The Impact Of Omega-3 Fatty Acids On Inflammatory Pathways In The Vessel Wall That Promote Atherosclerosis

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ABSTRACT OF THESIS

Despite the advances that have occurred in the prevention and management of coronary heart disease, there remains a substantial residual risk in the general population. Inflammation has received considerable attention as a target for therapies, given the inflammatory nature of atherosclerosis. Omega-3 fatty acids have anti-inflammatory properties, and recent clinical trial evidence has demonstrated a reduction in major adverse cardiovascular events with fish oil. The studies presented in this thesis investigated the effects of omega-3 fatty acids on vascular inflammation as a possible mechanism of atheroprotection.

A review of the literature was performed, focusing on the inflammatory mechanisms of atherosclerosis, the effects of omega-3 fatty acids on inflammation, particularly vascular inflammation, and the results of cardiovascular outcome trials of fish and fish oil. This provided a theoretical basis for the studies presented.

A systematic review of high-quality randomised controlled trials catalogued in the Cochrane Library was performed to evaluate the impact of omega-3 fatty acids on the circulating mediators of atherosclerosis. These are among the earliest inducers of endothelial injury and vascular inflammation. Omega-3 fatty acids reduced levels of atherogenic mediators in all four categories.

A combined randomised controlled trial and cell culture study was performed to evaluate the effects of omega-3 fatty acids on the gene expression of markers of acute vascular inflammation (AVI). Healthy volunteers were supplemented with 4 grams daily of either EPA, DHA, fish oil with a 2:1 EPA:DHA ratio, or placebo for 30 days. Serum before and after supplementation was added to TNF-stimulated HUVECs in culture. The serum from those supplemented with high-dose EPA reduced the gene expression of MCP-1. The gene

expression of VCAM-1 and MCP-1 correlated positively with HDL-C levels, suggesting a pro-inflammatory effect at high HDL-C levels.

To study the impact of omega-3 fatty acids on the protein expression of markers of AVI, C57Bl/6 mice had non-occlusive collars applied surgically to their right carotid arteries after receiving 30 days of pre-treatment with either EPA, DHA, an oil control, or no treatment, by oral gavage. The intense inflammatory response was reduced by EPA, manifested by reduced expression of VCAM-1 and MCP-1 in the artery wall.

ApoE-deficient mice were fed an atherogenic diet for 16 weeks to induce advanced atherosclerotic lesions and chronic vascular inflammation. In the last 8 weeks they were randomised to either EPA, DHA, olive oil, or no treatment by oral gavage. EPA reduced gene expression of markers of chronic inflammation in the aorta, however no treatment altered the burden, characteristics or lipid content of plaque. EPA and DHA stabilised cholesterol levels and reduced triglyceride levels.

In all experimental studies, blood levels of omega-3 fatty acids, especially EPA, correlated inversely with markers of vascular inflammation.

The findings of this body of work demonstrate the suppressive effects of omega-3 fatty acids on acute and chronic vascular inflammation, with EPA being superior to DHA. These findings provide a rationale for the reduction in major adverse cardiovascular events seen recently with EPA therapy, and provide a mechanistic basis to guide future clinical trials.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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Anthony David Pisaniello

July 2019

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SCHOLARSHIPS, ABSTRACTS AND PRIZES

Scholarships and Grants

- 2014 – Statewide Research Scholarship – 1 year
- 2014 – Travel Grant to attend World Congress of Cardiology, Melbourne
- 2015 – Cardiac Society of Australia and New Zealand Research Scholarship – 1 year
- 2016 – National Health and Medical Research Council Postgraduate Scholarship – 1 year
- 2016 – Australian Academy of Science Douglas and Lola Douglas Scholarship in Medical Science top-up scholarship – 1 year
- 2016 – Travel Grant to attend AAS, ACvA and HBPRCA conference, Hobart
- 2017 – CSANZ Travelling Fellowship to attend CSANZ ASM, Perth

Abstracts

- **Pisaniello AD**, Di Bartolo BA, Liu G, Gibson RA, Kim SW, Psaltis PJ, Nicholls SJ. "*The impact of oral omega-3 fatty acid supplementation on acute vascular inflammation in a mouse model"*. Presented at ASMR ASM June 2016. Presented at Florey Research Foundation Conference Sept 2016. Presented at European Atherosclerosis Society conference April 2017.
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- 2017 – Adelaide Medical School Research Prize
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LIST OF ABBREVIATIONS

ABCA1	ATP-Binding Cassette Transporter A2
ABCG1	ATP-Binding Cassette Transporter G2
ACAT1	Acyl Coenzyme A:Cholesterol Acyltransferase-1
ACS	Acute Coronary Syndrome
ADMA	Asymmetric Dimethylarginine
AGEs	Advanced Glycation Endproducts
ALA	Alpha-Linolenic Acid
ANCOVA	Analysis Of Covariance
ANOVA	Analysis Of Variance
ATF	Activating Transcription Factor
ATP	Adenosine 5' Triphosphate
AVI	Acute Vascular Inflammation
BHT	Butylated Hydroxytoluene
CCL2	C-C Motif Chemokine Ligand 2
cDNA	Complementary Deoxyribonucleic Acid
CETP	Cholesteryl Ester Transfer Protein
CHD	Coronary Heart Disease
CI	Confidence Interval
cm	Centimetres
CO2	Carbon Dioxide
COX	Cyclooxygenase
CRP	C-Reactive Protein
CSE	Cystathionine-Γ-Lyase
CVI	Chronic Vascular Inflammation
DAB	3,3'-Diaminobenzidine
DAMP	Damage Associated Molecular Pattern
DBS	Dry Blood Spot
DHA	Docosahexaenoic Acid
DNA	Deoxyribonucleic Acid
DPA	Docosapentaenoic Acid
DPX	Distyrene, Plasticiser and Xylene

EC	Endothelial Cell
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
eNOS	Endothelial Nitric Oxide Synthase
EPA	Eicosapentaenoic Acid
EVOO	Extra-Virgin Olive Oil
FBS	Foetal Bovine Serum
FDG	Fluorodeoxyglucose
g	Grams
GC	Gas Chromatography
H&E	Haematoxylin And Eosin
HAECs	Human Aortic Endothelial Cells
HDL	High-Density Lipoprotein
HMG Co-A	3-Hydroxy-3-Methyl-Glutaryl-Coenzyme A
hs-CRP	High Sensitivity C-Reactive Protein
HUVECs	Human Umbilical Vein Endothelial Cells
ICAM-1	Intercellular Adhesion Molecule 1
IDL	Intermediate Density Lipoprotein
IFN-γ	Interferon-Gamma
IHC	Immunohistochemistry
iNOS	Inducible Nitric Oxide Synthase
IOD	Integrated Optical Density
IQR	Interquartile Range
KC	Keratinocyte Chemoattractant
kg	Kilograms
LDL	Low-Density Lipoprotein
LOX	Lipoxygenase
Lp(a)	Lipoprotein(a)
LPS	Lipopolysaccharide
MACE	Major Adverse Cardiovascular Events
MAPK	Mitogen-Activated Protein Kinase
MAT	Methionine Adenosyltransferase
MCP-1	Monocyte Chemoattractant Protein 1

mm	Millimetres
MMP	Matrix Metalloproteinases
MRTF	Myocardin-Related Transcription Factor
MTHFR	Methyltetrahydrofolate Reductase
MUFA	Monounsaturated Fatty Acid
NBF	Neutral-Buffered Formalin
nCEH	Neutral Cholesteryl Ester Hydrolase
NET	Neutrophil Extracellular Traps
NF-kB	Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells
NHS	Normal Horse Serum
NLRP3	Nucleotide-Binding Domain Leucine-Rich-Containing Family Pyrin Domain-Containing-3
O3FA	Omega-3 Fatty Acids
OCT	Optical Coherence Tomography
PAMP	Pattern-Associated Molecular Pattern
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PLA2	Phospholipase A2
PMNs	Polymorphonuclear Cells
PPAR-γ	Peroxisome Proliferator-Activated Receptor Gamma
PPR	Pattern Recognition Receptor
PUFA	Polyunsaturated Fatty Acid
RCT	Randomised Controlled Trials
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SAHMRI	South Australian Health And Medical Research Institute
SD	Standard Deviation
SEM	Standard Error Of The Mean
SFA	Saturated Fatty Acid
SMC	Smooth Muscle Cell
SOP	Standard Operating Procedure
SR-BI	Scavenger Receptor Bi
TCFA	Thin-Cap Fibroatheroma
TIMP1	Tissue Inhibitor Of Matrix Metalloproteinase

TLR-2	Toll-Like Receptor 2
TNFR1	Tumour Necrosis Factor Receptor 1
TNFR2	Tumour Necrosis Factor Receptor 2
TNF-α	Tumour Necrosis Factor-Alpha
TRAIL	Tumour Necrosis Factor-Related Apoptosis-Inducing Ligand
VCAM-1	Vascular Cells Adhesion Molecule 1
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low-Density Lipoprotein

“I know the human being and fish can coexist peacefully”

George W. Bush, 29 September 2000

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Atherosclerosis is an inflammatory disease

Cardiovascular disease is the leading cause of death worldwide and contributed to 44% of non-communicable disease deaths in 2016[1]. The most common diseases of the cardiovascular system are those caused by atherosclerosis. These include coronary artery disease, cerebrovascular disease, and peripheral vascular disease. Atherosclerosis can be described in simple terms as the accumulation of fatty plaque in the arterial wall that can progress to cause luminal narrowing. When sufficient luminal narrowing occurs, the supply of blood and therefore oxygen does not meet the metabolic demand of tissues, and ischaemia or infarction occurs. Atherosclerosis has traditionally been viewed as the consequence of cumulative injury from a number of risk factors, such as hyperlipidemia, hypertension, diabetes mellitus and tobacco smoking. Significant advances in the prevention and treatment of atherosclerotic diseases have led to major population-wide reductions in morbidity and mortality[2]. Despite these advances, there remains a substantial residual cardiovascular risk[3]. Inflammation has received considerable attention as a target of atherosclerosis, given the substantial evidence demonstrating its inflammatory nature. The response-to-injury hypothesis of atherosclerosis was first proposed by Russell Ross, who recognised the presence of multiple diverse inflammatory cell types in atherosclerotic plaques, revealing a complex milieu of inflammatory activity[4]. Inflammation is integral to all six stages of the continuum of atherosclerosis, which are diagrammatically represented in Figure 1.1, and described below.

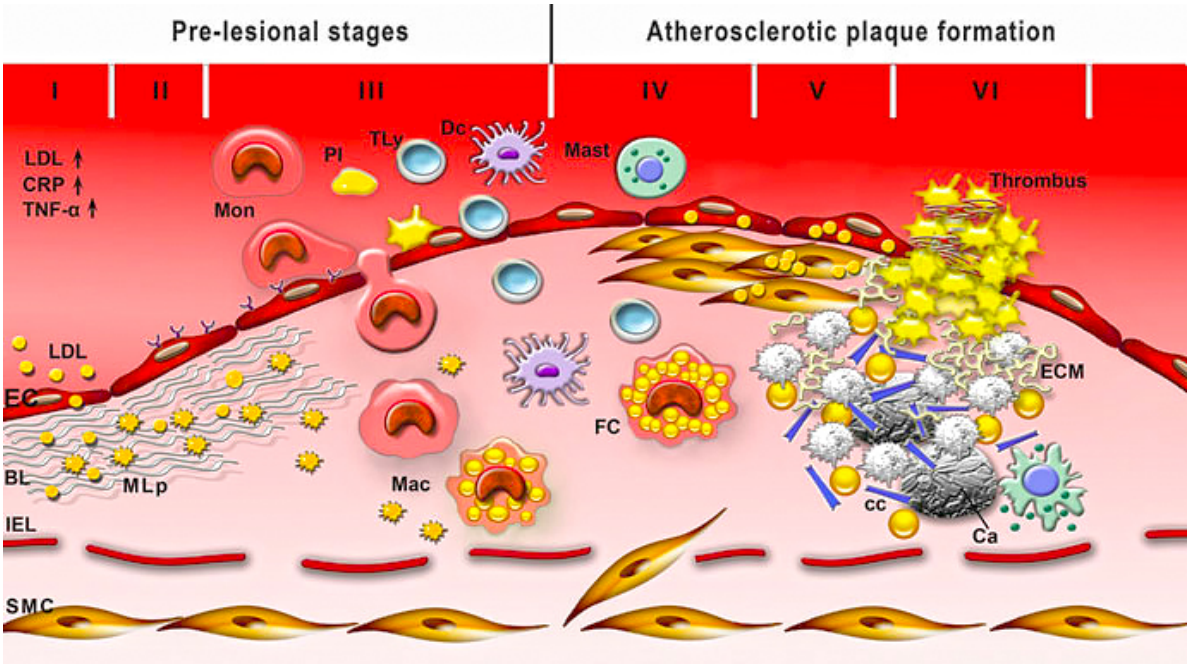


Figure 1.1: Diagrammatic representation of atherogenesis divided into six stages. Adapted from Wick, G., & Grundtman, C. (2012). *Inflammation and atherosclerosis*. Wien: Springer-Verlag/Wien[5].

1.1.1 Atherosclerosis stage I – Increased endothelial cell permeability

Minute changes in circulating lipoproteins, glucose, and inflammatory markers lead to modulation of endothelial cell (EC) function and increased permeability. This process involves changes in three components: (1) the "glycocalyx", which is the surface layer of glycoproteins, proteoglycans, and glycosaminoglycans that together create a scaffold on the endothelial surface; (2) energy-dependent vesicular trafficking, also called the "transcellular pathway"; and (3) and the opening or rearrangement of cell-to-cell junctions, also called the "paracellular pathway"[6]. Enhanced vesicular trafficking and/or widened intercellular spaces in the presence of a disarrayed or overtly disintegrated glycocalyx may all facilitate the transendothelial flux of low-density lipoprotein (LDL), which may ultimately become trapped in the subendothelium. Trapped LDL is oxidised by macrophages. Oxidised LDL (ox-LDL)

generates free radicals (which are toxic to ECs) leading to the generation of monocyte chemoattractant protein 1 (MCP-1). There is subsequent attraction and migration of monocytes to the subendothelium. In response to this, antioxidant heme oxygenase 1 is released, which reduces monocyte transmigration and toxicity to endothelial cells[7].

1.1.2 Atherosclerosis stage II – Endothelial cell dysfunction

Concurrent with increased endothelial cell permeability, ECs switch to a secretory phenotype, producing a hyperplastic, multilayered basal lamina, which further traps LDL in the subendothelium[8]. ECs express von-Willebrand factor, which binds to glycoprotein 1b on platelets and recruits them to the endothelial cell surface. Upon adhesion, platelets secrete a variety of proinflammatory cytokines and chemoattractants including platelet factor 4, RANTES, P-selectin, soluble CD40 ligand and matrix metalloproteinases. Platelet P-selectin interacts with monocyte P-selectin glycoprotein ligand-1, forming platelet-monocyte complexes. These activated platelets become more adhesive to vascular cell adhesion molecule 1 (VCAM-1) and inflamed or atherosclerotic endothelium.

ECs also lose their net negative surface charge that contributes to the characteristic non-thrombogenic surface of the endothelium[9]. Circulating cells have a negatively-charged surface, and hence are more easily able to attach and migrate through the endothelium.

Endothelial dysfunction activates nuclear factor kappa-B (NF-κB), a transcription factor which induces both pro-inflammatory and anti-inflammatory genes[10]. NF-κB is also activated by adverse conditions such as hypertension, low shear stress, and the presence of pro-inflammatory cytokines, ox-LDL, and reactive oxygen species (ROS). There are five subunits of the nuclear factor kappa B (NF-κB) family, and the main subunit is p65 which mediates transcriptional activation of target genes. When NF-κB is activated in endothelial cells (eg. during endothelial dysfunction), the response is pro-inflammatory. There is expression of genes

that recruit inflammatory cells (eg. VCAM-1, intercellular adhesion molecule 1 [ICAM-1], E-selectin and P-selectin), cytokines (eg. tumour-necrosis factor-alpha [TNF-α], interleukin-1 [IL-1β], interleukin-6 [IL-6], and interleukin-8 [IL-8]), chemokines (eg. MCP-1), and matrix metalloproteinases [MMPs])[11]. This set of molecular and cellular changes is a defence reaction assisting the vascular endothelium to recruit blood inflammatory cells.

1.1.3 Atherosclerosis stage III – Leucocyte transmigration and fatty streak formation

Through the EC expression of MCP-1, adhesion molecules, and through their interactions with monocyte integrins, monocytes are captured, roll and adhere to the endothelium, then transmigrate to the subendothelium. They subsequently differentiate into macrophages and phagocytose ox-LDL and become foam cells, the hallmark of the fatty streak. The differentiation process of monocytes to macrophages is normally a coordinated process that involves upregulation of scavenger receptors, e.g. SR-A and CD-36. When the macrophages encounter modified lipoproteins such as ox-LDL, advanced glycation endproducts, anionic phospholipids and apoptotic cells, the receptors are activated. The phagocytosis of ox-LDL is normally followed by esterification of cholesterol by Acyl coenzyme A:cholesterol acyltransferase-1 (ACAT1) and neutral cholesteryl ester hydrolase (nCEH), and then the efflux of cholesterol out of the cell by Adenosine 5' Triphosphate (ATP)-binding cassette transporters A1 (ABCA1) and G1 (ABCG1), and scavenger receptor BI (SR-BI). In atherogenic conditions, there is increased ox-LDL influx, increased cholesterol esterification, and decreased cholesterol efflux, hence the macrophages are ultimately transformed into lipid-laden foam cells[12]. Fatty streaks are macroscopically-visible aggregates of foam cells.

Concurrent with the transmigration of monocytes, circulating CD4+ T cells migrate into atherosclerotic lesions, where they bind to antigens such as modified lipoproteins, and

proliferate. Dendritic cells are specialised antigen presenting cells, required for activation of CD4 cells, and their transmigration from the circulation is augmented by EC dysfunction and inhibition of nitric oxide synthase[13]. Activated CD4 cells release cytokines such as TNF-α, which are pro-inflammatory and contributed to macrophage activation[14].

Other inflammatory cells that migrate into the subendothelium include polymorphonuclear cells (PMNs) and mast cells. The extravasation of PMNs is a normal part of immune surveillance, induced by selectin-mediated rolling and integrin-mediated adhesion[15]. The transmigration is augmented by the upregulation of ICAM-1 in acute vascular inflammation[16]. Mast cells are thought to be recruited to atherosclerotic plaque via the chemokine eotaxin-1 (CCL-11) expressed in plaque via the mast cell receptor CCR3[17]. Mast cells accumulate in the medial and adventitial tissues and cluster around neovessels. When activated, they release the contents of their granules such as histamine and proteases, which induce vascular leakage and promote intraplaque haemorrhage[18].

1.1.4 Atherosclerosis stage IV – Development of fibrous plaque

Foam cells secrete cytokines, growth factors, tissue factor, interferon-gamma, MMPs, and reactive oxygen species. With digestion of the internal elastic lamina, there is migration of smooth muscle cells (SMCs) from the media to the intima, forming intimal thickenings. The migrated SMCs switch to a secretory phenotype, resulting in a hyperplastic, multilayered basal lamina and enlarged extracellular matrix (ECM), enriched with collagen bundles and fibrils[19]. These cellular changes form a fibrous cap.

1.1.5 Atherosclerosis stage V – Development of calcified atherosclerotic fibro-lipid plaque

The switch of SMCs from a contractile to a secretory phenotype is associated with a significant reduction in their ability to metabolise LDL[20]. SMCs become lipid-laden, and

along with lipid-laden macrophages and extracellular lipid deposits in fibrous plaques, form fibro-lipid plaques. Free cholesterol also accumulates within plaque, and the oxidised forms are potent inducers of apoptosis of foam cells and SMCs[21]. The release of the cytotoxic contents of these cells results in formation of the necrotic core encapsulated by fibrous tissue[22]. Excess extracellular unesterified cholesterol nucleates into cytotoxic crystals. Plaque that is surrounded by a robust fibrous cap is considered "stable". Plaque with a thin fibrous cap, accumulation of cholesterol crystals, and with a large necrotic core are considered "unstable".

1.1.6 Atherosclerosis stage VI – Plaque rupture

Progressive deterioration and thinning of the EC layer overlying the fibrous cap results in exposure of the ECM to circulating blood cells. Circulating macrophages infiltrate the ECM and secrete proteases, which digest and destabilise the ECM and contribute to fibrous cap thinning. Local foam cells also secrete proteolytic enzymes. SMCs also contribute to fibrous cap thinning due to a decrease in collagen synthesis. Local mast cells, among other cells, release proteases that destabilise the plaque. The plaque ruptures at its weakest point, either spontaneously or in the setting of haemodynamic stress, and platelets aggregate and become activated, leading to thrombosis. Ninety-five percent of ruptured fibrous caps are less than 65 μm in thickness[23], which is the threshold for defining thin-cap fibroatheroma (TCFA)[24].

A summary of the fundamental processes involved in atherogenesis is represented in Figure 1.2.

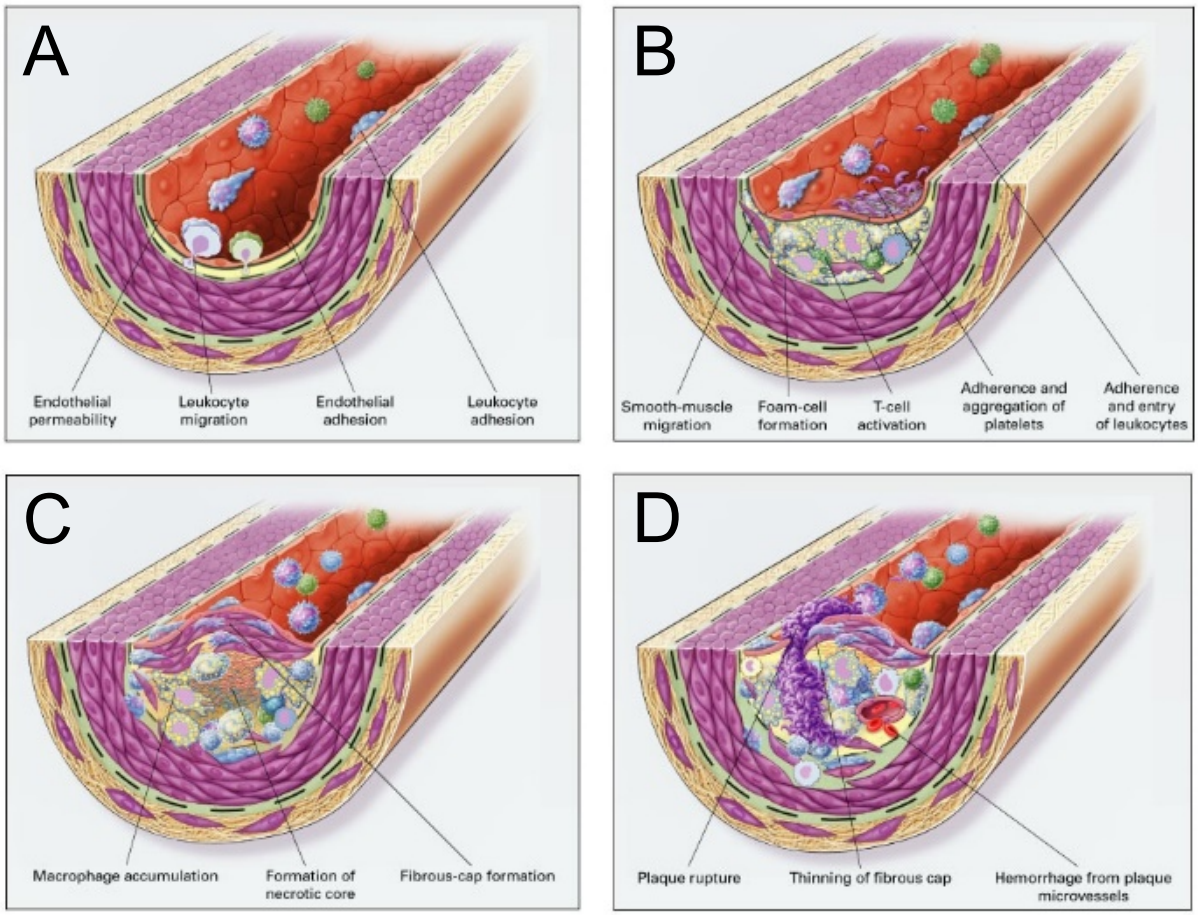


Figure 1.2: A schematic representation of the fundamental processes of atherogenesis, including endothelial dysfunction: (A), fatty streak formation (B), formation of advanced lesions (C), and plaque rupture (D). Reproduced with permission from (Ross, R. (January 01, 1999). Atherosclerosis--an inflammatory disease. *The New England Journal of Medicine, 340,* 2, 115-26[(4)]), Copyright Massachusetts Medical Society.

1.1.7 An alternative fate of atherosclerotic plaques – Erosion

An alternative fate of atherosclerotic plaques is superficial erosion leading to thrombosis. The lesions associated with superficial erosion are abundant with smooth muscle cells and extracellular matrix, and have a paucity of lipid and foam cell accumulation[(25)]. They are also much less inflammatory than lesions associated with plaque rupture[(26)].

The pathology of plaque erosion is not completely understood. Emerging data, including histopathological as well as from intracoronary imaging modalities such as optical coherence tomography (OCT) have provided valuable mechanistic insights[27]. The characteristic feature is a breach in endothelial cell integrity at the site of proteoglycan and smooth muscle cell-rich plaque with a thick fibrous cap[28]. Collagen and the necrotic core are then exposed, which allows platelet aggregation and thrombosis to occur. Plaque erosion tends to occur at sites of low shear stress, especially at arterial bifurcations[29]. Endothelial apoptosis is more prevalent at sites of low shear stress[30], and this apoptosis may be triggered by myeloperoxidase release from neutrophils[31] and from the expression of toll-like receptor 2 (TLR-2)[32]. A proposed sequence of events involves a two-hit hypothesis. Firstly, increased TLR-2 expression by endothelial cells overlying plaque increases the susceptibility of endothelial cells to injury. Secondly, when injury occurs, neutrophils aggregate, and the increased TLR-2 expression impairs healing. Endothelial injury continues, and the secreted tissue factor by dying endothelial cells promotes thrombosis. The dying endothelial cells have contact with the underlying subendothelial matrix, and granulocytes such as neutrophils become trapped in fibrin strands and form neutrophil extracellular traps (NETs)[32]. The prominent role of neutrophils and presence in plaque erosion differentiates it from plaque rupture, where macrophages are predominant. Similarly, endothelial cell apoptosis in plaque erosion contrasts with destruction of the ECM in plaque rupture.

Plaque erosion as a cause of thrombosis is becoming more prevalent, contributing to more than one quarter of acute coronary deaths[33]. This is likely due to the increasing success of LDL-C-lowering therapies[25], thereby lowering the prevalence of lipid-rich plaques. Studying plaque erosion has been hampered by the lack of reliable animal models[34], and research is ongoing in this field.

1.2 Adhesion molecules, cytokines and inflammatory cells in atherosclerosis

A vast number of inflammatory mediators contribute to atherogenesis. The predominant mediators in acute vascular inflammation are adhesion molecules, endothelium-derived cytokines, monocytes and neutrophils. In the setting of chronic vascular inflammation, prominent mediators include IL-1, IL-6 and TNF-α, which modulate the immune response and propagate inflammation. Macrophages are the dominant inflammatory cells, and are directly atherogenic.

1.2.1 Acute vascular inflammation

1.2.1.1 Vascular Cell Adhesion Molecule 1

VCAM-1, also known as CD106, is an immunoglobulin-like adhesion molecule that is expressed on the luminal surface of endothelial cells, and binds to the integrin α4β1, which is constitutively expressed on lymphocytes, monocytes and eosinophils[35]. VCAM-1 is not present in basal conditions, and its expression is stimulated in pro-inflammatory conditions. These include the presence of TNF-α, IL-1β, lipopolysaccharide (LPS), reactive oxygen species, oxLDL, hyperglycaemia, toll-like receptor agonists, and in the context of shear stress[36]. The gene expression of VCAM-1 is regulated by two specific subunits of the NFκB transcription pathway, namely p65 and p50 with low stimulation, and p65 alone with high stimulation[37]. Upon encountering the integrin α4β1, VCAM-1 mediates rolling of, or firm adhesion to, circulating leucocytes, and facilitates their transmigration to the subendothelium[38, 39].

1.2.1.2 Intercellular Adhesion Molecule 1

ICAM-1, also known as CD54, is an immunoglobulin-like adhesion molecule that is expressed constitutively on the luminal surface of endothelial cells. It binds to two integrins of the β2 family on the surface of leucocytes, including CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1). Its expression is regulated by the following transcription factors: NFκB (specifically

the p65 subunit), AP-1, CCAAT/enhancer binding protein family, ETS, signal transducer and activator of transcription-1 (STAT-1), and Sp1[40]. Amongst these transcription factors, NFκB activation is always required for ICAM-1 upregulation. ICAM-1 is induced by broad range of pro-inflammatory molecules in endothelial cells, including cytokines TNF-α, IL-1β, IL-6, and interferon-γ, LPS, thrombin, substance P, endothelin 1-3, oxLDL, hydrogen peroxide, and cyanate[41, 42]. In addition, shear stress induces ICAM-1 expression[41]. ICAM-1 functions similarly to VCAM-1; upon encountering integrins, it mediates rolling and transmigration of leucocytes into the subendothelium.

1.2.1.3 P-selectin

P-selectin is a cell adhesion molecule present in the Weibel-Palade bodies of endothelial cells. Upon stimulation of ECs, it is translocated to the external plasma membrane where it functions as a receptor for monocytes and neutrophils[43]. It is also present in platelets, where it is found in α-granules. Stimulants for P-selectin include TNF-α, IL-1β, oxygen free radicals, and histamine. The direct translocation to the cell surface after EC stimulation occurs within minutes as there is no requirement for transcription and translation[44]. P-selectin binds to P-selectin glycoprotein ligand 1 (PSGL-1), expressed on almost all leucocytes, as well as the sialyl-Lewis X ligand, and there is subsequent leucocyte rolling and transmigration to the subendothelium. The expression of P-selectin on the EC surface is initially transient, lasting approximately 10 minutes, with the protein then being internalised inside the cell, where it is degraded or recycled[45]. A more sustained expression occurs after continued EC stimulation, with *de novo* synthesis of P-selectin occurring within 2 hours[45].

1.2.1.4 E-selectin

E-selectin is an adhesion molecule present in endothelial cells, and rapidly expressed after EC stimulation. The pro-inflammatory mediators TNF-α, interferon-γ, IL-1β and LPS all stimulate E-selectin[46]. In addition, disturbed and oscillatory shear stress, rather than laminar

shear stress, stimulate E-selectin[47, 48]. It is transcriptionally regulated by both NFκB and Activating Transcription Factor (ATF)[49], and once on the EC surface recognises the sialyl-Lewis X ligand on leucocytes[50]. E-selectin mediates rolling and firm adhesion of leucocytes onto the EC surface and subendothelial transmigration. E-selectin is functionally similar to P-selectin, however its expression occurs a minimum of 3 hrs after stimulation and subsequently wanes, despite continued cytokine stimulation[51].

1.2.1.5 Monocyte Chemoattractant Protein 1

Also known as CCL2, MCP-1 is a chemokine (chemoattractant cytokine) secreted by numerous cell types including endothelial, fibroblasts, epithelial, smooth muscle, mesangial, astrocytic, monocytic, and microglial cells. Monocytes and macrophages are the dominant source of circulating MCP-1[52]. Stimulants for MCP-1 expression by endothelial cells include TNF-α, IL-1β, IL-6, and/or brain-derived neurotrophic factor. MCP-1 is transcriptionally regulated by NFκB and mitogen activated protein kinase (MAPK). MCP-1 binds to the CCR2 receptor on monocytes leading to the activation of intracellular signalling cascades that prompt migration toward the chemokine source (endothelial cell). Migration of monocytes from the blood stream across the vascular endothelium is required for routine immunological surveillance of tissues, as well as in response to inflammation. MCP-1 is also chemotactic for memory T lymphocytes and natural killer cells[53, 54].

1.2.1.6 Monocytes

Non-classical monocytes slowly patrol the endothelium of blood vessels, and upon endothelial cell activation and subsequent monocyte chemotaxis, are able to rapidly respond to local perturbations. In homeostatic conditions, arterioles, capillaries, and postcapillary venules are populated by patrollers, perhaps one third of the marginal pool of non-classical monocytes[55]. Once in the subendothelium, they are able to phagocytose debris and recruit neutrophils to mediate focal necrosis of endothelial cells[56]. These monocytes (with surface

markers $CD14^{hi}CD16^{-}$) produce high levels of pro-inflammatory cytokines such as TNF-α, attracting further monocytes to sites of injury by setting up a chemoattractant gradient[57]. They also produce proteases including matrix metalloproteinases (MMPs) that cleave collagen and other matrix components[58]. Hence, they amplify both the inflammatory response and local injury. Over hours to days, monocytes differentiate into macrophages with their ultimate phenotype depending on different levels of trophic factors, mainly M-CSF and GM-CSF[59].

Macrophages that differentiate in an environment dominated by pathogen associated molecular patterns (PAMPs), interferon-γ, IL-1, and TNF-α from damaged tissues become classically activated. Such macrophages have amplified phagocytic and cytotoxic activity, secretion of pro-inflammatory mediators and expression of scavenger receptors. As sites of injury heal, with reductions in pro-inflammatory mediators, there is a reduction in macrophage activity. Newly-recruited macrophages may differentiate into alternatively-activated macrophages, which facilitate tissue repair and promote granuloma formation[60].

1.2.1.7 Neutrophils

Neutrophils play a significant role in early acute vascular inflammation (AVI), and their accumulation in the vessel wall is a hallmark feature of AVI. Circulating neutrophils may be directed to a site of endothelial inflammation either by nearby monocytes using paracrine signalling with IL-1β, keratinocyte chemoattractant (KC), TNF-α, CCL3, or IL-6[56], or via chemotactic gradients from more distant sites of injury. At the sites of endothelial injury, adhesion molecules are upregulated. The interactions between these molecules, especially selectins, with P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophils, results in neutrophil tethering and rolling[61]. This is followed by firm adhesion to the endothelium, typically mediated by the integrin CD11a/CD18 (LFA-1)[62]. Neutrophils then crawl along the endothelium, in a slow, CD11b (Mac-1)-dependent meandering motion[62]. Following adhesion and crawling, neutrophils emigrate from the vasculature, and at the site of injury phagocytose

debris and recruit more inflammatory cells [63]. The presence of inflammation inhibits the apoptosis of neutrophils, and with the continued recruitment of neutrophils, the magnitude of the inflammatory response is increased[64, 65].

Neutrophils can also regulate the function of dendritic cells, lymphocytes and natural killer cells[63]. After entry into the inflammatory tissue site, in response to pro-inflammatory stimuli in the tissue, neutrophils become fully activated, a state characterised by release of granule proteins, acquisition of phagocytic capabilities, and production of NETs. Neutrophils are relatively nonresponsive to a single stimulus, but exposure to one stimulus enhances the ability of the cell to mount an enhanced activation response to a second individual stimulus[66]. The activation mechanisms that are beneficial for pathogen killing can also be detrimental in the context of sterile injury such as in AVI. For example, despite a targeted inflammatory response by neutrophils, there is typically local collateral tissue damage from leakage of cytosolic contents and oxidative products outside of the neutrophil from phagocytic activity[67].

The transmigration process of neutrophils across the endothelial cell barrier can induce signalling changes in endothelial cells, causing them to contract and thereby generating intercellular gaps. This allows serum proteins (such as cytokines, antibodies, and complement) to pass through the endothelial barrier, further aggravating the inflammatory state[68]. Such changes promote a continued inflammatory state that can ultimately progress to chronic vascular inflammation.

1.2.2 Chronic vascular inflammation and atherosclerosis

1.2.2.1 Interleukin 1-α and β

Interleukin 1-α (IL-1α) and interleukin 1-β (IL-1β) are members of the IL-1 cytokine superfamily and are produced by macrophages, monocytes, fibroblasts, and dendritic cells. They are both produced as precursor proteins that are subsequently cleaved, although IL-1β in

its precursor form is not biologically active. The precursor of IL-1α acts as a damage-associated molecular pattern (DAMP) molecule, which is recognised by innate immunity cells by pattern recognition receptors (PRRs) and functions as a danger signal for the immune system. IL-1α and β are produced in response to inflammatory stimuli and bind to interleukin-1 receptors. IL-1α generally remains associated with the cell surface or is released by dying cells and usually acts at short distances by juxtacrine or paracrine signalling. IL-1β on the other hand, can either act in a paracrine manner or systemically[69].

The main function of IL-1-type cytokines is to control proinflammatory reactions in response to tissue injury by pathogen-associated molecular patterns (PAMPs, such as bacterial or viral products) or (DAMPs)[70]. IL-1α and IL-1β rapidly induce the mRNA expression of hundreds of genes in multiple different cell types, such as monocytes, macrophages, endothelial and epithelial cells. In parallel, IL-1α and IL-1β also induce expression of their own genes, which serves as a positive-feedback loop that amplifies the IL-1 response in an autocrine or paracrine manner[71]. IL-1α and IL-1β can also increase the expression of the IL-1 receptor antagonist (IL-RA), enabling negative feedback inhibition, a mechanism that resists unrestricted IL-1 signalling[72].

Another method by which IL-1 signalling is regulated is through the requirement for activation of another molecule, caspase-I, for cleavage of pro-IL-1β to IL-1β. Caspase-I is present in an inactive form in the cytosol of many phagocytic cells, and is activated after stimulation by a myriad of microbial and endogenous signals. These signals stimulate the assembly of an inflammasome, specifically Nucleotide-Binding Domain Leucine-Rich-Containing Family Pyrin Domain-Containing-3 (NLRP3), a macromolecular complex that forms a scaffold for pro-caspase-I activation[73]. Notable effects of IL-1α and β on cells include induction of prostaglandin production through the induction of cyclooxygenase-2; the elaboration of nitric oxide by elevation of levels of the inducible isoform of nitric oxide

synthase; induction of the expression of many cytokines, including augmenting its own gene transcription; increased expression of leucocyte adhesion molecules and thrombogenic mediators; and activation of cells involved in innate immunity, prominently including the mononuclear phagocytes[70].

There are numerous stimulators of the inflammasome in atherosclerotic plaques, including cholesterol crystals and inflammatory cytokines. Indeed, there are significant amounts of NLRP3, caspase-I and IL-1β in plaques[74]. Hence, the requirement of a two-step activation process for the inflammasome and subsequent IL-1β generation is not a hindrance for continued inflammatory activity in atherosclerotic plaques. Other stimuli for the inflammasome in atherosclerotic plaques include altered shear stress, hypoxia and acidosis[75-77]. IL-1β is present in much larger concentrations and is a much greater contributor to persistent, chronic inflammation in plaques than IL-1α. Indeed, IL-1β stimulates ICAM-1 and VCAM-1 which recruit other leucocytes, stimulates production of MCP-1 and other chemokines, and also strongly induces smooth muscle cells to secrete IL-6[78]. Il-1β contributes to intimal thickening, increases inflammation in the vessel wall, and promotes aneurysm formation[79-81].

1.2.2.2 Interleukin 6

IL-6 is a cytokine with both pro- and anti-inflammatory properties, and is produced by a variety of cells, most notably monocytes and macrophages at inflammatory sites. Stimulants for IL-6 production include IL-1β, TNF-α, platelet-derived growth factor, LPS, and a variety of pathogens. In the acute inflammatory response, such as in AVI, large amounts of IL-6 can be produced. IL-6 binds to the IL-6 receptor (IL-6R), which is present on macrophages, neutrophils, some T cells, and hepatocytes. Following this, the IL-6/IL-6R complex binds to two molecules of GP130 (ubiquitously expressed on all cells), leading to signal transduction, which includes activation of the JAK/STAT, ERK, and PI3K signal transduction pathways. In

addition to binding to IL-6 receptors on cells, IL-6 may also bind to soluble IL-6 receptors (sIL-6R). Although this would be expected to dampen the immune response by scavenging circulating IL-6, it conversely amplifies the immune response, as IL-6/sIL-6R complexes may then bind to cells that express GP130, and then activate IL-6 signal transduction[82]. This form of "trans-signalling" as opposed to classical cell to cell signalling is crucial for rapid lymphocyte trafficking to sites of inflammation, and produces a much more robust inflammatory response. One mechanism by which sIL-6R is formed is by the cleavage of membrane-bound IL-6R, which can occur during the apoptosis of neutrophils[82].

The propagation of the effects of IL-6 can be controlled by the production of neutralising antibodies, or the release of soluble GP130, which can bind circulating IL-6/sIL-6R complexes. If these complexes do bind endothelial cells, they induce the secretion of adhesion molecules and MCP-1[83]. This results in a relative decrease in the secretion of cytokines that attract neutrophils, such as IL-8, in favour of monocytes, hence the inflammatory response becomes monocyte dominant[84]. This switch heralds the IL-6-driven transition from an acute to a chronic inflammatory response[85]. Furthermore, IL-6 plays a vital role in the initiation of specific rather than innate immune responses, such as end-stage B cell differentiation, immunoglobulin secretion and T cell activation.

The activity of IL-6 can be persistent and uncontrolled. This is particularly the case in the setting of vascular inflammation, where there is a continuous influx of monocytes that differentiate into macrophages in the inflammatory environment and propagate pro-inflammatory signalling. In addition, IL-6 acts upon naive T-lymphocytes resulting in their differentiation into helper or cytotoxic T cells, which are subsequently able to continue propagation of the inflammatory cascade[86]. IL-6 stimulates the proliferation of SMCs, and is produced by foam cells[87]. IL-6 has significant procoagulant activity, primarily through

inducing monocyte expression of tissue factor, which promotes thrombosis[88]. In addition, IL-6 promotes platelet aggregation[89].

Some of this pro-inflammatory activity is balanced by the anti-inflammatory properties of IL-6. IL-6 can suppress IL-1 and TNF-α production, induce the IL-1 receptor antagonist, and can induce tissue inhibitor of matrix metalloproteinase 1 (TIMP1), which impedes the activity of collagenase and therefore has anti-proteolytic activity[90]. However, the pro-inflammatory activity is dominant in the setting of vascular inflammation, and contributes to a chronic and atherogenic state.

1.2.2.3 Tumour necrosis factor-alpha

TNF-α is a cytokine that activates multiple transduction pathways, inducing or suppressing a wide variety of genes, including those encoding the production of cytokines, adhesion molecules, and inducible nitric oxide synthase (iNOS)[91]. TNF-α has many pro-inflammatory actions: orchestrating the inflammatory response through activation of pro-inflammatory cytokine genes, such as IL-1 and IL-6, as well as its own production[92]. The activated macrophage is the main source of TNF-α, containing both cell-associated and membrane-bound TNF-α, both of which are biologically active[93]. Other cells that release TNF-α include lymphocytes, fibroblasts, neutrophils, smooth muscle and mast cells.

TNF-α can bind to either TNF-receptor 1 (TNFR1), which is present on most cells, or TNF-receptor-2 (TNFR2), which is present on immune cells. The binding of TNF-α to its receptors results in either activation of the NFκB or MAPK transcription pathways, or induction of apoptosis[94]. The activation of NFκB and its translocation to the nucleus leads to the production of a multitude of pro-inflammatory proteins, many of which are relevant to vascular inflammation. These include but are not limited to: pro-inflammatory interleukins[95], adhesion molecules[96], interferon-γ[97], MCP-1[98], tumour necrosis factor-related apoptosis-

inducing ligand (TRAIL)[99], toll-like receptors 2 and 9[100, 101], tissue factor-1[102] and vascular endothelial growth factor (VEGF)[103]. The MAPK pathways control a large number of fundamental cellular processes including growth, proliferation, differentiation, motility, stress response, survival and apoptosis[104]. The MAPK signalling pathways include ERK5, p38, JNK, and ERK1/2 pathways. The p38 and JNK pathways may be the most relevant to atherosclerosis. They are both necessary for foam cell formation[105, 106]. They are both activated during vascular endothelial injury, inducing neointima formation[107], and p38 stimulates the proliferation of SMCs[108]. Although TNF-α is an inducer of apoptosis, in atherosclerotic plaque apoptosis is reduced in favour of cell necrosis, contributing to the necrotic core[109]. This effect may be partly due to the stimulation of macrophages and SMCs to synthesise matrix proteases[110]. Furthermore, the presence of TNF-α promotes more advanced, unstable lesions[109].

1.2.2.4 Macrophages

Monocytes that have transmigrated into the subendothelial space may either differentiate into classical, "M1", pro-inflammatory macrophages, or alternative, "M2", anti-inflammatory macrophages, depending on the local environment. Macrophages may also be derived from the adventitia, either from the vasa vasorum or as resident, locally-derived, macrophage progenitors[111]. Although the M1/M2 concept is an oversimplification of the role of the macrophage, it is practical and for the most part accurate. Macrophages are polarised towards an M2 phenotype when in a pro-resolving environment, such as during resolution of inflammation, where the emphasis is on tissue repair. Conversely, they are polarised to an M1 phenotype when in pro-inflammatory environment. Early atherosclerotic lesions have a predominance of M2 macrophages, and more advanced lesions, with high levels of inflammatory activity, have a predominance of M1[112]. The typical activating stimuli for M1 macrophages are interferon-γ and LPS[60]. The activating stimuli for the subclasses of M2

macrophages are: IL-4 and IL-13 for M2a, immune complexes plus either IL-1β or LPS for M2b, and IL-10, transforming growth factor-β or glucocorticoids for M2c[113].

The atherogenic roles of M1 macrophages in the vessel wall include: (1) secretion of pro-inflammatory cytokines (eg. IL-6, IL-12, and IL-23), (2) secretion of growth factors that induce neovascularisation and vessel permeability (eg. VEGF), (3) production of matrix metalloproteinases that digest the matrix and solubilise the fibrous cap, (4) production of reactive oxygen species which promote macrophage recruitment, impair efferocytosis of apoptotic cells and oxidise LDL, and (5) acting as antigen presenting cells, where the activation of antigen-specific T cells results in the amplification of the macrophage response [114]. This last role contributes to the chronicity of the inflammatory response.

As stated previously, macrophages are critical to the production of lipidic plaques. The ability of macrophages to remove oxidised LDL can be overwhelmed when the ability to remove ingested oxLDL is exceeded by the rate of ox-LDL influx and cholesterol esterification. This forms the basis for inflammatory, cholesterol-rich plaques.

1.3 Inflammation as a therapeutic target in atherosclerosis

Inflammation in the vessel wall has received considerable attention as a target for new therapies for atherosclerosis. A conundrum has existed for many years, since numerous anti-inflammatory agents increase the risk of cardiovascular events, such as non-steroidal anti-inflammatory drugs[115] and corticosteroids[116]. Furthermore, the recent CIRT study, which compared the anti-inflammatory agent methotrexate to placebo in patients with coronary artery disease, did not reduce cardiovascular events[117]. The LoDoCo study, however, demonstrated a 67% reduction in the composite incidence of acute coronary syndrome, out-of-hospital cardiac arrest, or noncardioembolic ischemic stroke in patients with stable coronary artery disease taking the anti-inflammatory agent colchicine compared to placebo ($p<0.001$)[118].

Colchicine's anti-inflammatory properties include an antitubulin effect that inhibits neutrophil function[119]. In addition, the potential for cardiovascular benefit by targeting the inflammatory nature of atherosclerosis was highlighted by the CANTOS study[120]. CANTOS compared canakinumab, a monoclonal antibody targeting interleukin-1β, to placebo in patients with a previous myocardial infarction and an elevated C-reactive protein (CRP) level. Canakunimab at a dose of 150 mg every three months reduced cardiovascular events by 15% compared to placebo at 48 months ($p=0.021$)[120].

The reductions in cardiovascular events seen in LoDoCo and CANTOS occurred in addition to the effects of statins, as more than 90% of patients in both studies were taking statins. Statins themselves have been clearly shown to have anti-inflammatory effects amongst their numerous pleiotropic effects. The PRINCE trial (2001) was an early study that demonstrated the CRP-lowering properties of pravastatin, which occurred independently of changes in LDL cholesterol[121]. Since then, statins have been shown to increase endothelial nitric oxide synthase (eNOS) gene expression and activation[122, 123]; reduce NFκB activation by pro-inflammatory stimuli[124]; upregulate antioxidant enzymes such as heme oxygenase-1[125]; inhibit pro-inflammatory cytokine release such as IL-6[126]; and upregulate anti-inflammatory cytokines such as IL-10[127]. The pleiotropic effects of statins have been shown to be clinically significant, as several clinical trials have demonstrated reductions in cardiovascular events beyond the effects of lipid lowering. For example, in the LIPID trial pravastatin reduced cardiovascular events by 24% in patients with a past history of an acute coronary syndrome, throughout the continuum of LDL-C levels ($p<0.001$)[128]. In the Heart Protection Study, patients with coronary, other occlusive arterial disease, or diabetes, were randomised to receive simvastatin or placebo; simvastatin reduced major vascular events by 24% irrespective of initial cholesterol concentrations ($p<0.0001$)[129].

Statins have been successfully used to reduce cardiovascular events by targeting systemic inflammation in addition to their established lipid-lowering effects. In the JUPITER study, apparently healthy men and women with low LDL-C levels (<3.4mmol/L) but elevated hs-CRP levels (>2.0 mg/L) were randomised to rosuvastatin or placebo; the trial was stopped early after a median of 1.9 years due to a 44% reduction in the occurrence of major adverse cardiovascular events in the rosuvastatin group ($p<0.00001$)[130]. Similarly, the anti-inflammatory properties of omega-3 fatty acids have led to considerable research into their therapeutic potential for a range of conditions.

1.4 The effects of omega-3 fatty acids on inflammation

1.4.1 Fatty acids in health and disease

1.4.1.1 Overview of fatty acids

Fatty acids are organic acids with a hydrocarbon chain and a terminal carboxyl group (see Figure 1.3). They are the major components of fats, oils, and waxes. Although there are over 1000 naturally-occurring fatty acids, only 20-30 are widely distributed in nature. These typically have between 10 and 22 carbon atoms. Fatty acids can be classified by the number of carbon atoms present, i.e.: "short-chain" if ≤ 5 carbon atoms, "medium-chain" if 6 to 12 carbon atoms, "long-chain" if 13 to 21 carbon atoms, and finally "very long chain" if ≥ 22 carbon atoms. A common form of nomenclature for fatty acids is "lipid number" in the form of (C:DωN), where C corresponds to the number of carbon atoms, D is the number of double bonds, and ωN is the position of the last double bond, if present, in the hydrocarbon chain. The position is defined as the number of carbon atoms away from the methyl (CH_3) end of the structure. For example, (20:5ω3) refers to eicosapentaenoic acid (EPA), which has 20 carbon atoms, 5 double bonds, and the position of the last double bond is 3 carbon atoms away from the methyl end. The hydrocarbon chain of a fatty acid can have either single carbon-carbon bonds with two flanking hydrogen atoms, or double bonds, of which there can be 0 to 6 in any

single structure. The double carbon-carbon bonds typically have *cis* geometry, i.e. the continuing carbon chains after the double bond face in the same direction. This creates a kink in the structure. In the case of *trans* geometry, the continuing carbon chains face the opposite direction, and the absence of a kink allows them to be pack closer together (see Figure 1.4). It is important to distinguish trans-fatty acids from cis-fatty acids, and therefore "trans N" or "cis N" may be used in nomenclature for this purpose. "*Cis*" is implied if this distinction is not made. "Trans-fatty acids" have the *trans* geometry, and are much less commonly found in nature than in the commercial food industry. They have a much higher melting point than their *cis* counterparts, and hence can be cooked as liquids at much higher temperatures while maintaining chemical stability[131].

"Saturated fatty acids" are defined by the absence of double bonds in the hydrocarbon chain (see Figure 1.5). This makes them very chemically stable, as oxidation and free radial damage to fatty acids occur at the sites of double bonds. Saturated fatty acids tend to be solids at room temperature due to their very high melting points. They can be found in both plant and animal sources, although animal products have the highest saturated fatty acid content. They have the maximum number of hydrogen atoms in the hydrocarbon chain, and a higher calorie content than unsaturated fatty acids.

"Monounsaturated fatty acids" (MUFAs) have a single double bond in the hydrocarbon chain, and are predominantly comprised of the omega-7 and omega-9 fatty acids. MUFAs are abundant in plant and vegetable oils, nuts, milk products, red meat, high fat fruits, and avocados. "Polyunsaturated fatty acids" (PUFAs) have at least two double bonds in the hydrocarbon chain. PUFAs are predominantly comprised of omega-6 and omega-3 fatty acids. Both omega-6 and omega-3 fatty acids are found in plant oils, however the omega-3 fatty acids EPA (20:5ω3) and DHA (22:6ω3) are abundant in fish and algal oils. Fatty acids that are obtained from plant, animal or fish sources are in the form of mixtures rather than as individual

fatty acids. Furthermore, they are not found naturally as free fatty acids, but as either triglycerides, phospholipids or less commonly, as cholesterol esters. This is necessary because fatty acids, being a form of lipid, are non-polar and hence do not dissolve in aqueous solutions.

Fatty acids are essential for basic cellular structure and function, and are important sources of fuel. The term "essential fatty acid" defines fatty acids that are required for normal biological processes in humans and animals, but cannot be synthesisedt[132]. These are linoleic acid (18:2ω6) and alpha-linolenic acid (18:3ω3), which cannot be synthesised due to the lack of the Δ12 and Δ15 desaturase enzymes responsible for converting oleic acid (18:1ω9) into linoleic acid (18:2ω6) and alpha-linolenic acid (18:3ω3)[133].

Table 1.1 summarises the lipid number, name, and composition of the most abundant fatty acids including saturated, trans-fatty, monounsaturated and polyunsaturated.

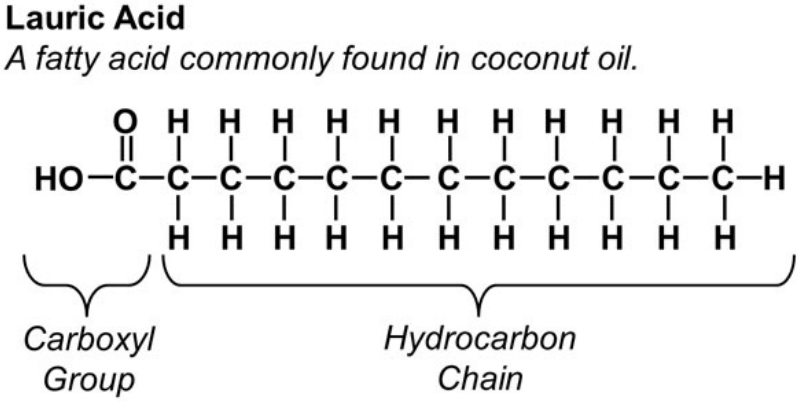


Figure 1.3: The basic structure of a fatty acid, using the saturated fatty acid lauric acid (12:0) as an example. Downloaded from the Dallas County Community College website: https://dlc.dcccd.edu/biology1-3/lipids on 7/12/18.

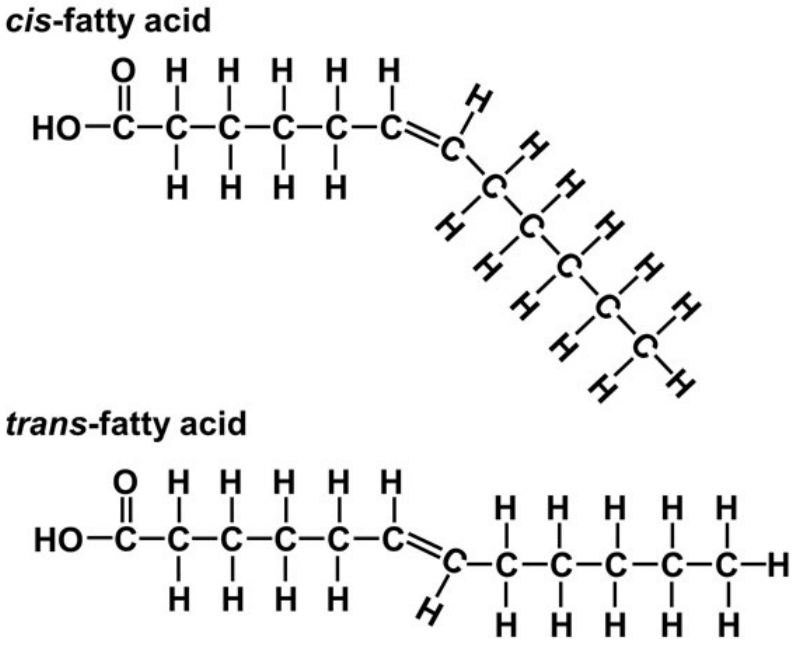


Figure 1.4: The structure of *cis*- compared to *trans*-fatty acids, demonstrating the direction of the continuing carbon chain after a double bond. Downloaded from the Dallas County Community College website: https://dlc.dcccd.edu/biology1-3/lipids on 7/12/18.

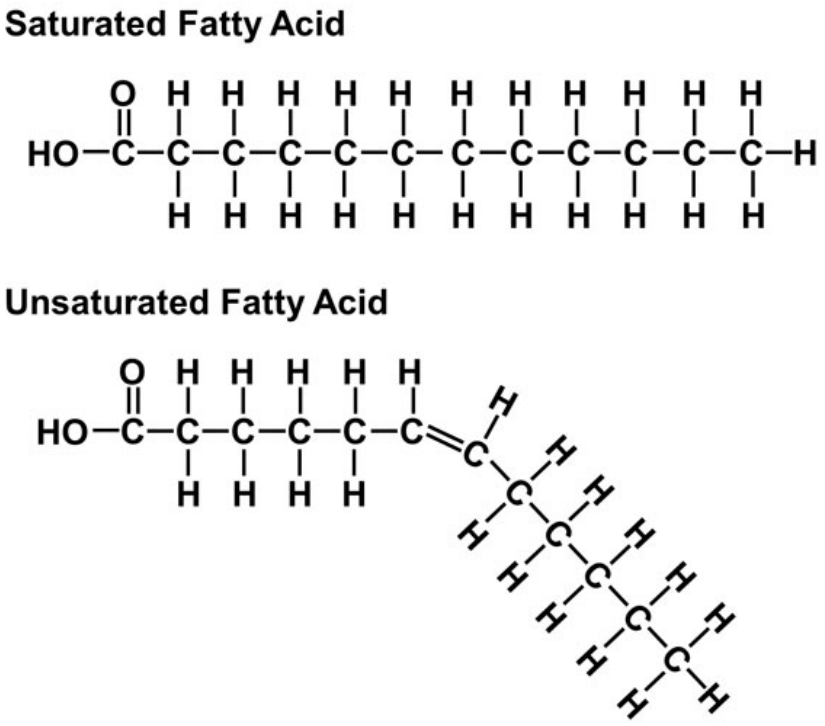


Figure 1.5: The structure of a saturated fatty acid compared to an unsaturated fatty acid. The absence of double bonds in the hydrocarbon chain defines a fatty acid as saturated. Downloaded from the Dallas County Community College website: https://dlc.dcccd.edu/biology1-3/lipids on 7/12/18.

Table 1.1: Summary of the type, lipid number, and common name of the most abundant fatty acids

Type of fatty acid	Lipid number	Common name
Saturated	C12:0	lauric acid
	C14:0	myristic acid
	C15:0	pentadecylic acid
	C16:0	palmitic acid
	C17:0	margaric acid
	C18:0	stearic acid
	C20:0	arachidic acid
	C22:0	behenic acid
	C24:0	lignoceric acid
Trans-fatty	t16:1ω-7	trans-palmitoleic acid
	t18:1ω-7	trans-vaccenic acid
	t18:1ω-9	trans-oleic acid
	t18:2ω-6	trans-linoleic acid
Monounsaturated	16:1ω-7	palmitoleic acid
	18:1ω-7	vaccenic acid
	18:1ω-9	oleic acid
	20:1ω-9	eicosenoic acid
	22:1ω-9	erucic acid
	24:1ω-9	nervonic acid
Polyunsaturated	18:3ω-3	alpha-linolenic acid
	20:5ω-3	eicosapentaenoic acid
	22:5ω-3	docosapentaenoic acid
	22:6ω-3	docosahexaenoic acid
	18:2ω-6	linoleic acid
	18:3ω-6	gamma-linolenic acid
	20:2ω-6	eicosadienoic acid
	20:3ω-6	dihomo-gamma-linolenic acid
	20:4ω-6	arachidonic acid

1.4.1.2 Saturated fatty acids

Saturated fatty acids (SFAs) are obtained from both plant and animal sources, but can also be created from hydrogenation of a corresponding (same carbon chain length) unsaturated fatty acid. Of the SFAs, palmitic acid (C16:0) is the most widely occurring in both animal fats and vegetable oils, whilst stearic acid (C18:0) is found in lesser quantities in vegetable oils.

There has for decades been concerns about the effects of SFAs on serum cholesterol[134]. The effects have been shown to be fatty acid-dependent, with myristic acid (C14:0), lauric acid (C12:0) and palmitic acid raising both LDL and HDL, with no effect on LDL seen with stearic acid[135, 136].However, despite these effects, a meta-analysis of 21 prospective cohort studies concluded that there is no association between saturated fat consumption and cardiovascular disease risk[137]. There is still a consensus that SFAs should be substituted for healthier nutrients where possible[138]. This is based a pooled analysis of 11 cohort studies which concluded that substitution of SFAs with PUFAs reduces the risk of coronary events by 13%[139].

The discrepancy between effects of SFAs on lipids and cardiovascular risk could be explained by effects on other atherogenic mediators. However, the effects on other atherogenic mediators are still overall detrimental, and hence this area requires continued investigation. For example, SFAs have been shown to promote insulin resistance[140], induce low-grade systemic inflammation[141], and directly stimulate inflammatory gene expression by way of TLR4 signalling[142].

1.4.1.3 Trans-fatty acids

Trans-fatty acids in the human diet are mostly encountered following partial hydrogenation of vegetable oils in the food industry (in particular, trans-oleic acid [t18:1ω-9]). This gives food desirable physical and chemical characteristics, as well as distinctive flavour, crispness, creaminess, plasticity and oxidative stability[143]. Trans-fatty acids are used extensively in the preparation of fast foods and in baked goods. A small percentage of trans-fatty acids is obtained from dairy products and ruminant meats (in particular, trans-vaccenic acid [t18:1ω-7]). The contribution of trans-fatty acids to the Australian diet is estimated to be 0.6% of total energy[144]. This compares to 2 to 3% in the United States[145].

The widespread production of trans-fatty acids has raised significant health concerns given their association with cardiovascular disease. Consumption of trans-fatty acids has been shown in a meta-analysis of 60 randomised controlled trials to raise LDL-C, lower HDL-C, raise triglycerides, and raise lipoprotein(a) levels[146]. In addition, trans-fatty acids also reduce LDL particle size, increasing its atherogenicity[147]. Trans-fatty acids are pro-inflammatory, and their consumption is associated with higher circulating levels of IL-6, TNF-α, CRP and MCP-1[148, 149]. They also induce endothelial dysfunction, as evidenced by raised soluble adhesion molecules and impaired flow-mediated dilatation[148, 150].

A number of observational studies have demonstrated a significantly higher cardiovascular risk associated with an increase in trans-fatty acid consumption. On a per-calorie basis, coronary heart disease (CHD) risk is raised more by trans-fatty acids than any other macronutrient[151]. In a meta-analysis of four prospective cohort studies involving nearly 140,000 subjects, a 2 percent increase in energy intake from trans-fatty acids was associated with a 23 percent increase in the incidence of CHD[151-155]. One study also demonstrated a positive correlation between trans-fatty acid levels in erythrocyte membranes and the risk of sudden cardiac death, with an adjusted odds ratio of 1.47 (95% CI: 1.01 to 2.13)[156]. The mechanisms of the hazardous effects of trans-fatty acids have not been fully established, however their detrimental effects on lipids may be due to an increase in the activity of cholesteryl ester transfer protein (CETP), the main enzyme that transfers cholesterol esters from HDL to LDL and VLDL[157].

The public health implications of high trans-fatty acid consumption are enormous. Since the estimated consumption of trans-fatty acids in the Australian diet is well within the World Health Organisation's target of <1%, there has not been a strong impetus to reduce the consumption further. There is no requirement for declaration of trans-fatty acid content on food labels in Australia, however the 2009 Australia and New Zealand QSR Industry Survey

suggested that a number of strategies were being employed by industries to reduce trans-fatty acid levels in foods[158].

1.4.1.4 Monounsaturated fatty acids

MUFAs are found in high quantities in most commodity oils, and the vast majority are omega-7s or omega-9s. The most prevalent naturally occurring MUFA is oleic acid (18:1ω9), found in high concentrations in olive, peanut, palm, canola (rapeseed), and sunflower oils.

The consumption of MUFAs has been shown to have health benefits, but an important consideration is their source and quality. There is convincing observational evidence that the cardiovascular benefits of MUFAs of plant origin are greater than those of animal origin[159]. One large meta-analysis divided studies into tiers of dietary MUFA quality, and found that the MUFA diets of the highest quality, considering percentage of olive oil, oleic acid and MUFA:SFA ratio, had the lowest rates of all-cause mortality and major adverse cardiovascular events[160].

MUFAs have also been shown to reduce inflammation. A diet rich in MUFAs contributes to a more anti-inflammatory gene expression profile in human adipose tissue compared with a diet high in SFAs[161]. Furthermore, adherence to a Mediterranean diet in healthy adults reduces levels of plasma IL-6, CRP, and homocysteine[162]. However, since the Mediterranean diet is highly abundant in other health-promoting constituents in addition to MUFAs, the results obtained from human dietary studies cannot be fully attributable to MUFAs. This was exemplified in the landmark PREDIMED study, which randomised individuals at high cardiovascular risk to a Mediterranean diet supplemented with extra-virgin olive oil (EVOO), a Mediterranean diet supplemented with mixed nuts, or a control diet (advice to reduce dietary fat). During a median of 4.8 years of follow-up, the intervention diets significantly reduced the primary endpoint of a composite of myocardial infarction, stroke, or

death from cardiovascular causes by approximately 30% compared to the control diet (hazard ratio of 0.70 [95% CI, 0.53 – 0.91, p=0.009] for the EVOO group and 0.70 [95% CI, 0.53 – 0.94, p=0.02] for the mixed nuts group)[163]. The fatty acid composition of the plasma of PREDIMED participants before and after treatment was analysed, and demonstrated that in addition to a significant increase in oleic acid in the EVOO group, the mixed nut group had a reduction in the omega-6 PUFA gamma-linolenic acid (18:3ω6), and both interventions had a reduction in the saturated fatty acid margaric acid (C17:0)[164]. The original PREDIMED was subsequently retracted due to protocol deviations, particularly related to instances of lack of randomisation, and the study was republished in 2018, accepted as being a non-randomised trial[165]. In the republished manuscript, the hazard ratios for the primary endpoint were 0.69 (95% CI, 0.53–0.91) for the EVOO group and 0.72 (95% CI, 0.54–0.95) for the mixed nuts group, however levels of significance were not reported.[166]

There is ongoing debate about what defines a Mediterranean diet, and the compositions of these diets in human studies have differed. MUFA-enriched high fat diets in animal studies have resulted in reduced IL-1β secretion from adipose tissue and improved insulin sensitivity compared to SFA-enriched high fat diets[167]. The reduction in IL-1β is likely due to the inhibitory effects that MUFAs have on the NLRP3 inflammasome [168].

In addition to plant and animal-derived MUFAs, there has recently been interest in marine-derived MUFAs. These long chain fatty acids, eg. gadoleic acid (20:1ω11) and cetoleic acid (22:1ω11), are found in fish such as soury, pollock, and herring. Analysis of the diets of the Greenland Inuits from the original studies from Bang and Dyerberg demonstrated levels of long-chain MUFAs 13 times higher than that of the Danish controls[169]. There is evidence in animal studies for beneficial effects on glucose and lipid metabolism[170], reduced triglyceride and VLDL levels[171], upregulation of the anti-inflammatory transcription pathway PPAR-γ[172], and reduced atherogenesis[173] with marine-derived MUFAs. With the continued interest

in the cardiovascular benefits of marine-derived PUFAs, clinical trials focusing on marine-derived MUFAs are expected.

1.4.1.5 Polyunsaturated fatty acids

The polyunsaturated fatty acids (PUFAs) consist of omega-6 and omega-3 fatty acids. These are long-chain molecules with typically 2 to 6 non-contiguous double bonds. In plants, the number of double bonds rarely exceeds three, although in algae and fish the fatty acids can contain up to six double bonds. The omega-6 and omega-3 fatty acids are present in most plant, animal, and commodity oils and fats. As outlined previously, the PUFAs include the essential fatty acids, linoleic acid (18:2ω6) and alpha-linolenic acid (18:3ω3). The most common omega-6 fatty acids encountered in the human diet are linoleic acid and arachidonic acid (20:4ω6). Since mammalian cells lack the omega-3 desaturase enzyme, omega-6 fatty acids cannot be converted *in vivo* to omega-3 fatty acids. Closely-related omega-6 and omega-3 fatty acids act as competing substrates for the same enzymes[174], outlining the importance of the proportion of omega-6 to omega-3 fatty acids in diet (discussed in Section 1.4.4).

Omega-6 fatty acids

Linoleic acid (18:2ω6), the shortest and most common omega-6 PUFA, is metabolised to the longer and more unsaturated arachidonic acid (20:4ω6) via the intermediates gamma-linolenic acid (18:3ω6) and dihomo-gamma-linolenic acid (20:3ω6). Arachidonic acid is metabolised by the cyclooxygenase (COX) enzymes to the 2-series of prostaglandins (i.e. PGE_2, PGI_2, PGD_2, $PGF_{2\alpha}$) and thromboxanes (A_2 and B_2). Arachidonic acid is also metabolised via the lipoxygenase (LOX) enzymes to the 4-series of leukotrienes (i.e. LTA_4, LTB_4, LTC_4, LTD_4, and LTE_4). Collectively, the prostaglandins, thromboxanes and leukotrienes are referred to as eicosanoids. Although the eicosanoids have important homeostatic functions in regulating both the promotion and resolution of inflammation in the immune response, they

predominantly have properties that are atherogenic. For example, PGD_2 and LTB_4 are pro-inflammatory, TXA_2 and TXB_2 are pro-aggregatory and vasoconstrictive, LTB_4 is chemotactic for neutrophils, and PGE_2 induces IL-6[175]. These properties predict a hazardous effect of excessive omega-6 consumption. Indeed, PGE_2 and PGI_2 are also proarrhythmic[175]. However cardiovascular outcome studies have not demonstrated an elevated cardiovascular risk associated with high omega-6 consumption. Conversely, the aggregate of randomised trials, case-control, and cohort studies indicate reduced cardiovascular risk[176]. This may be partly due to the increasingly recognised anti-inflammatory and atheroprotective effects of omega-6 PUFAs. For example, omega-6 PUFAs are a substrate for the anti-aggregatory and vasodilatory prostacyclin[177], the anti-chemotactic and pro-resolving lipoxin A_4[178], and the vasodilatory and anti-adhesive epoxyeicosatrienoic acids[179]. One human study showed a negative correlation between serum omega-6 PUFA levels and IL-6, and a negative correlation with the anti-inflammatory transforming growth factor β[180].

Individual studies of the effects of omega-6 PUFAs on serum lipids have produced mixed results, however a meta-analysis of 60 controlled fatty acid intervention trials indicated a beneficial effect on blood lipid levels with lower LDL and higher HDL levels[146]. Increasingly, when considering the physiological effects of omega-6 consumption, there has been a shift in focus to omega-6 as a proportion of, or relative to, other fatty acid species, and this continues to be studied extensively.

Omega-3 fatty acids

The omega-3 fatty acids are alpha-linolenic acid (ALA, 18:3ω3), eicosapentaenoic acid (EPA, 20:5ω3), docosahexaenoic acid (DHA, 22:6ω3), and docosapentaenoic acid (DPA, 22:5ω3). The latter is the intermediate species between EPA and DHA. ALA is found predominantly in plant oils, whereas EPA and DHA are best sourced from marine sources, i.e.

oily fish, fish oils and algal oil. In humans, there is a small amount of conversion from the essential fatty acid ALA to EPA (6%) via the enzymes Δ6 desaturase, elongase, and Δ5 desaturase, with an even smaller amount ultimately converted to DHA (3.8%)[181]. With a high omega-6 intake, these conversion rates are further decreased, leading some to consider EPA and DHA to be "conditionally essential"[182].

The consumption of omega-3 PUFAs leads to their incorporation into cell membranes, where they form an essential part of the phospholipid bilayer and alter cell physiology. Cellular membranes from some tissues, for example the brain, retina, and myocardium are particularly enriched in omega-3 PUFAs. DHA is the predominant fatty acid of membrane phospholipids in the brain grey matter and in the retina, and the accretion of DHA in their membranes is required for the optimum development of retinal and cerebral functions[183]. Despite this, supplementation of pregnant women, or fortification of infant formula, with DHA has not clearly resulted in improvements in neuropsychological performance in children[184]. Furthermore, although omega-3 PUFAs are purported to have positive effects on mood[185], systematic reviews of the literature have demonstrated mixed results for the prevention and treatment of depression[186-188]. The enrichment of the myocardium with omega-3 PUFAs suggests that they may be important for normal cardiac myocardial function and recovery as well as electrical stability. Recent evidence indicates that there is a reduction in adverse left ventricular remodelling and myocardial fibrosis following myocardial infarction in those supplemented with high-dose omega-3 PUFAs[189]. However, despite an increase in electrical stability with omega-3 supplementation[190], it has not been shown to reduce atrial or ventricular arrhythmias in those at high risk[191-193].

There has been considerable interest in the lipid-lowering effects of omega-3 PUFAs, and the overall effects could be best summarised as follows. Triglycerides are moderately decreased (25 to 30%), HDL-C is increased minimally (1 to 3%), and LDL-C is increased

mildly (5 to 10%)[194]. Given the modest effects of omega-3 PUFAs on atherogenic lipoproteins, there has been substantial interest in their anti-inflammatory effects as a mechanism for their cardioprotective effects.

1.4.3 The anti-inflammatory effects of omega-3 fatty acids

Omega-3 fatty acids have been shown to reduce inflammation in the *in vitro* and *in vivo* settings. Omega-3s are able to partly inhibit a number of aspects of inflammation including leucocyte chemotaxis, adhesion molecule expression and leucocyte–endothelial adhesive interactions, production of eicosanoids like prostaglandins and leukotrienes from the omega-6 fatty acid arachidonic acid, production of inflammatory cytokines, and T-helper 1 lymphocyte reactivity[195]. In addition, EPA and DHA give rise to inflammation-resolving resolvins, protectins and maresins[196]. Furthermore, omega-3s have been showed to attenuate oxidative stress-induced DNA damage[197]. These anti-inflammatory effects have translated into clinical benefit for a range of inflammatory conditions. One of the earliest conditions studied extensively was rheumatoid arthritis, given the understanding of the role of arachidonic acid metabolites in the disease process, which are clearly reduced by omega-3 fatty acids. One meta-analysis included data from 10 trials and concluded that dietary fish oil supplementation for 3 months significantly reduced morning stiffness and tender joint count in patients with rheumatoid arthitis[198]. There is some evidence for a beneficial effect of omega-3 fatty acids in asthma, which is characterised by high levels of pro-inflammatory cytokines, arachidonic acid-derived eicosanoids, and prostaglandins. Some studies have demonstrated improved lung function and a decline in disease scores after omega-3 supplementation[199, 200], however these findings have not been consistently reported[201].

Oxidative stress has been implicated as a pathogenic mechanism for the chronic, inflammatory neurodegenerative disease multiple sclerosis. In a randomised controlled trial, omega-3 fatty acids supplemented at a dose of 2 grams per day significantly reduced Expanded

Disability Status Scores (EDSS) in patients with multiple sclerosis, associated with reductions in serum CRP and the oxidative stress marker malondialdehyde[202]. Omega-3 fatty acids have been trialled in the setting of inflammatory bowel diseases (IBD), which are associated with increases in inflammatory eicosanoids, pro-inflammatory cytokines, and both CD4 and CD8 T cell activity[203, 204]. Despite omega-3s reducing all of these factors, a systematic review of clinical trials of IBD did not show clinical improvement with omega-3 supplementation[205]. The lack of a predictable clinical benefit based on known biochemical effects is a frequent scenario that necessitates further investigation. This is indeed the case for studies of the effects of omega-3 fatty acids on cardiovascular disease. In addition to the potential to modulate cardiovascular risk by targeting inflammation, omega-3 fatty acids have beneficial effects on established cardiovascular risk factors, including high levels of triglycerides[206-208] and blood pressure[209-211]. However, the results of cardiovascular outcome trials of omega-3 fatty acids have been overall neutral.

1.4.4 The omega 6:3 ratio

Conventional cardiovascular risk factors such as lipid parameters, blood pressure and inflammatory markers, have established and standardised tools for their measurement, as well as reference ranges that may define normal or associate with higher or lower risk. This is not the case for PUFAs. A common method for quantifying omega-3 "status" is to measure the proportion of total blood fatty acids comprised of omega-3s. This value, when measured in whole blood, has an inverse association with sudden death[212], and is the basis for the Omega-3 Index. The Omega-3 Index is the percentage of EPA and DHA in red blood cell fatty acids, and based on an analysis of 10 cohort studies, a value of < 4% predicts higher CHD mortality, and > 8% predicts lower CHD mortality[213]. However, indices such as this do not provide insights into the total blood fatty acid composition and the relative amounts of harmful versus

beneficial components. Furthermore, factors such as age and gender alter the absorption of fatty acids, and consequently the cut-offs for the Omega-3 index[214].

An alternative, arguably more informative measure is the ratio of pro-inflammatory and atherogenic omega-6 fatty acids to anti-inflammatory and atheroprotective omega-3 fatty acids, i.e. the Omega 6:3 ratio. The ratio of omega-6 PUFAs to omega-3 PUFAs in the human diet has increased markedly from hunter-gatherer times, when it was as low as 1:1, to now, when a typical Western diet can provide a ratio of 20:1[215]. An important concept exemplified by the Omega 6:3 ratio is that of fatty acid substitution. Since omega-6 and omega-3 PUFAs compete with each other for incorporation into cell membranes[216, 217], a higher omega-6 intake relative to omega-3 would be expected to shift healthy physiology to pathophysiology[218].

A high omega 6:3 ratio is a predictor of poorer outcomes for a number of chronic diseases. There is a positive association with obesity, as omega-6 and omega-3 PUFAs have divergent effects on adipogenesis, the brain-gut-adipose tissue axis, and systemic inflammation[215]. There is also a positive association with disease activity for several malignancies including colorectal and breast[219]. From a cardiovascular perspective, a lower omega 6:3 ratio is associated with reduced platelet aggregation and circulating inflammatory markers[219]. The Lyon Heart Study compared a Mediterranean-style diet to no dietary advice in patients after their first myocardial infarction, and achieved an omega 6:3 ratio in the intervention group as low as 4:1. Those receiving the Mediterranean diet had a 70% lower mortality rate at 2 years[220].

1.5 Clinical trials of omega-3 fatty acids

1.5.1 Dietary studies of omega-3 fatty acids

Bang and Dyerberg first proposed a cardioprotective effect of fish consumption after observing low lipid levels and rates of ischaemic heart disease in Greenland Inuit who

consumed a diet high in fish[221]. Since that time, numerous cohort studies have been published comparing the effects of fish consumption on outcomes. A meta-analysis of 17 such studies including 315 812 participants in the primary prevention setting and an average follow-up of 15.9 years, demonstrated a dose-response effect of fish intake on survival. Compared to low fish intakes (< 1 fish meal per week), the consumption of 1 fish meal per week resulted in a 16% reduction in mortality due to CHD[222]. The dose-response analysis indicated that every 15 g per day increment of fish intake decreased the risk of CHD mortality by 6%. Musa-Veloso et al performed a systematic review of 8 prospective studies comparing an estimated total omega-3 intake of < 250 mg versus ≥ 250 mg per day by fish consumption on fatal and non-fatal CHD in those with no prior history of CHD[223]. The higher intake was associated with a significant 35.1% reduction in the risk of sudden cardiac death and a near-significant 16.6% reduction in the risk of total fatal coronary events, while the risk of non-fatal myocardial infarction was not significantly reduced. The risk of CHD death was again found to be dose-dependently reduced by omega-3 intake.

One of the first randomised controlled trials of dietary omega-3 consumption on cardiovascular outcomes in the secondary prevention setting was the DART study (1989)[224]. Men admitted to hospital with an acute myocardial infarction were randomised to receive dietary advice to either reduce fat consumption, increase fatty fish or fish oil consumption, or increase fibre consumption. Those in the fish/fish oil group had a 29% reduction in all-cause mortality after 2 years[224].

Dietary studies of omega-3 intake in the primary and secondary prevention setting have demonstrated a lower mortality rate with fish intake that appears to be dose-responsive.

1.5.2 Cardiovascular outcome trials of fish oil

Supplementation with omega-3 fatty acids in the form of fish oil provides a convenient and efficient method of raising blood omega-3 levels. Fish oil is generally well-tolerated with minimal side effects, the predominant being gastrointestinal and an anecdotal increase in bleeding tendency. Studies have evaluated the risk of bleeding with fish oil supplementation, and have demonstrated no clinically significant increase, in a variety of settings[225]. Fish oils are available either as over the counter supplements or as prescription formulations, the latter being typically in the form of carboxylic acids or ethyl esters. Prescription formulations are purported to be superior based on having higher omega-3 concentrations, higher manufacturing standards, and their need for rigorous evaluation of efficacy and safety.

The GISSI-Prevenzione trial (1999) studied the impact of fish oil supplementation on outcomes in patients with a recent myocardial infarction. In this multi-centre trial, patients were randomly assigned to either fish oil alone (900mg of omega-3 per day), vitamin E alone, fish oil plus vitamin E, or no supplement (control) for 6 months. There was a 10% reduction in death, non-fatal myocardial infarction, and stroke seen in the fish oil group compared to the control group[226]. A summary of the major CVOTs of omega-3 fatty acid supplementation is presented in Table 1.2. In the JELIS study (2007), hypercholesterolaemic patients (total cholesterol ≥ 6.5 mmol/L) in Japan were recruited and randomised to either a statin or statin plus EPA (1.8g per day) for a mean duration of 4.6 years. There was a significant, 19% reduction in major coronary events in the EPA + statin group compared to statin therapy alone[227]. The reduction was only significant in patients who had a prior history of coronary artery disease, and was independent of effects on LDL levels. The Alpha Omega Trial (2010) used a much smaller dose of omega-3 fatty acids, as participants were randomised to one of four margarines[228]. Patients with a prior history of myocardial infarction and on state of the art medical therapy, were randomised to consumption of margarines fortified with either EPA

+ DHA, EPA + DHA + ALA, ALA alone, or placebo. The average dose of EPA + DHA was 376mg. There were no significant differences between the groups in the primary endpoint of major cardiovascular events. Although the comparative lack of benefit compared to earlier studies could be attributed to a low omega-3 dose used, this study raised questions about the magnitude of additional benefit that fish oil could provide in the setting of contemporary anti-atherosclerotic therapies. In the OMEGA trial (2010), published in the same year and with a similar population group, i.e. patients with a history of acute myocardial infarction, omega-3 ethyl esters (1g per day) were initiated and compared with placebo (1g of olive oil per day) for 1 year[229]. On top of standard medical therapy, there was no difference between the treatment groups in either sudden cardiac death, total mortality, major adverse cerebrovascular and cardiovascular events, or revascularisation. This trend continued in the ORIGIN trial (2012), where 1g of fish oil (≥ 900mg of omega-3 fatty acids) daily was compared with placebo (1g of olive oil daily), in patients with, or at risk of, diabetes mellitus, and with a previous MI or heart failure[230]. Over a median follow-up period of 6.2 years, there was no significant difference in death from cardiovascular causes between the treatment groups.

These large CVOTs had significant variability in omega-3 doses and preparations used, despite growing interest in the role of the omega-3 dose-effect relationship[231]. Two primary prevention studies in 2018, the ASCEND study and the VITAL study similarly used an intervention dose of 1g of fish oil daily. In the ASCEND study, diabetics without a history of atherosclerotic cardiovascular disease were randomised to either 1g of fish oil per day (840mg of EPA + DHA) or placebo (1g of olive oil per day), and were followed for a median duration of 2.5 years.[232] No significant difference was seen in the primary endpoint of vascular events or vascular death between the treatment groups. In the VITAL study, men of at least 50 years of age, and women of at least 55 years of age, without a history of cardiovascular disease, were randomised to either 1g of fish oil (840mg of EPA + DHA) per day or a matching placebo for

a median period of 5.3 years[233]. Fish oil did not reduce either of the primary outcomes of major adverse cardiovascular events or invasive cancer.

A major shift in fish oil trial outcomes occurred with the REDUCE-IT trial (2018). This study used a much higher dose of omega-3, with 4 grams daily of the prescription EPA form "icosapent ethyl"[234]. The high dose is required for an optimum triglyceride-lowering effect. The higher fish oil dose of 4 grams per day has similarly been used in the ongoing STRENGTH trial[235], in the form of EPANOVA®, which contains mixed omega-3 carboxylic acids. REDUCE-IT compared icosapent ethyl with placebo in patients with established cardiovascular disease or diabetics with other cardiovascular risk factors. Despite statin treatment, the patients were required have a triglyceride level between 1.52 and 5.63 mmol/L and an LDL level between 1.06 and 2.59mmol/L. Icosapent ethyl reduced the primary endpoint of the first episode of a composite of cardiovascular death, nonfatal MI, nonfatal stroke, coronary revascularisation and unstable angina by 25% compared to placebo[234]. It reduced total events at 4.9 years by 30%. Although the success of the higher doses of omega-3 fatty acids used in REDUCE-IT could be considered attributable to beneficial effects on lipid parameters, the difference in rates of the primary endpoint compared to placebo was independent of effects on triglycerides and LDL levels. This suggests an alternative anti-atherosclerotic mechanism of omega-3 fatty acids.

Given the inflammatory nature of atherosclerosis, it is logical to consider that a potential anti-atherosclerotic mechanism of omega-3 fatty acids is the amelioration of vascular inflammation. Indeed, canakinumab, which reduced MACE in the CANTOS study, targets interleukin 1-β, which plays an important role in the pathogenic mechanisms leading to vascular inflammation[236]. Phospholipase A2 (PLA_2), a superfamily of enzymes that liberate free fatty acids such as arachidonic acid from phospholipid membranes, has also been targeted to reduce vascular inflammation. The by-products of arachidonic acid oxidation, eicosanoids,

can induce microvascular dysfunction, oxidative stress and vascular inflammation[237]. Several families of PLA_2 exist, and two of them have been the subject of cardiovascular outcome trials. An inhibitor of secretory PLA_2, varespladib, was compared with placebo in the VISTA-16 trial (2014), which examined its cardioprotective effects in patients with a recent acute coronary syndrome[238]. The trial was stopped early due to futility and possible harm, with a hazard ratio for the primary endpoint of MACE of 1.25 (95% CI, 0.97 – 1.61, p=0.08). In addition, darapladib, an inhibitor of lipoprotein-associated PLA_2, was studied in the STABILITY trial (2014), which compared it to placebo in patients with stable coronary artery disease[239]. Darapladib did not significantly reduce the primary endpoint of MACE (HR 0.94 [95% CI, 0.85 – 1.03]), however did significantly reduce the rate of major and total coronary events by 10% and 9% respectively.

The cardioprotective effects of agents that target vascular inflammation deserve further exploration. In the case of omega-3 fatty acids, this may provide a mechanistic explanation for positive CVOTs.

Table 1.2: Major CVOTs of omega-3 fatty acid supplementation

Trial name and year	Study population	No. of subjects	Intervention and form	Omega-3 dose per day	Comparator	Effect of intervention
GISSI-Prevenzione (1999)[226]	Recent MI	11324	Fish oil (ethyl esters)	900mg	No supplement	HR = 0.90 (95% CI, 0.82-0.99) for MACE
JELIS (2007)[227]	Total cholesterol ≥ 6.5*	18645	EPA (ethyl esters) + statin	1800mg	Statin alone	HR = 0.81 (95% CI, 0.69-0.95) for MACE
Alpha Omega (2010)[228]	Previous MI	4837	Margarine fortified with EPA + DHA	400mg	Margarine without fortification	HR = 1.01 (95% CI, 0.87-1.17) for MACE
OMEGA (2010)[229]	Previous MI	3851	Fish oil (ethyl esters)	1000mg	olive oil	HR = 0.95 (95% CI, 0.56-1.60) for SCD
ORIGIN (2012)[230]	Dysglycemia	12536	Fish oil (ethyl esters)	900mg	olive oil	HR = 0.98 (95% CI, 0.87-1.10) for CV death
ASCEND (2018)[232]	Diabetes mellitus	15480	Fish oil (unspecified)	840mg	olive oil	HR = 0.97 (95% CI, 0.87-1.08) for vascular events
VITAL (2018)[240]	Men ≥ 50, Women ≥ 55	25871	Fish oil (ethyl esters)	840mg	olive oil	HR = 0.92 (95% CI, 0.80-1.06) for MACE
REDUCE-IT (2018)[234]	CVD or diabetes mellitus with trigs 1.52 to 5.63*	8179	Fish oil (ethyl esters)	4000mg	mineral oil	HR = 0.74 (95% CI, 0.65-0.83) for MACE

MI = Myocardial infarction
HR = Hazard ratio
MACE = Major adverse cardiovascular events
CV = Cardiovascular
CVD = Cardiovascular disease
SCD = Sudden cardiac death
* = mmol/L

1.6 Effects of omega-3 fatty acids on acute vascular inflammation

The role of vascular cells during the inflammatory response is critical. Multiple cytokines, leucocytes, and growth factors are present at sites of inflammation, and each of these can potentially influence the nature of the inflammatory response[241]. Endothelial cells and smooth muscle cells must integrate the signals generated by these multiple factors to effectively regulate the immunoinflammatory response through the expression of adhesion molecules, cytokines, chemokines, MMPs, and growth factors. This requires changes in signal transduction, which are mediated through NFκB, JAK/STAT and AP-1 signalling pathways.

Several studies have investigated the impact of omega-3 fatty acids on early (acute) vascular inflammation, with strong evidence for a reduction in adhesion molecule expression based on cell culture studies. For example, the DHA derivative maresin-1 has been shown to downregulate E-selectin expression in cultured endothelial cells stimulated with TNF-α[242]. Conversely, the EPA-derived Resolvin E1 did not alter circulating E-selectin, VCAM-1 or MCP-1 in ApoE*3Leiden mice, although this was in the setting of prolonged high fat feeding which can confound studies of acute inflammation[243]. De Caterina et al added DHA to human saphenous vein endothelial cells in culture stimulated with either TNF-α or IL-1, and demonstrated a reduction in protein expression of VCAM-1 and E-selectin[244]. DHA was not directly compared to EPA for these experiments. Yates et al. made direct comparisons of EPA with DHA and demonstrated a reduction in neutrophil adhesion to TNF-stimulated human umbilical vein endothelial cells (HUVECs) with DHA, but not with EPA[245]. This was achieved through the modulation of E-selectin expression, independent of effects on transcriptional regulation. This was one of the first studies to identify a differential effect of these two fatty acids on acute vascular inflammation. Both DHA and EPA have been shown to suppress protein expression of VCAM-1 and ICAM-1 in LPS-stimulated human aortic endothelial cells (HAECs) in a study by Huang et al, with a greater reduction seen with DHA[246]. The mechanisms were concluded to be (1) inhibition of the translocation of TLR4

into lipid raft domains, (2) suppression of TAK1 phosphorylation, (3) attenuation of NFκB activity by suppressing p38 and IκBα activation, and (4) induction of the expression of the anti-inflammatory and NFκB-suppressor gene A20 for EPA but not DHA[246]. Similar results were published by Wang et al, who reported a reduction in protein expression of VCAM-1 in TNF-stimulated HAECs co-incubated with DHA, and to a much lesser extent, EPA[247]. ICAM-1 was suppressed by DHA only at very high doses, and not at all by EPA. The superior effect of DHA in this study may be related to the modification of the structure and composition of membrane rafts and the membrane bilayer that occurs when PUFAs are incorporated into cell membrane phospholipids[248]. Specifically, DHA incorporates into (sphingomyelin and cholesterol-rich) membrane rafts with more than twice the affinity of EPA, making them more disordered and dysfunctional[249].

Based on the above results of studies of acute vascular inflammation, further investigation into the differential effects of EPA versus DHA is required. Furthermore, despite the results and mechanistic insights that cell culture studies of PUFAs have provided, there remains a significant limitation in the translatability of the results. Specifically, the addition of pure fatty acids to cell culture does not model the conditions that occur *in vivo*. Since PUFAs undergo metabolism and oxidation after oral consumption, the genetic, humoral and cellular responses *in vivo* are likely to be different to what is measured in the cell culture setting. Indeed, oxidised omega-3 fatty acids have different actions to pure unoxidised fatty acids, and are still beneficial, if not superior[250]. Sethi et al demonstrated that oxidised, but not native unoxidised EPA significantly inhibited human neutrophil and monocyte adhesion to endothelial cells *in vitro* by inhibiting endothelial adhesion receptor expression. In transcriptional coactivation assays, oxidised EPA potently activated the peroxisome proliferator-activated receptor α (PPAR-α)[251]. Similarly, Mishra et al demonstrated that oxidised but not unoxidised EPA and DHA inhibit cytokine-induced endothelial expression of MCP-1 and IL-8[252]. In this study, oxidised EPA potently inhibited cytokine-induced activation of NFκB expression (although

this was not caused by prevention of phosphorylation of IκBα as concluded by Huang et al). It is evident that the favourable effects of pure, unoxidised omega-3 fatty acids on acute vascular inflammation identified in a cell culture need to be replicated in a more physiological setting.

1.7 Effects of omega-3 fatty acids on chronic vascular inflammation and atherosclerosis

The continuum of atherogenesis, as outlined in Section 1.1.1, presents a challenge in studying the late stages of atherosclerosis in isolation, i.e. independent of inflammatory processes that have occurred earlier. Nonetheless, it is highly relevant to the translational potential of omega-3 fatty acids, as most of their demonstrated clinical efficacy and ultimately practical utility is in secondary prevention. The vast majority of studies of omega-3 fatty acids in chronic vascular inflammation and atherosclerosis have been in animal models. However, studies of human atherosclerosis have been performed, such as that by Thies et al, who studied the inflammatory composition of plaque after fish oil consumption[253]. Patients awaiting carotid endarterectomy were randomised to receive fish oil or a placebo oil leading up to surgery. The macrophage content of plaques was significant lower in the fish oil group, and higher concentrations of EPA and DHA were found within these plaques[253]. Xu et al measured the burden of atherosclerotic plaque and lipids in the aortas of ApoE-deficient mice fed an atherogenic diet and randomised to the addition of fish oil (1% w/w of diet) or unaltered diet[254]. No difference was seen in either parameter between treatment groups despite high measured circulating omega-3 levels. In this study, the dose of fish oil was low, triglyceride levels were significantly and unexpectedly increased in the fish oil group, and there was no oil control, which were limitations. EPA alone was studied by Matsumoto et al, who measured lipid and plaque burden in the aortas of ApoE-deficient and LDLR-knockout mice who were randomised to EPA (5% of diet by weight) or a standard atherogenic diet[255]. EPA reduced aortic lipid and plaque burden, reduced macrophage accumulation in plaque, increased smooth

muscle cell content, and increased plaque collagen content. These changes were seen in the absence of effects on circulating lipids. No comparison was made with DHA.

Li et al studied the functional aspects of plaque inflammation, including the ability of plaque macrophages to clear apoptotic cells (efferocytosis)[256]. Macrophage efferocytosis was defective in ob/ob;LDL-receptor deficient mice fed an atherogenic diet for 6 weeks, and this was reversed by dietary fish oil supplementation for a further 6 weeks, compared to an olive oil control. Another functional aspect of plaque inflammation, studied by Altenburg et al, is the uptake of oxidised LDL (oxLDL), which is mediated by IFN-γ-induced CXCL16 expression. DHA reduced CXCL16 expression by human aortic smooth muscle cells, measured by flow cytometry[257]. Furthermore, oxLDL uptake was reduced as measured by fluorescent labelling. Foam cell formation, the internalisation of modified lipoproteins such as oxLDL, is also reduced by omega-3 fatty acids. Song et al cultured THP-1 monocytes in oxLDL and phorbol myristate acetate to induce foam cell formation, and added various ratios of EPA to (the omega-6) arachidonic acid[257]. The conditions with the highest omega-3 content had the lowest cholesterol levels detected in foam cells, and a lower concentration of IL6 and TNF-α in the supernatant. DHA was not used in this study. The LPS-induced expression of toll-like receptor 4 (TLR4) on the lipid rafts of murine macrophages (RAW264.7 cells), is a marker of macrophage activation. In *in vitro* studies, both DHA alone and a combination of EPA and DHA reduced TLR4 expression, but EPA alone did not[258].

Further to studies of the functional aspects of plaque inflammation, the regression of atherosclerosis has been studied in LDL-receptor deficient mice by Nakajima et al[259]. After 8 weeks on an atherogenic diet, mice were fed with either a standard chow diet or one with the addition of EPA (5% w/w) for 4 weeks. The mice in the EPA group had a 22% reduction in atherosclerosis burden compared to the chow-fed mice, as well as a reduction in the content of macrophages, CD4+ T cells and dendritic cells in atherosclerotic lesions[259]. No comparison was made with DHA. Although much attention has been focused on EPA, the anti-

atherosclerotic effects of DHA were studied by Tan et al, by esterifying phytosterols with DHA[260]. In ApoE-deficient mice fed an atherogenic diet with or without phytosteryl DHA added (2% w/w) for 7 weeks, those in the DHA group had lower plasma cholesterol levels and three times smaller atherosclerotic lesions. No comparison was made with EPA.

A study comparing the effects of EPA and DHA in different ratios on atherosclerosis in ApoE-deficient mice did not demonstrate a clear difference in plaque or lipid burden in atherosclerotic aortas[261]. Nor was there a difference in markers of oxidative stress, markers of acute and chronic inflammation, or oxLDL uptake. Markers of chronic inflammation included IL1-β, IL6 and TNF-α. Takashima et al compared EPA with 1:1 combinations of EPA and DHA at different doses[258]. ApoE-deficient mice fed an atherogenic diet had the burden and characteristics in the aortic arch quantified after randomisation to either a control atherogenic diet, a diet supplemented with 5% EPA (w/w), one supplemented with 2.5% EPA + DHA (w/w), or one supplemented with 5% EPA + DHA (w/w). The latter group had the lowest burden of plaque and the highest plaque stability, which was significantly different to all other groups. The results of these studies of chronic inflammation and atherosclerosis demonstrate differential and beneficial effects of EPA and DHA. Despite much attention being focused on the anti-atherosclerotic effects of EPA, these studies highlight the potential benefits of combination therapy, which may be synergistic and/or take advantage of their unique mechanisms of action.

1.8 Areas for further investigation

1.8.1 Methodological considerations

The unique anti-inflammatory and anti-atherosclerotic mechanisms of action of EPA and DHA are yet to be fully elucidated, and there is evidence that different EPA/DHA combinations produce divergent effects. To further evaluate the effects of omega-3 fatty acids on acute vascular inflammation, it is prudent to first establish the effects of individual fatty acids. Modelling AVI *in vitro* requires a physiologically-appropriate inflammatory stimulus

applied to vascular endothelial cells. Omega-3 fatty acids, being non-polar molecules, do not dissolve readily in aqueous solutions such as cell culture media, and hence are commonly bound to albumin (with polar and non-polar sites), and subsequently ethanol (polar) before use in cell culture experiments. This process is not only highly challenging, but is temperature-sensitive, is not physiological, and produces variable results. Studies of AVI performed *in vitro* should ideally use systems whereby fatty acids are presented to cells in a form that replicates the *in vivo* setting.

Studies of AVI performed *in vivo* such as animal studies, must use models of pure acute vascular inflammation that do not induce atherosclerosis. For animal studies, omega-3 fatty acids are typically added to diet. This strategy results in animal-to-animal variations in both total omega-3 fatty acid consumption as well as total fatty acid oxidation. Furthermore, there is no adjustment for weight. A more accurate method that mimics human fish oil consumption, is to provide precise quantities of unoxidised fish oil directly to animals by oral gavage. An important consideration for human studies is ensuring adequate dosing. The studies quoted above have demonstrated the dose-dependent nature of the effects of omega-3 fatty acids, with oral doses directly correlating with blood and tissue concentrations[262-264]. At present, there is no upper limit for omega-3 dosing in humans, and dosing up to 5 grams per day is considered safe by the European Food Safety Authority[265].

1.8.2 Knowledge gap analysis

The *in vitro*, animal, and human studies of fish oil have used highly heterogeneous omega-3 preparations and dosing, leading to inconsistencies in results. With emerging evidence of differential mechanistic effects of EPA and DHA, it has become imperative to identify optimal preparations, either in combination or isolation, for specific indications. Currently, there is no clearly superior omega-3 fatty acid for the amelioration of acute vascular inflammation. The beneficial effects of EPA demonstrated in the JELIS study led to a focus on EPA for studies of atherogenesis, without sufficient comparisons with DHA or combinations.

There is a paucity of data on the effects of individual fatty acids on the nature as well as total burden of atherosclerotic plaque. Their impact on the inflammatory characteristics, stability, and lipid content of plaque need further evaluation. Since almost 10% of whole body omega-3 fatty acids are circulating in the bloodstream, it is likely that an important component of their anti-atherosclerotic effects is on circulating factors. It is important, therefore, to establish their effects on circulating atherosclerotic mediators.

1.9 Aims of research study

The aims of this body of work are:

1. To systematically review the literature on the impact of omega-3 fatty acids on the circulating mediators of atherosclerosis. These include atherogenic lipoproteins, inflammatory cytokines and adipokines, atherogenic amino acids and derivatives, and advanced glycation end products.
2. To determine the effects of omega-3 fatty acids on acute vascular inflammation in an *in vitro* model using serum from healthy volunteers supplemented with fish oil. Comparisons will be made between fish oils with different EPA:DHA ratios.
3. To determine the impact of individual omega-3 fatty acids on acute vascular inflammation in an *in vivo* animal model.
4. To determine the impact of individual omega-3 fatty acids on chronic vascular inflammation and atherosclerosis in an *in vivo* animal model.

1.10 Hypotheses

The overarching hypotheses for this examination of the impact of omega-3 fatty acids on the inflammatory mediators of atherosclerosis are:

1. Fish oils in various preparations and doses reduce levels of the established circulating mediators of atherosclerosis.

2. Omega-3 fatty acids that are delivered to cultured endothelial cells in a physiological manner will reduce markers of acute vascular inflammation measured at the gene level after cellular stimulation.
3. Omega-3 fatty acid pre-treatment reduces the protein expression of markers of acute vascular inflammation in an animal model.
4. In an animal model of atherosclerosis, omega-3 fatty acid supplementation reduces plaque lipid burden, the vulnerability and inflammatory cell content of plaque measured histologically and at the protein level, and reduces chronic vascular inflammation at the gene level, independent of changes in blood lipid levels.
5. EPA has a superior effect compared to DHA on ameliorating acute and chronic vascular inflammation and atherogenesis.

1.11 Outline of thesis

Chapter 1: Introduction and literature review

Chapter 2: General methods

Chapter 3: The impact of omega-3 fatty acids on circulating mediators of atherosclerosis – A systematic review

Chapter 4: Fish Oil Cell Uptake Study of INflammation (FOCUS IN). A randomised controlled trial of fish oil supplementation in healthy volunteers

Chapter 5: The impact of omega-3 fatty acids on acute vascular inflammation in a mouse model

Chapter 6: The impact of omega-3 fatty acids on atherosclerosis and chronic vascular inflammation

Chapter 7: Discussion

CHAPTER 2: GENERAL METHODS

2.1 Isolation of serum and plasma from whole blood

Whole blood was collected in either clot-activating collection tubes (for serum) or ethylenediaminetetraacetic acid (EDTA) tubes (for plasma). For serum isolation, blood was allowed to sit undisturbed for 30 mins at room temperature for clotting to occur, following by either immediate centrifugation or storage at 4ºC until centrifugation could be performed, which was no more than 4 hours in all cases. Blood collected for plasma isolation was stored at 4ºC for no more than 4 hours and then centrifuged. In both cases, centrifugation was performed at 1900 x *g* for 15 mins at 4ºC. Supernatant was aspirated using a pipette and immediately stored at -80ºC.

2.2 Fatty acid analysis by dry blood spot

Whole blood was spotted onto pre-made dry blood spot (DBS) cards for fatty acid analysis. DBS cards were hand-made using cardboard and PUFACoat paper (developed by Dr. Ge Liu and Professor Robert Gibson at the FoodPlus Research Centre, University of Adelaide)[266]. PUFACoat paper stabilises fatty acids in biological samples for at least 9 weeks when stored at room temperature, and for at least 18 months when stored at -20ºC. Immediately after blood collection, 30 µl of whole blood was pipetted directly onto the PUFACoat paper, and the DBS cards were then stored in sealed foil bags with desiccant at -20ºC prior to processing.

2.2.1. Preparation of DBS cards

PUFACoat paper was created as follows. Using a sonicator, 70 ml of pure ethanol was mixed with 200 mg of butylated hydroxytoluene (BHT) in a beaker. Then 3 ml of 0.5 M EDTA was added to 27 ml of distilled water, and brought to a pH of 8.0. This solution was then added to the ethanol/BHT solution, and sonicated for 5 mins. Filter paper cut to size was dipped into this final solution and allowed to air-dry. This paper was stapled to thin cardboard with holes punched out that allow blood to be spotted on to the paper underneath.

2.2.2. Fatty acid extraction from DBS specimens by transmethylation

Forceps and scissors were first sonicated in isopropanol and then dried. The blood-stained area of the PUFACoat paper was cut out and placed in 5 ml capped scintillation vials filled with 2 ml of 1% H_2SO_4. The vials were then heated to 70ºC for 2.5 hours. After the first 30 mins, the caps were released briefly to expel gas and the vials were vortexed. After each subsequent 30-min period the vials were vortexed only. The vials were allowed to cool to room temperature, and then 250 µl of distilled water and 700 µl of heptane were added to the vials, which were then vortexed. Using a Pasteur pipette, the top layer was transferred to a gas chromatography (GC) vial. The GC vial was sealed and then stored at -20ºC until GC analysis was performed.

2.2.3 Gas chromatography analysis

Gas chromatographic analysis was performed semi-quantitatively for the following fatty acids:

Saturated fatty acids

C14:0 (myristic acid)
C15:0 (pentadecylic acid)
C16:0 (palmitic acid)
C17:0 (margaric acid)
C18:0 (stearic acid)
C20:0 (arachidic acid)
C22:0 (behenic acid)
C24:0 (lignoceric acid)

Trans-fatty acids

t16:1ω-7 (trans-palmitoleic acid)
t18:1ω-7 (trans-vaccenic acid)
t18:1ω-9 (trans-oleic acid)
t18:2ω-6 (trans-linoleic acid)

Monounsaturated fatty acids

16:1ω-7 (palmitoleic acid)
18:1ω-7 (vaccenic acid)
18:1ω-9 (oleic acid)
20:1ω-9 (eicosenoic acid)
22:1ω-9 (erucic acid)
24:1ω-9 (nervonic acid)

Polyunsaturated fatty acids

18:3ω-3 (alpha-linolenic acid)
20:5ω-3 (eicosapentaenoic acid)
22:5ω-3 (docosapentaenoic acid)
22:6ω-3 (docosahexaenoic acid)
18:2ω-6 (linoleic acid)
18:3ω-6 (gamma-linolenic acid)
20:2ω-6 (eicosadienoic acid)
20:3ω-6 (dihomo-gamma-linolenic acid)
20:4ω-6 (arachidonic acid)

Results were expressed as a percentage of total specimen fatty acid content. GC analysis was performed using a Hewlett-Packard 6890 system (Palo Alto, CA, USA) equipped with a BPX70 capillary column 50 m × 0.32 mm, film thickness 0.25 μm (SGC Pty Ltd., Victoria, Australia), programmed temperature vaporisation injector and a flame ionisation detector (FID). The injector temperature was set at 250 °C and the FID temperature at 300 °C, a programmed temperature ramp (140–240 °C) was used. Helium gas was utilised as a carrier at a flow rate of 35 cm per second in the column and the inlet split ratio was set at 20:1. Quantification was achieved by comparing the retention times and peak area values of unknown samples to those of commercial lipid standards (Nu-Chek Prep Inc., Elysian, MN, USA) using the Hewlett-Packard Chemstation data system.

2.3 Preparation and maintenance of human umbilical vein endothelial cells (HUVECs)

HUVECs were obtained from fresh umbilical cords donated by the Women's and Children's Hospital, North Adelaide. They were cultured and used up to passage 5. For culture, HUVECs were plated in gelatin-coated flasks at a density of 10000 cells per cm^2. For general culture and experiments requiring serum, HUVECs were grown in MesoEndo Cell Growth Medium (Cell Applications, San Diego, CA, USA) supplemented with an extra 5% foetal bovine serum (FBS) to make a total of 10% FBS. For experiments requiring serum-free media, EBM-2 basal media plus SingleQuot kit supplements and growth factors (Lonza, Basel, Switzerland), with serum omitted, were used. Cells were cultured in incubators at 37ºC with 5% CO_2. Cells were passaged when they reached 90% confluency, and experimental conditions were added when cells were 80% confluent. Cells were passaged using Accutase® (BD Biosciences, Franklin Lakes, NJ, USA) by first washing the cells with room-temperature phosphate-buffered saline (PBS), then adding room-temperature Accutase®. Once cells were fully detached, Accutase® was neutralised with warm media. Centrifugation was performed at 220 x *g* for 5 mins, and after supernatant was removed, cells were resuspended in growth media. Cells were counted using Trypan blue exclusion. For experiments, HUVECs were cultured in 6-well plates.

2.4 Polymerase Chain Reaction

Reverse transcription polymerase chain reaction (RT-PCR) was performed after extraction of ribonucleic acid (RNA) from either cultured cells or tissues, making complementary deoxyribonucleic acid (cDNA), and then amplification and semi-quantification of DNA. RNA extraction from cells was performed using either a Bio-Rad (Hercules, CA, USA) Aurum™ Total RNA Mini Kit or using the TRI Reagent® method. RNA extraction of tissues was performed using the Qiagen (Venlo, Netherlands) AllPrep DNA/RNA/Protein Mini Kit.

2.4.1 RNA Extraction from cultured cells using the Bio-Rad Aurum™ Total RNA Mini kit

Cell culture media was aspirated, and then cells were washed with PBS at room temperature. Cells were detached from culture plates using room-temperature Accutase® (BD Biosciences, Franklin Lakes, NJ, USA). After neutralisation of Accutase® with warm media, cell solutions were centrifuged at 220 x *g* for 5 mins at 4ºC. Supernatant was aspirated, and then cells were immediately processed for RNA extraction using the Bio-Rad (Hercules, CA, USA) Aurum™ Total RNA Mini Kit. The Spin Protocol was followed as per the Instruction Manual (Catalogue # 732-6820). In summary, cell pellets were treated with the lysis solution and then 70% (w/v) ethanol. Subsequently, cell solutions were added to an RNA binding column, and high-speed centrifugation was performed. The RNA binding column was washed with low stringency and high stringency wash solutions, and then 40 µl of elution solution was added to the RNA binding column for 1 min, followed by high-speed centrifugation. The eluted RNA solution was quantified using the NanoDrop™ 8000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA was either used immediately for cDNA synthesis or stored at -80ºC for later use.

2.4.2 RNA extraction from cultured cells using the TRI-Reagent method

The media from cultured cells was aspirated, and then the cells were washed with 1 ml of cold (4ºC) PBS. After the PBS was aspirated, 500 µl of TRI Reagent® (Sigma-Aldrich, St. Louis, MO, USA) was added to each well of a 6-well plate and immediately frozen at -80ºC. After thawing at room temperature, cells were scraped off of 6-well plates using a cell scraper, and the cell/TRI reagent solution was transferred to sterile 1.5 ml microcentrifuge tubes. One tenth of the TRI reagent volume (50 µl) of 1-Bromo-3-chloropropane (Sigma-Aldrich, St. Louis, MO, USA) was added, and the mixture was vortexed for 15 seconds, ensuring complete mixing of both phases. The solution was centrifuged for 15 mins at 19000 x *g* at 4ºC. The aqueous phase was transferred to another sterile 1.5 ml microcentrifuge tube, and 250 µl of

isopropanol was added. This solution was transferred to a -20ºC freezer for RNA precipitation. At least 24 hours later, the solution was vortexed, and centrifuged at 19000 x *g* at 4ºC for 15 mins. The supernatant was removed using a pipette, and the RNA pellet was then washed by adding 250 µl of ice-cold 75% ethanol. The solution was vortexed and centrifuged at 19000 x *g* for 10 mins at 4ºC. The ethanol was removed, and the RNA pellet was air-dried for 10 mins. Pre-warmed (60ºC) nuclease-free water (20 µl) was added to the RNA pellet, followed by vortexing and brief centrifugation. The RNA solution was kept on ice until RNA quantification was performed, using the NanoDrop 8000 spectrophotometer. RNA was stored at -80ºC until use.

2.4.3 RNA, DNA, and protein extraction from tissues using the Qiagen AllPrep DNA/RNA/Protein Mini Kit

RNA was extracted from tissues (mouse aortas) using the Qiagen AllPrep DNA/RNA/Protein Mini kit, and DNA and protein were extracted simultaneously. The protocol followed was from the kit's handbook. In summary, snap-frozen mouse aortas were thawed on ice and then transferred to a 1.5 ml microcentrifuge tube. "Buffer RLT" (600 µl) was added to tube, and then the aortas were homogenised using a Precellys24 Homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France). The lysate was centrifuged for 3 mins at full speed, and the supernatant was then transferred to an AllPrep DNA spin column placed in a 2 ml microcentrifuge tube. The tube was centrifuged for 30 seconds at >8000 x *g*, and the spin column was placed in a new 2ml collection tube and stored at 4ºC for later DNA purification. The flow-through was used for RNA purification.

Pure ethanol (430 µl) was added to the flow-through, and mixed well by pipetting. Up to 700 µl of sample was transferred to an RNeasy spin column placed in a 2 ml collection tube, which was then centrifuged for 15 seconds at >8000 x *g*. The flow through was transferred to a 2 ml collection tube for later protein purification. The RNeasy spin column was washed by adding 700 µl of "Buffer RW1" and centrifuging at >8000 x *g* for 15 seconds. After discarding

the flow-through, 500 µl of "Buffer RPE" was added to the spin column, centrifugation was performed for 15 seconds at >8000 x *g*, and then this was repeated with a 2-minute spin. The RNeasy spin column was transferred to a new 1.5 ml microcentrifuge tube, and 30 µl of pre-warmed (70^{o}C) nuclease-free water was added to the spin column membrane. After 5 mins, centrifugation was performed for 1 min and > 8000 x *g* to elute the RNA. The RNA was then quantified using the NanoDrop 8000 Spectrophotometer, and stored at -80^{o}C until future use.

Protein purification was performed by adding 1000 µl of "Buffer APP" to the flow-through collected earlier. The solution was mixed vigorously and thoroughly by vortexing, and incubated for 10 mins at room temperature to precipitate protein. The solution in the 2 ml collection tube was centrifuged at full-speed for 10 mins, and the supernatant was discarded. Following this, 500 µl of 70% (w/v) ethanol was added to the protein pellet, centrifugation at full speed for 1 minute was performed, and the supernatant was again discarded. The protein pellet was dried for 10 mins at room temperature, then 250 µl of "Buffer ALO" and 10 µl of 8 M urea were added. The protein pellet solution was dissolved by prolonged vortexing and heating to 95 degrees for 5 minutes using an Eppendorf Thermomixer (Hamburg, Germany). The solution was cooled to room temperature and centrifuged for 1 min at full speed, and the protein solution and residual pellet were stored immediately at -80^{o}C.

For genomic DNA purification, 500 µl of "Buffer AW1" was added to the AllPrep DNA spin column, which was then centrifuged for 15 seconds at >8000 x *g*. This step was repeated with 500 µl of "Buffer AW2", with 2 mins of centrifugation. The AllPrep DNA spin column was placed in a sterile 1.5 ml microcentrifuge tube, and then 100 µl of prewarmed (70^{o}C) nuclease-free water was added to the spin column membrane. After 5 mins, centrifugation was performed for 1 min at > 8000 x *g*. The eluted DNA was stored at 4^{o}C.

2.4.4. Conversion of RNA to complementary DNA (cDNA)

Prior to conversion to cDNA, all RNA samples to be used for the same experiment were normalised to the same concentration. RNA was converted to cDNA using iScript™ Reverse Transcription Supermix for RT-PCR (Bio-Rad, Hercules, CA, USA). A desired quantity of RNA for the cDNA synthesis reaction was determined. RNA was thawed on ice, and the required volume was added to iScript RT Supermix, which comprised 20% of the total reaction mix (i.e. 4 µl iScript for a 20 µl cDNA reaction). The remaining volume was nuclease-free water. The mix was vortexed and centrifuged, and then incubated in a thermal cycler (T100™ Thermal Cycler, Bio-Rad) using the following protocol: priming – 5 mins at 25ºC, reverse transcription – 30 mins at 42ºC, and reverse transcription inactivation – 5 mins at 85ºC. cDNA was stored at 4ºC until further use.

2.4.5 Reverse Transcription Polymerase Chain Reaction

RT-PCR was performed using a Bio-Rad CFX Connect™ Real-Time PCR Detection System. Reactions were 20 µl in volume and were performed in 96-well plates. Reaction mixes consisted of 10 µl of Bio-Rad SsoAdvanced™ Universal SYBR® Green Supermix, 6 µl of nuclease-free water, 1 µl of forward primer, 1 µl of reverse primer, and 2 µl of cDNA. The reaction protocol was set at: 50ºC for 2 mins, 95ºC for 15 mins, then 40 cycles of: 94ºC for 15 seconds, 60-64ºC (primer-specific based on optimisation) for 30 seconds, 72ºC for 30 seconds, then 65ºC to 95ºC at 0.5ºC increments for 5 seconds each. Primer stocks were diluted to a concentration of 100 µM, and the working solution was 10 µM. All RT-PCR reactions were performed with reference genes (B2M for human, and 36B4 for mouse).

2.5 Primers for Polymerase Chain Reaction

For RT-PCR experiments, the primers used are listed in Table 2.1 below. All primers were optimised prior to use in experiments.

Table 2.1: List of human primers used in RT-PCR experiments

Human primers	**5' to 3' sequence**
Vascular Cell Adhesion Molecule 1 (VCAM-1) Forward	AAGGCAGGCTGTAAAAGAATTGC
Vascular Cell Adhesion Molecule 1 (VCAM-1) Reverse	AGGTCATGGTCACAGAGCCACC
Intercellular Adhesion Molecule 1 (ICAM-1) Forward	CAGAGTTGCAACCTCAGCCT
Intercellular Adhesion Molecule 1 (ICAM-1) Reverse	GGACACAGATGTCTGGGCATT
Monocyte Chemoattractant protein 1 (MCP-1) Forward	GATCTCAGTGCAGAGGCTCG
Monocyte Chemoattractant protein 1 (MCP-1) Reverse	TGCTTG TCCAGGTGGTCCAT
Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells p65 Subunit (NFkBp65) Forward	ACTGCCGGGATGGCTTCTAT
Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells p65 Subunit (NFkBp65) Reverse	AGGTCCCGCTTCTTCACACA
Beta-2 microglobulin (B2M) Forward	GAGTATGCCTGCCGTGTGAAC
Beta-2 microglobulin (B2M) Reverse	CCAATCCAAATGCGGCATCTTC
Peroxisome Proliferator-Activated Receptor Gamma (PPAR-γ) Forward	CACAATGCCATCAGGTTTGG
Peroxisome Proliferator-Activated Receptor Gamma (PPAR-γ) Reverse	GCTGGTCGATATCACTGGAGATC

Table 2.2: List of mouse primers used in RT-PCR experiments

Mouse primers	5' to 3' sequence
Interleukin 1-beta (Il-1beta) Forward	TGGATGCTCTCATCAGGACAG
Interleukin 1-beta (Il-1beta) Reverse	GAAATGCCACCTTTTGACAGTG
Tumour necrosis factor alpha (TNF-α) Forward	CAGGCGGTGCCTATGTCTC
Tumour necrosis factor alpha (TNF-α) Reverse	CGATCACCCCGAAGTTCAGTAG
Monocyte chemoattractant protein 1 (MCP-1) Forward	GCTGGAGCATCCACGTGTT
Monocyte chemoattractant protein 1 (MCP-1) Reverse	ATCTTGCTGGTGAATGAGTAGCA
Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells p65 Subunit (NFkBp65) Forward	ATTGCTGTGCCTACCCGAAA
Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells p65 Subunit (NFkBp65) Reverse	GATGCTGGGAAGGTGTAGGG
Peroxisome Proliferator-Activated Receptor Gamma (PPAR-γ) Forward	CACAATGCCATCAGGTTTGG
Peroxisome Proliferator-Activated Receptor Gamma (PPAR-γ) Reverse	GCTGGTCGATATCACTGGAGATC
Ribosomal protein, large, P0 (Rplp0), a.k.a 36B4 Forward	CAACGGCAGCATTTATAACCC
Ribosomal protein, large, P0 (Rplp0), a.k.a 36B4 Reverse	CCCATTGATGATGGAGTGTGG
Nucleotide-Binding Domain, Leucine-Rich-Containing Family, Pyrin Domain-Containing-3 (NLRP3) Forward	ATCAACAGGCGAGACCTCTG
Nucleotide-Binding Domain, Leucine-Rich-Containing Family, Pyrin Domain-Containing-3 (NLRP3) Reverse	GTCCTCCTGGCATACCATAGA

2.6 Mouse husbandry

Mice were housed and managed at the South Australian Health and Medical Research Institute (SAHMRI), in accordance with the Australian code for the care and use of animals for scientific purposes (National Health and Medical Research Council (2013) Australian code for the care and use of animals for scientific purposes, 8th edition. Canberra: National Health and Medical Research Council)[267]. Mice were housed in cages with siblings of the same gender. There were up to 5 mice per cage. Mice were monitored daily. C57Bl/6 mice and ApoE$^{(-/-)}$ mice on a C57Bl/6 genetic background were obtained from The Jackson Laboratory (ME, USA). Homozygous ApoE deficiency was confirmed on-site using the Genetic Engineering and

Archiving Services (GENEAS) genotyping service. Mice were given food and water ad-libitum. C57Bl/6 mice were fed a standard rodent chow diet (Teklad Global 18% Protein Rodent Diet [Harlan Laboratories, Madison, WI, USA]). This diet contains 18.6% protein, 6.2% fat, 44.2% carbohydrates, and no cholesterol. ApoE$^{(-/-)}$ mice were fed the Teklad Global 18% Protein Rodent Diet until 8 weeks of age, and were then fed a high cholesterol, high fat diet (SF00-219) from Specialty Feeds (Glen Forrest, Western Australia). This diet consists of 22% fat and 0.15% cholesterol. This diet has previously been demonstrated by our group to significantly increase plasma cholesterol levels and lead to accelerated atherosclerosis development[268]. Ethics approval for all animal work was obtained from both the SAHMRI Animal Ethics Committee and the University of Adelaide Animal Ethics Committee.

2.7 Oral gavaging of mice

The equipment used to gavage mice orally with either EPA, DHA, or olive oil included three 50 µl SGE GT LL syringes (SGE Analytical Science Pty Ltd, Ringwood, Victoria, Australia), three stainless steel 20-gauge ball-tipped animal feeding needles (Sigma-Aldrich catalogue number CAD7902 (Sigma-Aldrich, St. Louis, MO, USA), sterile gauze, water, 70% w/v ethanol, and disposable gloves. The EPA and DHA used for the study of acute vascular inflammation were >99% pure free fatty acids, purchased from Nu-Chek Prep, Inc (Elysian, MN, USA). The EPA and DHA used for the study of atherosclerosis and chronic inflammation were >97% pure ethyl esters donated by Bizen Chemical Co. (Okayama, Japan). The Olive Oil used for all experiments was Bertolli Olive Oil (Florence, Italy). Each needle and syringe pair was used to deliver a single liquid, either EPA, DHA, or olive oil, and there was no cross-contamination.

Mice were gavaged in accordance with the SAHMRI Standard Operating Procedure (SOP) (SOP9/098). The animal feeding needle was attached to the syringe, and the required volume was slowly drawn up. The mouse was scruffed with the thumb and index finger, and the middle finger was used to pull the head back to the neutral position. When the oropharynx

and body were aligned and the mouse was still, the feeding needle was advanced into the oropharynx, and into the oesophagus. The liquid was delivered, and the mouse was returned to its cage and monitored for any signs of distress that may represent aspiration. The animal feeding needle was flushed and rinsed with 70% ethanol, and then flushed and rinsed with tap water. After drying, the needle was used for the next mouse.

2.8 Superficial facial vein bleeding (submandibular cheek bleeding) in mice

Superficial facial vein bleeding was performed using sterile 5 mm Goldenrod (Beacon Falls, CT, USA) animal lancets, following the SAHMRI Standard Operating Procedure (SOP) (4/087). In brief, the volume of blood to be taken without rehydration was determined beforehand based on weight, in accordance with the SAHMRI SOP (4/086), with one drop of blood being estimated to be 50 µl. No more than 150 µl was taken from any mouse at any time. Mice were restrained by scruffing at the neck, using the thumb and index finger. The location of the inferior branch of the superficial facial vein was determined as lying underneath the visible sebaceous gland near the jaw. The intended puncture site was the common superficial facial vein. This was estimated to be 2 mm superior and 2 mm posterior to the sebaceous gland. A single pass was made with a lancet to elicit venous blood, which was collected for plasma into Sarstedt (Nümbrecht, Germany) Microvette® 500 K3E 500 µl EDTA collection tubes. After the predetermined blood volume was taken, pressure was applied with gauze until haemostasis was achieved. Mice were observed for ill-health for 10 mins after this procedure, and then returned to their cages if well. Blood was stored at 4^{o}C until centrifugation, plasma isolation, and plasma storage at -80^{o}C.

2.9 Mouse general anaesthesia

Prior to general anaesthesia, mice were pre-oxygenated with 100% oxygen for 5 mins in a closed chamber. Induction of general anaesthesia was performed using 3% isoflurane plus supplemental oxygen delivered in the same closed chamber. Anaesthesia was confirmed using the toe-pinch test. Maintenance anaesthesia was achieved with 1.5% isoflurane plus

supplemental oxygen, delivered via a nose cone. To minimise dry eye from the nose cone, eye drops were administered to both eyes immediately after induction. After surgery, isoflurane was ceased, and 100% oxygen was delivered for 5 mins. Mice were allowed to recover alone in pre-warmed (37^{o}C) recovery boxes, and when behaviour and activity levels returned to normal they were returned to their cages.

2.10 Mice humane killing and terminal cardiac puncture

Mice were humanely killed under general anaesthesia by both terminal cardiac puncture and vital organ harvesting (heart, lungs and major blood vessels). After anaesthesia was confirmed, betadine was applied to the chest and abdomen, and terminal cardiac puncture was performed using a 25-gauge needle attached to a 1 ml syringe. The puncture was made 1 mm left of the midline, below the ribcage. The needle was advanced until flashback was seen, and after slight further advancement, complete exsanguination was performed. The heart, lungs, and major blood vessels were removed routinely, and isoflurane was continued for the entire procedure.

2.11 Cholesterol and triglyceride assays in mice

Plasma total cholesterol and triglyceride levels in mice were measured by colorimetric assays, using LabAssay™ Cholesterol and LabAssay™ Triglyceride kits (Wako Pure Chemical Industries, Osaka, Japan). The assays were performed in 96-well plates. Plasma samples of 2 µl were added to 300 µl of chromogen reagent, and concentrations were calculated after generating a standard curve from standards of known concentrations. Plates were mixed and incubated for 5 mins at 37^{o}C. They were then read using a GloMax® Discover microplate reader (Promega Corporation, Madison, Wisconsin, USA) using a main wavelength of 600 nm and a subtracted wavelength of 700 nm.

2.12 Assessment and quantification of plaque burden, collagen content and medial expansion

Mouse specimens of brachiocephalic artery and heart were stored in 10% neutral-buffered formalin after harvesting, followed by dehydration, clearing and embedded in paraffin blocks. Specimens were cut at the level of the proximal brachiocephalic artery and the aortic sinus into 5 μm sections using a microtome, and then mounted onto silane-coated slides. The slides were then deparaffinised and rehydrated and subsequently stained with either haematoxylin and eosin (H&E) for plaque quantification and assessment of medial thickness, or the Masson's trichrome stain for assessment of collagen content. Slides were mounted, coverslipped and photographed using the Nanozoomer C9600-12 slide scanner (Hamamatsu Photonics, Shizuoka, Japan). Image resolution was 228 nm per pixel. Images were saved uncompressed and then converted to .tiff files using ImagePro Premier 9.1 (Rockville, MD, USA) for analysis using ImageJ 1.51 (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA). To quantify plaque area, the total artery area or aortic sinus area was measured using the Freehand selection tool. The area of plaque was then measured and expressed as a percentage of total artery or aortic sinus area. The thicknesses of the intimal and medial layers were measured using the Freehand selection tool, and expressed as a percentage of total artery area ("intimal and medial thickness"). The collagen content of plaque in the brachiocephalic artery or aortic sinus was quantified using a macro in ImageJ 1.51 published by Kennedy DJ et al[269]. The macro is detailed below:

```
run("Brightness/Contrast..."); set brightness and contrast to automatic
run("RGB Stack"); convert RGB image to a stack
run("Convert Stack to Images"); split stack to three images, Red, Blue and Green
```

run("Image Calculator...", "image1=Blue operation=Divide image2=Red create 32-bit"); divide blue by red

run("Brightness/Contrast..."); set brightness and contrast to automatic

run("Subtract...", "value=1.2"); discard all image where blue intensity is not 120% of red intensity

run("Multiply...", "value=10000000"); convert all decimal values to integers

run("Brightness/Contrast..."); set brightness and contrast to automatic

run("Max...", "value=1"); set max value to be 1

run("Min...", "value=0"); set min value to be 0

run("Measure"); add up 1s and express as fraction of total area

The collagen content was hence expressed as a percentage of total plaque area.

2.13 Immunohistochemical staining

Immunohistochemical staining of mouse specimens was performed using the Avidin-Biotin peroxidase technique. Harvested organs were initially stored in 10% neutral-buffered formalin for fixation, followed by dehydration, clearing and embedding in paraffin blocks. Glass slides were coated with silane. Thermo Scientific Menzel X72 SuperFrost® Plus (Thermo Fisher Scientific, Waltham, MA, USA) slides were loaded onto racks and rinsed in 100% ethanol. They were dipped in a silane solution comprised of a 1 in 50 dilution of 3-aminopropyl-triethoxy-silane in 100% ethanol, and subsequently rinsed in 100% ethanol and then deionised water before being dried. Specimens were cut into 5 µm sections using a microtome, and placed in a waterbath filled with deionised water, and then mounted onto silane-coated slides. The slides were dried in a 56^{o}C oven for 1 hour.

To deparaffinise and rehydrate the slides, they were heated to 70ºC for 10 mins and then sequentially incubated twice in 100% xylene for 3 mins each time, and then sequentially incubated twice in 100% ethanol for 3 mins each time. Endogenous peroxidase was then blocked by incubating slides in 0.5% hydrogen peroxide in methanol for 30 mins at room temperature. The slides were then rinsed twice in PBS for 5 mins each time, and then placed in the antigen retrieval buffer (10 mM sodium citrate buffer, pH 6). This was heated in a microwave on the highest setting until the solution boiled. The solution was then transferred to a second, calibrated microwave, which heated the solution to 98ºC for 10 mins. The solution was allowed to cool at room temperature. This was followed by two sequential PBS washes for 5 mins each. Using a Pasteur pipette, the specimens were coated with 3% normal horse serum (NHS) for 30 mins. The NHS was drained from the slides, and specimens were then coated with the primary antibody at a concentration determined by prior optimisation. Incubation occurred overnight. The following day the slides were rinsed twice with PBS for 5 mins each time, and then the specimens were coated with the secondary antibody for 30 mins at a concentration determined by prior optimisation. The slides were washed twice in PBS for 5 mins each time. The tertiary antibody, a 1:1000 streptavidin peroxidase:NHS solution, was added to the specimens for 60 mins. The specimens were washed twice in PBS for 5 mins each time. A 75 mg/ml of 3,3'-diamino benzidine (DAB)/PBS solution was added to the specimens for exactly 7 mins, and then the slides were thoroughly rinsed in running water for 10 mins. The DAB was subsequently neutralised by adding an equal volume of 0.2 M potassium permanganate and the same volume of 2.0 M sulphuric acid. The mixture was allowed to stand overnight, and then 5% (w/v) of ascorbic acid was added until the colour disappeared. The solution was then discarded. The slides were counterstained in haemotoxylin and coverslipped using distyrene, plasticiser and xylene (DPX) Mounting Media (Labworks, Victoria, Australia) and Deckgläser 24 x 50mm cover slips (Menzel-Gläser GmbH, Braunscheig, Germany).

2.14 Antibodies used for immunohistochemical staining

Table 2.3: Antibodies used for mouse acute vascular inflammation study

Company and catalogue no.	Name	Host	Reactivity	Conjugation	Clonality	Isotype
Primary antibodies						
Abcam - ab134047	Anti-VCAM1 antibody	rabbit	Mouse, rat, human	Unconjugated	Monoclonal	IgG
Abcam - ab119871	Anti-ICAM1 antibody	rat	Mouse	Unconjugated	Monoclonal	IgG2b
Abcam - ab8101	Anti-MCP1 antibody	rat	Mouse	Unconjugated	Monoclonal	IgG1
Abcam - ab119830	Anti-CD18 antibody	rat	mouse	Unconjugated	Monoclonal	IgG2A
Isotype controls						
Abcam - ab18450	Rat IgG2A kappa isotype control	rat			Monoclonal	IgG2A
Abcam - ab18407	Rat IgG1 kappa isotype control	rat			Monoclonal	IgG1 k
Abcam - ab18541	Rat IgG2b kappa isotype control	rat			Monoclonal	IgG2b k
Abcam - ab125938	Rabbit IgG isotype control	rabbit			Monoclonal	IgG
Secondary antibodies						
Abcam - ab7096	Goat anti-rat IgG H&L Biotin preadsorbed	goat	rat	Biotin	Polyclonal	IgG
Abcam - ab7082	Donkey anti-rabbit IgG H&L Biotin preadsorbed	donkey	rabbit	Biotin	Polyclonal	IgG

Table 2.4: Antibodies used for mouse atherosclerosis and chronic inflammation study

Company and catalogue no.	Name	Host	Reactivity	Conjugation	Clonality	Isotype
Primary antibodies						
BD Biosciences - 550292	Purified Rat Anti-Mouse CD107b (Mac-3)	rat	mouse	Unconjugated	Monoclonal	IgG1 k
Abcam - ab32575	Anti-actin alpha smooth muscle antibody	rabbit	mouse, rat, human	Unconjugated	Monoclonal	IgG
Isotype controls						
Abcam - ab18412	Rat IgG kappa isotype control	rat			Monoclonal	IgG1 k
Abcam - ab172730	Rabbit IgG monoclonal isotype control	rabbit			Monoclonal	IgG
Secondary antibodies						
Vector – BA9400	Biotinylated Goat Anti-Rat IgG Antibody (H+L)	goat	rat	Biotin	Polyclonal	IgG
Vector – BA1000	Biotinylated Goat Anti-Rabbit IgG Antibody (H+L)	goat	rabbit	Biotin	Polyclonal	IgG

2.15 Immunohistochemical analysis using ImagePro Premier 9.1 and ImageJ

Slides were photographed using a Nanozoomer C9600-12 slide scanner (Hamamatsu Photonics, Shizuoka, Japan). Image resolution was 228 nm per pixel. Images were saved uncompressed and then analysed in either ImagePro Premier 9.1 (Rockville, MD, USA) or converted to .tiff files and analysed using ImageJ 1.51.

2.15.1 Analysis using ImagePro Premier 9.1

After selecting and cropping a region of interest for analysis, the DAB analysis app was used to quantify the degree of antibody staining. Staining intensity was expressed as Integrated Optical Density (IOD) in units of lumens x pixels^2 and corrected for total area.

2.15.2 Analysis using ImageJ

Antibody staining was quantified in ImageJ using the Colour Deconvolution plugin, based on the technique described by Helps SC, et al[270]. The original .ndip files were first opened in ImagePro Premier 9.1 and cropped and saved into .tiff format. Cropped sizes were either 7000 x 7000 pixels for quantification in brachiocephalic artery specimens, or 20000 x

20000 pixels for quantification in aortic sinus specimens. The .tiff images were then opened in Adobe Photoshop CS6 (Adobe Systems, San Jose, CA, USA), and the Magic Wand tool was used to select the background (canvas). The background colour changed to white (RGB 255,255,255) using the paint bucket tool, to maximise contrast. The image was saved and then opened in ImageJ. Uneven illuminated background was removed using the "rolling ball" method as follows. The background was subtracted using the following commands: Process → Subtract background → rolling ball radius of 50.0 pixels. DAB staining was quantified using the Colour Deconvolution Plugin version 3.0.1 using the following commands: Plugins → Colour Deconvolution → H DAB. Three separate images are returned, and Image 2 with brown DAB staining visible being the image of interest. The region of interest for DAB analysis was then traced from Image 2, and then subjected to histogram analysis using commands Analyze → Histogram. The histogram list was then imported into a Microsoft Excel worksheet. The histogram list provides the number of pixels at each pixel intensity. The darkest pixels, which represent positive DAB staining are worth 0, whereas the lightest pixels are worth 255. These values were inverted so that maximum DAB staining was worth 255, and absence of staining was worth 0. To estimate the amount of DAB in a section, each pixel intensity was multiplied by the number of pixels at that intensity (0 to 255) and then summed. Hence, this dimensionless weighted DAB value (DABwt) = Σ[(255-histogramvalue) x (histogramvaluecount)]. To express this as a percentage, this value was divided by the maximum theoretical DAB, where each pixel in the image has a histogram value of 255, and multiplied by 100, obtain "DABwt%".

Therefore, DABwt% values obtained represent an estimate of the amount of DAB (and thus antigen) on the original tissue section. The DABwt% can then be compared with different sections (images) and the results subjected to statistical analysis.

2.16 Oil Red O staining and analysis using ImageJ

Lipid content in mouse aortas was assessed and quantified by Oil Red O staining. Harvested aortas were stored in formalin and then adventitial fat was carefully and thoroughly removed under stereo microscopy. Aortas were then cut and opened *en-face* for staining and returned to formalin until Oil Red O staining was performed. The Oil Red O solution for staining consisted of 1 g of Oil Red O powder (Sigma-Aldrich), 250 ml of propan-2-ol, and 150 ml of MilliQ water (Millipore Corporation, Billerica, MA, USA). First, the Oil Red O powder was added to the propan-2-ol while stirring. After 10 mins of stirring, the MilliQ water was added, and stirring continued for another minute. The solution was left to stand for 6 mins, and then filtered through a 0.2 μm vacuum hose. The solution was used to stain aortas immediately.

2.16.1 Oil Red O staining method

Aortas with adventitial fat removed and cut for *en-face* staining were first rinsed in distilled water and then dipped in 60% propan-2-ol solution, then incubated in the Oil Red-O solution for 15 mins. They were then dipped in 60% propan-2-ol again, and then rinsed in distilled water, before being transferred to 10% neutral-buffered formalin again, for later pinning and photography.

2.16.2 Photography of stained aortas

Aortas were placed in 100 mm petri dishes filled with paraffin wax for pinning. Aortas were pinned *en-face* using 0.15 mm rod diameter Minutien Pins (Fine Science Tools, North Vancouver, British Columbia, Canada), to expose as much luminal surface area as possible. Aortas were photographed using a Zeiss Axio Scope A1 microscope polarised light microscope (Carl Zeiss AG, Oberkochen, Germany). All aortas were photographed with a colour temperature of 5500K, and an exposure time of 5 milliseconds, which were found to be the optimal settings. Images were saved in .tiff format. Three to four overlapping segments of each aorta were photographed separately.

2.16.3 Analysis of Oil Red O staining

Segments of images were merged in Adobe Photoshop CS6 using the Photomerge tool. They were then opened in ImageJ 1.51 and then the background was subtracted using the command Process → Subtract background, and selecting a rolling ball radius of 5.0 pixels, which was found to be optimal. The region of interest was selected by tracing around the aorta using the Freehand selection tool. An RGB stack was created, and the green channel was selected. A threshold was set using the commands Image → Adjust → Threshold. The minimum and maximum thresholds were set at 0 and 120 respectively as these settings were found to be optimal. The total area staining red was then determined by selecting Results → Area, and was expressed as a percentage of total aortic luminal surface area.

2.17 Laboratory analytes in humans

Venepuncture was performed on fasting human study participants by accessing forearm veins using the needle and syringe method. Blood was collected in EDTA tubes for plasma, and clot activating tubes for serum. Serum specimens were initially allowed to clot for 30 mins at room temperature, and both serum and plasma specimens were stored at 4^{o}C until laboratory analysis was performed. Blood was sent to SA Pathology laboratories for commercial analysis for lipid profile (total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglycerides), lipoprotein (a), and high sensitivity C-reactive protein (hs-CRP). Human studies were performed at SAHMRI and were approved by the University of Adelaide Human Research Ethics Committee, and authorised after a SAHMRI Site Specific Assessment.

2.18 Statistical analysis

GraphPad Prism 7 (GraphPad Software Inc, La Jolla, CA, USA), SPSS 19 (IBM Corporation, Armonk, New York, USA), and Microsoft Excel 2016 (Microsoft, Albuquerque, NM, USA) were used to analyse data. Categorical data were analysed using the Chi-Square

Test or Fisher's Exact Test if $n < 5$ for any group. The D'Agostino-Pearson normality test was performed to determine whether continuous data was normally-distributed. Normally-distributed data were analysed using either the T-test if comparing means between two groups, or the One-way Analysis of Variance (ANOVA) if comparing means between multiple groups. If correcting for multiple comparisons, the Dunnett test was used. If comparing means between multiple groups adjusted for a covariate, an Analysis of Covariance (ANCOVA) was performed. The Two-way ANOVA test was used to compare means between multiple groups depending on two independent categorical variables. If continuous data were not normally-distributed, analysis was performed between two groups using the Mann-Whitney U Test, and the Kruskal-Wallis test if more than two groups were being compared. If correcting for multiple comparisons, Dunn's test was used. Statistical correlations were analysed using a linear regression model. Statistical significance was set at the 0.05 level.

CHAPTER 3: THE IMPACT OF OMEGA-3 FATTY ACIDS ON CIRCULATING MEDIATORS OF ATHEROSCLEROSIS – A SYSTEMATIC REVIEW

ABSTRACT

Background: Atherosclerosis is a systemic disease with multiple established systemic risk factors. Recent randomised trial evidence has demonstrated a reduction in major adverse cardiovascular events with omega-3 fatty acid supplementation. Their high concentrations in blood after ingestion present substantial opportunity to favourably affect the circulating mediators of atherosclerosis. The aim of this systematic review was to evaluate the impact of omega-3 fatty acids (O3FAs) compared to placebo on measured levels of circulating mediators of atherosclerosis in humans. These fall into the following four classes: atherogenic lipoproteins (native LDL, oxidised LDL, VLDL, IDL, Lp(a)), which are components of non-HDL cholesterol; inflammatory cytokines and adipokines (IL-1β, IL-6, IL-8, TNF-α, MCP-1, IFN-γ, leptin, resistin); atherogenic amino acids and derivatives (homocysteine, asymmetric dimethylarginine); and advanced glycation endproducts (AGEs).

Methods: To obtain the highest quality evidence from human clinical trials of O3FA supplementation, only randomised, placebo-controlled trials from the Cochrane Library with an NHMRC Evidence Level of I or II were included. Search dates were from database inception until January 2018, with independent record screening by two authors. Atherogenic mediators were required to be measured in blood either directly *in vivo*, or *ex vivo* in cell culture after human omega-3 supplementation. The heterogeneity of omega-3 formulations available, and the inclusion of *in vivo* and *ex vivo* studies, required qualitative rather than quantitative assessments of effect sizes for each mediator.

Results: 1012 results were obtained, with 109 studies eligible for inclusion, comprised of 1 Cochrane systematic review, 6 non-Cochrane systematic reviews, and 102 randomised controlled trials. It was concluded that O3FAs reduce atherosclerotic mediators from all four classes. Specifically, significant reductions were noted for oxLDL, VLDL-C, non-HDL-C, leptin, homocysteine, and AGEs. Mild reductions were noted for IL-6, and TNF-α. No significant changes were noted for the other mediators evaluated.

Conclusions: This systematic review of the literature demonstrates that omega-3 supplementation in humans has favourable effects on all four classes of circulating atherogenic mediators. This provides mechanistic insights for the reduction in cardiovascular events seen in a number of clinical trials of both fish and fish oil consumption. There is a need to further elucidate the effects of O3FAs at the tissue level, especially those of EPA compared to DHA, and to correlate these with clinical outcomes.

I, Anthony Pisaniello, conceived, designed, executed and analysed all of the work included in this chapter.

3.1 Introduction

3.1.1 Introduction and rationale for systematic review

Atherosclerosis is a complex inflammatory disease[4], and the factors leading to its initiation and progression have been extensively studied but not been fully elucidated. As a systemic disease, the mediators of atherosclerosis include systemic factors such as hypertension[271-274], hyperlipidemia[273, 275-277], diabetes mellitus[278-280], tobacco smoking[281-283], and hyperhomocysteinaemia[284-286]. There has been considerable interest in circulating atherogenic factors, particularly those that are pro-inflammatory, as new pathogenic roles for cellular signalling molecules, chemokines, and lipoproteins are elucidated. It is predictable that compounds with anti-inflammatory, anti-hypertensive, and lipid-modulating effects would have favourable effects on atherosclerosis. Omega-3 fatty acids are such compounds[195, 287-289], and there is evidence for anti-atherogenic effects, with reductions in atherosclerotic plaque volume seen in animal studies[258, 261]. Advances in plaque imaging have allowed changes in plaque volume to be measured *in vivo*, however few such studies have been performed studying the effects of omega-3s, and these have produced inconsistent results[290, 291].

Human cardiovascular outcome trials (CVOTs) of dietary omega-3 intake (i.e. fish consumption) have demonstrated a dose-dependent reduction in mortality from coronary heart disease[222]. However, CVOTs of fish oil supplementation have produced inconsistent results and have frequently suffered from methodological issues such as the use of low omega-3 doses. Both the JELIS (2007) and GISSI Prevenzione (1999) studies demonstrated reductions in major adverse cardiovascular events (MACE) in the primary and secondary prevention settings respectively[226, 227]. The later OMEGA (2010) and ORIGIN (2012) secondary prevention studies, and the ASCEND (2018) and VITAL (2018) primary prevention studies, reported no reduction in MACE, and thus challenged the purported cardioprotective effects of omega-3 fatty acids[229, 230, 232, 233]. In contrast to these studies which mostly treated with no more than 1g of omega-3 fatty acids per day, the REDUCE-IT study (2018) evaluated the effects of 4 g

per day of the prescription EPA formulation icosapent ethyl[234]. In this study of hypertriglyceridemic patients on statin therapy with a history of cardiovascular disease or diabetes mellitus, EPA reduced the primary endpoint of a composite of cardiovascular death, nonfatal MI, nonfatal stroke, coronary revascularisation and unstable angina by 25% compared to placebo. This occurred irrespective of changes in triglyceride levels, and hence there are likely to be alternative mechanisms for the benefits observed. EPA (as ethyl ester) was provided at a dose of 1.8 g per day to statin-treated hyperlipidemic patients in the JELIS study, and a significant, 19% relative reduction in the primary endpoint of any major coronary event was seen, compared to placebo[227]. REDUCE-IT and JELIS used higher-than-average omega-3 doses, and provided EPA only. The cardioprotective effects of omega-3 fatty acids are likely to be both dose and formulation dependent, however the mechanisms for this observation are at present unclear.

Omega-3 fatty acids are known to impact on multiple atherogenic conditions, such as inflammation, hypertension and hyperlipidemia. Omega-3 fatty acids are known to modulate inflammation through several mechanisms. These include incorporation into the phospholipids of inflammatory cells[292, 293], reduced eicosanoid production[294], synthesis of resolvins[295], inhibition of the pro-inflammatory NF-κB transcription pathway[296-298], induction of the anti-inflammatory PPAR-γ transcription pathway[299], disruption of lipid rafts[300, 301], and binding to the G-protein coupled receptor GPR120[302] which initiates an anti-inflammatory signalling cascade. Omega-3 fatty acids have been shown to reduce systolic and diastolic blood pressure[303], as well as triglycerides[304]. The effects of omega-3 fatty acids on lipoproteins is less pronounced, with increases in HDL-C and LDL-C seen with combinations of EPA and DHA[194, 305, 306].

After oral consumption, omega-3 fatty acids appear in the bloodstream within an hour, and are in their free forms for up to 8 hours before they are redistributed[307]. Their incorporation into tissues, however, including the time taken to do this and maximum

concentration achievable, is highly variable[308]. Indeed, after prolonged, high dose oral omega-3 consumption, total concentrations in tissues can be as little as 1 per cent of total fatty acids[308]. This is in contrast to blood, in which omega-3s can comprise almost 10% of total body fatty acids[309]. Hence, there is substantial opportunity for omega-3 fatty acids to impact the circulating mediators of atherosclerosis. The vascular endothelium has direct, constant contact with the bloodstream, and minute changes in the levels of circulating atherogenic mediators affect endothelial function. The atherogenicity of all of these factors involves induction of inflammation in the vessel wall[310-316]. If omega-3 fatty acids do indeed have clinically-significant anti-atherogenic properties, it is likely that an important component of these is their impact on circulating atherosclerosis mediators. This would be the earliest mechanism of altering vascular inflammation. This has not been systematically investigated.

3.1.2 Objectives

The aim of this systematic review is to evaluate the current literature on the impact of omega-3 fatty acids on circulating mediators of atherosclerosis. These mediators all have clear evidence for their atherogenicity and can be categorised into four classes:

(i) **Atherogenic lipoproteins**, including (native) LDL[317, 318], oxidised LDL[319], VLDL[320, 321], IDL[320], and Lp(a)[322]. These lipoproteins are components of non-HDL cholesterol.

(ii) **Inflammatory cytokines and adipokines**, including IL-1β[323-325], IL-6[326], IL-8[327], TNF-α[328], MCP-1 (CCL2) [329, 330], IFN-γ[331], leptin[332, 333] and resistin[334, 335].

(iii) **Atherogenic amino acids and derivatives**, including homocysteine[336, 337] and asymmetric dimethylarginine (ADMA)[315, 338, 339].

(iv) **Advanced glycation endproducts** (AGEs)[340, 341].

This systematic review aims to incorporate the highest quality of evidence from human clinical trials of omega-3 supplementation, through accessing only high-quality clinical trial databases.

3.2 Review Protocol

This study was performed based on the preferred reporting items for systematic reviews and meta-analyses (PRISMA) guidelines[342].

3.2.1 Sources

A comprehensive search of the medical literature was performed from database inception until January 2018 using the Cochrane Library to obtain only the highest quality clinical trials.

3.2.2 Search strategy

The following search string was used for the Cochrane Library search:

> ("MeSH descriptor: [Fish Oils] explode all trees" OR "MeSH descriptor: [Fishes] explode all trees" OR "MeSH descriptor: [Fatty Acids, Omega-3] explode all trees" OR "MeSH descriptor: [Fatty Acids, Unsaturated] explode all trees" OR "MeSH descriptor: [Eicosapentaenoic Acid] explode all trees" OR "MeSH descriptor: [Docosahexaenoic Acids] explode all trees" OR "MeSH descriptor: [alpha-Linolenic Acid] explode all trees" OR "Docosapentaenoic acid")
>
> AND
>
> ("MeSH descriptor: [Cholesterol, LDL] explode all trees" OR "Oxidized LDL" OR "oxidized low density lipoprotein" OR "MeSH descriptor: [Cholesterol, VLDL] explode all trees" OR "MeSH descriptor: [Lipoproteins, IDL] explode all trees" OR "MeSH descriptor: [Lipoprotein(a)] explode all trees" OR "non-HDL" OR "non-HDL Cholesterol" OR "MeSH descriptor: [Interleukin-1beta] explode all trees" OR "MeSH descriptor: [Interleukin-6] explode all trees" OR "MeSH descriptor: [Interleukin-8] explode all trees" OR "MeSH descriptor: [Tumor Necrosis Factor-alpha] explode all trees" OR "MeSH descriptor: [Chemokine CCL2] explode all trees" OR "MeSH descriptor: [Interferon-gamma] explode all

trees" OR "MeSH descriptor: [Leptin] explode all trees" OR "MeSH descriptor: [Resistin] explode all trees" OR "MeSH descriptor: [Homocysteine] explode all trees" OR "dimethylarginine" OR "MeSH descriptor: [Glycation End Products, Advanced] explode all trees")

3.2.3 Eligibility criteria

Only articles written in English were included. Only studies that measured the pre-specified mediators in the context of omega-3 exposure were included. Studied had to be high quality (NHMRC Evidence level I or II[343]) randomised placebo-controlled studies of omega-3 supplementation. Atherogenic mediators were required to be measured in blood either directly *in vivo*, or *ex vivo* in cell culture after human omega-3 supplementation. Studies in which omega-3 levels were measured in the absence of omega-3 supplementation were excluded. Studies were eligible if omega-3 supplementation included at least one of EPA, DPA or DHA. Studies of gene expression of atherogenic mediators were not included.

3.2.4 Study selection

The titles, abstracts and keywords of every record were retrieved and separately screened by two authors (AP and JA) to find potentially relevant studies for the full review. Any discrepancies were resolved by discussion. Full text articles were retrieved if records indicated that eligibility criteria were likely to be met. Duplicate records were excluded.

3.2.5 Data collection process

Data were extracted from the studies independently by AP and JA. Disagreements were resolved by discussion. The data extracted were: study type, study design, study quality, sample size, method and nature of omega-3 exposure, controls used, treatment duration, analytes (mediators) measured, and results including changes in measured levels with intervention/exposure. When studies included more than one dose or duration of an omega-3 supplement, the results from the maximum dose and maximum duration were included. When studies included separate EPA and DHA treatment arms, results for both were reported.

3.2.6 Quality of studies

The quality of each study was appraised, and the level of evidence was graded from I to IV based on the NHMRC Evidence Hierarchy[343] for human studies.

3.2.7 Summary measures

Changes in the levels of circulating atherogenic mediators were expressed as either as a concentration change or a percentage change. Summaries of studies that measured percentage changes were graphed separately to those that measured concentration changes.

3.2.8 Synthesis of results

Studies of omega-3 supplementation are heterogeneous, with different study designs, omega-3 formulations, doses, durations, and placebo controls used. In this systematic review, circulating atherogenic mediators were measured either *in vivo* or *ex vivo*, adding another source of heterogeneity. Therefore, an overall effect size of omega-3 supplementation on any individual mediator was assessed qualitatively, as it was not possible to accurately do so quantitatively.

3.3 Results

3.3.1 Study characteristics and selection

The Cochrane Library search yielded 1012 results, of which 2 were Cochrane systematic reviews, 14 were non-Cochrane systematic reviews and meta-analyses, 992 were randomised controlled clinical trials, and 4 were economic evaluations. In total there were 1012 results to review.

One Cochrane systematic review, 6 non-Cochrane systematic reviews and meta-analyses, 102 randomised controlled trials, and 0 economic evaluations were eligible for inclusion in this systematic review. Hence, 109 of 1012 studies were included in the analysis. Reasons for study exclusion are itemised in Table 3.1 below.

Table 3.1: Reasons for exclusion of studies from systematic review

Reason for study exclusion	No. of studies
Not a study of omega-3 supplementation	602
Results do not include pre-specified circulating mediators	132
Full text article not available	51
Multiple omega-3 formulations compared in the same study	25
Results are not clearly or suitably reported	22
Lack of a suitable choice or dose of a placebo	21
Omega-3 supplementation includes ALA only (no EPA, DPA or DHA)	16
Study of gene expression	7
Article not written in English	6
Unsuitable study design	5
Economic evaluation only	4
Lack of suitable omega-3 intervention or sufficient supplementation	3
Duplicate data from another article	3
Significant confounders present	3
Multiple subject groups for same intervention	2
Outcome measures are not relevant	1
TOTAL	903

3.3.2 Quality of studies

All studies, aside from the four economic evaluations, had an NHMRC evidence level of I or II.

3.3.3 Synthesis of results

The studies analysed for each atherogenic mediator were not compared to each other in the form of a meta-analysis due to the clinical and methodological heterogeneity observed across studies. For example, measurements of atherogenic mediators in human serum versus in cell culture media supplemented with human serum are not comparable. Weighting systems such as that described by Deeks et al[344] cannot be applied. Effect sizes and the homogeneity of studies were also not comparable. Instead, the results of studies have been reported and graphically represented. On each graph, a qualitatively-estimated overall effect for each mediator is indicated. Systematic reviews and meta-analyses have suffixes of "S/R" and "M/A" added to author names on forest plots to highlight the higher level of evidence. Studies with a statistically significant overall effect are presented in blue. Studies are graphed together in the same units. A 95% confidence interval is presented when reported, or when it can be calculated from the original manuscript[345].

A summary of the included studies is presented in Appendix A.

3.3.3.1 Atherogenic lipoproteins

Sixty-one studies measured (native) LDL-C, including two systematic reviews and one meta-analysis. In 16 studies, the net effect of omega-3s was a statistically significant increase in LDL-C. In 7 studies, the net effect was a significant decrease in LDL-C. No significant change was seen in 38 studies. Overall, omega-3s were concluded to not significantly alter LDL-C, with the average change amongst positive studies of +0.11 mmol/L, and an average change amongst all studies of +0.02 mmol/L, see Figure 3.1.

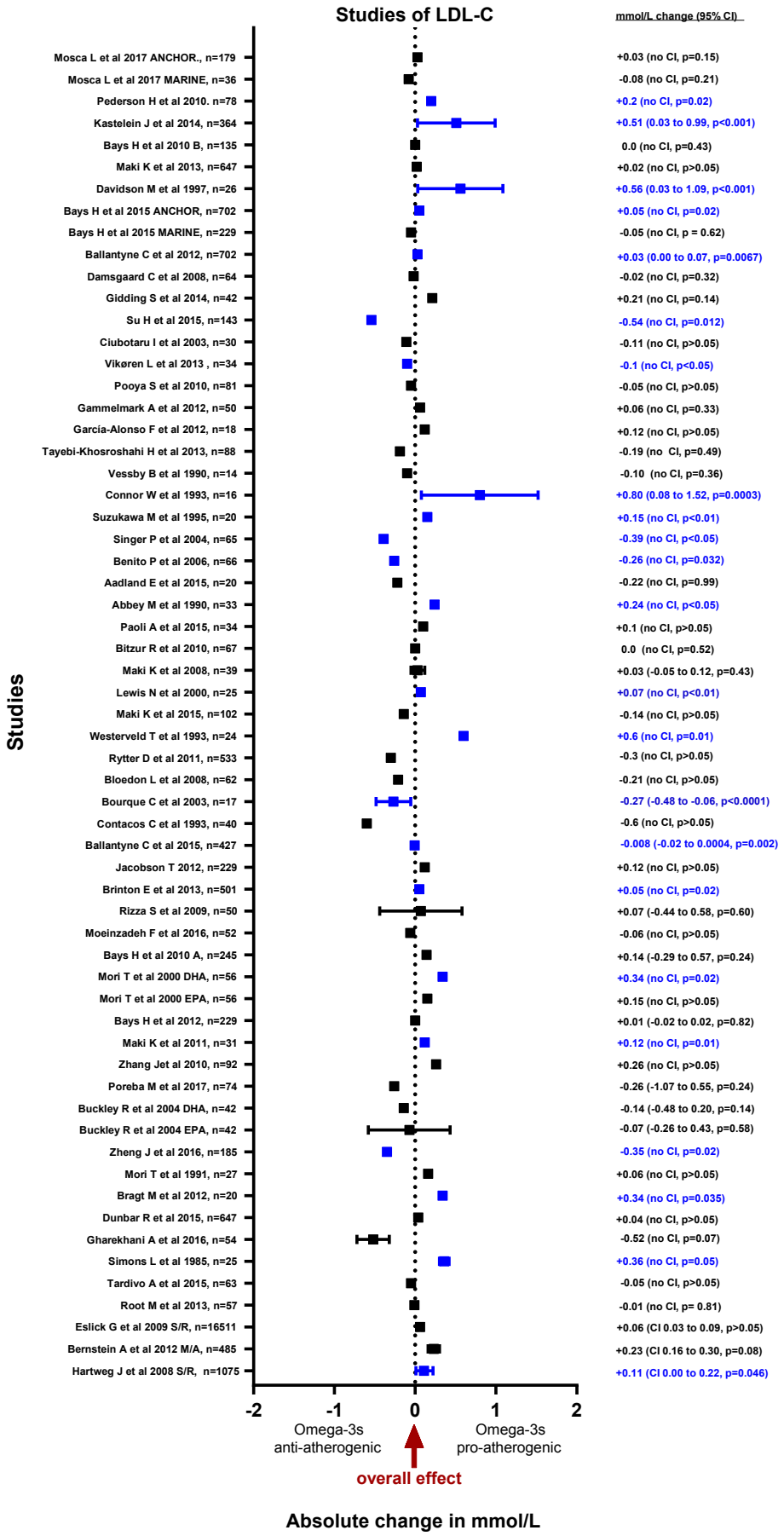


Figure 3.1: Summary of studies of omega-3 fatty acids and LDL-C. Studies with statistically significant changes are highlighted in blue. n=61 studies.

Thirteen studies measured oxidised LDL (oxLDL), and in 6 studies omega-3 fatty acids significantly reduced oxLDL levels. In no studies did omega-3 fatty acids significantly increase oxLDL levels. Overall, omega-3 fatty acids were concluded to decrease oxLDL, with the average change amongst positive studies of -7.7%, and an average change amongst all studies of -2.5%, see Figure 3.2.

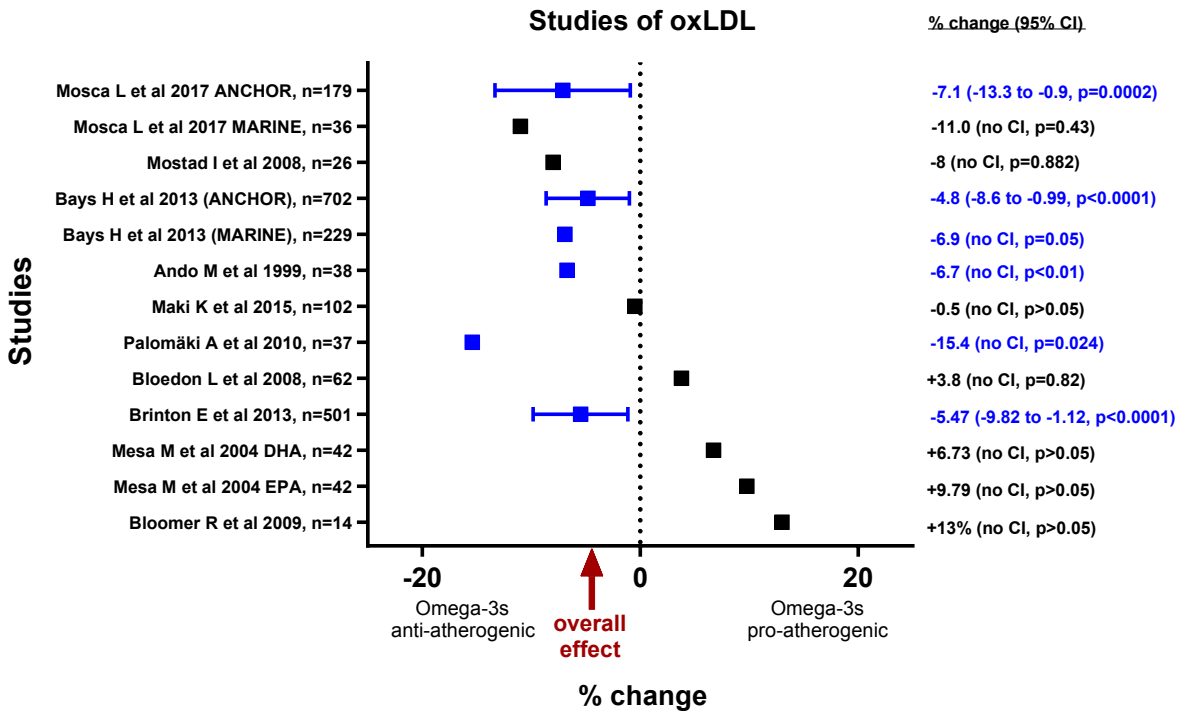


Figure 3.2: Summary of studies of omega-3 fatty acids and oxidised LDL. Studies with statistically significant changes are highlighted in blue. n=13 studies.

Twenty studies measured VLDL-C, and in 14 studies omega-3 fatty acids significantly reduced VLDL-C levels. In no studies did omega-3 fatty acids significantly increase VLDL-C levels. Overall, omega-3 fatty acids were concluded to decrease VLDL-C levels, with the average change amongst positive studies of -0.33 mmol/L, and an average change amongst all studies of -0.25 mmol/L, see Figure 3.3.

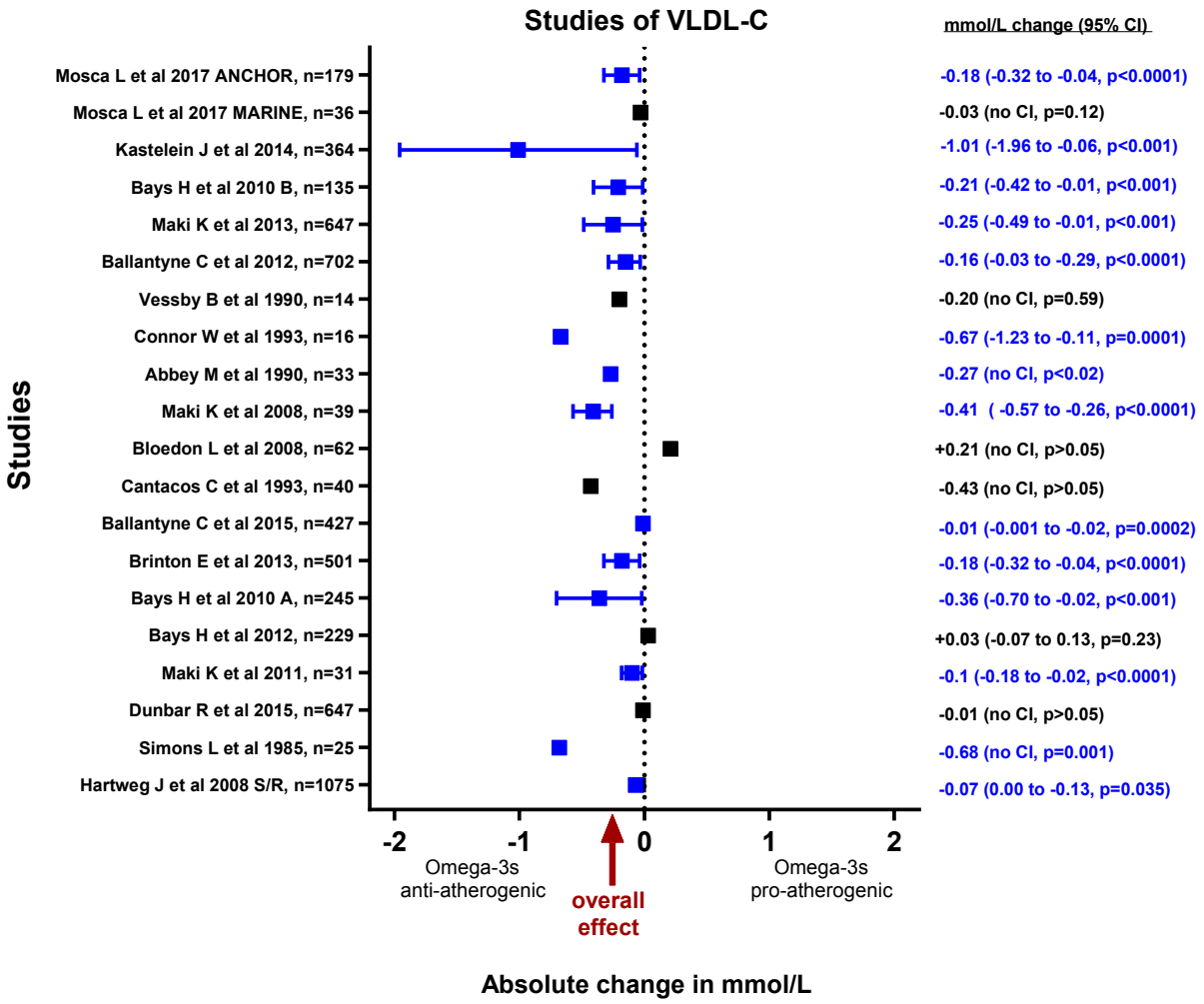


Figure 3.3: Summary of studies of omega-3 fatty acids and VLDL-C. Studies with statistically significant changes are highlighted in blue. n=20 studies.

Five studies measured IDL-C, and in one study omega-3 fatty acids significantly reduced IDL-C levels, with a change of -0.04 mmol/L. The average change amongst all studies was -0.03 mmol/L. There was no significant effect of omega-3 fatty acids on IDL-C demonstrated, see Figure 3.4.

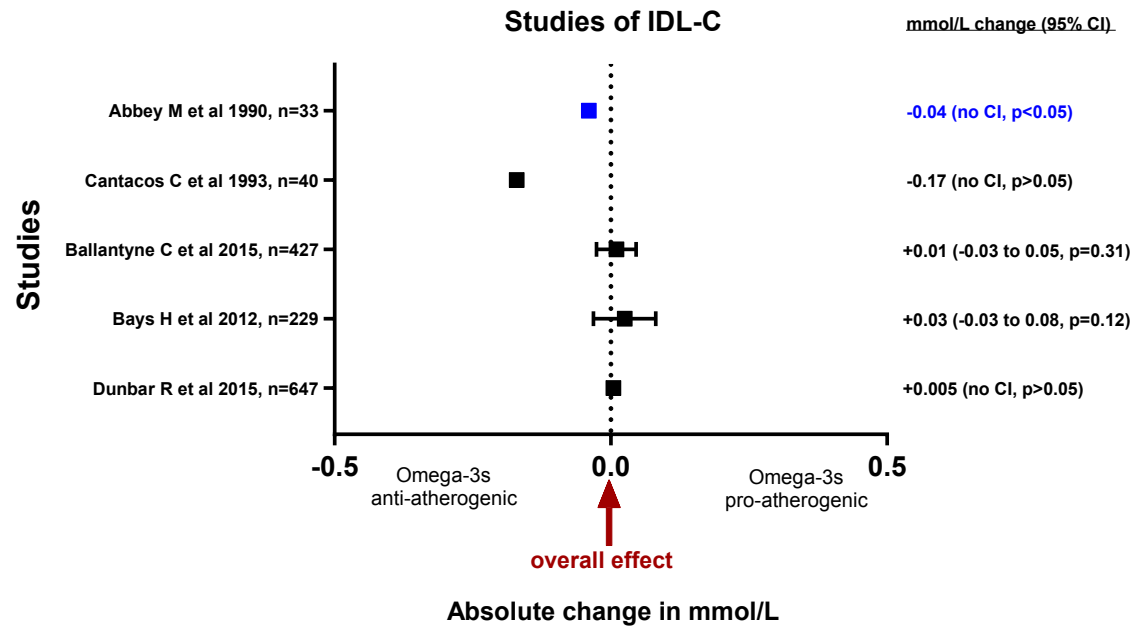


Figure 3.4: Summary of studies of omega-3 fatty acids and IDL-C. Studies with statistically significant changes are highlighted in blue. n=5 studies.

Six studies measured Lp(a), and in two studies omega-3 fatty acids significantly reduced Lp(a) levels, with an average change of -14.3%. Four studies showed no change in Lp(a). The overall change from all studies was -1.8%, see Figure 3.5. Omega-3 fatty acids were concluded to mildly reduce Lp(a) levels.

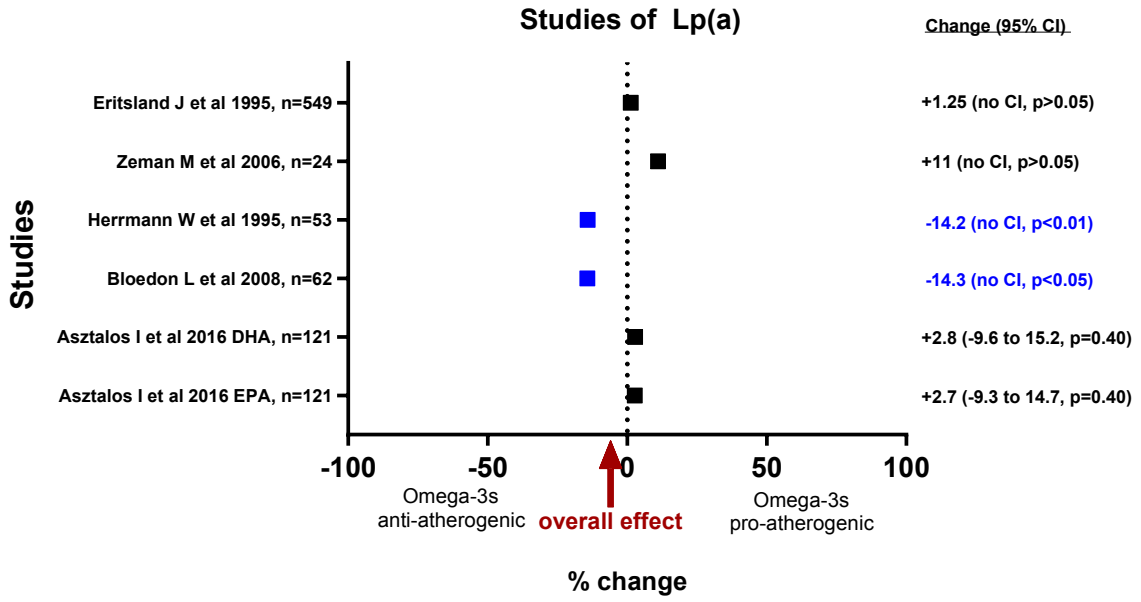


Figure 3.5: Summary of studies of omega-3 fatty acids and Lp(a). Studies with statistically significant changes are highlighted in blue. n=6 studies.

Total non-HDL cholesterol was measured in 17 studies, and in 12 studies there was a significant reduction with omega-3 fatty acids. In 2 studies, non-HDL-C was significantly increased. The average change was -0.24mmol/L, see Figure 3.6. Omega-3 fatty acids were concluded to reduce non-HDL-C.

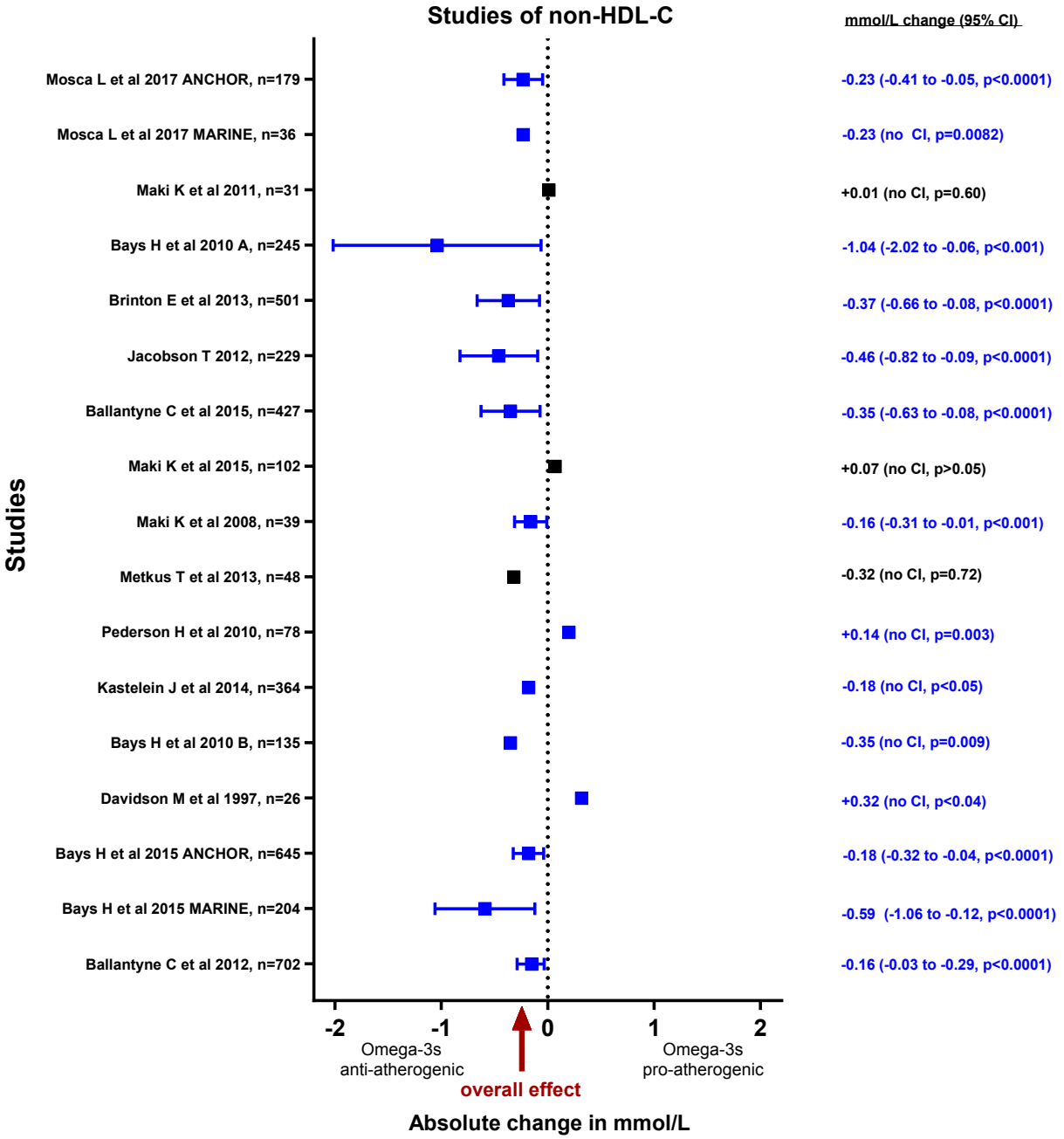


Figure 3.6: Summary of studies of omega-3 fatty acids and non-HDL-C. Studies with statistically significant changes are highlighted in blue. n=17 studies.

Atherogenic lipoproteins were overall reduced by omega-3 fatty acid supplementation, both as a collective non-HDL-C class, and individually for VLDL-C and Lp(a). Notably, oxLDL was reduced, and no change was seen in with LDL-C.

3.3.3.2 Inflammatory cytokines and adipokines

Changes in IL-1β with omega-3 supplementation were reported in either pg/ml units (6 studies) or as a percentage change (5 studies). In the former, 3 out of 6 studies demonstrated a significant reduction in IL-1β with omega-3 supplementation, with an overall average change of -0.6 pg/ml. In the latter, omega-3 supplementation did not significantly reduce IL-1β in any of the studies, with an average change of +7.0%, see Figures 3.7 and 3.8. It was concluded that omega-3 fatty acids did not significantly alter IL-1β.

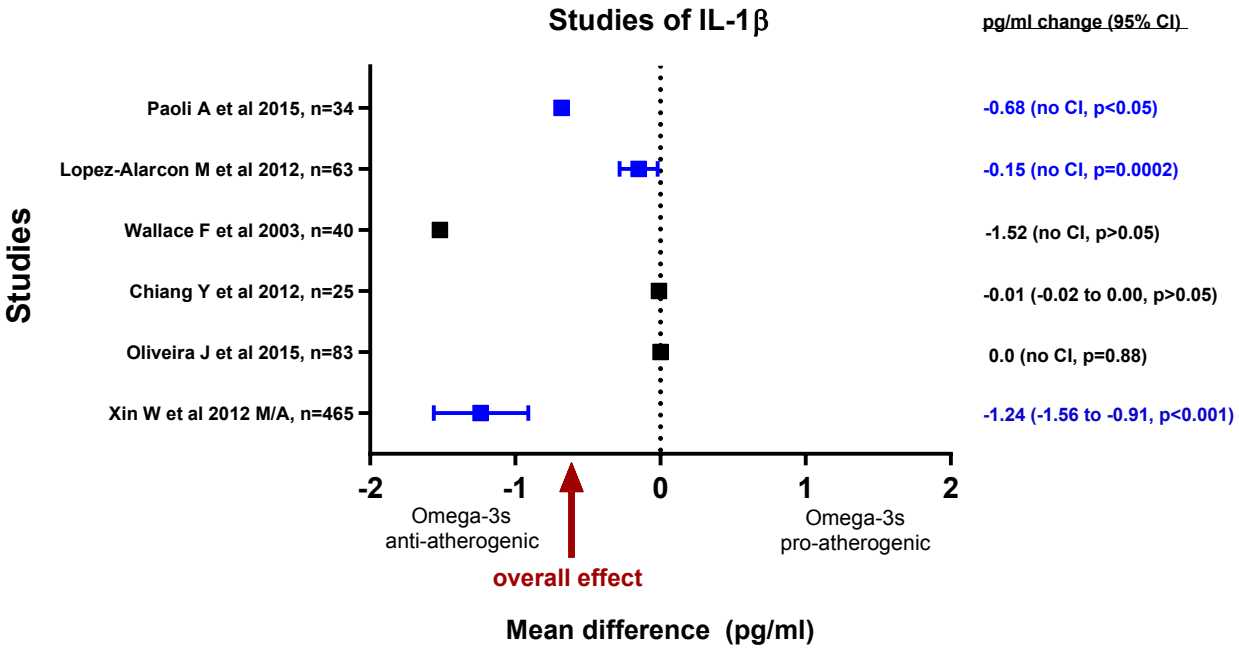


Figure 3.7: Summary of studies of omega-3 fatty acids and IL-1β reported as pg/ml. Studies with statistically significant changes are highlighted in blue. n=6 studies.

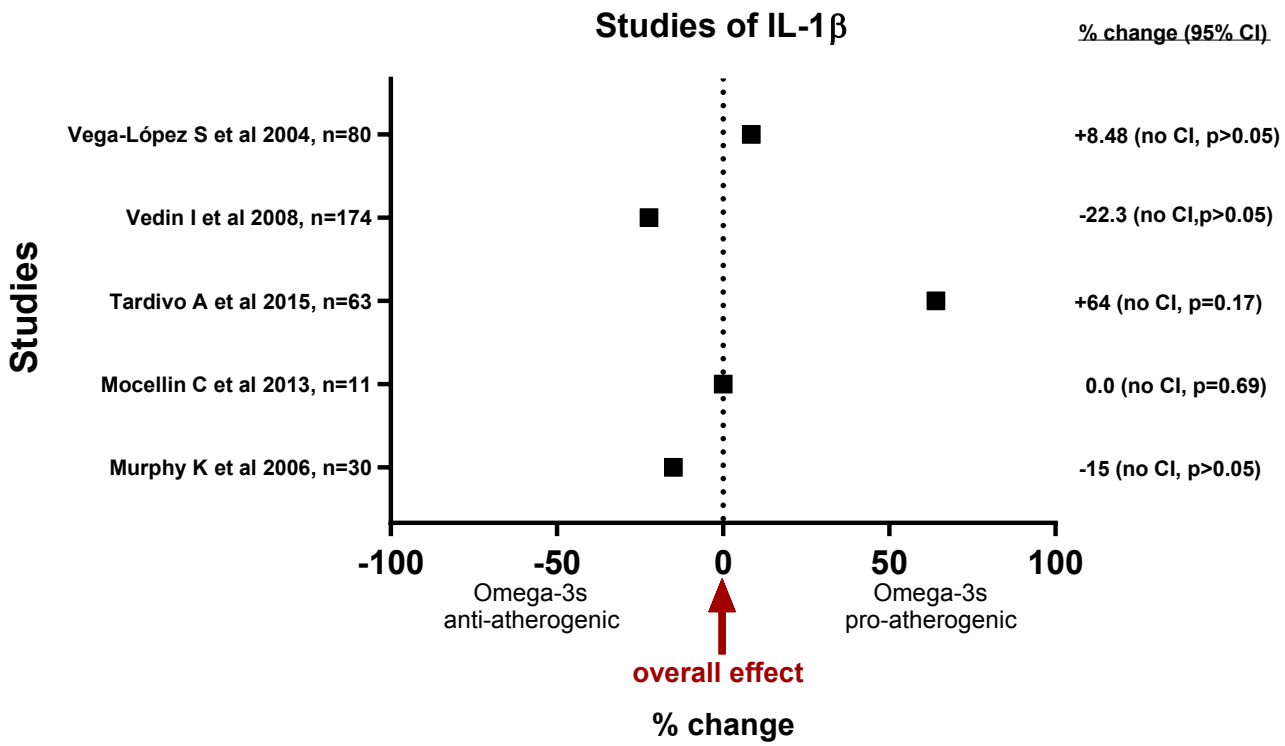


Figure 3.8: Summary of studies of omega-3 fatty acids and IL-1β reported as %. No studies had statistically significant changes. n=5 studies.

Changes in IL6 with omega-3 supplementation were likewise measured in both pg/ml and % change. In the former, 9 out of 26 studies demonstrated a significant reduction after omega-3 supplementation, with an average change of -0.3 pg/ml. In the latter, omega-3 fatty acids reduced IL6 in 5 out of 14 studies, and increased it in 2, with an average change of -17.6%. Omega-3 fatty acids were concluded to mildly reduce IL6, see Figures 3.9 and 3.10.

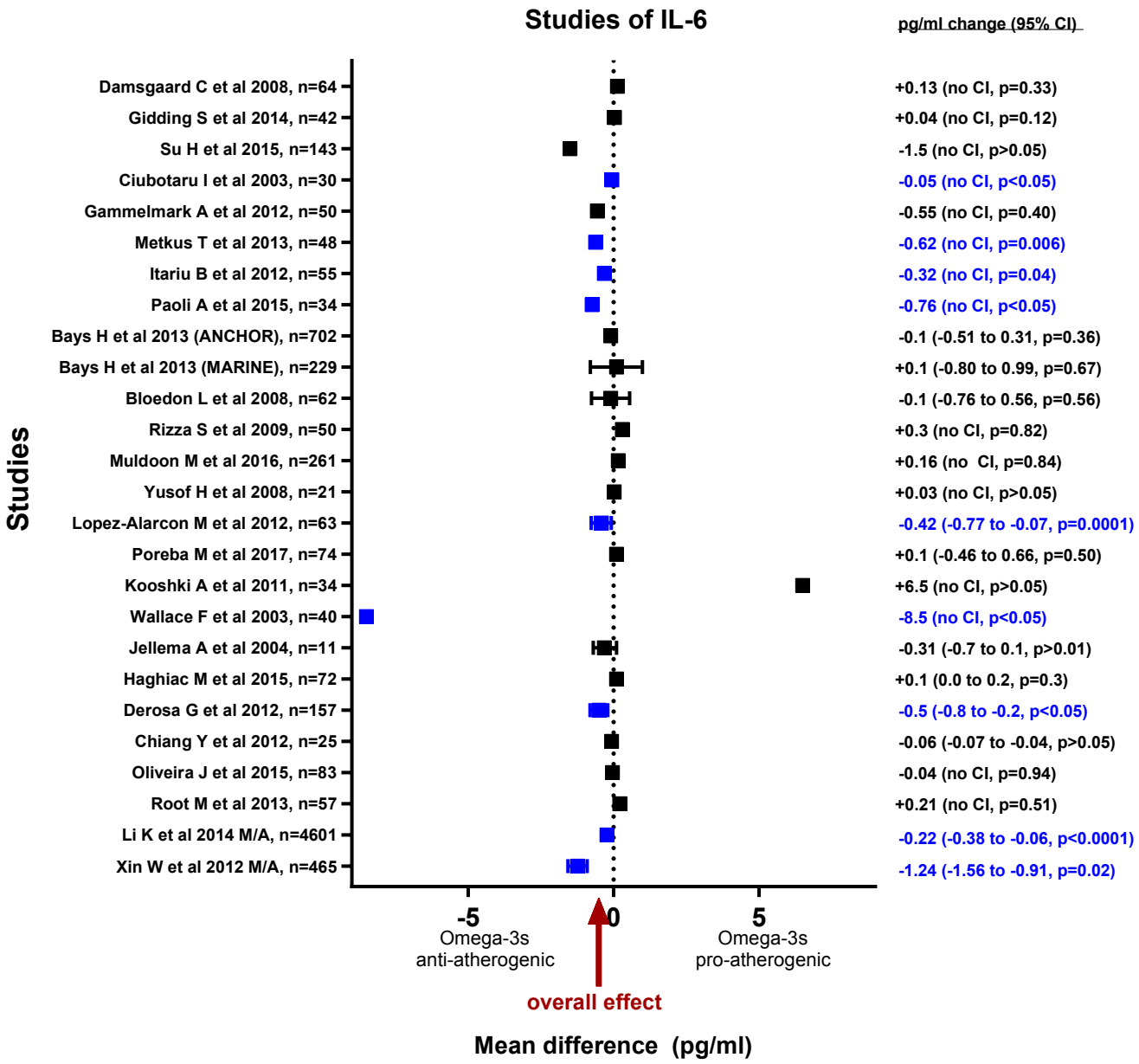


Figure 3.9: Summary of studies of omega-3 fatty acids and IL6 reported as pg/ml. Studies with statistically significant changes are highlighted in blue. n=26 studies.

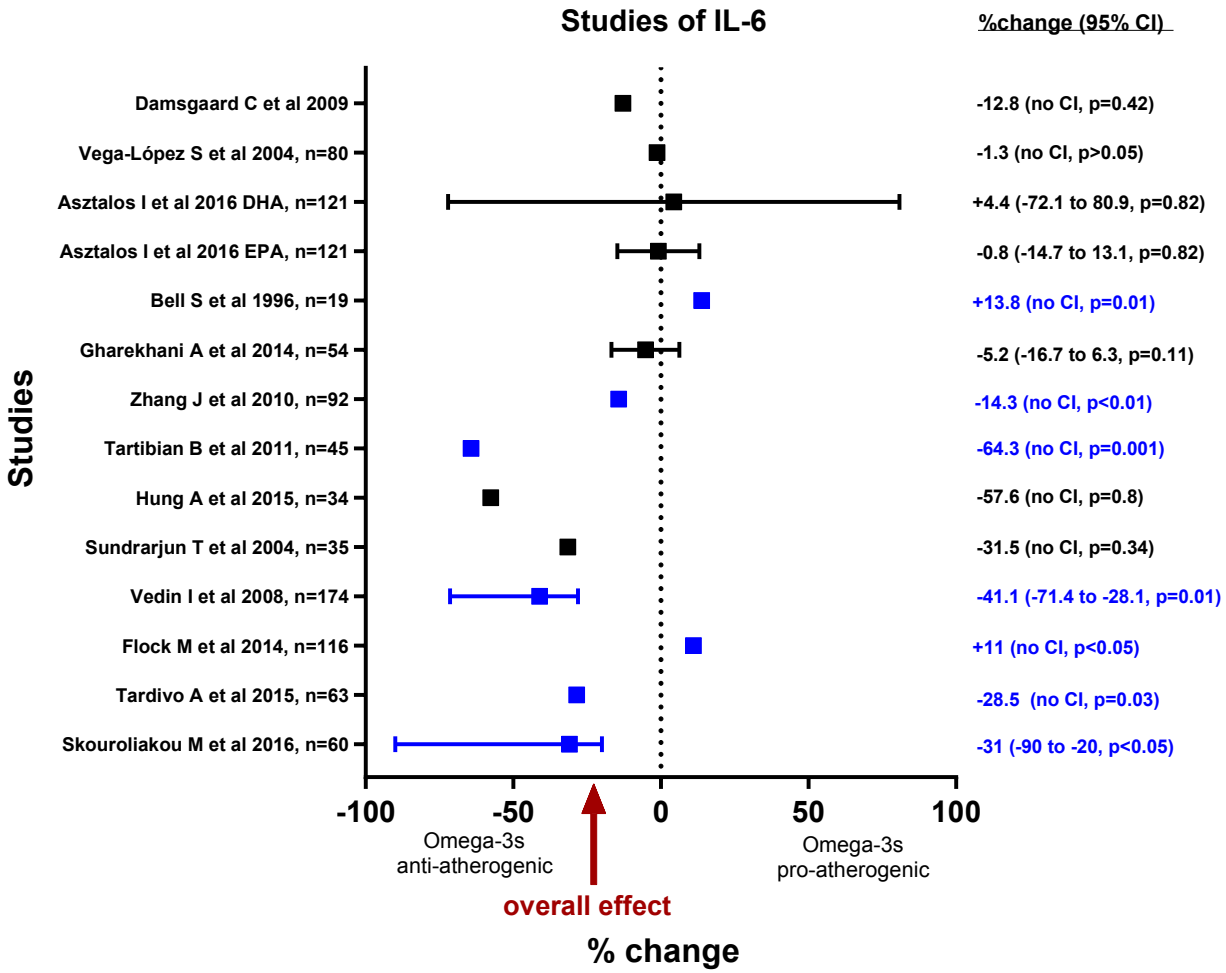


Figure 3.10: Summary of studies of omega-3 fatty acids and IL6 reported as %. Studies with statistically significant changes are highlighted in blue. n=14 studies.

The effect of omega-3 supplementation on IL8 was measured in both pg/ml (1 study) and % change in 2 studies. One study in the latter demonstrated a statistically significant reduction. No overall effect of omega-3s could be established (see Figure 3.11).

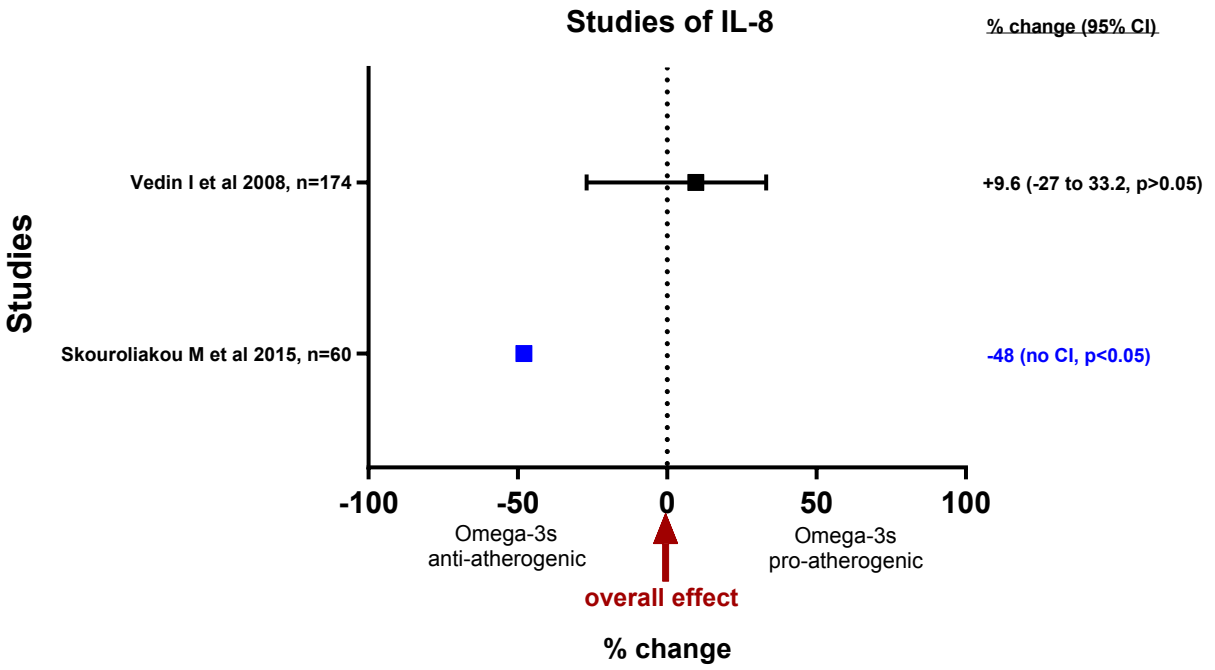


Figure 3.11: Summary of studies of omega-3 fatty acids and IL-8. The study with a statistically significant change is highlighted in blue. n=2 studies.

Changes in TNF-α were measured in pg/ml and %, and for the former, 6 out of 17 studies demonstrated a reduction with omega-3 supplementation, with an average change of -0.6 pg/ml. For the latter, omega-3s significantly reduced TNF-α in 3 out of 16 studies, and increased TNF-α in one study. The average change was -5.0% (see Figures 3.12 and 3.13). Omega-3s were concluded to mildly reduce TNF-α.

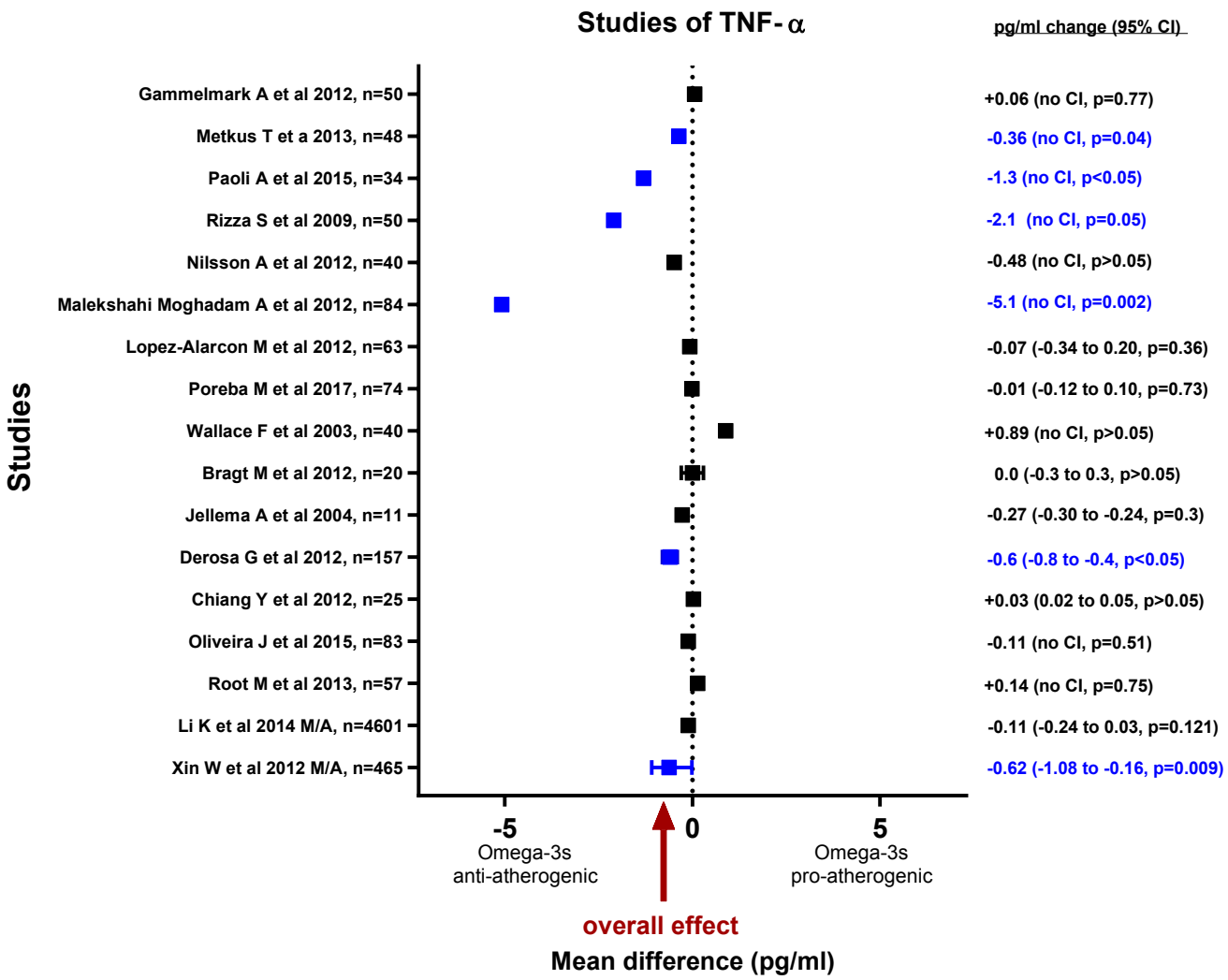


Figure 3.12: Summary of studies of omegas-3 fatty acids and TNF-α reported in pg/ml. Studies with statistically significant changes are highlighted in blue. n=17 studies.

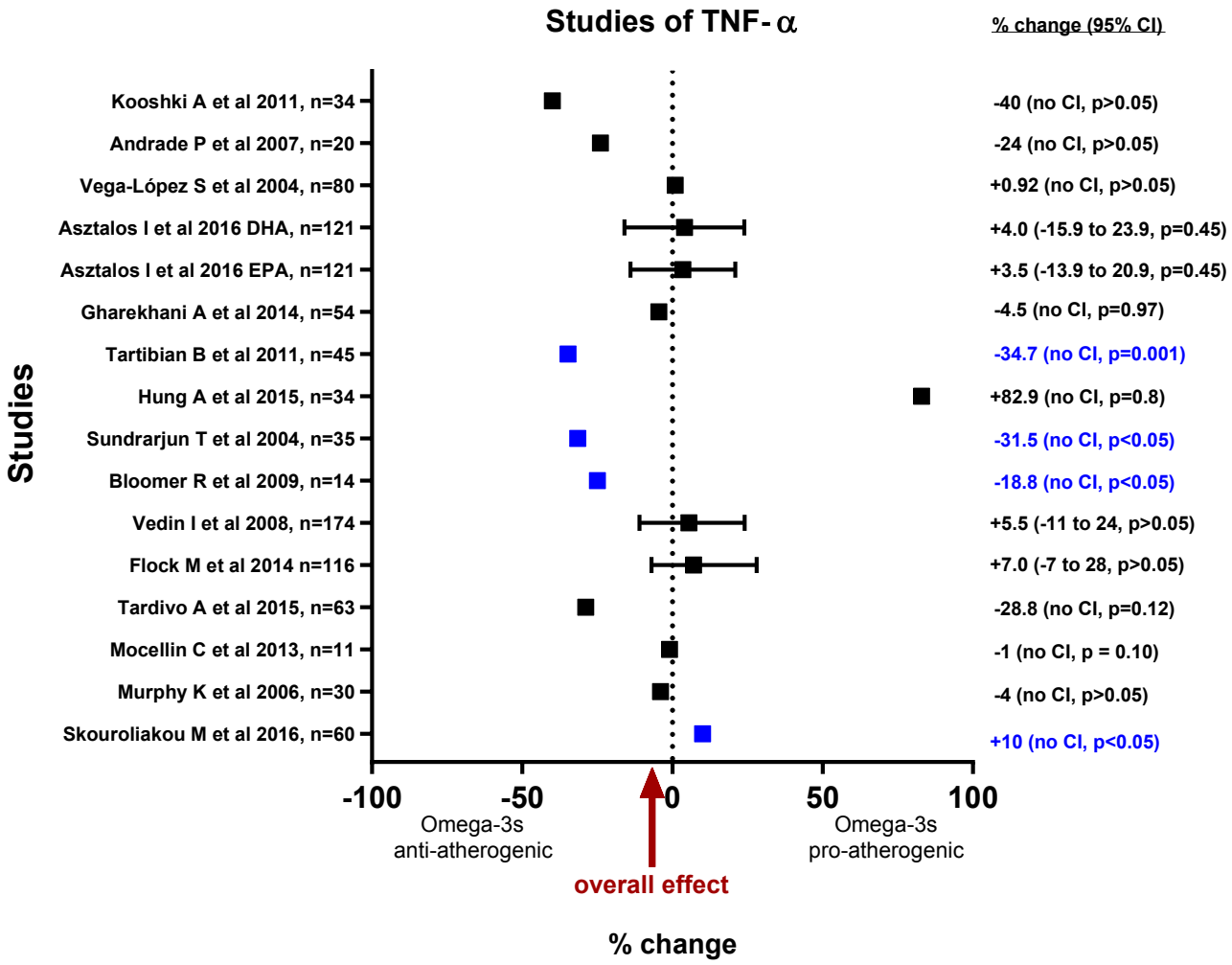


Figure 3.13: Summary of studies of omegas-3 fatty acids and TNF-α reported in %. Studies with statistically significant changes are highlighted in blue. n=16 studies.

MCP-1 was measured in 3 studies, in both pg/ml and % changes. In the former, 1 out of 2 studies demonstrated a significant reduction with omega-3 fatty acids, and there was no significant change with the latter (see Figures 3.14 and 3.15). No significant effect of omega-3s on MCP-1 could be demonstrated.

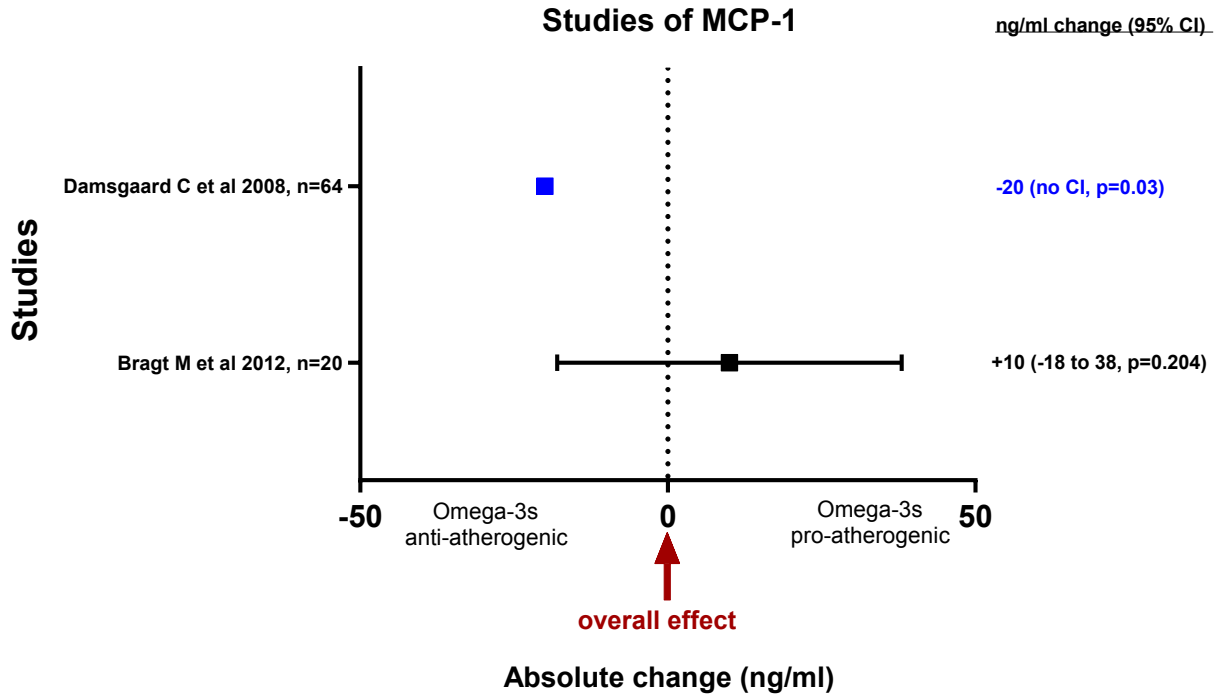


Figure 3.14: Summary of studies of omega-3 fatty acids and MCP-1 reported in ng/ml. The study with a statistically significant change is highlighted in blue. n=2 studies.

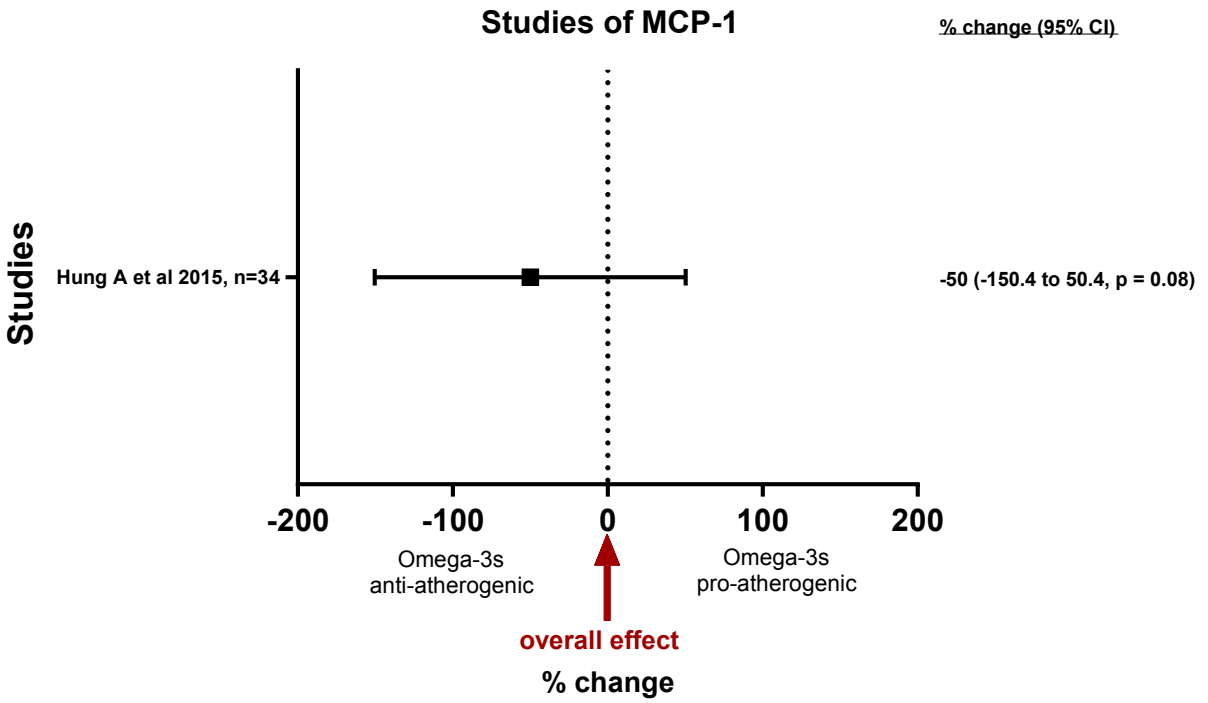


Figure 3.15: Summary of studies of omega-3 fatty acids and MCP-1 reported in %. The single study did not have a statistically significant change.

The effects of omega-3 fatty acids on IFN-γ were measured in 2 studies as % change. In one study there was a statistically-significant reduction, with an overall change of -53% (see Figure 3.16). Given the small sample size, no significant effect of omega-3s could be demonstrated.

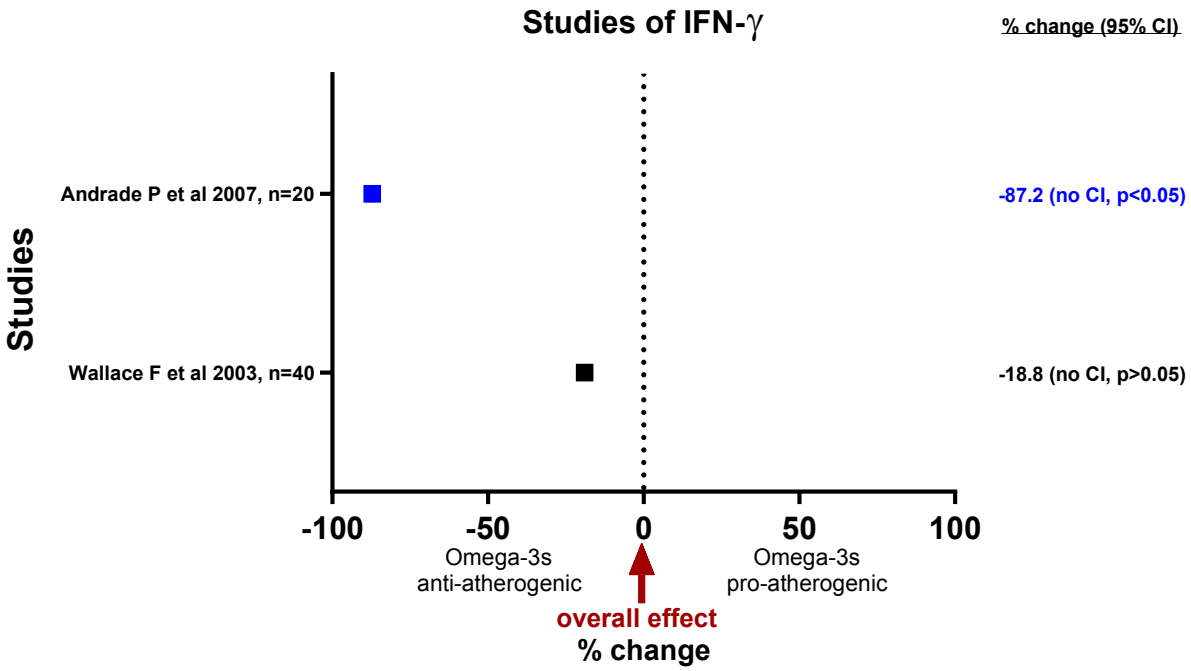


Figure 3.16: Summary of studies of omega-3 fatty acids and IFN-γ. The single study with a statistically significant change is highlighted in blue. n=2 studies.

The adipokine leptin was measured in 9 studies, and in 4 there was a significant reduction with omega-3 fatty acids. The average change was -0.22 ng/ml (see Figure 3.17). It was concluded that omega-3 fatty acids reduced leptin levels.

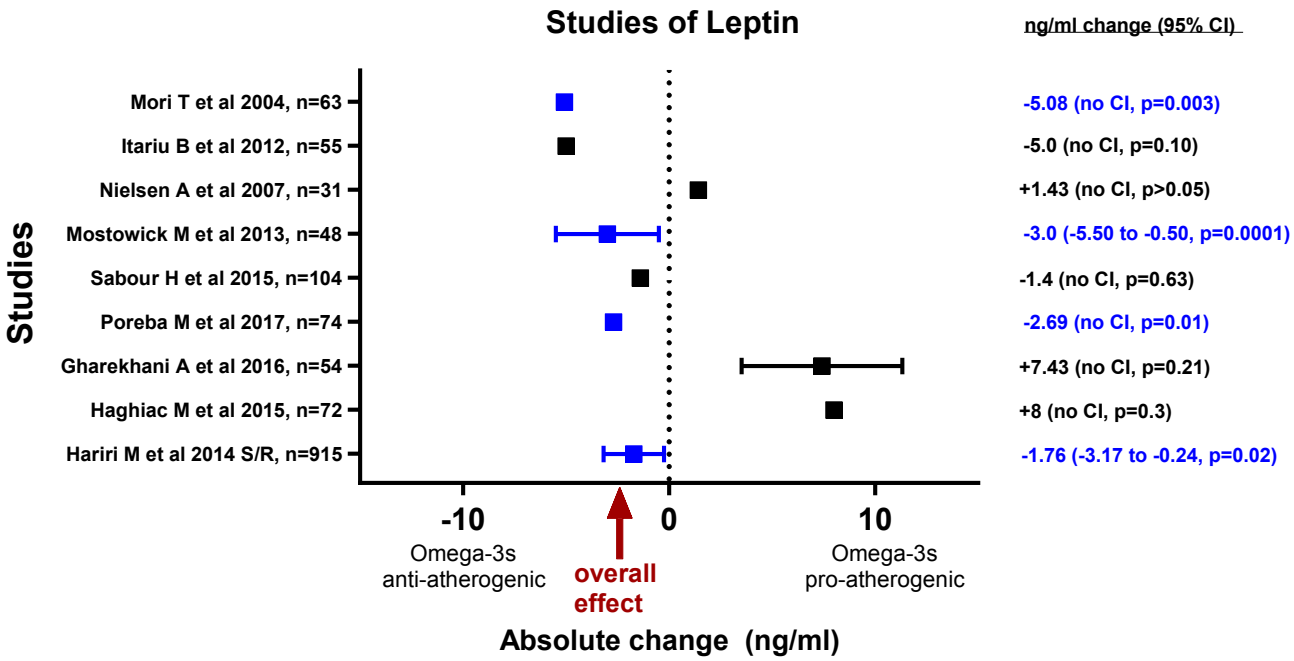


Figure 3.17: Summary of studies of omega-3 fatty acids and leptin. Studies with statistically significant changes are highlighted in blue. n= 9 studies.

The adipokine resistin was not measured in any of the studies.

The atherogenic inflammatory mediators were overall mildly reduced by omega-3 fatty acids, with effects on the cytokines IL6 and TNF-α, as well as the adipokine leptin.

3.3.3.3 Atherogenic amino acids and derivatives

Homocysteine was evaluated in 11 studies, and in 8 studies omega-3 fatty acids significantly reduced circulating homocysteine levels. In one study homocysteine was significantly elevated, and the overall average change was -1.8 µmol/L (see Figure 3.18). Omega-3 fatty acids were concluded to significantly reduce homocysteine levels.

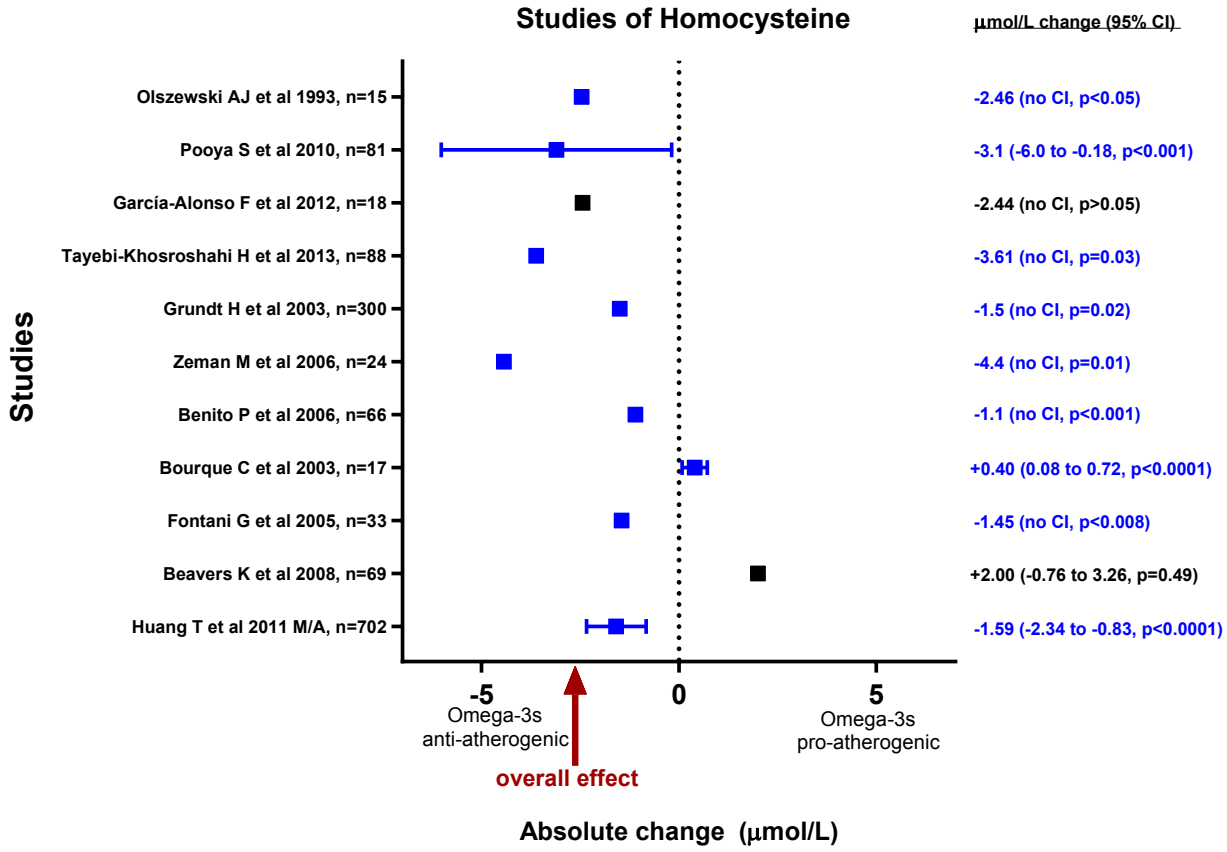


Figure 3.18: Summary of studies of omega-3 fatty acids and homocysteine. Studies with statistically significant changes are highlighted in blue. n= 11 studies.

The impact of omega-3 fatty acids on asymmetric dimethylarginine (ADMA) was evaluated in two studies. No significant changes were seen, with an average change of +3 µmol/L (see Figure 3.19)

Omega-3 fatty acids significantly reduced the atherogenic amino acid homocysteine, however there was insufficient evidence in the literature to draw conclusions regarding effects on ADMA.

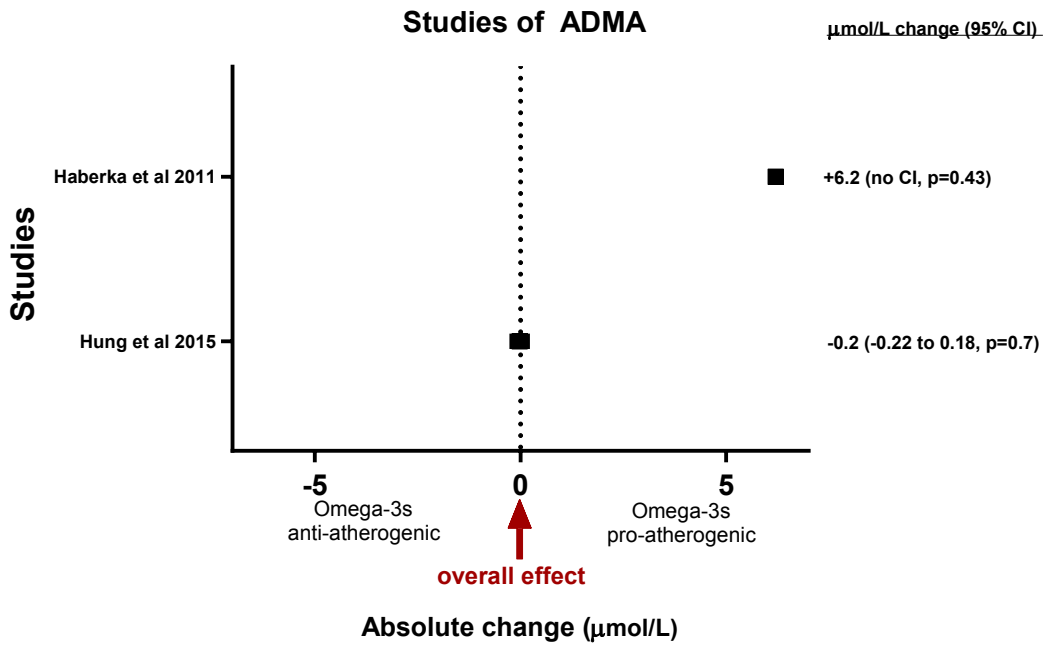


Figure 3.19: Summary of studies of omega-3 fatty acids and ADMA. The two studies did not have statistically significant changes. n=2 studies.

3.3.3.4 Advanced glycation end products

Changes in levels of advanced glycation end products (AGEs) with omega-3 fatty acid supplementation were investigated in a single study. AGEs are conventionally measured in arbitrary units (AUs), and a 2.1 AU reduction was seen (see Figure 3.20).

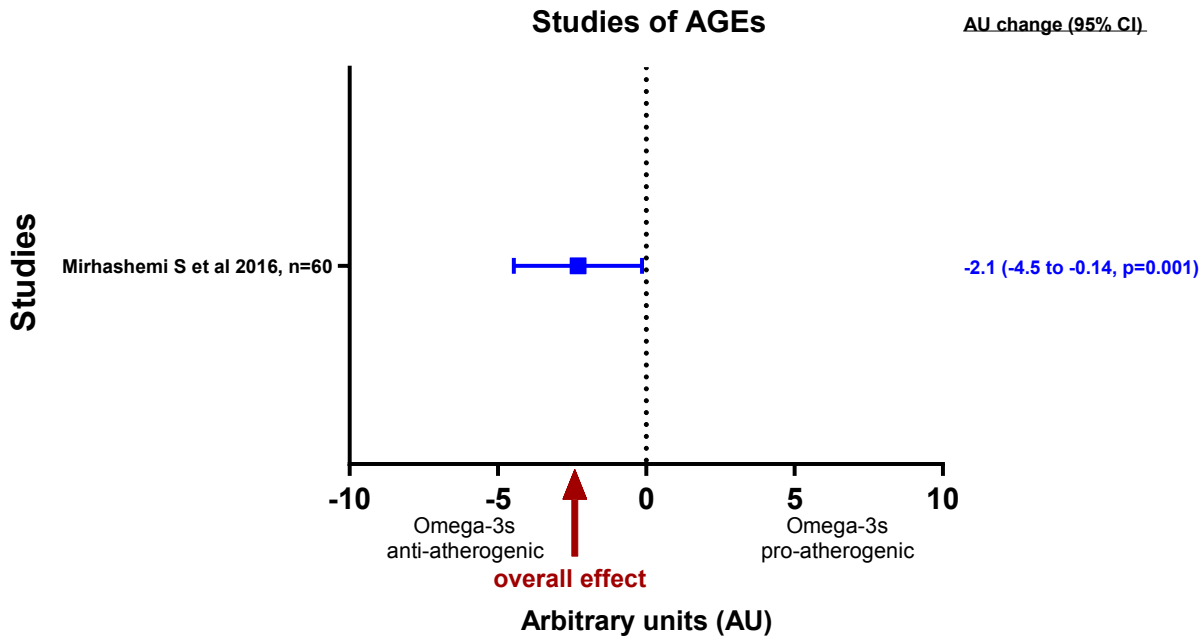


Figure 3.20: Single study of omega-3 fatty acids on advanced glycation endproducts (AGEs). The result was statistically significant and presented in blue.

3.4 Discussion

This systematic review evaluated the impact of omega-3 fatty acids on circulating atherogenic mediators, and included only high-quality studies catalogued in the Cochrane library. Systematic reviews, meta-analyses, and randomised placebo-controlled trials were included exclusively. The recently-published clinical trial REDUCE-IT provided evidence for an atheroprotective effect of omega-3 fatty acids[234], however previously published CVOTs of omega-3 fatty acids, especially those of fish oil supplementation, produced variable results and suffered from limitations of study design[346]. To understand the cardioprotective effects of omega-3 fatty acids, and the possible reasons for disparity amongst studies, it is essential to directly study their effects on atherogenic mediators. Despite the associations of innumerable circulating factors with atherosclerosis, only mediators with clear evidence for direct

atherogenicity were evaluated. Given the heterogeneity of study design, which included both *in vivo* and *in vitro* studies, it was not possible to apply a weighting to individual studies as is commonly performed in systematic reviews. Nonetheless, general conclusions could be made from the results generated.

Omega-3 fatty acids reduced levels of circulating atherogenic markers in all four classes. Among the atherogenic lipoproteins studied, non-HDL cholesterol, oxLDL, VLDL-C and Lp(a) were reduced by omega-3 fatty acids. LDL-C levels were not significantly altered by omega-3 fatty acids. It is important to note that triglycerides were not included in this review as they are not directly atherogenic[347]. Rather, high triglyceride levels associate with higher cardiovascular risk by virtue of triglyceride-rich lipoproteins and remnant particles[347]. The mechanisms by which omega-3s reduce levels of atherogenic lipoproteins are not completely understood. There is evidence that omega-3 fatty acids reduce VLDL production rate[348], and this may be due to their ability to reduce hepatic fat content[349] or reduce fatty acid availability for triglyceride and subsequent VLDL production by means of increased fatty acid oxidation[350, 351]. Omega-3 fatty acids have been shown to increase hepatic production of LDL particles[348], which likely explains the increased LDL-C levels seen in some studies.

Omega-3 fatty acids reduced the cytokines IL6 and TNF-α to a mild degree. This is likely due to the inhibitory effects of omega-3s on the pro-inflammatory NF-κB transcription pathway[195], as well as being a natural ligand for the anti-inflammatory nuclear receptor PPAR-γ[352]. Despite the known inhibitory effects of omega-3 fatty acids on activation of the NLRP3 inflammasome[353-355], no overall effect was seen on IL-1β levels. This suggests additional effects of omega-3s on IL-1β regulation. This is particularly relevant given the reduction in major adverse cardiovascular events seen with IL-1β lowering seen in the CANTOS study[120]. In this study, canakinumab reduced recurrent cardiovascular events in patients with a history of myocardial infarction and elevated C-reactive protein levels, without lowering lipid levels. The effects of omega-3 fatty acids on the inflammasome, in particular the regulation of IL-1β,

require further study. Insufficient studies of IL8, MCP-1, IFN-γ and resistin were included in this systematic review to allow conclusions to be made.

Omega-3 fatty acids significantly reduced homocysteine levels, likely due to regulatory effects on genes associated with homocysteine metabolism[356]. The upregulation of cystathionine-γ-lyase (CSE) with both EPA and DHA, the upregulation of 5-methyltetrahydrofolate reductase (MTHFR) with DHA and ALA, and the downregulation of methionine adenosyltransferase (MAT) with EPA, DHA and ALA, have all been demonstrated, and likely contribute to the significant reductions in homocysteine levels seen in this systematic review[356]. Only two studies measured ADMA levels, with no effects observed.

A single study measured advanced glycation endproducts, demonstrating a significant reduction. The mechanisms by which omega-3 fatty acids may reduce AGEs are unclear, although NFκB has been implicated in their formation[357]. Given the significant role of AGEs in atherogenesis, the lack of high-quality studies yielded by this review suggests an important area of future investigation. This may identify new roles for omega-3 fatty acids in the management of patients with diabetes mellitus.

This systematic review provides mechanistic insights into the atheroprotective effects of omega-3 fatty acids. They reduce levels of all four classes of circulating atherogenic mediators and hence this is their earliest mechanism of reducing vascular inflammation after ingestion. Given that the atheroprotective effects of omega-3 fatty acids appear to be dose-responsive based on dietary studies, and have been demonstrated with high omega-3 doses in fish oil studies, there may now be further impetus to routinely measure omega-3 levels in blood and aim for high levels. Thus far, no correlation has been shown between the concentration of blood omega-3 levels and the degree of suppression of circulating atherogenic mediators, although this would be predicted. Several methods exist for measuring and expressing blood omega-3 levels. Firstly, measuring the proportion of total fatty acids in whole blood comprised of omega-3s, which has an inverse association with sudden death[212]. Secondly, measuring the

total omega-3 content of the red cell component of blood, which forms the basis of the Omega-3 Index. This value has cut-offs that predict a higher mortality (< 4%) or a lower mortality (> 8%)[213]. Thirdly, measuring the ratio of pro-inflammatory omega-6s to anti-inflammatory omega-3s in whole blood, i.e. the omega 6:3 ratio. It is conceivable that since omega-6 and omega-3 fatty acids compete with each other for incorporation into cell membranes[216, 217], a higher relative omega-6 content could dampen the beneficial effects of omega-3s even if they were present in high concentrations. The measurement of blood omega-3 fatty acid content and its relationship with circulating atherosclerotic mediators, and ultimately cardiovascular outcomes requires further investigation.

This systematic review has several limitations. The literature search was restricted to studies with an NHMRC Class I or II level of evidence, and were extracted from a single database (Cochrane). This was to maximise the quality of articles retrieved, and to ensure that the conclusions drawn were informed by robust evidence. This limited the number of studies that could be included. In view of the heterogeneity of the study designs, individual studies could not be weighted against each other. Hence, studies with a higher level of evidence and those with a high number of participants could not be appropriately apportioned greater significance. The possible effects of concomitant use of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG Co-A) reductase inhibitors by subjects were not accounted for in this review, however this is unlikely to significantly confound the results given the randomised nature of the included studies. This review did not investigate the relative effects of the two main omega-3 fatty acids, EPA and DHA, as the vast majority of studies used combinations of EPA and DHA.

3.5 Conclusions

This systematic review of the literature demonstrates that omega-3 fatty acids have favourable effects on all four classes of circulating atherogenic mediators. They modestly reduce atherogenic cytokines and adipokines, and this likely reflects the complexities of their

regulation. Atherogenic lipoproteins as well as homocysteine are reduced by omega-3s to a greater degree, and a single study of advanced glycation endproducts demonstrated a significant reduction. The findings of this systematic review provide mechanistic insights for the reduction in cardiovascular events seen in a number of CVOTs of fish and fish oil consumption. The reduction in circulating atherogenic mediators by omega-3s, suggests that there may be value in targeting specific blood omega-3 levels, and further research is required in this area. Moreover, there is a need to elucidate the effects of omega-3 fatty acids, especially the differential effects of EPA versus DHA on mediators of atherogenesis at the tissue level, and to correlate these with clinical outcomes.

3.6 Appendix A: Summary of included studies

AUTHORS	COMORBIDITIES	STUDY TYPE	SAMPLE SIZE	NATURE OF INTERVENTION	CONTROL	TREATMENT DURATION	ANALYTES MEASURED
Aadland E et al 2015[358]		RCT placebo controlled	20	EPA + DHA	control diet	4 weeks	LDL
Abbey M et al 1990[359]		RCT placebo controlled	33	EPA + DHA	safflower oil	6 weeks	IDL, VLDL, LDL
Ando M et al 1999[360]	ESKD on haemodialysis	RCT placebo controlled	38	EPA	control capsules	7 months	oxLDL
Andrade PM et al 2007[361]		RCT placebo controlled	20	EPA + DHA	mineral oil	6 weeks	IFN, TNF
Asztalos I et al 2016[362]		RCT placebo controlled	121	EPA or DHA	olive oil	6 weeks	TNF, IL6, LDL, Lp(a)
Ballantyne C et al 2012[363]		RCT placebo controlled	702	ICOSAPENT ETHYL (EPA)	control capsules	12 weeks	LDL, non-HDL, VLDL, HDL
Ballantyne C et al 2015[364]		RCT placebo controlled	427	ICOSAPENT ETHYL (EPA)	control capsules	12 weeks	VLDL, LDL, IDL, non-HDL
Bays H et al 2010 A[365]	Hyperlipidemia	RCT placebo controlled	245	EPA + DHA	corn oil	16 weeks	VLDL, LDL, non-HDL
Bays H et al 2010 B[366]	Hypertriglyceridemia	RCT placebo controlled	135	EPA + DHA	control capsules	8 weeks	LDL, VLDL
Bays H et al 2012[367]	Hypertriglyceridemia	RCT placebo controlled	229	ICOSAPENT ETHYL (EPA)	control capsules	12 weeks	IDL, VLDL, LDL
Bays H et al 2013[368]		RCT placebo controlled	931	ICOSAPENT ETHYL (EPA)	control capsules	12 weeks	oxLDL, IL6
Bays H et al 2015[369]	Metabolic syndrome	RCT placebo controlled	849	EPA	control capsules	12 weeks	LDL, non-HDL
Beavers K et al 2008[370]	End stage kidney disease	RCT placebo controlled	69	EPA + DHA	corn oil	6 months	homocysteine
Bell S et al 1996[371]	HIV	RCT placebo controlled	19	EPA + DHA	safflower oil	6 weeks	IL6, TNF
Benito P et al 2006[372]	Metabolic syndrome	RCT placebo controlled	66	EPA + DHA	milk	3 months	LDL, homocysteine
Bernstein A et al 2012 M/A[373]		Meta-analysis of 11 RCTs	485	Algal oil (41% DHA)	multiple	4-17 weeks	LDL
Bitzur R et al 2010[374]		RCT placebo controlled	67	EPA + DHA	corn oil	12 weeks	LDL
Bloedon L et al 2008[375]		RCT placebo controlled	62	EPA + DHA	control diet	10 weeks	Lp(a), LDL, VLDL, IL6
Bloomer R et al 2009[376]		RCT placebo controlled	14	EPA + DHA	soybean oil	6 weeks	TNF, oxLDL
Bourque C et al 2003[377]		RCT placebo controlled	17	EPA + DHA	control diet	27 days	homocysteine, LDL
Bragt M et al 2012[378]	Overweight and obese	RCT placebo controlled	20	EPA + DHA	sunflower oil	6 weeks	LDL, MCP1,

Brinton E et al 2013[379]	Type 2 DM and hypertriglyceridemia	RCT placebo controlled	501	ICOSAPENT ETHYL (EPA)	control capsules	12 weeks	oxLDL, LDL, VLDL, non-HDL
Buckley R et al 2004[380]		RCT placebo controlled	42	EPA + DHA	olive oil	4 weeks	LDL
Chiang Y et al 2012[381]		RCT placebo controlled	25	EPA + DHA	low omega-3 diet	4 weeks	IL1b, TNF, IL6
Ciubotaru I et al 2003[382]		RCT placebo controlled	30	EPA + DHA	sunflower oil	5 weeks	LDL, IL6
Connor W et al 1993[383]	Type 2 DM	RCT placebo controlled	16	EPA + DHA	olive oil	6 months	VLDL, LDL
Contacos C et al 1993[384]		RCT placebo controlled	40	EPA + DHA	control capsules	18 weeks	VLDL, LDL, IDL
Damsgaard C et al 2008[385]		RCT placebo controlled	64	EPA + DHA	olive oil	8 weeks	IL6, LDL
Damsgaard C et al 2009[386]		RCT placebo controlled	64	EPA + DHA	olive oil	8 weeks	IL6
Davidson M et al 1997[387]	Elevated triglycerides and LDL	RCT placebo controlled	26	DHA	vegetable oils (corn and soybean)	6 weeks	LDL, non-HDL
Derosa G et al 2012[388]		RCT placebo controlled	157	EPA + DHA	sucrose, mannitol, mineral salts	6 months	IL6, TNF
Dunbar R et al 2015[389]	Hypertriglyceridemia	RCT placebo controlled	647	EPA + DHA	olive oil	6 weeks	LDL, VLDL, IDL
Eritsland J et al 1995[390]	Coronary artery disease	RCT placebo controlled	549	EPA + DHA	control capsules	6 months	Lp(a)
Eslick G et al 2009 S/R[391]		Systematic review of 47 RCTs	16511	EPA ± DHA	multiple	3-182 weeks	LDL
Flock M et al 2014[392]		RCT placebo controlled	116	EPA + DHA	soybean oil	5 months	IL6, TNF
Fontani G et al 2005[393]		RCT placebo controlled	33	EPA + DHA	olive oil	70 days	homocysteine
Gammelmark A et al 2012[394]		RCT placebo controlled	50	EPA + DHA	olive oil	6 weeks	IL6, TNF, LDL
García-Alonso F et al 2012[395]		RCT placebo controlled	18	EPA + DHA	tomato juice	2 weeks	homocysteine, LDL
Gharekhani A et al 2014[396]	ESKD on haemodialysis	RCT placebo controlled	54	EPA + DHA	paraffin oil	4 months	IL6, TNF
Gharekhani A et al 2016[397]	ESKD on haemodialysis	RCT placebo controlled	54	EPA + DHA	paraffin oil	4 months	LDL, leptin
Gidding S et al 2014[398]	Hypertriglyceridemia	RCT placebo controlled	42	EPA + DHA	corn oil	8 weeks	LDL, IL6
Grundt H et al 2003[399]		RCT placebo controlled	300	EPA + DHA	corn oil	12 months	homocysteine

Haberka M et al 2011[400]		RCT placebo controlled	40	EPA + DHA	control therapy	1 month	ADMA
Haghiac M et al 2015[401]	Obesity and pregnancy	RCT placebo controlled	72	EPA + DHA	wheat germ oil	until delivery	IL6, IL8, TNF, leptin
Hariri M et al 2015 S/R[402]		Systematic review of 14 RCTs	915	EPA + DHA	multiple	4-24 weeks	leptin
Hartweg J et al S/R 2008[403]	Type 2 DM	Systematic review of 23 RCTs	1075	EPA + DHA	multiple	3-24 weeks	LDL, VLDL
Herrmann W et al 1995[404]		RCT placebo controlled	53	EPA + DHA	rapeseed oil	4 weeks	Lp(a)
Huang T et al 2011 M/A[405]		Meta-analysis of 11 RCTs	702	EPA + DHA	multiple	6-48 weeks	homocysteine
Hung A et al 2015[406]		RCT placebo controlled	34	EPA + DHA	control capsules	6 weeks	ADMA, IL6, TNF, MCP1
Itariu B et al 2012[407]	Severe obesity	RCT placebo controlled	55	EPA + DHA	butterfat	8 weeks	leptin, IL6
Jacobson T 2012[408]		RCT placebo controlled	229	EPA	control capsules	12 weeks	oxLDL, LDL, VLDL, non-HDL
Jellema A et al 2004[409]	Obesity and pregnancy	RCT placebo controlled	11	EPA + DHA	sunflower oil	6 weeks	IL6, TNF
Kastelein J et al 2014[206]	Hypertriglyceridemia	RCT placebo controlled	364	EPA + DHA	olive oil	12 weeks	VLDL, HDL, LDL, non-HDL
Kooshki A et al 2011[410]	ESKD on haemodialysis	RCT placebo controlled	34	EPA + DHA	medium chain trigs	10 weeks	IL6, TNF
Lewis N et al 2000[411]		RCT placebo controlled	25	EPA + DHA	control diet	6 weeks	LDL
Li K, et al 2014 M/A[412]		Meta-analysis of 68 RCTs	4601	EPA + DHA	multiple	2-26 weeks	IL6, TNF
Lopez-Alarcon M et al 2012[413]	Septic neonates	RCT placebo controlled	63	DHA	olive oil	14 days	IL6, IL1b, TNF
Maki K et al 2008[414]	Mixed dyslipidemia	RCT placebo controlled	39	EPA + DHA	vegetable oil	6 weeks	VLDL, LDL, non-HDL
Maki K et al 2011[415]		RCT placebo controlled	31	EPA + DHA	soy oil	6 weeks	VLDL LDL
Maki K et al 2013[416]	Hypertriglyceridemia on statins	RCT placebo controlled	647	EPA + DHA	olive oil	6 weeks	LDL, VLDL
Maki K et al 2015[417]	Hypertriglyceridemia	RCT placebo controlled	102	EPA	olive oil	8 weeks	LDL, oxLDL, non-HDL
Malekshahi Moghadam A et al 2012[418]	Type 2 DM	RCT placebo controlled	84	EPA + DHA	sunflower oil	8 weeks	TNF
Mesa M et al 2004[419]		RCT placebo controlled	42	EPA + DHA	olive oil	4 weeks	oxLDL
Metkus T et al 2013[420]	HIV with hypertriglyceridemia	RCT placebo controlled	48	EPA + DHA	corn oil	8 weeks	TNF, IL6, non-HDL

Mirhashemi S et al 2016[421]	Type 2 DM with nephropathy	RCT placebo controlled	60	EPA + DHA	control capsules	12 weeks	AGEs
Mocellin C et al 2013[422]	Colorectal Cancer	RCT placebo controlled	11	EPA + DHA	not stated	9 weeks	IL1b, TNF
Moeinzadeh F et al 2016[423]	ESKD on haemodialysis	RCT placebo controlled	52	EPA + DHA	control capsules	6 months	LDL
Mori T et al 1991[424]		RCT placebo controlled	27	EPA	olive oil	3 weeks	LDL
Mori T et al 2000[288]	Hyperlipidemia	RCT placebo controlled	56	EPA or DHA	olive oil	6 weeks	LDL
Mori T et al 2004[211]	Overweight and HT	RCT placebo controlled	63	EPA + DHA	control diet	16 weeks	leptin
Mosca L et at 2017[425]		RCT placebo controlled	215	EPA + DHA	not stated	12 weeks	LDL, VLDL, oxLDL
Mostad I et al 2008[426]	Type 2 DM	RCT placebo controlled	26	EPA + DHA	corn oil	9 weeks	oxLDL
Mostowik M et al 2013[427]		RCT placebo controlled	48	EPA + DHA	soybean oil	30 days	leptin
Muldoon M et al 2016[428]		RCT placebo controlled	261	EPA + DHA	soybean oil	18 weeks	IL6
Murphy KJ et al 2006[429]		RCT placebo controlled	30	EPA + DHA	olive oil	6 weeks	IL1b, TNF
Nielsen A et al 2007[430]	Crohn's disease	RCT placebo controlled	31	EPA + DHA	omega-6 enriched supplement	9 weeks	leptin
Nilsson A et al 2012[431]		RCT placebo controlled	40	EPA + DHA	olive oil	5 weeks	TNF
Oliveira J et al 2015[432]	HIV	RCT placebo controlled	83	EPA + DHA	soy oil	24 weeks	IL1b, IL6, TNF
Olszewski A et al 1993[433]		RCT placebo controlled	15	EPA + DHA	olive oil	3 weeks	homocysteine
Palomäki A et al 2010[434]		RCT placebo controlled	37	EPA + DHA	butter	8 weeks	oxLDL
Paoli A et al 2015[435]		RCT placebo controlled	34	EPA + DHA	control diet	4 weeks	IL6, IL1b, TNF, LDL
Pedersen H et al 2010[436]		RCT placebo controlled	78	EPA + DHA	vegetable oil	16 weeks	LDL, non-HDL
Pooya S et al 2010[437]	Type 2 DM	RCT placebo controlled	81	EPA + DHA	sunflower oil	2 months	homocysteine, LDL
Poreba M et al 2017[438]	Type 2 DM and cardiovascular disease	RCT placebo controlled	74	EPA + DHA	control therapy	3 months	LDL, IL6, TNF, leptin
Rizza S et al 2009[439]		RCT placebo controlled	50	EPA + DHA	olive oil	12 weeks	IL6, TNF, LDL
Root M et al 2013[440]		RCT placebo controlled	57	EPA + DHA	safflower oil	4 weeks	IL6, IL8, TNF
Rytter D et al 2011[441]		RCT placebo controlled	533	EPA + DHA	olive oil	3 months	LDL
Sabour H et al 2015[442]	Spinal cord injury	RCT placebo controlled	104	EPA + DHA	control capsules	14 months	leptin
Simons L et al 1985[443]		RCT placebo controlled	25	EPA + DHA	olive oil	3 months	VLDL LDL
Singer P et al 2004[444]		RCT placebo controlled	65	EPA + DHA	olive oil	6 months	LDL

Skouroliakou M et al 2016[445]		RCT placebo controlled	60	EPA + DHA	soybean oil	30 days	IL6, TNF
Su H et al 2015[446]	Metabolic syndrome	RCT placebo controlled	143	EPA + DHA	control diet	12 weeks	LDL, IL6
Sundrarjun T et al 2004[447]		RCT placebo controlled	35	EPA + DHA	control diet	24 weeks	IL6, TNF
Suzukawa M et al 1995[448]		RCT placebo controlled	20	EPA + DHA	corn oil	12 weeks	LDL
Tardivo A et al 2015[449]	Metabolic syndrome	RCT placebo controlled	63	EPA + DHA	control diet	6 months	LDL, IL1b, TNF, IL6
Tartibian B et al 2011[450]		RCT placebo controlled	45	EPA + DHA	soybean plus corn oil	48 hours	IL6, TNF
Tayebi-Khosroshahi H et al 2013[451]	ESKD on haemodialysis	RCT placebo controlled	88	EPA + DHA	control capsules	8 weeks	homocysteine, LDL
Vedin I et al 2008[452]	Alzheimers disease	RCT placebo controlled	174	EPA + DHA	corn oil	6 months	IL1b, IL6, IL8, TNF
Vega-Lopez S et al 2004[453]		RCT placebo controlled	80	EPA + DHA	control capsules	12 weeks	IL1b, IL6, TNF
Vessby B et al 1990[454]	Type 2 DM	RCT placebo controlled	14	EPA + DHA	olive oil	16 weeks	VLDL, LDL
Vikøren L et al 2013[455]		RCT placebo controlled	34	EPA + DHA	control capsules	8 weeks	LDL
Wallace F et al 2003[456]		RCT placebo controlled	40	EPA + DHA	linolenic acid	12 weeks	IFN, TNF, IL1b, IL6
Westerveld T et al 1993[457]	Type 2 DM	RCT placebo controlled	24	EPA	olive oil	8 weeks	LDL
Xin W, et al 2012 M/A[458]	Congestive heart failure	Meta-analysis of 7 RCTs	465	EPA + DHA	multiple	3-12 months	TNF, IL1b, IL6
Yusof H et al 2008[459]		RCT placebo controlled	21	EPA + DHA	medium chain saturated fatty acids	8 weeks	IL6
Zeman M et al 2006[460]		RCT placebo controlled	24	EPA + DHA	olive oil	3 months	Lp(a), homocysteine
Zhang J et al 2010[461]		RCT placebo controlled	92	EPA + DHA	control diet	8 week	IL6, LDL
Zheng J et al 2016[462]		RCT placebo controlled	185	EPA + DHA	corn oil	180 days	LDL

CHAPTER 4: Fish Oil Cell Uptake Study of INflammation (FOCUS IN). A RANDOMISED CONTROLLED TRIAL OF FISH OIL SUPPLEMENTATION IN HEALTHY VOLUNTEERS

ABSTRACT

Background: Acute vascular inflammation (AVI) is an early and critical stage of atherogenesis. Omega-3 fatty acids have anti-inflammatory properties, and although they have been studied in the setting of AVI, the study settings have typically not reflected physiological conditions. With the findings of the recent REDUCE-IT study that omega-3 fatty acids reduce cardiovascular events in high-risk patients, there is a need to understand the factors underlying this benefit. This study aimed to examine the effects of omega-3 fatty acids on AVI on a cellular level, in an *in vitro* setting that was physiological and translatable.

Methods: Forty healthy volunteers with a low baseline omega-3 fatty acid consumption were randomised to treatment with either (1) fish oil high in EPA (86% of total mass), (2) fish oil high in DHA (90% of total mass), (3) fish oil with a standard 2:1 EPA:DHA ratio, or (4) placebo oil, for 30 days. Baseline and post-treatment heart rate (HR), blood pressure (BP), lipids (including total cholesterol, LDL-C, HDL-C, and triglycerides), fatty acid profile, and inflammatory markers (high-sensitivity CRP and lipoprotein[a]) were measured. Serum taken pre- and post-treatment was added to tumour necrosis factor-alpha-stimulated human umbilical vein endothelial cells (HUVECs) in culture. Gene expression of markers of AVI: VCAM-1, ICAM-1, MCP-1 and NFκBp65 were measured by RT-PCR. To identify any associations between gene expression and participant characteristics, correlations were made between the expression of inflammatory markers and lipid parameters, omega 6:3 ratio, blood EPA content, blood DHA content, and saturated fats.

Results: The baseline parameters of participants included: age 38.5±11.0 years, 70% female, LDL-C 2.9±0.8 mmol/L, HDL-C 1.6±0.3 mmol/L, and triglycerides 1.1±0.5 mmol/L. Blood EPA and DHA levels increased significantly and predictably in the different fish oil groups compared to placebo. DHA reduced triglycerides by 27% (p=0.02 compared to placebo, see Abstract Table). None of the fish oil treatments significantly altered cholesterol parameters,

heart rate or systolic BP. DHA reduced diastolic BP by 4.1±1.8 mmHg, which was significantly more than placebo (p=0.05, corrected for baseline values). The gene expression of MCP-1 by TNF-stimulated HUVECs reduced by 59% in the EPA group compared to baseline (p=0.02), and was 25% lower than in the placebo group (p=0.03, corrected for baseline values). No significant correlation was present between the expression of markers of AVI and blood omega-3 levels. A positive, significant association was present between HDL-C levels and both VCAM-1 (r=0.36, p=0.02) and MCP-1 (r=0.40, p=0.01) expression.

Conclusions: The administration of serum from healthy volunteers supplemented with EPA to TNF-stimulated endothelial cells reduced gene expression of MCP-1, indicating a favourable effect of EPA on AVI. Higher fasting HDL-C levels associated with greater serum-induced expression of endothelial inflammatory factors implicated in early atherosclerosis. The positive correlation between HDL-C and endothelial inflammatory factors is consistent with HDL dysfunctionality at very high HDL-C levels, and this warrants further investigation.

Abstract Table: Changes in lipid parameters and inflammatory markers

	High EPA	High DHA	EPA:DHA 2:1	Placebo	P value
LDL-C	0%	-7%	-7%	-4%	0.49
HDL-C	-1%	+7%	+1%	+6%	0.37
TG	+3%	-27% P=0.02*	-18%	+8%	0.03
CRP	+22%	+22%	-17%	-25%	0.28
VCAM-1	+5%	-31%	-23%	+4%	0.41
ICAM-1	+27%	-69%	-42%	-21%	0.19
MCP-1	-59% p=0.02*	-22%	0%	+9%	0.03
NFκB-p65	-6%	-40%	-40%	-7%	0.51
*P value compared with baseline					

I, Anthony Pisaniello, conceived, designed, executed and analysed all of the work included in this chapter.

4.1 Background

Since the time a cardioprotective effect of polyunsaturated fatty acids was first proposed[221], researchers have been considering and investigating potential anti-atherosclerotic mechanisms. In the randomised controlled trials of fish oil in the primary and secondary prevention setting that demonstrated a reduction in major adverse cardiac events, such as JELIS[227] and GISSI-Prevenzione[463], the benefits were observed independent of cholesterol levels. Given the inflammatory nature of atherosclerosis[4], it may be the anti-inflammatory effects of fish oil that have contributed to their atheroprotective effects. Although the anti-inflammatory effects of fish oil are wide-ranging, it is likely to be the effects on vascular inflammation that are most important in the setting of cardiovascular disease.

Previous studies have examined the effects of omega-3 fatty acids on markers of vascular inflammation, particularly in the *in vitro* setting. Acute vascular inflammation (AVI) can be induced by stimulating vascular endothelial or smooth muscle cells with pro-inflammatory agents such as TNF-α and lipopolysaccharide. The adhesion molecules and cytokines produced can be measured at the gene and protein level. Chronic vascular inflammation (CVI) as it pertains to atherosclerosis is more complex, as it occurs in conjunction with plaque formation and not in isolation[464]. To summarise the effects of omega-3 fatty acids on *in vitro* models of AVI from less than 10 published studies, both EPA and DHA reduce adhesion molecule expression, with a greater effect seen with DHA compared to EPA[242, 244-247]. In these studies, EPA and DHA were added in purified forms. Although these studies have provided mechanistic insights into the effects of omega-3 fatty acids on AVI, they have limited translatability as they do not model *in vivo* conditions. Since omega-3 fatty acids undergo metabolism and oxidation after oral consumption, the genetic, humoral and cellular responses *in vivo* are likely to be different to what is measured in the cell culture setting. Indeed, oxidised omega-3 fatty acids have different actions to pure unoxidised fatty acids, and are still

beneficial, if not superior[250]. Sethi et al demonstrated that oxidised, but not native unoxidised EPA significantly inhibited human neutrophil and monocyte adhesion to endothelial cells *in vitro* by inhibiting endothelial adhesion receptor expression[251]. Similarly, Mishra et al demonstrated that oxidised but not unoxidised EPA and DHA inhibit cytokine-induced endothelial expression of MCP-1 and IL-8[252]. A translatable, physiological method of studying the effects of omega-3 fatty acids is to deliver them to cells after oral consumption. The concept of adding serum from humans to endothelial cells in culture and studying its effects has been utilised previously and demonstrated to be effective[465].

Fish oil is available in numerous formulations commercially, including with high concentrations of EPA or DHA. The most commonly available preparations have an EPA:DHA ratio of between 1.5:1 and 3:1[466]. The availability of a wide range of fish oil products allows the relative effects of EPA and DHA on AVI to be studied.

4.1.1 Aims and rationale of study

The primary objective of the FOCUS IN study was to evaluate and compare the effects of different omega-3 fish oil preparations on endothelial cell markers of acute vascular inflammation after supplementation in healthy volunteers. FOCUS IN should identify an optimal omega-3 fish oil formulation which could be further studied in a primary and secondary prevention setting.

The omega-3 formulations studied in FOCUS IN would be (1) high in EPA, (2) high in DHA, and (3) have a standard 2:1 ratio of EPA:DHA. These formulations would be compared with a placebo. Although the cardioprotective effects of omega-3 fatty acid supplementation have been questioned in a recent meta-analysis[467], the formulations and doses evaluated in that meta-analysis were highly heterogeneous. In high-risk patients, daily doses of $\geq$ 4 grams of omega-3 fatty acids have been shown to optimally reduce blood pressure, triglycerides, total

cholesterol, and circulating inflammatory markers[209, 368, 468]. Furthermore, this dose has been shown to reduce major adverse cardiac events[234].

4.1.2 Hypotheses

The hypotheses of this study were that:

- Omega-3 fatty acids would reduce endothelial cell markers of AVI, with EPA having a superior effect over DHA.
- Changes in endothelial cell markers of acute vascular inflammation with omega-3 fatty acids would occur independent of any effects on plasma cholesterol and triglyceride levels.
- Blood levels of omega-3 fatty acids would correlate inversely with the gene expression of markers of AVI.

4.2 Methods

4.2.1 Study outline

Forty healthy adult volunteers were recruited from the staff and students of the South Australian Health and Medical Research Institute for participation in the study. The volunteers were required to fulfil the eligibility criteria listed below:

Eligibility criteria

Healthy males and females aged ≥ 18 years

Has not taken omega-3 supplements or eaten more than one fish meal per week in the preceding 6 months.

No bleeding tendency or significant health problems

Not allergic to fish or fish oil supplements

Not currently taking medications that are anti-inflammatory or increase bleeding risk.

Not currently pregnant or breastfeeding

No anticipated need to stop taking fish oil in the next 30 days, such as upcoming surgery.

Volunteers who satisfied the eligibility criteria underwent a baseline health assessment at a clinic visit, which included measurement of height, weight, blood pressure and heart rate. A blood sample (20 mL) was taken by venepuncture; half of the sample was used to extract serum (as per Section 2.1) and then stored at -80ºC, and half was used for biochemical analysis. For this, 30 microlitres of whole blood was spotted onto dry blood spot cards for fatty acid analysis (as per Section 2.2), and the remainder was analysed for serum levels of total cholesterol, LDL-C, HDL-C, high-sensitivity C-reactive protein (hs-CRP) and lipoprotein(a) (Lp(a)).

The forty participants were randomised in a double-blind fashion to one of four treatment groups using random number generation by an unblinded investigator.

The four treatment groups (10 participants each) were:

(1) A high EPA-containing fish oil, "PharmEPA® Step 1 Restore" (Igennus HealthCare Nutrition, Cambridge, UK). Each 580mg capsule contains 500mg of EPA (86% of total mass) and no detectable DHA. Omega-3 is in triglyceride form. Participants were give 8 capsules per day to achieve 4 g of omega-3.

(2) A high DHA-containing fish oil, "Nature's Own™ Red Algal Omega-3" (Sanofi Consumer Healthcare, Virginia, QLD, Australia). Each 787.5mg capsule contains 707.5mg of DHA (90% by mass), and 30mg of EPA (4% by mass). Omega-3 is in triglyceride form. Participants were given 6 capsules per day to achieve 4 g of omega-3.

(3) Fish oil containing a standard EPA:DHA ratio of 2:1, "AlaskOmega® 400200 TG" (Bioriginal, Den Bommel, Netherlands). Each 1000mg capsule contains 420mg of EPA (42%

by mass) and 209mg of DHA (21% by mass). Omega-3 is in triglyceride form. Participants were given 7 capsules per day to achieve 4 g of omega-3.

(4) Placebo capsule (Bioriginal, Den Bommel, Netherlands). Each 1000mg capsule contains a mixture of palm oil, sunflower oil, rapeseed oil, and fish oil to preserve the smell and taste of fish oil. Each capsule contained 1% EPA by mass and 0.6% DHA by mass. Participants were given 7 capsules per day.

The participants took the capsules daily for 30 days. During this period, participants kept a diary of capsule consumption and fish intake. After study completion a second health assessment was performed and a blood sample was taken, as before.

4.2.2 Cell culture experiments

A cell culture model of acute vascular inflammation was used to study the impact of the different omega-3 treatments on this process. The stored serum from each participant, taken before and after treatment, was added to cells in culture with the method described below.

Human Umbilical Vein Endothelial Cells (HUVECs) were obtained from fresh umbilical cords donated by the Women's and Children's Hospital, North Adelaide. They were plated in gelatin-coated flasks at a density of 10000 cells per cm^2. They were cultured using MesoEndo Cell Growth Medium (Cell Applications, San Diego, CA, USA) supplemented with an extra 5% of foetal bovine serum (FBS) to make a total of 10% FBS. Passage 3 HUVECs were plated onto 6-well plates until they reached 75% confluence, with 2 ml of media used per well. They were then washed twice with warm sterile PBS and then cultured for 24 hours in EBM-2 basal media plus SingleQuot kit supplements and growth factors without the serum aliquot (Lonza, Basel, Switzerland). The serum was taken from participants at baseline and again at end of study, and was added for 24 hours at a concentration of 10%. Control conditions used 10% foetal bovine serum. All wells were then washed twice with warm sterile PBS, and

then fresh EBM-2 basal media plus SingleQuot kit supplements and growth factors except for serum was added again. For each condition, there was either TNF or no TNF added for 4 hours in serum-free media. For TNF conditions, human TNF-α (Sigma-Aldrich, St. Louis, MO, USA) was added at a concentration of 10 ng/ml. This dose and duration have been demonstrated to significantly increase cell adhesion molecule expression[469, 470].

The cell culture media was then aspirated from each well, immediately placed on dry ice and stored at -80ºC. The cells were washed with PBS at 4ºC, and the Tri-reagent method was then used to extract RNA from the cells (as per Section 2.4.2). The RNA was quantified using a NanoDrop™ 8000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The concentration of RNA was normalised between all samples and then RNA was reverse transcribed to cDNA (as per Section 2.4.4). Reverse transcription PCR was performed (as per Section 2.4.5) to measure the relative expression of the following human genes: VCAM-1, ICAM-1, MCP-1 and NFkBp65, with B2M being used as a reference gene (see Section 2.5 for primers). All PCR reactions used 100ng of cDNA. Due to the large number of 96-well PCR plates used for this experiment, control conditions were used on each PCR plate. These conditions were the culture conditions of (1) HUVECs cultured in 10% FBS, and (2) HUVECs cultured in 10% FBS plus TNF-α. Inter-PCR-plate calibration was performed using Bio-Rad CFX Manager™ software version 3.0.1224.1015 (Hercules, CA, USA). Data analysis was performed using GraphPad Prism 7 (La Jolla, CA, USA). To identify any associations between gene expression and participant characteristics, correlations were made between the expression of inflammatory markers and lipid parameters (total cholesterol, LDL, HDL, triglycerides), omega 6:3 ratio, blood EPA content, blood DHA content, and saturated fats.

4.2.3 Statistical and data analysis

4.2.3.1 Sample size calculation

The sample size of 10 participants per group was based on a study by Jiang et al[471]. The most important factor for calculating sample size was the gene expression of VCAM-1. In Jiang et al's study, the active treatment, irbesartan, reduced TNF-α-induced VCAM gene expression by 45% and this was statistically significant. Using a one-way ANOVA power calculation for 4 groups with a significance level of 0.05, with 80% power, for an effect size of 0.45, a sample size of 10 subjects per group was required.

4.2.3.2 Statistical methods

Participant data were de-identified, and statistical analysis was performed using GraphPad Prism 7 software. This analysis was performed blinded to treatment allocation, and according to the intention-to-treat principle. The D'Agostino-Pearson normality test was performed to determine whether continuous data were normally-distributed. Normally-distributed data were analysed using the One-way Analysis of Variance (ANOVA) to compare means between multiple groups. If correcting for multiple comparisons, the Dunnett test was used. Results were expressed as mean ± standard error of the mean (SEM). If continuous data were not normally-distributed, analysis was performed using the Kruskal-Wallis test, with Dunn's test used to correct for multiple comparisons. Results were expressed as median + interquartile range (IQR). Statistical correlations were analysed using a linear regression model. Statistical significance was set at the 0.05 level.

4.2.4 Ethical and site approval

Ethics approval was obtained from the University of Adelaide Human Research Ethics Committee. The trial was registered with the Australian New Zealand Clinical Trials Registry

(Trial ID: ACTRN12616000928415). Site specific authorisation was obtained from the South Australian Health and Medical Research Institute.

4.3 Results

4.3.1 Baseline participant characteristics

The demographics of the participants are summarised in Table 4.1. Seventy percent of participants were female, and the mean age of participants was 38.5 years (see Table 4.1). Participants in the EPA group were older, but otherwise participants in all four groups were evenly matched. The baseline body mass index (BMI), resting heart rate, systolic and diastolic BP, lipid parameters, high-sensitivity CRP, Lp(a), and percentage of EPA and DHA in blood are presented in Table 4.1. These parameters were not significantly different between allocated treatment groups at baseline. Participants on average were mildly overweight with a normal blood pressure and mildly elevated resting heart rate. Total cholesterol and LDL-C levels were at the upper end of normal, and the inflammatory markers were low. EPA and DHA levels were consistent with a low omega-3 intake.

Table 4.1 – Summary of baseline participant characteristics by treatment group

	Placebo	Std Fish Oil*	EPA	DHA	p value
Number of participants	10	10	10	10	NA
Male (%)	40	30	30	20	0.36
Age (SD)	34.6 (7.1)	32.7 (7.8)	47.5 (10.2)	39.3 (12.6)	0.02
BMI (SD)	25.4 (2.8)	24.6 (2.2)	25.4 (4.8)	26.1 (3.9)	0.81
Resting HR bpm (SD)	77.8 (8.5)	69.2 (11.4)	70.2 (7.4)	67.4 (11.4)	0.11
Systolic BP mmHg (SD)	126.7 (19.5)	124.3 (12.3)	116.5 (14.2)	119.9 (12.3)	0.43
Diastolic BP mmHg (SD)	80.6 (9.3)	78.8 (6.2)	76.6 (9.5)	76.2 (10.4)	0.67
Tchol mmol/L (SD)	5.0 (0.8)	4.5 (0.8)	5.3 (0.7)	5.3 (1.2)	0.23
LDL-C mmol/L (SD)	3.0 (1.1)	2.4 (0.6)	3.1 (0.6)	3.2 (0.8)	0.11
HDL-C mmol/L (SD)	1.5 (0.2)	1.7 (0.4)	1.7 (0.3)	1.6 (0.4)	0.24
Trigs mmol/L (IQR)	1.3 (1.1-1.5)	0.9 (0.7-1.3)	0.9 (0.7-1.1)	1.0 (0.7-1.2)	0.4
hs-CRP mg/L (IQR)	1.1 (0.9-1.9)	0.7 (0.5-1.4)	0.4 (0.3-2.1)	1.0 (0.8-2.9)	0.5
Lp(a) g/L (IQR)	0.1 (0-0.3)	0.1 (0-0.2)	0.1 (0-1.4)	0.4 (0.1-0.6)	0.72
EPA % (IQR)	0.5 (0.4-0.8)	0.7 (0.6-0.8)	0.8 (0.5-0.9)	0.5 (0.4-0.7)	0.35
DHA % (IQR)	1.7 (1.5-1.8)	1.9 (1.6-2.3)	1.9 (1.6-2.3)	1.7 (1.4-1.8)	0.12

*Std Fish Oil = standard fish oil

Capsule compliance was assessed by an unblinded investigator based on the return of a capsule diary. Capsule compliance in the study was high (94±5%). There was no significant difference in capsule compliance between treatment groups (p=0.63). The average number of treatment days was 30.5±4, and did not differ significantly between treatment groups (p=0.38). One participant discontinued the capsules after 6 days due to gastrointestinal side effects, however completed the follow up assessment and investigations.

4.3.2 Effect of fish oil treatment on physical and biochemical measures

Effects on physical measures

None of the fish oil treatments had a significant effect on resting heart rate (p=0.33, corrected for baseline values, see Figure 4.1).

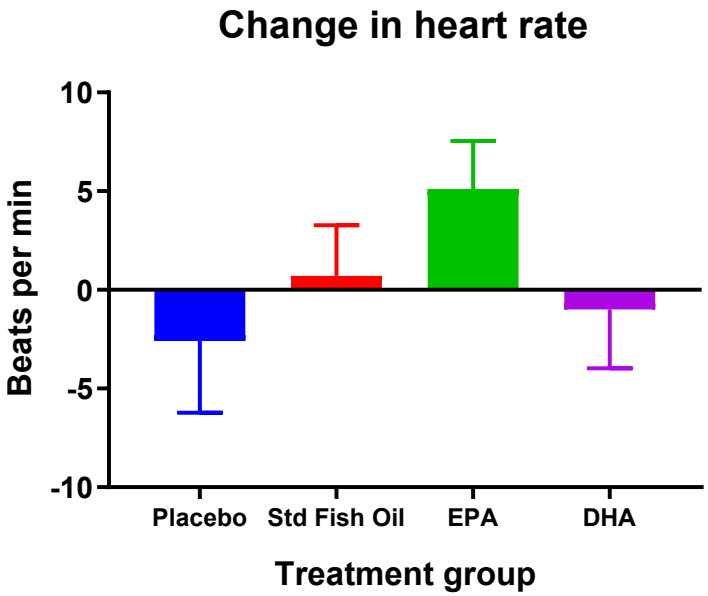


Figure 4.1: Effect of fish oil supplementation on resting heart rate. n = 10 per group. Results expressed as mean ± SEM.

No fish oil treatment had a significant effect on systolic blood pressure (p=0.54). DHA reduced diastolic blood pressure by 4.1±1.8 mmHg, which was significantly more than placebo (p=0.05, corrected for baseline values, see Figure 4.2).

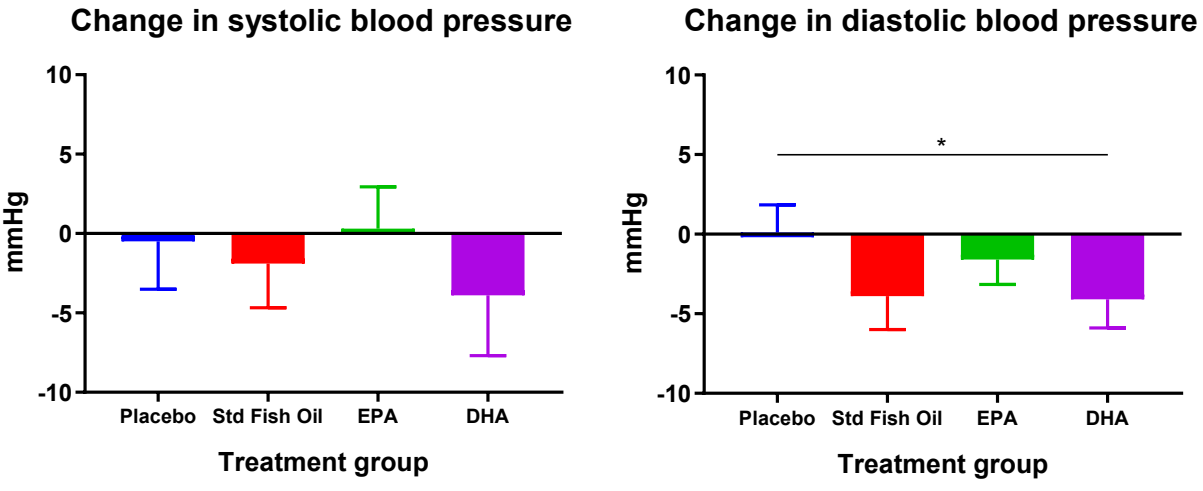


Figure 4.2: Effect of fish oil supplementation on systolic and diastolic blood pressure. n = 10 per group. *p=0.05. Results expressed as mean ± SEM.

None of the fish oil treatments resulted in a significant change in body weight (p=0.80 corrected for baseline values, see Figure 4.3).

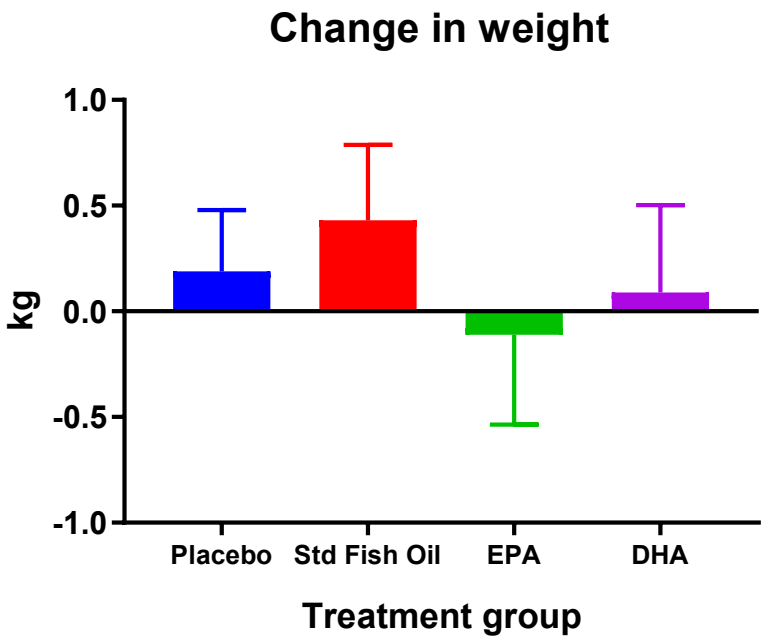


Figure 4.3: Effect of fish oil supplementation on body weight. n = 10 per group. Results expressed as mean ± SEM.

Effects on blood fatty acid levels

The distribution of fatty acids in blood was significantly altered by the fish oil treatments. Both EPA and standard fish oil (which has a predominance of EPA) significantly increased EPA levels in the blood by an average of 253% each ($p<0.001$ for each, corrected for baseline values, see Figure 4.4). There was a slight but non-significant increase in the DHA treatment group.

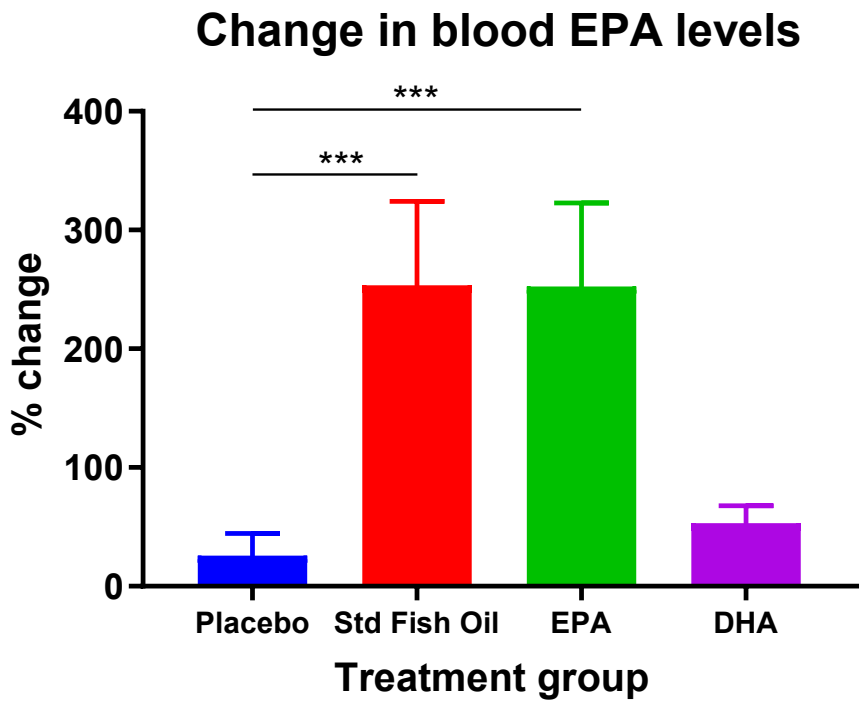


Figure 4.4: Effect of fish oil supplementation on EPA levels in blood, expressed as a percentage of total fatty acids. ***p<0.001. n = 10 per group. Results expressed as mean ± SEM.

Both DHA and standard fish oil supplementation significantly increased DHA levels in blood by 145% ($p<0.001$) and 28% ($p=0.02$) respectively, corrected for baseline values (see Figure 4.5). The changes seen in EPA and DHA levels after fish oil supplementation confirm the efficacy of, and compliance with, the respective capsules.

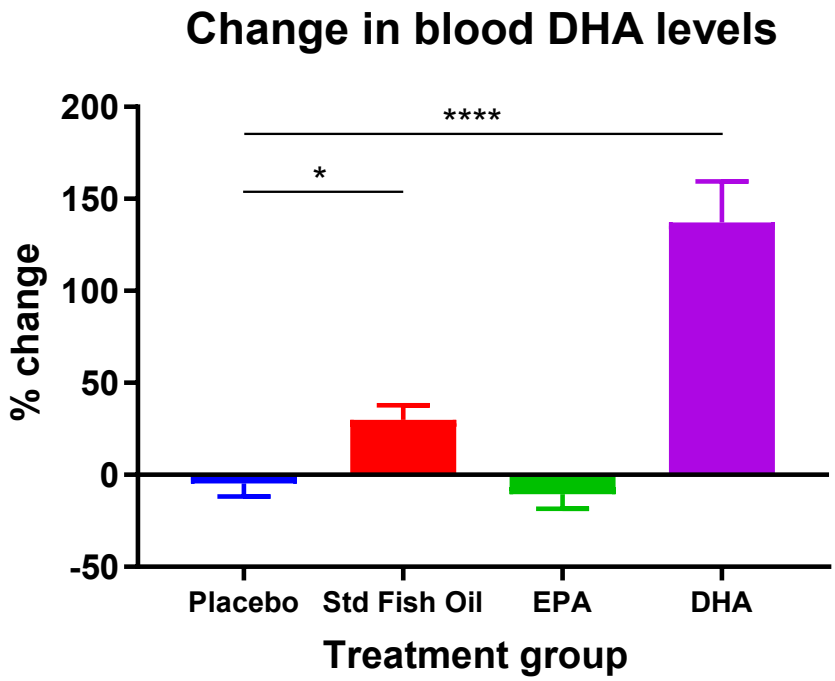


Figure 4.5: Effect of fish oil supplementation on DHA levels in blood, expressed as a percentage of total fatty acids. ****p<0.0001, *p<0.05. n = 10 per group. Results expressed as mean ± SEM.

The omega 6:3 ratio, which has a U-shaped association with mortality[472], was not significantly different between treatment groups at baseline (p=0.79), with a mean ratio of 7.3±1.1. After supplementation, the omega 6:3 ratio was significantly lower in all fish oil treatment groups compared to placebo (43 – 49% reductions, p<0.0001 for all comparisons, see Figure 4.6).

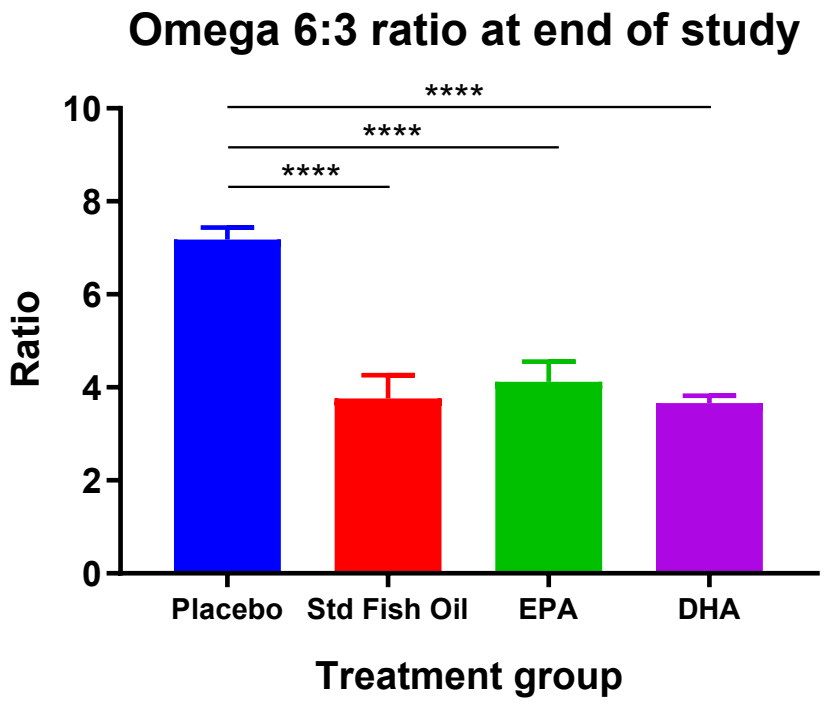


Figure 4.6: Ratio of omega-6 to omega-3 at the end of the study. ****p<0.0001. n = 10 per group. Results expressed as mean ± SEM.

Effects on serum lipids and inflammatory markers

There were no significant differences between treatment groups in levels of either total cholesterol (p=0.29), LDL (p=0.49), or HDL (p=0.37) when correcting for baseline values (see Figure 4.7). DHA significantly reduced triglyceride levels, by an average of 0.31 mmol/L (27% reduction, p=0.018 corrected for baseline values). There was a numerical but non-significant reduction in triglycerides in the standard fish oil group of 0.18 mmol/L (p=0.08, corrected for baseline values, see Figure 4.7).

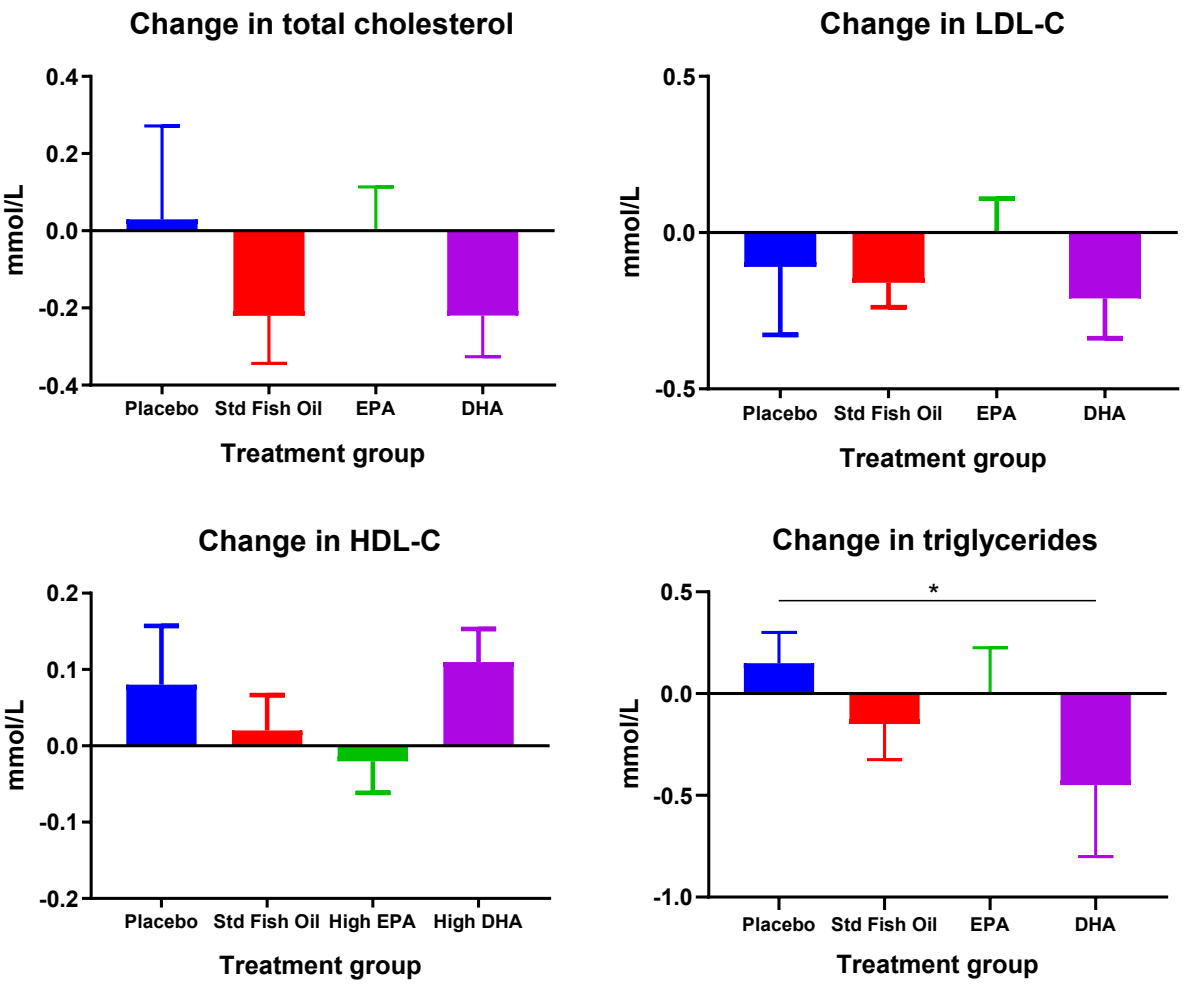


Figure 4.7: Effect of fish oil supplementation on total cholesterol, LDL-C, HDL-C and triglycerides. *p<0.05. n = 10 per group. Results expressed as mean ± SEM, except for triglycerides, which is median + interquartile range.

Levels of lipoprotein(a), which is a particle associated with increased cardiovascular risk[473] and is pro-inflammatory[474], were measured before and after treatment. Complete results were only obtained for 4 to 7 participants per group, because on multiple occasions the request for this uncommonly-performed test was incorrectly interpreted as another lipoprotein or as lipase. Lp(a) levels were low in this cohort, with a median level at baseline of 0.15 (IQR

0.00 – 0.47) g/L (reference range 0 - 0.3 g/L). Fish oil supplementation did not significantly alter Lp(a) levels (p=0.65, corrected for baseline values, see Figure 4.8).

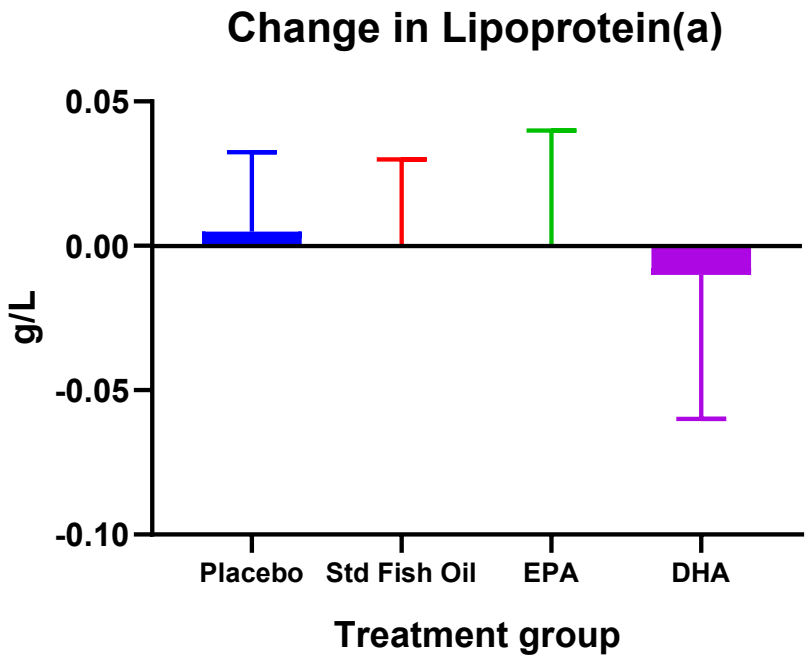


Figure 4.8: Effect of fish oil supplementation on lipoprotein(a) levels. n = 4-7 per group. Results expressed as median + interquartile range.

High-sensitivity C-reactive protein (hs-CRP) levels were generally low, with a median of 0.9 (IQR 0.42 – 2.1) mg/L (reference range 0 - 2mg/L). Fish oil supplementation did not significantly alter hs-CRP levels (p=0.28, corrected for baseline values, see Figure 4.9). There was a trend towards an increase in hs-CRP levels in the EPA group (p=0.10).

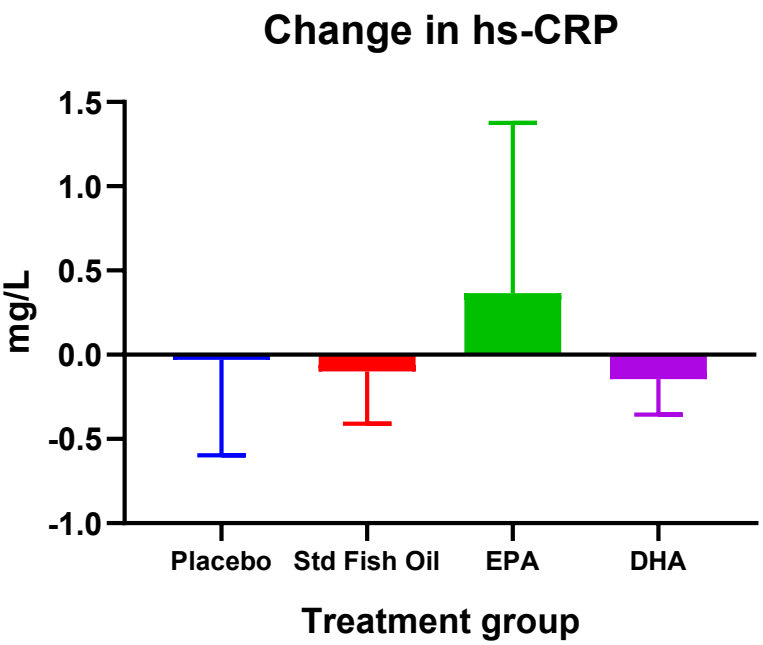


Figure 4.9: Effect of fish oil supplementation on hs-CRP levels. n = 10 per group. Results expressed as median + interquartile range.

4.3.3 In vitro studies of vascular inflammation

Gene expression of VCAM-1, ICAM-1, MCP-1 and NFκB-p65 was measured by RT-PCR in HUVECs co-incubated with serum from participants before and after treatment, and with or without TNF stimulation. As expected, only conditions with TNF stimulation resulted in significantly increased gene expression. This gene expression was compared between the different treatment group allocations at baseline, and was not significantly different (p=0.68 for VCAM-1, p=0.65 for ICAM-1, p=0.16 for MCP-1, and p=0.83 for NFκBp65). This was predictable as the participants were healthy and had comparable characteristics as evident in Table 4.1. Relative gene expression was set at 100% for the TNF-stimulated expression with serum taken at baseline.

The VCAM-1 expression of TNF-stimulated HUVECs co-incubated with serum obtained at study completion was not significantly different between treatment groups. There was a trend towards a reduction in the standard fish oil group compared to placebo (35% reduction, p=0.11 corrected for baseline values, see Figure 4.10).

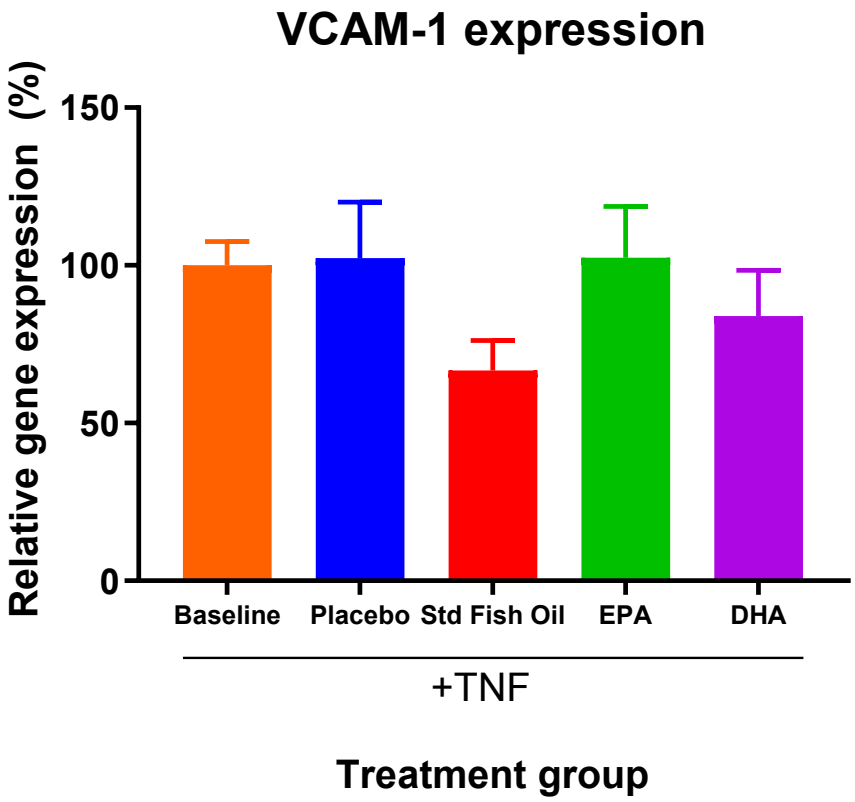


Figure 4.10: Relative gene expression of VCAM-1 by TNF-stimulated HUVECs co-incubated with serum obtained at study completion, relative to baseline. n = 40 for combined baseline and n = 10 for treatment groups. Results expressed as mean ± SEM.

ICAM-1 expression was also not significantly different between treatment groups (p=0.25, corrected for baseline values, see Figure 4.11).

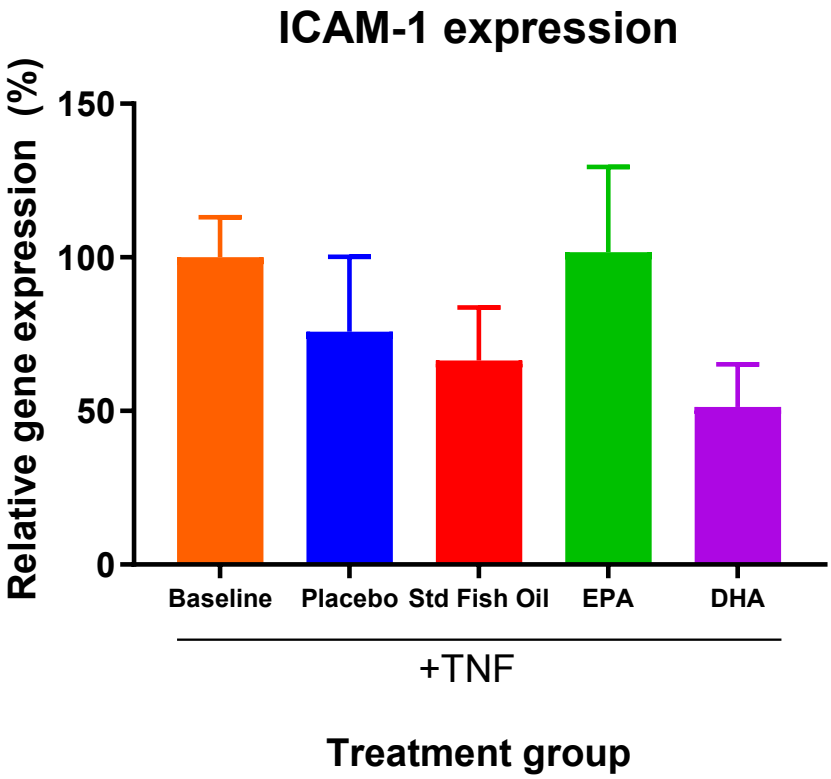


Figure 4.11: Relative gene expression of ICAM-1 by TNF-stimulated HUVECs co-incubated with serum obtained at study completion, relative to baseline. n = 40 for combined baseline and n = 10 for treatment groups. Results expressed as mean ± SEM.

MCP-1 expression was 25% lower in the EPA group compared to placebo (p=0.03 corrected for baseline values, see Figure 4.12). There was a trend towards a reduction in the standard fish oil group compared to placebo (19% reduction, p=0.10 corrected for baseline values).

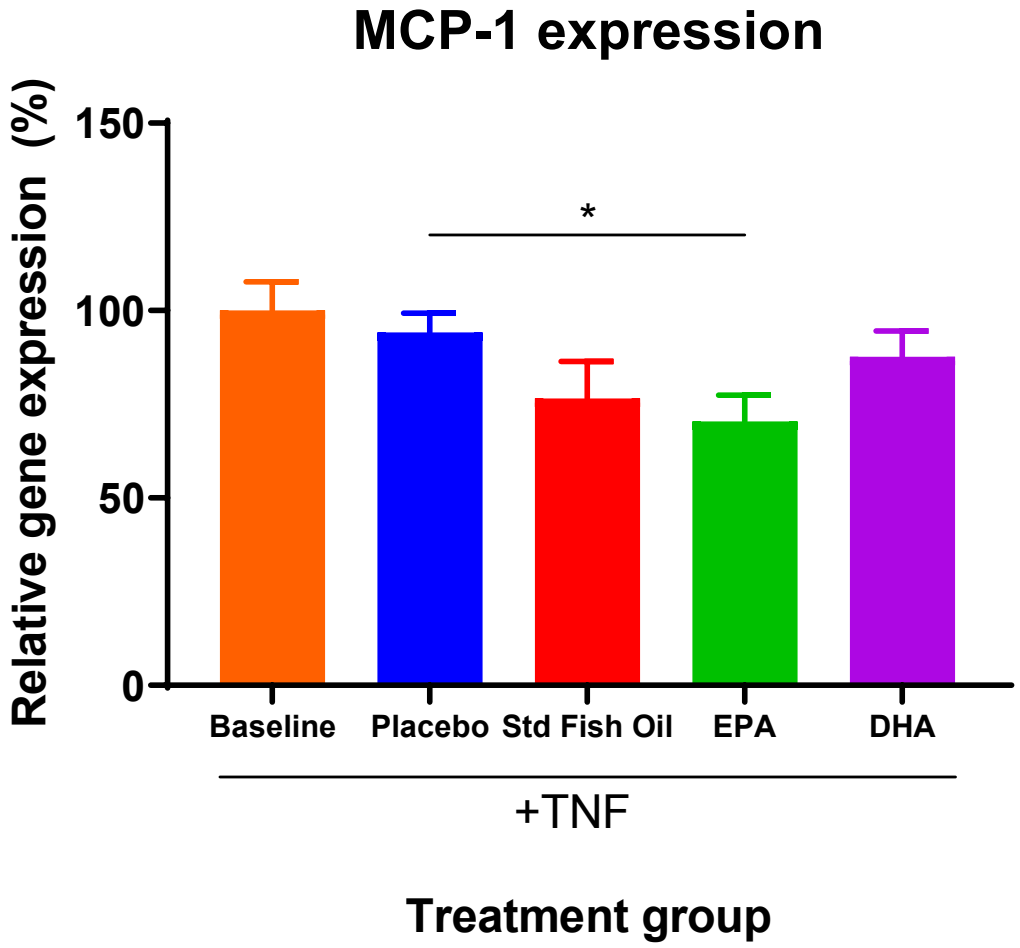


Figure 4.12: Relative gene expression of MCP-1 by TNF-stimulated HUVECs co-incubated with serum obtained at study completion, relative to baseline. *p<0.05. n = 40 for combined baseline and n = 10 for treatment groups. Results expressed as mean ± SEM.

Gene expression of the pro-inflammatory transcription factor NFκBp65 was not significantly altered by any fish oil supplement (p=0.63 corrected for baseline values, see Figure 4.13).

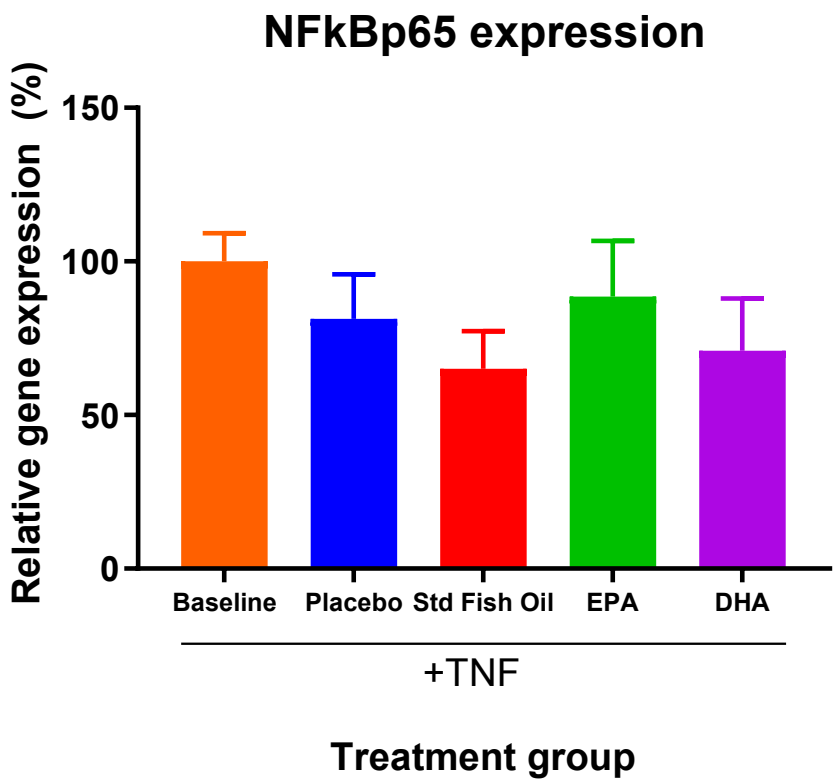


Figure 4.13: Relative gene expression of NFκBp65 by TNF-stimulated HUVECs co-incubated with serum obtained at study completion, relative to baseline. n = 40 for combined baseline and n = 10 for treatment groups. Results expressed as mean ± SEM.

4.3.4 Correlations between inflammatory markers and circulating factors

The expression of VCAM-1, ICAM-1, MCP-1 and NFκBp65 by stimulated HUVECs was correlated with the following patient characteristics to determine whether any significant associations exist: total cholesterol, LDL-C, HDL-C, triglycerides, omega 6:3 ratio, blood EPA levels, blood DHA levels, and saturated fats.

Total cholesterol

No significant correlations were present between total cholesterol and VCAM-1, ICAM-1, MCP-1 or NFκBp65 (see Figure 4.14).

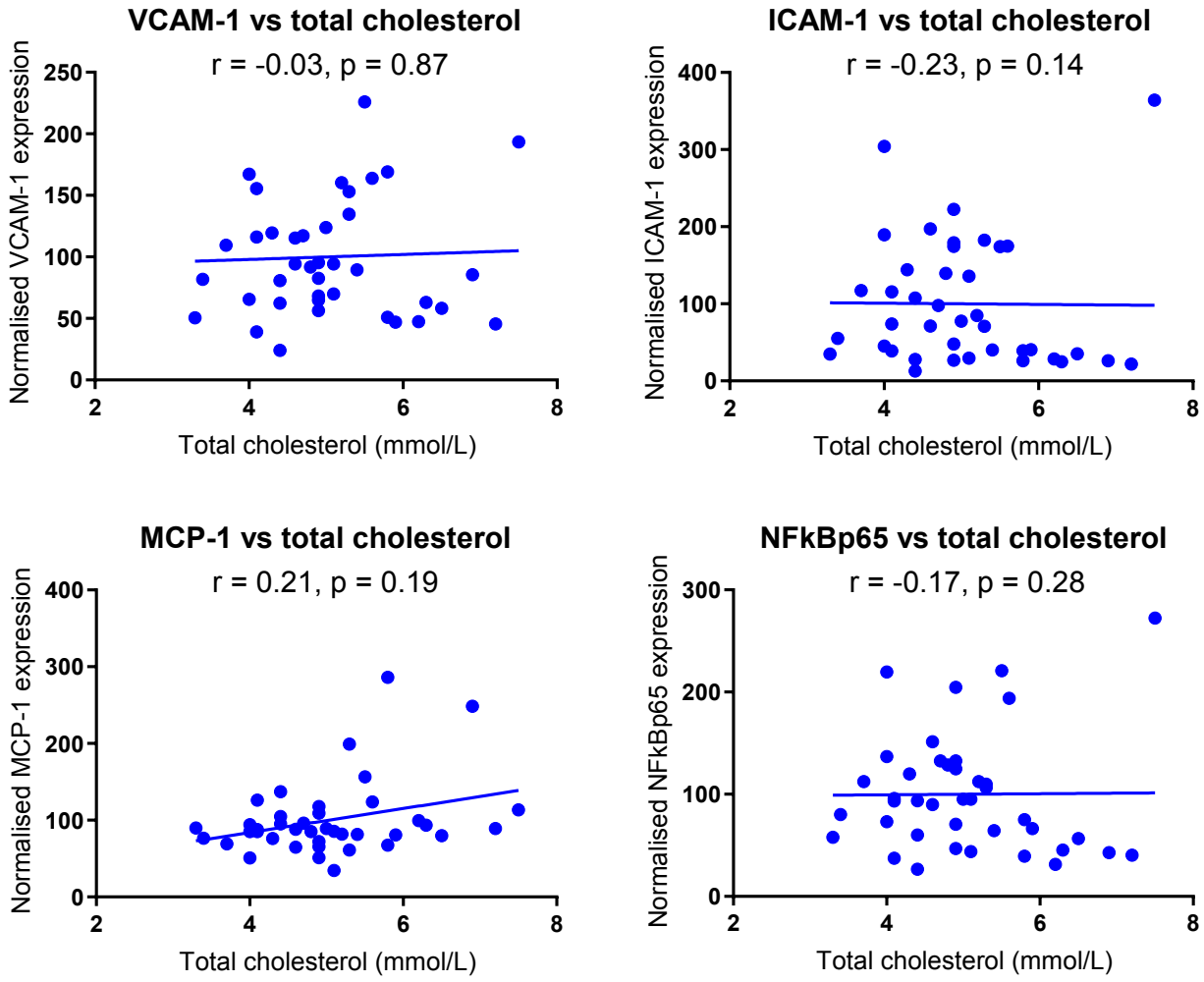


Figure 4.14: Correlations of gene expression of VCAM-1, ICAM-1, MCP-1, NFκBp65 and total cholesterol. n = 40 for each graph.

LDL-C

No significant correlations were present between LDL-C and VCAM-1, ICAM-1, MCP-1 or NFκBp65 (see Figure 4.15).

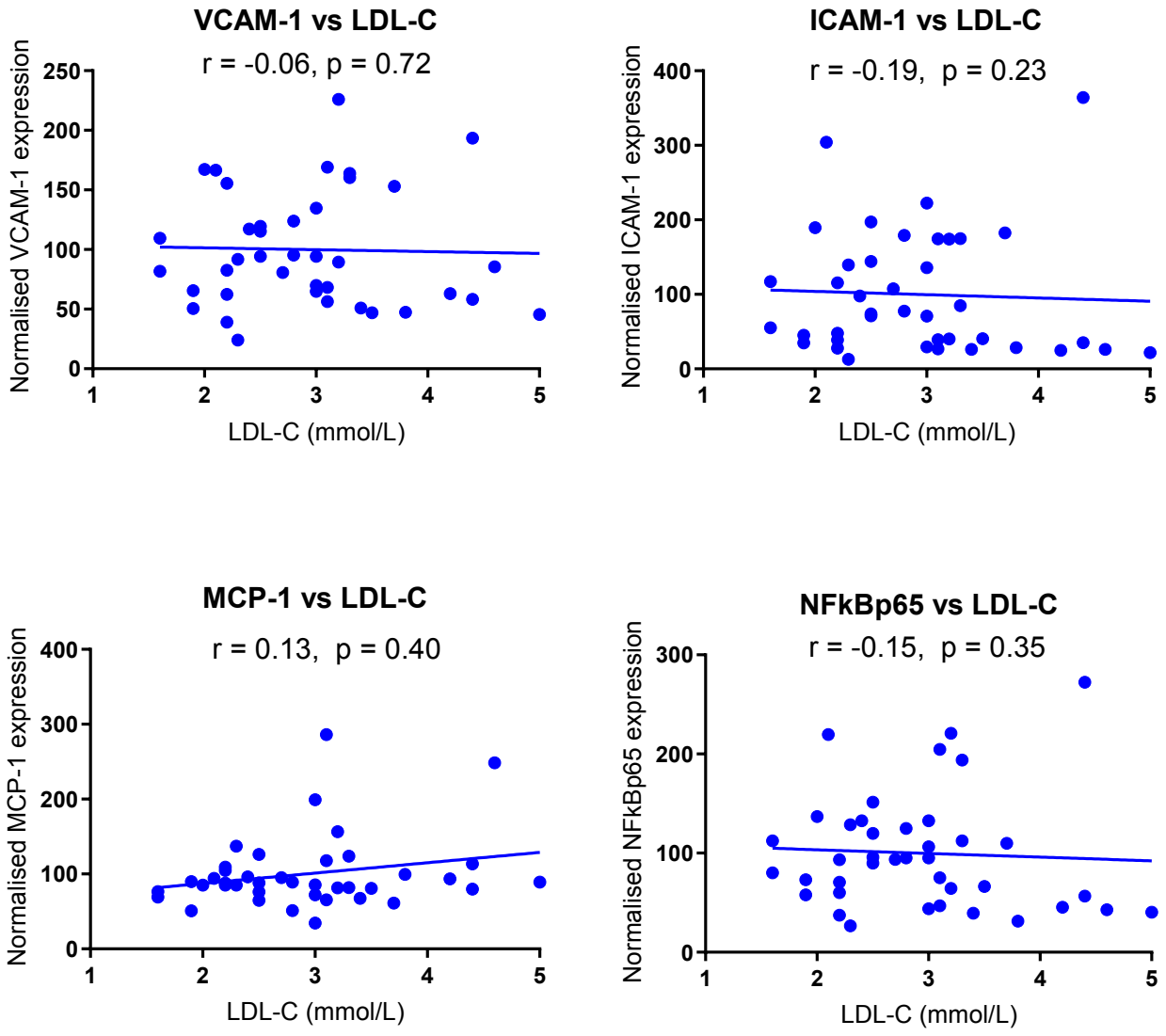


Figure 4.15: Correlations of gene expression of VCAM-1, ICAM-1, MCP-1, NFκBp65 and LDL-C. n = 40 for each graph.

HDL-C

A positive, significant association was present between HDL-C levels and both VCAM-1 and MCP-1 expression, but not ICAM-1 or NFκBp65 expression (see Figure 4.16).

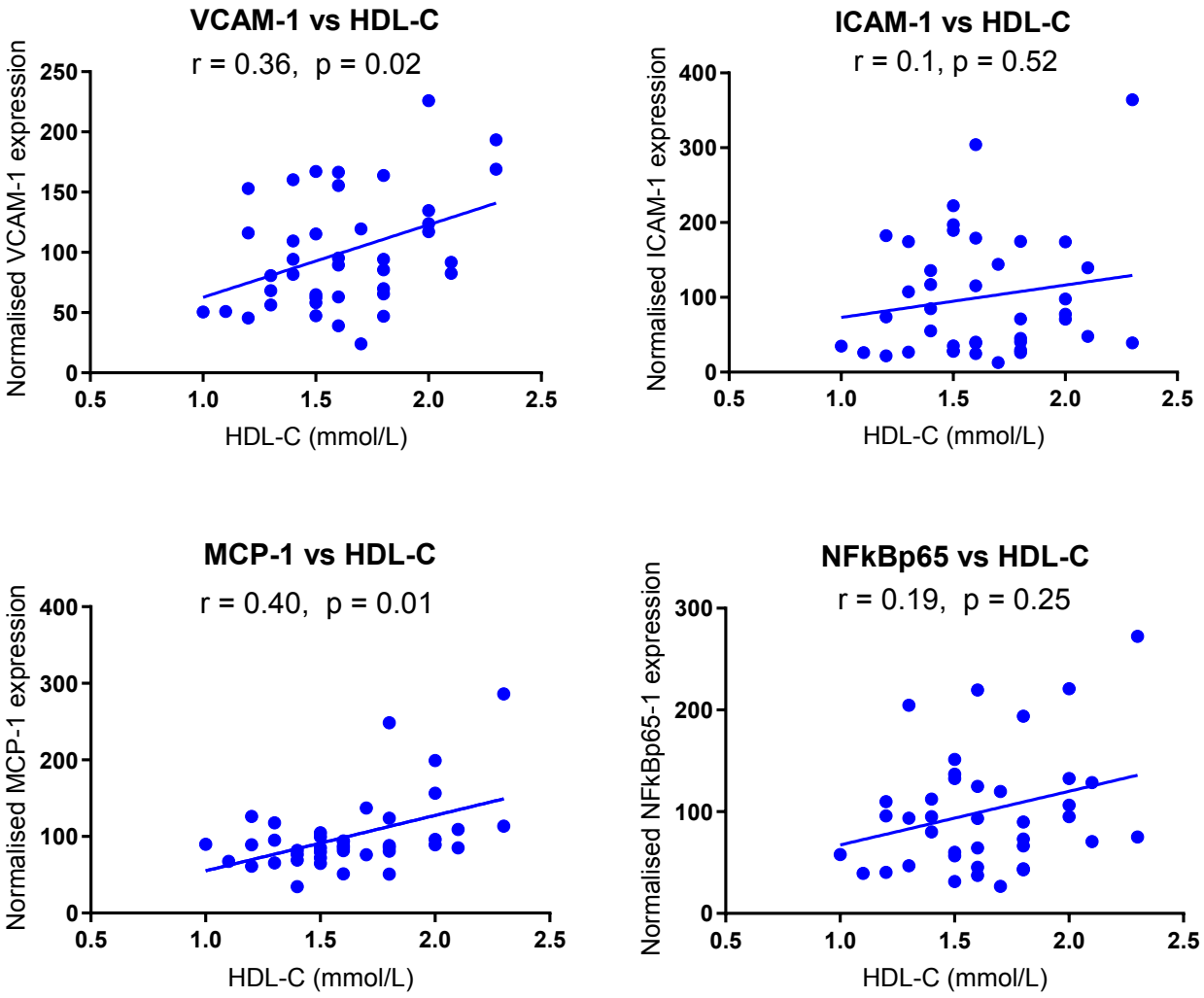


Figure 4.16: Correlations of gene expression of VCAM-1, ICAM-1, MCP-1, NFκBp65 and HDL-C. n = 40 for each graph.

Triglycerides

A non-significant inverse correlation (r = -0.3, p=0.06) was present between VCAM-1 and triglyceride levels. No significant correlations were present between triglycerides and either ICAM-1, MCP-1 and NFκBp65 (see Figure 4.17).

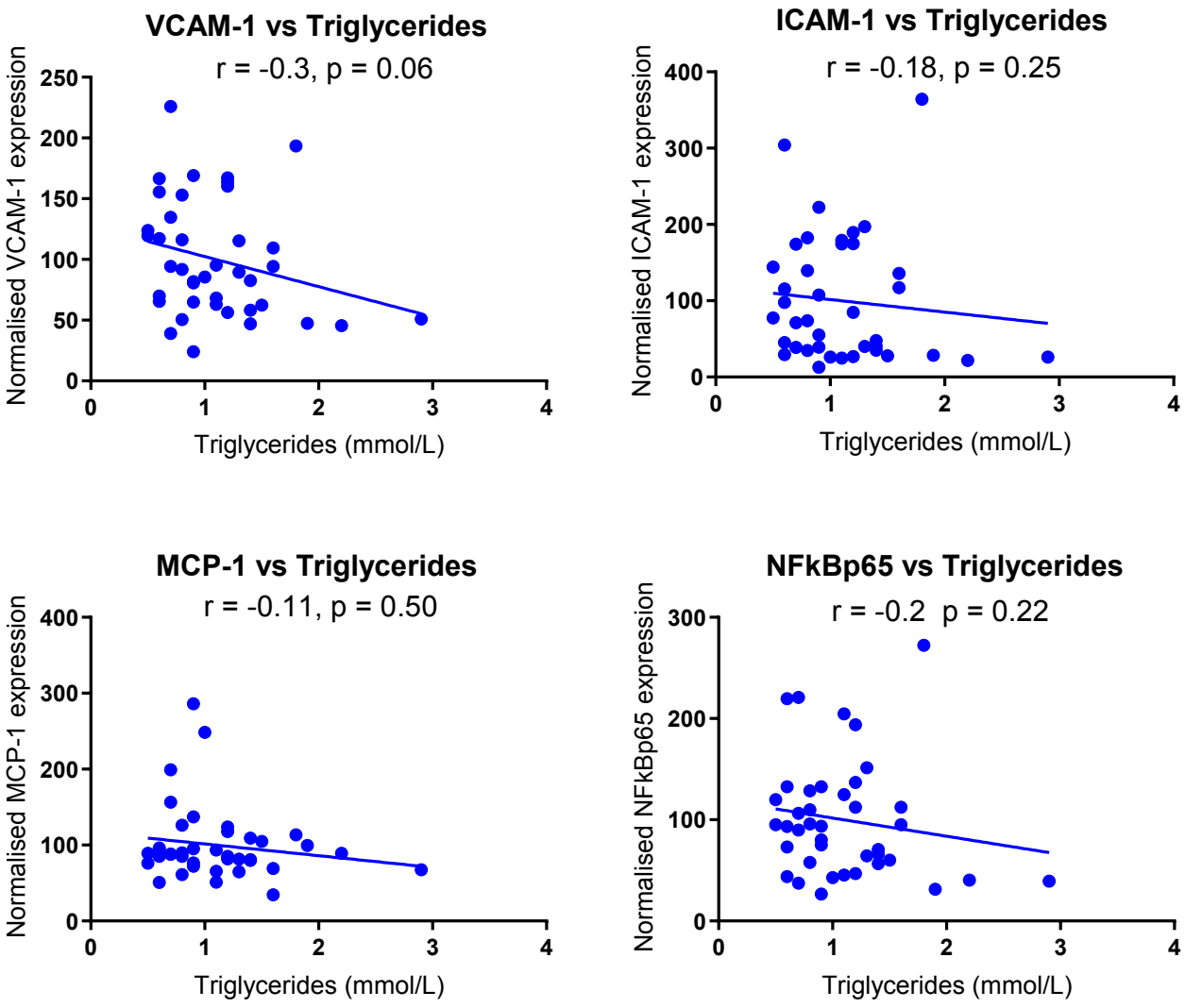


Figure 4.17: Correlations of gene expression of VCAM-1, ICAM-1, MCP-1, NFκBp65 and triglycerides. n = 40 for each graph.

Omega 6:3 ratio

Despite the omega-6 and omega-3 fatty acids having pro- and anti-inflammatory properties, respectively, there was no significant association between the omega 6:3 ratio and any of the inflammatory markers (see Figure 4.18).

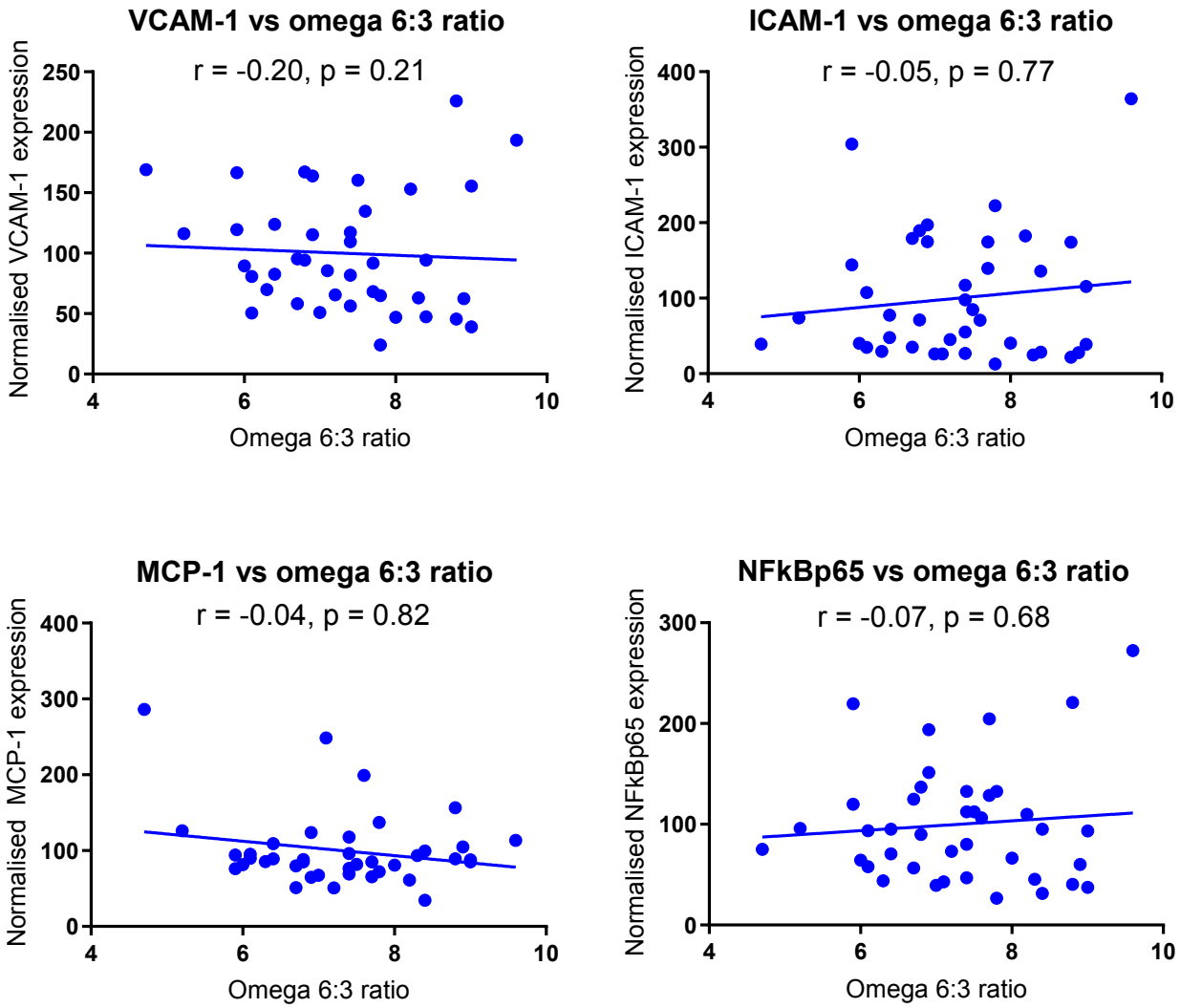


Figure 4.18: Correlations of gene expression of VCAM-1, ICAM-1, MCP-1, NFκBp65 and omega 6:3 ratio. n = 40 for each graph.

Saturated fats

Despite the proinflammatory nature of saturated fatty acids[475], no significant association was found between the percentage of saturated fats in blood and gene expression of inflammatory markers (see Figure 4.19).

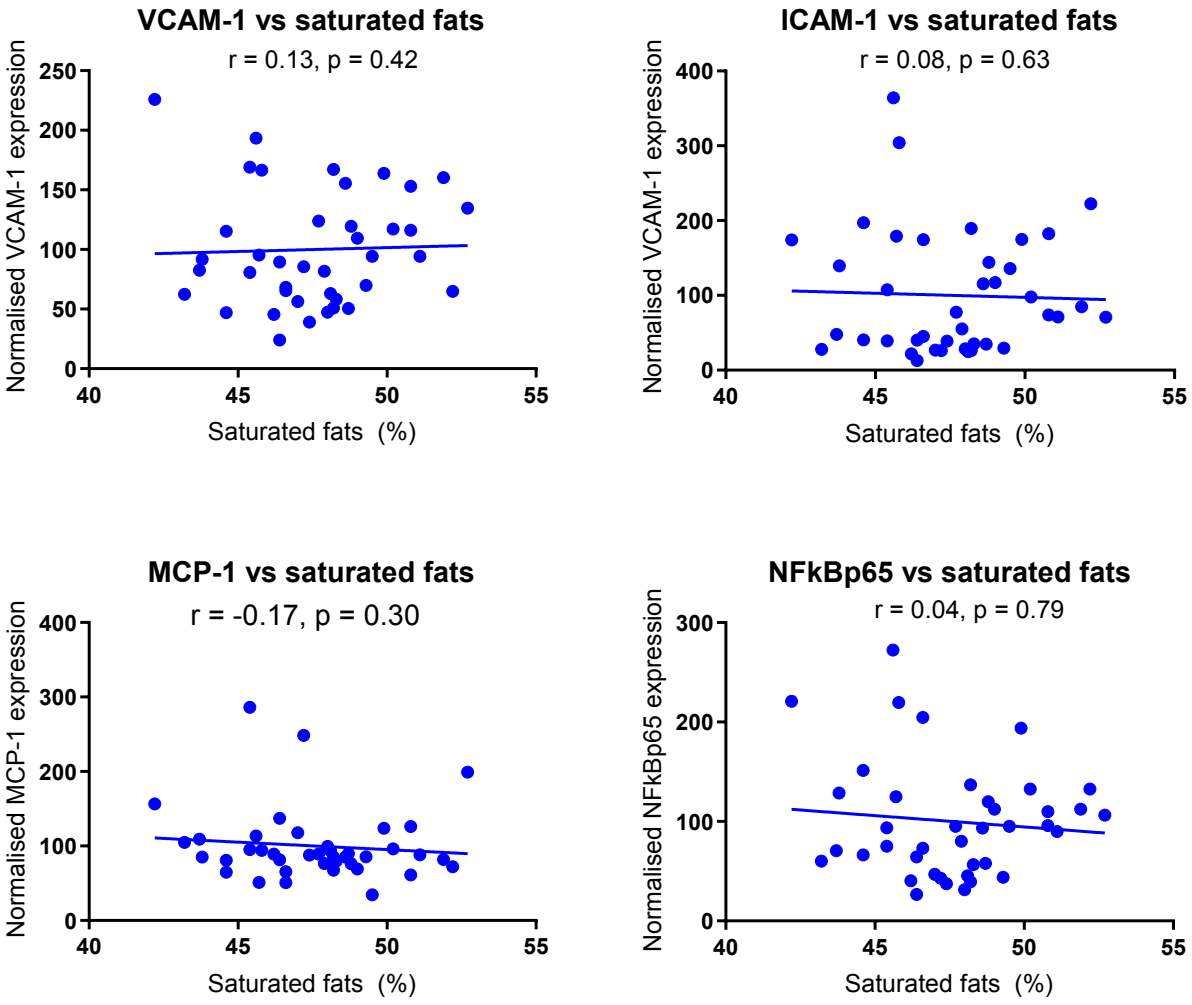


Figure 4.19: Correlations of gene expression of VCAM-1, ICAM-1, MCP-1, NFκBp65 and saturated fats. n = 40 for each graph.

4.4 Discussion

The FOCUS IN study evaluated the impact of high dose omega-3 fatty acids on markers of acute vascular inflammation in an *in vitro* model. By supplementing healthy volunteers with 4 grams of either EPA, DHA, standard fish oil or placebo daily for 30 days, the differential effects of EPA versus DHA were studied. To minimise confounders, participants were required to have a low baseline omega-3 intake, and have no significant medical illnesses or take regular medications such as anti-inflammatories. The addition of serum from participants to TNF-stimulated HUVECs before and after fish oil supplementation achieved two goals: (1) the omega-3s were delivered to cells in a physiological manner rather than in a chemically-pure state, and (2) the differential effects of EPA versus DHA could be studied and compared with combination therapy, all at a sufficient dose. One of the major limitations of previous human fish oil studies has been insufficient dosing. In the recently published ASCEND study, which assessed the effect of fish oil on cardiovascular events in patients with diabetes mellitus, only one capsule of fish oil was given per day, providing less than 1 g of omega-3 fatty acids[232]. Similarly, in the recently-published meta-analysis by Aung et al of omega-3 trials assessing cardiovascular outcomes[467], 6 out of the 10 trials used less than or equal to 1 g of omega-3 per day. Such doses are widely considered to be insufficient to produce a clinical benefit. The negative conclusions from these studies therefore need to be interpreted with caution.

Participants in the FOCUS IN study were generally well-matched at baseline, aside from those in the EPA group being older. The dose of 4 g of omega-3 per day was well tolerated, with only one participant experiencing side effects, and it achieved the desired goal of significantly increasing blood omega-3 levels. Those supplemented with either EPA or standard fish oil had a >200 % increase in EPA levels. Those supplemented with DHA had an almost 150 % increase in circulating DHA levels. These changes also translated into a reduced omega 6:3 ratio. Although a lower omega 6:3 ratio has long been considered desirable for

reducing the risk of chronic diseases[476], the recently-identified U-shaped association with mortality[472] indicates that this interpretation of the ratio may be an oversimplification. Indeed, there is evidence that higher levels of specific omega-6 fatty acids (namely linoleic acid)[477] confer more mortality benefit than others, and this likely to also be the case for omega-3s. Hence, the individual fatty acid contributors to the omega 6:3 ratio may be more important than the value.

At follow-up, DHA significantly reduced diastolic blood pressure, and produced a numerical but non-significant reduction in systolic blood pressure. Previous studies have demonstrated a blood pressure lowering effect of omega-3 fatty acids[209-211], but this is the first study to compare EPA with DHA, and identified a superior effect with DHA. DHA significantly reduced triglyceride levels, although none of the fish oil supplements affected total cholesterol, LDL or HDL levels. The 27% reduction in triglyceride levels is consistent with the magnitude of effect previously described, of 25 to 34%[194, 478]. This greater reduction in triglycerides with DHA compared to EPA is consistent with previous studies that have compared the two fatty acids directly[479-483]. Lp(a) levels were reduced to the greatest extent in the DHA group, although not significantly, and this should be investigated in future studies.

In the cell culture experiments, serum from healthy volunteers supplemented with one of three types of fish oil or with placebo was added to TNF-stimulated HUVECs in culture, and the effects on gene expression of inflammatory markers were measured. Fatty acids in blood are either incorporated into the phospholipids of red blood cell membranes[484] or circulate freely or bound to albumin[485]. Serum is therefore an effective method of fatty acid delivery to cells in culture. EPA significantly reduced the gene expression of MCP-1 in TNF-stimulated HUVECs. The oral administration of 4 g of EPA per day to healthy adults is sufficient to reduce markers of acute vascular inflammation and is independent of effects on circulating inflammatory markers and lipids. There were trends towards reductions in both

VCAM-1 and MCP-1 with standard fish oil, which contains a predominance of EPA, and which increased EPA levels to a similar extent as the EPA treatment in this study. The mechanisms by which EPA and DHA demonstrate differential effects on acute vascular inflammation are not fully understood, and require further investigation.

Correlations were studied between gene expression of inflammatory markers and levels of total cholesterol, LDL-C, HDL-C, triglycerides, omega 6:3 ratio, blood EPA, blood DHA, and saturated fats taken at baseline. A striking positive association was present between HDL-C levels and both VCAM-1 and MCP-1 expression. Although the HDL molecule has anti-inflammatory properties[486], with higher levels being associated with lower rates of cardiovascular events and mortality[487, 488], there is evidence that extremely high circulating HDL-C levels are paradoxically associated with increased mortality[489]. The reasons for this are unclear and require further investigation, however the results from this study suggest a possible harmful, pro-inflammatory effect at the highest HDL-C levels. This could be consistent with a hypothesis that the functionality of HDLs is compromised at extremely high levels, with the concentration of HDL cholesterol no longer reflecting HDL function, and such dysfunctional HDLs being harmful[490]. If this is the case, this would have implications for the interpretation of the results of studies of HDL-raising therapies on cardiovascular outcomes[491-494].

The limitations of this study include the small sample size of 10 participants per group. The numerical reductions seen in the gene expression of VCAM-1, ICAM-1 and MCP-1 after different fish oil treatments may have reached statistical significance in the setting of a larger cohort. However, the high threshold for a demonstrable reduction in gene expression in this small study highlighted the superior effect of EPA over other treatments. The requirement for healthy volunteers in this study allowed the effects of the fish oil preparations on inflammation to be demonstrated with minimal confounding factors. However, physical ambulatory

measures such as heart rate and blood pressure, as well as circulating factors such as lipids and inflammatory markers, were largely in the normal range for these healthy volunteers. Therefore, the effects of fish oil preparations on these variables were not expected to significantly reduce them even though significant beneficial effects may have been demonstrated in individuals at high cardiovascular risk. The changes seen in the gene expression of markers of AVI do not necessarily predict the changes that would occur *in vivo* or even at the protein level. Studies in the *in vivo* setting are required to confirm the *in vitro* findings.

There are several clinical implications of this study. In a healthy population, high dose omega-3 fatty acids would not be expected to significantly reduce heart rate, body weight, or systolic blood pressure. DHA would likely reduce diastolic blood pressure modestly, however the requirement of 4 g per day of omega-3 to achieve this would not be economical or practical. Furthermore, the predicted 0.5 mmol/L reduction in triglycerides seen with DHA would be unlikely to translate into a significant benefit. High dose EPA reduced the inflammatory response to endothelial injury in this study, and in a healthy population the number needed to treat to prevent an ischaemic event would be high. For the general population, there is not a strong indication for daily high dose omega-3 fatty acid consumption based on this study. Those at high cardiovascular risk and those with established atherosclerotic disease may well derive benefit, and indeed these groups were the target of the recent REDUCE-IT study, which evaluated 4 grams of EPA daily in the form of icosapent ethyl[234]. The 25% reduction in the incidence of first major cardiovascular event, and 30% reduction in total events occurred in statin-treated patients with hypertriglyceridemia and history of atherosclerosis or diabetes[495]. Icosapent ethyl is EPA in ethyl ester form which differs from EPA in triglyceride form used in FOCUS IN. The benefits seen in FOCUS IN and REDUCE-IT suggest that the anti-inflammatory and anti-atherosclerotic effects of EPA are not limited to a specific form.

The association seen between high plasma HDL-C levels and a greater inflammatory response in HUVECs to TNF-α seen in FOCUS IN is consistent with a hypothesis of disturbed HDL functionality at high HDL-C levels. Future studies will be required to redefine a target HDL-C range, however the concept of HDL-C being invariably regarded as "good cholesterol" in patient and population education needs to be reconsidered.

In conclusion, the results of the FOCUS IN study provide evidence for a beneficial effect of EPA on markers of acute vascular inflammation by virtue of a reduction in MCP-1 by stimulated endothelial cells. The magnitude of the anti-inflammatory effect is modest, as the other inflammatory markers studied were not significantly reduced. DHA on the other hand, is superior for triglyceride reduction as previously described, as well as blood pressure lowering, which is a new finding. The results of FOCUS IN provide a mechanistic rationale for the reduction in major adverse cardiovascular events with high-dose EPA seen in the recently-published REDUCE-IT study[234]. This study's findings demonstrated *in vitro* need to be confirmed in an *in vivo* model of AVI.

CHAPTER 5: THE IMPACT OF OMEGA-3 FATTY ACIDS ON ACUTE VASCULAR INFLAMMATION IN A MOUSE MODEL

ABSTRACT

Background: Acute vascular inflammation (AVI) is an early and critical stage of atherogenesis. The anti-inflammatory properties of omega-3 fatty acids have been demonstrated in a number of settings, however their effects on AVI, and in particular, the relative effects of EPA compared to DHA, have not been well defined. This is particularly relevant, given the recently published findings of a reduction in major adverse cardiovascular events with EPA treatment. Previous *in vitro* and *in vivo* studies have had methodological limitations, whereby omega-3s have not been administered in a physiological manner, have been subject to oxidation, or have not been provided in a pure form. The aim of this study was to determine the effects of pure EPA versus DHA on AVI in an established animal model.

Methods: Forty, 8-week-old C57BL/6 chow-fed mice were randomised to supplementation with 600mg/kg/day of either EPA, DHA, olive oil as an oil control, or no treatment, for 30 days by oral gavage. Non-occlusive collars were surgically implanted around the right carotid artery to induce AVI. After 48 hours, the mice were humanely killed. The carotids underwent immunohistochemical staining for the inflammatory factors VCAM-1, ICAM-1, MCP-1 and CD18. Blood was analysed for cholesterol, triglycerides, and fatty acids. Correlations were made between blood omega-3 levels and the protein expression of markers of AVI.

Results: Thirty-eight mice (95%) had successful carotid collaring. The collared carotids of mice had significantly more expression of all inflammatory factors compared to uncollared carotids which were used as internal controls ($p<0.01$ for all comparisons). EPA reduced the expression of VCAM-1 and MCP-1 by 43% and 38% respectively in collared carotids compared to no treatment ($p<0.05$ for both comparisons). There were numerical reductions in ICAM-1 and CD18 expression with EPA, which did not reach statistical significance. Furthermore, there was a numerical reduction in all four markers of AVI with DHA, which was less than EPA in each case, and not statistically significant. Plasma cholesterol and triglycerides

did not differ between treatment groups. EPA and DHA supplementation increased their respective blood levels by 272% and 62% ($p<0.0001$). There were significant inverse correlations observed between blood levels of both EPA and DHA and the expression of all four markers of AVI on immunohistochemistry. Furthermore, significant inverse correlations were also observed between the blood EPA:DHA ratio and all four markers of AVI.

Conclusions: Pre-treatment with EPA, but not DHA, significantly reduced acute vascular inflammation in a mouse model. This protective effect was present independent of plasma cholesterol and triglyceride levels, which were not significantly altered by any of the treatments. The greater effect of EPA compared to DHA in reducing AVI was further supported by significant inverse correlations observed between the EPA:DHA ratio and all four markers of AVI studied. The findings of this study may provide a mechanistic contribution underlying the significant cardiovascular benefit seen with EPA treatment in the recent REDUCE-IT study. Further studies of the effects of omega-3 fatty acids on vascular inflammation, including different ratios of EPA:DHA, are warranted.

I, Anthony Pisaniello, conceived, designed, executed and analysed all of the work included in this chapter.

5.1 Background

In recent decades, there has been increasing interest in the role of omega-3 polyunsaturated fatty acids in inflammation. Preclinical studies have identified a number of anti-inflammatory as well as pro-resolving properties of omega-3 fatty acids[195, 196]. The anti-inflammatory properties of the omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been demonstrated on multiple levels, including cellular, humoral, signal transduction, and gene expression[496], although there is a paucity of quality data on the impact on acute vascular inflammation (AVI).

AVI is an early and critical stage of atherogenesis[4]. For example, minute changes in LDL, glucose, blood pressure, and inflammatory markers, lead to increased endothelial cell permeability, which permits the migration of circulating LDL into the subendothelial space. Concurrent with this, endothelial cells switch to a secretory phenotype, producing a hyperplastic, multilayered basal lamina, which further traps LDL in the subendothelium[8]. Trapped LDL is oxidised by macrophages, generating pro-inflammatory free radicals and monocyte chemoattractant protein 1 (MCP-1). The recruitment of monocytes to the subendothelium, and of platelets to the endothelial cell surface (after expression of von-Willebrand factor by activated endothelial cells), triggers a plethora of changes; these include the release of pro-inflammatory cytokines, activation of pro-inflammatory signal transduction pathways, and an increase in the adhesiveness of the endothelial cell surface[9-11, 57]. These changes of AVI promote the formation of atherosclerotic lesions, ultimately culminating in either plaque rupture or erosion.

Physiological mechanisms exist to partly counteract the atherogenic effects of AVI, such as the efflux of LDL from the subendothelial space[497], and the release of heme oxygenase-1 (HO-1) which reduces monocyte transmigration and toxicity to endothelial cells[7]. The implications of preventing or reducing AVI and hence retarding atherogenesis are

significant, given that cardiovascular disease is the leading cause of death worldwide[1]. Since macroscopic evidence of atherosclerosis has been identified in humans as early as the foetal stage, i.e. in the form of fatty streaks[498], supplementation with a safe agent that reduces AVI has the potential for use in primary cardiovascular prevention in all age groups.

Previous studies have explored, with limitations, the effects of omega-3 fatty acids, found abundantly in fish and fish oil, on markers of AVI. These include measuring the expression of cell adhesion molecules and endothelium-derived cytokines, and using various methods of exposure to omega-3s, such as direct addition to cell culture media, oral supplementation in the form of capsules or the addition to food, and the use of transgenic animals that can synthesise omega-3s due to the encoding of omega-3 fatty acid desaturase[244, 499-503]. Endothelial cell permeability, adhesion molecule expression, and chemotaxis of leucocytes have all been studied in the setting of omega-3 supplementation. Although omega-3 fatty acids have not been shown to reduce lipopolysaccharide-induced endothelial cell permeability[504], they do reduce adhesion molecule expression by endothelial cells in culture[244, 505, 506] and chemotaxis of monocytes[507]. However, the direct administration of purified omega-3 fatty acids to cells cultured *in vitro* is not physiological, and hence does not model *in vivo* exposure to fatty acids which have undergone modification during metabolism. In Chapter 4 of this thesis, omega-3 fatty acids were delivered to endothelial cells by adding human serum obtained before and after fish oil consumption. The omega-3 fatty acids were therefore presented to cells after ingestion and metabolism. Fish oil that was high in EPA (86% total mass) reduced gene expression of MCP-1 by TNF-stimulated human umbilical vein endothelial cells. EPA was superior to both DHA and standard fish oil (2:1 EPA:DHA ratio). The results of FOCUS-IN need to be confirmed at a protein level and in an *in vivo* setting.

Previous animal studies of the effects of omega-3s on AVI have been limited by difficulties in eliciting a local endothelial insult without inducing atherosclerotic lesions.

Kockx M et al described a method of inducing AVI in the arterial wall of rabbits by surgically implanting a non-occlusive silicone elastic collar around the carotid arteries, and demonstrated intense infiltration of polymorphonuclear leucocytes from 6 hours to 3 days post-surgery[508]. Short-term collar application did not induce atherosclerotic lesions, and this method was safely and effectively replicated in mice by von der Thusen et al[509]. Application of a periarterial collar to the carotid artery increases the production of reactive oxygen species, and stimulates neutrophil recruitment, adhesion molecule expression, and inflammatory cytokine production[510]. Previous studies have most commonly administered omega-3 fatty acids to animals by means of dietary supplementation, leading to difficulties with quantifying intake, and also resulting in fatty acid oxidation. Oral gavage of unoxidised and appropriately stored fatty acids overcomes this problem. The use of the short-term carotid collar model in animals supplemented with omega-3 fatty acids by oral gavage is a physiological way to study their effects on pure vascular inflammation. Furthermore, direct comparisons can be made between the effects of EPA versus DHA.

5.1.1 Aims and rationale of study

The aim of this study was to determine the impact of individual omega-3 fatty acids, administered in a physiological manner, on acute vascular inflammation in an established animal model. The non-occlusive periarterial collar model elicits features of pure vascular inflammation when used for less than 72 hours[503, 510]. Chow-fed C57Bl/6 mice do not have a propensity to develop atherosclerosis, and hence were used for this experiment. The pre-treatment of mice with omega-3 fatty acids and then subsequent induction of AVI mimics the primary prevention clinical setting. In this animal study, the collared carotids could be harvested so that protein expression of markers of AVI could be measured and compared between different omega-3 formulations.

5.1.2 Hypotheses

The primary hypotheses of this study were that:

(1) Pre-treatment with high-dose omega-3 fatty acids reduces the degree of acute vascular inflammation induced by periarterial collaring.

(2) There is a differential effect among omega-3 fatty acids, with EPA reducing markers of acute vascular inflammation more than DHA.

(3) Blood levels of omega-3 fatty acids, especially EPA, have an inverse correlation with the degree of acute vascular inflammation.

5.2 Methods

5.2.1 Study outline

Forty 8-week-old C57Bl/6 mice fed a standard rodent chow diet were randomised equally to supplementation with either EPA, DHA, olive oil, or no treatment for 30 days, by oral gavage. EPA and DHA were >99% pure free fatty acids purchased from Nu-Chek Prep, Inc (Elysian, Minnesota, USA), and all treatments were administered at a dose of 600 mg/kg/day. Periarterial collaring of the right carotid artery was then performed under general anaesthesia. The collar was left in place for 48 hours, and then mice were subsequently humanely killed (See Figure 5.1). Blood was harvested for plasma total cholesterol and triglyceride levels, and fatty acid analysis. Both carotid arteries were harvested for immunohistochemical staining for VCAM-1, ICAM-1, MCP-1 and CD18. The left carotid artery was a within-animal control.

This study was approved by the animal ethics committees of both the South Australian Health and Medical Research Institute, and the University of Adelaide.

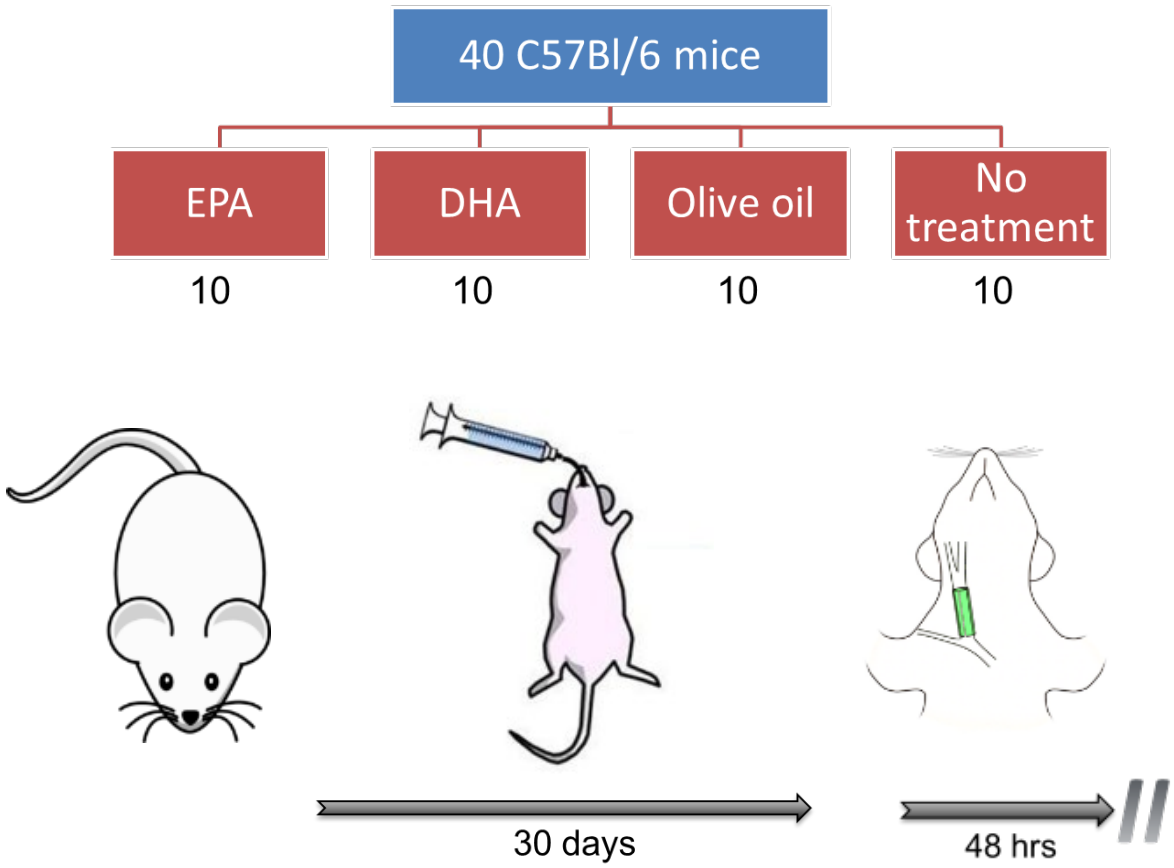


Figure 5.1: Schematic representation of the study outline. After randomisation to one of four treatment groups, the mice were gavaged daily for 30 days, then had 48 hours of carotid collaring before being humanely killed.

5.2.2 Diet and preparation of EPA, DHA, and olive oil

Mice were fed the Teklad Global 18% Protein Rodent Diet (Harlan Laboratories, Madison, WI, USA) and had free access to food and water. Free fatty acids were aliquoted into 200 µl microcentrifuge tubes, which were sealed under nitrogen gas using paraffin film. Tubes were stored at -20ºC, protected from light, in between uses. Each tube was resealed under

nitrogen gas using paraffin film after each use. Olive oil was prepared as above although was stored at room temperature as it solidifies when refrigerated.

5.2.3 Periarterial collaring method

1. Silicone tubing with internal diameter 0.64 mm and outer diameter 1.2 mm (BlueSky Scientific, Burnside, SA, Australia) was cut to a length of 4 mm. Two cuts were then made into the tubing lengthwise, 0.5 mm apart, so that a sliver of tubing was removed.
2. The mouse was pre-oxygenated with 100% FiO2 for five minutes, and then anaesthetised with 3% isoflurane for induction and 1.5% isoflurane for maintenance.
3. The anterior neck was shaved, and povidone-iodine was applied to the skin. After administration of subcutaneous buprenorphine (0.1 mg/kg) and intradermal lignocaine (50 μl) into the area to be incised, a midline incision was made into the skin of the neck and thorax. After blunt dissection and retraction of deep tissues, the right common carotid artery was exposed.
4. The right common carotid artery was gently separated from the jugular vein and the vagus nerve, and then lifted by passing suture material underneath and suspending the vessel. The silicone tubing was slipped around the carotid artery from beneath while suspended, using haemostats. The tubing was placed proximal to the aorta, away from the carotid bifurcation, to avoid contact with local microscopic nerve bundles (see Figure 5.2).
5. A small metal clip applicator (Weck Hemoclip® Plus EZ Load Applier (Teleflex Medical, North Carolina, USA) was used to gently apply a metal clip to the tubing to secure its position without compressing the vessel.

6. The midline incision was closed with 6-0 nylon sutures, and isoflurane was ceased. The mouse was given supplemental oxygen again, 100% FiO2 for 5 minutes, and after the recovery period was returned to its cage.

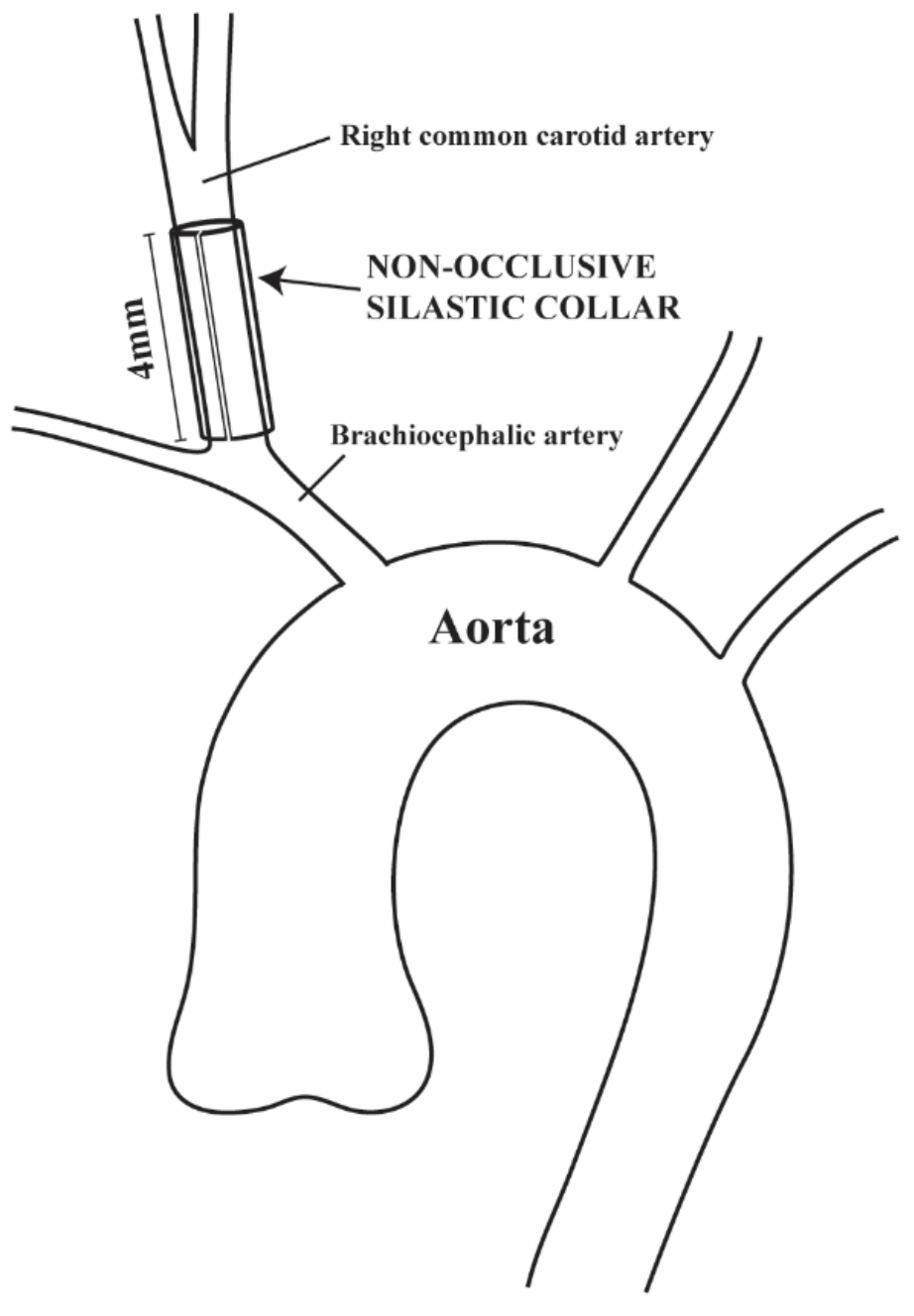


Figure 5.2: Schematic representation of collar placement on right carotid artery.

5.2.4 Fatty acid analysis

Whole blood was spotted onto pre-made dry blood spot (DBS) cards for fatty acid analysis. DBS cards were hand-made using cardboard and PUFACoat paper (developed by Dr. Ge Liu and Professor Robert Gibson at the FoodPlus Research Centre, University of Adelaide). PUFACoat paper stabilises fatty acids in biological samples for at least 9 weeks when stored at room temperature, and for at least 18 months when stored at -20ºC. Immediately after blood collection, 30 µl of whole blood was pipetted directly onto the PUFACoat paper, and the DBS cards were then stored in sealed foil bags with desiccant at -20ºC prior to processing. The blood-stained area of the PUFACoat paper was cut out and placed in 5 ml capped scintillation vials filled with 2 ml of 1% H_2SO_4. The vials were then heated to 70ºC for 2.5 hours. After the first 30 mins, the caps were released briefly to expel gas and the vials were vortexed. After each subsequent 30-minute period the vials were vortexed only. The vials were allowed to cool to room temperature, and then 250 µl of distilled water and 700 µl of heptane were added to the vials, which were then vortexed. Using a Pasteur pipette, the top layer was transferred to a gas chromatography (GC) vial. The GC vial was sealed and then stored at -20ºC until GC analysis was performed. GC analysis was performed using a Hewlett-Packard 6890 system (Palo Alto, CA, USA) equipped with a BPX70 capillary column 50 m×0.32 mm, film thickness 0.25 µm (SGC Pty Ltd., Vic, Australia), programmed temperature vaporisation injector and a flame ionisation detector (FID). The injector temperature was set at 250 °C and the FID temperature at 300 °C, a programmed temperature ramp (140–240 °C) was used. Helium gas was utilised as a carrier at a flow rate of 35 cm per second in the column and the inlet split ratio was set at 20:1. Quantification was achieved by comparing the retention times and peak area values of unknown samples to those of commercial lipid standards (Nu-Chek Prep Inc., Elysian, MN, USA) using the Hewlett-Packard Chemstation data system.

5.2.5 Tissue harvesting

Blood from terminal cardiac puncture was spotted onto dry blood spot cards (40 µl in total), which were then stored at -20 ºC for later fatty acid analysis as per Section 2.2. The remaining blood was collected in EDTA tubes for plasma extraction, and later analysed for plasma total cholesterol and triglycerides as described in Section 2.11. The left ventricle was flushed with normal saline, and both carotid arteries were harvested and stored in 10% neutral buffered formalin for later immunohistochemical analysis as described in Section 2.13.

5.2.6 Immunohistochemistry

Sections (5mm) of the vessels were cut, dewaxed, and rehydrated as described[511]. Sections were stained with rabbit anti-mouse/rat/human VCAM-1 (1:1000; Abcam ab134047), rat anti-mouse ICAM-1 (1:200; Abcam ab119871), rat anti-mouse MCP-1 (1:50; Abcam ab8101), and rat anti-mouse CD18 (1:1000; Abcam ab119830) antibodies to assess endothelial expression of markers of AVI.

Quantification was performed using ImagePro Premier v9.1 (Media Cybernetics, Silver Spring, MD, USA). The threshold for positive staining was determined by a blinded pathologist. Image analysis results, which represent the average positive staining above the threshold for individual arterial sections, are expressed as the Integrated Optical Density in units of lumens x pixels^2. All IgG controls were negative.

5.2.7 Statistical and data analysis

5.2.7.1 Sample size calculation

The sample size of 40 mice was calculated based on data from the paper by Borissoff et al[512]. The neutrophil count in the vessel wall was considered the most important factor for calculating sample size. In Borissoff et al's study, perivascular carotid collars were applied, with a resulting density of neutrophils of 151±48 per standardised area of vessel wall measured

by immunofluorescence staining for $Ly6G^+$ cells. This was statistically significantly decreased after pharmacological intervention to 83±28 neutrophils per standardised area. To detect a significant difference in neutrophil count in the artery wall assuming a power of 80% and alpha set at 0.05, a one-way ANOVA power calculation derived a sample size of 9 per group, with 4 groups in total. To account for a predicted 10% failure rate based on a pilot study of the procedure, one extra mouse per group was added.

5.2.7.2 Statistical methods

GraphPad Prism 7 (GraphPad Software Inc, La Jolla, CA, USA) and Microsoft Excel 2016 (Microsoft, Albuquerque, NM, USA) were used to analyse data. The D'Agostino-Pearson normality test was performed to determine whether continuous data were normally-distributed. Normally-distributed data were analysed using the One-way Analysis of Variance (ANOVA) to compare means between multiple groups, and results were expressed as mean ± standard error of the mean (SEM). If correcting for multiple comparisons, the Dunnett test was used. If continuous data were not normally-distributed, analysis was performed using the Kruskal-Wallis test, and results were expressed as median + interquartile range (IQR). If correcting for multiple comparisons, Dunn's test used. Statistical correlations were analysed using a linear regression model. Statistical significance was set at the 0.05 level.

5.3 Results

Thirty-eight out of forty mice (95%) completed the study. One mouse died during anaesthesia prior to the commencement of surgery, and another developed a reduced respiratory rate in the recovery period and was culled on humane grounds.

5.3.1 Omega-3 supplementation in mice modulates fatty acid profiles

A complete fatty acid profile of blood taken at study completion was obtained. Individual fatty acid levels in whole blood were expressed as a percentage of total fatty acid content. The relative proportions of all major fatty acids, i.e. saturated fatty acids, trans-fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids (including omega-3 and omega-6) for mice in each treatment group are presented in Figure 5.3. Saturated fatty acids and omega-6 polyunsaturated fatty acids comprised the majority of fatty acids in each group. Trans-fatty acids comprised less than 1% of total fatty acids in each group.

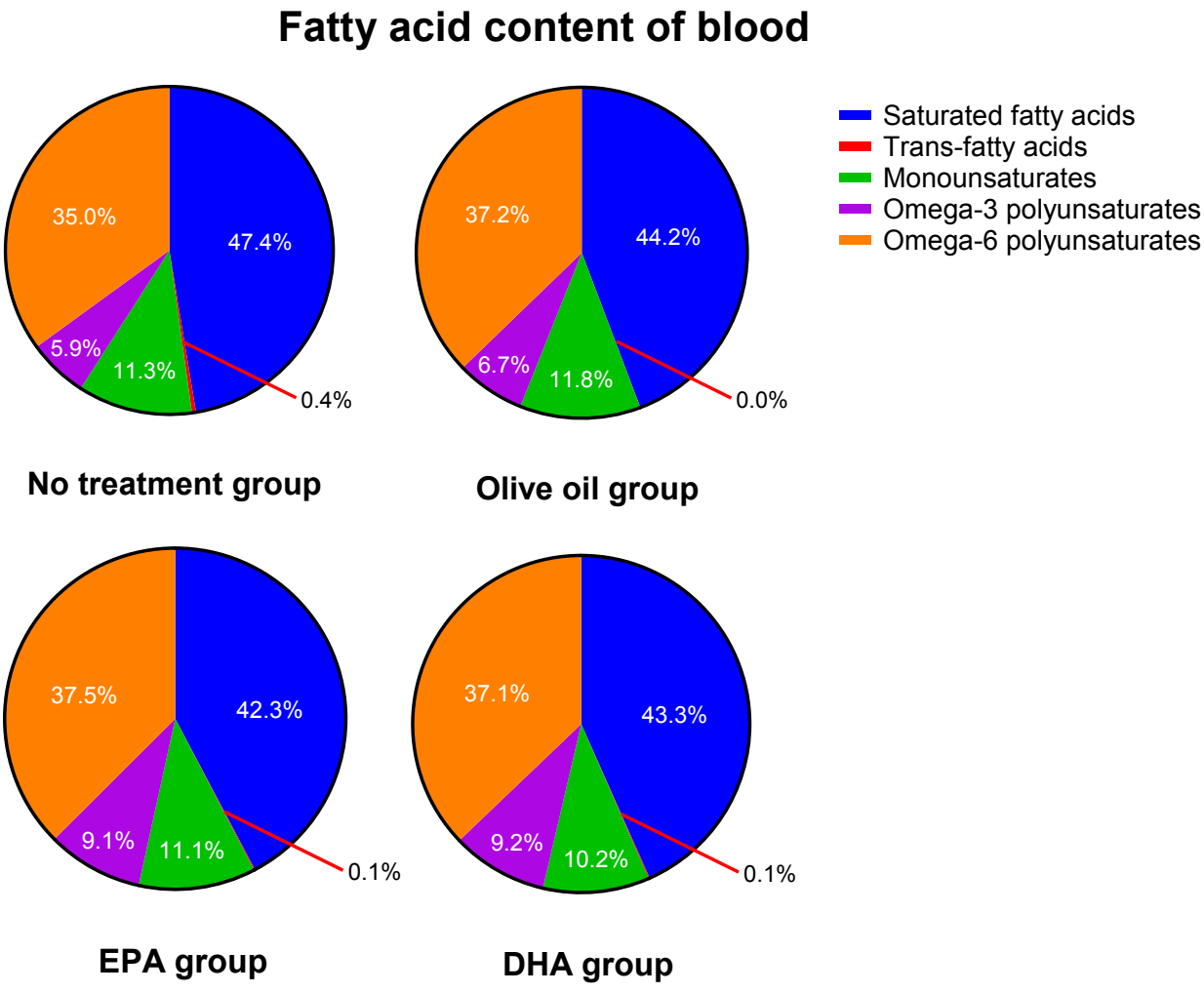


Figure 5.3 Relative proportions of all major types of fatty acids in blood in all treatment groups at study completion, as measured by dry blood spot analysis.

5.3.1.1 Polyunsaturated omega-3 fatty acids

Polyunsaturated fatty acids are omega-3 or omega-6 fatty acids that have more than one double bond in the fatty acid chain. As expected, the total omega-3 fatty acid content in blood was significantly higher in the EPA- (9.07±0.22%, p=0.0001) and DHA-supplemented groups (9.20±0.38%, p=0.0001) compared to the no treatment group (5.90±0.45%), see Figure 5.4. There was no significant difference in the olive oil group (6.72±0.40%, p=0.29) compared to the no treatment group. The same trend was also observed for EPA and DHA blood levels. That is, EPA levels were significantly higher in the EPA- (1.07±0.04%, p<0.0001) and DHA-supplemented groups (0.56±0.06%, p<0.0001) compared to the no treatment group (0.23±0.05%). DHA levels were significantly higher in the EPA- (6.32±0.17%, p=0.0012) and DHA-supplemented groups (7.64±0.34%, p<0.0001) compared to the no treatment group (4.71±0.36%), see Figures 5.5 and 5.6.

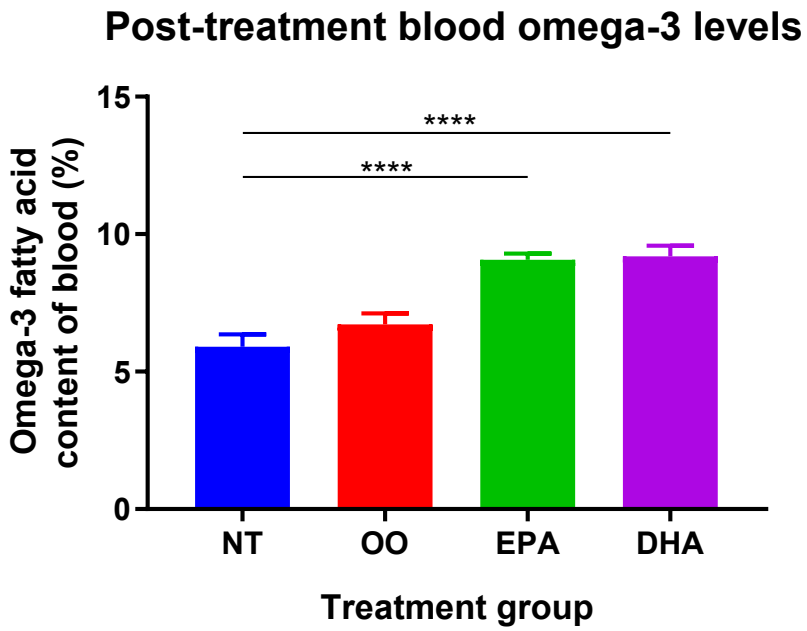


Figure 5.4: Total omega-3 fatty acid content in blood from mice in each treatment group. Data are presented as mean ± SEM. n = 9-10 per group. NT = No treatment. OO = Olive oil. ****p≤0.0001

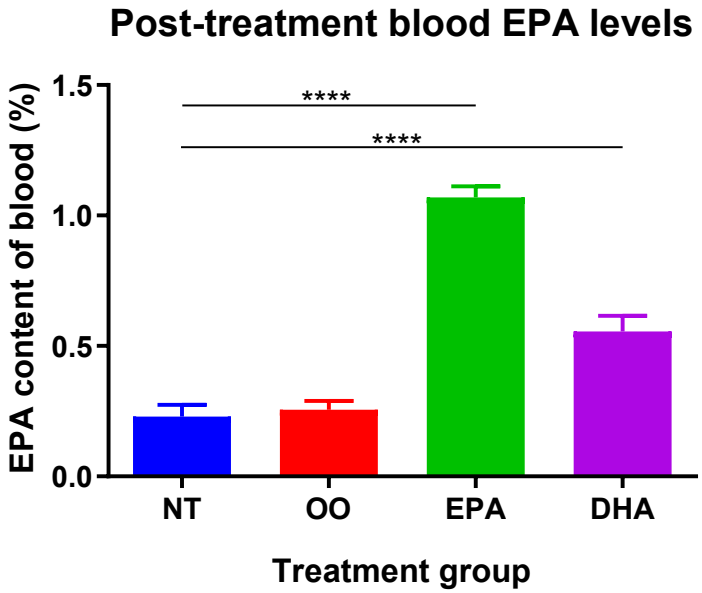


Figure 5.5: Blood levels of EPA for mice in each treatment group. Data are presented as mean ± SEM. n = 9-10 per group. ****p<0.0001

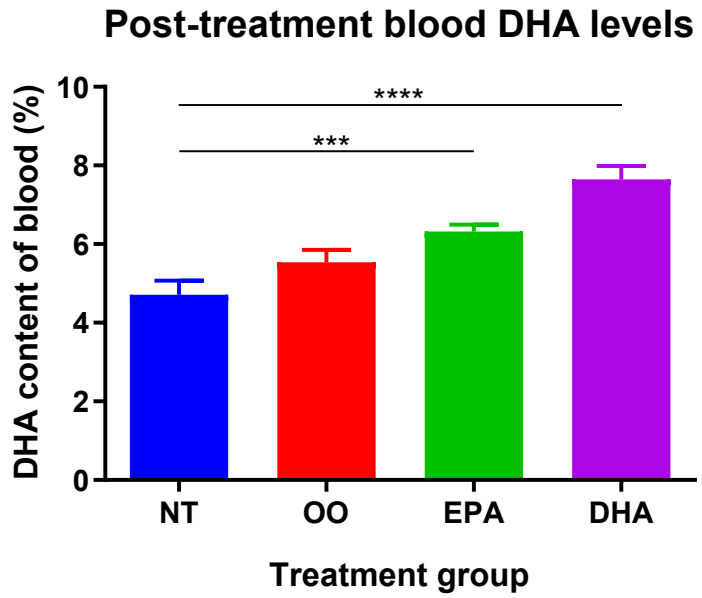


Figure 5.6: Blood levels of DHA for mice in each treatment group. Data are presented as mean ± SEM. n = 9-10 per group. ****p<0.0001, ***p<0.001

Levels of docosapentaenoic acid (DPA) were significantly higher in mice treated with EPA (1.21±0.05%) compared to those in the no treatment group (0.57±0.04%, p<0.0001), see

Table 5.1. This was expected, given that DPA is the intermediary product during conversion of EPA to DHA.

The essential omega-3 fatty acid alpha-linolenic acid (ALA), obtained by rodents from their diet, comprised only 0.43±0.06% of total blood fatty acids in the no treatment group. This was not significantly different in the olive oil (0.39±0.06%, p=0.87), EPA (0.47±0.04%, p=0.91) or DHA (0.47±0.04%, p=0.94) treatment groups, see Table 5.1.

Table 5.1: Post-treatment levels of fatty acids in blood as a percentage of total fatty acid content.

Fatty acids	Treatment Groups			
	NT	Olive Oil	EPA	DHA
Total Omega-3 (%)	5.90±0.45	6.72±0.40	9.07±0.22****	9.20±0.38****
EPA (%)	0.29±0.03	0.26±0.03	1.07±0.04****	0.56±0.06****
DHA (%)	4.71±0.36	5.53±0.32	6.32±0.17***	7.64±0.34****
DPA (%)	0.57±0.04	0.56±0.05	1.21±0.05****	0.53±0.02
ALA (%)	0.43±0.06	0.39±0.06	0.47±0.04	0.47±0.04
Total Omega-6 (%)	35.02±1.21	37.22±1.08	37.51±0.31	37.13±0.51
Linoleic acid (%)	20.35±0.55	20.34±0.65	23.05±0.43*	23.27±0.69*
Arachidonic acid (%)	12.22±0.71	14.28±0.59*	12.16±0.27	11.56±0.47
Total Monounsaturated Fats (%)	11.31±0.33	11.83±0.37	11.07±0.28	10.23±0.35^^
Total Trans-fatty Acids (%)	0.4±0.11	0±0**	0.05±0.04**	0.13±0.09*
Total Saturated Fats (%)	47.37±1.37	44.22±1.34*	42.29±0.31**	43.31±0.53*

***p<0.05, **p<0.01, ***p<0.001, ****p≤0.0001 compared to NT. ^^p<0.01 compared to OO.**

5.3.1.2 Polyunsaturated omega-6 fatty acids

Omega-6 fatty acids comprised 35.02±1.21% of total blood fatty acids in the no treatment group. This was not significantly different in the olive oil (37.22±1.08%, p=0.19), EPA (37.51±0.31%, p=0.11), or DHA (37.13±0.51%, p=0.22) treatment groups, see Figure 5.7. The most prevalent omega-6 fatty acids were linoleic acid and arachidonic acid. Linoleic

acid comprised 20.35±0.55% of total fatty acids in the no treatment group, and this was statistically significantly greater in the EPA (23.05±0.65, p=0.005) and DHA (23.27±0.69, p=0.003) treatment groups, although the numerical differences were modest, see Table 5.1. Arachidonic acid comprised 12.22±0.71% of total fatty acids in the no treatment group. This was significantly greater in the olive oil treatment group (14.28±0.59, p=0.03), but was not significantly different in either the EPA (12.16±0.27, p=0.94) or DHA (11.56±0.47, p=0.62) treatment groups, see Table 5.1.

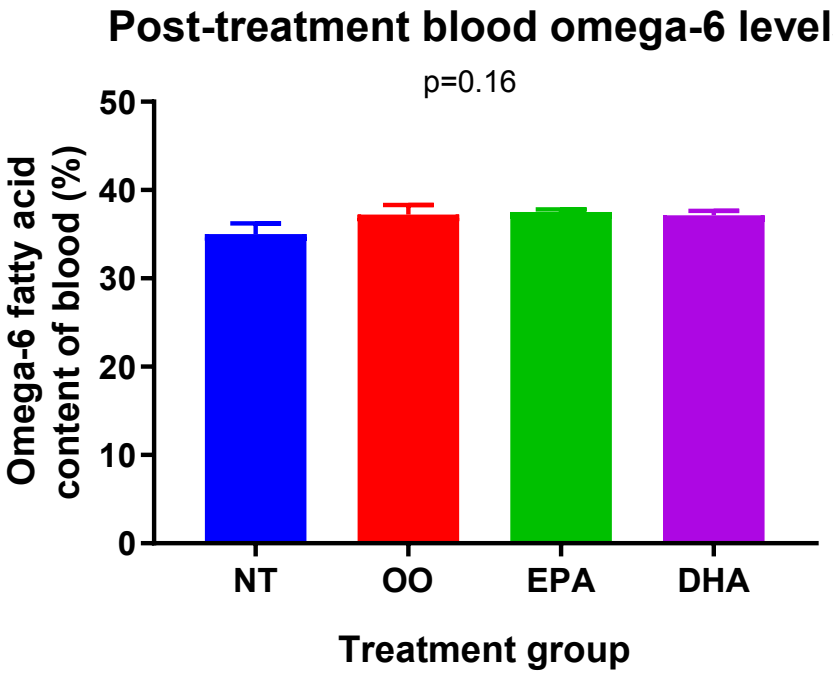


Figure 5.7: Total omega-6 levels in blood expressed as a percentage of total fatty acids. Data are presented as mean ± SEM. n = 9-10 per group.

5.3.1.3 Ratio of omega 6:3

The ratio of omega-6 to omega-3, which in humans has a positive association with cardiovascular mortality[220], was 6.15±0.31 : 1 in the no treatment group. This was not significantly different in the olive oil group (5.63±0.20 : 1, p=0.21). As expected, the omega

6:3 ratio was significantly lower in mice supplemented with EPA (4.16±0.11 : 1, p=0.0001) and DHA (4.07±0.13 : 1, p=0.0001), see Figure 5.8.

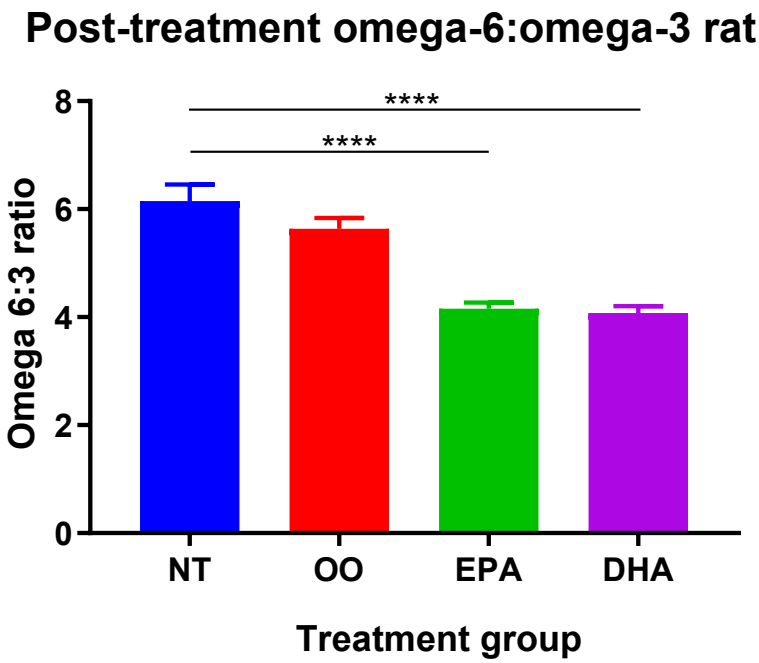


Figure 5.8: Comparison of omega-6/omega-3 ratios in blood between treatment groups. Data are presented as mean ± SEM. n = 9-10 per group. p≤0.0001.

5.3.1.4 Monounsaturated fatty acids

Monounsaturated fatty acids contain a single double bond in the fatty acid chain, and consist of omega-7 and omega-9 fatty acids. In the no treatment group, monounsaturated fatty acids comprised 11.31±0.33% of total fatty acid content. Given the high proportion of the oleic acid (omega-9) in olive oil, the olive oil treatment group had the highest proportion (11.83±0.37%) of monounsaturates, although the numerical differences were modest. The EPA (11.07±0.88%, p=0.91) and DHA (10.23±0.35%, p=0.07) treatment groups did not have significantly different levels of monounsaturates, see Table 5.1.

5.3.1.5 Trans-fatty acids

Trans-fatty acids comprised less than 1% of total blood fatty acids in all groups. This value was 0.4±0.11% in the no treatment group. All supplements reduced trans-fatty acid levels, demonstrating the effect of fatty acid substitution. The olive oil (0.0±0.00%, p=0.001), EPA (0.05±0.04%, p=0.003) and DHA (0.13±0.07%, p=0.03) treatment groups had significantly lower levels, see Table 5.1.

5.3.1.6 Saturated fatty acids

Saturated fatty acids were the major fatty acid component of blood, comprising 47.37±1.37% of all fatty acid content in the no treatment group. All supplements reduced saturated fatty acid levels. The olive oil treatment group (44.22±1.34%, p=0.034), the EPA treatment group (42.29±0.31%, p=0.003), and the DHA treatment group (43.31±0.53%, p=0.015) all had significantly lower levels, see Table 5.1.

5.3.2 Plasma cholesterol and triglycerides were not altered with omega-3 supplementation

Plasma total cholesterol and triglyceride levels, obtained at the time of death, were low in all treatment groups, consistent with the use of a non-atherogenic mouse model. Total cholesterol was 2.01±0.09 mmol/L in the no treatment group, and supplementation did not significantly alter this. Total cholesterol levels were 1.68±0.26 mmol/L in the olive oil group (p=0.37), 1.82±0.12 mmol/L in the EPA group (p=0.73), and 1.72±0.14 mmol/L in the DHA group (p=0.45), see Figure 5.9.

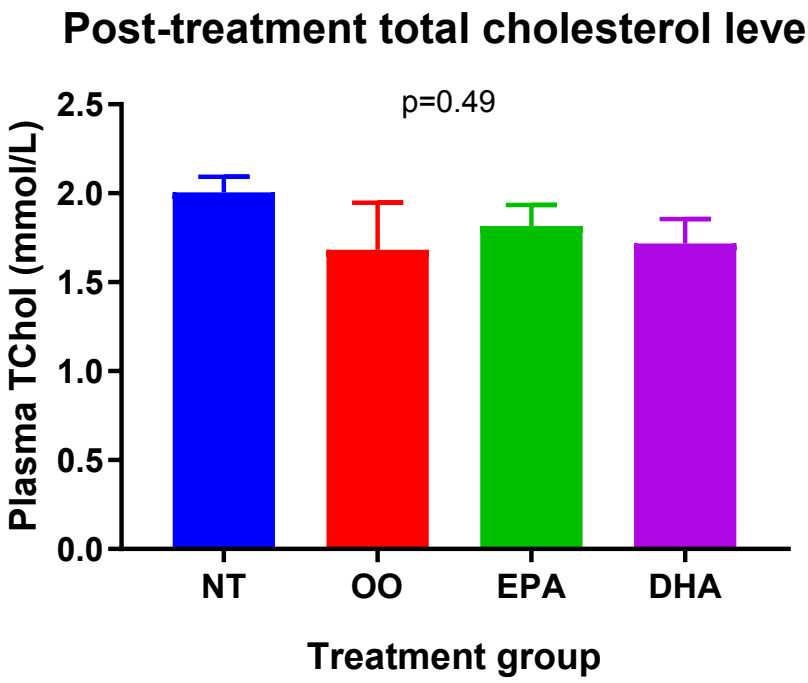


Figure 5.9: Total plasma cholesterol levels in different treatment groups at end of study. Data are presented as mean ± SEM. n = 9-10 per group.

Plasma triglyceride levels were 0.53±0.05 mmol/L in the no treatment group, and did not significantly differ in the olive oil (0.48±0.07 mmol/L, p=0.90), EPA (0.61±0.07 mmol/L, p=0.64), or DHA (0.63±0.06 mmol/L, p=0.52) treatment groups, see Figure 5.10.

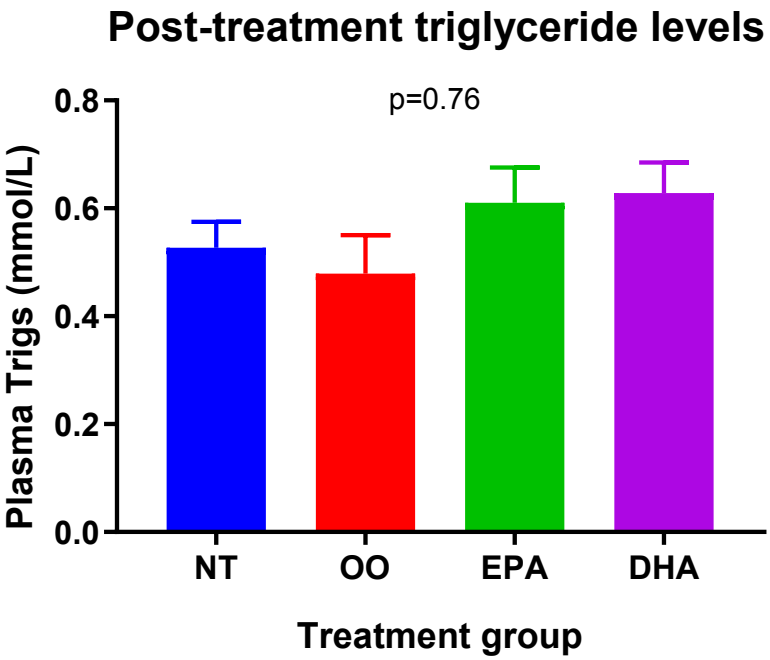


Figure 5.10: Plasma triglyceride levels in different treatment groups at study completion. Data are presented as mean ± SEM. n = 9-10 per group.

5.3.3 Endothelial expression of markers of acute vascular inflammation were significantly reduced with omega-3 supplementation

Immunohistochemical (IHC) staining of carotid arteries was performed for the following markers of acute vascular inflammation: VCAM-1, ICAM-1, MCP-1 and CD18. Staining of collared carotids was markedly increased compared to uncollared carotids, demonstrating the intense vascular inflammatory response produced by the collaring method. As demonstrated in Figure 5.11, the expression of all four markers was significantly increased in collared (right) carotids compared to uncollared (left) carotids.

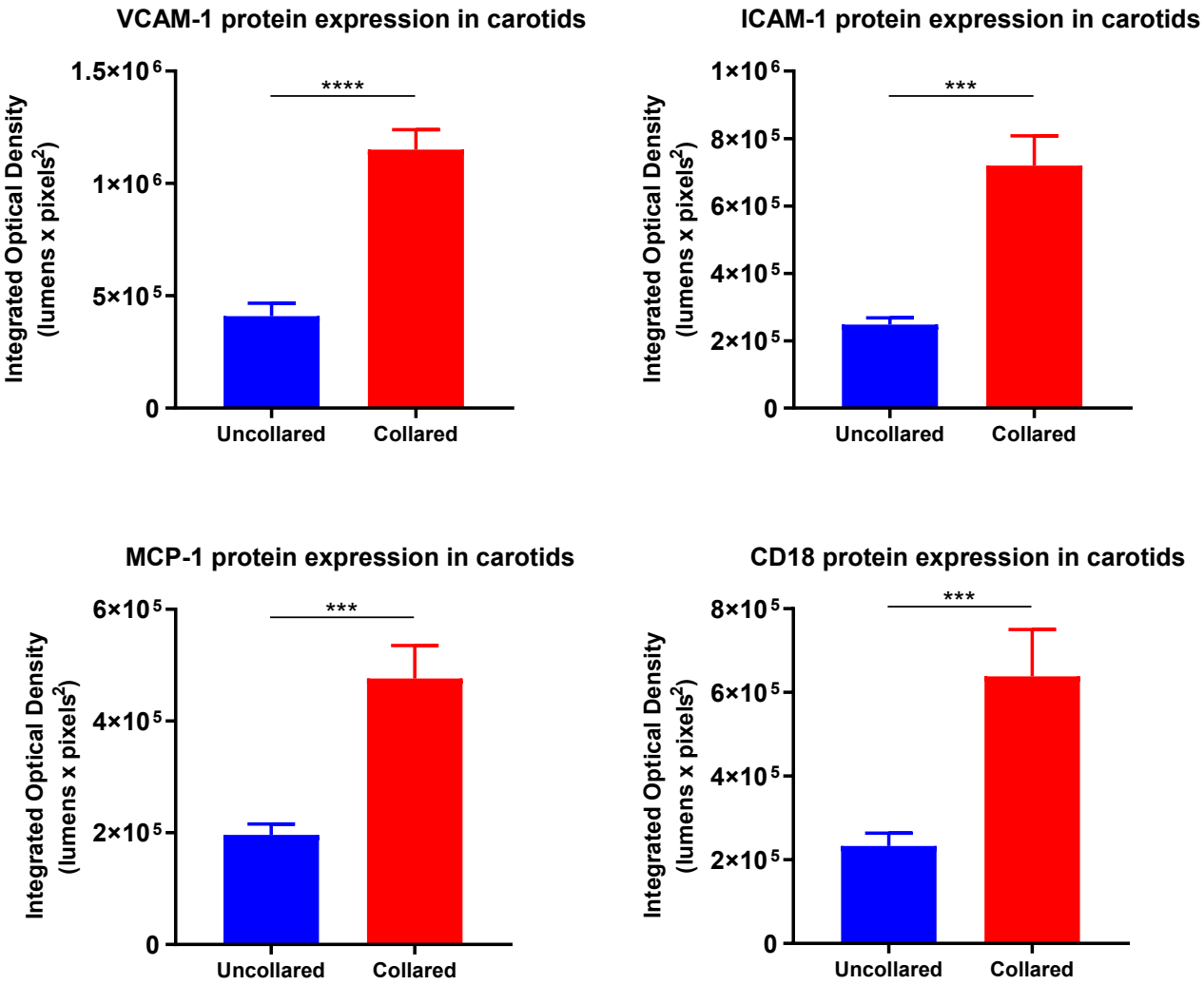


Figure 5.11: Protein expression of VCAM-1, ICAM-1, MCP-1 and CD18 measured by IHC of collared compared to uncollared carotid arteries. Data are presented as mean ± SEM. n = 9-10 per group. ****p<0.0001, ***p<0.001

VCAM-1

Compared with collared carotids from mice on no treatment, EPA-treated mice had a 42.8±3.38% reduction in VCAM-1 expression (p=0.02). There was no significant change in VCAM-1 expression in the olive oil group (+5.78±7.40%, p=0.97). There was a notable reduction in the DHA (-27.5±7.82%) group, however it did not reach statistical significance (p=0.25), see Figures 5.12 and 5.13.

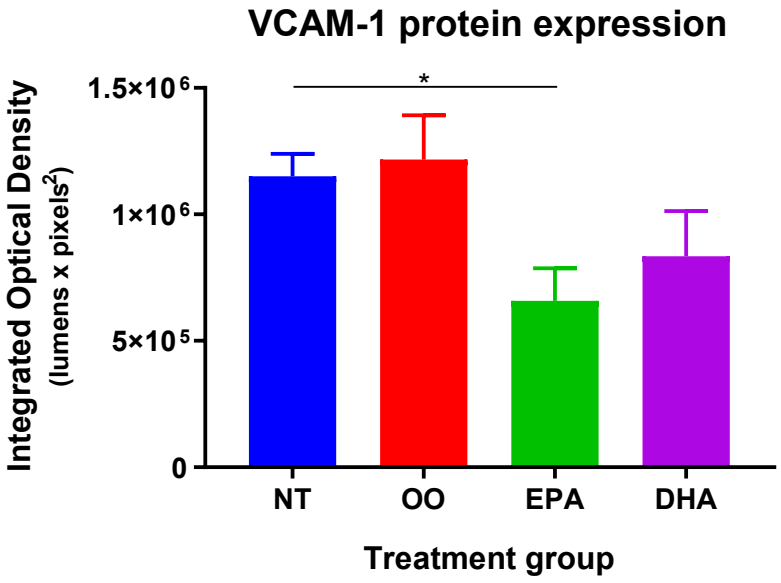


Figure 5.12: Quantification of VCAM-1 staining by IHC in collared carotids for each treatment group. Data are presented as mean ± SEM. n = 6-10 per group. *p<0.05

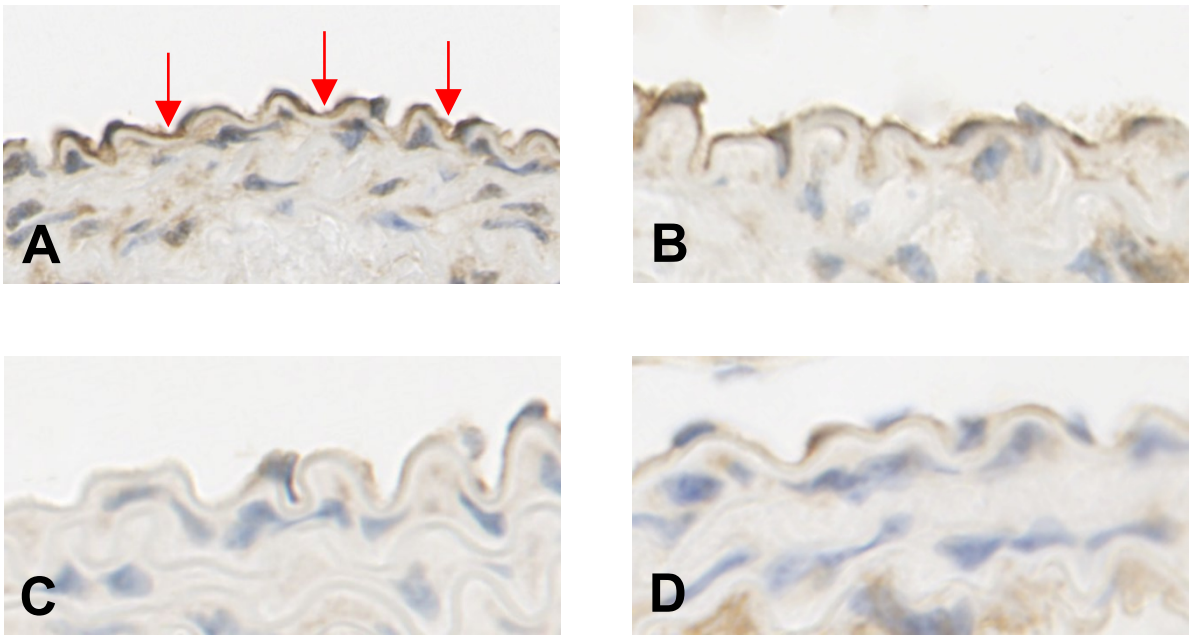


Figure 5.13: Representative IHC staining for VCAM-1 in collared carotid arteries. Staining is visible in the endothelium (arrows). Carotid specimens are from mice treated with (A) No Treatment, (B) Olive Oil, (C) EPA, and (D) DHA. The EPA group has much less endothelial staining compared to the No Treatment group.

ICAM-1

ICAM-1 expression was not significantly different between treatment groups. There were numerical but non-significant reductions in ICAM-1 expression in the olive oil (-13.2±2.01%, p=0.79), EPA (-28.8±0.33%, p=0.18), and DHA (-27.9±2.01%, p=0.28) treatment groups compared to the no treatment group, see Figure 5.14.

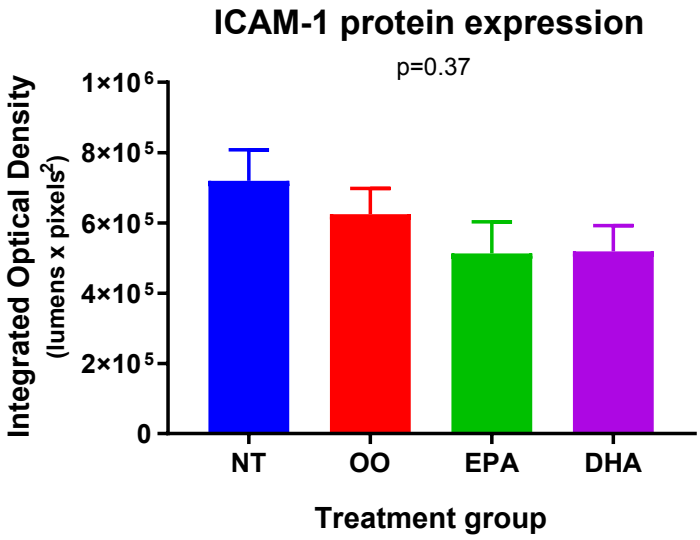


Figure 5.14: Quantification of ICAM-1 expression by IHC in collared carotids for each treatment group. Data are presented as mean ± SEM. n = 6-10 per group.

MCP-1

Compared with mice in the no treatment group, mice in the EPA-treated group had a significant reduction (-40.9±6.59%, p=0.03) in MCP-1 expression in collared carotid arteries. There was no significant change in MCP-1 expression in the olive oil- (+13.5±2.68%, p=0.75) or DHA- (-24.3±1.87%, p=0.33) treated groups compared with the no treatment group, see Figures 5.15 and 5.16.

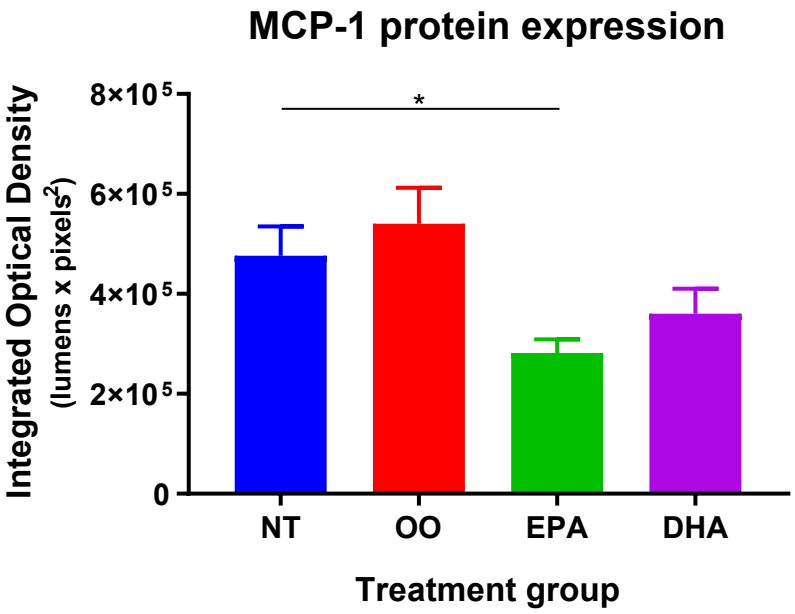


Figure 5.15: Quantification of MCP-1 expression by IHC in collared carotids for each treatment group. Data are presented as mean ± SEM. n= 6-10 per group. *p<0.05

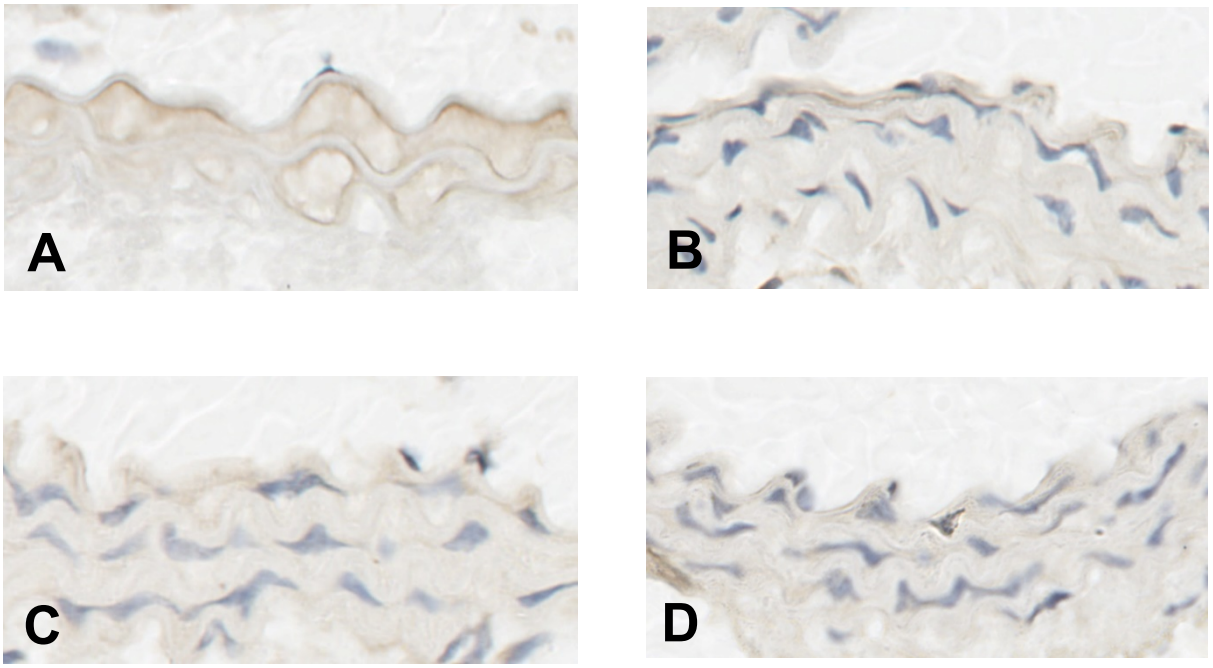


Figure 5.16: Representative IHC staining for MCP-1 in collared carotid arteries from mice treated with (A) No Treatment, (B) Olive Oil, (C) EPA, and (D) DHA. Endothelial staining is least visible in the EPA-treated group.

CD18

CD18 expression was not significantly different between treatment groups. There were numerical but non-significant reductions in CD18 expression in the EPA (-27.1±9.27%, p=0.32) and DHA (-26.1±13.00%, p=0.44) treatment groups compared to the no treatment group. The olive oil-treated group had a numerical but non-significant increase in CD18 expression (+21.1±0.85%, p=0.60), see Figure 5.17.

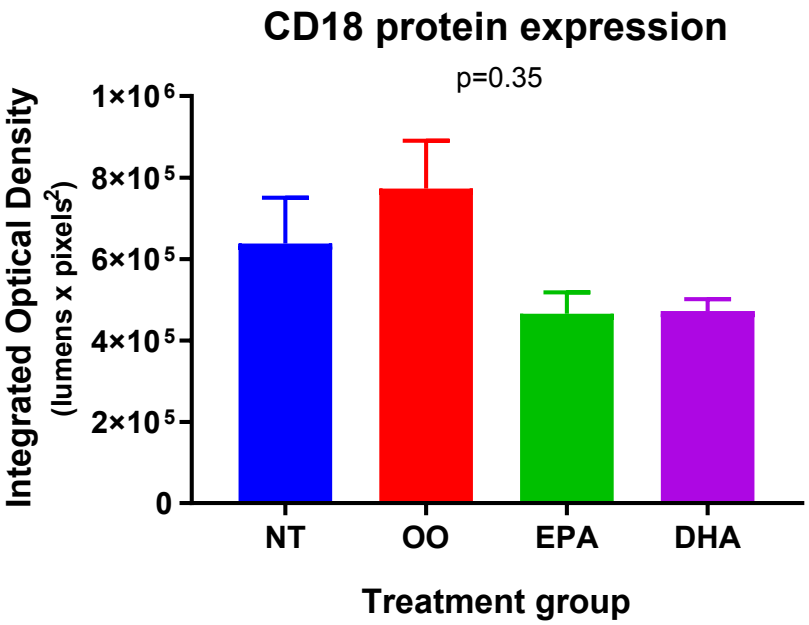


Figure 5.17: CD18 expression by IHC in collared carotids by treatment group. Data are presented as mean ± SEM. n = 6-9 per group.

5.3.4 Both EPA and DHA blood levels had an inverse correlation with inflammatory markers

The markers of AVI measured by IHC were correlated with blood levels of EPA and DHA, and a significant inverse relationship was demonstrated. There were significant inverse correlations present between blood levels of EPA, and all four inflammatory markers, see Figure 5.18.

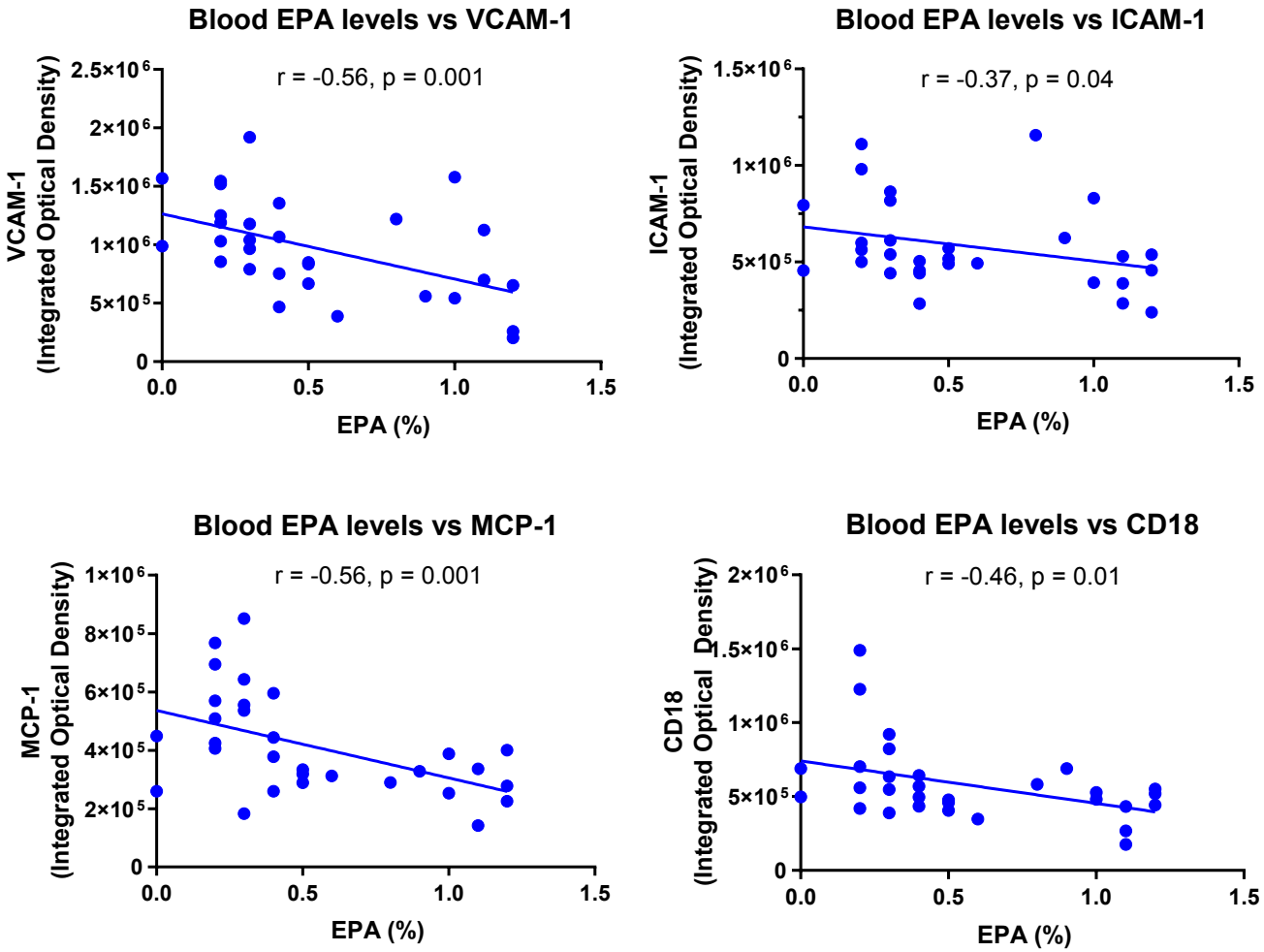


Figure 5.18: Correlations between blood levels of EPA and the protein expression of VCAM-1, ICAM-1, MCP-1 and CD18 as measured by IHC.

Similarly, there were significant inverse correlations between blood levels of DHA and the protein expression of all four markers of AVI in the vessel wall, see Figure 5.19. This was in the context of a numerical but non-significant reduction in VCAM-1 and MCP-1 protein expression in the collared carotid of DHA-treated mice. This likely reflects a genuine anti-inflammatory effect that has not been fully demonstrated due to a modest study sample size.

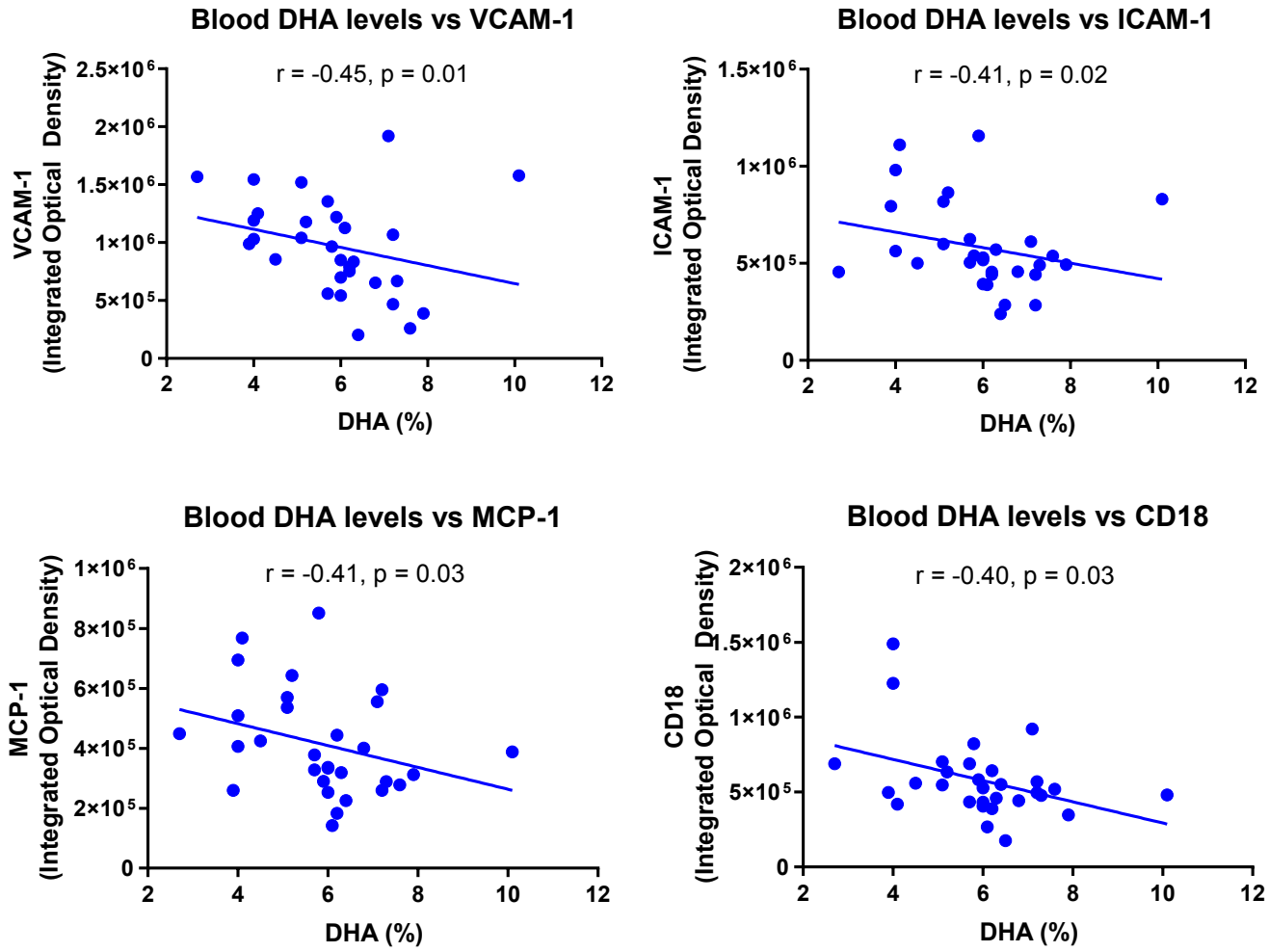


Figure 5.19: Correlations between blood levels of DHA and the protein expression of VCAM-1, ICAM-1, MCP-1 and CD18 as measured by IHC.

The superior anti-inflammatory properties of EPA compared to DHA was again demonstrated when correlating the protein expression of markers of AVI with the ratio of EPA to DHA in blood. There was a significant inverse correlation with all four markers, consistent with the greater anti-inflammatory effect seen with EPA, see Figure 5.20.

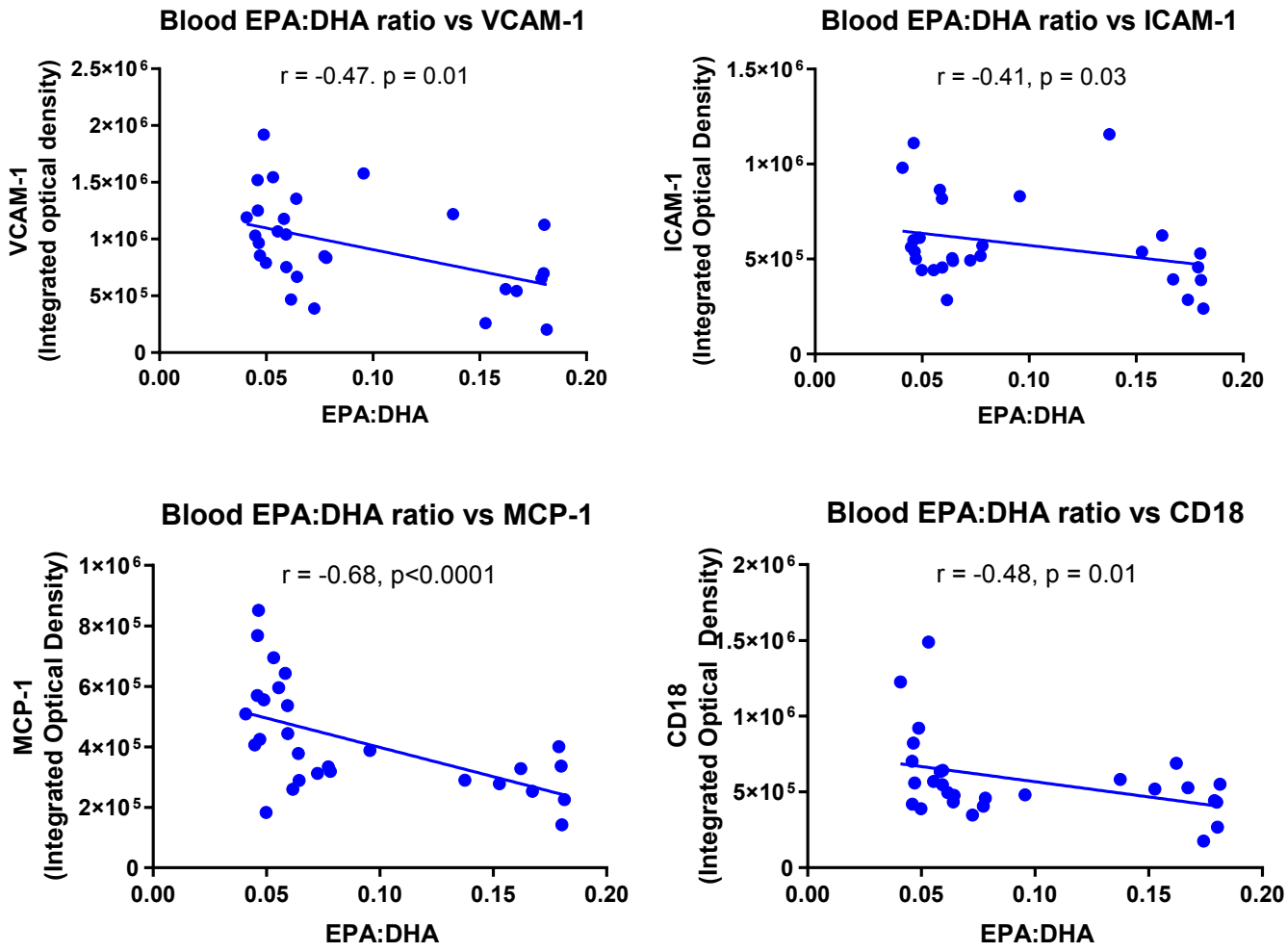


Figure 5.20: Correlations between the EPA:DHA ratio in blood and the protein expression of VCAM-1, ICAM-1, MCP-1 and CD18 as measured by IHC.

5.4 Discussion

In this study, we sought to determine the impact of omega-3 fatty acids, in particular the comparative effects of EPA and DHA, on AVI in an *in vivo* model. The omega-3 fatty acid EPA has been shown in this study to reduce periarterial collar-induced acute vascular inflammation (AVI), as evidenced by reduced endothelial protein expression of VCAM-1 and MCP-1. Notable reductions in markers of AVI were seen with DHA, which did not achieve statistical significance and may reflect the low sample size of 10 mice per treatment group.

These outcomes were in the context of EPA and DHA supplementation having no effect on cholesterol or triglyceride levels, which were low in all mice.

The effectiveness of the oral gavage method and the method of supplement preparation were confirmed by the significant increase in blood omega-3 levels after 30 days of omega-3 supplementation. Omega-3 fatty acids can be administered in various biochemical forms, including phospholipid, free fatty acid, triglyceride, and ethyl ester forms. The free fatty acid form is the most bioavailable[513] and was used in this study. The dose chosen was equivalent to ten times the dose used in many recent human clinical trials of 4 grams per day. Four grams per day equates to 60mg/kg/day for a 70kg human, and hence 600mg/kg/day was used in this mouse study to ensure sufficient dosing.

In eukaryotes, fatty acids may undergo enzymatic conversion to other fatty acid species, by such processes as elongation, desaturation, chain shortening and beta-oxidation. This allows multiple diverse species of fatty acids to be generated from the presence of a single fatty acid. These processes however, are inefficient, and hence supplementation with a specific fatty acid produces the highest blood and tissue concentrations of that fatty acid. EPA (20:5ω3) can be elongated, Δ6 desaturated, and beta-oxidised to DHA (22:6ω3) via the intermediary, DPA (22:5ω3)[514]. Likewise DHA can be retroconverted directly back to EPA, but without an intermediary[515]. It follows that supplementation with either EPA or DHA in this study significantly increased both EPA and DHA levels, with a natural predominance of the fatty acid being supplemented. Likewise, DPA, which is formed by the elongation of EPA, was significantly increased in the EPA-supplemented mice, but not in the DHA-supplemented mice.

The essential omega-3 fatty acid alpha-linolenic acid (18:3ω3) was not increased by supplementation with either EPA or DHA, as EPA does not readily undergo carbon chain

shortening. Blood levels of alpha-linolenic acid hence reflect the presence of this fatty acid in diet. Supplementation with olive oil, rich in the omega-9 oleic acid (18:1ω9), did not alter total omega-3 levels or total omega-6 levels. Arachidonic acid (20:4ω6) was increased by olive oil, and this is likely because oleic acid is a precursor of arachidonic acid.

A low omega-6/omega-3 ratio is associated with lower levels of oxidative stress, inflammation, and endothelial dysfunction[516]. Reductions of >30% were seen in the EPA- and DHA-supplemented mice, despite the high background blood levels of omega-6 fatty acids. The levels of monounsaturated fatty acids, comprised of omega-7 and omega-9 fatty acids, were naturally highest in the mice supplemented with olive oil. Trans-fatty acids, which are atherogenic and promote inflammation and oxidative stress[517] are present in very low levels in standard rodent diets, and this was reflected in the trans-fatty acid blood levels. Supplementation with large quantities of alternative fatty acids, in the form of EPA, DHA and olive oil in this study, reduced trans-fatty acid levels to nearly undetectable in each case. Saturated fats were present in high concentrations in all mice in the study, which is consistent with previous fatty acid profiling in mice[518, 519]. Supplementation with high doses of unsaturated fatty acids in this study led to reductions in the proportions of saturated fatty acids in all groups. Thus, it is emphasised that the omega-6/omega-3 ratio, and levels of trans-fatty and saturated fatty acids, are lowered by substitution by more favourable fatty acids, namely monounsaturated fatty acids and omega-3 polyunsaturated fatty acids.

Periarterial, non-occlusive collaring for 48 hours produced an intense inflammatory response throughout the vessel wall. VCAM-1 and ICAM-1, which are adhesion molecules that are upregulated during early vascular inflammation[520], were present in significant concentrations in the endothelium of collared carotids. MCP-1, another marker of early vascular inflammation, which regulates migration of monocytes into the vessel wall[521], was similarly induced in the endothelium by collaring. Leucocyte recruitment to the vessel wall is

a key, early event in acute vascular inflammation[522], and this was studied using the pan-leucocyte marker CD18. CD18 was also induced in the endothelium by collaring. The numerical reductions in endothelial protein expression of all factors by both EPA and DHA is consistent with the known anti-inflammatory effects of omega-3 fatty acids[195]. The significant reductions seen in VCAM-1 and MCP-1 in the mice supplemented with EPA provides evidence for a differential anti-inflammatory effect on acute vascular inflammation between EPA and DHA. Significant inverse correlations were present between blood levels of both EPA and DHA and the expression of VCAM-1, ICAM-1, MCP-1, and CD18. However, a higher EPA:DHA ratio in blood correlated with lower levels of all four inflammatory markers. This further demonstrates the superior anti-inflammatory effect of EPA compared to DHA, while also providing evidence of the anti-inflammatory properties of DHA. The superior anti-inflammatory effect of EPA may simply reflect a more potent anti-inflammatory effect, or different mechanisms of action.

Omega-3 fatty acids are known to modulate inflammation through several mechanisms. These include incorporation into the phospholipids of inflammatory cells[292, 293], reduced eicosanoid production[294], synthesis of resolvins[295], inhibition of the pro-inflammatory NF-κB transcription pathway[296-298], induction of the anti-inflammatory PPAR-γ transcription pathway[299], disruption of lipid rafts[300, 301], and binding to the G-protein coupled receptor GPR120[302], which initiates an anti-inflammatory signalling cascade. The expression of VCAM-1, ICAM-1 and MCP-1 are regulated via the NF-κB transcription pathway[523]. Additionally, ICAM-1 is regulated by the MRTF-A/B pathway[524] and is inhibited by mIR-22[525], both of which are not known to be a target of omega-3 fatty acids. This may explain the lack of a significant reduction in ICAM-1 expression observed. Moreover, the recruitment of leucocytes to the vessel wall, measured by CD18, is regulated by several factors[526], some of which are not a target of omega-3 fatty acids. There is a paucity of quality data on the

differential effects of EPA versus DHA on inflammation. Some studies that have examined this *in vitro* have noted comparable downregulation of gene expression of inflammatory markers with both EPA and DHA[527-529], or seen differential effects between the two fatty acids that have been discordant between studies[530, 531]. Other *in vitro* studies have demonstrated differential effects of EPA compared to DHA on the expression of regulatory genes by concanavalin A-treated T-lymphocytes (DHA superior)[532], and in lipopolysaccharide-stimulated THP-1 macrophages (EPA superior)[527]. The specific pathways studied were cytokines and related receptors, signal transduction pathways, transcription factors, immune response, cell cycle, defence and repair, apoptosis, DNA synthesis, cell adhesion, cytoskeleton, and hormone receptors[527, 532]. These studies have provided some mechanistic clues to explain differential effects on inflammation but much further study is required.

Whether there is a differential effect of EPA compared to DHA on chronic vascular inflammation and atherosclerosis is yet to be determined. This is relevant since there is now clear evidence that certain anti-inflammatory therapies reduce cardiovascular events. The CANTOS study evaluated the effects of canakinumab, a monoclonal antibody that targets interleukin 1-β, which plays an important role in the pathogenic mechanisms leading to vascular inflammation[236]. Canakinumab reduced cardiovascular death, nonfatal MI and nonfatal stroke compared to placebo in patients with a previous history of MI and an elevated C-reactive protein, independent of lipid levels. Thrombosis is a major pathophysiological event leading to these cardiovascular endpoints, with plaque rupture being the most common cause[533]. The intense inflammatory milieu present in ruptured plaques is a potential target for therapies such as omega-3 fatty acids that reduce acute inflammation. Although EPA has not been compared directly with DHA in cardiovascular outcome trials, high-dose EPA has been shown to significantly reduce major adverse cardiovascular events. The recently-published REDUCE-IT study evaluated high-dose EPA (4 grams daily of icosapent ethyl) in

hypertrigyceridemic patients who had established cardiovascular disease or were diabetics with other cardiovascular risk factors. Icosapent ethyl reduced the primary endpoint of a composite of cardiovascular death, nonfatal MI, nonfatal stroke, coronary revascularisation and unstable angina by 25% (first event) and 30% (all events) compared to placebo[234]. The reductions in triglyceride levels that occurred with icosapent ethyl did not influence its efficacy in reducing the primary endpoint. This further highlights the importance of inflammation reduction as a mediator of atheroprotection in this study, and the likelihood that targeting acute inflammation impacts all stages of atherogenesis from endothelial dysfunction to thrombosis. Future well-designed studies of omega-3 fatty acids on acute vascular inflammation are warranted, and will have implications for a number of acute and chronic vascular diseases.

The limitations of this study include a small sample size of 10 mice per group. There were numerical reductions in the protein expression of all four markers of acute vascular inflammation with both EPA and DHA supplementation. EPA had a greater anti-inflammatory effect than DHA, however only reached statistical significance for VCAM-1 and MCP-1. It is likely that with a greater sample size, significant reductions would have been demonstrated for both EPA and DHA. This study was designed to directly compare pure EPA with pure DHA, and no mice were supplemented with formulations with different ratios of EPA and DHA. Therefore this study was unable to identify an optimal EPA:DHA ratio to reduce acute vascular inflammation. The design of this study best modelled a primary prevention setting, as wildtype mice fed a standard rodent diet were pre-treated with omega-3s and then given an acute inflammatory insult. An additional treatment arm in which mice were first collared and then treated with omega-3s would have permitted a comparison of primary prevention versus acute therapy. However, attempting to orally gavage mice after neck surgery would have significantly increased the risk of wound dehiscence and aspiration and therefore was not attempted.

This is the first animal study of the differential effects of EPA versus DHA supplementation on pure acute vascular inflammation. Pre-treatment with high dose EPA significantly reduced protein expression of VCAM-1 and MCP-1 in an established perivascular collar model of AVI. This occurred independent of lipid levels, and was supported by strong inverse correlations seen between blood EPA levels as well as the EPA:DHA ratio with protein expression of all four markers of AVI studied. This was consistent with the findings of Chapter 4, where gene expression of MCP-1 was reduced by EPA in an *in vitro* model of AVI. The recently published REDUCE-IT study demonstrated a significant reduction in major adverse cardiovascular events with high dose EPA supplementation, and the effects of EPA on AVI may contribute to this. Moreover, the effects of EPA and DHA on chronic vascular inflammation and atherogenesis require further study, to further elucidate a mechanistic rationale for the results of REDUCE-IT.

5.5 Appendix A – Periarterial collaring materials

1. Normal saline
2. Betadine
3. Isoflurane for anaesthesia
4. Anaesthetic machine with nose cone and oxygenation chamber
5. Dissecting microscope
6. Small metal clip applicator [Weck Hemoclip® Plus EZ Load Applier (Teleflex Medical, North Carolina, USA)], with small metal clips
7. Silicone tubing with 0.64 mm internal diameter, 1.2 mm outer diameter (Catalogue number: JHS06412, Blue Sky Scientific, Burnside, SA, Australia).

8. Clippers for fur removal

9. Lignocaine for injection, diluted to 0.5% in normal saline

10. Buprenorphine

11. 6/0 nylon sutures (Dyloc D602D sutures, Dynek, SA, Australia).

12. Heatpad

13. Cotton tips

14. 25 gauge or smaller needles for injection

15. 1 ml syringes

16. Metal retractors

17. Scalpel

18. Small, blunt forceps x 2

19. Suture holder

20. Ligation aid (hook-shaped)

21. Ruler

22. Blunt haemostats for blunt dissection

23. Small, fine scissors

24. Lubricating eye drops

25. Recovery box

CHAPTER 6: THE IMPACT OF OMEGA-3 FATTY ACIDS ON ATHEROSCLEROSIS AND CHRONIC VASCULAR INFLAMMATION

ABSTRACT

Background: Despite the significant advances that have occurred in the prevention and management of cardiovascular disease, there remains a significant residual risk in the general population. Inflammation has received considerable attention as a target for new therapies. Omega-3 fatty acids have anti-inflammatory properties, and clinical evidence is emerging that supports an atheroprotective role, especially when provided at a high dose. Previous animal studies have attempted to evaluate the effects of omega-3 fatty acids on atherogenesis, which have frequently had methodological limitations, and have not explored the relative effects of EPA versus DHA. This study aimed to determine the effects of omega-3 fatty acids on chronic vascular inflammation and atherogenesis, specifically the burden and characteristics of atherosclerotic plaque, in an animal model. The relative effects of EPA were to be compared with DHA.

Methods: Forty 8-week-old ApoE-deficient mice, 20 of each sex, were fed an atherogenic diet for 16 weeks to induce chronic vascular inflammation and advanced atherosclerotic plaques. After the first 8 weeks, a blood sample was taken for fatty acid and lipid analysis. The mice were then randomised equally to supplementation with 600mg/kg/day of either (1) EPA, (2) DHA, (3) olive oil as an oil control, or (4) no treatment, by oral gavage, to be given for the final 8 weeks. The mice were subsequently humanely killed and a terminal blood sample was taken for fatty acid and lipid analysis. Organs were harvested, and plaque burden, intimal and medial layer expansion, plaque collagen content, smooth muscle cell content and inflammatory cell content were measured in the aortic sinuses and brachiocephalic arteries. The aortas were stained for lipid using Oil Red O. Aortas were snap-frozen, and gene expression of markers of chronic inflammation and regulatory transcription factors were measured by RT-PCR (including IL-1β, TNF-α, MCP-1, NFκB-p65, and PPAR-γ). Correlations were made between

the blood concentrations of EPA, DHA, and the EPA:DHA ratio at study completion and the plaque, lipid, and inflammatory measures.

Results: 38 out of 40 mice completed the study. Blood EPA and DHA levels increased significantly in the respective treatment groups. Plasma cholesterol levels were high in all mice after 8 weeks (17.96±5.4 mmol/L), continued to increase in the no treatment and placebo groups, but stabilised in the EPA and DHA treatment groups. Triglyceride levels were elevated in all mice after 8 weeks (2.27±0.70 mmol/L), and were significantly reduced by EPA and DHA treatment. All mice developed advanced atherosclerotic plaques, however the burden of plaque in the aortic sinuses and brachiocephalic arteries did not differ among treatment groups. Although the combined thickness of the intima and media was similar between treatment groups, there was an inverse correlation between blood EPA levels and intimal plus medial thickness ($r=-0.49$, $p=0.04$). Collagen content, a marker of plaque stability, did not significantly differ among treatment groups in the aortic sinus or brachiocephalic artery plaques. In these sites, the density of smooth muscle actin staining and macrophage (CD107b) staining on immunohistochemistry was similar among treatment groups. Lipid was present throughout the aorta in all mice, with 6.3 (IQR 5.0 – 10.6) % of the aortic surface staining positively for Oil Red-O in the no treatment group; this did not differ significantly among treatment groups. In aortic tissue, the gene expression of markers of chronic inflammation, IL-1β and TNF-α, were significantly lower in the mice treated with EPA (-44.3% [$p=0.04$] and -48.8% [$p=0.04$], respectively). There were significant inverse correlations present between IL-1β and TNF-α expression and both blood EPA levels and the EPA:DHA ratio.

Conclusions: In an atherogenic mouse model, supplementation with both EPA and DHA stabilised cholesterol levels and reduced triglyceride levels. Despite this, neither EPA nor DHA had a significant effect on plaque burden, lipid burden, markers of plaque stability, or the inflammatory cell content of plaque. EPA significantly reduced gene expression of the markers

of chronic vascular inflammation IL-1β and TNF-α in the arterial wall, with a strong inverse correlation present between these markers and blood EPA levels and the EPA:DHA ratio. The findings of this study, in particular the reduction in chronic vascular inflammation by EPA supplementation, may provide a mechanistic contribution to the reduction in major adverse cardiac events seen with EPA in the recently-published REDUCE-IT trial. The pharmacodynamics of individual omega-3 fatty acids and their effects on all stages of atherogenesis require continued investigation.

I, Anthony Pisaniello, conceived, designed, executed and analysed all of the work included in this chapter.

6.1 Background

Cardiovascular disease is the leading cause of death worldwide, with the largest contribution being from atherosclerotic diseases[1]. Significant advances in the prevention and treatment of atherosclerotic diseases have led to major reductions in morbidity and mortality[2]. Despite these advances, there remains a substantial residual cardiovascular risk in the general population[3]. There have been continued efforts to identify and target new atherogenic pathways, and much attention has been focused on the role of inflammation, which is present in all stages of atherogenesis[534]. Given the essential contribution of acute and chronic vascular inflammation to atherogenesis, agents that attenuate these processes are likely to have anti-atherosclerotic properties and reduce cardiovascular risk. This has recently been demonstrated using canakunimb, a monoclonal antibody to interleukin-1β, which contributes significantly to the inflammatory state of plaques, especially in the vessel wall[79-81]. Canakinumab reduced cardiovascular events in patients with a previous myocardial infarction and a raised C-reactive protein levels[120]. The same may be applicable to omega-3 fatty acids, which have beneficial effects on inflammation[535, 536], as well as on endothelial function[537, 538], oxidative stress[197, 539], and lipids[540, 541]. As demonstrated in Chapter 4 and 5 of this thesis, omega-3 fatty acids, in particular eicosapentaenoic acid (EPA), reduce acute vascular inflammation (AVI). Since AVI is an early and critical stage of atherogenesis, it is predictable that omega-3 fatty acid supplementation would reduce the volume and inflammatory nature of atherosclerotic plaques. Moreover, inflammation in plaque promotes vulnerability[542, 543], and hence omega-3s may also have a favourable effect on plaque stability. The effects of omega-3 fatty acids on the pathology of chronic vascular inflammation and atherosclerosis have been studied predominantly in animal models of atherosclerosis. In a study by Matsumoto et al, EPA improved plaque and lipid burden in ApoE-deficient mice, and improved plaque stability and inflammatory cell content in LDLR-deficient mice[255]. However a similar study by Xu et al,

which assessed the effects of combined EPA and DHA in the form of fish oil on plaque and lipid burden in ApoE-deficient mice demonstrated no benefit[254]. Favourable effects of combined EPA and DHA on the inflammatory state were seen in a study by Li et al, where efferocytosis by macrophages was increased in the atherosclerotic plaques of ob/ob mice[256]. The prevalence of inflammatory cells, namely macrophages, dendritic cells and CD4+ T cells in atherosclerotic plaques, was reduced by EPA supplementation in LDLR-deficient mice in a study by Nakajima et al[259]. In that study, EPA was also shown to significantly regress atherosclerotic plaque. DHA on the other hand, has been less studied in the setting of chronic vascular inflammation and atherosclerosis, yet was shown in one study to reduce atherosclerotic lesion size in ApoE-deficient mice when added to their diet[260]. These studies have provided evidence for beneficial effects of omega-3 fatty acids on chronic vascular inflammation and atherosclerosis, which require further investigation. These studies have not directly compared EPA with DHA. The method of supplementation used in these animal studies, i.e. the addition of omega-3 fatty acids to food has limitations such as a propensity for both fatty acid oxidation and variability in dosing.

Cardiovascular outcome trials (CVOTs) of omega-3 supplementation in the primary and secondary prevention settings have produced variable results[226-230, 232, 233], despite overall positive results from dietary studies[222-224]. Some of the limitations of those CVOTs were overcome by the well-designed REDUCE-IT trial, which demonstrated a 25% reduction in major adverse cardiovascular events with EPA supplementation[234]. Although there was no comparison with DHA in REDUCE-IT, data from a recent cohort study by Veno et al provided evidence for a superior atheroprotective effect of EPA compared to DHA[544]. Middle-aged adult subjects had adipose tissue analysed for fatty acid content at baseline, and were followed for a median period of 13.5 years for the development of ischaemic strokes. There was a significant inverse correlation between EPA content and ischaemic stroke risk (HR 0.74, 95%

CI, 0.62–0.88), which was not present for DHA or for total omega-3 fatty acid content[544]. The evidence for a cardioprotective effect of omega-3 fatty acids mandates further evaluation of mechanisms of benefit. This may include beneficial effects on chronic vascular inflammation and on the characteristics and burden of plaque. Given the results of REDUCE-IT, the comparative effects of EPA compared to DHA in a model of secondary prevention would be of significant interest.

6.1.1 Aims and rationale of study

The aims of this study were to:

(1) Determine in an animal model of atherosclerosis the impact of omega-3 fatty acids on (i) plaque burden, characteristics, inflammatory content and stability, (ii) arterial lipid burden and circulating lipids, and (iii) markers of chronic inflammation in the vessel wall.

(2) Compare the relative effects of EPA versus DHA supplementation on the above measures.

An ideal animal model for this study is the ApoE-deficient mouse fed an atherogenic diet, which rapidly develops advanced atherosclerotic lesions that resemble those of humans[545]. The very high cholesterol levels induce endothelial dysfunction as early as four weeks of age, followed by fatty streak formation by 6 weeks, and early inflammatory atherosclerotic plaques by 10 weeks of age[546]. By 10-15 weeks of age, foam cell formation and smooth muscle cell proliferation are evident, and after 20 weeks of age advanced plaques are seen, progressing from the proximal aorta distally throughout the arterial tree, characterised by a necrotic core and fibrous cap[547]. Precise quantities of omega-3 fatty acids can be administered to mice by oral gavage, and mice do not have a natural aversion to the taste or odour of fish unlike other animals such as rabbits, which are often used to study atherosclerosis[548].

6.1.2 Hypotheses

The hypotheses of this study were that:

(1) Omega-3 fatty acids alter the development of atherosclerotic plaques by reducing total plaque content, reducing intimal and medial proliferation, reducing inflammatory cell content, and increasing collagen and smooth muscle cell content.

(2) Omega-3 fatty acids reduce the lipid content of aortic lesions independent of effects on plasma cholesterol and triglyceride levels.

(3) Omega-3 fatty acids reduce markers of chronic vascular inflammation in the arterial wall.

(4) For all of the above measures, EPA will have a greater effect compared to DHA.

(5) A significant inverse correlation will be present between blood omega-3 levels, especially EPA, and the above measures of plaque, lipid and vascular inflammation.

6.2 Methods

6.2.1 Study outline

Forty 8-week-old chow-fed ApoE-deficient mice, 20 of each sex, previously fed a standard rodent chow diet, were commenced on an atherogenic diet (22% fat, 0.15% cholesterol, SF00-219, Specialty Feeds, Glen Forrest, WA, Australia) for 16 weeks. Mice were weighed at baseline and then weekly thereafter. After 8 weeks, a blood sample was taken by superficial facial vein bleeding. Whole blood was spotted onto dry blood spot cards for fatty acid analysis. Blood was also collected for plasma total cholesterol and triglycerides. The mice were then randomised equally to daily supplementation with either EPA, DHA, olive oil (OO), or no treatment for the last 8 weeks of the 16-week period, by oral gavage. EPA and DHA were

in the form of ethyl esters (>97% purity), and were provided by Bizen Chemical Company (Okayama, Japan). The olive oil was Bertolli brand (Florence, Italy). The dose administered was 600 mg/kg/day. At the end of the treatment period, the mice were humanely killed as per Section 2.10, and the blood that was obtained from terminal cardiac puncture was analysed for fatty acids, plasma total cholesterol and plasma triglycerides. The study outline is presented schematically in Figure 6.1. Organ harvesting was then performed. Organs were stored in 10% neutral-buffered formalin (NBF) or snap-frozen in liquid nitrogen.

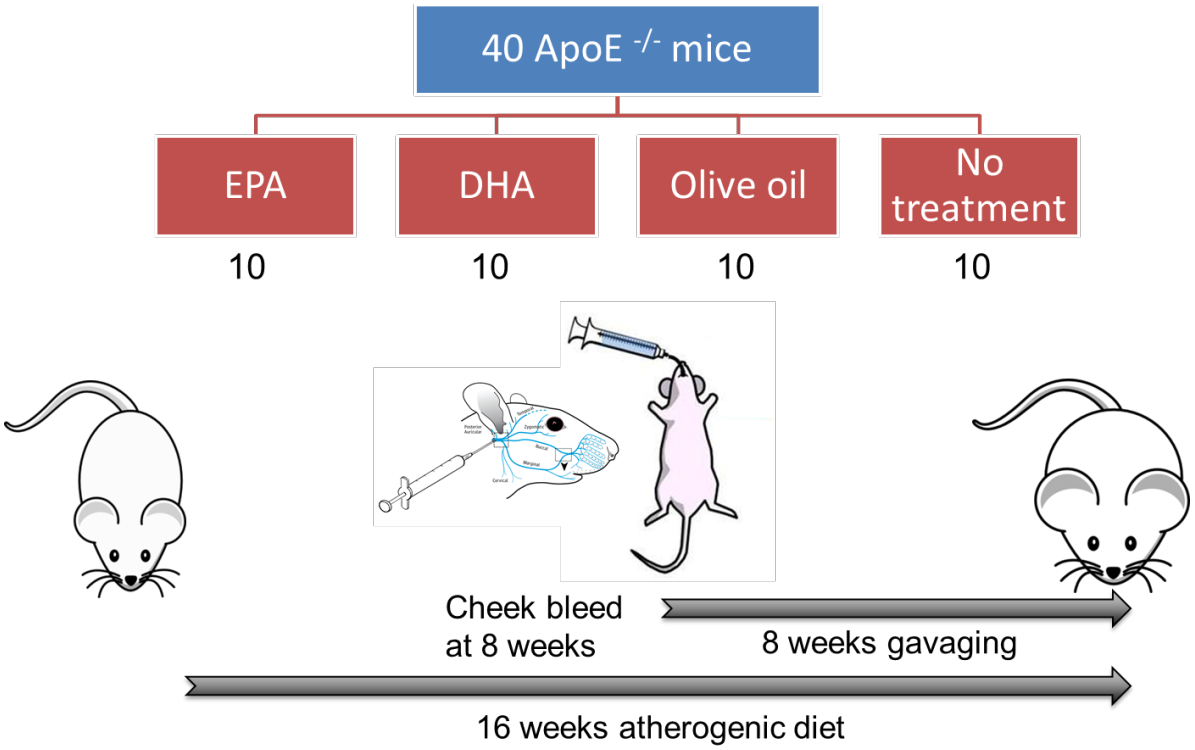


Figure 6.1: Schematic representation of study outline. Forty ApoE-deficient mice were randomised equally to one of four treatment groups. After 8 weeks on an atherogenic diet, a cheek bleed was performed and treatment by oral gavage was commenced. After a further 8 weeks the mice were humanely killed.

The harvested hearts were sectioned parallel to the aortic valve, through its entire depth, in 5 μm increments. Sections were stained for haematoxylin and eosin, as well as the Masson's trichrome stain, to allow quantification of plaque burden, intimal and medial expansion, and plaque collagen content. In addition, sections were assessed for the presence of smooth muscle cells by immunohistochemical staining for smooth muscle actin. Quantification of all immunohistochemical staining was performed using ImageJ software as per Section 2.15. The inflammatory content of plaque was assessed by immunohistochemical staining for macrophages using the CD107b (Mac3) stain. The brachiocephalic artery was sectioned through its entire length, and staining and quantification were as per the aortic valves. The aortas were stained for lipid using the Oil Red O and quantified using ImageJ software as per Section 2.16. The snap-frozen aortas were homogenised, and gene expression of markers of chronic inflammation and regulatory transcription factors were measured by PCR. These comprised IL-1β, TNF-α, MCP-1, NFκB-p65, and PPAR-γ. Correlations were made between the blood concentrations of EPA, DHA, and the EPA:DHA ratio at study completion and the plaque, lipid, and inflammatory measures.

6.2.2 Statistical methods

6.2.2.1 Sample size calculation

The sample size calculation for this study was based on a paper by Liu M et al[549]. The macrophage content of plaques was deemed to be the most important factor in demonstrating differences between treatment groups. In Liu M et al's study, 17% of the atherosclerotic plaque area of ApoE-deficient mice fed an atherogenic diet stained positively for macrophages. Treatment with the active drug (simvastatin) reduced this to 10%, and was significantly greater than an inactive vehicle. Using a one-way ANOVA power calculation for 4 groups with a significance level of 0.05, with 80% power, and a standard deviation of 10, a sample size of 40 was chosen, i.e. 10 mice per group.

6.2.2.2 Statistical analysis

GraphPad Prism 7 (GraphPad Software Inc, La Jolla, California, USA), SPSS 19 (IBM Corporation, Armonk, New York, USA), and Microsoft Excel 2016 (Microsoft, Albuquerque, New Mexico, USA) were used to analyse data. The D'Agostino-Pearson normality test was performed to determine whether continuous data were normally-distributed. Normally-distributed data were analysed using either the T-test if comparing means between two groups, or the One-way Analysis of Variance (ANOVA) if comparing means between multiple groups. If correcting for multiple comparisons, the Dunnett test was used. If comparing means between multiple groups adjusted for a covariate, an Analysis of Covariance (ANCOVA) was performed. Results were expressed as mean ± standard error of the mean (SEM). If continuous data were not normally-distributed, analysis was performed using the Kruskal-Wallis test, with Dunn's test used to correct for multiple comparisons. Results were expressed as median + interquartile range (IQR). Statistical correlations were analysed using a linear regression model. Statistical significance was set at the 0.05 level.

6.3 Results

Thirty-eight out of 40 mice completed the study. Two mice were culled on humane grounds; one after pulmonary aspiration occurred following oral gavage in the 9th week of supplementation (DHA group), and another due to the development of significant dermatitis (olive oil group).

6.3.1 Mouse body weight increased appropriately in the omega-3 treatment groups

At baseline, the 8-week-old ApoE-deficient mice fed a standard chow diet had a mean weight of 22.63±0.51 g. On the atherogenic diet, this increased by approximately 1.2 g per week to 32.40±0.13 g by 8 weeks. There were no significant differences between mice

allocated to different treatment groups at baseline (p=0.65) or after 8 weeks (p=0.52). In the no treatment group, the mice gained 5.90±0.50 g from weeks 9 to 16. This did not significantly differ in the EPA or DHA treatment groups (p=0.50 and p=0.10 respectively, adjusted for differences in weight after 8 weeks). Olive oil reduced further weight gain, with only a 2.22±0.47 g increase over the last 8 weeks of the study, which was notably less than that of the no treatment group (p=0.004), see Figure 6.2.

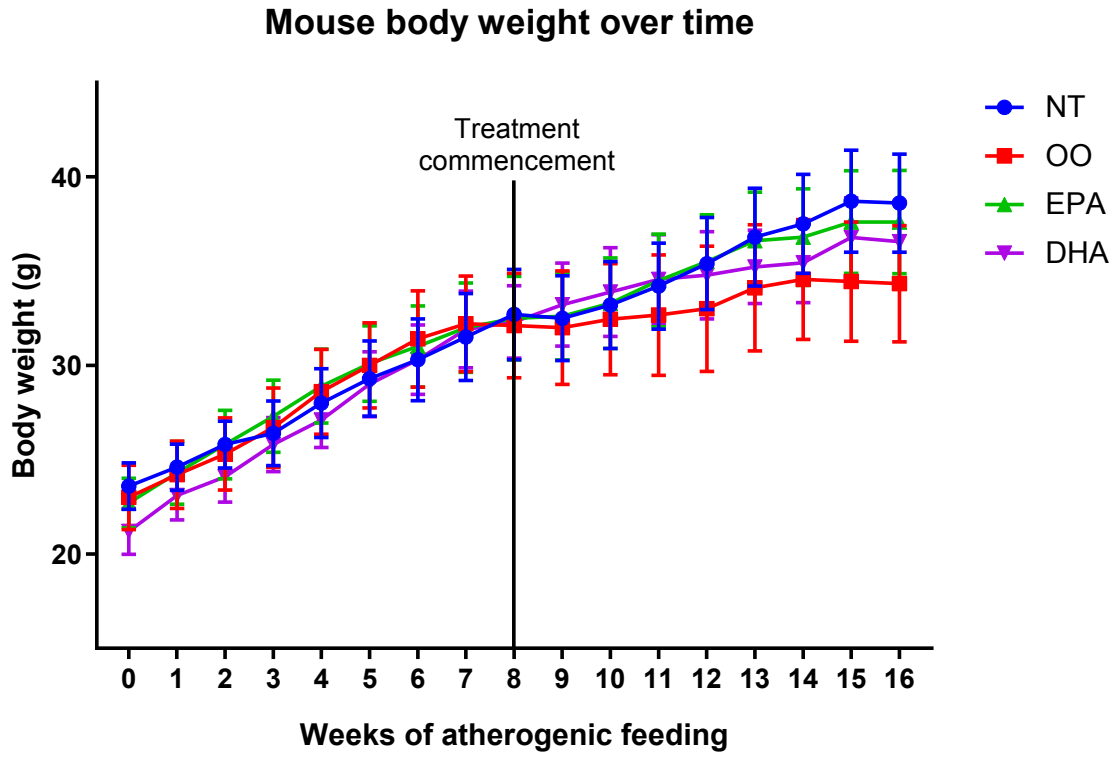


Figure 6.2: Weight of mice in each treatment group over the 16-week atherogenic feeding period. Note that treatment began after 8 weeks on this diet. n = 9-10 per group. Results expressed as mean ± SEM. NT = No treatment, OO = Olive oil.

6.3.2 Fatty acid analysis of whole blood

6.3.2.1 Summary of fatty acid content of blood by treatment group

A complete fatty acid profile of blood was taken after 8 weeks and then again after 16 weeks of atherogenic feeding. This was to record the fatty acid content of blood before and after supplementation, and to confirm the effectiveness of the oral gavage delivery method. Fatty acid levels in whole blood were expressed as a percentage of total fatty acid content. The highest proportion of fatty acids in blood were monounsaturated and saturated fatty acids. Omega-6 fatty acids were more prevalent than omega-3s, and trans-fatty acids were the least prevalent overall. The relative proportions of all major fatty acids at study completion are presented in Figure 6.3.

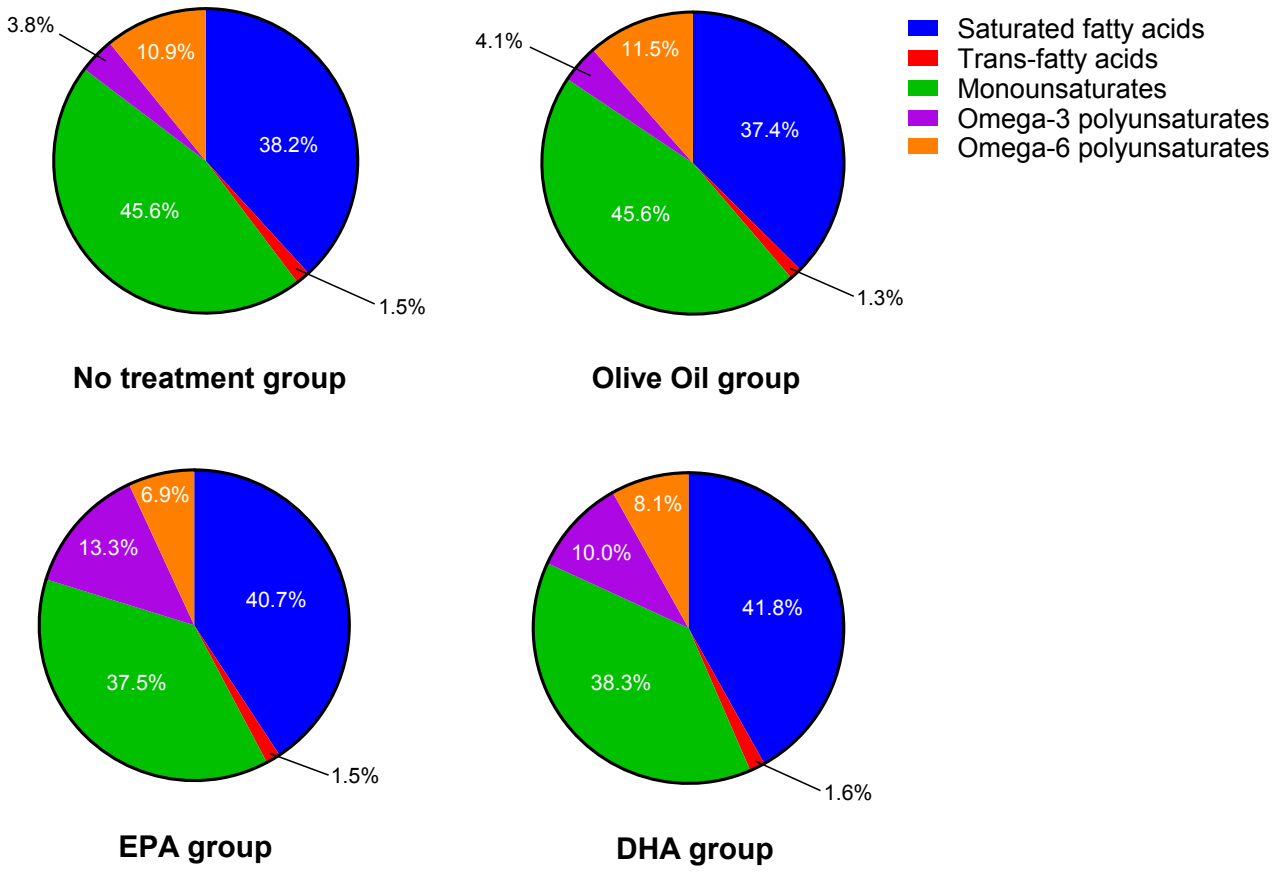


Figure 6.3: Relative proportions of all major fatty acid groups in blood at study completion, separated by treatment group.

6.3.2.2 Polyunsaturated omega-3 fatty acids

Total omega-3 fatty acid content

The SF00-219 rodent atherogenic diet has very low levels of omega-3 fatty acids (0.35% of total), and amongst those, it is predominantly composed of alpha-linolenic acid. After 8 weeks on this diet, the mean omega-3 content of blood for all mice was 2.60±0.11%. In the no treatment group, with continued feeding of the atherogenic diet, the total omega-3 content of blood increased by study completion to 3.79± 0.28%. A similar outcome was seen in the olive oil group, with an increase to 4.06±0.21%. As predicted, EPA and DHA supplementation markedly increased total omega-3 levels, with significantly higher values compared to that of the no treatment group (13.34±0.96%; p=0.001, and 9.98±0.67%; p<0.0001, respectively), see Figure 6.4.

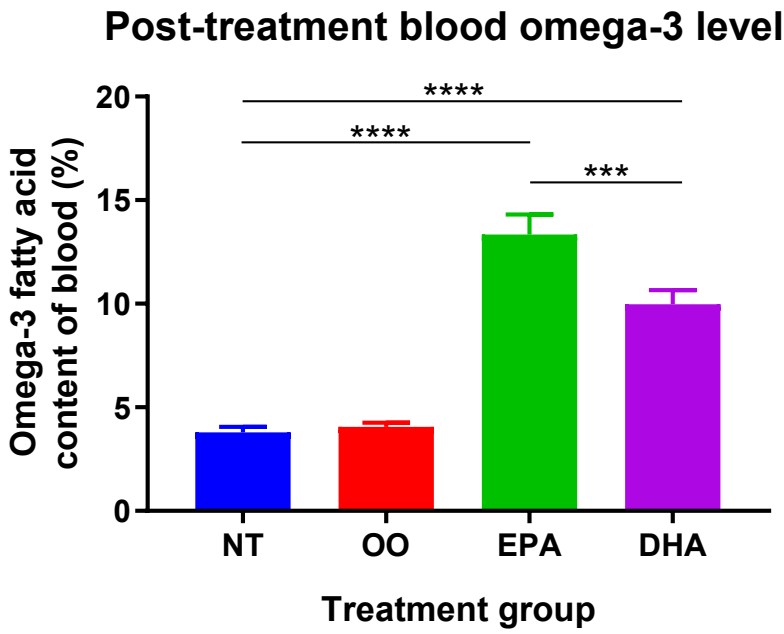


Figure 6.4: Proportion of total blood fatty acids comprised of omega-3s at study completion. n = 9–10 per group. Results expressed as mean ± SEM. ****p<0.0001. ***p<0.001.

Eicosapentaenoic acid levels in blood

The omega-3 fatty acids with established anti-inflammatory properties are EPA, DPA and DHA. In rodents, the plant-derived omega-3 fatty acid ALA (18:3ω3), is not efficiently converted to longer chain omega-3 fatty acids. Hence, blood levels of EPA (20:5ω3), DPA (22:5ω3) and DHA (22:6ω3) reflect their respective supplementation in a dose-dependent manner. Favourable properties of EPA include its superior hypolipidemic effect, and its effect on endothelial function. After 8 weeks, which was before randomisation, blood EPA levels were 0.60±0.02% of total fatty acid content in the 40 mice. Over the subsequent 8 weeks, blood EPA content increased mildly in the no treatment and olive oil groups to 0.97±0.07% and 0.98±0.06%, respectively. Blood EPA levels were markedly increased in the EPA (9.27±0.81%) and DHA (3.68±0.29%) groups ($p<0.0001$ for both comparisons with the no treatment group). EPA supplementation naturally produced significantly higher levels of EPA than did DHA supplementation ($p<0.0001$), see Figure 6.5.

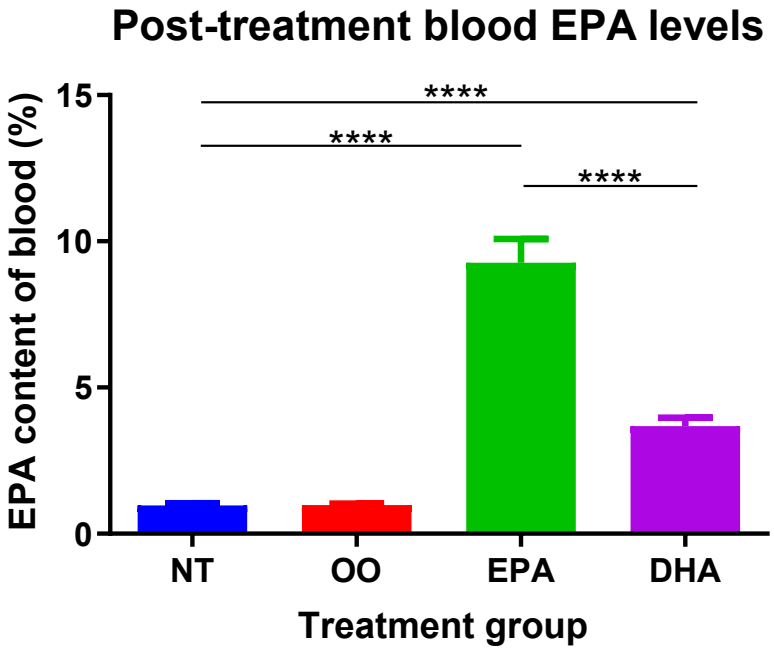


Figure 6.5: The percentage of EPA in whole blood at study completion. n = 9-10 per group. Results expressed as mean ± SEM. ****p<0.0001.

Docosahexaenoic acid levels in blood

DHA is the most unsaturated omega-3 fatty acid, and the one present in highest concentrations in blood and tissue. After 8 weeks of atherogenic feeding, DHA comprised 1.41±0.07% of total fatty acids in the 40 mice. By study completion, those in the no treatment and olive oil groups had a mild increase in DHA levels, to 2.13±0.18% and 2.3±0.15%, respectively. EPA supplementation only mildly increased DHA levels (to 2.70±0.16%; p=0.23 for comparison with the no treatment group). DHA supplementation was highly effective at increasing blood DHA levels, which were significantly greater than those in all other groups (5.52±0.38%; p<0.0001 for all comparisons), see Figure 6.6.

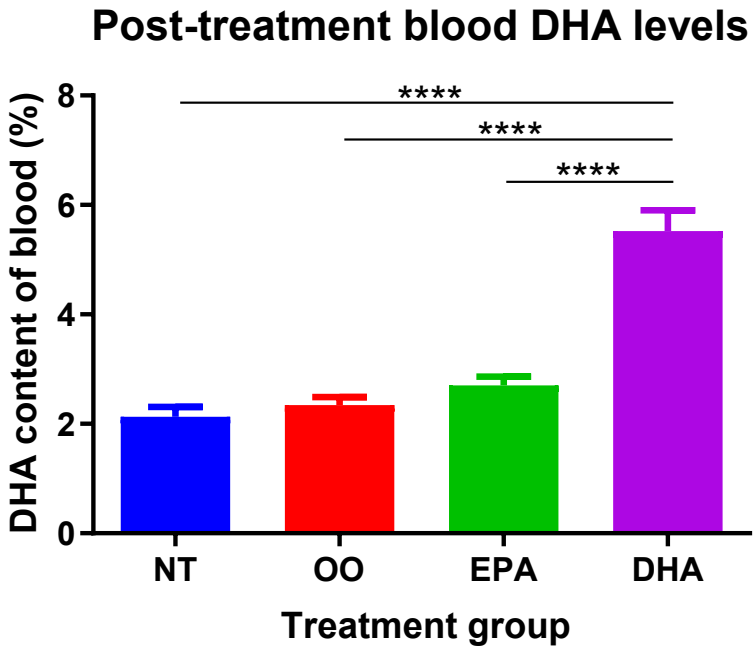


Figure 6.6: The percentage of DHA in whole blood at study completion. n = 9-10 per group. Results expressed as mean ± SEM. ****p<0.0001.

Docosapentaenoic acid (DPA) levels in blood

DPA is an intermediary between the conversion of EPA to DHA, although the retroconversion of DHA to EPA does not have the DPA intermediary in rodents. It is present in lower concentrations in blood and tissue, and has received considerably less attention than EPA and DHA. Consequently, its effects on inflammation and atherosclerosis are largely unknown. After 8 weeks of atherogenic feeding, DPA comprised 0.32±0.01% of total fatty acids in the 40 mice. This remained low in the no treatment and olive oil groups for the duration of the study, with levels of 0.38±0.03% and 0.40±0.02%, at study completion, respectively. There was a predictable increase in DPA levels (231%) in the EPA-treated mice to 1.06±0.05%, see Figure 6.7. As expected, pure DHA supplementation did not significantly alter DPA levels.

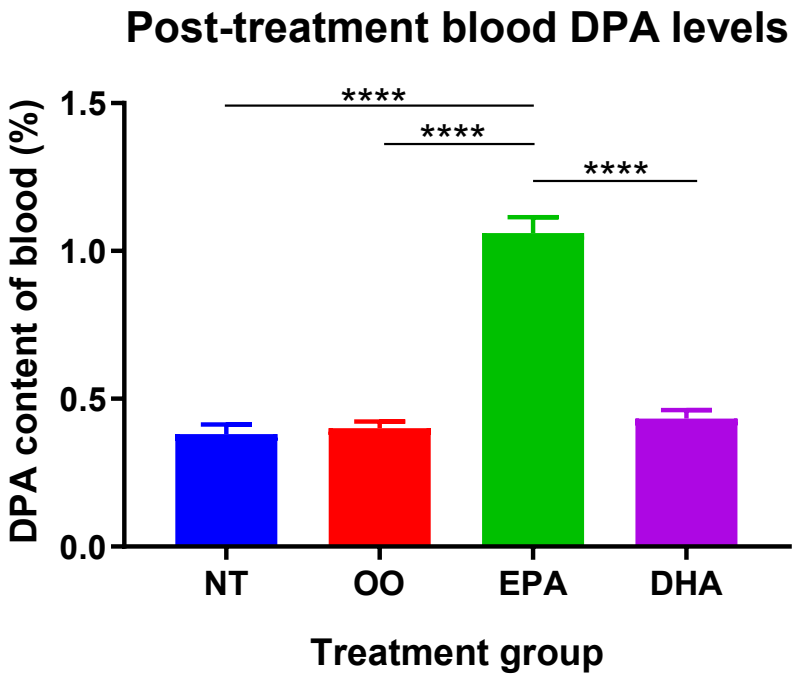


Figure 6.7: The percentage of DPA in whole blood at study completion. n = 9-10 per group. Results expressed as mean ± SEM. ****p<0.0001.

Alpha-linolenic acid (ALA)

ALA (18:3ω3) is present in low quantities in the bloodstream of rodents, and much of the ALA that is present is used as a substrate for the synthesis of EPA (20:5ω3) and DHA (22:6ω3). Longer chain omega-3s are not readily converted back to ALA, but despite this, small increases in ALA levels are expected after supplementation with longer chain omega-3s. After 8 weeks, ALA comprised 0.27±0.01% of total fatty acids in the 40 mice. Small increases occurred in the no treatment and olive oil groups by the end of the study, to 0.32±0.02% and 0.34±0.02%, respectively. Despite the prolonged, high-dose omega-3 supplementation, there were no significant increases in ALA levels in the EPA (0.27%±0.03%; p=0.43) or DHA (0.37±0.03%; p=0.51) treatment groups, see Table 6.1. ALA levels were higher in those treated with DHA compared to EPA (p=0.03), despite the higher total omega-3 levels in the EPA group.

Table 6.1: Summary of blood fatty acids levels in each treatment group at study completion.

Fatty acids in blood	Treatment Groups			
	NT	Olive Oil	EPA	DHA
Total omega-3 (%)	3.79±0.28	4.06±0.21	13.34±0.96****	9.98±0.67****
EPA (%)	0.97±0.07	0.98±0.06	9.27±0.81****	3.68±0.29****
DHA (%)	2.13±0.18	2.34±0.15	2.70±0.16****	5.52±0.38****
DPA (%)	0.38±0.03	0.40±0.02	1.06±0.05****	0.43±0.03
ALA (%)	0.32±0.02	0.34±0.02	0.27±0.03	0.37±0.03
Total omega-6 (%)	10.9±0.53	11.57±0.51	6.92±0.24****	8.14±0.29****
Linoleic acid (%)	5.23±0.19	5.48±0.14	4.16±0.14****	5.04±0.10
Arachidonic acid (%)	4.67±0.33	4.96±0.32	2.13±0.11****	2.34±0.16****
Total Monounsaturated Fats (%)	45.58±0.69	45.64±0.76	37.53±0.82****	38.32±0.59****
Total Trans-fatty Acids (%)	1.52±0.09	1.34±0.11	1.54±0.12	1.61±0.11
Total Saturated Fats (%)	38.15±0.63	37.38±0.73	40.70±0.97	41.82±0.94*

***p<0.05, ****p<0.0001 compared to NT. Results expressed as mean ± SEM.**

6.3.2.3 Polyunsaturated omega-6 fatty acids

Total omega-6 fatty acid content

Omega-6 fatty acids have pro-inflammatory properties, and are highly prevalent in "Western-type" diets. After 8 weeks, omega-6 comprised 9.59±0.24% of total fatty acids in the 40 mice. With the continued atherogenic feeding, this increased to 10.90±0.53% and 11.57±0.51% in the no treatment and olive oil groups, respectively. Omega-3 supplementation had a favourable effect by significantly reducing omega-6 levels, likely by simple fatty acid substitution. In the EPA and DHA groups, the relative proportions of omega-6 significantly decreased, to 6.92±0.24% and 8.14±0.29%, respectively ($p<0.0001$ for both comparisons with no treatment), see Figure 6.8.

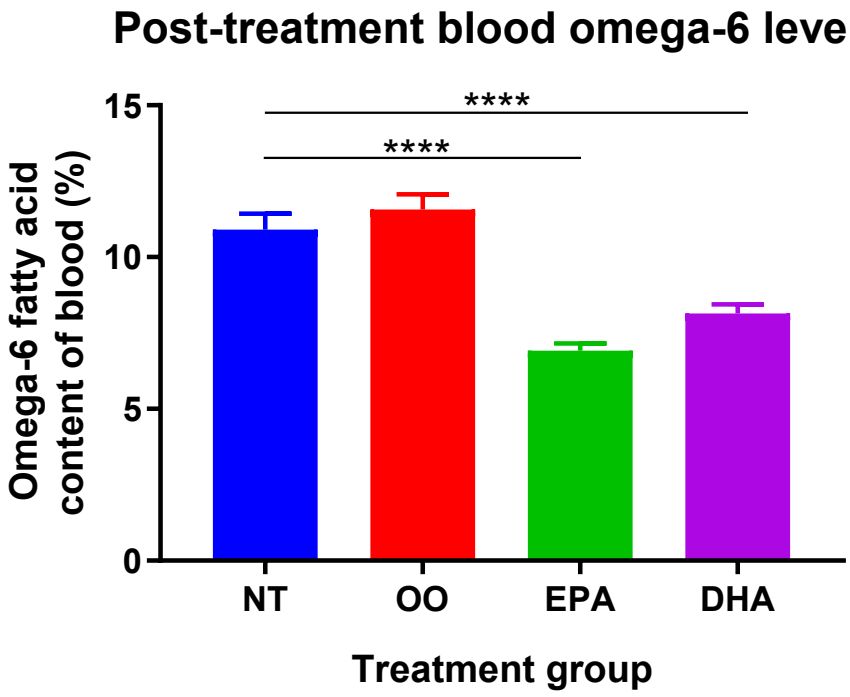


Figure 6.8: The proportion of total fatty acids in blood comprised of omega-6 fatty acids at study completion. n = 9-10 per group. Results expressed as mean ± SEM. ****p<0.0001.

Linoleic acid

The two main omega-6 fatty acids are linoleic acid (LA) and arachidonic acid (AA). The enzymes that convert LA (18:2ω6) to AA (20:4ω6) are the same as those that convert ALA to EPA and DHA. Therefore, higher LA levels result in less conversion of ALA to EPA and DHA. After 8 weeks, linoleic acid comprised 5.03±0.10% of total fatty acids in the 40 mice. This did not increase with ongoing atherogenic feeding, and was 5.23±0.19% in the no treatment group and 5.48±0.14% in the olive oil group. DHA supplementation did not alter LA levels (5.08±0.14%; p=0.999), however EPA supplementation significantly reduced LA levels to 4.16±0.12% (p<0.0001), see Table 6.1.

Arachidonic acid

Arachidonic acid (AA) is a biologically important fatty acid as it is a substrate for the production of eicosanoids, hormone-like mediators of tissue inflammation. After 8 weeks, arachidonic acid comprised 3.65±0.14% of total fatty acids in the 40 mice. With the ongoing atherogenic diet, this increased in the no treatment (4.67±0.33%) and olive oil (4.96±0.32%) groups. EPA and DHA supplementation reduced the proportion of arachidonic acid in blood to 2.13±0.11% and 2.34±0.16% respectively, and these were significantly lower than in the no treatment group (p<0.0001 for both comparisons), see Table 6.1.

Omega-6/Omega-3 ratio

The omega-6/omega-3 ratio is a summary of the ratio of pro-inflammatory omega-6 to anti-inflammatory omega-3 fatty acids. Higher levels favour a pro-inflammatory state and confer a higher mortality risk[472]. After 8 weeks on the atherogenic diet, the 40 mice had a mean omega-6/omega-3 ratio of 3.81±0.07. In the no treatment and olive oil groups, the omega-6/omega-3 ratios reduced by the end of the study to 2.95±0.14 and 2.86±0.08, respectively. In

the EPA and DHA groups, there were major reductions, to 0.54±0.03 and 0.84±0.04 respectively ($p<0.0001$ for comparisons with the no treatment group), see Figure 6.9.

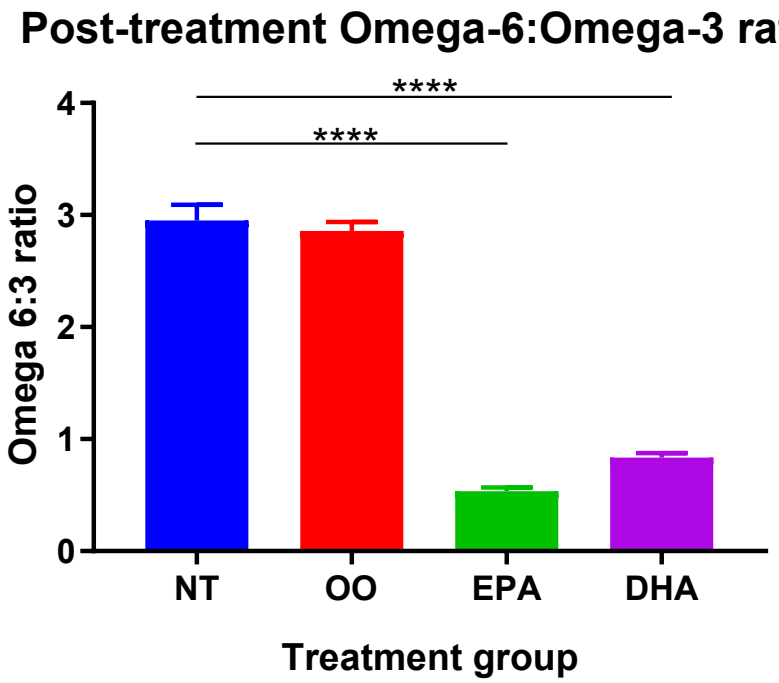


Figure 6.9: The blood omega-6:omega-3 ratio for all treatment groups at study completion. n = 9-10 per group. Results expressed as mean ± SEM. ****p<0.0001.

6.3.2.4 Monounsaturated fatty acids

Total monounsaturated fatty acid content

The monounsaturated fatty acids (omega-7 and omega-9) are the most biologically neutral of the fatty acids from an inflammatory perspective. However, mere substitution of unfavourable fatty acids with monounsaturates, leads to a reduction in inflammation. After 8 weeks, monounsaturated fatty acids comprised 43.76±0.33% of all blood fatty acids in the 40 mice. This was unchanged in both the no treatment (45.6±0.69%) and olive oil (45.6±0.76%) groups, despite the high concentration of omega-9s in olive oil. By comparison, there were

significant reductions, in the EPA (37.5±0.82%; p<0.0001) and DHA (38.3±0.59%; p<0.0001) treatment groups, see Table 6.1.

6.3.2.5 Trans-fatty acids

Total trans-fatty acids

Trans-fatty acids, which promote inflammation and increase cardiovascular risk, are present in low levels in rodent diets. After 8 weeks, trans-fatty acids comprised only 1.46±0.04% of total blood fatty acids in the 40 mice. There was no significant change by the end of the study regardless of treatment group (no treatment: 1.52±0.09%, olive oil: 1.34±0.11%, EPA: 1.54±0.12%, and DHA: 1.61±0.11%; p=0.88 for comparison between treatment groups, see Table 6.1).

6.3.2.6 Saturated fats

Total saturated fatty acids

Saturated fats lack double bonds and do not readily undergo conformational changes to other fatty acid species. Saturated fats are pro-inflammatory although have not been conclusively shown to increase cardiovascular risk[137]. After 8 weeks, saturated fatty acids comprised 42.6±0.36% of total blood fatty acids of the 40 mice. In the no treatment and olive oil groups, this decreased to 38.2±0.63% and 37.4±0.73%, respectively. This value was 40.7±0.97% in the EPA group (p=0.10), and 41.8±0.94% in the DHA group, which was significantly higher than the no treatment group (p=0.02), see Table 6.1.

6.3.3 Plasma cholesterol and triglycerides

Total cholesterol

All mice developed hypercholesterolaemia after 8 weeks on the atherogenic diet with a mean total cholesterol level of 17.96±0.9 mmol/L. Before treatments commenced, there were no significant differences in total cholesterol levels between mice allocated to each treatment

group (p=0.53). In the no treatment and olive oil groups, total cholesterol levels continued to increase for the last 8 weeks of the study, to 20.13±1.1 mmol/L and 27.21±2.9 mmol/L, respectively, with significant increases from their 8-week levels (p=0.005 and p=0.02, respectively). Both EPA and DHA stabilised total cholesterol levels, with an increase by 0.0±2.8 mmol/L to 20.8±1.6 mmol/L in the EPA group (p=0.999 for change from the levels at 8 weeks), and an increase by 2.75±1.9 mmol/L to 19.96±1.3 mmol/L in the DHA group (p=0.16 for change from the levels after 8 weeks), see Figure 6.10.

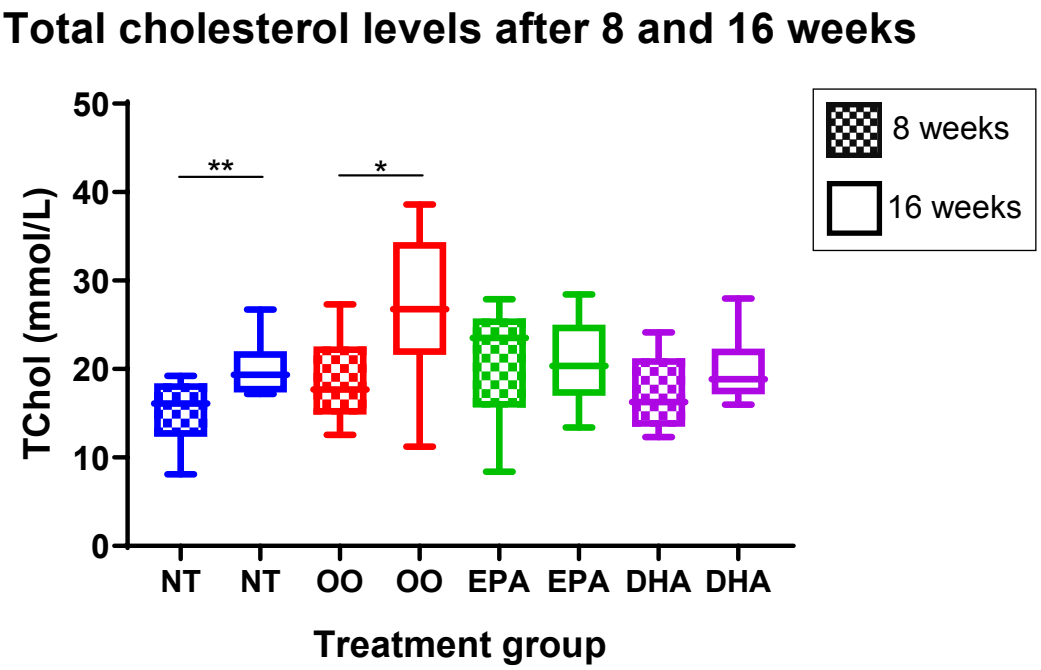


Figure 6.10: Changes in plasma total cholesterol levels for mice in each treatment group from 8 weeks until study completion. n = 9-10 per group. Results are expressed as mean ± SEM. **p<0.01. *p<0.05. NT = no treatment, OO = olive oil.

Triglycerides

Triglyceride levels were mildly elevated after 8 weeks, with a mean level of 2.27±0.70 mmol/L. There were no significant differences between allocated treatment groups (p=0.25). In the no treatment group, the mean triglyceride level after 16 weeks was not significantly different to that after 8 weeks, with a -0.11±0.30 mmol/L change (p=0.71). In the olive oil group, there was mild, non-significant reduction of 0.56±0.30 mmol/L (p=0.08). This reduction is consistent with the known triglyceride-lowering effects of diets high in monounsaturated fatty acids. In the EPA and DHA groups, triglyceride levels reduced by 0.73±0.27 mmol/L (p=0.02), and 1.02±0.22 mmol/L (p=0.0002), respectively, see Figure 6.11.

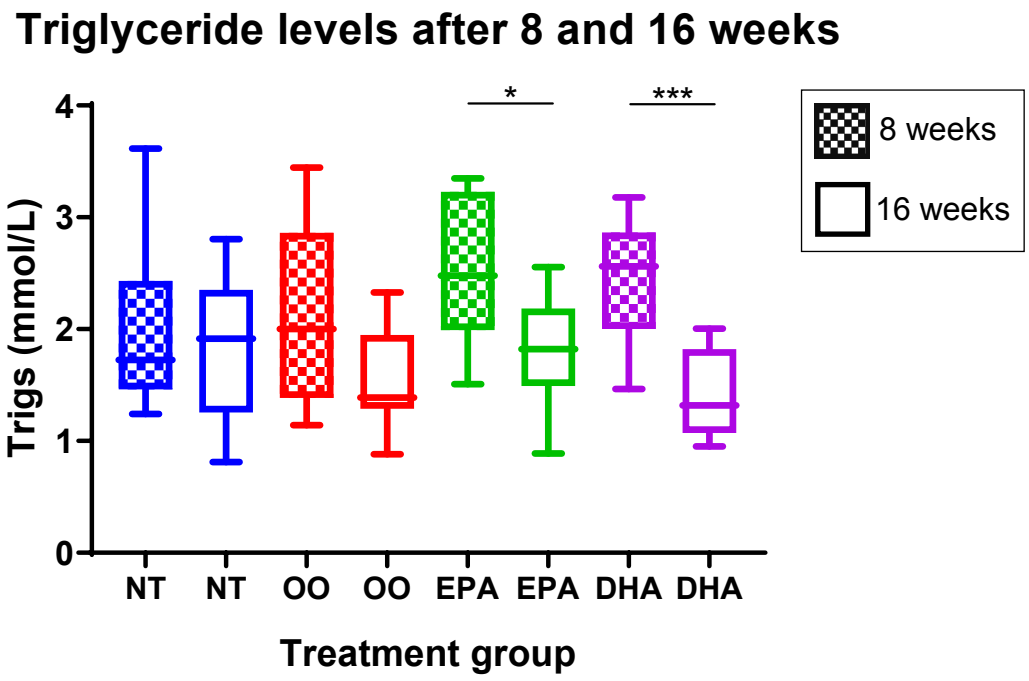


Figure 6.11: Changes in plasma triglyceride levels for mice in each treatment group from 8 weeks until study completion. n = 9-10 per group. Results expressed as mean ± SEM. ***p<0.001. *p<0.05. NT = no treatment, OO = olive oil.

6.3.4 Atherosclerotic plaque quantification

Brachiocephalic artery

The brachiocephalic artery is a site of predilection for plaque formation in ApoE-deficient mice fed an atherogenic diet. In this study, brachiocephalic arteries were harvested from male mice, resulting in a maximum of 5 mice per treatment group. When plaque was present it was quantified and expressed as a percentage of total cross-sectional artery area. In the no treatment group, plaque comprised 32.0 (IQR 24 - 40) % of total cross-sectional artery area. There were no significant differences in plaque burden in the olive oil [30.3 (13-38) %, p=0.46] and EPA [27.1 (16.3 – 51.5) %, p=0.54] groups. Mice in the DHA group had notably less plaque compared to the no treatment group [18.1 (16.3 – 20.7) %], although it did not reach statistical significance in the context of a small sample size (p=0.09), see Figure 6.12. Representative images from haematoxylin and eosin-stained sections are presented in Figure 6.13.

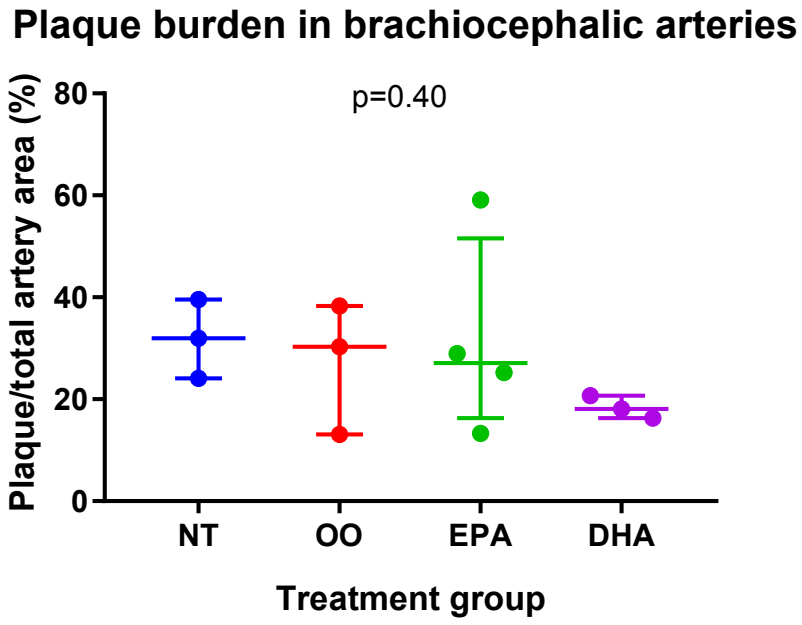
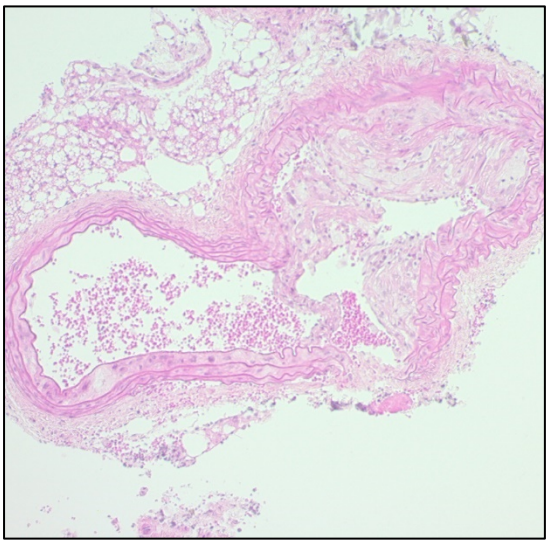
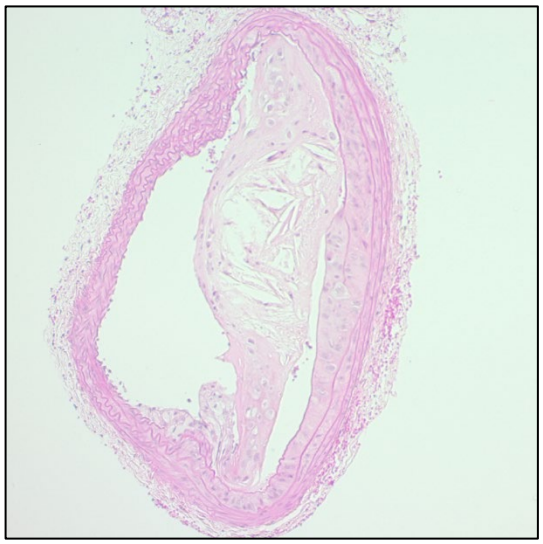


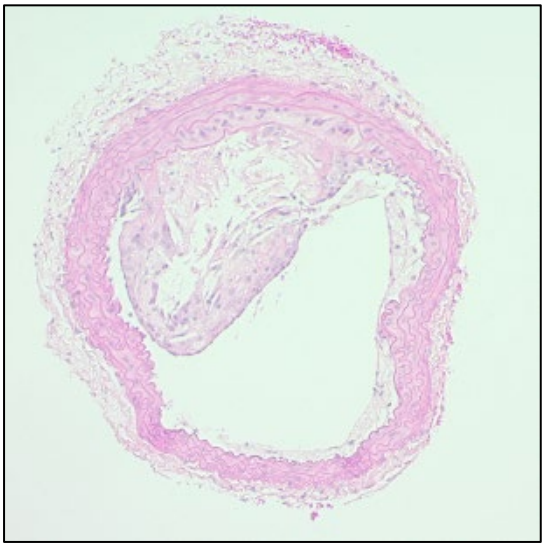
Figure 6.12: Comparison of brachiocephalic artery plaque burden as a percentage of total cross-sectional area. n= 3-4 per group. Results expressed as median + interquartile range.



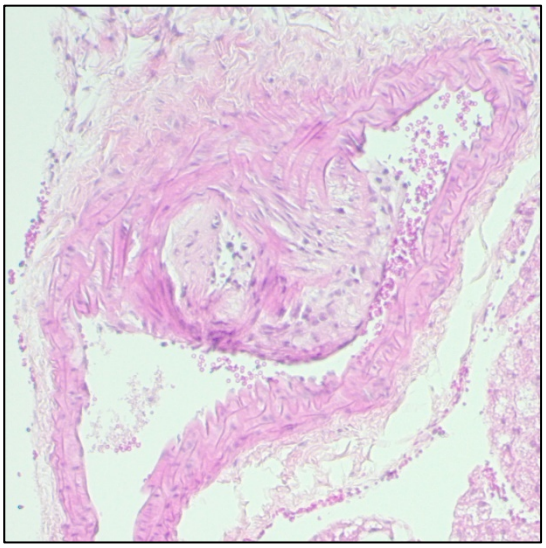
No treatment



Olive oil



EPA



DHA

Figure 6.13: Representative haematoxylin and eosin-stained sections of brachiocephalic arteries from mice in each treatment group. Advanced plaques are seen, including cholesterol crystals and intimal and medial expansion. No significant differences are present between treatment groups.

Aortic sinuses

Plaque burden in the aortic sinuses was expressed as the percentage area of each sinus comprised of plaque. The plaque burden in each sinus was averaged for each mouse. In the no treatment group, plaque comprised 34.2 (IQR 19 – 35) % of total sinus area. This was similar to the plaque burden seen in the brachiocephalic arteries. There were no significant differences among treatment groups (p=0.95 for the overall comparison), see Figure 6.14. In the olive oil group, plaque comprised 33.5 (26.1 – 39.6) % of total sinus area, and in the EPA and DHA groups plaque comprised 32.8 (20.1 – 37.3) % and 30.5 (26.1 – 35.6) % of total sinus area, respectively. Representative images from haematoxylin and eosin-stained slides are presented in Figure 6.15.

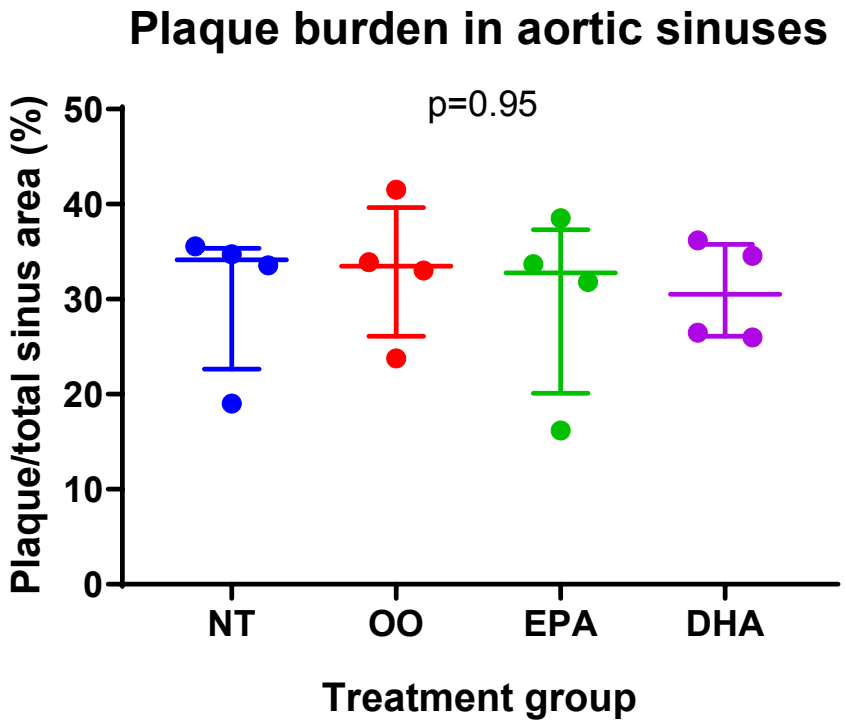
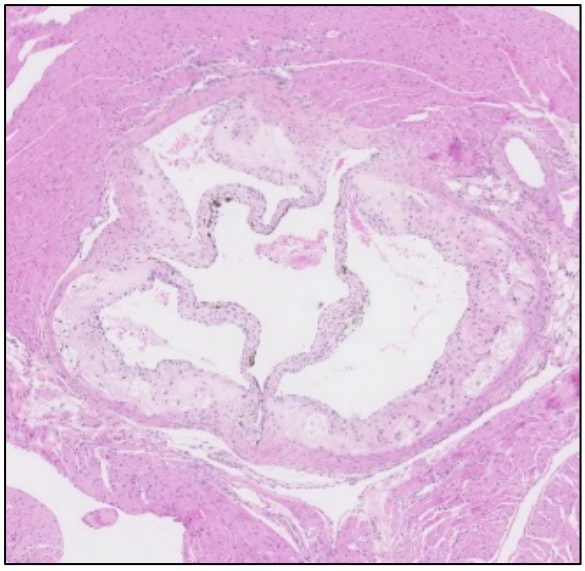
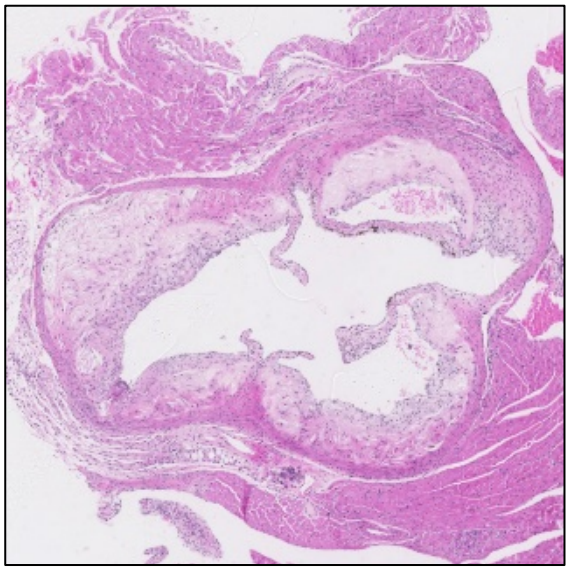


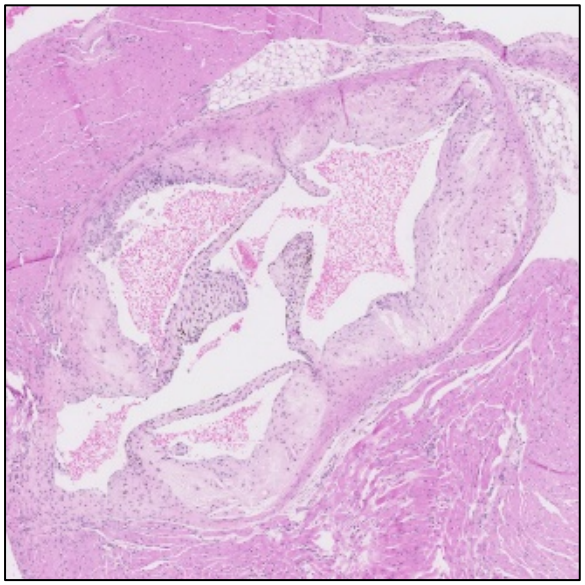
Figure 6.14: Comparison of plaque burden in the aortic sinuses between treatment groups. n = 4 per group. Results expressed as median + interquartile range.



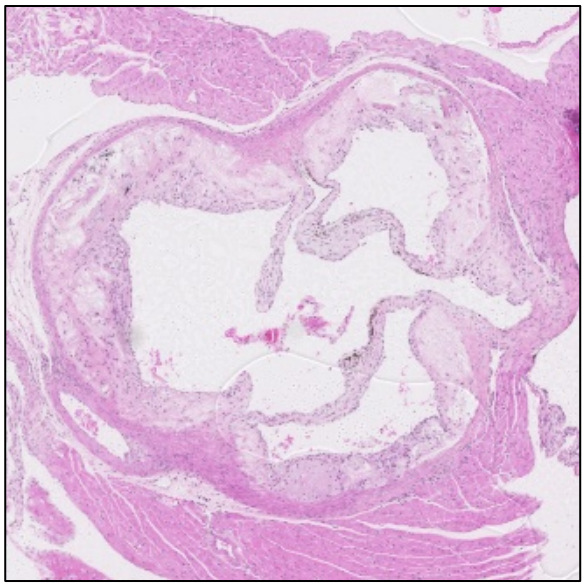
No Treatment



Olive Oil



EPA



DHA

Figure 6.15: Representative haematoxylin and eosin-stained sections of the aortic sinuses from mice in each treatment group. Advanced plaques with inflammatory infiltrates and cholesterol crystals can be seen. No significant differences were present between treatment groups.

6.3.5 Expansion of the intima and media in the artery wall

Following endothelial injury, an early event in atherogenesis, smooth muscle cells (SMCs) are recruited to the intima and proliferate. Over time, this expands the extracellular

matrix and results in thickening of the intimal and medial layers of the arterial wall. This thickening narrows the lumen. The impact of omega-3 supplementation on this process was quantified histologically in the brachiocephalic artery specimens. The area comprised by the intimal and medial layers was measured and expressed as a proportion of the total area of the artery wall. In the no treatment group, the median value was 30.7 (IQR 26.1 – 37.2) %. In the olive oil group, this was significantly greater at 48.2 (38.6 – 52.0) % (p=0.02). However, there were no significant differences noted in the EPA [36.2 (28.5 – 42.6) % (p=0.42)] or DHA [39.9 (34.5 – 49.2) % (p=0.10)] treatment groups, see Figure 6.16). The representative H & E sections of plaque in Figure 6.13 also demonstrate expansion of the intimal and medial layers.

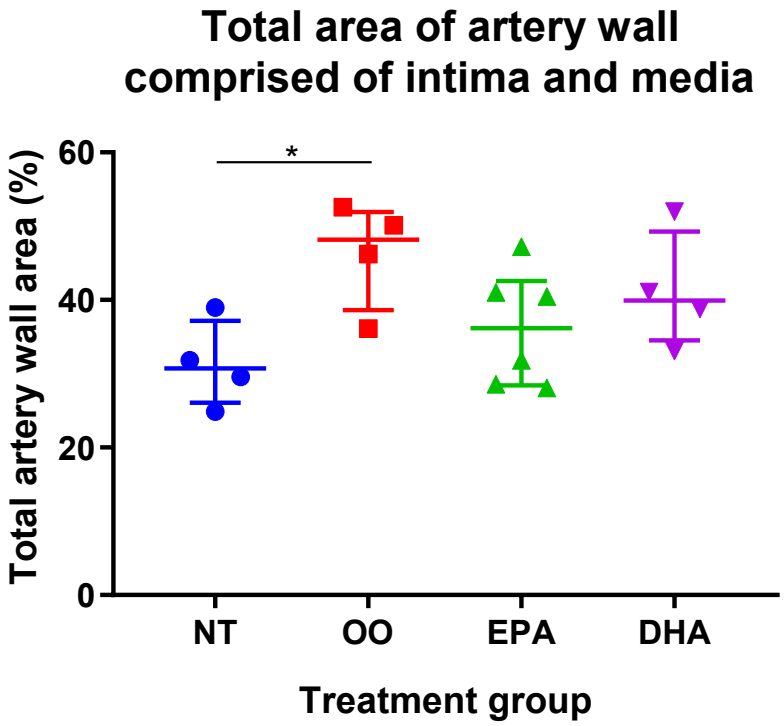


Figure 6.16: The percentage of total artery wall area comprised of the intima and media, compared between treatment groups. n = 4-6 per group. Results expressed as median + interquartile range. *p<0.05.

6.3.6 Collagen content of plaque as a measure of stability

Atherosclerotic plaques that are stable are less prone to rupture. The amount and organisation of matrix collagen is positively associated with the mechanical stability of the fibrous cap[550]. In advanced plaques, inflammatory cells release metalloproteinases (MMPs), which degrade collagen and cause apoptosis of collagen-producing SMCs. The collagen content of plaques in the brachiocephalic arteries and aortic sinuses were quantified using the Masson's trichrome stain. Collagen comprised 60.6 (IQR 55.1 – 68.6) % of brachiocephalic artery plaque in the no treatment group. There were no significant differences in collagen content amongst different treatment groups, with mean values for the olive oil, EPA, and DHA treatment groups of 60.1 (38.1 – 70.3) %, 65.6 (46.2 – 71.8) %, and 48.8 (36.9 – 62.5) %, respectively (p=0.65 for differences between the groups), see Figure 6.17. Representative images of slides stained with Masson's trichrome stain from each treatment group are presented in Figure 6.18.

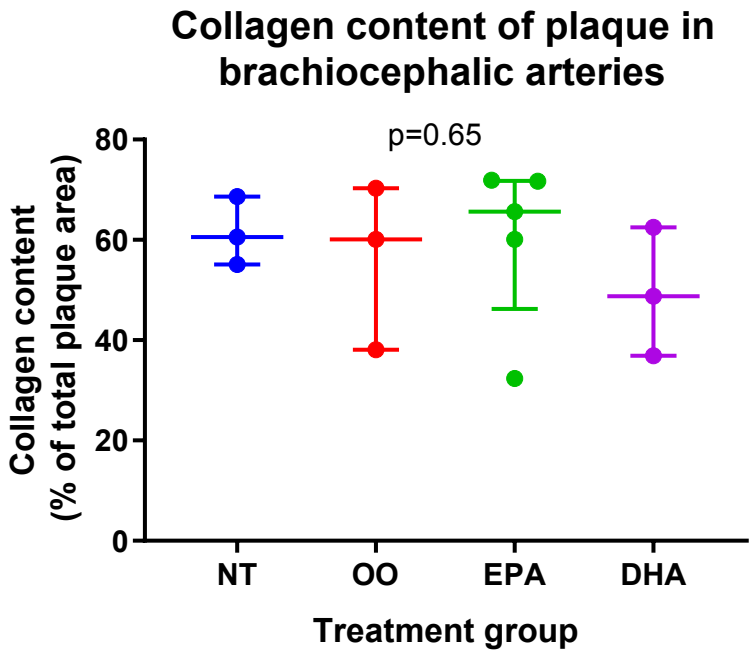
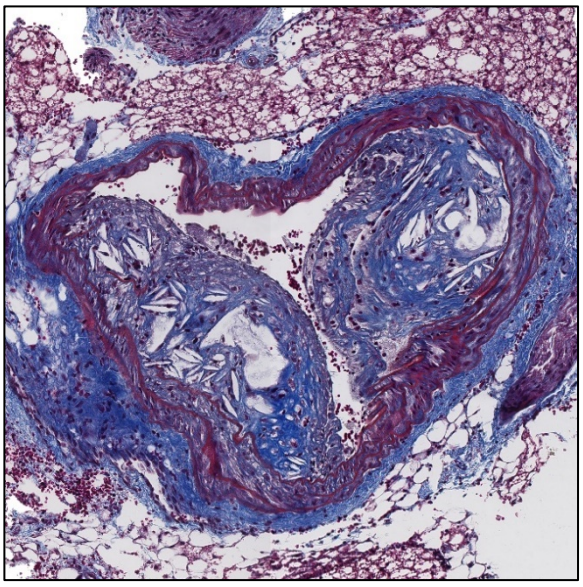
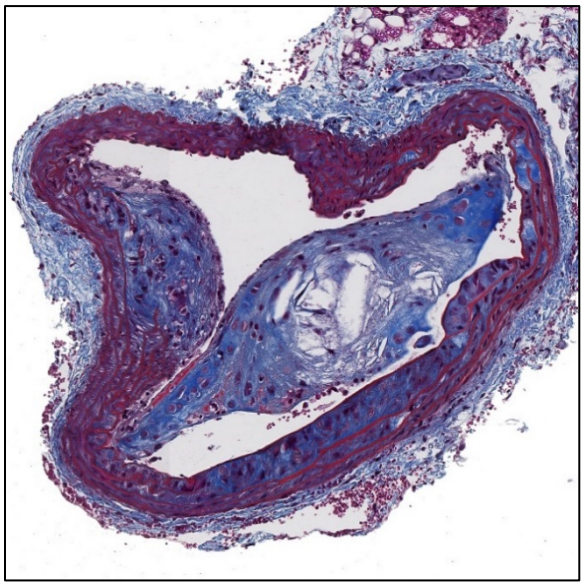


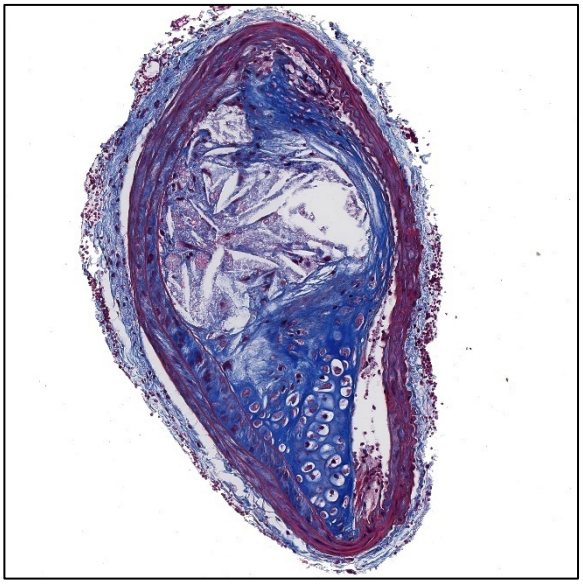
Figure 6.17: Comparison of the collagen content of plaque in the brachiocephalic arteries between treatment groups. n = 3-5 per group. Results expressed as median + interquartile range.



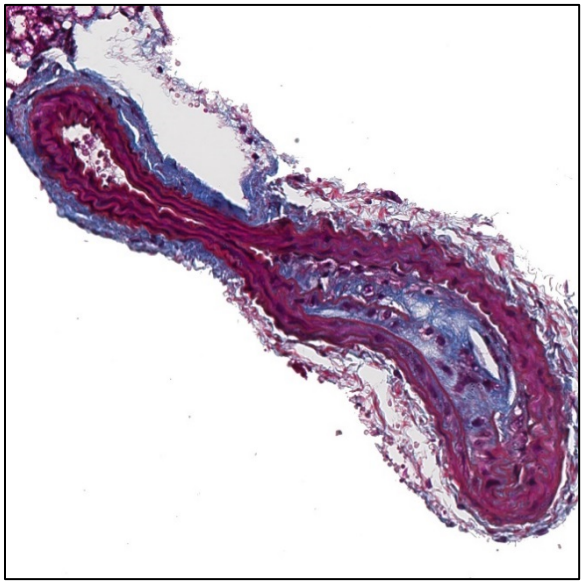
No Treatment



Olive Oil



EPA



DHA

Figure 6.18: Representative sections of brachiocephalic arteries from mice in each treatment group stained with the Masson's Trichrome stain. The collagen content of plaques was high, and not significantly different between treatment groups.

Plaque in the aortic sinuses had a higher collagen content than that in the brachiocephalic arteries. In the no treatment group this was 83.4±1.1%, and not significantly different in the EPA (72.9±5.4%, p=0.11) or DHA (73.5±2.5%, p=0.11) treatment groups. It was significantly reduced in the olive oil treatment group (70.9±4.0%, p=0.04), see Figure 6.19. Representative images of aortic sinus plaques stained with the Masson's trichrome stain are presented in Figure 6.20.

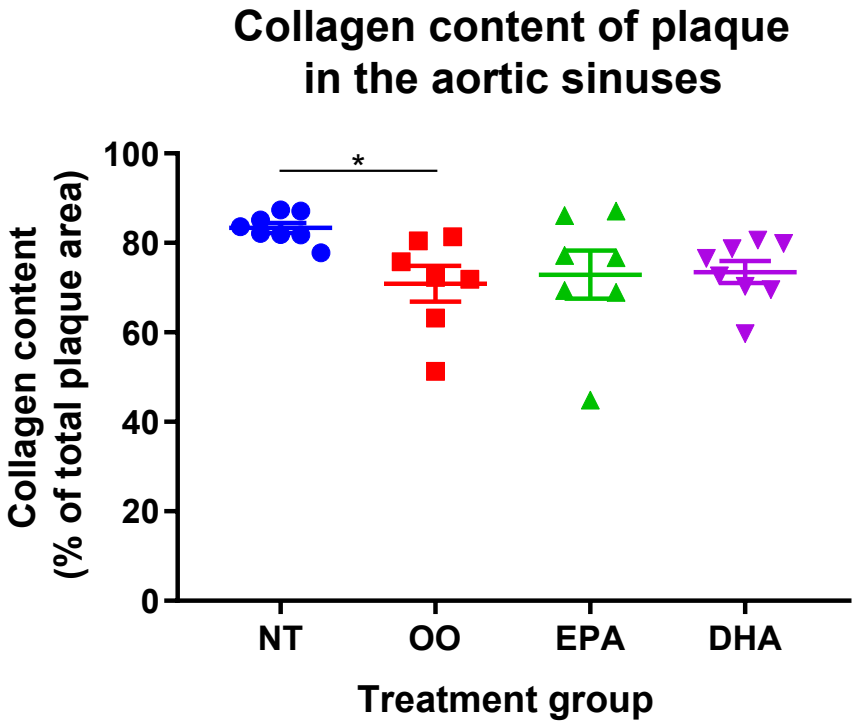
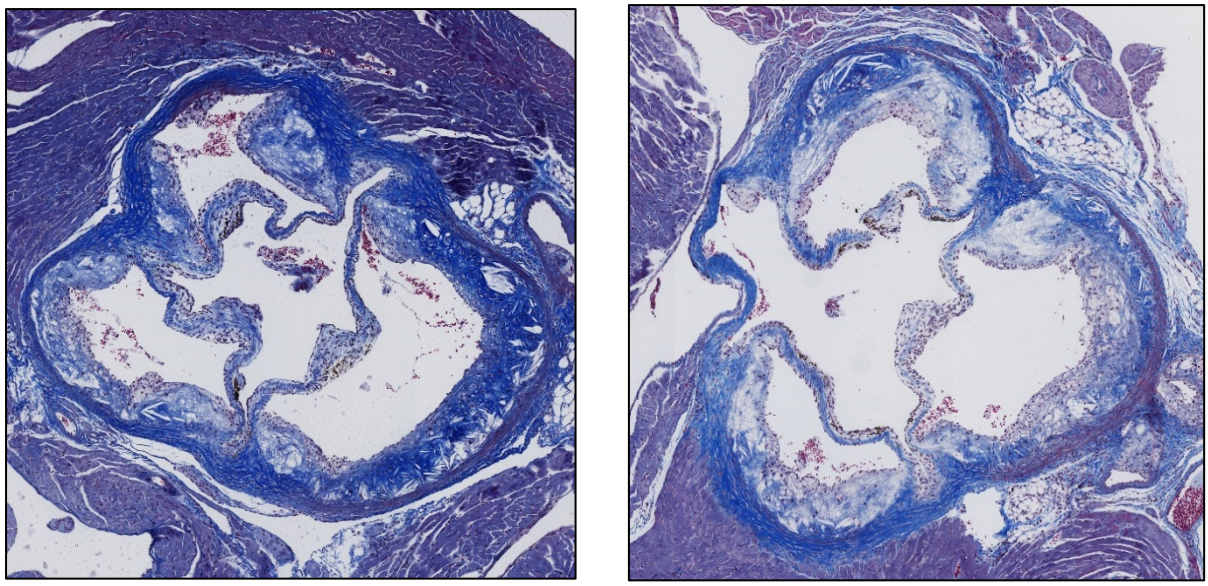


Figure 6.19: Comparison of the collagen content of aortic sinus plaques between treatment groups. n = 7-8 per group. Results expressed as mean ± SEM. *p<0.05



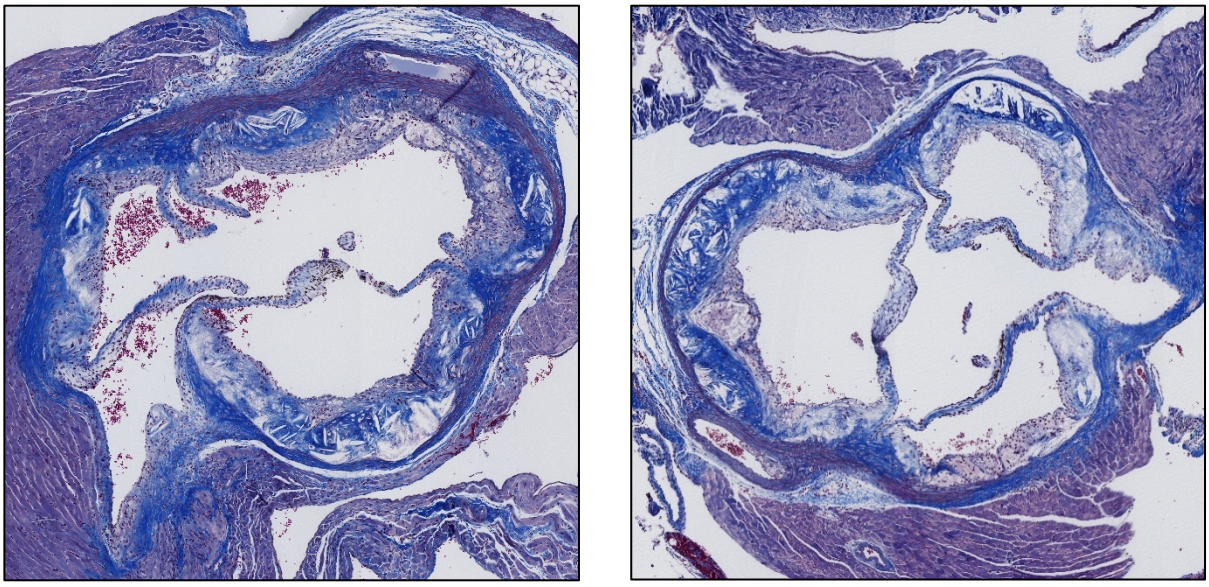


Figure 6.20: Representative sections of aortic sinuses from mice in each treatment group stained with the Masson's Trichrome stain. The content of collagen, stained blue, was higher than that of the brachiocephalic artery plaque, and was not significantly different between treatment groups.

6.3.7 Smooth muscle cell content of plaque

In atherosclerotic plaques SMCs contribute to the formation of the fibrous cap. With degradation of SMCs and collagen by MMPs in inflammatory plaques, SMC content and plaque stability are reduced. Brachiocephalic artery and aortic sinus sections underwent immunohistochemical staining for SMCs in plaque using the smooth muscle actin (SMA) antibody. The density of SMA staining in the brachiocephalic arteries in the no treatment group was 43.2 (IQR 38.8 – 56.3) % of the maximum possible. No significant differences were seen between treatment groups (p=0.45), with 25.5 (25.2 – 54.1) % in the olive oil group, 32.2 (24.4 – 54.1) % in the EPA group, and 51.7 (33.2 – 59.0) % in the DHA group, see Figure 6.21. Representative images are shown in Figure 6.22.

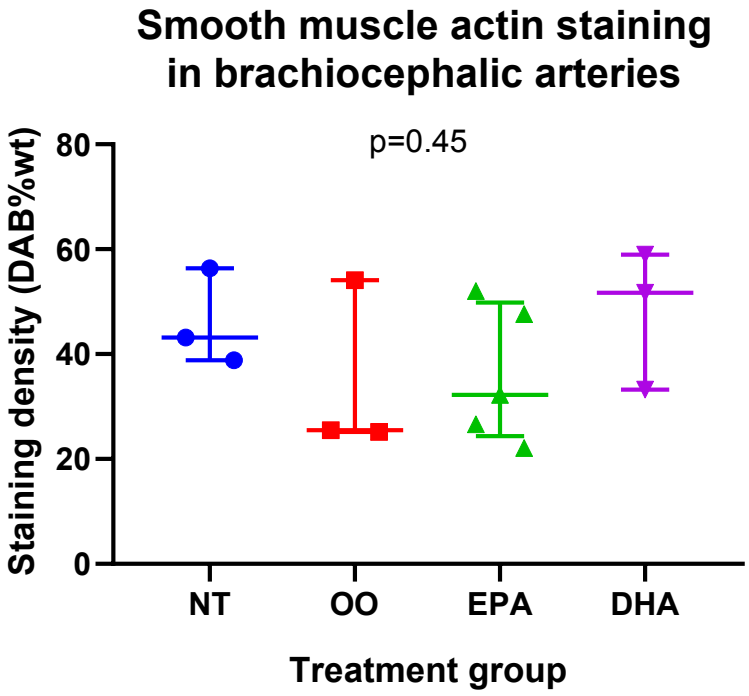


Figure 6.21: Smooth muscle cell content of brachiocephalic artery plaques, measured by IHC staining for smooth muscle actin (SMA). n = 3-5 per group. Results expressed as median + interquartile range.

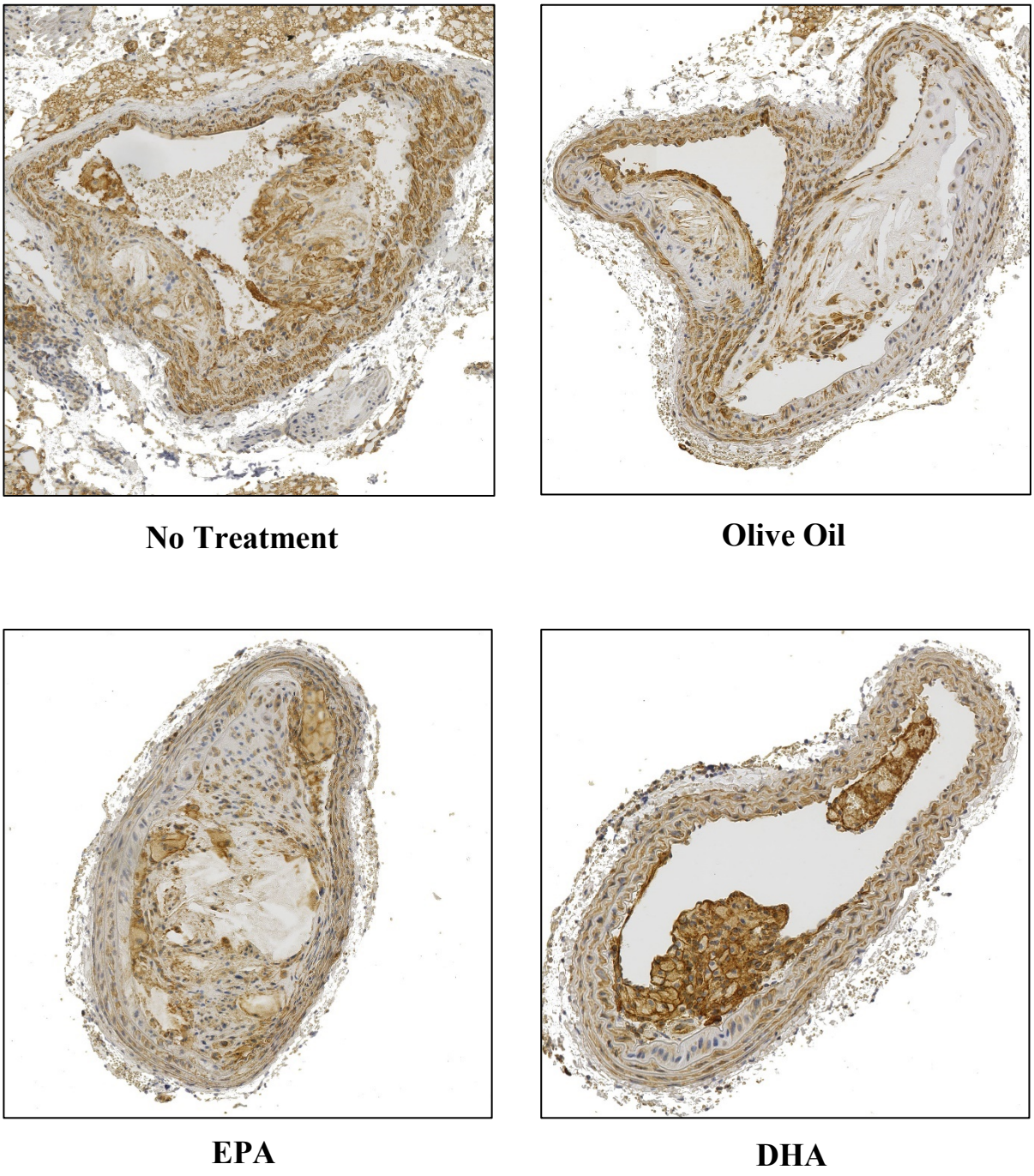


Figure 6.22: Representative images of brachiocephalic arteries in each treatment group stained for smooth muscle actin. Staining was positive throughout plaques and did not significantly differ between treatment groups.

The density of SMA staining in aortic sinus plaques was less than that of the brachiocephalic artery plaques. In the no treatment group, the staining density of SMA was 19.1 (IQR 18.2 – 21.4) % of the maximum possible. No significant differences were present

between treatment groups (p = 0.94). In the olive oil, EPA, and DHA treatment groups, the staining density was 17.3 (16.0 – 25.1) %, 20.4 (18.4 – 21.9) %, and 19.1 (13.7 – 21.6) %, respectively, see Figure 6.23. The pattern of staining was diffuse in the brachiocephalic artery plaques. The aortic sinus plaques had a striking intensity of staining localised to the plaque surface, consistent with the location of the fibrous cap, see Figure 6.24.

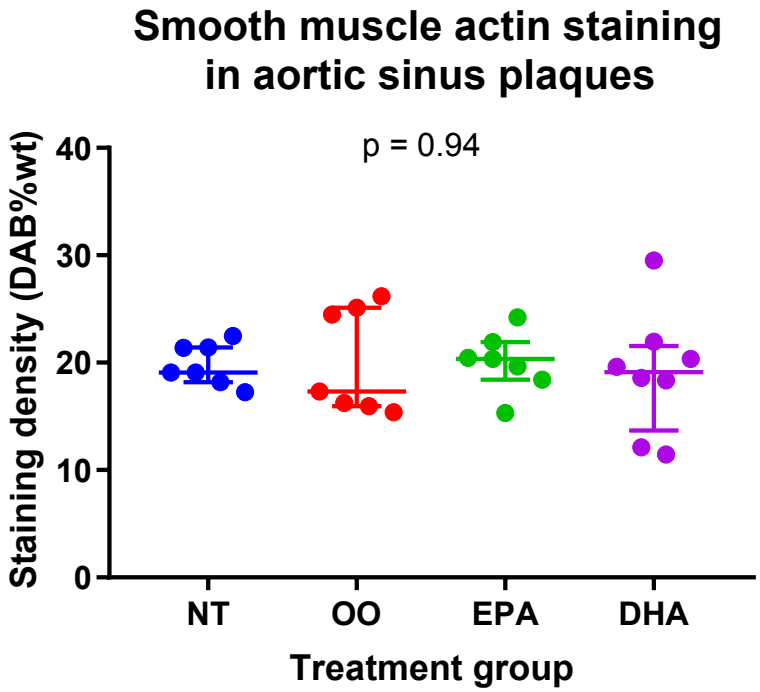
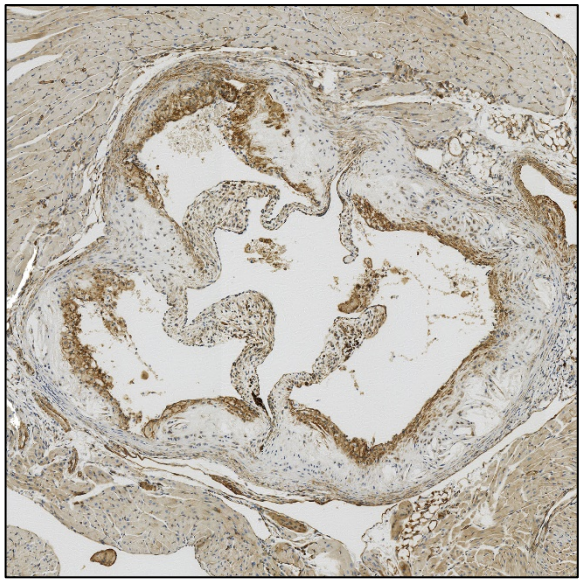
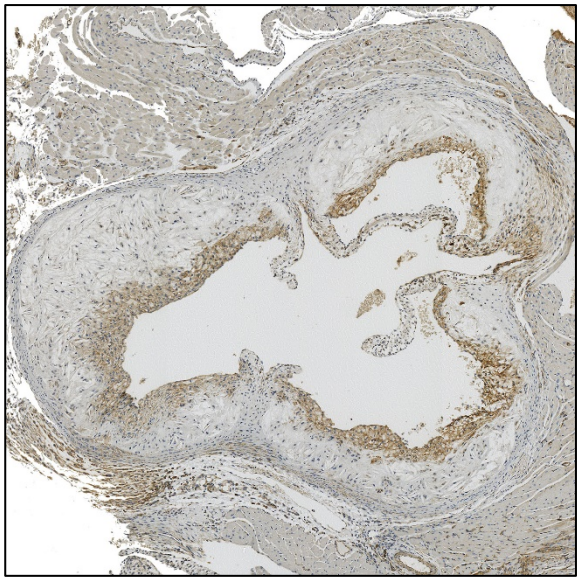


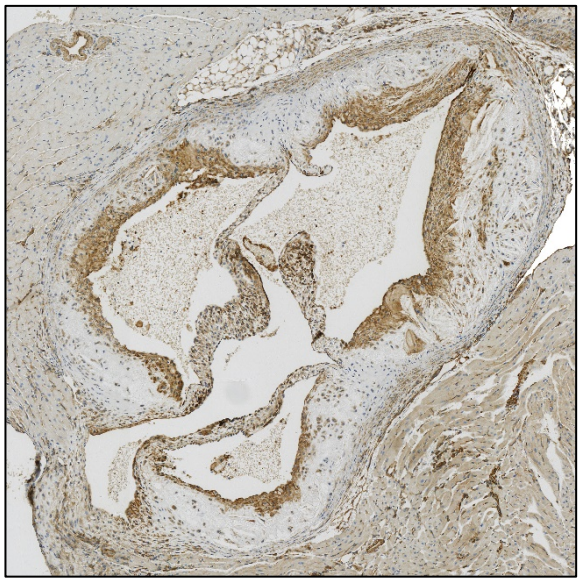
Figure 6.23: Density of IHC staining for smooth muscle actin (SMA) in aortic sinus plaques. n = 7-8 per group. Results expressed as median + interquartile range.



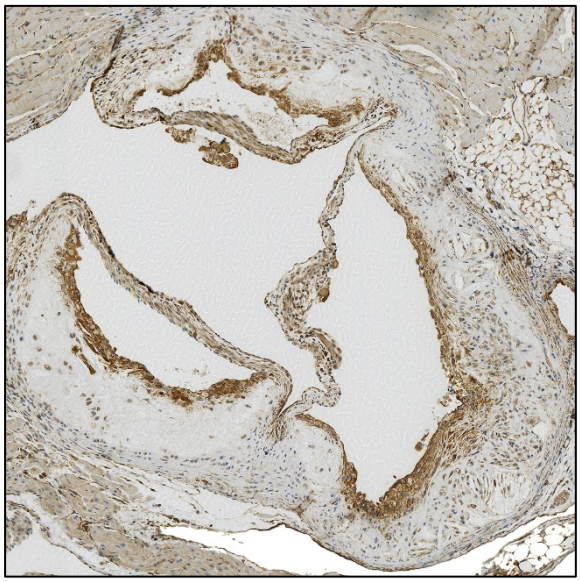
No Treatment



Olive Oil



EPA



DHA

Figure 6.24: Representative images of aortic sinus plaques stained for smooth muscle actin. Intense staining is present on the surface of plaques. No differences in staining density were present between treatment groups.

6.3.8 Chronic inflammation in plaque and in the vessel wall

6.3.8.1 Macrophage content of plaques

Atherosclerotic plaques with a high inflammatory cell content are more biologically active and vulnerable[551]. Macrophages are highly prevalent at sites of chronic inflammation, and play a critical role in all stages of atherosclerosis. The macrophage content of plaques was measured by performing immunohistochemical staining of brachiocephalic arteries and aortic sinuses for CD107b (Mac3), which is expressed on the surface of macrophages. In the no treatment group, the staining density of CD107b in brachiocephalic artery plaques was 22.1 (IQR 21.5 – 27.8) % of the maximum possible. This was not significantly different amongst treatment groups (p=0.14), with 26.2 (16.9 – 46.7) % in the olive oil group, 20.1 (16.4 – 25.5) % in the EPA group, and 39.3 (36.1 – 45.9) % in the DHA group, see Figures 6.25 and 6.26.

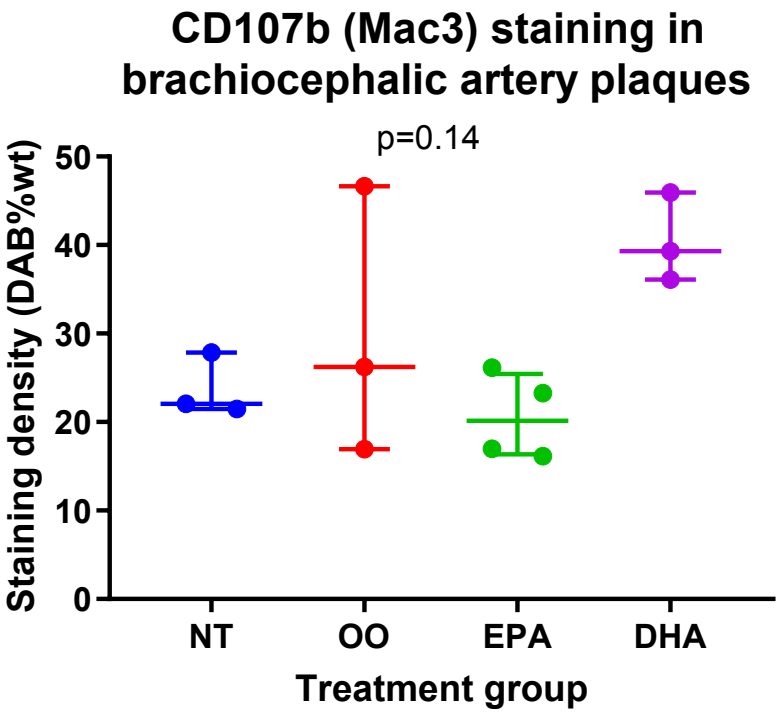


Figure 6.25: Density of IHC staining for CD107b (Mac3) in brachiocephalic artery plaques. n = 3-4 per group. Results expressed as median + interquartile range.

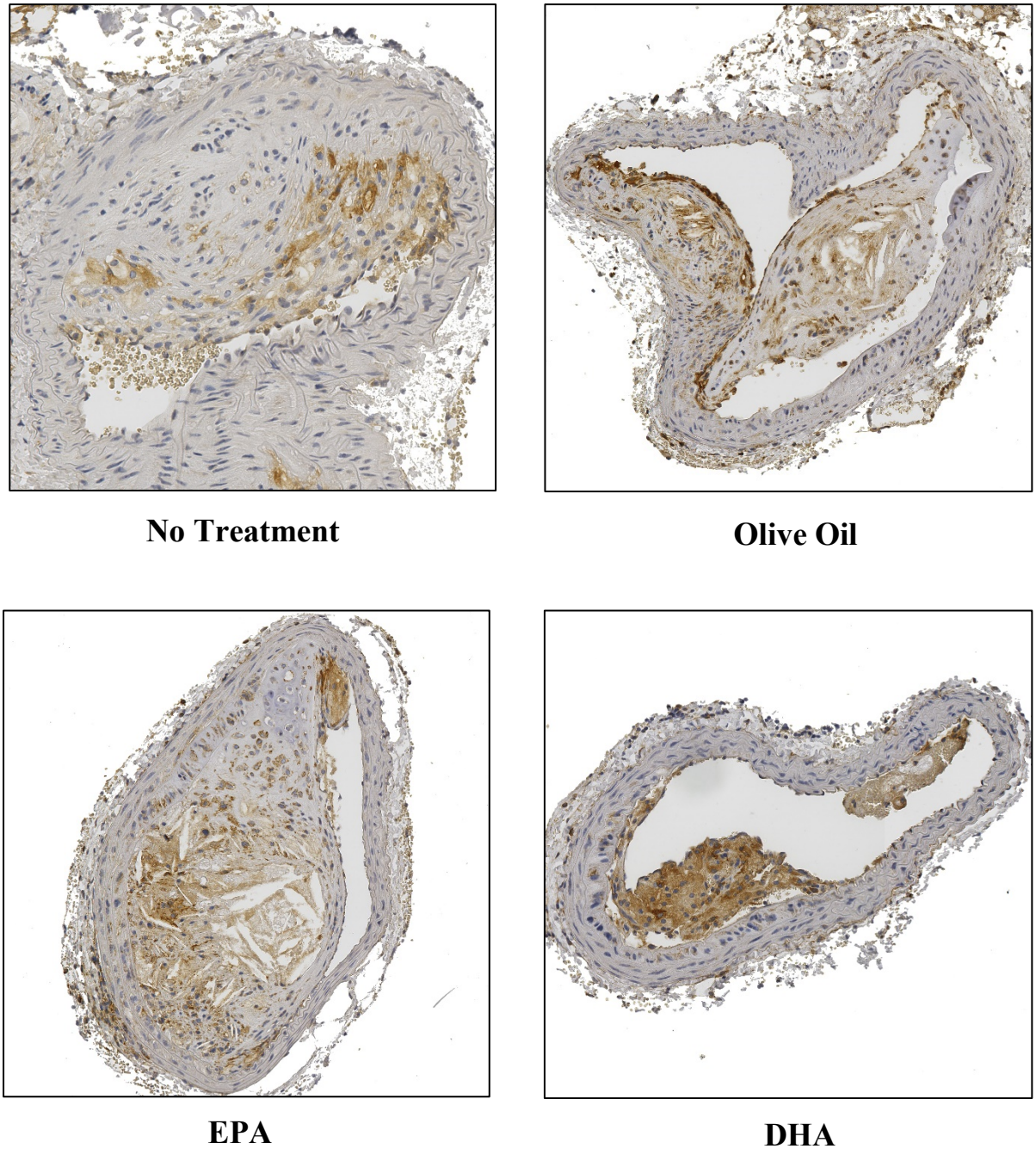


Figure 6.26: Representative images of brachiocephalic artery plaques stained for the macrophage marker CD107b (Mac3). Staining is present diffusely throughout plaque, and the density of staining is no different between treatment groups.

Aortic sinus plaques had less CD107b staining than the brachiocephalic plaques, with a median plaque staining density of 6.1 (IQR 5.4 – 7.5) % of the maximum possible in the no

treatment group. This was consistent with other measures of plaque stability, indicating that brachiocephalic artery plaques were more vulnerable than aortic sinus plaques. There were no significant differences in the olive oil and EPA groups, with staining densities of 7.1 (6.8 – 8.1) % and 7.0 (6.3 – 10.5) %, respectively. Staining was unexpectedly higher in the DHA group [9.7 (7.2 – 12.4) %], and reached borderline significance (p = 0.05), see Figures 6.27 and 6.28.

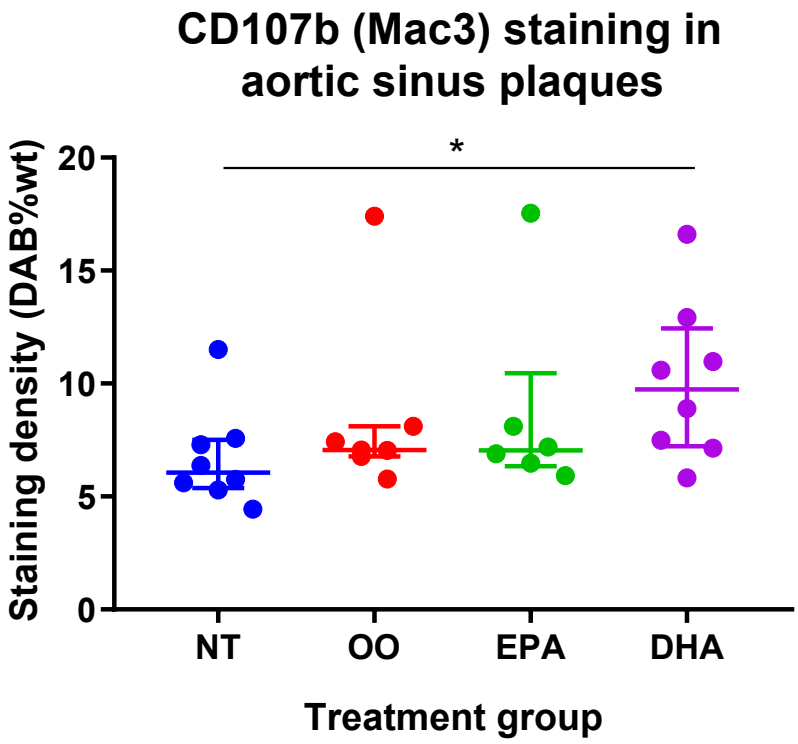
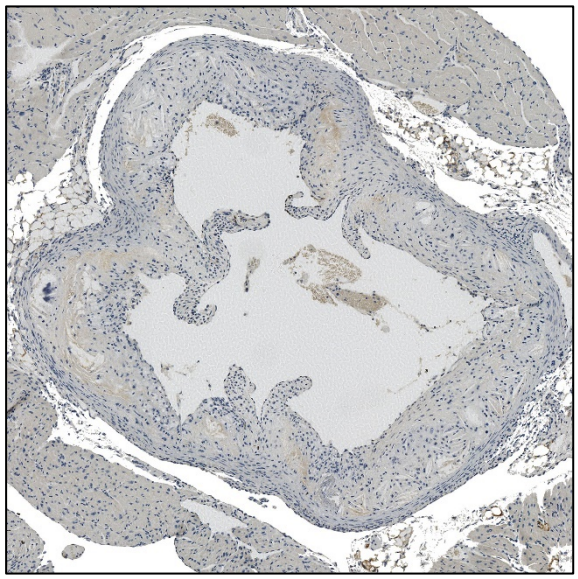
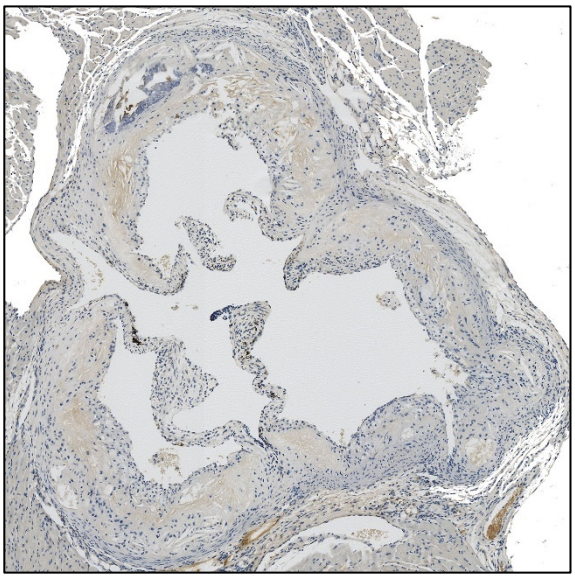


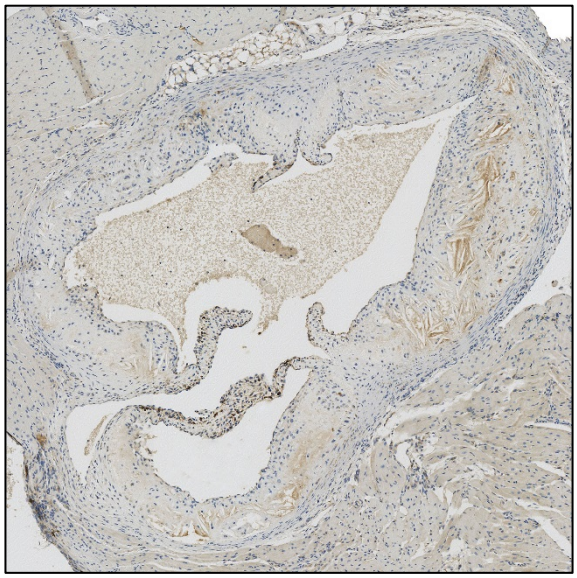
Figure 6.27: Density of IHC staining for CD107b (Mac3) in aortic sinus plaques. Results expressed as median + interquartile range. n = 6-8 per group. *p=0.05



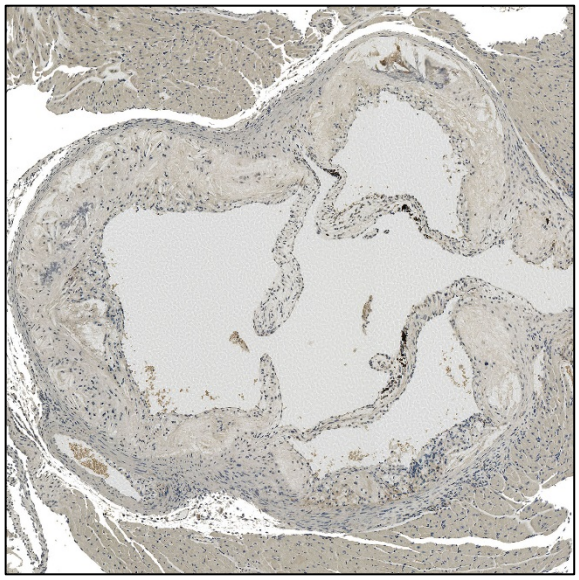
No Treatment



Olive Oil



EPA



DHA

Figure 6.28: Representative images of aortic sinus plaques stained for CD107b (Mac3). Staining is diffuse throughout plaque but weaker than that seen in brachiocephalic artery plaques. There was a greater density of staining in the DHA group which reached borderline significance.

6.3.8.2 Chronic inflammation in the vessel wall

Prolonged atherogenic feeding in ApoE-deficient mice induces a chronic inflammatory state. The following markers and transcriptional regulators of chronic inflammation were measured at the gene level by RT-PCR in snap-frozen aortas: IL-1β, TNF-α, MCP-1, NFκB-p65, and PPAR-γ. IL-1β is a potent pro-inflammatory cytokine that is produced after NFκB-dependent activation of the inflammasome, and is prevalent in states of chronic inflammation. It is produced by endothelial cells and macrophages in the setting of atherosclerosis. TNF-α is a cytokine secreted by numerous immune cells including those in the vessel wall at sites of acute and chronic injury. Its presence stimulates other inflammatory mediators that contribute to a chronic inflammatory milieu. MCP-1 is a chemotactic cytokine which is highly expressed in atherosclerotic lesions. It recruits leukocytes to sites of endothelial injury and induces chronic vascular inflammation. The transcription factor NFκB regulates the transcription of pro-inflammatory genes. NFκB is detectable in atherosclerotic plaques, with higher levels seen in plaques that are unstable, and p65 is its most important subunit. PPAR-γ regulates the transcription of anti-inflammatory genes, and is also detectable in plaques.

Compared to the no treatment group, the relative gene expression of IL-1β in the EPA group was 44.3 (IQR 33.4 – 51.8) % lower (p=0.04). In the DHA group it was 48.8 % (25.2 – 51.8) lower, but it did not reach statistical significance (p=0.06). Gene expression was 33.5 (16.4 – 80.8) % greater in IL-1β expression in the olive oil group, but was not statistically significant (p=0.56), see Figure 6.29.

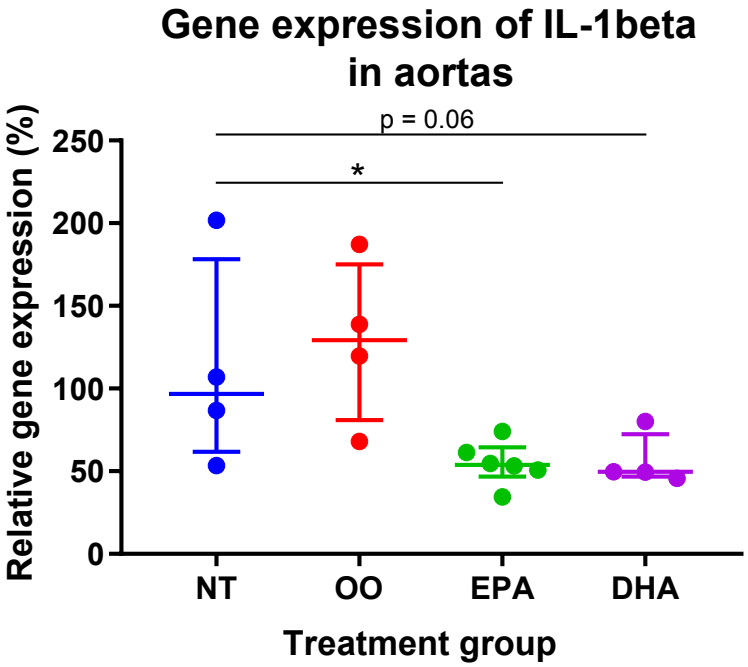


Figure 6.29: Relative gene expression of IL-1β in mouse aortas, measured by RT-PCR. Notable reductions were seen in the EPA and DHA treatment groups. n = 4-6 per group. Results expressed as median + interquartile range. *p<0.05.

Similarly, the EPA and DHA groups had significantly lower TNF-α expression compared to the no treatment group. Gene expression was 47.9 (IQR 31.0 – 63.2) % lower in the EPA group (p=0.04), and 49.8 (27.4 – 70.1) % lower in the DHA group (p = 0.05). Gene expression was 20.3 (-22.2 – 46.6) % lower in the olive oil group, which was not statistically significant (p = 0.26), see Figure 6.30.

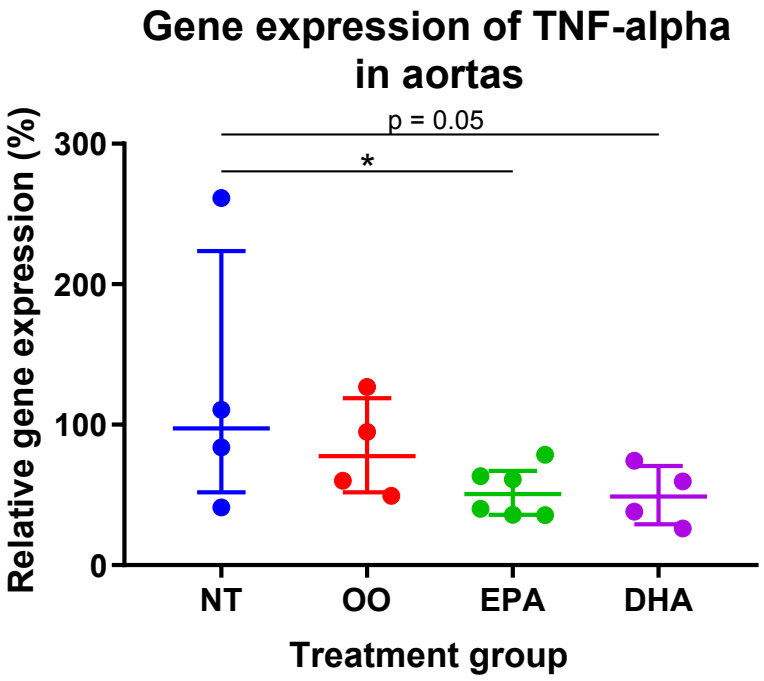


Figure 6.30: Relative gene expression of TNF-α in mouse aortas, measured by RT-PCR. TNF-α was significantly reduced by EPA and DHA. n = 4-6 per group. Results expressed as median + interquartile range. *p<0.05.

Despite the reductions in IL-1β and TNF-α seen with EPA and DHA, there were no significant differences between treatment groups seen for MCP-1 expression. Compared to the no treatment group, MCP-1 gene expression was 6.2 (-25.8 – 37.5) % lower in the EPA group (p=0.79), and 36.3 (24.0 – 51.0) % lower in the DHA group (p=0.15). Gene expression was 4.0 (-47.4 - 95) % greater in the olive oil group (p=0.79), see Figure 6.31.

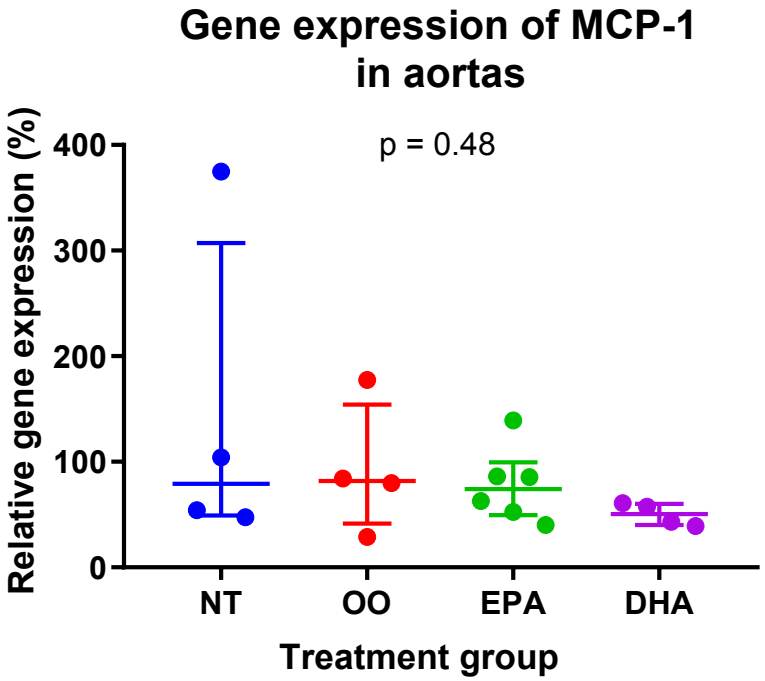


Figure 6.31: Relative gene expression of MCP-1 in mouse aortas, measured by RT-PCR. n = 4-6 per group. Results expressed as median + interquartile range.

Gene expression of the transcription factors NFκBp65 (pro-inflammatory) and PPAR-γ (anti-inflammatory) were similar in all treatment groups, see Figures 6.32 and 6.33. Compared to the no treatment group, the gene expression of NFκBp65 was 5.6 (-16.7 – 44.7) % greater in the EPA group (p=0.65), 21.4 (-16.1 – 50.9) % greater in the DHA group (p=0.43), and 11.3 (-31.1 – 19.8) % greater in the olive oil group (p=0.96). Compared to the no treatment group, the gene expression of PPAR- γ was 15.7 (-30.1 – 67.2) % greater in the EPA group (p=0.98), 41.8 (-20.1 – 92.3) % greater in the DHA group (p=0.69), and 55.3 (27.3 – 107.0) % greater in the olive oil group (p=0.29). Functional changes in transcription factors are likely to be more important than the magnitude of gene expression.

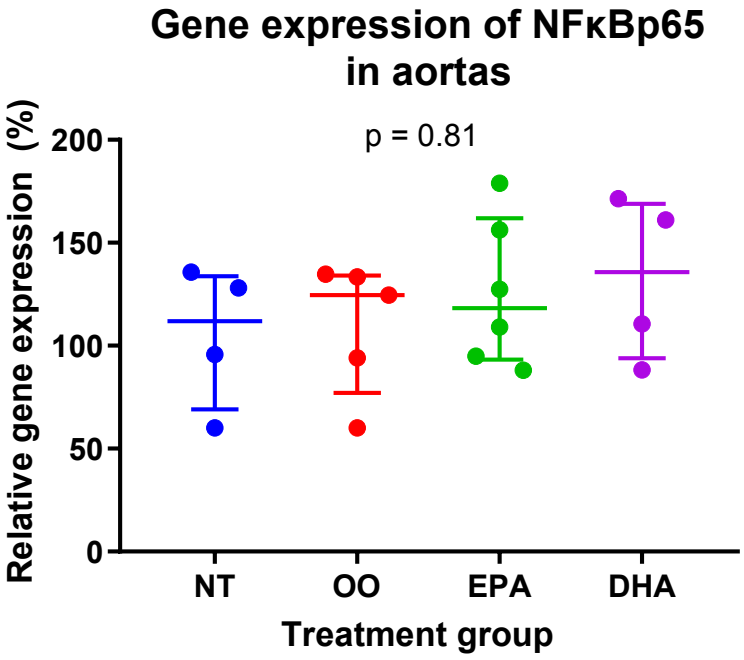


Figure 6.32: Relative gene expression of NFκBp65 in mouse aortas, measured by RT-PCR. n = 4-6 per group. Results expressed as median + interquartile range.

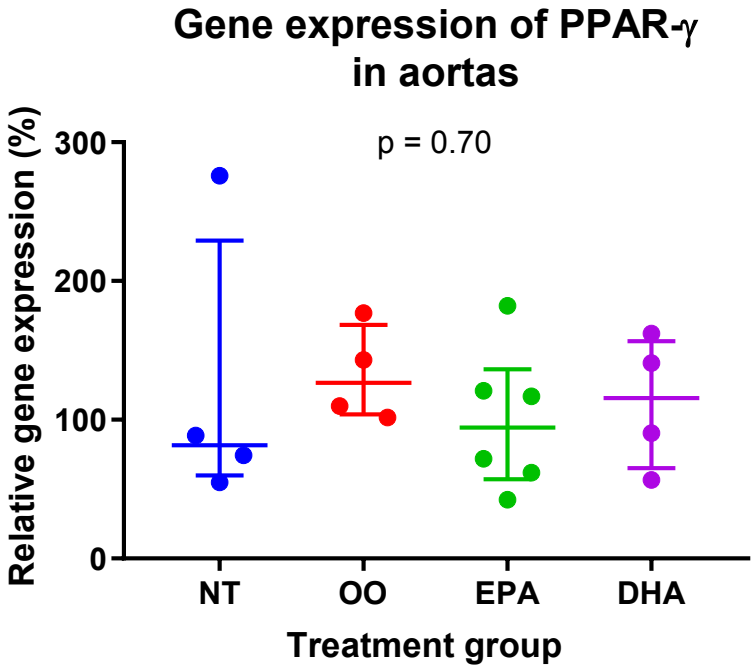


Figure 6.33: Relative gene expression of PPAR-γ in mouse aortas, measured by RT-PCR. n = 4-6 per group. Data are presented as median + interquartile range.

6.3.9 Lipid content of the aorta

After 16 weeks of atherogenic feeding, the aortas of all ApoE-deficient mice stained positively for Oil Red-O. Lipid was present diffusely throughout the aorta in all mice. In the no treatment group, 6.3 (IQR 5.0 – 10.6) % of the aortic surface area stained positively for Oil Red-O. Treatment with neither olive oil, EPA or DHA significantly modified aortic lipid content, with 7.1 (5.2 – 13.0) %, 6.9 (6.6 – 8.1) %, and 4.4 (4.1 – 8.1) % of aortic surface area staining positively, respectively ($p>0.99$ for all comparisons with no treatment), see Figures 6.34 and 6.35. The lack of effect of EPA or DHA on aortic lipid burden was in spite of their favourable effects on plasma cholesterol and triglycerides.

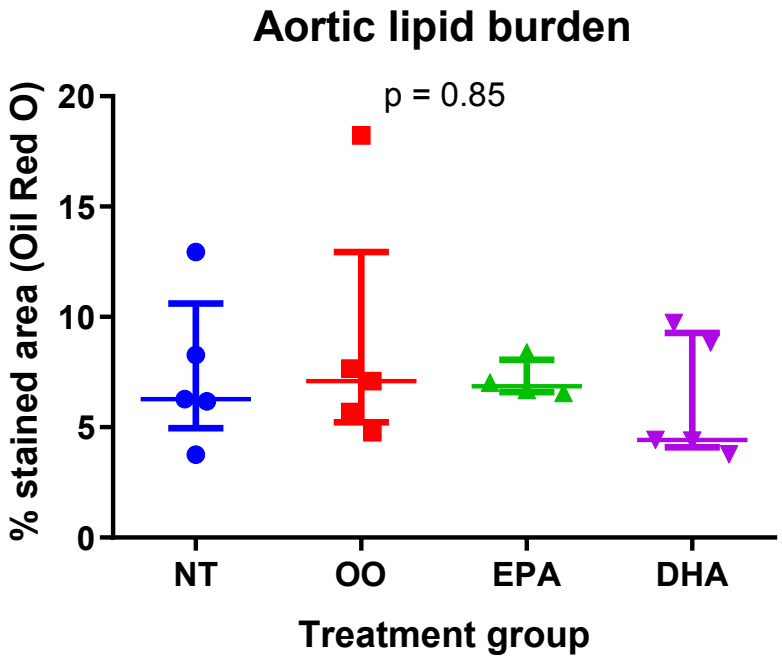


Figure 6.34: Burden of lipid in the aorta, as a proportion of total aortic surface area. n = 4-5 per group. Results expressed as median + interquartile range.

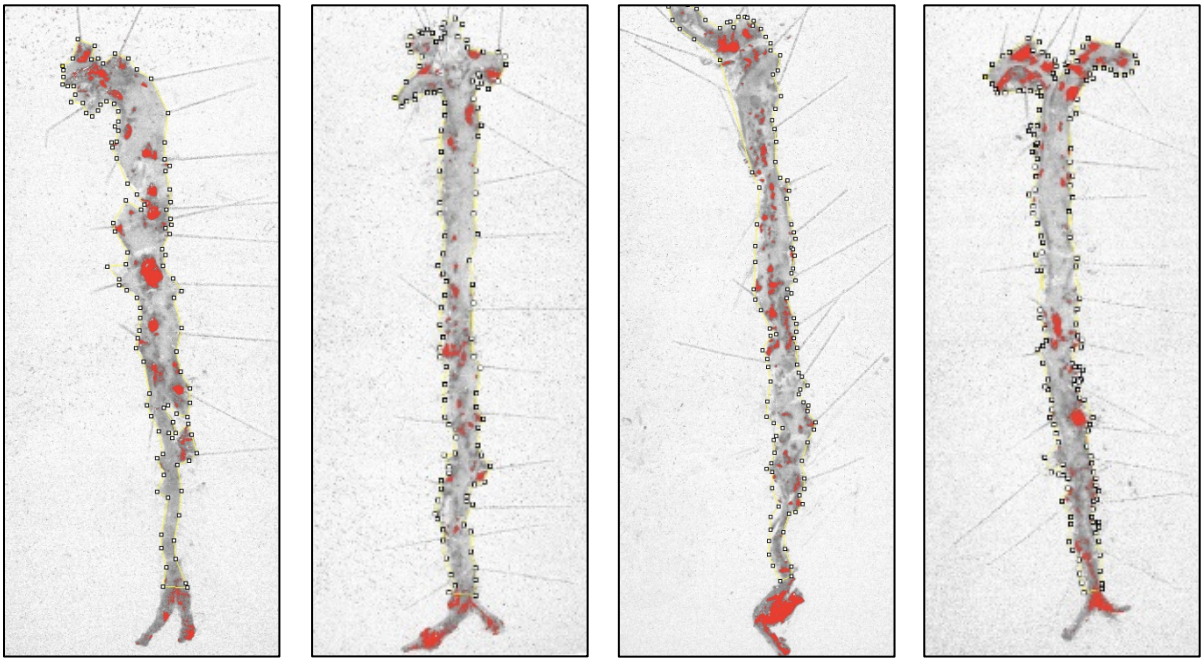


Figure 6.35: Representative images from analysis of Oil Red-O staining in aortas pinned *en-face*. Lipid deposition spans the entire length of the aorta, with a similar lipid burden amongst the different treatment groups.

6.3.10 Correlations between blood omega-3 levels and measures of plaque burden, plaque characteristics, lipid burden and vascular inflammation

The blood concentrations of EPA, DHA and the EPA:DHA ratio at study completion were correlated with (1) all measures of plaque burden, stability, inflammatory cell content, (2) gene expression of markers of inflammation in the vessel wall, and (3) the burden of lipid in the aorta. A summary of the correlation (Spearman) coefficient and level of statistical significance for each correlation is presented in Table 6.2. No significant correlation was seen between blood omega-3 concentrations and the burden of plaque in the aortic sinuses or brachiocephalic arteries, however there was a significant inverse correlation between EPA levels and the thickness of the intima and media in the arterial wall (see Figure 6.36).

Table 6.2: Summary of correlations between blood concentrations of EPA, DHA, and the ratio of EPA to DHA with measures of plaque burden and characteristics, lipid burden and vascular inflammation.

Parameter	Correlation coefficient and significance level		
	EPA	DHA	EPA:DHA
Aortic sinus plaque burden	r = -0.07, p = 0.82	r = 0.11, p = 0.70	r = 0.05, p = 0.86
BCA plaque burden	r = 0.10, p = 0.74	r = -0.36, p = 0.22	r = 0.08, p = 0.79
Arterial intimal + medial thickness	**r = -0.49, p = 0.04**	r = -0.11, p = 0.67	r = -0.39, p = 0.11
Aortic sinus plaque collagen	r = -0.22, p = 0.25	r = -0.34, p = 0.06	r = -0.12, p = 0.53
BCA plaque collagen	r = -0.06, p = 0.85	r = -0.47, p = 0.09	r = -0.13, p = 0.65
SMA staining in aortic sinus plaque	r = 0.06, p = 0.76	r = 0.09, p = 0.65	r = 0.04, p = 0.83
SMA staining in BCA plaque	r = 0.13, p = 0.66	r = 0.56, p = 0.54	r = 0.12, p = 0.68
CD107b staining in aortic sinus plaque	r = 0.32, p = 0.09	**r = 0.58, p = 0.0008**	r = 0.05, p = 0.79
CD107b staining in BCA plaque	r = -0.04, p = 0.89	**r = 0.66, p = 0.01**	r = -0.29, p = 0.32
IL-1β expression in arterial wall	**r = -0.63, p = 0.009**	r = -0.47, p = 0.06	**r = -0.67, p = 0.004**
TNF-α expression in arterial wall	**r = -0.50, p = 0.04**	r = -0.45, p = 0.06	**r = -0.47, p = 0.049**
MCP-1 expression in arterial wall	r = -0.13, p = 0.61	r = -0.43, p = 0.07	r = 0.02, p = 0.93
NFκBp65 expression in arterial wall	r = -0.11, p = 0.66	r = -0.13, p = 0.62	r = -0.02, p = 0.95
PPAR-γ expression in arterial wall	r = -0.31, p = 0.21	r = -0.13, p = 0.60	r = -0.35, p = 0.16
Lipid burden in aorta (Oil Red-O)	r = -0.21, p = 0.39	r = -0.40, p = 0.09	r = 0.18, p = 0.46

BCA = brachiocephalic artery. SMA = smooth muscle actin.

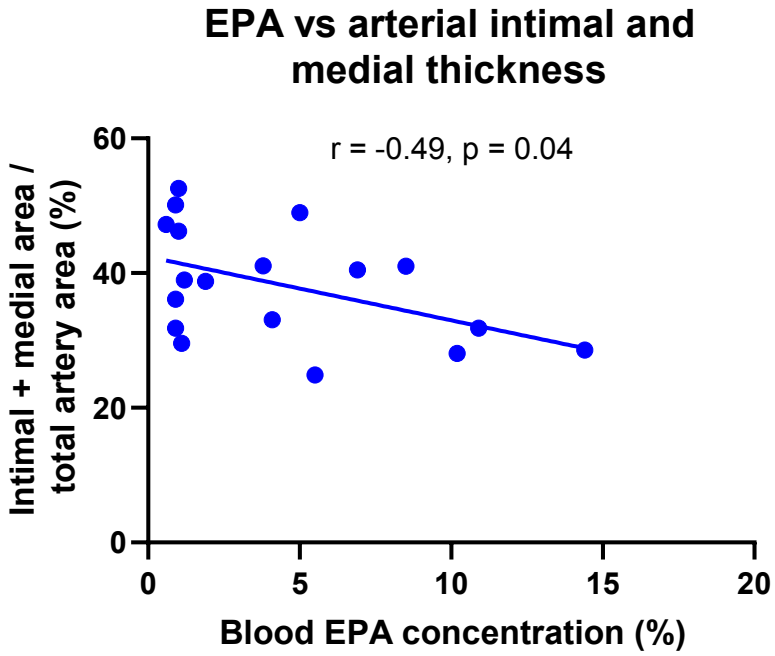


Figure 6.36: Correlation between blood EPA concentration at study completion and intimal plus medial thickness in the artery wall. n = 18 mice.

No significant correlation was present between omega-3 levels and two important measures of plaque stability, i.e. collagen content and smooth muscle actin staining (See Table 6.2). The macrophage content of both aortic sinus and brachiocephalic artery plaques correlated positively and significantly with blood DHA levels, indicating that DHA may have a pro-inflammatory effect in atherosclerotic plaques (See Figures 6.37 and 6.38).

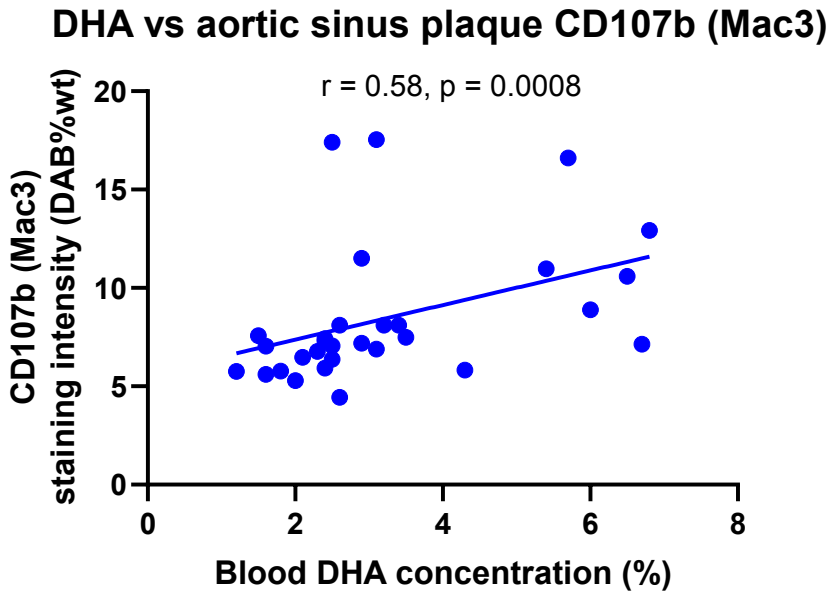


Figure 6.37: Correlation between blood DHA concentration at study completion and staining for CD107b (Mac3) in aortic sinus plaques. n = 30 mice.

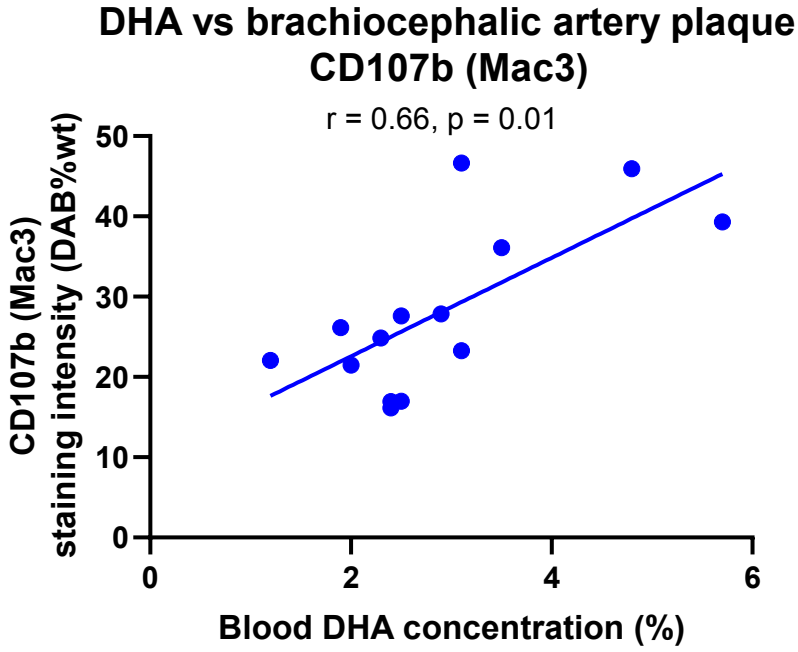


Figure 6.38: Correlation between blood DHA concentration at study completion and staining for CD107b (Mac3) in brachiocephalic artery plaques. n = 14 mice.

Consistent with the reductions in gene expression of IL-1β and TNF-α in the arterial wall occurring with EPA supplementation, there was a significant inverse correlation present between (1) blood EPA levels and (2) the EPA:DHA ratio with the expression of those genes (see Figures 6.39 to 6.42). No significant correlations were demonstrated between blood omega-3 levels and the gene expression of MCP-1, NFκBp65, or PPAR-γ in the arterial wall (see Table 6.2). Furthermore, no significant correlations were demonstrated between blood omega-3 levels and the burden of lipid in the aorta (see Table 6.2).

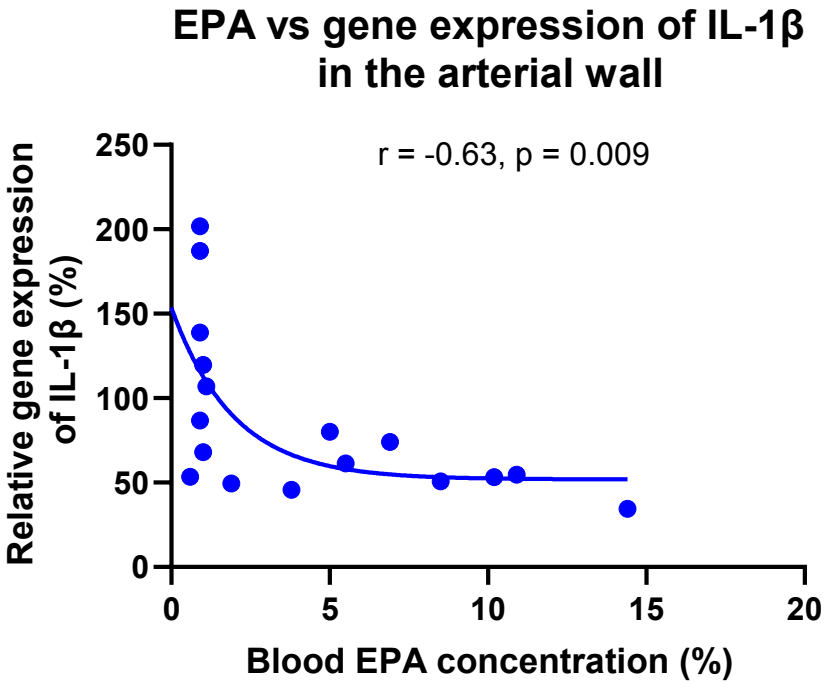


Figure 6.39: Correlation between blood EPA concentration at study completion and the gene expression of IL-1β in the arterial wall. n = 17 mice.

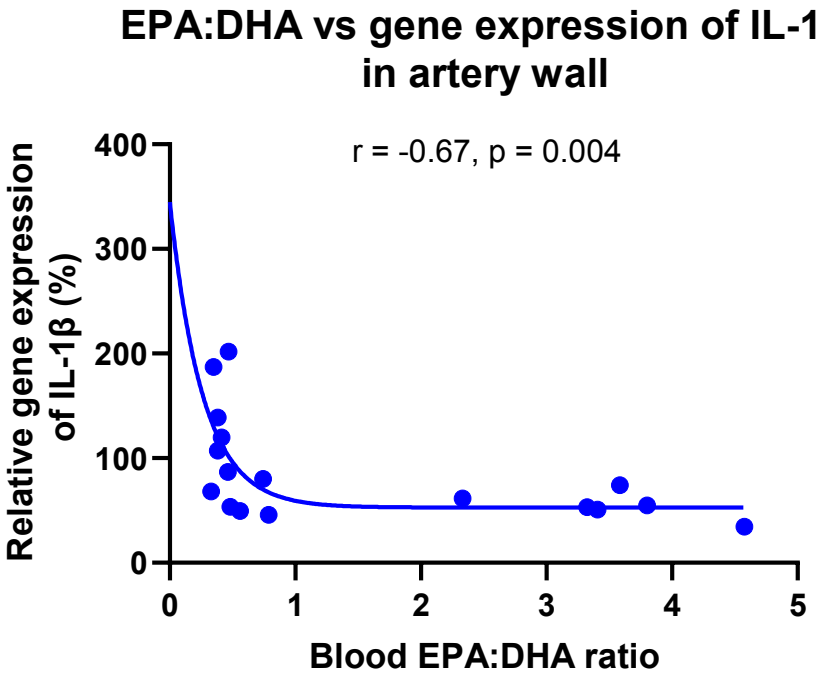


Figure 6.40: Correlation between blood EPA:DHA ratio at study completion and the gene expression of IL-1β in the arterial wall. n = 17 mice.

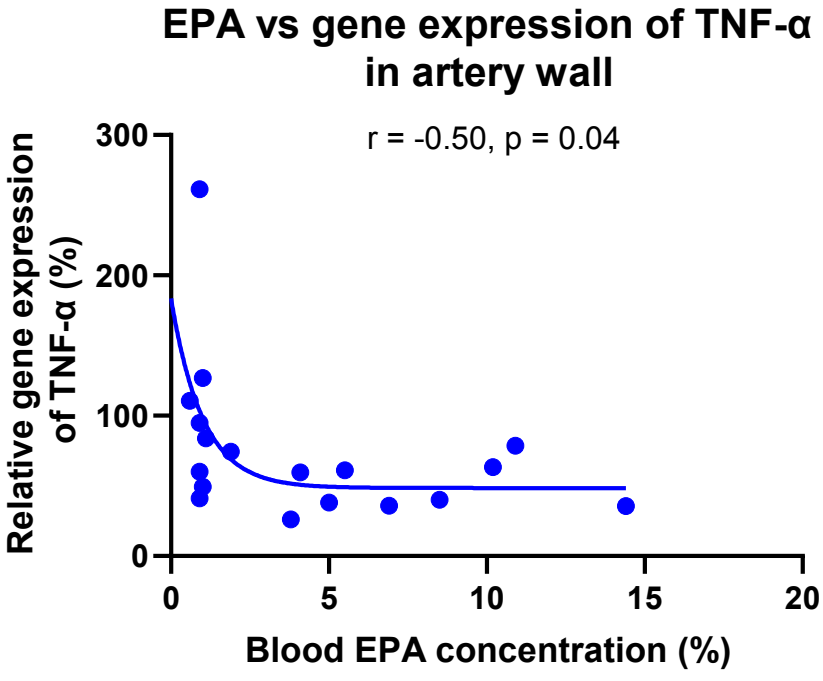


Figure 6.41: Correlation between blood EPA concentration at study completion and the gene expression of TNF-α in the arterial wall. n = 18 mice.

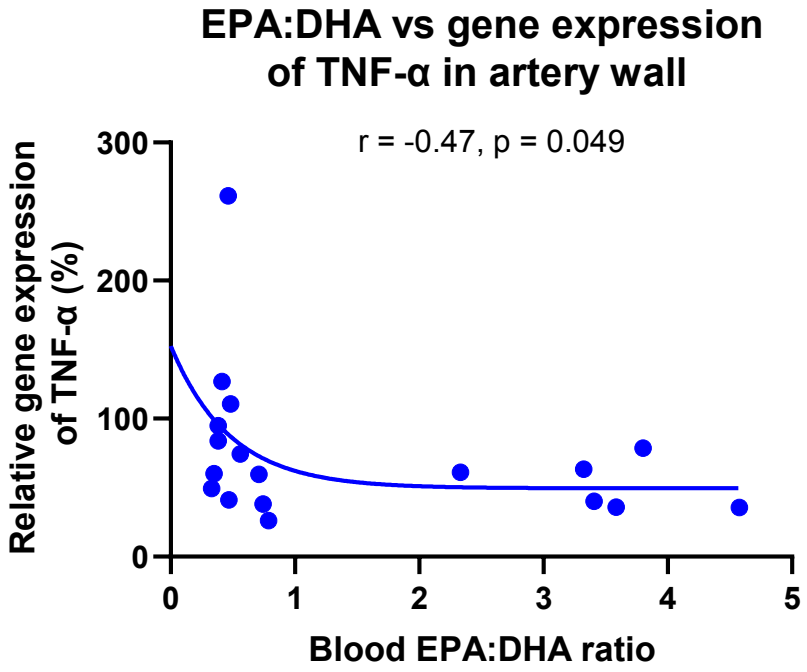


Figure 6.42: Correlation between blood EPA:DHA ratio at study completion and the gene expression of TNF-α in the arterial wall. n = 18 mice.

6.4 Discussion

This is the first study comparing direct supplementation of purified EPA with DHA on atherogenesis and chronic vascular inflammation in a mouse model. Previous studies have administered combinations of EPA and DHA, or individual fatty acids alone, typically by their addition to diet. Chapters 4 and 5 of this thesis demonstrated that EPA reduces acute vascular inflammation and has a superior effect over DHA. This chapter extends the study of omega-3 fatty acids to their effects on the development of advanced atherosclerotic lesions and on chronic vascular inflammation. ApoE-deficient mice fed an atherogenic diet were supplemented with high dose EPA, DHA, olive oil, or had no treatment, for the last 8 weeks of a 16-week atherogenic feeding period prior to being humanely killed. The dose chosen was equivalent to ten times the dose used in many recent human clinical trials of 4 grams per day.

Four grams per day equates to 60 mg/kg/day for a 70kg human, and hence 600 mg/kg/day was used in this mouse study to ensure sufficient dosing. The effects of treatments on the rate of weight gain, lipid levels, the burden of plaque and lipid, the inflammatory characteristics and stability of atherosclerotic plaque, and on chronic inflammation of the vessel wall were quantified.

The neutral effects of EPA and DHA on the rate of weight gain in this study are consistent with previous studies showing no effect of omega-3 fatty acid supplementation on body weight[552-554]. Olive oil reduced the rate of weight gain, and this may be because oleic acid, its dominant fatty acid, is a substrate for the lipid messenger oleoylethanolamide, which induces satiety after fat consumption[555].

The effect of fish oil on total cholesterol levels has been studied extensively, but this is one of few studies using pure EPA and DHA. The stabilising effect of EPA and DHA on cholesterol levels demonstrated in this study is consistent with a study by Guo et al, where EPA, DPA, and DHA were administered to C57Bl/6 mice fed an atherogenic diet, and all three fatty acids reduced total cholesterol and LDL levels[556]. These cholesterol-lowering effects of omega-3 fatty acids are in contrast to the small increase typically seen in humans[541], and even differ between rodent species[557]. Mechanisms for this effect in mice may be the activation of AMP-activated protein kinase[558], activation of fatty acid oxidation genes[559], and suppression of liver fatty acid synthesis[560].

Triglyceride levels were naturally elevated in all mice after 8 weeks, as ApoE-deficient mice have reduced triglyceride clearance[561]. Both EPA and DHA significantly reduced these levels by the end of the study. This is consistent with findings from Guo et al's study, with EPA, DPA, and DHA all reducing triglyceride levels. Previous animal studies have also shown reductions in plasma triglycerides with combination omega-3 (EPA plus DHA)

supplementation[558, 562], and a triglyceride-lowering effect with DHA administered alone[563]. Human studies have likewise consistently demonstrated triglyceride lowering with omega-3s[207, 564]. This has been attributed to reduced triglyceride production and increased clearance[565]. Specifically, the stimulation of PPAR-α causes beta-oxidation of fatty acids, which reduces their availability for VLDL production[559]. The superior effect of DHA in reducing triglyceride levels compared to EPA in the current study is also consistent with human studies[288, 479, 480, 482, 483, 566].

Neither EPA nor DHA altered the burden of atherosclerotic plaque. Atherosclerotic plaques were quantified at sites of predilection for advanced plaque formation, namely the aortic sinuses and brachiocephalic arteries. Omega-3 fatty acids have numerous pleiotropic effects beyond lipid lowering, which lower cardiovascular risk[567], but which were clearly insufficient to lead to a measurable anti-atherosclerotic effect. In the current study, plaque in the aortic sinuses and brachiocephalic arteries was extensive and morphologically advanced. The burden of plaque was similar in both locations (32-34% of total area), however the stability of plaque was higher in the aortic sinuses as evidenced by a higher collagen content, a high concentration of smooth muscle cells on the plaque surface, and a lower inflammatory cell (macrophage) content. Blood flow at arterial branch points (such as the brachiocephalic artery) is subject to abnormal haemodynamic shear stress, which induces endothelial dysfunction and promotes atherosclerosis[568] compared to sites of more laminar flow, such as the aortic root. This explains the discrepant findings at the two sites. It is plausible that omega-3s would have a favourable effect on arterial shear stress given the reductions in arterial stiffness[569] and increases in local nitric oxide production[570] previously demonstrated. However, this has not been studied directly using omega-3 fatty acids and warrants further investigation. Furthermore, neither EPA nor DHA reduced the degree of expansion of the arterial intima and media. There was, however, a significant inverse correlation between the content of EPA in

blood and the thickness of the intima and media. This is consistent with evidence for an association between higher blood omega-3 levels and lower carotid intima-media thickness in certain population groups, however a greater association of EPA compared to DHA has not been previously reported[571, 572]. The collagen content, smooth muscle cell content, and inflammatory cell (macrophage) content were not altered by any of the treatments. However, there was a significant positive correlation between DHA levels and the macrophage content of plaques. This would naturally be considered to represent a pro-inflammatory relationship of DHA with plaque. This is in contrast to evidence of a macrophage-lowering effect of omega-3 fatty acids, which has been demonstrated in carotid endarterectomy specimens after fish oil supplementation[253]. It has been suggested that omega-3 fatty acids lower macrophage content in plaque by inducing their death by apoptosis[253]. Macrophages in plaque contribute to plaque instability, both pathologically[573] and clinically[574], and hence their apoptosis would generally be considered beneficial. However, apoptotic macrophages in plaques are incompletely scavenged, and their death results in the release of extracellular lipid into plaque. The cellular debris and extracellular lipid may propagate the chronic inflammatory response in plaque[575]. Therefore, the positive correlation of DHA with plaque macrophage content demonstrated in this study suggests, but does not necessarily imply, a pro-atherosclerotic relationship, with EPA being more protective than DHA.

Lipid was detected using Oil Red-O staining throughout the aorta. EPA and DHA did not reduce lipid burden despite lowering serum cholesterol and triglyceride levels. Although the positive and continuous association between serum cholesterol and atherosclerosis has been well-established[275, 576], only recently has the lipid content of plaque become quantifiable *in vivo*, using near-infrared spectroscopy (NIRS)[577-579]. Unlike total plaque burden, the lipid content of plaque does not correlate directly with serum cholesterol levels. Rather, changes in the lipid content of plaque has been associated with changes in HDL-C but not other lipid

parameters[580]. This may explain the discordance between serum cholesterol levels and aortic lipid burden. Given that only 4-5 mice per treatment group had aortic Oil Red-O staining performed, an effect on plaque lipid by omega-3 fatty acids cannot be excluded. Larger studies will be required to further elucidate the differential effects of EPA and DHA on plaque lipid, and the clinical implications of this.

EPA significantly reduced the gene expression of two important markers of chronic vascular inflammation in plaque, IL-1β and TNF-α. There was also a significant inverse correlation between the gene expression of both IL-1β and TNF-α and blood levels of EPA as well as the EPA:DHA ratio. This provides evidence for a differential and superior effect of EPA compared to DHA on chronic vascular inflammation. The suppressive effect of EPA on IL-1β gene expression may be due to its effects on the inflammasome. There are numerous stimulators of the inflammasome in atherosclerotic plaques, including cholesterol crystals and inflammatory cytokines. Indeed, there are significant amounts of NLRP3, caspase-I and IL-1β in plaques[74]. Hence, the requirement for a two-step activation process for the inflammasome and subsequent IL-1β generation is not a hindrance for continued inflammatory activity in atherosclerotic plaques. Dietary fatty acid composition is sensed by the NLRP3 inflammasome in human macrophages, and omega-3 fatty acids (studied in the forms of fish oil and DHA) have been shown to have an inhibitory effect[353, 354, 581]. Williams-Bey et al demonstrated that inhibition of the G-Protein-coupled Receptor, GPR120, also known as Free Fatty Acid Receptor 4, was necessary for this to occur, which suppressed the nuclear translocation of NF-κB[581]. It is likely that EPA inhibits the inflammasome by the same mechanisms demonstrated for fish oil and DHA. Based on the success of IL-1β inhibition on reducing cardiovascular events in high risk patients[120], it is likely that the cardioprotective effects of high-dose EPA seen in the REDUCE-IT trial are partly due to this mechanism[234].

The suppressive effect of omega-3 fatty acids on TNF-α has been demonstrated for both circulating and tissue forms. Circulating TNF-α has been reduced with fish oil supplementation in both mice[582] and humans[583]. *In vitro* studies have corroborated this with reductions seen in endotoxin-induced TNF production by monocytes[296, 584] after the addition of omega-3 fatty acids. This was shown to be due to inhibition of NFκB activation[297, 298]. TNF-α has been measured in the vessel wall by Western Blot after EPA supplementation in a rabbit model of acute vascular inflammation. In this study, pre-treatment of Japanese white rabbits for 1 week with high dose EPA resulting in a reduction in TNF-α levels in the vessel wall both before and 3 days after carotid cuff placement[585]. In the setting of chronic vascular inflammation and atherosclerosis, a study of LDL-R-deficient mice fed an atherogenic diet demonstrated a reduction in gene expression of TNF-α in established atherosclerotic plaques after EPA supplementation[259]. A proposed mechanism elucidated by Vassiliou et al is that the omega-3 metabolite Resolvin D1 inhibits T cell proliferation (a source of TNF-α) through increasing indoleamine 2,3-dioxygenase expression in dendritic cells[586]. This is the first study to compare the effects of EPA with DHA on the gene expression of TNF-α in vessel wall in the setting of chronic vascular inflammation, and hence the finding of a superior effect of EPA is a novel finding.

An important limitation of this study is the small sample size. Although a power calculation guided the overall allocation of 10 mice per treatment group, several analyses of plaque, inflammation and lipid were performed with only 5 mice per treatment group because they included only mice of a single sex. Hence, these analyses per underpowered, and the negative results should be interpreted with caution.

Supplementation of omega-3 fatty acids for the final 8 weeks of a 16-week atherogenic feeding period in ApoE-deficient mice models a clinical scenario of the commencement of fish oil supplementation in an adult with atherosclerotic cardiovascular disease. Nakashima et al

demonstrated that by 15 weeks of age, approximately the same age that the mice in the current study were randomised to treatments, ApoE-deficient mice fed an atherogenic diet have developed foam cell lesions, smooth muscle cell proliferation and early fibrous plaques[546]. Neither EPA nor DHA significantly altered the burden of plaque or several measures of its stability. Nor did EPA or DHA alter lipid burden in the aorta. Consistent with the reduction in acute vascular inflammation in collared carotid arteries by EPA seen in Chapter 5, the current study demonstrates a reduction in markers of chronic vascular inflammation in aortas with EPA treatment. Furthermore, these reductions were significantly inversely correlated with blood EPA levels. These findings may contribute to the mechanisms underlying the reduction in major adverse cardiac events seen with EPA in the recently-published REDUCE-IT trial[234]. The results of the current study predict a superior atheroprotective effect of EPA compared to DHA, however this will need to be confirmed in clinical trials. Although atherosclerosis is fundamentally an inflammatory disease[4], the neutral effect of omega-3s on atherogenesis despite a clear anti-inflammatory effect highlights the complex mechanisms underlying this disease process. The pharmacodynamics of individual omega-3 fatty acids and their effects on atherogenic mediators require continued investigation.

CHAPTER 7: DISCUSSION

7.1 Rationale for body of work

The effects of omega-3 fatty acids on atherosclerosis have been studied extensively, including mechanistic studies, observational studies, clinical trials, and epidemiological research. Throughout the period of investigation, which has continued for approximately half a century, there have been discrepancies in the findings of these studies, with numerous examples. Mechanistic studies typically demonstrated beneficial effects of omega-3 fatty acids on inflammation and atherogenesis. Observational studies, both prospective and retrospective, have most commonly demonstrated inverse associations between fish and fish oil consumption as well as blood omega-3 levels on cardiovascular events and mortality. Notably, these inverse associations were more consistent with fish compared to fish oil consumption. Clinical trials of fish oil have produced varied results, however, from an overall neutral effect to significant reductions in cardiovascular events and mortality. Neutral effects were seen more commonly, and identified a paradox of basic research studies identifying cardioprotective properties of omega-3 fatty acids that did not reliably translate into clinical benefit. Moreover, omega-3 fatty acids have consistently been shown to reduce inflammation and triglyceride levels, with both associated with increased cardiovascular risk.

The omega-3 formulations used in basic and clinical studies have been highly heterogeneous, including a variety of doses and EPA:DHA ratios. There is a possibility that an optimal ratio of EPA:DHA exists, and that a dose threshold may be required to achieve an atheroprotective effect. Furthermore, the form in which omega-3 fatty acids are consumed may also play an important role. In most oily fish, omega-3 fatty acids are primarily in triglyceride form, and to a lesser extent phospholipid and free fatty acid forms. However, fish oil supplements are predominantly in either ethyl ester or triglyceride forms. The doses of omega-3 fatty acids used in prospective clinical studies have often been ≤ 1 gram per day, with issues of intolerance, compliance, bleeding risk, and cost all being considered in study design.

However, in pre-clinical studies, omega-3 fatty acids have commonly been supplemented at comparatively high doses, such as 5% of total food intake for animal studies. The latter is an example of a commonly-implemented method of omega-3 supplementation, which has limitations both due to the imprecision of measuring total omega-3 intake and also due to oxidation of fatty acids that occurs upon exposure to air.

The question of whether fish oil consumption is atheroprotective has been confounded broadly by the limitations and heterogeneity of previous study designs, the temporal changes in background anti-atherosclerotic therapies, and the lack of specific omega-3 target levels. The current body of work aimed to answer several important questions about the role of omega-3 fatty acids for atheroprotection by focusing on vascular inflammation. There has been increasing recognition of the role of inflammation in all stages of atherogenesis, and given the established anti-inflammatory properties of omega-3 fatty acids, it was important to investigate their impact on the inflammatory processes in the vessel wall that promote atherosclerosis.

7.2 Findings of individual studies

7.2.1 The impact of omega-3 fatty acids on circulating mediators of atherosclerosis – a systematic review

This systematic review, which included only high-quality (NHMRC Evidence Level I or II) randomised, placebo-controlled omega-3 fatty acid studies indexed in the Cochrane Library, demonstrated that supplementation with omega-3 fatty acids reduced levels of all four classes of atherogenic mediators. These four classes were: atherogenic lipoproteins, atherogenic cytokines and adipokines, atherogenic amino acids and derivatives, and advanced glycation endproducts. Atherogenic mediators were required to be measured in blood either directly *in vivo*, or *ex vivo* in cell culture after human omega-3 supplementation. The Cochrane Library search yielded 1012 results, of which 109 studies were eligible for inclusion in the final

analysis. These comprised 1 Cochrane systematic review, 6 non-Cochrane systematic reviews and meta-analyses, and 102 randomised controlled trials. Each mediator was assessed individually, and the overall effect of omega-3 fatty acids was evaluated qualitatively due to the heterogeneity of study types included. The fatty acid contents of the supplements studied were highly variable, although mostly included mixtures of EPA and DHA, such as what occurs in standard fish oil. Significant reductions were noted for oxLDL, VLDL-C, non-HDL-C, leptin, homocysteine, and advanced glycation endproducts. Mild reductions were noted for IL-6, and TNF-α. No significant changes were noted for the other mediators evaluated.

Amongst included studies, there was significant heterogeneity in sample size, duration of supplementation, the choice of placebo, and omega-3 formulation. Although this systematic review did not attempt to segregate studies based on omega-3 dose or measured blood omega-3 levels, it is predictable that the degree of suppression of atherogenic mediators would correlate with blood omega-3 concentrations. This, as well as the relative effects of EPA versus DHA, and the effects on vascular inflammation measured at the tissue level, requires further evaluation.

7.2.2 Fish Oil Cell Uptake Study of INflammation (FOCUS IN). A randomised controlled trial of fish oil supplementation in healthy volunteers.

The FOCUS IN study enrolled 40 healthy adult volunteers with a low baseline omega-3 intake, and randomised them in a double-blind fashion to four grams per day of either (1) fish oil high in EPA, (2) fish oil high in DHA, (3) fish oil with a standard 2:1 EPA: DHA ratio, or (4) a placebo oil as an oil control, for 30 days. Participants in the EPA group were older, however the groups were otherwise evenly matched at baseline in terms of age, gender, body mass index, resting heart rate and blood pressure, lipid profile, inflammatory profile and omega-3 status.

There were no significant differences in resting heart rate or systolic blood pressure between the 4 treatment groups, although DHA significantly reduced diastolic blood pressure compared to placebo. Total cholesterol, LDL-C and HDL-C did not significantly differ between treatment groups, although DHA significantly reduced triglyceride levels. High-sensitivity CRP and lipoprotein (a) levels did not significantly differ between treatment groups.

Serum collected before and after treatment was added to human umbilical vein endothelial cells (HUVECs) in culture at a concentration of 10%, before and after TNF-α stimulation (10ng/ml). Changes in the gene expression of the following markers of vascular inflammation were measured by RT-PCR: VCAM-1, ICAM-1, MCP-1 and NFκBp65. Serum from participants taking EPA reduced the gene expression of MCP-1 by TNF-stimulated HUVECs by 25% compared to placebo (p=0.03). No significant differences were seen between treatment groups for the other markers.

The expression of VCAM-1, ICAM-1, MCP-1 and NFκBp65 by stimulated HUVECs was correlated with the following patient characteristics to determine whether any significant associations exist: total cholesterol, LDL-C, HDL-C, triglycerides, omega 6:3 ratio, blood EPA levels, blood DHA levels, and blood saturated fats. HDL-C levels correlated positively and significantly with both VCAM-1 (r=0.36, p=0.02) and MCP-1 (r=0.40, p=0.01) expression. This is consistent with HDL dysfunctionality at very high HDL-C levels, and this warrants further investigation.

7.2.3 The impact of omega-3 fatty acids on acute vascular inflammation in a mouse model

In this study, eight-week-old C57Bl/6 mice fed a chow diet were randomised to supplementation with 600mg/kg/day of either EPA, DHA, olive oil as an oil control, or no treatment, for 30 days by oral gavage. Subsequently, pure acute vascular inflammation was induced using the surgical application of non-occlusive silicon elastic collars to the right carotid

artery, which remained in place for 48 hours. Subsequently, the mice were humanely killed and both carotid arteries were harvested for immunohistochemical analysis for markers of acute vascular inflammation, namely VCAM-1, ICAM-1, MCP-1 and CD18. A blood sample was obtained by terminal cardiac puncture.

Carotid collaring significantly upregulated the protein expression of all markers measured. EPA reduced the protein expression of VCAM-1 and MCP-1 by 43% and 38% respectively in collared carotids compared to no treatment ($p<0.05$ for both comparisons). There were numerical reductions in ICAM-1 and CD18 expression with EPA, which did not reach statistical significance. Furthermore, there was a numerical reduction in all four markers of AVI with DHA, which was less than EPA in each case, and not statistically significant.

Plasma total cholesterol and triglycerides were low, and did not differ significantly between treatment groups. EPA and DHA supplementation increased their respective blood levels by 272% and 62% ($p<0.0001$). There were significant inverse correlations observed between blood levels of both EPA and DHA and the expression of all four markers of AVI on immunohistochemistry. Furthermore, significant inverse correlations were also observed between the blood EPA:DHA ratio and all four markers of AVI, consistent with the superior effect of EPA compared to DHA.

The reduction in protein expression of markers of AVI by EPA supplementation measured in the arterial wall, is consistent with the *in vitro* gene expression findings of FOCUS IN.

7.2.4 The impact of omega-3 fatty acids on atherosclerosis and chronic vascular inflammation

In this study, forty 8-week-old ApoE-deficient mice were fed an atherogenic diet for 16 weeks to induce chronic vascular inflammation and advanced atherosclerotic plaques. After 8

weeks, a blood sample was taken and the mice were randomised to supplementation with 600mg/kg/day of either: (1) EPA, (2) DHA, (3) olive oil as an oil control, or (4) no treatment. Supplementation was performed by oral gavage, and continued for the last 8 weeks of the 16-week treatment period, after which time the mice were humanely killed and blood and organ harvesting were performed.

The body weight of mice in all four treatment groups increased over the atherogenic feeding period, with no effect of EPA or DHA. Plasma total cholesterol levels were high in all mice after 8 weeks, and were stabilised by both EPA and DHA. Plasma triglyceride levels were elevated in all mice after 8 weeks, and EPA and DHA significantly reduced these levels by study completion.

Atherosclerotic plaque was measured in the aortic sinuses and brachiocephalic arteries. Although advanced plaques had developed by study completion, no differences in plaque burden were demonstrated between treatment groups. Similarly, the thickness of the intimal and medial layers, and the collagen content of plaques did not differ among treatment groups. The smooth muscle cell content and macrophage content of plaques, measured by immunohistochemical staining for smooth muscle actin and CD107b, did not differ between treatment groups. Hence, omega-3 supplementation did not impact on measures of plaque stability. The lipid content of aortas was assessed by Oil Red-O staining, and in all mice lipid was distributed diffusely throughout the aorta. Omega-3 supplementation did not significantly impact the burden of lipid.

In aortic tissue, the gene expression of markers of chronic inflammation, IL-1β and TNF-α, were significantly lower in the mice treated with EPA (-44.3% [p=0.04] and -48.8% [p=0.04], respectively). Consistent with this beneficial effect on EPA on chronic vascular inflammation, there were significant inverse correlations present between IL-1β and TNF-α

expression and both blood EPA levels and the EPA:DHA ratio. Blood EPA levels also correlated inversely with intimal and medial thickness in the artery wall. This finding suggests a signal for a favourable effect of EPA on plaque, that may not have been demonstrated due to the low sample size in this study (n < 5 mice for some measures).

7.3 Overarching conclusions

Omega-3 fatty acids reduce vascular inflammation from the earliest stages of endothelial injury to the advanced stages of atherosclerosis. In the *in vitro* study FOCUS IN, and in the acute and chronic mouse studies, EPA was consistently shown to be superior than DHA. Omega-3 fatty acids reduce triglyceride levels and stabilise cholesterol levels. Nonetheless, the beneficial effects of omega-3 fatty acids on vascular inflammation in the experimental studies have largely occurred independent of effects on both cholesterol and triglyceride levels. Despite the critical role of vascular inflammation in atherogenesis, and the reduction in circulating atherogenic mediators by omega-3 fatty acids established in the systematic review, omega-3 fatty acids did not improve plaque burden or characteristics, nor lipid burden. In view of the small sample size of the chronic mouse study, which was less than 5 mice for some analyses, an atheroprotective effect has not been excluded. Moreover, the findings of the recent REDUCE-IT study demonstrated a significant reduction in major adverse cardiovascular events with high-dose EPA supplementation[234].

In the experimental studies presented in this thesis, omega-3 fatty acids were supplemented at high doses. In FOCUS IN, this was 4 grams of omega-3 per day. In both mouse studies, a daily omega-3 dose of 600mg/kg was given (equivalent to 40 grams per day for a weight of 70kg). Consistent with the results of previous dietary omega-3 studies[222, 223], which demonstrated dose-dependent effects, and the high dose of EPA ethyl esters (4 grams per day) utilised in the positive REDUCE-IT trial, it is likely that high doses of omega-3 fatty acids are required to demonstrate beneficial effects.

7.4 Clinical implications of research findings

7.4.1 Omega-3 fatty acids for primary prevention

In this body of work, the beneficial effects of omega-3 fatty acids on vascular inflammation have been demonstrated in experimental studies that model primary and secondary prevention settings. In FOCUS IN, serum from healthy volunteers supplemented with fish oil was added to HUVECs in culture for a duration of 24 hours, prior to the addition of an inflammatory stimulus (TNF-α). Similarly, in the mouse study of AVI, omega-3 fatty acids were supplemented for 30 days prior to the induction of AVI by carotid collaring. However, in the chronic mouse study, the ApoE-deficient mice were 16 weeks of age and had had 8 weeks of atherogenic feeding prior to the commencement of omega-3 supplementation. By this stage, foam cell lesions, smooth muscle cell proliferation and early fibrous plaques would likely have developed[546].

Regular high-dose consumption of omega-3 fatty acids, whether in dietary or supplement form, is likely to have a role in the primary prevention of cardiovascular disease. Since macroscopic evidence of atherosclerosis has been identified in humans as early as the foetal stage, i.e. in the form of fatty streaks[498], omega-3 supplementation has the potential for use in primary cardiovascular prevention in all age groups. High-dose omega-3 consumption on a population level for primary prevention is not feasible, however, for several reasons. Firstly, the number needed to treat in order to prevent one cardiovascular event has not been established and is likely to be high. Secondly, to achieve 4 grams of omega-3 fatty acids per day would be prohibitively expensive and potentially cumbersome for the consumer. For example, in the FOCUS IN study, 6-8 capsules per day were required for each of the supplements. Thirdly, as the main source of omega-3 fatty acids is marine-derived, consideration must be given to sustainability of the aquaculture industry and the risk of extinction to fish populations[587]. In recent years, Australian scientists have been among those

pioneering the development of genetically-modified crops that produce omega-3 oils, as a sustainable source[588-590]. Finally, although generally well-tolerated, fish oil consumption is associated with a number of mild adverse effects that may lead to discontinuation, in particularly gastrointestinal complaints[591].

A more feasible approach for the use of omega-3 fatty acids for primary prevention would be to target specific population subgroups. Conceivably, these could include individuals with risk factors that have been shown to be ameliorated by omega-3 fatty acids. Based on the results of the systematic review, individuals with elevated VLDL-C and non-HDL-C levels, hyperhomocysteinemia, the overweight or obese (likely to have elevated leptin levels), and those with hyperglycaemia or vascular diabetic complications (expected to have circulating advanced glycation endproducts) could have a slowed progression of atherogenesis. The two experimental studies that modelled primary prevention (FOCUS IN and the mouse collar study) were conducted using subjects with minimal cardiovascular risk factors (healthy volunteers and wild-type mice), however some additional target groups could be surmised. For example, individuals with very high levels of HDL-C, which correlated positively with VCAM-1 and MCP-1, may be a target for therapies that reduce these inflammatory markers, namely EPA. In addition, among these subgroups, individuals with low blood omega-3 levels, especially EPA, are likely to have a greater inflammatory response to a future vascular insult, which could be reduced by omega-3 supplementation. Triglyceride levels, although not a focus of this body of work, were lowered by omega-3 fatty acids in the experimental studies - their atherogenic effects being exerted by virtue of their incorporation into atherogenic triglyceride-rich lipoproteins. Prospective studies will be required to demonstrate the effectiveness of targeting these population subgroups for primary prevention, however REDUCE-IT produced encouraging findings. Almost 30% of patients in REDUCE-IT were in the primary prevention setting, i.e. were diabetics with elevated triglyceride levels and other risk factors but no

established history of cardiovascular disease. Although not statistically-significant, the hazard ratio for the primary endpoint for these patients was 12% lower in the icosapent ethyl group compared to placebo[234].

7.4.2 Omega-3 fatty acids for secondary prevention

The results of the chronic mouse study suggest promising roles for the use of omega-3 fatty acids in the secondary prevention setting. In the context of established atherosclerosis, omega-3 fatty acids did not significantly reduce plaque burden or favourably modify plaque characteristics seen histologically. Furthermore, atheromatous lesions were no less lipidic. These findings would naturally suggest that in patients with atherosclerotic cardiovascular disease, omega-3 fatty acids would not modify the risk of future acute plaque events or progressive myocardial ischaemia. However, markers of chronic inflammation in the vessel wall were significantly reduced by EPA. Given the intensely inflammatory nature of ruptured plaques, it is possible that the significant reduction in acute plaque events with icosapent ethyl reported in REDUCE-IT was partially driven by a reduction in vascular inflammation. If indeed modifications in the cytokine profile of the vessel wall in the setting of atherosclerosis are sufficient to contribute to a reduction in major adverse cardiovascular events, then this may require a paradigm shift in cardiovascular risk assessment, in particular the role of plaque imaging. The traditional focus on the assessment of the characteristics of intracoronary plaque using intravascular ultrasound and optical coherence tomography, could be complemented in future by the increased use of functional molecular imaging. Indeed, several radionuclide tracers are available such as ^{18}F fluorodeoxyglucose (FDG) and ^{18}F sodium fluoride that can detect and measure aspects of plaque inflammation when combined with imaging modalities such as positron emission tomography and computed tomography[592, 593].

In the days immediately following an acute coronary syndrome (ACS), culprit plaques are highly inflamed and may be most responsive to anti-inflammatory therapies[594, 595].

Features of both acute and chronic vascular inflammation are present in these plaques, and hence high-dose EPA supplementation may be beneficial in the early post-ACS period. A reduction in vascular inflammation could contribute to reduced coronary spasm and infarct size in the acute period, and a reduction in recurrent events in the long term.

7.5 Suggestions for future research

7.5.1 Elucidating the differential mechanistic effects of EPA compared to DHA

The experimental studies demonstrated a superior effect of EPA compared to DHA for the reduction of vascular inflammation. The mechanisms underlying these differences need to be further elucidated. Although structurally very similar, there is emerging evidence for different pharmacodynamic effects of these two molecules. Differences in effects on adhesion molecule expression with EPA compared to DHA, for example, may relate to differences in (1) the inhibition of the translocation of TLR4 into lipid raft domains, (2) the suppression of TAK1 phosphorylation, (3) the attenuation of NFκB activity by suppressing p38 and IκBα activation, and (4) the induction of the expression of the anti-inflammatory and NFκB-suppressor gene A20[246]. The differences in the magnitude of effects may partly relate to their concentration and assembly in cell membrane phospholipids; for example DHA is incorporated into (sphingomyelin and cholesterol-rich) membrane rafts with more than twice the affinity of EPA[249]. Hence, although much of the focus of the effects of fatty acids has been on their pharmacodynamics, differences in pharmacokinetics may also be relevant.

Future mechanistic omega-3 studies should use purified forms of EPA and DHA provided for sufficient duration to allow for incorporation into the cell membranes of interest, and with care taken to minimise oxidation.

7.5.2 Targeting blood omega-3 concentrations

The inverse correlations between blood omega-3 levels and markers of vascular inflammation demonstrated in the experimental studies provide further evidence for a dose-dependent effect. As with blood pressure and lipid targets, there is a rationale for establishing a therapeutic target for blood omega-3 levels. The Omega-3 Index, which is the percentage of EPA and DHA in red blood cell fatty acids, identified a cut-off value (< 4%) that associates with coronary heart disease mortality[213], but this index does not profile individual fatty acids. This is critically important, given the consistent demonstration of stronger correlations with EPA levels in the experimental studies. A sub-study of the JELIS trial examined the relationships between various plasma fatty acid concentrations and the risk of coronary events using a Cox proportional hazard model. High plasma EPA concentrations, but not DHA concentrations, were significantly inversely associated with major coronary events, with a hazard ratio of 0.71 for those with the highest tier of EPA concentrations[596]. Ohnishi et al examined this association further, and found that a ratio of EPA to arachidonic acid of > 0.75 also significantly inversely associated with major coronary events[597]. This provided further evidence for the prognostic value of blood omega-6:omega-3 ratios, with a focus on individual fatty acids.

To establish therapeutic blood omega-3 concentrations, future cardiovascular outcome trials will need to achieve both high omega-3 levels and a range of levels, and hence consider both the dose and bioavailability of omega-3 sources. For the currently ongoing STRENGTH trial, the treatment group has received a high dose of omega-3 (4 grams per day) provided as free fatty acids (omega-3 carboxylic acids), which do not require hydrolysis by pancreatic lipase and therefore have optimal intestinal absorption[235]. This large (>13000 participants recruited) randomised, placebo-controlled trial, is investigating the effects of omega-3 carboxylic acids on the risk of cardiovascular events in statin-treated patients with high

triglyceride and low HDL-C levels. Fatty acid levels in both plasma and red blood cells have been taken at baseline, during treatment, and at the end of treatment, and will be correlated with clinical events[235].

Future clinical studies should evaluate the therapeutic effects of achieving specific blood omega-3 concentrations in the context of the complete fatty acid profile in a range of clinical settings.

7.5.3 The concept of dysfunctionality

Fatty acids undergo metabolism, oxidation, shortening, elongation and conversion to other fatty acids after oral ingestion. Oxidation changes the structure and function of fatty acids, and longer chain polyunsaturated fatty acids are more susceptible to oxidation due to the higher number of double bonds present. It has been proposed that oxidised products in fish oils may attenuate their beneficial effects[598]. However, Mishra et al provided evidence that the anti-inflammatory effects of fish oil on the endothelium after cytokine stimulation result from the inhibitory effects of oxidised omega-3 fatty acids on NF-κB activation[252]. It is not clear at present to what extent conformational changes in omega-3 fatty acids after oral ingestion may lead to dysfunction, and further research is needed in this area.

The incorporation of fatty acids into cellular membranes is critical to their effects on downstream signalling. Fatty acids incorporate into the membrane rafts of the most abundant phospholipids, initially phosphatidylcholine and phosphatidylethanolamine[599]. Fatty acid uptake is an active process regulated by fatty acid transporters[600]. Hence, there may be pathophysiological conditions which reduce fatty acid uptake by fatty acid transporters, leading to a state of "fatty acid resistance" akin to insulin resistance. The conditions which may contribute to a fatty acid-resistant state and limit the functional effects of omega-3 supplementation would be worthy of exploration.

FOCUS IN demonstrated a strongly-positive correlation between blood levels of HDL-C and the gene expression of VCAM-1 and MCP-1 by stimulated HUVECs. The correlations were not a primary focus of the study, but occurred on a background of a recent report from two prospective cohort studies of extremely high levels of HDL-C being associated with increased all-cause mortality[489]. This paradoxical association raises the possibility of HDL dysfunctionality at very high levels. If this is the case, HDL may develop pro-inflammatory properties. The effects of HDL particles on the vessel wall in the setting of extreme HDL-C levels warrant further investigation.

Despite the advances that have occurred in our understanding of the impact of omega-3 fatty acids on atherogenesis over the last half-century, there remain significant knowledge deficits that will lead to fertile and exciting areas for continued investigation. The findings of this body of work provide mechanistic insights into the atheroprotective effects of omega-3 fatty acids, particularly EPA, and may inform the design of future clinical studies.

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