The impact of fish oil fatty-acids on post-prandial vascular reactivity.

Seán Christopher McManus B.A. (HONS)

Submitted to the Norwich Medical School, University of East Anglia in fulfilment of the requirements for the degree of Doctor of Philosophy, September 2014

Project supervisors:

Prof. Anne Marie Minihane
Prof. Aedín Cassidy
Dr. David Vauzour

Table of Contents

Abbreviations

HEPE	hydroxyeicosapentanoic acid
AF-2	activating function-2
AT-RvD1	aspirin triggered resolving D1
BADGE	bisphenol-A-diglycidyl ether
BSA	Bovine Serum Albumin
CAT	Cysteine aminotransferase
CBS	Cystathionine-β-synthetase
CHD	Coronary heart disease
CL	Cysteine lyase
COX	Cyclooxygenase
CPT-1	Carnitine palmitoyl transferase 1
CSE	Cystathionine-γ-lyase
CVD	Cardiovascular disease
DHA	Docosahexaenoic acid
DiHETE	Dihidroxy-eicosatrienoic acids
DiHDPA	Dihydroxy-docapentaenoic acid
DPA	Docosapentaenoic acid
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EpETE	Epoxy-eicosatetraenoic acid
EPA	Eicosapentaenoic acid

EpDPE	Epoxy-docosapentaenoic acid
e-NOS	Endothelial Nitric Oxide Synthase
GPCR	G-protein coupled receptor
GTN	Glyceryl trinitrate/nitroglycerin
HBM	Hepes Buffered Medium
HDL-C	High density lipoprotein cholesterol
HEPE	Hydroxyeicosapentaenoic acid
HEPTE	Hydroperoxyeicosa-tetraenoic acid
HUVEC	Human umbilical cord vascular endothelial cells
ICAM	Intercellular Adhesion Molecule 1
IL	Interleukin
JNK	c-JUN N-terminal protein kinase
LDL-C	Low density lipoprotein cholesterol
LC n-3 PUFA	Long chain n-3 polyunsaturated fatty acids
M-199	Medium-199
MAG- DHA	Monoacylglyceride-DHA
MAPK	Mitogen activated protein kinase
MMP	Matrix metalloproteinase
MLC-phosphatase	Myosin light chain-phospatase
MYPT	Myosin phosphatase targeting protein
NFκB	Nuclear factor-κB
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
RhoA	Ras homolog gene family, member A

RR	Relative risk
RvD1/2	Resolvin D1/2
RvE1/2	Resolvin E1/2
RXR	Retinoid X Receptor
SRC-1	Steroid receptor co-activating factor-1
LTB	Leukotriene-B
M-199	Media-199
PA	Palmitic acid
PAI-1	Plasminogen activator inhibitor-1
PD	Protectin
PGH	Prostaglandin
PKA/B/C	Protein Kinase A/B/C
PMN	Polymorphonuclear neutrophils
PPAR	Peroxisome proliferator activated receptor
PPRE	Peroxisome proliferator response element
SNP	Single nucleotide polymorphism
TXA	Thromboxane
VCAM	Vascular cell adhesion protein
VEGF	Vascular endothelial growth factor

Declaration

I confirm that the work presented in this thesis is my own, contributions from other individuals and institutions are fully acknowledged below.

Mrs. Ciara McCormack, of University College Dublin, Ireland, assisted in cell culture for the whole blood culture experiments.

Dr. Khader Awwad, of University of Frankfurt, Germany, conducted the quantification of epoxide and diol derivatives of EPA and DHA.

Abstract

Progressive loss of vascular reactivity and increased vascular tone with age are being increasingly recognised as significant cardiovascular disease (CVD) risk factors. The vasculature has emerged as a target for dietary strategies to modify these progressions. Our previous data suggest that inclusion of fish oil in a high-fat test meal improves postprandial vascular reactivity in healthy men. The primary aim of this project was to determine the individual effects of the fish oil fatty acids, eicosapentaenoic acid (EPA) versus docosahexaenoic acid (DHA) on post-prandial vascular reactivity and to identify underlying molecular mechanisms of these effects.

In this study, the acute effects of a single dose (4.16g) of EPA, DHA and placebo oil on postprandial vascular reactivity were determined in men categorised as being at a ≥ 1.5 relative risk of CVD in a double-blind randomised crossover trial. Measures of vascular tone and function, alongside an endothelium dependent measure of vascular reactivity, were taken along with blood samples, at baseline and at 4 hours in order to coincide with the anticipated peak plasma concentration of these fatty acids. These blood samples were utilised for assessment of biomarkers associated with changes in vascular tone and postprandial whole blood culture (WBC). WBC was conducted to investigate the effect of changes in the postprandial lipidome, dependent on EPA and DHA consumption, on cytokine production. Response to intervention according to genotype was also determined retrospectively.

We observed that DHA (p=0.04) but not EPA (p=0.06) significantly reduced Augmentation Index (AI) postprandially when compared to control. AI was reduced by 13.3% and 11.3% in response to DHA and EPA respectively. In addition, our data shows for the first time, that levels of the vasoactive n-3 PUFA epoxide and diol metabolites are subject to large changes post consumption of physiological levels of EPA and DHA. Our data also suggests there is wide inter-individual variability in circulating levels of these compounds, which may in part explain inter-individual responsiveness to EPA and DHA. We were not able to observe changes in plasma nitrite or H_2S levels postprandially, suggesting that EPA and DHA dependent changes in vascular tone may be mediated in part by their vasoactive epoxide and diol metabolites. However, whole Blood Culture experiments did not show a significant effect on any of the cytokines or growth factors investigated, before or after correction for BMI and AGE. Finally, our investigations of response to treatment by genotype suggested a novel interaction between the PPAR-γ rs1801282 polymorphism, DHA consumption and improvements in postprandial triglyceridemia.

The findings of this thesis emphasise the differential effects of EPA and DHA in the vasculature and the inter-individual responsiveness to these nutrients.

Acknowledgements

First and foremost, I want to thank Prof. Anne Marie Minihane, who was my main academic guidance throughout this process. Anne Marie has always had an open door and has been a tireless mentor throughout my time as her student. Anne Marie – thank you, I feel like I've grown as a scientist and as a professional under your guidance and beyond that, I've always enjoyed our conversations on science, our shared passion for Adidas Top Tens and beyond! It goes without saying that this work would not be possible without your guidance.

Dr. David Vauzour and Prof. Aedin Cassidy were my secondary supervisors during this project and I owe them many thanks for their help and insight throughout the process. Aedin was a huge help in her input on our clinical studies and David in his assistance and guidance in the lab. Thank you both!

On a personal note, I want to thank my parents, Josephine Leonard and Jim McManus, and Brendan Ball, for instilling in me the worth of education. Your support and love has put me in a position to follow my passions in life. My father was one of the first in his family to go to University and paid his way through by summers working in Philadelphia and Algeria. My mother finished her University education just 3 years ago as a mature student with dyslexia. Despite the challenges she finished with honours. Whenever, I need some inspiration it's you two that I think of. Brendan, you've been like a third parent to me and were my first exposure to scientific research. I love you all very much and I wouldn't be pursuing a PhD if it wasn't for you all. I also want to thank my partner, Megan Good, for her patience throughout these 3 and a half years. Doing a PhD can be an inherently lonely endeavour at times. You were always by my side and kept my spirits up the whole way through. It's very hard for me not to do my best when you're in my corner.

I would like to give thanks to the UEA Nutrition Department as a whole for providing me with the platform and the resources to do this work and particular thanks to a few special people who have helped me along the way namely: Noemi Tejera Hernandez (UEA), Ciara McCormack (Formerly of UCD, now Arryzyta), Catherine Wright (NHS, UEA Clinical Research and Trials Unit) and all the participants who participated in the clinical study.

Finally, I would like to dedicate this work to my son Tadhg McManus. It's exciting to think that someday when you're grown up you might read this. I wrote most of this in the first five months of your life. It's been a tiring five months, but by far the most exciting and happiest of my life. I love you – this is for you!

Chapter 1

Literature review: The impact of fish oil fatty acids on postprandial vascular reactivity

Chapter 1: Literature Review

1.1.   Cardiovascular disease (CVD) mortality and underlying pathology

Cardiovascular disease (CVD) remains the main cause of morbidity in the UK; in addition to this it claims an estimated 17.1 million lives a year worldwide (1). A central tenet of CVD is the presence of atherosclerotic plaque. Atherosclerosis (the process that leads to the build-up of these plaques) is characterised by arterial wall thickening as a result of cholesterol and other lipid build up. This process eventually leads to the formation of atherosclerotic atheromas - abscess like deposits of lipids, macrophages, calcium and fibrous connective tissue (see Figure 1.2) (2). Rupturing of the fibrous connective tissue, which forms a barrier between the atheroma and the blood in the artery, can induce a rapid platelet and clotting response. Critically, this response can cause a narrowing or occlusion of arteries, which may cause a loss of blood flow to organs downstream of this occlusion. This clotting response to rupturing of an atheroma underlies the pathology of stroke, heart attack and other related cardiovascular complications.

The initial events which occur in the development of atheroma are monocyte recruitment and migration into the sub-endothelial space which is mediated by a number of adhesion molecules expressed on the surface of vascular endothelial cells. The next step is the development of fatty streaks. The development of these fatty streaks is a critical stage in the progression towards the formation of atheroma and is dependent on differentiation of monocytes to macrophages which "ingest" low density lipoprotein cholesterol (LDL-C) and form the foam cells. The accumulation of foam cells in an artery leads to the formation of these fatty streaks. The final progression towards an atheroma is the formation of a fibrous cap. This fibrous cap is made up of collagen, which has its deposition mediated by the migration of vascular smooth muscle cells towards the intima of the artery, and extracellular matrix proteins. These processes are detailed below in Figure 1.1.
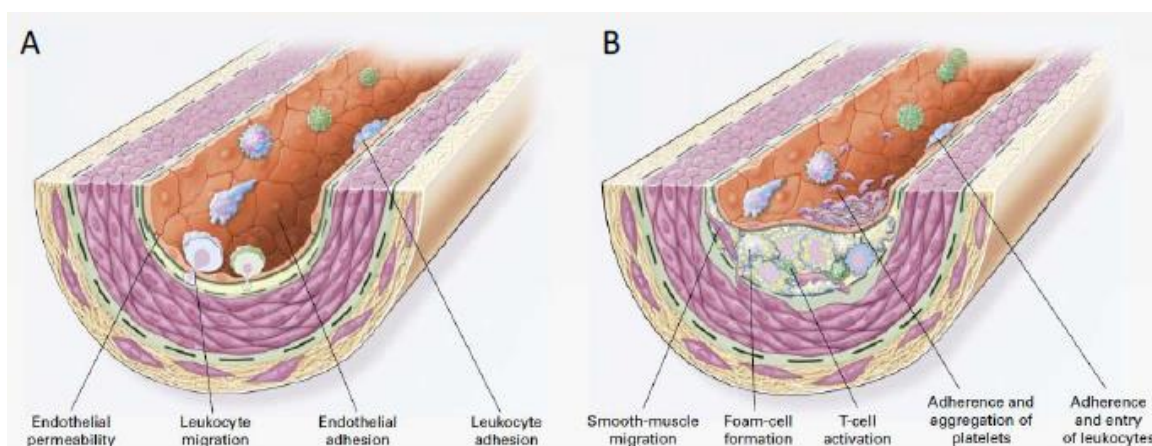
Figure 1.1 Diagrammatic representation of atherosclerosis. (Adapted from Ross 1999 (2)).

1.2. Vascular function and its impact on CVD pathogenesis

The highest level of evidence for linking modifiable elements of diet with reduced incidences of CVD events are long term randomised control trials which look at hard endpoints, such as incidences or mortality from CVD, such as myocardial infarction or stroke, or death from CVD. However, long term investigations which measure end points such as cardiovascular events and CVD mortality necessitate investigations of prohibitively long time spans with large numbers of participants. Accordingly, it is increasingly common for investigators to utilise surrogate markers that are associated with changes in risk. These measures typically measure early events associated with changes in blood vessel physiology (3), such as vascular and endothelial function. Vascular function can be broadly defined as a capacity of blood vessels to adequately modify and control blood supply to organs and tissue as according to local demand. Dysfunction of the vasculature can be mediated through structural changes or earlier functional changes to blood vessels, which can include changes to the endothelial cell layer which line blood vessels. Alterations in vascular tone, or the degree of constriction of a blood vessel, is one of these early changes; the impact of which is increased vascular resistance to blood flow. These changes are increasingly recognised as a significant early stage CVD risk factor and highly predictive of future CVD events. Endothelial dysfunction, which will be discussed in more detail later in this review, is also known as a key early step in the manifestation of overt aspects of CVD.

There are a number of validated and clinically accepted measures of vascular and endothelial dependent vascular function and vascular tone currently available to researchers. Examples of such, which will be utilised in the clinical trial undertaken as part of this project, are Pulse Wave Analysis, Pulse Wave Velocity (PWV) and Endopat, which are used to assess vascular compliance, arterial stiffness and endothelial function respectively. Another key methodology utilised in this context is flow mediated dilation (FMD). Flow mediated dilation is considered the gold standard of endothelium function (4), which is typically measured by imaging of the brachial artery, before and after a 5 minute occlusion, with ultrasound technology. This allows the user to establish an index which is indicative of this conduit artery's capacity to dilate in response to hyperaemia. FMD is noted as being prone to poor reproducibility unless strict guidelines are followed (5), and requires a skilled and trained user in order to guarantee rigorous, reliable and reproducible results. Due to this consideration, use of FMD was beyond the scope of this PhD, due to the length of time required to establish the technique and train users on the proper use this methodology.

Endopat is a measure of endothelial function (see section 2.7.3.3) which has been shown to correlate with multiple known CVD risk factors, including obesity, diabetes mellitus and ratio of total to high

density lipoprotein cholesterol (HDL-C) (6). Endopat Reactive Hyperaemic Index (RHI) measurement is obtained by measuring the increase in blood flow in response to the stimulus of shear stress. This shear stress is caused by occlusion of the brachial artery, subsequent release of the occlusion causes a sudden increase in blood flow to the finger tips. The amount of hyperaemia in response to this stimulus is indicative of the capacity of the endothelium to produce vasodilatory substances. Endopat has also been shown to be predictive of cardiac death, myocardial infarction, revascularization or cardiac hospitalization (7). Specifically, individuals with ln(RHI)<0.4 (or RHI<1.04) had a 7 year rate of 48% for incidences of cardiac death, myocardial infarction, revascularization or cardiac hospitalization compared to 28% for individuals with ln(RHI)>0.4.

PWV is the measurement of time taken for a pulse wave to travel from one anatomical site to another, the current "gold standard" considered to be the carotid and femoral sites. PWV progressively worsens with age, with unwanted increases in PWV reflective of the progression of arterial stiffness. The progression of arterial stiffness typically occurs due to elastic fibres fraying due to continual mechanical stress and alongside other changes to the structure of blood vessels, with chronic modifications to endothelium produced vasodilatory compounds likely having an impact on this process. A meta-analysis has shown PWV to be an independent predictor of CVD risk (8), with each 1m/s increase in PWV correlating with a 15% increase in risk of each of total CV events, CV mortality, and all-cause mortality. The Vicorder equipment utilised to undertake PWV measurements also allows several other non-invasive measurements to be taken simultaneously including augmentation index (AI), an indirect measure of the enhancement of central aortic pressure by reflected pulse waves. The Augmentation index measurement is likely to be affected by alterations of vascular smooth muscle tone in peripheral muscular arteries. AI has also been shown to be associated with increased European Society of Cardiology (ESC) risk levels (9). Based on the results of this study by Nurnerberger *et al*, a 4% increase in AI would correlate to an equivalent 1% increase in CVD risk as per the ESC risk levels. Critically, AI has also been shown to be modified acutely upon administration of vasodilatory compounds, such as nitro-glycerine, and vasoconstrictory agents, such as angiotensin II (10).

Also of consideration, is the measurement of blood pressure, which is associated with increased vascular tone and systematic vascular resistance and is the most widely utilised and most widely recognised predictor of CVD among these measures. Vascular tone alone is not the only determinant of blood pressure. Blood pressure is also dependent on the physiology of conduit arteries, peripheral arteries and the heart itself, heart rate, strength of ventricular contractions and many other factors.

1.3. Inflammation in CVD

It is known that the innate inflammatory response plays a critical role in the pathogenesis of CVD (11). As mentioned in section 1.1, monocytes and macrophages play a key role in the pathogenesis of atherosclerotic plaque. A key consideration here is the infiltration of monocytes into the sub-endothelial space by recruitment through adhesion molecule expression on endothelial cells. Subsequently, the accumulation of monocytes and differentiation to macrophages leads to the formation of foam cells, which play a central role in the formation of fatty streaks in the vasculature.

The production of inflammatory cytokines is central to initiating, and perpetuating, the sequence of events that leads to the eventual formation of these fatty streaks. A key effector protein in this process is the redox sensitive transcription factor nuclear factor-κB (NFκB). NFκB is expressed and active in most cell types. In this context its activation in the endothelium is important due to its role in upregulating the expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM1) and vascular cell adhesion molecule-1 (VCAM), which leads to the recruitment of monocytes to the site of expression.

This NFκB initiated recruitment of monocytes plays a key role in the development of fatty streaks and ultimately, atherosclerotic plaques. Subsequently, the accumulation of monocytes and differentiation to macrophages leads to the formation of foam cells, which accumulate to form fatty streaks in the vasculature. These monocytes and macrophages contribute to local production of pro-inflammatory cytokines such as IL-4, IL-6, IL-8 and TNF-α which propagate the inflammatory response and in turn lead to recruitment of more monocytes. Dysregulation of production of IL-10, an anti-inflammatory cytokine, by monocytes is also an important consideration in this context and can lead to increases in NFκB activity (12). It is also worth considering that cytokine concentrations in the systemic circulation, are also likely to play a role and increased plasma concentrations can be indicative of CVD risk (13).

Ultimately, the activation of the innate immunity response through NFκB and production of pro-inflammatory cytokines is meant to act as an important response to cellular injury; however, in the context of CVD this process can become dysregulated and lead to collateral damage in the vasculature. This damage can manifest itself as stiffened arteries, which is a product of both mechanical fatigue and overexpression of matrix metalloproteinases (MMPs). These MMPs are induced as part of the above described cellular injury response by monocytic production of factors such as vascular endothelial growth factor (VEGF) (14). As mentioned previously, increased monocytic recruitment and the formation of fatty streaks are also a manifestation of dysregulation of the immune response and this plays a central role in the development of atherosclerosis.

1.4. An introduction to the fish-oil long chain n-3 polyunsaturated fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)

Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) are n-3 polyunsaturated fatty acids (commonly referred to as omega-3 fatty acids), typified by the presence of a double carbon-carbon bond at the third carbon from the methyl-end of their carbon chains (or at positions 18 and 20 respectively, as per IUPAC nomenclature, see figure 1.4). As can be seen in Figure 1.3, both are carboxylic acids which differ in chain length (DHA possesses a 22 carbon chain and EPA a 20 carbon chain), by the number of cis-double bonds (6 in DHA and 5 in EPA) and the positioning of these cis-double bonds. The most common dietary source of these fatty acids are cold water oily fish, such as salmon, kipper, trout, mackerel, anchovies and sardines which have high concentrations of EPA and DHA in their tissues due to the algae and plankton which these fish consume. Consumption of these fish typically provides 1.5-3.0g of EPA and DHA per portion (15). In contrast, white fish such as cod and halibut, primarily store EPA and DHA in the liver, which is used to provide EPA and DHA rich cod and halibut liver oil.
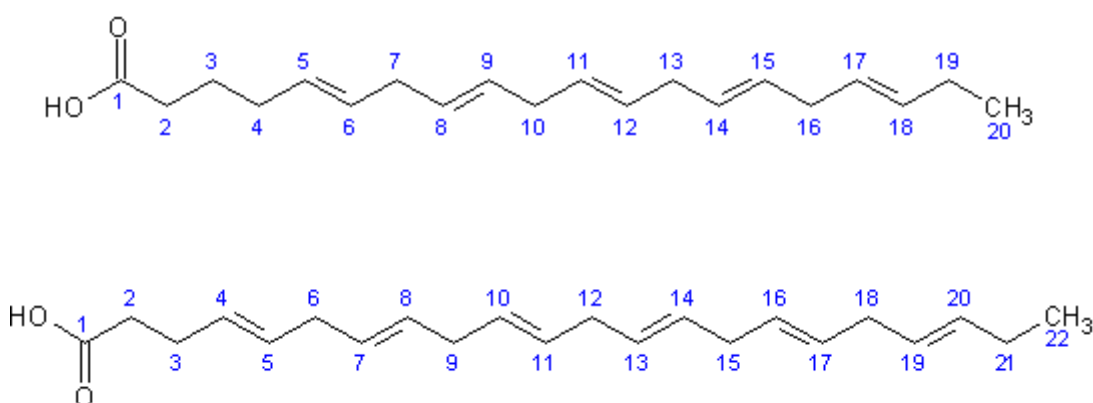
Figure 1.4. Above – EPA (eicosa-5,8,11,14,17-pentenoic acid) and below - DHA (docosa-4,7,10,13,16,19-hexa-enoic acid).

In human metabolism, DHA can be synthesised from EPA with docosapentaenoic acid (DPA) as an intermediate. Synthesis via DPA occurs via an initial chain elongation to 24:5n-3 acid, which is in turn desaturated at position 6 to yield a 24:6n-3 acid. The chain of this 24:6n-3 acid is then shortened via beta-oxidation to yield DHA (16, 17). EPA can also be synthesised in human metabolism via α-linolenic acid. However, it has been shown that the pathways capable of converting other fatty acids (namely α-linolenic acid) to EPA and DHA are non-efficient (18-20) (see Figure 1.4.1 for a diagrammatic depiction of EPA and DHA synthesis). Accordingly, it is known that improvement in EPA and DHA status is best achieved through increased intakes.
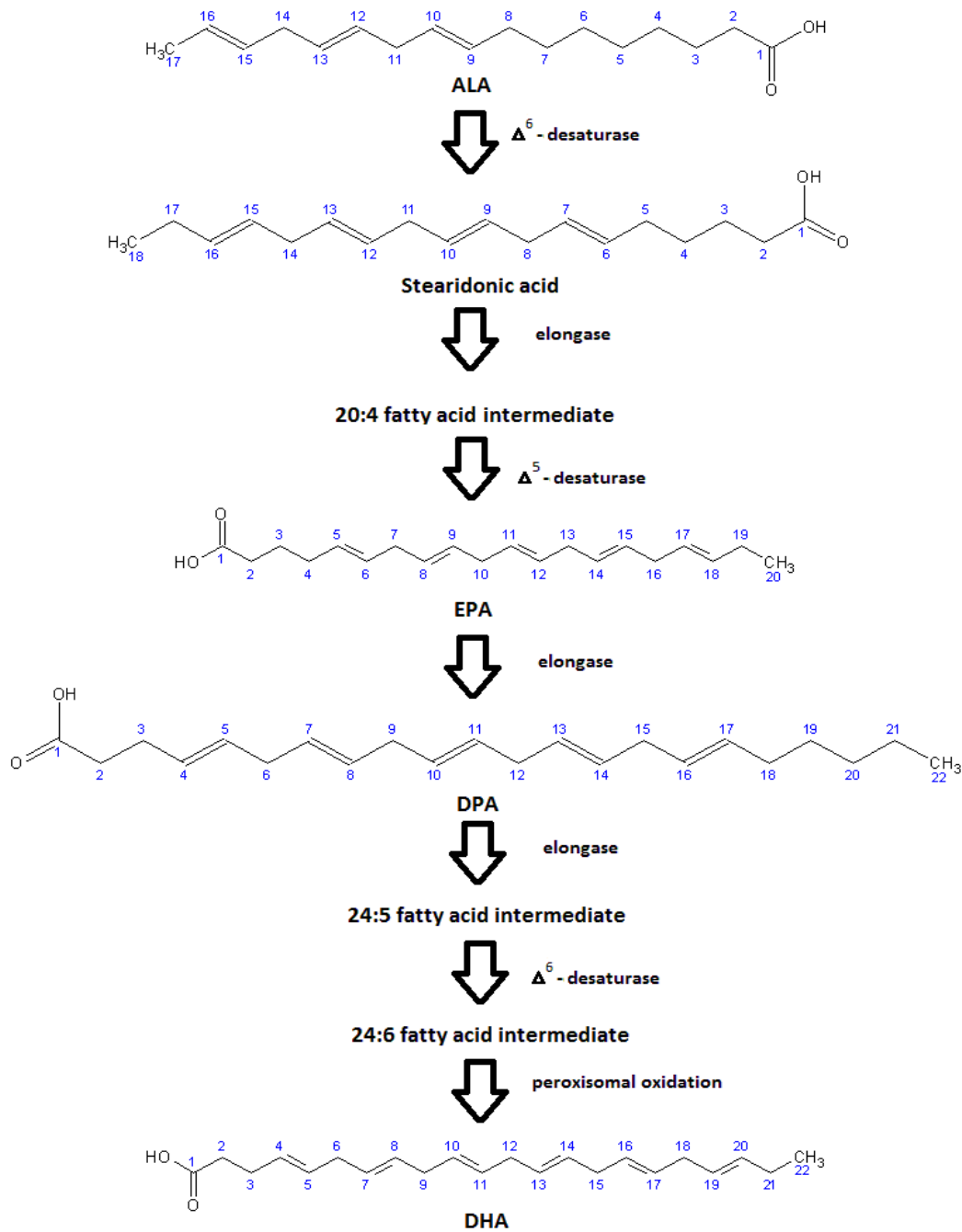
Figure 1.4.1. Pathway for human EPA and DHA synthesis.

Currently, the recommended level of EPA and DHA in the United Kingdom is a minimum of 450 mg per day (21), a recommendation which is largely based on the cardio-protective actions of EPA and DHA as detailed in section 1.5. The equivalent of 450mg per day of EPA and DHA can be achieved by consuming one portion of oily fish per week. EPA and DHA intake in adults of 19-64 years of age in the United Kingdom is estimated as 244mg per day, about 50% of the recommended intake (21). It is worth noting that this average intake is highly skewed with skewed with approximately 70% of the population being non-oily fish consumers, with intakes <50mg per day (22, 23).

1.5. Impact of fish oil fatty acids on CVD risk and incidence

The development of cardiovascular disease is a complex and dynamic process which is affected by a large number of potentially modifiable variables. Diet (24, 25) and in particular, the lipid composition of diet, has long been understood to be a modifiable aspect of CVD pathogenesis (25). It has been known for over 40 years that increased intakes of fish derived long chain n-3 fatty polyunsaturated fatty acids (LC n-3 PUFA) are associated with improved blood lipid profiles and also decreased incidences of cardiovascular disease (26). Since these initial observations, the effect of LC n-3 PUFA intake on CVD risk has been studied extensively. For instance, a number of large randomised control trials have demonstrated these CVD risk reducing effects of EPA and DHA – including the JELIS (27) and GISSI (28) trials. The JELIS and GISSI trials recruited 18,645 and 11,324 participants respectively. GISSI, a secondary prevention investigating the effects of LC n-3 PUFA (1 g/day) , vitamin E (300mg/day), both , or control (no treatment) for 3.5 years showed decrease in risk of cardiovascular death by 17% by two-way analysis and 30% by four-way analysis for LC n-3 PUFA. The JELIS trial was a primary prevention trial conducted in Japan, which investigated the effects of EPA ethyl esters on Japanese hypercholesterolemic participants. In JELIS participants were randomised either to EPA (1.8 g/day) and statin treatment or statin treatment alone. The results showed reductions of 24% in the frequency of unstable anginas. Furthermore, major coronary events were reduced by 19% in the EPA treated group relative to the control group. It is important to note that the effects of EPA in this trial were evident even with concurrent statin administration and in the Japanese population, who habitually consume high amounts of LC n-3 PUFA (estimated as 750 mg/day on average).

A number of other studies, summarised in pooled meta-analysis, have shown EPA and DHA intake to be an important modifiable aspect of diet which influences CVD risk (29-33). For instance, meta-analysis of randomized controlled trials undertaken in populations with coronary heart disease have shown that polyunsaturated n-3 fatty acids (average intake of 3.8g/day) are associated with a 20% decreased risk of myocardial infarction and 30% decreased risk of fatal myocardial infarction. These effects were accompanied by an average 20% decrease in blood triglyceride levels (30). Meta-analysis has also shown that studies which recruited healthy populations showed an average 37% decrease risk of mortality from coronary heart disease (CHD) with an average daily intake of 566 mg/d of EPA and DHA (33), indicating that lower doses may also decrease CVD risk. However, it is worth noting that there isn't currently a consensus among clinical trials that have investigated the effect of EPA and DHA on CVD outcomes. A recent large scale trial, the Risk and Prevention Study, did not show an effect for 850mg per day of EPA and DHA over 5 years on CVD morbidity or mortality in a population of individuals (n=6250 per group) with multiple CVD risk factors (34). However, it should be noted that

due to the rate of stroke and myocardial infarction being lower than expected, this study may have been underpowered. On a more general level, the lack of consensus in these studies may be attributable to variances in dosage, ratio of EPA and DHA administered, endpoints measured and populations investigated. Evidence in the literature suggests that EPA and DHA might be particularly efficacious in secondary prevention post MI (27, 28) and possibly that doses of approximately 2g per day, or greater than 2g per day, may be required to reduce incidences of CVD events (29, 30).

It is likely that the positive effects seen in these randomised controlled trials are mediated by the anti-arrhythmic (35), hypolipidemic (36-39), improved vascular function and blood pressure lowering (40), anti-inflammatory and plaque stabilising (41) and cholesterol profile modulating (42, 43) effects of LC n-3 PUFA. As previously suggested, there is considerable variation in the LC n-3 PUFA dosage needed to exert these effects. For example, LC n-3 PUFA intake as low as 0.5g/day may have potential anti-arrhythmic effects (44, 45). Whereas studies have shown 4g/day of DHA but not EPA to lower both diastolic and systolic blood pressure (SBP) by approximately 5% in hyperlipidemic individuals. Meta-analysis has shown a median dose of 1.68g/day of DHA to increase HDL-C by an estimated 5% (43). Doses in the range of 0.7g to 1.8g/day of EPA and DHA have been shown to lower fasting TAG typically by 10-20% (39, 46), but also as large as 50% (39); with evidence suggesting a larger effect for DHA in this regard (47). Accordingly, low doses of EPA and DHA (0.5g/day) mediate anti-arrhythmic effects, moderate doses (1-2g/day) are known to modulate lipid profiles whereas higher doses (>3/4g/day) of EPA or DHA may be needed to modulate vascular function and blood pressure. It should be noted that limited studies have been conducted to assess the effects of lower doses in this regard.

1.6. A role for dietary fat, and EPA and DHA, in the modulation of innate inflammatory responses

A number of factors can contribute to the establishment of a dysregulated inflammatory state in the vasculature including exposure to biological and chemical agents, genetic, diet and lifestyle factors. Diet in particular is a well-documented modifier of chronic inflammation in the vasculature. It is thought that amount and the type of dietary fat plays a role in modifying innate inflammatory status (48). For instance, certain common fatty acids, such as palmitic acid and oleic acid, are known to activate Toll-like receptor 4 (TLR4) signalling and increase inflammatory cytokine production (49). TLR-4 plays a fundamental role in innate immunity activation in CVD (see Figure 1.6) (50), and can be involved as a trigger for NFκB activation. Accordingly, this mechanism is one, among many, that contributes to dietary fat's capacity to modify inflammatory state in CVD progression. Another important consideration in this context, is that DHA and in particular EPA, have been indicated to have a positive effect on inflammatory status (51). This is thought to occur through the action of the eicosanoid metabolites, which will be discussed in further depth in section 1.9.4 of this review.
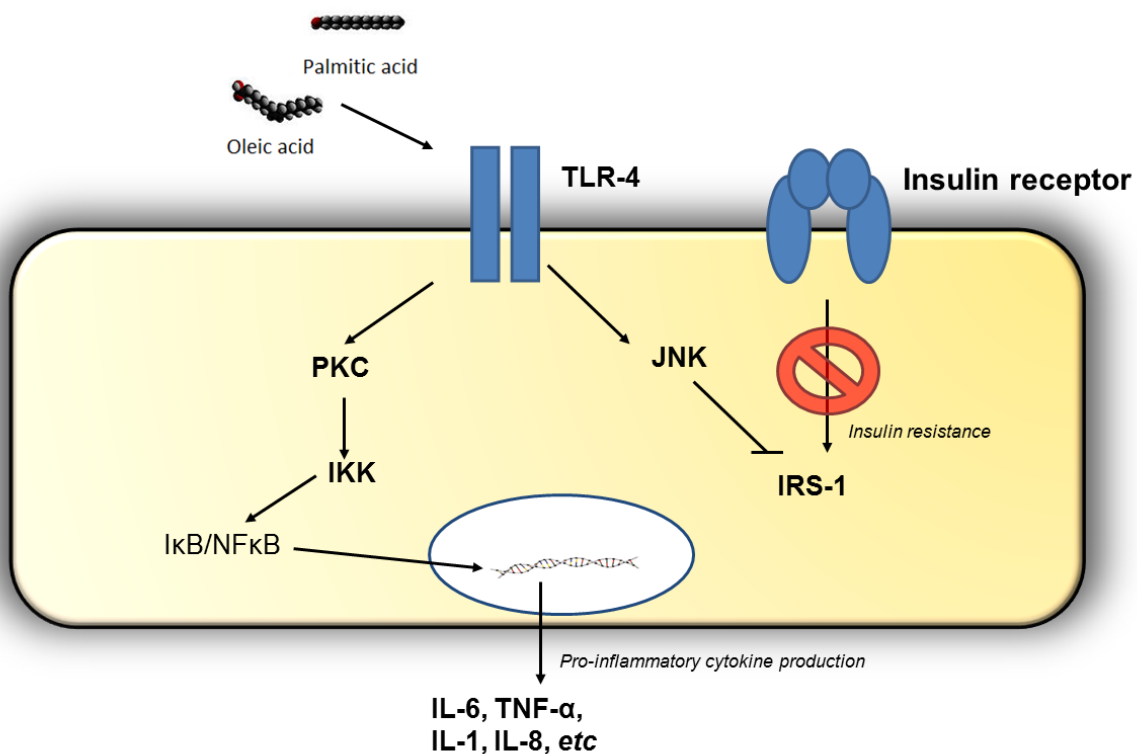
Figure 1.6. An overview of the interaction between TLR-4, inflammatory cytokine production and dietary saturated and monounsaturated fatty acids.

Examples of EPA, and to some extent DHA's capacity to modify inflammatory status can be seen in studies of monocytic cytokine production. For instance, studies on monocytic cytokine production in LPS stimulated whole blood culture (WBC) models have indicated that 8 weeks of fish-oil consumption (3.1g per day of EPA and DHA, ratio of EPA:DHA not provided) yield modest reductions in IL-6 (52). *In-vitro* studies have indicated that EPA (114µM) rich media modifies TNF-α transcription through modification of NFκB activation in a macrophage cell line (53). Similar experiments conducted subsequent to this observation have indicated a similar effect when macrophage cultures are exposed to EPA and DHA (54). Finally, a 90 day supplementation of 6g/day of DHA in 11 healthy men has been shown to decrease IL-1β and TNF-α secretion in peripheral blood mononuclear cells isolated post intervention (55). However, the relative effects of EPA and DHA in their capacity to modify monocytic cytokine production have not been well described in the literature. Furthermore, the majority of experiments in this context have investigated the effects of longer term EPA and DHA supplementation (56) and it remains unknown how postprandial alterations in the lipidome may affect *ex-vivo* cytokine production.

1.7. EPA and DHA's effects on vascular and endothelial function

1.7.1. The impact of EPA and DHA on BP and vascular tone: Evidence from human studies.

A number of studies are suggestive of the LC n-3 PUFA having a capacity to modulate vascular tone, both in response to chronic habitual consumption of these fats and in the acute state. Longer term studies have shown that over a 6 week intervention period DHA (4g/day) but not EPA reduced ambulatory blood pressure and heart rate in 59 mildly hyperlipidemic men (40) relative to placebo. Specifically, the DHA intervention resulted in a significant 4.5% reduction in systolic and diastolic blood pressure; this equated to a 5.8/3.3mmHg decrease in systolic and diastolic blood pressure respectively.

Further to this, a 6 week intervention in which hyperlipidemic obese men were randomised to receive either 4 g/day of purified EPA, DHA (equivalent to the n-3 fatty acid content of approximately 2 ½ servings of oily fish per day) or olive oil showed DHA to be more efficacious in enhancing vasodilator mechanisms, as measured via forearm blood flow, relative to placebo (57). Specifically, these investigations showed that DHA supplementation improved vasodilatory responses to acetylcholine with L-NNMA and nitroprusside, suggesting that this effect is mediated in an endothelium independent manner. DHA supplementation also attenuated vasoconstrictory responses to norepinephrine but not to basal blood flow responses to L-NMMA infusion (an eNOS inhibitor). Taken together this evidence is suggestive of these vasodilatory effects of DHA being independent of the endothelium.

With regards to current understanding of the post-prandial effects of fish-oil on vascular tone, data generated by our research group indicates that inclusion of fish oil (2.16g EPA and 3.24g DHA) in a high-fat test meal improves postprandial endothelium independent vasodilatory responses (by approximately 20%) in 25 healthy men (58). The effect for endothelium dependent vasodilatory responses did not reach significance in this study. Other studies have suggested that inclusion of 1g of EPA and DHA preserves endothelial function in healthy men and women, as measure by FMD, after a high fat meal. Other studies on AI responses indicate that inclusion of 2.0g of EPA and 2.7g of DHA in a high fat meal (30g of palm oil and soybean oil in a 4:1 ratio in the control meal, with control fat being displaced by EPA and DHA in the test meal) improve postprandial measures of AI in healthy males and females (59). Finally, a study has also shown improvements in postprandial FMD response to a high fat meal containing EPA (2.8g) and DHA (1.2g) in diabetics at risk of CVD complications (60). However, these effects were also observed post consumption to a high fat MUFA control meal containing α-linolenic acid. In short, the evidence in the literature is strongly supportive of an effect for meals

containing mixed doses of both EPA and DHA to improve vasodilatory responses in the postprandial state; however, it is not clear whether this effect is mediated by endothelium dependent or independent mechanisms.

With regards the individual effects of EPA and DHA in the postprandial state, acute studies which have compared the effects of EPA and DHA have shown systemic vascular resistance to be decreased by 5% in 22 healthy males in the postprandial state during exercise, but not at rest, when preceded by 4.7g of DHA but not EPA (61). Furthermore, a recently published study has investigated the effects of inclusion of EPA and DHA (5g in a 4:3 ratio) relative to the effects of inclusion of DHA (5g) only in a meal high in MUFA (75g of total fat) in 16 healthy males (62). This study showed no effect for either the EPA and DHA meal, or the DHA meal, when compared to control on AI. A trend for greater decreases in Digital Volume Pulse Reflective-Index (indicative of changes in vascular tone of peripheral resistance arteries) was observed postprandially for the DHA meal; however, these effects did not reach significance.

Accordingly, given the evidence provided from longer term studies and post-prandial studies which have investigated the effects of EPA and DHA on cardiovascular hemodynamics during exercise, we anticipate that DHA will be more efficacious than EPA in improving postprandial vascular tone. Since individuals tend to spend the majority of the day in the post-prandial state (63) and impaired vascular tone is intrinsically associated with the accelerated progression of atherosclerosis (64), it is possible that the impact of EPA or DHA on the postprandial response may correlate with the effects of these fats when habitually consumed.

In summary, there is a growing body of evidence to suggest an effect for the LC n-3 PUFA in modulating vascular tone, and in turn BP. When considering both the chronic and acute data, the balance of the literature is suggestive of this effect being mediated in an endothelium independent manner. However, there is not a consensus as a number of studies have shown modulations to endothelium dependent measures of vascular tone. Furthermore, there remains a paucity of information on the individual effects of EPA and DHA on the post-prandial response in the vasculature in particular in individuals at risk of CVD. This thesis therefore aims to expand on these findings by comparing the effects of consuming a DHA rich meal to an EPA rich meal and control meal on postprandial vascular function and tone in a population at an increased risk of CVD.

1.7.2. Regulation of endothelial function by NO and H$_2$S

Endothelial dysfunction is a key step in the progression of both macro and microvascular disorders such as atherosclerosis, stroke and hypertension (65, 66). Endothelial dysfunction can be broadly defined as an imbalance in the production of vasodilatory and vasoconstrictory paracrine signalling molecules, however, it can also be characterised by modifications to adhesion molecule expression, cytokine release, and ROS production. Two vasodilatory signalling molecules whose productions are perturbed in endothelial dysfunction are Nitric Oxide (NO) and the novel vasodilatory signalling molecule, Hydrogen Sulfide (H$_2$S).

NO is a key mediator of vasodilation and insufficient NO production or bioavailability is known to be a central tenant of endothelial dysfunction. Further to this, NO also has been shown to exert important anti-inflammatory and anti-thrombotic properties (67). NO is released by the endothelium and decreased production has been indicated in the pathogenesis of cardiovascular diseases (68, 69). NO is produced by Nitric Oxide Synthase (NOS) which catalyses the formation of NO from L-arginine. NO then migrates to vascular smooth muscle cells where it acts on soluble guanylyl cyclase, which acts to increase the production of cyclic-guanosine monophosphate which in turn activates Protein Kinase G. The downstream effect of this is the closing of vascular smooth muscle cell calcium channels causing decreases in intra-cellular Ca^{2+} and accordingly, decreases in smooth muscle contraction (see Fig. 1.7.2) Production of NO in the endothelium is mostly mediated by NOS III (eNOS), however NOS II (inducible-NOS or iNOS, also known as high-output NOS) is also known to contribute to NO production in the endothelium. However, increased iNOS expression and activity, in contrast with that of eNOS, can be associated with increased inflammatory states in the vasculature (70).

It should also be noted that hydrogen sulfide (H$_2$S) has emerged as a novel and important endothelium produced vasodilator (71, 72). H$_2$S is produced from L-cysteine by at least four separate enzymes: cystathionine-β-synthetase (CBS), cystathionine-γ-lyase (CSE), cysteine aminotransferase (CAT) and cysteine lyase (CL). CSE-mediated H$_2$S biosynthesis is known to occur in vascular endothelial cells of mice (73) and in human umbilical cord vein endothelial cells (HUVECs) (74). Of these CSE and CBS mediated H$_2$S production are understood the best and are thought to be the predominant producers of H$_2$S in the vasculature (75). With regards H$_2$S*CVD interactions, it has been shown that blood levels of H$_2$S are lowered in individuals with coronary heart disease, and suggested that lower H$_2$S levels reflect increases in disease severity (76). Further to this, plasma H$_2$S levels have been shown to be inversely associated with diastolic and systolic blood pressure in men (77). Associations between

adiposity and diabetes, which are known CVD risk factors also supports a role for H_2S in the modifying the progression of cardiovascular disease.

H_2S modulates vascular tone through stimulation of plasma membrane K_{ATP} channels in vascular smooth muscle cells. Stimulation of these K_{ATP} channels modulates the resting membrane potential of cells and this modifies the tendency of these vascular smooth muscle cells to contract, leading to net increases in vasorelaxation (78). It is currently unknown whether these effects are mediated directly or by derived species that also exist at physiological pH. Species derived from H_2S include HS^- and S^{2-}. It is unlikely S^{2-} mediates any of these effects, as it is primarily present at high and non-physiological pH ranges; however it is possible that HS^- may mediate some of these effects. As it is currently unknown if the H_2S derived species are responsible for the observed bioactivity of H_2S, 'H_2S' is used to encompass the sum of H_2S derived species present under physiological conditions.
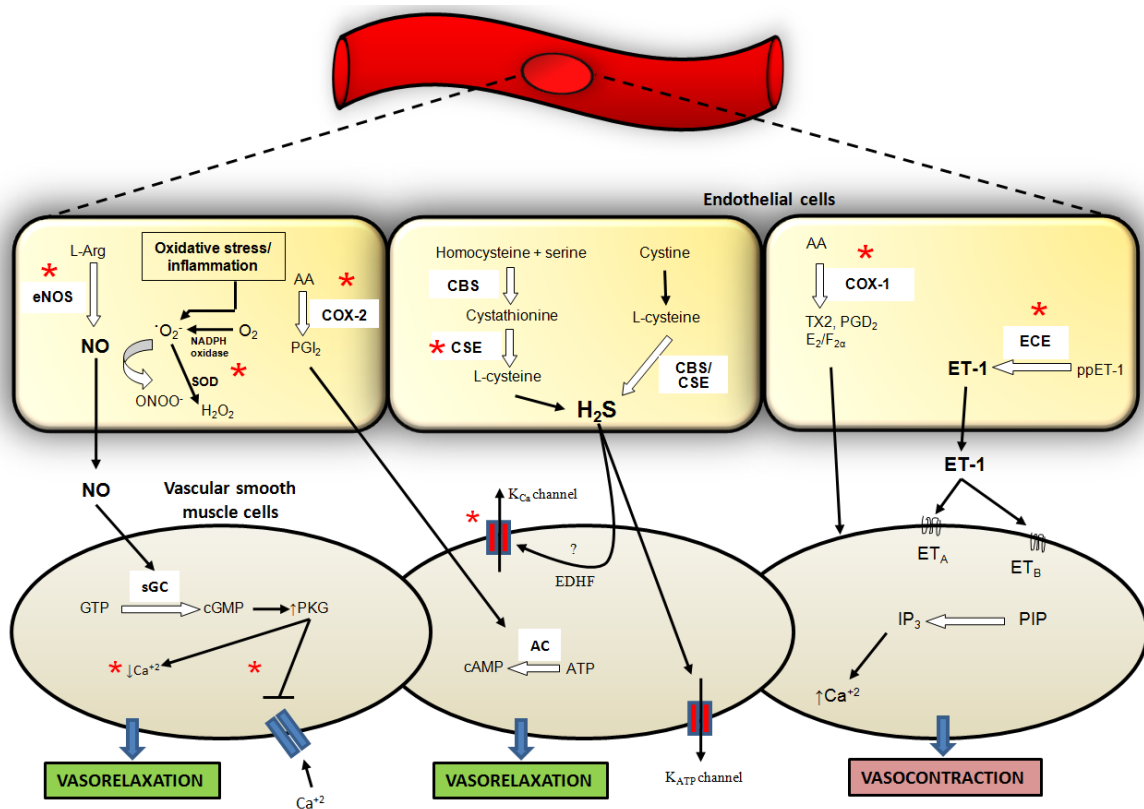
Figure 1.7.2. Endothelium-derived nitric oxide synthesis and action (on left), along with endothelial derived H_2S synthesis (middle) and COX-1 and ET-1's vasoconstrictor effects.

1.7.3. Effect of EPA and DHA on endothelium produced vasodilators

As discussed in section 1.6, there is some evidence from clinical trials to suggest that EPA and DHA have beneficial effects on measures of vascular tone dependent on NO production (57, 58, 79). Investigations on plasma levels of markers of NO metabolism have indicated an increase in total plasma NOx (a surrogate marker for NO production) in response to consumption 3.24g of DHA and 2.16g of EPA. Furthermore, *ex-vivo* investigations on HUVECs showed an increase in eNOS mRNA expression in response to exposure of triacylglycerol rich lipoproteins derived from plasma after consumption of 3.24g DHA and 2.16g EPA (58). However, another human study showed no effect on nitrite, nitrate or NO_x plasma concentrations in response to consumption of 5g of a mixed EPA and DHA dose or 5g of DHA within a high fat meal (62). A further study investigating the effects of inclusion of EPA (5g) in a high fat meal showed that both control and EPA meals decreased total NO_x with no significant difference between meals (80).

A number of investigations in cell culture models have also investigated the effects of EPA and DHA on NO production via eNOS. For example, *in-vitro* studies on retinal microvascular endothelial cells have indicated that DHA (50µM) but not EPA increases NO production (81), and that this effect is mediated by increases in phosphorylation of the serine 1179 residue on e-NOS and also localisation of e-NOS away from caveolin-1 containing caveolae. Caveolin-1 is a well-recognised negative regulator of e-NOS; it regulates these effects by limiting calcium-calmodulin binding (82), which is known to effect e-NOS activity (83). Accordingly, modifications to e-NOS/caveolin-1 co-localisation or caveolin-1 activity or expression have important implications for e-NOS activity. Other *in-vitro* studies have suggested that EPA but not DHA may be responsible for activation of eNOS in porcine coronary artery endothelial cells by mediating phosphorylation at serine1177 (84). Finally, a study on a permanent HUVEC cell line (EA.hy926) has shown that EPA (50µM) improves histamine induced eNOS activity whereas DHA (50µM) has a deleterious effect. In this study, EPA (90µM) but not DHA (90µM) was sufficient to correct lyso-phosphatidylcholine dependent decreases in eNOS serine 1177 phosphorylation. Neither treatment effected basal phosphorylation status in contrast to studies on microvascular endothelial cells. In summary, *in-vitro* studies seem to indicate some role for EPA and DHA on modulating eNOS activity. However, there is a lack of consensus, and a degree of confusion, within the literature with regards to the individual effects of these fats on eNOS activity and NO production with some studies indicating EPA to have an effect but not DHA and vice-versa. This may be reflective of the array of cell culture lines used in these investigations, which somewhat hampers comparison between studies.

Although, the impact of EPA and DHA on H_2S production is unknown, a recent study has reported an activation of CSE (cystathionine-γ-lyase) in response to DHA-rich tuna-oil supplementation in lung tissue of Sprawgue-Dawley rats (85). Furthermore, given that CSE utilises homocysteine as a substrate to produce H_2S (75) and that consumption of fish derived LC n-3 PUFA have been shown to be associated with decreases in plasma homocysteine (86), this offers a tentative link to suggest that fish derived n-3 fatty acids may interact with the expression or activity of CSE. To date, no human studies have investigated if H_2S plays a role in mediating EPA or DHA dependent changes in vascular tone.

A growing body of evidence also suggests cross-talk and involvement of H_2S in the in regulation of NO signalling pathways (87). Our human study will investigate the impact of EPA and DHA meals on both NO and H_2S plasma levels to establish efficacy of these fats in modulating the concentration of these blood borne modulators of vascular tone.

1.7.4. Effects of EPA and DHA on Nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) expression and activity

It has been proposed that an ability to modulate oxidative stress status may be responsible for EPA and DHA's effects on CVD progression. This is an important consideration as oxidative stress is linked with the development of atherosclerotic lesions (88) and increased production of ROS is known to cause sequestering of vasoactive compounds such as NO. Animal models support the theory of an effect for the LC n-3 PUFA in modulating oxidative stress as mice models of atherosclerosis have shown reduced oxidative stress load in response to diets containing EPA and DHA (89). As hyperlipidemia is associated with increased levels of oxidative stress, it is possible that the hypolipidemic effects of EPA and DHA (36-38) may mediate a decrease in oxidative stress.

These decreases may also occur via decreases in Nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) expression and activity. NADPH oxidase is the major source of the superoxide anion in the vasculature and a decrease in its production would be associated with decreased oxidative stress and also decreased NO degradation and hence, increased NO bioavailability. When the NADPH Oxidase p47[phox] component becomes phosphorylated, it's translocation to the plasma membrane is promoted to form an active enzyme complex (90).

A number of studies have shown a role for DHA and to some extent, EPA in modulating NADPH oxidase activity and expression. For instance, a study has shown that endothelial cells exposed to DHA (in concentrations up to 25µM) have decreased translocation of p47[phox] to the plasma membrane and accordingly, decreased NADPH oxidase activity (91). It is also interesting to note that work by this group has shown DHA's effects to be more potent than EPA (92, 93). Evidence from studies investigating the effects of EPA and DHA (50µM) on micro vascular endothelial cells have also shown

decreased ROS production in response to EPA and DHA, with DHA having a greater effect than EPA (81). Expression of NOX-4 mRNA, the NADPH oxidase subunit expressed in endothelial cells, has also been shown to be down regulated in HUVECs exposed to triacylglycerol rich lipoproteins rich in EPA and DHA (9g) (58).

In the context of EPA and DHA dependent changes in vascular tone, it is important to consider that any decreases in ROS production would likely lead to decreased sequestering of NO by ROS and this may contribute to increased net vasodilation, and possibly, decreases in systematic resistance and blood pressure.

1.7.5. Summary

The literature strongly supports an effect for EPA and DHA in modulating vascular tone in the postprandial state and suggests that this effect may play a part in reducing blood pressure in the longer term. There is evidence (mainly from chronic feeding studies) that DHA is more efficacious in this regard, however, there is lack of information with regards to the individual effects of EPA and DHA in the postprandial state; Particularly, in individuals at increased risk of CVD. Furthermore, there is a lack of consensus within the literature on the mechanisms for this effect with some studies indicating that the effect is endothelium dependent and others suggesting otherwise. This thesis aims to address this by comparing the effects of an EPA rich meal, DHA rich meal and control meal on measures of postprandial vascular function and tone. We will also investigating EPA and DHA dependent changes in circulatory concentrations of vasomediators such as NO (via quantification of nitrite) and H_2S using state of the art methodologies.

1.8. Mechanisms for a EPA and DHA dependent changes in vascular tone

1.8.1. EPA and DHA as Peroxisome proliferator-activated receptor (PPAR) family activators

A property of EPA and DHA which is likely to be partly responsible for part of their CVD risk reducing capacity is their ability to directly modify the activity and expression of the peroxisome proliferator-activated receptor (PPAR) family of molecules. The PPAR family includes the isoforms α, β and γ which are expressed in a number of tissues. Dysregulation of PPAR activity or expression is implicated in a number of complex non-communicable diseases such as CVD (94-98), Parkinson's Disease (99, 100), Alzheimer's Disease (99), type-II diabetes (101), Lupus and the associated nephritic syndrome (102).

The PPAR family members are ligand-dependent transcription factors, which form homodimers with the retinoid X receptor (RXR) and bind other co-activators such as steroid receptor co-activating factor-1 (SRC-1) in order to bind to the peroxisome proliferator response elements (PPRE) in the promoter region of genes containing these PPREs (see Figure 1.5). Genes containing these PPREs are involved in lipid and glucose metabolism and inflammation (103, 104).

1.8.2. Modification of PPAR family activation and expression by EPA and DHA

The ligand binding domain of the PPAR family members is buried in the core of three key α-helices. Binding of EPA and DHA occurs through their carboxylic acid groups interacting with key histidine residues (H323, H473 and H449) located within this ligand binding domain (105, 106) with hydrophobic interactions stabilizing this configuration (106). This interaction of EPA and DHA with the PPAR family has been shown to increase activity of PPAR-γ (107-109) , PPAR-α (110-112) and PPAR-β (111, 113) in a number of cell types and tissues in both *in-vitro* and animal model experiments. Current evidence suggests that this increase in activation occurs by inducing changes in conformation of a key activating function-2 (AF-2) helix which is important for ligand dependent transcription. Upon these conformational changes in AF-2 taking place, co-activator proteins such as SRC-1 may bind (105), allowing the PPAR family to induce expression of those target genes containing a PPRE.

Of note is reports of increases in PPAR-γ mRNA in response to DHA but not EPA in 3T3-L1 adipocytes (114). The study also showed that both EPA and DHA increased secreted adiponectin (a hormone involved in glucose regulation and fatty acid catabolism) concentration relative to control (44% and 102% respectively). Further to this incubation with a PPAR-γ antagonist, bisphenol-A-diglycidyl ether (BADGE), inhibited the observed increase in adiponectin secretion in cells which were incubated with

DHA but not those incubated with EPA. These results indicate that the mechanism underlying DHA's affects may be more PPAR-γ dependent than EPA.

It is important to consider, that members of the PPAR family do indeed bind other fatty acids (106) and not just EPA and DHA, but there is little evidence to suggest that other fatty acids are as potent activators of the PPAR family as EPA and DHA. It has been proposed by Xu *et al* (106) that the carbon chain length and degree of unsaturation of fatty acids may be key in determining the interactions of fatty acids with the PPAR family. It has also been suggested that the PPAR family members only interact with fatty acids of a certain length due to the fact that short chain fatty acids cannot make the hydrophobic interactions required for stable binding, further to this they propose that excessively long chain fatty acids may be subject to destabilizing effects of solvent due to an inability to fit into the ligand binding domain. Unsaturation of long chain fatty acids also plays a key role in determining the ability of fatty acids to bind and activate the PPAR family members. A greater amount of double cis-bonds facilitates the formation of kinks in the hydrocarbon chain and accordingly, allows fatty acids containing greater number of cis-bonds to adopt a contorted conformation that is needed to enter into the ligand binding domain to bind and activate the PPAR family. Accordingly, it is likely a combination of optimal amounts of double cis-bonds and optimal chain length confers EPA and DHA with a capacity to bind and active the PPAR family members. Differential activation of these PPAR family members by EPA and DHA may some role in the differential effects of EPA and DHA *in-vivo*.

1.8.3. Potential PPAR family dependent modulation of endothelial function

Putative evidence exists to suggest that PPAR activation may confer part of the suggested ability of EPA and DHA to modulate NO production in the endothelium. PPAR-γ agonists have been shown to activate eNOS phosphorylation (115) and increase eNOS mRNA expression in *in-vitro* models of hemodynamic conditions (116). Furthermore, PPAR-α agonists have shown to increase activity of eNOS via phosphorylation (117) and expression of eNOS (118). PPAR-α (117, 118) and PPAR-β (119, 120) activation has also shown a capacity to reduce hypertension (119, 121), perhaps via increased NO production by the endothelium (117, 120). Taken together, given that EPA and DHA are known PPAR family agonists, this evidence tentatively suggests that a PPAR-α, PPAR-β and PPAR-γ dependent mechanism may play a role in any potential increases in NO production in response to EPA and DHA. It is interesting to note, however, that the human eNOS gene does not contain a PPRE. It is plausible, that events downstream from PPAR-α, PPAR-β and PPAR-γ activation may influence alterations in eNOS activity. These events could potentially include binding to other regions within the eNOS promoter or alteration of eNOS mRNA stability (122), thereby increasing eNOS expression. It has also

been shown that caveolin-1 expression is modified by PPAR-γ (104). As mentioned earlier in this review, caveolin-1 is a known negative regulator of eNOS activity.

PPAR-γ agonists have also been shown to down regulate P47^{phox} in macrophages and monocytes (a major source of superoxide production in the vasculature) (123). Accordingly, DHA dependent activation of PPAR-γ may play a role in modulation of ROS production and subsequently NO bioavailability. Animal studies have supplied further evidence, that EPA and DHA may modulate superoxide production through a PPAR dependent modification in NADPH expression/activity in the vasculature. For instance, it has been shown that PPAR-γ agonists prevent ET-1 activation of pro-inflammatory pathways (including NADPH oxidase activation) in vascular smooth muscle cells from normotensive and hypertensive rats (124) (see Figure 1.7.2 for an overview of ET-1's effect on the vasculature). The observations that DHA is more efficacious in modulating NADPH activity than EPA (92, 93) could again potentially be explained by a PPAR-γ dependent model for these effects, given the reports indicating increases in PPAR-γ mRNA in response to DHA but not EPA (114).
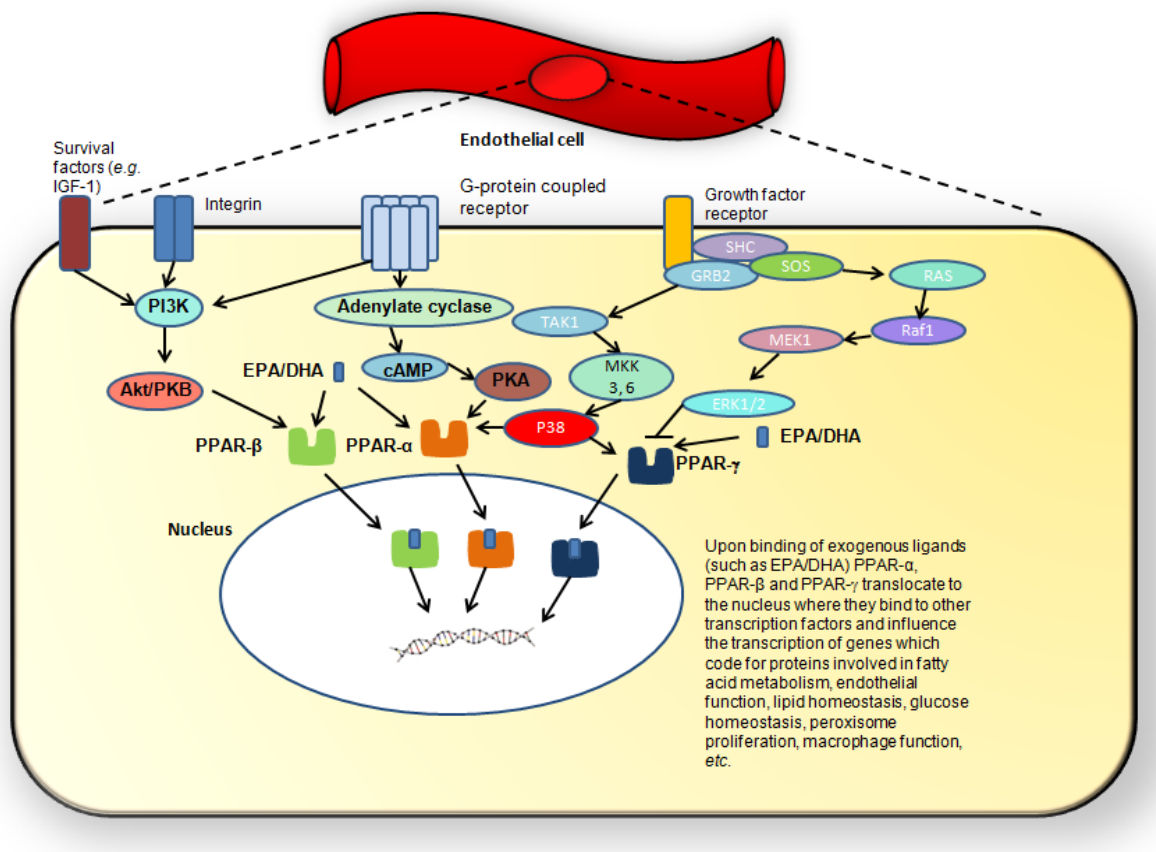
Figure 1.8.3. An overview of pathways influencing PPAR-γ, α and β activity.

1.8.4. Summary

In summary, the observations suggesting DHA is be more efficacious in modulating NO production than EPA (81) could potentially be explained by a PPAR-γ dependent mechanism. It must be noted that at the moment these are putative links and further work must be undertaken to confirm whether this mechanism contributes to EPA and DHA dependent modulations to vascular tone. These mechanistic investigations were beyond the scope of this PhD. Never the less, the interaction of EPA and DHA with the PPAR family is an important consideration in understanding the effects of EPA and DHA on the vasculature and on lipid metabolism and accordingly in chapter 5 genotype*treatment interactions for a common variant in the PPAR-γ gene were examined.

1.9. A role for EPA and DHA metabolites in the modulation of vascular tone

There is strong evidence to suggest a role for the metabolites of EPA and DHA in their ability to modulate vascular tone. These metabolites are produced by both discrete enzymatic and non-discrete enzymatic pathways, have a wide range of actions and a relatively wide range of receptors, some of which may yet to be described.

EPA supplementation is known to have effects on "classical" eicosanoid production. Eicosanoids are autocrine and paracrine hormones produced via oxidation of the 20-carbon fatty acids - EPA, AA, and dihomo-γ-linoleic acid. Eicosanoids include leukotrienes, prostanoids (prostaglandins and prostacyclins) and thromboxanes – all of which possess vasoconstrictory properties. It is well established that the production of EPA derived "classical" eicosanoids species in favour of the AA and dihomo-γ-linoleic acid derived species results in the production of metabolites with decreased vasoconstrictory properties. For instance, fish-oil intake has been shown to modulate leukotriene production *in-vivo* with LTB_5 (containing 5 double bonds) being produced in favour of LTB_4 (containing 4 double bonds) (125). Interestingly, LTB_5 has been shown to have a decreased capacity to illicit smooth muscle contraction (126). Prostaglandin H_3 (PGH3) is also derived from EPA and is formed at a slower rate than AA-derived Prostaglandin H_2 (127). It has been shown that increased production of PGH_3 is associated with a reduced risk of CVD and its effect on vasoconstriction are less potent than its AA derived counterparts (128). The molecules derived from PGH_3 such as TXA_3 have also been shown to have less potent vasoconstrictory effects than TXA_2 (derived from AA) (128). It is likely that some of the beneficial effects of EPA intake on vascular tone are mediated through EPA's capacity to increase PGH_3 and subsequently, LTB_5 and TXA_3 production, however, the effects of these metabolites alone are unlikely to explain in full the vasodilatory effects of EPA or in particular, DHA. Please see Figure 1.9, for an overview of the range of bioactive metabolites produced from EPA and DHA.

A current gap in the literature with regards to EPA and DHA's beneficial effects on the vasculature is establishing the contribution of the novel EPA and DHA epoxymetabolite and resolvin/protectin groups to EPA and DHA's capacity to modulate vascular tone *in-vivo*. There is also evidence to suggest genotype*nutrient interactions may influence the production of these novel and potent vasodilatory epoxymetabolites produced from EPA and DHA. Curiously, this area is particularly understudied in the context of human nutrition. The following sections will cover the bioactivity of these novel EPA and DHA metabolites in the vasculature.

1.9.1. EPA and DHA derived epoxymetabolites

The CYP450 enzymes are a large and diverse group of enzymes involved in the oxidation of organic compounds in phase 1 metabolism. The CYP450 enzymes were first identified as being central to the processing and detoxification of xenobiotics (129). It is also known that these enzymes act on fatty acids and other lipid compounds and of this substantial group of enzymes the 2J2, 1A1, 1A2, 4A11, 4B1, 4F12 CYP450s, among others, act on EPA, DHA and other fatty acids as their major substrates. EPA and DHA are among the fatty acids that these CYP450 enzymes act upon.

Several oxygenated metabolites generated by cytochrome P450 enzymes from EPA and DHA have been shown to modulate vascular muscle tone (130). It has been shown that these metabolites, referred to as epoxymetabolites, due to the presence of an epoxide group (a cyclic ether with a three atom ring), activate large Ca^{2+} - activated K^+ channels (131). The effect of the activation of these K^+ channels by EPA and DHA derived epoxymetabolites is hyperpolarization and relaxation of vasculature smooth muscles cells (132-134). The effects of these metabolites on vascular tone will be discussed in sections 1.9.2 and 1.9.3.
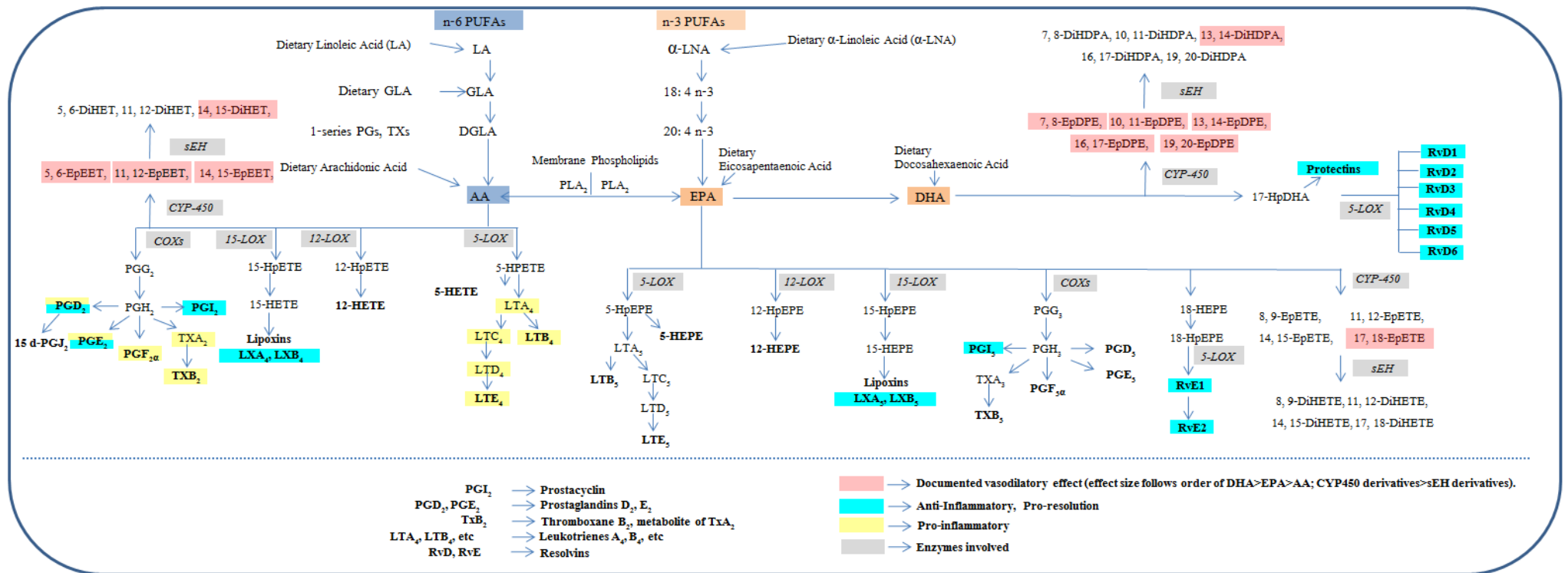
Figure 1.9. Diagram depicting the diverse range of metabolites derived from EPA, DHA and AA.

1.9.2. Effect of EPA and DHA epoxymetabolites on thromboxane production

In accordance with the evidence that suggests DHA to have more potent vasodilatory effects than EPA, a number of recent studies suggest CYP450 epoxide metabolites derived from DHA to be more potent than those derived from EPA (135). Evidence also exists to suggest that DHA derived epoxy-docosapentaenoic acid (EpDPEs) isomers have an inhibitory effect on platelet aggregation and the production of the potent vasoconstrictor thromboxane A_2 (135). In this study, DHA derived EpDPEs (19,20 EpDPE, 16,17 EpDPE, 13,14 EpDPE, 10,11 EpDPE, and 7,8 EpDPE; see Fig. 1.9.2) had IC_{50} values for inhibition of platelet aggregation in the range of 0.71 to 1.5µM and IC_{50} values for inhibition of thromboxane production in the range of 6 to 75µM. Interestingly, the epoxyeicosatetraenoic acid (EpETE) epoxymetabolites, derived from EPA, had IC_{50} values for inhibition of platelet aggregation in the range of 3.2 to 5.4µM, *i.e.* 3-4 fold higher than the DHA epoxides. This effect was mirrored in the EpETE capacity to reduce thromboxane production, which was approximately 33% decreased relative to the DHA derived epoxides, with IC_{50} values in the range of 9.7 to >100µM. Accordingly, when considering the effects of DHA and EPA epoxy-metabolites on platelet function DHA epoxy-metabolites are likely to be the more efficacious than EPA. This effect on platelet function is likely to mitigate some effect on vascular tone through thromboxane A2, a potent vasoconstrictor which acts through the G-protein couple thromboxane receptors. This evidence once again suggests that DHA is potentially more efficacious than EPA in beneficially modulating vascular tone.
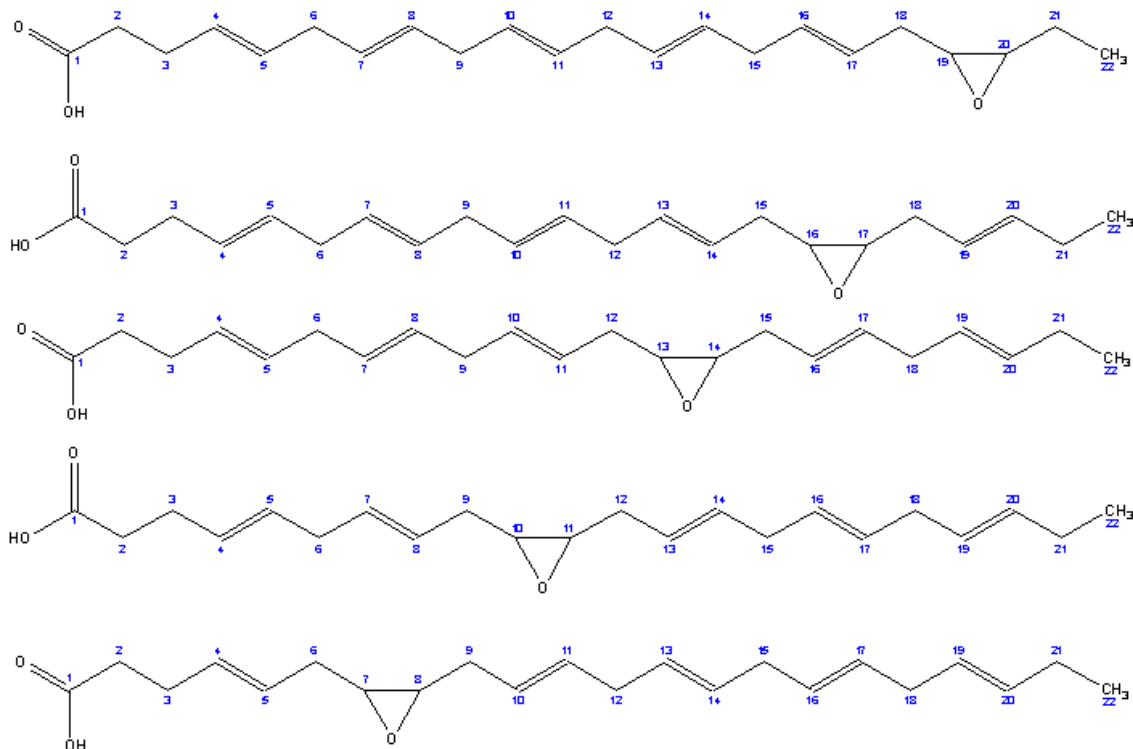
Figure 1.9.2. The DHA derived epoxymetabolites.

1.9.3. Direct effects of EPA and DHA epoxymetabolites on vascular smooth muscle tone

It has also been shown that 19, 20 EpDPE has potent direct effects on vascular smooth muscle tone (131). In this study pulmonary arteries were treated with 0.01-100μM MAG-DHA or 19, 20-EpDPE (0.001–10μM). Experiments revealed a concentration dependent effect for MAG-DHA on arterial relaxation in arteries challenged with U-44619 (30nM), a thromboxane A_2 agonist which induces vasoconstriction. These experiments were then repeated with 19, 20-EpDPE which showed a similar concentration dependent relationship for decreases in vascular tone. These experiments also showed that when treated with U-44619 the arteries had enhanced Ca^{2+} sensitivity (increasing vascular tone) and that pre-treatment with MAG-DHA caused a marked inhibition of this effect. Interestingly, experiments conducted in order to establish if MAG-DHA had effects on Ca^{2+} sensitivity in non-challenge arteries showed non-significant results. These results suggest that the metabolites of DHA offer a protective effect for arterial reactivity when the arteries are subject to non-optimal conditions, but they do not improve vascular reactivity beyond normal baseline levels.

In these experiments significant reductions in RhoA activity were also observed in response to both 3μM and 30μM MAG-DHA. Western-blotting revealed that phosphorylation of MLC and myosin phosphatase targeting protein (MYPT) was increased in response to U-44619 and reduced in response to pre-treatment with MAG-DHA. RhoA protein is known to activate Rho kinase, which leads to inhibition of myosin light chain phosphatase (MLC-phosphatase). When myosin light chains are phosphorylated they actively contract. Accordingly, the net effect of these reductions in RhoA is decreased contraction of vascular smooth muscle cells via RhoA dependent increases in MLC-phosphatase activity. Finally, this study (131) also investigated the effect of the selective CYP-450 epoxygenase inhibitor N-(methylsulfonyl)-2-(2-propynyloxy)-benzenehexanamide (MS-PPOH) on MAG-DHA's ability to modulate vascular reactivity in these *in-vitro* pulmonary arteries. It was shown during the course of these experiments that MS-PPOH treatment alongside MAG-DHA resulted in an approximately 75% decrease in MAG-DHA's ability to modulate vascular tone. This data provides further evidence that DHA's ability to modulate vascular tone *in-vitro* is dependent on the production of these epoxymetabolites, such as 19, 20 EpDPE, which are produced by CYP450 epoxygenases.

In-vitro studies of animal blood vessels also indicate DHA derived epoxymetabolites to be particularly efficacious in eliciting vasodilation. Some studies have suggested these DHA metabolites to be up to 2-3 orders of magnitude more potent than the arachidonic acid derived epoxymetabolites and 1-2 orders of magnitude more potent than the EPA derived epoxymetabolites (EpETEs) with regard to their ability to modulate BK channels in porcine coronary arterioles (134). *In-vitro* studies of human

PMA have shown IC50 values for the vasodilatory effects of the EPA epoxymetabolite, 17, 18-EpETE to be in the low µM range (132) in human PMA pre-contracted by methacholine (1µM). Experiments with the DHA epoxymetabolite 19,20 EpDPE showed an IC50 for its vasodilatory effects in human PMA's to be of one order of magnitude lower than that of 17,18-EpETE (approximately 0.1µM) (131). It should be noted that in the 19, 20 EpDPE experiments the arteries were challenged with U-46619 (30nM), whereas methacholine (1µM) was used in the previous study, and this inconsistency makes it difficult to directly compare the results; however, this does suggest that the DHA derived epoxymetabolites have greater vasodilatory capacity than their EPA counterparts. Consideration should be given to the fact that blood flow is regulated differentially in different vascular beds and the effects of these compounds in pulmonary arteries is likely to differ in peripheral arteries and large conduit arteries. It is also worth noting that studies in porcine coronary arteries have shown IC50 values for the DHA epoxymetabolites to be in the pM to nM range (134), but it should be noted that it may not be advisable to extrapolate these results from animal *in-vitro* blood vessel models to those from human blood vessels models.

Further work is warranted to establish if the effects shown for these epoxymetabolites in these *in-vitro* studies contribute to the effects of EPA and DHA to modulate vascular tone. Furthermore, further mechanistic work is warranted to establish the relative bioactivity of the EPA and DHA derived epoxy metabolites on vascular reactivity. Although data from clinical studies have shown a potential for circulatory concentrations of these compounds to be modulated after a 4 week supplementary period (136), in-vivo studies documenting their contributions to the improvements in vascular tone in response to either acute or chronic interventions are non-existent. Furthermore, the only demonstration in the literature of short term modulation of the concentration of these compounds is in response to a dosage of 22g of DHA and 3g of EPA and DHA, consumption of this amount of DHA and EPA through diet would require consumption of 1.5kg of salmon. Accordingly, given the potential impact of these compounds on human health further research is required on the modulation of these compounds to physiological consumption of EPA and DHA, the inter-individual variability in the production of these compounds and the determinants of any variability in the production of these compounds.

Specifically, given that allelic variants of the CYP450 enzymes which produce these metabolites have been shown to produce differential epoxymetabolite profiles from EPA (137), it is possible that genotype*nutrient interactions may also influence an individual's capacity to produce these epoxymetabolites and this may have an impact on the chronic and acute vascular response to EPA and

DHA. Given the health benefits of EPA and DHA, the diversity in the bioactive lipids produced from these n-3 PUFA and the heterogeneity of the response to EPA and DHA (138) establishment of the aetiology of the variability in response to intake is of wide public health interest.

1.9.4. An introduction to the resolvins and protectins

Resolvins are a group of bioactive compounds derived from EPA (E-series resolvins) and DHA (D-series resolvins) by the cyclooxegenase-2 (COX-2) pathway and lipoxygenase enzymes (see Fig. 1.9.4), which have been shown to modulate inflammation in the vasculature (139). The resolvins were first identified in *in-vivo* experiments of murine dorsal air pouches treated with aspirin (most widely known for exerting its anti-inflammatory effects via the COX-2 pathway) and EPA or DHA.
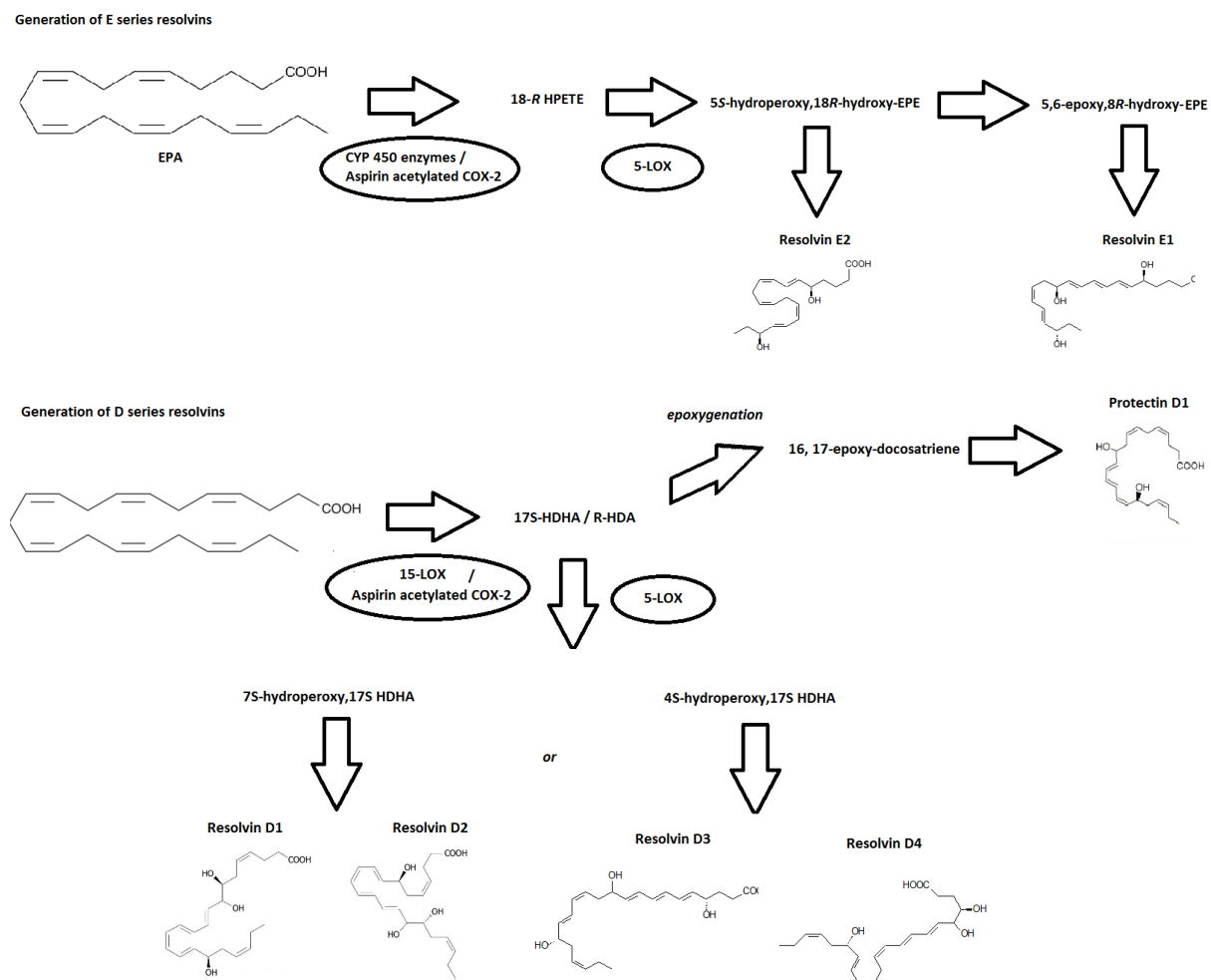
Figure. 1.9.4. Generation of E and D-series resolvins and protectin D1.

The E-series resolvins are generated from 18*R*-hydroxyeicosapentanoic acid (18 *R*-HEPE). 18 *R*-HEPE has been shown to be generated in endothelial cells with upregulated COX-2 expression treated with aspirin. This generation of 18 *R*-HEPE has been shown to be blocked in response to COX-2 inhibition, indicating the critical role COX-2 plays in the generation of the resolvins (140). 18 *R*-HEPE can then be

released by endothelial cells to be acted upon by leukocytes which convert 18 *R*HEPE to Resolvin E1 (RvE1) or Resolvin E2 (RvE2) by 5-LOX via an intermediate possessing a 5(6) epoxide group (140, 141). D-series resolvins, such as aspirin-triggered RvD1 (AT-RvD1) and RvD1 are synthesised via a pathway involving sequential oxygenations. AT-RvD1 production is initiated by aspirin-acetylated COX-2 and RvD1 production is initiated by 15-LOX in the microvascular. In a similar manner to the E-series resolvins, 5-LOX present in leukocytes plays a role in the generation of the D-series resolvins.

DHA can also generate protectins (PDs). These PDs are synthesised enzymatically by 15-LOX which generates a 17*S*-hydroperoxide containing intermediate, which is rapidly converted by human leukocytes into a 16(17)-epoxide. This 16(17) epoxide is then enzymatically converted to a 10, 17-dihydroxy-containing compound, such as protectin D1 (PD1 (142).

1.9.4.1. Bioactivity of resolvins and protectins

To date, studies on the bioactivity of the resolvins have focused mainly on their anti-inflammatory effects. For example, studies have shown RvE1 to inhibit trans-endothelial migration of polymorphonuclear neutrophils (PMNs) (140). These PMNs play a role in the atherogenesis through their capacity to produce cytokines which help induce and maintain the inflammatory state associated with atherosclerosis. PMNs are also likely to have involvement in this process through their production of the superoxide ion, which concurrently decreases NO bioavailability, and mediates their capacity to oxidise LDL (143).

Another plausible mechanism by which these neutrophils may contribute to a pro-atherogenic state is through induction of CD36 expression in macrophages via IL-4. Induction of CD36 expression results in increased oxLDL uptake and it has been shown that increased levels of CD36 are present in individuals with advanced atherosclerosis. It is likely that the RvE1's effects are mediated through the orphan G-protein couple receptor (GPCR) – ChemR23 (144). Activation of ChemR23 down-regulates the activity of NF-κB and also activates signalling pathways involved in initiating mitogen-activated protein kinase (MAPK). Critically, it has been shown that this GPCR is expressed on endothelial cells (145) and that it's activation is likely to induce production of NO (146). It has also been shown that RvE1 protects against ox-LDL induced injury in HUVECs and that this effect is inhibited by the PI3K inhibitor wortmanin (147). This would suggest that some of the beneficial effects of RvE1 on the vasculature are likely to be mediated through the PI3K-AKT signalling pathway. RvE1 has also shown to modulate PDGF-receptor activation and the chemotaxis in in-vitro vascular smooth muscles cells (148). This PDGF-receptor activation and chemotaxis is associated with vascular cell migration in the formation of atheromas. Interestingly RvD1 was shown to be less efficacious in exerting these effects.

Less is known regarding the mechanisms through which RvE2 may act but it has been shown to have similar actions as RvE1 on PMNs (131). The receptor which RvE2 binds to has yet to be established, since RvE2 effects have been shown to be additive to RvE1 (149), it has been hypothesised that it acts on a distinct receptor.

The D-series resolvins have been shown to act in a similar manner to the E-series resolvins, in particular with reference to their effects on neutrophil migration (150, 151). RvD1, like RvE1, has also shown to modulate leukotriene B4 signalling. This modulation of leukotriene B4 signalling reduces the pro-inflammatory response to increased cytokine production and blocks the function of the β-2 integrin adhesion molecules which play a role in neutrophil adhesion (152). It is interesting to note that both RvE1 and RvD1 have been shown to have no effect on PPAR-α, PPAR-β or PPAR-γ in PMN at nano-molar concentrations indicating that the effects of these EPA and DHA metabolites are likely to be independent of the PPAR family (152).

As illustrated in Figure. 1.9.4, DHA also acts a precursor for PD1. PD1 has also shown that it may offer some capacity to resolve the inflammation associated with oxidative stress and the atherogenic phenotype. This likely occurs through modulation of neutrophil migration and increasing macrophage ingestion of apoptotic PMNs (150).

Direct contribution of these compounds to EPA and DHA changes in vascular tone have not been documented in the literature and it is unknown if the concentrations of these compounds are modified in the acute state. The quantification of the resolvins and protectins profile in response to EPA and DHA rich test meals was beyond the scope of this PhD, due to a lack of in-house capacity. However, this may be undertaken retrospectively.

1.9.5. Summary

A broad range of bioactive compounds are derived from EPA and DHA and these compounds are thought to account for some of EPA and DHA's beneficial effects in the vasculature.

With specific reference to potential modulation of vascular tone, a robust mechanism for DHA and EPA dependent changes in vascular tone has emerged through investigations of the effects of CYP450 generated epoxide metabolites on *in-vitro* whole artery models with evidence suggesting that the DHA derived epoxymetabolites are more active than their EPA counterparts. *In-vitro* experiments have suggested that 19, 20-EpDPE accounts for approximately 75% of DHA's capacity to induce changes in vascular tone in *in-vitro* experiments in human pulmonary arteries. It is important to consider that the bioavailability of these compounds is thought to be low and it is unknown if circulatory

concentrations of these compounds changes acutely in response to physiological dosages of EPA and DHA. Furthermore, demonstrations of changes in the circulatory concentrations of these compounds *in-vivo* alongside demonstrations of changes in vascular tone have not been demonstrated. Finally, it has been shown that allelic variants of the CYP450 enzymes that produce these metabolites impact the epoxymetabolite profiles from EPA (137). It is possible that genotype*nutrient interactions may influence an individual's capacity to produce these epoxymetabolites and this may have an impact on the chronic and acute vascular response to EPA and DHA. This may in part contribute to the heterogeneity in response to EPA and DHA (138). Although the effects of the EPA and DHA derived resolvins and protectins have been well described and likely contribute to EPA and DHA's longer term effects in the vasculature, a strong line of evidence on their effects on vascular tone does not emerge in the literature. Accordingly, these compounds were not a main focus of this project.

We hypothesise that these epoxide compounds may contribute to EPA and DHA's capacity to modulate vascular tone in the postprandial state. Accordingly, our study will investigate the production of these novel metabolites in response to physiological doses of EPA and DHA for the first time. Given the bioactivity of these compounds, it is of critical importance to establish their bioavailability post consumption and also to generate data regarding inter individual variation in the capacity to produce these compounds.

1.10. Hypothesis and objectives

There is evidence from a number of avenues of research which indicate that DHA may be more efficacious than EPA in modulating vascular tone. Longer term chronic feeding clinical trials have indicated DHA to be more efficacious in modulating fasting vascular tone (40, 57) and this has been supported more recently by a single acute feeding studies which investigate the impact of EPA and DHA on cardiac and vascular function during exercise (61). A current research gap is establishing the relative efficacy of EPA and DHA to modulate post-prandial vascular tone in at risk populations. We hypothesise that DHA may be more efficacious than EPA in modulating vascular tone and a primary aim of this project will be to elucidate the relative impact of EPA versus DHA on postprandial vascular tone in a population at an increased risk of CVD.

Further to this, although it is known that LC n-3 PUFA modulate vascular tone, the mechanisms mediating these effects have not been fully elucidated. Investigations on how these effects are exerted are another novel aim of this project. It is interesting to note that studies which give insight into potential mechanisms of these effects suggest DHA to be more efficacious than EPA in modulating vascular tone. Finally, it is evident that a significant contribution to EPA and DHA's beneficial effects on the vasculature are mediated by their metabolites. Current knowledge indicates that the epoxy metabolites generated by the CYP450 enzymes act directly on vascular smooth muscle cells to elicit dilatation via activation of large Ca^{2+} - activated K^+ channels (131), with the DHA derived epoxy-metabolites known to be particularly potent (132-134). Our study will investigate a broad range of blood borne vasomediators via state of the art methodology in order to compare the capacity of EPA and DHA to influence the concentration of vasoactive compounds in the postprandial state.

Our study will also investigate the impact of meal dependent changes to the postprandial blood fat profile on innate inflammatory responses by utilisation of an LPS stimulated WBC model. Previous investigations using this model have shown that a 6 week fish-oil intervention has been shown to alter production of IL-6 in LPS stimulated WBC (52). We will investigate the impact of our EPA and DHA interventions compared to control on LPS stimulated WBC responses from blood drawn postprandially. We hypothesise that EPA and DHA may have differential effects in this regard. Furthermore, information on the impact of post meal alterations to the whole lipidome on monocytic cytokine production haven't been described in the literature and our study will allow us to address this consideration.

Finally, we will investigate the interactions between meal fat content, a select number of candidate genes and vascular and triglyceride responses as a pilot study. These candidate genes include SNPs in

the genes coding for PPAR-γ, NAPDH oxidase and eNOS. An introduction to these candidate genes is provided in Section 5.1.

In summary, to date, the characterisation and comparison of the individual effects of EPA and DHA on postprandial responses has been insufficiently described in the literature. Given the evidence suggesting a role for the LC n-3 PUFAs in modulating CVD risk, establishing the relative efficacy of EPA and DHA to modulate postprandial responses are important given that most individuals spend the majority of the day in the postprandial state. It is possible that such day to day "acute" changes to postprandial responses, when habitually repeated, have implications for long term vascular health and CVD risk. Our study aims to address these considerations by investigating the effects of EPA and DHA rich meals, when compared to a high-fat control meal, on vascular and inflammatory responses.

Chapter 2

A clinical investigation of the impact of fish-oil fatty acids on postprandial vascular reactivity – Trial design and methodology

Chapter 2: A clinical investigation of the impact of fish-oil fatty acids on postprandial vascular reactivity – Trial design and methodology

2.1. Introduction

The primary aim of this study, which will be hence forth referred to as the (F.O.F.A) Fish Oil Fatty Acid Study, was to determine the impact of the fish oil fatty acids, eicosapentaenoic acid (EPA) versus docosahexaenoic acid (DHA) on vascular reactivity and to identify underlying physiological and molecular mechanism of any observed effects in a group of males aged 35-55 years (Chapter 3). In addition, LPS stimulated WBC was undertaken on blood drawn postprandially to compare the effect of EPA and DHA consumption on WBC responses (Chapter 4). Response to intervention according to genotype was also determined retrospectively (see Chapter 5). The following chapter details the protocol of this human intervention study.

2.2. Approach

In this study the ability of EPA and DHA to acutely affect PWV, AI and Endopat RHI was tested in men estimated to be at a 1.5 increased relative risk of developing CVD. Further to this, the effect of EPA and DHA inclusion in meals on whole blood culture of blood drawn postprandially was investigated.

To this end, a randomised controlled acute study, with 3 arms, was designed to test the relative efficacy of a single dose of EPA and DHA (4.16g) provided in a milkshake form; see Table 2.6.1 for meal design. This dosage was chosen on the basis of results indicating that similar doses of EPA and DHA modulate vascular tone in human intervention studies (40, 57, 58).

Subjects were randomised to one of the three study groups 1 to 3 outlined below, and consumed a single dose of each of the following treatments in random order:

1) High fat meal containing 4.2g EPA

2) High fat meal containing 4.2g DHA

3) High fat meal containing placebo fat

Each test meal was assessed separately (3 study days in total) at the Clinical Research and Trials Unit at the University of East Anglia, which is an embedded NHS facility under the auspices of the Norfolk and Norwich University Hospital (NNUH). A 4 week wash-out period between each visit to the unit was observed. This 4 week wash out period between treatments is consistent with other acute phase

studies with fish-oil (153). Clearance of test fats from circulation was confirmed by quantification of the fatty acid profile at baseline at each visit (see section 3.3.2.5). An overview of the enrolment and assessment process is detailed in Figure 2.2.1 and 2.2.2.
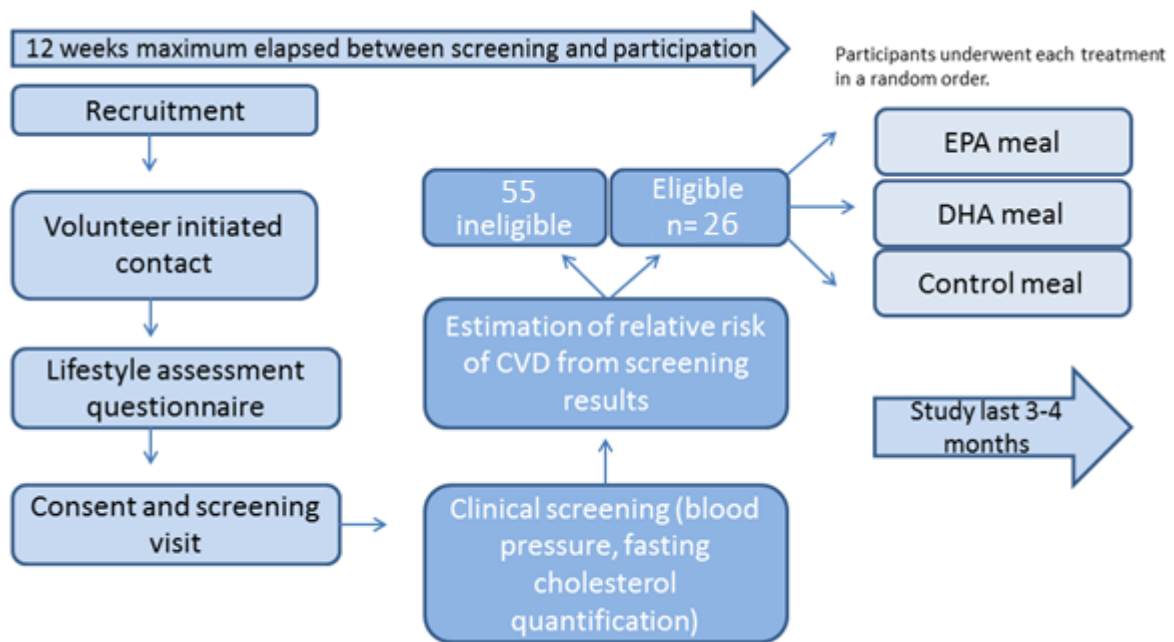
Figure 2.2.1. Overview of schedule of participant recruitment, screening and randomisation to the acute intervention.
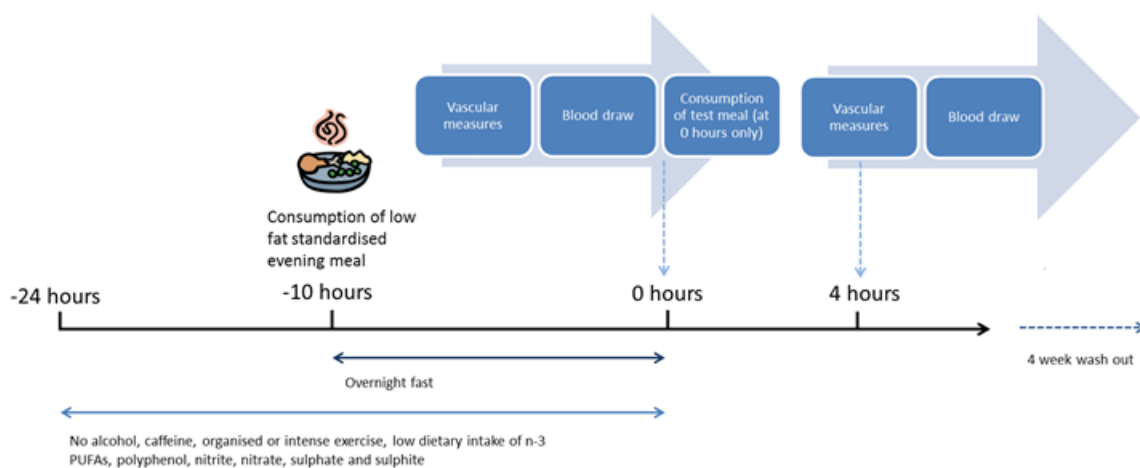
Figure 2.2.2. Order of meal and assessments on study days – including pre-study day restrictions.

In brief, participants attended each assessment visit in a fasted state, with blood samples and vascular measurements taken before administration of the test food. All measurements were then repeated

at 4 hour postprandially. This time point was chosen as 4 hours postprandially is the point at which exogenous lipids reach their peak in plasma post consumption (154) and also the point at which postprandial reductions in vascular reactivity tend to occur after consumption of a high fat meal (155).

Prior to the visit participants were asked to fast for 10 hours and to consume a standardised meal as the last meal before their visit to the clinic. Furthermore, for 24 hours before each assessment visit, participants abstained from consuming specific EPA and DHA rich foods, alcohol and caffeinated products. A list of dietary restrictions and alternative foods that were freely consumed are provided in Annex 2. Participants also consumed a standardised low fat meal (<10g total fat) the night before each assessment. For instance, for 48hr prior to each assessment, participants also refrained from strenuous exercise and moderated the intake of nitrate and nitrite rich foods (including leafy green vegetables, broccoli, beetroot, radishes, carrots, sausages, other processed, cured meats, bottled waters (excluding Buxton bottled water, which contains <0.1mg/L nitrate) and sulphite and sulphate rich foods (such as onions, garlic, wine, dried fruits or meats, nut/trail mixes, shredded coconut, brussel sprouts, cabbage, broccoli, red peppers). This was undertaken as these dietary components could potentially affect our measures of blood H_2S, NO and NO oxidation products.

Ethics submission, recruitment, screening, clinical measures and all aspects of protocol compliance in this study were the responsibility of Seán McManus. Phlebotomy was performed by staff at the University of East Clinical Trials and Research Unit.

2.3. Study population

In order to recruit a population at a relative risk of developing CVD, males aged 35-55 years, who had one of the following risk factors for CVD were recruited through local advertisement:

- Total cholesterol $\geq$ 6mmol/L
- High density lipoprotein cholesterol (HDLC) $\leq$ 1.0mmol/L
- Systolic blood pressure > 140 mmHg
- Diastolic blood pressure > 90 mmHg
- Waist circumference > 102cm

The rationale for these selection criteria was based on evidence suggesting that individuals with these measures are at 1.5 relative risk of developing cardiovascular disease relative to other adult males of the same age. This approach was utilised in order to select for a study population with an increased relative risk of CVD complications who were otherwise healthy and whose cardiovascular status was otherwise considered pre-clinical. The rationale for this was two fold; we reasoned that we would be unlikely to observe any nutrient dependent changes in vascular tone in individuals with poor vascular reactivity, such as would be expected in individuals with clinically diagnosed CVD. Furthermore, it was reasoned that the study would be more clinically relevant if undertaken in a population with pre-clinical risk factors for CVD. Accordingly, the above described approach was utilised.

Based on power calculations of Endopat and Pulse Wave Velocity (measures of vascular tone) a target of 25 participants was required to complete the study (see section 2.4). Individuals matching the following criteria were deemed ineligible for the study:

- A history of clinically diagnosed vascular or circulatory disease, diabetes, systemic lupus erythematosus, hepatic, renal, digestive, haematological, neurological, cancer or thyroidal disease.
- Allergy to intervention foods (dairy or fish products)
- Individuals unprepared or unable to adhere to dietary restrictions during the 3 days preceding each assessment visit.
- Current smokers, or ex-smokers ceasing <3 months before screening
- Prescribed medication that could affect lipoprotein metabolism, liver enzyme function, blood clotting or blood pressure. Including vasodilators such as Viagra (see Annex 1)
- Had taken antibiotics or vaccinations within 3 months of enrolment into the study.
- Taking dietary fish-oil supplements (>1g of EPA and DHA per day) or high dose of anti-oxidant

vitamins (>800µg vitamin A, 60mg of vitamin C, 50 µg selenium,10mg of vitamin E or 400µg β-carotene).

- Heavy drinkers (>30 units of alcohol per week).
- Individuals with a sustained BP ≥160/100 mmHg at screening.
- Individuals with a Body Mass Index (BMI) >30kg/m^2.
- Parallel participation in another research project which involves concurrent dietary intervention and/or sampling of biological fluids/material.
- Habitual consumption of more than one portion of oily fish per week (as defined as 140g of any oil fish, including salmon, trout, mackerel, sardines, pilchards, herring, kipper, eel, whitebait, *etc*).

Screening methodology is detailed in full in section 2.8.

2.4.   Sample size considerations

Sample size calculations were undertaken with a power of 0.80 to detect clinically relevant changes at the 5% significance level for 2 assessments in the trial, the measurement of endothelial function by Endopat (primary outcome) and Pulse Wave Velocity (secondary outcome). The calculations assumed no correlation between baseline and end of the intervention. For Endopat, given a standard deviation of 0.4 (156), a clinically relevant difference of 0.25 between treatments would be detectable in 21 subjects per treatment group. For Pulse Wave Velocity, given a standard deviation of 1.2m/sec (157), a clinically relevant difference of 1.5m/sec would be detectable with a sample size of 11 participants per treatment group. For Augmentation Index (secondary outcome), a clinically relevant 4% decrease in AI would be detectable in a population of 13 individuals, assuming a populational standard deviation of 5% in AI. As the sample size needed to detect a clinically relevant difference for Endopat was greater than that for Pulse Wave Velocity or AI, we aimed to recruit 25 participants. This number was chosen to allow for a potential drop-out rate of 15% and to allow for 21 participants to complete the study.

2.5.   Volunteer randomisation

The treatment order for assessment days 1 to 3 was randomised for each participant by use of an internet based random number generator by an independent member of staff not involved with the data analysis or data generation (Dr. Peter Curtis). The details of the treatment order were provided (by Dr. Peter Curtis) to staff who were assigned the duty of preparing the treatment foods. In order to ensure no bias was introduced the staff assigned the responsibility of preparing the treatment foods

were not involved in data collection or analysis. The coding of the treatment order was only broken at the analysis stage of the study.

2.6.   Test meal composition

The test meal was designed in order to fulfil the following conditions:

1. The test meal was to be composed in such a manner to reduce inter-individual variations in absorbance as much as possible.

2. The test meals were designed so as to deliver a total dose of either 4.16g EPA, DHA or control fat.

3. The test meals were designed to have a high fat content and so that the non-EPA and DHA portion of this fat would be broadly representative of the fat composition of the UK diet.

4. The test meal was to be designed in such a way so as to mask the smell and flavour of the EPA and DHA oils.

To meet these criteria, it was decided to administer the intervention materials in a liquid form in order to ensure homogeneity in absorption. It was decided to administer our test fats in this matrix in preference to capsulation, due to the inherent difficulty in encapsulating our control fat mixture which exists in a semi-solid state at room temperature. A peppermint oil extract was also included in the test meal in order to mask the flavour and smell of the fish-oils and ensure both the participants and research scientists were blinded to the treatment order.

Justification for dosage was on the basis of previous studies showing doses in the range of 4-5g to be sufficient to modify vascular tone (40, 57, 58).

Justification for meal fat composition was on the basis that the non-n-3 fatty acid fat composition of this meal closely reflects that of the fat composition of the UK diet and also that this meal fat composition has been previously shown to induce postprandial increases in vascular tone (58). As high-fat meals are associated with impairment of vascular tone when compared to low fat meals, and inclusion of fish-oil fatty acids in high fat-meals is known to have a protective effect on vascular tone, we deemed that this meal design was optimal to investigate any differences exist between EPA and DHA's efficacy in contributing to this effect.

Treatment	Meal composition
Control	40g palm oil/soybean oil (4:1) made up in a milkshake form (skimmed milk (150g), Nesquik chocolate flavoured powder (25g), Skimmed milk powder (15g)), 2g Peppermint Oil Extract
EPA	33.06g palm oil/soybean oil mixture (4:1) + 6.94g of EPA rich oil made up in a milkshake form (skimmed milk (150g), Nesquik chocolate flavoured powder (25g), Skimmed milk powder (15g)), 2g Peppermint Oil Extract
DHA	31.67 g palm oil/soybean oil mixture (4:1) + 8.33g of DHA rich oil made up in a milkshake form (skimmed milk (150g), Nesquik chocolate flavoured powder (25g), Skimmed milk powder (15g)), 2g Peppermint Oil Extract

Table. 2.6.1. The intervention meal design.
*All intervention materials given to volunteers with toasted white bread (73g) and jam (30g).

Nutritional content	Control meal	EPA meal	DHA meal
Protein	17.8g	17.8g	17.8g
Fat	42.4g	42.4g	42.4g
of which SFA	15.1g	12.9g	12.5g
of which MUFA	17.3g	15.4g	15.3g
of which PUFA	10.0g	14.1g	14.6g
Carbohydrates	73.8g	73.8g	73.8g
of which sugars	45.0g	45.0g	45.0g
Sodium	832mg	832mg	832mg
Vitamin A	1.5µg	1.5µg	1.5µg
Vitamin C	2mg	2mg	2mg
Vitamin E	12mg	30mg	30mg
Fibre	1.56g	1.56g	1.56g
Total calories	747.6kcal	747.6kcal	747.6kcal

Table 2.6.2. Meal nutritional information.

Fatty acid	Control meal (%)	EPA meal (%)	DHA meal (%)
Palmitic acid C16:0	*32.15*	*27.36*	*26.49*
Palmitoleic acid C16:1	n/a	0.46	0.76
Stearic acid C18:0	*3.62*	*3.02*	*2.90*
Oleic acid C18:1	*40.47*	*35.32*	*34.87*
Linoleic acid C18:2	*22.27*	*18.40*	*17.63*
α-Linoleic acid C18:3	*1.35*	*1.58*	*1.84*
Stearidonic acid C18:4	n/a	0.46	0.76
Arachidic acid C20:0	0.13	0.11	0.11
Gondoic acid C20:1	n/a	0.46	0.55
Arachidonic acid C20:4	n/a	0.46	0.76
EPA C20:5	*n/a*	*9.82*	*1.96*
Heneicosapentaenoic acid C21:5	n/a	0.46	0.76
DPA C22:5	n/a	0.46	0.76
DHA C22:6	*n/a*	*1.64*	*9.82*

Table 2.6.3. Estimated fatty acid composition of meal as derived from manufacturers data.

2.7.   Recruitment strategy

Individuals matching the characteristics of our target population (see 2.3) were identified through open and targeted recruitment through local advertising, media calls and targeted web based social network advertising.

2.7.1. Assessment visits

Assessment visits were undertaken after an overnight fast, beginning at 7.30am and finishing at 2.30pm. Participants were asked to consume a standardised meal before the visit in order to ensure that variance in pre-assessment dietary intake did not impact individual response to the intervention. At the start of each assessment a medical check and recall of diet over the preceding 24 hours was taken to ensure adherence to the dietary restrictions (as detailed in Annex 1 and Annex 2). It was also ensured that participants had not taken any medication on the prohibited medications list including non-prescribed non-steroidal anti-inflammatory medications.

Subsequent to these checks, baseline vascular measures (see Section 2.9) and blood draws (see section 2.10.2) were taken. Participants were then asked to consume one of the intervention meals, which were composed daily by a third party researcher who was not involved in study design or analysis. Accordingly, both participants and study researchers were blinded to meal identity. 4 hours post meal consumption postprandial vascular measures and blood draws were taken.

2.8.   Screening methodology

2.8.1. Introduction

All participants were screened in order to determine that they fell within the study population criteria defined in section 2.3. Screening visits were undertaken after a 10 hour fast. At the screening, questionnaires on medical history and dietary habits were taken in order to establish if any of the potential participants were to be excluded on the basis of fitting the exclusion criteria detailed in section 2.3. Those individuals who were not excluded on these criteria then had their blood pressure taken by an automated blood pressure measuring device (Omron 705 CP-II). Blood pressure measures were taken 3 minutes apart and in triplicate after a 15 minute resting period. Subsequent to blood pressure measurements, a blood draw was taken via a standard butterfly gauge needle and subsequently centrifuged at 3300rpm at 4°C for 10 minutes. Serum samples were then stored in monitored -80°C until analysis. Serum isolated at screening was analysed within 2 weeks for total cholesterol and HDL-cholesterol via an automated I-Lab 3000 Clinical Chemistry analyser. These methods, which are based on spectrophotometric methodologies, are detailed in Section 2.8.2.2.

2.8.2. Materials

All Materials for screening analysis were obtained from Instrumentation Laboratories (Warrington, UK) unless noted otherwise.

2.8.3. Methods

2.8.3.1. Screening sample analysis

Samples generated at screening were taken via venous puncture through a standard butterfly gauge needle and subsequently centrifuged at 3300rpm at 4°C for 10 minutes. Blood was then stored in a monitored -80°C until analysis.

2.8.3.2. Serum total cholesterol and HDL-C analysis at screening

Total cholesterol was determined in serum samples generated at screening via spectrophotometric analysis in an ILAB 650 (Instrumentation Laboratory UK Ltd). The analysis is based on the utilisation of an enzymatic pathway (see Figure 2.8.2.2) facilitating the production of quinoneimine dyes.

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{cholesterol esterase}} \text{Cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{Cholest-4-en-3-one} + H_2O_2$$

$$2\,H_2O_2 + \text{4-aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{Quinoneimine} + 4\,H_2O$$
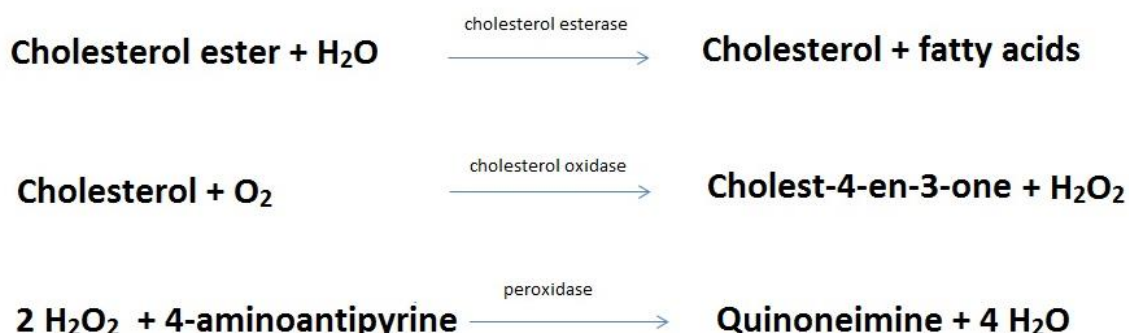
Figure 2.8.2.2. As described above 1 molecule of Quinoneimine is produced for every 2 cholesterol esters.

HDL-C was determined in serum samples in a similar fashion. HDL-C quantification was based on the principal of anti-human β-lipoprotein binding to all lipoproteins other than HDL. The antigen-antibody complexes that form block enzyme reactions with these lipoproteins. Upon addition of cholesterol esterase and cholesterol oxidase there will be selective reaction with HDL-C. Accordingly, allowing one to quantify the levels of HDL-C in a sample in the range of 40-994 mg/dL.

The above reactions allowed the quantification of total cholesterol and HDL-C levels due to the fact that quinoneimine production is proportional to the levels of total cholesterol and HDL-C in the sample

(1 mole of quinoneimine to every 2 moles of cholesterol). The primary and secondary wavelengths at which the samples were measured were 510nm and 700nm respectively as quinoneimine absorption peaks at these wavelengths.

The ILAB 600 was calibrated every 14 days, or in the cases where an analysis fell greater than 14 days after the previous calibration, before analysis. Calibration for total cholesterol measurement was undertaken via utilisation of a Referr IL G Calibrator (Instrumentation Laboratory UK Ltd). The Referr Il G Calibrator is a multicomponent calibrator for which total cholesterol values had been pre-established by the supplier. Calibration for HDL-C measurement was undertaken via utilisation of a Referr IL HDL-C Calibrator (Instrumentation Laboratory UK Ltd). Total cholesterol values were pre-established against a human serum reference which had an assigned value obtained via the Abell-Kendall method (158) in a laboratory which participates in the Cholesterol Reference Method Laboratory Network.

Serachem Control Level 1 and Serachem Control Level 2 (instrumentation Laboratory UK Ltd) were utilised as quality controls in order to ensure the accuracy and precision of the analysis. Serachem controls were constituted from lyophilized bovine serum with cholesterol concentrations lower (Serachem Control Level 1) and higher (Serachem Control Level 2) than observed concentrations in serum isolated at screening. Accordingly, these quality controls allowed us to ensure the concentrations established for the screening samples were within the range of linearity for this analysis.

Calibration and quality controls were established and the coefficient of variation was confirmed to be below 2.5% for both analyses. Subsequent to addition of samples cuvettes, total cholesterol levels were quantified by measuring absorption at 510nm and 700nm, whereas HDL-C levels were quantified by measuring absorption at 600nm and 700nm. Differences in wavelength measured in these two techniques are due to the presence of a phenolic component in the total cholesterol measure. Previously established standard curves were utilised to establish concentration from absorption values for both analysis.

2.8.3.3. Screening blood pressure measurement

As detailed in section 2.8.1, blood pressure was measured at screening visits via an automated blood pressure measuring device (Omron 705 CP-II). Blood pressure measures were taken 3 minutes apart and in triplicate after a 15 minute resting period.

2.9. Clinical vascular measures

2.9.1. Introduction

A battery of vascular measures was taken at 0H and 4H postprandially in a temperature controlled (21 to 22°C) room. These measures were all chosen based on a selection criteria described below:

- must have a sound physiological and mechanistic basis that links it to atherosclerotic risk.
- must be reproducible and easily standardized.
- an improvement in the test should predict an improvement of subsequent cardiovascular risk, likewise, a worsening in the test should correlate to increased cardiovascular risk.
- must be variable over the course of 4-5 hours dependent on the production of vasoactive mediators.
- must be non-invasive.
- capable of measuring either endothelium dependent or endothelium independent vascular tone.

The vascular measures taken included the following measures, the physiological basis for these methods are introduced in full in the following sub-sections, whereas methods are described in full in section 2.9.2.

- Blood pressure measurements

- Augmentation index

- Pulse wave velocity (PWV)

- Reactive hyperaemia index via Endopat

- Reactive hyperaemia index in response to 400μg nitroglycerine administration

2.9.1.1. Augmentation Index

Augmentation Index (AI) is a non-invasive method which utilises the pulse profile as a proximal measure of arterial stiffness and acute vascular tone. Specifically, a 10 second snapshot is taken at the radial artery via a tonometer probe. This snapshot is subsequently utilised to glean information on the extent of resistance against which the heart has to pump. Contributions to this resistance include

arterial and acute vascular tone. In addition to this measure, the system provides details of central blood pressure, aortic augmentation index, ejection duration and sub-endocardial viability ratio.

Critically, the AI outcome is dependent on the elastic effects of the entire arterial tree. Changes in these elastic effects is one of the causes of increasing blood pressure with advancing age and occurs in other diseases states that increase arterial stiffness prematurely. The AI has also been shown to decrease acutely upon administration of vasodilatory compounds such as nitroglycerin and increase upon administration of compounds with vasoconstrictive effects, such as angiotensin II (10). Accordingly, it is known that this measure is sensitive to acute changes in vascular tone mediated by blood borne vasoactive components.

The mathematical calculation of AI is detailed below, in Figure 2.9.1.1, with diagrammatic aid. In short, AI is calculated from the pulse wave by expressing augmentation pressure as a percentage of total pulse pressure. This gives an indication of the degree by which systolic blood pressure is augmented by late reflected waves in the peripheral arterial tree. Late reflected waves tend to result in extra left ventricular load due to blood returning to the left ventricular in timing that is out of sync with left ventricular ejection; this in turn acts to increase systolic blood pressure. As mentioned, this measure is affected by both arterial stiffness, which does not change acutely, and changes in peripheral artery tone which is affected acutely by blood borne vascular mediators, such as NO, H_2S, thromboxane and the EET, ETE and EpDPE family of molecules, among others. Critically, AI has been shown to be associated with increased CVD risk (9).
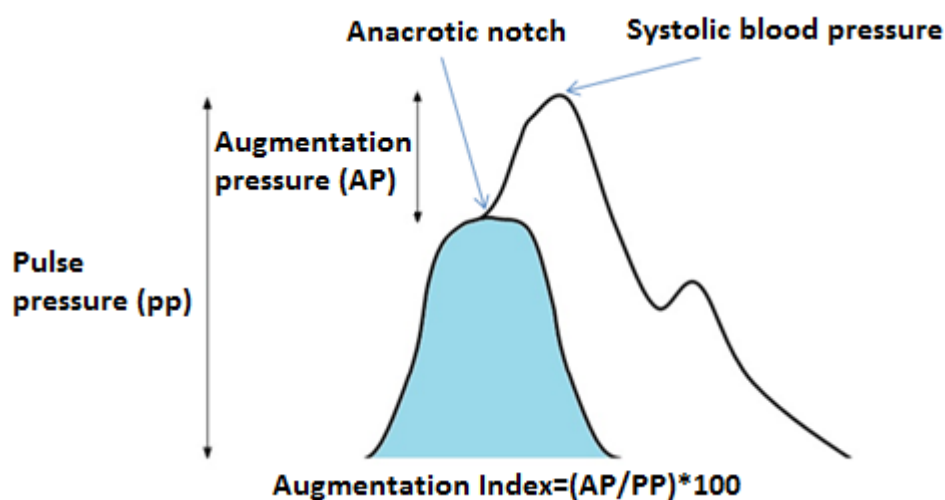
Figure 2.9.1.1. Diagrammatic representation of pulse wave form showing pulse pressure and augmentation pressure.

2.9.1.2. Pulse Wave Velocity

Pulse wave velocity (PWV) is a non-invasive method to measure arterial stiffness. Arterial stiffness can occur due to aging as elastic fibres made from elastin begin to fray due to continual mechanical stress. Critically, PWV has been shown to be an independent predictor of CVD risk (8).

Specifically PWV, measures the time for the pulse wave to travel from one site on the body to another. The gold standard for anatomical measurement sites is considered to be from the carotid to femoral artery. The calculation of PWV is depicted below in Figure 2.9.1.2.
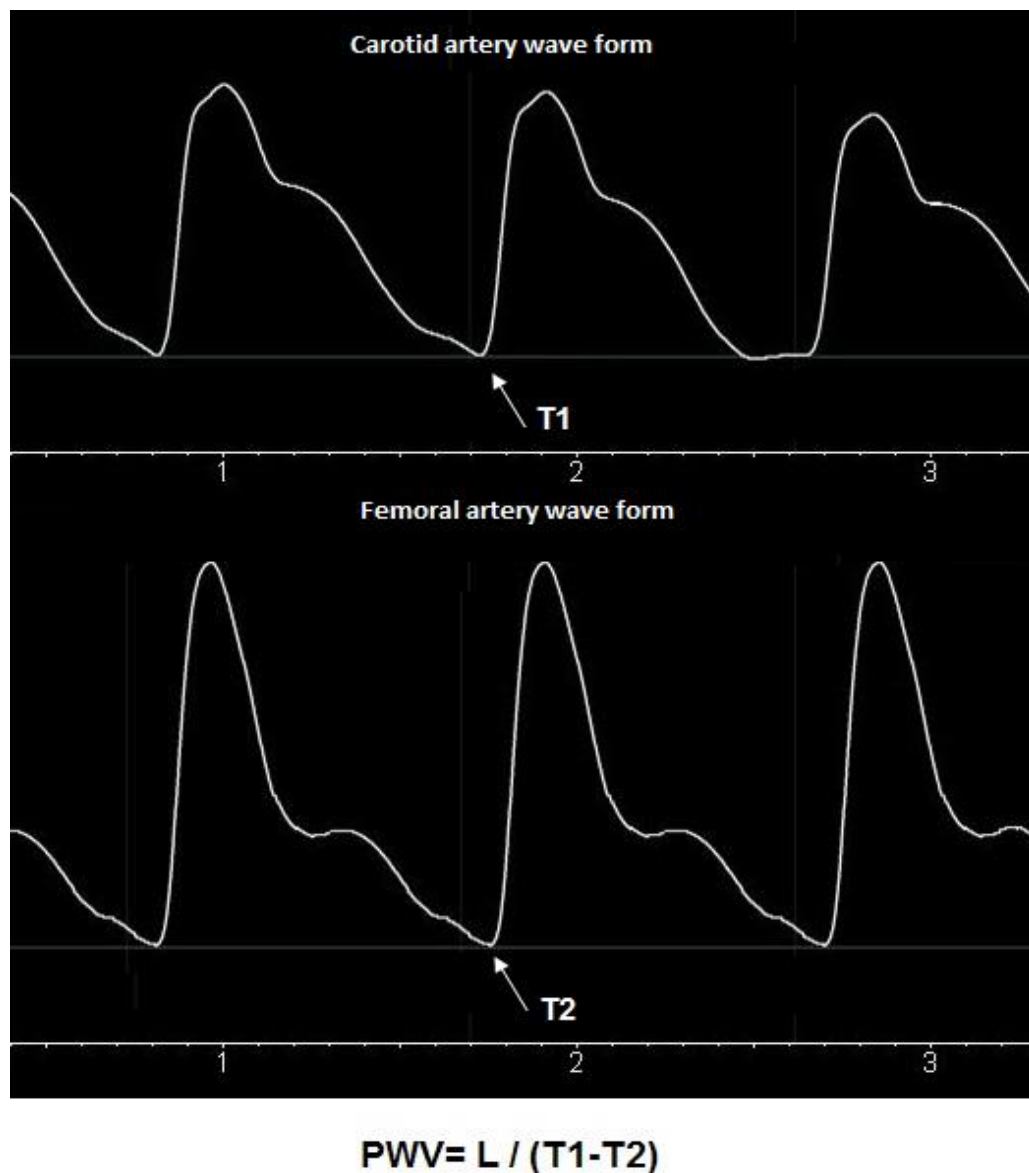
Figure 2.9.1.2. Diagrammatic representation of carotid artery and femoral artery wave form and calculation of PWV

2.9.1.3. Endopat

Endopat is a measure of endothelial function which has been shown to be associated with multiple known CVD risk factors (6) and to be predictive of future cardiovascular events (7). The Endopat Reactive Hyperaemic Index (RHI) measurement is obtained by measuring the increase in blood flow in response to the stimulus of shear stress. This shear stress is caused by occluding the brachial artery for 5 minutes, and subsequent releasing of the occlusion, causing sudden increase in blood flow to the finger tips. The relative amount of hyperaemia is then measured by pneumatic sensors placed on the fingers in order to establish a RHI measurement. This RHI measurement is indicative of the capacity of the endothelium to produce vasodilatory substances, in particular NO.

A measurement of RHI was also undertaken postprandially in response to 400µg nitroglycerine administration at the postprandial timepoint. This was undertaken in order to utilise nitroglycerine's capacity as NO donor. Effectively, this procedure allowed us to measure RHI in a state in which the relative contribution of NO at the postprandial timepoint for each treatment was normalized. Accordingly, any changes in this measure dependent on treatment would suggest the contribution of vasoactive factors other than NO.

$$RHI = \frac{A}{B} / \frac{C}{D} \times \text{Baseline correction factor}$$
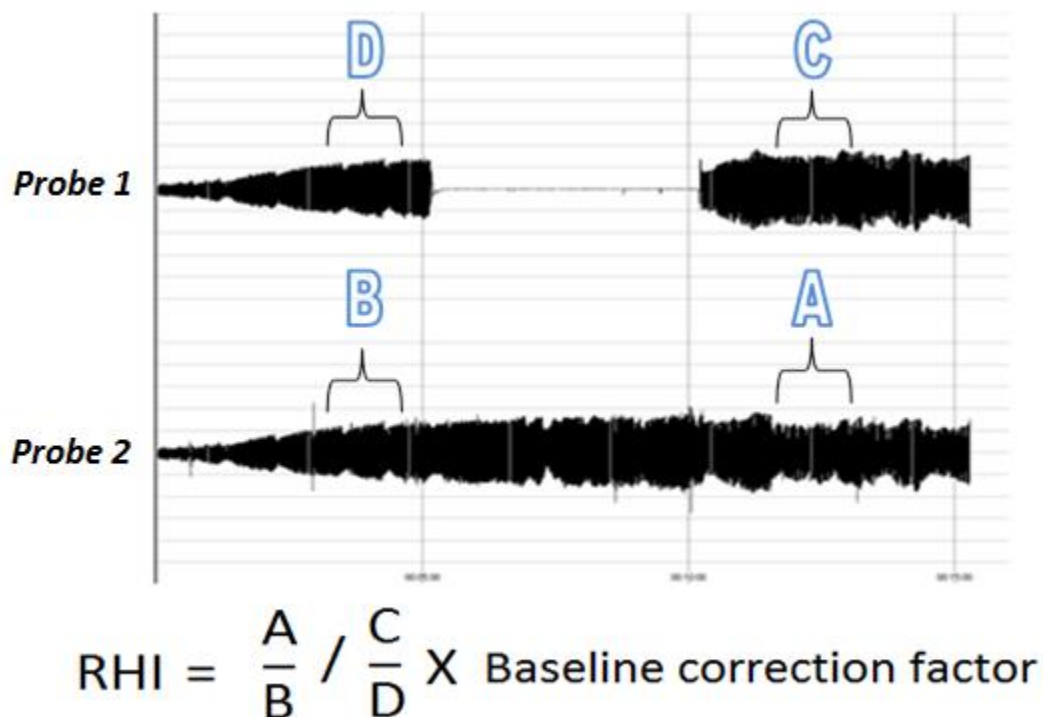
Figure 2.7.4.3. A diagrammatic representation of the Endopat RHI measurement. The letters, A, B, C and D represent the mean amplitude of the peripheral arterial tone measurements in the control arm (probe 2) and test arm (probe 1).

2.9.2. Methods

2.9.2.1. Measurement of Augmentation index and Pulse wave velocity

AI and PWV measurements were both taken in triplicate via a Vicorder device (Skidmore Medical). Before the beginning of the measures the measurements were explained in full to participants. Participants were then asked to lie down in a supine position for period of 15 minutes in order to ensure the measures were taken at rest and to allow full acclimatization to room temperature (21 to 22°C). Subsequent to this 15 minute resting period, resting blood pressure measurements were taken via an Omron 750 CP-II blood pressure measuring device. The Vicorder arm cuff was then attached half way between the elbow and the shoulder over the brachial artery in order to take the measure of augmentation index. AI was calculated via the Vicorder software by analysis of the blood pressure wave form. Specifically, Augmentation Index was calculated from the ratio of augmentation pressure to pulse pressure, as detailed in section 2.9.1. The measures were repeated in triplicate with a 1 minute interlude between each measure.

Subsequent to the Augmentation Index measures, measures of PWV were taken via placement of two blood pressure cuffs at the carotid artery (neck) and at the femoral artery (thigh) respectively. To ensure adequate recording of the pressure wave form at the carotid artery, volunteers were first asked to sit up and to turn to face the opposite side of the room as the PWV measurer so as to expose the carotid artery for optimal placement of the carotid artery pressure cuff. Volunteers were then asked to lie down in a supine position and to bend the knee on the same side as the carotid artery cuff placement. The femoral artery cuff was then placed as close to the hip as possible. A measurement of length was then taken from the upper sternal notch to the mid-point of the femoral artery cuff in order to approximate the difference in length by which the pulse wave would have to travel. Participants were then asked to lie down and to breathe gently for the period of measurement. These measurements were taken 3 times with the average of the three measures used as the PWV input. This average measure of length was then utilised by the Vicorder software to output the PWV measurement. PWV measurements were taken in triplicate with a 1 minute interlude between each measure.

2.9.2.2. Measurement of Endopat Reactive Hyperaemia Index

Endopat Reactive Hyperaemia Index (RHI) was measured by arterial tonometry via an Endopat device (Itamar Medical Ltd).

The RHI measurement was taken subsequent to the PWA and PWV measurements. Participants once again remained in a supine position for this measurement. In brief it was explained to participants in

lay terms that the measurement would take 15 minutes in total, with 5 minutes at rest, 5 minutes with a brachial occlusion cuff inflated and subsequently another 5 minutes at rest. It was explained to participants that the brachial occlusion cuff would be quite tight and that they might feel some numbness or tingling akin to "pins and needles" and that this sensation would subside once the cuff was removed. Participants were also reminded to remain as still as possible during the duration of the measurement.

Before the measurement, participant's index fingers were checked in order to ensure that their fingernails did not protrude more than 1mm beyond the tip of their finger. Once this had been confirmed finger tips were checked to ensure they were dry and free of any dirt. If this was not the case fingertips were washed and dried with wet and dry paper cloth prior to the measurement. Once these checks were completed, participants were asked to insert their index fingers into the uninflated Endopat finger probes which were inserted into a probe place holder. An occlusion cuff was then attached to the participant's left upper arm. Once the measuring apparatus was fully prepared the measure was started by inflating the Endopat finger probes. The participant was then asked to remove their index fingers from the probe place holder, the place holder was then slid back so as to allow participants to rest their index fingers freely over the edge of the of the place holder (as seen below in Figure 2.9.2.2).

Figure 2.9.2.2. Depiction of the correct positioning of arm at rest during Endopat measurement.

Once fingers were placed at rest and proper probe placement was checked, the participant was informed that the 15 minute measurement period was to commence. At the 5 minute mark the brachial occlusion cuff was inflated to either 200mmHg or 60mmHg above resting systolic blood

pressure for 5 minutes. Preference was given to the higher of these two pressures. After a period of 5 minutes the brachial occlusion cuff pressure was release and the arterial tonometry measure was taken for a further 5 minutes. Once completed the fingers probes and occlusion cuffs were removed and the participant was informed that the measurement period was completed.

2.9.2.3. Measurement of post prandial Endopat RHI measurement in response to nitroglycerin administration

During the battery of vascular measurements at the 4 hour postprandial time point an additional measurement of RHI in response to nitroglycerin administration was taken. This measure was taken as described in Section 2.9.2.2, with the exception that a 400µg dose of a standard nitroglycerine spray was administered by trained nursing staff immediately prior to the measurement. Nitroglycerine (a NO donor) was administered in order to blind any effect of endogenously produced NO so that we could investigate any NO independent differences in the RHI measure in response to treatment.

2.10. Biochemical analysis of biological samples at baseline and in response to intervention

2.10.1. Materials

All materials were obtained from Sigma (Dorset, UK) unless noted otherwise.

2.10.2. Methods

2.10.3.1. Blood sample processing at assessment visits

Blood draws were taken at baseline (0H) and at 4 hours postprandially on study days. These blood draws were taken immediately following the barrage of vascular measurements and were always undertaken on the left arm. An overview of the order of these blood draws and the various plasma and blood treatments utilised immediately following the blood draw are detailed in Table 2.10.3.1.

Vacutainer order	Treatment
#1 – Lithium Heparin (Ice Cold)	The vacutainer was spun at 3300rpm at 4°C for 10 minutes. 30µL of plasma was then removed and mixed with 70 µl of Tris–HCl (100 mM, pH 9.5, 0.1 mM DTPA), and 50 µl of MBB solution (10 mM, in CH_3CN) for analysis of plasma H_2S levels. Samples were stored at -80°C until analysis.
#2 - Lithium Heparin (Ice Cold)	The vacutainer was spun at 3300rpm at 4°C for 10 minutes. Plasma was then alliquoted into labelled tubes for analysis of biomarkers pertaining to vascular function. Alliquots for the analysis of labile biomarkers (such as NO and its oxidation products) were snap frozen and placed in the dark immediately. Samples were stored at -80°C until analysis.
#3 and 4 - Lithium Heparin	The vacutainer were spun at 3300rpm at 4°C for 10 minutes and plasma stored at -80°C until analysis.
#5 and 6 - Serum	The vacutainer was left to sit at room temperature for 30 minutes in order to facilitate coagulation of blood. The vacutainer was then spun at 3300rpm at 4°C for 10 minutes. Samples were stored at -80°C until analysis.
#7, 8, 9 and 10 – EDTA	These vacutainer was spun at 3300rpm at 4°C for 10 minutes. Plasma was then alliquoted into labelled tubes for analysis of biomarkers pertaining to vascular function. Aliquots for the analysis of potentially labile biomarkers were snap frozen and placed in the dark immediately. Samples were stored at -80°C until analysis.
#11 – Lithium Heparin (only taken postprandially)	1ml of whole blood was utilised for whole blood culture as detailed in Chapter 4.

Table 2.10.3.1. Blood draw treatment details.

2.10.3.2. Serum Triglyceride and NEFA quantification at baseline and in response to intervention

Serum triglyceride and NEFA levels were determined in plasma samples generated during the intervention period via spectrophotometric analysis in an ILAB 650 (Instrumentation Laboratory UK Ltd). Both analyses are based on the formation of quinoneimine from Hydrogen Peroxide whose production is coupled in these assays to the presence of triglycerides and NEFA. Quinoneimine is measured in this assay at 510nm and 700nm and the absorbance at these wavelengths is proportional to the amount of Hydrogen Peroxide produced, which in turn is dependent on the amount of triglycerides and NEFA present. Accordingly, these assays allowed us to measure the concentration of triglycerides and NEFAs in our samples.

The ILAB 600 was calibrated every 14 days, or in the cases where an analysis fell greater than 14 days after the previous calibration, before analysis. Calibration was undertaken via utilisation of a Referr IL G Calibrator (Instrumentation Laboratory UK Ltd). The Referr II G Calibrator is a multicomponent calibrator for which triacylglycerol values had been pre-established by the supplier.

Serachem Control Level 1 and Serachem Control Level 2 (instrumentation Laboratory UK Ltd) were utilised as quality controls in order to ensure the accuracy and precision of the analysis. Serachem controls were constituted from lyophilized bovine serum with triacylglycerol concentrations lower (Serachem Control Level 1) and higher (Serachem Control Level 2) than observed concentrations in serum isolated at screening. Accordingly, these quality controls allowed us to ensure the concentrations established for the screening samples were within the range of linearity for this analysis.

Once calibration and quality controls were established and the coefficient of variation was confirmed to be under 2.5% samples were added to cuvettes for quantification of total cholesterol by measuring absorption at 510nm and 700nm and correlating this against a standard curve previously established during calibration.

2.10.3.3. Serum Glucose analysis at baseline and in response to intervention

Serum triacylglycerol levels were determined in serums samples generated during the intervention period via spectrophotometric analysis in an ILAB 650 (Instrumentation Laboratory UK Ltd). The quantification of Glucose was based on an endpoint, bichromatic hexokinase based methodology. Specifically, hexokinase catalyzes the phosphorylation of hexoses including glucose and subsequently glucose-6-phosphate dehydrogenase catalyses the formation of 6-phosphogluconate, NADH and a lone proton from Glucose-6-Phosphate and NAD^+. The production of NADH, which has an absorbance peak at 340nm, is dependent and directly proportional to the concentration of glucose in a 1:1 ratio. Accordingly, the quantification of glucose in serum was based on an increase in absorbance at 340nm.

The ILAB 600 was calibrated every 14 days, or in the cases where an analysis fell greater than 14 days after the previous calibration, before analysis. Calibration was undertaken via utilisation of a Referr IL G Calibrator (Instrumentation Laboratory UK Ltd). The Referr II G Calibrator is a multicomponent calibrator for which triacylglycerol values had been pre-established by the supplier.

Serachem Control Level 1 and Serachem Control Level 2 (instrumentation Laboratory UK Ltd) were utilised as quality controls in order to ensure the accuracy and precision of the analysis. Serachem controls were constituted from lyophilized bovine serum with glucose concentrations lower (Serachem Control Level 1) and higher (Serachem Control Level 2) than observed concentrations in serum isolated at screening. Accordingly, these quality controls allowed us to ensure the concentrations established for the screening samples were within the range of linearity for this analysis.

Once calibration and quality controls were established and the coefficient of variation was confirmed to be under 2.5% samples were added to cuvettes for quantification of total cholesterol by measuring absorption at 510nm and 700nm and correlating this against a standard curve previously established during calibration.

2.10.3.4. Plasma nitrite analysis at baseline and in response to intervention

Plasma nitrite levels were determined using a chemiluminescence detector (CLD88), designed to measure levels of nitric oxide in an inert carrier gas. This detector was utilised by injecting plasma samples into a purge vessel containing reductive solutions, designed to reduce nitrite (NO_2^-), nitrate (NO_3^-) and other oxidative by products of nitric oxide, back to nitric oxide for measurement. It is known that both nitrite and nitrate can be reduced *in-vivo* to produce nitric oxide. Accordingly, due to the short lived nature of nitric oxide, measurement of nitrite, nitrate and other oxidation products can be utilised as a good proximal measure of *in-vivo* nitric oxide production.

For measurement of nitrite, a triiodide solution was utilised. Specifically, a 45mM solution of I^- and 10mM solution of I_2 was dissolved in a 93% glacial acetic acid as per Freelisch (159). Nitrogen gas was passed through the reductive solution to carry nitric oxide through to a scrubbing bottle containing sodium hydroxide (1M at 0°C) and finally, through a sampling probe and the detector. The scrubbing bottle was utilised in order to trap traces of acid and iodine before transfer into the detector. To maintain consistent results at trace level range nitrogen flow was kept constant throughout the entire measurement cycle. This was achieved by utilisation of a pressure gauge to ensure flow rates between the scrubbing bottle and sampling probe remained constant.

A standard curve ($R^2>0.99$) was established by integration of peaks generated after injection of solutions of Sodium Nitrite ranging from 100nM to 0nM. Once coefficient of variation for standards, and plasma quality controls was confirmed to be under 10%, standards were run with duplicate injections. The triiodide solution was replaced every 40 injections, under our conditions this yielded optimal reproducibility for peaks derived from both standards and samples. Plasma nitrite concentrations in samples were generated via utilisation of standard curves. A representative standard curve is shown in Annex 4, Figure B.

2.10.3.5. Plasma Hydrogen sulfide analysis at baseline and in response to intervention

Plasma H_2S concentrations were determined via utilisation of monobromobimane (MBB) to derivitise Hydrogen Sulfide to sulphide dibimane (SDB). This protocol was adapted from publications from Winter *et al* (160) and Shen *et al* (161) and was developed and validated as part of this PhD project. Specifically, this methodology utilises the reaction between MBB and hydrogen sulfide under basic conditions at room temperature to produce SDB. SDB contains two bimane cores, and due to bimane being hydrophobic, SDB is more hydrophobic than most physiological thiols. This characteristic allows SDB to be separated by reversed phase-HPLC with a gradient elution. Subsequently, SDB can be quantified via flouresence detection.

On each study day, free plasma H_2S was derivitised to SDB immediately after centrifugation and separation of the plasma. In short, 30µL of plasma was added to 70µL of Tris-HCl (100 mM, pH 9.5, 0.1mM DTPA) and 50µL of MBB solution (10 mM, in CH_3CN) in an eppendorf tube wrapped in tin foil, as bromobimanes are sensitive to photolysis. The solution was incubated for 30 minutes at room temperature, before addition of 50µL of ice cold sulfosalicylic acid (200mM). Sulfosalicylic acid was added to stop the reaction and to precipitate any protein. The mixture was then placed on ice for ten minutes before centrifugation at 12,000 rpm at 4 °C for 10. Supernatant was removed and stored at -80°C until analysis. At analysis, standards and samples were run in an Agilent C:18 4.6x250mm column (particle size, 5µm) under the conditions detailed in Figure 2.10.2.4.

Time (min)	% Phase A	% Phase B
0	85	15
5	65	35
16	45	55
23	30	70
24	10	90
26	10	90
28	85	15
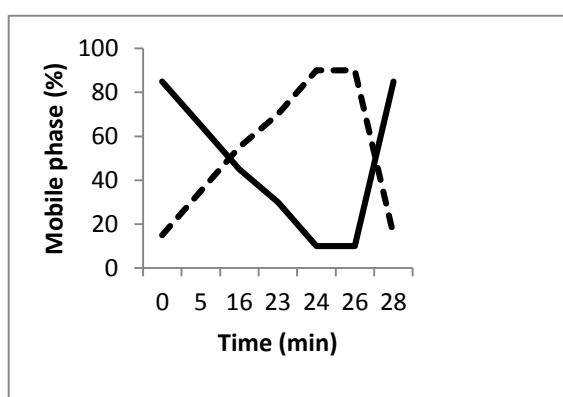
Figure 2.10.2.4. Mobile phase gradient for the quantification of H_2S derivitised SDB. Phase A was made up of 99.9% H_2O + 0.1% TFA (v/v). Phase B was made up of 99.9% CH_3CN + 0.1% TFA (v/v).

SDB was synthesised in our laboratory by reaction of MBB with excess Sodium Sulfide. SDB was subsequently purified by extraction in ethyl acetate, evaporation under nitrogen stream and subsequent re-constitution in HPLC grade water, as described by Shen *et al* (161). Purity of SDB was

subsequently assessed by qualification of a single absorbance peak between 300 and 800nm at 380nm consistent with reports by Shen *et al*. Under the conditions described in Figure 2.10.2.4, purified SDB in HPLC grade water eluted at 11.6 minutes. We confirmed no matrix effect on retention times by spiking purified SDB in plasma, to qualify SDB elution in plasma samples at 11.6 minutes. SDB concentration in our samples was subsequently calculated from a standard curve, generated from reaction of known quantities of Sodium Sulfide with MBB. Quantification of plasma H_2S concentrations was undertaken using a standard curve with R^2 values greater than 0.99. A standard curve is shown in Annex 4, Figure C. A weakness of the analysis is that the range for the standard curve utilized extended beyond the biological range of plasma H_2S concentrations. Previous work utilizing this method to quantify circulating H_2S concentrations has utilized standard curves of similar range, which have shown to be linear (160). Although the graph utilized in our study had R^2 values greater than 0.99, the possibility remains that a standard curve generated specifically within the biological range of our analysis may have differing slope than the standard curve utilized in our analysis. It should be noted that this is a weakness of the current analysis which should be addressed in future analyses.

Limit of detection for analysis was below 0.2µM as previously reported by Wintner *et al* (160). Inter assay and intra assay coefficient of variation for spiked samples and standards was below 10%.

2.10.3.6. Plasma fatty acid analysis

Total lipids were extracted from 500µl of plasma with chloroform/methanol (2:1 v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant (162). The organic solvent was evaporated under a stream of nitrogen and the lipid content was determined gravimetrically. Total lipid fraction (TL) was subjected to acid-catalyzed transmethylation for 16 h at 50 ºC, using 1 ml of toluene and 2 ml of 1% sulphuric acid (v/v) in methanol. The resultant fatty acid methyl esters (FAME) were purified by thin layer chromatography (TLC), and visualised under spraying with 1% iodine in chloroform. After elution, FAME were separated and quantified by gas-liquid chromatography using a Hewlett Packard 5890 GC and a SGE BPX70 capillary GC column (30 m x 0.22 mm I.D.; SGE UK Ltd) with helium as carrier gas and using on-column injection. The temperature gradient started at 115 °C for 3 min, then went to 200 °C at 2 °C/min, 2 minutes at 200 °C, and then to 240 °C at 60 °C/min. After 5 minutes at 240°C, it cooled to 115 °C and equilibrated for 3 minutes before the next injection. Individual methyl esters were identified by reference to authentic standards and to well-characterized fish oil (PUFA-3 from menhaden oil, SUPELCO, Supelco Park, Bellefonte, USA). Data were collected and processed using GC Chemstation (version B04-02).

2.10.3.7. Plasma analysis of fatty acid epoxides and diols at baseline and in response to intervention

Plasma samples were spiked with the corresponding deuterated internal standards (Cayman Europe, Tallinn, Estonia) and lipid epoxide and diol derivatives were extracted twice into ethyl acetate (0.5 ml). After evaporation of the solvent in a vacuum block under a gentle stream of nitrogen, samples were reconstituted with 50µl of methanol-water (1:1, vol/vol). Lipid concentrations were subsequently determined with a Sciex API4000 mass spectrometer operating in the multiple reaction monitoring mode (163, 164). For the chromatographic separation a Gemini NX C18 column and precolumn were used (150 mm × 2 mm i.d, 5µm particle size and 110Å pore size (Phenomenex Aschaffenburg, Germany). A linear gradient was employed at a flow rate of 0.5 ml/min mobile phase with a run time of 18.5 minutes. Mobile phase A was water/ammonia (100:0.05, v/v) and B acetonitrile/ammonia (100:0.05, v/v). The gradient started from 85% A to 10% within 12 min. This was held for another 1 min at 10% A. Within 0.5 min the mobile phase shifted back to 85% A and was held for 4.5 min to equilibrate the column for the next sample. The injection volume of samples was 20µl. Quantification was performed with Analyst Software V 1.4.2 (Applied Biosystems, Darmstadt, Germany) employing the internal standard method (isotope-dilution mass spectrometry). Ratios of analyte peak area and internal standard area (y-axis) were plotted against concentration (x-axis) and calibration curves were calculated by least square regression with 1/concentration^2 weighting. For each lipid a calibration curve from 0.5 to 2500 ng/mL was constructed.

Chapter 3

Screening and clinical results from the Fish Oil Fatty Acid (F.O.F.A) Study and insights into the biochemical basis of LC n-3 PUFA dependent changes in postprandial vascular reactivity

Chapter 3 – Screening and clinical results from the Fish Oil Fatty Acid (F.O.F.A) Study and insights into the biochemical basis of LC n-3 PUFA dependent changes in postprandial vascular reactivity

3.1. Screening of the study population in the F.O.F.A Study

3.1.1. Introduction

In this study we investigated postprandial modifications in vascular tone in response to EPA or DHA consumption in a population estimated to be at a 1.5 relative risk of developing CVD. This population was selected according to the inclusion (and exclusion) criteria detailed in section 2.3 (page 50). This approach was utilised as we reasoned that any EPA or DHA dependent modifications to vascular tone would be of more clinical relevance if observed in a population at an increased risk of cardiovascular disease.

The following section details the results of the screening process employed in the study and subsequent discussion on the results of this screening criteria.

3.1.2. Screening results
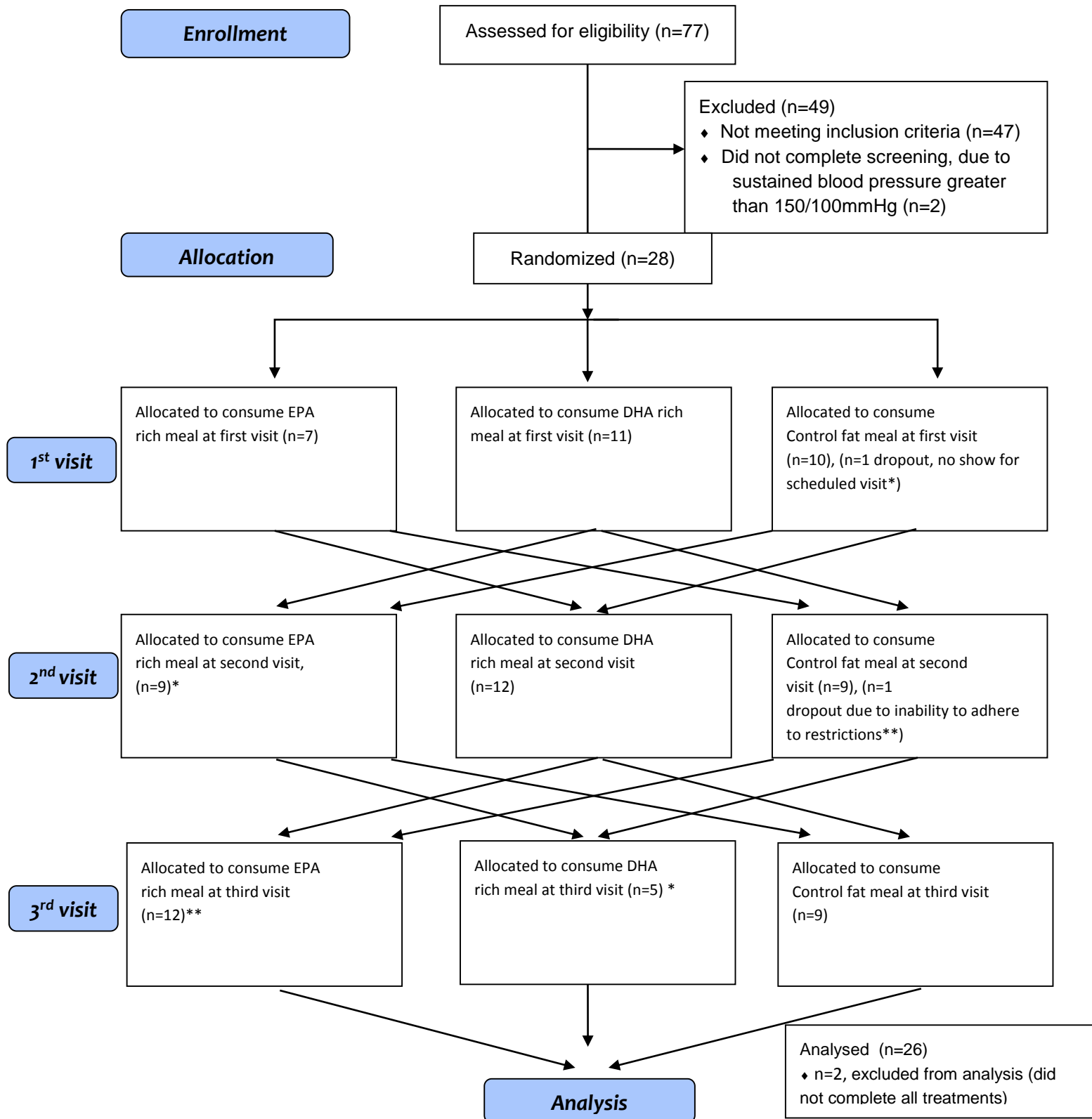
Figure 3.1.2.1. Consort diagram detailing trial enrolment, allocation, randomisation of treatments and data included in analysis.

Total cholesterol and HDL-cholesterol screening results

Median total cholesterol levels are presented below (Figure 3.1.2.2) for all individuals screened, volunteers deemed non-eligible and our study population. The average total cholesterol concentration of the study participants (5.95mmol/L) was 11% higher than that for all individuals screened (5.36mmol/L) and 19% higher than the average for individuals who were deemed ineligible (4.99mmol/L).

Median HDL-cholesterol levels are also presented below (Figure 3.1.2.2) for all individuals screened, volunteers deemed non-eligible and our study population. HDL-cholesterol levels were 5% and 3% lower in our study participants when compared to the mean for non-eligible individuals and the entire group who were screened.
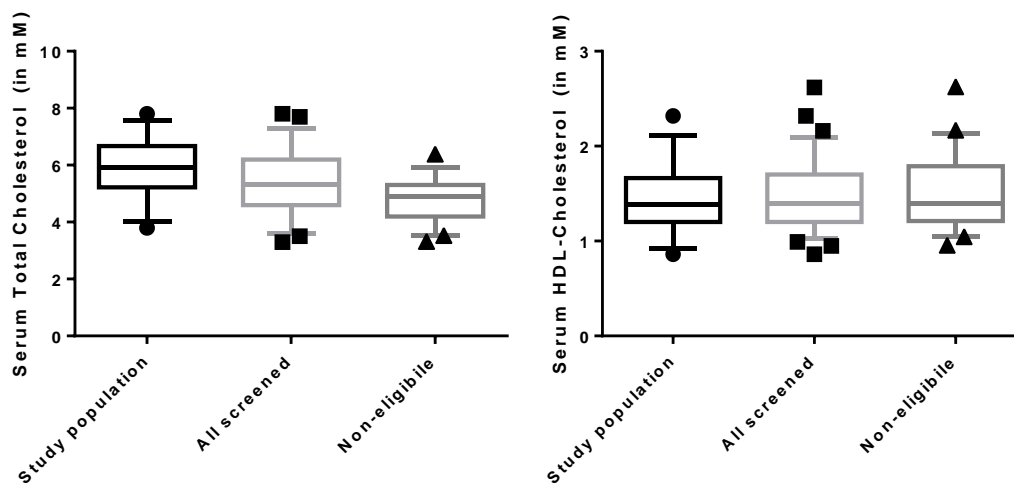
Figure 3.1.2.2. Box-plots of 5th and 95th percentiles showing median values and outliers (<5th percentile or >95th percentile) for total cholesterol and HDL-cholesterol (high density lipoprotein cholesterol) in non-eligible (n=47), all individuals screened who proceeded for blood cholesterol tests (n=75) and eligible participants (n=26). Data for n=2 dropouts is not presented.

Screening blood pressure values

Median systolic blood pressure values are shown below (Figure 3.1.2.3) for all individuals screened, volunteers deemed non-eligible and our study population. The average systolic blood pressure values at screening for the study population was 136mmHg, which was 5.3% and 10.0% higher relative to the average for all individuals screened and ineligible individuals respectively.

Median diastolic blood pressure values are also shown below (Figure 3.1.2.3) for all individuals screened, volunteers deemed non-eligible and our study population. The average diastolic blood pressure values in our study population was 86mmHg, which was 5.5% and 10.5% higher relative to averages for all individuals screened and ineligible individuals.
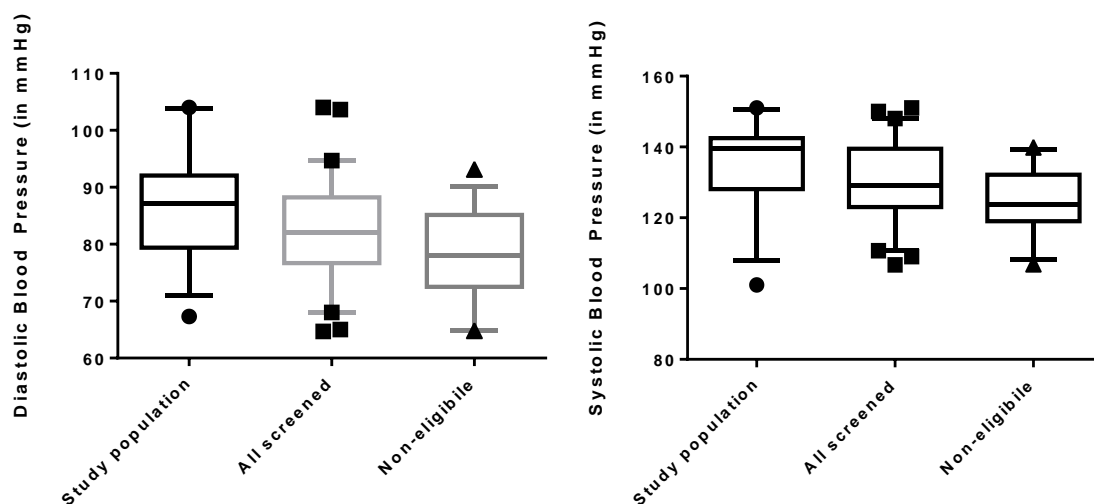
Figure 3.1.2.3. Box-plot of 5th and 95th percentiles showing median values and outliers (<5th percentile or >95th percentile) for systolic and diastolic blood pressure in non-eligible (n=47), all screened (n=77) and study population (n=26). Data for n=2 dropouts is not presented.

Study population characteristics	
Age	45 +/- 5 (36 - 54)
Total cholesterol (mmol/L)	5.84 +/- 0.90 (3.8-7.3)
HDL-cholesterol (mmol/L)	1.42 +/- 0.28 (0.86 – 2.32)
Systolic Blood pressure (mmHg)	136 +/- 10 (101-150)
Diastolic Blood pressure (mmHg)	86 +/- 7 (67.33 – 104)
Weight (kg)	87.07 +/- 11.51 (73.5 - 129.4)
BMI (kg/m^2)	27.4 +/- 3.3 (20.4 – 39.9
Waist circumference	95.5 +/- 10.4 (76.8 – 128.0)

Table 3.1.2. Study population characteristics with range. Data are presented as mean±SEM.

3.1.3. Discussion

The aim of the screening exercise in this study was two-fold; to select study participants that were at an increased relative risk of cardiovascular disease relative to the general population and to exclude individuals with conditions or lifestyle practices that might interfere with accurately establishing nutrient dependent changes in vascular tone. Our rationale for these inclusion criteria was that the clinical significance of any nutrient dependent changes in vascular tone would be greater in a population with pre-clinical risk factors for CVD.

When compared to the average population values for total cholesterol in males in the UK, our study participant's average total cholesterol levels were 8.1% higher.

With reference to HDL-cholesterol results presented in Figure 3.1.2.2, we were successful in selecting for a population with lower HDL-C levels relative to the full screening population. The average study population HDL-C concentrations did not fall below 1mmol/L and accordingly, it is unlikely that the HDL-C values in this population were low enough to deem the population to be at an increased relative risk of CVD on the basis of their HDL-C values alone.

As can be seen in figures 3.1.2.2 and 3.1.2.3, both systolic and diastolic blood pressure measures were higher in our study group relative to the excluded individuals and the whole screening population. For SBP, the average value was 135mmHg. When compared to average population values for males between the ages of 19-64 years in the UK, average systolic blood pressure values in our study population were 6.1% higher (165). Similarly, average diastolic blood pressure values of 86mmHg in our study population were 14.9% higher than the expected average populational levels for males between the ages of 19 and 64 the UK. Accordingly, when viewed together it can be deemed that our screening criteria was successful in selecting a population with clinically significant increases in three of the most well established traditional cardiovascular risk factors (total cholesterol, systolic blood pressure and diastolic blood pressure), but without overt health complications.

3.2.   Vascular measures at baseline and in response to intervention

3.2.1. Introduction

As detailed in Chapter 1, the effect of LC n-3 PUFA intake on CVD risk has been studied extensively. Evidence exists to suggest anti-arrhythmic (35), hypolipidemic (36-39), anti-inflammatory and plaque stabilising (41) and cholesterol profile modulating (42, 43) effects for the LC n-3 PUFA. One weakness in these investigations is that, due to the lack of availability of pure sources of EPA and DHA, EPA and DHA have traditionally been grouped together in investigations of their effects on human health. Evidence is beginning to emerge that these two dietary fats may have differential effects on human health, with specific reference to their effects on vascular tone and blood pressure. Specifically, chronic intervention studies have suggested a potential hypertensive effect for DHA but not EPA (40), which corresponds with a capacity for DHA, but not EPA, to modulate fasting vascular tone (57).

A number of studies have also investigated the effect of EPA and DHA on postprandial vascular tone. As mentioned previously, the postprandial state is of particular physiological relevance due to the fact that individuals spend the majority of the day in the postprandial state. Recent studies in this context have shown that mixed doses of EPA and DHA beneficially modulate postprandial vascular tone (58-60) and that DHA but not EPA reduces systemic vascular resistance in the postprandial state (61).

A current research gap is establishing the relative efficacy of EPA and DHA to modulate post-prandial vascular tone in at risk populations. We hypothesise that DHA may be more efficacious than EPA in modulating postprandial vascular tone. Accordingly, a primary aim of this project was to elucidate the relative impact of EPA versus DHA on postprandial vascular tone in a population at an increased risk of CVD.

As mentioned in Chapter 2 and as detailed in Section 2.9.1, a number of vascular measures were chosen in order to investigate the effects of EPA and DHA meals on vascular tone and function in our study. These measures included Augmentation index, Pulse Wave Velocity, Endopat and Endopat*GTN methodologies.

This section details the results of these investigations on the effects of high fat meals containing EPA and DHA on vascular tone.

3.2.2. Statistical Analysis

All data are presented as mean +/- standard error of the mean. Statistical analysis was undertaken using the SPSS statistical software (version 22; Chicago, Illinois, USA). Prior to analysis all datasets were checked for normality via visual inspection of normal Q-Q Plots. Outliers were identified by analysis of normal Q-Q Plots and variance in values greater than +/- two standard deviations. Repeated measures ANOVA was utilised in order to quantify the significance level for time, treatment and time*treatment interactions for measures. For Repeated Measures ANOVA, assumptions of sphericity were assessed via Mauchly's Test of Sphericity. In cases where the assumptions of sphericity were not met a Greenhouse-Geisser correction was applied. In cases where significance was reached via initial Repeated measures ANOVA, subsequent *post-hoc* analysis was undertaken in order to investigate treatment dependent changes from baseline using repeated measure ANOVA and Bonferroni adjustment on change scores.

For Endopat*GTN investigations a reduced sample size of n=20 was utilised due to loss of data due to electricity cuts (n=1), patient discomfort in response to nitroglycerine treatment (n=2) and errors in Endopat software reporting output in response to GTN stimulus for some individuals (n=3).

3.2.3. Results

3.2.3.1.   Pulse Wave Analysis results

Analysis showed that the DHA meal significantly improved AI when compared to control.

Specifically, repeated measures ANOVA showed a significant treatment*time interaction (p=0.005, $F(2, 50)$ = 5.88, partial η^2 = 0.19), time (p<0.01, $F(1, 25)$ =40.63, partial η^2 = 0.62) but not treatment (p=0.81, $F(2, 50)$ = 0.21, partial η^2 = 0.00) effect.

Subsequent *post-hoc* analysis on change from baseline showed that the DHA meal significantly decreased mean AI measures by 3.19% when compared to the Control meal (95% CI, 0.03 to 6.35%, *p*=.047). AI is itself measured in percentage and accordingly, the noted 3.19% change is equivalent to a 13.3% improvement for the DHA treatment when compared to control. The EPA treatment did not significantly decrease mean AI when compared to the control meal (95% CI, -.119 to 5.986%, *p*=.063). Likewise, there were no significant differences between EPA and DHA treatments (95% CI, -2.435to 1.927%, *p*=1.000).
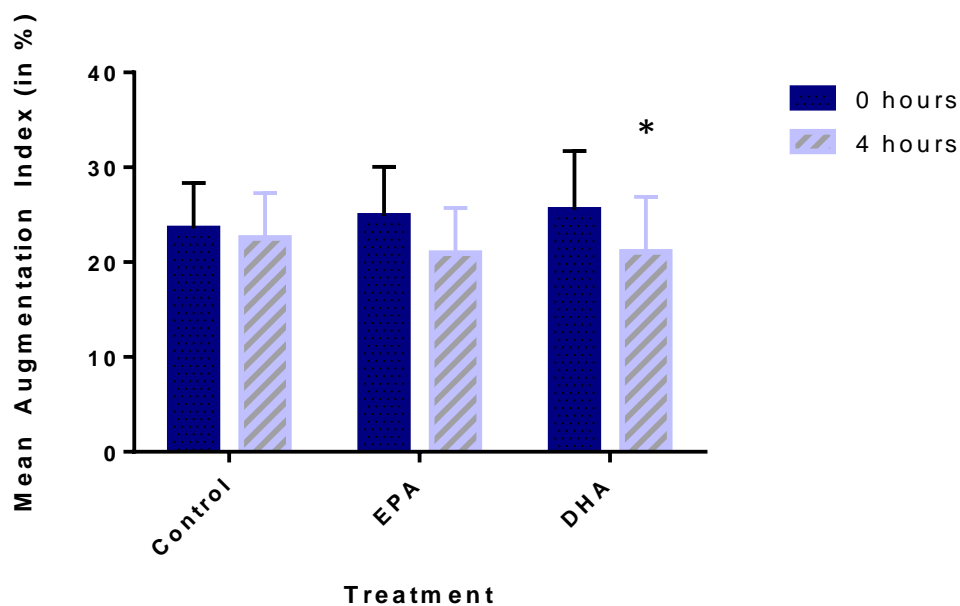
Figure 3.2.3.1. Mean Augmentation Index values (in %) measured at baseline (0 hours) and 4 hours postprandially in response to intervention. Asterisks indicates a significant difference (* p<0.05) in change from baseline in Augmentation Index relative to control. Data are presented as mean±SEM.

3.2.3.2. Pulse Wave Velocity results

Intervention had no effect on PWV. In this analysis, the assumption of sphericity for the treatment*time interaction was violated, as assessed by Mauchly's Test of Sphericity, $\chi^2(2) = 7.362$, $p = 0.025$, accordingly a Greenhouse-Geisser correction was applied ($\varepsilon = 0.791$).

Analysis showed no significant treatment*time interaction, (p=0.32, $F(1.58, 39.58) = 1.15$, partial $\eta^2 = 0.04$), treatment (p=0.15, $F(2, 50) = 1.98$, partial $\eta^2 = 0.07$) or time (p=0.85, $F(1, 25) = 0.04$, partial $\eta^2 = 0.00$) effect.
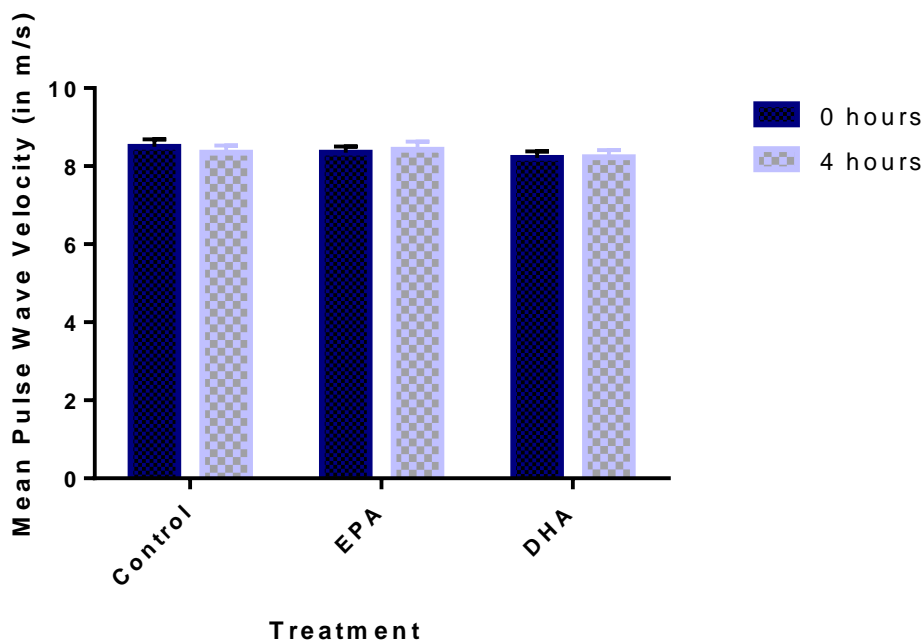
Figure 3.2.3.2. Mean Pulse Wave Velocity values (in m/s) measured at baseline (0H) and 4 hours postprandially in response to intervention. Data are presented as mean±SEM.

3.2.3.3. Endopat results

None of the intervention meals impacted the Endopat RHI measure.

Specifically, repeated measures ANOVA showed no significant treatment*time interaction for the RHI measure (p=0.92, $F(2, 48)$ = .082, partial η^2 = 0.003), or time (p=0.75 , $F(1, 24)$ = 0.10, partial η^2 = 0.00) or treatment (p=0.56, $F(2, 48)$ =0.59, partial η^2 = 0.02) effect.
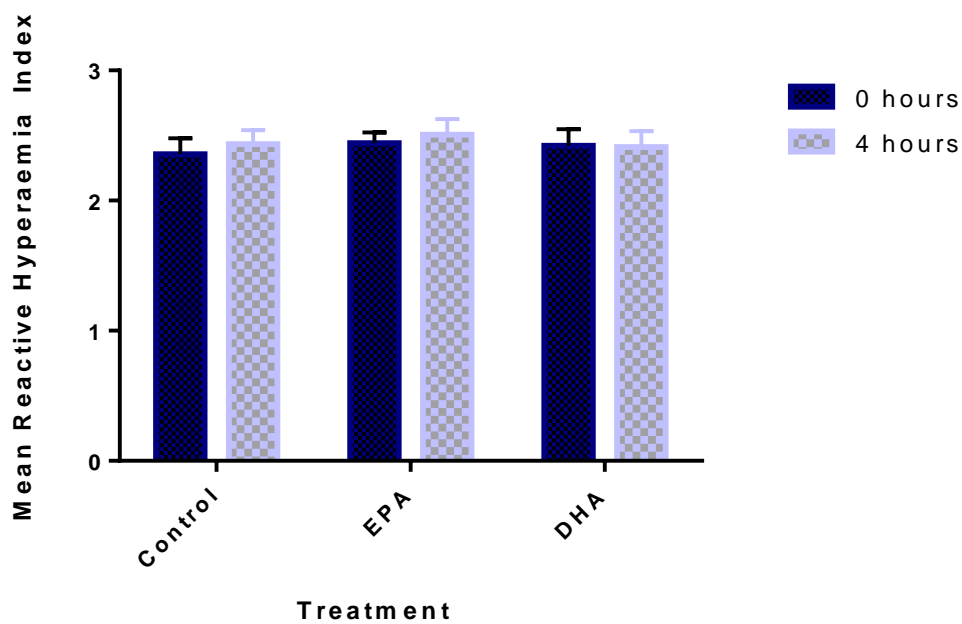
Figure 3.2.3.3. Mean Reactive Hyperaemia values measured at baseline (0H) and 4 hours postprandially in response to intervention. Data are presented as mean±SEM.

3.2.3.4. Endopat*GTN results

Repeated Measures ANOVA showed no significant effect for treatment (p=0.59, $F_{(2, 38)}$=0.55, partial η^2 = 0.03), accordingly post-hoc analysis was not undertaken.
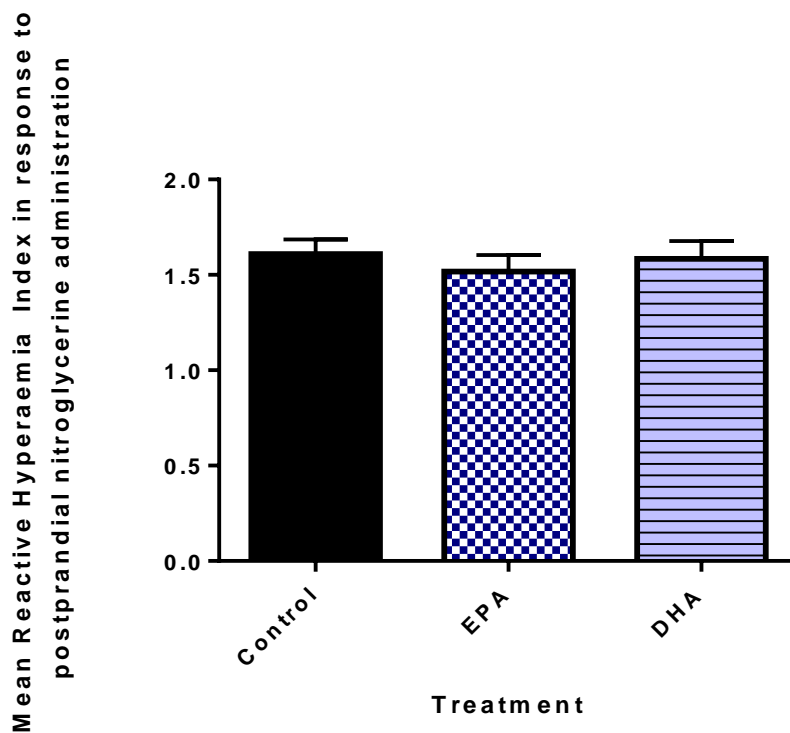
Figure 3.2.3.4. Mean Reactive Hyperaemia values measured in response to nitrogylcerine (400µg) administration at 4 hours postprandially. Data are presented as mean±SEM.

3.2.3.5. Blood pressure results

Interventions had no effect on systolic or diastolic blood pressure.

In this analysis, the assumption of sphericity was violated for the treatment effect for systolic and treatment*time interaction for diastolic blood pressure. Accordingly, Green-Geisser corrections were applied (ε = 0.79 and 0.76 respectively).

Analysis showed no significant treatment*time interaction (p=0.38, $F(2, 50)$ = 0.98, partial η^2 = 0.04), treatment (p=0.09, $F(2, 50)$ = 2.54, partial η^2 = 0.09) or time (p=0.93, $F(1, 25)$ =0.08, partial η^2 = 0.00) effect for systolic blood pressure. Similarly, analysis showed no significant treatment*time interaction (p=0.15, $F(1.51, 37.82)$ = 2.05, partial η^2 = 0.076), time (p=0.25, $F(1, 25)$ = 1.38, partial η^2 = 0.05) or treatment (p=0.35, $F(2, 50)$ =1.07, partial η^2 = 0.04) effect for diastolic blood pressure.
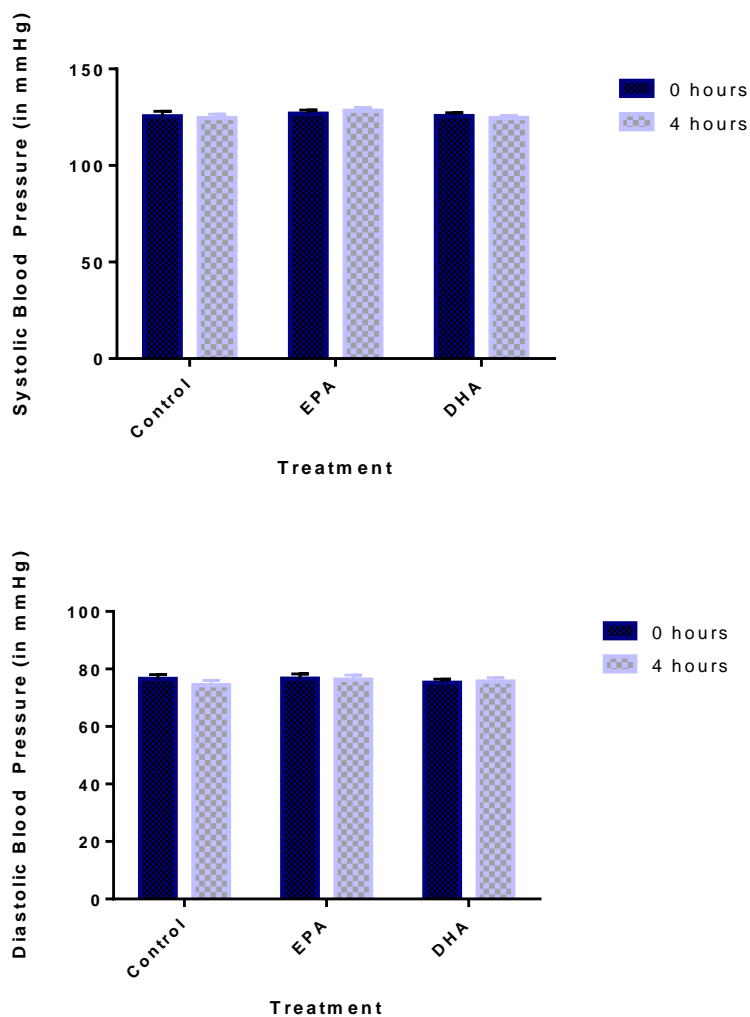
Figure 3.2.3.5. Mean diastolic blood and systolic blood pressure values measured at baseline and in response to intervention. Data are presented as mean±SEM.

3.2.4. Discussion

Our results indicate that although EPA and DHA both improve AI measures postprandially, this effect only reaches significance for DHA and this is reflective of a greater effect size for DHA (13.3% when accounting for changes in response to control meal) when compared to EPA (11.3% when accounting for changes in response to control meal). We did not observe an effect for any of our treatments on PWV, Endopat RHI, Endopat RHI in response to GTN, SBP or DBP. These results are discussed in depth in the following sub-sections.

3.2.4.1.   Discussion of Pulse Wave Analysis results

As shown in Figure 3.2.3.1, Augmentation Index measures decreased postprandially in each treatment. Analysis, showed a significant treatment*time interaction. Accordingly, subsequent *post-hoc* analysis was undertaken in order to investigate treatment dependent changes from baseline using repeated measure ANOVA and Bonferroni adjustment. Testing revealed that the DHA meal significantly improved mean AI measures by 13.3% when compared to the Control meal. Although there was a trend towards an improvement in mean AI measures in response to EPA treatment when compared to the control meal, this effect did not reach statistical significance (p=0.06). It is also worth noting that there were no significant differences between EPA and DHA treatment.

As mentioned in section 2.7.4, the Augmentation Index outcome is dependent on the elastic effects of the entire arterial tree. Changes in these elastic effects is one of the causes of increasing blood pressure with advancing age and in diseases states that increase arterial stiffness prematurely. Augmentation Index has also been shown to decrease acutely upon administration of vasodilatory compounds such as nitroglycerin and increase upon administration of compounds with vasoconstrictive effects, such as angiotensin II (10). Accordingly, it is known that this measure is sensitive to acute changes in vascular tone.

Taken as a whole, this evidence suggests that the inclusion of DHA in a high fat meal improves postprandial vascular reactivity. DHA's bioactivity in this regard is consistent with investigations on the impact of chronic DHA supplementation on fasting vascular tone (166). For example a 6 week intervention of DHA (4g/day) but not EPA (4g/day) enhances vasodilation in the fasting state (57). Specifically, these investigations showed that DHA supplementation improved endothelium independent vasodilatory response as measured by forearm blood flow in response to a number of

agonists and antagonists of vascular tone, including acteylcholine+L-NNMA, nitroprusside and norepinephrine but not L-NNMA alone.

Our evidence corroborates the results in our group's previously published paper that showed inclusion of fish oil containing both EPA and DHA in a high fat meal had a positive effect on microvascular tone as assessed by Laser Doppler Iontophoresis (58). Furthermore, our study corroborates investigations by Chong *et al* (59), which have reported that inclusion of EPA (2.0g) and DHA (2.7g) in a high fat meal (30g of palm oil and soy bean oil in a 4:1 ratio) improves AI and Digital Volume Pulse-stiffness index in both males and females. Our findings are also in part consistent with those of Rontoyanni *et al*, who showed that the consumption of DHA (4.7g) but not EPA (4.7g) in a high fat meal impacts exercise induced changes in systemic vascular resistance (SVR), but does not impact changes to SVR at rest 5 hours postprandially (61). In this study a finger arterial BP monitor, namely the Finometer, was used to estimate SVR from mean arterial pressure divided by cardiac output. Finometer strength lies in its capacity to accurately measure of blood pressure during exercise (167). However, it has been noted that arterial pressure waveforms distort between brachial and finger arteries and accordingly, correction, through utilisation of a waveform filter, is required to improve systolic blood pressure tracking with this method. Accordingly, in this context AI can be considered more sensitive in the information it yields on changes to vascular tone and wave reflection. Further to this, AI has been shown to be associated with increased European Society of Cardiology (ESC) risk levels (9). However, it should be noted that AI as measured in our study, in contrast to Finometer measurement of SVR, is not an appropriate methodology for use in the context of exercise.

It should also be noted that our results contrast somewhat with a recently published paper by Purcell *et al* (62), which has indicated that neither inclusion of EPA and DHA (5g in a 4:3 ratio) or inclusion of DHA (5g) in a meal high in MUFA (75g of total fat) impacted AI significantly when compared to a high MUFA reference meal in 16 healthy males (62). This study showed no effect for either the EPA and DHA meal, or the DHA meal, when compared to the reference high MUFA meal on AI. A trend for greater decreases in Digital Volume Pulse-Reflective Index (indicative of changes in vascular tone of peripheral resistance arteries) was observed postprandially for the DHA meal, however, these effects did not reach significance. Our investigations differed somewhat in study design which may account for some of the discrepancy between these results. These studies differed with specific reference to sample size (n=14 in the study by Purcell *et al* for the AI analysis as compared to n=26 in our study), study population and meal design. Of these considerations study population and meal design are probably of the most relevance although the impact of study power may also have played a part.

Specifically, as detailed in section 3.1, our study population was comprised of individuals estimated to be at a 1.5 increased relative risk of CVD based on fitting into a profile of being either pre-hypertensive/hypertensive, having central obesity or possessing pre-clinical cholesterolemia, as compared to the study population in the study by Purcell *et al* who were not an "at risk" population but did have increased fasting triglyceride levels relative to our study population (mean SBP, DBP, triglyceride and total cholesterol values were 122 mmHG, 80 mmHg, 1.88mM and 5.25mM respectively in the study by Purcell *et al*). In addition, meal design differed significantly between these studies. This study by Purcell *et al* utilised a meal which contained 75g total fat in each meal, 92.8 g carbohydrate, and 14.4 g protein. This compared to 42.4 g total fat, 73.75g carbohydrate and 17.75 g protein in our study. With regards to meal fat composition, the reference meal in the above cited study contained primarily oleic acid (18:1) whereas our control meal fat content composed of primarily oleic acid, palmitic acid (16:0) and linoleic acid (18:2 n6) (please see Table 2.6.3). Interestingly in the study by Purcell *et al*, a fourth meal high in linoleic acid (18:2 n6) seemed to have a negative effect on postprandial AI when compared to the mixed EPA and DHA, DHA and reference MUFA meal. These results might be suggestive of the presence of a relatively high amount of linoleic acid (22.3% of total fat) in our control meal contributing to the significant differences seen between DHA and control meals in our study. As mentioned previously, previous reports in the literature (59) have shown a capacity for a mixed dose of EPA (2.0g) and DHA (2.7g) in a high fat meal (30g of palm oil and soybean oil in 4:1 ratio) to improve postprandial AI in a high fat meal. Our study corroborates these reports, and suggests that DHA is slightly more efficacious than EPA in its capacity to positively influence postprandial vascular tone. When taken as a whole, the differences in these studies might suggest that individuals may respond differentially dependent on basal blood pressure status and potentially, that the n-6 fatty acids may play a role in increasing postprandial vascular tone. This consideration warrants further attention in future studies.

Our knowledge of arterial constitution and function suggests that the effects of DHA on AI are mediated in small arteries and arterioles, which tend to exert a greater effect in determining systemic resistance in the arterial tree. If this was to be investigated in the future, direct imaging of arteries via ultrasound techniques, such as FMD, may yield further detail on where these changes on wave reflections and Augmentation Index are physiologically determined in the arterial tree.

From a clinical stand point, a habitual 13.3% improvement of AI score would be considered clinically significant. Based on previous studies which have investigated associations between AI and European Society of Cardiology risk levels (9) a 13.3% habitual improvement in this measure would equate

approximately to a decrease in 10 year relative risk of CVD from 3.25% to 2.83%. It is important to consider that this improvement in AI, likely mediated by decreased vascular tone and accordingly, decreased systematic resistance in the arterial tree, is also indicative of decreased left ventricular load. Indications suggest that modifications to left ventricular load dependent on microvascular tone are associated with the development of left ventricular hypertrophy and the pathogenesis of ischemic heart disease (168). Accordingly, overtime a habitual 13.3% improvement in AI would not only be indicative of improved vascular tone, but may also correlate with decreased instances of left ventricular hypertrophy. Insights from the Framingham Study suggest that cardiac hypertrophy, in particular left ventricular hypertrophy, is a particularly strong predictor of congestive heart failure and is an "ominous harbinger" of this condition (169).

It could be hypothesised, on the basis of these results that long term DHA consumption might be slightly more efficacious in improving vascular tone than EPA and this in turn, might correlate with decreased cardiovascular risk in individuals who habitually consume greater amounts of DHA. This finding adds to the body of evidence which suggests that establishing the individual long term effects of DHA and EPA, as distinct dietary fats, on CVD outcomes is warranted.

3.2.4.2. Discussion of Pulse Wave Velocity Results

Our results did not indicate an effect for meal fat composition on changes on changes to PWV postprandially. Our finding is similar to other previous investigations which have also shown that PWV does not change acutely dependent on the composition of meal fat (170). Specifically this study investigated whether replacement of a stearic acid rich fat source by an oleic acid rich fat source impacted measures of vascular function, including PWV. However, it is possible that long-term consumption of DHA or EPA might slow the increase of PWV with age. A cohort study looking at differences in PWV in age matched individuals from fishing and farming villages in Japan has suggested that this may be the case (171). Meta-analysis of ten LC n-3 PUFA trials (with a pooled sample size of n=550), defined as chronic to sub-chronic in length, is also supportive of such an effect for EPA and DHA on PWV (172). However, it remains unknown what the exact contributions of EPA and DHA are.

As previously discussed in Section 2.7.4, the Pulse Wave Velocity measure is a proximal measure of gross arterial stiffness determined by the speed at which pulse wave travels through the arterial tree. As arteries are exposed to mechanical and chemical stress as we age, the elastic fibres in the arteries tend to fray and stiffen. This has a number of implications for cardiovascular disease, with regards to its impact on the Pulse Wave Velocity measure, the increase in arterial stiffness tends to increase the

speed at which ejected pulse waves travel throughout the arterial tree (173). In turn, these changes in pulse wave velocity have a tendency to negatively augment the arrival of reflected waves to the heart leading to a positive contribution to central SBP, which in turns increases the force needed for the left ventricle to maintain adequate coronary blood flow. Viewed broadly, increases in PWV can be considered an intrinsic part of the pathogenesis of myocardial infarction.

With regards to potential acute changes to PWV, the Moens-Korteweg equation that defines PWV, which is shown below in Figure 3.2.4.2, indicates that arterial radius does contribute to PWV. Furthermore, arterial radius is intrinsically linked to vascular tone. However, it should be noted that as according to the Moens-Korteweg equation any decrease in radius (R) as a result of an increase in vascular tone will be counterbalanced by an increase in arterial thickness (h) and an intrinsic alteration of the elasticity of the vessel (E).

$$PWV = \sqrt{Eh/2\rho R}$$

E = Young's modulus of the arterial wall
h = Arterial wall thickness
ρ = Blood density
R = Arterial radius (at the end of diastole)

Figure 3.2.4.2. The formula for PWV.

On the basis of these findings, it can be reasonably deducted that EPA and DHA consumption, or indeed meal fat content, do not impact PWV acutely, however, this does not necessarily correlate with their efficacy in modulating vascular tone of peripheral conduit arteries, small arteries and arterioles, whose tone, do not tend to impact PWV acutely. As mentioned, the possibility of differential effects of EPA and DHA on PWV in the longer term should not be discounted and warrants further investigation.

3.2.4.3. Discussion of Endopat and Endopat*GTN Results

We were not able to detect any changes from baseline in response to meal fat dependent on time, treatment effects or treatment*time interactions (see Figure 3.2.3.3). Previous research in this

context has yielded mixed results, one study has shown that inclusion of EPA and DHA (0.07g/kg bodyweight, 1:9.5 ratio) in a saturated fat meal (0.45g/kg bodyweight) improves postprandial endothelium dependent vascular reactivity, as measured by FMD, relative to a saturated fat control meal (0.52g/kg) (174). Previous studies by our group suggest no effect for EPA and DHA consumption on postprandial endothelium dependent vascular reactivity as measured by Laser Doppler Iontophoresis in response to acetycholine (58). Other studies have observed that chronic responses to DHA (1.6g/day for 16 weeks) do not impact fasting FMD measures (175). Finally a study comparing the effects of 4/g day of either EPA or DHA or olive oil showed that DHA improved fasting endothelium independent forearm blood flow responses but not fasting endothelium dependent forearm blood flow responses (57). Due to the broad array of methodologies employed in these studies a proper comparison of these effects requires careful attention to the methodologies employed.

With regards specific considerations for the Endopat RHI measure utilised in our study, previous investigation of the Endopat RHI output has indicated that it correlates with the gold standard measurement of endothelial function FMD (176). Furthermore, it is reasonably operator independent. However, it should be noted that during our use of the technique a number of challenges were encountered. In this study we observed high intra-individual variance between individuals at baseline, which were consistent with reports from other vascular studies undertaken in parallel at the University of East Anglia. As noted in the methods section, our measures were undertaken in a temperature controlled environment and individuals were given a period to rest and adjust to room temperature before measurement. These considerations were taken into account to control for any potential effects of temperature on the cutaneous vascular beds, which is a weakness of the Endopat technique, although perhaps not a weakness specific to Endopat as FMD and the Laser Doppler iontophoresis technique are also subject to temperature dependent changes in their outputs. Critically, other indications have since emerged in the literature which suggest that Endopat is not sufficiently sensitive to assess changes in endothelial function in early stage clinical pharmacological interventions (177). Specifically, this study indicated that Endopat is not able to detect changes in endothelial function in renally impaired and type-II diabetics, when compared to a healthy population. Furthermore, Endopat was not able to detect changes in endothelial function in robust interventions in healthy populations, including investigations on glucose load and smoking. This information, when taken into consideration with the findings of our study, is suggestive that the Endopat measure may be suited towards qualitatively establishing the presence of endothelial dysfunction, but may not be ideally designed for the quantitative measurement of endothelial function, in particular in the context of measuring acute, non-chronic subtle responses to intervention.

In short, taken on face value, our data suggests that neither EPA nor DHA meals in our study impacted postprandial endothelial dependent vascular tone. This is broadly in line with other indications in the literature but it should be noted that there isn't a clear consensus in this regard, as discussed in this section and section 1.7.1. Furthermore, we suggest caution in interpretation of this result due to differences in methodologies employed, with specific reference to the methodological considerations for Endopat described above.

We were also unable to detect any changes in response to Endopat in response to nitroglycerine administration dependent on treatment (see Figure 3.2.3.4). This experimental approach was developed in order to estimate contributions independent of NO production on the RHI response. It should be noted that we did observe an increase in amplitude of the peripheral arterial tone measurement used to calculate the Endopat RHI output in response to nitroglycerine administration (400mg) as expected. This is indicative of the peripheral arterial tone measurement in Endopat being dependent on circulating NO and corroborates the suggestions that the increase in peripheral arterial tone in the Endopat measure in response to brachial artery occlusion is dependent on increased NO production.

In summary, we were not able to pick up changes in vascular tone via the Endopat measure for EPA, DHA or control meals. We feel that that although the Endopat measure, from a theoretical perspective, is based on sound physiological principles, it may not be sensitive enough to pick up on acute physiological changes in endothelial function. As mentioned, previous indications in the literature suggest that this may be the case (177). We further suggest that Endopat's utility in measuring acute changes in endothelial function should be evaluated carefully.

3.2.4.4. Discussion of blood pressure results

Our results did not indicate any changes in systolic or diastolic blood pressure dependent on time, treatment or treatment*time effects (see Figure 3.2.3.5). Furthermore, we did not observe any significant differences in the baseline measures between the 3 visits. It is however worth noting, that we did observe a modest decrease in both diastolic and systolic blood pressure at the assessment visits when compared to screening visits. This effect was likely due to habituation to the surroundings of the clinic and the patients being more comfortable at follow up visits, given that blood pressure measurement protocol was kept consistent across visits. However, it is not implausible that dietary restrictions in the lead up to assessment visits, which prohibited caffeine consumption, impacted these measurements favourably due to caffeine's capacity to raise blood pressure acutely (178).

Our observations are corroborative of previous intervention studies of similar design which have indicated neither mixed doses of EPA and DHA (1g total, ratio not disclosed) (79), or 4.7g of EPA or DHA within a high fat meal effects postprandial measures of blood pressure (61). However, it should be noted that the previous study by Rontoyanni *et al*, suggests an effect for DHA in modulating diastolic BP response to exercise in the postprandial state (61), which was not assessed in our study. Another recently published study by Purcell *et al (62),* has also indicated that neither inclusion of EPA and DHA (5g in a 4:3 ratio) or inclusion of DHA (5g) in a meal high in MUFA (75g of total fat) impacted SBP or DBP 16 healthy males (62).

3.2.4.5.   Summary

In summary, our data indicates that although EPA and DHA both improve AI measures postprandially, this effect only reaches significance for DHA (p=0.04) and this is reflective of a slightly greater effect size for DHA when compared to EPA. These effect sizes equated to a 13.3% and 11.3% improvement in AI relative to control for DHA and EPA respectively. As mentioned previously, it is known that increases in AI correlate with increases in CVD risk (9). A habitual 13.3% improvement in this measure would equate approximately to a decrease in 10 year relative risk of CVD from 3.25% to 2.83% according to previously established correlations between the European Society of Cardiology Risk Levels and AI (9). Our findings suggest for the first time, that DHA is more efficacious than EPA in modulating resting postprandial vascular tone. Furthermore, when considering the impact of these effects in the longterm, these findings are suggestive of DHA having a greater capacity to chronically improve vascular function and associated health conditions. These findings should be considered in the context of potential therapeutic uses of these fatty acids, and further inform us of the unique properties of these individual dietary nutrients.

Given that individuals tend to spend the majority of the day in the postprandial state, these changes in postprandial vascular reactivity are likely to correlate with the chronic effects of EPA and DHA on vascular function, when habitually consumed. Also it should be noted, that measurement of fasting vascular tone in response to dietary intervention, although useful in part for standardisation purposes, cannot be considered to correlate fully with the normal physiological state of the average individual; who tends to spend 16 to 18 hours of the day in the postprandial state.

With regards to potential mechanisms, this effect on AI is likely indicative of the activity of blood borne vasoactive factors in the small peripheral arteries and arterioles of the arterial tree, as these tend to more reactive to changes in vascular tone. We also know that AI tends to be sensitive to changes in vascular tone in this part of the arterial tree. The following section (3.3) of this chapter, describes our

investigations on the blood borne vasoactive factors which may be involved in mediating these effects in the peripheral arterial tree.

We did not detect any changes in PWV or blood pressure in this acute study. We were also unable to detect changes in the Endopat RHI measure. As mentioned in Section 4.2.5.3, although the physiological basis of Endopat seems sound, there are indications that it may not be sufficiently sensitive to pick up on acute or early changes in endothelial function and its use in this context warrants further investigation.

3.3. Biochemical analysis of biological samples at baseline and in response to intervention

3.3.1. Introduction

Although a number of studies have investigated modifications to blood borne vasomediators in response to EPA and DHA consumption, the literature has failed to come to a consensus on how EPA and DHA dependent changes in vascular tone are mediated. Some investigations have observed postprandial changes to total NO_x in response to a mixed dose of EPA and DHA (58), however there has not been a consensus in this regard with some other studies showing no effect on NO_x, nitrite or nitrate (62, 80). This is similarly reflected in a lack of consensus of whether changes in vascular tone dependent on EPA and DHA are mediated in an endothelium dependent or endothelium independent manner with some studies suggesting the former (60, 174) and others suggesting the latter (57, 58).

We opted to investigate NO, via quantification of plasma nitrite levels in our study via chemiluminescent reaction with O_3, which is thought to be the most sensitive clinically feasible measure of changes in NO. Quantification of plasma nitrite has been shown to be the most reliable measure of NO production and to correlate more reliably than NO_x or nitrate with changes in vascular tone (179). Furthermore, given that studies have shown links between DHA and tissue levels of CSE (an enzymatic producer of H_2S) in Sprawgue-Dawley rats, and reports of potential modifications to cysteine metabolism dependent on LC n-3 PUFA (86), we investigated changes In plasma levels of H_2S in response to EPA and DHA consumption. This was undertaken to investigate if H_2S may play a role in mediating EPA or DHA's capacity to modulate vascular tone. Finally given that the LC n-3 PUFA epoxide metabolites have been shown to robustly and efficaciously directly modify vascular tone in *in-vitro* whole blood vessel models (131-134), we hypothesised that these compounds may contribute, or potentially be responsible for, EPA and DHA dependent changes in vascular tone. To the best of our knowledge, our study is the first to examine the production of these compounds in the postprandial state in response to physiological consumption of EPA and DHA. Furthermore, our study is the first to compare changes in the profile of these LC n-3 PUFA epoxides and diols in response to EPA and DHA rich meals.

Serum triglyceride, NEFA and glucose concentrations were also established at baseline and in response to meal consumption to investigate how meal composition affected triglyceride, NEFA and glucose metabolism at 0h and 4h postprandially.

The following section details the biochemical analysis conducted on blood samples generated at baseline and at 4 hours postprandially (*i.e.* in response to intervention) during the F.O.F.A study.

3.3.2. Statistical Analysis

All data are presented as mean +/- standard error of the mean. Statistical analysis was undertaken using the SPSS statistical software (version 22; Chicago, Illinois, USA). Prior to analysis all datasets were checked for normality via visual inspection of normal Q-Q Plots. Outliers were identified by analysis of normal Q-Q Plots and confirmed due to variance +/- two standard deviations. Repeated measures ANOVA was utilised in order to quantify the significance level for time, treatment and time*treatment interactions for measures. For Repeated Measures ANOVA, assumptions of sphericity were assessed via Mauchly's Test of Sphericity. In cases where the assumptions of sphericity were not met a Greenhouse-Geisser correction was applied. In cases where significance was reached via initial Repeated measures ANOVA, subsequent *post-hoc* analysis was undertaken in order to investigate treatment dependent changes from baseline using repeated measure ANOVA and Bonferroni adjustment on change scores.

For serum glucose analysis and plasma H_2S analysis n=1 and n=2 samples were lost during analysis respectively. For serum triglyceride analysis data for n=3 individuals was omitted on the basis of variance in values +/- 2 standard deviations. For plasma H_2S analysis and nitrite analysis data for n=2 and n=4 individuals were omitted due to variance greater than 2 standard deviations at baseline.

3.3.3. Results

3.3.3.1. Serum triglyceride and NEFA results at baseline and in response to intervention

Analysis showed no significant differences between treatments on serum triglyceride concentrations.

Specifically, analysis showed no significant treatment*time interaction for serum triglycerides, p=0.36, $F(1, 22) = 59.14$, partial $\eta^2 = 0.04$. Accordingly, no further investigation was undertaken for treatment*time effects. Analysis showed significant differences dependent on time as expected, p<0.001, $F(2, 44) = 1.01$, partial $\eta^2 = 0.73$, with 40% higher levels at 4h vs 0h, but no significant effect of treatment alone, p=0.36, $F(2, 44)=1.12$, partial $\eta^2=0.05$.

Figure 3.3.3.1.1. Mean serum triacylglycerol concentration measured at baseline and in response to treatment. Data are presented as mean ± SEM.

Analysis showed no significant difference between treatments on serum NEFA concentrations at 4 hours postprandially.

Specifically, repeated measures ANOVA showed no significant treatment*time interaction for NEFA concentration (p=0.90, $F_{(2, 50)} = 0.11$, partial $\eta^2 = 0.004$) or treatment (p=0.47, $F_{(2, 50)} = 0.76$, partial $\eta^2 = 0.03$). As expected, we saw a significant effect for time (p=0.02, $F_{(1, 25)} = 5.75$, partial $\eta^2 = 0.19$). Average concentrations (for 3 treatment groups combined) were 0.33 mM and 0.38 mM at 0h and 4h respectively.

Figure 3.3.3.1.2. Mean serum NEFA concentration measured at baseline and in response to treatment. Data are presented as mean ± SEM.

3.3.3.2. Serum glucose results at baseline and in response to intervention

Analysis showed no difference between treatments on serum glucose concentrations at 4 hours postprandially.

Analysis showed no significant effect for treatment*time interaction (p=0.49, $F(2, 48) = 0.73$, partial $\eta^2 = 0.03$) or for treatment (p=0.22, $F(1, 24) = 1.55$, partial $\eta^2 = 0.06$). There was a significant effect for time (p<0.001, $F(1, 24) = 27.37$, partial $\eta^2 = 0.53$), with glucose concentrations decreasing by 9% postprandially.
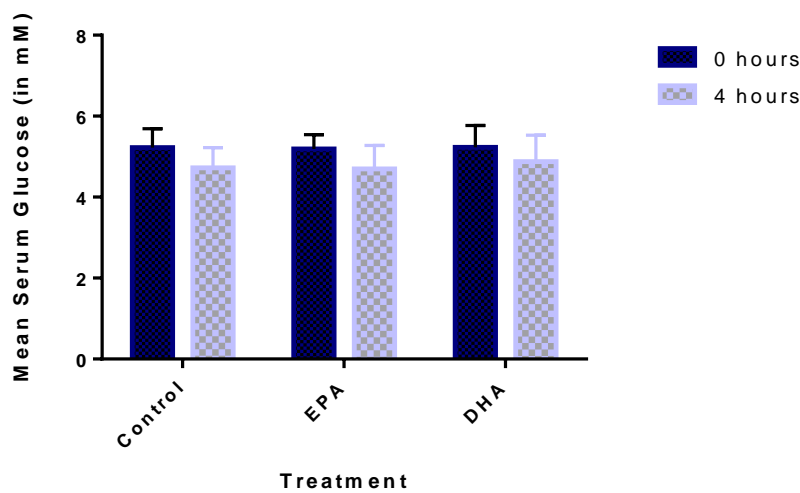
Figure 3.3.3.2. Mean serum glucose concentration measured at baseline and in response to treatment. Data are presented as mean ± SEM.

3.3.3.3. Plasma Hydrogen sulphide analysis at baseline and in response to intervention

Analysis showed no effect of meal consumption or any significant differences between meals on plasma H_2S concentrations.

Specifically, Repeated Measures Anova showed no significant effect for the treatment*time interaction for plasma H_2S concentrations, (p=0.35, $F_{(2, 42)}$ = 1.08, partial η^2 = 0.05), treatment (p=0.80, $F_{(2, 42)}$=0.23, partial η^2 = 0.01) or time effects (p=0.76, $F_{(1, 21)}$=0.10, partial η^2 = 0.01). Accordingly, differences in meal fat content had no significant effect on postprandial H_2S concentration and no further post-hoc analysis was undertaken.
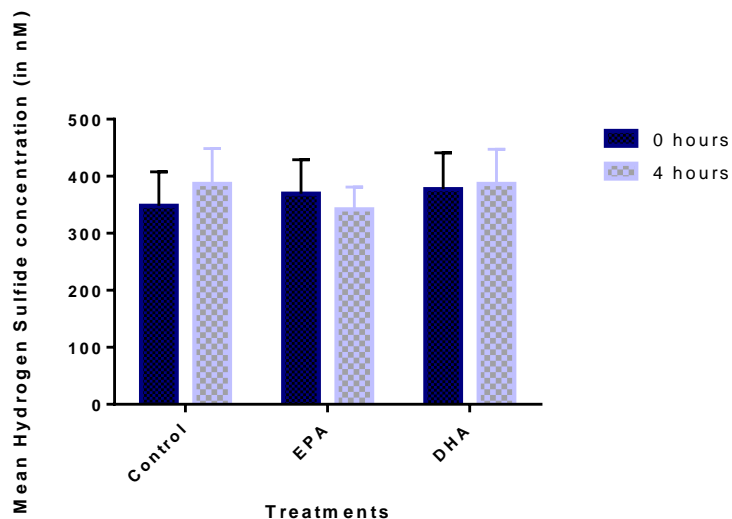
Figure 3.3.3.3. Mean plasma H_2S concentration measured at baseline and in response to treatment. Data are presented as mean ± SEM.

3.3.3.4. Plasma nitrite levels at baseline and in response to treatment

Analysis showed no effect of meal consumption or any significant differences between meals on plasma nitrite concentrations.

Specifically, Repeated Measures Anova showed no significant effect for the treatment*time interaction for plasma nitrite concentrations, (p=0.46, F(2, 42) = 0.78, partial η^2 = 0.04), treatment (p=0.76, F(2, 42)=0.28, partial η^2 = 0.01) or time effects (p=0.37 F(1, 21)=0.89, partial η^2 = 0.04). Accordingly, differences in meal fat content had no significant effect on postprandial nitrite concentrations and no further post-hoc analysis was undertaken.
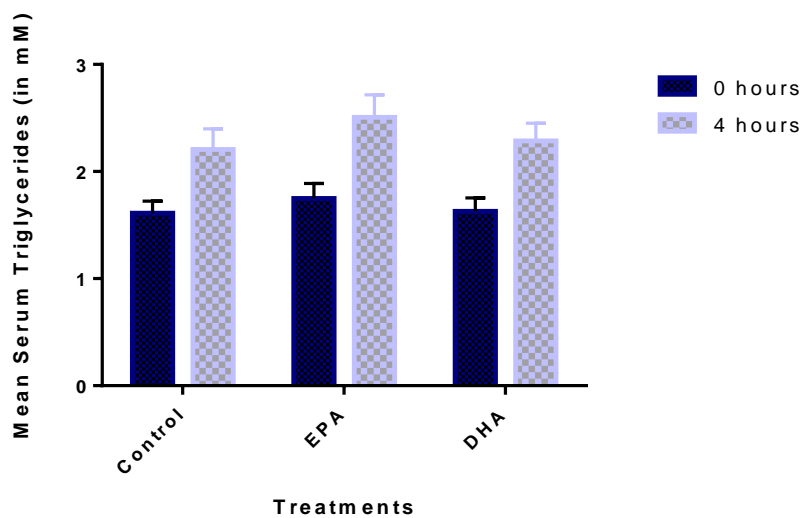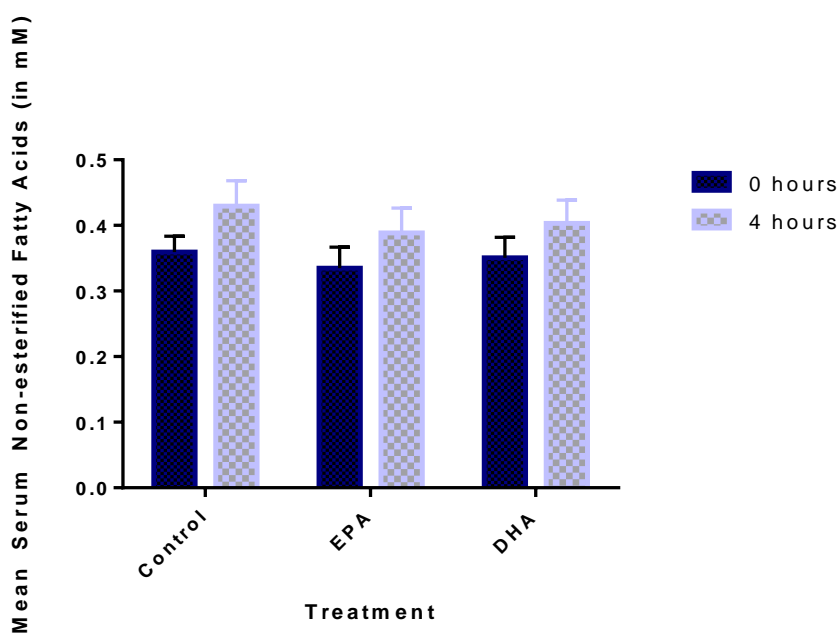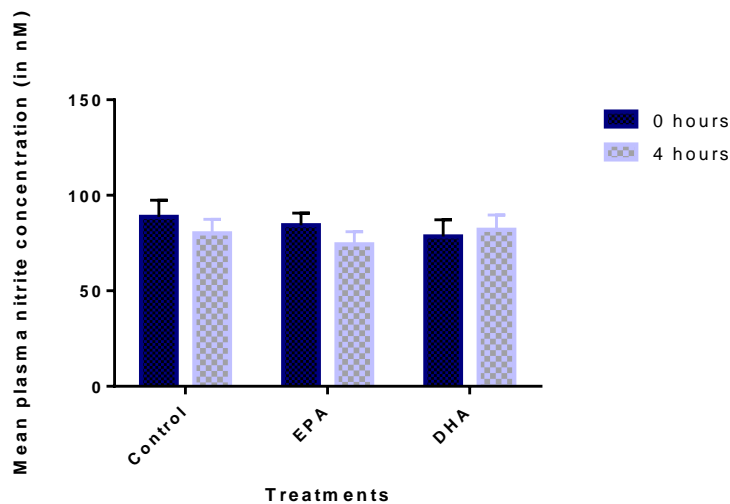

Figure 3.3.3.4. Mean plasma nitrite concentration measured at baseline and in response to treatment. Data are presented as mean ± SEM.

3.3.3.5. Plasma fatty acid analysis

The fatty acid data was expressed in both % of total fatty acid (see supplementary Annex 4, Table A) and concentration (µg/ml plasma) terms. Here in the main text the data is given in concentration, which is a more physiologically meaningful way of considering the data in the current context, where absolute amounts are likely to provide a better insight into the association of plasma fatty acids and vascular tone.

Plasma fatty acid concentrations at baseline and in response to treatment

Fatty acid	Control meal (0h)	Control meal (4h)	EPA meal (0h)	EPA meal (4h)	DHA meal (0h)	DHA meal (4h)
16:0 (µg/ml)	87.01±14.93	89.89±7.81	89.87±8.35	90.58±7.65	86.54±10.00	91.72±7.89
16:1 (µg/ml)	8.68±2.05	7.12±0.57	8.71±0.93	7.84±0.85	7.80±1.01	7.57±0.80
18:0 (µg/ml)	28.30±4.78	29.30±2.65	28.48±2.80	27.33±2.25	27.92±3.36	28.11±2.36
18:1 (µg/ml)	94.98±4.78	98.27±6.88	95.66±10.03	95.31±8.72	95.17±10.89	102.62±9.01
18:2n6 (µg/ml)	105.92±19.43	103.48±8.33	101.97±10.63	99.46±8.76	95.30±7.94	100.66±8.29
18:3n3 (µg/ml)	2.50±0.39	2.88±0.23	2.24±0.31	2.94±0.33	2.84±0.62	3.07±0.33
18:3n6 (µg/ml)	1.64±0.22	1.70±0.20	1.66±0.20	1.81±0.20	2.17±0.48	1.78±0.20
20:3n6 (µg/ml)	6.35±1.28	5.83±0.57	6.44±0.69	5.97±0.49	5.90±0.66	5.90±0.53
20:4n6 (µg/ml)	24.79±3.50	24.17±2.23	25.45±3.03	23.72±2.29	23.90±3.57	24.07±2.40
20:5n3[1,2,3] (µg/ml)	3.00±0.41	3.49±0.62	3.31±0.38	8.49±1.46*	3.66±0.74	5.08±0.64
22:5n3[3] (µg/ml)	0.88±0.14	1.04±0.15	1.07±0.16	0.74±0.12	0.80±0.12	0.92±0.13
22:6n3[1,2,3] (µg/ml)	6.59±1.28	6.44±0.66	6.70±0.78	7.54±0.83	6.44±0.91	12.00±1.28*

Table 3.3.3.5. Plasma fatty acid concentration at baseline and in response to treatment. Data presented as mean±SEM. [1]Indicates significant effect for time by Repeated Measures Anova, [2]Indicates significant effect for treatment by Repeated Measures Anova, [3]Indicates significant effect for time*treatment effect by Repeated Measures Anova. *Indicates a significant difference in change from baseline when compared to control.
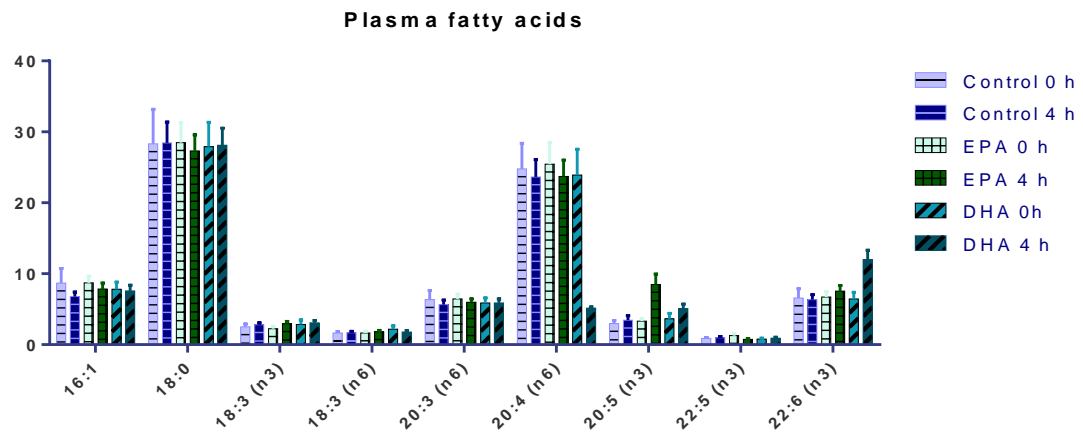
Figure 3.3.3.5. Graph showing plasma fatty acid concentrations of minor plasma fatty acids (<10% of total fatty acids) at baseline and in response to treatment. Data are presented as mean±SEM.
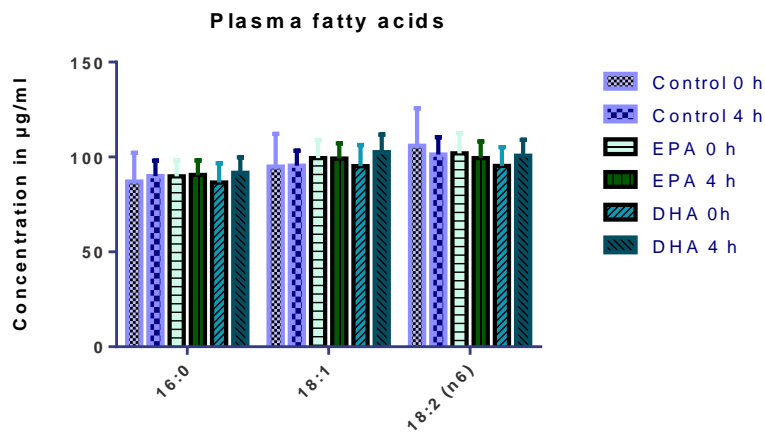
Figure 3.3.3.5.1. Graph showing plasma fatty acid concentrations of major plasma fatty acids (>10% of total fatty acids) at baseline and in response to treatment. Data presented as mean±SEM.

No significant effect of time, treatment or a time*treatment interactions was evident for C16:0, C16:1, C18:0, C18:1, C18:6n6, C18:3n3, C20:3n6, C20:4n6 (Table 3.3.4.5).

20:5 n3 (EPA)

Analysis showed significant treatment*time interactions (p=0.008, $F_{(2, 50)}$=5.32, partial η^2 = 0.18). Analysis also showed significant differences dependent on treatment (p=0.004, $F_{(2, 50)}$=6.10, partial η^2 = 0.20) and time (p<0.001, $F_{(1, 25)}$=17.61, partial η^2=0.41).

Post-hoc analysis revealed that the EPA meal significantly increased mean EPA concentrations (by 156%) when compared to the control meal (95% CI, 0.44 to 8.94, *p*=0.03) but not the DHA meal (95% CI, -0.58 to 8.10, p=0.10). Although a 58% increase in EPA from baseline was evident, the DHA meal did not significantly change EPA concentrations when compared to the control meal (95% CI, -2.05 to 3.91, *p*=1.00).

22:5 n3 (DPA)

Analysis showed significance for treatment*time interactions (p=0.02, $F_{(2, 50)}$=4.03, partial η^2 = 0.14) but no significant differences dependent on treatment alone (p=0.66, $F_{(2, 50)}$=0.42, partial η^2 = 0.02) or time (p=0.86, $F_{(1, 25)}$=0.03, partial η^2=0.00).

Post-hoc analysis revealed that after bonferonni correction, no treatment had a significant impact on changes in DPA concentration from baseline. P=0.07 for the control meal and EPA meal comparison, p=1.00 for the control meal and DHA meal comparison and p=0.12 for the DHA meal and EPA comparison. No further analysis analysis was undertaken and it was concluded that meal fat content did not impact plasma DPA concentrations significantly.

22:6 n3 (DHA)

Analysis showed significant treatment*time interactions (p=0.007, $F_{(2, 50)}$=5.412, partial η^2 = 0.18). Analysis also showed significant differences dependent on treatment (p=.005, $F_{(2, 50)}$=5.89, partial η^2 = 0.19) and time (p=0.006, $F_{(1, 25)}$=9.19, partial η^2=0.27).

Post-hoc analysis revealed that the DHA meal significantly increased mean DHA concentrations (by 100%) when compared to the control meal (95% CI, -0.03 to 11.46, *p*=0.05) and to the EPA meal (95% CI, 0.35 to 9.11, p=.03). Although there was an increase of 12% the EPA meal did not significantly change DHA concentrations when compared to the control meal (95% CI, -4.97 to 3.00, *p*=1.00).

3.3.3.6. Plasma epoxide and diol metabolites at baseline and in response to treatment

A repeated measures ANOVA was conducted to determine the significance of treatment and time effects and treatment*time interactions for all epoxide and diol metabolite concentrations.
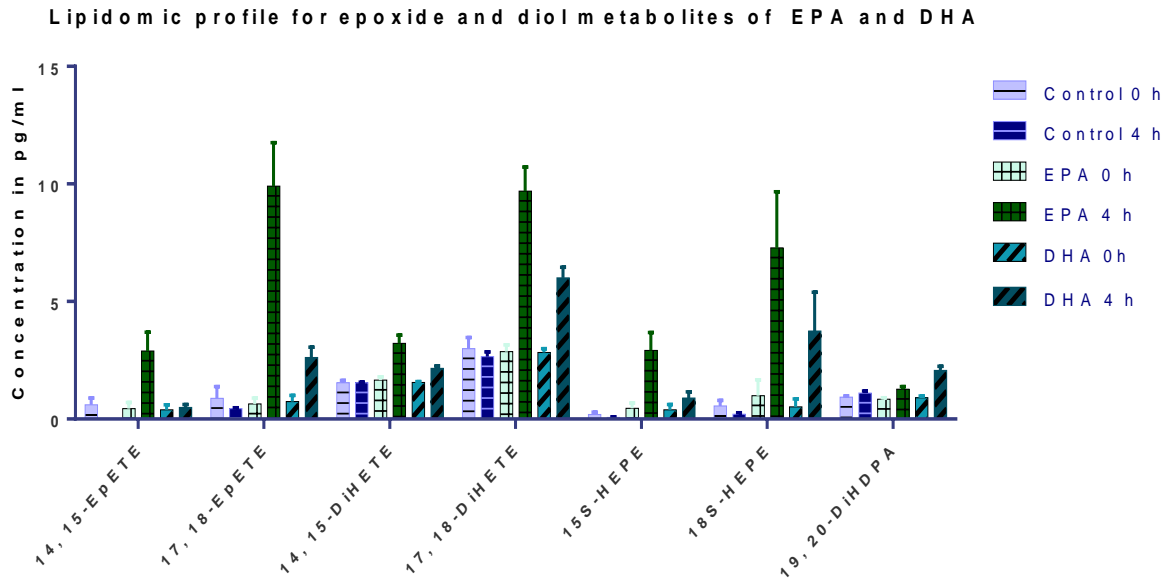
Figure 3.3.3.6. Mean plasma n-3 PUFA epoxide and diol concentrations at baseline and in response to treatment. Data are presented as mean±SEM.

	Control meal (0h)	Control meal (4h)	EPA meal (0h)	EPA meal (4h)	DHA meal (0h)	DHA meal (4h)
14, 15-EpETE[2,3] (pg/ml)	0.60±0.29	0.00±0.00	0.45±0.27	2.89±0.78*	0.39±0.21	0.48±0.13
14, 15-DiHETE[1,2,3] (pg/ml)	1.54±0.10	1.54±0.03	1.66±0.14	3.22±0.35*	1.56±0.04	2.16±0.10*
17, 18-EpETE[1,2,3] (pg/ml)	0.88±0.50	0.45±0.03	0.65±0.25	9.90±1.81*	0.74±0.26	2.62±0.42*
17, 18-DiHETE[1,2,3] (pg/ml)	2.99±0.47	2.65±0.19	2.87±0.28	9.7±1.00*	2.83±0.17	6.00±0.44*
15S-HEPE[1,2,3] (pg/ml)	0.20±0.09	0.07±0.02	0.80±0.48	2.57±0.67*	0.40±0.22	0.89±0.26
18S-HEPE[1,2] (pg/ml)	0.55±0.23	0.21±0.06	1.80±1.00	6.47±2.29	0.52±0.33	3.75±1.61
19, 20-DiHDPA[1,2,3] (pg/ml)	0.94±0.05	1.11±0.08	0.84±0.07	1.27±0.11	0.91±0.06	2.07±0.17*

Table 3.3.3.6. Mean plasma n-3 PUFA epoxide and diol concentration measured at baseline and in response to treatment. Data are presented as mean±SEM. [1]Indicates significant effect for time by Repeated Measures Anova, [2]Indicates significant effect for treatment by Repeated Measures Anova, [3]Indicates significant effect for time*treatment effect by Repeated Measures Anova. *Indicates a significant difference in change from baseline when compared to control.

Analysis showed that post EPA meal consumption increases in 14,15-EpETE, 14,15-DiHETE, 17,18-EpETE, 17, 18-DiHETE and 15S-HEPE concentrations reached statistical significance when compared to control. Further to this, increases in 14,15-EpETE, 14,15-DiHETE, 17,18-EpETE, 17,18-DiHETE, 15S-HEPE but not 18S-HEPE post consumption of EPA meal reached statistical significance when compared to the DHA meal.

Post consumption of the DHA meal 19,20-DiHDPA, 14,15-DiHETE, 17,18-EpETE, 17,18-DiHETE increased significantly compared to control the meal. 19, 20-DiHDPA also increased significantly post consumption of the DHA meal when compared to the EPA meal.

14, 15-EpETE

Analysis showed significant treatment*time interactions ($p=0.001$, $F_{(2, 50)}=8.09$, partial $\eta^2 = 0.24$). Analysis also showed a significant effect of treatment ($p<0.001$, $F_{(2, 50)}=9.21$, partial $\eta^2 = 0.27$), but not time ($p=0.06$, $F_{(1, 25)}=4.04$, partial $\eta^2=0.14$).

Post-hoc analysis revealed that the EPA meal significantly increased mean **14, 15-EpETE** when compared to the control meal (95% CI, 0.57 to 5.529, *p*=.012) and the DHA meal (95% CI, 0.107 to 4.589, p=.038). The DHA treatment did not significantly change 14, 15-EpETE when compared to the control meal (95% CI, -0.187 to .433, *p*=0.38). Likewise, there were no significant differences between EPA and DHA treatments (95% CI, -2.435 to 1.927, *p*=1.000). See table 3.3.4.5 for mean concentrations by timepoint and treatment.

14, 15-DiHETE

Analysis showed a significant treatment*time interaction ($p<0.001$, $F_{(1.24, 30.96)}=11.15$, partial $\eta^2 = 0.31$), treatment effect ($p<0.0001$, $F_{(1.24, 30.91)}=13.34$, partial $\eta^2 = 0.35$) and time effect ($p<0.001$, $F_{(1, 25)}=25.66$, partial $\eta^2 =0.51$).

Post-hoc analysis revealed that the EPA (95% CI, 0.532 to 2.57, *p*<0.005) and DHA (95% CI, 0.193 to 0.99, *p*<0.005) meals significantly increased **14, 15-DiHETE by 94% and 38%** from baseline, as compared to the control meal in which 15-15-DiHETE did not change from baseline. The EPA treatment did not significantly increase 14, 15-DiHETE when compared to the DHA meal (95% CI, -0.026 to 1.951, p=0.06). See table 3.3.4.5 for mean concentrations by timepoint and treatment.

17, 18-EpETE

Analysis showed significant treatment*time interactions (p<0.001, $F_{(1.21, 30.24)}=18.665$, partial $\eta^2 = 0.427$), treatment effects (p<0.001, ($F_{(1.24, 30.95)}=18.79$, partial $\eta^2=0.43$ and time effects (p<0.001, $F_{(1, 25)}=28.2$, partial $\eta^2=.053$).

Post-hoc analysis revealed that the EPA meal significantly increased mean **17, 18 EpETE** when compared to the control meal (95% CI, 4.584 to 14.798, p<0.0005) and when compared to the DHA meal (95% CI, 2.409 to 12.334) p<0.005). Mean 17, 18-EpETE concentrations increased 15 fold and 3.5 fold post EPA and DHA meals respectively, whereas mean 17, 18-EpETE concentrations decreased from 0.88ng/ml to 0.45ng/ml post control meal. The DHA treatment also significantly increased 17, 18 EpETE when compared to the control meal (95% CI, 0.452 to 4.19, p=0.01). See table 3.3.4.5 for mean concentrations by timepoint and treatment.

17, 18-DiHETE

Analysis showed a significant treatment*time interaction (p<0.001, $F_{(1.59, 39.78)}=27.86$, partial $\eta^2 = 0.527$), treatment effect (p<0.001, $F_{(1.46, 36.37)}=25.49$, partial $\eta^2 = 0.51$) and time effect (p<0.001, $F_{(1.59, 39.78)}=27.86$, partial $\eta^2 = 0.527$).

Posthoc analysis revealed that the EPA meal (95% CI, 4.357 to 9.97, p<0.0005) and DHA meal (95% CI, 1.776 to 5.245) significantly increased mean **17, 18**-DiHETE by approximately 3 and 2 fold when compared to the control meal. 17, 18-DiHETE values were also significantly higher following the EPA vs. DHA meal (95% CI, 0.948 to 6.356, p<0.05). See table 3.3.4.5 for mean concentrations by timepoint and treatment.

15S-HEPE

Analysis showed significant effects for the treatment*time interaction (p=0.03, $F_{(1.221, 30.528)}=4.617$, partial $\eta^2 = 0.156$), treatment (p=0.001, $F_{(1.19, 29.65)}=11.77$, partial $\eta^2 = 0.32$) and time (p=0.05, $F_{(1, 25)}=4.15$, partial $\eta^2 = 0.14$).

Post-hoc analysis revealed, that the EPA meal increased mean **15S-HEPE** when compared to the control (95% CI, 0.213 to 3.599), p=0.029) and when compared to the DHA meal. (95% CI, -0.084 to 2.639, p=0.07). The DHA treatment also increased 15S-HEPE when compared to the control meal but this effect did not reach significance. See table 3.3.4.5 for mean concentrations by timepoint and treatment.

18S-HEPE

Analysis, showed no significant treatment*time interaction, (p=0.07, $F_{(2, 50)}$ = 2.75, partial η^2 = 0.1), but a significant effect for treatment (p=0.007, $F_{(1.40, 35.06)}$=5.55, partial η^2 = 0.18) and time (p=0.02, $F_{(1, 25)}$=6.69, partial η^2 = 0.21). See table 3.3.4.5 for mean concentrations by timepoint and treatment.

19, 20-DiHDPA

Analysis, showed a significant effects for the treatment*time interaction (p<0.001, $F_{(2, 50)}$ = 22.599, partial η^2 = 0.475), treatment (p<0.001, $F_{(1.58, 39.42)}$=17.70, partial η^2 = 0.42 and time (p<0.001 $F_{(1, 25)}$=37.06, partial η^2 = 0.60).

Testing revealed that the DHA meal significantly increased mean 19, 20-DiHDPA when compared to the control meal (95% CI, 0.589 to 1.376, *p*<0.0005) and the EPA meal (95% CI, 0.282 to 1.180, p=0.001). Specifically, the DHA meal increased 19, 20-DiHDPA concentrations from baseline by 127%. The EPA treatment also increased 19, 20-DiHDPA when compared to the control meal but this effect did not reach significance. See table 3.3.4.5 for mean concentrations by timepoint and treatment.

3.3.3.7. Bivariate correlations between EPA and DHA epoxide and diol concentrations and Augmentation Index

Correlations between AI values (at baseline and postprandial time points) with concentrations of EPA and DHA epoxides and diols were investigated via 1-tailed bivariate correlations. AI was chosen as the vascular measure of interest as it is the main vascular measure influenced by treatment. Results showed that associations between plasma nitrite, H_2S, 17,18-DiHETE, 19,20-DiHDPA, EPA and DHA and AI reached statistical significance. In order of decreasing magnitude, Nitrite, H_2S, 17,18-DiHETE and 19,20-DiHDPA emerged as being the most strongly associated with improved AI measures.

	Pearson correlation with AI	P-value
14, 15-EpETE	0.001	0.48
14, 15-DiHETE	0.03	0.357
17, 18-EpETE	-0.05	0.279
17, 18-DiHETE	-0.22**	0.003
15S-HEPE	0.01	0.477
18S-HEPE	0.01	0.425
19, 20-DiHDPA	-0.20*	0.006
H_2S^2	-0.21**	0.008
Nitrite[1]	-0.33**	<0.001
EPA	-0.16*	0.02
DHA	-0.16*	0.025

Figure 3.3.3.7. Pearson correlation of EPA and DHA epoxide and diol concentrations with Augmentation Index (AI). *marks significance at the p≤0.05 level, **marks significance at the p≤0.01 level. [1]As per nitrite analysis in section 3.3.3.4, four outliers were omitted in this analysis.[2]As per H_2S analysis in section 3.3.3.3, two outliers were omitted in this analysis.

3.3.4. Discussion

3.3.4.1. Quantification of serum markers of metabolic state

There were no significant differences in serum NEFA, triglycerides or glucose at baseline between study days (see Figures 3.3.3.1.1, 3.3.3.1.2 and 3.3.3.2). Compared to baseline there was significant increase in triglycerides postprandially for all treatments, with an overall 40% increase from baseline. This results was as expected, as the 4h time point was chosen to approximately coincide with peak lipaemia and concentration of meal derived fatty acids in the plasma. Similar increases of 70% from baseline have been observed in other postprandial studies of comparable design (58). There was a non-significant trend towards a decrease in serum glucose levels postprandially, likely due to the fact that sampling was not concordant with the postprandial glucose peak, typically 60-120 minutes after a mixed high-fat/carbohydrate meal. Serum NEFA concentrations also significantly increased postprandially, however there were no differences between treatments. A previous study of similar meal and intervention design showed no significant differences between treatment group effects on these markers in response to a combined 5.4g dose of DHA and EPA (in a ratio of 3:2) compared to a mixed oil control meal (58). One study has shown similar results for changes in glucose and triglyceride concentration in response to a mixed meal with or without EPA inclusion (5g) (80). Another study which compared the effects of 4.7g EPA or 4.7g DHA in a high fat meal had consistent results with our study for the postprandial NEFA, triglyceride and glucose response (61). It should be noted that in this study individuals were considerably younger (average 23 years of age) and the postprandial measures were taken at 5 hours as opposed to 4 hours.

In summary, in this study neither EPA nor DHA inclusion influenced gross markers of postprandial lipaemia in our whole study population and this is consistent with previous observations in the literature. Of note, indications in the literature have suggested that genotype may play a role in the postprandial lipaemic response to meal fat content and this is explored in the context of PPAR-γ genotype in Chapter 6.

3.3.4.2. Plasma Hydrogen sulphide analysis at baseline and in response to intervention

As discussed in section 1.7.3, there have been indications of CSE activation by DHA. Given that H_2S acts as a vasodilator in the vasculature, we hypothesised, that changes in H_2S concentration dependent on CSE activation may contribute to DHA's capacity to elicit vasodilation. To this end we investigated if H_2S concentrations change acutely and according to meal fat content. This investigation is the first to investigate H_2S in the context of the postprandial state.

Our results suggest modest increases in H_2S concentrations after consumption of the high fat control meal (11%) and modest decreases in response to EPA (7.5%) but not DHA (see Figure 3.3.3.3). However, the time*treatment interaction and time and treatment effects in this analysis did not reach statistical significance. It is worth noting that the modest increases following intake of the control high fat meal although non-significant were not consistent with our hypothesis. Evidence is emerging that indicates H_2S production can be increased in response to high fat diets and that H_2S may play a role in signalling for reduction in the rate of lipolysis (180, 181). It is possible that the slightly increased H_2S concentrations observed in this study in response to the high fat meal are in response to a postprandial hyper-lipaemic state and may be involved in signalling for inhibition of lipolysis. It is also possible that these modest non-significant increases are attributable to normal biological variance.

With regards potential interactions between EPA or DHA and H_2S production, although never examined in the context of postprandial lipaemia, a study has demonstrated an activation of CSE (cystathionine-γ-lyase) in response to DHA-rich tuna-oil supplementation in lung tissue of Sprawgue-Dawley rat (85). With regards to our findings, within the context of the postprandial state, our data did not support the hypothesis that EPA or DHA modified H_2S production significantly.

In summary, we were not able to detect any statistically significant differences in plasma H_2S in response to treatment in this study. Accordingly, it is unclear whether H_2S is involved as a secondary messenger in the response to either n-3 fatty acids or the hyper-lipaemic state. Further work may be warranted to qualify these interactions.

3.3.4.3. Plasma nitrite analysis at baseline and in response to intervention

In our investigations we did not observe changes in plasma nitrite concentrations dependent on time, treatment or time*treatment effects. This observation is not consistent with our previous study investigating inclusion of a mixed dosage of DHA and EPA (5.2g total in a ratio of 3:2) in a high fat meal (58). In the absence of other data it is difficult to come to a definitive conclusion on what may be driving the discrepancy between these studies. The two most notable difference in study design, in these investigations of NO production in response to EPA and DHA consumption, are the different proximal measures of NO production quantified nitrite vs NO_x (nitrite + nitrate), the methodologies used to measure the nitrite/NO_x and the utilisation of a 3:2 ratio of DHA:EPA in the previous study. The previous study utilised a modified Griess reaction which also incorporated a reduction of nitrate to nitrite in order include nitrate's contribution as a potential marker of NO production. The most likely explanation is that the additional contribution from nitrate explains the discrepancy between these studies. The final consideration in the comparison of these studies is that EPA and DHA consumption have an additive effect and the ratio of EPA:DHA has an important role in this effect. Given that we did not show any change from baseline in plasma nitrite concentration post EPA and DHA meals in our current study it is more likely that the discrepancy between these observations are described by methodological considerations described above.

In summary, our study of these suggested changes in NO production in response to EPA and DHA consumption is novel in its use of methodology that accurately reflects changes to eNOS activity and in its comparison of the relative efficacy of EPA vs DHA. In these studies we were not able to observe postprandial changes in plasma nitrite concentrations in response to EPA or DHA inclusion in a high fat meal.

3.3.4.4. Plasma fatty acid analysis at baseline and in response to intervention

Analysis of plasma samples revealed that that the only plasma fatty acids to change markedly in concentration in response to treatment were EPA and DHA (see table 3.3.3.5 and figure 3.3.3.5). We also saw increases in DPA for all treatments. Analysis of fatty acids as a percentage of total lipid weight revealed that the relative percentage of palmitoleic acid (16:1), dihomo-gamma-linoleic acid (20:3 n6) and arachidonic acid (20:4 n6) decreased postprandially (see Table 3.3.4.5.1) but this was not reflective of significant changes in their concentrations.

Somewhat unexpectedly we did not observe marked increases in the concentration of other fatty acids measured. Palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), alpha (18:3 n3) and gamma-linoleic

acid (18:3 n6) all tended to increase but we were not able to observe significance for these effects in our analysis.

In our analysis, EPA concentrations increased 2.6 fold in response to the EPA meal and DHA concentrations increased nearly 2 fold in response to the DHA meal. These effect sizes are broadly in line with previous investigation which investigated the effects of comparable doses of EPA and DHA on the postprandial response to high fat meals which have shown approximately 4 fold increases postprandially (61, 80). Specifically these previous studies investigated the effects of inclusion of EPA (5g) in a high fat meal in health male volunteers (80) and inclusion of 4.7g of EPA or DHA within a high fat meal in healthy male volunteers (61). Differences in effect sizes may be in part due to smaller EPA and DHA (4.16g) doses in our study or possibly the effect of meal matrix.

3.3.4.5. Plasma epoxide and diol metabolite response and associations with vascular tone

As discussed in section 1.9, it is known that vascular reactivity is directly affected by metabolites derived from EPA and DHA and therefore the concentration of these metabolites is more likely to provide mechanistic insight into the impact of meal fatty acid composition on vascular tone compared to the parent fats. These metabolites are produced by both discrete and non-discrete enzymatic pathways. Examples of these metabolites in the literature include the eicosanoid family. It is well documented in the literature that production of EPA derived eicosanoids in favour of the AA and dihomo-y-linoleic derived eicosanoids can beneficially modulate excessive inflammatory states, and may also beneficially influence vascular tone through the generation of thromboxane A3 over the A2 AA derivative which has greater vasoconstrictory capacity. However, there are still gaps in our knowledge with regards how the full array of EPA and DHA derived metabolites contribute to the effects of the lipidome in influencing vascular and metabolic health. As noted in section 1.9, there are several EPA and DHA metabolites derived from the CYP450 enzymes which are known to be potent modulators of vascular tone (131, 132, 182). Curiously, these metabolites have been understudied in the context of human health and their potential effects on vascular health and function *in-vivo* in human subjects have not been documented either in response to chronic EPA and DHA consumption or in the postprandial state.

Interestingly, it has been previously shown that high fat meals alter circulating concentrations of certain oxylipins in the postprandial state (183). This previous study investigated postprandial changes in a broad range of circulating oxylipins in response to a very high fat SFA meal, MUFA meal and n-3 PUFA meal. Specifically, the intervention meals were comprised of SFA (95g Palm Oil), MUFA (95g of oleic acid rich sunflower oil) and n-3 PUFA (55g of fish oil equivalent to 22g of DHA and 3g of EPA

alongside 40g of palm oil) respectively in a milkshake form. In the context of normal human diets, the dosage of DHA used in this study can be considered supra-physiological; an equivalent dosage achieved through consumption of an oily fish, such as salmon, would require consumption of 1.5kg of salmon. Furthermore, it should be noted that anecdotal evidence [1] suggests that long term consumption of this amount of EPA and DHA causes disaggregation of platelets and spontaneous internal haemorrhaging. Accordingly, although these results demonstrate a novel postprandial modulation of this compartment of the lipidome, they are of limited value in terms of translatability and there remains a need to investigate modulation of this compartment of the lipidome in response to physiological doses of EPA and DHA.

We hypothesised that the epoxide metabolites derived from the CYP450 enzymes might in part explain the impact of EPA and DHA on postprandial vascular reactivity (58, 61) and that a more physiologically relevant dosage (4.16g) may be sufficient to significantly alter the concentration of these compounds acutely. Accordingly, we opted to investigate the profile of epoxide metabolites and their diol derivatives in order to qualify if a more physiological dose of EPA and DHA (4.16g) would be sufficient to alter this portion of the oxylipin profile. Our data is the first to investigate postprandial changes in the concentration of these metabolites in response to physiological EPA vs DHA dosage.

In our investigations, we showed that the concentration of epoxides and diols in plasma change profoundly in the postprandial state in response to high but physiological relevant doses of EPA and DHA. Of note, we showed highly significant increases in the vasoactive epoxides derived from EPA in response to the EPA meal (see Figure and Table 3.3.3.6). Specifically, we saw a 6.5 fold increase in 14, 15 EpETE concentration and a 15 fold increase in 17, 18 EpETE concentration in response to the EPA meal. These effects were not observed for the DHA or control meal indicating that changes in the concentration of these epoxides is highly dependent on meal fat consumption. We also observed that the concentration of these compounds decreased in response to the control meal. This is likely explained by the epoxide profile shifting to be more reflective of the presence of n-6 fatty acids in the control meal. Given the reported increased capacity of these EPA metabolites relative to their n-6 counterparts to induce vasodilation these changes in concentration may in part explain the improvement in AI measures we saw in response to EPA meal when compared to control meal. These decreases in n-3 epoxide concentrations in response to the control meal may also play a role in the postprandial decline in vascular tone which have previously been shown in response to this control meal (58). It is also worth noting that post consumption of the DHA meal we saw 3.5 fold increases 17, 18 EpETE concentrations. This effect was modest when compared to the EPA meal and is likely

---

[1] This evidence stems from the unpublished "inuit diet" self-experimentation of Dr. Hugh Sinclair, documented in "Fine Wines and Fish Oil: The Life of Hugh Mc Donald Sinclair".

explained by the inclusion of small amounts of EPA in the DHA meal and also possible retroconversion of DHA to EPA (see table 2.6.2).

We also saw significant concurrent increases in the diol derivatives of the EPA epoxides, 17, 18 DiHETE and 14, 15 DiHETE. Specifically, we observed 3.4 fold increase in concentration of 17, 18 DiHETE and a 1.94 fold increase in concentration of 14, 15 DiHETE in response to the EPA meal. Both of these increases were highly significant when compared to the control meal. We also saw modest increases in the concentration of these compounds in response to the DHA meal. This effect reached significance for 17, 18 DiHETE and 14, 15 DiHETE when the DHA meal was compared to control. Curiously, little is known about the bioactivity of these diols compounds although it seems that they play a role in both the excretion of EPA and DHA (184) and may play a role in angiogenesis and immune function (185). However, there is no evidence to suggest these compounds contribute to vascular tone and accordingly, it is unlikely that they contribute to the vasoactive effects of their parent molecules.

Concentrations of 15S-HEPE and 18S-HEPE also changed markedly postprandially. Specifically, we observed 6.4 fold increases in 15S-HEPE concentration in response to the EPA meal and a 2.3 fold increase in response to the DHA meal (non-significant). 18S-HEPE concentration also increased 7.3 fold in response to EPA and 7.2 fold in response to the DHA meal. It should be noted that the mean 18S-HEPE concentration reached 7.27ng/ml after the EPA meal compared to 3.74ng/ml after the DHA meal. The similarity in percentage increases but differences in concentration at 4 hours, were attributed to one individual having raised levels of 18S-HEPE at baseline for EPA treatment. Curiously, the acute changes observed for 18S-HEPE concentrations fell out of significance for treatment*time effects in our study despite the large changes in concentration in response to EPA and DHA meals. This lack of significance was likely due to very large variances in the data due to huge inter-individual differences in responsiveness. Specifically, in the greatest responder we observed blood levels of 18S-HEPE by close to 100 fold, whereas in some individuals there was no increase in the concentration of this compound acutely. This observation alone warrants further investigation as 18S-HEPE acts as pre-cursor for the E-series resolvins described in section 1.9.4 and inter-individuals variation in the production of this compound may have consequences for human health. It is of note that to the best of our knowledge, specific biological activity has not been discerned for 15S-HEPE and 18S-HEPE. Given that the bioactivity of these compounds has been scarcely explored, the possibility remains that the lack of documented bioactivity is as much a product of a lack of exploration as an actual lack of bioactivity. Currently, it is difficult to discern how changes in these compounds may affect vascular tone postprandially or indeed other aspects of human health.

We also observed highly significant increases in the DHA derived diol 19, 20 DiHDPA concurrent with treatment. Interestingly, this metabolite has been indicated in the promotion of angiogenesis (186). In the long term, this effect may contribute to DHA's effect on cardiovascular progression and further work is warranted to investigate these possibilities. However, there is currently little evidence to suggest 19, 20 DiHDPA contributes to the short term effects on vascular tone of its parent molecule. As indicated in section 1.9, the DHA derived epoxide, 19, 20 EpDPE, is noted as being a particularly efficacious vasodilator. Keeping in mind that 19, 20 DiHDPA is derived from 19, 20 EpDPE, the marked and highly significant increase in concentration of 19, 20 DiHDPA in response to the DHA meal is likely reflective of increases in 19, 20 EpDPE concentrations. However, the concentration of 19, 20 EpDPE fell beyond the limit of detection in our analysis, therefore no data is available for this metabolite.

With regards, to changes in concentration of the diol derivative of 19, 20 EpDPE, postprandial concentrations of 19, 20 DiHDPA increased by 127% relative to baseline. Modest increases were seen in response to EPA treatment, however, this change did not reach significance when compared to control. Results from the human serum metabolome project suggest that the molar ratio of 19, 20 EpDPE to 19, 20 DiHDPA is in the range of 1 in 8 (187). This ratio is reflective of previous studies which have indicated that the concentration of the DHA derived epoxides in blood is below 0.1µM or beyond the limit of detection (187, 188). These estimates put the serum concentration of 19, 20 EpDPE in the low picomolar range, which may explain why this compound fell beyond the limit of detection in our analysis.

As noted, given that the epoxide derived diol 19, 20 DiHDPA was detectable in our sample and doubled in response to DHA consumption, it is highly likely that this is reflective of changes in 19, 20 EpDPE concentration. Accordingly, any assumption that these DHA epoxides are not present, does not change acutely and/or do not contribute to the lipidome's effects on vascular tone postprandially should be interpreted with due caution. When interpreting these results it is also worth noting that soluble epoxide hydrolase (sEH), the enzyme that generates these diols from their epoxide derivatives, has shown slightly lesser affinity for the DHA derived epoxides when compared to the EPA derived epoxides (189). In our study we showed that the molar ratio of 17, 18 EpETE and 14, 15 EpETE to their diol counterparts was roughly 1:1 post EPA meal consumption. Assuming these ratios remain broadly consistent for the EpDPEs and DiHDPAs, we would expect a doubling in concentration of 19, 20 EpDPE. It should be noted that this assumption is based on the assumption that the CYP450 enzymes have equal propensity for EPA and DHA. As mentioned, it has been previously observed that human sEH has a relatively low K_M for 19, 20 EpDPE when compared to other fatty acid epoxides, and an

intermediate K_{CAT} which might infact suggest that we see more than a doubling of the DHA derived epoxides postprandially (189).

In summary, it is likely that the concentration of the DHA derived epoxides increase postprandially. Given their increased capacity to illicit vasodilation relative to their EPA counterparts this increase may explain the observation that the DHA meal improved AI to a greater degree than the EPA meal. Unfortunately, we were unable to detect 19, 20-EpDPE or other DHA derived epoxides in our plasma samples due to the low concentration of these compounds in plasma. Given these considerations, and the likely contribution of these DHA derived epoxides to the parent nutrient's effect on vascular tone and health, future work in this area would be well advised to consider utilisation of a combination of sample concentration and higher sensitivity methods in order to accurately quantify changes in the concentration of the DHA derived epoxides in response to acute or chronic DHA consumption.

3.3.4.6. Bivariate correlations between EPA and DHA derived epoxide and diols and Augmentation Index

We conducted a bivariate correlation between concentrations of the EPA and DHA epoxides and diols measured in our analysis and augmentation index, in order to investigate if changes in the concentration of these compounds were correlated with changes in AI (see table 3.3.3.7).

Our analysis showed that 17, 18-DiHETE and 19, 20-DiHDPA acid were significantly negatively correlated with Augmentation Index. As decreases in Augmentation Index are associated with decreases in vascular tone, these results tentatively link these compounds to decreases in vascular tone. It should be noted that it is unknown whether 17, 18-DiHETE or 19, 20-DiHDPA activity on vascular smooth muscle tone. We hypothesise a number of explanations for these observations. In the case of 19, 20-DiHDPA, it is likely that if this correlation is indicative of a genuine link between this molecule and improvements in vascular tone, that in this instance the increases in 19, 20-DiHDPA are indicative of increases in 19, 20-EDP (the parent molecule of 19, 20-DiHDPA which is responsible for the causation of improvements in vascular tone. In the case of 17, 18-DiHETE this correlation may be indicative of some yet undocumented bioactivity. Previous indications have suggested that the diol derivatives can have similar capacities to induce dilation of blood vessel as their parent epoxides (190), so this possibility is not without precedent. It is worth noting that generally the epoxides have been shown to elicit a more potent vasodilatory response than the diols, and accordingly this hypothesis should be interpreted with due caution. The alternative explanation for these observations is that that these correlations are merely indicative of a correlation between the decreased AI values we saw in response to the EPA and DHA meal and do not indicative causation. It is also worth noting that we also

observed a significant negative correlation between EPA and DHA concentrations in our study, although the size of the correlation was decreased when compared to the diol metabolites.

Interestingly, although nitrite did not change in our intervention it did correlate significantly with decreased AI measures in our analysis. Other investigations in this context have shown pharmacological modulation of NO metabolism to impact plasma nitrite levels, which in turn correlate with regional changes in vascular tone (191). These correlations are in line with this finding. Similarly, although we did not observe any significant changes in plasma H_2S concentrations in response to our interventions we also observed a correlation for the novel vasodilator H_2S with AI measures.

In interpretation of these results, it should be noted that these correlations are, at best, a crude estimate of the contribution of these compounds to changes in vascular tone. The control of vascular tone is a complex process dependent on the nervous system and a plethora of chemical mediators of autocrine, paracrine, exocrine and endocrine origin and also exogenously produced compounds (drugs and xenobiotics). Accordingly, these correlations are not adequate for the purposes of establishing causation. Most notably the epoxide metabolites of EPA and DHA have been shown to be efficacious and robust modulators of vascular tone in a number of models in studies undertaken by a number of independent groups (131-134, 192). We feel the contribution of these compounds likely accounts for a sizable portion of DHA dependent changes in vascular tone.

In summary, despite the limitations of this analysis, this data, in particular the correlations with the epoxide derived diols 19, 20-DiHDPA and 17, 18-DiHETE, provides some insight into the changes in vascular tone which have been observed in our study and others in response to DHA and EPA consumption.

3.3.4.7. Summary

Our results for serum glucose, triglyceride and NEFA levels did not suggest any differential effects for control EPA or DHA containing meals on these biomarkers. These results are consistent with the results for studies which investigated the inclusion of EPA or DHA (61) in high fat meals and also those which included mixed doses of EPA/DHA in high fat meals (58). We observed changes in the concentration of EPA and DHA postprandially dependent on treatment, and increases in DPA independent of treatment, but no marked increases in any other plasma fatty acids.

With references to our measurement of blood borne vasomediators, our study provided novelty in its investigation of the relative efficacy of EPA vs DHA to modulate a broad array of vasomediators in the postprandial state, these included H_2S (previously uninvestigated in this context), nitrite as a marker of NO production and the vasoactive epoxy and diol derivatives of EPA and DHA. In short, using the

most reliable techniques clinically available, we did not observe changes in plasma H_2S or nitrite levels dependent on meal consumption, or EPA or DHA inclusion in the meal. Accordingly, we were not able to establish a role for these vasomediators in contributing to EPA or DHA dependent changes in vascular tone. Finally, we were able to establish that EPA derived epoxides and diols and DHA derived diols change profoundly in the postprandial state in response to physiologically relevant doses of EPA and DHA. Although we were unable to qualify changes in the concentration of the DHA epoxides (due to analytes falling beyond the range of detection), given that the concentrations of the 19, 20-DiHDPA changed >2 fold in response to the DHA meal it is likely that changes in the concentration of the parent epoxide also occur. Bivariate correlations undertaken between these epoxy and diol metabolites linked 17, 18-DiHETE and 19, 20-DiHDPA to Augmentation Index measures (see 3.3.5.5 and 3.3.5.6 for a thorough discussion of these results).

Chapter 4

Whole Blood Culture investigations on the impact of postprandial blood fat profile on the innate

inflammatory response

Chapter 4 – Whole Blood Culture investigations on the impact of postprandial blood fat profile on the innate inflammatory response

4.1. Introduction

Whole blood culture (WBC) has been utilised in a number of human studies to measure leukocytic cytokine production in response to a range of treatment (193-195). Comparison of this methodology to responses in isolated monocytes and PBMCs have validated WBC as a reliable, and low cost, proximal measure of monocytic cytokine production (193). This approach also has the benefit of including a complex mixture of effector cells, including granulocytes, monocytes, lymphocytes and potentially other minor contributors to blood cytokine production including circulating endothelial progenitor cells and fibroblasts. Accordingly, this model is likely more physiologically comparable to the *in-vivo* heightened systemic inflammation observed in cardio-metabolic disease states, than one single monocytic cell line (such as THP-1) or PBMC isolation.

In our model, we stimulated whole blood cytokine production with LPS. Stimulation of whole blood culture with LPS is known to induce cytokine signalling primarily through TLR4 (196), which plays a critical role in innate immunity and is known to be activated above normal, basal levels in obesity and cardiovascular diseases. Furthermore, certain free fatty acids, such as palmitic acid and oleic acid, all contained within the control meal (Table 2.6.3), are known to activate TLR4 signalling and increase inflammatory cytokine production (49). Given the integral role that inflammation plays in the pathology of obesity and cardiovascular disease, and the likely impact of the postprandial dyslipidemia on inflammation, our whole blood culture experiments were designed to investigate the impact our interventions had on cytokine production in cultures of whole blood taken at 4 hours postprandially.

Previous experiments utilising this model have shown modest reductions in LPS stimulated IL-6 production after 8 weeks of fish-oil consumption (3.1g per day of EPA and DHA, ratio of EPA:DHA not provided) (52). However, to the best of our knowledge, no studies have been undertaken which have investigated how acute changes to the lipidome, dependent on meal fat content, might impact whole blood LPS stimulated cytokine production responses. As mentioned previously in this thesis, we hypothesise that controlled investigation of the postprandial state (which is associated with 'heightened' inflammation) is more likely to generate informative and physiologically relevant data on how individual dietary components such as EPA or DHA might exert their effects *in-vivo* given than investigation of the fasting state (which only represent approximately 25% of the day for individuals

who eat regular meals). Accordingly, we aimed to utilise this model in order to investigate how changes in the lipidome postprandially, after the consumption of EPA or DHA, might affect cytokine production in this whole blood culture model. This approach allowed us to assess the impact of the whole profile of lipids, and lipid derivatives, found in the blood postprandially in response to each treatment (a comprehensive overview of meal nutrient information and fat composition can be found in Section 3.6) in a physiologically relevant model of inflammation. A number of cytokines were measured in our model, including IL-6, IL-10, IL-1α, Il-4, IFN-y, IL-1β, IL-8, MCP-1 and the growth factors epidermal growth factor (EGF) and VEGF. A more thorough review of these cytokines and growth factors, and their interplay with the conditions which arise in vascular dysfunction and cardiovascular disease is given in Section 1.3 and 1.6.

4.2.　Materials

All materials were obtained from Sigma (Dorset, UK) unless noted otherwise.

4.3.　Methods

The main study design and blood sampling procedure are given in chapter 2, section 2.7.5. Here the specific details of the whole blood culture and analysis of cytokines and growth factors are given.

4.3.1. Whole blood culture

LPS stimulated whole blood culture was carried out as per Damsgaard *et al* (52). At 4 hours postprandially blood was drawn into a 6ml Lithium Heparin vacutainer for whole blood culture in order to investigate aspects of how the postprandial inflammatory response was modified by meal fat content. 6-well plates were pre-alliquoted with 200 µL of 10 µg/mL of LPS stock solution (final concentration 1µg/mL LPS). Blood drawn at 4 hours was mixed with RPMI 1640 media supplemented with HEPES, L-glutamine and 0.05ug/ml of benzyl penicillin and streptomycin sulphate (Lonza Wokingham) in a ratio of 1:1 (4ml of blood to 4ml of media), mixed gently and subsequently 1.8ml was added to the well plates pre-alliquoted with LPS and RPMI 1640. The resulting solution was incubated for 24 hours at 37°C in a 5% CO_2 incubator. At the end of the culture period, well contents were removed, transfer to three eppendorf tubes and centrifuged at 1000g for 4 minutes. The supernatant was removed and frozen at -20°C until analysis.

4.3.2. Analysis of cytokine production in whole blood culture in response to intervention

The production of cytokines and growth factors in the whole blood culture model was investigated via the Evidence Investigator (Randox Laboratories Ltd, County Antrim, Northern Ireland) in a cytokine and growth factor array (Cat No. EV3513). For VEGF which fell out of the range of analysis concentrations were determined by single ELISA kits (Sigma Aldrich, Dorset, United Kingdom).

For the cytokine and growth factor array, 200µl of assay buffer and 100µl of samples are added into each biochip in the array. The biochips were then placed in a thermoshaker at 37°C at 370RPM for 60 minutes. The biochips were subsequently rinsed twice using a washing buffer, followed by four two minute washes in wash buffer. 300µl of a conjugate solution was then added to each reaction well before placing the biochips in a thermoshaker at 4°C at 370 RPM for 60 minutes. Following this, 250µl of Luminol-EV701 and peroxide solution was added to each biochip before loading into the Evidence Investigator for analysis.

For the VEGF ELISA, pre-prepared standards and samples were added to wells and incubated for 150 minutes at room temperature with gentle shaking. Standards and samples were then discarded and wells were washed 4 times with wash solution. 100µl of prepared biotinylated antibody was then added to each well and left to incubate for 60 minutes with gentle shaking. The solution was then discarded, and wells were washed as before. 100µl of streptavidin solution added to each well and left to incubate for 45 minutes with gentle shaking. The streptavidin solution was discarded after incubation, wells were washed as before and 100µl 3,3',5,5'-tetramethylbenzidine solution was added and incubated for 30 minutes, before final addition of stop solution. Absorbance was subsequently read at 450nm.

4.3.3. Statistical analysis

The impact of meal fat composition on *ex-vivo* cytokine production was tested via 1-way ANOVA. Data was also subsequently tested to investigate the effect of a number of covariates on cytokine production in our model via use of ANCOVA. SPSS (version 22; Chicago, Illinois, USA) was used for all statistical analysis. Further to this, a summative score of inflammatory cytokine production was also generated in order to investigate the net effect of treatment on all inflammatory cytokines measured in our model. This summative score was generated by normalising data via use of z-scores which were generated by pooling responses to all treatments (see Fig 4.2.3.).

$$z = (x - \mu)/\sigma$$

z=z-score, x=mean concentration, σ=standard deviation

Figure 4.2.3. Formula for generation of z-score

Average z-scores for inflammatory cytokines were then summed as described to provide an indication of treatment effect on overall inflammatory cytokine production (see Figure 4.2.3.1.). This approach has previously been utilised in nutrition studies in order to elucidate the subtle effect of individual nutrients on related biological outcomes (197).

$$\text{Total inflammatory cytokine production score (TICPS)} = Z_{IL\text{-}4} + Z_{IL\text{-}6} + Z_{IL\text{-}8} + Z_{IL\text{-}1\alpha} + Z_{IL\text{-}1\beta} + Z_{IFN\text{-}y} + Z_{MCP\text{-}1}$$

Figure 4.2.3.1. Formula for investigative total inflammatory cytokine production score.

Finally, Pearson bivariate correlations were established between postprandial triglyceride concentrations and cytokine concentrations for control cultures alongside bivariate correlations between EPA and DHA concentrations and cytokine concentrations for all cultures.

Outliers were identified by visual inspection of normal Q-Q Plots and by variance greater than +/- two standard deviations or between visit coefficient of variation falling above 30%. IL-6, IL-10, IL-1α, IL-4, IFN-γ, IL-1β, IL-8, MCP-1 and EGF were all analysed by Evidence Investigator Biochip arrays. Sample size for these investigations was n=20, but was reduced to n=18 due to removal of data for n=2 individuals due to presence of an outlier >2 SD from populational mean for one individual and due to average between visit coefficient of variations falling above 30% for another individual. Sample size for IL-8 and MCP-1 was further reduced to n=17 and EGF (epidermal growth factor) to n=8, this was due to readings falling out of range of analysis for each of these analytes.

VEGF was quantified via a single VEGF strip ELISA. Sample size for this analysis was n=19. One outlier was omitted due to a value falling >2 SD from population mean.

4.4. Results

One-way ANOVA showed no significant effect of treatment on WBC cytokine production, or total inflammatory cytokine production score (TICPS) (see table 4.3.1, Figure 4.3.2). Subsequent analysis was undertaken in order to correct for age and BMI levels as covariates via ANCOVA. BMI significantly influenced IL-6, IL-4 and IL-10 However, adjusted p-values did not reach significance for the effect of treatment on any individual cytokines following the age and BMI adjustment.

For the TICPS although there was some evidence of a trend towards a reduced inflammatory z-score for EPA and DHA relative to control, no significant effect was evident.
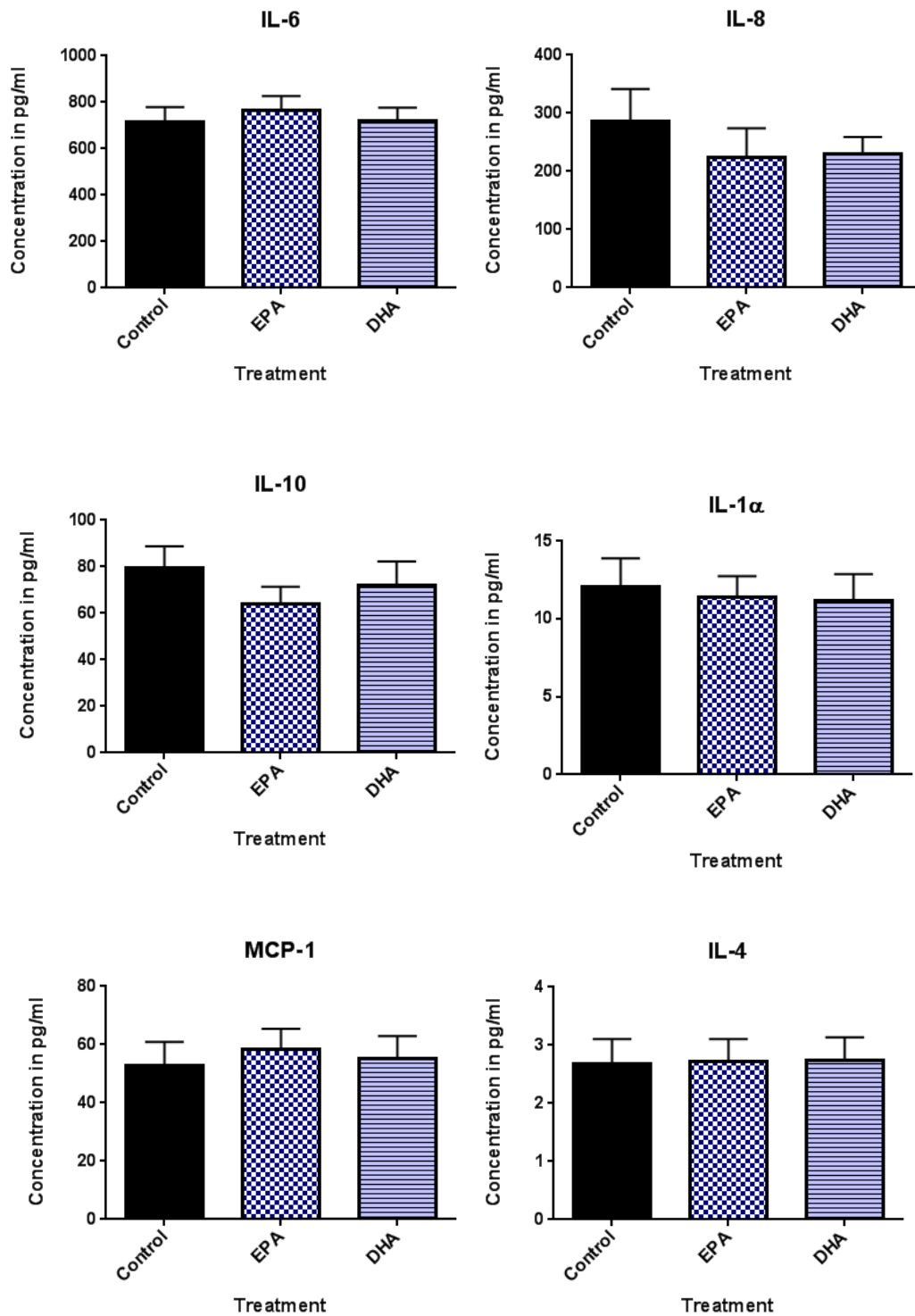
Figure 4.3.2.a. Cytokine concentrations in the supernatant from 24-hour culture of whole blood drawn 4-hour post-test meal consumption, data are presented as mean ± SEM. Continued, on following page.
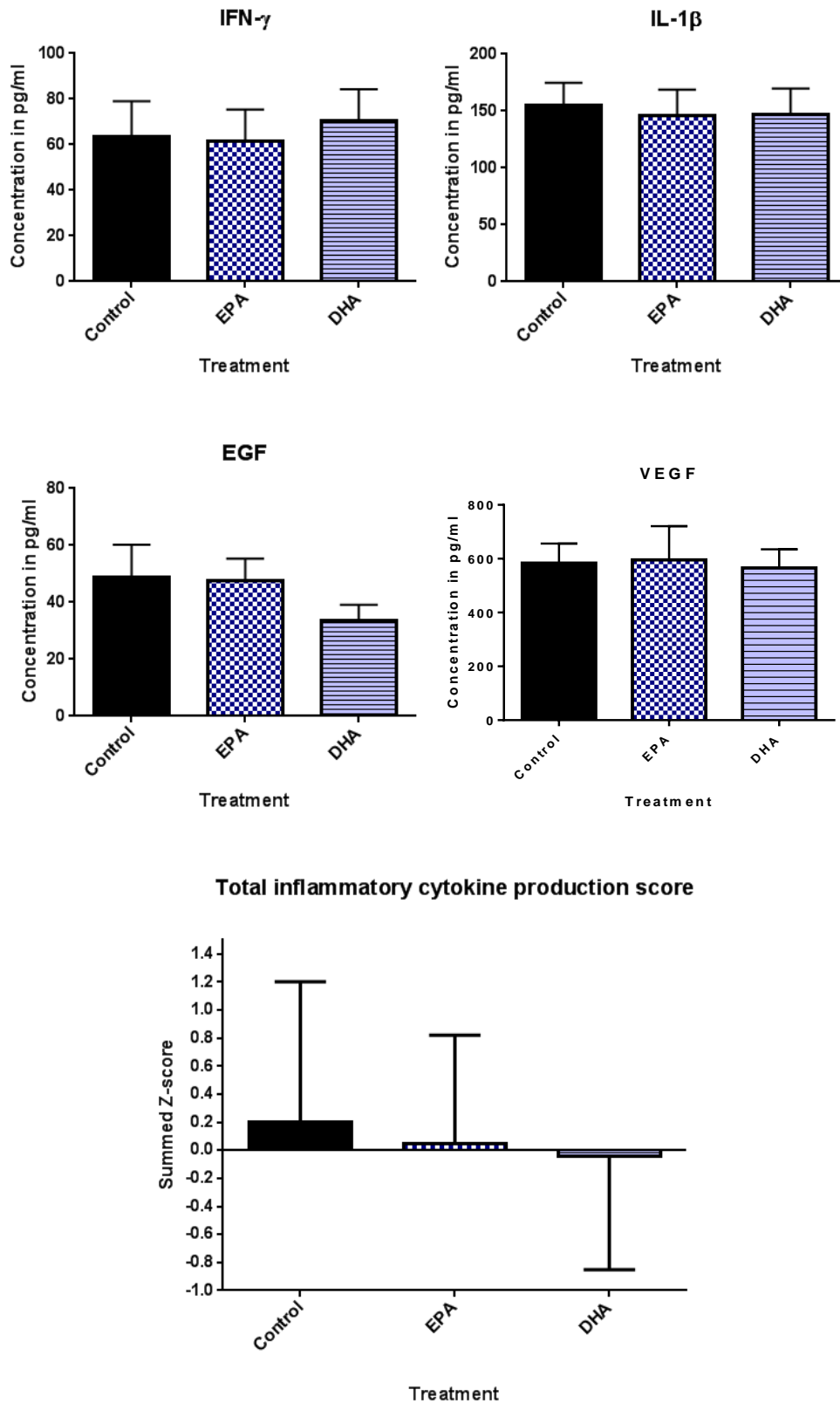
Figure 4.3.2.b. Cytokine concentrations in the supernatant from 24-hour culture of whole blood drawn 4-hour post test meal consumption, data are presented as mean ± SEM.

ANOVA showed no statistically significant effect for treatment for any of the cytokines or growth factors measured. Furthermore, after adjustment for age and BMI by ANCOVA, treatment did not reach statistical significance.

Cytokine	P-non adjusted[1]	P-Age[2]	P-BMI[3]	P-adjusted[4]
IL-6	0.83	0.67	.04*	0.83
IL-8	0.58	0.74	0.32	0.58
IL-1α	0.93	0.31	0.54	0.93
MCP1	0.91	0.34	0.82	0.91
IL-4	0.99	0.41	0.03*	0.99
IFN-γ	0.90	0.50	0.37	0.90
IL-1β	0.95	0.25	0.54	0.95
IL-10	0.49	0.33	>0.01**	0.39
EGF	0.39	0.22	0.94	0.37
VEGF	0.97	0.09	0.05	0.40
TICPS	0.98	0.71	0.11	0.97

Table 4.3.1. P-values for ANOVA[1] and between subject effects for ANCOVA after adjustment for Age[2], BMI[3] and Age and BMI[4] for treatment effect on cytokine production and TICPS.

Pearson bivariate correlations with plasma triglyceride, EPA and DHA concentrations.

Pearson bivariate correlations showed no significant association between any of the cytokines or growth factors measured and postprandial triglyceride levels or EPA and DHA concentrations.

Cytokine	Pearson correlation with triglycerides	P-value	Pearson correlation with EPA	P-value	Pearson correlation with DHA	P-value
IL-6	0.19	0.44	0.45	0.10	0.16	0.24
IL-8	-0.33	0.20	-0.03	0.82	-0.05	0.73
IL-1α	0.23	0.35	0.14	0.32	-0.05	0.71
MCP1	-0.06	0.81	0.07	0.63	0.18	0.20
IL-4	0.15	0.54	0.18	0.18	0.27	0.06
IFN-γ	-0.02	0.94	0.13	0.34	0.25	0.07
IL-1β	0.02	0.93	0.04	0.79	0.04	0.80
IL-10	-0.37	0.13	-0.23	0.10	-0.23	0.10
EGF	-0.26	0.54	-0.06	0.77	-0.34	0.11
VEGF	0.12	0.61	0.03	0.80	0.03	0.80
TICPS	0.05	0.84	0.19	0.18	0.23	0.09

Table 4.3.2. Pearson bivariate correlation between postprandial triglyceride, EPA and DHA concentrations and cytokine concentrations. Bivariate correlations were established between postprandial triglyceride concentrations and cytokine concentrations for control cultures, whereas for bivariate correlations between EPA and DHA concentrations and cytokine concentrations data from all cultures was utilised.

4.5. Discussion

This investigation was conducted to examine how postprandial alterations to the whole lipidomic profile, post consumption of EPA or DHA, impacts inflammatory cytokine production in a LPS stimulated whole blood culture model. The whole blood culture model was utilised as it is a physiologically relevant method for measuring whole blood cytokine production, which is thought to be reflective of mainly monocytic cytokine production (193), but also is dependent on contributions from granulocytes, monocytes, lymphocytes and other minor contributors such as endothelial progenitor cells and fibroblasts.

In these studies we were not able to establish a statistically significant effect of treatment on the production of any of the cytokines measured, however, some over-arching trends were noted. In particular it was noted that of the pro-inflammatory cytokines, IL-8 was 22% and 20% lower in response to the EPA and DHA meals when compared to control. Somewhat in contrast to this we also observed concurrent 20% and 10% decreases in the production of the anti-inflammatory cytokine IL-10 in response to EPA and DHA. Concurrently, we also observed an 11% relative increase in production of MCP-1 in response to EPA treatment and an 11% relative increase in IFN-y in response to DHA production. No other responses for any of the inflammatory cytokines differed markedly from control.

With regards to comparisons with other investigations, one trial (n=15) has shown that a 30 day 3.4g EPA and DHA intervention lowers serum IL-10 levels (198). However, larger scale epidemiological investigations have shown positive correlations between total serum n-3 fatty acids and IL-10 concentrations (199). Although it is difficult to compare the findings of our *ex-vivo* investigations with these *in-vivo* observations, our current understanding of EPA and DHA's effect on innate immunity signalling cascades might be suggestive that these results indicating a dampening of TLR4 dependent LPS response. EPA and DHA have also been shown to modulate or have a differential effect on IL-8 expression compared to other dietary fats in a number of contexts, including in epithelial cell lines (200), endothelial progenitor cells (201) and fibroblasts (202), with suggestions that DHA is more efficacious in mediating this effect (202). Given that our whole blood culture model would have accounted for a broad range of effector cells, including endothelial progenitor cells and fibroblasts, our data are somewhat supportive of these previous finding. It should be noted that in contrast to these studies we were not able to establish significance and our data did not suggest marked difference between EPA and DHA treatment in this regard. One other study has investigated the effect of mixed doses of EPA and DHA in this whole blood culture model. Specifically, this study involved a 2x2 factorial 8 week intervention in which individuals (n=64) were randomised to either 5ml/day of

fish-oil (corresponding to 3.1g of n-3 PUFA of unknown EPA:DHA ratio) or olive oil. Within these groupings individuals also received instructions to consume either high or low linoleic acid spreads (52). In this study, after 8 weeks IL-6 levels were significantly lower in LPS stimulated whole blood culture from individuals randomised to the fish-oil treatment when compared to the olive oil intervention. In contrast, we were not able to establish a role for either EPA or DHA treatments in reducing IL-6 production in our model, however, it is possible that these effects are dependent on incorporation of EPA and DHA into PBMC membranes, which likely would not have occurred within the 4 hours post-consumption.

In interpreting these results it is important to consider that production of these cytokines is a co-ordinated, auto-regulatory process which, when functioning optimally, contributes to maintenance of a homeo-static environment. In this light, and in the context of our experiments, the observation of trends towards decreased IL-10 production alongside decreased IL-8 should not necessarily be considered diametrically opposed. Most likely, these changes are explained by some decrease in the inflammatory response to LPS stimulation which leads to decreased production of inflammatory cytokines in the initial response to LPS, but also decreases in IL-10 production in the latter part of culture.

With regards mechanisms for these trends, as mentioned, a plausible explanation for this observation is that EPA and DHA dampened the LPS response somewhere downstream of TLR4, causing diminished production of IL-8 during the initial response to LPS exposure, but also leading to diminished IL-10 production during the latter stages of culture. It is important to remember that up-regulation of anti-inflammatory cytokines, such as IL-10, is delayed in response to LPS in monocytes (203) and production of IL-10 is often a product of ERK1/2, p38 and NF-κB signalling (204). Accordingly, it is likely that a greater degree of the IL-10 produced in our investigation was in the latter half of the culture, whereas the pro-inflammatory cytokines would have been produced in the initial exposure to LPS. Appropriately designed time course experiments would be informative on how these treatments differentially affect cytokine production as a product of time and this information could in turn yield insight into the mechanistic workings of the treatments in our model. It is also important to consider the crosstalk between LPS induced ERK1/2 activation, the subsequent activation of eicosanoid synthesising enzymes and substrate availability. These considerations have important consequences for these experiments as substrate availability for eicosanoid production would have differed significantly in our model dependent on treatment. The amount and type of eicosanoids produced likely play a role in any immune function modulation dependent on EPA or DHA in this model. Finally, there are also a number of other target molecules in the TLR4 kinase cascade which could also play a

role in these responses. For example, a plausible effector molecule which could play a role is SOCS3, which is known to decrease JAK1 activity (involved downstream of ligand binding in the IL-10 signalling cascade) and MKK3 activity (involved downstream of ligand binding in the TLR-4/LPS signalling cascade). Notably, SOCS3 is known to be activated by DHA in a PPAR-γ dependent manner (205) and it is likely that EPA has similar although possibly diminished effects.

We also saw a trend towards a decrease in VEGF (9%) and EGF (31%) production in response to DHA. VEGF is involved in neoangiogenesis and vasculogenesis and also in migration of vascular smooth muscle cells into the atheroma in later stage atherosclerosis (206) and is also chemotactic for macrophages and granulocytes (207, 208). Accordingly, it has a causal link to CVD. EGF is also implicated in migration of vascular smooth muscle cells into the atheroma, and activation of its receptor has been shown to cause vasoconstriction (209-211) and there are links between the abnormal vascular smooth muscle tone in type I and II diabetes and dysregulation of EGFR signalling (212).

Previous reports have reported a number of interactions between EPA, DHA, their metabolites and VEGF signalling in a number of contexts. These have included reports of reduced expression in colon cancer cells *in-vivo* and *in-vitro* in response to DHA via a ERK1/2 dependent mechanism (213), decreased VEGF dependent cell migration in a HUVEC model in response to DHA via a GPR120, ERK1/2 dependent mechanism (214), decreased VEGF dependent angiogenesis in Met-1 breast cancer models in response DHA epoxides and sEH inhibitors (215) and EPA but not DHA inhibited VEGF induced proliferation in bovine carotid artery endothelial cells (216). Accordingly, there are a number of studies reporting evidence of interactions between DHA and its metabolites with VEGF signalling and one study which suggests these effects extend to EPA but not DHA in a bovine endothelial cell model. There are also a number of lines of evidence that are supportive of this effect occurring through some modification of ERK1/2 phosphorylation state. Our data is suggestive of the possibility of these interactions involving some modification of VEGF expression in monocytes and possibly other blood borne cytokine producing cells. With regards to differences in EGF concentrations dependent on treatment, a previous study has shown changes in mesangial cell expression of EGF in response to DHA but not EPA (217). We are not aware of other studies which have investigated the differential effects of EPA and DHA in this context. It is plausible that the effect of treatment may reach significance with greater sample size (retrospective power calculation give a required n=20, at a power of 0.80 to distinguish the effect size observed as significant). Given the implications of EGF's role in the development of dysfunction of the endothelium, abnormal smooth muscle cell tone, atherosclerosis and diabetes mellitus (218), future studies on these interactions may be warranted.

Despite a number of cytokines decreasing in response to EPA and DHA, we were not able to establish statistical significance for any of the 10 cytokines measured. In order to account for covariates which may have contributed to some of the variance in our analysis, we utilised ANCOVA which adjusted for BMI and age. We hypothesised that due to positive associations between increased BMI and inflammation (219, 220) that BMI may be a causal factor in inter-individual variations systematic innate immune response. Similarly, we hypothesised that links between aging and increased inflammation (221) may influence our data. However, after adjusting for BMI and age as covariates, treatment dependent changes in cytokine production, growth factor production or TICPS did not reach significance. BMI did emerge as modifier of response for IL-10 and IL-4 and IL-6 production (see Table 4.3.1). However, BMI did not interact with treatment to a sufficient degree to impact significance in our adjusted ANCOVA model. Although this study was not designed to investigate these interactions, these results can be considered supportive of previous indications of associations between BMI and increased inflammation (219, 220), and might suggest that increased BMI is associated with functional changes in circulatory productions of these cytokines.

We also investigated correlations between postprandial triglyceride, EPA and DHA concentrations and cytokine and growth factor production (see Table 4.3.2). This was undertaken as triglyceride rich lipoproteins have been shown to bind and modulate LPS activity (222) and we hypothesised that this may play a role in modulating LPS stimulated cytokine production in our model. Furthermore, we reasoned that if a significant impact of treatment was evident then absolute concentrations of EPA and DHA in plasma would be associated with the WBC cytokine response. Accordingly, this analysis was undertaking to see if postprandial triglyceride, EPA or DHA concentrations correlated with any changes in cytokine production. We did not show significant bivariate correlations between postprandial triglyceride, EPA or DHA concentrations for any of the variants analysed.

With regards limitations of this study, it is worth noting that, as expected, there was significant difference in inter-individual responsiveness to LPS stimulated cytokine production and this likely played a part in mean responses to treatment falling outside of statistical significance. Previous investigations have shown that although individual cytokine production responses to LPS in this model are reproducible over long time periods, there are large inter-individual variations in response (193). In this regard our observations are consistent with previous investigations. This consideration also played a role in a lack of power to detect any significance in this study, which was originally powered to examine impact of treatment on vascular responses. Retrospective power calculations indicate that n numbers of 169, 99 and 76 would be required to detect significance at a power of 0.80 for the effect sizes observed for IL-8, IL-10 and VEGF analyses respectively.

Some other aspects of our experimental design may have contributed to the lack of significance in this analysis. Specifically, due to aspects of our trial design we were unable to run a baseline blood culture as a comparison. Although our experiments accounted for an appropriate control fat comparison, a baseline culture would have allowed us to completely rule out any interday differences in baseline cytokine concentrations or responsiveness of the effector cells in this model to LPS. We accounted for these concerns in our trial design by checking for signs or reports of infection at each visit before baseline measures and blood draws, along with checking medication usage with particular emphasis on any other medications which might affect nutrient absorption and metabolism, vascular function or immune function (see Annex 3). Despite this, the only way to fully account for potential interday variations in inflammatory status and responsiveness to LPS would have been to perform a baseline culture. Future experiments in this context would be well advised to perform a baseline culture to account for these factors.

Other considerations for future work include investigation of concentrations of eicosanoid and other lipid mediators at baseline and in response to LPS exposure. Further to this, western blotting of targets downstream of TLR4 and the IL-10 receptor in an appropriate monocytic cell model may yield important information on mechanisms of any effects in monocytes, which are the major contributor to cytokine production in this model. As mentioned, a potential explanation for the dichotomy of our observation of decreased IL-8 production in response to EPA and DHA alongside decreased IL-10 production is that the EPA and DHA effect the propagation of the signal downstream of the TLR4 and this dampens both initial inflammatory cytokine production in response to LPS and also the production of anti-inflammatory cytokines in the resolution phase of LPS exposure. Accordingly, it would be advisable to perform a time course analysis in order to see how the cytokine response varies as a product of both time and treatment.

In conclusion, we were not able to show any modification of cytokine or growth factor production dependent on treatment, before or after adjustment for BMI and age, or dependent on postprandial triglyceride levels. However, it is plausible that some effects of treatment in particular for EPA and DHA's effect on IL-8 and IL-10 and DHA's effect on EGF and VEGF might reach significance with greater sample size (approximations put a sample size with 0.80 power in the order of magnitude of 2).

Chapter 5

Impact of genotype on the postprandial response to EPA and DHA in a high fat meal - a pilot study

Chapter 5 – Impact of genotype on the postprandial response to EPA and DHA in a high fat meal - a pilot study

5.1. Introduction

As discussed throughout this thesis, there are numerous indications in the literature that suggest EPA and DHA have beneficial effects in the vasculature (40, 58-60, 79). Evidence suggests that EPA and DHA improve postprandial vascular reactivity (58) and this may contribute to improvement in the integrity of both peripheral and conduit arteries in the longer term which in turn, may contribute to improvements in cardiovascular health outcomes (40). However, there is a dearth of data from human investigations on the relative effects of EPA vs DHA in general, and on their effects in the postprandial state. Furthermore, it is also suggested that the postprandial and chronic response to EPA and DHA in the vasculature varies significantly according to genotype (46, 174). There is also a lack of information on the influence of genotype in the response to these dietary fats. Accordingly, in order to generate pilot data for future work investigating nutrient*genotype interactions which may affect inter individual responses to EPA and DHA in the vasculature, a candidate gene approach was taken. SNPs were initially identified on the basis of pre-existing data in the literature suggesting that interactions between mixed EPA and DHA supplementation and SNP genotype influence vascular responses or on the basis of significant theoretical links between SNP variation and change of function/efficiency of enzymes which may impact the vasculature response to EPA and DHA. Given our sample size, in order to produce meaningful data, we restricted our options to those with a minor allelic frequency of >10%.

A list of the four SNPs to emerge is found below in Table 5.1.

SNP	Protein coded by gene	BP upstream and downstream of SNP	SNP location in gene	Minor allele frequency
rs1799983	eNOS	…ATGA[G/T]CCCC…	Exon	35%
rs4673	NADPH Oxidase	…GAAG[C/T]ACAT…	Exon	31%
rs1801282	PPAR-y	…TGAC[C/G]CAGA…	Exon	9%
rs1048943	CYP1A1	…GACC[A/G]TTGC..	Exon	4%

Figure 5.1. SNPs selected for investigation in the F.O.F.A study.

With regards to indications in the literature linking these SNPs to altered responses to EPA and DHA, the rs1799983 SNP (also known as the eNOS, Glu298Asp polymorphism) has been previously linked to alterations in the vasculature response to fish-oil (174, 223). Specifically, both males and female carriers of the homozygous for the ASP298 isoform have shown 2-fold greater improvement in measures of endothelium dependent vascular reactivity (FMD) in the postprandial state in response to a dose of 0.46g of EPA and 4.18g DHA alongside heparin infusion which was utilised to artificially

raise NEFA levels. A cohort study, on a population of male and female individuals undergoing routine diagnostic cardiac catheterization for unclear chest pain (n=97), has also shown positive correlations between plasma n-3 fatty acid concentrations and FMD in GLU298 carriers, but not in ASP298 carriers (223). On a mechanistic level, the minor allelic variant of the ASP298 polymorphism has been linked to perturbations in normal vasoregulation, specifically increased coronary vascular resistance (224), and the development of endothelial dysfunction (225) independent of EPA and DHA. Our study aimed to investigate for the first time whether EPA or DHA rich meals differed in their capacity to alter vascular tone dependent on the rs1799983 SNP. We reasoned that these investigations would help expand the base of knowledge on these interactions as previous studies have not compared the individual effects of EPA vs DHA in this regard.

NADPH Oxidase genotype was investigated as presence of the *242T* allele has been demonstrated to have a negative impact on vascular superoxide production (226). Specifically, the nucleotide change at this position gives rise to a histidine residue instead of a tyrosine residue in the expressed protein. The impact of this change in amino acid on enzyme functionality has been insufficiently evaluated, however, evidence is suggestive that it may confer an increased capacity for production of superoxide to NADPH oxidase. Interestingly, this SNP has been linked by meta-analyses with modest increases in risk of coronary artery disease in Caucasians (227). We hypothesized that given that reactive oxygen species are involved in the sequestering of NO, by reaction of superoxide with nitric oxide to produce peroxynitrite, it is possible that this genotype may play a role in bioavailability of NO. Although there isn't existing evidence in the literature to suggest an interaction for this SNP with EPA or DHA, we reasoned that due to previous observations suggesting a role for NO in EPA and DHA's effects in the vasculature (58), this SNP could theoretically influence vascular responsiveness to EPA or DHA. Accordingly, we investigated this SNP to see if vascular responsiveness to EPA and DHA may be influenced by a capacity of this SNP to impact oxidative stress dependent sequestering of NO.

As discussed in Chapter 1, it is known that PPAR-γ plays a pivotal role in the metabolic response to EPA and DHA. Furthermore, the 2 allelic variants at position rs1801282 (the Pro12Ala polymorphism) in the gene coding for PPAR-γ have been shown to alter the serum triglyceride response in a 3 month n-3 fatty acid supplementation (228). Specifically, carriers of the ALA12(G:C) polymorphism had greater triglyceride decreases in response to consumption of 2.4g per day of n-3 PUFA (ratio of EPA:DHA was not provided in the original study) than PRO12 carriers (C:C) in this 3 month intervention. Furthermore, the minor allelic variant has been shown to be associated with greater degrees of postprandial hyper-triglyceridemia (229). Accordingly, the status of this polymorphism was investigated in order to see if the postprandial triglyceridemic responses to high fat meal consumption

were altered dependent on rs1801282 status by inclusion of EPA or DHA content of the intervention meals.

Finally, given that the CYP1A1 rs1048943 SNP has been observed to contribute to differential production of EPA epoxymetabolites in *in-vitro* investigations (230), we reasoned that this SNP may impact *in-vivo* production of the EPA and DHA epoxymetabolites in our trial, for which we observed large inter-individual variation. Unfortunately, validation of genotyping for this SNP failed in our investigations. This data may in the future be utilised for retrospective genotyping pilot studies on the genetic contribution to variations in LC n-3 PUFA epoxide production.

5.1. Materials

Fully details of the study design can be found in Chapter 2, blood sampling procedure is described in section 2.7.5.

All materials for genotyping were provided by LGC Genomics (Herts, United Kingdom) and Qiagen (Manchester, United Kingdom) unless otherwise noted.

5.2. Methods

5.2.1. Genomic DNA Isolation

Genomic DNA was isolated from buffy coat samples generated at screening for the 26 individuals who completed the trial. DNA was isolated using the QIAamp DNA blood kit. In brief, 25 µL of a protease solution (QIAGEN Protease) was added to 1.5ml eppendorf tubes. 200 µL of buffy coat samples was then added to the protease solution. 200 µL of lysis buffer (QIAGEN Buffer AL) was then added and eppendorfs were briefly vortexed and then incubated at 56°C for ten minutes. 200 µL of ethanol was added after incubation and samples were once again vortexed. Subsequent to addition of ethanol, samples were applied to a QIAamp spin column and were centrifuged at 8000rpm and 14,000rpm for 1 minute and 3 minutes respectively with wash buffers in order to remove impurities. Genomic DNA was then eluted from the column by incubating the column with 200 µL of elution buffer (10mM Tris-HCl, 0.5mM EDTA, pH 9.0) before centrifugation at 8000rpm for 1 minute. Genomic DNA content was subsequently quantified by nanodrop and then stored at -20°C until use.

5.2.2. KASP Genotyping

All DNA samples were genotyped via KASP genotyping. In short, KASP genotyping relies on polymerase chain reaction (PCR) process in a cyclical process in which genomic DNA is denatured, primers are annealed, and complement allele specific tails with FAM or HEX fluorescent tags are elongated dependent on which allelic variants are present. The end product of this process being exponential production of labelled DNA strands which can be measured by fluorescence in order to qualify genotype. Fluorescence is subsequently measured in order to qualify which allelic variant has been amplified and genotype is subsequently qualified via this process.

Specifically, KASP Master Mix and KASP Primer Mix were thawed and vortexed. 300 µL of KASP Master Mix and 8.4 µL of KASP Primer Mix were mixed and made up to 600 µL with PCR grade water.

Isolated genomic DNA 5 µL (concentration >5ng/ml) for each individual was added to wells in a 96 well plate. 5 µL of the previously prepared KASP Master and Primer Mix was subsequently added to each well. Plates were sealed with an optimally clear seal to prevent evaporation. Thermal cycling was conducted immediately, as described below in Table 5.2.2, was utilised to denature DNA and allow for subsequent cycles of annealing/elongation.

Step		Temperature	Time	Number of cycles per step
1.	Activation	94°C	15 min	1
2.	A)Denaturation B)Annealing/Elongation	94°C 61-55°C	20 sec 60 sec (drop 0.6°C per cycle)	10 cycles
3.	A) Denaturation B)Annealing/Elongation	94°C 55°C	20 sec 60 sec	26 cycles

Table 5.2.2. Thermal cycling conditions.

After thermal cycling plates were left to cool below 40°C and fluorescence was subsequently measured in each well in order to qualify genotype of each individual.

5.2.3. Statistical Analysis

The impact of genotype at baseline was assessed by 2-tailed heteroscedastic student t-tests (rs1801282 and rs1799983) or one-way ANOVA (rs4673) for BMI, serum total cholesterol, triglycerides, AI, PWV, RHI, DBP and SBP. For triglyceride, AI, PWV and RHI for each individual an

average value was taken from their three baseline (pre-meal) measures and used in the statistical analysis. BMI, serum total, and cholesterol values were taken from screening values.

In order to assess the impact of genotype on response to treatment, three-way repeated measures ANOVA were conducted for rs1799983 and rs4673. Three-way repeated measures ANOVA were not possible for rs1801282 due to unequal group sizes, accordingly for rs1801282 one-way repeated measures ANOVA were conducted on change scores. SPSS (version 22; Chicago, Illinois, USA) was used for all statistical analysis.

5.3. Results

5.3.1. Baseline vascular measures, blood pressure, markers of lipid metabolism and anthropometric measures as according to genotype

2-tailed students T-test showed significant differences in baseline serum triglyceride concentrations dependent on rs1801282 genotype (p=0.041). Specifically, baseline triglyceride levels were 26% higher in rs1801282 12ALA (G:C) carriers when compared to 12PRO (C:C) carriers. None of the other variables investigated differed significantly by rs1801282, rs1799983 or rs4673 genotype in our analysis.

	PPAR-γ - rs1801282		eNOS - rs1799983		NADPH oxidase - rs4673		
	C:C (Pro/Pro) (n=26)	G:C (Pro/Ala) (n=3)	G:G (Glu/Glu) (n=14)	T:G (Glu/Asp) (n=12)	C:C (Tyr/Tyr) (n=12)	C:T (Tyr/His) (n=11)	T:T (His/His) (n=3)
Age (years)	44.9±1.2	45.7±2.2	44.4±1.7	45.7±1.3	44.4±1.8	44.6±1.4	48.1±1.1
BMI (kg/m^2)	27.0±0.8	28.8±1.1	-	-	-	-	-
Total Cholesterol (mmol/L)	5.71±0.19	6.93±0.29	-	-	-	-	-
Triglycerides (mmol/L)	1.74±0.14	2.19±0.09*	-	-	-	-	-
AI (%)	-	-	23.2±1.3	24.9±1.1	21.2±2.5	26.1±1.5	24.2±1.2
PWV (m/s)	-	-	8.4±0.2	8.1±0.2	8.6±0.4	8.4±0.2	8.2±0.3
RHI	-	-	2.50±0.14	2.32±0.09	2.25±0.22	2.45±0.12	2.39±0.14
Diastolic BP (mmHg)	-	-	85±2	92±42	87±1	83±3	89±4
Systolic BP (mmHg)	-	-	133±2	140±2	137±2	132±4	138±2

Table 5.3.1. Baseline age, BMI, lipids and vascular measures as according to genotype.
– variables not analysed as according to this genotype
* significant difference from major allelic variant.
Data are presented as mean ± SEM.
BMI, Body mass index; AI, Augmentation Index; PWV, Pulse Wave Velocity; BP, Blood Pressure.
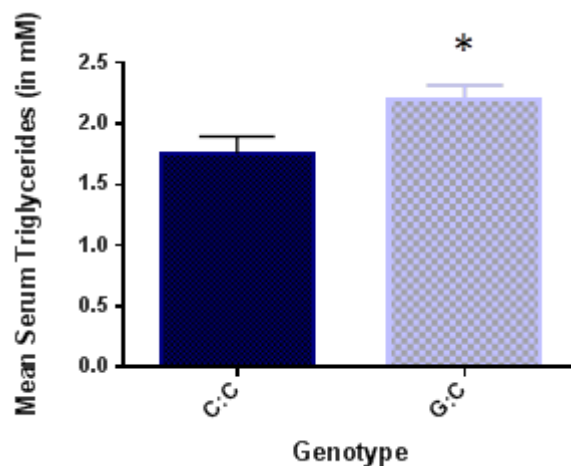
Figure 5.3.1.1. Mean serum triglyceride levels at baseline as according to PPAR-y rs1801282 genotype. Data are presented as mean ± SEM.

5.3.2. Postprandial triglyceridemic response to treatment according to PPAR-ɣ genotype

The absolute triglyceride concentration at 0h and 4h according to genotype and treatment is given in Figure 5.3.2.1. One-way ANOVA undertaken on change scores showed that there were significantly greater increases in triglyceride concentrations in response to consumption of the EPA and control meals in Pro12Ala carriers (G:C) when compared to Pro12Pro carriers (C:C), p=0.02 and 0.007 respectively. This effect did not reach significance for DHA, p=0.2, indicating that DHA treatment negated the effect of this genotype on postprandial triglyceridemia (see Figure 5.3.2.2.).

With regards treatment effect sizes, triglyceride concentrations increased postprandially by 64% and 69% in response to control and EPA treatments for the Pro12Ala carrier. In Pro12Pro carriers these increases were 30% and 40% respectively. In contrast, in response to the DHA treatment triglyceride levels increase by 33% in Pro12Pro carriers and by 54% in the Pro12Ala carriers.
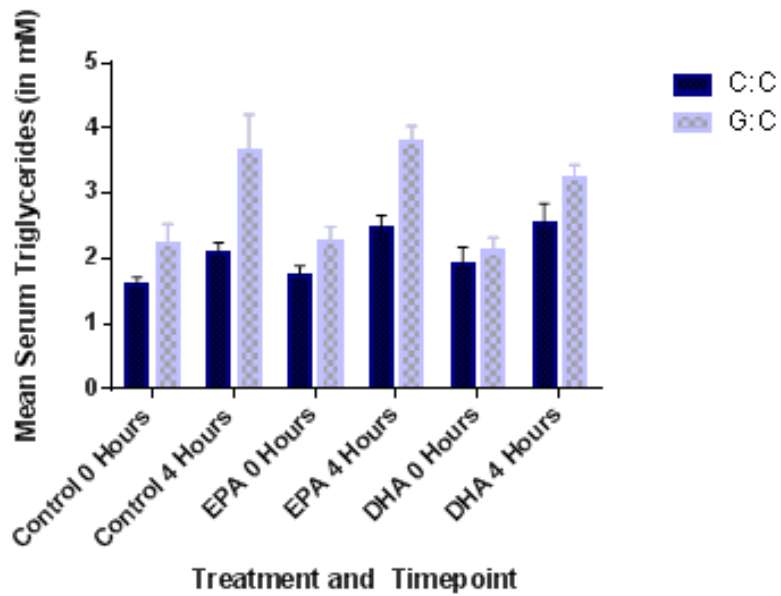
Figure 5.3.2.1. Serum triglyceride levels at baseline and in response to treatment as according to PPAR-γ rs1801282 genotype. Data are presented as mean ± SEM.
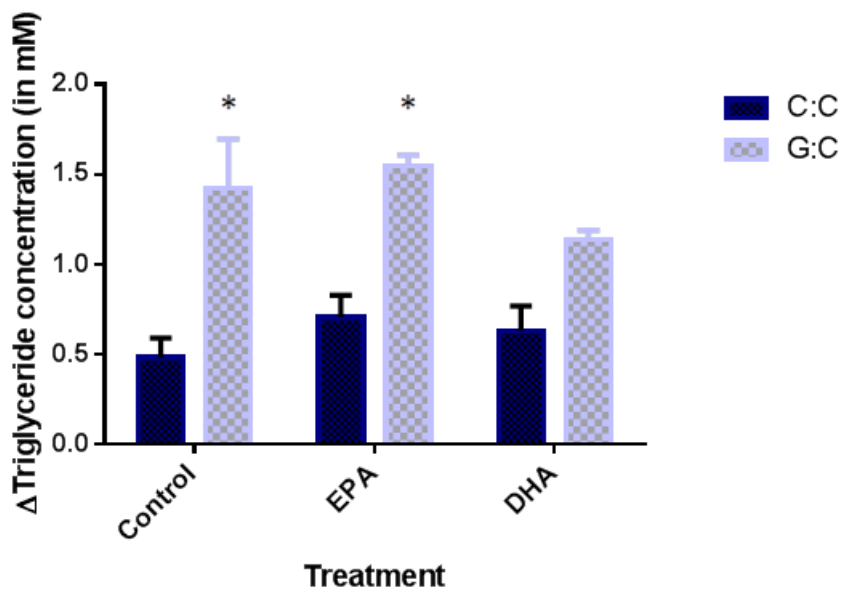
Figure 5.3.2.2. Mean change in serum triglyceride concentration from baseline dependent on treatment and rs1801282 genotype. * indicates significant difference in change score when compared to major allelic variant (C:C). Data presented as mean ± SEM.

5.3.3. Vascular response as according to eNOS rs1799983 genotype

Analysis did not show an interaction between intervention and the eNOS rs1799983 genotype for any of the vascular measures in our study.

Specifically, by Repeated Measures Anova treatment*time*genotype interactions did not reach significance for PWV (p=0.54, $F(2, 22) = 0.64$, partial $\eta^2 = 0.14$), PWA (p=0.79, $F(2, 22) = 0.24$, partial $\eta^2 = 0.08$) or RHI (p=0.35, $F(2, 20) = 1.09$, partial $\eta^2 = 0.21$) for the rs1799983 genotype. Accordingly, no post-hoc analysis was undertaken.

Data for PWV, PWA and RHI are presented below in Figures 5.3.3.1, 5.3.3.2 and 5.3.3.3 respectively.
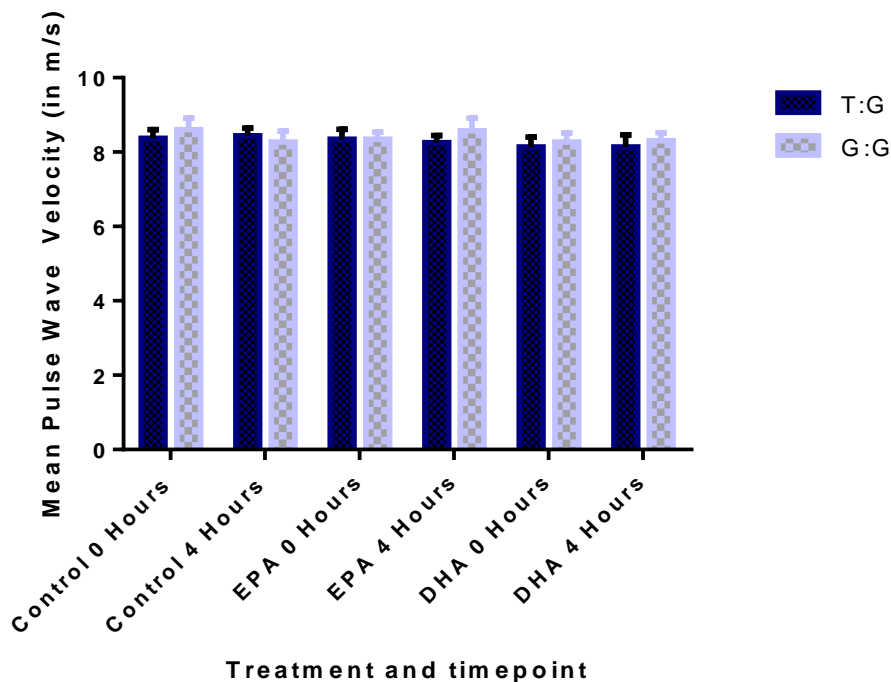
Figure 5.3.3.1. PWV response to treatment as according to eNOS rs1799983 genotype. Data presented as mean ± SEM.
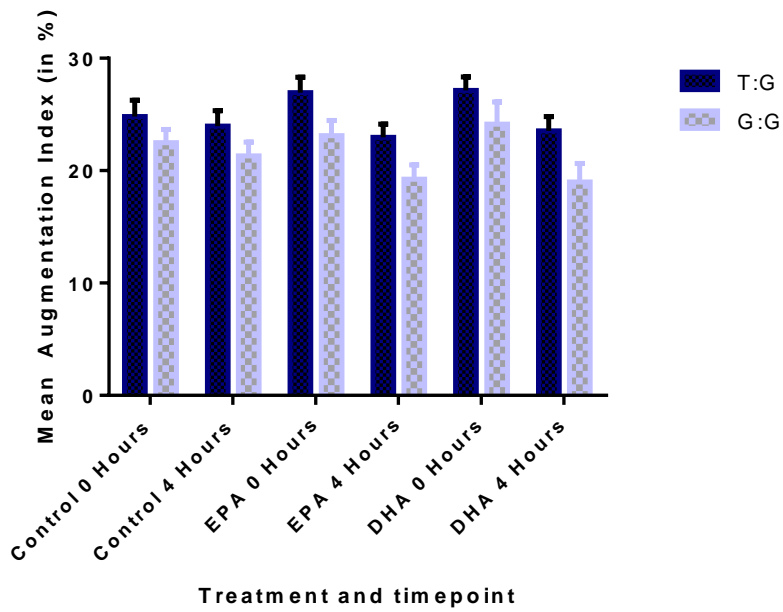
Figure 5.3.3.2. AI response to treatment as according to eNOS rs1799983 genotype. Data presented as mean ± SEM.
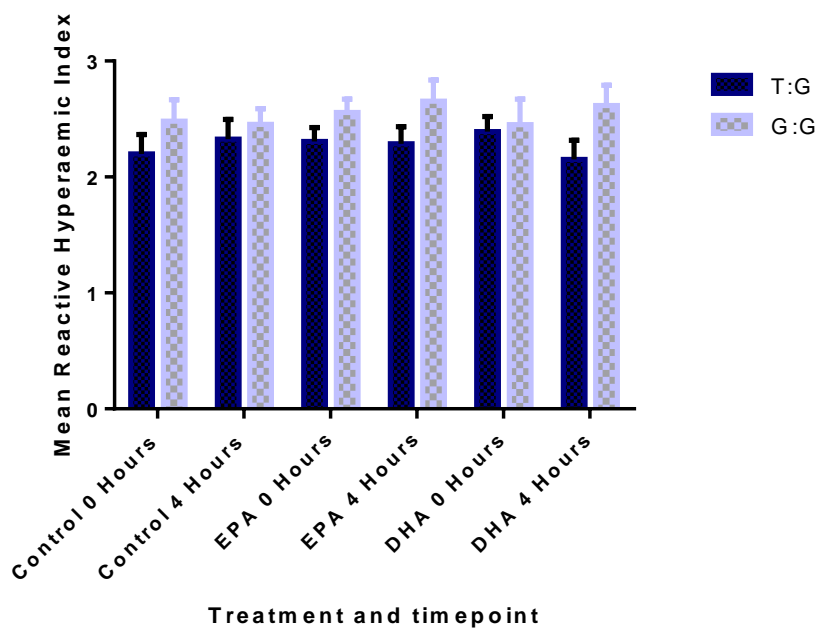
Figure 5.3.3.3. RHI response to treatment as according to eNOS rs1799983 genotype. Data presented as mean ± SEM.

5.3.4. Vascular response as according to NADPH rs4673 genotype

Analysis did not show an interaction between intervention and the NADPH rs4573 genotype on any of the vascular measures in our study.

Treatment*time*genotype interactions did not reach significance for PWV (p=0.69, F(4, 8) = 0.58, partial η^2 = 0.13), PWA (p=0.07, F(4, 8) = 3.33, partial η^2 = 0.58) or RHI (p=0.73, F(4, 8) = 0.51, partial η^2 = 0.12) for the rs4673 genotype. Accordingly, no post-hoc analysis was undertaken.

Data for PWV, PWA and RHI are presented below in Figures 5.3.4.1, 5.3.4.2 and 5.3.4.3 respectively.
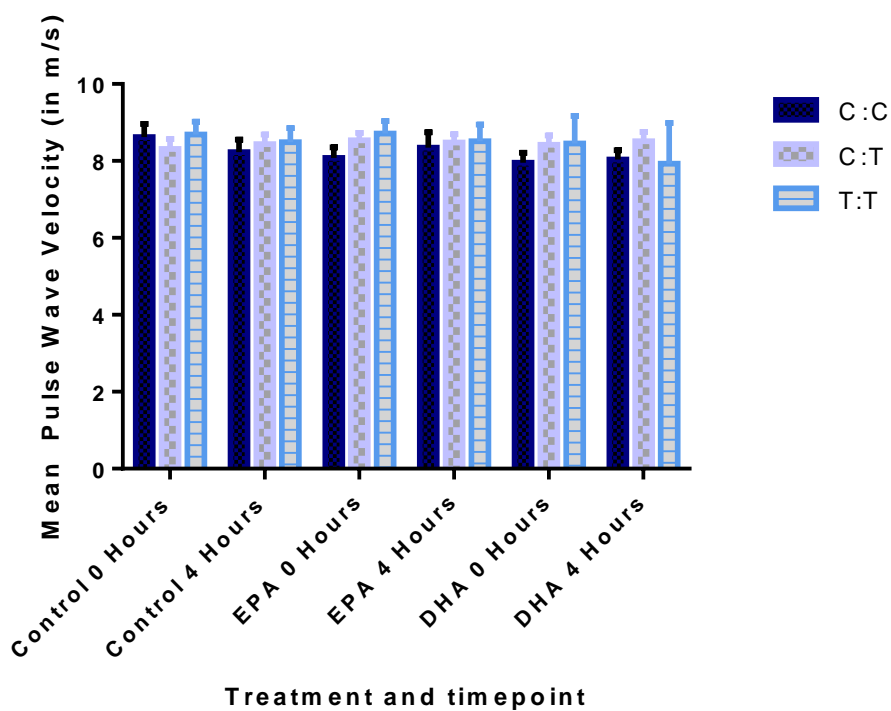
Figure 5.3.4.1. PWV response to treatment as according to NADPH rs4673 genotype. Data presented as mean ± SEM.

.
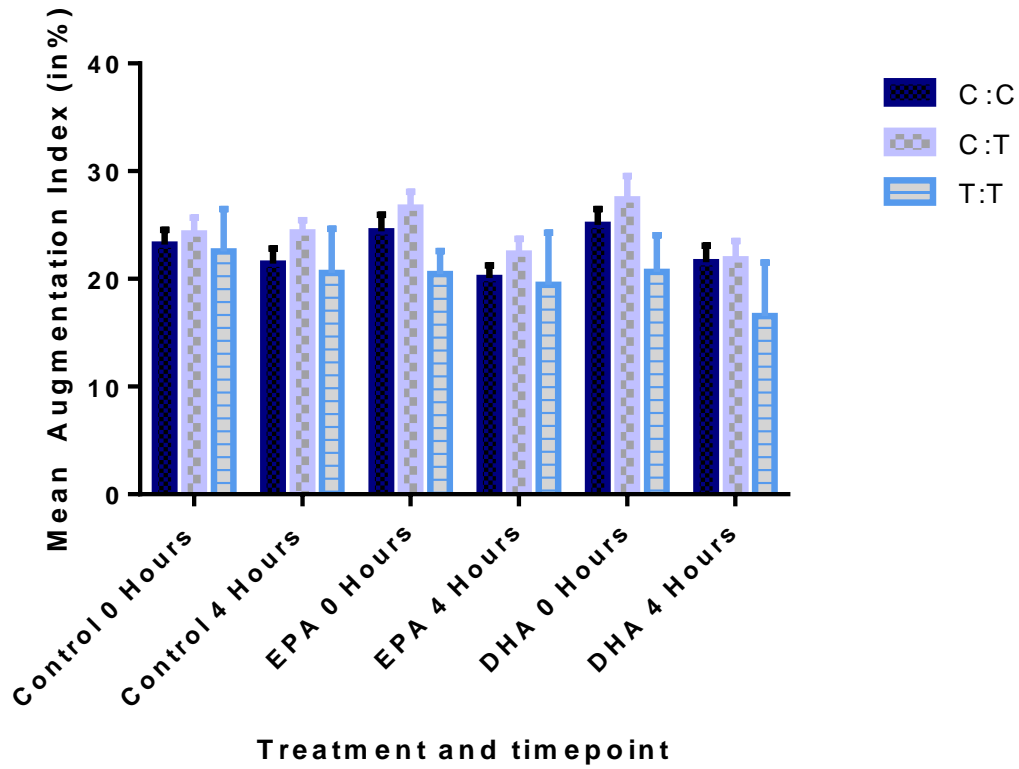
Figure 5.3.4.2. AI response to treatment as according to NADPH rs4673 genotype. Data presented as mean ± SEM.
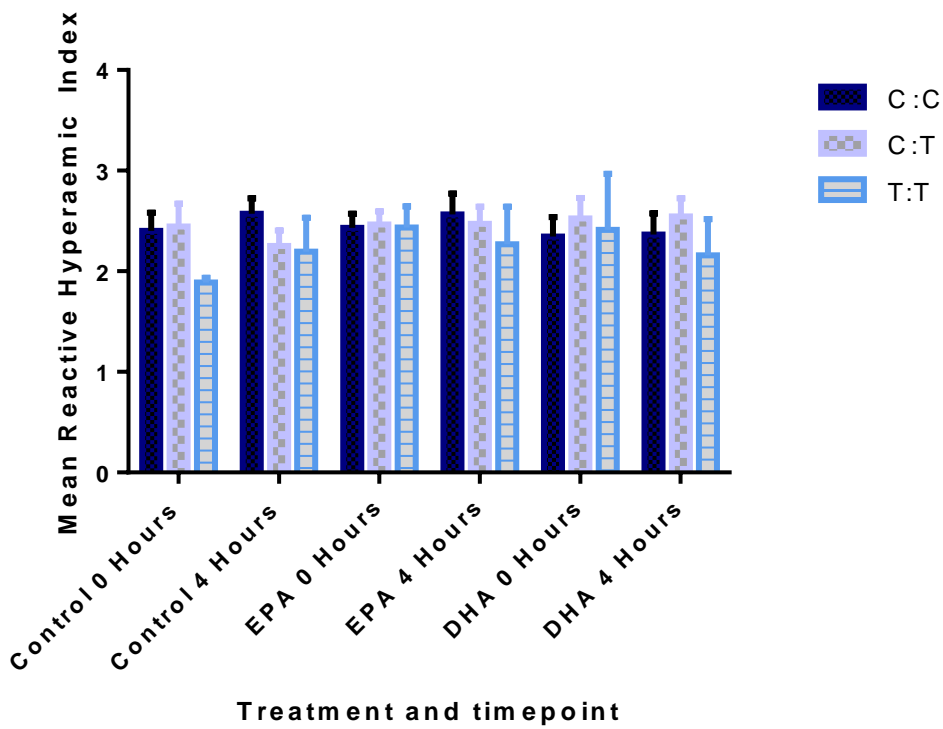
Figure 5.3.4.3. RHI response to treatment as according to NADPH rs4673 genotype. Data presented as mean ± SEM.

5.4. Discussion

5.4.1. Baseline vascular measures, blood pressure, markers of lipid metabolism and anthropometric measures as according to genotype

In our pilot analyses we investigated whether PPAR-γ rs1801282 genotype, eNOS rs1799983 genotype or NADPH rs4673 genotype influenced baseline vascular or plasma lipid measures. As mentioned, in our investigations we utilised a "candidate gene" approach and baseline status was investigated with a hypothesis driven approach in order to minimise the risk of type-1 errors. Accordingly, markers of lipid metabolism along with body weight and BMI were analysed dependent on PPAR-γ rs1801282 genotype; whereas blood pressure and vascular measures were analysed dependent on eNOS rs1799983 genotype and NADPH rs4673 genotype.

In this study, baseline serum triglyceride concentrations were significantly higher in rs1801282 ALA12 (G:C) carriers when compared to PRO12 (C:C) carriers (see Table 5.3.1). However, we draw conclusions from our findings with caution given that we had only three participants in our rare allele group. Nevertheless this observation is consistent with a meta-analysis (n=52,998) which investigated the effect of this genotype on fasting triglyceride concentrations (231). We also observed higher total cholesterol levels in the ALA12 group; however, this effect fell just out of significance in our analyses. As before, this observation is consistent with previous findings from a meta-analysis (231), which indicate that there is an interaction between this genotype and fasting total cholesterol concentrations.

Fasting vascular measures and blood pressure did not differ depending on eNOS rs1799983 genotype or NADPH rs4673 genotype in our analyses. With regards to the eNOS rs1799983 previous studies have observed that although lifestyle factors (smoking status) and dietary factors (plasma n-3 fatty acid concentrations) interact with rs1799983 to influence endothelial function (namely FMD), a relationship does not hold when considering only the impact of this genotype alone (223). With regards potential interactions between blood pressure and rs1799983, a previous cohort study of Caucasian women (n=18,436) did not show a relationship between this SNP and incident hypertension or progression of hypertension (232). Our investigations corroborate these observations and suggest that these previous findings also hold in males. It should be noted that although AI and RHI did not differ significantly depending on rs1799983 genotype, these measures tended to be improved in G:G carriers. However, this trend did not hold for PWV.

With regards, the NADPH rs4673 genotype a previous indication suggests that the rs4673 polymorphism does not influence baseline endothelial dependent or independent vascular function (233). Similarly to the interactions with rs1799983 our results are corroborative of previous findings, and once again suggest that these findings hold in males aged 35-55 years who phenotypically are at an increased risk of CVD.

5.4.2. Triglyceride response to treatment according to PPAR-ɣ genotype

In our present study we demonstrated that after consumption of a high fat meal, postprandial changes in triglyceride concentrations responses vary dependent on PPAR-ɣ rs1801282 genotype (see Figure 5.3.2.1). Further to this, DHA but not EPA seems to have a corrective effect in individuals who are heterogeneous for the minor allelic variant of rs1801282 (see Figure 5.3.2.2).

A previous study has shown an interaction between this genotype and changes in serum triglyceride concentrations in response to a mixed n-3 fatty acid intake. Specifically, the previous demonstration which compared the effects of 2.4g of EPA and DHA per day to an olive oil control, showed that carriers of the ALA12(G:C) polymorphism had greater triglyceride decreases than PRO12 carriers (C:C) in a 3 month intervention (228). Although the ratio of EPA:DHA was not explicitly stated in the paper, information available elsewhere suggests that the ratio of EPA and DHA in the supplement used was 1:1. Our work builds on these demonstrations by comparing the interaction of this genotype with plasma triglyceride levels in response to an EPA rich meal and DHA rich meal when compared to control fat in the context of the postprandial state. Our data suggests that the interaction of LC n-3 PUFA and the ALA12 polymorphism is extended into the postprandial state and also that this effect may be dependent on DHA, rather than EPA.

Another study has also demonstrated a greater propensity towards postprandial hyper-triglyceridemia in ALA12 carriers when compared to PRO12 carriers (odds ratio, 7.6; $p<0.001$) (229). This study defined postprandial hyper-triglyceridemia as a >1.71mmol/L increase in plasma triglyceride levels postprandially, a quite marked increase in triglyceride levels. Our data, which showed a 64% (1.43 mmol/l) increase in triglyceride concentration in response to the control meal in ALA12 carriers as compared to a 30% (0.48mmol/l) increase in PRO12 homozygotes, supports this previous observation. It is also of note that baseline triglyceride concentrations in the ALA12 individuals were 26% higher when compared to PRO12 carriers. As mentioned in section 5.4.1., this difference at baseline reached significance.

In summary, our data demonstrates that inclusion of DHA in high fat meals significantly dampens the postprandial hyper-triglyceridemia after consumption of a high fat meal in rs1801282 ALA12 carriers.

Specifically, the control meal increased triglyceride concentrations postprandially by 20% more than the DHA meal. Although proper interpretation of these results is hampered by small sample sizes (n=3 for ALA12 carriers and n=23 for PRO12 carriers), the consistency, size and significance of the responses warrants further investigation. In particular in the light of evidence that suggests that postprandial lipaemia is a highly significant CVD risk factor (234). It is thought that BMI, age and gender are among the most significant physiological determinants for postprandial lipaemia, however, genotypes that influence this response, and specifically those that may be modifiable dependent on dietary or lifestyle modification, are likely to be of wide spread public health relevance.

When considering these results, it is worth noting that DHA is suggested to be a more effective PPAR-γ activator than EPA and indeed other fatty acids (106, 114). It is also worth noting that this polymorphism codes for an amino acid in the ligand binding domain of PPAR-γ. Of particular importance are observations that have previously shown the ALA12 polymorphism to have an almost 2 fold decreased affinity for the PPAR-γ promoter response element in electrophoretic mobility shift assay experiments (235). Another investigation which utilised thialozinediones to compare the activity of the PPAR-γ rs1801282 isoforms upon ligand binding suggest that the presence of the minor allelic variant may impact the effect of ligand binding on induction of PPAR-γ activity (236). Our results are suggestive of DHA in part compensating for the impact of defective enzyme activity on the postprandial TG response. From a mechanistic standpoint it follows logically that DHA's greater activation of PPAR-γ when compared to other fats, could play a role in compensating for the effect on the minor allelic variant on PPAR-γ promoter binding affinity. In short, given the impact of this polymorphism on PPAR-γ activity, and the significant role PPAR-γ plays in extracellular fat metabolism, and indeed triglyceride clearance, a plausible mechanism for this observation exists, which provides further support for future investigations of these interactions.

In summary, this data provides a base for future investigations on the interactions between EPA and DHA with the rs1801282 polymorphism. From a theoretical standpoint, it plausible, given that DHA has been shown to be an effective activator of PPAR-γ and that PPAR-γ plays a role in lipid clearance, that DHA may play a greater role than EPA in contributing to increased LC n-3 PUFA dependent changes in triglyceride concentrations in ALA12 carriers when compared to PRO12 carriers (228). The possibility that this interaction could be leveraged to offer a therapeutic dietary strategy to overcome previously indicated increases in postprandial hyper-triglyceridemia in ALA12 carriers (229) is an interesting prospect. When considering that previous findings indicate an interaction between this SNP and modifications to basal triglyceride concentration in longer term n-3 PUFA interventions (228),

the potential impact of an interaction between this SNP and chronic DHA consumption on basal triglyceride concentration also warrants consideration.

5.4.3. Vascular response to treatment as according to eNOS rs1799983 genotype

In contrast with previous studies (174, 223, 237) we did not observe any differences in the vascular response to EPA and DHA when compared to control dependent on rs1799983 genotype, see Figures 5.3.3.1 to 5.3.3.2. There are a number of factors which may in part explain these observations, including the power of the studies, differences in the vascular measures undertaken and differences in total n-3 dosage and indeed differences in the amounts of individual EPA and DHA ingested.

With regards interactions between postprandial PWV measures and meal fat content, our results showed no impact for any treatment on PWV. Given that PWV is more dependent on physical changes in artery structure, rather than acute changes in artery diameter dependent on vascular tone, it is also likely that any interactions between meal fat content, genotype and changes in postprandial vascular tone are not measurable by PWV.

Changes in AI and RHI also did not reach statistical significance for genotype*treatment interactions. With reference to eNOS rs1799983 genotype, a previous study showed a 2-fold greater improvement in FMD in response to an intervention similar in scope to the DHA arm of our study (150). Unpublished work in our lab has also suggested that the vasodilatory response to EPA and DHA in individuals carrying ASP298 may be greater than non-carriers (237). A cohort study has also shown interactions between plasma n-3 PUFA concentrations, rs1799983 and basal vascular reactivity as measured by FMD (223). There are a number of notable differences between our study and the previous studies which may explain the discrepancies observed. These differences include the measures involved (FMD in the previously published trial as compared to AI and RHI in our study), study design (the previously published trial utilised infused heparin to artificially raise NEFA levels and also utilised a meal design that differed significantly in meal fat composition), sample size and health status, age and gender of the study population. It is plausible that each of these differences contributed to the discrepancy between results.

With regards to the measurements undertaken in our study, AI can be considered to be dependent on both endothelium dependent and independent changes in vascular tone and longer term mechanical changes to artery structure which influence arterial stiffness, whereas RHI can be considered a measure of an endothelial dependent measure of peripheral arterial tone. The FMD measure used in this previous study is considered the gold standard of endothelium dependent

measures of vascular reactivity. A number of physiological determinants distinguish this measurement from our measure of RHI. It is important to consider that FMD is measured via ultrasound, most commonly in the brachial artery, a major blood vessel, whereas RHI utilises measures of peripheral arterial tone in the tip of the index fingers, these measures are more dependent on the tone of the smaller arterioles of the fingertip than the FMD measure. The gross differences in structure and physiology of these blood vessels may well contribute to the discrepancy in observations between these studies, as may the difference in the responsiveness of these blood vessels to NO, n-3 epoxides and other vasomediators. The AI measurement also differs significantly to the FMD measure and it is also likely that these differences contribute to the discrepancy in these observations.

Finally, with specific references to our study, it is worth noting that these investigations were designed as pilot studies and accordingly, the original study was more adequately powered than ours. Furthermore, our study investigated the vascular responses to EPA and DHA in individuals with a higher CVD risk profile than that of the previous study and our individuals were significantly older (mean age of 45 in our study vs 28 in original study). It is plausible that the contribution of any 'subtle' impact of rs1799983 genotype to the postprandial vascular response to DHA and EPA changes with age and the baseline reactivity of the vasculature (which would tend to decrease as CVD risk increases).

In summary, we were unable to demonstrate a significant impact of this SNP on the vascular responses to EPA and DHA in the postprandial state. As noted this was a retrospective study, designed with the aim of generating pilot data, and accordingly was not sufficiently powered to generate definitive answers to these research questions. Also previous demonstrations of the interaction between rs1799983 and fish-oil intake in the literature have focused on younger populations (174, 223, 237). It is plausible that the effect for rs1799983 may hold in younger populations but not in older populations, or those with a higher CVD risk profile. This could be attributable to differences in basal vascular reactivity, which tends to worsen with increased CVD risk. Future work on rs1799983's impact on EPA and DHA's effect on postprandial vascular reactivity might consider investigating the effect of age on these interactions.

5.4.4. Vascular response to treatment as according to NADPH oxidase rs4673 genotype

Our investigations of NADPH Oxidase rs4673 genotype were based on previous indications that the presence of the T allele at this position is associated with decreased superoxide production (226). Accordingly, given the links between superoxide production and NO bioavailability, and the suggested links between the vascular response to fish-oil and NO (58), we investigated if this SNP may influence vascular responsiveness to fish-oil.

In our investigation, we did not observe a relationship between rs3673 genotype and inter-individual vascular responsiveness to EPA or DHA when compared to control for PWV (Figure 5.3.4.1), AI (Figure 5.3.4.2) or RHI measures (Figure 4.3.4.2).

It is likely that the lack of effect for PWV is attributable to the lack of change in PWV in response to meal consumption. As mentioned, this in turn is likely due to the fact that PWV is more dependent on physical changes in artery structure, rather than acute changes in artery diameter dependent on vascular tone. As discussed in section 5.4.1, rs4673 genotype also had no effect on baseline PWV measures. AI and RHI, which are modifiable by NO bioavailability also did not change dependent on rs4673 genotype or for treatment*time*genotype interactions for rs4673. Only one other study has investigated rs4673 in this context, which has shown that rs4673 genotype does not impact forearm blood flow in response to acetylcholine in hypercholesterolemic individuals (233).

Although we would emphasise prudency in interpretation of these data due to sample size considerations, in the absence of other data, the investigative work presented in this chapter does not support further investigations on the contribution of this SNP to inter-individual differences in vascular response to EPA or DHA.

5.4.5. Conclusion

The scope of the investigative work in this chapter was to derive pilot data to inform future work. To this end, the data presented in this chapter have generated two main findings which may warrant further investigation.

Previous investigations have suggested PPAR-γ rs1801282 minor allele carriers have a greater degree of postprandial hyper-triglyceridemia (229) and that there is an interaction between this SNP, n-3 PUFA intake and the degree by which n-3 PUFA intake reduces basal triglyceride concentrations in chronic interventions (228). Our data support the previous observation that minor allele carriers are subject to greater degrees of postprandial hyper-triglyceridemia. Furthermore, our data adds to the current evidence of interactions between this SNP and n-3 PUFA consumption by comparing the effects of meals high in EPA or DHA and by investigating the postprandial state, which is more physiologically relevant than the fasting state. Specifically our data suggests that inclusion of DHA in a high fat meal has a compensatory effect for the postprandial hyper-triglyceridemia associated with the minor allelic variant of rs1801282. In the context of LC n-3 fatty acid dependent modifications to blood lipid levels, it seems that increased DHA intake may be particularly beneficial to ALA12 carriers. This data, presents a new and novel perspective which should inform future research. Should these findings hold in larger populations these observations could potentially lead to better targeting and more efficacious therapeutic strategies for reduction of blood lipid levels in the proportion of the population (approximately 10%) that carries the minor allelic variant of this SNP.

As discussed in section 5.4.3, we were unable to show any interaction between rs4673 and rs1799983, the EPA, DHA and control meals and AI, PWV and RHI. The main suggestion to come forward from this data is that future investigations on interactions between n-3 PUFA and rs1799983 should account for age as a variable which may alter the degree by which this SNP influences the interaction between EPA, DHA and vascular and endothelial function.

Chapter 6

Conclusions and future directions of work

Chapter 6 – Conclusion

6.1. Conclusion

The first documentation of associations between LC n-3 PUFA and modifications to blood lipid profiles and decreased incidences of cardiovascular disease are over 40 years old (26). Since this initial observation, the effect of LC n-3 PUFA intake on CVD risk has been studied extensively, with evidence suggesting anti-arrhythmic (35), hypolipidemic (36-39), anti-inflammatory and plaque stabilising (41) and cholesterol profile modulating (42, 43) effects for LC n-3 PUFA. As they are usually consumed together in fish and or fish oil products, EPA and DHA have traditionally been grouped together in investigations of their effects on human health. However, evidence is emerging that they may have differential effects with specific reference to different effects on vascular tone and blood pressure. Specifically, chronic intervention studies have suggested a potential hypertensive effect for DHA but not EPA (40), which corresponds with a capacity for DHA, but not EPA, to modulate fasting vascular tone (57).

Although studies on dietary dependent changes to fasting vascular tone are meaningful and informative in their own regard, we hypothesise that changes in the postprandial state may be more important given that individuals spend the majority of the day in the postprandial state. Recent studies in this context have shown that mixed doses of EPA and DHA beneficially modulate postprandial vascular tone (58-60) and that DHA but not EPA reduces systemic vascular resistance in the postprandial state (61). However, it remains unknown how DHA and EPA compare with regards their capacity to induce these effects in the postprandial state in individuals at increased risk of CVD.

The main finding of this thesis is that acute intake of a single dose (4.16g) of DHA modifies postprandial vascular tone to a greater degree than EPA in men 45-55 years of age at an increased risk of CVD. Although both treatments had some utility in this regard only the effect of the DHA treatment reached significance (p=0.04 as compared to 0.06 for EPA). The importance of these findings is significant, given that individuals spend the majority of the day in the postprandial state and the significant role of vascular reactivity in the pathogenesis of CVD. The significant effect observed for DHA, by current estimates would correlate with a decrease in 10 year CVD risk from 3.25% to 2.83%, as according to estimates established between AI and ESC risk levels (9). The fact that new emerging technologies have been shown to have a capacity to produce high quality DHA from plant sources (238), underpins the relevance and importance of these investigations. Also of consideration is that these technologies may offer more affordable sources of DHA in the future, which will aid not only with research efforts which aim to elucidate the unique effects of these distinct dietary fats on human health, but also the

potential translatability of any observed effects. In the future, it may be possible that the EPA/DHA blend recommended to individuals may depend on the physiological target of interest.

To the best of our knowledge, our study represents the most thorough profiling of changes to acutely modifiable vasomediators, including H_2S, NO and the epoxy and diol metabolites of EPA and DHA, in response to EPA rich, DHA rich and control meals. Furthermore, our data is the first *in-vivo* indication of a link between changes in n-3 PUFA epoxide and diol metabolites to these changes in vascular reactivity. Other novel findings in this regard are the large variations and the sizable changes in the profile of n-3 PUFA epoxide and diol metabolites postprandially in response to physiological consumption of EPA and DHA. The large inter-individual variability in changes in the concentration of these compounds is an important finding, which is suggestive of inter-individual functional differences in either the CYP450 enzymes that generate the epoxides or the sEH enzymes that hydrolyse them, or both. Given the well documented capacity of these compounds to modulate vascular tone (131-134), a reduced capacity to produce the n-3 PUFA epoxides may have an impact on CVD outcomes. Qualifying the genetic variants that contribute to these effects may also aid in establishing which individuals respond the most beneficially to EPA and DHA consumption. Furthermore, given the ongoing attempts to develop new sEH inhibiting drugs to enhance the stability of these compounds (239), establishing the genetic variants that contribute to these large inter-individual differences may be important in establishing individuals who may respond the most to these drugs. The final consideration in this context is that individuals, who are particularly effective producers of the n-3 PUFA epoxides, may be prone to adverse effects in response to sEH inhibiting drugs due to over production of the n-3 PUFA epoxides.

Our investigation was also novel both in its investigation of H_2S production in the postprandial state and in response to EPA and DHA, and in its utilisation of a reliable proximal measure of NO production to investigate EPA and DHA dependent changes in NO production in the postprandial state. Our findings suggest that neither NO or H_2S play a role in mediating the effects of an acute dosage of EPA or DHA on vascular tone in the postprandial state.

Our analysis of markers of glucose and lipid metabolism in our whole study population, indicate no differential effect for EPA or DHA in modulating the glucose, triglyceride or NEFA response and are consistent with previous observations in the literature. Likewise our analysis of plasma fatty acid profiles were as expected and indicated that concentrations of EPA and DHA increased, approximately 1.4 fold and 2 fold respectively, upon consumption. Of note, upon investigation of interactions between genotype and the lipidemic response, our data suggested a previously undocumented beneficial effect of DHA consumption on postprandial triglyceridemia in carriers of the minor allelic

variant of PPAR-γ rs1801282. As noted in Chapter 5 this work was undertaken as part of a pilot study and further work is required to establish if this effect holds in a larger population.

Finally our LPS stimulated whole blood culture investigations were not able to determine a significant differential effect of EPA, DHA or control on IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, MCP-1, VEGF, EGF production or summative score of total inflammatory cytokine production. We also we were not able to establish any significant associations between postprandial triglyceride, EPA or DHA concentrations and cytokine concentrations in whole blood culture. As discussed in chapter 4, due to the high inter-individual variance in WBC responses it is likely that this investigation was underpowered and also it would be of benefit to conduct a baseline culture of blood to account for any effect of interday variation in LPS stimulated cytokine production. These considerations may have contributed to the lack of significance in this investigation.

6.2. Future work

Our observations suggest that DHA is a more potent modulator of postprandial vascular tone than EPA. Due to the fact that individuals spend a large proportion of the day in the postprandial state, we hypothesise that this capacity of DHA to modulate vascular tone may impact CVD risk. This data may also be indicative of chronic habitual intake of DHA, having different effects to chronic habitual intake of EPA. To date there have been no adequately powered, long term clinical trials of the effect of DHA consumption on hard CVD outcomes. The lack of long term studies in this context is in part explained by economic considerations, due to the expense of the currently commercially available oils high in DHA. This fact has ramifications for the cost of running an adequately powered trial and also for the translatability of any findings of such a trial, as the high cost of DHA rich oils is a barrier to the commercial viability of these products. This consideration impacts the likelihood of commercial funding for this research and also the likelihood for the public to utilise these products. However, given the emergence of GM technologies that have the capacity to produce high quality DHA oils from plant sources at lower costs, studies on the long term effects of DHA supplementation are likely to be of warrant in the near future. Our data supports the hypothesis that dietary DHA has unique and beneficial effects in the vasculature, different to those of EPA. Accordingly, we feel our data adds support for the justification of adequately powered long term studies on the effect of DHA on hard CVD outcomes.

More immediately feasible studies on soft outcomes in this context could include investigation of the longer term effects of DHA doses of between 0.5g to 4.0g per day on haemodynamic and vascular outcomes measures, including BP, FMD and AI in both hyper-triglyceridemic and hypertensive

populations, when compared to appropriate control fat treatments. Alongside these investigations parallel exploration of the genetic determinants of inter-individual variation in LC n-3 PUFA epoxide and diol production are advisable as these genetic determinants of changes in LC n-3 PUFA epoxide and diol concentrations may in turn influence inter-individual differences in BP and vascular responses to LC n-3 PUFA. In these studies, the interaction between the genes that influences LC n-3 PUFA epoxide and diol production and changes in BP and vascular responses should also be conducted.

This work would be well advised to also investigate if long term DHA supplementation modifies postprandial responses to high fat challenge meals both with and without DHA inclusion. Investigations in this context should consider that postprandial changes in the concentration of these compounds may be more pronounced than the long term changes in concentration due to the efficiency of sEH in hydrolysing the epoxide derivatives of EPA and DHA. Once again, due to the fact that individuals spend the majority of the day in the postprandial state, investigation of the genetic determinants of changes in the concentration of these bioactive LC n-3 PUFA epoxide derivatives in the postprandial state may be informative of some of the longer term differences in inter-individual responsiveness to these dietary fats. Specifically, variation in the capacity to produce the LC n-3 PUFA epoxide compounds in response to LC n-3 PUFA consumption may correspond with variations in reductions to BP or improvement in vascular tone, in particular in response to DHA. Information on the genetic determinants of inter-individual variation in changes to circulatory concentrations of the LC n-3 PUFA epoxides in response to LC n-3 PUFA consumption may be informative in the context of other conditions also. For instance, evidence suggests these compounds exert effects on a number of physiological and patho-physiological processes including, but not limited to, angiogenesis (215) and tumor growth (215). Genes of interest in these studies could include rs1048943 (CYP1A1) and tagging SNPs or SNPs which induce functional changes in genes coding for other enzymes in the CYP450 family, including CYP1A2, CYP2C19, CYP2C11, CYP2J2, CYP4A11, CYP4F2, CYP4B1 and CYP4F12 and others in the 2C/2J family which have been shown to act on LC n-3 PUFA (240).

Finally, given the efforts to develop sEH inhibitors for use in a number of therapeutic indications (239), establishing the genetic component of variability in production of n-3 PUFA epoxide and diol may aid in selective targeting of these drugs. As mentioned, in section 6.1, these considerations may also aid in prediction of individuals who may react adversely to these drugs.

The final recommendation for future research based on the results presented in this thesis, is a larger scale study studying interactions between the PPAR-y rs1801282 minor allelic variant, DHA consumption and postprandial triglyceridemia and also fasting triglyceride levels. Based on our the effect size in our observations, while accounting for a high degree of variance in postprandial

triglyceride levels, a trial which prospectively carriers of the rs1801282 minor allelic variant would have 0.80 power to reliably detect an interaction between DHA and reductions in postprandial triglyceridemia with a sample size of n=17 in each group. This endeavour would require screening of an estimated n=90 individuals based on estimates of minor-allelic frequency in Northern European populations. However, it may be prudent to expand on these still modest sample sizes to account for the possibility that the putative effect of DHA in this context was inflated in our analysis due to small sample size.

With regards investigations of the effects of EPA and DHA on whole blood culture production of cytokines, as discussed in chapter 4, future investigations utilising this model would be well advised to use sample sizes in the region of n=100 due to issues regarding inter individual variability in WBC responses. Furthermore, baseline cultures may aid in accounting for some non-treatment inter-day variations in cytokine production.

6.3. Concluding remarks

In conclusion, the major findings of this thesis support our hypothesis that DHA is a more efficacious modifier of post-prandial vascular reactivity than EPA. However, it is worth nothing that the EPA treatment also had some utility in it's capacity to reduce the AI measure although the effect did not reach statistical significance (p=0.06). Furthermore, from these investigations it seems likely that these effects are mediated through the epoxide and diol metabolites rather than NO or H_2S.

This work also emphasises the importance of investigating how genotype influences responsiveness to EPA and DHA.

The findings of this thesis are likely to be of growing importance as high quality and affordable EPA and DHA supplements become available due to the utilisation of emerging recombinant plant technologies. Our research extends our knowledge of the differential and unique effects of EPA and DHA in the vasculature and sets the scene for future research on the longer term individual effects of EPA and DHA consumption in this context.

References

1. Stephenson J. Global Mortality Trends. JAMA: The Journal of the American Medical Association 2008;299(23):2737. doi: 10.1001/jama.299.23.2737-a.
2. Ross R. Atherosclerosis — An Inflammatory Disease. New England Journal of Medicine 1999;340(2):115-26. doi: doi:10.1056/NEJM199901143400207.
3. McCall DO, McKinley MC, Noad R, McKeown PP, McCance DR, Young IS, Woodside JV. The assessment of vascular function during dietary intervention trials in human subjects. Br J Nutr 2011;106(7):981-94. doi: S0007114511002996
4. Ellins EA, Halcox JPJ. Where Are We Heading with Noninvasive Clinical Vascular Physiology? Why and How Should We Assess Endothelial Function? Cardiology Research and Practice 2011;2011:9. doi: 10.4061/2011/870132.
5. Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, et al. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. J Am Coll Cardiol 2002;39(2):257-65.
6. Hamburg NM, Keyes MJ, Larson MG, Vasan RS, Schnabel R, Pryde MM, Mitchell GF, Sheffy J, Vita JA, Benjamin EJ. Cross-Sectional Relations of Digital Vascular Function to Cardiovascular Risk Factors in the Framingham Heart Study. Circulation 2008;117(19):2467-74. doi: 10.1161/circulationaha.107.748574.
7. Rubinshtein R, Kuvin JT, Soffler M, Lennon RJ, Lavi S, Nelson RE, Pumper GM, Lerman LO, Lerman A. Assessment of endothelial function by non-invasive peripheral arterial tonometry predicts late cardiovascular adverse events. European Heart Journal 2010;31(9):1142-8. doi: 10.1093/eurheartj/ehq010.
8. Vlachopoulos C, Aznaouridis K, Stefanadis C. Prediction of cardiovascular events and all-cause mortality with arterial stiffness: a systematic review and meta-analysis. J Am Coll Cardiol 2010;55(13):1318-27. doi: S0735-1097(10)00280-9
9. Nurnberger J, Keflioglu-Scheiber A, Opazo Saez AM, Wenzel RR, Philipp T, Schafers RF. Augmentation index is associated with cardiovascular risk. J Hypertens 2002;20(12):2407-14. doi: 10.1097/01.hjh.0000045501.82010.fa.
10. Kelly RP, Millasseau SC, Ritter JM, Chowienczyk PJ. Vasoactive Drugs Influence Aortic Augmentation Index Independently of Pulse-Wave Velocity in Healthy Men. Hypertension 2001;37(6):1429-33. doi: 10.1161/01.hyp.37.6.1429.
11. Libby P. Inflammation in Atherosclerosis. Arteriosclerosis, Thrombosis, and Vascular Biology 2012;32(9):2045-51. doi: 10.1161/atvbaha.108.179705.
12. Driessler F, Venstrom K, Sabat R, Asadullah K, Schottelius AJ. Molecular mechanisms of interleukin-10-mediated inhibition of NF-kappaB activity: a role for p50. Clin Exp Immunol 2004;135(1):64-73.
13. Tedgui A, Mallat Z. Cytokines in atherosclerosis: pathogenic and regulatory pathways. Physiol Rev 2006;86(2):515-81. doi: 10.1152/physrev.00024.2005.
14. Heo SH, Choi YJ, Ryoo HM, Cho JY. Expression profiling of ETS and MMP factors in VEGF-activated endothelial cells: role of MMP-10 in VEGF-induced angiogenesis. J Cell Physiol 2010;224(3):734-42. doi: 10.1002/jcp.22175.
15. Minihane AM. Fish oil omega-3 fatty acids and cardio-metabolic health, alone or with statins. Eur J Clin Nutr 2013;67(5):536-40. doi: 10.1038/ejcn.2013.19.
16. Voss A, Reinhart M, Sankarappa S, Sprecher H. The metabolism of 7,10,13,16,19-docosapentaenoic acid to 4,7,10,13,16,19-docosahexaenoic acid in rat liver is independent of a 4-desaturase. Journal of Biological Chemistry 1991;266(30):19995-20000.
17. De Caterina R, Basta G. n-3 Fatty acids and the inflammatory response — biological background. European Heart Journal Supplements 2001;3(suppl D):D42-D9. doi: 10.1016/s1520-765x(01)90118-x.

18.   Burdge GC, Jones AE, Wootton SA. Eicosapentaenoic and docosapentaenoic acids are the principal products of α-linolenic acid metabolism in young men. British Journal of Nutrition 2007;88(04):355. doi: 10.1079/bjn2002662.

19.   Burdge GC, Wootton SA. Conversion of α-linolenic acid to eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in young women. British Journal of Nutrition 2007;88(04):411. doi: 10.1079/bjn2002689.

20.   Pawlosky RJ, Hibbeln JR, Novotny JA, Salem N. Physiological compartmental analysis of α-linolenic acid metabolism in adult humans. Journal of Lipid Research 2001;42(8):1257-65.

21.   Ian Givens D, Gibbs RA. Current intakes of EPA and DHA in European populations and the potential of animal-derived foods to increase them. Proceedings of the Nutrition Society 2008;67(03):273-80. doi: doi:10.1017/S0029665108007167.

22.   Welch AA, Lund E, Amiano P, Dorronsoro M, Brustad M, Kumle M, Rodriguez M, Lasheras C, Janzon L, Jansson J, et al. Variability of fish consumption within the 10 European countries participating in the European Investigation into Cancer and Nutrition (EPIC) study. Public health nutrition 2002;5(6B):1273-85. doi: 10.1079/PHN2002404.

23.   Welch AA, Shakya-Shrestha S, Lentjes MA, Wareham NJ, Khaw KT. Dietary intake and status of n-3 polyunsaturated fatty acids in a population of fish-eating and non-fish-eating meat-eaters, vegetarians, and vegans and the product-precursor ratio [corrected] of alpha-linolenic acid to long-chain n-3 polyunsaturated fatty acids: results from the EPIC-Norfolk cohort. Am J Clin Nutr 2010;92(5):1040-51. doi: 10.3945/ajcn.2010.29457.

24.   Hjermann I, Holme I, Byre KV, Leren P. EFFECT OF DIET AND SMOKING INTERVENTION ON THE INCIDENCE OF CORONARY HEART DISEASE: Report from the Oslo Study Group of a Randomised Trial in Healthy Men. The Lancet 1981;318(8259):1303-10. doi: 10.1016/s0140-6736(81)91338-6.

25.   Shekelle RB, Shryock AM, Paul O, Lepper M, Stamler J, Liu S, Raynor WJ. Diet, Serum Cholesterol, and Death from Coronary Heart Disease. New England Journal of Medicine 1981;304(2):65-70. doi: doi:10.1056/NEJM198101083040201.

26.   Bang HO, Dyerberg J, Nielsen AB. Plasma lipid and lipoprotein pattern in Greenlandic West-coast Eskimos. Lancet 1971;1(7710):1143-5.

27.   Yokoyama M, Origasa H, Matsuzaki M, Matsuzawa Y, Saito Y, Ishikawa Y, Oikawa S, Sasaki J, Hishida H, Itakura H, et al. Effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic patients (JELIS): a randomised open-label, blinded endpoint analysis. Lancet 2007;369(9567):1090-8. doi: S0140-6736(07)60527-3.

28.   Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. The Lancet 1999;354(9177):447-55. doi: 10.1016/s0140-6736(99)07072-5.

29.   Ishikawa Y, Yokoyama M, Saito Y, Matsuzaki M, Origasa H, Oikawa S, Sasaki J, Hishida H, Itakura H, Kita T, et al. Preventive Effects of Eicosapentaenoic Acid on Coronary Artery Disease in Patients With Peripheral Artery Disease. Circulation Journal 2010;74(7):1451-7. doi: 10.1253/circj.CJ-09-0520.

30.   Bucher HC, Hengstler P, Schindler C, Meier G. N-3 polyunsaturated fatty acids in coronary heart disease: a meta-analysis of randomized controlled trials. The American Journal of Medicine 2002;112(4):298-304. doi: 10.1016/s0002-9343(01)01114-7.

31.   Daviglus ML, Stamler J, Orencia AJ, Dyer AR, Liu K, Greenland P, Walsh MK, Morris D, Shekelle RB. Fish Consumption and the 30-Year Risk of Fatal Myocardial Infarction. New England Journal of Medicine 1997;336(15):1046-53. doi: doi:10.1056/NEJM199704103361502.

32.   Hu FB, Bronner L, Willett WC, Stampfer MJ, Rexrode KM, Albert CM, Hunter D, Manson JE. Fish and Omega-3 Fatty Acid Intake and Risk of Coronary Heart Disease in Women. JAMA: The Journal of the American Medical Association 2002;287(14):1815-21. doi: 10.1001/jama.287.14.1815.

33.   Harris WS, Kris-Etherton PM, Harris KA. Intakes of long-chain omega-3 fatty acid associated with reduced risk for death from coronary heart disease in healthy adults. Curr Atheroscler Rep 2008;10(6):503-9.

34.   Risk, Prevention Study Collaborative G, Roncaglioni MC, Tombesi M, Avanzini F, Barlera S, Caimi V, Longoni P, Marzona I, Milani V, et al. n-3 fatty acids in patients with multiple cardiovascular risk factors. N Engl J Med 2013;368(19):1800-8. doi: 10.1056/NEJMoa1205409.

35.   Calo L, Martino A, Tota C. The anti-arrhythmic effects of n-3 PUFAs. Int J Cardiol 2013;170(2 Suppl 1):S21-7. doi: 10.1016/j.ijcard.2013.06.043.

36.   Cussons AJ, Watts GF, Mori TA, Stuckey BGA. Omega-3 Fatty Acid Supplementation Decreases Liver Fat Content in Polycystic Ovary Syndrome: A Randomized Controlled Trial Employing Proton Magnetic Resonance Spectroscopy. Journal of Clinical Endocrinology & Metabolism 2009;94(10):3842-8. doi: 10.1210/jc.2009-0870.

37.   Mori TA, Bao DQ, Burke V, Puddey IB, Watts GF, Beilin LJ. Dietary fish as a major component of a weight-loss diet: Effect on serum lipids, glucose, and insulin metabolism in overweight hypertensive subjects. American Journal of Clinical Nutrition 1999;70(5):817-25.

38.   Derosa G, Maffioli P, D'Angelo A, Salvadeo S, Ferrari I, Fogari E, Gravina A, Mereu R, Randazzo S, Cicero A. Effects of long chain ω-3 fatty acids on metalloproteinases and their inhibitors in combined dyslipidemia patients. Expert Opinion on Pharmacotherapy 2009;10(8):1239-47. doi: doi:10.1517/14656560902865601.

39.   Damsgaard CT, Frokiaer H, Andersen AD, Lauritzen L. Fish oil in combination with high or low intakes of linoleic acid lowers plasma triacylglycerols but does not affect other cardiovascular risk markers in healthy men. J Nutr 2008;138(6):1061-6. doi: 138/6/1061 [pii].

40.   Mori TA, Bao DQ, Burke V, Puddey IB, Beilin LJ. Docosahexaenoic Acid but Not Eicosapentaenoic Acid Lowers Ambulatory Blood Pressure and Heart Rate in Humans. Hypertension 1999;34(2):253-60.

41.   Thies F, Garry JM, Yaqoob P, Rerkasem K, Williams J, Shearman CP, Gallagher PJ, Calder PC, Grimble RF. Association of n-3 polyunsaturated fatty acids with stability of atherosclerotic plaques: a randomised controlled trial. Lancet 2003;361(9356):477-85. doi: 10.1016/S0140-6736(03)12468-3.

42.   Kelley DS, Adkins Y, Woodhouse LR, Swislocki A, Mackey BE, Siegel D. Docosahexaenoic acid supplementation improved lipocentric but not glucocentric markers of insulin sensitivity in hypertriglyceridemic men. Metab Syndr Relat Disord 2012;10(1):32-8. doi: 10.1089/met.2011.0081.

43.   Bernstein AM, Ding EL, Willett WC, Rimm EB. A meta-analysis shows that docosahexaenoic acid from algal oil reduces serum triglycerides and increases HDL-cholesterol and LDL-cholesterol in persons without coronary heart disease. J Nutr 2012;142(1):99-104. doi: jn.111.148973.

44.   von Schacky C. Omega-3 Fatty acids: anti-arrhythmic, pro-arrhythmic, or both? Front Physiol 2012;3:88. doi: 10.3389/fphys.2012.00088.

45.   Skuladottir GV, Heidarsdottir R, Arnar DO, Torfason B, Edvardsson V, Gottskalksson G, Palsson R, Indridason OS. Plasma n-3 and n-6 fatty acids and the incidence of atrial fibrillation following coronary artery bypass graft surgery. Eur J Clin Invest 2011;41(9):995-1003. doi: 10.1111/j.1365-2362.2011.02497.x.

46.   Caslake MJ, Miles EA, Kofler BM, Lietz G, Curtis P, Armah CK, Kimber AC, Grew JP, Farrell L, Stannard J, et al. Effect of sex and genotype on cardiovascular biomarker response to fish oils: the FINGEN Study. The American Journal of Clinical Nutrition 2008;88(3):618-29.

47.   Buckley R, Shewring B, Turner R, Yaqoob P, Minihane AM. Circulating triacylglycerol and apoE levels in response to EPA and docosahexaenoic acid supplementation in adult human subjects. Br J Nutr 2004;92(3):477-83.

48. Calder PC, Ahluwalia N, Brouns F, Buetler T, Clement K, Cunningham K, Esposito K, Jonsson LS, Kolb H, Lansink M, et al. Dietary factors and low-grade inflammation in relation to overweight and obesity. The British journal of nutrition 2011;106 Suppl 3:S5-78. doi: 10.1017/S0007114511005460.

49. Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid–induced insulin resistance. The Journal of Clinical Investigation 2006;116(11):3015-25. doi: 10.1172/JCI28898.

50. Moghimpour Bijani F, Vallejo JG, Rezaei N. Toll-like receptor signaling pathways in cardiovascular diseases: challenges and opportunities. Int Rev Immunol 2012;31(5):379-95. doi: 10.3109/08830185.2012.706761.

51. Calder PC. n-3 fatty acids, inflammation and immunity: new mechanisms to explain old actions. Proc Nutr Soc 2013;72(3):326-36. doi: 10.1017/S0029665113001031.

52. Damsgaard CT, Lauritzen L, Calder PC, Kjær TR, Frøkiær H. Reduced ex Vivo Interleukin-6 Production by Dietary Fish Oil Is Not Modified by Linoleic Acid Intake in Healthy Men. The Journal of Nutrition 2009;139(7):1410-4. doi: 10.3945/jn.108.102269.

53. Lo CJ, Chiu KC, Fu M, Lo R, Helton S. Fish oil decreases macrophage tumor necrosis factor gene transcription by altering the NF kappa B activity. J Surg Res 1999;82(2):216-21. doi: 10.1006/jsre.1998.5524.

54. Novak TE, Babcock TA, Jho DH, Helton WS, Espat NJ. NF-kappa B inhibition by omega -3 fatty acids modulates LPS-stimulated macrophage TNF-alpha transcription. Am J Physiol Lung Cell Mol Physiol 2003;284(1):L84-9. doi: 10.1152/ajplung.00077.2002.

55. Kelley DS, Taylor PC, Nelson GJ, Schmidt PC, Ferretti A, Erickson KL, Yu R, Chandra RK, Mackey BE. Docosahexaenoic acid ingestion inhibits natural killer cell activity and production of inflammatory mediators in young healthy men. Lipids 1999;34(4):317-24.

56. Sijben JW, Calder PC. Differential immunomodulation with long-chain n-3 PUFA in health and chronic disease. Proc Nutr Soc 2007;66(2):237-59. doi: 10.1017/S0029665107005472.

57. Mori TA, Watts GF, Burke V, Hilme E, Puddey IB, Beilin LJ. Differential Effects of Eicosapentaenoic Acid and Docosahexaenoic Acid on Vascular Reactivity of the Forearm Microcirculation in Hyperlipidemic, Overweight Men. Circulation 2000;102(11):1264-9.

58. Armah CK, Jackson KG, Doman I, James L, Cheghani F, Minihane AM. Fish oil fatty acids improve postprandial vascular reactivity in healthy men. Clinical Science 2008;114(11):679. doi: 10.1042/cs20070277.

59. Chong MF, Lockyer S, Saunders CJ, Lovegrove JA. Long chain n-3 PUFA-rich meal reduced postprandial measures of arterial stiffness. Clin Nutr 2010;29(5):678-81. doi: S0261-5614(10)00030-0.

60. West S, Hecker K, Mustad V, Nicholson S, Schoemer S, Wagner P, Hinderliter A, Ulbrecht J, Ruey P, Kris-Etherton P. Acute effects of monounsaturated fatty acids with and without omega-3 fatty acids on vascular reactivity in individuals with type 2 diabetes. Diabetologia 2005;48(1):113-22. doi: 10.1007/s00125-004-1600-7.

61. Rontoyanni VG, Hall WL, Pombo-Rodrigues S, Appleton A, Chung R, Sanders TA. A comparison of the changes in cardiac output and systemic vascular resistance during exercise following high-fat meals containing DHA or EPA. Br J Nutr 2012:1-8. doi: S0007114511005721.

62. Purcell R, Latham SH, Botham KM, Hall WL, Wheeler-Jones CP. High-fat meals rich in EPA plus DHA compared with DHA only have differential effects on postprandial lipemia and plasma 8-isoprostane F2alpha concentrations relative to a control high-oleic acid meal: a randomized controlled trial. Am J Clin Nutr 2014. doi: 10.3945/ajcn.114.091223.

63. Jackson KG, Armah CK, Minihane AM. Meal fatty acids and postprandial vascular reactivity. Biochem Soc Trans 2007;35(Pt 3):451-3. doi: BST0350451.

64. Heitzer T, Schlinzig T, Krohn K, Meinertz T, Munzel T. Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. Circulation 2001;104(22):2673-8.

65. Shimokawa H. Primary Endothelial Dysfunction: Atherosclerosis. Journal of Molecular and Cellular Cardiology 1999;31(1):23-37. doi: 10.1006/jmcc.1998.0841.

66. Mombouli J-V, Vanhoutte PM. Endothelial Dysfunction: From Physiology to Therapy. Journal of Molecular and Cellular Cardiology 1999;31(1):61-74. doi: 10.1006/jmcc.1998.0844.

67. Landmesser U, Hornig B, Drexler H. Endothelial Function: A Critical Determinant in Atherosclerosis? Circulation 2004;109(21_suppl_1):II-27-33. doi: 10.1161/01.CIR.0000129501.88485.1f.

68. Kobayashi T. The PI3-KAkt pathway roles related to alterations in vasomotor responses in diabetic models. J Smooth Muscle Res 2005;41

69. Pieper GM. Review of Alterations in Endothelial Nitric Oxide Production in Diabetes : Protective Role of Arginine on Endothelial Dysfunction. Hypertension 1998;31(5):1047-60.

70. Virdis A, Colucci R, Fornai M, Polini A, Daghini E, Duranti E, Ghisu N, Versari D, Dardano A, Blandizzi C, et al. Inducible Nitric Oxide Synthase Is Involved in Endothelial Dysfunction of Mesenteric Small Arteries from Hypothyroid Rats. Endocrinology 2009;150(2):1033-42. doi: 10.1210/en.2008-1112.

71. Mustafa AK, Sikka G, Gazi SK, Steppan J, Jung SM, Bhunia AK, Barodka VM, Gazi FK, Barrow RK, Wang R, et al. Hydrogen sulfide as endothelium-derived hyperpolarizing factor sulfhydrates potassium channels. Circ Res 2011;109(11):1259-68. doi: CIRCRESAHA.111.240242.

72. Wang R. Signaling pathways for the vascular effects of hydrogen sulfide. Curr Opin Nephrol Hypertens 2011;20(2):107-12. doi: 10.1097/MNH.0b013e3283430651.

73. Yang G, Wu L, Jiang B, Yang W, Qi J, Cao K, Meng Q, Mustafa AK, Mu W, Zhang S, et al. H2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase. Science 2008;322(5901):587-90. doi: 322/5901/587.

74. Lee M, Schwab C, Yu S, McGeer E, McGeer PL. Astrocytes produce the antiinflammatory and neuroprotective agent hydrogen sulfide. Neurobiology of Aging 2009;30(10):1523-34. doi: 10.1016/j.neurobiolaging.2009.06.001.

75. Whiteman M, Le Trionnaire S, Chopra M, Fox B, Whatmore J. Emerging role of hydrogen sulfide in health and disease: critical appraisal of biomarkers and pharmacological tools. Clin Sci (Lond) 2011;121(11):459-88. doi: CS20110267.

76. Jiang HL, Wu HC, Li ZL, Geng B, Tang CS. [Changes of the new gaseous transmitter H2S in patients with coronary heart disease]. Di Yi Jun Yi Da Xue Xue Bao 2005;25(8):951-4.

77. Whiteman M, Gooding KM, Whatmore JL, Ball CI, Mawson D, Skinner K, Tooke JE, Shore AC. Adiposity is a major determinant of plasma levels of the novel vasodilator hydrogen sulphide. Diabetologia 2010;53(8):1722-6. doi: 10.1007/s00125-010-1761-5.

78. Whiteman M, Moore PK. Hydrogen sulfide and the vasculature: a novel vasculoprotective entity and regulator of nitric oxide bioavailability? J Cell Mol Med 2009;13(3):488-507. doi: JCMM645.

79. Fahs. The effect of acute fish-oil supplementation on endothelial function and arterial stiffness following a high-fat meal. Applied Physiology, Nutrition, and Metabolism 2010;35(3):294-302. doi: doi:10.1139/H10-020.

80. Hall WL, Sanders KA, Sanders TAB, Chowienczyk PJ. A High-Fat Meal Enriched with Eicosapentaenoic Acid Reduces Postprandial Arterial Stiffness Measured by Digital Volume Pulse Analysis in Healthy Men. The Journal of Nutrition 2008;138(2):287-91.

81. Matesanz N, Park G, McAllister H, Leahey W, Devine A, McVeigh GE, Gardiner TA, McDonald DM. Docosahexaenoic acid improves the nitroso-redox balance and reduces VEGF-mediated

angiogenic signaling in microvascular endothelial cells. Invest Ophthalmol Vis Sci 2010;51(12):6815-25. doi: iovs.10-5339.

82. Williams TM, Lisanti MP. The Caveolin genes: from cell biology to medicine. Ann Med 2004;36(8):584-95.

83. Michel JB, Feron O, Sacks D, Michel T. Reciprocal regulation of endothelial nitric-oxide synthase by Ca2+-calmodulin and caveolin. J Biol Chem 1997;272(25):15583-6.

84. Zgheel F, Alhosin M, Rashid S, Burban M, Auger C, Schini-Kerth VB. Redox-Sensitive Induction of Src/PI3-kinase/Akt and MAPKs Pathways Activate eNOS in Response to EPA:DHA 6:1. PLoS One 2014;9(8):e105102. doi: 10.1371/journal.pone.0105102.

85. Huang T, Wahlqvist ML, Li D. Docosahexaenoic acid decreases plasma homocysteine via regulating enzyme activity and mRNA expression involved in methionine metabolism. Nutrition 2010;26(1):112-9. doi: S0899-9007(09)00236-6.

86. Huang T, Zheng J, Chen Y, Yang B, Wahlqvist ML, Li D. High consumption of Omega-3 polyunsaturated fatty acids decrease plasma homocysteine: a meta-analysis of randomized, placebo-controlled trials. Nutrition 2011;27(9):863-7. doi: S0899-9007(11)00009-8.

87. Predmore BL, Julian D, Cardounel AJ. Hydrogen sulfide increases nitric oxide production from endothelial cells by an akt-dependent mechanism. Front Physiol 2011;2:104. doi: 10.3389/fphys.2011.00104.

88. Witztum JL, Steinberg D. Role of oxidized low density lipoprotein in atherogenesis. The Journal of Clinical Investigation 1991;88(6):1785-92.

89. Wang H-H, Hung T-M, Wei J, Chiang A-N. Fish oil increases antioxidant enzyme activities in macrophages and reduces atherosclerotic lesions in apoE-knockout mice. Cardiovascular Research 2004;61(1):169-76. doi: 10.1016/j.cardiores.2003.11.002.

90. Ray R, Shah AM. NADPH oxidase and endothelial cell function. Clin Sci 2005;109(3):217-26. doi: 10.1042/cs20050067.

91. Massaro M, Habib A, Lubrano L, Turco SD, Lazzerini G, Bourcier T, Weksler BB, De Caterina R. The omega-3 fatty acid docosahexaenoate attenuates endothelial cyclooxygenase-2 induction through both NADP(H) oxidase and PKC inhibition. Proceedings of the National Academy of Sciences 2006;103(41):15184-9. doi: 10.1073/pnas.0510086103.

92. De Caterina R, Bernini W, Carluccio MA, Liao JK, Libby P. Structural requirements for inhibition of cytokine-induced endothelial activation by unsaturated fatty acids. Journal of Lipid Research 1998;39(5):1062-70.

93. De Caterina R, Cybulsky M, Clinton S, Gimbrone M, Libby P. The omega-3 fatty acid docosahexaenoate reduces cytokine-induced expression of proatherogenic and proinflammatory proteins in human endothelial cells. Arteriosclerosis, Thrombosis, and Vascular Biology 1994;14(11):1829-36. doi: 10.1161/01.atv.14.11.1829.

94. Kanda T, Brown JD, Orasanu G, Vogel S, Gonzalez FJ, Sartoretto J, Michel T, Plutzky J. PPARγ in the endothelium regulates metabolic responses to high-fat diet in mice. Journal of Clinical Investigation 2008. doi: 10.1172/jci36233.

95. Lee TI, Kao YH, Chen YC, Pan NH, Lin YK, Chen YJ. Cardiac peroxisome-proliferator-activated receptor expression in hypertension co-existing with diabetes. Clinical Science 2011;121(7):305-12. doi: 10.1042/cs20100529.

96. Yu JZ, Z.; Li, Z.; Feng, X.; He, L.; Liu, S.; Mao, J.; Wang, G.; Wang, X. Peroxisome Proliferator-activated Receptor-(PPAR) Agonist Improves Coronary Artery Endothelial Function in Diabetic Patients with Coronary Artery Disease. The Journal of International Medical Research 2010;38:86-94.

97. Martina Victoria Schmidt. The Nuclear Hormone Receptor PPARfgamma as a Therapeutic Target in Major Diseases. TheScientificWorldJOURNAL 2010;10:2181–97. doi: 10.1100/tsw.2010.213.

98. Nicol CJ, Adachi M, Akiyama TE, Gonzalez FJ. PPAR[gamma] in endothelial cells influences high fat diet-induced hypertension. Am J Hypertens 2005;18(4):549-56.

99.   Luna-Medina R, Cortes-Canteli M, Alonso M, Santos A, Martínez A, Perez-Castillo A. Regulation of Inflammatory Response in Neural Cells in Vitro by Thiadiazolidinones Derivatives through Peroxisome Proliferator-activated Receptor γ Activation. Journal of Biological Chemistry 2005;280(22):21453-62. doi: 10.1074/jbc.M414390200.

100.   Carta AR, Pisanu A, Carboni E. Do PPAR-Gamma Agonists Have a Future in Parkinson's Disease Therapy? Parkinson's Disease 2011;2011:1-14. doi: 10.4061/2011/689181.

101.   Zeggini E, Parkinson JRC, Halford S, Owen KR, Walker M, Hitman GA, Levy JC, Sampson MJ, Frayling TM, Hattersley AT, et al. Examining the relationships between the Pro12Ala variant in PPARG and Type 2 diabetes-related traits in UK samples. Diabetic Medicine 2005;22(12):1696-700. doi: 10.1111/j.1464-5491.2005.01717.x.

102.   Zuo Y, Yang H-C, Potthoff SA, Najafian B, Kon V, Ma L-J, Fogo AB. Protective effects of PPARγ agonist in acute nephrotic syndrome. Nephrology Dialysis Transplantation 2011. doi: 10.1093/ndt/gfr240.

103.   Rakhshandehroo M, Knoch B, Muller M, Kersten S. Peroxisome proliferator-activated receptor alpha target genes. PPAR Res 2010;2010. doi: 612089.

104.   Tian J, Smith A, Nechtman J, Podolsky R, Aggarwal S, Snead C, Kumar S, Elgaish M, Oishi P, Goerlach A, et al. Effect of PPARgamma inhibition on pulmonary endothelial cell gene expression: gene profiling in pulmonary hypertension. Physiol Genomics 2009;40(1):48-60. doi: 00094.2009.

105.   Nolte RT. Ligand binding and coactivator assembly of peroxisome proliferetor activator receptor. NATURE 1998;VOL 395(10 SEPTEMBER 1998):13-143.

106.   Xu HE. Molecular recognition of fatty acids by Peroxisome Proliferator–Activated Receptors. Molecular Cell, 1999,; Vol. 3:, 397–403,.

107.   Li H, Ruan XZ, Powis SH, Fernando R, Mon WY, Wheeler DC, Moorhead JF, Varghese Z. EPA and DHA reduce LPS-induced inflammation responses in HK-2 cells: Evidence for a PPAR-[gamma]-dependent mechanism. Kidney Int 2005;67(3):867-74.

108.   Wang D, Hirase T, Nitto T, Soma M, Node K. Eicosapentaenoic acid increases cytochrome P-450 2J2 gene expression and epoxyeicosatrienoic acid production via peroxisome proliferator-activated receptor [gamma] in endothelial cells. Journal of Cardiology 2009;54(3):368-74. doi: 10.1016/j.jjcc.2009.06.004.

109.   Banga A, Unal R, Tripathi P, Pokrovskaya I, Owens RJ, Kern PA, Ranganathan G. Adiponectin translation is increased by the PPARγ agonists pioglitazone and ω-3 fatty acids. American Journal of Physiology - Endocrinology And Metabolism 2009;296(3):E480-E9. doi: 10.1152/ajpendo.90892.2008.

110.   Sethi S, Ziouzenkova O, Ni H, Wagner DD, Plutzky J, Mayadas TN. Oxidized omega-3 fatty acids in fish oil inhibit leukocyte-endothelial interactions through activation of PPARα. Blood 2002;100(4):1340-6. doi: 10.1182/blood-2002-01-0316.

111.   Di Nunzio M, Danesi F, Bordoni A. n-3 PUFA as Regulators of Cardiac Gene Transcription: A New Link between PPAR Activation and Fatty Acid Composition. Lipids 2009;44(12):1073-9. doi: 10.1007/s11745-009-3362-y.

112.   Mishra A, Chaudhary A, Sethi S. Oxidized Omega-3 Fatty Acids Inhibit NF-{kappa}B Activation Via a PPAR{alpha}-Dependent Pathway. Arterioscler Thromb Vasc Biol 2004;24(9):1621-7. doi: 10.1161/01.atv.0000137191.02577.86.

113.   Yu YH, Wang PH, Cheng WTK, Mersmann HJ, Wu SC, Ding ST. Porcine peroxisome proliferator-activated receptor δ mediates the lipolytic effects of dietary fish oil to reduce body fat deposition. Journal of Animal Science 2010;88(6):2009-18. doi: 10.2527/jas.2009-2597.

114.   Oster RT. Docosahexaenoic acid increases cellular adiponectin mRNA and secreted adiponectin protein, as well as PPARγ mRNA, in 3T3-L1 adipocytes. Applied Physiology, Nutrition, and Metabolism 2010;35(6):783-9. doi: doi:10.1139/H10-076.

115. Gonon A, Bulhak A, Labruto F, Sjöquist PO, Pernow J. Cardioprotection mediated by rosiglitazone, a peroxisome proliferatoractivated receptor gamma ligand, in relation to nitric oxide. Basic Research in Cardiology 2007;102(1):80-9. doi: 10.1007/s00395-006-0613-4.

116. Dancu M, Berardi D, Vanden Heuvel J, Tarbell J. Atherogenic Endothelial Cell eNOS and ET-1 Responses to Asynchronous Hemodynamics are Mitigated by Conjugated Linoleic Acid. Annals of Biomedical Engineering 2007;35(7):1111-9. doi: 10.1007/s10439-007-9290-1.

117. Okayasu T, Tomizawa A, Suzuki K, Manaka K-i, Hattori Y. PPAR[alpha] activators upregulate eNOS activity and inhibit cytokine-induced NF-[kappa]B activation through AMP-activated protein kinase activation. Life Sciences 2008;82(15-16):884-91. doi: 10.1016/j.lfs.2008.02.002.

118. Goya K, Sumitani S, Xu X, Kitamura T, Yamamoto H, Kurebayashi S, Saito H, Kouhara H, Kasayama S, Kawase I. Peroxisome Proliferator-Activated Receptor {alpha} Agonists Increase Nitric Oxide Synthase Expression in Vascular Endothelial Cells. Arterioscler Thromb Vasc Biol 2004;24(4):658-63. doi: 10.1161/01.atv.0000118682.58708.78.

119. Zarzuelo MJ, Jiménez R, Galindo P, Sánchez M, Nieto A, Romero M, Quintela AM, López-Sepúlveda R, Gómez-Guzmán M, Bailón E, et al. Antihypertensive Effects of Peroxisome Proliferator-Activated Receptor-β Activation in Spontaneously Hypertensive Rats. Hypertension 2011;58(4):733-43. doi: 10.1161/hypertensionaha.111.174490.

120. Jiménez R, Sánchez M, Zarzuelo MJ, Romero M, Quintela AM, López-Sepúlveda R, Galindo P, Gómez-Guzmán M, Haro JM, Zarzuelo A, et al. Endothelium-Dependent Vasodilator Effects of Peroxisome Proliferator-Activated Receptor β Agonists via the Phosphatidyl-Inositol-3 Kinase-Akt Pathway. Journal of Pharmacology and Experimental Therapeutics 2010;332(2):554-61. doi: 10.1124/jpet.109.159806.

121. Relou IAM, Damen CA, van der Schaft DWJ, Groenewegen G, Griffioen AW. Effect of culture conditions on endothelial cell growth and responsiveness. Tissue and Cell 1998;30(5):525-30. doi: 10.1016/s0040-8166(98)80032-3.

122. Wang Y, Yang Q, Yan JT, Zhao C, Cianflone K, Wang DW. Effects of bezafibrate on the expression of endothelial nitric oxide synthase gene and its mechanisms in cultured bovine endothelial cells. Atherosclerosis 2006;187(2):265-73. doi: 10.1016/j.atherosclerosis.2005.09.008.

123. von Knethen A, Brüne B. Activation of Peroxisome Proliferator-Activated Receptor γ by Nitric Oxide in Monocytes/Macrophages Down-Regulates p47phox and Attenuates the Respiratory Burst. The Journal of Immunology 2002;169(5):2619-26.

124. Montezano AC, Amiri F, Tostes RC, Touyz RM, Schiffrin EL. Inhibitory effects of PPAR-[gamma] on endothelin-1-induced inflammatory pathways in vascular smooth muscle cells from normotensive and hypertensive rats. Journal of the American Society of Hypertension;1(2):150-60. doi: 10.1016/j.jash.2007.01.005.

125. Grimm H, Mertes N, Goeters C, Schlotzer E, Mayer K, Grimminger F, Fürst † P. Improved fatty acid and leukotriene pattern with a novel lipid emulsion in surgical patients. European Journal of Nutrition 2006;45(1):55-60. doi: 10.1007/s00394-005-0573-8.

126. Andazola JJ, Underwood JA, Chiono M, Torres OA, Herman CA. Leukotriene B4 and leukotriene B5 have binding sites on lung membranes and cause contraction of bullfrog lung. Journal of Pharmacology and Experimental Therapeutics 1992;263(3):1111-7.

127. Lands W. Biochemistry and physiology of n-3 fatty acids. The FASEB Journal 1992;6(8):2530-6.

128. Needleman P, Raz A, Minkes MS, Ferrendelli JA, Sprecher H. Triene prostaglandins: prostacyclin and thromboxane biosynthesis and unique biological properties. Proc Natl Acad Sci U S A 1979;76(2):944-8.

129. Mueller GC, Miller JA. The metabolism of 4-dimethylaminoazobenzene by rat liver homogenates. J Biol Chem 1948;176(2):535-44.

130. Roman RJ. P-450 metabolites of arachidonic acid in the control of cardiovascular function. Physiol Rev 2002;82(1):131-85. doi: 10.1152/physrev.00021.2001.

131. Morin C, Fortin S, Rousseau E. 19,20-EpDPE, a bioactive CYP450 metabolite of DHA monoacygyceride, decreases Ca(2) sensitivity in human pulmonary arteries. Am J Physiol Heart Circ Physiol 2011;301(4):H1311-8. doi: ajpheart.00380.2011.

132. Morin C, Sirois M, Echave V, Rizcallah E, Rousseau E. Relaxing effects of 17(18)-EpETE on arterial and airway smooth muscles in human lung. Am J Physiol Lung Cell Mol Physiol 2009;296(1):L130-9. doi: 90436.2008.

133. Konkel A, Schunck WH. Role of cytochrome P450 enzymes in the bioactivation of polyunsaturated fatty acids. Biochim Biophys Acta 2011;1814(1):210-22. doi: S1570-9639(10)00258-X.

134. Ye D, Zhang D, Oltman C, Dellsperger K, Lee HC, VanRollins M. Cytochrome p-450 epoxygenase metabolites of docosahexaenoate potently dilate coronary arterioles by activating large-conductance calcium-activated potassium channels. J Pharmacol Exp Ther 2002;303(2):768-76.

135. VanRollins M. Epoxygenase metabolites of docosahexaenoic and eicosapentaenoic acids inhibit platelet aggregation at concentrations below those affecting thromboxane synthesis. Journal of Pharmacology and Experimental Therapeutics 1995;274(2):798-804.

136. Keenan AH, Pedersen TL, Fillaus K, Larson MK, Shearer GC, Newman JW. Basal omega-3 fatty acid status affects fatty acid and oxylipin responses to high-dose n3-HUFA in healthy volunteers. J Lipid Res 2012;53(8):1662-9. doi: 10.1194/jlr.P025577.

137. Schwarz D, Kisselev P, Chernogolov A, Schunck W-H, Roots I. Human CYP1A1 variants lead to differential eicosapentaenoic acid metabolite patterns. Biochemical and Biophysical Research Communications 2005;336(3):779-83. doi: 10.1016/j.bbrc.2005.08.172.

138. Madden J, Williams CM, Calder PC, Lietz G, Miles EA, Cordell H, Mathers JC, Minihane AM. The impact of common gene variants on the response of biomarkers of cardiovascular disease (CVD) risk to increased fish oil fatty acids intakes. Annu Rev Nutr 2011;31:203-34. doi: 10.1146/annurev-nutr-010411-095239.

139. Serhan CN, Hong S, Gronert K, Colgan SP, Devchand PR, Mirick G, Moussignac RL. Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. J Exp Med 2002;196(8):1025-37.

140. Serhan CN, Clish CB, Brannon J, Colgan SP, Chiang N, Gronert K. Novel functional sets of lipid-derived mediators with antiinflammatory actions generated from omega-3 fatty acids via cyclooxygenase 2-nonsteroidal antiinflammatory drugs and transcellular processing. J Exp Med 2000;192(8):1197-204.

141. Arita M, Clish CB, Serhan CN. The contributions of aspirin and microbial oxygenase to the biosynthesis of anti-inflammatory resolvins: novel oxygenase products from omega-3 polyunsaturated fatty acids. Biochem Biophys Res Commun 2005;338(1):149-57. doi: S0006-291X(05)01684-0.

142. Serhan CN. Novel chemical mediators in the resolution of inflammation: resolvins and protectins. Anesthesiol Clin 2006;24(2):341-64.

143. Fuller CJ, Agil A, Lender D, Jialal I. Superoxide production and LDL oxidation by diabetic neutrophils. Journal of Diabetes and its Complications 1996;10(4):206-10. doi: 10.1016/1056-8727(95)00002-x.

144. Arita M, Bianchini F, Aliberti J, Sher A, Chiang N, Hong S, Yang R, Petasis NA, Serhan CN. Stereochemical assignment, antiinflammatory properties, and receptor for the omega-3 lipid mediator resolvin E1. The Journal of Experimental Medicine 2005;201(5):713-22. doi: 10.1084/jem.20042031.

145. Kaur J, Adya R, Tan BK, Chen J, Randeva HS. Identification of chemerin receptor (ChemR23) in human endothelial cells: Chemerin-induced endothelial angiogenesis. Biochemical and Biophysical Research Communications 2010;391(4):1762-8. doi: 10.1016/j.bbrc.2009.12.150.

146. Zhao R-j, Wang H. Chemerin/ChemR23 signaling axis is involved in the endothelial protection by KATP channel opener iptakalim. Acta Pharmacol Sin 2011;32(5):573-80.
147. Chen YF, Jiang H, Gong X, Wan JY. [Resolvin E1 protects against ox-LDL-induced injury on vascular endothelial cells]. Zhonghua Xin Xue Guan Bing Za Zhi 2011;39(11):1039-43.
148. Ho KJ, Spite M, Owens CD, Lancero H, Kroemer AH, Pande R, Creager MA, Serhan CN, Conte MS. Aspirin-triggered lipoxin and resolvin E1 modulate vascular smooth muscle phenotype and correlate with peripheral atherosclerosis. Am J Pathol 2010;177(4):2116-23. doi: S0002-9440(10)60260-X.
149. Chen L, Zhao Q, Wang X-L, You R, Zhang Y-H, Ji H, Lai Y-S. ZLJ-6, a novel COX/5-LOX inhibitor, attenuates TNF-α-induced endothelial E-selectin, ICAM-1 and VCAM-1 expression and monocyte–endothelial interactions via a COX/5-LOX-independent mechanism. Vascular Pharmacology 2011;55(5–6):135-42. doi: 10.1016/j.vph.2011.07.003.
150. Schwab JM, Chiang N, Arita M, Serhan CN. Resolvin E1 and protectin D1 activate inflammation-resolution programmes. Nature 2007;447(7146):869-74.
151. Poeckel D, Funk CD. The 5-lipoxygenase/leukotriene pathway in preclinical models of cardiovascular disease. Cardiovascular Research 2010;86(2):243-53. doi: 10.1093/cvr/cvq016.
152. Krishnamoorthy S, Recchiuti A, Chiang N, Yacoubian S, Lee C-H, Yang R, Petasis NA, Serhan CN. Resolvin D1 binds human phagocytes with evidence for proresolving receptors. Proceedings of the National Academy of Sciences 2010;107(4):1660-5. doi: 10.1073/pnas.0907342107.
153. Schuchardt JP, Schneider I, Meyer H, Neubronner J, von Schacky C, Hahn A. Incorporation of EPA and DHA into plasma phospholipids in response to different omega-3 fatty acid formulations - a comparative bioavailability study of fish oil vs. krill oil. Lipids Health Dis 2011;10:145. doi: 1476-511X-10-145.
154. Jackson KG, Clarke DT, Murray P, Lovegrove JA, O'Malley B, Minihane AM, Williams CM. Introduction to the DISRUPT postprandial database: subjects, studies and methodologies. Genes Nutr 2010;5(1):39-48. doi: 10.1007/s12263-009-0149-y.
155. Vogel MDRA, Corretti MDMC, Plotnick MDGD. Effect of a Single High-Fat Meal on Endothelial Function in Healthy Subjects. The American Journal of Cardiology 1997;79(3):350-4. doi: Doi: 10.1016/s0002-9149(96)00760-6.
156. Martin EA, Nelson RE, Felmlee-Devine MD, Brown TE, Lerman A. Comparing EndoPAT and BIOPAC measurement of vascular responses to mental stress. Cell Biochemistry and Function 2011;29(4):272-8. doi: 10.1002/cbf.1745.
157. Asmar RG, London GM, O'Rourke ME, Safar ME, coordinators ftRP, investigators. Improvement in Blood Pressure, Arterial Stiffness and Wave Reflections With a Very-Low-Dose Perindopril/Indapamide Combination in Hypertensive Patient. Hypertension 2001;38(4):922-6. doi: 10.1161/hy1001.095774.
158. Abell LL, Levy BB, Brodie BB, Kendall FE. A SIMPLIFIED METHOD FOR THE ESTIMATION OF TOTAL CHOLESTEROL IN SERUM AND DEMONSTRATION OF ITS SPECIFICITY. Journal of Biological Chemistry 1952;195(1):357-66.
159. Feelisch M, Rassaf T, Mnaimneh S, Singh N, Bryan NS, Jourd'Heuil D, Kelm M. Concomitant S-, N-, and heme-nitros(yl)ation in biological tissues and fluids: implications for the fate of NO in vivo. FASEB J 2002;16(13):1775-85. doi: 10.1096/fj.02-0363com.
160. Wintner EA, Deckwerth TL, Langston W, Bengtsson A, Leviten D, Hill P, Insko MA, Dumpit R, VandenEkart E, Toombs CF, et al. A monobromobimane-based assay to measure the pharmacokinetic profile of reactive sulphide species in blood. Br J Pharmacol 2010;160(4):941-57. doi: 10.1111/j.1476-5381.2010.00704.x.
161. Shen X, Pattillo CB, Pardue S, Bir SC, Wang R, Kevil CG. Measurement of plasma hydrogen sulfide in vivo and in vitro. Free Radic Biol Med 2011;50(9):1021-31. doi: 10.1016/j.freeradbiomed.2011.01.025.

162. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957;226(1):497-509.

163. Fromel T, Jungblut B, Hu J, Trouvain C, Barbosa-Sicard E, Popp R, Liebner S, Dimmeler S, Hammock BD, Fleming I. Soluble epoxide hydrolase regulates hematopoietic progenitor cell function via generation of fatty acid diols. Proc Natl Acad Sci U S A 2012;109(25):9995-10000. doi: 10.1073/pnas.1206493109.

164. Michaelis UR, Fisslthaler B, Barbosa-Sicard E, Falck JR, Fleming I, Busse R. Cytochrome P450 epoxygenases 2C8 and 2C9 are implicated in hypoxia-induced endothelial cell migration and angiogenesis. J Cell Sci 2005;118(Pt 23):5489-98. doi: 10.1242/jcs.02674.

165. England PH. National Diet and Nutrition Survey. Results from Years 1-4 (combined) of the Rolling Programme . 2014:64-9.

166. Mori TA. Dietary n-3 PUFA and CVD: a review of the evidence. Proc Nutr Soc 2014;73(1):57-64. doi: 10.1017/S0029665113003583.

167. Gizdulich P, Imholz BP, van den Meiracker AH, Parati G, Wesseling KH. Finapres tracking of systolic pressure and baroreflex sensitivity improved by waveform filtering. J Hypertens 1996;14(2):243-50.

168. Treasure CB, Klein JL, Vita JA, Manoukian SV, Renwick GH, Selwyn AP, Ganz P, Alexander RW. Hypertension and left ventricular hypertrophy are associated with impaired endothelium-mediated relaxation in human coronary resistance vessels. Circulation 1993;87(1):86-93.

169. Kannel WB, Levy D, Cupples LA. Left ventricular hypertrophy and risk of cardiac failure: insights from the Framingham Study. J Cardiovasc Pharmacol 1987;10 Suppl 6:S135-40.

170. Berry SEE, Tucker S, Banerji R, Jiang B, Chowienczyk PJ, Charles SM, Sanders TAB. Impaired Postprandial Endothelial Function Depends on the Type of Fat Consumed by Healthy Men. The Journal of Nutrition 2008;138(10):1910-4.

171. Hamazaki T, Urakaze M, Sawazaki S, Yamazaki K, Taki H, Yano S. Comparison of pulse wave velocity of the aorta between inhabitants of fishing and farming villages in Japan. Atherosclerosis 1988;73(2-3):157-60.

172. Pase MP, Grima NA, Sarris J. Do long-chain n-3 fatty acids reduce arterial stiffness? A meta-analysis of randomised controlled trials. Br J Nutr 2011;106(7):974-80. doi: 10.1017/S0007114511002819.

173. Oliver JJ, Webb DJ. Noninvasive Assessment of Arterial Stiffness and Risk of Atherosclerotic Events. Arteriosclerosis, Thrombosis, and Vascular Biology 2003;23(4):554-66. doi: 10.1161/01.atv.0000060460.52916.d6.

174. Thompson AK, Newens KJ, Jackson KG, Wright J, Williams CM. Glu298Asp polymorphism influences the beneficial effects of fish oil fatty acids on postprandial vascular function. Journal of Lipid Research 2012;53(10):2205-13. doi: 10.1194/jlr.P025080.

175. Singhal A, Lanigan J, Storry C, Low S, Birbara T, Lucas A, Deanfield J. Docosahexaenoic acid supplementation, vascular function and risk factors for cardiovascular disease: a randomized controlled trial in young adults. J Am Heart Assoc 2013;2(4):e000283. doi: 10.1161/JAHA.113.000283.

176. Kuvin JT, Patel AR, Sliney KA, Pandian NG, Sheffy J, Schnall RP, Karas RH, Udelson JE. Assessment of peripheral vascular endothelial function with finger arterial pulse wave amplitude. American Heart Journal 2003;146(1):168-74. doi: 10.1016/s0002-8703(03)00094-2.

177. Evaluation of the EndoPAT as a Tool to Assess Endothelial Function. International Journal of Vascular Medicine 2012;2012. doi: 10.1155/2012/904141.

178. Karatzis E, Papaioannou TG, Aznaouridis K, Karatzi K, Stamatelopoulos K, Zampelas A, Papamichael C, Lekakis J, Mavrikakis M. Acute effects of caffeine on blood pressure and wave reflections in healthy subjects: should we consider monitoring central blood pressure? International Journal of Cardiology 2005;98(3):425-30.

179. Lauer T, Preik M, Rassaf T, Strauer BE, Deussen A, Feelisch M, Kelm M. Plasma nitrite rather than nitrate reflects regional endothelial nitric oxide synthase activity but lacks intrinsic vasodilator action. Proc Natl Acad Sci U S A 2001;98(22):12814-9. doi: 10.1073/pnas.221381098.

180. Hwang SY, Sarna LK, Siow YL, O K. High-fat diet stimulates hepatic cystathionine beta-synthase and cystathionine gamma-lyase expression. Can J Physiol Pharmacol 2013;91(11):913-9. doi: 10.1139/cjpp-2013-0106.

181. Geng B, Cai B, Liao F, Zheng Y, Zeng Q, Fan X, Gong Y, Yang J, Cui QH, Tang C, et al. Increase or decrease hydrogen sulfide exert opposite lipolysis, but reduce global insulin resistance in high fatty diet induced obese mice. PLoS One 2013;8(9):e73892. doi: 10.1371/journal.pone.0073892.

182. Zhang Y, Oltman CL, Lu T, Lee HC, Dellsperger KC, VanRollins M. EET homologs potently dilate coronary microvessels and activate BK(Ca) channels. Am J Physiol Heart Circ Physiol 2001;280(6):H2430-40.

183. Strassburg K, Esser D, Vreeken RJ, Hankemeier T, Muller M, van Duynhoven J, van Golde J, van Dijk SJ, Afman LA, Jacobs DM. Postprandial fatty acid specific changes in circulating oxylipins in lean and obese men after high-fat challenge tests. Mol Nutr Food Res 2013. doi: 10.1002/mnfr.201300321.

184. Knapp HR, Miller AJ, Lawson JA. Urinary excretion of diols derived from eicosapentaenoic acid during n-3 fatty acid ingestion by man. Prostaglandins 1991;42(1):47-54.

185. Ramstedt U, Serhan CN, Lundberg U, Wigzell H, Samuelsson B. Inhibition of human natural killer cell activity by (14R,15S)-14,15-dihydroxy-5Z,8Z,10E,12E- icosatetraenoic acid. Proc Natl Acad Sci U S A 1984;81(22):6914-8.

186. Hu J, Popp R, Fromel T, Ehling M, Awwad K, Adams RH, Hammes HP, Fleming I. Muller glia cells regulate Notch signaling and retinal angiogenesis via the generation of 19,20-dihydroxydocosapentaenoic acid. J Exp Med 2014;211(2):281-95. doi: 10.1084/jem.20131494.

187. Psychogios N, Hau DD, Peng J, Guo AC, Mandal R, Bouatra S, Sinelnikov I, Krishnamurthy R, Eisner R, Gautam B, et al. The human serum metabolome. PLoS One 2011;6(2):e16957. doi: 10.1371/journal.pone.0016957.

188. Quehenberger O, Armando AM, Brown AH, Milne SB, Myers DS, Merrill AH, Bandyopadhyay S, Jones KN, Kelly S, Shaner RL, et al. Lipidomics reveals a remarkable diversity of lipids in human plasma. J Lipid Res 2010;51(11):3299-305. doi: 10.1194/jlr.M009449.

189. Morisseau C, Inceoglu B, Schmelzer K, Tsai HJ, Jinks SL, Hegedus CM, Hammock BD. Naturally occurring monoepoxides of eicosapentaenoic acid and docosahexaenoic acid are bioactive antihyperalgesic lipids. J Lipid Res 2010;51(12):3481-90. doi: 10.1194/jlr.M006007.

190. Larsen BT, Miura H, Hatoum OA, Campbell WB, Hammock BD, Zeldin DC, Falck JR, Gutterman DD. Epoxyeicosatrienoic and dihydroxyeicosatrienoic acids dilate human coronary arterioles via BKCa channels: implications for soluble epoxide hydrolase inhibition, 2006.

191. Lauer T, Preik M, Rassaf T, Strauer BE, Deussen A, Feelisch M, Kelm M. Plasma nitrite rather than nitrate reflects regional endothelial nitric oxide synthase activity but lacks intrinsic vasodilator action. Proceedings of the National Academy of Sciences 2001;98(22):12814-9. doi: 10.1073/pnas.221381098.

192. Ulu A, Stephen Lee KS, Miyabe C, Yang J, Hammock BG, Dong H, Hammock BD. An omega-3 epoxide of docosahexaenoic acid lowers blood pressure in angiotensin-II-dependent hypertension. J Cardiovasc Pharmacol 2014;64(1):87-99. doi: 10.1097/FJC.0000000000000094.

193. Damsgaard CT, Lauritzen L, Calder PC, Kjaer TM, Frokiaer H. Whole-blood culture is a valid low-cost method to measure monocytic cytokines - a comparison of cytokine production in cultures of human whole-blood, mononuclear cells and monocytes. J Immunol Methods 2009;340(2):95-101. doi: 10.1016/j.jim.2008.10.005.

194. He CS, Fraser WD, Gleeson M. Influence of vitamin d metabolites on plasma cytokine concentrations in endurance sport athletes and on multiantigen stimulated cytokine production by whole blood and peripheral blood mononuclear cell cultures. ISRN Nutr 2014;2014:820524. doi: 10.1155/2014/820524.

195. Miles EA, Z, Zouboui P, Calder PC. Differential anti-inflammatory effects of phenolic compounds from extra virgin olive oil identified in human whole blood cultures. Nutrition 2005;21(3):389-94. doi: 10.1016/j.nut.2004.06.031.

196. Desch CE, Kovach NL, Present W, Broyles C, Harlan JM. Production of human tumor necrosis factor from whole blood ex vivo. Lymphokine Res 1989;8(2):141-6.

197. Stonehouse W, Conlon CA, Podd J, Hill SR, Minihane AM, Haskell C, Kennedy D. DHA supplementation improved both memory and reaction time in healthy young adults: a randomized controlled trial. Am J Clin Nutr 2013;97(5):1134-43. doi: 10.3945/ajcn.112.053371.

198. Block RC, Dier U, Calderonartero P, Shearer GC, Kakinami L, Larson MK, Harris WS, Georas S, Mousa SA. The Effects of EPA+DHA and Aspirin on Inflammatory Cytokines and Angiogenesis Factors. World J Cardiovasc Dis 2012;2(1):14-9. doi: 10.4236/wjcd.2012.21003.

199. Ferrucci L, Cherubini A, Bandinelli S, Bartali B, Corsi A, Lauretani F, Martin A, Andres-Lacueva C, Senin U, Guralnik JM. Relationship of plasma polyunsaturated fatty acids to circulating inflammatory markers. J Clin Endocrinol Metab 2006;91(2):439-46. doi: 10.1210/jc.2005-1303.

200. Tanaka S, Saitoh O, Tabata K, Matsuse R, Kojima K, Sugi K, Nakagawa K, Kayazawa M, Teranishi T, Uchida K, et al. Medium-chain fatty acids stimulate interleukin-8 production in Caco-2 cells with different mechanisms from long-chain fatty acids. J Gastroenterol Hepatol 2001;16(7):748-54.

201. Spigoni V, Lombardi C, Cito M, Picconi A, Ridolfi V, Andreoli R, Anelli N, Gnudi L, Goldoni M, Zavaroni I, et al. N-3 PUFA increase bioavailability and function of endothelial progenitor cells. Food Funct 2014. doi: 10.1039/c3fo60641d.

202. Storey A, McArdle F, Friedmann PS, Jackson MJ, Rhodes LE. Eicosapentaenoic acid and docosahexaenoic acid reduce UVB- and TNF-alpha-induced IL-8 secretion in keratinocytes and UVB-induced IL-8 in fibroblasts. J Invest Dermatol 2005;124(1):248-55. doi: 10.1111/j.0022-202X.2004.23543.x.

203. Moore KW, O'Garra A, de Waal Malefyt R, Vieira P, Mosmann TR. Interleukin-10. Annu Rev Immunol 1993;11:165-90. doi: 10.1146/annurev.iy.11.040193.001121.

204. Saraiva M, O'Garra A. The regulation of IL-10 production by immune cells. Nat Rev Immunol 2010;10(3):170-81. doi: 10.1038/nri2711.

205. Berger H, Vegran F, Chikh M, Gilardi F, Ladoire S, Bugaut H, Mignot G, Chalmin F, Bruchard M, Derangere V, et al. SOCS3 transactivation by PPARgamma prevents IL-17-driven cancer growth. Cancer Res 2013;73(12):3578-90. doi: 10.1158/0008-5472.CAN-12-4018.

206. Cucina A, Borrelli V, Randone B, Coluccia P, Sapienza P, Cavallaro A. Vascular endothelial growth factor increases the migration and proliferation of smooth muscle cells through the mediation of growth factors released by endothelial cells. J Surg Res 2003;109(1):16-23.

207. Hayashibara T, Yamada Y, Miyanishi T, Mori H, Joh T, Maeda T, Mori N, Maita T, Kamihira S, Tomonaga M. Vascular Endothelial Growth Factor and Cellular Chemotaxis: A Possible Autocrine Pathway in Adult T-Cell Leukemia Cell Invasion. Clinical Cancer Research 2001;7(9):2719-26.

208. Murdoch C, Giannoudis A, Lewis CE. Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues, 2004.

209. Berk BC, Brock TA, Webb RC, Taubman MB, Atkinson WJ, Gimbrone MA, Jr., Alexander RW. Epidermal growth factor, a vascular smooth muscle mitogen, induces rat aortic contraction. J Clin Invest 1985;75(3):1083-6. doi: 10.1172/JCI111772.

210. Kim J, Lee CK, Park HJ, Kim HJ, So HH, Lee KS, Lee HM, Roh HY, Choi WS, Park TK, et al. Epidermal growth factor induces vasoconstriction through the phosphatidylinositol 3-kinase-mediated mitogen-activated protein kinase pathway in hypertensive rats. J Pharmacol Sci 2006;101(2):135-43.

211. Kawanabe Y, Masaki T, Hashimoto N. Involvement of epidermal growth factor receptor-protein tyrosine kinase transactivation in endothelin-1-induced vascular contraction. J Neurosurg 2004;100(6):1066-71. doi: 10.3171/jns.2004.100.6.1066.

212. Matrougui K. Diabetes and microvascular pathophysiology: role of epidermal growth factor receptor tyrosine kinase. Diabetes Metab Res Rev 2010;26(1):13-6. doi: 10.1002/dmrr.1050.

213. Calviello G, Di Nicuolo F, Gragnoli S, Piccioni E, Serini S, Maggiano N, Tringali G, Navarra P, Ranelletti FO, Palozza P. n-3 PUFAs reduce VEGF expression in human colon cancer cells modulating the COX-2/PGE2 induced ERK-1 and -2 and HIF-1alpha induction pathway. Carcinogenesis 2004;25(12):2303-10. doi: 10.1093/carcin/bgh265.

214. Chao CY, Lii CK, Ye SY, Li CC, Lu CY, Lin AH, Liu KL, Chen HW. Docosahexaenoic Acid Inhibits Vascular Endothelial Growth Factor (VEGF)-Induced Cell Migration via the GPR120/PP2A/ERK1/2/eNOS Signaling Pathway in Human Umbilical Vein Endothelial Cells. J Agric Food Chem 2014. doi: 10.1021/jf5007165.

215. Zhang G, Panigrahy D, Mahakian LM, Yang J, Liu JY, Stephen Lee KS, Wettersten HI, Ulu A, Hu X, Tam S, et al. Epoxy metabolites of docosahexaenoic acid (DHA) inhibit angiogenesis, tumor growth, and metastasis. Proc Natl Acad Sci U S A 2013;110(16):6530-5. doi: 10.1073/pnas.1304321110.

216. Yang SP, Morita I, Murota SI. Eicosapentaenoic acid attenuates vascular endothelial growth factor-induced proliferation via inhibiting Flk-1 receptor expression in bovine carotid artery endothelial cells. J Cell Physiol 1998;176(2):342-9. doi: 10.1002/(SICI)1097-4652(199808)176:2<342::AID-JCP12>3.0.CO;2-5.

217. Yusufi AN, Cheng J, Thompson MA, Walker HJ, Gray CE, Warner GM, Grande JP. Differential effects of low-dose docosahexaenoic acid and eicosapentaenoic acid on the regulation of mitogenic signaling pathways in mesangial cells. J Lab Clin Med 2003;141(5):318-29. doi: 10.1016/S0022-2143(03)00005-2.

218. Makki N, Thiel KW, Miller FJ, Jr. The epidermal growth factor receptor and its ligands in cardiovascular disease. Int J Mol Sci 2013;14(10):20597-613. doi: 10.3390/ijms141020597.

219. Motie M, Evangelista LS, Horwich T, Lombardo D, Zaldivar F, Hamilton M, Fonarow GC. Association between inflammatory biomarkers and adiposity in obese patients with heart failure and metabolic syndrome. Exp Ther Med 2014;8(1):181-6. doi: 10.3892/etm.2014.1673.

220. Siervo M, Ruggiero D, Sorice R, Nutile T, Aversano M, Iafusco M, Vetrano F, Wells JC, Stephan BC, Ciullo M. Body mass index is directly associated with biomarkers of angiogenesis and inflammation in children and adolescents. Nutrition 2012;28(3):262-6. doi: 10.1016/j.nut.2011.06.007.

221. Chung HY, Cesari M, Anton S, Marzetti E, Giovannini S, Seo AY, Carter C, Yu BP, Leeuwenburgh C. Molecular inflammation: underpinnings of aging and age-related diseases. Ageing Res Rev 2009;8(1):18-30. doi: 10.1016/j.arr.2008.07.002.

222. Barcia AM, Harris HW. Triglyceride-rich lipoproteins as agents of innate immunity. Clin Infect Dis 2005;41 Suppl 7:S498-503. doi: 10.1086/432005.

223. Leeson CP, Hingorani AD, Mullen MJ, Jeerooburkhan N, Kattenhorn M, Cole TJ, Muller DP, Lucas A, Humphries SE, Deanfield JE. Glu298Asp endothelial nitric oxide synthase gene polymorphism interacts with environmental and dietary factors to influence endothelial function. Circ Res 2002;90(11):1153-8.

224. Naber CK, Baumgart D, Altmann C, Siffert W, Erbel R, Heusch G. eNOS 894T allele and coronary blood flow at rest and during adenosine-induced hyperemia. Am J Physiol Heart Circ Physiol 2001;281(5):H1908-12.

225. Wattanapitayakul SK, Mihm MJ, Young AP, Bauer JA. Therapeutic implications of human endothelial nitric oxide synthase gene polymorphism. Trends in Pharmacological Sciences 2001;22(7):361-8.

226. Guzik TJ, West NEJ, Black E, McDonald D, Ratnatunga C, Pillai R, Channon KM. Functional Effect of the C242T Polymorphism in the NAD(P)H Oxidase p22phox Gene on Vascular Superoxide Production in Atherosclerosis. Circulation 2000;102(15):1744-7. doi: 10.1161/01.cir.102.15.1744.

227. Wu Z, Lou Y, Jin W, Liu Y, Lu L, Chen Q, Xie Y, Lu G. Relationship of the p22phox (CYBA) gene polymorphism C242T with risk of coronary artery disease: a meta-analysis. PLoS One 2013;8(9):e70885. doi: 10.1371/journal.pone.0070885.

228. Lindi V, Schwab U, Louheranta A, Laakso M, Vessby B, Hermansen K, Storlien L, Riccardi G, A. Rivellese A. Impact of the Pro12Ala polymorphism of the PPAR-γ2 gene on serum triacylglycerol response to n−3 fatty acid supplementation. Molecular Genetics and Metabolism 2003;79(1):52-60. doi: 10.1016/s1096-7192(03)00065-9.

229. Cardona F, Morcillo S, Gonzalo-Marín M, Garrido-Sanchez L, Macias-Gonzalez M, Tinahones FJ. Pro12Ala Sequence Variant of the PPARG Gene Is Associated with Postprandial Hypertriglyceridemia in Non-E3/E3 Patients with the Metabolic Syndrome. Clinical Chemistry 2006;52(10):1920-5. doi: 10.1373/clinchem.2006.069690.

230. Schwarz D, Kisselev P, Chernogolov A, Schunck WH, Roots I. Human CYP1A1 variants lead to differential eicosapentaenoic acid metabolite patterns. Biochem Biophys Res Commun 2005;336(3):779-83. doi: 10.1016/j.bbrc.2005.08.172.

231. Huang X, Zhao J, Zhao T. Effects of peroxisome proliferator activated receptor-gamma 2 gene Pro12Ala polymorphism on fasting blood lipids: A meta-analysis. Atherosclerosis 2011;215(1):136-44.

232. Conen D, Glynn RJ, Buring JE, Ridker PM, Zee RY. Association of renin-angiotensin and endothelial nitric oxide synthase gene polymorphisms with blood pressure progression and incident hypertension: prospective cohort study. J Hypertens 2008;26(9):1780-6. doi: 10.1097/HJH.0b013e3283077eef.

233. Schneider MP, Hilgers KF, Huang Y, Delles C, John S, Oehmer S, Schmieder RE. The C242T p22phox polymorphism and endothelium-dependent vasodilation in subjects with hypercholesterolaemia. Clin Sci (Lond) 2003;105(1):97-103. doi: 10.1042/CS20030003.

234. Jackson KG, Poppitt SD, Minihane AM. Postprandial lipemia and cardiovascular disease risk: Interrelationships between dietary, physiological and genetic determinants. Atherosclerosis 2012;220(1):22-33. doi: 10.1016/j.atherosclerosis.2011.08.012.

235. Deeb SS, Fajas L, Nemoto M, Pihlajamaki J, Mykkanen L, Kuusisto J, Laakso M, Fujimoto W, Auwerx J. A Pro12Ala substitution in PPARgamma2 associated with decreased receptor activity, lower body mass index and improved insulin sensitivity. Nat Genet 1998;20(3):284-7. doi: 10.1038/3099.

236. Masugi J, Tamori Y, Mori H, Koike T, Kasuga M. Inhibitory effect of a proline-to-alanine substitution at codon 12 of peroxisome proliferator-activated receptor-gamma 2 on thiazolidinedione-induced adipogenesis. Biochem Biophys Res Commun 2000;268(1):178-82. doi: 10.1006/bbrc.2000.2096.

237. Armah CK. Physiological responsiveness of cardiovascular risk factors to long chain n-3 polyunsaturated fatty acids: Impact of genotype, gender and age. Hugh Sinclair Department of Nutrition: The University of Reading, 2008:154-80.

238. Ruiz-Lopez N, Haslam RP, Napier JA, Sayanova O. Successful high-level accumulation of fish oil omega-3 long-chain polyunsaturated fatty acids in a transgenic oilseed crop. Plant J 2014;77(2):198-208. doi: 10.1111/tpj.12378.

239. Shen HC, Hammock BD. Discovery of inhibitors of soluble epoxide hydrolase: a target with multiple potential therapeutic indications. J Med Chem 2012;55(5):1789-808. doi: 10.1021/jm201468j.

240. Arnold C, Markovic M, Blossey K, Wallukat G, Fischer R, Dechend R, Konkel A, von Schacky C, Luft FC, Muller DN, et al. Arachidonic Acid-metabolizing Cytochrome P450 Enzymes Are Targets of ω-3 Fatty Acids. Journal of Biological Chemistry 2010;285(43):32720-33. doi: 10.1074/jbc.M110.118406.

Annex 1 – List of prohibited medications

Ca2+-channel blockers

Adalat Capsules (Nifedipine), Adalat LA (Nifedipine), Adipine XL (Nifedipine) ,Amlodipine (Amlodipine) ,Angitil SR (Diltiazem), Beta-Adalat (Atenolol, Nifedipine), Calchan MR (Nifedipine), Cardene (Nicardipine), Cardene SR (Nicardipine), Cardioplen XL (Felodipine), Coracten XL (Nifedipine), Diltiazem (Diltiazem), Dilzem XL (Diltiazem), Exforge (Amlodipine, Valsartan), Fortipine LA (Nifedipine), Istin (Amlodipine), Lercanidipine, Motens (Lacidipine), Nicardipine (Nicardipine), Nifedipine (Nifedipine), Nimotop (Nimodipine), Plendil (Felodipine), Prescal (Isradipine), Securon I.V. (Verapamil), Securon SR (Verapamil), Sevikar (Amlodipine, Olmesartan), Slozem (Diltiazem), Syscor MR (Nisoldipine), Tarka (Trandolapril, Verapamil), Tenif (Atenolol, Nifedipine), Tensipine MR (Nifedipine), Tildiem LA (Diltiazem), Triapin (Felodipine, Ramipril), Univer (Verapamil), Valni XL (Nifedipine), Verapamil (Verapamil), Vertab SR (Verapamil), Viazem XL (Diltiazem), Zanidip (Lercanidipine), Zemtard XL (Diltiazem), Zolvera (Verapamil)**.**

α-blockers

Baratol (Indoramin), Cardura XL (Doxazosin), Carvedilol (Carvedilol), Combigan (Brimonidine, Timolol), Combodart (Dutasteride, Tamsulosin hydrochloride), Contiflo XL (Tamsulosin), Doralese (Indoramin), Doxazosin (Doxazosin), Eucardic (Carvedilol), Flomaxtra XL (Tamsulosin), Hypovase (Prazosin), Hytrin (Terazosin), Indoramin (Indoramin), Labetalol (Labetalol), Opilon (Moxisylyte), Pinexel PR (Tamsulosin), Raporsin XL (Doxazosin), Rogitine (Phentolamine), Tabphyn MR (Tamsulosin), Tamsulosin (Tamsulosin), Terazosin (Terazosin), Trandate (Labetalol), Xatral XL (Alfuzosin).

Antibiotics

Beta Lactam Medicines

Amoxicillin, Amoxicillin + clavulanic acid, Ampicillin, Benyathine benzylpenicillin, Benzylpenicillin, Cefalexin, Cefazolin, Ceftriaxone, Cloxacillin, Phenoxymethylpenicillin, Procaine benzylpenicillin, Cefotaxime, Ceftazidime, Imipenem + cilastatin.

Other antibacterials

Azithromycin, Chloramphenicol, Ciprofloxacin, Doxycycline, Erythromycin, Nitrofurantoin, Spectinomycin, Sulfamethoxazole + trimethoprim, Clindamycin, Vancomycin.

Thiazolidinediones

Rosiglitazone, Pioglitazone, Troglitazone.

Liver enzyme inducing drugs

Barbiturates**,** Carbamazepine, Phenytoin, Primidone, Ritonavir, Sulfinpyrazone, St. John's wort.

β-blockers

Acebutolol (Acebutolol), Atenolol (Atenolol), Azarga (Brinzolamide, Timolol), Beta-Adalat (Atenolol, Nifedipine), Beta-Cardone (Sotalol), Betagan (Levobunolol), Celectol (Celiprolol), Celiprolol (Celiprolol), Combigan (Brimonidine, Timolol), Corgard (Nadolol), Cosopt (Dorzolamide, Timolol), Co-tenidone (Co-tenidone), DuoTrav (Timolol, Travoprost), Emcor (Bisoprolol), Eucardic (Carvedilol)Ganfort (Bimatoprost, Timolol), Inderal Injection (Propranolol), Inderal LA (Propranolol), Kalten (Amiloride, Atenolol, Hydrochlorothiazide), Labetalol (Labetalol), Levobunolol, Lopresor (Metoprolol), Metoprolol (Metoprolol), Nebilet (Nebivolol), Nyogel (Timolol), Prestim (Bendroflumethiazide, Timolol maleate), Propranolol (Propranolol), Sectral (Acebutolol), Slow-Trasicor (Oxprenolol), Sotacor (Sotalol), Syprol (Propranolol), Tenif (Atenolol, Nifedipine), Tenoret 50 (Atenolol, Chlortalidone, Co-tenidone), Tenoretic (Atenolol, Chlortalidone, Co-tenidone), Tenormin (Atenolol), Teoptic (Carteolol), Timolol (Timolol), Timoptol-LA (Timolol), Trandate (Labetalol), Trasicor (Oxprenolol), Trasidrex (Co-prenozide, Cyclopenthiazide, Oxprenolol), Viskaldix (Clopamide, Pindolol), Visken (Pindolol), Xalacom (Latanoprost, Timolol maleate)**.**

ACE Inhibitors

Accupro (Quinapril), Accuretic (Hydrochlorothiazide, Quinapril), Capoten (Captopril), Capozide (Captopril, Co-zidocapt, Hydrochlorothiazide), Captopril (Captopril), Carace 10 Plus (Hydrochlorothiazide, Lisinopril), Coversyl Arginine (Perindopril), Coversyl Arginine Plus (Indapamide, Perindopril), Enalapril (Enalapril), Enalapril + hydrochlorothiazide (Enalapril, Hydrochlorothiazide), Fosinopril (Fosinopril), Gopten (Trandolapril), Innovace (Enalapril), Innozide (Enalapril, Hydrochlorothiazide), Lisinopril (Lisinopril), Lisinopril + hydrochlorothiazide (Hydrochlorothiazide, Lisinopril), Perdix (Moexipril), Perindopril (Perindopril), Quinapril (Quinapril), Ramipril (Ramipril), Tanatril (Imidapril), Tarka (Trandolapril, Verapamil), Triapin (Felodipine, Ramipril), Tritace (Ramipril), Vascace (Cilazapril), Zestoretic 20 (Hydrochlorothiazide, Lisinopril), Zestril (Lisinopril).

Lipid lowering medication

Statins

Lescol XL (Fluvastatin), Lipitor (Atorvastatin), Lipostat (Pravastatin), Pravastatin (Pravastatin), Crestor (Rosuvastatin), Fluvastatin, Simvastatin (Simvastatin), Zocor (Simvastatin).

Annex 2 – Dietary Restrictions

All participants partaking in the dietary intervention assessments in the trial were provided with the following guidelines on dietary restrictions. Adherence to these restrictions was subsequently checked at the beginning of the assessment visit.

Table 1: Food restriction for 3 days prior to each assessment visit

Foods to avoid	Restrict		Alternatives (*see table 2, for foods to be avoided 24hr before assessment)
Seafood, fish, fish products and other omega-3 rich foods			
• Any oily fish such as salmon, tuna, mackerel, kippers, salmon, sardines, herring, bream. • Eel, lamprey, caviar, roe or any shellfish (mussels, oysters, prawns, crab, lobster, etc) • Omega-3 enriched eggs.	• Pork • Omega-3 rich margarines	1 serving per day	Chicken, beef, non-oily fish (cod, haddock) and other red meats. Replace margarine with butter.
Vegetables, salad and products (e.g. quiche, pizza, casserole) containing:			
Garlic, onion, red peppers, carrot, Broad bean, red onion, aubergine, red cabbage, red skinned potatoes, beetroot, purple carrot, dried fruits, nuts and trail mixes, shredded coconut, dehydrated meat products (such as beef jerky), brussel sprouts.	Black bean and black olives Kidney beans	2 handful per 3 days 1 handful per 3 days 4 per 3 days	Shallot, green bean, tomato, carrot, cauliflower, cucumber, peas, potato (excluding red potato), pumpkin, parsnip, pepper, lentil, sweetcorn, lettuce, green olive
Other foods: including products (e.g. drinks, desserts, vegetarian meat alternatives) containing the following:			
Dark chocolate, baking chocolate and cocoa products Soy, soy milk and soy containing products (e.g. quorn, tofu) Cider, red wine, teas (of any kind)			Sugar based and/or dairy desserts and snacks, egg based desserts (e.g. plain biscuits, cereal bar, ice cream, custard) Milk and milk containing products (e.g. non berry fruit yoghurt, cheese) Water, carbonated drinks (e.g. coca-cola, sprite), beer, white wine, spirits

Please observe the following food restrictions for the 3 days before your assessment visit.

- Foods listed in the red box are not to be eaten.
- Foods listed in the orange box can be eaten as per the guideline
- Foods listed in the green box can be eaten freely.

Table 2: Further food restriction 24 hrs prior to each assessment visit

Foods to avoid	Alternatives
Vegetables, salad and products (e.g. quiche, pizza, casserole) containing:	
All fruits and most vegetables.	Rice, white potatoes, spaghetti, bread.
Other foods: including products (e.g. drinks, desserts, vegetarian meat alternatives) containing the following:	
Drinks containing caffeine e.g. tea, coffee, hot chocolate, coke, energy drinks (such as RedBull) Drinks containing berry fruit and citrus fruit (including cordials) Bottled water (excluding Buxton mineral water) Drinks / foods containing alcohol Cured and canned meat (e.g. bacon, ham, sausages, corned beef) Smoked fish	Milk (excluding soya milk), Buxton water (provided by the researchers for the 24hr before you attend an assessment visit) Fresh meat (e.g. chicken, turkey, beef, pork, lamb) Fresh fish (excluding oily fish)

The restrictions above are to be followed for the last 24 hours before the assessment visit alongside those in Table 1. Those foods in table 1 listed under the green may still be freely consumed for the last 24 hours unless stated otherwise above (*i.e.* fruits and vegetables and caffeinated drinks may not be consumed)

Please note these additional lifestyle restrictions:

- For 48 hours before your visit we ask that you refrain from strenuous exercise or sports.
- For 3 days prior to your visit please refrain from using anti-inflammatory medications such as ibuprofen, aspirin, naproxen, etc, unless otherwise advised by your GP or health care provider.

Annex 3 – Pre-assessment health check, 24 hour diet recall and restriction adherence checks

Assessment day general health check and adherence to study restrictions

	YES	NO
Has the subject observed a 10h fast?	☐	☐
Has the subject felt sick, unusually tired or weak within the last week? (if yes, fill out an AE form)	☐	☐
Does the subject feel sick, unusually tired or weak today? (if yes, fill out an AE form)	☐	☐
Has the subject recently taken medication that may affect the assessment measurements? e.g. antibiotics or medication in Annex 23	☐	☐

Details of sickness / ill health:

_____
_____
_____
_____
_____

If any of the above answers fall <u>outside</u> the 'bold' boxes – reschedule visit

	YES	NO
Has the subject refrained from strenuous exercise in the last 48h?	☐	☐
Has the subject consumed alcohol in the last 24h?	☐	☐
Has the subject consumed nitrate/nitrite rich food, sulphate/sulphite rich foods, EPA/DHA rich foods or chocolate, berries, caffeinated beverage, citrus fruits or soy products in the last 24 h?	☐	☐
Did the subject consume the evening meal provided?	☐	☐

Ticks in non-bold boxes = protocol violations: add details of action taken in comments section below

Name of scientist / research nurse (PRINT): ……………………………………….

Signature of the scientist / research nurse: ……………………………………….

DATE:	D	D	M	M	M	Y	Y	Y	Y
E.g.	0	1	J	A	N	2	0	1	1

COMMENTS:

24 hour dietary recall sheet

VOL ID:	Date:					Day of week (circle below)			Typical? (circle)		Other important detail:
	Mon	Tue	Wed	Thur	Fri	Sat	Sun	YES	NO	
Meal / eating event	Time (24hr format)	location	Food Item – identify full description, brand, type (i.e. low fat, sugar free etc.)			Preparation method (e.g. boiled, grilled etc.)	Estimate amount	Leftovers? ..how much		Additional info identified during multiple pass

*Additional sheets will be used to elucidate all dietary information

Instructions to the researcher
The recall should follow these steps

- **Snapshot-** Volunteer is asked to recall main meals, food and beverages consumed the day before the interview (morning to morning).

- **Playback** – recall the foods consumed back to the participant – remembering to include time and location;

  - Look for long gaps between eating events and ask specifically about this

  - Look for food items that are usually eaten together i.e. toast + fat spread + preserve

  - Drinks, snacks, accompaniments (i.e. sauce, salt, pepper), alcoholic beverages are often missed – ask about these if they are not on the list

- **Detail Cycle** - For each food, a detailed description, amount eaten, and additions to the food are collected.

- **Usual dietary practices / forgotten foods** – volunteer is asked about their normal eating habits, with the intention to cross-reference normal habits against reported 24hr intake. Consumption of foods commonly forgotten during the snapshot and probes will be used as time and eating occasion are collected for each food (See table 1 below)

- *Final Probe - Additional foods not remembered earlier are collected (further probe questions)*

- **List of questions for further food intake detail:**

Select (√)	List of probes questions (for all questions, cross-check responses against reported 24hr intake)
	Do you normally eat breakfast? Did you do that yesterday?
	Do you drink hot drinks? If so, what type and how many do you usually have? Did you drink that many yesterday?
	Do you drink milk in hot drinks and / or on cereal? What type?
	Do you add sugar / artificial sweetener to hot drinks? If so, what type and how much?
	Do you normally have biscuits or snacks with hot drinks? If yes, did you do that yesterday? (cross check with dietary restrictions)
	What fat spread do you use? Do you add this to sandwiches etc.
	What oils do you cook with? Do you deep fry items?
	Do you tend to buy full fat, reduced fat or low fat foods? Were any of the foods you ate yesterday any of the reduced or low fat type?
	Did you drink any alcohol yesterday? Do you usually drink? What kind (e.g. beer, red wine)? How much in glasses/ pints?
	Was the amount of food consumed on the recall day more than usual, usual, or much less than usual?

Restricted foods cross-check:

Food	Consumed in last 1 to 2 days (circle)		Amount consumed during last 1 to 2 days
Oily fish (e.g. tuna, kippers, mackerel, salmon, sardines, herring, bream, trout) or sea-food (e.g. crab, eel, octopus, squid, prawns, oysters, mussels) If yes; which:	Yes	No	
Citrus fruits (e.g. orange, tangerine, satsuma, Clementine, grapefruit, lime, lemon) If yes, which;	Yes	No	
Soy and soy based products (e.g. soya milk, soya spreads, tofu, vegetarian meat) If yes, which;	Yes	No	
Dark chocolate and cocoa products If yes, which;	Yes	No	
Red wine or tea? If yes, which;	Yes	No	
Berries, berry products (e.g. Blueberry, strawberry, raspberry, cranberry, blackcurrant, redcurrant), If yes, which; Or any of the following fruit and vegetables: Black / red grapes, raisins, plum, prune, cherry, pomegranate, Broad bean, red onion, aubergine, red cabbage, red skinned potatoes, purple carrot If yes, which;	Yes	No	

Annex 4 - Supplementary Data

Plasma fatty acids as % of total lipid weight at baseline and in response to treatment

Fatty acid	Control meal (0h)	Control meal (4h)	EPA meal (0h)	EPA meal (4h)	DHA meal (0h)	DHA meal (4h)
16:0	23.33±0.48	23.63±0.37	23.07±0.39	23.24±0.30	23.21±0.42	23.08±0.37
16:1[2]	2.09±0.10	1.85±0.09	2.27±0.14	1.99±0.12	2.05±0.10	1.86±0.09
18:0	7.45±0.17	7.46±0.19	7.39±0.18	7.07±0.13	7.59±0.23	7.19±0.17
18:1	24.94±0.50	25.57±0.52	25.61±0.47	25.56±0.44	25.53±0.34	25.57±0.34
18:2n6	26.43±0.47	26.00±0.66	25.73±0.62	25.31±0.52	25.76±0.62	25.30±0.59
18:3n3[2]	0.66±0.04	0.75±0.03	0.59±0.04	0.74±0.03	0.70±0.06	0.76±0.04
18:3n6	0.51±0.05	0.46±0.05	0.45±0.04	0.48±0.04	0.53±0.05	0.45±0.04
20:3n6[2]	1.58±0.06	1.47±0.06	1.68±0.07	1.56±0.05	1.57±0.05	1.46±0.05
20:4n6[2]	6.54±0.30	6.09±0.31	6.44±0.27	6.17±0.22	6.33±0.25	6.01±0.19
20:5n3[123]	0.78±0.05	0.86±0.11	0.87±0.07	2.03±0.19*	0.90±0.06	1.23±0.03
22:5n3	0.27±0.03	0.26±0.03	0.27±0.03	0.20±0.03	0.23±0.03	0.24±0.03
22:6n3[123]	1.61±0.08	1.65±0.10	1.68±0.07	1.92±0.08	1.70±0.0	2.90±0.16*

Table A) Plasma fatty acids from total lipids (% weight) at baseline and in response to treatment. Data is presented as mean±SEM. [1]Indicates significant effect for time by Repeated Measures Anova, [2]Indicates significant effect for treatment by Repeated Measures Anova, [3]Indicates significant effect for time*treatment effect by Repeated Measures Anova.* indicates a significant difference in change from baseline when compared to control.
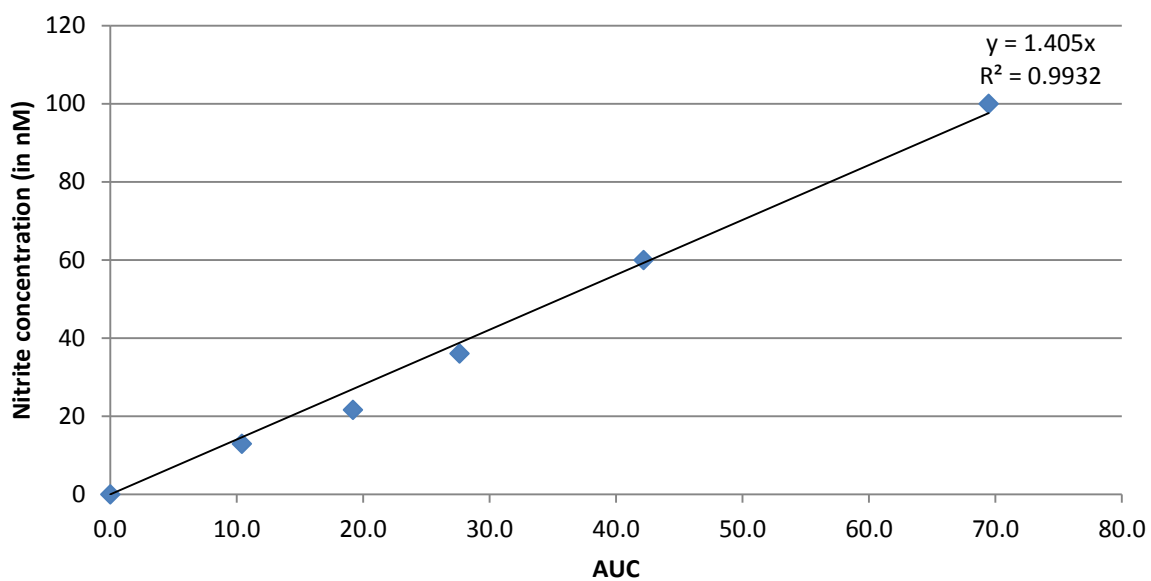
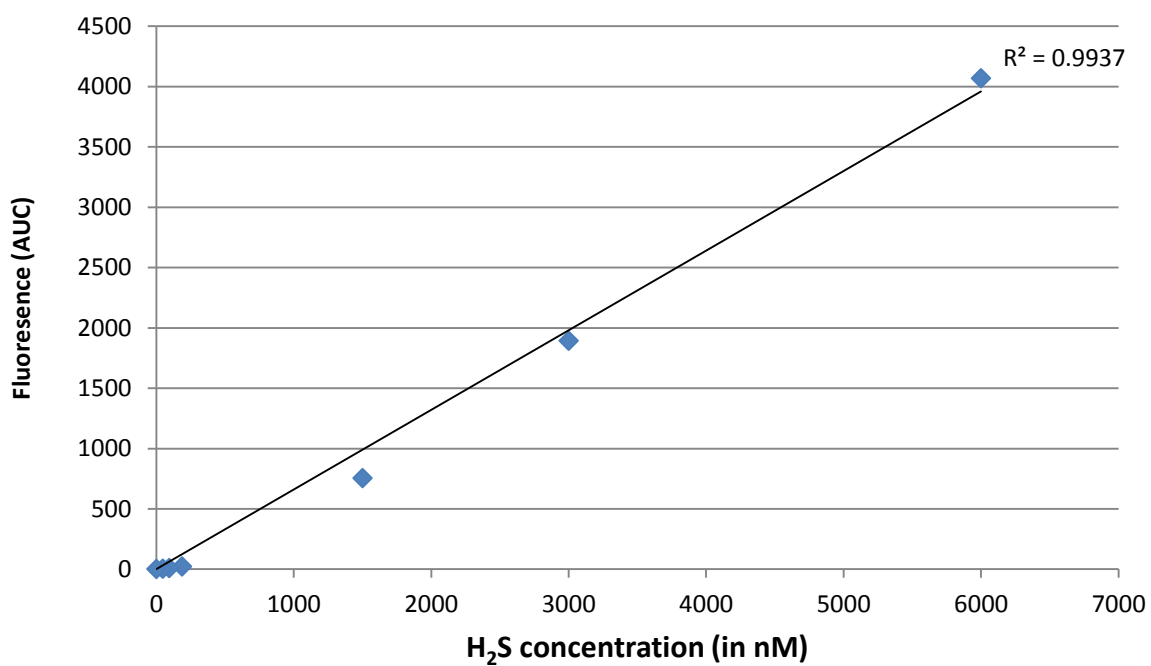**Figure b.** Representative calibration curve for nitrite analysis.

Figure c. Standard curve for H_2S quantification via SDB derivatisation.