual nutraceuticals are capable of attenuating lipid peroxidation, indicating a possible interaction occurring within the novel mixture of CardioWise and contributing to an increase in lipid peroxidation (Kesavulu *et al.*

2002; Tak *et al.* 2016; Ali *et al.* 2015). Due to the high number of mitochondria present within brown fat, its activation has been associated with increased *SOD1* expression and lipid peroxidation (Barja de Quiroga *et al.* 1991; Pan *et al.* 2005; Jastroch *et al.* 2010). Therefore the increased expression of *SOD1* and increased levels of lipid peroxidation may be further evidence of brown fat activation in wild type mice following CardioWise treatment while receiving a high fat diet.

Another blood parameter measured during the study was the level of pro- and anti-inflammatory cytokines. After 21 days of CardioWise treatment in wild type mice fed a high fat diet, the levels of pro-inflammatory cytokines CXCL1, IL-2 and TNF-α all showed a trend of decrease (Fig. 4.8). CXCL1 plays a major role in monocyte and macrophage migration, therefore reductions in the circulating levels of this cytokine indicate that CardioWise would attenuate cell recruitment *in vivo* which would coincide with the *in vitro* result (Fig. 3.10). CardioWise treatment appears to attenuate the levels of IL-2 within the plasma of mice. However this result is based on a reduced number of mice due to undetectable assay readings and therefore requires further investigations before any firm conclusions can be drawn. As previously discussed, TNF-α is a major pro-inflammatory cytokine involved in the progression of atherosclerosis (Ramji and Davies 2015; Moss and Ramji 2016a). The circulating levels of TNF-α were reduced by a human equivalent physiological dose of CardioWise treatment in wild type mice. Dietary supplementation with ω-3 PUFAs, catechin and phytosterols have been previously shown to reduce the circulating levels of CXCL1 and TNF-α in mice (Guruvayoorappan and Kuttan 2008; Nordgren *et al.* 2014; Vaidya *et al.* 2017). IL-2 secretion was found to be reduced following ω-3 PUFA treatment whereas (+)-catechin supplementation has been shown to be able to induce its production (Guruvayoorappan and Kuttan 2008; Bilal *et al.* 2011). The results presented here, and from previous studies, indicate that CardioWise has the potential to exert strong anti-inflammatory effects. However despite CardioWise treatment resulting in the reduction in the circulating levels of pro-inflammatory cytokines, it also produced a reduction in the levels of anti-inflammatory cytokines, in particular IL-5 and IL-10. Increased levels of IL-5 and IL-10 have been associated with reduced atherosclerosis disease progression (Ramji and Davies 2015). Previous studies have shown ω-3 PUFA treatment to reduce the levels of IL-5 and IL-10 in mice, whereas catechin treatment in mice receiving a high fat diet failed to alter IL-10 protein levels (Bilal *et al.* 2011; Cunha *et al.* 2013; Li *et al.* 2014a). The effects of phytosterol supplementation in mice appears to be more complex with IL-5 levels being attenuated following treatment while IL-10 levels were unaffected (Yuk *et al.* 2007; Vaidya *et al.* 2017). In this study the levels of both IL-5 and IL-10 were non-significantly reduced following CardioWise treatment for 21 days (Fig. 4.8). The cytokine IL-6 has been shown to exert both pro- and anti-inflammatory effects (Ramji and Davies 2015). The levels of IL-6 in the plasma of wild type mice fed a high fat diet for 21 days were non-significantly decreased following CardioWise treatment (Fig. 4.8). This result

correlates with previous data within the literature which also found that ω-3 PUFAs, catechin and phytosterols are all capable of reducing IL-6 levels (Guruvayoorappan and Kuttan 2008; Nordgren *et al.* 2014; Vaidya *et al.* 2017). The results of CardioWise treatment imply a general dampening of immune activation and systemic inhibition of cytokine production rather than a specific reduction in pro-atherogenic cytokine levels. However this needs to be assessed in greater detail in atherosclerotic mouse models as changes in the systemic cytokine profile may not reflect the changes occurring at a local arterial level.

The changes in circulating cytokine levels following CardioWise treatment also indicate that it may potentially promote brown fat activation. Adipose tissue is recognised as a powerful endocrine organ due to its ability to secrete a variety of chemokines and cytokines including TNF-α and IL-6 (Kershaw and Flier 2004; Coelho *et al.* 2013). During obesity, the adipose tissue becomes dysfunctional and results in the altered secretion of cytokines. Circulating levels of CXCL1, IL-5, IL-10 and IL-6 have all been shown to be increased in obese individuals (Nunemaker *et al.* 2014; Schmidt *et al.* 2015; Manna and Jain 2015). Treatment with CardioWise in wild type mice reduced the plasma levels of these cytokines, indicating CardioWise is capable of protecting against high fat diet induced obesity and restore normal adipose tissue function. Furthermore TNF-α is thought to play a role in the deterioration and dysfunction of brown fat (Nisoli *et al.* 2000; Markelic *et al.* 2011). CardioWise may protect brown fat and re-establish its physiological function during a high fat diet by attenuating the circulating levels of TNF-α. However IL-2 levels are attenuated during obesity and was also observed following CardioWise dietary supplementation (Vargas *et al.* 2016). Overall these results indicate that CardioWise restores the normal function of adipose tissue during a high fat diet.

4.4.3 The effect of CardioWise on gene expression in the liver of wild type mice

To determine whether the changes caused by CardioWise treatment discussed so far were due to changes in gene expression, a qPCR array containing 84 atherosclerosis related genes was used to assess gene expression changes within the liver. A total of 49 genes were found to have their expression altered by at least 10% (Fig. 4.9). Of those 49 genes, 8 were found to be significantly altered and those genes were; *CFLAR*, *CXCL1*, *IFN-γ*, *LIF*, *LYPLA1*, *NR1H3*, *PPAR-γ* and *SOD1*. *CFLAR* is considered a master regulator in preventing cell apoptosis by switching cell signalling from pro-apoptotic to anti-apoptotic by blocking caspase 8 activation and stimulating NFκB and extracellular signal-regulated kinase (ERK) signalling (Kavurma *et al.* 2008; Safa 2012; Wang *et al.* 2017a). The expression of *CFLAR* has been shown to be elevated in healthy hearts and reduced in individual's with heart failure (Micheau 2003). Furthermore the presence of oxLDL has been shown to attenuate *CFLAR* expression in vascular endothelial cells leading to apoptosis and tissue destruction (Micheau 2003). The upregulation of *CFLAR* may therefore protect the blood vessels from oxLDL exposure and

retard the progression of atherosclerosis. The expression of *CFLAR* was found to be significantly upregulated following CardioWise treatment in wild type mice fed a high fat diet for 21 days (Fig. 4.10B). This result would indicate that CardioWise is capable of preventing vascular endothelial damage which may occur during exposure to oxLDL and reduce atherosclerosis disease development. Previous research has shown that ω-3 PUFAs are capable of promoting *CFLAR* expression, however the effects of catechin and phytosterols still remains unknown (Suzuki *et al.* 2003). The role of BCL2A1A during apoptosis and atherosclerosis is still unclear, however FGF2 is thought to signal through it, and BCL2A1A appears to play a role in B-cell maintenance (Kim *et al.* 2012; Ottina *et al.* 2012). The levels of *BCL2A1A*, *FGF2* and B-cell population were all reduced following CardioWise treatment for 21 days (Figs. 4.10A, 4.12B and 4.22C respectively).

The expression of the chemokine *CXCL1* was significantly attenuated following CardioWise treatment (Fig. 4.11B), which correlates with the reduced plasma CXCL1 levels previously seen (Fig. 4.8A). CXCL1 plays an important role in the recruitment of monocytes to the site of oxLDL accumulation by allowing them to attach to the activated endothelium before migrating into the wall of the artery (Wan and Murphy 2013; van der Vorst *et al.* 2015). Reductions in the expression of this chemokine may explain the decrease in monocyte migration observed *in vitro* (Fig. 3.9). The expression of *CXCL1* is ubiquitous within human and mouse atherosclerotic lesions and its circulating levels have been found to be increased in patients with coronary artery disease (CAD; Breland *et al.* 2008; Wan and Murphy 2013). Furthermore *LDLr* deficient mice also lacking *CXCL1* develop smaller atherosclerotic plaques, highlighting the major pro-inflammatory role that CXCL1 plays during atherosclerosis development (Boisvert *et al.* 2006). The gene expression levels of *CD44*, *ITGB2* and *SELPLG*, which all play a role in cell recruitment during atherosclerosis, were also found to be reduced by CardioWise (Fig. 4.11; Merched *et al.* 2010; McEver 2015; Saha *et al.* 2017). These results provide evidence that CardioWise would be able to reduce the size of atherosclerotic lesions by decreasing cell recruitment to the site of oxLDL accumulation, however this needs to be studied in future longer term feeding studies and mouse atherosclerosis models.

The expression levels of the cell proliferation gene *LIF* was also reduced by CardioWise treatment (Fig. 4.12D). This result is not surprising as flavanol treatment has previously been shown to attenuate *LIF* gene expression (Akhtar and Haqqi 2011). LIF is considered as an anti-atherogenic cytokine due to its ability to attenuate endothelial cell proliferation and induce vasorelaxation within arteries by increasing NO production (Demyanets *et al.* 2012). The amount of intimal thickening which occurs following blood vessel injury due to increased level of VSMC proliferation can be inhibited by LIF (Demyanets *et al.* 2012). Therefore the decreased expression of *LIF* within the liver of wild type mice due to CardioWise treatment would indicate that CardioWise exerts potential pro-atherogenic effects (Fig. 4.12D). However

reductions in the expression of other pro-atherogenic genes involved in cell proliferation including *CSF2*, *FGF2* and *IL-3* were observed in the CardioWise treated mice compared to the vehicle control treated mice (Fig. 4.12; Liu *et al.* 2013; Subramanian *et al.* 2015; Ramji and Davies 2015). CardioWise appears to be a strong anti-proliferative nutraceutical, however the effect of CardioWise on cell proliferation *in vivo* still needs to be determined in future studies.

The results of reduced cell recruitment and proliferation related genes in the CardioWise treated mice in comparison to the vehicle control treated mice (Figs. 4.11 and 4.12) indicates that dietary supplementation with CardioWise would potentially reduce lesion size by attenuating cellular build-up within the atherosclerotic plaque. Previous studies have shown that ω-3 PUFAs, flavanol and phytosterol treatment is capable of decreasing lesion size within mice (Moghadasian *et al.* 1999; Yeganeh *et al.* 2005; Moghadasian 2006; Chyu 2004; Nakajima *et al.* 2011; Brown *et al.* 2012; Minatti *et al.* 2012; Morrison *et al.* 2014). The results presented in this study may provide a mechanism of action by which ω-3 PUFAs, catechin and phytosterols achieve a reduction in atherosclerotic lesion size formation in the studies mentioned above. Atherosclerotic plaques with reduced cellular content are considered to be more stable and less likely to rupture when compared to those with a high number of cells, in particular macrophages, within the lesion (Wilson 2010; Seneviratne *et al.* 2013). Furthermore CardioWise treatment was also found to increase the expression of the cell apoptosis inhibitor gene *CFLAR* (Fig. 4.10B). Preventing apoptosis in the macrophages present in the plaque would reduce the amount of lipid content and ECM modulating enzymes they release and therefore also contribute to improved plaque stability. These results infer that CardioWise has the potential to reduce atherosclerotic lesion formation and reduce the speed of disease development, however this needs to be assessed in greater detail in long term feeding studies involving atherosclerotic mouse models.

The enzyme APT1 is encoded by the gene *LYPLA1* which was found to have its expression enhanced by CardioWise in the livers of wild type mice fed a high fat diet (Fig. 4.13). The function of APT1 during atherosclerosis disease development remains unclear but it is thought to be involved in lipid metabolism due to its ability to remove fatty acid groups from cell surface proteins (Vujic *et al.* 2016). The removal of a fatty acid group from the proteins causes them to return to the Golgi apparatus in a process known as depalmitoylation (Vujic *et al.* 2016). As CardioWise results in an increase in the expression of APT1, it therefore has the potential to reduce the number of proteins expressed on the surface of cells due to amplified cell surface protein removal. This means CardioWise is capable of influencing intracellular signalling.

The expression of several genes involved in stress responses was attenuated by CardioWise treatment *in vivo* (Fig. 4.14). As previously discussed, IFN-γ is a major pro-inflammatory cytokine and considered to be the main regulator of atherosclerosis disease development (Moss and Ramji 2015; Moss and Ramji 2016a; Ramji and Davies 2015; McLaren and Ramji

2009). CardioWise treatment significantly reduced *IFN-γ* expression indicating that it was capable of exerting strong anti-inflammatory effects. A reduction in *IFN-γ* expression has also been seen in previous studies which assessed the effect of the nutraceuticals within the CardioWise formulation in isolation (Nakajima *et al.* 2011; Leong *et al.* 2009; Valerio *et al.* 2011). Despite changes at the gene expression level, there was no observable reduction in the circulating levels of IFN-γ (Fig. 4.8B). A lack of reduction in the protein levels of IFN-γ would imply CardioWise does not possess anti-inflammatory properties, however the mRNA expression and protein levels of two other pro-inflammatory genes, *IL-1β* and *TNF-α*, were attenuated in the mice which received treatment compared to the vehicle control receiving mice. Furthermore reduced IFN-γ and TNF-α signalling have been associated with improved brown fat activation, thereby providing additional evidence for the possible activation of brown fat by CardioWise (Nisoli *et al.* 2000; Moisan *et al.* 2015). The expression of *SOD1* was significantly increased following CardioWise treatment when compared to the vehicle control mice (Fig. 4.14C). As previously discussed, this may explain the lack of a significant increase in ROS production levels *in vivo* that were observed during the *in vitro* assay (Figs. 4.7A and 3.14). Overall the changes in the stress response gene expression observed following CardioWise treatment would suggest it exerts strong anti-inflammatory effects which would protect against atherosclerosis plaque formation.

CardioWise treatment also increased the expression of two transcriptional regulators *NR1H3* and *PPAR-γ* (Fig. 4.15). NR1H3, also known as LXRα, protects against cholesterol accumulation within cells by enhancing the expression of genes involved in cholesterol efflux and transportation (Parikh *et al.* 2014; Michael *et al.* 2012a). Activation of *NR1H3* has also been shown to attenuate the expression of pro-inflammatory genes including *TNF-α* and *IL-1β* (Zelcer and Tontonoz 2006). Indeed CardioWise treatment was found to increase the expression of *NR1H3* (Fig. 4.15A) which correlated with decreases in the expression of *TNF-α* and *IL-1β* (Fig. 4.14). Furthermore increased NR1H3 activation is associated with reduced dietary cholesterol absorption from the intestine via NPLC1L1 (Alvaro *et al.* 2010). Unfortunately the levels of circulating total cholesterol were unaffected during this short term feeding study (Fig. 4.5). However NR1H3 is capable of increasing plasma HDL cholesterol levels and this was seen as a result of CardioWise treatment (Fig. 4.5B). Perhaps the increased expression of *NR1H3* is the mechanism by which CardioWise achieves its HDL raising properties. The other regulator of transcription to have its expression increased by CardioWise treatment was *PPAR-γ* (Fig. 4.15B). NR1H3 and PPAR-γ are transcription factors that are considered to play important roles during adipocyte differentiation (Ohshima *et al.* 2012; Laurencikiene and Rydén 2012). Indeed activation of NR1H3 and PPAR-γ has been associated with increased brown fat activation (Steffensen *et al.* 2003; Ohno *et al.* 2012). Furthermore increased PPAR-γ activation has been shown to reduce high fat diet induced inflammation in mice by decreasing the expression and secretion of IL-1β, IL-6 and TNF-α, all

of which were also observed in this study (Figs. 4.8 and 4.14; Seed *et al.* 1998; Engstrom *et al.* 2010). Increased PPAR-γ activation may possibly be the mechanism by which CardioWise is cable of attenuating pro-inflammatory gene expression and circulating pro-inflammatory cytokine levels *in vivo*. This result concurs with that of previous studies which have shown that ω-3 PUFAs and catechin are capable of activating PPAR-γ (Calder 2012; Shin *et al.* 2009). The effect of CardioWise on the expression of transcriptional regulators appears to be anti-inflammatory and may provide a mechanism of action by which it exerts its other anti-inflammatory effects previously discussed.

Based on the changes in gene expression in the liver of wild type mice fed a high fat diet for 21 days, CardioWise appears to exert strong anti-inflammatory effects. This can be seen by attenuating the expression of several pro-inflammatory genes which are key in the development of atherosclerosis including *CXCL1*, *SELPLG*, *IFN-γ*, *IL-1β* and *TNF-α*. Furthermore CardioWise was able to increase the expression levels of a variety of anti-inflammatory genes including *CFLAR*, *NR1H3* and *SOD1*. Future studies are required to fully evaluate these gene expression changes and their possible effects on atherosclerosis disease development in long term feeding studies using either *ApoE* or *LDLr* deficient mice.

4.4.4 The possible activation of brown fat following CardioWise treatment

As previously discussed, there is evidence to suggest that CardioWise treatment in wild type mice is capable of enhancing brown fat activation. CardioWise has been shown to affect *NR1H3*, *PPAR-γ*, *SOD1* and pro-inflammatory gene expression as well as circulating levels of cytokines, HDL cholesterol and markers of lipid peroxidation, which have all been associated with brown fat activation (summarised in Fig. 4.23; Barja de Quiroga *et al.* 1991; Nisoli *et al.* 2000; Steffensen *et al.* 2003; Nedergaard *et al.* 2005; Pan *et al.* 2005; Jastroch *et al.* 2010; Markelic *et al.* 2011; Moisan *et al.* 2015; Hoeke *et al.* 2017). It is possible that CardioWise is capable of activating brown fat as previous studies have shown that treatment with ω-PUFAs or flavanols activates brown fat (Quesada-López *et al.* 2016; Pahlavani *et al.* 2017; Yoneshiro *et al.* 2017). Since the turn of the century, research into the possible use of activating brown fat to protect individuals against obesity and CVD has exploded (Harms and Seale 2013; Peirce *et al.* 2014; Villarroya *et al.* 2016). The primary function of brown fat is to produce heat and maintain body temperature through a process known as non-shivering thermogenesis. Heat production is achieved by the high number of mitochondria present within the brown adipocytes and the high expression levels of uncoupling protein 1 (*UCP1*), which uncouples substrate oxidation and ATP production resulting in heat production (Harms and Seale 2013; Peirce *et al.* 2014; Villarroya *et al.* 2016). Brown fat activation is thought to protect against obesity and CVD due to non-shivering thermogenesis being a high energy dependent process and consequently resulting in fat being used as an energy source rather than being stored in white fat deposits (Harms and Seale 2013; Peirce *et al.* 2014; Villarroya *et al.* 2016). Brown fat

is also capable of functioning like an endocrine organ and therefore its activation may alter the circulating cytokine profile of an individual to a more anti-inflammatory one (Villarroya *et al.* 2016). Furthermore it has been found that during brown fat activation, the levels of *UPC1* expressing, thermogenic-capable adipocytes increase within the white fat deposits (Harms and Seale 2013; Peirce *et al.* 2014; Villarroya *et al.* 2016; Jeremic *et al.* 2017). These adipocytes are often referred to as beige adipocytes. During brown fat activation, the number of beige adipocytes present in white fat deposits increases in a process known as 'browning' (Harms and Seale 2013; Peirce *et al.* 2014; Villarroya *et al.* 2016; Jeremic *et al.* 2017). As a result of the discovery of brown fat deposits within adult humans, the activation of brown fat and the browning of white fat have become novel therapeutic targets. Further studies are required to explore the possibility of CardioWise treatment activating brown fat or enhancing the browning of white adipose tissue. These future studies must investigate gene expression changes and markers such as *UCP1*, zic family member (ZIC)1 and myosin light chain, phophorylatable, fast skeletal muscle (*MYLPF*) which are associated with brown fat activation (Harms and Seale 2013; Peirce *et al.* 2014). Additionally the gene expression levels of *UCP1*, *CD137* and short stature homeobox (SHOX)2 needs to be assessed in white adipose tissue as they are indicators of beige adipocytes (Harms and Seale 2013; Peirce *et al.* 2014). Furthermore due to the role of brown fat in non-shivering thermogenesis, the temperature of the mice must be monitored as an increase in body temperature may also indicate brown fat activation (Crane *et al.* 2014; Meyer *et al.* 2017).

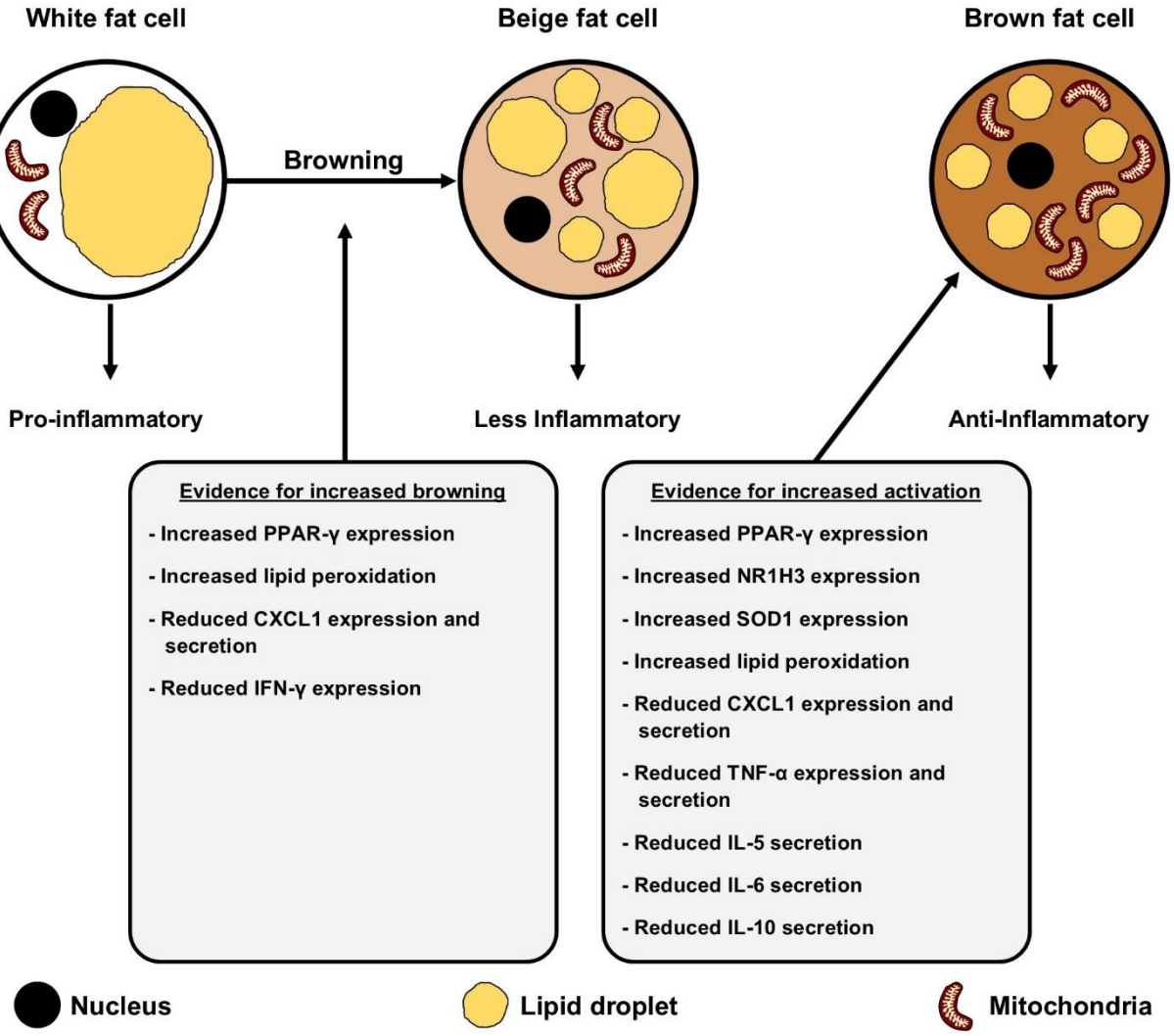

Fig. 4.23. The different shades of fat and the possible effects of CardioWise treatment. The above figure summarises the possible evidence that CardioWise treatment in wild type mice fed a high fat diet for 21 days may activate brown fat and enhance the browning of white adipose tissue. See text for further details.

4.4.5 The effect of CardioWise on bone marrow cell populations in wild type mice

A high fat diet has been associated with changes in the proportion of bone marrow cell populations which can lead to atherosclerosis (Chan *et al.* 2012; Wu *et al.* 2013; Adler *et al.* 2014; van den Berg *et al.* 2016; Lang and Cimato 2014; Murphy *et al.* 2014; Ma and Feng 2016). Furthermore dietary supplementation with nutraceuticals has been shown to also alter the cell populations within bone marrow (Takano *et al.* 2004a; Betiati *et al.* 2013; Chang and Deckelbaum 2013). Therefore the bone marrow of wild type mice fed a high fat diet for 21 days was analysed to determine whether CardioWise treatment could alter the proportion of cell populations and retard atherosclerosis progression. The summary of the changes within the bone marrow following CardioWise treatment can be found in Fig. 4.24.

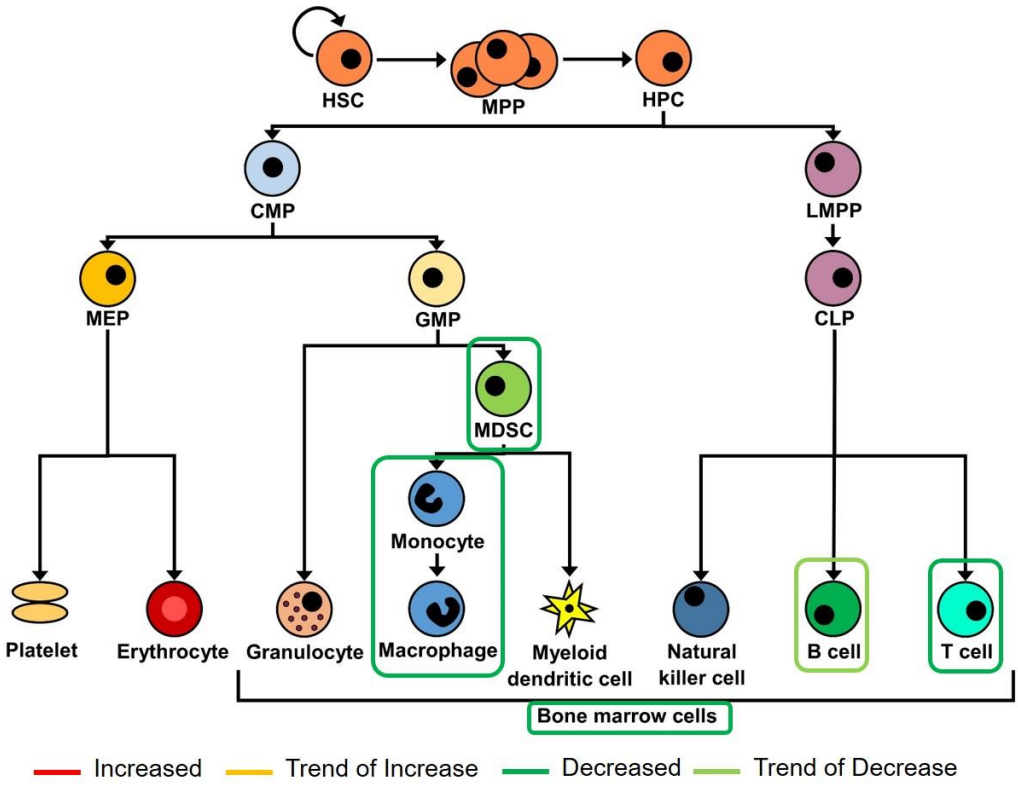

Fig. 4.24. The CardioWise induced changes in proportions of cell populations in the bone marrow. The bone marrow of wild type mice were harvested after they had received either CardioWise or vehicle control treatment while being fed a high fat diet for 21 days. HSC, haematopoietic stem cell; MPP, multipotent progenitors; HPC, haematopoietic progenitor cell; CMP, common myeloid progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-macrophage progenitor; MDSC, myeloid-derived suppressor cells; LMPP, lymphomyeloid progenitor; CLP, common lymphoid progenitor.

The levels of an individual's white blood cells can be seen as an indicator of their inflammatory state. Previous studies have shown that the levels of white blood cells normally increase during atherosclerosis disease progression (Ates *et al.* 2011; Mozos *et al.* 2017). A reduction in the proportion of bone marrow cells was observed in the bone marrow of wild type mice following CardioWise treatment, indicating a possible reduction in the inflammatory state and reduced atherosclerosis development (Fig. 4.16). This change in bone marrow cell number is a result of a reduction in cell populations such as macrophages, MDSCs, B-cells and T-cells which will be discussed in greater detail below.

Previous studies have demonstrated a correlation between increased cholesterol levels and increased number of haematopoietic stem/progenitor cells (HSPCs) which leads to an increase in the number of white blood cells (Ma and Feng 2016). During this study, CardioWise did not significantly alter total serum cholesterol levels but did increase HDL cholesterol concentration (Fig. 4.5). Therefore FACS analysis was performed in order to determine whether CardioWise treatment results in altered cell population proportions within the bone marrow of wild type mice.

During atherosclerosis disease progression, it has been found that the HSCs and LK cell populations begin to proliferate and relocate to the spleen where they begin to differentiate into monocytes which specifically target the atherosclerotic lesion (Robbins *et al.* 2012). Previous studies have observed increases in CMP and GMP cell populations within the LK cell group in mice following a high fat diet (Gao *et al.* 2014). CardioWise had no effect on either the HSC and LK cell populations indicating no anti-atherogenic shift in cell populations within the bone marrow (Figs. 4.18 and 4.19). Both CSF2 and IL-3 are essential in the differentiation of HSPCs, especially in the formation of monocytes from its precursors (Lang and Cimato 2014; Seita and Weissman 2010; Schmid *et al.* 2010; Wu *et al.* 2017; Francisco-Cruz *et al.* 2014). The expression of *CSF2* and *IL-3* was reduced in mice receiving CardioWise (Fig. 4.12). This result indicated CardioWise has the potential to increase proliferation of HSPCs but attenuate their differentiation into monocytes and exert anti-inflammatory effects. Indeed CardioWise treatment results in a significant decrease in the number of monocytes and macrophages found within the bone marrow of wild type mice (Fig. 4.21). This reduction in monocyte and macrophage number could eventually result in decreased foam cell formation and smaller atherosclerotic plaques forming due to a smaller number of monocytes which can be recruited to the site of oxLDL accumulation within the artery wall (Hansson and Libby 2006). Although there is a decrease in the number of monocytes and macrophages, there is no indication on whether these macrophages are pro-atherogenic M1 macrophages or anti-atherogenic M2 macrophages. Further studies are required to determine the consequences of the observed decrease in monocyte numbers following CardioWise treatment and which phenotype the resulting macrophage develop into.

Increases in serum HDL levels have been shown to attenuate HPSC proliferation (Murphy *et al.* 2011; Chen *et al.* 2014; Yvan-Charvet *et al.* 2010). The circulating levels of HDL cholesterol was found to be increased in the mice which received CardioWise when compared to the vehicle control mice (Fig. 4.5). Perhaps this increase in HDL cholesterol is responsible for the non-significant reductions observed in the HPC and CLP progenitor cell populations (Figs. 4.18 and 4.20).

Changes within the haematopoietic cell populations may also arise due to changes in gene expression. Both B- and T-cells are rare within the atherosclerotic lesion and depending on their phenotype they can exert both pro- and anti-atherogenic effects (Andersson *et al.* 2010; Kyaw *et al.* 2011). The rate of proliferation of both the B- and T-cell populations can be influenced by a variety of genes including *CFLAR* and *BCL2A1A* (Safa 2012; Ottina *et al.* 2012; Métais *et al.* 2012). As previously discussed, dietary supplementation with CardioWise resulted in the altered expression of these genes (Fig. 4.10). This altered gene expression may explain the decrease observed in the proportion of B- and T-cells found within the bone marrow following CardioWise treatment (Fig. 4.22). The reduction in the expression of *BCL2A1A* is of

particular interest as decreases in the expression of this gene have been previously associated with attenuated early T-cell formation and reduced B-cell populations due to its role in preventing apoptosis (Ottina *et al.* 2012; Métais *et al.* 2012). However one of the major genes involved in regulating B- and T-cell proliferation is *PPAR-γ*. This gene negatively regulates both B- and T-cell proliferation, and previous studies have found that a deficiency in *PPAR-γ* expression results in the increased proliferation of B- and T-cells (Choi and Bothwell 2012; Garcia-Bates *et al.* 2009). Therefore the increase in *PPAR-γ* expression observed following CardioWise treatment might explain the changes in B- and T-cell populations. Furthermore an increase in the expression of *PPAR-γ* has been shown to promote the survival and activity of regulatory T-cells (Tregs), which have anti-atherogenic actions, therefore even though CardioWise reduces T-cell numbers it may enhance their differentiation into the anti-atherogenic phenotype (Choi and Bothwell 2012). This altered gene expression following CardioWise treatment may provide a mechanism of action by which previous studies have shown that treatment with ω-3 PUFAs *in vitro* and *in vivo* can result in reduced T-cell proliferation (Calder 2015).

Wild type mice fed a high fat diet and treated with CardioWise had reduced number of MDSCs within their bone marrow when compared to the vehicle control treated mice (Fig. 4.19). Not much is currently known about the role of MDSCs but they have been found to be capable of suppressing T-cell differentiation (Talmadge and Gabrilovich 2013). However CardioWise resulted in the decrease in the MDSC and T-cell population. This result may indicate that CardioWise treatment caused the reduction of both MDSCs and T-cell populations via two separate mechanisms. The cytokines CSF2, IL-6 and IL-10 have been shown to be involved in the differentiation of MDSCs (Mundy-Bosse *et al.* 2011; Dolcetti *et al.* 2009). CardioWise treatment reduced the gene expression and circulating levels of these cytokines which may explain the reduced number of MDSCs found in this study (Figs. 4.8 and 4.12). MDSCs have been found to be involved in the progression and metastasis of tumours by releasing a variety of cytokines capable of influencing cell invasion, proliferation, survival and adhesion (Talmadge and Gabrilovich 2013). Furthermore angiogenesis during tumour growth has been found to be promoted by MDSCs (Talmadge and Gabrilovich 2013). As previously discussed, angiogenesis within the atherosclerotic plaque can result in it becoming unstable and rupturing, therefore the reduction in the MDSC population as a result of CardioWise treatment may potentially result in improved plaque stability (Greenberg and Jin 2013). Few studies have investigated the role of MDSCs during atherosclerosis disease progression but those which did have observed that a reduction in population number was associated with an increase in inflammation and atherosclerosis disease progression (Xia *et al.* 2011; Foks *et al.* 2016). Additional studies are required to determine the specific role of MDSCs within atherosclerosis disease development in order to better understand the implications of CardioWise treatment reducing this cell population.

The changes in bone marrow cell populations following CardioWise treatment appear to be anti-atherogenic. Further studies are required to determine the consequences of these alterations in haematopoietic cell populations which resulted from CardioWise treatment. Future long term feeding studies also need to analyse the cell populations within the atherosclerotic plaque directly. This would overcome the limitation associated with this study which was restricted to only assessing the cell populations with the bone marrow. Without determining the cell population changes within an atherosclerotic plaque, we are unable to assess whether the changes within the bone marrow correlate with changes within the plaque or whether the changes in cell populations within this study are due to an altered secretion of lineage cells from the bone marrow into the blood stream. For example, decreased monocyte and macrophage numbers in the bone marrow may actually represent increased monocyte and macrophage release into the blood stream. This would alter the conclusions drawn in this study. The reduction in monocyte and macrophage number within the bone marrow would then represent a pro-inflammatory effect of CardioWise rather than an anti-inflammatory one.

4.4.6 Summary of the effect of CardioWise in wild type mice

This is the first *in vivo* study to assess the effect of a unique nutraceutical combination containing ω-3 PUFAs, flavanols and phytosterols at a human physiological equivalent dose in mice receiving a high fat diet. CardioWise appeared to exert strong anti-atherogenic effects by increasing serum HDL cholesterol levels, promoting anti-inflammatory gene expression and altering circulating levels of pro-inflammatory cytokines (summarised in Fig. 4.25). Furthermore this study generated a lot of indirect evidence which indicated that CardioWise is potentially capable of enhancing brown fat activation as indicated by changes in gene expression and several other blood parameters. A reduction in the number of B-, T-cells and macrophages present within the bone marrow of mice which received CardioWise implies an anti-atherogenic effect. Overall the results of this study indicate that CardioWise is capable of promoting an anti-inflammatory state within wild type mice receiving a high fat diet. This initial study justifies the use of CardioWise in longer term *in vivo* feeding studies which involve atherosclerotic mouse models such as *ApoE* or *LDLr* deficient mice to fully understand its anti-atherogenic potential. Using atherosclerotic mouse models would also allow the effect of CardioWise treatment on plaque size and formation, lesion cell composition, plaque stability and plaque regression to be determined. Previous studies have demonstrated that ω-3 PUFAs, flavanols and phytosterols are capable of attenuating plaque formation (Moghadasian *et al.* 1999; Yeganeh *et al.* 2005; Moghadasian 2006; Chyu 2004; Nakajima *et al.* 2011; Brown *et al.* 2012; Minatti *et al.* 2012; Morrison *et al.* 2014). These previous studies indicate that CardioWise would also result in a smaller atherosclerotic plaque.

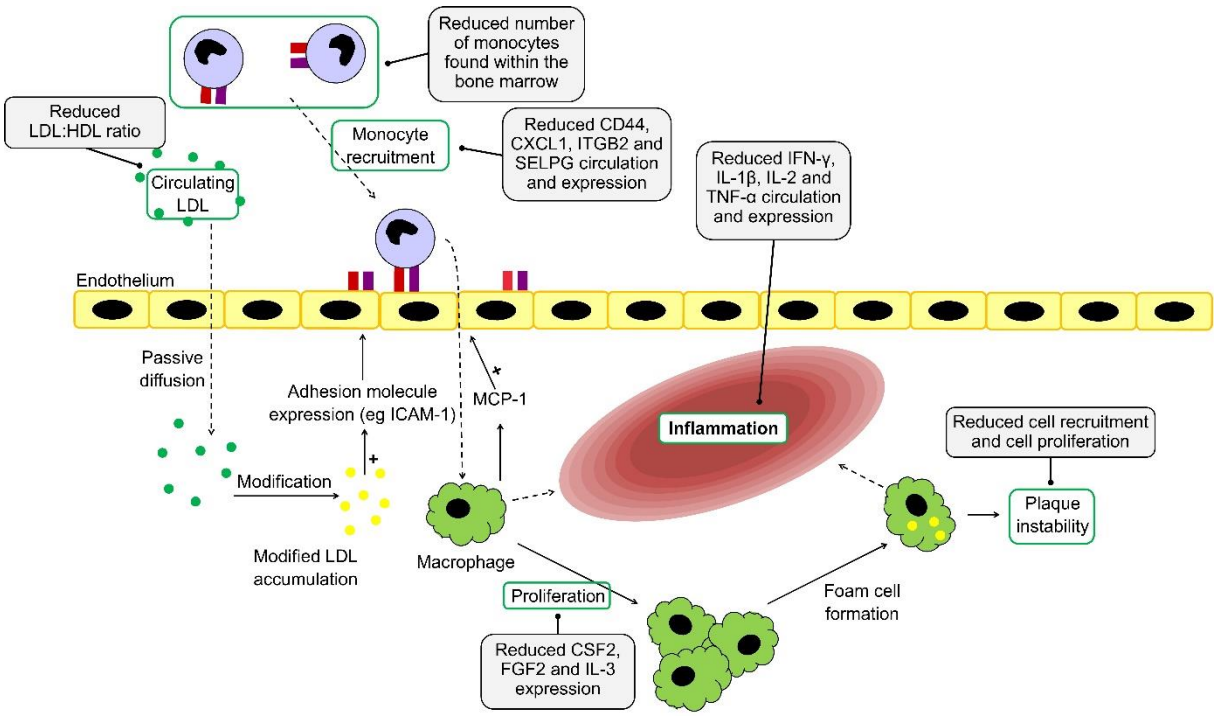

Fig. 4.25. Summary of the potential anti-atherogenic properties of CardioWise *in vivo*. The steps potentially reduced in atherosclerosis disease progression following CardioWise treatment are highlighted in green. Possible mechanisms for these changes are also included.

Chapter 5

Assessing the main bioactive components of CardioWise *in vitro*

5.1 Introduction

Due to CardioWise being a combination of fish oil, cocoa flavanols and phytosterols, it was important to investigate whether any of the individual bioactive ingredients had specific mechanistic actions. Studying the nutraceuticals within CardioWise would not only aid in explaining how it exerts its anti-atherogenic effects but it would help in refining the product by identifying the most effective compounds and altering the proportions of the nutraceuticals within the mixture. The major nutraceuticals within CardioWise were determined to be EPA, DHA, catechin, stigmasterol, campesterol and β-sitosterol.

These nutraceuticals were used in a variety of initial assays at the concentrations they can be found within CardioWise in order to find an individual compound that would be investigated in further detail. Initially the levels of secreted pro-inflammatory cytokines MCP-1 and IL-1β were measured by ELISA. The role of MCP-1 during atherosclerosis lesion formation has been discussed in previous sections. The innate immune system response can be activated by IL-1β (Moss and Ramji 2016a). The importance of IL-1β during disease progression can be demonstrated in atherosclerotic mouse models. *ApoE* deficient mice which were also lacking either IL-1β or its receptor were found to develop smaller atherosclerotic plaques (Kamari *et al.* 2011; Shemesh *et al.* 2012). Additionally, the CANTOS trial demonstrated that inhibition of IL-1β with a neutralising antibody was capable of reducing an individual's risk of a recurrent CVD-related event (Ridker *et al.* 2017a). Furthermore IL-1β is a major cytokine released by the NLRP3 inflammasome and therefore the level of IL-1β can be used as an indirect measurement of inflammasome activation (Hoseini *et al.* 2017). The individual nutraceuticals were also evaluated in monocyte migration, ROS production and cholesterol efflux assays (details on the rationale of these assays can be found in Chapter 3).

While assessing the individual nutraceuticals from CardioWise, an additional potential bioactive compound was included within the initial assays. Butyrate is a short chain fatty acid which is formed from dietary fibre fermentation within the gut (Moss and Ramji 2016b). Preclinical studies have started to observe anti-inflammatory effects following butyrate treatment. The expression of the pro-inflammatory cytokines *IL-1β* and *TNF-α* was significantly reduced by butyrate in murine macrophages (Liu *et al.* 2012a). Furthermore *ApoE* deficient mice fed a standard chow diet which was supplemented with 1% butyrate were found to develop smaller and more stable atherosclerotic plaques (Aguilar *et al.* 2014). The size of the

atherosclerotic lesions were reduced by approximately 50% due to decreased monocyte and macrophage accumulation, which correlated with attenuated expression of the cell recruitment genes *MCP-1* and *ICAM-1* (Aguilar *et al.* 2014). Furthermore the plaques were found to have increased ECM deposition, a sign of increased plaque stability (Aguilar *et al.* 2014). Additionally several epidemiological studies have found a correlation between increased dietary fibre intake and a reduced risk of suffering a CVD-related event (Liu *et al.* 2002; Merchant *et al.* 2003; Oh *et al.* 2005a). However the effectiveness of a fibre rich diet, and which component of the diet exerts the potential cardiovascular protective effects, is still debated within the literature (Moss and Ramji 2016b). Therefore butyrate was included within the initial assays to determine its potential as a nutraceutical.

Following the results of the monocyte migration and ROS production assays, catechin was chosen as the nutraceutical which would be investigated in greater detail. It was also chosen as relatively little is known about its potential anti-atherogenic effects and mechanism of action when compared to other nutraceuticals such as ω-3 PUFAs. Therefore this study presented an ideal opportunity to assess catechin in greater detail using our robust *in vitro* models of atherosclerosis disease development steps. Initially a mini-dose response was performed on gene expression, monocyte migration and ROS generation assays to ensure an effective dose of catechin was being utilised. A dose of 1.5 µg/ml was selected as it is the same concentration of catechin found with CardioWise. Additional doses of 5, 10 and 20 µg/ml were used to provide a good range of catechin concentrations in the initial assays. Once an optimal dose of catechin had been determined, its effect on cell viability was tested with an LDH assay to ensure it did not exert any detrimental effects before using it in further assays.

5.2 Experimental Aims

Details of specific methodologies for each experimental aim are outlined in Chapter 2 and the experimental strategies for Chapter 5 are presented in Figs. 5.1 – 5.4.

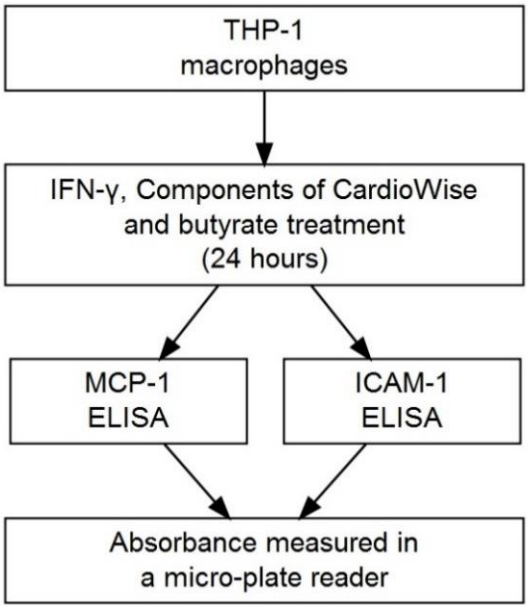

Fig. 5.1. Strategy for assessing pro-inflammatory cytokine production. ELISA, enzyme-linked immunosorbent assay; ICAM, intercellular adhesion molecule; MCP, monocyte chemotactic protein.

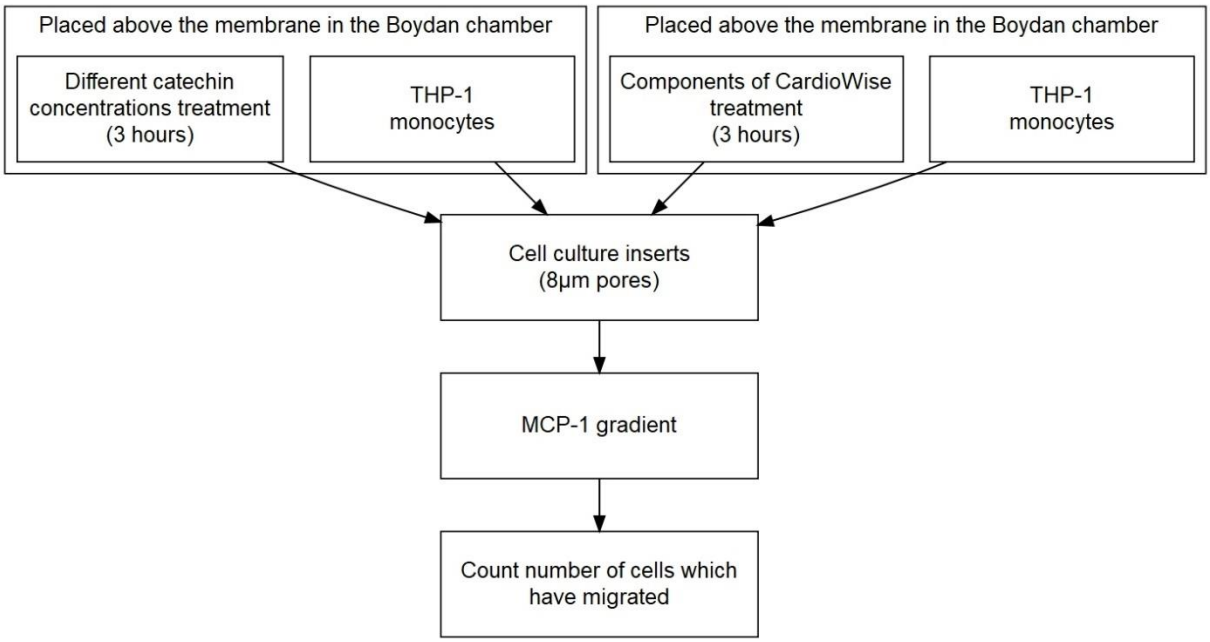

Fig. 5.2. Monocyte migration experimental strategy. MCP, monocyte chemotactic protein.

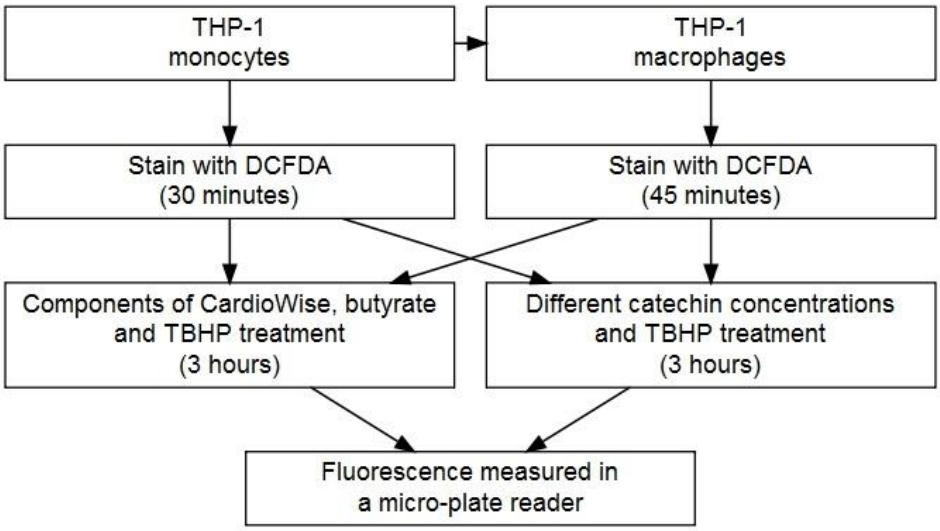

Fig. 5.3. Strategy for assessing ROS production. A longer incubation period is required for macrophages as it takes more time for the DCFDA compound to diffuse into adherent cells. DCFDA, dichlorofluorescin diacetate; TBHP, tert-butyl hydrogen peroxide.

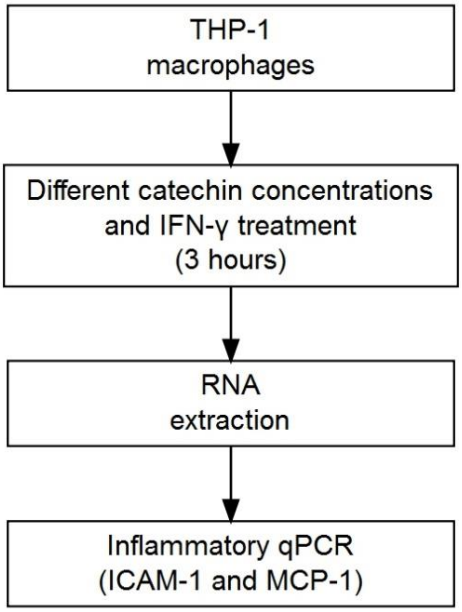

Fig. 5.4. Strategy for assessing gene expression. ICAM, intercellular adhesion molecule; IFN, interferon; MCP, monocyte chemotactic protein.

5.3 Results

5.3.1 The effect of nutraceuticals on the secreted levels of pro-inflammatory cytokines following IFN-γ stimulation in human macrophages

The production of two major pro-inflammatory cytokines, MCP-1 and IL-1β, can be induced by IFN-γ (Moss and Ramji 2016a). PMA differentiated THP-1 macrophages were incubated with IFN-γ (250 U/ml) for 24 hours to induce MCP-1 and IL-1β expression. The concentration of secreted MCP-1 was significantly increased by 5.8 fold (p=0.050), whereas IL-1β levels showed a non-significant trend of increase of 1.5 fold in human THP-1 derived macrophages (p=0.079; Fig. 5.5). Following EPA and DHA treatment the levels of MCP-1 were reduced by 45.6% (p=0.065) and 24.9% (p=0.417) respectively, however these changes were not significant (Fig. 5.5A). Furthermore catechin, stigmasterol, campesterol, β-sitosterol and butyrate did not affect the secretion of MCP-1. IL-1β secretion was significantly increased by 39.5%, 48.2%, 62.0% and 67.9% after treatment with catechin, stigmasterol, campesterol and β-sitosterol (p=0.023, p=0.003, p<0.001 and p<0.001 respectively; Fig. 5.5B). EPA, DHA and butyrate failed to significantly affect IL-1β levels (p=0.995, p=0.995, p=0.999 respectively). Overall these results are not very informative about the active ingredient of CardioWise and hence monocyte migration and ROS production assays were used for subsequent studies.

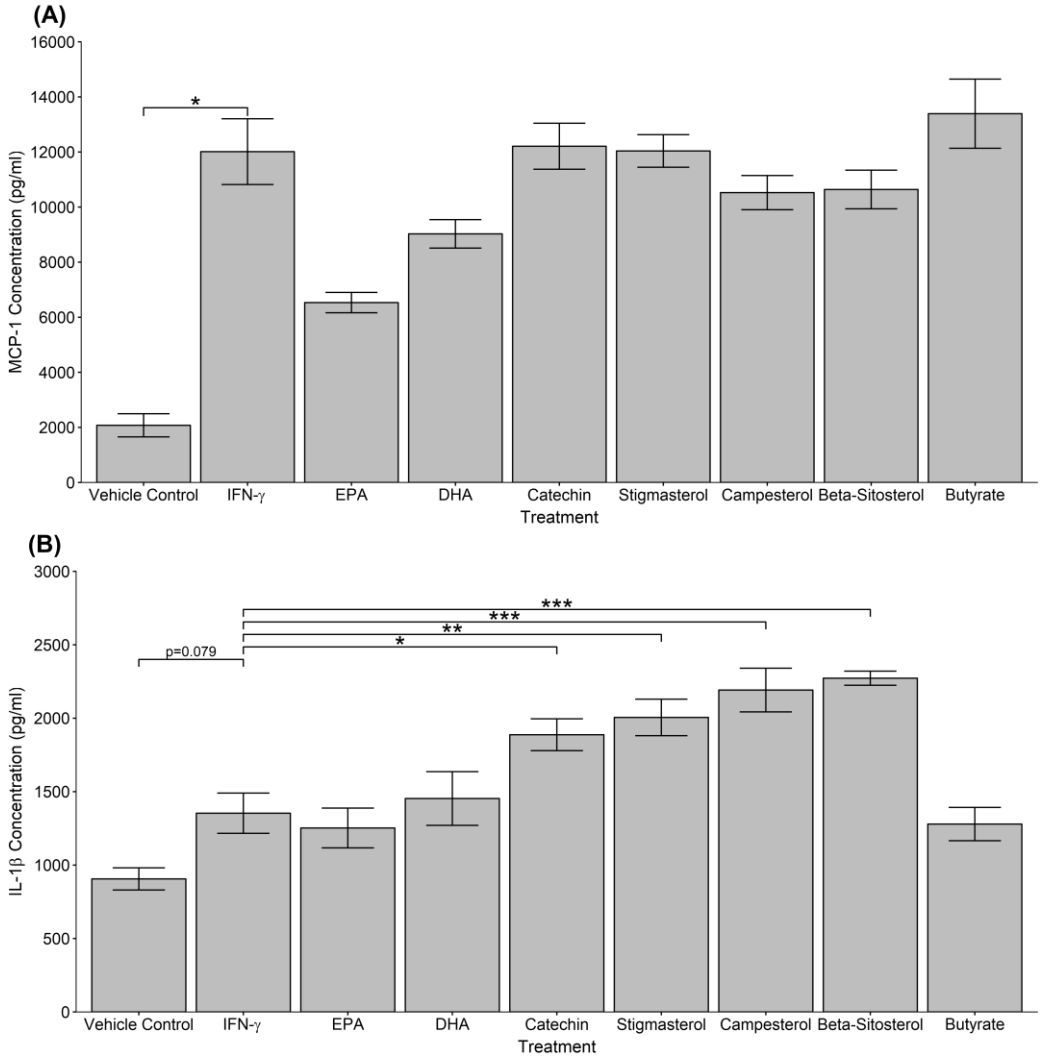

Fig. 5.5. Bioactive compounds within CardioWise can affect MCP-1 and IL-1β protein secretion levels in human macrophages. The protein concentrations of MCP-1 (A) and IL-1β (B) were assessed in PMA differentiated THP-1 macrophages that were either treated with vehicle (vehicle control) or with IFN-γ (250 U/ml) or IFN-γ (250 U/ml) in the presence of a variety of nutraceuticals (EPA 30 µg/ml; DHA 19.7 µg/ml; catechin 1.5 µg/ml; stigmasterol 10 µg/ml; campesterol 13.9 µg/ml; β-sitosterol 27.2 µg/ml; butyrate 44.1 µg/ml) for 24 hours. Protein concentrations were assessed using an ELISA. The data are presented as the mean ± SEM from three independent experiments. Statistical analysis was performed using a one-way ANOVA with either a Games-Howell (A) or Dunnett (B) post-hoc analysis where * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

5.3.2 Key bioactive ingredients of the complete formulation can inhibit the migration of human monocytes towards MCP-1

A 9.61 fold ($p<0.001$) increase in monocyte recruitment was observed in cells stimulated with MCP-1 (20 ng/ml) in comparison to the vehicle control after 3 hours (Fig. 5.6). Both EPA and catechin were able to significantly attenuate MCP-1-driven monocyte migration by 52.7% ($p=0.006$) and 47.5% ($p=0.016$) respectively. Decreased monocyte recruitment was observed following DHA, stigmasterol, campesterol and β-sitosterol treatment, however these changes were not found to be significant ($p=0.192$, $p=0.659$, $p=0.143$, $p=0.122$ respectively). Butyrate treatment had no effect on monocyte migration ($p=1.000$).

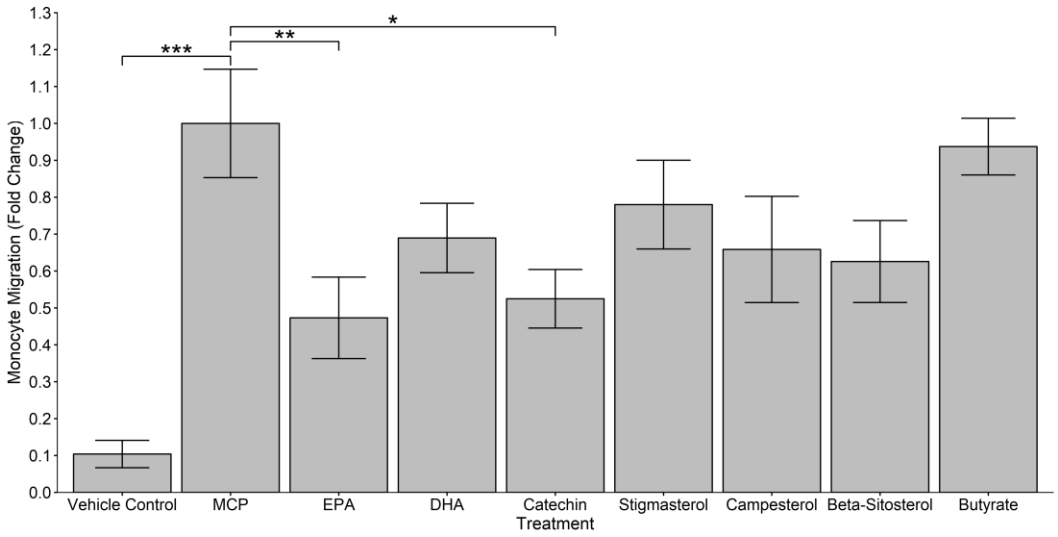

Fig. 5.6. Nutraceuticals within CardioWise can reduce human monocyte migration towards MCP-1. Cellular migration was assessed using THP-1 monocytes that were treated with either vehicle (vehicle control) or with MCP-1 (20 ng/ml) or with MCP-1 (20 ng/ml) in the presence of a variety of nutraceuticals (EPA 30 µg/ml; DHA 19.7 µg/ml; catechin 1.5 µg/ml; stigmasterol 10 µg/ml; campesterol 13.9 µg/ml; β-sitosterol 27.2 µg/ml; butyrate 44.1 µg/ml) for 3 hours. Monocyte migration was then determined as described in Materials and Methods. Monocyte migration is expressed as a fold-change compared to the proportion of cells that moved from the apical compartment into the basolateral compartment in response to MCP-1 alone, which has been arbitrarily set as 1. The data are presented as the mean ± SEM from four independent experiments. Statistical analysis was performed using a one-way ANOVA with Dunnett post-hoc analysis on Log transformed data where * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

5.3.3 Key bioactive ingredients of CardioWise can alter ROS production in human monocytes and macrophages

The level of ROS generation increased in both THP-1 monocytes and macrophages by 61% and 40% respectively ($p<0.001$ and $p<0.001$) following 50 µM TBHP stimulation for 3 hours (Fig. 5.7). In THP-1 monocytes a significant increase in ROS levels occurred following treatment with both EPA (192.6%; $p<0.001$) and DHA (53.1%; $p<0.001$; Fig. 5.7A). On the other hand treatment with Catechin and β-sitosterol reduced ROS production by 24.3% and 12.1% respectively ($p<0.001$ and $p=0.028$). Stigmasterol, campesterol and butyrate did not significantly alter TBHP induced ROS generation. Comparable results were observed in THP-1 monocyte-derived macrophages (Fig. 5.7B). EPA, DHA and campesterol treatment significantly increased ROS production by 141.2%, 42.1% and 10.7% respectively ($p<0.001$, $p<0.001$ and $p=0.003$). The only nutraceutical to reduce ROS generation was catechin (20.9%; $p<0.001$), whereas neither stigmasterol, β-sitosterol nor butyrate had any significant effects.

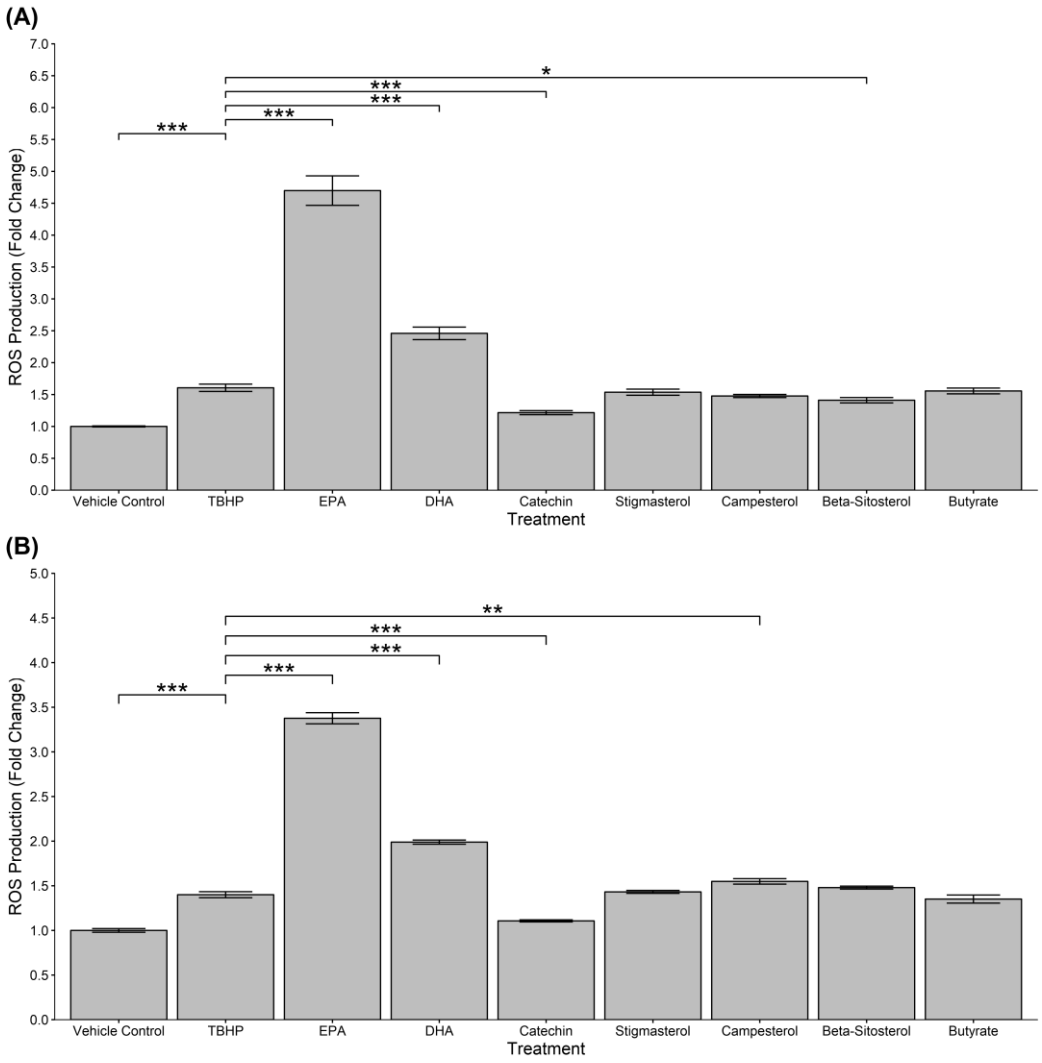

Fig. 5.7. Individual nutraceuticals within CardioWise can modify ROS production in human monocytes and macrophages. ROS production was assessed in THP-1 monocytes (A) and PMA differentiated THP-1 macrophages (B) that were either treated with vehicle (vehicle control) or tert-Butyl hydroperoxide (TBHP; 50 µM) or TBHP (50 µM) and a variety of nutraceuticals (EPA 30 µg/ml; DHA 19.7 µg/ml; catechin 1.5 µg/ml; stigmasterol 10 µg/ml; campesterol 13.9 µg/ml; β-sitosterol 27.2 µg/ml; butyrate 44.1 µg/ml) for 3 hours. ROS production was measured in a fluorescence microplate reader, with excitation at 485 nm and emission detected at 535 nm. Vehicle control was given an arbitrary value of 1. The data are presented as the mean ± SEM from three independent experiments. Statistical analysis was performed on the log-transformed data using a one-way ANOVA with a Dunnett post-hoc analysis where * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

5.3.4 Catechin has no effect on the expression of pro-inflammatory genes following IFN-γ stimulation in human macrophages

Due to the positive results induced by catechin presented above, it was investigated further at a range of concentrations in order to assess its effects on other key stages of atherosclerosis progression. The expression of *MCP-1* in human PMA differentiated THP-1 macrophages showed a non-significant increase of 35.2 (p=0.127) fold following IFN-γ stimulation for 3 hours (250 U/ml; Fig. 5.8A). Furthermore the transcript level of *ICAM-1* was increased by 2.9 fold (p<0.001) when compared to the vehicle control (Fig. 5.8B). When treated with catechin at

doses 1.5, 5, 10 or 20 µg/ml, there were no significant alterations in IFN-γ induced expression of either *MCP-1* or *ICAM-1*.

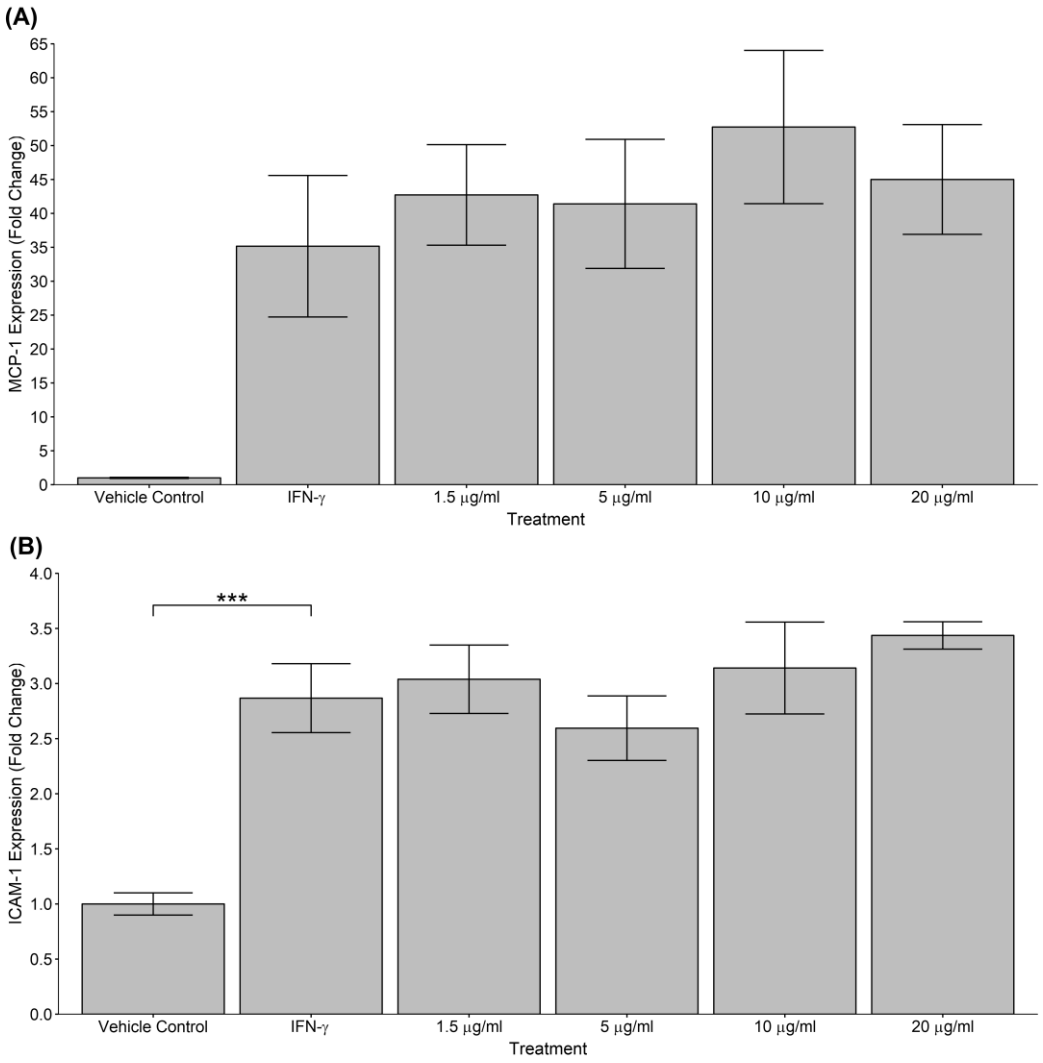

Fig. 5.8. IFN-γ induced expression of *MCP-1* and *ICAM-1* is not affected be catechin in human macrophages. The expression of *MCP-1* (A) and *ICAM-1* (B) was assessed in PMA differentiated THP-1 macrophages that were either treated with vehicle (vehicle control) or with IFN-γ (250 U/ml) or IFN-γ (250 U/ml) in the presence of the indicated concentrations of catechin for 3 hours. Gene expression levels were assessed using qPCR and calculated using the comparative Ct method and normalised to GAPDH levels with values from vehicle treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from three independent experiments. Statistical analysis was performed using a one-way ANOVA with either a Games-Howell (A) or Dunnett T3 (B) post-hoc analysis where *** $p \leq 0.001$.

5.3.5 Catechin attenuates human monocyte migration

MCP-1 (20 ng/ml) induced the recruitment of THP-1 monocytes by 5.4 fold ($p<0.001$) when compared to the vehicle control lacking MCP-1 after 3 hours (Fig. 5.9). A 36% reduction in monocyte recruitment occurred following treatment with catechin at a dose of 1.5 µg/ml ($p=0.049$). Decreases of 23.2% ($p=0.326$), 26.6% ($p=0.236$) and 20.0% ($p=0.494$) were observed for catechin doses 5, 10 and 20 µg/ml respectively, however none of these decreases were significant.

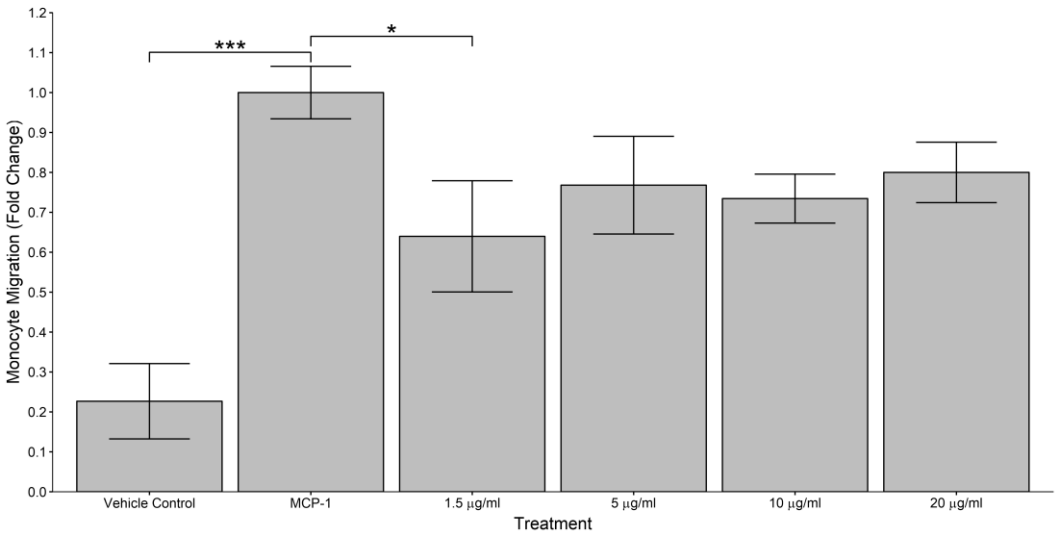

Fig. 5.9. The migration of human monocytes towards MCP-1 is inhibited by physiologically relevant doses of catechin. THP-1 monocytes were treated with vehicle (vehicle control) or treated with MCP-1 (20 ng/ml) alone or with MCP-1 (20 ng/ml) in the presence of one indicated concentrations of catechin for 3 hours. Monocyte migration was then determined as described in Materials and Methods. Data were normalised to the percentage of cells that migrated from the apical compartment of the modified Boyden chamber into the basolateral compartment in response to MCP-1 only treatment. This proportion was arbitrarily assigned as 1. The data are presented as the mean ± SEM from three independent experiments. Statistical analysis was performed using a one-way ANOVA with a Dunnett post-hoc analysis where * $p \leq 0.05$ and *** $p \leq 0.001$.

5.3.6 Catechin attenuates ROS production in a dose dependent manner in human monocytes and macrophages

The presence of TBHP (50 μM) induced ROS production in both THP-1 monocytes and macrophages by 70% and 127% respectively ($p<0.001$ and $p<0.001$) when compared to the vehicle control after 3 hours (Fig. 5.10). Catechin at doses 1.5, 5, 10 and 20 μg/ml was able to attenuate ROS production in THP-1 monocytes by 27.6%, 36.2%, 39.9% and 41.5% respectively when compared to cells treated with TBHP alone (all $p<0.001$; Fig. 5.10A). Similar results were seen in PMA differentiated THP-1 macrophages, decreases of 34.3%, 48.7%, 51.4% and 53.0% were observed for the same doses of catechin ($p=0.021$, $p=0.001$, $p<0.001$ and $p<0.001$ respectively; Fig. 5.10B).

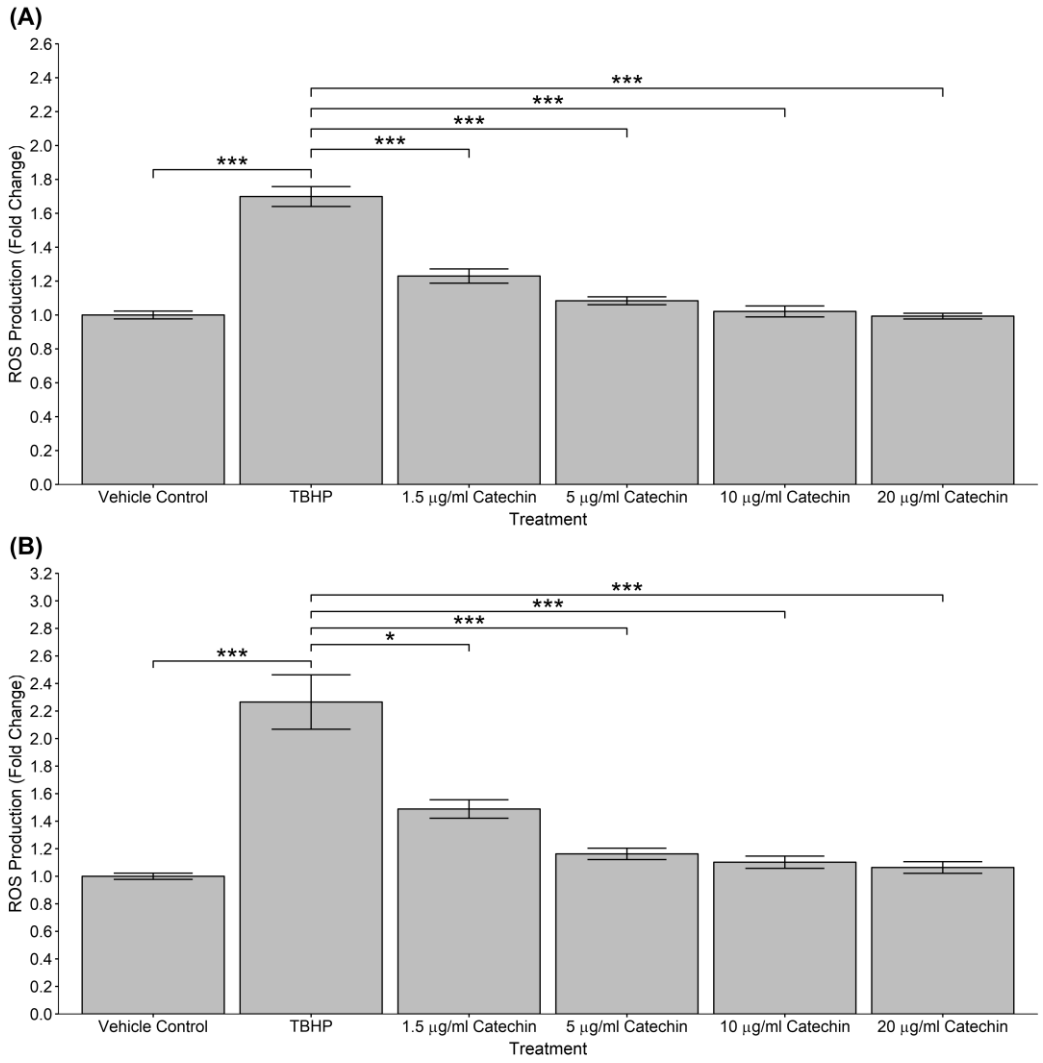

Fig. 5.10. Catechin attenuates ROS production in human monocytes and macrophages in a dose dependant manner. ROS production was assessed in THP-1 monocytes (A) and PMA differentiated THP-1 macrophages (B) that were either treated with vehicle (vehicle control) or tert-Butyl hydroperoxide (TBHP; 50 μM) or TBHP (50 μM) and the indicated concentrations of catechin for 3 hours. ROS production was measured in a fluorescence microplate reader, with excitation at 485 nm and emission detected at 535 nm. Vehicle control was given an arbitrary value of 1. The data are presented as the mean ± SEM from three independent experiments. Statistical analysis was performed on the log-transformed data using a one-way ANOVA with either a Tukeys (A) or Games-Howell (B) post-hoc analysis where * $p \leq 0.05$ and *** $p \leq 0.001$.

5.3.7 The viability of human macrophages is unaffected by catechin *in vitro*

A catechin concentration of 1.5 μg/ml was used for subsequent experiments because it was found to effectively attenuate both monocyte migration (Fig. 5.9) and ROS production (Fig. 5.10). Prior to subsequent experiments, the effect of the chosen concentration of catechin on cell viability was determined using human THP-1 monocyte-derived macrophages and a LDH assay. As shown in Fig. 5.11 there were no significant differences in cell viability observed following treatment with catechin for 24 hours in comparison to the vehicle control.

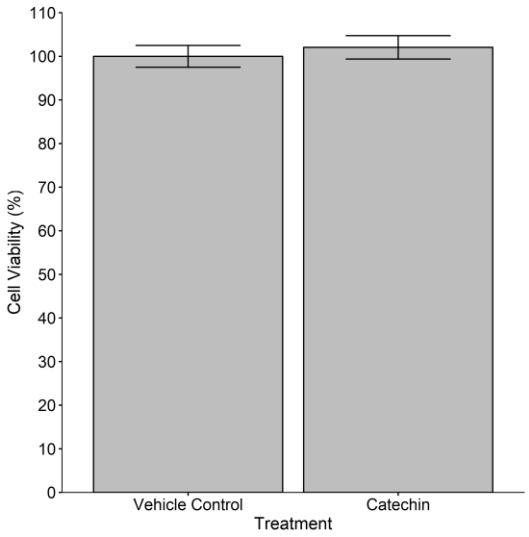

Fig. 5.11. No detrimental effects were observed in human macrophages following treatment with catechin *in vitro*. Cell viability was assessed by a LDH assay in PMA differentiated THP-1 macrophages that were treated with vehicle (vehicle control) or catechin (1.5 µg/ml) for 24 hours. Data were normalised to the vehicle control that has been arbitrarily assigned as 100%. The data are presented as the mean ± SEM from three independent experiments. Statistical analysis was performed using a t-test.

5.4 Discussion

The results of atherosclerosis-associated assays used for the numerous studies presented in this chapter show that the individual nutraceuticals within CardioWise had different effects on a variety of stages in atherosclerosis disease progression, including pro-inflammatory cytokine production, monocyte migration, ROS generation and cholesterol efflux in human monocytes and macrophages. These studies allow for a greater understanding of which compounds within CardioWise are potentially the most important and require further investigation. The assays also provide some insight into the mechanisms by which CardioWise exerts its anti-atherogenic effects.

As MCP-1 is a key chemoattractant during the initial atherosclerotic lesion formation and its expression can be induced by IFN-γ, the levels of MCP-1 secreted by THP-1 human macrophages was assessed by a sandwich ELISA (Moss and Ramji 2016a). In this study we show that both EPA and DHA are capable of reducing the concentration of MCP-1 secreted by human macrophages, however the result was found not to be significant (Fig. 5.5A). This result correlates with the reduced *MCP-1* gene expression in macrophages following treatment with a physiologically relevant dose of CardioWise (Figs. 3.8 and 3.9). Neither catechin, phytosterols nor butyrate were capable of attenuating MCP-1 secretion. These results indicate that it is the EPA and DHA present in CardioWise which make it capable of reducing pro-inflammatory gene expression. Previously EPA and DHA have been found to reduce MCP-1 secretion in LPS stimulated human kidney cells, however another study found only DHA

significantly reduced MCP-1 levels in TNF-α stimulated human adipocytes whereas EPA caused a non-significant trend of increase (Li *et al.* 2005; Romacho *et al.* 2015). Both *in vitro* and *in vivo* studies have observed decreases in MCP-1 secretion in a variety of cell types and in response to a range of pro-inflammatory stimuli following treatment with either catechin, β-sitosterol or butyrate (Fusunyan *et al.* 1999; Bustos *et al.* 2008; Melgarejo *et al.* 2009; Haramizu *et al.* 2011; Wang *et al.* 2014b). These previous results in combination with the observations in this study may indicate that the effectiveness of ω-3 PUFAs, catechin, phytosterols and butyrate in reducing MCP-1 secretion may depend on cell type and pro-inflammatory stimuli used, resulting in some of the contrasting results seen in this study and the literature.

IL-1β is a major pro-inflammatory cytokine during atherosclerosis disease progression and its expression can be induced by oxLDL, meaning atherosclerosis disease initiation and IL-1β expression are closely linked (Moss and Ramji 2016a; Liu *et al.* 2014b). Previous studies have shown that IL-1β not only induces the expression of several pro-inflammatory genes but it is also capable of promoting the pro-inflammatory M1 macrophage phenotype (Chinetti-Gbaguidi *et al.* 2015; Leitinger and Schulman 2013; Van Tassell *et al.* 2013; Ridker 2016). Smaller atherosclerotic lesions have been observed in *ApoE* deficient mice that also lacked either IL-1β or its receptor (Kamari *et al.* 2011; Shemesh *et al.* 2012). As activated inflammasomes convert IL-1β and IL-18 into their mature forms, measuring IL-1β secretion is an indirect way of measuring inflammasome activation. The results in this study show that catechin and phytosterols cause an increase in IL-1β secretion and therefore increase inflammasome activation (Fig. 5.5). Catechin and other flavanols have been shown to attenuate inflammasome activation in both endothelial cells and mice (Tsai *et al.* 2011; Wu *et al.* 2014; Jhang *et al.* 2015). As far as we are aware this is the first study to investigate the effects of stigmasterol, campesterol and β-sitosterol individually on inflammasome activation. This assay shows that phytosterols are capable of exerting detrimental effects during atherosclerosis disease progression and potentially increase the pro-inflammatory response. EPA, DHA and butyrate treatment had no effect on IL-1β secretion. This result contradicts previous studies in the literature which found ω-3 PUFAs are capable of attenuating inflammasome activation in both THP-1 macrophages and bone marrow-derived macrophages (BMDMs; Yan *et al.* 2013; Williams-Bey *et al.* 2014). The failure of butyrate to reduce inflammasome activation observed in this study has also been seen in LPS stimulated BMDMs (Youm *et al.* 2015). However due to the small number of studies investigating the effects of butyrate there is no consensus within the literature, as another study has shown that butyrate is capable of attenuating inflammasome activation (Bian *et al.* 2017). The primary limitation with our study is that IFN-γ was used to stimulate inflammasome activation. The major inflammasome class involved in atherosclerosis disease development appears to be the NLRP3 inflammasome, which is activated by oxLDL and cholesterol crystals (Hoseini *et al.* 2017). Therefore a greater insight

into the effects of ω-3 PUFAs, catechin, phytosterols and butyrate on inflammasome activation in relation to atherosclerosis would be achieved if either oxLDL or cholesterol crystals were used to stimulate inflammasome activation instead of IFN-γ.

Both EPA and catechin were capable of attenuating MCP-1 driven monocyte migration (Fig. 5.6). Furthermore DHA, stigmasterol, campesterol and β-stigmasterol showed trends of decrease, however none of these trends were significant. These results show all the components within CardioWise are capable of attenuating monocyte recruitment to some degree. All the nutraceuticals were used at the same concentration they are found in CardioWise, however when tested individually both EPA and catechin resulted in similar reductions in migration that were achieved by using the full combination in CardioWise (Fig. 3.10). These results would imply that there were no additive or synergistic effects on monocyte migration achieved from using the nutraceuticals ω-3 PUFAs, flavanols and phytosterols in combination. A future study could assess this by using different combinations of EPA, DHA, catechin and phytosterols and comparing any reductions in monocyte recruitment to the nutraceuticals individually. The observations in this study are similar to those previously seen when assessing the effect of ω-3 PUFAs, flavanols and phytosterols on monocyte migration (Brown *et al.* 2012; Melgarejo *et al.* 2009; Bustos *et al.* 2008). Butyrate appeared to have no effect on monocyte migration.

As previously discussed, the aberrant ROS production can lead to increased lipid peroxidation, and hence an increase in oxLDL levels and thereby more atherosclerosis disease development (Chen *et al.* 2017c). Therefore reducing ROS production would decrease one of the triggers of atherosclerosis disease development. As both monocyte and macrophages are present within the atherosclerotic plaque, ROS generation was assessed in both to ensure the effects of the nutraceuticals occurred in both cell types. Only catechin was found to reduce ROS formation in both monocytes and macrophages (Fig. 5.7). This is no surprise as catechin is a well-known anti-oxidant (Zielinski *et al.* 2014; Higdon and Frei 2003; Kumar and Pandey 2013). β-sitosterol decreased ROS production in macrophages which agrees with previously published results that show it reduced oxidative stress by increasing *SOD1* expression (Vivancos and Moreno 2005). This result goes some way to explaining the difference in ROS production observed following treatment with CardioWise in the presence or absence of phytosterols in human macrophages (Fig. 3.14). However the reduction in ROS generation by β-sitosterol is not the same as the difference between CardioWise with or without phytosterols, implying the possibility of additive or synergistic effects occurring in the combination. Testing a combination of catechin and β-sitosterol as well as the other phytosterols in future studies would be able to establish any extra antioxidant benefits when the nutraceuticals are combined. Both EPA and DHA significantly increased ROS production in human monocytes and macrophages, indicating that these nutraceuticals were responsible for the increased ROS

levels following incubation with CardioWise. Previous studies have also observed increases in ROS production following treatment with ω-3 PUFAs (Yin *et al.* 2017; Di Nunzio *et al.* 2016). In this study butyrate was found to have no effect on ROS production.

Following the results of the initial assays, catechin was chosen as the nutraceutical that would be investigated in further detail. Catechin was chosen as it was found to be very effective at reducing monocyte migration (Fig. 5.6) and ROS production (Fig. 5.7), two key steps in atherosclerosis disease progression. Phytosterols and butyrate were eliminated as possible choices due to the lack of cardiovascular protective effects being observed in the initial assays. Both EPA and DHA were not chosen as nutraceuticals to explore in greater detail as they were found to be capable of increasing ROS generation (Fig. 5.7). Furthermore EPA and DHA were not chosen due to the fact extensive research has already been performed on these two nutraceuticals. Over the last 5 years, 126 and 113 results can be found on pubmed.gov after searching for “EPA atherosclerosis” and “DHA atherosclerosis”, whereas only 57 can be found for “catechin atherosclerosis”. To ensure an optimal concentration was used for catechin, initial testing was performed with a mini-dose response. A dose of 1.5 µg/ml was selected as it is the concentration of catechin present in CardioWise. Doses of 5, 10 and 20 µg/ml were selected to ensure that a broad range of concentrations were tested.

The importance of IFN-γ induced gene expression of *MCP-1* and *ICAM-1* has been previously discussed (Moss and Ramji 2016a; Moss and Ramji 2015; Ramji and Davies 2015). In this study we show that catechin is unable to attenuate IFN-γ stimulated expression of *MCP-1* or *ICAM-1* at any of our selected concentrations (Fig. 5.8). These results indicate that catechin did not play a role in reducing *MCP-1* or *ICAM-1* expression following CardioWise treatment (Figs. 3.8 and 3.9). Previous studies have shown a reduction in *MCP-1* and *ICAM-1* expression following catechin treatment, however the biggest changes in gene expression were observed at 50 µg/ml a concentration much higher than the highest dose used in our study (Nakanishi *et al.* 2010; Wang *et al.* 2014b). The effect of catechin on gene expression in response to other pro-inflammatory stimuli needs to be assessed to determine whether the results presented in this study are specific to IFN-γ or represent a general response.

Despite catechin being unable to attenuate IFN-γ induced *MCP-1* expression, it was capable of decreasing monocyte migration to a MCP-1 stimuli (Fig. 5.9). All doses of catechin tested showed a reduction in monocyte recruitment, however only the lowest dose was found to be significant. There is some suggestion that catechin concentration has an inverse relationship with monocyte migration, however the difference between the lowest and highest dose of catechin is minor and therefore may be as a result of natural variation within the assay. A previous study observed catechin attenuating LPS stimulated migration of RAW264.7 macrophages in a dose dependent manor (Ren *et al.* 2014). Catechin has also been found to attenuate the migration of THP-1 monocytes in response to MCP-1 (Melgarejo *et al.* 2009).

Furthermore due to its antioxidant properties catechin reduced ROS production in a dose dependent manner (Fig. 5.10).

The only dose of catechin found to be significant in both the monocyte migration and ROS production was 1.5 µg/ml, for this reason it was selected as the concentration of catechin for future assays. Another advantage of selecting this dose of catechin is that it is the same concentration of catechin found within CardioWise, therefore it will allow for comparisons to be made between the two treatments. Before catechin could be used in further assays, its effects on cell viability on human macrophages *in vitro* was assessed (Fig. 5.11). These results show that catechin has no detrimental effect on cell viability at 1.5 µg/ml, therefore all results observed in future assays are as a result of catechin treatment rather than due to a decrease in cell viability. Any potential anti-atherogenic effects of catechin are assessed in the next chapter.

Chapter 6

Assessing the potential anti-atherogenic properties of catechin *in vitro*

6.1 Introduction

Polyphenols are a large class of anti-oxidants which occur naturally in a wide variety of plants and plant products, including nuts, herbs, tea, fruit and vegetables (Moss and Ramji 2016b; Bahramsoltani *et al.* 2017). Since their discovery thousands of polyphenols have been uncovered, and early epidemiological studies have revealed positive associations between polyphenol intake and reduced risk of a CVD-related event (Moss and Ramji 2016b; Bahramsoltani *et al.* 2017). The largest trial which investigated the potential cardiovascular protective effects of polyphenols was a dietary intervention study known as the PREDIMED study (Tresserra-Rimbau *et al.* 2014a; Tresserra-Rimbau *et al.* 2014b). A total of 7,477 high risk Spanish CVD patients were recruited for the study and randomly assigned one of three diets which were either low fat control diet; Mediterranean diet supplemented with mixed nuts and fruit; and Mediterranean diet supplemented with extra virgin olive oil (Tresserra-Rimbau *et al.* 2014a; Tresserra-Rimbau *et al.* 2014b). Patients with the highest intake of polyphenols had a 37% reduction in their risk of all-cause mortality when compared to the patients with the lowest dietary intake of polyphenols (Tresserra-Rimbau *et al.* 2014b). Further in-depth analysis of the PREDIMED study has shown increased polyphenol dietary intake to be associated with reduced blood pressure, possibly by enhancing vasodilation via an increase in NO production, and reduced serum TG levels but no changes in HDL or LDL serum levels (Medina-Remón *et al.* 2015; Guo *et al.* 2016; Medina-Remón *et al.* 2017).

Flavonoids are the largest subgroup of polyphenols found within a typical diet due to their ubiquity in plants (Spencer 2008). Catechin contains two chiral carbons and hence has four possible isomers; (+)-catechin, (-)-catechin, (+)-epicatechin and (-)-epicatechin (Fig. 6.1). Due to the fact there are several possible forms of the compound, catechin and epicatechin are the key flavanols (a major subclass of flavonoids) in fruit (Manach *et al.* 2004). Furthermore the consumption of (+)-catechin and (-)-epicatechin has been associated with a reduced CVD-related event risk (Arts *et al.* 2001). The abundance and potential health benefits of flavanols provided Cultech Limited with the rationale for including them within their CardioWise formulation. (+)-catechin was determined to be the major flavanol within the complete CardioWise formulation and therefore this form of catechin was used throughout the study. After identifying catechin as a safe potential nutraceutical for atherosclerosis (Chapter 5) by observing reductions in both monocyte migration (Fig. 5.6) and ROS production (Fig. 5.7) it

was studied in greater detail in our *in vitro* model systems of atherosclerosis disease development.

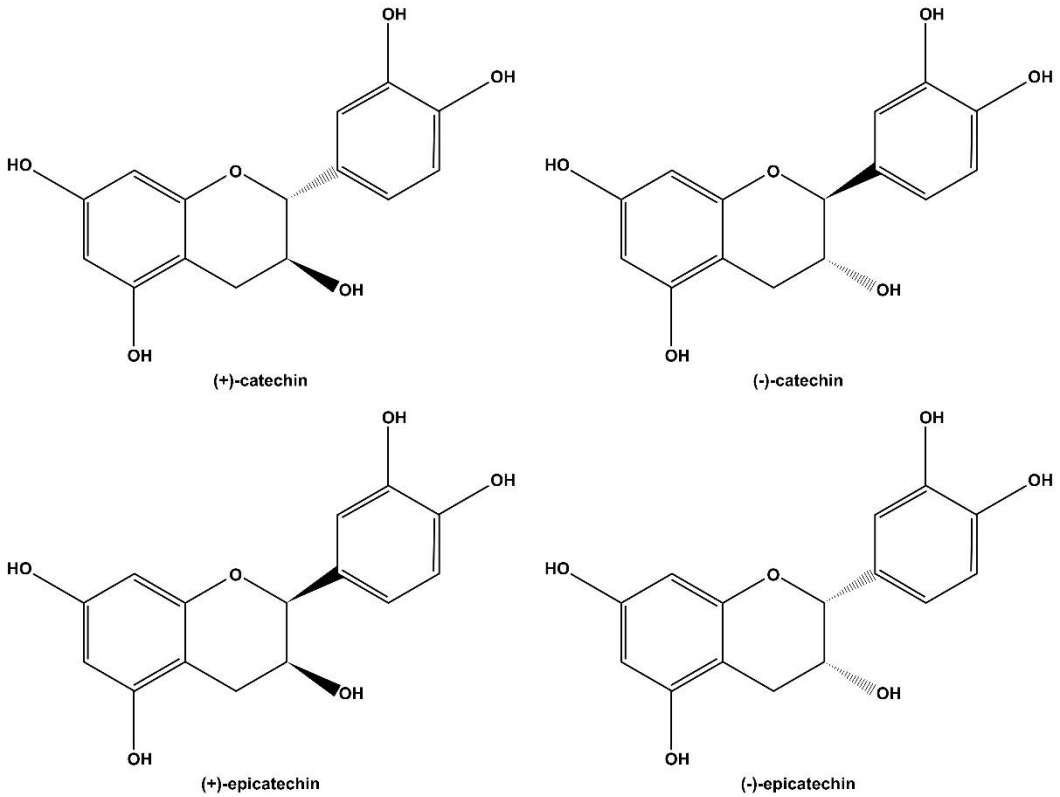

Fig. 6.1. The different isomers of catechin. Due to catechin having two chiral carbons, it has four possible isomers as shown above.

Initially the effect of catechin on basal gene expression levels (i.e. no pro-inflammatory stimuli used to induce gene expression) was assessed by qPCR array. The rationale for qPCR arrays is discussed in Chapter 4. Unlike the qPCR arrays performed in Chapter 4 and 8 which use an average gene expression baseline derived from all of the control mice, the *in vitro* qPCR array performed in this Chapter used the same analysis strategy that is used for regular qPCRs. However due to the cost of the qPCR array plates, independent experiments could not be performed in triplicate for *in vitro* samples unlike regular qPCRs. This leads to no error bar for the vehicle control cells as it is always arbitrarily set to 1 during analysis. A total of six independent experiments were carried out, however a CT value could not be obtained for every repeat due to the low expression levels of some genes and therefore some of data does not contain the maximum possible number of independent results. Due to the variation within the data, resulting in a smaller chance of differences in gene expression emerging as significant, any difference with a p value of less than or equal to 0.1 was considered to be showing a trend of change and included in the results section (see Sections 6.3.1 - 6.3.7). Results of the qPCR array were also validated against results of regular qPCRs.

Due to the large volume of gene expression data generated by the qPCR arrays, it was able to guide the study to new areas to explore *in vitro* that would have previously not been addressed. The genes were classified by their function; apoptosis, blood coagulation, cell adhesion, cell proliferation, lipid metabolism, stress response and transcriptional regulation. These gene classes were investigated in more detail due to their functional importance in the progression of atherosclerosis. During early atherosclerotic lesion formation, damaged endothelial cells undergo cell apoptosis which results in the recruitment of monocytes (Stoneman and Bennett 2004). As macrophages become foam cells, their survivability is influenced by a variety of pro-inflammatory cytokines and eventually they undergo apoptosis causing their intracellularly stored lipids to become released into the intima of the artery (Stoneman and Bennett 2004; Moss and Ramji 2016a). The presence of oxidised lipids triggers further recruitment of monocytes resulting in a continuous pro-inflammatory response. As the plaque continues to develop, the amount of apoptosis continues to increase and contributes to the growth of the lipid-rich necrotic core (Stoneman and Bennett 2004). In mature plaques, there is some evidence that macrophage apoptosis can eventually cause VSMCs in the fibrous cap to also undergo apoptosis (Stoneman and Bennett 2004). As VSMC apoptosis increases, the fibrous cap becomes weaker and eventually ruptures. However whether macrophage apoptosis directly influences plaque stability was previously debated (Stoneman and Bennett 2004). TNF-α is a major pro-inflammatory cytokine which is able to induce macrophage and foam cell apoptosis (Moss and Ramji 2016a). *ApoE* deficient mice also lacking TNF-α were found to develop smaller atherosclerotic lesions as a result of decreased lipid accumulation (Xiao *et al.* 2009). Furthermore previous studies have demonstrated that serum starvation induces THP-1 macrophage apoptosis (Iida *et al.* 2002), as a result a combination of serum starvation and TNF-α stimulation was used to induce macrophage apoptosis. The level of apoptosis was measured by using a fluorescently labelled annexin V protein. This protein is able to bind to cell markers of apoptosis, therefore the intensity of the fluorescent signal is proportional to the amount of cell apoptosis. Reducing cell apoptosis by catechin may lead to a retardation of atherosclerosis disease development.

Unregulated cell proliferation within the intima of arterial walls can lead to the growth of atherosclerotic plaques (Fuster *et al.* 2010). Mouse models have shown that macrophage proliferation could contribute to lesion size and growth as much as monocyte recruitment does (Robbins *et al.* 2013). Therefore targeting macrophage proliferation within atherosclerotic lesions may represent a promising therapeutic target in order to slow disease progression. In this study, the effect of catechin on cell proliferation was determined by three *in vitro* assays, crystal violet assay, bromouridine ELISA, and cell counting for 7 days. The rationale and brief explanation on how the crystal violet assay works can be found in Chapter 3. Bromouridine is a uridine molecule with a bromine substituting the fifth carbon. It is incorporated into RNA in exactly the same way as uridine but it can be detected by fluorescently labelled antibodies.

Therefore the more the RNA is present, the greater the amount of bromouridine incorporation, however increased levels of RNA would also be a sign of more cells and more cell proliferation. Consequently the stronger the fluorescent signal from the anti-bromouridine antibodies, the more cell proliferation has occurred. The final method to assess cell proliferation was by counting the number of monocytes over 7 days. As there are two possible factors that may affect cell number in this assay, time and treatment type, statistical analysis was performed using a GLM. The advantage of using a GLM is that it is able to assess both continuous variables (i.e. time) and categorical variables (i.e. treatment type) simultaneously. Additionally it can determine if there is a significant interaction between time and treatment type and provide answers for more complex hypothesises, for example, the longer the cells are exposed to catechin the more effective it becomes. Upon further investigation, the interaction between time and treatment type was found to be insignificant and as a result the final statistical analysis is based on the effects of both variables in isolation.

Disruption of cholesterol homeostasis to favour cholesterol uptake and reduce cholesterol efflux in macrophages can lead to increased intracellular cholesterol accumulation, resulting in the formation of foam cells (McLaren *et al.* 2011a; Buckley and Ramji 2015). Therefore using nutraceuticals to influence cholesterol homeostasis, and attenuated uptake and enhanced efflux, would reduce foam cell formation and retard disease progression. After determining the effect of catechin on cholesterol uptake and efflux, its effect on cholesterol storage was assessed by TLC, a technique that allows major lipid classes to be separated and the proportion of total intracellular lipids calculated.

Mature plaques rely on having a strong fibrous cap, consisting of ECM and VSMCs, in order to prevent rupturing and triggering a thrombotic reaction and potentially causing a CVD-related event (Newby 2006; Schonbeck *et al.* 1997; Lusis 2000). If the balance of ECM production remains stable or in favour of deposition then the plaque would continue to be relatively stable and less likely to rupture and lead to a CVD-related event (Newby 2006). However a wide variety of pro-inflammatory cytokines including, IFN-γ and IL-18, can either activate MMPs or stimulate their release from macrophages and VSMCs (Schonbeck *et al.* 1997; Moss and Ramji 2016a). As MMP production continues to increase, the more ECM is destroyed in the fibrous cap leading to a reduction in the stability of the atherosclerotic plaque and increase the risk of rupture. Therefore reducing MMP activity would potentially represent a sign of improved plaque stability and reduced risk of a CVD-related event.

The effect of catechin on M1 phenotype polarisation was also assessed, the rationale and background for this assay can be found in Chapter 3.

6.2 Experimental Aims

Details of specific methodologies for each experimental aim are outlined in Chapter 2 and the experimental strategies for Chapter 6 are presented in Figs. 6.2 - 6.8.

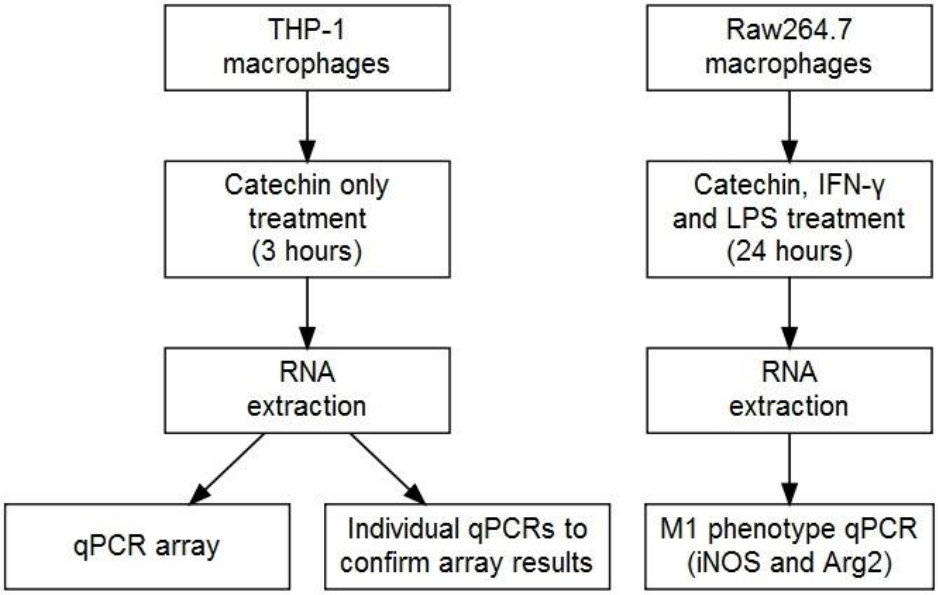

Fig. 6.2. Experimental strategy used to assess gene expression. Previous studies have suggested that differentiating THP-1 monocytes into macrophages with PMA can result in an established M1 phenotype (Park et al. 2007). For this reason the murine RAW264.7 macrophages were used to assess the effect of catechin on M1 macrophage phenotype formation in this study. Arg2, arginase 2; IFN, interferon; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide.

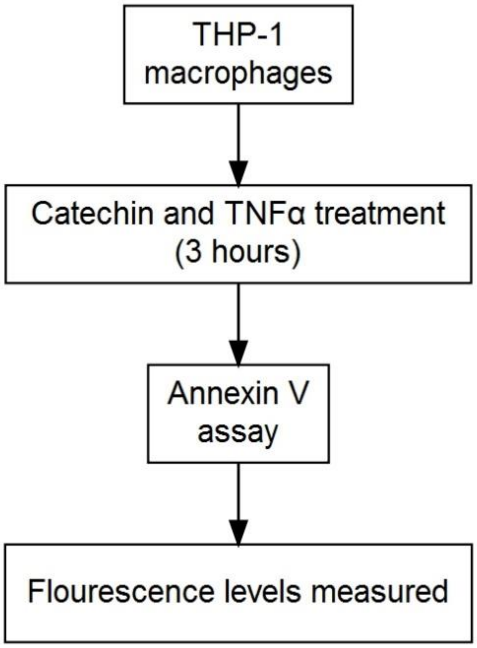

Fig. 6.3. Apoptosis experimental strategy. TNF-α, tumour necrosis factor α.

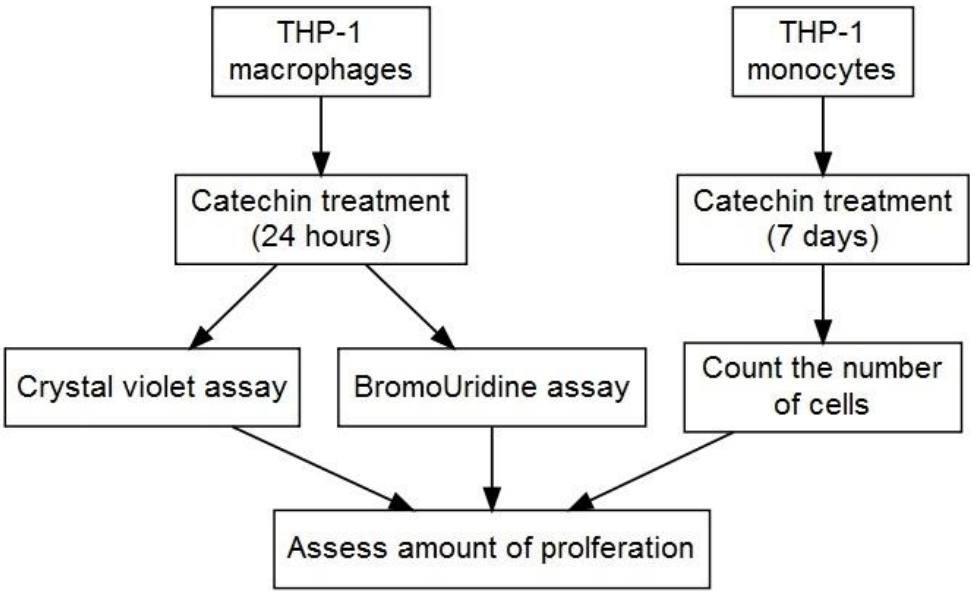

Fig. 6.4. Strategies for cell proliferation assays.

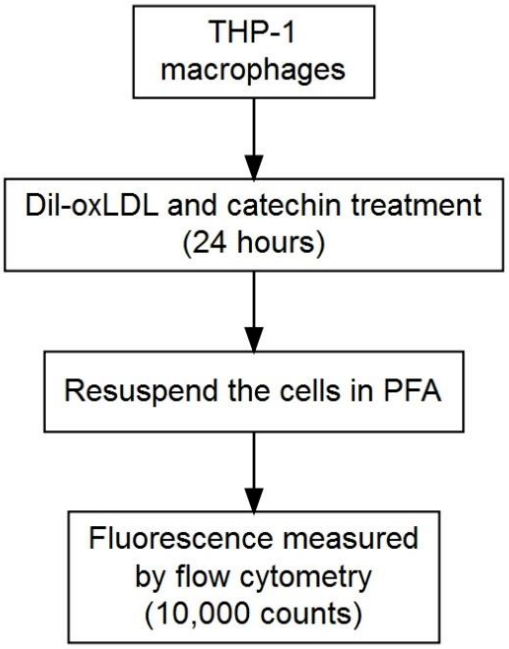

Fig. 6.5. Cholesterol uptake experimental strategy. PFA, paraformaldehyde.

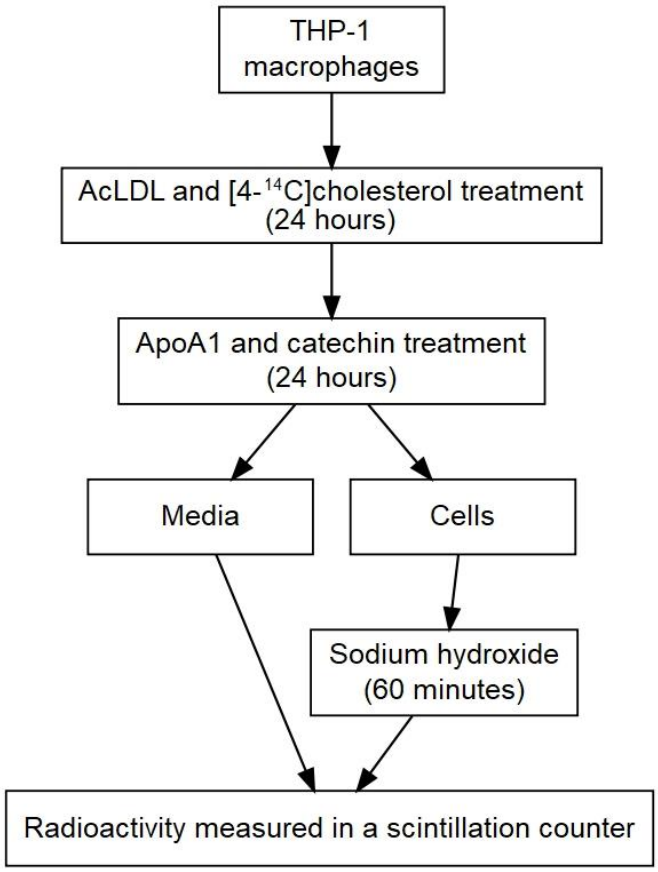

Fig. 6.6. Experimental strategy for assessing cholesterol efflux. AcLDL, acetylated LDL; ApoA1, apolipoprotein A1.

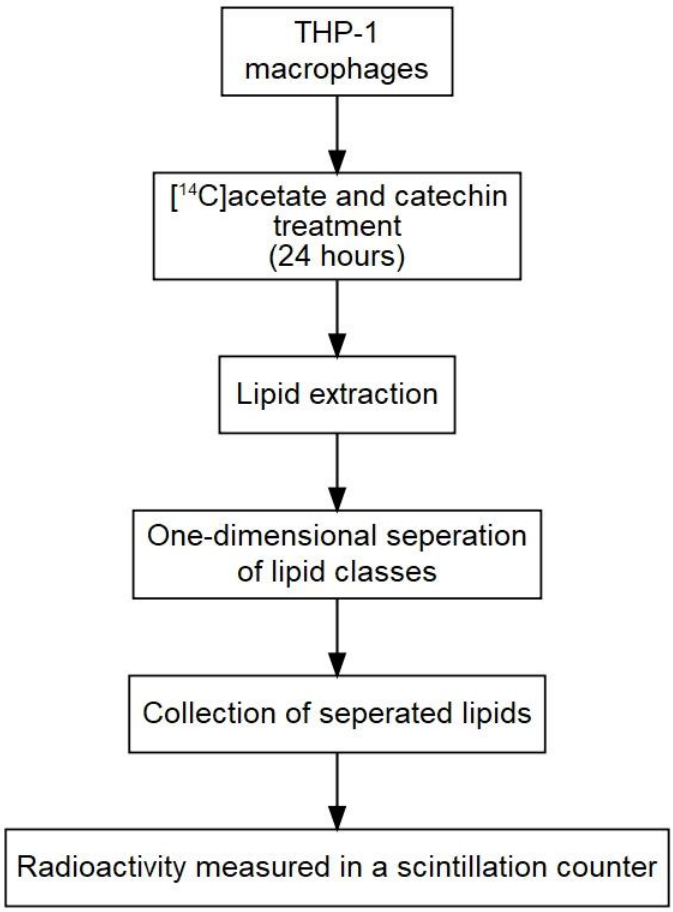

Fig. 6.7. Strategy for determining the proportion of intracellular lipid classes.

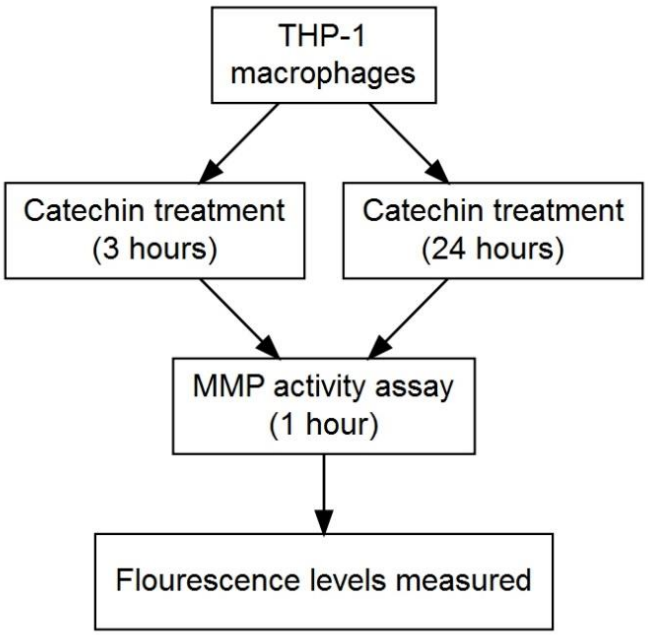

Fig. 6.8. MMP activity experimental strategy. MMP, matrix metalloproteinases.

6.3 Results

6.3.1 The expression of all genes present on the qPCR array following catechin treatment

Fig. 6.9 shows the global significant and non-significant gene expression changes in human THP-1 derived macrophages following treatment with 1.5 µg/ml of catechin for 3 hours. The housekeeping genes: *B2M*; *GAPDH*; hypoxanthine phosphoribosyltransferase (HPRT)1; and ribosomal protein lateral stalk subunit P0 (*RPLP0*); were found to be stable during the assay and were therefore used to calculate gene expression fold changes induced by CardioWise. A total of 37 genes were found to have their expression altered by at least 10% and 3 of those genes were found to be significantly altered. The genes have been classed by their function (according to the literature provided by Qiagen which accompanied the Atherosclerosis RT^2 Profiler PCR Arrays) and will be explored in greater detail in the subsequent sections.

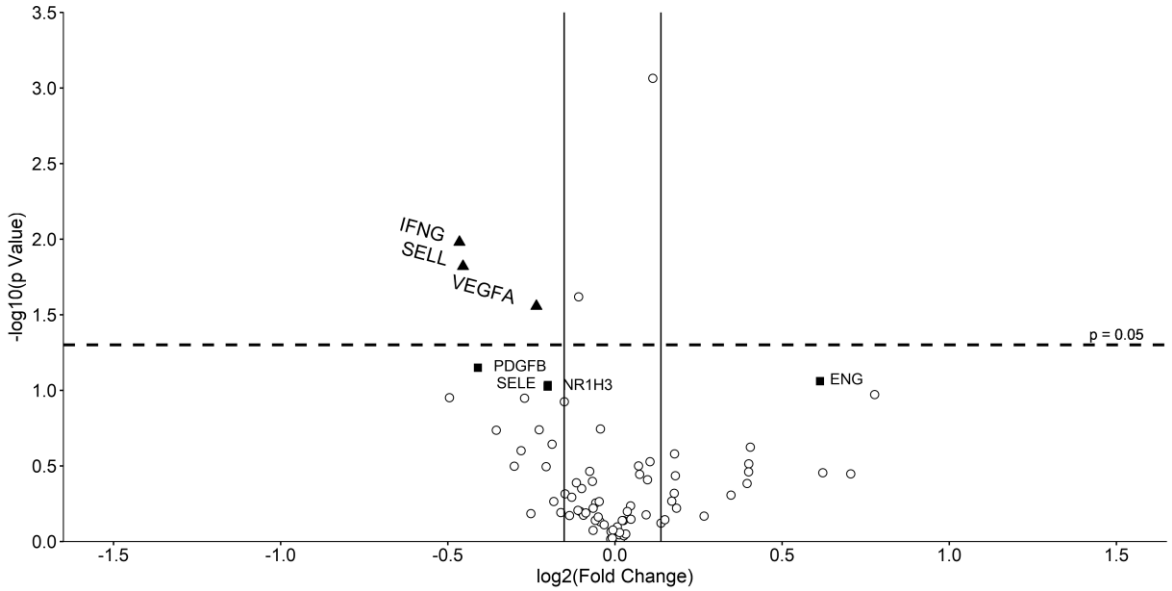

Fig. 6.9. Volcano plot showing global gene expression changes in human macrophages treated with catechin vs vehicle control cells. Gene transcript levels of 84 genes were assessed in human THP-1 derived macrophages which were treated with either a vehicle control or a catechin (1.5 µ/ml) for 3 hours. All genes present on the Qiagen RT2 profiler PCR Array were plotted. The log fold change in the treated cells versus the vehicle control cells is represented on the x-axis. The y-axis shows the -log10 of the p value. A p value of 0.05 (dashed line) and a fold changes of ±10% (solid lines) are indicated. Significantly altered gene expressions are indicated by a black triangle and non-significant trends of change (approximately p<0.1) are indicated by a black square.

6.3.2 The expression of a gene involved in the regulation of apoptosis is attenuated by catechin in human macrophages

First apoptosis signal (FAS) receptor is known as a death receptor present on the surface of cells and its activation can lead to apoptosis, therefore changes in *FAS* expression may correlate with possible changes in cell apoptosis. In the presence of catechin, there was a trend of decrease in the expression of *FAS* of 21.4% when compared to cells treated with the

vehicle control (Fig. 6.10). However these results are based on two independent experiments and must be repeated before any firm conclusions can be drawn.

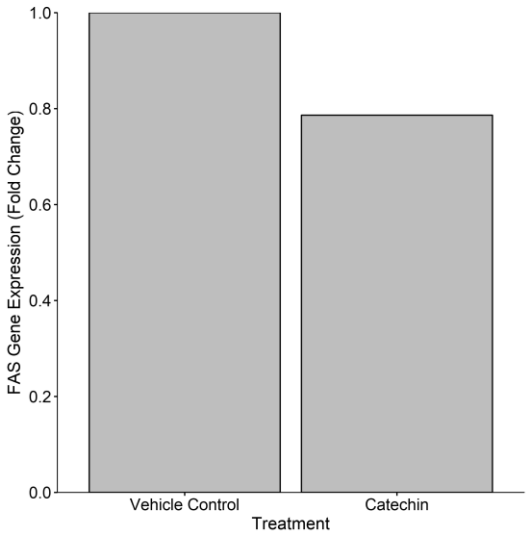

Fig. 6.10. Catechin attenuates the expression of a gene involved in the regulation of apoptosis. Gene transcript levels of *FAS* were assessed in PMA differentiated THP-1 macrophages that were either treated with vehicle (vehicle control) or catechin (1.5 µg/ml) for 3 hours. Gene transcript levels were calculated using the comparative Ct method and normalised to four housekeeping genes (*B2M*, *GAPDH*, *HPRT1* and *RPLP0*) with values from the vehicle treated cells given an arbitrary value of 1. The data are presented as the mean from two independent experiments.

6.3.3 Catechin alters the expression of genes involved in blood coagulation and circulation control in human macrophages

Endoglin (*ENG*) and platelet-derived growth factor subunit B (*PDGFB*) are two genes involved in the regulation of blood coagulation and circulation as both are able to play key roles in angiogenesis. Catechin treatment increased *ENG* expression by 53.0% (p=0.087; Fig. 6.11A), whereas the expression *PDGFB* was attenuated by 24.7% (p=0.071; Fig. 6.11B) compared to the vehicle control treated cells. However neither result was found to be significant.

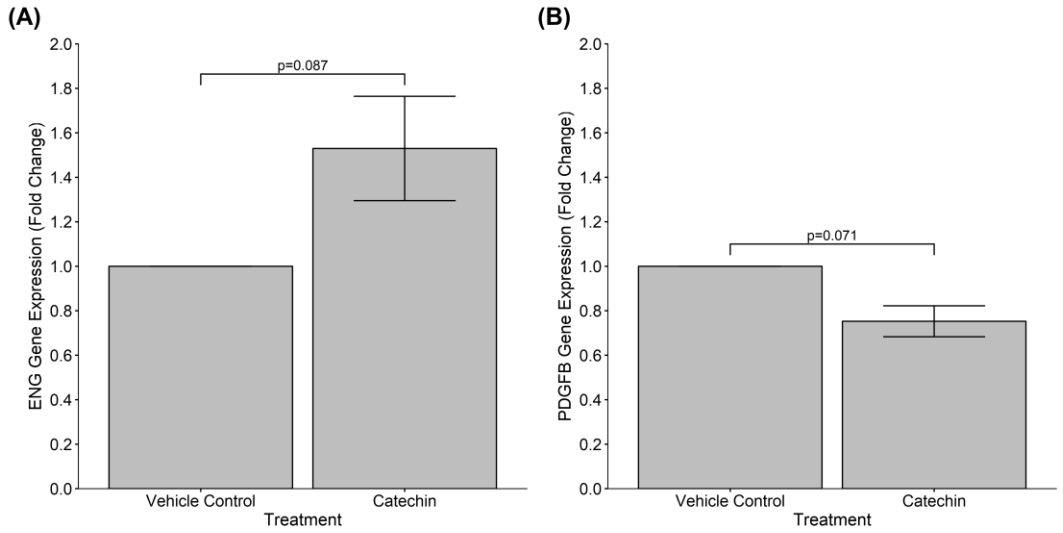

Fig. 6.11. The expression of genes involved in blood coagulation and circulation are altered by catechin. Gene transcript levels of *ENG* (A) and *PDGFB* (B) were assessed in PMA differentiated THP-1 macrophages that were either treated with vehicle (vehicle control) or catechin (1.5 µg/ml) for 3 hours. Gene transcript levels were calculated using the comparative Ct method and normalised to four housekeeping genes (*B2M*, *GAPDH*, *HPRT1* and *RPLP0*) with values from the vehicle treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from three (B) or five (A) independent experiments. Statistical analysis was performed using a t- test (unequal variances).

6.3.4 The expression of cell adhesion molecules is down-regulated in human macrophages in the presence of catechin

Laminin subunit alpha 1 (*LAMA1*), E-selectin (*SELE*) and L-selectin (*SELL*) are all genes which are able to influence cell recruitment and migration, therefore reducing their expression may lead to attenuated atherosclerosis plaque formation. *LAMA1* and *SELE* showed trends of decrease in expression following catechin treatment of 68.2% and 13.0% (p=0.092) respectively when compared to the vehicle control treated cells (Fig. 6.12A and B). The expression of *SELL* was significantly attenuated by 27.0% (p=0.015) in the presence of catechin (Fig. 6.12C).

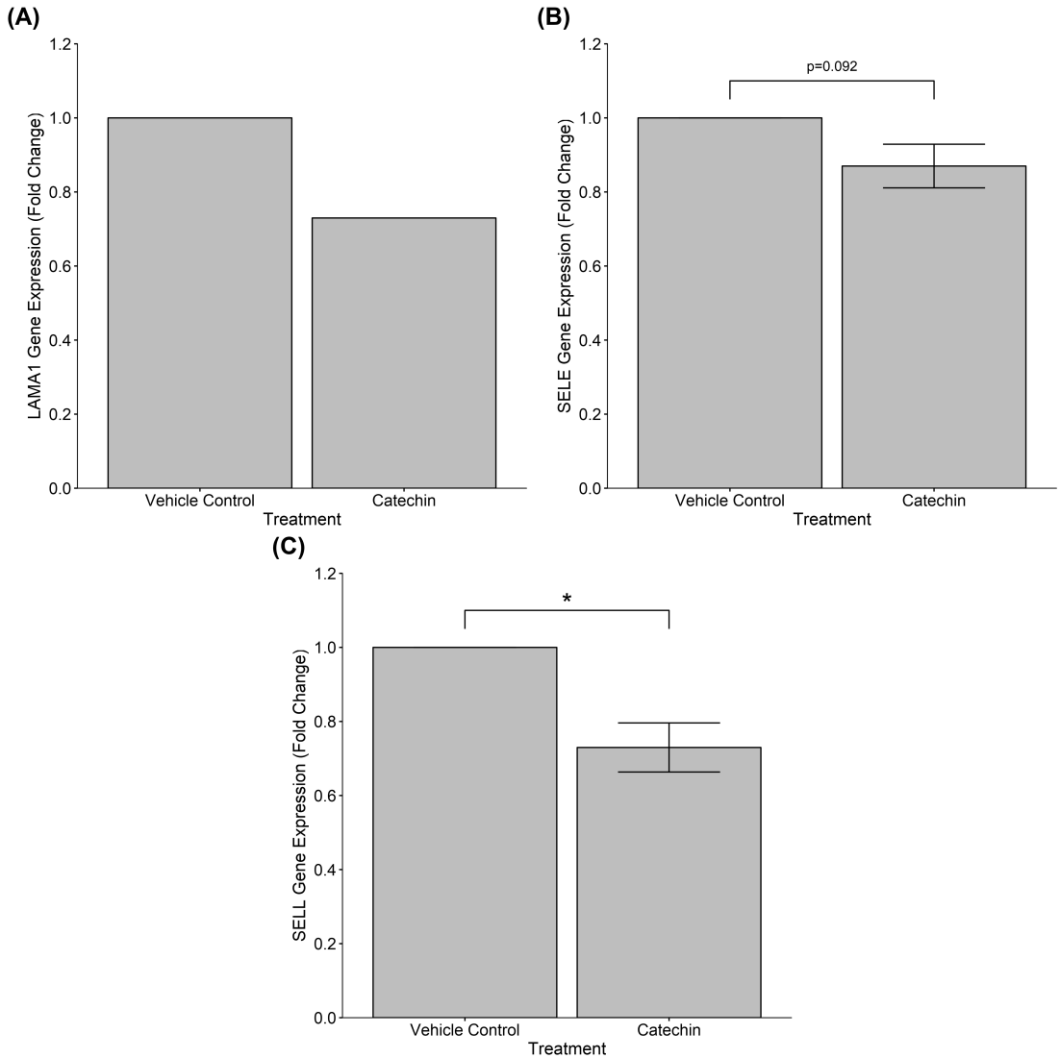

Fig. 6.12. Catechin attenuates the expression of cell adhesion molecule genes. Gene transcript levels of *LAMA1* (A), *SELE* (B) and *SELL* (C) were assessed in PMA differentiated THP-1 macrophages that were either treated with vehicle (vehicle control) or catechin (1.5 µg/ml) for 3 hours. Gene transcript levels were calculated using the comparative Ct method and normalised to four housekeeping genes (*B2M*, *GAPDH*, *HPRT1* and *RPLP0*) with values from the vehicle treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from two (A) or five (B and C) independent experiments. Statistical analysis was performed using a t- test (unequal variances) where * p <0.05.

6.3.5 Catechin attenuates the expression of a gene involved in regulating cell growth and proliferation in human macrophages

Vascular endothelial growth factor (VEGF)A is a growth factor that can influence a variety of physiological processes including cell proliferation, angiogenesis and cell migration. Catechin attenuated *VEGFA* expression by 15.0% (p=0.028) when compared to the vehicle control only treated cells (Fig. 6.13).

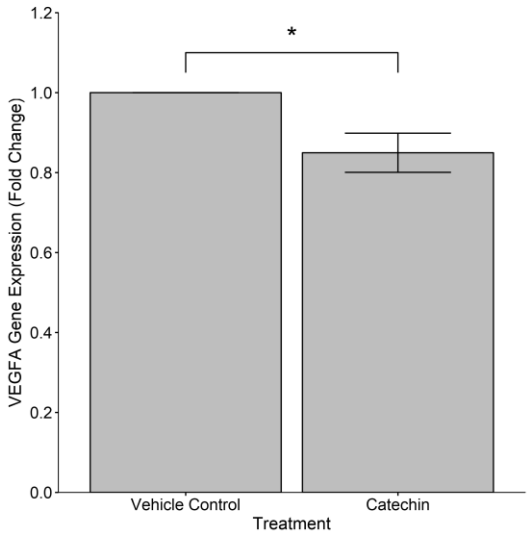

Fig. 6.13. The expression of a gene associated with cell growth and proliferation is down-regulated by catechin. Gene transcript levels of *VEGFA* were assessed in PMA differentiated THP-1 macrophages that were either treated with vehicle (vehicle control) or catechin (1.5 µg/ml) for 3 hours. Gene transcript levels were calculated using the comparative Ct method and normalised to four housekeeping genes (*B2M*, *GAPDH*, *HPRT1* and *RPLP0*) with values from the vehicle treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from six independent experiments. Statistical analysis was performed using a t- test (unequal variances) where * p <0.05.

6.3.6 Catechin treatment decreases the expression of a lipid transport and metabolism gene

The LDLr is a key receptor in LDL uptake, therefore changes in *LDLr* expression may represent changes in cholesterol metabolism. Catechin treatment reduced *LDLr* expression by 7.3% (*p*=0.024) when compared to the vehicle control cells (Fig. 6.14).

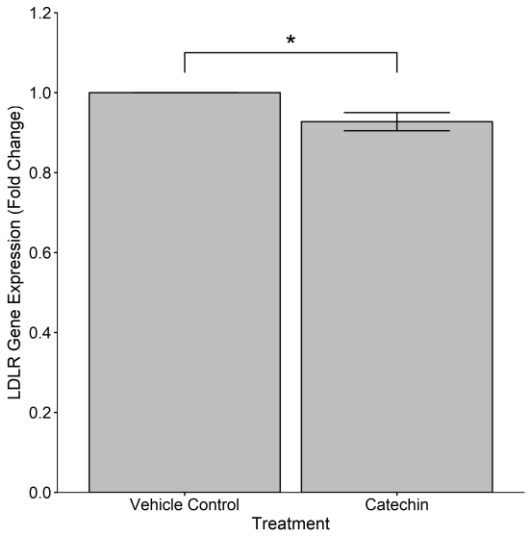

Fig. 6.14. Catechin attenuates the expression of a key gene implicated in the control of lipid transport and metabolism. Gene transcript levels of *LDLR* were assessed in PMA differentiated THP-1 macrophages that were either treated with vehicle (vehicle control) or catechin (1.5 µg/ml) for 3 hours. Gene transcript levels were calculated using the comparative Ct method and normalised to four housekeeping genes (*B2M*, *GAPDH*, *HPRT1* and *RPLP0*) with values from the vehicle treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from six independent experiments. Statistical analysis was performed using a t- test (unequal variances) where * $p < 0.05$.

6.3.7 The expression of a gene involved in stress responses is down-regulated by catechin

As previously discussed, IFN-γ is often considered a master regulator of atherosclerosis disease development, therefore changes in the expression of this cytokine may affect the speed of disease progression. The expression of *IFN-γ* was significantly decreased by 27.6% (p=0.011) following catechin treatment when compared to the vehicle control cells (Fig. 6.15).

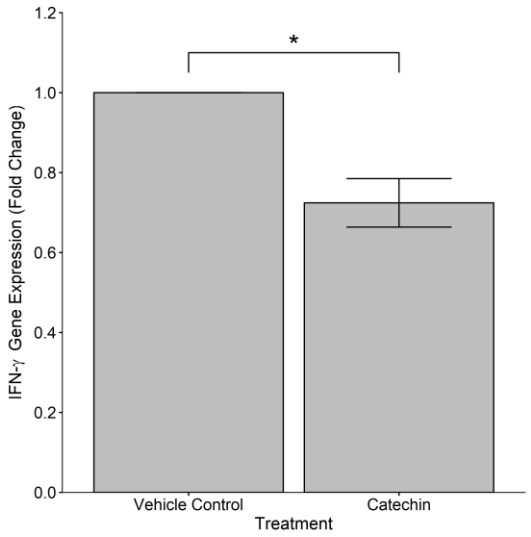

Fig. 6.15. Catechin attenuates the expression of a stress response associated gene. Gene transcript levels of *IFN-γ* were assessed in PMA differentiated THP-1 macrophages that were either treated with vehicle (vehicle control) or catechin (1.5 µg/ml) for 3 hours. Gene transcript levels were calculated using the comparative Ct method and normalised to four housekeeping genes (*B2M*, *GAPDH*, *HPRT1* and *RPLP0*) with values from the vehicle treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from five independent experiments. Statistical analysis was performed using a t- test (unequal variances) where * p <0.05.

6.3.8 The expression of transcriptional regulators can be reduced by catechin

Both NR1H3 and Peroxisome proliferator activated receptor delta (PPARD) are regulators of transcription and have been associated with atherosclerosis disease progression (see Chapter 4 for further details). Catechin treatment caused a non-significant trend of decrease in *NR1H3* expression of 13.0% (p=0.094; Fig. 6.16A) when compared to the vehicle control cells. On the other hand the expression of *PPARD* was significantly increased by 8.2% (p<0.001) in comparison to the vehicle control cells (Fig. 6.16B).

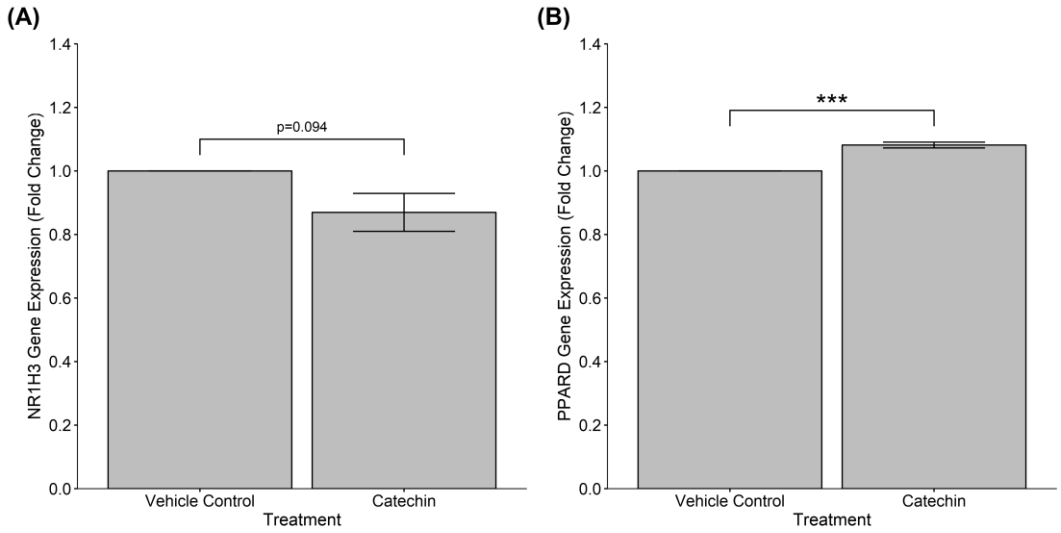

Fig. 6.16. The expression of transcriptional regulators is down-regulated by catechin. Gene transcript levels of *NR1H3* (A) and *PPARD* (B) were assessed in PMA differentiated THP-1 macrophages that were either treated with vehicle (vehicle control) or catechin (1.5 µg/ml) for 3 hours. Gene transcript levels were calculated using the comparative Ct method and normalised to four housekeeping genes (*B2M*, *GAPDH*, *HPRT1* and *RPLP0*) with values from the vehicle treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from five independent experiments. Statistical analysis was performed using a t- test (unequal variances) where *** p <0.001.

6.3.9 The results of the qPCR array were confirmed by regular qPCRs

The expressions of *ICAM-1* (Fig. 6.17A), *MCP-1* (Fig. 6.17B) and macrophage scavenger receptor (*MSR*)*-1* (Fig. 6.17C) were simultaneously assessed by regular qPCR in order to confirm the accuracy of the qPCR arrays. Catechin was found to attenuate *ICAM-1*, *MCP-1* and *MSR-1* expression by 6.5%, 12.2% and 20.8% respectively. The same genes in qPCR array analysis were reduced by 2.1%, 6.7% and 13.3%, therefore similar trends are seen in the regular qPCRs and qPCR arrays.

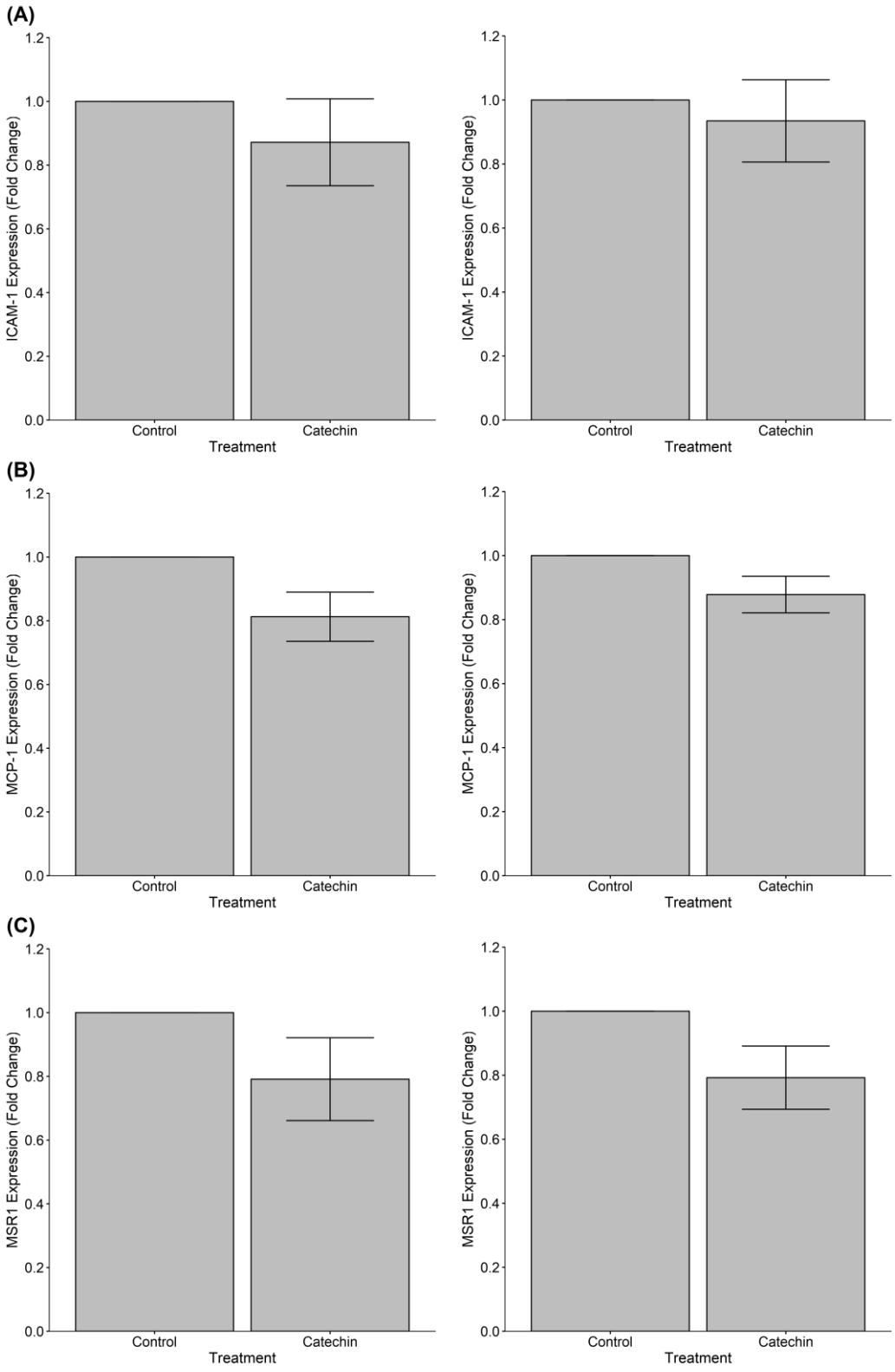

Fig. 6.17. A comparison between the expression of ICAM-1, MCP-1 and MSR1 from regular qPCR and qPCR array results. Gene transcript levels of *ICAM-1* (A), *MCP-1* (B) and *MSR-1* (C) were assessed in PMA differentiated THP-1 macrophages that were either treated with vehicle (control) or catechin (1.5 µg/ml) for 3 hours. Results on the left are from regular qPCRs whereas the results on the right are from qPCR arrays. The same cDNA was used for both qPCR techniques. Gene transcript levels were calculated using the comparative Ct method and normalised to either *GAPDH* (regular qPCR) or four housekeeping genes (*B2M*, *GAPDH*, *HPRT1* and *RPLP0*; qPCR array) with values from the vehicle treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from at least three independent experiments. Statistical analysis was performed using a t- test (unequal variances).

6.3.10 Cell apoptosis in human macrophages is unaffected by catechin treatment

Due to the reduction in the expression of the cell surface death receptor *FAS* (Fig. 6.10), the effect of catechin treatment on apoptosis was assessed. During the progression of atherosclerotic plaque formation, the apoptosis of foam cells causes them to release their lipid-rich contents into the wall of the artery which accumulates to form the lipid-rich necrotic core. A combination of serum starvation and TNF-α stimulation (100 ng/ml) was found to significantly increase cell apoptosis in our *in vitro* model by 1.39 fold (p<0.001) in comparison to the vehicle control (Fig. 6.18). There was no significant reduction in cell apoptosis following treatment with catechin.

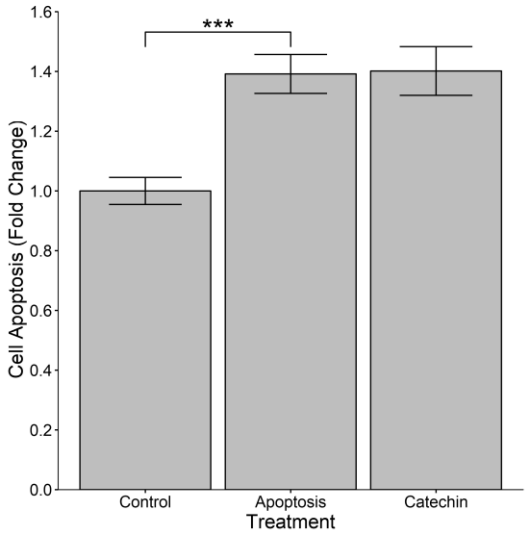

Fig. 6.18. Cell apoptosis in human macrophages is not prevented by catechin. PMA differentiated THP-1 macrophages were treated with vehicle (vehicle control), or a combined treatment of serum starvation and stimulation with TNF-α (100 ng/ml; Apoptosis) or serum starved and stimulated with TNF-α (100 ng/ml) in the presence of catechin (1.5 µg/ml) for 3 hours to evaluate cell apoptosis. The vehicle control was arbitrarily assigned as 1 and remaining data normalised to this. The data are presented as the mean ± SEM from three independent experiments. Statistical analysis was performed using a one-way ANOVA with a Dunnett post-hoc analysis where *** p <0.001.

6.3.11 The proliferation of human monocytes and macrophages is attenuated following catechin treatment

Catechin treatment was found to attenuate the expression of *VEGFA*, a gene capable of stimulating cell proliferation (Fig. 6.13). As a result, the effect of catechin on macrophage proliferation was determined by either a crystal violet assay (Fig. 6.19A) or bromouridine ELISA (Fig. 6.19B). A significant reduction in cell proliferation of 12.6% and 13.8% was observed following treatment with catechin after 24 hours using crystal violet assay or bromouridine ELISA respectively (p=0.029 and p=0.034). A 24 hour time point was chosen for this assay to ensure sufficient time for determination of changes in the level of cell proliferation. Monocyte proliferation was also assessed in THP-1 monocytes which were grown for 7 days and fresh media containing either catechin or vehicle control added on days 0, 1, 2, 3 and 4 (Fig. 6.19C).

Cell proliferation had a significant positive association with time (p<0.001; adj-R^2=0.57), furthermore cell proliferation was significantly lower in cells which had received catechin by 21.9% (p=0.001). There was no significant effect on the interaction between time and treatment type.

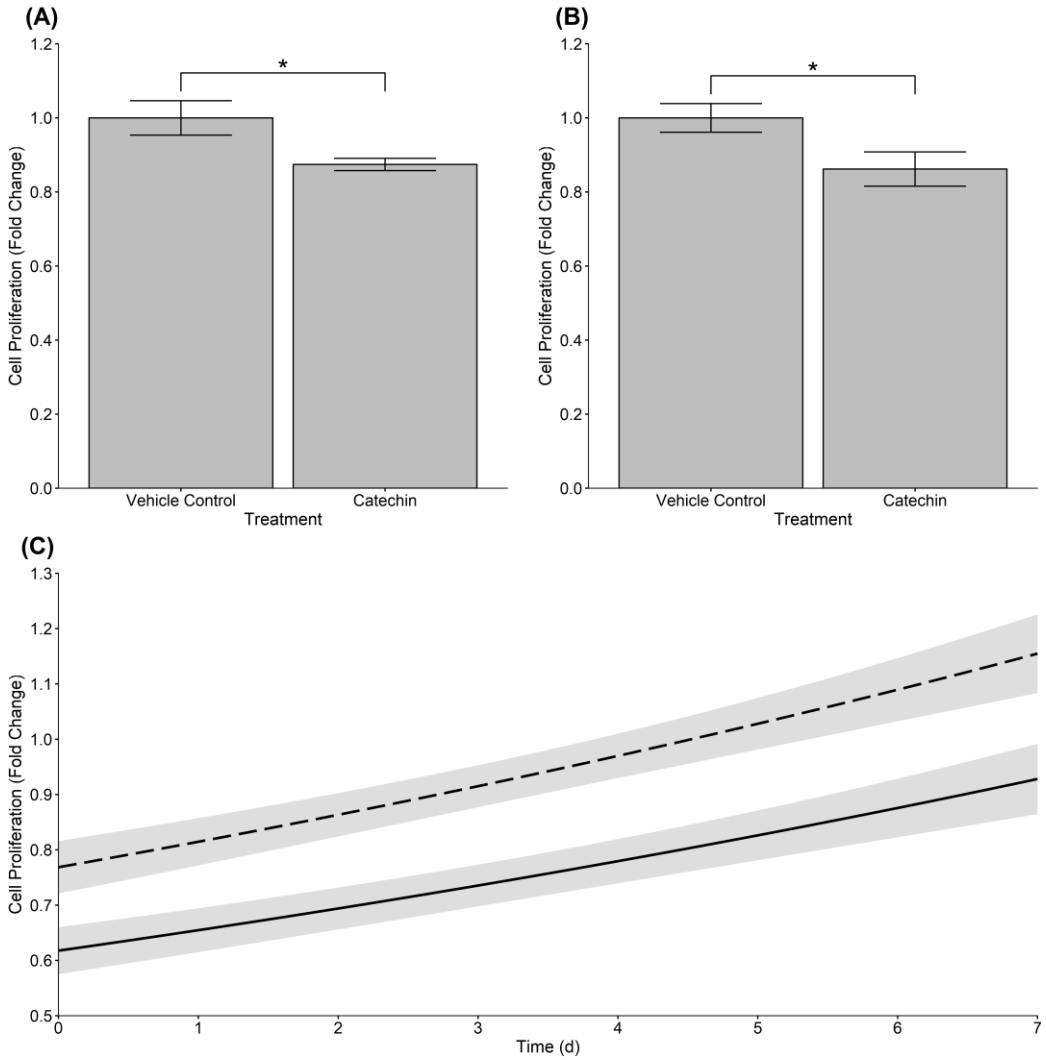

Fig. 6.19. Catechin reduces cell proliferation in human monocytes and macrophages. Cell proliferation was assessed by either crystal violet assay (A) or bromouridine ELISA (B) in THP-1 derived macrophages that were treated with vehicle (vehicle control) or catechin (1.5 µg/ml) for 24 hours. Values from vehicle treated cells were given an arbitrary value of 1 and all other data are normalised to this (A - B). Cell proliferation was also assessed in THP-1 monocytes (C) that were either treated with vehicle (dashed line) or catechin (1.5 µg/ml; solid line) for 7 days. Values from vehicle treated cells on day 0 were given an arbitrary value of 1 and all other data are normalised to this (C). The data are presented as the mean ± SEM from three (A) or four (B) independent experiments. Statistical analysis was performed using a t-test (equal variances) where * p ≤0.05. The prediction of average monocyte proliferation fold change as calculated by a generalised linear model with a Gaussian error distribution and log link function is displayed ± SEM from three independent experiments (C).

6.3.12 Cholesterol uptake and efflux are unaffected by catechin in human macrophages

Due to the attenuated expression of *LDLr* (Fig. 6.14), the effect of catechin treatment on cholesterol uptake was assessed. Human PMA differentiated THP-1 macrophages were

treated with Dil-oxLDL in the presence of vehicle control or catechin for 24 hours. Following Dil-oxLDL only treatment, the THP-1 macrophages were able increase their cholesterol uptake by 1.87 fold (p<0.001; Fig. 6.20A). Catechin did not significantly alter cholesterol uptake.

Simultaneously the levels of cholesterol efflux were also measured to better understand the effect of catechin treatment on foam cell formation. Differentiated THP-1 macrophages, pre-loaded with cholesterol, were treated with ApoA1 (10 µg/ml) for 24 hours to stimulate the efflux of their intracellular radiolabelled cholesterol. ApoA1 stimulation caused the cells to release 19.4% of their cholesterol into the surrounding media (Fig. 6.20B), an increase of 1.44 fold (p<0.001). No significant changes in cholesterol efflux were observed following treatment with catechin.

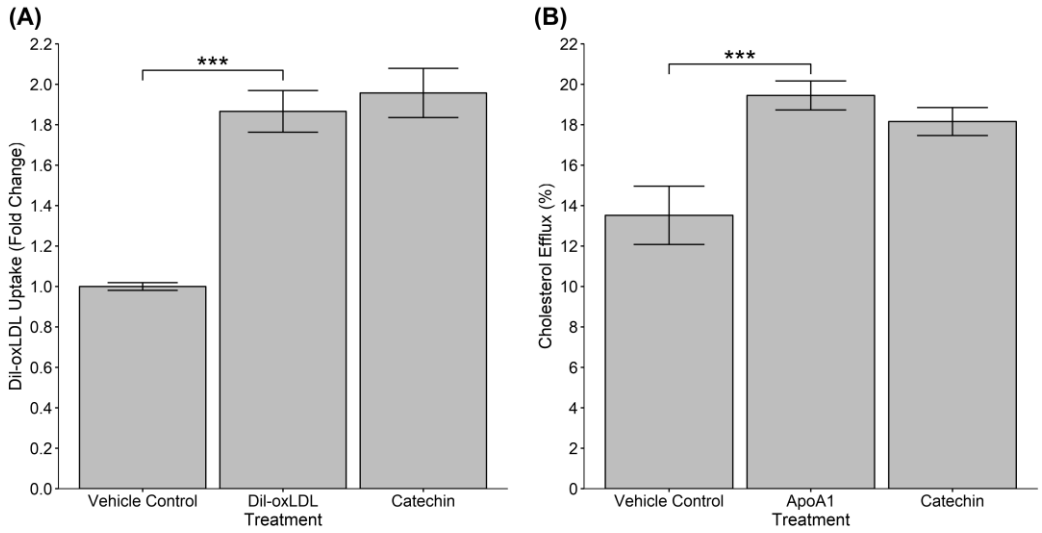

Fig. 6.20. Catechin does not alter cholesterol uptake or efflux from human macrophages. (A) PMA-differentiated THP-1 macrophages were loaded with Dil-oxLDL for 24 hours in the presence of vehicle or catechin (1.5 µg/ml). The vehicle control was arbitrarily assigned as 1 and the remaining data normalised to this. The data are presented as the mean ± SEM from three independent experiments. Statistical analysis was performed using a one-way ANOVA with a Games-Howell post-hoc analysis where *** p ≤0.001. (B) PMA-differentiated THP-1 macrophages were loaded with [4-^{14}C]cholesterol and acLDL for 24 hours prior to a further 24 hour treatment with ApoA1 (10 µg/ml) in the presence of vehicle or ApoA1 in the presence of catechin (1.5 µg/ml). The data are presented as the mean ± SEM from three independent experiments. Statistical analysis was performed using a one-way ANOVA with a Dunnett post-hoc analysis on squared-transformed data where *** p ≤0.001.

6.3.13 Catechin did not alter lipid accumulation within murine macrophages

To determine the effect of catechin on cholesteryl ester accumulation in macrophages, the cells were treated with vehicle control, acLDL (25 µg/ml) or acLDL and catechin. The TLC assay was performed in RAW264.7 macrophages as previous work has demonstrated that they are capable of producing higher levels of intracellular lipids when compared to THP-1 macrophages (Gallagher 2016). The different lipid fractions were separated by TLC and the activity of incorporated [^{14}C]acetate was measured by a scintillation counter (Fig. 6.21). The levels of lipids present in the TPL fraction increased by 1.62 fold following stimulation with

acLDL, whereas FC, FFA and TG fractions were reduced by 55%, 36% and 71% respectively. There were no observable change in the accumulation within the CE fraction following acLDL treatment. Catechin did not affect the proportions of the lipid fractions when compared to the acLDL only treated cells. However these results are based on one independent experiment and must be repeated before any firm conclusions can be drawn. Due to the lack of changes observed in cholesterol uptake and efflux (Fig. 6.20), it was decided not to proceed further with this assay.

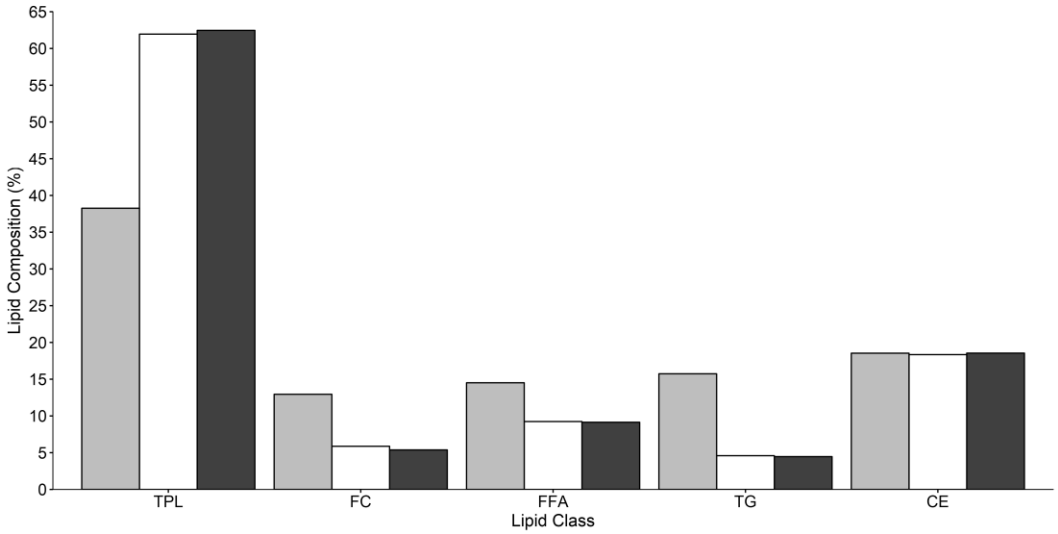

Fig. 6.21. Catechin does not influence lipid accumulation or proportions in murine macrophages. Murine macrophages were loaded with [^{14}C]acetate for 24 hours in the presence or absence of acLDL (25 µg/ml). Cells were treated with either vehicle control (grey), acLDL and vehicle control (white) or acLDL and catechin (1.5 µg/ml; black). The data are displayed as a percentage of the total amount of lipid extracted. The data are presented as the mean from one independent experiment performed in triplicate. TPL, total polar lipid; FC, free cholesterol; FFA, free fatty acid; TG, triacylglycerol; CE, cholesterol ester.

6.3.14 Catechin reduces MMP activity in human macrophages

The activity and expression of MMPs has previously been shown to be influenced by IFN-γ (Moss and Ramji 2015). As *IFN-γ* expression was attenuated following catechin treatment (Fig. 6.15), its effects on MMP activity were determined. The stability of mature atherosclerotic plaques depends on the deposition of ECM in the fibrous cap which covers the lesion. If MMP activity become unregulated and increases ECM degradation it can lead to the plaque becoming unstable and rupturing. The effects of catechin on MMP activity was determined at both 3 and 24 hours to delineate both short-term and long-term actions. THP-1 macrophages were found to have reduced MMP activity of 28.8% and 29.6% respectively (p=0.036 and p=0.033; Fig. 6.22) when compared to the vehicle control cells.

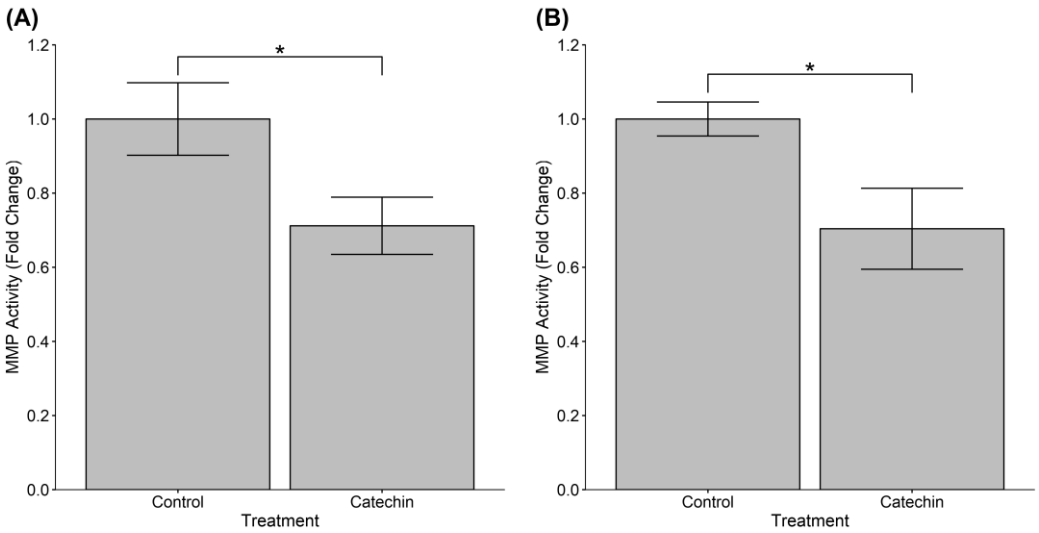

Fig. 6.22. MMP activity in human macrophages is attenuated by catechin. PMA differentiated THP-1 macrophages were treated with vehicle (vehicle control) or catechin (1.5 µg/ml) for either 3 (A) or 24 (B) hours to evaluate MMP activity. The vehicle control was arbitrarily assigned as 1 and remaining data normalised to this. The data are presented as the mean ± SEM from three independent experiments. Statistical analysis was performed using a t- test (equal variances) where * p ≤0.05.

6.3.15 M1 Macrophage polarisation is not altered by catechin in murine macrophages

The expression of five M1 macrophage markers, *Arg2*, *IL-1β*, *IL-6*, *iNOS* and *MCP-1*, were assessed in murine RAW264.7 macrophages following co-stimulation with IFN-γ (250 U/ml) and LPS (100 ng/ml) to determine the effect of catechin on macrophage phenotype polarisation. There are concerns within the literature which suggest that using PMA to differentiate THP-1 monocytes results in an established M1 macrophage phenotype, therefore this assay was performed in murine RAW264.7 macrophages (Park *et al.* 2007). The expression of *Arg2* (p<0.001), *IL-1β* (p<0.001), *IL-6* (p<0.001), *iNOS* (p<0.001) and *MCP-1* (p<0.001) was significantly increased following co-stimulation treatment (Fig. 6.23). Incubation with catechin increased *Arg2* expression by 92.0% (p=0.014). No changes in *IL-1β*, *IL-6*, *iNOS* or *MCP-1* expression was observed following catechin treatment when compared to the co-stimulation only cells.

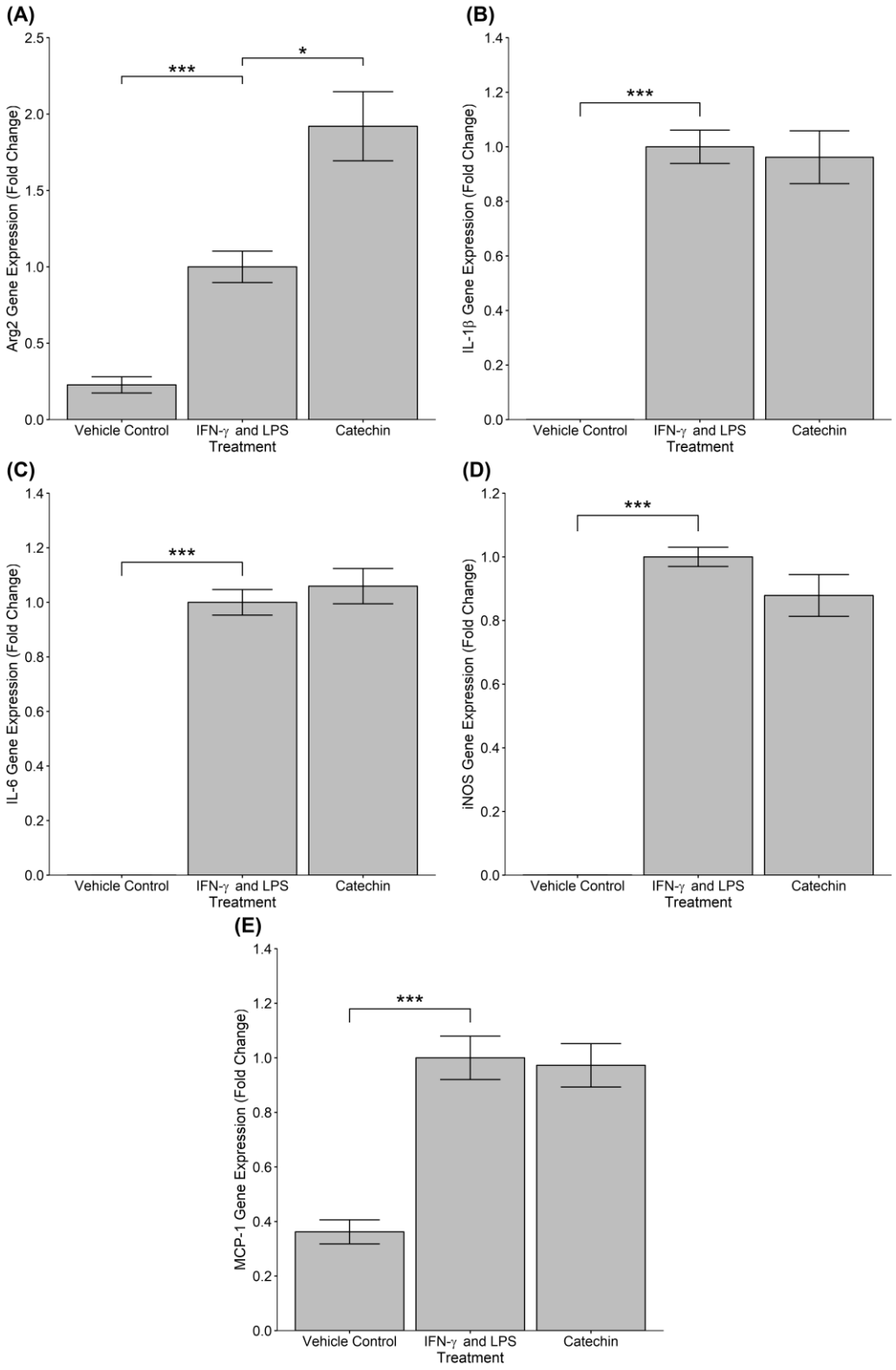

Fig. 6.23. M1 polarisation in murine macrophages is not influenced by catechin. The expression of *Arg2* (A), *IL-1β* (B), *IL-6* (C), *iNOS* (D) and *MCP-1* (E) was assessed in murine RAW264.7 macrophages that were either treated with vehicle (vehicle control); with IFN-γ (250 U/ml) and LPS (100 ng/ml); with IFN-γ (250 U/ml) and LPS (100 ng/ml) in the presence of catechin (1.5 µg/ml) for 24 hours. Gene expression levels were assessed using qPCR and calculated using the comparative Ct method and normalised to *β-actin* levels with values from IFN-γ and LPS treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from three independent experiments. Statistical analysis was performed using a one-way ANOVA with either Games-Howell (A, B, C and D) or a Tukey (E) post-hoc analysis where * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

6.4 Discussion

The results from this study show that catechin has some potential as a nutraceutical for preventing atherosclerosis disease development but the results from *in vitro* studies still remain a little mixed. Catechin was found to attenuate six pro-atherogenic actions, including; cell adhesion molecule expression, monocyte migration, cell proliferation, ROS production, MMP activity and *IFN-γ* gene expression. However catechin was found to have no effect on lipid transport, lipid storage, cell apoptosis or M1 phenotype polarisation. These assays highlight the possible use of catechin as a preventative or an add-on treatment to target specific steps within atherosclerosis plaque formation rather than as a single standalone therapy. The current literature remains unclear about the efficacy of catechin as an anti-atherogenic nutraceutical and relatively little is known about its mechanism of action (Moss and Ramji 2016b). Our study provided an ideal opportunity to explore the effects of catechin in our *in vitro* models and explore possible mechanisms of action.

The qPCR array assessed the expression of 84 genes simultaneously and observed changes of at least 10% in 37 genes, of which 3 were found to be significant (Fig. 6.9). Those three genes were *IFN-γ*, *SELL* and *VEGFA*. The significant role IFN-γ plays during atherosclerosis development has previously been discussed in Chapter 1 and extensively covered in recent reviews (Moss and Ramji 2016a; McLaren and Ramji 2009; Moss and Ramji 2015; Ramji and Davies 2015). Catechin was found to significantly reduce the expression of *IFN-γ* and should be seen as a potential dampening of the whole pro-inflammatory setting which enhances atherosclerosis plaque formation. However this hypothesis needs to be tested *in vivo* before any conclusions can be drawn. The effect of catechin on *IFN-γ* gene expression in THP-1-derived macrophages has not previously been assessed *in vitro* but other studies have shown that catechin and other flavanols in green tea are capable of attenuating IFN-γ stimulated gene expression, possibly via blocking IFN-γ signalling through the JAK-STAT pathway (Leong *et al.* 2009). Two key IFN-γ inducible genes are *MCP-1* and *ICAM-1*, which were not found to have their expression significantly altered in the qPCR array. Furthermore there were no reductions observed in MCP-1 or ICAM-1 secretion following catechin treatment (Fig. 5.8). Despite a reduction in *IFN-γ* gene expression, the results of our study do not indicate any major cardiovascular protective effects further downstream based solely on changes in IFN-γ signalling through the JAK-STAT pathway. However there are a variety of JAK-STAT independent IFN-γ signalling pathways which should be investigated in future studies (Green *et al.* 2017).

The second gene found to be significantly reduced in the qPCR array was *SELL*. Selectins are transmembrane protein molecules capable of influencing leucocyte adhesion and rolling on activated endothelial cells (McEver 2015; Galkina and Ley 2007). They also play a key role in the recruitment of immune cells to the initial site of atherosclerotic lesion formation (McEver

2015; Galkina and Ley 2007). There are a variety of selectins, however the three predominantly involved in cell recruitment are SELL, which is expressed on leucocytes, SELE which is expressed on the surface of activated endothelial cells and P-selectin (SELP) which is expressed on platelets (McEver 2015; Galkina and Ley 2007). Generally it is thought that SELL is pro-atherogenic as mice models deficient in this selectin have shown a reduction in the recruitment of monocytes, lymphocytes and neutrophils in an inflammatory setting (Tedder *et al.* 1995). Furthermore, the transplantation of *SELL*$^{-/-}$ lymphocytes into *ApoE* deficient mice showed that B- and T-cell migration was attenuated by 57% and 50% respectively when compared to transplantation of wild type lymphocytes (Galkina *et al.* 2006). However there are also other studies which have shown SELL to have cardiovascular protective effects. *ApoE* deficient mice that were also lacking *SELL* were found to have accelerated atherosclerosis development due to a 1.3 fold increase in the number of leucocytes within the aorta (Galkina *et al.* 2014). Although the study observed a decrease of 2 fold in the number of B-cells within the aorta, the number of macrophages present increased by 1.5 fold (Galkina *et al.* 2014). This study suggests that SELL may actually exert anti-atherogenic effects. The results of our study indicate that catechin is capable of attenuating *SELL* expression in human macrophages (Fig. 6.12). Furthermore catechin also showed trends of decrease in the expression of *SELE* and *LAMA1*. Smaller atherosclerotic lesions have been found to develop in mouse model systems which are deficient in *SELE* (Collins *et al.* 2000). On the other hand, LAMA1 is an important glycoprotein within the basement membrane and provides it with structural stability, and has additionally been implicated in cell recruitment (Colognato and Yurchenco 2000). The reduced expression of *LAMA1*, *SELE* and *SELL* following catechin treatment may provide a mechanism of action by which reduced cell recruitment was achieved previously (Fig. 5.9). As the previous assay didn't involve any ECM, it is therefore most likely that catechin reduces monocyte migration by reducing the expression of *SELE* and *SELL*. Previous studies have observed a reduction in SELL protein levels in obese individuals following 4 weeks of flavanol-rich cocoa consumption (McFarlin *et al.* 2015). The level of secreted SELE by HUVECs has also been found to be reduced following treatment with a mixture of epicatechin and catechin (Carnevale *et al.* 2014). The results of our study show similar trends to the current literature.

VEGFA is a growth hormone which plays a major role in promoting angiogenesis (Greenberg and Jin 2013; Smith *et al.* 2015; Camaré *et al.* 2017). Not only is it vital for the maintenance of vascular function and tissue homeostasis, it has also been found to influence gene expression, metabolism, migration, survival and proliferation of a variety of cell types including macrophages (Greenberg and Jin 2013; Smith *et al.* 2015; Camaré *et al.* 2017). However unregulated over expression of *VEGF* has been implicated in a variety of diseases including atherosclerosis (Greenberg and Jin 2013; Camaré *et al.* 2017). The anaerobic conditions within the lipid-rich necrotic core stimulates the production and release of VEGF, which in turn promotes angiogenesis within the plaque (Greenberg and Jin 2013). Eventually these newly

formed blood vessels can either haemorrhage or cause the plaque to rupture, though it remains unclear if interplaque haemorrhage directly causes plaque rupturing (Greenberg and Jin 2013). *ApoE* deficient mouse models have shown VEGF to enhance atherosclerosis disease development (Heinonen *et al.* 2013; Celletti *et al.* 2001). In our study, we found catechin to significantly reduce the expression of *VEGFA* (Fig. 6.13), indicating possible downstream benefits such as reduced monocyte migration (Fig. 5.9), reduced proliferation (Fig. 6.19) and improved plaque stability. Although the effect of catechin on *VEGFA* has not been explored in THP-1 macrophages before, previous studies using human cells have also shown other flavanols to inhibit the expression of *VEGFA* (Nakanishi *et al.* 2015; Zhu 2011; Neuhaus *et al.* 2004). The results from the qPCR array can be considered accurate as similar trends in gene expression changes were seen by regular qPCR (Fig. 6.17). The main aim of the qPCR array was to generate data from genes we would not normally assess with a regular qPCR in order to guide us into new *in vitro* assays to explore the possible cardiovascular protective effects of catechin in as greater detail as possible.

As atherosclerotic plaques continue to mature, macrophages and foam cells undergo apoptosis and necrosis which causes them to expel their lipid-rich contents into the wall of the artery and increase the size of the lipid-rich necrotic core (Stoneman and Bennett 2004). As well as releasing their lipid contents, macrophages also secrete a variety of proteins and enzymes, including MMPs, into the intracellular space (McLaren *et al.* 2011a; Ruddy *et al.* 2016). As well as activating MMPs, their expression is also induced by several pro-inflammatory cytokines and lead to a weakening of the fibrous cap by degradation of the ECM present (Moss and Ramji 2016a; Ruddy *et al.* 2016). Therefore targeting both cell apoptosis and MMP activation may represent a potential therapeutic window to improve plaque stability. FAS is a cell surface receptor that causes apoptosis when activated (Peter *et al.* 2015). A trend of decrease in *FAS* expression was found following catechin treatment (Fig. 6.10), therefore the effect of catechin on cell apoptosis was assessed (Fig. 6.18). Previous studies have shown that catechin and other flavanols are capable of attenuating cell apoptosis both *in vitro* and *in vivo* (Tanigawa *et al.* 2014; Mohan *et al.* 2017; Yu *et al.* 2017). However this study was unable to observe any changes in cell survivability following catechin treatment during serum starvation and TNF-α induced apoptosis (Fig. 6.18). As catechin is an anti-oxidant perhaps apoptosis should have been induced by oxidative stress in a manner similar to the study performed by Tanigawa *et al.* (2014), which did observe improved cell survivability following (+)-catechin treatment of murine macrophages.

Previous studies have demonstrated that IFN-γ is capable of inducing the expression and activation of MMPs in macrophages (Moss and Ramji 2015). As catechin was found to be capable of attenuating the expression of *IFN-γ* (Fig. 6.15), its effect on MMP activity was determined. Catechin decreased MMP activity in human macrophages after both 3 and 24

hours (Fig. 6.20). The reduction in MMP activity was consistent and there were no further reductions from prolonged exposure to catechin meaning the maximum reduction was achieved in the first 3 hours *in vitro*. The reduction in MMP activity also correlates with reduced *IFN-γ* expression (Fig. 6.15). Previous work has shown IFN-γ to be a pro-inflammatory cytokine capable of regulating MMP activity (Moss and Ramji 2015; Moss and Ramji 2016a). The results of this study fit with the current literature which shows that catechin and other flavanols are capable of attenuating the expression and activation of MMPs (Kim-Park *et al.* 2016; Nowakowska and Tarasiuk 2016; Wang *et al.* 2016; Roomi *et al.* 2017; Owczarek *et al.* 2017; Sato *et al.* 2017). MMP9, a key protease in atherosclerosis disease development, was found to have its production and release from PMA differentiated THP-1 macrophages reduced following treatment with epigallocatechin gallate (Wang *et al.* 2016). These results imply that catechin would improve plaque stability, however this still needs to be assessed *in vivo*. Furthermore, our study investigated general MMP activity and future studies should investigate the effect of catechin on specific MMPs. Additionally as MMP gene expression was unaffected in our study, it implies that the reduction of MMP activity was due to inhibition of the enzyme itself rather than at the expression level, therefore future assays should also explore how catechin achieves this and whether it exerts this effect indirectly by increasing the production and secretion of tissue inhibitors of metalloproteinases (TIMPs). Previous studies have found flavanols to increase the secretion of TIMPs and this therefore represents a possible mechanism by which catechin attenuates MMP expression (Garbisa *et al.* 2001; Lewandowska *et al.* 2013).

The reduction in *VEGFA* gene expression (Fig. 6.13) implied that catechin may be able to attenuate cell proliferation. Uncontrolled cell proliferation within the atherosclerotic lesion can lead to it increasing in size, as a consequence attenuating macrophage proliferation would potentially retard disease progression (Fuster *et al.* 2010; Robbins *et al.* 2013). A significant decrease in human monocyte and macrophage proliferation was observed as a result of incubation with catechin (Fig. 6.19). Furthermore periodic treatment with catechin was found to maintain this reduction in cell proliferation, however accumulative catechin treatment did not affect the rate of proliferation (Fig. 6.19C). The results of this study coincide with results in the current literature which have found catechin and other flavanols to reduce cell proliferation *in vitro* (Baker and Bauer 2015; Papademetrio *et al.* 2013).

Due to catechin being found to reduce monocyte recruitment (Fig. 5.9) and cell proliferation (Fig. 6.19) *in* vitro, it implies that catechin has the potential to reduce atherosclerotic lesion size *in vivo* by reducing the cellular content within the lesion. Indeed others have observed treating mice with green tea extracts results in smaller atherosclerotic lesions (Chyu 2004; Minatti *et al.* 2012; Morrison *et al.* 2014). *ApoE* deficient mice fed epigallocatechin gallate for either 21 or 42 days were found to have plaque formation reduced by 55% and 73%

respectively (Chyu 2004). A significant reduction of 35% in the area of atherosclerotic lesions was found in LDLr knockout mice following treatment with green tea extract after 4 weeks (Minatti *et al.* 2012). Furthermore epicatechin treatment for 20 weeks in ApoE*3-Liden mice resulted in smaller lesion formation (Morrison *et al.* 2014). Our results of reduced cell recruitment and proliferation may represent the mechanism behind smaller lesion formation following flavanol treatment in the studies mentioned above. Atherosclerotic plaques which are considered vulnerable to rupturing tend to have higher cellular content, especially macrophages, when compared to more stable plaques (Wilson 2010; Seneviratne *et al.* 2013). When considering the reduced MMP activity (Fig. 6.22) and *VEGFA* expression (Fig. 6.13) in addition to our cell recruitment and proliferation assays, the results suggest catechin has the potential ability to improve plaque stability and reduce the risk of rupture. However the effect of catechin on plaque stability *in vivo* remains unclear at this present time and needs to be assessed in future studies.

The imbalance of cholesterol homeostasis to favour cholesterol uptake over cholesterol efflux in macrophages leads to increased intracellular cholesterol accumulation and foam cell formation (McLaren *et al.* 2011a). Catechin treatment of human PMA differentiated THP-1 macrophages had no effect on either cholesterol uptake or cholesterol efflux (Fig. 6.20), despite a significant reduction in *LDLr* expression (Fig. 6.14), indicating that catechin is unable to attenuate foam cell formation. Few studies have assessed the effect of flavanols on cholesterol homeostasis *in vitro*. Previous studies have shown epigallocatechin gallate is capable of attenuating intracellular cholesterol accumulation (Bursill and Roach 2006). A more recent study has observed decreases in oxLDL uptake in both human THP-1 derived and primary macrophages following epigallocatechin gallate treatment (Chen *et al.* 2017d). As far as we are aware this is the first *in vitro* study to directly assess the effect of catechin on cholesterol uptake and efflux. Unsurprisingly as catechin did not affect cholesterol uptake and efflux, there were also no observable changes in the proportions of the major intracellular lipid classes (Fig. 6.21). However the results are based on a single independent experiment and requires further investigation in future studies.

Formation of M1 phenotype macrophages should be seen as an important therapeutic target for retarding atherosclerosis disease progression. The expression of four markers of M1 macrophage phenotype, *IL-1β*, *IL-6*, *iNOS* and *MCP-1*, were all unaffected following catechin treatment in murine macrophages (Fig. 6.23). However the expression of *Arg2* was significantly increased (Fig. 6.23A). Earlier studies also found that (+)-catechin had no effect on *IL-1β* or *IL-6* expression in primary white blood cells from cows (Sehm *et al.* 2005). More recent studies have found the expression and release of IL-1β, IL-6 and iNOS was significantly reduced following treatment with either polyphenol-rich cocoa or epicatechin in both THP-1 macrophages and human neutrophils (Marinovic *et al.* 2015; Dugo *et al.* 2017). It is worth

noting that neither of these studies used (+)-catechin specifically as part of their treatment and as a result their results may be compound specific. Epicatechin has also been shown to reduce the expression of *Arg2* after 24 hours (Schnorr *et al.* 2008). The significant increase in *Arg2* expression following (+)-catechin treatment appears to be gene specific rather than an indication of increased M1 phenotype formation as the other four genes assessed were unaffected. The effect of (+)-catechin on *Arg2* expression should be investigated further in future studies. Overall catechin treatment had no effect on M1 phenotype polarisation in murine macrophages.

This is the first study to assess the potential cardiovascular health benefits of (+)-catechin in such great detail. Although it appears affect only some steps in atherosclerosis disease development rather than all of them, it did attenuate monocyte migration, cell proliferation, ROS generation, MMP activity and *IFN-γ* expression (summarised in Fig. 6.24). Despite not preventing either cell apoptosis, M1 macrophage polarisation or foam cell formation, catechin never exerted any detrimental effects. Catechin was only found to have anti-inflammatory effects and did not appear to possess any pro-atherogenic properties, this surely makes (+)-catechin an exciting novel nutraceutical worth pursuing. This study has also helped our understanding about the mechanism of action by which catechin and other flavanols may exert their anti-atherogenic effects. Many other studies have found flavanols to reduce atherosclerotic lesion size (Chyu 2004; Minatti *et al.* 2012; Morrison *et al.* 2014). This reduction in lesion size may be due to the reduced cell migration and proliferation observed in this study. Reduced circulating oxLDL levels have been seen in several *in vivo* studies following catechin and other flavanol treatment, possibly due to reduced ROS generation found within this study (Babu *et al.* 2008; Tinahones *et al.* 2008). The results of our study also suggest that catechin would improve plaque stability due to reduced cellular content within the plaque as a result of less cell migration and proliferation, in addition to better maintenance of the fibrous cap due to reduced MMP activity. Catechin treatment may also provide a systemic dampening of the inflammatory response to oxLDL accumulation within the wall of arteries due to a decrease in *IFN-γ* expression. However the effect of catechin on plaque stability and reduced *IFN-γ* expression still need to be explored in future *in vivo* studies before any conclusions can be drawn. These *in vitro* results justify further exploration of catechin, both *in vitro* and *in vivo*, to fully assess its potential cardiovascular protective effects.

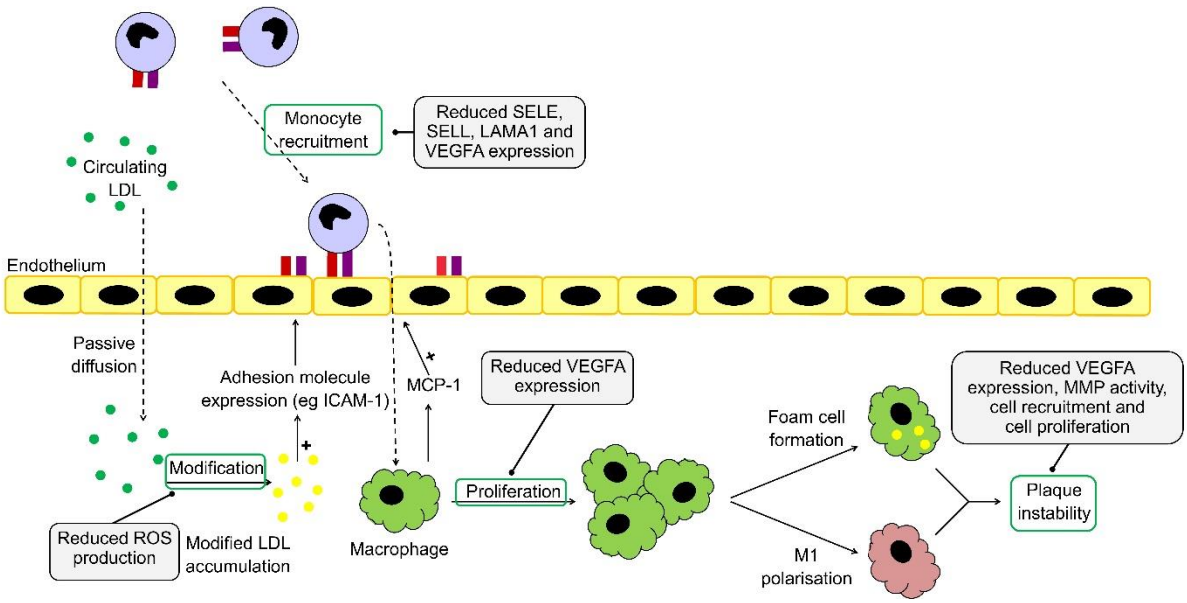

Fig. 6.24. Summary of the potential anti-atherogenic properties of catechin. The steps potentially reduced in atherosclerosis disease progression following (+)-catechin treatment are highlighted in green. Possible mechanisms for these reductions are also included.

Chapter 7

The effects of catechin in wild type mice fed a high fat diet

7.1 Introduction

The *in vitro* results presented in Chapter 6 showed that catechin has great potential as an anti-inflammatory nutraceutical therapy for slowing the progression of atherosclerosis disease development. Therefore an initial *in vivo* study was established to further explore the cardiovascular protective effects of catechin and generate more substantial evidence for the use of catechin as a nutraceutical which currently cannot be achieved *in vitro*. However due to the cost of using pure (+)-catechin for 21 days in 8 mice being too high (~£15,000), (+)-catechin hydrate was used as an alternative. Catechin hydrate is simply a catechin molecule which has an additional water molecule attached (Fig. 7.1). A dosage of 200 mg/kg was used in this study due to it being employed in previous *in vivo* studies which delivered catechin by gavage for a similar time period of 3 weeks (Potenza *et al.* 2007).

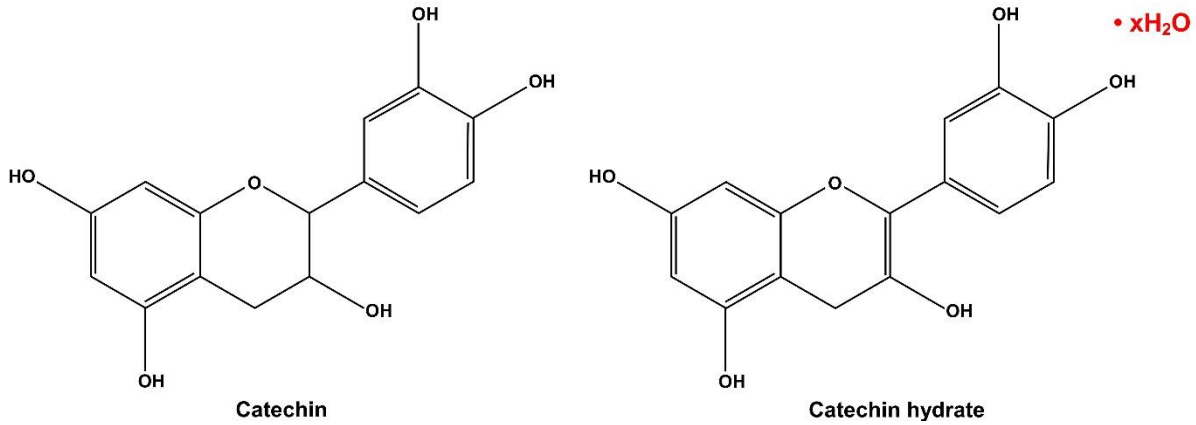

Fig. 7.1. The structure of catechin and catechin hydrate. The structure of catechin and catechin hydrate are almost identical apart from an additional water molecule being present (highlighted in red).

The same assays used to study the effect of CardioWise *in vivo* were employed to assess the potential cardiovascular protective effects of catechin. Details on their rationale can be found in Chapter 4. Initially both mouse and fat pad weight were recorded. Subsequently the levels of several risk factors, including cholesterol, TG, ROS and circulating cytokines, within the plasma were assessed. To determine whether changes in these parameters were brought about by changes in gene expression, RNA was extracted from the liver to study the effect of catechin on gene expression. Finally the bone marrow was harvested in order to study the effect of catechin on haematopoietic cell populations. Unfortunately due to unknown problems during cell extraction, high quality samples were only recovered from four control and four

catechin treated mice. Therefore the results of cell population analysis are based on a reduced sample size.

7.2 Experimental Aims

Details of the specific *in vivo* methodologies are outlined in Chapter 2 and a brief experimental strategy for Chapter 7 is presented in Fig. 7.2.

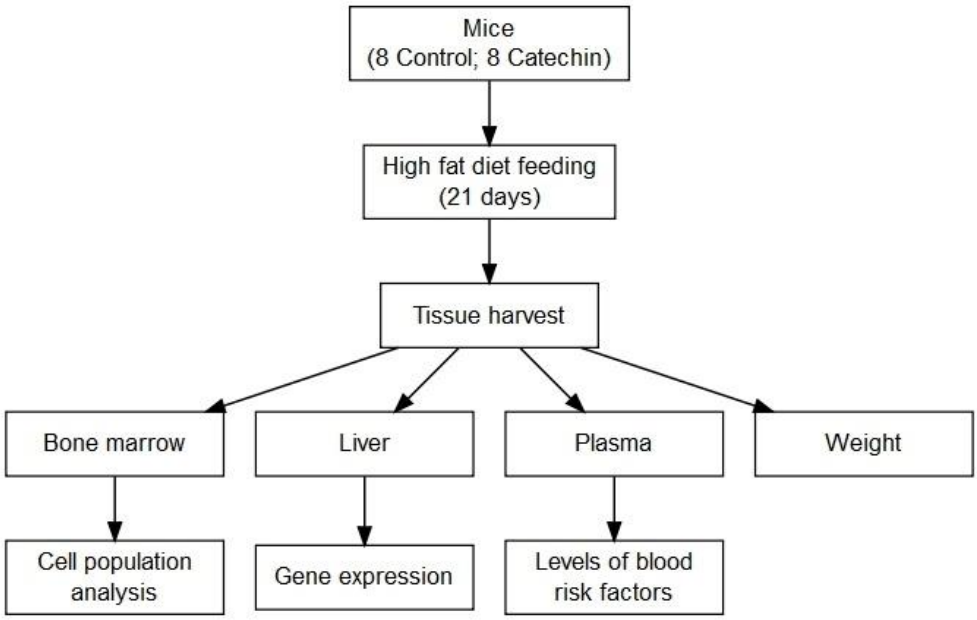

Fig. 7.2. Experimental strategy used to assess the effect of catechin *in vivo*.

7.3 Results

7.3.1 The migration of human monocytes is prevented by catechin hydrate *in vitro*

To ensure catechin hydrate exerted the same anti-atherogenic properties of catechin, its effect on monocyte migration was assessed. During the progression of atherosclerosis disease development, the recruitment of monocytes to the affected area is a key mechanism in lesion formation. Therefore using this *in vitro* assay could be used to compare the effects of catechin hydrate to catechin. The chemokine MCP-1 is capable of recruiting monocytes and this was confirmed in our *in vitro* model as a significant 498.3% increase ($p<0.001$) in monocyte migration occurred in comparison to the vehicle control (Fig. 7.3). A 1.5 µg/ml dose of catechin hydrate reduced monocyte recruitment to MCP-1 by 41.8% ($p=0.005$) in comparison to MCP-1 only. A similar decrease in monocyte migration was seen following 1.5 µg/ml dose of catechin treatment of human THP-1 monocytes (Fig. 5.9).

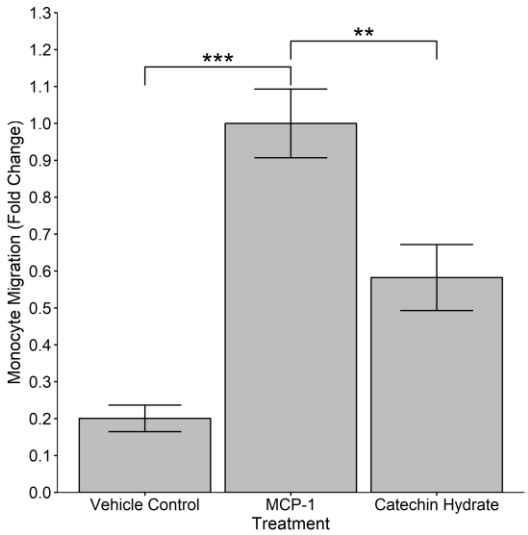

Fig. 7.3. The migration of human monocytes towards MCP-1 is inhibited by catechin hydrate. THP-1 monocytes that were treated with vehicle (vehicle control) or treated with MCP-1 (20 ng/ml) alone or with MCP-1 (20 ng/ml) in the presence of catechin hydrate (1.5 µg/ml) for 3 hours were used to evaluate monocyte migration. Data were normalised to the percentage of cells that migrated from the apical compartment of the modified Boyden chamber into the basolateral compartment in response to MCP-1 only treatment. This proportion was arbitrarily assigned as 1. The data are presented as the mean ± SEM from three independent experiments. Statistical analysis was performed using a one-way ANOVA with a Dunnett post-hoc analysis where ** p<0.01 and *** p <0.001.

7.3.2 Catechin caused wild type mice receiving a high fat diet to increase weight gain

The interaction between treatment type and time was positively correlated with mouse weight gain (Fig. 7.4), with catechin treated mice gaining an extra 0.06 g per day compared to the vehicle control treated mice (p<0.001; adj-R^2=0.91). After 21 days, the catechin receiving mice were on average 2.14 g heavier than the control mice (p=0.029).

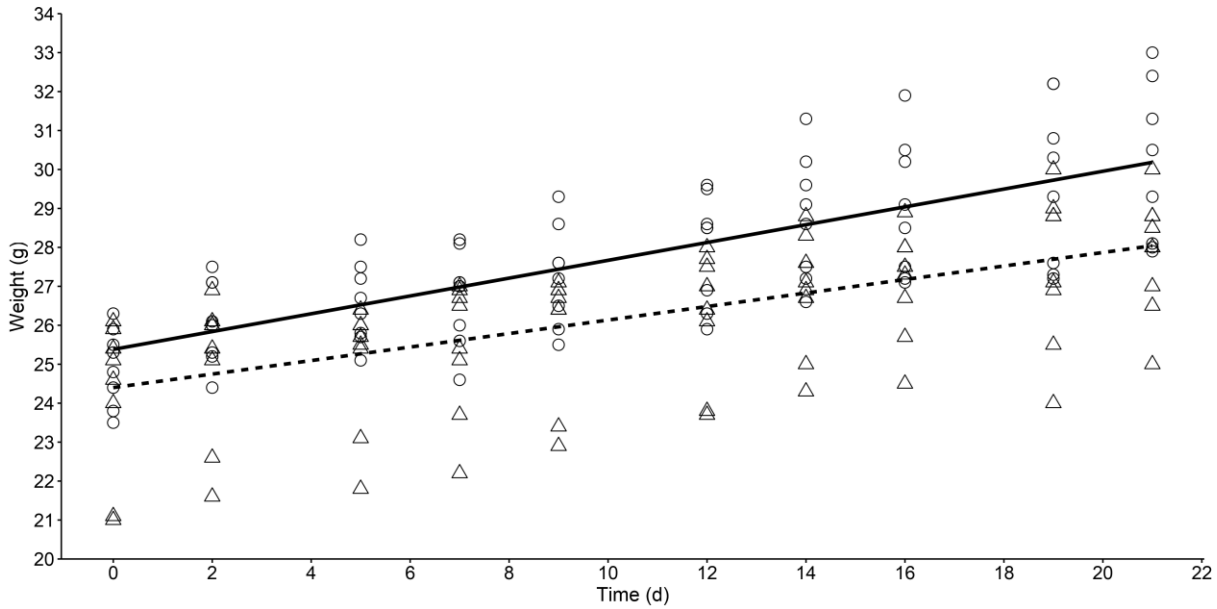

Fig. 7.4. The rate of weight gain in wild type mice fed a high fat diet was increased by catechin treatment. The weight of mice was measured every 2 or 3 days for the entire duration of the study (21 days). Mice were fed a high fat diet which was either supplemented with vehicle control (triangle; dashed line) or 200 mg/kg of catechin hydrate (circle; solid line). The prediction of the average rate of weight gain as calculated by a generalised linear mixed model with a Gamma error distribution and log link function is displayed from 16 mice (control 8; catechin 8). To avoid pseudoreplication the identity of each mouse was included within the model as a random term. The raw data from each mouse is also presented to provide a visual representation of the spread within the data.

7.3.3 Catechin significantly increases white fat accumulation in wild type mice fed a high fat diet

As shown in Fig. 7.5, the increase in mouse weight gain following catechin treatment was a result of increased fat accumulation within the subcutaneous, gonadal and renal fat deposits. These fat deposits were significantly increased by 70%, 78% and 94% respectively (p=0.002, p=0.002 and p=0.015). This increase in fat accumulation caused a 0.78 g increase (p=0.003) in the total fat weight in catechin treated mice compared to the vehicle control receiving mice (Fig. 7.5G). The weight of the inguinal, brown and PVAT fat deposits was unaffected by catechin treatment.

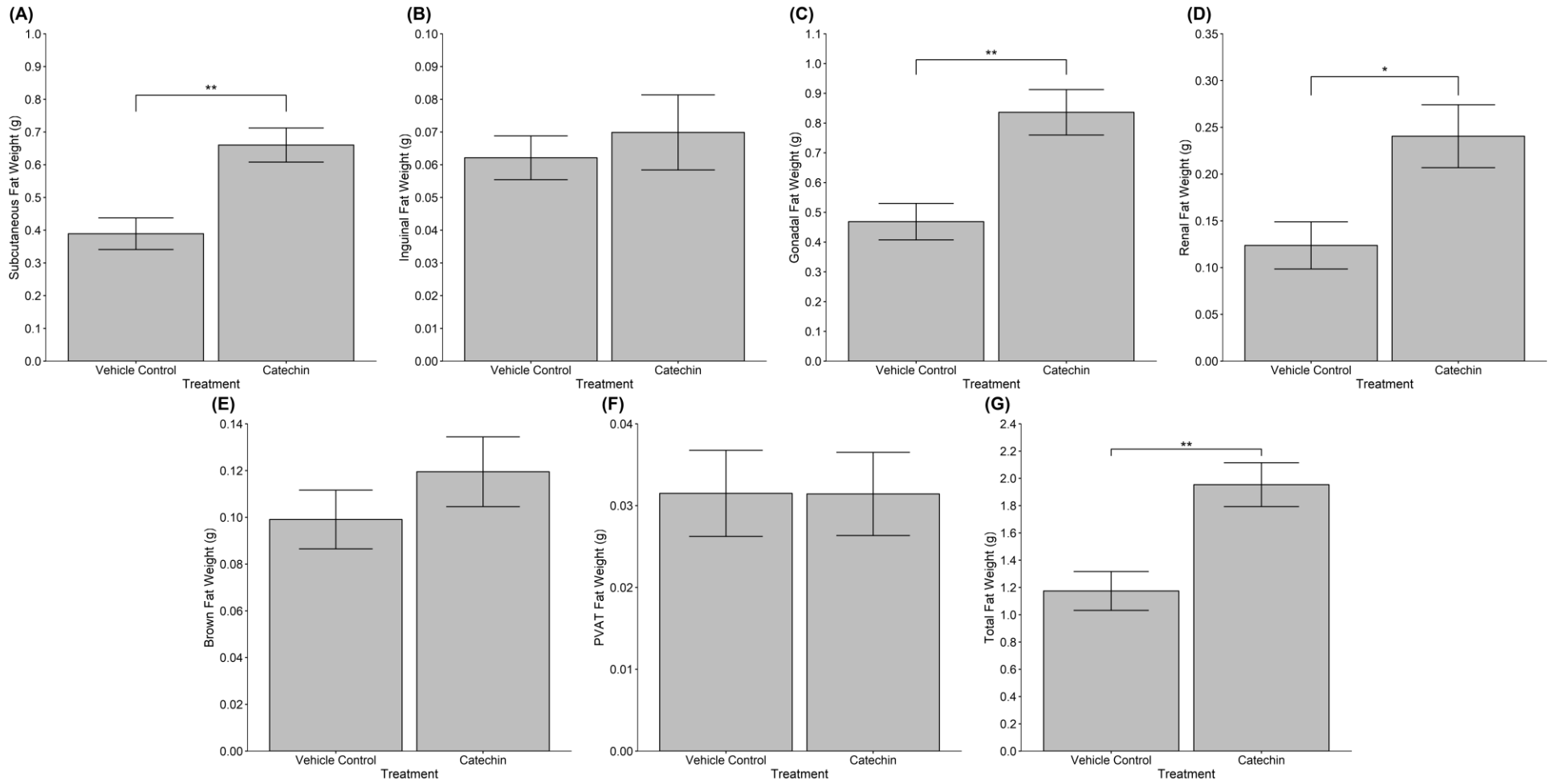

Fig. 7.5. White fat accumulation increased in wild type mice fed a high fat diet which received catechin treatment. Subcutaneous (A), inguinal (B), gonadal (C), renal (D), brown (E), PVAT (F) and total (G) fats from wild type mice were weighed after 21 days of a high fat diet and treatment with either a vehicle control or 200 mg/kg of catechin hydrate. The data are presented as the mean ± SEM from eight control mice and either seven (F) or eight (A, B, C, D, E and G) catechin treated mice. One mouse was removed from the PVAT catechin group (F) due to a possible transcription error during recording the weight of the fat pad recovered. Statistical analysis was performed using a t-test (equal variance) on untransformed data where * $p \leq 0.05$ and ** $p \leq 0.01$.

7.3.4 Catechin treatment shows a trend of increase in circulating HDL cholesterol levels in wild type mice fed a high fat diet

Catechin treatment in wild type mice fed a high fat diet for 21 days had no significant effect on the level of circulating total cholesterol and LDL cholesterol or their ratios relative to each other (Fig. 7.6). However a non-significant increase of 36.1% in serum HDL cholesterol levels was observed in mice receiving catechin compared to the vehicle control treated mice (p=0.133; Fig. 7.6B). This increase in HDL cholesterol levels results in a 23.1% and 23.8% non-significant decrease in both total cholesterol/HDL and LDL/HDL cholesterol ratios respectively (p=0.165 and p=0.190; Figs. 7.6D and F). This result indicates that catechin is capable of reducing the amount of total cholesterol and LDL relative to circulating HDL levels.

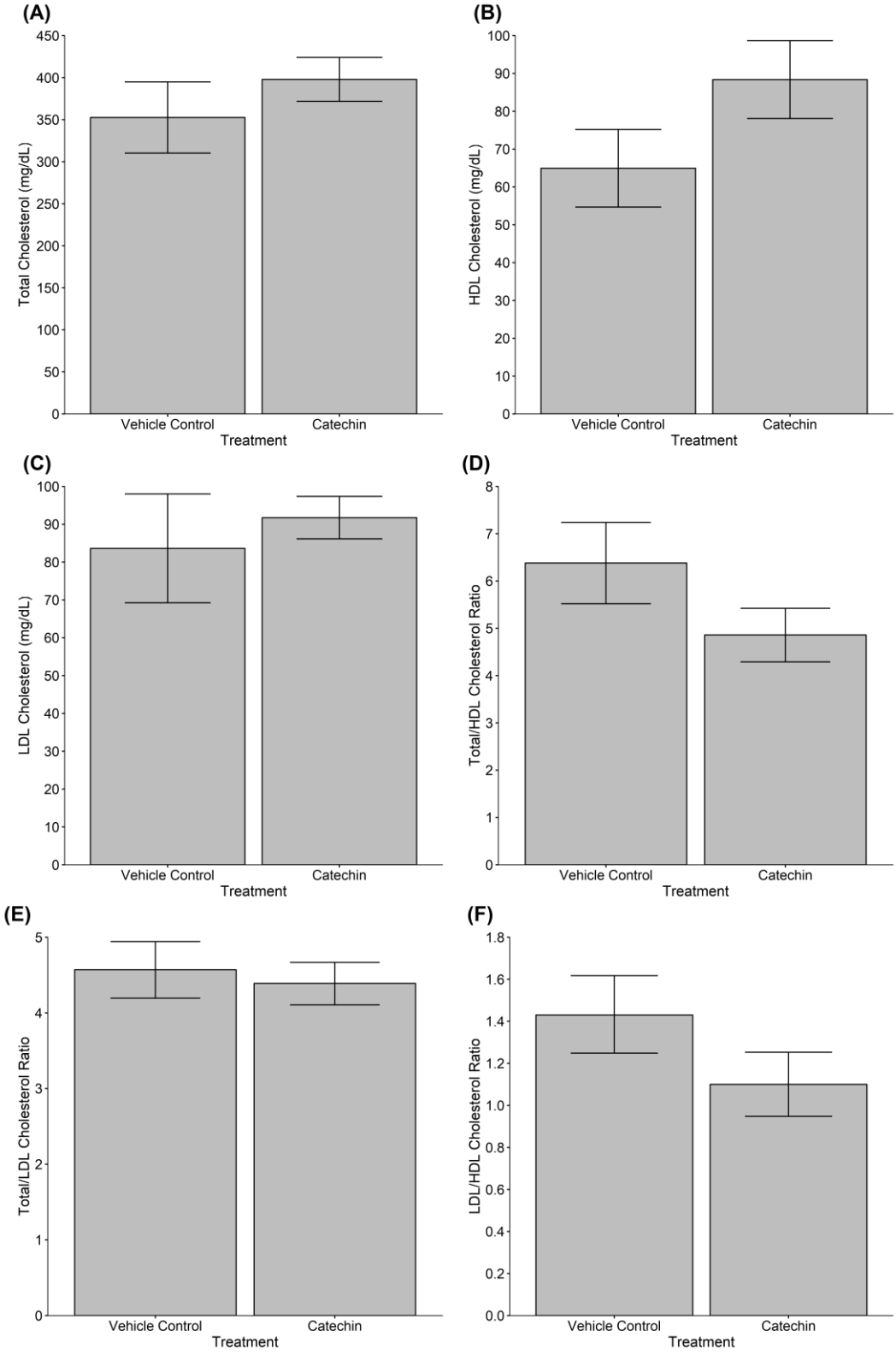

Fig. 7.6. Wild type mice on a high fat diet and treated with catechin had increased HDL cholesterol levels. Total cholesterol (A), HDL (B) and LDL (C) from wild type mice was measured after 21 days of a high fat diet and treatment with either a vehicle control or 200 mg/kg of catechin hydrate. The ratios of total cholesterol/HDL (D), total cholesterol/LDL (E) and LDL/HDL (F) were also assessed. The data are presented as the mean ± SEM from either seven (B, D and F) or eight (A, C and E) control mice and catechin treated mice. Mice with undetectable assay readings were removed before statistical analysis. Statistical analysis was performed using either a t-test (equal variance; A, B, D, E and F) or a t-test (unequal variance; C) on untransformed data (B, C, D, E and F) or squared-transformed data (A).

7.3.5 Catechin does not affect TG levels in wild type mice fed a high fat diet

Serum TG levels were unaffected by catechin treatment of high fat diet fed mice in comparison to vehicle control treatment of high fat diet receiving mice (Fig. 7.7A). Additionally no significant changes in TG/total, TG/HDL or TG/LDL cholesterol ratios were observed following catechin treatment (Figs. 7.7B, C and D).

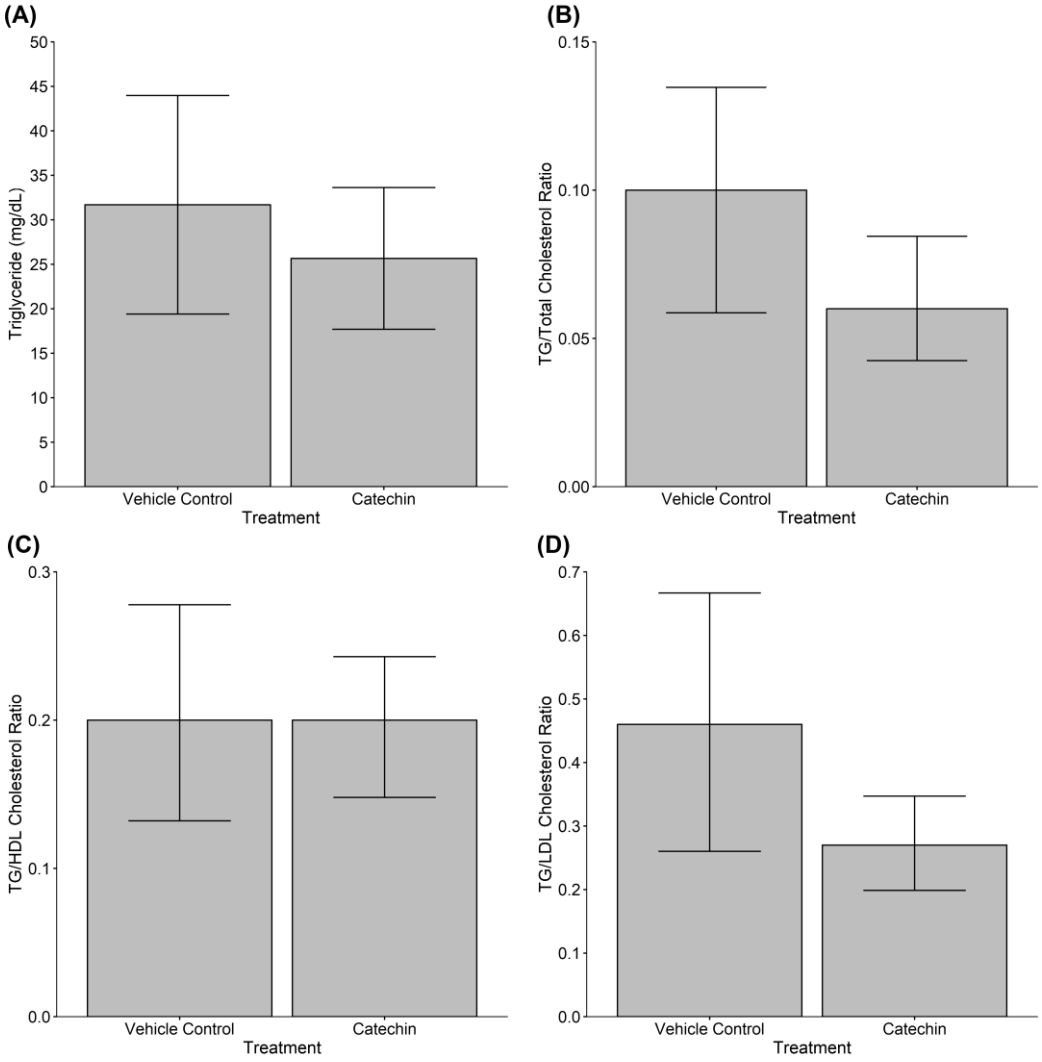

Fig. 7.7. Catechin treatment in wild type mice did not alter TG levels while receiving a high fat diet. TG (A) levels from wild type mice were measured after 21 days of a high fat diet and treatment with either a vehicle control or 200 mg/kg of catechin hydrate. The ratios of TG/total cholesterol (B), TG/HDL cholesterol (C) and TG/LDL cholesterol (D) were also assessed. The data are presented as the mean ± SEM from three (C) or five (A, B and D) vehicle control mice and either five (C) or six (A, B and D) catechin treated mice. Mice with undetectable assay readings were removed before statistical analysis. Statistical analysis was performed using a t-test (equal variance) on either untransformed data (C) or square root-transformed data (A, B and D).

7.3.6 Catechin treatment fails to attenuate ROS production and lipid peroxidation in wild type mice fed a high fat diet

Treatment with catechin did not appear to alter ROS production or lipid peroxidation, measured by MDA levels, in comparison to the control mice (Fig. 7.8).

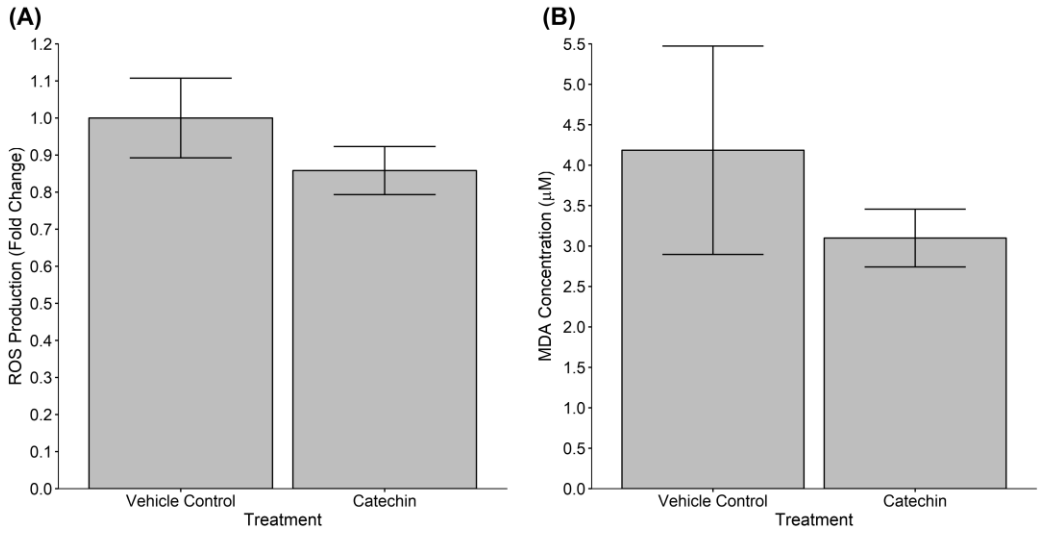

Fig. 7.8. Catechin treatment did not alter ROS or MDA production in wild type mice receiving a high fat diet. ROS production (A) and MDA levels (B) were assessed in wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or 200 mg/kg of catechin hydrate. ROS production was measured in a fluorescence microplate reader, with excitation at 485 nm and emission detected at 535 nm. Vehicle control was given an arbitrary value of 1. MDA levels were measured in a fluorescence microplate reader, with excitation at 535 nm and emission detected at 590 nm. The data are presented as the mean ± SEM from six (B) or eight (A) control mice and either seven (B) or eight (A) catechin treated mice. Mice with undetectable assay readings were removed before statistical analysis. Statistical analysis was performed using a t-test (equal variance) on log-transformed data.

7.3.7 Catechin reduces the levels of several pro- and anti-inflammatory cytokines in wild type mice fed a high fat diet

The circulating serum levels of the pro- and anti-inflammatory cytokines CXCL1, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10 and TNF-α (see Table 4.1 for further details) following catechin treatment were measured by a Luminex Cytokine Assay via the Central Biotechnology Service (Figs. 7.9 and 7.10). A non-significant decrease of 45% (p=0.263) and 32% (p=0.163) was found in the circulating levels of CXCL1 and TNF-α respectively following catechin treatment when compared to the vehicle control receiving mice. All the circulating levels of the IL measured during this study appeared to show a trend of decrease following catechin treatment. The levels of IL-2 were significantly decreased by 37% (p=0.005) in mice which were treated with catechin (Fig. 7.10B). The levels of IL-1β, IL-4, IL-5, IL-6 and IL-10 were all non-significantly reduced by 50% (p=0.077), 54% (p=0.180), 26% (p=0.231), 61% (p=0.456) and 26% (p=0.060) respectively. Catechin treatment did not appear to alter circulating IFN-γ levels.

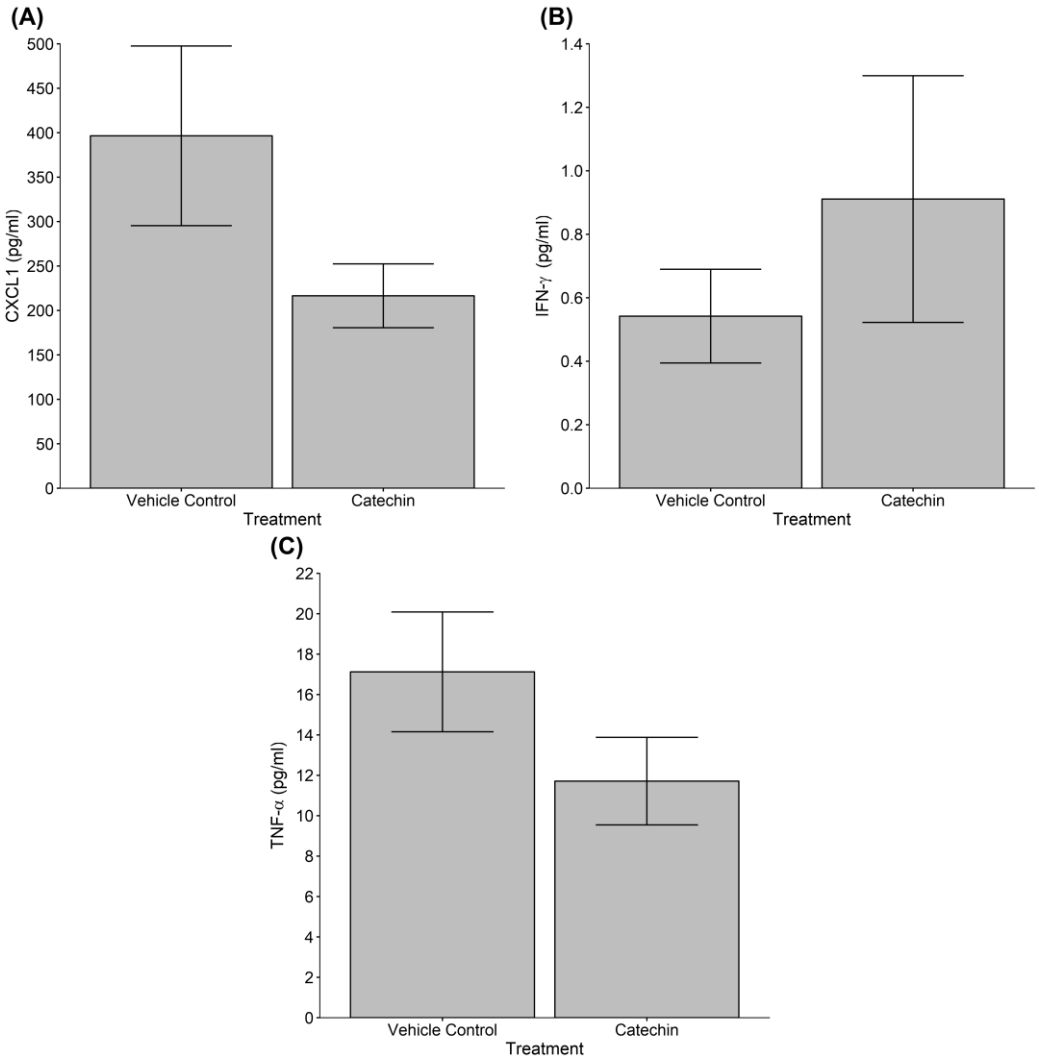

Fig. 7.9. Wild type mice on a high fat diet and treated with catechin had a trend of reduced levels of several cytokines. CXCL1 (A), IFN-γ (B), and TNF-α (C) levels within the plasma of wild type mice were measured after 21 days of a high fat diet and treatment with either a vehicle control or 200 mg/kg of catechin hydrate. The data are presented as the mean ± SEM from seven (B) or eight (A and C) control mice and either six (A) or eight (B and C) catechin treated mice. Mice with undetectable assay readings were removed before statistical analysis. Statistical analysis was performed using a t-test (equal variance) either on untransformed data (C) or log-transformed data (A and B).

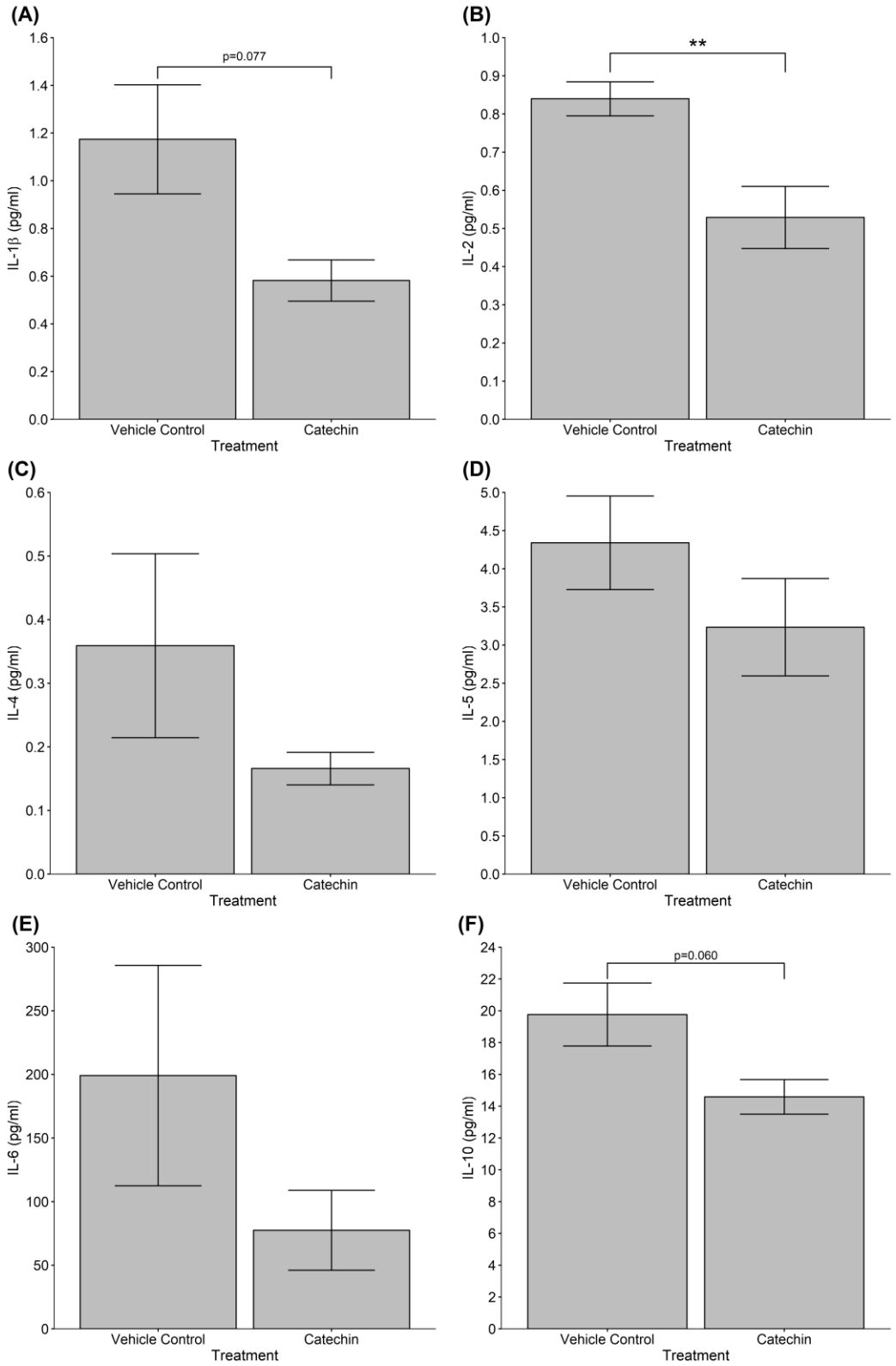

Fig. 7.10. The levels of several interleukin cytokines are reduced in wild type mice following catechin treatment. IL-1β (A), IL-2 (B), IL-4 (C), IL-5 (D), IL-6 (E) and IL-10 (F) levels within the plasma of wild type mice were measured after 21 days of a high fat diet and treatment with either a vehicle control or 200 mg/kg of catechin hydrate. The data are presented as the mean ± SEM from three (C), seven (A and B) or eight (D, E and F) control mice and either four (C), five (B), seven (A and F) or eight (D and E) catechin treated mice. Mice with undetectable assay readings were removed before statistical analysis. Statistical analysis was performed using a t-test (equal variance) either on untransformed data (B and D) or log-transformed data (A, C, E and F).

7.3.8 The expression of all genes present on the qPCR array following catechin treatment in wild type mice fed a high fat diet

Fig. 7.11 shows the global significant and non-significant gene expression changes in the livers of wild type mice fed a high fat diet for 21 days following treatment with catechin compared to vehicle control treated mice. The housekeeping genes *GUSB* and *HSP90AB1* were found to be stable during the assay and were therefore used to calculate gene expression fold changes induced by catechin. A total of 55 genes were found to have their expression altered by at least 10% and 12 of those genes were found to be significantly altered. The genes have been classed by their function (according to the literature provided by Qiagen which accompanied the Atherosclerosis RT^2 Profiler PCR Arrays) and will be explored in greater detail in the subsequent sections.

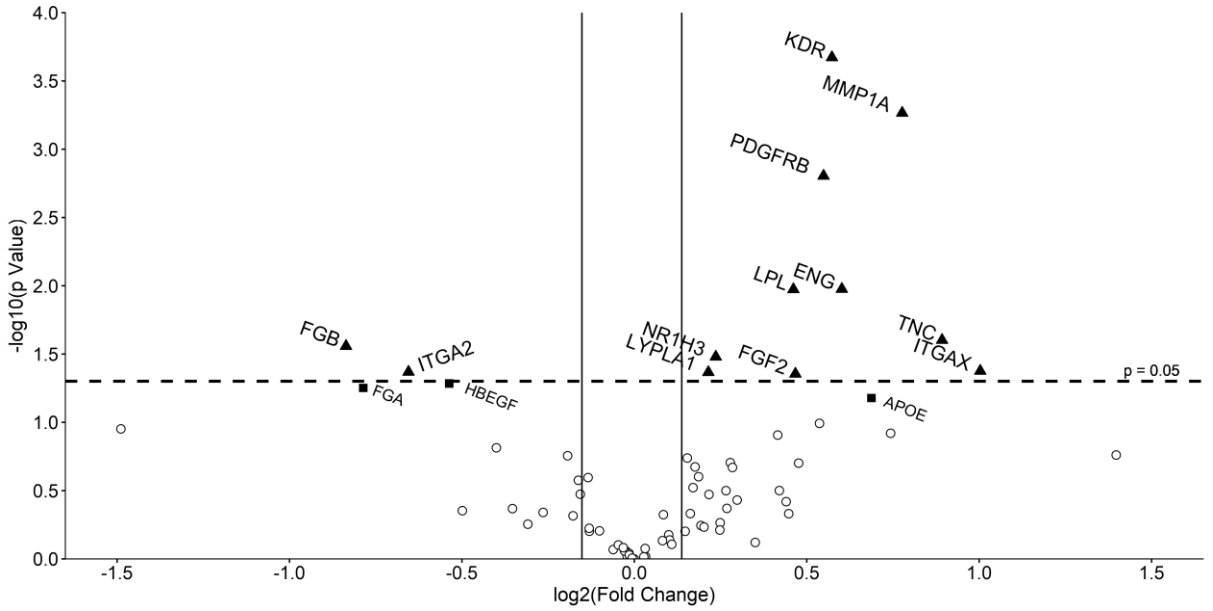

Fig. 7.11. Volcano plot showing global gene expression changes in the liver of wild type mice treated with catechin vs vehicle control. Gene transcript levels of 84 genes were assessed in the livers of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or 200 mg/kg of catechin hydrate. The data are presented as the mean catechin induced fold change when compared to the vehicle control treated mice. Results are based on at least three control and three catechin treated mice. All genes present on the Qiagen RT^2 profiler PCR Array were plotted. The log fold change in the treated cells versus the vehicle control cells is represented on the x-axis. The y-axis shows the -log10 of the p value. A p value of 0.05 (dashed line) and a fold changes of ±10% (solid lines) are indicated. Significantly altered gene expressions are indicated by a black triangle and non-significant trends of change ($p<0.1$) are indicated by a black square.

7.3.9 Catechin increases the expression of a gene involved in the regulation of blood coagulation and circulation in wild type mice fed a high fat diet

The protein endoglin is encoded by the gene *ENG* and it is capable of influencing the formation of new blood vessels. Following treatment with catechin, the expression of *ENG* was significantly increased by 51.8% (p=0.011) when compared to the vehicle control treated mice (Fig. 7.12).

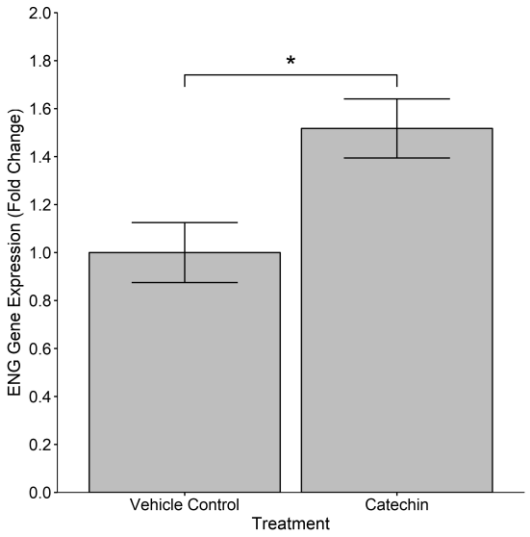

Fig. 7.12. Catechin treatment alters the expression of a gene involved in blood coagulation and circulation *in vivo*. Gene transcript levels of *ENG* was assessed in the livers of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or 200 mg/kg of catechin hydrate. Gene transcript levels were calculated using the comparative Ct method and normalised to two housekeeping genes (*GUSB* and *HSP90AB1*) with values from the vehicle treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from eight control and eight catechin treated mice. Statistical analysis was performed using a t-test (unequal variances) where * $p \leq 0.05$.

7.3.10 Catechin alters the expression of cell adhesion molecules in wild type mice fed a high fat diet

Due to the role of cell adhesion molecules, such as ITGA2, ITGAX and thrombospondin (THBS)-4, during monocyte recruitment and migration in atherosclerotic plaque formation, altering their expression may lead to reduced atherosclerosis disease progression. Catechin treatment was found to reduce *ITGA2* expression by 36.5% (p=0.043) when compared to mice treated with the vehicle control (Fig. 7.13A). However catechin treatment resulted in the increased expression of *ITGAX* and *THBS4* by 100.4% (p=0.042) and 90.4% respectively (Figs. 7.13B and C). However due to the low expression levels of *THBS4*, the result is based on one vehicle control and two catechin treated mice and therefore requires repeating before any solid conclusions can be drawn.

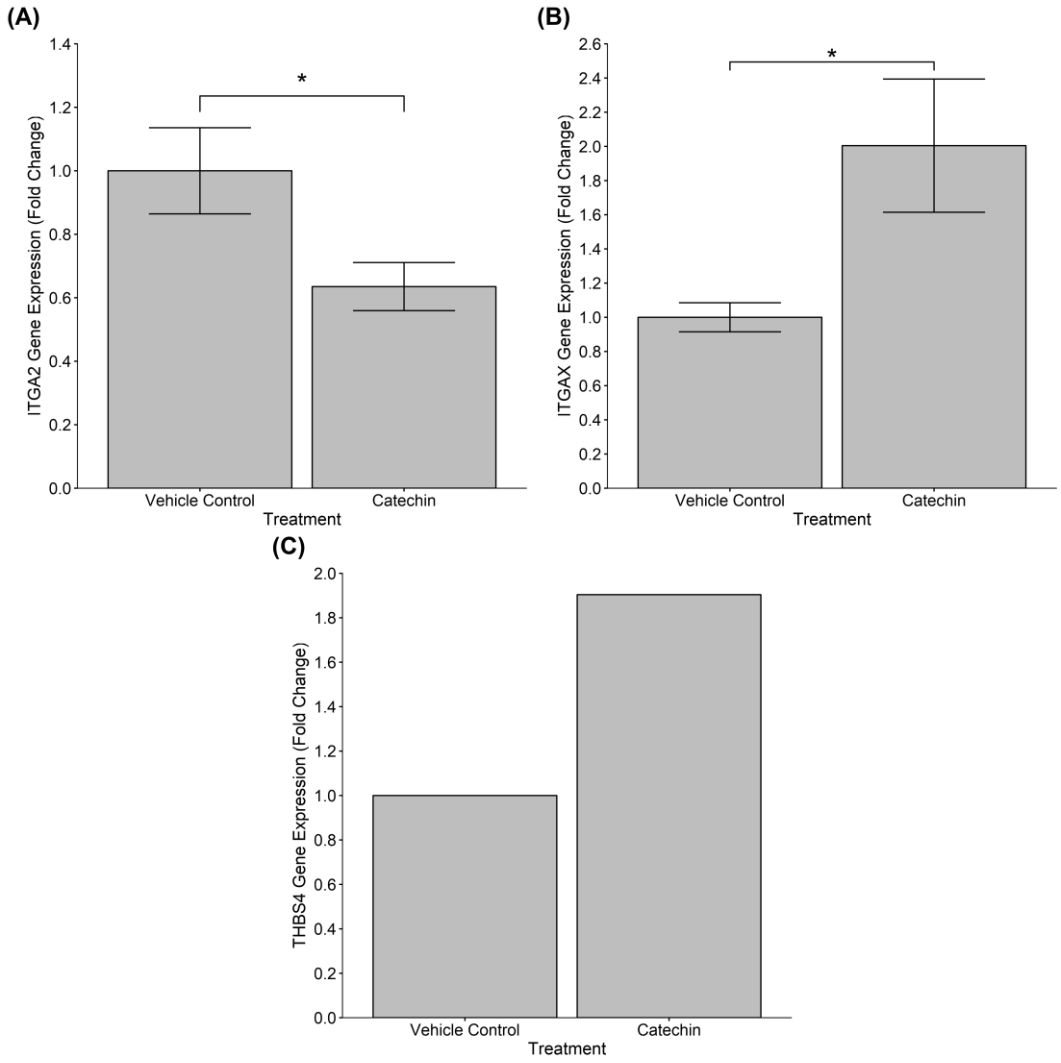

Fig. 7.13. Catechin treatment alters the expression of cell adhesion molecules *in vivo*. Gene transcript levels of *ITGA2* (A), *ITGAX* (B) and *THBS4* (C) were assessed in the livers of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or 200 mg/kg of catechin hydrate. Gene transcript levels were calculated using the comparative Ct method and normalised to two housekeeping genes (*GUSB* and *HSP90AB1*) with values from the vehicle treated mice given an arbitrary value of 1. The data are presented as the mean ± SEM from one (C) or seven (A and B) control mice and either two (C), six (A) or seven (B) catechin treated mice. Mice with undetectable Ct readings were removed before statistical analysis. Statistical analysis was performed using a t-test (unequal variances) where * $p \leq 0.05$.

7.3.11 Catechin alteres the expression of cell growth and proliferation regulators in wild type mice fed a high fat diet

The largest group of genes which were found to have their expression altered by catechin treatment *in vivo* are involved in cell growth and proliferation (Fig. 7.14). A variety of genes including *FGF2*, *IL-2*, *IL-5*, kinase insert domain receptor (*KDR*) and *PDGFB2* were found to have their expression increased in the liver of catechin treated wild type mice fed a high diet. The levels of expression of *FGF2*, *KDR* and *PDGFB2* were increased by 38.2% (p=0.044), 48.8% (p<0.001) and 46.3% (p=0.002). On the other hand, trend of increases of 129.9% and 262.2% were observed for *IL-2* and *IL-5* respectively. However due to the low expression levels

of *IL-2 and IL-5*, the assay requires repeating before any solid conclusions can be drawn. The only cell proliferation gene expression found to be reduced by catechin *in vivo* was heparin-binding EGF-like growth factor (*HBEGF*) which showed a trend of decrease of 31.1% (p=0.052) in contrast to the vehicle control treated mice.

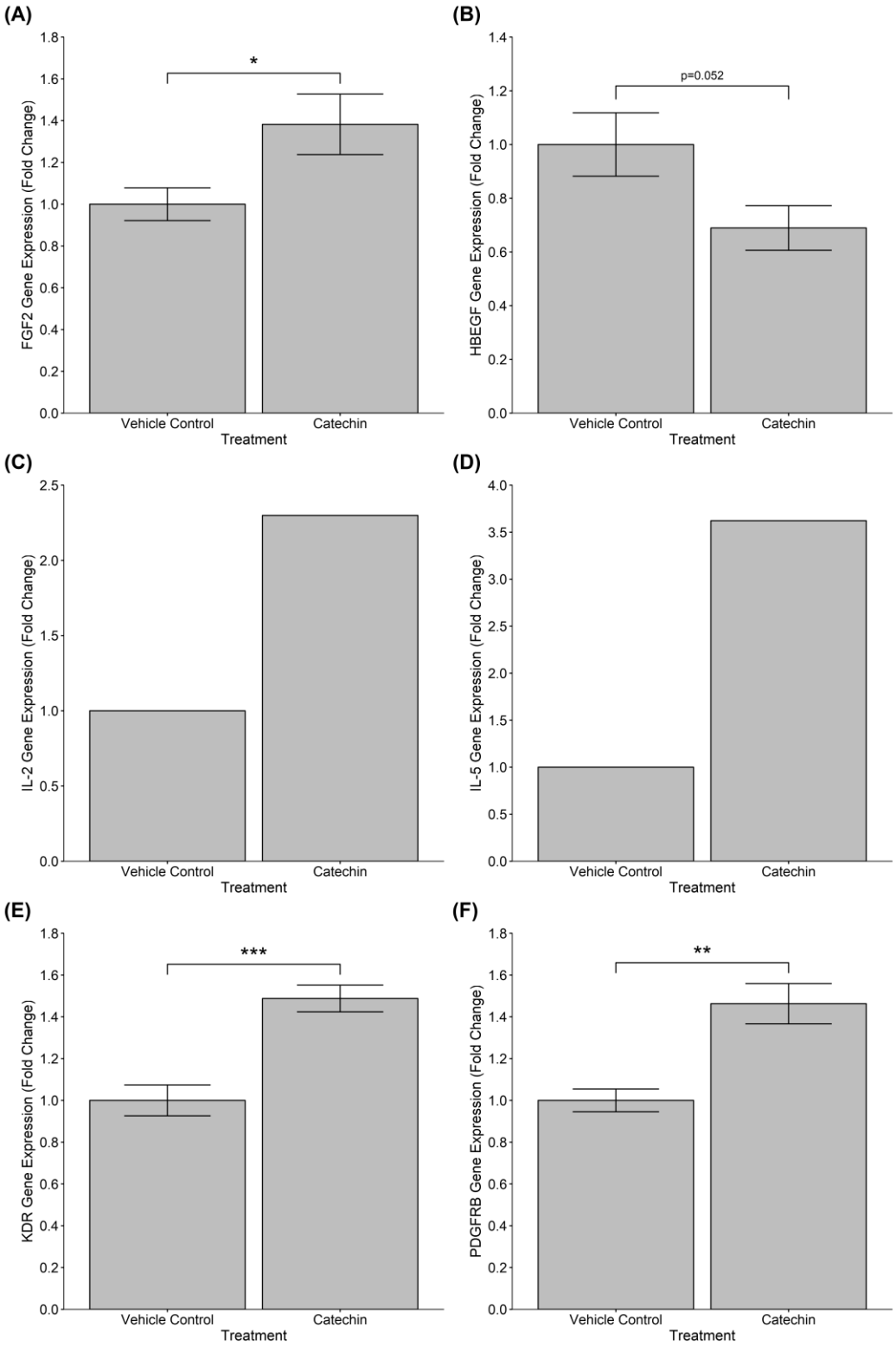

Fig. 7.14. Catechin treatment alters the expression of cell growth and proliferation regulators *in vivo*. Gene transcript levels *FGF2* (A), *HBEGF* (B), *IL-2* (C), *IL-5* (D), *KDR* (E) and *PDGFRB* (F) were assessed in the livers of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or 200 mg/kg of catechin hydrate. Gene transcript levels were calculated using the comparative Ct method and normalised to two housekeeping genes (*GUSB* and *HSP90AB1*) with values from the vehicle treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from one (C), three (D), seven (F) or eight (A, B and E) control mice and either one (D), two (C), seven (A and B) or eight (E and F) catechin treated mice. Mice with undetectable Ct readings were removed before statistical analysis. Statistical analysis was performed using a t-test (unequal variances) where * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

7.3.12 Catechin treatment affectes the expression of ECM genes in wild type mice fed a high fat diet

Fibrinogen (*FG*)*A*, *FGB*, *MMP1A* and tenascin-C (*TNC*) genes encode proteins which can influence ECM deposition and degradation as well as affecting intercellular signalling. The expression of both *FGA* and *FGB* was reduced in the livers of wild type mice receiving catechin by 42.0% and 44.0% respectively when compared to the vehicle control mice (p=0.056 and p=0.028; Figs. 7.15A and B). In contrast to the control mice, catechin induced the expression of *MMP1A* and *TNC* by 71.3% and 85.6% respectively (p=0.001 and p=0.025; Figs. 7.15C and D).

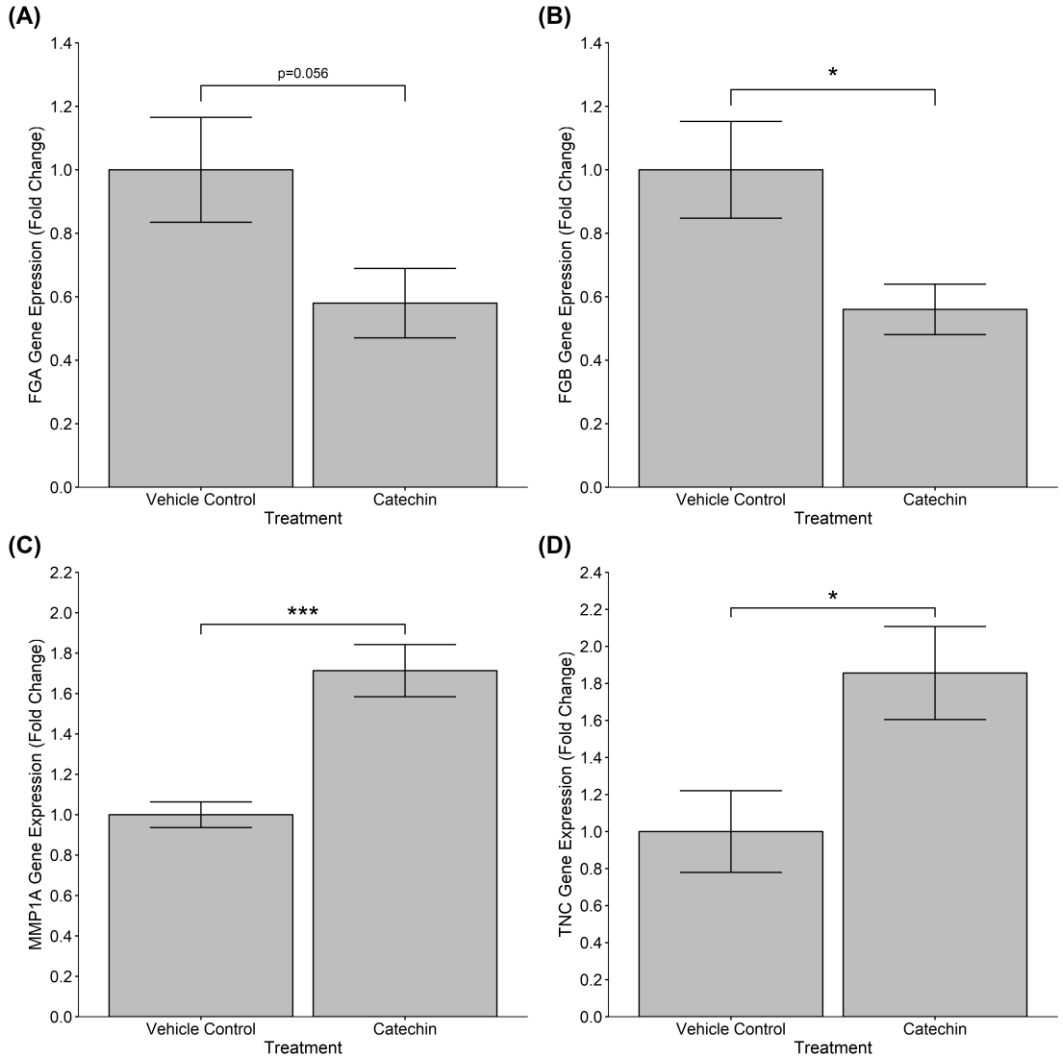

Fig. 7.15. Catechin treatment alters the expression of genes involved in regulating ECM synthesis *in vivo*. Gene transcript levels of *FGA* (A), *FGB* (B), *MMP1A* (C) and *TNC* (D) were assessed in the livers of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or 200 mg/kg of catechin hydrate. Gene transcript levels were calculated using the comparative Ct method and normalised to two housekeeping genes (*GUSB* and *HSP90AB1*) with values from the vehicle treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from six (D) or eight (A, B and C) control mice and either seven (A and B) or eight (C and D) catechin treated mice. Mice with undetectable Ct readings were removed before statistical analysis. Statistical analysis was performed using a t-test (unequal variances) where * $p \leq 0.05$ and *** $p \leq 0.001$.

7.3.13 Catechin increases the expression of genes involved in lipid transport and metabolism in wild type mice fed a high fat diet

The genes *APOE*, *LPL* and *LYPLA1* are all capable of influencing lipid transport and metabolism. An increase of 61.1% (p=0.066), 37.7% (p=0.011) and 16.0% (p=0.043) was observed in *APOE*, *LPL* and *LYPLA1* gene expression respectively in wild type mice which had received catechin treatment in comparison to the vehicle control mice (Fig. 7.16).

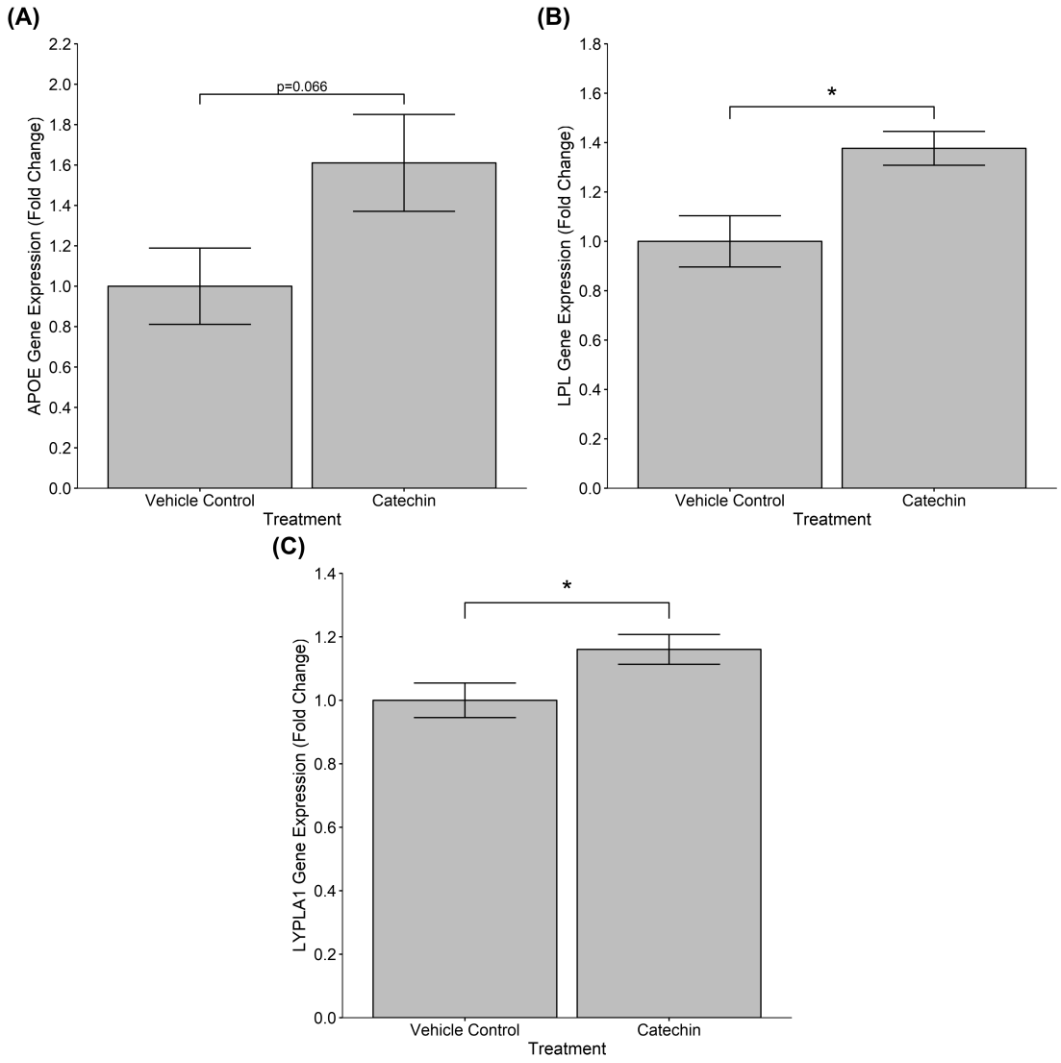

Fig. 7.16. Catechin treatment increases the expression of genes involved in the regulation of lipid transport and metabolism *in vivo*. Gene transcript levels of *APOE* (A), *LPL* (B) and *LYPLA1* (C) were assessed in the livers of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or 200 mg/kg of catechin hydrate. Gene transcript levels were calculated using the comparative Ct method and normalised to two housekeeping genes (*GUSB* and *HSP90AB1*) with values from the vehicle treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from eight control mice and either seven (B) or eight (A and C) catechin treated mice. Mice with undetectable Ct readings were removed before statistical analysis. Statistical analysis was performed using a t-test (unequal variances) where * $p \leq 0.05$.

7.3.14 Catechin increases the expression of the transcriptional regulator *NR1H3* in wild type mice fed a high fat diet

NR1H3 is an anti-inflammatory regulator of gene transcription, therefore increasing its expression with catechin would represent strong anti-inflammatory effects. There was a significant increase of 17.8% (p=0.033) in *NR1H3* gene expression within the liver of wild type mice treated with catechin when compared to the control mice (Fig. 7.17).

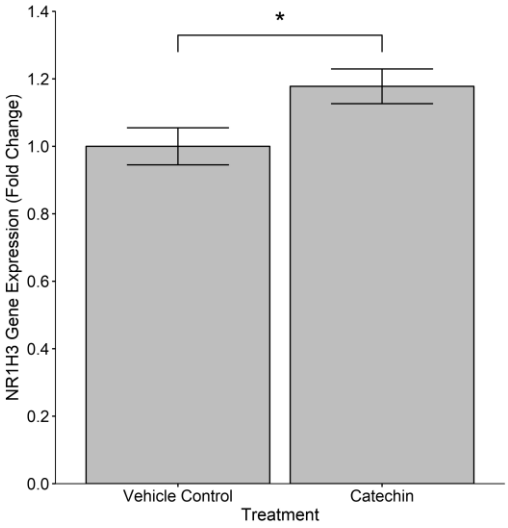

Fig. 7.17. Catechin treatment increases the expression of a gene involved in transcriptional regulation *in vivo*. Gene transcript levels of *NR1H3* were assessed in the livers of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or 200 mg/kg of catechin hydrate. Gene transcript levels were calculated using the comparative Ct method and normalised to two housekeeping genes (*GUSB* and *HSP90AB1*) with values from the vehicle treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from eight control and catechin treated mice. Statistical analysis was performed using a t-test (unequal variances) where * $p \leq 0.05$.

7.3.15 Catechin treatment increases the number of total bone marrow cells in wild type mice fed a high fat diet

The inflammatory state of an individual can often be indicated by the levels of white blood cells within their bone marrow. Wild type mice treated with catechin were found to have the proportion of white blood cells within their bone marrow increased non-significantly by 1.20 fold in contrast to the mice treated with the vehicle control (p=0.101; Fig. 7.18).

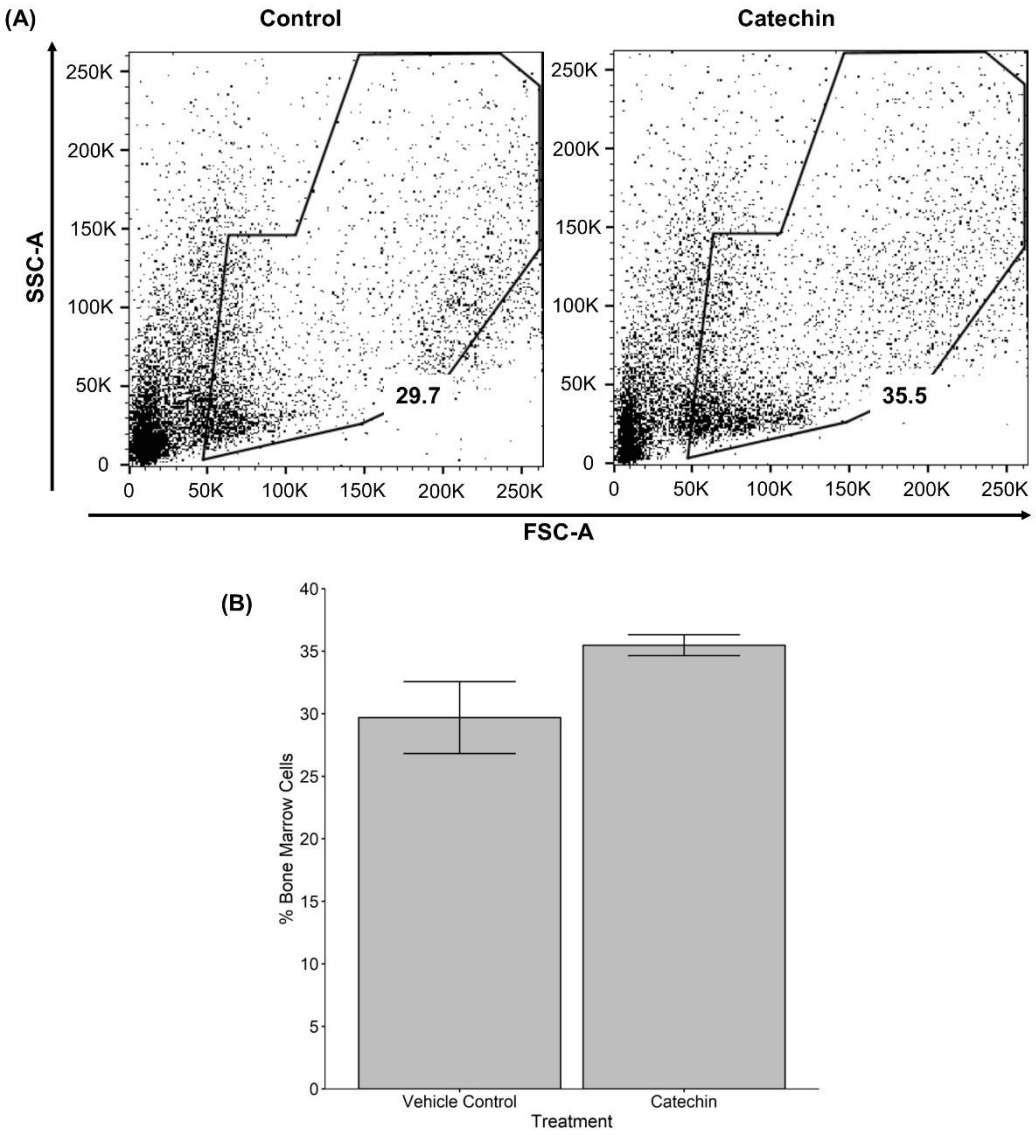

Fig. 7.18. Catechin increases the proportion of total bone marrow cells. Cell populations were assessed in the bone marrow of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or 200 mg/kg of catechin hydrate. Representative flow plots of the total cell population in the bone marrow (A). Numbers on the flow plots indicate the proportion of the cell population as a percentage of total bone marrow cell count. The forward scatter (FSC-A) is a measurement of cell size by determining the amount of light which passes around it. The side scatter (SSC-A) is a measurement of the amount of light which is reflected by particles within the cells and therefore can be used to determine the granularity of cells. Cumulative bar graphs showing the frequency of total bone marrow cells as a percentage of total cell count (B). The data are presented as the mean ± SEM from four vehicle control and four catechin treated mice. Statistical analysis was performed using a t-test (equal variances).

7.3.16 The number of SLAM and progenitor cells within the bone marrow of wild type mice fed a high fat diet following catechin treatment

The flow plots for the number of SLAM and progenitor cells present in the bone marrow of wild type mice fed either a vehicle control or catechin are displayed in Fig. 7.19 and the results are explored in greater detail in the subsequent sections.

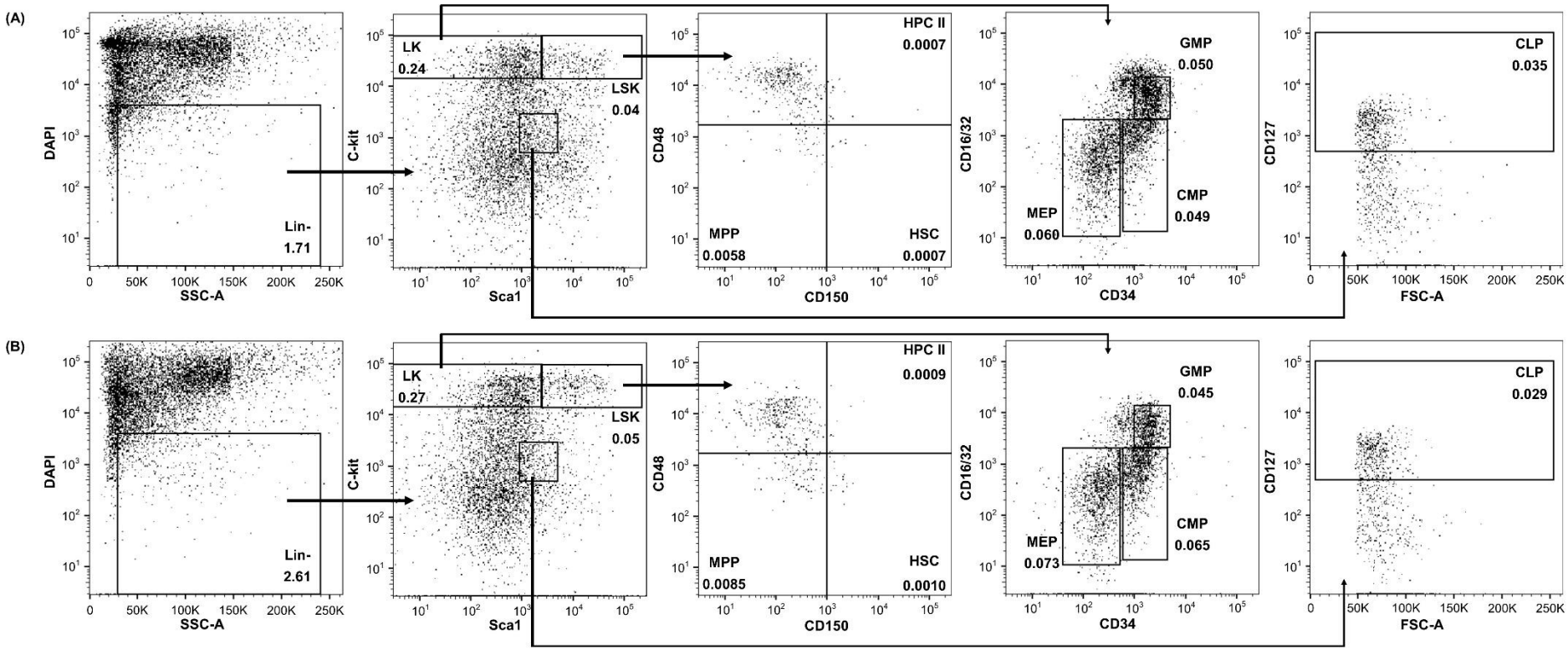

Fig. 7.19. The effect of catechin on the proportion of SLAM and progenitor cells present in the bone marrow. Cell populations were assessed in the bone marrow of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control (A) or 200 mg/kg of catechin hydrate (B). Representative flow plots of the SLAM and progenitor populations in the bone marrow are presented. Numbers on the flow plots indicate the proportion of the cell population as a percentage of total bone marrow cell count. Cumulative bar graphs showing the cell populations as a percentage of total bone marrow cell count are shown in Figs. 7.20 - 7.22. The arrows indicate how each haematopoietic stem/progenitor cell population can be identified within the preceding gating strategy. The axis represent the various stains used to separate the cell populations (see Table 2.8 for further details). The forward scatter (FSC-A) is a measurement of cell size by determining the amount of light which passes around it. The side scatter (SSC-A) is a measurement of the amount of light which is reflected by particles within the cells and therefore can be used to determine the granularity of cells. HSC, haematopoietic stem cell; MPP, multipotent progenitors; HPC, hematopoietic progenitor cell; CMP, common myeloid progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-macrophage progenitor; MDSC, myeloid-derived suppressor cells; CLP, common lymphoid progenitor; LIN, lineage.

7.3.17 Catechin treatment increases the number of Live Lin- and MPP cells within the bone marrow of wild type mice fed a high fat diet

The live lineage negative cell population represent the total proportion of non-terminally differentiated blood cells found within the bone marrow, whereas the LSK cell population represents the total number of multipotent blood cells. A significant increase of 1.53 fold (p=0.005) was observed in the live lineage negative (Live Lin-) cell population in mice treated with catechin when compared to the vehicle control mice (Fig. 7.20A). The change in the proportion of LSK cells within the bone marrow of catechin treated mice was found to be not significant when compared to the vehicle control treated mice (p=0.310; Fig. 7.20B). All myeloid and lymphocyte cells within the bone marrow are derived from the HSC, MPP and HPC II cell populations. A non-significant trend of increase was seen in the MPP cell population of 1.45 (p=0.086) fold. The proportion of HSC and HPC II cell populations within the bone marrow were unaffected by catechin treatment 1.38 (p=0.329) and 1.32 (p=0.287; Fig. 7.20).

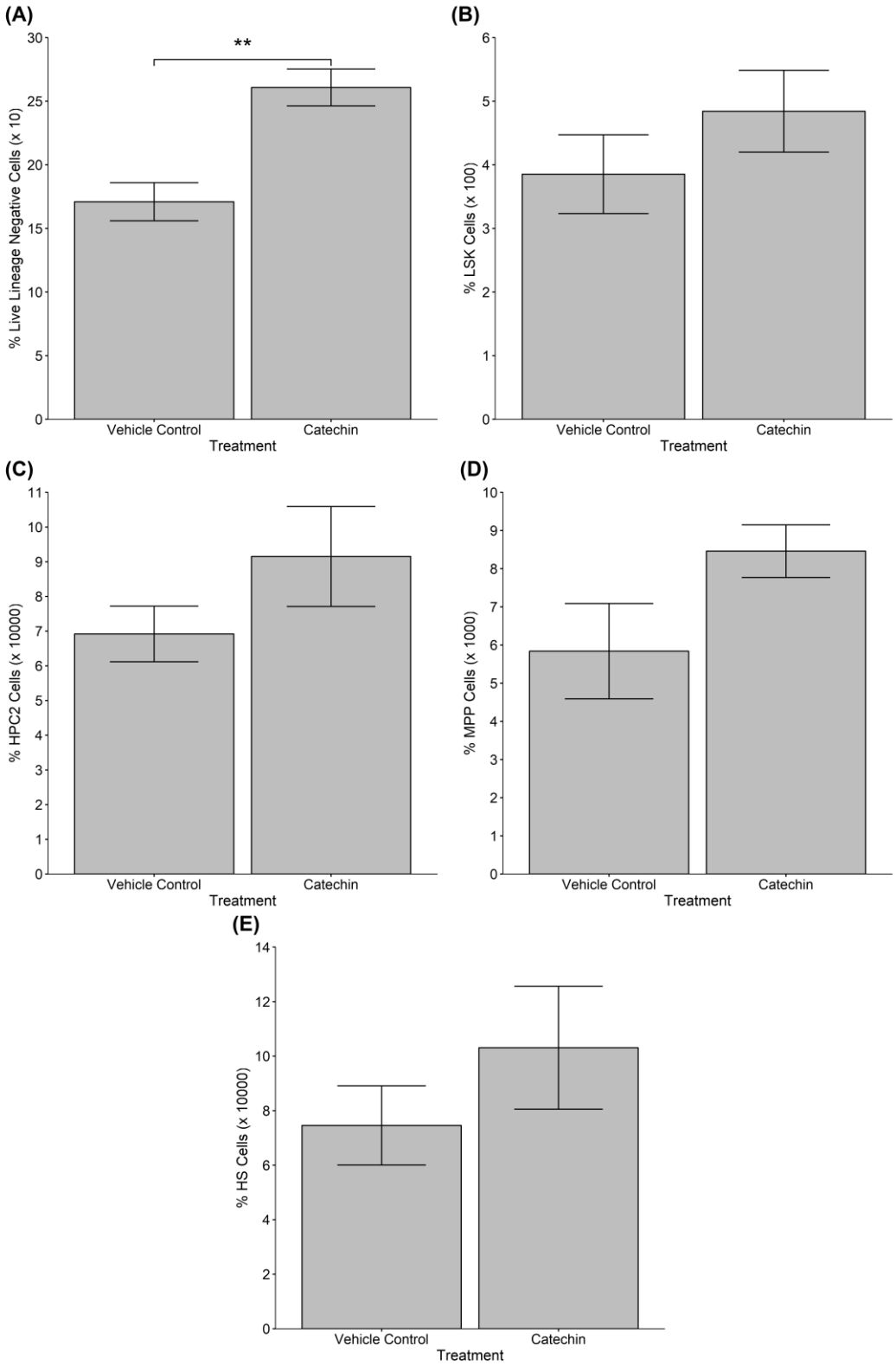

Fig. 7.20. Catechin increases the proportion of Live Lin-, LK, LSK, HPC, MPP and HSC cells present in the bone marrow. Cell populations were assessed in the bone marrow of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or 200 mg/kg of catechin hydrate. Cumulative bar graphs showing the frequency of live lineage negative (Live Lin-; A), LSK (B), HPC II (C), MPP (D) and HSC (E) populations in the bone marrow as a percentage of total bone marrow cell count. Where appropriate, the data has been multiplied by powers of 10 for ease of presenting the result clearly. The data are presented as the mean ± SEM from four control mice and four catechin treated mice. Statistical analysis was performed using a t-test (equal variances) on either log-transformed (C), squared-transformed (D) or untransformed (A, B and E) data where ** $p \leq 0.01$.

7.3.18 Catechin treatment increases the number of CMP cells within the bone marrow of wild type mice fed a high fat diet

All myeloid cells are derived from the LK cell population. Wild type mice treated with catechin showed no change in the proportion of LK cells within their bone marrow (Fig. 7.21A). The CMP, MEP and GMP cell populations are found within the LK class of cells. Catechin treatment resulted in a non-significant 1.33 fold increase in the proportion of CMP cells found within the bone marrow (p=0.112; Figs. 7.21B). No changes were observed in the proportion of MEP and GMP cells within the bone marrow of mice treated with catechin when compared to the vehicle control mice (Fig. 7.21C and D).

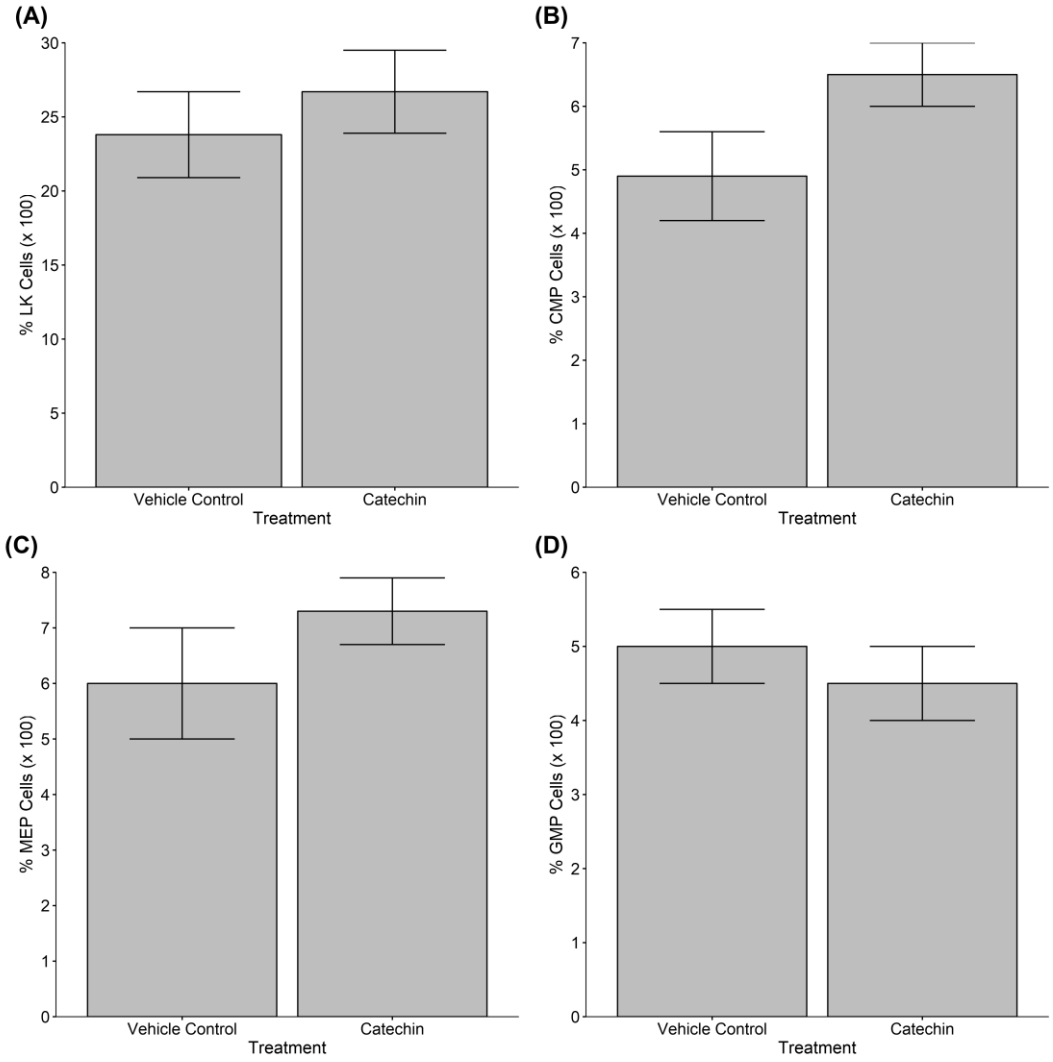

Fig. 7.21. Catechin increases the proportion of CMP and MEP cells present in the bone marrow. Cell populations were assessed in the bone marrow of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or 200 mg/kg of catechin hydrate. Cumulative bar graph showing the frequency of LK (A), CMP (B), MEP (C) and GMP (D) populations in the bone marrow as a percentage of total bone marrow cell count. Where appropriate, the data has been multiplied by powers of 10 for ease of presenting the result clearly. The data are presented as the mean ± SEM from four vehicle control and four catechin treated mice. Statistical analysis was performed using a t-test (equal variances).

7.3.19 Catechin treatment does not affect the number of CLP cells within the bone marrow of wild type mice fed a high fat diet

Both T- and B-cells are derived from the CLP cell population. No change was observed in the proportion of CLP cells found within the bone marrow of catechin treated wild type mice when compared to the vehicle control treated mice (Fig. 7.22).

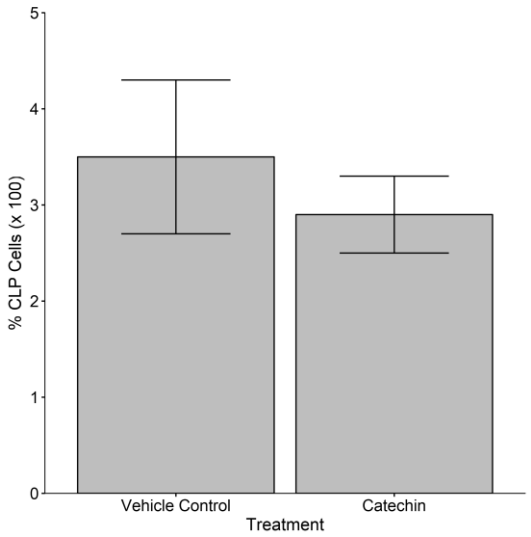

Fig. 7.22. Catechin does not affect the proportion of CLP cells present in the bone marrow. Cell populations were assessed in the bone marrow of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or 200 mg/kg of catechin hydrate. Cumulative bar graphs showing the frequency of the CLP populations in the bone marrow as a percentage of total bone marrow cell count. The data has been multiplied by 100 for ease of presenting the result clearly. The data are presented as the mean ± SEM from four control and four catechin treated mice. Statistical analysis was performed using a t-test (equal variances) on square root-transformed data.

7.3.20 Catechin treatment reduces the number of MDSCs within the bone marrow of wild type mice fed a high fat diet

Wild type mice treated with catechin showed a non-significant trend of decrease in the proportion of MDSCs within the bone marrow by 32% (p=0.200) in contrast to the mice treated with the vehicle control (Fig. 7.23C). In contrast, catechin treatment was found to have no effect on the proportion of granulocytes or macrophages within the bone marrow when compared to the vehicle control treated mice (Fig. 7.23B and D).

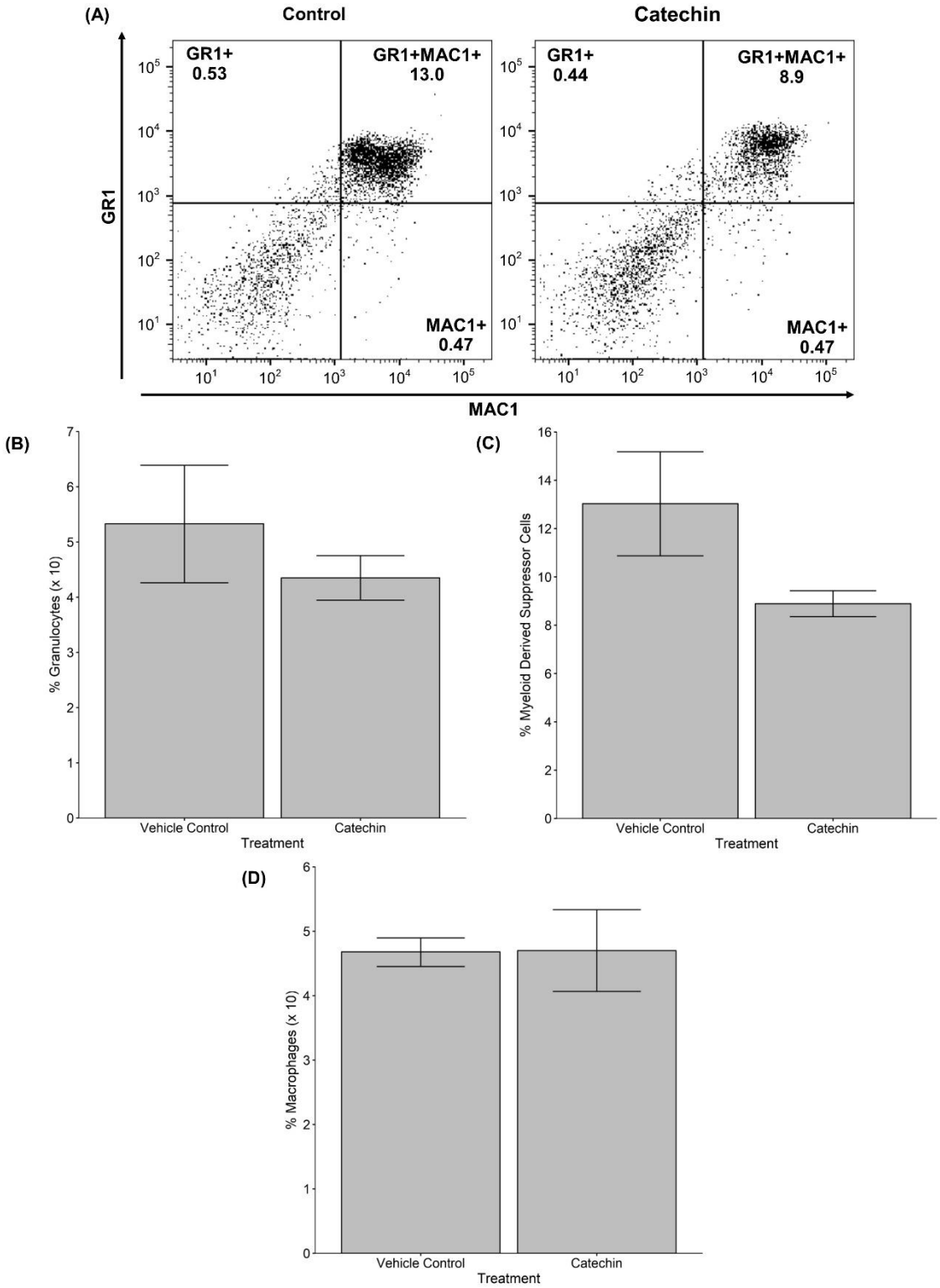

Fig. 7.23. Catechin reduces the proportion of granulocytes and MDSCs present in the bone marrow. Cell populations were assessed in the bone marrow of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or 200 mg/kg of catechin hydrate. Representative flow plots of the granulocyte, macrophage and MDSC populations in the bone marrow (A). Numbers on the flow plots indicate the proportion of the cell population as a percentage of total bone marrow cell count. Cumulative bar graphs showing the frequency of granulocytes (B), MDSCs (C) and macrophage (D) populations in the bone marrow as a percentage of total bone marrow cell count. Where appropriate, the data has been multiplied by powers of 10 for ease of presenting the result clearly. The data are presented as the mean ± SEM from four vehicle control and four catechin treated mice. Statistical analysis was performed using either a t-test (equal variances; B and D) or a Mann-Whitney test (C).

7.3.21 Catechin treatment affects the number of B- and T-cells within the bone marrow of wild type mice fed a high fat diet

A non-significant trend of increase of 61% (p=0.089) was observed in the proportion of B-cells found within the bone marrow of wild type mice treated with catechin when compared to the vehicle control treated mice (Fig. 7.24C). On the other hand, catechin treatment did not significantly alter the proportion of T-cells within the bone marrow of wild type mice (p=0.336; Fig. 7.24B).

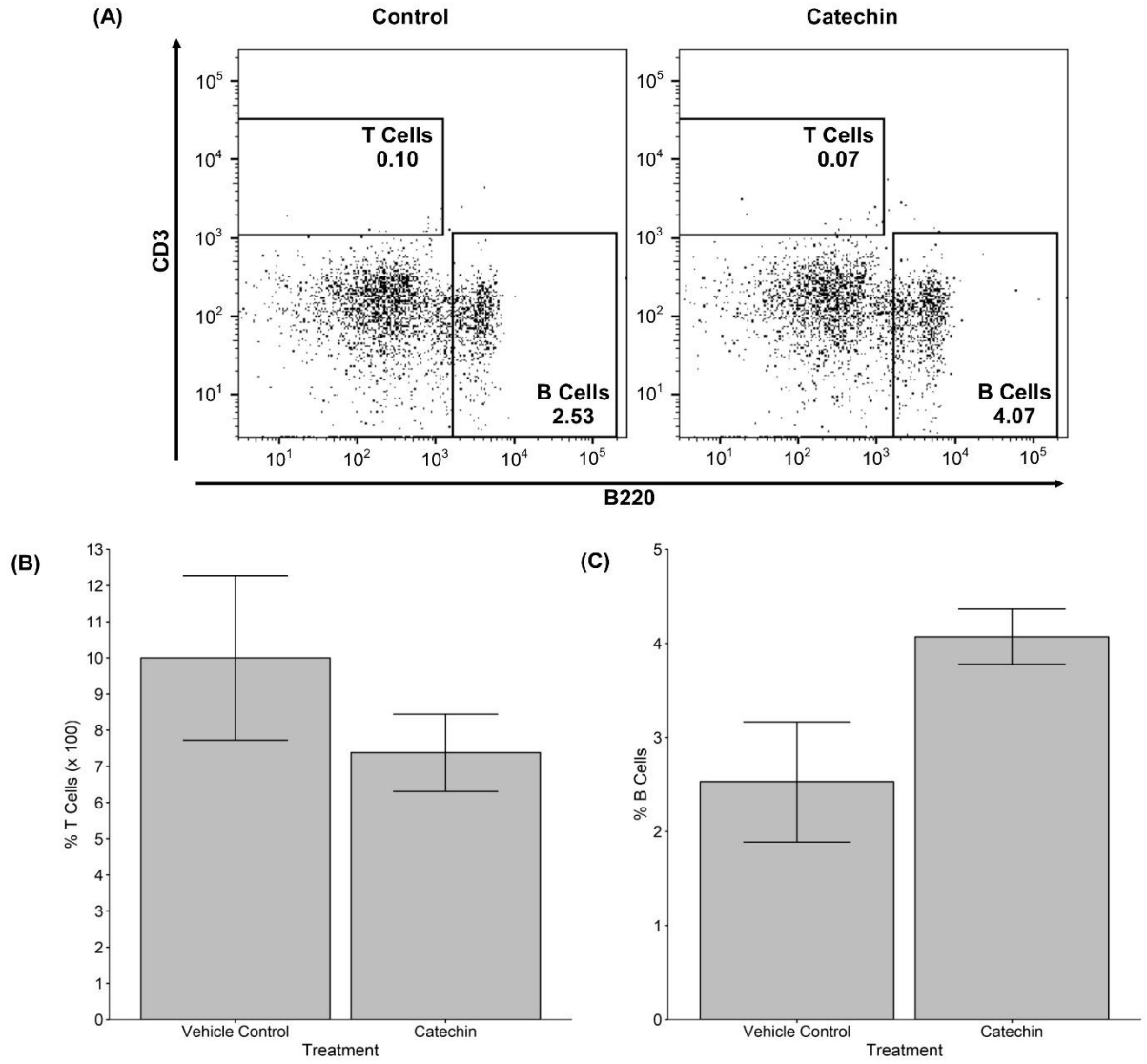

Fig. 7.24. Catechin affects the proportion of B- and T-cells present in the bone marrow. Cell populations were assessed in the bone marrow of wild type mice after 21 days of a high fat diet and treatment with either a vehicle control or 200 mg/kg of catechin hydrate. Representative flow plots of the B and T-cell populations in the bone marrow (A). Numbers on the flow plots indicate the proportion of the cell population as a percentage of total bone marrow cell count. Cumulative bar graphs showing the frequency of T-cells (B) and B-cells (C) in the bone marrow as a percentage of total bone marrow cell count. Where appropriate, the data has been multiplied by powers of 10 for ease of presenting the result clearly. The data are presented as the mean ± SEM from four vehicle control and four catechin treated mice. Statistical analysis was performed using either a t-test (equal variances; B) or a t-test (unequal variances; C).

7.4 Discussion

7.4.1 The effect of catechin on physical parameters in wild type mice

The possibility of using catechin as an anti-atherogenic nutraceutical and decreasing an individual's chance of suffering a CVD-related event has been explored in great detail in this *in vivo* study. The levels of HDL cholesterol and LDL/HDL cholesterol ratio were found to be improved following catechin hydrate treatment in wild type mice fed a high fat diet. Furthermore the level of ROS generation and circulating level of pro-inflammatory cytokines were reduced. These changes could potentially be explained by catechin treatment resulting in an anti-atherogenic gene expression profile within the livers of these mice. Mice which received catechin also had anti-atherogenic changes in the haematopoietic stem and progenitor cells within their bone marrow, including reductions in the proportion of granulocytes, MDSCs and T-cells. This *in vivo* study provides additional evidence which confirms the anti-atherogenic properties of catechin which were observed during our *in vitro* assays (Chapters 5 and 6).

After 21 days of a high fat diet, the first notable effect of catechin treatment was its action on rate of weight gain. In comparison to the vehicle control treated mice, those which had received catechin gained weight at a faster rate and after 21 days they were found to be significantly heavier (Fig. 7.4). Previous studies have shown a mixed effect of flavanol dietary supplementation on the rate of weight gain in murine studies. Short term feeding studies have shown green tea extracts to have no effect on weight gain (Murase *et al.* 2002; Sugiura *et al.* 2012). Long term studies involving dietary supplementation with green tea extracts has shown them to be capable of attenuating weight gain in mice (Murase *et al.* 2002). The increase in weight gain observed in this study was driven by an increased accumulation of fat within the subcutaneous, gonadal and renal deposits (Fig. 7.5). This result contradicts current literature which has found green tea extract to reduce white adipose tissue accumulation (Tokimitsu 2004; Osada *et al.* 2006; Nagao *et al.* 2007). However these studies used a mixture of green tea catechins and therefore the reduction in white fat accumulation may be the result of another catechin present in the mixture rather than (+)-catechin. During obesity white adipose tissue expands and becomes dysfunctional, leading to inflammation and increased lipid levels within the blood (Hotamisligil 2006; Nordestgaard 2016). However white adipose tissue expansion is not always associated with increased atherosclerosis disease progression (van Dam *et al.* 2017). Indeed during this study, catechin was found to reduce the levels of circulating pro-inflammatory cytokines, including CXCL1, IL-5, IL-6 and IL-10, which are normally increased during obesity (Figs. 7.9 and 7.10; Nunemaker *et al.* 2014; Schmidt *et al.* 2015; Manna and Jain 2015). Additionally LPL is required for TG turnover within the fatty deposits and catechin treatment also significantly increased *LPL* expression in the liver (Fig. 7.16; Cinti 2009; van Dam *et al.* 2017). Increased *LPL* expression within the adipose and muscle tissue is considered to be anti-atherogenic, however LPL secreted by macrophages and SMCs is considered to be pro-atherogenic by promoting lipid accumulation and inflammation (Li *et al.*

2014b; Mead *et al.* 2002). Therefore the effect of catechin on *LPL* expression needs to be assessed in greater detail. The results of this study indicate that despite catechin leading to an increase in white fat accumulation, it is capable of maintaining its physiological function via increased *LPL* expression and therefore potentially also maintaining TG turnover. The lack of changes in the weight of brown fat and PVAT deposits means catechin treatment had no effect on brown fat activation (Figs. 7.5E and F).

7.4.2 The effect of catechin on blood parameters in wild type mice

The circulating levels of cholesterol, TG, ROS and cytokines within the plasma can all be used as risk markers of atherosclerosis disease development. An increase in serum HDL cholesterol is a sign of increased RCT and therefore is thought of as anti-atherogenic (McLaren *et al.* 2011a). Catechin treatment in wild type mice receiving a high fat diet for 21 days was found to non-significantly increase HDL levels (Fig. 7.6B). A possible mechanism by which catechin can boost HDL cholesterol levels may be achieved through the increased expression of *ApoE* (Fig. 7.16A) and *NR1H3* (Fig. 7.17). Previous studies have demonstrated that raised expression of *ApoE* and *NR1H3* result in improved circulating HDL levels (Alvaro *et al.* 2010; Ramasamy 2016). Catechin treatment in rats receiving a standard chow diet has been shown to increase HDL cholesterol levels and simultaneously attenuate the levels of circulating LDL cholesterol (Chisaka *et al.* 1988; Babu *et al.* 2006; Babu *et al.* 2008). However in this study, catechin hydrate treatment failed to significantly alter serum LDL levels (Fig. 7.6C). Despite this lack of changes in LDL cholesterol levels, catechin treatment was found to show a trend of improved LDL/HDL cholesterol ratio (Fig. 7.6F). Due to the importance of the LDL/HDL cholesterol ratio as clinical risk indicator of CVD, changes in this marker are more important than assessing the levels of LDL or HDL in isolation (Millán *et al.* 2009; Kunutsor *et al.* 2017). Indeed previous human studies have demonstrated that catechin treatment is capable of enhancing the LDL to HDL cholesterol ratio (Babu *et al.* 2008). The results of this study show that catechin treatment is capable of altering a lipid profile to be more anti-atherogenic while receiving a high fat diet *in vivo*.

The circulating levels of TGs within the plasma can be an indicator of the level of VLDL, chylomicron remnants and TG-rich lipoproteins, all of which are capable of exacerbating atherosclerosis disease progression (Talayero and Sacks 2011). This study found catechin was unable to affect circulating TG levels (Fig. 7.7A). This lack of reduction in TG levels following catechin treatment is similar to previous studies, using rats receiving a high fat diet or obese children, which were unable to observe decreased circulating serum TGs (Matsuyama *et al.* 2008; Friedrich *et al.* 2012; Ahmad *et al.* 2015). As previously discussed, the TG/HDL ratio may actually provide a clearer indication on CVD risk when compared to using TG levels individually (Hulley *et al.* 1980; Miller *et al.* 2011; da Luz *et al.* 2008). However catechin treatment also failed to improve the TG/HDL cholesterol ratio (Fig. 7.7C). Although

catechin treatment resulted in improved HDL and LDL/HDL cholesterol levels, it failed to attenuate TG levels following a high fat diet. Therefore additional long term studies are required to fully establish the potential anti-atherogenic effect of catechin treatment on the lipidomic profile.

Flavanols are a well-known class of anti-oxidants, as demonstrated by previous studies and our *in vitro* results which found catechin treatment to attenuate ROS generation in human THP-1 cells (Fig. 5.10; Moss and Ramji 2016; Bahramsoltani *et al.* 2017; Tak *et al.* 2016; Yu *et al.* 2017; Oyama *et al.* 2017). Furthermore due to the effect of aberrant ROS production leading to lipid peroxidation and thrombus formation, the level of MDA within the plasma of wild type mice was also measured (Kinnunen *et al.* 2012; Ayala *et al.* 2014; Chen *et al.* 2017c). Catechin treatment did not reduce ROS formation in mice fed a high fat diet for 21 days (Fig. 7.8A). This result contradicts with the current literature which shows that flavanols are potent anti-oxidants (Zielinski *et al.* 2014; Higdon and Frei 2003; Kumar and Pandey 2013). Previous studies have also shown flavanols to attenuate lipid peroxidation which was not observed in this study when mice were treated with catechin hydrate (Fig. 7.8B; Tak *et al.* 2016). Thus reduction in ROS generation may be the mechanism by which reduced circulating oxLDL levels have been reported in previous human studies (Babu *et al.* 2008; Tinahones *et al.* 2008). The lack of reduction in ROS production following catechin treatment needs to be assessed further.

Catechin treatment in wild type mice fed a high fat diet for 21 days showed a non-significant decrease in the plasma levels of pro-inflammatory cytokines CXCL1 and TNF-α (Fig. 7.9). As previously discussed, CXCL1 is a potent chemoattractant and plays a major role during monocyte recruitment and migration during atherosclerosis disease development (Wan and Murphy 2013). This decrease in the circulating levels of CXCL1 may explain the reduced monocyte migration observed following catechin treatment *in vitro* (Fig. 5.6). Due to its role in systemic inflammation, TNF-α is considered to be a key pro-inflammatory cytokine during atherosclerotic plaque formation (Ramji and Davies 2015; Moss and Ramji 2016a). Furthermore *TNF-α* and *ApoE* deficient mice have been found to develop fewer foam cells and as a result of reduced atherosclerotic burden (Ramji and Davies 2015). The reduction in TNF-α levels due to catechin treatment may indicate that it is also capable of attenuating foam cell formation *in* vivo. However this requires further assessment in future *in vivo* studies as our *in vitro* studies failed to show any alteration in cholesterol uptake or efflux following catechin treatment (Fig. 6.20). The results of this study coincide with the current literature which has previously shown flavanol treatment in rats and mice receiving a standard chow diet to attenuate the circulating levels of CXCL1 and TNF-α (Takano *et al.* 2004b; Guruvayoorappan and Kuttan 2008). Despite these observed anti-inflammatory effects of catechin, it fails to lower the circulating levels of the major pro-inflammatory cytokine IFN-γ.

The serum levels of several IL cytokines including, IL-1β, IL-2, IL-4, IL-5, IL-6 and IL-10, were all non-significantly reduced, however IL-1β and IL-10 showed strong trends of decrease following catechin treatment in wild type mice (Fig. 7.10). IL-1β is a potent pro-inflammatory cytokine which is capable of activating the innate immune response and in turn contributes to the development of atherosclerosis (Ramji and Davies 2015; Moss and Ramji 2016a). Indeed *ApoE* deficient mice which also lack IL-1β or its receptor develop smaller atherosclerotic plaques (Kamari *et al.* 2011; Shemesh *et al.* 2012). Additionally, as previously discussed the circulating levels of IL-1β can be used as a marker of NLRP3 inflammasome activation. The NLRP3 inflammasome is associated with disease progression and plaque stability (Zheng *et al.* 2013; Zheng *et al.* 2014; Guo *et al.* 2015; Hoseini *et al.* 2017). Due to the pro-inflammatory nature of IL-1β, it is also capable of influencing macrophage polarisation into their pro-atherogenic M1 phenotype (Moss and Ramji 2016a). The reduction in serum levels of IL-1β following catechin treatment observed in this *in vivo* study has been previously reported in mice receiving a standard chow diet (Tsai *et al.* 2011; Jhang *et al.* 2015). This result would indicate that catechin is potentially capable of attenuating atherosclerotic plaque formation, improve plaque stability and reduce pro-inflammatory differentiation of macrophages by possibly reducing inflammasome activation. However catechin treatment *in vitro* failed to attenuate inflammasome activation (measured indirectly via IL-1β release) or influence the expression of M1 macrophage markers (Figs. 5.5B and 6.23) and therefore requires further investigation in future *in vivo* studies. IL-2 is a cytokine shown to enhance the development of atherosclerosis (Ramji and Davies 2015). An increase in IL-2 production due to catechin dietary supplementation has been previously reported in mice receiving a standard chow diet (Guruvayoorappan and Kuttan 2008), however in this study catechin treatment was found to reduce serum IL-2 levels in wild type mice receiving a high fat diet. The role of IL-4 in atherosclerosis is still debated in the literature, however deficiency of the cytokine in *ApoE* atherosclerotic mouse models has been shown to result in smaller plaque formation (Lee *et al.* 2010b). Therefore reductions in this cytokine following catechin treatment highlights another mechanism by which catechin may result in smaller atherosclerotic plaques (Morrison *et al.* 2014). Indeed it has been previously observed that serum IL-4 levels are reduced following flavanol treatment in mice fed a standard chow diet (Liu *et al.* 2014a). The reductions in pro-inflammatory cytokines found in this *in vivo* study indicate that dietary supplementation with catechin may retard atherosclerosis disease progression and attenuate lesion formation. This requires further investigation in future studies.

Despite the reductions in pro-inflammatory serum cytokine levels as a result of catechin dietary supplementation in wild type mice receiving a high fat diet, catechin was also found to attenuate the levels of anti-inflammatory ILs. Both IL-5 and IL-10 are considered to be anti-inflammatory cytokines as their genetic depletion in atherosclerotic mouse models results in enhanced atherosclerosis disease development (Ramji and Davies 2015). The levels of both

IL-5 and IL-10 were reduced following catechin treatment (Fig. 7.10D and F), which was not observed in previous studies (Cunha *et al.* 2013). Due to the anti-inflammatory effects associated with these two cytokines, the results of this study would suggest that catechin has the capacity to enhance atherosclerosis disease development. The atherosclerotic nature of IL-6 remains unclear as it has previously been shown to exert both pro- and anti-inflammatory effects (Ramji and Davies 2015). The treatment of wild type mice fed a high fat diet with catechin was found to decrease serum IL-6 levels (Fig. 7.10E). This result was to be expected as it has previously been demonstrated in mice fed a standard chow diet (Guruvayoorappan and Kuttan 2008; Liu *et al.* 2014a). Catechin appears to induce a systemic inhibition of cytokine production as opposed to inhibiting pro-inflammatory cytokine release. However this needs to be assessed in greater detail in atherosclerotic mouse models as changes in the systemic cytokine profile does not reflect the changes occurring at a local vascular or atherosclerotic plaque level. The results of the *in vivo* catechin study are similar to those found within CardioWise *in vivo* study (Chapter 4), possibly indicating that the changes in cytokine levels observed due to CardioWise treatment are due to catechin being present in the mixture.

During obesity, the white adipose tissue deposits can expand and become dysfunctional, resulting in an alteration in the level of cytokines produced and released (Kershaw and Flier 2004; Coelho *et al.* 2013). Previous studies have reported that obesity is associated with increased serum levels of CXCL1, IL-4, IL-5, IL-6 and IL-10 (Nunemaker *et al.* 2014; Schmidt *et al.* 2015; Manna and Jain 2015). However the level of IL-2 is normally decreased during obesity, a result that was also seen following catechin treatment. Overall the decrease in the concentration of these cytokines found within the plasma of wild type mice would suggest that catechin treatment maybe capable of maintaining the normal physiological function of white adipose tissue despite contributing to its expansion.

7.4.3 The effect of catechin on gene expression in the liver of wild type mice

Changes in gene expression within the liver were assessed to ascertain whether they related to changes in both physical and blood parameters previously discussed in this chapter. A qPCR array containing 84 atherosclerosis related genes was used to determine gene expression levels. A total of 55 genes were found to have their expression altered by at least 10% (Fig. 7.11) and 12 genes were significantly different. Those genes were *ENG*, *FGB*, *FGF2*, *ITGA2*, *ITGAX*, *KDR*, *LPL*, *LYPLA1*, *MMP1A*, *NR1H3*, *PDGFRB* and *TNC*. The *ENG* gene encodes the protein endoglin, also known as CD105, which is capable of binding to the anti-inflammatory cytokine TGF-β (Rathouska *et al.* 2015; Moss and Ramji 2016a). ENG is predominately expressed in proliferating endothelial and VSMCs within the atherosclerotic plaque (Conley *et al.* 2000; Prashar *et al.* 2017). This has led to both increased *ENG* and *TGF-β* expression levels being associated with improved atherosclerotic plaque stability due to increased collagen and VSMC content. During this study, it was found that catechin treatment

significantly increased *ENG* expression (Fig. 7.12), suggesting that catechin treatment could potentially improve plaque stability. The major function of ENG appears to be its role in angiogenesis and vasodilation (Núñez-Gómez *et al.* 2017). The expression of *ENG* is increased during angiogenesis, especially at the sites of new blood vessel formation (Núñez-Gómez *et al.* 2017). More importantly ENG has been implicated in vasodilation. Studies have demonstrated that reduced endoglin protein levels is associated with decreased NO production due to a reduction in the expression of endothelium NOS (eNOS; Jerkic *et al.* 2004; Jerkic *et al.* 2006; Park *et al.* 2013). The co-localisation of endoglin and eNOS proteins on the cell surface improves their stability and allows for the proper function of eNOS proteins to occur (Núñez-Gómez *et al.* 2017). Reduced endoglin protein levels leads to eNOS uncoupling, which in turn causes an increase in ROS generation and reduced NO production (Núñez-Gómez *et al.* 2017). As previously discussed, catechin treatment in wild type mice resulted in a reduction in ROS generation (Fig. 7.8). The increase in *ENG* expression may represent the mechanism by which catechin and other green tea extracts are capable of attenuating ROS production, increasing NO levels and improving vasodilation and vascular function in previously reported studies (Fisher *et al.* 2003; Babu *et al.* 2008; Tinahones *et al.* 2008; Rassaf *et al.* 2016; Heiss *et al.* 2015; Sansone *et al.* 2015). This will require further assessment in future *in vivo* studies using green tea extracts.

Catechin treatment was found to have mixed effects on the expression of cell adhesion molecules (Fig. 7.13). ITGA2, also known as CD49b, is a subunit of the integrin α2β1 (Habart *et al.* 2013; Finney *et al.* 2017; Yurdagul *et al.* 2016). It is expressed in a variety of cells including endothelial and VSMCs and integrin α2β1 signalling is linked with the inactive phenotypes of both cells and allows them to interact with collagens and laminins present in the ECM (Yurdagul *et al.* 2016; Finney *et al.* 2017). Catechin treatment may result in improved plaque stability as it was found to attenuate *ITGA2* expression (Fig. 7.13A), meaning reduced integrin α2β1 signalling may alter the VSMC phenotype from a quiescent one into an active migratory one and result in a thicker fibrous cap and restored vessel function. The effect of catechin on plaque stability needs to be assessed in future studies. Integrin α2β1 has also been found to be involved in platelet activation and *ITGA2* deficient mice were found to have a reduced platelet response to collagen without any detrimental haemostatic effects (Chen *et al.* 2002; Holtkötter *et al.* 2002; Habart *et al.* 2013). Previous studies have observed an inverse relationship between catechin treatment and platelet activation (Mangels and Mohler 2017). The reduction in *ITGA2* expression following catechin treatment in this study provides a possible mechanism by which catechin and other flavanols exert their anti-platelet activation effects. ITGAX is a member of the β2 integrin family and forms a heterodimeric receptor with CD18 (Yashiro *et al.* 2017). In mice *ITGAX* is predominately expressed by dendritic cells (DCs) and therefore can be used as a marker to identify this cell population (Sándor *et al.* 2016; Yashiro *et al.* 2017).

Atherosclerosis disease progression is associated with an increase in the number of DCs present at atherosclerotic prone sites and within lesions (Libby *et al.* 2008; Zernecke 2015). DCs are considered pro-atherogenic due to their ability to form foam cells as well as influencing the microenvironment within the shoulder regions of plaques to make them more vulnerable to rupturing (Zernecke 2015). Catechin treatment was found to increase the expression of *ITGAX* (Fig. 7.13B) which may be a marker of increased number of DCs and therefore represent a pro-inflammatory effect. The expression of *THBS4* was found to be increased following dietary supplementation of catechin in wild type mice (Fig. 7.13C). THBS4 is a glycoprotein secreted from ECs into the ECM following TGF-β stimulation (Muppala *et al.* 2017). Once in the ECM, THBS4 is thought to be capable of influencing ECM remodelling and inducing the formation of new blood vessels and restoring EC function (Muppala *et al.* 2017). The protective role of THBS4 has been demonstrated by an increase in the amount of scarring which occurred in the hearts of pressure overloaded *THBS4* deficient mice (Frolova *et al.* 2012; Rienks *et al.* 2014). Overall these results suggest that the altered gene expression profile of cell adhesion molecules due to catechin treatment would result in improved endothelial function. However the effect of induced expression of *ITGAX* needs to be assessed in greater detail in future studies.

The largest class of genes to have their expression altered by catechin treatment in wild type mice were those that are involved in cell proliferation. Following catechin treatment, the expression of *FGF2*, *IL-2*, *IL-5*, *KDR* (also known as VEGFR2) and *PDGFRB2* were all increased (Fig. 7.14). This result was unexpected as catechin treatment *in vitro* had been found to attenuate cell proliferation and decrease the expression of *VEGF* which is a gene capable of inducing cell proliferation (Fig. 6.13 and 6.19). However these results may also indicate that catechin treatment has different effects on hepatocytes and macrophages, which needs to be investigated in greater detail in future studies. Furthermore the circulating levels of IL-2 and IL-5 were found to be attenuated following catechin dietary supplementation (Fig. 7.10). Interestingly all the cell proliferation related genes which were induced by catechin *in vivo* are also capable of triggering angiogenesis and maintaining endothelium function (Andrae *et al.* 2008; Bouchentouf *et al.* 2011; Liu *et al.* 2013; Park *et al.* 2017; Camaré *et al.* 2017).

As previously discussed, aberrant angiogenesis within the atherosclerotic plaque can lead to haemorrhaging or rupture (Greenberg and Jin 2013). Therefore these results indicate that catechin treatment is capable of increasing the risk of a CVD-related event or show that it should be limited to being used as a preventative rather than being used in those with established atherosclerotic plaques. However this increased expression of angiogenic genes may represent a mechanism by which catechin treatment is capable of restoring vascular function and inducing vasodilation and reducing blood pressure observed in human trials (Fisher *et al.* 2003; Babu *et al.* 2008; Tinahones *et al.* 2008; Rassaf *et al.* 2016; Heiss *et al.*

2015; Sansone *et al.* 2015). Furthermore increased KDR signalling has been found to improve blood supply and tissue perfusion in white fat deposits (Robciuc *et al.* 2016). This was associated with increased insulin delivery and improved adipose tissue function (Robciuc *et al.* 2016). This fits with the previously described hypothesis that despite catechin treatment increasing white fat accumulation, it also improved white fat function as indicated by the reduction in associated circulating cytokines. Perhaps catechin improves white fat function by increasing the amount of angiogenesis occurring within the tissue.

The only cell proliferation related gene to have its expression attenuated by catechin treatment was *HBEGF* (Fig. 7.14B). *HBEGF* is actively expressed and secreted from adipocytes during obesity and has been shown to induce VSMC proliferation (Matsuzawa 2005). During obesity the plasma levels of HBEGF have been found to be increased, which has been associated with reduced risk of a CVD-related event due to a more stable atherosclerotic plaque (Matsuzawa 2005; Rattik *et al.* 2015). These studies suggest that HBEGF is secreted during obesity in order to perform a protective role. If catechin is capable of restoring white fat function during obesity perhaps there is no need for the protective role of HBEGF, which may explain its reduced expression levels.

Dietary supplementation with catechin for 21 days was found to significantly alter the expression of several ECM related genes (Fig. 7.15). The genes *FGA* and *FGB* encode the alpha and beta peptide chains of the fibrinogen molecule. During coagulation both the alpha and beta chains are cleaved by thrombin, resulting in the exposure of multiple polymerisation sites and the formation of fibrin fibres (Davalos and Akassoglou 2012). These fibrin fibres can then interact with platelets and form a blood clot. Fibrinogen forms the largest component of blood clots as measured by mass (Davalos and Akassoglou 2012). Increased serum levels of fibrinogen can be an indicator of an individual's inflammatory state as increased levels have been associated with inflammation, hypertension and atherosclerosis (Adams *et al.* 2004; Sándor *et al.* 2016). Mice deficient for fibrinogen were found to have reduced platelet activation and attenuated serum levels of IL-10 and TNF-α (Iwaki *et al.* 2006; Zacharowski *et al.* 2007).

Catechin treatment was also found to attenuate the expression of both *FGA* and *FGB*, indicating a reduction in the formation of fibrinogen, which was also found to be associated with reduced IL-10 and TNF-α circulating levels (Figs. 7.9 and 7.10). A study by *Rabadán-Chávez et al.* (2016) also demonstrated that epicatechin treatment was capable of reducing the amount of circulating fibrinogen in rats fed a high fat diet. Furthermore increased levels of fibrinogen are associated with vasoconstriction, therefore reductions in the expression of *FGA* and *FGB* may provide another mechanism by which flavanol treatment has been previously found to induce vasodilation (Davalos and Akassoglou 2012; Lominadze *et al.* 2010).

Another anti-atherogenic effect of catechin treatment was the increased expression of *TNC* (Fig. 7.15D). The *TNC* gene encodes the ECM protein tenascin-C, which is capable of influencing cell adhesion, inflammation and tissue remodelling (Chiquet-Ehrismann 2004; Franz *et al.* 2015). Mice lacking the *TNC* gene were found to have increased monocyte and macrophage infiltration (Song *et al.* 2017). The cardiovascular protective effects of TNC have been demonstrated in *ApoE* deficient mice which were also lacking *TNC* (Wang *et al.* 2012). These mice were found to have enhanced atherosclerosis disease progression due to larger lesions forming, possibly due to increased cell infiltration because of increased *VCAM-1* expression. Furthermore these mice were also found to suffer from more interplaque haemorrhaging in comparison to the control mice (Wang *et al.* 2012). Therefore catechin treatment may reduce atherosclerotic lesion size by attenuating cell recruitment and increase lesion stability by preventing interplaque haemorrhaging as a results of increased *TNC* expression.

Despite these benefits of catechin treatment, it also appeared to exert pro-atherogenic effects as measured by increased *MMP1A* expression (Fig. 7.15C). Increased MMP expression is normally associated with the weakening of the fibrous cap due to the increased destruction of ECMs present within it (McLaren *et al.* 2011a; Moss and Ramji 2016a; Ruddy *et al.* 2016). This result was not expected as previous studies have demonstrated that catechin and other flavanol treatments are capable of attenuating MMP expression and activation (Kim-Park *et al.* 2016; Nowakowska and Tarasiuk 2016; Wang *et al.* 2016; Roomi *et al.* 2017; Owczarek *et al.* 2017; Sato *et al.* 2017). Furthermore our *in vitro* study observed a reduction in MMP activity following catechin treatment (Fig. 6.22). Catechin treatment has also been found to increase the expression of TIMPs and therefore although catechin treatment increased the expression of *MMP1A*, it may also increase the expression of TIMPs and actually attenuate MMP activity (Garbisa *et al.* 2001; Lewandowska *et al.* 2013). The effect of catechin on MMP expression and activity *in vivo* requires further exploration in future feeding studies, as the current study determined the gene expression levels of *MMP1A* in the liver rather than macrophages.

Indirect evidence points to the possibility of catechin treatment being capable of reducing the size of an atherosclerotic lesion and improve its stability. The reduction in the circulating or expression levels of CXCL1, fibrinogen and TNC (Figs. 7.9 and 7.15) in addition to *in vitro* monocyte migration assays (Fig. 5.6) indicate that catechin treatment is capable of attenuating monocyte recruitment during atherosclerotic plaque formation. Furthermore cell proliferation was found to be attenuated *in vitro* (Fig. 6.19), therefore the cellular content within a plaque may be reduced following catechin treatment accompanied by increased plaque stability (Wilson 2010; Seneviratne *et al.* 2013). Additionally, the reduced expression of *ITGA2* and fibrinogen following catechin treatment indicates reduction in the risk of thrombosis due to a reduction in platelet activation (Figs. 7.13 and 7.15). This would coincide with previous studies

which have observed reductions in plaque size and attenuated platelet activation following catechin treatment (Morrison *et al.* 2014). However an increase in the expression of *ITGAX* and *MMP1A* (Figs. 7.13 and 7.15) may be markers of an increase in the number of DCs within the plaque as well as increased fibrous cap degradation, both of which play a role in plaque destabilisation (Zernecke 2015; Ruddy *et al.* 2016). Furthermore raised expression of angiogenesis genes (Fig. 7.14) may be an indicator of increased interplaque vessel formation which can also contribute to plaque destabilisation (Greenberg and Jin 2013). However increased expression of *TNC* may help to protect against this (Wang *et al.* 2012). On the other hand, the increased expression of angiogenesis genes may actually represent improved endothelium function following catechin treatment. The effect of catechin dietary supplementation on atherosclerotic plaque size and stability needs to be assessed in greater detail in future long term feeding studies using atherosclerotic mouse models.

Catechin treatment appeared to exert anti-atherogenic effects on lipid transport and metabolism related genes by increasing their expression (Fig. 7.16). ApoE is an essential apolipoprotein in the RCT process as it allows HDL particles to transfer excess cholesterol back from peripheral tissues to the liver for excretion (van der Velde 2010; McLaren *et al.* 2011a). The importance of ApoE in preventing atherosclerosis development has been well established in mouse models which lack the apolipoprotein as they rapidly develop atherosclerosis plaques (Chistiakov *et al.* 2017). Therefore increasing *ApoE* expression following catechin treatment implies strong anti-atherogenic effects. Furthermore an increase in ApoE implies an increase in the RCT process which may explain the observed increase in HDL levels (Fig. 7.6B). However if increasing *ApoE* expression is a mechanism by which catechin treatment exerts its cardiovascular protective effects, it must be taken into account when using traditional atherosclerotic mouse models of atherosclerosis as they will be lacking either *ApoE* or its receptor *LDLr*.

LPL is also an essential component of cholesterol metabolism and homeostasis. It is capable of breaking down the TGs present in chylomicrons and VLDL particles, resulting in HDL particle formation (Rip *et al.* 2005). Additionally LPL is capable of increasing the removal of pro-atherogenic lipoproteins by the LDLr within the liver (Rip *et al.* 2005). For this reason, *LPL* deficiency has been associated with increased serum TG levels and reduced HDL levels, resulting in increased atherosclerosis and risk of a CVD-related event (Benlian *et al.* 1996; Rip *et al.* 2005; Ramasamy 2016). However the effect of LPL on atherosclerosis development depends on where it is expressed as increased levels within adipose and muscle tissue is considered to be anti-atherogenic, whereas it is considered to be pro-atherogenic within macrophages and SMCs as it promotes lipid accumulation and inflammation (Li *et al.* 2014b). The effect of catechin treatment on *LPL* expression needs to be assessed further in future studies to determine its expression levels in these tissues/cells in order to determine whether

it is exerting a pro- or anti-inflammatory effect. Unlike previous studies which have observed a decrease in the levels of *LPL* following catechin and green tea extract treatment, the gene expression levels of *LPL* was found to be increased within the liver of wild type mice following catechin treatment in our study (Auclair *et al.* 2009; Chen *et al.* 2009; Lee *et al.* 2009). Furthermore LPL is required for TG turnover in adipose tissue, therefore increasing *LPL* expression may be another indicator that catechin treatment is capable of restoring normal adipose tissue function during a high fat diet (Stolarczyk 2017).

As previously discussed, the role of APT1, encoded by *LYPLA1*, during atherosclerosis disease development remains uncertain. However it is known to play a role in lipid metabolism and cell signalling due to its ability to influence the removal of cell surface proteins in a process known as depalmitoylation (Vujic *et al.* 2016). The observed increase in *LYPLA1* gene expression indicates that catechin treatment results in the increased removal of receptors and other proteins from the cell surface, and is therefore capable of influencing cell signalling. Overall catechin exerts anti-atherogenic effects on cholesterol homeostasis by increasing the gene expression of both *ApoE* and *LPL* which potentially resulted in the observable increase in HDL cholesterol levels.

The expression of the transcriptional regulator *NR1H3*, also known as LXRα, is capable of upregulating the expression of genes involved in cholesterol efflux and transportation as well as attenuating the expression cholesterol uptake genes (Parikh *et al.* 2014). Catechin treatment was found to increase *NR1H3* gene expression within the liver of wild type mice (Fig. 7.17). On the other hand, our *in vitro* studies found that catechin treatment attenuated *NR1H3* expression in THP-1 macrophages (Fig. 6.16A), however this may be a cell specific effect. NR1H3 is thought to alter cholesterol homeostasis by attenuating the expression of NPC1L1, a protein involved in the uptake of cholesterol in the intestine (Alvaro *et al.* 2010). However catechin treatment failed to alter total serum cholesterol levels (Fig. 7.6A). Increased *NR1H3* expression is associated with increased plasma HDL cholesterol levels and indeed catechin was found to be capable of increasing HDL cholesterol levels (Fig. 7.6B), another possible mechanism by which catechin exerts its anti-atherogenic effects. Furthermore *NR1H3* activation is linked to reduced *TNF-α* and *IL-1β* expression (Zelcer and Tontonoz 2006). Catechin treatment also resulted in the reduced circulating levels of TNF-α and IL-1β (Figs. 7.9 and 7.10). The increased expression of *NR1H3* following catechin treatment may represent an underlying mechanism behind some of the cardiovascular protective effects of catechin.

Overall, it appears that catechin treatment exerts anti-atherogenic gene expression changes. This can be seen by its ability to attenuate the expression of several pro-inflammatory genes such as *FGA* and *FGB*. Furthermore catechin was also capable of increasing the expression of *ApoE*, *ENG*, *KDR*, *NR1H3*, *THBS4* and *TNC*, which are all considered to be anti-atherogenic

genes. This altered gene expression profile may explain and provide mechanistic insight into the anti-atherogenic effects of catechin observed in this study.

7.4.4 The effect of catechin on bone marrow cell populations in wild type mice

As previously discussed a high fat diet and obesity are capable of altering the proportion of cell populations within the bone marrow (Chan *et al.* 2012; Wu *et al.* 2013; Adler *et al.* 2014; van den Berg *et al.* 2016; Lang and Cimato 2014; Murphy *et al.* 2014; Ma and Feng 2016). Furthermore the cell population profile of an individual can be altered by dietary supplementation with a nutraceutical (Takano *et al.* 2004a; Betiati *et al.* 2013; Chang and Deckelbaum 2013). As catechin resulted in an increase in weight gain and fat accumulation in wild type mice, it was expected that catechin treatment may induce pro-atherogenic changes within the cell populations. The summary of changes to the bone marrow cell populations as a result of catechin treatment are summarised in Fig. 7.25.

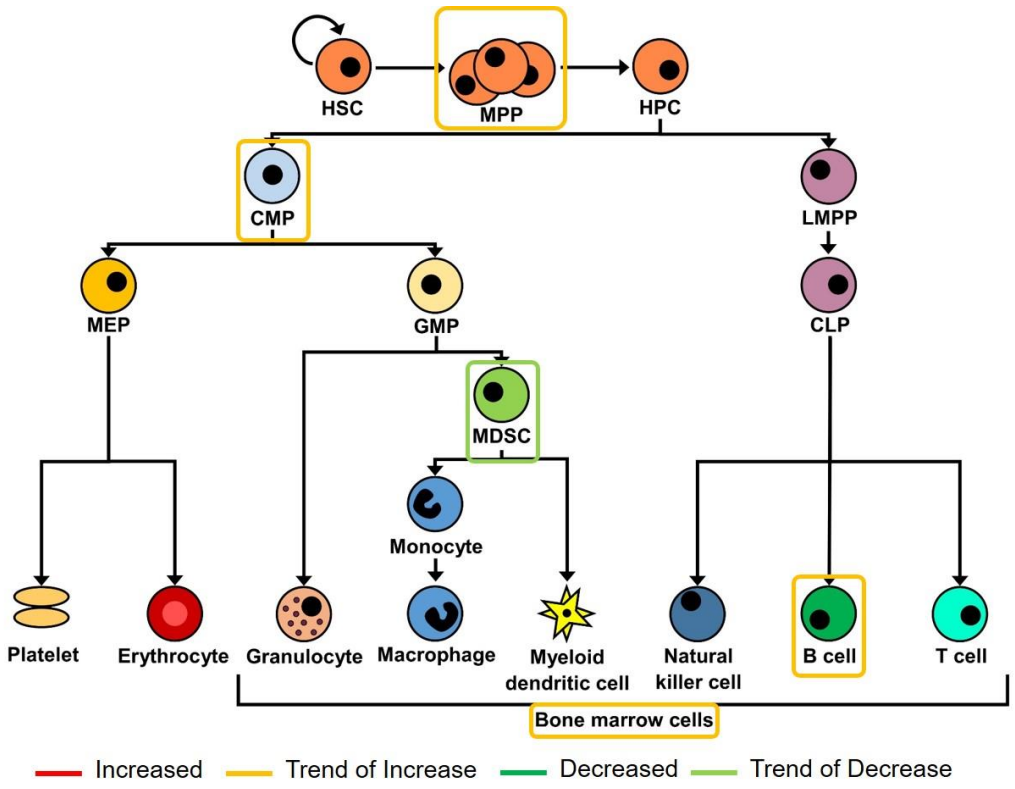

Fig. 7.25. The catechin induced changes in the proportions of cell populations within the bone marrow. The bone marrow of wild type mice were harvested after they had received either catechin or vehicle control treatment while being fed a high fat diet for 21 days. HSC, haematopoietic stem cell; MPP, multipotent progenitors; HPC, haematopoietic progenitor cell; CMP, common myeloid progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-macrophage progenitor; MDSC, myeloid-derived suppressor cells; LMPP, lymphomyeloid progenitor; CLP, common lymphoid progenitor.

Atherosclerosis disease progression is positively correlated with an increase in white blood cell number and therefore can be used as a marker of an individual's inflammatory state (Ates *et al.* 2011; Mozos *et al.* 2017). Catechin treatment resulted in an increase in the proportion of

total bone marrow cells recovered from the bone marrow of wild type mice (Fig. 7.18). This result would imply that catechin exerts a pro-inflammatory effect and potentially contributes towards atherosclerosis development. Indeed this was confirmed when the level of live-lineage negative cell population, a population which contains all HSPCs in the bone marrow, was also found to be increased by catechin (Fig. 7.20A). This increase was driven by the expansion of the MPP cell population (Fig. 7.20). The increased expression of *FGF2*, *IL-2*, *IL-5*, *KDR* and *PDGFRB* have been implicated in stem cell proliferation and therefore the increased expression of these gene following catechin treatment may explain the increase observed in HSPC populations (Fig. 7.14; Horikawa and Takatsu 2006; Choi *et al.* 2008; Rodrigues *et al.* 2010; Liao *et al.* 2011).

The LK cell population usually expands during atherosclerosis development and relocates to the spleen where they differentiate into monocytes and macrophages, which are capable of targeting the atherosclerotic lesion (Robbins *et al.* 2012). Catechin treatment had no effect on the size of the LK population found within the bone marrow, however it did non-significantly increase the CMP subpopulation (Figs. 7.21). In contrast, the MEP and GMP subpopulations was found to be unaffected following catechin treatment (Fig. 7.21). Previous studies have found that a high fat diet is capable of inducing the expansion of the CMP and GMP subpopulation, unfortunately these results indicate that catechin is not capable of protecting against an obesity driven expansion of the CMP population (Gao *et al.* 2014).

Although the results show no change in the monocyte and macrophage cell population (Fig. 7.23D), it is not clear whether these macrophages are the pro-atherogenic M1 or anti-atherogenic M2 phenotype. The expression of several pro-angiogenic genes was found to be increased as a result of catechin and recently M2 macrophages have been implicated in promoting angiogenesis (Owen and Mohamadzadeh 2013). The changes in angiogenic genes may provide some insight into the phenotype of the macrophages which develop following catechin treatment. However, the full effect of catechin treatment on macrophage phenotype needs to be assessed in further detail as our *in vitro* study found that catechin was unable to induce the M2 macrophage phenotype (Fig. 6.23).

In contrast to the vehicle control treated mice, those which received catechin treatment were found to have a non-significant decrease in their MDSC population within the bone marrow (Fig. 7.23C). The role of the MDSC population during atherosclerosis disease development remains unclear, although they are known to supress T-cell differentiation (Talmadge and Gabrilovich 2013). However it is worth noting that despite a decrease in the MDSC population, there was no change in the size of the T-cell population (Fig. 7.24B). Previous studies have found that the cytokines IL-6 and IL-10 are involved in the differentiation of MDSCs and therefore the reduction in the circulating levels of these cytokines may explain the reduction in the MDSC population (Fig. 7.10; Mundy-Bosse *et al.* 2011; Dolcetti *et al.* 2009). Much of our

knowledge on the role of MDSCs has been generated from tumour progression studies. MDSCs were found to be capable of inducing invasion, proliferation, survival and adhesion of tumours during their development and metastasis (Talmadge and Gabrilovich 2013). Furthermore angiogenesis has been found to be promoted by the MDSC population, however this decrease in the MDSC population contradicts the increase in the expression of angiogenic related genes following catechin treatment (Talmadge and Gabrilovich 2013). These mixed and contradictory results only highlight our lack of understanding of the general function of MDSCs as well as their role within atherosclerosis disease development. Studies which has tried to assess their function found that a reduction in the MDSC population was associated with increased atherosclerosis and inflammation (Xia *et al.* 2011; Foks *et al.* 2016). Therefore studies designed to uncover the specific role of MDSCs during atherosclerosis disease progression are required to fully evaluate the consequences of catechin treatment in reducing this population.

Catechin treatment was associated with a non-significant increase in the B-cell population (Fig. 7.24C). Increased B-cell proliferation is associated with IL-5 levels (Horikawa and Takatsu 2006). Catechin treatment was found to increase *IL-5* gene expression, explaining the increase in B-cell numbers (Fig. 7.14D). Although rare within the atherosclerotic lesion, B-cells are capable of exerting both pro- and anti-atherogenic effects depending on the phenotype (Andersson *et al.* 2010; Kyaw *et al.* 2011). Without future studies determining the specific phenotypic changes within the B-cell population it is not possible to determine whether the increase in B-cell number following catechin treatment is beneficial or detrimental.

The changes in bone marrow cell populations following catechin treatment indicates a slight pro-atherogenic shift. This result coincides with a previous study which found (+)-catechin treatment caused a significant increase in the number of white blood cells in mice receiving a standard chow diet (Takano *et al.* 2004a). Additional studies are needed to assess the effect of these HSPC population changes on atherosclerosis disease development. Future studies are required to determine the cell changes which occur directly within the atherosclerotic plaque to overcome the limitations associated with only assessing the populations within the bone marrow (discussed in Chapter 4). The major limitation of this study was the loss of four mice per treatment group, limiting the study to four independent values. This study needs to be repeated in order to achieve a better understanding of the effect of catechin treatment on the changes in HSPCs within the bone marrow of wild type mice.

7.4.5 Summary of the effect of catechin in wild type mice

As far as we are aware, this is the first *in vivo* study to assess the cardiovascular protective effects of (+)-catechin treatment in a highly rigorous and detailed manner. Catechin treatment was found to induce a variety of anti-atherogenic effects such as increasing HDL cholesterol levels, promoting anti-inflammatory gene expression and reducing circulating pro-inflammatory

cytokine levels (summarised in Fig. 7.26). However this study also implies a slight pro-inflammatory state within the bone marrow cell populations by catechin treatment. Overall this initial study justifies the use of catechin in long term feeding studies using atherosclerotic mouse models to fully uncover the nutraceutical potential of catechin for attenuating the rate of atherosclerosis disease development. The use of atherosclerotic models would also allow the effect of catechin on the plaque size, cellular composition and stability to be assessed *in vivo*. Furthermore the effect of catechin on vasorelaxation needs to be assessed during future studies in order to link it to changes in gene expression. Previous studies have shown that catechin and other flavanols are capable of attenuating plaque formation (Chyu 2004; Minatti *et al.* 2012; Morrison *et al.* 2014). Therefore catechin holds great promise for use as a cardiovascular protective treatment.

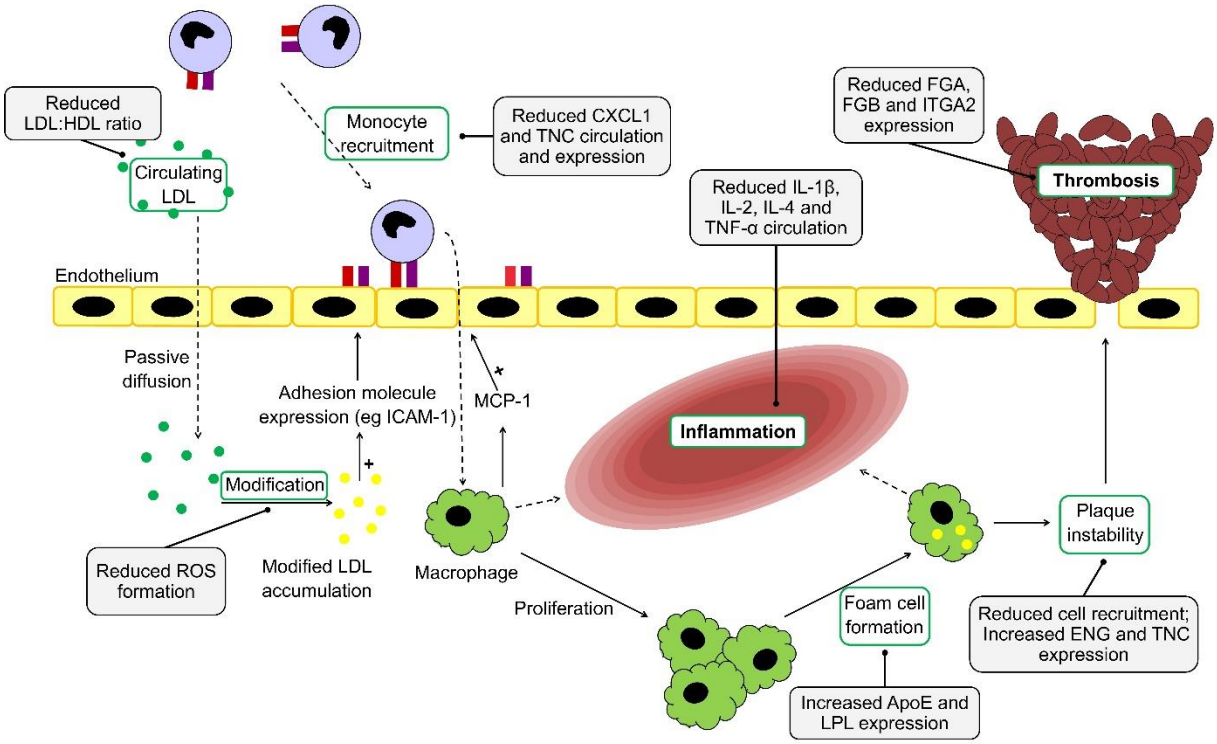

Fig. 7.26. Summary of the potential anti-atherogenic properties of catechin *in vivo*. The steps potentially involved in the reduction of atherosclerosis disease progression following catechin treatment are highlighted in green. Possible mechanisms for these changes are also included.

Chapter 8

General Discussion

Due to the sustained increase in the global rates of obesity and diabetes as well as the continual westernisation of diets in developing countries, the global prevalence of CVD-related events will only continue to rise (WHO 2017). Traditional pharmaceuticals, such as statins, are not always capable of sufficiently lowering an individual's circulating LDL levels despite receiving the highest statin dosage (Leitersdorf 2001; McLaren *et al.* 2011a). This means some patients have a considerable residual cardiovascular risk despite receiving statins. Therefore there is a large demand for alternative anti-inflammatory therapies that could be used either in isolation or in combination with statins to treat or prevent atherosclerosis.

Compounds within healthy diets have been identified as possessing additional health benefits that go beyond their nutritional value and have been given the term 'nutraceutical' (Moss and Ramji 2016b). The nutraceuticals ω-3 PUFAs, flavanols and phytosterols have emerged as compounds possessing anti-inflammatory properties and therefore may provide cardiovascular protective effects (extensively reviewed in *Moss and Ramji* 2016). Therefore investigating these nutraceuticals in greater detail may provide more insight into their anti-atherogenic potential and lead to the identification of a novel preventative or treatment for atherosclerosis. Furthermore combining these nutraceuticals in a mixture may result in synergistic effects and result in stronger anti-atherogenic effects when compared to assessing them in isolation. CardioWise is a novel nutraceutical combination designed by Cultech Limited. The main aim of this project was to assess whether this nutraceutical and the components within it were capable of exerting anti-inflammatory effects and attenuate the key stages of atherosclerosis disease development.

CardioWise was found to have many anti-atherogenic actions on monocytes/macrophages *in vitro*, including attenuated pro-inflammatory gene expression, monocyte recruitment, foam cell formation and M1 macrophage polarisation (see Chapter 3). These anti-atherogenic actions extended *in vivo* to mice fed a high fat diet (see Chapter 4). CardioWise treatment increased serum HDL levels as well as reducing the circulating levels of pro-inflammatory cytokines. Furthermore CardioWise produced an anti-atherogenic gene expression profile in addition to decreasing the proportion of macrophages found within the bone marrow. Catechin was identified as a key nutraceutical within CardioWise due to its anti-oxidant properties and had the ability to attenuate monocyte recruitment and macrophage proliferation *in vitro* (see Chapters 5 and 6). The anti-oxidant effects of catechin were also identified *in vivo* in mice fed a high fat diet (see Chapter 7). Mice treated were catechin were also found to have reduced

levels of pro-inflammatory cytokines and increased serum HDL cholesterol levels. Additionally catechin treatment induced the expression of anti-inflammatory genes as well as reducing the proportion of MDSCs, granulocytes and T-cells within the bone marrow of mice.

8.1 The cardiovascular protective effects of CardioWise

The results presented in Chapter 4 represent the first *in vivo* study to treat wild type mice fed a high fat diet with a novel nutraceutical combination containing ω-3 PUFAs, flavanols and phytosterols at a human physiological equivalent dose. The increased circulating levels of HDL cholesterol, reduced pro-inflammatory gene expression and reduced plasma pro-inflammatory cytokine levels show that CardioWise exerts strong anti-atherogenic effects *in vivo* (summarised in Fig. 4.25). Changes within the gene expression profile and altered lipid and cytokine levels within the mice receiving CardioWise indicate a possible activation of brown adipose tissue deposits and the browning of white fat (summarised in Fig. 4.23). This would indicate that CardioWise treatment could be used in obese individuals to activate their brown fat deposits and enhance lipid clearance by non-shivering thermogenesis. This hypothesis requires further exploration in future studies in order to gain a better understanding of the effect of CardioWise treatment on the function of fat deposits (see Section 8.6 for further details). As well as CardioWise exerting these anti-atherogenic effects, it was found to decrease the proportion of monocytes and macrophages found within the bone marrow when compared to the vehicle control treated mice, a sign of reduced inflammation (summarised in Fig. 4.24). ω-3 PUFAs, flavanols and phytosterols have all been previously shown to be capable of attenuating atherosclerotic plaque formation (Moghadasian *et al.* 1999; Yeganeh *et al.* 2005; Moghadasian 2006; Chyu 2004; Nakajima *et al.* 2011; Brown *et al.* 2012; Minatti *et al.* 2012; Morrison *et al.* 2014). This reduction in lesion size may arise due to the ability of CardioWise treatment to reduce the expression of cell recruitment and proliferation related genes (Figs. 4.11 and 4.12). The altered gene expression profile in combination with previous studies showing that ω-3 PUFAs, flavanols and phytosterols are capable of retarding atherosclerosis disease progression indicate that CardioWise treatment has the potential to reduce atherosclerotic plaque size (Moghadasian *et al.* 1999; Yeganeh *et al.* 2005; Moghadasian 2006; Chyu 2004; Nakajima *et al.* 2011; Brown *et al.* 2012; Minatti *et al.* 2012; Morrison *et al.* 2014). Future studies using atherosclerotic related mouse models are required to assess the effect of CardioWise treatment on plaque formation (see Section 8.6 for further details). Overall the results presented in this thesis demonstrate that CardioWise possesses anti-inflammatory properties *in vitro* using human derived macrophages as well as protecting wild type mice from the pro-inflammatory actions of a high fat diet.

8.2 The cardiovascular protective effects of catechin

As shown in Chapter 7, catechin treatment resulted in a variety of cardiovascular protective effects, including increased HDL cholesterol levels, enhanced anti-inflammatory gene expression and reduced circulating pro-inflammatory cytokine levels (summarised in Fig. 7.26). Furthermore the study also revealed that catechin induces the expression of several genes involved in maintaining the function of the endothelium (Fig. 7.14). Previous studies have observed improved vascular function, vasodilation and reduced blood pressure following catechin treatment (Fisher *et al.* 2003; Babu *et al.* 2008; Tinahones *et al.* 2008; Rassaf *et al.* 2016; Heiss *et al.* 2015; Sansone *et al.* 2015). The increased expression of these gene may represent the underlying mechanism by which catechin and other flavanol treatments are capable of inducing vascular function improvements seen in previous human trials (Fisher *et al.* 2003; Babu *et al.* 2008; Tinahones *et al.* 2008; Rassaf *et al.* 2016; Heiss *et al.* 2015; Sansone *et al.* 2015). Additional *in vivo* studies are required to confirm the potential link between altered gene expression and reduced blood pressure following catechin treatment (see Section 8.6 for further details). Although catechin treatment resulted in several anti-atherogenic effects, it was also found to shift the bone marrow cell populations to a slightly pro-inflammatory one (summarised in Fig. 7.25). However due to unforeseen problems with the cell extraction from the bone marrow, the results of the cell population analysis are based on a reduced sample size and therefore requires repetition to fully evaluate the effect of catechin on haematopoietic cell populations. Catechin treatment was found to attenuate the circulation and expression of a variety of monocyte recruitment factors *in vivo* (Figs. 7.9 and 7.15) in addition to decreasing the amount of monocyte migration *in vitro* (Fig. 5.6). This may represent the mechanism by which previous studies have shown that catechin and other flavanols are capable of reducing atherosclerotic plaque size (Chyu 2004; Minatti *et al.* 2012; Morrison *et al.* 2014). The direct link between the effect of catechin on circulating cytokine levels as well as the expression of cell adhesion molecules and the size of the plaque needs to be assessed in future studies to confirm the mechanism of action (see Section 8.6 for further details). This study has shown that catechin treatment is capable of exerting cardiovascular protective effects in both human macrophages and wild type mice.

8.3 Similarities and differences between CardioWise and catechin treatment

As catechin is a major component within the CardioWise combination, it is useful to compare and contrast the cardiovascular protective effects generated by each treatment. This would aid in determining the role of catechin within the mixture or identifying additional protective effects which cannot be achieved by catechin treatment in isolation. A comparison of the effects of CardioWise and catechin treatment on foam cell formation *in vivo* is summarised in Fig. 8.1. There is indirect evidence to suggest that CardioWise and catechin treatment are capable of

restoring the physiological function of brown and white fat respectively (see Chapters 4 and 7). Previous studies have demonstrated that ω-3 PUFAs and other GPR120 agonists are capable of activating brown fat, which explains the additional benefit of brown fat activation following treatment with the mixture (Quesada-López *et al.* 2016; Pahlavani *et al.* 2017). The CardioWise mixture was also capable of increasing circulating HDL levels and improving the LDL/HDL cholesterol ratio (Fig. 4.5). This result was expected as ω-3 PUFAs, flavanols and phytosterols have all been found to increase serum HDL levels in murine studies (Chisaka *et al.* 1988; Babu *et al.* 2006; Moghadasian *et al.* 1999; Nakajima *et al.* 2011). Catechin treatment in isolation was also found to improve HDL levels and the LDL/HDL ratio (Fig. 7.6), however the changes were comparable to the combined treatment indicating that there were no additional benefits on circulating cholesterol levels from the extra nutraceuticals. Catechin treatment also resulted in a reduction in the circulating levels of several atherosclerosis related cytokines (see Chapter 7). CardioWise treatment resulted in similar trends of reduction in circulating cytokine levels which were observed following catechin treatment (see Chapter 4), therefore it indicates that main compound capable of reducing cytokine levels within the mixture is catechin. However this requires further exploration in future studies.

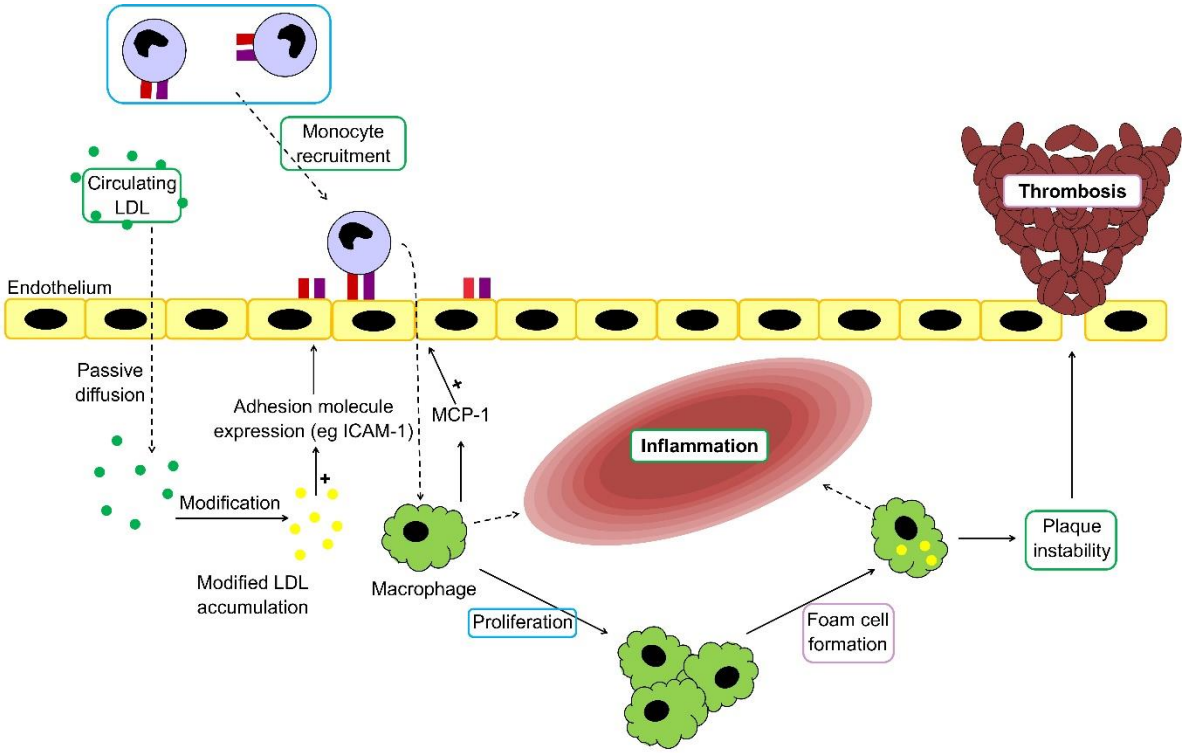

Fig. 8.1. The similarities and differences between CardioWise and catechin treatment on foam cell formation *in vivo*. The steps altered by both CardioWise and catechin treatment during atherosclerosis disease progression are highlighted in green. The steps altered by CardioWise treatment only during atherosclerosis disease progression are highlighted in blue. The steps altered by catechin treatment only during atherosclerosis disease progression are highlighted in purple.

Both catechin and CardioWise treatment resulted in a decrease in the proportion of MDSCs within the bone marrow of wild type mice, whereas CardioWise treatment also reduced the number of macrophages (Fig. 4.21 and 7.23).

As well as exerting similar effects, catechin and CardioWise treatments resulted in cardiovascular protective effects that were unique to each treatment. An anti-atherogenic gene expression profile was induced by both catechin and CardioWise treatment but interestingly the genes which were found to be altered by catechin treatment were not the same genes altered by CardioWise treatment (see Chapters 4 and 7). These results suggest that either another component within the mixture masked the effects of catechin or an interaction between the nutraceuticals caused changes in the expression of different genes. This may be due to the increased *PPAR-γ* expression observed following CardioWise treatment which was not seen when catechin was used in isolation (Fig. 4.15B and 7.17). This coincides with previous studies which have shown that ω-3 PUFAs cause the activation of *PPAR-γ* (Calder 2012). As *PPAR-γ* is a key regulator of transcription, its activation following CardioWise treatment may explain why different genes are either activated or attenuated when compared to the mice receiving catechin only (Derosa *et al.* 2013; Derosa *et al.* 2018).

Previous studies have demonstrated that flavanols are unable to alter circulating TG levels and this was confirmed in our study (Fig. 7.7; Matsuyama *et al.* 2008; Friedrich *et al.* 2012). However both ω-3 PUFAs and phytosterols have been found to reduce the serum levels of TGs (Kamat and Roy 2016; Schonewille *et al.* 2014; Perez-Ternero *et al.* 2017). It was therefore unexpected when CardioWise treatment resulted in an increase in TG levels (Fig. 4.6A). As previously discussed there is evidence that CardioWise potentially induces brown fat activation which in turn can raise TG levels (Hoeke *et al.* 2017). Another possible explanation for this increase in TG levels is due to an interaction between all of the nutraceuticals present within the mixture which therefore requires further investigation. Furthermore CardioWise resulted in a decrease in the number of B- and T-cell present within the bone marrow, whereas catechin treatment used in isolation only resulted in a reduction in the number of T-cells (Fig. 4.22). This change may be a result of the altered gene expression changes observed following dietary supplementation with CardioWise (see Chapter 4) or it is a result of an additional nutraceutical present within the mixture. Indeed previous studies have demonstrated that ω-3 PUFAs are capable of reducing the number of T-cells and therefore is the most likely nutraceutical responsible for the decrease in B- and T-cells (Calder 2015). As previously discussed MDSCs reduce the proliferation of T-cells, however in CardioWise treated mice both the MDSC and T-cell population were found to be decreased. This led to the hypothesis that the two populations had their numbers reduced by two independent mechanisms (see Chapters 4).

The ω-3 PUFAs and phytosterols present within the CardioWise mixture prevented the weight gain which occurred in mice receiving the dietary supplementation of catechin only (Figs. 4.3 and 7.4). However as this was a short term feeding study, a longer time period is required to fully assess the effects of CardioWise and catechin treatment on weight gain in wild type mice. Previous studies have demonstrated that flavanols are strong anti-oxidants which are capable of attenuating ROS generation (Moss and Ramji 2016b; Bahramsoltani *et al.* 2017; Tak *et al.* 2016; Yu *et al.* 2017; Oyama *et al.* 2017). However ROS generation was not attenuated in mice receiving CardioWise or catechin (Fig. 4.7 and 7.8). Overall these results demonstrate that the unique mixture of ω-3 PUFAs, flavanols and phytosterols present in CardioWise has different and additional cardiovascular protective effects when compared to catechin only treatment in wild type mice. This study shows that combinations of nutraceuticals can be used as potential therapies for CVD and atherosclerosis and should therefore should be assessed in greater detail in future studies. Studies are required to explore the potential effects of mixing previously known cardiovascular protective nutraceuticals to try and establish a potent anti-inflammatory therapy for retarding the progression of atherosclerosis in a similar manner to the development of CardioWise.

8.4 From bench to mouse to bedside?

This study has demonstrated that both CardioWise and catechin treatment are effective anti-inflammatory nutraceuticals which have the potential to exert anti-atherogenic effects in our atherosclerotic related *in vitro* models and wild type mice (see Chapters 3- 7). The results from these initial studies justify the further exploration of CardioWise and catechin dietary supplementation in atherosclerotic related mice models. Both *ApoE* and *LDLr* deficient mice have previously been used to show that the individual components of CardioWise, ω-3 PUFAs, flavanols and phytosterols, are capable of retarding atherosclerosis disease development (Table 1.2; Moss and Ramji 2016). Therefore it is predicted that both CardioWise and catechin treatment would be capable of attenuating atherosclerosis plaque formation.

As the majority of nutraceutical therapies are most likely going to be used to treat individuals who are already receiving statins, the effect of CardioWise and catechin dietary supplementation needs to be assessed in atherosclerotic mice also receiving statins. This would determine whether there were any additional cardiovascular protective effects or any detrimental effects associated with receiving a combination of traditional pharmaceuticals and nutraceutical therapies for atherosclerosis as add on treatments for statins. Only once CardioWise and catechin dietary supplementation have been found safe and effective in these studies should it progress into preliminary human clinical trials for assessment as add-on therapies for statins. Results will be able to determine whether CardioWise and catechin are most effective when used as a preventative (i.e. in low to medium risk patients who do not

initially qualify for statin treatment but will in the future without some form of intervention) or used in combination with statins to achieve greater cardiovascular protective effects which cannot be achieved by statins in isolation. Furthermore as CardioWise is a combination of nutraceuticals, results from the *in vivo* murine and human studies should always be used to determine whether the mixture would benefit from additional nutraceuticals being added, or changing the concentrations of the individual components within the emulsion to result in a more effective combination. These changes could easily be assessed within our *in vitro* atherosclerotic models before use in clinical trials in an effective bench-to-bedside approach to anti-atherogenic nutraceutical research.

Another issue to consider when designing potential nutraceutical therapies for the prevention or treatment of atherosclerosis is the bioavailability of the compounds. The bioavailability of all nutrients can be affected by a variety factors including, their physicochemical properties, the presence or absence of other nutrients which may boost or attenuate absorption, and how they are metabolised once taken up by the body (Schuchardt and Hahn 2013). Studies have shown that following the consumption of EPA or DHA, plasma levels rapidly increase and they also begin to aggregate in mononuclear cells and adipose tissue (Browning *et al.* 2012; Browning *et al.* 2014). Furthermore, the half-life of EPA has been shown to be approximately 3 days (Braeckman *et al.* 2013). These studies show that ω-3 PUFAs are able to reach the target tissues and at high concentrations for a prolonged period of time.

On the other hand, the bioavailability of flavonoids is extremely variable (Thilakarathna and Rupasinghe 2013). Catechin is known to be efficiently absorbed within the gut following consumption, as demonstrated by an increase in the concentration of catechin metabolites in urine (Manach *et al.* 2004; Manach *et al.* 2005; Del Rio *et al.* 2010). The plasma concentrations of flavanols has been show to peak approximately 1.5 hours after consumption and they have a half-life of 2 to 3.5 hours (Lee *et al.* 2002). Relatively little is known about what concentrations catechin or its metabolites are able to reach in different tissues around the body. This needs to be investigated in greater detail in future studies to ensure catechin reaches the target tissues at sufficient concentrations. The bioavailability of phytosterols is extremely poor as there is relatively little absorption within the gut (Ostlund 2002), however this is how they exert their potential cardiovascular health benefits. As phytosterols attenuate cholesterol uptake in the gut by out competing its ability to bind to the necessary receptors, its bioavailability within the body is not as important as the ω-3 PUFAs and catechin.

8.5 The use of nutraceuticals for other inflammatory based diseases

Chronic inflammation is not limited to atherosclerosis and is involved in several diseases including arthritis, asthma and inflammatory bowel disease (IBD). Therefore the anti-

inflammatory effects observed following CardioWise and catechin treatment both *in vitro* and *in vivo* means they also have the potential to be used as nutraceutical therapies for these diseases. Several studies have explored the use of nutraceuticals, especially ω-3 PUFAs, for a variety of inflammatory based conditions with success (Tanaka and Takahashi 2013; Calder 2015). A brief overview of the role of the inflammatory response in arthritis, asthma, IBD and Abdominal aortic aneurysm is discussed below along with evidence for the protective effects of nutraceuticals.

8.5.1 Arthritis

There are many forms of arthritis but the two most prevalent forms are osteoarthritis (OA) and rheumatoid arthritis (RA). Arthritis occurs when the cartilage and neighbouring bone are broken down within the joint, disrupting the protected smooth movement of the joint which leads to joint pain. OA is an age related condition and is usually triggered by mechanical stress, whereas RA is a chronic inflammatory autoimmune disorder (Hoxha 2018). RA is characterised by the increased production and secretion of pro-inflammatory cytokines and cell adhesion molecules within the joint (Hoxha 2018). The major cytokine thought to be involved in promoting the destruction of the joint is TNF-α (Hoxha 2018). Current therapies for RA which target the cytokines TNF-α and IL-6 are beyond the scope of this project but have been extensively reviewed in *Chaabo and Kirkham* (2015) and *Kim et al.* (2015). Furthermore an increase in the number of B- and T-cells infiltrating the joint is also associated with RA (Hoxha 2018).

CardioWise treatment in wild type mice receiving a high fat diet was found to be capable of attenuating the expression and secretion of cytokines and cell adhesion molecules, including TNF-α (Figs. 4.8, 4.11 and 4.14). Furthermore the proliferation of B- and T-cells was reduced in mice treated with CardioWise (Fig. 4.22). These results indicate that CardioWise also has the potential to be used as a nutraceutical therapy for RA. Indeed some of the components of CardioWise, ω-3 PUFAs and flavanols, have been shown to attenuate RA development in mice (Tang *et al.* 2007; Ierna *et al.* 2010; Calder 2015). Dietary supplementation with EPA and DHA in the form of fish or krill oil was found to attenuate the formation of arthritis in mice when compared to the vehicle control diet (Ierna *et al.* 2010). A reduction in arthritis score, joint pathology, inflammatory cell invasion and paw swelling was observed in mice receiving ω-3 PUFAs compared to the control mice (Ierna *et al.* 2010). Catechin treatment in rats with induced arthritis was found to reduce secondary inflammatory paw swelling in addition to attenuating the levels of TNF-α (Tang *et al.* 2007).

The RA protective benefits of ω-3 PUFAs dietary supplementation has also been observed in human trials (Boe and Vangsness 2015; Calder 2015; Fattori *et al.* 2016). The most common treatment for RA is the use of non-steroidal anti-inflammatory drugs (NSAIDs). These drugs work by targeting the inflammatory reaction which occurs during the destruction of the articular

cartilage (Boe and Vangsness 2015). After treatment with ω-3 PUFAs, patients were found to need less NSAID treatment (Boe and Vangsness 2015; Calder 2015; Fattori *et al.* 2016). Furthermore randomised clinical trials have shown that patients who receive ω-3 PUFA treatment for 3 to 4 months reported less joint pain, reduced joint swelling and improved grip strength (Boe and Vangsness 2015; Calder 2015; Fattori *et al.* 2016). These anti-RA health benefits are thought to be due to a reduction in the levels of pro-inflammatory cytokines which occurred as a result of ω-3 PUFA treatment (Boe and Vangsness 2015). The changes in cytokine levels following CardioWise treatment presented in this study in combination with the evidence of the benefits of ω-3 PUFA dietary supplementation from previous studies, indicate that CardioWise has the potential to relieve joint pain in RA suffers.

8.5.2 Asthma

Asthma is a chronic inflammatory disease within the airways of the lung. It can be characterised by airway spasms and a reversible obstruction of airflow due to an increase in the contractility of the surrounding smooth muscles. This means asthma can result in coughing, wheezing and shortness of breath, therefore it is important that all possible therapies are explored to relieve asthma suffers of their discomfort. The inflammatory reaction during asthma is primarily associated with an increase invasion of eosinophils within the lung (Calder 2015). Additionally mast cells, neutrophils, macrophages and T-cells are all present in increased numbers. Furthermore the levels of several pro-inflammatory cytokines including TNF-α, IL-5 and IL-6 are known to be increased in asthma sufferers (Calder 2015).

CardioWise treatment in wild type mice receiving a high fat diet results in decreased monocyte proliferation (Fig. 4.21), in addition to reducing the number of T-cells present within the bone marrow (Fig. 4.22). Furthermore a reduction in the circulating levels of TNF-α, IL-5 and IL-6 was observed in mice following treatment with CardioWise when compared to the vehicle control treatment mice (Fig. 4.8). Catechin treatment was also found to attenuate the circulating levels of these cytokines (Figs. 7.9 and 7.10). These results would indicate that both CardioWise and catechin dietary supplementation have the potential to reduce the inflammatory response which occurs during asthma. Previous studies using the Fat-1 mouse model (mice which should have a ω-3:ω-6 ratio of 1:1) have reduced airway inflammation, white blood cell invasion and IL levels including pro-inflammatory IL-5 when compared to wild type mice (Bilal *et al.* 2011).

A small clinical trial involving 23 participants was found to have reduced airway inflammation following 5 weeks of ω-3 PUFA dietary supplementation (Schubert *et al.* 2008). Patients were pre-treated with ω-3 PUFAs before being challenged with low level mite allergen for the remaining 2 weeks. However lung function was shown to be unchanged between the two groups, indicating that despite a reduction in inflammatory biomarkers there was not a significant improvement in airflow in the lung (Schubert *et al.* 2008). Furthermore meta-analysis

of 6 and 9 randomised clinical trials has shown that ω-3 PUFA dietary supplementation has little benefit in child or adult sufferers of asthma (Thien *et al.* 2002; Anandan *et al.* 2009). Early studies may have suggested that ω-3 PUFA treatment was capable of relieving the symptoms of asthma, however recent studies have questioned their efficacy and therefore further research is required to fully understand the effects of ω-3 PUFA supplementation in asthma sufferers (Calder 2015).

A limited number of studies have also explored the effect of flavanol treatment in asthma sufferers. Both epidemiological and cross-sectional studies have demonstrated a correlation between diets rich in fruit and vegetables, which contain flavonoids, and reduced asthma severity (Tanaka and Takahashi 2013). Mice treated with flavanols have also shown reduced levels of IL-4 and IL-5 which was associated with reduced symptoms of asthma (Tanaka and Takahashi 2013). Large clinical trials are needed to assess the effectiveness of both ω-3 PUFAs and flavanol treatment in reducing the inflammatory response in asthma patients. As previous studies have only been able to inconsistently demonstrate the anti-asthmatic benefits of ω-3 PUFA and flavanol treatment in isolation, CardioWise treatment should also be explored due it being a combination of potentially beneficial nutraceuticals and therefore may be more effective.

8.5.3 IBD

IBD is a general term for ulcerative colitis and Crohn's disease which are two chronic inflammatory diseases which affect the digestive system. Colitis primarily affects the colon and is frequently associated with increased neutrophil invasion and increased production of IL-5 and IL-10 (Calder 2015). In contrast the entire digestive system can be affected by Crohn's disease, which is also accompanied by an increased invasion of neutrophils and elevated levels of IFN-γ, IL-1β, IL-6 and TNF-α (Calder 2015). The treatment for IBD typically involves constant pharmaceutical and surgical intervention to improve a patient's quality of life (Vezza *et al.* 2016). However long term pharmaceutical intervention is associated with several side effects and therefore there is a need to develop alternative therapeutics (Vezza *et al.* 2016). CardioWise and catechin treatment in wild type mice receiving a high fat diet was found to attenuate the levels of several pro-inflammatory cytokines, including those associated with colitis and Crohn's disease (Figs. 4.8, 7.9 and 7.10). Therefore they represent potential nutraceuticals which could be used to reduce the inflammatory response in IBD.

A variety of murine studies have demonstrated that ω-3 dietary supplementation induced a reduction in chemically triggered colitis inflammation in comparison to those mice treated with ω-6 PUFAs (Calder 2015). Fat-1 mouse models have shown to have reduced gut inflammation and damage compared to wild type mice following 10 weeks of a ω-6 rich diet (Hudert *et al.* 2006). Furthermore these mice were found have reduced expression of *TNF-α* and *IL-1β* within the gut. Green tea extract has also been used to reduce in the inflammatory response in murine

models of colitis by attenuating the level of ROS production and subsequent lipid peroxidation (Brückner *et al.* 2012; Oz *et al.* 2013). ROS generation was also found to be reduced by catechin treatment in our study (Fig. 7.8).

The beneficial effects of ω-3 PUFA dietary supplementation for the treatment of IBD remains controversial. Several clinical trials have shown that a diet enriched with EPA and DHA is capable of reducing gut inflammation, in addition to improving a variety of markers including clinical score and gut mucus histology (Calder 2008; Calder 2015). The patients receiving the dietary supplementation were also able to reduce their need for traditional pharmaceutical intervention (Calder 2008; Calder 2015). However other studies have observed no change in the levels of gut inflammation following ω-3 PUFA treatment (Calder 2008; Calder 2015). Therefore further clinical trials are required to fully evaluate the effectiveness of ω-3 PUFA and catechin dietary supplementation on gut inflammation.

8.5.4 Abdominal aortic aneurysms

Abdominal aortic aneurysm is an inflammatory condition which results in the enlargement of the aorta by at least one and a half times its original diameter (Meital *et al.* 2017). The aorta continually expands due to the weakening of the arterial wall caused by ECM degradation until it eventually ruptures and results in death (Meital *et al.* 2017). The inflammatory reaction during aortic aneurysm formation is primarily associated with an increased accumulation of macrophages, granulocytes, B- and T-cells within the walls of the artery (Meital *et al.* 2017). Furthermore the levels of several pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α are known to be increased in patients with an aortic aneurysm (Meital *et al.* 2017).

CardioWise treatment in wild type mice receiving a high fat diet results in decreased monocyte proliferation (Fig. 4.21), whereas catechin treatment resulted in a reduction in proportion of granulocytes found within the bone marrow (Fig. 7.23). Additionally both treatments reduced the number of T-cells present within the bone marrow (Figs. 4.22 and 7.24). Furthermore a reduction in the circulating levels of IL-1β, IL-6 and TNF-α was observed in mice treated with CardioWise when compared to the vehicle control treatment mice (Fig. 4.8). Catechin treatment was also found to attenuate the circulating levels of these cytokines (Figs. 7.9 and 7.10). These results would indicate that both CardioWise and catechin dietary supplementation have the potential to reduce the inflammatory response which occurs during aortic aneurysm.

At the time of writing there has only been one human clinical trial performed which investigated the effect of ω-3 PUFA dietary supplementation on aortic aneurysm formation (Berger *et al.* 2008). The small trial involved 24 patients recovering from abdominal aortic aneurysm surgery receiving either ω-3 PUFA supplementation or a vehicle control for 4 days (Berger *et al.* 2008). The dietary intervention was found to be ineffective as both sets of patients had increased serum CRP and lipid peroxidation levels after 4 days, indicating no difference in their

inflammatory state (Berger *et al.* 2008). However the study did not investigate the effect of ω-3 PUFA supplementation on the circulating levels of pro-inflammatory cytokines, which would have provided more information about the patient's inflammatory state (Meital *et al.* 2017).

A recent study using rats fed a standard chow diet found that green tea extract is capable of attenuating abdomen aortic aneurysm development (Setozaki *et al.* 2017). The rats were fed green tea extract for 2 weeks before an aortic aneurysm was surgically induced. Dietary supplementation continued for a further 4 weeks (Setozaki *et al.* 2017). In comparison to the vehicle control receiving rats, those which received the green tea extract had a smaller aortic diameter and a thicker arterial wall (Setozaki *et al.* 2017). Furthermore these rats had attenuated TNF-α, IL-1β and MMP9 gene expression levels, as well as reduced MMP activity (Setozaki *et al.* 2017). Catechin was found to exert similar effects in our study as it was capable of reducing the serum levels of IL-1β and TNF-α (Figs. 7.9 and 7.10), in addition to decreasing MMP activity (Fig. 6.22). Due to the limited number of studies which have investigated the potential benefits of using nutraceutical to retard the development of abdominal aortic aneurysms, both CardioWise and catechin should be used in future *in vivo* studies to explore their potential benefits in greater detail.

8.6 The limitations and future directions of this study

The major limitation associated with this study is the lack of a standard chow diet control mice during the *in vivo* assays. This was due to the limited availability of mice at the time of the study. Mice fed a chow diet would establish a baseline by which the high fat diet mice could be compared to. It would provide a better understanding of the effect of a high fat diet on all *in vivo* parameters measured, especially the effect of a high fat diet on cell populations within the bone marrow. A baseline would also allow a better interpretation of the changes induced by CardioWise and catechin treatment (i.e. Do the nutraceutical therapies reverse the impact of a high fat diet on wild type mice?). Therefore all future studies using CardioWise and catechin treatment should also include chow fed mice.

This study was performed using wild type mice. Although this is not strictly a limitation as this study was designed to generate initial data to evaluate the potential of performing long term feeding studies. However future studies should use atherosclerotic mouse models, either *ApoE* or *LDLr* deficient mice, as these mice are capable of developing plaques unlike wild type mice. The CardioWise and catechin treatment period should also be increased from 3 to 12 weeks. This is to allow adequate time for plaques to develop as this time point has been shown to be effective in previous studies (Chen *et al.* 2017a; Shamsuzzaman *et al.* 2017; Tang *et al.* 2017). The number of weeks also needs to be increased as patients will be expected to receive nutraceutical therapy for a prolonged period of time. Therefore it is essential to determine no

detrimental effects occur from sustained CardioWise and catechin treatment. By using atherosclerosis prone mice, it would allow the effects of CardioWise and catechin treatment to be assessed on several other parameters of atherosclerosis development that could not be determined in this study. For example the effect of CardioWise and catechin treatment on plaque size, stability and cellular composition could be assessed. This would provide a greater insight into the cardiovascular protective effects of CardioWise and catechin as well as develop a better understanding of their mechanism of action.

Another aspect which could be changed in future studies is the time point at which CardioWise and catechin treatment is delivered. As one of the possible use of nutraceutical therapy is to delay a patient's need for traditional pharmaceutical interventions, studies need to be designed with this aspect in mind. CardioWise and catechin treatment should be given to atherosclerotic prone mice for a short time period while receiving a standard chow diet before switching to a high fat diet. A delay in the development of atherosclerotic plaques would represent a preventative effect. Other studies should do the reverse and move mice onto a high fat diet for several weeks before CardioWise or catechin intervention (Chistiakov *et al.* 2017). This would allow plaques to develop to equal size. Once atherosclerotic lesions have formed, treating the mice with either CardioWise or catechin would determine whether either nutraceutical intervention is capable of inducing plaque regression and restore blood vessel function in individuals with advanced atherosclerosis.

Another potential use of nutraceutical therapies is to use them in combination with traditional pharmaceuticals as an additional way to achieve health targets. For this reason studies should also treat atherosclerotic mice with CardioWise or catechin in combination with statins. In all likelihood, this scenario would represent the majority of patients who would use nutraceutical therapies. Therefore it is essential to assess the effects of CardioWise and catechin as an additional add-on therapy to statins to ensure there are no detrimental effects and whether further reductions in atherosclerosis development can be achieved.

Throughout this study, indirect evidence showed that CardioWise treatment is capable of activating non-shivering thermogenesis in brown adipose tissue. Therefore studies should also be designed to investigate this aspect by testing the levels of markers of brown fat activation within the fat deposits of mice (Harms and Seale 2013; Peirce *et al.* 2014). The body temperature of the mice should also be measured routinely throughout the study as an increase in body temperature can be a sign of increased brown fat activation (Crane *et al.* 2014; Meyer *et al.* 2017). Furthermore due to the ability of catechin to lower blood pressure, future studies should determine the blood pressure of the mice periodically over the entire time course of the trial by using a tail cuff (Wang *et al.* 2017b).

Some of the results produced during this study were only found to result in non-significant trends of change. Therefore care must be taken when interpreting these results and drawing conclusions. Future studies should investigate these trends further in order to produce more definitive conclusions.

Once these studies have been performed, the anti-atherogenic properties of CardioWise and catechin and their mechanism of action should be well established. However to fully determine the efficacy of CardioWise and catechin treatment for atherosclerosis, long term human trials need to be conducted in participants who are and are not receiving statins over an extensive treatment time period. Only after randomised clinical trials can the effectiveness of CardioWise and catechin treatment be fully determined.

8.7 The limitations within the nutraceutical field and future directions

Research into the cardiovascular protective effects of nutraceuticals has grown over the years. Nutraceuticals have emerged from both preclinical and clinical studies as possible anti-atherogenic compounds which have the potential to reduce an individual's risk of suffering a CVD-related event such as MI or stroke (Moss and Ramji 2016b). It is essential that the health benefits of nutraceuticals continue to be explored as current and emerging pharmaceutical therapies are either not fully effective or fail to progress from clinical trials to approval. However, research on nutraceuticals has often lagged behind pharmaceutical therapies for a variety of reasons.

Clinical trials for nutraceuticals often occur on a small scale and within a population from a single country, which fails to account for differences in lifestyle and diets (Moss and Ramji 2016b). This heterogeneity within the populations used for different nutraceutical clinical trials may explain why some are found to be successful while others fail to find any additional health benefits from dietary supplementation. To overcome this issue, large, robust randomised clinical trials which recruit participants from multiple countries should be performed. This is currently the rationale behind the REDUCE-IT and STRENGTH clinical trials which are assessing the cardiovascular protective effects of ω-3 PUFAs (U.S. National Institutes of Health 2017a; U.S. National Institutes of Health 2017c). Furthermore consistency is essential in human trials. Studies often differ in nutraceutical composition, concentration and duration of intervention, which only contributes to the discrepancy that often occurs within the published literature in relation to nutraceutical's effects and efficacy.

To reduce the failure rate at the human clinical trial level, an improvement in the design of preclinical studies would aid in the identification of the most effective nutraceuticals. The major limitation of preclinical nutraceutical research is the dosage used as they are often too high. Therefore studies should perform experiments with a concentration of the desired nutraceutical

at a dosage which would be found within the target tissue after consumption. Furthermore the use of dose-response experiments should be utilised whenever possible to ensure the most effective dosage is delivered to patients. Additionally preclinical studies should also focus on the mechanism of action behind potential cardiovascular protective effects of nutraceuticals. Developing a better understanding of how nutraceuticals are capable of exerting their anti-atherogenic effects may aid in the development of novel pharmaceutical therapies which are capable of mimicking the key responses. For example, GPR120 was identified as the potential receptor by which ω-3 PUFAs mediated their anti-inflammatory and insulin sensitising effects (Oh *et al.* 2010). This led to the development of an orally administered cpdA, which is a highly selective small molecule GRP120 agonist capable of improving insulin resistance and chronic inflammation in obese mice (Oh *et al.* 2014).

8.8 Conclusion

Using our *in vitro* models of atherosclerosis disease progression, we were able to show that CardioWise, which is a unique nutraceutical combination of ω-3 PUFAs, flavanols and phytosterols, was capable of exerting anti-atherogenic effects. These anti-atherogenic effects were also observed in wild type mice fed a high fat diet. The major flavanol, (+)-catechin, was identified as a potent anti-atherogenic compound *in vitro* and *in vivo*. Future studies are required to explore the effect of both CardioWise and catechin as add-on therapies for statins in atherosclerotic mouse models. This study has shown that nutraceuticals are effective anti-inflammatory therapies capable of providing cardiovascular protective effects and therefore represent promising emerging therapies for atherosclerosis. We are entering an exciting phase in nutraceutical research.

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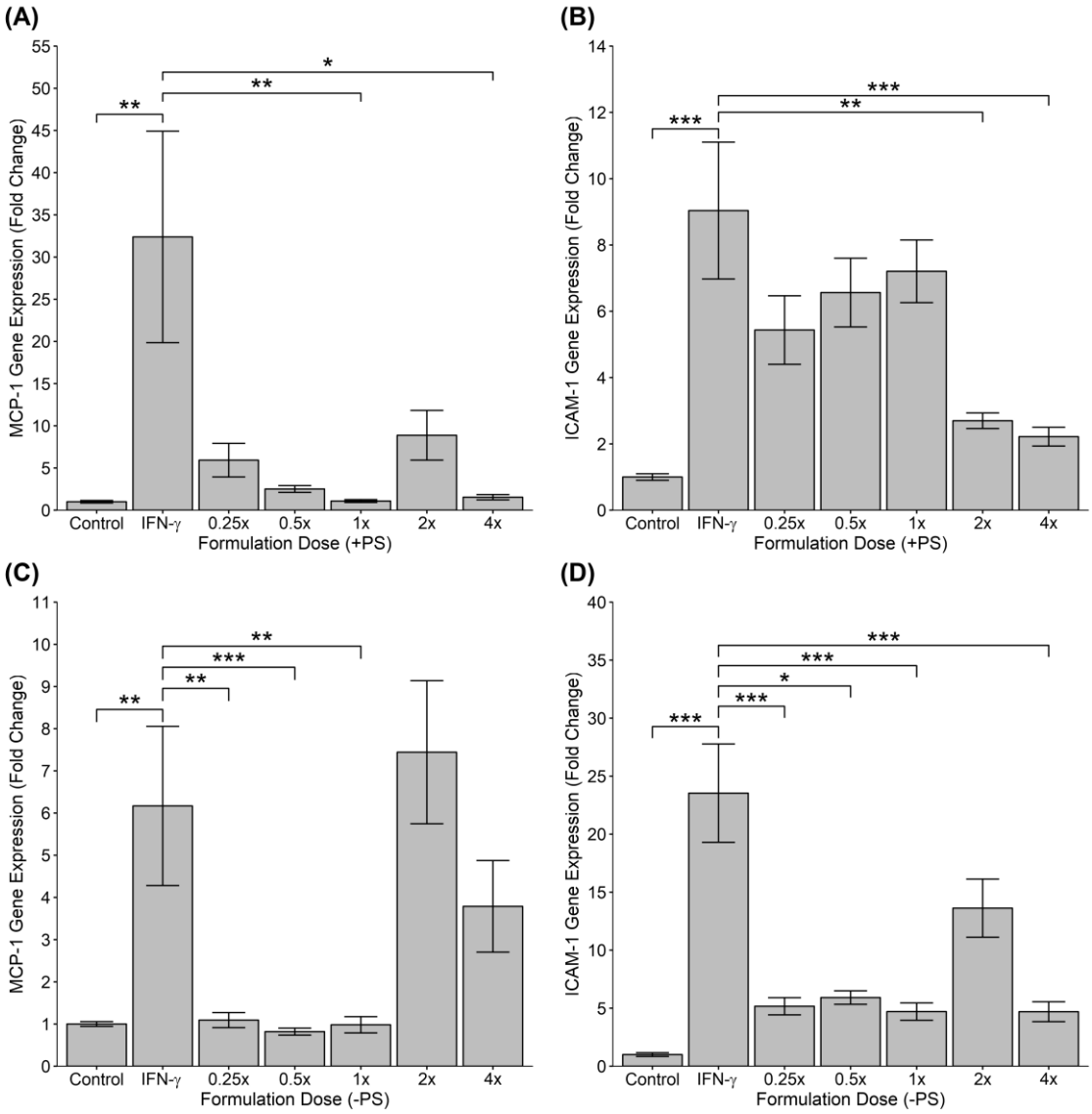
Supplementary

S.1 CardioWise attenuates the expression of pro-inflammatory genes following IFN-γ stimulation in murine macrophages

IFN-γ is capable of inducing the expression of two key markers of inflammation, MCP-1 and ICAM-1, which also play major roles in the development of atherosclerosis development (Moss and Ramji 2016a; Li *et al.* 2010). The expression of MCP-1 in RAW264.7 macrophages was significantly increased by 32.39 and 6.17 fold (p=0.006 and p=0.004) following IFN-γ stimulation (250 U/ml; Supplementary Fig. S.1A and S.1C). Furthermore the transcript levels of ICAM-1 were increased by 9.04 and 23.54 fold (p<0.001 and p<0.001) when compared to the vehicle control (Supplementary Fig. S.1B and S.1D). When treated with either a 1x or 4x physiological dose of the complete CardioWise formulation, the IFN-γ stimulated MCP-1 expression was attenuated by 96.6% (p=0.010) and 95.2% (p=0.021) respectively (Supplementary Fig. S.1A). A decrease of 70.1% (p=0.009) in IFN-γ induced expression of ICAM-1 was observed when cells were incubated with 2x dose of complete CardioWise, whereas the 4x dose reduced ICAM-1 expression by 75.5% (p=0.001; Supplementary Fig. S.1B). Similar observations were apparent in murine RAW264.7 macrophages which were treated with CardioWise in the absence of phytosterols. The expression of MCP-1 was reduced by 82.3% (p=0.004), 86.7% (p=0.001) and 84.1% (p=0.003) when treated with the 0.25x, 0.5x or 1x physiological dose respectively (Supplementary Fig. S.1C). In comparison to the IFN-γ stimulated cells, the expression of ICAM-1 was attenuated in the presence of CardioWise lacking phytosterols at the 0.25x, 0.5x, 1x and 4x dose by 78.0% (p=0.001), 74.9% (p=0.014), 80.0% (p<0.001) and 80.1% (p<0.001) respectively (Supplementary Fig. S.1D).



Supplementary Fig. S.1. CardioWise can inhibit the IFN-γ induced expression of MCP-1 and ICAM-1 at physiologically relevant doses in murine macrophages. The expression of MCP-1 (A and C) and ICAM-1 (B and D) was assessed in RAW264.7 macrophages that were either treated with vehicle (vehicle control) or with IFN-γ (250 U/ml) or IFN-γ (250 U/ml) in the presence of the complete CardioWise formulation (+PS; A and B) or with IFN-γ (250 U/ml) in the presence of CardioWise lacking phytosterols (-PS; C and D) for 3 hours. Gene expression levels were assessed using qPCR and calculated using the comparative Ct method and normalised to β-actin levels with values from vehicle treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from four (A and C) or five (B and D) independent experiments. Statistical analysis was performed on the log-transformed data using a one-way ANOVA with either a Dunnett (D) or Games-Howell (A, B and C) post-hoc analysis where * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

S.2 First authored publications

The following publications were written and accepted during the project time period.

S.3.1 Interferon-γ: Promising therapeutic target in atherosclerosis

World Journal of Experimental Medicine

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
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EDITORIAL

Interferon-γ: Promising therapeutic target in atherosclerosis

Joe WE Moss, Dipak P Ramji

Joe WE Moss, Dipak P Ramji, Cardiff School of Biosciences, Cardiff University, Cardiff CF10 3AX, United Kingdom

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Correspondence to: Dr. Dipak P Ramji, Cardiff School of Biosciences, Cardiff University, Sir Martin Evans Building, Museum Avenue, Cardiff CF10 3AX, United Kingdom. ramji@Cardiff.ac.uk
Telephone: +44-29-20876753
Fax: +44-29-20874116

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Abstract

Atherosclerosis is a chronic inflammatory disorder of the vasculature and is the primary cause of cardiovascular disease (CVD). CVD is currently the world's leading cause of death and the numbers are predicted to rise further because of a global increase in risk factors such as diabetes and obesity. Current therapies such as statins have had a major impact in reducing mortality from CVD. However, there is a marked residual CVD risk in patients on statin therapy. It is therefore important to understand the molecular basis of this disease in detail and to develop alternative novel therapeutics. Interferon-γ (IFN-γ) is a pro-inflammatory cytokine that is often regarded as a master regulator of atherosclerosis development. IFN-γ is able to influence several key steps during atherosclerosis development, including pro-inflammatory gene expression, the recruitment of monocytes from the blood to the activated arterial endothelium and plaque stability. This central role of IFN-γ makes it a promising therapeutic target. The purpose of this editorial is to describe the key role IFN-γ plays during atherosclerosis development, as well as discuss potential strategies to target it therapeutically.

Key words: Atherosclerosis; Interferon-γ; Inflammation; Neutralization; MicroRNA

Core tip: Atherosclerosis is an inflammatory disorder of the vasculature and studies in mouse model systems have highlighted the beneficial effects of counteracting inflammation in limiting the progression of this disease. Due to its key role in inflammation and atherosclerosis development, interferon-γ (IFN-γ) is seen as a promising therapeutic target. In this editorial we discuss the role of IFN-γ in atherosclerosis together with potential therapeutic approaches against this cytokine and its key downstream targets.

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INTRODUCTION

Atherosclerosis is the underlying cause of cardiovascular disease (CVD) such as myocardial infarction (MI) and

stroke. The World Health Organisation estimated that there were 17.5 million deaths from a CVD-related event in 2012, equating to approximately 1 in 3 global deaths[1]. The number of global deaths related to CVD has been predicted to increase due to rises in the incidences of obesity and diabetes and the acquisition of a westernised diet in developing countries. The disease is a major healthcare and economic burden and therefore there is a need to understand the disease in more detail and to develop new therapeutic approaches.

ATHEROSCLEROSIS DEVELOPMENT

Atherosclerosis is a chronic, inflammatory disease characterized by the formation of foam cells in initial atherosclerotic lesions which then progress into advanced plaques. Low-density lipoprotein (LDL) can become trapped in the intima of medium and large arteries and modified to oxidized LDL (OxLDL). The presence of OxLDL triggers an inflammatory response in the neighbouring endothelial cells (ECs), causing the release of a variety of pro-inflammatory cytokines and chemokines, and expression of adhesion molecules on the cell surface (activation of ECs). These factors include macrophage chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) as well as P- and E-selectins[2,3]. Such pro-inflammatory molecules guide circulating monocytes in the blood stream to the OxLDL accumulation in the intima of arterial walls and aid the progression of atherosclerosis development[4-6]. Once in the intima the monocytes become exposed to macrophage colony-stimulating factor, triggering their differentiation into macrophages as well as inducing scavenger receptor (SR) expression on their surface[2,7]. Macrophages are then able to uptake OxLDL by SR-mediated endocytosis, macropinocytosis or phagocytosis and develop into foam cells, causing the appearance of the initial lesions and fatty streaks in arteries, which can then progress into mature plaques[8,9].

Mature atherosclerotic plaques are made up of vascular smooth muscle cells (VSMCs) and extracellular matrix (ECM), as well as accumulated OxLDL, cholesterol and apoptotic cells, which form a lipid-rich necrotic core[10]. During plaque progression VSMCs proliferate and migrate towards the LDL accumulation and form a fibrous cap, which is tightly controlled and influenced by the nearby macrophages, ECs and T-cells[2,11]. As the fibrous cap continues to develop it forms a stable lesion by covering the large lipid-rich necrotic core, therefore the balance of ECM production and degradation can affect the stability of the lesion[2]. If the plaque ruptures it triggers a thrombotic reaction and in turn platelet aggregation, which can quickly impede or obstruct blood flow through the artery[7]. Depending on the location of the rupture it can potentially cause a MI or stroke. Therefore acute CVD events may be manageable by affecting plaque stability and preventing them from rupturing[7,12]. Amongst the cytokines involved in the development of atherosclerosis, interferon-γ (IFN-γ) is potentially a master regulator and will therefore be addressed in more detail.

IFN-γ

IFN-γ is a key pro-inflammatory cytokine in atherosclerosis development as it is capable of inducing the expression of approximately a quarter of genes expressed in macrophages[3]. Immune cells present in the atherosclerotic lesions, including T-lymphocytes, natural killer T-cells, macrophages and other antigen presenting cells, secrete IFN-γ at pronounced levels[13,14]. Stimulation of many signaling pathways that regulate the immune and inflammatory responses can be induced by IFN-γ. The major signaling pathway that IFN-γ signals through is the Janus kinase (JAK)-Signal Transducers and Activators of Transcription (STAT) pathway[3].

JAK-STAT pathway

The IFN-γ cell surface receptor complex (IFN-γR) is made up of two subunit pairs (IFN-γR1:IFN-γR) which dimerize upon binding of the cytokine[13]. Bound to each subunit are two JAKs 1 and 2, which become activated by phosphorylation of tyrosine residues in the N-terminus in a mainly JAK2-dependent process[15]. Once activated, the JAKs phosphorylate the tails of the IFN-γR which triggers the recruitment of STAT1 monomers from the cytoplasm that then interact with the receptor *via* their src-homology 2 domains[16]. The recruited STAT1 monomers are then phosphorylated by the JAKs at tyrosine 701 and dissociate from the receptor complex to form STAT1:STAT1 homodimers[3]. The dimer is then able to translocate into the nucleus and stimulate the transcription of IFN-γ target genes, such as MCP-1 and ICAM-1, by binding to γ-activated sequence (GAS) elements in their promoters[13,15]. Furthermore, extracellular signal-regulated kinase (ERK) and other kinases are capable of phosphorylating the homodimer at serine 727 for maximal activity[17].

ROLE OF IFN-γ IN ATHEROSCLEROSIS DEVELOPMENT

Therapeutically targeting IFN-γ in order to reduce the incidence of CVD represents a promising avenue due to its pro-inflammatory functions during atherosclerotic plaque formation, including the recruitment of immune cells to the site of OxLDL accumulation, foam cell formation, and plaque development and stability. A 2-fold increase in the size of atherosclerotic lesions has been reported in the Apolipoprotein E (ApoE) deficient mouse model that was injected with recombinant IFN-γ every day, even with a 15% reduction in plasma cholesterol levels[18]. Furthermore, ApoE deficient mice which also lacked IFN-γR showed a reduction in atherosclerosis development, as well as a 60% decrease in lipid

build up in the lesions when fed on a western diet[19]. Deficiency of STAT1 in mouse model systems is also associated with reduced atherosclerosis development and foam cell formation, highlighting the key role of the JAK-STAT1 pathway in IFN-γ signaling during plaque progression[20,21].

Recruitment of immune cells

IFN-γ is a key recruiter of immune cells in the development of atherosclerosis and therefore important in the growth of lesions[22]. IFN-γ has been shown to be localized in atherosclerotic lesions and mice models lacking either IFN-γ or its receptor have been reported to have a reduced cellular content in their lesions[19,23,24]. The expression of key pro-atherogenic chemokines and their receptors, such as MCP-1 that has been detected in atherosclerotic lesions by immunohistochemistry and *in situ* hybridization, can be induced by IFN-γ[25,26]. Mouse models which were deficient for either MCP-1 or its receptor showed a reduced cellular content in lesions, as well as a reduction in the size of the lesions without changes in circulating lipid or lipoprotein levels[25]. IFN-γ can also influence the recruitment of immune cells by inducing the expression of adhesion molecules, such as ICAM-1 and VCAM-1, in ECs during the early stages of atherosclerosis development[27,28].

Foam cell formation

Cholesterol uptake and efflux is carefully balanced during homeostasis of this sterol in healthy cells. The formation of foam cells can be regarded as a pathological imbalance in favour of reduced cholesterol efflux and increased uptake of OxLDL[7,29]. The expression levels of a number of key genes involved in cholesterol metabolism are regulated by IFN-γ, including ApoE, ATP-binding cassette transporter A1 (ABCA1) and acetyl-CoA acetyltransferase 1 (ACAT1)[22]. *In vitro* studies that have incubated macrophage-derived foam cells with IFN-γ have shown a reduction in cholesterol efflux *via* increasing the expression of ACAT1 and attenuating the expression of ABCA1, resulting in increased accumulation of intracellular cholesteryl esters which promote the formation of foam cells[30]. Furthermore, the expression of several key SRs in foam cell development, including SR-A and SR that binds phosphatidylserine and oxidized lipids (SR-SPOX; also known as CXCL16), have been shown to be increased in human THP-1 and primary macrophages stimulated with IFN-γ, resulting in an increased uptake of OxLDL[31-33]. Therefore IFN-γ is capable of altering cholesterol homeostasis towards lower cholesterol efflux and higher retention of OxLDL in macrophages and contributes to foam cell formation.

Plaque progression and stability

IFN-γ can influence a variety of processes involved in the development of the early atherosclerotic lesions into mature plaques as well as their stability. Part of plaque development involves the migration of VSMCs and the formation of the fibrous cap. IFN-γ induces the expression of integrins on the surface of VSMCs which are capable of binding to fibronectin in ECM, triggering the VSMCs to differentiate from their inactive to their proliferative phenotype allowing migration towards the lesion to form the fibrous cap[34]. The stability of atherosclerotic plaques relies on the balance of ECM production and degradation which can also be affected by IFN-γ[2,22]. Foam cell apoptosis is also promoted by IFN-γ and causes them to expel their contents into the intima, contributing to the lipid-rich necrotic core and ECM degradation[35,36]. The balance can be tipped further towards ECM degradation by IFN-γ-mediated inhibition of the expression of several collagen genes, thereby suppressing matrix synthesis by VSMCs and resulting in reduced plaque stability and increased risk of a rupture[7]. ECM degradation can also be triggered by matrix metalloproteinases (MMPs) which are found in atherosclerotic plaques and are often localized to the shoulder regions where a rupture is more likely to occur[37]. MMPs are released by macrophages and VSMCs and their expression can be induced by IFN-γ stimulation[38].

THERAPEUTICALLY TARGETING IFN-γ

Due to the high prevalence of CVD there are a variety of therapeutics designed to reduce various aspects of atherosclerosis development, including decreasing serum cholesterol levels and altering the expression of genes that are involved in cholesterol metabolism or the inflammatory response[3,39]. Statins, the most widely used and successful cholesterol lowering therapy class of drugs, are primarily designed to inhibit the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMG CoA reductase)[3]. HMG CoA reductase catalyses the rate limiting step in cholesterol biosynthesis, thereby lowering the levels of circulating LDL[40]. However there is a marked residual risk of CVD in patients on statin therapy, with a significant proportion unable to attain their target LDL levels even when receiving the highest recommended dosage, stressing the importance of developing new therapeutics[2,41].

One new potential therapeutic target is IFN-γ due to its key roles in atherosclerosis development. There are currently two strategies that have been developed that either target IFN-γ directly (IFN-γ neutralization) or inhibit its signaling pathways. Statins and agonists of nuclear receptors also attenuate IFN-γ actions in part by modulating its signal transduction pathways[42-44]. In human macrophages, IFN-γ-induced phosphorylation of STAT1 on serine 727 can be blocked using adenosine[45]. Work by Lee *et al*[46] has shown that stimulation of the adenosine A3 receptor with a novel agonist, thio-CL-IB-MECA, resulted in attenuated IFN-γ-induced STAT1-dependent gene expression. Furthermore a naturally occurring phenol in plant extract, resveratrol, is capable of preventing STAT1 phosphorylation at tyrosine 701

or serine 727 as well as JAK2 activation in human macrophages *in vitro*[47]. These compounds represent promising avenues for therapies targeted at the downstream signaling events in the JAK-STAT pathway in order to reduce the pro-inflammatory effects of IFN-γ. Other therapies target IFN-γ *via* alternative signaling pathways, for example, ACS14 (a hydrogen sulphide releasing aspirin) is capable of attenuating the expression of IFN-γ-stimulated CX3 chemokine receptor 1 (CX3CR1) *via* a peroxisome proliferator-activated receptor-γ-dependent mechanism[48]. Hydrogen sulphide has previously been shown to exert anti-atherogenic effects and its use in ACS14 has been shown to reduce atherosclerosis development in ApoE mice models[48,49].

IFN-γ neutralization involves the use of a soluble IFN-γR (sIFN-γR) which acts as decoy receptor to prevent the activation of IFN-γR and in turn the phosphorylation of STAT1 in the JAK-STAT pathway, in effect "neutralizing" the IFN-γ. The approach was first developed by Koga *et al*[50], and demonstrated in ApoE mice which were fed a high fat diet for 8 wk and given two intramuscular injections of a plasmid encoding sIFN-γR at weeks 4 and 6. Compared to the control mice, those that received the sIFN-γR injections had dramatically reduced atherosclerotic lesion size as well as greater plaque stability. This increase in plaque stability was found to be due to an increase in the number of VSMCs in the fibrous cap in addition to greater collagen deposition. Additionally, there was also a decrease in the amount of lipid accumulation and number of macrophages in the necrotic core, which further improved plaque stability and reduced the risk of rupture. Furthermore, neutralizing antibodies have been used for other cytokines such as IL-1β and show great therapeutic promise[51,52], therefore similar strategies could potentially be developed to use antibodies to achieve IFN-γ neutralization.

Although targeting IFN-γ in atherosclerosis development may result in reduced lesion size and improved plaque stability, there are potential drawbacks that need to be assessed before IFN-γ targeting can be recommended therapeutically. The major concern involves the systemic inhibition of IFN-γ due to the major role it performs in the immune response[53]. Sustained universal inhibition of IFN-γ may increase an individual's risk of acquiring intracellular infections and tumour development[53]. On the other hand it may benefit those high-risk patients who are unable to achieve target LDL plasma levels using currently available therapeutics. A possible solution to overcome universal inhibition would be to try and develop a drug delivery system, for example using nanoparticles, that would allow IFN-γ-targeted therapeutics to be delivered to a specific location rather than system wide[53,54].

Another possible solution would be to target further downstream targets of the IFN-γ signaling pathways, either alone or in combination with therapies that target IFN-γ directly. IFN-γ is known to induce the expression of several microRNAs (miRNAs) in addition to having its own expression regulated by miRNAs[55]. miRNAs are short non-coding single-stranded RNAs approximately 19-25 nucleotides in length that are evolutionary conserved in eukaryotic organisms[56]. Evidence is continuously accumulating that indicates that miRNAs are capable of regulating gene expression by inhibiting translation or inducing targeted mRNA degradation[57]. miRNAs have also been found to regulate a number of key steps during atherosclerosis development, including the inflammatory response triggered by IFN-γ[58-60]. One miRNA that is thought to play a key role in atherosclerosis development is miR-155. Evidence for the role of miR-155 in the inflammatory response was found by O'Connell *et al*[61]. miR-155 was the only miRNA out of 200 tested that was considerably up-regulated in primary murine macrophages after being treated with pro-inflammatory stimulants. Additional evidence for the involvement of miR-155 in the inflammatory response comes from studies which have shown its levels to be up-regulated in macrophages in atherosclerotic lesions as well as having an association with increased pro-inflammatory cytokine expression, potentially due to its ability to repress the expression of the Suppressor of Cytokine signaling 1 (*SOCS1*) gene[62-64]. However the specific role miR-155 plays during atherosclerosis is still being debated, with a number of studies reporting miR-155 to exert pro-atherogenic effects in ApoE deficient mouse models[65,66]. Targeting miRNAs, which are either regulated by IFN-γ and are known to be involved in atherosclerosis development or regulate the expression of IFN-γ, may provide an excellent therapeutic avenue that allows specific arterial targeted treatment to reduce atherosclerosis development and improve plaque stability without potential consequences from systemic IFN-γ inhibition.

CONCLUSION

Due to the central role of IFN-γ during atherosclerosis development and plaque stability, along with the expected rise in global rates of CVD-related events, this cytokine represents a promising therapeutic target. Targeting either IFN-γ directly or its signaling pathways in both *in vitro* and *in vivo* studies has shown that directed therapies have the potential of reducing atherosclerosis development. However the potential side effects of long term IFN-γ inhibition still needs to be assessed.

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S.3.2 A Unique Combination of Nutritionally Active Ingredients Can Prevent Several Key Processes Associated with Atherosclerosis *In Vitro*

PLOS | ONE

RESEARCH ARTICLE

A Unique Combination of Nutritionally Active Ingredients Can Prevent Several Key Processes Associated with Atherosclerosis *In Vitro*

Joe W. E. Moss[1], Thomas S. Davies[1], Iveta Garaiova[2], Sue F. Plummer[2], Daryn R. Michael[2]*, Dipak P. Ramji[1]

1 School of Biosciences, Cardiff University, Cardiff, United Kingdom, **2** Cultech Limited, Baglan Industrial Park, Port Talbot, United Kingdom

* darynm@cultech.co.uk

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Abstract

Introduction

Atherosclerosis is the underlying cause of cardiovascular disease that leads to more global mortalities each year than any other ailment. Consumption of active food ingredients such as phytosterols, omega-3 polyunsaturated fatty acids and flavanols are known to impart beneficial effects on cardiovascular disease although the combined actions of such agents in atherosclerosis is poorly understood. The aim of this study was to screen a nutritional supplement containing each of these active components for its anti-atherosclerotic effect on macrophages *in vitro*.

Results

The supplement attenuated the expression of intercellular adhesion molecule-1 and macrophage chemoattractant protein-1 in human and murine macrophages at physiologically relevant doses. The migratory capacity of human monocytes was also hindered, possibly mediated by eicosapentaenoic acid and catechin, while the ability of foam cells to efflux cholesterol was improved. The polarisation of murine macrophages towards a pro-inflammatory phenotype was also attenuated by the supplement.

Conclusion

The formulation was able to hinder multiple key steps of atherosclerosis development *in vitro* by inhibiting monocyte recruitment, foam cell formation and macrophage polarisation towards an inflammatory phenotype. This is the first time a combination these ingredients has been shown to elicit such effects and supports its further study in preclinical *in vivo* models.

responsible for project conception. DRM was involved in the design of the study and contributed to manuscript writing. The Innovation voucher (2014/BIV/340 R SS) provided additional research materials.

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Introduction

Cardiovascular disease (CVD) related events, such as myocardial infarction (MI) and stroke, are the leading causes of global death every year. The World Health Organisation estimated that there were approximately 17.5 million deaths from CVD-related events in 2012 [1] and this figure has been predicted to rise to 23.3 million by 2030 due to the global increase in obesity and diabetes, and the incorporation of a westernised lifestyle in developing countries [1]. Atherosclerosis is the major underlying cause of CVD, emphasising the need to develop novel approaches to support disease prevention.

Atherosclerosis is a chronic, inflammatory disease of the vasculature characterised by the formation of lipid laden foam cells [2–5]. The initial trigger of atherosclerosis is the accumulation and trapping of ApoB-containing lipoproteins, such as low-density lipoprotein (LDL), in the intima of medium and large arteries [3–5]. Modification of the trapped LDL, particularly oxidised (ox)-LDL, instigates an inflammatory response in the nearby endothelial cells (ECs) [3–5]. Activated ECs then release pro-inflammatory cytokines and chemokines, such as macrophage chemoattractant protein-1 (MCP-1), which direct circulating monocytes and T lymphocytes to the site of oxLDL accumulation [3–5]. Additionally, the ECs also begin to express cell adhesion molecules on their surface, including intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), as well as P- and E-selectins, which aid the adhesion of circulating monocytes to the site of oxLDL accumulation [3–5]. Once in the intima, the monocytes differentiate into macrophages which are then able to uptake oxLDL and develop into foam cells [3–5]. Over time, the continued and aberrant recruitment of macrophages to the site of insult results in the accumulation of foam cells that eventually develop into an unstable atherosclerotic plaque that, upon rupture, leads to thrombosis, MI and stroke [3–5].

Numerous macrophage phenotypes reside within atherosclerotic lesions and this population is largely composed of classical M1 or alternative M2 polarised macrophages [3, 5]. Under normal physiological conditions, M2 polarisation occurs as a result of IL-4 stimulation and is usually associated with the resolution of inflammation and repair whereas M1 macrophages are activated by stimuli such as interferon-γ (IFN-γ), lipopolysaccharide (LPS) and oxLDL and participate in pro-inflammatory actions towards bacterial infections during the innate immune response [6]. However, due to their pro-inflammatory nature it is thought that the presence of M1 macrophages in atherosclerotic plaques can drive disease progression [7, 8] and it has been shown that this macrophage subset often localises to rupture-prone shoulder areas of atherosclerotic plaques [8]. Approaches to reduce M1 accumulation may result in more stable atherosclerotic plaques and also represent an ideal target for disease intervention.

It has been estimated that approximately 70% of cardiovascular disease related events are not prevented by the current therapies which includes the use of statins [9]. These are the most extensively used class of cholesterol lowering therapy for CVD and their primary function is to inhibit the enzyme 3-hydroxy-3-methylglutaryl-CoA, which catalyses the rate-limiting step in cholesterol biosynthesis [4]. However, a significant minority of people receiving statins are unable to lower their LDL levels even at the maximal dose [4] and are prone to adverse side effects such as increased risk of type 2 diabetes [10, 11] stressing the need to develop novel approaches to prevent atherosclerosis. It has been known for some time that the consumption of various active food ingredients can improve cardiovascular health. For example, numerous epidemiological studies [12, 13] and clinical trials [14, 15] examining the effects of fish oil consumption, particularly the omega-3 polyunsaturated fatty acids (ω-3 PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have reported lower plasma cholesterol levels and a reduced risk of a MI. EPA and DHA have also been shown to dampen the inflammatory

response by macrophages by directly binding to G protein-coupled receptor (GP)-120 present on the cell surface [16] which is likely to contribute to the observed anti-foam cell effects of these fatty acids [17]. Flavanoids, a group of secondary plant metabolites that can be found in foods such as cocoa beans and specific flavonoid subgroups, such as flavanols (catechins), have also been shown to reduce the expression of pro-inflammatory genes [18], and circulating LDL levels *in vivo* [19]. An abundant form of catechin, epigallocatechin-3-gallate, has been shown to impart an anti-inflammatory effect on macrophages by directly binding to the cell surface laminin receptor (67LR) [20] and by regulating the expression of a range of trancription factors including nuclear factor κB (NF-κB) (reviewed extensively in [21]). Phytosterols (PS) are cholesterol homologues found in plants that have been shown to reduce plasma LDL levels in both observational and clinical studies [22, 23] by competitive exclusion of cholesterol from micellar space in the intestinal lumen and modulation of enterocyte cholesterol trafficking [24]. PS also have a direct effect on cholesterol homeostasis in macrophage-derived foam cells by regulating the expression of key cholesterol transport genes such as ATP-binding cassette transporter (ABC)A-1 and ABCG-1 [25].

The objective of this study is to assess the ability of a dual-action nutritional supplement comprised of mixed PS, ω-3 PUFA-rich fish oil and flavanol rich cocoa extract in a readily absorbable emulsified format [26, 27] to prevent the development of atherosclerosis by, firstly, reducing the amount of cholesterol absorbed in the intestines by the action of PS, followed by inhibition of the cellular mechanisms driving the disease after absorption. The supplement has been designed to present physiologically relevant doses of the active ingredients in a series of established *in vitro* models of atherosclerosis in order to demonstrate efficacy.

Methods

Regents and cell culture

All reagents were purchased from Life Technologies (Paisley, UK), unless otherwise stated. EPA, DHA, campesterol and β-sitosterol were purchased from Cambridge Biosciences (Cambridge, UK). Stigmasterol and (+)-catechin were purchased from Sigma-Aldrich (Poole, UK). Human acute monocytic leukemia cell line (THP-1; obtained directly from ECACC, Salisbury, UK (Catalogue number: 88081201)), primary human monocyte derived macrophages (HMDM) and Raw264.7 murine macrophage (obtained directly from Sigma-Aldrich, Poole, UK (Catalogue number: 85062803-VL)) were grown in complete RPMI-1640 (Lonza, Manchester, UK) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (2 mmol/l) at 37°C in a humidified atmosphere containing 5% (v/v) CO_2. THP-1 monocytes were differentiated into macrophages by incubation with 160 nM phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich, Poole, UK) for 24 hours to ensure high expression levels of genes implicated in the control of macrophage foam cell formation [28, 29]. HMDM were isolated from buffy coats supplied by the Welsh Blood service using Ficoll-Hypaque purification described elsewhere [29]. Ethical approval and informed consent for each donor was granted by the Welsh Blood Service for the use of human blood samples.

Formulation dosage and application to cells

The nutritional supplement formulation (S1 Table) was provided by Cultech Limited (Port Talbot, UK) as a concentrated stock (500x) designed to deliver each active ingredient at doses approximating to reported *in vivo* serum levels with a 1x dose composed of 180 μg/ml fish oil (delivering 30 μg/ml EPA, 19.7 μg/ml DHA [26]), 56 μg/ml of mixed PS (delivering 10 μg/ml Stigmasterol [30], 13.9 μg/ml Campsterol, 27.2 μg/ml Sitosterol and 1.672 μg/ml Brassicasterol)

and 7.25 μg/ml cocoa extract (delivering 5 μmol/ml catechins [31]). A PS-free version of the formulation was also provided. Fatty acid free bovine serum albumin (BSA; 100 μg/ml per 1x dose) was included in both formulations to act as a physiologically relevant protein carrier and to facilitate the emulsification of the formulations and allow their natural dispersion in an aqueous medium. BSA alone was used as the vehicle control at an appropriate concentration and the formulation was applied to the cells diluted in complete culture media unless otherwise stated. EPA, DHA, (+)-catechin, stigmasterol, campsterol and β-sitosterol were applied to the cells (at doses equivalent to those present in the complete formulation (x1)) in dimethyl sulfoxide (DMSO) that was also included at the relevant concentration as the vehicle control for these experiments.

Cell viability and proliferation assays

The cell supernatants from THP-1 macrophages ($4.11x10^5$ cells/cm^2) were removed and assayed for lactate dehydrogenase (LDH) content using the Pierce LDH cytotoxicity assay (Thermo Scientific, Waltham, USA) in accordance with the manufacturer's instructions. The remaining cells were then stained with 0.2% (w/v) crystal violet solution (in 10% (v/v) ethanol) for 5 minutes at room temperature before washing the cells three times in warm PBS (pH 7.4). Intracellular crystal violet was then solubilised in 0.1 M NaH_2PO_4 (in 50% (v/v) ethanol) and the absorbance was read at 570 nm using a colorimetric spectrophotometer. Viability (LDH assay) and proliferation (crystal violet) were expressed as a percentage of the BSA vehicle control that was arbitrarily assigned as 100%.

RNA extraction, reverse transcription, and quantitative PCR

RNA was extracted from THP-1 macrophages ($1.28x10^5$ cells/cm^2), HMDM ($1.28x10^5$ cells/cm^2) or Raw264.7 macrophages ($5.13x10^4$ cells/cm^2) using RiboZol™ RNA extraction reagent (Amresco, Solon, US) in accordance with the manufacturer's instructions and total RNA concentrations were quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, USA). cDNA was then reverse transcribed from 1000 ng of RNA as previously described [32]. Quantitative polymerase chain reaction (qPCR) was performed on 10 ng cDNA using SYBR Green JumpStart™ Taq Readymix™ and 50 nM of each oligonucleotide primer was used (see S2 Table). Fold changes in gene expression were calculated using $2^{-(\Delta Ct1-\Delta Ct2)}$, where ΔCt represents the difference between the threshold cycle (Ct) for each target gene and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript levels for human studies or β-actin mRNA transcript levels for murine studies. Initial melting (94°C for 120 seconds) was followed by 40 cycles of melting (95°C for 30 seconds), annealing (60°C for 60 seconds (66°C for Inducible Nitrogen Oxide Synthase (iNOS) and Arginase-2 (Arg2)), and extension (72°C for 60 seconds)). The inclusion of reverse transcriptase negative controls and melting curve analysis in each experiment confirmed there was no amplification from genomic DNA. The amplicon size of all primers sets were confirmed by agarose gel electrophoresis (data not shown).

Monocyte Migration

Monocyte migration was assessed by adding undifferentiated THP-1 monocytes (alone or with formulation or with individual bioactive components; $1x10^6$ cells/ml) to the apical compartment of Falcon® cell culture inserts (8 μm pore size) that were housed in Falcon® 12-well companion plates (VWR Jencons, Lutterworth, UK) containing complete RPMI media supplemented with MCP-1 (20 ng/ml; Peprotech, London, UK). Following incubation, apical medium was aspirated and the underside of the porous membrane washed into the wells to capture all migrating cells. Monocyte numbers present in the basolateral (lower) compartment

were counted using a haemocytometer and monocyte migration is expressed as a fold-change compared to the proportion of cells that moved from the apical compartment into the basolateral compartment in response to MCP-1 alone and has been arbitrarily set as 1.0.

Cholesterol efflux assay

Cholesterol efflux assays were carried out following a previously described protocol with minor adaptations [32]. Briefly, THP-1 macrophages ($1.28x10^5$ cells/cm^2) were incubated with acetylated (Ac)LDL (25 mg/ml; Biotrend, Cologne, Germany) and [4-^{14}C]cholesterol (0.5 mCi/ml; Amersham, Buckinghamshire, UK) in media containing 0.2% (v/v) fatty acid free BSA for 24 hours before they were treated for a further 24 hours with the previously described media containing Apolipoprotein A-I (ApoA-I) (10 mg/ml; Sigma-Aldrich, Poole, UK) with or without the formulation. Cell supernatants were then collected and the remaining cells were solubilised in 0.2 M NaOH. Cholesterol efflux was calculated as the percentage radioactivity in the supernatant versus total radioactivity (cells and supernatant) as measured by a liquid scintillation counter. Cholesterol efflux was expressed as a fold change of the vehicle control that was arbitrarily assigned as 1.0.

Data analysis

The normality of all data sets was tested using Shapiro-Wilk test for normality and confirmed with histograms and Q-Q plots. Any required data transformations are stated in the figure legends. *P* values were determined using one-way ANOVA with Dunnett T3 (unequal variance) or Dunnett (equal variance) post-hoc analysis and significance was defined when $p<0.05$. All statistics were performed using SPSS statistical software package version 20.0. Data are presented as means (± standard error of the mean (SEM)).

Results

The formulation does not impart a cytotoxic effect on human macrophages *in vitro*

The relative health and activity of the human THP-1 monocyte-derived macrophages in the presence of the formulation was confirmed by two independent assays. As shown in Fig 1, no significant differences in cell viability (Fig 1a: LDH assay) or cell proliferation/activity (Fig 1b: crystal violet assay) were observed in response to 24 hours incubation with the complete formulation (+PS) at a range of doses when compared to the vehicle control.

The formulation inhibits the IFN-γ induced expression of MCP-1 and ICAM-1 mRNA transcripts in macrophages

MCP-1 and ICAM-1 are both considered to be robust markers of inflammation that are associated with atherosclerosis [5] and their expression is known to be induced by IFN-γ [33]. To this end, IFN-γ stimulation significantly increased the transcript levels of MCP-1 (Fig 2a; 7.83-fold increase ($p<0.001$), Fig 2c; 10.13-fold increase ($p<0.001$)) and ICAM-1 (Fig 2b; 2.55-fold increase ($p = 0.014$), Fig 2d; 1.99-fold increase ($p = 0.001$)) in human THP-1 monocyte-derived macrophages when compared to the vehicle control. However, in the presence of 1x and 2x doses of the complete formulation, significant 71.3% ($p = 0.004$) and 73.6% reductions ($p = 0.001$), respectively, in IFN-γ induced MCP-1 transcript levels were observed when compared to cells treated with IFN-γ alone (Fig 2a). Likewise, 51.1% ($p = 0.108$) and 57.5% ($p = 0.010$) reductions were observed in IFN-γ induced ICAM-1 expression in response to the same doses, although the 1x dose failed to reach significance (Fig 2b). As seen is Fig 2c,

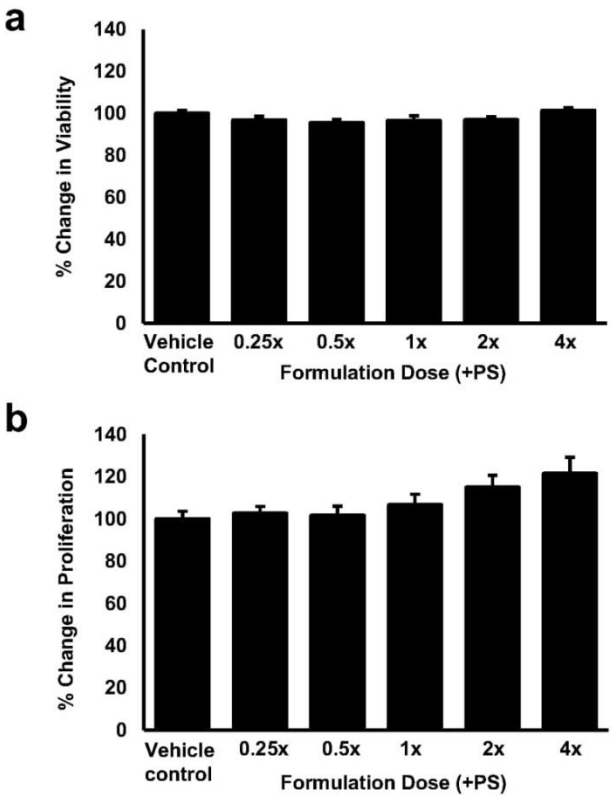

Fig 1. Physiologically relevant doses of the formulation have no detrimental effect on human macrophage viability or proliferation. Cell viability (a) or proliferation (b) was assessed in PMA differentiated THP-1 macrophages that were treated with vehicle (vehicle control) or various doses of the complete formulation (+PS) for 24 hours. Data were normalised to the vehicle control that has been arbitrarily assigned as 100%. The data are presented as the mean ± SEM from three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnett T3 post-hoc analysis.

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comparable effects were also apparent in response to a formulation that lacked the PS component where the 1x and 2x doses were able to significantly reduce IFN-γ induced MCP-1 transcript levels by 79.1% ($p = 0.001$) and 78.2% ($p = 0.002$), respectively, when compared to the IFN-γ treated cells. IFN-γ induced ICAM-1 expression was also significantly reduced in the presence of 1x and 2x doses lacking PS (by 52.8% ($p<0.001$) and 64.7% ($p<0.001$) respectively) when compared to the IFN-γ treated cells (Fig 2d). These effects were partly confirmed in primary HMDM where IFN-γ induced expression of ICAM-1 (1.75-fold increase ($p = 0.016$)) was significantly decreased the presence of the complete formulation and that lacking PS by 63.9% ($p = 0.002$) and 50.6% ($p = 0.013$) respectively (Fig 2e). No significant differences were observed between the complete formulation and that lacking PS for either gene in THP-1 or HMDM. In addition, the observed changes in MCP-1 and ICAM-1 gene expression also appear to be conserved in murine Raw264.7 macrophages although slight differences in dosing are apparent (S1 Fig).

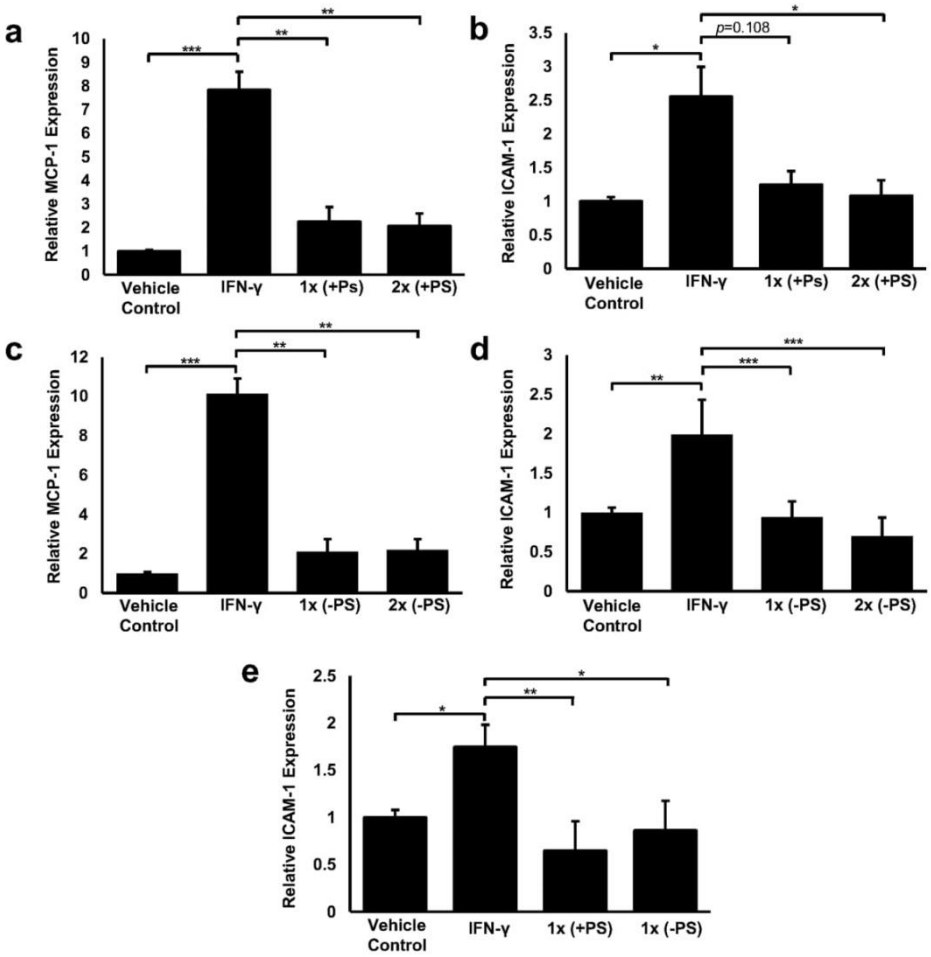

Fig 2. Physiologically relevant doses of the formulation can inhibit the IFN-γ induced expression of MCP-1 and ICAM-1 in human macrophages. Gene transcript levels of MCP-1 (a and c) and ICAM-1 (b, d and e) were assessed in PMA differentiated THP-1 macrophages (a, b, c and d) or HMDM (e) that were either treated with vehicle (vehicle control) or with IFN-γ (250 U/ml) or IFN-γ (250 U/ml) in the presence of the complete formulation (+PS; a, b and e) or with IFN-γ (250 U/ml) in the presence of the formulation lacking PS (-PS; c, d and e) for 3 hours. Gene transcript levels were calculated using the comparative Ct method and normalised to GAPDH levels with values from vehicle treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from three (a, b, c and d) or four (e) independent experiments. Statistical analysis was performed using a one-way ANOVA with Dunnett T3 post-hoc analysis on log-transformed data where * p <0.05, ** p <0.01 and *** p <0.001.

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The formulation inhibits the migration of human monocytes towards MCP-1

The recruitment of monocytes to the activated endothelium in response to chemokines such as MCP-1 is a critical early step in the development of atherosclerosis [5] and these actions were confirmed in our system as a significant 6.84-fold induction ($p<0.001$) in monocyte recruitment in cells treated with MCP-1 alone was observed when compared to the vehicle control (Fig 3). Upon the inclusion of the complete formulation (1x), monocyte recruitment in

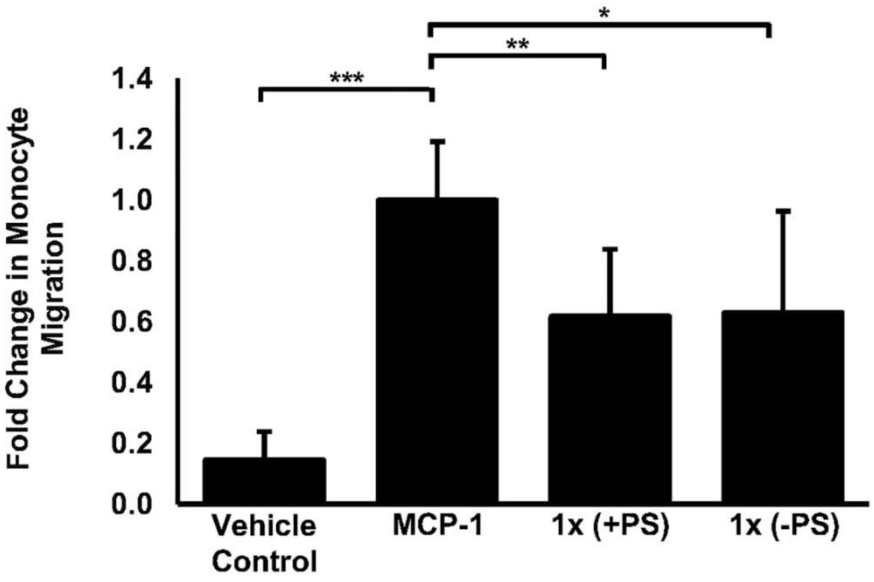

Fig 3. A physiologically relevant dose of the formulation can inhibit MCP-1 induced migration of human monocytes. Cellular migration was assessed using THP-1 monocytes that were treated with vehicle (vehicle control) or treated with MCP-1 (20 ng/ml) alone or with MCP-1 (20 ng/ml) in the presence the complete formulation (+PS) or with MCP-1 (20ng/ml) in the presence the formulation lacking PS (-PS) for 3 hours. Monocyte migration is expressed as a fold-change compared to the proportion of cells that moved from the apical compartment into the basolateral compartment in response to MCP-1 alone that has been arbitrarily set as 1. The data are presented as the mean ± SEM from four independent experiments. Statistical analysis was performed using a one-way ANOVA with Dunnett T3 post-hoc analysis where ** p <0.01 and *** p <0.001.

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response to MCP-1 was significantly reduced (38.2%, $p = 0.001$) when compared to cells treated with MCP-1 alone. Similarly, incubation with the formulation lacking PS resulted in a 37% ($p = 0.023$) reduction in migration when compared to MCP-1 alone treated cells. No significant difference in monocyte migration was observed between the complete formulation and that lacking PS.

The formulation improves the efflux of cholesterol from human macrophages

After 24 hours stimulation with ApoA-I, human THP-1 macrophage-derived foam cells were able to efflux approximately 20.2% of their intracellular radiolabelled cholesterol content (Fig 4). Significant fold changes in cholesterol efflux were observed in cells which were either co-incubated with the complete formulation (1x) or the formulation lacking PS (1x) with ApoA-I, 1.61 ($p = 0.002$) and 1.53 (p = 0.001) respectively, when compared to those treated with ApoA-I alone. No significant differences in cholesterol efflux were observed between the complete formulation and that lacking PS.

The formulation attenuates the expression of M1 phenotype markers in murine macrophages

Co-stimulation of Raw264.7 macrophages with IFN-γ and LPS significantly induced the expression of iNOS (Fig 5a, $p<0.001$) and Arg2 (Fig 5b, $p<0.001$), two robust markers of M1 phenotype [34]. Inclusion of the complete formulation (1x) significantly decreased the

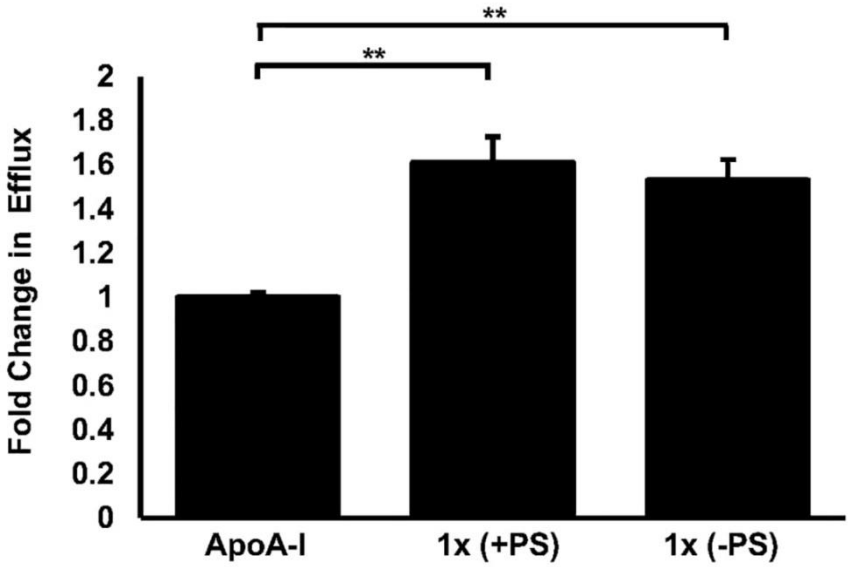

Fig 4. A physiologically relevant dose of the formulation can induce cholesterol efflux from human macrophage foam cells. Cholesterol efflux was assessed in 24 hour [4-^{14}C]cholesterol loaded PMA-differentiated THP-1 cells in the presence of AcLDL that were treated with ApoA-I (10 μg/ml) in the presence of vehicle or ApoA-I in the presence of the complete formulation (+PS) or ApoA-I in the presence of the formulation lacking PS (-PS) for 24 hours. Data were normalised to the ApoA-I, vehicle treated sample that has been arbitrarily assigned as 1. The data are presented as the mean ± SEM from three independent experiments. Statistical analysis was performed using a one-way ANOVA with Dunnett T3 post-hoc analysis where ** p <0.01.

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expression of both iNOS (Fig 5a) and Arg2 (Fig 5b) by 47.6% (p = 0.004) and 41.5% (p = 0.015), respectively, when compared to the IFN-γ and LPS treated cells. Likewise, incubation with the formulation lacking PS (1x) significantly reduced the IFN-γ and LPS mediated induction of iNOS (Fig 5a) by 44.6% (p = 0.002) whereas a trend towards a 37.2% decrease (p = 0.056) was observed for Arg2 (Fig 5b). No significant differences in iNOS or Arg2 expression were observed between the complete formulation and that lacking PS. It should be noted that murine Raw264.7 macrophages were used in these experiments rather than human THP-1 monocyte-derived macrophages in light of evidence suggesting that PMA differentiation promotes an established population of M1 phenotype [35].

Key bioactive ingredients of the complete formulation can inhibit the migration of human monocytes towards MCP-1

Similar to that observed in Fig 3, MCP-1 significantly induced (9.61-fold, p<0.001) monocyte recruitment when compared to the vehicle control (Fig 6). Significant inhibitions of MCP-1 induced monocyte migration were observed in the presence of EPA (52.7%, p = 0.009) or (+)-catechin (47.5%, p = 0.02) when compared to cells treated with MCP-1 alone. While they did not reach significance, a trend towards a reduction in monocyte migration was observed in response to DHA, stigmasterol (SS), campsterol (CS) and β-sitosterol (β-SS).

Discussion

This study shows that a combination of food ingredients can attenuate the expression of two key pro-atherogenic genes, reduce MCP-1 driven monocyte migration, induce cholesterol

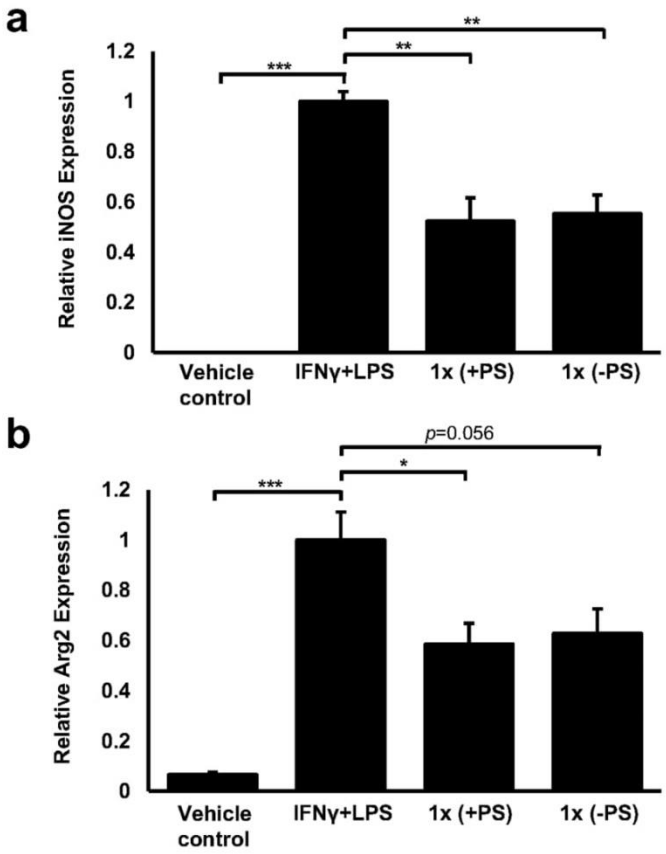

Fig 5. A physiologically relevant dose of the formulation can hinder M1 polarisation in murine macrophages. Gene transcript levels of iNOS (a) and Arg2 (b) were assessed in Raw264.7 murine macrophages that were treated with either vehicle (vehicle control); with IFN-γ (250 U/ml) and LPS (100 ng/ml); with IFN-γ (250 U/ml) and LPS (100 ng/ml) in the presence of the complete formulation (+PS) or with IFN-γ (250 U/ml) and LPS (100 ng/ml) in the presence of the formulation lacking PS (-PS) for 24 hours. Gene transcript levels were calculated using the comparative Ct method and normalised to β-actin levels with the values from IFN-γ and LPS treated cells given an arbitrary value of 1. The data are presented as the mean ± SEM from five independent experiments. Statistical analysis was performed using a one-way ANOVA with Dunnett T3 post-hoc analysis on square root-transformed data where * p <0.05, ** p <0.01 and *** p <0.001.

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efflux from macrophage foam cells and retard the polarisation of macrophages towards an M1 (pro-inflammatory) phenotype when assessed using a series of well-established *in vitro* models of atherosclerosis. The ability of physiologically relevant doses of this unique formulation to beneficially modulate multiple key atherosclerotic events highlight the possibility of a nutritionally orientated approach to support the prevention of early disease development.

To date, no other studies have examined the effect of a similar combination of active ingredients on atherosclerosis or key processes associated with this disease although a plethora of *in vivo* studies have demonstrated the anti-atherosclerotic actions of fish oils [36], cocoa extracts [18, 19] and PS [22, 23] in isolation. However, it should be noted that polyphenols, fish

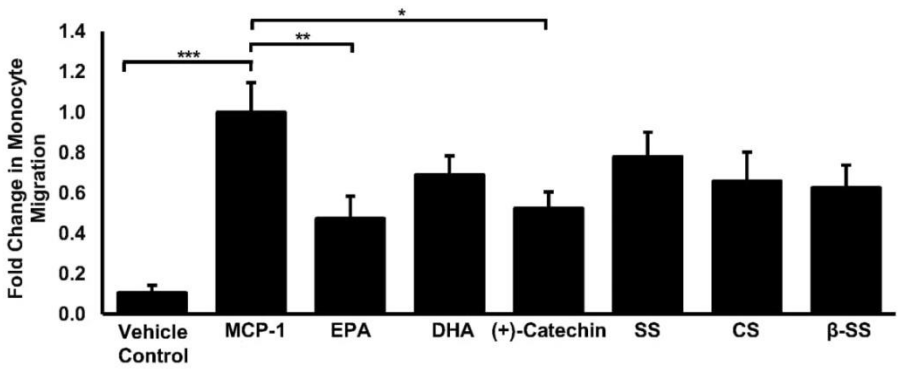

Fig 6. Major bioactive components associated with the complete formulation can inhibit MCP-1 induced migration of human monocytes. Cellular migration was assessed using THP-1 monocytes that were treated with either vehicle (vehicle control) or with MCP-1 (20 ng/ml) or with MCP-1 (20 ng/ml) in the presence of EPA (30 μg/ml), DHA (19.6 μg/ml), (+)-catechin (1.45 μg/ml), stigmasterol (SS, 10 μg/ml), campsterol (CS, 13.9 μg/ml) or β-sitosterol (β-SS, 27.2 μg/ml) for 3 hours. Monocyte migration is expressed as a fold-change compared to the proportion of cells that moved from the apical compartment into the basolateral compartment in response to MCP-1 alone that has been arbitrarily set as 1. The data are presented as the mean ± SEM from four independent experiments. Statistical analysis was performed using a one-way ANOVA with Dunnett post-hoc analysis on Log transformed data where * p <0.05, ** p <0.01 and *** p <0.001.

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oils and PS have all been shown to impart undesirable effects in numerous studies [25, 37–39] although such observations may be the result of the inclusion of single agents in *in vitro* studies that do not consider the potential impact of all other natural food source components, or, in *in vivo* studies, other contributing factors such as the genetic make-up and the presence of various risk factors such as elevated plasma cholesterol levels or a disrupted inflammatory state. In light of this, and due to their ability to directly affect macrophage function [16, 20, 25], the food ingredients used in our study were applied primarily in their natural or most available format, although in some instances individual bioactive components were also used, in a well-established monocyte derived THP-1 macrophage model that shows a highly conserved response with primary HMDM and *in vivo* evidence [29, 32, 40] and, considering the involvement of macrophages at all stages of atherosclerosis [4], in order to facilitate a holistic screening approach.

We first confirmed that no adverse effects on cell viability or proliferation were apparent in response to the application of the food ingredients to human macrophages *in vitro* (Fig 1). These data also ensured that any observed changes in gene expression and/or cell behaviour reported were not as a consequence of altered cell health. In addition, it is worthy of note that PS have been included in the formulation to utilise their ability to reduce the absorption of dietary cholesterol from the intestinal lumen upon regular consumption of the supplement [24]. However, PS are known to cross the intestinal barrier albeit at low levels [41] and individual PS such as stigmasterol and sitosterol, both of which are present in the supplement, have been shown to elicit sometimes opposing atherosclerotic effects on macrophages *in vitro* [25]. Therefore, a formulation lacking PS was assessed in all of our *in vitro* models to determine the potential atherosclerotic effects of systemic PS. To this end, Figs 2, 3, 4, 5 and 6 show that no masked anti-atherosclerotic effect can be attributed to PS and support its inclusion in the formulation.

IFN-γ is a pro-inflammatory cytokine and is considered to be a key regulator of atherosclerosis [3, 5, 42]. It is produced at prominent levels in atherosclerotic lesions by a variety of immune cells including T-lymphocytes, natural killer T-cells and macrophages [43] and is

considered a promising therapeutic target due to its key role in all stages of the disease [44]. Studies by our own group have showed that IFN-γ can induce the expression of MCP-1 and ICAM-1 in macrophages that are known to play prominent roles during the recruitment of monocytes to the inflamed endothelium [33]. Here we show that physiologically relevant doses of the formulation, with or without PS, can attenuate the IFN-γ induced ICAM-1 and MCP-1 expression in THP-1 monocyte derived macrophages, physiologically relevant primary HMDM (Fig 2) and murine macrophages (S1 Fig) and may therefore prevent the recruitment of monocytes to the inflamed endothelium—a key stage of early atheroma development. In addition, incubation with either formulation clearly inhibits the movement of monocytes towards MCP-1 (Fig 3) which also alludes to reduced numbers of macrophages at the site of atheroma development and adds functionality to our observed changes in gene expression (Fig 2). Analysis of individual bioactive components contained in the formulation suggest that the effects on monocyte migration maybe mediated by EPA and (+)-catechin (Fig 6), an observation supported by other studies demonstrating reduced monocyte migration/adhesion in response to various catechin isomers [45, 46] and fish oil or ω-3 PUFAs [47, 48]. Reduced serum ICAM-1 levels in response to cocoa extract supplementation [49] and reduced transcript levels of ICAM-1 in EPA stimulated monocytes [50] has also been previously documented. Interestingly, our study also suggests the lack of antagonistic or synergistic effects between the individual formulation components as both EPA and (+)-catechin (Fig 6) appear to attenuate monocyte migration at levels comparable with the complete formulation (Fig 3) though further experiments will be required for firm conclusions.

The formation of macrophage derived foam cells is also considered a major step in atherosclerosis development [4] and in recent years the underlying mechanisms have become a common target for preliminary intervention studies [29, 32]. Here we report that exposure of cholesterol-loaded human macrophages, or foam cells, to either formulation (with or without PS) can increase ApoA-I mediated cholesterol efflux from the cells and possibly reverse the development of foam cells. PS and ω-3 PUFAs have both been implicated as regulators of macrophage cholesterol homeostasis in other *in vitro* studies [17, 25] suggesting that these may be responsible for the changes observed in our study, however, no differences in cholesterol efflux were observed between the formulations with or without PS in our model.

It is now emerging that once in the arterial intima, macrophages can polarise into either pro-inflammatory M1 or anti-inflammatory M2 subgroups and an the accumulation of M1 macrophages may have a detrimental effect on fibrotic plaque stability [8]. In this study we clearly show that either formulation can hinder Raw264.7 macrophage polarisation towards the M1 phenotype as depicted by a reduction in the expression of iNOS and Arg2; two robust markers of the M1 phenotype [34]. This suggests that the formulation may have the potential to help stabilise the fibrotic cap in established atherosclerosis and supports earlier studies that have shown strong links between ω-3 PUFAs and reduced M1 phenotype formation [51, 52].

In conclusion, this is the first *in vitro* study to examine the effect of this combination of nutritional ingredients and provides preliminary evidence to suggest a potential anti-atherosclerotic effect during numerous processes associated with the disease. Furthermore, this study provides support for future *in vivo* animal and/or human studies to elucidate its ability to prevent atherosclerosis.

Supporting Information

S1 Fig. Physiologically relevant doses of the formulation can inhibit IFN-γ induced expression of MCP-1 and ICAM-1 in murine macrophages.
(DOCX)

S1 Data. Excel spreadsheet of experimental data.
(XLSX)

S1 Table. Active ingredient inclusion levels of the supplement.
(DOCX)

S2 Table. Oligonucleotide sequences.
(DOCX)

Author Contributions

Conceived and designed the experiments: SFP IG DPR DRM JWEM. Performed the experiments: JWEM TSD. Analyzed the data: JWEM. Wrote the paper: DPR JWEM TSD DRM.

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S.3.3 Cytokines: Roles in atherosclerosis disease progression and potential therapeutic targets

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Cytokines: Roles in atherosclerosis disease progression and potential therapeutic targets

Joe W. E. Moss[a] and **Dipak P. Ramji**[a,+]

[a]Cardiff School of Biosciences, Cardiff University, Sir Martin Evans Building, Museum Avenue, Cardiff, CF10 3AX, United Kingdom

Abstract

Atherosclerosis, the primary cause of cardiovascular disease (CVD), is a chronic inflammatory disorder in the walls of medium and large arteries. CVD is currently responsible for about one in three global deaths and this is expected to rise in the future due to an increase in the prevalence of obesity and diabetes. Current therapies for atherosclerosis mainly modulate lipid homeostasis and whilst successful at reducing the risk of a CVD-related death, they are associated with considerable residual risk and various side effects. There is therefore a need for alternative therapies aimed at regulating inflammation in order to reduce atherogenesis. This review will highlight the key role cytokines play during disease progression as well as potential therapeutic strategies to target them.

Keywords

Atherosclerosis; Cytokines; Chemokines; Neutralisation; Nanoparticles; Inflammation; Cardiovascular disease

1 Introduction

The World Health Organization (WHO) has estimated that one in three global deaths are as a result of cardiovascular disease (CVD)-related events such as myocardial infarction (MI) and stroke [1]. In 2012 there were approximately 17.5 million global deaths due to CVD-related events. As developing countries adopt a more westernized lifestyle and the incidences of diabetes and obesity continue to increase worldwide, the estimated number of CVD-related deaths is expected to rise to 23.3 million by 2030 [1]. CVD is a significant problem for the healthcare systems around the world and thus a major economic burden [2]. Therefore, there is a great need to discover new targets and to develop potential therapies for CVD.

Atherosclerosis is the primary cause of CVD-related events and associated morbidity and mortality from this disease. Atherosclerosis is a chronic inflammatory disease of the large and medium arteries that can be triggered by several risk factors including a diet rich in saturated fats, lack of exercise and smoking. The progression of atherosclerosis is influenced

[+]Corresponding author. Tel: +44 29 208 76753; Fax: +44 29 208 74116; Ramji@Cardiff.ac.uk.

by the innate and adaptive immune system responses that are both regulated by a variety of cytokines [3,4]. Atherosclerosis is initiated by endothelial cell (EC) dysfunction/activation that is often triggered by the accumulation of low-density lipoprotein (LDL) and other apolipoprotein (Apo)B-containing lipoproteins in the walls of large and medium arteries [4–6]. An inflammatory response is triggered in the ECs neighbouring the LDL accumulation as it becomes oxidized into oxidized (ox)-LDL [4–6]. The activated ECs begin to release cytokines and chemokines into the blood stream as well as express cell adhesion molecules on their surface in order to recruit circulating monocytes and other immune cells to the site of oxLDL build-up [4–6]. Once the monocytes migrate into the walls of the arteries they differentiate into macrophages which are able to uptake oxLDL and form foam cells [4–6]. Atherosclerotic plaques develop due to the continuous and uncontrollable recruitment of macrophages and build-up of foam cells at the site of oxLDL accumulation and defective clearance of apoptotic cells/debris (efferocytosis) that leads to a chronic inflammatory response [4–6]. As the plaque continues to develop it can become unstable and rupture, leading to thrombosis, stroke or MI depending on the location of the rupture [4–7].

2 Cytokines are key orchestrators of inflammation in atherosclerosis

Cytokines are a board range of small proteins involved in cellular signaling pathways and can be divided into several categories including the interferons (IFN), colony-stimulating-factors (CSF), interleukins (IL), chemokines, transforming growth factors (TGF) and tumour necrosis factors (TNF) [3,5,6]. Furthermore, they can either aid atherosclerotic plaque development (pro-atherogenic) or attenuate plaque formation (anti-atherogenic). However some cytokines do not have a definite function and can be either pro- or anti-atherogenic depending on the surrounding environment [3,5,6]. More details regarding the role of specific cytokines and how they can be therapeutically targeted will be discussed in greater detail in the subsequent sections of this review. Due to their key roles in influencing the inflammatory response during atherosclerosis, targeting cytokines and their signaling pathways represents a promising therapeutic strategy for attenuating the development of this disease by inhibiting those that augment atherogenesisis as well as promoting those which retard plaque formation. A brief description of the key cytokines involved in atherosclerosis development and their signaling pathways are summarized in Table 1. This review will highlight some of the key cytokines and their roles in the different stages of atherosclerosis development and discuss the emerging therapeutics designed to target cytokines as well as their receptors. A comprehensive coverage of the roles of the full spectrum of cytokines in atherosclerosis is beyond the scope of the current article and the reader is directed to more recent reviews on this topic [3, 6].

3 Key cytokines associated with the inflammatory response in atherosclerosis

Cytokines are able to influence all stages of atherosclerosis development ranging from the initial recruitment of circulating monocytes and other immune cells from the bloodstream all the way through to mature plaque formation and stability (Figure 1). The use of atherosclerotic mouse models have greatly improved our understanding of the role of

cytokines and their signaling during disease development. Normally wild type mice do not develop atherosclerosis, however ApoE knockout mice ($ApoE^{-/-}$) and LDL receptor knockout mice ($LDLr^{-/-}$) are able to develop atherosclerotic lesions while being fed a standard chow diet. Plaque formation in these mice can be accelerated by feeding them a high fat diet.

3.1 Key role of cytokines in monocyte recruitment

During the initial formation of an atherosclerotic lesion, the reorganization of the actin and tubulin cytoskeletons of ECs can be triggered by IFN-γ and TNF-α [6]. This reorganization leads to changes in the shape of the ECs and creates gaps between neighbouring cells, making the endothelial layer permeable and allowing LDL to passively diffuse into to the walls of the arteries and accumulate. The LDL can then be oxidized to oxLDL which triggers an immune response in the nearby ECs. The activated ECs begin to release a variety of cytokines and chemokines. Chemokines is a term given to a sub-group of cytokines which are capable of attracting cells to a desired location [6]. Chemokines are able to exert their effects by interacting with cell surface receptors and activating heterotromeric G proteins and related intracellular signaling pathways [6].

In atherosclerosis development, chemokines play an important role in the recruitment of circulating monocytes and other immune cells to the site of oxLDL retention [5,30–32]. For circulating monocytes to enter into the intima of the artery they must first slow their speed through the blood by rolling along the endothelium before coming to rest and moving through the endothelium [30,31]. Two chemokines that are key to the initial rolling phase of monocyte recruitment are chemokine C-X3-C motif ligand (CX_3CL)1 and chemokine C-C motif ligand (CCL)5 [6,30,31]. These chemokines interact with proteoglycans and P-selectins on the surface of ECs, allowing them to bind to their corresponding receptors on the circulating monocytes [6,30,31]. The rolling monocytes come to rest when adhesion molecules such as vascular cellular adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 also bind to their receptors on the monocytes, creating a firm interaction between the monocyte and the endothelial layer and allowing the monocytes to migrate into the intima [6,30,31].

Targeting chemokines therapeutically can often be challenging as there is frequently functional redundancy in their signaling, with numerous interacting with several receptors or a receptor interacting with several agonists [33–35]. However there appears to be at least three major chemokines (and their corresponding receptors) whose deficiency completely abrogates the development of atherosclerosis in mouse model systems [6,36–38]. These chemokines are CCL2, CCL5 and CX_3CL1 [6,30]. CCL2, also known as monocyte chemoattractant protein (MCP)-1, is thought to be a major chemokine involved in monocyte recruitment during atherosclerosis development. Atherosclerotic mouse models with attenuated expression of CCL2 or its receptor CCR2 resulted in smaller atherosclerotic lesions due to reduced monocyte recruitment [15,38,39]. A recent study has highlighted a possible new mechanism for the action of some chemokines which involves the remodelling of the actin cytoskeleton resulting in CD36 clustering, which increases foam cell formation by augmenting the responsiveness of the receptor for oxLDL [40].

Targeting CCL2, CX_3CL1 and CCL5 or the chemokine network may represent promising therapeutic avenues in order to attenuate the development and progression of atherosclerosis. In addition, as cytokines are capable of influencing all stages of atherosclerosis development, it is important to understand the roles of key cytokines in further downstream events in the disease following the recruitment of immune cells, such as foam cell formation, and identify further therapeutic targets.

3.2 Key role of cytokines in lesion formation

Once in the intima of the arteries, the monocytes are exposed to several cytokines, including macrophage colony stimulating factor (M-CSF), and differentiate into macrophages [5]. The local microenvironment together with signaling from additional cytokines will affect whether the macrophages are classically activated (M1 phenotype) or alternatively activated (M2 phenotype) [5,9–11]. M1 macrophages are generally considered to be pro-inflammatory due to their role in promoting an inflammatory response to bacterial infection in the innate immune response [11]. The M1 phenotype has been found to be induced by the pro-inflammatory cytokines IFN-γ and IL-1β [9–11]. These M1 macrophages are able to exert their deleterious actions by releasing the pro-inflammatory cytokines IL-6, IL-12 and TNF-α [6]. M2 macrophages on the other hand are thought of as anti-inflammatory due to their role in repair and the resolution of inflammation [11]. Cytokines including IL-4 and IL-13 are able to induce the formation of M2 macrophages, which are then capable of producing the anti-inflammatory cytokines IL-10 and TGF-β [9–11]. A number of other macrophage phenotypes have also been identified though these are relatively poorly characterized [9,41]. These include M2b, M2c and M2d macrophages which can be induced by IL-1 receptor ligands, IL-10 and co-stimulation of Toll-like receptor together with adenosine A_{2A} receptor agonists respectively [41]. The cytokine CXCL4 has been identified as being able to differentiate macrophages into a pro-inflammatory phenotype known as M4 in human atherosclerotic plaques [42].

The role cytokines play in macrophage polarization makes them a promising therapeutic target to switch the balance of M1:M2 formation in favour of the M2 phenotype in order to change the atherosclerotic plaque environment from a pro-inflammatory to an anti-inflammatory one to attenuate disease progression. It is important to try and achieve this due to the effects that are exerted by the cytokines they produce. For example increasing the expression of IL-6 in $ApoE^{-/-}$ mice by using a lentivirus resulted in an unstable plaque [43] whereas macrophage specific over expression of IL-10 in $LDLr^{-/-}$ mice resulted in reduced atherosclerosis development by a decrease in cholesteryl ester accumulation [44]. Such pro- and anti-inflammatory effects of the cytokines produced by the different macrophage phenotypes highlight the rationale for targeting cytokine signaling involved in macrophage polarization in order to inhibit M1 formation and promote the M2 phenotype. However it should be noted that one recent study has shown that M2 macrophages increase their expression of scavenger receptors (SRs) CD36 and SR-A1 following endoplasmic reticulum stress, which correlated to increased foam cell formation [45]. Further studies are required to fully determine the role of different macrophage phenotypes in atherosclerosis disease development.

In order to become foam cells the macrophages must take up modified LDL, in particular oxLDL [4,6,7]. There are several mechanisms by which macrophages are able to take up lipoproteins including macropinocytosis, pinocytosis and phagocytosis, however the major route for the uptake of oxLDL and other modified forms of this lipoprotein is via SR-mediated endocytosis [5,6]. Unlike LDL uptake via LDLr which is highly regulated by a negative feedback loop by intracellular cholesterol levels, oxLDL uptake by SRs such as SR-A1 and CD36, is unregulated resulting in uncontrolled uptake [5,6]. Foam cells begin to form when the balance of cholesterol uptake and efflux is tipped in favour of uptake and retention [5,6]. Cytokines are important in maintaining this balance as they are able to influence the expression of several key genes involved in cholesterol uptake and efflux [5].

The pro-inflammatory cytokine IFN-γ can induce foam cell formation by increasing cholesterol retention by attenuating the expression of ATP-binding cassette, sub-family A (ABCA1), a key transporter in cholesterol efflux, as well as inducing the expression of acyl-CoA acyltransferase 1 (ACAT1), which is involved in cholesterol esterification [6]. Human THP-1 and primary macrophages stimulated with IFN-γ showed an increase in the expression of key SRs, such as SR-A1, leading to an increase in oxLDL uptake [12,13]. Additionally, ApoE$^{-/-}$ mice also deficient for TNF-α develop smaller atherosclerotic lesions due to a decrease in lipid accumulation after 6 weeks [46]. Furthermore the expression of pro-inflammatory cytokines and adhesion molecules such as IFN-γ, ICAM-1, VCAM-1 and CCL2, were significantly decreased at the mRNA level in these mice [46]. These genes were also found to be down regulated after 8 weeks feeding of a high fat diet in ApoE$^{-/-}$ mice which also had the p55 TNF receptor (p55 TNFR) knocked out [47]. The lesions that developed in these mice were also smaller compared to controls [47], highlighting the pro-apoptotic role TNF-α plays during early lesion formation.

The cytokines IL-1α, IL-β and IL-18 are pro-inflammatory and play a major role in atherosclerosis disease progression [6,18]. ApoE$^{-/-}$ mice which were also deficient for IL-1α and IL-β were found to develop smaller atherosclerotic lesions compared to the control mice [48]. Furthermore a bone marrow transfer (BMT) of IL-1$^{+/+}$, IL-1α$^{-/-}$ or IL-β$^{-/-}$ cells into ApoE$^{-/-}$ mice found that those receiving IL-1$^{+/+}$ cells developed larger plaques by approximately 52% and 32% when compared to those mice that received IL-1α$^{-/-}$ and IL-β$^{-/-}$ cells respectively [48]. Similar results were observed in ApoE$^{-/-}$ mice which also lacked the IL-1 receptor type-1 (IL-1R1) [49]. Smaller atherosclerotic lesions were found in mice which lacked IL-1R1 compared to the ApoE$^{-/-}$ control mice. This correlated to reduced expression of monocyte recruitment genes including CCL2, ICAM-1 and VCAM-1 in the vascular wall [49], demonstrating the pro-inflammatory effects of IL-1 during atherosclerosis disease development. One study using ApoE-/- mice fed a standard chow diet while receiving daily injections of recombinant IL-18 developed larger and more unstable lesions by increasing the expression of CD36 and matrix metalloproteinase (MMP)-9, possibly via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [50]. However another study which fed ApoE$^{-/-}$IL-18$^{-/-}$ mice a high fat diet for 12 weeks developed more and larger lesions compared to ApoE$^{-/-}$ mice [51]. These lesions were also less stable due to an increased lipid content [51], indicating that IL-18 may actually attenuate atherosclerosis development. Other pro-inflammatory cytokines that promote foam

cell formation include IL-6 [52], IL-15 [53], TNF-related weak inducer of apoptosis (TWEAK) [54] and TNF-like protein 1A (TL1A) [55].

The over expression of TGF-β in ApoE$^{-/-}$ mice leads to smaller and more stable plaques compared to controls after receiving a high fat diet for 24 weeks [23]. Inhibition of TGF-β signaling by introducing a functional inactivation mutation in the TGF-β receptor II (TGFβR2) in ApoE$^{-/-}$ mice resulted in a two fold increase in atherosclerotic lesion size, as well as increasing the presence of inflammatory markers such as IFN-γ [24]. Although LDLr$^{-/-}$ mice which were also deficient for Smad7 (an inhibitor of TGF-β signaling) developed larger lesions, their fibrous caps contained more collagen which correlates to a more stable plaque [56]. Therefore targeting TGF-β signaling may represent a promising therapeutic avenue to reduce early lesion formation while simultaneously making already developed plaques more stable. However a recent study found that specific inhibition of TGF-β signaling in T cells had no effect on atherosclerosis development [57]. TGF-β also attenuates macrophage foam cell formation by inhibiting the expression of key genes implicated in the uptake of lipoproteins and stimulating those that are involved in the efflux of cholesterol [58].

Another anti-inflammatory cytokine capable of attenuating lipid uptake is IL-33. ApoE$^{-/-}$ mice injected with IL-33 twice a day for 6 weeks had decreased build-up of foam cells compared to the control group [26]. Furthermore the same study also found that IL-33 was capable of increasing the expression of key genes implicated in cholesterol efflux, including ABCA1 and ApoE, as well as attenuating the expression of SR genes such as CD36 [26]. This altered pattern of gene expression correlated to a decrease in modified LDL uptake and intracellular cholesterol accumulation in addition to an increase in cholesterol efflux *in vitro* [26]. In addition, studies in ApoE$^{-/-}$ mice in which the action of IL-33 was inhibited by injection of a soluble decoy receptor or augmented by injection of the cytokine revealed its protective role in atherosclerosis [29].

Other cytokines such as IL-10 and IL-13 have also shown cardiovascular protective effects. Overexpressing IL-10 in LDLr$^{-/-}$ mice resulted in an increase in cholesterol efflux, reduced apoptosis as well as a reduction in the expression of pro-inflammatory molecules including TNF-α and CCL2 [44]. These results indicate an attenuation of the disease progression, which correlates to the observed reduction in atherosclerotic lesion size observed in these mice [44]. Biweekly injections of IL-13 in LDLr$^{-/-}$ mice which were fed a high fat diet for 16 weeks showed a reduced number of macrophages and increased collagen content in their plaques compared to control mice [59], two indicators of plaque stability. Furthermore, it was found that the IL-13 injections resulted in a decrease in the pro-inflammatory M1 macrophage and increased M2 macrophage differentiation. The greatest evidence for the anti-atherogenic properties of IL-13 comes from LDLr$^{-/-}$ mice which are also deficient for IL-13. These mice developed larger atherosclerotic plaques, which correlated to an increase in the size of the necrotic core, as well as a reduction in the number of anti-inflammatory M2 macrophages found within the plaque compared to the control mice [59].

3.3 Key role of cytokines in plaque stability

Although anti-inflammatory cytokines such as TGF-β can promote plaque stability, a prolonged inflammatory response and continuous stimulation in the plaque by pro-inflammatory cytokines can lead to an unstable plaque which can rupture and cause a MI or stroke [6]. Pro-inflammatory cytokines such as TNF-α, IFN-γ and IL-1β can induce macrophage and foam cell apoptosis, causing them to release their lipid content into the intima of the artery and contribute towards the size of the lipid core in the plaque [6,14]. As well as lipids the macrophages also release enzymes that can influence extracellular matrix remodelling including MMPs and their inhibitors known as TIMPs (tissue inhibitor of metalloproteinases) [60]. The activity of both MMPs and TIMPs are highly regulated by a range of cytokines [60]. Inhibition of IFN-γ signaling postnatally in $ApoE^{-/-}$ mice by over expressing a soluble decoy receptor also resulted in smaller lesions as well as increasing mature plaque stability by increasing the number of SMCs present in the fibrous cap [61]. However, the relationship between pro-inflammatory cytokines and plaque instability is not always clear-cut. For example, $ApoE^{-/-}$ mice which are also deficient in the IL-1 receptor type 1 (IL-1R1) developed plaques with lower amounts of collagen and fewer smooth muscle cells (SMCs), both of which are indicators of increased plaque instability [62].

4 Cytokine targeted therapeutic approaches

Statins are a class of cholesterol lowering therapeutics which target and inhibit the enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG CoA) reductase. This enzyme plays a major role in the rate limiting step of cholesterol biosynthesis. Therefore targeting HMG CoA reductase with statins results in a decrease in serum cholesterol levels, in particular LDL, reducing an individual's risk of suffering a CVD-related event. In addition, statins have other pleotropic effects such as acting in an anti-inflammatory manner and reducing endothelial cell dysfunction [63]. However long term usage of statins is known to cause several side effects ranging from minor effects, such as nose bleed and headaches, to major ones including an increased risk of developing diabetes and muscle pain [64–66]. In addition, statin therapy is associated with considerable residual risk for CVD. Furthermore there is a significant minority of individuals who are unable to achieve recommended plasma cholesterol levels even with the maximum dose of statins [5].

Due to the pro-inflammatory characteristics of some cytokines and their key roles in atherosclerosis development they represent promising therapeutic targets in the treatment of this disease. An excellent example of cytokine therapeutics is a neutralizing antibody targeted against IL-1β. This cytokine is produced by the activation of the inflammasome and is a key regulator of the innate immune response [18,19]. Not only can IL-1β induce the expression of several pro-inflammatory mediators, it can also induce its own expression due to its auto-inflammatory nature [20]. Therefore IL-1β represents an ideal cytokine to target and retard the progression of atherosclerosis.

A human monoclonal antibody against IL-1β known as canakinumab provides some hope for specifically targeting cytokines. Canakinumab is currently approved for use in two auto-inflammatory diseases, Muckle Wells syndrome and cryopyrin-associated periodic syndrome [20]. The promising outcome lead to its use in a phase II clinical trial involving

556 diabetic participants with high risk for developing CVD [67]. Although this study found that canakinumab did not alter LDL or HDL concentrations, it did reduce the levels of key downstream pro-inflammatory mediators including IL-6 and C-reactive protein (CRP) in a concentration dependent manner. CRP is a well-known and recognized indicator of inflammation that often correlates with CVD [68]. Furthermore canakinumab has been found to exert long term anti-inflammatory effects due to decreased levels of IL-6, CRP and IL-1β several months after individuals receive only a single dose [67]. To further investigate the effectiveness of canakinumab, a large scale trial known as Canakinumab Anti-inflammatory Thrombosis Outcomes study (CANTOS) was set up in 2011 [20]. The trial involves 10,065 post-myocardial patients worldwide who receive either canakinumab or a placebo every 3 months [20]. The CANTOS trial is expected to end in 2017. There is hope that this trial will provide the first evidence of targeting IL-1β directly using neutralizing antibodies reduces an individuals' risk of suffering a CVD-related event and open up similar avenues with other cytokines. Another ongoing study is the Cardiovascular Inflammation Reduction Trial (CRIT) [69]. Due to the success of using low dose methotrexate therapy in patients with rheumatoid arthritis and psoriasis to dampen the inflammatory response, it is now being investigated as a potential therapeutic to reduce a patient's risk of suffering a CVD-disease related event. As a generic anti-inflammatory compound, which is likely to affect the expression of several cytokines, the CRIT trial may open multiple avenues for broad anti-inflammatory agents.

Several approaches have been pursued in relation to IFN-γ due to the central role of this cytokine in atherosclerosis disease progression. The strategies can be broadly defined as targeting the IFN-γ pathway or neutralizing the cytokine directly [7]. IFN-γ signals through the Janus Kinase (JAK)-Signal Transducer and Activator of Transcription (STAT) pathway which requires the phosphorylation of STAT1 at tyrosine 701 and serine 727 for maximal activity [7,70]. Some potential therapeutics have been designed to inhibit the phosphorylation of STAT1 in order to prevent the pro-inflammatory effects of IFN-γ. For example adenosine is known to inhibit the phosphorylation of serine 727 on STAT1 in human macrophages [7]. This has led to the creation of a novel compound called thio-CI-IB-MECA, which is designed to be an agonist for the adenosine A3 receptor and has been found to reduce IFN-γ-induced, STAT1-dependent gene expression [71]. Furthermore *in vitro* work using human macrophages has shown that phosphorylation of tyrosine 701 or serine 727 on STAT1 as well as JAK2 activation can be inhibited by resveratrol, which is a phenol compound that occurs naturally in plant extracts [72].

Unlike IL-1β neutralization that directly targets the cytokine with antibodies, studies on IFN-γ neutralization so far involves the use of a soluble decoy receptor. The soluble IFN-γ receptor (sIFN-γR) works by out competing the normal IFN-γR for binding to the cytokine and thereby preventing the activation of the JAK-STAT pathway [7]. This then prevents the downstream expression of IFN-γ-induced pro-inflammatory mediators. The first use of a sIFN-γR was in ApoE$^{-/-}$ mice which were fed a high fat diet for 8 weeks, while also receiving intramusclular injections containing a sIFN-γR encoding plasmid on weeks 4 and 6 [61]. The sIFN-γR treated mice developed smaller atherosclerotic plaques when compared to the control mice [61]. Furthermore, due to increased levels of collagen accumulation and vascular smooth muscle cells (VSMCs) in the fibrous cap, the plaques which developed

were more stable [61]. Soluble decoy receptors have also been used to inhibit IL-6 signalling [52]. Soluble glycoprotein 130 (sgp130) has been used in $LDLr^{-/-}$ mice to attenuate IL-6 signaling which resulted in decreased atherosclerosis disease progression due to reduced monocyte recruitment and EC activation [52]. These studies show the potential of using a cytokine decoy receptor to attenuate atherosclerosis development.

Due to the importance of the chemokines CCL2, CCL5 and CX_3CL1 during the initial monocyte recruitment phase of atherosclerosis development, targeting their corresponding receptors pharmaceutically in order to prevent activation and reduce disease progression has also been explored. Several approaches have shown success in mouse model systems, including viral proteins that act as broad spectrum inhibitors [73,74], pharmacological inhibitors [75], neutralizing antibodies against chemokines or their receptors [33–35], decoy ligands that bind to the receptors without causing activation [33–35] and RNA intereference-mediated knockdown strategies [76]. A major problem with targeting chemokine and their receptors is that they are also crucial for the resolution of an inflammatory response triggered by infection or other stresses. It will therefore be necessary to target them specifically to the arterial wall by use of nanoparticles. $ApoE^{-/-}$ mice which also expressed human CCR2 did not develop smaller atherosclerotic lesions following a high fat diet for 5 weeks while receiving a daily dose of a novel CCR2 antagonist [77]. However the mice did have reduced macrophage content within their plaques, an indicator of increased plaque stability [77]. Specifically targeting CCR2 in monocytes with a siRNA linked to a nanoparticle was used to reduce monocyte recruitment during MI inflammation in $ApoE^{-/-}$ mice [76]. A phase II clinical trial involving 112 patients at risk of CVD who received the antibody MLN1202, which is capable of directly blocking CCR2, found that their circulating CRP levels were reduced compared to the placebo group [78]. Targeting CCR5 with an antagonist retroviral drug, known as maraviroc, reduced both ritonavir-induced atherosclerotic plaque size and the number of macrophages recruited to the plaque in $ApoE^{-/-}$ mice [79]. Furthermore the expression of VCAM-1, ICAM-1, CCL2 and TNF-α were all significantly reduced within the plaque [79].

A recent *in vitro* study has found that a known HIV infection blocking anti-body, RoAb13, is also capable of reducing monocyte migration towards CCR5 stimuli in a transwell system [80]. An antagonist for CX_3CR1 known as F1 is capable of reducing the lesion size which develops in $ApoE^{-/-}$ mice following treatment three times a week for 10 weeks after the mice had already been receiving a high fat diet for 5 weeks [81]. The reduction in plaque size was due to a decrease in the number of monocytes and macrophages recruited to the site of lesion formation [81]. These studies highlight the possibility of specifically targeting chemokine receptors to attenuate monocyte recruitment during atherosclerosis progression and reduce the size of the plaques that develop. Targeting the chemokine receptors would potentially attenuate atherosclerosis plaque formation before initial lesions have time to mature into large plaques. Another anti-atherogenic strategy being pursued is the blocking of chemokine activity by viral proteins [73,74]. The vaccinia virus produces a generic CC chemokine inhibitor known as 35K. $ApoE^{-/-}$ mice which were fed a standard chow diet and injected with a lentivirus encoding 35K had reduced chemokine activity, correlating to a decrease in atherosclerotic plaque size compared to the control mice after 12 weeks [74]. Viral proteins

may be a potential therapeutic avenue capable of providing long-term attenuation of chemokine activity in order to slow atherosclerosis progression.

Although targeting cytokines and chemokines appears to be an exciting therapeutic avenue to explore in order to reduce atherosclerosis progression there are major limitations that must be first overcome. The biggest limitation to targeting cytokines is the potential consequences of systemic inhibition. Due to the major role cytokines play during the immune response [82–84], targeting them therapeutically may result in severe adverse effects. For example using antibodies to target pro-inflammatory cytokines systemically will result in a dampening of the patients' inflammatory response causing them to be at a greater risk of developing infections. Furthermore cytokines act in short range cell to cell signaling manner, therefore the administration of anti-inflammatory cytokines would result in unspecified systemic responses and would also need to be given at potentially toxic levels in order to mimic the physiological paracrine response at the intended target location [85]. Therefore the use of therapies that target cytokines systemically should be reserved for those considered high risk of suffering a CVD-related event. The development of nanoparticles and micelles (a spherical structure which is formed of lipid molecules when they are in aqueous solutions due to their hydrophobic nature) has led to the ability of directly targeting atherosclerotic plaques for imaging and therapeutic delivery [86–89]. Although nanoparticles and micelles are capable of directing antibodies and antagonists for cell surface receptors to atherosclerotic plaques for imaging [86,87], very little has been developed for directing cytokine therapies [88]. However nanoparticles have been shown to be able to target the anti-inflammatory cytokine IL-10 and improve *ex vivo* imaging of plaques from AopE$^{-/-}$ mice [90]. This study demonstrates that it is possible to target cytokines with nanoparticles and highlight the potential to use them to deliver cytokine-targeted therapies directly to atherosclerotic plaques. Indeed this potential therapeutic avenue has started to be explored. A recent study which treated LDLr$^{-/-}$ mice with a nanoparticle-directed short peptide designed to mimic annexin A1, resulted in improved inflammation resolution [91]. The advanced plaques in the LDLr$^{-/-}$ mice developed a thicker fibrous cap as well as reducing oxidative stress and necrosis within the plaque, indicating improved lesion stability compared to the control mice [91].

Another potential strategy to slow atherosclerosis disease progression is to accelerate the resolution of inflammation. This can be done by using natural products derived from food sources known as nutraceuticals [92–94]. Fish oils, which contain omega-3 polyunsaturated fatty acids (ω-3 PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have many anti-atherogenic actions [95–98]. Monocyte migration has shown to be attenuated both *in vitro* and *in vivo* with ω-3 PUFA treatment [95]. Furthermore, foam cell formation is reduced by ω-3 PUFAs, due to their ability to alter the expression of key genes implicated in the uptake and efflux of cholesterol [96,97]. There are several murine and human studies which highlight the importance of using nutraceuticals in the resolution and prevention of atherosclerosis [95,99,100]. Both EPA and DHA have been found to be metabolised via the COX and lipoxygenase pathways into a new class of lipid mediators known as resolvins [101]. Resolvins are powerful anti-inflammatory agents thought to be capable of accelerating the resolution of inflammation [101], highlighting one possible mechanism by which ω-3 PUFAs are capable of exerting their anti-inflammatory effects. It

should be noted that although the consumption of ω-3 PUFAs has shown some promise in attenuating atherosclerosis disease progression, their effectiveness as nutraceuticals is still debated in the current literature. Several meta-analyses have been unable to demonstrate any association between ω-3 PUFA consumption and reduced risk of suffering a CVD-related event [102–104]. Numerous other nutraceuticals including flavanols [105,106], phytosterols [107] and polyphenols [108,109] have also been shown to exert anti-inflammatory actions. Furthermore, a unique combination of nutraceuticals has recently been shown to exert anti-inflammatory effects on several key processes associated with atherosclerosis *in vitro* [110]. Due to the anti-inflammatory effects of nutraceuticals and their ability to reduce the expression of pro-inflammatory cytokines, they can potentially be given without the systemic-inhibition risks associated with cytokine targeting therapies. In addition nutraceuticals can also be taken safely over an individual's lifetime, a major advantage when compared to traditional pharmaceutical therapies which are only prescribed once an individual is considered to be at high risk of suffering a CVD-related event.

5 Conclusion

Due to cytokines being the main orchestrators of atherosclerosis development and the driving force behind many of the key steps involved in disease progression, including immune cell recruitment, foam cell formation and plaque stability, they represent promising therapeutic targets. As cytokines and their receptors have functional redundancy, the majority of current cytokine targeted therapies use antibodies to neutralize and prevent pro-inflammatory cytokine signaling in order to dampen the inflammatory response observed in atherosclerotic lesions and slow plaque growth. However the administration of anti-cytokine antibodies and potentially decoy receptors in patients would mean the cytokine would be targeted systemically and leave the individual immunocompromised and at a greater risk of infections. Therefore their use should be limited to those who are considered to be at high risk of suffering a CVD-related event. Despite the current limitations of cytokine-targeted therapies, they are still a therapeutic avenue worth pursuing and provide hope of attenuating atherosclerosis development by targeting the key drivers of disease progression. The development of drug delivery systems which will direct cytokine therapies to an atherosclerotic plaque will make anti-cytokine therapies an even more viable therapeutic option. However to achieve the full potential of cytokine-targeted therapies, more studies focused on developing a greater understanding of both the role and signaling pathways of cytokines in atherosclerosis development must be carried out. Once a more complete insight into cytokine signaling pathways has been developed, novel therapeutic targets may be identified.

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Future Perspective

Over the next 5 to 10 years as drug delivery systems such as nanoparticles and micelles continue to be developed and refined there will be an explosion in atherosclerotic targeted therapies. This will be particularly true for cytokine-targeted therapies. Drug delivery systems will allow for the use of cytokine-targeted therapies without the risk of potential systemic side effects. Not only will they allow the direct delivery of antibodies, decoy receptors and inhibitors of pro-inflammatory cytokine signaling directly to the plaque without the risks of systemic inhibition, they will also allow for the delivery of anti-inflammatory cytokines/agents at non-toxic levels in order to accelerate the resolution of inflammation. Furthermore, over the next 5 to 10 years there will be an increase in the number of studies focusing on nutraceuticals in order to speed up the resolution of inflammation. Nutraceuticals such as omega-3 fatty acids, flavanols, polyphenols and combinations of nutraceuticals have already been shown to exert anti-atherosclerotic effects with little to no side effects. As previously discussed some patients taking current pharmaceutical therapies are unable to achieve lower plasma cholesterol levels and sustained use of traditional pharmaceutical therapies can result in side effects, stressing the need to investigate alternative therapies such as nutraceuticals and cytokine targeted therapies.

Executive Summary

Atherosclerosis

- Atherosclerosis is characterized as the build-up of fatty deposits in the walls of medium and larger arteries.
- This build-up triggers an inflammatory response which recruits immune cells to the affected site.
- Over time the inflammatory response becomes chronic and a plaque begins to form.
- If this plaque ruptures it can lead to a myocardial infarction or stroke.

Role of cytokines in disease development

- Cytokines are small protein molecules involved in cellular signalling and can be broadly classified as either pro- or anti-inflammatory (with some able to exert characteristics of both).
- Chemokines play a major role during the initial development of atherosclerosis. They are the main driver of monocyte and other immune cell recruitment to the fatty deposit build-up.
- Once in the walls of the arteries, these monocytes become exposed to pro-inflammatory cytokines which causes them to differentiate into the pro-inflammatory M1 macrophage phenotype.
- Cytokines are then able to influence modified lipoprotein uptake and cholesterol efflux, leading to increased intracellular cholesterol levels and the development of foam cells.
- These foam cells then undergo apoptosis or necrosis and “spill” their lipid-rich content into the walls of the arteries and contribute to the size of the atherosclerotic plaque.
- The stability of the plaque is then influenced by cytokines, with pro-inflammatory cytokines leading to the increased release of MMPs from macrophages which can lead to plaque destabilization.

Targeting cytokines therapeutically

- Due to the inability of statins to reduce cholesterol levels in some individuals and potential side effects of long-term usage, targeting cytokines represents a promising therapeutic strategy.
- Neutralizing pro-inflammatory cytokine signaling by targeting either the cytokine or receptor is showing some promise.
- Neutralization can be achieved with antibodies against the cytokine/receptor to stop the two interacting and triggering the signalling pathway or by outcompeting the receptor by using a decoy receptor.

- However due to the major role of cytokines in the immune response, systemic administration of cytokine targeted therapies may result in severe adverse effects.
- To overcome this problem, the use of drug delivery systems such as nanoparticles may allow the delivery of cytokine therapies directly to the atherosclerotic plaque without any systemic side effects.

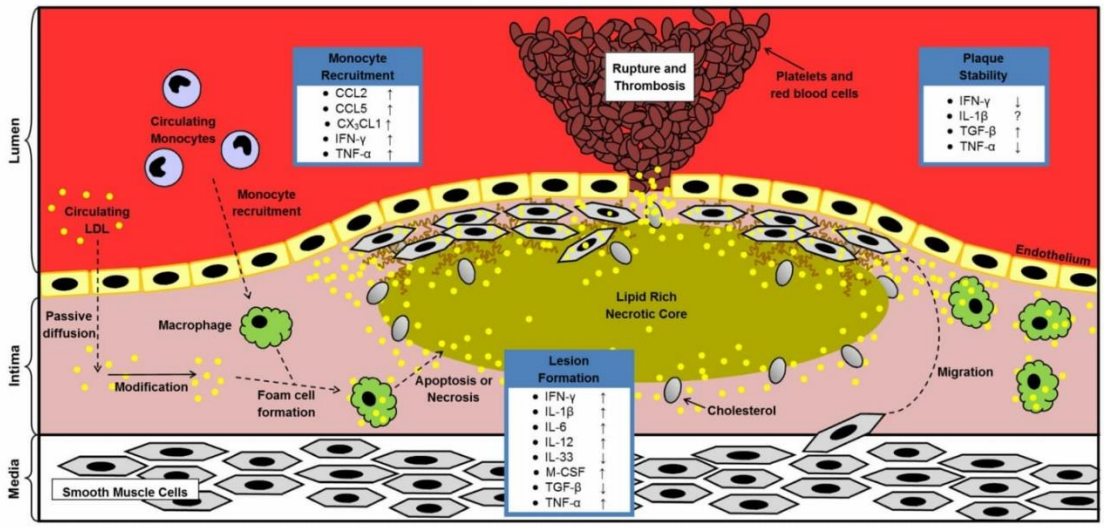

Figure 1. Atherosclerosis disease progression and the role of key cytokines.

The accumulation of modified LDL within the intima of the artery triggers an inflammatory response in the nearby endothelial cells. The activated endothelial cells begin to express cell-adhesion molecules on their surface as well as secrete pro-inflammatory cytokines and chemokines which are able to recruit circulating monocytes to the affected region. Once in the intima, the monocytes differentiate into macrophages and begin to take up modified LDL and become foam cells. Overtime these foam cells can accumulate and form the initial atherosclerotic lesion. During atherosclerotic plaque maturation, foam cells start to undergo apoptosis or necrosis, which causes the formation of a necrotic core. To stabilise the necrotic core, smooth muscle cells migrate from media to intima and form a fibrous cap over the core by depositing extra cellular matrix. The chronic inflammatory response of atherosclerosis eventually causes the mature atherosclerotic plaque to become unstable and rupture, leading to a thrombotic reaction, which can cause a myocardial infarction or stroke depending on the location of plaque formation. ↑, increase; ↓, decrease; ?, unclear. CCL, Chemokine (C-C motif) ligand; CX_3CL1, Chemokine (C-X3-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.

Table 1

The role and signaling pathway of crucial cytokines in atherosclerosis development.

Cytokine	Pro- or anti-atherogenic	Role in atherosclerosis development	Signaling pathway	Reference
IFN-γ	Pro-	Can induce the pro-inflammatory M1 phenotype in macrophages. It is able to increase foam cell formation by attenuating the expression of key genes implicated in macrophage cholesterol efflux and increase the expression of those involved in the uptake of cholesterol. Contributes to the continuously growing plaque size by inducing foam cell apoptosis which causes them to 'spill' their lipid content into the core of the plaque.	JAK-STAT	[7–14]
CCL2	Pro-	Also known as MCP-1. It is one of the major chemokines involved in monocyte recruitment during atherosclerosis development. Deficiency of CCL2 in atherosclerotic mouse models markedly attenuates disease development when CX_3CL1 and CCL5 are also knocked out. Mouse models lacking CCL2 or its receptor develop smaller atherosclerotic plaques.	PKC/ERK1/2/NF-κB	[15,16]
IL-1β	Pro-	It is able to induce the pro-inflammatory M1 macrophage phenotype. oxLDL has been shown to stimulate IL-1β secretion in human macrophages. It is a major activator of the innate immune response. The expression of several pro-inflammatory genes can be induced by IL-1β. It also exerts an auto-inflammatory response by inducing its own expression.	NF-κB/JNK/p38 MAPK	[9–11,17–21]
TGF-β	Anti-	The anti-inflammatory M2 macrophage releases TGF-β. Its over expression in $ApoE^{-/-}$ mice results in smaller plaque, whereas the inverse occurs in $ApoE^{-/-}$ mice that are deficient for the TGF-β receptor.	SMAD-dependent or SMAD-independent	[9–11,22–24]
IL-33	Anti-	IL-33 is capable of attenuating foam cell formation by stimulating macrophage cholesterol efflux and attenuating cholesterol uptake. $ApoE^{-/-}$ mice injected with IL-33 have a reduced number of foam cells present in their atherosclerotic lesions. Neutralization of IL-33 increases atherosclerosis.	ST2/IL-1RAcP/MyD88 or ERK1/2 or p38 MAPK or NF-κB	[6,25–29]

ApoE, Apolipoprotein E; CCL, Chemokine (C-C motif) ligand; PKC, Protein kinase C; CX3CL, Chemokine (C-X3-C motif) ligand; ERK, Extracellular signal-regulated kinase; IFN, Interferon; IL, Interleukin; IL-1RAcP, IL-1 receptor accessory protein; JAK, Janus kinase; JNK, c-JUN N-terminal kinases; MAPK, Mitogen activated protein kinases; MCP-1, Monocyte chemoattractant protein-1; MyD88, Myeloid differentiation primary response gene 88; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; oxLDL, oxidized low density lipoprotein; STAT, Signal transducer and activator of transcription; TGF, Transforming growth factor.

S.3.4 Nutraceutical therapies for atherosclerosis

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Nutraceutical therapies for atherosclerosis

Joe W.E. Moss and **Dipak P. Ramji**
Cardiff School of Biosciences, Cardiff University, Sir Martin Evans Building, Museum Avenue, Cardiff, CF10 3AX, UK

Abstract

Atherosclerosis is a chronic, inflammatory disease affecting large and medium arteries and is considered to be a major underlying cause of cardiovascular disease (CVD). Although the development of pharmacotherapies to treat CVD has resulted in a decline in cardiac mortality in the past few decades, CVD is estimated to be the cause of one in three global deaths. Nutraceuticals are natural nutritional compounds that are beneficial for the prevention or treatment of disease and, therefore, represent a possible therapeutic avenue for the treatment of atherosclerosis. The purpose of this review is to highlight potential nutraceuticals for use as anti-atherogenic therapies, with evidence from *in vitro*, *in vivo*, clinical, and observational studies.

In 2015, the WHO reported that approximately one third of global deaths were attributable to a cardiovascular disease (CVD)-related event1. Atherosclerosis, an inflammatory disorder of the vasculature, is the primary cause of CVD-related events, including myocardial infarction (MI) and stroke. Given the increase in prevalence of obesity and diabetes in developing countries, the global incidence of CVD is predicted to increase and impose a greater economic burden on the health-care services around the world.

Under normal healthy conditions, the metabolism and transport of cholesterol, including influx and efflux within cells, is highly regulated. The development of atherosclerosis can begin when these homeostatic mechanisms become unbalanced in favour of either increased cholesterol influx or decreased efflux. Within the blood, there are several lipoproteins that each has a different function in lipid transportation. LDL is one of the most important lipoproteins found in the bloodstream and its function is to transport cholesterol from the liver to the peripheral tissues2. LDL particles enter cells primarily by receptor-mediated endocytosis using the LDL receptor (LDLr). In order to maintain a balance in cholesterol metabolism, HDL transports excess cholesterol from the peripheral tissues back to the liver for excretion via the bile system by a process known as reverse cholesterol transport2. However, only 5% of the biliary cholesterol is excreted in faeces as the rest is reabsorbed in the intestine2. Given that high LDL-cholesterol and low HDL-cholesterol levels have been associated with reduced endothelial function, increased LDL-cholesterol and HDL-cholesterol levels are thought to be pro-atherogenic and anti-atherogenic, respectively3.

Corresponding author to D. P. R. Ramji@Cardiff.ac.uk.

Author contributions
Both authors researched data for the article, discussed its content, and wrote, reviewed, and edited the manuscript before submission.

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Therefore, strategies for treating atherosclerosis should be aimed at lowering plasma LDL levels and increasing serum HDL levels.

Research on mouse models in the past twenty years have improved our understanding of the pathophysiology of atherosclerosis. Mice do not naturally develop atherosclerosis, but LDLr-deficient and apolipoprotein E (ApoE)-deficient mouse models are prone to atherosclerotic lesion formation on a high-fat or high-cholesterol diet and are able to mimic several aspects of the disease seen in humans. Atherosclerosis is often characterised by the build-up of fatty deposits and the formation of plaques in the walls of large and medium arteries, followed by a strong immunological response to the fatty deposit accumulation (Figure 1). This initial fatty deposit build-up in the intima of arteries, often referred to as a fatty streak, is composed of ApoB containing lipoproteins, in particular LDL and other lipoprotein remnants2. This trapped LDL can then become oxidised to form oxidised LDL (oxLDL)2. The presence of oxLDL within the intima of the artery triggers an inflammatory response in the neighbouring endothelium cells which start producing pro-inflammatory cytokines and chemokines2,4. The roles of different cytokines and chemokines in atherosclerosis have been extensively reviewed elsewhere and these can be generally classified as either pro-inflammatory or anti-inflammatory4,5.

Once monocytes have migrated into the intima of the arteries, they become exposed to macrophage colony-stimulating factor and differentiate into macrophages, a process that is associated with increased expression of scavenger receptors (SRs) on their cell surface2. The uptake of LDL via the LDLr is controlled by a negative feedback loop, whereas oxLDL uptake via SRs, such as MSR1 and CD36, is unregulated6. Pro-inflammatory cytokines are capable of inducing foam cell formation by altering the expression of key genes implicated in the regulation of cholesterol metabolism and transport including *APOE*, *ABCA1*, *ACAT1*, and *MSR1*2,4,5,7,8. Foam cells subsequently begin to accumulate and form an initial lesion that matures into an atherosclerotic plaque2,4,5,8.

During maturation of the atherosclerosis lesion, the accumulated foam cells begin to undergo apoptosis and necrosis, causing them to release their fatty contents into the intima of the arteries. The apoptotic cells and the fatty contents accumulate to form a lipid-rich necrotic core. During the latter stages of plaque progression, macrophages, endothelial cells, and T cells stimulate the proliferation and migration of vascular smooth muscle cells from the media to the intima of arteries, resulting in the formation of a fibrous cap over the lipid core2,9. The fibrous cap is then strengthened by the extracellular matrix (ECM) produced by the vascular smooth muscle cells2,10. Given that the fibrous cap stabilises the lesion, the balance of ECM deposition and degradation is critical in dictating the clinical progression of atherosclerosis. ECM degrading enzymes are released particularly from macrophages that are undergoing apoptosis, shifting the balance towards ECM degradation and increasing the risk of a plaque rupture11. Clinical symptoms of plaque development are usually not observable until the plaque ruptures. Upon rupture, platelet aggregation rapidly occurs, which can quickly impede or obstruct blood flow though the artery2, resulting in a coronary event. Statins are the most commonly used cholesterol-lowering agents. Statins reduce circulating LDL-cholesterol by inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase (HMG CoA reductase), the enzyme involved in the rate limiting step during cholesterol

biosynthesis12. However, patients taking statins still harbour a discernible residual risk of a CVD-related event and a small proportion of patients are unable to achieve target plasma cholesterol levels, despite receiving the maximum recommended dosage of statin2,13. Furthermore, high-dose statin therapy is associated with side effects such as muscle pain and hepatic abnormalities14,15. Therefore, new therapeutics are needed that can either be taken alone or in combination with statins2,13. Despite emerging therapies such as ezetimibe16–18 and antibodies targeting proprotein convertase subtilisin/kexin type 9 (PCSK9)19–22 and certain pro-inflammatory cytokines23 being explored, further research should be carried out on alternative approaches that limit inflammation and other pro-atherogenic changes in atherosclerosis.

One potential therapeutic avenue being explored for the prevention of atherosclerosis is natural products, known as nutraceuticals that are thought to have anti-inflammatory properties. Nutraceuticals can be classified as either functional foods or dietary supplements with health benefits beyond their basic nutritional value. Diets that are rich in fruit, vegetables, fish, cereal grains or olive oil have all been associated with cardiovascular health benefits24–26. The aim of this review is to assess the key nutraceutical components in these diets and to discuss their possible uses for the prevention of atherosclerosis development with evidence from both pre-clinical and clinical studies found within the current literature. Figure 2 provides a summary of the stages of atherosclerosis development at which different nutraceuticals exert their potential beneficial effects.

Omega-3 polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFAs) are capable of regulating blood pressure and clotting, and are involved in the formation of eicosanoids, mediators that can modulate the inflammatory response25. PUFAs contain two or more carbon-carbon double bonds and can be classified as either omega-6 or omega-3 depending on the position of the carbon-carbon double bond closest to the methyl terminus of the molecule25. Dietary intake of PUFAs is vital as they cannot be synthesised *in vivo*; fish oils, flax seeds and nuts are a rich source of omega-3 PUFAs25, whereas vegetable oils and animal fat are the major source of omega-6 PUFAs25.

The cardiovascular health benefits of omega-3 PUFAs have been shown through several epidemiological and clinical studies over the past 60 years27,28. The American Heart Association (AHA) recommend eating two portions of oily fish every week, where one portion is defined as at least 100g29. The AHA also advise individuals who are unable to boost their omega-3 intake through diet alone to discuss with their doctor about the possibility of taking omega-3 supplements29. An epidemiological study published in 1980 found a reduced incidence of CVD-related events that could be attributed to lower serum cholesterol levels in the Inuit population of Greenland, despite their diet being rich in saturated fats (in the form of fish and whale meat) and low in fruit and vegetables30. The omega-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are both known to exert cardiovascular health benefits.

The omega-3:omega-6 PUFAs ratio is generally considered as a major determinant of CVD-related events. Consumption of omega-6 PUFAs in Europe, particularly linoleic acid (LA), has increased by approximately 50% in the past 20 years, correlating with the increased rates of inflammatory-based diseases, particularly CVD31. While the ideal dietary intake of omega-3:omega-6 PUFAs is 1:4, the actual ratio consumed is considered to be closer to 1:15 in developed countries, owing to the increased consumption of omega-6-rich vegetable oils32. Furthermore, diets high in omega-6 PUFAs and deficient in omega-3 PUFAs have been linked to increased production of pro-inflammatory eicosanoids33. Although diets high in omega-6 PUFAs have generally been linked to increased susceptibility of oxLDL formation *ex vivo*34, there is growing evidence that some are also capable of exerting anti-inflammatory effects and reducing atherosclerosis development35 (addressed below in detail under omega-6 polyunsaturated fatty acids).

Many *in vitro* and *in vivo* studies have shown that omega-3 PUFAs are capable of attenuating several key steps involved in atherosclerotic plaque development. Omega-3 PUFAs can reduce the expression of key pro-atherogenic markers in both murine and human macrophages stimulated with pro-inflammatory cytokines36,37. The attenuation of monocyte migration to the plaque has also been demonstrated in both *in vitro* and *in vivo* assays after omega-3 PUFA supplementation38. Furthermore, omega-3 PUFA treatment has been shown to both reduce the expression of genes implicated in the uptake of LDL and increase the expression of genes involved in cholesterol efflux39, which might explain in part the observation of reduced cholesterol uptake40 and accelerated cholesterol efflux41 *in vitro*.

LDLr deficient mice fed on a high fat diet to mimic human atherosclerosis were supplemented with fish oil for 16 weeks in a study by Brown and colleagues38. The fish oil-treated mice had a significant decrease in plasma cholesterol levels and atherosclerotic plaque size compared with the control group38, attributable to a 50% reduction in monocyte migration into the atherosclerotic lesion. However, the same study reported no differences in lesion size or monocyte migration after fish oil supplementation in ApoE-deficient mice38. In a separate study, investigators fed LDLr-deficient mice with a high fat diet for 8 weeks before switching to a normal diet with or without 5% EPA for an additional 4 weeks42. EPA supplementation increased plasma HDL levels and caused the plaque to regress by 20.9%. Furthermore, the expression of several pro-inflammatory factors including IFN-γ, IL-12, tumour necrosis factor (TNF)-α, and intercellular adhesion molecule (ICAM)-1, were all significantly reduced in the atherosclerotic plaques in the EPA-treated mice42.

The importance of the ratio of omega-3 to omega-6 has been demonstrated in an ApoE -deficient mouse model that also expressed a *fat-1* gene from *Caenorhabditis elegans*43. *Fat-1* transgenic mice are able to metabolise omega-6 into omega-3 PUFAs using an omega-3 fatty acid desaturase and, therefore, should have an approximate 1:1 ratio of omega-3 fatty acids to omega-6 fatty acids43. After being fed a high-fat diet for 14 weeks, the *apoE*$^{-/-}$/*fat-1* mice had smaller atherosclerotic lesions and reduced expression of IFN-γ and monocyte chemoattractant protein-1 (MCP-1; also known as C-C motif chemokine 2) compared with the *apoE*$^{-/-}$ littlermates43. However, no differences in the plasma levels of LDL, HDL, or cholesterol were observed between the two groups. Together, these preclinical data support

the use of EPA and DHA dietary supplementation to repress pro-inflammatory eicosanoid production and reduce the incidence of CVD.

Relevant clinical data in humans

The cardiovascular benefit of omega-3 PUFAs has also been demonstrated in humans. A meta-analysis published in 2015 reported that increased EPA and DHA consumption, through either supplementation or consumption of enriched foods, was associated with decreased blood triacylglycerol levels in healthy patients or in patients with marginal hyperlipidaemia44. Omega-3 PUFAs have very few detrimental side effects, and have been shown to be beneficial for individuals suffering from hypertriglycaeridemia45. Furthermore, a cohort of 600 men with CVD receiving fish oil supplementation showed reduced markers of atherothrombotic risk46. A study involving 160 Japanese patients found that low serum levels of DHA correlated with reduced endothelial function, as measured by flow-mediated dilatation47. This observation confirms the results seen in an earlier study that reported an improvement in endothelial function and arterial stiffness, as measured by flow-mediated dilatation and pulse wave velocity respectively in 29 participants after 12 weeks of daily omega-3 PUFA intake48.

Over the past 30 years there have been three well known trials performed to assess the cardiovascular benefits of EPA and DHA supplementation: the DART trial49, GISSI-Prevenzione trial50, and the JELIS trial51. The DART trial, published in 1989, recruited 2,033 men who had recently suffered a MI and randomly allocated them to receive advice or no advice on each of three dietary factors: reduced fat intake to increase the ratio of polyunsaturated fat to saturated fat; increased omega-3 PUFAs intake either in the form of oily fish or fish capsules; and increased cereal fibre intake49. After a 2-year follow up, patients who were advised to increase omega-3 PUFAs in their diet showed a significant 29% reduction in mortality compared with those who did not49, which was largely attributable to a reduction in CVD-related events. No differences were found in the mortality of patients allocated to receive the other dietary advice49.

Investigators of the subsequent GISSI-Prevenzione trial,50 published in 1999, recruited 11,324 patients with recent MI and randomly assigned them to receive omega-3 supplements (1g daily), vitamin E, both, or none for 3.5 years. The primary endpoint of the study was a composite of death, nonfatal MI, and stroke. After 6 months, the study found no clinically important changes in the serum levels of total cholesterol, LDL-cholesterol, and HDL-cholesterol50. However, one year after initial treatment, patients who had received omega-3 PUFA supplementation, but not vitamin E, showed a 15% reduction in the primary endpoint of the study. Furthermore, sudden cardiac death was 45% lower in the treatment group compared to the control group50. Together, these two large trials support the use of omega-3 PUFAs in the context of MI.

The benefit of adding EPA to statin therapy has been evaluated in several trials. In a study published in 2007, a total of 18,645 patients with hypercholesterolaemia recruited for the JELIS trial51 were randomly assigned to statin therapy combined with EPA supplementation, or statin-only therapy. After an average follow up of 4.6 years, a 19% relative reduction in major CVD-related events was observed in patients receiving EPA and

statin, compared with the statin-only group. However, EPA supplementation did not increase serum HDL levels or reduce serum LDL levels51. In another study published in 2016, 95 patients who had been receiving statin treatment for a minimum of 6 months were randomised to receive EPA supplementation (1,800 mg/day) or no additional treatment for 6 months52. Compared with the statin-only therapy group, the atherosclerotic plaques of patients who had received EPA had fibrous caps with increased collagen content as well as a reduction in lipid volume, indicating increased plaque stability52. Furthermore, patients receiving EPA showed reduced levels of pro-inflammatory cytokines, including MCP-152. These clinical trials provide support that increasing omega-3 PUFA levels, especially EPA and DHA, alone or in combination with statin therapy can substantially reduce an individual's risk of a major CVD-related event.

Despite the promising results discussed thus far, the benefits of omega-3 PUFA supplementation on cardiovascular health remain inconclusive, given the conflicting results in the literature. In 2014, a meta-analysis that included five trials enrolling 396 participants found no significant reduction in CVD-related events in individuals with peripheral arterial disease53. A systematic review that included 48 randomised controlled trials (36,913 individuals) and 41 cohort studies also did not detect any significant reductions in CVD-related mortality in patients receiving omega-3 supplementation for 6 months54. A meta-analysis that specifically focused on patients with a history of CVD was also unable to identify any substantial protective effects of omega-3 PUFA supplementation in 14 randomised double blind trials that recruited 20,485 participants55. A further meta-analysis also failed to demonstrate any association between omega-3 supplementation and mortality risk after evaluation of 20 randomised clinical studies that included 68,680 individuals in total56. However, care must be taken when interpreting the results of clinical trials owing to the heterogeneity within the designs of the studies53. One difference between the trials is whether omega-3 PUFAs were given alone or in combination with statins, which together might have exerted synergistic effects. In addition, dose and intervention time period differs between the trials. Furthermore, one key difference between the trials is the populations used. The consumption of omega-3 PUFAs is approximately 15 times lower among Western populations compared with the Japanese population57,58, which might affect studies that use omega-3 PUFA dietary supplementation. All of these factors are likely to affect the outcomes of the trials, and result in the inconsistent results found within the clinical trials and meta-analysis.

Two trials are currently ongoing that use omega-3 PUFA supplementation: the REDUCE-IT59 and STREGTH60. The REDUCE-IT trial, which is expected to be completed in 2017, has an estimated enrolment of 8,000 participants and is designed to investigate the effect of Vascepa® (icosapent ethyl), a purified ethy ester of EPA for the treatment of hyperglyceridaemia59. The primary aim of the REDUCE-IT trial is to evaluate whether Vascepa® and statins are able to further reduce the incidence of CVD-related events compared with statin-only treatment. The secondary aim of the study is to evaluate its effects on serum lipid and lipoprotein levels59. The STREGTH trial, which is scheduled to be completed in 2019, is designed to assess the effect of combined statin and Epanova® (ω-3 carboxylic acids) therapy in an estimated 13,000 individuals60. The main aim of the STREGTH trial is to assess whether Epanova® can reduce the number of CVD-related

events compared with those who received the statin-only treatment. These two new large clinical trials will hopefully be able to provide more insight into whether omega-3 PUFA supplementation can reduce the residual risk of CVD present in users of statin.

Omega-6 polyunsaturated fatty acids

Although a high intake of omega-6 PUFAs is traditionally thought to promote inflammation and contribute to the pathogenesis of many diseases, including CVD, not all omega-6 PUFAs are associated with detrimental effects. The AHA currently recommend that omega-6 PUFAs should generally make up 5% to 10% of the energy intake of an individual's diet, provided other AHA dietary and lifestyle guidelines are followed, as it is thought that lowering omega-6 intake any further is more likely to increase the risk of suffering a CVD-related event rather than decrease it61. One key omega-6 PUFA that is considered to have anti-atherogenic effects is dihomo-γ-linolenic acid (DGLA) that can be metabolised after consumption into prostaglandin E1 (PGE1), a potent anti-atherogenic compound35. Pre-treating murine macrophages with DGLA resulted in a dose-dependent increase in prostaglandin levels, primarily PGE1 and prostaglandin D1, following lipopolysaccharide stimulation62. PGE1 has been shown to improve atherosclerotic plaque stability by increasing the thickness of fibrous cap in a dose-dependent manner in rabbits with a vulnerable plaque induced by balloon injury and a high cholesterol diet63. The right balance of omega-3 and omega-6 PUFAs is essential for optimal cardiovascular health, as they are capable of interacting and influencing the metabolism of one another35. DGLA can increase the metabolism of EPA into prostaglandin I3, a potent vasodilator and platelet anti-aggregator64, whereas EPA inhibits DGLA conversion to arachidonic acid, resulting in higher tissue levels of DGLA35. This is subsequently metabolised into a variety of products, in particular PGE1. ApoE deficient mice receiving a 0.5% DGLA diet for 6 months showed a significant increase in vasodilatation and a reduction in mRNA levels of ICAM-1 and vascular cell adhesion molecule (VCAM)-165. DGLA supplementation was also associated with a decrease in plaque size, exemplified through a reduction in lipid accumulation, monocyte and macrophage number, and migration of vascular smooth muscle cells65. Furthermore, diets that are enriched in γ-linolenic acid (GLA), a precursor of DGLA during omega-6 metabolism, have also been shown to reduce blood pressure in spontaneously hypertensive rats66.

Relevant clinical data in humans

Several observational studies have shown a reduction in omega-6 PUFAs in patients with atherosclerosis. Luostarinena and colleagues compared the make-up of fatty acids in the phospholipid fraction of human coronary arteries between aged-matched patients who died of ischaemic heart disease and patients who died of other non-cardiovascular causes67, and found a reduced proportion of both omega-3 and omega-6 PUFAs in those who had died from a CVD-related event. In a separate study, the lipid profile of 668 aortic plaques from 30 men who died of ischaemic heart disease were analysed and compared with their undisrupted plaques68. The concentration of all fatty acids was significantly increased at the edge of disrupted plaques compared with the center; however, the proportion of omega-6

PUFAs as a percentage of total fatty acid concentration was significantly lower, suggesting possible oxidation of PUFAs.

Low serum levels of GLA has been correlated with peripheral arterial disease in a cohort of 474 participants69. Treatment of 120 individuals suffering from lower limb atherosclerosis with a combination of GLA and EPA also significantly improved their blood pressure after 2 years compared to those receiving the placebo70. Additionally there was a small but non-significant reduction in the number of non-fatal CVD-related events70. Furthermore a smaller study has observed a decrease in serum triacylglycerol, total cholesterol and LDL levels as well as an increase in serum HDL levels following daily GLA consumption for 4 months in hyperlipidemic patients71. By contrast, an epidemiological study involving 2,206 Japanese men found that increased serum levels of omega-6 PUFAs was associated with increased arterial stiffness, in addition to higher serum C-reactive protein (CRP) levels72. An additional study involving 501 participants also linked increased serum levels of omega-6 PUFAs with increased arterial stiffness73, whereas a smaller randomised, double-blind trial reported that daily DGLA administration for 4 weeks did not exert any anti-thrombotic effects74. Given these mixed findings, whether DGLA or GLA can contribute to the prevention of atherosclerosis or reduce the risk of a CVD-event in individuals who already have atherosclerosis remains controversial, emphasising the need for further studies.

Although DGLA and GLA are products of linoleic acid metabolism, linoleic acid supplementation has not always convincingly been associated with cardioprotective effects. Linoleic acid did not improve arterial stiffness, blood pressure, serum lipid concentrations, or serum CRP levels after 6 months of supplementation in overweight individuals75. However recent re-evaluation of the Minnesota Coronary Experiment (MCE), performed in 1968 involving 9570 participants, found that replacing saturated fat with linoleic acid reduced serum cholesterol levels76. Despite lowering serum cholesterol levels, linoleic acid supplementation was unable to reduce the risk of a CVD-related event76. In contrast, an epidemiological study involving 1,813 individuals found an association between higher tissue levels of linoleic acid and a decreased risk of MI77. However, given that linoleic acid is metabolised into GLA and DGLA, this higher linoleic acid tissue level might actually represent increased GLA and DGLA formation. DGLA and GLA might thus be more suitable for use as nutraceuticals than linoleic acid.

Together, the data presented suggests that DGLA or its precursor GLA as a nutraceutical might be as effective as EPA and DHA supplementation for preventing atherosclerotic development, owing to their direct actions or the need to maintain an optimal ratio of omega-3 to omega-6 PUFA. However, the observation that omega-6 PUFAs might be associated with increased arterial stiffness is concerning, and requires further investigation.

Hydroxytyrosol

The Mediterranean diet has long been associated with reduced incidence of CVD-events78. Individuals living in countries within the Mediterranean basin consume a greater amount of olive oil compared with those elsewhere around the world. Several epidemiological studies have reported a correlation between increased levels of olive oil in the diet and a lower risk

of developing atherosclerosis and other CVD26,79. Numerous polyphenol compounds in olive oil exert anti-inflammatory effects, including oleuropein, tyrosol and hydroxytyrosol. Oleuropein has been shown to reduce reactive oxygen species (ROS)-mediated expression of matrix metalloproteinase (MMP)-9 and cyclooxygenase 2 (COX-2) in human umbilical vein endothelial cells (HUVEC)80. Furthermore, oleuropein and hydroxytyrosol have been shown to inhibit lipopolysaccharide-induced expression of VCAM-1, ICAM-1, and E-selectin in a dose-dependent manner in HUVEC81. However, oleuropein undergoes almost complete degradation during olive ripening and, therefore, is unlikely to contribute to the cardiovascular health benefits associated with the Mediterranean diet82. By contrast, hydroxytyrosol levels increase throughout the ripening process82 and therefore it is often considered as one of the major anti-atherogenic polyphenol compounds in olive oil.

Numerous *in vitro* and *in vivo* studies have assessed the use of hydroxytyrosol as a nutraceutical for atherosclerosis. Co-incubation of hydroxytyrosol with pro-inflammatory cytokines in HUVEC *in vitro* resulted in a significant reduction in the expression of cell surface adhesion molecules such as VCAM-1 and ICAM-1 compared with incubation with cytokines alone83. Furthermore, hydroxytyrosol has been shown to reduce the production of several pro-inflammatory markers in cultures of primary human monocytes84. A murine study involving 32 Wistar rats that were fed olive oil-based diets for 6 weeks demonstrated that a phenol-enriched olive oil was able to significantly increase plasma HDL levels85. The same study also showed that the non-enriched virgin olive oil did not significantly alter HDL levels, indicating that cardioprotective effects of the olive oil was dependent on the phenol compounds85. However, neither the virgin olive oil nor the enriched olive oil was able to reduce plasma LDL levels. A subsequent study involving 60 Wistar rats did observe a decrease in total cholesterol and plasma LDL-cholesterol levels in those fed both virgin olive oil and cholesterol, compared with rats fed with only cholesterol after 4 weeks86. Hydroxytyrosol was also able to reduce atherosclerotic plaque size and improve antioxidant status in hyperlipaemic rabbits fed an atherogenic diet87.

Increasing dietary intake of hydroxytyrosol might be a strategy to increase serum HDL levels, as well as decreasing serum oxLDL levels. However, this approach might not be effective for those already on a low-cholesterol diet. ApoE-deficient mice that were given a standard chow diet and daily hydroxytyrosol supplementation for 10 weeks showed larger atherosclerotic lesions compared with the control group88, in addition to a decrease in ApoA1 levels, and an increase in total cholesterol levels, with no changes in plasma HDL levels. These results indicate that hydroxytyrosol might actually enhance atherosclerosis development in those on a low-cholesterol diet. Given that the majority of patients at risk of CVD are likely to already be on a low-cholesterol diet, further *in vivo* studies are required to understand the effects of hydroxytyrosol supplementation when taken in combination with a low-cholesterol diet.

Relevant clinical data in humans

Many clinical trials have been performed to investigate the potential health benefits of hydroxytyrosol supplementation. The randomized, crossover, controlled EUROLIVE study involving 200 healthy male individuals that were assigned to receive olive oil with low,

medium, or high phenolic content found a linear relationship between the phenolic content of olive oil and an increase in serum HDL levels, which resulted in a decrease in the ratio of total cholesterol to HDL cholesterol89. This increase in HDL was also accompanied by a decrease in triacylglycerol levels, as well as a reduction in the markers of oxidative stress89. Consistent with this finding, two additional studies have shown that hydroxytyrosol is also capable of decreasing serum oxLDL concentration in a dose-dependent manner in both healthy individuals and patients with coronary heart disease (CHD)90,91. Hydroxytyrosol has also been shown to exert anti-inflammatory effects in 28 patients with stable CHD who received a daily dose of virgin olive oil (50ml) for 21 days92. Daily intake of virgin olive oil intake reduced IL-6 and CRP levels, key markers of inflammation and predictors of CVD92. Furthermore, a randomised, controlled, double-blind, crossover study involving 13 prehypertensive or hypertensive individuals found that olive oil enriched with its own polyphenols significantly improved endothelial function and decreased oxLDL levels compared with non-enriched olive oil alone93.

The PREDIMED study, involving 7447 participants considered to be at high CVD risk, found that receiving a Mediterranean diet supplemented with either extra-virgin olive oil or nuts for 5 years significantly reduced an individual's risk of suffering a CVD-related event compared to those on the low-fat control diet94. However it should be noted that there was no difference in the total number of CVD-related events between the olive oil and nut diet receiving groups94. Earlier analysis of the PREDIMED study in 187 asymptomatic high CVD risk patients, identified a significant reduction in the intima-media thickness in those with an initial baseline of 0.9 mm or thicker after one year on a Mediterranean diet supplemented with either olive oil or nuts95. However no changes in the intima-media thickness were observed in patients whose baseline was less than 0.9 mm, indicating a possible role for the use of the Mediterranean diet in order to reduce subclinical atherosclerosis in those at a greater initial risk95. The PREDIMED study would have benefited from a Mediterranean diet without supplementation group to fully determine whether olive oil and nut supplementation exerted additional cardiovascular protectives compared to the base diet.

Another trial randomly assigned 90 participants into three treatment groups, patient's regular diet, Mediterranean diet and virgin olive oil (328 mg/kg polyphenols) or Mediterranean diet and washed virgin olive oil (55 mg/kg polyphenols) for 3 months96. A significant reduction in serum levels of LDL, HDL and total cholesterol were observed after 3 months when compared to baseline levels in those receiving the Mediterranean plus virgin olive oil diet with no changes in total cholesterol:HDL or LDL:HDL ratios96. The serum cholesterol levels of those receiving the Mediterranean and washed olive oil were also not significantly altered when compared to baseline, however both Mediterranean dietary interventions significantly reduced serum CRP levels when compared to their respective baselines96. Furthermore, the Mediterranean diet with virgin olive oil significantly reduced the expression of several pro-atherogenic genes, including IFN-γ, compared to the control group96. Additionally a trial involving 52 participants who received polyphenol enriched olive oil for 4 months showed signs of significant improvement in endothelium function as well as a decrease in several inflammatory markers, including serum ICAM-1 levels and monocyte number97. However it should be noted that some individuals also received an

olive oil enriched with epigallocatechin 3-gallate, however it did not provide any additional benefits when compared to the polyphenol enriched oil group97. These clinical studies together highlight the potential anti-atherogenic properties of hydroxytyrosol.

Allicin

Allicin (diallyl thiosulfinate) is a natural organic sulphur-containing compound found in garlic (*Allium sativum*). When fresh garlic is crushed, alliin is converted into allicin by the enzyme alliinase. The newly formed allicin is highly unstable and rapidly breaks down into several smaller polysulphides which are able to form hydrogen sulphide (H_2S) in a thiol-dependent manner in cells98. The anti-atherogenic and anti-inflammatory health benefits of garlic are attributable to this formation of H_2S.

The benefits of treatment with H_2S donors (compounds capable of being broken down into H_2S) have been observed in both *in vitro* and *in vivo* studies. The treatment of lipopolysaccharide-stimulated murine macrophages with sulphur-containing compounds originating from garlic has been shown to attenuate the expression of several pro-inflammatory cytokines, including IL-1β, IL-6, and TNFα99. The anti-inflammatory abilities of H_2S donors have also been observed *in vivo* using murine models, which demonstrate inhibition of leukocyte adherence to the endothelium, indicative of a reduction in the inflammatory response100. In addition to diminishing the initial inflammatory response, H_2S has also been shown to attenuate p38 mitogen-activated protein kinase activation and caspase-3 cleavage, which results in accelerated resolution of inflammation by stimulating the short-term survival of neutrophils101.

Potent antioxidant effects have been associated with H_2S, with many studies showing that H_2S donors are capable of reducing lipopolysaccharide-stimulated inducible nitric oxide synthase and cyclooxygenase (COX)-2 expression, which consequentially diminishes ROS production *in vitro*99,102. Furthermore, H_2S donors have been shown to reduce foam cell formation by attenuating the expression of MSR1, sterol O-acyltransferase 1 (also known as ACAT1), and CD36 in human monocyte-derived macrophages, possibly through the ATP-sensitive K^+ channel (K_{ATP}), and mitogen-activated protein kinase 1 and 3 pathways103. ACS14 (2-acetyloxybenzoic acid 4-(3-thioxo-3H-1,2-dithiol-5-yl)phenyl ester) is a novel H_2S-releasing aspirin that has been used to study the effects of H_2S donors on atherosclerotic plaque development in ApoE-deficient mice104. Mice supplemented with ACS14 developed smaller atherosclerotic lesions compared with mice receiving the equivalent dose of regular aspirin, possibly attributable to reduced monocyte migration into the plaque104. Administration of pure allicin in ApoE-deficient and LDLr-deficient mice has also been shown to reduce plaque size by approximately 69% and 57%, respectively, after 8 weeks compared with placebo105.

Relevant clinical data in humans

The benefits of garlic supplementation have also been observed in clinical studies. In a study involving 152 participants, high-dose dietary garlic supplementation (900 mg garlic powder/day) for 48 months significantly attenuated lesion volume by 6–18%106. A subsequent preliminary study showed that treatment with statin plus aged garlic extract was capable of

slowing the rate of atherosclerotic development by reducing coronary calcification compared with statin-only therapy107. A meta-analysis of 45 trials found that garlic supplementation was also able to reduce serum levels of LDL, triacylglycerol and cholesterol after 1-3 months but not after 6 months108. The study also found that garlic was unable to significantly improve blood pressure. The effect of garlic supplementation on clinical outcomes was not analysed due to the lack of robust, long-term trials, stressing the need for large clinical trials to fully evaluate the potential of garlic as a nutraceutical. Notably, a randomised clinical study involving 192 participants found no differences in LDL or HDL levels between patients receiving garlic in three different forms (aged garlic extract, raw garlic or garlic powder) and the patients receiving placebo109. However, allicin might exert its cardioprotective effects via other mechanisms, such as reducing ROS production and attenuating pro-inflammatory gene expression, rather than directly altering the ratio of LDL to HDL in the bloodstream.

Phytosterols

Phytosterols are steroid compounds found in plant sources and are similar in structure to cholesterol. Diets rich in phytosterols have long been associated with reduced plasma-LDL levels110,111. Phytosterols are thought to exert their cardioprotective effects by competing with cholesterol in the lumen of the intestine during dietary and biliary cholesterol uptake111. Murine macrophages treated with phytosterols *in vitro* have shown changes in the expression of genes implicated in cellular cholesterol homeostasis, including an increase in ABCA1 and a decrease in LDLR112. Furthermore, phytosterols also increase cholesterol efflux in response to ApoA1 and HDL in human THP-1 macrophages, consistent with the observed changes in gene expression112.

Phytosterols have also been shown to mediate strong anti-inflammatory effects *in vivo*. This effect has been demonstrated in ApoE-deficient mice fed a high-fat diet supplemented with 2% phytosterols for 2 weeks, and then injected with ovalbumin to trigger an inflammatory response to a foreign antigen113. The spleen cells from phytosterol-treated mice showed reduced production of pro-inflammatory cytokines IL-6 and TNF-α, and increased production of the anti-inflammatory cytokine IL-10 compared with the spleen cells from mice on the control diet113. In addition, atherosclerotic lesion size was 60% smaller in mice on the phytosterol-enriched diet113. After 14 weeks on a diet supplemented with 2% phytosterols, ApoE-deficient mice showed alterations in the expression of 132 genes, including several hepatic genes associated with the regulation of sterol metabolism114. The changes in gene expression in this study may provide a greater insight into how phytosterols mechanistically exert their cardiovascular protective effects. However, further studies are required to link specific altered gene expression patterns to the anti-atherogenic properties of phytosterols. In a separate study, the atherosclerotic lesions of ApoE-deficient mice fed a high-fat diet supplemented with a 2% phytosterols mixture for 20 weeks were reduced by approximately 50% compared with the high-fat diet-only control group115. Furthermore, phytosterol supplementation was also associated with reduced hepatic lipase activity and plasma fibrinogen concentrations, in addition to a small increase in HDL-cholesterol levels115. Consistent with these findings, later studies also reported smaller atherosclerotic

lesions and lower plasma LDL-levels in ApoE-deficient mice fed a 2% phytosterol-supplemented diet after 12 and 14 weeks116,117.

Relevant clinical data in humans

An epidemiological study involving 22,256 participants found a correlation between diets with high levels of phytosterols and low levels of serum LDL, supporting the role of phytosterols in LDL lowering111. Consistent with this finding, a study involving 233 participants demonstrated a significant reduction in serum LDL levels with 12 weeks of phytosterol supplementation, though no changes in flow-mediated dilatation or pulse-wave velocity was found118. Recent meta-analysis, involving 20 randomised control trials and 1308 participants, found an association between regular phytosterol intake and reduced serum LDL levels119. However, the study failed to find any significant correlation between phytosterol consumption and plasma CRP levels119, highlighting the need for further research to assess the effects of phytosterol dietary supplementation on inflammation. Nevertheless, because of the LDL lowering effects of phytosterols, the European Atherosclerosis Society (EAS) consensus panel has recommended the use of phytosterol supplementation in individuals who are either: at low/intermediate risk of CVD but fail to meet requirements for traditional pharmaceutical therapies; suffering from familial hypercholesterolemia; or unable to achieve target LDL levels while receiving statin therapy120,121.

The type of phytosterol-delivery system has also been shown to impact their LDL lowering properties. For example, one study found that treatment of hypercholesterolemia with phytosterol capsules did not result in a reduction in plasma LDL levels122. However other studies have reported that phytosterol capsules and phytosterol-rich foods do not differ in their LDL lowering properties123. These studies emphasise the need for further trials to evaluate whether the LDL lowering properties of phytosterols are altered by the chosen delivery system.

Despite the reported beneficial effects of phytosterols, other studies have also suggested that high levels of phytosterol in the diet might actually be detrimental and contribute towards atherosclerotic development124. In a study involving 109 postmenopausal women, an increased ratio of phytosterol to cholesterol was associated with a higher risk of developing CHD125. However, many studies claiming that phytosterols can increase the risk of CVD-events lack appropriate controls or fail to match serum LDL levels between cases and controls; therefore, their findings must be taken with caution124. For example, Assmann and colleagues reported that serum phytosterol levels were significantly higher in 159 participants who had suffered from a MI or sudden cardiac death compared with 318 healthy individuals126. However, the study failed to match LDL-cholesterol, total cholesterol, and triacylglycerol levels, in addition to blood pressure levels between the two groups, all of which are risk factors for CVD-events124. Given that the ratio of phytosterol to cholesterol between the two groups was not significantly different, the study fails to provide conclusive evidence that the CVD-events were directly linked to increased phytosterol levels124.

Flavanols

Flavanols, a subclass of flavonoids, are secondary plant metabolites that are commonly found in fruit and vegetables127. Given that a diet rich in fruit and vegetables is linked with cardiovascular health benefits, flavanol supplementation represents a promising avenue as a nutraceutical for the prevention of atherosclerosis24. Catechin is a major flavanol present in green tea and cocoa that has been found to reduce endothelial exocytosis128, a process by which activated endothelial cells are able to release pro-inflammatory cytokines and chemokines, which are usually stored in intracellular endothelial granules, into the extracellular space128. Catechins might therefore have a role in reducing vascular inflammation during the development of atherosclerosis.

ApoE*3-Leiden mice fed a high-fat diet supplemented with 0.1% epicatechin (*cis* configuration isomer of catechin) for 20 weeks showed attenuation of atherosclerotic lesion area with no effect on plasma lipids129. Furthermore, a microarray analysis also revealed that epicatechin supplementation resulted in 173 genes being differently expressed compared with no supplementation, including 77 that appeared to be inversely regulated129. A substantial number of these 173 genes were involved in cell migration129, highlighting a possible mechanism by which epicatechin is able to reduce lesion size.

Relevant clinical data in humans

An increase in nitric oxide production has also been observed in a small clinical study in which 27 healthy individuals consumed a flavanol-rich diet consisting of cocoa (epicatechin and catechin) for 5 days130. This increase in nitric oxide production was accompanied by an increase in vasodilatation, providing an insight into another mechanism by which flavanols exert their cardioprotective effects130. Other studies have since confirmed the vasodilatory properties of flavanols, in addition to observing a reduction in circulating oxLDL levels after 5 weeks of flavanol supplementation in the form of green tea extract131,132. Daily catechin consumption for 24 weeks has also been shown to significantly reduce circulating LDL levels in obese or near-obese children compared with those who did not receive supplementation133. In addition, the consumption of cocoa flavanol-rich supplements for 30 days improved flow-mediated dilatation in 57 patients with end-stage renal disease134, highlighting its use in a population with endothelial dysfunction at high risk of developing CVD. Furthermore in a trial with 20 patients with congestive heart failure, who were randomly assigned flavanol-rich chocolate or control chocolate for 4 weeks, found that flavanols significantly improved flow-mediated dilation135.

Many other studies have also demonstrated the beneficial effects of cocoa flavanols in healthy individuals. Daily cocoa flavanol supplementation for 30 days resulted in improved vascular function in 100 healthy individuals without a prior history of CVD136. Additionally, the consumption of cocoa flavanols for 14 days improved flow-mediated vasodilation and reduced arterial stiffness in both young and elderly participants137. Furthermore, catechin has been shown to exert anti-inflammatory effects, as consumption of a green tea extract attenuated the levels of several pro-inflammatory mediators, including Fas ligand, IL-6 receptor, IL-8, soluble TNF-receptor 2, and neutrophil-activating peptide138. The ratio of total cholesterol to HDL cholesterol is also significantly reduced in

17 healthy men after daily catechin supplementation for 3 weeks underlining its possible use in the prevention of atherosclerosis139. However the same study found no reductions in other cardiovascular disease risk biomarkers such as blood pressure139. Although another study investigating the intake of daily cocoa flavanol for 4 weeks failed to show a decrease in blood pressure and flow-mediated dilatation in 30 overweight adults, a significant improvement in arterial stiffness was found in the female participants140. The lack of reduction in blood pressure in these studies contradicts the decreases found in the previously mentioned trials130–132. This discrepancy might be attributable to the small number of individuals used in the trials.

Together, these data suggest that flavanols might exert their cardiovascular health benefits by lowering circulating LDL levels and possibly blood pressure, both of which are key risk factors of atherosclerosis development.

Vitamin C and E

Given that the human body is unable to store vitamin C (also known as ascorbic acid), it is vital that foods rich in vitamin C, such as oranges, orange juice, broccoli and blackcurrants, form part of the daily diet. Increased intake of vitamin C has long been associated with a decrease in the prevalence of coronary artery disease141. Numerous *in vivo* studies have shown that vitamin C supplementation can improve endothelial function142,143. ApoE-deficient mice supplemented with 1% vitamin C for 26 to 28 weeks were found to have restored endothelial nitric oxide synthase activity and increased tetrahydrobiopterin levels in the aorta compared with the control mice142. A later study in ApoE-deficient mice fed a high-fat diet supported these results by demonstrating that chronic treatment with vitamin C inhibited endothelial dysfunction of the carotid artery induced by hypercholesterolaemia143. Despite such promise, the use of vitamin C as a nutraceutical for the prevention of atherosclerosis remains controversial because many studies have failed to show any benefit on plaque lesions or lipid profiles. Dietary supplementation with a cocktail of anti-oxidants (vitamin E, vitamin C, and β-carotene) in ApoE deficient mice did not reduce lesion size or alter plasma lipid profile144. However, this study involved older mice (20 weeks old), whereas the previously mentioned positive mouse studies were performed in much younger mice (4–5 weeks142,143), suggesting that age might influence the cardiovascular health benefits of vitamin C supplementation in ApoE-deficient mice. Consistent with this observation, vitamin C and E supplementation in ApoE-deficient mice aged 50–60 weeks failed to significantly reduce angiotensin II induced plaque rupture145. By contrast, vitamin E supplementation in 26-week-old ApoE-deficient mice prevented angiotensin II mediated plaque rupture146.

Relevant clinical data in humans

Lower serum vitamin C levels have been linked with a greater risk of a CVD-event in humans147. Vitamin C can exert its cardiovascular health benefits by mitigating inflammatory and oxidative stresses mediated by a high-fat and high-carbohydrate diet, by preventing endotoxin increase and Toll-like receptor expression148. Others have reported that vitamin C is able to improve vasodilatation in patients with coronary artery disease149

and in smokers150, thereby resulting in reduced blood pressure, and consequently reduced risk of CVD-related events. In 2014, a meta-analysis based on 44 clinical trials found a positive association between vitamin C supplementation and improved endothelial function in patients with atherosclerosis151. In addition, the ASAP trial, involving 520 participants, showed a significant attenuation in the progression of atherosclerosis in men following a treatment with a combined supplementation of vitamin C and E twice a day for 3 years152. However, individual supplementation with vitamin C or E failed to reduce intima-media thickness, and the combined supplementation did not reduce atherosclerosis progression in women152. Pooled analysis of 9 studies by Knekt *et al.*153 found an association between high vitamin C supplementation and a reduced risk of CVD-related event. However, the same analysis also found that high vitamin E intake was not associated with any cardiovascular protective effects153. By contrast, the CHAOS trial involving 2,002 patients with established atherosclerosis found that daily vitamin E supplementation reduced the risk of suffering a non-fatal MI compared to those receiving the placebo after 1 year154. However the study also found that vitamin E supplementation was unable to reduce CVD-related deaths154. Furthermore, the treatment of 30 hypertensive men with a combined vitamin C and E supplement every day for 8 weeks significantly improved arterial stiffness and flow-mediated dilation, as well as reducing their oxidative stress levels155.

Despite numerous positive findings, the inconsistencies in the results assessing vitamin C and vitamin E supplementation are also evident in many other clinical trials. A randomised study that used an initial 2g dose followed by a daily intake of 1g of vitamin C in 20 young adult smokers showed improved vasodilation after the first 2 hours, but there were no sustained beneficial effects after 8 weeks156. In addition, a large-scale study involving 20,536 adults in the UK with either coronary artery disease, peripheral occlusive arterial disease, or diabetes that were randomly assigned a daily dietary supplement containing either vitamin E, vitamin C, β-carotene, or placebo reported no observable benefits in terms of all-cause mortality or CVD-events at the 5-year follow up157. Furthermore, several studies have failed to demonstrate any cardiovascular protective effects following vitamin E consumption. The previously mentioned GISSI-Prevenzione trial found that daily consumption of vitamin E (300 mg) was not associated with a reduced risk of CVD-related events50. The HOPE study, which involved 9,541 participants considered to be at high risk of a CVD-related event, was also unable to find any significant reductions in cardiovascular deaths following daily vitamin E consumption for 4.5 years158. On the other hand, the VEAPS trial observed a decrease in plasma oxLDL levels and a reduction in the vulnerability of LDL to oxidation in 353 individuals following daily vitamin E supplementation for 3 years159. However, this trial also demonstrated that vitamin E supplementation was unable to reduce the intima-media thickness compared to the placebo159, indicating that it was unable to prevent atherosclerosis development. In conclusion, although both vitamin C and E were once considered ideal nutraceuticals for the prevention of atherosclerosis owing to their antioxidative and vasodilatory properties, they have not been proven to be consistently effective in long-term prevention of CVD. This position is consistent with the AHA whose advisory panel in 2004 recommended against using vitamin supplements to reduce the risk of CVD-related events160.

Dietary fibre

Dietary fibre can be fermented by the gut microbiota in the intestine to produce a variety of short chain fatty acids that are capable of exerting anti-atherogenic properties. Butyrate is a key short chain fatty acid produced during fibre fermentation that has been shown to prevent inflammation161,162. Butyrate treatment of murine macrophages stimulated with lipopolysacchardie have reduced pro-inflammatory cytokine production, including IL-1β, IL-6, and TNF-α, and attenuated nitric oxide production162. Furthermore, HUVEC treated with butyrate for 24 hours resulted in increased ICAM-1 expression, but no changes in VCAM-1 expression163,164. However, preincubation of HUVEC with butyrate attenuated TNF-α induced expression of VCAM-1, which correlated with a decrease in monocyte adhesion to endothelial cells164.

In vivo studies have also demonstrated the benefits of butyrate in atherosclerosis. ApoE-deficient mice fed a chow diet supplemented with 1% butyrate for 10 weeks developed smaller and more stable lesions compared to the control mice165. Lesions were reduced by approximately 50% owing to attenuated monocyte and macrophage migration towards the site of the plaque, together with lower levels of VCAM-1 and MCP-1 expression in the lesion165. Furthermore, the lesions in the butyrate-supplemented mice were composed of more ECM compared with the control mice, which is an indicator of increased plaque stability.

Relevant clinical data in humans

The relationship between increased fibre intake and reduced cardiovascular disease has been well established. A 6-year follow-up study involving 39,876 female participants found higher fibre intake was associated with a lower risk of MI and CVD after adjusting for age and other treatments received166. However, this relationship was no longer found to be significant after controlling for other confounding variables. Another study involving 46,032 men found that increased dietary intake of fibre was significantly linked with reduced risk of peripheral arterial disease over a 12 year follow up, even after adjusting for all other factors167. Increased dietary fibre has also been correlated with a lower risk of haemorrhagic stroke168. Furthermore, a meta-analysis of 10 cohort studies involving 91,058 men and 245,186 women reported an inverse relationship between increased dietary fibre intake and the risk of suffering a CVD- event169. For every 10g increase of dietary fibre per day, there was a 14% and 27% decrease in the risk of suffering a CVD-event and coronary death, respectively, over a 6–10 year follow-up period169. However, addition of fibre to statin and/or ezetimibe treatment did not provide extra cardiovascular health benefits to patients with hypercholesterolaemia, but improved blood glucose levels and reduced BMI170. Notably, given that this study did not include a fibre-only treatment group, it is not possible to delineate the effects of fibre that is independent of the lipid-lowering therapy.

Other less-studied nutraceuticals

Carnosine

Carnosine, a known anti-oxidant171, is a dipeptide formed from histidine and beta-alanine and is commonly found in meat given its abundance in animal proteins. Carnosine has been shown to reduce the glycation of LDL in human monocyte-derived macrophages, resulting in reduced intracellular cholesterol accumulation and attenuated foam cell formation172. This process is important in patients with diabetes as they are at increased risk of developing atherosclerosis. ApoE-deficient mice with diabetes receiving a carnosine dietary supplementation showed an improvement in key indicators of atherosclerotic plaque stability after 20 weeks173. Although carnosine did not reduce plaque size, it stabilised the lesion by increasing the collagen content by 50% and reduce the area of the plaque filled by lipids by 60%173. However, the number of macrophages within the plaque was also increased by approximately 70%,173 an indicator of plaque instability. Furthermore, carnosine supplementation in Sprague-Dawley rats for 6 weeks significantly improved serum HDL levels as well as reducing serum LDL levels, however the levels of total cholesterol and triacylgycerols were unaffected174. The same study also found that carnosine supplementation increased serum levels of superoxide dismutase while simultaneously decreasing plasma malondialdehyde (a marker of lipid peroxidation) levels174. This study highlights carnosine's strong anti-oxidant properties and may explain how it exerts some of its cardiovascular protective effects. A small double-blind randomised trial also found that carnosine supplementation every day for 12 weeks significantly improved patient's insulin resistance, however there was no improvement in blood pressure, serum cholesterol or CRP levels175. Carnosine might therefore represent a promising nutraceutical for patients with diabetes at risk of atherosclerosis, but further studies are required to elucidate its effect on plaque stability. Given the lack of *in vivo* and clinical data directly linking carnosine supplementation with anti-atherogenic effects, its use as a nutraceutical for patients with atherosclerosis should remain limited, until sufficient clinical data has been gathered.

Coenzyme CoQ_{10}

Coenzyme Q10 (CoQ_{10}), an antioxidant that is present in many food sources, has an important role in the electron transport chain within the mitochondria. Given that CoQ_{10} and cholesterol synthesis share the same intermediate steps in their respective biosynthetic pathways, patients receiving statin treatment also experience a reduction in CoQ_{10}176. In an *in vivo* study involving ApoE-deficient mice receiving CoQ_{10} dietary supplementation for 4 weeks, CoQ_{10} treatment attenuated LDL oxidation and reduced foam cell formation177. These effects were achieved by enhancing the reverse cholesterol transport process via the microRNA miR-378, resulting in increased cholesterol efflux from the cell and decreasing the formation of foam cells177. Furthermore, the sizes of the plaques from the mice receiving CoQ_{10} were significantly smaller compared with the control group. By contrast, in another *in vivo* study involving ApoE-deficient mice, CoQ_{10} supplementation for 15 weeks was unable to reduce lesion size in cigarette smoke-enhanced atherosclerotic development178. The ability of CoQ_{10} to increase cholesterol efflux has also been observed in human monocyte-derived macrophages *ex vivo*179. In a small study with 20 healthy participants, who were either given placebo or CoQ_{10} supplements twice a day for 1 week,

CoQ_{10} consumption significantly increased cholesterol efflux from macrophages, which correlated with an increase in the expression of the *ABCG1* gene implicated in the promotion of cholesterol efflux179.

In patients with multiple sclerosis, CoQ_{10} supplementation was linked with a reduction in the plasma levels of the pro-inflammatory markers such as TNF-α, IL-6, and MMP-9, but did not alter anti-inflammatory markers such as IL-4 and transforming growth factor-β180. A meta-analysis of five trials involving a total of 194 participants concluded that CoQ_{10} supplementation significantly improved endothelial function181. In addition, daily dose of CoQ_{10} for 8 weeks in participants with left ventricular systolic dysfunction improved flow-mediated dilatation182. However, CoQ_{10} did not lower blood pressure or serum CRP levels182. CoQ_{10} supplementation for 12 weeks also failed to improve arterial stiffness or serum levels of oxLDL and CRP in obese percipients183. A random, double-blind FAITH trial involved 65 fire-fighters considered to have a high CVD risk (occupational stress) taking a daily combined CoQ_{10} and garlic supplement for a year184,185. The study found that the combined supplement was able to significantly reduce serum CRP levels as well as improve both pulse wave velocity and endothelium function compared to the placebo184,185. However, as the study did not include a garlic or CoQ_{10} only group, it is not possible to conclude whether the cardiovascular protective effects were due to one of the nutraceuticals or the combined supplement. Overall, given that statin therapy reduces its *de novo* synthesis, CoQ_{10} might be a promising nutraceutical to take in combination with statins to further reduce atherosclerotic development. However, the lack of consistent studies demonstrating the benefit of CoQ_{10} supplementation for prevention of atherosclerosis has limited its use as a nutraceutical at present.

Curcumin

Curcumin is the active component of turmeric and is the dietary pigment which gives curry its orange colour. Curcumin has been shown to reduce phorbol-12-myristate-13-acetate (PMA) and lipopolysaccharide-induced expression of key proatherogenic cytokines such as MCP-1, IL-1β, and TNF-α in primary human monocytes186. Further *in vitro* studies have also demonstrated that curcumin is also capable of mediating the polarisation of the anti-inflammatory M2 phenotype in murine macrophages187. An *in vivo* study involving rabbits fed a diet containing lard and cholesterol found that LDL was less susceptible to oxidation in those receiving turmeric extract for 7 weeks188. In addition, 30-day turmeric supplementation in high-fat fed rabbits resulted in a smaller fatty streak compared with the untreated control189. Furthermore, a reduction in atherosclerotic lesion size has also been observed in ApoE and LDLr double knockout mice after a daily dose of 0.3 mg of curcumin for 4 months190. Lesion area was reduced by approximately 50% compared with the control group190.

The benefit of curcumin in patients at risk of atherosclerosis has also been described. A randomised double-blind trial involving 240 individuals with type 2 diabetes reported a decrease in CVD risk with 6 months of curcumin dietary supplementation, exemplified through a lower pulse wave velocity and improved metabolic profile191. Furthermore, the use of curcumin for 8 weeks improved flow-mediated dilatation in 32 postmenopausal

women192. Interestingly the same study also found that the improvement in flow-mediated dilatation was similar to those who did not receive supplement, but who exercised for 8 weeks instead192. A major limitation to using curcumin as a nutraceutical is its poor bioavailability, owing to inadequate absorption in the gut and as it is rapidly broken down and quickly excreted from the body193. Several strategies are being pursued in an attempt to increase the bioavailability of curcumin, including the use of liposomal curcumin, nanoparticles, and a curcumin phospholipid complex193.

Lycopene

Lycopene is the carotenoid that gives tomatoes their bright red colour. Several epidemiological studies have found an association between diets rich in lycopene and a reduced incidence of CVD194,195, leading to several studies to further investigate its potential cardioprotective effects. Lycopene might exert its anti-atherogenic effects by inhibiting *de novo* cholesterol synthesis, as demonstrated *in vitro* using murine macrophages196. By contrast, another *in vitro* study reported that LDL isolated from human donors which was then enriched with lycopene before being co-incubated with human endothelial cells actually increased its susceptibility to oxidation197.

In a randomised clinical study involving 144 participants with subclinical atherosclerosis, a combined dietary supplementation of 20mg lutein (another carotenoid with potential cardioprotective effects) and 20mg lycopene for 12 months significantly reduced the thickness of the intima and media in the carotid artery198. Given that the combination of lutein and lycopene supplementation was more effective than lutein alone, synergistic effects of lutein and lycopene might exist. Reduced serum levels of lycopene have also been linked with increased arterial stiffness199. Flow-mediated dilatation was also improved in patients receiving combination lycopene and statin therapy compared with statin alone200. However, no changes in dilatation were observed in healthy participants given the lycopene supplementation, possibly indicating an additive or synergistic effect of lycopene when taken in combination with statins, and highlighting a potential role as a secondary prevention nutraceutical200. In the same study, arterial stiffness, CRP serum levels, and blood pressure levels were also unchanged by lycopene in either the healthy participants or patients with CVD200. Another clinical trial involving 225 healthy participants also found that lycopene supplementation did not reduce blood pressure or improve arterial stiffness201. The possible dual effect of lycopene and statin therapy requires further investigation in studies with a larger cohort.

Resveratrol

Although increased alcohol consumption is associated with hypertension and elevated plasma cholesterol levels, the phenomena known as the ‘French paradox’ has been used to explain why the incidence of CVD is lower in France, despite a similar westernised diet high in fat and carbohydrates202. Resveratrol is a natural phenol commonly found in the skin of grapes and is considered to be one of the key active compounds responsible for these cardiovascular protective effects. Resveratrol has also been shown to reduce foam cell formation by inhibiting oxLDL uptake as well as increasing cholesterol efflux in human THP-1 macrophages203. This increase in efflux corresponded to an elevation in the

expression of key proteins involved in the regulation of cholesterol efflux203. ApoE*3-Leiden.CETP mice fed a high-cholesterol diet with a 0.01% dietary supplementation of resveratrol were also found to have smaller atherosclerotic lesions by approximately 50% compared with control mice, in addition to improved lesion stability due to increased ratio of collagen to macrophages204. However, the cardioprotective benefits were similar between the reservatrol-only and statin-only groups, and the combination of treatments was unable to provide any benefit204.

After adjusting for other risk factors, one epidemiological study concluded that the higher average alcohol consumption in France (particularly wine) was attributable to a lower incidence of CVD202. The GISSI-Prevenzione trial also found an association between daily consumption of wine and a reduced risk of a CVD-event and all-cause mortality205. Although resveratrol might be useful in the prevention of atherosclerosis, it might not enhance the anti-atherogenic effects of statins. Large clinical trials involving statin-only treatment versus statin-plus-resveratrol treatment are required to determine its potential as a nutraceutical.

Berberine

Berberine is a cholesterol-lowering plant alkaloid known for its anti-inflammatory and anti-diabetic effects206. *In vitro* studies have reported that berberine can attenuate the expression of lipopolysaccharide-induced pro-inflammatory genes such as *MCP-1*, *iNOS*, *IL-1β,* and *IL-6* in mouse macrophages207. Furthermore, berberine is capable of reducing macrophage migration208, indicating a potential role for retarding the progression of atherosclerotic development. oxLDL accumulation within human macrophages is also reduced, owing to an upregulation of expression of *ABCA1*, a key gene implicated in the promotion of cholesterol efflux, after berberine treatment209. Although the expression of SRs were unaffected, the capability of berberine to increase cholesterol efflux from human macrophages might make it a possible nutraceutical for reducing foam cell formation. ApoE-deficient mice fed a western diet and berberine for 8 weeks developed less atherosclerotic lesions compared with those on the control diet, in addition to a reduction in the levels of ICAM-1 and VCAM-1, and decreased oxidative stress210. In addition, high-fat fed obese mice treated with berberine for 36 days were found to have lower levels of serum total cholesterol211, which correlates with an earlier study in human hepatic cells that described a reduction in PCSK9 expression after berberine treatment212. As PCSK9 is an inhibitor of LDLr expression, berberine treatment was also found to increase the mRNA levels of LDLr212. These data highlight a possible mechanism by which berberine can exert its cardioprotective effects.

Berberine has also been shown to reduce serum cholesterol levels in several clinical studies. In a study involving 91 Chinese patients with hypercholesterolaemia, berberine supplementation twice a day for 3 months reduced serum levels of total cholesterol and LDL cholesterol, but did not alter serum HDL levels213. In a separate study, berberine treatment twice a day for 3 months lowered serum total cholesterol and LDL levels and increased serum HDL levels in 144 patients who were considered to have a low cardiovascular risk214. The effect of berberine treatment in combination with statins has also been assessed215. Participants with hypercholesterolaemia received either berberine, statins, or a

combination of the two for 2 months. Both statins and berberine were individually able to lower serum total cholesterol and LDL-cholesterol levels. Furthermore, the combination of the two therapies provided an additive effect, reducing total cholesterol and LDL further compared with the individual therapies215. A daily combination of berberine, red yeast rice, and policosanol for 6 weeks in 50 individuals was also effective in reducing serum levels of both total cholesterol and LDL, as well as improving flow-mediated dilatation216. Together, these studies show that berberine has the potential of being used as cholesterol-lowering nutraceutical either to prevent the development of atherosclerosis or to be taken in combination with statins to enhance LDL-lowering capability.

Limitations and future directions

The potential cardioprotective effects of all the nutraceuticals mentioned in this review from either preclinical studies or human studies are summarised in Table 1 and 2, respectively. One of the major challenges involved in nutraceutical research is identifying whether the cardioprotective effects of an individual's diet is attributable to a specific compound or as a result of a combination of elements. Therefore, when a potential nutraceutical is identified, its effectiveness needs to be assessed using robust randomised, controlled trials before it can be recommended as a dietary supplement. The majority of current clinical trials only compare the nutraceutical to a placebo or a nutraceutical in combination with other pharmaceutical therapies to a placebo. We therefore suggest that future clinical trials focusing on patients with subclinical atherosclerosis should investigate the effect of a nutraceutical alone or in combination with other pharmaceuticals and compare the outcomes to both a placebo group as well as those only receiving pharmaceutical intervention. This would allow the effectiveness of nutraceuticals to be directly compared to pharmaceutical only strategies in addition to identifying any additional/synergistic benefits that may occur from taking a combination.

Another possible strategy to improve the identification of novel nutraceuticals is to include preclinical studies that focus on plaque regression rather than the prevention of atherosclerosis. The outcomes of such studies would be much more translatable to humans, as those requiring medicinal intervention are likely to already have established atherosclerosis. Another limitation of preclinical studies is that the doses of nutraceuticals used are sometimes much higher than those used in clinical trials, meaning the nutraceuticals often show no beneficial effect when they reach the clinical trial phase. Preclinical studies should employ a dose that is physiologically relevant to humans (i.e. a dose that is found within the bloodstream following consumption), rendering the outcomes of mechanistic studies more relevant with clinical studies. Furthermore, such trials need to be designed to also investigate clinically relevant end points as well as surrogate markers, and might consider including using younger participants and investigating markers of atherosclerosis regression. The development of new imaging techniques that allow the measurement of atherosclerotic plaques sizes within arteries or the identification of new biomarkers that predict atherosclerotic development will help in the design of these trials.

A major advantage of nutraceuticals is that they can be taken safely over the life time of an individual, whereas pharmaceutical strategies are only administered once an atherosclerotic

risk has been identified and can result in adverse effects with prolonged use. The effects of using multiple nutraceuticals in combination must be examined further to determine whether any synergistic effects takes place and can lead to a greater reduction in atherosclerosis development compared to the individual components. Finally, nutraceuticals should not be seen as alternatives to current atherosclerosis therapies but rather as an additional complementary strategy to ensure both prevention and treatment of atherosclerosis in order to further reduce its global prevalence.

Conclusions

There is growing evidence that nutraceuticals are able to exert cardiovascular protective effects and reduce an individual's risk of suffering a CVD-related event such as a MI or stroke. Further studies are required to fully evaluate the effectiveness of some of the nutraceuticals mentioned in this review. Such advances in our understanding of nutraceutical actions will lead to the identification of novel treatment and prevention strategies in order to reduce the global prevalence of CVD.

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Key points

- Atherosclerosis is a chronic inflammatory disease of the arterial walls and is the primary cause of cardiovascular disease.
- Statins therapy are not effective in reducing cholesterol levels in a small proportion of users and prolonged use of statins can increase the risk of adverse effects.
- Nutraceuticals are natural compounds derived from food sources that are known be beneficial against disease.
- Several nutraceuticals have been shown to potentially exert anti-inflammatory effects making them promising compounds to explore for novel anti-atherogenic therapies.
- Although nutraceuticals are showing some promise, large, robust clinical trials are required to determine their full effectiveness in attenuating/regressing atherosclerosis disease progression.

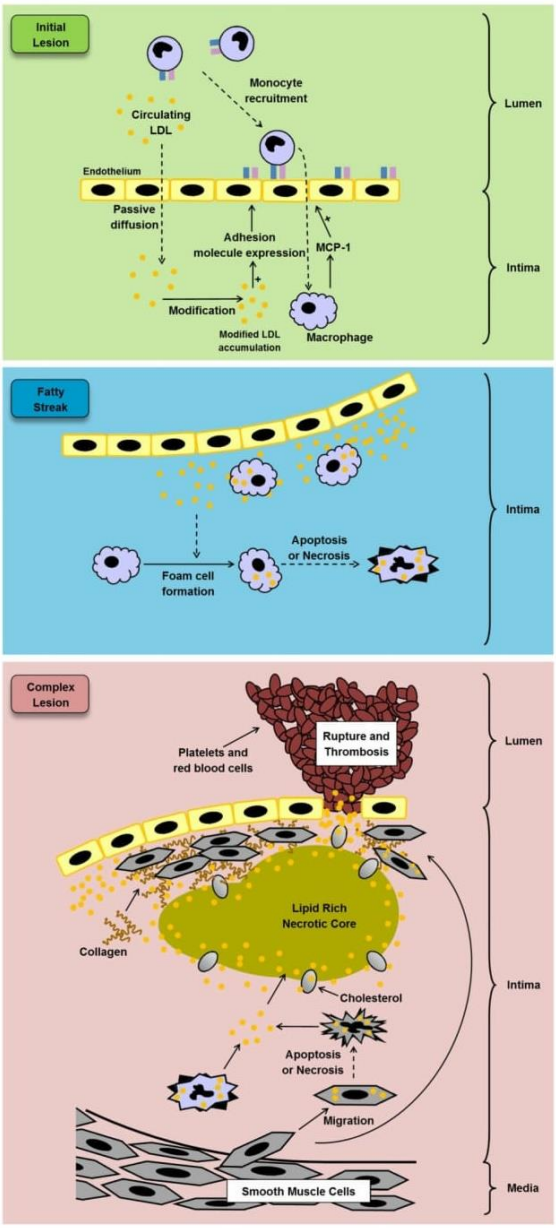

Figure 1. Formation of an atherosclerotic plaque.

The expression of pro-inflammatory genes, including ICAM-1 and MCP-1, is triggered by the build-up of modified LDL in the neighbouring endothelial cells during the development of the initial lesion. Circulating monocytes are then recruited to the modified LDL accumulation and migrate into the intima and differentiate into macrophages. Once in the walls of the artery, the macrophages are able to take up the modified LDL and become lipid-laden foam cells, which can accumulate and form a fatty streak. During complex lesion formation, foam cell lysis by apoptosis and necrosis leads to the formation of a necrotic

core, and together with defective efferocytosis, leads to the amplification of the inflammatory response. SMCs begin to migrate from the media to the intima and the ECM produced by them forms fibrous cap and stabilises the plaque. SMCs also transform to foam cells. During later stages of the complex lesion the plaque can become unstable owing to the inflammatory response, resulting in an inhibition of ECM formation, particularly collagen production by SMCs. The remaining ECM can then start to be degraded by proteases released by macrophages, resulting in an unstable lesion that can rupture and lead to thrombosis. These events can cause a myocardial infarction or stroke, depending on the location of plaque formation. ECM, Extracelular matrix; ICAM-1, Intercellular adhesion molecule-1; LDL, Low density lipoprotein; MCP-1, Macrophage chemoattractant protein-1; SMCs, Smooth muscle cells.

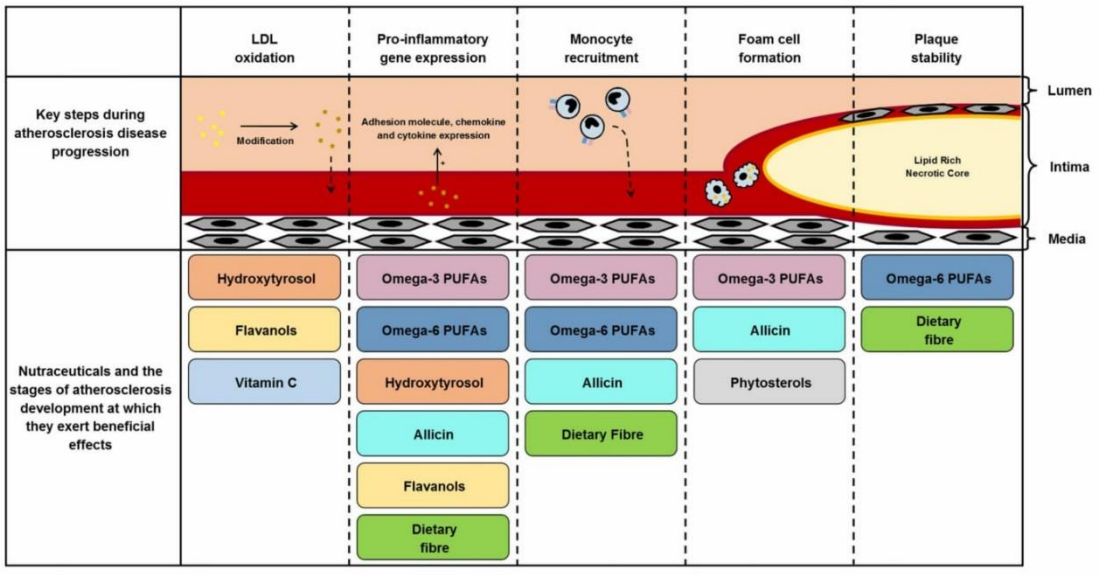

Figure 2. The stages of atherosclerosis development at which different nutraceuticals exert their potential beneficial effects.

There are several major steps involved in the development of atherosclerosis including LDL oxidation, pro-inflammatory gene expression, monocyte migration, foam cell formation, and plaque stability. This figure highlights the stages at which the major nutraceuticals discussed in this review could aid in reducing atherosclerosis disease progression. LDL, Low density lipoprotein; PUFAs, Polyunsaturated fatty acids.

Table 1

Summary of potential cardiovascular benefits of nutraceuticals in preclinical studies

Nutraceutical	Cardiovascular health benefits	References
Allicin	• Reduced the expression of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) in murine macrophages stimulated with lipopolysaccharide	99
	• Decreased the inflammatory response by reducing leukocyte adherence	100
	• Attenuated the expression of MSR1, ACAT1 and CD36 in human monocyte-derived macrophages, resulting in reduced foam cell formation.	103
	• Together with supplementation of H_2S donors resulted in slowing of atherosclerosis development via reduction of lesion size in ApoE-deficient mice	104
Berberine	• Attenuated lipopolysaccharide-induced pro-inflammatory gene expression, including MCP-1, iNOS, IL-1β and IL-6, in mouse macrophages	207
	• Reduced macrophage migration	208
	• Induced the expression of cholesterol efflux gene ABCA1, resulting in reduced intracellular accumulation of oxidised LDL in human macrophages	209
	• Reduced serum total cholesterol levels and the number of atherosclerotic lesions in ApoE-deficient mice	210,211
Butyrate	• Attenuated nitric oxide and pro-inflammatory cytokine production in lipopolysaccaride-stimulated murine macrophages	162
	• Reduced plaque size by attenuating monocyte and macrophage migration in ApoE-deficient mice	165
Carnosine	• Protected against foam cell formation *in vitro*	172
	• Improved key factors associated with plaque stability in murine diabetes-associated atherosclerosis models	173
	• Increased serum HDL and reduced those of LDL in rats as well as increasing serum superoxide dismutase levels	174
Coenzyme Q_{10}	• Promoted macrophage reverse cholesterol transport and slowed the development of atherosclerosis, possibly via miR-378	177
	• Increased cholesterol efflux in human monocyte-derived macrophages, which correlated with increased expression of the cholesterol efflux gene ABCG1	179
Curcumin	• Decreased the production of pro-inflammatory cytokines in primary human monocytes	186
	• Stimulated an anti-inflammatory M2 macrophage phenotype *in vitro*	187
	• Reduced oxidative stress and LDL oxidation, in addition to reducing aortic fatty streak development	188,189
	• Dietary supplementation in ApoE and LDL receptor double knockout mice resulted in smaller atherosclerotic lesions after 4 months	190
Flavanols	• Reduced the size of atherosclerotic plaques in ApoE*3-Leiden mice after 20 weeks	129
	• Attenuated endothelial exocytosis (the process of releasing pro-inflammatory cytokines and chemokines into the extracellular space) in HUVEC	128
Hydroxytyrosol	• Reduced the expression of the pro-inflammatory adhesion proteins VCAM-1 and ICAM-1 in HUVEC	83
	• Wistar rats fed a diet containing hydroxytyrosol had higher plasma HDL levels and lower plasma LDL levels compared to control rats	85,86
	• Attenuated atherosclerosis disease development in hyperlipaemic rabbits, shown by smaller atherosclerotic lesions	87
Lycopene	• Inhibited LDL oxidation and cholesterol synthesis *in vitro*	196
ω-3 PUFAs	• Attenuated the expression of several key atherosclerotic markers in both murine and human macrophages	36,37
	• Increased the expression of cholesterol efflux genes and decreased the expression of LDL-uptake genes	39

Nutraceutical	Cardiovascular health benefits	References
	• Reduced atherosclerotic lesion size and increased plasma HDL levels in LDLr deficient mice	38,42
ω-6 PUFAs	• PGE1, a metabolite of DGLA, improved plaque stability in rabbits by increasing the thickness of the fibrous cap	63
	• GLA-enriched diets after 7 weeks reduced blood pressure in hypertensive rats	66
	• DGLA dietary supplementation decreased the size of atherosclerotic plaque in murine models after 6 months	65
Phytosterols	• Increased cholesterol efflux in human THP-1 macrophages	112
	• Decreased the size of the lesions as well as plasma LDL levels in mouse model systems	113,115–117
Resveratrol	• Reduced foam cell formation by increasing cholesterol efflux in human THP-1 macrophages	203
	• Reduced atherosclerotic lesions by approximately 50% in ApoE*3-Lieiden.CEPT mice	204
Vitamin C	• Improved eNOS activity and enhanced endothelial function	142,143

ACAT-1, Acyl-CoA acyltransferase; ApoE, Apolipoprotein E; CHD, Coronary heart disease; CVD, Cardiovascular disease; DGLA, Dihomo-γ-linolenic acid; DHA, Docosahexaenoic acid; eNOS, Endothelial nitrogen oxide synthase; EPA, Eicosapentaenoic acid; GLA, γ-linolenic acid; H_2S, Hydrogen sulphide; HDL, High density lipoprotein; HUVEC, human umbilical vein endothelial cells; ICAM-1, Intercellular adhesion molecule-1; IL, Interleukin; LDL, Low density lipoprotein; LDLr, LDL receptor; LPS, Lipopolysaccharide; MSR1, Macrophage scavenger receptor 1; MCP-1, Monocyte chemotactic protein-1; miR, Micro RNA; MMP, Matrix metalloproteinases; NO, Nitric oxide; oxLDL, Oxidised LDL; PGE1, Prostaglandin E1; PUFAs, Polyunsaturated fatty acids; TNFα, Tumour necrosis factor α; VCAM-1, Vascular cellular adhesion molecule-1.

Table 2
Summary of potential cardiovascular benefits of nutraceuticals in human studies

Nutraceutical	Study type	Size	Observations	References
Allicin	Clinical	152	Reduced lesion volume	106
	Preliminary	19	Reduced coronary calcification, resulting in a diminished rate of atherosclerosis development	107
	Clinical	192	No change in LDL and HDL levels	109
	Meta-analysis	2,987	Short term reduction in levels of serum LDL, total cholesterol and triacylglycerols but no long term benefits	108
Berberine	Clinical	91	Reduced serum total cholesterol and LDL-cholesterol levels. No change in serum HDL-cholesterol levels	213
	Clinical	144	Reduced serum total cholesterol, LDL-cholesterol, and HDL-cholesterol levels	214
	Clinical	63	Reduced serum total cholesterol levels and LDL-cholesterol levels. An additive effect was observed when berberine was taken in combination with statins	215
Butyrate	Epidemiological	39,876	Trend for reduced risk of CVD-events, which were no longer significant after controlling for other confounding variables	166
	Epidemiological	46,032	Reduced risk of peripheral arterial disease	167
	Epidemiological	78,779	Reduced risk of haemorrhagic stroke	168
	Meta-analysis	336,244	Reduced risk of a CVD-event	169
	Clinical	116	No cardiovascular benefits	170
Carnosine	Clinical	30	Improved insulin resistance with no change in blood pressure, serum cholesterol or CRP levels	175
Coenzyme Q_{10}	Clinical	45	Reduced plasma levels of pro-inflammatory markers, and no changes in plasma levels of anti-inflammatory markers	180
	Meta-analysis	194	Improved endothelial function	181
	Clinical	56	Enhanced endothelial function, but no changes in blood pressure and serum CRP levels	182
	Clinical	51	No changes in arterial stiffness, serum oxLDL levels, and serum CRP levels	183
	Clinical	65	CoQ_{10} and garlic supplementation reduced serum CRP levels, improved arterial stiffness and endothelial function	184,185
Curcumin	Clinical	240	Reduced arterial stiffness	191
	Clinical	32	Improved endothelial function	192
Flavanols	Clinical	27	Increased vasodilatation	130
	Clinical	14	Increased vasodilatation, reduced oxLDL levels	132
	Clinical	40	Reduced circulating LDL levels	133
	Clinical	60	Reduced expression of pro-inflammatory cytokines	138
	Clinical	17	Reduced ratio of total cholesterol to HDL cholesterol, but no change in any other CVD risk biomarkers	139
	Clinical	57	Enhanced endothelial function	134
	Clinical	100	Enhanced endothelial function	136
	Clinical	42	Enhanced endothelial function, reduced arterial stiffness	137
	Clinical	30	Reduced arterial stiffness, but no changes in blood pressure or endothelial function	140

Nutraceutical	Study type	Size	Observations	References
	Clinical	20	Enhanced flow-mediated dilation in patients with congestive heart failure	135
Hydroxytyrosol	Epidemiological	12,763	Reduced risk of CVD-event	79
	Clinical	200	Correlation between phenolic content of olive oils and increased serum HDL levels	89
	Clinical	40	Reduced serum oxLDL levels	90
	Clinical	30	Reduced serum oxLDL levels	91
	Clinical	28	Reduced expression of inflammatory biomarkers	92
	Clinical	26	Improved endothelial function, reduced serum oxLDL levels	93
	Clinical	7447	Reduced risk of CVD-event	94
	Clinical	187	Reduced subclinical atherosclerosis in high risk patients	95
	Clinical	90	Reduced serum LDL, HDL and total cholesterol levels, decreased pro-atherogenic gene expression, but no changes in CRP levels	96
	Clinical	52	Improved endothelial function, reduced inflammatory markers	97
Lycopene	Clinical	144	Reduced thickness of the intima and media in the carotid artery	198
	Epidemiological	264	Low serum levels of lycopene were associated with increased arterial stiffness	199
	Clinical	72	Improved endothelial function in those also receiving statins, but no change in arterial stiffness, serum CRP levels, or blood pressure	200
	Clinical	225	No change in blood pressure or arterial stiffness	201
ω-3 PUFAs	Clinical	600	Reduced atherothrombotic risk	46
	Clinical	2,033	Reduced number of CVD-events	49
	Clinical	11,323	Reduced number of sudden cardiac deaths, but no change in serum total cholesterol, LDL, or HDL levels	50
	Clinical	18,645	Reduced number of major CVD-events, but no change in serum HDL or LDL levels	51
	Clinical	95	Increased plaque stability and reduced levels of pro-inflammatory cytokines	52
	Meta-analysis	396	No change in number of CVD-events	53
	Meta-analysis	36,913	No change in cardiac mortality	54
	Meta-analysis	20,485	No cardiovascular protective effects associated with omega-3 PUFA supplementation	55
	Meta-analysis	68,680	No change in mortality risk	56
	Epidemiological	160	Low serum DHA levels correlated to reduced endothelial function	47
	Clinical	29	Increased endothelial function and reduced arterial stiffness	48
ω-6 PUFAs	Epidemiological	59	Reduced omega-3 and omega-6 PUFA levels associated with spontaneous CVD death	67
	Epidemiological	30	Reduced levels of omega-6 PUFAs found in the shoulder regions of plaques and in ruptured plaques compared to non-ruptured plaques	68
	Epidemiological	474	Reduced serum GLA levels correlated with increased risk of peripheral arterial disease	69
	Clinical	120	Reduced systolic blood pressure	70
	Clinical	33	No anti-thrombotic effects observed	74
	Epidemiological	2206	Increased arterial stiffness and serum CRP levels	72
	Epidemiological	501	Increased arterial stiffness	73
	Clinical	12	Reduced serum LDL, total cholesterol and triacylglycerol levels	71

Nutraceutical	Study type	Size	Observations	References
	Clinical	9570	Reduced serum cholesterol levels, but no reduction in the risk of a CVD-related event	76
Phytosterols	Epidemiological	22,256	Reduced levels of serum LDL	111
	Epidemiological	48	Raised ratio of serum phytosterol to cholesterol associated with higher risk of developing CHD	125
	Epidemiological	477	Increased number of sudden CVD deaths, however, the study failed to match other CVD risk factors between the different groups	124,126
	Clinical	233	No change in endothelium function and arterial stiffness. Reduced serum LDL levels	118
	Meta-analysis	1,308	Reduced levels of serum LDL, but no change in serum CRP levels	119
Resveratrol	Epidemiological	11,282	Reduced risk of CVD-event	205
Vitamin C & E	Epidemiological	19,496	Vitamin C reduced risk of CVD-event	147
	Epidemiological	85,118	Vitamin C reduced prevalence of CAD	141
	Clinical	46	Vitamin C increased vasodilatation.	149
	Clinical	20	Vitamin C increased vasodilatation	150
	Meta-analysis	1,129	Vitamin C increased endothelial function	151
	Clinical	20	Vitamin C had no long-term cardiovascular protective effects	156
	Clinical	20,536	No change in number of CVD-events	157
	Clinical	520	Reduced atherosclerosis progression in men	152
	Meta-analysis	293,172	Vitamin C but not vitamin E was associated with reduced risk of CVD-event	153
	Clinical	2,002	Vitamin E supplementation reduced risk of non-fatal CVD-events, but no change in CVD-related deaths	154
	Clinical	30	Combined vitamin C and E supplementation improved arterial stiffness, flow-mediated dilation and oxidative stress levels	155
	Clinical	11,323	Vitamin E did not reduce risk of CVD-event	50
	Clinical	9,541	Vitamin E did not reduce CVD deaths	158
	Clinical	353	Vitamin E reduced serum oxLDL levels, but no change in intima-media thickness	159

CAD, coronary artery disease; CHD, Coronary heart disease; CRP, C-reactive protein; CVD, Cardiovascular disease; DHA, Docosahexaenoic acid; GLA, γ-linolenic acid; HDL, High density lipoprotein; LDL, Low density lipoprotein; oxLDL, Oxidised LDL; PUFAs, Polyunsaturated fatty acids.